

Comparative study of pharmacological activities in methanol and aqueous extract of aegle marmelos

Submitted in the partial fulfilment of the requirements for the award of Bachelor
of Technology degree in Biotechnology

By

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SATHYABAMA



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We, S.SANGAVI (37230075) hereby declare that the Project Report entitled “Comparative study of pharmacological activities in methanol and aqueous extract of aegle marmelos” was done by me under the guidance of Dr. Sudha is submitted in the partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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We are glad to permit your student Ms. SANGAVI.S, B.Tech, DEPARTMENT OF BIOTECHNOLOGY, SATHYABAMA INSTITUTE OF SCIENCE & TECHONOLOGY to carry out her Project for Three months (December to March) in our research laboratory. We assure to make necessary lab facilities for your student and assist in the successful completion of her research work.

Thanking you.



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ABSTRACT

Aegle marmelos is commonly called bael. They belong to Rutacear family. Aegle marmelos is small or medium sized tree. The bark of the tree is pale brown or greyish in colour. The bark has long straight spines which when cur slimy sap oozes out. The leaf of aegle is trifoliate with pointed tip and round base the young leave are pale green in colour and mature leave are dark green. The flowers are yellowish or pale green with sweet scent the flower of aegal marmelos are bisexual. The fruit is slightly pear shaped with a thick outed and does not split upon ripening. Aegle marmelos is a native species to Indian subcontinent and south-east Asia. It is present in India , sir Lanka, Thailand and Malesia. Aegla marmelos is extensively used in traditional Indian system of medicine. Aegal marmelos has many medical benefits like anti diarrhoea, anti microbial, antiviral, radioprotective, chemopreventive etc... this study is to perform anticancer activity, to investigate antioxidant activity and GC-MS on aegla marmelos in aqueous and methanoic extract.

Keywords: antioxidant, anticancer, GC-MS, methanoic, aqueous

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LIST OF SYMBOLS AND ABBREVIATIONS

DPPH - 2, 2-diphenyl-1-picrylhydrazyl

Fe³⁺ - Ferric ion

H₂SO₄ - Sulphuric acid or Hydrogen sulphate

K₂ Fe(CN)₆ - Potassium ferrocyanide

mM - millimolar

µg/mL - microgram/milliliter

W/V - weight/volume

UV-Vis - Ultra violet – Visible spectrophotometer

pH - Potential of Hydrogen

µL – microliter

µM - micro molar

°C - Degree Celsius

nm - nanometer

FeSO₄ - Ferrous sulphate

H₂ O₂ - Hydrogen peroxide

NBT - Nitro blue tetrazolium dye

EDTA - Ethylene diamine tetra acetic acid

DMBA - 7,12-dimethylbenz(a)anthracene

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Chapter 1

Introduction

Aegle marmelos belonging to family Rutaceae, is commonly known as Bael in indigenous systems of medicine and has been regarded to possess various medicinal properties. The bael is one of the sacred trees of the Hindus. Leaves are offered in prayers to Shiva and Parvathi since ancient times¹. Bael is a deciduous sacred tree, associated with Gods having useful medicinal properties, especially as a cooling agent. This tree is popular in Shiva and Vishnu temples and it can be grown in every house. Its leaves are trifoliate symbolizing the Thrimurthies-Brahma, Vishnu, Shiva, with spear shaped leaflets resembling Thrisoolam the weapon of Lord Shiva. Many legends, stories and myths are associated with this tree. The leaflets are given to devotees as prasadam in Shiva temples and as Tulasi in Vishnu temples. In India flowering occurs in April and May soon after the new leaves appear and the fruit ripens in 10 to 11 months from bloom March to June of the following year

Scientific Classification

- Kingdom- Plantae.
- Order- Sapindales.
- Family- Rutaceae.
- Subfamily- Aurantioideae.
- Genus- Aegle.
- Species- Aegle Marmelos.
- Botanical name- Aegle marmelos.



Figure 1.1 Aegle marmelos

1.1 Medicinal uses

Leaves: Leaves are used as mild laxative, or the inflammation of the mucous membrane having a free discharge and for asthma . The decoction of the leaves is febrifuge, or helps in eliminating fever and is an expectorant, or promotes the removal of mucous secretion from the bronchial tubes. The leaf juice is given in dropsy or the abnormal accumulation of liquid in the cellular tissue accompanied with constipation and jaundice. A hot poultice of the leaves is applied in ophthalmia or severe inflammation of conjunctiva with acute bronchitis and inflammation of the other body parts.

Root: The decoction of the root and sometimes the stem bark is useful in intermittent fever, also in hypochondriasis and palpitation of the heart. The decoction of root is given with sugar and fried rice for checking diarrhoea and gastric irritability in children. Root is a one of the ingredients of Dasamoola a standard Ayurvedic remedy for loss of appetite and puerperal diseases e.g. Inflammation of uterus

Flower: Distillation of flowers yielded a drug used as tonic for stomach and intestine, anti-dysenteric, antidiabetic, diaphoretic and as local anaesthetic.

Fruit: Fruit is eaten during convalescence after diarrhea. It is valid for its mild astringency and as remedy for dysentery. The traditional healers of southern Chhattisgarh use dry powder of fruit with mustard oil for the treatment of burn cases. One part of powder and two part of mustard oil are mixed and are applied externally. Fruits are also used in gastric troubles, constipation, laxative, tonic, digestive, stomachic, brain and heart tonic, ulcer, antiviral, intestinal parasites, gonorrhoea, epilepsy.

Ripe fruit: The ripe fruit promotes digestion and is helpful in treating inflammation of rectum. The ripe fruit extract showed antiviral activity against ranikhet disease virus. Pulp of ripe fruit is sweet, cooling, aromatic and nutritive when taken fresh. Fruit pulp marmalade is used as prevention during cholera epidemics, also given to prevent the growth of piles, useful in patients suffering from chronic dysenteric condition characterised by alternate diarrhea and constipation relieves flatulent colic from a condition of chronic gastrointestinal eatarrh. Fresh juice is bitter and pungent fruit extract lower the blood sugar

Unripe fruit: Fine powder of unripe fruit showed significant effect on intestinal parasites and also effective against *Entamoeba histolytica* and *Ascaris lumbricoides*. Unripe fruit is used as an astringent in dysentery, stomachache in diarrhea, tonic, digestive, demulcent, described as cardiacal, restorative, given in piles, Decoction of unripe fruit is astringent, useful in diarrhea.

1.2 Antibacterial activity

Antibacterial activity of a molecule is completely associated with the compounds that provincially kill bacteria or slow down their rate of growth, without being extensively toxic to nearby tissues. Most recently discovered antimicrobial agents are modified natural compounds and this modification is done through chemical mode, for example, b-lactams (penicillins), carbapenems, or cephalosporin. Pure natural products, such as aminoglycosides, and other entirely synthetic antibiotics, for example, sulfonamides, are also frequently used. The antimicrobial agents could be classified as the agents that can either be bactericidal, which kill bacteria, or bacteriostatic, which slow down the growth of bacteria. Antibacterial agents are the most important in fighting infectious diseases. But, with their wide use as well as abuse, the appearance of bacterial resistance toward antibacterial agents has become a major problem for today's pharmaceutical industry. Resistance is most commonly based on developmental processes taking place, for example, antibiotic therapy, that leads to inheritable resistance.

This increasing resistance of the microorganisms toward antibacterial agents has been responsible in recent years for serious health issues. Most infectious bacteria are resistant to a minimum of one of the antibiotics that are generally used to eliminate the infection. This problem motivates the study of new agents that can efficiently inhibit the growth of microorganisms.(Kushagri Singh, Kavita Singh 2019)

The antimicrobial properties of plants have been investigated by a number of researchers world wide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatments. It was documented that among the compounds extracted from these plants, twelve inhibited the growth of *Staphylococcus aureus*, ten

inhibited *Escherichia coli*, and four inhibited *Aspergillus niger* and also reported that the most potent compound was one extracted from *Tabebuia impetiginosa*. The antimicrobial properties of compounds obtained from *Parthenum argentatum* against *Candida albicans*, *Torulopsis*, *Hansenula*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was detected. Work done was observed that the substances extracted from nine known plants in Uruguai did not show any activity against *C. albicans* and *Saccharomyces cerevisiae*, but inhibited the growth of *Bacillus subtilis*, *E. coli* and *P. aeruginosa*. (Braz. J. Microbiol. vol.31 no.4 São Paulo Oct./Dec. 2000)

1.3 Phytochemical

Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoid, alkaloids and phenolic compounds. Terpenoids exhibit various important pharmacological activities i.e., anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities. Terpenoids are very important in attracting useful mites and consume the herbivorous insects. Alkaloids are used as anaesthetic agents and are found in medicinal plants. (Wadood et al., Biochem Anal Biochem 2013).

The secretion of these compounds is varying from plant to plant some produce more and some produce in minimal quantity. Sometimes they can be harmful and sometimes they can be very helpful. There is evidence from laboratory studies that phytochemicals in fruits and vegetables may reduce the risk of cancer, possibly due to dietary fibers, polyphenol antioxidants and anti-inflammatory effects. (Tariq. A. L 2013)

1.4 Antioxidant

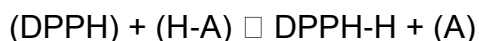
1.4.1 DPPH assay (2, 2-diphenyl-1-picrylhydrazyl)

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al. (2001). The decrease in the absorption of the DPPH solution

after the addition of an antioxidant was measured at 517nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

Principle

1, 1 Diphenyl 2- Picryl Hydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as,



Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

1.4.2 Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto (1999).

Principle

This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour.

1.4.3 Super oxide anion radical scavenging activity

Superoxide dismutase (SOD) is a metalloenzyme that catalyze the dismutation of superoxide radical into hydrogen peroxide (H₂O₂) and molecular oxygen (O₂) and consequently provide an important defense mechanism against superoxide radical toxicity (Nishikimi et al., 1972).

Principle

The principle involved in this assay is the conversion of Nitroblue Tetrazolium (NBT) into NBT diformazan via superoxide radical. SOD utilizes the highly water-soluble tetrazolium

salt and that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD.

1.4.4 Metal chelating activity

Principle

Ferrozine can quantitatively chelate with Fe^{2+} and form a red coloured complex. This reaction is limited in the presence of other chelating agents and results in a decrease of the red colour of the ferrozine- Fe^{2+} complex. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000).

The antioxidants present in plant extract forms a coordinate complex with the metal ions(chelating activity) and inhibit the transfer of electrons. Thus oxidation reaction is arrested and no free radicals are produced.

1.5 Gcms

GC-MS can be used to study liquid, gaseous or solid samples. Analysis begins with the gas chromatograph, where the sample is effectively vaporized into the gas phase and separated into its various components using a capillary column coated with a stationary (liquid or solid) phase. The compounds are propelled by an inert carrier gas such as helium, hydrogen or nitrogen. As components of the mixture are separated, each compound elutes from the column at a different time based on its boiling point and polarity. The time of elution is referred to as a compound's retention time. GC has the capacity to resolve complex mixtures or sample extracts containing hundreds of compounds.

Once the components leave the GC column, they are ionized and fragmented by the mass spectrometer using electron or chemical ionization sources. Ionized molecules and fragments are then accelerated through the instrument's mass analyzer, which quite often is a quadrupole or ion trap. It is here that ions are separated based on their different mass-to-charge (m/z) ratios. GC-MS data acquisition can be performed in either full scan mode, to cover either a wide range of m/z ratios, or selected ion monitoring (SIM) mode, to gather data for specific masses of interest.

The final steps of the process involve ion detection and analysis, with fragmented ions appearing as a function of their m/z ratios. Peak areas, meanwhile, are proportional to the quantity of the corresponding compound. When a complex sample is separated by GC-MS, it will produce many different peaks in the gas chromatogram and each peak generates a unique mass spectrum used for compound identification. Using extensive commercially available libraries of mass spectra, unknown compounds and target analytes can be identified and quantified.

1.6 Breast cancer

Breast cancer is a type of cancer that starts in the breast. Cancer starts when cells begin to grow out of control. Breast cancer cells usually form a tumor that can often be seen on an x-ray or felt as a lump. Breast cancer occurs almost entirely in women, but men can get breast cancer, too.

It's important to understand that most breast lumps are benign and not cancer (malignant). Non-cancerous breast tumors are abnormal growths, but they do not spread outside of the breast. They are not life threatening, but some types of benign breast lumps can increase a woman's risk of getting breast cancer. Any breast lump or change needs to be checked by a health care professional to determine if it is benign or malignant (cancer) and if it might affect your future cancer risk.

Breast cancers can start from different parts of the breast.

- Most breast cancers begin in the ducts that carry milk to the nipple (ductal cancers)
- Some start in the glands that make breast milk (lobular cancers)
- There are also other types of breast cancer that are less common like phyllodes tumor and angiosarcoma
- A small number of cancers start in other tissues in the breast. These cancers are called sarcomas and lymphomas are not really thought of as breast cancers.

Although many types of breast cancer can cause a lump in the breast, not all do. Any breast cancers are also found on screening mammograms, which can detect cancers at an earlier stage, often before they can be felt, and before symptoms develop.

There are many different types of breast cancer and common ones include ductal carcinoma in situ (DCIS) and invasive carcinoma. Others, like phyllodes tumors and angiosarcoma are less common.

Once a biopsy is done, breast cancer cells are tested for proteins called estrogen receptors, progesterone receptors and HER2. The tumor cells are also closely looked at in the lab to find out what grade it is. The specific proteins found and the tumor grade can help decide treatment options.

Chapter 2

Review of literature

Azad Rajaram et.al.,(2017) The present study is aimed to compare the anti-inflammatory properties of different extracts of young roots (year wise) and mature parts of Bilva plants collected from different geographical locations in India, so as to identify a sustainable source for Ayurvedic formulation. A total of 191 extracts (petroleum ether, ethyl acetate, ethanol and aqueous) of roots, stems and leaves of *A. marmelos* (collected from Gujarat, Maharashtra, Odisha, Chhattisgarh, Karnataka and Andhra Pradesh region) were tested for anti-inflammatory effects in vitro on isolated target enzymes cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), lymphocyte proliferation assay (LPA), cytokine profiling in LPS induced mouse macrophage (RAW 264.7) cell line and in vivo carrageenan induced paw edema in mice. The present study reveals that young roots of Bilva plants from Gujarat and Odisha region could form a sustainable source for use in Ayurvedic formulations with anti-inflammatory activities. The present study also indicates that the region in which the plants are grown and the age of the plants play an important role in exhibiting the anti-inflammatory effect.

Farina Mujeeb et.al.,(2014) The present study is focused on phytochemical screening of aqueous and methanolic leaf extracts of 18 varieties/accessions of *A.marmelos*. The crude extracts of *A.marmelos* revealed the presence of several biologically active phytochemicals with the highest quantity of alkaloids, flavonoids, and phenols in Pant Aparna variety. The antibacterial efficacy was investigated against pathogenic bacterial strains and the highest inhibitory activity of aqueous extract was obtained against *S.epidermidis*, whereas methanolic extract was found to be most potent against *S.Aureus* at 40 mg/mL concentration. However, in aqueous : ethanol, the best results were observed against *E.Aerogenes* followed by *K.pneumonia* and *S.epidermidis*. The MIC of aqueous and methanol extract of *Aegle marmelos* ranged from 10 mg/mL to 40 mg/mL whereas in aqueous : ethanol it ranged between 40 mg/mL and 160 mg/mL. The GC-MS analysis revealed the presence of many bioactive compounds such as flavonoids, alcohols, aldehydes, aromatic compounds, fatty acid methyl esters, terpenoids, phenolics, and steroids that can be postulated for antibacterial activity.

Shahedur Rahman and Rashida Parvia (2014) Extensive experimental and clinical studies prove that *Aegle marmelos* possesses antidiarrhoeal, antimicrobial, antiviral, radioprotective, anticancer, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties, which help it to play role in prevention and treatment of many disease. Therefore, it is worthwhile to review its therapeutic properties to give an overview of its status to scientist both modern and ancient. This review also encompasses on the potential application of the above plant in the pharmaceutical field due to its wide pharmacological activities.

N.P.Atul et.al.,(2012) This extensive investigations have been carried out on different parts of *Aegle marmelos* and as a consequence, varied classes of compound viz., alkaloids, coumarins, terpenoids, fatty acids and amino acids have been isolated from its different parts. Various phytochemical, ethnobotanical and pharmacological evaluations have been reported in this literature for the important potential of the *Aegle marmelos*.

S.Brijesh et.al.,(2009) In study the decoction of the unripe fruit pulp of *A. marmelos*, despite having limited antimicrobial activity, affected the bacterial colonization to gut epithelium and production and action of certain enterotoxins. These observations suggest the varied possible modes of action of *A. Marmelos* in infectious forms of diarrhoea thereby validating its mention in the ancient Indian texts and continued use by local communities for the treatment of diarrhoeal diseases.

Gupta et.al.,(2011) All the parts of this tree including stem, bark, root, leaves, fruit and seeds at all stages of maturity has medicinal virtues and has been used in Ethno-medicine to exploits its medicinal properties including antidiarroheal, antidysentric, antipyretic and anti inflammatory activities. Compounds purified from bael fruit have been proven to have biological potential against several diseases like diabetes, gastric ulcer and hyperlipidaemia. It should also be indicated that the therapeutic activities of few isolated constituents have also proven to possess antibacterial, antiviral, antioxidant and radioprotective activities. Thus the purpose of this abstract is to explore the pharmacological potential of some crude extracts of bael fruit.

Dinesh Kumar Sekar et.al.,(2011) *A. marmelos* is extensively studied for its medicinal properties by advanced scientific techniques and a variety of bioactive compounds have been isolated from the different part of plant and were analysed pharmacologically. The medicinal properties of this plant represent it as a valuable source of medicinal compound. This report is a summarized information concerning the morphology, distribution, phytochemistry, traditional uses and biological activities of the *A. marmelos*.

Shail Bala Sanghi et.al.,(2017) In this various parts of this plant such as leaves, roots, seed, bark and fruit, possess anti-microfilarial, antifungal, immunomodulatory, anti-proliferative, wound healing, antifungal, analgesic, anti-inflammatory, antipyretic, hypoglycaemic, antidyslipidemic, antifertility, and insecticidal activity. Various phytopharmacological evaluations have been reported in this writing for the important potential of the *Aegle marmelos*.

Vivek Akhouri et.al.,(2017) the present research work has been aimed to study the anticancer activity of *Aegle marmelos* fruit extract on 7,12-dimethylbenz(a)anthracene (DMBA) induced breast cancer in rats. Female Charles Foster rats, 55–60 days old weighing around (150 ± 10g) were used for the study and were induced DMBA (20 mg/mL dissolved in Olive oil) orally. After the development of breast tumors (about 0.5cm), the rats were treated with *Aegle marmelos* ethanolic fruit pulp extract (200mg/kg b.w./day) orally for 5 weeks and then volume of tumor was measured. *Aegle marmelos* treatment showed significantly reduced mammary tumor volume ($P < 0.05$), along with significant reduction ($P < 0.0001$) in the different serum biomarkers such as TNF- α level, serum malondialdehyde (MDA) level and glucose levels. Significant ($P < 0.0001$) improvement in both, the kidney and liver serum biomarker parameters were also observed after the treatment with *Aegle marmelos* ethanolic fruit pulp extract. From the entire study, taking everything into account it can be interpreted that *Aegle marmelos* ethanolic fruit pulp extract possesses anti-proliferative activity by suppressing the progression of breast tumors in rat model. The plant extract also possesses hepato-renal protective effect. Hence, it can be targeted as novel and safe anti-cancer drug against breast cancer.

Chapter 3

Aim and scope

Aim:

the aim of this work is to analysis and compare two different type of extract of the same sample aegle marmoles and obtain the best results for breast cancer line and GC_MS.

Scope:

- to evaluate Anti-oxidant
- Anti bacterial
- phytochemical analysis
- GC-MS activities
- 'Anti-cancer activities' with cell lines of 'MDA MB 231- Human breast cancer cell lines

Chapter 4

Material and methods

4.1 *In vitro* antioxidant assays

4.1.1 DPPH[•] radical scavenging activity

The antioxidant activity of aqueous and methanoic extract of aegle marmelos was measured by DPPH (1, 1-diphenyl 2-picrylhydrazyl) free radical scavenging activity⁸. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (20-120 µg/mL) of aqueous Rose petals extract. The mixture was then allowed to stand for 30 min incubation in dark. One mL of DPPH solution mixed with 1 mL of methanol was used as the control. The decrease in absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of DPPH}^{\bullet} \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}} \right] \times 100$$

4.1.2 Superoxide radical scavenging activity

Superoxide radical scavenging activity was carried out by the method of Ravishankara et al⁹. The reaction mixture contains different concentrations (20-120 µg/mL) of aqueous and methanoic extract of aegle marmelos, 1.5 mM of riboflavin (200 µL), 12 mM of EDTA (100 µL), and 50 mM of NBT (50 µL). All the reagents should be prepared in 50 mM of phosphate buffer (pH 7.8). The reaction was started by illuminating the reaction mixture for 90 seconds. Immediately, the absorbance was measured at 590 nm and ascorbic acid was used as the positive control.

$$\% \text{ of superoxide radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}} \right] \times 100$$

4.1.3 Phosphomolybdenum reduction assay

The antioxidant capacity of aqueous and methanoic extract of aegle marmelos was assessed as described by Prieto *et al*¹⁰. Extract with different concentrations (20 - 120 µg/mL) was combined with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 95°C for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of Phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}} \right] \times 100$$

4.1.4 Ferric (Fe³⁺) reducing power assay

The reducing power of aqueous and methanoic extract of aegle marmelos was determined by the slightly modified method of Yen and Chen¹¹. One mL of extract with different concentrations (20 - 120 µg/mL) was mixed with 1 mL of 1% (w/v) potassium ferricyanide [K₃Fe (CN)₆] solution and 1 mL of phosphate buffer (0.2 M, pH 6.6). The mixtures were then incubated at 50°C in a water bath for 20 min. Five hundred µL of 10% (w/v) trichloroacetic acid solution was added to each mixture followed by 1 mL of 0.1% (w/v) freshly prepared FeCl₃ solution was added and shaken well. The absorbance was measured at 700 nm and ascorbic acid was used as the standard reference. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of Fe}^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}} \right] \times 100$$

4.2 Antibacterial activity

The microorganisms of Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus subtilis* were used for the evaluation of the antibacterial activity.

4.2.1 Agar well diffusion method

Nutrient broth agar medium was prepared according to the standard methods (peptone-5 g, yeast extract-3 g, NaCl-5 g, distilled water- 1000 mL, agar-20 g). The required amount of ingredients was weighed and suspended in the required volume of 100 mL of distilled water and then autoclaved at 15 lbs and 121°C for 15 min. The hot medium was poured in sterile petri plates which were kept in the aseptic Laminar chamber. The medium was allowed to solidify for 15 min.

Antibacterial activity of aqueous and methanoic extract of aegle marmelos was carried out using agar well diffusion method ¹². The solidified nutrient agar in the petri plates was inoculated by dispensing the inoculum using sterilized cotton swabs which are previously immersed in the inoculum containing test tube and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The root tuber extract was then poured into each well containing 250, 375, and 500 µg/mL concentrations. All the plates with extract loaded wells were incubated at 37°C for 24 h and the antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the well. Tetracycline (25 µg) was used as the positive control.

4.3 Gas chromatography–Mass Spectrometry (GC–MS)

The methanoic extract of aegle marmelos were injected into an HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following chromatographic conditions were used: Helium as the carrier gas, the flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of

250°C; interface temperature of 250°C; the mass range of 50-600 mass units¹³. The database of the National Institute of Standard and Technology (NIST) was used for the interpretation of the mass spectrum of GC-MS.

4.4 Anticancer assay:

Keratinocytes (KCs) represent 95% of the epidermal cells. Primarily, they play the structural and barrier function of the epidermis, but their role in the initiation and perpetuation breast cancer is recognized. **MDA MB 231** cells are used in this assay. The cells were cultured in Dulbecco's modified Eagle medium (DMEM). Supplemented with 10% FetalBovine serum with 2% L-Glutamine, 100 U/ml penicillin and 100µg/ml streptomycin. Then, incubate the cultures with trypsin/EDTA for 15min to split them.

Chapter 5

Results

5.1 Preparation of extracts

For the preparation of extracts fresh sample of aegle marmelos was collected. Sliced into pieces and soaked in water, then gently heated at 100°C. Then filter thus aqueous extract is obtained and another fresh sample is take and cut into small pieces and soaked in methanal for 48 hr. Then filter thus methanoic extract is obtained.



Figure 5.1 Aqueous extract



Figure 5.2 methanoic extract

5.2 DPPH[•] radical scavenging activity

Scavenging of DPPH free radicals is one of the popular antioxidant methods. DPPH radical scavenging assay is a decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH[•] radicals¹⁷. The ability of aqueous and methanoic extract of aegle marmelos to scavenge free radicals was assessed by the DPPH method. The maximum DPPH[•] radical scavenging activity of was 82.59±0.043% for methanolic extract and 39.28±0.119 for aqueous extract at 120 µg/mL concentration and the IC₅₀ was 80.33 and 0.117 µg/mL concentration respectively. It was compared with the standard of ascorbic acid (IC₅₀ = 11.98 µg/mL concentration). In this method, the purple colour 1,1-

diphenyl-2-picrylhydrazyl (DPPH) free radical was reduced to yellow colour 1,1-diphenyl-2-picrylhydrazine, and the reducing capacity increases with increasing concentration of the extract.

S.no	Concentration (μ l)	Absorbance at 517nm	% of inhibition
1	0	0.247	
2	20	0.214	13.36
3	40	0.186	24.69
4	60	0.140	43.31
5	80	0.124	49.797
6	100	0.063	74.49
7	120	0.043	82.59

Table 5.1 DPPH' radical scavenging activity(methanoic extract)

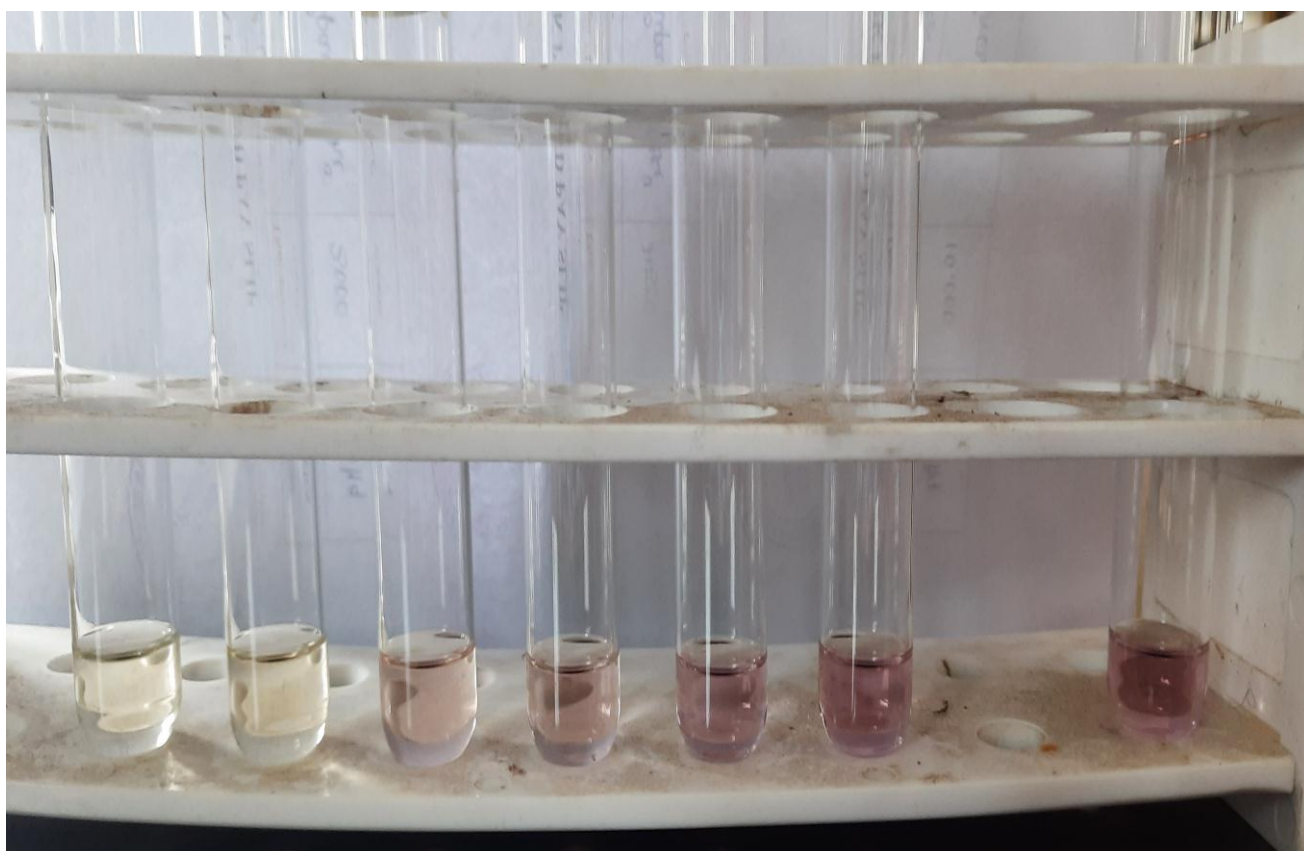


figure 5.3 DPPH' radical scavenging activity (methanoic extract)

S.no	Concentration (μl)	Absorbance at 517nm	% of inhibition
1	0	0.196	
2	20	0.134	31.63
3	40	0.131	33.16
4	60	0.129	34.98
5	80	0.123	37.24
6	100	0.121	38.26
7	120	0.119	39.28

Table 5.2 DPPH[•] radical scavenging activity (aqueous extract)

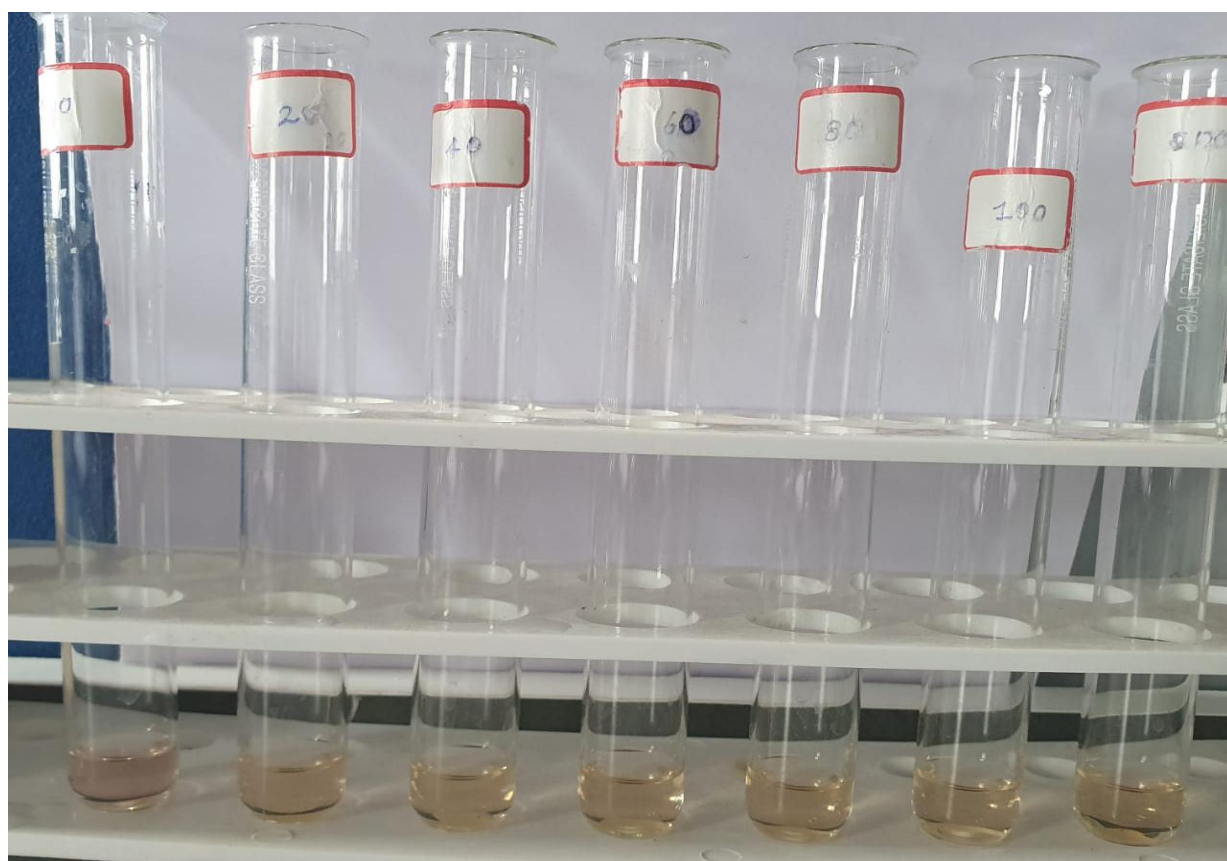


figure 5.4 DPPH[•] radical scavenging activity (aqueous extract)

5.3 Superoxide ($O_2^{\cdot-}$) radical scavenging activity

Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590 nm. Antioxidants can inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The maximum superoxide radical scavenging activity of aqueous dried methanolic and aqueous extract was $61.11 \pm 0.035\%$ and $79.1146 \pm 0.069\%$ at 120 $\mu\text{g/mL}$ concentration. the IC_{50} was 85.72 and 14.80 $\mu\text{g/mL}$ concentration. It was compared with the standard of ascorbic acid ($IC_{50} = 9.65 \mu\text{g/mL}$ concentration).

S.no	Concentration (μl)	Absorbance at 590nm	% of inhibition
1	0	0.090	
2	20	0.075	16.66
3	40	0.061	32.22
4	60	0.053	41.11
5	80	0.048	46.66
6	100	0.039	56.66
7	120	0.035	61.11

Table 5.3 Superoxide radical scavenging activity (methanol extract)

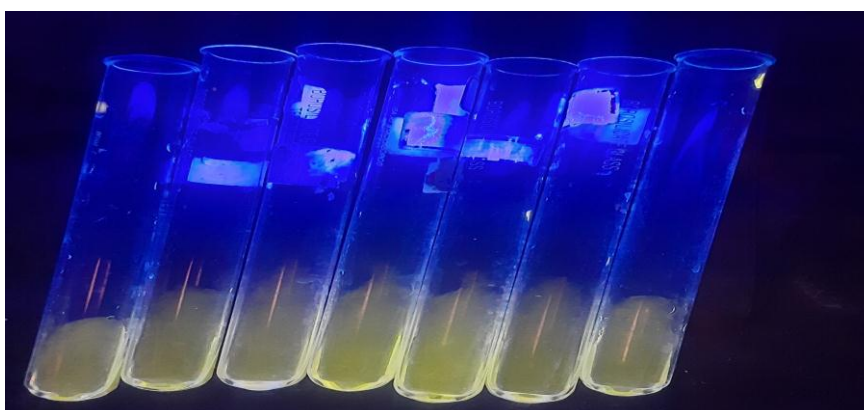


figure 5.5 Superoxide radical scavenging activity (methanol extract)

S.no	Concentration (µl)	Absorbance at 590mm	% of inhibition
1	0	0.336	
2	20	0.109	67.55
3	40	0.087	74.10
4	60	0.076	77.38
5	80	0.075	77.67
6	100	0.071	78.86
7	120	0.069	79.46

Table 5.4 Superoxide radical scavenging activity (aqueous extract)

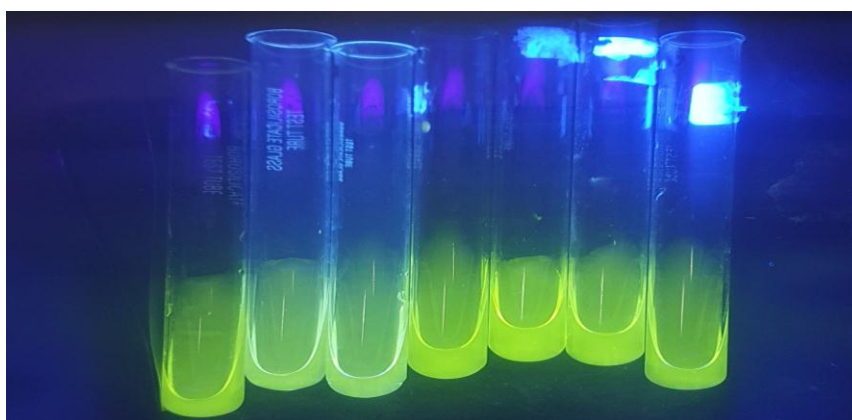


Figure 5.6 Superoxide radical scavenging activity (aqueous extract)

5.4 Phosphomolybdenum reduction activity

The total antioxidant activity of methanoic and aqueous extract was measured by phosphomolybdenum reduction assay method. In this method, Mo (VI) ion reduced to Mo(V) by the extract and the formation of green phosphate/Mo (V) complex at acidic pH¹⁹. The maximum phosphomolybdenum reduction was 89±0.212% for methanoic extract and 88,48±0.466% for aqueous extract at 120 µg/mL concentration and the RC₅₀ was 17.85 and 13.42 µg/mL concentration receptively. It was compared with the standard ascorbic acid (RC₅₀ = 6.34 µg/mL concentration). PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant, and molybdenum ligand. It involves

thermally generating auto-oxidation during the prolonged incubation period at a higher temperature

S.no	Concentration (μl)	Absorbance at 695mm	% of reduction
1	0	0.022	
2	20	0.050	56
3	40	0.121	81
4	60	0.133	83
5	80	0.157	85
6	100	0.177	87
7	120	0.212	89

Table 5.5 Phosphomolybdenum reduction activity(methanol extract)

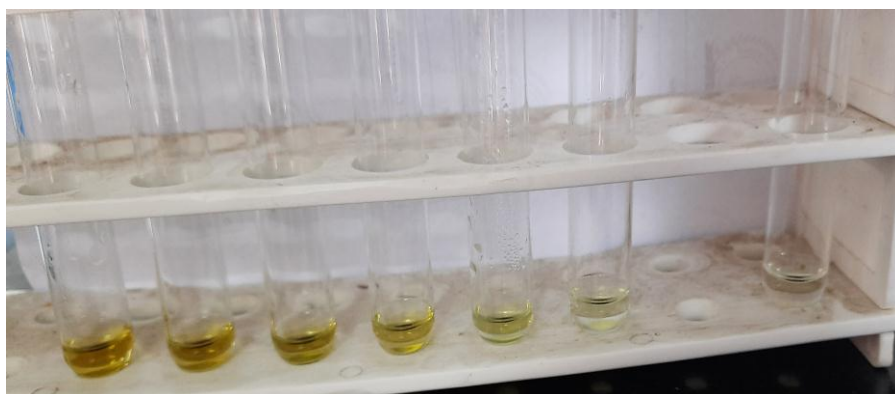


Figure 5.7 Phosphomolybdenum reduction activity(methanol extract)

S.no	Concentration (μl)	Absorbance at 695mm	% of inhibition
1	0	0.052	
2	20	0.204	74.50
3	40	0.259	79.92
4	60	0.303	82.83
5	80	0.362	85.63
6	100	0.401	87.03
7	120	0.466	88.48

Table 5.6 Phosphomolybdenum reduction activity(aqueous extract)

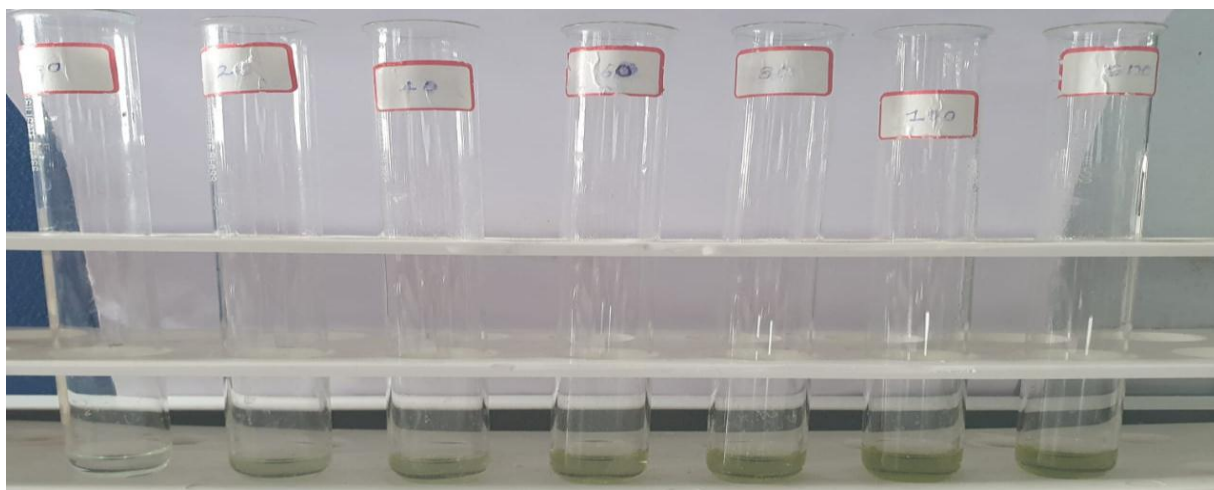


Figure 5.8 Phosphomolybdenum reduction activity(aqueous extract)

5.5 Ferric (Fe^{3+}) reducing power activity

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the aqueous dried Rose petals extract and the subsequent formation of Ferro-ferric complex. The reduction ability increases with an increase in the concentration of the extract²⁰. The maximum Fe^{3+} reduction was $51.33 \pm 0.144\%$ for methanoic extract and 65.52 ± 1.114 for aqueous extract at $120 \mu\text{g/mL}$ concentration and the RC_{50} was 85.47 and $48.96 \mu\text{g/mL}$ concentration respectively. It was compared with the standard ascorbic acid ($\text{RC}_{50} = 7.72 \mu\text{g/mL}$ concentration). Also in this assay, higher absorbance of the reaction mixture indicates higher reduction potential and a significant indicator of its potential antioxidant activity. The reducing capacity of the extract was performed using Fe^{3+} to Fe^{2+} reduction assay as the yellow colour changes to green or blue colour depending on the concentration of antioxidants. The antioxidants such as phenolic acids and flavonoids were present, a considerable amount in methanoic and aqueous extract, and showed the reducing capacity in a concentration dependant manner.

S.no	Concentration (μL)	Absorbance at 720nm	% of reduction
1	0	0.200	
2	20	0.294	31.97
3	40	0.297	32.65
4	60	0.322	37.88
5	80	0.376	46.80
6	100	0.386	48.18
7	120	0.411	51.33

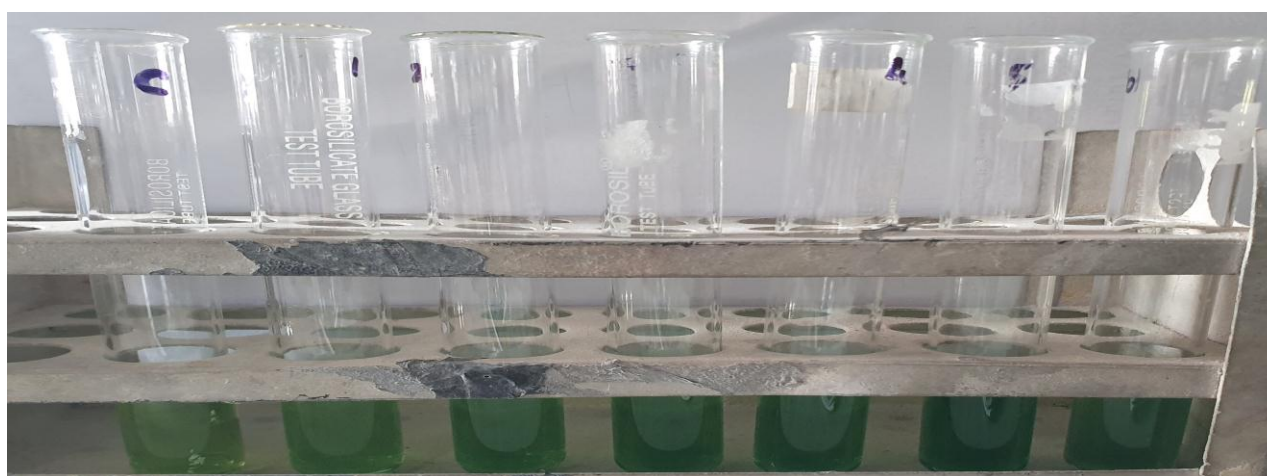
Table 5.7 Fe³⁺ reducing power activity (methanol extract)



figure 5.9 Fe³⁺ reducing power activity (methanol extract)

S.no	Concentration (μl)	Absorbance at 720nm	% of reduction
1	0	0.384	
2	20	0.569	32.51
3	40	0.680	48.82
4	60	0.834	53.95
5	80	0.871	55.91
6	100	0.934	58.88
7	120	1.114	65.52

Table 5.8 Fe³⁺ reducing power activity (aqueous extract)



5.6 Antibacterial activity by agar well diffusion method

The methanoic and aqueous extract of aegle marmelos was tested for its antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, and *Bacillus subtilis*. The antibacterial activity showed a maximum zone of inhibition of 39 mm and 25 mm against *Enterococcus faecalis* at 500 µg/mL concentration respectively of the extract. The antibacterial activity may be due to the presence of phenolic compounds, terpenoids, which inhibit bacterial cell wall biosynthesis by binding to a highly conserved motif of lipid II and lipid III, two key precursors of bacterial cell-wall polymers as peptidoglycan and teichoic acid that adversely affect the growth of microbes.

Organism	standard	250µg	375µg	500µg
Escherichia coli	36mm	14mm	13mm	18mm
Pseudomonas aeruginosa	10mm	9mm	16mm	28mm
Staphylococcus aureus	16mm	17mm	23mm	31mm
Enterococcus faecalis	15mm	17mm	21mm	39mm

Table 5.9 antibacterial against methanoic extract

Organism	standard	250µg	375µg	500µg
Escherichia coli	24mm	12mm	15mm	16mm
Pseudomonas aeruginosa	8mm	10mm	13mm	18mm
Staphylococcus aureus	14mm	16mm	20mm	32mm
Enterococcus faecalis	12mm	16mm	19mm	25mm

Table 5.10 antibacterial against aqueous extract

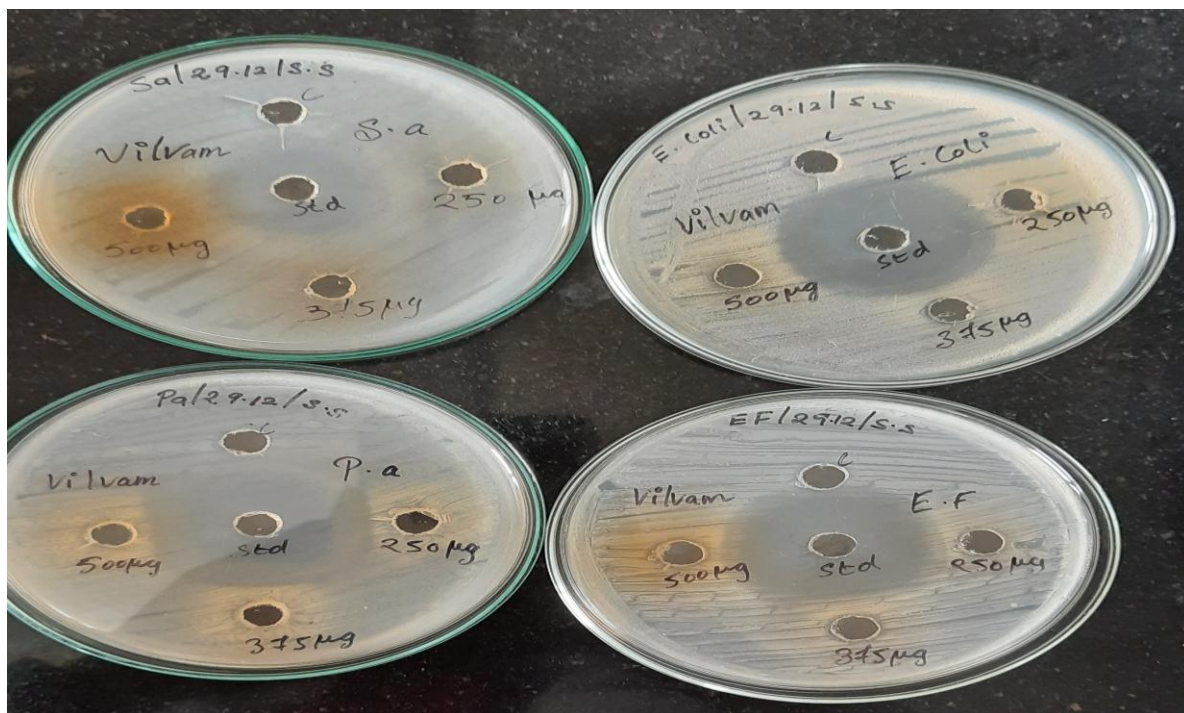


Figure 5.11 Antibacterial for methanoic extract of aegle marmoles

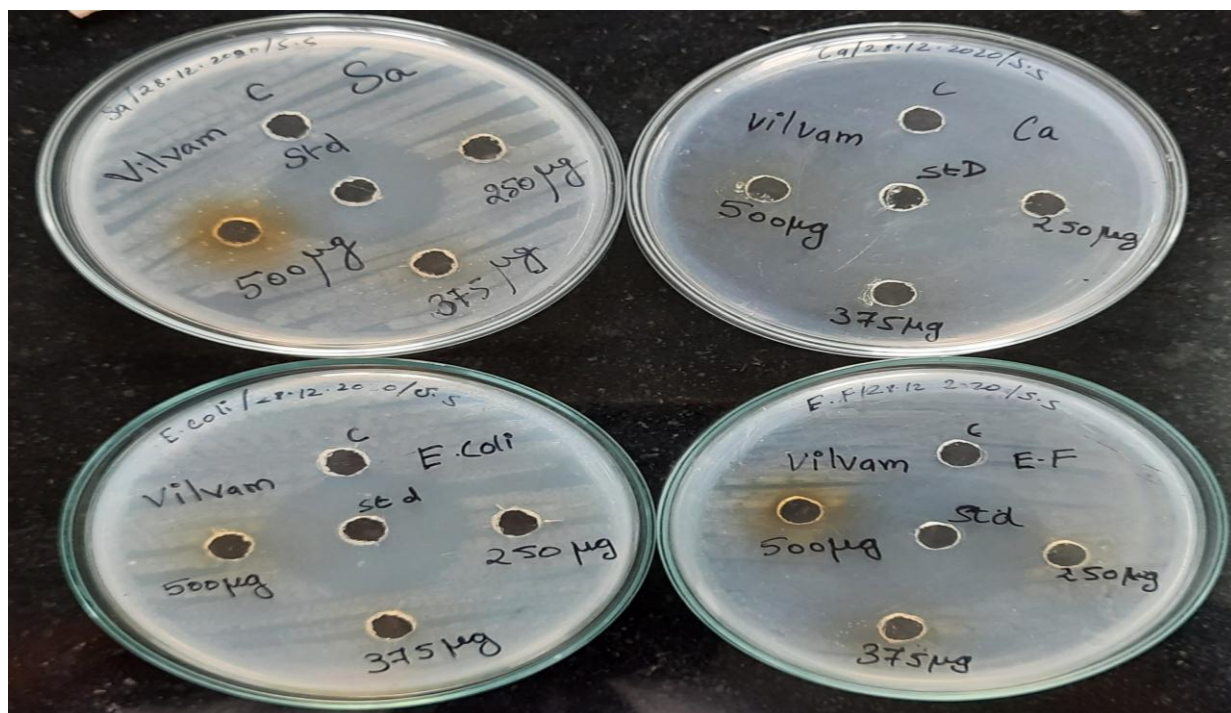


figure 5.12 Antibacterial for aqueous extract of aegle marmoles

5.7 GC-MS analysis

GC-MS analysis of methanoic of aegle marmelos was shown in Table 11 and the chromatogram was represented in Figure 11. Antioxidant compounds such as flavone (5,7-dihydroxy-3-phenylchromen-4-one), oleic acid, and antibacterial compound Pyrimidine,5-ethyl-2-[4-[4-ethylcyclohexyl)phenyl] were eluted and recorded.

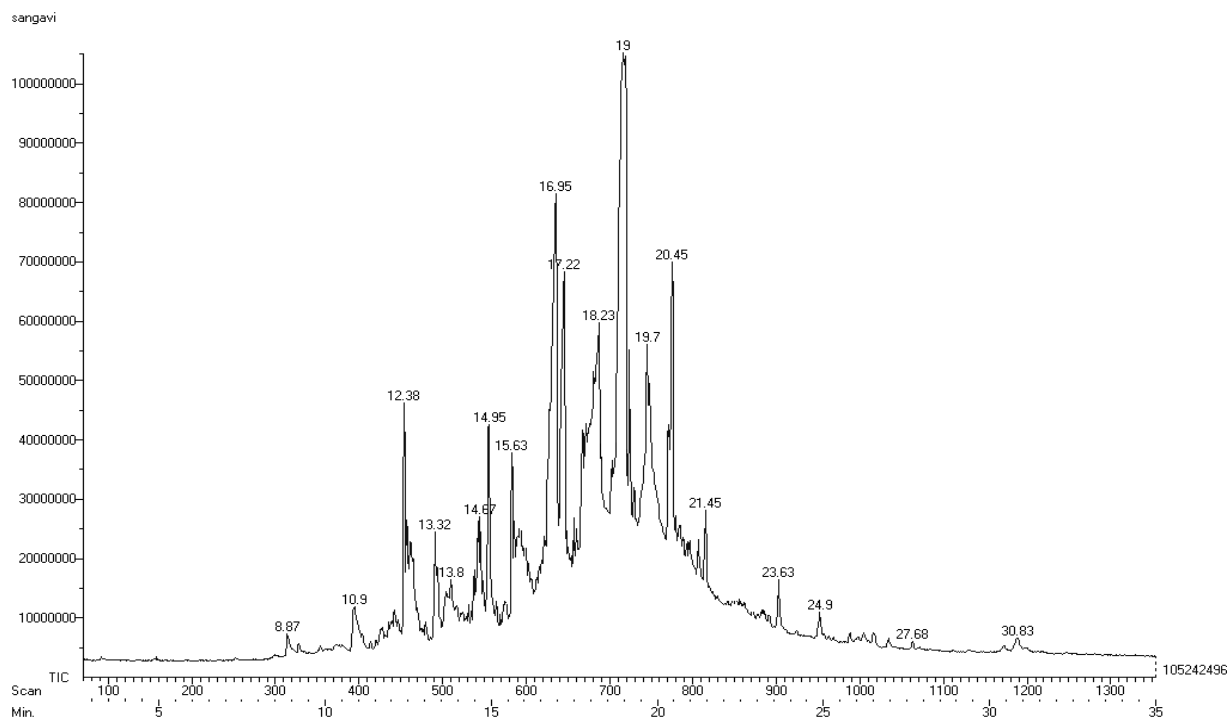


Figure 5.13 GC-MS Graph

Peak no	Peak RT time (in secs)	Details
1	8.87	á-Pinene
2	12.38	Eugenol
3	10.9	Cyclohexanol,1-methyl-4--[1-methylethylidene]-
4	13.32	6-Undecanone
5	13.8	Himachala-2,4-diene
6	14.67	Phenol,2,4-bis(1,1-dimethylethyl)-
7	14.95	Flavone
8	15.63	3-Hexadecene,[Z]

9	16.95	N-Hexadecanoic acid
10	17.22	Pentadecanoic acid, 13-methyl-,methyl ester
11	18.23	Z,E-2-Methyl-3,13-octadecadien-1-ol
12	19	Oleic Acid
13	19.7	4H-1-Benzopyran-4-one,5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-
14	20.45	Retinoyl fluoride (all-trans)
15	23.63	1-tridecene,2-decyl-
16	27.68	Benzoic acid,2,4-dimethoxy-6-methyl-,(8,8-dimethoxy-2-octyl)ester
17	30.83	Ergocalciferol
18	24.9	Vismiquinone
19	21.45	3-methoxy-12-methyl-D-homoestra-1,3,5(10),8,14-pentaen-17-one

Table 5.11 peak report

5.8 Phytochemical analysis of plant extract

The phytochemical analysis of methanolic and aqueous extract of *Aegle marmelos* showed the presence of flavonoids, tannins, phenol, glycosides, steroids and alkaloids. Whereas saponins are present in aqueous extract and not present in methanolic extract.

ASSAYS	AQUEOUS	METHANOL
FLAVONOIDS	+	+
TERPENOIDS	+	+
SAPONINS	+	-
TANNINS	+	+
PHENOL	+	+
GLYCOSIDES	+	+
STEROIDS	+	+
ALKALOIDS	+	+

Table 5.12 phytochemical

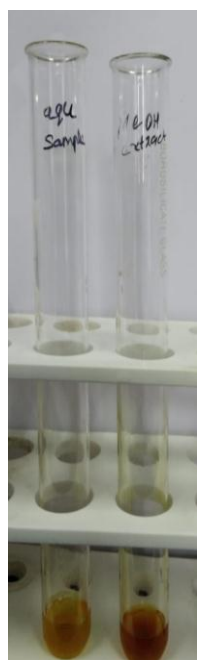


Figure 5.14
flavonoids



Figure 5.15
alkaloids

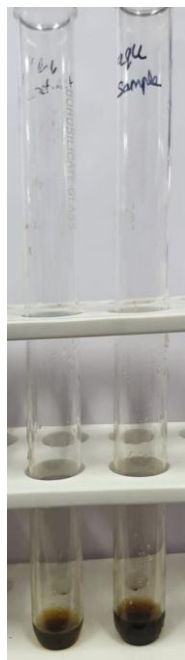


Figure 5.16
phenolic



Figure 5.17
Saponins

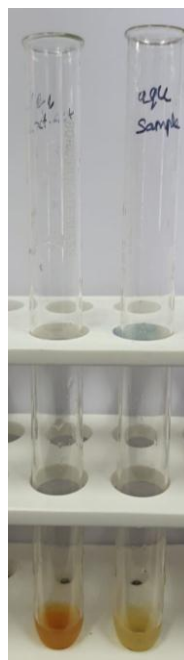


Figure 5.18
tannins



Figure 5.19
terpenoids

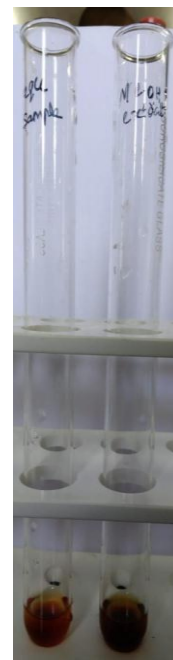


Figure 5.20
glycosides



Figure 5.21
steroids

5.9 Anticancer

Plant extracts would help cells to survive under conditions which progressively break intracellular machinery (free radical scavenging activities). On the other hand, the most efficient modern drugs kill cells, without discriminating normal and cancerous cells (free radicals attack). No matter that cancer cell, due to their extraordinary proliferative activity, are more vulnerable to DNA damaging or cell cycle blocking agents. A single healthy cell mutation can provoke its transformation into a malignant phenotype. In any case, in vitro

experiments on cancer cell lines have to be confirmed by clinical studies. This study showed the anticancer potentials of methanoic extract on MDA MB 231. The maximum cell death of MDA MB 231 cells by extract was $25.85 \pm 0.96\%$ at $250 \mu\text{g/mL}$ concentration and the IC_{50} was $1.57 \mu\text{g/mL}$. The microscopic observations revealed the dye was to be having outstanding effect on treated MDA MB 231 cells compared to untreated cells. The number of dead 39 cells increased correspondingly with increasing concentration of the extracts. At high extract concentration, enlargement of the cells was conspicuously observed.

Concentration ($\mu\text{g/mL}$)	Mean	SD	% Live cell
250	74.15	0.96	25.85
100	70.75	0.59	29.25
50	67.35	0.89	32.65
25	63.95	1.50	36.05
12.5	60.54	2.18	39.46
6.25	57.14	2.88	42.86
3.13	53.74	3.58	46.26
1.56	50.34	4.29	49.66
0.78	46.94	5.00	53.06

Table 5.13 Anticancer activity

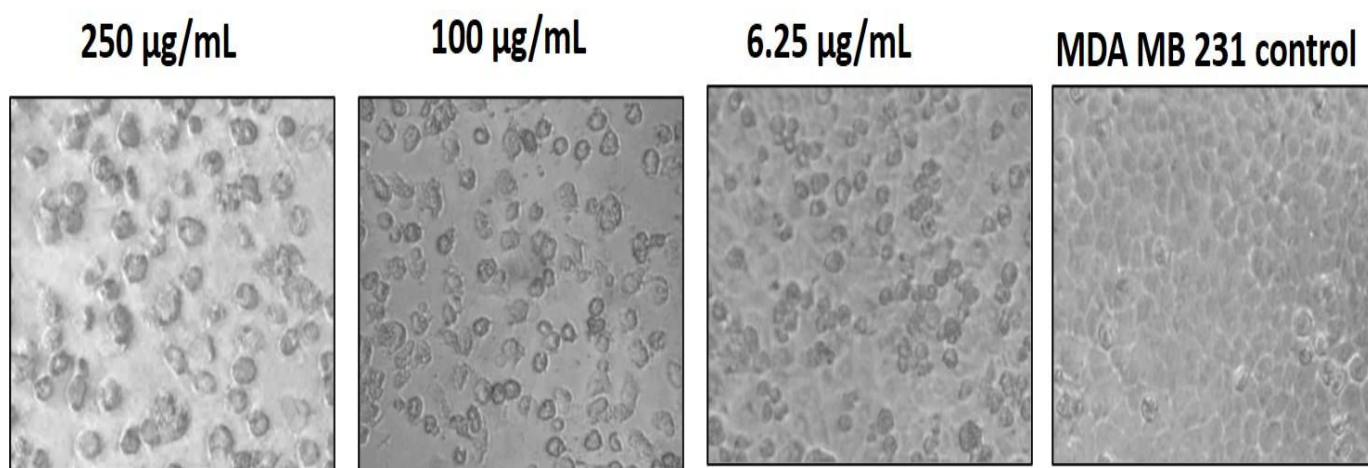


Figure 5.22 Different concentration of methanoic extract

CHAPTER 6

SUMMARY AND CONCLUSION

Antioxidants are substances that significantly delay or prevent the oxidation of an oxidizable substrate when present in low concentrations. Antioxidants help protect human body cells from the formation of radicals. Radicals, also known as free radicals are aggressive and toxic and are produced typically during cell metabolism. They are common transient intermediaries in chemical reactions with cell components, causing permanent damage and they are believed to be the source of ageing and the cause of several degenerative diseases. Plants are potential sources of natural invaluable food antioxidants. Antioxidants react with free radicals and form a complex that prevents cell destruction and is easily cleaned out. The results of the present study indicate that methanoic and aqueous extract of *Aegle marmelos* has significant antioxidant activities to reduce the harmful effect of radicals. The results of the present study provide promising hope to use extracts of *Aegle marmelos* as a natural antioxidant as well as antibacterial agents. As an outcome of its antibacterial activity, methanoic and aqueous extract of *Aegle marmelos* are regarded as a very promising antibiotic candidate for the treatment of Gram-positive infections. The most optimistic result was obtained in methanoic extract of *Aegle marmelos*, thus anticancer and GC-MS was done. Anticancer activity gives favourable results against breast cancer line for methanoic extract.

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