PHYTOCHEMICAL SCREENING, GC-MS ANALYSIS, ANTIBACTERIAL, ANTIOXIDANT, TPC, TFC, AND CYTOTOXICITY ANALYSIS OF EXTRACTS AND ESSENTIAL OIL OF *AEGLE MARMELOS* (L.) CORREA

A DISSERTATION

SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE IN CHEMISTRY

By

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BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation entitled "**Phytochemical screening, GC-MS Analysis, Antibacterial, Antioxidant, TPC, TFC and Cytotoxicity Analysis of Extracts and Essential Oil of *Aegle marmelos* (L.) Correa**" was prepared and submitted by Homa Karki, under the supervision of Asst. Prof. Dr. R. L. (Swagat) Shrestha, Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu, Nepal is hereby approved for the award of Master of Science (M.Sc.) Degree in Chemistry.

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RECOMMENDATION LETTER

This is to recommend that **Ms. Homa Karki** has carried out dissertation work entitled "**Phytochemical Screening, GC-MS Analysis, Antibacterial, Antioxidant, TPC, TFC and Cytotoxicity Analysis of Extracts and Essential Oil of *Aegle marmelos* (L.) Correa**" for partial fulfillment of the requirements of Master of Science Degree in Chemistry under my supervision. To the best of my knowledge, this work has not been submitted to any other degree.

She has fulfilled all the requirements laid down by the Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Lainchour for the submission of the dissertation for the partial fulfillment of the requirements for the Master of Science Degree in Chemistry.

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DECLARATION

I, **Homa Karki**, hereby declare that the dissertation entitled "**Phytochemical Screening, GC-MS Analysis, Antibacterial, Antioxidant, TPC, TFC and Cytotoxicity Analysis of Extracts and Essential Oil of *Aegle marmelos* (L.) Correa**" is being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Kathmandu, Nepal for the partial fulfillment of the requirements for the Master of Science Degree in Chemistry, presented herein is my genuine work carried out under the supervision of Asst. Prof. Dr. R. L. (Swagat) Shrestha, Department of Chemistry, Amrit Campus, Kathmandu. This dissertation is done originally by me and has not been published or submitted elsewhere for the requirement of a degree program. Any literature, data, or works done by others and cited in this dissertation has been given due acknowledgment and listed in the reference section.

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Homa Karki
September 2021

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Homa Karki

September 2021

LIST OF ABBREVIATIONS

AM	*Aegle marmelos*
AMLE	*Aegle marmelos* leaf extract
AgNPs	Silver nanoparticles
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
FTIR	Fourier Transform Infrared
GAC	Gallic acid concentration
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
IC_{50}	Inhibitory concentration for killing 50% cells
IR	Infrared
NMR	Nuclear Magnetic Resonance
QC	Quercetin concentration
QE	Quercetin equivalent
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Activity
S. aureus	*Staphylococcus aureus*
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TLC	Thin Layer Chromatography
UV	Ultra-violet
ZOI	Zone of Inhibition

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ABSTRACT

Aegle marmelos (L.) Correa (Rutaceae family), also known as the Bel fruit tree, is a widely distributed plant and is found in Nepal, Bangladesh, India, China, Sri Lanka, Myanmar, Pakistan, etc. The objective of the present study is to evaluate the phytochemicals and bio-activities of extracts and essential oil of *A. marmelos*. The *A. marmelos* leaves were successively extracted using methanol, hexane, and chloroform organic solvents. Qualitative phytochemical analysis of methanol and chloroform extracts of *A. marmelos* leaves confirmed the presence of alkaloids, saponins, glycosides, phenols, flavonoids, protein, and tannin. GC-MS analysis of crude chloroform extract showed 9 different major compounds among which Limonene dioxide (27.78 %) and Germacrene B (20.65 %) were the most abundant. The IC_{50} value of the chloroform extract against DPPH was found to be 308.21 μg/mL. The result showed that the total phenolic and flavonoid content in *A. marmelos* leaves with the value 58.36 mg gallic acid equivalent and 142.29 mg quercetin equivalent/g of dry extract respectively. The extract also exhibited higher toxicity against brine shrimp with the LC_{50} value of 157.50 μg/mL. The essential oil by hydrodistillation was analyzed by GC-MS for its composition and exhibited the presence of 12 different compounds. The most abundant were Limonene (25.06 %) and Germacrene B (18.63 %). Antibacterial activity was performed against six bacterial species and *Bacillus subtilis* was susceptible to the essential oil showing zone of inhibition (ZOI) 7 mm. The IC_{50} value of the essential oil against DPPH was found to be 13.63 mg/mL. The total phenolic content and flavonoid content in the essential oil of *A. marmelos* leaves were found to be 10.65 mg gallic acid equivalent and 3.27 mg quercetin equivalent/g of essential oil respectively. The toxicity of essential oil of *A. marmelos* tested by brine shrimp lethality (LC_{50}) was found to be 58.17 μg/mL.

Keywords: *Aegle marmelos*, extracts, oil, phytochemical screening, GC-MS, Antibacterial, Antioxidant, TPC, TFC, Cytotoxicity.

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CHAPTER 1: INTRODUCTION

1.1. About Diversity of flora in Nepal

Nepal is a mountainous country in the central Himalayas situated in the lap of the great Himalayas in South East Asia, which occupies about one-third (800 km) of the entire length of the Himalayan Mountain range. Nepal alone claims eight out of the top ten tallest mountains in the world, including Mount Everest (8,848.86 m). Apart from the mountains, deep gorges, river valleys and, flatlands, it provides a unique assemblage of very different habitats and great biodiversity within a small geographical area. The 147,516 km^2 that makeup Nepal, is slightly less than 0.1 % of the global landmass but contains a disproportionately large diversity of plants and animals [1].

Nepal is ranked ninth among the Asian countries for its floral wealth with an estimated 9,000 species of flowering plants. The country's 118 ecosystems harbor over 2 % of the flowering plants, 3% of the pteridophytes, and 6% of the bryophytes in the world's flora [1]. A total of 807 algae, 2,025 fungi, 771 lichens, 1,150 bryophytes, 534 pteridophytes, 28 gymnosperms, and 6,653 species of angiosperms have been reported in Nepal [2]. Out of those, about 370 species of flowering plants are considered endemic to Nepal. According to the Nepal biodiversity strategy published in 2002 by the Ministry of Forests and Soil conservation, the British Museum has over 40,000 specimens, the University of Tokyo about 100,000 specimens, and the National Herbarium and Plant Laboratories of Kathmandu, 150,000 specimens. In addition, approximately 10,000 specimens are housed in different institutions of Tribhuvan University. However, many parts of Nepal have not been well explored yet.

1.2. *Aegle marmelos* (L.) Correa

Aegle marmelos (L.) Correa, locally known as Bel (Rutaceae family) is a moderate-sized, slender, aromatic tree with a height of 6.0-7.5 m and a girth of 90-120 cm. It is native to the subcontinents of India and is primarily found in tropical and subtropical regions [3]. *A. marmelos* plant grows from sea level up to an altitude of 1,200 m. *A. marmelos* is a widely distributed plant and is found

in Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, India, China, etc [4]. Traditionally, *A. marmelos* is used in diarrhea treatment and any part of plants has medicinal properties such as its fruits, stem, bark, and leaves, and plant leaf is considered one of the highest accumulator components containing bioactive compounds synthesized as secondary metabolites. In traditional and folk medicine, *A. marmelos* has many ethnomedicinal applications. Leaves of this plant are used in females to induce infertility/abortion [5]. The roots and fruits of *A. marmelos* are used as an anti-asthmatic agent. *A. marmelos* have anti-amoebic activity and hypoglycemic activity. In the treatment of diseases such as diarrhea, gastric disorders, tonic, and constipation, intestinal, stomach, and laxative, the fruit of *A. marmelos* has several healing properties [6]. Data on the biosynthetic pathways and the encoding enzymes present in the *A. marmelos* leaves would be extremely useful for functional genomics through transgenic and metabolic engineering approaches. Also, for the green synthesis of gold and silver nanoparticle extract, *A. marmelos* leaf extract is used [7]. This plant's leaves, roots, bark, seeds, and fruits have several medicinal qualities. Bioactive compounds such as phenolic, alkaloids, coumarins, carotenoids, flavonoids, terpenoids, and other antioxidants are likely to protect us from chronic conditions. The endophytes of different medicinal plants generate various bioactive compounds, such as Taxol, flavonoids, terpenoids, and enzymes such as asparaginase [8].

Essential oil is a mixture of a volatile, aromatic, hydrophobic compound from any part of a plant, most commonly sourced from higher plant leaves, wood pulp, or bark tissue, but also commonly found in bryophytes, such as liverworts. The aqueous solubility of individual components of an essential oil varies. While essential oils are only slightly soluble in water, it is commonly predicted that components with more polar functional groups would be more soluble in water compared to other components [9,10]. Anti-fungal and anti-bacterial activities have been shown in the essential oil extracted from the bel tree leaves. The radioprotective, anti-cancerous and anti-inflammatory activities of the essential oil of the bel tree leaves have been demonstrated by numerous studies [11]. Although the extraction, value addition, and marketing of essential oil from its leaves have many limitations. Alternatively, essential oils can play a

less evident environmental role, such as in fire tolerance, seed dispersal attraction of pollinators and/or herbivores, drought tolerance, or plant-to-plant biosemiotics [12].

1.3. Morphology of *Aegle marmelos*

Bel is a medium-sized deciduous tree greater than 8 m tall. The tree has unusual branches with aromatic leaves, sweet-scented, and greenish-white flowers. The leaves are alternate, pale green, trifoliate, having a long petiole which is 3.2 cm long and the two lateral leaflets are almost sessile, 4.1 cm long, 2.2 cm wide along with terminal leaflet, 5.7 cm long, 2.8 cm broad, ovate to lanceolate, reticulate pinnate venation. The flowers are greenish-white, sweetly scented, and have a bisexual, actinomorphic, bracteates, hypogynous stalk, which holds the flower is 8 mm long. Bel has gamosepalous and five-lobed calyx and polypetalous with 5 petals, imbricate, leathery, pale yellow from above, and green from beneath Corolla. Similarly, androecium is stamens of length 4 mm with the polyandrous condition, numerous, basified, dehiscence longitudinally. The gynoecium is light green with capitate stigma hosting terminal style. It has golden-colored resembles a golden apple, very smooth, woody in nature, 5–15 cm in diameter. Numerous seeds are present which are densely enclosed with fibrous hair and are entrenched in a thin aromatic pulp [13]. The flowering season of plants is May-June and fruits season is May-June of the following year. It has 2n=18 chromosomes [7].

Figure 1: Plant leaves of *A. marmelos*

Figure 2: Flower of *A. marmelos*

1.4. Classification of *Aegle marmelos*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Subfamily: Aurantioideae

Tribe: Clauseneae

Genus: *Aegle*

Species: *marmelos*

Binomial name*: Aegle marmelos* (L.) Correa [4]

1.5. Common name of *Aegle marmelos*

Latin	:	*Aegle marmelos*
Sanskrit	:	Bilwa
Nepali	:	Bel, Gudu
English	:	Wood/Stone apple, Bengal Quince, Indian Quince, holy fruit, golden apple, elephant apple, sriphal
Hindi	:	baelputri, bela, sriphal, kooralam
Burmese	:	Opesheet, ohshit, bel Indian
German	:	Belbaum, Schleimapfelbaum
French	:	Oranger du Malabar, cognassier du bengale, bel Indian
Gujrat	:	Billi
Indonesia	:	maja batuh, maja
Vietnam	:	tar imam, mbau nau
Arab	:	Bull, Quiththa el hind
Urdu	:	bel
Tamil	:	Vilvam

1.6. Traditional use of *Aegle marmelos*

A. marmelos is commonly considered a sacred tree by the Hindus as its leaves are offered to Lord Shiva during worship, and leaves, fruit, stem, and roots of this tree are used as ethnomedicine against different human ailments at all times. The unripe dried fruit is astringent, digestive, stomachic, and used to cure diarrhea and dysentery. Fair and easy treatment for dyspepsia is ripe fruit. The roots and barks of the tree are used by making a decoction of them in the treatment of fever. For the treatment of inflammation, asthma, hypoglycemia, febrifuge, hepatitis, and analgesics, the leaf portion of the plants is used. It is primarily used for conjunctivitis and styes, rhinitis, coccygodynia, nocturnal seminal emission with amorous visions, and chronic dysentery in homeopathic treatments [14]. *A. marmelos* leaves are medically adventitious and beneficial in the cure of various diseases such as diabetes, dysentery, jaundice, gastralgia, constipation, recurrent diarrhea, stomach ache, fever, asthma, inflammation, febrile delirium, acute bronchitis, snakebite, gastrointestinal pain, acidity, burning sensation, epilepsy, indigestion, leprosy, myalgia, smallpox, and spermatorrhoea [15]. The bark is used for diarrhea and bone fracture therapy. The roots are used for treating heart palpitation. Fruits are used as a laxative [16].

1.7. Nutritional use of *Aegle marmelos*

Bel fruit is rich in nutritional value, physicochemical studies reveal. Bel pulp is a rich source of glucose, sugar, and fiber and is used as an energy drink with milk in the traditional medicine scheme. This drink is very good for removing hair from the stomach. Nutrition, fat, minerals, fibers, carbohydrates, calcium, phosphate, potassium, iron, vitamin A, vitamin B1, nicotinic acid, riboflavin, vitamin C are other nutrients of Bel [17]. Bel fruit studies suggest that it consists of 61.5 % moisture, 1.7 % minerals, 2.9 % fiber, 0.3 % fat, 1.8 % protein, and 31.8 % carbohydrates per 100 grams of edible part. Its calorific value is equivalent to 137 [13]. Leucine and aspartic acid are prominent amino acids in leaves, seeds, and fruits pulp of bel [18].

1.8. Objectives of the study

The objectives of this study are as follows:

General objective

- To find out the phytochemical constituents and medicinal value of plant extracts and essential oil of *A. marmelos*

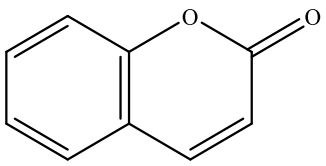
Specific objectives

- To extract constituents in the leaf with the different solvent systems (Methanol, Hexane, and Chloroform) and perform phytochemical screening
- To conduct GC-MS chromatography and study the GC-MS chromatogram of plant extracts and essential oil
- To study the antibacterial, antioxidant, TPC, TFC, Toxicity analysis of the plant extracts and essential oil

CHAPTER 2: LITERATURE REVIEWS

A. marmelos has rare branches with aromatic leaves, sweet-scented, and greenish-white flowers and is a medium-sized deciduous tree larger than 8 m tall. The leaves are alternating, light green, trifoliate, 3.2 cm long with a long petiole, and two lateral leaflets are almost sessile, 4.1 cm long, 2.2 cm wide, and 5.7 cm long, 2.8 cm wide, ovate to lanceolate, reticulate, pinnate venation. Major phytochemicals such as alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids, and biologically active steroids, are the primary source of treatment for various diseases [13].

Bajpai *et al.* (2014) conducted the study with aqueous and methanolic extracts of *A. marmelos* to demonstrate the existence of medicinally important phytochemicals such as tannins, saponins, flavonoids, alkaloids, terpenoids, carotenoids, cardiac glycosides, and sugar reduction. The highest antibacterial activity against *Staphylococcus epidermidis* (14.3 mm) was shown by the aqueous extract, followed by *Staphylococcus aureus* (13 mm) and *Klebsiella pneumoniae* (10.6 mm). The most potent methanolic extract was against *S. aureus*. The highest sensitivity of *S. aureus* may be due to its cell wall structure and outer membrane also to the presence of tannins, flavonoids, and terpenoids. The methanol extracts of *A. marmelos* leaves and flowers have reported substantial *in vitro* antimicrobial activity. Several bioactive compounds were identified from *A. marmelos*, namely marmin and marmelosin, coumarin derivatives, and angelin [5].

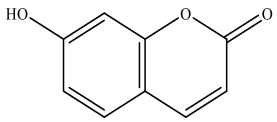


Coumarin

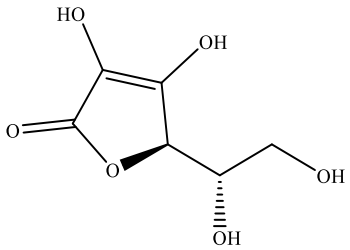
Patil *et al*. (2015) studied the isolation, classification, and biological activities of endophytic fungi of *A. marmelos*. One of the isolates, named L7, was classified using morphology and ITS gene sequence as *Aspergillus flavus*. The

endophytic *flavus*, collected from *A. marmelos* should have been explored as an economic and probable natural resource with diverse biological and medicinal operations [8].

Patel *et al*. (2018) reported several Coumarins, alkaloids, steroids, and essential oils in Bel. Coumarins such as scoparone, scopoletin, and umbelliferone, are present in roots and fruits. It also contains polysaccharides that can be obtained after hydrolyses, such as galactose, arabinose, uronic acid. *A. marmelos* has been reported to contain different forms of carotenoids which are responsible for imparting a yellow pale color to the fruit. Minor constituents, including leukemic K562, Tlymphoid3, ascorbic acid, sitosterol, crude fibers, tannins, α-amyrine, a carotenoid. 400 mg/kg extract administration has shown an anticancer effect in an animal model of Ehrich ascites carcinoma and the anticancer potential of folk medicine used in Bangladesh, India has been documented [17].



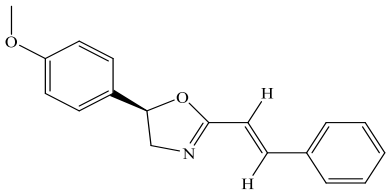
Umbelliferone



Ascorbic acid

Kothari *et al*. (2011) showed the antibacterial activity of three *A. marmelos* leaf extracts tested for potential against five bacterial strains: *Lactobacillus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and three *Pestolotia foedans*, *Paecilomyces variotii*, and *Fusarium oxysporum* fungal strains. Chloroform extract has demonstrated outstanding antibacterial and antifungal properties against *E. coli* and the *Oxysporum fusarium* [19].

Faizi *et al*. (2009) revealed that leaves contain alkaloids, mermesinin, rutin, and b-sitosterol -*β* -D glucoside. A rare alkaloid, shahidine showed activity against a few Gram-positive bacteria, having an unstable oxazoline core has been isolated as a major constituent from the fresh leaves of *A. marmelos*. It is moisture-sensitive and found to be the parent compound of aegeline and other amides, however, it is stable in dimethyl sulfoxide. Its structure was established by spectroscopic analysis [20].

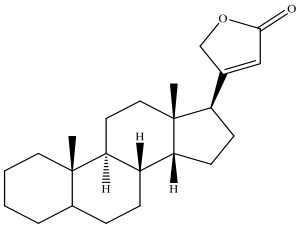


Shahidine

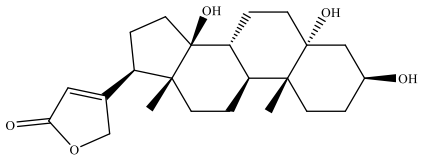
Lambole *et al*. (2010) identified a novel anthraquinone, 1-methyl-2-(3-methyl-but-2-enyloxy)-anthraquinone from *A. marmelos* seeds and characterized by spectral analysis (UV, IR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, and mass spectroscopy). In the disc diffusion assay (MIC value of 6.25 μg/disc), micro broth dilution, and percent spore germination inhibition assays (MIC value of 31.25–62.5 μg/ml), the compound exhibited substantial antifungal activity against pathogenic strains of *Aspergillus* species and *Candida albicans*. Using spore germination assays, the antifungal activity of essential oil isolated from the leaves of bel was evaluated. The oil exhibited variable efficacy against various fungal isolates and 500 ppm was observed at 100 % inhibition of spore germination of all the fungi studied. However, at 400 ppm, *Fusarium udum*, the most resistant fungus, was 80 % inhibited [21].

Panda *et al*. (2009) evaluated the ability of cardenolide, periplogenin, isolated from *A. marmelos* leaves to protect doxorubicin-induced cardiotoxicity and lipid peroxidation (LPO) in rats. Cardiac and hepatotoxicity-induced doxorubicin was characterized by major biochemical changes including an increase in serum creatine kinase-MB (CK-MB), glutamate-pyruvate transaminase (SGPT). However, 25 mg/kg proved to be the most effective out of three different concentrations (12.5, 25, and 50 mg/kg) in the periplogenin

test. The isolated compound demonstrated better therapeutic potential when its effectiveness was compared with that of vitamin E. Periplogenin, isolated from the leaves of *A. marmelos* could theoretically inhibit cardiovascular problems caused by doxorubicin in rats. Its moderate dose was, however, found to be the most effective [22].



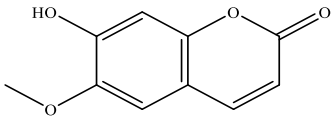
Cardenolide



Periplogenin

Gupta *et al*. (2018) has been investigated the antibacterial activity of leaf extracts by using crude methanolic and chloroform extracts via the agar disc diffusion process. For the research, two Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) and three Gram-negative bacteria *(Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) were used. The inhibition zone obtained from the method showed that maximum activity 15 mm against *P. aeruginosa* and 18 mm against *S. aureus* was exhibited by methanolic and chloroform leaf extracts respectively [23].

Pratibha *et al*. (2020) reported antioxidant activity of the extracts by using the DPPH free radical scavenging and power reduction process, while the antimicrobial activity was well diffused. The presence of flavonoids was suggested by phytochemical screening, and phenols were abundantly present in methanolic and aqueous extracts of *A. marmelos*. Methanolic extract demonstrated considerable potential for antioxidants, which was, however, lower than ascorbic acid. The maximum inhibition zone against *S. aureus* was shown by the methanolic extract. It was (18 mm) and higher than the standard tetracycline. The presence of scopoletin, marmelosin, and umbelliferone, which may contribute to their pharmacological activity, was predicted by HPLC data [24].

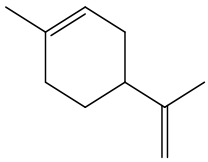


Scopoletin

Diana *et al*. (2015) designed the study to classify the possible antioxidant fraction of *A. marmelos* rind. TLC bioautography analyzed the rind extract of *A. marmelos*. Preparative TLC ran further on the fraction showing DPPH reduction property in TLC bioautography, and the positive fraction was extracted and analyzed using GC-MS. Anti-oxidant compounds such as Phenol 3,5-Bis (1,1-Dimethylethyl) have been discovered in *A. marmelos* rind [25].

Sivakumar *et al*. (2020) performed phytochemical analysis and discovered different bioactive compounds in aqueous, methanolic, ethyl acetate, chloroform, and petroleum ether extracts of *A. marmelos*. Protein denaturation against bovine serum albumin and egg albumin was screened for *in vitro* anti-arthritic activity of different *A. marmelos* extracts. At rising concentrations, the fruit extract of *A. marmelos* demonstrated significantly higher anti-arthritic activity. The explanation for this antiarthritic behavior may be the flavonoids and triterpenoids present in *A. marmelos* [26].

Using the gas chromatography-mass spectrometry (GC-MS) process, volatile compounds in bel fruit pulp were analyzed. There was a total of 28 volatile compounds reported, and monoterpenes and sesquiterpenes were the dominant components. The key constituent generating the characteristic bel fruit flavor was limonene among these components [27].



Limonene

Poonkodi *et al*. (2019) reported that (GC-MS) analyzed the hydro distilled essential oil and the oil yield was (0.9 % v/w). A total of 31 components, representing 97.44 % of the oil, were identified. p-mentha-1,4(8)-diene (33.2 %), limonene (13.1 %), p-cymen-alpha-ol (9.5 %), γ-gurjunene- (7.9 %), β-phellandrene (4.3 %), β-pinene (2.0 %), were the major components of Western Ghats. Significant anticancer activity against human cervical cancer is produced by essential oil [9].

The distilled leaf essential oil collected in Nepal was analyzed for the chemical composition by GC–MS showing various phytochemical constituents. The oil obtained was rich in sesquiterpenes, primarily β-caryophyllene (26 %), while there were small amounts of monoterpenes. The oil was tested against several human tumor cell lines, including pancreatic, colon, lung, and ovarian, because of the richness of sesquiterpenes that are promising as anticancer drugs [28].

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Solvents

Methanol, hexane, and chloroform were the solvents used in the extraction procedure. All the solvents were of analytical grade and manufactured by Fisher chemical company, India.

3.1.2. Chemicals, plant materials, and test organisms

- TLC Aluminium sheets Silica gel 60 F_{254}
- Concentrated HCl
- Concentrated H_2SO_4
- Ammonia solution
- 2, 2-Diphenyl-1- picrylhydrazyl (DPPH)
- Mercuric chloride
- Potassium iodide
- NaOH
- Dimethyl Sulfoxide (DMSO)
- Ethyl acetate
- Hexane
- Methanol
- Chloroform

Plant material: Leaf of *A. marmelos*

Test organisms: The test microorganisms used for this research were four gram-positive bacteria *Staphylococcus aureus* KCTC 1916, *Bacillus subtilis* KACC 17047, *Micrococcus luteus* KACC 13377, and *Enterococcus species*. KACC 13002, two gram-negative bacteria; *Pseudomonas aeruginosa* KACC 10232 and *Klebsiella pneumonia* KCTC 2242.

3.1.3. Instruments

The following instruments were used.

- Grinder

- Electronic balance
- Refrigerator
- Rotary evaporator
- UV-Chamber for TLC
- Digital Water Bath
- Hot Air Oven
- Double Beam UV Spectrophotometer

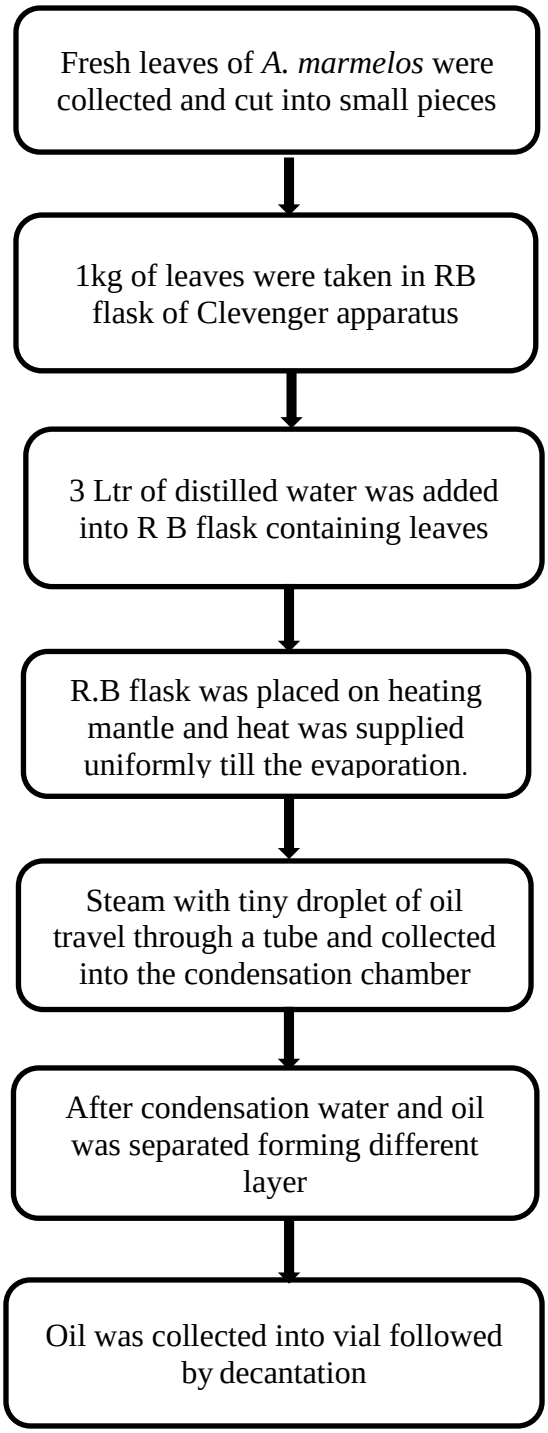
3.2. Methods

3.2.1. Collection of the plant parts

About 10 kg of leaves of *A. marmelos* were collected from Dhading, Nepal at about 1200 m altitude in March 2019.

3.2.2. Extraction of essential oil

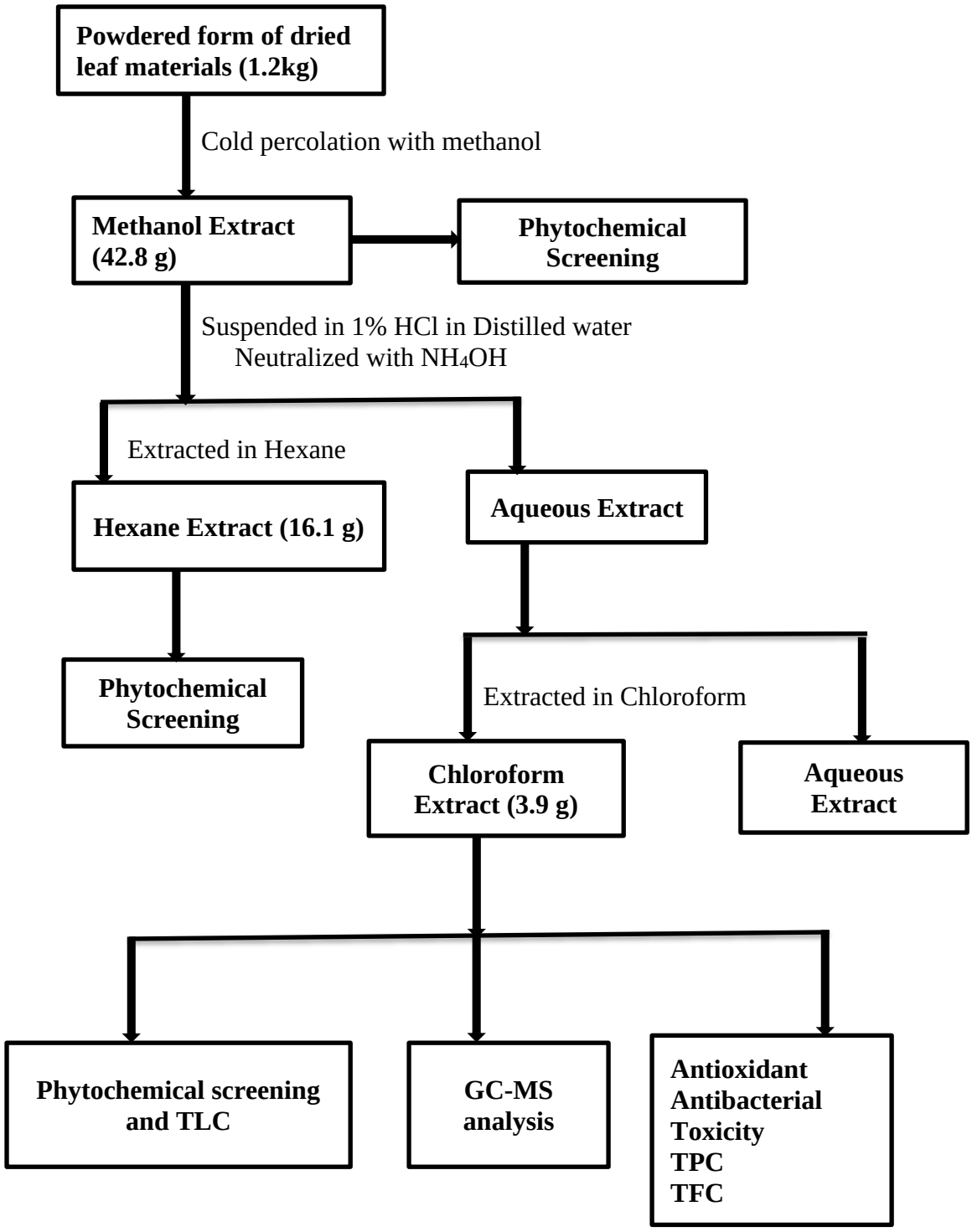
Extraction of essential oil was carried out by the hydrodistillation process. Fresh leaves of *A. marmelos* were collected and shade dried then cut into small pieces. 1 kg of leaves was taken into a round bottom flask (5 Ltr) of Clevenger apparatus. 3 Liters of distilled water were added into the leaves in the RB flask of Clevenger apparatus. RB flask was placed in the heating mantle, and the heat was supplied uniformly till evaporation. A tiny droplet of oil travelled through a tube and collected into the condensation chamber. After condensation, water was separated forming a different layer. Finally, the essential oil was collected into a vial followed by decantation.



Scheme 1 : Flow chart diagram showing essential oil extraction

3.2.3. Extraction procedure with different solvents

The powder of the leaves was first exhaustively extracted with 4 L of methanol by cold percolation process in a gas jar for 30 days. The content was filtered using Whatman filter paper no. 1. The filtrate i.e., methanol extract was concentrated using a Rota evaporator. The small portion of methanol extract was subjected to various phytochemical tests. The remaining large portion was further suspended in 1% HCl in distilled water then proceeded for successive extraction with n-Hexane in a separating funnel and shaken vigorously with continuous release of air. The light hexane fraction remained at the top and the heavy aqueous fraction at the bottom was separated. The crude hexane extract was obtained from hexane fraction using Rota evaporator and subjected to various phytochemical tests. The aqueous fraction was mixed with chloroform for further extraction in the separating funnel. Similar to extraction by hexane, the mixture was shaken vigorously with continuous release of air with great care. The heavy chloroform fraction and light aqueous fraction were separated. The separated chloroform fraction was concentrated using a Rota evaporator and subjected to various phytochemical tests and the qualitative analysis of constituents in the chloroform extract was carried out through thin layer chromatography.



Scheme 2*:* Procedure for the extraction of *A. marmelos*

3.2.4. Phytochemical screening

The chemicals which occur naturally in plants are phytochemicals. Due to their innumerable medicinal uses and zero side effects, they are more common. Due to their disease-curing abilities without any damage, they are considered "man-friendly medicines" [28].

Following normal procedures, phytochemical screening was performed for methanol, hexane, and chloroform extracts [26,29,30].

3.2.4.1. Test for Alkaloids

Extracts were dissolved individually in dil. HCl and filtered.
Mayer's test: Mayer's reagent was treated with 1 mL of filtrate. The formation of a yellow-coloured precipitate suggests that alkaloids are present.

Dragendorff's test: A few drops of Dragendorff's reagent were added to the 2-3 mL of filtrate. The development of an orange-brown precipitate suggests that alkaloids are present.

Wagner's test: 2-3 mL extract was applied with a few drops of Wagner's reagent. The formation of a reddish-brown precipitate suggests that alkaloids are present.

3.2.4.2. Test for Flavonoids

Alkaline reagent test: The extracts were treated and shaken well with a few drops of sodium hydroxide solution. The presence of flavonoids implies the development of an intense yellow colour, which becomes colourless when diluted.

Lead acetate test: A few drops of lead acetate solution were used to treat the extracts. The development of a precipitate of a yellow colour suggests the presence of flavonoids.

Shinoda test: With 2-3 mL extract, a few pieces of magnesium were mixed, followed by dropwise addition of HCl concentrate, and boiled for 5 minutes. The magenta colour formation suggests the presence of flavonoids.

3.2.4.3. Test for Glycosides

The extracts have been hydrolyzed by dil. HCl, and then screened with modified Born Trager's reagent and Legal's reagent for glycosides to treat them.

Modified Borntrager's test: The extracts were hydrolyzed with dil. HCl, and then subjected to glycoside test to treat them with modified Borntrager's reagent and Legal's reagent. Extracts were treated with a solution of ferric chloride and heated, cooled, and shaken with an equal volume of benzene by immersing them in boiling water for about 5 minutes. An ammonia solution was used to treat the resulting solution. The presence of anthranol glycosides is suggested by the development of rose-pink colour in the ammonia layer.

Legal's test: The extracts were treated with pyridine-based sodium nitroprusside and sodium hydroxide. The pink to blood-red colour formation suggests the presence of cardiac glycosides.

3.2.4.4. Test for Phenols

Ferric chloride test: Extracts were treated with 3-4 drops of ferric chloride solution. The presence of phenols is demonstrated by the formation of bluish-black colour.

3.2.4.5. Test for Quinone

A few drops of the Conc.H_2SO_4 or aqueous NaOH solution were added to 2 mL of the extract. The presence of the quinoid compound is suggested by colour formation.

3.2.4.6. Test for Tannins

Lead acetate test: Extracts were combined with 10 % lead acetate solution for a few drops. White precipitate formation shows the presence of tannins.

3.2.4.7. Test for Saponins

Approximately 1 mL of plant extract was diluted with 2 mL of distilled water, shaken vigorously, and allowed to stand for a few minutes, indicating the existence of saponins during foam growth.

3.2.4.8. Test for Proteins

A few drops of extract were diluted in 10 mL of distilled water, the extract was filtered with Whatman no. 1 filter paper. With a few drops of Millon's reagent, 2 mL of filtrate was heated (the reagent is made by dissolving metallic mercury in nitric acid and diluting with water). The presence of tyrosine residue that happens in almost all proteins is suggested by a reddish-brown colouration or precipitate.

3.2.4.9. Test for Carbohydrates

With the extract, an equal volume of Fehling A and Fehling B mixture was heated gently. The formation of red colour shows the presence of carbohydrates.

3.2.4.10. Terpenoids (Salkowski test)

2 mL of chloroform ($CHCl_3$) and 3 mL of concentrated sulphuric acid (H_2SO_4) were carefully applied to about 1 mL of plant extract. The presence of terpenoids was signified by a reddish-brown colouration.

3.2.5. Thin-layer chromatography

The collective term for a group of laboratory techniques for the separation of mixtures into their components is chromatography. All the forms of chromatography operate on the same principle and have a stationary phase that is a solid or a liquid that can be a liquid or a gas, accompanied by a solid and a mobile phase. The mixture to be separated is dissolved in a liquid called the

mobile phase, which carries it through a structure holding another material called the stationary phase. The mobile phase moves through the stationary phase, taking the components of the mixture with it. The various rates of movement allow the different components of the mixture to be separated. The separation is based on the differential division between the stationary and mobile phases [31].

Chromatography can be analytical or preparatory. Preparative chromatography is intended to isolate the components of a mixture for more advanced use (and is thus a form of purification). Analytical chromatography is usually conducted with smaller quantities of material and is used in a mixture to calculate the relative concentrations of analytes. Both are not limited to each other [32].

A chromatography technique used to separate non-volatile mixtures is thin-layer chromatography (TLC). Thin-layer chromatography can be used to track the progress of a reaction, assess the purity of a substance, and classify compounds contained in a given substance. TLC is a widely employed laboratory technique. TLC is one of the most useful instruments in phytochemistry and biotechnology for tracking the progress of organic chemical reactions and for testing the purity of organic compounds. In TLC, the different affinity of the analyte with the mobile and stationary phases helps to achieve the separation of complex mixtures of organic molecules. A TLC plate is a sheet of glass, metal, or plastic that is coated with a thin layer of a solid adsorbent (stationary phase). Near the bottom of this plate, a small amount of the mixture to be analyzed is spotted and the plate is put in a shallow pool of a solvent in a forming chamber [33].

TLC can be categorized by the separation mechanism: adsorption (physical sorption of the solutes on the sorbent particles), partition (dissolution of the solutes on the sorbent into a stationary liquid), ion exchange (attraction of ions to groups with opposite loads on the sorbent) and exclusion of size or permission of gel (rejection or retention based on size or shape). Adsorption and partition are most commonly involved in TLC as both involve the same form of forces, dipole-dipole induced and hydrogen bonding. TLC is also a more flexible,

inexpensive, and simple process than HPLC for analytical and preparatory applications [34].

To observe the qualitative analysis of constituents, present in that extract, the chloroform extract of *A. marmelos* was performed via thin-layer chromatography. TLC was conducted here on TLC aluminum sheet Silica gel 60 F_{254} pre-coated TLC sheet E. Merck Enterprise. Pre-coated, 0.2 mm thick TLC aluminum plates. The plates were formed by gradually increasing the polarity of hexane to ethyl acetate in various solvent ratios. The solvent system concentration for TLC is shown in Table 1. The plates were visualized in a UV fluorescence lamp.

Table 1: The concentration of the solvent system for TLC

S. No.	Solvent system of TLC
1	10% ethyl acetate in hexane
2	20% ethyl acetate in hexane
3	30% ethyl acetate in hexane
4	40% ethyl acetate in hexane
5	50% ethyl acetate in hexane
6	60% ethyl acetate in hexane
7	70% ethyl acetate in hexane
8	80% ethyl acetate in hexane
9	90% ethyl acetate in hexane
10	100% ethyl acetate

3.2.6. Gas Chromatography-Mass Spectrometry

This method is referred to as a hyphenated technique in which mass spectrometry is combined with gas chromatography. Based on their molecular mass and volatility, this approach identifies the structure of a mixture of organic compounds. Two instrumental elements are the gas chromatograph and the mass spectrometer, GC-MS. The first instrumental element is a gas chromatograph that separates the components of a mixture using a temperature-controlled

capillary column. Based on the boiling points (volatility) and molecular weights, the gas chromatograph distinguishes the constituents. The high volatility rate component passes from the column earlier, and those components that have high boiling points and high molecular weights pass later. The mass spectrometer is the second instrumental component. In this segment, each pulse is broken down and offers the pattern of mass fragmentation (mass spectra). Then the mass spectra for structure validation are compared to the available database [35].

3.2.6.1. Analytical condition for GC-MS

GC-MS analysis was performed on a gas chromatography-mass spectrometer GCMS-QP 2010 under the following condition:

Injection volume	1μL
Split ratio	1: 90
Carrier gas	Helium
Dimension of column	30m× 0.25mm×0.25 μm

Identification was accompanied by a comparison of MS.

The analytical line for GC-MS analysis

Column oven temperature	50.0℃
Injection temperature	220.00℃
Injection mode	Split
Flow control mode	Linear 24
Pressure	58.3 kPa
Total flow	56.2 mL/min
Linear velocity	37.4 cm/sec
Purge flow	2.0 mL/min
Split ratio	50.0
High-pressure injection	OFF
Carrier gas saver	OFF
Splitter hold	OFF
Ion source temperature	200.00℃

Interface temperature	250.00℃
Solvent cut time	3.00 min
Detector gain mode	Relative
Detector gain	1.05 kV
Threshold	0

Analytical Line for MS

Start time	3.00 min
End Time	22.60 min
ACQ mode	Scan
Event Time	0.50 sec
Scan Speed	1000
Start m/z	40.00
End m/z	500.00

3.2.6.2. Analysis of chloroform extract

The little amount of concentrated extract of chloroform obtained from the Rota evaporator was dissolved in chloroform and subjected to GC-MS analysis. The GC-MS analysis of chloroform extract and essential oil has been carried out at the Department of Food Technology and Quality Control, Babar Mahal, Kathmandu, Nepal.

3.2.7. Antibacterial activity

Antibacterial activity has determined the capacity of plant extracts/fractions/compounds to kill or prevent the growth of pathogenic microorganisms. It offers the rationale for the choice of potentially bioactive compounds. The antibacterial screening of plant extracts and essential oil was performed based on the procedure provided by the agar well diffusion process [42,43]. In this procedure, for the estimation of the antibacterial activity of the extract, and essential oil, the average diameter of the zone of inhibition (ZOI) formed by plant extracts and essential oil extract for a specific pathogenic bacterium was calculated. The capacity of an antibacterial agent to prevent *in*

vitro bacterial growth is assessed by antibacterial susceptibility tests. There are primarily two approaches for checking for antibacterial susceptibility. These are the mechanism of diffusion and the system of dilution. Of these, the approach based on diffusion is generally known as the method of Kirby-Bauer. The antibacterial activity test is very important. The following procedures follow this method:

3.2.7.1. Preparation of stock/ working solution

25 mg of plant extract (chloroform extract) was dissolved in 500 µL DMSO to form the concentration of 50 mg/mL stock solution in an Eppendorf tube. The extract was diluted from a stock solution in autoclaved distilled water and developed a 25 mg/mL working solution concentration. The pipes were sealed after making a stock/working solution and kept in the incubator at 37°C before use. The tube was capped, sealed, and kept cool until it was used.

3.2.7.2. Collection of standard culture

Active cultures of six standard strains of bacteria were provided by Research Institute for Bioscience and Biotechnology (RIBB), Nakhkhu, Lalitpur, Nepal. The following organisms were included in the study; four gram-positive bacteria *Staphylococcus aureus* KCTC 1916, *Bacillus subtilis* KACC 17047, *Micrococcus luteus* KACC 13377, and *Enterococcus sp.* KACC 13002, two gram-negative bacteria; *Pseudomonas aeruginosa* KACC 10232 and *Klebsiella pneumonia KCTC 2242*. In Nutrient Broth, all micro-organisms were cultured and maintained viable by sub-culturing in Nutrient Agar. By sub-culturing using the steak plate method, the integrity of the species was preserved.

3.2.7.3. Preparation of standard culture inoculum

As mentioned below, it was prepared from primary culture plates. The isolated colony was aseptically sub-cultured on nutrient agar plates with an inoculating loop. Then it was moved to a tube containing 9 mL of broth of sterile nutrients and incubated at 37°C for 24 hours.

3.2.7.4. Preparation of media

A) Nutrient agar

It was applied to distilled water at a ratio of 28 g/liter in a suitable conical flask size and boiled with continuous shaking and autoclaved for 15 minutes at 121°C. Sterilized media was allowed to cool at about 50°C. They were distributed aseptically in sterile 90 mm diameter Petri dishes at a ratio of 25 mL per dish and properly labeled. For solidification, plates were left as such.

B) Nutrient broth

For growing these pathogenic bacteria, the nutrient broth is used. In some distilled water, 1.3 g of nutrient was dissolved and diluted to 100 mL. It was sterilized for 15 minutes at 121°C by autoclaving. It was cooled and poured 9 mL of it inside the screwed capped bottle and sterilized again.

C) Muller Hinton agar

3.42 g of media was dissolved in 100 mL of distilled water and sterilized for 15 minutes at 121°C by autoclaving. It was then allowed to cool about 50°C and poured in 15 mL per plate into Petri-plates and the plates were left for solidification as well.

3.2.7.5. Screening and evaluation of an antibacterial activity

Sterile Muller-Hinton agar plates, already prepared, were dried to extract excess moisture from the surface of the media. The sterile cotton swab was dipped into the prepared inoculums, and by pressing and rotating against the upper inner wall of the tube above the liquid mark, the excess inoculums were collected and then carefully swabbed all over the plates. After every swabbing, the plate was rotated at an angle of 60°. The swab was eventually passed around the edges of the surface of the Agar. The inoculated plates, closed with a lid, were left to dry for minutes.

The wells were made with the aid of a sterile cork borer (4 mm) in the incubated media plates and properly numbered. Then, with the aid of a micropipette, 15 µL of working solution of plant extracts was loaded into the respective wells.

The solvent (DMSO) in the separate well was tested for its operation as a control at the same time. The plates were then left with the lid closed for half an hour so that samples were disseminated to the media. At 37°C, the plates were incubated for 6 hours. After proper incubation, the plates were observed for the inhibition zone around the well, which is indicated by a clean zone without growth was noted. The ZOI was calculated and the mean was recorded for the estimation of the potency of antibacterial substances with the help of the ruler. Also, the same procedure was applied for the calculation of ZOI of essential oil of *A. marmelos*.

3.2.8. Antioxidant activity

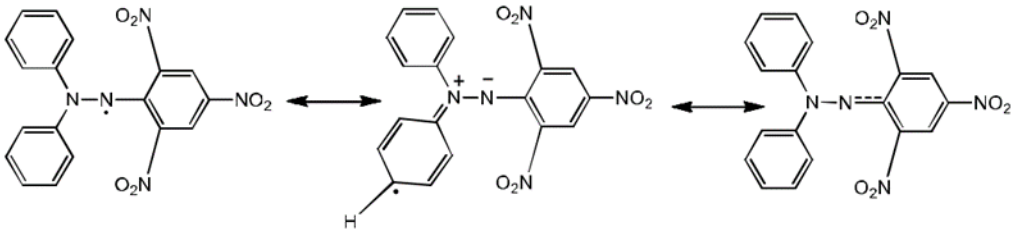
Compounds capable of either delaying or inhibiting the oxidation processes that occur under the influence of atmospheric oxygen or reactive oxygen species are antioxidants. They are used to stabilize polymeric materials, petrochemicals, foodstuffs, pharmaceuticals, and cosmetics [44]. Free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), are known to cause aging-related damage to lipids, proteins, enzymes, and nucleic acids, resulting in injury to cells or tissues. Degenerative disorders such as inflammation, cancer, atherosclerosis, diabetes, liver damage, Alzheimer's, Parkinson's, and coronary heart diseases are caused by these free radicals and oxidative stress. Some evidence suggests that oxidative stress can lead to damage to cells and tissues. Antioxidant degradation occurs during oxidative stress. Superoxide (O_2^-.), hydroxyl (OH.), peroxyl (ROO.), peroxynitrite (.$ONOO^-$), and nitric oxide (NO.) radicals, as well as non-free radical species, such as hydrogen peroxide (H_2O_2), nitrous acid (HNO_2) and hypochlorous acid (HOCl), are various reactive entities found in the ROS and RNS [45].

Superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione (GSH), beta-carotene, vitamin A, ascorbic acid (vitamin C), and alpha-tocopherol (vitamin E) have a broad variety of enzymatic and non-enzymatic antioxidant defenses. Both the behaviors and the intracellular levels of these metabolites are interrelated, shielding themselves from oxygen toxicity [46]. The stable free radical method of DPPH is a simple, fast, and sensitive

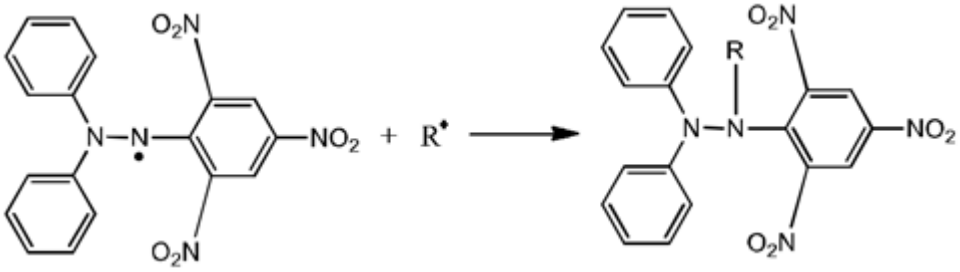
way of investigating the antioxidant activity of a particular compound or plant extract [47].

3.2.8.1. Principle of DPPH assay

The free-radical method of DPPH (2, 2-Diphenyl-1-picrylhydrazyl-hydrate) is an electron-transfer dependent antioxidant assay that creates a violet solution in alcohol and, due to the presence of an antioxidant molecule, changes to a colorless solution [48]. Because of the delocalization of the lone pair of electrons over the molecule as a whole, molecule 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is defined as a stable free radical to avoid dimerization, as would be the case for most other free radicals. The delocalization results in deep violet color, distinguished by an absorption band centered at about 517 nm in the methanol solution. When a DPPH solution is combined with a product that may donate a hydrogen atom, due to the still presence of pecryl residue, this gives decreased shape with the transition of the violet color to the pale color at the end [49,50].



Stable DPPH free radical



DPPH(dark purple) Derivatives of DPPH(pale yellow)

The reaction of DPPH free radical

3.2.8.2. Preparation of DPPH solution

The molecular weight of 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) is 394.32 g/mol. Therefore, 100 mL of 0.1 mM solution of DPPH was prepared by weighing 4 mg of the DPPH carefully and dissolving it with methanol, and finally, maintaining the volume to 100 mL.

3.2.8.3. Measurement of DPPH free radical scavenging activity

The percentage of radical scavenging activity was calculated using the following formula:

Percentage scavenging = $\frac{A_o - A_T}{A_o} \times 100\%$

Where A_o = Absorbance of the DPPH

A_T = Absorbance of the DPPH free radical solution containing the sample extract.

The 50 % inhibitory concentration value IC_{50} is indicated as the effective concentration of the sample required to scavenge 50 % of the DPPH free radicals. IC_{50} values were determined using the dose inhibition curve in the logarithm range by plotting the concentration of extract versus the corresponding scavenging effect.

3.2.8.4. General protocol for antioxidant assay

When 1 mg of the sample is dissolved in 1 mL of solvent, gives a solution of 1 mg/mL concentration and 2 mg in 1 mL of solvent gives a solution of 2 mg/mL concentration. Therefore, 10 mg of the sample (*A. marmelos* chloroform extract) to be tested was dissolved in 5 mL of methanol to achieve a concentration of 2 mg/mL (2000 µg/mL) stock solution. Different concentrations of 1000 µL (1 mL) extracts (1500, 1000, 500, 250 and 125 µg/mL) were prepared using stock solution by two-fold dilution process.

Table 2: Preparation of different concentration test samples for antioxidant assay

Concentration (µg/mL)	**Extract Solution**	**Distilled water (µL)**	**Final Volume(µL)**
1500	750 µL (2000 µg/mL)	250	1000
1000	500 µL (2000 µg/mL)	500	1000
500	500 µL (1000 µg/mL)	500	1000
250	500 µL (500 µg/mL)	500	1000
125	500 µL (250 µg/mL)	500	1000

For 1500 µL (1.5 mL) of 0.1 mM DPPH (4 mg DPPH in 100 mL methanol), 500 µL (0.5 mL) of these solutions were applied differently. By coating with aluminum foil, the solutions were prevented from illumination and the solution was vigorously shaken for around 2 minutes. The solution was left in a dark room at room temperature for 30 minutes. After 30 minutes, their absorbance against methanol was taken as a blank at 517 nm. The solution was prepared by 0.5 mL methanol and 1.5 mL DPPH solution were taken as control and its absorption was taken spectrophotometrically at 517 nm. Moreover, a calibration curve was prepared.

The essential oil of antioxidant assay was conducted against DPPH. At first, 1 mg of the sample to be tested was dissolved in 1 mL of methanol to prepare a stock solution of 1 mg/mL concentration. Then 100 µL of these solutions were applied to 100 µL of 0.1 mM (methanol-prepared) DPPH and left in the darkroom for 30 minutes. After 30 minutes, their absorption was assessed against DPPH at 517 nm and the result was calculated.

3.2.9. Total phenol content assay

Phenolic compounds are secondary metabolites derived from the plant pathways of pentose phosphate, shikimate, and phenylpropanoid [51]. Approximately 8000 phenolic compounds have been classified as biologically active ingredients [52]. For different purposes, plants produce polyphenols, such as plant pigmentation, reproduction, and protection from bacterial pathogens or UV light. The chemically specified "phenolic" or "polyphenol" is a material having an aromatic ring with one (phenol) or more hydroxyl substituents (polyphenol) [53].

The most naturally occurring phenolic compounds are present as mono- and polysaccharide conjugates, connected to one or more of the phenolic groups, and can also occur as functional derivatives such as glycosides, esters, and methyl esters [54]. A broad variety of physiological properties are displayed by phenolic substances, such as anti-allergenic, anti-inflammatory, anti-microbial, antioxidant, cardioprotective, antimutagenic, anticarcinogenic, and gene expression modification capacities. The health benefits resulting from eating high amounts of fruits and vegetables have been correlated with phenolic compounds [55].

3.2.9.1. Principle of the total phenolic content assay

The contents of different total phenolic compounds in the plant are calculated by the Folin-Ciocalteau reagent. Indeed, the Folin-Ciocalteau reagent tests the reduction capacity of a sample. The exact chemical composition of the Folin-Ciocalteau reagent is not known, but the Folin-Ciocalteau phenol reagent is assumed to contain a mixture of heteropoly acids, phosphomolybdic acids, and phosphotungstic acids in which the oxidation state of molybdenum and tungsten is 6^{+}. Reversible one or two-electron reduction sequences contribute to the formation of molybdenum blue and tungsten blue, probably (Phenol-$MoW11O40)_4$, and the average metal oxidation state is between 5 and 6. In essence, molybdenum is assumed to be easier to reduce in the complex and electron transfer reaction occurs between reductants and Mo(VI) [56,57].

3.2.9.2. Preparation of Folin-Ciocalteau reagent

1 mL of Folin-Ciocalteau reagent was taken in a beaker and 10 times dilution was done with distilled water.

3.2.9.3. Preparation of standard Gallic acid solution

In 1 mL of distilled water, 1 mg of Gallic acid was dissolved, so the solution concentration was 1 mg/mL or 1000 µg/mL. The solution is called a stock solution. The different concentrations of Gallic acid solution (100, 80, 60, 40, 20, and 10 µg/mL) were then prepared using a two-fold dilution process.

3.2.9.4. Measurement of total phenolic content

The concentration of total phenolic content in the sample was determined as milligram of Gallic acid equivalent by using the following equation:

$$TPC = \frac{C \times V}{m}$$

Where C = concentration of Gallic acid from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

3.2.9.5. General protocol for total phenolic content

1 mL of Folin-Ciocalteu phenol reagent (1:10 dilution with water) and 0.8 mL of aqueous 1 M Na_2CO_3 solution were combined with 0.1 mL of sample (1 mg/mL methanol). The reaction mixture was allowed to stand in the dark for about 15 minutes and then the absorption of the reactants was measured against the blank at 765 nm (methanol). Gallic acid was used as standard. The total phenolic content is expressed as mg of equivalent Gallic acid per gram of dried extract.

3.2.10. Total flavonoid content assay

Flavonoids are a diverse family distributed widely in plant leaves, seeds, bark, and bulbs. The flavonoids have two benzene rings separated by a propane unit.

The most commonly distributed of all phenolic are flavones and flavonols [58]. Diphenyl propane (C_6-C_3-C_6) skeletons, secondary plant phenolic distinguished by the flavone nucleus, are a large class of low molecular weight polyphenols with flavonoids. Flavonoids include six major subgroups that are found in higher plants: the chalcones, flavones, flavandiols, anthocyanin, and condensed tannins (or proanthocyanidins) and aurones [53]. Such compounds protect against ultraviolet radiation, bacteria, and herbivores in plants. The majority of beneficial health effects of flavonoids are due to their antioxidant, chelating ability, and decreased heart disease incidence [59]. The pharmacological effects of flavonoids include their ability to inhibit histamine release, blood platelet adhesion, and lens aldose reductase action, to block the inflammatory effects of hepatotoxins, and function as heart stimulants [46].

3.2.10.1. Principle of total flavonoid content assay

The amount of the total flavonoids of the plant extract was estimated by the well-known colorimetric method of aluminum chloride. In this approach, with hydroxyl groups of flavonoids present in the sample, aluminum chloride forms complexes. At 420 nm, this complex has maximum absorbance.

3.2.10.2. Preparation of standard quercetin solution

In 1 mL of distilled water, 1 mg of quercetin was dissolved, so the solution concentration is 1 mg/mL or 1000 μg/mL. This is called a stock solution. Then, the different quercetin solution concentrations (80, 60, 40, 20, 10, and 5 μg/mL) were prepared using the two-fold dilution process.

3.2.10.3. Measurement of total flavonoid content

The concentration of total phenolic content in the sample was determined as milligram of quercetin equivalent by using the following equation:

$$\text{TFC} = \frac{\text{C} \times \text{V}}{m}$$

Where C= concentration of quercetin from curve (μg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

3.2.10.4. General protocol for total flavonoid content

1 mL of sample (0.1 mg/mL in methanol) was mixed with 1 mL of 2 % $AlCl_3$ (dissolved in methanol) and kept for 1 hour and absorbance was measured at 415 nm against the blank (methanol). Quercetin was used as standard. Total flavonoid content is expressed as mg of quercetin equivalents per gram of dried sample.

3.2.11. Brine shrimp lethality bioassay

To investigate the cytotoxicity of plant extracts, the brine shrimp lethality bioassay was carried out. The bioassay for brine shrimp lethality is a quick, inexpensive, and comprehensive bioassay that has allowed bioactive compounds of natural origin to be discovered [60]. It is a preliminary toxicity screening of plant extracts, fungal toxins, heavy metals, toxins for cyanobacteria, pesticides, dental substance cytotoxicity monitoring, and nanostructures. In a basic zoological organism (brine nauplii), the approach utilizes *in vivo* lethality as a convenient control for screening and fractionation in the discovery of new bioactive natural products [61]. This research was first suggested by Michael *et al.* in 1956 and later updated by others. This lethality assay has been successfully employed as a bioassay guide for active cytotoxic and antitumor agents. It is a rapid (24 hours), and simple test as no aseptic techniques are required. A large number of species are easily used for statistical validation and do not require special equipment and relatively small amounts of samples (2-20 mg or less). Antifungal effects, pesticide effects, teratogenic effects, environmental toxicity, and many more are also indicated in the brine shrimp lethality bioassay [62,63].

3.2.11.1. Preparation of seawater

3.8 g sea salt (without iodine) was weighed, dissolved in 100 mL of distilled water, and filtered off to get a clear solution.

3.2.11.2. Hatching of brine shrimp

The research organism *Artemia salina* leach (brine shrimp eggs) was hatched inside a conical flask of seawater. The shrimp were allowed to hatch for two days, and to mature as nauplii. Through the hatching time, a continuous oxygen supply was carried out. The hatched shrimps were drawn to the light (phototaxis). The nauplii were extracted by a pipette from the fish tank and dissolved in clear fresh seawater to improve visibility, and 10 nauplii were taken carefully by micropipette.

Figure 3: Brine shrimp

3.2.11.3. Preparation of test solutions with samples of experimental plants

10 mg of the test sample (chloroform extract of *A. marmelos*) was taken and dissolved in 1 mL of pure dimethyl sulfoxide (DMSO) and finally, the volume was made to 10 mL with seawater. Thus, the concentration of the stock solution was 1000 µg/mL. The solution was then serially diluted to 500, 250, 125, 62.5, 31.25, 15.625 µg/mL with seawater. Then, in 2.5 mL of seawater containing 10 nauplii, 2.5 mL of plant extract solution was added.

Table 3: Preparation of different concentration test samples for brine shrimp lethality assay

Concentration (µg/mL)	**Extract Solution**	**Seawater containing 10 nauplii (mL)**	**Final Volume (mL)**
500	2.5mL (1000 µg/mL)	2.5	5
250	2.5mL (500 µg/mL)	2.5	5
125	2.5mL (250 µg/mL)	2.5	5
62.5	2.5mL (125 µg/mL)	2.5	5
31.25	2.5mL (62.5 µg/mL)	2.5	5
15.625	2.5mL (31.25 µg/mL)	2.5	5

3.2.11.4. Preparation of control group

In the cytotoxicity analysis, control groups were used to verify the test method and ensure that the findings obtained were only attributable to the behavior of the test agent and nullification of the effects of the other potential variables. In each of three pre-marked test tubes containing 4.95 mL of simulated seawater and 10 shrimp nauplii for use as control groups, 50 µL of DMSO was applied. If a rapid mortality rate is shown by the brine shrimps in these vials, then the test is regarded as invalid because the nauplii died for some reason other than the cytotoxicity of compounds.

3.2.11.5. Counting of nauplii

After 24 hours, the test tubes were inspected against a black background using a magnifying glass and the number of nauplii surviving in each tube was counted. From this data, for each concentration, the percent (%) of the lethality of brine shrimp nauplii was determined. Plant product efficacy or concentration-mortality relationship is typically expressed as a median lethal concentration

(LC_{50}). This reflects the concentration of the chemical that causes death after a certain exposure period in half of the test subjects and is measured by the process of logarithm regression from the % mortality plot against the corresponding concentration [64].

CHAPTER 4: RESULT AND DISCUSSION

4.1. Plant extracts

The extracts of *A. marmelos* in methanol, hexane, and chloroform were prepared, concentrated using Rota-evaporator, and dried using a water bath. The weight of different extracts of the plant is shown below:

Table 4: Weight of plant extract

Plant extract	**Weight(g)**
Methanol	42.8
Hexane	16.1
Chloroform	3.9

4.2. Phytochemical screening analysis

Any chemical compounds that have a definite physiological impact on the human body lie in the medicinal value of plants [65]. The results of phytochemical screening of *A. marmelos* are shown below:

Table 5: Phytochemical screening of plant extracts

Phytochemical constituents	Qualitative analysis			Conclusion
	Methanol	Hexane	Chloroform	
Alkaloids	+	-	+	Alkaloids were present in methanol and chloroform extracts
Flavonoids	+	-	+	Flavonoids were present in methanol and chloroform extract
Phenols	+	-	-	Phenols were present in methanol extracts
Glycosides	+	-	+	Glycosides were present in methanol chloroform extracts
Tannins	+	-	+	Tannins were present in methanol and chloroform extracts
Quinones	-	-	-	Quinones was absent in all three extracts
Saponins	+	+	+	Saponins were present in all three extracts
Protein	+	+	+	Protein was present in all three extracts
Carbohydrates	+	-	-	Carbohydrate was present in methanol extracts
Emoline	+	-	-	Emoline were present in methanol extracts

'+' indicates presence and '- ' indicates the absence

4.3. TLC analysis

The qualitative analysis of chloroform extract of *A. marmelos* was done by TLC during which the polarity of ethyl acetate to hexane was increased gradually. The plates were created in different solvents and visualized in a UV-fluorescence lamp. The visualized plates were shown in the following figure:

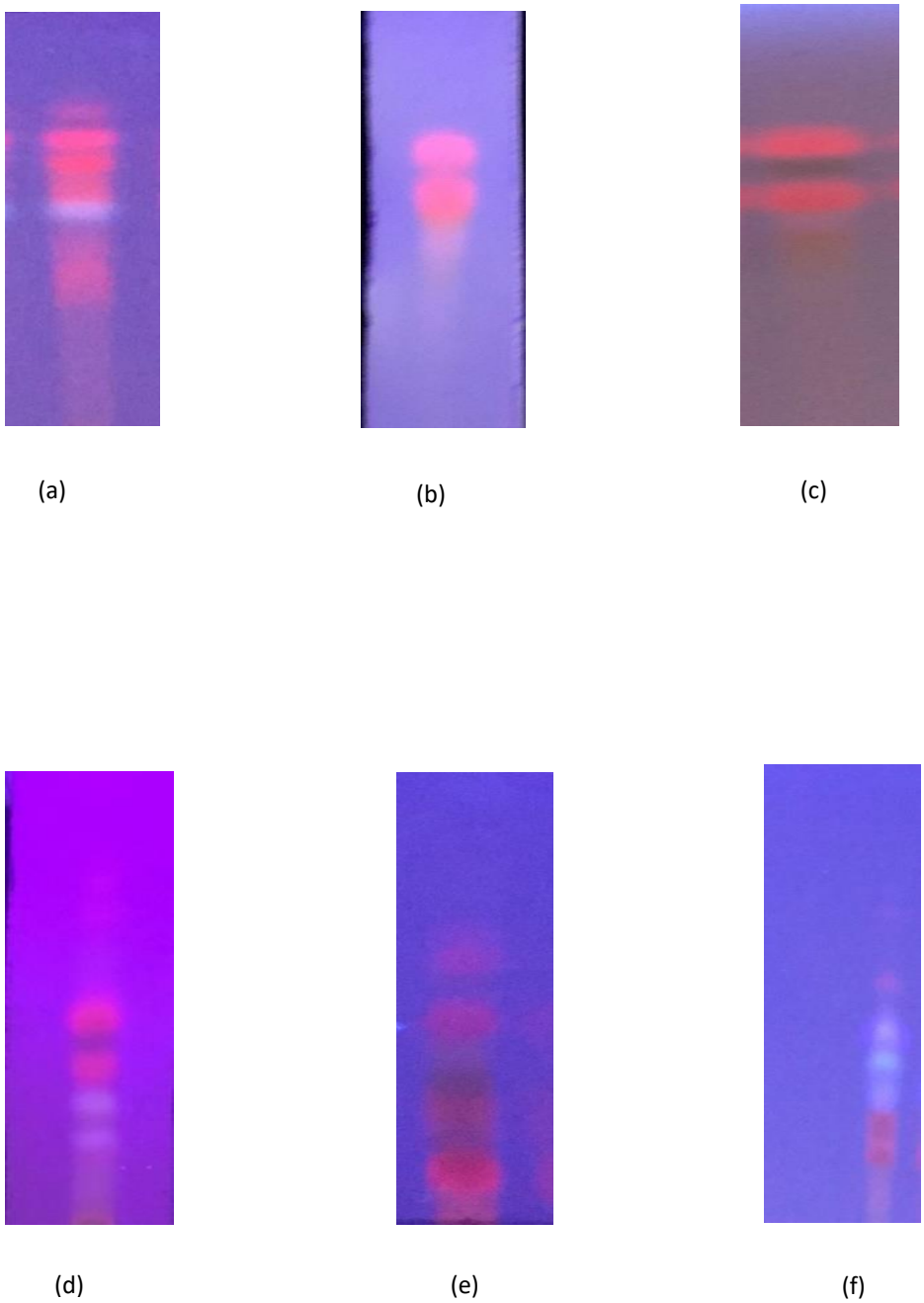


Figure 4: TLC showing spots of extracts of *A. marmelos* under UV florescence (a) 20 % (b) 30 % (c) 40 % (d) 50 % (e) 60 % and (f) 70 % ethyl acetate in hexane solvent systems

The TLC reports are illustrated in the following Table 6:

Table 5: TLC of different fractions of chloroform extract of *A. marmelos*

S.N	Solvent of TLC	TLC report
1	10% ethyl acetate in hexane	No movement
2	20% ethyl acetate in hexane	4 spots with tailing
3	30% ethyl acetate in hexane	2 spot with tailing
4	40% ethyl acetate in hexane	2 clear spot
5	50% ethyl acetate in hexane	4 spot
6	60% ethyl acetate in hexane	4 spot with tailing
7	70% ethyl acetate in hexane	Spot with tailing
8	80% ethyl acetate in hexane	Spot with tailing
9	90% ethyl acetate in hexane	Spot with tailing
10	100% ethyl acetate	Spot with tailing

4.4. GC-MS spectra analysis

GC-MS chromatogram of the chloroform extract of *A. marmelos* shows the presence of 9 major compounds. The GC-MS chromatogram of chloroform extract of *A. marmelos* is presented below:

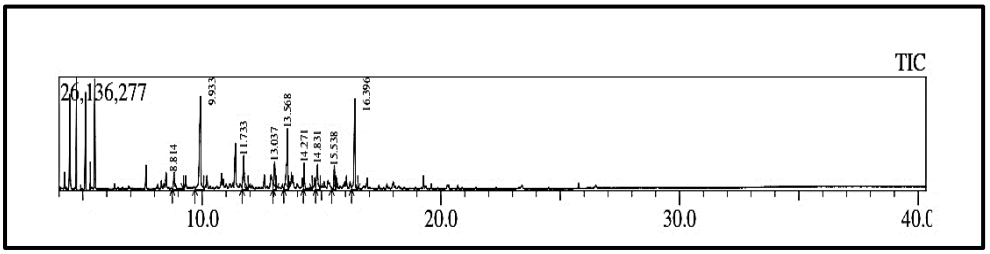


Figure 5: Chromatogram of chloroform extract of *A. marmelos*

The composition of chloroform extract of *A. marmelos* was analyzed by GC-MS coupled with mass library search revealed the presence of 9 major compounds which are shown below:

Table 6: Percentage composition of chloroform extract of *A. marmelos*

S.N.	Name of Compound	Retention time	Molecular formula	Molecular Weight	Area (%)
1	4(10)-Thujen-2-ol,acetate	8.814	$C_{12}H_{18}O_2$	194	4.85
2	Limonene dioxide	9.933	$C_{10}H_{16}O_2$	168	27.78
3	Cyclohexanone,2-(1-methyl-oxopropyl)	11.733	$C_{10}H_{11}O_2$	168	8.38
4	(1S,2S,3R,5S)-(+)-Pinanediol	13.037	$C_{10}H_{18}O_2$	170	6.40
5	Cis-caryophyllene	13.568	$C_{15}H_{24}$	204	15.14
6	1,4,7,-cycloundecatriene,1,5,9,9, tetramethyl	14.271	$C_{15}H_{24}$	204	5.50
7	GermacreneD	14.831	$C_{15}H_{24}$	204	6.79
8	Cubenol	15.538	$C_{15}H_{26}O$	222	4.48
9	GermacreneB	16.396	$C_{15}H_{24}$	204	20.65

4.4.1. Mass spectral data of constituents of plant extract identified by GC-MS

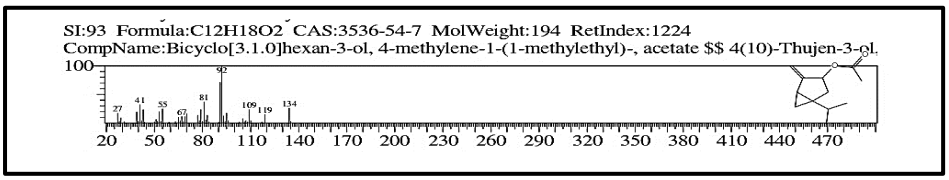


Figure 6: Mass spectral data of 4(10)-Thujen-3-ol,acetate

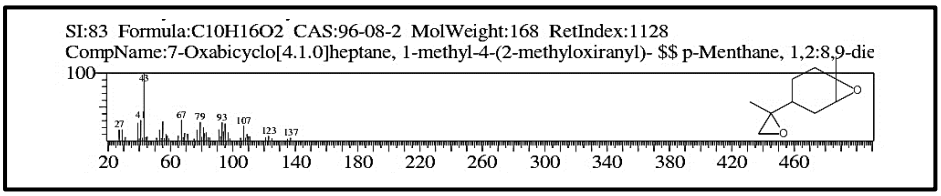


Figure 7: Mass spectral data of Limonene dioxide

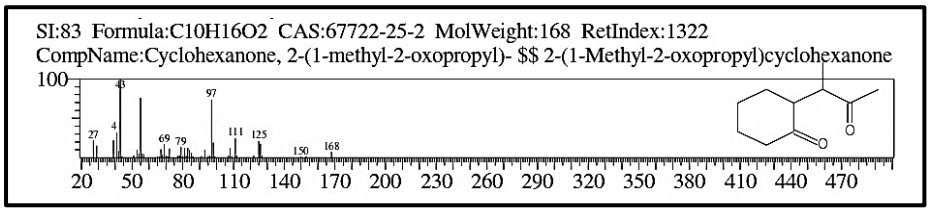


Figure 8: Mass spectral data of Cyclohexanone, 2-(1-methyl-oxopropyl)

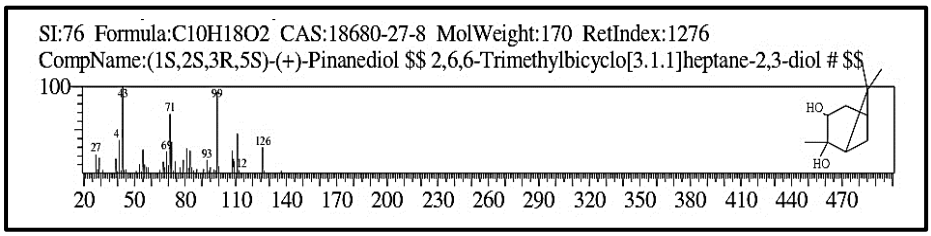


Figure 9: Mass spectral data of (1S, 2S, 3R, 5S)-(+)-Pinanediol

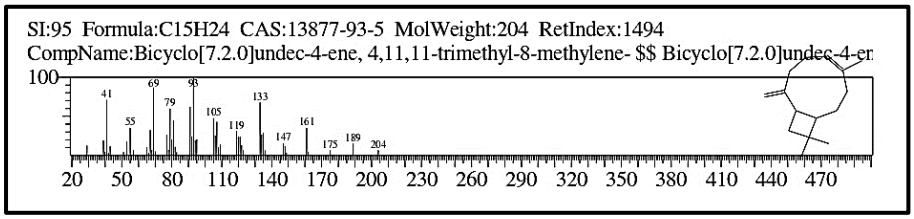


Figure 10: Mass spectral data of Cis-caryophyllene

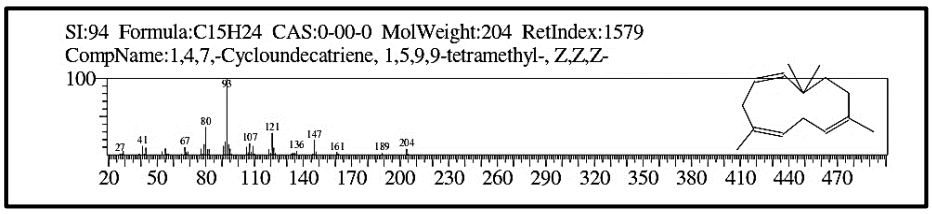


Figure 11: Mass spectral data of 1,4,7, cycloundecatriene,1,5,9,9,tetramethyl

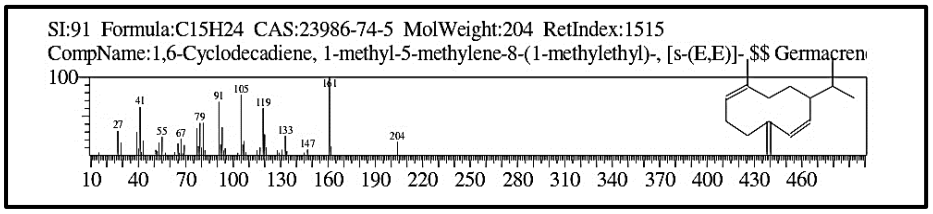


Figure 12: Mass spectral data of GermacreneD

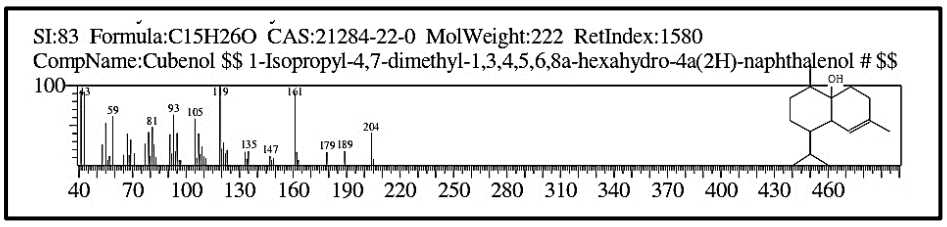


Figure 13: Mass spectral data of Cubenol

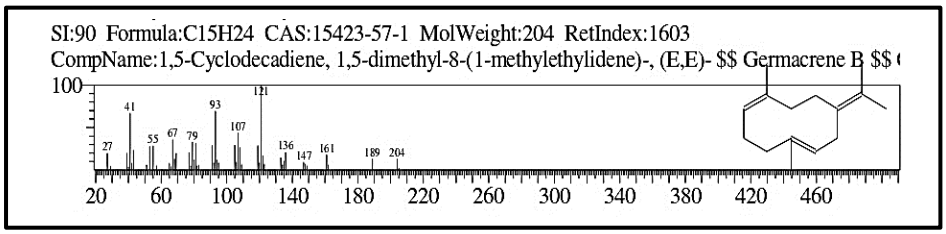


Figure 14: Mass spectral data of GermacreneB

The gas chromatographic analysis of essential oil resulted in the identification of a total of 12 different constituents. The essential oil was found to contain Limonene (25.06 %) and GermacreneB (18.63 %) as presented in Table 8:

Table 7: Percentage composition of essential oil of *A. marmelos*

S.N.	Name of Compound	Retention time	Molecular formula	Molecular Weight	Area (%)
1	4-ethoxybicyclo[3.2.0]hept-2-ene	7.643	$C_9H_{14}O$	138	3.39
2	4(10)-Thujen-3-ol	8.814	$C_{12}H_{18}O_2$	194	4.38
3	Limonene dioxide	9.933	$C_{10}H_{16}O_2$	168	25.06
4	Cyclohexanone,2-(1-methyl-oxopropyl)	11.733	$C_{10}H_{16}O_2$	168	7.56
5	Pinanediol	13.037	$C_{10}H_{18}O_2$	170	5.78
6	Cis-caryophyllene	13.568	$C_{15}H_{24}$	204	13.66
7	(1Z,4Z,7Z)-1,5,9,9-tetramethylcycloundeca-1,4,7-triene	14.271	$C_{15}H_{24}$	204	4.96
8	Cubenol	15.538	$C_{15}H_{26}O$	222	4.48
9	GermacreneB	16.396	$C_{15}H_{24}$	204	18.63
10	GermacreneD	14.831	$C_{15}H_{24}$	204	6.13
11	Dipentene dioxide	15.272	$C_{10}H_{16}O_2$	168	3.30
12	Diosphenol	13.738	$C_{10}H_{16}O_2$	168	3.11

4.4.2. Mass spectral data of constituents of essential oil identified by GC-MS

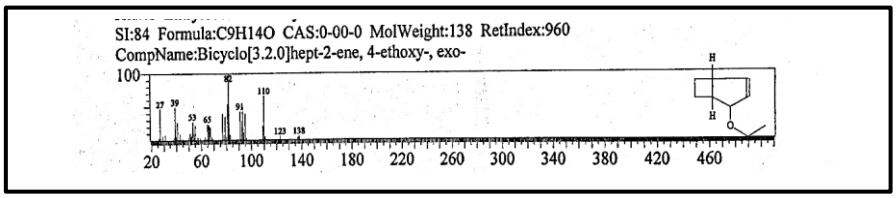


Figure 15: Mass spectral data of 4-ethoxybicyclo [3.2.0]hept-2-ene

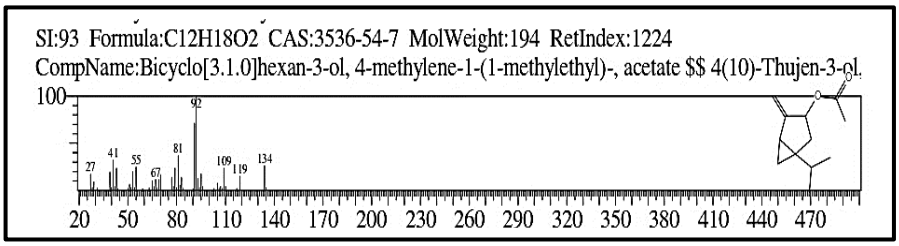


Figure 16: Mass spectral data of 4(10)-Thujen-3-ol, acetate

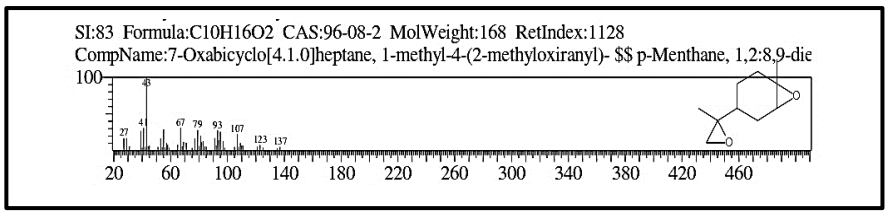


Figure 17: Mass spectral data of Limonene dioxide

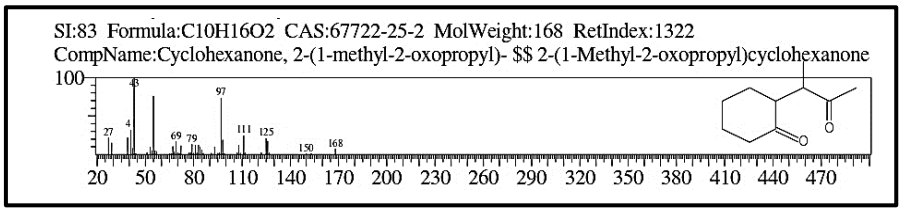


Figure 18: Mass spectral data of Cyclohexanone,2-(1-methyl-oxopropyl)

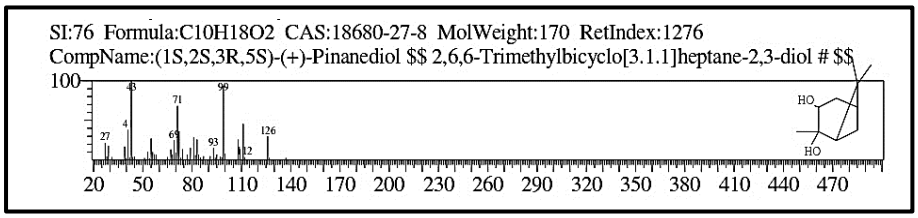


Figure 19: Mass spectral data of (1S,2S,3R,5S)- (+)-Pinanediol

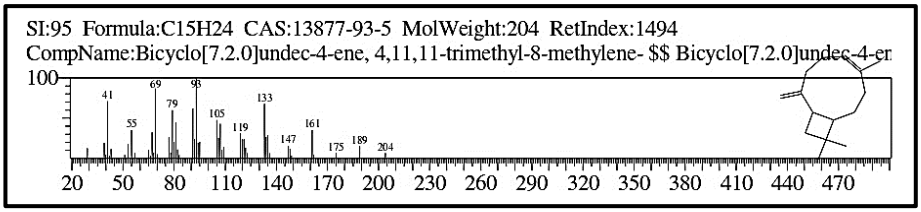


Figure 20: Mass spectral data of Cis-caryophyllene

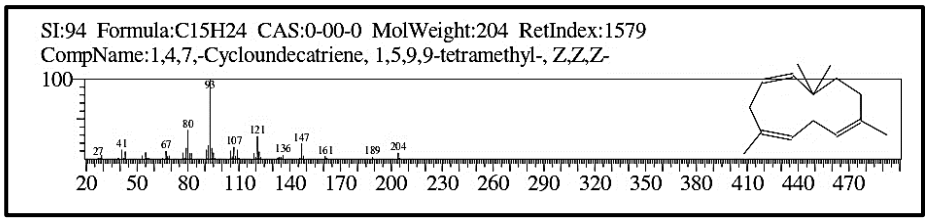


Figure 21: Mass spectral data of (1Z,4Z,7Z)-1,5,9,9-tetramethylcycloundeca-1,4,7-triene

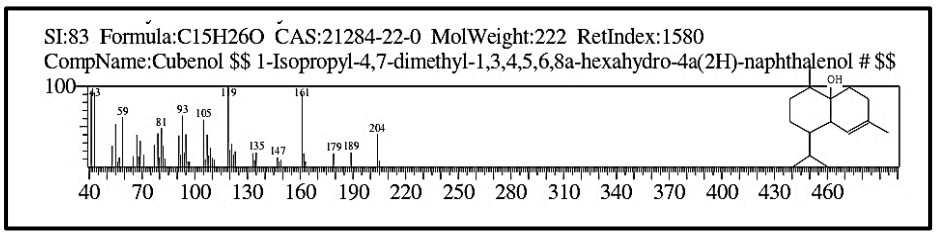


Figure 22: Mass spectral data of Cubenol

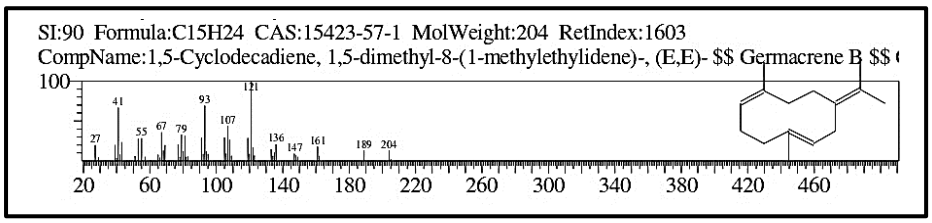


Figure 23: Mass spectral data of GermacreneB

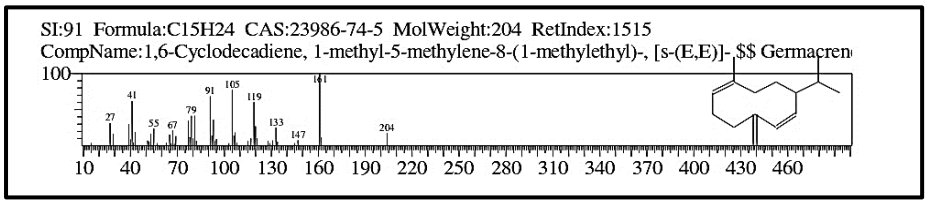


Figure 24: Mass spectral data of GermacreneD

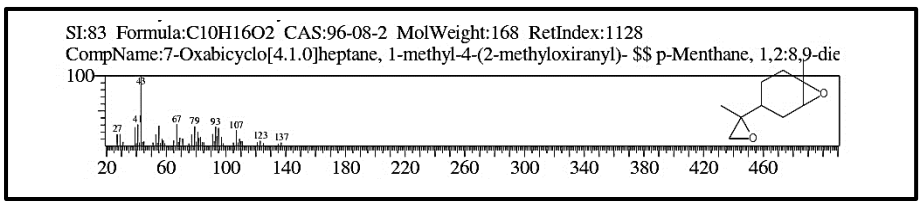


Figure 25: Mass spectral data of Dipentene dioxide

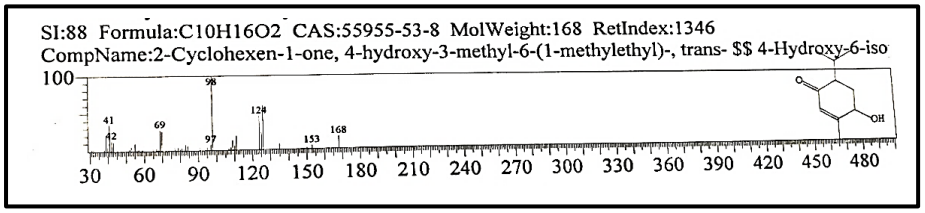
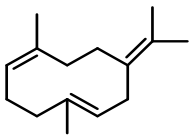
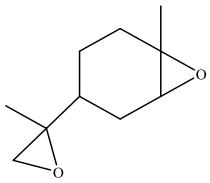


Figure 26: Mass spectral data of Diosphenol

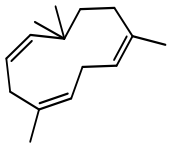
4.4.3. Structure of compounds detected from GC-MS analysis of chloroform extract and essential oil of *A. marmelos*



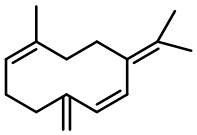
GermacreneB



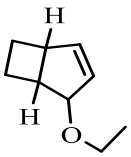
Limonene dioxide



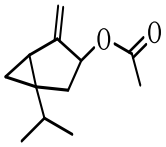
(1Z,4Z,7Z)-1,5,9,9-tetramethylcloundeca-1,4,7-triene



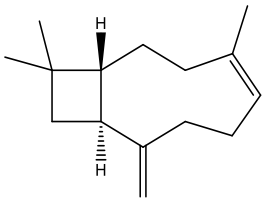
GermacreneD



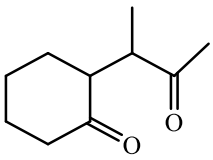
4-ethoxybicyclo[3.2.0]hept-2-ene



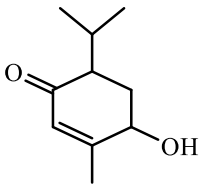
4(10)-Thujen-3-ol



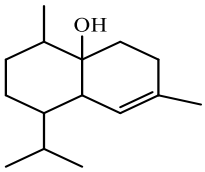
Cis-Caryophyllene



2-(3-oxobutan-2-yl)cyclohexane-1-one



Diosphenol



Cubenol

4. 5. Antibacterial screening analysis

The chloroform extract of *A. marmelos* was examined against six bacteria samples; *Staphylococcus aureus, Enterococcus sp., Bacillus subtilis, Klebsiella pneumoniae, Micrococcus luteus,* and *Pseudomonas aeruginosa* for the antibacterial potential of the chloroform extract. The plant extract did not show any antibacterial activity.

The zone of inhibition of essential oil for six bacteria was tested. The ZOI of *B. subtilis* was observed 7 mm present in Table 9:

Table 8: Antibacterial activity test of essential oil

Bacteria tested	Zone of inhibition (15 µL)
Staphylococcus aureus KCTC 1916	No activity
Bacillus subtilis KACC 17047	7 mm
Micrococcus leutus KACC 13377	No activity
Pseudomonas aeruginosa KACC 10232	No activity
Enterobacter cloaceae subsp. disolvens KACC 13002	No activity
Klebsiella pneumonia KCTC 2242	No activity

The result shows that the essential oil of *A. marmelos* is slightly effective for antibacterial activities. The essential oil isolated from *A. marmelos* showed antibacterial activity against *B. subtilis* (7 mm) among all tested microorganisms, for others, no activity was detected.

4.6. Antioxidant screening analysis

The antioxidant potential is inversely related to the IC_{50} value, which can be determined from the linear regression of % inhibition versus antioxidant activity. Lower the IC_{50} value indicates high antioxidant activity. All of the measurements are based on the normal procedure. Absorbance was measured at 517 nm [35,68].

The absorbance of each solution was measured and recorded as follows:

Table 9: Result of DPPH assay of chloroform extract

Sample	Concentration (µg/mg)	Absorbance(nm)			Average Absorbance (nm)	Percentage Scavenged
Control		0.672	0.671	0.673	0.672	
A. marmelos (Chloroform Extract)	1500	0.098	0.096	0.099	0.097	85.438
	1000	0.132	0.130	0.133	0.131	80.505
	500	0.228	0.226	0.226	0.226	66.369
	250	0.388	0.387	0.391	0.388	42.261
	125	0.489	0.491	0.483	0.487	27.529

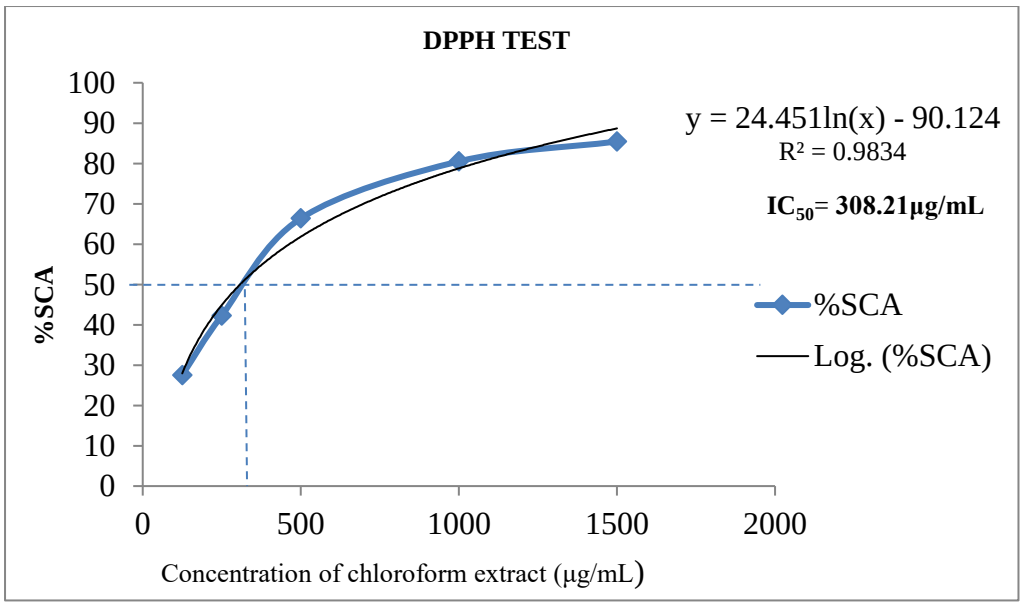


Figure 27: Graphical representation of DPPH assay of the extract

This quantitative measure shows how much of a specific drug or other substance (inhibitor) is required to inhibit a given biological process (or process part, i.e., enzyme, cell, cell receptor, or microorganism). Usually, the values are

expressed as molar concentration. In pharmacological science, it is widely used as an indicator of antagonist drug potency. IC_{50} represents the concentration of a drug that is needed for 50 % inhibition *in vitro*, according to the FDA.

This study shows that the IC_{50} value of chloroform extract of *A. marmelos* was 308.21 µg/mL. This demonstrates that the chloroform extract of *A. marmelos* was average towards an antioxidant activity.

Table 10: Result of DPPH assay of essential oil of *A. marmelos*

Concentration (mg/mL)	Percentage scavenge
12	37.037
14	52.490
16	69.621
18	77.103
20	83.099

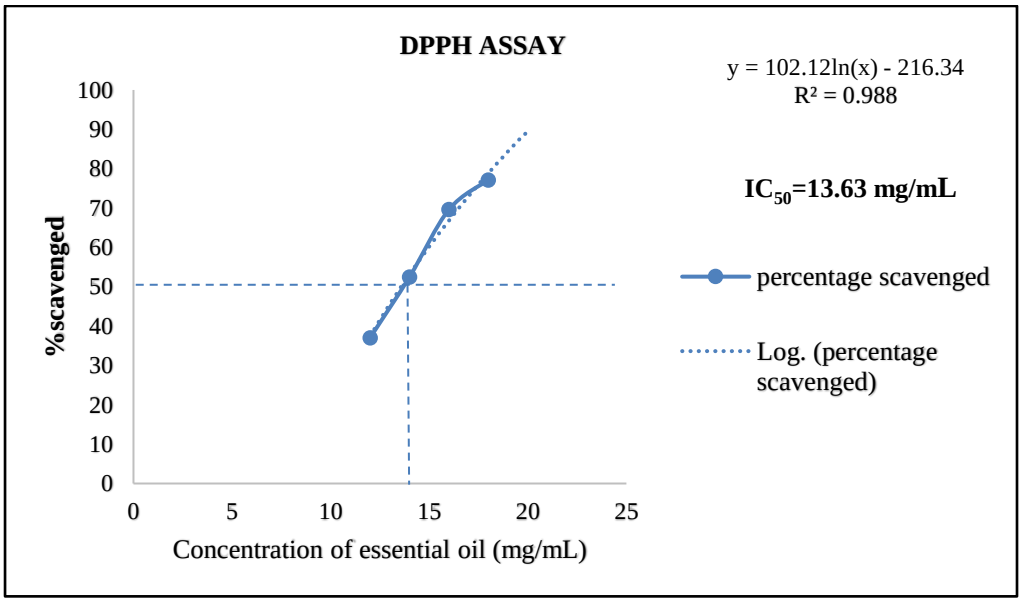


Figure 28: Graphical representation of DPPH assay of the essential oil

The IC_{50} of the oil was found to be 13.63 mg/mL. From this result, it is known that the essential oil of *A. marmelos* is slightly average for antioxidant activity.

4.7. Total phenolic content analysis

The total phenolic content was determined as a milligram of Gallic acid equivalent using the calibration curve of Gallic acid.

The absorbance of each solution was measured and recorded as follows:

Table 11: Absorbance of Gallic acid

Concentration(µg/mL)	Absorbance (nm)			Average Absorbance (nm)
10	0.088	0.086	0.088	0.087
20	0.177	0.174	0.173	0.174
40	0.324	0.323	0.323	0.323
60	0.488	0.483	0.489	0.486
80	0.671	0.663	0.631	0.657
100	0.831	0.854	0.861	0.846

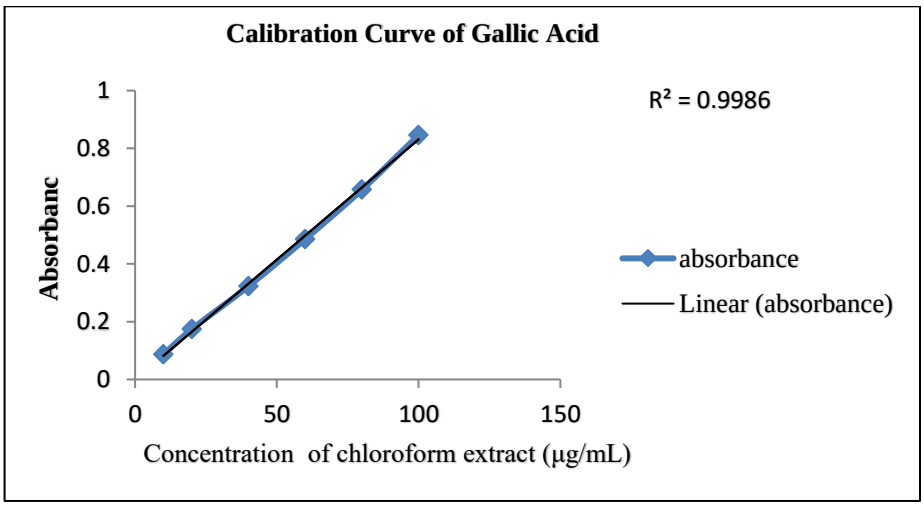


Figure 29: Calibration curve of gallic acid for total phenolic content determination

The concentration of gallic acid in chloroform extract of *A. marmelos* was determined by using an equation that was obtained from the standard Gallic acid curve in figure 29. The equation is given below:

$y = 0.0083x - 0.0014$

$R^2 = 0.9986$

Where, y = absorbance

x = Gallic acid concentration (GAC) (µg/mL)

m = slope = 0.0083

c = y-intercept = -0.0014

$\therefore X = \frac{y+0.0014}{0.0083}$

Table 12: Total phenolic content in leaves of *A. marmelos*

Sample concentration (mg/mL)	**Weight of extract per mL m(g)**	**Absorbance**	**GAC, C (µg/mL)**	**GAC, C (mg/mL)**	**TPC as GCE** $= \frac{C \times V}{m}$ **(mg/g)**
1	0.001	0.483	58.361	0.05836	58.36

Table 13 shows the total phenolic contents of *A. marmelos* leaf tested as Gallic acid equivalent by reference to a standard curve ($y = 0.0083x - 0.0014$, $R^2 = 0.996$). The total phenolic content in the leaf of *A. marmelos* was 58.36 mg Gallic acid equivalent / g of dry extract.

The total phenolic content of essential oil of *A. marmelos* was determined as a milligram of phenol standard using the calibration curve of Gallic acid.

Table 13: Absorbance of phenol standard for essential oil

Concentration (µg/mL)	**Absorbance (nm)**
10	0.03
20	0.117
40	0.266
60	0.429
80	0.6
100	0.789

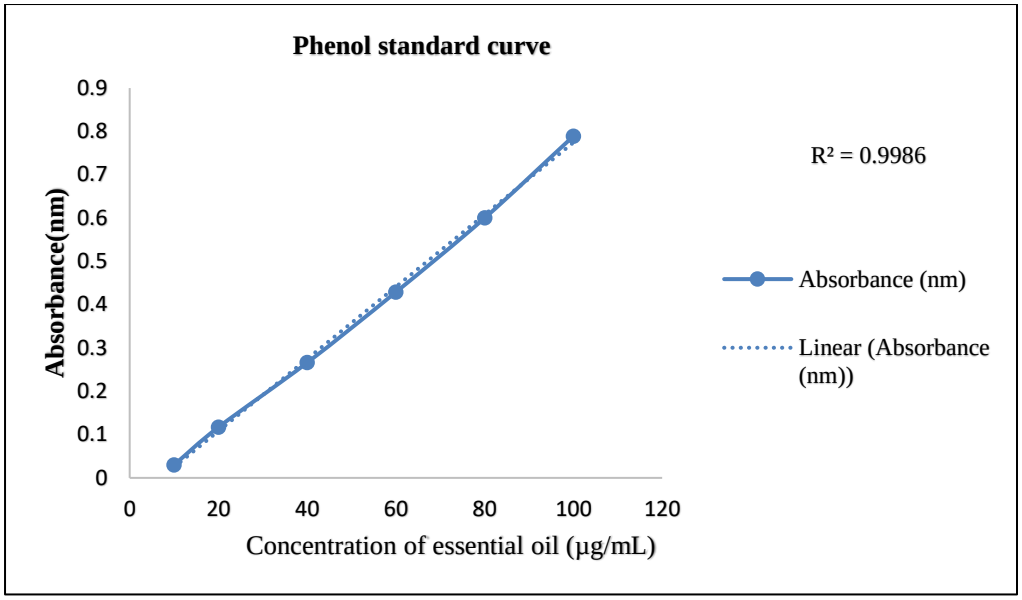


Figure 30: Calibration curve of phenol standard for total phenolic content determination of essential oil

Table 14: Total phenolic content in the essential oil of *A. marmelos*

Sample concentration (mg/mL)	**Weight of extract per mL m(g)**	**Absorbance**	**GAC, C (µg/mL)**	**GAC, C (mg/mL)**	**TPC as GCE** $= \frac{C \times V}{m}$ **(mg/g)**
1	0.001	0.03	10.65	0.01065	10.65

Table 15 shows the total phenolic contents of essential oil of *A. marmelos* tested as Gallic acid equivalent by reference to a standard curve (y = 0.0083x – 0.0584, R^2 = 0.9986). The total phenolic content in the leaf essential oil of *A. marmelos* was 10.65 mg Gallic acid equivalent / g of dry extract.

4.8. Total flavonoid content analysis

Flavonoids are capable of inhibiting enzymes such as prostaglandin synthase closely linked to tumorigenesis, and inducing detoxifying enzyme systems such as glutathione S-transferase. Quercetin prevents low-density lipoprotein *in-vitro* degradation and cytotoxicity and helps to reduce the risk of coronary heart

disease or cancer[69]. Total flavonoid content is expressed as mg of quercetin equivalents per gram of dried sample:

Table 15: Absorbance of quercetin

Concentration (µg/mL)	Absorbance (nm)			Average absorbance (nm)
5	0.0411	0.0430	0.0431	0.0431
10	0.158	0.151	0.147	0.152
20	0.452	0.458	0.454	0.455
40	0.993	0.996	0.985	0.991
80	1.972	1.935	1.889	1.93

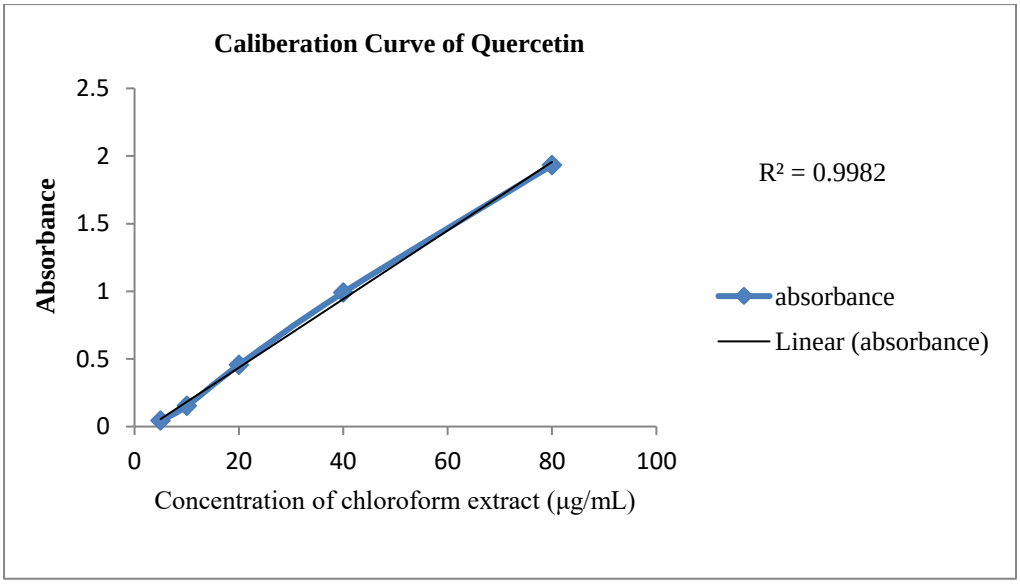


Figure 31: Calibration curve of quercetin for total flavonoid content determination

The concentration of quercetin in chloroform extract of *A. marmelos* was determined by using an equation that was obtained from the standard quercetin curve in figure 31. The equation is given below:

$y=0.0253x-0.071$

$R^2 = 0.9982$

Where, y = absorbance

x = Quercetin concentration (µg/m)

m = slope = 0.0253

c = y-intercept = -0.071

$\therefore X = \frac{y+0.071}{0.0253}$

Table 16: Total flavonoid content in leaves of *A. marmelos*

Sample concentration (mg/mL)	**Weight of extract per mL m(g)**	**Absorbance**	**QC, C (µg/mL)**	**QC, C (mg/mL)**	**TFC as QE** $= \frac{C \times V}{m}$ **(mg/g)**
0.1	0.0001	0.289	14.22	0.014229	142.29

The total flavonoid content of *A. marmelos* is demonstrated in table 17. By relation to a standard curve, *A. marmelos* leaf is measured as a quercetin counterpart (y = 0.0253x-0.071, R^2 = 0.9982). The total flavonoid content in the leaf of *A. marmelos* was 142.29 mg quercetin equivalent / g of dry extract.

The total flavonoid content of essential oil of *A. marmelos* is expressed as mg of quercetin equivalents per gram of oil sample.

Table 17: Absorbance of quercetin

Concentration(µg/mL)	**absorbance (nm)**
5	0.060
10	0.122
20	0.425
40	0.96
80	1.901

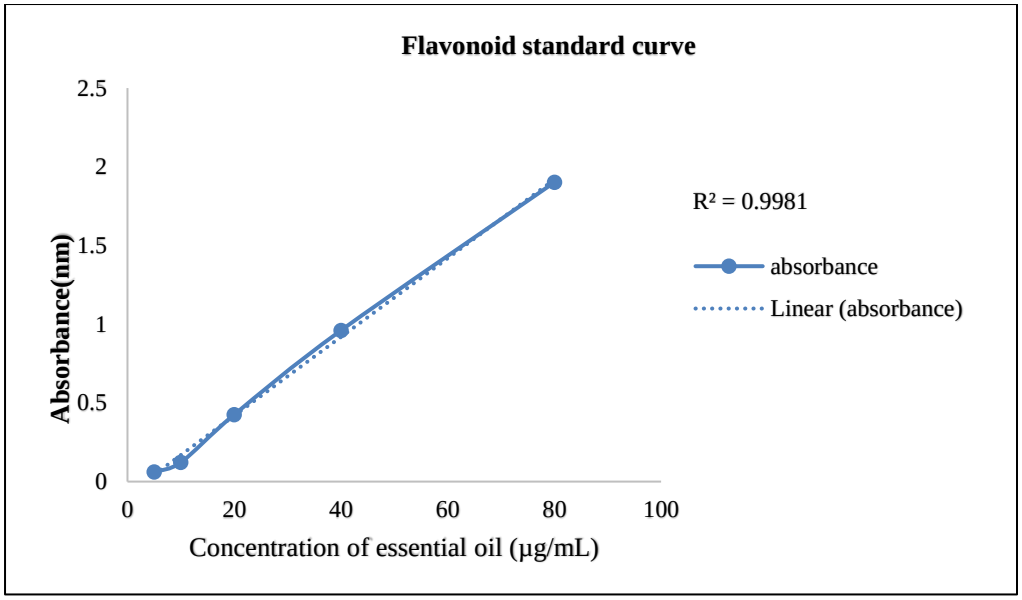


Figure 32: Calibration curve of quercetin for total flavonoid content determination in the essential oil of *A. marmelos*.

Table 18: Total flavonoid content in the essential oil of *A. marmelos*

Sample concentration (mg/mL)	**Weight of extract per mL m(g)**	**Absorbance**	**QC, C (µg/mL)**	**QC, C (mg/mL)**	**TFC as QE** $= \frac{C \times V}{m}$ **(mg/g)**
0.1	0.0001	0.00033	3.2652	0.0032652	3.2652

The total flavonoid content of *A. marmelos* is demonstrated in table 19. By relation to a standard curve, *essential oil* is measured as a quercetin counterpart ($y = 0.0253x-0.0813$, $R^2 = 0.9981$). The total flavonoid content in the essential oil of *A. marmelos* was 3.27 mg quercetin equivalent / g of dry extract.

4.9. Brine shrimp lethality analysis

In the toxicity assessment of plant extracts by the brine shrimp lethality bioassay, LC_{50} values lower than 1000 µg/mL are considered bioactive [70]. The important association between *in vitro* growth inhibition of human solid tumor cell lines and the brine shrimp assay has been demonstrated by the

National Cancer Institute (NCI, USA) and emphasized the importance of this bioassay as a pre-screening method for antitumor drug research [64].

Table 19: Effect of chloroform extract of *A. marmelos* in brine shrimp

Concentration(µg/mL)	Percentage Mortality
500	70
250	70
125	50
62.5	20
31.25	10
15.625	0

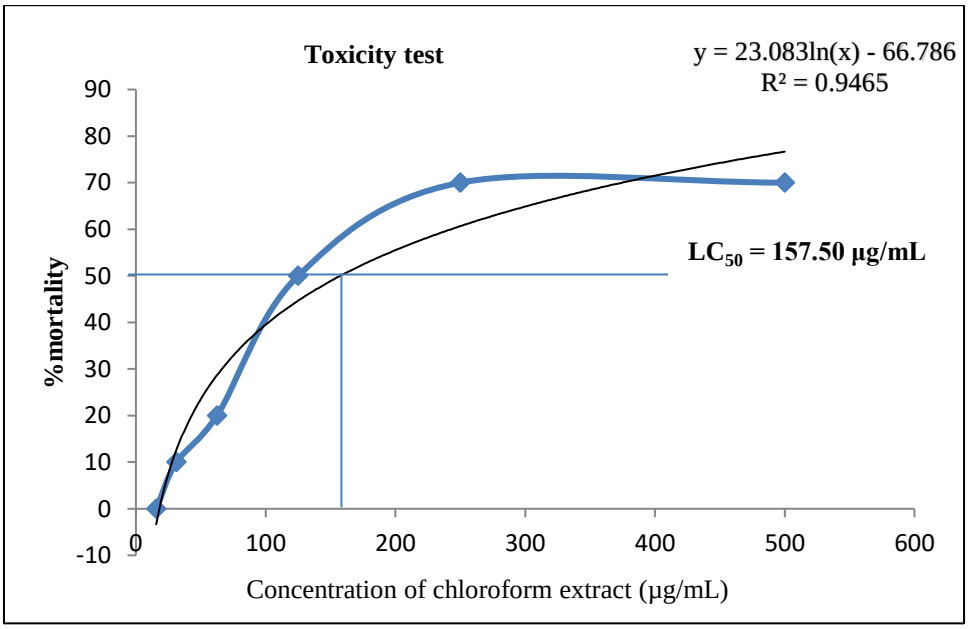


Figure 33: Plot of concentration of chloroform extract of *A. marmelos* versus percent shrimp mortality after 24 hours of exposure

Table 20 shows the lethality of chloroform extract of *A. marmelos* to the brine shrimp nauplii. The degree of lethality exhibited by the extractives was found to be directly proportional to the concentration of the extractives varying from the minimum concentration (15.62 µg/mL) to the maximum concentration (500 µg/mL). This concentration-dependent increment in percent mortality of Brine shrimp nauplii produced by the *A. marmelos* indicates the presence of cytotoxic principles in these extractives. This study shows that the LC_{50} value of chloroform extract of *A. marmelos* was 157.50 µg/mL.

Brine shrimp assay is based on the capability to kill larvae (nauplii) cultured in the laboratory The nauplii of brine shrimp (*Artemia salina*) were exposed to different concentrations of essential oil of *A. marmelos* for 24 hours. The number of motile nauplii was calculated that represented the effectiveness of the oil. The result of the brine shrimp lethality assay of essential oil is given in the table below;

Table 20: Effect of essential oil of *A. marmelos* in brine shrimp

Concentration(µg/mL)	Percentage Mortality
500	100
250	80
125	70
62.5	60
31.25	40
15.625	10

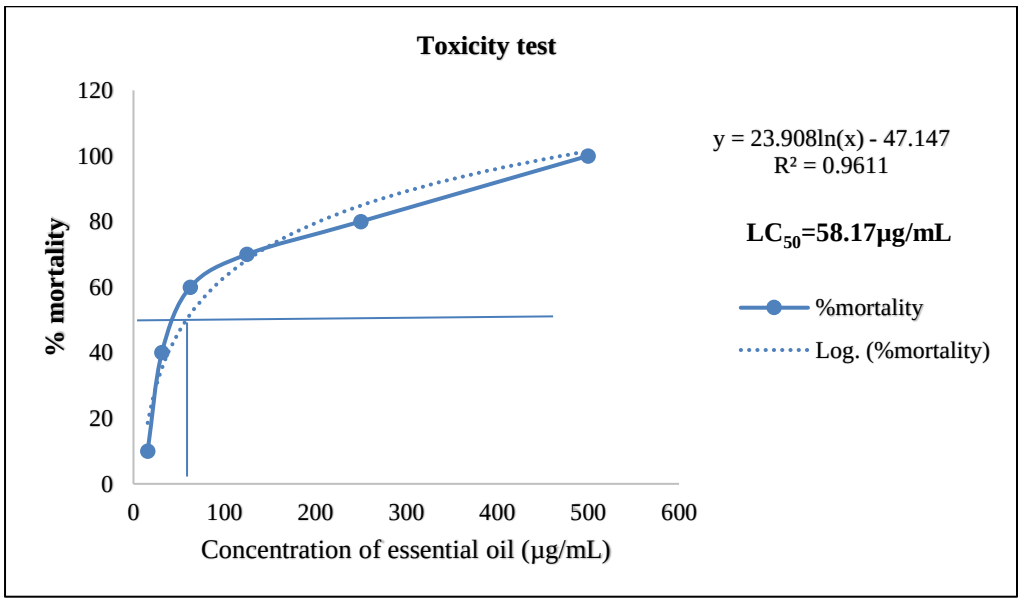


Figure 34: Plot of concentration of essential oil of *A. marmelos* versus percent shrimp mortality after 24 hours of exposure

The presence of a toxic effect in the essential oil is shown by the percentage of brine shrimp mortality caused by *A. marmelos* with increasing concentration. This study shows that the LC_{50} value of essential oil of *A. marmelos* was 58.17 µg/mL.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

From the result of this study, it can be concluded that various phytochemicals, including alkaloids, phenols, flavonoids, saponins, proteins were present in the leaves of the *A. marmelos*. GC-MS analysis of chloroform extract showed the presence of 9 major compounds among which Limonene dioxide (27.78 %) and Germacrene B (20.65 %) were the most abundant. Antibacterial activity was not shown by the chloroform leaf extract. However, the Antioxidant activity of chloroform extracts of *A. marmelos* was investigated using DPPH radical scavenging method and showed average inhibition with an IC_{50} value of 308.21 µg/mL. The plant also had average total phenolic and flavonoid content with the value of 58.26 mg Gallic acid equivalent/g of dry extract and 142.29 mg quercetin equivalent/ g of dry extract respectively. The leaf extracts of *A. marmelos* exhibited toxic activity against the brine shrimp with an LC_{50} value of 157.50 µg/mL were considered.

GC-MS analysis of essential oil showed the presence of 12 different compounds. The most abundant were limonene (25.06 %) and germacrene (18.64 %). Among the tested organisms, *Bacillus subtilis* having ZOI 7 mm was susceptible to the essential oil. The IC_{50} value of the oil against DPPH was calculated as 13.63 mg/mL from the data. The oil also had total phenolic and flavonoid content with the value of 10.65 mg Gallic acid equivalent/g of essential oil and 3.27 mg quercetin equivalent/g of essential oil extract respectively. The LC_{50} value against brine shrimp was calculated as 58.17 µg/mL.

Further investigations are required to isolate and identify the active compounds in the extracts. The study also recommends the need for extensive study on effective, safe, cheap, and non-toxic drug formulations, which would not only increase value to our resources but also generates a rational approach to exploit our resources.

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APPENDICES

Reagents used for Phytochemical Screening

Mayer's Reagent: 1.358 g of $HgCl_2$ was dissolved in 60 mL of water and mixed with a solution of 5 g of Potassium iodide (KI) in 10 mL of water.

Benedict's reagent:

Solution I: 50 g of crystalline sodium carbonate, 50 g of crystalline sodium citrate, and 31.25 g of potassium thiocyanate were dissolved in 200 mL hot distilled water.

Solution II: 4.5 g of $CuSO_4$ was dissolved in 25 mL water.

Solution III: 5 % solution of potassium Ferro cyanate was prepared by dissolving 5 g potassium Ferro cyanate in 100mL water.

Finally, Benedict's reagent was prepared by mixing solution I, solution II and

Fehling's Reagent:

Fehling A: 31.66 g of $CuSO_4$ was dissolved in water to produce a 500 mL solution.

Fehling B: 176 g of sodium-potassium tartrate and 77 g of sodium hydroxide was dissolved in water to produce a 500 mL solution.

Finally, an equal volume of solutions I and II was mixed to prepare Fehling's solution.

Gelatin Solution (1%): 1 g of gelatin was dissolved in 100 mL of hot water.

Concentrated Sulfuric Acid Solution: 36 N concentrated sulfuric acid.
The solution was used.

Dilute Sulfuric Acid Solution: Concentrated sulfuric acid was diluted 10 times with water to produce a dilute sulfuric acid solution.

Concentrated Hydrochloric Acid: 36 N concentrated hydrochloric acid solution was used.

1% Dilute Hydrochloric Acid: 1 mL of conc. HCl acid was dissolved in 100mL of water.

Ferric Chloride Solution: 15 g of ferric chloride hexahydrate ($FeCl_3.6H_2O$) was dissolved in 100 mL of distilled water.

Ammonia Solution: 25% of ammonia solution was used.

Copper Acetate Solution: 19.97 g of copper acetate was dissolved in 100 mL of distilled water.

Sodium Hydroxide Solution: 20 g of NaOH was dissolved in 100 mL of distilled water.

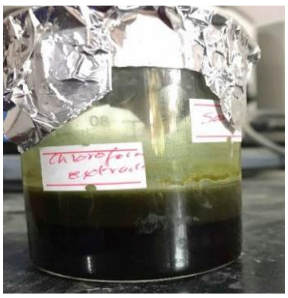
Lead Acetate Solution: 10g of lead acetate was dissolved in 100 mL of CO_2-free water.

1 M Na_2CO_3 Solution: 10.6 g of Na_2CO_3 was dissolved in little distilled water in a 100 mL volumetric flask and diluted to the mark .by adding distilled water.

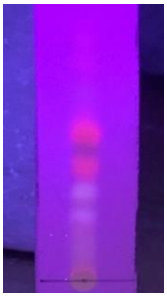
Preparation of 2% $AlCl_3$ Solution: 2 g of $AlCl_3$ crystals were dissolved in little distilled water in a 100 mL volumetric flask and diluted to the mark by adding distilled water.


Aegle marmelos plant with fruit and fresh leaves and dried leaves


Powdered leaves and extraction of leaves by cold percolation in methanol

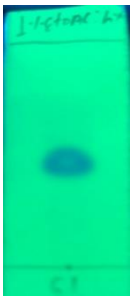



The methanol extract and chloroform extract

Phytochemical Test, TLC spots in UV florescence

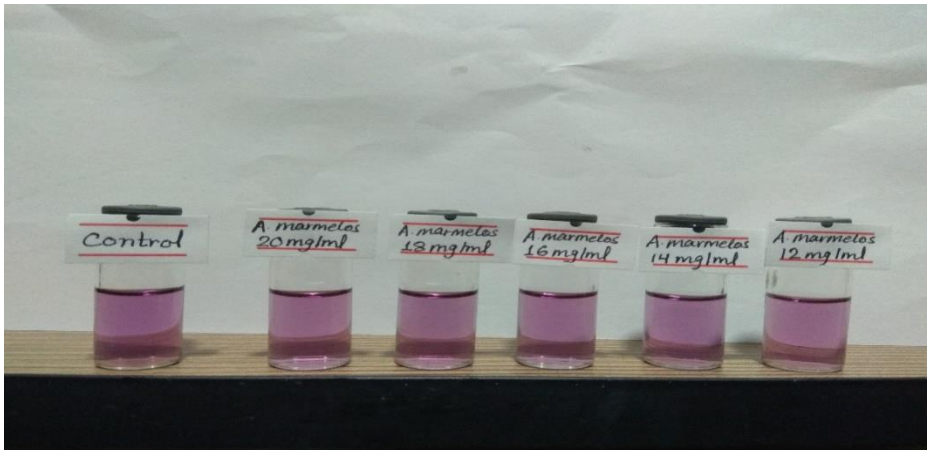
Hydrodistillation process by Clevenger apparatus and essential oil



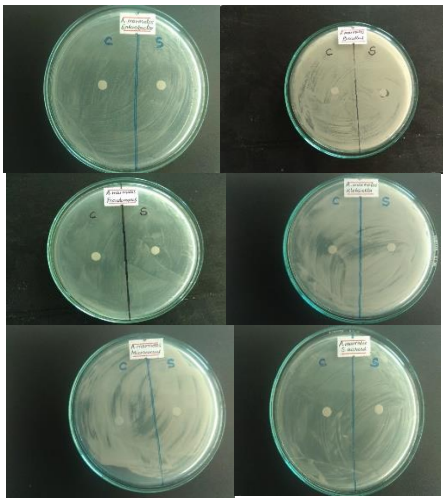
Column-chromatographic separation, a collected fraction of plant extract, and TLC spot in UV florescence

Working in Ascol Research lab.

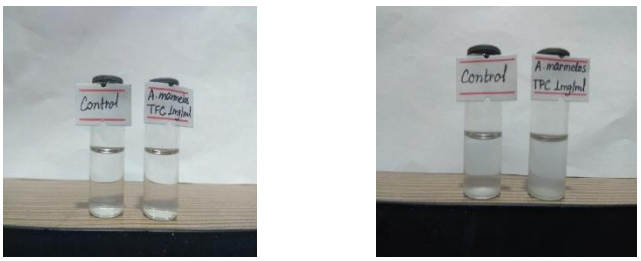
Different concentration solutions of plant extract for DPPH assay



Different concentration solutions of essential oil extract for DPPH assay



Antibacterial analysis of essential oil extract



TFC and TPC of essential oil