THE BIOLOGICAL PROPERTIES OF AEGLE MARMELOS(L.) CORREA AND HR-LCMS BASED METABOLITES PROFILING

A THESIS SUBMITTED TO THE:

DEPARTMENT OF CHEMISTRY

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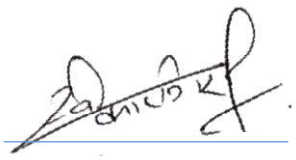
OCTOBER 2023

DECLARATION

I declare that this dissertation entitled **"The Biological Properties of Aegle marmelos (L.) Correa and HR-LCMS based metabolites profiling,"** are my own research work. This work has not been published or accepted and submitted for any degree award.Plagiarismchecked at Birendra Multiple Campus library also confirmed that the work is original and genuine.



Mr. SwikarGiri

October 2023

RECOMMENDATION

This dissertation entitles **"The Biological Properties of Aegle marmelos (L.) Correa and HR-LCMS based metabolites profiling," is** submitted by **Mr. Swikar Giri** for the partial fulfillment of M.Sc. degree in Chemistry at Birendra Multiple Campus. The entire work is completed under our supervision. All the reports presented here are his finding. We confidently recommend this thesis for final evaluation.

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FOREWORD

The thesis work **"The Biological Properties of Aegle marmelos (L.) Correa and HR-LCMS based metabolites profiling,"** submitted by **Mr. Swikar Giri** as a part of M.Sc. Course work in Chemistry at the department of Birendra Multiple Campus is carried out under my supervision. Any part of this thesis work has not been submitted for any other degree award.

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Date: 11 /10/2023

On the recommendation of **Ganga Raj Pokhrel, Ph.D. (Assoc. Prof.)** and **Bodh Babu Bhattrai, Ph.D. (Asst. Prof.)** this M.Sc. thesis submitted by **Swikar Giri** entitled **"The Biological Properties of Aegle marmelos (L.) Correa and HR-LCMS based metabolites profiling,"** is forwarded by Department of Chemistry, Birendra Multiple Campus (BMC) to the office of Dean, IOST, T. U.

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This dissertation entitled **"The Biological Properties of Aegle marmelos (L.) Correa and HR-LCMS based metabolites profiling,"** by **Swikar Giri**, under the supervision of ***Ganga Raj Pokhrel, Ph.D. (Assoc. Prof.)*** and ***Bodh Babu Bhattarai, Ph.D. (Asst. Prof.)*** Department of Chemistry, Tribhuvan University is submitted for the partial fulfillment of the Master of Science (M.Sc.) degree in Chemistry. It is importantly note that this work has not been previously presented to any other institution for pursue any degree.

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To sum up, I want to express my appreciation to the Central Department of Chemistry, Department of chemistry (BMC), Department of microbiology (BMC) whohad provide me all the required facilities during the course of this study. I am grateful not only with all Faculties but also with administrative office staffs too. I am alsothankful with my colleagues SachinSilwal, SabiThapa, SushmaSubedi and family who supported me through the entire journey, believing me and my work.

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Thank you
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ABSTRACT

The Biological Properties as well as HR-LCMS analysis was carried out on the methanolic extract of Aegle marmelos (L.)Correa Leaves.Extract of the leaves was prepared using Soxhlet extractor. The leaves of Aegle marmelos has been employed as therapeutics for various kinds of disease traditionally over the years. This study employed the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, a sustainable and cost-effective method, to assess the antioxidant properties of the plant extract. This plant extractwas evaluated across six biological samples, namely S. typhi, E.Coli, K. pneumonia, P. aureginosa, B. subtilis, and S. aureus, revealing a substantial inhibition of growth in all of these biological specimens.

To investigate further insights into the plant extract,Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Antioxidant Activity (TAA) and DPPH scavenging activitiesweremeasured using a double-beam UV-visible spectrophotometer. This analysis unveiled the antioxidant and antimicrobial activitiesinherent in the selected plant parts.

Additionally, High-Resolution Liquid Chromatography-Mass Spectrometry (HR-LCMS) analysis of the sample was carried out for the selected plant parts.

TheRotary evaporator was used to evaporate the solvent from the extract and18.45% yield extract was obtained from the dried leaves. The methanolic extract was subjected on the analysis of Total Phenolic Content (TPC) (59.307 ± 1.058 mg GAE/g dryweight extract), Total Flavonoid Content (TFC) (132.8±1mg QR/g dry weightextract), Total Antioxidant Activity (TAA)(309.444±2.341mg AAE/g dryweight extract) and IC_{50} value of DPPH scavenging activity was found to be 238 µg/mL

Notably, antibacterial activity of the extract against *Bacillus subtilis*and *Salmonella typhi*was found to be with zone of inhibition (ZOI)of 26±4.242mm and 14±1.414mm respectively.

Intriguingly, the High-Resolution Liquid Chromatography-Mass Spectrometry (HR-LCMS) analysis of the crude extract identified a total of 60 compounds among them 45 are known and 15v are unknown compounds. Some of these compounds have potential applications. So that the plant extract can be used for the preparation of medicine and natural product-based therapeutics. 3-beta,6-betaDihydroxynortropane, Manumycin A, Cepharenthine, Ritterazine A, and Saphenamycin has potential effect for the treatment of diseases like leukemia, cell cancer, constipation, skin allergy, inflammatory diseases etc.

To sum up, plant extract has 15 unknown compounds that may exhibit potential biological activities. So further works are necessary for the characterization of these unknown molecules and precisely disclose their biological activity.

Keywords: HR-LCMS, Anti-microbial, TAC, TFC, TPC, Phytochemical screening.

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LIST OF SYMBOLS

°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
M	Mu
%	Percentage
µL	Microliter
µg/mL	Microgram per milliliter

LIST OF ABBREVIATIONS AND ACRONYMS

A1	Trail 1
A2	Trail 2
A3	Trail 3
GEN	Gentamycin
AMT	Anti microbial Testing
HEp	Human epithelial
ATCC	American Type Culture Collection
ºC	Degree Celsius
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
Cm	Centimeter
DMSO	Dimethyl Sulphoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
D/W	Distilled Water
EC50	Effective concentration, 50%
FCR	Folin-Ciocalteau Reagent
FDA	Food and drug Administration
Fe (II)	Ferrous
g	Gram
g/L	Gram per Liter
GAC	Gallic acid concentration

GAE	Gallic Acid Equivalent
h	Hours
IC50	Inhibitory Concentration for 50% inhibition
kg	kilogram
L	Liter
LC-MS	Liquid chromatography - mass spectrometry
M	Meter
Mcg	Microgram
MHA	Muller Hinton Agar
mg	Milligram
ml	Milliliter
Mm	Millimeter
Mm	Milli-molar
mol.wt.	molecular weight
nm	Nanometer
ppm	Parts per million
RE	Rutin Equivalent
rpm	Revolution per minute
RT	Room Temperature
SD	Standard Deviation
TAC	Total antioxidant capacity
TFC	Total Flavonoid Content
TPC	Total Phenolic Content

UV	Ultraviolet
V/v	volume by volume
w/v	weight by volume
WHO	World Health Organization
ZOI	Zone of Inhibition

TABLE OF CONTENTS

1. CHAPTER I: INTRODUCTION

1.1 Geography of Nepal

Nepal is landlockedHimalayancountry situated in south East Asia and lies between two huge nationsIndia and China. Due to wide geographical variation, Nepal is rich in its biodiversity. Nepal is well known for its prosperous biodiversity having an area of 147,181 km². The altitude variation starts from almost sea level (~70 meter) to the top of the world (8848 meter) having climate difference, varied ecological habitats, rich off flora and fauna life. According to the report, Nepal covers 0.1% globe landmass out of which 3.2% of flora and 1.2% of fauna respectively (GON, 2014). The country has its total 6973 plants species out of which 10% (700) are of medicinal kind (Schippmann, et al., 2002), Which are distinct with specific adaptation features.

1.2 About Aegle marmelos

Aegle marmelos (L.) Correa commonly called 'Stone apple' or 'Bel' in Nepal belongs to the family Rutaceae. *A. marmelos* is native in terai region of Nepal, but widely found throughout the Indian Peninsula and in Ceylon, Burma, Bangladesh, Thailand, India and Indo-China (phytochemicals, 2022, June 19). According to Charaka (1500 BC), the high priest of Ayurveda, Bel is one of the most important medicinal plants in Ayurveda, which has been in existence for a long time and is extensively used by inhabitants of India (Culie I, 1982). The Juice of ripen as well as unripen fruit of Bel shows significant role for treating diarrhea, constipation and stomach ache (Culie, 1982). Bel fruit, leaves, roots, bark and seeds are used as a folk medicine in Ayurveda for the treatment various ailments (National trust conservation, 2020). In additionstudies have also shown that the new activated carbon prepared from non-usable Bel- fruit shell is an efficient low cost adsorbent to remove the toxic metal Chromium from aqueous phase, thereby preventing environmental pollution (Boning, Charles, 2006). Bel also reduced the cumulative number of tumors, the tumor burden per animal and tumor yield suggesting its usefulness as a chemo preventive agent (Behera, et al., 2014).

The extract obtained from wild varieties of *A. marmelos*is used to control diarrhea and sun burn of the skin. Studies also showed that it has anticancer andantipyreticcharacteristics. Leaves extract is supposed to have ulcer healing, chemoprevention, anti-genotoxic, diuretic, anti-inflammatory, and anti-fertility (Behera, et al., 2014)characteristics. But, Elaborated Anti-oxidant activity, anticancer activity, Anti-microbial and LC-MS analysis of *A. marmelos* extract in methanol has not been reported yet. In this study *A. marmelos* leaves extract in methanol and water as solvent is subjected to anti-oxidant, anti-microbial and anti-cancer test along with LC-MS analysis.

1.2.1 Classification of Aegle marmelos

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Sub family: Aurantioideae

Tribe: Clauseneae

Genus: *Aegle*

Species: *marmelos*

Binomial name: *Aegle marmelos* (L.) Correa (Sekar,et al., 2011)

1.2.2 Morphology of Aegle marmelos L. correa

A.Marmelos is a deciduous plant which may grow up to 13m in height with slender drooping branches and rather open, irregular crown. The tree has unusual branches with aromatic leaves, sweet-scented, and greenish-white flowers. Bark of A. marmelos is pale brown or grayish, smooth or finely fissured armed with long straight spines. Leaf is trifoliate, alternate; each leaflet is 5-4cmlong, ovate with tapering or pointed tip and round base. Each leaf has 4 to 12 pairs of side veins which are joined at the margin.

Flower is bisexual, actinomorphic, bracteates, hypogynous, gamosepalous,having five lobed calyx, polypetalous with 5 petals, imbricate. Likewise, stamen having 4 mm of lengthwith polyandrous condition, numerous, basified anddehiscence longitudinally. Gynoecium has capitate stigma hosting terminal style. The fibrous yellow pulp is very aromatic. Boning (2006) indicates that the flavor is "sweet, aromatic and pleasant, although tangy and slightly astringent in some varieties. It resembles a marmalade made, in part, with citrus and, in part, with tamarind." (Boning & Charles, 2006).

1.2.3Oxidative stress and antioxidant:

Oxidative stress arises from an imbalance between the production of oxidizing agents and the body's ability to neutralize them with antioxidants. This imbalance causes cellular damage and is linked to various health conditions. Neurodegenerative diseases like Parkinson's and Alzheimer's, gene mutations leading to cancers, chronic fatigue syndrome, fragile X syndrome, heart and vascular issues including atherosclerosis, heart failure, heart attacks, as well as inflammatory diseases, are some of the pathophysiological outcomes attributed to oxidative stress. Oxygen, while essential for life, can also generate reactive oxygen species that harm living organisms (Upadhya N et al. 2013). Reactive Oxygen Species (ROS) are highly reactive and unstable ions generated within living organisms due to oxidative stress, both from normal cell metabolic processes and pathological factors. Exogenous pollutants such as cigarette smoke, ultraviolet rays, ionizing radiation, and toxic chemicals also contribute to their formation. Examples of ROS include hydrogen peroxide (H2O2), free radicals like the hydroxyl radical (•OH), superoxide anion (O2-), alkoxy, and proxy radicals (RO• and ROO•). These ROS pose significant harm by interacting with various cellular components such as DNA, proteins, lipids, and fatty acids. This interaction primarily leads to lipid peroxidation, reduces membrane fluidity, induces DNA mutations, and plays a major role in the progression of life-threatening conditions like cancer, inflammation, cardiovascular diseases, and infections(Lahlou, 2013). The DPPH free radical (DPPH•) scavenging assay is widely adopted by researchers due to its simplicity, reliability, and reproducibility in assessing the in-vitro antioxidant activity of both individual compounds and plant extracts. DPPH (1,1-diphenyl-2-picrylhydrazyl) is

recognized as a stable radical owing to the paramagnetism created by its unpaired electron. This assay serves as a quick and efficient method for evaluating the capacity of substances to neutralize or scavenge free radicals, offering valuable insights into their potential antioxidant properties. Its gram molecular weight is 394.32g. Its solution in methanol appears as a deep violet color solution and shows a strong absorption band at 517 nm (Molyneux, 2002) DPPH assay relies on the principle of DPPH radicals being reduced and losing color in the presence of antioxidants. Antioxidants exhibit this effect by either donating a hydrogen atom or an electron to the DPPH free radical, thereby neutralizing its free radical nature and converting it into a stable diamagnetic molecule. This reduction in color or decolorization is indicative of the antioxidant's ability to counteract free radicals, demonstrating its potential in neutralizing oxidative stress. The scavenging of these free radicals reflects the antioxidant capacity or potential of the tested sample, showcasing its effectiveness in preventing, interpreting, and even repairing damage within biological systems caused by oxidative stress (Jamuna et al. 2012)

1.2.4 Antimicrobial Activity:

Among the pivotal discoveries of the 20th century in medicine, antimicrobial agents stand out significantly. Pathogenic microbes like fungi, bacteria, viruses, and nematodes pose substantial health risks worldwide, causing severe infections in humans. Unfortunately, due to the misuse and overuse of antimicrobial agents, these microbes have developed resistance to numerous antibiotics, leading to significant challenges in effectively treating infectious diseases. Moreover, antibiotics come with a range of side effects. For instance, Staphylococcus aureus, a gram-positive bacterium, is accountable for various skin conditions like pimples, boils, and wound infections. While some common antibiotics are effective against S. aureus, the urgency remains to discover alternative drugs to combat it before it develops resistance to existing treatments (Fruq et al. 2010).

Plants generate secondary metabolites that exhibit activity against pathogenic microbes. These compounds offer advantages such as reduced side effects, cost-effectiveness, and enhanced patient tolerance due to their lower molecular weight. When faced with microbial threats, plants synthesize these phytochemicals in response to infections. This

suggests their potential effectiveness against a wide array of microorganisms(Panghal et al. 2011).

Plant pheno serve multiple defensive roles, displaying potent antibacterial and antifungal capabilities. These compounds, found within the cytoplasm of epidermal cells and on plant surfaces, act as shields against pathogens. Phenolic acids like pyrogallol and gallic acid, as well as flavonoids such as rutin, myricetin, and daidzein, demonstrate significant efficacy as antibacterial agents. While the precise mechanism behind the antimicrobial activity of secondary metabolites remains somewhat unclear, phenolic compounds primarily exert their antimicrobial effects by acting as nonionic surface-active agents. This action disrupts the lipid-protein interface, leading to protein denaturation and enzyme inactivation within the pathogens. Additionally, phenols modify membrane permeability, which inhibits active transport, disrupts oxidative phosphorylation coupling, and causes membrane damage, ultimately resulting in the loss of vital metabolites (Manoj et.al. 2012).

1.2.5 Objective of the Study:

The objectives of this research are as follows:

i. To collect *Aegle marmelos L. correa* plant sample.
ii. To prepare the methanolic extract of plant using Soxhlet extractor.
iii. To estimate the amount of total phenolic content (TPC) and total flavonoids content of the crude extract.
iv. To analyze the biological properties of the plant extract on the Pathogenic bacteria.
v. To determine antioxidants properties of extract.
vi. To make chemical profiling of the plant extract using HR-LC-MS.

1.2.5 Rationale of the study:

In Nepal, medicinal plants stand as the second most valuable bio-resource following water resources. For many years, over 80 percent of drugs have been derived and formulated using natural products sourced from medicinal plants. However, in the

Arghakhanchi district, certain plants remain largely unexplored, particularly in relation to their local and traditional uses for treating various non-fatal diseases.

One such plant under investigation is *Aegle marmelos,* which holds significant ethnobotanical relevance. Studies suggest the presence of crucial phytoconstituents like alkaloids, flavonoids, phenolic compounds, and terpenoids within this plant species. Additionally, there's speculation regarding potential biological activities associated with this plant, including antioxidant, antidiabetic, and antibacterial properties. These suppositions form the primary hypothesis for this research, aiming to uncover and validate the medicinal potential of *Aegle marmelos,* based on its traditional use and phytochemical composition.

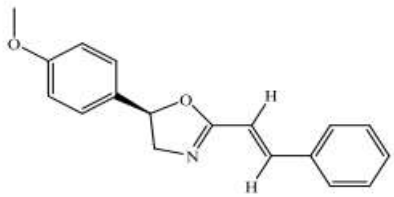
2 CHAPTER II: LITERATURE REVIEW

A. marmelos is distinguished by its unique branching pattern, fragrant leaves with a sweet aroma, and the presence of greenish-white flowers. This deciduous tree reaches a height of over 8 meters, making it of medium size. The leaves are arranged alternately and have a pale green color, featuring a trifoliate pattern with a length of 3.2 cm and an elongated petiole. Additionally, there are two lateral leaflets, almost sessile, measuring 4.1 cm by 2.2 cm and 5.7 cm by 2.8 cm respectively. These leaflets have an ovate to lanceolate shape and are adorned with reticulate, pinnate venation. Notably, A. marmelos contains a variety of important phytochemicals, including alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids, and biologically active steroids. These compounds serve as a valuable resource for the treatment of various medical conditions (Behera, et al., 2014).

Padalia, et al prepared the extract ofleaves, stem and fruit of A. Marmelos by decoction extraction method. The extracts obtained from A. marmelos leaves and stems, when combined with PE, exhibited a modest level of synergy in their activity against S. aureus, while the fruit extract did not display any synergistic effects against the four bacteria tested. On the other hand, A. marmelos leaf, stem, and fruit extracts, in combination with GEN, demonstrated significant synergistic activity against all four bacteria, with the highest activity observed against S. aureus. When evaluated with AMT, the leaf and stem extracts did not exhibit any synergistic antifungal activity, while the fruit extract displayed a minor degree of synergy against C. albicans. In contrast, when KT was employed, A. marmelos leaf, stem, and fruit extracts exhibited substantial synergistic activity against both fungi, with the maximum activity observed against C. neoformans (Padalia, et al., 2016).

It was discovered that the leaves of A. marmelos contain several noteworthy compounds, including alkaloids, mermesinin, rutin, and β-sitosterol-β-D glucoside. Among these, a rare alkaloid called shahidine exhibited activity against select Gram-positive bacteria. This alkaloid possesses an unstable oxazoline core and is prominently present as a major constituent in the fresh leaves of A. marmelos. Interestingly, it is moisture-sensitive but

remains stable when dissolved in dimethyl sulfoxide. Its chemical structure was meticulously elucidated through spectroscopic analysis (Faizi, et al., 2009).



Shahidine

The cytotoxicity of methanol and acetone extracts of *Aegle marmelos* was investigated against HEp-2, MDA-MB-231, and Vero cell lines. The IC_{50} value for the methanol extract of *Aegle marmelos* was found to be 47.08 µg/mL, while the IC_{50} value for the acetone extract of Aegle marmelos was 79.62 µg/mL. This indicates that HEp-2 cells exhibited greater sensitivity to the acetone extract. Both extracts of *Aegle marmelos* displayed cytotoxic effects on cancer cells; however, Vero cells demonstrated resistance and were able to survive for 24 hours under these conditions (seemaisamy,et al., 2019)

In a live animal study, Swiss albino mice afflicted with Ehrlich ascites carcinomaisadministered an intraperitoneal injection of a hydro alcoholic extract of *Aegle marmelos* at a dosage of 400 mg/kg. This treatment led to a notable and statistically significant extension of the median survival time, reaching 28 days following tumor inoculation, as compared to the control group that received a saline injection (Jagetia, et al., 2005).

A lectin extract derived from fruit, exhibiting an IC_{50} value of 3.36 µg/mL, demonstrated superior effectiveness in enhancing glucose uptake by yeast cells when compared to the conventional medication, metformin. This investigation revealed that the fruit extract from *Aegle marmelos* exhibited hypoglycemic properties, which could be ascribed to its anti-oxidative capabilities and its substantial concentration of bioactive components (Abdallah,et al., 2017).

Additionally, in the study of Ahmad, W., et al. the alcoholic extract obtained from *Aegle marmelos* leaves exhibited notable inhibitory activity against the enzymes α-amylase and α-glycosidase, as evidenced by IC_{50} values of 46.21µg/mL and 42.07 µg/mL,

respectively. Furthermore, *Aegle marmelos* significantly reduced the elevated levels of reactive oxygen species (ROS) induced by high glucose and enhanced glucose consumption in HepG2 cells ($p < 0.05$) (Ahmad, et al., 2021; Amir, et al., 2021).

In the study, the methanol extract derived from *Aegle marmelos* leaves displayed significant in vitro antimalarial activity, particularly against Plasmodium falciparum. Notably, this extract exhibited low cytotoxicity. The anti-plasmodial activity of *Aegle marmelos*, as indicated by its IC_{50} value, was determined to be 7.00µg/mL, highlighting its promising potentialityas an antimalarial agent(Kamaraj et al., 2012). Mice infected with the parasite and administered C. longa treatment did not exhibit a suppressive effect. In contrast, treatment with *Aegle marmelos* at dosages of 20 and 40 mg/kg body weight successfully inhibited the parasite infection in these mice. Thus*Aegle marmelos*exhibits potent antioxidant and anti-plasmodial properties (KettawanAikkarach, 2012).

Anti-microbial and anti-fungal activitiestested*on Candida albicans, Aspergillus niger, Aspergillus fumigatus, and Staphylococcus aureus* displayed different minimum inhibitory concentrations (MIC) values of 19.50 g/mL, 39.00 g/mL, 62.5 g/mL, and 1.25 g/mL, respectively (Gheisari, et al., 2011). When tested against *Candida albicans and Aspergillus niger,* the substance exhibited practical Minimum Fungicidal Concentration (MFC) values of 2.50 mg/mL and 5.00 mg/mL, respectively. In this review, it was observed that the decoction displayed greater efficacy against fungi compared to foodborne pathogenic bacteria. The control drug ampicillin was found to be similarly effective in inhibiting the growth of pathogenic bacterial strains, akin to the ethanolic extract derived from *A. marmelos* fruit pulp (Behera, et al., 2014). The antibacterial efficacy of various *A. marmelos*leaf extracts was evaluated through the disc diffusion method, employing multi-resistant bacterial strains. The results indicate that the ~~pet~~ ether extract displays a more pronounced antibacterial effect when compared to standard streptomycin (Gavimath, 2008).

In contrast to the standard Gallic acid with an IC_{50} value of 1.10 ± 0.08 µmmarmelosindemonstrated significant antioxidant activity, displaying an IC_{50} value of approximately 15.40 ± 0.32 µM in the ethyl acetate extract of Bel fruit. Notably,

marmelosin was found to exhibit superior antioxidant properties compared to the standard Gallic acid (Pynam et al., 2008).

The *A. marmelos* fruit decoction demonstrated noteworthy antioxidant activity, with an IC_{50} value of 17.37 ± 2.71 mg/mL. Additionally, its antioxidant capacity was measured at 379.9 ± 28.28 mg AEAC (Ascorbic Acid Equivalent)/ 100g, which was compared to the standard ascorbic acid(Gheisari, et al., 2011).

An evaluation of the dried fruit pulp of *A. marmelos* was conducted to assess its topical characteristics. Swiss albino mice were subjected to an acute oral toxicity test using an ethanol extract of the dried fruit pulp from *A. marmelos* at doses of 550.00 mg/kg and 1250.00 mg/kg. The results of the study indicate that the extract poses no discernible hazards at these dose levels. Throughout the 14-day trial period, the behavior and physiological activity of the mice remained unchanged (Rakulini, 2019).

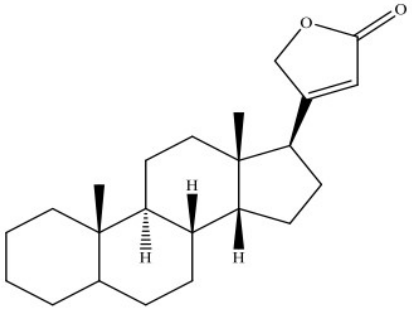
The findings further revealed that the LD_{50} (median lethal dose) of the test extract is highly significant. In the oral acute toxicity study, no toxic symptoms, alterations in behavior, or mortality were observed even at the higher dose of 1250.00 mg/kg. Consequently, it can be concluded that the ethanolic extract of *A. marmelos* dried fruit pulp has no biologically significant toxic effects on the mice below the LD_{50} threshold (Rakulini, 2019).

Yield of the hydro-distilled essential oil from a particular sourcewas determined to be 0.90% (v/w). A total of 31 different components were identified in the essential oil, collectively representing 97.44% of its composition. Notable components included p-mentha-1, 4(8)-diene (33.2%), limonene (13.1%), p-cymen-alpha-ol (9.5%), γ-gurjunene (7.9%), β-phellandrene (4.30%), and β-pinene (2.00%). These components were found to be the major constituents of the essential oil sourced from the Western Ghats region. Furthermore, it was reported that this essential oil demonstrated significant anticancer activity against human cervical cancer (Panda, et al., 2009)

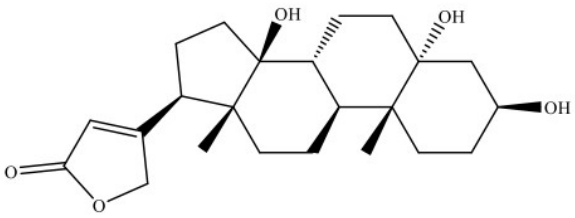
Leaves extract of *A. marmelos*that contained cardenolideperiplogenin, exhibited protective effect against doxorubicin-induced cardiotoxicity and lipid peroxidation (LPO) in rats. The administration of doxorubicin led to cardiac and hepatotoxicity, which was

characterized by significant biochemical changes, including elevated levels of serum creatine kinase-MB (CK-MB) and glutamate-pyruvate transaminase (SGPT)(Panda, et al., 2009).

The study of three different concentrations of periplogenin (i.e.12.50 mg/kg, 25.00 mg/kg, and 50.00 mg/kg) ~~and~~ found that the 25.00 mg/kg dosage proved to be the most effective in mitigating the adverse effects induced by doxorubicin. Periplogenin, ~~as~~ isolated from *A. marmelos* leaves, exhibited superior therapeutic potential when compared to vitamin E, a well-known antioxidant. This suggests that periplogenin, at a moderate dose, holds promise for preventing cardiovascular problems associated with doxorubicin in rats, (Panda, et al., 2009).



Cardenolide



Periplogenin

Phytochemicals are those chemical constituents that are produced by plants that help them to thrive pathogens and predator. They are bioactive chemicals of plant origin regarded as secondary metabolites because the plants that make them may have little need for them. These constituents work with different nutrients produced in plant parts to form natural defense system in plants. They are naturally produced by plant in their different parts such as leaves, root, stem, fruit, flower etc. The crude extract of plant is then subjected to test the secondary metabolites such as alkaloids, flavonoids, tannis, saponins, phenolic compounds, cardiac glycosides, etc. Phytochemical screening test method was employed by the procedure given by I. Culie, 1982 (Methods for studying Drugs, personal communication).

The quantification of total flavonoid content is achieved through the Aluminum Chloride colorimetric method. This method operates on the fundamental principle that aluminum chloride establishes a stable complex with the C-4 keto group and, alternatively, either the C-3 or C-5 hydroxyl group of flavonoids.

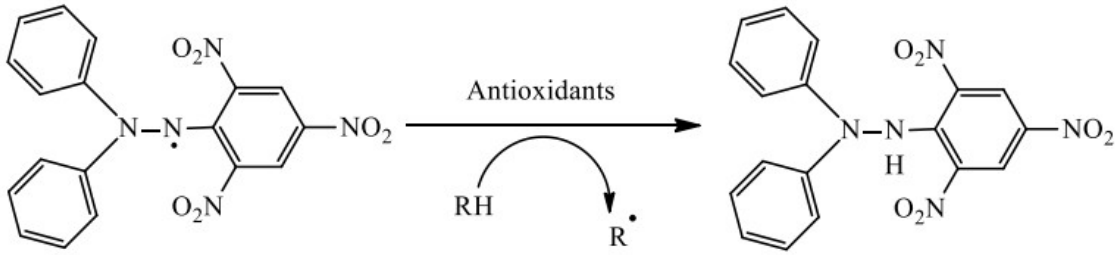
The determination of total phenolic content (TPC) relies on the utilization of the Folin-Ciocalteu (F-C) method. This particular technique serves as a means to quantify the presence of phenolic compounds within natural substances, such as extracts from various plants. Employing a UV spectrophotometer, the F-C method gauges the absorbance of these phenolic compounds. The measured absorbance corresponds directly to the TPC, expressed in terms of Gallic acid equivalence. In an alkaline environment, the F-C reagent is reduced by phenolic compounds, giving rise to a distinctive blue complex with its peak absorbance occurring at 765 nm. To ensure precision in the results, various factors warrant thorough investigation, including the optimal absorption wavelength, the duration required for color development, and the appropriate volume ratio between alkali and the F-C reagent.

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl-hydrate) free-radical method is an antioxidant assay that relies on electron transfer reactions. Initially, it produces a violet solution in alcohol, but in the presence of an antioxidant molecule, this violet solution transitions into a colorless one. Unlike most other free radicals, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is considered a stable free radical due to the delocalization of its lone pair of electrons across the entire molecule. This delocalization is responsible for the deep violet color exhibited by DPPH, characterized by an absorption band centered at approximately 517 nm in a methanol solution (Prieto, et al., 1999).

When a DPPH solution encounters a substance capable of donating a hydrogen atom, owing to the continued presence of the picryl residue, a reduction reaction occurs. This reduction reaction leads to a noticeable change in the solution, as the violet color gradually fades into a pale color, indicating the reduction of DPPH and the antioxidant activity of the tested compound (Karthika, et al., 2016).

The assessment of the free radical scavenging capability of plant extracts was conducted using the stable free radical known as DPPH. This particular method is both

uncomplicated and highly sensitive. The underlying principle of this assay hinges on the idea that a substance capable of donating hydrogen functions as an antioxidant. It gauges the presence of compounds that can effectively scavenge radicals. The figure below illustrates the mechanism through which DPPH˙ accepts hydrogen atoms from an antioxidant. The efficacy of the antioxidant is directly proportional to the reduction in DPPH˙ levels. DPPH˙ exhibits a prominent absorption peak at 517 nm, characterized by a purple hue. This color undergoes a transition to yellow as DPPH transforms back into its original form upon absorbing hydrogen from an antioxidant. Notably, this reaction adheres to a stoichiometric relationship with respect to the number of hydrogen atoms assimilated. Consequently, one can readily assess the antioxidant's effectiveness by monitoring the reduction in UV absorption at 517 nm (Molyneux et al., 2003).



DPPH free radical (Violet color) DPPH non radical (Pale yellow color)

RH= Antioxidant radical scavenger R˙= anti-oxidant radical

Figure 1: Reaction mechanism of DPPH

The Total Antioxidant Capacity (TAC) by the phosphomolybdenum method is a common laboratory technique used to assess the overall antioxidant potential of a

sample, such as a biological fluid or a plant extract. This method is based on the reduction of molybdenum (Mo) VI to molybdenum (Mo) V by the antioxidants present in the sample. The reduction reaction results in the formation of a green phosphate/Mo (V) complex, which can be quantified spectrophotometrically.

3 CHAPTER III: MATERIALS AND METHOD

3.1 Plant collection and Identification.

Leaves of *A. marmelos* were collected from Tilottama-1, Rupandehi, Nepal (Latitude: 27°37'9.44"N; Longitude: 83°28'30.09"E and Altitude: 426 ft from sea level). Plant was officially verified by National Herbarium and Plant Laboratories (NHPL),Godawari, Lalitpur, Nepal with Specimen voucher number 198198.

3.2 Bacterial cultures

Six bacterial strains were collected from Department of Microbiology, Bharatpur Hospital, Chitwan.

3.3 Extract preparation

Freshleaves of plant free from fungal infection was taken and washed with tap water followed by distilled water. Then, it was subjected for shade dry in dark room for 10 days with continuous air flow through fan at normal room temperature. After that it was grinded into fine powder of mess size 40 with the help of grinder, 186.4 gm of powder was packed on thimble and soxhlet was allowed to run with 420mL of methanol as solvent at controlled temperature till the original color of methanol appears on siphon tube of soxhlet.

The extract was filtered through Whatmann no. 1 filter paper and concentrated through Rotary evaporator (Model: IKA RV 10). The extract was stored in sterile bottle at refrigerator at -4^{o}C for further analysis.

3.4 Phytochemical Screening

3.4.1Test for Alkaloid's

3.4.1.1Preparation of Stock Solution

0.450g of crude extract was dissolved in 18mL of 1% HCl and kept in water bath at temperature of 35^{o}C for 5 minutes. Then, the stock solution was stored at room temperature.

3.4.1.2Mayers Test

Reagent preparation

Firstly, Mercuric chloride solution was prepared by dissolving 1.358g of mercuric chloride in 60mL of distilled water.

Secondly Potassium iodide solution was prepared by dissolving 5g of potassium iodide in 10 mL of distilled water.

Finally working solution was prepared by mixing both of the above solution and distilled water was added to make the final volume of 100mL.

Test

2mL of Extract solution was mixed with 1mL of Mayer's solution. Test was done on three trail basis and result was analyzed.

\3.4.1.3 Hager's Test

Preparation of reagent

1gm of picric acid was dissolved in 100mL of distilled water

Test

2mL of extract solution was mixed with 1mL Hager's solution. Test was done on three trail basis and result was analyzed.

3.4.1.4 Wagner's Test

Preparation of reagent

1.27g of iodine and 2 g of potassium iodide were mixed well with 100mL of distilled water.

2mL of extract solution was mixed with 1mL of Wagner's solution.Three replicate experiments were performed and resultswere analyzed.

3.4.2 Test for Flavonoids

3.4.2.1 Preparation of Stock solution

0.304gm of crude extract was dissolved in 36mL of distilled water and heated in water bath for 5minutes at 35^{o}C. Then, it is stored at normal room temperature.

3.4.2.2 Alkaline reagent test

Preparation of reagent

1.0319gm of NaOH(97%purity) was dissolved in 50mL of water to make 2% solutionofNaOH.

2mL of extract solution was mixed with few drops of 2% NaOH solution. Three replicate tests were performed and result was analyzed.

3.4.2.3 Lead Acetate Test

Preparation of reagent

5.0789gm of lead acetate having the purity 98.5% was dissolved in 50mL of water to make 10% of lead acetate solution.

2mLof extract solution was mixed with few drops of 10% Lead acetate solution. Three replicate tests were performed and result was analyzed..

3.4.2.4 Pew's Test

0.1gm of zinc powder was added to 2mL of extract solution followed by addition of 8ml Conc. HCl. Three replicate tests were performed and result was analyzed.

3.4.3 Test for Phenols

3.4.3.1 Preparation of Stock solution

0.304gm of crude extract was dissolved in 36mL of distilled water and heated in water bath for 5minutes at 35^{o}C. Then, it is stored at normal room temperature.

3.4.3.2 $Fecl_3$ Test

Preparation of reagent

5.101gm of ferric chloride having the purity of 98.5% was dissolved in 50mL water to make 10% ferric chloride solution.

2mL of Extract solution was dissolved in few drops of 10% $FeCl_3$ solution. Three replicate tests were performed and result was analyzed.

3.4.3.3 Liebermann's Test

0.1gm of sodium nitrite was dissolved in 2mL of extract solution followed by addition of 8mL of Conc. HCL. Then it was heated to its boiling point followed by addition of excess 10% NaOH. Three replicate tests were performed and result was analyzed.

3.4.4 Lead Acetate Test

Preparation of reagent

5.0789gm of lead acetate having the purity 98.5% was dissolved in 50mL of water to make 10% of lead acetate solution.

2mL of extract solution was mixed with few drops of 10% Lead acetate solution. Three replicate tests were performed.

3.4.5 Test for carbohydrates

Preparations of stock solutionof the plant extract.

0.304gm of crude extract was dissolved in 18mL of distilled water and kept on water bath at temperature of 35^{o}C for 5 minutes and stored in normal room temperature.

3.4.5.1 Molish's Test

Preparation of reagent

3.75gm of α-Nepthanol was dissolved in 25mL of 99% pure ethanol.

2mL of extract solution was mixed with 2mL of alpha Nepthanol reagent followed by the addition of 8mL conc. H_2SO_4 along the side of test tube. Three replicate tests were performed and result was analyzed.

3.4.5.2 Benedict's test

2mL of extract solution was mixed with few drop of Benedict's reagent followed by gentle heating.Three replicate tests were performed and result was analyzed.

3.4.5.3 Fehling's Test

Preparation of 10% NaOH

10.309gm of NaOH was dissolved in 100mL of distilled water to make 10% NaOH.

Preparation of 10% HCl

27.027mL of HCl was dissolved in 100mL of distilled water to make 10% HCl.

2mL of extract solution was mixed with 1mL of 10% HCl followed by the addition of 10% NaOH. Then, 1mL Fehling solution A and 1ml Fehling solution B was added and heated gently for 2mins. Three replicate tests were performed and result was analyzed.

3.4.6 Testing for amino acid

Preparationsof stock solutionof the plant extract.

0.304gm of crude extract was dissolved in 18mL of distilled water and kept on water bath at temperature of 35^{o}C for 5 minutes and stored in normal room temperature.

3.4.6.1 Millon's Test

2mL of extract solution was mixed with 2mL of Millon's reagent. Test was done on three trail basis and result was analyzed.

3.4.6.2 Ninhydrin Test.

2mL of extract solution was dissolved in 2mL of Ninhydrin solution. Three replicate tests were performed and result was analyzed.

3.4.7 Test for protein

3.4.7.1 Xanthoproteic Test

2mL of extract solution was mixed with 2mL of Conc. HNO_3. Three replicate tests were performed and result was analyzed.

3.4.8 Test for tannin's

Preparation of stock solutions of the extract.

0.304gm of crude extract was dissolved in 18mL of distilled water and kept on water bath at temperature of 35^{o}C for 5 minutes and stored in normal room temperature.

3.4.8.1 $FeCl_3$ Test

2mL of extract solution was mixed with 2 drops of $Fecl_3$ solution. Three replicate tests were performed and result was analyzed.

3.4.8.2 Potassium Dichromate Test

Preparation of 10 % $KMnO_4$Solution.

10.10gm of potassium dichromate was dissolved in 100 mLof distilled water to make 10% potassium dichromate.

3mL of extract solution was dissolved in 1mL of 10% aqueous potassium dichromate solution. Three replicate tests were performed and result was analyzed.

3.4.9 Gelatin Test

Preparation of stock solutionof the extract.

0.304gm of crude extract was dissolved in 18mL of distilled water and kept on water bath at temperature of 35^{o}C for 5 minutes and stored in normal room temperature.

Preparation of 10% NaCl

10.10gm of NaCl was dissolved in 100mL of distilled water in volumetric flask and stored in normal room temperature.

Preparation of 1% gelatin solution

1gm of gelatin was dissolved in 100mL of distilled water

2mL of extract solution was mixed with 1% gelatin solution followed by addition of 10% NaCl solution. Test was done on three trail basis and result was analyzed.

3.4.10 Test for Quinone's

Preparation of extract stock solution

10mg of crude extract was dissolved in 6mL of isopropyl alcohol and stored in normal room temperature in dark room.

2mL of extract solution was mixed with 3 drops of conc. H_2SO_4 followed through side wise addition.Three replicate tests were performed and result was analyzed.

3.4.11 Test for Anthraquniones

Preparation of extract stock solution

3mL of aqueous extract was dissolved with 3mL of benzene and filtered with Whatsmann No.1 filter paper and subject to testing.

3.4.11.1 Brontrager's Test

1 mL of extract solution was mixed with 5% H_2SO_4. Boil the mixture and filter it. After then, add 2mL of chloroform and allow it to stand for 5 minutes followed by addition of dilute ammonia solution to lower layer. Three replicate tests were performed and result was analyzed

3.5 Total Phenolic Compounds Determination

3.5.1 Preparation of Folin-Ciocalteau reagent.

1.00 mL of FCR was diluted 10 timeswith distilled water.

3.5.2 Preparation of standard Gallic acid.

1.00 g of Gallic acid was dissolved in 1.00 L of distilled water to make a stock solution of concentration of 1000µg/mL. Then different concentrations of Gallic acid solution (40, 80,120,160,200 and 240 µg/mL was prepared.

3.5.3 Measurement of Total Phenolic content.

The concentration of total phenolic content in sample was determined by following equation by expressing in milligram of Gallic acid.

$$TPC = (C \times V)/ m$$

Where C = concentration of Gallic acid from curve

V = volume of extract (mL)

m = mass of plant extract (g)

3.5.4 General protocol for total phenolic content

TPC were determined using the protocol mentioned in 2.00 mLof extract was added to 2.00 mL of Folin- Ciocalteu reagent and the mixture was incubated for 7 min in room temperature. Then, 2.00 mL of 0.01M Sodium carbonate was added to the mixture and was again incubated for 5 more minutes. After the incubation, 12.5mL of distilled water was added to resulting mixture and the absorbance was measured at 790nm using UV/VIS Spectrophotometer (T80+, pg instruments). Gallic acid was used as standard and calibration curve were prepared (40, 80,120,160,200,240 mg/mL). The amount of TPC in extract was calculated and expressed as mg of Gallic acid equivalent per mg of dried sample (mg GAE/mg ds)(Alvarado-Lopez et al.2019)

3.6 Total Flavonoid Compound Determination

3.6.1 Preparation of standard quercetin solution

2.00g of quercetin was dissolved in 1.00 L of distilled water to make a stock solution of concentration of 1000µg/mL.Then different concentrations of quercetin solution (250, 500, 750, 1000, 1500, 2000µg/mL) was prepared.

3.6.2 Measurement of Total Flavonoid content

The concentration of total flavonoid content in sample was determined by following equation by expressing in milligram of quercetin.

$$TFC = (C \times V)/m$$

Where C = concentration of quercetin from curve

V = volume of extract (mL)

m= mass of plant extract (g)

3.6.3 General protocol of total flavonoid content

TFC was determined by following the protocol of (Zhishen, et al., 1999). In the analysis, 500 µL of the extract was combined with 1 ml of distilled water, and then 75 µL of a 5% sodium nitrite ($NaNO_2$) solution was introduced. Following this step, the reaction mixture was allowed to sit undisturbed for approximately 6 minutes at a temperature of 25°C. Subsequently, 150 µL of a 10% aluminum chloride ($AlCl_3$) solution was added, and the mixture was left undisturbed for an additional 5 minutes at room temperature. Afterward, 500 µL of 1.00 M sodium hydroxide (NaOH) was incorporated into the mixture. To complete the process, the total volume of the reaction mixture was adjusted to 2.5 mL using distilled water, and the absorbance was measured at 510 nm employing a double beam UV-VIS spectrophotometer (T80+, pg instruments). The determination of flavonoid content was carried out by referencing a standard curve prepared using quercetin in the range of 250,500,750,1000,1500,2000µg/mL. The amount of TFC in extract was calculated and expressed as mg of quercetin equivalent per mg of dried sample (mg QUE/mg ds) (Zhisen et al. 1999).

3.7 Total Antioxidant capacity

3.7.1 Preparation of Molybdate reagent solution

In a 50.00 mL solution, 1.00 mL of 0.6 M sulfuric acid, 1.00 mL of 28.00 mM sodium phosphate, and 1.00 mL of 4.00mM ammonium molybdate were gently mixed with

distilled water. This solution was prepared by adding the components to a total volume of 20 mL and then diluting it further with distilled water.

3.7.2 Preparation of Standard Ascorbic acid solution

1.50g of Ascorbic acid was dissolved in 1l of distilled water to make a stock solution of concentration of 1500µg/mL. Then different concentrations of ascorbic acid solution (376, 528, 660, 792, 924 and 1056µg/mL) was prepared.

3.7.3 Measurement of Total Antioxidant capacity

The concentration of total antioxidant capacity in sample was determined by following equation by expressing in milligram of ascorbic acid.

$$TFC = (C \times V)/m$$

Where C = concentration of ascorbic acid from curve

V = volume of extract (mL)

m = mass of plant extract (g)

3.7.4 General protocol for total antioxidant capacity

The assessment of total antioxidant capacity was conducted using the phosphomolybdenum method, following the protocol outlined by (Prieto et al. 1999). 0.1 mL aliquot of the extract was combined with 1.00 mL of a reagent solution consisting of 0.6 M sulfuric acid, 28.00mM sodium phosphate, and 4.00mM ammonium molybdate. The reaction mixtures were then placed in test tubes and subjected to incubation at 95ºC for a duration of 90 minutes. Following the incubation, the absorbance of each solution was measured at a wavelength of 695 nm utilizing a double-beam UV-visible spectrophotometer (T80+, pg instruments), with a blank reference containing 0.10 mL of methanol in place of the extract. This reference was used to account for background absorbance.

The antioxidant activity of the extract was then determined by comparing its absorbance to the calibration curve ranging the concentration from 376µg/mL, 528µg/mL, 660µg/mL, 792µg/mL, 924µg/mL, 1056µg/mL and expressing it in equivalents of

ascorbic acid. To quantify the antioxidant activity, the results were expressed in terms of the gram equivalent of ascorbic acid (Prieto, et al., 1999).

3.8 DPPH scavenging activity.

3.8.1 Preparation of standard 0.01M DPPH solution

A 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by dissolving 3.9 mg of DPPH in a 100.00 mL volumetric flask filled with methanol and covered with aluminum foil.

3.8.2 Preparation of Standard Ascorbic acid solution

2.00g of Ascorbic acid was dissolved in 1.00L of distilled water to make a stock solution of concentration of 2000.00µg/mL. Then different concentrations of ascorbic acid solution (1000, 1200, 1400, 1600, 1800 and 2000µg/mL) was prepared.

3.8.3 Preparation of Extract solution

2.00g of sample extract was dissolved in 1.00Lof distilled water to make a stock solution of concentration of 2000µg/mL. Then different concentrations of extract solution (1000, 1200, 1400, 1600, 1800 and 2000µg/mL) was prepared.

3.8.4 General protocol of determination of DPPH scavenging activity.

Free radical scavenging activity of theextract was measured as described by Brand, et al., 1995 and Williams, et al., 1995). 3.00mL of DPPH solution was mixed with 77.00 mL of an extract solution. This resulted in a final mass ratio of extracts to $DPPH^{\cdot}$ of approximately 3:1, 1.5:1, or 0.75:1, depending on the specific assay. Subsequently, the samples were kept in a dark environment at room temperature for 15 minutes, after which the reduction in absorption was measured by UV spectrophotometer (T80+, pg instruments) at 515nm and quantified the result. To establish a baseline, a blank sample containing the same quantity of methanol and $DPPH^{\cdot}$ solution was prepared and assessed. This experiment was conducted in triplicate to ensure accuracy. The radical scavenging activity was determined using the following formula.

$$\% \text{ Inhibition} = [(A_B - A_A)]/ A_B] \times 100$$

Where: A_B= absorption of blank sample (t= 0 min)

A_A = absorption of extract solution (t= 15min)

3.9 Anti-microbial Test

Anti-microbial activity of extract of *Aegle marmelos* against five bacterial species were performed using agar well diffusion method. Zone of Inhibition (ZOI) of extract against different bacteria was determined by the method (Cavalieri, et al., 2005; Valgas, et al., 2007).

3.9.1 Preparation of Stock solution

100.00 mg/mL of stock solution was prepared in volumetric flask by dissolving 1000mg of plant extract in 10.00mL of 100% methanol as solvent with continuous shaking over vortex. After complete dissolution of the extract, it was stored in refrigerator at -4^{o}C.

3.9.2 Testing bacteria

Two gram positive bacteria (*Bacillus subtilis and Staphylococcus aureus)* and four gram negative bacteria (*Escherichia coli, Salmonella typhi, and Pseudomonas aeruginosa and Klebsiellapneumoniae)* were collected from Department of microbiology, Bharatpur Hospital, Chitwan, Nepal.

3.9.3 Preparation of Standard Culture Inoculum

First, the bacteria to be tested were aseptically collected using an inoculating loop from the primary culture plate. Subsequently, these bacterial samples were carefully transferred into a test tube containing 10.00 mL of sterile Muller Hinton broth. The test tube was then placed in an incubator and incubated overnight at a temperature of 37 ºC. This incubation process was necessary to adjust the turbidity of the bacterial suspension to the 0.50 McFarland standards, resulting in a final inoculum concentration of 1.50×108 CFU/mL.

3.9.5 Preparation of Mueller Hinton Agar (MHA) Plates

To create the culture medium, 19.00 grams of media were suspended in 500 mL of distilled water ~~with~~in ~~a~~ conical flask. The mixture was heated until it boils to facilitate

dissolution and ensure sterility. Subsequently, the medium was sterilized by autoclaving at 121.00°C for duration of 15 minutes. After autoclaving, the medium was allowed to cool to approximately 50.00°C. At this point, it was poured into sterile petri-plates in volumes of 20.00 mL per plate. The plates were then left undisturbed to allow for solidification of the medium.

3.9.6 General protocol of anti-microbial qualitative screening

Sterile Mueller-Hinton Agar (MHA) plates, each with an approximate thickness of 4.00mm, were carefully dried at the appropriate temperature to eliminate any excess moisture on the surface of the agar medium. These agar plates, intended for the assay, were meticulously labeled with the name of the bacteria and the corresponding code for the testing disc.

To initiate the testing process, fresh inoculums were prepared to match the established turbidity standard. A sterile cotton swab was selected and immersed into the prepared bacterial inoculum. Subsequently, the swab, now containing the bacteria, was transferred to the surface of the Petri dish. Any surplus inoculum was meticulously removed by pressing and gently rolling the swab against the upper inner wall of the tube, just above the liquid level. The swab was then carefully spread across the entire surface of the agar plate. To ensure even distribution, the plate was rotated at a 60-degree angle after each swabbing. Finally, the swab was gently run along the edges of the agar surface. After this thorough inoculation process, the plates were left undisturbed to air-dry for a few minutes at room temperature, with their lids securely closed.

In each inoculated media plate, precisely three wells were created using a sterile cork borer, specifically No. 6. These wells had a standardized diameter of 6.00 mm and were accurately labeled. Subsequently, three different solutions were loaded into these wells using a micropipette:

1. A 50 µL volume of the working solution of the plant extract.

2. Another 50 µL volume of 100% methanol, designated as the negative control (N).

3. Lastly, 50mcg Erythromycin (antibiotic) for *Klebsiella pneumonia*, *Salmonella typhi* and *Bacillus subtilis*, 25µg Vancomycin (antibiotic) for *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and 10µgof Gentamicin for *Escherichia coli* was assigned as the positive control (P).

The plateshaving plant extract, negative control and positive control were then left undisturbed for approximately half an hour, with their lids securely closed, to allow for the diffusion of the extract into the agar medium. Following this, the plates were subjected to overnight incubation at a temperature of 37°C.

After the appropriate incubation period (approximately 24 hours), the plates were meticulously examined for any signs of bacterial growth inhibition, which was indicated by the presence of a clear zone around the wells. The size of this zone of inhibition was measured, and the antibacterial activity was quantified in terms of the average diameter of the zone, measured in millimeters. If no zone of inhibition was observed, it was interpreted as the absence of antibacterial activity. The measurements of the zone of inhibition were taken using a millimeter ruler, and the mean value was recorded for analysis.

4 CHAPTER IV: RESULT AND DISCUSSION

4.1 Extraction Yield

The total percentagesyield of *Aegle Marmelos* (L.) *Correa* methanol extract is found to be 18.863 % (w/w) which is represented below.

Table 1:Extraction yield percentage of methanolic extract of Aegle Marmelos (L.) Correa

SN	**Name of plant**	**Dry Weight of plant**	**Weight of crude extract**	**Yield %**
1.	*Aegle Marmelos* (L.) *Correa*	186.4	35.161	18.863

4.2 Phytochemical Screening

Phytochemicals screeningof the methanolic extract of Aegle Marmelos (L.) Correa are presented in the table below.

Table 2: Phytochemical screening of methanolic extract of Aegle Marmelos (L.) Correa

SN.	Phytochemicals	Result	Conclusion
1	Alkaloids	+	Alkaloids is present
2	Flavonoids	+	Flavonoid is present
3	Phenols	+	Phenols is present
4	Carbohydrates	-	Carbohydrates is absent
5	Protein and amino acid	+	Protein and amino acid is present
6	Tanis	+	Tannin is present
7	Gelatin	-	Gelatin is absent
8	Quinone's	+	Quinone's is present
9	Anthraquinone's	-	Anthraquinones is absent
10	Cameron's	+	Cameron's is present
11	Fixed oil and Fats	+	Fixed oil and fats is present
12	Phlobatannis	-	Phlobatannins is absent
13	Gum and Resin	-	Gum and Resin is absent
14	Saponins	+	Saponins is present.

4.3 Total Phenolic content analysis

The quantification of total phenolic content was carried out by assessing it in milligrams of Gallic acid equivalent, utilizing the Gallic acid calibration curve. The absorbance of each solution was then meticulously measured and documented in the subsequent manner:

Table 3:Absorbance of Gallic acid for TPC

S.N	Concentration (µg/mL)	A1	A2	A3	Avg	S.D	AVG±S.D
1	0	0	0	0	0	0	0
2	40	0.066	0.056	0.069	0.064	0.007	0.064±0.007
3	80	0.132	0.148	0.142	0.141	0.008	0.141±0.008
4	120	0.163	0.16	0.16	0.161	0.002	0.161±0.002
5	160	0.254	0.237	0.243	0.245	0.009	0.245±0.009
6	200	0.291	0.288	0.295	0.291	0.004	0.291±0.004
7	240	0.325	0.391	0.392	0.369	0.038	0.369±0.038

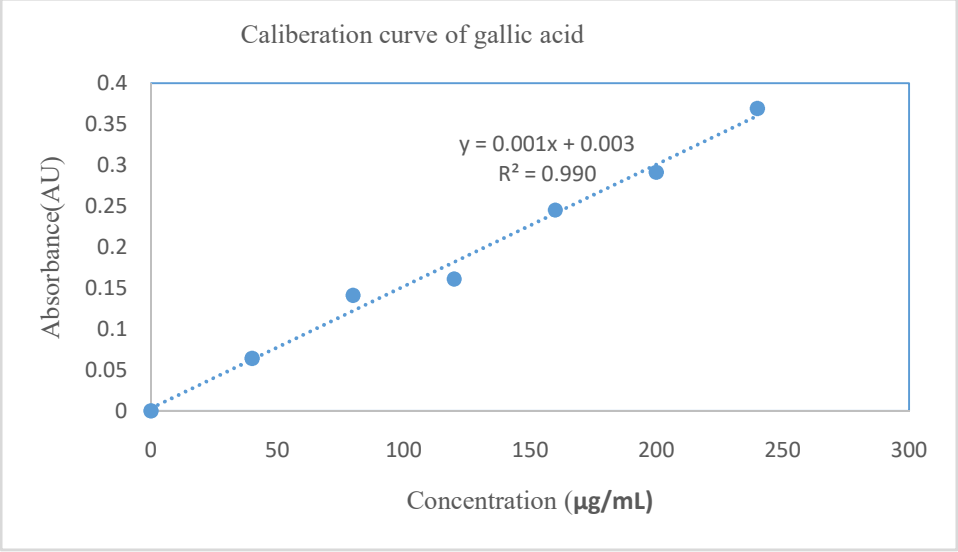


Figure 2:Calibration curve of Gallic acid at different concentration

The assessment of Gallic acid concentration in the methanolic extract of *A. marmelos* was conducted through the application of an equation derived from the standard Gallic acid curve, as illustrated in Figure. The equation used for this determination is as follows:

$$y = 0.0015x + 0.0032$$

$$R^2 = 0.9902$$

In this equation:

- Y represents the absorbance.
- X signifies the Gallic acid concentration (GAC) measured in µg/mL.
- The slope, denoted as 'm,' is equal to 0.0015.
- The y-intercept, referred to as 'c,' is 0.0032.

$$\text{Thus, } X = \frac{y+0.0032}{0.0015}$$

Table 4: TPC absorption of plant extract

S.N	Concentration	A1	A2	A3	TPC1	TPC2	TPC 3	AVE TPC±SD
1	Sample (1 mg/mL)	0.439	0.451	0.454	58.107	59.707	60.107	59.307±1.058

Above Table displays the total phenolic content *of A. marmelos* leaves, quantified in Gallic acid equivalent units by referencing a standard curve represented by the equation y = 0.0015x + 0.0032, with an R^2 value of 0.9902. The determined total phenolic content within the *A. marmelos* leaf was found to be 59.307±1.058mg Gallic acid equivalent per gram of dry extract.

4.4 Total Flavonoid content analysis

The determination of the total flavonoid content in the Leaves extract is evaluated on the basis of milligrams of quercetin equivalent (employing the quercetin calibration curve). Subsequently, the absorbance of each solution was carefully measured and recorded in the following manner:

Table 5:Total absorbance of quercetin for TFC

S.N	Concentration(µ/ml)	A1	A2	A3	Avg	SD	AVG±SD
1	0	0	0	0	0	0	0
2	250	0.21	0.19	0.196	0.199	0.01	0.199±0.01
3	500	0.388	0.395	0.371	0.385	0.012	0.385±0.012
4	750	0.551	0.509	0.518	0.526	0.022	0.526±0.022
5	1000	0.6	0.639	0.684	0.641	0.042	0.641±0.042
6	1500	0.965	0.969	1.01	0.981	0.025	0.981±0.025
7	2000	1.188	1.2	1.175	1.188	0.013	1.188±0.013

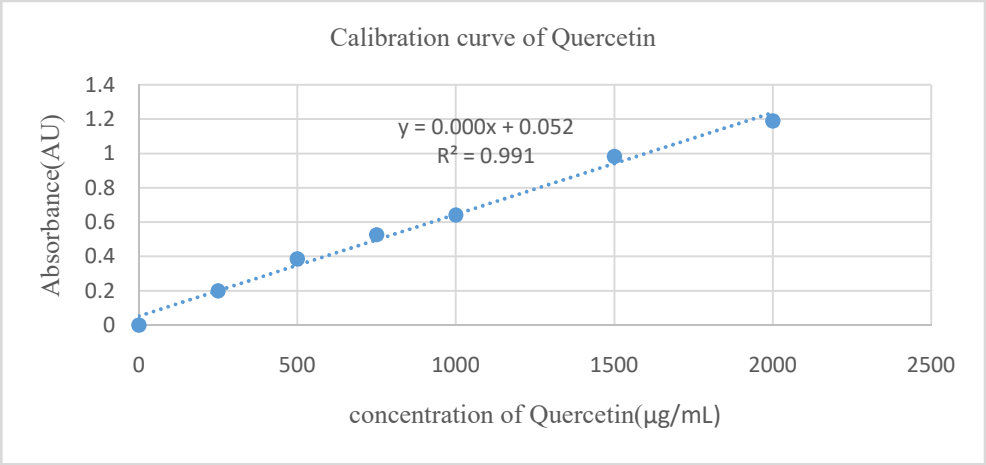


Figure 3: Calibration curve of quercetin.

The assessment of quercetin concentration in the methanol extract of *A. marmelos* was conducted through the application of an equation derived from the standard quercetin curve, as illustrated in Figure. The equation used for this determination is as follows:

$$y = 0.0006x + 0.0526$$

$$R^2 = 0.9915$$

In this equation:

- Y represents the absorbance.

- X signifies the quercetin concentration (QC) measured in µg/mL.

- The slope, denoted as 'm,' is equal to 0.0059.

- The y-intercept, referred to as 'c,' is 0.0526.

$$\text{Thus, } X=\frac{y+0.0526}{0.0006}$$

Table 6: TFC absorption of plant extract

S.N	Concentration	A1	A2	A3	TFC1	TFC2	TFC3	AVER TFC±SD
1.	sample(1mg/mL)	0.439	0.451	0.454	131.8	132.8	133.8	132.8±1

Above Table displays the total flavonoid content *of A. marmelos* leaves, quantified in quercetin equivalent units by referencing a standard curve represented by the equation y = 0.0006x + 0.0526, with an R^2 value of 0.9915. The determined total flavonoid content within the *A. marmelos* leaf was found to be 132.80 ±1mg quercetin pergram of dry extract.

4.5 Total antioxidant capacity analysis

The determination of the total flavonoid content involved quantifying it in milligrams of ascorbic acid equivalent, employing the ascorbic acid calibration curve. Subsequently, the absorbance of each solution was carefully measured and recorded in the following manner:

Table 7: Absorbance of ascorbic acid for TAC

S.N	Concentration(µ/ml)	A1	A2	A3	Average	SD	AVG±SD
1	0	0	0	0	0	0	0
2	376	0.492	0.499	0.515	0.502	0.012	0.502±0.012
3	528	0.741	0.744	0.749	0.745	0.004	0.745±0.004
4	660	1.01	0.98	1.05	1.013	0.035	1.013±0.035
5	792	1.154	1.153	1.149	1.152	0.003	1.152±0.003
6	924	1.411	1.42	1.35	1.394	0.038	1.394±0.038
7	1056	1.661	1.556	1.661	1.626	0.061	1.626±0.061

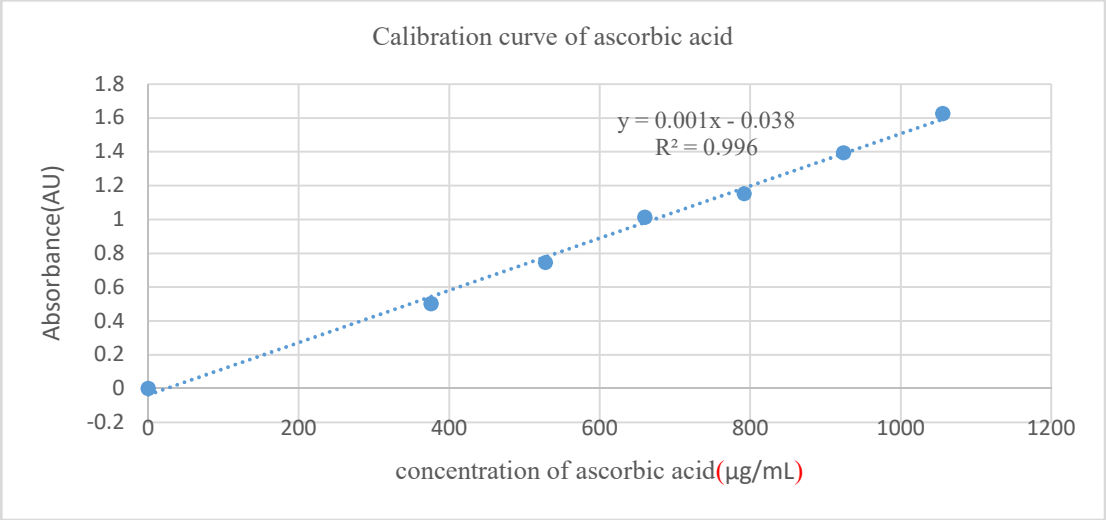


Figure 4: Calibration curve of ascorbic acid

The assessment of ascorbic acid concentration in the methanolic extract of *A. marmelos* was conducted through the application of an equation derived from the standard ascorbic acid curve, as illustrated in Figure. The equation used for this determination is as follows:

$$y = 0.0015x - 0.0385$$

$$R^2 = 0.996$$

In this equation:

- Y represents the absorbance.
- X signifies the ascorbic acid concentration (AAC) measured in µg/mL.
- The slope, denoted as 'm,' is equal to 0.0103.
- The y-intercept, referred to as 'c,' is -0.048.

$$\text{Thus, } X = \frac{y+0.0385}{0.0015}$$

Table 8: TAC absorbance of plant extract

S.N	Concentration	A1	A2	A3	TAC1	TAC2	TAC3	AV TAC±SD
1.	Sample (1 mg/mL)	0.426	0.422	0.469	309.667	307	311.667	309.444±2.341

Above table displays the total antioxidant content *of A. marmelos* leaves, quantified in ascorbic acid equivalent units by referencing a standard curve represented by the equation $y = 0.0015x - 0.0385$, with an R^2 value of 0.996. The determined total flavonoid content within the *A. marmelos* leaf was found to be 309.444±2.341mg ascorbic acid per gram of dry extract.

4.6 DPPH radical scavenging analysis

The antioxidant potential is inversely correlated with the IC_{50} value, which is derived through linear regression analysis of % inhibition versus antioxidant activity. A lower IC_{50} value signifies a higher level of antioxidant activity. These assessments were conducted using the standard procedure, with absorbance measurements taken at 517 nm (Perumal et al. 2018) (Williams et al. 1995). The determination of the DPPH radical scavenging activity involved quantifying it in milligrams. Subsequently, the absorbance of each standard ascorbic acid solution and plant extract was carefully measured and recorded in the following manner:

Table 9: Absorbance of Sample DPPH Scavenging

Concentration	Absorbance(Sample)					
	T1	T2	T3	Average	SD	% inhibition
1000	0.451	0.45	0.451	0.450	0	33.757
1200	0.388	0.387	0.388	0.387	0.000577	43.018
1400	0.301	0.3	0.302	0.301	0.001	55.756
1600	0.25	0.251	0.251	0.250	0.000577	63.155
1800	0.154	0.154	0.153	0.153667	0.000577	77.413
2000	0.099	0.1	0.101	0.1	0.001	85.301
Control	0.68	0.68	0.681	0.680333	0.000577	

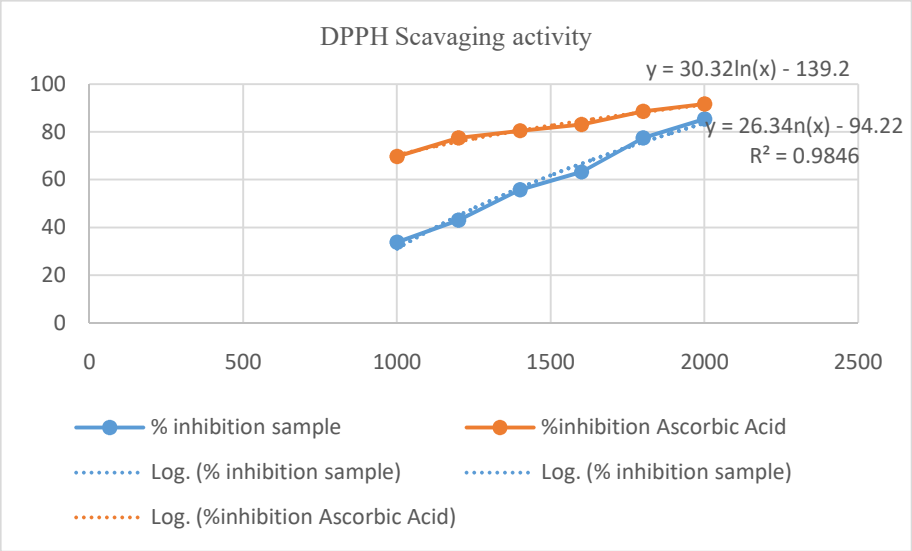


Figure 5:% inhibition of DPPH scavaging activity of sample and standard.

This research study reveals that the IC_{50} value for the methanol extract derived from *Aegle marmelos* was determined to be 238.00µg/mL. Thisresearch reveals that the antioxidant activity of the methanol extract of *A. marmelos*is of moderate range relative to the standard used.

4.7 Antimicrobial analysis

The in-vitro antimicrobial activity of the methanolic extract of *Aegle marmelos*is assessed against two gram-positive and four gram-negative bacteria using the agar well diffusion method. The resulting zones of inhibition (ZOI) were measured and are presented in tabular form in the respective table.

Table 10: Anti-microbial screening of methanolic extract of Aegle marmelos on different microbes

S.N.	Name of Bacteria	Standard (antibiotics)/Positive control ZOI(mm)	Average (mm)	Standard Deviation	Average ± SD(mm)
1.	*Klebsiella pneumonia*	11 (Erythromycin)	8.33	0	8.33 ± 0
2.	*Pseudomonas aeruginosa*	23 (vancomycin)	-	-	-
3.	*Staphylococcus aureus*	14 (Vancomycin)	10.33	0.707	10.33±0.707
4.	*Escherichia coli*	17 (Gentamicin)	-	-	-
5.	*Salmonella typhi*	25 (Erythromycin)	14	1.414	14±1.414
6.	*Bacillus subtilis*	24 (Erythromycin)	26	4.242	26±4.242

The results indicate that the methanolic extract of *Aegle marmelos* demonstrated significant inhibition in the growth of two gram-positive bacterium (*Staphylococcus aureus* and *Bacillus subtilis*) and two gram-negative bacterium (K*lebsiella pneumonia* and *Salmonella typhi*), with corresponding zone of inhibition (ZOI) of 10.33 ± 0.707, 26 ± 4.242 and 8.33 ± 0, 14 ± 1.414 mmrespectively. Notably, the plant extract exhibited a stronger antibacterial effect against two gram-negative bacterium (K*lebsiella pneumonia* and *Salmonella typhi*) and two gram-positive bacterium (*Staphylococcus aureus* and *Bacillus subtilis*). This suggests that the plant extract possesses potent antibacterial properties.

However, it is important to note that the methanolic extract of *Aegle marmelos* did not exhibit any inhibitory effect on *Pseudomonas aeruginosa* and *Escherichia coli*. This lack of inhibition could be attributed to the presence of an outer lipid membrane in gram-negative bacteria, which serves as an additional protective barrier, making them less susceptible to the extract's antibacterial activity.

4.8 HR-LC MS spectra analysis of methanolic extract of *Aegle marmelos*

The LC-MS chromatogram of the methanolic extract derived from *Aegle marmelos* reveals the detection of 36 identified compounds and 16 unidentified compounds with matching mass library data. Among these known compounds, 10 compounds were found to have significant medicinal value. Analysis identified the presence of 3beta,6 beta-Dihydroxynortropane (constituting 40.56% of the extract) and 3-O-Caffeoyl-1-O-methylquinic acid (making up 33.48% of the extract), as detailed below:

Table 11: List of most medicinal component identified in HR-LC MS Spectra of Aegle marmelos

S.N	Name of compound	Molecular Formula	Mass	Retention Time	Volume(%)
1	Phosphinothricin	$C_5 H_{12} N O_4 P$	181.2284	1.097	0.43
2	3beta,6beta-Dihydroxynortropane	$C_7 H_{13} N O_2$	143.0946	1.236	40.56
3	Aegle marmelos Alkaloid C	$C_{23} H_{27} N O_3$	365.1991	15.436	3.51
4	Tryptophyl-Aspartate	$C_{15} H_{17} N_3 O_5$	319.1168	10.299	14.34
5	Manumycin A	$C_{31} H_{38} N_2 O_7$	550.2679	10.364	1.55
6	3-O-Caffeoyl-1-O-methylquinic acid	$C_{17} H_{20} O_9$	368.1107	5.963	33.48
7	Saphenamycin	$C_{23} H_{18} N_2 O_5$	402.1216	3.509	4.09
8	Cepharanthine	$C_{37} H_{38} N_2 O_6$	606.2802	22.072	1.11
9	Tropisetron	$C_{17} H_{20} N_2 O_2$	284.1525	15.434	2.6
10	Promazine sulfoxide	$C_{17} H_{20} N_2 O S$	300.1296	16.805	4.09

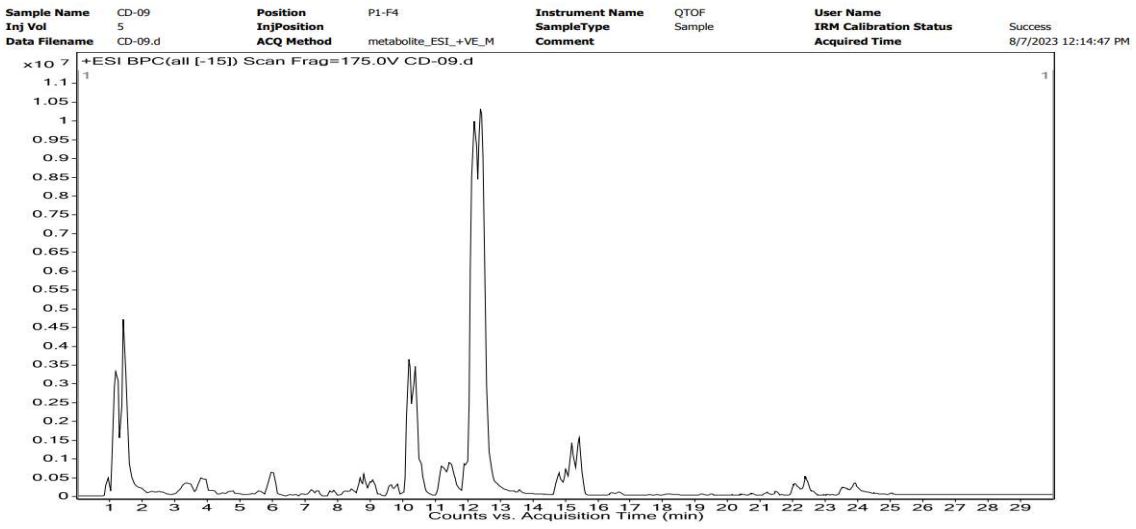


Figure 6: LCMS chromatogram of Aegle marmelos extract

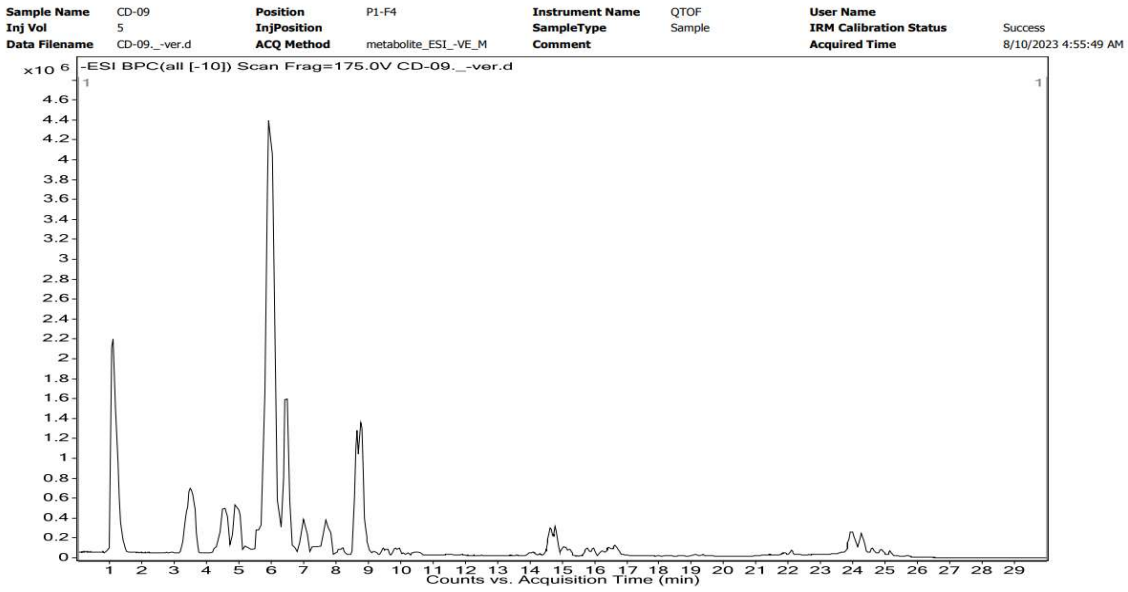


Figure 7:LC MS chromatogram of Aegle marmelos extract

Likewise, on further analysis of 36 identified compounds it was found 6 were alkaloids, 3 were flavonoids and 3 were glycosides. Further, details of groups and phytochemicals of identified compounds in +ESI and -ESI modeare tabulated below:

Table 12: List of –ESI mode compounds in HR-LC MS Spectra of Aegle marmelos with their chemical compositions

S.N	Name of compound	Chemical compositions
1	3-Fucosyllactose	Trisaccharide
2	TosyllysineChloromethyl Ketone	Alkylating Reagent
3	Pyriftalid	Herbicides(Lactones Class)
4	Saphenamycin	Antibiotics
5	Vinpocetine	Alkaloid
6	Mecarbinzid	Benzimidazole Fungicide
7	Gancaonin U	Flavanoid
8	6alpha-Fluoroprednisolone	Synthetic Steriods
9	Phthalocyanine	Tetra Pyyrole
10	Quinacridone	Fusion Acridone And Quinoline
11	3-O-Caffeoyl-1-O-methylquinic acid	Methyl Chlologenic Acid Ester
12	Calendoflavobioside	Flavanoids And Glycosides
13	Isoacteoside	Glycosides

14	Biorobin	Flavanoids
15	5-O-p-Coumaroylnigrumin	Carbohydtraes
S.N	**Name of compound**	**Chemical compositions**
16	3-Mercapto-3-methylbutyl formate	Carboxylic Ester
17	9-Hydroxy-7-megastigmen-3-one glucoside	Glycosides
18	17,21-Epoxy-9-fluoro-11beta-hydroxypregn-4-ene-3,20-dione	Steroids Ester
19	10-Oxo-11-octadecen-13-olide	Fatty Acids
20	LysoPE(0:0/14:1(9Z))	Lysophospholipid
21	Ritterazine A	Bis-Steroidal Pyrazine Alkaloid.

Table 13: : List of +ESI mode compounds in HR-LC MS Spectra of Aegle marmelos with their chemical compositions

S.N	Name of Compounds	Chemical Constituents
1	Phosphinothricin	Glutamic Acid
2	N-Acetyl-leucyl-leucine	Amino Acid Leucine
3	3beta,6beta-Dihydroxynortropane	Alkaloid
4	Pirimicarb	Carbamide Insecticide
5	Valganciclovir	L-Valyl Ester Of Ganciclovir
6	5-Methyl-THF	Folic Acid
7	Tryptophyl-Aspartate	Protein
8	Manumycin A	Microbial Metabolite
9	(+/-)-gamma-Lycoran	Unknown
10	1xi,3xi)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-3-carboxylic acid	B-Carboline Alkaloid
11	N4-(b-N-Acetyl-D-glucosaminyl)-L-asparagine	Carbohydrates
12	Tropisetron	Indolyl Carboxylate Ester,
13	Aegle marmelos Alkaloid C	Alkaloids
14	Promazine sulfoxide	Phenothiazines
15	Cepharanthine	Alkaloid

3-beta,6-beta-Dihydroxynortropane

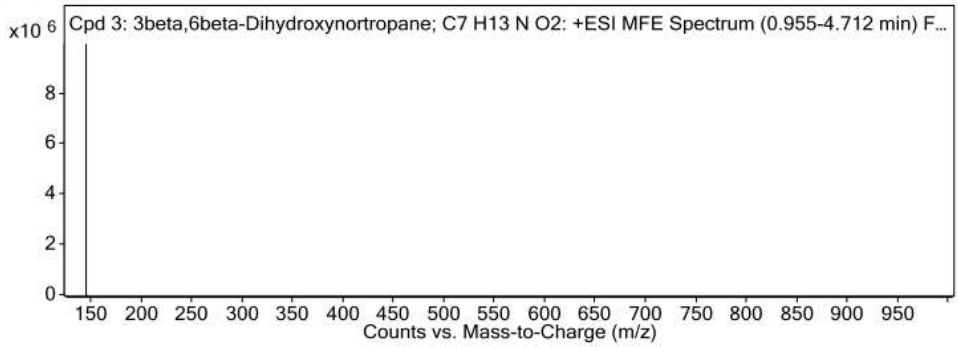
3-beta,6-beta-Dihydroxynortropane is classified as one of the organic compounds falling under the tropane alkaloids category. Tropane alkaloids are a group of organic compounds characterized by the presence of the nitrogenous bicyclic alkaloid parent molecule known asN-Methyl-8-azabicyclo[3.2.1]octane.

3-beta,6-beta-Dihydroxynortropane has been identified in fruits, although its precise quantification remains unreported. It is suggested that 3-beta,6-beta-

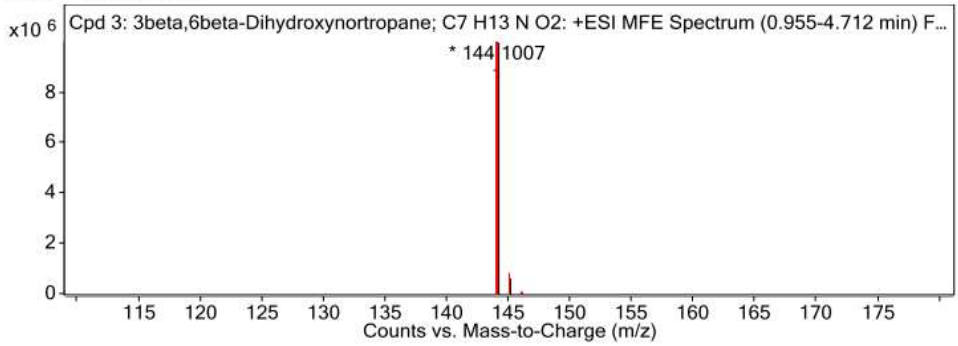
Dihydroxynortropane could serve as a potential biomarker for assessing the consumption of these food items.(HMDB. 2011).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 3: 3beta,6beta-Dihydroxynortropane; C7 H13 N O2	**3beta,6beta-Dihydroxynortropane**	144.1007	1.236	Find by Molecular Feature	143.0935

MFE MS Spectrum



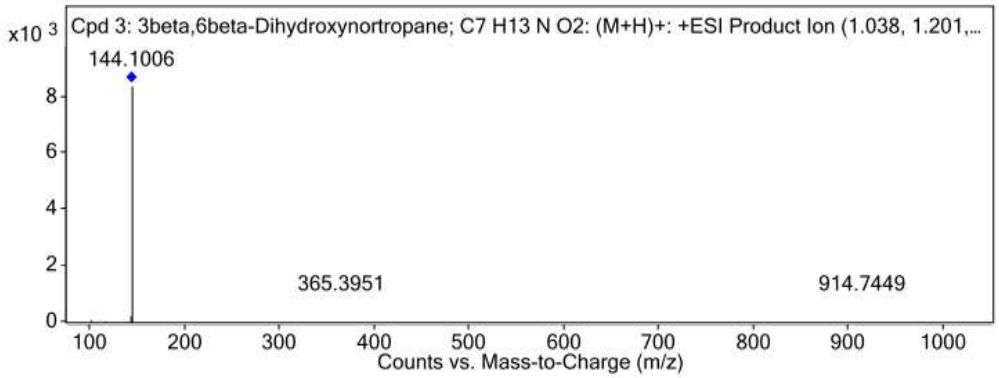
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
144.1007	1	9983482	C7 H13 N O2	(M+H)+
145.104	1	691204.96	C7 H13 N O2	(M+H)+
146.1057	1	53499.57	C7 H13 N O2	(M+H)+

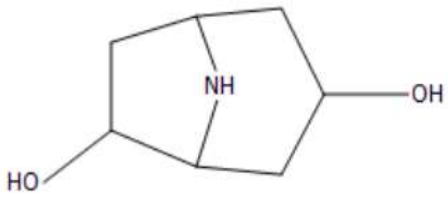
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
102.0533	1	113.9
115.0526	1	20.68
117.0679	1	28.31
124.9353	1	24.56
142.9447	1	35.64
143.0796	1	241.43
144.0799	1	123.09
144.1006	1	8387.32
144.1658	1	55.87
145.0648	1	18.65

Compound Structure

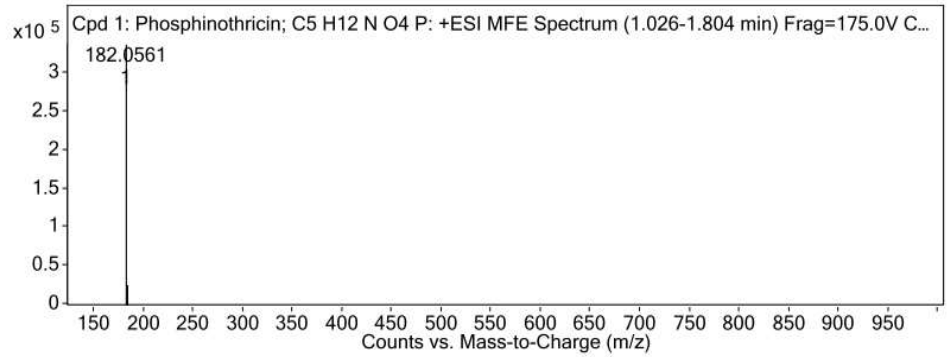


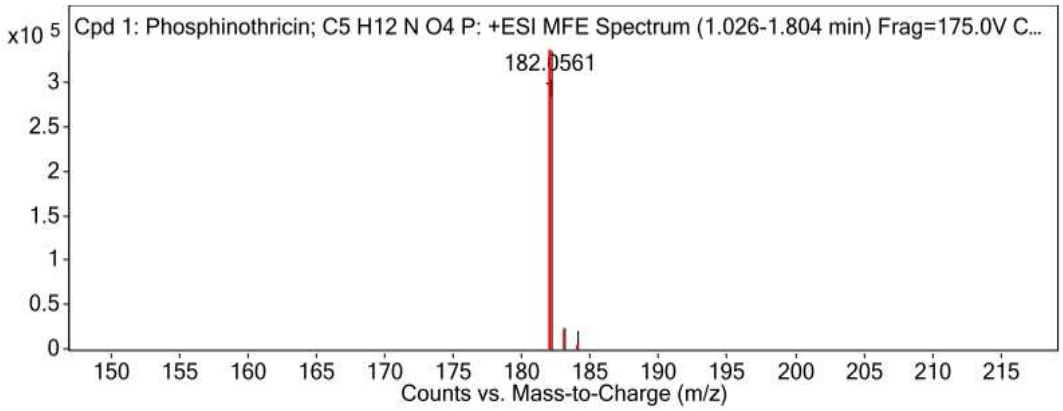
Phosphinothricin

Phosphinothricin is a wide-ranging herbicide that functions as a competitive inhibitor of glutamine synthetase. This herbicide is an excellent choice for selecting transformed plants. Phosphinothricin rapidly degrades in both soil and water, it possess a minimal risk of environmental contamination (Dragićević, et al., 2012).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 1: Phosphinothricin; C5 H12 N O4 P	**Phosphinothricin**	182.0561	1.097	Find by Molecular Feature	181.0485

MFE MS Spectrum

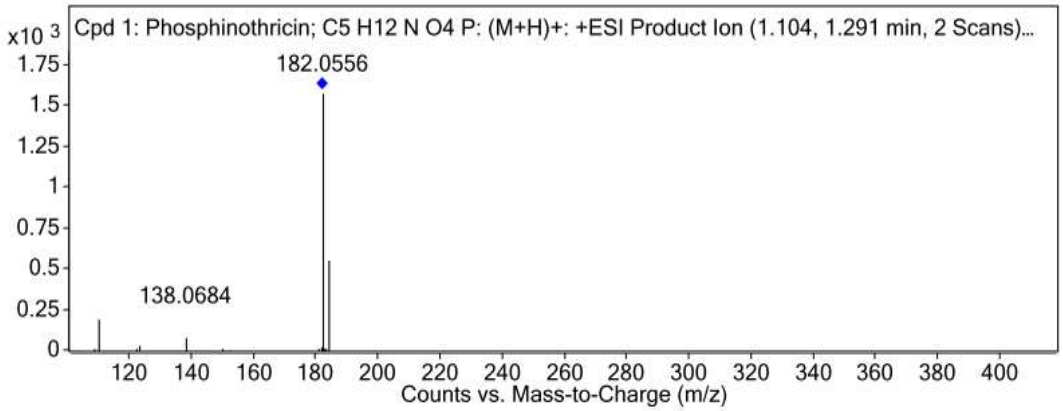




MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
182.0561	1	336882.53	C5 H12 N O4 P	(M+H)+
183.0593	1	25267	C5 H12 N O4 P	(M+H)+
184.0545	1	23345.59	C5 H12 N O4 P	(M+H)+

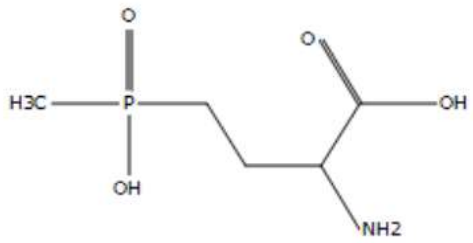
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
108.804	1	17.82
110.0961	1	196.19
122.0964	1	17.61
123.0774	1	34.26
138.0684	1	80.96
181.4519	1	24.52
182.0556	1	1579.77
182.1187	1	45.79
183.1117	1	21.81
184.1312	1	561.58

Compound Structure



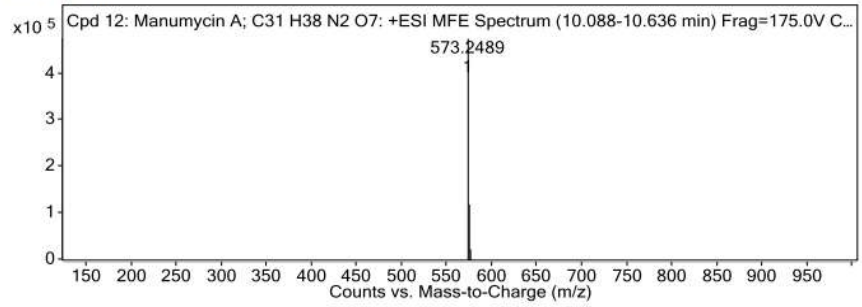
Manumycin A

Manumycin A (Man A) is a bacterial secondary metabolite initially discovered through a serendipitous screening process aimed at identifying inhibitors of farnesyl transferase (FTase)(Hara, et al., 1993). FTase plays a crucial role in the post-translational farnesylation of proteins, including the Ras protein family. Ras proteins are responsible for governing a multitude of cellular functions, such as cell growth, proliferation, and cell signaling. Their activity involves binding to and activating various effector proteins, which, in turn, oversee essential cellular processes such as transcription, translation, cell-cycle progression, and calcium signaling.

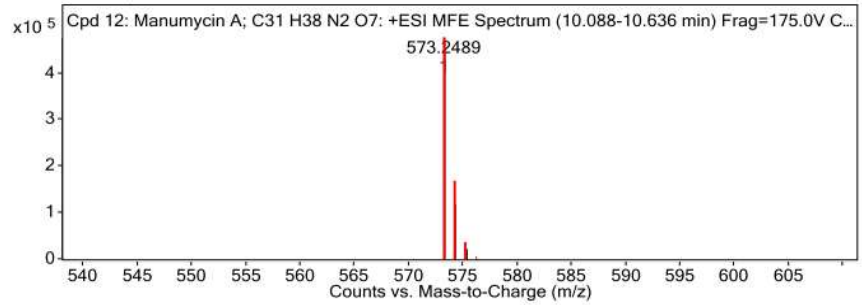
Manumycin A (Man A) demonstrates time-dependent inhibition of mammalian cytosolic thioredoxin reductase 1 (TrxR-1). Its IC50 is measured at 272 nmpreincubation and 1586 nmwithout preincubation. Thus it can be concluded that the anticancer properties of manumycin A (Man A) have been ascribed to its ability to inhibit farnesyl transferase (FTase), an enzyme responsible for the post-translational modification of Ras proteins. This inhibition of FTase can disrupt Ras protein function, contributing to its anticancer effects. (Tuladhar, et al., 2018).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 12: Manumycin A; C31 H38 N2 O7	**Manumycin A**	573.2489	10.364	Find by Molecular Feature	550.2595

MFE MS Spectrum



MFE MS Zoomed Spectrum

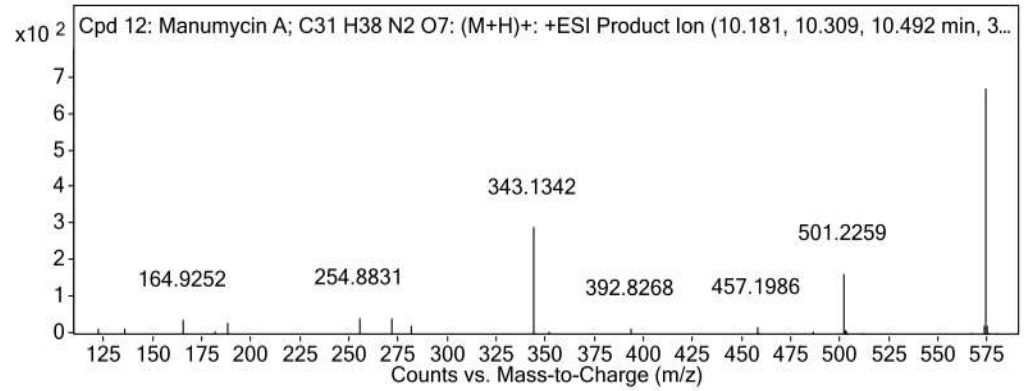


MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
573.2489	1	474655.41	C31 H38 N2 O7	(M+H)+
574.2517	1	120611.06	C31 H38 N2 O7	(M+H)+
575.2538	1	25517.77	C31 H38 N2 O7	(M+H)+
576.2558	1	3394.45	C31 H38 N2 O7	(M+H)+

MSMS Spectrum

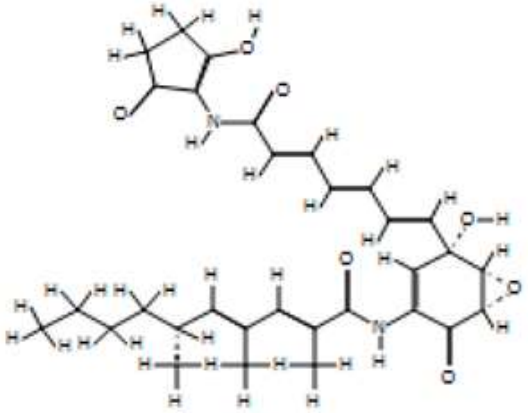
MSMS Spectrum



MS/MS Spectrum Peak List

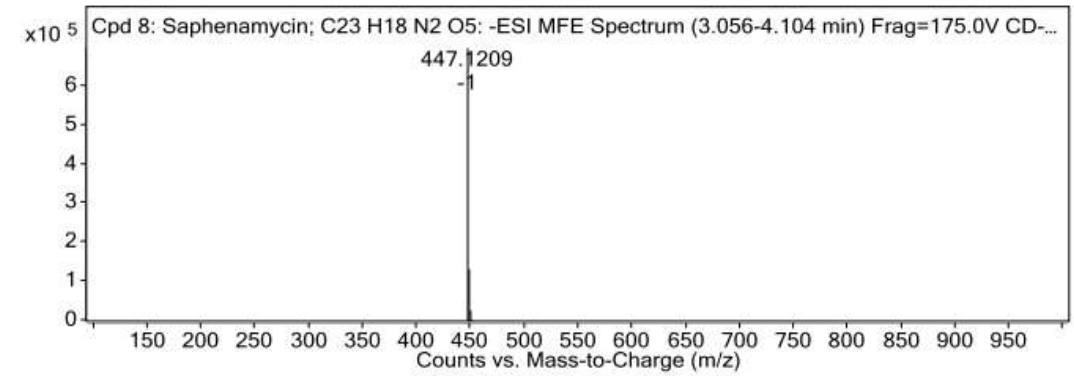
m/z	z	Abund
164.9252	1	38.64
188.069	1	31.99
254.8831	1	44.38
271.1094	1	42.25
281.0941	1	24.21
343.1342	1	291.29
501.2259	1	165.77
572.2078	1	25.29
573.2475	1	671.83
574.179	1	24.83

Compound Structure

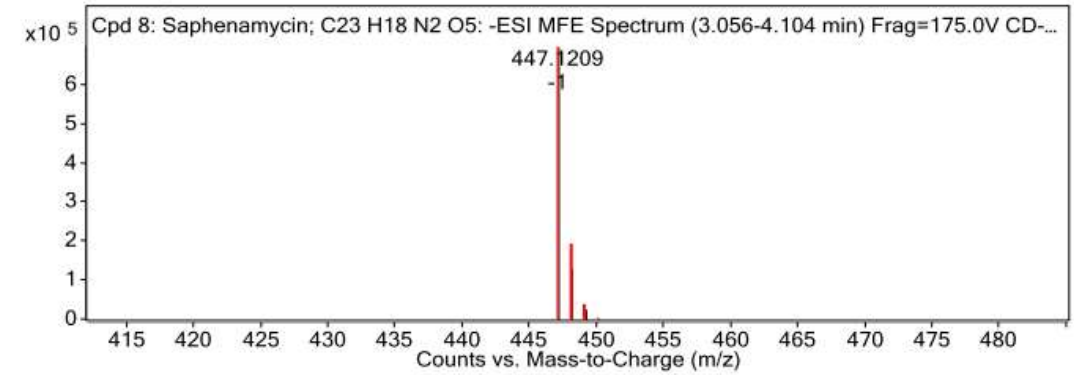


Saphenamycin

Saphenamycin, an antibiotic with the chemical composition $C_{23}H_{18}N_2O_5$, is naturally synthesized by various Streptomyces species, including Streptomyces canaries (kitahara, et al., 1982).In a study done on leukemia of mouse, Saphenamycin showed a significant effect on the survival period of mouse of leukemia L1210 cells.(kitahara, et al., 1982).

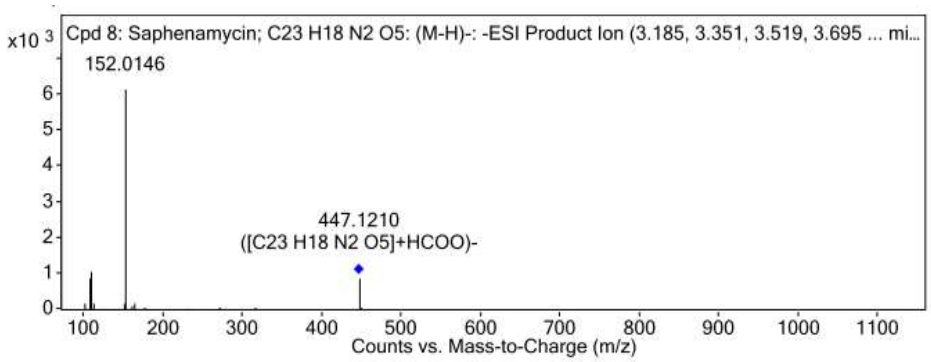


MFE MS Zoomed Spectrum



MS Spectrum Peak List

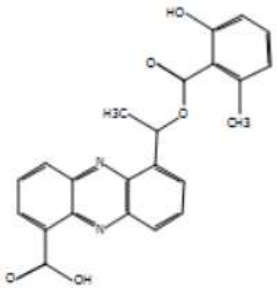
m/z	z	Abund	Formula	Ion
447.1209	-1	696050.06	C23 H18 N2 O5	(M-H)-
448.1237	-1	132044.77	C23 H18 N2 O5	(M-H)-
449.1249	-1	26999.86	C23 H18 N2 O5	(M-H)-
450.128	-1	3058.84	C23 H18 N2 O5	(M-H)-



MS/MS Spectrum Peak List

m/z	*Calc m/z*	Diff (ppm)	z	Abund	Formula	Ion
101.0271			1	191.53		
108.0244			1	893.07		
109.0316			1	1076.93		
113.0277			1	190.8		
151.0427			1	173.02		
152.0146			1	6162.41		
161.0469			1	107.14		
163.0407			1	184.08		
447.0882			1	103.03		
447.121	447.1198	-2.77	1	866.86	C23 H18 N2 O5	(M+HCOO)-

Compound Structure



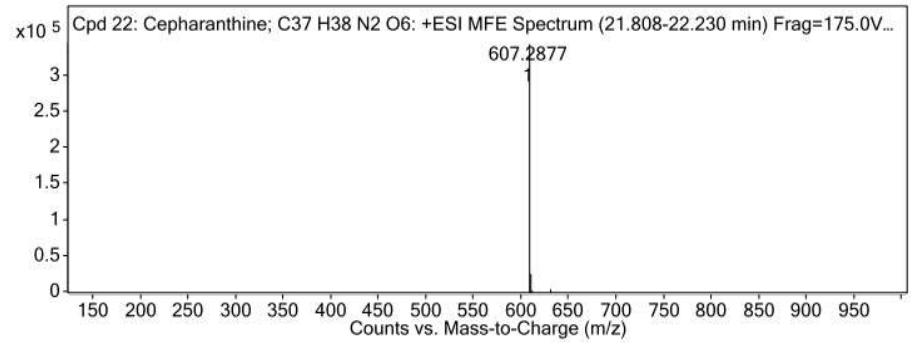
Cepharanthine

Cepharanthine is a naturally occurring compound obtained through the isolation and extraction process from StephaniacepharanthaHayata, a plant belonging to the Menispermaceae family. This bioactive substance, categorized as a bisbenzylisoquinoline alkaloid, possesses a wide array of pharmacological attributes, encompassing its role as an antioxidant, anti-inflammatory agent, immune-modulator, and contributor to anti-tumor and antiviral activities(Liang, et al., 2022).

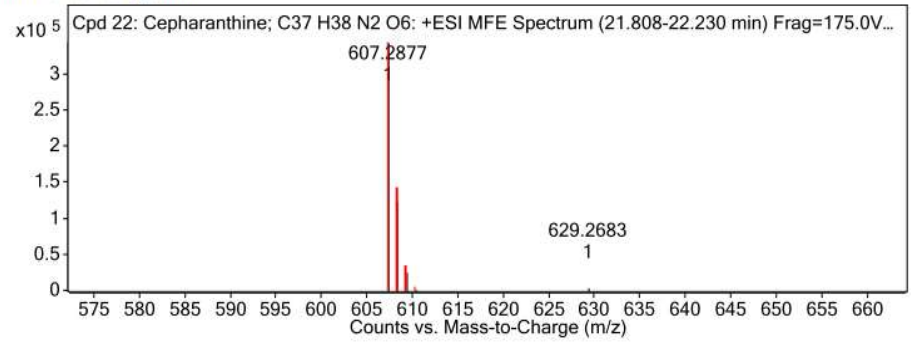
Cepharanthine (CEP) has been employed as a therapeutic agent in Japan for over seven decades, dating back to the 1950s. This versatile drug serves as an effective remedy for a diverse range of both acute and chronic medical conditions. Its applications encompass the treatment of conditions such as leukopenia, snake bites, xerostomia, and alopecia. Notably, Cepharanthine holds the distinction of being the sole approved medication for human use within the extensive category of bisbenzylisoquinoline alkaloids (Bailly, 2019) .

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 22: Cepharanthine; C37 H38 N2 O6	**Cepharanthine**	607.2877	22.072	Find by Molecular Feature	606.2802

MFE MS Spectrum



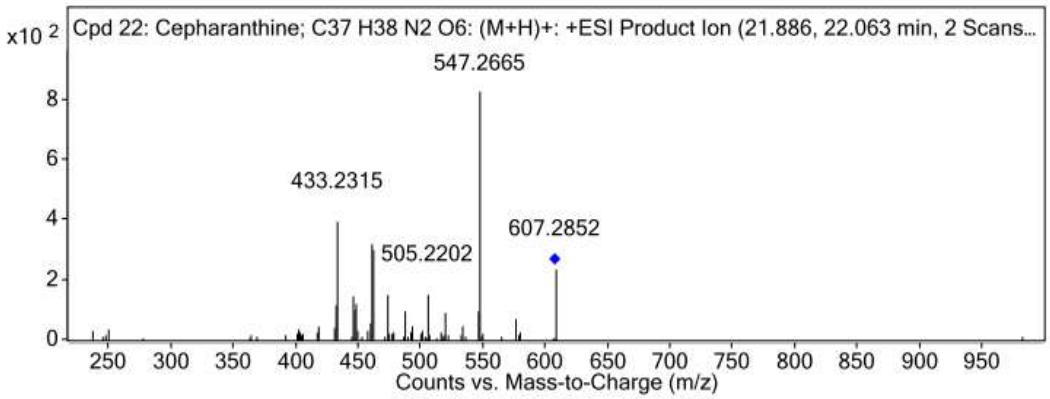
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
607.2877	1	344321.22	C37 H38 N2 O6	(M+H)+
608.2904	1	123898.97	C37 H38 N2 O6	(M+H)+
609.2928	1	25941.3	C37 H38 N2 O6	(M+H)+
610.2957	1	3524.83	C37 H38 N2 O6	(M+H)+
629.2683	1	6106.69		(M+Na)+

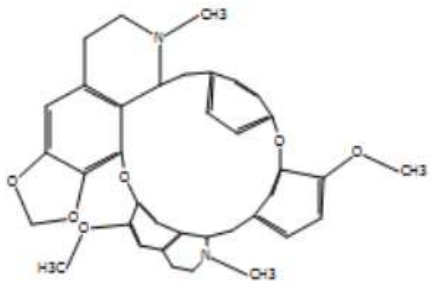
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
431.1804	1	116.61
433.2315	1	395.54
445.201	1	150.59
447.2115	1	123.03
460.2197	1	322.71
461.2298	1	304.46
473.233	1	152.01
505.2202	1	152.8
547.2665	1	830.62
607.2852	1	237.98

Compound Structure

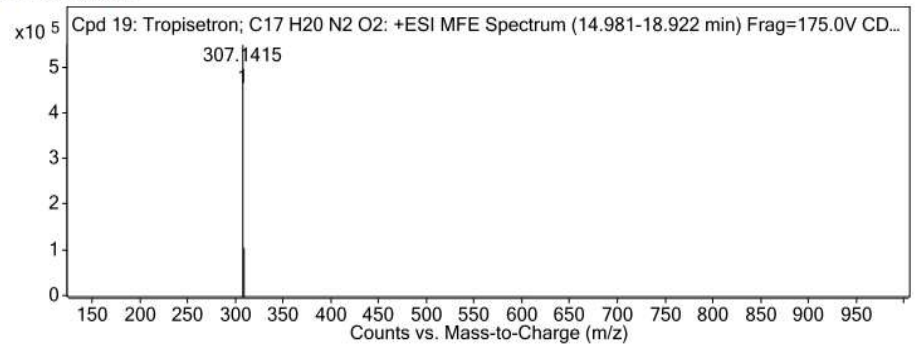


Tropisetron

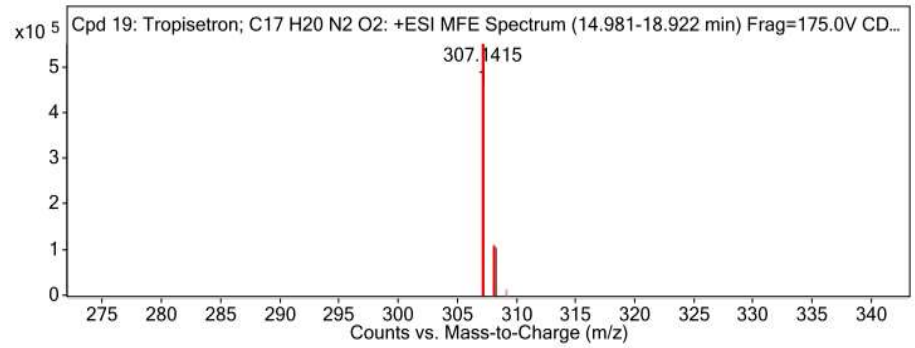
Tropisetron is a compound derived from indole and possesses potent antiemetic properties. Its mechanism of action involves acting as a selective antagonist at serotonin receptors, specifically the 5HT3 receptors, where it competitively inhibits the effects of serotonin (DrugBank. (2018)). This action effectively alleviates the nausea and vomiting induced by chemotherapy and radiotherapy treatments. Tropisetron is primarily employed as an antiemetic to manage the unpleasant side effects of chemotherapy-induced nausea and vomiting. In experimental settings, it has been explored for its potential as an analgesic in the context of fibromyalgia (Müller and Stratz, 2004).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 19: Tropisetron; C17 H20 N2 O2	**Tropisetron**	307.1415	15.434	Find by Molecular Feature	284.1522

MFE MS Spectrum



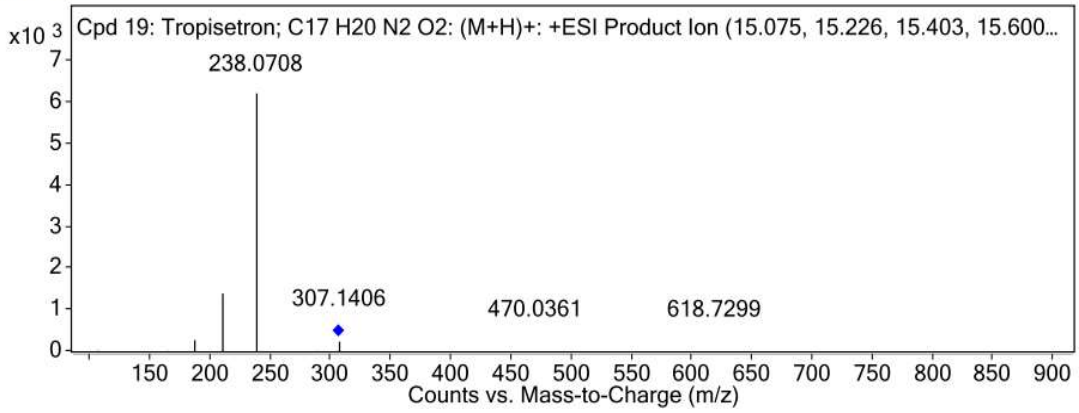
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
307.1415	1	550132.63	C17 H20 N2 O2	(M+H)+
308.1444	1	108509.76	C17 H20 N2 O2	(M+H)+

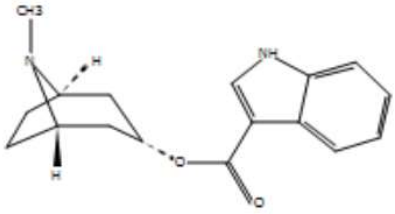
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
107.0487	1	34.91
164.9262	1	48.33
186.9083	1	298.22
210.0761	1	1433.69
238.0708	1	6240.96
288.856	1	44.76
306.1519	1	26.42
306.2757	1	34.81
307.1406	1	265.84
307.1978	1	95.82

Compound Structure



5. CHAPTER V: CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Based on the results of this study, it is concluded that the leaves of A. marmelos contain a diverse array of phytochemicals. These phytochemicals include alkaloids, phenols, flavonoids, saponins, Quinone's, protein and amino acid, tannins and Cuamarin's. While extracting the methanolic extract of leaves using soxhlet, the yield was found to be 18.863%.HR-LCMS analysis of crude methanol extract disclosed 60 different kinds of molecules present in the extract out of which 36were identified compounds and 16 were unidentified compounds.3-beta,6-beta-Dihydroxynortropane serves as potential bio maker, Phosphinothricin is used as herbicide, manumycin A (Man A) has potential of anti cancer properties that have been ascribed to its ability to inhibit farnesyl transferase.Like wiseTropisetron is primarily employed as an antiemetic to manage the unpleasant side effects of chemotherapy-induced nausea and vomiting.Cepharanthine shows significant effect on acute chronic disease. Saphenamycin showed a significant effect on the survival period of mouse of leukemia.Out of 36 identified molecules, 6 are alkaloids, 3 areflavonoids and 3 areglycosideswhereas 6 different molecules exhibit significant biological activities and potency.In this research, Methanol extract of *A. marmelos* shows the TPC content of 59.307 ± 1.058 mg Gallic acid equivalent/g of dry extract, TFC content 132.80±1 mg Quercetin equivalent/g of dry extract and TAC content of 309.444±2.341 mg Ascorbic acid equivalent/g of dry extract. The IC_{50} value of methanol extract of *A. mamelos* was found to be238 micro gram/ mL. On microbial test of methanol extract on gram positive and gram negative bacteria, it was found that extract was highly effective against *Bacillus subtilis* with ZOI of 26.00±4.242mm and S*almonella typhii*with ZOI of 14.00±1.414mm.

5.2 Recommendation

Further investigations are crucial to isolate and identify the active compounds present in the extracts. Identifying these specific bioactive compounds can lead to a deeper understanding of their individual properties and potential medicinal applications.

Moreover, the study strongly recommends the need for comprehensive research into the development of drug formulations that are safe, cost-effective, and non-toxic. Such formulations may be potential to enhance the value of our natural resources. This approach aligns with a rational and sustainable strategy for harnessing the benefits of our resources, offering both economic and environmental advantages.

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APPENDIX I

HRLCMS acquisition method

Acquisition Method Report Agilent Technologies

Acquisition Method Info

Method Name	metabolite_ESI_+VE_MSMS.m
Method Path	D:\MassHunter\Methods\2022\metabolite_ESI_+VE_MSMS.m
Method Description	Default Method

Device List

HiP Sampler
Binary Pump
Column Comp.
Q-TOF

TOF/Q-TOF Mass Spectrometer

Component Name	MS Q-TOF	**Component Model**	G6550A
Ion Source	Dual AJS ESI	**Stop Time (min)**	30.00
Can wait for temp.	Enable	**Fast Polarity**	N/A
MS Abs. threshold	200	**MS Rel. threshold(%)**	0.010
MS/MS Abs. threshold	5	**MS/MS Rel. threshold(%)**	0.010
Tune File	AutoTune (3).tun		

Time Segments

Time Segment #	Start Time (min)	Diverter Valve State	Storage Mode	Ion Mode
1	0	MS	Both	Dual AJS ESI

Time Segment 1

Acquisition Mode AutoMS2

MS Min Range (m/z)	126
MS Max Range (m/z)	1200
MS Scan Rate (spectra/sec)	1.00
MS/MS Scan Rate (spectra/sec)	1.00
Isolation Width MS/MS	Medium (~4 amu)

Ramped Collision Energy

Charge	Slope	Offset
1	8	-2.6
2	6	-2.6
3	4	-2.6

Auto MS/MS Preferred/Exclude Table

Mass	Delta Mass (ppm)	Charge	Type	Retention Time (min)	Delta Ret. Time (min)	Isolation Width	Collision Energy
197.8075	500	1	Exclude	0		Medium (~4 amu)	

Precursor Selection

Max Precursors Per Cycle	10
Threshold (Abs)	10000
Threshold (Rel)(%)	0.010
Precursor abundance based scan speed	Yes
Target (counts/spectrum)	25000.000
Use MS/MS accumulation time limit	Yes
Use dynamic precursor rejection	No
Purity Stringency (%)	100.000
Purity Cutoff (%)	30.000
Isotope Model	Common
Active exclusion enabled	Yes
Active exclusion excluded after (spectra)	1
Active exclusion released after (min)	0.20
Sort precursors	By abundance only

Charge State Preference

Selected Charges
1
2
Unk

Source Parameters

Parameter	Value
Gas Temp (°C)	250
Gas Flow (l/min)	13
Nebulizer (psig)	35
SheathGasTemp	300
SheathGasFlow	11

Scan Segments

Scan Seg #	Ion Polarity	Collision Energy
1	Positive	0

Scan Segment 1

Scan Source Parameters

Parameter	Value
VCap	3500
Nozzle Voltage (V)	1000
Fragmentor	175
Skimmer1	65
OctopoleRFPeak	750

ReferenceMasses

Ref Mass Enabled	Disabled
Ref Nebulizer (psig)	

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	15	10000000

Acquisition Method Report

Agilent Technologies

Name: HiP Sampler **Model:** G4226A

Auxiliary	
Draw Speed	100.0 µL/min
Eject Speed	100.0 µL/min
Draw Position Offset	0.0 mm
Wait Time After Drawing	2.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	Yes
Injection	
Injection Mode	Injection with needle wash
Injection Volume	5.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	3.0 s
High throughput	
Automatic Delay Volume Reduction	No
Overlapped Injection	
Enable Overlapped Injection	No
Valve Switching	
Valve Movements	0
Valve Switch Time 1	
Switch Time 1 Enabled	Yes
Switch Time 1	0.01 min
Valve Switch Time 2	
Switch Time 2 Enabled	No
Valve Switch Time 3	
Switch Time 3 Enabled	No
Valve Switch Time 4	
Switch Time 4 Enabled	No
Stop Time	
Stoptime Mode	As pump/No limit
Post Time	
Posttime Mode	Off

Name: Binary Pump **Model:** G4220B

Flow	0.300 mL/min
Use Solvent Types	Yes
Stroke Mode	Synchronized
Low Pressure Limit	0.00 bar
High Pressure Limit	1200.00 bar
Max. Flow Ramp Up	100.000 mL/min²
Max. Flow Ramp Down	100.000 mL/min²
Expected Mixer	No check
Stroke A	
Automatic Stroke Calculation A	Yes
Stop Time	
Stoptime Mode	Time set
Stoptime	35.00 min
Post Time	
Posttime Mode	Off

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Ch2 Solv.	Name 2	Selected	Used	Percent
1	A	100.0 % Water V.02	0.1% FA in water	100.0 % Water V.02	0.1% FA in water	Ch. 2	Yes	95.00 %
2	B	100.0 % Methanol V.03		100.0 % Acetonitrile V.02		Ch. 2	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	1.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar
2	25.00 min	0.00 %	100.00 %	0.300 mL/min	1200.00 bar
3	30.00 min	0.00 %	100.00 %	0.300 mL/min	1200.00 bar
4	31.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar
5	35.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar

Name: Column Comp. **Model:** G1316C

Ready when front door open	Yes
Left Temperature Control	
Temperature Control Mode	Temperature Set
Temperature	40.00 °C
Enable Analysis Left Temperature	
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.80 °C
Right Temperature Control	
Right temperature Control Mode	Temperature Set
Right temperature	40.00 °C
Enable Analysis Right Temperature	
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.80 °C
Stop Time	
Stoptime Mode	As pump/injector
Post Time	
Posttime Mode	Off

APPENDIX II

HR-LCMS Profiling of compound in positive ESI mode

Qualitative Compound Report

Data File	CD-09.d	**Sample Name**	CD-09
Sample Type	Sample	**Position**	P1-F4
Instrument Name	QTOF	**User Name**	
Acq Method	metabolite_ESI_+VE_MSMS.m	**Acquired Time**	8/7/2023 12:14:47 PM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125.3)

Compound Table

Compound Label	RT	Mass	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 1: Phosphinothricin; C5 H12 N O4 P	1.097	181.0485	Phosphinothricin	C5 H12 N O4 P	C5 H12 N O4 P	C5 H12 N O4 P	10.62	6
Cpd 2: N-Acetyl-leucyl-leucine; C14 H26 N2 O4	1.227	286.1875	N-Acetyl-leucyl-leucine	C14 H26 N2 O4	C14 H26 N2 O4	C14 H26 N2 O4	6.14	7
Cpd 3: 3beta,6beta-Dihydroxynortropane; C7 H13 N O2	1.236	143.0935	3beta,6beta-Dihydroxynortropane	C7 H13 N O2	C7 H13 N O2	C7 H13 N O2	8.21	3
Cpd 4: 3beta,6beta-Dihydroxynortropane; C7 H13 N O2	1.349	143.0932	3beta,6beta-Dihydroxynortropane	C7 H13 N O2	C7 H13 N O2	C7 H13 N O2	9.88	3
Compound 5	1.369	313.2455						
Compound 6	3.378	210.1462						
Cpd 7: Pirimicarb; C11 H18 N4 O2	3.865	238.1412	Pirimicarb	C11 H18 N4 O2	C11 H18 N4 O2	C11 H18 N4 O2	7.44	1
Compound 8	5.99	390.0907						
Cpd 9: Valganciclovir; C14 H22 N6 O5	6.045	354.1633	Valganciclovir	C14 H22 N6 O5	C14 H22 N6 O5	C14 H22 N6 O5	5.35	10
Cpd 10: 5-Methyl-THF; C20 H25 N7 O6	8.783	459.1873	5-Methyl-THF	C20 H25 N7 O6	C20 H25 N7 O6	C20 H25 N7 O6	-1.39	2
Cpd 11: Tryptophyl-Aspartate; C15 H17 N3 O5	10.299	319.116	Tryptophyl-Aspartate	C15 H17 N3 O5	C15 H17 N3 O5	C15 H17 N3 O5	2.5	10
Cpd 12: Manumycin A; C31 H38 N2 O7	10.364	550.2595	Manumycin A	C31 H38 N2 O7	C31 H38 N2 O7	C31 H38 N2 O7	15.2	1
Cpd 13: (+/-)-gamma-Lycorane; C16 H19 N O2	10.415	257.1414	(+/-)-gamma-Lycorane	C16 H19 N O2	C16 H19 N O2	C16 H19 N O2	0.63	10
Compound 14	10.529	446.218						
Cpd 15: (1xi,3xi)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-3-carboxylic acid; C13 H14 N2 O2	11.233	230.1058	(1xi,3xi)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-3-carboxylic acid	C13 H14 N2 O2	C13 H14 N2 O2	C13 H14 N2 O2	-1.34	10
Compound 16	11.577	460.2334						
Cpd 17: N4-(b-N-Acetyl-D-glucosaminyl)-L-asparagine; C12 H21 N3 O8	12.427	335.1374	N4-(b-N-Acetyl-D-glucosaminyl)-L-asparagine	C12 H21 N3 O8	C12 H21 N3 O8	C12 H21 N3 O8	-13.58	2
Compound 18	13.361	227.2591						
Cpd 19: Tropisetron; C17 H20 N2 O2	15.434	284.1522	Tropisetron	C17 H20 N2 O2	C17 H20 N2 O2	C17 H20 N2 O2	0.98	5
Cpd 20: Aegle marmelos Alkaloid C; C23 H27 N O3	15.436	365.196	Aegle marmelos Alkaloid C	C23 H27 N O3	C23 H27 N O3	C23 H27 N O3	8.6	3
Cpd 21: Promazine sulfoxide; C17 H20 N2 O S	16.805	300.1313	Promazine sulfoxide	C17 H20 N2 O S	C17 H20 N2 O S	C17 H20 N2 O S	-5.5	5
Cpd 22: Cepharanthine; C37 H38 N2 O6	22.072	606.2802	Cepharanthine	C37 H38 N2 O6	C37 H38 N2 O6	C37 H38 N2 O6	-11.91	1
Cpd 23: Cepharanthine; C37 H38 N2 O6	22.401	606.2802	Cepharanthine	C37 H38 N2 O6	C37 H38 N2 O6	C37 H38 N2 O6	-11.9	1
Compound 24	23.918	337.3312						

APPENDIX III

Zone of inhibition of different bacteria

SN	Name of bacteria	Standard (antibiotics)/Positive control ZOI(mm)	Plant extract ZOI(mm)			Average(mm)	Standard Deviation	Average ± SD(mm)
			T 1	T 2	T3			
1.	*Klebsiella pneumonia*	11 (Erythromycin)	9	8	8	8.330	0	8.33 ± 0.000
2.	*Pseudomonas aeruginosa*	23 (Vancomycin)	-	-	-	-	-	-
3.	*Staphylococcus aureus*	14 (Vancomycin)	10	10	11	10.330	0.707	10.33± 0.707
4.	*Escherichia coli*	17 (Gentamicin)	-	-	-	-	-	-
5.	*Salmonella typhi*	25 (Erythromycin)	16	14	12	14	1.414	14±1.414
6.	*Bacillus subtilis*	24 (Erythromycin)	26	29	23	26	4.242	26±4.242

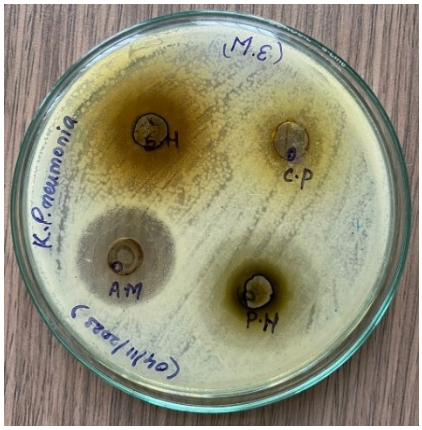
APPENDIX IV

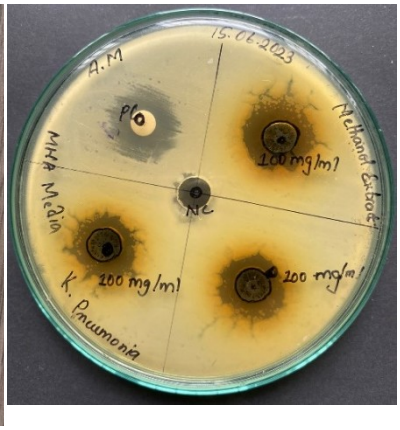
Absorbance of Standard ascorbic acid

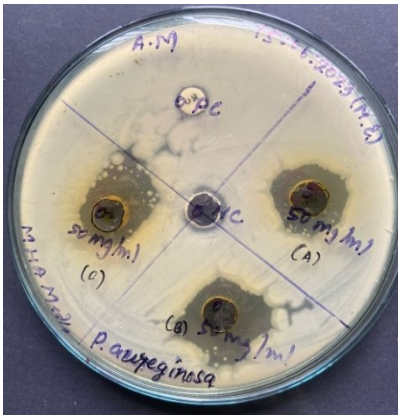
Concentration (µg/mL)	Absorbance(Ascorbic Acid)					
	T1	T2	T3	Average	SD	% inhibition
0	0	0	0	0	0	100
1000	0.122	0.122	0.122	0.122	0	69.6013289
1200	0.09	0.091	0.091	0.090667	0.000577	77.40863787
1400	0.079	0.079	0.078	0.078667	0.000577	80.3986711
1600	0.068	0.067	0.069	0.068	0.001	83.05647841
1800	0.047	0.047	0.044	0.046	0.001732	88.53820598
2000	0.032	0.035	0.034	0.033667	0.001528	91.61129568
Control	0.402	0.401	0.401	0.401333	0.000577	

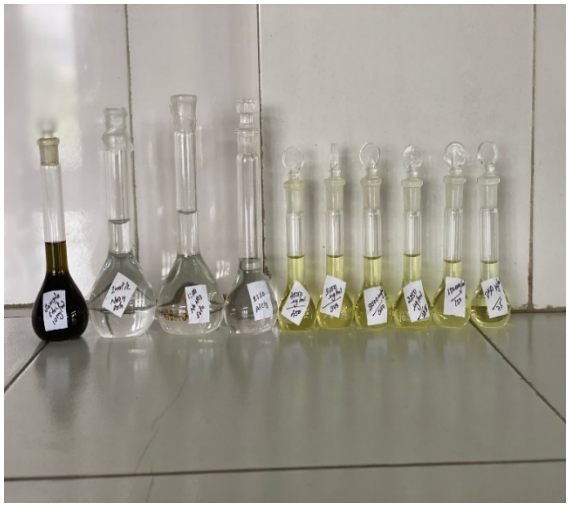
APPENDIX V

Photographs of some research work









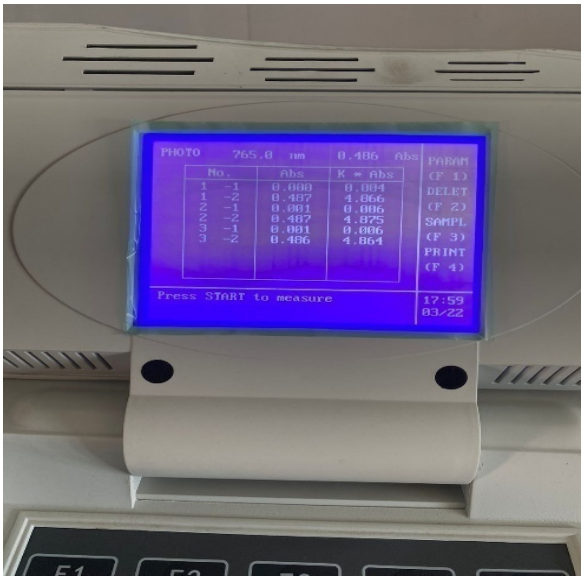


PHOTO 765.0 nm 0.486 Abs
No. Abs K * Abs
1 -1 0.000 0.004
1 -2 0.487 4.866
2 -1 0.001 0.006
2 -2 0.487 4.875
3 -1 0.001 0.006
3 -2 0.486 4.864
PARAM (F 1)
DELET (F 2)
SAMPL (F 3)
PRINT (F 4)
Press START to measure
17:59
03/22