PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF SOME SELECTED MEDICINAL PLANTS OF NEPAL

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BY

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DECLARATION

Thesis entitled **"Phytochemical Analysis and Biological Activities of Some Selected Medicinal Plants of Nepal"** which is being 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 Prof. Dr. Sangeeta Rajbhandary, Central Department of Botany, Tribhuvan University, Nepal and Co supervised by Dr. Deepak Raj Pant, Associate Professor, Central Department of Botany, Tribhuvan University, Nepal and Dr. Stefano Dall'Acqua, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy.

This research work is original 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.

Date: July 04, 2022 .....

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RECOMMENDATION

This is to recommend that **Mr. Shyam Sharan Shrestha** has carried out research entitled **"Phytochemical Analysis and Biological Activities of Some Selected Medicinal Plants of Nepal"** for the award of Doctor of Philosophy (Ph.D.) in **Botany** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He 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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On the recommendation of Prof. Dr. Sangeeta Rajbhandary, Supervisor; Associate Prof. Dr. Deepak Raj Pant, Co-Supervisor and Associate Prof. Dr. Stefano Dall'Acqua, Co-Supervisor, this Ph. D. thesis submitted by Shyam Sharan Shrestha, entitled **"Phytochemical Analysis and Biological Activities of some selected Medicinal Plants of Nepal"** is forwarded by Central Department Research Committee (CDRC) to the Dean, Institute of Science and Technology, Tribhuvan University.

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ABSTRACT

Nepal is a habitat for diverse ethnomedicinal plants. In this present study, the intensive phytochemical, cyotxicity and biological assays were done in ethnomedicinal plants *Girardinia diversifolia* (Link) friis, *Tectaria coadunata* (J. Smith) c. Christensen and *Melia azedarach* L. extracts.

Girardinia diversifolia, locally known as Allo in Nepali. In english it was named as Himalayan nettle. The plant is perennial. The plant is also used to treat different ailments in rural as well as urban areas of Nepal. Stem of the plant have fibre which are used to prepare different house hold items. *T. coadunata*, is known as Kalo neuro in Nepali. The plant is used for various types of disease typically in rural areas of Nepal. The plant is usually found all over Nepal. *M. azedarach* is a common tree used as ethnomedicine for different ailments as well as wood for furniture, cooking and household purposes in many countries and in Nepal. Up to now limited information is available on Nepalese ethnomedicinal plants. Phytochemical analysis, cytotoxicity and biological assays on these plants are scarce. The present study was focused to analyse different types of phytochemical compounds which are available in the medicinal plants. Plants were extracted by methanol initially. Nuclear magnetic resonance was used as tools for the study. Besides that Liquid chromatography mass spectrophotometry was also used. High performance liquid chromatography, gas chromatography mass spectrophotomety were also used to determine secondary metabolites of the medicinal plants. With the help of these study, it could be suggested that medicinal plants could be used to make pharmaceutical products, cosmetic items and nutraceutical products.

The intensive phytochemical analysis in the present study revealed that there were presence of phytosterols and fatty acids in high amount. It was also found carotenoids, phenolics and saponins in low quantity. *G. diversifolia* contained high amount of phyto compounds which were beta-sitosterol (11% dw) and gamma sitosterol (9% dw). The linoleic acids were found to be 22 mg/g and linolenic acids 9.7 mg/g. Vitamin c was also obtained which was 2.9 mg/g and vitamin B2 was 0.12 mg/g. The antioxidant capacity was also calculated. Enzyme inhibitory activities were also

performed. Antioxidant capacity was reported due to presence of phenolics and enzyme inhibitory activities were found to be acetylcholinesterase (AChE), butyrilcholinesterase (BuChE), tyrosinase, α-amylase and α-glucosidase. Priliminary cytotoxic activities were analysed. It was reported more potent for hepatic and pancreatic cancer cell lines. Plant extracts can effect to hepaticcarcinoma cells in down regulation of LDRL.

The phytochemical analysis of *T. coadunata* showed that it contained high amount of PACs. The obtained PACs were eriodictyol-7-O-glucuronide and luteolin-7-O-glucoronide. Antioxidant capacity were also analysed in the study which showed that it was effective against different metabolic disorders. The study of enzyme inhibitory activities revealed that it could be used to treat Alzheimer's diseases. Tyrosinase inhibition was also done which indicated that it could be used in skin related diseasses. The cytotoxic study revealed that the extract could be used to treat pancreatic cancer.

Phytochemical investigations in *M.azedarach* revealed large amount of fatty acids, phenolic mostly flavonoid glycosides (1%) and Limonoids (0.7%) in the leaves. As significant bioactive compounds with Meliatoosein L (2.145 mg/g) and Masendanin A (2.084 mg/g) were identified as major limonoid derivatives. Considering the pehnolics rutin was the most abundant, while quercetin, quercetin derivatives and keampferol derivatives were also present. Most abundant phytosterol constituent was beta-sitosterol but significant amounts of fucostanol, campesterol and stigmasterol were detected. The methanolic leaves extract exhibit IC_{50} of 26.4 ug/mL 2008 showing mild cytotoxic activities on human ovarian cell line (2008). The antioxidant activities and the inhibition of some key-enzymes were moderate except for tyrosinase inhibition that resulted in 131.57 ± 0.51 mg Kojic Acid Equivalents/g suggesting possible usefulness in skin hyperpigmentation conditions. Over all findings showed potential usefulness of all three ethnomedicinal plants as a source of phytochemicals for cosmetics, neutraceuticals and pharmaceuticals uses.

Keywords: NMR; LC-MS; Phytosterols; Procyanidins; Limonoids; Tyrosinase inhibition; Cytotoxicity

LIST OF ACRONYMS AND ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AD	Alzheimer disease
ACAE	Acarbose equivalent
AChe	Acetylcholinesterase
APCI	Atmospheric pressure chemical ionization
BChE	Butyrylcholinesterase
COSY	Correlation spectroscopy
CUPRAC	Cupric reducing antioxidant
CH_2Cl_2	Dichloromethane
DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Fluorescence recovery after photobleaching
FLD	Fluorescence detector
GAE	Gallic acid equivalent
GALAE	Galantamine equivalent
GC-MS	Gas Chromatography Mass spectrophotometry
HMBC	Heteronuclear multiple quantum coherence
1H-NMR	Proton nuclear magnetic resonance
HSQC	Heteronuclear Single Quantum Coherence
HSQC-DEPT	Heteronuclear Single Quantum Coherence-distortionless enhancement by polarization transfer
KAE	Kojic acid equivalent

KATH	National Herbarium and Plant Laboratories, Godavari, Lalitpur
LC-DAD-MS	Liquid chromatography, diode array detector, Mass spectrophotometry
LDLR	Low-density lipoprotein receptor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
PAC	Procyanidin
RE	Rutin equivalent
SRB	Sulphorhodamine B
TC-MeOH	*T. coadunata* Methanolic extract
TC-EtoAC	*T. coadunata* ethyl acetate extract
TC-H2O	*T. coadunata* water extract
TDDS	Turbo data-dependent scanning
TE	Trolox equivalent
TLC	Thin Layer Chromatography
TUCH	Tribhuvan University Central Herbarium

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CHAPTER 1

1.1 INTRODUCTION

Plants form the main ingredients of medicines in traditional systems of healing and have been the source of inspiration for several major pharmaceutical drugs. Therefore, plants have been one of the important sources of medicines ever since the dawn of human civilization. All over the world, especially in developing countries traditional systems of medicine are important health sources. A World Health Organisation (WHO) Expert Group defined 'Traditional Medicine' as the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (WHO, 1976). WHO (2004) have also stated that the use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare setting.

Many developed and developing countries still rely on ethnobotanical remedies for the treatment of many diseases. In Nepal also medicinal plants are indispensable components especially in the rural areas as they are used to cure different infectious diseases. Plants used in traditional medicine are important sources of novel bio-molecules (Heinrich & Gibbons, 2010), with application for the manufacture of pharmaceuticals and cosmeceuticals. Every plant is identified by its own different therapeutic properties due to active bioactive molecule. Therapeutic effects of medicinal plants are associated with their chemical peculiarities, which are in reality components of the defense strategies of plants. Even at the dawn of 21st century, 11% of the 252 drugs considered as basic and essential by the WHO were exclusively of flowering plant origin (Veeresham, 2012). The extracts and phytochemicals derived from plant and plant products have strong health significance. Medicinal plants contain phytochemical substances like alkaloids, flavonoids, glycosides, bioflavonoid, bezophenones, xanthones as well as some metabolites such as tannins, saponins, cyanates, oxalate etc. which are used against different diseases and relieve pain (Hena *et al*., 2010).

More than 8000, plant secondary metabolites like flavonoids and the other phenolic compounds which are naturally occurring substances in plants have been reported. Among these secondary metabolites half of these are phenolic compounds like flavonoids presenting as aglycone, glycosides and methylated derivatives (Kumar & Pandey, 2013). Both of these phytochemical substances, flavonoids and many other phenolic components have been reported on their effective antioxidants, anticancer, antibacteria, cardioprotective agents, anti-inflammation, immune system promoting, skin protection from UV radiation, have been important substance for pharmaceutical and medical application (Kumar & Pandey, 2013; Meng *et al*., 2018). In recent years probably due to insignificant side effects compared to synthetic drugs and antibiotic there has been an increase in the demand for herbal medicines.

Nepal Himalayas due to its unique topography showcases vast varieties of medicinal plants, many of which are still beyond the domain of contemporary research. Therefore, very little information is known about biochemical specificities of Himalayan medicinal plants, but some evidence suggests that such plants offer great potential for discovery of novel molecules and new sources of active compounds, mainly because of the environmental stress to which they are subjected (Jackson & Dewick, 1984), for example, found that the content of podophyllotoxin, which is isolated from podophyllin (a resin produced by species of the genus *Podophyllum*, commonly known as 'may-apple'), is much higher (4.3% of dry weight)in the Himalayan species *Podophyllum hexandrum* than in the American species *Podophyllum peltatum (*0.25%). Likewise, many Himalayan herbs have served as an importance source of synthetic drugs like taxol (*Taxus wallichiana*), diosgenin (*Dioscorea bulbifera*), podophyllotoxin (*Podophyllum hexandrum*), ephedrine (*Ephedra gerardiana*), reserpine (*Rauvolfia serpentina*), and picrorhizin (*Picrorhiza kurrooa*) (Rawal, 1998). From medicinal plants 78% of new chemical constituents being natural or natural product-derived molecules are being used as a promising alternative treatment for infectious diseases (Lokhande *et al*., 2007). Plants, therefore, have been fundamental sources of medicines throughout human history (Rajbhandary & Ranjitkar, 2006).

Phytochemical analysis and assessment of bioactivity of phyto-constituents are essential for evaluating medicinal efficacy of ethnomedicinal plants. Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines (Sofowora *et al*., 2013). Ethnomedicinal

studies play a vital role to discover new drugs from indigenous medicinal plants. In recent past, search for plants used in disease prevention or general ethnobotanical surveys which probably emphasized more enquiries on the use of plants to treat disease was carried out in the world. At the same time, there has been a tremendous increase in the use of plant based health products in developing as well as developed countries resulting in an exponential growth of herbal products globally (Jain *et al*., 2006; Zafar, 2009; Hasan, 2014).

In Nepal also ethnomedicinal uses of Nepalese plants have been well documented (Rajbhandary, 2001; Manandhar, 2002;Baral & Kurmi, 2006), however, information related to phytochemical composition and bioactivity of the phytoconstituents are lacking for a large fraction of ethnomedicinal plants. The situation becomes more serious when this information are lacking for highly used species which are mostly collected from wild population.

In the present study, phytochemical composition has been analysed along with cytotoxic activities assays from extracts of three medicinal plants *Girardinia diversifolia*, *Tectaria coadunata* and *Melia azedarach*, which are highly used species. *Girardinia diversifolia* is largely used in traditional medicine for different diseases like constipation (Thapa, 2013), gastric disorders, chest pain (Rana *et al*., 2015), rheumatism, tuberculosis (Nath *et al*., 2011), headache, joints aches (Manandhar, 2002; Kunwar *et al*., 2012), eczema, intestinal worms and inflamed pancreas (Joshi, 2008; Hasan *et al*., 2013) and diabetes (Balami, 2004; Gurung *et al*., 2012; Bhattarai & Tamang, 2017; Tamang *et al*., 2017). *G. diversifolia* contained bioactive components with potential antibacterial and antifungal activities. β-Sitosterol, 3-Hydroxystigmast-5-en-7-one, 7-Hydroxysitosterol were isolated from *Girardinia diversifolia* by Njogu *et al*. (2011).

Girardinia diversifolia is widely distributed in different parts of Nepal. It occurs abundantly in the forests of hills in moist and damp soil at altitude of 1200 to 3000 meters. It is also found in northern India, Bhutan, Sri Lanka, eastward to central China, Myanmar, Malaysia, Indonesia and Africa (Manandhar, 2002). Apart from the medicinal use young leaves and inflorescences are cooked as a green vegetables (Manandhar, 2002; Joshi, 2008; Kunwar *et al*., 2012). It is also traditionally used as best fibre for making varieties of clothing, ropes, mats, sacks and various other

domestic implements and one of the most important non timber forest products used for income generation among rural communities in the Himalaya region of Nepal (Singh & Shrestha, 1985; Singh & Shrestha, 1988; Clarke, 2007; Barakoti & Shrestha, 2009; Pyakurel & Baniya, 2011; Gurung *et al*., 2012; Bhandari, 2019).

Tectaria coadunata is used to treat various ailments like stomach pain and giardiasis, gastrointestinal disorders, jaundice, diarrhea and dysentery, cuts and wounds and to eradicate worms (Joshi *et al*., 2011; Uprety *et al*., 2011; Thapa, 2013; Subba *et al*., 2016; Subedi, 2017; Tamang *et al*., 2017). Rhizomes were used against hyperlipidemia and considered to have no toxicity at therapeutic dose level (Mori *et al*., 2016). Fresh rhizome and frond are used in case of insect and centipede bites, while the extract of dried rhizome, stem and stripe is used in respiratory disorders like cold cough, asthma and bronchitis (Malviya *et al*., 2012). Plant is widely distributed in north facing slopes. It is usually found on mossy rocks along stream banks and on muddy rocks in streamlets throughout Nepal (Rajbhandary, 2016).

Studies on *T. coadunata* were limited on phytochemical study, twenty-one compounds were detected by Dubal and Kale (2019) from GC-MS analysis. Other constituents were indicated as general classes of compounds comprising carbohydrate, tannins, phenols, anthraquinone glycosides, coumarin glycosides, flavonoids and steroids (Pawar *et al*., 2016).

Melia azedarach is a common tree generally used as traditional medicinal to treat various ailments like, diarrhea, dysentery, spleen disorder, as an anthelmintic (Manandhar, 1991; Joshi & Joshi, 2000; Balami, 2004; Burlakoti & Kunwar, 2008; Khan *et al*., 2014) and are also used for treating vomit, blood impurities, urinary discharge, to treat diabetes and gastrointestinal diseases (Acharya & Acharya, 2009).

Melia azedarach plant has been considered tube used as a source of antioxidant and antimicrobial for pharmacological preparations (Pokhrel *et al*., 2015). Various bioactivities of *M. azedarach* have been attributed to different principal compounds present in this plant. Several important compoundslike azaridin, azadirachtin, bakalactone, bakayanin, benzoic acid, deacetylsalanin, dihydronimocinol, fraxinellone, quercetin, meliacarpinin, meliacine, meliotannic acid, melazolide, nimbolinin, rutin, salanin, salannal, vilasinin have been reported from bark and leaves of *M. azedarach* (Watanabe *et al*., 2005).

1.2 Rationale and implications

The use of medicinal plants has been rooted in Nepalese culture since ancient times as mentioned above, but this traditional knowledge is threatened by the loss of oral tradition and the use of synthetic drugs. But due to scarce availability and high costs of synthetic medicines, people in rural areas of Nepal are still dependent on medicinal plants (Shrestha *et al*., 2019). In this context, the exploration of Nepalese flora used in traditional medicine is a unique opportunity to study bioactive extracts as sources of new natural products. The ethnopharmacology of several species is still poorly considered and can offer new research opportunities.

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their uses in traditional medicine (Gurung *et al*., 2012). The medicinal actions of plants are unique to particular plant species or groups are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct (Ghimire *et al*., 2008). One major asset of medicinal plant-based drug discovery is the existence of ethnopharmacological information providing hints for compounds therapeutically effective in humans. Numerous drugs have been introduced to international markets through validations of traditional medicines, indigenous therapies and ethno-pharmacological practices (Olsen & Overgaard, 2003).

Phytochemicals constituents in plants have defense mechanism and show therapeutic role in disease cure. Therefore, it is worthwhile to use modern science and technology tools for verifying therapeutic potential of medicinal plants as antioxidant as per international standards. Such information may be of potential value in the design of further studies to unravel novel treatment strategies for disorders associated with free radicals-induced tissue damage (Abi Beaulah *et al*., 2011). Historically plants used in traditional medicine by the indigenous populations across the world have produced some of the most useful modern day pharmaceuticals. Despite many studies on medicinal plant resources of Nepal, a large number of medicinal plants and associated indigenous uses still wait proper documentation and evaluation of their therapeutic properties.

The proposed species *Girardinia diversifolia, Tectaria coadunata* and *Melia azedarach* have multiple ethnomedicinal uses. However, they were selected based on their common use as antidiabetics in traditional medicine in Nepal. This study has thus been carried out to find out the effectiveness of these plants in different biological activities *in vitro* and also to isolate and to characterize the phytochemicals responsible for such activity.

1.3 Hypothesis

Extracts of *G. diversifolia, T. coadunata* and *M. azedarach* possess different bioactive phytochemicals for pharmaceuticals uses.

1.4 Objectives

The general objective of the study is to conduct phytochemical analysis from extracts of traditionally used important medicinal plants of Nepal: *G. diversifolia, T. coadunata* and *M. azedarach.*

Specific objectives are:

1. To identify different bioactive compounds from the extract of *G. diversifolia, T. coadunata,* and *M. azedarach*
2. To assess *in vitro* biological activities (Antioxidant, Enzyme Inhibitory Assays and Cytotoxic activities) from extracts of *G. diversifolia, T. coadunata,* and *M. azedarach*

1.5 Limitations

- As the study was focused on phytochemical and biological assays, ecological and biodiversity parameter were excluded

CHAPTER 2

2. LITERATURE REVIEW

2.1 Medicinal plants and ethnobotanical study

Nepal, because of its geological, ecological and climatic variation have resulted in environmental diversity that gave a unique wealth in the form of various green vegetation including valuable crude drugs, which has never ceased to lure people from all over the world. These valuable crude drugs are well known for their purity and effectiveness since time immemorial and have provided good opportunity to our ancestors to develop an indigenous system of medicine, the Ayurveda (Manandhar, 1980).

In Nepal, a hand written herbal encyclopaedia, Bir Nighantu was prepared by Pandit Ghana Nath Devkota based on ancient manuscripts about the utilization of medicinal plants and traditional knowledge. Later under the instruction of Prime Minister Chandra Shamsher this manuscript was compiled as "*Chandra Nighantu*", in the 19th and the beginning of 20th century, based mainly on Ayurveda, which provided information on 800 different medicinal plant species and about 200 animals and mineralsis probably the first hand written effort towards a compilation of the traditional knowledge about medicinal plants.

Apart from the historical manuscript, the history of ethno-medico-botany in Nepal is quite long and an outcome of the research, the first comprehensive account of medicinal plants and their utilisation was published in the book "Medicinal Plants of Nepal" in the year 1970, by Department of Medicinal Plants (DMP) now known as Department of Plant Resources (DPR). It provides information on 393 medicinal plants occurring in Nepal that are used in traditional as well as Ayurvedic medicine systems and many of them are also included in the pharmacopoeias of different countries. It was supplemented by another volume in 1984 with additional information on 178 species of plants. (Bajracharya, 1979) compiled 175 medicinal plants commonly used in Ayurvedic system. (Manandhar, 1980) complied 300 important medicinal plants available in Nepal (Rajbhandary & Winkler, 2015).

Subsequently medicinal plants continued to be the major focus (Dash, 1976; Dobremez, 1976; Malla, 1977; Bajracharya, 1979; Peter, 1979; Sharma, 1979;

Shrestha, 1989; Singh *et al*., 1979; Lecup, 1980; Manandhar, 1980) on medicinal ferns (Gurung, 1979) and on veterinary medicine (Joshi, 1979), with some works dealing with the chemical constituents of certain species of medicinal plants (Dhoubhadel & Manandhar 1979; Dhoubhadel & Shrestha, 1979) and others with pharmacology (Adhikari & Shakya, 1977; Manandhar & Khatri Chhetri, 1980).

Ethnobotany, more than a century old independent discipline, studies the relationship between plant and people. Several studies have been made on ethnobotany and based on those researches (Rajbhandary, 2001) compiled the information in a book entitled *Ethnobotany of Nepal* that provides important insights into ethnobotanical studies in the past. It enumerates the ethnobotanical information, local name and use of 562 species of medicinal plants. Whereas, (Manandhar, 2002) published book on *Plants and people of Nepal* that covered the information of 1002 medicinal plants used by different ethnic community.

Shrestha *et al. (*2002) compiled database of 1624 species of Medicinal Plants of Nepal. (Baral & Kurmi, 2006) compiled 1,792 species of MPs (including lichens and fungi). Now, Nepal comprises 1,950 species of medicinal plants, including 1,614 native species (Ghimire *et al*., 2008), of which 143 species are of trade value (Bhattarai & Ghimire, 2006). Most of the medicinal plant species traded from Nepal is wild, while few species are exotic, naturalised or cultivated since long (Olsen, 2005; Ghimire *et al*., 2016). Till the year 2014, out of 800 references 44% dealt with medicinal plants (Rajbhandary & Winkler, 2015).

2.2 Phytochemistry from Nepalese medicinal plants

Pharmacological screening of 17 indigenous medicinal plants of Nepal have been observed with a number of interesting antispasmodic, uterine stimulants, hypothermic, hypotensive, etc. (Adhikari & Shakya, 1979) and on six species by (Regmi, 1989). Talapatra *et al. (*1989) worked on the chemical investigations of some medicinal plants of Nepal. Phytochemical and antimicrobial screening of different plants has been focused on *Swertia angustifolia* (Dhoubhadel, 1979). *Astilbe rivularis* (Dhoubhadel & Manandhar, 1979), *Acorus calamus (*Dhoubhadel and Shrestha, 1978), on the leaves of *Aegle marmelos* (Shrestha & Manandhar, 1979), *Alangium chinense (*Manandhar & Khatri Chhetri, 1980), *Quercus spicata, Rubus ellipticus, Polygonum amplexicaule*, *Meconopsis regia* and *Melia azedarach* (Shrestha, 1989),

Boenninghausenia albiflora (Prasain *et al*., 1994) and *Coriaria nepalensis* (Joshi & Joshi, 2000). Two hundred species of medicinal and aromatic plants belonging to 60 families, collected from different parts of the country, were screened (Shrestha *et al*., 1994; Karanjit & Singh, 2003) conducted phytochemical screening of 14 medicinal plants containing flavanoids.

Cyanogenesis is a property, which is exhibited by certain plants to liberate HCN on disruption of plant tissue by the interaction of cyanogenic glycoside and β-glucosidase. More than 353 plants were screened for cynogenic activity. About 45 new cynogenic plants have been recorded by (Agrawal, 1989) and a survey of cyanogenic plants was carried out in different regions of Nepal by Shrestha & Agrawal (1994). Compounds 6-0-methylacrylplenolin, 6-0-isobutylplenolin and 6-0-angeloylplenolin were isolated from *Centipeda minima*by (Taylor & Towers, 1998). Besides, some of the laboratory work on screening was also reported by (Khatri Chhetri *et al*., 2000;Kumar *et al.*,2000).

Watanabe *et al. (*2005) have also attempts phytochemical analysis of some Nepalese medicinal plants. Phytochemical screening of 47 Nepalese medicinal plants belonging to 45 genera and 35 families was carried out by Gyawali *et al. (*2008). The result obtained showed that there was definite co-relation between the traditional application of plants and possession of secondary metabolites, which supported the scientific basis for the traditional medicinal system.

As a result of collaborative work which started during 2009, two diterpenoid alkaloids (Navirine B and C) as well as three flavonoid glycoides and four phenol glyocides were isolated from *Aconitum naviculare*. Likewise, two phenolic glycosides (Curculigoside D and Orchioside E) were isolated from rootstock of *Curculigo orchioides* (Shrestha, 2010).

Similarly, the phytochemical analysis of *Centella asiatica* was carried out to obtain bioactive triterpenes (asiaticoside and asiatic acid) and phenol derivatives (flavonoids and caffeoyl esters). It was found that the highest amount were found samples from Gorkhaand Chitwan districts (Devkota *et al*., 2010). Qualitative phytochemical analysis of *Achyranthes bidentata* showed the presence of alkaloids, glycosides, terpenoids, steroids and reducing sugar (Joshi *et al*., 2011).

Two major compounds were isolated from *Dendrobium moniliforme.* Dimethylsulfoxonium formylmethylide and 2, 3-dihydro-3, 5-dihydroxy-6-methyl-

4H-pyran-4-one were detected and identified from methanolic extract and two, 5-(hydroxymethyl)-2-furancarboxaldehydeand 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-onefrom ethanol extract by (Paudel *et al*., 2018).

Various secondary metabolites, including alkaloids, flavonoids, reducing sugars, saponins, terpenoids and tannins were screened from *Buddleja asiatica* leaf extract. Among them total phenolics and flavonoids were found to be the highest (Sai *et al*., 2019).

2.3 Ethnomedicinal uses and phytochemistry of *Girardinia diversifolia* (Link) friis

Different parts of *G. diversifolia* have been reported to cure various types of ailments, which are as follows:

2.3.1 Use in gastrointestinal ailments

Roots of *G. diversifolia* are reported to be used in the treatment of constipation, gastric disorder, chest burn (Manandhar, 2002; Balami, 2004; Gurung *et al*., 2008; Nath *et al*., 2011; Thapa, 2013; Subedee *et al*., 2020), and worm killer (Shrestha *et al*., 2000; Hasan *et al*., 2013). Similarly, roots and leaves of *G. diversifolia* along with *Centella asiatica* (Malla *et al*., 2015) or roots of *G. diversifolia* along with *Oxalis corniculata* (Manandhar, 2002) are reported to be used in the treatment of gastric troubles. (Limbu & Rai, 2013) have reported the use of the stem of *G. diversifolia* in the treatment of stomachache.

2.3.2 Use in rheumatism and pain

Different parts of *G. diversifolia* are also reported to be used in rheumatism (Nath *et al*., 2011; Kumar Rana *et al*., 2015), bone fractures (Saha *et al*., 2011), dog bite and joint pain (Pande *et al*., 2007; Subedee *et al*., 2020). In addition, *G. diversifolia* is also reported to cure hot feel/over heat (Subba *et al*., 2016), headache and fever (Shrestha *et al*., 2000).

Piece of stem is attached to the painful part to relief from pain (Sharma *et al*., 2014). Leaves were used for joint pain and headache. Roots paste of both *Cirsium argyracanthum* and *G. diversifolia* were also reported to be used for the same purpose

(Balami, 2004). The paste of shoot, root and inflorescence of the plant was given orally to treat bone fracture. Juice of the leaves was applied to treat headache and joint aches (Kunwar *et al*., 2012; Hasan *et al*., 2013; Malla *et al*., 2015). Leaf juice is also used for sprain, fracture or broken bones by mixing with other herbs. Leaf paste of *G. diversifolia* was reported to be applied externally on swollen body parts (Rokaya *et al*., 2010). Root and leaf paste of allied species like *G. heterophylla* plant are also reported to be used in the treatment of bone fracture (Jaiswal *et al*., 2004). Root decoction and fresh root is used for the treatment of boils, swelling and joint pain (Singh *et al*., 2017).

2.3.3 Use for skin ailments

Cloth made from the fibre of allo is believed to have an anti-allergic effect on skin (Kaneria *et al*., 2017; Subedee *et al*., 2020). Paste and ash of whole plant, root and leaves of *G. diversifolia*, were used to treat eczema (Shrestha *et al*., 2000; Hasan *et al*., 2013). Paste of fresh leaf is applied locally for scabby until cure (Jagtap *et al*., 2009).

2.3.4 Use in diabetes and blood pressure

Raw buds are given to treat diabetics (Balami, 2004; Gurung *et al*., 2012; Malla *et al*., 2015). The paste of shoot, root and inflorescence of the plant is used to treat high pressure and diabetes (Saha *et al*., 2011). The preparation of thick soup of inflorescence of the plant is used to treat blood pressure (Moktan & Das, 2016).

Infusion of leaves is given orally for treatment of anemia, inflamed pancreas and diabetes. Leaf powder is applied on scarifications for edema (Tacham *et al*., 2015).

2.3.5 Miscellaneous ailments

Juice of root was applied to treat cuts and wounds and plant juice was given to treat fever (Manandhar, 2002), while leaf juice and paste was given for headache, fever, joint aches, tuberculosis and asthma (Balami, 2004; Gurung *et al*., 2012; Malla *et al*., 2015; Subedee *et al*., 2020). Root powder or juice was given with hot water to treat internal injury and blood purification (Rokaya *et al*., 2010).

Plant leaves are used to conduct smooth delivery (Pande *et al*., 2007). Decoction of the leaf of the plant was used to treat in Gonorrehea and delivery problems (Bhatti &

Vashishtha, 2008). Leaves are used for snakebite and dog bites (Bhat *et al*., 2012; Kichu *et al*., 2015).

Apart from medicinal use, fresh leafy parts of *G. diversifolia* are used as vegetables. It is also used for preparation of snacks. Inflorescence are used as vegetables soup and considered to have high nutritional value (Misra *et al*., 2008; Kunwar *et al*., 2012b; Subedee *et al*., 2020).

Bast fiber obtained from the bark is applied for production of different articles such as porter strap Namlo, fishing nets, bags, sacks clothing materials, weaving rugs, jackets (Subedee *et al*., 2020).

2.3.6 Biological activities

Biological activities of the plant are important for the particular treatment for the ailments. This shows that plants have high affinity to treat ailments. Various extracts of *G. diversifolia* are reported to exhibit many biological activities like antiulcer, anti-diabetic, anti-inflammatory etc. (Kumar *et al*., 2013). The treatment with hydroethanolic extract resulted into antihyperglycemic effect as well as improved body weight in STZ-NA induced diabetic rats (Dhungyal & Jha, 2019).

Ethanolic extract, aqueous and chloroform extracts has exhibited significant anti-diabetic activity to control diabetic due to presence of various active constituents (Gusain & Upadhyaya, 2016).

2.3.7 Phytochemistry

Phytochemistry is the important part of the present work. Review shows some work has been carried out previously but not adequately. Phytochemical compounds were isolated from swollen base of root of *G. heterophylla*. Bioactive compounds were 0.008% β-sitosterol, 0.010% γ-sitosterol, and a pentacyclictriterpene acid having 0.001% (Ursolic acid) from roots using petroleum ether extract (Kumar *et al*., 2013). Glycosides, flavones, tannins, phenols, carbohydrates were isolated from the *G.heterophylla* (Kumar *et al*., 2013).

2.4 Ethnomedicinal uses and phytochemistry of *T. coadunata (*J.Smith) c. Christensen

2.4.1 Use in gastrointestinal ailments

Tectaria coadunata is one of the ethnomedicinally important pteridophyte used to cure stomach pains, gastrointestinal disorders, eradication of worms in children and as anthelmintic activity. The fresh rhizome and frond is used on insect bites or getting relief in centipede bites while the extraction of dried rhizome, stem and stipe is used in respiratory disorders like cold cough, asthma and bronchitis (Malviya *et al*., 2012). Rhizome decoction is also useful in colitis (Upreti *et al*., 2009).

Rhizome decoction was used to treat stomachache and gastritis, diarrhea and dysentery (Jain, 1994; Bharti, 2011; Tamang & Singh, 2015; Timilsina & Singh, 2015; Subedi, 2017; Tamang *et al*., 2017; Gubhaju & Gaha, 2019). Similarly, decoction of leaves was also given for diarrhea, dysentry and snake bites (Jain, 1994; Timilsina & Singh, 2015; Subedi, 2017; Verma & Kanwar, 2020). Young fronds are used for curing stomach trouble (Benjamin & Manickam, 2007; Singh *et al*., 2008; Benniamin, 2011; Bharti, 2011). Rhizome have also been reported to be used to treat giardiasis (Joshi *et al*., 2011), and jaundice (Subba *et al*., 2016). Young shoot boiled and given as soup for a week to treat dysentery (Uprety *et al*., 2016).

2.4.2 Use in respiratory ailments

Plant has been reported to be used in antibacterial, asthma, bronchitis (Singh *et al*., 2008). Extraction of dried rhizome, stem and stipe were used in respiratory disorders like cold cough, asthma and bronchitis (Malviya *et al*., 2012). The leaves mixed with honey or decoction of leaves was given to asthma and bronchitis patients (Parihar & Parihar, 2006).

2.4.3 Miscellaneous ailments

Leaf powder mixed with *Ricinus communis* oil is given to goats and sheep for running stomach, young leaves are chewed by cow after delivery to accelerate the expulsion of the placenta, and root paste is applied externally to stop foot itching caused by fungal growth and root is used against gonorrhea (Nwosu, 2002). Paste of fresh rhizome and fronds are used in stings of insect, honey bee and centipede (Sharma & Vyas, 1985; Parihar & Parihar, 2006; Malviya *et al*., 2012). Leaves juice is applied to the cut

wounds to stop bleeding (Jain, 1994; Timilsina & Singh, 2015; Subedi, 2017). Young shoots of *T. coadunata* are used for food (Uprety *et al*., 2012; Bhattarai & Rajbhandary, 2017).

2.4.4 Biological activities

Leaves of *Tectaria zeilanica* showed high antioxidant activity and minimum free radical scavenging activity (Maridass & Ravichandran, 2009). Methanol/methylene chloride extracts of *T. singaporeana* leaves, roots and stems showed cytotoxic potential against human breast cancer cells. Similarly, the extracts of plant proved to be useful in cancer treatment and prevention (Aini *et al*., 2008). *T. gemmifera* showed the antimicrobial properties from acetone extract (Neel *et al*., 2018). Karade & Jadhav (2018) reported that *Tecatria cicutaria* has a promising anti-cancer activity against the selected cell line.

2.4.5 Phytochemistry

The preliminary phytochemical analysis were not done well by using modern equipments which are essential to determine the secondary metabolites. However, following literatures provides basic information about secondary metabolites of the plants. GC-MS analysis of *T. coadunata* extracts exhibited abundant Palmitic acid in the leaves and Decenediol, Dodecanoic acid and Methyl stearate is in the rhizomes.

The phytochemical screening of methanol and hexane extracts of *T. coadunata* revealed the presence of polyphenols, terpenoids, saponins, tannins, alkaloids, quinones, glycosides, steroids, phenolic compounds and flavonoids. The percentage composition of different nutrition parameters (moisture, total ash, crude fat, protein, crude fiber, carbohydrate) were found to be 87.23, 1.40, 0.14, 1.76, 2.42, and 6.97% in young edible fronds (Marahatta *et al*., 2019).

T. cicutaria exhibit different compounds, 2-propanone, Silanediol, di hydroxy-dimethylsilane, hexa-siloxane, phthalicd acid, dibutylester, n-hexadecanoic acid, oleic acid, methyl ester, steric acid, 9-octadecenoic acid, 13-octadecenal and hepatadecaneby GC-MS analysis (Karade & Jadhav, 2018).

Preliminary phytochemical screening of *T. coadunata* from methanolic, aqueous and petroleumether extracts indicated the presence of carbohydrates, tannins,

anthraquinone and coumarin glycosides, steroids and flavonoids (Pawar *et al*., 2016). *T. gemmifera*, exhibited the presence of steroid, glycoside, flavonoid, tannin, phenol, terpenoids, phytosterol and saponin in rhizome while absent in leaf extract (Neel *et al*, 2017). *T. zeylanica* consists of abundant alkaloids and flavonoids (Maridass & Ravichandran, 2009).

2.5 Ethnomedicinal uses and phytochemistry of *M. azedarach* L

2.5.1 Use in gastrointestinal ailments

Melia azedarach is important ethnomedicinal plants and used in varities of ailments from the ancient time. Several works were done previously by different scientists. Plant parts were used for different traditional medicinal purposes (Sharma & Paul, 2013). Flowers oil are used to treat antidiarrheal, deobstruent, diuretic (Joy *et al*., 1998), used in nausea, vomiting and general debility, loss of appetite, stomache (Rahmatullah *et al*., 2010). Paste of bark is used to treat piles, used as lotion on ulcers, syphilitic (Sen *et al*., 2010). Decoction of bark orally used along with water in empty stomach to treat diarrhoea (de Wet *et al*., 2010; Singh *et al*., 2011), and bowel (Tamang, 2003). Bark is also used forcholera and constipation (Bhattarai *et al*., 2009). Bark and leaves are used for spleen disorders (Burlakoti & Kunwar, 2008).

Leaves paste are applied externally on burns, used as mouth wash for gingivitis; pyrexia and bloody piles (Khan *et al.*, 2011). For intestinal worms, fresh fruits without seeds were eaten (Joshi & Joshi, 2000). Seeds were used as expectorant, anthelmintic and aphrodisiac and also useful in helminthiasis, pain in the pelvic region and scrofula (Sen *et al*., 2010).

Fruit is ground and its juice is mixed with oil and given as anthelmintic (Khan *et al*., 2014), powerful vermifuge (Cropley & Haseqawa, 2007) and also used to treat vomiting, blood impurities and urinary discharge (Balami, 2004; Acharya & Acharya, 2009; Joshi & Joshi, 2009). While, dried ripe fruit used as external parasiticide (Sultana *et al*., 2011; Khan *et al*., 2014).

2.5.2 Use in pain managements

Bark is used as a remedy for fever, aches and pains (Dharani *et al*., 2010). Stems are used in toothache, rheumatic pain, fever, insecticide (Rahmatullah *et al*., 2010).

2.5.3 Use in skin ailments

Seed are used to treat skin diseases like scabies (Rahmatullah *et al*., 2010) hysteria (Husain *et al*., 2008; Kaneria *et al*., 2009). Seed oil used as antiseptic for sores and ulcers. It is also used for rheumatism and skin diseases such as ring worm and scabies. The oil is also useful in malaria fever and leprosy (Khan *et al*., 2011). Flowers are effective against bacterial skin diseases in children including cellulitis, pustules, and pyogenic infections (Joshi & Joshi, 2009; Rahmatullah *et al*., 2010).

Leaves were used on affected part (Joshi & Joshi, 2000) and hair strength (Bharali *et al*., 2018; Hussain *et al*., 2018). Flowers are used to eradicate lice and to treat skin diseases (Manandhar, 1991). Roots are used in pimples, and blood purification (Sultana *et al*., 2011).

2.5.4 Use in diabetes

Roots are used to treat diabetes (Sultana *et al*., 2011). Pericarp of fruit is very effective phytotherapy for the treatment of diabetes (Sultana *et al*., 2011; Khan *et al*., 2014).

2.5.5 Miscellaneous ailments

Stem bark were used for gonorrhea, treat malaria and to expel parasitic worms (Dharani *et al*., 2010). Stems are used for asthma (Sen *et al*., 2010). Fruits and leaves are used to treat external parasites like lice and bugs and also used for insecticides and pesticides (Jha *et al.,* 2008; Rana *et al*., 2015). The paste of leaves is used to treat boils/blisters (Kumar & Bharati, 2014).

Leaves were used to treat Snake bite (Handa *et al.,* 2006). Seeds of *M. azedarach* is a potent larvicide against vector mosquito species (Selvaraj & Mosses, 2011). They are used as astringent, refrigerant, anodyne, diuretic, resolvent, deobstruent and alexipharmic (Sen *et al.,* 2010). Leaves were used asdiuretic and was also used in emmenagogue (Manandhar, 1991).

Roots are used as astringent, anodyne, depurative, vulnerary, antiseptic, expectorant, febrifuge, antiperiodic andtonic in low doses (Sen *et al.,* 2010). Root and bark used to treat malaria (Handa *et al.,* 2006; Dharani *et al*., 2010).

2.5.6 Biological activities

Petrol fraction shows maximum inhibition against *Bacillus subtilis, Proteus mirabilis* and *Shigella flexneri* (Zone of inhibition 6 mm/1 mg/ml/disk). Benzene extract inhibited the growth of bacteria (Khan *et al*., 2008).

Seeds of *M. azedarach* contained active substances responsible for their anthelmintic action against *Haemonchus contortus*. *M. azedarach* was the most active in the inhibition of egg hatching of *H.contortus* (Maciel *et al*., 2006). Meliacine extracted from *M. azedarach* can be used as a therapeutic agent against herpes simplex virus (HSV-1) ocular infection (Petrera & Coto, 2009).

M. azedarach can be used as a source of antioxidant and antimicrobial for pharmacological preparations (Pokhrel *et al*., 2015). *M. azedarach* leaf extract had hepatoprotective activity against CCl4 (Rao Avanapu *et al*., 2013). Ethanolic extractof leaves showed alkaloids, carbohydrates, flavanoids, glycosides, saponins from mgC-MS analysis (Krishnaiah & Prashanth, 2014).

Bakayanin, Rutin, Quercitrin, Backalactone, Cystine, Serine, Arginine, Glycine, Glutamic acid and Proline, were isolated from leaves, root and seeds. It was also used for Leprosy, Scrofula, Anthelmintic, Antilithic, Diuretic, Deobstruent, Resolvent, Sores, Ulcers, Ringworm, Scabies, and Malaria (Qasim *et al.,* 2016).

M. azedarach leaves have potent antiurolithiatic activity against ethylene glycol-induced calcium oxalate urolithiasis in male albino rats (Dharmalingam *et al*., 2016). The whole plant of *M. azedarach* is a good source for the synthesis of zinc oxide nanoparticles (Manokari *et al*., 2016).

Limonoids and triterpenes were isolated from the seeds of *M. azedarach*. Antimicrobial activities were also measured (Liu *et al*., 2011). The plant was used as biopesticide, cholinesterase and antioxidant activity. Phenolic acids were also isolated. Btyrylcholinesterase inhibition was observed in the extracts (Orhan *et al*., 2012).

The presence of rutin and quercetin in ethonolic leaves extract of *M. azedarach* could be a potential source of natural anti-oxidant (Rao *et al*., 2013).The leaves of the plant is potential candidate for development of anti-hyperglycemic formulation (Sulistiyani *et al*., 2016).

Limonoids showed significant inhibitory activities against tested cell lines (Yuan *et al*., 2012). The dichloromethane fraction of the stem barkand methanolic extract of root, stem bark and leaves showed a broad spectrum of antibacterial activity (Khan *et al*., 2001). From fruit of the plant, the cytotoxicity of the isolated compounds toward the human lung adenocarcinoma epithelial cell line A549 was determined (Ntalli *et al*., 2010). Biological activities are observed as antifeedant activity (Nakatani *et al*., 1998).

2.5.7 Phytochemistry

Three compounds (20S)-5, 24 (28)-ergostadiene-3β, 7α, 16β, 20-tetrol, (20S)-5-ergostene-3β, 7α, 16β, 20-tetrol and 2α, 3β-dihydro-5-pregnen-16-one were isolated from *M. azedarach.* The isolated compounds showed significant cytotoxic effects against two human cancer cell lines A549 andH460 (Wu *et al*., 2009).

A novel cinnamoyl glycoside, cinnamoyl-1-A-L-rhamnoside, and a novel acylated quercetin triglycoside, quercetin-3-O-[rhamnosyl 1→6 (4″-lactoyl glucoside)]-4′-O-glucoside have been isolated from the leaves of plant. Kaempferol-3-O-rutinoside, 3-O-rhamnoside, quercetin-3-O-rutinoside, 3-O-rhamnoside, the aglycones quercetin and kaempferol were isolated compounds from leaves (Salib *et al*., 2008).

Two limonoids, one triterpenoid, one steroid, and one sesquiterpenoid, along with other known limonoids, were isolated from the bark of *M. azedarach*. *M. azedarach* contains highest amount of phenolic compounds exhibited the greatest anti-oxidant activity (Rao *et al*., 2013). Flavonols are abundant in the leaves of *M. azedarach* as reported by Jafari *et al*. (2013) and azedaralide and 12 alpha acetoxyfraxinellone were also isolated (Nakatani *et al*., 1998).

Watanabe *et al. (*2005) isolated compounds (Azaridin, azadirachtin, bakalactone, bakayanin, benzoic acid, deacetylsalanin, dihydronimocinol, fraxinellone, quercetin, meliacarpinin, meliacine, meliotannic acid, melazolide, nimbolinin, rutin, salanin, salannal, vilasinin) from leaves of *M. azedarach.*

Y-Sitosterol, Dithiocarbamate, Smethyl-, N- (2methyl-3-oxobutyl), Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl) ethy, Trichloromethane, 2-Piperidimethanamine 6 etc were isolated from *M. azedarach* (Ali *et al*., 2015).

Bakayanin, Rutin, Quercitrin, Backalactone, Cystine, Serine, Arginine, Glycine, Glutamic acid and Proline were isolated from leaves, roots and seeds (Qasim *et al*., 2016).

CHAPTER 3

3. MATERIALS AND METHODS

Following plants are selected for the present research work.

3.1 Plant materials

Girardinia diversifolia (Link) Friis

Family: Urticaceae

Nepali: *Allo*; Gurung: *Paasyar*; Rai: *Rakchiki*; Sherpa: *Longjo*; Tamang: *Paachyar*

Photo plate 1: *Girardinia diversifolia (*Link) Friis

Tall and bushy perennial herb, up to 2 m high, bearing stinging hairs throughout, occurring in subtropical and temperate zones, with leaves broadly ovate, palmately 3-7 lobed, sometimes lower leaves unlobed, and flowers unisexual, male and female flowers in separate plants, in panicles. Plants could be easily identified due to its palm shaped leaves and stinging in nature (Photo Plate 1).

Tectaria coadunata (J. Sm.) C. Chr

Family: Dryopteridaceae. Tectariaceae

Nepali: *Kaali Nyuro, ball kuthurke;* Chepang: *Gorgan;* Gurung: *Mhro-kuta, Yolpakuta;* Tamang: *Toplign devil*

Photo plate 2: *Tectaria coadunata (*J. sm.) c. Chr.

Terrestrial, erect fern about 70 cm high with dark brown stalk and creeping rhizomes. Fronds 25-35 cm long, 14-20 cm wide, deltoid-oblong, bipinnatifid at the base and apex pinnatifid, pinnae large with blunt lobes, sori conspicuous, bright brown in two rows near the main vein (Photo Plate 2).

Melia azedarach Linnaeus

Family: Meliaceae

Nepali: *Bakaino;* Chepang: *Bakaina;* Danuwar: Bakainu; Gurung: *Bakainu*; Magar: *Bakainu*; Tamang: *Bakaina*

Photo plate 3: *Melia azedarach*Linnaeus

Deciduous tree about 10 m high. Leaves stalked, large, bi-or tripinnate, leaflets 3-11 in each pinna, short-stalked, 3-9 cm long, 0.8-2.5 cm wide, ovate to lanceolate, acuminate, serrate or subentire, glabrous. Flowers lilac-blue with fragrance. Fruit a drupe, ellipsoidal, yellowish when ripe, wrinkled and remaining long on the tree after ripening. The plant is distributed throughout Nepal at 700-1700 m in open places around the villages (Photo Plate 3).

3.2 Collection of plant materials

Plants were collected from Sindhuli Gadi, Sinduli, south of Kathmandu and Suryabinayak, Bhaktapur as tabulated in the table with altitude and GPS locations (Table 1). Aerial parts of *Girardinia diversifolia* were collected during October from Sindhuli Gadi, Sinduli area (1332-1394 m). The collected material included mostly leaves and flowers. Rhizomes of *Tectaria coadunata* were collected from Dakshinkali, Kathmandu area (1400-1509 m) in November. Fibrous roots were completely removed and the rhizomes were washed properly to remove soil particles. Leaves of *Melia azedarach* were collected during October from Suryabinayak, Bhaktapur area (1290-1301 m) (Voucher specimen: Appendix 1). Fresh plant material of all three species was air dried by cutting into small pieces and spreading in a shaded area for 2-3 weeks. After the plant material was completely dry it was grinded into fine powder with the help of an electric grinder.

Table 1: Collection of plants from different areas of Nepal

SN	Name of plant	Collection area (Collection no.)	Altitude	GPS
1.	*Girardinia diversifolia* (Link) friis	Sindhuli Gadi, Sindhuli (S001)	1332-1394	27°16'43"N 85°57'31"E
2.	*Tectaria coadunate* (J.Sm.) C. Christensen	Dakshinkali, Kathmandu (S002)	1400-1509	27°36'02"N 85°15'04"E
3.	*Melia azedarach* L.	Suryabinayak, Bhaktapur (S003)	1290-1301	27°39'09"N 85°22'49" E

3.3 Identification

After the collection and drying of the plant material, herbarium was prepared following standard herbarium techniques (Bridson & Forman, 1998). Plant materials were identified by tallying with the herbarium specimens deposited at National Herbarium and Plant Laboratory (KATH), Godawari, Lalitpur, Nepal. The herbarium has been submitted to TUCH (Tribhuvan University Central Herbarium), Kirtipur. Ethnobotanical information was collected through different literatures.

3.4 Extraction

The extract was extracted from the powder of plant material.The solvent was methanol. Intermittent sonication was used for percolation. A quantity of 10 g of plant powder was taken for the extraction. On the other hand 100 mL of methanol was measured and mixed in the ratio of 10:1. The mixture was taken into tube with lid so that it could not poured out from the tube. The tube was subjected into intermittent sonication for 2 hours. The sonication cycle was fixed at 30 kHz for 30 min (with 10 min interruption). What man filter paper no.1 was used to filter the solution, the company was Whatman Ltd., Kent, UK. The filtrate was evaporated with the help of rotary evaporator at reduced pressure. Finally the extract was obtained. It was kept inside glass tube for further experiments. The exact weight was taken. Then it was stored at 4 degree celcius .This is called methanolic extract. *G. diversifolia* extract yield was 14%. 15.5% for *T. coadunata* extract yield *was 15.5%. M. azedarach* extract yield was 24.21%. Methanol extracts of plant materials was used for phytochemical analysis for all three species.

For *G. diversifolia*, further separation techniques was used by methanolic extract. The obtained dried extract was mixed with water in the ratio of 9:2. The other solvents were cyclohexane, dichloromehtane and ethyl acetate. The separated solvents were collected .Drying method was used for dry with the help of sodium sulphate. The

cyclohexane yield was 18%. Dichloromethane was 8%. Ethyl acetate yield was 6%. The obtained extracts were store in 4 degree celcius temperature for further experiments.

Extraction of plant materials of all three species were done at Central Department of Botany, Tribhuvan University, Nepal, while fractionation, isolation of phytoconstituents, identification, quantification and purification, total phenolic content, antioxidant, and enzyme inhibitory assays and cytotoxicity were all done at Natural Product Lab, Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Italy (Photo plate 4).

3.5 Phytochemical analysis for *G. diversifolia, T. coadunata*and *M. azedarach*

3.5.1 Isolation of phytoconstituents

For all three plant materials Column chromatography (CC) and thin layer chromatography (TLC) were used to get crude compounds. Purification of these compounds was done using high performance liquid chromatography (HPLC). Structures of isolated compounds were elucidated by spectroscopic methods using ultraviolet (UV), infra red (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS). Methanolic extract of *G. diversifolia (*8 g) was taken. Column chromatography was used as separation technique. One litre of dichloromethane and methanol was taken in the ratio of 95:5. The mixture was tappered in the following way. In the ratio of 90:10 in 500 mL like wise in the ratio of 80:20 in 500 mL. Another ratio of 50:50 of 500 mL was also taken for separation. The methanol was used as moblile phase (Dall'Acqua *et al*., 2017) with slight modification.

There were 85 fraction obtained from chromatographic separation. TLC was used to separate fractions. The solvents for TLC was dichloromethane and methanol 5%. The fractions were mixed in same flask with their similar behaviour. The Fractions obtained were 11-13 (250 mg) and 14-16 (450 mg). Due to their similar behaviour they were collected in same flask. The amount was 750 mg. The method used was silicagel column chromatography by dichloromethane (2L) as solvent. TLC was used for identification of similar behaviour of the compounds. They were collected in round bottom flask and dried with the help of rotavapour.

Preparative TLC (silica gel 60 F-254) was used for separation of compounds. The two fractions were obtained with the help of praparative TLC. Beta sitosterol (32mg) and

gamma sitosterol (11 mg) was obtained. Both were characterised with the help of nuclear magnetic resonance and mass spectrometry.

Similarly, methanolic extract of *T. coadunata (*15 g) was taken for separation process. Silica gel column chromatography was used for separation process. The moble phase solvent was methanol 1%. (Dall'Acqua *et al*., 2017). TLC was used to analyse compound identification with the help of ethyl acetate cylclohexane mixture in the ratio of 2:1 and chloroform and methanol in the ratio of 99:1. The obtained similar colours were collected in the same flask.There were altogether 100 fractions of 20 mL were obtained. The similar behaviour mixture were collected in same flask. Fractions collected from 23 to 45 were collected in same flask. At the end of the experiments, the ethyl acetate fractions were 3.15 g and water fractions were 11.83 g were obtained.

Water extract (8g) was further purified to get clear picture of the various compounds. For this methanol water 50% (0.5mL/min), column 2cm×40cm volume was used for separation. The separated Fractions were collected with the help of their similar behavious in the TLC. Further, for the separation technique, preparative HPLC Varian 920-LC was used. The HPLC was equipped with column oven and UV-Vis detector. The separation was achieved through Agilent ZORBAX SB-C-18 (21.2×150 mm, particle size 5 µm) as stationary phase. The injection volume was 200 µL, the flow was 3 mL/min, and the temperature was set at 35°C. The UV and Vis lamps were set at 280 and 454 nm, respectively. The mobile phase was 2% formic acid in water (A) and acetonitrile (B). A gradient program was used as follows: (0→30 min: A:B (95:5)→A:B (50:50)30→50 min: A:B (50:50)→A:B (0:100)50→55 min: A:B (0:100)→A:B (0:100)55→60 min: A:B (0:100)→A:B (95:5)).

Luteoline-7-O-glucuronide was obtained (10 mg) from the water extract. The structure was also identified with the help of 1D-2D-NMR and using mass spectrometry. Eridictiol-7-O-glucuronide (5 mg) was alos separated from ethyacetate fraction with allowing to same procedure. The structure was confirmed by 1D 2D NMR and using mass spectrometry. NMR spectra were obtained on a Bruker Avance III 400 Ultrashield spectrometer with a superconducting 400-MHz magnet. Data were acquired in MeOD-d4 (Sigma-Aldrich) using Durian® 4.95-MM NMR tubes (Durian Group). Chemical shifts are expressed in δ values in ppm. 1H-NMR and HSQC-DEPT, HMBC, and COSY experiments were acquired using standard Bruker sequences measuring p1 and d1 for each acquired sample.

The methanolic extract of *M. azedarach (*10 g) was dissolved in 50 ml of CH_2Cl_2 was separated through a silica gel column chromatography using 500 ml of CH_2Cl_2, then 500 ml of 1% MeOH in CH_2Cl_2, 500 ml of 5% MeOH in CH_2Cl_2, 500 ml of 10% MeOH in CH_2Cl_2 and finally MeOH and CH_2Cl_2 50:50. 112 fractions were collected and analyzed via TLC using 1% of MeOH in CH_2Cl_2 as mobile phase.The fractions presenting similar behaviour were pooled on the basis of chromatographic behaviour, the solvent was removed, and they were analyzed through NMR using $CDCl_3$ as solvent (Dall'Acqua *et al*., 2017) with some modification. Samples from M7 to M25 were combined, dissolved in 15 ml of methanol and further separated by gel permeation chromatography. The stationary phase was TELOS Sephadex LH20 and the mobile phase was 300 ml of acetone and then 100 ml of methanol, with a flow of 0.5 ml/min.

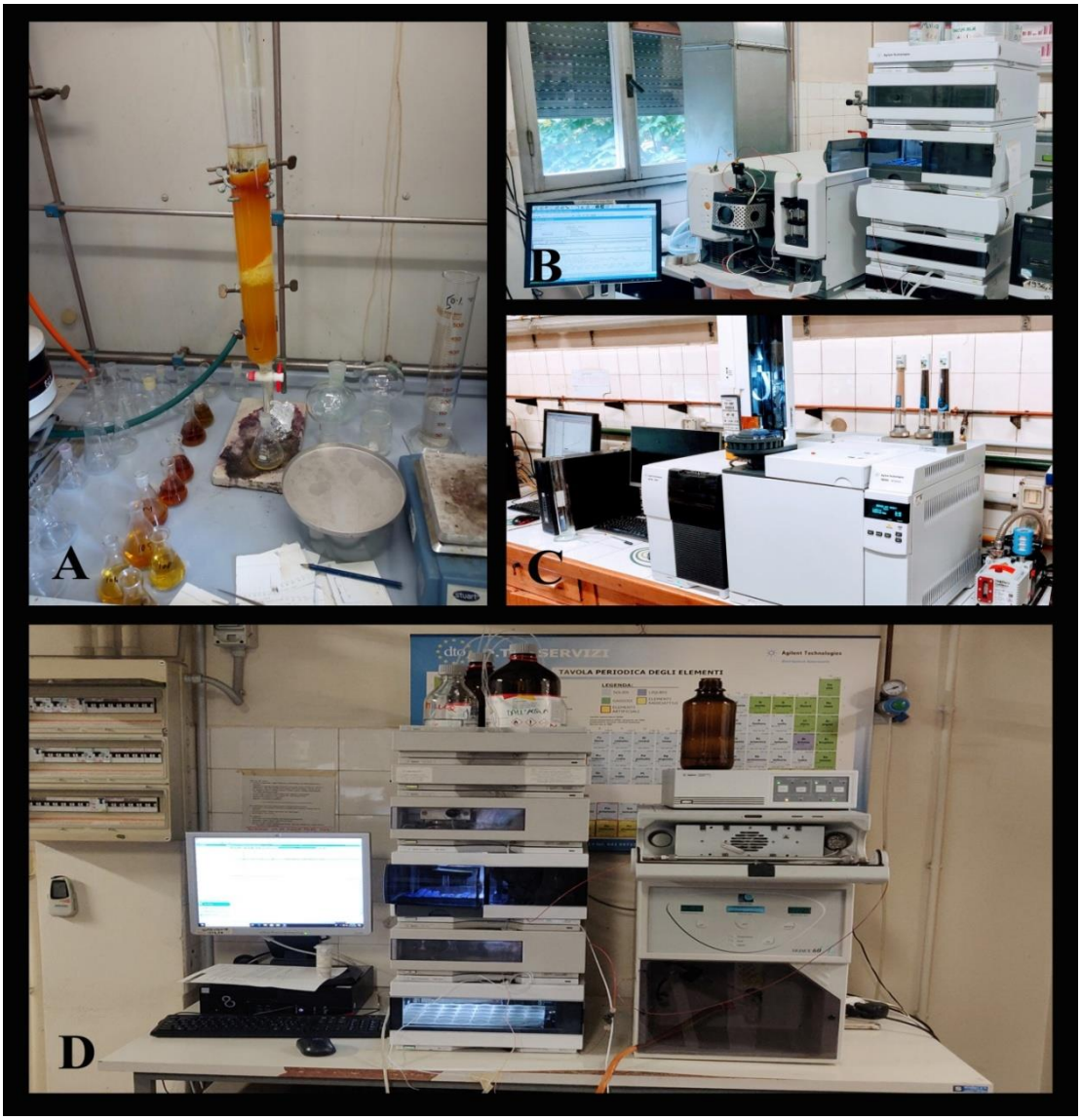


Photo plate 4: A. Chromatographic process; **B.** Liquid Chromatography (LC)-MS; **C.** Gas Chromatography (GC)-MS; **D.** High Performance Liquid Chromatography (HPLC)

3.5.2 HPLC analysis for compound identification, quantification and purification of *G. diversifolia, T. coadunata* and *M. azedarach*

a. HPLC-DAD-APCI-MS of phytosterols, HPLC-DAD-APCI for carotenoids, and HPLC-DAD-ESI-MS for phenolic and saponins of *G. diversifolia*

HPLC is High performance liquid chromatography. It is also called liquid chromatography because mobile phase is liquid. Agilent 1260 (Santa Clara, CA, USA) was used to analysis of the obtained extracts. It was associated with 1260 diode array dector. A Varian MS-500 ion trap mass spectrometer was also associated with ESI and APCI ion resources. There was T shaped splitter which separated the flow rate. The half of the mobile phase went to DAD and another half went to ion trap mass spectrometer. This allowed separation of the compounds. This was placed at the terminal side of the column. This acts to purify the compounts in the extracts. Ultravoilet visible spectra were obtained in 190-400 nm of range. With the help of ESI positive and negative ion mode, mass spectrometry spectra were obtained. APCI is also obtained in positive ion mode. A turbo data-dependent scanning (TDDS) was also used to obtain ionic mode of the compounds. This provides mass fragmentation i.e. positive and negative ions. Ethyl acetate solvent was used to analyze phytosterols and terpene alcohols in *G. diversifolia*. For it 100 mg of plant material was taken and 30 mL of the ethyl acetate solvent was used.

The obtained liquid was collected and filtered. The total volume was 5 mL. The obtained solvent was concentrated (Dall'Acqua *et al*., 2017) with slight modification. The stationary phase is usually inside the instrument. Agilent ZORBAX Eclipse XDB-C18 column (3.0 mm x 150 mm, 3.5 µm) was the stationary phase column. This is used to obtain terpene and phytosterol compounds. The mobile phase was water (0.1% formic acid) (A), acetonitrile (B) and methanol (C). A gradient program was used as follows: [0→8th min: A:B:C (2:95:3)→ A:B:C (2:95:3)8 → 10^{th} min: A:B:C (2:95:3)→ A:B:C (2:0:98)10→28^{th} min: A:B:C (2:0:98)→ A:B:C (2:0:98)28→30th min: A:B:C (2:0:98)→A:B:C (2:95:3)30→35th min: A:B:C (2:95:3)→A:B:C (2:95:3)]. The flow of the mobile phase was adjusted in such a way that the rate was 0.4 mL/min, injectable volume was 10 µL and the temperature was maintained at 30°C. Mass spectrometry spectra were obtained from the range of m/z 100-2000, using an APCI ion source operating in positive ion mode. At the end identification of compounds was carried out with the help of literature data and information from

reference compounds. The reference compounds were available at the market. Beta-sitosterol (176 µg/mL) and stigmasterol (185.6 µg/mL) were used as standard reference compounds. The quantification of the standard compounds were obtained by calibration curve. For the quantification of the beta sitosterol, beta sitosterols was applied as reference compounds. To quantify fucosterol, gamma sitosterol was used as reference compounds. Calibration curves were as follows: y = 0.63x+0.2705 (R2=0.99611) for β-sitosterol; y = 2.1153x−15.216 (R2 = 0.9949) for stigmasterol. For Saponins and polyphenols analysis, Synergi Polar-RP (Phenomenex, Italy Bologna) was carried out as the stationary phase (3.0×150 mm; 4 micron); water, and 1% formic acid (A) and methanol (B), were used as eluents. The gradient began using 95% of solvent 'A' and went to 25% of 'A' in 15min, then 0% 'A' in 20min. The flow rate was adjusted as 0.4 mL/min. Diod array dectector chromatograms and Ultravoilet visible spectra were obtained in the range of 200-650 nm. Mass spectrometry spectra were recorded in negative or in positive ion mode in the 50-2000 Darange, using an ESI ion source. Quantification of compound was obtained using DAD for hydroxycinnamic acid derivatives (chlorogenic, caffeic, syringic) chlorogenic acid solution was used (100-1 µg/mL) as a reference standard and the used wave length was 330 nm.

The calculation of the calibration curve was Y=1.325x+0.236 (R2 =0.9989). For flavonols, vitexin solutions (20-1 µg/mL) were used at 350 nm and the calibration curve was Y = 12.3x + 0.325. For saponins, escin was used as a reference compound and a 205 nm wavelength was used (100-10 µg/mL) Y = 0.325x + 0.023 (R2 = 0.9986). For the analysis of carotenoids, a YMC carotenoid column (4.6×300 mm, 5 micron) was used as the stationary phase. Mobile phases were methylterbuthyl ether/methanol 90/10 (A) and methanol (B). The gradient started with 10% 'A' and in 12 min reached 100% 'A'. The flow rate was adjusted in such a way as 1.3 mL/min. The reference compounds used were β-Carotene and zeaxanthin, preparing solution in the range 40-0.5 µg/mL. For the acquisition of Mass spectrometry spectra, APCI was used in positive ion mode, acquiring spectra in the m/z range of 365-1000 atomic mass unit in TDDS ion mode.

b. HPLC HILIC-DAD-FLD-ESI-MS for *T. coadunata*

10 mg of the extracts were taken. The taken extracts were methanolic, water and ethyl acetate. They were mixed with 1 mL of methnol. The ratio was 1:10. Vials were

collected and 1mL of diluted solution was kept inde the vial. All the samples were prepared for three times. In order to analyze MeOH, EtOAc, and H_2O extracts, HPLC-DAD-FLD-ESI-MS was performed using a Chromatograph Agilent 1260 apparatus (Santa Clara, CA, USA) equipped with a 1260 auto sampler, column oven, diode array detector (DAD), and fluorescence detector (FLD). The column was prepared. The flow was divided by two T shped connector. Diode array dector received 50% of the mobile phase, 25% to FLD, and finally VarianMS-500 ion trap mass spectrometer received er 25%. Separation was carried out with the help of TOSOH TSK gel amide-80 (2.1×150 mm, particle size 3.5 µm) as stationary phase. The initial sample injection volume was 10 µL, the flow rate was 0.25 mL/min, and 35°C was the temperature inside the column. Ultravoilet visible spectra were obtained from the range of 190-640 nm. 1% formic acid in water (A) and acetonitrile (B) were used as mobile phase. A gradient program was used as follows: (0→20 min: A:B (1:99)→A:B (20:80)20→25 min: A:B (20:80)→A:B (20:80)25→45 min: A:B (65:35)→A:B (65:35)45→ 67 min: A:B (85:15)→A:B (1:99)69→75 min: A:B (1:99)→A:B (1:99). Mass spectrometer spectra were collected in the mass charge ratio of 100-2000, using ESI ion source operating in negative ion mode. Positive and negative ions were fragmented. Fragmentation of the ionic mode were obtained using the turbo data dependent scanning (TDDS) instrument function. The literatures and referece compounds were used to identify various compounds. The fragmented ions also purify the obtained compounds. DAD and FLD detectors were used to estimate the amount of PAC s and flavonoids and to acquire spectral data of eluted compounds. As reference compounds, PAC A2 (Sigma Aldrich, St. Louis, MO, USA) and luteolin (Sigma Aldrich) were used. The chromatograms were monitored in FLD for Procyanidins (excitation 230 nm, emission 321 nm; scan range 200-500 nm), whereas flavonoids were monitored at 350 nm; Ultravoilet visible spectra were acquired from the range of 190-640 nm. Calibration curve was used for the quantification of the obtained compounds: Procyanidin A2 was used as external standard for Procyanidins quantification, while luteolin was used for flavonoids. Calibration curves were as follows: $y = 6.6721x + 8.6153$ ($R2 = 0.9991$) for PAC A2; $y = 127.77x-2.4$ ($R2 = 0.9998$) for luteolin.

c. HPLC-DAD-ESI-MS for *M. azedarach*

Qualitative analysis of the compounds for *M. azedarach* in the methanolic extract was carried out by HPLC-DAD-ESI-MS. The measurements were performed using an

Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with 1260 diode array detector (DAD) and Varian MS-500 ion trap mass spectrometer. Sample was analysed by using an Agilent Eclipse XDB C-18 (3.0 × 150 mm, particle size 3.5 μm) as stationary phase (Dall'Acqua *et al*., 2017). The mobile phase was acetonitrile (A) and water (0.1% formic acid) (B). To obtain the good results, the procedure was followed as: [0 → 30th min: A:B (10:90)→ A:B (100:10)30 → 35th min: A:B (100:10)→ A:B (100:10)35 → 36th min: A:B (100:10)→ A:B (10:90)36→40th min: A:B (10:90)→ A:B (10:90)]. Flow rate was adjusted in 0.4 mL/min, injection volume was 20 μL and the temperature was set at 40°C. At the end of the column, a "T" splitter separated the flow rate to DAD and MS. UV-Vis spectra were acquired in the range of 190-640 nm. MS spectra were recorded in the range of *m/z* 100-2000, using ESI ion source operating in negative and positive ion mode. Turbo data dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species. Identification of compounds was obtained based on fragmentation spectra as well as the comparison with the literature and reference compounds, when available.

To detect limonoids, azadirachtin was used as reference compound. A HPLC-DAD-APCI-MS was performed using the same instrument. Sample analysis was achieved using an Agilent C18 Eclipse (3.0 × 150 mm, particle size 3.5 μm) as stationary phase. The mobile phase was acetonitrile (A) and water (0.1% formic acid) (B). The procedure was prepared and followed as: [0 → 28th min: A:B (20:80)→ A:B (99:1)28 → 32th min: A:B (99:1)→ A:B (99:1)32 → 33th min: A:B (99:1)→ A:B (20:80)].Flow rate was 0.4 mL/min, injection volume was 20 μL and the temperature was set at 40°C. At the end of the column, a "T" splitter separated the flow rate to DAD and MS. UV-Vis spectra were acquired in the range of 190-640 nm. MS spectra were recorded in the range of *m/z* 300-1000, using APCI ion source operating in positive ion mode. Spray chamber was set to 50°C, drying gas 15psi, nebulizer 25 psi, drying gas temperature start at 285°C and decrease to 270°C in 25 minutes, vaporizing temperature from 295°C at 270°C in 25 minutes. Corona current was set to 5 microamperes, spectra were acquired using turbo data dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species. Capillary 80V, RF loading was 80%. As reference a standard solution of azadirachtin was used with a concentration range of 100-0.2 μg/mL.

To measure the phytosterols content in the extract, HPLC-APCI-MS method was used (Sut *et al*., 2018). Briefly as stationary phase an Agilent Eclipse XDB-C18 column (3.0 mm x 150 mm, 3.5 µm) was used. The mobile phase was composed by water (0.1% formic acid) (A), acetonitrile (B) and methanol (C). A gradient program was used as follows: [0 → 8th min: A:B:C (2:95:3)→ A:B:C (2:95:3)8 → 10th min: A:B:C (2:95:3)→ A:B:C (2:0:98)10 → 28th min: A:B:C (2:0:98)→ A:B:C (2:0:98)28 → 30th min: A:B:C (2:0:98)→ A:B:C (2:95:3)30 → 35th min: A:B:C (2:95:3)→ A:B:C (2:95:3)] with a flow rate of 400 uL/min, the injection volume was 10 µL and the oven column temperature was 30°C. MS spectra were recorded in the range of m/z 100-2000, using Atmospheric Pressure Chemical Ionization (APCI) ion source working in positive ion mode. The turbo data-dependent scanning (TDDS) instrument function was used reveal the fragmentation of the main ionic species. Compounds were identified by comparison with the literature data and using reference compounds. As standards, solutions of β-sitosterol at four different levels (concentration ranges 176-1.76 µg/mL) and stigmasterol (concentration ranges 185.6-1.856 µg/mL) were used.

Compound quantification was obtained with the method of calibration curve: β-sitosterol was used as the external standard for β-sitosterol and γ-sitosterol quantification, while stigmasterol was used for fucosterol. Calibration curves were as follows: $y = 0.64x + 0.2302$ ($R2 = 0.9993$) for β-sitosterol; $y = 2.0987 x - 13.816$ ($R2 = 0.9989$) for stigmasterol.

3.5.3 GC-MS analysis for *G. diversifolia* and *M. azedarach*

Methanolic extract of *G. diversifolia* and *M. azedarach* was derivatized with MeOH in presence of $H2SO4$, for Gas chromatography and mass spectrometry analysis of the fatty acid content. Fatty acid methyl esters were stabled in Gas chromatography analysis, they were obtained from esterification fo fatty acids (Dall'Acqua *et al*., 2017). A quantity of 139.1 mg of *G. diversifolia* and 77 mg of *M. azedarach* extract was added, with 15mL of Methanol, 1mL of dichloromethane, 3 drops of sulphuric acid and 25.7 mg of methyl pentadecanoate (Sigma Aldrich, St. Louis, MO, USA), used as internal standard. Under the reflux condition, the mixture was heated for 1hour then cooled in a ice bath. A liquid-liquid partition was performed with10mL of water and 5 mL of diethyl ether, and then the organic phase was collected and dried. The residue was re-dissolved with1.5mL of diethylether and put in vial. Gas

chromatography and mass spectrometry analysis was performed through an Agilent 7820A coupled with a single quadrupole mass spectrometer Agilent 5977B MSD, using a HP88 (60 m×0.25 mm, 0.2 μm film thickness) stationary phase. Helium was taken as a carrier gas, with a column head pressure of 14.1 psi. The flow rate through the column was 1.19 mL/min. The injector was set at 300°C with a split ratio of 20:1, the split flow was 23.9 mL/min and 1 μL injections were made. The temperature gradient started with a 120°C initial temperature with a linear increase to240°Cat 3°C/min. The total run time was 55 min. MS spectra were recorded in the range of m/z 40-650, using an EI ion source operating in positive ion mode.

3.6 Assays for total phenolic and flavonoid contents for *G. diversifolia, T. coadunata* and *M. azedarach*

The total phenolic content was carried out by applying the procedure which were provided in the previous literatures with some slight modification. Diluted Folin-Ciocalteu reagent (1 mL, 1:9, v/v) sample was taken and it was mixed with sample solution (0.25 mL). The samples were thoroughlly mixed. Na_2CO_3 solution (0.75 mL, 1%) was added after 3 minutes. The absorbance was taken at 760 nm after 2 hrs. incubation at room temperature. The total phenolic content was expressed as milligrams of gallic acid equivalents (mg Gallic acid E/g extract) (Uysal *et al*., 2017) with some modification.

The $AlCl_3$ method was used to calculate the total flavonoid content. The plant extracts sample solution (1 mL) was taken. It was mixed with equal amount of aluminum trichloride (2%) in methanol. Sample solution (1 mL) to methanol (1 mL) without $AlCl_3$ was as blank solution.The reading at 415 nm after 10 min incubation at room temperature were taken of both sample and blank solution. The absorbance of the blank was subtracted from that of the sample. Rutin was used as a reference standard and the total flavonoid content was expressed as milligrams of rutin equivalents (mg RutineE/g extract) (Uysal *et al*., 2017) with some modification.

3.7 Determination of antioxidant and enzyme inhibitory effects for *G. diversifolia, T. coadunata* and *M. azedarach*

Antioxidant (DPPH and ABTS radical scavenging, reducing power (CUPRAC and FRAP), phosphomolybdenum and metal chelating (ferrozine method)) and enzyme inhibitory activities (cholinesterase (Elmann's method), tyrosinase (dopachrome

method), α-amylase (iodine/potassium iodide method), and α-glucosidase (chromogenic PNPG method) were calculated. The previously mentioned antioxidant and enzyme inhibitory activities were carried out according to Uysal *et al*. (2017) and Grochowski *et al*. (2017) with some slight modification.

Initially antioxidant activities were carried out with the help of DPPH (1, 1-diphenyl-2-picrylhydrazyl). DPPH solution with 0.004% in methanol was added to 4 mL extract solution. The absorbance was taken at 517 nm in spectrophotometer. The reading was taken after 30 min of incubation at room temperature inside dark. The antioxidant capacity DPPH was taken in milligrams of trolox equivalent (mg TroloxE/g extract). The other experiment was followed by ABTS antioxidant activities.

ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline) 6-sulfonic acid) was taken for the assay.All the experiments were carried out inside dark room inorder to protect exposure from the sun light. Potassium persulfate amounting 2.45mm was taken. It was added with ABTS+ which was carried out instantly by mixing 7 mm ABTS solution. Initially ABTS solution was taken. It was diluted with MeOH. The absorbance of 0.700 ± 0.02 at 734 nm was taken. The sample plant extract solution also taken. It was mixed with ABTS solution (2 mL). After 30 minute of incubtion period at room temperature, the absorbance was taken at 734 nm with the help of spectrophotometer. The ABTS antioxidant activities was taken as milligram of trolox equivalents (mg TE/g extract).

CUPRAC (cupric ion reducing activity) experiment was under taken for finding out antioxidant activities in plant extract. The extract solution of 1 mL was taken. It was premixed with $CuCl_2$ (10 mm), neocuproine (1 mL, 7.5 mm) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Then blank was prepared for the further study. The blank was prepared adding with plant sample solution (0.5 mL) and premixed reaction mixture (3 mL) without $CuCl_2$. The added solutions were kept for incubation for 30 minute inside the dark room. The absorbances were taken at 450 nm in spectrophotometry. Finally, CUPRAC antioxidant activity was calculated by subtrating the blank from extract sample readings.

Premixed FRAP reagent (2 mL) with acetate buffer (0.3 M, pH 3.6), 2, 4, 6-tris (2-pyridyl)-S-triazine (TPTZ) (10 mm) in 40 mm HCl and ferric chloride (20 mm) in a

ratio of 10:1:1 (v/v/v) was taken. The plant extract sample solution was mixed in it. The whole experiment was kept inside the room for 30 minutes. The temperature was maintained at room temperature. The plant extract sample absorbance was taken at 593 nm by spectrophotometer. FRAP antioxidan activity was taken as milligrams of trolox equivalents (mg TE/g extract).

Phosphomolybdenum antioxidant was calculated in the next experiment. The plant extract solution was taken. This was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mm ammonium molybdate). The incubation period was fixed for 90 minutes at 95 degree celcius. After then plant extract sample absorbance was taken at 695 nm from spectrophotometer. The antioxidant activities was calculated in millimoles of trolox equivalents (mmol TE/g extract).

The another experiment was metal chelating antioxidant activity. The plant extract sample was taken. It was mixed with $FeCl_2$ solution (0.05 mL, 2 mm). 5 mm ferrozine (0.2 mL) solution was already added in the mixture for proper reaction completed. The plant extract sample solution (2 mL) to $FeCl_2$ solution (0.05 mL, 2 mm) and water (0.2 mL) without ferrozine were mixed. This was taken as a blank. Finally incubation period was fixed for 10 minutes at room temperature. The observations of absorbance were taken at 562 nm in spectrophotometer. Both the plant extracts sample and blank observations were taken for the further calculation to determine metal chelatin antioxidant activities. The metal chelatin antioxidant was calculated by milligrams of EDTA (disodium edetate) equivalents (mg EDTAE/g extract).

Enzyme inhibition activites were calculated in further experiments. Cholinesterase (ChE) inhibitory activites were under taken initially. The plant extract sample solution (was added in DTNB (5, 5-dithio-bis (2-nitrobenzoic) acid, Sigma, St. Louis, MO, USA) (125 µL) and AChE (acetylcholines-terase (Electric ell acetylcholinesterase, Type-VI-S, EC 3.1.1.7, Sigma)), or BChE (butyrylcholinesterase (horse serum butyrylcholinesterase, EC 3.1.1.8, Sigma)) solution (25 µL) in Tris-HCl buffer (pH 8.0). The solution were transfered in a 96-well microplate. Finally incubation period was fixed for 15 min at 25°C. The experiment was under taken mixing with acetylthiocholine iodide (ATCI, Sigma) or butyrylthiocholine chloride (BTCl, Sigma) (25 µL). After then blank was taken. Blank was obtained by mixing plant extract sample solution and reagents without enzyme (AChE or BChE) solution. The

incubation period was fixed for 10 minutes at 25 degree celcius inside the room. Absorbances at 405 nm were taken both of plant extract sample and cholinesterase solution. The cholinesterase inhibitory activity was calculated by subtracting from the plant extract sample to cholinesterase inhibitory readings. The experiment was calculated by galanthamine equivalents (mgGALAE/g extract).

Tyrosinase inhibitory activity was also calculated. The plant extract sample solution was added with tyrosinase solution (40 μL, Sigma) and phosphate buffer (100 μL, pH 6.8). The mixture was transfered into 96-well microplate. The incubation period was fixed for 15 min at 25°C. Finally, L-DOPA (40 μL, Sigma) was added for the expected reaction. The blank was prepared. It was added plant extract solution and reagents which was without tyrosinase enzymes. The incubation was fixed for 10 minutes at 25°C.The observation of absorbances were taken at 492 nm. The observations were taken both of plant extract sample and blank. The blank observations were calculated by subtrating from plant extract sample. The tyrosinase inhibitory activities were calculated by kojic acid equivalents (mgKAE/g extract).

Alpha-amylase inhibitory activity was carried out. The plant extract sample solution was added with α-amylase solution (ex-porcine pancreas, EC 3.2.1.1, Sigma) (50 μL) in phosphate buffer (pH 6.9 with 6 mm sodium chloride).The solution was transferred in a 96-well microplate. The incubation period was fixed for 10 min at 37°C. Before incubation, pre-incubated, starch solution (50 μL, 0.05%) was added for the proper reaction. Finally, blank was prepared. It was mixture of plant extract sample and reagents without enzyme (α-amylase) solution. The solution was fixed for incubation for 10 min at 37°C. Immediately after by putting HCl (25 μL, 1 M), the reaction was terminated. Then again it was added iodine-potassium iodide solution (100 μL) for terminating reaction. The blank was prepared to obtain absorbances at 630 nm. The absorbances were also taken of plant extract samples. The observations of the blank were subtracted from plant extract samples. The alpha amylase inhibitory activities were calculated as acarbose equivalents (mmol ACE/g extract).

Alpha-glucosidase inhibitory activities were calculated in further experiments. The plant extract sample were added with glutathione (50 μL), α-glucosidase solution (from Saccharomyces cerevisiae, EC 3.2.1.20, Sigma) (50 μL) in phosphate buffer (pH 6.8) and PNPG (4-N-trophenyl-α-D-glucopyranoside, Sigma) (50 μL). The

obtained solution was transferred in a 96-well microplate. The incubation period was fixed for 15 min at 37°C. The blank was also prepared for the further experiment. It was a mixture of plant extract sample and all reagents without enzyme (alpha glucosidase) solution. Sodium carbonate (50 µL, 0.2 M) was added to terminate the reaction. The blank was prepared. Then the observations of both blank and plant extract absorbances were taken at 400 nm. The blank absorbances were calculated by subtracting form plant extract sample. The alpha glucosidase inhibitory activities were calculated as acarbose equivalents (mmol ACE/g extract).

3.8 Cytotoxicity studies of *G. diversifolia, T. coadunata* and *M. azedarach*

Preliminary cytotoxic acitvities were done for *G. diversifolia*, *T. coadunata* and *M. azedarach*. To evaluate the cytotoxic study, Human pancreatic ovarian (2008) and pancreatic (BxPC3) carcinoma cell lines were taken from Prof. A. Marverti, Department of Biomedical Science, Modena University, Modena, Italy, obtained from American Type Culture Collection (ATCC, Rockville, MD). The obtained cell lines were preserved in the logarithmic phase at 37°C. To preserve cell lines for the experiment 5% carbon dioxide atmosphere using RPMI-1640 medium containing 10% fetal calf serum obtained from Euroclone, Milan, Italy, antibiotics (100 units/mL penicillin and 100 g/mL streptomycin), and 2 mm l-glutamine were used. Then the MTT assay was done with the help of 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazoliumbromide). This was used to calculate relative measure of cell viability (Dall'Acqua *et al*., 2017) with slight modification. After then, 10^3 cells/well were prepared. Then 96 well microplates were also prepared. The cell lines were transferred to all wells with growth medium. The plates were prepared in such a way that it fit with growth characteristics of the cell lines. The growth medium was taken out after 24 hours. The fresh growth medium was replaced with the compounds which was applied for the study. The appropriate concentration was (0.1-30 µM) for isolated compounds, 1-100 µg/mL for EO). The study was done for the three times in order to get fine results. It didnot allow error during the experiments. After all, for the further experiment the prepared solutions were incubatied for 72 hours. Then every well were mixed with 10µL of a 5mg/mL MTT solution in phosphate-buffered saline (PBS). Then the incubation was fixed for 4 hours. After all 100µL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl were mixed in each well. The incubation period was

fixed for overnight.Then MTT reduction was quantified spectrophotometrically using a microplate reader Bio Rad 680, by absorbance measurement at 540 nm. The observed absorbances were calculated to obtain mean absorbance. The mean absorbance for each drug dose was expressed as a percentage of the control, untreated, well absorbance and plotted vs drug concentration. Cytotoxicity acitvities was expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values were obtained, the drug concentrations that decrease the mean absorbance at 570 nm to 50% of that of untreated control wells, were calculated using Graph Pad Prism 4 (Graph Pad Software, S. Diego, CA). The obtained final value was the mean ± Standard deviation. As there were triplicte experiments, these performed less chance of error during experiments. The morphological features were also observed with the help of inverted phase contrast microscopy (Olympus BX41).The photographs were taken with the help of a canon digital camera (Canon 6D).

3.9 Cell culture

The further experiments were again under taken for cell culture, cell viability assay and western blot analysis. The experiments were done due to presence of beta sitosterol obtained from LC-MS which was confirmed by NMR in *G. diversifolia.* Hepatocarcinoma cell lines were also used for the study of cytotoxic effect of plant extract samples.

Further, to study the cytotoxic activities of plant extracts samples, Human pancreatic (BxPC3) carcinoma cells and human ovarian (2008) carcinoma cells were taken. It was stored in the logarithmic phase at 37°C in a 5% carbon dioxide atmosphere using a RPMI-1640 cell culture medium containing 10% foetal calf serum obtained from (Euroclone, Milan, Italy), antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin) and 2 m M l-glutamine. Human hepatic cancer cell line (Huh7) was cultured in MEM supplemented with 10% FCS, L-glutamine, sodium-pyruvate and non-essential amino acids, and with penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (Ferri *et al*., 2007). After then the cells were added in MEM/10% FCS. Then they were kept for incubation for 72 hours. DMSO solution was added in all *G. diversifolia* extracts. The terminal concentration of the DMSO was not exceed to 0.25% v/v. This obtained solvents were mixed to all the experiments.

3.10 Cell viability assay

MTT experiment was performed to evaluate inhibitory growth effect towards pancreatic and ovarian tumor cell lines. A four-parameter logistic (4-PL) model were used to calculate IC_{50} value. Sulphorhodamine B (SRB) experiment was done to evaluate the cell viability of the hepatic cells after treatment with SRB. The experiments were done for three times in total of 8000 cells/well. All samples were kept in 96-well tray (Keepers *et al*., 1991). The experiments were incubated for 24 hours. The experimenting cells were treated with different concentrations of compounds.

SRB experiment were undertaken after 48 hrs. because it require incubation period as previously described (Rimoldi *et al*., 2017). After then the cells were treated with the protein precipitation with 50% trichloroacetic acid at 4°C (50 µL per well, final concentration 10%) for 1 hour. Then it was washed with with tap water for five times, the cells were stained for 15 min with 0.4% SRB dissolved in 1% acetic acid (50 µL per well). Then immediately washed with 1% acetic acid for four times to remove unbound stain. The plates were air-dried, and bound protein stain was solubilized with 150 µL 10 mmol/L unbuffered Tris base. The optical density observations were taken at 540 nm. Data were expressed as mean ± Standard deviation of quadruplicate values of relative absorbance compared to control.

3.11 Western blot analysis

The obtaind cells were washed twice with PBS and lysed with a solution of 50 m MTris pH 7.5, 150 m MNaCl, 0.5% Nonidet-P40, containing protease and phosphatase inhibitor cocktails (SIGMA, Milan, Italy) for 30 min in ice. Twenty µg of proteins and a molecular mass marker (Thermo Scientific, Monza, Italy) were divided in 4%-12% SDS-PAGE (BIO-RAD, Segrate (MI) italy) under denaturing and reducing conditions (Ferri *et al*., 2007). The obtained proteins were then transferred into a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (BIO-RAD). Membranes were washed with Tris-buffered saline-Tween 20 (TBS-T, Milan, Italy), and non-specific binding sites were blocked in TBS-T containing 5% non fat dried milk for 60 min at room temperature. The blots were then incubated for overnight at 4°C with a diluted solution (5% nonfat dried milk) of the following

human primary antibodies: anti LDLR (mouse monoclonal antibody, Millipore clone 2H7.1; dilution 1:1000), and anti-α-tubulin (mouse monoclonal antibody, Sigma clone DM1A; dilution 1:2000). TBS-T was used to wash membranes. Then it was kept for incubation for 90 min at room temperature to a diluted solution (5% non fat dried milk) of the secondary antibodies (peroxidase-conjugate goat anti-rabbit, and anti-mouse, Jackson immuno Research). Immunoreactive bands were detected by exposing the membranes to Clarity TM Western ECL chemiluminescent substrates (Bio-Rad) for 5 min, and images were acquired with an Azure c400 Imaging System (Aurogene). Densitometric readings were observed using the ImageLabTM software (Bio-Rad, Image Lab Software 6.0.1).

3.12 Statistical analysis for *G. diversifolia, T. coadunata* and *M.azedarach*

Statistical analysis was performed by using the Prism statistical analysis package for *G. diversifolia*, version 5.01 (Graph Pad Software, San Diego, CA, USA). p values were determined by Student's t test. Aprobability value of $p < 0.05$ was considered statistically significant.

The obtained data of total phenolic content, antioxidant, and enzyme inhibitory assays were taken as mean ± Standard deviation.Then the statistical analysis was under taken. Graph Pad Prism 8 software was taken for statistical analysis. The correlation values (Pearson's correlation co-efficients) were tabulated between total components (TPC, TFC, and PAC) and biological abilities (antioxidant and enzyme inhibitory properties). R software v.3.6.1 was used to evaluate Correlation co-efficients.

CHAPTER 4

4. RESULTS AND DISCUSSION

Medicinal plants are an important source of phytochemicals, which plays an important role in different biological activities. Nepal being rich in biodiversity, there are thousands of species of medicinal value which has been used by different ethnic community. Ethnobotanical information of these medicinal plants has been reported in different publication from historical documents to till date.The extracts from these plants provide enormous therapeutic potential to treat many diseases. The diseases are cured due to phytochemicals constituents in plants that have defence mechanism and show therapeutic role. But to evaluate medicinal efficacy of ethnomedicinal plants, phytochemical analysis and assessment of bioactivity of phyto-constituents are essential. Medicinal and aromatic plants of Nepal could be important resource for the discovery of bioactive compounds.

4.1 *Girardinia diversifolia* (Link) Friis (Urticaceae)

There are very few informations regarding phytochemical content of *Geradinia diversifolia*. Sterol derivatives like beta-sitosterol, 7-hydroxysitosterol and 3-hydroxystigmast-5-en-7-1, has been obtained from *G. diversifolia* collected in Kenia, and they also showed the antimicrobial activity (Njogu *et al*., 2011).

The main bioactive phytochemicals of this plant have been accessed. The study was carried out by using Liquid chromatography-diode array detector-tandem mass spectrometry and Gas chromatography-mass spectrometry. The result obtained were further analysed with *in vitro* bioassays. The *in vitro* bioassay was done for study of enzymes effect in the different ailments like central nervous system and skin problems. The enzymes tested was namely acetylcholinesterase (AchE) and butyrilcholinesterase (BchE) related with nervous system and tyrosinase for skin problems. Similarly, key enzymes that manages glucose levels, like α-amylase and α-glucosidase, were also tested. As these enzymes play important role on metabolic syndrome. Along with antioxidant properties of the plant extract were performed. Other biological properties were assessed. It was also studied on cytotoxicity capacity of the three plant extracts in different cancer cell lines.

4.1.1 Phytochemical analysis

For the phytochemical analysis of *G. diversifolia* extract, two types of liquid chromatography mass spectrometry namely LC-APCI-MSn and LC-DAD-ESI-MSn was used. Further, several techniques were applied for the seperation and quantification of different compounds. For the quantification of compounds like terpenoids and phytosterols a specifc method C-18 stationary phase was used coupling with chromatography and mass spectrometry (MS) combined with atmospheric pressure chemical ionization (APCI). While, to assess and quantify the carotenoid derivatives a C-30 stationary phase combined with a diode array detector (DAD) and APCI-MS was uused. C-18 column and electrospray (ESI-MS) were used to seperate more non polar constituents, such as phenolics and saponins. Most abundant compounds identified from these methods are provided in Table 2 and obtained chromatograms are presented in Figures 1 and 2.

Table 2: Identified lipophilic compounds in *G. diversifolia* extract by LC-APCI-MS. Compounds were identified on the basis of literature data (CIT)and injection of authentic standards (*)

RT (min)	$[M+H-H_2O]^+$	fragments	identification	mg/g
6.7	425	405-389-365-299-251	Erythrodiol	0.50 ± 0.05
7.8	425	405-389-365-299-251	Uvaol	0.54± 0.05
8.5	427	409-391-255-173	Hydroxy cycloartenol	0.25 ± 0.05
9.2	409	391-339-297-269-173	Cycloartenol	0.35 ± 0.05
21.7	395	297-255-241-199-159	Fucosterol*	23.5± 0.05
21.8	397	315-299-285-257-243-203-189	γ-Sitosterol*	91.0 ± 0.05
22.5	381	297-255-227-173-159	Brassicasterol*	3.14 ± 0.05
23.1	429	165-137-122-67	α-Tocopherylquinone	n.d.
24.7	383	273-257-243-215-161	Campesterol	32.5± 0.05
27.6	397	257-175-161	β-sitosterol*	112.4±0.07
29.6	399	316-257-243-190-175-149	Sitostanol*	16.6± 0.05
5.07	553		α-Cryptoxanthin	3.9± 0.1
5.91	549.5		Carotenoid	3.4± 0.1
6.02	545.5	489-435-339	Phytoene	1.3± 0.1
6.23	555.1	534-460-442	β-Carotene epoxide	4.0± 0.1
6.43	597.5	534-460-442	Zeaxhantin*	6.1± 0.1
7.05	553.5	534-460-442	Cryptoxanthin	8.7± 0.1
7.58	551.4	535-558-471-444	Ketocarotenoid	3.8± 0.1
8.53	551.5	535-558-471-444	Ketocarotenoid	3.2± 0.1
9.62	551.5	535-558-471-444	Ketocarotenoid	2.5± 0.1
9.78	551.5	535-558-471-444	Ketocarotenoid	1.6± 0.1
10.05	537.5	457-445-413	β-Carotene*	1.9± 0.1

Major compounds detected from the application of different techniques are Phytosterols and triterpenes, with beta- sitosterol, gamma sitosterol, campesterol,

fucosterol and sitostanol (Figure 3). Among different Phytosterols, β-sitosterol was detected with highest amount (112.4 mg/g) and the structure of beta-sitosterol was determine by separating the structure and than verifying it by comparing the obtained 1D and 2D Nuclear Magnetic Resonance data and also with previous studies done by Caabatediaz *et al*., (2007) and Sheng & Chen (2009) (Appendix 2).

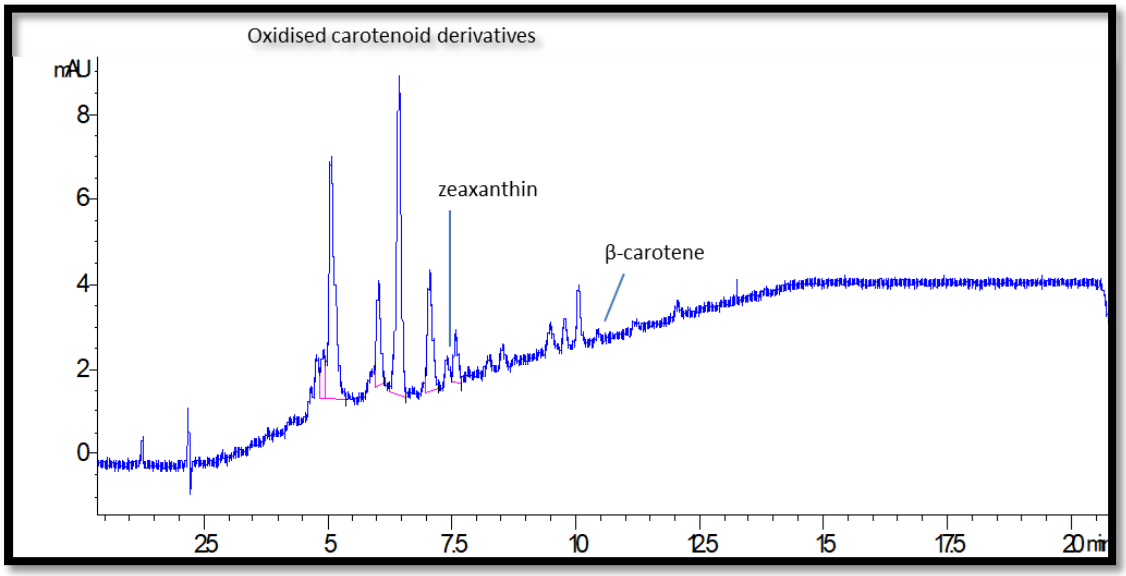


Figure 1: LC-DAD chromatogram related to the analysis of carotenoids and derivatives 425 nm

Out of the total methanol extract, 26% of phytosterols content was obtained. It was analysed to estimate the presence of carotenoids. Certain amount of such constituents were detected with a total amount of 0.47 mg/g. Low amount of beta-carotene and zeaxanthin were traced in comparison with the standard reference, while other derivatives of carotenoids, particularly beta-carotene epoxide was obtained and they were separated on the basis of MS spectra and it was compared with previous finding by Rivera *et al.*, (2014). The obtained carotenoids product might be because of the error during the extraction and drying process of plant. The Liquid chromatography diode array detector at 425 nm is given in figure 1.

LC-DAD-ESI-MS^n was use to analyze polar constituents and obtained compounds are tabulated in Tables 3, with the amounts of the major constituents. Major constituents obtained were ascorbic acid with 2.9 mg/g along with some citric and quinic acids. These compounds are reported to be quite common in vegetable sources. Gluconic acid was also observed as detectable but not in quantifiable amount.The detected compounds can be seen in the Chromatograms represented by several peaks (Figure 2).

Table 3: Identifiedpolar compounds in *G. diversifolia* extract by LC-DAD-ESI-MS, *indicate comparison with authentic standard

RT (min)	$[M-H]^-$	fragments	identification	mg/g
1.8	341	179	Sucrose*	nd
1.9	191	179 85	Quinic acid*	0.138 ± 0.021
2.2	191		Citric acid*	0.235± 0.045
2.3	195	177 129 85	Gluconic acid	nd
2.7	353	191 179 135	3-Caffeoyl quinic acid	0.056± 0.045
3.7	371	209 191 85	*trans*-Syringin (eleuteroside B)	0.503± 0.046
5.2	315	153	Protocatecuic acid glucoside	0.121± 0.031
6.3	365	211 153	Protocatecuic ester with sinapoylalchol	0.103± 0.031
6.6	447	357 315 271	Isoorientin	0.090± 0.032
7.85	175	157 115 85	Ascorbic acid	2.901± 0, 031
8.1	371	147 209	Synapoilquinic acid	0.162± 0.051
9.2	385	224	Synapoyl acid hexosideisomer 1	0.081± 0.028
10.3	447	285 199 243 175	Kaempferol-3-O-glucoside*	0.033± 0.002
10.9	367	191	5-O-feruloyl quinic acid	0.191± 0.029
11.5	385	224	Synapoyl acid hexosideisomer 2	0.151± 0.031
11.8	841	779 679 617, 547, 529	3-[Xyl]-28-Glc-11-Hydroxyphytolaccagenin	0.154± 0.021
12.0	431	341 311 283	Vitexin	0.058± 0.023
12.8	841	679 517 473 457 437	Scabran G	0.035± 0.011
13.6	563	443 413 323	Vitexin-2''*O*-xyloside	0.171± 0.028
14.2	593	473 413 341 311	Vitexin-2''*O*-glucoside	0.085± 0.006
14.3	679	517 473 455 438	**Ganoderic acid C2 hexoside isomer 1**	0.079± 0.007
14.9	679	517 499 473 455 438	**Ganoderic acid C2 hexoside isomer 2**	0.074± 0.007
16.3	679	518 499 473 455 438	**Ganoderic acid C2 hexoside isomer 3**	0.037± 0.007
18.9	517	499 437 304	Ganoderic acid C2	0.038± 0.0071
19.4	327	309 291 229	oxoDihydroxy-octadecanoic acid	0.390± 0.008
15.4	805	643	Malvidin-3-O-glucoside-4-vinyl-catechin	0.032± 0.008
8.7	377	243	Vitamin B2	0.122± 0.021

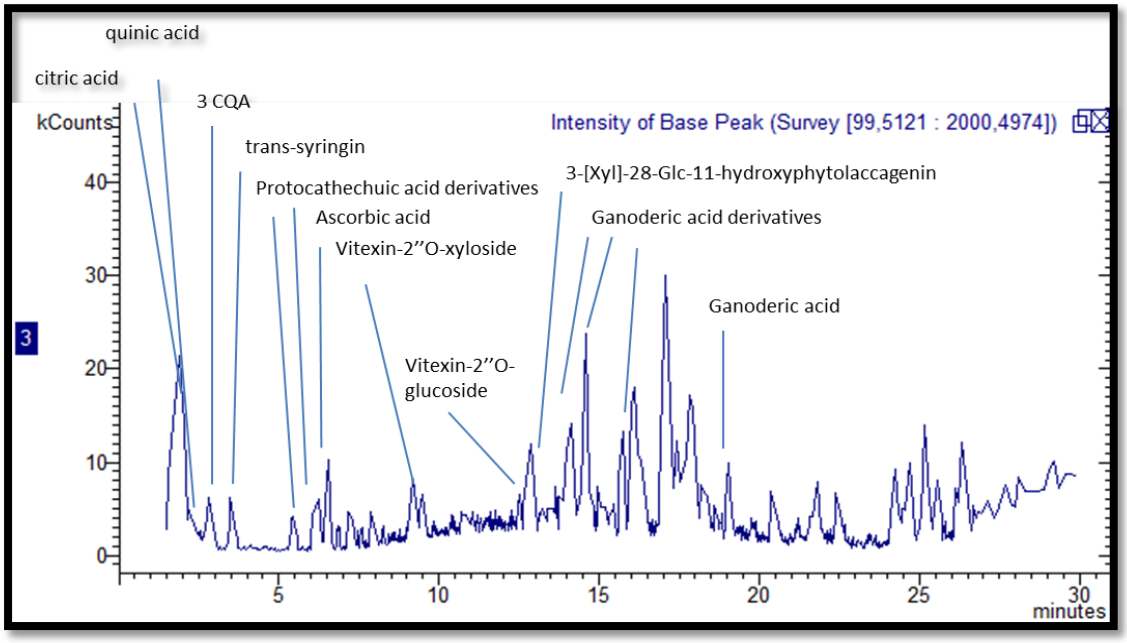


Figure 2: LC-MS chromatogram in negative ion mode; some of the identified peaks reported in Table 3 are highlighted

Some of the peaks corresponded to flavonoids such as iso-orientin, kaempferol glucoside, and vitexin and its derivatives (Gouveia-Figueira & Castilho, 2015; Frii *et al*., 2016). Other minor phenolic derivatives were also found, including caffeoylquinic acid esters (Maggi *et al*., 2019), syringic, protocatecuic, and sinapoyl acid derivatives (Frii *et al*., 2016), which have been described from various other plant species.Traces of complex malvidin glycoside coupled to catechin unit were detected in positive ion mode (Flamini, 2013).

Similarly, ganoderic acid derivatives were detected in different peaks represented by*m/z* values and fragmentation pattern. The peak at retention time 18.9 min with molecule ion $[M-H]^-$ at mass charge ration (*m/z*) 517could be observed MS^2 fragments due to the removal of water (-18 amu), consequent loss of water and CO_2 (-62 amu) (Figure 3). At other three peaks at retention time 14.3, 14.9 and 16.3 min presenting mass charge ration value of 679 similar behavior was also observed. Particular fragmentation and MS^n spectrum of derivative mass charge ratio 679, tentatively designated as glycosidic derivative of ganoderic acid can be seen in Figure 3. Even though, the presence of ganoderic acid derivative in *Giradinia diversifolia* was found at very low concentration it has been reported in different literatures (Gouveia-Figueira and Castilho, 2015; Wei *et al*., 2017). Eventhough ganoderic acid metabolites were typical found in *Ganoderma* fungi recently it has also been reported in *Rubus* fruits, leaves and flowers by Gouveia-Figueira & Castilho (2015).

Presence of ganoderic acid derivative has not been reported before in any of the species belonging to Urticaceae family. From this, it could be suggested that secondary metabolites could be obtained from the fungal metabolism, if symbiotic fungi could be grown in association with higher plant. This gives a very good reason to further investigate to assess the origin of these metabolites in *G. diversifolia*. Apart from ganoderic acid derivative, vitamin B2 was also measured which was very low but in detectable amount (Table 3).

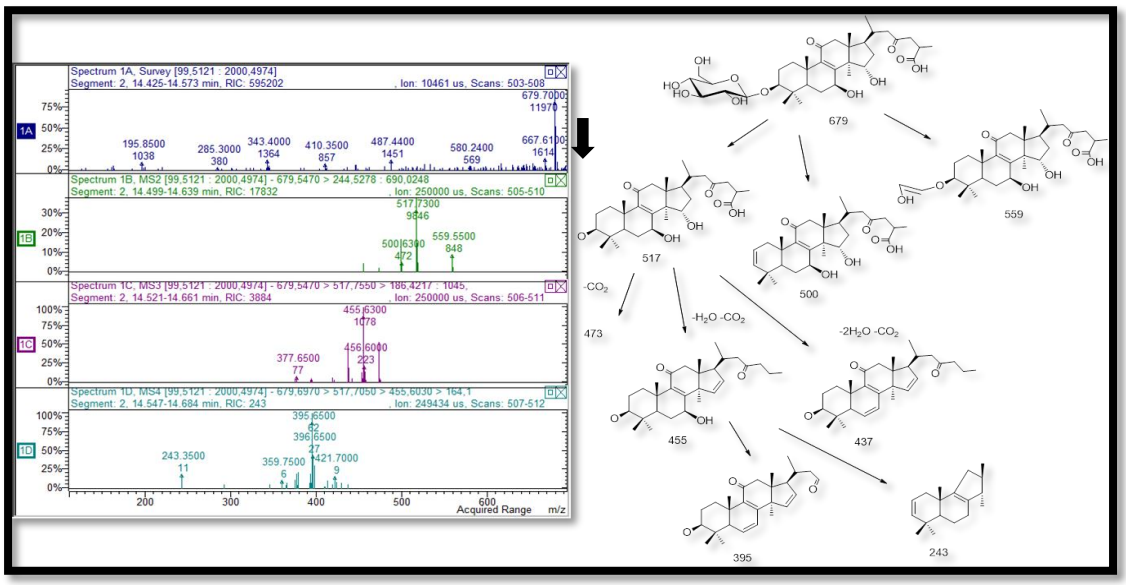


Figure 3: Proposed fragmentation scheme and MS^n spectrum of derivative *m/z* 679, tentatively assigned to glycosidic derivative of ganoderic acid

As there was large amount of lipophilic compounds, to reveal the presence of other lipophilic constituents after derivatization with methanol, Gas chromatography mass spectrometry was used (Table 4). The methanolic extract contained 13 percent lipidic fraction, along with 46 percent saturated derivatives and 32 percent unsaturated fatty acids. Palimitic acid was the major fatty acid, while on the other hand linoleic acid was the major compound (22 mg/g) among unsaturated fatty acids. Phytol was the main constitutes of the lipophilic fraction 27 milligram per gram (Table 4; Figure 3). The structures of the major compounds of the *G. diversifolia* extract are given in Figure 4.

Table 4: Constituents in *G. diversifolia* extract by GC-MS after derivatization

RT (min)	Identification	MW (Da)	CAS	mg/g
14.2	Phytol	296	102608-53-7	26.72± 0.05
18.4	Myristic acid, methyl ester (C16:0)	242	124-10-7	0.70± 0.03
23.1	Palmitic acid, methyl ester (C10:0)	270	112-39-0	37.18± 0.05
24.3	7-hexadecenoic acid, methyl ester (C16:1)	268	5687-67-3	2.06± 0.06
27.6	Stearic acid, methyl ester (C18:0)	298	112-61-8	8.98± 0.06
28.7	Elaidic acid, methyl ester (C18:1)	296	112-62-9	8.66± 0.06
30.4	Linoleic acid, methyl ester (C18:2 ω-6)	294	112-63-0	21.95± 0.08
31.8	Arachidic acid, methyl ester (C20:0)	326	1120-28-1	6.01± 0.05
32.3	Linolenic acid, methyl ester (C18:3 ω-3)	292	301-00-8	9.69± 0.06
33.5	Heneicosanoic acid, methyl ester (C21:0)	340	6064-90-0	1.22± 0.03
35.6	Docosanoic acid, methyl ester (C22:0)	354	929-77-1	2.94± 0.03
37.3	Tricosanoic acid, methyl ester (C23:0)	368	2433-97-8	0.65± 0.02
38.5	1-docosanol	326	661-19-8	0.36± 0.02
39.1	Lignoceric acid, methyl ester (C24:0)	382	2442-49-1	2.42± 0.04
42.3	Cerotic acid, methyl ester (C26:0)	410	5802-82-4	0.80± 0.02
45.5	Montanic acid, methyl ester (C28:0)	438	55682-92-3	0.52± 0.02

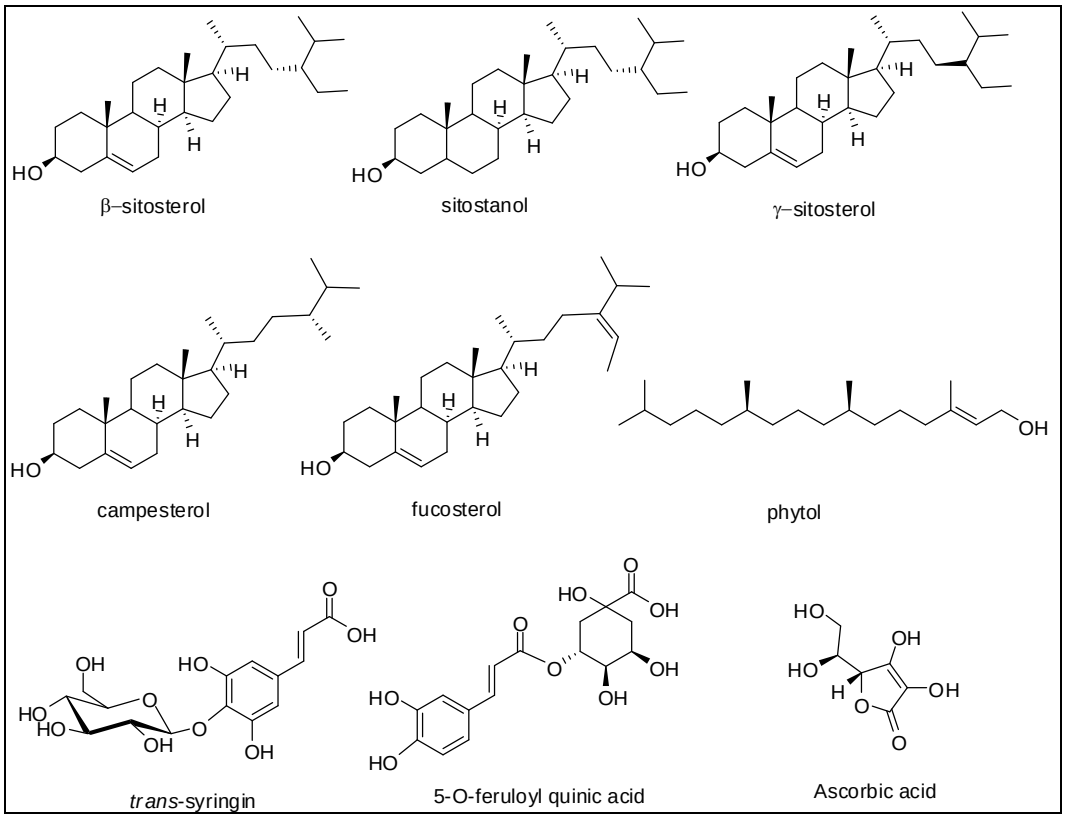


Figure 4: Structure of the most abundant constituents found in the *G. diversifolia* extract

The overall phytochemical composition suggests that the phytocomplex of *G. diversifolia* might be useful. The compounds could be used as nutraceutical.

4.1.2 *In vitro* bioassays

In most of the literatures alkaloids extracted from the plants have been known for their activity, but at the same time their use has lead to unpleasant side effects (Brühlmann *et al*., 2004; Dall'Acqua *et al*., 2010). According to Sharma *et al*., (2019) in healthy human brain, enzymes like AchE predominates while BchE plays minor role to control brain acetylcholine levels. Both these enzymes, therefore, represent legitimate therapeutic targets for enhancing cholinergic deficit which is responsible for the neurological malfunction that results into Alzheimer's disease. Therefore, it is essential to look for non-nitrogen containing Acetylcholinesterase and Butyrylcholinesterase inhibitors that might have different interaction with the target enzymes which will provide an opportunity to determine other pharmacological characters (Brühlmann *et al*., 2004) that could be used for these enzymes.

The phytochemical analysis of *G. diversifolia* extract indicates that there is the presence of some non-alkaloidal inhibitors. Significant *in vitro* enzyme

(Acetylcholinesterase) inhibition activity by *G. diversifolia* methanolic extract can be seen in the results given in Table 5.

Table 5: Results of *in vitro* enzyme inhibition assays on *G. diversifolia* methanolic extract

Samples	AchE inhibition (mgGALAE/g)	BchE inhibition (mgGALAE/g)	Tyrosinase inhibition (mgKAE/g)	Amylase inhibition (mmolACAE/g)	Glucosidase inhibition (mmolACAE/g)
G. diversifolia	4.08 ± 0.21	7.21 ± 0.61	138.14 ± 1.36	1.19 ±_ 0.05	5.42± 0.03

Values are reported as mean ± SD of three replicates. GALAE: Galantamine equivalent; ACAE: Acarbose equivalent; KAE: Kojic acid equivalent

Tyrosinase is a major compound in the synthesis of melanin. Significant inhibition result (138.14 ± 1.36) showed that the extract could be used for cosmetic as well as for skin related problems (Table 5). The activity in this case may be related with the presence of high levels of phytosterols present in the extract. Jun *et al*. (2012) has also reported that significant effect of gamma-oryzanol in cellular melanogenes is due to the inhibition on enzyme (tyrosinase) activity that has reduced MITF and target genes in the PKA dependent pathway.

Postprandial glucose levels in diabetes mellitus patients could be controlled by two enzymes A-Amylase and α-glucosidase which helps in carbohydrate digestion and their inhibition. According to different literatures, young shoots paste of *G. diversifolia* have been given to treat diabetics (Balami, 2004; Saha *et al*., 2011; Gurung *et al*., 2012; Malla *et al*., 2015). This may be due to an inhibition of alpha amylase (1.92 mmol ACAE/g) and alpha glucosidase (5.4 mmol ACAE/g) by *G. diversifolia* extract (Table 5), however, the obtained activities could be considered moderate demonstrating the limited activity of the extract.

Significant *in vitro* enzyme inhibitory activities and antioxidant properties on Acetylcholinesterase and Butyrylcholinesterase of *G. diversifolia* methanolic extract may be related to the high content in phytosterols and low amount of polyphenols as seen in the phytochemical composition. In previous papers also the phytosterol mixture was reported to be better Acetylcholinesterase inhibitor than its pure constituent on the Acetylcholinesterase of *Culex quinquefasciatus* (Gade *et al*., 2017), while Sultana & Khalid (2010) reported that AchE inhibition with IC_{50} of 644 micro molar (265 mg/L) is better.

Beta-sitosterol have also been examined for its capacity to inhibit AchE and IC_{50} of 24.1 ± 0.7 microgram pre milliliter (58.1 ± 1.6 µM) by Bahadori *et al*., (2016). While in some previous findings beta-sitosterol have been reported as better inhibitor of BchE, with IC_{50} value of 0.56 micromolar than compared to AchE (IC_{50} 14,57 µM) due to the positions of side chain and hydroxyl group in enzyme active site (Bahadori *et al*., 2016). Therefore, further investigation of *G. diversifolia* methanolic extract for *in vitro* enzyme inhibition and antioxidant properties may lead to new findings.

The data obtained from the chromatographic measurements could be related with significant result on the antioxidant and total phenolic constituents tests. But, the amount of polyphenols present is moderate and the effect of antioxidant properties is also limited. Results of *in vitro* antioxidant activities on *G. diversifolia* methanolic extract exhibited scavenging abilities on DPPH (14.37 mg TE/g extract) and ABTS (28.33 mg TE/g extract) as well (Table 6). Similarly, reducing power exhibit the capacity of electron-donation of an extract in CUPRAC (105.27 mg TE/g extract) and FRAP (42.63 mg TE/g extract) test. In addition, the metal chelating activity (25.70 milligram EDTAE per gram) seems to be significant, as it shows potential utility result in the protection of metal based reactions that generate free radicals (Table 6).

Table 6: Results of *in vitro* antioxidant test on *G. diversifolia* methanolic extract

Extract	***G. diversifolia***
Total phenolic content (mgGAE/g)	24.30±0.15
DPPH (mgTE/g)	14.37 ± 0.72
ABTS (mgTE/g)	28.33 ± 1.21
CUPRAC (mgTE/g)	105.27 ± 0.65
FRAP (mgTE/g)	42.63± 0.69
Metal chelating (mgEDTAE/g)	25.70±2.35
Phosphomolybdenum (mmolTE/g)	2.53±0.25

Values are reported as mean ± SD of three replicates. GAE: Gallic acid equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent

Another constituent which was in significant amount in *G. diversifolia* extract is phytol, which may be responsible for enzyme inhibitory properties on both AchE and BchE. Previous paper by Moodie *et al*. (2019) also reported that phytol isolated from marine organisms obtained phytol IC_{50} of 2.7 and 5.8 micromolar and by using

molecular docking studies, activity of phytol was confirmed which suggest that it was capable of forming strong interactions with Arg177 an ion outside the binding pocket of AchE. This study thus confirmed that presence of 2.7 percent Phytol in an extract showed significant activities on cholinesterase.

Regarding the polar constituents syringin was reported being active having IC_{50} of 31.47±2.2 and 52.77±0.12 μM respectively on AchE and BchE, that showed significant activities on BchE enzyme than on AchE (Yang *et al*., 2015). Therefore, the presence of trans-syringin in the methanol extract at 0.5 mg/g (Table 3) can be considered to clarify the efficacy of *G. diversifolia* in having inhibitory properties on these enzymes.

In *G. diversifolia* methanolic extract phytol and phytosterols was contained in high amount, while other compounds like phenolics, carotenoids, saponins were found in limited amount along with vitamin C and vitamin B2. Because of the presence of phytosterols, phytol and syringing in the extract of *G. diversifolia*, it might be useful ingredients for nutraceuticals targeted for neuroprotection. In a recent paper also efficient neuroprotective role of phytosterols was seen on cognitive deficit of aged rats when it was induced by a high-cholesterol diet (Rui *et al*., 2017).

Significant inhibition of cholinesterase was seen in the result of chemical bioassays. This may be due to the presence of phytosterol, that have inhibitory properties on enzymes like AchE and BuChE, which has also been reported in several other research papers (Yoshida & Niki, 2003; Ali *et al*., 2017; Rui *et al*., 2017; Walker *et al*., 2017) and phytol has also been studied in this regard by Walker *et al*. (2017). While, Jabir *et al*., (2018), have reported that phenolic constituents also could inhibit these enzymes and suggested that the presence of multiple phytocomplex compounds in *G. diversifolia* may be responsible for the above activities. But in case of tyrosinase activity, oryzanol a phytosterol fraction has significant inhibitory properties on tyrosinase (Jun *et al*., 2012).

On the other hand, the result obtained from different assays showed that the antioxidant property exhibited by *G. diversifolia* methanolic extract is moderate. This result may be due to the limited presence of non-phenolic antioxidants such as

phytosterols, vitamin C, phytol and carotenoids. There are several papers that have reported similar findings (Yoshida & Niki, 2003; Young & Lowe, 2018; Caritá *et al*., 2020). Whereas, Wang *et al*. (2009) have reported that metal chelating ability reveals the inhibition of production of free radicals in the Fenton reaction and also of some non-phenolic compounds (ascorbic acid, polysaccharides and peptides).

4.1.3 Cytotoxic activity

Potential bioactivity of *G. diversifolia*, was tested using *in vitro* cellular models. Three tumor cell lines, 2008 (ovarian cancer), BxPC3 (pancreatic cancer) and Huh7 (hepatocarcinoma) was used for the preliminary cytotoxicity tests of the methanolic extract (Shrestha *et al*., 2020), which showed significant cytotoxic effect on BxPC3 (IC50 = 12.5 µg/mL) and Huh7 cells (IC50 of 7.2±2.5 µg/mL) (Table 7). These results indicated that antioxidant capacity of the extracts has a strong relationship with cytotoxic capacity towards cancer cells.

Table 7: Cytotoxicity tests of extracts of *G. diversifolia* on 2008, BxPC3 and Huh7 cell lines (nd not determined)

	IC_{50} (µg/mL)		
Extract	**2008**	**BxPC3**	**Huh7**
G. diversifolia MeOH	47.5±4.1	12.5±3.5	7.2±2.5
G. diversifolia Ethyl acetate	nd	nd	19.9±1.5
G. diversifolia Cyclohexane	nd	nd	38.8±1.2
G. diversifolia Dichloromethane	nd	nd	Not Active

Out of the three cancer cell line used for the cytotoxic activity, methanolic extract showed the most potent cytotoxic effect on Huh7 cells with an IC50 of 7.2±2.5 µg/mL, compared to ethyl acetate extract with 19.9±1.5 µg/mL and cyclohexane extracts with 38.8±1.2 µg/mL respectively (Table 7 and Figure 5). Cytotoxic activity were more potent on Huh7 cells than on ovarian cancer (2008), pancreatic cancer (BxPC3).

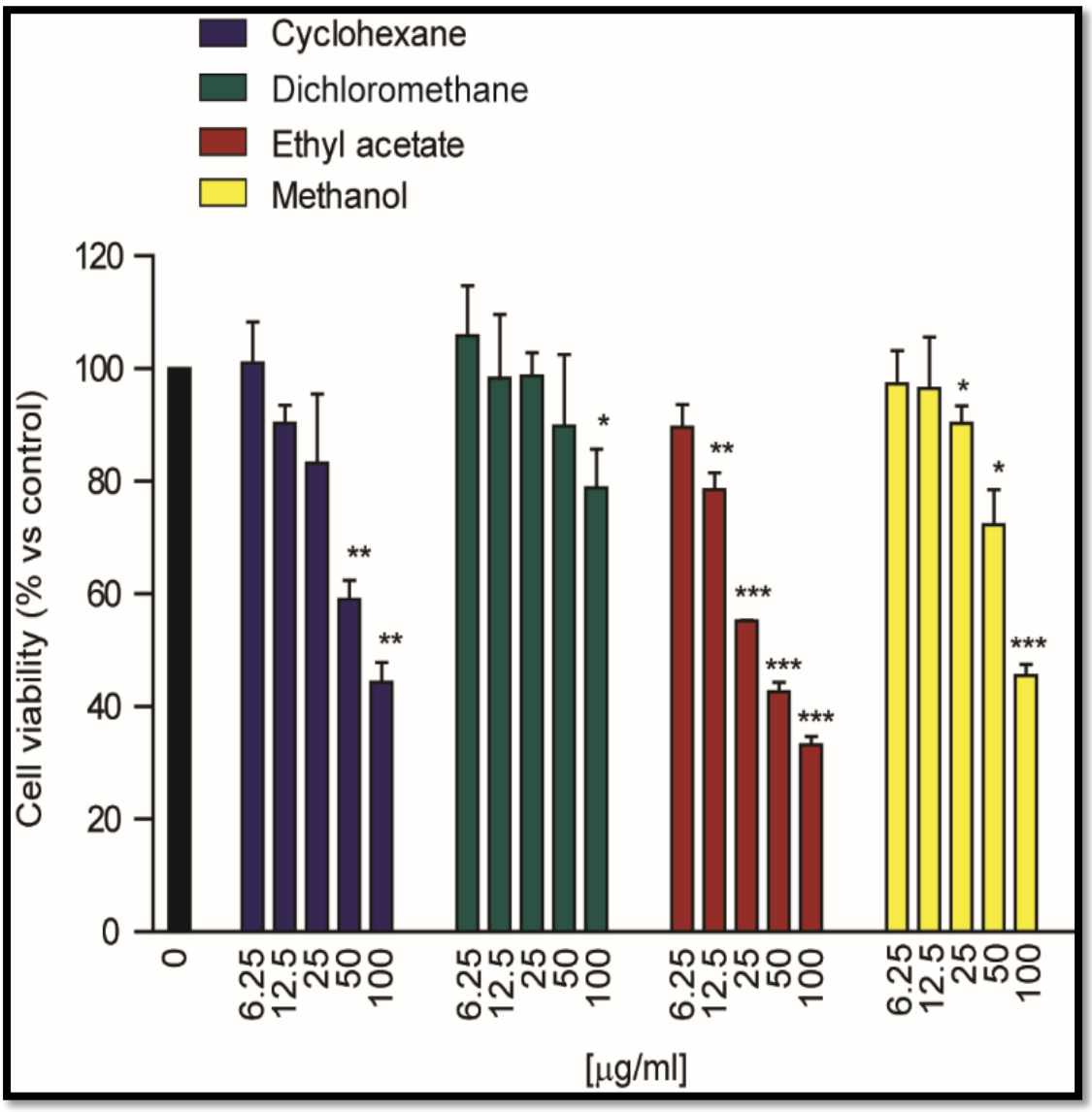


Figure 5: Cytotoxic effect of different solvent extracts of *G. diversifolia* on the Huh7 cell line
* p< 0.05; ** p < 0.01; *** p < 0.001. Student's t-test

Having complex phytochemical composition the extract of *G. diversifolia* was further fractionated in order to separate lipid constituents from more hydrophilic compounds using different solvent cyclohexane, dichloromethane, ethyl acetate as well as methanolic extract. Quantitative data for the most abundant non-polar lipophilic portion showed distinct difference in phytosterol composition (Table 8). Beta-sitosterol being more concentrated in hexane fraction (417.52 ± 0.15 µg/mL) than in dichloromethane and ethyl acetate fraction, while gamma-sitosterol was more concentrated in the dichloromethane fraction (230.77±0.07 µg/mL), but phenolic constituents were not measurable in these three extracts (Table 8).

Table 8: Quantitative data of phytosterol in fractionated extracts of *G. diversifolia*, * for phytol quantitative data were obtained by GC-MS

Ion	identification	hexane extract (mg/g)	dichloromethane extract (mg/g)	Ethyl acetate extract (mg/g)
$[M+H-H_2O]+425$	Erythrodiol	0.35± 0.02	2.26±0.04	3.38± 0.02
$[M+H-H_2O]+425$	Uvaol	0.28 ± 0.02	1.46± 0.02	1.96±0.04
$[M+H-H_2O]+427$	Hydroxy cycloartenol	0.24 ± 0.05	5.22±0.03	0.24± 0.02
$[M+H-H_2O]+409$	Cycloartenol	2.61 ± 0.02	5.65±0.07	4.52±0.04
$[M+H-H_2O]+395$	Fucosterol*	134.39± 0.10	165.27±0.11	7.23±0.07
$[M+H-H_2O]+397$	γ-Sitosterol*	0.92± 0.10	230.77±0.07	55.42±0.09
$[M+H-H_2O]+381$	Brassicasterol*	29.14 ± 0.04	32.40±0.09	5.78± 0.02
$[M+H-H_2O]+429$	α-Tocopherylquinone	n.d.		
$[M+H-H_2O]+383$	Campesterol	31.53 ± 0.07	29.66±0.07	4.36±0.06
$[M+H-H_2O]+397$	β-sitosterol*	417.52 ± 0.15	112.2±0.07	0.20± 0.02
$[M+H-H_2O]+395$	Stigmasterol	70.29 ± 0.21	50.72±0.17	4.31± 0.01
$[M+H-H_2O]+399$	Sitostanol*	6.11 ± 0.05	6.83±0.03	0.06± 0.02

Exemplificative LC-MS chromatograms related to the phytosterol content are reported in Figure 6

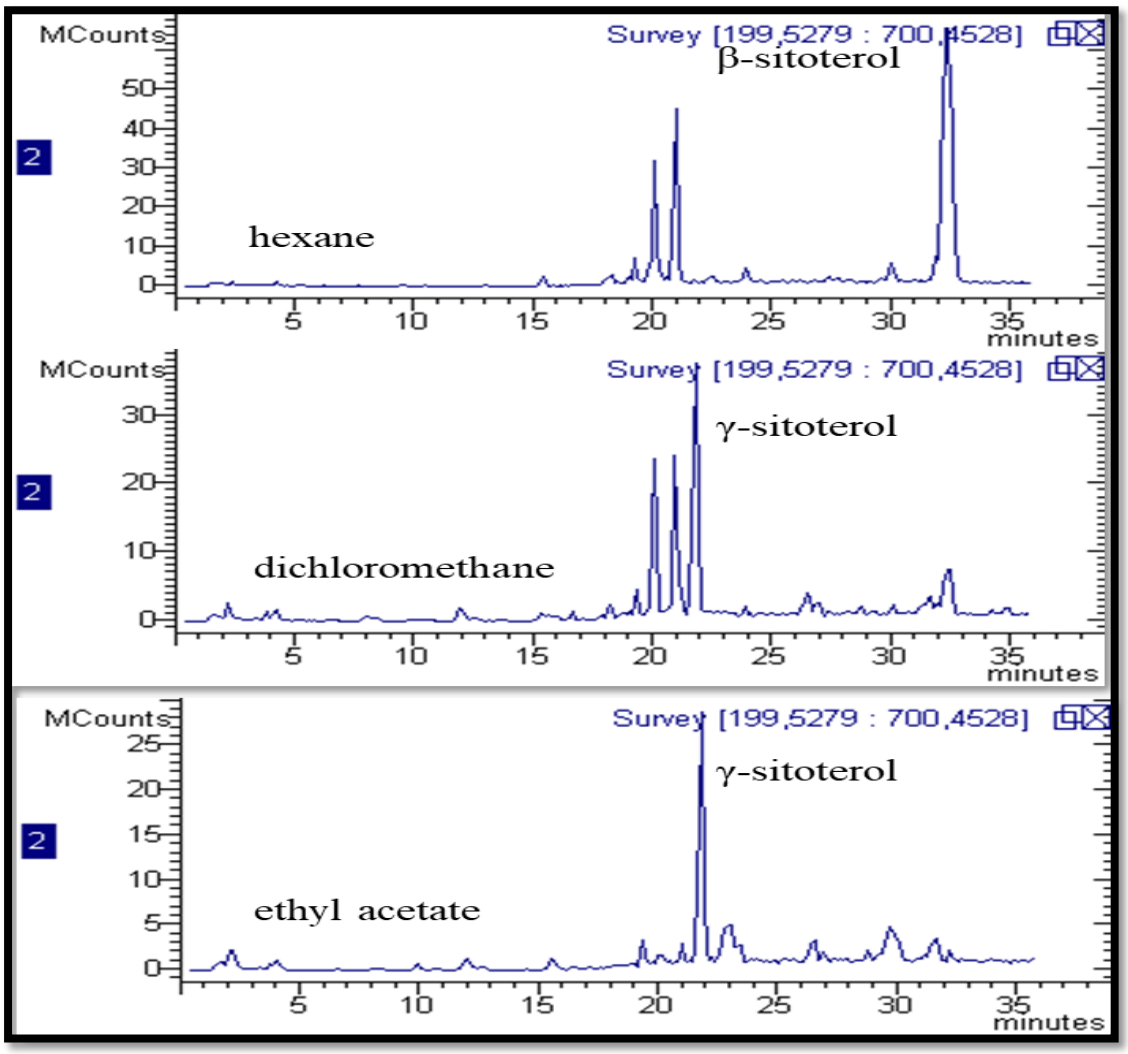


Figure 6: HPLC-MS chromatograms (base peak ion) of the three fractions obtained from the methanol extract by liquid-liquid partition, hexane, dichloromethane and ethyl acetate

Out of three extract only dichloromethane extracts showed a significant result of cell viability in the hepatocarcinoma (Huh7) cell line with only 0.1 mg/mL concentrations (Figure 6). However, the IC_{50} value could not be determined, since the amount of the effect was less than 50% (Table 7). From this preliminary result it can be concluded that the whole phytocomplex was more cytotoxic than the fraction enriched in lipophilic constituents for the hepatocarcinoma (Huh7) cell line. Similar findings have also been reported in previous publication that indicates cytotoxic activity for phytosterols, fucosterol and flavonoids (Park *et al*., 2007; Malek *et al*., 2009; Khanavi *et al*., 2012; Ali *et al*., 2017) due to their different modes of action. In case of hepatocarcinoma cell line the cytotoxic effect observed might be due a possible mode of action where a significant reduction of LDLR expression after 72 hrs exposure can profoundly alter intracellular cholesterol homeostasis. Apart from the hepatocarcinoma cell line, the cytotoxic activity was also tested in ovarian cancer (2008), and pancreatic cancer (BxPC3) using *in vitro* cellular models. Out of these two cancer cell line, methanolic extract showed the most potent cytotoxic effect on pancreatic cancer (BxPC3) cells with an IC50 of 12.5±3.5 µg/mL, compared to ovarian cancer (2008) 47.5±4.1 µg/mL (Table 7) which is clearly visible in the Photo plate 5 (A, B, C, D).

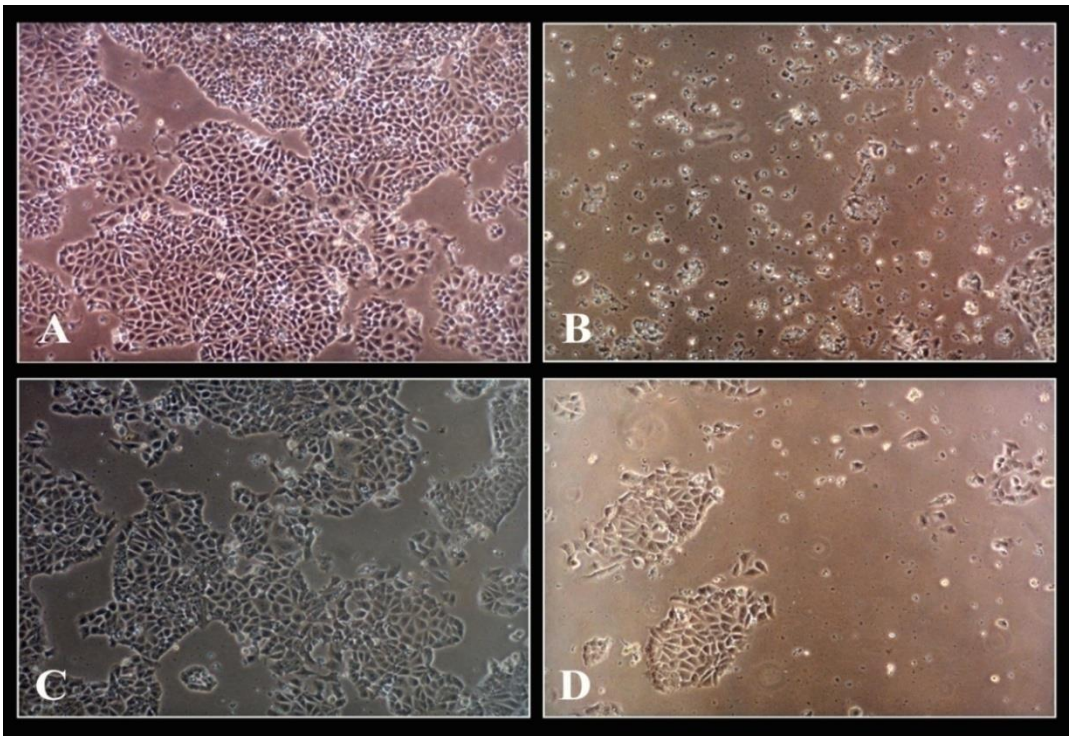


Photo plate 5: A. Ovarian (2008) cell lines CTR and **B.**Ovarian (2008) GDM Treatment. **C.** Pancreatic cell lines (BxPC3) CTR and **D.** Pancreatic cell lines (BxPC3) GDM Treatment. Pictures (40xmagnification)

4.1.4 Effect on LDLR expression in hepatoma cell line Huh7

The extract of *G. diversifolia* was tested to observe efficiency of the LDL receptor in hepatocyte. Different extracts of *G. diversifolia* showed significant reduction of LDLR expression after 72 hrs of exposure (Figure 7). Even though there is a strong limitation of *in vitro* analysis, this result shows that the extract of *G. diversifolia* does not have significant lipid-lowering properties but may profoundly alter the intracellular cholesterol homeostasis. According to Vasseur & Guillaumond (2016) have pointed out that cancer cells internalize cholesterol from circulating LDL through the LDLR on the cell surface, which leads to inter-tumoral cholesterol imbalance and improvement of chemotherapy efficiency, this justifies our work. In addition, LDL-R expression was significantly increased in hepatocarcinoma, whereas it was less expressed in the adjacent liver tissue (Bhat *et al*., 2015). This evidence indicates that the *G. diversifolia* extract might apply their cytotoxic effects by reducing the expression of LDLR. In addition, in hepatocarcinoma, the increased levels of LDLR had been associated with Proprotein Convertase Subtilisin/Kexin Type9 (PCSK9), a new pharmacological target for controlling hypercholesterolemia (Ferri *et al*., 2016).

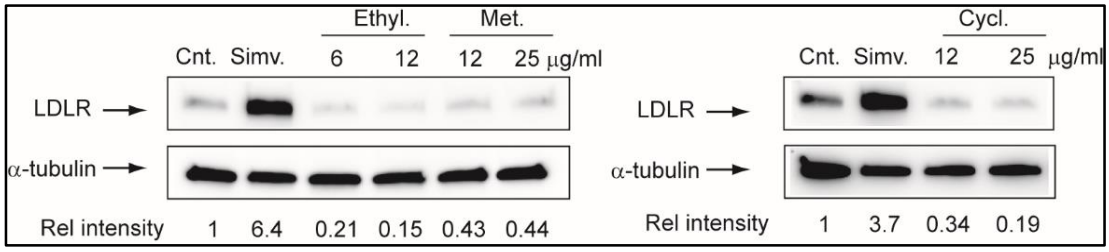


Figure 7: Effect of different solvent extracts of *G. diversifolia* on LDLR expression in Huh7 cell line

The methanolic extract fraction was most effective against hepatocarcinoma cellines. The obtained cytotoxic activities were due to presence of secondary metabolites like phytosterols (beta-sitosterol, gamma-sitosterol, fucosterol and flavonoids) (Table 8).

Beta-sitosterol showed significant cytotoxic activities in different tumor cell lines (Malek *et al*., 2009), as well as for leukaeima cells (Park *et al*., 2007). While, fucosterol extracted from marine algae *(Sargassum angustifolium*) exhibited cytotoxicity effect in colon carcinoma, colorectal adenocarcinoma and breast ductal carcinoma cell lines (Khanavi *et al.*, 2012).

Like Beta-sitosterol, vitexin-2-O-xyloside's a phenolic compound having anti-proliferative properties showed cytoxicity against $CaCo_2$ cells and its activity was increased when phenolic compound was co-administrated with betalains (Khanavi *et al*., 2012). From the above results it can be concluded that extract of Himalayan nettle (*Giradinia diversifolia*) having various complex chemical compound and its activities can be considered as an important species. But to know the exact compound, it requires further experiment to purify and test specific compounds and fractions to study the possible mode and potential cytotoxic effect on different cancer cell line.

4.2 *Tectaria coadunata (*J. Smith) C. Christensen

Lower group of plants like lycophytes and ferns are neglected and ignored in the modern era, but they still possess very important role in traditional medicine. Several species of fern and fern allies are commercially used in different systems of medicines like Ayurvedic, Unani, and Homeopathic, etc (Reddy *et al*., 2020). Dubal & Kale (2019) have reported twenty one phytoconstituents in *T. coadunata* by gas chromatography-mass spectrophotometry (GC-MS) analysis. The constituents have been indicated to involve general classes of compounds such as carbohydrates, tannins, phenols, anthraquinone glycosides, coumarin glycosides, flavonoids, and steroids (Pawar *et al*., 2016).

The information on phytochemical composition of *Tectaria coadunata* was obtained by using different tools where crude extract was run through Nuclear magnetic resonance (NMR) along with liquid chromatography-diode array detector-mass spectrophotometry (LC-DAD-FLDESI-MS) and chromatographic separations were carried out. The process was used to get the result on the phytochemical compounds and to see the possible bioactivity of this traditional medicine in order to bridging the gap between the use and its effect.

Likewise as in *G. diversifolia*, extract of Tectaria coadunata have been used to study the phytochemical constituents and bioactive activities. Antioxidant and enzymatic assays on five enzymes was evaluated by using in vitro assay. Preliminary

cytotoxicity text was also done on cancer cell lines to assess potential activities of the extract. The result is expected to get new information on this medicinal plant.

4.2.1 Phytochemical analysis

For the characterization of *Tectaria coadunata* extract NMR technique was used. This technique helps to identify secondary metabolites available in the extract, which is a procedure followed by most of the researchers for other plant extracts (Sut *et al*., 2019a; Sut *et al*., 2019b). As the extract gave many complex phytoconstituents, it was further analyzed to get simple compounds.

The 1H-NMR spectrum presents two broad signals in the deshielded proton region, one being δ 6.50-7.50, ascribable to aromatic signals of phenolics, and another in the range δ 5.85-6.20, supporting other less deshielded aromatics or double bonds. Further signals were the two doublets at δ 5.11 (J = 3.95) and 4.48 (J = 7.75) and several multiplets in the spectral region from δ 3.20 to 4.10; all these signals suggest the presence of carbohydrates (Figure 8A). Due to less populated, but presence of multiple groups of aliphatic region in the spectrum it can be suggested the occurrence of aliphatic chains (Figure 8B). The sugar region in the spectrum may be due to the presence of saccharose and glucose (Figure 8B).

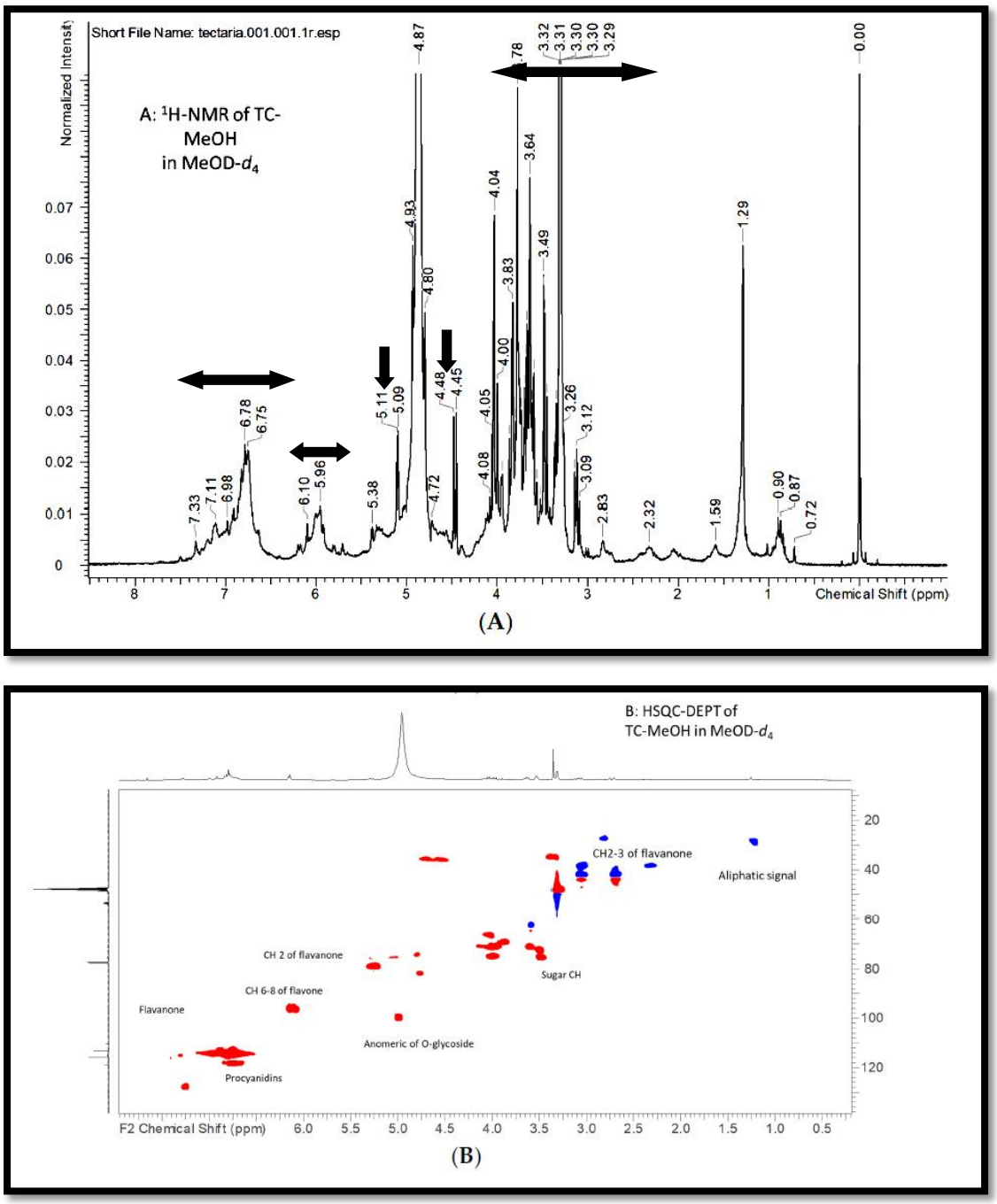


Figure 8: 1H-NMR of *Tectaria coadunata (*TC)-MeOH (A) and heteronuclear single quantum coherence spectroscopy-distortionless enhancement by polarization transfer (HSQC-DEPT) of TC-MeOH (B) in MeOD-d4

Tectaria coadunata ethylacetate extract was also analyzed along with *Tectaria* methanolic extract to identify the secondary metabolites. The 1H-NMR spectrum result shows that methanolic extract is more resolved than ethylacetate extract as the spectrum shows sharp peaks in both aromatic and sugar regions as well as in the aliphatic region (Figure 8B). More data was observed in the spectrum (Figure 8A) when the data from HSQC-DEPT (hetero nuclear single quantum coherence

spectroscopy-distortion less enhancement by polarization transfer was combined. This result has given the value of the chemical shift of both Hydrogen and Carbon of all non-quaternary positions, and allowed to observe the long-range correlations from hydrogen to carbon distances of 3-4 bonds. Connectivity data were obtained from the correlation spectroscopy (COSY) spectrum, showing proton couplings. The presences of different classes of constituents are summarized in Tables 9 and 10. The data shows that *Tectaria* methanolic extract mostly contained phenolics polymers such as procyanidins, flavanol and glycosides (Table 9, Figure 8B).

Recent publication has also reported diverse group of bioactive/chemical components such as sugars, sugar alcohol, flavonoids, terpenoids and phenolics from *T. coadunata,* which supports our findings (Choudhari *et al*., 2013; Reddy *et al*., 2020). Phytochemical screening of rhizomes of *Tectaria cicutaria* also confirmed the presence of triterpenoid, flavanoids, saponins, tannins, phenols, and carbohydrate (Karade & Jadhav, 2018). Phenols, saponins, tannins, xanthoproteins, coumarins and carbohydrates were also screened from *T. zeylanica* (Sukumaran *et al*., 2012) and presence of alkaloids and flavonoids in *Tectaria zeylanica* was also reported by (Maridass & Ravichandran, 2009b).

Table 9: Nuclear magnetic resonance (NMR) assignments of TC-MeOH

δH	δC	Correlations	T-MeOH rentative assignments
7.45	115.3	150.5 125.3	aromatic phenol ring of procyanidin or tannin
7.31	114.5		aromatic phenol ring of procyanidin or tannin
6.76	114.5	129.4 118.7 116.5	aromatic phenol ring of procyanidin or tannin
7.25	127.7	156.8 125.7 79.5	flavanol moiety
6.90-6.99	114.2-117.2	144.7 118.5 79.5	flavanol moiety
6.16-6.15	94.5-95.8	196.3 163.5 103.5 94.2	flavanol moiety position H-6/8
5.26	78.3	196.3 128.5 113.5	flavanol moiety CH position 2
2.47 dd		196.3 127.8 79.5	flavanol moiety CH_2 position 3
3.11 dd		196.3 127.8 79.5	flavanol moiety CH_2 position 3
5.01	98.9	163.4	anomeric proton of O-glycoside residue
4.77	80.5		flavonol or procyanidin CH
3.50	74.1-72.2	98.6 75.6	sugar residue CH
3.62	71.3		sugar residue CH
3.86	68.8		sugar residue CH
4.00	70.0		sugar residue CH
4.05	74.6	70.0	sugar residue CH
4.02	66.7	75.0	sugar residue CH
2.32	37.3	172.6	organic acid CH_2
1.25	28.6		aliphatic

Procyanidins are the main component of *T. coadunata* ethyl acetate extract fraction as given in Table 10. The Presence of procyanidin is represented by aromatic hydrogen bond at 6 and 8 for the upper units and H-6 for the lower units. Different signals were obtain for position 2 of monomers, that is represented by the signals at δ H and δ C with the value of 5.31, 79.3 as well as 4.81 and 74.3 respectively (Table 10). Other related signals were recognized at different C-3 positions and some signals were detected at CH2 at δH and δC position as C4 terminal units (Table 10). Presence of procyanidin in acetone-eluted fraction has also been reported by Ezaki-Furuichi *et al. (*1986).

Table 10: NMR assignments of TC-EtOAc. Data were obtained from H, HSQC-DEPT, COSYand HMBC spectra in MeOD-d4

δH	δC	Correlations	T-EtOAc tentative assignments
7.28	126.5	144.5 119.4	aromatic phenol ring of procyanidin or tannin
7.00	115.0	144.5 120.0 73.6	catechin moietyH-2' or H-6'
6.80	118.2	144.0 129.4 116.5	catechin H-5'
5.98-6.01	95.0-93.0	156.0 101.0 93	H-6/8 of catechin units
5.31	79.3		H-2 of upper unit of catechin/epicatechin moieties
4.81	74.2	67.5 113.3 119.8 129.5	H-2 of lower units of catechin/epicatechin moieties
4.07	74.7		H-2 of lower units of catechin/epicatechin moieties
3.84	68.9	101.5 37.4 38.5	H-3 of upper units of catechin/epicatechin moieties
3.31	47.6		C-4 of upper units of catechin/epicatechin moieties
3.14-2.72	42.6		C-4 of terminal units
3.05-2.39	37.4		C4 terminal units

NMR technique have verified that *Tectaria coadunata* extracts contains different types of glycosidic flavone and various kinds of procyanidins. Therefore to verify it further, liquid chormatography and mass spectrometry was used.

4.2.2 Quali-quantitative analysis

Methanolic, Ethylacetate and water extracts of *T. coadunata* were investigated by using HPLC-DAD-MS, for the detection and characterization of phytoconstituents, according to their retention time (Table 11). There were different degree of procyanidins and glycosylated flavone were obtained and results were verified with NMR analysis. The obtained flavonoids were naringenin-7-O-glucuronide (Figure 11), eriodictyol-7-O-glucuronide (Figures 9 and 11), and luteolin-7-O-glucuronide (Figure 11). The structures of the compounds were elucidated by NMR and MS.

In all three extracts, procyanidin (PAC) dimers, trimers, and tetramers, as well as larger polymers, were obtained. Separation, identification and characterization of different procyanidins were obtained from MS data. Both A-and B-type procyanidins

was confirmed, the B-type procyanidin dimer was reported at 19.5 min, while A-type procyanidin trimers was reported at 17.9, 19.1, and 20.8 min (Table 11). With reference to quantitative aspect, dimers and trimers were in significant amount in ethylacetate fraction (Figure 9). In methanolic extract high amount of A-type procyandin tetramer and bigger polymers of both A and B types was contained (Figure 10). Sephadex was use to purify procyanidin structure. The significant constituent in methanolic extract was A-type procyanidin tetramer with one unit of (epi) afzelechin (25.2 mg/g). In ethylacetate and water extract high amount of A-type procyanidin trimer (38.69 milligram per gram and 2.58milligram per gram, respectively) was present (Table 11).

Table 11: Identified compounds in TC-MeOH, TC-EtOAc, and TC-H_2O extracts by HPLC HILIC-DAD-FLD-ESI-MS

Tr	[M-H]-	Identification	Fragmentation	mg/g in MeOH extract	mg/g in EtOAc extract	mg/g in H_2Oextract
14.6	447	Naringenin-7-O-glucuronide	MS2 [447]: 271 (100) MS3 [271]: 151 (100)-175 (25) MS4 [151]: 107 (100)	*	0.24±0.06	0.006±0.0003
16.0	463	Eriodictyol-7-O-glucuronide	MS2 [463]: 287 (100) MS3 [287]: 151 (100) MS4 [151]: 107 (100)	0.57±0.09	7.64±0.8	0.48±0.06
17.9	847	A-type proanthocyanidin trimer with one unit of (epi) afzelechinIsomer 1	MS2 [847]: 711 (98)-559 (100)-327 (7)	0.95±0.06	7.40±0.4	0.06±0.003
19.1	847	A-type proanthocyanidin trimer with one unit of (epi) afzelechinIsomer 2	MS2 [847]: 711 (92)-559 (100) MS3 [711]: 585 (100)-559 (75)-423 (60) MS4 [585]: 423 (100) MS3 [559]: 389 (100) MS4 [389]: 362 (50)-345 (100)-273 (3)	8.96±0.45	11.7±2.1	0.05±0.007
19.5	577	B-type procyanidin dimer	MS2 [877]: 425 (100)-407 (60)-289 (30) MS3 [425]: 407 (100)-273 (10)-281 (8) MS4 [407]: 389 (20)-339 (30)-285 (100)-281 (98)-256 (40)-269 (20)-243 (22)-213 (10)	0.39±0.07	11.13±0.3	0.48±0.02

20.3	461	Luteolin-7-O-glucuronide	MS2 [461]: 285 (100) MS3 [285]: 257 (45)-243 (25)-241 (90)-213 (50)-199 (100)-175 (90)-151 (35)	2.13±0.2	16.4±1.2	1.25±0.07
20.8	863	A-type procyanidin trimer	MS2 [863]: 711 (100)-573 (50)-451 (70)-411 (70) MS3 [711]: 559 (100)-407 (27) MS4 [559]: 415 (90)-327 (60)-255 (100)	9.73±0.91	38.69±2.6	2.58±0.21
22.4	1135	A-type proanthocyanidintetramer with one unit of (epi) afzelechin	MS2 [1135]: 999 (70)-847 (100)-707 (70)-634 (58)	25.2±0.17	5.70±1.8	0.09±0.004
25.1	1151	A-type proayanidintetramer	MS2 [1151]: 1025 (60)-863 (100)-709 (60)-573 (25)	6.5±0.76	0.44±0.8	*
25.3	1424 [M-2H]2-	B-type proanthocyanidindecamer with two units of (epi) afzelechin	MS2 [1424]: 1271 (100)	0.69±0.09	4.74±0.1	0.02±0.005
26.1	1151 [M-2H]2-	A-type procyanidin octamer	MS2 [1151]: 863 (42)-777 (55)	8.97±0.06	0.62±0.05	0.09±0.03
26.9	1438 [M-2H]2-	A-type procyanidin decamer with two A bonds	MS2 [1438]: 1191 (100)	11.4±0.1	0.61±0.04	0.013±0.001
28.5	720 [M-2H]2-	B-type procyanidin pentamer	MS2 [720]: 643 (100) MS3 [643]: 559 (65)-407 (25)	5.67±0.1	5.97±0.08	0.09±0.006
28.6	719 [M-2H]2-	A-type procyanidin pentamer	MS2 [719]: 567 (50)-451 (20)	10.8±1.1	0.99±0.09	0.07±0.007
29.8	1440 [M-2H]2-	B-type procyanidin decamer	MS2 [1440]: 1313 (100)-961 (55)-817 (70)	0.51±0.2	2.79±0.06	0.14±0.004
33.0	864 [M-2H]2-	B-type procyanidin esamer	MS2 [864]: 779 (90)-575 (70)-532 (75)-411 (100)-289 (20)	5.85±0.4	5.44±0.1	0.15±0.003
34.3	1008 [M-2H]2-	B-type procyanidin heptamer	MS2 [1008]: 777 (55)	9.87±1.3	1.78±0.03	0.08±0.005

*detectable but not quantifiable

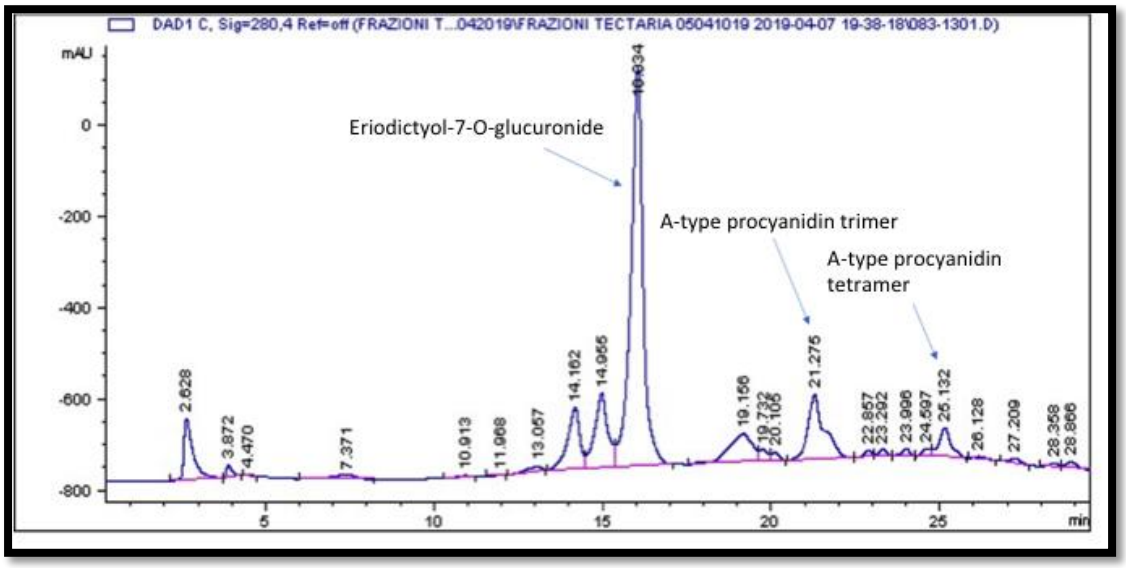


Figure 9: Chromatogram of Ethyl acetate extract at 280 nm

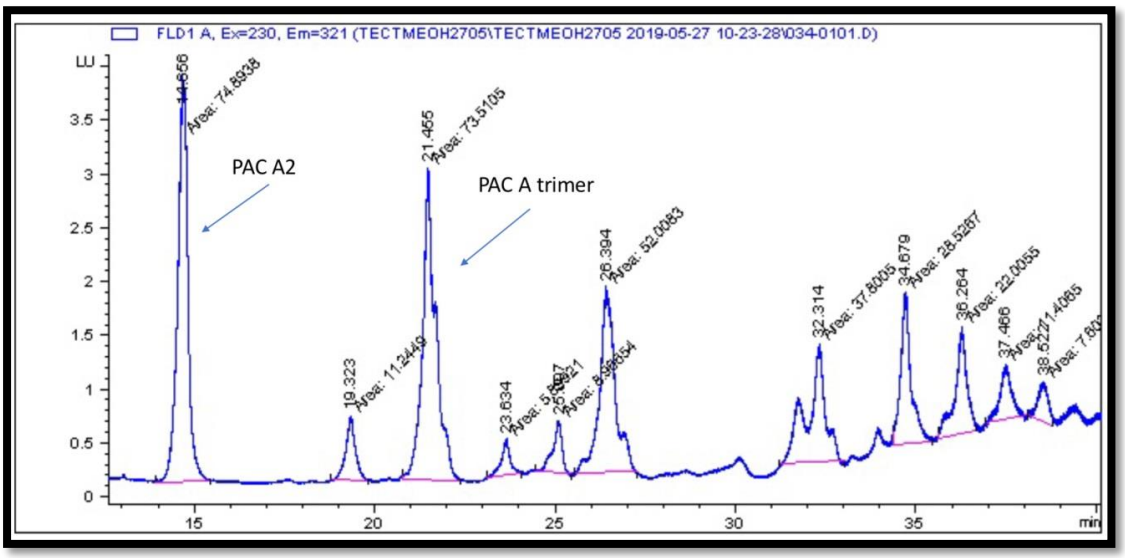


Figure 10: Chromatogram of methanolic extract in FLD (ex 230; em 321)

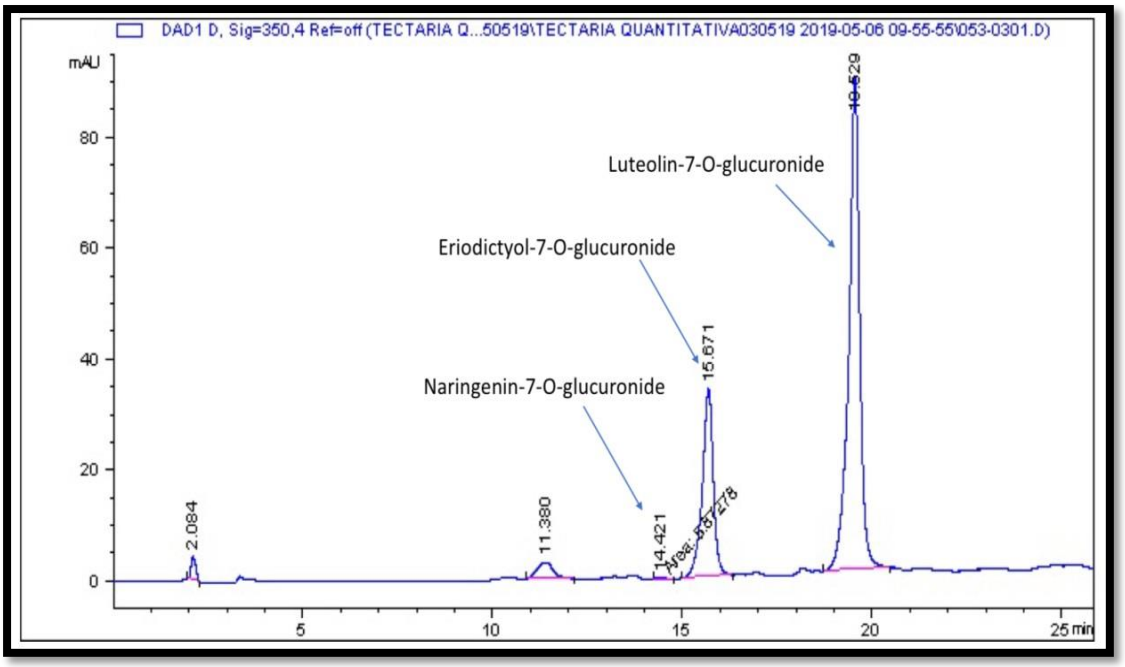


Figure 11: Chromatogram of H_2O extract, 350 nm

Flavonoids were abundant in all extracts (Table 12). Ethylacetate fraction of *T. caodunata* contained more amounts of flavonoid (24.28 milligram per gram) (Table 12). In methanolic extract, flavonoids were present in an amount comparable to that of water extract (2.70 and 1.74 milligram per gram), apart from naringenin-7-O-glucuronide which was not obtained.

HPLC-HILIC-FLD was used for the verification of quantitative analysis of procyanidins and the results is provided in Table 12. The chromatogram of methanolic extract is presented in Figure 10. Methanolic and Ethylacetate fractions of

T. coadunata achieved the highest amount of total procyanidins (105.49and 98.0 milligram per gram), resulting for more than fifty percentage of total polymers in methanolic and more than fifty percent of the total as trimers in ethylacetate extract. Only low amounts of procyanidin were obtained in water fraction (3.91±0.5 milligram per gram), because they are less soluble in water (Table 12).

Table 12: Quantitative results of total flavonoids, total procyanidin (PAC), and PACs divided on the basis of different degrees of polymerization in TC-MeOH, TC-EtOAc, and TC-H_2O extracts

Sample	Totalflavonoid (mg/g)	Total PAC (mg/g)	PAC dimers (mg/g)	PAC trimers (mg/g)	PAC tetramersand polymers (mg/g)
TC-MeOH	2.70±0.05	105.49±0.15	0.39±0.01	19.64±0.13	85.46±0.16
TC-EtoAc	24.28±0.15	98.00±0.12	11.13±0.15	57.79±0.15	29.08±0.15
TC-H_2O	1.74±0.05	3.91±0.05	0.48±0.01	2.69±0.05	0.74±0.02

High concentration of polymers was obtained in methanolic extract, while procyanidins with lower molecular weights was obtained in high amount in ethylacetate fraction, because ethylacetate and water fractions were obtained from methanolic extract. This may be due to procyanidins having higher molecular weight are less soluble in water and in ethylacetate fraction. Mitchell *et al. (*2017) have also reported that procyanidin oligomers were soluble than big ones and there is interactions between solute and solvent.

Phytochemical constituents of the ethylacetate fraction confirmed that there were high amount of condensed tannins, some flavonols and dihydroflavone.

4.2.3 *In vitro* bioassays

4.2.3.1 Antioxidant activity

There were several phenolic compounds present in *T. coadunata* extracts. These phenolic and polyphenolic constituents exihibit biological properties, which is presented in Table 13. Antioxidant activities from DPPH and ABTS, CUPRAC, FRAP and metal chelating aand enzyme inhibition data are provided in Table 14. DPPH and ABTS were used to find out antioxidant properties. Table 13 showes the range of antioxidant activities which was found to be 762.62 milligram Trolox equivalent per gram of methanolic extract, to 933.97 and 948.59 of water and ethylacetate extracts, respectively. ABTS antioxidant activity was found to be from

1097.10 milligram Trolox equivalent per gram of the methanolic extract to 1661.21 of ethylacetate extract. The data showed that ethylacetate fraction exhibit highest antioxidant activities that was correlated by the presence of polyphenols amounting 276.70 milligram Gallic acid equivalant per gram (Table 13). The study was verified by the correlation coefficient analysis which is present in Figure 12. The total phenolic content and 2,2-diphenyl-1-picrylhydrazyl acitvities showed excellent result with reducing sugars and the phytochemical compounds that was experimented for oxidation of the compunds with the help of phenolics.

Table 13: Results of total phenolic content and in vitro antioxidant assays on *T. coadunata* extracts

Samples	Total phenolic content (mgGAE/g)	DPPH (mgTE/g)	ABTS (mgTE/g)	CUPRAC (mgTE/g)	FRAP (mgTE/g)	Metal chelating mgEDTAE/g)	Phosphomolybdenum (mmolTE/g)
TC-EtoAc	276.70 ± 2.58	948.59 ± 30.92	1661.21 ± 9.01	1510.63 ± 31.55	931.18 ± 17.74	na	6.32 ± 0.41
TC-H_2O	235.85 ± 1.82	933.97 ± 12.12	1269.30 ± 21.75	1108.66 ± 4.44	713.07 ± 11.98	6.26 ± 0.73	6.25 ± 0.18
TC-MeOH	234.30 ± 0.99	762.62 ± 34.65	1097.10 ± 14.02	1089.99 ± 6.42	645.59 ± 4.83	2.61 ± 0.34	5.70 ± 0.59

Values are reported as mean ± SD of three replicates. ABTS: 2, 20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); CUPRAC: cupric-reducing antioxidant; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; FRAP:ferric-reducing antioxidant power; GAE:gallic acid equivalent; TE:Troloxequivalent; EDTAE:EDTA equivalent; na: notactive. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

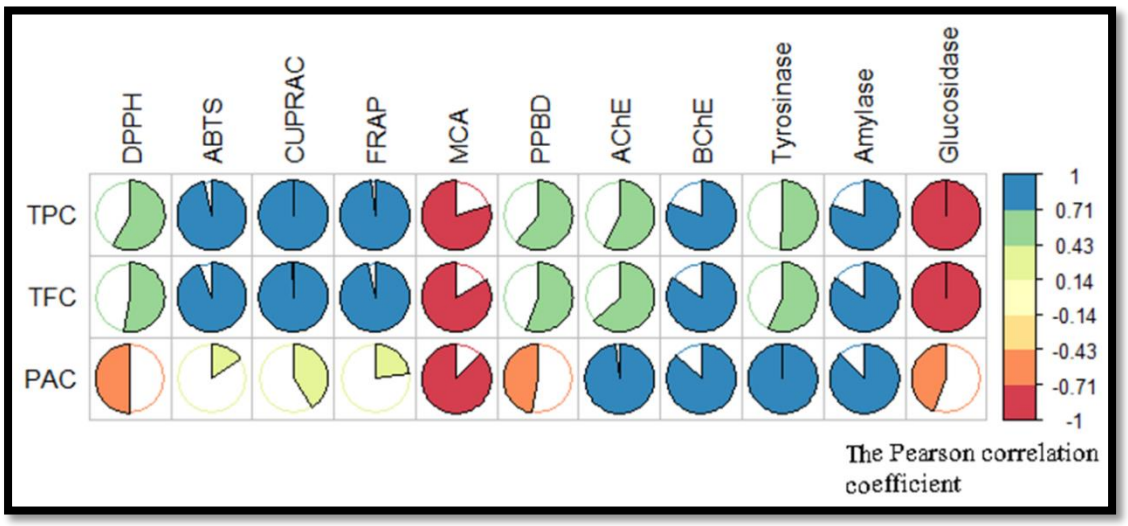


Figure 12: Correlation coefficients between total bioactive compounds and biological activities (Pearson correlation coefficient (R), $p < 0.05$). TPC: total phenolic content; TFC: Total flavonoid content; PAC: procyanidin; PPBD: phosphomolybdenum assay. MCA: metal-chelating assay

Varities of polyphenolics extracted from *T. coadunata* exhibited antioxidant properties, which was coherent with different literatures. Several literatures showed that procyanidins were responsible for oxidation of the compounds, that exhibit defense mechanism against oxidation stress (Wang *et al.*, 2015). Ugartondo *et al.* (2007) also reported that antioxidant activities depends on polymerization and enhance with galloylation. However, Ricardo-da-Silva *et al.* (1991) showed that the degree of polymerization was not directly proportional to antioxidant properties, but with the presence of hydroxyl groups the process of polymerization could increase. While, Pietta, (2000) reported that the antioxidant capacity may also be due to presence of catechol group present in ring B, which could reduce highly oxidizing free radicals (superoxide, peroxyl, alkoxyl, and hydroxyl) by donating hydrogen atom.

From the above result, it can be concluded that *Tectaria coadunata* ethyl acetate extract exhibited high antioxidant activities, may be due to the presence of procyanidins dimers (11.13 milligram per gram), trimers (57.79 milligram per gram), tetramers and polymers (29.08 milligram per gram) (Table 12). Likewise, various experiments have proved that phospolipids heads groups are responsible for oxidation as, PACs, catechin and epicatechin could absorb through membranes, which could lead to exhibit antioxidant reaction. Likewise other external factor of oxidants also helps to participate in antioxidant activities (Erlejman *et al.*, 2004). Pazos *et al.* (2006) also reported that galloylated catechins could control the membrane pattern by forming complex structure that limit the access of prooxidants.

Reducing power activities, particularly ferric reducing antioxidant power (from Fe3+ to Fe2+), Cupric reducing antioxidant capacity (from Cu2+ to Cu+), and phosphomolybdenum (from Mo (4) to Mo (5) acitivites, were carried out to show similar electron giving capacity of the extracts and the result are presented in Table 13. The activities showed that ethyl acetate fraction showed highest antioxidant properties than water and methanolic extract. The results could be due to the existence of phenolics, especially procyanidins ethyl acetate extracts. Several papers have also reported that antioxidant properties was responsible due to the presence of phenolics (Zhou *et al.*, 2018; Chang *et al.*, 2019). However, ethyl acetate extract was not active in the metal-chelating assay, while water extract was more active than methanolic

extracts (Table 13). The finding could be explained due to the presence of non-phenolic chelators.The experiment done by Rice-Evans *et al*. (1996) showed that metal-chelating capacity was a small antioxidant properties of phenolics.

4.2.3.2 Test of inhibitory effect against degenerative and metabolic enzymatic activities: cholinesterases, α-amylase, α-glucosidase and tyrosinase

Like in *Giradinia diversifolia* the inhibitory activity was experimented both on acetylcholinesterase (AChE) and butyryl cholinesterase (BChE), which were hydrolytic enzymes performing on acetylcholine (ACh) to finish its actions within the synaptic cleft by cleaving the neurotransmitter to choline and acetate. According to Sharma *et al*. (2019) both enzymes are found in the brain and are responsible for neurological decline characteristic of Alzheimer's disease. It had been advised that acetylcholinesterase enzymes predominates within the healthy brain, with butyryl cholinesterase considered to play small role in changing brain ACh levels. During this disorder, with the progress of the disease AChE activity reduces to 33-45% of normal values, while the activity of BChE increases by the maximum amount as 40-90% specifically in the brain areas. This alteration of AChE to BChE ratio causes a change within the normally supportive role of BChE in hydrolyzing excess ACh. As a result, it can be concluded that BChE inhibition might provide an appropriate therapeutic target to treat AD (Mushtaq *et al*., 2014).

Therefore, the inhibitory activities of tested extracts against cholinesterases (acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase was done since enzyme inhibition is considered as one possible approach to manage some chronic conditions of health problems. A well known treatment regime for Alzheimer's disease (AD) includes cholinesterase inhibitors like donepezil, galantamine, and rivastigmine. Current treatment options to manage type-2 diabetes consistof α-amylase and α-glucosidase inhibitors like acarbose, miglitol, andvoglibose. Similaly, epidermal tyrosinase inhibition by kojic acid was recently used to manage skin hyper pigmentation. However, the adverse effects related to the utilization of recently available enzyme inhibitors, researchers have showed interest in finding novel therapeutic agents (Sut *et al*., 2019).

Among different extract, *T. coadunata* water extract showed lowest activity against all enzymes, while the ethyl acetate extract inhibition activities showed the highest values, especially for acetylcholinesterase (6.22 mg GALAE/g), butyrylcholinesterase (9.82 mg galantamine equivalent (GALAE)/g), and tyrosinase (153.89 mg GALAE/g) (Table 14). *T. coadunata* methanolic extract and ethyl acetate extract demonstrated prominent inhibitory effects against acetylcholinesterase with the 5.58 mg GALAE/g and 6.22 mg GALAE/g respectively (Shrestha *et al.*, 2019). The results thus show that phenolic compounds have cholinesterase inhibitory activities as reported by Orhan *et al*. (2007).

Table 14: Results of in vitro enzyme inhibition assays on *T. coadunata* extracts

Samples	AChE inhibition (mgGALAE/g)	BChE inhibition (mgGALAE/g)	Tyrosinase inhibition (mgKAE/g)	Amylase inhibition (mmolACAE/g)	Glucosidase inhibition (mmolACAE/g)
TC-EtoAc	6.22±0.06	9.82±0.68	153.89±1.61	1.50±0.02	5.46±0.05
TC-H_2O	1.35±0.03	1.70±0.67	66.85±1.22	0.42±0.04	5.48±0.01
TC-MeOH	5.58±0.10	6.31±0.71	149.41±0.96	1.04±0.05	5.48±0.01

*Values are reported mean ± SD of three replicates. GAE: Gallic acid equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; na: not active. Different superscripts indicate significant differences in the extracts ($p<0.05$)

T. coadunata ethyl acetate extract showed significant AChE inhibitory activities in the result. This may be due to the non-detectability of nitrogen-containing compounds which suggest that there may be the presence of non-alkaloidal inhibitors in the extract. The finding for non-nitrogen containing acetylcholinesterase and butyrylcholinesterase inhibitors was important since alkaloids is reported to cause some common side effects (Mishra *et al.*, 2019). Furthermore, non-alkaloidal inhibitors probably have different kinds of interaction with the target enzyme because of the shortage of a charged part, thus showing the chance to find other pharmacological properties (Jing *et al*., 2019).

In this regard, a paper showed that phenolic compounds were capable to interact with organic compound residues showing the site of AChE via a chemical bond, hydrophobic, and π-π interaction (Jabir *et al*., 2018). Multiple hydroxyl groups within the phenolic compound were suggested to boost the inhibitory action of AChE due

to strong binding capacity. These inhibitory actions suggest the inhibitory potential of most of the phenolic compounds but not all show the equal mode of action. Correlation analysis have also determined this fact (Figure 12). Based on the correlation analysis, an perfect correlation was achieved between total bioactive components and also the inhibitory activities of cholinesterases phenolics (R: 0.57 for AChE and R: 0.80 for BChE), flavonoids (R: 0.63 for AChE and R: 0.85 for BChE), and procyanidin (R: 0.98 for AChE and R:0.87 for BChE) (Table 15).

Table 15: Correlation coefficients between total bioactive compounds and biological activities

Variables	TPC	TFC	PAC	DPPH	ABTS	CUPRAC	FRAP	MCA	PPBD	AChE	BChE	Tyrosinase	Amylase	Glucosidase
TPC	1	0.998	0.412	0.586	0.964	1.000	0.981	-0.795	0.612	0.575	0.806	0.512	0.801	-0.999
TFC	0.998	1	0.475	0.528	0.943	0.997	0.965	-0.836	0.556	0.631	0.845	0.570	0.841	-0.999
PAC	0.412	0.475	1	-0.496	0.154	0.406	0.227	-0.880	-0.468	0.982	0.872	0.994	0.875	-0.442
DPPH	0.586	0.528	-0.496	1	0.781	0.592	0.733	0.025	0.999	-0.326	-0.007	-0.396	-0.015	-0.560
ABTS	0.964	0.943	0.154	0.781	1	0.966	0.997	-0.605	0.801	0.336	0.618	0.264	0.613	-0.955
CUPRAC	1.000	0.997	0.406	0.592	0.966	1	0.982	-0.791	0.618	0.569	0.802	0.506	0.797	-0.999
FRAP	0.981	0.965	0.227	0.733	0.997	0.982	1	-0.662	0.754	0.405	0.675	0.335	0.670	-0.974
MCA	-0.795	-0.836	-0.880	0.025	-0.605	-0.791	-0.662	1	-0.008	-0.953	-1.000	-0.928	-1.000	0.814
PPBD	0.612	0.556	-0.468	0.999	0.801	0.618	0.754	-0.008	1	-0.295	0.025	-0.366	0.018	-0.587
AChE	0.575	0.631	0.982	-0.326	0.336	0.569	0.405	-0.953	-0.295	1	0.948	0.997	0.950	-0.601
BChE	0.806	0.845	0.872	-0.007	0.618	0.802	0.675	-1.000	0.025	0.948	1	0.921	1.000	-0.824
Tyrosinase	0.512	0.570	0.994	-0.396	0.264	0.506	0.335	-0.928	-0.366	0.997	0.921	1	0.924	-0.539
Amylase	0.801	0.841	0.875	-0.015	0.613	0.797	0.670	-1.000	0.018	0.950	1.000	0.924	1	-0.820
Glucosidase	-0.999	-0.999	-0.442	-0.560	-0.955	-0.999	-0.974	0.814	-0.587	-0.601	-0.824	-0.539	-0.820	1

In Azheimer's disease, it is suggested to know the structural requirements of flavonoids as inhibitors of enzymes. For this AChE and BChE, were tested along with the established structure-activity relationships (SARs) of flavonoids as reactive oxygen species (ROS) scavengers and metal chelators. According to Ji & Zhang (2006) flavonoids, like quercetin that could efficiently bind with enzymes can act as AChE and BChE inhibitors, which was showed by docking experiments. The reason behind AChE inhibition by flavonoids may be due to the presence of phenylchroman in the backbone along with the position, number, substitution of hydroxyl groups, and the oxidation number of C-ring of the flavonoid structure. Jabir *et al*. (2018) also stated that an in depth inspection showed that the binding depends not only on the OHs at positions 5 and/or 7, but also on the catechol in ring B, which might be the reason for the effectiveness of AChE inhibition by flavonoids.

4.2.3.3 Amylase and glucosidase inhibitory activity related to phytochemical composition of the *T. coadunata* extracts

The *T. coadunata* extract was also tested for tyrosinase, α-amylase, and α-glucosidase, apart from the inhibitory activities tested against cholinesterases (acetylcholinesterase (AChE), BChE (butyrylcholinesterase), and the result is provided in Table 14. Amylase and glucosidase inhibition was quite low with values around 1 mmol acarbose equivalent (ACAE)/g for amylase inhibition and 5.5 mmol ACAE/g for glucosidase, showing limited activity of *T. coadunata* extracts. The present result is therefore, very different with the findings of several researchers that showed significant inhibition abilities of procyanidin (PAC) against amylase (Yilmazer-Musa *et al*., 2012; Kato *et al*., 2017; Dai *et al*., 2018). The contradictory results might be because of the antagonistic actions of phytochemicals in the extracts. At this point, the isolated compounds from *T. coadunata* could be specially tested as diabetic agents in further studies.

All of the *T. coadunata* extracts (with the exception of the aqueous one) displayed significant tyrosinase inhibitory activity ranging from 149.41 mg kojic acid equivalent (KAE)/g for TC-MeOH extract to 153.89 mg KAE/g for the TC-EtOAc extract, related to their high total phenolic content. High phenolic content in the extract, especially the flavonoids containing a 3-hydroxy, 4-keto group, like eriodictyol, may have demonstrated significant tyrosinase inhibitory activity. According to a recent paper on flavonols showed that aglycones derivates perform tyrosinase inhibitory activity, that supports the function of the 3-hydroxyl group, which supports our findings. However, according to Kim & Uyama (2005), the 3-hydroxyl radical is not found in several flavones, like luteolin and luteolin7-O-glucoside, but still show tyrosinase inhibitory activity.

Cytotoxicity tests

Several anti-cancer agents have been discovered from plants in the last decades. The existence of phytoconstitutents *viz*., alkaloids, phenolics and terpenoids in plant extracts has been associated with anticancer and cytotoxic activity (Meyer *et al*., 1982; Dushimemaria *et al*., 2017). Flavonoids show anti-allergic, anti-inflammatory, anti-microbial and anticancer activity (Manivannan & Johnson, 2020).

The phytochemical analysis has confirmed the presence of phenolics compounds in the extract of *T. coadunata*, especially the procyanidins and glycosylated flavonoids. The presence of variation in the metabolites contents may be responsible for all the biological activities tested in the present study. In this regard, the antioxidant, enzyme inhibitory and cytotoxic activities gave results with varied frequencies. Therefore, preliminary cytotoxicity tests was also performed on two human tumor cell lines, ovarian cancer (2008) and pancreatic cancer (BxPC3) and the results are present in Table 16.

Table 16: Cytotoxicity tests (concentration of compound inhibiting cell growth by 50% (IC_{50}, _g/mL) of TC-MeOH, TC-EtOAc, and TC-H2O extracts on 2008 and BxPC3 cell lines

Samples	2008	BxPC3
TC-EtOAc	28.7	12.5
TC-H2O	>50	>50
TC-MeOH	>50	>50

Cytotoxity was tested for all three extract of *T. coadunata (*TC-MeOH, TC-EtOAc and TC-H2O). The result shows that methanolic extract ((TC-MeOH) and water extract (TC-H2O) exhibited a amount of compound inhibiting cell growth by 50% (IC_{50})> 50 µg/mL on both cell lines, while ethyl acetate extract (TC-EtOAc) showed a significant cytotoxicity, with a IC_{50} of 12.5 and 28.7 µg/mL against human pancreatic and ovarian cancer cell lines, respectively (Table 16). The activity of ethyl acetate extract can be related with the content of procyanidin in the extract. In most of the literature it has been reported that the fractions with the highest degree of polymerization and galloylation can exert the toxic effect against cancer cells. The result was in agreement shown by various researchers who also recognized the best level of cytotoxicity is due to the presence of polyphenolic compounds (Touriño *et al*., 2005). In most of the previous studies it has been suggested that procyanidins showed different apoptotic mechanisms. When pancreatic cell lines was treated with procyanidin present in ethyl acetate extract it exerted anti-proliferative and anti-invasive effects in the present study (Photo plate 6: C, D). From this it can be suggested that *Tectaria* ethyl acetate extract application as a potent chemo-preventive or therapeutic agent for carcinoma treatment as also reported by Lee (2017). On the opposite hand, previous studies in ovarian neoplastic cell lines (Photo plate 6: A, B) showed that procyanidin exert cytotoxic activity via several mechanisms, inducing apoptosis with DNA damage and caspase-3 mediation (Taparia & Khanna, 2016).

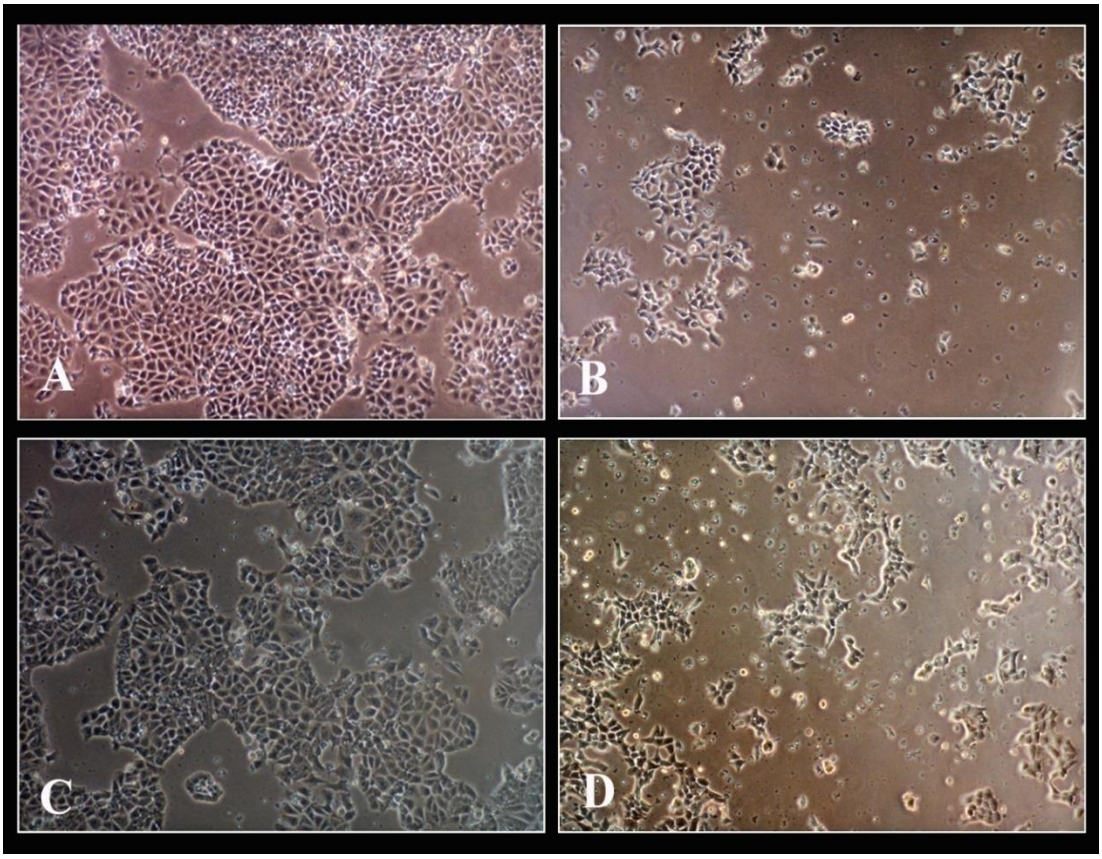


Photo plate 6: A. Photos of Ovarian (2008) cell lines CTR and **B.** Ovarian (2008) cell lines TCM Treatment. **C.** Photos of Pancreatic cell lines (BxPC3) CTR and **D.** Pancreatic cell lines (BxPC3) TCM Treatment. Pictures (40xmagnification)

Several species of *Tectaria* like *T. singaporeana* showed potential cytotoxic effect against MCF-7wt cell lines with IC_{50} value 28.57 µg/ml (Aini *et al*., 2008). *T. zeylanica* leaves showed the high antioxidant activity. *T. cicutaria* also showed potential cytotoxic effect against K562 cancer cell lines and the cell cycle progression and inducing apoptosis (Karade & Jadhav, 2018) all these findings supports our results.

4.3 *Melia azedarach* L

Melia azedarach is a common tree and one of the well known medicinal plants used in the traditional medicine of Nepal. It is an important ethnomedicinal plants in Nepal used by different ethnic communities for various ailments. It is used in headaches, healing arthritis, in rheumatic pain, stomach troubles, diarrhoea and dysentery and to treat skin diseases. Various parts of this plant have anthelmintic, stimulant,

antispasmodic, antimalarial, and emetic properties (Manandhar, 2002). All these medicinal value of this plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun *et al.*, 2007). Many sources on *M. azedarach* revealed that this plant contains many phytochemical constituents including alkaloids, phenols, flavonoids, glycosides, terpinoids, saponins, rutins, tannins and phenolic compounds (Abbas *et al*., 2017; Farook *et al.*, 2019). Phytochemicals such as phenol and flavonoids are well known for their antioxidant potential. Therefore, cytotoxic evaluation of leaves extracts can gives us better insight into cytotoxic effects of whole plants. For detail investigation of the leaves, their main fractions were also studied and the methanol leaf fraction of *M. azedarach* was selected for isolation of active compounds.

4.3.1 Phytochemical analysis

Melia azedarach have highly complex chemical composition. Therefore, it has been a subject of interest to evaluate the presence of bioactive constituents. Exploration of the phytochemicals was performed from leaves combining the data obtained with different analytical techniques. The methanol fraction as most polar fraction of the leaves is expected to contain foresaid active components more than others. NMR analysis of the methanolic extract revealed a high amount of signals in the hydrocarbons region of the spectra, which suggests presence of lipids not allowing the recognition of relevant aromatic signals. In order to purify the extract, different chromatographic separations, both on silica gel and on sephadex, as well as preparative TLC were performed; nevertheless a high amount of lipids still remains in each fraction. For the preliminary study of chemical composition, 1D and 2D NMR analysis were used. The main compound identified and quantified by HPLC-DAD-MSn approaches were flavonoid glycosides and limonoids. Later to study the phytosterol and lipid composition, HPLC-APCIMSn and Gas Chromatography/Mass spectrophotometry analysis were used. The extract, revealed a variety of flavonoids, especially flavonols, both as aglycones and as glycoside derivative. In fact, the main peaks in the chromatogram are represented by rutin, quercetin-3-O-glucoside and kaempferol (Figure 13).

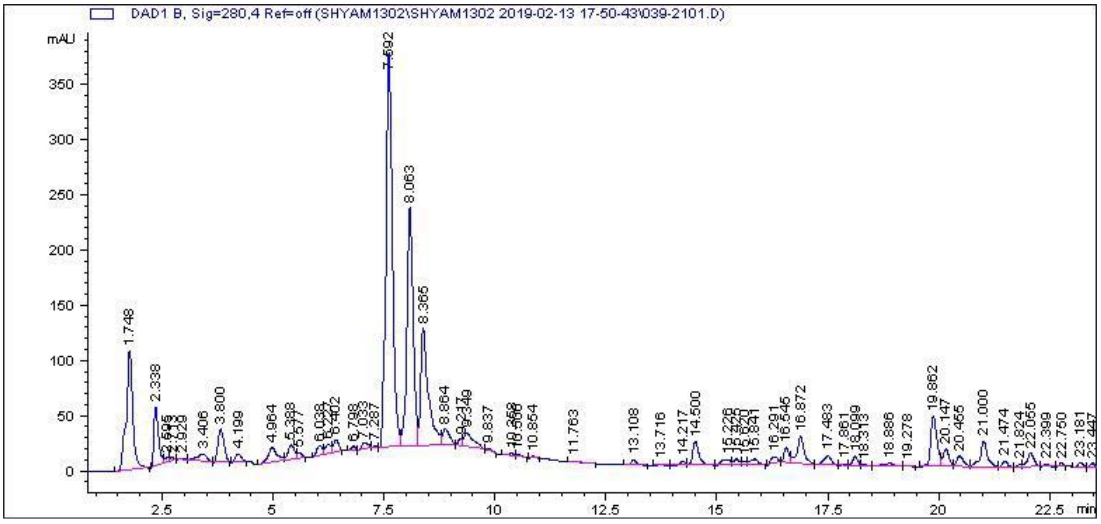


Figure 13: Chromatogram of MeOH extract of *M. azedarach* at 280 nm

1H-NMR analysis of the methanolic extract revealed a high amount of signals in the aliphaticregion of the spectrum (0.5-2.00ppm), intense signals ascribable to terminal methyl groups, (0.89-0.91 ppm) aliphatic CH_2 of the fatty acid chains (1.25-1.28 ppm) and triplets ascribable to CH_2 nearby double bond (1.60 ppm) and nearby carbonyl function (2.30 ppm) support the presence of lipids in large amount in the extract. Broad signals were detected in carbohydrate region of the spectrum allowing no identification of compounds, while in aromatic region large signals precluded the observation of any significant compound. Liquid-liquid partition was then used to obtain more simplified fraction and to acquire more detailed information on constituents. Partition was performed using solvents in increasing polarity.

1H-NMR of the hexane and chloroform fractions appear very similar and showed, as expected, large presence of lipid constituents. Combination of 1D and 2D homonuclear and heteronuclear experiments allowed the observation of diagnostic signals that allowed the tentative identification of some classes of phytoconstituents. In the spectrum region δ 6.0-7.5 signals were observed both in the ^1H and HSQC-DEPT that could be ascribed to furan ring namely the CH at δ 7.34 (δc 144.8), 7.28

(δc 138.8) and 6.23 (δ 110.0) suggesting the presence of limonoid derivatives as reported in Table 17 (Shrestha *et al*., 2021). Data are obtained from HSQC-DEPT, correlation spectroscopy, and HMBC spectra in MeOD-d4.

Table 17: Nuclear magnetic resonance (NMR) assignments of TC-MeOH

δH	δC	Correlations	M-MeOH assignment
6.23	110.0		Limonoid signal
7.34	144.8	138.8	Limonoid signals related to the furan ring
7.28	138.8	144.8	Limonoid signals related to the furan ring
6.0	115.5	145 143	aromatic signal of phenolic compounds
6.23	110.0		Limonoid signal
6.80	100.5	150 110	aromatic signal of phenolic compounds
6.85	99.1	149 151	aromatic signal of phenolic compounds
5.07	120.2	23.5	sp2 of unsaturated fatty acid
4.47	68.5	72.5 77.1	Sugar signal
2.80	28.3	175.5 22.5	Methylene of fatty acid nearby carbonyl group
1.44-1.60	22.5-23.5	120.5 20.2	Methylene of fatty acid vicinal to sp2
1.25-1.28	20.2	19.0-23.0	Methylene of fatty acid
0.89-0.91	18.5-19.5	20.2-22.5	Terminal group of aliphatic chains of fatty acids

Also in the methanol fraction obtained after partition with lipophilic solvents the ^{1}H-NMR spectrum revealed several doublets with small coupling constant (J= 1.0-1.5) in the region δ 7.4-8.0 and a broad signal formed by superimposition of several protons at δ 6.80 (Figure 14). These signals could support the presence of a furan ring typical of limonoid type compounds that have been reported for such species in other papers (Vieira *et al*., 2014). The doublets at δ 6.16-6.35 that correlate with aromatic CH at δ 93-98 ppm in HSQC-DEPT could be assigned to the H-6.8 of flavonoids while the signal at δ 6.85 correlating with δ 99.1 could be assigned to H-5 ring A of flavonoid derivative. The methanol extract showed the presence of numerous signals in the sugar regions supporting the presence of monosaccharides, oligosaccharides, furthermore of glycoside residue linked to the flavonoids.

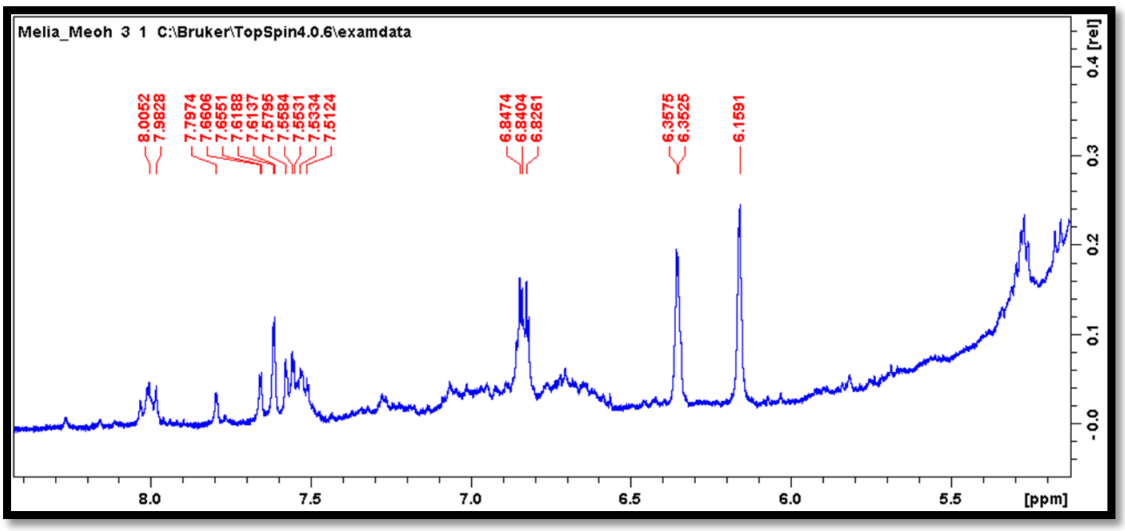


Figure 14:1H-NMR spectrum of melia methanol extract with enlargement of spectrum region presenting signals ascribable to furane ring of limonoids

Other constituents could be detected in the water/methanol fraction, in the aliphatic region of the spectrum singlet at δ 2.50 could be ascribed to succinic acid, while singlet at δ 1.44 could support the presence of alanine. Rhamnose was detected by the doublet at δ 1.33 and the singlet at δ 5.07, while alpha and beta glucose were detected based on the anomeric protons at δ 4.47 and 5.07.

All this initial information suggests that the leaves of *M. azedarach* contained lipids, flavonoids and other phenolics, limonoids thus further investigations were performed using different approaches by GC-MS, LC-DAD-MS^n.

4.3.1a Lipids and phytosterol constituents

The analysis of fatty alcohols, fatty acids and lipophilic compounds were performed by GC/MS and data obtained are presented in Table 18. The lipid constituents in leaves were mostly palmitic, linolenic, and linoleic acids accounting for 19, 15 and 4.5% of total lipophilic constituents (Table 18). In previous article also leaves of *M. azedarach* lipid constituents from Turkey were measured and large amount of linolenic acid (35%), linoleic acid (20%) and palmitic acid (26%) were observed (Orhan *et al*., 2012), this supports our result. This indicates that different geographic origins of plant material can influence the fatty acid composition. *M. azedarach* leaves are source of unsaturated lipids, accounting 8.1% of monounsaturated fatty acid of total lipophilic compounds and 21.0% of polyunsaturated fatty acid. They are source of different classes of unsaturated lipids including ω-3 represented by linolenic

acid (15% of total lipophilic constituents), ω-6 by linoleic and linolealidic acids (5.8%) and ω-9 by gonodic and erucic acid (4.9%). Unusual fatty acid, heneicosanoic acid, with 21 carbon atom was detected in significant amount (4.35% of total lipophilic compounds) (Table 18). Previous article reported significant levels of such constituents in hexane extract of leaves (Rutkauskis *et al*., 2015).

Table 18: Fatty acid, fatty alcohols composition of methanolic extract of *M. azedarach* obtained by GC/MS

tR	Compounds	Methanol extract (mg/g)
15.1	Capric acid methyl ester	0.53±0.02
15.6	4-Tetradecyne	0.30±0.02
16.2	8-hexadecine	0.36±0.03
17.2	Phytadiene	0.20±0.02
19.3	γ-n-Amylbutyrolactone	0.17±0.02
19.8	Methyl miristate	0.43±0.02
23	Methyl myristoleate	0.935±0.002
24.5	Methyl palmitate	11.4±0.20
25.4	Methyl heptanoate	0.18±0.02
25.8	Methyl palmitoleate	0.87±0.03
26.8	Methyl heptadecanoate	0.22±0.02
29.1	Methyl stearate	1.68±0.10
30.2	Methyl oleate	0.46±0.02
31.2	Methyl linoleate	2.72±0.01
33.2	Methyl linolealidate	0.91±0.02
33.9	Methyl linolenate	8.21±0.02
36.2	Phytol	1.84±0.03
37	Methyl arachidate	1.23±0.02
38	Hexacosanol	0.71±0.03
38.9	Methyl gondonate (cis-11-eicoseonic acid methyl ester)	1.930±0.02
39.9	Heptacosanol	8.19±0.12
40.7	Methyl heneicosanate	0.614±0.02
41.6	Tetracosanol	2.93±0.02
42.6	Methyl erucate (methyl docosenoate)	1.213±0.02
43.9	2-methylhexadecan-1-ol	9.21±0.04
44	Methyl tricosanoate	1.326±0.04
	Total lipid constituent (mg/g)	76.5

Results are expressed as mean ± standard deviation (n=4). tR: retention time

The lipid constituents in leaves were mostly palmitic, linolenic and linoleic acids, furthermore significant levels of long chain fatty alcohols were observed in particular heptacosanol (8.19) tetracosanol (2.93). Also, significant amount of the diterpene alcohol phytol (1.84) were revealed (Table 18).

Due to the high abundance of lipids content phytosterol contents analysis was performed by using LC-APCI-MS in dry leaves in water extract, and as methanol extract and the results are reported in Table 19.

Total amount of phytosterols was 1.3% in dried leaves of *M. azedarach* (Table 19) with β-sitosterol (0.56) being the larger constituent, accounting 43% of total phytosterols. Minor constituents were fucostanol and campesterol and stigmasterol (Shrestha *et al*., 2021). In case of methanol extract comparing with dried leaves, β-sitosterol (4.26) is with 7.6 fold extra and total phytosterols (9.59%) was of 7 fold extra (Table 19). This result indicates that methanol is efficient for phytosterol extraction from *M. azedarach* leaves.

Table 19: Amount of phytosterols of dried leaves and the methanolic extract of *M. azedarach*. Results are expressed as mean ± standard deviation (n=4)

Compound	[M+H-H2O]+	Leaves (% w/w)	Methanol extract (% w/w)
Hydroxycicloartanol	427	0.10	0.81
Cycloartanol	409	0.01	0.07
Fucostanol	395	0.29	1.71
Stigmasterol	395	0.13	1.15
β-sitosterol	397	0.56	4.26
Campesterol	383	0.18	1.37
Sitostanol	399	0.03	0.22
Total amount (%)		1.30	9.59

4.3.1b Phenolic and limonoid constituents

Methanolic extract of *M. azedarach* leaves were analyzed by LC-DAD-MSn. DAD detector showed the presence of many peaks that present the UV/VIS spectrum ascribable to phenolic and flavonoid. The evaluation of the UV data, MS, and fragmentation as well as the comparison with reference compounds allowed annotating 20 different derivatives comprising simple phenolic acids, hydroxycinnamic derivative and flavonoids (Figure 15). Methanol has been proven as effective solvent to extract phenolic compounds.

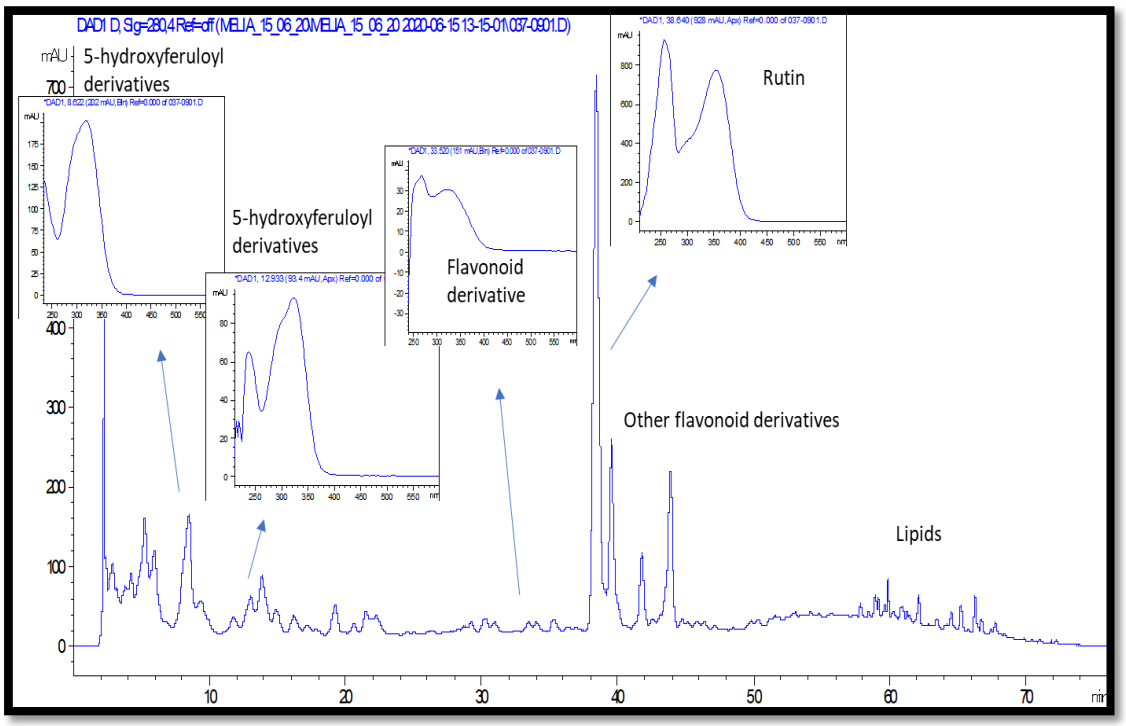


Figure 15: Exemplificative HPLC chromatogram (280nm) of *Melia* leaves methanol extract principal peaks and respective UV spectrums are highlighted

The most abundant compounds in methanol extracts were rutin (37.87), quercetin hexoside (8.88) and kaempferol-7-O-rutinoside (3.71) (Table 20). Similar qualitative pattern of phenolic were observed comparing dried leaves and extract composition, while the amount of phenolic in the methanol extract was increased nearly eight times higher than the dried leaves (Table 20). The isolation of four flavonoids including rutin, kaempferol-3-O-rutinoside, Kaempferol-7-O-rutinoside and isoquercetin was also reported by (Jafari *et al*., 2013) from the leaves of *M. azedarach*, which supports our findings. The presence of rutin and quercetin in ethonolic leaves extract of *M. azedarach* could be a potential source of natural anti-oxidant (Rao *et al*., 2013).

Table 20: Phenolic constituents in *M. azedarach* methanol extract

tR	Ion	Fragmentation	Annotation	Dried plant material	Methanol extract (mg/g)
5.7	315	153, 121	Protocatecuic acid hexoside	0.04±0.01	0.10±0.01
5.1	371	209, 191	5-hydroxyferuloyl-glucoside I	0.18	0.03
8.5	371	209, 191	5-hydroxyferuloyl-glucoside II	0.06	0.09
8.5	355	173, 191 (100)	Dyhydroxy caffeic quinic acid isomer I	0.04	0.04
9.5	355	174, 191 (100)	Dyhydroxy caffeic quinic acid isomer II	0.02	0.02
13.5	355	209, 191, 173	5-hydroxyferuloyl-deoxyhexoside	0.03	0.11
14.2	499	337, 191	p-cumaroyl, caffeoyl quinic acid	0.04	0.04
13.1	385	147, 173, 191 (100), 323, 348	5-hydroxydihydroferuloyl-quinic acid isomer I	0.04	0.11
14.1	385	148, 173, 191 (100), 323, 348	5-hydroxydihydroferuloyl-quinic acid isomer II	0.02	0.15
19.3	385	173, 191 (100), 209, 248, 327, 338	5-hydroxyferuloyl-methylhexoside	0.04	0.15
32.6	625	301	Quercetin di-O-hexoside	0.04	0.16
34.1	625	463, 301	Quercetin-O-hexoside-O-hexoside	0.08	0.31
33.6	771	609, 463, 301	Quercetin dihexoside deoxyhexoside	0.09	1.47
35.4	755	609, 447, 301	Quercetin trideoxyhesoside	0.07	0.71
38.5	609	301	Rutin*	4.88	37.87
40	463	301	Quercetin hexoside	1, 61	8.88
42.2	593	285, 284	Kaempferol-3-O-rutinoside*	0.81	2.20
43.8	593	285	Keampferol-7-O-rutinoside*	1.34	3.71
43.4	505	463, 301	Quercetin acetyl hexoside	0.43	0.99
44.8	447	357, 327, 284, 255	Keampferol-C-hexoside	0.08	0.16
51.5	301	151, 179	Quercetin*	0.02	0.30
			Total phenolic amount	9.94	75.70

Results are expressed as mean± standard deviation (n=4). t_R: retention time, *comparison with reference standard

The LC/MS analysis in positive ion mode allowed observing a series of peaks that were not significantly detected by DAD. These peaks could be ascribed to limonoids observing the MS and fragmentation pathways. Most of the former studies also reported limonoids as responsible compounds for mentioned activities of *M. azedarach* (Zargari, 1990; Carpinella *et al*., 2006). Figure 16 represent the chromatogram recorded in positive ion mode (intensity of base peak) showing the corresponding *m/z* values for each peak. Reference compounds related to *Melia* limonoids are not commercially available, but in previous article some authors (Haldar *et al*., 2014) reported general rules for the assignments of limonoid structures on the basis of MS data. Detected compounds were annotated and tentatively assigned.

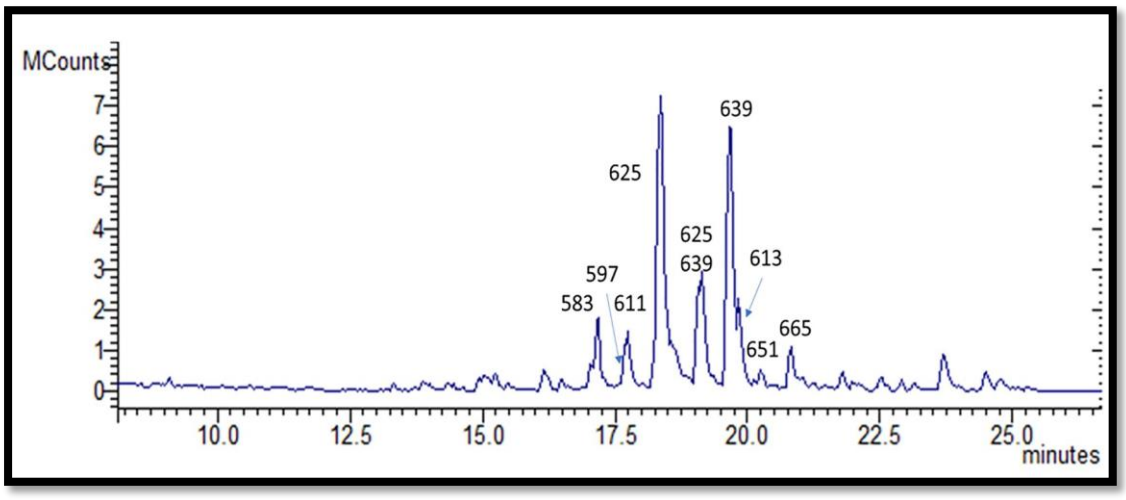


Figure 16: LC/MS of the leaves methanol extract showing the peaks and corresponding m/z values ascribable to limonoids

^{1}H-NMR also revealed the presence of aldehydic signal as well as the proton ascribable to furane ring thus suggesting the presence of limonoids presenting aldehydic function (Figure 17).

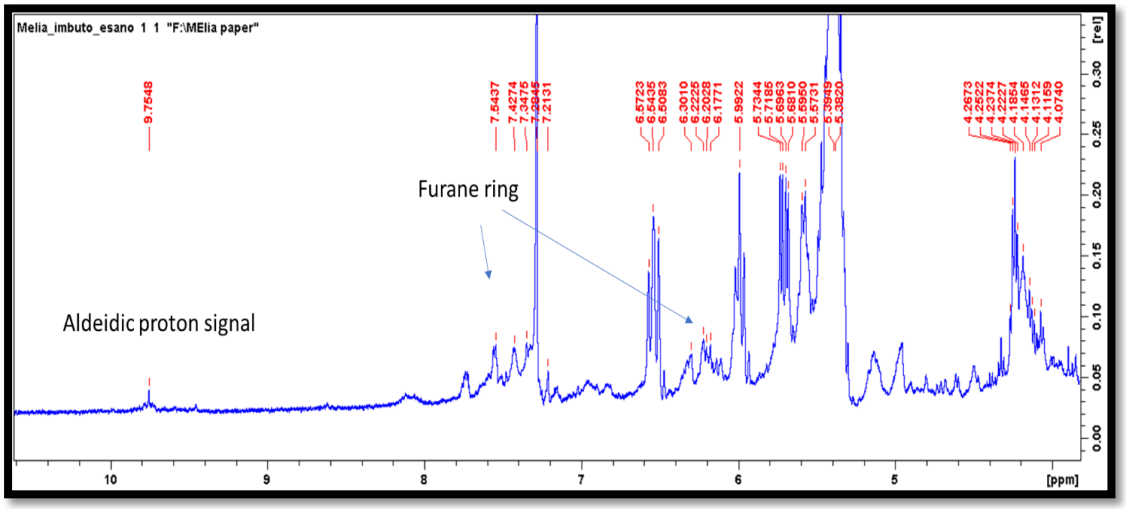


Figure 17: H-NMR of the leaves extract showing the signals ascribable to limonoids namely the aldheidic signal and signals that can be assigned to furane ring

On the basis of LC/MS data, limonoids were detected in both dried leaves and methanol extract of *M. azedarach* as reported in (Table 21). The absence of reference compound for *Melia* limonoids suggested a semi-quantitative analysis using as reference compound the limonoid azadirachtin. The quantitative data showed that methanol extract present almost complete loss of limonoids, probably caused by concentration procedures due to their partial volatility or by oxidative and/or hydrolytic reactions.

Table 21: Tentative identification of the limonoid constituents of dried leaves and the methanolic extract of *M. azedarach*

tR	Ion	Fragmentation	Annotation	Dried leaves (mg/g)	Methanol extract (mg/g)
17.1	583	483	Meliatoosorin R	0.44±0.02	0.03±0.01
17.8	553	467, 431, 389, 371, 353	Tinosposinenside A	0.06±0.01	0.03±0.01
19.8	554	468, 431, 389, 371, 353	Tinosposinenside B	0.05±0.01	0.08±0.02
17.7	597	565, 537, 497, 437, 419, 291, 273, 245	Salannin	0.08±0.01	0.10±0.02
19.7	613.3	584, 525, 465, 405, 387, 369	Salannal	0.60±0.02	0.44±0.02
19	639.4	607, 539, 479, 419, 401, 387, 273, 245	Meliatoosein L	0.95±0.03	-
19.5	639.4	607, 539, 501, 479, 419, 401, 387, 273, 245, 185, 153	Meliatoosein L isomer	2.15±0.05	-
20.1	635.4	603, 575, 454, 435, 375	1, 7 ditygloyl-3-acetyl vilasinin	0.10±0.01	-
18.5	625.4	593, 525, 465, 405, 387, 259, 185, 145	Masendanin A	2.08±0.02	-
19.1	625.4	593, 539, 525, 479, 465, 419, 405, 387, 273, 245	Masendanin A isomer	0.85±0.02	-
			Limonoids Total amount	7.36	0.69

Peak at minute 17.7 present $[M+H]^+$ at *m/z* 597 and were assigned to salannin (Table 21). Characteristic fragments were observed at m/z 565 (-32 Methanol), 537 (-60 Acetic acid), 497 (-100 Tygloyl), 437 (-60,-100), 419 (-18,-60,-100) and characteristic low-mass key fragments at m/z 291, 273 and 245. Main observed fragments and proposed structures are reported in Figure 15. Salannin waspreviously reported in *M. azedarach* by Haldar *et al. (*2014), this supports our findings.

Peak at minute 19.7 with *m/z* 613 was assigned to the $[M+H]^+$ of salannal. The main observed fragments were ions at *m/z* 553 (-60) corresponding to the loss of $C_2H_4O_2$ ascribable to neutral loss of acetic acid (substituent in C-6) or methoxyformiate moiety (chain in position C-11); *m/z* 513 (-100) tyglic acid $C_5H_8O_2$ (C-1), *m/z* 581 (-32) corresponding to the loss of formaldhyde (substituent at C-4). Other significant

fragments were observed at *m/z* 453 (-60,-100), 435 (-18,-60,-100) and 407 (-18,-29,-60,-100) confirming the presence of free hydroxyl group, aldehydic function, acetic and tiglic acid esterification (Figure 18).

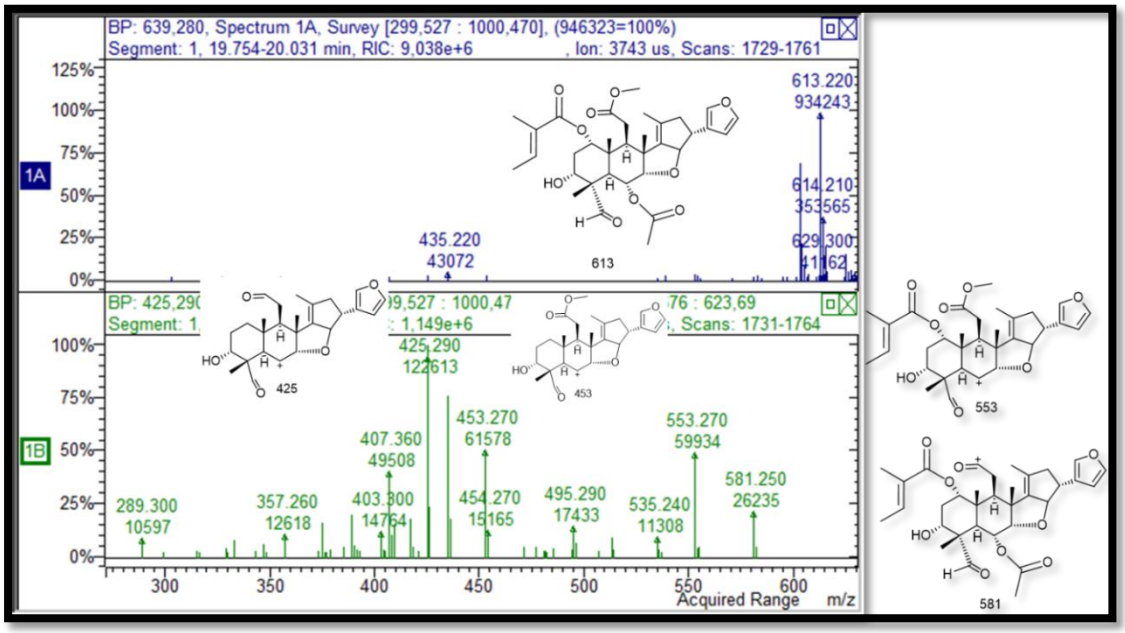


Figure 18: MS spectra of salannal, main fragments and proposed structures

Other significant peaks could be ascribed to the breaking of the diterpenoid moiety leading to fragment ions at m/z 289, 261 and 229 could support the loss of the methoxy substituent and of the oxygenated function in position 1 (Figure 19).

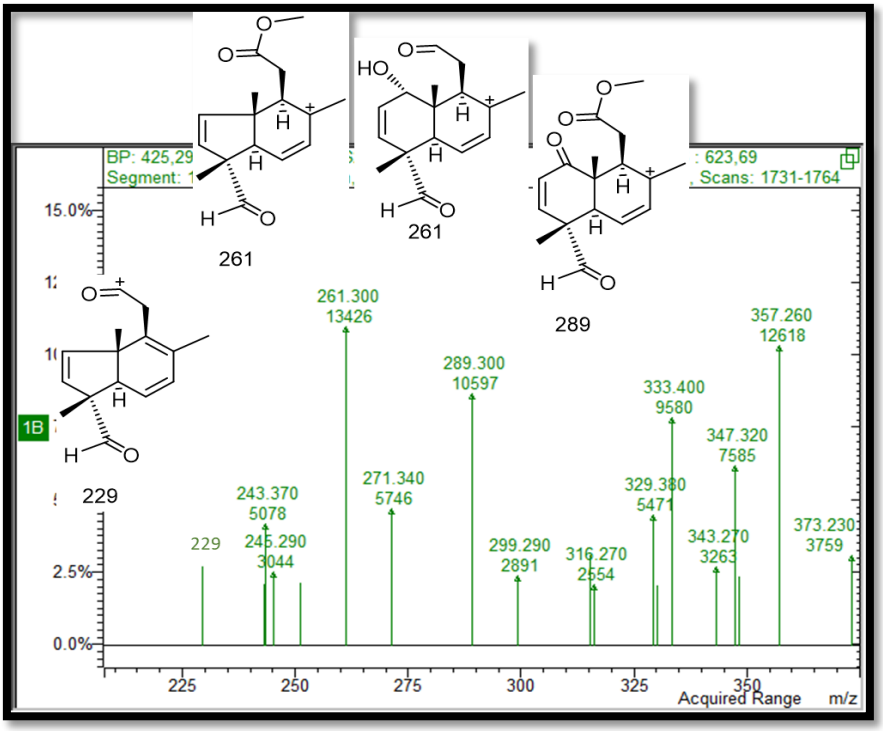
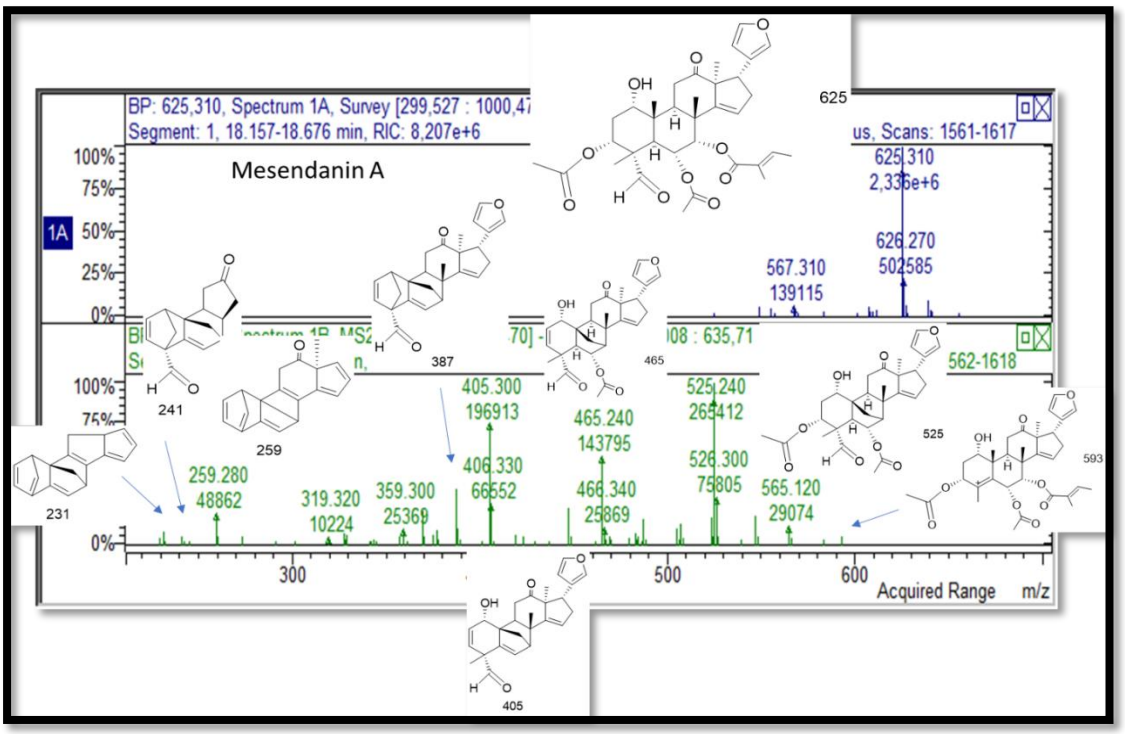


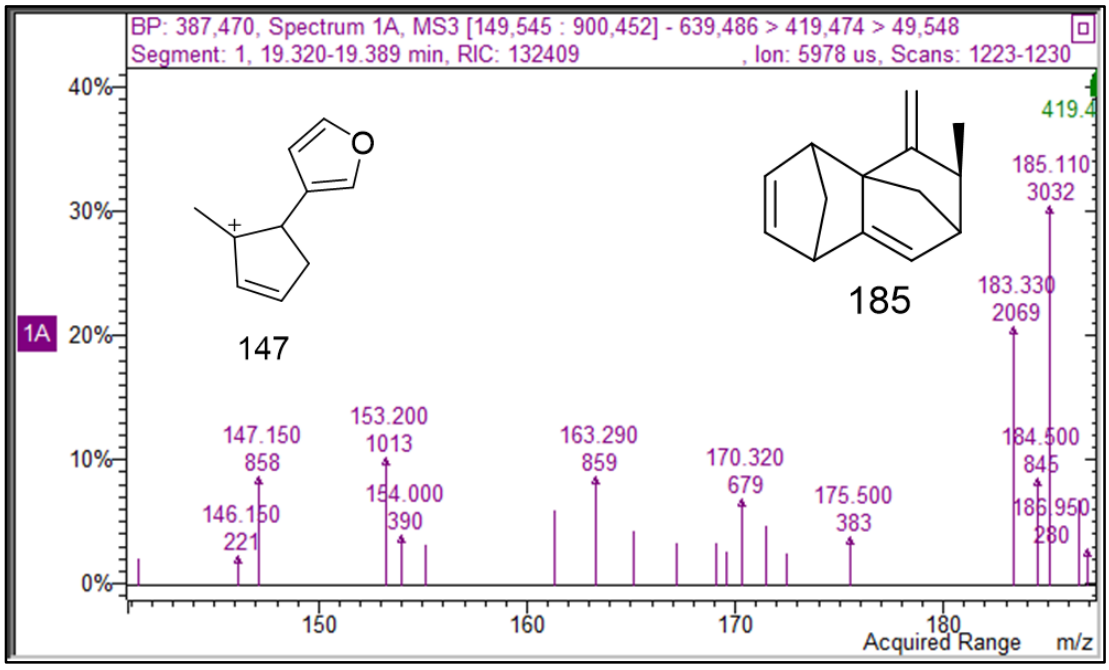
Figure 19: Proposed structures of the low molecular weight fragments of salannal

On the basis of the fragmentation schemes and comparing the proposed fragmentation previously described (Haldar *et al*., 2014). It could be proposed that the structure of the species at *m/z* 289 deriving from the ion after the loss of water and ester bonds and due to the breaking of bond C-8, C-14 and C-7 with oxygen. Other significant ion could beone at m/z 261 that correspond to the previous but with further loss of CO. Proposed structures are indicated in figure 18 and werealso deduce by Haldar *et al. (*2014). Similar findings from the fruit of *Melia toosendan* was also reported by (Wang *et al*., 2020).

Other compounds ascribable to limonoid derivatives have been observed. Peak at rt 18.5 present $[M+H]^+$ at m/z 625, fragments at m/z 593 (-32) supporting the loss of formaldehyde, m/z 525 (-100) corresponding to the loss of tyglic acid substituent, 465 (-60,-100) corresponding to the loss of tiglic acid and acetic acid, 405 (-100,-120) due to two units of acetic acid and tiglic acid and m/z 387 (-18,-100,-120) corresponding to the previous loss and a further water loss (Figure 20A). These fragments suggested the presence of two acetylation and one tygloylation as well as one free hydroxyl group and one aldehydic function in the molecule. Low-mass fragments were observed by Haldar *et al. (*2014)and were considered as deriving by the partial rearrangement of the tetracyclic nucleus of the limonoid. In particular the ions at *m/z* 259 can be originated by the loss of all the ester substituents as well as water molecule from free OH, as well as by the loss of C_4H_4O (ascribable to the neutral loss of furane in position 17), CH_2O ascribable to substituent in position 4, and CH_3 ascribable to a methylene. Further fragment ions related to the breaking of the cyclic moiety were the ions at m/z 185 ascribable to hexahydronaphtalene derivative related to the two hexa atomic cycle of the limonoid nucleus. As well as the ion at *m/z* 147 deriving from the methylcyclopentadienyl furane moiety originating from the two penta atomic cycle of the compound. A possible fragmentation pathway is reported in figure 20 A&B.



(A)



(B)

Figure 20: MS spectra of Mesendanin A, main fragments

NMR of the hexane fraction present signals that could support the presence of such compounds especially due to the aldehydic protons at δ 9.76 as well as the tigloyl and acetyl signals. Thus, the compound could be tentatively ascribed to Mesendanin A, this limonod was previously reported from *M. toosendan* by Dong *et al*. (2010), that supports our findings.

Two different peaks presents [M+H]+ at *m/z* 639 at 19 and 20 minutes, the fragmentation scheme were the same for the two isomers and the main observed ions suggest the loss of tygloyl (-100), acetyl (-60), the sum of the two above mentioned (-160). Furthermore, ions at m/z 419 as well as small fragments at m/z 245 and 273 suggest similar structure to those of other limonoids. Literature search suggest that the only structure reported were Meliatoosenin L and Nimboinin-type limonoidthat were previously isolated from *M. toosenin* and *M. azedarach (*Zhang *et al*., 2016). Main fragments observed in MS were consistent with this assignment (Figure 21).

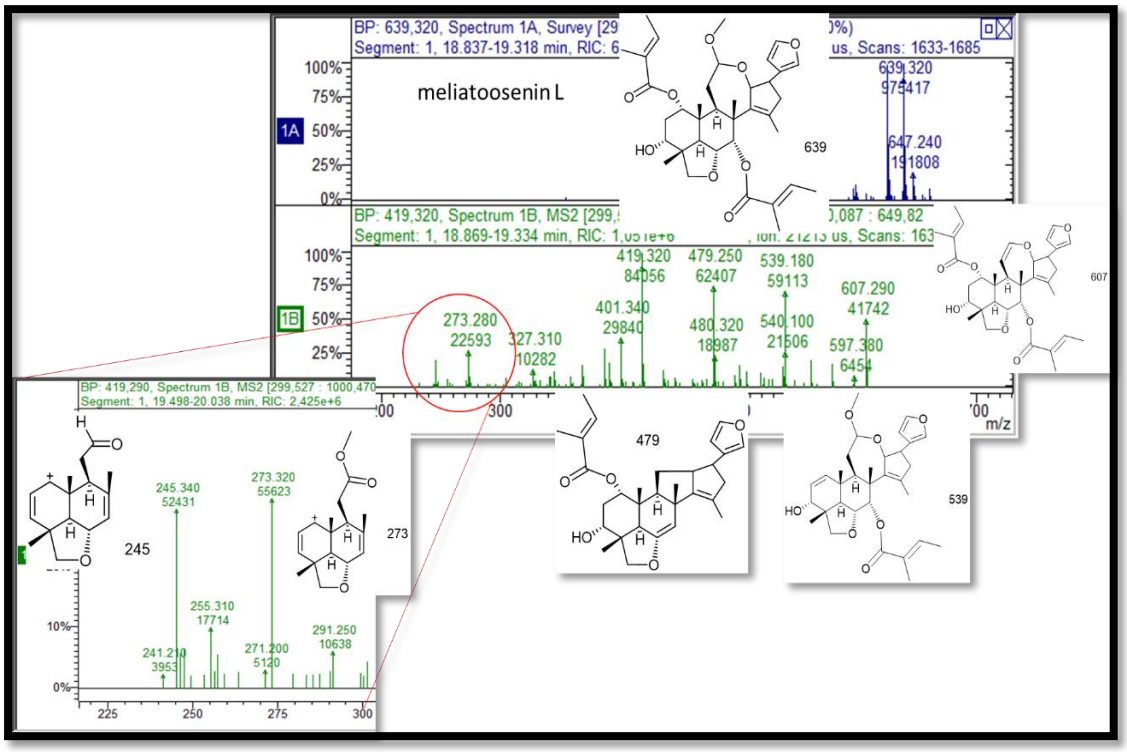


Figure 21: MS spectra of Meliatoosein L, main fragments

Results indicated that the Nepalese sample of *M. azedarach* contains limonoids, as confirmed by the NMR and LC-MS data. This information's are a good starting point for future phytochemical investigation on this plant to isolate constituents and confirm the proposed structures. From a quantitative point of view the tentatively assigned as Meliatoosein L (2.15 mg/g) and Masendanin A (2.084 mg/g) were the most abundant in leaves. The phytochemical analysis showed that Nepalese *M. azedarach* sample leaves contains nearly 1% of phenolic mostly flavonoid glycosides and significant amount of limonoids (0.7%) with Meliatoosein L and Masendanin A as major derivatives.

The comparison of the composition of the extract obtained by methanol and concentrated that was obtained in a ponderal yield of 24.2±1.0% showed that the extraction and subsequent concentration allowed the phenolic to be obtained in higher yields while limonoids were in large part lost probably during concentration procedures caused by their partial volatility or by oxidative and/or hydrolytic reactions. This should be considered during the preparation of extracts that want to concentrate limonoids. Literature indicate that tissues containing the larger amount of limonoids in *M. azedarach* were fruits (Dong *et al*., 2010) and in less extent leaves, which supports our results.

4.2.3 *In vitro* bioassays

Antioxidant and enzyme inhibitory properties

The phytochemial analysis revealed that the plant extract contained various amounts of phenolics and phytosterols. There were also obtained high amount of lipids. Due to presence of those phytochemicals, the biological activities were undertaken for the further analysis. The total phenolic content was carried out by Folin-ciocalteu colorimetric method. The observations were obtained by spectrophotometer. The total phenolic content was 33.53 mg gallic acid/g (Table 22). The study was also done in Melia. This provided various observations. Total phenolic content was 59.1 mg GAE/g of leaves extract (M'rabet *et al*., 2017) and 50.01 mg GAE/g (Kaneria *et al*., 2009). The difference in the result was due to the climatic and geographical condition of the particular region. The results were also affected by altitude of the particular area.The another reason was peptides and the reducing sugars could react with phenolics which allow to reduce the activity (Gulcin, 2020). Due to the limitations in colorimetric methods, chromatographic methods were used for the identificatin of phenolics and their biological activities. The study were focused in antioxidants and metabolic disorder like Alzheimer's diseases (Pellegrini *et al*., 2020). These information provided by the study suggested that there could be more study in the natural product which are important for human beings.

The antioxidant capacity was carried out. Different phytochemicals were responsible for the activity. The results were tabulated in table no 22. The antioxidant capacity was found to be in DPPH and ABTS were 47.56 and 65.34 mg TE/g, respectively. CUPRAC and FRAP capacity were calculated. The obtained results were 109.89 mg

and 65.02 mg TE/g, respectively (Table 22). Different investigatin in *M. azedarach* were done by (Kaneria *et al*., 2009; Pokhrel *et al.*, 2015; M'rabet *et al*., 2017).

The paper showed that antioxidant capacity were due to the presence of various phenolics and flavoinds (Yu *et al*., 2005; Tundis *et al*., 2014; Panche *et al*., 2016). This investigation showed that *M. azedarach* were good potential for natural antioxicant capacity. Enzyme inhbitory activity was carried out in order to manage metabolic disorders (Balbaa & EI Ashry, 2012; Copeland, 2013; Rauf & Jehan, 2017). However. Those chemical have severe side effects such as gastrointestinal disorders. The further study should carried out for specific target molecules for various diseases. Because of those consequences, enzyme inhibitory test were done for different enzymes in *M. azedarach* (Table 22). AChE and BChE inhibitory activities were obtained to be 5.05 and 2.43 mg GALAE/g, respectively (Table 22). The extract showed a major tyrosinase inhibition acitvities (131.57 mg KAE/g). Induced melanogenesis in B16F10 mouse melanoma cell lines were carried out and results were no effect in intracellular tyrosinase activity (Yao *et al*., 2015). Regarding amylase and glucosidase inhibition ability, the results were found to be 0.90 and 5.46 mmol ACAE/g. To the simplest of our knowledge, the data on the enzyme inhibitory properties of *M. azedarach* remains limited (Shahwar *et al*., 2013). Thus, the plant could be used as important biological active agents for various diseases.

Table 22: Total phenolic content, antioxidant, and enzyme inhibitory properties of methanolic extract of *M. azedarach*

Parametersers	**Results**
Total phenolic content (mg GAE/g)	33.53±0.39
DPPH (mgTE/g)	47.56±0.34
ABTS (mgTE/g)	65.34±3.14
CUPRAC (mgTE/g)	109.89±0.84
FRAP (mgTE/g)	65.02±3.09
Metal chelating (mgEDTAE/g)	12.26±0.32
Phosphomolybdenum (mmolTE/g)	2.05±0.13
AChE inhibition (mgGALAE/g)	5.05±0.02
BChE inhibition (mgGALAE/g)	2.43±0.77
Tyrosinase inhibition (mgKAE/g)	131.57±0.51
Amylase inhibition (mmolACAE/g)	0.90±0.02
Glucosidase inhibition (mmolACAE/g)	5.46±0.01

Values are reported mean ± SD of three replicates. GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; GALAE: Galantamine equivalent; ACAE: Acarbose equivalent; KAE: Kojic acid equivalent

4.2.4 Cytotoxicity test

Preliminary cytotoxicity analysis was performed on two tumor cell lines, namely human ovarian 2008 cancer cells and human pancreatic BxPC3 cancer cells (Photo plate 7). The methanolic leaves extract exhibit an IC_{50}>50 µg/mL on BxPC3 cell line while on 2008IC_{50} decreases to 26, 4, revealing a mild ability to compromise the viability of ovarian cancer cells (Table 23). In case of ovarian cancer cells there appeared to be different from those of the control from a morphological point of view (Photo plate 7 A-B). On the contrary, minor differences in term of cell morphology were evident in pancreatic cancer cells (Photo plate 7 C-D), even though micrographs of MAM treated pancreatic cancer cells revealed a slightly lower number of cells with respect to control cells (Shrestha *et al*., 2021).

Table 23: Cytotoxicity tests (concentration of compound inhibiting cell growth by 50% (IC50, µg/mL) of MeOH extracts of *M. azedarach*on 2008 and BxPC3 cell lines

Samples	**2008 IC_{50} (µg/mL)**	**BxPC3 IC_{50} (µg/mL)**
MAM	26.4	>50

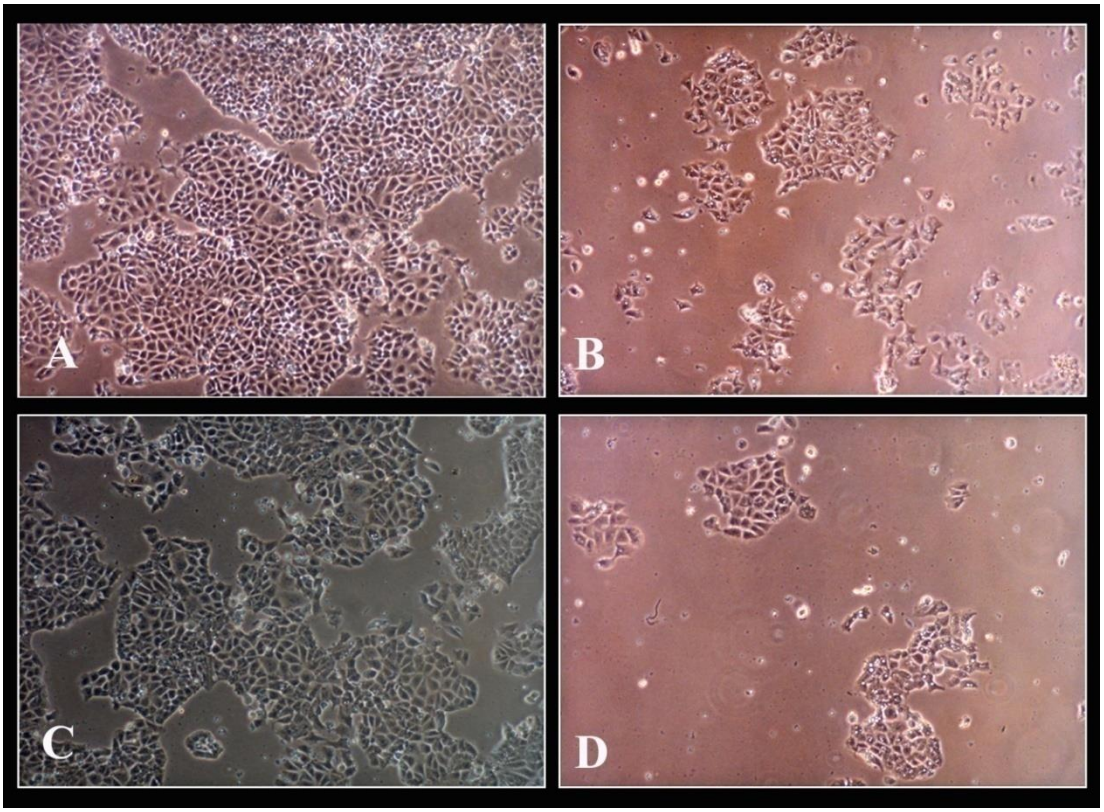


Photo plate 7: A. Photos of Ovarian (2008) cell lines CTR and **B.** Ovarian (2008) cell lines MAM treatment. **C.** Photos of Pancreatic cell lines (BxPC3) CTR and **D.** *Pancreatic* cell lines (BxPC3) MAM treatment. Pictures (40x magnification)

The observed cytotoxic effect could be explained by the presence of phytol (Jianqing Yu *et al*., 2007) and *β*-sitosterol (Malek *et al*., 2009) due to previous published articles. Rutin can be partially related to the observed cytotoxic effect, as report in previous article (Alonso-Castro *et al*., 2013). Rutin demonstrated to possess *in vitro* cytotoxic effects on tumor colon (SW480) cells and was able to induce an *in vivo* antitumor effects, without significant toxic effects on mice bearing SW480 tumor (Alonso-Castro *et al*., 2013). In the same article, the compound also showed anti-angiogenic properties (Alonso-Castro *et al*., 2013).

This capacity is due to presence of limonoids content, in fact limonoids affect in the process of carcinogenesis interfering in multiple signal transduction pathways and thus limiting proliferation, angiogenesis and metastasis or increasing apoptosis so it could be promising candidates for anticancer drug development also reported by Wu *et al*. (2009). In the previous study, the most active cell growth inhibitor liminoids was cedrelone, exhibiting IC_{50} values below 5 μM in all breast adenocarcinoma (MCF-7), non small lung cancer (NCI-H460) and melanoma (A375-C5) cell lines. limonoids whose structure possessing the lactone moiety on the ring D, gedunin was the only member which exhibited strong growth inhibitory activity (Cazal *et al*., 2010). Limonoids showed significant inhibitory activities against tested cell lines (Yuan *et al*., 2012). Limonoids were found to target and stop neuroblastoma cells (Jacob *et al*., 2000).

In particular, as reported by Abotaleb *et al*. (2018), flavonols as quercetin have the tendency to induce both apoptosis, via increase of Bax, caspase-3 and decrease in Bcl-2, and necroptosis through over expression of RIPK1 and RIPK3 as well as to trigger off intrinsic mitochondrial apoptotic pathway. Instead, kampferol is active against hormonal based cancers, like ovarian one, through the induction of intrinsic apoptosis via mitochondrial pathway, in which increase of caspase-3, caspase-7, p53, Bax, Bad and decrease in Bcl-xL is included. Kaempferol also down regulates VEGF, preventing in this way angiogenesis. Morover, delphinidin, the most abundant anthocyanidin in *M. azedarach* extract, is reported to reduce proliferation of ovarian cancer cells through inactivation of PI3K/AKT and ERK1/2 mitogen-activated protein kinase signaling cascades.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The present intensive phytochemical analysis, *in vitro* biological assays and cytotoxicity studies of three medicinal plants (*G. diversifolia*, *T. coadunata*, *M. azedarach*) showed that they are important both ethnobotanically as well as phytochemically. The phytochemicals constituents present in plants showed defense mechanism that was proved by the antioxidant properties seen in biological assays and cytotoxicity test, which might play an important therapeutic role in disease cure.Based on the literatures review and the obtained results for all the three plant the hypotheses of the present study have been justified.

Girardinia diversifolia known as Himalayan nettle was used to treat various ailments in the rural areas of Nepal. It was used as also traditional type of medicine having no side effects. It was also used to make fibre. Anti-cholinesterase and anti-tyrosinase enzymes activities was carried out. This was due to the presence of phenolic contents. The obtained phenolics were phytosterols, phytol, and oryzanol. The antioxidant activities were also oberved. The study showed that the antioxidant capacity was poor. This was due to low content of phenolics and carotenoids. The metalchelating activity was showed in high amount. This activity could be correlated with metal based red/oxreactions. The study showed that it could be used for that reactions.

The *in vitro* cytoxicity analysis revealed that the plant extract could be used against Pancreatic and hepatocarcinoma cell lines. The result of *G. diversifolia* extracts also revealed that it was able to reduce the expression of LDLR. This result could be correlated with anticancer activities of the plant extract. Finally the study showed that the himalayan nettle could rich in phytosterols and used for the various biological activities. The overall phytochemical composition suggests that the phytocomplex of *G. diversifolia* might be useful in the preparation of nutraceuticals.

Tectaria coadunata known as *Kaalo Nyuro* is used as medicine by several ethnic communities for different diseases and young fronds are eaten as vegetables. Phytochemical investigations were well studied. Procyanidins were major compounds

obtained from the investigation. The procyanidins were obtained with various degree of polymerization. The polymerized compounds were eriodictyol-7-O-glucuronide, luteolin-7-O-glucuronide, and naringenin-7-O-glucuronide. Further, the study was focused in biological activities of the plants extracts. *In vitro* antioxidant capacity was strong. The study was also focused on metabolic disorder. The extract also showed significant affect on enzymes which were responsible for different diseases. AChE, BChE, tyrosinase, and amylase inhibitory activities were carried out. Water extract was poor in the test where as methanolic and ethylacetate fractions were more effective. The study showed that lower two extracts provides rich amount of phenolics which were responsible for varities of the biological activities.

The obtained phenolics compounds suggested that *T. coadunata* extracts could be used for developing co-treatments for Alzheimer's disease. Further more specific studied should be under taken for targeted enzymes inhibition. In vivo study shoud be also undertaken for the further study so that it could be used specifically. Due to the significant activities for antioxidant, it could be used for the various disease of metabolic. Furthermore, the plant extract was more potent for pancreatic cancer cell lines which was shown more effective in ethyl acetate fraction. This may be due to the abundance of phenolic compounds like procyanidins in *Tectaria coadunata.*

The preliminary phytochemical study on Nepalese traditional medicinal plant *M. azedarach* was carried out. Phytochemical characterization, cytotoxicity and biological assay were investigated from the methanolic extract of leaves showed significant amounts of Limonoids and Masendanin A and Meliatoosein L as major derivatives. Phenolic flavonoids like rutin, quercetin, quercetin derivativea and keampferol derivatives are significant. Most abundant phytosterol constituents were beta-sitosterol with significant amounts of fucostanol, campesterol and stigmasterol.Phytochemicals such as phenol and flavonoids are well known for their antioxidant potential. An *in vitro* biological assay of methanolic extract of leaves of *M. azedarach* showed moderate activities in phenolic, antioxidants, Metal chelating and Phospholybdenum activities. Enzyme inhibition assays like AchE, BuChE, Amylase and Glucosidase were moderate where as tyrosinase inhibition by kojic acid was significant showing utility in skin hyperpigmentation conditions. Cytotoxic effects on ovarian (2008) and pancreatic (BxPC3) cell line revealed their mild effect on ovarian cell line where as no effects on pancreatic cell lines. *M. azedarach* leaves

can be considered as valuable starting material for the extraction of phenolics and phytosterols, yielding extracts with possible cosmetic and pharmaceutical applications. Thus, the findings could support the utilization of this plant as a putative natural source of biologically-active agents.

The phytochemical findings of all the three medicinal plants indicate the high possibility of isolating new compounds in further phytochemical analysis. Preliminary cytotoxicity assays on tumor cell lines were performed to assess potential activities of the extract of all the three plants (*G. diversifolia*, *T. coadunata*, *M. azedarach*) offering new information on these medicinal plant. Current research in these three plants has confirmed that plants rich in antioxidants play an essential role in preventing diseases like cancers. Therefore, plant-derived antioxidants are receiving particular attention as they enhance the body's immune system to recognize and destroy cancer cells as well as inhibit the development of angiogenesis necessary for tumor growth. They appear to have both preventative and therapeutic potential in combating cancers, which is one of the main outcomes of the present study.

5.2 Recommendations

- Further experiments are needed to purify and test specific compounds and fractions in order to deeply investigate the potential cytotoxic effect and the possible mode

- Vitamin B2, vitamin C obtained in *Girardinia* could be used as neutraceuticals

- Procyanidin treatment exerted anti-proliferative and anti-invasive effects in pancreatic cell lines. Therefore, further in depth studies is required in *Tectaria*, so that it can be suggested as one of the important medicinal plant that can be used as a potent chemo-preventive or therapeutic agent for pancreatic cancer treatment

- Further studies are required to access new dyanamic on the usefulness of *Melia azedarach* particularly on cytotoxicity, cosmetics, pharmceuticals and nutraceutical applications, as leaves of *Melia* can be considered as source of valuable phytoconstituents

CHAPTER 6

6. SUMMARY

6.1 Introduction

Traditional medicine practices are based on hit, trail and success. There are so many traditional medicinal plantsthat were using by local people since long time ago and still using it. In so many cases they are useful to treat different ailments in different parts of Nepal. Especially in urban areas people use modern medicine and people in rural areas they are still dependent on traditional medicine practices.The current study is focused to specify the traditional medicinal practices are also useful to treat different ailments as they are constituents of different secondary metabolites.

Girardinia diversifolia, *Tectaria coadunata* and *Melia azedarach* are the Nepalese ethnomedicinal plants used to treat various ailments from ancient time. *G. diversifolia* is used to treat different diseases like gastric disorders, chest pain, rheumatism, tuberculosis, headache, joint aches, diabetes, asthma, gastritis, headache, joint pain, tuberculosis, gonorrhea and delivery problems.

Tectaria coadunata is used to treat various ailments like stomach pain and giardiasis, gastrointestinal disorders, jaundice, diarrhea and dysentery, cuts and wounds and to eradicate worms.The rhizome of *T.coadunata* is used for its anthelmintic activity and against stomach pains and gastrointestinal disorders.

Fruits and leaves of *M.azedarach* were used to treat different ailments like, diarrhea, dysentery, intestinal worm and spleen disorder. Fruits were anthelmintic, also used for treating vomit, blood impurities and urinary discharge.Extract of leaves were applied to treat skin disease and fruits were used as anthelmintic. Leaves were traditionally used to control insect, mite and nematode pests, for skin disease, for gingivitis mouthwash, to treat diabetes, fever and stomach ache.

The present study reveals the presence of various secondary metabolites in all ethnomedicinal plants as well as having *in-vitro* biological activites and cytotoxic activities.

6.2 Methods

All the plants were collected from different parts of Nepal. Plants were washed thoroughly and dried properly under the shade. Plant materials were taken for the identification. Herbarium was carried to identify. KATH herbarium, Godawari, Lalitpur, Nepal was the authorised office for the identification of the plant. Herbarium was prepared after mounting on the herbarium sheets. The obtained plant powder was used for the extraction procedure. The methanol was taken as solvent. Intermittent sonication was used for the extraction procedure. The percentage yield of the extract was taken. The obtained dried extract was sotred in the vial and sealed properly. The temperature was maintained at 4°C. This was now ready for further analysis. Chromatography, LC-MS, GC-MS, HPLC, NMR tools were used to identify and quantify various secondary metabolites.

Enzyme inhibitory activities were analysed by cholinesterases, tyrosinase, amylase, and glucosidase inhibition procedures. Standard enzyme inhibitor compounds were used to evaluate the results. These compounds were galantamine (GALAE, for cholinesterases), Kojic acid (KAE, for tyrosinase), and acarbose (ACAE, for amylase and glucosidase). All experimental procedures were performed with 96 wells microplate.

Total phenolic was carried out by Folin-ciocalteu colorimetric procedure. The procedure was also used for total flavonoid content. The standard compound was taken for the study. The reference compound was Gallic acid (GAE). The antioxidant activities were determined. This signify that the plant could be used for antioxidant related ailments. The antioxidant activities were taken from the spectrophotometric procedure. The obtained data showed that plant extract exhibit strong antioxidant activity.

Ovarian (2008) and pancreatic (BxPC3) carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazoliumbromide) assay (MTT) was used as a relative measure of cell viability.The mean absorbance for each drug dose was expressed as a percentage of the control, untreated, well absorbance and plotted vs. drug concentration. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}).

6.3 Results

Girardinia diversifolia showed presence of phytosterols, fatty acids, carotenoids, polyphenols and saponins. The secondary metabolites obtained were beta and gamma-sitosterol (11 and 9% dw, respectively), and trans syringin (0.5 mg/g) was the most abundant phenolic. Fatty acids with an abundant portion of unsaturated derivatives (linoleic and linolenic acid at 22.0 and 9.7 mg/g, respectively), vitamin C (2.9 mg/g) and vitamin B2 (0.12 mg/g) were also present. The antioxidant activity was moderate while a significant ability to inhibit acetylcholinesterase (AChE), butyrilcholinesterase (BuChE), tyrosinase, alphaamylase and alphaglucosidase was observed. A cytotoxic effect was observed on human ovarian, pancreatic andhepatic cancer cell lines. The effect in hepatocarcinoma cells was associated to a downregulation ofthe low-density lipoprotein receptor (LDLR), a pivotal regulator of cellular cholesterol homeostasis.

The analysis in *T. coadunata,* significant amount of procyanidins, with different degree of polymerization, other than eriodichtyol derivatives and flavonolsdespite the lack of alkaloids in the extracts AchE and BuChE activity was observed. *T. coadunata* extracts contained higher number of phenolic compounds. The study showed that it could be used as enzyme inhibitory activities. The statistical data showed that it could be used against different metabolic disorder. In the future, it could be study to develop co-treatment for Alzheimer management due to significant antioxidant. The cytotoxicity study could lead to go further in the treatment of pancreatic cancer cell lines (BxPC3).

Phytochemical investigations for *M. azedarach* revealed large amount of fatty acids, flavonoid glycosides (1%) and limonoids (0.7%) in the dried leaves. Methanol extract was prepared, and flavonoids appear to be highly concentrated reaching 7.5% total amount. The extract is rich mostly in beta-sitosterol fucostanol, campesterol and stigmasterol. Plant material contains as limonoids, withMasendanin A and Meliatoosein L as major derivatives.

Phenolic content and antioxidant activity for the methanol extract were assessed. Tyrosinase inhibition was significant resulting 131.57±0.51 mg Kojic Acid

Equivalents/g suggesting possible usefulness in skin hyperpigmentation conditions. It can be used as starting material for the extraction of phenolics obtaining extracts with possible applications in cosmetics and pharmaceuticals.The methanolic extracts revealed that the plant extract were more potent for human ovarian cancer cell lines (2008). The calculated IC_{50} was of 26.4 µg/mL.

6.4 Conclusions

The present study showed the potential usefulness of plant species for possible applications in pharmaceuticals, nutraceuticals and cosmetics.The research on purified constituents will go on for further assessing potential usefulness also in this therapeutic field.

The present study revealed that the ethnomedicinal plants could be used for various ailments. Plants are important sources of natural ingridents for natural treatments. Hit and trail method of traditional medicinal practices can be used as a source of Knowledge for scientific validation.

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APPENDICES

Appendix 1 : Voucher specimen

A. ***Girardinia diversifolia*** (Link) Friis : Bagmati, Sindhuli, SindhuliGadi. 1332-1394 mts. October 2016, S.S. Shrestha.S001 (TUCH)

B. ***Tectaria coadunata*** (J. Sm.) C. Chr.: Bagmati, Kathmandu, Dakshinkali, 1400-1509 mts, November 2016, S.S. Shrestha. S002 (TUCH)

C. ***Melia azedarach*** Linnaeus: Bagmati, Bhaktapur, Suryabinayak, 1290-1301mts, October 2016, S.S. Shrestha. S003 (TUCH)

Appendix 2 : NMR assignment for relevant signal of γ-sitosterol

	^{1}H	^{13}C			^{1}H	^{13}C
1				16		
2				17		
3	3.48	71		18	0.61	11.96
4				19	0.95	19.09
5	-	140		20		
6	5.28	120		21	0.85	18.90
7				22		
8				23		
9				24		
10				25		
11				26	0.74	19.28
12				27	0.74	19.28
13				28		
14				29	0.76	11.06
15						

Appendix 3: List of scientific publications from Ph.D. thesis

1. **Shrestha, S. S.**, Sut, S., Di Marco,S. B., Zengin, G., Gandin, V., De Franco, M.,Pant, D. R., Mahomoodally, M. F., Dall'Acqua, S., & Rajbhandary, S. (2019). Phytochemical Fingerprinting and In Vitro Bioassays of the Ethnomedicinal Fern *Tectaria coadunata* (J. Smith) C. Christensen from Central Nepal. *Molecules* 24 (24). https://doi.org/10.3390/molecules24244457

2. Shrestha, S. S., Sut, S., Ferrarese, I., Di Marco, S. B., Zengin, G., de Franco, M., Pant, D. R., Mahomoodally, M. F., Ferri, N., Biancorosso, N., Maggi, F., Dall'Acqua, S., & Rajbhanday, S. (2020).Himalayan Nettle *Girardinia diversifolia* as a Candidate Ingredient for Pharmaceutical and Nutraceutical Applications—Phytochemical Analysis and In Vitro Bioassays." Molecules 25 (7), 1563.https://doi.org/10.3390/molecules25071563

3. Shrestha, S. S., Ferrarese, I., Sut, S., Zengin, G.,Grana, S.,Ak, G., Pant, D. R., Dall'Acqua, S., & Sangeeta Rajbhandary, S. (2021). Phytochemical Investigations and In Vitro Bioactivity Screening on *Melia azedarach* L. Leaves Extract from Nepal. Chemistry &Biodiversity 18 (5), e2001070.https://doi.org/10.1002/cbdv.202001070

Appendix 4: List of conference attended training/workshop/award during Ph.D

1. International Conference on "Biodiversity, Climate Change Assessment and Impacts on Livelihood" held in Kathmandu from January 10-12, 2017
2. Participated on Training program on "Research Methodology for PhD Scholars", Conducted by The Central Department of Botany, T. U. and Supported by University Grant Commission, Nepal from June 20-26, 2017 (Ashad 6-12, 2074).
3. Screening of Natural products (Microbes and Plants) and their anti-infective activities" and Seminar on "Natural Products and Drug Discovery" organized by Central Department of Biotechnology, TU, Kirtipur, Ethnobotanical Society of Nepal (ESON) and Society for Biological Applications Nepal (SBAN) held in Kathmandu from February 9-11, 2017
4. Attended the International Seminar in Biodiversity, Bioprospecting and Territories from 9-10 September, 2019 and presented paper entitled "Phytochemical analysis and cytotoxic activities of *Girardinia diversifolia*". The seminar was organized by Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy
5. Conference "National Conference on Integrating Biological Resources for Prosperity", Venue: Biratnagar, Date: 23-24, Magh, 2076 (6-7 February, 2020) attained with Paper presentation on *Tectaria coadunata*.The title is Phytochemical Fingerprinting and In Vitro Bioassays of the Ethnomedicinal Fern *Tectaria coadunata* (J. Smith) C. Christensen from Central Nepal
6. Biochemical extraction and analysis at Natural product Lab, Department of Pharmaceutical and Pharmacological Sciences.University of Padova, Italy. Feb 8-22, 2019.
7. Erasmus+KA107 International Credit Mobility Program (NPLKATHMAN02, 2019) Erasmus + programme received from the University of Padova, Italy, exchange program between Tribhuvan University and University of Padova, Italy on Feb-July, 2019
8. UGC Grant received PhD award No.-73/74-S&T-11entilted "Phytochemical analysis and bioactive compounds of some selected medicinal plants of nepal" by University Grant Comission, Kathmandu, Nepal
9. Research Fellowship Award received entitled "Medicinal Plants enriched with active compounds through Biostimulation" from August to December 2019 provided by Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy

molecules

MDPI

Article

Phytochemical Fingerprinting and In Vitro Bioassays of the Ethnomedicinal Fern *Tectaria coadunata* (J. Smith) C. Christensen from Central Nepal

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Abstract: *Tectaria coadunata*, an ethnomedicinal fern used in Nepal to treat a large number of diseases, has been poorly studied with regard to its phytochemical composition and possible bioactivity. This study was performed with the aim of supporting traditional medicine as a new source of bioactive constituents. Phytochemical compositions of methanol extracts were determined by nuclear magnetic resonance (NMR), liquid chromatography–diode array detector–mass spectrophotometry (LC-DAD-MS), and liquid chromatography–fluorescence–mass spectrometry. Quali-quantitative data revealed large amount of procyanidins, mainly of the A-type, as well as eriodictyol-7-*O*-glucuronide and luteolin-7-*O*-glucoronide as main constituents. The antioxidant, cytotoxic, and inhibitory activity of five enzymes that are implicated in human diseases was evaluated for the extract and fractions. High free-radical scavenging activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays and inhibitory activities against cholinesterases and tyrosinase were observed. Furthermore, a moderate cytotoxic effect was observed on the 2008 and BxPC3 cell lines. Overall results showed potential usefulness of this fern as a source of phytochemicals for pharmaceutical uses.

Keywords: *Tectaria coadunata*; antioxidant; medicinal fern; chromatography; procyanidins; flavonols

1. Introduction

The use of medicinal plants has been rooted in Nepalese culture since ancient times, but this traditional knowledge is threatened by the loss of oral tradition and the use of synthetic drugs. The richness of Nepalese flora is well described and is mainly related to the peculiar geographic and pedoclimatic conditions of this country, characterized by large altitudinal variations ranging from nearly 59 m up to 8848 m in height (Mount Everest). In this context, the exploration of Nepalese flora used in traditional medicine is a unique opportunity to study bioactive extracts as sources of new

natural products. The ethnopharmacology of several species is still poorly considered and can offer new research opportunities. In rural areas of Nepal people are still dependent on medicinal plants because of the scarce availability and high costs of synthetic medicines [1]. In fact there are 8.4 million indigenous people living in different parts of Nepal that rely on traditional medicine. The study of ethnopharmacology is fundamental to providing scientific support to the users, to preserve the knowledge in this field, and also to offer the opportunity to explore scarcely studied medicinal plants. It has been calculated that 1792–2331 medicinal and aromatic plants are used for different diseases. In Nepal, 293 species of fern and its allies have been reported [2], one of which is *Tectaria coadunata*, a terrestrial fern 30–100 cm tall. Its rhizome is creeping and erect, thick, and densely scaled, and its color is dark brown. Fronds clustered and stipe are stramineous to pale, glossy, and glabrescent. A sorus is found on each side of mid rib in a single row at the vein. Indusia are large, brown, entire, clypeate, membranous, glabrous, or hairy [3]. It is found in dense forests ranging from 500 to 2500 m in Chinese regions including Guangdong, Guangxi, Guizhou, Sichuan, Taiwan, Xizang, and Yunnan. It is also found in other countries such as Bhutan, India, Laos, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand, Vietnam, and Madagascar. The plant is widely distributed in north-facing slopes.

T. coadunata is used to treat various ailments such as stomach pain and giardiasis [4], gastrointestinal disorders, diarrhea and jaundice [5], and to eradicate worms. Leaves are crushed and juice applied to the cut wounds to stop bleeding. The rhizome of *T. coadunata* is used for its anthelmintic activity and against stomach pains and gastrointestinal disorders. During diarrhea and dysentery, leaves are taken and prepared as vegetables, or about three spoons of root paste are taken in half a glass of water twice a day after filtration with cotton cloth for 3–4 days. Today this species is not cultivated in Nepal but is largely used in herbal medicinal practice.

Previous chemical investigations on *T. coadunata* are limited to a single paper in which 21 compounds were detected by gas chromatography–mass spectrophotometry (GC-MS) analysis [6]. The constituents have been indicated to involve general classes of compounds such as carbohydrates, tannins, phenols, anthraquinone glycosides, coumarin glycosides, flavonoids, and steroids [7].

The present paper aimed to study *T. coadunate*, bridging the gap between its common medicinal use in Nepalese traditional medicine and the scarce knowledge related to its constituents and possible bioactivity. In this paper, comprehensive analytical approaches have been applied. Nuclear magnetic resonance (NMR) on crude extract, as well as liquid chromatography–diode array detector–mass spectrophotometry (LC-DAD-FLD-ESI-MS) and chromatographic separations were performed to collect information on phytochemical composition and to isolate the most abundant constituents. In vitro antioxidant and enzymatic assays on five enzymes that are implicated in human diseases were evaluated for the extract and fractions. Preliminary cytotoxicity assays on tumor cell lines were performed to assess potential activities of the extract, offering new information on this medicinal plant. Overall results aimed to assess the potential importance of such a plant as source of bioactive constituents and to explore its chemical constituents, offering a starting point for further studies focused on supporting the traditional uses with scientific data. Moreover, chemical characterization offers the opportunity to use the identified constituents as a marker compound to prepare standardized herbal remedies such as herbal teas with antioxidant properties or tinctures or dry extracts to be used as more concentrated supplements.

2. Results and Discussion

2.1. NMR Analysis of Tectaria coadunata Extracts

As starting point for extract characterization, NMR was chosen as a technique due to its ability to detect all kinds of phytoconstituents and their response factors, which are independent depending on the detected compound, following procedures previously used for other plant extracts [8,9]. Due to the complex composition of the extracts, some partially fractionated extracts have also been considered in order to simplify samples.

Concerning *T. coadunata* (TC)-MeOH, this extract may represent in large part the whole phytocomplex of the plant due to the efficacy of methanol in dissolving and extracting secondary metabolites from plant materials. The ^{1}H-NMR spectrum presents two broad signals in the deshielded proton region, one being δ 6.50–7.50, ascribable to aromatic signals of phenolics, and another in the range δ 5.85–6.20, supporting other less deshielded aromatics or double bonds. Further signals are the two doublets at δ 5.11 (J = 3.95) and 4.48 (J = 7.75) and several multiplets in the spectral region from δ 3.20 to 4.10; all these signals suggest the presence of carbohydrates (Figure 1). The aliphatic region of the spectrum appears less populated, presenting a group of multiplets that can suggest the presence of aliphatic chains. Protons of the sugar part can be ascribed to saccharose and glucose on the basis of comparison with the spectral database.

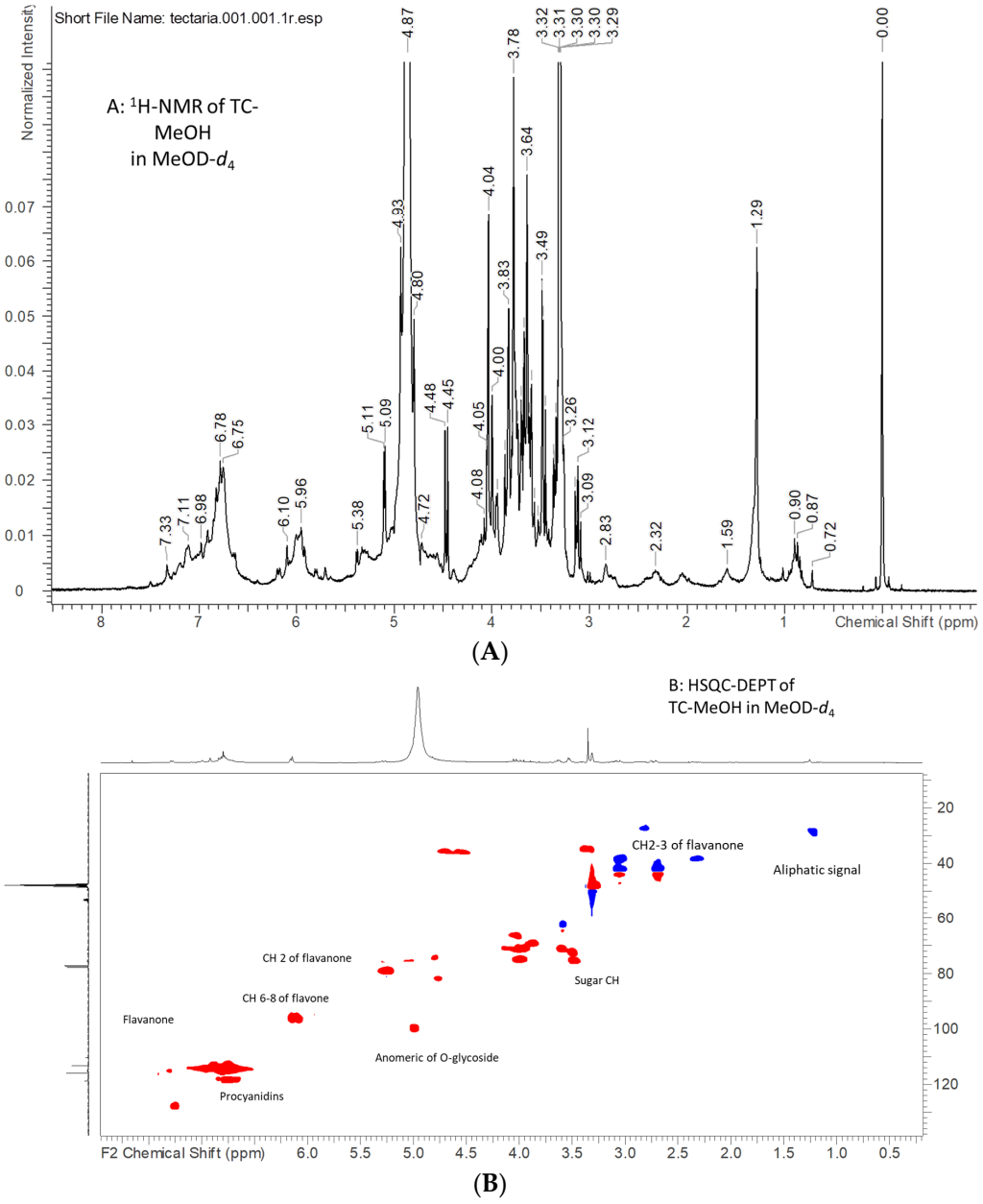

Figure 1. ^{1}H-NMR of *Tectaria coadunata* (TC)-MeOH (**A**) and heteronuclear single quantum coherence spectroscopy–distortionless enhancement by polarization transfer (HSQC-DEPT) of TC-MeOH (**B**) in MeOD-d_4.

To increase the signal intensity of secondary metabolites, partition with organic solvent was used and the TC-EtOAc extract was also analyzed. The ^{1}H-NMR spectrum of this extract was more resolved compared to the TC-MeOH extract, allowing the observation of more sharp peaks in the aromatic and in the sugar regions as well as in the aliphatic parts. More information can be obtained combining the data obtained from the heteronuclear single quantum coherence spectroscopy–distortionless enhancement by polarization transfer (HSQC-DEPT) spectrum, allowing us to assign the value of the chemical shift of both H and C of all non-quaternary positions, and heteronuclear multiple quantum coherence (HMBC), which allows us to observe long-range correlations from H to C with a distance of 3–4 bonds. Further connectivity data were obtained from the correlation spectroscopy (COSY) spectrum, showing proton couplings. Data are summarized in Tables 1 and 2, and allow us to substantiate the presence of different classes of constituents. Exemplificative spectra are reported in the Supplementary Materials. Thus, TC-MeOH mostly contains phenolics ascribable to flavone or catechin-type polymers such as procyanidins, and glycosides of flavonoids.

Table 1. Nuclear magnetic resonance (NMR) assignments of TC-MeOH. Data are obtained from H, heteronuclear single quantum coherence spectroscopy-distortionless enhancement by polarization transfer (HSQC-DEPT), correlation spectroscopy (COSY), and heteronuclear multiple quantum coherence (HMBC) spectra in MeOD-d_4.

			TC-MeOH
δ_H	**δ_C**	**Correlations**	**Assignments**
7.45	115.3	150.5, 125.3	Aromatic phenol ring of procyanidin or tannin
7.31	114.5		Aromatic phenol ring of procyanidin or tannin
6.76	114.5	129.4, 118.7, 116.5	Aromatic phenol ring of procyanidin or tannin
7.25	127.7	156.8, 125.7, 79.5	Flavanol moiety
6.90–6.99	114.2–117.2	144.7, 118.5, 79.5	Flavanol moiety
6.16–6.15	94.5–95.8	196.3, 163.5, 103.5, 94.2	Flavanol moiety position H-6/8
5.26	78.3	196.3, 128.5, 113.5	Flavanol moiety CH position 2
2.47 dd		196.3, 127.8, 79.5	Flavanol moiety CH_2 position 3
3.11 dd		196.3, 127.8, 79.5	Flavanol moiety CH_2 position 3
5.01	98.9	163.4	Anomeric proton of O-glycoside residue
4.77	80.5		Flavonol or procyanidin CH
3.50	74.1–72.2	98.6, 75.6	Sugar residue CH
3.62	71.3		Sugar residue CH
3.86	68.8		Sugar residue CH
4.00	70.0		Sugar residue CH
4.05	74.6	70.0	Sugar residue CH
4.02	66.7	75.0	Sugar residue CH
2.32	37.3	172.6	Organic acid CH_2
1.25	28.6		Aliphatic

Fractionation with Sephadex allowed us to obtain a methanol-eluted fraction that appears to be mostly composed of eriodictyol-7-*O*-glucuronide. On the other hand, the acetone-eluted fraction resulted to be mostly formed of procyanidin fraction [10]. The diagnostic signals that support the presence of procyanidin are aromatic H-6 and H-8 for the upper units and H-6 for the lower units; furthermore, signals ascribable to position 2 of monomers were observed at δ_H 5.31 and δ_C 79.3 as well as δ_H 4.81 and δ_C 74.3, ascribable to position 2 of the lower units. Other relevant signals can be ascribed to other C-3 positions (Table 1) and signals ascribable to CH_2 were detected at δ_H 3.14–2.72, δ_C 42.6, and δ_H 3.05, 2.39, and δ_C 37.4. A summary of main resonance ascribable to procyanidins in the TC-EtOAc fraction is shown in Table 2.

Table 2. NMR assignments of TC-EtOAc. Data were obtained from H, HSQC-DEPT, COSY, and HMBC spectra in MeOD-d_4.

			TC-EtOAc
δ_H	δ_C	Correlations	Assignments
7.28	126.5	144.5, 119.4	aromatic phenol ring of procyanidin or tannin
7.00	115.0	144.5, 120.0, 73.6	catechin moiety H-2′ or H-6′
6.80	118.2	144.0, 129.4, 116.5	catechin H-5′
5.98–6.01	95.0–93.0	156.0, 101.0, 93	H-6/8 of catechin units
5.31	79.3		H-2 of upper unit of catechin/epicatechin moieties
4.81	74.2	67.5, 113.3, 119.8, 129.5	H-2 of lower units of catechin/epicatechin moieties
4.07	74.7		H-2 of lower units of catechin/epicatechin moieties
3.84	68.9	101.5, 37.4, 38.5	H-3 of upper units of catechin/epicatechin moieties
3.31	47.6		C-4 of upper units of catechin/epicatechin moieties
3.14–2.72	42.6		C-4 of terminal units
3.05–2.39	37.4		C-4 terminal units

The overall NMR analysis on *T. coadunata* extracts revealed a composition mostly formed by glycosidic flavanone and procyanidins. Thus, further investigations were undertaken applying LC-MS-based approaches.

2.2. Quali-Quantitative Analysis

HPLC Coupled with Diode Array, Mass Spectrometry, and Fluorescence for the Analysis of Phenolic Constituents

TC-MeOH, TC-EtOAc, and TC-H_2O extracts of *T. coadunata* were analyzed by HPLC-DAD-MS, allowing the identification of secondary metabolites according to their retention time and mass fragmentation (Table 3, Figure 2). The three extracts, as shown in Figure 3, revealed the presence of procyanidins and glycosylated flavonoid and results were in agreement with NMR data. The identified flavonoids were namely naringenin-7-*O*-glucuronide, eriodictyol-7-*O*-glucuronide, and luteolin-7-*O*-glucuronide; for the latter two constituents, structures were elucidated by NMR and MS analysis after purification, while comparison with the reference standard confirmed the identity of the first compound. Procyanidin (PAC) dimers, trimers, and tetramers, as well as larger polymers, were detected in all the extracts. Identification of different PACs was tentatively done by MS data and revealed the presence of both A- and B-type PACs; in particular, the B-type procyanidin dimer was detected at 19.5 min and different A-type procyanidin trimers at 17.9, 19.1, and 20.8 min. From a quantitative point of view, when comparing the three extracts the dimers and trimers were more abundant in TC-EtOAc. An A-type procyandin tetramer and bigger polymers of both A and B types were mostly abundant in TC-MeOH. To assess the structures of procyanidin, purification by Sephadex was performed. The most abundant compound in TC-MeOH was an A-type procyanidin tetramer with one unit of (epi)afzelechin (25.2 mg/g), and the most abundant compound in the TC-EtOAc and the TC-H_2O extracts was the A-type procyanidin trimer (38.69 mg/g and 2.58 mg/g, respectively).

Table 3. Identified compounds in TC-MeOH, TC-EtOAc, and TC-H_2O extracts by HPLC HILIC-DAD-FLD-ESI-MS.

Tr	$[M-H]^-$	Identification	Fragmentation	UV s (nm)	mg/g in TC-MeOH	mg/g in TC-EtOAc	mg/g in TC-H_2O
14.6	447	Naringenin-7-*O*-glucuronide	MS^2 [447]: 271(100) MS^3 [271]: 151(100)-175(25) MS^4 [151]: 107(100)	200, 280	*	0.24 ± 0.06	0.006 ± 0.0003
16.0	463	Eriodictyol-7-*O*-glucuronide	MS^2 [463]: 287(100) MS^3 [287]: 151(100) MS^4 [151]: 107(100)	230, 280	0.57 ± 0.09	7.64 ± 0.8	0.48 ± 0.06
17.9	847	A-type proanthocyanidin trimer with one unit of (epi)afzelechin Isomer 1	MS^2 [847]: 711(98)-559(100)-327(7)	280	0.95 ± 0.06	7.40 ± 0.4	0.06 ± 0.003
19.1	847	A-type proanthocyanidin trimer with one unit of (epi)afzelechin Isomer 2	MS^2 [847]: 711(92)-559(100) MS^3 [711]: 585(100)-559(75)-423(60) MS^4 [585]: 423(100) MS^3 [559]: 389(100) MS^4 [389]: 362(50)-345(100)-273(3)	280	8.96 ± 0,45	11.7 ± 2.1	0.05 ± 0.007
19.5	577	B-type procyanidin dimer	MS^2 [877]: 425(100)-407(60)-289(30) MS^3 [425]: 407(100)-273(10)-281(8) MS^4 [407]: 389(20)-339(30)-285(100)-281(98)-256(40)-269(20)-243(22)-213(10)	280	0.39 ± 0.07	11.13 ± 0.3	0.48 ± 0.02
20.3	461	Luteolin-7-*O*-glucuronide	MS^2 [461]: 285(100) MS^3 [285]: 257(45)-243(25)-241(90)-213(50)-199(100)-175(90)-151(35)	225, 280	2.13 ± 0.2	16.4 ± 1.2	1.25 ± 0.07
20.8	863	A-type procyanidin trimer	MS^2 [863]: 711(100)-573(50)-451(70)-411(70) MS^3 [711]: 559(100)-407(27) MS^4 [559]: 415(90)-327(60)-255(100)	280	9.73 ± 0, 91	38.69 ± 2.6	2.58 ± 0.21
22.4	1135	A-type proanthocyanidin tetramer with one unit of (epi)afzelechin	MS^2 [1135]: 999(70)-847(100)-707(70)-634(58)	280	25.2 ± 0.17	5.70 ± 1.8	0.09 ± 0.004
25.1	1151	A-type procyanidin tetramer	MS^2 [1151]: 1025(60)-863(100)-709(60)-573(25)	280	6.5 ± 0.76	0.44 ± 0.8	*
25.3	1424 $[M-2H]^{2-}$	B-type proanthocyanidin decamer with two units of (epi)afzelechin	MS^2 [1424]: 1271(100)	280	0.69 ± 0.09	4.74 ± 0.1	0.02 ± 0.005
26.1	1151 $[M-2H]^{2-}$	A-type procyanidin octamer	MS^2 [1151]: 863(42)-777(55)	280	8.97 ± 0.06	0.62 ± 0.05	0.09 ± 0.03
26.9	1438 $[M-2H]^{2-}$	A-type procyanidin decamer with two A bonds	MS^2 [1438]: 1191(100)	280	11.4 ± 0.1	0.61 ± 0.04	0.013 ± 0.001
28.5	720 $[M-2H]^{2-}$	B-type procyanidin pentamer	MS^2 [720]: 643(100) MS^3 [643]: 559(65)-407(25)	280	5.67 ± 0.1	5.97 ± 0.08	0.09 ± 0.006
28.6	719 $[M-2H]^{2-}$	A-type procyanidin pentamer	MS^2 [719]: 567(50)-451(20)	280	10.8 ± 1.1	0.99 ± 0.09	0.07 ± 0.007

Table 3. *Cont.*

Tr	$[M-H]^-$	Identification	Fragmentation	UV s (nm)	mg/g in TC-MeOH	mg/g in TC-EtOAc	mg/g in TC-H_2O
29.8	1440 $[M-2H]^{2-}$	B-type procyanidin decamer	MS ^{2} [1440]: 1313(100)-961(55)-817(70)	280	0.51 ± 0.2	2.79 ± 0.06	0.14 ± 0.004
33.0	864 $[M-2H]^{2-}$	B-type procyanidin esamer	MS ^{2} [864]: 779(90)-575(70)-532(75)-411(100)-289(20)	280	5.85 ± 0.4	5.44 ± 0.1	0.15 ± 0.003
34.3	1008 $[M-2H]^{2-}$	B-type procyanidin heptamer	MS ^{2} [1008]: 777(55)	280	9.87 ± 1.3	1.78 ± 0.03	0.08 ± 0.005

* detectable but not quantifiable.

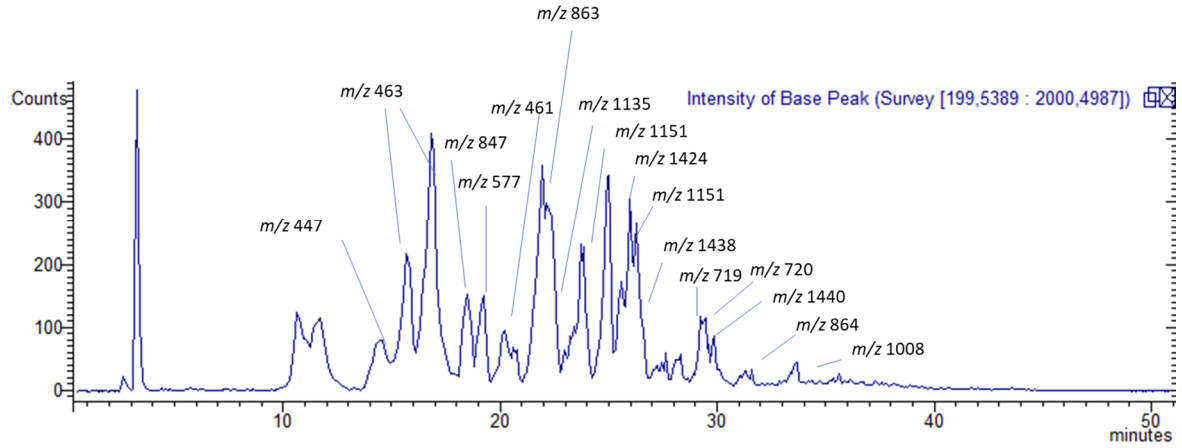

Figure 2. Base peak ion LC-MS chromatogram of TC-EtOAc showing the *m*/*z* values and corresponding peaks of identified compounds.

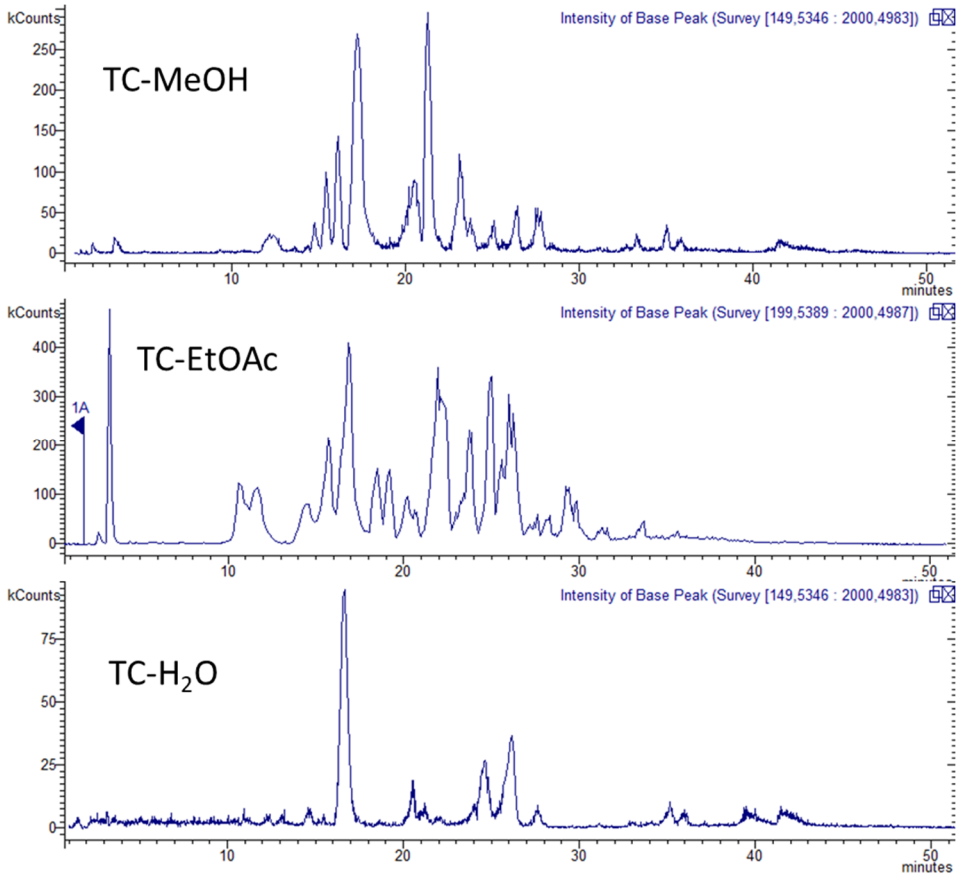

Figure 3. Base peak ion LC-MS chromatogram of TC-MeOH, TC-EtOAc, and TC-H_2O extracts.

Flavonoids are present in significant amounts in all three extracts, as reported in Table 4. TC-EtOAc contains higher amounts of flavonoid (24.28 mg/g). In TC-MeOH, flavonoids are present in an amount comparable to that of TC-H_2O (2.70 and 1.74 mg/g), except for naringenin-7-*O*-glucuronide which in TC-MeOH is not detectable.

Table 4. Quantitative results of total flavonoids, total procyanidin (PAC), and PACs divided on the basis of different degrees of polymerization in TC-MeOH, TC-EtOAc, and TC-H_2O extracts.

Sample	Total Flavonoid (mg/g)	Total PAC (mg/g)	PAC Dimers (mg/g)	PAC Trimers (mg/g)	PAC Tetramers and Polymers (mg/g)
TC-MeOH	2.70 ± 0.05	105.49 ± 0.15	0.39 ± 0.01	19.64 ± 0.13	85.46 ± 0.16
TC-EtOAc	24.28 ± 0.15	98.00 ± 0.12	11.13 ± 0.15	57.79 ± 0.15	29.08 ± 0.15
TC-H_2O	1.74 ± 0.05	3.91 ± 0.05	0.48 ± 0.01	2.69 ± 0.05	0.74 ± 0.02

With regard to the quantitative analysis, PACs were analyzed using HPLC-HILIC-FLD and results are summarized in Table 4. An exemplifying chromatogram of methanolic extract is reported in Figure 2. TC-MeOH and TC-EtOAc fractions present the highest amount of total PACs (105.49 mg/g and 98.0 mg/g), accounting for more than 50% of total polymers in TC-MeOH and more than 50% of the total as trimers in TC-EtOAc. PACs are found in TC-H_2O in low amounts (3.91 ± 0.5 mg/g), as expected due to their nature as poorly water-soluble compounds. PACs presenting lower molecular weights are more abundant in TC-EtOAc, while larger polymers concentrate in TC-MeOH. Since TC-EtOAc and TC-H_2O extracts were obtained by the fractionation of TC-MeOH, we could argue that PACs with increasing molecular weight are less soluble in water and in ethyl acetate, and thus can precipitate during the liquid–liquid partition. Previous studies reported that, in methanol solvent, smaller PAC oligomers are more soluble than bigger ones because the solute surface area exposed to the extraction solvent is larger and thus more solute–solvent interactions are present [11].

Phytochemical composition of the TC-EtOAc revealed a composition rich in condensed tannins, with the presence of flavonols and dihydroflavone.

2.3. In Vitro Bioassays

2.3.1. Antioxidant Activity

The interest in the pharmacological potential of medicinal plants might be due to their polyphenol compounds, in particular to flavonoids. From this perspective, some biological activities including the antioxidant activity of *T. coadunata* extracts were evaluated using total antioxidant capacity or phosphomolybdenum, radical scavenging (2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)), reducing power (cupric-reducing antioxidant (CUPRAC) and ferric-reducing antioxidant power (FRAP)), and metal chelating assays.

Results of in vitro bioassays are reported in Table 5 with regard to antioxidant activity, and in Table 6 with regard to enzyme inhibition.

The free radical scavenging activity of *T. coadunata* was evaluated using the DPPH and ABTS radical scavenging assays. As shown in Table 5, the values of DPPH radical scavenging activity for the three extracts of *T. coadunata* range from 762.62 mg TE/g of TC-MeOH extract to 933.97 and 948.59 of TC-H_2O and TC-EtOAc extracts, respectively. In ABTS radical scavenging activity *T. coadunata* ranges from 1097.10 mg Trolox equivalent (TE)/g of the TC-MeOH extract to 1661.21 of TC-EtOAc. Additionally, both in DPPH assay and ABTS, the TC-EtOAc extract had the highest radical scavenging activity among all samples, which is coherent with its high amount of polyphenols (276.70 mg GAE/g). This fact also was observed by correlation analysis and the analysis results are given in Figure 4. In any case, it is to be remembered that most TPC and DPPH assays can also present positive results with reducing sugars and other chemical constituents that can be subjected to oxidation other than phenolics.

Table 5. Results of total phenolic content and in vitro antioxidant assays on *T. coadunate* extracts.

Samples	Total Phenolic Content (mg GAE/g)	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Metal chelating (mg EDTAE/g)	Phosphomolybdenum (mmol TE/g)
TC-EtOAc	276.70 ± 2.58 [a]	948.59 ± 30.92 [a]	1661.21 ± 9.01 [a]	1510.63 ± 31.55 [a]	931.18 ± 17.74 [a]	na	6.32 ± 0.41 [a]
TC-H_2O	235.85 ± 1.82 [b]	933.97 ± 12.12 [a]	1269.30 ± 21.75 [b]	1108.66 ± 4.44 [b]	713.07 ± 11.98 [b]	6.26 ± 0.73 [a]	6.25 ± 0.18 [a]
TC-MeOH	234.30 ± 0.99 [b]	762.62 ± 34.65 [b]	1097.10 ± 14.02 [c]	1089.99 ± 6.42 [b]	645.59 ± 4.83 [c]	2.61 ± 0.34 [b]	5.70 ± 0.59 [a]

Values are reported as mean ± SD of three parallel experiments. ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CUPRAC: cupric-reducing antioxidant; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric-reducing antioxidant power; GAE: gallic acid equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; na: not active. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

Table 6. Results of in vitro enzyme inhibition assays on *T. coadunata* extracts.

Samples	AChE Inhibition (mg GALAE/g)	BChE Inhibition (mg GALAE/g)	Tyrosinase Inhibition (mg KAE/g)	Amylase Inhibition (mmol ACAE/g)	Glucosidase Inhibition (mmol ACAE/g)
TC-EtOAc	6.22 ± 0.06 ^{a}	9.82 ± 0.68 ^{a}	153.89 ± 1.61 ^{a}	1.50 ± 0.02 ^{a}	5.46 ± 0.05 ^{a}
TC-H_2O	1.35 ± 0.03 ^{c}	1.70 ± 0.67 ^{c}	66.85 ± 1.22 ^{c}	0.42 ± 0.04 ^{c}	5.48 ± 0.01 ^{a}
TC-MeOH	5.58 ± 0.10 ^{b}	6.31 ± 0.71 ^{b}	149.41 ± 0.96 ^{b}	1.04 ± 0.05 ^{b}	5.48 ± 0.01 ^{a}

Values are reported as mean ± SD of three parallel experiments. GALAE: galantamine equivalent; ACAE: acarbose equivalent; KAE: kojic acid equivalent; AChE: acetylcholinesterase; BChE: butyrylcholinesterase. Different superscripts indicate significant differences in the extracts ($p < 0.05$).

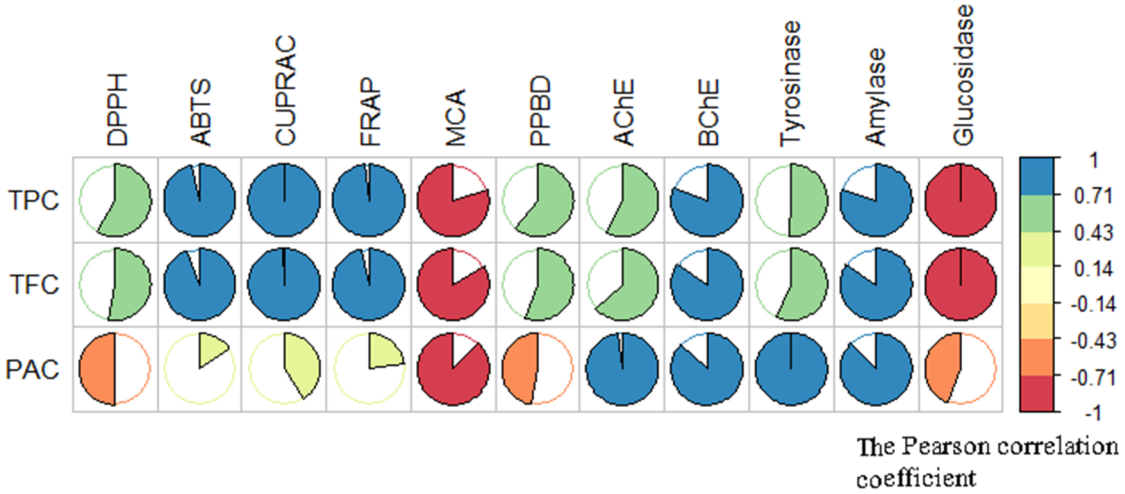

Figure 4. Correlation coefficients between total bioactive compounds and biological activities (Pearson correlation coefficient (R), $p < 0.05$). TPC: total phenolic content; TFC: total flavonoid content; PAC: procyanidin; PPBD: phosphomolybdenum assay. MCA: metal-chelating assay.

The results obtained for *T. coadunata* are coherent with the literature; in fact, polyphenolics are well-known antioxidant agents. In particular, among secondary plant metabolites procyanidins are the most liable to oxidation, and their activity is closely related to plant defense systems against oxidative stress [12]. It has also been reported that the antioxidant activity depends on polymerization and increases with galloylation [13]. However, a previous paper [14] reported that the increase of the antioxidant activity is not directly proportional with the degree of polymerization, but relies mainly on the number of hydroxyl groups, which can increase as a consequence of polymerization. The major determinant for radical-scavenging capability is the presence of a catechol group in ring B that is able to reduce highly oxidizing free radicals such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation [15].

In the light of this mechanism, it can be concluded that the high antioxidant activity of *T. coadunata* extract is due to its PAC content, and TC-EtOAc extract has the highest activity, presenting indeed the highest amount of PAC dimers (11.13 mg/g), trimers (57.79 mg/g), tetramers, and polymers (29.08 mg/g). Moreover, several investigations have shown that flavonoids such as epicatechin, catechin, and their related procyanidins can absorb through membranes through associations with the polar head groups of phospholipids, generating a flavonoid coat which would provide protection against oxidants as well as other external aggressors by limiting the access of oxidants to the bilayer and/or controlling the rate of propagation of free radical chain reactions occurring in the hydrophobic core membranes [16]. Particularly, galloylated catechins could affect the membrane configuration by forming more compact structures that limit the access of pro-oxidants [17].

Reducing power assays, namely FRAP (from Fe^{3+} to Fe^{2+}), CUPRAC (from Cu^{2+} to Cu^{+}), and phosphomolybdenum (from Mo (VI) to Mo (V)) assays, were performed to evaluate electron-donating abilities of the tested extracts and the results were similar to the results of radical scavenging assays (EtoAC > H_2O > MeOH). The results can be attributed to the presence of phenolics, especially PAC. Our findings were also supported by several researchers who reported that phenolics have great potential as reducing agents [18,19]. In contrast to radical scavenging and reducing power assays, TC-EtOAc was not active in the metal-chelating assay. TC-H_2O was more active than TC-MeOH. This contradictory finding could be explained with the non-phenolic chelators such as peptides or polysaccharides. This view is supported by Rice-Evans et al. [20] who reported that metal-chelating ability is a minor antioxidant property of phenolics.

2.3.2. Test of Inhibitory Effect Against Degenerative and Metabolic Enzymatic Activities: Cholinesterases, α-Amylase, α-Glucosidase, and Tyrosinase

The inhibitory activities of tested extracts against cholinesterases (acetylcholinesterase (AChE) and BChE (butyrylcholinesterase)), α-amylase, α-glucosidase, and tyrosinase were tested since enzyme inhibition is considered as one possible strategy to manage some chronic conditions of health problems. A well-known treatment regime for Alzheimer's disease includes cholinesterase inhibitors such as donepezil, galantamine, and rivastigmine. Current treatment modalities to manage type-2 diabetes consist of α-amylase and α-glucosidase inhibitors such as acarbose, miglitol, and voglibose. Epidermal tyrosinase inhibition by kojic acid is currently used to manage skin hyperpigmentation conditions. However, the adverse effects associated with the use of currently available enzyme inhibitors have fueled interest in finding novel therapeutic agents and this is why the extracts were tested in this context [8].

Generally, as presented in Table 6, TC-H_2O demonstrated the lowest activity against all enzymes, while the TC-EtOAc extract presented the highest values, especially for AChE (6.22 mg GALAE/g), BChE (9.82 mg galantamine equivalent (GALAE)/g), and tyrosinase (153.89 mg GALAE/g) inhibition. TC-MeOH and TC-EtOAc showed prominent inhibitory effects against AChE (TC-EtOAc: 6.22 mg GALAE/g, TC-MeOH: 5.58 mg GALAE/g), which is consistent with the literature, indicating that phenolic compounds have cholinesterase inhibitory activities [21].

2.3.3. Discussion of the Results of Acetyl and Butyril Cholinesterases Related to Phytochemical Composition of the *T. coadunata* Extracts

With respect to cholinesterase, the inhibitory activity was tested both on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are hydrolytic enzymes acting on acetylcholine (ACh) to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline and acetate. Both enzymes are present in the brain and have been detected in neurofibrillary tangles and neuritic plaques. It was suggested that AChE predominates in the healthy brain, with BChE considered to play a minor role in regulating brain ACh levels [22]. Both enzymes represent legitimate therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the neurological decline characteristic of Alzheimer's disease (AD). In this disorder, AChE activity decreases to only 33–45% of normal values as the disease progresses, while the activity of BChE increases by as much as 40–90% in certain brain areas, suggesting that this alteration of AChE to BChE ratio causes a change in the normally supportive role of BChE in hydrolyzing excess ACh. This implies that also BChE inhibition may serve as an appropriate therapeutic target to treat AD [23].

Results indicate significant AChE inhibitory activity for TC-EtOAc, and the non-detectability of nitrogen-containing compounds suggests the presence of non-alkaloidal inhibitors in the extract. The search for non-nitrogen containing AChE and BChE inhibitors is of interest since alkaloids lead to common side effects [24]. Furthermore, non-alkaloidal inhibitors probably have different types of interaction with the target enzyme due to the lack of a charged part, thus offering the opportunity to find other pharmacological properties [25].

In this regard a paper suggested that phenolic compounds are able to interact with amino acid residues defining the active site of AChE via a hydrogen bond, hydrophobic, and π-π interaction [26]. Multiple hydroxyl groups in the phenolic compound are believed to enhance the inhibitory action of AChE because of stronger binding capacity. These inhibitory actions explain the inhibitory potential of most of the phenolic compounds but not all follow the same mode of action. This fact was also confirmed by correlation analysis (Figure 4). Based on the correlation analysis, a strong correlation was found between total bioactive components and the inhibitory activities of cholinesterases (phenolics (R: 0.58 for AChE and R: 0.81 for BChE), flavonoids (R: 0.63 for AChE and R: 0.85 for BChE), and procyanidin (R: 0.98 for AChE and R:0.87 for BChE)).

The structural requirements of flavonoids as inhibitors of enzymes implicated in Azheimer's disease, like AChE and BuChE, were previously investigated in combination with the established structure-activity relationships (SARs) of flavonoids as reactive oxygen species (ROS) scavengers and metal chelators. For example same flavonoids, such as quercetin, act as AChE and BuChE inhibitors, and docking experiments showed that they can efficiently bind with enzymes [27]. In particular, the presence of phenylchroman backbone present in flavonoids could be the reason behind AChE inhibition and, in addition, the position, number, substitution of hydroxyl groups, and the oxidation state of C-ring of the flavonoid structure could also determine the effectiveness of AChE inhibition. A close inspection revealed that the binding depends not only on the OHs at positions 5 and/or 7, but also on the catechol in ring B [26].

However, due to the complexity of polyphenolics and a limited understanding of their bioactivity, absorption, metabolism, and distribution to brain tissues, the development of effective polyphenolic compounds suitable for clinical application has been rather limited. In a previous study, Wang et al. demonstrated that fractions of procyanidins, namely monomers and oligomers, in vitro, interfere with the generation of soluble neurotoxic Aβ oligomer species implicated in neuronal dysfunction in AD [28]. However, in vivo studies on eight-week-old male Sprague Dawley rats placed on a polyphenol-free AIN-93M diet revealed that only the monomer (catechin derivative) was able to improve spatial memory function and to reduce Aβ-mediated neuropathology in the brain at a concentration of 400 nM following oral administration. This can be related to the bioavailability of monomeric (catechin type) derivatives and its metabolites, while oligomers are largely not bioavailable as intact molecules. Pharmacokinetic studies indicated that catechin and epicatechin glucuronides and methylated glucuronide metabolites are the most abundant metabolites after intake of polyphenol-rich fractions or extracts [28]. The same research group demonstrated that repeated dosing of monomers resulted in the accumulation of catechin and epicatechin metabolites in the brain with concentrations reaching >300 pmol/g. Moreover, a biosynthetic brain-targeted PAC metabolite, 3-*O*-methyl-epicatechin-5-*O*-β-glucuronide, at a physiologically relevant concentration, can significantly improve basal synaptic transmission and maintenance of long-term potentiation through mechanisms associated with activation of cAMP response element binding protein (CREB) signaling, a pathway involved in synaptic plasticity essential for learning and memory [28].

The potential central nervous system actions of flavonoids strongly depend also on their ability to enter the central nervous system (CNS) and so distribution studies in this tissue should be performed. It was observed that flavonoids of different sub-classes (flavanones and anthocyanins) are able to access and transverse the endothelial cell layer [29]. In addition, the major flavonoid metabolites found in the blood circulation, glucuronides and *O*-methylated derivatives, are also incorporated into endothelial cells, where they are deconjugated, forming aglycones which may then be able to enter glial cells and possibly the brain. For catechin and quercetin, the hypothesis of transport by diffusion can be raised due to their hydrophobicity, while for glucoside derivatives, this hypothesis is not reasonable due to the presence of the glucose moiety [30]. In this context, GLUT1 is a possible transporter, concurrent with the finding that intestinal GLUT2 may be involved in the transport of these compounds [30]. In another study by Faria et al., the isomers (+)-catechin and (−)-epicatechin were found to be capable of crossing the BBB layer, with, a significant difference between the transport

of these two isomers reported, suggesting the involvement of a stereo-selective process [31]. This was demonstrated by studying the transport efficiency of 30 μM solution of catechin and epicatechin through rat brain endothelial cell (RBE4) in the presence of some transporter inhibitors. The results showed that phloridzin, an inhibitor of the sodium-dependent glucose transporter (SGLT1), affects only catechin transport, which is in agreement with a stereospecific effect. In the presence of cyclosporine A, a P-glycoprotein inhibitor, an increase in epicatechin transport, but not in catechin, was noted, suggesting the possibility that these compounds have different affinities for this transporter.

2.3.4. Discussion of the Results of Amylase and Glucosidase Inhibitory Activity Related to Phytochemical Composition of the *T. coadunata* Extracts

Considering the other enzymatic activity reported in Table 6, amylase and glucosidase inhibition is quite low for each of the tested extracts, with values around 1 mmol acarbose equivalent (ACAE)/g for amylase inhibition and 5.5 mmol ACAE/g for glucosidase, demonstrating limited activity of *T. coadunata* extracts in this targets. This fact contrasts with the findings of several researchers who indicated significant inhibition abilities of PAC against amylase [32–34]. The contradictory results may be due to the antagonistic actions of phytochemicals in the extracts. At this point, the isolated compounds from *T. coadunata* could be individually tested as diabetic agents in further studies.

All of the *T. coadunata* extracts (with the exception of the aqueous one) displayed tyrosinase inhibitory activity ranging from 149.41 mg kojic acid equivalent (KAE)/g for TC-MeOH extract to 153.89 mg KAE/g for the TC-EtOAc extract. This can be related to their high total phenolic content. Polyphenolics are able to act as cofactors or substrates of tyrosinase and in particular flavonoids containing a 3-hydroxy, 4-keto group, like eriodictyol, show significant tyrosinase inhibitory activity, which may be explained in terms of similarity with the di-hydroxyphenyl group in L-DOPA. Previously published data on flavonols indicated that aglycones but not their 3-glycoside derivates exhibit tyrosinase inhibitory activity, suggesting a role for the 3-hydroxyl group. However, this hydroxyl group may not be essential because several flavones, such as luteolin and luteolin 7-*O*-glucoside, which lack this 3-hydroxyl group, still present tyrosinase inhibitory activity [35].

2.4. Cytotoxicity Tests

Preliminary cytotoxicity tests were performed on two human tumor cell lines, 2008 (ovarian cancer) and BxPC3 (pancreatic cancer). Results are reported in Table 7.

Table 7. Cytotoxicity tests (concentration of compound inhibiting cell growth by 50% (IC_{50}, μg/mL)) of TC-MeOH, TC-EtOAc, and TC-H_2O extracts on 2008 and BxPC3 cell lines.

Samples	2008	BxPC3
TC-EtOAc	28, 7	12, 5
TC-H_2O	>50	>50
TC-MeOH	>50	>50

TC-MeOH and TC-H_2O exhibited a concentration of compound inhibiting cell growth by 50% (IC_{50}) > 50 μg/mL on both cell lines, while TC-EtOAc showed a significant cytotoxicity, with a IC_{50} of 12.5 and 28.7 μg/mL against human pancreatic and ovarian cancer cell lines, respectively. This relevant activity of TC-EtOAc can be related to the content of PACs. Actually, the fractions with the highest degree of polymerization and galloylation have been reported in literature to exert the most toxic effect against cancer cells [13]. This result is in agreement with those of other authors who also attributed the greatest level of cytotoxicity to polyphenolic compounds with these characteristics [36]. Previous mechanistic studies suggested that procyanidins show different apoptotic mechanisms. In particular, procyanidins arrest BxPC-3 cells in the G1 phase, which is mediated by decreases of cyclin D1, E, A, and B1 and by an increase in the level of Cip1/p21, and inhibited MMP-2 expression. Thus, procyanidin treatment exerted anti-proliferative and anti-invasive effects in pancreatic cell lines, suggesting its

application as a potent chemo-preventive or therapeutic agent for pancreatic cancer treatment [37]. On the other hand, previous studies in ovarian cancer cell lines showed that PACs exert cytotoxic activity via several mechanisms, inducing apoptosis with DNA damage and caspase-3 mediation; besides, down-regulation of pro-MMP-2 and a reduction in active MMP-2 levels imply a decreased invasive potential of the cells [38].

3. Materials and Methods

3.1. Plant Material

Plant twigs and rhizomes of *T. coadunata* were collected from Dakshinkali (27°36′24.88′′N and 85°15′40.01′′E), Kathmandu, Nepal. Elevation ranged from 1400 to 1509 m.

The plant twigs were dried carefully by proper pressing and the herbarium were prepared after mounting on the herbarium sheets. They were cross-checked with the herbarium deposited at the National Herbarium and Plant Laboratories, Godavari, Lalitpur (KATH). A voucher specimen was deposited with TU Herbarium number TC2018. Rhizomes were broken down into small pieces and air dried over 2–3 weeks inside the room until completely dried. The shade dried samples were grinded into fine powder with the help of an electric grinder. The powder obtained were preserved into zipper bag for extraction.

3.2. Extraction

The powder of plant material were subjected to extraction using methanol through percolation with intermittent sonication. Here, 50 g of plant powder were extracted three times with 500 mL of methanol such that the ratio of solvent in volume (mL) to the weight (g) of plant material would be 10:1. Then the solution was subjected to intermittent sonication for 2 h, i.e., continuous cycle of sonication at 30 kHz for 30 min (with 10 min interruption). After the completion of the cycle the solution was filtered with Whatman no.1 filter paper (Whatman Ltd., Kent, UK) and the filtrate was then subjected to evaporation at reduced pressure in rotary evaporator (IKA RV 10). The concentrated extract thus obtained was transferred to clean, dried and weighed glass vials. The obtained extract was called methanol extract (TC-MeOH). The resulting dried extract was then sealed and stored at 4 °C until use.

3.3. Isolation of Main Constituents

The TC-MeOH (15 g) was separated through a silica gel column chromatography, using 1% methanol in chloroform as mobile phase. Different fractions were obtained, analyzed by TLC using as eluents EtoAC:cyclohexane 2:1 and chloroform/methanol 99:1, and those presenting similar behavior were pooled. One-hundred fractions of 20 mL were collected and pooled on the basis of the chromatographic behavior. The fractions presenting similar TLC (fractions 23–45) spots were pooled. A liquid–liquid partition was performed with ethyl acetate and water, respectively, obtaining two different fractions after solvent removal: 3.15 g of ethyl acetate extract (TC-EtOAc) and 11.83 g of aqueous extract (TC-H_2O). In order to assess the structure of the most abundant compounds, the aqueous extract (8 g) was separated by a Sephadex column eluting with methanol–water 50% (0.5 mL/min), column volume 2 cm × 40 cm. Fractions were pooled on the basis of their TLC behaviors. Further preparative HPLC using Varian 920-LC, equipped with column oven and UV-Vis detector was done. The separation was achieved through Agilent ZORBAX SB-C-18 (21.2 × 150 mm, particle size 5 µm) as stationary phase. The injection volume was 200 µL, the flow was 3 mL/min, and the temperature was set at 35 °C. The UV and Vis lamps were set at 280 and 454 nm, respectively. The mobile phase was 2% formic acid in water (A) and acetonitrile (B). A gradient program was used as follows: (0 → 30 min: A:B (95:5) → A:B (50:50) 30→ 50 min: A:B (50:50) → A:B (0:100) 50→ 55 min: A:B(0:100) → A:B (0:100) 55→60 min: A:B (0:100) → A:B (95:5)).

From water extract, luteoline-7-*O*-glucuronide was obtained (10 mg) and its structure was confirmed by 1D- 2D-NMR and using mass spectrometry. With the same protocol, from the ethyl

acetate fraction (4 g) eridictiol-7-*O*-glucuronide (5 mg) was isolated, and its structure was confirmed by 1D 2D NMR and using mass spectrometry. NMR spectra were obtained on a Bruker Avance III 400 Ultrashield spectrometer with a superconducting 400-MHz magnet. Data were acquired in MeOD-d_4 (Sigma-Aldrich) using Durian® 4.95-mm NMR tubes (Durian Group). Chemical shifts are expressed in δ values in ppm. ^{1}H-NMR and HSQC-DEPT, HMBC, and COSY experiments were acquired using standard Bruker sequences measuring p1 and d1 for each acquired sample.

3.4. Quali-Quantitative Analysis: HPLC HILIC-DAD-FLD-ESI-MS

Exact weights of methanolic, ethyl acetate and water (MeOH, EtOAc, and H_2O) extracts (10 mg) were dissolved in 1 mL of methanol diluted 1:10 with the same solvent, and 1 mL was put in a vial; samples were prepared in triplicated.

In order to analyze MeOH, EtOAc, and H_2O extracts, HPLC-DAD-FLD-ESI-MS was performed using a Chromatograph Agilent 1260 apparatus (Santa Clara, CA, USA) equipped with a 1260 autosampler, column oven, diode array detector (DAD), and fluorescence detector (FLD). After the column, the flow was separated by two "T" connectors: 50% of the liquid was split to DAD, 25% to FLD, and the other 25% to a Varian MS-500 ion trap mass spectrometer. Separation was achieved using a TOSOH TSK gel amide-80 (2.1 × 150 mm, particle size 3.5 μm) as stationary phase. The sample injection volume was 10 μL, the flow was 0.25 mL/min, and the temperature of column was set at 35 °C. UV–vis spectra were acquired in the range of 190–640 nm. The mobile phase was 1% formic acid in water (A) and acetonitrile (B). A gradient program was used as follows: (0 → 20 min: A:B (1:99) → A:B (20:80) 20 → 25 min: A:B (20:80) → A:B (20:80) 25 → 45 min: A:B (65:35) → A:B (65:35) 45 → 67 min: A:B (85:15) → A:B (1:99) 69 → 75 min: A:B (1:99) → A:B (1:99)). MS spectra were collected in the *m/z* 100–2000 range, using ESI ion source operating in negative ion mode. Fragmentation of the ionic species was obtained using the turbo data dependent scanning (TDDS) instrument function. Identification of compounds was obtained based on fragmentation spectra as well as the comparison with the literature and reference compounds, when available.

DAD and FLD detectors were used to estimate the amount of PACs and flavonoids and to acquire spectral data of eluted compounds. As reference compounds, PAC A2 (Sigma Aldrich, St. Louis, MO, USA) and luteolin (Sigma Aldrich) were used. The chromatograms were monitored in FLD for PACs (excitation 230 nm, emission 321 nm; scan range 200–500 nm), whereas flavonoids were monitored at 350 nm; UV–vis spectra were acquired in the range of 190–640 nm. Compounds quantification was obtained with the method of calibration curve: PAC A2 was used as external standard for PACs quantification, while luteolin was used for flavonoids. Calibration curves were as follows: $y = 6.6721x + 8.6153$ ($R^2 = 0.9991$) for PAC A2; $y = 127.77x - 2.4$ ($R^2 = 0.9998$) for luteolin.

3.5. Total Phenolic Content, Antioxidant, and Enzyme Inhibitory Assays

Total phenolic and flavonoid content (TFC) was detected by Folin–Ciocalteu colorimetric method [39]. Briefly, sample solution (50 μL) was mixed with the reagent of Folin–Ciocalteu reagent (100 μL, 1:9, *v/v*). The mixture was kept for 3 min at the room temperature and then sodium carbonate (75 μL, 2%) was added. The mixture was incubated for 2 h at the room temperature. After that, the absorbances were recorded at 765 nm. The results were expressed as standard compounds (gallic acid (GAE)).

For antioxidant capacity, chemical different assays including free radical scavenging (DPPH and ABTS), reducing power (CUPRAC and FRAP), ferrous ion-chelating (ferrozine method), and phosphomolybdenum assay were performed. The results were recorded on the basis of the spectrophotometric measurements. The methods details were described in our earlier paper [39]. To explain the results, we used standard equivalent way and thus Trolox and EDTA (for ferrous ion chelation) were selected as standards

For enzyme-inhibitory assays, we selected on some enzymes related global health problems, namely cholinesterases, tyrosinase, amylase, and glucosidase. The experimental procedures were given

in our earlier paper [39]. Standard enzyme inhibitor compounds were used to evaluate the results. These compounds were galantamine (GALAE, for cholinesterases), kojic acid (KAE, for tyrosinase), and acarbose (ACAE, for amylase and glucosidase). All experimental procedures were performed with 96 wells microplate.

3.6. Cytotoxicity Studies

Ovarian (2008) and pancreatic (BxPC3) carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using RPMI-1640 medium (Euroclone) containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (100 units/mL penicillin and 100 g/mL streptomycin), and 2 mM L-glutamine.

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazoliumbromide) assay (MTT) was used as a relative measure of cell viability. Briefly, 10^3 cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium. After 24 h, the medium was removed and replaced with fresh medium containing the compound to be studied at the appropriate concentration (0.1–30 μM for isolated compounds, 1–100 μg/mL for EO). Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 μL of a 5 mg/mL MTT solution in phosphate-buffered saline (PBS) and, after 4 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl were added. After an overnight incubation, the extent of MTT reduction was quantified spectrophotometrically using a microplate reader BioRad 680, by absorbance measurement at 540 nm. The mean absorbance for each drug dose was expressed as a percentage of the control, untreated, well absorbance and plotted vs. drug concentration. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values, the drug concentrations that decrease the mean absorbance at 570 nm to 50% of that of untreated control wells, were calculated using GraphPad Prism 4 (GraphPad Software, S. Diego, CA). The final value is the mean ± S.D. of at least three independent experiments performed in triplicate.

3.7. Statistical Analysis

The data of total phenolic content, antioxidant, and enzyme inhibitory assays were presented as mean ± SD and the statistical procedures were performed using GraphPad Prism 8 software. One-way ANOVA followed by Tukey's multiple range was conducted to measure differences ($p < 0.05$) between the tested samples. The correlation values (Pearson's correlation coefficients) between total components (TPC, TFC, and PAC) and biological abilities (antioxidant and enzyme inhibitory properties) were determined using R software v. 3.6.1.

4. Conclusions

In this paper the composition of the ethnomedicinal fern *T. coadunata*, spontaneously grown and collected in Nepal, was considered and the phytochemical investigations revealed significant amounts of procyanidins with different degrees of polymerization (with the exceptions of eriodictyol-7-*O*-glucuronide, luteolin-7-*O*-glucuronide, and naringenin-7-*O*-glucuronide). To assess the potential usefulness of the extract, in vitro antioxidant tests as well as inhibitory tests on five enzymes that are related to degenerative and metabolic diseases were explored, showing significant activities. In particular, AChE, BChE, tyrosinase, and amylase inhibitory activities were observed, and TC-MeOH and TC-EtoAc resulted more active compared to TC-H_2O. These observations suggest that the higher amount of polyphenols in the two first extracts could be partly related to the measured activities. Results indicated that *T. coadunata* extracts, being a significant source of phenolics, could be considered for further studies aimed at developing co-treatments for Alzheimer's disease using more complex in vitro and in vivo tests due to their significant antioxidant activity and partial target enzyme inhibition. In addition, their significant antioxidant effect could be an advantage in the management of metabolic diseases. Assays studying the anti-inflammatory effect of the extracts and isolated

compounds may be added and implemented. Furthermore, significant cytotoxic activity was observed for ethyl acetate extract, mainly with respect to pancreatic cancer cells. Some of the constituents could thus be further studied, and research on the purified constituents will aid in assessing their potential usefulness in the therapeutic field. This medicinal fern could be suitable for cultivation in Nepal with the aim of obtaining plant material for the extraction of bioactive fractions for medicinal and pharmaceutical purposes.

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Abbreviations

ABTS	2:2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CUPRAC	cupric-reducing antioxidant
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	ferric-reducing antioxidant power
FLD	fluorescence detector
ESI-LC-DAD-MS	electrospray ionization source–liquid chromatography–diode array detector–mass spectrophotometry
NMR	nuclear magnetic resonance
PAC	procyanidin
TC-MeOH	*Tectaria coadunata* methanolic extract
TC-EtOAc	*T. coadunata* ethyl acetate extract
TC-H_2O	*T. coadunata* water extract
GC-MS	gas chromatography–mass spectrophotometry
AChE	acetylcholinesterase
BuChE	butyrylcholinesterase
AD	Alzheimer's disease
TLC	thin layer chromatography
TDDS	turbo detection data scanning
HSQC-DEPT	heteronuclear single quantum coherence spectroscopy–distortionless enhancement by polarization transfer
HMBC	heteronuclear multiple quantum coherence
COSY	correlation spectroscopy

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Sample Availability: Extracts and plant powder are available from the authors. The samples are stored in Department of Pharmaceutical and Pharmacological Sciences, University of Padova.

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Himalayan Nettle *Girardinia diversifolia* as a Candidate Ingredient for Pharmaceutical and Nutraceutical Applications—Phytochemical Analysis and In Vitro Bioassays

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Abstract: *Girardinia diversifolia*, also known as Himalayan nettle, is a perennial herb used in Nepal to make fiber as well as in traditional medicine for the treatment of several diseases. To date, phytochemical studies and biological assays on this plant are scarce. Thus, in the present work, the *G. diversifolia* extracts have been evaluated for their potential pharmaceutical, cosmetic and nutraceutical uses. For this purpose, detailed phytochemical analyses were performed, evidencing the presence of phytosterols, fatty acids, carotenoids, polyphenols and saponins. The most abundant secondary metabolites were β- and γ-sitosterol (11 and 9% dw, respectively), and trans syringin (0.5 mg/g) was the most abundant phenolic. Fatty acids with an abundant portion of unsaturated derivatives (linoleic and linolenic acid at 22.0 and 9.7 mg/g respectively), vitamin C (2.9 mg/g) and vitamin B2 (0.12 mg/g) were also present. The antioxidant activity was moderate while a significant ability to inhibit acetylcholinesterase (AChE), butyrilcholinesterase (BuChE), tyrosinase, α-amylase and α-glucosidase was observed. A cytotoxic effect was observed on human ovarian, pancreatic and hepatic cancer cell lines. The effect in hepatocarcinoma cells was associated to a downregulation of the low-density lipoprotein receptor (LDLR), a pivotal regulator of cellular cholesterol homeostasis. These data show the potential usefulness of this species for possible applications in pharmaceuticals, nutraceuticals and cosmetics.

Keywords: phytosterols; LC-MS; polyphenols; NMR; cytotoxicity; LDLR; low-density lipoprotein receptor

1. Introduction

Girardinia diversifolia (Link) Friis (Urticaceae) is a stout, erect, perennial herb, 25–200 cm tall, with a perennial rootstock. The aerial parts are armed with numerous slender stinging hairs and the leaves have 3–5 deep lobes, and are saw-toothed with bristles. The flowers are yellowish, clustered in a panicle; the male ones are white and borne in lower axillary panicles; the female ones are grouped in upper bristly axillary and terminal panicles. Flowers appear from July to September and fruits from September to November [1]. The plant is found on a clump with many stems, whose bark contains strong, smooth and light fibers, largely used for textiles in Nepal. *G. diversifolia* occurs abundantly in different parts of Nepal, notably in hill forests on moist and damp soil, at altitudes of 1200–3000 m; it is also found in northern India, Bhutan, Sri Lanka, eastward of central China, Myanmar, Malaysia, Indonesia and Africa. In Nepali, the plant is known as "allo" or "chalnesisno" as its leaf is broad and palm-like. In English it is commonly known as "Himalayan nettle" due to the presence of stinging hairs that cause irritation on the skin when touched.

G. diversifolia is largely used in traditional medicine for the treatment of several diseases such as gastric disorders, chest pain [2], rheumatism, tuberculosis [3], headache, joint aches [4], diabetes [1,5], asthma, gastritis, headache, joint pain, tuberculosis [6], gonorrhea and delivery problems [5]. Other traditional uses are related to the treatment of bone fracture, internal injury and blood purification [7]. It is also famous for the uses of the bast fiber of its bark, for making varieties of clothing, ropes, mats, sacks and other domestic implements. In this context, it is one of the most important non-timber forest products used for income generation among rural communities in the Himalaya region of Nepal for their livelihood. Young leaves and inflorescences are cooked as a green vegetable. Roasted seeds are consumed pickled [4]. Other in formation about this specie are available in supplementary informations.

There is a renewed interest in the study and evaluation of botanicals as active ingredients of herbal medicines and pharmaceutical, cosmetic and nutraceutical preparations. In particular, the area of nutraceuticals, naturally derived mixtures that are claimed to improve health and to prevent diseases [8], can be an innovative area of research for the discovery of useful remedies. In this regard, Nepalese medicinal and aromatic plants can be a valuable source for the discovery of bioactive extracts and constituents.

A literature search revealed limited information about the phytochemical content of *G. diversifolia*. Sterol derivatives were isolated from plants collected in Kenia, namely β-sitosterol, 7-hydroxysitosterol and 3-hydroxystigmast-5-en-7-one, and the antimicrobial activity was determined [9].

Thus, with the aim to consider *G. diversifolia* as a starting material for the preparation of new nutraceutical or pharmaceutical products, in this paper, we evaluated this plant as a source of bioactive phytochemicals. The work was performed combining LC-DAD-MS^n (liquid chromatography–diode array detector–tandem mass spectrometry) and GC-MS (gas chromatography–mass spectrometry) analysis with in vitro bioassays focused on some key enzymes involved in global health problems. Some of them are related to the central nervous system, namely acetylcholinesterase and butyrilcholinesterase. Others (e.g., tyrosinase) are involved in skin disorders highlighting potential use in cosmetics. Furthermore, due to possible importance of phytochemicals acting on metabolic syndrome, key enzymes in managing glucose levels, such as α-amylase and α-glucosidase, were taken into account. To complete the pattern of considered bioactivities, metal chelating and antioxidant capacity of the extract were performed, and preliminary cytotoxic activity was assayed on three different tumor cell lines. Finally, the capability to interfere with the synthesis of LDL receptors was studied in hepatocarcinoma cells. All the data were corroborated by detailed phytochemical fingerprinting, offering an overview of chemical composition and preliminary bioactivity of this species.

2. Results

2.1. Phytochemical Analysis

For screening purposes, the extraction of secondary metabolites was performed on dried plant material, using methanol as the solvent due to its ability to extract both lipophilic and hydrophilic constituents. Due to the different chemical constituents that can be present in plant material, different analytical methods were used. *G. diversifolia* extract was analyzed by LC-APCI-MS^n (liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry)and LC-DAD-ESI-MS^n (liquid chromatography–diode array detector–electrospray-tandem mass spectrometry) to obtain information on the phytochemical composition of the plant material sampled in Nepal. A specific method, using C-18 stationary phase coupling, with chromatography equipped with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS), was used for the identification and quantification of terpenoids and phytosterols. A C-30 stationary phase coupled with a diode array detector (DAD) and APCI-MS was used to assess the presence and to quantify carotenoid derivatives. Furthermore, a C-18 column and electrospray (ESI-MS) were used to identify more hydrophilic constituents, such as, for example, phenolics and saponins. The identified compounds are reported in Tables 1 and 2, along with the amounts of the most abundant compounds. Figures 1 and 2 represent the obtained chromatograms.

Table 1. Identified lipophilic compounds in *G. diversifolia* extract by LC-APCI-MS. Compounds were identified on the basis of literature data (CIT) and injection of authentic standards (*).

RT(min)	$[M + H\text{-}H_2O]^+$	Fragments	Identification	mg/g
6.7	425	405-389-365-299-251	Erythrodiol	0.50 ± 0.05
7.8	425	405-389-365-299-251	Uvaol	0.54 ± 0.05
8.5	427	409-391-255-173	Hydroxy cycloartenol	0.25 ± 0.05
9.2	409	391-339-297-269-173	Cycloartenol	0.35 ± 0.05
21.7	395	297-255-241-199-159	Fucosterol *	23.5 ± 0.05
21.8	397	315-299-285-257-243-203-189	γ-Sitosterol *	91.0 ± 0.05
22.5	381	297-255-227-173-159	Brassicasterol *	3.14 ± 0.05
23.1	429	165-137-122-67	α-Tocopherylquinone	n.d.
24.7	383	273-257-243-215-161	Campesterol	32.5 ± 0.05
27.6	397	257-175-161	β-sitosterol *	112.4 ±0.07
29.6	399	316-257-243-190-175-149	Sitostanol *	16.6 ± 0.05
5.07	553		α-Cryptoxanthin	3.9 ± 0.1
5.91	549.5		Carotenoid	3.4 ± 0.1
6.02	545.5	489-435-339	Phytoene	1.3 ± 0.1
6.23	555.1	534-460-442	β-Carotene epoxide	4.0 ± 0.1
6.43	597.5	534-460-442	Zeaxhantin *	6.1 ± 0.1
7.05	553.5	534-460-442	Cryptoxanthin	8.7 ± 0.1
7.58	551.4	535-558-471-444	Ketocarotenoid	3.8 ± 0.1
8.53	551.5	535-558-471-444	Ketocarotenoid	3.2 ± 0.1
9.62	551.5	535-558-471-444	Ketocarotenoid	2.5 ± 0.1
9.78	551.5	535-558-471-444	Ketocarotenoid	1.6 ± 0.1
10.05	537.5	457-445-413	β-Carotene *	1.9 ± 0.1

Phytosterols and triterpenes, with β-sitosterol, γ-sitosterol, campesterol, fucosterol and sitostanol as the most abundant constituents, were detected in the plant extract. To confirm the structure of γ-sitosterol, this compound was isolated, and the structure was elucidated by comparing the obtained 1D and 2D NMR data with the literature [10,11].

Overall, the phytosterols content is notable, considering their amount corresponded to 26% of total methanol extract. Analysis was also performed to assess the presence of carotenoids. The results revealed a limited amount of such compounds in the prepared extract, with a total content of 0.47 mg/g. In comparison with the reference standard, a low amount of β-carotene and zeaxanthin were detected, while other derivatives that were assigned to oxidized products of carotenoids, mostly β-carotene epoxide, were observed and tentatively identified on the basis of their MS spectra and comparison

with the literature [12]. The LC-DAD at 450 nm is reported in Figure 3. The presence of degradation products of carotenoids may be due to extraction procedures or drying of plant material.

Table 2. Identified polar compounds in *G. diversifolia* extract by LC-DAD-ESI-MS, * indicate comparison with authentic standard.

RT(min)	$[M-H]^-$	Fragments	Identification	mg/g
1.8	341	179	Sucrose *	nd
1.9	191	179 85	Quinic acid *	0.138 ± 0.021
2.2	191		Citric acid *	0.235 ± 0.045
2.3	195	177 129 85	Gluconic acid	nd
2.7	353	191 179 135	3-O-Caffeoyl quinic acid	0.056 ± 0.045
3.7	371	209 191 85	*trans*-Syringin (eleuteroside B)*	0.503 ± 0.046
5.2	315	153	Protocatecuic acid glucoside	0.121 ± 0.031
6.3	365	211 153	Protocatecuic ester with sinapoyl alchol	0.103 ± 0.031
6.6	447	357 315 271	Isoorientin	0.090 ± 0.032
7.85	175	157 115 85	Ascorbic acid	2.901 ± 0,031
8.1	371	147 209	Synapoilquinic acid	0.162 ± 0.051
9.2	385	224	Synapoyl acid hexoside isomer 1	0.081 ± 0.028
10.3	447	285 199 243 175	Kaempferol-3-*O*-glucoside *	0.033 ± 0.002
10.9	367	191	5-*O*-Feruloyl quinic acid	0.191 ± 0.029
11.5	385	224	Synapoyl acid hexoside isomer 2	0.151 ± 0.031
11.8	841	779 679 617, 547, 529	3-[Xyl]-28-Glc-11-Hydroxyphytolaccagenin	0.154 ± 0.021
12.0	431	341 311 283	Vitexin*	0.058 ± 0.023
12.8	841	679 517 473 457 437	Scabran G	0.035 ± 0.011
13.6	563	443 413 323	Vitexin-2″*O*-xyloside*	0.171 ± 0.028
14.2	593	473 413 341 311	Vitexin-2″*O*-glucoside*	0.085 ± 0.006
14.3	679	517 473 455 438	Ganoderic acid C2 hexoside isomer 1	0.079 ± 0.007
14.9	679	517 499 473 455 438	Ganoderic acid C2 hexoside isomer 2	0.074 ± 0.007
16.3	679	518 499 473 455 438	Ganoderic acid C2 hexoside isomer 3	0.037 ± 0.007
18.9	517	499 437 304	Ganoderic acid C2	0.038 ± 0.0071
19.4	327	309 291 229	oxo-dihydroxy-octadecanoic acid	0.390 ± 0.008
	$[M+H]^+$			
15.4	805	643	Malvidin-3-*O*-glucoside-4-vinyl-catechin	0.032 ± 0.008
8.7	377	243	Vitamin B2	0.122 ± 0.021

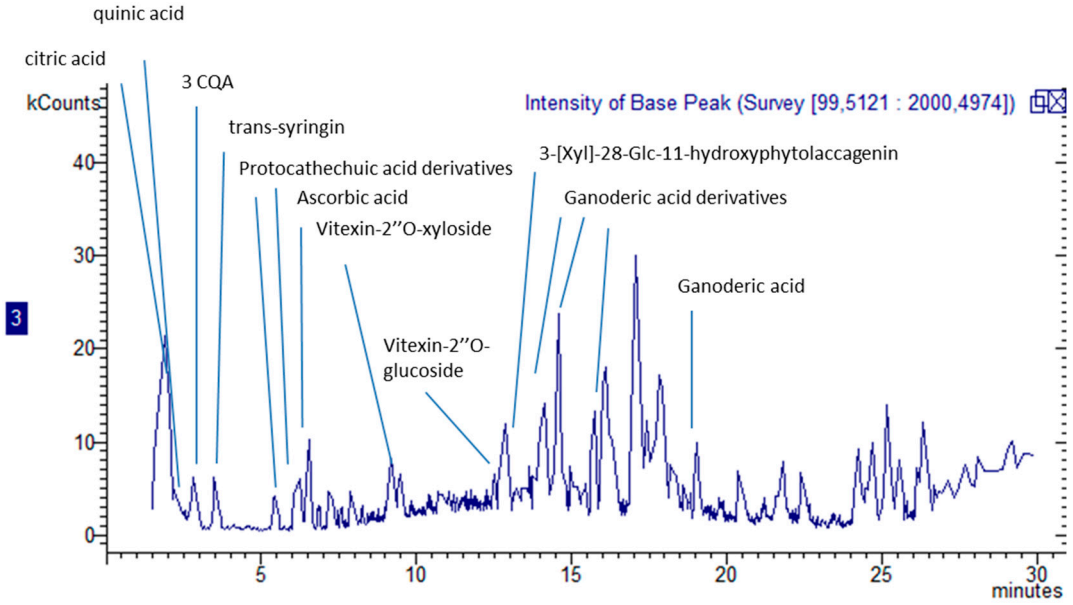

Figure 1. LC-MS chromatogram in negative ion mode; some of the identified peaks reported in Table 1 are highlighted.

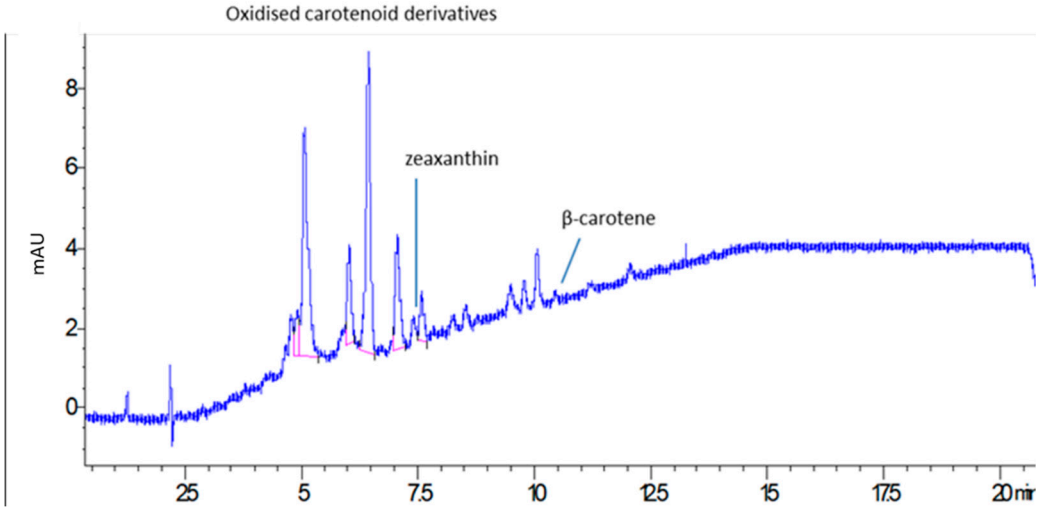

Figure 2. LC-DAD chromatogram related to the analysis of carotenoids and derivatives (425 nm).

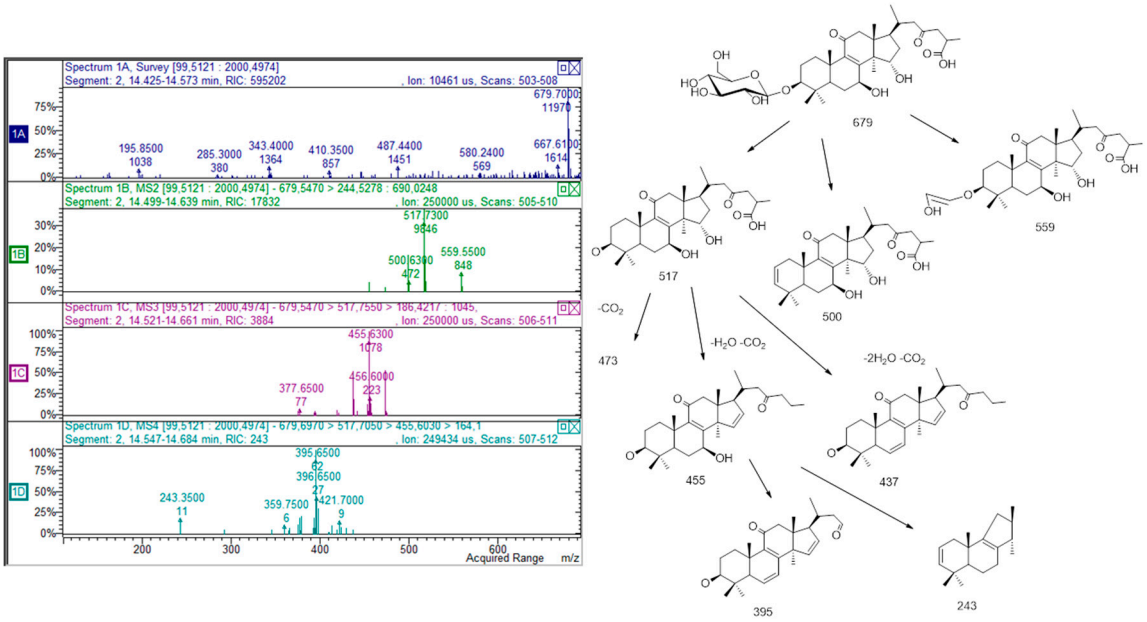

Figure 3. Proposed fragmentation scheme and MS^n spectrum of derivative *m/z* 679, tentatively assigned to a glycosidic derivative of ganoderic acid.

Polar constituents were analyzed by LC-DAD-ESI-MS^n, and some organic acids, namely citric and quinic acids were detected; the most abundant was ascorbic acid, assessed at 2.9 mg/g. These compounds are quite common in vegetal sources. A detectable but not quantifiable amount of gluconic acid was also observed.

Some peaks ascribable to flavonoids were detected as iso-orientin, vitexin and its derivatives, and kaempferol glucoside [13,14]. Other minor phenolic derivatives, mostly caffeoylquinic acid esters [15], syringic, protocatecuic and sinapoyl acid derivatives [13] were also present. In positive ion mode, traces of complex malvidin glycoside linked to catechin units was observed [16].

LC-MS^n data made it possible to reveal several peaks that present *m/z* values, and a fragmentation pattern that can be ascribed to saponins: in particular, a peak at retention time of 13 min with *m/z* 841 and fragmentations suggesting that the structure can be assigned to 3-[Xyl]-28-Glc-11-hydroxyphytolaccagenin [17]. An isomer of this compound presenting a different pattern of fragmentation is observed at retention time of 11.8 min and was tentatively assigned to an

iridoid derivative [18]. Furthermore, different peaks presenting *m*/*z* values and fragmentation patterns that suggest the presence of ganoderic acid derivatives were detected. In particular, from the peak at retention time of 18.9 min with molecular ion $[M-H]^-$ at *m*/*z* 517 we can observe MS^2 fragments due to the elimination of water (−18 amu) and subsequent loss of water and CO_2(−62 amu). Similar behavior is also observed for three other peaks at retention times of 14.3, 14.9 and 16.3 min, presenting *m*/*z* values of 679. The MS^n spectrum, as well as a proposed scheme of fragmentation, is reported in Figure 3 as an example. These data, when compared with the literature [14,19], suggest the presence of ganoderic acid derivative in *G. diversifolia* samples although at very low concentration. These metabolites are typical of *Ganoderma* fungi but have recently been reported in *Rubus* fruits, leaves and flowers [14]. To the best of our knowledge, no other papers have reported the presence of such derivatives in the Urticaceae family. We can suggest that symbiotic fungi can be grown in association with plants and that these metabolites can be derived from the fungal metabolism. However, further investigation should be performed to assess the origin of these metabolites in the Himalayan nettle. Finally, a low but detectable amount of vitamin B2 was measured.

Due to the large amount of lipophilic compounds, GC-MS analysis was also performed after derivatization with methanol to study fatty acid composition and to reveal the presence of other lipophilic constituents. The results are reported in Table 3. The methanolic extract of *G. diversifolia* contained a 13% lipidic fraction, including 46% saturated derivatives and 32% unsaturated fatty acids. The most abundant fatty acid was palimitic acid, while linoleic acid was the most abundant one (22 mg/g) among unsaturated fatty acids. Phytol was the main constituent of the lipophilic fraction (27 mg/g). Results are summarized in Table 3. Figure 4 presents the structures of the most abundant constituents of the *G. diversifolia* extract.

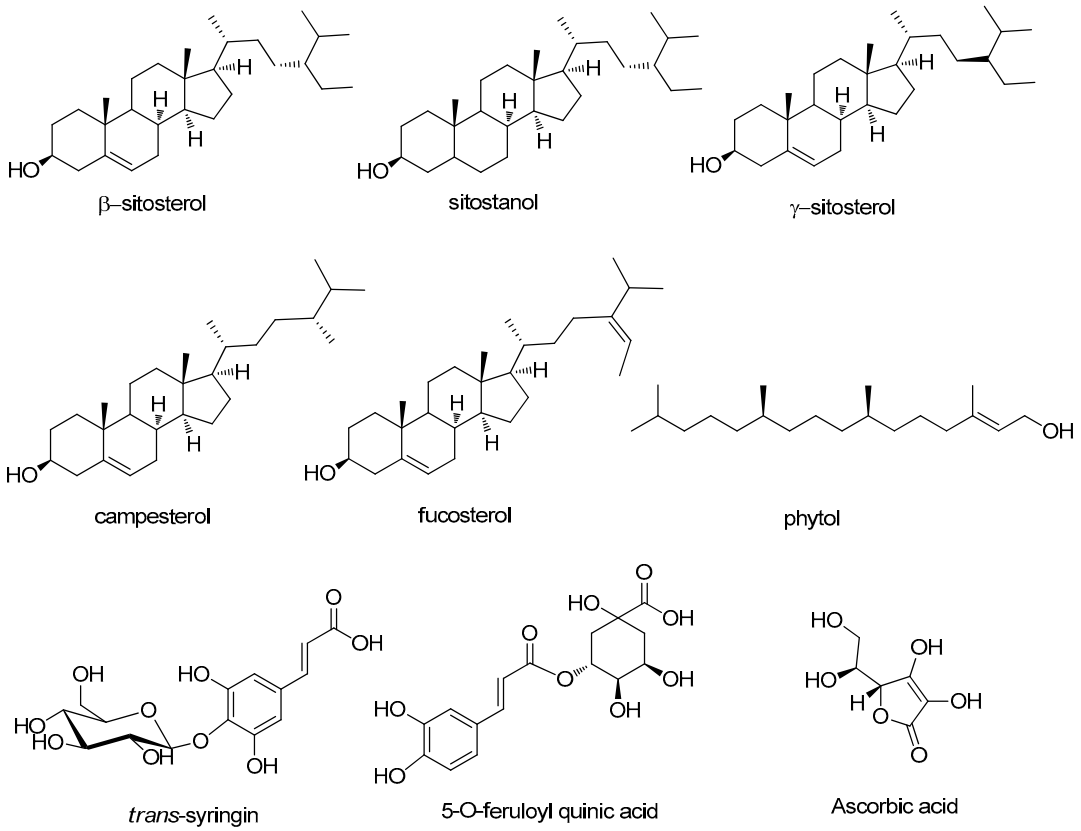

Figure 4. Structure of the most abundant constituents found in the *G.diversifolia* extract.

Table 3. constituents in *G. diversifolia* extract by GC-MS after derivatization.

RT (min)	Identification	MW (Da)	CAS	mg/g
14.2	Phytol	296	102608-53-7	26.72 ± 0.05
18.4	Myristic acid, methyl ester (C16:0)	242	124-10-7	0.70 ± 0.03
23.1	Palmitic acid, methyl ester (C10:0)	270	112-39-0	37.18 ± 0.05
24.3	7-hexadecenoic acid, methyl ester (C16:1)	268	5687-67-3	2.06 ± 0.06
27.6	Stearic acid, methyl ester (C18:0)	298	112-61-8	8.98 ± 0.06
28.7	Elaidic acid, methyl ester (C18:1)	296	112-62-9	8.66 ± 0.06
30.4	Linoleic acid, methyl ester (C18:2 ω-6)	294	112-63-0	21.95 ± 0.08
31.8	Arachidic acid, methyl ester (C20:0)	326	1120-28-1	6.01 ± 0.05
32.3	Linolenic acid, methyl ester (C18:3 ω-3)	292	301-00-8	9.69 ± 0.06
33.5	Heneicosanoic acid, methyl ester (C21:0)	340	6064-90-0	1.22 ± 0.03
35.6	Docosanoic acid, methyl ester (C22:0)	354	929-77-1	2.94 ± 0.03
37.3	Tricosanoic acid, methyl ester (C23:0)	368	2433-97-8	0.65 ± 0.02
38.5	1-docosanol	326	661-19-8	0.36 ± 0.02
39.1	Lignoceric acid, methyl ester (C24:0)	382	2442-49-1	2.42 ± 0.04
42.3	Cerotic acid, methyl ester (C26:0)	410	5802-82-4	0.80 ± 0.02
45.5	Montanic acid, methyl ester (C28:0)	438	55682-92-3	0.52 ± 0.02

2.2. *In Vitro* Bioassays

The results indicate significant AchE inhibitory activity for *G. diversifolia* extract as reported in Table 4. The phytochemical analysis indicates the presence of some non-alkaloidal inhibitors. The search for non-nitrogen-containing AchE and BuChE inhibitors is of interest, since alkaloids are known for their activity in this regard but their use leads to unpleasant side effects [20,21]. Furthermore, non-alkaloidal inhibitors probably have different types of interaction with the target enzyme due to the lack of a charged part, thus offering the opportunity to find other pharmacological properties [20].

Table 4. Results of in vitro enzyme inhibition assays on *G. diversifolia* methanolic extract. Values are reported as mean ± SD of three parallel experiments. GALAE: Galantamine equivalent; ACAE: Acarbose equivalent; KAE: Kojic acid equivalent.

Extract	AchE Inhibition (mgGALAE/g)	BuChE Inhibition (mgGALAE/g)	Tyrosinase Inhibition (mgKAE/g)	Amylase Inhibition (mmolACAE/g)	Glucosidase Inhibition (mmolACAE/g)
G.diversifolia	4.08 ± 0.21	7.21 ± 0.61	138.14 ± 1.36	1.19 ± 0.05	5.42 ± 0.03

AchE predominates in the healthy brain, with BuChE considered to play a minor role in regulating brain acetylcholine levels [22]. Both enzymes represent legitimate therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the neurological decline occurring in Alzheimer's disease (AD). Tyrosinase is a main enzyme in the synthesis of melanin and its inhibition was found to be significant, showing potential usefulness of the extract for cosmetic uses and skin related diseases.

A-Amylase and α-glucosidase are enzymes related to carbohydrate digestion and their inhibition could help to control postprandial glucose levels in diabetes mellitus patients. Considering the α-amylase and α-glucosidase inhibition, the observed activities can be considered moderate, with values around 1 mmol acarbose equivalent (ACAE)/g for α-amylase inhibition and 5.4 mmol ACAE/g for α-glucosidase, demonstrating limited activity of *G. diversifolia* extract as reported in Table 4.

The tests on the antioxidant and total phenolic constituents are in agreement with the obtained data from the chromatographic measurements and are summarized in Table 5. The levels of polyphenols appear to be moderate and also the antioxidant effects. The extract exhibited scavenging abilities on DPPH (2,2-diphenyl-1-picrylhydrazyl) (14.37 mg TE/g extract) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (28.33 mg TE/g extract). Reducing power reflects the electron-donation ability of the extract in CUPRAC (cupric reducing antioxidant capacity) (105.27 mg TE/g extract) and FRAP (ferric ion reducing antioxidant power) (42.63 mg TE/g extract) assays. In addition, the metal chelating activity (25.70 mg EDTAE/g) appears to be significant, showing potential usefulness in the protection of metal-based reactions producing free radicals.

Table 5. Results of in vitro antioxidant assays on *G. diversifolia* methanolic extract. Values are reported as mean ± SD of three parallel experiments. GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; na: not active.

Extract	Total Phenolic Content (mgGAE/g)	DPPH (mgTE/g)	ABTS (mgTE/g)	CUPRAC (mgTE/g)	FRAP (mgTE/g)	Metal Chelating (mgEDTAE/g)	Phosphomolybdenum (mmolTE/g)
G. diversifolia	24.30 ± 0.15	14.37 ± 0.72	28.33 ± 1.21	105.27 ± 0.65	42.63 ± 0.69	25.70 ± 2.35	2.53 ± 0.25

Thus, taking into account the phytochemical composition and the in-vitro-enzyme-inhibitory properties and antioxidant capacity of *G. diversifolia* methanolic extract, we observed a significant effect on AchE and BuChE. Due to the low amount of polyphenols, we can suggest that this activity may be related to the high content of phytosterols. In a previous paper, stigmasterol was considered as an insecticidal constituent of the extract of the plant Chromolaena odorata or Eupatorium odoratum. Authors also reported that the phytosterol mixture was a better AchE inhibitor than its pure compound on C. quinquefasciatus AchE [23]. Other authors reported non-activity of stigmasterol in a mice model and in an vitro test at a dose of 0.3–30 μM (0.123 mg/L, 12.3 mg/L) [24], while other authors reported AchE inhibition with IC_{50} of 644 μM (265 mg/L) [25].

Additionally, β-sitosterol has been evaluated for its ability to inhibit AchE, and IC_{50} of 24.1 ± 0.7 μg/mL (58.1 ± 1.6 μM) was reported as showing significant ability to inhibit this enzyme [26]. Furthermore, other authors have reported that β-sitosterol was an efficient inhibitor of BuChE, with an IC_{50} value of 0.56 μM and also presenting significant selectivity compared to AchE (IC_{50}14.57 μM). Authors explained this activity by studying the docking positions of side chains and hydroxyl groups at the enzyme's active site [26].

Another compound that is present in a high quantity in *G. diversifolia* extract is phytol and its presence can also be related to the observed inhibitory properties, both on AchE and BuChE. A previous paper reported, for phytol that was isolated from marine organisms, an IC_{50} of 2.7 and 5.8 μM, respectively. The significant activity was corroborated by molecular docking studies that suggest that phytol is capable of forming strong interactions with Arg177, which is a residue outside the binding pocket of AchE [27]. Thus, the contribution of phytol to the *G. diversifolia* extract inhibitory activity on the two cholinesterase should be taken into account due to the high levels observed (2.7%) in the extract.

Considering the more polar constituents, syringin was reported to be active, having IC_{50} values of 31.47 ± 2.2 and 52.77 ± 0.12 μM, respectively, for AchE and BuChE [28], and showing significant selectivity on the latter enzyme. Thus, also, trans-syringin that is present in the methanol extract at 0.5mg/g can be considered to explain the efficacy of *G. diversifolia* inhibitor properties on these enzymes.

The presence of phytosterols, phytol and syringin make the extract of *G. diversifolia* a promising ingredient for nutraceuticals targeted for neuroprotection. Considering the lipophilic portion, a recent paper demonstrated an efficient neuroprotective role of phytosterols against high-cholesterol diet-induced cognitive deficits in aged rats [29].

Related to the tyrosinase activity, in this case the presence of high levels of phytosterols can also be related to the observed effect. A previous paper showed a significant effect for γ-oryzanol in cellular melanogenesis owing to its inhibition of tyrosinase enzyme activity and reduction of MITF and target genes in the PKA-dependent pathway [30].

2.3. Cytotoxic Activity

To obtain further information on the potential bioactivity of *G. diversifolia*, we considered in vitro cellular models. As a starting point, preliminary cytotoxicity tests of *G. diversifolia* methanolic extract were performed on three tumor cell lines, 2008 (ovarian cancer), BxPC3 (pancreatic cancer) and Huh7 (hepatocarcinoma). The *G. diversifolia* extract was tested on three different tumor cell lines, showing a significant cytotoxic effect on BxPC3 (IC_{50} = 12.5 μg/mL) and Huh7 cells (IC_{50} of 7.2 ± 2.5 μg/mL).

Furthermore, due to the complex phytochemical composition of the extract, we performed further fractionation, and cyclohexane, dichloromethane and ethyl acetate soluble fractions of methanolic extract were obtained in order to separate lipid constituents from the more hydrophilic compounds.

Exemplificative LC-MS chromatograms related to the phytosterol content are reported in Figure 5.

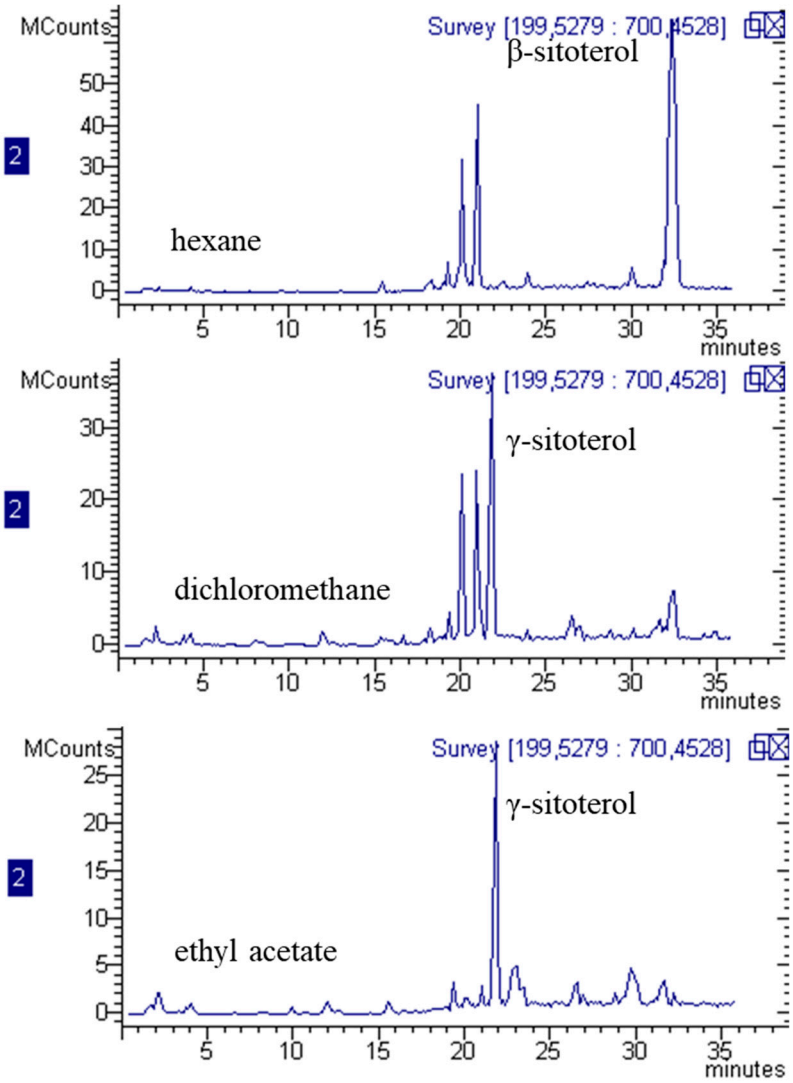

Figure 5. HPLC-MS chromatograms (base peak ion) of the three fractions obtained from the methanol extract by liquid–liquid partition, hexane, dichloromethane and ethyl acetate.

The lipophilic portion showed differences in phytosterol composition, with β-sitosterol being more concentrated in the hexane fraction and γ-sitosterol in the other ones; phenolic constituents were not detectable in these three extracts. Quantitative data for the most abundant lipophilic secondary metabolites derivatives is reported in Table 6.

The cytotoxic activity of the three fractions was determined using the Huh7 cells due to the lower observed IC_{50}. The methanolic extract showed the most potent cytotoxic effect in Huh7 cells with an IC_{50} of 7.2 ± 2.5 μg/mL, compared to 19.9 ± 1.5 and 38.8 ± 1.2 μg/mL for ethyl acetate and cyclohexane extracts, respectively (Table 7 and Figure 6). Finally, dichloromethane extracts showed a significant effect of cell viability in the Huh7 cell line only at concentrations of 0.1 mg/mL (Figure 6); however, the IC_{50} value could not be determined, since the extent of the effect was less than 50%. Thus, these preliminary data showed that for the Huh7 cells the whole phytocomplex is more cytotoxic than the fraction enriched in lipophilic constituents.

Table 6. Quantitative data on phytosterol in fractionated extracts of *G. diversifolia*, *for phytol, quantitative data were obtained by GC-MS.

Ion	Identification	Hexane Extract (mg/g)	Dichloromethane Extract (mg/g)	Ethyl Acetate Extract (mg/g)
$[M + H\text{-}H_2O]^+425$	Erythrodiol	0.35 ± 0.02	2.26 ± 0.04	3.38 ± 0.02
$[M + H\text{-}H_2O]^+425$	Uvaol	0.28 ± 0.02	1.46 ± 0.02	1.96 ± 0.04
$[M + H\text{-}H_2O]^+427$	Hydroxy cycloartenol	0.24 ± 0.05	5.22 ± 0.03	0.24 ± 0.02
$[M + H\text{-}H_2O]^+409$	Cycloartenol	2.61 ± 0.02	5.65 ± 0.07	4.52 ± 0.04
$[M + H\text{-}H_2O]^+395$	Fucosterol *	134.39 ± 0.10	165.27 ± 0.11	7.23 ± 0.07
$[M + H\text{-}H_2O]^+397$	γ-Sitosterol *	0.92 ± 0.10	230.77 ± 0.07	55.42 ±0.09
$[M + H\text{-}H_2O]^+381$	Brassicasterol *	29.14 ± 0.04	32.40 ± 0.09	5.78 ± 0.02
$[M + H\text{-}H_2O]^+429$	α-Tocopherylquinone	n.d.		
$[M + H\text{-}H_2O]^+383$	Campesterol	31.53 ± 0.07	29.66 ± 0.07	4.36 ±0.06
$[M + H\text{-}H_2O]^+397$	β-sitosterol *	417.52 ± 0.15	112.2 ± 0.07	0.20 ± 0.02
$[M + H\text{-}H_2O]^+395$	Stigmasterol	70.29 ± 0.21	50.72 ± 0.17	4.31 ± 0.01
$[M + H\text{-}H_2O]^+399$	Sitostanol *	6.11 ± 0.05	6.83 ± 0.03	0.06 ± 0.02

Table 7. Tests of extracts of *G. diversifolia* on 2008, BxPC3 and Huh7 cell lines (nd: not determined).

	IC_{50} (μg/mL)		
Extract	**2008**	**BxPC3**	**Huh7**
*G. diversifolia*MeOH	47.5 ± 4.1	12.5 ± 3.5	7.2 ± 2.5
G. diversifolia Ethyl acetate	nd	nd	19.9 ± 1.5
G. diversifolia Cyclohexane	nd	nd	38.8 ± 1.2
G. diversifolia Dichloromethane	nd	nd	Not Active

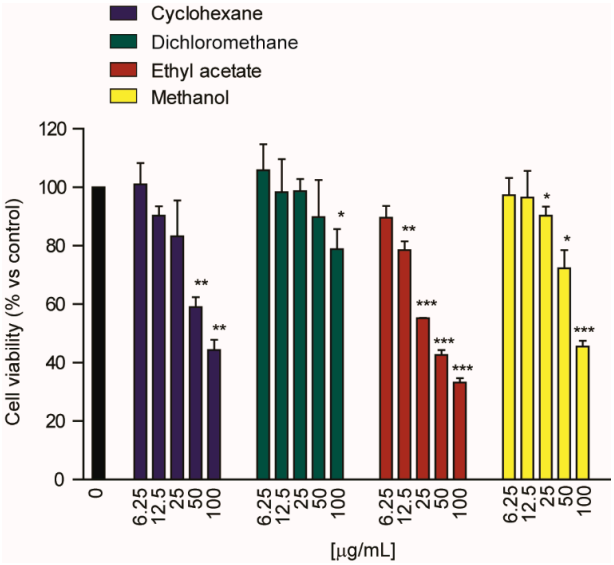

Figure 6. Cytotoxic effect of different solvent extracts of *G. diversifolia* on the Huh7 cell line. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Student's t-test.

2.4. Effect on LDLR Expression in Hepatoma Cell Line Huh7

To assess one possible mode of action in hepatocarcinoma cells, a test considering the ability of *G. diversifolia* extracts to induce the synthesis of LDLR in hepatocyte was performed. As shown in Figure 7, all different extracts of *G. diversifolia* showed a significant reduction of LDLR expression after 72 h of exposure. Considering the strong limitations of this in vitro analysis, it is conceivable to predict that *G. diversifolia* extract would not have significant lipid-lowering properties but may profoundly alter the intracellular cholesterol homeostasis. Tumor cells internalize cholesterol from circulating LDL

through the LDLR on the cell surface, and it has been hypothesized that disruption of LDLR leads to intertumoral cholesterol imbalance and improvement of chemotherapy efficiency [31]. In addition, LDL-R expression is significantly increased in hepatocarcinoma, whereas it is less expressed in the adjacent liver tissue [32]. This evidence indicates that the extracts of *G. diversifolia* may exert their cytotoxic effects by reducing the expression of LDLR. In addition, in hepatocarcinoma, the increased levels of LDLR have been associated with the downregulation of Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9), a new pivotal pharmacological target for controlling hypercholesterolemia [33]. Further studies will be required in order to address this hypothesis.

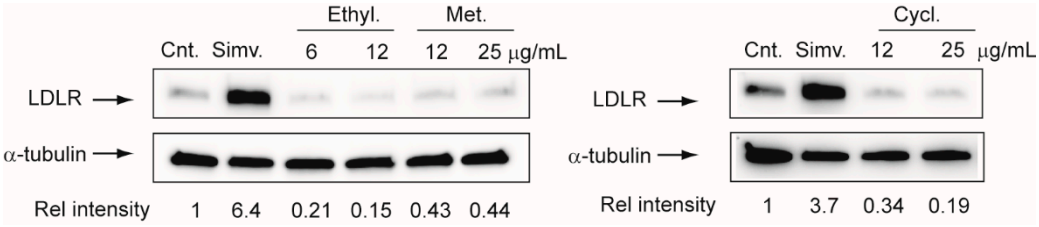

Figure 7. Effect of different solvent extracts of *G. diversifolia* on LDLR expression in Huh7 cell line.

Human hepatoma cells Huh7 were incubated for 72 h with indicated concentrations of *G. diversifolia* extracts, and LDLR reductase protein expression was evaluated by western blot analysis. α-Tubulin was used as a loading control. Densitometric readings were evaluated using the ImageLab™ software and expressed as relative intensity (Rel. Intensity) compared with control. Cnt.: Control; Simv. Simvastatin (5 µM); Ethyl.: Ethyl acetate; Met.: methanol; Cycl. Cyclohexane.

Fractionation of the extract with lipophilic solvent made it possible to obtain three fractions, but the total methanolic extract was the most active on Huh7 cells. The observed cytotoxic effect could be explained by the presence of phytosterols, such as β-sitosterol, γ-sitosterol, fucosterol and flavonoids. Previously published research showed that β-sitosterol exhibited significant ability on different cancer cell lines [34]. Furthermore, cytotoxic and proapoptotic properties of β-sitosterol were observed against leukaeima cells [35]. As previously reported, fucosterol extracted from Sargassum angustifolium marine algae showed cytotoxicity on colon carcinoma, colorectal adenocarcinoma and breast ductal carcinoma cell lines [36]. However, phenolic compounds also contribute to the toxicity. Recently, vitexin-2-O-xyloside's antiproliferative properties have been studied, and this compound presented toxicity against CaCo2 cells and its activity was increased by coadministration of betalains [36]. However, considering the complex chemical nature of Himalayan nettle extract, further experiments are needed to purify and test specific compounds and fractions in order to deeply investigate the potential cytotoxic effect and the possible mode of action.

3. Discussion

G. diversifolia methanolic extract contains a high amount of phytol and phytosterols while phenolics, carotenoids, saponins are in a limited amount. Furthermore, the plant contains vitamin C and vitamin B2. The structures of the most abundant constituents are reported in Figure 4. The overall phytochemical composition suggests that the phytocomplex may be useful in the preparation of nutraceuticals. In particular, the results obtained in the chemical bioassays showed significant inhibition of cholinesterase. A previous paper indicated that phytosterols can have inhibitory properties on AChE and BuChE [24,29,37,38] and phytol has also been studied in this regard [24]. Previous published literature showed that phenolic constituents can act as inhibitors of these enzymes [39], thus suggesting that multiple compounds in the phytocomplex of *G. diversifolia* may be the responsible for the observed activity. Regarding the tyrosinase activity, literature data indicate that the phytosterol fraction indicated as oryzanol has significant inhibitory properties on this enzyme [30].

Regarding the antioxidant properties, the *G. diversifolia* methanolic extract exhibited moderate effects as revealed by different assays. Similar to enzyme inhibitory results, the observed antioxidant

properties may be linked to non-phenolic antioxidants such as phytosterols, vitamin C, phytol and carotenoids. In some recent papers, important antioxidant mechanisms of non-phenolic compounds have been reported [38,40,41] and this is in accordance with our approach. Metal chelating ability reflects the inhibition of production of free radicals in the Fenton reaction and some non-phenolic compounds such as ascorbic acid, polysaccharides and peptides [42,43] have this ability.

The *G. diversifolia* methanolic extracts were tested for cytotoxicity against three tumor cell lines, and the most potent cytotoxic effect was observed in Huh7 cells (IC_{50} of 7.2 ± 2.5 μg/mL). Extract fractionation reduced the cytotoxicity, thus indicating that the whole mixture presented a higher effect than the obtained fractions. Considering the phytochemical composition of the methanol extract, the cytotoxic activity can be ascribed to the overall phytocomplex, and in fact, a previous published paper indicates cytotoxic activity for phytosterols, fucosterol and flavonoids [34–36]. These different compounds may act by different modes of action. In this regard, our study considered a possible mode of action; in fact we observed a significant reduction of LDLR expression after 72 h of exposure and this effect can profoundly alter intracellular cholesterol homeostasis. Thus, the LDLR reduction can be considered as a possible mode of action to explain the observed cytotoxic effect on hepatocarcinoma cell lines.

The overall results make it possible to have a deeper knowledge of the phytochemical composition of Himalayan nettle, and show its usefulness for cosmetic, pharmaceutical and nutraceutical applications.

4. Materials and Methods

4.1. Plant Material

Shoot tips of *Girardinia diversifolia* were collected from SindhuliGadi, Sindhuli, elevation range 1332–1394, GPS coordinates 27° 16′ 43″ N and 85° 57′ 31″ E, central part of Nepal. Plant materials were identified and authenticated with the KATH herbarium, Godawari, Lalitpur, Nepal.

The herbarium specimens were prepared following standard herbarium techniques (Bridsonand and Forman, 1992). The plants were dried carefully by proper pressing and the herbarium was prepared after mounting on the herbarium sheets. They were crosschecked with the herbarium, deposited at National Herbarium and Plant Laboratories, Godavari, Lalitpur (KATH), and submitted to TUCH (Tribhuvan University Central Herbarium), Kirtipur. Ethnobotanical information was collected from the literature. The working samples were collected and dried after making into small pieces. The sample was air dried over 2–3 weeks inside the room. The plant was grinded into fine powder with the help of an electric grinder.

4.2. Extraction

The dried powder of plant material was subjected to extraction using methanol through percolation with intermittent sonication. An quantity of 10 g of plant powder was extracted three times with 100 mL of methanol so that the ratio of solvent in volume (mL) to the weight (g) of plant material would be 10:1. The solution was then subjected to intermittent sonication for 2 h, i.e., continuous cycle of sonication at 30 kHz for 30 min (with 10 min interruption). After the completion of the cycle, the solution was filtered with Whatman no.1 filter paper (Whatman Ltd., Kent, UK) and the filtrate was then subjected to evaporation at reduced pressure in a rotary evaporator (IKA RV 10, Milan, Italy). The concentrated extract thus obtained was transferred to clean, dried and weighed glass vials. The obtained extract was indicated as a methanol extract, and yield was 14%. The resulting dried extract was then sealed and stored at 4 °C until use. For the fractionation of the total methanol extract, the dried residue was dissolved in a mixture of 9:2 water: methanol and partitioned with cyclohexane, then dichloromethane then ethyl acetate. Organic solvents were collected and dried with sodium sulphate and then dried in rotavapor. Yields were 18%, 8%, 6% for cyclohexane, dichloromethane and ethyl acetate respectively.

4.3. HPLC-DAD-APCI-MS of Phytosterols, HPLC-DAD-APCI for Parotenoids, and HPLC-DAD-ESI-MS for Phenolic and Saponins

The extracts were analyzed using different methods. The instrumentation was an Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with a 1260 diode array detector (DAD), and a Varian MS-500 ion trap mass spectrometer equipped with ESI and APCI ion sources. At the end of the column, one "T" splitter separated the flow rate: half of the liquid was split to DAD and half to Agilent/Varian MS-500 ion trap mass spectrometer. UV-Vis spectra were acquired in the range of 190–400 nm.

MS spectra were recorded using ESI in positive and negative ion mode, and APCI in positive mode. A turbo data-dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species.

To specifically analyze phytosterols and terpene alcohols in *G. diversifolia*, ethyl acetate was used as a solvent and 100 mg of plant material was extracted using 30 mL of solvent. The liquid was filtered and the solvent was concentrated, and the volume was adjusted to 5 mL.

For the terpene and phytosterol constituents, an Agilent ZORBAX Eclipse XDB-C18 column (3.0 mm x 150 mm, 3.5 μm) was used as stationary phase. The mobile phase was water (0.1% formic acid) (A), acetonitrile (B) and methanol (C). A gradient program was used as follows: [0 → 8th min: A:B:C (2:95:3) → A:B:C (2:95:3) 8 → 10th min: A:B:C (2:95:3) → A:B:C (2:0:98) 10 → 28th min: A:B:C (2:0:98) → A:B:C (2:0:98) 28 → 30th min: A:B:C (2:0:98) → A:B:C (2:95:3) 30 → 35th min: A:B:C (2:95:3) → A:B:C (2:95:3)]. The flow rate was 0.4 mL/min, injection volume was 10 μL and the temperature was set at 30 °C. MS spectra were recorded in the range of *m*/*z* 100–2000, using an APCI ion source operating in positive ion mode. A turbo data-dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species. Identification of compounds was obtained based on comparison with the literature and reference compounds, when available. As standards, known solutions of β-sitosterol (176 μg/mL) and stigmasterol (185.6 μg/mL) were used.

Compound quantification was obtained with the method of calibration curve: β-sitosterol was used as the external standard for β-sitosterol and γ-sitosterol quantification, while stigmasterol was used for fucosterol. Calibration curves were as follows: $y = 0.63x + 0.2705$ ($R^2 = 0.99611$) for β-sitosterol; $y = 2.1153x - 15.216$ ($R^2 = 0.9949$) for stigmasterol.

For the analysis of polar constituents, such as polyphenol and saponins, Synergi Polar-RP (Phenomenex, Italy Bologna) was used as the stationary phase (3.0 × 150 mm; 4 micron); water, and 1% formic acid (A) and methanol (B), were used as eluents. The gradient began using 95% of solvent A and went to 25% of A in 15 min, then 0% A in 20 min. The flow rate was 0.4 mL/min. DAD chromatograms and UV-VIS spectra were acquired in the range of 200–650 nm. MS spectra were recorded in negative or in positive ion mode in the 50–2000 Da range, using an ESI ion source. Quantification of compound was obtained using DAD for hydroxycinnamic acid derivatives (chlorogenic, caffeic, syringic) chlorogenic acid solution was used (100–1 μg/mL) as a reference standard and the used wavelength was 330 nm. The calibration curve was $Y = 1.325x + 0.236$ ($R^2 = 0.9989$). For flavonols, vitexin solutions (20–1 μg/mL) were used at 350 nm and the calibration curve was $Y = 12.3x + 0.325$. For saponins, escin was used as a reference compound and a 205 nm wavelength was used (100–10 μg/mL) $Y = 0.325x + 0.023$ ($R^2 = 0.9986$).

For the analysis of carotenoids, a YMC carotenoid column (4.6 × 300 mm, 5 micron) was used as the stationary phase. Mobile phases were methylterbuthyl ether/methanol 90/10 (A) and methanol (B). The gradient started with 10% A and in 12 min reached 100% A. The flow rate was 1.3 mL/min. β-Carotene and zeaxanthin were used as a reference, preparing solution in the range 40–0.5 μg/mL. For the acquisition of MS spectra, APCI was used in positive ion mode, acquiring spectra in the *m*/*z* range of 365-1000 amu in TDDS ion mode.

4.4. GC-MS Analysis

For GC-MS analysis of the fatty acid content, a sample of *G. diversifolia* methanolic extract was derivatized with MeOH in presence of H_2SO_4, leading to the esterification of fatty acids to fatty acid

methyl esters, which offer excellent stability for GC analysis. A quantity of 139.1 mg of the extract was added, with 15 mL of MeOH, 1 mL of CH_2Cl_2, 3 drops of H_2SO_4 and 25.7 mg of methylpentadecanoate (Sigma Aldrich, St. Louis, MO, USA), used as internal standard. The mixture was heated under reflux for 1 hour then cooled in an ice bath. A liquid–liquid partition was performed with 10 mL of water and 5 mL of diethyl ether, then the organic phase was collected and dried. The residue was re-dissolved with 1.5 mL of diethyl ether and put in vial. GC-MS analysis was performed through an Agilent 7820A coupled with a single quadrupole mass spectrometer Agilent 5977B MSD, using a HP88 (60 m × 0.25 mm, 0.2 μm film thickness) stationary phase. Helium was the carrier gas, with a column head pressure of 14.1 psi. The flow rate through the column was 1.19 mL/min. The injector was set at 300 °C with a split ratio of 20:1, the split flow was 23.9 mL/min and 1 μL injections were made. The temperature gradient started with a 120 °C initial temperature with a linear increase to 240 °C at 3 °C/min. The total run time was 55 min. MS spectra were recorded in the range of *m*/*z* 40–650, using an EI ion source operating in positive ion mode.

4.5. Isolation of Phytoconstituents

Part of the methanolic extract (18 g) was separated through a silica gel column chromatography, using 1 L of a mixture of dichloromethane and methanol at 95:5, then 500 mL of 90:10, 500 mL at 80:20, 500 mL at 50:50, and finally 500 mL with 100% methanol as the mobile phase. Different fractions were obtained and analyzed by TLC (5% MeOH in CH_2Cl_2) and pooled on the basis of chromatographic behavior obtaining 85 fractions.

Fractions 11–13(250 mg) and 14–16 (450 mg) presented significant spots, so they were pooled (750 mg) and separated through silica gel column chromatography, using 2 L of dichloromethane. Obtained fractions were analyzed through TLC, and the ones presenting similar behavior were collected and dried. Further purification was performed by preparative TLC (silica gel 60 F-254). In this way, two fractions were obtained and further purified by preparative TLC, allowing the isolation of β-sitosterol (32 mg) and γ-sitosterol (11 mg) respectively, which were identified on the basis of their NMR and MS measurements.

4.6. Bioassays

To obtain the total level of phenolic in the extract, colorimetric assay was used as described in our previous paper [44]. Gallic acid (GAE) was used as one standard, and the result was expressed as equivalent of gallic acid (mg GAE/g extract).

The antioxidant potential of the extracts was evaluated by phosphomolybdenum, antiradical (DPPH and ABTS), reducing power (FRAP and CUPRAC) and ferrous chelating assays as described [44]. Trolox equivalents were used for the expression of antioxidant activities. EDTA was employed as a reference compound for the metal chelating assay. The key enzymes inhibition activity of the extracts against AChE ((E.C. 3.1.1.7), from *Electrophorus electricus* (electric eel), Sigma-Aldrich, Darmstadt, Germany), BuChE ((E.C. 3.1.1.8), from equine serum, Sigma-Aldrich), tyrosinase ((E.C.1.14.18.1), from mushroom, Sigma-Aldrich), α-glucosidase ((E.C. 3.2.1.20), from *Saccharomyces cerevisiae*, Sigma-Aldrich) and α-amylase ((E.C. 3.2.1.1), from porcine pancreas, Sigma-Aldrich) were measured using the protocols previously reported [44]. Galanthamine (GALAE, for cholinesterases), kojic acid (KAE, for tyrosinase) and acarbose (ACAE, for amylase and glucosidase) were used as standard inhibitors in the enzyme assays.

4.7. Cell Cultures

A human pancreatic (BxPC3) carcinoma cell line was obtained by American Type Culture Collection (ATCC, RocKville, MD, USA). A total of 2008 human ovarian carcinoma cells were kindly provided by Prof. A. Marverti (Dept. of Biomedical Science of Modena University, Modena, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using a

RPMI-1640 cell culture medium containing 10% foetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin) and 2 mM l-glutamine.

Human hepatic cancer cell line Huh7 was cultured in MEM supplemented with 10% FCS, L-glutamine, sodium-pyruvate and non-essential amino acids, and with penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, the cells were seeded in MEM/10%FCS and then the treatments were continued for an additional 72 h. All *G. diversifolia* extracts were dissolved in DMSO. The final concentration of DMSO did not exceed 0.25% *v*/*v* and the same amount of solvent was added at all experimental points.

4.8. Cell Viability Assay

The growth inhibitory effect towards pancreatic and ovarian tumor cell lines was evaluated by means of MTT assay, as previously described. IC_{50} values were calculated by a four-parameter logistic (4-PL) model.

For the hepatic cells, Sulphorhodamine B (SRB) assay was performed to assess the cell viability after treatment. A total of 8000 cells/well were seeded in a 96-well tray in triplicate [45]. After 24 h of incubation, the cells were treated with different concentrations of compounds. SRB assays were performed after 48 h as previously described [46]. Briefly, the cells were fixed by means of protein precipitation with 50% trichloroacetic acid at 4 °C (50 μL per well, final concentration 10%) for 1 h. After five washing with tap water, the cells were stained for 15 min with 0.4% SRB dissolved in 1% acetic acid (50 μL per well) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried, and bound protein stain was solubilized with 150 μL 10 mmol/L unbuffered Tris base. The optical density was read at 540 nm. Data were expressed as mean ± SD of quadruplicate values of relative absorbance compared to control.

4.9. Western Blot Analysis

Cells were washed twice with PBS and lysed with a solution of 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet-P40, containing protease and phosphatase inhibitor cocktails (SIGMA, Milan, Italy) for 30 min. on ice. Twenty μg of proteins and a molecular mass marker (Thermo Scientific, Monza, Italy) were separated on 4%–12% SDS-PAGE (BIO-RAD, Segrate (MI) Italy) under denaturing and reducing conditions [47]. Proteins were then transferred to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (BIO-RAD). Membranes were washed with Tris-buffered saline-Tween 20 (TBS-T, Milan, Italy), and nonspecific binding sites were blocked in TBS-T containing 5% nonfat dried milk for 60 min at room temperature. Blots were incubated overnight at 4 °C with a diluted solution (5% nonfat dried milk) of the following human primary antibodies: anti LDLR (mouse monoclonal antibody, Millipore clone 2H7.1; dilution 1:1000), and anti-α-tubulin (mouse monoclonal antibody, Sigma clone DM1A; dilution 1:2000). Membranes were washed with TBS-T and then exposed for 90 min at room temperature to a diluted solution (5% nonfat dried milk) of the secondary antibodies (peroxidase-conjugate goat anti-rabbit, and anti-mouse, Jackson ImmunoResearch). Immunoreactive bands were detected by exposing the membranes to Clarity™Western ECL chemiluminescent substrates (Bio-Rad) for 5 min, and images were acquired with an Azure c400 Imaging System (Aurogene**)**. Densitometric readings were evaluated using the ImageLab™ software (Bio-Rad, Image Lab Software 6.0.1).

4.10. Statistical Analysis

Statistical analysis was performed using the Prism statistical analysis package, version 5.01 (GraphPad Software, San Diego, CA, USA). p values were determined by Student's t test. A probability value of $p < 0.05$ was considered statistically significant.

5. Conclusions

Girardinia diversifolia is a Himalayan nettle employed in traditional medicine for the treatment of several diseases as well as to make fiber. This study was designed to evaluate this plant for its potential pharmaceutical, cosmetic and nutraceutical uses. The methanolic extract presented several activities. Anti-cholinesterase and anti-tyrosinase activities can be mainly related to phytosterols, phytol, and oryzanol, as well as to some of the phenolic constituents. Moderate antioxidant effects are observed, probably due to the low abundance of phenolics and carotenoids, known as highly active antioxidant compounds. The metal chelating activity was significant, showing potential usefulness in the protection of metal based red/ox reactions. An in vitro test of total methanol extract showed a significant cytotoxic effect on BxPC3 and Huh7 cells. *G. diversifolia* extracts were able to reduce the expression of LDLR, and this may explain, in part, their cytotoxic effects on this cell line. Thus, Himalayan nettle can be considered as a valuable source of extracts rich in phytosterols, hydroxycinnamic acids and with potential bioactivities; nevertheless, further studies are needed to assess its efficacy and safety for the development of pharmaceutical, cosmetical or nutraceutical ingredients.

Supplementary Materials: File with information on plant material is included in supplementary.

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FULL PAPER

CHEMISTRY & BIODIVERSITY

Phytochemical Investigations and *In Vitro* Bioactivity Screening on *Melia Azedarach* L. Leaves Extract from Nepal

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Melia azedarach is a common tree used in the traditional medicine of Nepal. In this work, leaves were considered as source of bioactive constituents and composition of methanol extract was evaluated and compared with starting plant material. Flavonoid glycosides and limonoids were identified and quantified by HPLC-DAD-MS^n approaches in dried leaves and methanolic extract, while HPLC-APCI-MS^n and GC/MS analysis were used to study phytosterol and lipid compositions. β-Sitosterol and rutin were the most abundant constituents. HPLC-APCI-MS^n and HPLC-DAD-MS^n analysis revealed high levels of phytosterols and flavonoids in methanolic extract accounting 9.6 and 7.5% on the dried weight, respectively. On the other hand, HPLC/MS^n data revealed that limonoid constituents were in minor amount in the extract <0.1%, compared with leaves (0.7%) indicating that degradation occurred during extraction or concentration procedures. The methanol extract was subjected to different bioassays, and antioxidant activity was evaluated. Limited inhibitory activity on acetyl and butyryl cholinesterase, as well as on amylase were detected. Moreover, tyrosinase inhibition was significant resulting in 131.57 ± 0.51 mg kojic acid equivalents/g of dried methanol extract, suggesting possible use of this *M. azedarach* extract in skin hyperpigmentation conditions. Moderate cytotoxic activity, with IC_{50} of 26.4 μg/mL was observed against human ovarian cancer cell lines (2008 cells). Our findings indicate that the Nepalese *M. azedarach* leaves can be considered as valuable starting material for the extraction of phenolics and phytosterols, yielding extracts with possible cosmetic and pharmaceutical applications.

Keywords: *Melia azedarach*, polyphenols, phytosterols, traditional Nepalese medicine, enzyme inhibitory assays.

Introduction

Melia azedarach (Meliaceae) is a common tree, known as 'Bakaino', generally used as traditional medicine and firewood for cooking purpose in Nepal. Wood part is mostly used for furniture and other household items.[1] It is a fast growing plant and trees are 7–12 m in height. It is found in mixed evergreen broad-leaved, deciduous forests, field margins and roadsides ranging altitude 500–2100 m. This species is cultivated in many warm-temperate and tropical parts of the world. Because of its extensive cultivation and tendency to become naturalized in disturbed habitats, its original wild distribution is uncertain. Different plant parts are used in Nepal for medicinal purpose and in other countries. Seed powder is used to treat diabetes and gastrointestinal diseases and bark decoction to treat diarrhea.[2] Fruits are used to treat diarrhea, dysentery, intestinal worm,[3] spleen disorder.[4] Fruits juice mixed with oil are anthelmintic,[5] and are used to treat vomit, blood impurities and urinary discharge.[6] The leaves are used to treat skin disease as traditional practice, to control insect, mite and nematode pests, for gingivitis

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mouthwash, diabetes, fever and stomach ache.[7] In Nepalese communities leaves are used as fodder.[8]

In the proceeding of our studies on Nepalese medicinal plants,[9,10] we considered *M. azedarach* leaves due to the use in the traditional medicine in the rural as well as urban parts of Nepal. Leaves can be a valuable source to extract bioactive compounds because this material is easily available and multiple collections can be obtained during the year.

The chemical composition of *M. azedarach* is highly complex and have been subject to several investigations aimed to evaluate the presence of bioactive constituents.[7,11–17] Previous article revealed that leaves contains phenylpropanoids and flavonoids like myricetin, quercetin, kaempferol and isorhamnetin derivatives.[13] Previous studies on the genus *Melia* have led to the isolation of a variety of structurally diverse compounds including triterpenoids, steroids, and limonoids. Limonoids have been mainly isolated from fruits and bark, showing the presence of highly diverse structures and broad range of bioactivities.[17–21] In this regard the information about the leaves composition of Nepalese *M. azedarach* are still scarce and the evaluation of the potential usefulness of such material as extraction source of bioactive constituents is still missing.

The aim of this study is the evaluation of *M. azedarach* leaves as potential source of extractable phytoconstituents. Exploration of the phytochemicals that can be extracted from leaves was performed combining the data obtained with different analytical techniques. 1D and 2D NMR analysis were used for preliminary study of chemical composition. Flavonoid glycosides and limonoids were identified and quantified by HPLC-DAD-MS^n approaches, while HPLC-APCI-MS^n and GC/MS analysis were used to study phytosterol and lipid composition. The composition of methanolic extract was compared with the dried leaves. Finally, *in vitro* bioassays were performed on the extract focusing on antioxidants and enzyme inhibition tests. Preliminary cytotoxicity study was also carried out on a human pancreatic (BxPC3) carcinoma and human ovarian (2008) cancer cells.

Results and Discussion

NMR Screening of M. Azedarach *Leaves*

NMR techniques were used to investigate the presence of different classes of constituents in *M. azedarach* leaves. Few milligrams of dried leaves powder were subjected to sequential extraction with deuterated chloroform and deuterated methanol.

In the chloroform soluble fraction intense signals were detected in the aliphatic region of the spectrum (δ 0.5 – 2.0), ascribable to terminal methyl groups, (δ 0.89–0.91) aliphatic CH_2 of the fatty acid chains (δ 1.25–1.28) and triplets ascribable to CH_2 nearby double bond (δ 1.60) and nearby carbonyl function (δ 2.30), supporting the presence of lipids. Combination of 1D and 2D homonuclear and heteronuclear experiments allowed the observation of diagnostic signals ascribable to limonoids. In the spectrum region δ 6.0–7.5, signals in the ^{1}H and HSQC-DEPT could be ascribed to furan ring namely the CH at δ 7.34 (δc 144.8), 7.28 (δc 138.8) and 6.23 (δ 110.0) suggesting the presence of limonoid derivatives[22] (*Figure 1*).

^{1}H-NMR analysis of the methanol soluble fraction revealed limited number of signals ascribable to limonoid derivatives.[22] Furthermore, other signals indicate the presence of flavonoids, namely the doublets at δ 6.16–6.35, that correlate with aromatic CH at δ 93–98 ppm in HSQC-DEPT that could be assigned to the H-6/8 of flavonoids; the signal at δ 6.85, correlating with δ 99.1, that could be assigned to H-5 ring A of flavonoid derivative. The methanol soluble fraction showed the presence of numerous signals in the sugar regions supporting the presence of monosaccharides, oligosaccharides, or glycoside residue linked to the flavonoids. Other constituents could be detected in the methanol soluble fraction, in the aliphatic region of the spectrum singlet at δ 2.50 could be ascribed to succinic acid, while singlet at δ 1.44 could support the presence of alanine. Rhamnose was detected by the doublet at δ 1.33 and the singlet at δ 5.07, while *alpha* and *beta* glucose were detected based on the anomeric protons at δ 4.47 and 5.07.

On the basis of the preliminary results the leaves appear to contain lipids, limonoids, phenolics and sugars. Methanol extract was prepared from 50 grams of dried leaves using maceration, and different type of chemical analysis were performed to investigate this extract as source of bioactive constituents. Methanolic extract composition was compared with dried leaves.

Lipids and Phytosterol Constituents in the Leaves and in the Methanol Extract

Total lipid contents in leaves was 1.77% on dried weight. The analysis of fatty alcohols, fatty acids and lipophilic compounds were performed by GC/MS and

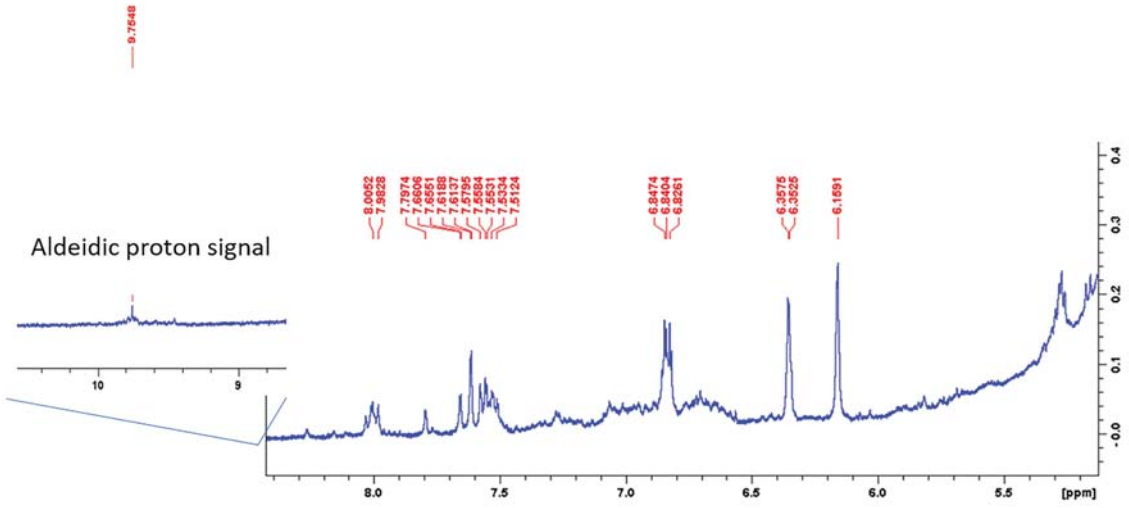

Figure 1. [1]H-NMR spectrum of *M. azedarach* chloroform soluble fraction with enlargement of spectrum region presenting aldehydic proton signals.

methanolic extract composition was compared with dried leaves (*Table 1*).

The lipid constituents in leaves were mostly palmitic, linolenic, and linoleic acids accounting for 19, 15 and 4.5% of total lipophilic constituents. In previous article, leaves *M. azedarach* lipid constituents from Turkey were measured and large amount of linolenic acid (35%), linoleic acid (20%) and palmitic acid (26%) were observed.[23] This indicate that different geographic origins of plant material can influence the fatty acid composition. Nepalese leaves are source of unsaturated lipids, accounting 8.1% of monounsaturated fatty acid of total lipophilic compounds and 21.0% of polyunsaturated fatty acid. Nepalese leaves are source of different classes of unsaturated lipids including ω-3 represented by linolenic acid (15% of total lipophilic constituents), ω-6 by linoleic and linolealidic acids (5.8%) and ω-9 by gonodic and erucic acid (4.9%). Unusual fatty acid, heneicosanoic acid, with 21 carbon atom was detected in significant amount (4.35% of total lipophilic compounds). Previous article reported significant levels of such constituents in hexane extract of leaves.[24]

Long chain fatty alcohols as hexacosanol, heptacosanol, tetracosanol and 2-methylhexadecan-1-ol were identified, usually present in cuticular waxes of vegetal material. Also, significant amount of the diterpene alcohol phytol was revealed. The analysis of the methanol extract revealed that composition remains unchanged from dried leaves, and the extract concentrated the lipophilic compounds from 4 to 6 times compared to the amount detected in leaves.

Due to the high abundance of lipids in leaves and due to our objective to use this plant material as source of valuable phytoconstituents with potential interest in pharmaceutical and nutraceutical applications, analysis for the phytosterol contents were performed using LC-APCI-MS method.[9]

Total amount of phytosterols was 1.3% in dried leaves of *M. azedarach* (*Table 2*) with β-sitosterol being the larger constituent, accounting 43% of total phytosterols. Minor constituents were fucostanol and campesterol and stigmasterol. A general concentration of such compounds were observed in the extract comparing with dried leaves, with 7.6 fold of β-sitosterol and 7 fold of total phytosterols. This result indicate that methanol is efficient for phytosterol extraction from *M. azedarach* leaves.

Phenolic and Limonoid Constituents

M. azedarach leaves and methanolic extract were analyzed by LC-DAD-MS^n. DAD detector showed the presence of many peaks that present the UV/VIS spectrum ascribable to phenolic and flavonoid. The evaluation of the UV data, MS, and fragmentation as well as the comparison with reference compounds allowed to annotate 20 different derivatives comprising simple phenolic acids, hydroxycinnamic derivatives and flavonoids (*Figure 2*).

Table 1. Fatty acid, fatty alcohols composition of dried leaves (extracted with hexane) and the methanolic extract of *M. azedarach* obtained by GC/MS. Results are expressed as mean ± standard deviation ($n = 4$). t_R: retention time.

t_R	Compounds	Dried leaves (mg/g)	Methanol extract (mg/g)
15.1	Capric acid methyl ester	0.172 ± 0.002	0.53 ± 0.02
15.6	4-Tetradecyne	0.063 ± 0.002	0.30 ± 0.02
16.2	8-Hexadecene	0.081 ± 0.002	0.36 ± 0.03
17.2	Phytadiene	0.051 ± 0.002	0.20 ± 0.02
19.3	γ-n-Amylbutyrolactone	0.037 ± 0.002	0.17 ± 0.02
19.8	Methyl myristate	0.111 ± 0.002	0.43 ± 0.02
23.0	Methyl myristoleate	0.183 ± 0.002	0.935 ± 0.002
24.5	Methyl palmitate	2.696 ± 0.002	11.4 ± 0.20
25.4	Methyl heptanoate	0.038 ± 0.002	0.18 ± 0.02
25.8	Methyl palmitoleate	0.170 ± 0.002	0.87 ± 0.03
26.8	Methyl heptadecanoate	0.080 ± 0.002	0.22 ± 0.02
29.1	Methyl stearate	0.352 ± 0.002	1.68 ± 0.10
30.2	Methyl oleate	0.083 ± 0.002	0.46 ± 0.02
31.2	Methyl linoleate	0.632 ± 0.002	2.72 ± 0.01
33.2	Methyl linolealidate	0.184 ± 0.002	0.91 ± 0.02
33.9	Methyl linolenate	2.127 ± 0.002	8.21 ± 0.02
36.2	Phytol	0.346 ± 0.002	1.84 ± 0.03
37	Methyl arachidate	0.394 ± 0.002	1.23 ± 0.02
38	Hexacosanol	0.149 ± 0.002	0.71 ± 0.03
38.9	Methyl gondonate (cis-11-eicoseonic acid methyl ester)	0.410 ± 0.002	1.930 ± 0.02
39.9	Heptacosanol	1.809 ± 0.002	8.19 ± 0.12
40.7	Methyl heneicosanoate	0.614 ± 0.002	0.614 ± 0.02
41.6	Tetracosanol	0.700 ± 0.002	2.93 ± 0.02
42.6	Methyl erucate (methyl docosenoate)	0.280 ± 0.002	1.213 ± 0.02
43.9	2-Methylhexadecan-1-ol	1.865 ± 0.002	9.21 ± 0.04
44	Methyl tricosanoate	0.277 ± 0.002	1.326 ± 0.04
	Total lipophilic constituent	13.9	76.5

Table 2. Amount of phytosterols of dried leaves and the methanolic extract of *M. azedarach*. Results are expressed as mean ± standard deviation ($n = 4$).

Compound	$[M+H-H_2O]^+$	Dried leaves % w/w	Methanol extract % w/w
Hydroxycycloartanol	427	0.10 ± 0.02	0.81 ± 0.02
Cycloartanol	409	0.01 ± 0.02	0.07 ± 0.02
Fucostanol	395	0.29 ± 0.02	1.71 ± 0.02
Stigmasterol	395	0.13 ± 0.02	1.15 ± 0.02
β-Sitosterol	397	0.56 ± 0.02	4.26 ± 0.02
Campesterol	383	0.18 ± 0.02	1.37 ± 0.02
Sitostanol	399	0.03 ± 0.02	0.22 ± 0.02
Total amount		1.30	9.59

The most abundant compounds in dried leaves sample were rutin, quercetin-3-*O*-glucoside and kaempferol-7-*O*-rutinoside (*Table 3*). Similar qualitative pattern of phenolic were observed comparing dried leaves and extract composition, while the amount of phenolic in the extract was increased nearly eight time higher than the dried leaves.

The LC/MS analysis in positive ion mode allowed to observe a series of peaks that were not significantly detected by DAD. These peaks could be ascribed to limonoids observing the MS and fragmentation pathways. *Figure 3* represent the chromatogram recorded in positive ion mode (intensity of base peak) showing the corresponding *m/z* values for each peak.

Reference compounds related to *Melia* limonoids are not commercially available, but in previous article some authors[25] reported general rules for the assignments of limonoid structures on the basis of MS data. Detected compounds were annotated and tentatively assigned.

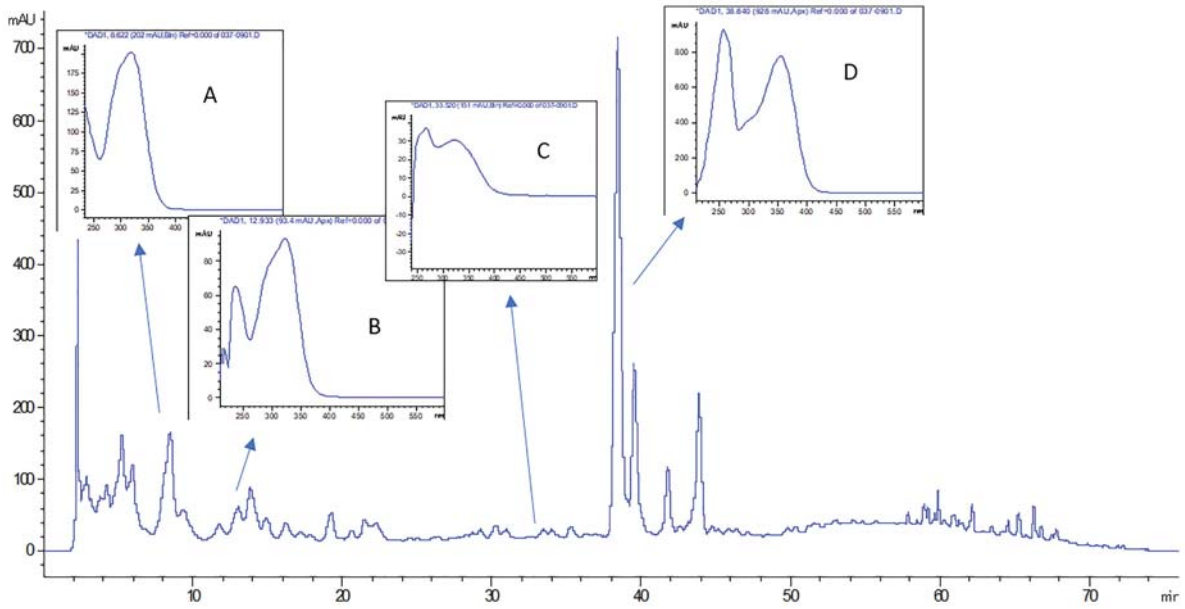

Figure 2. HPLC chromatogram (280 nm) of *M. azedarach* leaves methanol extract. Principal peaks with respective UV spectrum are A), B) 5-hydroxyferuloyl derivative; C) flavonoid derivative and D) rutin.

Table 3. Phenolic constituents in *M. azedarach* dried leaves and methanol extract. Results are expressed as mean ± standard deviation ($n = 4$). t_R: retention time, *comparison with reference standard.

t_R	UV_{max} (nm)	$[M-H]^-$	Fragmentation	Annotation	Dried leaves (mg/g)	Methanol extract (mg/g)
5.7	265	315	153, 121	Protocatechuic acid hexoside	0.04 ± 0.01	0.10 ± 0.01
5.1	321	371	209, 191	Hydroxyferuloylglucoside isomer 1	0.18 ± 0.01	0.03 ± 0.01
8.5	321	371	209, 191	Hydroxyferuloylglucoside isomer 2	0.06 ± 0.01	0.09 ± 0.01
8.5	280	355	173, 191 (100)	Dihydroxy caffeic quinic acid isomer 1	0.04 ± 0.01	0.04 ± 0.01
9.5	280	355	174, 191 (100)	Dihydroxy caffeic quinic acid isomer 2	0.02 ± 0.01	0.02 ± 0.01
13.5	321	355	209, 191, 173	Hydroxyferuloyldeoxyhexoside	0.03 ± 0.01	0.11 ± 0.01
14.2	325	499	337, 191	*p*-Coumaroyl, caffeoyl quinic acid	0.04 ± 0.01	0.04 ± 0.01
13.1	280	385	147, 173, 191 (100), 323, 348	Hydroxydihydroferuloylquinic acid isomer 1	0.04 ± 0.01	0.11 ± 0.01
14.1	280	385	148, 173, 191 (100), 323, 348	Hydroxydihydroferuloylquinic acid isomer 2	0.02 ± 0.01	0.15 ± 0.01
19.3	321	385	173, 191 (100), 209, 248, 327, 338	Hydroxyferuloylmethylhexoside	0.04 ± 0.01	0.15 ± 0.01
32.6	350	625	301	Quercetin di-*O*-hexoside	0.04 ± 0.01	0.16 ± 0.01
34.1	350	625	463, 301	Quercetin-*O*-hexoside-*O*-hexoside	0.08 ± 0.01	0.31 ± 0.01
33.6	350	771	609, 463, 301	Quercetin dihexoside deoxyhexoside	0.09 ± 0.01	1.47 ± 0.01
35.4	350	755	609, 447, 301	Quercetin trideoxyhexoside	0.07 ± 0.01	0.71 ± 0.01
38.5	350	609	301	Rutin*	4.88 ± 0.01	37.87 ± 0.01
40	350	463	301	Quercetin hexoside	1.61 ± 0.01	8.88 ± 0.01
42.2	340	593	285, 284	Kaempferol-3-*O*-rutinoside*	0.81 ± 0.01	2.20 ± 0.01
43.8	340	593	285	Keampferol-7-*O*-rutinoside*	1.34 ± 0.01	3.71 ± 0.01
43.4	350	505	463, 301	Quercetin acetyl hexoside	0.43 ± 0.01	0.99 ± 0.01
44.8	340	447	357, 327, 284, 255	Kaempferol-*C*-hexoside	0.08 ± 0.01	0.16 ± 0.01
51.5	350	301	151, 179	Quercetin*	0.02 ± 0.01	0.30 ± 0.01
				Total phenolic amount	9.94	75.70

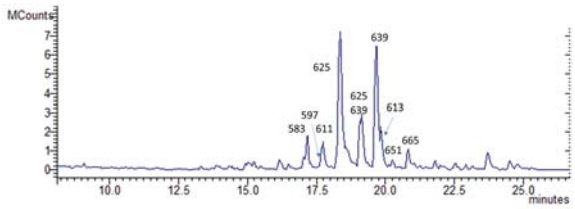

Figure 3. LC/MS of the leaves methanol extract showing the peaks and corresponding *m/z* values ascribable to limonoids.

Peak at 17.7 min presents $[M+H]^+$ at *m/z* 597 and was assigned to salannin. Characteristic fragments were observed at *m/z* 565 (−32 methanol), 537 (−60 acetic acid), 497 (−100 tiglate group), 437 (−60, −100), 419 (−18, −60, −100) and characteristic low-mass key fragments at *m/z* 291, 273 and 245 (*Supporting Information* S1).[25]

Peak at 19.7 min with $[M+H]^+$ at *m/z* 613 was tentatively assigned to of salannal.[17] The main observed fragments were ions at *m/z* 553 (−60) corresponding to the loss of $C_2H_4O_2$ ascribable to neutral loss of acetic acid (substituent in C-3) or methoxy formate moiety (chain in position C-11); *m/z* 513 (−100) tiglic acid $C_5H_8O_2$ (C-1), *m/z* 581 (−32) corresponding to the loss of formaldehyde (substituent at C-4). Other intense fragments were observed at *m/z* 453 (−60, −100), 435 (−18, −60, −100) and 407 (−18, −29, −60, −100) supporting the presence of free hydroxy group, aldehydic function, acetic and tiglic acid esterification (*Supporting Information* S2). Other significant peaks are ascribed to the breaking of the diterpenoid moiety leading to fragment ions at *m/z* 289, 261 and 229 supporting the loss of the methoxy substituent and the oxygenated function in position 1. The structure of the specie at *m/z* 289 derived from the father ion after the loss of water and ester bonds and due to the breaking of bond C-8, C-14, and C-7 with oxygen.[25] Other significant ion at *m/z* 261 correspond to the previous loss with further loss of CO (*Supporting Information* S3).

Peak at 18.5 min was annotated as mesendanin A and present $[M+H]^+$ at *m/z* 62. Observed fragments were at *m/z* 593 (−32) supporting the loss of formaldehyde, *m/z* 525 (−100) corresponding to the loss of tiglic acid substituent, *m/z* 465 (−60, −100) corresponding to the loss of tiglic acid and acetic acid, *m/z* 405 (−100, −120) due to two units of acetic acid and tiglic acid and *m/z* 387 (−18, −100, −120) corresponding to the previous loss and a further water loss (*Supporting Information* S4). These fragments suggested the presence of two acetylation and one tigloylation as well as one free hydroxy group and one aldehydic function in the molecule. Low-mass fragments were observed[25] and were considered as deriving by the partial rearrangement of the tetracyclic nucleus of the limonoid. In particular, the ion at *m/z* 259 can be originated by the loss of all the ester substituents as well as water molecule from free OH, as well as by the loss of C_4H_4O (ascribable to the neutral loss of furane in position 17), CH_2O ascribable to substituent in position 4, and CH_3 ascribable to a methylene. Further fragment ions related to the breaking of the cyclic moiety was the ion at *m/z* 185 ascribable to hexahydro naphthalene derivative related to the two hexa atomic cycle of the limonoid nucleus. The ion at *m/z* 147 derived from the methylcyclopentadienyl furane moiety originating from the two penta atomic cycle of the compound (*Supporting Information* S5).

Two different peaks presents $[M+H]^+$ at *m/z* 639 at 19 and 19.5 min and both shared the same fragmentation scheme. The main observed fragments suggested the loss of tigloyl (−100), acetyl (−60), the sum of the two above mentioned (−160). Furthermore, fragment ions at *m/z* 419, 245 and 273 suggested structure of Nimboinin-type limonoid (*Supporting Information* S6). This isomers were annotated as Meliatoosenin L that were previously isolated from *M. toosenin* and *M. azedarach*.[20]

On the basis of LC/MS data, limonoids were detected in both dried leaves and methanol extract of *M. azedarach* as reported in (*Table 4*). The absence of reference compound for *Melia* limonoids suggested a semi-quantitative analysis using as reference compound the limonoid azadirachtin. The quantitative data showed that methanol extract present almost complete loss of limonoids, probably caused by concentration procedures due to their partial volatility or by oxidative and/or hydrolytic reactions. Literature indicate that tissues containing the larger amount of limonoids in *M. azedarach* were fruits[18] and in less extent leaves.

Antioxidant and Enzyme Inhibitory Properties

Chemical analysis revealed that methanolic extract contains significant amounts of lipids, phytosterols and phenolics, therefore, was subjected to preliminary bioactivity assays. Total phenolic content was determined by the spectrophotometric Folin-Ciocalteu method and the content was found to be 33.53 mg GAE/g.

Table 4. Tentative identification of the limonoid constituents of dried leaves and the methanolic extract of *M. azedarach*. Results are expressed as mean ± standard deviation ($n = 4$). t_R: retention time.

t_R	Ion	Fragmentation	Annotation	Dried leaves (mg/g)	Methanol extract (mg/g)
17.1	583	483	Meliatoosorin R	0.07 ± 0.02	0.03 ± 0.01
17.8	553	467, 431, 389, 371, 353	Tinosposinenside A	0.06 ± 0.01	0.03 ± 0.01
19.8	554	468, 431, 389, 371, 353	Tinosposinenside B	0.05 ± 0.01	0.08 ± 0.02
17.7	597	565, 537, 497, 437, 419, 291, 273, 245	Salannin	0.08 ± 0.01	0.10 ± 0.02
19.7	613.3	584, 525, 465, 405, 387, 369	Salannal	0.60 ± 0.02	0.44 ± 0.02
19.0	639.4	607, 539, 479, 419, 401, 387, 273, 245	Meliatoosenin L	0.95 ± 0.03	–
19.5	639.4	607, 539, 501, 479, 419, 401, 387, 273, 245, 185, 153	Meliatoosenin L isomer	2.15 ± 0.05	–
20.1	635.4	603, 575, 454, 435, 375	1,7-Dityglyol-3-acetylvilasinin	0.10 ± 0.01	–
18.5	625.4	593, 525, 465, 405, 387, 259, 185, 145	Masendanin A	2.08 ± 0.02	–
19.1	625.4	593, 539, 525, 479, 465, 419, 405, 387, 273, 245	Masendanin A isomer	0.85 ± 0.02	–
			Limonoids total amount	7.36	0.69

Previous article regarding total phenolic content of *M. azedarach* leaves reported different results. Total phenolic content of leaves extract reported by M'rabet (2017)[13] was 59.1 mg GAE/g and 50.01 mg GAE/g in an earlier study conducted by Kaneria (2009).[26] Observed differences could be explained by geographical and climatic factors. The results from Folin-Ciocalteu method has some limitations, because not only phenolic, but other compounds (peptides, reducing sugars, etc.) can react.[27] For these reason advanced chromatographic techniques, such as LC-DAD-MS^n have been used to corroborate results.

Many studies have been reported that a negative correlation between the intake of dietary antioxidants and mortality and morbidity of chronic and degenerative diseases including cardiovascular disease, diabetes, and Alzheimer's diseases.[28] In the lights of these information, the discovery of natural and novel antioxidant sources has a great interest in the scientific platform. In the current work, antioxidant properties of methanolic extract of *M. azedarach* were assayed by different chemical methods including reducing power (CUPRAC and FRAP), radical quenching (ABTS and DPPH), metal chelating and phosphomolybdenum assays (*Table 5*). The radical quenching ability in DPPH and ABTS was observed as 47.56 and 65.34 mg TE/g, respectively. CUPRAC and FRAP assays were evaluated the electron-donating ability of the extract and the results were 109.89 mg and 65.02 mg TE/g, respectively. According to literature search, several articles investigate antioxidant properties of *M. azedarach* parts (leaves, stem, or fruits).[13,15,26] In these articles, the extract exhibited significant antioxidant effects that can be correlated with compound, like flavonoids and hydroxy cinnamic acids, with antioxidant properties.[29,30]

Enzymes are considered as the main pharmaceutical target board to manage global health problems including obesity, diabetes mellitus and Alzheimer's disease.[31–33] In this sense, several enzyme inhibitors have been chemically synthesized but most of them have significant side effects such as toxicity and gastrointestinal disturbances. Thus, the search of novel and safe enzyme inhibitors is becoming an interesting

Table 5. Total phenolic content, antioxidant, and enzyme inhibitory properties of methanolic extract of *M. azedarach*. Values are reported mean ± SD of three parallel experiments. GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; GALAE: Galantamine equivalent; ACAE: Acarbose equivalent; KAE: Kojic acid equivalent.

Parameters	Results
Total phenolic content (mg GAE/g)	33.53 ± 0.39
DPPH (mg TE/g)	47.56 ± 0.34
ABTS (mg TE/g)	65.34 ± 3.14
CUPRAC (mg TE/g)	109.89 ± 0.84
FRAP (mg TE/g)	65.02 ± 3.09
Metal chelating (mg EDTAE/g)	12.26 ± 0.32
Phosphomolybdenum (mmol TE/g)	2.05 ± 0.13
AChE inhibition (mg GALAE/g)	5.05 ± 0.02
BChE inhibition (mg GALAE/g)	2.43 ± 0.77
Tyrosinase inhibition (mg KAE/g)	131.57 ± 0.51
Amylase inhibition (mmol ACAE/g)	0.90 ± 0.02
Glucosidase inhibition (mmol ACAE/g)	5.46 ± 0.01
Metal chelating (mg EDTAE/g)	12.26 ± 0.32
Phosphomolybdenum (mmol TE/g)	2.05 ± 0.13
AChE inhibition (mg GALAE/g)	5.05 ± 0.02
BChE inhibition (mg GALAE/g)	2.43 ± 0.77
Tyrosinase inhibition (mg KAE/g)	131.57 ± 0.51
Amylase inhibition (mmol ACAE/g)	0.90 ± 0.02
Glucosidase inhibition (mmol ACAE/g)	5.46 ± 0.01

research topic in recent years. For this reason, enzyme inhibitory effects of methanolic extract of *M. azedarach* were tested by several enzymes (*Table 5*). AChE and BChE inhibitory properties were found to be 5.05 and 2.43 mg GALAE/g, respectively. The extract exhibited a significant tyrosinase inhibition effect (131.57 mg KAE/g). Previous work reported that *M. azedarach* extract induced melanogenesis in B16F10 mouse melanoma cell lines without affect intracellular tyrosinase activity.[34] Regarding amylase and glucosidase inhibition ability, the results were found to be 0.90 and 5.46 mmol ACAE/g. To the best of our knowledge, the information on the enzyme inhibitory properties of *M. azedarach* is still limited.[35] Thus, the findings could support the utilization of this plant as a putative natural source of biologically-active agents.

Cytotoxicity Test

Preliminary cytotoxicity analysis was performed on two tumor cell lines, namely human ovarian 2008 cancer cells and human pancreatic BxPC3 cancer cells. The methanolic extract of *M. azedarach* (MAM) exhibited an $IC_{50} > 50$ μg/mL on BxPC3 cell lines while on human 2008 cancer cells IC_{50} decreases to 26.4 μg/mL, revealing a moderate ability of the extract to compromise the viability of ovarian cancer cells. These results were also confirmed by microscopy analysis (*Figure 4*).

When treated with MAM, human 2008 cancer cells appeared to be different from those of the control from a morphological point of view (*Figure 4*,*c* and *d*). In addition, a significantly decreased number of cells was detected in the photomicrograph of treated 2008 cancer cells (*Figure 4*,*d*). On the contrary, no differences in term of cell morphology were evident in pancreatic

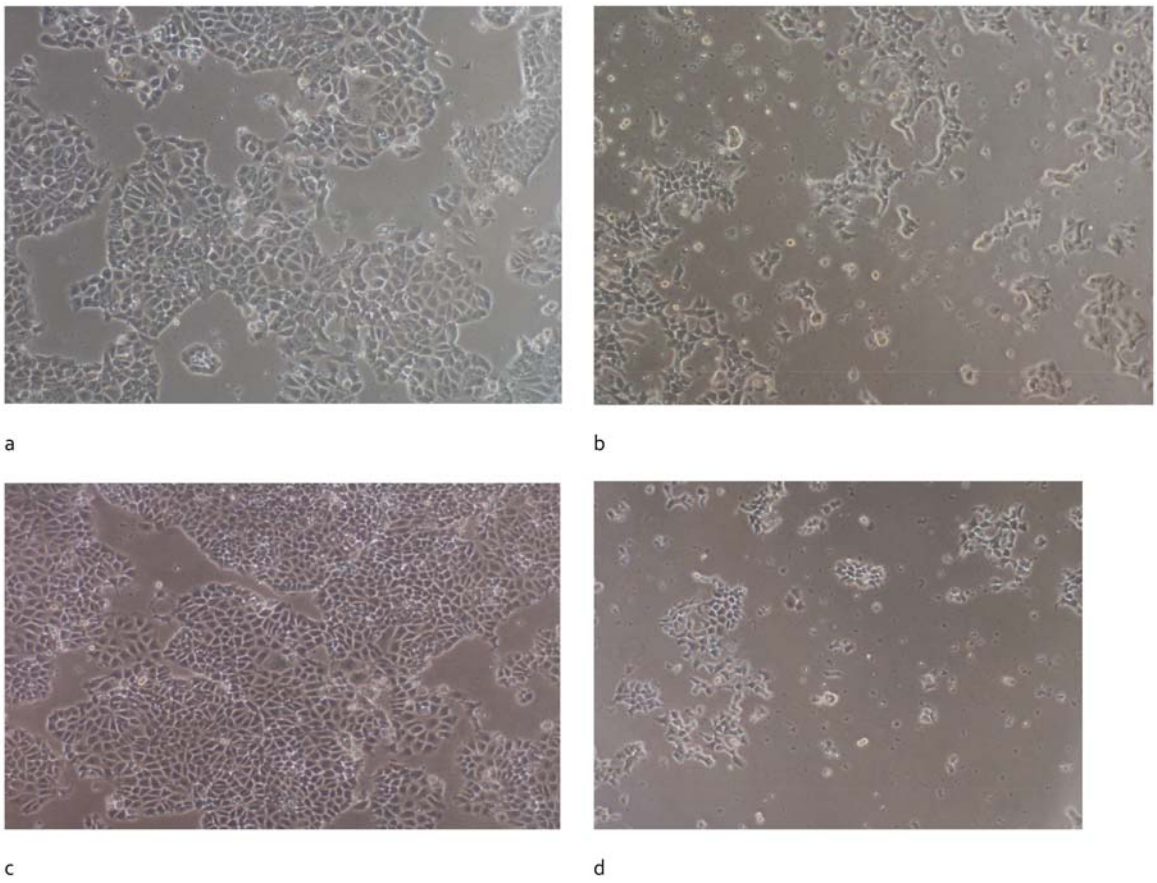

Figure 4. Microscopy analysis of pancreatic and ovarian cancer cell lines after treatment with MAM. Pictures (40×magnification) of a) Control BxPC3 cells; b) MAM treated BxPC3 cells; c) Control 2008 cells; d) MAM treated 2008 cells.

cancer cells after treatment with MAM with respect to control cells (*Figure 4,a* and *b*), even though micrographs of MAM treated pancreatic cancer cells revealed a slightly lower number of cells with respect to control cells. The observed cytotoxic effect could be explained by the presence of phytol[36] and β-sitosterol[37] due to previous published articles. Rutin can be partially related to the observed cytotoxic effect, as report in previous article.[38] Rutin demonstrated to possess *in vitro* cytotoxic effects on tumor colon (SW480) cells and was able to induce an *in vivo* antitumor effects, without significant toxic effects on mice bearing SW480 tumor.[38] In the same article, the compound also showed antiangiogenic properties.[38]

Conclusions

Nepalese traditional medicinal plant *M. azedarach* leaves was subjected to phytochemical characterization showing significant contents in lipids, phytosterols, flavonoids, and limonoids. Phytochemical composition of the methanolic extract was compared with the dried leaves. Results revealed that lipids, phytosterols and flavonoids were efficiently concentrated, while limonoids were almost completely loss during extraction procedures. Limited enzyme inhibition on AChE, BuChE, amylase and glucosidase were observed while significant tyrosinase inhibition was measured suggesting potential usefulness in skin hyperpigmentation conditions. Extract was subjected to cytotoxic assay revealing a moderate ability to reduce the viability of ovarian cancer cells 2008. *M. azedarach* leaves can be considered as source of valuable phytoconstituents with potential interest in pharmaceutical and nutraceutical applications.

Experimental Section

Plant Materials

Leaves of *M. azedarach* were collected during October 2017. Altitude is 1290 m. Location is at Central region of Nepal 27°39′9″ N and 85°22′49″ E. Bhaktapur. The plant leaves were dried carefully by proper pressing and the herbarium samples were prepared; voucher number was indicated as MAL17. Plant material was cross checked with Herbarium deposited at National Herbarium and Plant Laboratories, Godavari, Lalitpur (KATH). Leaves were air dried over 2—3 weeks inside the room until completely dried. The shade dried leaves were grinded into fine powder with the help of electric grinder. The powder obtained were preserved into zipper bag for extraction.

Extraction

The powder of plant material was subjected to extraction using methanol through percolation with intermittent sonication. 50 g of plant powder was taken into falcon tube and was added 500 ml of methanol such that the ratio of solvent in volume (ml) to the weight (gram) of plant material would be 10:1. Then the solution was subjected to intermittent sonication for 2 h, i.e., continuous cycle of sonication at 30 kHz for 30 min (with 10 min interruption). After the completion of the cycle the solution was filtered with Whatman No. 1 filter paper (Whatman Ltd., Kent, UK) and the filtrate was then subjected to evaporation at reduced pressure in rotating vacuum evaporator (IKA RV 10). Methanolic extract was transferred to clean, dried and weighed glass vials. The percentage yield of the extract was 24.21 ± 1.05 %. The resulting dried extract was then sealed and stored at 4 °C until use.

NMR Analysis of Dried Leaves of M. Azedarach

1D- and 2D- NMR spectra were obtained on a Bruker Avance III 400 Ultrashield spectrometer with 400 MHz magnet. NMR spectra were acquired in deuterated chloroform and deuterated methanol (Sigma-Aldrich) with TMS as an internal standard. Durian® 4.95 mm NMR tubes (Durian Group) were used. Chemical shifts are expressed in δ values in ppm. ^{1}H-NMR and HSQC-DEPT, HMBC, COSY, ^{13}C-NMR experiments were acquired using standard Bruker sequences measuring p1 and d1 for each acquired sample. For the acquisition of NMR spectra powdered plant material (100 mg) was subjected to extraction with deuterated chloroform (3 mL) at room temperature sonicated for 5 min. Mixture was centrifuged (3 min, 13000 rpm) and surnatant was used for NMR-experiment. From the methanol extract, solid residues were extracted in deuterated methanol (3 mL) and sonicated at room temperature for 5 min, solution was centrifuged (3 min, 13000 rpm) and surnatant used for analysis.

GC/MS of Fatty Acid

For GC/MS analysis of the fatty acids content, sample of *M. azedarach* plant material was extracted with hexane. Then the extract was dried under vacuum and derivatized with MeOH in presence of H_2SO_4, leading

to the esterification of fatty acids to fatty acid methyl esters, which offer excellent stability for GC analysis. 77 mg of the extract were added with 15 mL of MeOH, 1 mL of CH_2Cl_2, 3 drops of H_2SO_4 and 12 mg of methyl pentadecanoate (Sigma Aldrich, St. Louis, MO, USA), used as internal standard. The mixture was heated under reflux for 1 h, and then, cooled in ice bath. A liquid/liquid partition was performed with 10 mL of water and 5 mL of diethyl ether, then, the organic phase was collected and dried. The residue was re-dissolved with 1.5 mL of diethyl ether and put in vial. For the analysis of methanolic extract, 100 mg were exactly weighted and was subjected to the same procedure. GC/MS analysis was performed through an Agilent 7820 A coupled with a single quadrupole mass spectrometer Agilent 5977B MSD, using a HP88 (60 m×0.25 mm, 0.2 μm film thickness) as stationary phase. Helium was the carrier gas with a column head pressure of 14.1 psi. The flow rate through the column was 1.19 mL/min. The injector was set at 300 °C with a split ratio of 20:1, the split flow was 23.9 mL/min and 1 μL injections were made. The temperature gradient started with a 120 °C initial temperature with a linear increase to 240 °C at 3 °C/min. The total run time was 55 min. MS spectra were recorded in the range of *m/z* 40–650, using EI ion source operating in positive ion mode. The identification of the constituents was performed by combining computer library search based on comparison of mass spectral fragmentation patterns with those reported in NIST library and visual interpretation of the mass spectra, and retention indices determined by reference to a homologous series of n-alkanes.

Liquid Chromatography Coupled with Mass Spectrometry Methods Used for Qualitative Quantitative Analysis of Phenolics, Limonoids, Phytosterols

Qualitative analysis of the compounds in the methanolic extract was obtained by HPLC-DAD-ESI-MS. 50 mg of dried leaved of *M. azedarach* were added to 2 ml of methanol, sonicated for 10 min, and centrifuged 5 min. For methanolic extract, 10 mg were exactly weighted and procedure above mentioned were used. Surnatant was transferred in vial and used for phenolic, limonoid and phytosterol analysis. The measurements were performed using an Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with 1260 diode array detector (DAD) and Varian MS-500 ion trap mass spectrometer. Sample analysis was achieved using an Agilent Eclipse XDB C-18 (3.0× 150 mm, particle size 3.5 μm) as stationary phase. The mobile phase was acetonitrile (A) and water (0.1% formic acid) (B). A gradient program was used as follows: [0→30th min: A/B (10:90)→A/B (100:10) 30→35th min: A/B (100:10)→A/B (100:10) 35→36th min: A/B (100:10)→A/B (10:90) 36→40th min: A/B (10:90)→A/B (10:90)]. Flow rate was 0.4 mL/min, injection volume was 20 μL and the temperature was set at 40 °C. At the end of the column, a 'T' splitter separated the flow rate to DAD and MS. UV/VIS spectra were acquired in the range of 190–640 nm. MS spectra were recorded in the range of *m/z* 100–2000, using ESI ion source operating in negative and positive ion mode. Turbo data dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species. Identification of compounds was obtained based on fragmentation spectra as well as the comparison with the literature. Quercetin, rutin, kaempferol-3-*O*-rutinoside, kaempferol-7-*O*-rutinoside and chlorogenic acid were used as reference compounds.

To detect limonoids, azadirachtin was used as reference compound. A HPLC-DAD-APCI-MS was performed using the same instrument. Sample analysis was achieved using an Agilent C18 Eclipse (3.0× 150 mm, particle size 3.5 μm) as stationary phase. The mobile phase was acetonitrile (A) and water (0.1% formic acid) (B). A gradient program was used as follows: [0→28th min: A/B (20:80)→A/B (99:1) 28→32nd min: A/B (99:1)→A/B (99:1) 32→33rd min: A/B(99:1)→A/B (20:80)]. Flow rate was 0.4 mL/min, injection volume was 20 μL and the temperature was set at 40 °C. At the end of the column, a 'T' splitter separated the flow rate to DAD and MS. UV/VIS spectra were acquired in the range of 190–640 nm. MS spectra were recorded in the range of *m/z* 300–1000, using APCI ion source operating in positive ion mode. Spray chamber was set to 50 °C, drying gas 15psi, nebulizer 25 psi, drying gas temperature start at 285 °C and decrease to 270 °C in 25 min, vaporizing temperature from 295 °C at 270 °C in 25 min. Corona current was set to 5 microamperes, spectra were acquired using turbo data dependent scanning (TDDS) instrument function was used to acquire mass fragmentation pathways of the main ionic species. Capillary 80 V, RF loading 80%. As reference a standard solution of azadirachtin was used with a concentration range of 100–0.2 μg/mL.

To measure the phytosterols content in the extract we used a previously publisher method by HPLC-APCI-MS.[9] Briefly as stationary phase an Agilent

Eclipse XDB-C18 column (3.0 mm × 150 mm, 3.5 μm) was used. The mobile phase was composed by water (0.1% formic acid) (A), acetonitrile (B) and methanol (C). A gradient program was used as follows: [0→8th min: A/B/C (2:95:3)→A/B/C (2:95:3) 8→10th min: A/B/C (2:95:3)→A/B/C (2:0:98) 10→28th min: A/B/C (2:0:98)→A/B/C (2:0:98) 28→30th min: A/B/C (2:0:98)→A/B/C (2:95:3) 30→35th min: A/B/C (2:95:3)→A/B/C (2:95:3)] with a flow rate of 400 μL/min, the injection volume was 10 μL and the oven column temperature was 30 °C. MS spectra were recorded in the range of *m/z* 100–2000, using Atmospheric Pressure Chemical Ionization (APCI) ion source working in positive ion mode. The turbo data-dependent scanning (TDDS) instrument function was used reveal the fragmentation of the main ionic species. Compounds were identified by comparison with the literature data and using reference compounds. As standards, solutions of *β*-sitosterol at four different levels (concentration ranges 176–1.76 μg/mL) and stigmasterol (concentration ranges 185.6–1.856 μg/mL) were used. Compound quantification was obtained with the method of calibration curve: *β*-sitosterol was used as the external standard for *β*-sitosterol and *γ*-sitosterol quantification, while stigmasterol was used for fucosterol. Calibration curves were as follows: $y = 0.64 \times + 0.2302$ ($R^2 = 0.9993$) for *β*-sitosterol; $y = 2.0987 \times -13.816$ ($R^2 = 0.9989$) for stigmasterol.

Total Phenolic Content, Antioxidant, and Enzyme Inhibitory Assays

Total phenolic and flavonoid content (TFC) were detected by Folin-Ciocalteu colorimetric method.[39] Briefly, sample solution (50 μL) was mixed with the reagent of Folin-Ciocalteu reagent (100 μL, 1:9, v/v). The mixture was kept for 3 min at room temperature and then, sodium carbonate (75 μL, 2%) was added. The mixture was incubated for 2 h at room temperature. After that, the absorbance's were recorded at 765 nm. The results were expressed as standard compounds (gallic acid (GAE)). For antioxidant capacity, chemical different assays including free radical scavenging (DPPH and ABTS), reducing power (CUPRAC and FRAP), ferrous ion-chelating (ferrozine method), and phosphomolybdenum assay were performed. The results were recorded based on the spectrophotometric measurements. The methods details were described in our earlier article.[39] To explain the results, we used standard equivalent way and thus Trolox and EDTA (for ferrous ion chelation) were selected as standards for enzyme-inhibitory assays, we selected on some enzymes related global health problems, namely cholinesterase, tyrosinase, amylase, and glucosidase. The experimental procedures were given in our earlier article.[39] Standard enzyme inhibitor compounds were used to evaluate the results. These compounds were galantamine (GALAE, for cholinesterase), kojic acid (KAE, for tyrosinase), and acarbose (ACAE, for amylase and glucosidase). All experimental procedures were performed with 96 wells microplate.

Cytotoxicity Studies

Cytotoxic activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay (MTT). Human pancreatic (BxPC3) carcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human ovarian (2008) cancer cells were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Modena, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using RPMI-1640 medium (Euroclone) containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (100 units/mL penicillin and 100 g/mL streptomycin), and 2 mM L-glutamine. The MTT assay was used as a relative measure of cell viability. Briefly, 10^3 cells/well, dependent upon the growth characteristics of the cell lines, were seeded in 96-well microplates in growth medium. After 24 h, the medium was removed and replaced with fresh medium containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 μL of a 5 mg/mL MTT solution in phosphate-buffered saline (PBS) and after 4 h of incubation, 100 μL of a sodium dodecyl sulfate (SDS) solution in 0.01 M HCl were added. After an overnight incubation, the extent of MTT reduction was quantified spectrophotometrically, by using a microplate reader (Bio Rad 680) at 540 nm. The mean absorbance for each drug dose was expressed as a percentage of the control, untreated, well absorbance and plotted vs. drug concentration. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values, the drug concentrations that decrease the mean absorbance at 570 nm to 50% of that of untreated control wells. The final value is the mean ± S.D. of at least three independent experiments performed in triplicate.

Cell Morphological Analysis

BxPC3 and 2008 human cancer cells were plated into 5 cm Petri dishes (1×10^5). After 24, cells were treated with IC_{50} of MAM. The morphological features were evaluated via inverted phase contrast microscopy (Olympus BX41) and pictures were obtained by a Canon digital Camera (Canon 6D).

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Author Contribution Statement

Conceptualization, S.D.A., S.R. and S.S.S.; methodology, I.R., S.S. and G.Z.; software, G.A. and G.Z.; validation, I.R. and G.Z.; formal analysis, S.S.S., I.F., S.S. and S.D.A.; investigation, S.S.S. and S.D.A.; resources, D.R.P. and S.R.; data curation, I.R., and S.S.; writing – original draft preparation, S.S.S. and S.D.A.; writing – review and editing, S.S.; visualization, S.D.A.; supervision, S.R., and project administration, S.D.A.. All authors have read and agreed to the published version of the manuscript.

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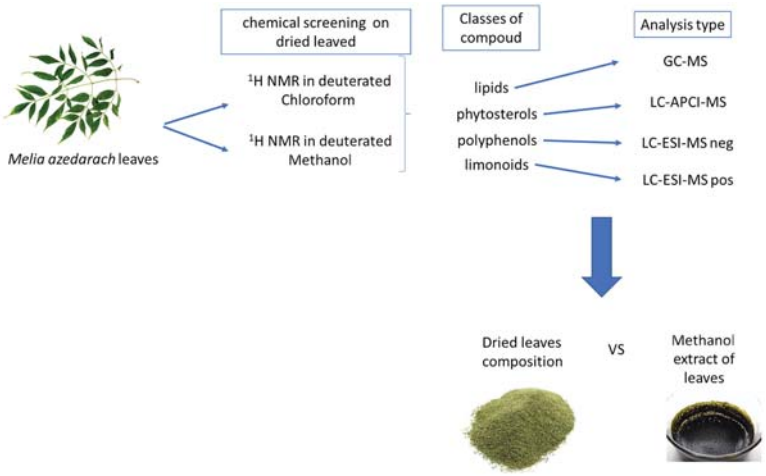
FULL PAPER



S. S. Shrestha, I. Ferrarese, S. Sut, G. Zengin, S. Grana, G. Ak, D. R. Pant, S. Dall'Acqua, S. Rajbhandary*

Phytochemical Investigations and *In Vitro* Bioactivity Screening on *Melia Azedarach* L. Leaves Extract from Nepal

Phytochemical investigations and in vitro bioactivity screening on Melia azedarach L. leaves extract from Nepal by S. Dall'Acqua et al., @UniPadova

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Gokhan Zengin
Gunes Ak
Sara Grana
Stefania Sut
Stefano Dall'Acqua
Irene Ferrarese
Sangeeta Rajbhandary
Shyam Sharan Shrestha

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms.

Shyam Sharan Shrestha

has presented paper/poster/attended

the International Conference on Biodiversity, Climate Change Assessment and Impacts on Livelihood

at Kathmandu on January 10-12, 2017.

Dr. Bishwa Nath Oli
Secretary, Ministry of Population and Environment

Dr. Nir Krakauer
Co-Chair, Conference Organizing Committee
PI: USAID-IPM-IL-Biodiversity Project

Prof. Naba R Devkota
Director of Research
Agriculture and Forestry University

Prof. Mohan Siwakoti
Secretary, Conference Organizing Committee
Head, Central Dept. of Botany, TU

Prof. Pramod K Jha
Chair, Conference Organizing Committee
Nepal Coordinator & Co-PI
USAID-IPM-IL-Biodiversity Proj

Tribhuvan University
Institute of Science and Technology
Central Department of Botany
Kirtipur, Kathmandu, Nepal

Training Program

This certificate is awarded to Mr. Shyam Sharan Shrestha in due recognition of participating in the Training Program on '***Research Methodology for PhD Scholars***', conducted by the Central Department of Botany, Tribhuvan University, and supported by University Grants Commission, Nepal from June 20-26, 2017 (Ashad 6-12, 2074)

Prof. Dr. Mohan P Panthi
Acting Head
Central Department of Botany, T.U.

Dr. Chitra Bahadur Baniya
Co-ordinator

Prof. Dr. Parashar Prasad Koirala
Chairman
University Grants Commission, Nepal

July 5, 2017 (Ashad 21, 2074)

Workshop on

NATURAL PRODUCTS AND DRUG DISCOVERY

Central Department of Biotechnology
Tribhuvan University, Kirtipur, Nepal
9th – 11th February, 2017

This is to certify that

Shyam Sharan Shrestha

has successfully completed hands on training on screening of natural products (plants and microbes) and their anti- infective activities to drug discovery.

Skills Acquainted with

- *Phytochemical extraction*
- *Isolation of Streptomyces, Myxobacteria, and Endophytes*
- *Mammalian cell culture*
- *Quantitative estimation of Phyto constituents*
- *Extraction of bacterial, fungal DNA*
- *Antibacterial assay of plant extracts*

Prof. Krishna K Shrestha,
President, Ethnobotanical Society of Nepal (ESON)

Prof. Krishna D Manandhar
Head, Central Department of Biotechnology (CDBT-TU)

Mr. Bivek Timalsina
Vice - President, Society for Biological Applications of Nepal (SBAN)

This course was organized by
Central Department of Biotechnology, Tribhuvan University - Ethnobotanical Society of Nepal - Society for Biological Applications Nepal

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Padova 20/09/2019

This letter is to certify that

SHRESTHA SHYAM SHARAN has successfully attended the International Seminar on Biodiversity, Bioprospecting and Territories from September 9-10, 2019 and presented paper entitled "Phytochemical analysis and cytotoxic activities of *Girardinia diversifolia*". The seminar was organized by Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy.

Prof. Stefano Dall'Acqua

National Conference on Integrating Biological Resources for Prosperity

Certificate of Participation

This is to certify that

Mr. Shyamsharan Shrestha

has presented Paper / Poster / Attended the

National Conference on Integrating Biological Resources for Prosperity

held at Biratnagar on February 6-7, 2020.

Prof. Dr. Mohan Siwakoti	**Prof. Dr. Sasinath Jha**	**Dr. Bishwa Nath Oli**	**Hon. Jagadish Prasad Kusiyait**
Chairman	Chairman	Secretary	Minister
Conference Organizing Committee	Nepal Biological Society	Ministry of Forests and Environment	Ministry of Industry, Tourism, Forests and Environment
President, Botanical Society of Nepal			Province No. 1

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Padova 23 /07/2019

To whom who may concern

International office, Univertisty of Padova , Prof. Ram Kailash Prasad Yadav, Head of Department .Central Department of Botany, Tribhuvan University and Prof. Sangeeta Rajbhandary, Supervisor, Central Department of Botany, Tribhuvan University

Object: Ph D Student Shyam Sharan Shrestha

With the present letter I declare that PhD student Shyam Sharan Shrestha was present in Natural Product Laboratory at the Department of Pharmaceutical and Pharmacological Sciences of the University of Padova during the period 7 February 2019 to July 31 2019.

During this period the PhD student Shyam Sharan Shrestha has performed research activities related to Nepalese medicinal plant joining the Natural Product Laboratory group under my supervision. During this period prof Sangeeta Rajbhandary has been constantly in contact to share results and to support the student research and study during this PhD period abroad. My opinion of the work and participation of Shyam Sharan Shrestha in the PhD research project dealing with Nepalese medicinal plant is excellent, during this period he was able to perform measurements and experiments, participate to data, discussion and paper preparation with very good attitude in group work.

Prof Dall'Acqua Stefano

Stefano Dall'Acqua

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Padova, 04/12/2018

RE: Programme ERASMUS + KA107 "International Credit Mobility" – Academic year 2018/19

TO WHOM IT MAY CONCERN

In the framework of the European Programme ERASMUS+ KA107 "International Credit Mobility", coordinated by Università degli Studi di Padova (Italy) and funded by the European Commission,

Mr **SHYAM SHARAN SHRESTHA**

born on 2/10/1975 in Bhaktapur (Nepal), citizen of Nepal, passport nr. 09898272, enrolled at the Tribhuvan University (Nepal) will spend a study period of 6 months at the Università degli Studi di Padova – School of Agricultural Sciences and Veterinary Medicine – starting from 1/2/2019.

The academic supervisor will be Prof. Antonio MASI; the administrative contact person at the International Office is Ms Elisa Zambon (elisa.zambon@unipd.it, Tel. +39 049 827 3741, Fax +39 049 827 3060).

The European Commission will pay a total grant of Euro 5100 and a contribution for the travel expenses of Euro 820. The civil responsibility and health insurance will be purchased by the student.

For the accommodation in Padua Mr SHRESTHA will be supported by SASSA service/ESU Accommodation. This service, on the bases of an agreement between Università degli Studi di Padova and ESU/Padova (Regional office for university students' economic benefits), helps foreign students for their accommodation in Padua.

For the enrolment of international exchange students the Università degli Studi di Padova does not require the Declaration of value of the foreign diploma or the knowledge of Italian language.

More information about the programme are available at the following web site:
http://www.unipd.it/en/node/1482

This document is delivered to the person concerned for the purposes granted by law.

Elisa Zambon
International Officer

UNIVERSITY GRANTS COMMISSION
Sanothimi, Bhaktapur, Nepal

***The UGC PhD Fellowship and Research Support* Agreement**
(FY 2073-74)

A.N:- 74-SBT-11

This agreement is made on this 25th day of Jan in the year 2017 by and between:

1. University Grants Commission,

2. Shyam Sharan Shrestha, the PhD Fellow, with the following details;
Program: PhD in ~~Science and Technology~~ ~~[illegible]~~ Botany
Host Institution: CDB, TU
University registered to: 27/2072-73
Date of registration: 2073/4/9
Date of the acceptance of PhD Research Proposal by the registered university: 2073-4-9
UGC PhD Fellowship award no.:

3. Prof. Dr Sangeeta Rajbhandary (the Supervisor of the PhD Fellow), and

4. CDB, TU (the Host Institution)

(hereafter, referred to as "parties")

in connection with the UGC PhD Fellowship and Research Support grant awarded by the UGC to the fellow Shyam Sharan Shrestha on 16th of November 2017 (2074 Kartik 30) for the PhD research project Phytochemical Analysis and Bioactive compounds of some medicinal plats of Nepal in the year 2073-74 (2016-2017).

The parties hereby agree to abide by the following terms and conditions:

Terms and Conditions

A. Obligations of the UGC

1. Provide Fellowship and Research Support grant to the Fellow according to the funding and disbursement scheme outlined in Section B of this agreement.
2. Periodically monitor the progress and provide suggestions.
3. Take action in the case of unsatisfactory progress, negligence or misconduct on part of the Fellow and any lack of cooperation to the research project on part of the Supervisor and the Host Institution.
4. Assist the Host Institution to have code of academic integrity and policy on research misconduct, and on maintaining of research ethics.
5. Provide a Certificate of Fellowship and Research Grant to the Fellow at the completion of the programs marked by the submission of thesis approved by the concerned university and duly completed final report of the research project to the UGC.

B. PhD Fellowship and Research Support Grant disbursement

DEPARTMENT OF PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

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To the attention of
Shyam Sharan Shrestha
Suryabinayak Municipality-1
Sirutar
Bhaktapur - Nepal

Padova, 30.07.2019
Ref. no. 2244
Year 2019 Tit. III Cl. 12

SUBJECT: Selection procedure for awarding a research fellowship, issued by record no.2162 dated 18/07/2019 - Conferral

Dear Dr Shrestha,

we are pleased to let you know that your application has placed first in the ranking order referred to above. Thus, you have been awarded the fellowship with the following title: ***Medicinal Plants enriched with active compounds through Biostimulation*** to carry out related research activities.

The following provisions are envisaged on acceptance of the research fellowship:

- the research activities will take place at the Department of Pharmaceutical and Pharmacological Sciences under the Research Project Supervisor, Prof. Stefano Dall'Acqua;
- the research fellowship will start from 1st August 2019 for the duration of 5 months;
- a notice period of 15 days must be given if you decide to renounce;
- the gross amount of the research fellowship, € 5.900,00 shall be transferred to your bank account in monthly installments at the end of each month, unless otherwise advised by your Research Project Supervisor for nonfulfillment of planned research activities;
- insurance cover for on-the-job injuries and third-party civil liability will be the responsibility of the Department you will be attached to under the terms stipulated in the University's insurance policies;
- Upon completion of the project, you will submit a report, previously approved by the Research Project Supervisor, to the Department / Centre's Head on the activities carried out and the outcomes achieved.

We also remind you that conferral of the fellowship involves compliance with the University of Padova's Code of Ethics and Code of Conduct, as well as the University's Regulations.

The awarding of the research fellowship does not constitute subordinate employment nor does it entitle the Research Fellowship Holder to a permanent position at the University.

We invite you to report to the Department to sign the acceptance letter within and no later than ten days from receipt of this notice, under penalty of forfeiture.

Financial Officer
Dott.ssa Daniela Rosin

Head of Department
Prof. Paolo Caliceti