ECOLOGY AND PHYTOCHEMISTRY OF *Zanthoxylum armatum* DC.

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BOTANY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BOTANY

BY

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AUGUST 2022

DECLARATION

Thesis entitled "**Ecology and Phytochemistry of *Zanthoxylum armatum* DC.**" submitted to the Central Department of Botany, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph. D.), is a research work carried out by me under the supervision of Professor Dr. Sangeeta Rajbhandary, Central Department of Botany, Tribhuvan University and co-supervised by Professor Emeritus Pramod Kumar Jha, Central Department of Botany, Tribhuvan University and Dr. Pankaj Prasad Raturi, Dabur India Private Limited, India. I declare that the work is my own and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree. All information cited in this work has been specifically acknowledged and credited to the respective authors or institutions as references.

Date: August, 2022

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RECOMMENDATION

This is to recommend that **Ms. Nirmala Phuyal** has carried out research entitled "**Ecology and Phytochemistry of *Zanthoxylum armatum* DC.**" for the award of Doctor in Philosophy (Ph. D.) in **Botany** under our supervision. To the best of our knowledge, this work has not been submitted for any other degree. She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph. D. degree.

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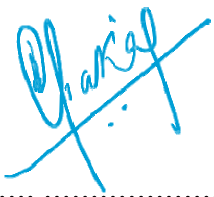

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LETTER OF APPROVAL

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On the recommendation of Prof. **Dr. Sangeeta Rajbhandari**, Prof. Emeritus **Dr. Pramod Kumar Jha** (Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu) and **Dr. Pankaj Prasad Raturi** (Dabur India Private Limited, Delhi, India), this Ph.D. thesis submitted by **Ms. Nirmala Phuyal** entitled **"Ecology and Phytochemistry of *Zanthoxylum armatum* DC."** is forwarded by Central Department Research Committee (CDRC) to the Dean, Institute of Science and Technology (IOST), Tribhuvan, University.

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Nirmala Phuyal

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ABSTRACT

Medicinal and aromatic plants are one of the major bio-resources of Nepal; their wise use can contribute to the local economy and subsistence health needs. *Zanthoxylum armatum* DC. is an important ethno-medicinal plant having several medicinal, pharmaceutical and biological properties and also a promising source of several secondary metabolites. It is also one of the prioritized medicinal plants for economic development and its demand is also increasing, not only in national but also in international markets. In this regard, the present study has been carried out to generate baseline information of *Z. armatum* regarding its ecological status including seed attributes, propagation techniques, and phytochemical variations.

Population, distribution, and regeneration of *Z. armatum* was studied in six different localities along different altitudes of Salyan district. Altogether 50 plant species belonging to 44 genera under 34 families were found to be associated with *Z. armatum*. Mean population density of *Z. armatum* in the study area was found to be 913.33 individuals/ha. The regeneration of *Z. armatum* in the study area was fair with the average seedling and sapling densities of 150 individuals/ha. and 100 individuals/ha respectively.

The overall yield was found to be increasing along with increasing elevation up to 1800 m. The average yield per plant was 4.18 kg (fresh wt.) with the lowest yield of 3.4 kg (fresh wt.), 2.16 kg (dry wt.) at 1000-1200 m and the highest of 5.7 kg (fresh wt.) at 1600-1800 m elevation. Similarly, the size and weight of seeds also showed an increasing pattern with the increase in altitude up to 1800 m. The soil nutrients: organic carbon, nitrogen, phosphorus, and potassium were found to have a strong positive correlation with the fruit yield.

The seeds of *Z. armatum* do not germinate easily. Chemical and hormonal treatments had relatively some effects on the germination, while hot water, cold water, and chilling treatment did not induce germination. Nitric acid had comparatively better germination rate than hydrochloric acid and sulfuric acid. Germination percentage increased with the increase in concentration of GA_3 with the highest 54.67% at 1500 ppm and decreased thereafter. The most effective mode of increasing germination rate in *Z. armatum* is sowing freshly harvested seeds at proper time. 62.44% germination was achieved by sowing seeds harvested during September 16-October 15.

The effect of different growth hormones, their concentrations, and different rooting media on the rooting and sprouting of *Z. armatum* stem cuttings was studied. The cuttings were treated with two types of auxins namely IBA and NAA at different concentrations (2000 ppm, 3000 ppm and 5000 ppm) and planted in three different rooting media: sand, neopeat and mix. Hormone concentration and growth media significantly affected the rooting and shooting ability of *Z. armatum* stem cuttings. IBA was found to be more effective than NAA. Neopeat medium was better than sand and mix media.

Total phenolic and flavonoid contents (TPC & TFC) were comparatively lower in the the extract of leaf than the extracts of fruit, seed and bark. Highest TPC value was 226.3 ± 1.14 mg/g GAE in the extract of the fruit and the lowest was 63 mg/g GAE in the extract of leaf. Similarly, the highest TFC value was 135.17 ± 2.02 mg/g QE in fruits and the lowest was 46 mg/g QE in leaves. The extracts showed variable antioxidant properties. The fruits exhibited excellent antioxidant properties with IC_{50} values of 40.62 µg/mL and 45.62 µg/mL for cultivated and wild fruits respectively. The least antioxidant capacity was shown by the extract of the seed with IC_{50} values of 86.75 µg/mL and 94.49 µg/mL for wild and cultivated seeds respectively.

The yield of essential oil of dried leaf obtained from hydro-distillation ranged from 0.16% to 0.50% with a total of 17 compounds. The major components were linalool, limonene, and undecan-2-and other components tridecan-2-one, myrcene, methyl cinnamate and α-bergamotene were also identified in most of the samples but in lower proportions. Similarly, the yield of essential oil of fruits ranged from 2.72 to 8.2 %. The maximum yield 8.2 % was obtained from September harvested fruits. This is the maximum recorded essential oil yield from *Z. armatum* fruits. A total of 13 volatile compounds were identified from the essential oil of fruits and the major components were linalool, methyl cinnamate, limonene, myrcene, sabinene and terpinen-4-ol.

Crude methanol extracts of fruits, seeds, leaves, and bark and essential oil of leaf and fruit of *Z. armatum* were investigated in vitro for antimicrobial activities against eleven different bacterial and two fungal strains and the minimum bactericidal concentration (MBC) was determined. The extracts exhibited antibacterial properties against five bacteria only ie. *Bacillus subtillis, Escherichia coli*, Methicilin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus, and Staphylococcus epidermidis*, while no activity for the fungi was observed. *S. aureus* was found to be more susceptible for all the extracts compared to other strains. The maximum Zone of inhibition of 20.72 mm was produced by fruits (wild) and 18.10 mm (cultivated) against *S. aureus*. Similarly, the lowest MBC of 0.78 mg/mL was obtained for the extract of fruit against MRSA, 1.56 mg/mL for extracts of fruits, seeds and bark against *B. subtilis*, MRSA and *S. aureus* and highest value of 50 mg/mL for extracts of fruit and seed against *S. epidermidis.*

Keywords: Distribution; Altitude; Regeneration; Vegetative Propagation; Germination; Essential oil; Antibacterial activities; *Zanthoxylum*

LIST OF ACRONYMS AND ABBREVIATION

°C	Degree centigrade
ATCC	American Type Culture Collection
BAP	Benzyl amino purine
CCA	Cannonical Correspondence Analysis (CCA)
CCl4	Carbon tetrachloride
CITES	Convention on International Trade in Endangered Species of Wild Fauna & Flora
cm	Centimeter
DCA	Detrended Correspondence Analysis
DMRT	Duncan's Multiple Range Test
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
e.g.	for example
et al.	and others
etc.	Et cetera
FCR	Folin-Ciocalteu Reagent
g	Gram
GA_3	Gibberellic acid
GAE	Gallic acid equivalent
GC-MS	Gas chromatography–mass spectrometry
GPS	Global Positioning System
hr	Hour
HW	Hard-wood
IAA	indole-3-acetic acid
IBA	Indole-3-butyric acid
LSD	Least Signinificant Difference
KATH	National Herbarium and Plant Laboratories
kg	Kilogram
km	Kilometer
LSD	Least significance difference
m asl	meter above sea level
m	Meter

MAPs	Medicinal and Aromatic Plants
MBC	Minimum bactericidal concentration
mg	Milligram
MHA	Mueller-Hinton agar
MHA.GMB	Muller-Hinton Agar with Glucose and Methylene Blue
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
mL	Milliliter
mm	Millimeter
MRSA	Methilicin Resistant *Staphyloccus aureus*
MS	Murashige and Skoog medium
NA	Nutrient agar
NAA	Naphthaleneacetic acid
NARC	Nepal Agricultural Research Council
NB	Nutrient broth
nm	Nano meter
NRs	Nepali Rupees
NTFPs	Non-Timber Forest Products
PDA	Potato dextrose agar
PGRs	Plant Growth Regulators
Ppm	Parts per million
QE	Quercetin equivalent
RI	Retention index
SHW	Semi-hard wood
SOC	Soil organic carbon
sp	Species
t	ton
TFC	Total flavonoid contents
TPC	Total phenolic contents
TUCH	Tribhuvan University Central Herbarium
UV	Ultra violet
WHO	World Health Organization
ZOI	Zone of inhibition

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CHAPTER 1

1. INTRODUCTION

1.1 Background

Plants form the preliminary ingredients of medicines in traditional systems of healing and are the source of many major pharmaceutical drugs. Plants, therefore, have been regarded as the fundamental sources of medicines throughout human history (Rajbhandary & Ranjitkar, 2006). People have used medicinal plants in health care since the time of earliest human evolution. They are still the source of medication for a wide range of ailments. Traditional systems of medicinal practices are still the significant sources of healthcare around the world, particularly in the developing countries (WHO, 2019). Plants used in traditional medicine are important sources of bio molecules. Different phenolics and flavonoids, having antimicrobial, antioxidant, and anticancer properties have been recognized and isolated from various plants (Greenberg et al., 2008; Kale et al., 2010).

Medicinal plants are second most valuable bio-resources of Nepal after water resources (Rawal et al., 2009). The history of medicinal plants in Nepal can be traced back to the Vedic period, where Nepal Himalaya was mentioned as a sacred heaven of potent medicinal plants (Malla, 2002). The total number of medicinal and aromatic plants reported in Nepal varies according to various authors (DPR, 1970, 1983; Baral & Kurmi, 2006). Ghimire (2008) reported 1950 species of medicinal plants in Nepal. Many highly demanded and globally important medicinal plants are harbored in various geo-climatic regions of the country. Medicinal and aromatic plants in the mountains of Nepal can contribute to the local economy and subsistence health needs while conserving the ecosystem and biodiversity of an area. About 1600 species of higher plants representing 25% of the vascular flora have been recorded to be used in traditional medicine. Every year about 20,000 t of medicinal plant products comprising of about 70 species valued at 25-30 million US dollars are collected in the wild (Bhattarai & Karki, 2013). More than two thirds of the world's plant species, at least 35,000 of which are estimated to have medicinal value, come from the developing countries. At least 7000 medical compounds in the modern pharmacopeia are derived from plants (Srivastava, 2011). High value medicinal plants have been the basis for modern allopathic drug development. Because individual plants and therefore species can only function physiologically and successfully complete their life cycles under specific environmental conditions. Changes to climatic and soil conditions are likely to have significant impact on plants from the level of individual right through the level of ecosystem or biome. Knowledge about distribution and ecological features of the plants helps to decrease over exploitation by encouraging sustainable and discrete collection of medicinal plant (Bhattacharya & Sharma, 2008).

Zanthoxylum armatum is valuable economically as the source oil, fruit, wood, raw materials for industry and it also has ornamental and culinary applications (Adesina, 2005; Seidemann, 2005). With a diverse range of household, commercial, and ethno-medicinal uses, it is one of the important medicinal plants. Different plant parts leaf, fruit, stem, bark, seed, and root are used in indigenous medicine preparation against various diseases (Singh & Singh, 2011). The diverse pharmacological applications of the plant have been attested by several studies (Phuyal et al., 2019a). Alongside the pharmaceutical uses, it is also widely used in flavoring and fragrance industries.

The fruit of *Z. armatum* is used as a carminative, and in the treatments of dyspepsia, stomachache, as well as toothache. Seeds are used as condiment and flavoring agent (Arshad & Ahmad, 2005; Abbasi et al., 2010a). Further, its seeds and fruits are given as aromatic tonic in dyspepsia, fever, stomachache, and as anthelmintic, particularly in treating roundworm infestation (Verma & Khosa, 2012; Tiwary et atl, 2007; Kalia et al., 1999). The essential oil of fruits has a good tenacity and is appreciated for its fixative qualities. Since almost all the plant parts are aromatic, the entire plant body is assumed to contain essential oil. In fact the composition of the essential oil can provide more insight regarding the active constituents of this plant, and thereby its medicinal properties. The volatile oil of the plant species is also used as anticataerhal, antiseptic, antidiarrheal, and as deodorant (Neetu et al., 2001; Bhattacharya & Zaman, 2009; Bhattacharya et al., 2010).

Because of its diversified and various uses in different sectors, from pharmaceutical to flavoring industries, the plant can be developed into an economically important product with a good scope of scaling up of the production by adoption of proper management system, improving harvesting techniques and proper post-harvest methods as important significance for its trade in Nepal because of its multiple functions and potential for rural livelihood improvement. The product has been established as a locally usable commodity and as an integral source of income benefiting the women, landless and unemployed people in Nepal. It can be grown on less fertile soil, marginal lands and can be harvested after three years of planting with less chances of pest infestation (ANSAB, 2011; MoAD, 2011; DoF, 2014a). The Agricultural Commodity Export Program established under the Department of Agriculture, Agri-business Promotion and market Development Directorate, of the Ministry of Agricultural Development of the Government of Nepal, can be instrumental in linking the national market of an agricultural product like *Z. armatum* with the international markets (Phuyal et al., 2018).

1.2 Justification of the study

Z. armatum has been listed as one of the 33 prioritized medicinal plants that can contribute to the economic development of Nepal (DPR, 2006). Its trade value is also very high and is one of the top five NTFP species in trade in terms of royalty collection (DoF, 2015). During the last two decades, the market price of *Z. armatum* has been increasing considerably in national as well as international markets. This consistent rise in demand, unsustainable modes of collection, inadequate technical know-how and lack of correct techniques for harvesting and post-harvest practices have posed serious threat to the native populations effecting a steep decline of the species in the wild. This has adversely affected the regeneration of the species. Thus, commercial farming of *Z armatum* by developing suitable agro-technology could be the key for reducing the threat to natural populations, at the same time contributing towards economic upliftment of the marginalized and disadvantaged communities. The genus *Zanthoxylum* has great significance because of its ethnobotany, phytochemistry and biological activities, and is a promising source of several secondary metabolites and alkaloids.

Numerous studies on the medicinal plants focusing on their botany, ethnobotany, and ecology have been undertaken. Further, pharmacology and phytochemistry of quite a few medicinal plants have also been accomplished. Similarly, regarding *Z. armatum* also, a significant volume of studies have been conducted in the Indian subcontinent on the plant’s phytochemistry, pharmacology, biological activities (Gilani et al., 2010; Waheed et al., 2011; Negi et al., 2012; Barkatullah et al., 2013; Bharti & Bhusan, 2015; Kanwal et al., 2015), germination behavior (Tiwary et al., 2007; Ramdas et al., 2012), etc. In the Nepalese context, however, the studies in *Zanthoxylum* are still few and far between. With a study based on western part of Salyan district in mid-western Nepal, an analysis was done by Wiersum (2000) on different management systems of *Z. armatum* production in Nepalese forests. Also, Phuyal et al. (2018, 2019a, b, 2020 a, b, c) studied the vegetative propagation methods, antioxidant and antibacterial properties and the composition of the essential oil of the leaf and fruit of *Z. armatum.* However, studies regarding the relationship between the different environmental factors as altitude, habitat conditions, populations etc. and the active phyto-constituents of *Z. armatum* is still meager in Nepal.

Conservation and cultivation of any species requires the in-depth knowledge about their ecology. The present study focuses on the detailed study of *Z. armatum* regarding its ecological status including seed attributes and propagation, phytochemical variations. One of the prominent threats to many medicinal and aromatic plants (MAPs) including *Zanthoxylum* is the unsustainable harvesting and lack of appropriate management practices. Hence, knowing the ecology and biology of these species is very important for the subsequent development of agro-technology and cultivate them commercially, which ensures the steady supply of raw materials without hampering their natural population.

Salyan is regarded as a hub for *Z. armatum* farming since very long. The government also prioritized *Z. armatum* as one village one product (OVOP) for Salyan. The provincial and local level governments are also promoting commercial farming and enterprise development and the farmers are also shifting from conventional farming to commercial cultivation of *Z. armatum*. It is gaining huge popularity with a distinct change in the livelihood of the local farmers. Certain areas have been declared as pocket areas for *Z. armatum* cultivation and the Division Forest Office and Plant Research Centers have also started to work with the farmers' cooperatives to promote its cultivation. The trade and royalty collection of the species are also high from this region (DoF, 2014b).

1.3 Hypothesis

The present study has been based on the following hypotheses:

- There is a significant impact of habitat types and altitudes on the distribution, and phytochemical constituents of *Z. armatum.*

- The different plant parts of *Z. armatum* possess antioxidant and antimicrobial activities.
- Different treatments could break dormancy and induce germination process in seeds of *Z. armatum.*

1.4 Objectives

The specific objectives of this study are:

- To study the population status and distribution of *Z. armatum* in Salyan district.
- To study the seed attributes, germination behavior and vegetative propagation of *Z. armatum.*
- To analyze the phytochemical analysis and antimicrobial activities of leaves, fruits, and seeds of *Z. armatum* from different altitudes.
- To quantify the essential oil content in leaf and fruit of *Z. armatum* from different altitudes.

1.5 Limitations

The major limitations of the present work were

- Only the major *Z. armatum* production areas have been selected for the study. Limited resources and time did not allow covering entire range of the district for sampling and sample collection.
- The sample size for phytochemical studies was relatively small due to resource limitation.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Morphology and anatomy

Zanthoxylum armatum (Timur in Nepali) is an aromatic shrub up to 6 m high; branches: glabrous, usually armed with straight or slightly compressed, reddish brown stipular spines; leaves: imparipinnate, with 3-5 pairs of leaflets, elliptic-lanceolate, acuminate, base rounded or cuneate, sessile, margins mostly entire, with a large gland associated with each tooth; petiole and rachis: often winged between leaflets, at times bearing a spine at the point of insertion; inflorescence: terminal panicles on short lateral shoots; flowers: minute, polygamous, borne on short cymes; male flower: with 6-8 stamens, filaments 2 mm long arranged around globose pistillode; female flower: with 1-3 ovoid-subglobose carpels with two ovules attached to inner angle of axis; fruit: a small drupe, reddish, ovoid and with glandular wart that splits into two when ripe; fruits contain single rounded, shining black seeds, 2-3 mm in size (Grierson & Long, 1991; Nair & Nayar, 1997).

Barkatullah et al. (2014) studied the anatomical characters of the leaf, stem, bark, and fruit of *Z. armatum*. The internal structure of leaf shows a single layer of epidermis and palisade mesophyll. The vein-islets are squarish, elongated, polygonal, or irregular with forked and unforked vascular branches. The internal structure of leaf was unique with the complete absence of any kind of trichomes or any other appendages. Nine types of stomata were recorded, among them brachparatetracytic was the most frequent one. Special leaf epidermal feature, the stomatal cluster was also observed. Bark and fruit anatomy of *Z. armatum* showed different tissue arrangement. The seed was non endospermic and contained an elongated embryo. Oggero et al. (2016) also studied the anatomical characters of the leaf and stem of *Z. armatum* from central Argentina. The peculiar characteristics are the presence of secretory cavities, glandular trichomes and hypostomatic leaves. The transverse section of stem bark shows the presence of phellogen, phelloderm, cortex, phloem, and medullary rays (Jothi et al., 2019).

2.2 Distribution and ecology

Z. armatum is found in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), north-east India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, north and south Korea, north Vietnam, Taiwan, Lesser Sunda Islands, Philippines, Malaya peninsula and Sumatra (Nair & Nayar, 1997). In Nepal, it is distributed from west to east at an elevation range of 1000 to 2500 m asl in open places or in forest undergrowth (DPR, 2016). The distribution range of *Z. armatum* in Nepal based on herbarium specimens at National Herbarium (KATH) and Tribhuvan University Central Herbarium (TUCH) is presented in Figure 1.

The plant grows well in open pastures, wastelands, and secondary scrub forests with adequate rainfall. Moist areas with deep soils exposed to sun and degraded slopes, shrub lands, natural forests, and wastelands are the suitable habitat for *Z. armatum*. For the cultivation of this species, clay or loam soil with high organic matter is preferable. The flowering starts in April-May, fruiting in August-October and can be harvested from October to January. Flowering is greatly affected by hails and storm (Kunwar & Pokharel, 2012).

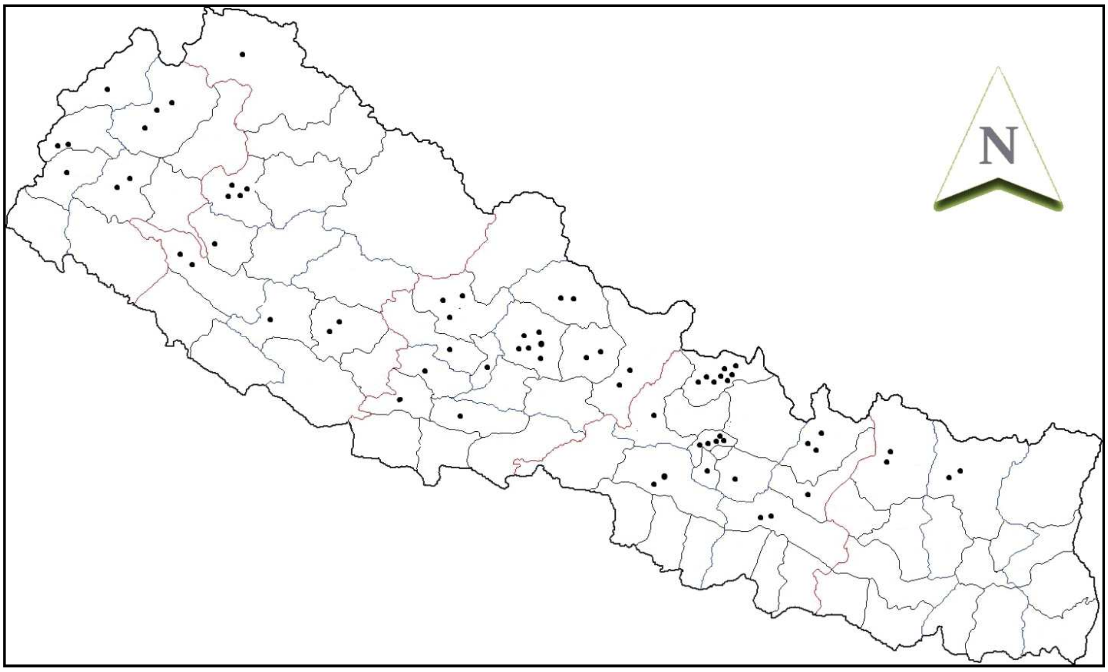


Figure 1: Distribution of *Z.armatum* in Nepal (based on herbarium specimens deposited at KATH & TUCH)

2.3 Propagation

The common method of propagation is through seeds but seed germination in *Z. armatum* is very low due to the presence of hard seed coat (Chadha, 1976) which might be a great hurdle for large scale production of plantlets. Natural regeneration usually occurs through seeds but the seeds undergo strong dormancy and may take few months to years for germination. Furthermore, the solitary seeds in the fruit also limit the quantity of seeds in *Z. armatum* (Singh & Rawat, 2017). Hence, vegetative propagation through stem cuttings could be a viable option for mass scale nursery production of quality planting materials of required genotypes.

It is also propagated from vegetative parts through soft wood cuttings. Stem cuttings may also be planted in the nursery during monsoon in July-August. Propagation from the stem cuttings is more suitable than seed sowing for *Z. armatum* because seed germination process is very slow in the species. Daudi et al. (2016) conducted propagation techniques in *Zanthoxylum alatum* through stem cuttings and seed germination. The cuttings treated with the concentration 5000 ppm of IBA exhibited better sprouting and rooting than the concentrations 4000 ppm and 6000 ppm of IBA. In an experiment conducted by Singh and Rawat (2017), the semi-hard wood (SHW) and hard-wood (HW) branch cuttings of *Z. armatum* exhibited greater success in root and shoot growth with IBA at 3000 and 4000 ppm concentrations whereas lower concentrations completely failed to root.

The plants are ready for harvest after around three years of plantation and the average annual yield of a five years plant is about 3.5 kg (Anonymous, 2011). The crop is generally free from any disease, insect or nematode attack, and physiological disorders. However, Tara et al. (2011) investigated 7 insect pests on *Z. armatum* that mostly caused the defoliation.

2.3.1 In-vitro propagation

In vitro propagation by tissue culture has been considered as an important tool for conservation and propagation of rare and threatened plants. An in-vitro propagation protocol for *Z. armatum* was developed by Purohit et al. (2016) through culture of nodal explants. Murashige and Skoog (MS) medium, supplemented with 12.0 µM 6-benzyl amino purine (BAP), 0.5 µM indole-3-acetic acid (IAA), and 0.5 µM gibberellic acid (GA_3), resulted in good shoot proliferation. One hundred percent rooting was achieved through pulse treatment of shoots on full-strength MS medium supplemented with 50.0 µM indole-3-butyric acid (IBA) for 12 h, followed by subsequent transfer to half strength MS medium without plant growth regulators (PGRs). The plant survival rate following acclimatization was 75%.

Purohit et al. (2019) developed an efficient protocol for high rate of adventitious shoot regeneration in *Z. armatum* through indirect organogenesis from callus induced from leaf explants and 100% rooting was also obtained. The explants were soaked for different time duration (12, 24 or 36 h) in liquid woody plant medium (WPM) supplemented with various concentrations (15.0, 25.0 or 50.0 µM) of thidiazuron (TDZ) and transferred for callus induction onto WPM supplemented with different concentrations of TDZ (2.0, 4.0, 6.0, 8.0 and 10.0 µM) either alone or in combination with varied concentrations (0.5, 1 and 1.5 µM) of naphthalene acetic acid (NAA). The callus clumps transferred onto WPM fortified with different concentrations (2.0-9.0 µM) of benzyl amino purine (BAP), indole-3-acetic acid (IAA, 1.0 µM) and gibberellic acid (GA_3, 0.5-3.0 µM) induced maximum shooting after 8 weeks of incubation. 80% survival rate after 60 days was recorded for the regenerated plants.

2.4 Vernacular information

Commonly called as Winged Prickly Ash or Prickly Ash, *Z. armatum* is a popular spice plant of Nepal. In Nepali, it is known as Timur. In different languages and regions, it is known by the following names (Manandhar, 2002).

Vernacular names among the various ethnic groups in Nepal

Bhojpuri	Timur
Chepang	Timpur, Upur
Danuwar	Timur, Tirkene
Gurung	Prumo
Lepcha	Sungrukung, Timbur
Limbu	Midimba, Warekpa
Newari	Tebu
Rai	Khakchan, Terkane

Sherpa	Yerma
Sunwar	Sekkren
Tamang	Prumo
Tharu	Timur
Tibetan	Gyer-ma

Z. armatum is known in other countries as following (Bachwani et al., 2012; Bharti & Bhusan, 2015)

Bengali	Gaira
Burmese	Gawra Kha Nan Nan, Teza Bo
Chinese	Ci Zhu Ye Hua Jiao, Qin Jiao (Taiwan), Zhu Ye Jiao
English	Bamboo-Leaved Prickly Ash, Nepal Pepper, Prickly Ash, Prickly Ash bark, Toothache Tree, Winged Prickly Ash,
German	Nepal pfeffer
Japanese	Fuyu Zanshou, Fuyu-Sansh
Korean	Gae San Cho
Thai	Mak Kak
Hindi	Tejphal, Tumru, Darmar, Trimal, Nepali dhaniya
Manipuri	Mukthrubi
Kannada	Dhiva, Tumburudu, Jimmi
Malayalam	Tumpunal, Tumpuni
Marathi	Chirphal, Naepaali dhane
Mizoram	Arhrikreh
Oriya	Arhrikreh, Ranabelli
Pashtu	Dambara
Sanskrit	Saurabha, Tejovati, Tumbaru, Vanaja
Tamil	Tumpunalu
Telugu	Gandhalu, Konda-kasimi, Konda, Kaasimanda
Urdu	Dambrary, Tamur

2.5 Ethnobotanical uses/importance

Different ethnic communities in Nepal have been extensively using *Z. armatum* in traditional indigenous medicinal practices. The different ethnomedicinal uses of *Z. armatum* in several types of diseases have been well documented by several ethnobotanical studies. The bark and seed are useful in fever, dyspepsia as aromatic, carminative, and tonic (DPR, 1970).

The powder of the seeds is taken with warm water in stomach problems. The seeds and fruits are used as leech repellant and also for curing tooth ache and cholera (Shrestha, 1985, 1988; Manandhar, 1986; Joshi & Edington, 1990; Joshi & Joshi, 2000; Balami et al., 2004). The fruits are used to treat cough, cold, fever and indigestion (DPR, 1983). The dried seeds can be used as an effective pesticide to control tiny insects on wheat plants. The paste of the leaves of *Artemisia vulgaris* and seed paste of *Z. armatum* are effective in repelling termites and other wood-eating insects from wooden structures and furniture (Turin, 2003). In the case of liver dysfunction, seed powder with lukewarm water is taken orally twice a day for a week (Rai & Pokharel, 2006). The fruits, bark and thorns are used as fish poison (Joshi, 2004; Kunwar et al., 2009, 2013; Malla et al., 2014). In toothache, the seeds are chewed to relieve pain, they are also added in vegetables for detoxification. The seeds are fermented and the steam (liquid) collected afterwards is taken twice daily to cure tuberculosis. The seeds cooked with water, wheat flour and oil are taken during edema (Subedi, 2017).

It is a valuable plant with several pharmacological, biological and medicinal properties. The fruits are used to treat dyspepsia, stomachache, toothaches and as a carminative, also used as a flavour and condiments (DPR, 1970; Arshad & Ahmad, 2005; Iqbal & Hamayun, 2005; Abbasi et al., 2013). The bark, branches, seeds and fruits are commonly used as anthelmintic, stomachic, and carminatives, and the branches are also used as toothbrushes. Berries and the decoctions of the fruit are used to treat various ailments in Nepal like abdominal pain, skin illnesses, rheumatism, diabetes, asthma and cholera (Singh et al., 2016). Fresh fruits are used as seasonings as well as in pickles (Joshi & Joshi, 2000; Manandhar, 2002; Balami, 2004; Malla et al., 2014).

The seeds and fruits of *Z. armatum* are widely used to expel roundworms, they are also used as a tonic in indigestion, fever, stomachache, toothache (Kalia et al., 1999; Rajbhandari et al., 2001; Tiwary et al., 2007; Uprety et al., 2010; Verma & Khosa, 2012). The fruits may contain essential oil ranging from 2-7.6%, with an average output of 5%, depending on the environmental conditions (Manandhar, 2002; Phuyal et al., 2020c). The essential oil is used as an antiseptic, anticatarrhal, deodorant and antidiarrheal (Neetu et al., 2001; Bhattacharya & Zaman, 2009; Bhattacharya et al., 2010). The fruits are commonly used by the different pharmaceutical industries to make various types of toothpaste and gel. Table 1 lists the several ethnomedicinal uses of *Z. armatum*.

Table 1: Different ethno-medicinal uses of *Z. armatum*

SN	Ailments/use	Parts used	References
1	Abdominal pain	Fruit decoction	Rajbhandari et al. (2001), Rijal (2011)
2	Alcohol preparation	Fruits	Kala et al. (2005)
3	Anthelmintic	Fruits and seeds	Tiwary et al. (2007), Ramanujan and Ratha (2008), Verma and Khosa (2010)
4	Antipyretic	Fruits	Akhtar et al. (2013)
5	Antispasmodic	Fruits	Baral and Kurmi (2006)
6	Appetizer	Fruits	Balami (2004), Kala (2005)
7	Aromatic tonic	Seeds	Alamgeer et al. (2013)
8	Asthma	Fruits	Kirtikar and Basu (1993), Kanjilal (1997), Baral and Kurmi (2006)
9	Bronchitis	Stem, bark, fruits, seeds	Kirtikar and Basu (1993), Kanjilal (1997), Naeemuddin et al. (2010)
10	Carminative	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2005), Baral and Kurmi (2006), Tiwary et al. (2007), Abbasi et al. (2010a), Verma and Khosa (2010), Alamgeer et al. (2013), DPR (2016)
11	Chest infection: Fruit powder mixed with *Mentha* sp. and table salt is taken with boiled egg	Fruits	Islam et al. (2009)
12	Cholera	Fruits, bark	Joshi and Edington (1990), Baral and Kurmi (2006), Abbasi et al. (2010 a), Rijal (2011), Alamgeer et al. (2013), Malla et al.(2014), DPR (2016)
13	Cold and cough	Fruits	Joshi (2004), Kala (2005), Gewali and Awale (2008), Bhatt and Chhetri (2009), Kunwar et al. (2009), Singh et al. (2011)

14	Condiments and flavoring agents	Fruits	Joshi and Joshi (2000), Arshad and Ahmad (2005), Balami (2004), Kala et al. (2005), Abbasi et al. (2010 b), Malla et al. (2014)
15	Depression	Seeds	Zaidi et al. (2009)
16	Diabetes	Fruit	Baral and Kurmi (2006), Khan and Yadava (2010)
18	Diuretic	Bark	CSIR (2005)
19	Dizziness	Fruit pickle	Gewali and Awale (2008)
20	Dysentery	Seeds	DPR (1983), Subedi (2017)
21	Dyspepsia	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2005), Tiwary et al. (2007), Verma and Khosa (2010), DPR (2016)
22	Fever	Bark and seeds	Kala (2005), Tiwary et al. (2007), Gewali and Awale (2008), Verma and Khosa (2010), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016)
23	Fish poison	Fruits, thorns, branches	DPR (1983), Rasaily (2003), Kunwar et al. (2009), Malla et al. (2014), DPR (2016), Tamang et al. (2017)
24	Flatulence	Seed	Zaidi et al. (2009)
25	Gastrointestinal disorders	Fruits	Joshi and Joshi (2000), Shrestha and Dhillion (2003), Naeemuddin et al. (2010), Uprety et al. (2010), Singh and Singh (2011), Abbasi et al. (2013)
26	Gum diseases	Young shoots, fruits	Hamayun (2003), Ahmed et al. (2004), Arshad and Ahmad (2005), Iqbal and Hamayun, 2005), Kala et al. (2005)
27	Headache	Fruit pickle	Gewali and Awale (2008)
28	High altitude sickness	Fruit pickle	Gewali and Awale (2008)
29	Houseflies repellent	Fruits	Gaur (1999)
30	Indigestion	Fruits, seeds	DPR (1983), Kirtikar and Basu (1993), Kanjilal (1997), Balami (2004), Rajbhandary and Ranjitkar (2006), Zaidi et al. (2009)
32	Insecticides/pesticides	Branches	Subedi (2017)
33	Leech repellent	Fruits	Balami (2004), Kala et al. (2005), Manandhar (1986)
34	Limb numbness	Fruit pickle	Gewali and Awale (2008)
35	Piles	Fruits	Abbasi et al. (2013)
36	Piscicide (Catching fishes)	Root, fruit, bark, leaves	Mathur et al. (1961), Zaidi et al. (2009)
37	Pneumonia (Cattles: sheep)	Aerial parts	Sindhu et al. (2010)
38	Rheumatism	Fruits	Kirtikar and Basu (1993), Kanjilal (1997), Baral and Kurmi (2006)
39	Skin diseases	Fruits	Baral and Kurmi (2006)
40	Stomach ache	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2005), Joshi (2004), Tiwary et al. (2007), Gewali and Awale (2008), Verma and Khosa (2010), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016)

41	Tick infestation (Cattles: buffalo)	Aerial parts	Sindhu et al. (2010)
42	Timur hag (soup): Made from dried fruits is consumed to keep the body warm.	Fruits	Kala et al. (2005)
43	Tonsillitis	Fruit pickle	Gewali and Awale (2008)
44	Tooth brush	Young shoots/branches	Hamayun (2003), Ahmed et al. (2004), Arshad and Ahmad (2005), Kala et al. (2005), Abbasi et al. (2010)
45	Toothache	Fruit/seeds	Kirtikar and Basu (1993), Kanjilal (1997), Arshad and Ahmad (2005), Kala et al. (2005), Kunwar et al. (2009), Kunwar et al. (2013), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016), Tamang et al. (2017)
46	Tuberculosis	Seeds	Gurung (2002), Subedi (2017)
47	Varicose veins	Stem bark, fruit, seed	Kirtikar and Basu (1993), Kanjilal (1997)
48	Vermicide	Fruits	Kala et al. (2005)
49	Walking sticks	Wood	Arshad and Ahmad (2005), Iqbal and Hamayun (2005)

2.6 Phytochemistry

Various phytochemical constituents like terpenoids, flavonoids, alkaloids, phenolics, lignins, coumarins, glycosides and benzoids, steroids, fatty acids, alkenoic acids, amino acids have been extracted from different parts of *Z. armatum*, i.e., seed, leaf, fruit, root and bark (Li et al., 2006; Tiwary et al., 2007; Negi et al., 2011; Waheed et al., 2011; Bachwani et al., 2012; Negi et al., 2012; Joshi & Gyawali, 2012; Barkatullah et al., 2013; Brijwal et al., 2013; Bharti & Bhusan, 2015; Kayat et al., 2016; Singh et al., 2016).

Monoterpenes like linalool and limonene are the major constituents of the essential oil. Seeds contain hydroxylic (*4Z*) enolic acid and various volatile compounds (Ahmad et al., 1993). The various alkaloids, flavonoids, flavonol glycosides, lignins, phenolics, sterols, terpenoids, fatty acids, alkenic acids, amino acids, various aromatic and volatile and a variety of other compinents have been recognized and extracted from *Z. armatum* essential oil in good quantity (Waheed et al., 2011; Bhatt et al., 2018). 72 different compounds have been identified in the essential oil of stem bark of *Z. armatum* with the major constituents as α-Pinene, germacrene-D, E-caryophyllene, α-cadinol, 2-undecanone, limonene, 2-tridecanone, β-myrcene, and α-humulene (Dhami et al., 2019).

Kayat et al. (2016) enlisted 36 different components in the hexane extract of fruits of *Z. armatum* through the gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the essential oil extracted by hydro-distillation identified the main components in the oil as β-linalool (53.05%), bergamot mint oil (12.73%), α-limonene diepoxide (11.39%), α-pinene (4.08%), β-Myrcene (3.69%) and d-limonene (3.10%) (Barkatullah et al., 2013).

Several coumarins and alkaloids have also been identified and isolated from the different parts of *Z. armatum*: berberine (stem-bark), alkaloids (g-fagarine, b-fagarine, magnoflorine, laurifoline, nitidine, chelerythrine, tambetarine and candicine), coumarins (xanthyletin, zanthoxyletin, alloxanthyletin), and resin, tannin and volatile oil (Bachwani et al., 2012; Joshi & Gyawali, 2012).

The bark of *Z. armatum* yielded a novel amide called armatamide, as well as two lignans fargesin and asarinin. Lupeol, α and β-amyrins, and β-sitosterol-beta-D-glucoside have also been extracted from the bark. A novel flavonoid glycoside has also been isolated from the alcoholic extract of the stem bark (Sati et al., 2011a). Table 2 enlists the different secondary components found in *Z. armatum.*

Table 2: Secondary compounds present in *Z. armatum*

SN	Class	Compound	Plant parts	References
Terpenoids				
1		(allo-Aromadendrene	Seed	Ahmad et al. (1993)
2		(*E*)-Carveol	Seed	Tiwary et al. (2007)
3		(*E*)-Linalool oxide	Seed	Tiwary et al. (2007)
4		(E)-Nerolidol	Seed	Tiwary et al. (2007)
5		(*Z*)-Linalool oxide	Seed	Tiwary et al. (2007)
6		(*Z*)-Pinene hydrate	Seed	Tiwary et al. (2007)
7		(*Z*)-Sabinene hydrate	Seed	Tiwary et al. (2007)
8		1,8-Cineole	Seed	Ahmad et al. (1993)
9		1-α-Phellandrene	Seed	Perry (1980)
10		Bornyl acetate	Leaf oil	Negi et al. (2012)
11		Camphene	Seed, leaf oil	Ahmad et al. (1993), Negi et al. (2012), Barkatullah et al. (2013)
12		Carvone	Seed	Ahmad et al. (1993), Singh et al. (2013)
13		Cis-β-Ocimene	Seed, leaf oil	Ahmad et al. (1993), Barkatullah et al. (2013)
14		Citral	Dry fruit	CSIR (1976)
15		Citronellal	Seed	Yoshihito et al. (2000)
16		Citronellol	Seed	Yoshihito et al. (2000)

17	cymene	Leaf oil	Negi et al. (2012)
18	d-Limonene	Leaf oil	Barkatullah et al. (2013)
19	Eucalyptol	fruit	Kayat et al. (2016)
20	Geraniol	Dry fruit	CSIR (1976)
21	Limonene	Seed/leaf	Ahmad et al. (1993), Negi et al. (2012), Singh et al. (2013)
22	Linalool	Seed/leaf oil	Ahmad et al. (1993), Negi et al. (2012), Singh et al. (2013)
23	Linanyl acetate	Dry fruit	CSIR (1976)
24	Lupeol	Bark	Kalia et al. (1999)
25	Myrcene	Seed	Ahmad et al. (1993), Singh et al. (2013)
26	Nerol	Seed	Tiwary et al. (2007)
27	p-Cymene	Seed	Ahmad et al. (1993), Singh et al. (2013)
28	Piperitone	Seed	Tiwary et al. (2007), Singh et al. (2013)
29	Sabinene	Seed	Tiwary et al. (2007), Barkatullah et al. (2013), Singh et al. (2013)
30	Tagetonol	Seed	Ahmad et al. (1993)
31	Terpinen-4-ol	Seed	Tiwary et al. (2007)
32	Terpinolene	Leaf oil	Barkatullah et al. (2013)
33	*trans*-caryophyllene	Leaf oil	Negi et al. (2012)
34	*Trans*-β-Ocimene	Leaf oil	Barkatullah et al. (2013)
35	α-Amyrins	Bark	Tiwary et al. (2007)
36	α-Caryophyllene	Seed	Ahmad et al. (1993)
37	α-Copaene	Leaf oil	Negi et al. (2012)
38	α-Fenchol	Seed	Ahmad et al. (1993)
39	α-phellandrene	Leaf oil	Barkatullah et al. (2013)
40	α-Pinene	Seed	Tiwary et al. (2007), Barkatullah et al. (2013), Singh et al. (2013)
41	α-Terpinene	Seed	Tiwary et al. (2007)
42	α-Terpineol	Seed	Ahmad et al. (1993), Singh et al. (2013)
43	α-Terpinolene	Leaf oil	Negi et al. (2012)
44	α-Thujene	Seed	Ahmad et al. (1993), Singh et al. (2013)
45	α-Thujone	Seed	Tiwary et al. (2007)
46	β-Amyrins	Bark	Kalia et al. (1999)
47	β-Amyrone	Bark	Li et al. (1996)
48	β-Cymene	Leaf oil	Luong et al. (2003)
49	β-Myrcene	Leaf oil	Barkatullah et al. (2013)
50	β-Ocimene	Leaf oil	Negi et al. (2012)
51	β-Phellandrene	Seed	Neetu et al. (2001), Singh et al. (2013), Shah (1991)
52	β-Pinene	Seed	Tiwary et al. (2007), Singh et al. (2013)
53	β-Terpeneol	Leaf oil	Luong et al. (2003)
54	γ-Terpinene	Seed/Leaf oil	Ahmad et al. (1993), Negi et al. (2012)

Flavonoids			
55	3,5,3’,4’-Tetrahydroxy-7, 8-dimethoxy flavone	Seed	Ramidi and Ali (1999)
56	3,5,3’-Trihydroxy-6,7-dimethoxy-4’-(7”-hydroxygeranyl-1”-ether) flavone	Seed	Ramidi and Ali (1999)
57	3,5-Diactyltambulin	Bark	Li et al. (2006)
58	Catechin	Leaf	Bhatt et al. (2016)
59	Hesperidine	Leaf	Bhatt et al. (2016)
60	Isovitexin	Leaf	Bhatt et al. (2016)
61	Kaempferol	Bark	Li et al. (2006)
62	Tambuletin	Seed	Ramidi and Ali (1999), Nair et al.1982
63	Tambulin		Muller et al. (1996)
64	Vitexin	Leaf	Bhatt et al. (2016)
Alkaloids			
65	Berberine	Bark	Ranawat et al. (2010)
66	b-Fagarine	Bark	Vashist et al. (2016)
67	Chelerythrine	Bark	Vashist et al. (2016)
68	Dictamnine	Root	Perry (1980)
69	g-Fagarine	Bark	Vashist et al. (2016)
70	Magnoflorine	Root	Perry (1980)
71	Nevadensin	Seed oil	Ramidi et al. (1998)
72	Skimmianine	Bark	Li et al. (1996)
73	Zanthonitrile	Bark	Li et al. (1996)
Lignins			
74	Asarinin	Bark leaf	Ranawat et al. (2010), Bhatt et al. (2016)
75	Eudesmin	Leaf	Bhatt et al. 2016
76	Fargesin	Bark leaf	Kalia et al. (1999), Singh et al. (2013), Bhatt et al. (2016)
77	Kobusin	Leaf	Bhatt et al. (2016)
79	*L*-Asarinin	Bark	Li et al. (1996), Rao and Singh (1994)
81	*L*-Planinin	Bark	Li et al. (1996), Rao and Singh, 1994
80	*L*-Sesamin	Bark	Li et al. (1996), Rao and Singh, 1994
82	Magnolin		Neetu et al. (2001)
78	Planispine-A	Leaf	Bhatt et al. (2016)
83	Sesamin	Leaf/Bark	Muller et al. (1996), Bhatt et al. (2016), Vashist et al. (2016)
Sterols and Steroids			
84	Stigmasta-5-en-3β-D-Glucopyranoside	Seed	Akhtar et al. (2009)
85	β-Daucosterol	Bark	Li et al. (1996)
86	β-Sitosterol	Bark	Li et al. (1996), Vashist et al. (2016)
87	β-Sitosterol-β-D-Glucoside	Bark	Ranawat et al. (2010)

Amides			
88	Armatamide	Bark	Kalia et al. (1999)
89	Hydroxyl-α-sanshooil	Pericarp	Khare (2007)
90	α-Sanshool	Leaf	Bhatt et al. (2016)
Coumarins			
91	Alloxanthtin	Bark	Vashist et al. (2016)
92	Bergapten	Bark	Li et al. (1996)
93	p-Soralen	leaf	Bhatt et al. (2016)
94	Umbelliferone	Bark	Li et al. (1996)
95	Xanthyletin	Bark	Vashist et al. (2016)
96	Zanthoxyletin	Bark	Vashist et al. (2016)
Carbonyl Compounds			
97	2-Tridecanone	Leaf-oil	Luong et al. (2003)
98	Cuminaldehyde	Seed	Tiwary et al. (2007)
99	Cuminol	Bark/Fruit	Kayat et al. (2016)
100	Phellandral	Seed	Tiwary et al. (2007), Singh et al. (2013)
101	Undecan-2-one	Aerial parts	Weyerstahl et al. (1999)
Aromatic compounds			
102	(*E*)-Methyl cinnamate	Seed	Tiwary et al. (2007), Singh et al. (2013)
103	(*Z*)-Methyl cinnamate	Seed	Neetu et al. (2001)
104	1-Hydroxy-6,13-anthraquinone	Seed	Akhtar et al. (2009)
105	1-Methoxy-1,6,3-anthraquinone	Seed	Akhtar et al. (2009)
106	2-Hydroxy-4-methoxy benzoic acid	Seed	Akhtar et al. (2009)
107	2-Hydroxybenzoic acid	Seed	Akhtar et al. (2009)
108	3,5,6,7-Tetrahydroxy-3’,4’-dimethoxyflavone-5-β-(D)-xylopyranoside	Seed	Akhtar et al. (2009)
109	3-Methoxy-11-hydroxy-6,8-dimethylcarboxylate biphenyl	Seed	Akhtar et al. (2009)
110	Methyl cinnamate	Seed/fruit	Ahmad et al. (1993), Kayat et al. (2016)
111	Monoterpenetriol-3,7-dimethyl-1-octane-3,6,7-triol	Seed oil	Ramidi and Ali (1998)
112	*trans*-Cinnamic acid	Seed oil	Ramidi and Ali (1998)
113	Vanillic acid	Bark	Li et al. (1996)
Other Aliphatic Compounds			
114	2,6-Dimethyl-1,3,5,7-octatetraene	Leaf oil	Luong et al. (2003)
115	6-Hydroxynonadec-(*4Z*)-enoic acid	Seed	Ahmad et al. (1993)
116	6-Methylheptanoic	Seed	Yoshihito et al. (2000)

117	7-Hydroxy-7-vinylhexadec-(*4Z*)-enoic acid	Seed	Ahmad et al. (1993)
118	8-Hydroxypentadec-(*4Z*)-enoic acid	Seed	Ahmad et al. (1993)
119	8-Methylnonanoic acid	Seed	Yoshihito et al. (2000)
120	*cis*-10-Octadecenoic acid		Kokate et al. (2001)
121	*cis*-9,12,15-Octadecatrienoic acid		Kokate et al. (2001)
122	*cis*-9,12-Octadecadienoic acid		Venkatachalam et al. (1996)
123	*cis*-9-Hexa-decenoic	Seed oil	Ahmad et al. (1980)
124	Hexadec-(*4Z*)-enoic acid	Seed	Ahmad et al. (1993)
125	Methyl palmitate	Seed	Tiwary et al. (2007)
126	Oleic acid	Seed	Tiwary et al. (2007), Singh et al. (2013)
127	Palmitic acid	Seed/Fruit	Tiwary et al. (2007), Kayat et al. (2016), Singh et al. (2013)
128	Palmitolic acid	Seed	Tiwary et al. (2007)

2.6.1 Essential oil

The main components of the oil are oleic acid, palmitic acid, linoleic acid methyl ester, limonene, and linalool (Shah, 1991; Negi et al., 2012; Kayat et al., 2016). The essential oil in fruits of *Z. armatum* consists of linalool, methyl cinnamate, limonene, myrcene, sabinene and terpinen-4-ol; likewise, the main components of essential oil in leaf are linalool, limonene, undecan-2-one, tridecan-2-one, myrcene, cinnamate (*E*)-methyl and α-bergamotene. The essential oil from the seeds consists entirely over 85% of the hydrocarbon 1-α-phellandrene and a small quantity of linalool and an unidentified sesquiterpene (Waheed et al., 2011). The stem and root contain α-amyrin, α-sitosterol, L-asarinin, L-planinin, and zanthobungeanine (Verma & Khosa, 2012). Bark yields a bitter crystalline principle, identical to berberine, dictamnine, volatile oil and resin. The carpels yield a volatile oil, resin, yellow acid principle, and crystalline solid body, xanthoxylin (CSIR, 2005). A total of 17 components were identified from the essential oil of leaves and 13 components from the essential oil in fruits of *Z. armatum* (Phuyal et al., 2019b, 2020c).

Various studies have proved that the phyto-constituents of plants are affected by a variety of factors such as edaphic, climatic, topography, elevation and also an interaction of various factors (Loziene & Venskutonis, 2005; Rahimmalek et al., 2009). The composition of the essential oil might be affected by different factors like the time of collection, season, types of habitat, management procedures thinning, pruning, trimming, and harvesting process. Elevation and soil chemistry significantly affect the essential oil yield and composition of *Z. armatum* (Phuyal et al., 2019b, 2020c).

2.7 Biological activities

Different studies have shown that various extracts (dichloromethane, acetone, aqueous, ethanol, methanol, petroleum ether, etc.) of *Z. armatum* possesses different pharmacological and biological activities like larvicidal, antifungal, hepato-protective, keratolytic, antiviral, antiprotozoal, pesticidal/insecticidal, antibacterial, anthelminthic, allelopathic.

2.7.1 Antibacterial activities

The essential oil and the extracts of leaf, fruit, seed and bark of *Z. armatum* have been found to exhibit considerable amount of antibacterial activities in different bacterial strains. The aqueous extract of the fruit exhibited significant antibacterial activity against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli, Salmonella typhi*) (Zaidi et al., 2009; Guleria et al., 2013). The essential oil in leaf of *Z. armatum* showed inhibitory effect against different strains of bacteria *Micrococcus leutus, Escherichia coli, Staphylococcus aureus, Pasteurella multocida, Pseudomonas aeruginosa, Bacillus subtilis* and *Streptococcus viridans*. *B. subtilis* and *S. viridans* were more susceptible as compared to other strains tested (Barkatullah et al., 2013). The ethanol extracts of *Z. armatum* leaves were found to be more effective than hot water extracts against different pathogens *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus*, and *Salmonella typhimurium* (Akbar et al., 2014).

The crude methanol extracts of the bark, fruit and seed of *Z. armatum* showed considerable antibacterial properties against several infectious pathogens (Bacillus *subtilis*, *Escherichia faecalis*, MRSA, *Staphylococcus aureus* and *Staphylococcus epidermidis*) causing several diseases in humans. (Phuyal et al., 2020b).

2.7.2 Antifungal activities

Several in-vitro antifungal studies verified the antimycotic potential of *Z. armatum* against various fungal strains: *Aspergillus parasiticus* (Dube et al., 1990), *Candida* *albicans*, *Cryptococcus neoformans* (Goel et al., 2002), *Microsporum gypseum*, *Trichophyton mentagrophytes* (Dikshit et al., 1986), *Sclerotium rolfsii*, *Rhizoctonia bataticola* (Sharma et al., 2009), *Alternaria brassicicola* (Parajuli et al., 2005), *Bipolaris sorokiniana* (Manandhar & Tiwari, 2005).

2.7.3 Cytotoxic and phytotoxic effects

Barkatullah et al. (2013) carried out Brine shrimp toxicity bioassay (a preliminary study for the detection and development of anti-cancer drugs) to investigate the preliminary cytotoxic potential of *Z. armatum*. The crude ethanolic and n-hexane extract of leaf, bark, fruit, and essential oil of leaf exhibited outstanding mortality rate (100%) at a dose of 1000 μg/mL. In the same experiment, these extracts also showed significant phyto inhibition at higher dose.

Similarly, in another experiment, the ethanolic extract and subsequent fractions of the fruits of *Z. armatum* were evaluated for Brine shrimp lethality test using concentrations 5, 50, and 500 mg/mL. The crude extract exhibited significant toxicity with LC_{50} value of 6.66 ± 1.1 mg/mL (Alam & Saqib, 2017). In another experiment, different concentrations of the crude extract, n-hexane, chloroform and aqueous-methanolic fractions of fruits of *Z. armatum* were evaluated for in-vitro phytotoxic activity against *Lemna minor* L. Remarkable phytotoxicity was observed at the highest concentration causing complete inhibition of the plant growth (Alam & Saqib, 2017).

2.7.4 Antioxidant activity

The antioxidant potential of *Z. armatum* has been demonstrated by several in-vitro/in-silico antioxidant experiments by different authors (Batool et al., 2010; Upadhyaya & Kumar, 2010; Negi et al., 2012; Prakash et al., 2012; Barkatullah et al., 2013; Guleria et al., 2013; Kanwal et al., 2015; Karmakar et al., 2015). However, several scientific journals are skeptical about such in-vitro/in-silico studies. All plants possess antioxidant activity so it is insignificant unless evidenced by in-vivo studies (Gafner, 2018).

Karmakar et al. (2015) carried out in-vivo antioxidant assay of methanol extract of leaves of *Z. armatum* in male Wister albino rats. The oral administration of the extract significantly enhanced the activities of antioxidant enzymes (SOD, CAT and GSH) in the treated animals.

2.7.5 Hepatoprotective activities

Several studies have shown the hepatoprotective activities of *Z. armatum* (Ranawat et al., 2010; Verma & Khosa, 2012). The ethanol extract of leaves was used to study the in-vivo hepatoprotective effect against Carbon tetrachloride (CCl_4) induced liver damage in Wister albino rats. CCl_4 intoxication in normal rats elevated the levels of SGOT, SGPT, ALKP, SBLN and liver inflammation were observed significantly indicating acute hepatocellular damage and biliary obstruction. Oral administration of the extract showed a significant decrease in all the SGOT, SGPT, ALKP, SBLN levels and liver inflammation, by normalizing the elevated levels of the hepatic enzymes. The results obtained support the use of this plant for the treatment of hepatitis in oriental traditional medicine (Verma & Khosa, 2012).

2.7.6 Anti-inflammatory activities

In-vivo anti-inflammatory activity of ethanolic extract of stem bark of *Z. armatum* was evaluated by carrageenan induced paw edema method in male Wister rats (Sati et al., 2011b). The degree of inhibition was 19.12%, at 250 mg/kg dose after 4 hours of administration. Ibuprofen (10 mg/kg of body weight) was used as positive control. Fruits extract also showed considerable anti-inflammatory activity with the inhibition of carrageenan that induced paw edema in Wister rats (Alam et al., 2020). It has analgesic activity also due to the presence of lignan components (Guo et al., 2011).

2.7.7 Antispasmodic effect

The crude extract of *Z. armatum* was used to study the in-vivo spasmolytic effects against castor-oil-induced diarrhea in mice. Pre-treatment of animals with the extract showed 20% protection from diarrhea at 300 mg/kg and 60% protection at 1000 mg/kg (Gilani et al., 2010). In another experiment, the essential oil of leaves of *Z. armatum* was evaluated for probable antidiarrheal effect on spontaneous and potassium chloride induced contracted smooth muscle of the isolated rabbit jejunum. The extracts relaxed the contracted muscle, suggesting the possible mode of action of this plant as either blocking the release of stored calcium from the sarcoplasmic reticulum or blocking the calcium channel (Barkatullah et al., 2013).

2.7.8 Anti-cancer activity

The compounds ZP-amide A, C, D, E, hydroxyl α and β sanshool, and Timuramide A, B, C and D present in the methanolic extract of fruit of *Z. armatum* inhibited the growth of mouse glioma cells that were deficient of tumor suppressor genes NF1 homolog-Nf1 (Devkota et al., 2013). Similarly the compounds Apigenin and Kaempferol-7-O-glucoside, present in the ethyl acetate extract of dried root of *Z. armatum* were found to possess anti-cancerous property against A-549, MIA-PaCa, MCF-7, and CACO2 cancer colon cell lines (Mukhija et al., 2015). In a study by Singh et al., 2015, the methanol extract of leaf of *Z. armatum* induced apoptosis in cervical cancer cell lines (HeLA) at IC50 (60 μg/mL) through Caspase 3-independent and extracellular signal-regulated kinases (ERK)-dependent mitogen activated protein kinases (MAPK) apoptosis pathways. In another study using the mechanism of apoptosis, Alam et al., 2017 demonstrated that the methanol extracts of leaves, fruit, and bark of *Z. armatum* have a potential of exerting a cytotoxic effect on breast (MDA-MB-468, MCF-71) and colorectal cancer (Caco-21) cell lines. Tambulin, a flavonoid isolated from the fruit exhibited anti-proliferative action on certain cancer cell lines like MCF-7, WRL-68, COLO-205, MDAMB-231, with an IC_{50} ranging from 37.96 to 48.7 μg/mL (Nozaki et al., 2016).

2.7.9 Antiviral/antiprotozoal activities

The methanol and aqueous extract of the dried fruits of *Z. armatum* showed inhibition of HSV-1/Vero cells, Influenza A/MDCK cells (Rajbhandari et al., 2009), Japanese B encephalitis virus (Goel et al., 2002). The aqueous extract of the leaves showed antiprotozoal effect on *Giardia lamblia* and *Plasmodium berphei* (Goel et al., 2002).

2.7.10 Mosquito repellant

In combination, seed oil of *Z. armatum*, vanillin and fruit oil of *Z. piperitum* have been able to enhance repellent activity against female *Aedes aegypti* and the effect was compared with N, N diethyl-3 methyl benzamide (DEET) repellent (Kwon et al., 2011). The essential oil was found to significantly repel mosquitoes at 0.57 mg/cm^2 concentrations *viz*. 445 min in mustard oil and 404 min in coconut oil base (Das et al., 1999).

Tiwary et al. (2007) studied in-vitro larvicidal activities of essential oil of the seeds of *Z. armatum* against three species of mosquitoes: *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus. Culex quinquefasciatus* was the most sensitive with LC_{50} and followed by *Aedes aegypti, Anopheles stephensi* with LC_{50} values in the range of 54-58 ppm. Similar larvicidal effects were also studied against *Culex pipiens, Culex quinquefasciatus* by Peng et al. (2009) and against *Aedes albopictus* and *Culex pipiens* by Zhang et al. (2010).

2.7.11 Allelopathic effects

Different concentrations (i.e., 2, 5, and 10%) of aqueous extracts of leaf, bark, and fruit pulp of *Z. armatum* were found to have significant allelopathic effect on some important winter field crops (*Triticum aestivum, Hordeum vulgare, Brassica campestris* and *Lens culinaris*). A study carried out in Garhwal Himalaya region of India showed significant effects of these bioassays on germination and growth of all the test crops. In an average, 83.6%, 52.6% and 84.9% reduction in radicle growth was observed in *Triticum aestivum, Hordeum vulgare* and *Lens culinaris,* respectively (Singh et al., 2007).

2.7.12 Soothing effect on skin

The lipophilic extract of *Z. armatum* with alcohol gives remarkable soothing effect based on inhibition of sensory irritation from sun bathing, shaving depilation, insect bites and chemical treatment (Guglielmini & Cristoni, 2002).

2.8 Trade

The tradition of collection and sale of *Z. armatum* in Nepal has a long history and can be dated back to the early 1980s, when the trade started with India, before which it was used by the rural communities for domestic purposes (Malla et al., 1993). Historically, the rural people traded different medicinal plants including *Z. armatum* as a source of their income (Manandhar, 1986; Kunwar et al., 2018). There was a social mechanism of exchange and distribution of Timur, and the fruits were bartered for grains, millets, pulses, and other items (Kala, 2005). With the increasing demand of the fruits by different pharmaceutical and other companies, the value of this plant has raised surprisingly (DoF, 2014a). The rural people have also started to commercially cultivate the plants in their farmyards, as a secondary source of income.

The market price of *Z. armatum* has been increasing every year. The price per kg was NRs. 1.8 in 1980s and Rs. 44 in 2007 (Anonymous, 2011). According to the price list provided by ANSAB (http://www.ansab.org/mis/price-list/), the market price of the *Z. armatum* fruits was NRs. 100/- per kg in 2010, 265/- in 2011, 235/- in 2012, 300/- in 2014, 250/- in 2015, 340/- in 2016, 600/- in 2017 and 2018 and reached a maximum of NRs. 1100/- per kg in 2019 with a sharp decline to Nrs. 375/- in 2020. The main market of Timur is India, where there is a high demand of the dried fruits (Edwards, 1996; Hertog & Wiersum, 2000). Only a small portion is processed inside Nepal while most of the quantity (more than 90%) is exported to India in raw form, where it is further processed and used for different pharmaceutical and industrial purposes (DoF, 2014a). Several Ayurved companies and other industries inside Nepal also consume some amount of *Z. armatum* in their various products. Small quantity of *Zanthoxylum* oil is also exported to some European countries. China is another probable country for market expansion as the fruits are extensively used there as a flavoring agent in food (Anonymous, 2011). It is one of the many other MAPs that are traded from Nepal to China and the commerce is not only limited to just traditional users in Tibet but also to the mega-cities of Central China (He et al., 2018).

As it requires less fertile soil, it can be cultivated around croplands with very less impact to the cultivated crops, in the barren lands and different forests lands. It is regarded as a prioritized commodity with huge export possibilities not only to the Indian market but also to the lucrative European market, where there is a high demand of the essential oil obtained from the fruit. According to a report published by the Department of Forest, Government of Nepal, the total revenue collected from the sale of *Z. armatum* in 2011 was NRs. 281,568, with a total production of 17,896 kg while it was 240,206 kg in 2013 amounting to NRs. 1,921,648/-, 72290 kg with a total revenue of 578320/- in 2014 and in 2015 it was 418,179 kg with the royalty of NRs. 3,345,432 (DoF, 2014b, 2015). The amount was 355,500 kg with total revenue of NRs. 2,834,000/- in Salyan district alone (DFO, 2015).

Because of its wide commercial potential, farmers of not only Salyan but also neighboring districts have started to cultivate *Z. armatum* in their farms, fallow lands, and patches of forests. Because it has become a good source of income for farming families, *Zanthoxylum* cultivation has received priority from all the three levels of government ie. local, provincial and federal. The Karnali Provincial government has also proposed a *Z. armatum* Year Program in its hilly districts including Salyan. The federal government announced that it would celebrate fiscal year 2019-2020 as the year of *Z. armatum* plantation. Furthermore, various herb-based industries have been proposed for Salyan, Rukum (East, West), and Jajarkot districts of Karnali Province. Chhatreshwori village of Salyan has been designated as a pocket area for *Z. armatum* farming. The market demand and purchase price for *Z. armatum* are extremely high. By growing and selling it, farmers can make regular, decent income. *Z. armatum* farming has become an enterprise that is helping reduce the migration of youths to India and the Gulf countries. Therefore, it can be developed as an alternative cash crop to increase the income of small local farmers, thereby improving the livelihoods, which would ultimately help to reduce rural poverty. This endeavor howerver needs collegial efforts from various governmental, non-governmental, public, and private organizations

Despite the huge possibilities of *Zanthoxylum* in the rural economy, only a little study has been conducted in Nepal regarding the genus *Zanthoxylum*. These information are crucial for the successful development and commercialization of any medicinal plant used in various indigenous healthcare systems. Therefore, future development of plans and policies related to these sectors specifically depends on the generation of empirical data on distribution, abundance, plant-environment interactions, and phytochemical composition. Hence, this study was carried out to generate information which could provide further impetus for the overall development of this valuable species. The propagation and germination techniques as explored in this study could address potential problems in cultivation practices.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study area/collection sites

Two districts Salyan and Kavrepalanchowk were selected for the ecological study and sample collection. However, ecological study was conducted in Salyan district only, while vegetative propagation and germination studies were conducted at Kavrepalanchowk district.

3.1.1 Salyan district

Salyan district was selected for the study of population density, distribution pattern and comparative phytochemical studies of *Z. armatum*. All the samples required for phytochemical studies and essential oil composition of *Z. armatum* were collected from different localities of Salyan. Salyan district, a part of Karnali province is located at 28°22'31 N 82°9'42 E in the mid-west hills of Nepal. The district with Salyan or Salyan Khalanga at its headquarters is in the western part of Rapti zone. The political boundary of the district is associated with Rolpa in the east, Surkhet and Banke in west, Jajarkot and Rukum in north and Dang and Banke in the south. It is situated in the height of 457 to 3049 m from sea level and its area is 1929.55 sq km (DFRS, 2018). The economic base of the district is pre-dominantly agricultural where above 80% of the population is engaged in agriculture and deriving their livelihoods from this sector. The major sources of income of the district are vegetable production, ginger, fruit production, non-timber forest products (NTFPs) and resin collection. NTFPs and resin collection are the major sources of revenue generation in the district. Due to high level of poverty and lack of employment opportunities in the district, seasonal and permanent migration are increasing. People from remote areas go to India, gulf countries and in different parts of Nepal as an un-skilled laborer for seeking their livelihoods.

Out of the ten municipalities of Salyan district, four municipalities were selected based on the availability and cultivation of *Z. armatum* in the district. Salyan is rich in forest area. Out of the total area of the district, the area covered by forest is 1929.55 sq km (61.5%) (DFRS, 2018) which, is very high compared to the national average. The details of land cover, forest area of the study sites is presented in Table 3. The forests in the lower altitudes are dominated by *Pinus roxburghii*, which sometimes have an understory of *Aesculus indica* and *Bassia latifolia*. In the upper parts, the pine forests usually merge with oak forests. Two oak species are characteristic of this zone, *Quercus incana* and *Q. lanuginosa*. In the second story, they may be associated with species such as *Rhododendron arboreum* and *Lyonia ovalifolia.* In both pine and oak forests, *Z. armatum* grows naturally as an understory species.

Z. armatum is the main non timber forest product (NTFP) of Salyan District with 400-600 t collected per year. The fruits of this species account for about 70% of the overall value of the NTFPs collected from the district. If one working day is needed for collecting 4 kg of Timur, approximately 100,000-150,000 man days per year are required to pick the fruits during the collecting season in Salyan district (Hertog & Wiersum, 2000). Regarding the availability and importance of Timur in Salyan, the Department of Plant Resources under the Ministry of Forests and Soil Conservation has been promoting and facilitating farmers for its commercial cultivation.

Average monthly minimum temperature of the study area (Salyan district) was 6.57 °C during the month of January and average monthly maximum temperature was 29.5 °C during month of May. The study area receives the monthly maximum rainfall 285 mm during July and minimum 1.71 mm in November (Figure 2).

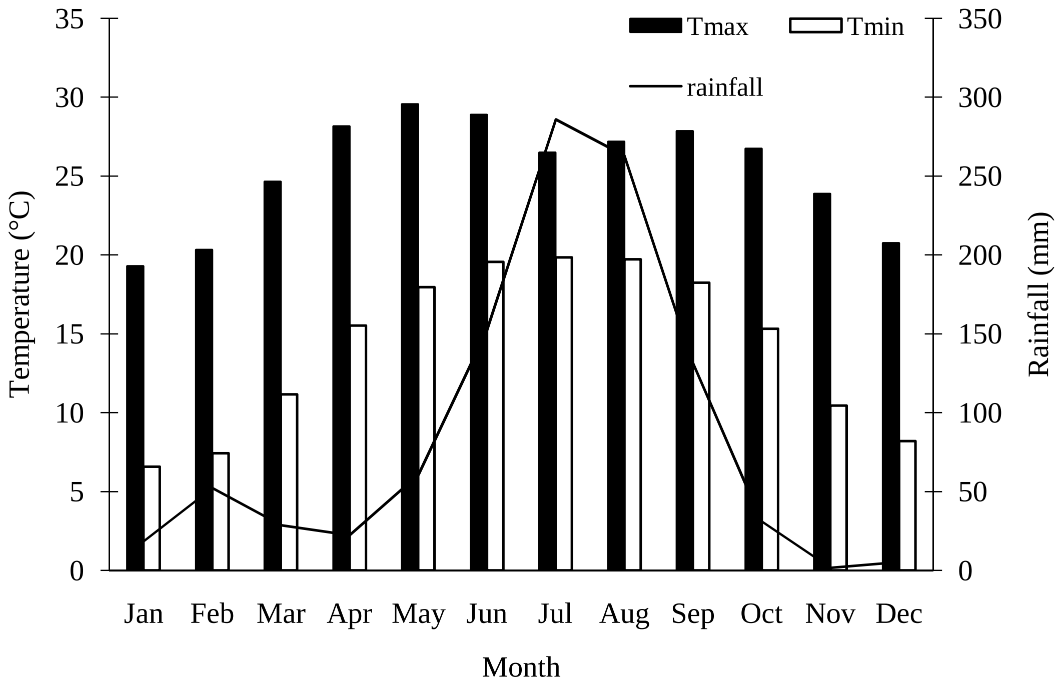


Figure 2: Average monthly mean temperature (°C) and rainfall (mm) 2007 to 2018 recorded at Ginger Research Program (GRP) Station, Kapurkot, Salyan (28° 13' 26" N, 82°21' 09" E, 1480 m)

Source: Department of Hydrology and Meteorology, Government of Nepal, Kathmandu

3.1.2 Kavrepalanchok district

Vegetative propagation and seed germination experiments were conducted at Dabur Nursery Private Limited, Banepa. Similarly, the samples for studying comparative yield and composition essential oil of fruits based on harvesting time were also collected from Dabur Nursery, Banepa, Kavrepalanchok district. Kavrepalanchok, 31 km east of Kathmandu Valley, is a part of Bagmati Province and one of the 77 districts, in central mid-hills region of Nepal (27°37" and 85°33" E). The district, with Dhulikhel as its district headquarters, covers an area of 1,396 sq km with elevation range of 280 (Dolalghat/Sunkoshi River) to 3018 m (Bethanchok Narayan Danda). It is bordered to the east by Ramechhap and Dolakha, west by Kathmandu valley, north by Sindhupalchowk and south by Sindhuli and Makawanpur. This region has a subtropical climate and the average temperature ranges from 10 °C to 31 °C. Vegetation of the region is characterized by the forest of *Schima wallichii*, *Castanopsis* sp., *Pinus roxburghii* and *Alnus nepalensis* at the lower belt, while broad leaved oak forests of *Quercus* spp are found at upper belt.

The mean yearly temperature of the district ranges from 26.61 °C to 11.82 °C, with the maximum temperature of 26.61 °C during June and the minimum of 3.58 °C during January. The average annual precipitation of the district is 1091.94 mm the highest precipitation occurs in July (315.84 mm) (Figure 3).

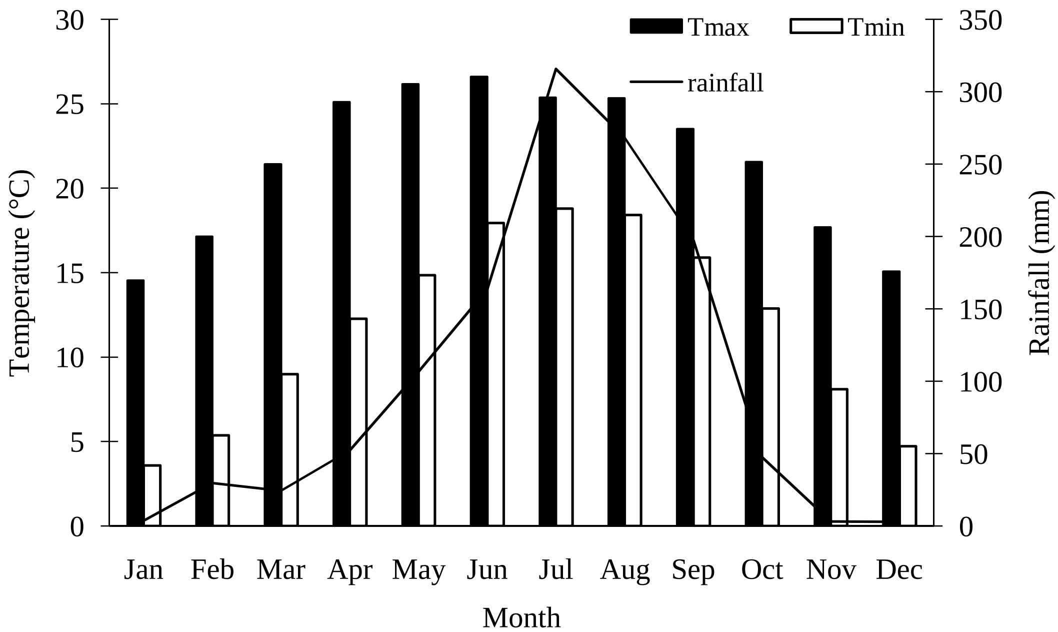


Figure 3: Average monthly mean temperature (°C) and rainfall (mm) 2007 to 2018 recorded at Dhulikhel Station, Kavre District (27.37° N, 85.24° E, 1532 m)

Source: Department of Hydrology and Meteorology, Government of Nepal, Kathmandu

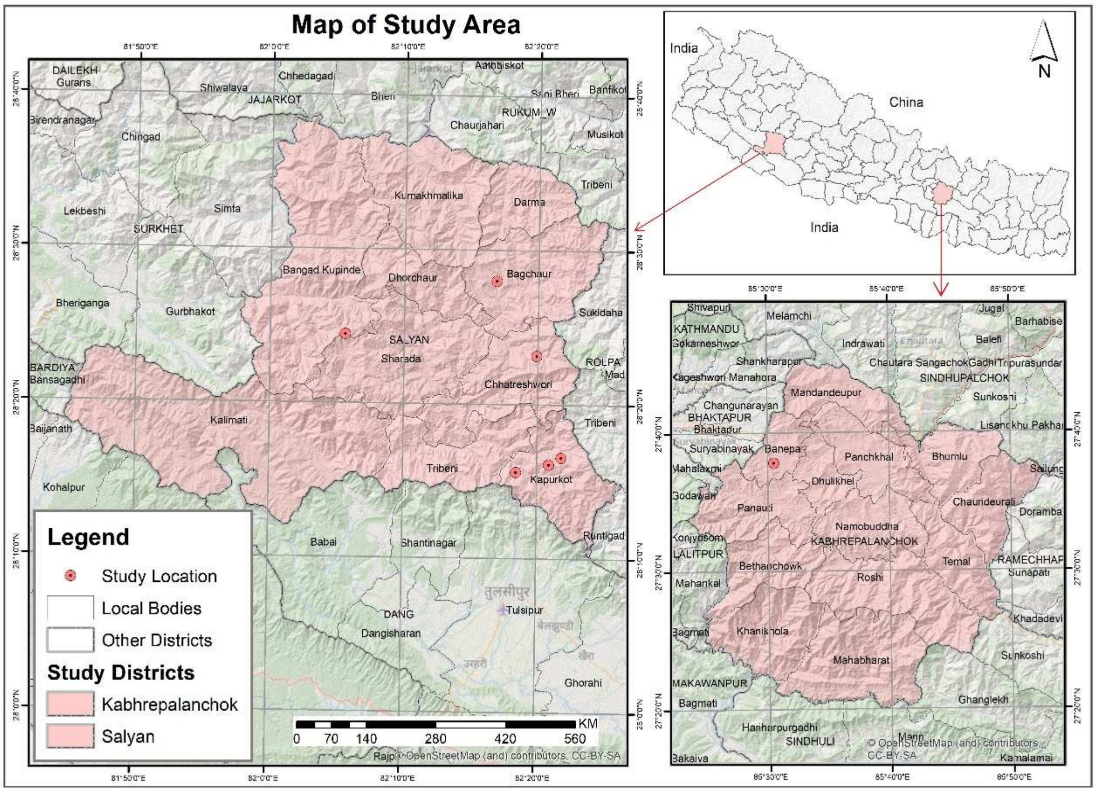


Figure 4: Map of Nepal showing study area and sampling/collection sites of *Zanthoxylum armatum*

3.2 Study species

Zanthoxylum armatum DC., belonging to family Rutaceae is a common spice plant of Nepal and is popularly known as Timur in Nepali (English: prickly ash or Nepal pepper). Eight species of *Zanthoxylum* have been identified from Nepal till date and they are: *Z. acanthopodium* DC., *Z. armatum* DC., Z. *floribunda* Wall., *Z. nepalense* Babu, *Z. nitidum* (Roxb.) DC., *Z. oxyphyllum* Edgew., *Z. simularis* Hance and *Z. tomentellum* Hook. f. (DPR, 2011a, 2016; Rajbhandari et al., 2015). Despite the fact that Nepal has eight species of *Zanthoxylum*, only five of them have been acknowledged taxonomically by The Plant List. *Z. floribunda* Wall and *Z. simularis* Hance are not recognized taxa and *Z. nepalense* Babu has been categorized as unresolved name. (The Plant List, 2013). *Zanthoxylum armatum* DC., among these eight species is the most common one of the 33 prioritized MAPs by the Government of Nepal for economic development emphasizing on the cultivation and agro-technology development (DPR, 2006; DPR, 2011b).

The plant is a perennial shrub growing up to 6 m high with thick glabrous leaves and straight prickles on stem. The fruit is a small drupe, ovoid, green when young, reddish and splitting into two when ripe (Figure 5, a-c). The branches, barks, fruits, and seeds are extensively used in indigenous system of medicine as carminative, stomachic and anthelmintic. The fruits are found to contain 2-7.6% essential oils (Manandhar, 2002, Phuyal et al., 2020c). In Nepal, the fruit decoctions and berries are used for abdominal pain, carminative, antispasmodic, rheumatism, skin diseases, cholera, diabetes, and asthma (Singh et al., 2016).

(a) (b) (c)

Figure 5: *Zanthoxylum armatum* (a) A mature flowering plant (b) Young fruits (c) Ripe fruits

3.3 Field sampling

A preliminary filed survey was carried out in April 2017 to select the study site and sampling areas, gather general information about the study species viz. *Z. armatum,* and rapport building with the local people and concerned authorities. The principal visit was conducted during the months of May 2017 and October 2018. All necessary data and samples were collected during that period. The details of study sites and map of the study area are presented in Figure 4 and Table 3.

Table 3: Details of sampling sites of *Zanthoxylum armatum* in the study area

SN	Municipality	Total area (ha)	Forest area (ha)	Forest cover (%)	Study site	Altitude (m)	Latitude (N) Longitude (E)	Aspect	Land use/Forest type
1	Kapurkot	11875	7542	63.5	Dhanwang	1000-1200	28.26875, 82.30842	NE	Forest near village settlement
2					Kapurkot	1200-1400	28.2707, 82.35038	NW	Near roadside on edges of farmyard
3					Rim	1400-1700	28.27611, 2.36361	SW	Mixed Quercus forest
4	Baghchaur	16251	8453	52	Baghchaur	1400-1600	28.46694, 2.28139	NE	Mixed forest
5	Bangad Kupinde	33678	22709	67.4	Kupinde	1600-1800	28.41319, 82.0935	NE	Disturbed forest due to road construction
6	Chhatreshwori	15011	9841	65.6	Chhatreshwori	1800-2000	28.38611, 2.36361	NE	Moist and dense forest

Source: 1. Forest cover and land cover: DFRS (2018), 2. Field survey

The study was mainly based on primary data collection. Necessary information was collected through extensive field observation of the area. The data was collected through physical measurement in the field and review of relevant literature on similar previous studies. Systematic random sampling design was applied in which plots were selected by a random or stratified random plan (Misra, 1968). The sampling sites were selected from six localities to cover all the possible habitats and associated vegetation types of *Z. armatum* so that a comparative study can be done based on disturbance factors, altitudinal difference, etc.

Vegetation sampling was done along the elevation of 1000 m to 2000 m. In each locality, four transect lines were set up at 30-50 m in *Z. armatum* available sites. In each transect line five plots of 5m x 5m were laid down at a distance of 10 m. The number of individuals of *Z. armatum* and other tree and shrub species (excluding grasses) in the sample plot associated with *Z. armatum* were recorded. Vegetation attributes, including frequency, density, and richness, were recorded, along with environmental coordinates such as latitude, longitude, and elevation of each sample plot using a global positioning system (Garmin model 2000) (Khan et al., 2011).

3.3.1 Density and abundance

Both the term refers to the number of species in a community. Abundance of any individual species is expressed as percentage of the total number of species present in the community and therefore it is a relative measure (Khan et al., 2014). In sampling the abundance of species, the individual of species is counted instead of just noting their presence or absence.

Density and relative density were calculated using Zobel et al. (1987), whereas abundance was determined based on the formula of Kilewa and Rashid (2014).

$$\text{Density (D) (plants/ha)} = \frac{\text{Total no.individuals of a species in all quadrats}}{\text{Total no.of plots studied}\times\text{size of the plot (m2)}} \times 10000$$

$$\text{Relative Density (RD\%)} = \frac{\text{Density of individual species}}{\text{Total density of all species}} \times 100$$

$$\text{Abundance (A)} = \frac{\text{Total no.of individuals of a species in all quadrats}}{\text{Toal no.of quadrats in which the species occured}}$$

$$\text{Relative Abundance (RA\%)} = \frac{\text{Total no.of a particular species}}{\text{Total no.of individuals of all species recorded}} \times 100$$

3.3.2 Frequency

Occurrence of trees and shrub species within each major plots of the study area were recorded to assess their distribution pattern in. Then, frequencies of these species were obtained by following formula (Zobel et al., 1987). Relative frequency is the frequency of a species in relation to other species

$$\text{Frequency (F) (\%)} = \frac{\text{No.of quadrats in which an individual species occured}}{\text{Total no.of quadrats studied}} \times 100$$

$$\text{Relative Frequency (RF\%)} = \frac{\text{Frequency of individual species}}{\text{Sum of frequencies of all species}} \times 100$$

3.3.3 Distribution pattern (A/F ratio)

Abundance and frequency taken together are of great importance in determining the structure of a community. High frequency and low abundance indicate regular distribution whereas the converse indicates contiguous distribution. The ratio of abundance to frequency (A/F) for different species was determined for eliciting the distribution pattern. Spatial distribution of plant species was determined following Whitford index WI (Singh & Singh, 1987) as

Distribution (WI) $= \frac{\text{Abundance}}{\text{Frequency}}$ (A/F Ratio)

If value is <0.025 = regular distribution, value lies between 0.025-0.05 = random distribution and value > 0.05 = clumped distribution (Whitford, 1949).

3.3.4 Importance Value Index (IVI)

The Importance Value Index (IVI) was calculated to understand the species' share in the community (Cottam & Curtis, 1956). Species with the highest importance value are the leading dominant species of the specified vegetation (Shibru & Balcha, 2004). This considers density, frequency, and abundance of the species present in the community. For each species, the relative density, relative frequency, and relative abundance were calculated and summed. It gives the overall importance of each species in the community structure. The Importance Value Index (IVI) for all the species was calculated by adding the sum of relative values of density, frequency and abundance. It was caculated following Bhadra and Patnayak (2016) as

Importance Value Index (IVI) = Relative Density + Relative Frequency + Relative Abundance

3.3.5 Regeneration

Population structure of naturally emerged seedlings of *Z. armatum* reported in each sample plot was studied. Density of all the individuals of seedlings, saplings and adult were determined. The size classes of individuals of *Z. armatum* were broadly defined according to plant height. Plant height less than 0.1 m were classified as seedlings. Plant height ranging from 0.1 m to 1.0 m were classified as saplings and plant height usually more than 1 m and also bearing reproductive structures were classified as adult (Schemske et al.,1994).

Regeneration status of species was totally based on population size of seedlings and saplings (Khan et al. 1987; Saha et al., 2016). Good regeneration if seedlings >saplings >adults; fair regeneration, if seedlings> or ≤ saplings ≤ adults; poor regeneration, if the species survives only in sapling stage, but no seedlings (saplings may be or = adults). If a species is present only in an adult form, it is considered as not regenerating. Species is considered as new if the species has no adults but only seedling or saplings. The status of natural regeneration was determined based on the values as shown in Table 4 (Bhuyan et al., 2003; Khumbongmayum et al., 2006).

Table 4: Different regeneration status

SN	Regeneration status	Seedling (Se)	Sapling (Sa)	Compared to adult
1	Good regeneration	Present	Present	Se > Sa > adults
2	Fair regeneration	Present	Present	Se > or < Sa; Sa ≤ adult
3	Poor regeneration	Absent	Present	Sa > or < or = adult
4	No regeneration	Absent	Absent	only adult

As shown in the above table, ‘good regeneration’ is defined as the condition in which an ample or adequate number of seedlings and saplings contribute to the mature population, while ‘fair regeneration’ is defined as the condition in which there were a fair number of seedlings, but the percentage of saplings was either lower than or close to that of the mature trees. ‘Poor regeneration’ is the condition in which individuals were found at either the seedling or sapling stage only, in greater numbers than the mature trees. The fourth regeneration status is termed as ‘no regeneration,’ in which a species presented only at the mature stage and did not occur in either seedling or sapling stages.

3.3.6 Species richness and diversity

The species richness in this study was obtained by counting the number of species present in each 5m×5m sample plot. In this study, species richness has been defined as, the number of species per plot and expressed as species/m2.

Diversity in species refers to the combined effect of richness and evenness in species. While richness pertains to the number of species in each sampling unit evenness implies to the distribution of individuals among the species. Species richness is a biologically appropriate measure of diversity and the total number of species in any ecological community, landscape, or region relative to the total number of all individuals in that community.

A diversity index depicts the structure of biological community in terms of numerical value. It gives more information on community composition than simply species richness. Further, it offers insights into rarity and commonness of species in a community, thereby diversity index acts as important tool for biologists in the understanding of community structure (Muthulingam & Thangavel, 2012). Several indices are used to quantify the species diversity of which Simpson's index (Simpson, 1949) and Shannon-Wiener's index are the most commonly used. Shannon's diversity index (H) and Simpson's Index (1−D) in terms of density for each plot were calculated using the following indices:

Shannon's diversity index (H) $= -\sum_{i=1}^{n}(pi \times ln\, pi)$ (Shannon & Weaver, 1963)

Simpson's diversity Index (1 − D) = $1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$ (Simpson, 1949)

Where, pi= (n/N),

n = density of individual species in a plot

N = Total density of all species in a plot

ln = Natural logarithm values

The Shannon diversity index ranges typically from 1.5 to 3.5 and rarely reaches 4.5 (Gaines et al., 1999). The Simpson's Index values range from 0 to 1. The closer the value of Simpson's Index to 0, the more diverse the plot will be. A plot with only one species would have a Simpson's Index value of 1. Trends are opposite to those found for Shannon Weaver values since Simpson's Index values decrease with increased diversity (Reich et al., 2001). In practice, the values below 0.5 indicate a relatively even community, while high values are indicative of communities dominated by one or a few species.

3.4 Soil analysis

Soil samples were collected from the four corners and center of each quadrat to a depth of 5-10 cm. The subsamples were mixed thoroughly, and about 100 g soil were collected, air dried in shade (Zobel et al., 1987), kept in zipper plastic bags, properly labeled, and brought to the Laboratory for the analysis of pH, soil organic carbon, nitrogen, phosphorus, and potassium. Soil organic carbon (SOC) was calculated by following Walkley and Black (1934), nitrogen (N) by Kjeldahl method (Bremner & Mulvaney, 1982), phosphorous (P) by a modified Olsen's method following Gupta, (2000), potassium (Flame photometer method following Trivedi & Goel, 1986 and pH using a digital pH meter with 1:5 soil water ratio (Gupta, 2000). These soil samples were analyzed in the Laboratory of Forests Research and Training Centre, Babarmahal Kathmandu under the Ministry of Forests and Environment. Details of soil nutrient analysis protocols are presented in Annex 1.

3.5 Fruit yield

For estimating the fruit yield per plant, *Z. armatum* cultivated in farmland of the farmers were selected as yield estimation from the wild was misleading. Twenty mature and healthy plants from each locality were selected and tagged for the estimation of yield. The fruits were harvested along with the farmers during harvesting period. Fresh weight of the fruits was taken immediately after harvesting. Then the fruits were shade dried separately; traditionally the berries are dried in the shadow of trees or under the awnings of the houses. Dry weight of the fruits was taken after they were completely dried. Soil samples were also collected from the farmland for analyzing organic carbon, nitrogen, phosphorus, potassium and pH.

3.6 Seed biology

3.6.1 Seed size and weight

After estimating the fruit yield, seeds were removed from the fruits, packed in butter paper, labelled, and brought to Dabur Nursery, Banepa. One hundred seeds from each locality were selected randomly for their mass and area. Individual seed was measured in a digital balance and the length and breadth of the seeds were also measured using a vernier caliper (ISMA, 2019).

3.6.2 Seed germination

Since the seed germination in *Z. armatum* is very low and the seeds may take from few months to years for germination, several experiments were conducted for three consecutive years; 2017, 2018 and 2019, which included various treatments as well.

Ripe fruits of *Z. armatum* were collected from healthy and vigorous plants growing in natural habitats during their harvesting period. The seeds were carefully removed from the pericarp, and stored at 4 °C in refrigerator in separate paper bags until the initiation of germination experiment.

Treatment consisted of four different methods (Table 5). Before applying the treatment all the seeds were placed in a beaker containing water for 24 h. Empty seeds consistently floated in the water surface where as non-empty seeds settled down in the bottom of a beaker. The floating seeds were discarded and the submerged seeds were used for germination experiment. This was done in order to exclude all seeds that may not have been properly ripened and/or those that might have been infected with pests. After rinsing, all the seeds were dried using blotting paper and were surface sterilized with 5% sodium hypochlorite (NaOCl) solution for 5 min and subsequent rinsing in distilled water.

The experiments were conducted using a completely randomized design. Each treatment was replicated thrice and each replicate consisted of 50 seeds making a total of 150 seeds per treatment. The experiment was conducted at Laboratory of Dabur Nepal Private Limited Nursery, Banepa and in the seed lab of NARC.

Fifty seeds for each replicate were placed in 90 mm diameter sterilized petri dishes. Each petri dish was lined with two layers of sterilized No. 5 Whatman filter papers. About 20 mL of distilled water was initially added to each petri dish to moisten the filter papers. Seeds were incubated in a growth chamber at 25±3 °C temperature and humidity at 70-80%. (ISTA, 2016). The internal environment of petri dishes was maintained saturated with moisture by frequently adding distilled water at a 5-day interval. Seed germination was observed up to 45 days after sowing Emergence of radicle from seed was considered as seed germinated. Percent germination was recorded as number of seeds germinated over 45 days.

Germination (%) was calculated by using the following formula (Krishnaswamy & Seshu, 1990)

$$\text{Germination (\%)} = \frac{\text{number of germinating seeds}}{\text{number of viable seeds initiated}} \times 100$$

Table 5: Various treatments given to seeds of *Zanthoxylum armatum* during germination experiments

Treatment		Description	References
A.	Mechanical		
1	Sand scarification	Seeds were rubbed vigorously with sand and washed with distilled water	Morais et al., 2014; Rusdy 2015
2	Sandpaper scarification	Each seed was vigorously rubbed on both sides with 80 no. sandpaper	Morais et al., 2014; Rusdy 2015
3	Hot water treatment 65° C and 95 °C	Seeds were immersed in hot water for 5 min	Giasson et al., 2019
4	Cold treatment 5°C: 12 hr, 24 hr, 36 hr.	Seeds were immersed in cold water for different time durations.	Omran, 2013
5	Stratification: Cold stratification at 4 °C for 3 months	Seeds were placed between moist paper towels in plastic storage bags, which were then placed in a 4°C refrigerator for the prescribed stratification period.	Hopkins and Gravatt, 2019
B.	Acid		
1	Sulphuric acid H_2SO_4 (60%.): 1 min, 2 min, 5 min	Seeds were immersed in a beaker containing concentrated sulphuric acid for 1 min, 2 min and 5 min Then the seeds were washed free of acid under running tap water tap in a steel sieve, and dried with blotting paper.	Arowosegbe, 2016
2	Hydrochloric acid HCL (36%)	As in above treatment	Arowosegbe, 2016
3	Nitric acid HNO_3 (60%)	As in above treatment	Younis et al., 2007
C.	Hormone		
1	Gibberellic acid GA_3: Ppm: 100, 500, 1000, 1500, 2000, 2500 For 24 h	The seeds were pretreated with different concentrations (100, 500, 1000, 1500, 2000, 2500 ppm) of Gibberellic acid GA3 for 24 h	Keshtkar *et al*, 2008
D.	Control	Seeds were untreated	

3.6.3 Germination behavior study based on harvesting period

Similarly, another experiment was also conducted with seeds collected from the plants cultivated at Dabur Nursery to study the germination behavior of *Z. armatum* based on harvesting period. The fresh fruits were collected at various months (early August,

August 15-September 15, September 16-October 15, October 16-November 15, November 16-December 15 and late December). The fruits were dried for 2-3 days and all the seeds were separated from the fruits and they were used immediately for germination experiment. The seeds were scarified with sand and soaked in Gibberellic acid (GA_3) 100 ppm for 24 hours. The pretreated seeds were sown in seeding trays (18×12×5 inches) containing mix growth media with a mixture of sand, neopeat and perlite. The seeds soaked in water only and not scarified or treated were used as control. For every treatment, five replicates were used. The experiment was set up as complete randomized design at controlled green-house conditions at Dabur Nepal Private Limited Nursery, Banepa, Kavre. The humidity was maintained at 70% and temperature 28 °C throughout the germination period.

3.7 Vegetative propagation

Vegetative propagation experiment was conducted at the green house of Dabur Nursery, for two consecutive years in 2017 and 2018. Semi hard wood branches were taken from 4-5 years old healthy plants of *Z. armatum* growing at the nursery. They were cut into 15 cm long pieces containing 2-3 nodes and all the leaves were removed from the branches. All the cuttings were surface sterilized by dipping them in freshly prepared 1% fresh Bordeaux mixture (calcium hydroxide and copper sulphate) for 10-15 min.

Two plant growth regulators namely indole butyric acid (IBA) and naphthalene acetic acid (NAA) at different concentrations 2000, 3000 and 5000 ppm were used to study the rooting and shooting behaviour of *Z. armatum* following Hartmann et al. (2002). The cut ends of the surface sterilized cuttings were dipped for 24 hours in a bucket having hormone solutions to enhance the absorption of hormones.

The rooting behavior of *Z. armatum* was studied using three distinct growth media (rooting media), namely sand, neopeat (coconut fiber), and mix (a 2:1:1 mixture of sand, soil, and vermi-compost). The cuttings were planted straight into the rooting media after being dipped in hormones. The cuttings were planted in plastic trays (No. 21) with 20 cells/cavities and holes in the bottom. The tray's length and width are 54 cm and 28 cm, respectively. Each cell is 6.8 cm long, with a top diameter of 6 cm and a bottom diameter of 2.7 cm. The trays' voids were filled with the appropriate medium. A single cutting was put obliquely into each hollow up to 3 cm.

Completely randomized design was used for the experiment. Total number of stem cuttings of *Z. armatum* used in the experiment was 1080 for 18 treatments in three replicates (20 cuttings×18 treatments×3 replicates). Hundred cuttings treated with Bordeaux mixture and washed with distilled water were used as control. The experiment was carried out during February 2017 at the nursery's green house.

After planting the cuttings, all the planting trays were labeled clearly and transferred to the green house and placed in the controlled environment. The relative humidity and the temperature were maintained at 21.9 ºC and 75%, respectively throughout the research/study period. Inside the greenhouse, agro-meteorological parameters were recorded through the sensor system run by ARGUS Control software and the data were recorded in the computer. Relative humidity was maintained by the means of misting.

After 90 days of planting, the number of roots, root length, and shoot length of each cutting were measured. If a cutting had at least one major root that was about one millimeter long, it was deemed rooted. The cutting was gently uprooted for measuring, and then carefully scraped from the rooting material so that the roots were not broken. Using a ruler, the length of the root and shoot were counted and the number of primary roots was also counted.

The collected data were analyzed statistically using R-program with Agricola. Least significant difference (LSD) and Duncan's multiple Range Test (DMRT), as mean separation technique was applied to identify the most efficient treatment in the rooting and shooting behavior of *Z. armatum* (Gomez & Gomez, 1984).

3.8 Phytochemical analysis

Fresh fruits, seeds, matured leaves, and bark of *Z. armatum* were collected from 1000 to 2000 m altitudes from the wild and cultivated populations. Leaves were collected during their active growing season in May 2017 and fruits, seeds and bark were collected during their harvesting period in May 2018 (Table 6). Leaves were collected from different altitudes and populations (wild and cultivated) but altitude wise collection of fruits, seeds and bark was not possible because of resource limitations and other social factors. The plant samples were collected with the permission of Department of Plant Resources, Ministry of Forests and Environment, Government of

Nepal in accordance to article no. 10 (B) of Plant Resource Research Procedure 2013 and revised 2016. *Z. armatum* is not enlisted in the CITES and protected plant list of Nepal. The plant was identified by Nirmala Phuyal. Herbarium of voucher specimens were prepared, and deposited at National Herbarium and Plant Laboratories (KATH; NPZA 20-NPZA 50). The samples were cleaned and shade dried for a week before the extraction procedure.

Table 6: Details of collection sites of different samples of *Zanthoxylum armatum*

SN	Locality	Altitude (m)	Latitude	Longitude	Habitat
1	Dhanwang	1000	28.2687	81.6916	Cultivated
2	Dhanwang	1060	28.2684	81.6916	Wild
3	Kapurkot	1390	28.2427	81.6477	Cultivated
4	Kapurkot	1400	28.2432	81.6473	Wild
5	Rim	1650	28.4355	81.4144	Cultivated
6	Rim	1700	28.6842	81.8403	Wild
7	Kupinde	1730	28.4348	81.3813	Cultivated
8	Kupinde	1770	28.4348	81.3813	Wild
9	Chhatreshwori	1990	28.6694	81.4029	Wild
10	Chhatreshwori	2000	28.2687	81.6916	Cultivated

The dried samples were then powdered separately in a grinder. Known weight of the powdered samples were loaded in thimble and put inside the Soxhlet apparatus. They were then successively extracted with hexane, ethyl acetate and methanol by hot Soxhlet extraction method. The apparatus was run for 72 h until the appearance of the colored solvent in the siphon for obtaining the crude extracts of the samples. After complete extraction, the solvents, i.e., hexane, ethyl acetate and methanol were evaporated in a rotary vacuum evaporator at 65 °C or below the boiling points of the solvent under reduced pressure and thus obtained extracts were finally dried in water bath. The dried extracts were sealed inside 20 mL sterilized culture tubes and stored in refrigerator at 2-8 °C for further analysis (Tiwari et al., 2011).

3.8.1 Composition of leaf, fruit, seed and bark extracts (GC-MS analysis)

Quantitative analysis of the chemical constituents in the methanol extracts of fruit, seed, leaf and bark of *Z. armatum* was carried out using a Shimadzu Gas Chromatograph (GC 2010) with Rtx-5MS column (25 m×0.25 mm×0.25 µm). The initial column was maintained at 40 °C and the injection temperature was 250 °C.

Qualitative analysis of the extract was further continued in a Shimadzu GCMSQP 2010 Plus. The ion source temperature and the interface temperature were kept at 200 °C and 250 °C, respectively. One µL of the extract diluted with spectroscopic grade hexane (10:1) was injected into the GC inlet maintaining column flow rate of 1 mL/min and purge flow 3 mL/min after fixing the split ratio at 120, using Helium as a carrier gas. Detector scanning start time was 4 min and end time was 68 min, mass spectra were scanned from m/z 40-350, with the scanning speed of 666. The extracts components were identified by the determination of their retention indices (RI), by comparison with authentic reference compounds and by comparison of mass spectra using the NIST 11 (National Institute of Standards and Technology, Gaithersburg, MD) and FFNSC 1.3 library. The relative percentage of each constituent present in the extract was calculated according to the area of the chromatographic peaks.

3.8.2 Total phenolic content (TPC)

Total phenolic content (TPC) in the different extracts (fruit, seed, leaf, and bark) were determined by Folin-Ciocalteu colorimetric (F-C) method as described by Chang et al. (2001) with some modifications. Standard gallic acid solution was prepared by dissolving 10 mg of it in 10 mL of methanol (1 mg/mL). Various concentrations of gallic acid solutions in methanol (25, 50, 75, 100 µg/mL) were prepared from the standard solution. To each concentration, 5 mL of 10% Folin-Ciocalteu Reagent (FCR) and 4 mL of 7% Na_2CO_3 were added making a final volume of 10 mL. Thus, obtained blue colored mixture was shaken well and incubated for 30 min at 40 °C in a water bath. Then, the absorbance was measured at 760 nm against blank. The FCR reagent oxidizes phenols in plant extracts and changes into dark blue color, which was then measured by UV-Visible Spectrophotometer. All the experiments were carried out in triplicates and the average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

The extracts were prepared at different concentrations (25, 50, 75, and 100 g/mL). Following the the same process for standard gallic acid, the absorbance was measured for each concentration of the extracts. For each measurement, triplicate samples were made and the calibration curve was used to plot the quantity of phenols in the extracts. The extracts' total phenolic content was measured in mg gallic acid equivalents

(GAE) per gram of sample in dry weight (mg/g). The total phenolic content of all of the samples was estimated using the formula:

$$C = c\ V/m$$

where, C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram.

3.8.3 Total Flavonoid Content (TFC)

An aluminum chloride colorimetric test was used to measure the total flavonoid content in the extracts following Chandra et al. (2014). Quercetin stock solution (4 mg/mL) was made by dissolving 4 mg quercetin in 1 mL methanol. This standard solution was serially diluted to get solutions with concentrations of 0.25, 0.5, 0.75, and 1 mg/mL. In a test tube containing four mL of distilled water, one mL of each concentration of quercetin was added. 0.3 mL of 5% $NaNO_2$ was added to the test tube at the same time, followed by 0.3 mL of 10% $AlCl_3$ after 5 minutes. After six minutes, two mL of 1 M NaOH was added to the mixture. By immediately adding 4.4 mL distilled water, the volume of the mixture was made 10 mL. Using the linear equation based on the calibration curve, the total flavonoids content was expressed as quercetin equivalent (QE)

Total flavonoid content in the extracts were determined by aluminum chloride colorimetric assay (Chandra et al., 2014). The stock solution (4 mg/mL) of quercetin was prepared by dissolving 4 mg of quercetin in 1 mL of methanol. This standard solution was diluted serially to make various concentrations of 0.25, 0.5, 0.75, and 1 mg/mL solutions. One mL quercetin of each concentration was added to the test tube containing four mL of distilled water. At the same time, 0.3 mL of 5% $NaNO_2$ was added to the test tube and 0.3 mL of 10% $AlCl_3$ after 5 min. Then two mL of 1 M NaOH was added to the mixture after six min. The volume of the mixture was made ten mL by immediately adding 4.4 mL of distilled water. The total flavonoids content was expressed as quercetin equivalent (QE) using the linear equation based on the calibration curve.

Stock solutions of 4 mg/mL concentration in methanol of the extracts were prepared and diluted serially to make different concentrations (0.25, 0.5, 0.75, and 1 mg/mL) solutions. Similar procedure as described for quercetin was followed for the extracts also and the absorbance was measured by spectrophotometer at 510 nm. Readings were taken in triplicate and the average value of absorbance was used to calculate total flavonoid content. The flavonoid content was expressed as quercetin equivalent (mg QE/g) using the linear equation based on the standard calibration curve.

3.8.4 Antioxidant activity (DPPH (2, 2-Diphenyl-1-picrylhydrazyl radical scavenging activity)

In-vitro antioxidant activity of the extracts was determined using the DPPH (2, 2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay as described by Nithianantham et al. (2011) with some modifications. This is a quick and easy method to analyze the scavenging potential of antioxidants. DPPH in oxidized form gives a deep violet color in methanol. An antioxidant compound donates the electron to DPPH thus causing its reduction and in reduced form its color changes from deep violet to yellow. DPPH solutions show a strong absorbance at 517 nm appearing as deep violet color. Scavenging of DPPH free radical determines the free radical scavenging capacity or antioxidants potential of the test samples, which shows its effectiveness, prevention, interception, and repair mechanism against injury in a biological system.

DPPH solution (0.1 M) was prepared by dissolving 0.39 mg of DPPH in a volumetric flask, dissolved in methanol and the final volume was made 100 mL. Thus, prepared purple colored DPPH free radical solution was stored at-20 °C for further use.

A stock solution of different extracts of one mg/mL was prepared by dissolving required quantity of each extract in required volume of methanol. From the sample stock solution 25, 50, 75, 100 μg/mL (for fruits, seeds, and bark extracts) and 25, 50, 75, 100, 125, 150, 175, 200 μg/mL (for leaves extracts) solutions of each extract were prepared from the stock solution.

To the sample solutions of different concentration, one mL DPPH solution was added and incubated at room temperature for 30 min in dark. A control was prepared by mixing one mL methanol and one mL DPPH solution. Finally, the absorbance of the solutions was measured by spectrophotometer at 517 nm. Ascorbic acid was used as the standard. The 50% inhibitory concentrations (IC_{50} values) of the extracts were calculated from graph as concentration versus percentage inhibition. The radical-scavenging activity was expressed as percentage of inhibition. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical. Measurements were taken in triplicate. IC_{50} of the extracts indicates the corresponding concentration in which the radical scavenging potential is 50%. The IC_{50} of the extract and standards were determined graphically.

Percentage of inhibition was calculated by using the following formula,

$$IC_{50}\,(\%) = \frac{AC - AO}{AC} \times 100$$

where,

IC_{50} (%) = Inhibition (%)

AC = absorbance of the control (1 mL methanol + 1 mL DPPH solution)

AO = absorbance of the sample solution, and

Radical scavenging activities of the extracts are expressed in terms of their IC_{50} values. The data were presented as mean values ± standard deviation (n = 3).

3.9 Essential oil composition

The fresh fruits and leaves of *Z. armatum* were collected from healthy and vigorous plants from different populations (wild and cultivated) and elevation ranging from 1000 to 2000 m asl from Salyan district. Herbarium of the voucher specimens were prepared and deposited at National Herbarium and Plant Laboratories (KATH) NPZA 20-NPZA 50. The details of collection site are presented in Table 3. A total of ten samples from different altitudes and localities of Salyan district have been used for this study.

The collected samples were shade dried at room temperature for a week before the extraction of the oil. Seeds were separated from the pericarp. For the extraction, 100 g of dried samples were subjected to hydro-distillation for 6 h using modified Clevenger-type apparatus. The protocol was followed according to Anonymous (1988). Volume of the essential oil was measured directly in the extractor. The essential oil thus collected was then dehydrated over anhydrous sodium sulphate and stored in sealed, labeled glass vials at 4 °C until further analysis. Total yield was calculated as volume (mL) essential oil per 100 g of plant dry matter using the following formula (AOAC, 1990).

$$\text{Essential oil yield (\%)} = \frac{\text{Weight of oil}}{\text{Total weight of samples used for extraction}} \times 100$$

Quantitative analysis of the chemical constituents in the essential oils was carried out using a Shimadzu Gas Chromatograph (GC 2010) with Rtx-5MS column (25 m×0.25 mm×0.25 µm). The initial column was maintained at 40 °C and the injection temperature was 250 °C. Qualitative analysis of the essential oil was further continued in a Shimadzu GCMSQP 2010 Plus. The ion source temperature and the interface temperature were kept at 200 °C and 250 °C, respectively. One µL of the essential oil diluted with spectroscopic grade hexane (10:1) was injected into the GC inlet maintaining column flow rate of 1 mL/min and purge flow 3 mL/min after fixing the split ratio at 120, using Helium as a carrier gas. Detector scanning start time was 4 min and end time was 68 min, mass spectra were scanned from m/z 40-350, with the scanning speed of 666. The oil components were identified by the determination of their retention indices (RI), relative to C_8-C_{32} n-alkane series under identical experimental condition, by comparison with authentic reference compounds as well as with published mass spectra (Adams, 2007) and by comparison of mass spectra using the NIST 11 (National Institute of Standards and Technology, Gaithersburg, MD) and FFNSC 1.3 library. The relative percentage of each constituent present in essential oil was calculated according to the area of the chromatographic peaks.

3.9.1 Essential oil composition of fruits based on harvesting period

Essential oil composition of fruits based on harvesting period was also analyzed to determine whether storing of fruits have any effect on the composition of essential oil components. Fruits were harvested from the cultivated plants of Dabur Nursery, during August, September, October, November and December. The fruits were shade dried for a week and put in cotton bags and labelled. Some of the seeds were extracted immediately and some were stored for a month and extracted. The essential oil yield was calculated and the quantitative analysis of the essential oil was carried out by GC-MS for the freshly harvested and stored fruits.

3.10 Antimicrobial activity

Antimicrobial activities of the extracts of fruit, seed, leaf, bark and essential oil of *Z. armatum* from different elevations and populations were evaluated. Since the hexane and ethyl acetate extracts did not show any considerable results (alkaloids, flavonoids contents and antioxidants), only the methanol extracts of leaf, fruit, seed, and bark and the essential oil of fruit were screened against the microorganisms using agar well diffusion methods following Perez et al. (1990).

The extracts and the essential oil were screened against eleven different bacterial and two fungal strains.

Bacteria: *Bacillus subtillis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 739, *Klebsiella pneumonia* ATCC 700603, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typh*i Clinical sample, *Shigella dysenteriae* Clinical sample, *Staphylococcus aures* ATCC 6538P, Methilicin Resistant *Staphyloccus aureus* (MRSA) Clinical sample, and *Staphylococcus epidermi*dis ATCC 1228.

Fungi: *Saccharomyces cerevisiae* and *Candida albicans*

The medium used for bacterial culture was nutrient agar (NA) and that for fungi was potato dextrose agar (PDA). The required amount of media was prepared, autoclaved and respective media were melted, cooled to 40 °C then poured to sterilized petri dishes and allowed to solidify. Required numbers of colonies of test organisms were cultured in the respective plates and kept inside incubator for 18-24 h at 35 °C for bacteria and 25 °C for fungi prior to inoculation. All these experiments were carried out aseptically in a biosafety cabinet.

Required colonies (1.5×108 CFU/mL) of the freshly cultured test organisms were inoculated aseptically to glass vials containing normal saline. The suspension was homogenized by vortexing the solution and compared with the turbidity of 0.5 Mc Farland Nephelometer standard recommended by WHO (1991) for antimicrobial susceptibility test.

The prepared cell suspensions were uniformly spread with a cotton swab (carpet culture) over the dry surface of Muller-Hinton Agar (MHA) plates for bacteria and

Muller-Hinton Agar with Glucose and Methylene Blue (MHA, GMB) platted for fungi. Swabbing was done three times and the plates were rotated through an angle of 60° after each swabbing and the inoculated plates were left for maximum 15 min to allow absorption of excess surface moisture. Four wells, each of 6 mm diameter were bored in the inoculated plates using sterile cork borer. Fifty µl of the test solution of the extracts, positive control, and negative control were poured into the well. Antibiotic chloramphenicol was used as the standard antibacterial agent (positive control) and methanol as negative control. The inoculated plates were dried and put inside incubator at suitable temperature (35±2 °C) for bacteria and (25±2 °C) for fungi. After proper incubation (18-24 h for bacteria and 24-48 h for fungi) the plates were then examined for zone of inhibition (ZOI) around the well determined with clear area and no growth of microorganisms. Diameter of each ZOI was measured in millimeter by digital Vernier Caliper.

3.10.1 Minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined by observing the visible growth of the test organisms in two-fold serial diluted antimicrobial substances in nutrient broth (NB) culture media as the methods described by Sahm and Washington (1991) and Akinpelu and Kolawole (2004). A set of twelve sterilized labelled vials containing one mL of pre-sterilized Mueller-Hinton Broth (MHB) were prepared. One mL of the respective extract solution added to eleven vials only and the first vial was used as negative growth control. After complete homogenization, one mL of the solution containing nutrient broth and extract solution was transferred to the second vial. In the same manner, two-fold serial dilution was prepared up to the 10^{th} vial. Thereafter, one mL of content was discarded from the 10^{th} vial and the 11^{th} vial was used as positive control. Now all the vials except the 1^{st} and last contain equal volume, i.e., one mL gradually decreasing concentration of the solution. To all these vials 20 µL of bacterial suspension (turbidity equal to a McFarland Standard 0.5, supposed to have (1.5×106 CFU/mL) was put and mixed thoroughly. The vials were then incubated at 37 °C for 24 h. The MIC was taken as the lowest concentration that prevented the growth of bacterial culture (Wayne, 2015).

3.10.2 Minimal bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent required to kill the microorganism. The MBC values were determined by sub-culturing all the test dilutions of the extracts on the fresh nutrient agar (NA) medium and incubating further for 24 h at 37 °C (Forbes et al., 2007). The lowest concentration of the extract (mg/mL) that did not result in the appearance of a single bacterial colony on the solid medium was regarded as the MBC (Abu-Shanab et al., 2006).

3.11 Statistical analysis

All the analyses were carried out in R Studio (R Studio Team, 2016) in R platform (R Core Team, 2020). The normality (Shapiro-Wilk test) for all the parameters were tested before choosing a parametric or non-parametric tool for analyzing the data. All the parameters were tested by Analysis of Variance (ANOVA), Tukey’s test for normal data, and non-parametric Kruskal-Wallis one-way ANOVA, Duncan multiple comparison test for non-normal data.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Vegetation analysis

4.1.1 Vegetation composition

Altogether fifty plant species (trees and shrubs) belonging to 44 genera under 34 families were found to be associated with *Z. armatum* in the study area. Rosaceae was the dominant family with seven species, followed by Fagaceae with five species. *Quercus* was the largest genera with 4 species while the genera *Castanopsis* and *Prunus* had 2 species each (Table 7).

Table 7: List of trees and shrub species associated with *Z. armatum* in the study area

SN	Name of the species	Family	Local name	Habit
1	*Albizzia procera* (Roxb.) Benth.	Fabaceae	Sirish	Tree
2	*Adhatoda vesica* Nees	Acanthaceae	Asuro	Shrub
3	*Aesculus indica* (Wall. ex Cambess.) Hook.	Sapindaceae.	Lekh pangre	Tree
4	*Alnus nepalensis* D. Don	Betulaceae	Uttis	Tree
5	*Bauhinia variegate* L.	Fabaceae	Badahar	Tree
6	*Benincasa hispida* (Thunb.) Cogn.	Cucurbitaceae	Kubindo	Climber
7	*Berberis aristata* DC.	Berberidaceae	Chutro	Shrub
8	*Castanopsis hystrix* Hook. f. & Thomson ex A. DC.	Berberidaceae	Patle katush	Tree
9	*Castanopsis indica* (Roxb. ex Lindl.) A.DC.	Fagaceae	Daale katush	Tree
10	*Clematis* sp.	Ranunculaceae	Sikari lahara	Climber
11	*Colebrookea oppositifolia* Smith	Lamiaceae	Dhusure	Shrub
12	*Cotoneaster microphyllus* Wall. ex Lindl.	Rosaceae	Khareto	Shrub
13	*Daphne bholua* Buch.-Ham. ex D.Don	Thymelaeaceae	Lokata	Shrub
14	*Ficus neriifolia* Sm.	Moraceae	Dudhilo	Tree
15	*Ficus semicordata* Buch.-Ham. ex Sm.	Moraceae	khaniu	Tree
16	*Fraxinus floribunda* Wall.	Oleaceae	Laakuri	Tree
17	*Grewia optiva* J.R. Drumm. ex Burret	Malvaceae	Bhimal	Tree
18	*Juglans regia* L.	Juglandaceae	Okhar	Tree
19	*Pistacia integerrima* Stew. ex Brand	Anacardiaceae	Kakadsinghi	Shrub
20	*Ligustrum confusum* Decne.	Oleaceae	Kanike	Shrub
21	*Lyonia ovalifolia* (Wall.) Drude	Ericaceae	Angeri	Shrub
22	*Maesa chisia* Buch.-Ham.ex D. Don	Primulaceae	Bilaune	Shrub
23	*Mallotus philippinensis* Muell. Arg	Euphorbiaceae	Sindure	Tree
24	*Murraya* koenigii (L.) Spreng.	Rutaceae	Karipatta	Shrub
25	*Myrica esculenta* Buch.-Ham. ex D. Don	Myricaceae	Kaphal	Tree
26	*Persea odoratissima* (Nees) Kosterm.	Lauraceae	Kaulo	Tree
27	*Pinus roxhburghii* Sarg.	Pinaceae	Khote salla	Tree
28	*Prinsepia utilis*	Rosaceae	Dhatelo	Shrub
29	*Prunus cerasoides* D.Don	Rosaceae	Paiyun	Tree

30	*Prunus persica* (L.) Batsch	Rosaceae	Aaru	Tree
31	*Pyracantha crenulata* (D. Don) M. Roeme	Rosaceae	Ghangaroo	Shrub
32	*Pyrus pashia* Buch.-Ham. ex D.Don	Rosaceae	Mayal	Tree
33	*Quercus leucotricophora* A.Camus	Fagaceae	Sano banjh	Tree
34	*Quercus glauca* Thunb.	Fagaceae	Phalat	Tree
35	*Quercus incana* Roxb. Hort. Beng.	Fagaceae	Thulo banjh	Tree
36	*Quercus semecarpifolia* Smith in Rees	Fagaceae	Khasru	Tree
37	*Reinwardtia indica* Dumort.	Linaceae	Pyauli	Shrub
38	*Rhododendron arboretum* Sm.	Ericaceae	Lali gurans	Tree
39	*Rhus javanica* L.	Anacardiaceae	Bhaki amilo	Tree
40	*Rubus ellipticus* Sm.	Rosaceae	Ainselu	Shrub
41	*Salix* sp.	Salicaceae	Bains	Tree
42	*Sapindus mukorossi* Gaertn.	Sapindaceae	Rithha	Tree
43	*Sapium insigne* (Royle) Trimen	Euphorbiaceae	Khirro	Tree
44	*Schima wallichii* Choisy	Theaceae	Chilaune	Tree
45	*Smilax* sp.	Smilacaceae	Kukurdaino	Climber
46	*Stephania* sp.	Menispermaceae	Batulpate	Climber
47	*Tinospora sinensis* (Lour.) Merr.	Menispermaceae	Gurjo	Climber
48	*Toona ciliate* M.Roem.	Meliaceae	Tuni	Tree
49	*Viburnum erubescens* Wall.	Adoxaceae	Asare	Tree
50	*Zanthoxylum armatum* DC.	Rutaceae	Timur	Large shrub

The species richness for each plot was the number of species per plot. Significantly higher species richness ($p<0.001$) was recorded at Rim (1400-1700 m) and Chhatreshwori (1800-2000 m) while the species richness was significantly lower at Kupinde (1600-1800 m) (Figure 6) (Tables 9-14).

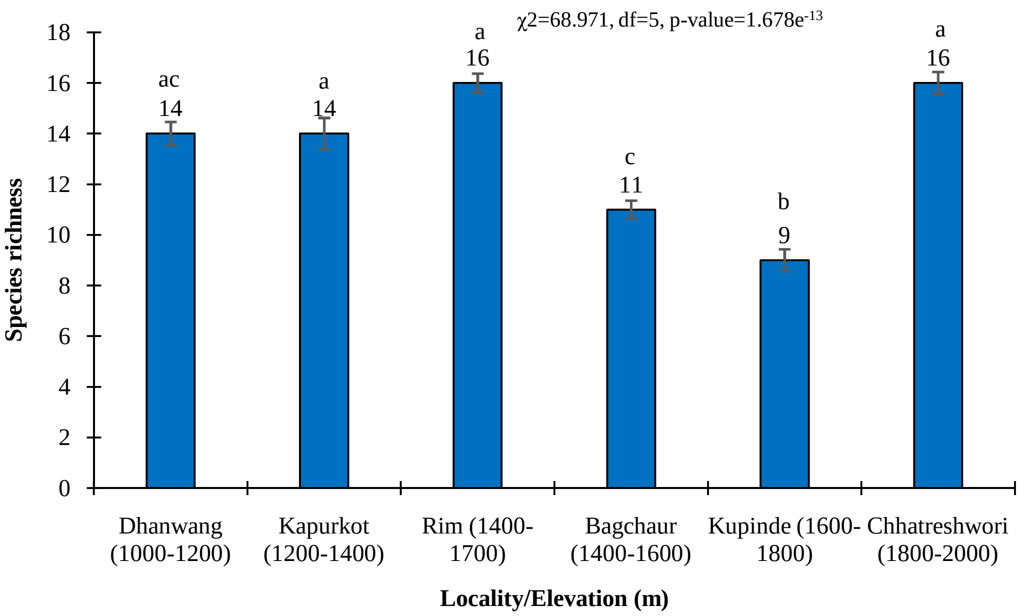


Figure 6: Species richness at different locality and elevation (m)

Note: Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$

For measuring the biodiversity of any area, species composition and species richness are considered as the important indices (Husch et al. 2002), and they may be heavily influenced or depend upon the different management techniques applied. The presence of 50 shrub and tree species (Table 7) in the study area demonstrates the richness of the area in terms of diversity. This situation can be compared with other similar researches conducted in Nepal, India and Bhutan in similar types of forest. 78 species of trees were recorded from Bhutan's west central region by Wangda and Ohsawa (2006), while 39 species of trees were identified by Buffum et al. (2008) from Bhutan's eastern region. Likewise, 81 species of trees were recorded from the temperate forest in Sikkim (Sundriyal and Sharma, 1996) and 31 and 37 plant species in two different localities between the elevation of 2650-2800 m in Nepal (Shrestha et al., 2013).

Because of the settlement and farm zones, the number of species recorded at Baghchaur (1400-1600 m) and Kupinde (1600-1800 m) (Figure 6) is lower than at the other sites. The total number of species in a highly disturbed site was only 16 whereas 47 species were found in a comparatively less disturbed area in the eastern region of India (Bhuyan et al., 2003). In the southern region of India, 34 species of trees were found in less disturbed area as compared to 14 species in highly disturbed areas (Sunil et al., 2011). Plantation forests in Nepal had the lowest number of trees with only 9 species (Webb & Sah, 2003). The intensity of disturbance produced by anthropogenic activities is attributed to the discrepancies in the outcomes in all of these studies.

There is generally a linear relationship between vegetation attributes like species richness, diversity, and ecological factors like altitude, aspect, and distance of the site from disturbance stimuli (Schuster & Diekmann 2005). A monotonic decline in the number of species with increasing elevation has often been considered a general pattern (Brown, 1988; Stevens, 1992). Inverse correlation between altitude and species richness in Himalayan alpines have also been established in several studies (Vetaas, 2000; Kala & Mathur, 2002; Panthi et al., 2007). However, the present study did not follow the similar pattern; maximum number of species was recorded at 1400-1700 m and 1800-2000 m. The high species richness may be attributed to less anthropogenic activities, higher soil moisture and greater topographic variations in habitat conditions.

4.1.2 Density

The mean population density of *Z. armatum* in the study area was found to be 913.33 individuals/ha. The density of *Z. armatum* among the different localities did not vary significantly (Figure 7A). Among the six localities studied, the total density of *Z. armatum* was maximum at Kupinde (1100 individuals/ha), followed by Baghchaur (1020 individuals/ha), Rim (1000 individuals/ha), Dhanwang (840 individuals/ha), Kapurkot (780 individuals/ha) and the lowest at Chhatreshwori (740 individuals/ha).

The highest relative density (15.45%) of *Z. armatum* was at Baghchaur and lowest (5.35%) at Chhatreshwori (Figure 7 B). It was found to be associated with different species at different localities and altitudes. Mostly it was found growing in the northern and northeastern slopes and had least occurrence on the south and southeastern slopes. A study in Indonesia showed that fully opened habitat with full sun exposure during daytime may not be the suitable habitat for the natural population of *Zanthoxylum acanthopodium* (Junaedi & Nurlaeni, 2019).

Among all the species, *Murraya koenigii* had the greatest density with a total of 1140 individuals/ha at Dhanwang, *Berberis aristata* at Rim with 980 individuals/ha and *Daphne bholua* at Chhatreshwori with with 1100 individuals/ha. Similarly *Z. armatum* had greatest density at Kapurkot, Baghchaur and Kupinde with 1000, 1100 and 1020 individuals/ha respectively (Tables 9-14). The species having lowest densities were *Ligustrum confusum, Tinospora cordifolia* (200 individuals/ha at Dhanwang), *Ficus semicordata* (260 individuals/ha at Dhanwang), *Clematis* sp. (200 individuals/ha at Rim).

Moist habitat, lower elevation, resource availability, moderate fragmentation along with variation in climate, level of disturbances, resource fluctuations, and limitation to dispersal are among the various factors that might influence the structure of population of any species (Shaheen et al., 2011a; Tiwari et al., 2012). The difference in the densities among different elevation and localities may be because of the differences in the composition of the soil and other climatic as well as abiotic factors. According to a study conducted in Indonesia, *Zanthoxylum acanthopodium* does not prefer to grow naturally in an open habitat exposed to full sun in the daytime (Junaedi & Nurlaeni, 2019).

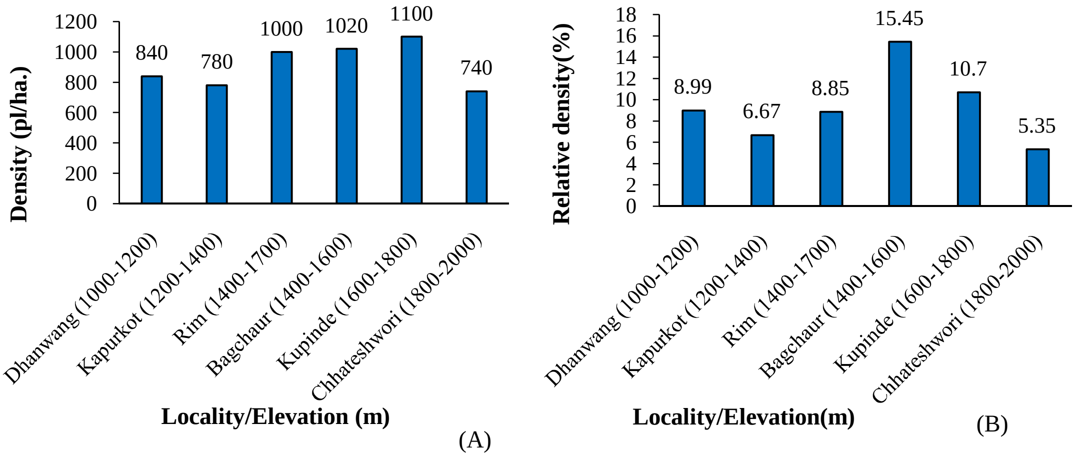


Figure 7: Density (A) and Relative density (B) of *Z. armatum* at different locality and elevation

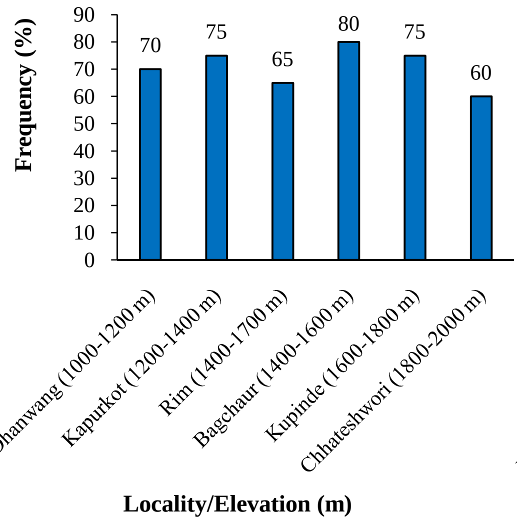
There have been ample studies on the population size and distribution of other medicinal plants in Nepal. But no study has been conducted previously on the population, distribution, frequency, and abundance of this species so no comparable data is available from Nepal. However, a study from India recorded a low population size of *Z. armatum* from the villages of Chamoli district of Uttarakhand, India; the average density was 368.2 individuals/ha. Due to low population size, it has been placed in the IUCN vulnerable category (Kala, 2010). The same study also recorded *Berberis aristata, Ficus, Grewia optiva, Pyrus pashia, Pyrecantha crenulata, Quercus*, etc. as the major associates of *Z. armatum* (Table 9-14).

The market price of the fruits is very good so the extensive demand of this species has put an enormous pressure on the natural population. Though the farmers have already started commercial cultivation of *Z. armatum*, the collection from wild has not yet decreased. Since the fruits are difficult to harvest because of the thorns, destructive harvesting without taking proper care is a common practice, creating a tremendous pressure on its existing population in the wild. Similar scenario also prevails in Uttarakhand, India where harvesting of the entire plant before setting even flowers along with the profuse invasion from woody weeds such as *Lantana* has negatively impacted the natural distribution of *Z. armatum* (Kala, 2010). Threats to *Z. armatum* from invasive species was however not evident at the study sites in the present study. Since the plant is thorny and aromatic, grazing by livestock is not a threat to the natural population of *Z. armatum,* instead it also provides shelter and protection to its associated species in the natural habitats by preventing from livestock grazing and browsing (Kala, 2010).

4.1.3 Frequency

The frequency of *Z. armatum* at different location and elevation in the study area did not vary significantly. The mean frequency was found to be 70.83% and the relative frequency was 5.61%. The highest frequency of *Z. armatum* was 80% at Baghchaur (1400-1500 m) and the lowest 60% was at Chhatreshwori (1800-2000 m) (Figure 8 A). In comparison with the other associated plant species, *Z. armatum* had the highest overall relative frequency of 8.02% at Kupinde (1600-1800 m) and had the lowest 3.82% at Chhatreshwori (1800-2000 m) (Figure 8 B). Table 9-14 lists the frequency and relative frequency of other associated plant species.

The mean frequency and relative frequency of *Z. armatum* in the study area were 70.83% and 5.61%, respectively. The highest relative frequency; 8.02% was at Kupinde (1600-1800 m) and lowest, 3.82 % at Chhatreshwori (1800-2000 m) as compared to its associates (Figure 8B). The frequency and relative frequency of other associates are presented in Table 9-14.



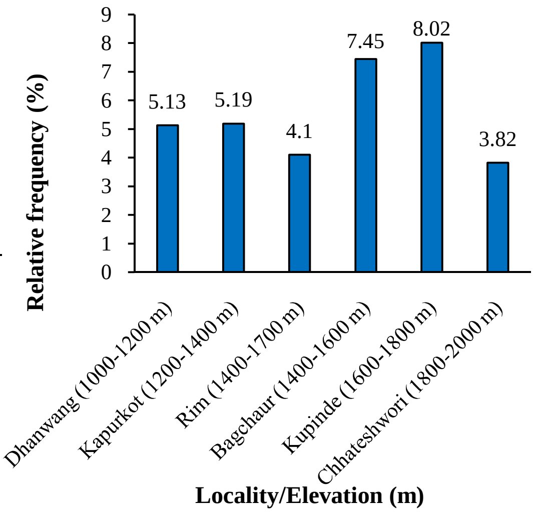
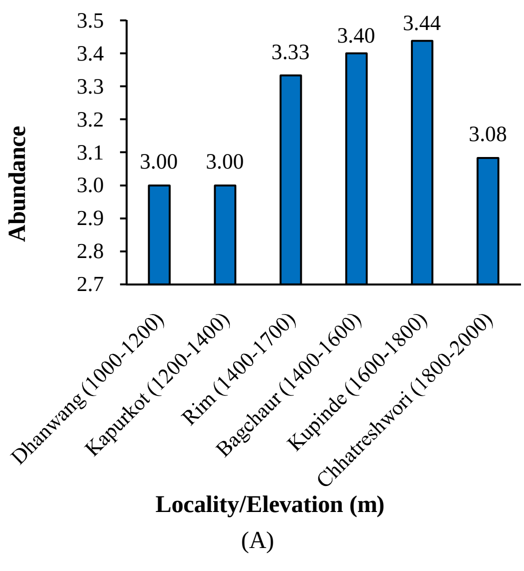


Figure 8: Frequency (%) (A) and relative frequency (%) (B) of *Z. armatum* at different locality and elevation.

The most frequently occurring associated plant species (with >70% of occurrence) in the study area at different elevation and locality were *Aesculus indica*, *Alnus nepalensis*, *Bauhinia variegata*, *Berberis asiatica*, *Castanopsis hystrix*, *Colebrookeaa oppositifolia*, *Daphne bholua*, *Ficus nerifolia*, *Fraxinus floribunda*, *Grewia optiva*, *Juglans regia*, *Lyonia ovalifolia*, *Maesa chisia*, *Murraya koenigii*, *Persea* *odoratissima, Pinus roxhburghii, Prinsepia utilis, Prunus cerasoides, Pyracantha crenulata, Pyrus pashia, Quercus glauca, Q. incana, Rhododendron arboreum*, and *Sapium insigne* (Table 9-14).

4.1.4 Abundance

The abundance of *Z. armatum* was almost similar and did not vary significantly in all the localities of the study area, the values ranging from 3.00 at Dhanwang and Kapurkot to 3.40 at Baghchaur and 3.44 at Kupinde (Figure 9A). Likewise, the highest total relative abundance (15.45%) was at Baghchaur and the lowest (5.35%) at Chhatreshwori (Figure 9B). Abundance of *Z. armatum* was highest (3.44) at Kupinde and relative abundance was highest (15.45%) at Baghchaur. The density and frequency values of *Z. armatum* were also high at Baghchaur and Kupinde. Nkoa et al. (2015) stated that the abundance is related to number (density) or frequency. The higher density and frequency might have influenced the abundance positively in this study also.



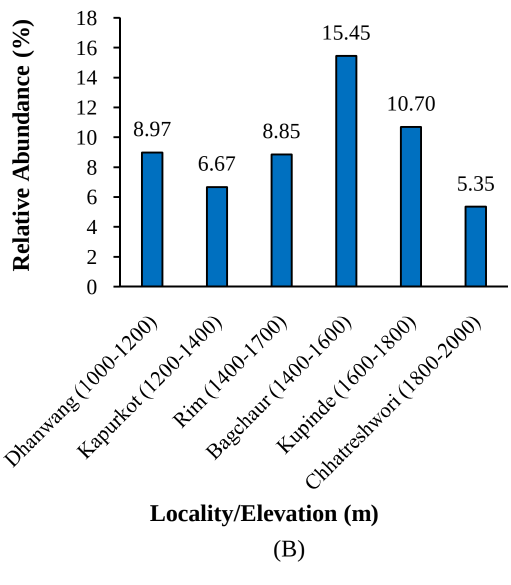


Figure 9: Abundance and relative abundance (%) of *Z. armatum* at different locality & elevation.

4.1.5 Importance Value Index (IVI)

The highest IVI value of *Z. armatum* was 38.35% at Baghchaur and the lowest value was 14.53% at Chhatreshwori (Figure 10). Based on IVI values, *Berberis asiatica* (22.88) and *Daphne bholua* (20.06) were the dominant species at Kapurkot and *Daphne bholua* at Chhatreshwori (Tables 9-14)). Likewise, *Z. armatum* was dominant at Baghchaur, Kupinde, Dhanwang, and Rim with the IVI values of 38.35, 29.42, 23.09, and 21.81 respectively (Figure 10).

The importance value index (IVI) depicts the significance of a species in relation to its ecological success and dominance (Misra, 1968). The variation in IVI between study sites in this study can be related to differences in the composition of the species, intensity of disturbance and elevation (Saravanan et al., 2019).

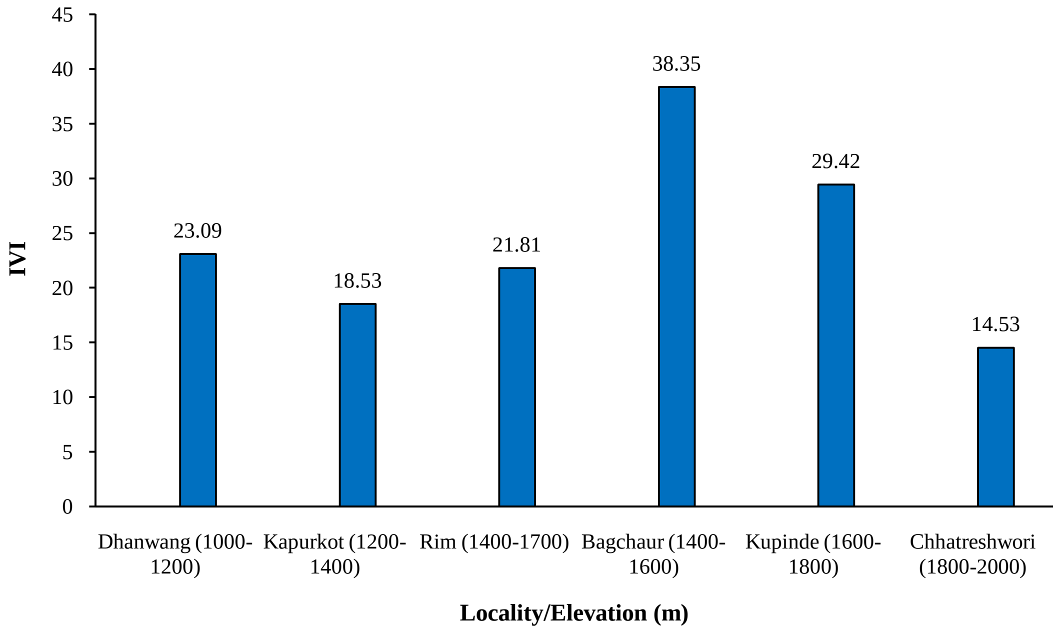


Figure 10: Importance Value Index (IVI) of *Z. armatum* at different locality and elevation.

4.1.6 Regeneration status

The natural regeneration potential of *Z. armatum* differed depending on elevation and location. The highest density of both the seedlings and saplings was at Kapurkot (1200-1400 m), while the lowest density was at Chhatreshwori (1800-2000 m). At Kapurkot, the total seedling and sapling densities were 200 and 140 individuals/ha, respectively, whereas at Chhatreshwori, the densities were 100 and 60 individuals/ha Likewise, the total seedling and sapling densities were 180 and 100 individuals/ha at Baghchaur (1400-1600 m) 160 and 100 individuals/ha at Dhanwang (1000-1200 m), 140 and 120 individuals/ha at Rim (1400-1700 m), 120 individuals/ha and 80 individuals/ha at Kupide (1600-1800 m) and that in Chhatreshwori were 100 and 60 individuals/ha, respectively (Table 8).

The average seedling and sapling densities of *Z. armatum* in the study area were 150 individuals/ha and 100 individuals/ha respectively. A study by Rawat & Chandhok (2009) reported saplings and seedlings layer densities ranging from 90 to 410 individuals/ha and 50 to 510 individuals/ha, respectively. In another study, the seedling and sapling layer density ranged from 340 to 1190 individuals/ha. for seedlings and 340 to 920 individuals/ha for saplings (Saha et al., 2016). The densities of seedlings and saplings in the present study did not show any specific pattern for elevation gradient. However, a study by Gairola et al. (2008) for different high altitude Himalayan forests showed maximum seedling density throughout the altitudinal strata suggesting that the slope and aspect favor regeneration of tree species. Similarly, significant difference was observed between top hill and bottom hill positions, with the highest amount of regeneration in the bottom and lowest amount of regeneration in the top hill (Nur et al., 2016).

Table 8: Seedling, sapling and adult density of *Z. armatum* at different locality/elevation.

SN	Samplig sites	Density (pl/ha)		
		Seedling	Sapling	Adult
1	Dhanwang (1000-1200)	160	100	840
2	Kapurkot (1200-1400)	200	140	780
3	Rim (1400-1700)	140	120	1000
4	Bagchaur (1400-1600)	180	100	1020
5	Kupinde (1600-1800)	120	80	1100
6	Chhatreshwori (1800-2000)	100	60	740
	Average	150	100	913.33

The quantity of seedlings and saplings determines the regeneration potential of any species (Dhar et al., 1997; Singh & Singh, 1992). The densities of seedlings and saplings of *Z. armatum* in the study area were much lower than the adult densities. One probable reason for this trend could be because the fruits were harvested early before they had even fallen off the seeds on the ground. This practice has a severe impact on the natural seed bank stock, as well as density of seedlings and saplings. Based on the regeneration status table (Table 4; Bhuyan et al., 2003; Khumbongmayum et al., 2006), it can be concluded that the regeneration status of *Z. armatum* is fair in the study locality. However, comparatively good regeneration potential was found at Kapurkot and the lower regeneration status of *Z. armatum* was observed at Chhatreshwori, among the six localities of the study area.

The continuous survival of any species of a community is governed by the process of natural regeneration. The ability of the species to generate new seedlings, their growth and survival are the three key components of effective regeneration (Khan and Saikia, 2013). Successful regeneration is defined as the presence of a sufficient number of seedlings, saplings, and young trees in any given population (Saxena & Singh, 1984), which is influenced regularly by the biotic interactions and anthropogenic disturbances. The possible state of regeneration of tree species in a forest stand in space and time determines the future composition of the forest in general (Henle et al., 2004).

Natural regeneration is a key process for the continued existence of a species in a community. The three major components of successful regeneration are the ability of species to initiate new seedlings, their survival and growth (Good & Good, 1972; Saikia & Khan, 2013). Presence of sufficient number of seedlings, saplings and young trees in a given population indicates successful regeneration (Saxena & Singh, 1984), which is frequently influenced by the biotic interactions and anthropogenic disturbances. Generally, regeneration of a species is usually affected by anthropogenic and natural factors. Seed germination rate in *Z. armatum* is very low (Phuyal et al., 2018) and hindered by the presence of hard seed coat; the seeds undergo a strong dormancy and may take few months to years for germination (Chadha, 1976). Furthermore, the solitary seeds in the fruit also limit the quantities of seed (Singh & Rawat, 2017) and lower the rate of germination. Because of the high demand of *Z. armatum*, its commercial cultivation in the study area is escalating during the last few years. There is also a high demand of plantlets but the supply is minimum due to lack of nurseries. District Forest and Plant Resources offices at Salyan provided free plantlets to the interested farmers, still the supply is inadequate to meet the demand. This has put a high pressure on the naturally regenerating seedling and saplings in the natural forests as the villagers uproot the seedlings and saplings from the forest to plant them in their farmland, which greatly alters the regeneration status of *Z. armatum* naturally. Furthermore, the fruits are prematurely harvested from the wild, probably affecting the seedbank of *Z. armatum*, leading to lower production of seedlings.

The reason behind less density, frequency, abundance and regeneration at Chhatreshwori might be due to the overexploitation by the local people as they collect bigger trees for their own consumption and extra income.

4.1.7 Distribution

Z. armatum had nearly identical pattern of distribution in all of the locality studied (Figure 11). Based on the abundance/frequency (A/F; Whitford index) values of 0.04 and 0.05 at all localities, it can be said that *Z. armatum* has random distribution in the study area. It was found to be growing in scattered patches in association with other species. There were no pure stands of *Z. armatum* anywhere in the study area. Considerable differences in the distribution of *Z. armatum* were observed most likely due to changes in the intensity of disturbance and management practices along with varied ecological parameters.

Due to anthropogenic disruptions, the natural distribution of this important species has been affected seriously in the recent years, a tendency that has been observed for other Himalayan medicinal plants as well (Vashistha et al., 2006). The native population of *Z. armatum* is under jeopardy because of the rising market demand and unsustainable exploitation. It has been identified one of many other MAPs that are collected with a strong preference for use in both market and locally (Kunwar et al., 2015).

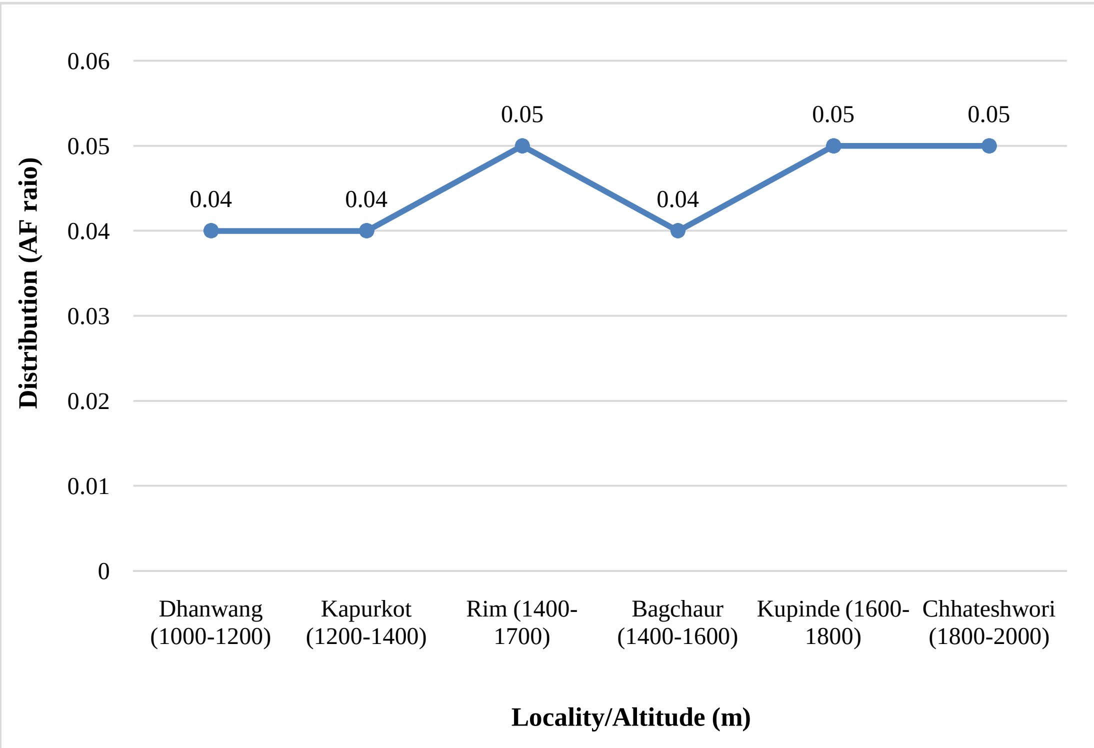


Figure 11: Distribution pattern (A/F ratio) of *Z. armatum* at different locality and altitude

Anthropogenic interferences like unsustainable harvesting and digging up of the saplings and seedlings to plant them in farmyard were found to be the major cause affecting the natural distribution of *Z. armatum* in the study area. Because it is a thorny plant, damage from grazing was not as noticeable as it is for many other medicinal plants. There was a huge discrimination in the harvesting pattern and collection of *Z. armatum* in the study area. During field visit at the fruiting season, it was a very common scene that the plants in farmers' farmyard were overloaded with ripe fruits while those in the wild were harvested prematurely. Plants in the farmyard were considered private and those in the forest were public so whoever saw them first would harvest haphazardly. Though the farmers were aware of the economic benefits of the species, there seems to be a lack of awareness towards the conservation and sustainable harvesting of the species from the wild.

Table 9: Total population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Dhanwang (1000-1200 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Albizzia procera*	300	3.21	60	4.40	1.25	3.21	10.82	0.02
2	*Bauhinia variegata*	280	3	50	3.66	1.40	2.99	9.65	0.03
3	*Berberis aristata*	640	6.85	70	5.13	2.29	6.84	18.82	0.03
4	*Castanopsis hystrix*	320	3.43	75	5.49	1.00	2.14	11.06	0.01
5	*Colebrookea oppositifolia*	500	5.35	75	5.49	1.14	3.42	14.26	0.02
6	*Ficus semicordata*	300	3.21	55	4.03	1.67	5.34	12.58	0.03
7	*Ligustrum confusum*	200	2.14	45	3.30	1.36	3.21	8.65	0.03
8	*Maesa chisia*	600	6.42	80	5.86	1.11	2.14	14.42	0.01
9	*Mallotus philippinensis*	400	4.28	45	3.30	1.88	6.41	13.99	0.04
10	*Murraya koenigii*	1140	12.21	85	6.23	2.22	4.27	22.71	0.03
11	*Myrica esculenta*	240	2.57	50	3.66	3.35	12.18	18.41	0.07
12	*Pinus roxhburghii*	1000	10.71	80	5.86	1.20	2.56	19.13	0.02
13	*Pyrus pashia*	280	3	50	3.66	3.13	10.68	17.34	0.06
14	*Salix* sp.	380	4.07	60	4.40	1.40	2.99	11.46	0.02
15	*Sapindus mukorossi*	260	2.78	60	4.40	1.58	4.06	11.24	0.03
16	*Sapium insigne*	240	2.57	55	4.03	1.08	2.78	9.38	0.02
17	*Schima wallichii*	380	4.07	60	4.40	1.09	2.56	11.03	0.02
18	*Smilax* sp.	320	3.43	60	4.40	1.58	4.06	11.89	0.03
19	*Stephania sp*	280	3	65	4.76	1.33	3.42	11.18	0.02
20	*Tinospora sinensis*	200	2.14	50	3.66	1.08	2.99	8.79	0.02
21	*Toona ciliata*	240	2.57	65	4.76	1.00	2.78	10.11	0.02
22	*Zanthoxylum armatum*	840	8.99	70	5.13	3.00	8.97	23.09	0.04

Table 10: Total Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Kapurkot (1200-1400 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Albizia procera*	300	2.65	55	3.81	1.36	2.65	9.12	0.02
2	*Alnus nepalensis*	500	4.42	65	4.50	1.92	4.42	13.35	0.03
3	*Benincasa hispida*	380	3.36	65	4.50	1.46	3.36	11.22	0.02
4	*Berberis aristata*	980	8.67	80	5.54	3.06	8.67	22.88	0.04
5	*Castanopsis indica*	480	4.25	70	4.84	1.71	4.25	13.34	0.02
6	*Clematis* sp.	300	2.65	70	4.84	1.07	2.65	10.15	0.02
7	*Daphne bholua*	840	7.43	75	5.19	2.80	7.43	20.06	0.04
8	*Ficus nerifolia*	560	4.96	75	5.19	1.87	4.96	15.10	0.02
9	*Ficus semicordata*	260	2.30	60	4.15	1.08	2.30	8.75	0.02
10	*Lyonia ovalifolia*	820	7.26	75	5.19	2.27	6.02	18.46	0.03
11	*Maesa chisia*	680	6.02	70	4.84	2.93	7.26	18.12	0.04
12	*Myrica esculenta*	360	3.19	70	4.84	2.43	6.02	14.05	0.03
13	*Persea odoratissima*	300	2.65	65	4.50	1.38	3.19	10.34	0.02
14	*Pinus roxhburghii*	600	5.31	60	4.15	1.25	2.65	12.12	0.02
15	*Prunus cerasoides*	300	2.65	75	5.19	2.14	5.31	13.15	0.03
16	*Prunus persica*	360	3.19	65	4.50	1.15	2.65	10.34	0.02
17	*Pyrecantha crenulata*	500	4.42	65	4.50	1.38	3.19	12.11	0.02
18	*Quercua leucotricophora*	400	3.54	75	5.19	1.67	4.42	13.15	0.02
19	*Quercus semecarpifolia*	680	6.02	65	4.50	1.54	3.54	14.06	0.02
20	*Rhododendron arboreum*	700	6.19	70	4.84	2.50	6.19	17.23	0.04
21	*Zanthoxylum aramtum*	780	6.67	65	4.19	3.00	6.67	17.53	0.04

Table 11: Total Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Rim (1400-1700 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Alnus nepalensis*	420	3.59	65	4.10	1.62	3.59	11.28	0.02
2	*Berberis aristata*	960	8.21	80	5.05	3.00	8.21	21.46	0.04
3	*Castanopsis indica*	340	2.91	65	4.10	1.31	2.91	9.91	0.02
4	*Clematis* sp.	200	1.71	50	3.15	1.00	1.71	6.57	0.02
5	*Cotoneaster microphyllus*	440	3.76	60	3.79	1.83	3.76	11.31	0.03
6	*Daphne bholua*	540	4.62	75	4.73	1.80	4.62	13.96	0.02
7	*Ficus neriifolia*	280	2.39	60	3.79	1.17	2.39	8.57	0.02
8	*Fraxinus floribundus*	400	3.42	70	4.42	1.43	3.42	11.25	0.02
9	*Lyonia ovalofolia*	740	6.32	75	4.73	2.47	6.32	17.38	0.03
10	*Maesa chisia*	320	2.74	65	4.10	1.23	2.74	9.57	0.02
11	*Myrica esculenta*	780	6.67	65	4.10	3.00	6.67	17.43	0.05
12	*Persea odoratissima*	440	3.76	65	4.10	1.69	3.76	11.62	0.03
13	*Pinus roxhburghii*	860	7.35	75	4.73	2.87	7.35	19.43	0.04
14	*Prunus cerasoides*	400	3.42	70	4.42	1.43	3.42	11.25	0.02
15	*Pyrecantha crenulata*	360	3.08	70	4.42	1.29	3.08	10.57	0.02
16	*Pyrus pashia*	560	4.79	80	5.05	1.75	4.79	14.62	0.02
17	*Quercus leucotricophora*	560	4.79	75	4.73	1.87	4.79	14.30	0.02
18	*Reinwardtia indica*	300	2.56	70	4.42	1.07	2.56	9.54	0.02
19	*Rododendron arboreum*	760	6.50	75	4.73	2.53	6.50	17.72	0.03
20	*Rubus ellipticus*	400	3.42	75	4.73	1.33	3.42	11.57	0.02
21	*Smilax* sp.	520	4.44	65	4.10	2.00	4.44	12.99	0.03
22	*Viburnum erubescens*	340	2.91	70	4.42	1.21	2.91	10.23	0.02
23	*Zanthoxylum armatum*	1000	8.85	75	5.19	3.33	8.85	22.89	0.05

Table 12: Total Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Baghchaur (1400-1600 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Alnus nepalensis*	820	7.98	75	6.64	2.73	7.98	22.59	0.04
2	*Bauhinia variegata*	520	5.06	80	7.08	1.63	5.06	17.20	0.02
3	*Berberis aristata*	760	7.39	85	7.52	2.24	7.39	22.31	0.03
4	*Fraxinus floribunda*	660	6.42	85	7.52	1.94	6.42	20.36	0.02
5	*Grevia optiva*	500	4.86	80	7.08	1.56	4.86	16.81	0.02
6	*Lyonia ovalifolia*	1060	10.31	80	7.08	3.31	10.31	27.70	0.04
7	*Persea odoratissima*	440	4.28	75	6.64	1.47	4.28	15.20	0.02
8	*Pinus roxhburghii*	680	6.61	90	7.96	1.89	6.61	21.19	0.02
9	*Prunus cerasoides*	740	7.20	80	7.08	2.31	7.20	21.48	0.03
10	*Pyrecantha crenualta*	460	4.47	75	6.64	1.53	4.47	15.59	0.02
11	*Pyrus pashia*	780	7.59	85	7.52	2.29	7.59	22.70	0.03
12	*Rhododendron arboreum*	1180	11.48	85	7.52	3.47	11.48	30.48	0.04
13	*Sapium insigne*	580	5.64	75	6.64	1.93	5.64	17.92	0.03
14	*Zanthoxylum armatum*	1020	15.45	75	7.94	3.4	15.45	38.84	0.04

Table 13: Total Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Kupinde (1600-1800 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Berberis aristata*	640	9.70	75	7.94	2.13	9.70	27.33	0.03
2	*Castanopsis hystrix*	580	8.79	85	8.99	1.71	8.79	26.57	0.02
3	*Daphne bholua*	480	7.27	85	8.99	1.41	7.27	23.54	0.02
4	*Ficus semicordata*	300	4.55	65	6.88	1.15	4.55	15.97	0.02
5	*Lyonia ovafolia*	440	6.67	80	8.47	1.38	6.67	21.80	0.02
6	*Myrica esculenta*	580	8.79	65	6.88	2.23	8.79	24.45	0.03
7	*Pinus roxhburghii*	420	6.36	70	7.41	1.50	6.36	20.13	0.02
8	*Princepia utilis*	280	4.24	60	6.35	1.17	4.24	14.83	0.02
9	*Pyrus pahsia*	400	6.06	75	7.94	1.33	6.06	20.06	0.02
10	*Quercus incana*	380	5.76	65	6.88	1.54	6.06	18.70	0.02
11	*Rhododendron arboreum*	400	6.06	75	7.94	2.27	10.30	24.30	0.03
12	*Rhus javanica*	680	10.30	70	7.41	1.36	5.76	23.47	0.02
13	*Zanthoxylum armatum*	1100	10.70	80	7.08	3.44	10.70	28.48	0.05

Table 14: Total Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Chhatreshwori (1800-2000 m asl)

SN	Name of the plant	Density	RD (%)	Frequency (%)	RF (%)	Abundance	RA (%)	IVI	Distribution (A/F ratio)
1	*Adhatoda vesica*	440	3.18	65	4.13	1.69	3.18	10.49	0.03
2	*Aesculus indica*	700	5.07	75	4.76	2.33	5.07	14.90	0.03
3	*Berberis aristata*	520	3.76	60	3.81	2.17	3.76	11.33	0.04
4	*Daphne bholua*	1100	7.96	75	4.76	3.67	7.96	20.68	0.05
5	*Fraxinus floribundus*	500	3.62	70	4.44	1.79	3.62	11.68	0.03
6	*Juglans regia*	520	3.76	80	5.08	1.63	3.76	12.60	0.02
7	*Pistacia integerrima*	420	3.04	60	3.81	1.75	3.04	9.89	0.03
8	*Lyonia ovalifolia*	680	4.92	70	4.44	2.43	4.92	14.28	0.03
9	*Maesa chisia*	940	6.8	80	5.08	2.94	6.80	18.68	0.04
10	*Myrica esculenta*	640	4.63	75	4.76	2.13	4.63	14.02	0.03
11	*Persia odoratissima*	500	3.62	60	3.81	2.08	3.62	11.05	0.03
12	*Princepia utilis*	700	5.07	75	4.76	2.33	5.07	14.90	0.03
13	*Prunus cerasoides*	320	2.32	65	4.13	1.23	2.32	8.76	0.02
14	*Pyrecantha crenulata*	480	3.47	60	3.81	2.00	3.47	10.75	0.03
15	*Pyrus pashia*	360	2.6	60	3.81	1.50	2.60	9.01	0.03
16	*Quercus glauca*	900	6.51	85	5.40	2.65	6.51	18.42	0.03
17	*Quercus incana*	460	3.33	75	4.76	1.53	3.33	11.42	0.02
18	*Quercus semecarpofolia*	340	2.46	60	3.81	1.42	2.46	8.73	0.02
19	*Rhododendron arboreum*	780	5.64	75	4.76	2.60	5.64	16.05	0.03
20	*Rhus javanica*	660	4.78	65	4.13	2.54	4.78	13.68	0.04
21	*Salix* sp.	640	4.63	75	4.76	2.13	4.63	14.02	0.03
22	*Sapium insigne*	480	3.47	50	3.17	2.40	3.47	10.12	0.05
23	*Zanthoxylum armatum*	740	5.35	60	3.82	3.08	5.35	14.53	0.05

4.1.8 Species diversity

Diversity values according to the Shannon and Simpson indices were higher at Rim and lower at Kupinde. The mean Shannon-Weaver diversity index (H’) which measures the diversity of *Z. armatum* along with its associates ranged from 2.5 to 3.08. Among the six study sites the highest species diversity (H’ = 3.08) was recorded from Chhatreshwori whereas the lowest species diversity (H’ = 2.5) was recorded at Kupinde. The mean Simpson's diversity index (1−D) value ranged from 0.92 to 0.95 (Figure 12). The higher value of the diversity indices is an obvious indication of high species diversity and abundance (Adekunle et al., 2013). This diversity index is comparable to that found in the tropical forest of Eastern Ghats ranging between 3.76 - 3.96 (Naidu & Kumar, 2016). Forests with Shannon index greater than 2 are considered as medium to highly diverse in terms of species (Giliba et al., 2011). Thus from the findings of this study it can be said that the study area falls in the category of forests with high diversity.

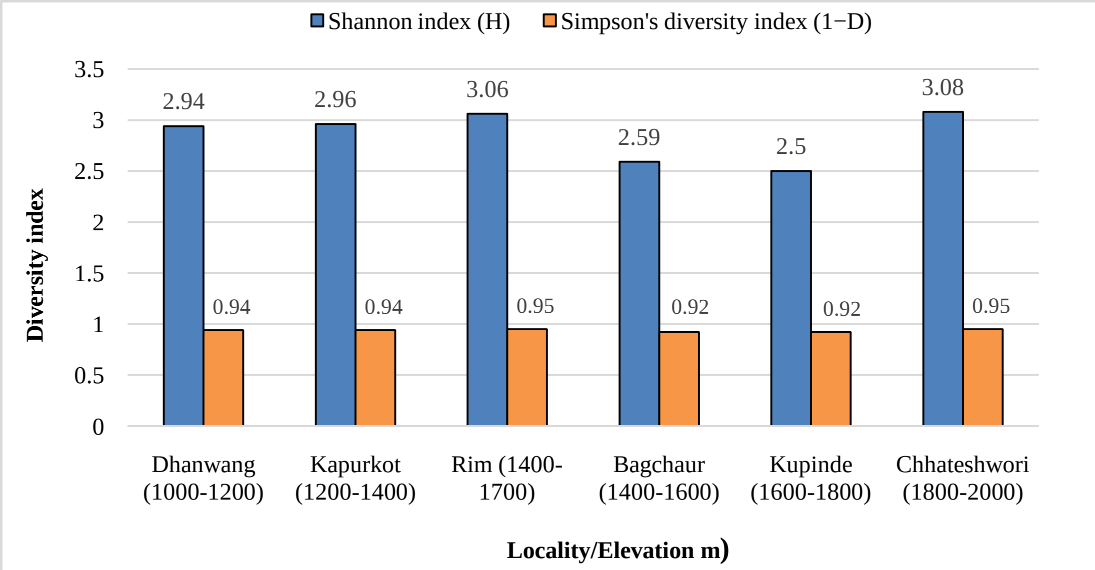


Figure 12: Simson's diversity index and Shannon-Weaver index for *Z. armatum* and its associates

The mean average species diversity of 2.8 recorded in the present study is comparable to the results of similar investigations in different Himalayan regions: 1.53-2.88 in the western Himalayas (Samant et al., 1998; Gaur & Joshi, 2006), 2.39-4.63 in the Gharwal Himalayas (Nautiyal & Guar, 1999), 2.5-3.10 in the trans-Himalayan alpines of Nepal (Panthi et al. 2007) and 3.13 in the alpine pastures of Kashmir, Pakistan (Shaheen et al., 2011b).

The government of Nepal has also prioritized *Z. armatum* as one of the most significant medicinal plants for economic development, with the prime focus on agro-technology development and cultivation (DPR, 2006). The fruits have a very good market is also extremely good, therefore there is a lot of demand for this species, putting a lot of pressure on the natural population. Despite the fact that the farmers have begun commercial farming in their farmland, the harvesting of fruits from the wild has not yet decreased.

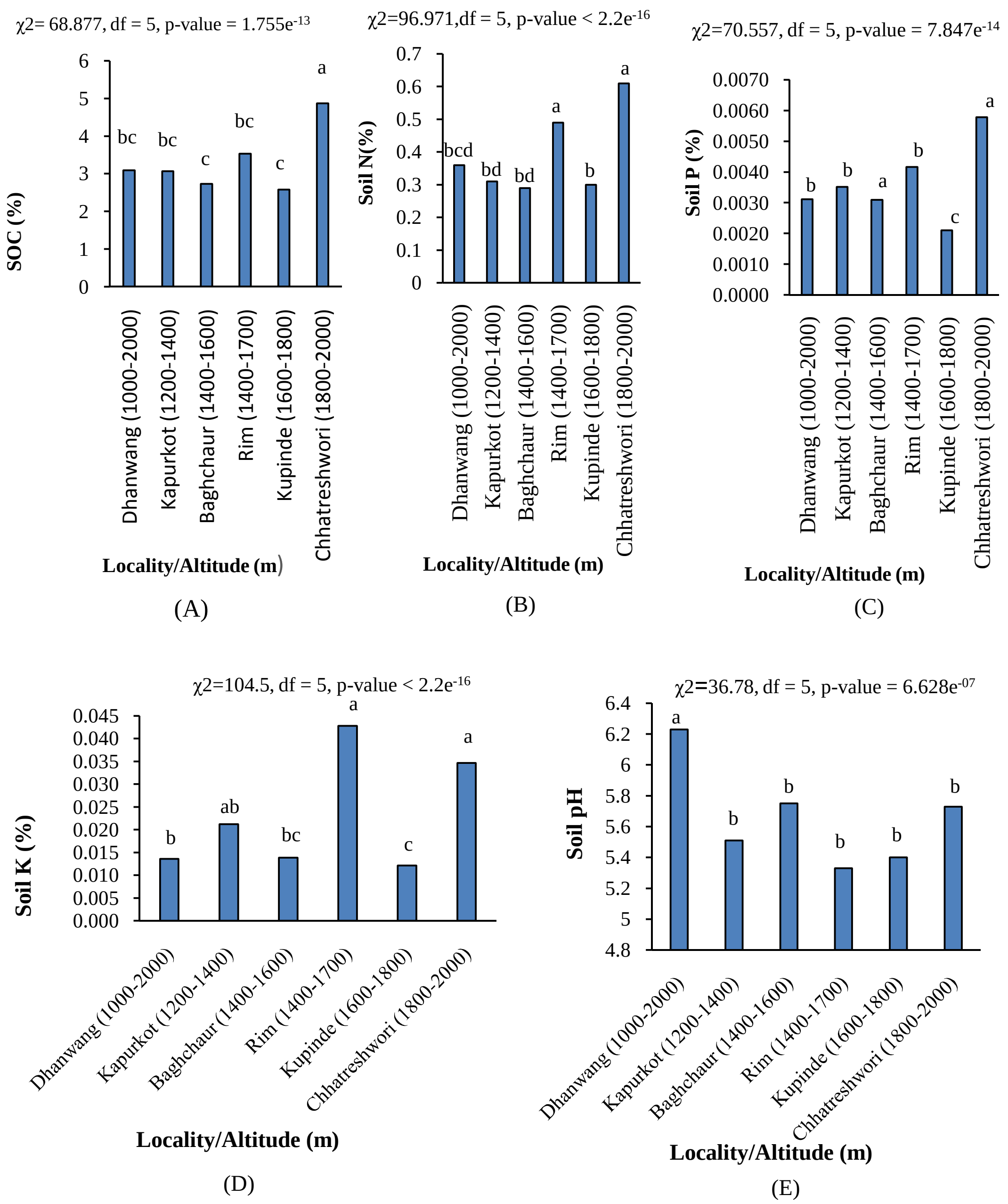


Figure 13: Variation in soil chemical properties: Soil Organic Carbon (A), Soil nitrogen (B), Soil phosphorus (C), Soil potassium (D), and Soil pH (E) at different locality and altitude

Note: Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$

Various factors like temperature, precipitation, latitude and area substantially affect the pattern of distribution and diversity of any species (Zhang et al., 2011). Climate also governs the large scale pattern of distribution of the species and physiognomy. Climate may be defined by a number of different variables, the majority of which impact the pattern of distribution of a species in any area (Bakkenes et al., 2002).

The variations in the types of soil, pattern of rainfall trends, change in land use pattern, anthropogenic disturbances among many other factors might have caused the differences in diversity among the different elevation and locality of the study area. The forest in Kupinde has been completely degraded due to road development and landslides, which could be a reason for the low diversity seen there. Road networks encourage resource extraction and forest encroachment, resulting in the loss of biodiversity (Sundriyal & Sharma 1996; Hitimana et al., 2004). Because of the relatively high soil nutrients, Chhatreshwori has a higher species richness and diversity. The direct link between soil nutrients and species diversity has been proved by various studies. Soil fertility has a significant impact on species diversity, as proved by Grime (1973), Tilman & Pacala (1992). Loreau et al. (2001) also stated that species diversity is often linked to soil fertility. At Chhatreshwori, the forest was relatively dense and moist. Moisture is also one of the important determining factor that influences the species composition and richness (Vetaas, 2000). The vegetation composition of any area is greatly influenced by the nutrients, organic matter and moisture present in the soil (Tang, 1990).

Overall, due to the increase in population size and the overexploitation of forests, change of land use among other factors; negative impacts can result on the forest ecology including reduction of plant stock, disruption of regeneration, and loss of nutrients in harvested materials (Murkherjee and Chaturvedi, 2017). Anthropogenic disturbances including premature harvesting and digging up of saplings was found to severely affect the natural distribution and regeneration in the study area. Other factors that may affect the sustainability of plants are collection of premature plants, grazing, and soil erosion. Therefore, deliberate efforts should be taken by all stakeholders to ensure that these plants are used in a sustainable way. Effective conservation and management initiatives are most important for conserving the wild genetic diversity of *Z. armatum* in the study area.

4.2 Fruit yield

The yield of *Z. armatum* fruits per plant varied significantly ($p<0.001$) at different altitudes (Figure 14). The overall yield was found to be increasing along with increasing elevation up to 1600-1800 m. The average yield per plant was 4.18 kg (fresh wt) and 2.91 kg (dry wt) with the lowest yield of 3.4 kg (fresh wt), 2.16 kg (dry wt) at 1000-1200 m and the highest of 5.7 kg (fresh wt), 3.22 kg (dry wt) at 1600-1800 m elevation. The fruit yield at 1800-2000 m elevation was 5.22 kg (fresh wt) and 3.15 kg (dry wt).

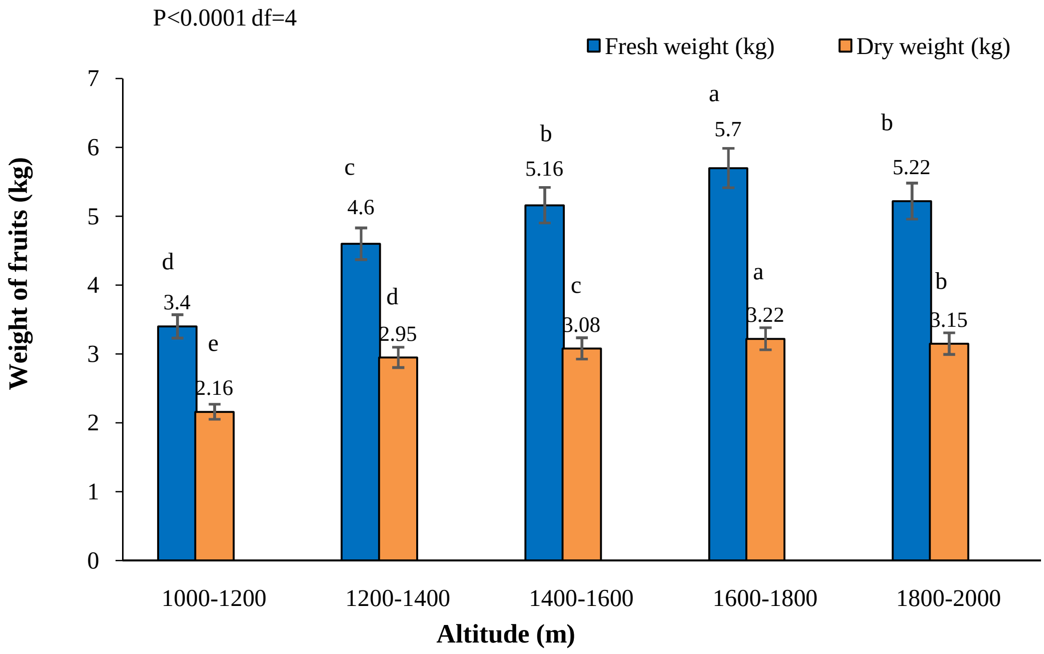


Figure 14: Variations in fruit yield of *Z. armatum* at different altitudes.

Note: Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$

The plants are ready for harvest after three years of plantation and the average annual yield of a five years plant is about 3.5 kg (Anonymous, 2011), which is consistent with the findings of the present study. The yield is a complex of many factors including the source of seedlings (whether germinated from seeds or vegetatively propagated), integrated manuring, management practices like thinning, pruning, harvesting period, post-harvest procedures as well as climatic conditions. Farmers do the periodic lopping of old and matured branches to increase the fruit production. The differential rate of fruit yield at different elevations might also be due to difference in nutrient contents in the soil and the manures used in the soil. The higher yield at 1600-1800 m elevation has correlation with higher content of soil nutrients (Table 16). Combination of soil and management factors has been found to affect yield; the crops that grow on soils with higher fertility often have higher yields (Fischer et al., 2020).

There was a strong correlation (p=0.05) between soil organic carbon, nitrogen, phosphorus and potassium and fruit yield (Table 15) indicating that the nutrients in soil were closely related to fruit yield. The soil nutrient elements directly affect growth, fruit setting, retention, yield, quality, and the sustainable production of orchard. So the efficient use of fertilizers to increase fruit yield is an important goal in all agricultural manipulation systems (Li et al., 2017).

Table 15: Pearson correlation between soil nutrients and weight of fruits (fresh and dry)

SN	Soil chemicals	Fresh Weight	Dry Weight
1	Organic carbon	0.57*	0.48*
2	Nitrogen	0.56*	0.45*
3	Phosphorus	0.57*	0.54*
4	Potassium	0.54*	0.48*
5	pH	0.82*	0.72*

* Correlation is significant at 0.05

4.3 Soil chemical analysis

The soil nutrient analysis (organic carbon, nitrogen, available phosphorus exchangeable potassium, and pH) of *Z. armatum* cultivated farmland at different altitudes is presented in Table 16. The soil organic carbon (SOC) increased with increasing elevation up to 1800 m, and then it decreased. Highest SOC (5.07±5.02%) was found at 1600-1800 m, followed by 4.13±0.27% at 1800-2000 m elevation and the lowest (2.63±0.24%) was at 1000-1200 m elevation. Rate of production of *Z. armatum* is higher above 1500 m in the study area, most of the farmers are engaged in commercial farming of the plant so they use a lot of farmyard manure in their fields.

Total nitrogen and available potassium did not show any specific trend with elevation. Total nitrogen was highest (6051.02±24.67 ppm) at 1600-1800 m and lowest (2612.58±189.04 ppm) at 1000-1200 m elevation. Similarly, available potassium was the highest (422.32±49.13 ppm) at 1600-1800 m and lowest at 1400-1600 m elevation, whereas the available phosphorus showed an increasing trend with increasing elevation with the highest (57.75±4.87 ppm) at 1800-2000 m and the lowest (12.60±0.32 ppm) at 1000-1200 m elevation. Soils at all the elevations were found slightly acidic, except at 1000-1200 m, with the pH value of 6.79±0.19. The acidity of soil was not consistent with the elevation trend. The soil of the farmlands had highest soil organic carbon and NPK.

Table 16: Soil chemical properties of *Z. armatum* cultivated farmlands along different altitude

SN	Altitude (m)	SOC (%)	Total N (%)	Available P (%)	Available K (%)	pH
1	1000-1200	2.63±0.24	0.26±0.01	0.0012±0.0003	0.013±0.002	6.79±0.19
2	1200-1400	3.39±0.07	0.37±0.02	0.0029±0.0002	0.021±0.002	6.27±0.09
3	1400-1600	3.19±0.35	0.32±0.02	0.0031±0.0002	0.011±0.002	5.8±0.14
4	1600-1800	5.07±5.02	0.6±0.17	0.0043±0.0001	0.042±0.001	5.62±0.09
5	1800-2000	4.13±0.27	0.37±0.01	0.0057±0.0001	0.035±0.001	6.19±0.03

4.4 Seed biology

4.4.1 Seed size and weight

Z. armatum fruit produces a solitary seed. The seeds are glossy black in color and roughly rounded in shape, with a very hard seed coat. The size of seeds at different altitudes varied significantly ($p<0.001$) (Figure 15 A). The average length and breadth of seeds are 4.6 mm and 3.9 mm, respectively. The size of seeds also showed an increasing pattern with the increase in altitude up to 1600-1800 m and decreased thereafter. The diameter of seeds was highest at 1600-1800 m and lowest at 1000-1200 m elevation. The weight of seeds were also significantly different ($p<0.001$) at different altitudes. Average weight of one air dried seed was 21.6 mg. The weight of seeds also showed similar pattern like the diameter, with the highest value at 1600-1800 m and lowest at 1000-1200 m elevation (Figure 15 B).

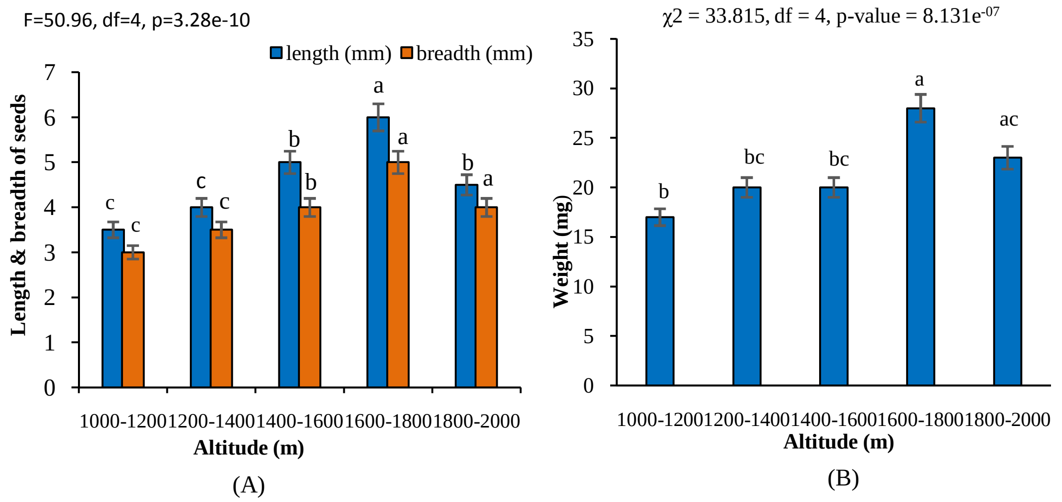


Figure 15: Length and breadth (mm) (A) and dry weight (mg) (B) of seeds of *Z. armatum* collected from different altitudes.

Note: Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$

The soil nutrient analysis of *Z. armatum* cultivated farmland showed that the soil organic carbon, total nitrogen, available phosphorus and potassium were comparatively higher at 1600-1800 m and lower at 1000-1200 m (Table 16), which could be a reason for increased seed shape and weight at 1600-1800 m altitude.

Seed size, seed mass, and seed germination are some of the ecological strategic traits of plants linking to the seed dispersal and plant regeneration (Hamilton et al., 2005). Seed size has an impact on germination and survival under stressful circumstances. Plants from larger seeds, compared with those from smaller seeds, show a competitive advantage on germination and seedling stress tolerance (Larios et al., 2014). Seed size is an important morphological trait that may affect the population regeneration process and the production of seedlings (Mao et al., 2019).

Seed mass can influence the probability of seedling establishment by affecting the distance to which seeds disperse and the probability of early survival (Westoby et al., 1992) as well as other traits like flower size, plant longevity (Guo et al., 2000; Leishman et al., 2000). Seedlings from larger seeds have been found to have a higher probability of survival and emergence from beneath the forest litter layer in comparison to seedlings emerging from relatively small seeds (Gross 1984; Tripathi & Khan, 1990). Larger reserve in seeds may allow more pre-photosynthetic growth of seedlings, which may contribute to better growth and survival (Burslem & Miller 2001).

4.4.2 Seed germination

It was found that the seeds of *Z. armatum* did not germinate easily. Pre-sowing treatments affected the germination rate to some extent but the results were not so encouraging. Different treatments showed differential results. Seed viability decreased with storage for long period. Chemical and hormonal treatments had relatively some effects on the rate of germination. The seeds in general started to germinate after 25 days of sowing.

4.4.2.1 Mechanical treatment

There was significant difference ($p<0.05$) in the germination of seeds of *Z. armatum* among various treatments (Table 17). Sand scarification was found to be the best among other mechanical treatments. The germination of sand scarified seeds was 35.33±2.90% and that of sandpaper was 22.67±2.90%. Soaking the seeds in hot water for 5 min had no significant effect on germination. The germination percentages were 10.00±1.15 and 10.67±0.66, respectively for seeds immersed in 150 and 200 °F water, respectively. Similarly, chilling treatment also did not show remarkable results. Germination was 3.33±0.66 and 4.67±0.66%, respectively for cold treated seeds for 12 h and 24/36 h, respectively.

Table 17: Effect of different mechanical treatments showing mean germination percentage of seeds of *Z. armatum*

SN	Treatments	Germination (%)
1	Sand scarification	35.33 ± 2.90 [a]
2	Sand paper scarification	22.67 ± 2.90 [b]
3	Hot water (150°F)	10.00 ± 1.15 [c]
4	Hot water (200°F)	10.67 ± 0.66 [c]
5	Chilling temperature (12 h)	3.33 ± 0.66 [d]
6	Chilling temperature (24 h)	4.67 ± 0.66 [d]
7	Chilling temperature (36 h)	4.67 ± 0.66 [d]
8	Control	4.20 ± 0.72 [d]

Note: Different letters in the same colulmn indicate statistically significant difference ($p < 0.05$)

Maximum seed germination in water soaking treatment was reported by Daudi et al. (2016), with 28.67% germination in seeds of *Zanthoxylum alatum* soaked in hot water for 12 h. It might be due to the differences in experimental conditions or genotype. Hard seed coat is the characteristic of seed of *Z. armatum*, which might be the most likely cause of dormancy. Therefore, coat-imposed dormancy might have prevented seed germination by blocking water entry to the embryo (Ortega et al., 2002). Dormancy in the seeds of tropical and subtropical tree species include combined effect of seed coat and physiological dormancy (Wolf & Kamondo, 1993) resulting in poor germination.

Male and female flowers borne on separate individuals, poor synchronization in flowering period and fruits formed without pollination may result in empty seeds in *Z. armatum* (Purohit et al., 2016). Furthermore, seed infestation with insect larvae also results in poor germination in several species of *Zanthoxylum* (WAC, 2005). Physical dormancy due to hard and impermeable seed coat hinders uptake of water and diffusion of oxygen into the embryo. Different pre-soaking treatments, like sulfuric acid, hot water and mechanical scarification have been known to overcome seed coat-imposed dormancy (Emongor et al., 2004; Zida et al., 2005). But in the present study, hot water treatment completely failed to germinate and sulfuric acid treatment did not break dormancy.

4.4.2.2 Stratification

Cold stratification at 4° C for 3 months did not induce germination of *Z. armatum* as none of the seeds crack. Since seeds did not germinate for this treatment, data regarding this was not included in the calculations.

Various other treatments improved germination in other species of *Zanthoxylum*, for example, cold stratification in *Zanthoxylum piperitum* from 28 to 74% (Goo et al., 1995) but in the present study, cold stratification did not help seed germinate.

4.4.2.3 Acid treatment

The germination percentage of seeds for acid treatments was significantly lower ($p < 0.05$) for sulfuric acid and hydrochloric acid than nitric acid (Table 18). The germination increased with the increase in the duration of soaking. The germination percentage of the seeds treated with nitric acid were 11.33±2.4, 12±2.3 and 18.00±3.05 for 1, 2 and 5 minutes respectively. For hydrochloric acid, it was 5.33±0.66, 6.67±1.33 and 8.10±1.15% respectively for 1, 2 and 5 minute. Likewise, the germination was 2.8±0.66, 3.33±0.66, and 5.33±1.33% for sulfuric acid treated seeds for 1, 2 and 5 minutes respectively. For the untreated (control) seeds, the germination was only 2.00±0.26%.

Table 18: Effect of different acid treatments showing mean germination of seeds of *Z. armatum*

SN	Treatments	Germination (%)
1	Nitric acid (1 min)	11.33 ± 2.4^{b}
2	Nitric acid (2 min)	12.10 ± 2.3^{b}
3	Nitric acid (5 min)	18.00 ± 3.05^{a}
4	HCL (1 min)	5.33 ± 0.66^{cde}
5	HCL (2 min)	6.67 ± 1.33^{cd}
6	HCL (5 min)	8.10 ± 1.15^{bc}
7	Sulfuric acid (1 min)	2.80 ± 0.66^{de}
8	Sulfuric acid (2 min)	3.33 ± 0.66^{de}
9	Sulphuric acid (5 min)	5.33 ± 1.33^{cde}
10	Control	2.00 ± 0.26^{e}

Note: Different letters in the same colulmn indicate statistically significant difference ($p < 0.05$)

Purohit et al. (2015) observed the rate of germination in *Z. armatum* seeds treated with diluted H_2SO_4 (50%) for 15 min resulted in maximum germination (93.3%) along with five months prolonged germination period due to poor germination and dormancy. In another study by Patade et al. (2019), the rate of germination of seeds of *Z. armatum* treated for 120 min with concentrated sulfuric acid was 11.33%, while the seeds treated with the same for 1 or 5 min failed to germinate. Failure in seed germination in concentrated acid-treated seeds for 1 or 5 minute has also been earlier reported by Purohit et al. (2015). Seed germination increased with the increase in time of acid treatment. However, in this study, treatment of the seeds with different acids for a longer duration destroyed the seed coat and the seeds totally failed to germinate. Decreasing germination at longer periods in acids could be related to possible damage of embryo (Majd et al., 2013).

Sulfuric acid treatment is a well-recognized effective method of softening impermeable seed coats. Acid treatment for breaking seed dormancy have been reported for several other plant species also (Keshtkar et al., 2008; Azazi et al., 2013; Majd et al., 2013; Arowosegbe, 2016; Giasson et al., 2019).

A study by Daudi et al. (2016) recorded a maximum germination of 34.33% in *Z. alatum* untreated seeds (soaked in normal water), while in this study the untreated seeds (control) showed significantly lower rate of germination than pre-treated seeds. In the study by Purohit et al. (2015) no germination was recorded in the untreated control seeds of *Z. armatum.*

4.4.2.4 Hormonal treatment

In contrast, hormonal treatment had significantly higher ($p < 0.05$) germination than for all other treatments (Table 19). Germination percentage increased with the increase in concentration of GA_3 upto 1500 ppm and then decreased thereafter. The maximum germination percentage was 54.67±8.11 at 1500 ppm, followed by 52.00±6.11 at 2000 ppm, 48.67±6.35 at 1000 ppm, 42.00±8.71 at 500 ppm, 34.67±5.45 at 100 ppm, and the lowest of 3.10±0.29% for untreated (control) seeds.

Table 19: Effect of different concentrations of GA_3 on mean germination percentage of seeds of *Z. armatum*

SN	GA_3 Concentrations	Germination (%)
1	100 ppm	34.67 ± 5.45 [b]
2	500 ppm	42.00 ± 8.71 [ab]
3	1000 ppm	48.67 ± 6.35 [a]
4	1500 ppm	54.67 ± 8.11 [a]
5	2000 ppm	52.00 ± 6.11 [a]
6	2500 ppm	46.67 ± 2.40 [ab]
7	Control	3.10 ± 0.29 [c]

Note: Different letters in the same colulmn indicate statistically significant difference ($p < 0.05$)

Contrary to the findings of present study, the germination was 72.5% in *Z. armatum* seeds treated with GA_3 200 ppm + kinetin 100 ppm in a study by Datt et al. (2017). Increasing the concentration of GA_3 resulted in an increase in germination percentage of seeds (Koyuncu, 2005). GA_3 has been found to be effective in increasing germination in several species and to break dormancy in dormant seeds (Ballington, 1984; Giba et al., 1993; Nicolás et al., 1996; Iralu & Upadhaya, 2018; Tang et al., 2019). GA_3 is known to affect physiological as well as metabolic activities of seeds and hence inducing early germination (Chuanren et al., 2004). GA_3 stimulates seed germination effectively and minimizes the differences in germination date attributing to increased activity of hydrolytic enzymes within the seeds and accelerate the regeneration process by mobilizing the nutrients in dormant seeds (Joshi & Dhar, 2003; Manjkhola et al., 2003).

4.4.3 Germination behavior based on harvesting period

Harvesting period and pre-sowing seed treatments significantly affect the seed germination process in *Z. armatum.* Freshly harvested seeds at proper time period exhibited better germination. Pretreated seeds with GA_3 (100 ppm) germinated better than untreated (control) seeds. The maximum germination, i.e., 62.44±8.94 % was obtained for the seeds collected during 16 September-15 October. Likewise, the germination for early August collected seeds was 4.36±0.79%, 15 August-15 September (26.52±5.08%), 16 October-15 November (54.12±6.26%), 16 November-

15 December (30.44±3.11%), and 20.92±2.21% for late December collected seeds (Table 20). The seeds showed better germination during mid-phase of harvesting than early or late harvesting (Figure 16). So, for getting optimum germination, the seeds should be collected at the appropriate time and should be sown immediately afterwards. Storage of seeds for longer period makes the seeds dormant and hard to germinate.

Table 20: Effect of harvesting period on germination seeds of *Z. armatum*

SN	Harvesting period	Germination (%)
1	Early August	4.36 ± 0.79^{c}
2	15 Aug-15 Sept	26.52 ± 5.08^{b}
3	16 Sept-15 Oct	62.44 ± 8.94^{a}
4	16 Oct-15 Nov	54.12 ± 6.26^{a}
5	16 Nov-15 Dec	30.44 ± 3.11^{b}
6	Late December	20.92 ± 2.21^{b}
7	Control	7.11 ± 1.49

Note: Different letters in the same column indicate statistically significant difference ($p < 0.05$)

The natural regeneration of *Z. armatum* is adversely affected by physiological dormancy and high emptiness nature of seeds as a result the seed germination is extremely rare in wild (Datt et al., 2017). Although many treatments have been attempted to study the germination rate of seeds of *Z. armatum*, it is apparent from this study that not all treatments are equal in efficacy. Sowing of pretreated seeds with GA_3 100 ppm at mid-phase of harvesting, 16 September to 15 December (in this study) was significantly better than mechanical or chemical treatments. Cold stratification and storage for longer period may cause the seeds to enter a pronounced phase of dormancy and halt the germination process. So, based on the data of the present study, it is concluded that sowing freshly harvested seeds at proper time is a simple, safe, reliable, low-cost efficient way to obtain higher germination rates in *Z. armatum* for obtaining uniform germination and seedling emergence for raising nursery.

Figure 16: Emergence of seedlings of *Z. armatum*

4.5 Vegetative propagation

The length of shoot, root and the quantity of roots growing from the stem cuttings of *Z. armatum* were significantly affected ($p<0.05$) by the different concentrations of IBA, NAA and rooting media (sand, mix, and neopeat). Although the statistics for IBA and NAA were similar, NAA was found to be less effective than IBA, while in case of the growth media, neopeat was superior to sand or mix. Furthermore, when the concentration of growth hormones increased from 2000 ppm to 5000 ppm, the measured parameters (root length, shoot length, and number of roots) indicated a constant increase. However, the scenario was somewhat different for NAA, the measured parameters showed increment with the increase in concentration of hormones from 2000 ppm to 3000 ppm and declined at 5000 ppm. But in case of root numbers, the highest value was at the highest concentration ie. 5000 ppm. Similarly, cuttings treated with 3000 ppm of IBA produced shorter shoots than those treated with 2000 ppm. In comparison to NAA treated cuttings, IBA treated cuttings developed more roots per cutting, and comparatively longer roots and shoots. The hormone treated stem cuttings of *Z. armatum* performed relatively better than the untreated cuttings or control.

Table 21: Effect of different hormones on the rooting and shooting of stem cuttings of *Z. armatum*

SN	Growth hormones	Root length (cm)	Shoot length (cm)	Number of roots
1	IBA	9.0a	28.0a	4.6a
2	NAA	8.1a	25.3b	4.4a
3	Control	5.4b	22.0c	2.4b

Note: Means with the same letter in the same column are not significantly different (P ≤ 0.05)

The types of growth hormones and their concentration and the types of rooting media seem to significantly affect the root number per cutting but it was not affected by interaction of the rooting media and concentration of the hormones. The root number in both the IBA and NAA treated cuttings were nearly identical. The number of roots in IBA treated cuttings was 4.6, while in NAA treated cuttings it was 4.4. (Table 21). IBA 5000 ppm treated cuttings produced the highest mean number of roots (5.9), whereas the lowest mean number of roots (2.4) was produced by untreated cuttings ie. control (Table 22). Similarly, the maximum average root number 5 was produced in the cuttings grown in neopeat, whereas in sand, it was 4.2. (Table 23). The maximum value for the number of roots was observed in the interaction between 5000 ppm concentration of IBA and the medium neopeat produced the maximum number of roots 6.5 whereas the untreated cuttings (control) grown in sand produced the lowest number of roots ie. 2 (Table 24).

Table 22: Effects of hormone concentrations on the stem cuttings of *Z. armatum*

SN	Hormone concentration	Root length (cm)	Shoot length (cm)	Number of roots
1	IBA2000	7.6b	28.6a	3.4c
2	IBA3000	8.3b	26.8b	4.5b
3	IBA5000	11.3a	28.7a	5.9a
4	NAA2000	7.6b	25.9b	3.2b
5	NAA3000	9.8a	26.1b	4.3c
6	NAA5000	6.9b	23.9c	5.2a
	Control	5.4c	22.0c	2.4c

Note: Means with the same letter in the same column are not significantly different (P ≤ 0.05)

Hormone concentration had significant effect on the root length of *Z. armatum* stem cuttings. The longest root length was 11.3 cm in cuttings treated with 5000 ppm IBA, whereas the shortest was 5.4 cm in untreated (control) cuttings (Table 22). Interaction between the concentration of hormones and rooting media was not affected significantly. From the interaction of hormone concentration and rooting media, it is clear that the cuttings treated with 5000 ppm IBA and grown in the media neopeat and mix had the highest mean root length of 11.6 cm each, while the lowest was 5 cm for untreated cuttings grown in sand (Table 24). Cuttings grown in neopeat produced the longest mean root length (9.2 cm) among the rooting media while the least 8.3 cm was for the cuttings grown in sand (Table 23).

Table 23: Effects of rooting media on the performance of stem cuttings of *Z. armatum*

SN	Growth media	Root length (cm)	Shoot length (cm)	Number of roots
1	Sand	8.3a	26.4a	4.2b
2	Neopeat	9.2a	27.0a	5.0a
3	Mix	8.8a	26.4a	4.4b

Note: Means with the same letter in the same column are not significantly different ($P \leq 0.05$)

The length of shoots was not significantly affected by different growth media and the interaction between different hormone types and concentration. The interaction between growth media and hormone concentration had the highest (29.7 cm) shoot length in the combination of IBA 2000 ppm and neopeat and the lowest (21.0 cm) mean shoot length in control with (Table 24). On the other hand, the different hormone types and concentration had significant effect on the shoot length. IBA had the highest (28 cm) mean shoot length and IBA 5000 ppm had the best effect on the shoot length with a mean value of 28.7 cm (Table 22).

Hormone concentration had significant effect on the root length of *Z. armatum* stem cuttings. The longest root length was 11.3 cm in cuttings treated with 5000 ppm IBA, whereas the shortest was 5.4 cm in untreated (control) cuttings (Table 22). Interaction between the concentration of hormones and rooting media was not affected significantly. From the interaction of hormone concentration and rooting media, it is clear that the cuttings treated with 5000 ppm IBA and grown in the media neopeat and mix had the highest mean root length of 11.6 cm each, while the lowest was 5 cm for untreated cuttings grown in sand (Table 24). Cuttings grown in neopeat produced the longest mean root length (9.2 cm) among the rooting media while the least 8.3 cm was for the cuttings grown in sand (Table 23).

Table 24: Effect of hormone concentration and growth media on the stem cuttings of *Z. armatum*

	Growth media								
	Root length (cm)			**Shoot length (cm)**			**Number of roots**		
	Sand	**Neopeat**	**Mix**	**Sand**	**Neopeat**	**Mix**	**Sand**	**Neopeat**	**Mix**
Hormone concentration									
IBA2000	6.9^{fg}	8.0^{cdef}	8.0^{cdef}	28.8^{abc}	29.7^{a}	27.8^{abcde}	2.7^{h}	3.3^{gh}	3.8^{defgh}
IBA3000	8.5^{bcdef}	9.1^{abcdef}	$7.4d^{efg}$	27.5^{bcdef}	27.2^{bcdef}	25.8^{efgh}	3.8^{defgh}	5.1^{abcdef}	$4.8^{abcdefg}$
IBA5000	10.7^{ab}	11.6^{a}	11.6^{a}	28.2^{abcd}	28.5^{abc}	29.0^{ab}	5.5^{abcd}	6.5^{a}	5.6^{abc}
NAA2000	7.4^{defg}	7.1^{fg}	8.3^{bcdef}	26.0^{efgh}	25.4^{fgh}	26.3^{defgh}	4.5^{bcdefg}	5.8^{ab}	$5.0^{abcdefg}$
NAA3000	7.0^{fg}	11.3^{a}	11.3^{a}	26.3^{defgh}	27.3^{bcdef}	24.8^{gh}	$4.4^{bcdefgh}$	5.9^{ab}	3.7^{efgh}
NAA5000	7.0^{fg}	8.2^{bcdef}	5.4^{g}	22.1^{i}	25.0^{gh}	24.4h	3.9^{cdefgh}	3.4^{fgh}	3.2^{gh}
Control	5.0^{g}	5.8^{g}	5.5^{g}	21.2^{i}	21.0^{i}	21.1^{i}	2.0^{i}	2.3^{i}	2.9^{i}

Note: Means with the same letter in the same column are not significantly different ($P \leq 0.05$)

Varied concentrations of IBA and NAA, as well as the different growing media *viz*. sand, neopeat, and mix had significant impacts on the rooting and sprouting behavior of the stem cuttings of *Z. armatum*. Several studies have validated that the exogenous application of growth hormones stimulate the production of roots on the stem cuttings (Leakey et al., 1994; Poupard et al., 1994; Hartman et al., 2002; Tchoundjeu et al., 2004). The various forms of auxins: IAA, IBA, and NAA, which are known to boost the rate of rooting as well as the quantity of roots per cutting, are the most extensively used sources of growth hormones for artificially inducing rooting in stem cuttings (Gehlot et al., 2014; Ibrahim et al., 2015).

Among the various IBA concentrations tested, on the rooting and shooting of stem cuttings of *Z. armatum*, 5000 ppm concentration exhibited better results. Similar result was also obtained by Daudi et al. (2016), while conducting the propagation experiment in the stem cuttings of *Zanthoxylum alatum*. They study concluded that the cuttings treated with 5000 ppm IBA sprouted and rooted better than those treated with 4000 ppm and 6000 ppm IBA. They also came to the conclusion that vegetative propagation is a viable option than sowing of seeds to get the optimum results as the process of seed germination is not so encouraging in *Z. alatum*. Another study by Singh and Rawat (2017) also concluded that the semi-hard wood (SHW) and hard-wood (HW) cutting of the branches of demonstrated excellent rooting and shooting at 0.3% and 0.4 % concentrations of the hormone IBA, while the cuttings trated with lower concentrations of the hormone did not produce any root or shoot.

One of the most important aspect that has a direct impact on the rooting behavior of the stem cuttings is the rooting or the growth medium (Ingram et al., 1993). The combination of a multitude of elements in the growth media, such as oxygen, water and the availability of nutrient directly affects the process of rooting of any cutting (Alikhani et al., 2011; Bhardwaj, 2014).

The findings of this study demonstrated that growing media had a substantial impact on the number of roots, length of root and shoot in the stem cuttings of *Z. armatum*. Neopeat was found to be the best growth medium over the sand medium. Compared to the sand and mix media, the cuttings grown in neopeat medium produced maximum roots as well the longer roots than the cuttings grown in the medium sand. The water holding capacity in sand is not so good because of its porosity, moreover it's deficient in nutrients as well. Contrary to this neopeat has a blend of all the necessary nutrients, greater aeration, and enough drainage (Akinyele, 2010). Cuttings may perish before the initiation of root due to poor aeration in waterlogged environments (Schmitz et al., 2013).

When it came to rooting and shooting, both the growth hormones IBA and NAA performed well, although IBA was more effective than NAA. In terms of average length of the root, shoot and per cutting root number, the stem cuttings treated with 5000 ppm of IBA outperformed the other concentrations. In case of the growth media, the neopeat outperformed the sand and mix media. As a result of this experiment, it can be concluded that the combination of 5000 ppm concentration of IBA with neopeat medium is the optimal treatment for enhancing rooting in the stem cuttings of *Z. armatum*. The findings of the present study could have a significant impact on the large scale production of high-quality plantlets and also the improvement of agroforestry systems.

4.6 Phytochemical analysis

4.6.1 Composition of extracts of leaf, fruit, seed and bark

The bioactive compounds present in methanol extracts of wild and cultivated leaves, fruits, seeds and bark of *Z. armatum* is presented in Table 25. The identification and characterization were based on their elution order in a DB5 column. The elution time and the amount of these bioactive compounds are also presented. Four compounds in wild and three in cultivated leaves were determined through GC-MS analysis of leaves. The major component cyclononasiloxane, octadecamethyl (41.86%) presented in wild samples was absent in cultivatead samples, while the major component heptasiloxane, hexadecamethyl (18.87%) in cultivated leaves was absent in wild samples (Table 25).

Table 25: Phyto-compounds of methanol extracts of wild and cultivated leaves, fruits, seeds and bark of *Z. armatum*

SN	Name of the compound	Retention time	Area (%)	
Leaf			Wild	Cultivated
1	Cyclohexasiloxane, dodecamethyl-	18.22	9.27	4.64
2	Pentasiloxane, dodecamethyl-	14.96	6.12	4.36
3	Heptasiloxane, hexadecamethyl-	30.01	23.25	18.87
4	Cyclononasiloxane, octadecamethyl-	34.77	41.86	-
Fruit			Wild	Cultivated
1	Linalool	6.53	9.30	11.49
2	Methyl cinnamate	12.7	25.52	28.71
3	Caryophyllene	13.49	-	0.70
4	Methyl palmitoleate	22.36	0.99	0.79
5	Methyl palmitate	22.69	0.49	-
6	Myrtenol	28.33	44.5	31.66
Seed			Wild	Cultivated
1	Linalool	6.53	1.69	0.34
2	Methyl cinnamate	12.72	-	0.40
3	Methyl palmitoleate	22.36	-	1.51
4	Palmitic acid, methyl ester	22.71	6.29	0.67
5	*trans*-Oleic acid	23.52	-	67.66
6	Linoleic acid, methyl ester	25.42	7.93	2.65
7	Linoleoyl chloride	25.49	-	7.45
8	*cis*-9-Hexadecenal	28.07	-	2.25
9	Monolein	31	-	6.33
10	Linoleic acid	25.48	7.93	-
11	Oleic acid, methyl ester	25.56	6.76	-
12	β-Sitosterol	33.36	9.70	-
13	α-Amyrin		8.39	-
14	Octadecamethylcyclononasiloxane		1.55	-
Bark			Wild	Cultivated
1	Linalool	6.53	2.37	7.89
2	*cis*-9-Hexadecenal	28.05	5.09	-
3	Heptacosane	35.02	-	6.15
4	Campesterol	33.36	28.44	-
5	Squalene	35.04	4.81	-
6	(*E*)-4-Benzylidene-3-phenyl-3,4-dihydronaph	37.67	-	47.60

Similarly, a total of six compounds were identified in bark extracts (four each in wild and cultivated), with the major components campesterol (28.44%), *cis*-9-Hexadecenal (5.09%) and squalene (4.81%) in wild; (*E*)-4-Benzylidene-3-phenyl-3, 4-dihydronaph (47.6%), linalool (7.89%) and heptacosne (6.15%) in cultivated bark (Table 25).

Many of these identified constituents are known to possess several pharmacological activities. Palmitic acid, linolenic, linoleic, oleic, major components of seeds is known to possess strong antimicrobial and antioxidant activities (Agoramoorthy et al., 2007; Tepe et al., 2011). Linalool has been reported to contain antioxidant and antibacterial activities against several bacteria (Negi et al., 2012). Likewise the other compounds are also known to possess several biological activites as well (Gilani et al., 2010; Barkatullah et al., 2013; Guleria et al., 2013; Misra et al., 2013; Bhartia & Bhusan et al., 2015).

4.6.2 Total phenolic content (TPC)

4.6.2.1 Leaf

The total phenolic content (TPC) was highest in methanolic extracts than in hexane and ethyl acetate extracts. The TPC value ranged from 63.0 to 76.29 mg/g GAE (Gallic Acid Equivalent) in the methanol extracts, while the value ranged from 22.22 to 30.81 mg/g GAE in hexane extracts (Figure 17). Hexane extracts contain relatively lower concentration of phenols. The total phenolic content in plant extracts depends on the type of extract, i.e., the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Zhou & Yu, 2004; Mohsen & Ammar, 2008). The extracts however did not show any remarkable variation in the total phenolic content according to altitude or population.

In this study, the TPC value was lowest in hexane extracts and highest in methanol extracts. Because of their potential antioxidant and antimicrobial properties, the polyphenols derived from plants have great importance. Phenolic compounds exhibit a considerable free radical scavenging (antioxidant) activity (Wojdylo et al., 2007). Thus, the phenolic compound present in the leaves of *Z. armatum* might be responsible for several biological activities and hence used in different indigenous medicinal practices in various ailments.

The phenolic composition in plant parts might be effected by the microenvironment variation. Temperature, elevation, soil, humidity and water availability are some of the important factors afecting metabolism and secondary metabolite accumulation in plants (Ahuja et al., 2010). Thus, as a survival strategy, the environmental variations at diferent sources might lead to the variation of the phenolic compounds of plants. The phenolic content in the leaves of *Z. armatum* might have varied in response to the combined environmental conditions.

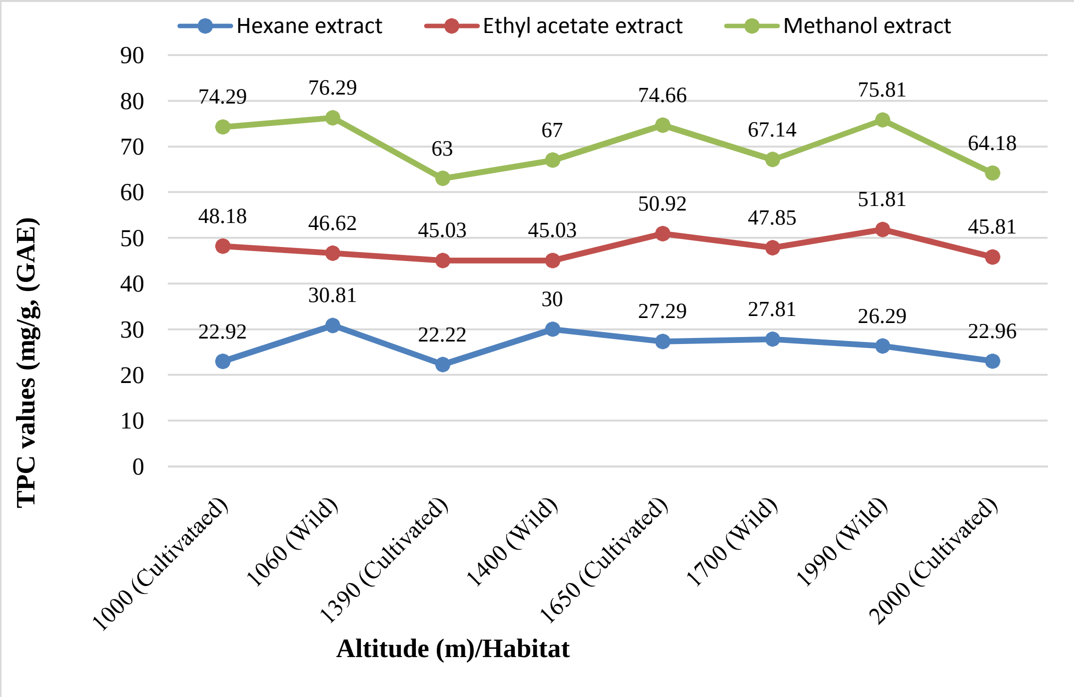


Figure 17: Comparative TPC values (mg/g GAE) of different extracts of leaves of *Z. armatum*

4.6.2.2 Fruit, seed and bark

There were significant differences ($p<0.001$) in the TPC values of fruit, seed and bark extracts of *Z. armatum* (Figure 18). Similarly, the extracts of the fruit and bark contained significantly higher ($p<0.001$) phenolic contents than that of the extract of the seed. The highest phenolic content was observed in the fruit followed by the bark and the lowest in the seed. The TPC values of the extracts of the cultivated and wild fruits were 226.3 ± 1.14 mg/g GAE and 185.02 ± 2.15 mg/g GAE respectively. Likewise, the TPC value of the extracts of the bark was 171.13 mg/GAE for cultivated and 185.15 ± 1.22 mg/GAE for wild samples. And the TPC values were 167.74 ± 2.63 mg/GAE and 137.72 ± 4.21 mg/GAE for the extracts of the wild and cultivated seeds respectively (Figure 18).

The findings of the present study can be correlated with the results obtained by Barkatullah et al. (2012). They found that the TPC values of the ethanol extracts of fruit and bark of *Z. armatum* were 21.68 ± 0.44 mg/g and 16.48 ± 1.33 mg/g respectively. In another study, The TPC value of the extract of fruit, seed and bark of *Z. armatum* were as 226.3 ± 1.14 mg GAE/g, 167.74 ± 2.63 GAE/g, 185.15 ± 1.22 GAE/g respectively (Phuyal et al., 2020a). Likewise, the TPC value of the methanol extract of fruit of *Z. armatum* was 366.3 mg of GAE/g in the study by Guleria et al. (2013). The antioxidant capacity of plants is closely connected to the amount of phenolic content present in them. The phenolic compounds serve as hydrogen donors, reducing agents, and are scavengers of free radicals (Wojdyo et al., 2007). The prevalence of a significant amount of phenolic compounds in the bark, fruit and seed extracts of *Z. armatum* contribute significantly to the antioxidant properties. Because of these properties this plant is being used in several traditional herbal medicinal practices.

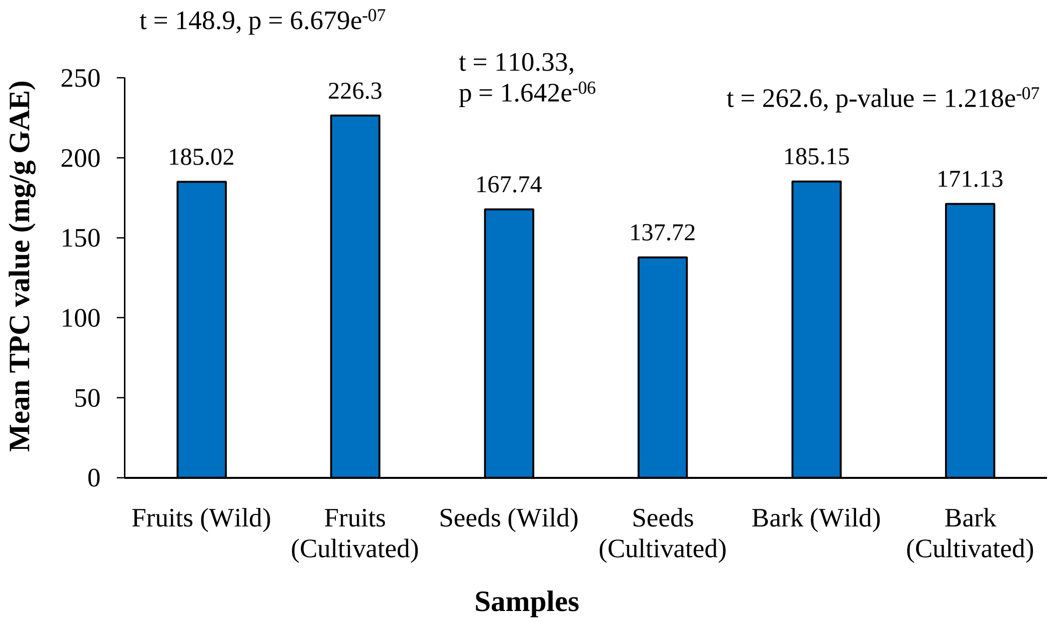


Figure 18: Comparative TPC values (mg/g GAE) of fruits, seeds, and bark extracts from of *Z. armatum*

The antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure (Gracia et al., 1997). In addition, there may be some interference rising from other chemical components present in the extract, such as sugars or ascorbic acid (Singleton & Rossi, 1965). In this study also there were differences in the total phenolic components of the wild and cultivated fruit, seed, and bark extracts. These differences could arise from variations in genetic backgrounds, environmental factors, and agronomic practices as well (Doshi et al., 2006).

Because of their ubiquitous presence in vascular plants, phenols have long been associated with passive and active defense responses of plants (Nicholson & Hammerschmidt, 1992.). Plants respond to various abiotic and biotic stresses such as salinity, heavy metal, drought, temperature, UV lights, disease progression, etc. by the increased biosynthesis of phenol and polyphenol compounds. Several studies have demonstrated that phenolic and flavonoid compounds increased in plants as a result of insect feeding, pathogen infection, or colonization of beneficial microorganism. (Wallis & Galarneau, 2020).

4.6.3 Total flavonoids content (TFC)

4.6.3.1 Leaf

Total flavonoid content in *Z.armatum* were relatively higher in methanol and ethyl acetate extracts than hexane extracts. The trend was almost similar for methanol and ethyl acetate extracts. TFC values ranged from 18 to 20 mg/g Quercetin equivalent (QE) for hexane extract, 32.27 to 49.9 mg/g QE for ethyl acetate extract and 41.36 to 57.18 mg/g QE for methanol extract in the measured samples (Figure 19). The total flavonoids content of the extracts was not affected by altitude or habitat. Barkatullah et al. (2012) reported 13.68±0.66 mg/g QE total flavonoid content in ethanolic extract of *Z. armatum* leaf. The concentration of flavonoids in plant extracts also depend on the polarity of solvents used in the extract preparation (Ali et al., 2010). Due to high polarity, methanol was found to exhibit better efficiency in extracting different polar phyto-compponents (phenolics and flavonoids) from the leaves of *Z. armatum.* Considerable amount of flavonoids could be of great value for the potential of this plant in several therapeutic uses.

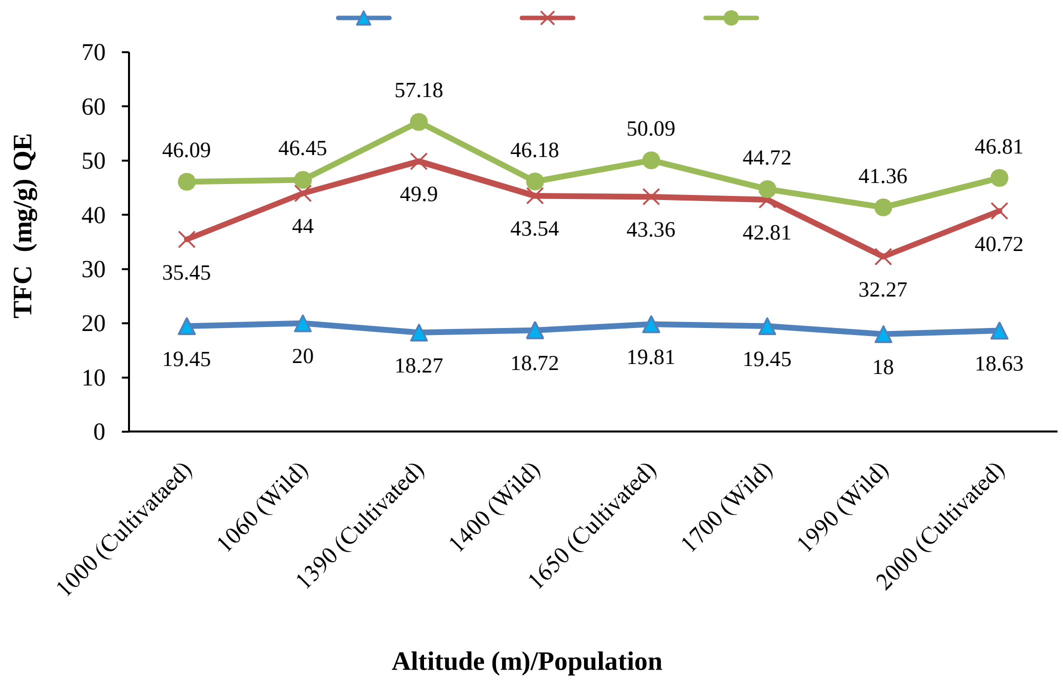


Figure 19: Comparative TFC values (mg/g, QE) of leaves of *Z. armatum* from different altitudes

4.6.3.2 Fruit, seed and bark

The TFC values followed a similar pattern to that of the TPC values. There were significant differences ($p<0.001$) between TFC values of the extracts of the fruit, seed and bark collected from wild and cultivated populations. The TFC value of the fruit extract was significantly higher compared to the TFC value of the seed and bark extracts ($p<0.001$) (Figure 20).The extract of the fruit had the highest TFC value, followed by the bark, and the seed. The extract of the cultivated fruit showed highest TFC value of 135.17±2.02 mg/g QE, while the TFC value for the extract of wild fruit was 103.7±1.39 mg/g QE. Similarly, the TFC values of the extracts of cultivated and wild barks were 111.2±3.67 mg/g QE and 91.27±3.13 mg/g QE, respectively. Likewise, the lowest TFC value was 76.58±4.18 mg/g QE, for the extracts of cultivated seeds and that for wild seed was 92.71±3.14 mg/g QE (Figure 20).

The TFC value as reported by in the ethanol extracts of fruit of *Z. armatum* was comparatively lower than the TFC values obtained in this study. The TFC values of the fruit and bark were 22.8±1.33 mg/g 18.33±1.22 mg/g respectively (Barkatullah et al., 2012). The total concentration of flavonoids and phenols in any extracts to a large extent depends upon the solvents used for the extraction procedure and their polarity (Jing et al., 2015).

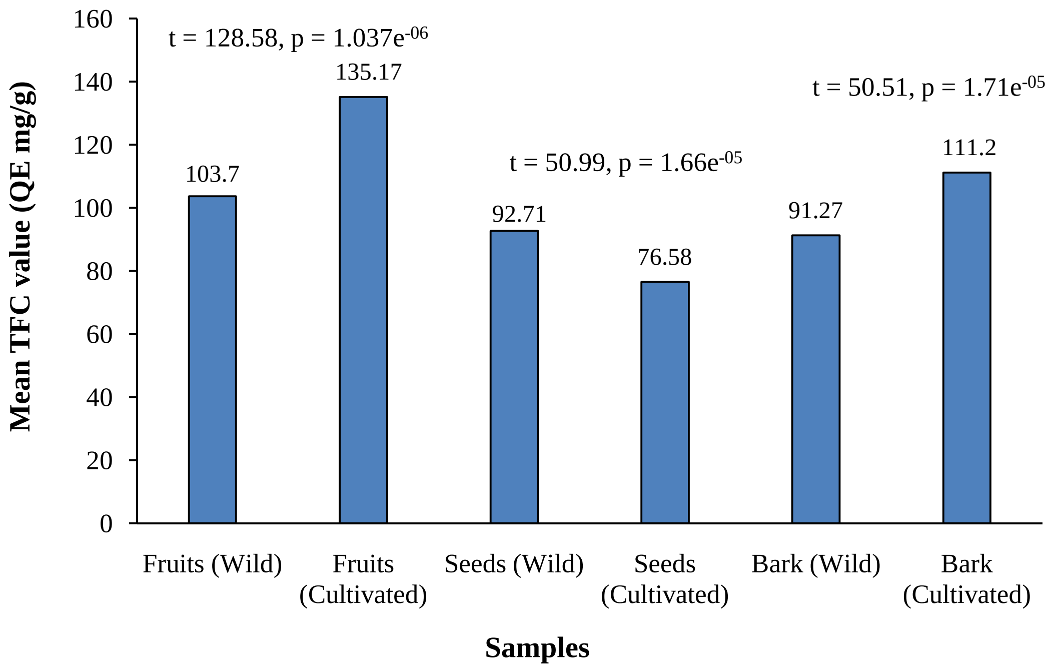


Figure 20: Comparative TFC values (mg/g QE) of fruits, seeds, and bark extracts of *Z. armatum*

The results showed that the TFC value was high in cultivated fruits than wild. It might be due to the defence mechanism of the plants to pest and pathogens. Plants produce secondary metabolites in response to infective agents and environmental factors. These compounds are biosynthesized by plants as protection mechanisms against environmental factors and infectious agents (Okagu et al., 2021). Several studies have shown that the defense mechanisms of plants result in an increased production of active compounds. In a study by Banaszczak et al. (2018), in the leaves of *Trollius europaeus*, the total polyphenols (TPC) was 34.5% higher in extracts from plant's leaves, which are fed by aphids comparaad to the leaf collected from healthy plants not infected with aphids.

4.6.4 Antioxidant activity

4.6.4.1 Leaf

From the analysis of Figure 21, it can be concluded that the scavenging effects of extracts ofd leaf of *Z. armatum* showed considerably different values for samples from different localities. The extracts exhibited antioxidant activities in a dose dependent manner and ascorbic acid showed better antioxidant activity than leaf extracts. The IC_{50} values of the samples from 1000 m (cultivated), 1700 m (wild) and

1060 m (wild) were excellent, i.e., 13.76 µg/mL, 16.76 µg/mL and 17.66 µg/mL, respectively, near to the IC_{50} value of ascorbic acid, showing strong antioxidant potential. The IC_{50} vlaue of ascorbic acid is 7.46. Likewise, the relatively higher IC_{50} values are 55.34 µg/mL and 50.74 µg/mL for the samples from 2000 m (cultivated) and 1400 m (wild), respectively, exhibiting their weak antioxidant activities.

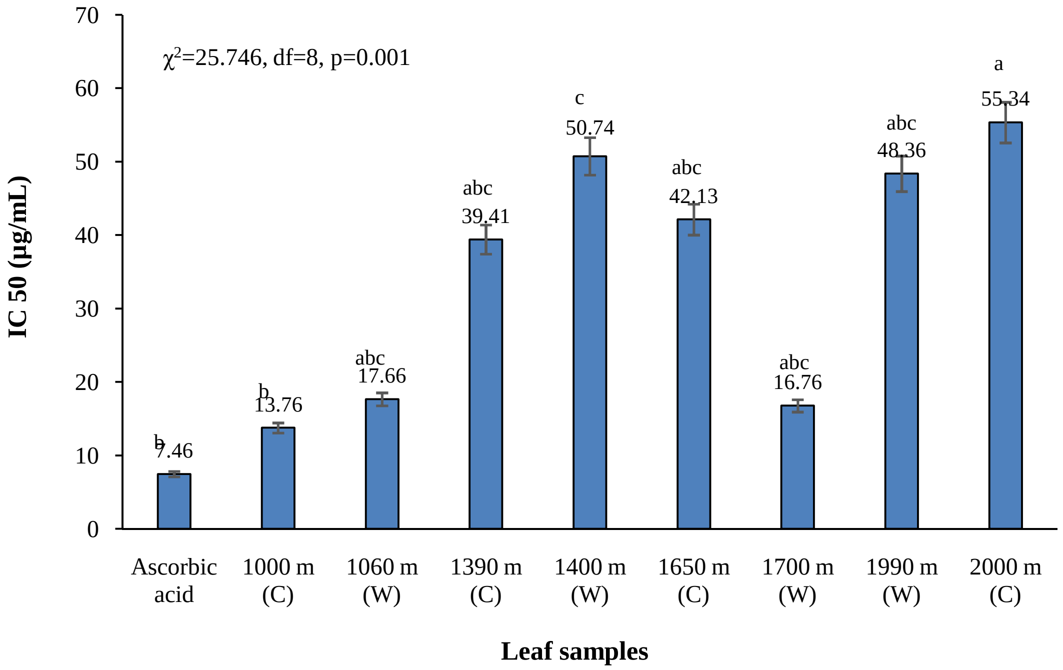


Figure 21: IC_{50} values of ascorbic acid and leaf extracts *of Z. armatum.*

Note: Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$

Antioxidant activity decreases the absorbance of DPPH in the presence of antioxidants, which correlates with the free radical scavenging potential of the antioxidants. The different phyto-compounds present in the extracts might be responsible for the scavenging activity. The antioxidant activity of the leaf extracts of *Z. armatum* is lower than that of ascorbic acid. Mixed type of scavenging activities was exhibited by the samples. There was no variation in the scavenging activities of the extracts of samples from different altitudes and populations.

4.6.4.2 Fruit, seed and bark

DPPH free-radical scavenging assay was used to determine the antioxidant properties of bark, fruit and seed extracts of *Z. armatum*, and their reducing power was calculated using the concentrations that provided 50% inhibition (IC_{50}) values, that means the quantity needed to scavenge 50% of DPPH free-radicals. Figure 22 shows the mean percentage of DPPH free-radical scavenging activity of the different extracts as well as the standard ascorbic acid, expressed as IC_{50} values. The radical scavenging activity of different extracts increased in a concentration dependent manner.

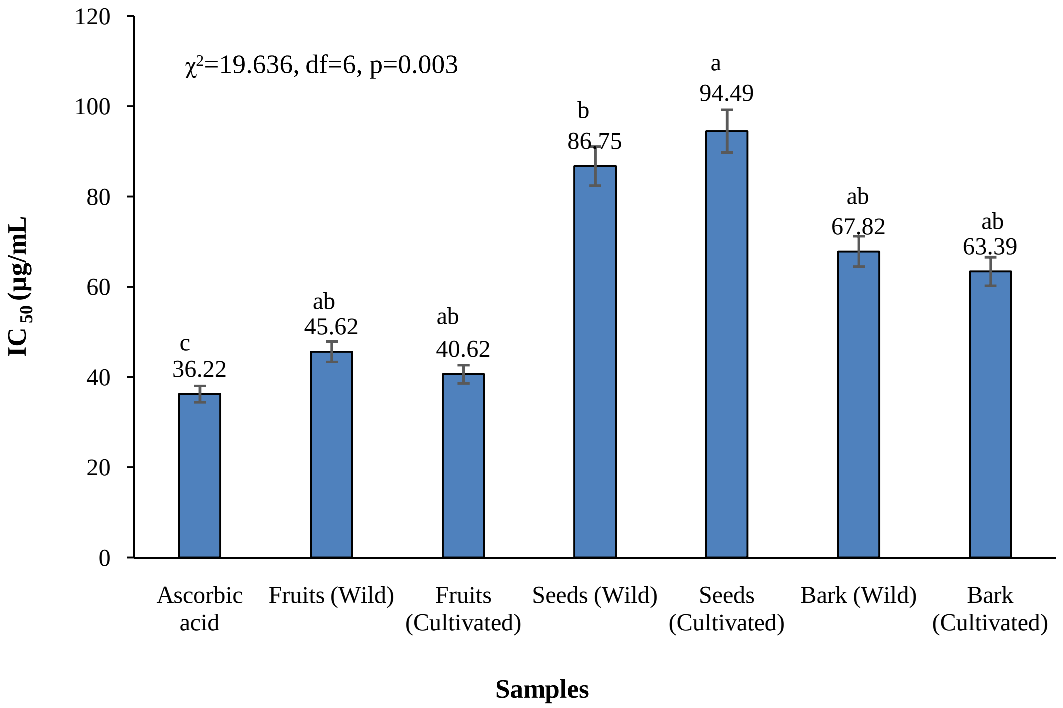


Figure 22: IC_{50} values of ascorbic acid and different extracts of *Z. armatum.*

Note: Different letters above bars indicate statistically significant difference between different elevation at $p < 0.001$

The different extracts showed variable antioxidant properties. Ascorbic acid used as the standard, whose IC_{50} value was 36.22 µg/mL. Lower antioxidant capability or lower radical scavenging activity is indicated by a higher IC_{50} value. In comparison to the seed and bark extracts, the fruit extract showed the highest antioxidant capacity. The IC_{50} values of the extracts of the fruits of *Z. armatum* and that of the standard used were similar, which indicates higher antioxidant potential. The IC_{50} values of the extracts of cultivated fruit and wild fruit were 40.62 g/mL and 45.62 g/mL respectively. Similarly, the IC_{50} values of the extracts of the wild and cultivated seeds were 86.75 g/mL and 94.49 g/mL, respectively, indicating that wild seeds have the least antioxidant effects. The bark also has a modest antioxidant potential, with IC_{50} values of 63.39 µg/mL for cultivated extracts and 67.82 µg/mL for wild extracts, respectively (Figure 22). The extracts of the cultivated fruits and bark showed higher antioxidant capacity compared to the cultivated ones, whereas the case was just reverse in seeds, with the higher antioxidant potential in the extracts of wild samples than the cultivated samples.

The antioxidant potential of *Z. armatum* has also been reported previously by various studies (Batool et al., 2010; Upadhyaya & Kumar, 2010; Negi et al., 2012; Prakash et al., 2012; Barkatullah et al., 2013; Guleria et al., 2013; Mukhijal & Kalia, 2014; Kanwal et al., 2015; Karmakar et al., 2015, Dhami et al., 2019). The free radical scavenging activity of methanol extract of fruit ranged from 59.56 to 64.85% (Nooreen et al., 2017). Similarly, the IC_{50} value of methanol extract of bark of *Z. armatum* was 149.26 µg/mL (Mukhijal & Kalia, 2014) but it was 63.39 µg/mL in the present study. Results obtained through DPPH assay in this study also confirmed redox protective activities of *Z. armatum*. Therefore, further use of this plant in drug development should be highlighted.

The antioxidant potential (scavenging of the free radicals) of the extracts of the different parts of *Z. armatum* may be attributed to the phenolic compounds, polyphenols and flavonoids present in the plant, and phenols primarily are the major antioxidants (Mansouri et al., 2005). Plant-based antioxidants are responsible for suppressing or avoiding the negative effects of oxidative stress. The DPPH assay, among many others, is one of the most practical methods for assessing the plant antioxidant potential. The solution of DPPH in methanol gets reduced because of the generation of non-radicals when there are antioxidant compounds containing hydrogen-donating groups, such as phenols and flavonoids (Mensour et al., 2011). Phenolic and flavonoid compounds not only possess the antioxidant activities but are also found to exhibit other biological activities as well such as anticancer, antiviral, antimicrobial etc. (Havsteen, 2002). These pharmacological and biological properties are mainly linked to their ability of protein binding and scavenge free radicals (Fotie, 2008).

Antioxidants are extremely important chemicals that can protect the body from the harm caused by oxidative stress generated by free radicals and can prevent from various diseases, such as obesity, cardiovascular and neurodegenerative diseases (Srinivasulu et al., 2018). Because their hydroxyl groups donate hydrogen, the polyphenols in plant perform as antioxidants and reducing agents (Aberoumand & Deokule, 2008). Due to the presence of these compounds in the various parts, this plant is useful and beneficial to human health.

The obtained results of the present study indicate that *Z. armatum* leaf, fruit, seed and bark from different altitudes and populations highlighted various levels of TPC, TFC and total antioxidant capacity. However, the highest TPC, TFC and antioxidant potential was found in fruit compared to the leaf, seed and bark. Furthermore, cultivated parts were found to contain more phenol, flavonoids and antioxidant potential. The large variability in TPC, TFC and antioxidant potential may refect the interaction between *Z. armatum* plants and their environment (soil). Flavonoids, as signal molecules have a vital role in promoting nodular formation in roots cell by symbiotic bacteria (Mandal et al., 2010). Flavonoids also act as intemediator between plant and environment and possess antioxidant and antimicrobial efects (Wink, 2013). Therefore, the fact that the highest TPC, TFC and antioxidant capacity found in cultivated fruits of *Z. armatum* might be a part of their defence strategy.

4.7 Essential oil

4.7.1 Leaf

The yield of essential oil (EO) in leaf of *Z. armatum* at different altitudes ranged from 0.16 to 0.5% (Table 26), with the highest 0.5% in wild samples collected from 1680 m and the lowest 0.16% also in the naturally growing samples at 1400 m altitude. The yield of essential oil is almost similar from leaves collected from all elevations, except for 1600-1700 m, where it was slightly higher. There was no significant difference between dry weight yield of the essential oil of leaf of cultivated and wild plants ($t = 0.2416$, $df = 15.528$, $p = 0.2416$) (Figure 23). This could probably because of the fact that all the samples were collected at the same time and they were extracted concurrently. Essential oils are present in all parts of the plant, however they are concentrated more in the fruits. The total yield of the essential oil is affected by several factors including the differences in environmental conditions, season, collection time and genetic variations (Rahimmalek et al., 2009). Variation in the essential oil yield of *Z. armatum* leaves ranged from 0.088% to 0.176% in samples of India (Negi et al., 2012). But in case of present study, the yield was high due to suitable conditions at the altitude of 1600 m).

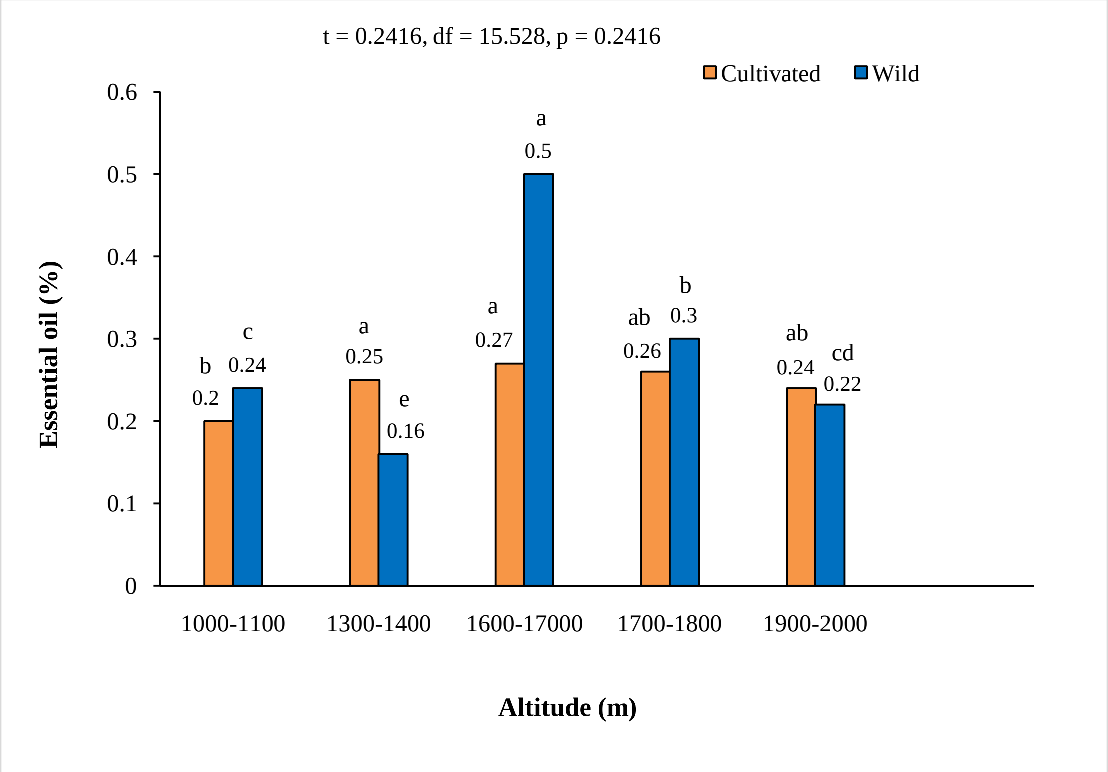


Figure 23: Essential oil yield in leaves of *Z. armatum*

There were considerable variations in the essential oil composition of leaf of *Z. armatum* from different altitude and population. A total of seventeen compounds were detected from GC-MS analysis in the essential oil of leaf; table 26 lists all of the compounds (excluding the trace elements) identified. Linalool, limonene, and undecan-2-one were the main components of the essential oil and they shared the highest percentage in all of the samples analyzed. Apart from that, methyl (E)-cinnamate, myrcene, tridecan-2-one and trans-alpha-bergamotene (Figure 25) were also present in most of the samples. There was no significant difference between number of components in essential oil of leaves of cultivated and wild plants ($t = 0.92045$, $df = 7.943$, $p = 3.844$). Highest (12) number of compounds were identified from the samples collected from naturally growing *Z. armatum* at 1990 m, 11 components from cultivated samples at 1000 m and wild samples at 1400 m. The lowest (7) number of components were detected from the samples collected from cultivated population at 2030 m and wild population at 1060 m (Figure 24). Among the compounds identified, some of the compounds as isophytol acetate, 2, 3-octanedione, isophytol and alpha-pinene were present in a smaller percentage in the essential oil of leaf collected from only one place at 1000 m altitude. The total number of phyto-components in the wild samples were relatively higher than in the cultivated samples.

Table 26: Essential oil composition of leaves of *Z. armatum*

SN	Name of the compound identified	RI [a]	RI [b]	Oil (%)										Average (%)
				1000 (C)	1060 (W)	1390 (C)	1400 (W)	1650 (C)	1680 (W)	1730 (C)	1770 (W)	1990 (W)	2030 (C)	
1	α-Pinene	939	948	0.42		-	-	-	-	-	-	-	-	0.42
2	Myrcene	991	991	2.08	2.35	7.19	4.24	3.31	4.25	2.88	3.67	4.12	2.16	3.62
3	Limonene	1024	1030	11.94	12.57	35.55	21.91	17.51	22.40	18.11	19.55	24.78	13.92	19.82
4	Linalool	1098	1101	62.77	64.48	16.01	43.26	48.00	27.68	21.87	50.06	20.14	33.07	38.73
5	2,3-Octanedione	1082	1115	-		-	-	-	-	-	-	0.59	-	0.59
6	3,7-Dimethylundecane	1218	1185	0.34		-		-	-	1.12	-	0.48	-	0.64
7	α-Terpineol	1189	1195	0.42		-	0.55	-	1.42	-	0.47	-	-	0.71
8	Dodecane	1199	1200	-		0.63		-	-	-		-	1.03	0.83
9	2-Undecanol	1287	1277	-		-	0.47	-	0.94	-	-	1.51	-	0.97
10	Undecan-2-one	1287	1294	11.74	9.55	24.88	16.58	16.98	31.03	34.63	15.67	33.72	32.73	22.75
11	Tridecane	1300	1300	-		1.36	0.20	-	-	1.22	-	0.70	-	0.87
12	Methyl (*E*)-Cinnamate	1379	1384	4.44	1.46	2.03	3.23	4.83	1.29	1.51	3.07	0.67	-	2.5
13	α-Bergamotene	1434	1430	1.26	1.42	2.07	1.36	1.91	2.40	3.56	1.73	2.62	6.10	2.44
14	Tridecan-2-one	1496	1495	2.03	1.79	7.47	5.62	4.30	5.36	13.03	3.51	8.06	8.91	6
15	*trans* Nerolidol	1534	1564	-		-	0.52	-	-	-	0.57	-	-	0.54
16	Phytol	2105	2106	-		-	-	2.12	1.00	-	-	1.28	-	1.46
17	Isophytol, acetate	2282	2286	0.77		-	-	-	-	-	-	-	-	0.77
Oil yield (%)				0.2	0.24	0.25	0.16	0.27	0.50	0.26	0.30	0.24	0.22	0.26
Total no of components				11	7	9	11	8	10	9	9	12	7	9.3
Total% of components				98.21	93.62	97.19	97.94	98.66	97.77	97.93	98.3	98.67	97.92	97.62

W: Wild, C: Cultivated; Compounds are listed in the order of elution on a DB5 column

RI[a] Retention index from Adams, 2007

RI[b] calculated by GC using n-alkane series under the same conditions as for samples.

Linalool was found in highest proportion in all of the samples analyzed, with an average of 38.73%, followed by undecan-2-one (22.75 %) and limonene (19.82 %) (Table 26). In most of the samples investigated, the principal components linalool, limonene, and myrcene were found in higher proportions in the samples collected from wild population, whereas components like tridecan-2-one and undecan-2-one were detected in lower proportion in wild samples than cultivated samples. At lower altitudes, the main component linalool was present in high percentage in the essential oil of leaf with 64.48% from wild population at 1060 m and 62.77% from the cultivated populations at 1000 m The overall number and proportion of components of the essential oil of leaf were higher in the samples from wild population than the samples from cultivated populations. Phytochemical changes between taxa are usually more noticeable than intraspecific variations of a single taxon at different elevations (Zidorn et al., 2005). The volatile oil in the leaf of *Z. armatum* showed significant changes in the amount of linalool and limonene in plants of various altitudes (Gupta et al., 2011). The difference in chemical composition may also be attributed to different geographical environment, growth, and physiological development of the plant (Guleria et al., 2013).

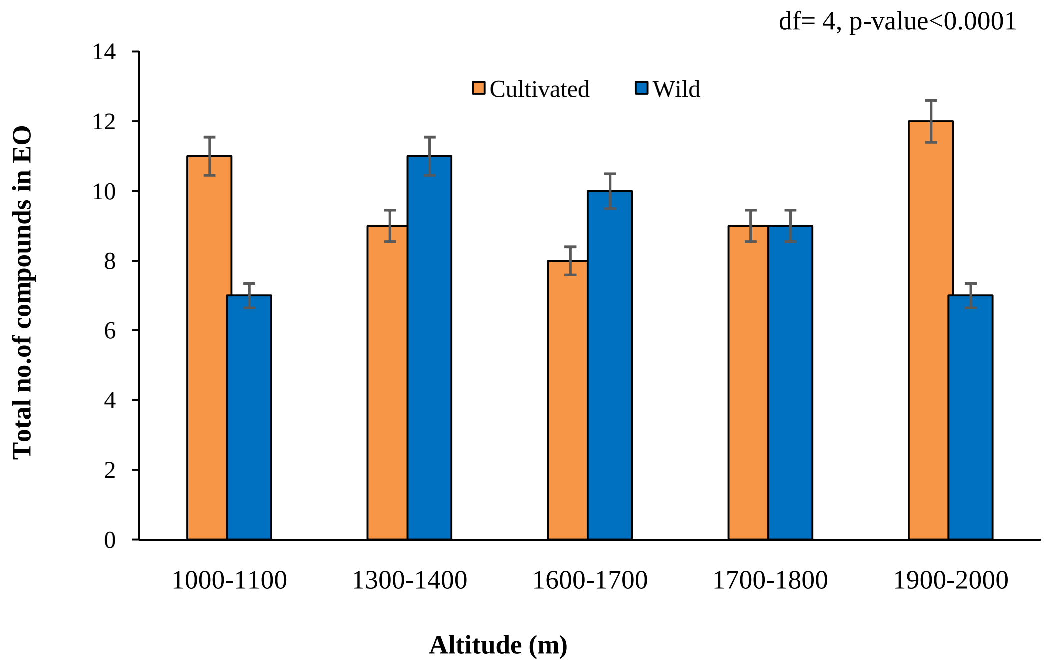
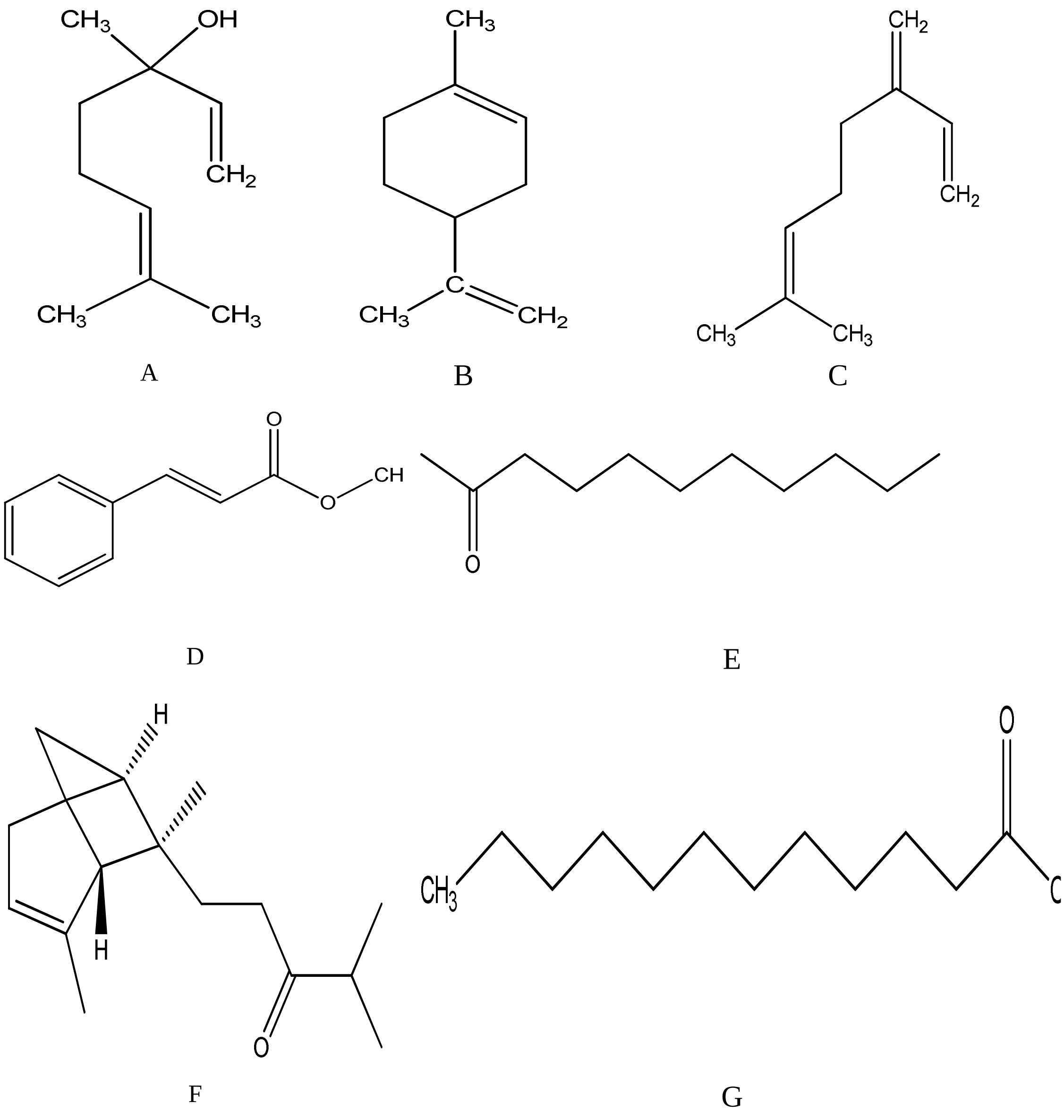


Figure 24: Total number of components in essential oil of leaves of *Z. armatum*

The essential oil of leaf of Z. armatum contained a total of thirty five components, with the major linalool (53.05%), α-limonene diepoxide (11.39%), α-pinene (4.08%), myrcene (3.69%) and limonene (3.1%) in a study by (Barkatullah et al., 2013). The major components like tridecan-2-one, *trans*-α-bergamotene and methyl (*E*)-cinnamate identified in the essential oil of leaf detected in this study were not identified by the previous study. Similarly, the various compounds in essential oil of leaf of *Z. armatum* identified by earlier studies have not been detected in the present study, e.g., *trans*-caryopctehyllene, bornyl acetate, α-copaene (Negi et al., 2012), sabinene (Luong et al., 2003), *β*-fenchol, *β*-phellandrene (Guleria et al., 2013), etc.

The main component in the essential oil of leaf of *Zanthoxylum alatum* from Vietnam was 1,8-cineole (41.0%), with the inclusion of sabinene (8.4%), β-terpineol (2.1%), linalool (4.5%), terpinen-4-ol (5.2%), β-terpineol (4.1%), β-cymene (1.3%), 2,6-dimethyl-1,3,5,7-octatetraene (1.5%) and 2-tridecanone (1.8%) (Luong et al., 2003). Guleria et al. (2013) reported fourteen compounds in the essential oil of *Z. armatum* with the main as linalool (30.58%), 2-decanone (20.85%), β-fenchol (9.43%), 2-tridecanone (8.86%), β-phellandrene (5.99%), sabinene (4.82%), and α-pinene (4.11%).



A: Linalool, B: Limonene, C: Myrcene, D: Methyl cinnamate, E: Undecan-2-one, F: *trans*-α-bergamotene, G: Tridecan-2-one

Figure 25: Structures of the major components in essential oil of leaf of *Z. armatum*

The differences in chemical composition of the essential oil among different altitudes may be attributed to the adaptation to habitats or different growing conditions. Different environmental parameters such as temperature, solar radiation, wind velocity, relative humidity, water availability etc. affect the eco-physiological relations in plants to a large extent. The variation in elevation might produce substantial changes in these parameters, which can lead to major differences in the secondary metabolites accumulation in plants (Sanli & Karadogan, 2017). Therefore the changes in chemical constituents in the essential oil contents at various elevations in this study might be a result of changing ecological niches.

4.7.2 Fruit

The yield of essential oil in fruits of *Z. armatum* at different altitude and population varied significantly ($p<0.001$) ranging between 2.72-7.6 % (Table 27). The maximum yield of essential oil was at 1600 and 1800 m altitude, which decreased thereafter. Similarly, the essential oil yield of wild fruits were higher than the cultivated fruits along all the altitudes. The highest (7.6%) yield of essential oil was from wild fruits collected from 1600-1800 m altitude, while the lowest was 2.72 % from cultivated fruits collected from 1000-2000 m. (Figure 26).

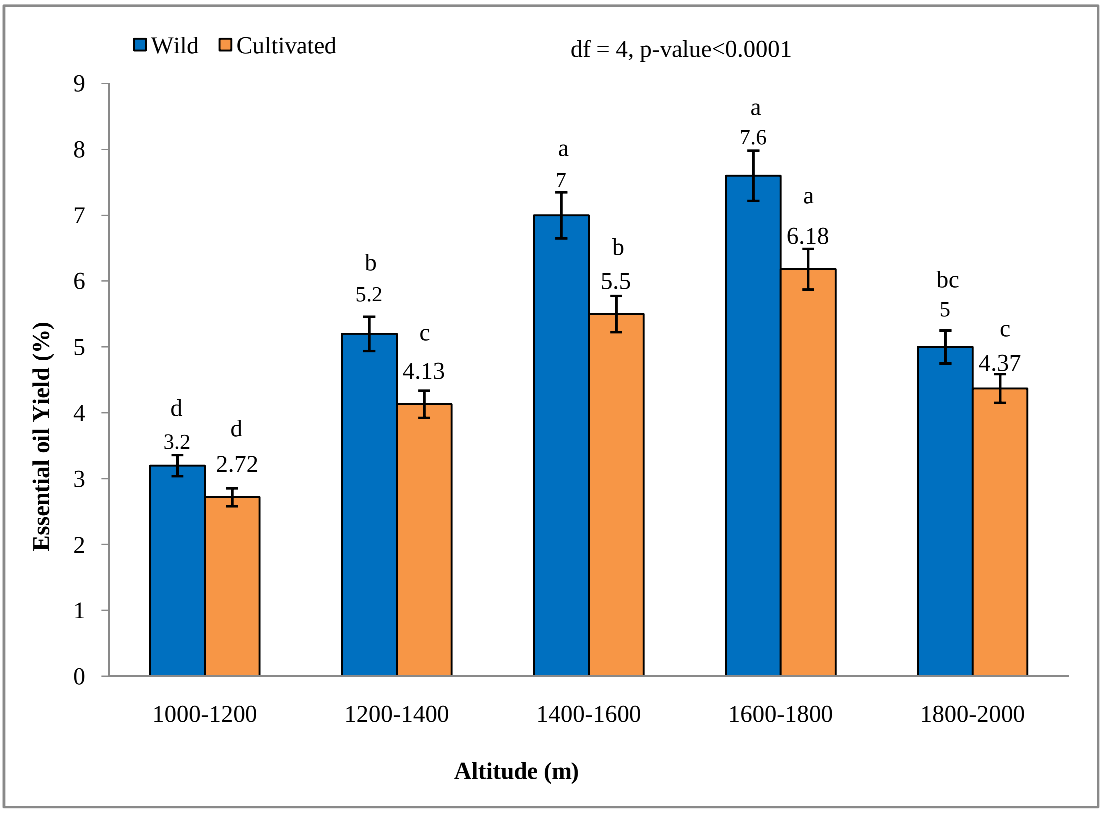


Figure 26: Yield of essential oil in fruits of *Z.armatum*

A total of 13 components were identified from the GC-MS analysis of the essential oil of the fruits of *Z. armatum* collected from different populations and habitats. Table 27 lists all of the identified constituents except the trace components. Linalool, limonene, methyl cinnamate, myrcene, sabinene, and terpinen-4-ol (Figure 25 & 27) were the principal components of the essential oil, and they were found in the highest percentage in all of the samples studied. Linalool was the most prevalent compound in all of the samples examined, followed by myrcene. The total share of linalool was 74.12 % in wild samples and 70.04% in cultivated samples collected from 1600-1800 m altitude while at 1400-1600 m altitude, it was 70.22 % in the wild fruits and 62.28% percentage in the cultivated fruits.

The lowest 47.7% of linalool was identified in cultivated populations at 1200-1400 m altitude. Terpinen-4-ol was detected in lowest percentage in all the samples, and it was not present in 1200-1800 m altitudes. Myrcene, limonene and methyl cinnamate were present in higher quantity at 1800-2000 m altitude whereas sabinene and terpinen-4-ol were highest at 1200-1400 m altitude. β-phellandrene, α-terpineol and piperitone were present in only 2 or 3 samples (Phuyal et al., 2020c). These variations in the quantity of the phyto-components might be because of the differences in genetic makeup, plant organ, and environmental factors (climate, harvesting seasons and geographical location) (Luís et al., 2016).

Table 27: Composition and yield of essential oil in *Z. armatum* fruits from different altitudes and habitats

SN	Name of the compound	RI[a]	RI[b]	Area%										Average (%)
				1000-1200 m		1200-1400 m		1400-1600 m		1600-1800 m		1800-2000 m		
				Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	
1	Sabinene	973	972	1.51	2.75	0.47	0.71	1.08	0.62	0.51	0.53	1.64	0.75	1.05
2	Myrcene	991	991	1.53	4.50	2.76	1.84	2.07	1.55	1.86	1.38	2.24	2.89	2.30
3	Limonene	1031	1030	12.82	31.82	20.49	19.11	12.19	6.52	12.70	5.67	19.99	25.29	16.95
4	β-Phellandrene	1031	1031	-	-	-	-	-	4.63	-	4.44	-		4.53
5	Terpinolene	1084	1086	-	-	-	-	-	-	-	-	-	0.37	0.37
6	Linalool	1098	1101	57.33	44.73	59.69	55.65	70.22	62.28	74.12	70.14	49.09	47.19	59.37
7	Nonanal	1108	1107	-	-	-	0.30		-	-	-	-	-	0.30
8	Terpinen-4-ol	1178	1180	0.62	1.17	0.42	-	0.66	-	0.36	-	0.65	0.49	0.62
9	Cryptone	1188	1187	-	-	-	-		-	-	-	0.49	0.30	0.39
10	α-Terpineol	1189	1195	0.49	-	-	-		-	-	-	0.43	-	0.46
12	Carvone	1242	1246	-	-	-	-	0.57	-	-	-	-	-	0.57
11	Piperitone	1282	1277	0.68	-	-	-		-	-	-	0.47	0.35	0.5
13	Methyl cinnamate	1379	1384	24.18	14.27	16.15	22.30	12.33	12.82	9.52	14.28	25.00	21.90	17.57
Essential oil yield (%)				3.2	2.72	5.2	4.13	7	5.5	7.6	6.18	5	4.37	5.09
Total no of components				8	6	6	6	7	6	6	6	9	9	6.9
Total% of components				99.16	99.24	99.98	99.91	99.12	88.42	99.07	96.44	100	99.53	98.09

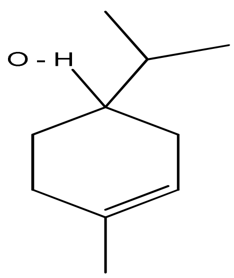
Compounds are listed in the order of elution on a DB5 column.

RI[a] Retention indices taken from Adams (2007).

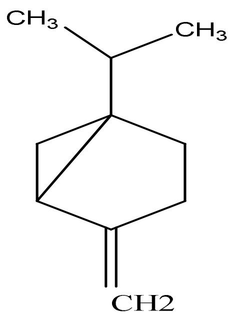
RI[b] calculated by GC using n-alkane series under the same conditions.

The concentrations of linalool and myrcene were highest in the wild populations than in cultivated at all elevations (myrcene was highest in cultivated populations at 1200-1400 m altitude). The variability of the compounds in the essential oil from different populations and altitudes could be attributed to the environmental factors as well as agronomic and management practices including irrigation, plant density and soil tillage (Lubbe & Verpoorte, 2011). Genetic modifications or physiological alterations occurring during growth stage might cause changes in the composition of oil (Németh, 2005).

Altitudinal variation can bring about considerable changes in the different environmental factors like solar radiation, temperature, relative humidity, wind velocity, water availability, etc. which in turn might cause significant differences in the accumulation of secondary metabolites production in plants (Sanli & Karadogan, 2017). Hence, the variations in the chemical constituents at different altitudes in this study could be a result of changing ecological niches. The fruits from natural habitats were in different stages of maturation. Although the fruits were collected during the same period, there were differences in maturity between populations, individuals and even fruits on the same branches.



Terpinen4-ol



Sabinene

Figure 27: Structures of the components of fruit essential oil of *Z. armatum*

Similar to the findings of this study, limonene, linalool, sabinene, myrcene and methyl cinnamate have been previously identified as the major compounds of the essential oil of *Zanthoxylum* (Yoshihito et al., 2000; Tiwary et al., 2007; Waheed et al., 2011; Barkatullah et al., 2013; Paudel et al., 2017; Bhatt et al., 2018). The major components reported by Bhatt et al. (2018) in fruits of *Z. armatum* essential oil were linalool (36.29-45.61%), β-phellandrene (19.93-38.38%), myrcene (4.13-8.73%), methyl cinnamate (3.12-22.14%) and sabinene (1.57-4.78%).

Linalool is an importnt compound used commenrcially in several industries as flavoring agents. In this study the essential oil in fruits from 1600-1800 m had the highest linalool content so the fruits of *Z. armatum* from the locality can be used as a commercial source of linalool for the potential use in relevant industries.

4.7.2.1 Yield and composition of essential oil in fruits based on harvesting period

The yield of essential oil in fruits of *Z. armatum* based on harvesting time was comparatively higher. The oil yield percentage was highest immediately after harvesting and decreased thereafter. The yield ranged from the maximum of 8.2% to the minimum of 4%. There were significant differencences between essential oil yield in fresh and stored fruits (t = 3.7276, df = 20.91, p-value = 0.001251) The yield was highest 8.2% in fruits harvested in September (when fruits were dark red in color) and decreased thereafter to the lowest 4% in fruits harvested in December (Figure 28). The essential oil yield of 8.2% from fruits of *Z. armatum* harvested in September in this study is higher than those reported by earlier researchers. The essential oil yield in fruits of *Z. armatum* was 7.6% (Phuyal et al., 2020c). The yield of essential oil recorded previously by various studies was comparatively lower; 2.3% (Sharma et al., 1996), 1.5% (Gupta et al., 2011), 2%, 5% (Paudel et al., 2017).

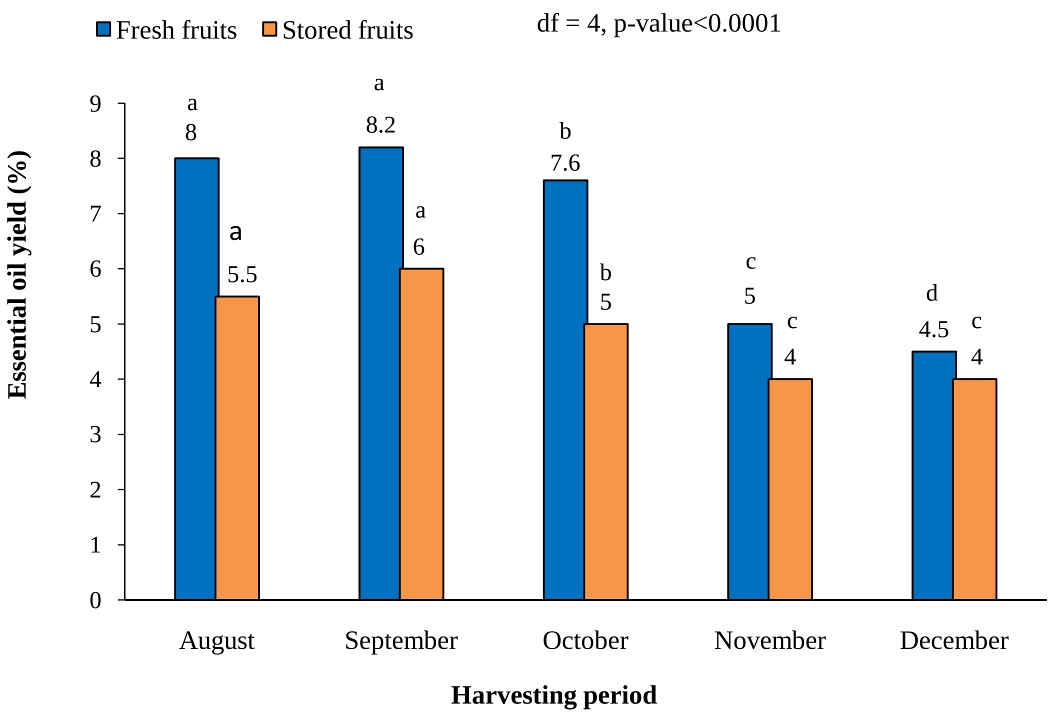


Figure 28: Essential oil yield in fruits of *Z. armatum*

Note: Different letters above bars indicate statistically significant difference between different harvesting time (p < 0.001)

GC-MS analysis of the essential oil in fruits of *Z. armatum* harvested at different period revealed a total of 13 compounds with the major linalool, limonene, methyl cinnamate, sabinene and myrcene. Table 28 lists all the compounds (excluding the trace components). The dominant compound was linalool followed by limonene and methyl cinnamate. The concentration of linalool was highest (82.7%) in September harvested fruits. The concentration of all the identified compounds decreased after storage for some period (Table 28).

Table 28: Composition and yield of essential oil in fruits of *Z. armatum* based on the harvesting period and storage

SN	Name of the compound identified	RI [a]	RI [b]	% Essential Oil										
				Aug	Sept	Oct	Nov	Dec		Aug	Sept	Oct	Nov	Dec
1	Sabinene	973	972	0.70	0.52	2.04	1.80	1.05	Oil was extracted after storing the fruits for a month		0.43	0.82	0.47	0.36
2	Pinene	939	948	1.53				0.34						
3	Myrcene	991	991	1.34	0.99	1.24	3.76	1.44			0.8	0.81	3.09	0.8
4	Cymene						0.47							
5	Limonene	1031	1030	6.05	14.76	20.83	4.45	5.8		5.23	5.54	14.32	3.43	4.76
6	Phellandrene	1031	1031	4.47	2.63	3.35	9.81	5.05		4.27			4.02	3.79
7	β-Ocimene			0.51										
8	Terpinolene	1084	1086				0.39							
9	Linalool	1098	1101	74.95	82.7	56.78	76.87	72.96		74.53	80.64	52.23	67	72.27
10	Ethyllinalool				0.32									
11	Terpinen-4-ol	1108	1107	0.31			0.44						0.41	
12	Methyl cinnamate	1379	1384	10.63	17.27	21.36	16.98	9.63		10.14	9.71	9.72	7.5	6.76
Essential oil yield (%)				8	8.2	7.6	5	4.5		5.5	6	5	4	4

Compounds are listed in the order of elution on a DB5 column.

RI[a] Retention indices taken from Adams (2007).

RI[b] calculated by GC using n-alkane series under the same conditions.

The oil from *Z. armatum* is the potential source of linalool (Kirtikar & Basu, 1993). In this study, the maximum linalool content is 82.7% (Table 28). Earlier reported maximum linalool content of essential oil from fruits of *Z. armatum* was 74.12% (Phuyal et al., 2020c); 64.1% (Sharma et al., 1996). The quantity of linalool in the essential oil of *Z. armatum* from Nepal is 62.2% (Yoshihito et al., 2000). The essential oil in *Zanthoxylum* is highly valued in international market. Linalool is a slightly volatile compound with pleasant aroma because of which it has been used in several industrial and pharmaceutical companies as flavoring agents, perfumes, and cosmetics, and linalool is generally considered as safe for these purposes (Bickers et al., 2003). Based on the present study it can be said that for getting optimum benefits, the fruits should be harvested earlier when they are just ripe and dark red in color and the oil should be extracted immediately. Storage of the fruits after harvesting reduces the quality and quantity of the essential oil. The fruits of *Z. armatum* from the particular locality can be used as a commercial source for the isolation of linalool for the potential use in relevant industries.

4.8 Antimicrobial activities

4.8.1 Leaf

To determine the antibacterial potential of the extract of the leaf of *Z. armatum*, the diameter of the Zone of Inhibition (ZOI) formed by the extracts against the particular microorganism was measured. The extracts produced ZOI only against *Bacillus subtilis*, *Escherichia coli*, MRSA (Methicillin-resistant *Staphylococcus aureus*), and *Staphylococcus aureus* among the organisms evaluated for antibacterial activities, whereas the extracts did not produce any ZOI for the remaining organisms (*Enterococcus faecalis, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typh*i, *Shigella dysenteriae,* and *Staphylococcus epidermidis*). The extracts showed good antibacterial property against MRSA with the maximum ZOI of 16.33±0.04 mm for the sample from 1000 m (cultivated) and 16.28±0.01 for sample from 1060 m (wild). Similarly, the extracts also showed good antibacterial property against *B. subtilis* and moderate activities against *S.s aureus.* Least activity occurred in the extracts against *E. coli*, with the minimum ZOI of 10.32±0.02 mm for sample from 1060 m (wild) and 10.87±0.03 mm for sample from 1400 m (wild) (Table 29). The extracts did not show any activity against the fungi tested (*Saccharomyces cerevisiae* and *Candida albicans*).

Table 29: ZOI produced by the extract of leaf of *Z. armatum* against different bacteria

Name of the organisms	**Zone of inhibition (ZOI) mm**								
	Chloram-phenicol	**1000 (C)**	**1060 (W)**	**1390 (C)**	**1400 (W)**	**1650 (C)**	**1700 (W)**	**1990 (W)**	**2000 (C)**
Bacillus subtilis	23.63 ± 0.06	16.11 ± 0.05	12.92 ± 0.02	13.24 ± 0.03	12.13 ± 0.06	14.06 ± 0.02	13.53 ± 0.05	11.29 ± 0.04	12.05 ± 0.03
Escherichia coli	24.62 ± 0.04	11.32 ± 0.05	10.32 ± 0.02	12.26 ± 0.03	10.87 ± 0.03	12.49 ± 0.07	12.11 ± 0.03	13.14 ± 0.01	11.5 ± 0.2
MRSA	25.07 ± 0.02	16.33 ± 0.04	16.28 ± 0.01	11.91 ± 0.08	12.27 ± 0.06	13.56 ± 0.02	15.21 ± 0.03	10.88 ± 0.02	11.55 ± 0.04
Staphylococcus aureus	24.38 ± 0.03	13.08 ± 0.03	10.65 ± 0.04	15.2 ± 0.02	12.86 ± 0.03	13.1 ± 0.08	12.53 ± 0.02	10.88 ± 0.03	12.71 ± 0.02

The antibacterial properties of the extracts of the leaf of *Z. armatum* against different bacterial strains were also found to be independent of the altitude and habitat factors. The minimum bactericidal concentration (MBC) value ranged from 50 mg/mL to 3.125 mg/mL. The lowest MBC value of 3.125 mg/mL was exhibited by the sample from 1000 m (cultivated) against MRSA and the highest, i.e., 50 mg/mL was for samples from 1990 m (cultivated) and 2000 m (wild) against *Staphyloccus aureus* (Table 30). Most of the MBC values were between 6.5 mg/mL to 12.5 mg/mL.

Table 30: Minimum Bactericidal Concentration (MBC) of the extracts of leaf of *Z. armatum* against different bacteria

SN	Organisms	MBC values (mg/ml)							
		1000 (Cultivated)	**1060 (Wild)**	**1390 (Cultivated)**	**1400 (Wild)**	**1650 (Cultivated)**	**1700 (Wild)**	**1990 (Wild)**	**2000 (Cultivated)**
1	*E. coli*	12.5	25	12.5	25	25	25	12.5	12.5
2	*S. aureus*	12.5	25	6.25	12.5	12.5	6.25	50	50
3	*MRSA*	3.125	6.25	12.5	12.5	12.5	6.25	25	25
4	*B. subtilis*	6.25	12.5	6.25	12.5	6.25	12.5	25	6.25

Similar results were obtained by Barkatullah et al. (2012) for *B. subtilis* and *E. coli* in the leaf extract of *Z. armatum*. But in another study by Guleria et al. (2013), the methanol extract of leaf of *Z. armatum* did not show any activity against *B. subtilis*, *E. coli* and *S. aureus.* Several experiments have demonstrated considerable amount of antibacterial activities of extracts of leaf *Z. armatum* against different bacterial strains (Joshi & Gyawali, 2012; Barkatullah et al., 2012, 2013; Bharti & Bhushan, 2015).

The antibacterial properties of the methanolic extracts of leaf may be because of the combined effects of different phytocompounds present in the crude extracts (Table 19). These studies substantiate future extensive in-vitro and in-vivo investigation of *Z.armatum* for its potential use in several human diseases including dysentery diarrhea, and the infection of skin and urinary tract.

4.8.2 Fruit, seed and bark

The extracts of the fruit and seed of *Z. armatum* were active against *B. subtilis*, *E. faecalis,* MRSA, *S. aureus*, and *S. epidermidis,* while the extract of the bark was active against MRSA and *S. aureus* only among all the tested organisms. For all of the extracts, the most sensitive strain was *S. aureus*, which had the highest ZOI when compared to the other strains. The extracts against *S. aureus* produced the highest ZOI; 20.72±0.04 mm for wild fruits and 18.10±0.28 mm for cultivated fruits, 17.83±0.04 mm for wild seeds and 16.33±0.01 mm for cultivated seeds, and 17.01±0.01 mm for wild bark and 16.44±0.04 mm for cultivated bark. The extract of the seed ad the least efficacy against *E. faecalis*, with a minimum ZOI of 11.29±0.08 mm for cultivated and 11.73±0.06 mm for wild samples (Table 31).

Table 31: Zone of inhibition (ZOI) produced by the extracts of fruit, seed, and bark of *Z. armatum* against different bacteria

SN	Name of the bacteria	Zone of inhibition (ZOI, mm)						
		Chloram-phenicol	Fruits		Seeds		Bark	
			Wild	Cultivated	Wild	Cultivated	Wild	Cultivated
1	*Bacillus subtilis*	25.52 ± 0.01	16.24 ± 0.05	17.04 ± 0.04	15.72 ± 0.07	16.28 ± 0.02	0	0
2	*Escherichia faecalis*	21.61 ± 0.03	14.28 ± 0.07	14.62 ± 0.05	11.73 ± 0.06	11.29 ± 0.08	0	0
3	*MRSA*	30.7 ± 0.1	15.02 ± 0.07	16.28 ± 0.06	17.79 ± 0.07	16.44 ± 0.02	14.30 ± 0.25	13.28 ± 0.03
4	*Staphylococcus aureus*	25.64 ± 0.07	20.72 ± 0.04	18.10 ± 0.28	17.83 ± 0.04	16.33 ± 0.01	17.01 ± 0.01	16.44 ± 0.04
5	*Staphylococcus epidermidis*	24.55 ± 0.02	16.38 ± 0.01	16.19 ± 0.01	15.58 ± 0.04	13.25 ± 0.07	0	0

The extracts of the fruit were found to inhibit the growth of the tested bacteria effectively than the extracts of the seed or bark. The fruit extract had a ZOI of 16.24±0.05mm and 17.04±0.04 mm against *B. subtilis* from wild and cultivated samples respectively. Similarly, the ZOI of the extracts of the wild and cultivated fruits against *E. faecalis* were 14.28±0.07 mm and 14.62±0.05 mm respectively and against MRSA were 15.02±0.07 mm for wild and 16.28±0.06 mm for cultivated; against *S. epidermidis*, the ZOI were 16.38±0.01 mm (wild) and 16.19±0.01 mm (cultivated). The extracts of the seed had moderate antimicrobial activity against the pathogens tested, with a ZOI of 15.72±0.07 mm and 16.28±0.02 mm for wild and cultivated extracts respectively against *B. subtilis*; 17.79±0.07 mm (wild) and 16.44±0.02 mm (cultivated) against MRSA and 15.58±0.04 mm (wild) and 13.25±0.07 mm (cultivated) against *S. epidermidis*. The extract of the bark produced a ZOI of 14.30±0.25 mm (wild) and 13.28±0.03 mm (cultivated) against against MRSA, and 17.01±0.01 mm (wild) and 16.44±0.04 (cultivated) against *S. aureus* (Table 31).

The different types of extracts of wild and cultivated plant samples exhibited differential antibacterial activities and were shown to be unaffected by the habitat factors. Some of the extracts of the wild plant sample had better antibacterial capabilities, while some of the cultivated plant samples were better antibacterials. Fruit, seed, and bark extracts were all shown to be less effective than the standard antibiotic used in this experiment.

Table 32: MBC values of extracts of fruit, seed, and bark of *Z. armatum a*gainst different bacteria

SN	Organisms	MBC values (mg/mL)					
		Fruits		Seeds		Bark	
		Wild	Cultivated	Wild	Cultivated	Wild	Cultivated
1	*B. subtilis*	1.56	1.56	1.56	6.25	0	0
2	*E. faecalis*	12.5	6.25	12.5	25	0	0
3	MRSA	0.78	0.78	1.56	1.56	6.25	6.25
4	*S. aureus*	1.56	1.56	3.12	3.12	1.56	1.56
5	*S. epidermidis*	50	50	12.5	50	0	0

The value of the the minimum bactericidal concentration (MBC) ranged from 0.78 mg/mL to 50 mg/mL. Fruit extracts had the lowest MBC value of 0.78 mg/mL against MRSA, whereas seeds (wild) and fruits had the highest MBC value of 50 mg/mL for *S. epidermidis*. Fruits (wild and cultivated) and seeds (wild) were found to have an MBC of 1.56 mg/mL against *B. Subtilis*; seeds (wild and cultivated) were found to have an MBC of 1.56 mg/mL against MRSA; fruits (wild and cultivated) and bark (wild and cultivated) were found to have an MBC of 1.56 mg/mL against *S. aureus*. Likewise, the MBC for seeds (wild and cultivated) against *S. aureus* was 3.12 mg/mL. The extracts' MBC values were fairly higher against *S. epidermidis* and *E. faecalis* compared to other organisms. (Table 32).

Several studies have shown that the extracts of different parts of *Z. armatum viz*. leaf, fruit, seed, and bark exhibit significant antibacterial activities against a variety of bacteria (Joshi & Joshi, 2000; Joshi & Gyawali, 2012; Barkatullah et al., 2013; Guleria et al., 2013; Bharti & Bhushan, 2015; Singh et al., 2016). In a study, the ZOI exhibited by the hexane and ethanolic extracts of the bark of *Z. armatum* demonstrated antibacterial activity against various bacteria. The ethanol extract developed a ZOI of 14.33 mm against *E. coli* and 10.33 mm against *B. subtilis* whereas the hexane extract produced a ZOI of 11.33 mm against *E. coli* and 11.33 against *B. subtilis*. Similarly ZOI for the hexane extract of the bark against *S. aureus* was 12.67 mm whereas the ethanol extract did not produce any ZOI. (Barkatullah et al., 2012). Similarly, in another investigation the ZOI of the methanol extracts of the fruits was 7 mm against *S. aureus* with the MBC value of 2.5 mg/mL and for *B.*

subtilis 23 mm ZOI with less than 10 (>10) MBC value (Joshi & Joshi, 2000). The methanol extract of bark showed 28.7 mm ZOI against *S. aureus* (Srivastava et al., 2013).

The antibacterial activity of various plant extracts against different organisms are influenced by a number of external as well as internal variables. The ability of agar media to diffuse the extract may allow it to produce less ZOI than its true efficiency. Therefore, the MBC value was calculated to determine the minimal concentration of extracts that is required to stop the growth of the microorganisms (Cheesbrough, 1993).

Antimicrobial, antiviral, antioxidant, and anticancer capabilities are just a few of the remarkable biological activity that flavonoids and other phenolics possess (Havsteen, 2002). The fruit extracts of *Z. armatum* were found to exhibit better antibacterial activity than the extracts of the seed or bark. This could be because fruits might possibly have more phenols and flavonoids than seeds or bark (Phuyal et al., 2020b).

The antibacterial properties of the crude extracts may be attributed to the collegial effects of several phyto-constituents present in the plant such as limonene, linalool, oleic acid, squalene, campestrol, etc. (Prakash et al., 2012; Liu et al., 2013; Dhami et al., 2019; Martins et al., 2020). *Z. armatum* has been reported to produce structurally diverse chemicals including terpenoids, flavonoids, coumarins, sterols, and alkaloids that show antibacterial activity. Many active components have been identified from the plant that might be developed into novel drugs.

All of the extracts studied were found to contain antibacterial activity against a variety of pathogens that cause a variety of infectious human diseases. These findings to some extent support the use of this plant in a variety of traditional ethno-medicinal methods to treat a variety of ailments. Hence the screening, isolation, and characterization of the individual compound that has a major role in various antibacterial activities along with their underlying mechanisms of action, should be emphasized. However, further extensive research is needed to validate the acute and chronic toxicity in animals before clinical trials may begin (Phuyal et al., 2018; Manandhar et al., 2019).

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The present study throws an insight into the various ecological and phytochemical aspects of *Z. armatum*, a common medicinal and aromatic plant of Nepal. The population structure, distribution pattern, regeneration potentiality, and seed germination behaviour along with phytochemical constituents, antioxidant and antimicrobial activities of different parts of the plant were analyzed. Based on the observed data and the review of pertinent literatures, all of the hypotheses have been justified through the generation of empirical data.

Species composition, density, distribution and regeneration status can be considered important factors to judge the status of a forest. *Z. armatum* was found to be distributed randomly in Salyan district and the lower number of seedlings and saplings indicates a fair regeneration pattern. Although the dependency on natural forests for the collection of berries is gradually being replaced by cultivation in private land in the recent years, unsustainable harvesting and collection of saplings from the wild has not been stopped completely. Anthropogenic disturbances including premature harvesting and digging up of saplings was found to severely affect the natural distribution and regeneration in the study area. Consistent rise in demand and unsustainable modes of collection have posed serious threat to the native populations of *Z. armatum*. Thus, effective conservation and management initiatives are most important for conserving the wild genetic diversity of *Z. armatum* in the study area. Assessment of diversity and regeneration status of species is important for their sustainable utilization, management, and conservation. Therefore, a systematic management plan is required for the conservation and sustainable utilization of this valuable species.

After around three years of planting, the plants are ready to harvest. The overall fruit yield increased along with elevation up to 1600-1800 m. The average yield per plant was 4.18 kg fresh wt (2.91 kg dry wt). Soil chemical properties and management practices were found to directly influence the overall yield and quality of fruits. The soil organic carbon, nitrogen, phosphorus and potassium were positively correlated with the fruit yield. Hence, an increase in the fruit could be achieved by adopting effective management practices and integrated manuring.

Seed germination in *Z. armatum* is not easy because of the presence of hard seed coat. Because of slow rate of germination, the natural regeneration of this species in the wild is also affected. Chemical and hormonal treatments induce the germination process, GA_3 being the most effective in promoting germination. The different treatments applied in this study to induce seed germination proved that not all treatments are equally effective. Sand scarification and GA_3 treatment were comparatively better options than other mechanical or chemical treatments for breaking the dormancy. However, from the findings of the present study, it can be concluded that sowing freshly harvested seeds at proper time period is a rather simple and efficient technique to obtain maximum germination in *Z. armatum* for uniform seedling emergence and for raising nursery. The seeds germinate better during mid-phase of harvesting than early or late harvesting. The longer period storage induces a strong phase of dormancy, which is quite difficult to break.

As the seed germination is very slow in *Z. armatum*, vegetative propagation through stem cuttings is a viable option for the mass production of elite plant materials. Different growth hormones, concentration, and growth media significantly affect the rooting and shooting performance of *Z. armatum* stem cuttings. Both IBA and NAA responded well in rooting and shooting, IBA was found to be more effective as compared to NAA. Similarly, the neopeat growth medium was found to be superior over the sand and mix media. So, it can be concluded from this study that IBA 5000 ppm concentration with neopeat medium is the best treatment for rooting the stem cuttings of *Z. armatum*. The results obtained in this study could be of relative significance for the commercial production of quality plantlets as well as for improving agroforestry systems. Establishment of high-tech nurseries and free distribution of saplings to the farmers could possibly reduce the pressure on natural population and also uplift the economic status of the marginalized and poor communities.

The GC-MS analysis of the methanol extracts of leaf, fruit, seed and bark of *Z. armatum* identified a total of four compounds in the extract of the leaf, five compounds in the extract of the fruit, fourteen components in the extract of the seed and six compounds in the extract of the bark. Some of the major components were cyclononasiloxane, octadecamethyl, myrtenol, trans-oleic acid, (E)-4-Benzylidene-3-phenyl-3, 4-dihydronaph, campesterol, β-Sitosterol, α-Amyrin, and methyl ester. Many of these compounds have been known to possess several pharmacological and biological activities. Total phenolic, flavonoid contents, and antioxidant properties of the fruits, seeds, leaves, and bark extracts of *Z. armatum* were considerably good. However, these parameters were remarkably better in fruit and bark extracts as compared to the seed and leaf extracts. Some of the wild samples showed excellent results, and some of the cultivated samples showed better results. The concentration of phenols and flavonoids were highest in the extracts obtained using solvents of high polarity. The methanol extract exhibited greater power of extraction for phenol and flavonoids from *Z. armatum*. The highest TPC, TFC and antioxidant capacity as exhibited in cultivated parts of *Z. armatum* might be a reflection of the interaction between the environment and a part of defense as a result of insect feeding or pest and pathogen infection. Because of the considerablae amount of phenolic and flavonoid contents and remarkable scavenging effects on DPPH, the fruits, seeds, leaves, and bark of *Z. armatum* could be the potent source of natural antioxidants. Further studies should be directed towards the extensive in vivo antioxidant activities of the plant and the relationship of individual phenolic compounds to antioxidant with different mechanisms and isolation, screening, and characterization of individual compounds responsible for antioxidant properties to authenticate their probable uses as sources of natural antioxidants as well as to validate their traditional uses in several medicinal practices.

Variation in elevation, growing conditions, and edaphic factors significantly affect the production and distribution of different active phytochemicals in *Z. armatum* grown at different altitudes. Among the 17 compounds identified from GC-MS, linalool, undecan-2-one, and limonene were the major constituents of the essential oil from leaves. Besides, alpha-bergamotene, myrcene, methyl cinnamate and tridecan 2-one were also identified in almost all the samples. Other components were present in trace amounts. Similarly, there were distinct variations in the composition of essential oil in fruits of *Z. armatum* from different altitudes and populations. The essential oil yield of 8.2% from September harvested fruits of *Z. armatum* in this study was higher than those reported by earlier researches. Among the 13 compounds identified from the fruits essential oil, linalool, methyl cinnamate, limonene, myrcene, sabinene and terpinen-4-ol were the major components. This study also recorded the highest quantity of linalool (82.7%), which was not reported previously. The essential oil yields along with the percentage of components were also comparatively higher in both leaves and fruits from wild populations. Hence it can be said that the various factors including environmental and altitude may also influence the phytochemical constituents. The characterization of volatile oil and the isolation of the major component linalool from the particular locality could be of commercial importance for the potential use in several industries.

The crude extracts of different parts of *Z. armatum* have significant antibacterial properties against several infectious pathogens causing several diseases in humans. The fruit extract was more active against the bacteria than seed, leaf or bark extract and had the highest ZOI against *Staphylococcus aureus*. Bark exhibited activities against MRSA and *Staphylococcus aureus* only. The extracts from leaves showed ZOI against *Bacillus subtilis, Escherichia coli,* MRSA and *Staphylococcus aureus*. The antibacterial activities of fruits extracts of *Z. armatum* were comparatively better than seeds and bark extracts. These experiments partially validate the use of this plant in several traditional medicinal practices to cure various diseases. Therefore, further emphasis should be on screening, isolation, and characterization of the individual components responsible for different antibacterial activities and their underlying mechanism of action. However, additional studies are required to quantify the acute and chronic toxicity in animals before clinical trials.

5.2 Recommendations

Based on the present study, following recommendations have been made:

- Future research should focus on promoting sustainable use and conservation of this valuable plant species incorporating the traditional knowledge with scientific findings.
- Establishment of high-tech nurseries and free distribution of plantlets to the farmers could possibly reduce the pressure on natural population.
- The seed germination and propagation results can be used by nurseries for producing fast and better saplings for commercial cultivation.

- *Z. armatum* can be a potent source of natural antioxidants, hence the isolation, screening, and characterization of individual compounds should be carried out to substantiate their potential as natural antioxidant sources

- The characterization of volatile oil and the isolation of the major component linalool from the locality could be of commercial importance for the potential use in several industries.

- The antibacterial properties as exhibited by *Z. armatum* suggest its potential use in several diseases. Therefore, future studies should focus on the extensive in-vivo and clinical studies as well as the mode of action underlying the antibacterial properties.

- Since *Z. armatum* is a high value plant, specific studies should be conducted for the development of advanced technologies to boost productivity in commercial and mass-scale production.

CHAPTER 6

6.1 SUMMARY

Z. armatum, commonly called as Timur, is a popular Nepalese spice plant with several ethno-medicinal uses. It is distributed in Nepal from west to east at an elevation of 1000 to 2500 m asl. It is one of the 33 prioritized medicinal and aromatic plants with high trade value. Despite its huge importance, the detailed study on ecology and the relation of ecological factors on the accumulation of active phytochemicals in *Z. armatum* is still meager. Understanding the ecology of individual species is important for conservation and for cultivation purposes. Hence, an attempt has been made in the present study to explore the various aspects of *Z. armatum* regarding its ecological status including propagation techniques and phytochemical variations from wild and cultivated populations.

The study was conducted at Salyan and Kavrepalanchok districts. Six different localities of Salyan district were selected to cover possible habitats for the study of population density, distribution pattern and comparative phytochemical studies. Samples for phytochemical studies and essential oil composition were collected from Salyan district. Vegetative propagation and seed germination experiments were conducted at, Dabur Nursery, Kavrepalanchowk district. Samples for studying comparative essential oil yield and composition of fruits essential oil based on harvesting time were collected from Dabur Nursery.

The population, distribution, frequency, abundance, and regeneration pattern of *Z. armatum* were studied. Altogether 50 species belonging to 44 genera and 34 families were recorded from the study area. Rosaceae was the dominant family with 6 species. The mean population density of *Z. armatum* in the study area was found to be 913.33 individuals/ha. It was found to be distributed randomly, scattered in patches associated with other species. Pure stands were not evident anywhere. It was most dominant at Kupinde and least dominant at Chhatreshwori. Regeneration status of *Z. armatum* in the study area was fair. Average seedlings and saplings densities were 150 and 100 individuals/ha respectively. Despite the steady increase in commercial cultivation, collection from wild has not yet decreased putting an enormous pressure on the natural population of *Z. armatum* in the study area. Anthropogenic disturbances mainly uprooting of seedlings and saplings to plant in private land was found to affect the natural distribution.

For estimating the average fruit yield, the plants cultivated in farmland were selected. The plants are usually ready for harvest after about 3 years of plantation and the average yield per plant was found to be 4.18 kg (fresh wt), 2.91 kg (dry wt). Periodic lopping of old and matured branches increases fruit production. Fruit yield was found to be positively correlated with soil nutrients. Higher soil organic carbon, nitrogen, phosphorus and potassium were found to increase the overall yield of fruits. *Z. armatum* produces solitary seeds, which are glossy black in color, roughly rounded with hard seed coat. The average length and breadth of a single seed was found to be 4.6 and 3.9 mm, respectively and the average weight of 1 air dried seed was 21.6 mg.

The effects of different growth hormones, their concentrations, and different rooting media on the rooting and sprouting behavior of stem cuttings of *Z. armatum* were investigated. The cuttings were treated with different concentration of hormones IBA and NAA (2000 ppm, 3000 ppm, and 5000 ppm), and the cuttings were subsequently planted in sand, neopeat, and mix rooting media. IBA 5000 ppm in neopeat medium produced the highest number of roots (6.5) and root length (11.6 cm).

Several experiments including various treatments were conducted to study the germination behavior in the seeds of *Z. armatum*. Pre-sowing treatments affected the germination to some extent. The germination percentage for sand scarified seeds was 35.33, which is comparatively higher than that of chemical treatments. Cold stratification and hot water treatment did not induce germination. Higher concentrations of GA_3 increased germination, with the maximum of 54.67% at 1500 ppm. Viability decreased with storage for long period. Among all the methods for inducing seed germination in *Z. armatum*, the most effective was sowing freshly harvested seeds at proper time. A maximum of 62.44% germination was obtained for the seeds collected during 16 September-15 October. Seeds germinated better during mid-phase of harvesting than early or late. Sowing seeds immediately after harvesting best is a simple, safe, reliable, low-cost efficient way to obtain higher germination in *Z. armatum.*

The quantitative evaluation of the bioactive compounds present in the methanol extracts of leaf, fruit, seed and bark of *Z. armatum* were carried out through GC-MS. Four compounds were identified in the extract of leaf; the major component was cyclononasiloxane, octadecamethyl (41.86%). Five compounds were identified in the extracts of fruits with the major component myrtenol (44.5%). The extract of seed were found to contain a total of fourteen components and the major were were trans-oleic acid (67.66%), β-Sitosterol (9.7%), α-Amyrin (8.39%),, methyl ester (7.93%), Similarly, a total of six compounds were identified in the extract of the bark with the major component (E)-4-Benzylidene-3-phenyl-3, 4-dihydronaph (47.6%) and campesterol (28.44%).

Total phenolic contents (TPC), Total flavonoid Contents (TFC) and antioxidant activities of the methanolic extracts of leaf, fruit, seed and bark from wild and cultivated populations were calculated. Phenols, flavonoids and antioxidant properties were higher in fruit compared to the leaf, seed and bark. The TPC value of the extract of cultivated fruit was 226.3 ± 1.14 mg/g GAE and that of wild fruit was 185.02 ± 2.15 mg/g GAE.). Similarly, the The highest TFC value was 135.17 ± 2.02 mg/g QE for the extract of cultivated fruit and for wild fruit it was 103.7+1.39 mg/g QE. Cultivated parts were found to contain more phenol, flavonoids and antioxidant properties.

Variation in the yield and composition of essential oil from leaf and fruit of *Z. armatum* in relation to altitude from wild and cultivated populations were analyzed. The essential oil components were analyzed through GC-MS. The essential oil yields were comparatively higher in wild leaves and fruits. The yield of essential oil yield ranged from 0.16 to 0.5% and altogether 17 components were identified in the essential oil of leaf. Similarly, the the yield of essential oil of fruits ranged from 2.72% to 7.6% and consisted of a total of 13 compounds. Essential oil yield was higher in wild fruits than cultivated fruits. The major components of the essential oil were linalool, limonene, sabinene, myrcene, methyl cinnamate undecan-2-one, trans-alpha-bergamotene, tridecan-2-one linalool, limonene, methyl cinnamate, and terpinen-4-ol. The yield and composition of essential oil of fruits based on harvesting period were also analyzed. The yield was comparatively higher and ranged from the maximum of 8.2% to the minimum of 4%. The yield percentage of the essential oil was highest immediately after harvesting and decreased thereafter. Concentration of all identified compounds decreased after storage for some period. Highest yield, 8.2% was in September collected fruits (when fruits were dark red). The essential oil yield of 8.2% in fruits of *Z. armatum* in this study was higher than those reported by earlier researches. This study also recorded the highest quantity of linalool, 82.7%, which was not reported previously.

The in-vitro antimicrobial activities of essential oil of leaves and fruit and the methanolic extracts of leaf, fruit, seed, and bark of *Z. armatum* were evaluated against eleven bacterial and two fungal strains using agar well diffusion method. The minimum inhibitory concentration and the minimum bactericidal concentration were also determined. The extracts of different parts were active against five bacteria only ie. *Bacillus subtillis, Enteroccus faecalis,* Methicilin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* and *Staphylococcus epididermis,* whereas no activity was observed against the tested fungi. Similarly, the essential oil was also found to be inactive against the tested organisms. *S. aureus* was the most sensitive strain for all the extracts. The maximum zone of inhibition was produced by the extracts of fruits against *S. aureus* (20.72 mm for wild and 18.10 mm for cultivated). The minimum bactericidal concentration value ranged from 50 mg/mL to 3.125 mg/mL. Antibacterial activities of fruits were comparatively better than seeds and bark. It might be due to the higher phenolics and flavonoids contents in fruits than seeds.

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APPENDICES

Appendix 1: Soil analysis protocol

Soil organic carbon (SOC)

Walkley and Black method was used to determine organic carbon and organic matter in soil. Dried and fine (passed through 0.5 mm sieve) soil (0.5g) was taken in a 500 mL conical flask and added 10 mL potassium dichromate (1N) with gentle swirling. Then, 20 mL of concentrated sulphuric acid was added and the mixture was allowed to cool down. After 30 min, 200 mL of distilled water was added to the mixture. After adding 10 mL phosphoric acid and 1mL diphenyl a mine indicator, the mixture was titrated with freshly prepared ferrous ammonium sulphate (0.5N) until the color changed from blue-violet to green. In every bath of 10 soil samples, a single blank (without soil) was run. Organic carbon was calculated as follows:

$$\text{Organic carbon (\%)} = \frac{1.3 \times 0.003 \times 100 \times \mathrm{N}\ (\mathrm{B}-\mathrm{C})}{\mathrm{M}}$$

Organic carbon (%): 1.3×0.003×100×N (B-C) M

Where, N = Normality of Ferrous ammonium sulphate

B = Volume of Ferrous ammonium sulphate consumed in blank titration (mL)

C = Volume of Ferrous ammonium sulphate consumed with soil sample (mL)

M = mass of soil (g)

Soil nitrogen (N)

Estimation of total nitrogen by micro-Kjeldahl method included three steps: digestion, distillation, and titration. In digestion, 1g air dried and sieved (passed through 0.5 mm sieve) soil was taken in a clean and dry kjeldahl digestion flask (300 mL). As a catalyst, mixture of 3.5 g potassium sulphate and 0.4 g copper sulphate was mixed with soil sample, and 6 mL concentrated sulphuric acid was added to the mixture with gentle shaking. The flask was heated at low temperature until the bubbles disappeared from the black mixture. Then the temperature was raised to about 350°C and heating was continued until the mixture turned to turquoise (greenish - blue). The flask was removed from the heating mantle and allowed to cool down for about 15 min Then 50 mL distilled water was added to the digest with shaking. For distillation, the digest mixture was transferred to distillation flask (300 mL). Boric acid indicator (10 mL) was taken in a small beaker (100 mL) and placed below the noggle of condenser in such a way that the noggle was dipped into the indicator solution. When the mixture in the distillation flask was slightly warm, 30 mL of sodium hydroxide solution (40%) was added. When distillate began to accumulate in the beaker with boric acid indicator, color of the indicator changed from pink to green. Distillation was continued until the volume of distillate reached to about 50 mL. Then the distillate was titrated with hydrochloric acid (0.1 N) and the volume of acid consumed was recorded. With each batch of soil samples, single blank (without soil) was run. Following formula was used to determine total nitrogen content of the soil samples.

$$\text{Nitrogen content (\%)} = \frac{14 \times N \times (S - B)}{M} \times 100$$

where, N = Normality of HCl

S = Volume of HCl consumed with sample (mL)

B = Volume of HCl consumed with blank (mL)

M = Mass of soil taken (mg)

Soil phosphorus (P)

Available phosphorus of soil samples was estimated by using procedures as described in Trivedy and Goel (1986). Turger's extract was made by mixing 2 g of soil with 200 ml of 0.002 N H_2SO_4 and the mixture was shaken (photo 15) in a vibrator (model: KCHVIBRAX-VXR) at speed of 1200/min for half an hour. Then the total suspension (200 ml) was filtered by using Whatmann No.1 filter paper to get a clear soil solution. Filtration was repeated until the filtrate was clear. Then after, 50 ml of the filtrate was taken in a clean beaker and 2 ml of ammonium molybdate solution was added to it followed by 5 drops of $SnCl_2$ solution. A blue color was observed in the mixture after addition of $SnCl_2$ solution. Then the reading of the solution was taken at 690 nm on a spectrophotometer using distilled water as blank solution with same amount of chemicals. The reading was taken after 5–12 minutes of the addition of $SnCl_2$. Similarly, for standard curve, various dilutions of the standard phosphate solution at the interval of 0.1 mg P/L were made and their absorbance at 690 nm was noted. Finally, a curve of absorbance and concentration of various dilutions of phosphorus was made and the equation for the curve was estimated by using MS Excel (Figure A).

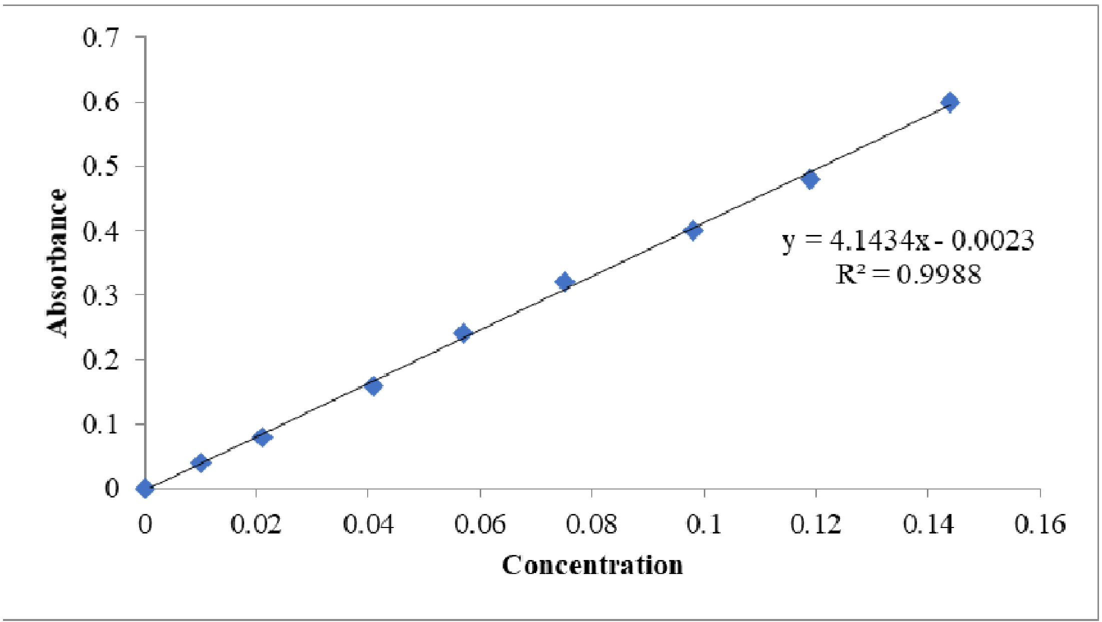


Figure A: Line graph showing calibration curve of available phosphorus

Thus, obtained equation from standard curve was used to estimate the concentration of available phosphorus. Finally, the percentage of available phosphorus in soil was calculated by using following formula:

$$\text{Phosphorus (P)} = \frac{\text{mg P/L soil solution}}{50}$$

Where, mg P/L soil solution was obtained with the help of standard curve.

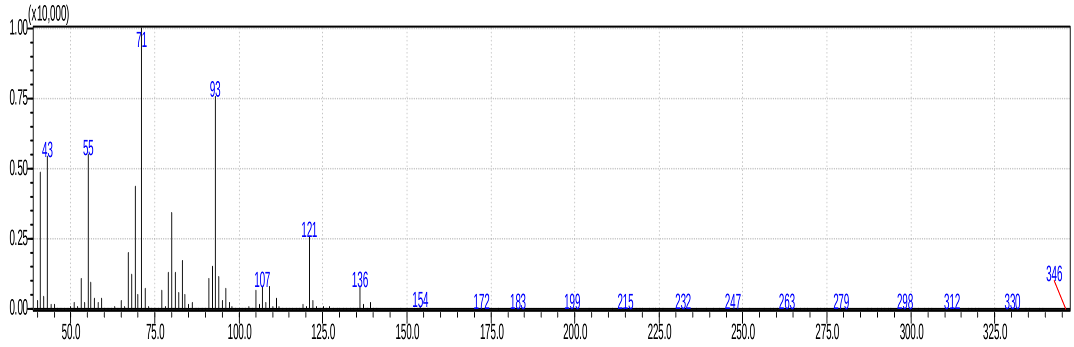
Soil potassium (K)

Exchangeable Potassium content of soil samples was determined by flame photometer method (Trivedy and Goel, 1986). Soil extract was prepared by mixing 2 g of soil sample with 20 ml of 1 N ammonium acetate solution. Then the mixture was shaken in a vibrator of KCH-VIBRAX-VXR model at speed of 1200/min for fifteen minutes. Afterwards, the total suspension of 20 ml was filtered by using Whatmann no.1 filter paper to get a clear soil solution. Filtration was repeated until the filtrate was clear. Thus, obtained clear filtrate was transferred to a clean rinsed test tube and its flame photometer reading was noted at filter of 768 nm.

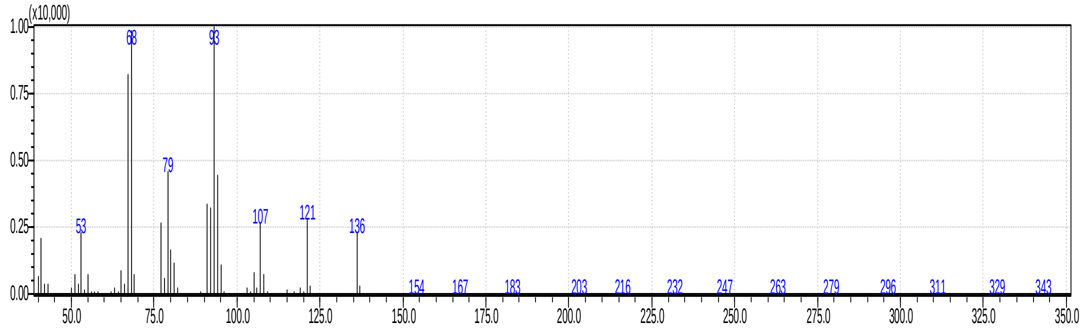
Soil pH

Soil pH was determined by the potentiometric method (Gupta 2000) using a pH meter (Digital pH meter, 802, systronics (89-92) Naroda Industrial Area, Ahmeda Bad, India). Before pH measurement, the electrode of the pH meter was dipped for 24 h in tap water. Then, buffer solutions of pH tablet 7.0 and 4.0 were prepared freshly. The pH meter was warmed up for 15 min before starting pH measurement. 10 g of air-dried fine soil was mixed in 100 mL of distilled water and stirred well by the help of glass rod. Then, the mixture of soil and water was left for decantation about half an hour and hence solution of soil sample was made ready for pH measurement. Now, the pH meter was calibrated through buffer solution of pH 4.0 and 7.0 and pH measurement was taken for each solution of soil sample. Electrode of pH meter was flushed by distilled water and wiped by cotton each time before dipping it from anyone solution either buffer or of soil sample to next.

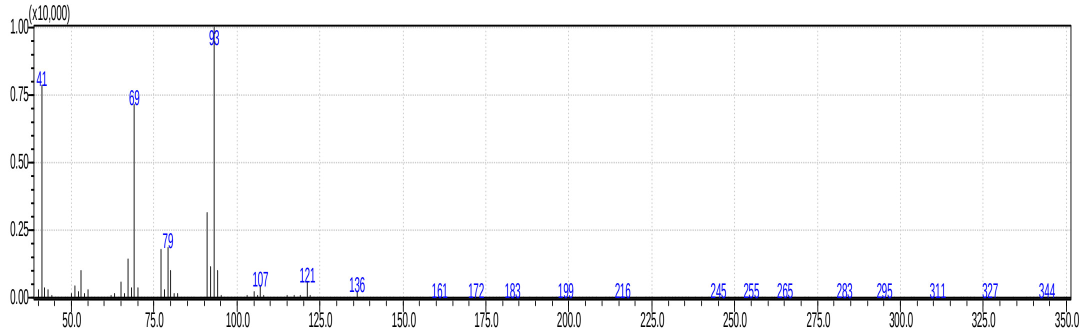
Appendix 2: Mass spectra of major components of essential oil of fruits and leaves of *Zanthoxylum armatum*



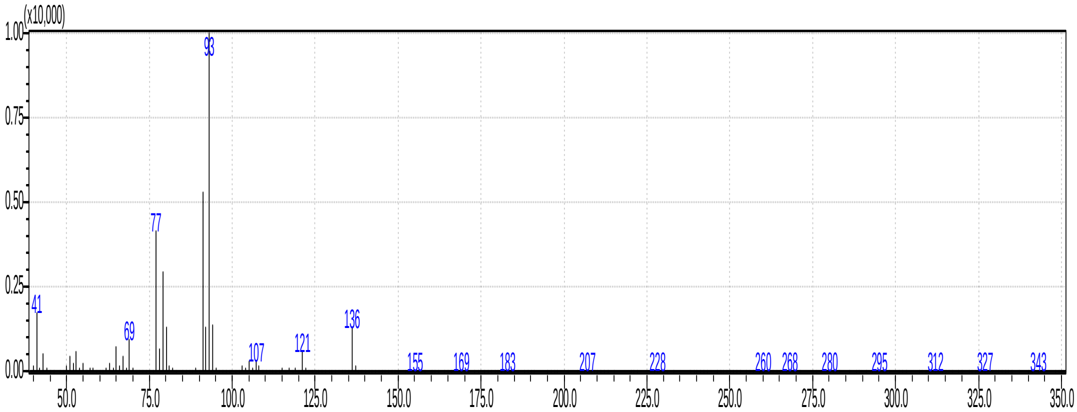
Linalool



Limonene

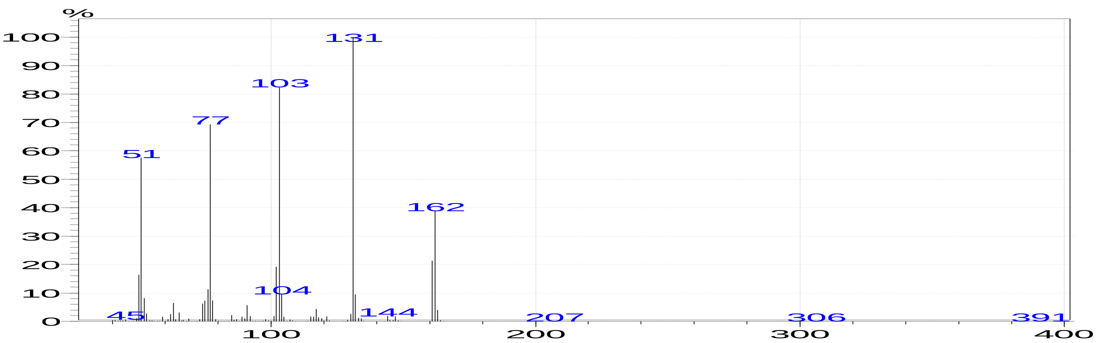


Myrcene

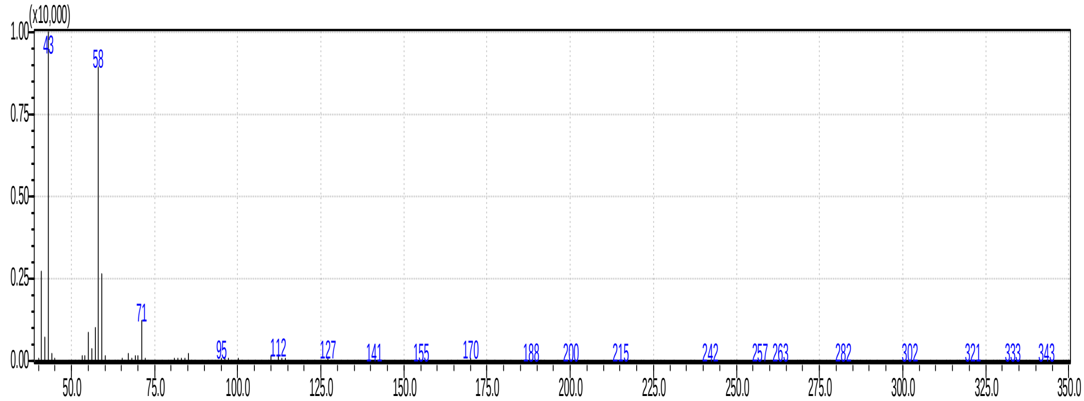


Sabinene

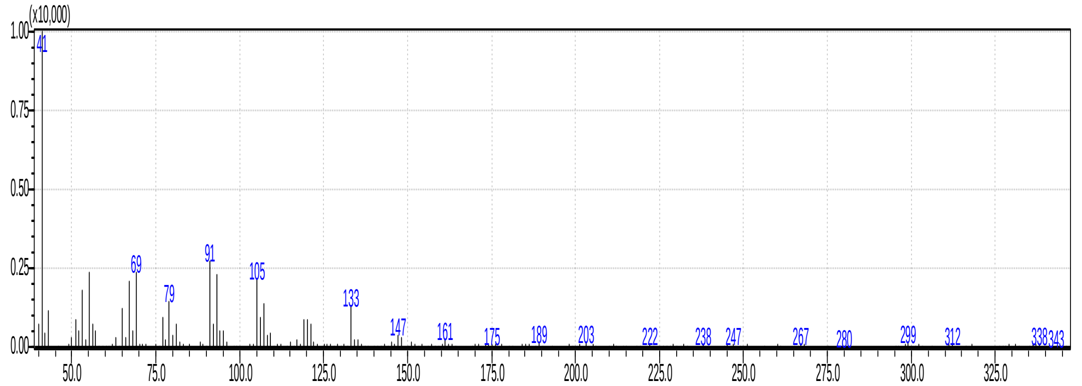
Terpinen-4-ol



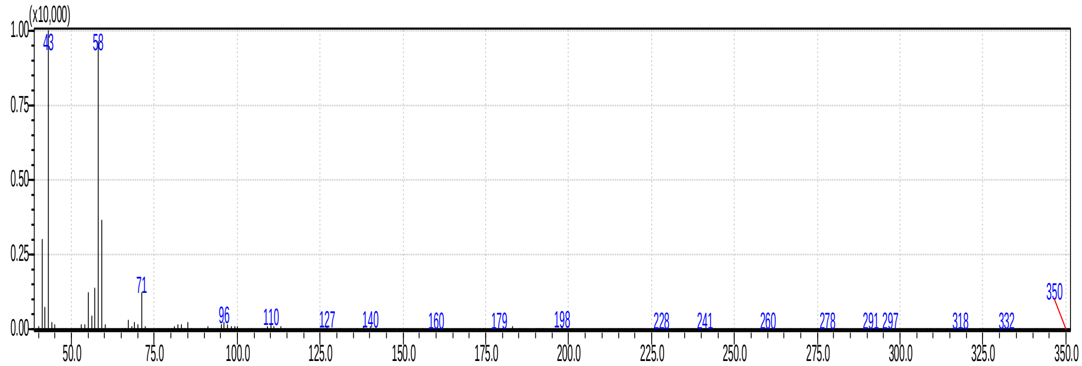
Methyl Cinnamate



Undecan-2-one



Trans-α-Bergamotene

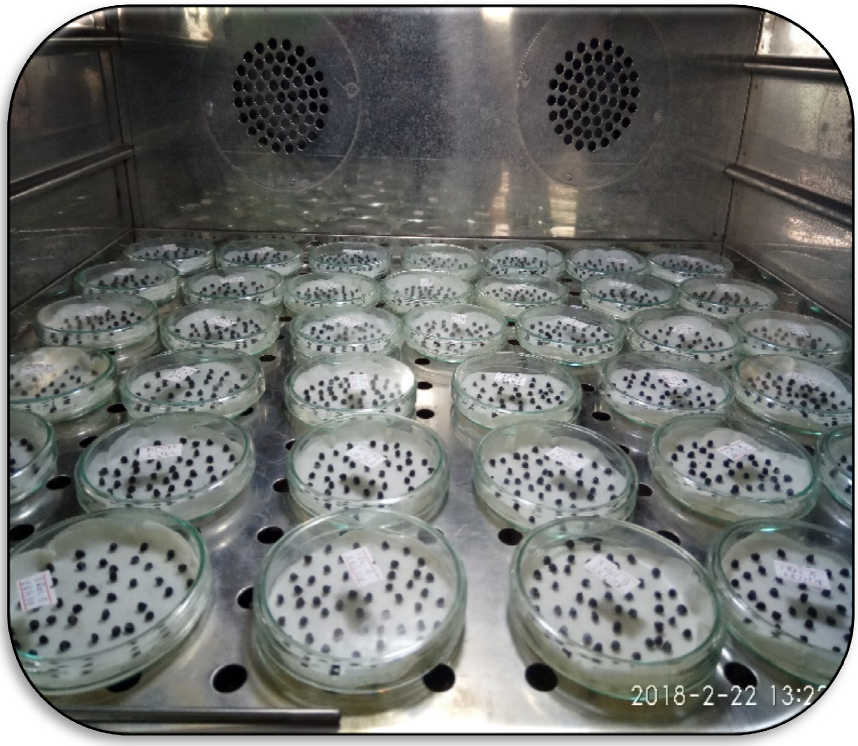


Tridecan-2-one

Appendix 3: Study area (Salyan district) and study species (*Zanthoxylum armatum)*

Appendix 4: Vegetative propagation experiment under green house condition at Dabur Nursery

Appendix 5: (A)Treated seeds of *Zanthoxylum armatum* inside seed germinator (B) Emergence of seedlings



(A)

(B)

Appendix 6: Antibacterial activities of different extracts of *Zanthoxylum armatum* (A) Working condition inside a biosafety cabinet (B) ZOI of methanol extracts of leaf against MRSA (B) MIC of fruit against Bacillus subtilis (C) MBC determination of fruit against MRSA

Appendix 7: List of publications and conference

Publications

1. Phuyal, N., Dahal M. S.., Jha, P. K., Raturi, P. P., Gurung, S., Rajbhandary, S. 2022. Effects of pre-sowing treatments and harvesting period on the seed germination of *Zanthoxylum armatum* DC. Journal of Applied Research on Medicinal and Aromatic Plants. 2022, 100435, ISSN 2214-7861, https://doi.org/10.1016/j.jarmap.2022.100435.

2. Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2022. Population structure and regeneration of *Zanthoxylum armatum.* Journal of Ecology and Environment 46:11.

3. Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2020. Comparison between essential oil compositions of *Zanthoxylum armatum* DC. fruits grown at different altitudes and populations in Nepal. *International Journal of Food Properties*. 23 (1): 1971-1978. (DOI: 10.1080/10942912.2020.1833032).

4. Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2020. In-vitro antibacterial activities of methanolic extracts of fruits, seeds, and bark of *Zanthoxylum armatum* DC. *Journal of Tropical Medicine*. Volume 2020, Article ID 2803063, 7 pages. (https://doi.org/10.1155/2020/2803063).

5. Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2020. Total phenolic, flavonoid contents, and antioxidant activities of fruit, seed, and bark extracts of *Zanthoxylum armatum* DC. *The Scientific World Journal.* Volume 2020, Article ID 8780704, 7 pages. (https://doi.org/10.1155/2020/8780704).

6. Phuyal, N., Jha, P. K., Raturi, P. P., Gurung, S., Rajbhandary, S. 2019. Essential oil composition of *Z. armatum* leaves as a function of growing conditions. *International Journal of Food Properties*. 22 (1) 1873-1885.

7. Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2019. *Zanthoxylum armatum* DC.: Current knowledge, gaps, and opportunities in Nepal. *Journal of Ethnopharmacology*. (doi:10.1016/j.jep.2018.08.010).

8. Phuyal, N., Jha, P. K., Raturi, P. P., Gurung, S. and Rajbhandary, S. 2018. Effect of growth hormone and growth media on the rooting and shooting of *Zanthoxylum armatum* stem cuttings. *Banko Janakari*. 28 (2): 3-12.

Conference/Seminar

Oral presentation

- Phuyal, N., Jha, P. K., Raturi, P. P., Gurung, S., Rajbhandary, S. 2022. Effects of pre-sowing treatments and harvesting period on the seed germination of *Zanthoxylum armatum* DC. International Conference on Biodiversity and Bioprospecting. 22-24 June, 2022, Kathmandu, Nepal
- Phuyal, N., Jha, P. K., Raturi, P. P., Rajbhandary, S. 2019. Zanthoxylum armatum DC. (Rutaceae), an important medicinal plant of Nepal. 11th HOPE Meetings with Nobel Laureates. 4 March (4-8 March, 2019). Okinawa, Japan.
- Phuyal, N., Jha, P. K., Raturi, P. P. and Rajbhandary, S. 2019. "Antibacterial activities of methanolic extracts of fruits, seeds and bark of Zanthoxylum armatum DC". International Youth conference on Science, Technology and Innovation, Research, and Innovation for Prosperity. 2019 (IYCSTI-2019) (21-23 October, 2019), Kathmandu, Nepal

Poster presentation

- Phuyal, N., Jha, P.K., Raturi, P.P. and Rajbhandary, S. (2019). *Zanthoxylum armatum* DC. (Rutaceae), an important medicinal plant of Nepal. 11th HOPE Meetings with Nobel Laureates. 6 March (4-8 March, 2019). Okinawa, Japan.
- Phuyal, N., Jha, P.K., Raturi, P.P., Gurung, S. and Rajbhandary, S. (2019). Effect of harvesting period on the seed germination behavior of *Zanthoxylum armatum* DC. South Asian Agro-forestry Conference. (2-3 July, 2019), Kathmandu, Nepal.

Award/Reward

- NAST Journal Award 2020 for the article "Comparison between essential oil compositions of *Zanthoxylum armatum* DC. fruits grown at different altitudes and populations in Nepal" published in International Journal of Food Properties
- NAST Journal Award 2018 for the article "*Zanthoxylum armatum* DC.: Current knowledge, gaps and opportunities in Nepal" published in Journal of Ethnopharmacology.
- First prize for the poster "Effect of harvesting period on seed germination of *Zanthoxylum armatum* DC" at South Asian Agro-forestry Conference, 2019, Kathmandu, Nepal).

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Zanthoxylum armatum DC.: Current knowledge, gaps and opportunities in Nepal

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Linalool (CID: 6549)
Limonene (CID: 440917)
α-Phellandrene (CID: 7460)
β –Phellandrene (CID: 11142)
Myrcene (CID: 31253)
P-Cymene (CID: 7463)
Camphene (CID: 6616)
β-Amyrin (CID: 73145)
Vitexin (CID: 5280441)
Tambulin (CID: 5281700)
Berberine (CID: 2353)
G-Fagarine (CID: 107936)
B-Fagarine (CID: 637520)
Asarinin (CID: 233333)
Sesamin (CID: 72307)
Bergapten (CID: 2355)
Xanthyletin (CID: 65188)
Methyl cinnamate (CID: 637520)
Armatamide (CID: 5320157)
β-Sitosterol-β-D-Glucoside (CID: 91884650)

Keywords:
Zanthoxylum armatum
Timur
Phytochemistry
Pharmacology
Biological activities

ABSTRACT

Ethnopharmacological relevance: *Zanthoxylum armatum* DC. possesses several medicinal properties and has been commonly used in different indigenous medicinal practices to cure several diseases because of its stomachic, carminative and anthelmintic properties.
Aim: This review paper aims to provide an update on and analysis of information about the ecology, uses, phytochemistry, pharmacology, trade opportunities, policy gaps for the commercialization of this species forming a basis for further scientific innovations
Materials and methods: Information was gathered through a search of different books, journals, articles, annual reports, proceedings and web-based materials.
Result: Alkaloids, sterols, phenolics, lignins coumarins, terpenoids and flavonoids have been identified from leaves, fruits, stem, bark and seeds. Its trade value is also very high with its manifold applications in Ayurveda, allopathy, general pharmacy, and other industries. Antimicrobial, antiviral, antioxidant, anti-inflammatory, cytotoxic, hepato-protective, insecticidal/larvicidal effects are of particular relevance.
Conclusion: It is one of the prioritized medicinal plants for economic development in Nepal. Owing to its diverse applications, the species can be developed as an important commodity for alleviation of poverty in rural areas. The various ethno-pharmacological applications of *Zanthoxylum armatum* have been verified by several related researches. More extensive study on the individual specific phyto-component can lead to novel innovations for the well-being of mankind.

1. Introduction

Zanthoxylum armatum DC. (Rutaceae), commonly called Timur in Nepal (English: Nepal pepper or prickly ash), is an important medicinal plant. Eight species of *Zanthoxylum* have been reported from Nepal till now: *Z. acanthopodium* DC., *Zanthoxylum armatum* DC., *Z. floribunda* Wall., *Z. nepalense* Babu, *Z. nitidum* (Roxb.) DC., *Z. oxyphyllum* Edgew., *Z. simularis* Hance and *Z. tomentellum* Hook. f. (DPR, 2011a, 2016;

Abbreviations: KATH, National Herbarium; TUCH, Tribhuvan University Central Herbarium; GC-MS, Gas chromatography-mass spectrometry; MIC, Minimal inhibitory concentration; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; SOD, Superoxide dismutase; CAT, Catalase; GSH, Glutathione; CCl4, Carbon tetrachloride, SGOT, serum glutamyl oxalacetic acid transaminase; SGPT, serum glutamyl pyruvate transaminases; ALKP, alkaline phosphatase; SBLN, serum bilirubin; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; DBil, direct bilirubin; TBil., Total bilirubin, DEET diethyl-3methylbenzamide, NRs Nepali Rupees; MAPs, medicinal and aromatic plants; NTFPs, non-timber forest products; WHO, World Health Organization; ISO, The International Organization for Standardization

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Rajbhandari et al., 2015). Even though eight species of *Zanthoxylum* have been included in this review as being used in Nepal, only five species have been accepted taxonomically according to The Plant List. *Z. nepalense* Babu is classed as an unresolved name, while *Z. floribunda* Wall and *Z. simularis* Hance are not recorded there (The Plant List, 2013). Among these species reported from Nepal, *Zanthoxylum armatum* DC. is the most common and one of the 30 medicinal plants of the country, which has been prioritized by the government of Nepal for economic development with a high emphasis on cultivation and agro-technology development (DPR, 2006).

The different parts of the plants: leaves, fruits, stem, bark, seeds have been used in several indigenous medicinal practices as carminative, antipyretic, appetizer, stomachic, toothache, dyspepsia (Manandhar, 2002; Kala et al., 2005; Singh et al., 2016). A wide array of chemical compounds including alkaloids, flavonoids, lignins, coumarins, phenols, terpenoids have been found in this plant. These compounds are responsible for various biological activities like anti-oxidative, antimicrobial, antiviral, hepato-protective, insecticidal/larvicidal etc., which have been demonstrated by several pharmacological studies. There is a huge demand of *Zanthoxylum armatum* in both domestic and international market due to which the market price has been escalating in the last two decades (Hertog and Wiersum, 2000).

Despite of the species' importance, a comprehensive review on *Zanthoxylum armatum* is still not available. Hence an effort has been made to gather all the fragmentary information of *Zanthoxylum armatum* regarding the uses, phytochemistry, pharmacology and to analyze the current state of knowledge and possible opportunities that can be tapped for the overall benefit of the rural communities. It is important to establish a strong linkage between the traditional knowledge and modern researches to authenticate the ages old traditional ethno-medicinal practices. Hence it is expected that this information will be of relative significance to all the stakeholders, students and researchers for future research prospects.

2. Morphology and anatomy

Zanthoxylum armatum (Fig. 1) is a small aromatic tree or large shrub up to 6 m high. Branches are glabrous, usually armed with straight or slightly compressed, reddish brown stipular spines. The leaves are imparipinnate with 3–5 pairs of leaflets, elliptic-lanceolate, acuminate, base rounded or cuneate, sessile, margins usually entire, with a large gland associated with each tooth. The petiole and rachis are often winged between leaflets and sometimes bearing a spine at the point of insertion. Inflorescences are terminal panicles on short lateral shoots. Flowers are minute and polygamous, borne on short cymes. Male flower has 6–8 stamens, filaments 2 mm arranged around globose pistillode. Female flower has 1–3 ovoid-subglobose carpels with two ovules attached to inner angle of axis. Fruit is a small drupe, reddish, ovoid and glandular warted, splitting into two when ripe (Fig. 1). Fruits contain single rounded and shining black seeds, 2–3 mm in size (Grierson and Long, 1991; Nair and Nayar, 1997)

Barkatullah et al. (2014) studied the anatomical characters of the leaf, stem bark and fruit of *Zanthoxylum armatum*. The internal structure of leaf shows a single layer of epidermis and palisade mesophyll. The vein-islets are squarish, elongated, polygonal or irregular with forked and unforked vascular branches. The internal structure of leaf was unique with the complete absence of any kind of trichomes or any other appendages. Nine types of stomata were recorded, among them brachparatetracytic was the most frequent one. Special leaf epidermal feature, the stomatal cluster was also observed. Bark and fruit anatomy of *Z. armatum* showed different tissue arrangement. The seed was non endospermic and contains an elongated embryo. Oggero et al. (2016) also studied the anatomical characters of the leaf and stem of *Zanthoxylum armatum* from central Argentina. The peculiar characteristics are the presence of secretory cavities, glandular trichomes and hypostomatic leaves.

3. Distribution and ecology

Zanthoxylum armatum is found in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), north-east India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, North & South Korea, north Vietnam, Taiwan, Lesser Sunda Islands, Philippines, Malaya peninsula and Sumatra (Nair and Nayar, 1997). In Nepal, it is distributed from west to east at an elevation range of 1000–2500 m in open places or in forest undergrowth (DPR, 2007). The distribution range of *Zanthoxylum armatum* in Nepal based on herbarium specimens deposited at National Herbarium (KATH) and Tribhuvan University Central Herbarium (TUCH) is presented in Fig. 2.

The plant grows well in open pastures, wastelands and secondary scrub forests with adequate rainfall. Moist areas with deep soils exposed to sun and degraded slopes, shrub lands, natural forests and wastelands

Fig. 1. *Zanthoxylum armatum* (a) A mature flowering plant (b) Young fruits (c) Ripe fruits.

Fig. 2. Distribution of *Z. armatum* in Nepal based on herbarium specimens deposited at KATH & TUCH.

are the suitable habitat for *Zanthoxylum armatum*. For the cultivation of this species, clay or loam soil with high organic matter is preferable. The flowering starts on five year old plants in April-May and fruiting in August-October and can be harvested from October to January. Flowering is greatly affected by hails and storm (Kunwar and Pokharel, 2012).

4. Propagation

It is generally propagated through seeds, but also from vegetative parts through soft wood cuttings. Natural regeneration usually occurs through seeds but the seeds undergo strong dormancy and may take few months to years for germination. Freshly harvested seeds are best for the large-scale cultivation. The seeds are sown in August-September in polybags in nursery or main field. The seeds germinate in 20–30 days after sowing. Stem cuttings may also be planted in the nursery during monsoon in July-August. The plants are ready for harvest after three years of plantation and the average annual yield of a five years plant is about 3.5 kg (ANSAB, 2011). The crop is generally free from any disease, insect or nematode attack, and physiological disorders. However Tara et al. (2011) investigated 7 insect pests on *Zanthoxylum armatum* that mostly caused the defoliation.

5. Vernacular information

Zanthoxylum armatum, commonly known as Prickly Ash, Winged Prickly Ash is a common Nepalese spice plant. It is called as Timur in Nepali. It is known by various names in different regions and languages.

Vernacular names in different ethnic groups of Nepal

Bhojpuri	Timur
Chepang	Timpur, Upur
Danuwar	Timur,Tirkene
Gurung	Prumo
Lepcha	Sungrukung, Timbur
Limbu	Midimba, Warekpa
Newari	Tebu
Rai	Khakchan, Terkane
Sherpa	Yerma
Sunwar	Sekkren
Tamang	Prumo
Tharu	Timur
Tibetan	Gyer-ma

Source: Manadhar, 2002.

In other countries/ regions

Bengali	Gaira
Burmese	Gawra Kha Nan Nan, Teza Bo
Chinese	Ci Zhu Ye Hua Jiao, Qin Jiao (Taiwan), Zhu Ye Jiao
English	Bamboo-Leaved Prickly Ash, Nepal Pepper, Prickly Ash, Prickly Ash bark, Toothache Tree, Winged Prickly Ash, Winged Prickly-Ash, Prickly Ash
German	Nepal pfeffer
Japanese	Fuyu Zanshou, Fuyu-Sansh
Korean	Gae San Cho
Thai	Mak Kak
Hindi	Tejphal, Tumru, Darmar, Trimal, Nepali dhaniya (Bharti and Bhushan, 2015)
Manipuri	Mukthrubi (Bharti and Bhushan, 2015)
Kannada	Dhiva, Tumburudu, Jimmi
Malayalam	Tumpunal, Tumpuni
Marathi	Chirphal, Naepaali dhane
Mizoram	Arhrikreh
Oriya	Arhrikreh, Ranabelli
Pashtu	Dambara
Sanskrit	Saurabha, Tejovati, Tumbaru, Vanaja
Tamil	Tumpunalu
Telugu	Gandhalu, Konda-kasimi, Konda, Kaasimanda
Urdu	Dambrary, Tamur

Source: Bachwani et al. (2012)

6. Ethnobotanical uses / importance

Zanthoxylum armatum has been used extensively in traditional indigenous medicinal practices in Nepal by different ethnic communities. Several ethnobotanical studies have documented the various ethnomedicinal uses in different types of ailments. The seeds and barks of *Z. armatum* are used as aromatic, carminative, tonic in fever, dyspepsia (Anonymous, 1970). In stomach problems, the seeds powder is taken with warm water. The fruits and seeds are used for curing cholera, tooth ache and as leech repellant (Shrestha, 1985, 1988; Manandhar, 1987; Joshi and Edington, 1990; Joshi and Joshi, 2000; Balami, 2004). The fruits are used in fever, cold, cough, indigestion and as tonic (DPR, 1983). The bark, thorns and fruits are used in fish poisoning (Kunwar et al., 2009, 2013; Joshi, 2004; Malla et al., 2014). The seeds are chewed to cure toothache, added in vegetables for detoxification. The liquid (steam) collected after the fermentation of seeds are taken twice a day to cure tuberculosis. The seeds cooked with water, wheat flour and oil is taken during edema (Subedi, 2017). 5–6 powdered seeds are taken orally with lukewarm twice daily for one week in malfunction of the liver (Rai and Pokharel, 2006). The dried seeds can act as effective pesticide against small insects of wheat plants. The paste of its seeds and leaves of *Artemisia vulgaris* are applied to wooden house and furniture to repel termites and other wood eating insects (Turin, 2003).

It is an important plant having various medicinal, pharmaceutical, biological properties. Fruits are used for toothache, dyspepsia, as a carminative and for stomachache. Fruits are used as condiment and flavoring agent (Anonymous, 1970; Arshad and Ahmad, 2004; Iqbal and Hamayun, 2005; Abbasi et al., 2013). The branches, barks, fruits and seeds are extensively used as a carminative, stomachic and anthelmintic, branches are used as toothbrush. In Nepal, the fruit decoctions and berries are used for abdominal pain, carminative, antispasmodic, rheumatism, skin diseases, cholera, diabetes and asthma (Singh et al., 2016). Fresh fruits are used as spices and also for making pickles (Joshi, 2000; Manandhar, 2002; Balami, 2004; Malla et al., 2014)

The fruits and seeds are employed as an aromatic tonic in fever, dyspepsia, tooth ache, stomach ache, and expelling roundworms (Verma and Khosa, 2012; Tiwary et al., 2007; Kalia et al., 1999; Rajbhandari et al., 2001; Uprety et al., 2010). Depending upon the

Table 1
Different ethnomedicinal uses of *Zanthoxylum armatum*.

S. No.	Ailments/Use	Parts used	References
1	Abdominal pain	Fruit decoction	Rajbhandari (2001), Rijal (2011)
2	Alcohol preparation	Fruits	Kala et al. (2005)
3	Anthelmintic	Fruits and seeds	Tiwary et al. (2007), Ramanujan and Ratha (2008), Verma and Khosa (2010)
4	Antipyretic	Fruits	Akhtar et al. (2013)
5	Antispasmodic	Fruits	Baral and Kurmi (2006)
6	Appetizer	Fruits	Balami (2004), Kala (2005)
7	Aromatic tonic	Seeds	Alamgeer et al. (2013)
8	Asthma	Fruits	Kirtikar, Basu (1933), Kanjilal (1997), Baral and Kurmi (2006)
9	Bronchitis	Stem bark, fruits, seeds	Kirtikar, Basu (1933), Kanjilal (1997), Naeemuddin et al. (2010)
10	Carminative	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2004), Baral and Kurmi (2006), Tiwary et al. (2007), Abbasi et al. (2010a), Verma and Khosa (2012), Alamgeer et al. (2013), DPR (2016)
11	Chest infection: Fruit powder mixed with *Mentha* sp. and table salt is taken with boiled egg.	Fruits	Islam et al. (2009)
12	Cholera	Fruits, bark	Joshi and Edington (1990), Baral and Kurmi (2006), Abbasi et al. (2010a), Rijal (2011), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016)
13	Cold and cough	Fruits	Joshi (2004), Kala (2005), Gewali and Awale (2008), Bhatt and Chhetri (2009), Kunwar et al. (2009), Singh and Singh (2011)
14	Condiments and flavoring agents	Seeds	Joshi and Joshi (2000), Arshad and Ahmad (2004), Balami (2004), Kala et al. (2005), Abbasi et al. (2010b), Malla et al. (2014)
15	Depression	Seeds	Zaidi et al. (2009)
16	Diabetes	Fruit	Baral and Kurmi (2006), Khan, Yadava (2010)
18	Diuretic	Bark	CSIR (2005)
19	Dizziness	Fruit pickle	Gewali and Awale (2008)
20	Dysentery	Seeds boiled in water	DPR (1983), Subedi (2017)
21	Dyspepsia	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2004), Tiwary et al. (2007), Verma and Khosa (2012), DPR (2016)
22	Fever	Bark and seeds	Kala (2005), Tiwary et al. (2007), Gewali and Awale (2008), Verma and Khosa (2012), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016)
23	Fish poison	Fruits, thorns, branches	DPR (1983), Rasaily (2003), Kunwar et al. (2009), Malla et al. (2014), DPR (2016), Tamang et al. (2017)
24	Flatulence	Seed	Zaidi et al. (2009)
25	Gastrointestinal disorders	Fruits	Joshi and joshi (2000), Shrestha and Dhillion (2003), Naeemuddin et al. (2010), Uprety et al. (2010), Singh and Singh (2011), Abbasi et al. (2013), Sigdel et al. (2013)
26	Gum diseases	Young shoots, fruits	Hamayun (2003), Ahmed et al. (2004), Arshad and Ahmad (2004), Iqbal and Hamayun (2005), Kala et al. (2005)
27	Headache	Fruit pickle	Gewali and Awale (2008)
28	High altitude sickness	Fruit pickle	Gewali and Awale (2008)
29	Houseflies repellent	Fruits	Gaur (1999)
30	Indigestion	Fruits, seeds	DPR (1983), Kirtikar, Basu (1933), Kanjilal (1997), Balami (2004), Rajbhandary and Ranjitkar (2006), Zaidi et al. (2009)
32	Insecticides /pesticides	Branches	Subedi (2017)
33	Leech repellent	Fruits	Balami (2004), Kala et al. (2005), Manandhar (1996)
34	Limb numbness	Fruit pickle	Gewali and Awale (2008)
35	Piles	Fruits	Abbasi et al. (2013)
36	Piscicide (Catching fishes)	Root, fruit, bark, leaves	Mathur et al. (1961), Zaidi et al. (2009)
37	Pneumonia (Cattles: sheep)	Aerial parts	Sindhu et al. (2010)
38	Rheumatism	Fruits	Kirtikar, Basu (1933), Kanjilal (1997), Baral and Kurmi (2006)
39	Skin diseases	Fruits	Baral and Kurmi (2006)
40	Stomach ache	Fruits	Ahmed et al. (2004), Arshad and Ahmad (2004), Joshi (2004), Tiwary et al. (2007), Gewali and Awale (2008), Verma and Khosa (2012), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016)
41	Tick infestation (Cattles: buffalo)	Aerial parts	Sindhu et al. (2010)
42	Timur hag (soup) : Made from dried fruits is consumed to keep the body warm.	Fruits	Kala et al. (2005)
43	Tonsillitis	Fruit pickle	Gewali and Awale (2008)
44	Tooth brush	Young shoots/ branches	Hamayun (2003), Ahmed et al. (2004), Arshad and Ahmad (2004), Kala et al. (2005), Abbasi et al. (2010a, 2010b)
45	Toothache	Fruit/seeds	Kirtikar, Basu (1933), Kanjilal (1997), Arshad and Ahmad (2004), Kala et al. (2005), Kunwar et al. (2009), Kunwar et al. (2013), Alamgeer et al. (2013), Malla et al. (2014), DPR (2016), Tamang et al. (2017)
46	Tuberculosis	Seeds	Gurung (2002), Subedi (2017)
47	Varicose veins	Stem bark, fruit, seed	Kirtikar, Basu (1933), Kanjilal (1997)
48	Vermicide	Fruits	Kala et al. (2005)
49	Walking sticks	Wood	Arshad and Ahmad (2004), Iqbal and Hamayun (2005))

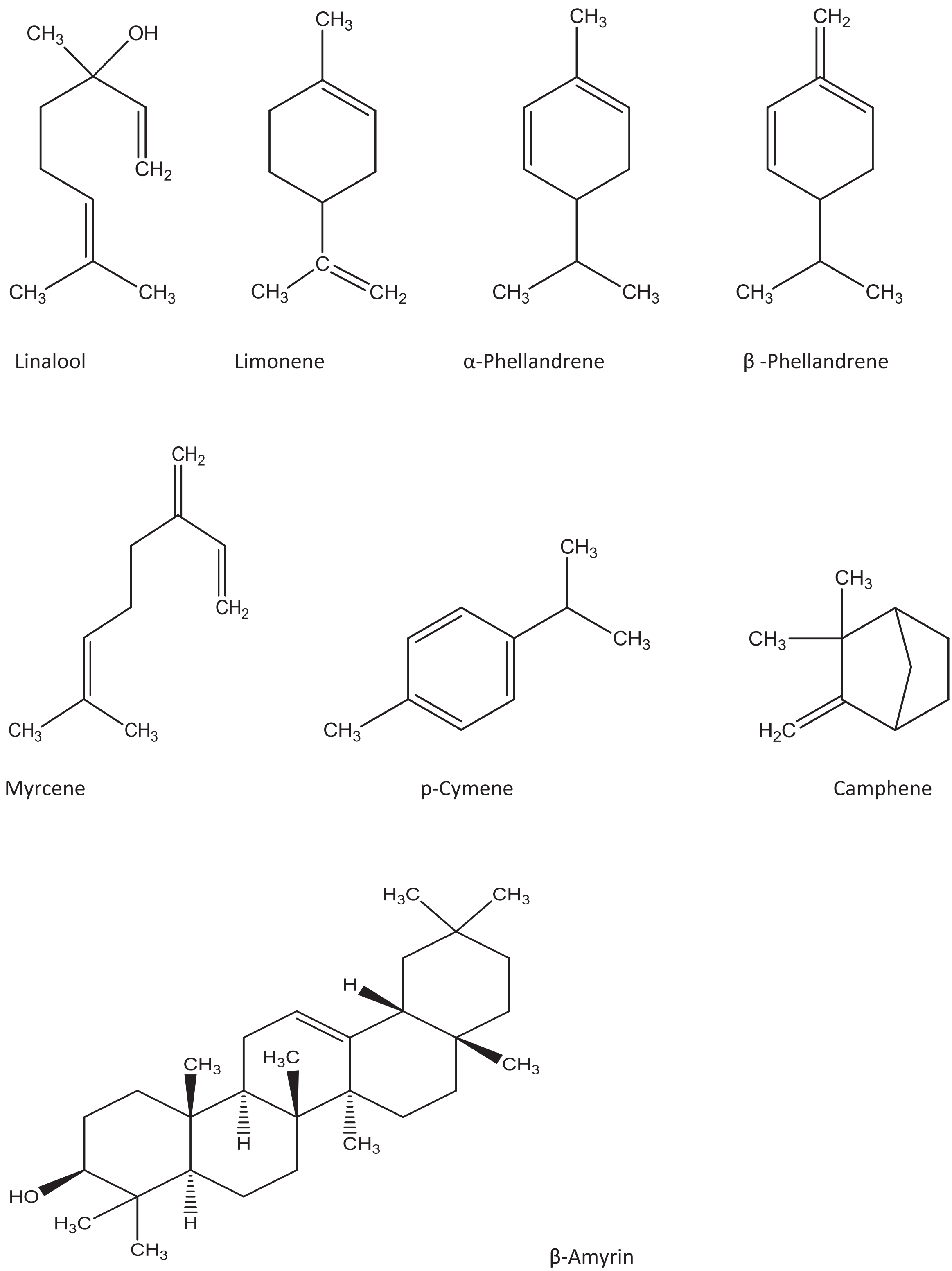

Fig. 3. Structures of different terpenoids isolated from seed/leaf oil of *Zanthoxylum armatum*.

environmental condition, the fruits may contain essential oil that ranged from 2% to 7% with an average yield of 5% (Manandhar, 2002; Paudel et al., 2017). The volatile oil is employed as an antidiarrheal, antiseptic, deodorant and anticatarrhal (Bhattacharya and Zaman, 2009; Bhattacharya et al., 2010; Neetu et al., 2001). The pharmaceutical companies generally use the fruits for making different types of toothpaste and gel. The different ethnomedicinal uses of *Zanthoxylum armatum* is presented in Table 1.

The essential oil, extracted from the fruits is commercially known as *Zanthoxylum* oil, which is highly valued for its vibrant aroma and used in perfumeries and has a high demand in international markets (DoF, 2014). It is frequently used in different aroma therapy because of its soothing effect. Almost all parts of the plant are aromatic and hence, supposed to possess essential oil. The essential oil composition has much more importance regarding the medicinal properties and its constituents (Bhattacharya and Zaman, 2009).

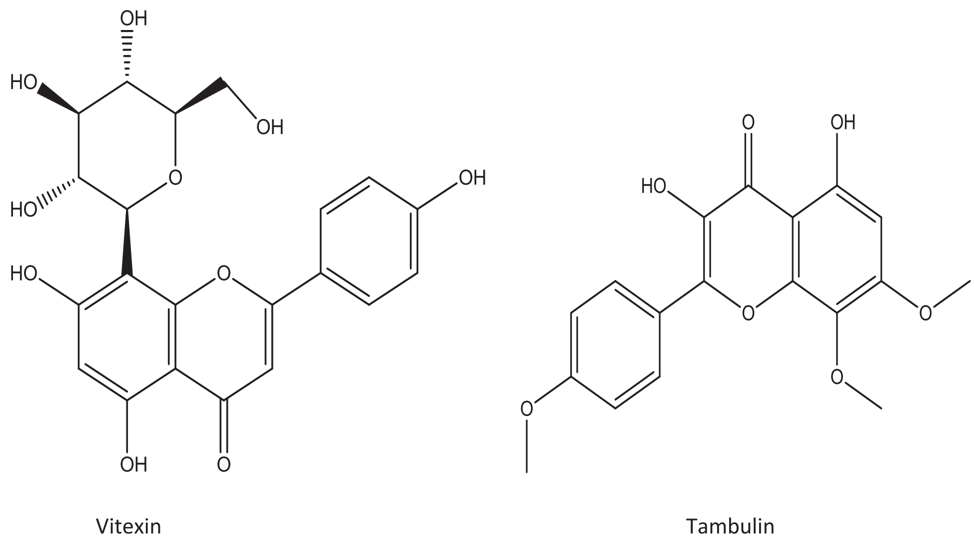

Fig. 4. Structures of flavonoids of *Zanthoxylum armatum*.

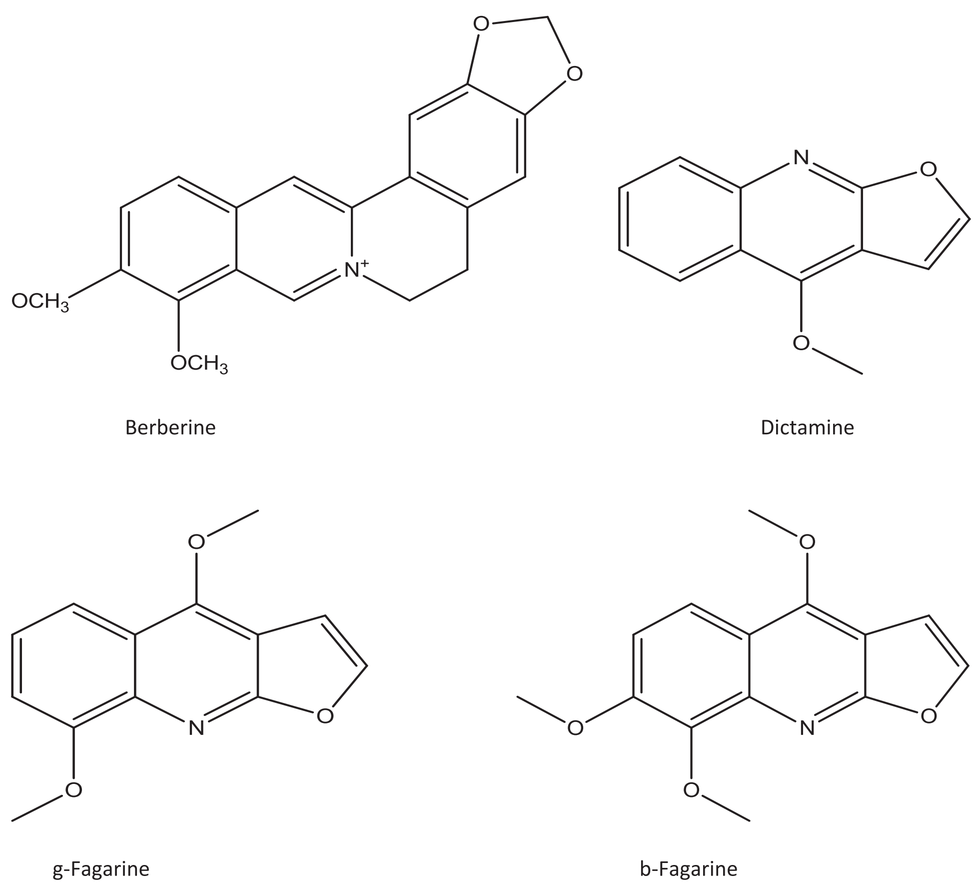

Fig. 5. Structures of alkaloids from bark of *Zanthoxylum armatum*.

7. Phytochemistry

Various phytochemical constituents like terpenoids (Fig. 3), flavonoids (Fig. 4), alkaloids (Fig. 5), phenolics, lignins (Fig. 6), coumarins (Fig. 7), glycosides and benzoids, steroids (Fig. 8), fatty acids, alkenoic acids, amino acids have been extracted from different parts of the plant i.e. seed, leaf, fruit, root and bark (Li et al., 2006; Tiwary et al., 2007; Negi et al., 2011, 2012; Waheed et al., 2011; Bachwani et al., 2012; Joshi and Gyawali, 2012; Barkatullah et al., 2013; Brijwal et al., 2013; Bharti and Bhushan, 2015; Kayat et al., 2016; Singh et al., 2016).

Monoterpenes like linalool and limonene (Fig. 3) are the major constituents of the essential oil. Seeds contain hydroxylic (4Z) enolic acid and various volatile compounds (Ahmad et al., 1993). The various alkaloids, flavonoids, flavonol glycosides, lignins, phenolics, sterols, terpenoids, fatty acids, alkenic acids, amino acids, various aromatic and volatile and number of other compounds have been identified and isolated from *Zanthoxylum armatum* essential oil in good quantity (Waheed et al., 2011; Bhatt et al., 2018).

Gas chromatography-mass spectrometry (GC-MS) analysis reveals that the hexane extract of fruits contains 36 different chemical

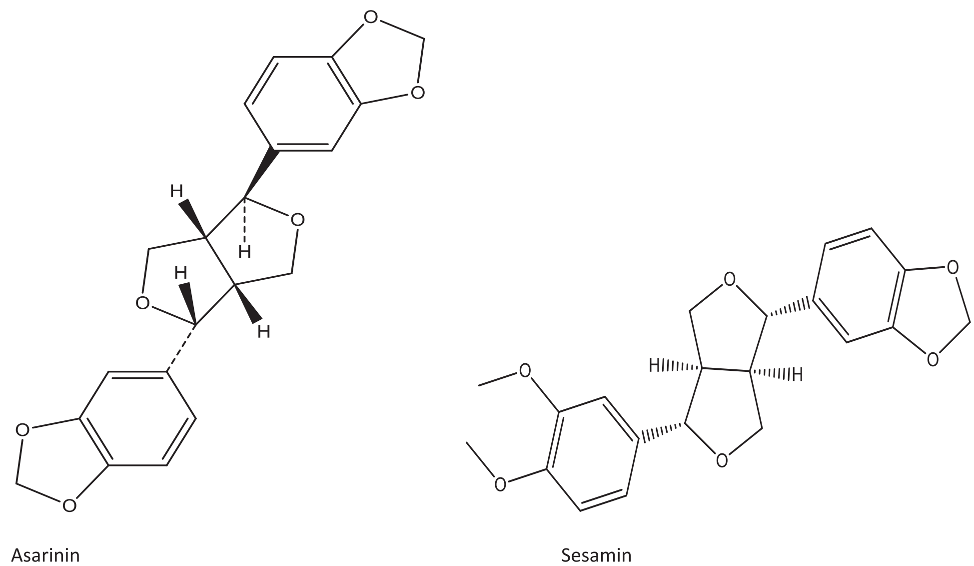

Fig. 6. Some lignins found in leaf and bark of *Zanthoxylum armatum*.

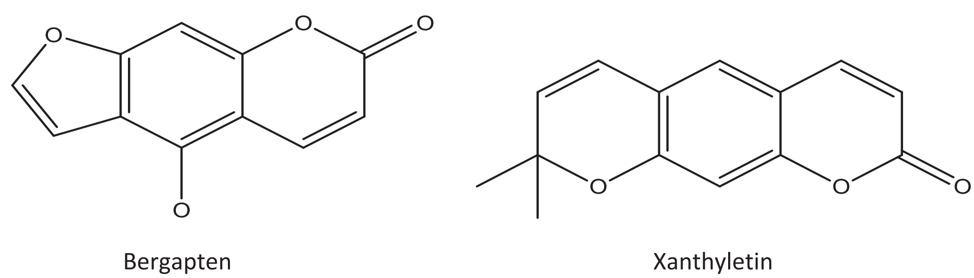

Fig. 7. Some coumarins derived from bark of *Zanthoxylum armatum*.

compounds (Kayat et al., 2016). The essential oil extracted through hydro-distillation through GC-MS revealed the major constituents as beta-Linalool (53.05%), Bergamot mint oil (12.73%), alpha-Limonene diepoxide (11.39%), alpha- pinene (4.08%), beta- Myrcene (3.69%) and D-Limonene (3.10%) (Barkatullah et al., 2013).

A number of alkaloids and coumarins have been isolated and reported from the various parts of *Zanthoxylum armatum:* berberine (stembark), alkaloids (g-fagarine, b-fagarine, magnoflorine, laurifoline, nitidine, chelerythrine, tambetarine and candicine), coumarins (xanthyletin, zanthoxyletin, alloxanthyletin), and resin, tannin and volatile oil (Bachwani et al., 2012; Joshi and Gyawali, 2012).

A new amide designated as armatamide (Fig. 8) along with two lignans, asarinin and fargesin, alpha and beta-amyrins, lupeol, and beta-sitosterol-beta-D-glucoside (Fig. 8) have been isolated from the bark of *Zanthoxylum armatum* (Kalia et al., 1999). From the alcoholic extract of the stem bark, a new flavonoidal glycoside has also been isolated (Sati et al., 2011a).

The main components of the oil are oleic acid, palmitic acid, linoleic acid methyl ester, limonene and linalool (Shah, 1991; Negi et al., 2012; Kayat et al., 2016). The essential oil from the seeds consists entirely over 85% of the hydrocarbon 1-α-phellandrene and also a small quantity of linalool and an unidentified sesquiterpene (Waheed et al., 2011). The stems and roots contain α-amyrin, α-sitosterol, L-asarinin, L-planinin, and zanthobungeanine (Verma and Khosa, 2012). Bark yields a bitter crystalline principle, identical to berberine, dictamnine, volatile oil and resin. The carpels yield a volatile oil, resin, yellow acid principle, and crystalline solid body, xanthoxylin (CSIR, 2005). Table 2 shows the different secondary compounds present in *Zanthoxylum armatum.*

8. Biological activities

Different studies have shown that *Zanthoxylum armatum* possesses different pharmacological and biological activities like larvicidal, antifungal, hepato-protective, keratolytic, antiviral, antiprotozoan, pesticidal/insecticidal, antibacterial, anthelminthic, allelopathic from different extracts i.e., dichloromethane, acetone, aqueous, ethanol, methanol, petroleum ether etc.

8.1. Antibacterial activities

In-vitro assessment of antibacterial activity of the ethanolic and *n*-hexane extracts of leaves, fruits and bark of *Zanthoxylum armatum* was evaluated against different bacterial strains: *Micrococcus luteus*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Streptococcus viridans* through agar well diffusion method (Barkatullah et al., 2012). The wells were supplied with l00 µl of 3 mg/mL of respective extract dissolved in dimethyl sulfoxide (DMSO). DMSO and Ciprofloxacin (10 µg well^{-1}) were used as negative and positive controls respectively. The maximum inhibition was exhibited by the ethanolic extract of the fruits extract against *M. luteus* with (21.33 ± 0.41 mm ZOI) and *P. multocida* (18.33 ± 0.41 mm), followed by the ethanolic extract of the leaves against *M. luteus* (18.00 ± 0.71 mm), *P. multocida* (18.00 ± 0.71 mm) and finally by hexane extract of the fruits against *M. luteus* (19.67 ± 0.41 mm). The ethanolic extract of the bark showed moderate activity against all tested bacteria while the hexane extract of the bark extract was found active against *M. luteus* (20.33 ± 0.41 mm), *P. multocida* (17.67 ± 0.41 mm). The minimum inhibitory concentration (MIC) values for most of the bacterial species were found to be 0.65 µg/

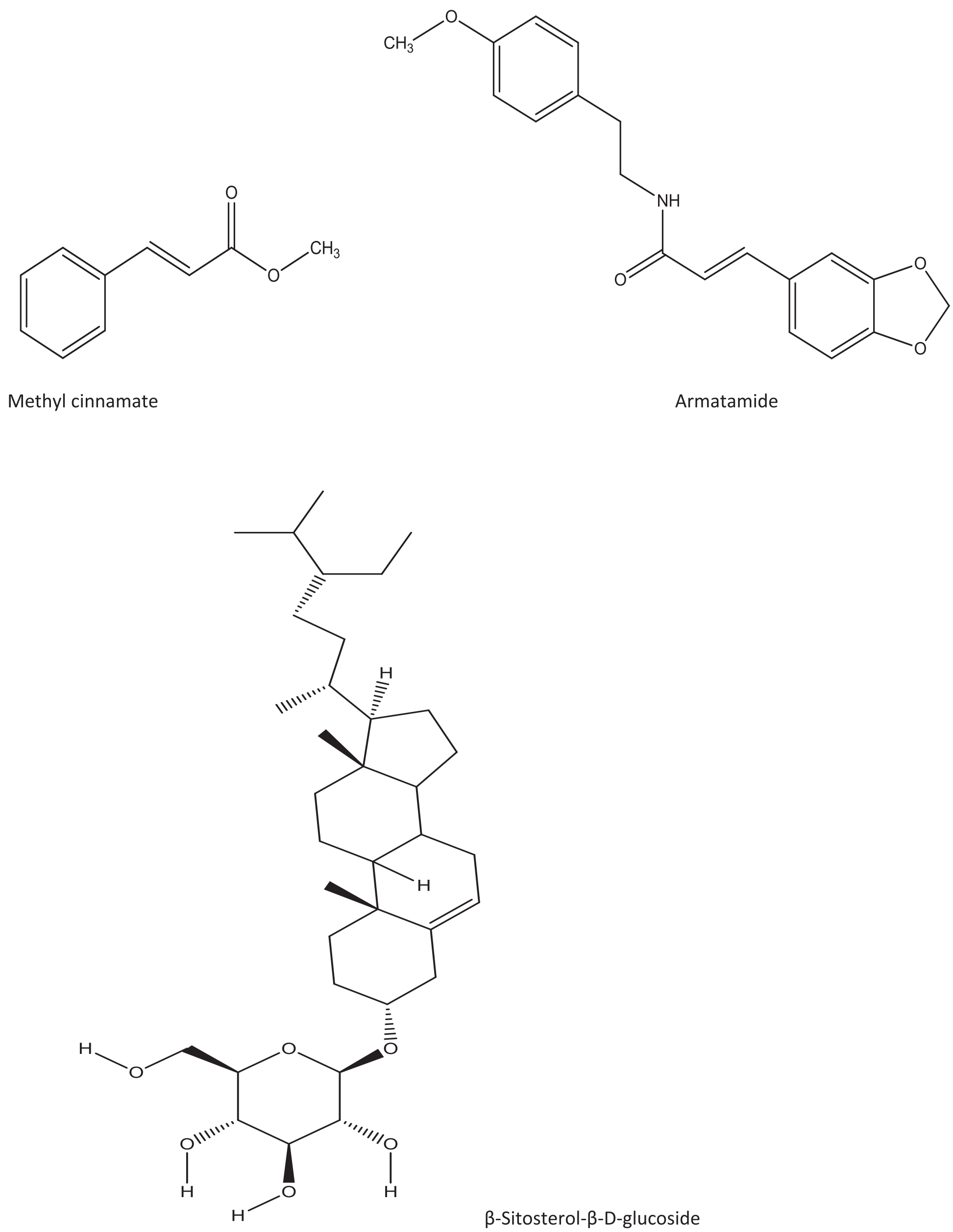

Fig. 8. Some structures of aromatic compound, amide & steroid found in *Zanthoxylum armatum*.

mL. The most resistant bacterial strain was *B. subtilis* with a maximum MIC value ranging from 1.25 to 5 mg/mL while the lowest MIC was observed for *S. aureus* ranging from 0.65 to 2.5 mg/mL.

In another experiment, in-vitro antibacterial activities of the leaf essential oil and methanol extract of *Zanthoxylum armatum* were tested against different bacteria using agar well diffusion method. The oil (diluted with DMSO 1:1 and 20 µl $well^{-1}$) exhibited strong activity against *Micrococcus luteus*, *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* with zones of inhibition ranging from 15 to 21 mm; the maximum was 21 mm against *Micrococcus luteus* while *Pseudomonas aeruginosa* showed moderate activity with 7 mm zone of inhibition. *Pseudomonas aeruginosa* was least sensitive to the oil with MIC value > 1000 µg/mL while it was 62.5 µg/mL for *Micrococcus luteus* and 125.5 µg/mL for *Staphylococcus aureus. Escherichia coli* and *Bacillus subtilis* were also sensitive to the oil with MIC values of 250 µg/mL and 500 µg/mL respectively. Methanol extract (2 mg $well^{-1}$)) was found to be ineffective against all the tested bacterial strains. Chloramphenicol (10 µl $well^{-1}$) was used as control (Guleria et al., 2013). The antibacterial properties may be attributed the collegial effects of several compounds present in their crude extracts. These experiments substantiates further research for exploiting the possible uses of *Zanthoxylum armatum* for treating different bacterial diseases like urinary tract infection and skin infection, diarrhea and dysentery.

8.2. Antifungal activities

In-vitro anti-mycotic test of the essential oil obtained from the leaves of *Zanthoxylum armatum* exhibited remarkable activities against various fungal strains like *Trichophyton longifilis, Candida albicans, Fusarium solani, Microsporum canis, Aspergillus flavus* and *Candida glabrata.* Maximum effect as a percent inhibition of mycelial growth was observed against *Candida albicans* (66.67 ± 0.57%) followed by *Aspergillus flavus* (55.33 ± 0.57%) and *Fusarium solani* (46.33 ± 0.33%) at 125 µg/mL concentration. DMSO and Miconazole were used as negative and positive control respectively (Barkatullah et al., 2013). In another experiment, the essential oil from the leaves

Table 2
Secondary compounds present in *Zanthoxylum armatum*.

S. No.	Class	Compound	Plant parts	References
	Terpenoids			
1		α-Fenchol	Seed	Ahmad et al. (1993)
2		α-Terpinene	Seed	Tiwary et al. (2007)
3		α-Thujene	Seed	Ahmad et al. (1993), Singh et al. (2013)
4		α-Thujone	Seed	Tiwary et al. (2007)
5		α-Pinene	Seed	Tiwary et al. (2007) Barkatullah et al. (2013), Singh et al. (2013)
6		α-copaene	Leaf oil	Negi et al. (2012)
7		α-Terpineol	Seed	Ahmad et al. (1993), Singh et al. (2013)
8		alpha-phellandrene	Leaf oil	Barkatullah et al. (2013)
9		β-Pinene	Seed	Tiwary et al. (2007), Singh et al. (2013)
10		beta- Myrcene	Leaf oil	Barkatullah et al. (2013)
11		β-Cymene	Leaf oil	Luong et al. (2003)
12		β-Phellandrene	Seed	Neetu et al. (2001), Singh et al. (2013), Shah (1991)
13		β-Terpeneol	Leaf oil	Luong et al. (2003)
14		Camphene	Seed, leaf oil	Ahmad et al. (1993), Negi et al. (2012), Barkatullah et al. (2013)
15		Carvone	Seed	Ahmad et al. (1993), Singh et al. (2013)
16		Citral	Dry fruit	CSIR (1976)
17		Citronellol	Seed	Yoshihito et al. (2000)
18		Citronellal	Seed	Yoshihito et al. (2000)
19		1,8-Cineole	Seed	Ahmad et al. (1993)
20		β-ocimene	Leaf oil	Negi et al. (2012)
21		Trans-beta-Ocimene	Leaf oil	Barkatullah et al. (2013)
22		cis-beta-Ocimene	Seed, leaf oil	Ahmad et al. (1993), Barkatullah et al. (2013)
23		Geraniol	Dry fruit	CSIR (1976)
24		γ-terpinene	Seed/Leaf oil	Ahmad et al. (1993), Negi et al. (2012)
25		*(E)*-Carveol	Seed	Tiwary et al. (2007)
26		*(E)*-Linalool oxide	Seed	Tiwary et al. (2007)
27		Limonene	Seed /leaf	Ahmad et al. (1993), Negi et al. (2012), Singh et al. (2013)
28		D-Limonene	Leaf oil	Barkatullah et al. (2013)
29		Linalool	Seed /leaf oil	Ahmad et al. (1993), Negi et al. (2012), Singh et al. (2013)
30		Bornyl acetate	Leaf oil	Negi et al. (2012)
31		Linanyl acetate	Dry fruit	CSIR (1976)
32		Myrcene	Seed	Ahmad et al. (1993), Singh et al. (2013)
33		Terpinolene	Leaf oil	Barkatullah et al. (2013)
34		α-terpinolene	Leaf oil	Negi et al. (2012)
35		Nerol	Seed	Tiwary et al. (2007)
36		1-α-Phellandrene	Seed	Perry (1980)
37		cymene	Leaf oil	Negi et al. (2012)
38		*p*-Cymene	Seed	Ahmad et al. (1993), Singh et al. (2013)
39		Piperitone	Seed	Tiwary et al. (2007), Singh et al. (2013)
40		Sabinene	Seed	Tiwary et al. (2007) Barkatullah et al. (2013), Singh et al. (2013)
41		Tagetonol	Seed	Ahmad et al. (1993)
42		Terpinen – 4-ol	Seed	Tiwary et al. (2007)
43		*(Z)*-Sabinene hydrate	Seed	Tiwary et al. (2007)
44		*(Z)*-Linalool oxide	Seed	Tiwary et al. (2007)
45		*(Z)*-Pinene hydrate	Seed	Tiwary et al. (2007)
46		*(allo*-Aromadendrene	Seed	Ahmad et al. (1993)
47		α-Caryophyllene	Seed	Ahmad et al. (1993)
48		*trans*-caryophyllene	Leaf oil	Negi et al. (2012)
49		(*E*)-Nerolidol	Seed	Tiwary et al. (2007)
50		α-Amyrins	Bark	Tiwary et al. (2007)
51		β-Amyrone	Bark	Li et al. (1996)
52		β-Amyrins	Bark	Kalia et al. (1999)
53		Lupeol	Bark	Kalia et al. (1999)
54		Eucalyptol	fruit	Kayat et al. (2016)
	Flavonoids			
55		Catechin	Leaf	Bhatt et al. (2016)
56		Hesperidine	Leaf	Bhatt et al. (2016)
57		Vitexin	Leaf	Bhatt et al. (2016)
58		Isovitexin	Leaf	Bhatt et al. (2016)
59		3,5-Diactyltambulin	Bark	Li et al. (2006)
60		Kaempferol	Bark	Li et al. (2006)
61		Tambulin		Muller et al. (1996)
62		3,5,3′-Trihydroxy – 6,7- dimethoxy – 4′-(7″-hydroxygeranyl – 1″-ether) flavone	Seed	Ramidi and Ali (1999)
63		3,5,3′,4′-Tetrahydroxy – 7, 8-dimethoxy flavone	Seed	Ramidi and Ali (1999)
64		Tambuletin	Seed	Ramidi and Ali (1999), Nair et al. (1982)

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Table 2 (*continued*)

S. No.	Class	Compound	Plant parts	References
	Alkaloids			
65		Berberine	Bark	Ranawat et al. (2010)
66		Dictamnine	Root	Perry (1980)
67		g-fagarine	Bark	Vashist et al. (2016)
68		b-fagarine	Bark	Vashist et al. (2016)
69		chelerythrine	Bark	Vashist et al. (2016)
70		Magnoflorine	Root	Perry (1980)
71		Nevadensin	Seed oil	Ramidi et al. (1998)
72		Skimmianine	Bark	Li et al. (1996)
73		Zanthonitrile	Bark	Li et al. (1996)
	Lignins			
74		Asarinin	Bark leaf	Ranawat et al. (2010), Bhatt et al. (2016)
75		Eudesmin	Leaf	Bohlin and Bruhn (1999), Bhatt et al. (2016)
76		Epieudesmin		Bohlin and Bruhn (1999)
77		Fargesin	Bark leaf	Kalia et al. (1999), Singh et al. (2013), Bhatt et al. (2016)
78		Kobusin	Leaf	Bohlin and Bruhn (1999), Bhatt et al. (2016)
79		Planispine-A	Leaf	Bhatt et al. (2016)
80		*L*-Asarinin	Bark	Li et al. (1996), Rao and Singh (1994)
81		*L*-Sesamin	Bark	Li et al. (1996), Rao and Singh (1994)
82		*L*-Planinin	Bark	Li et al. (1996), Rao and Singh (1994)
83		Magnolin		Neetu et al. (2001)
84		Phylligenin		Bohlin and Bruhn (1999)
85		Planinin		Bohlin and Bruhn (1999)
86		Sesamin	Leaf/Bark	Muller et al. (1996), Bhatt et al. (2016), Vashist et al. (2016)
	Sterols & Steroids			
87		β-Daucosterol	Bark	Li et al. (1996)
88		β-Sitosterol	Bark	Li et al. (1996), Vashist et al. (2016)
89		Stigmasta − 5-en − 3β-Dglucopyranoside	Seed	Akhtar et al. (2009)
90		β-Sitosterol-β-D-glucoside	Bark	Ranawat et al. (2010)
	Amides			
91		Armatamide	Bark	Kalia et al. (1999)
92		*α*-Sanshool	Leaf	Bhatt et al. (2016)
93		Hydroxyl-α-sanshooil	Pericarp	Khare (2007)
	Coumarins			
94		xanthyletin	Bark	Vashist et al. (2016)
95		zanthoxyletin	Bark	Vashist et al. (2016)
96		alloxanthtin	Bark	Vashist et al. (2016)
97		Bergapten	Bark	Li et al. (1996)
98		Umbelliferone	Bark	Li et al. (1996)
99		psoralen	leaf	Bhatt et al. (2016)
	Carbonyl Compounds			
100		Cuminol	Bark/Fruit	Kayat et al. (2016)
101		Cuminaldehyde	Seed	Tiwary et al. (2007)
102		Phellandral	Seed	Tiwary et al. (2007), Singh et al. (2013)
103		2-Tridecanone	Leaf-oil	Luong et al. (2003)
104		Undecan − 2-one	Aerial parts	Weyerstahl et al. (1999)
	Aromatic compounds			
105		1-Hydroxy − 6,13-anthraquinone	Seed	Akhtar et al. (2009)
106		2-Hydroxybenzoic acid	Seed	Akhtar et al. (2009)
107		2-Hydroxy − 4-methoxy benzoic acid	Seed	Akhtar et al. (2009)
108		*trans*-Cinnamic acid	Seed oil	Ramidi et al. (1998)
109		Vanillic acid	Bark	Li et al. (1996)
110		(*E*)-Methyl cinnamate	Seed	Tiwary et al. (2007), Singh et al. (2013)
111		Methyl cinnamate	Seed /fruit	Ahmad et al. (1993), Kayat et al. (2016)
112		(*Z*)-Methyl cinnamate	Seed	Neetu et al. (2001)
113		3-Methoxy − 11-hydroxy − 6,8-dimethylcarboxylate biphenyl	Seed	Akhtar et al. (2009)
114		3,5,6,7-Tetrahydroxy − 3′,4′-dimethoxyflavone − 5-β-D-xylopyranoside	Seed	Akhtar et al. (2009)
115		Monoterpenetriol − 3,7-dimethyl − 1-octane − 3,6,7-triol	Seed oil	Ramidi et al. (1998)
116		1-Methoxy − 1,6,3-anthraquinone	Seed	Akhtar et al. (2009)
	Other Aliphatic Compounds			
117		*cis* − 9-Hexa-decenoic	Seed oil	Ahmad et al. (1980)
118		*cis* − 10-Octadecenoic acid		Kokate et al. (2001)
119		*cis* − 9,12-Octadecadienoic acid		Venkatachalam et al. (1996)
120		*cis* − 9,12,15-Octadecatrienoic acid		Kokate et al. (2001)
121		2,6-Dimethyl − 1,3,5,7-octatetraene	Leaf oil	Luong et al. (2003)
122		6-Hydroxynonadec-(4*Z*)-enoic acid	Seed	Ahmad et al. (1993)
123		8-Hydroxypentadec-(4*Z*)-enoic acid	Seed	Ahmad et al. (1993)
124		7-Hydroxy − 7-vinylhexadec-(4*Z*)-enoic acid	Seed	Ahmad et al. (1993)
125		Hexadec-(4*Z*)-enoic acid	Seed	Ahmad et al. (1993)
126		6-Methylheptanoic	Seed	Yoshihito et al. (2000)
127		8-Methylnonanoic acid	Seed	Yoshihito et al. (2000)
128		Oleic acid	Seed	Tiwary et al. (2007), Singh et al. (2013)

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Table 2 (*continued*)

S. No.	Class	Compound	Plant parts	References
129		Palmitic acid	Seed /Fruit	Tiwary et al. (2007), Kayat et al. (2016), Singh et al. (2013)
130		Palmitolic acid	Seed	Tiwary et al. (2007)
131		Methyl palmitate	Seed	Tiwary et al. (2007)

showed antifungal activities against three crop pathogens namely *Alternaria alternata*, *Alternaria brassicae, Curvularia lunata* with antifungal indices of 35.6 ± 1.49%, 14.5 ± 0.36% and 42.0 ± 1.63% respectively. Similarly antifungal indices of methanol extract of leaves were 47.4% and 51.4% against *Alternaria alternata* and *Curvularia lunata* respectively. The methanolic extract did not show any activity against *Alternaria brassicae* at 2 mg/mL concentration (Guleria et al., 2013). The antimycotic potential of crude methanol extract could be because of the phenolic and flavonoid compounds present in the leaves.

Several in-vitro antifungal studies verified the antimycotic potential of *Zanthoxylum armatum* against various fungal strains; *Aspergillus parasiticus* (Dube et al., 1990), *Candida albicans, Cryptococcus neoformans* (Goel et al., 2002), *Microsporum gypseum, Trichophyton mentagrophytes* (Dikshit et al., 1986), *Sclerotium rolfsii, Rhizoctonia bataticola* (Sharma et al., 2009), *Alternaria brassicicola* (Parajuli et al., 2005), *Bipolaris sorokiniana* (Manandhar and Tiwari, 2005). These experiments necessities further specific researches for the possible use of *Z. armatum* against various crop pathogens.

8.3. Antioxidant activity

The antioxidant potential of *Zanthoxylum armatum* have been demonstrated by several in-vitro/in-silico antioxidant experiments by different authors (Batool et al., 2010; Upadhyaya and Kumar, 2010; Negi et al., 2012; Prakash et al., 2012; Barkatullah et al., 2013; Guleria et al., 2013; Karmakar et al., 2015; Kanwal et al., 2015 etc.). However such studies are not considered in this review as per the skeptical judgment of such studies by scientific journals. All plants possess antioxidant activity so it is insignificant unless evidenced by in-vivo studies (Gafner, 2018). (http://cms.herbalgram.org/heg/volume15/01January/JournalsSkepticalofAssays.html.)

Karmakar et al. (2015) carried out in-vivo antioxidant assay of methanol extract of leaves of *Zanthoxylum armatum* in male Wister Albino rats. The test animals were orally administered with the extract at a dose of 100 and 200 mg/kg body weight, whereas the normal control animals were given distilled water and positive control group was given standard Vitamin E. After five days, the test animals were given a mixture of 1:1 CCl_4 and olive oil for 2 days. The activities of SOD, CAT and GSH were decreased significantly ($P < 0.01$) in normal control groups, whereas lipid peroxidation level was found to increase in EAT bearing control group as compared to normal animals. Administration of the extract significantly enhanced the activities of antioxidant enzymes (SOD, CAT and GSH) in the treated animals. Treatment at the dose of 100 and 200 mg/kg increased the levels of SOD and CAT significantly ($P < 0.05$ and $P < 0.01$) and GSH was also increased, whereas the lipid peroxidation level was decreased at 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) respectively.

The excessive production of free radicals might cause cell and tissue damage, resulting in aging and untimely cell death (Hsieh et al., 2009). The prominent antioxidant enzymes to inhibit free radical formation are SOD, CAT and GSH (Liu et al., 2013). The results obtained in the present study indicated the potent antioxidant activity of *Zanthoxylum armatum*. Further research could validate the use of the plant as a natural antioxidant.

8.4. Hepatoprotective activities

The ethanol extract of leaves *Zanthoxylum armatum* was used to study the in-vivo hepatoprotective effect against Carbon tetrachloride (CCl_4) induced liver damage in Wister albino rats. Silymarin (100 mg/kg of body weight) was used as a positive control. CCl_4 intoxication in normal rats elevated the levels of SGOT, SGPT, ALKP, SBLN and liver inflammation were observed significantly indicating acute hepatocellular damage and biliary obstruction. Oral administration of the extract at 500 mg/kg showed a significant ($P < 0.001$) decrease in all the SGOT, SGPT, ALKP, SBLN levels and liver inflammation, by normalizing the elevated levels of the hepatic enzymes. The results obtained support the use of this plant for the treatment of hepatitis in oriental traditional medicine (Verma and Khosa, 2012). In another experiment, the hepatoprotective activity of ethanolic extract of bark of *Zanthoxylum armatum* in CCl_4 induced hepatotoxicity in male Wister rats was examined. 1:1 of CCl_4 in olive oil administration caused a significant increase in the serum activities of ALT, AST, ALP, DBil and TBil. The oral administration of doses (ethanol extract) at 100, 200, and 400 mg/kg once daily for 7 days significantly reduced the above elevated parameters. However, treatment with 400 mg/kg showed significant hepatoprotective activity that was comparable to silymarin (25 mg/kg) (Ranawat et al., 2010).

8.5. Anti-inflammatory activity

In-vivo anti-inflammatory activity of ethanolic extract of stem bark of *Zanthoxylum armatum* was evaluated by carrageenan induced paw edema method in male Wister rats (Sati et al., 2011b). The degree of inhibition was 19.12%, at 250 mg/kg dose after 4 h of administration. Ibuprofen (10 mg/kg of body weight) was used as positive control. Fruits extract also showed inhibition of carrageenan that induced paw edema in Wister rats (Mehta et al., 2011). It has analgesic activity also due to the presence of lignan components (Guo et al., 2010).

8.6. Antispasmodic effect

The crude extract of *Zanthoxylum armatum* was used to study the in-vivo spasmolytic effects against castor-oil-induced diarrhea in mice. Pre-treatment of animals with the extract showed 20% protection from diarrhea at 300 mg/kg and 60% protection at 1000 mg/kg. Loperamide (10 mg/kg) was used as positive control (Gilani et al., 2010). In another experiment, the essential oil of leaves of *Zanthoxylum armatum* was evaluated for possible antidiarrheal effect on spontaneous and potassium chloride induced contracted smooth muscle of the isolated rabbit jejunum. The spasmolytic effect of the oil started from 0.03 mg/mL and showed 100% effect at 10 mg/mL dose. The extracts relaxed the contracted muscle, suggesting the possible mode of action of this plant as either blocking the release of stored calcium from the sarcoplasmic reticulum or blocking the calcium channel (Barkatullah et al., 2013).

8.7. Inhibition of keratinocyte growth

The methanol extract of the bark of *Zanthoxylum armatum* was evaluated for the anti-proliferative activity against the growth of rapidly multiplying human keratinocytes (HaCaT cells). The HaCaT keratinocyte proliferation assay and determination of cell growth was carried out. The concentrations of the extracts used were between 0.008

and 0.4 mg/mL. The extract significantly inhibited the growth of keratinocytes. It was found to be highly active with an IC50 value of 11 mg/mL (Kumar, Müller, 1999)

8.8. Cytotoxic and phytotoxic effects

Barkatullah et al. (2013) carried out in-vitro toxicity bioassay in Brine shrimp to investigate the preliminary cytotoxic potential of the crude ethanolic and n-hexane extract of leaf, bark, fruits and leaf essential oil of *Zanthoxylum armatum*. The concentrations tested were 10, 100 and 1000 µg/ml. The oil exhibited remarkable mortality rate (100%) at a dose of 1000 µg/ml with LC50 value 15.90. However, a dose of 1000 µg/ml clearly is of no therapeutic relevance and concerns remain about today's the relevance of this test system. Similarly in another experiment, the ethanolic extract and subsequent fractions of the fruits of *Zanthoxylum armatum* were evaluated for Brine shrimp lethality test using concentrations 5, 50, and 500 mg/mL. Etoposide was used as standard cytotoxic drug. The crude extract exhibited significant toxicity with LC50 value of 6.66 ± 1.1 mg/mL, which probably indicates the distribution of synergistic activity of compounds within these two fractions. Similarly, the LC50 values of chloroform and aqueous-methanol fraction was 21.4 ± 3.3 and 29.6 ± 3.9 mg/mL respectively. The n-hexane fraction, exhibited lower toxicity level with LC50 value greater than1000 mg/mL. (Alam and us Saqib, 2017). Further research could possibly find out the active biochemical compounds.

In another experiment, different concentrations (1000, 100 and 10 mg/mL) of the crude extract, n-hexane, chloroform and aqueous-methanolic fractions of fruits of *Zanthoxylum armatum* was evaluated for in-vitro phytotoxic activity against *Lemna minor* L. Paraquat (0.9025 mg/mL) was used as positive control. Remarkable phytotoxicity was observed at the highest concentration (1000 mg/mL) causing complete inhibition of the plant growth. At 100 mg/mL concentration, the n-hexane extract caused complete inhibition while the crude extract and chloroform extract exhibited 63.3 ± 5% and 54.54 ± 7% inhibitions, respectively. Lowest effect was observed for the aqueous methanol extract with only 22.72 ± 4% inhibition (Alam and us Saqib, 2017).

8.9. Antiviral/antiprotozoal activity

Methanolic extracts of fruits of *Zanthoxylum armatum* (concentrations of 100, 50, 25, 12.5 and 6.25 mg/mL) have been investigated for in vitro antiviral activity against Herpes simplex virus type 1(HSV-1) and influenza virus A (100, 50, 25, 12.5 mg/mL) by dye uptake assay in the systems HSV-1/Vero cells and influenza virus A/MDCK cells. The extracts showed inhibition of HSV-1/ Vero cells, Influenza A/MDCK cells with CC50 value > 100 mg/mL and 36 mg/mL (Rajbhandari et al., 2009). The aqueous extract of the leaves showed antiprotozoal effect on *Giardia lamblia* and *Plasmodium berghei* (Goel et al., 2002).

8.10. Soothing effect on skin

The lipophilic extract with alcohol gives remarkable soothing effect based on inhibition of sensory irritation from sun bathing, shaving depilation, insect bites and chemical treatment (Guglielmini and Cristoni, 2002).

8.11. Mosquito repellant

In combination, *Zanthoxylum armatum* seed oil, vanillin and fruit oil of *Zanthoxylum piperitum* have been able to enhance repellent activity against female *Aedes aegypti* and the effect was compared with N,N diethyl-3methylbenzamide (DEET) repellent (Kwon et al., 2011). The essential oil was found to significantly repel mosquitoes at 0.57 mg/cm^2 concentrations. *viz*. 445 min in mustard oil and 404 min in coconut oil base (Das et al., 1999).

8.12. Larvicidal activities

Tiwary et al. (2007) studied in-vitro larvicidal activities essential oil of the seeds of *Zanthoxylum armatum* against three species of mosquitoes: *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. The test doses used for the study were 200, 150, 100, 50, 25 and 10 ppm. It was found that *Culex quinquefasciatus* was the most sensitive with LC50 and LC95 values of 49 and 146 ppm respectively followed by *Aedes aegypti, Anopheles stephensi* with LC50 values in the range of 54–58 ppm. Temephos, chemical larvicide used commonly for controlling mosquito larvae, was used as positive control at a range of 0.005–0.1 ppm concentration. Similar larvicidal effects were also studied by Peng et al. (2009) against *Culex pipiens, Culex quinquefasciatus* and Zhang et al. (2010) against *Aedes albopictus* and *Culex pipiens*.

8.13. Allelopathic effects

Different concentrations (i.e., 2%, 5%, and 10%) of aqueous extracts of leaf, bark, and fruit pulp were found to have significant allelopathic effect on some important winter field crops (*Triticum aestivum, Hordeum vulgare, Brassica campestris* and *Lens culinaris*). A study carried out in Garhwal Himalaya region of India showed significant effects of these bioassays on germination and growth of all the test crops. In an average, 83.6%, 52.6%, and 84.9% reduction in radicle growth was observed in *Triticum aestivum, Hordeum vulgare* and *Lens culinaris*, respectively (Singh et al., 2007).

9. Trade

The tradition of collection and sale of *Zanthoxylum armatum* in Nepal has a long history and can be dated back to the early 80s, when the trade started with India, before which it was used by the rural communities for domestic purposes (Malla et al., 1993). Historically, the rural people traded different medicinal plants including *Zanthoxylum armatum* as a source of their income (Manandhar, 1986; Kunwar et al., 2018). There was a social mechanism of exchange and distribution of Timur, and the fruits were bartered for grains, millets, pulses and other items (Kala, 2005). With the increasing demand of the fruits by different pharmaceutical and other companies, the value of this plant has raised surprisingly (DoF, 2014). The rural people have also started to commercially cultivate the plants in their farmyards, as a secondary source of income.

The market price of *Zanthoxylum armatum* has been increasing every year. The price per kg was NRs 1.8 in 1980s and Rs.44 in 2007 (ANSAB, 2011). According to the price list provided by ANSAB (http://www.ansab.org/mis/price-list/), the market price of the *Zanthoxylum armatum* fruits was NRs. 100/- per kg in 2010, NRs. 265/- in 2011, NRs. 235/- in 2012, NRs. 300/- in 2014, NRs. 250/- in 2015 and a maximum of NRs. 340/- per kg in 2016. The main market of Timur is India, where there is a high demand of the dried fruits (Edwards, 1996; Hertog and Wiersum, 2000). Only a small portion is processed inside Nepal while most of the quantity (more than 90%) is exported to India in raw form, where it is further processed and used for different pharmaceutical and industrial purposes (DoF, 2014). Several Ayurved companies and other industries inside Nepal also consume some amount of *Zanthoxylum armatum* in their various products. Small quantity of *Zanthoxylum* oil is also exported to some European countries. China is another probable country for market expansion as the fruits are extensively used there as a flavoring agent in food (ANSAB, 2011). *Zanthoxylum armatum* is one of the many other MAPs that are traded from Nepal to China and the commerce is not only limited to just traditional users in Tibet but also to the mega-cities of Central China (He et al., 2018).

According to a report published by the Department of Forest, Government of Nepal, the total revenue collected from the sale of *Zanthoxylum armatum* in 2011 was NRs 281,568, with a total production of 17,896 kg while it was 240,206 kg in 2013 amounting to NRs.

1921,648/- and in 2015 it was 418,179 kg with the royalty of NRs. 3345,432 (DoF, 2013, 2015). The amount was 272,200 kg with total revenue of NRs. 1959,850/- in Salyan district alone (DFO, 2015). Different regions rich in Timur population in Salyan district have been designated as pocket areas of Timur for the promotion and commercial cultivation. As it requires less fertile soil, it can be cultivated around croplands with very less impact to the cultivated crops, in the barren lands and different forests lands. It is regarded as a prioritized commodity with huge export possibilities not only to the Indian market but also to the lucrative European market, where there is a high demand of the essential oil obtained from the fruits. Hence, it can be developed as an alternative cash crop to increase the income of small local farmers, thereby improving the livelihoods, which would ultimately help to reduce rural poverty. Such initiative however requires collaborative efforts of different governmental, non-governmental, public and private sectors.

10. Opportunities

Species of *Zanthoxylum* are of great economic importance as source of edible fruit, oil, wood, raw materials for industries, medicinal plant, ornamental, culinary application (Adesina, 2005; Seidemann, 2005). Different plant parts leaves, fruits, stem, bark, seeds and root are used in indigenous medicine preparation against various diseases (Singh and Singh, 2011). This plant species is not only used for pharmaceutical purposes, but also in the flavoring and fragrance industries. The diverse pharmacological applications of *Zanthoxylum armatum* have been verified by various studies

Owing to its varied and diverse application in several sectors, *Zanthoxylum armatum* can be developed into an important commodity with a good scope of increase in production by adopting proper management system, improving harvesting tools/methods, and proper post-harvest handling as important significance for its trade in Nepal because of its multiple functions and potential for rural livelihood improvement. The product has been established as a locally useable commodity and as an integral source of income benefiting the women, landless and unemployed people in Nepal (MoAD, 2011; ANSAB, 2011; DoF, 2014). It can be grown on less fertile soil, marginal lands and can be harvested after three years of planting with less chances of pest infestation. Recognizing the importance of agricultural product, the Ministry of Agricultural Development of the Government of Nepal established the Agricultural Commodity Export Promotion Program under the Department of Agriculture, Agri-business Promotion and Market Development Directorate for the pre-requisite of the national market to be linked with international markets (Agricultural Commodity Export Promotion Program).

Regarding its importance as a valuable prioritized medicinal plant, The Department of Plant Resources under the Ministry of Forests and Environment has published Quality Standard, Good Agricultural and Collection Practices of *Zanthoxylum armatum* (DPR, 2011b) covering useful information of the species, its good collection and agricultural practices, post-harvest procedures and quality standards. Similarly the Department of Forests has also published a report on value chain and designing of timur in Panchase Protected Forest Area (DoF, 2014).

11. Gaps

The National Conservation Strategy (NPC, 1988) emphasized on the enforcement of legislations for sustainable extraction and utilization of MAPs of Nepal. Similarly, Master Plan for the Forestry Sector (DoF, 1989), Industrial Enterprises Act (GoN, 1992), Forest Act (GoN, 1993) and Regulations (GoN,1995), Herbs and Non-Timber Forest Products Development Policy (DPR, 2004) have emphasized on the subsequent development of the NTFPs including MAPs for uplifting the livelihood of the rural communities through sustainable and wise use of these valuable resources. Trade Policy in Nepal (GoN, 2009) has also prioritized Zanthoxylum armatum as an important commodity for export. However, there seems to be inadequate institutional coordination in the effective implementation of plans and policies. Similarly, there is no well-defined institution with a clear mandate to work in the sector of MAPs. The Herbs and NTFP Policy seems to be too ambitious to be implemented from grass root levels. There is also a huge gap in the baseline information on the demand and supply chain. The major impediment to achieving desired benefits from the commercialization of this valuable species is the paucity of scientific research.

There have been tremendous studies on the medicinal plants of Nepal regarding their botany, ecology, ethnobotany etc. Phytochemistry and pharmacology of many of them have also been investigated. A significant amount of research has also been carried out in *Zanthoxylum armatum* in the Indian subcontinent regarding its phytochemistry (Waheed et al., 2011; Negi et al., 2012; Barkatullah et al., 2013; Bharti and Bhushan, 2015), pharmacology (Bharti and Bhushan, 2015; Kanwal et al., 2015), biological activities (Gilani et al., 2010; Sati et al., 2011a, 2011b), germination behavior (Tiwary et al., 2007; Ramdas et al., 2012) etc. However there have been very few studies in *Zanthoxylum* in Nepalese context. Hertog and Wiersum (2000) conducted a study in western part of Salyan District in the mid- western region of Nepal that analyzed different management systems of Timur production in Nepalese forests. However, study of relation of ecological factors like altitude, habitat conditions, different populations etc. on the accumulation of active phytochemicals in the genus *Zanthoxylum armatum* in Nepal is still meager.

Increasing market demand and unsustainable harvesting procedures are posing serious threat to the natural population of *Zanthoxylum armatum*. It is one of the many other medicinal plants, that is collected with high preference for markets as well as local use (Kunwar et al., 2015). Due to insufficient knowledge and skill, the practice of early harvesting is prevalent, which is the main cause for pest infestation and subsequent decrease in production. Apart from that, the population size is low and is not a fast-growing species; the proliferation of woody weeds such as *Lantana* is also a major threat for the survival of the native population (Kala, 2005). Inadequate coordination among stakeholders, traders, bureaucrats, etc. and weak institutional initiatives for markets are major impediments for the sustainable management of this valuable plant species.

The quality standards of the commodity to be traded should comply with the international norms and standards. In Nepal, there is no provision for sanitary and phyto-sanitary measures as well as the compliance with the WHO guidelines. An ISO certified accredited laboratory is an utmost need today to get our products into the network of global competitive market.

12. Conclusion

Zanthoxylum armatum is one of the important medicinal plants having a wide array of household, commercial and ethno-medicinal applications. The fruits, leaves, seeds and stem bark are used in headache, fever, toothache, tonsillitis, diarrhea, dysentery, altitude sickness. The fruits contain essential oil that possesses antiseptic, disinfectant properties so it has its wide application in pharmaceuticals and flavoring industries. The main constituents of the essential oil are limonene and linalool.

Different chemical compounds like alkaloids, flavonoids, terpenoids, phenols, coumarins etc present in different parts of the plants have attributed to several biological activities like antimicrobial, antiviral, hepatoprotective, larvicidal, antioxidant etc. The different traditional ethno-medicinal practices have been validated by several in vitro and in vivo ethno-pharmacological studies, as evidenced in this review, suggesting for further potential biological applications of *Zanthoxylum armatum.*

Many active components have been identified from the plant that might be developed into novel drugs. Therefore further emphasis should be on screening, isolation and characterization of the individual components responsible for different pharmacological activities and their underlying mechanism of action. However, additional studies are required to quantify the acute and chronic toxicity in animals before clinical trials. The ethnopharmacological, phytochemical and pharmacological studies could establish a strong linkage between the ages old indigenous medicinal practices and modern scientific researches that could excavate the immense underlying capabilities of medicinal plants and their relevant therapeutic activities.

Based on its varied industrial uses, the demand of *Zanthoxylum armatum* is constantly increasing both in domestic and international markets. Increased market demands, devious modes of collection and insufficient technical knowledge and proper skills of harvest and post-harvest techniques have posed serious threats to the native populations attributing to a sharp decline of the species in the wild, due to which the regeneration of this species is adversely affected. Commercial farming by developing suitable agro-technology could be very crucial for the enhancement of the marginalized and disadvantaged rural communities. Specific studies should be conducted for the development of sophisticated technologies that would increase productivity for the commercial and mass scale production.

Detail information on ecology with respect to phytochemical variations is still meager in Nepal. Unsustainable harvesting from the wild without proper management practices is the major threat to most of the MAPs including *Zanthoxylum armatum.* Hence understanding the ecology and biology of the valuable plant is very important for development of agro-technology and commercial cultivation, which will ensure the steady supply of raw materials without hampering their natural population. Future research should focus on promoting sustainable use and conservation of this valuable plant species incorporating the traditional knowledge with scientific findings.

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Authors contributions

This review paper was written by Nirmala Phuyal. The manuscript was reviewed and edited by Pramod Kumar Jha and Sangeeta Rajbhandary. Pankaj Prasad Raturi provided necessary inputs and suggestions.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Effect of growth hormone and growth media on the rooting and shooting of *Zanthoxylum armatum* stem cuttings

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The common method of propagation is through seeds but seed germination in *Zanthoxylum armatum* is very low due to the presence of hard seed coat, which might be a great hurdle for large scale production of plantlets. So an attempt was made in this study to see the effect of different growth hormones, their concentrations and different rooting media on the rooting and sprouting of *Z. armatum*. The stem cuttings of *Z. armatum* were treated with two types of auxins namely Indole-3-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) at different concentrations (2000 ppm, 3000 ppm and 5000 ppm), while the untreated cuttings were used as control. The cuttings were planted in three different rooting media: sand, neopeat and mix (containing a mixture of sand, soil and vermin-compost). The completely randomized design was used for the experiment. The total number of stem cuttings of *Z. armatum* used in the experiment was 1080 for 18 treatments in three replicates (20 cuttings per treatment x 18 treatments x 3 replicates). The experiment was set up in controlled green-house conditions at Dabur Nepal Private Limited Nursery, Banepa, Kavre District. The parameters evaluated were root length, shoot length and number of roots per cutting. The collected data were analyzed statistically using R-program with Agricola. Least significant difference (LSD) and Duncan multiple Range Test (DMRT), as mean separation technique was applied to identify the most efficient treatment in the rooting and shooting behavior of *Z. armatum* (Gomez and Gomez, 1984). Hormone concentration and growth media significantly affected the rooting and shooting ability of *Z. armatum* stem cuttings. IBA was found to be more effective than NAA. Neopeat medium was better than sand and mix media. The highest number of roots (6.5) and root length (11.6 cm) were recorded under IBA 5000 ppm in neopeat medium.

Key words: growth hormones, growth media, sprouting, stem cuttings, rooting, *Zanthoxylum armatum*,

Zanthoxylum armatum DC. (Eng. winged prickly ash; Nep. Timur) belonging to family Rutaceae, is a popular Nepalese spice plant (Manandhar, 2002). The plant is an erect shrub or a small tree up to 6 m in height with dense glabrous foliage and straight prickles on stem, commonly occurring in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), north-east India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, North and South Korea, North Vietnam, Taiwan, Lesser Sunda Islands, Philippines, Malaya peninsula and Sumatra (Grierson and Long, 1991; Nair and Nayar, 1997). In Nepal, it is distributed from west to east at an elevation range of 1000 to 2500 m in open places or in forest undergrowth (DPR, 2007). It is an important medicinal plant with a high trade value having diverse uses in Ayurveda, pharmacy and industry. It has been used in several traditional medicinal practices to cure several diseases such as abdominal pain, carminative, antispasmodic, rheumatism, skin diseases, cholera, diabetes and asthma (Singh *et. al*, 2016). Among the eight species of *Zanthoxylum* found in Nepal, *Z. armatum* is the most common and one of the

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30 medicinal plants and it is prioritized by the government for cultivation and agro-technology development (DPR, 2006).

The plant grows well in open pastures, degraded slopes, shrub lands, natural forests and wastelands with adequate rainfall, deep soils exposed to sun. Clay or loam soil with high organic matter is suitable for the cultivation of this species. The flowering starts in five year old plants in April-May and fruiting in August-October and can be harvested from October to January (Anonymous, 2008). The plants are ready to harvest after three years of plantation and the average annual yield of five years old plant is about 3.5 kg (ANSAB, 2011). *Z. armatum* is generally free from disease, insect or nematode attack; however seven insect pests mostly causing defoliation were reported by Tara *et al.* (2011).

The growing demand of *Z. armatum* in both domestic and international markets, unsustainable harvesting from the wild and lack of proper conservation strategies have led to a sharp decline in the natural population of this valuable plant (Phuyal *et al.,* 2018). The common method of propagation is through seeds but seed germination in *Z. armatum* is very low due to the presence of hard seed coat (Chadha, 1976) which might be a great hurdle for large scale production of plantlets. Furthermore the solitary seeds in the fruit also limit the quantities of seed in *Z. armatum* (Singh and Rawat, 2017). Hence vegetative propagation through stem cuttings could be a viable option for mass scale nursery production of quality planting materials of required genotypes. The increased genetic gains through mass propagation have been obtained in several horticultural plants (Leakey *et al*., 1994; Poupard *et al.,* 1994; Swamy *et al.,* 2002). However, root initiation in cuttings is affected by various factors like plant growth regulators, age of the plant, growth media, size of the cuttings (Hartmann *et al*., 2002; Husen and Pal, 2006).

The information on the propagation techniques of *Z. armatum* is still meager. Therefore a low cost and reliable technology for the propagation for *Z. armatum* has to be developed to integrate its manifold applications into agroforestry systems for the overall benefit of the rural communities as well as the *ex-situ* and *in-situ* conservation of this important plant. Commercial farming by developing suitable agro-technology could be very crucial for enhancement of the marginalized and disadvantaged rural communities. Hence an attempt was made in this study to see the effect of different growth hormones, their concentrations and different rooting media on the rooting and sprouting of *Z. armatum*.

Materials and methods

The study was conducted at the green house of Dabur Nepal Nursery Private Limited, Banepa during February 2017.

Collection of plant materials

Fresh branches were collected from 4–5 years old healthy plants of *Z. armatum* grown at the Nursery premises. The semi-hard wood branches were cut into 15 cm long segment with 2–3 nodes, and all the leaves were removed.

Surface sterilization

All the selected cuttings were surface sterilized by soaking in freshly prepared 1 % Bordeaux mixture (Calcium Hydroxide and Copper Sulphate) for 10-15 minutes.

Hormone concentration

Different concentrations *i.e.* 2000, 3000 and 5000 ppm of two plant growth hormones IBA and NAA were prepared according to the procedure described by Hartmann *et al*. (2002). The surface sterilized cuttings (cut ends only) were then dipped in a bucket containing the respective hormones solutions for 24 hours so as to enhance the absorption of hormones.

Growth media

Three different growth media (rooting media) viz. sand, neopeat (coconut fiber) and mix (containing a mixture of sand, soil and vermi-compost, in the proportion of 2:1:1) were used for studying the rooting behavior of *Z. armatum*. After dipping in hormones, the cuttings were planted directly into the rooting media. Plastic trays (No.21) having 20 cells/cavities and holes at the bottom were used for planting the cuttings. The length and breadth of the tray are 54 cm and 28 cm, respectively. The length of each cell is 6.8 cm whereas the top diameter is 6 cm and the bottom diameter is 2.7cm. The cavities in the trays were filled with the respective rooting media. A single cutting per cavity was inserted obliquely up to a depth of 3 cm.

Experimental design

The completely randomized design was used for the experiment. The total number of stem cuttings of *Z. armatum* used in the experiment was 1080 for 18 treatments in three replicates (20 cuttings x 18 treatments x 3 replicates). Hundred cuttings treated with Bordeaux mixture and washed with distilled water were used as control.

Growth conditions

After planting the cuttings, all the planting trays were labeled clearly and they were transferred to the green house and placed in the controlled environment. The temperature and relative humidity were maintained at 21.9ºC and 75%, respectively throughout the research/study period. Inside the greenhouse, agro-meteorological parameters were recorded through the sensor system run by ARGUS Control and the data were recorded in the computer. Relative humidity was maintained through misting.

Data collection

Numbers of roots, root length and shoot length of individual cutting was recorded after 90 days of planting. A cutting was considered to be rooted if it had at least one primary root of about 1 mm long. For measurement, the cutting was uprooted gradually and then it was cleared off the rooting media carefully so that the roots do not get damaged. The number of primary roots was counted and root and shoot length were measured with a ruler.

Statistical analysis

The collected data were analyzed statistically using R-program with Agricola. Least significant difference (LSD) and Duncan multiple Range Test (DMRT), as mean separation technique was applied to identify the most efficient treatment in the rooting and shooting behavior of *Z. armatum* (Gomez and Gomez, 1984).

Results and discussion

Both growth hormones, IBA and NAA at different concentrations and different growth media *viz.* sand, mix and neopeat had a significant effect ($p< 0.05$) on the number of roots, length of roots and shoots of stem cuttings of *Z. armatum.* The values obtained for IBA and NAA were close to each other (Table 1), however, IBA was found to be more effective than NAA and neopeat was the best growth medium as compared to sand and mix media (Table 3). Furthermore, the measured values (root length, shoot length and number of roots) showed steady increment with the increase in concentration of the growth hormones from 2000 ppm to 5000 ppm. But for NAA, the values increased from 2000 ppm to 3000 ppm concentration and decreased in 5000 ppm concentration except for number of roots, which was the highest in concentration 5000 ppm of NAA. Similarly, the shoot length for concentration 3000 of IBA was lower than concentration 2000 ppm (Table 2). IBA produced more number of roots per cutting as compared to NAA as well as the root and shoot lengths were also longer in IBA than NAA. The values obtained in the treated groups were relatively higher than those of the untreated groups (control).

The number of roots per cutting was affected by the type and concentration of growth hormones and the different growth media but not by the interaction between hormone concentration and growth media. The number of roots produced by IBA and NAA are not significantly different. The value is 4.6 for IBA and 4.4 for NAA (Table 1). The concentration 5000 ppm of IBA had the highest mean number of roots *i.e.* 5.9,

Table 1: Effect of different hormones on the rooting and shooting of *Z. armatum* stem cuttings

Growth hormones	Root length (cm)	Shoot length (cm)	Number of roots
IBA	9.0a	28.0a	4.6a*
NAA	8.1a	25.3b	4.4a
Control	5.4b	22.0c	2.4b*

Means with the same letter in the same column are not significantly different ($P \geq 0.05$).

Table 2: Effects of hormone concentrations on *Z. armatum* stem cuttings

Hormone concentration	Root length (cm)	Shoot length (cm)	Number of roots
IBA2000	7.6b	28.6a	3.4c
IBA3000	8.3b	26.8b	4.5b
IBA5000	11.3a	28.7a	5.9a
NAA2000	7.6b	25.9b	3.2b
NAA3000	9.8a	26.1b	4.3c
NAA5000	6.9b	23.9c	5.2a
Control	5.4c	22.0c	2.4c

Means with the same letter in the same column are not significantly different ($P \geq 0.05$).

while control had the least mean number of roots *i. e.* 2.4 (Table 2). Likewise, the maximum average number of roots was 5 in the growth medium neopeat, while it was 4.2 in sand (Table 3). On the other hand, the highest number of roots was observed in the interaction of concentration 5000 ppm of IBA with neopeat medium with an average of 6.5 and the lowest value was 2 for the interaction between control and sand (Table 4).

Hormone concentration had significant effect on the root length of *Z. armatum* stem cuttings. The concentration 5000 ppm of IBA had the longest root length (11.3 cm) and control had root length of 5.4 cm (Table 2). There was no significant effect of growth media and interaction between hormone concentration and growth media. The interaction between hormone concentration and growth media showed that the concentration 5000 ppm of IBA with neopeat and mix media had the highest mean length of root of 11.6 cm each, while the least value was 5cm for control in sand (Table 4). Among the growth media, neopeat had the longest mean root length of 9.2 cm, while the least value was 8.3cm for sand (Table 3).

The length of shoots was not significantly affected by different growth media and the interaction between different hormone types and concentration. The highest mean shoot length (27cm) was in neopeat, while it was 26.4 cm in both sand and mix media (Table 3). Similarly the interaction between growth media and hormone concentration had the highest shoot length (29.7cm) in the combination of IBA 2000 ppm and neopeat and the lowest mean shoot length of 21.2cm in control with sand (Table 4). On the other hand, the different hormone types and concentration had significant effect on the shoot length. IBA had the highest mean shoot length (28 cm) (Table 1) and IBA 5000 ppm had the best effect on the shoot length with a mean value of 28.7cm (Table 2).

Table 3: Effects of rooting media on the performance of *Z. armatum* stem cuttings

Growth media	Root length (cm)	Shoot length (cm)	Number of roots
Sand	8.3	26.4	4.2b
Neopeat	9.2	27.0	5.0a
Mix	8.8	26.4	4.4ab

Means with the same letter in the same column are not significantly different ($P \geq 0.05$)(ns: not significant)

Table 4: Interaction of hormone concentration and growth media on *Z. armatum* stem cuttings

Growth media									
	Root length (cm)			**Shoot length (cm)**			**Number of roots**		
	Sand	**Neopeat**	**Mix**	**Sand**	**Neopeat**	**Mix**	**Sand**	**Neopeat**	**Mix**
Hormone Concentration									
IBA2000	6.9	8.0	8.0	28.8	29.7	27.8	2.7	3.3	3.8
IBA3000	8.5	9.1	7.4	27.5	27.2	25.8	3.8	5.1	4.8
IBA5000	10.7	11.6	11.6	28.2	28.5	29.0	5.5	6.5	5.6
NAA2000	7.4	7.1	8.3	26.0	25.4	26.3	4.5	5.8	5.0
NAA3000	7.0	11.3	11.3	26.3	27.3	24.8	4.4	5.9	3.7
NAA5000	7.0	8.2	5.4	22.1	25.0	24.4	3.9	3.4	3.2
Control	5.0	5.8	5.5	21.2	22.6	22.4	2.0	2.3	2.9

There were significant differences in effect of different concentrations of IBA and NAA and different growth media (sand, neopeat and mix) on the rooting and shooting of stem cuttings of *Z. armatum*.. The exogenous application of growth hormones to induce rooting on stem cuttings has been widely established by several researches (Leakey *et al*., 1994; Poupard *et al*., 1994; Hartman *et al*., 2002; Tchoundjeu *et al*., 2004). The widely used sources of growth hormones for rooting stem cuttings are the different types of auxins: IAA, IBA and NAA, which are known to increase the rate of rooting as well as number of roots per cutting (Gehlot *et al*., 2014; Ibrahim *et al*., 2015).

Auxins are responsible for the overall development in plants from cell division to cell expansion (Taiz and Zeiger, 1998). The initial cell division during root formation in the cuttings depends on the level of auxins, be it exogenous or endogenous (Ludwig, 2000; Kochhar *et al*., 2005). In this experiment also, IBA and NAA had a significant effect on the number of roots per cutting as well as the length of the roots and shoots as compared to the untreated groups. This might be due to the accumulation of metabolites at the auxins application site, cell enlargement, enhanced hydrolysis of carbohydrates, synthesis of new proteins, and cell division (Strydem and Hartman, 1960). IBA was found to be more effective than NAA in enhancing root formation. The exogenous application of adequate IBA might have caused the vascular differentiation of cells and production of more number of roots. Increase in length of the roots and shoots at higher concentrations might be due to the early formation of roots and more utilization of the nutrients (Banjara, 2017).

The effectiveness of IBA in enhancing root proliferation as well as root numbers have been well documented by several earlier studies in different species. The cuttings of *Jatropha curcas* treated with IBA had the highest mean number of roots than cuttings treated with NAA (Adekola and Akpan, 2012). The highest rooting rate was obtained in *Aesculus indica* cuttings treated with IBA (Majeed *et al*., 2009). *Stereospermum suaveolens* cuttings produced the longest root with IBA (Baul *et al*., 2008). Similar results were obtained for the cuttings of *Shorea leprosula* (Aminah *et al*., 1995), *Ulmus villosa* (Bhardwaj and Mishra, 2005), *Lippia javanica* (Soundy *et al*., 2008), *Buchholzia coriacea* (Akinyele, 2010), *Ficus hawaii* (Hassanein, 2013), *Massularia acuminate* (Usman and Akinyele, 2015), *Cyclopia subternata* (Mabizela *et al*., 2016) and *Toona ciliata* (Thakur *et al*., 2018).

Among the different concentrations of IBA, 5000 ppm showed the best result in both rooting and shootings of *Z. armatum* stem cuttings. This is in accordance with the findings of Daudi *et al*. (2016), who conducted propagation techniques in *Z. alatum* through stem cuttings and seed germination. They found that the cuttings treated with the concentration 5000 ppm of IBA exhibited better sprouting and rooting than the concentrations 4000 ppm and 6000 ppm of IBA. They also concluded that propagation from the stem cuttings is more suitable than seed sowing for *Z. armatum* because seed germination process is very slow in the species. Similar results were obtained by Singh and Rawat (2017) in *Z. armatum* semi-hard wood (SHW) and hard-wood (HW) branch cuttings. IBA at 0.3% and 0.4 % concentrations exhibited greater success in root and shoot growth, whereas lower concentrations completely failed to root.

Several studies have demonstrated the better rooting ability of IBA at higher concentrations. Majeed *et al.* (2009) proved that IBA at 4000 ppm concentration is an optimal plant growth regulator for rooting the cuttings of *Aesculus indica* . High rooting rate was obtained for *Celtis australis* cuttings treated with 3000 ppm IBA (Shameet *et al*.. 1989). *Dalbergia sisso* and *Dalbergia latifolia* also exhibited a very high rooting rate with the application of the concentration 5000 ppm of IBA (Sharma and Pandey, 1999). Maximum number of roots was produced in *Melissa officinalis* stem cuttings at 5000 ppm concentration of IBA (Sevik and Guney, 2013).

Thakur *et al.* (2018) concluded that the cuttings of *Toona ciliata* produced significantly maximum average length of sprouts, root and number of roots per cutting with the application of 8000 ppm of IBA in comparison to other formulations. The apical cuttings of *Berberis aristata* treated with 5000 ppm of IBA concentration demonstrated significantly better rooting and sprouting compared to other treatments (Ali *et al*., 2008). The higher concentration of IBA is required to compensate the low endogenous levels of auxin in the mature cuttings, otherwise difficult to root species like *Terminalia arjuna* (Banjara, 2017).

The growth medium or the rooting medium is one of the major factors affecting the rooting of stem cuttings (Ingram *et al.,* 1993). The rooting success in any cutting is affected by the interaction of a number of factors like water, oxygen, and nutrient availability in the growth media (Alikhani *et al*., 2011; Bhardwaj, 2014). The effect of growth media on the rooting ability of stem cuttings of several economically important plants have been demonstrated by several works (Wojtusik *et al*., 1994; Tchoundjeu *et al*., 2002; Akinyele, 2010; Jacygrad *et al*., 2012; Usman and Akinyele, 2015; Ibironke and Victor, 2016).

The results obtained in this study revealed that there was a significant effect of growth media on the root number, root length and the shoot length of *Z. armatum* stem cutings. The highest values were observed in neopeat medium and the lowest values in sand medium. Neopeat medium had more number of roots and the longest roots than sand and mix media. Sand is too porous and cannot retain water for a longer period of time as well as low in nutrient content whereas the neopeat consists of mixture of all the required nutrients, better aeration and adequate drainage (Akinyele, 2010). Poor aeration in waterlogged conditions may lead to decay of cuttings before root initiation (Schmitz *et al*., 2013).

This corroborated with the findings of Tchoundjeu *et al*. (1998) in the cuttings of *Prunus africana,* which rooted better in sawdust than in sand. Similar findings were described by Wojtusik *et al*., 1994 in *Prosopis juliflora* cuttings, which produced more number of roots and the longest roots in perlite medium than in compost medium. Therefore to enhance steady rooting, the best quality hormone and rooting media is crucial.

Conclusion

As the seed germination rate is very slow in *Z. armatum*, vegetative propagation through stem cuttings is a viable option for the mass production of elite plant materials. This study evaluated the effect of different growth hormone types, concentration and growth media on the rooting and shooting performance of *Z. armatum* stem cuttings. The results showed that growth media and hormonal concentration significantly affect the growth of root, shoot and the number of roots of *Z. armatum* stem cuttings. Both IBA and NAA responded well in rooting and shooting but IBA was found to be effective as compared to NAA. Among the various concentrations of IBA, 5000 ppm showed the best performance in terms of average root length, shoot length and number of roots per cutting. Similarly, the neopeat growth medium was found to be superior over the sand and mix media. So it can be concluded from this study that IBA 5000 ppm concentration with neopeat medium is the best treatment for rooting the stem cuttings of *Z. armatum*. The results obtained in this study could be of relative significance for the commercial production of quality plantlets as well as for improving agroforestry systems.

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Research Article

Total Phenolic, Flavonoid Contents, and Antioxidant Activities of Fruit, Seed, and Bark Extracts of *Zanthoxylum armatum* DC

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Natural antioxidants present in several medicinal plants are responsible for inhibiting the harmful effects of oxidative stress. These plants contain polyphenols and flavonoids that act as free radical scavengers and reduce oxidative stress and may be an alternative remedy to cure various harmful human diseases. This study aims to quantify the total phenolic and flavonoid contents (TPC and TFC) and antioxidant properties of methanol extracts of fruits, seeds, and bark of an important medicinal and aromatic plant, *Zanthoxylum armatum* collected from wild and cultivated populations in Nepal. TPC was determined by Folin–Ciocalteu colorimetric method using gallic acid as standard, and various concentrations of the extract solutions were measured at 760 nm. TFC was calculated by aluminum chloride colorimetric assay. Quercetin was used as standard, and the absorbance was measured at 510 nm. The antioxidant potential of the different extracts was estimated by DPPH free radical scavenging assay, and the absorbance was measured at 517 nm. The highest TPC value was 226.3 ± 1.14 mg GAE/g in wild fruits, and the lowest was 137.72 ± 4.21 mg GAE/g in cultivated seeds. Similarly, the highest TFC value was 135.17 ± 2.02 mg QE/g in cultivated fruits, and the lowest was 76.58 ± 4.18 mg QE/g in cultivated seeds. The extracts showed variable antioxidant properties. The fruits exhibited excellent antioxidant properties with IC_{50} values of 40.62 μg/mL and 45.62 μg/mL for cultivated and wild fruits, respectively. Similarly, the IC_{50} values of the bark were 63.39 μg/mL and 67.82 μg/mL, respectively, for cultivated and wild samples. And the least antioxidant capacity was shown by the seeds extract with IC_{50} values of 86.75 μg/mL and 94.49 μg/mL for wild and cultivated seeds, respectively. The IC_{50} value of the standard ascorbic acid was 36.22 μg/mL. Different extracts of *Z. armatum* contain considerable amount of phenols and flavonoids, including antioxidant properties, suggesting the potential use of this species in pharmacy and phytotherapy as a source of natural antioxidants.

1. Introduction

Medicinal plants have been used in several indigenous herbal practices since very old times to cure several diseases. Herbal medication still continues to serve as an important health care system even today despite the greater advancements in modern medication systems in the recent years. Their long uses in the folk medicine and their safer implications in human health have generated much interest in them, especially in developing countries. It has now been established that medicines derived from plant products are safer than their synthetic counterparts [1].

Plant and plant-based products are the natural sources of different phytochemicals such as phenols, flavonoids, alkaloids, glycosides, lignins, and tannins. Phenols and flavonoids are the most common phytoconstituents of different fruits, vegetables, and medicinal and aromatic plants, which are responsible for antioxidant activities [2]. Due to the potential toxicological effects of synthetic antioxidants [3], natural antioxidants such as phenols and flavonoid compounds from plant origin are gaining popularity these days [4]. An antioxidant is a substance that inhibit or delays oxidative damage to the cells of the organisms by scavenging the free radicals such as peroxide or hydroperoxide and thus reducing the risk of degenerative diseases [5]. Abnormal production of free radicals may cause several severe human diseases such as cancer; Alzheimer's disease; cardiac, kidney, and liver diseases; fibrosis; atherosclerosis; arthritis; neurodegenerative disorders; and aging. Several medicinal plants have been screened for their antioxidant and other biological activities [6–8].

Zanthoxylum armatum DC (family: Rutaceae), commonly called as *Timur* in Nepal, is an aromatic perennial shrub or a small tree up to 6 m height with dense glabrous foliage and straight prickles. It is distributed from Kashmir to Bhutan, north-east India and Pakistan, Laos, Myanmar, Thailand, China, Japan, North and South Korea, North Vietnam, and Taiwan [9]. In Nepal, it is found at 1000 m to 2500 m from east to west [10]. It is used in traditional medicinal systems for various ailments such as cholera, diabetes, cough, diarrhea, fever, headache, microbial infections, and toothache [11–14]. The dried fruits of the plant are used as condiment and have excellent spice value. Several phytocomponents such as alkaloids, flavonoids, terpenoids, phenols, and steroids have been extracted from different parts of the plant such as fruits, seeds, leaves, and bark [13, 15, 16]. These compounds are responsible for several pharmacological activities such as antibacterial, antifungal and antihelminthic, antioxidant, anti-inflammatory, hepatoprotective, cytotoxic, larvicidal, and antispasmodic [17–21].

A lot of experiments have been carried out in *Z. armatum* regarding their antioxidant properties [22]. But the comparative study from different habitats and different parts of the plants is still meager, so the present study was carried out to quantify the total phenolic and flavonoid contents and evaluate the antioxidant properties in methanolic extracts of the fruits, seeds, and bark of *Z. armatum* collected from wild and cultivated populations. The correlation between total phenolic and flavonoid content and antioxidant activity with the habitat conditions could help to establish foundation for further studies focusing on the development of safer and inexpensive natural antioxidants for their use in therapeutic and pharmaceutical preparations.

2. Methods

2.1. Collection and Processing of Samples. The fresh fruits, seeds, and bark of *Z. armatum* were collected from wild and cultivated populations from Salyan district of west Nepal during May 2018. The plants were collected with the permission of Department of Plant Resources, Ministry of Forests and Environment, Government of Nepal, in accordance with article no. 10(B) of Plant Resource Research Procedure 2013 and revised 2016. This plant is not mentioned in CITES and protected plant list of Nepal. The plant was identified by Nirmala Phuyal. Herbarium of voucher specimens was prepared and deposited at National Herbarium and Plant Laboratories (KATH); NPZA 20-NPZA 50. The samples were cleaned and shade dried for a week before the extraction procedure.

2.2. Extraction of the Samples. The dried samples were then powdered separately in a grinder. Known weight of the powdered samples was loaded in thimble and put inside the Soxhlet apparatus. They were then successively extracted with methanol by the hot Soxhlet extraction method. The apparatus was run for 72 hours till the colored solvent appeared in the siphon for obtaining the crude extracts of the samples. After complete extraction, the solvent was evaporated in a rotary vacuum evaporator at 65°C under reduced pressure. The obtained extracts were then dried in a water bath. The dried extracts were sealed inside 20 mL sterilized culture tubes and stored in refrigerator at 2–8°C for further analysis [23].

2.3. Determination of Total Phenolic Content (TPC)

2.3.1. Preparation of Standard Gallic Acid for Calibration Curve. Total phenolic contents (TPC) in the fruits, seeds, and bark extracts were determined by Folin–Ciocalteu colorimetric method as described by Singleton et al. [24] with some modifications. Standard gallic acid solution was prepared by dissolving 10 mg of it in 10 mL of methanol (1 mg/mL). Various concentrations of gallic acid solutions in methanol (25, 50, 75, and 100 μg/mL) were prepared from the standard solution. To each concentration, 5 mL of 10% Folin–Ciocalteu reagent (FCR) and 4 mL of 7% Na_2CO_3 were added making a final volume of 10 mL. Thus, the obtained blue colored mixture was shaken well and incubated for 30 min at 40°C in a water bath. Then, the absorbance was measured at 760 nm against blank. The FCR reagent oxidizes phenols in plant extracts and changes into the dark blue color, which is then measured by UV-visible spectrophotometer. All the experiments were carried out in triplicates, and the average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

2.3.2. Preparation of Samples for Total Phenolic Content. Various concentrations of the extracts (25, 50, 75, and 100 μg/mL) were prepared. The procedure as described for standard gallic acid was followed, and absorbance for each concentration of the extracts was recorded. The samples were prepared in triplicate for each analysis, and the average value of absorbance was used to plot the calibration curve to determine the level of phenolics in the extracts. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The total phenolic contents in all the samples were calculated by the using the formula:

$$C = c\frac{V}{m}, \tag{1}$$

where C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/mL, V = volume of extract in mL, and m = mass of extract in gram.

2.4. Determination of Total Flavonoid Content

2.4.1. Preparation of Standard Quercetin for Calibration Curve. Total flavonoid contents in the extracts were determined by aluminum chloride colorimetric assay. Stock solution (4 mg/mL) of quercetin was prepared by dissolving 4 mg of quercetin in 1 mL of methanol. This standard solution was diluted serially to make various concentrations of 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, and 1 mg/mL solutions. 1 mL quercetin of each concentration was added to the test tube containing 4 mL of distilled water. At the same time, 0.3 mL of 5% $NaNO_2$ was added to the test tube and 0.3 mL of 10% $AlCl_3$ after 5 min. Then, 2 mL of 1 M NaOH was added to the mixture after 6 min. The volume of the mixture was made 10 mL by immediately adding 4.4 mL of distilled water. The total flavonoids content was expressed as quercetin equivalents using the linear equation based on the calibration curve.

2.4.2. Preparation of Samples for Total Flavonoid Content. Stock solutions of 4 mg/mL concentration in methanol of the extracts were prepared, and they were diluted serially to make different concentrations (0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, and 1 mg/mL) solutions. Similar procedure as described for quercetin was followed for the extracts also, and the absorbance was measured by spectrophotometer at 510 nm. Readings were taken in triplicate, and the average value of absorbance was used to calculate the total flavonoid content. The flavonoid content was expressed as quercetin equivalent (mg QE/g) using the linear equation based on the standard calibration curve.

2.5. Antioxidant Activities

2.5.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Activity. In vitro antioxidant activities of the extracts were determined using the DPPH free radical scavenging assay described by Nithianantham et al. [25] with some modifications. This is a quick and easy method to analyze the scavenging potential of antioxidants. DPPH in oxidized form gives a deep violet color in methanol. An antioxidant compound donates the electron to DPPH, thus causing its reduction and in reduced form its color changes from deep violet to yellow. DPPH solutions show a strong absorbance at 517 nm appearing as deep violet color. Scavenging of DPPH free radical determines the free radical scavenging capacity or antioxidants potential of the test samples, which shows its effectiveness, prevention, interception, and repair mechanism against injury in a biological system.

2.5.2. Preparation of DPPH Solution (0.1 M). DPPH solution (0.1 M) was prepared by dissolving 0.39 mg of DPPH in a volumetric flask, dissolved in methanol, and the final volume was made 100 mL. Thus, prepared purple-colored DPPH free radical solution was stored at −20°C for further use.

2.5.3. Preparation of Extract Solutions. Stock solution of different extracts of 1 mg/mL was prepared by dissolving required quantity of each extract in required volume of methanol. From the sample stock solution, 25, 50, 75, and 100 μg/mL solutions of each extract were prepared.

2.6. Evaluation of Antioxidant Potential. To the sample solutions of different concentration, 1 mL DPPH solution was added and incubated at room temperature for 30 min in dark. A control was prepared by mixing 1 mL methanol and 1 mL DPPH solution. Finally, the absorbance of the solutions was measured by using a spectrophotometer at 517 nm. Ascorbic acid was used as the standard. 50% inhibitory concentrations (IC_{50} values) of the extracts were calculated from graph as concentration versus percentage inhibition. Radical scavenging activity was expressed as percentage of inhibition. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical. Measurements were taken in triplicate. IC_{50} of the extracts indicates the corresponding concentration in which the radical scavenging potential is 50%. The IC_{50} of the extract and standards were determined graphically.

The percentage of inhibition was calculated by using the formula:

$$I\% = \frac{AC - AO}{AC} \times 100\%, \qquad (2)$$

where AC = absorbance of the control (1 mL methanol + 1 mL DPPH solution), AO = absorbance of the sample solution, and I% = percentage of inhibition.

The radical scavenging activities of the extracts are expressed in terms of their IC_{50} values. The data were presented as mean values ± standard deviation ($n = 3$).

3. Results

3.1. Total Phenolic Contents (TPC). Total phenolic contents in different extracts of fruits, seeds, and bark of *Z. armatum* were determined by Folin–Ciocalteu (F–C) method using gallic acid as the standard. The absorbance values obtained at different concentrations of gallic acid were used for the construction of calibration curve. Total phenolic content of the extracts was calculated from the regression equation of calibration curve ($Y = 0.0108x$; $R^2 = 0.993$) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g).

TPC values were higher in the fruit and bark extracts than the seed extracts. The highest TPC value was observed for the fruits followed by the bark, and the lowest was for the seeds extracts. TPC value of the cultivated fruit extract was 226.3 ± 1.14 mg GAE/g and that for wild fruit was 185.02 ± 2.15 mg GAE/g. Similarly, the value was 185.15 ± 1.22 mg GAE/g for wild bark and 171.13 mg GAE/g for cultivated bark. And for the wild seeds, the TPC value was 167.74 ± 2.63 mg GAE/g and that for cultivated was 137.72 ± 4.21 mg GAE/g (Table 1).

This is in accordance with the study of Barkatullah et al. [18], where the TPC value of ethanolic extracts of *Z. armatum* fruits was found to be 21.68 ± 0.44 mg/g and that of the bark was 16.48 ± 1.33 mg/g. Similarly, in another study, the TPC value of methanol extract of fruits was 366.3 mg of GAE/g [19]. The phenolic content of any plants is directly related to their antioxidant properties. Phenolic compounds act as reducing agents, hydrogen donors, and are capable of scavenging free radicals [26]. Presence of considerably good

Table 1: Total phenolic contents (TPC) in different extracts of *Z. armatum*.

S. No.	Samples	25 (μg/mL)	50 (μg/mL)	75 (μg/mL)	100 (μg/mL)	Mean TPC value (GAE/g)
1	Fruits (wild)	181.48	185.18	186.41	187.03	185.02 ± 2.15
2	Fruits (cultivated)	225.92	227.77	224.69	226.85	226.3 ± 1.14
3	Seeds (wild)	166.66	166.66	165.43	172.22	167.74 ± 2.63
4	Seeds (cultivated)	133.33	135.18	144.44	137.96	137.72 ± 4.21
5	Bark (wild)	184.77	183.33	186.41	186.11	185.15 ± 1.22
6	Bark (cultivated)	162.93	168.51	171.6	181.48	171.13 ± 6.73

amount of phenolics in the fruits, seeds, and bark extracts of *Z. armatum* may contribute significantly to the antioxidant properties. Because of these properties, this plant might have been used in several traditional herbal medications.

The antioxidant response of phenolic compounds varies remarkably, depending on their chemical structure [27]. In addition, there may be some interference rising from other chemical components present in the extract, such as sugars or ascorbic acid [28]. In this study also, there were differences in the total phenolic components of the wild and cultivated fruits, seeds, and bark extracts. These differences could arise from variations in genetic backgrounds, environmental factors, agronomic practices as well [29].

3.2. Total Flavonoid Contents (TFC). Total flavonoid content of the extracts was calculated from the regression equation of the calibration curve ($Y = 0.0011x$; $R^2 = 0.992$) and expressed as mg quercetin equivalents (QE) per gram of sample in dry weight (mg/g). The TFC values also showed similar trends with that of TPC values. The highest TFC value was obtained for the fruits followed by the bark, and the lowest was for the seeds extracts. The highest TFC value was 135.17 ± 2.02 mg QE/g for cultivated fruit extract, and for wild fruit, it was 103.7 ± 1.39 mg QE/g. The TFC values were 111.2 ± 3.67 mg QE/g and 91.27 ± 3.13 mg QE/g for cultivated and wild barks, respectively. Similarly, the lowest TFC value was 76.58 ± 4.18 mg QE/g for cultivated seeds, and for wild seeds, it was 92.71 ± 3.14 mg QE/g (Table 2). In a previous study, the total flavonoid content of ethanolic extracts of *Z. armatum* fruit was 22.8 ± 1.33 mg/g and that of bark was 18.33 ± 1.22 mg/g [18], which is quite lower than that of the present study. The concentration of phenols and flavonoids also depends on the polarity of the solvents used for extraction [30].

3.3. Antioxidant Activity

3.3.1. DPPH Assay. Antioxidant activity of the fruits, seeds, and bark of *Z. armatum* was determined by DPPH free radical scavenging assay, and their reducing power was determined on the basis of their concentration providing 50% inhibition (IC_{50}) values or in other words, the amount required to scavenge 50% DPPH free radicals. The mean percentage of DPPH free-radical scavenging activity at different concentrations of extracts is shown in Table 3, Figure 1. The radical scavenging activity of different extracts increased in a concentration dependent manner.

Ascorbic acid used as the standard and the different extracts showed variable antioxidant properties. The IC_{50} value of ascorbic acid was 36.22 μg/mL. The higher IC_{50} value indicates lower radical scavenging activity or lower antioxidant potential. The fruits extracts had the highest antioxidant capacity compared to the seeds and bark extracts. The IC_{50} value of the fruits extracts was close to that of the standard, i.e., 40.62 μg/mL and 45.62 μg/mL, respectively, for the cultivated and wild fruits. Similarly, the IC_{50} value was 86.75 μg/mL and 94.49 μg/mL, respectively, for wild and cultivated seeds, showing least antioxidant properties. And the bark showed moderate antioxidant capacity with the IC_{50} value of 63.39 μg/mL and 67.82 μg/mL for cultivated and wild extracts, respectively. The antioxidant potential of fruits and bark extracts were higher in cultivated than in wild samples, whereas in seeds extracts, it was higher in wild than cultivated samples.

The antioxidant potential of different parts of *Zanthoxylum armatum* has been evaluated by various previous studies [20, 31–35]. The free radical scavenging activity of methanolic fruits extracts ranged from 59.56 to 64.85% [36], while in the present study, the scavenging percent ranged from 58.35 to 78.36% (Table 3). Similarly, the IC_{50} value of the methanolic bark extract of *Z. armatum* was 149.26 μg/mL [34], but it was 63.39 μg/mL in the present study.

The radical scavenging activity of different extracts of *Z. armatum* may be due to the presence of polyphenols, flavonoids, and phenolic compounds, and most of the antioxidant activity of plants is because of the phenols [37]. Natural antioxidants present in plants are responsible for inhibiting or preventing the harmful consequences of oxidative stress. DPPH assay among many other assays is one of the convenient methods for determining the antioxidant potential of plants. The presence of antioxidant substances containing hydrogen-donating groups such as flavonoids and phenols causes the methanolic DPPH solution to get reduced due to the formation of nonradical [38]. Apart from antioxidant properties, flavonoids and other phenolics also exhibit several biological activities such as antimicrobial, antiviral, and anticancer [39]. These biological and pharmacological activities are usually associated with their ability of binding proteins and free radical scavenging properties [40].

Antioxidants are tremendously important substances, which possess the ability to protect the body from damage caused by free radical-induced oxidative stress. Plant polyphenols act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups [41].

Table 2: Total flavonoid contents (TFC) in different extracts of *Z. armatum*.

S. No.	Samples	0.25 mg/mL	0.50 mg/mL	0.75 mg/mL	1 mg/mL	Mean TFC value (QE/g)
1	Fruits (wild)	101.8	105.44	103.02	104.54	103.7 ± 1.39
2	Fruits (cultivated)	138.16	134.54	132.54	135.45	135.17 ± 2.02
3	Seeds (wild)	94.52	94.54	87.26	94.54	92.71 ± 3.14
4	Seeds (cultivated)	72.72	74.54	83.62	75.45	76.58 ± 4.18
5	Bark (wild)	90.88	94.54	93.33	86.36	91.27 ± 3.13
6	Bark (cultivated)	116.36	109.08	106.66	112.72	111.2 ± 3.67

Table 3: Mean absorbance and IC_{50} values of extract and ascorbic acid at different concentrations.

	% Inhibition (scavenging capacity)						
Concentration (μg/mL)	Ascorbic acid	Fruits (wild)	Fruits (cultivated)	Seeds (wild)	Seeds (cultivated)	Bark (wild)	Bark (cultivated)
100	84.21	73.29	78.36	49.41	46.44	58.45	61.25
75	78.22	68.2	73.54	44.12	41.36	53.98	56.08
50	72.45	63.84	67.38	41.47	38.98	48.67	51.12
25	65.13	58.35	62.23	37.49	34.21	43.46	46.04
0	0	0	0	0	0	0	0
IC_{50} (μg/mL)	36.22	45.62	40.62	86.75	94.49	67.82	63.39

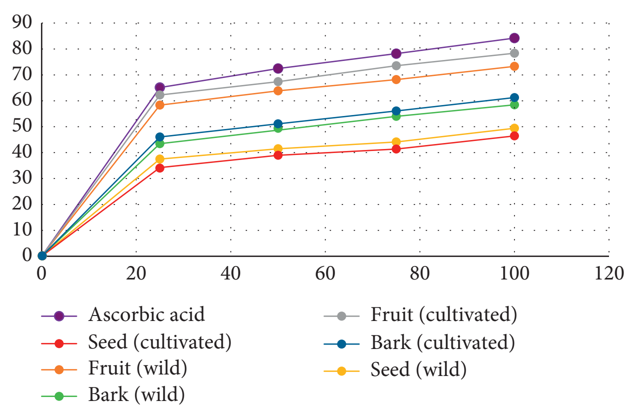

Figure 1: Plot of radical scavenging percentage between ascorbic acid and different samples.

4. Conclusions

Total phenolic, flavonoid contents, and antioxidant properties of the fruits, seeds, and bark extracts of *Z. armatum* were considerably good. However, these parameters were remarkably better in fruit and bark extracts as compared to the seed extracts. Some of the wild samples showed excellent results, and some of the cultivated samples showed better results. The differential TPC, TFC contents, and antioxidant properties from different habitat may plausibly be due to geographical variations in chemical constituents. The results of the present study suggested that the fruits, seeds, and bark of *Z. armatum* could be the potent source of natural antioxidants because of their phenolic and flavonoid contents and their remarkable scavenging effects on DPPH. So, this plant could be of greater significance in preventing several harmful human diseases. Further studies should be directed towards the extensive in vivo antioxidant activities of the plant and the relationship of individual phenolic compounds to antioxidant with different mechanisms and isolation, screening, and characterization of individual compounds responsible for antioxidant properties to authenticate their probable uses as sources of natural antioxidants as well as to validate their traditional uses in several medicinal practices.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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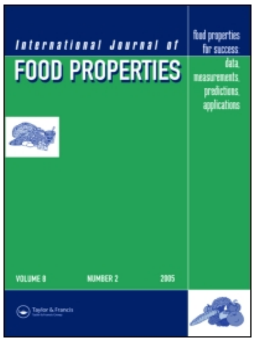

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Essential oil composition of *Zanthoxylum armatum* leaves as a function of growing conditions

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Essential oil composition of *Zanthoxylum armatum* leaves as a function of growing conditions

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ABSTRACT

Variation in the composition of leaf essential oils of *Zanthoxylum armatum* in relation to altitude and soil chemistry was analyzed in Nepal. The essential oil was extracted by Clevenger apparatus and the components were analyzed through GC-MS. The results showed that: Yield of the essential oil obtained from the hydro-distillation of dried leaves ranged from 0.16% to 0.50%. GC-MS analysis revealed total of 17 compounds in the essential oil from the dried leaves of *Z. armatum* from different altitudes and populations (wild and cultivated). The three major components, linalool, limonene and undecan-2-one, present in higher proportion in all the samples were analyzed. Other components tride-can-2-one, myrcene, cinnamate(E)-methyl and alpha-bergamotene were also identified in most of the samples but in lower proportions. The highest number of components (12) was identified from the wild samples collected from 1990 m and the lowest number (7) was from 1060 m (wild) and 2030 m (cultivated populations). Linalool, a major component was found in higher amount (64.88%) at the lowest altitude of 1060 m. Results of the present study showed that the altitude, soil chemistry, and growing conditions significantly affect the essential oil components in *Zanthoxylum armatum*.

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Introduction

Among the eight species of *Zanthoxylum* (family Rutaceae) reported from Nepal so far, *Zanthoxylum armatum*, commonly called as Timur is one of the important medicinal and aromatic plants. It is a small aromatic tree or large shrub up to 6 m high and found in the hot valleys of Himalayas (Kashmir to Bhutan), north-east India, Nepal, Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, and Japan.[1] In Nepal, it is distributed from west to east at an elevation range of 1000 m to 2500 m in open places or in forest undergrowth.[2] The plant is highly valued because of its medicinal, phytochemical and pharmacological properties and has been used in different traditional medicinal practices as carminative, antipyretic, appetizer, anthelmintic, stomachic, toothache, dyspepsia.[3–5]

Although all the parts of the plants possess essential oil, the fruits (pericarp) essential oil, commonly known as *Zanthoxylum* oil is highly valued for commercial purpose. Several studies have been carried out on the essential oil composition of the fruits, seeds, and leaves of *Zanthoxylum armatum*, which reveal that the main constituents of the oil are linalool and limonene.[6–8] Besides, several other compounds like myrcene, camphene, oleic acid, palmitic acid, methyl ester alpha- and beta-pinene, trans-beta-ocimene have also been identified from the volatile oil.[6,9–12] Similarly,

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several monoterpenes have also been identified from the leaves and the major ones are linalool, limonene cymene, myrcene, geraniol carvone, tridecanone, *trans*-caryophyllene, terpinolene, ocimene. One study claimed that 2-undecanone was identified as the major component of the leaf essential oil of *Z. armatum* for the first time from Kumaun, India.[13]

The accumulation of active substances in plants may be affected by several factors like the age of the plant, season, solar radiation, altitude, nutritional status, climatic and edaphic factors.[14,15] Several researches have proved that the different ecological, physiological, and genetic factors also affect the quality and quantity of secondary metabolites as well as their biological activities.[16–18] Among them, altitude, precipitation, soil texture, soil diversity, organic matters' rate, calcium phosphorus, and potassium elements are the most effective factors influencing the essential oil components.[19,20] Among all these factors, the most discussed one is the higher solar intensity at higher altitudes that has significant impact on secondary metabolite composition in higher plants.[21]

Literature shows that numerous works have been conducted on the composition of essential oil in *Z. armatum*, most of them on the fruit and very few on the leaf.[22] So far, no comprehensive study has been performed on the interrelationship between elevation and edaphic factors and the chemical composition of leaf essential oil in *Z. armatum* collected from different elevations and populations. Hence, this study endeavors to establish a linkage between the altitude, soil characters of the habitat and the constituents of the leaf essential oil of *Z. armatum*, the result of which could be of relative significance in providing new insights for future studies to find out the high yielding desired components of the essential oil.

Methods

Collection of leaves

The required samples were collected from Salyan district of Nepal during May 2018. The location was chosen for this study as Timur, is the main non-timber forest product (NTFP) of this district accounting to approximately 70% of the total value of the NTFPs collected.[23] Further, the government of Nepal has also prioritized the district for the commercial cultivation and facilitation of Timur.

Sampling was done during May 2018 and systematic random sampling method was applied to collect the leaves. Healthy and vigorous plants were selected from different populations (wild and cultivated) and altitudes ranging from 1000 m to 2030 m and fresh leaves of *Zanthoxylum armatum* were collected. The leaves were shade dried for a week before extraction of the oil. Herbarium of voucher specimens were prepared, and deposited at National Herbarium and Plant Laboratories (KATH) NPZA 20-NPZA 50. The details of the locality are presented in Table 1.

Table 1. Locality details.

S. No.	Locality	Altitude (m)	Latitude/Longitude	Aspect	Habitat	Land use/Forest Type
1	Aringalphedi	1000	N28°16′07.3″ E 082°18′30.3″	NE	Cultivated (C)	Fallow land near forest
2	Aringalphedi	1060	N28°16′06.3″ E 082°18′30.3″	NW	Wild (W)	Forest near village settlement
3	Aagarkhola	1390	N28°14′33.78″ E 82°21′08.35″	NE	Cultivated (C)	Agricultural land near forest
4	Aagarkhola	1400	N 28°14′35.63″ E082°21′09.61″	NW	Wild (W)	Mixed Forest near village
5	Rim, Saunepani	1650	N 28°26′763″ E 082°35′830″	NE	Cultivated (C)	On the edges of roadside
6	Kupinde	1680	N 28°41′319″ E 082°09′350″	NE	Wild (W)	Disturbed forest due to road construction
7	Kimichaur	1730	N 28°26′524″ E 082°37′741″	NW	Cultivated (C)	Near roadside on edges of farmyard
8	Rim, Rayale	1770	N 28°26′525″ E 082°37′740″	SW	Wild (W)	Mixed *Quercus* forest
9	Kopchikhola, Chhatreswori	1990	N 28°40′098″ E 082°35′497″	NW	Wild (W)	Moist shady mixed forest
10	Kopchikhola, Chhatreswori	2030	N 28°40′227″ E 082°35′688″	NE	Cultivated (C)	Cultivated on edge of farmyard

Collection and analysis of soil samples

While collecting the leaf samples, soil samples from different habitats and altitudes were also collected from a depth of 15–30 cm. Collected samples were brought to the laboratory, air dried and passed through a 2-mm sieve. Soil organic carbon, total N and pH of the soil were determined according to the Walkley and Black, Kjeldahl and potentiometry methods, respectively. Soil analysis was carried out at the laboratory of the Forest Research and Training Centre, Babarmahal, Kathmandu.

Extraction of essential oil

Total of 10 samples have been used for this study. The collected leaves were shade dried at room temperature. For the extraction of the oil, 100 g of the dried leaves were subjected to hydro-distillation for 6 h using modified Clevenger-type apparatus. The protocol was followed according to the British Pharmacopoeia.[24] The volume of the oil was measured directly in the extractor. The oil thus collected was then dehydrated over anhydrous sodium sulfate and stored in sealed, labeled glass vials at 4°C until further analysis. Total yield percentage was calculated as volume (ml) essential oil per 100 g of plant dry matter.

Analysis of essential oil samples by GC-MS

Quantitative analysis of the chemical constituents in the essential oils was carried out using a Shimadzu gas chromatograph (GC 2010) with Rtx-5MS column (25 m×0.25 mm× 0.25 μm). The initial column was maintained at 40°C and the injection temperature was 250°C. Qualitative analysis of the essential oil was further continued in a Shimadzu GCMSQP 2010 Plus. The ion source temperature and the interface temperature were kept at 200°C and 250°C, respectively. One μL of the essential oil diluted with spectroscopic grade hexane (10:1) was injected into the GC inlet maintaining column flow rate of 1mL/min and purge flow 3 mL/min after fixing the split ratio at 120, using Helium as a carrier gas. Detector scanning start time was 4 min and end time was 68 min, mass spectra were scanned from m/z 40–350, with the scanning speed of 666. The oil components were identified by the determination of their retention indices (RI), relative to C8–C32 n-alkane series under identical experimental condition, by comparison with authentic reference compounds as well as with published mass spectra[25–30] and by comparison of mass spectra using the NIST 11 (National Institute of Standards and Technology, Gaithersburg, MD) and FFNSC 1.3 library. The relative percentage of each constituent present in essential oil was calculated according to the area of the chromatographic peaks.

Statistical analysis

In order to compare the significantly different means to test the significant effects of altitude and edaphic factors in the percentage yield and chemical composition of leaf essential oil of *Z. armatum*, one-way ANOVA method was employed using R software package version 3.6.1.[31]

Results and discussion

Essential oil yield

In the present study, the yield of essential oil from leaves ranged from 0.16% to 0.5% at different altitudes (Table 2). The lowest yield was 0.16% at 1400 m altitude in wild population, while the highest was 0.5% at 1680 m altitude in wild population. The values are almost similar for all altitudes and populations. But in the samples of India, the variation in the essential oil yield of *Z. armatum* leaves ranged from 0.088% to 0.176%.[15] The yield depends on several factors like different environmental conditions, genetic differences or time of collection, season.[32] All the samples for this study were collected at the same period, during the phenological period and were extracted

Table 2. GC-MS analysis of leaf essential oil of *Z. armatum* from different elevation & populations.

S.No	Name of the compound	RI [a]	RI [b]	Area %										Average (%)
				1000 (C)	1060 (W)	1390 (C)	1400 (W)	1650 (C)	1680 (W)	1730 (C)	1770 (W)	1990 (W)	2030 (C)	
1	Alpha-Pinene	939[25]	948	0.42		-	-	-	-	-	-	-	-	0.42
2	Myrcene	991[25]	991	2.08	2.35	7.19	4.24	3.31	4.25	2.88	3.67	4.12	2.16	3.62
3	Limonene	1031[25]	1030	11.94	12.57	35.55	21.91	17.51	22.40	18.11	19.55	24.78	13.92	19.82
4	Linalool	1098[25]	1101	62.77	64.48	16.01	43.26	48.00	27.68	21.87	50.06	20.14	33.07	38.73
5	2,3-Octanedione	1082[26]	1115	-		-	-	-	-	-	-	0.59	-	0.59
6	3,7-Dimethylundecane	1218[25]	1185	0.34		-		-	-	1.12	-	0.48	-	0.64
7	Alpha-Terpineol	1189[25]	1195	0.42		-	0.55	-	1.42	-	0.47	-	-	0.71
8	Dodecane	1199[25]	1200	-		0.63		-	-	-		-	1.03	0.83
9	2-Undecanol	1287[25]	1277	-		-	0.47	-	0.94	-	-	1.51	-	0.97
10	Undecan-2-one	1287[27]	1294	11.74	9.55	24.88	16.58	16.98	31.03	34.63	15.67	33.72	32.73	22.75
11	Tridecane	1299[25]	1300	-		1.36	0.20	-	-	1.22	-	0.70	-	0.87
12	Methyl (E)-Cinnamate	1379[25]	1384	4.44	1.46	2.03	3.23	4.83	1.29	1.51	3.07	0.67	-	2.5
13	Alpha-Bergamotene	1434[28]	1430	1.26	1.42	2.07	1.36	1.91	2.40	3.56	1.73	2.62	6.10	2.44
14	Tridecan-2-one	1496[25]	1495	2.03	1.79	7.47	5.62	4.30	5.36	13.03	3.51	8.06	8.91	6
15	Trans Nerolidol	1564[25]	1564	-		-	0.52	-	-	-	0.57	-	-	0.54
16	Phytol	2105[29]	2106	-		-	-	2.12	1.00	-	-	1.28	-	1.46
17	Isophytol, acetate	2282[30]	2286	0.77		-	-	-	-	-	-	-	-	0.77
Oil Yield (%)				**0.2**	**0.24**	**0.25**	**0.16**	**0.27**	**0.50**	**0.26**	**0.30**	**0.24**	**0.22**	**0.26**
Total no. of components				11	7	9	11	8	10	9	9	12	7	9.3
Total % of components				98.21	93.62	97.19	97.94	98.66	97.77	97.93	98.3	98.67	97.92	97.62

Compounds are listed in the order of elution on a DB5 column.
RI[a] Retention index from different literatures.
RI[b] calculated by GC using n-alkane series under the same conditions.
Oil yield % are given in bold.

simultaneously so there might not have been significant differences in the dry weight essential oil yield.

Essential oil composition

The chemical composition of leaf essential oil of *Zanthoxylum armatum* from different populations and altitudes varied significantly. Altogether 17 components were identified by GC-MS analysis in the oil from various altitudes and localities. All the compounds (excluding the trace components) are presented in Table 2. The major components are linalool, limonene, and undecan-2-one (Figure 2), which were present in the highest percentage in all the samples. Besides, myrcene, methyl (E)-cinnamate, alpha-bergamotene and tridecan-2-one (Figure 2) were also present in most of the samples. Highest number of compounds i.e.,12 were present in sample from wild populations at 1990 m, 11 compounds were present at 1000 m (cultivated) and 1400 m (wild). The least number of compounds, i.e., seven was recorded at 2030 m altitude from cultivated populations and 1060 wild and some compounds like alpha-pinene, 2,3-octanedione, isophytol acetate were detected in lower percentage in only one locality/altitude (Table 2). Wild populations had comparatively higher number of components than cultivated populations.

The concentration of linalool was highest in all the samples, with an average value of 38.73% followed by undecan-2-one, 22.75% and limonene, 19.82% (Table 2). The major components linalool, limonene, and myrcene were present in higher proportion in the wild populations in most of the samples studied, whereas the components like tridecan-2-one and undecan-2-one were identified in lesser amount in wild samples than cultivated ones. The major component linalool shared the highest percentage at lower altitude; 64.48% in the wild population at 1060 m and 62.77% in the cultivated populations at 1000 m. The total number and percentage of components were comparatively higher in the samples from wild populations than in cultivated populations (Figure 1). Usually, there are marked phytochemical differences between different taxa than intraspecific variations of a particular taxon at different elevations.[33] Significant variations in the amount of linalool and limonene in the plants of different altitudes were observed in the leaf essential oil of *Z. armatum*.[34] If variations occur in the same taxon at different altitudes, it might be due to the differences in genetic makeup. Thus, the pronounced variations observed in the chemotypes of leaf essential oil of cultivated and wild populations of *Z. armatum* might be due to genetic variability, different geographical environment, growth and physiological development of the plant.[35] The differences in chemical composition of the essential oil among different altitudes in the present study may be attributed to adaptation to particular habitats or different growing conditions a result of changing ecological niches.

Thirty-four compounds were identified from the leaf essential oil of *Z. armatum* and the major were beta-Linalool (53.05%), alpha-Limonene diepoxide (11.39%), alpha-pinene (4.08%), beta-Myrcene (3.69%) and D limonene (3.1%).[36] However, compounds like methyl (E)-cinnamate, -alpha-bergamotene and tridecan-2-one, which have been identified as major components in this study were not reported by the previously mentioned study. Also, different components identified in leaf essential oil of *Z. armatum* by earlier studies have not been detected in the samples used in this study, for e.g., *trans*-caryophyllene, bornyl acetate, α-copaene[15], sabinene 37, β-fenchol β-phellandrene,[35] etc, indicating wider range of variability in chemotype.

The leaf essential oil of *Zanthoxylum alatum* of Vietnam constituted major component as 1,8-cineole (41.0%) and others were sabinene (8.4%), b-terpineol (2.1%), linalool (4.5%), terpinen-4-ol (5.2%), a-terpineol (4.1%), b-cymene (1.3%), 2,6-dimethyl-1,3,5,7-octatetraene (1.5%) and 2-tridecanone (1.8%).[37] Fourteen components were identified from the leaf oil of *Z. armatum* and the major were linalool (30.58%), 2-decanone (20.85%), β-fenchol (9.43%), 2-tridecanone (8.86%), β-phellandrene (5.99%), sabinene (4.82%), and α-pinene (4.11%).[35]

Eco-physiological relations in plants are greatly affected by different environmental factors by solar radiation, temperature, relative humidity, wind velocity, water availability, etc. Altitudinal

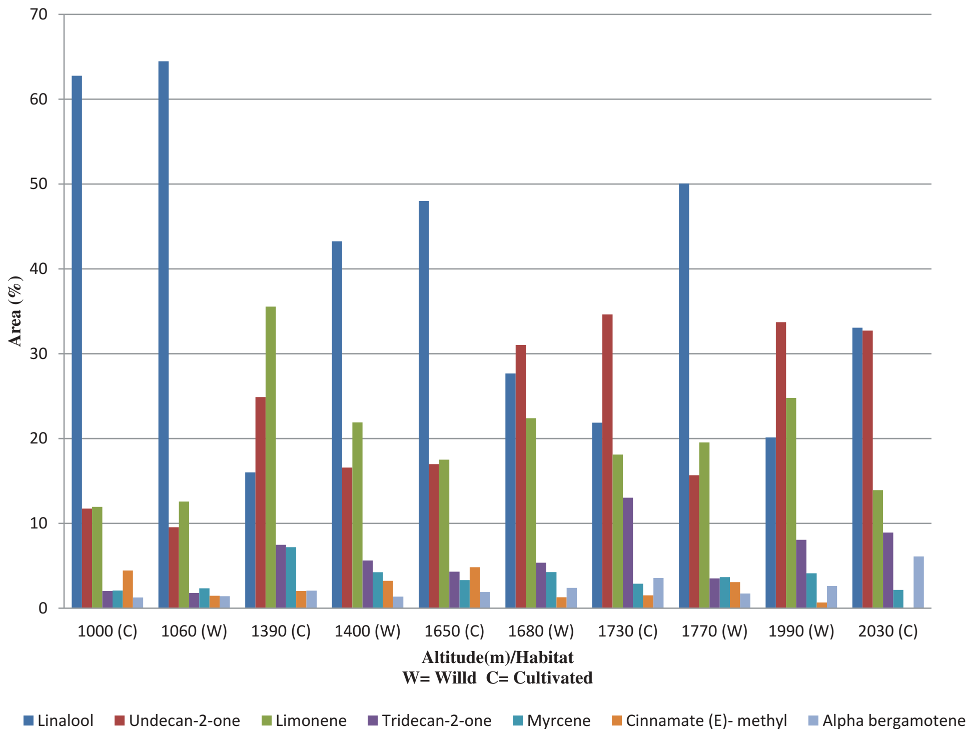

Figure 1. Composition of leaf essential oil of *Zanthoxylum armatum* at different altitudes and habitats.

variations can bring about significant changes in these factors, which in turn affect the secondary metabolites production in plants.[38] Different studies have shown that the harvesting time and season can also have an impact on the yield and the quantitative composition of essential oils components in plants.[39–41] Since all the samples for this study were collected around same time, the effect of harvesting season and time was not applicable in this study.

Soil parameters

Soil chemistry may influence the yield and chemical components of essential oils in aromatic bearing plants.[42] Nitrogen, as one of the important soil minerals, can alter the essential oil component through biosynthetic metabolic pathways.[43] It has been found to increase the essential oil components in some plants like Thyme.[44] The quality and quantity of essential oils of medicinal and aromatic plants may be affected not only by the elevation factors but also by the physicochemical properties of soil.[45] Various chemical elements in soil found in rhizosphere of plants enter into the composition of enzymes and affect the biochemical processes of plants[42]. Hence, the soil chemistry can influence the phytochemical composition.[46]

In the present study, the soil organic carbon, total nitrogen content, and pH of the soil varied among different habitats at different altitudes. Percentage of soil organic carbon was higher in most of the samples from cultivated populations, whereas the total nitrogen content and pH values were slightly higher in most of the wild populations (Table 3). The highest total nitrogen (0.656%) was e present in the soil sample from wild population at 1400 m, with neutral soil (pH value 6.78) (Table 3). The essential oil yield from the leaves collected from this site was the lowest (0.16%). The lowest value of organic carbon (2.478%) was at present in soil from 1990 m (wild population), where total numbers and percentages of

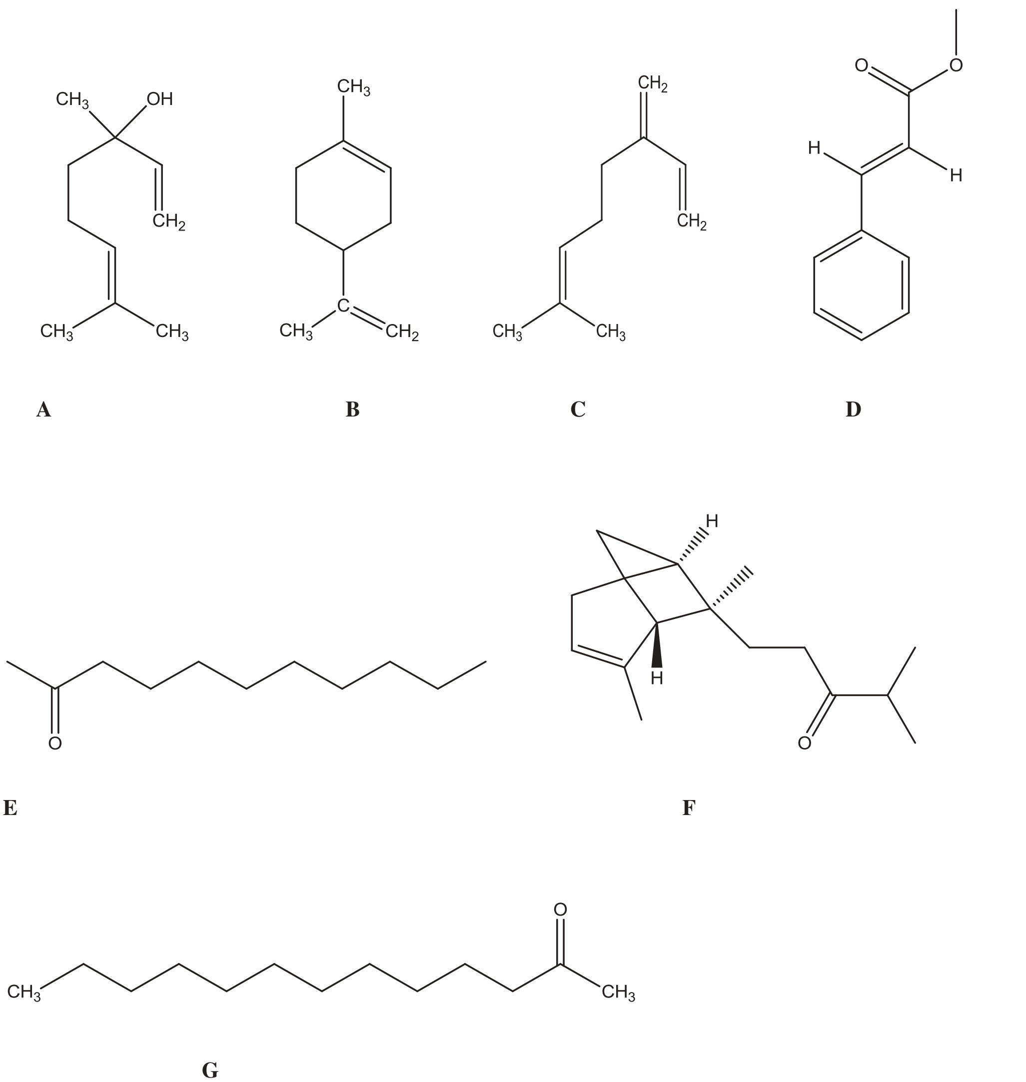

Figure 2. Structures of the major components of leaf essential oil of *Zanthoxhylum Armatum* **(A)** Linalool **(B)** Limonene **(C)** Myrcene **(D)** Methyl **(E)** Cinnamate **(E)** Undecan −2-One **(F)** Alpha-Bergamotene **G** Tridecan −2-One.

Table 3. Soil parameters at different altitudes.

S. No.	Altitude	SOC (%)	Total N (%)	pH
1	1000 (C)	3.344	0.300	6.48
2	1060 (W)	4.493	0.353	7.5
3	1390 (C)	4.629	0.432	7.52
4	1400 (W)	2.688	0.656	6.78
5	1650 (C)	4.854	0.373	5.92
6	1680 (W)	2.891	0.221	5.65
7	1730 (C)	3.548	0.602	5.5
8	1770 (W)	4.197	0.326	6.08
9	1990 (W)	2.478	0.555	7.04
10	2030 (C)	5.028	0.394	6.19

Table 4. ANOVA table for the effect and soil on the major components of *Z. armatum* essential oil.

S. N.	Name of the compound	Mean value
1	Linalool	621.9***
2	Undecan-2-one	190.37***
3	Limonene	97.86***
4	Tridecan-2-one	24.41***
5	Myrcene	4.38***
6	Methyl (E)- cinnamate	5.41***
7	Alpha-bergamotene	4.91***

*** P value < 0.01

components present were higher. Hence, it can be said that the soil components also have a major role to play in the chemical profiling of the essential oil components.

Result of ANOVA too revealed that different altitudes and soil characters have a significant effect on the essential oil composition and the dry matter yield of *Z. armatum*. All the major components linalool, limonene, undecan-2-one, tridecan-2-one, myrcene, and alpha-bergamotene were highly significant at $P > .01$ (Table 4). The production of secondary metabolites in plants is directly related to the climatic factors and the quality and quantity of active components is influenced by environmental and genetic factors.

The unlike distribution of chemicals may be attributed to the different environments of the habitats. The environmental factor favorable for one component might not be suitable for the dominance of another component.[42] For example, in this study also, at the altitude 1060 m, linalool was found in higher amount (64.88%) whereas undecan-2-one in lower proportion (9.55%)

Conclusion

The results of this study showed that variation in elevation, growing conditions, and edaphic factors significantly affect the production and distribution of different active phytochemicals in *Zanthoxylum armatum* collected from different populations. Among the 17 compounds identified, linalool, undecan-2-one, and limonene were the major constituents of the leaf essential oil, which were more prevalent. Besides, alpha-bergamotene, myrcene, methyl (E)-cinnamate and tridecan-2-one were also identified in almost all the samples. Other components were present in trace amounts. There were distinct variations in the chemical components of the essential oil from wild and cultivated populations. The oil yields as well as the total number and percentage components were also comparatively higher in wild populations than cultivated ones.

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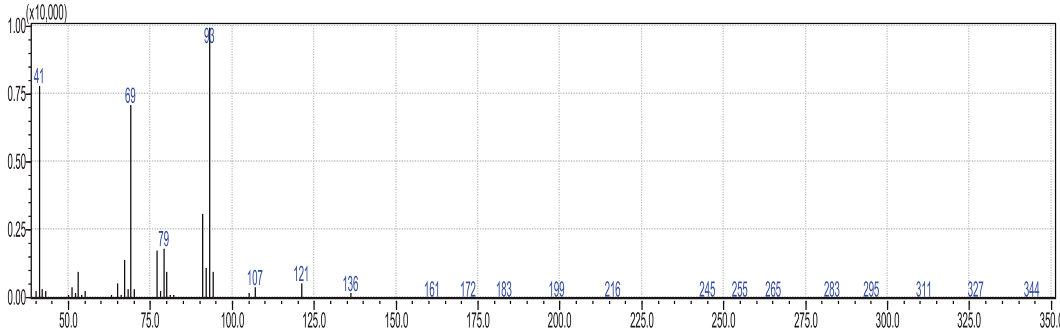
Appendix

Mass spectra of major components of essential oil of *Zanthoxylum armatum* leaves



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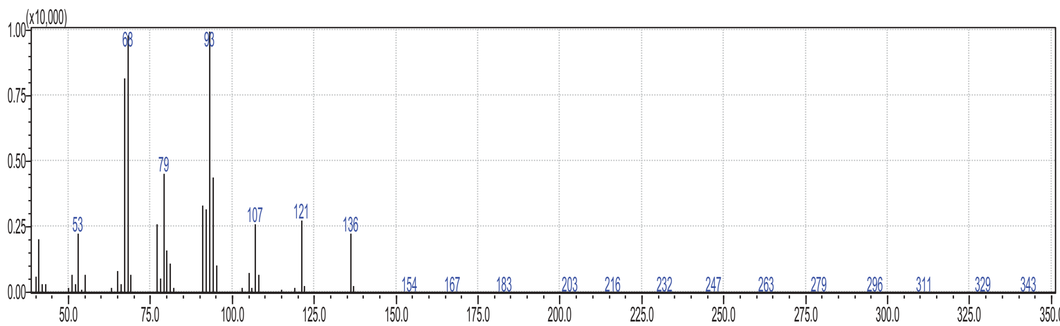

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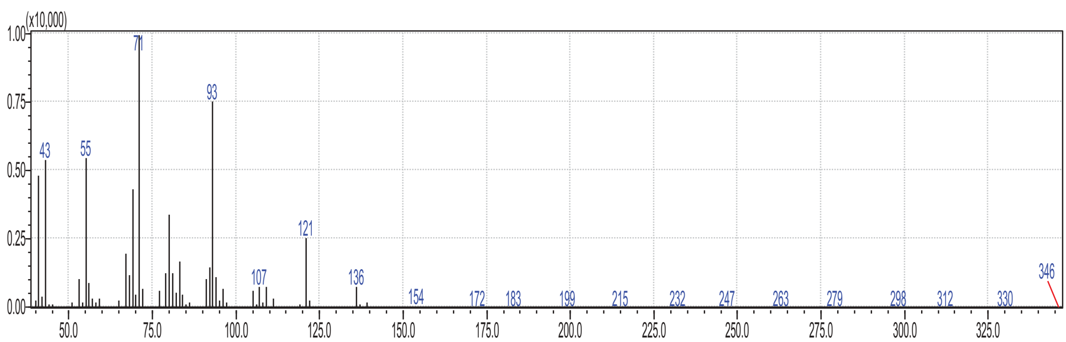

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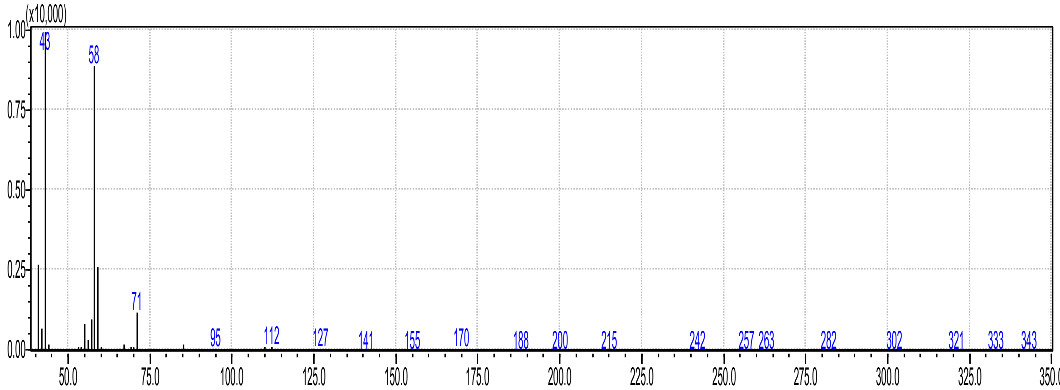

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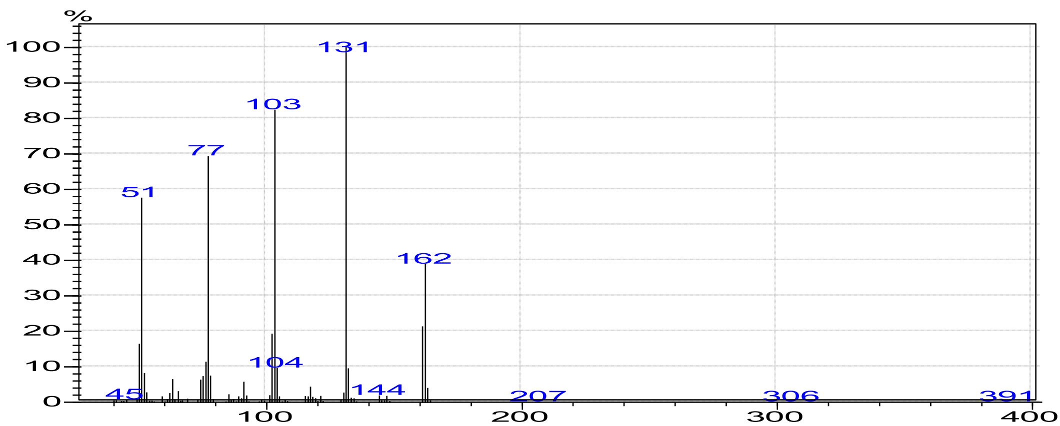

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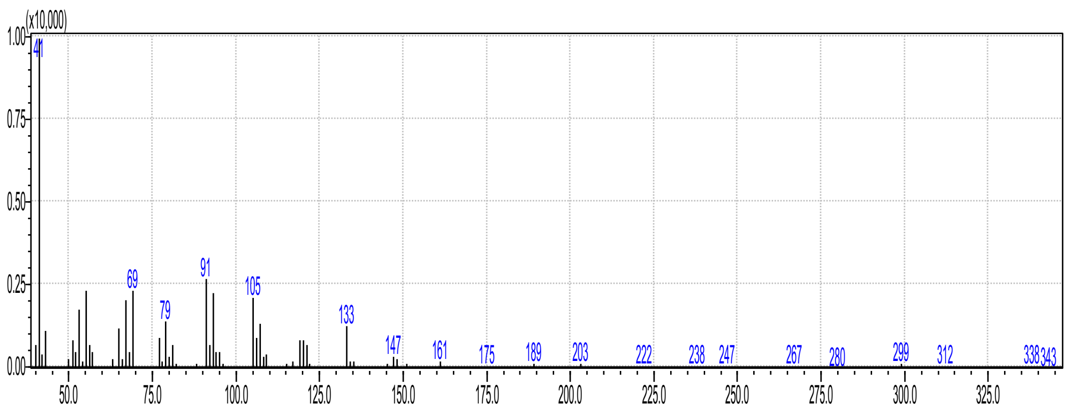
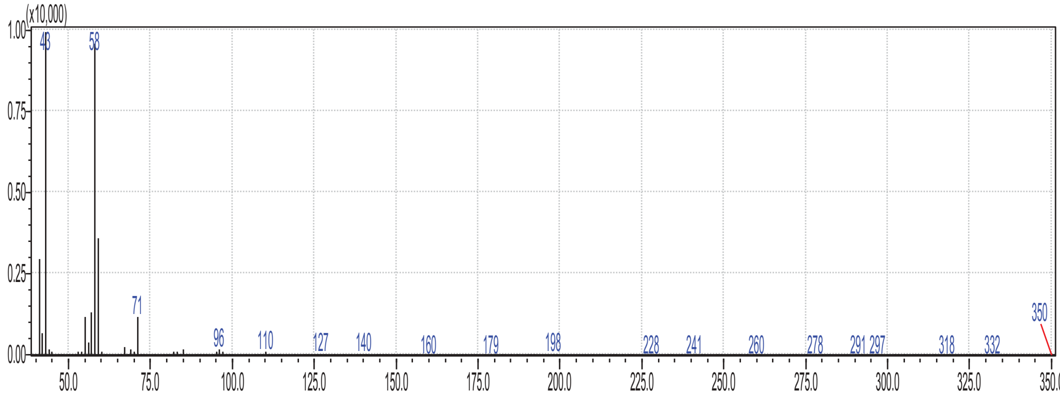


Alpha- Bergamotene



Tridecan-2-one

Hindawi
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Research Article

In Vitro Antibacterial Activities of Methanolic Extracts of Fruits, Seeds, and Bark of *Zanthoxylum armatum* DC

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Crude methanol extracts of fruits, seeds, and bark of *Zanthoxylum armatum* were investigated in vitro for antimicrobial activities against 9 different bacterial strains: *Bacillus subtilis*, *Enterococcus faecalis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella dysenteriae*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Staphylococcus epidermidis* using agar well diffusion method, and the MBC values were determined. Only 5 bacteria, i.e., *Bacillus subtilis, Enterococcus faecalis,* MRSA, *Staphylococcus aureus*, and *Staphylococcus epidermidis* exhibited antibacterial properties against the different extracts. The fruit and seed extracts showed activities against 5 bacteria, while the bark extract was active against 2 bacteria only (MRSA and *Staphylococcus aureus*). *Staphylococcus aureus* was found to be more susceptible for all the extracts compared to other strains. The maximum ZOI of 20.72 mm was produced by fruits (wild) and 18.10 mm (cultivated) against *Staphylococcus aureus.* Likewise, the least ZOI of 11.73 mm was produced by seeds (wild) and 11.29 mm (cultivated) against *Escherichia faecalis.* Similarly, the lowest MBC value of 0.78 mg/mL was obtained for fruit extracts against MRSA, 1.56 mg/mL for fruits, seeds, and bark extracts against *Bacillus subtilis*, MRSA, and *Staphylococcus aureus*, and highest value of 50 mg/mL for fruits and seeds extracts against *S. epidermidis.* The fruits, seeds, and bark extracts of *Z. armatum* exhibited remarkable antibacterial properties against different pathogenic bacteria causing several diseases, which suggests the potential use of this plant for treating different bacterial diseases such as skin infection, urinary tract infection, dental problems, diarrhea, and dysentery.

1. Introduction

Among the 8 species of *Zanthoxylum* (family Rutaceae) found in Nepal, *Zanthoxylum armatum* DC. is one of the most common spice plants with many medicinal values. Commonly known as timur, it is a subdeciduous aromatic shrub or small tree up to 6 m high. It is found in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), northeast India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, and Bhutan [1]. In Nepal, it is found from west to east in open places or in forest undergrowth at an altitude of 1000 m to 2500 m [2].

In modern medicine system, a wide range of antimicrobials are used for the treatment of several contagious diseases, which may result in undesirable side effects and serious medical problems [3, 4]. The indiscriminate use of commercial antimicrobials has also resulted in the development of multiple drug resistance. This has urged researchers to identify or extract natural antimicrobials from natural sources with less health severity [5]. Plants are the potential source of novel antimicrobial agents [6, 7]. Herbal medicinal practice, which uses plant sources to cure various infectious diseases, is also getting popularity these days. A number of flavonoids and phenolics, the potential agents for antimicrobial, antioxidant, and anticancer activities, have been identified and isolated from different plants [8, 9].

The leaves, fruits, seeds, and bark of *Z. armatum* possess various medicinal properties and have been used traditionally in several diseases as carminative, antipyretic, appetizer, stomachic, dyspepsia, and in toothache [2, 10–12]. A wide array of chemical compounds such as alkaloids, flavonoids, glycosides, terpenoids, steroids, phenols, lignins, coumarins, and benzoids [13–18] have been reported from different parts of the plant, which are responsible for several biological activities including antimicrobial, antioxidant, antipyretic, larvicidal, and anti-inflammatory properties. Young shoots are used as toothbrush and useful for curing gum diseases and toothache. The main components of the essential oil are linalool and limonene. The fruits and seeds are employed as an aromatic tonic in fever, dyspepsia, and expelling roundworms [2, 11]. The fruits are used as condiments and spices. Several indigenous medicinal practices of *Z. armatum* in various ailments indicate that the plant may have constituents of antimicrobial potential. Hence, an attempt has been made in this study to evaluate the potential antimicrobial activities of methanolic extracts of fruits, seeds, and bark of *Zanthoxylum armatum* collected from wild and cultivated populations in Nepal.

2. Materials and Methods

2.1. Collection and Processing of Samples. The fresh fruits, seeds, and bark of *Z. armatum* were collected from wild and cultivated populations from Salyan District of Nepal during May 2018. The samples were cleaned and shade dried for a week before the extraction procedure. The herbarium of the voucher specimens was prepared, which are deposited at the National Herbarium and Plant Laboratories (KATH) NPZA 20-NPZA 50.

2.2. Extraction of the Samples. The dried samples were then powdered separately in a grinder. Known weight of the powdered samples was loaded in a thimble and put inside the Soxhlet apparatus. They were then successively extracted by the hot Soxhlet extraction method. The apparatus was run for 72 hours till the colored solvent appeared in the siphon for obtaining the crude extracts of the samples. After complete extraction, the solvents were evaporated in a rotary vacuum evaporator at 65°C under reduced pressure. The obtained extracts were then dried in water bath. The dried extracts were sealed inside 20 mL sterilized culture tubes and stored in a refrigerator at 2–8°C for further analysis [19].

2.3. Antibacterial Activity. Methanolic extracts of the fruits, seeds, and bark of *Zanthoxylum armatum* from wild and cultivated populations were screened against different bacteria by agar well diffusion methods as described by Perez et al., 1990 [20].

2.3.1. Test Organisms. The extracts were screened against 9 different bacterial strains: *Bacillus subtilis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* clinical sample, *Shigella dysenteriae* clinical sample, *Staphylococcus aures* ATCC 6538P, methicillin-resistant *Staphylococcus aureus* (MRSA) clinical sample, and *Staphylococcus epidermidis* ATCC 1228. Chloramphenicol was used as the standard antibacterial agent.

2.3.2. Culture of the Bacteria. Nutrient agar (NA) media were used for culturing the bacteria. Required amount of the media was prepared, autoclaved, then cooled to 40°C and poured to sterilized Petri dishes, and allowed to solidify. Required numbers of colonies of test organisms were cultured in the respective plates and kept inside an incubator for 18–24 hours at 35°C prior to inoculation. All these experiments were carried out aseptically in a biosafety cabinet.

2.3.3. Preparation of Inoculum/Suspension. Required colonies (1.5×10^8 cfu·mL^{-1}) of freshly cultured bacteria were inoculated aseptically to glass vials containing normal saline. The suspension was homogenized by vortexing the solution and compared with the turbidity of 0.5 McFarland standard turbidity recommended by the WHO, 1991 [21], for the antimicrobial susceptibility test.

2.3.4. Screening and Evaluation of Zone of Inhibition (ZOI). Carpet culture of the prepared cell suspensions was done by uniformly spreading the suspensions with the help of cotton swabs over the dry surface of Mueller-Hinton Agar (MHA) plates. These processes were repeated thrice rotating the plate through an angle of 60°C between each streaking, and the inoculated plates were left for maximum 15 minutes to allow absorption of excess surface moisture.

Four wells, each of 6 mm diameter, were bored in the inoculated plates using a sterile cork borer. 50 μL of the 20% test solution of the extracts dissolved in methanol, positive control, and negative control was poured into the respective wells. Chloramphenicol and methanol were used as positive and negative controls, respectively. Thus inoculated plates were put inside the incubator at 35 ± 2°C. After 18–24 h, the plates were evaluated for the zone of inhibition (ZOI). The diameter of each ZOI was measured in millimeters by digital Vernier Caliper.

2.3.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Minimum inhibitory concentration (MIC) was determined by observing the visible growth of the test organisms in two-fold serial-diluted antibacterial substances in nutrient broth (NB) culture media while MBC was determined by subculturing the MIC cultures on suitable agar plates [22].

(1). Minimum Inhibitory Concentration (MIC). A set of 12 sterilized labelled vials containing 1 mL of presterilized Mueller-Hinton Broth (MHB) were prepared. 1 mL of the respective extract solution was added to 11 vials only, and the first vial was used as negative growth control. After complete homogenization, 1 mL of the solution containing nutrient broth and extract solution was transferred to the second vial containing 1 mL of nutrient broth. In the same manner, two-fold serial dilution was prepared up to the 10th

vial. Thereafter, 1 mL of content was discarded from the 10th vial and the 11th vial was used as positive control. Now, all the vials except the 1st and last contain equal volume, i.e., 1 mL, gradually decreasing concentration of the solution. To all these vials, 20 μL of bacterial suspension (turbidity equal to a 0.5 McFarland standard, supposed to have 1.5×106 CFU/mL) was put into each vial and mixed thoroughly. The vials were then incubated at 37°C for 24 h. MIC was taken as the lowest concentration that prevented the growth of bacterial culture [23].

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of organisms as detected by lack of visible turbidity. The clarity of the solution (absence of turbidity) indicates the inhibition of microbes. However, whether the turbidity was due to the growth of the bacteria or due to the turbidity of the plant extract itself was difficult to determine. Hence, MBC was determined to find out the minimum concentration of the extract that kills the microorganisms.

(2). Minimal Bactericidal Concentration (MBC). Minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent required to kill the microorganism. The MBC values were determined by subculturing all the test dilutions of the extracts on the fresh nutrient agar (NA) medium and incubating further for 24 hours at 37°C. The lowest concentration of the extract (mg/mL) that did not result in the appearance of a single bacterial colony on the solid medium was regarded as the MBC [24].

3. Results and Discussion

Methanolic extracts of fruits, seeds, and bark of *Z. armatum* were tested against various bacteria. Antibacterial properties of the extracts were compared with chloramphenicol as positive control. The diameter of zone of inhibition (ZOI) produced by the extracts on the particular microorganisms was measured in mm for the estimation of their antibacterial activities. Amongst the organisms tested for antibacterial properties, fruit and seed extracts showed antibacterial activity against *Bacillus subtilis, Escherichia faecalis*, MRSA, *Staphylococcus aureus*, and *Staphylococcus epidermidis* and the bark extract against MRSA and *Staphylococcus aureus* only, while the extracts did not produce any ZOI for the rest of the organisms.

Antibacterial properties of the fruits, seeds, and bark extracts against different bacterial strains and the ZOI are presented in Table 1 and Figures 1–3. The most sensitive strain was *Staphylococcus aureus* for all the fruits, seeds, and bark extracts as they showed maximum ZOI for *S. aureus* compared to other strains. The highest ZOI was produced by the extracts against *S. aureus*, 20.72 mm for wild fruits and 18.10 mm for cultivated fruits, 17.83 mm for wild seeds and 16.33 mm for cultivated, and the ZOI for bark was 17.01 mm for wild and 16.44 mm for cultivated. The least activity was shown by the seed extracts against *Escherichia faecalis,* with a minimum ZOI of 11.73 mm (wild) and 11.29 mm (cultivated).

The fruits extract showed highest inhibition against the bacterial strains than seed and bark extracts. The ZOI of fruits against *B. subtilis* was 16.24 mm (wild) and 17.04 (cultivated); *Escherichia faecalis* 14.28 mm (wild) and 14.62 mm (cultivated); MRSA 15.02 mm (wild) and 16.28 mm (cultivated); and *Staphylococcus epidermidis* 16.38 mm (wild) and 16.19 mm (cultivated). The seed extracts showed moderate activities against the tested organisms. The ZOI of seeds for *Bacillus subtilis* was 15.72 mm (wild) and 16.28 mm (cultivated); MRSA 17.79 mm (wild) and 16.44 mm (cultivated); and *Staphylococcus epidermidis* 15.58 mm (wild) and 13.25 mm (cultivated). The ZOI of bark extract against MRSA was 14.30 mm (wild) and 13.28 mm (cultivated), and that for *Staphylococcus aureus* was 17.02 mm (wild) and 16.44 (cultivated) (Table 1).

The antibacterial activities of different extracts showed variable results for wild and cultivated populations and were found to be independent of the habitat factors. Some of the extracts from wild populations showed good antibacterial properties, and some of the cultivated samples showed better results. All the extracts of fruits, seeds, and bark were found to be less effective than the standard antibiotic used in the present study (Figure 4).

The minimum bactericidal concentration (MBC) values ranged from 0.78 mg/mL to 50 mg/mL. The lowest MBC value of 0.78 mg/mL was exhibited by fruit extracts against MRSA and the highest, i.e., 50 mg/mL, was by seeds (wild) and fruits for *S. epidermidis.* The results are presented in Table 2. The MBC value of 1.56 mg/mL was obtained for fruits (wild and cultivated) and seeds (wild) against *B. subtilis;* seeds (wild and cultivated) against MRSA; and fruits (wild and cultivated) and bark (wild and cultivated) against *S. aureus*. Similarly, the MBC value was 3.12 mg/mL for seeds (wild and cultivated) against *S. aureus*. The MBC values of the extracts against *E. faecalis* and *S. epidermidis* were comparatively higher (Figure 5).

Several experiments have demonstrated considerable amount of antibacterial activities of *Z. armatum* leaf, fruit, seed, and bark extracts against different bacterial strains [25–30]. The ZOI produced by the ethanolic and hexane extracts of barks of *Z. armatum* showed antibacterial activities against different bacteria. The ZOI produced by the extract against *B. subtilis* and *E. coli* both were 11.67 mm and against *S. aureus* was 17.33 mm for ethanolic extract and 17 mm for hexane extract [26]. Similarly, in another study, the methanolic extracts of the fruits produced 7 mm ZOI against *S. aureus*, and the MBC value was 2.5 mg/mL and 23 mm ZOI and MBC value >10 for *B. subtilis* [30]. The methanolic extract of bark showed 28.7 mm ZOI against *S. aureus* [31].

The antimicrobial activities exhibited by the different plant extracts against a particular organism depend upon several extrinsic and intrinsic factors. The diffusion ability of agar media may cause the extract to produce less ZOI than its actual efficacy. Hence, the MBC value was determined to actually find out the minimum concentration of the extracts required inhibits the growth of the test organisms [32].

Flavonoids and other phenolics exhibit a wide range of fascinating biological activities such as antimicrobial,

Table 1: ZOI of methanolic extracts of *Zanthoxylum armatum* fruits, seeds, and bark.

S. No.	Name of the organisms	Chloramphenicol	Zone of inhibition (ZOI (mm)) Fruits		Seeds		Bark	
			Wild	Cultivated	Wild	Cultivated	Wild	Cultivated
1	*Bacillus subtilis*	25.52	16.24	17.04	15.72	16.28	0	0
2	*Escherichia faecalis*	21.61	14.28	14.62	11.73	11.29	0	0
3	MRSA	30.70	15.02	16.28	17.79	16.44	14.30	13.28
4	*Staphylococcus aureus*	25.64	20.72	18.10	17.83	16.33	17.01	16.44
5	*Staphylococcus epidermidis*	24.55	16.38	16.19	15.58	13.25	0	0
6	*Proteus vulgaris*	0	0	0	0	0	0	0
7	*Pseudomonas aeruginosa*	0	0	0	0	0	0	0
8	*Salmonella typhi*	0	0	0	0	0	0	0
9	*Shigella dysenteriae*	0	0	0	0	0	0	0

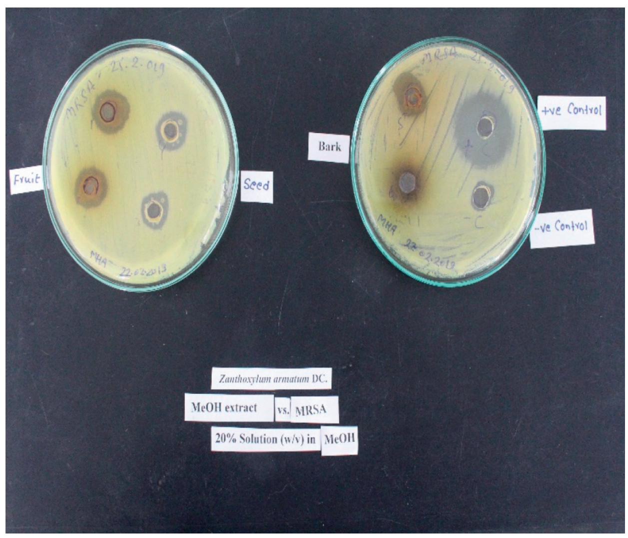

Figure 1: ZOI of different extracts against MRSA.

Table 2: MBC values of the extracts against different bacteria.

S. No.	Organisms	MBC values (mg/mL) Fruits		Seeds		Bark	
		Wild	Cultivated	Wild	Cultivated	Wild	Cultivated
1	*B. subtilis*	1.56	1.56	1.56	6.25	0	0
2	*E. faecalis*	12.5	6.25	12.5	25	0	0
3	MRSA	0.78	0.78	1.56	1.56	6.25	6.25
4	*S. aureus*	1.56	1.56	3.12	3.12	1.56	1.56
5	*S. epidermidis*	50	50	12.5	50	0	0

antiviral, antioxidant, and anticancer properties [33]. The antibacterial activities of fruit extracts of *Z. armatum* were comparatively better than seed and bark extracts. It might be due to the higher phenolic and flavonoid contents in fruits than seeds and bark [18]. The antibacterial properties of the crude extracts may be attributed to the collegial effects of several phytoconstituents present in the plant. *Z. armatum* has been reported to produce structurally diverse chemicals including terpenoids, flavonoids, coumarins, sterols, and alkaloids that show antibacterial activity. Many active components have been identified from the plant that might be developed into novel drugs. Therefore, further emphasis should be on screening, isolation, and characterization of the individual components responsible for different antibacterial activities and their underlying mechanism of action. However, additional studies are required to quantify the acute and chronic toxicity in animals before clinical trials [12, 34].

The tested extracts have potential antibacterial activities against different pathogens causing several infectious diseases in humans. These experiments partially validate the use of this plant in several traditional medicinal practices to cure various diseases. Hence, further research should be focused towards exploiting the possible uses of *Zanthoxylum armatum* for treating different bacterial diseases such as urinary tract infection, skin infection, diarrhea, dysentery, and tooth problems (Figures 1–3).

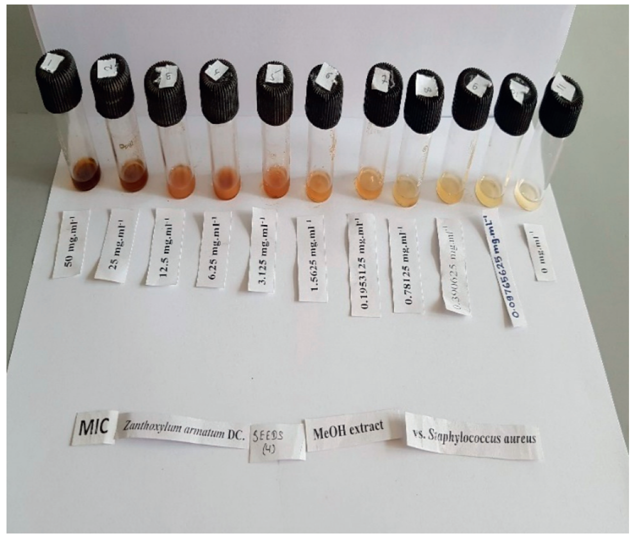

Figure 2: MIC of seeds (cultivated) against *Staphylococcus aureus*.

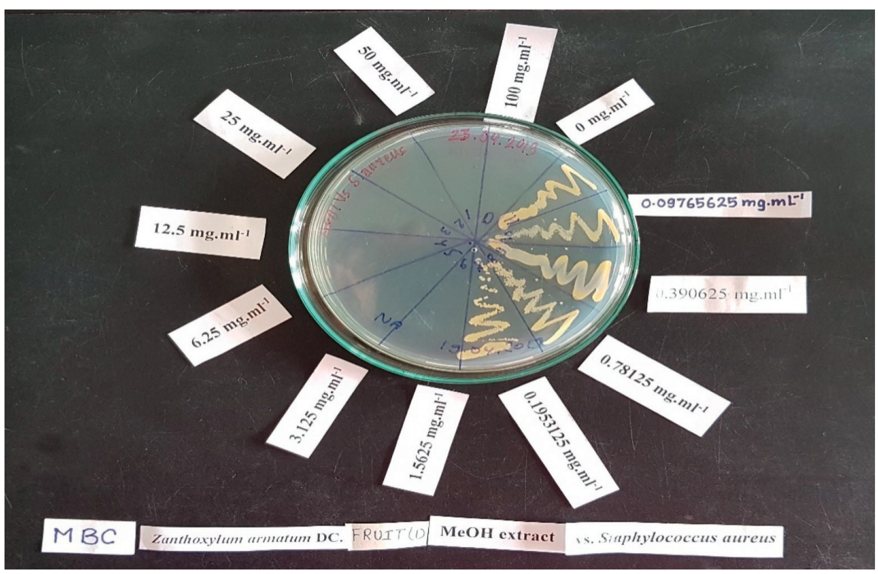

Figure 3: MBC determination of fruits (wild) against *Staphylococcus aureus*.

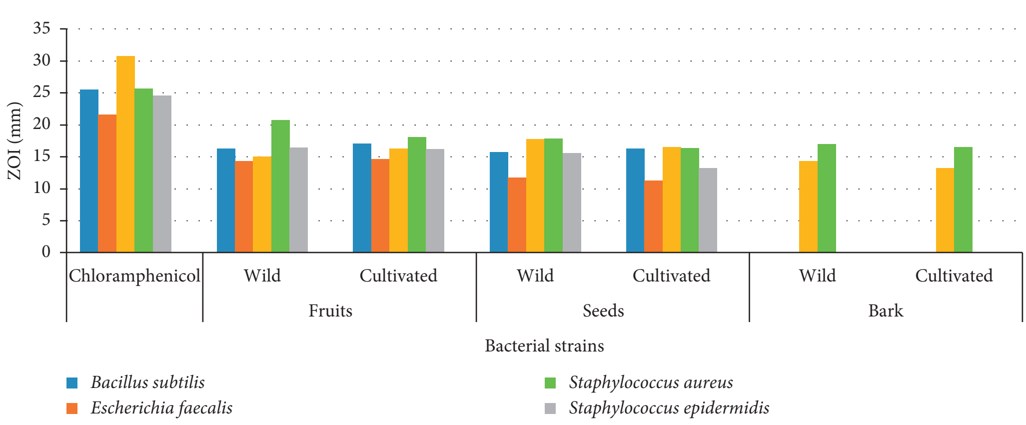

Figure 4: ZOI produced by the plant extracts against different bacteria.

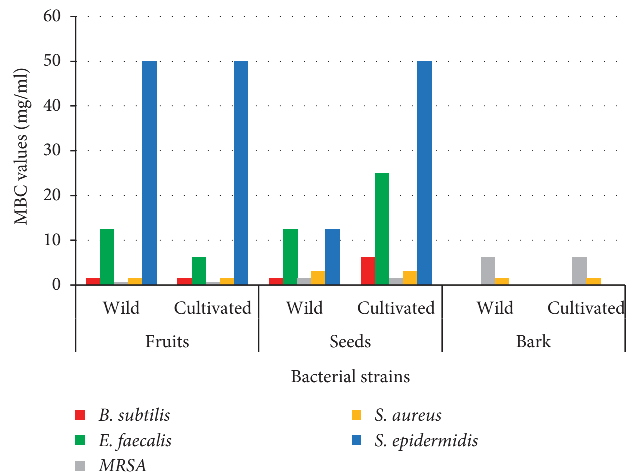

Figure 5: MBC values of the extracts against different bacteria.

4. Conclusions

The results of the study revealed that the crude extracts of fruits, seeds, and bark of *Zanthoxylum armatum* from different habitats have antibacterial properties against several infectious pathogens causing several diseases in humans. The fruits were found to be more active against the bacteria than seeds or bark, and the fruits showed highest ZOI against *Staphylococcus aureus*. Similarly, the seeds were also found to have good antibacterial properties. Bark exhibited activities against MRSA and *Staphylococcus aureus* only. The findings of this study support the traditional usage of the plants in herbal medicinal practices. Medicinal plants contain a variety of compounds that can be developed into several drugs for the safe usage and benefit to mankind. Since the indiscriminate use of modern antibiotics may cause severe health hazards, these plants can be safe alternatives to treat several diseases. The antibacterial properties as exhibited by the fruits, seeds, and bark of *Z. armatum* suggest the potential use of this plant in skin infection, urinary tract infection, dental problems, diarrhea, and dysentery. Hence, further research should be directed towards the extensive in vivo and clinical studies along with the mechanism of action of the antibacterial activities, which justifies the rational for their traditional uses and also leads to the development of novel plant-based antimicrobials for safe health care services.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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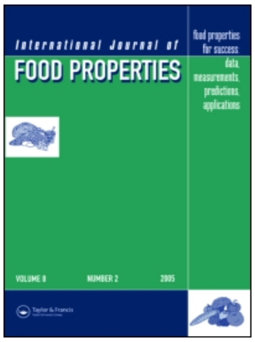

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Comparison between essential oil compositions of *Zanthoxylum armatum* DC. fruits grown at different altitudes and populations in Nepal

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Comparison between essential oil compositions of *Zanthoxylum armatum* DC. fruits grown at different altitudes and populations in Nepal

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ABSTRACT

The fruits of *Zanthoxylum armatum* DC. were collected from different altitudes (1000–2000 m) and populations (wild and cultivated) from Salyan district, Nepal. The essential oil was extracted by Clevenger apparatus and the components were analyzed through GC-MS. The yield of essential oil obtained from hydro-distillation of fruits ranged from 2.72 to 7.6%. The maximum yield was 7.6% from wild fruits at 1600–1800 m altitude and the minimum was 2.72% from cultivated fruits at 1000–1200 m altitude. This was the highest recorded essential oil yield from *Z. armatum* fruits. A total of 13 volatile compounds were identified from the essential oil by GC-MS analysis. The major components were linalool, cinnamate (E)methyl, limonene, myrcene, sabinene and terpinen-4-ol, which were present in higher proportion in all the samples. Other components were identified in a very low proportion. The main component linalool occurred in the highest proportion (74.12%) from wild populations at 1600–1800 m altitude and the lowest (44.73%) was from cultivated populations at 1000–1200 m altitude. The highest proportion of linalool was also reported for the first time in this study from *Z. armatum* fruits. Among the six major components of the essential oil, terpinen-4-ol was present in the lowest proportion. Results of the present study indicated that the altitude and habitat types could affect the essential oil composition in *Zanthoxylum armatum* fruits.

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Zanthoxylum armatum; essential oil; fruit; altitude; linalool; limonene

INTRODUCTION

Zanthoxylum armatum DC., commonly known as Timur in Nepal belongs to Rutaceae family. It is an evergreen, thorny shrub or small tree, attaining a height up to 6 m with dense foliage and pungent aromatic taste. It is distributed in Pakistan, Bangladesh, China, Bhutan, India, Indonesia, Japan, north and south Korea, Laos, Myanmar, Nepal, Pakistan, Philippines, Thailand, Vietnam Malaya peninsula and Sumatra.[1] In Nepal, it either occurs naturally or is cultivated from west to east at an elevation range of 1000–2500 m altitude .[2] The fruits, seeds and bark are used as carminative, antipyretic, appetizer, stomachic and in toothache, dyspepsia, fever, cough and cold in several indigenous medicinal practices.[3,4]

Almost all parts of the plant are aromatic and hence, supposed to possess essential oil. The essential oil of fruits is commercially known as *Zanthoxylum* oil, which is highly valued for its strong aroma, is used in perfumeries and has a high demand in international markets.[5] It is frequently used in different aromatherapy because of its soothing effect. Due to its appealing aroma and valuable perfume, it is used in the manufacture of several health-care products. The essential oil

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composition has much more importance regarding the medicinal properties and its constituents. The aromatic and medicinal properties of the plant have made it one of the most popular and important medicinal plants of Nepal. The volatile oil is employed as an anti-diarrheal, antiseptic, deodorant and anti-catarrhal.[6]

Several studies have been carried out on the essential oil composition of *Zanthoxylum armatum* fruits, seeds and leaves, which reveal that the main constituents of the oil are the monoterpenes like linalool and limonene.[7–10]. The main components of *Zanthoxylum* oil are oleic acid, palmitic acid, linoleic acid methyl ester, limonene and linalool.[11,12] GC-MS analysis of essential oils of *Z. armatum* fruits showed the presence of 20 different compounds.[13]

It has been established that the yield and the phyto-components of plants are determined by several factors like climate, edaphic, elevation, and topography, [14–16] genetics[17] and also an interaction of various factors.[18] The essential oil composition may also be affected by season, collection period, habitat types and management practices like pruning, trimming and harvesting. Altitude also affects in the amount of chemical constituents of plants, there is also a high correlation between altitude and essential oil yield and composition.[19,20]

Literature review shows that a lot of work has been carried out on the essential oil composition of *Z. armatum*[21] but no comprehensive study has been conducted till now on the inter-relationship between habitat and altitudinal factors and the essential oil components of *Z. armatum* fruits collected from different elevations and habitats in Nepal. Hence the objectives of this study were to (i) evaluate the effects of different habitats on qualities and quantities of essential oils and (ii) assess the relationship between altitudinal factor and the essential oil components of *Z. armatum* fruits in Nepal.

MATERIALS AND METHODS

Collection of plant materials

The fresh fruits of *Z. armatum* were collected from different populations (wild and cultivated) and altitudes ranging from 1000 m to 2000 m from Salyan district, Nepal. The collected fruits were shade dried for a week before the extraction process. Herbarium of the voucher specimens were prepared and deposited at National Herbarium and Plant Laboratories (KATH) NPZA 20-NPZA 50.

Essential oil extraction

A total of 10 samples have been used for this study. The collected fruits were separated from seeds and shade dried for a week at room temperature. For the extraction, 100 g of dried fruits were subjected to hydro-distillation for 6 hours using modified Clevenger-type apparatus. The protocol was followed according to British Pharmacopoeia, 1988 .[22] Volume of the essential oil was measured directly in the extractor. The essential oil thus collected was then dehydrated over anhydrous sodium sulphate and stored in sealed, labeled glass vials at 4°C until further analysis. Total yield percentage was calculated as volume (ml) essential oil per 100 g of plant dry matter[23]

Analysis of essential oil samples by GC-MS

Quantitative analysis of the chemical constituents in the essential oils was carried out using a Shimadzu gas chromatograph (GC 2010) with Rtx-5 MS column (25 m × 0.25 mm× 0.25 μm). The initial column was maintained at 40°C and the injection temperature was 250°C. Qualitative analysis of the essential oil was further continued in a Shimadzu GCMSQP 2010 Plus. The ion source temperature and the interface temperature were kept at 200°C and 250°C respectively. One μL of the essential oil diluted with spectroscopic grade hexane (10:1) was injected into the GC inlet maintaining column flow rate of 1 mL/min and purge flow 3 mL/min after fixing the split ratio at 120, using Helium as a carrier gas. Detector scanning start time was 4 min and end time was 68 min, mass spectra were scanned from m/z 40–350, with the scanning speed of 666.

The oil components were identified by the determination of their retention indices (RI), relative to C8–C32 n-alkane series under identical experimental condition, by comparison with authentic reference compounds as well as with published mass spectra[24] and by comparison of mass spectra using the NIST 11 (National Institute of Standards and Technology, Gaithersburg, MD) and FFNSC 1.3 library. The relative percentage of each constituent present in essential oil was calculated according to the area of the chromatographic peaks.

RESULTS AND DISCUSSION

Essential oil yield

The yield of essential oil from *Z. armatum* fruits from different altitudes and habitats ranged from 2.72% to 7.6% (Table 1). The essential oil yield increased as the altitude increased up to 1600–1800 m altitude and then decreased thereafter. Likewise, the yield of wild fruits was higher than cultivated fruits at all altitudinal ranges. The lowest yield was 2.72% from cultivated fruits at 1000–1200 m altitude, while the highest was 7.6% from wild fruits at 1600–1800 m altitude (Figure 1). The essential oil yield of 7.6% from fruits of *Z. armatum* recorded in this study has not been reported previously. The essential oil yield reported by previous works were comparatively lower; 2.3%[25] 1.5%,[26] 2%[3] and 5%.[27] The yield depends on several factors like different environmental conditions, genetic differences or time of collection, season, etc.[16]

Essential oil composition

GC-MS analysis of the essential oil of *Z. armatum* fruits from different populations and habitats identified a total of 13 compounds. All the compounds (excluding the trace components) are presented in Table 1.

The major components of the oil are linalool, limonene, cinnamate (E) methyl, myrcene, sabinene and terpinen-4-ol, which were present in highest percentage in all the samples (Table 2, Figure 2). Linalool was present in highest percentage in all the samples analyzed followed by myrcene. The percentage of linalool was 74.12 in wild and 70.04 in cultivated population at 1600–1800 m altitude, followed by 70.22% in wild and 62.28% in cultivated fruits at 1400–1600 m altitude. The lowest percentage of linalool (47.73) was identified in cultivated populations at 1200–1400 m altitude. Terpinen-4-ol was detected in lowest percentage in all the samples, and it was not present in 1200–1400, 1400–1600 and 1600–1800 m altitudes. Myrcene, limonene and cinnamate-E-methyl were present in higher percentages at 1800–2000 m altitude whereas sabinene and terpinen-4-ol were highest at 1200–1400 m altitude. beta-Phellandrene, alpha-terpineol and piperitone were present in only two or three samples (Figure 3). Percentage differences of the compounds may be due to genetic variation, plant organ, and different environmental factors (climate, harvesting seasons and geographical location.[28]

The concentration of linalool and myrcene were highest in wild populations than in cultivated at all elevations (myrcene was highest in cultivated populations at 1200–1400 m altitude). The variability of the compounds in the essential oils from different populations and altitudes could be attributed to the environmental factors as well as agronomic management practices including irrigation, plant density and soil tillage.[29] Genetic modifications or physiological alterations occurring during growth stage might cause changes in the composition of oil.[30]

Altitudinal variation can bring about considerable changes in the different environmental factors like solar radiation, temperature, relative humidity, wind velocity, water availability, etc., which in turn might cause significant differences in the accumulation of secondary metabolites production in plants.[31] Hence the variations in the chemical constituents at different altitudes in this study could

Table 1. Essential oil composition and yield of *Z. armatum* fruits from different altitudes & habitats.

S. no.	Name of the compound	RI[a]	RI[b]	Area %										Average (%)
				1000–1200 m		1200–1400 m		1400–1600 m		1600–1800 m		1800–2000 m		
				Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	Wild	Cultivated	
1	Sabinene	973	972	1.51	2.75	0.47	0.71	1.08	0.62	0.51	0.53	1.64	0.75	1.05
2	Myrcene	991	991	1.53	4.50	2.76	1.84	2.07	1.55	1.86	1.38	2.24	2.89	2.30
3	Limonene	1031	1030	12.82	31.82	20.49	19.11	12. 19.	6.52	12.70	5.67	19.99	25.29	16.95
4	beta-Phellandrene	1031	1031	-	-	-	-	-	4.63	-	4.44	-		4.53
5	Terpinolene	1084	1086	-	-	-	-	-	-	-	-	-	0.37	0.37
6	Linalool	1098	1101	57.33	44.73	59.69	55.65	70.22	62.28	74.12	70.14	49.09	47.19	59.37
7	Nonanal	1108	1107	-	-	-	0.30		-	-	-	-	-	0.30
8	Terpinen-4-ol	1178	1180	0.62	1.17	0.42	-	0.66	-	0.36	-	0.65	0.49	0.62
9	Cryptone	1188	1187	-	-	-	-		-	-	-	0.49	0.30	0.39
10	alpha-Terpineol	1189	1195	0.49	-	-	-		-	-	-	0.43	-	0.46
12	Carvone	1242	1246	-	-	-	-	0.57	-	-	-	-	-	0.57
11	Piperitone	1282	1277	0.68	-	-	-		-	-	-	0.47	0.35	0.5
13	Methyl cinnamate	1379	1384	24.18	14.27	16.15	22.30	12.33	12.82	9.52	14.28	25.00	21.90	17.57
Essential oil yield (%)				**3.2**	**2.72**	**5.2**	**4.13**	**7**	**5.5**	**7.6**	**6.18**	**5**	**4.37**	**5.09**
Total no. of components				**8**	**6**	**6**	**6**	**7**	**6**	**6**	**6**	**9**	**9**	**6.9**
Total % of components				**99.16**	**99.24**	**99.98**	**99.91**	**99.12**	**88.42**	**99.07**	**96.44**	**100**	**99.53**	**98.09**

Compounds are listed in the order of elution on a DB5 column.
RI[a] Retention indices taken from Adams.[24] RI[b] calculated by GC using n-alkane series under the same conditions.

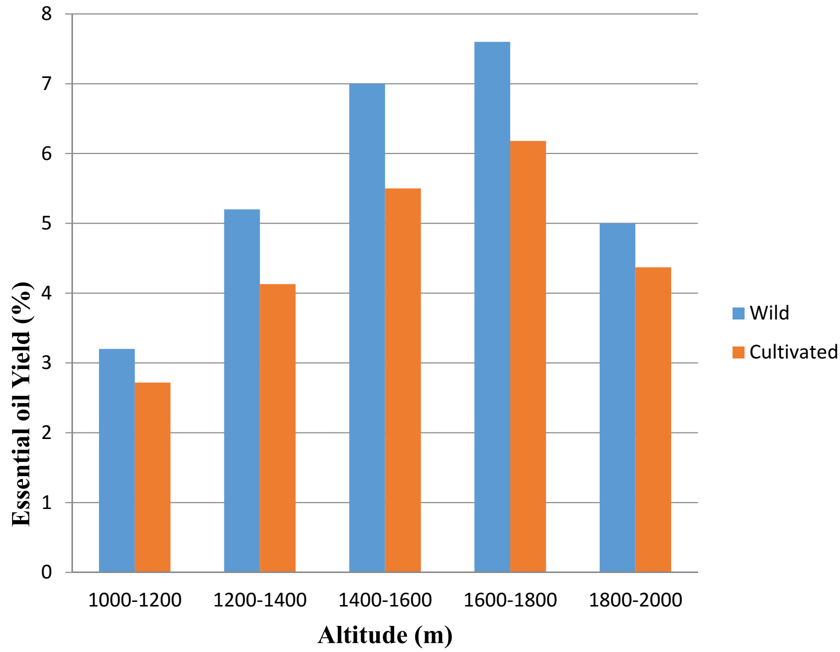

Figure 1. Essential oil yield of *Z. armatum* fruits at different altitudes and habitats.

be a result of changing ecological niches. The fruits from natural habitats were in different stages of maturation. Although the fruits were collected during the same period, there were differences in maturity between populations, individuals and even fruits on the same branches.

In accordance with the results of the present study, linalool, limonene, myrcene, sabinene, cinnamate (E)methyl have been previously reported as major components of *Zanthoxylum* volatile oil.[7,9,10,27,32,33] According to, [33] the major components of *Z. armatum* fruits oil were linalool (36.29–45.61%), beta-phellandrene (19.93–38.38%), myrcene (4.13–8.73%), methyl cinnamate (3.12–-22.14%) and sabinene (1.57–4.78%).

The oils from *Z. armatum* are potential sources of linalool.[34] In this study, the maximum linalool content is 74.12%. Earlier reported maximum linalool content of essential oil from fruits of *Z. armatum* was 64.1%.[25] As per, [7] the quantity of linalool in the essential oil of *Z. armatum* from Nepal is 62.2%. Linalool is a slightly volatile compound with pleasant aroma because of which it has been used in several industrial and pharmaceutical companies as flavouring agents, perfumes, and cosmetics, and linalool is generally considered as safe for these purposes.[35] The fruits of *Z. armatum* from the particular locality can be used as a commercial source for the isolation of linalool for the potential use in relevant industries.

CONCLUSION

The results of this study suggest that the variation in the essential oil yield and the chemical composition of *Zanthoxylum armatum* fruits could be attributed to the altitude and environmental factors. The highest recorded yield was 7.6% from the fruits collected from wild habitat at 1600–1800 m altitude and this is the highest recorded essential oil yield of *Z. armatum* fruits till now. Among the 13 compounds identified from the fruits essential oil, linalool, cinnamate (E) methyl, limonene, myrcene, sabinene and terpinen-4-ol were the major components. The main component was linalool sharing higher proportion in all the samples. This study also recorded the highest percentage of linalool, i.e., 74.12%, which was not reported previously. There were distinct variations

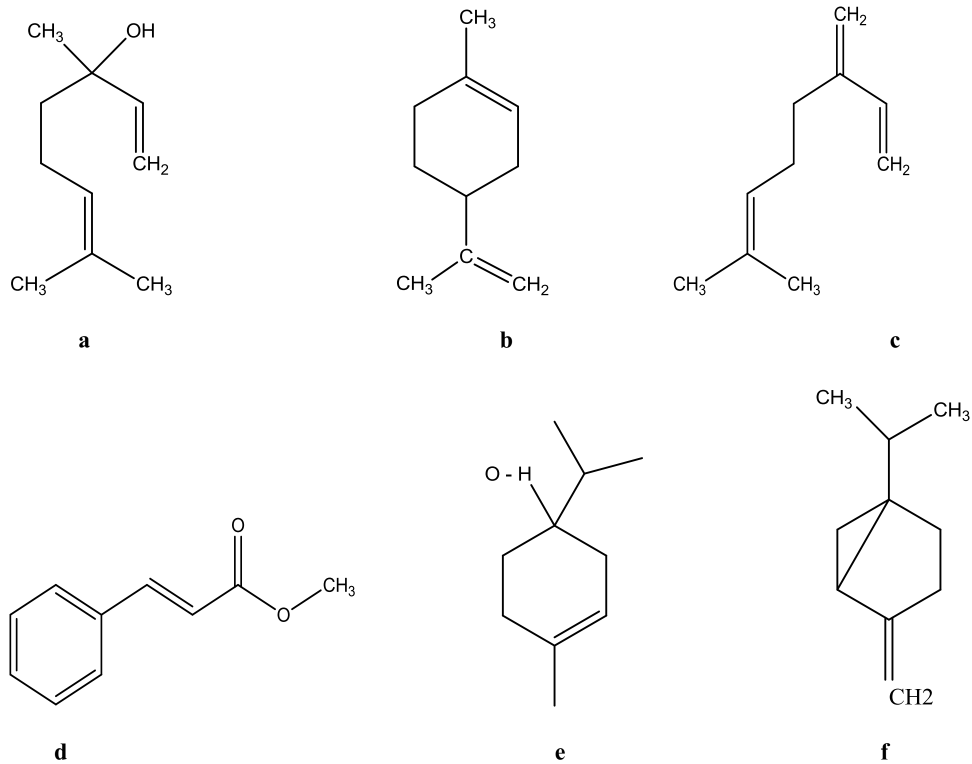

Figure 2. Structures of the major components of fruit essential oil of *Zanthoxylum armatum*. (A) Linalool, (B) Limonene, (C) Myrcene, (D) Methyl-E Cinnamate, (E) Terpinen4-ol and (F) Sabinene

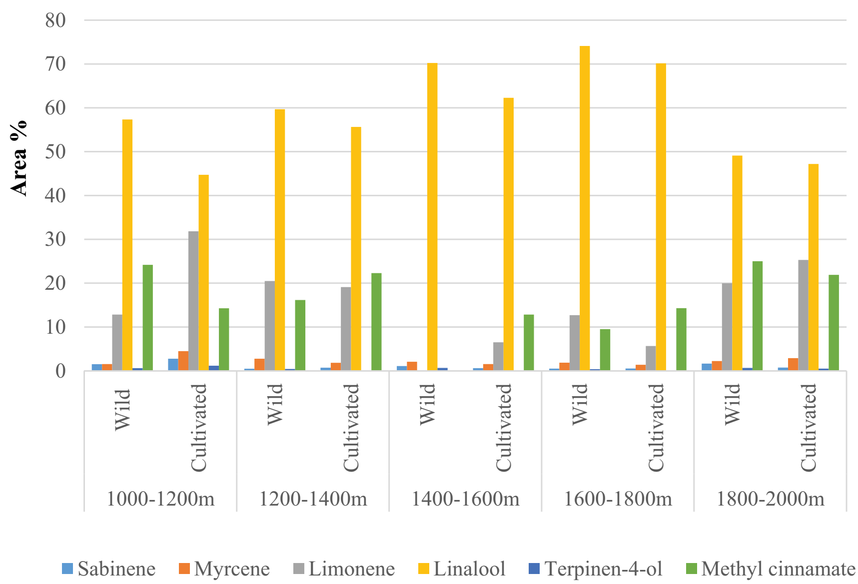

Figure 3. Main components in essential oil of *Z. armatum* fruits at different altitudes and habitats.

in the composition of fruits essential oil from different altitudes and populations (wild and cultivated). The yields along with the percentage of components were also comparatively higher in wild populations than cultivated ones. Hence it can be said that the various factors including environmental, altitude and genetic might also influence the phytochemical constituents of the plants. The characterization of volatile oil and the isolation of the major component linalool from the particular locality could be of commercial importance for the potential use in several industries.

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RESEARCH

Population structure and regeneration of *Zanthoxylum armatum* DC. in Salyan, Nepal

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Background: *Zanthoxylum armatum* is one of the 30 prioritized medicinal plants for economic development of Nepal with a high trade value. Understanding the ecology of individual species is important for conservation and cultivation purposes. However, relation of ecological factors on the distribution and populations of *Z. armatum* in Nepal remain unknown. To address this knowledge gap, an attempt has been made to study the population structure, distribution, and regeneration potentiality of *Z. armatum*. Vegetation sampling was conducted at six different localities of Salyan district along the elevation range of 1,000 m to 2,000 m.

Results: Altogether 50 plant species belonging to 44 genera under 34 families were found to be associated with *Z. armatum*. Significantly higher species richness was found at Rim (1,400–1,700 m) and Chhatreshwori (1,800–2,000 m) and lower at Kupinde (1,600–1,800 m). The highest population density of *Z. armatum* was at Kupinde (1,600–1,800 m) with a total of 1,100 individuals/ha. and the lowest at Chhatreshwori (1,800–2,000 m) with 740 individuals/ha. Based on the A/F value (Whitford index), it can be said that *Z. armatum* has random distribution in the study area. The plants were categorized into seedlings, saplings and adults based on plant height and the status of natural regeneration category determined. The regeneration potentiality of *Z. armatum* in the study area was fair with the average seedlings and saplings densities of 150 and 100 individuals/ha. Respectively. A Shannon–Weinner index mean value of 2.8 was obtained suggesting high species diversity in the study area.

Conclusions: The natural distribution and regeneration of *Z. armatum* is being affected in the recent years due to anthropogenic disturbances. Increasing market demand and unsustainable harvesting procedures are posing serious threat to *Z. armatum*. Thus, effective conservation and management initiatives are most important for conserving the natural population of *Z. armatum* in the study area.

Keywords: density, distribution, ecology, population, species diversity, regeneration

Introduction

Zanthoxylum armatum DC. (Rutaceae), commonly called Timur in Nepali (English: Nepal pepper or prickly ash), is an aromatic large shrub up to 6 m high. It is one of the 30 prioritized medicinal plants for economic development (DPR 2006). It is found in hot valleys of subtropical to temperate Himalayas (Kashmir to Bhutan), north-east India and Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, North & South Korea, North Vietnam, Taiwan, Lesser Sunda Islands, Philippines, Malaya peninsula and Sumatra (Nair and Nayar 1997). In Nepal, it is distributed from west to east at an elevation range of 1,000 m to 2,500 m in open places or in forest undergrowth (DPR 2007). The plant grows well in open pastures, wastelands and secondary scrub forests with adequate rainfall. Moist areas with deep soils exposed to sun and degraded slopes, shrub lands, natural forests and wastelands are the suitable habitat for *Z. armatum* (Phuyal et al. 2019).

Z. armatum (Fig. 1) has been used extensively in traditional indigenous medicinal practices in Nepal by different ethnic communities. Several ethnobotanical studies have documented the various ethno-medicinal uses in different types of ailments. The different parts of the plants: leaves, fruits, stem, bark, seeds have been used as carminative, antipyretic, appetizer, stomachic, toothache, dyspepsia (Kala

Fig. 1 *Zanthoxylum armatum.*

2005; Manandhar, 2002; Singh et al. 2016). This plant species is not only used for pharmaceutical purposes, but also used in the flavoring and fragrance industries (Phuyal et al. 2020). Based on its varied industrial uses, its demand is constantly increasing both in domestic and international markets. Increased market demands, devious modes of collection and insufficient technical knowledge and proper skills of harvest and postharvest techniques have posed serious threats to the native populations attributing to a sharp decline of the species in the wild, due to which the regeneration of this species is adversely affected (Phuyal et al. 2019).

The National Conservation Strategy (1988), Master Plan for the Forestry Sector (1988), Industrial Enterprises Act (GoN 1992), Forest Act (GoN 1993) and Regulations (GoN 1995), Herbs and Non-Timber Forest Products Development Policy (DPR 2004) have emphasized on the subsequent development and commercialization of the Non-timber Forest Products including Medicinal and Aromatic Plants (MAPs) for uplifting the livelihood of the rural communities through sustainable and wise use of these valuable resources. Trade Policy in Nepal (GoN 2009) has also prioritized *Z. armatum* as an important commodity for export.

There have been ample studies on the medicinal plants of Nepal regarding their botany, ecology, ethnobotany, population size, and distribution. A significant amount of research has also been carried out in Zanthoxylum in the Indian subcontinent. However, there have been very few studies in *Z. armatum* in Nepalese context and the study of relation of ecological factors on the distribution and population structure in Nepal is still meager (Phuyal et al. 2019). Understanding the ecology of individual species is important for conservation and for cultivation purposes. Unsustainable harvesting from the wild without proper management practices is the major threat to most of the MAPs including *Z. armatum*. So, understanding of ecology and biology of these valuable plants is very crucial for agro-technology development and commercial cultivation, which ensures the steady supply of raw materials without hampering their natural population. No study has been conducted previously regarding ecological status including the population, distribution, frequency, abundance, and regeneration status of *Z. armatum* from Nepal. The present study, therefore, aims to find the ecological status of *Z. armatum* in Salyan district regarding its distribution, density, frequency, abundance, diversity, and regeneration potentiality.

Materials and Methods

Field sampling

A preliminary filed survey was carried out in April 2017 to select the study site and sampling areas, gather general information about the study species viz. *Z. armatum*, and rapport building with the local people and concerned authorities. The principal visit was conducted during the months of May 2017 and October 2018. All necessary data and samples were collected during that period. The details of study sites (DFRS, 2018) and map of the study area are presented in Figure 2 and Table 1.

The study was mainly based on primary data collection. Necessary information was collected through extensive field observation of the area. The data was collected through physical measurement in the field and review of relevant literature on similar previous studies. Systematic random sampling design was applied in which plots were selected by a random or stratified random plan (Misra 1968). The sampling sites were selected from six localities to cover all the possible habitats and associated vegetation types of *Z. armatum* so that a comparative study can be done based on disturbance factors, altitudinal difference, etc.

Vegetation sampling was done along the elevation of 1,000 m to 2,000 m. In each locality, four transect lines were set up at 30–50 m in *Z. armatum* available sites. In each transect line five plots of 5 m × 5 m were laid down at a distance of 10 m. The number of individuals of *Z. armatum* and other tree and shrub species (excluding grasses) in the sample plot associated with *Z. armatum* were recorded.

Vegetation attributes, including frequency, density, and richness, were recorded, along with environmental coordinates such as latitude, longitude, and elevation of each sample plot using a global positioning system (Garmin model 2000) (Shaheen et al. 2011a).

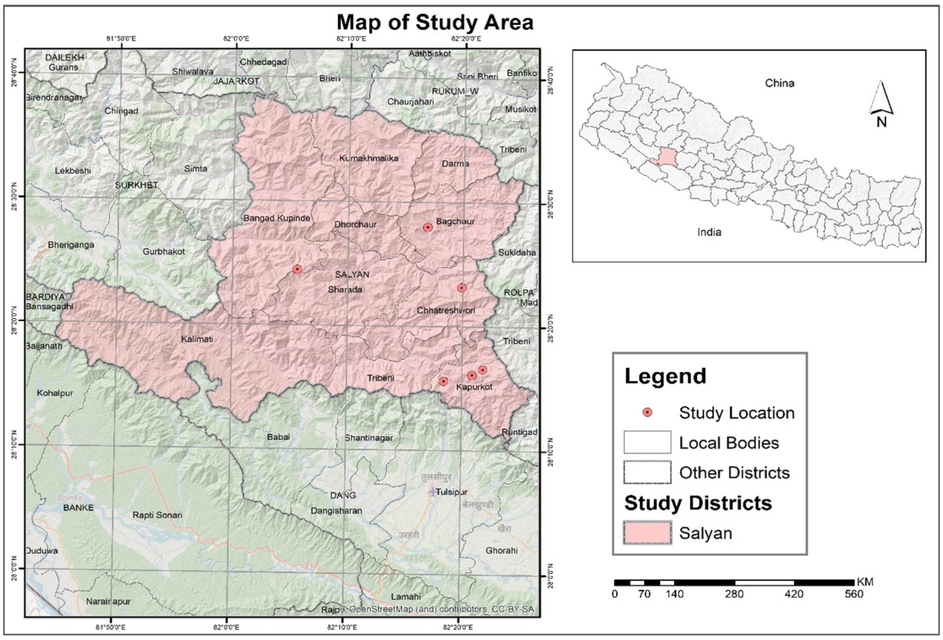

Fig. 2 Map of Nepal showing study area and sampling sites of *Zanthoxylum armatum*.

Table 1 Details of sampling sites of *Zanthoxylum armatum* in the study area

SN	Municipality	Total area (ha)	Forest area (ha)	Forest cover (%)	Study site	Altitude (m)	Latitude (N), Longitude (E)	Land use/Forest type
1	Kapurkot	11,875	7,542	63.5	Dhanwang	1,000–1,200	28.26875, 82.30842	Forest near village settlement
2					Kapurkot	1,200–1,400	28.2707, 82.35038	Near roadside on edges of farmyard
3					Rim	1,400–1,700	28.27611, 2.36361	Mixed *Quercus* forest
4	Baghchaur	16,251	8,453	52	Baghchaur	1,400–1,600	28.46694, 2.28139	Mixed forest
5	Bangad Kupinde	33,678	22,709	67.4	Kupinde	1,600–1,800	28.41319, 82.0935	Disturbed forest due to road construction
6	Chhatreshwori	15,011	9,841	65.6	Chhatreshwori	1,800–2,000	28.38611, 2.36361	Moist and dense forest

Source: 1. Forest cover and land cover: DFRS (2018), 2. Field survey.

Vegetation analysis

Species richness

The species richness in this study was obtained by counting the number of species present in each 5 m × 5 m sample plot. In this study, species richness has been defined as, the number of species per plot and expressed as species/m^2.

Density and abundance

Both this term refers to the number of species in a community. Abundance of any individual species is expressed as a percentage of the total number of species present in community and therefore it is a relative measure (Khan et al. 2014). In sampling the abundance of species, the individual of species is counted instead of just nothing their presence or absence was done while studying the frequency of a species.

Density and relative density were calculated using Yadav et al. (1987), whereas abundance was determined based on the formula of Kilewa and Rashid (2014).

Density (D) (plants/ha)

$$= \frac{\text{Total no.individuals of a species in all quadrats}}{\text{Total no.of plots studied} \times \text{size of the plot (m2)}} \times 10{,}000$$

Relative Density (RD%)

$$= \frac{\text{Density of individual species}}{\text{Total density of all species}} \times 100$$

Abundance (A)

$$= \frac{\text{Total no.of individuals of a species in all quadrats}}{\text{Toal no.of quadrats in which the species occured}}$$

Relative Abundance (RA%)

$$= \frac{\text{Total no.of a particular species}}{\text{Total no.of individuals of all species recorded}} \times 100$$

Frequency

Occurrence of trees and shrub species within each major plots of the study area were recorded to assess their distribution pattern in *Z. armatum* occurring areas. Then, frequencies of these species were obtained by following formula (Yadav et al. 1987). Relative frequency is the frequency of a species in relation to other species.

$$\text{Frequency (F) (\%)} = \frac{\text{No.of quadrats in which an individual species occured}}{\text{Total no.of quadrats studied}} \times 100$$

$$\text{Relative Frequency (RF\%)} = \frac{\text{Frequency of individual species}}{\text{Sum of frequencies of all species}} \times 100$$

Distribution pattern (A/F ratio)

Abundance and frequency taken together are of great importance in determining the structure of a community. High frequency and low abundance indicate regular distribution whereas the converse indicates contiguous distribution. The ratio of abundance to frequency (A/F) for different species was determined for eliciting the distribution pattern. Spatial distribution of plant species was determined following Whitford index WI (Singh and Singh, 1987) as

$$\text{Distribution (WI)} = \frac{\text{Abundance}}{\text{Frequency}} \text{ (A/F Ratio).}$$

If value is < 0.025 = regular distribution, value lies between 0.025–0.05 = random distribution and value > 0.05 = clumped distribution (Whitford 1949).

Importance Value Index (IVI)

The IVI was calculated to understand the species' share in the community (Curtis and Cottam 1956). Species with the highest importance value are the leading dominant species of the specified vegetation (Shibru and Balcha 2004). This considers density, frequency, and abundance of the species present in the community. For each species, the relative density, relative frequency, and relative abundance were calculated and summed. It gives the overall importance of each species in the community structure. The IVI for all the species was calculated by adding the sum of relative values of density, frequency and abundance. It was calculated following Bhadra and Pattanayak (2016) as

Importance Value Index (IVI) = Relative Density + Relative Frequency + Relative Abundance.

Regeneration

Population structure of naturally emerged seedlings of *Z. armatum* reported in each sample plot was studied. Density of all the individuals of seedlings, saplings and adult were determined. The size classes of individuals of *Z. armatum* were broadly defined according to plant height. Plant height less than 0.1 m were classified as seedlings. Plant height ranging from 0.1 m to 1.0 m were classified as saplings and plant height usually more than 1 m and also bearing reproductive structures were classified as adult (Schemske et al.1994).

Regeneration status was totally based on population size of seedlings and saplings (Khan et al. 1987; Saha et al. 2016). Good regeneration if seedlings > saplings > adults; fair regeneration, if seedlings > or ≤ saplings ≤ adults; poor regeneration, if the species survives only in sapling stage, but no seedlings (saplings may be or = adults). If a species is present only in an adult form it is considered as not regenerating. The status of natural regeneration was determined based on the values as shown in Table 2 (Bhuyan et al. 2003; Khumbongmayum et al. 2006).

As shown in the above table, 'good regeneration' is defined as the condition in which an ample or adequate number of seedlings and saplings contribute to the mature population, while 'fair regeneration' is defined as the condition in which there were a fair number of seedlings, but the percentage of saplings was either lower than or close to that of the mature trees. 'Poor regeneration' is the condition in which individuals were found at either the seedling or sapling stage only, in greater numbers than the mature trees. The fourth regeneration status is termed as 'no regeneration,' in which a species presented only at the mature stage and did not occur in either seedling or sapling stages.

Species diversity

Diversity in species refers to the combined effect of richness and evenness in species. While richness pertains to the number of species in each sampling unit, evenness implies to the distribution of individuals among the species. Species richness is a biologically appropriate measure of diversity and the total number of species in any ecological community, landscape, or region relative to the total number of all individuals in that community.

A diversity index can reveal the structure of biological community in terms of numerical value. It gives more information on community composition than simply species richness. Further, it offers insights into rarity and com-

Table 2 Different regeneration status

SN	Regeneration status	Seedling (Se)	Sapling (Sa)	Compare to adult
1	Good regeneration	Present	Present	Se > Sa > adults
2	Fair regeneration	Present	Present	Se > or < Sa; Sa ≤ adult
3	Poor regeneration	Absent	Present	Sa > or < or = adult
4	No regeneration	Absent	Absent	Only adult

monness of species in a community, thereby diversity index acts as important tool for biologists in the understanding of community structure (Muthulingam and Thangavel 2012). Several indices are used to quantify the species diversity of which Simpson's index (Simpson 1949) and Shannon-Wiener's index are the most commonly used. Shannon's diversity index (H) and Simpson's Index (1 – D) in terms of density for each plot were calculated using the following indices:

Shannon's diversity index (H) = $-\sum_{i=1}^{n}(pi \times ln\, pi)$ (Shannon and Weaver 1963)

Simpson's diversity Index (1 – D) = $1-\left(\frac{\sum n(n-1)}{N(N-1)}\right)$ (Simpson 1949)

Where, pi = (n/N),
n = density of individual species in a plot
N = Total density of all species in a plot
ln = Natural logarithm values

The Shannon diversity index ranges typically from 1.5 to 3.5 and rarely reaches 4.5 (Gaines et al. 1999). The Simpson's Index values range from 0 to 1. The closer the value of Simpson's Index to 0, the more diverse the plot will be. A plot with only one species would have a Simpson's Index value of 1. Trends are opposite to those found for Shannon Weaver values since Simpson's Index values decrease with increased diversity (Reich et al. 2001). In practice, the values below 0.5 indicates a relatively even community, while high values are indicative of communities dominated by one or a few species.

Soil analysis

Soil samples were collected from the four corners and center of each sample plot from the depth of 5–10 cm. The subsamples were mixed thoroughly, and about 100 g soil were collected, air dried in shade (Yadav et al. 1987), kept in zipper plastic bags, properly labeled, and brought to the Laboratory for the analysis of pH, soil organic carbon, total nitrogen, available phosphorus, and soil potassium. Soil organic carbon was determined by following Walkley and Black (1934), total nitrogen (N) by Kjeldahl method (Tsuji and Ohnishi 2001), available phosphorous (P) by a modified Olsen's method following Gupta (2000), soil potassium (Flame photometer method following Trivedy and Goel 1986 and pH using a digital pH meter with 1:5 soil water ratio (Gupta 2000).

Statistical analysis

All the analyses were carried out in R platform (R Core Team 2020). The normality (Shapiro–Wilk test) for all the parameters were tested prior to choosing a parametric or non-parametric tool to analyze. All the parameters were tested by Analysis of Variance (ANOVA), Tukey's test for normal data, and non-parametric Kruskal–Wallis one-way ANOVA, Duncan multiple comparison test for non-normal data.

Results

Species richness

Altogether fifty plant species (trees and shrubs) belonging to 44 genera under 34 families were found to be associated with *Z. armatum* in the study area. Rosaceae was the dominant family with seven species, followed by Fagaceae with five species and the families Berberideae, Fabaceae, Moraceae, Oleaceae, and Rutaceae had two species each. *Quercus* was the largest genera with 4 species while the genera *Castanopsis* and Prunus had 2 species each (Table 3).

The species richness for each plot was the number of species per plot. Significantly higher species richness ($p < 0.001$) was recorded at Rim (1,400–1,700 m) and Chhatreshwori (1,800–2,000 m) while the species richness was significantly lower at Kupinde (1,600–1,800 m) (Fig. 3, Tables S1–S6).

Density

The mean population density of *Z. armatum* in the study area was found to be 913.33 individuals/ha. The density among the different localities did not vary significantly (Fig. 4a). Among the six localities studied, the total density of *Z. armatum* was maximum at Kupinde (1,100 individuals/ha), followed by Baghchaur (1,020 individuals/ha), Rim (1,000 individuals/ha), Dhanwang (840 individuals/ha), Kapurkot (780 individuals/ha) and the lowest at Chhatreshwori (740 individuals/ha). The highest relative density (15.45%) of *Z. armatum* was at Baghchaur and lowest (5.35%) at Chhatreshwori (Fig. 4b). It was found to be associated with different species at different localities and altitudes. Mostly it was found growing in the northern and northeastern slopes and had least occurrence on the south and southeastern slopes.

Among all the species, *Murraya koenigii* had the greatest density with 1,140 individuals/ha at Dhanwang, *Berberis aristata* at Rim with 980 individuals/ha and *Daphne bholua* at Chhatreshwori with with 1,100 individuals/ha. Similarly *Z. armatum* had greatest density at Kapurkot, Baghchaur, and Kupinde with total of 1,000, 1,100, and 1,020 individuals/ha respectively (Tables S1–S6). The species having lowest densities were *Ligustrum confusum*, *Tinospora sinensis* (200 individuals/ha at Dhanwang), *Ficus semicordata* (260 individuals/ha at Dhanwang), *Clematis* sp. (200 individuals/ha at Rim).

Frequency

The mean frequency and relative frequency of *Z. arma-*

Table 3 List of plant species (trees and shrubs) recorded in the study area

SN	Name of the species	Family	Local name	Habit
1	*Albizzia procera* (Roxb.) Benth.	Fabaceae	Sirish	Tree
2	*Adhatoda vesica* Nees	Acanthaceae	Asuro	Shrub
3	*Aesculus indica* (Wall. ex Cambess.) Hook.	Sapindaceae.	Lekh pangre	Tree
4	*Alnus nepalensis* D. Don	Betulaceae	Uttis	Tree
5	*Bauhinia variegate* L.	Fabaceae	Badahar	Tree
6	*Benincasa hispida* (Thunb.) Cogn.	Cucurbitaceae	Kubindo	Climber
7	*Berberis aristata* DC.	Berberidaceae	Chutro	Shrub
8	*Castanopsis hystrix* Hook. f. & Thomson ex A. DC.	Berberidaceae	Patle katush	Tree
9	*Castanopsis indica* (Roxb. ex Lindl.) A.DC.	Fagaceae	Daale katush	Tree
10	*Clematis* sp.	Ranunculaceae.	Sikari lahara	Climber
11	*Colebrookea oppositifolia* Smith	Lamiaceae	Dhusure	Shrub
12	*Cotoneaster microphyllus* Wall. ex Lindl.	Rosaceae	Khareto	Shrub
13	*Daphne bholua* Buch.-Ham. ex D.Don	Thymelaeaceae.	Lokata	Shrub
14	*Ficus neriifolia* Sm.	Moraceae	Dudhilo	Tree
15	*Ficus semicordata* Buch.-Ham. ex Sm.	Moraceae	khaniu	Tree
16	*Fraxinus floribunda* Wall.	Oleaceae	Laakuri	Tree
17	*Grewia optiva* J.R. Drumm. ex Burret	Malvaceae	Bhimal	Tree
18	*Juglans regia* L.	Juglandaceae	Okhar	Tree
19	*Pistacia integerrima* Stew. ex Brand	Anacardiaceae	Kakadsinghi	Shrub
20	*Ligustrum confusum* Decne.	Oleaceae	Kanike	Shrub
21	*Lyonia ovalifolia* (Wall.) Drude	Ericaceae	Angeri	Shrub
22	*Maesa chisia* Buch.-Ham.ex D. Don	Primulaceae	Bilaune	Shrub
23	*Mallotus philippinensis* Muell. Arg	Euphorbiaceae	Sindure	Tree
24	*Murraya* koenigii (L.) Spreng.	Rutaceae	Karipatta	Shrub
25	*Myrica esculenta* Buch.-Ham. ex D. Don	Myricaceae	Kaphal	Tree
26	*Persea odoratissima* (Nees) Kosterm.	Lauraceae	Kaulo	Tree
27	*Pinus roxhburghii* Sarg.	Pinaceae	Khote salla	Tree
28	*Prinsepia* utilis	Rosaceae	Dhatelo	Shrub
29	*Prunus cerasoides* D.Don	Rosaceae	Paiyun	Tree
30	*Prunus persica* (L.) Batsch	Rosaceae	Aaru	Tree
31	*Pyracantha crenulata* (D. Don) M. Roeme	Rosaceae	Ghangaroo	Shrub
32	*Pyrus pashia* Buch.-Ham. ex D.Don	Rosaceae	Mayal	Tree
33	*Quercus leucotricophora* A.Camus	Fagaceae	Sano banjh	Tree
34	*Quercus glauca* Thunb.	Fagaceae	Phalat	Tree
35	*Quercus incana* Roxb. Hort. Beng.	Fagaceae	Thulo banjh	Tree
36	*Quercus semecarpifolia* Smith in Rees	Fagaceae	Khasru	Tree
37	*Reinwardtia indica* Dumort.	Linaceae	Pyauli	Shrub
38	*Rhododendron arboretum* Sm.	Ericaceae	Lali gurans	Tree
39	*Rhus javanica* L.	Anacardiaceae	Bhaki amilo	Tree
40	*Rubus ellipticus* Sm.	Rosaceae	Ainselu	Shrub
41	*Salix* sp.	Salicaceae	Bains	Tree
42	*Sapindus mukorossi* Gaertn.	Sapindaceae	Rithha	Tree
43	*Sapium insigne* (Royle) Trimen	Euphorbiaceae	Khirro	Tree
44	*Schima wallichii* Choisy	Theaceae	Chilaune	Tree
45	*Smilax* sp.	Smilacaceae	Kukurdaino	Climber
46	*Stephania* sp.	Menispermaceae	Batulpate	Climber
47	*Tinospora sinensis* (Lour.) Merr.	Menispermaceae	Gurjo	Climber
48	*Toona ciliate* M.Roem.	Meliaceae	Tuni	Tree
49	*Viburnum erubescens* Wall.	Adoxaceae	Asare	Tree
50	*Zanthoxylum armatum* DC.	Rutaceae	Timur	Shrub/Small tree

tum in the study area were 70.83% and 5.61%, respectively. The frequency at different locality and elevation did not vary significantly (Fig. 5a) with the highest (80%) at Baghchaur (1,400–1,500 m) and lowest (60%) at Chhatreshwori (1,800–2,000 m). The highest total relative frequency 8.02% was at Kupinde (1,600–1,800 m) and the lowest 3.82% at Chhatreshwori (1,800–2,000 m) as compared to its associates (Fig. 5b). The frequency and relative frequency of other associates are presented in Tables S1–S6.

The most frequently occurring associates (with > 70% of occurrence) at different localities were *Aesculus indica*, *Alnus nepalensis*, *Bauhinia variegata*, *Berberis asiatica*, *Castanopsis hystrix, Colebrookeaa oppositifolia, Daphne bholua, Ficus nerifolia, Fraxinus floribunda, Grewia optiva, Juglans regia, Lyonia ovalifolia, Maesa chisia, Murraya koenigii, Persea odoratissima, Pinus roxhburghii, Prinsepia utilis, Prunus cerasoides, Pyracantha crenulata, Pyrus pashia, Quercus glauca, Q. incana, Rhododendron arboreum*, and *Sapium insigne* (Tables S1–S6).

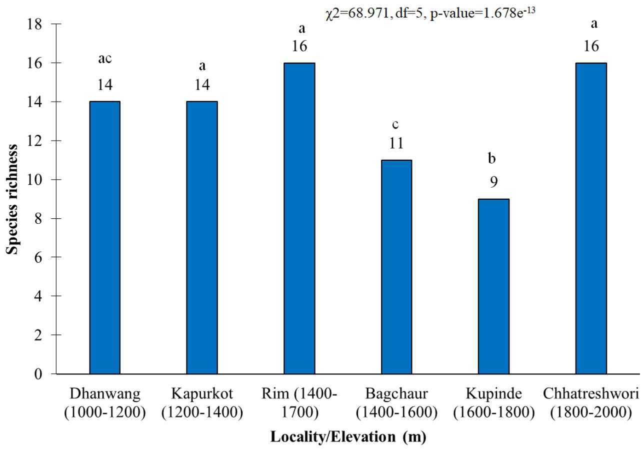

Fig. 3 Species richness at different locality and elevation (m). Different letters above bars indicate statistically significant difference between different altitudes at $p < 0.001$.

Abundance

The abundance of *Z. armatum* was almost similar and did not vary significantly in all the localities of the study area, the values ranging from 3.00 at Dhanwang and Kapurkot to 3.40 at Baghchaur and 3.44 at Kupinde (Fig. 6a). Likewise, the highest total relative abundance (15.45%) was at Baghchaur and the lowest (5.35%) at Chhatreshwori (Fig. 6b). Abundance of *Z. armatum* was highest (3.44) at Kupinde and relative abundance was highest (15.45%) at Baghchaur.

Importance Value Index (IVI)

Based on IVI values, *Z. armatum* was most dominant at Baghchaur and Kupinde and least dominant at Chhatreshwori. The highest IVI value of *Z. armatum* was 38.35% at Baghchaur and the lowest value was 14.53% at Chhatreshwori (Fig. 7). Based on IVI values, *Berberis asiatica* (22.88) and *Daphne bholua* (20.06) were the dominant species at Kapurkot and *Daphne bholua* at Chhatreshwori (Tables S1–S6). Likewise, *Zanthoxylum armatum* was dominant at Baghchaur, Kupinde, Dhanwang, and Rim with the IVI values of 38.35, 29.42, 23.09, and 21.81 respectively (Fig. 7).

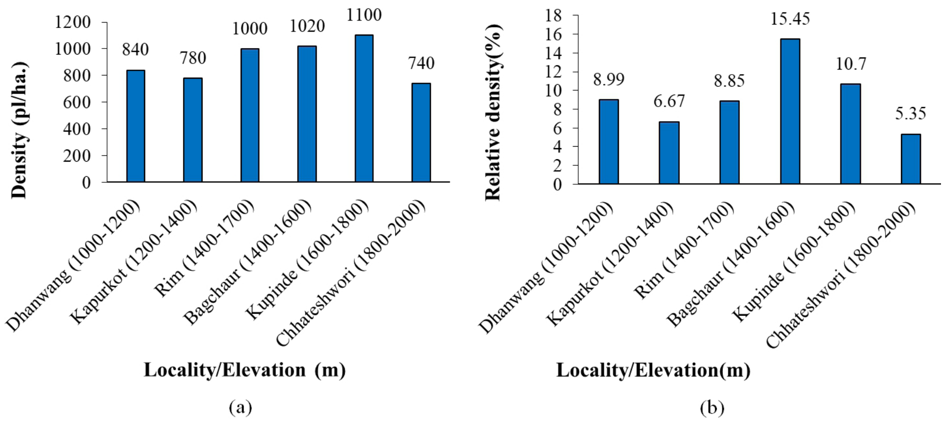

Fig. 4 Density (**a**) and Relative density (**b**) of *Z. armatum* at different locality and elevation.

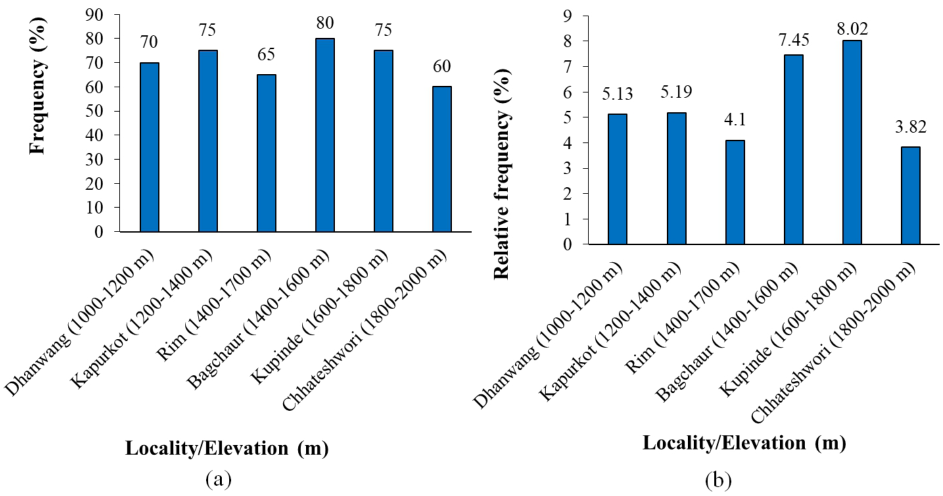

Fig. 5 Frequency (%) (**a**) and relative frequency (%) (**b**) of *Z. armatum* at different locality and elevation.

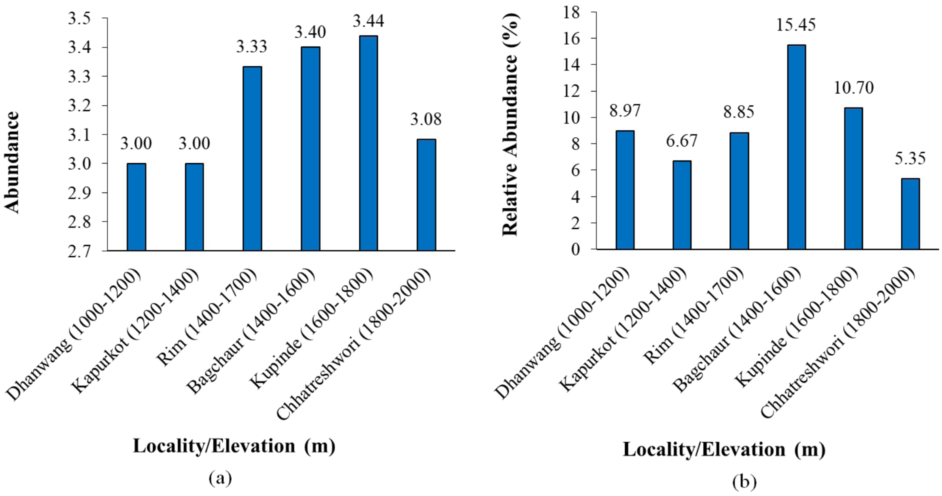

Fig. 6 Abundance (**a**) and relative abundance (%) (**b**) of *Z. armatum* at different locality & elevation.

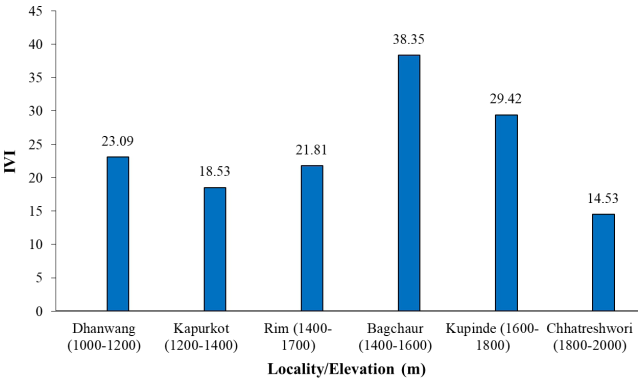

Fig. 7 Importance Value Index (IVI) of *Zanthoxylum armatum* at different locality and elevation.

Regeneration status

Natural regeneration of *Z. armatum* varied at different elevation/localities. Both seedlings and saplings at Kapurkot (1,200–1,400 m) had higher density and the lowest density was at Chhatreshwori (1,800–2,000 m). The total seedlings and saplings densities were 200 individuals/ha and 140 individuals/ha at Kapurkot and 100 individuals/ha and 60 individuals/ha at Chhatreshwori, respectively (Table 4). Similarly, the total seedlings and saplings densities were 180 individuals/ha and 100 individuals/ha, Baghchaur (1,400–1,600 m), 160 individuals/ha and 100 individuals/ha at Dhanwang (1,000–1,200 m), 140 individuals/ha and 120 individuals/ha at Rim (1,400–1,700 m), and 120 individuals/ha and 80 individuals/ha at Kupide (1,600–1,800 m), respectively.

Distribution

The distribution pattern of *Z. armatum* in all the localities studied were almost similar. The A/F ratio of 0.04 and 0.05 (Fig. 8) in all the localities showed that *Z. armatum* has random distribution in the study area. It was found scattered in patches associated with other species. Pure stands of *Z. armatum* were not evident anywhere in the study area. There were substantial differences in the distribution of *Z. armatum*, likely resulting from differences in the degree of management and disturbance as well as from different ecological factors.

Species diversity

Diversity values according to the Shannon and Simpson indices were higher at Rim and lower at Kupinde. The mean Shannon-Weaver diversity index (H') which measures the diversity of Z. armatum along with its associates ranged from 2.5 to 3.08. Among the six study sites the highest species diversity (H' = 3.08) was recorded from Chhatreshwori whereas the lowest species diversity (H' = 2.5) was recorded at Kupinde. The mean Simpson's diversity index (1 – D) value ranged from 0.92 to 0.95 (Fig. 9).

Soil nutrient analysis showed that soil organic carbon, soil nitrogen, and soil phosphorus were highest at Chhatreshwori than other localities (Table 5).

Discussion

Species richness

Species composition and species richness are important indicators for assessing the biodiversity (Husch et al. 2002) and may strongly depend and/or be influenced by the applied management practices. The listing of 50 shrub and tree species in the area shows the forest is rich in diversity (Table 3). This is comparable to other studies carried out in similar forest types of Bhutan, India, and Nepal. Wangda and Ohsawa (2006) listed 78 tree species in west central part of Bhutan and Buffum et al. (2008) reported 39 tree species from the eastern part of Bhutan. Sundriyal and Sharma (1996) recorded 81 tree species in the temperate forest in Sikkim, and Shrestha et al. (2013) recorded 31 and 37 plant species in the two sites within the elevation range of 2,650–2,800 m asl in Nepal.

The lower number of species recorded at Baghchaur

Table 4 Seedling, sapling and adult total density of *Z. armatum* at different locality

SN	Samplig sites	Density (pl/ha)		
		Seedling	Sapling	Adult
1	Dhanwang (1,000–1,200 m)	160	100	840
2	Kapurkot (1,200–1,400 m)	200	140	780
3	Rim (1,400–1,700 m)	140	120	1,000
4	Bagchaur (1,400–1,600 m)	180	100	1,020
5	Kupinde (1,600–1,800 m)	120	80	1,100
6	Chhatreshwori (1,800–2,000 m)	100	60	740
	Average	150	100	913.33

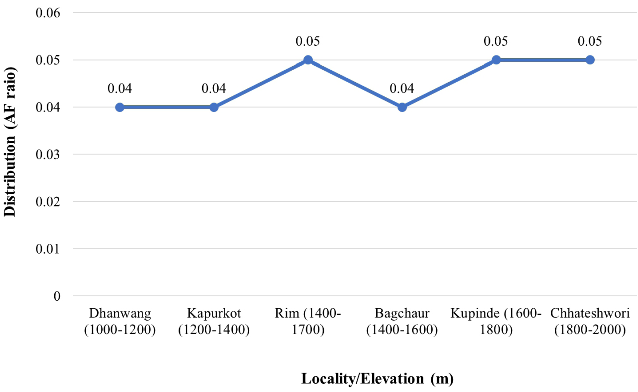

Fig. 8 Distribution pattern (abundance/frequency ratio, A/F ratio) of *Zanthoxylum armatum* at different locality and elevation.

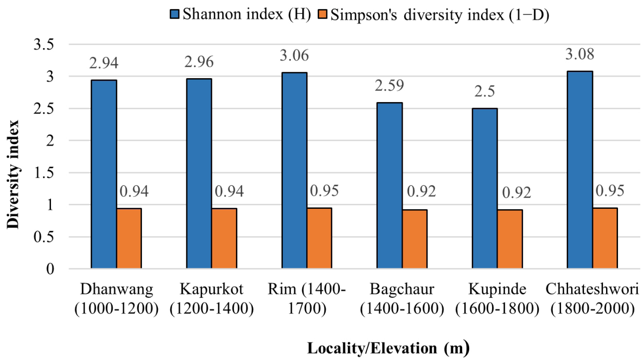

Fig. 9 Simson's diversity index and Shannon–Weaver index for *Z. armatum* and its associates.

(1,400–1,600 m) and Kupinde (1,600–1,800 m) (Fig. 3) is because of the the nearby settlement and agriculture zones as compared to the other sites. Bhuyan et al. (2003) reported only 16 species in highly disturbed site as compared to 47 species in the least disturbed site in the eastern part of India. Sunil et al. (2011) observed 34 tree species in the low disturbed sites against tree species of 14 in the highly disturbed sites in the southern part of India. The lowest was found in plantation forests, with only 9 species in Nepal (Webb and Sah 2003). All these studies attribute the differences in the results to the degree of disturbances caused by anthropogenic activities.

There is generally a linear relationship between vegetation attributes like species richness, diversity, and ecological factors like altitude, aspect, and distance of the site from disturbance stimuli (Schuster and Diekmann 2005). A monotonic decline in the number of species with increasing elevation has often been considered a general pattern (Brown 1988; Stevens 1992). Inverse correlation between altitude and species richness in Himalayan alpines have also been established in several studies (Kala and Mathur 2002; Panthi et al. 2007; Vetaas 2000). However, the present study did not follow the similar pattern; maximum number of species was recorded at 1,400–1,700 m and 1,800–2,000 m. The high species richness may be attributed to less anthropogenic activities, higher soil moisture and greater topographic variations in habitat conditions.

Density

Several factors as lower elevation, moist habitat, resource availability, disturbance levels, moderate fragmentation together with climatic variability, fluctuations to resources and dispersal limitation may influence the population structure (Shaheen et al. 2011a). The variation in the densities along the elevation gradient at different localities might be the result of the variations in the soil nutrients and other abiotic as well as climatic factors. A study in Indonesia showed that fully opened habitat with full sun exposure during daytime may not be the suitable habitat for the natural population of *Zanthoxylum acanthopodium* (Junaedi and Nurlaeni 2019).

Abundance and IVI

The density and frequency values of *Z. armatum* were also high at Baghchaur and Kupinde. Nkoa et al. (2015) stated that the abundance is related to number (density) or frequency. The higher density and frequency might have influenced the abundance positively in this study also. The IVI depicts the importance of the species in terms of its dominance and ecological success (Misra 1968). The change in IVI among the study sites can be attributed to the change in species composition and degree of disturbance and altitude (Saravanan et al. 2019).

Regeneration status

The average seedlings and saplings densities of *Z. arma-*

Table 5 Variation on soil chemical properties at different locality and elevation

SN	Locality	SOC (%)	N (%)	P (ppm)	K (ppm)	pH
1	Dhanwang (1,000–2,000)	3.09 ± 0.12	0.36 ± 0.05	31.11 ± 1.73	135.89 ± 1.43	6.23 ± 0.03
2	Kapurkot (1,200–1,400)	3.07 ± 0.07	0.31 ± 0.04	35.11 ± 2.57	212.27 ± 8.39	5.51 ± 0.07
3	Baghchaur (1,400–1,600)	2.73 ± 0.08	0.29 ± 0.10	30.91 ± 1.29	138.67 ± 3.46	5.75 ± 0.14
4	Rim (1,400–1,700)	3.53 ± 0.16	0.49 ± 0.08	41.65 ± 0.92	428.34 ± 15.22	5.33 ± 0.15
5	Kupinde (1,600–1,800)	2.58 ± 0.09	0.30 ± 0.15	21.03 ± 3.48	121.28 ± 1.92	5.4 ± 0.09
6	Chhatreshwori (1,800–2,000)	4.87 ± 0.04	0.61 ± 0.03	57.85 ± 1.83	346.84 ± 10.36	5.73 ± 0.1

Values are presented as mean ± standard error.
SOC, soil organic carbon; N, nitrogen; P, phosphorous.

tum in the study area were 150 individuals/ha and 100 individuals/ha. A study by Rawat and Chandhok (2009) reported saplings and seedlings layer densities ranging from 90 to 410 individuals/ha and 50 to 510 individuals/ha, respectively. In another study, the seedling and sapling layer density ranged from 340 to 1190 individuals/ha. for seedlings and 340 to 920 individuals/ha for saplings (Saha et al. 2016). The densities of seedlings and saplings in the present study did not show any specific pattern for elevation gradient. However, a study by Gairola et al. (2008) for different high altitude Himalayan forests showed maximum seedling density throughout the altitudinal strata suggesting that the slope and aspect favor regeneration of tree species. Similarly, significant difference was observed between top hill and bottom hill positions, with the highest amount of regeneration in the bottom and lowest amount of regeneration in the top hill (Nur et al. 2016).

Regeneration status of any species is determined by the number of saplings and seedlings (Dhar et al. 1997; Singh and Singh 1992). The seedlings and saplings densities of *Z. armatum* were comparatively very lower than the adult densities in the study area. One reason for this kind of pattern might be due to the prematurely harvesting of the fruits before even falling off of the seeds on the ground. This severely hinders the natural seed bank stock and thus affecting the seedling and sapling density. According to the regeneration status table (Table 4), it can be said that the regeneration status of *Z. armatum* in the study area is fair. However, among the six localities, Kapurkot had comparatively better regeneration status than other sites and Chhatreshwori had lower regeneration potential.

Natural regeneration is a key process for the continued existence of a species in a community. The three major components of successful regeneration are the ability of species to initiate new seedlings, their survival and growth (Saikia and Khan 2013). Presence of sufficient number of seedlings, saplings and young trees in a given population indicates successful regeneration (Saxena and Singh 1984), which is frequently influenced by the biotic interactions and anthropogenic disturbances. The future composition of the forests depends on the potential regenerative status of tree species within a forest stand in space and time (Henle et al. 2004). Generally, regeneration of a species is usually affected by anthropogenic and natural factors. Seed germination rate in *Z. armatum* is very low (Phuyal et al. 2018) and hindered by the presence of hard seed coat; the seeds undergo a strong dormancy and may take few months to years for germination (Chadha 1976). Furthermore, the solitary seeds in the fruit also limit the quantities of seed (Singh and Rawat 2017) and lower the rate of germination. Because of the high demand of *Z. armatum*, its commercial cultivation in the study area is escalating during the last few years. There is also a high demand of plantlets but the supply is minimum due to lack of nurseries. District Forest and Plant Resources offices at Salyan provided free plantlets to the interested farmers, still the supply is inadequate to meet the demand. This has put a high pressure on the naturally regenerating seedling and saplings in the natural forests as the villagers uproot the seedlings and saplings from the forest to plant them in their farmland, which greatly alters the regeneration status of *Z. armatum* naturally. Furthermore, the fruits are prematurely harvested from the wild, probably affecting the seedbank of *Z. armatum*, leading to lower production of seedlings.

Regeneration of any species is confined to a peculiar range of habitat conditions and the extent of those conditions is a major determinant of its geographic distribution (Grubb 1977) and the presence of saplings under the canopies of adult trees also indicates the future composition of a community (Pokhriyal et al. 2010). The natural regeneration of *Z. armatum* is adversely affected by physiological dormancy and high emptiness nature of seeds as a result the seed germination is extremely rare in wild.

The reason behind less density, frequency, abundance and regeneration at Chhatreshwori might be due to the overexploitation by the local people as they collect bigger trees for their own consumption and extra income.

Distribution

It was apparent that the natural distribution of this valuable species has been shrinking in the recent years due to anthropogenic disturbances, as a similar trend for other Himalayan medicinal plants as well (Vashistha et al. 2006). Increasing market demand and unsustainable harvesting are posing serious threat to the natural population of *Z.*

armatum. It is one of the many other medicinal plants that is collected with high preference for market as well as local use (Kunwar et al. 2015).

Anthropogenic disturbances mainly harvesting from the wild without proper care and uprooting of the seedlings and saplings for transplanting them in farmyard were found to be the major cause affecting the natural distribution of *Z. armatum* in the study area. Being a thorny plant, disturbance from grazing was however not apparent for the species as for many other medicinal plants.

There was a huge discrimination in the harvesting pattern and collection of *Z. armatum* in the study area. During field visit at the fruiting season, it was a very common scene that the plants in farmers' farmyard were overloaded with ripe fruits while those in the wild were harvested prematurely. Plants in the farmyard were considered private and those in the forest were public so whoever saw them first would harvest haphazardly. Though the farmers were aware of the enormous economic benefits of the species, there seems to be a lack of awareness towards the conservation and sustainable harvesting of the species from the wild.

Species diversity

The mean average species diversity (Shannon index, H) value of 2.8 recorded in the present study is comparable to the results of similar investigations in different Himalayan regions: 1.53–2.88 in the western Himalayas (Gaur and Joshi 2006; Samant et al. 1998), 2.39–4.63 in the Gharwal Himalayas (Nautiyal et al. 1999), 2.5–3.10 in the trans-Himalayan alpines of Nepal (Panthi et al. 2007) and 3.13 in the alpine pastures of Kashmir, Pakistan (Shaheen et al. 2011).

The higher value of the diversity indices is an obvious indication of high species diversity and abundance (Adekunle et al. 2013). This diversity index is comparable to that found in the tropical forest of Eastern Ghats ranging between 3.76–3.96 (Naidu and Kumar 2016). Forests with Shannon index greater than 2 are considered as medium to highly diverse in terms of species (Giliba et al. 2011). Thus from the findings of this study it can be said that the study area falls in the category of forests with high diversity.

The differences of diversity between different localities and altitudes of the study area could be because of variations in the soil type, rainfall trends, anthropogenic action, land use change, and so forth. Lowest diversity noted in Kupinde could be explained by the fact that the forest is totally degraded due to road construction and land slides. Road networks increase resource extraction and encroachment into the forest leading to a reduction in biodiversity (Hitimana et al. 2004; Sundriyal and Sharma 1996). Higher species diversity and species richness at Chhatreshwori could be because of relatively high soil nutrients. Several studies have established a direct relationship between soil nutrients and species diversity. Grime (1973), Tilman and Pacala (1993) demonstrated that soil fertility has a considerable impact on species diversity. Similarly, Loreau et al. (2001) also suggested that species diversity is usually related to soil fertility. The forest was comparatively dense and moist at Chhatreshwori. Moisture is also one of the important determinants of species richness and composition (Vetaas 2000). Soil organic matter, nutrients and moisture plays an important role in the vegetation composition of any area (Tang 1990).

There have been ample studies on the population size and distribution of other medicinal plants in Nepal. But no study has been conducted previously on the population, distribution, frequency, and abundance of this species so no comparable data is available from Nepal. However, a study from India recorded a low population size of *Z. armatum* from the villages of Chamoli district of Uttarakhand, India; the average density was 368.2 individuals/ha. Due to low population size, it has been placed in the International Union for Conservation of Nature (IUCN) vulnerable category (Kala 2010). The same study also recorded *Berberis aristata, Ficus, Grewia optiva, Pyrus pashia, Pyracantha crenulata, Quercus,* etc. as the major associates of *Z. armatum* (Tables S1–S6).

The market price of the fruits is very good so the extensive demand of this species has put an enormous pressure on the natural population. Though the farmers have already started commercial cultivation of *Z. armatum*, the collection from wild has not yet decreased. Since the fruits are difficult to harvest because of the thorns, destructive harvesting without taking proper care is a common practice, creating a tremendous pressure on its existing populations in the wild. Similar scenario also prevails in Uttarakhand, India where harvesting of the entire plant before setting even flowers along with the profuse invasion from woody weeds such as Lantana has negatively impacted the natural distribution of *Z. armatum* (Kala 2010). Threats to *Z. armatum* from invasive species was however not evident in the present study. Since the plant is thorny and aromatic, grazing by livestock is not a threat to the natural population of *Z. armatum*, instead it also provides shelter and protection to its associated species in the natural habitats by preventing from livestock grazing and browsing (Kala 2010).

Z. armatum has been prioritized by the government of Nepal as one of the important medicinal plants for economic development with a high emphasis on cultivation and agro-technology development (DPR 2006). Owing to its great economic potential, the Karnali Provincial government, which includes Salyan, has announced a *Zanthoxylum* Year Program in the province's hilly districts and all the three levels of government: federal, provincial, and local, have prioritized *Z. armatum* farming. The federal government also announced to celebrate fiscal year

2019–2020 as the year of *Z. armatum* plantation. The market price of the fruits is also very good so the extensive demand of this species has put an enormous pressure on the natural population. Though the farmers have already started commercial cultivation, the collection from wild has not yet decreased.

Diversity and distribution patterns of species are greatly affected by various factors, including area, latitude, precipitation, and temperature (Zhang et al. 2011). The large scale pattern in species distribution and physiognomy is also governed by the climate. Climate can be characterized by different variables which mainly determines the distribution pattern of species distribution in any area (Bakkenes et al. 2002).

Overall, due to the increase in population size and the overexploitation of forests, change of land use among other factors; negative impacts can result on the forest ecology including reduction of plant stock, disruption of regeneration, and loss of nutrients in harvested materials (Murkherjee and Chaturvedi 2017). Other factors that may affect the sustainability of plants are collection of premature plants, grazing, and soil erosion. Therefore, deliberate efforts should be taken by all stakeholders to ensure that these plants are used in a sustainable way.

Conclusions

Species composition, density, distribution and regeneration status can be considered important factors to judge the status of a forest. *Z. armatum* was found to be distributed randomly in Salyan district and the lower number of seedlings and saplings indicates a fair regeneration pattern. Although the dependency on natural forests for the collection of berries is gradually being replaced by cultivation in private land in the recent years, unsustainable harvesting and collection of saplings from the wild has not been stopped completely. Anthropogenic disturbances including premature harvesting and digging up of saplings was found to severely affect the natural distribution and regeneration in the study area. Increasing market demand and unsustainable harvesting procedures are posing serious threat to the natural population of *Z. armatum*. Thus, effective conservation and management initiatives are most important for conserving the wild genetic diversity of *Z. armatum* in the study area. Establishment of high-tech nurseries and free distribution of saplings to the farmers could possibly reduce the pressure on natural population and also uplift the economic status of the marginalized and poor communities. Assessment of diversity and regeneration status of species is important for their sustainable utilization, management, and conservation. Therefore, a systematic management plan is required for the conservation and sustainable utilization of this valuable species.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.1186/jee.22.015

Table S1. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Dhanwang (1000–1200 m). **Table S2**. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates. **Table S3**. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates. **Table S4**. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Baghchaur (1400–1600 m asl). **Table S5**. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Kupinde (1600–1800 m asl). Table S6. Population density (in ha), frequency, abundance, and distribution of *Z. armatum* and its major associates at Chhatreshwori (1800–2000 m asl).

Abbreviations

NTFPs: Non-timber Forest Products
MAPs: Medicinal and Aromatic Plants
RD: Relative Density
RF: Relative Frequency
RA: Relative Abundance
IVI: Importance Value Index
SOC: Soil organic carbon

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Authors' contributions

PKJ and SR conceptualized and supervised the research. NP collected and analyzed the data and wrote the manuscript. PKJ, PPR, and SR critically commented and approved the final version of the manuscript. The authors read and approved the final version of the manuscript.

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All data involved in this study are available from the corresponding authors upon request.

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The authors declare that they have no competing interests.

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