Title

Chemical characterization of *Aegle marmelos* and its micropropagation.

A Dissertation- II submitted by

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ABSTRACT

Aegle marmelos is a medicinal plant which is commonly known as Bael plant. It has been used in many traditional medicines to treat various diseases. Present study deals with the chemical characterization of bael plant and its micropropagation. Qualitative analysis of bael had shown presence of alkaloids, phenols, flavonoids and reducing sugars. For characterization plant parts used are leaves and bark. The extraction was done in four different solvents. Water had given maximum yield of crude extract. The extractive value in water was 27%. Out of all the extracts, leaf extract in methanol had shown more number of phytochemicals then the others. The quantitative analysis had shown that the phenolic compounds are present in high amount in methanol extract of leaf. The flavonoids were present in high amount in chloroform extract of leaf. The antimicrobial activity was checked using different test organisms. The petroleum ether extract had shown significant inhibition of growth of microbes. The TLC analysis had shown more number of compounds in case of methanol and chloroform extract of leaf and bark. The samples showing significant number of compounds were subjected to HPLC. HPLC was done using four standards i.e. Marmelosin, Marmesin, Quercetin and Kaempferol . In all the four extracts the standard compounds were found. But out of these the leaf extract in leaf is having high yield of compounds. In indicates that the methanol extract of leaf is most significant.

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Maninder Kaur

DECLARATION

I, Maninder Kaur, student of M.Sc (Hons.) Biotechnology under department of biotechnology, Lovely Professional University, Punjab hereby declares that all the information furnished in this dissertation is based on my all intensive research and is genuine.

This dissertation to the best of my knowledge contains part of my work which has been submitted for the award of my degree**.**

Maninder Kaur

CERTIFICATE

I hereby declare that Maninder Kaur has worked on project entitled **"Chemical characterization of *Aegle marmelos* and its micropropagation**" under my guidance at department of biotechnology, Lovely Professional University, Punjab.

To the best of my knowledge, the present work is the result of her original investigation and study. No part of the dissertation has ever been submitted for any other degree at any University. The dissertation is fit for submission and the partial fulfillment of the conditions for the award of degree.

Dr. Kuldip Chandra Verma

Project advisor

INDEX

LIST OF FIGURES

LIST OF TABLES

TERMINOLOGY

Serial no.	Abbreviations	Full form
1.	Mg	Milligrams
2.	Ml	Millimeters
3.	Rpm	Revolutions per minute
4.	^{o}C	Degree Celsius
5.	µl	Micro liter
6.	Mm	Millimeter
7.	Psig	Pounds per square inch gage
8.	MH	Muller Hilton agar
9.	Nm	Nanometer
10.	Hr	Hours
11.	TPC	Total phenolic content
12.	TFC	Total flavonoid content
13.	W.H.O	World Health Organization

CHAPTER 1

INTRODUCTION

Aegle marmelos belongs to family Rutaceae and it is considered to have many medicinal and nutraceutical properties. It is commonly known as Bael or wood apple plant and is used in various traditional medicines. The medicinal properties of bael indicate that it is a valuable source of variety of bioactive compounds. Plant and the medicines derived from plant are used by humans to treat and to relief physical and mental illness **(Dinesh *et al*., 2011).** Researchers are now aiming to identify and validate the substances from plants to treat various diseases. It has been found that different parts of plant like leaves, fruits, seeds etc are helpful to human body as they provide health and nutritional promoting compounds **(Ganesh *et al*., 2011).**

Bael is a medium sized tree (25-30 feet) slender, aromatic tree, and it grows slowly. Plant is having short stem, flaking soft bark and branches are sometimes spiny. Older plants have straight and stiff spines and sharp spikes of about one inch length. The leaflets are 4-10 cm long and 2-5 cm in width and are oval or lancet in shape. Leaves are further divided into 3-5 leaflets and mature leaves are having particular fragrance. Fruit is of diameter 2-4 inches and are spherical or oval in shape. Shell of fruit is woody and hard **(Dinesh *et al*., 2011).**

It is found that more than 25% of medicines which are used now days are derived indirectly or directly from plants. From the availability and safety point of view, the medicines derived from plants are considered good over the medicines derived from animals and from chemically synthesized medicines. Adverse effects and many side effects were observed in people using the synthetic drugs but these effects were less in medicines which are derived from plants. In home remedies, plants found in India are considered the main source of active constituents and used to treat many diseases. In India the rural population is more likely to use traditional ways of treatment because these kinds of treatment are easily available and are of cheap price. In present time W.H.O is also encouraging the use of herbal medicines which are used traditionally from last many years. Recently, W.H.O conducted a survey in which they found that around 20,000 medicinal plants are used either in pharmaceutical industry or in traditional medicine system. People are using these herbal medicines because they believe that natural medicines are more effective and are safer to use **(Pushpendra *et al*., 2012).** The wood of bael plan when freshly cut gives strong aroma and is used for carts, carving, tool, knife handles, pestles and

combs etc. Gum of bael is used as household glue, in watercolors, as protective coating on paintings and jewelers use it as adhesive. Bael plants also have insecticidal activity against brown plant hopper which is known as important pest of rice in Asia. *Aegle marmelos* is used to prepare different traditional medicines for treatment of various diseases like respiratory tract infections, tumors, nausea, smallpox, mental illness, eye disorders, bronchitis, leprosy, asthma, abdominal problems, fever inflammation, burning sensation, diarrhea, jaundice, constipation, acute bronchitis, snakebite, acidity, leucoderma, thyroid disorders, burning sensation, epilepsy, spermatorrhoea etc. In Indonesia, leaves and shoots of bael are used as green vegetables **(Dinesh *et al*., 2011).**

After distillation of flowers, a drug is yielded which is used against anti- dysenteric, diaphoretic, intestine and stomach diseases. It was found that its leaf extract is able to regenerate s-cells of pancreas in diabetic rats. For the treatment of intestinal parasites, like *Ascaris lumbricoides* and *Entamoeba histolytica*, powder of unripe fruit is used. Antimicrobial activity against some microorganisms like *Escherichia coli* was found in oil obtained from seeds. Leaf oil is used against cold and some respiratory infections **(Sandeep *et al*., 2010).**

In last few years *Aegle marmelos* has been studied with the help of advanced scientific techniques and it was found that *Aegle marmelos* is having many medicinal properties like anti-inflammatory activity, antidiabetic activity, antibacterial activity, anticancer activity, antioxidant activity, haemolytic activity, larvicidal activity, antifungal activity, hepatoprotective activity etc **(Dinesh *et al*., 2011).** In *Aegle marmelos* many bioactive compounds are found like phenolics, alkaloids, flavonoids, carotenoids, coumarins, reducing sugars, terpenoids etc. The bioactive compounds found in plants are generally accumulated as secondary metabolites in all the cells of various plant parts. The concentration of these bioactive compounds changes as which plant part is being used, season, climate and the growth phase etc.

In different parts of plant different bioactive compounds are found. But for extraction purpose the solvent should be properly selected. Because some compounds are soluble in one solvent but cannot be in other solvents. In other words the yield may also differ with change in solvent. Different parts of plant are having various nutritional values. Leaves of bael are used in asthma and a laxative for mucous membrane having a free discharge. To reduce or dispel fever decoction of plant leaves is used and also enhances the secretion f mucous from bronchial tube.

Medicinal properties of Aegle marmelos are shown in Fig.1

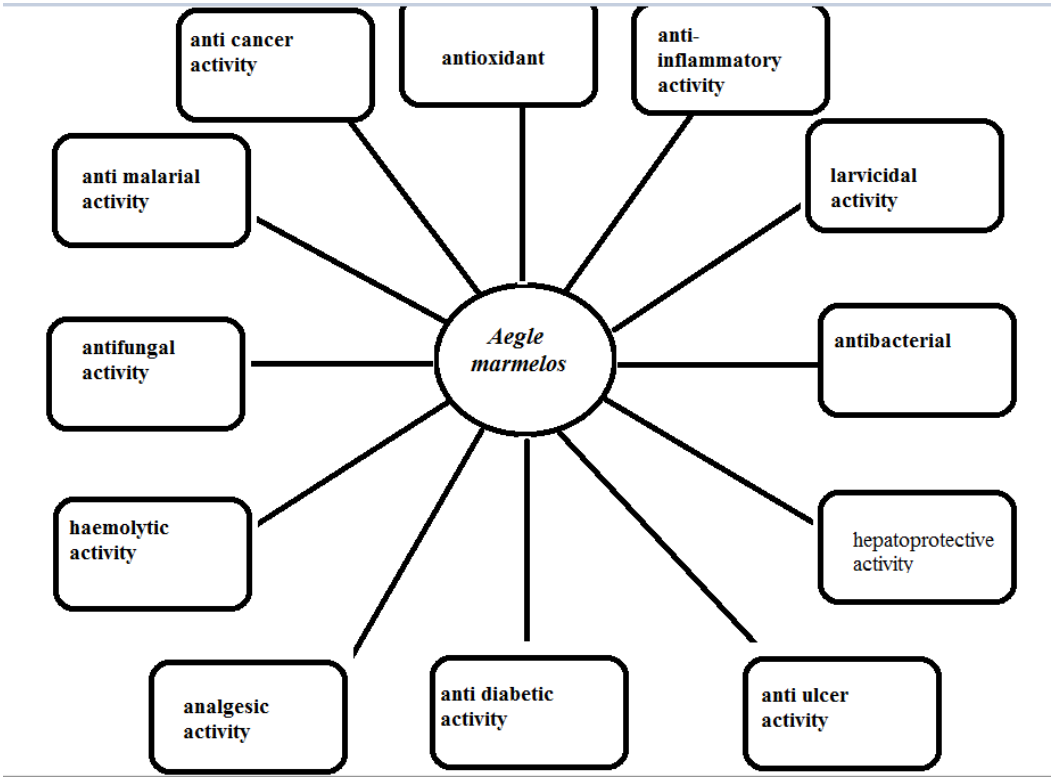

Fig.1 Medicinal properties of *Aegle marmelos*.

For inflammation of body parts and severe conjunctival inflammation, hot poultice of leaves is used. Juice of bael leaves is used to treat dropsy and jaundice. Leaf extract of bael is helpfull in restring blood glucose, and to keep the body weight to normal values. Leaves of bael works same as insulin works i.e. it promotes the ability to utilize the externally available glucose in body by stimulating glucose uptake. Extract of bael is used to cure some other problems like it helps in lowering blood urea, reduce lipid peroxidation and cholesterol and increased level of super dioxide dismutase, catalase, glutathione peroxidase level in serum and als in liver in experimental diabetic animals **(Sharma *et al*., 2007).** Fresh and young leaves of bael are supposed to cause sterility and abortion. Leaves of bael are used to prepare medicated oil which helps to give relief from recurrent cold and respiratory infections. Leaves are used in backpain, abdominal disorders, beriberi, acute brnchitis, child birth, hair tonic, abscess, cut and wounds, nervous disorders, cardio tonic etc **(Gaur *et al*., 1999).**

Fig. 2 *Aegle marmelos* plant leaves and fruit (bioinfo.bsir.res.in)

The fruit of bael is having high nutritional values. Fruit is used to prepare jam, juice jelly, syrup, toffee and some other products. In reports it was found that pulp of fruit contain water, protein, fat, sugar, fibres, potassium, calcium, minerals and iron **(Dinesh *et al*., 2011).** Simple and good cure of dyspepsia is use of ripe bael fruit. Oil prepared from fruit of bael is used to remove peculiar burning sensation on soles. Fruit of bael is prescribed by ayurveda for heart, chronic constipation, intestinal tonic, stomach, dysentery, indigestion, typhoid, intermittent fever, cholera and for heart palpitation. Medically it was found that unripe fruit is better than ripe fruit **(Ganesh *et al*., 2011).** Ripe fruit of bael enhances digestion and also helps to treat inflammation of rectum. Fruit pulp of bael is used to prevent growth of piles. Fresh juice of bael is bitter in taste and helps to lower blood sugar **(Vyas *et al.*, 1979).** Fruit of bael contain coumarins, carotenoids, alkaloids, phenolics, terpenoids and many other antioxidants which help in protection against chronic diseases. Bael, as food, is used in different forms in different countries. In India, ripe fruit is used as fresh fruit and also used to prepare squash, nectar, jam, sherbet and cream etc. In Indonesia, it is common practice to use fruit of *A. marmelos* as breakfast. Bael fruit pulp is also used to make beverage with tamarind. In Thailand bael fruit is cut into pieces and dried and packed in bags and preserved in syrup. In Thailand, young leaves and shoots are consumed as vegetable because they believe that it reduces appetite. It is also used as dessert or in preparation of cake **(Charoensiddhi *et al.*, 2008).** Similarly other plant parts like flower, seeds, roots, bark etc are used to treat various diseases.

Thin layer chromatography was used to check the presence of bioactive secondary metabolites in plant extracts. TLC is performed to separate a mixture into their components. These compounds are identified when the bands of unknown samples are compared with standard. In different extracts the

bioactive compounds are identified and the extracts are quantitatively analyzed using HPLC (High Performance Liquid Chromatography).

Different methods have been used and various chemical constituents like alkaloids, coumarins and steroids have been extracted and identified from seeds, leaf, fruit, bark etc. The alkaloids comprise the main class of secondary plant substances. Recently new alkaloids like ethyl cinnamamide etc are isolated. In the month of January maximum tannin content was recorded in bael fruit. In the pulp of wild fruit about 9 % tannin was found. Tannin is also present in leaves in different forms like skimmianine, it is also known as 4, 7, 8-trimethoxyfuro, quinoline. Pale color of fruit is due to the presence of Carotenoids. From leaf oil alpha-Phellandrene (56%) and p-cymene (17%) were reported from leaf oil. Limonene (82.4%) was reported as main constituents from leaves. The phenolic compounds present in bael are the main group of compounds that acts as major bioactive compounds. Now a day's more emphasis is given on use of plant material as source of medicine because of fast population, inadequate supply of drugs, side effect caused by allopathic drugs and increase in resistance in pathogens.

As bael plant contains a lot of antioxidants so it requires to micro propagate the plant using tissue culture techniques. In South India Bael is found as red-listed medicinal species. In developing countries about 70% world population is dependent on these treatments for their good health. It was found that seed progeny of bael are not uniform and easily attacked by insects and they have less survival potential. Vegetative propagation through roots and other conventional methods is very slow, difficult and season dependent. So micropropagation technique is used in mass multiplication of bael plant and other fruit species. The most widely used technique in plant tissue culture is micropropagation. The present study was carried out to develop an efficient in vitro regeneration system via callus phase by using various plant growth regulators and secondary metabolites are extracted from dried powder of leaves and bark several solvents **(Abirami *et al*., 2013).**

CHAPTER 2

REVIEW OF LITERATURE

The bioactive compounds found in bael showed following values of extracts - total flavonoids 15.20±0.51 (mg CEd/ g dw), total carotenoids 32.98±0.51 (µg/ g dw), ascorbic acid 26.17±0.85 (mg/ 100 g dw) **(Charoensiddhi *et al*., 2008)**.Antimicrobial activity of different plant parts i.e. leaf, bark and fruit was checked some microorganisms. The extract of these plant parts was prepared in different solvents i.e. methanol, chloroform and water. These effects were compared with standard. It was found that methanolic extract of leaves, root and bark were less effective when compared with antibiotics that are commercially available. The aqueous extract showed no activity against *Klebsiella pneumoniae*. It was observed that leaf extract in methanol was having maximum activity as compared to the chloroform and aqueous extract of plant leaves. It indicates that there are some antibacterial chemicals which can be either polar or no polar and these chemicals can be efficiently extracted through organic solvent medium. Test organisms to check the antimicrobial activity were *Klebsiella* species, *Proteus mirabilis, Staphylococcus aureus, Salmonella paratyphi A, Salmonella paratyphi B* and *E. coli* ***(*Poonkothai *et al*., 2008).*** Total phenolic and flavonoid content was estimated in leaf, stem and root of Aegle marmelos. Total phenolic content in leaf, stem and root was 9.8 mg/kg, 7.4 mg/kg and 1.7 mg/kg respectively. Total flavonoid content in leaf, stem and root was 8.2 mg/kg, 1.4 mg/kg and 1.08 mg/kg respectively **(Nadeem *et al.*, 2010).** The anti microbial activity was checked using well diffusion method for ethanol extract. Test organisms to check antimicrobial activity were *E. coli, Klebsiella pneumonia, Proteus vulgaris, Micrococcus luteus, Bacillus subtilis, Enterococcus faecalis and Streptococcus faecalis.* It was observed that Bacillus subtilis is resistant against hexane, cold methanol and hot methanol. But the standard showed the zone of clearance in B. subtilis. All the leaf extracts in hexane, cold methanol and hot methanol showed their effect against all other microorganisms i.e. *E. coli, Klebsiella pneumonia, Proteus vulgaris*, *Micrococcus luteus*, *Enterococcus faecalis and Streptococcus faecalis*. These extracts were most effective against *Streptococcus faecalis* **(Saradha *et al*., 2010).** Previous studies revealed that some compounds including cinnamic acid and coumarins derivates and alkaloids have been isolated from *Aegle marmelos.* Aegeline was isolated from leaves and skimmianine was obtained from the roots of plant. Melting points (uncorrected) were determined on Kohfler melting points apparatus **(Tiwari *et al*., 2010).**

There are different methods to check the Total Phenolic content, antioxidant activity of bael plant. The plant is having different compounds in different amounts like it is having content of tannin

(0.985%) and riboflavin (0.005%) **(Yogita *et al.*, 2011).**Total Flavonoid content was estimated using standard curve of Quercetin. Standard equation curve was having regression value, R^2= 0.91 y= 0.0009X +0.0011. The total amount of flavonoid in methanol and aqueous extract was found as 12.933 mg/g and 3.267 mg/g, respectively **(Sharma *et al.*, 2011).** From unripe fruit of Aegle marmelos salicylate and other three new compounds were isolated. Out of these three compounds, two were esters and one was acid. Thin layer chromatography was performed for their isolation with the help of Silica gel G **(Ganesh *et al.*, 2011)**. Antimicrobial activity of different extracts was checked against different microorganisms. Disc diffusion method was used to check the antimicrobial activity of extracts. Maximum inhibition zone was observed against gram positive bacteria i.e. *Streptococcus haemolyticus, Staphylococcus aureus.* Antimicrobial activity was also observed against some gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa*. It was observed that petroleum ether extract was having maximum efficiency against these test microorganisms. For this activity the standard antibiotic used was cefuroxime. When compared with standard it was found that petroleum ether extract was moderate against *Proteus mirabilis* and its mild effect was observed in *Proteus mirabilis* and *Klebsiella.* Chloroform extract showed maximum antimicrobial activity against *Klebsiella pneumonia and Proteus mirabilis.* When this extract was compared with standard then it was found that chloroform extract was moderate against *Escherichia coli* and *Pseudomonas aeruginosa.* Chloroform extract was showing mild effect against *Salmonella typhi*. Methanol extract showed maximum activity against *Salmonella typhi* which showed maximum zone of clearance which indicates that methanol extract was having maximum efficiency against *Salmonella typhi.* Phytochemical screening of leaves of bael showed the presence of various phytochemicals. In ether extract of bael only phenols and sterols were observed and other compounds like flavonoids, tannins, coumarins, saponins and triterpenoids were absent. In chloroform extract of bael phenols and sterols were observed and other compounds flavonoids, tannins, coumarins, saponins and triterpenoids were absent. In methanol extract most of the phytochemicals were observed. Methanol extract showed the presence of flavonoids, tannins, coumarins, saponins and triterpenoids, sterols and phenols **(Saroj *et al.*, 2011**). It was found that leaves contain a large number of constituents like marmine, alkaloids, aegeline, coumarins, sitosterol, sterols and some essential oils like d-limonene. From literature it was found that alkaloids present in bael are responsible for pharmacological properties **(Biresh *et al.*, 2011).** It was found that leaves contain a large number of constituents like marmine, alkaloids, aegeline, coumarins, sitosterol, sterols and some essential oils like d-limonene. From literature it was

found that alkaloids present in bael are responsible for pharmacological properties **(Biresh *et al*., 2011).**

For reducing sugars it was observed that reducing sugars are present in water and methanol extract of leaf and petroleum ether. But reducing sugars are absent in petroleum ether and chloroform extract **(Vanitha et al., 2012).** In toluene extract of bael alkaloids, coumarins, flavonoids, carboxylic acids and xanthoproteins were absent and only anthocyanins, phenols and sterols were present. In chloroform extract of bael alkaloids, phenols, xanthoproteins, carboxylic acids were present and most of the phytochemicals like coumarins, anthocynins, flavonoids and steroids were absent. In methanol extract only xanthoproteins, phenols and anthocyanins were observed and most of the compounds like alkaloids, carboxylic acids, flavonoid, coumarins and steroids were absent. Similarly, in aqueous extract of bael only phenol and anthocyanins were observed and carboxylic acids, alkaloids, flavonoids, coumarins. Sterols and xanthoproteins were absent **(Naresh *et al*., 2012).** Agar diffusion method was used to check the antimicrobial activity of leaf extract in solvents i.e. petroleum ether, chloroform and water. These extracts showed antimicrobial activity against gram positive and gram negative bacteria. Chloroform extract showed much efficiency as compared to water and petroleum ether extract. This comparison was made by comparing the zone of inhibition of all the extracts. Leaf extract of bael showed maximum activity against *Klebsiella* species. Minimum activity of these extracts was observed against *Pseudomonas* species (**Elavarasi *et al*., 2012).** In methanolic extract of bael total phenolic and flavonoid content are 504.9µmol/ml and 0.25µg/ml respectively **(Madhura *et al*., 2012)**. Phytochemical screening of leaf extract of *Aegle marmelos* showed the presence of alkaloids, carboxylic acids, anthocyanin, phenol, sterols, xanthoproteins and the absence of some bioactive compounds like coumarins and flavonoids. Solvent used for the extraction were Toluene, Chloroform, methanol and Aqueous. **(Chavda *et al*., 2012)**.As this plant has many active oxidants so different methods are there to isolate them from plant parts. Phytochemical screening was done with the help of petroleum ether, chloroform, ethanol and aqueous extracts and various phytochemicals like carbohydrates, tannins, saponins, flavonoids, alkaloids, anthocyanin, betacyanin, quinones, glycosides, terpenoids, phenols, coumarins, and triterpenoids were identified. For HPLC they used Urslic acid, Marmesin, Isorhamnetin, Farmarixetin, Quercetin and Rutin. In ethanol extract of leaves they observed that the extract is having Marmesin, rutin, Quercetin and ursolic acid **(Umadevi *et al*.,2012).** Studies showed that leaves of *Aegle marmelos* are rich in beta-carotene, ascorbic acid and polyphenols **(Vanitha *et al*., 2012).** In micropropagation different combinations of hormones is used. But among all

those combinations 0.5 mg BA/l was found best for maximum growth of shoots and for mean shoot length. The best response explants was found in media containing BA, gibberellins and KN. As the concentration of these hormones is increased the response of explants decreases accordingly **(Puhan *et al*., 2012).**

For *Aegle marmelos* qualitative and quantitative analysis was done in different organic and inorganic solvents. It was found that ethanol extract is having most of the compounds. These compounds are saponins, terpenoids, flavonoids, alkaloids, indoles, cardiac glycosides, phenols, steroids. Alkaloids, flavonoids, steroids, saponins, cardiac glycosides, terpenoids, tannins were found in methanol extract of *Aegle marmelos*. *Aegle marmelos* in chloroform showed the presence of phenols, flavonoids, steroids, cardiac glycosides and terpenoids. Flavonoid content in *Aegle marmelos* was found to be 104 mg/g and phenolic content was found to be 2.978 mg/g (**Mariya *et al*., 2013).** For *Aegle marmelos* in solvents i.e. water, acetone and chloroform, extractive values were found to be 7.5 %, 5.3 %and 6.4 % respectively. It was found that most of the phytochemicals are present in aqueous extract. In all the extracts phenolic and flavonoid compounds were observed (**Shailesh *et al*., 2013).** In methanol extract of leaves of *Aegle marmelos* the amount of phenol was found to be 63.01 mg GAE/ gm sample **(Tupe *et al*., 2013).** Quantitative analysis of leaves of bael was done in ethanol, ethyl acetate and distilled water. Ethanol extract was having maximum amount of phenolic compounds i.e. 1.92 mg/g. In ethyl acetate total phenolic compounds were 1.74 mg/g and in distilled water the amount of phenolic compounds was least i.e. 1.5 mg/g **(Sathya *et al*., 2013).** The total phenolic content found in bael was evaluated using Folin-Ciocalteu method. Total phenolic content of *Aegle marmelos* leaf extract in water, ethanol, methanol and hexane was 486, 289.9, 247.8, 85 mg GAE/ gm dry weight respectively (**Garima *et al.*, 2013).** Leaf extracts in solvents like water, acetone and chloroform showed that Phenolic compounds, Alkaloids, saponins and Flavonoids are present in leaf. It was found that out of these three solvents the phytochemicals were more in aqueous extracts. Leaves of bael were crushed in pestle and mortar. At room temperature the leaves were extracted for 12 h and extraction with acetone and chloroform was for the time period of 72 hours. The extract was filtered using whatmann filter paper**.** Total phenolic compound in alcoholic extract and water extract was found to be 13.55 and 15.69 mg GAE/100 gm respectively. Similarly, total flavonoid compounds in alcoholic and water extract was 1.59 & 1.96 mg QE/ 100 gm respectively (**Kumar *et al*., 2013).** It was found that there is difference in percentage yield of extract products with change in solvent used for extraction. This difference might be due to difference in solubility of various constituents of extract in solvents.

Aqueous extract of unripe fruit showed the presence of phenols, protein, carbohydrate, flavonoids, steroids, terpenoids, triterpenoids, saponins and cardiac glycoside and also showed the absence of tannins and alkaloids. Aqueous extract of ripe fruit showed the presence of tannins, phlobatannins, phenols, protein, carbohydrates, alkaloids, steroids, terpenoids, saponins and absence of flavonoids and triterpenoids. For pharmacological study ethanol fruit extract is considered suitable. In acetone extract least compounds were extracted **(Varughese *et al*., 2013).** In immature bark of bael qualitative analysis was done and it was found that bark contain most of the compounds like terpenoids, alkaloids, flavonoids, saponins, steroids, tannin and glycosides. In chloroform extract of *Aegle marmelos*, total phenolic content was estimated by Folin- Ciocalteu method. Total phenolic content in chloroform extract was found to be 898 mg/g **(Venkatesh *et al*., 2014).** Phenols and flavonoidal compounds have been found effective in many activities like antioxidant, free radical scavenging activity, anti-inflammatory, antimicrobial, anti-mutagenic, anti-carcinogenic etc. Pharmacological properties of a. marmelos shower various activities like anti-diarrhoeal activity, antimicrobial and antiviral activity, radioprotective effect, anticancer activity, chemopreventive, anti-pyretic, ulcer healing potential, anti-genotoxic activity, diuretic activity, anti-fertility activity and anti-inflammatory activity **(Shahedur *et al*., 2014).**

There are some standardized techniques like HPLC, TLC and GC-MS etc to separate and detect which are present in *Aegle marmelos*. From bark of Aegle marmelos the bioactive compounds named marmemin and faganine are identified. Two pharmacologically active compounds i.e. 1,2-benzenedicarboxilic and Di-n-octyl phthalate are proven to show antimicrobial activity. Terpenes are the phenolic compounds and from bael mono and tri terpenes are also found **(Diana *et al*., 2014).**

Chromatography has many categories and out of these HPLC is mostly used as analytical technique. Over the past decades HPLC has become a method of choice to analyze various compounds. As all the plants parts are used in preparation of traditional medicines so the extracted plant parts are subjected to HPLC to identify the active compounds. Main phytoconstituents found in leaves are Marmesinine, Citronella, Aegeline, Eugenol, Citral, Skimmianine, Cuminaldehyde, Cineol and Lupeol **(Nitu *et al*., 2015)**

CHAPTER 3
SCOPE OF THE STUDY

As we know that plants are the main source of ayurvedic medicines. Almost 70% people are dependent upon ayurvedic treatment because it is easily available and cheap. So the present study deals with the chemical characterization of medicinal plant i.e. *Aegle marmelos* and its micropropagation. Pharmacological properties of *Aegle marmelos* shower various activities like anti-diarrhoeal activity, antimicrobial and antiviral activity, radio protective effect, anticancer activity, chemo preventive, anti-pyretic, ulcer healing potential, anti-genotoxic activity, diuretic activity, anti-fertility activity and anti-inflammatory activity. Bael plants need to be micro propagated because it contains many phytochemicals like phenols, protein, carbohydrate, flavonoids, steroids, terpenoids, triterpenoids, saponins, tannins and alkaloids etc. These bioactive compounds can be isolated and used in medicine. The synthesized bioactive compounds are suspected to be carcinogenic so more emphasis is given to the micropropagation of medicinal plants. The other processes of vegetative propagation are very slow and the seed thus produced have less viability so researchers are trying to make the micropropagation process to be easily and convenient**.** Using micropropagation techniques more number of plants can be produced in less time. Further these micro propagated plants can be used as isolation of bioactive compounds. This can be further used for preparation of ayurvedic medicines.

CHAPTER 4

OBJECTIVES OF THE STUDY

The present study was undertaken for –

1. Chemical characterization of *Aegle marmelos*.
2. To check antimicrobial activity of extracts.
3. Thin layer chromatography and High performance liquid chromatography.
4. Standardization and micropropagation protocol.

CHAPTER 5

MATERIAL AND METHOD

Laboratory Equipments-

(a). Glass wares, apparatus and instruments-Conical flasks, Beakers, Test tubes, Micropipettes, Measuring cylinders, Petri dishes, Scissors, Scalpels, Blade, Forceps Aluminium foil, PH meter, Weighing Balance, Autoclave, Incubators, Pestle Mortar, Laminar air flow, Freezer, Whatman Filter Paper, Microwave oven, Hot Plate, TLC plates.

(b). Chemicals required- All the chemicals and solvents used during the project work were of high purity obtained from different companies. Chemicals used for chemical characterization and micro propagation are- Methanol, Petroleum ether, Chloroform, hydrochloric acid, Mayer's Reagent, Wagner's reagent, Dragendorff's reagent, ferric chloride, Ammonium hydroxide solution, Teepol solution, Sucrose, mercuric chloride, agar, M S medium (Murashige and Skoog), 2,4-D , BA ,IAA, IBA, Folin-ciocalteu's phenol reagent, Sodium carbonate, Gallic acid, Quercetin, silica gel G, toluene, ethyl acetate and formic acid.

Material required

The leaves and bark of *Aegle marmelos* were collected in September from Nawanshahar, Punjab, India. Pathological disorders and contamination of plants were checked after washing with distilled water.

Preparation of Leaf extracts

The fresh leaves and bark of bael were collected and dried under shade. These shade dried leaves and bark were grinded in electrical grinder. 10 grams of powdered plant part was dissolved in 4 different solvents i.e. petroleum ether, distilled water, methanol, chloroform (100 ml each) (Fig 3). These flasks were kept in orbital shaker at 85 rpm for 24 hours and then centrifuged at 5000 rpm for 20 minutes. The extracts were filtered using whatmann filter paper 1 and clear supernatant were collected in beakers

Fig. 3 leaf and bark powder in different solvents i.e. Petroleum ether, distilled water, methanol and chloroform.

Fig. 4 Leaf and bark extracts on hot plate to make the extract free from solvents. (Beakers 1, 2, 3, 4, 5, 6, 7 and 8 are having leaf extract in petroleum ether, water, methanol, chloroform and bark extract in petroleum ether, water, methanol, chloroform respectively).

These extracts were concentrated by keeping the beakers on hot plate at 80^0C as shown in Fig. 4. These extracts were heated till they became solvent free. The solvent free extracts were weighed and stored at 4^0C in refrigerator for further analysis.

For Chemical Characterization- All the plant extracts were subjected to phytochemical analysis. All the extracts were analyzed for the presence of phytochemicals like alkaloids, phenols, saponins flavonoids and reducing sugars.

Test for alkaloids-

In solvent free extract 5 ml of dilute hydrochloric acid was added and mixed properly and then this extract was filtered using filter paper 1. The filtrate obtained was used for detection of alkaloids using alkaloidal reagents as follows-

(a)Wagner's test

1 ml of filtrate was taken and few drops of Wagner's reagent were added. Formation of red brown precipitates was observed.

(b) Dragendorff's test

1 ml of filtrate was taken and few drops of Dragendorff's reagent were added. Formation of yellow colored precipitations was observed.

Test for phenolic compounds

5 ml distilled water was dissolved in the solvent free extract. 100µl of ferric chloride (5%) solution was added. Formation of dark green color was observed.

Test of saponins

10 ml of distilled water in added in 2ml of extract. And shake the test tube for 10 minutes. Formation of layer of foam at the top of mixture was observed.

Test of Flavonoids

Aqueous solution of extract (1 ml) is added with 5ml of ammonia solution (10%). Formation of yellow colored fluorescence was observed.

Test of reducing sugars

The presence of reducing sugars was checked using Fehling solution A & B. 1 ml of mixture of Fehling solution A & B was added to extracts .Brick red colored were observed which shows the presence of reducing sugars in extract.

Total phenolic content in crude extracts-

1 ml crude extract were added to 5 ml distilled water and 2 ml of Folin-ciocalteu's phenolic reagent (diluted with distilled water in the ratio 1:1). Mix them and keep at room temperature for 5 min. In this mixture 2 ml sodium carbonate (20% in 0.1 N NaOH Solution). Keep them at water bath for 30 min. Cool the test tubes and check the absorbance at 680 nm in spectrophotometer. Gallic acid is used as standard for calibration of curve.

Total flavonoid content in crude extracts –

Take 0.5 ml of plant extract and mix it with 1.5 ml of methanol and add 0.1 ml of 10% aluminium chloride and mix it. Now add 0.1 ml of potassium acetate (1M). Now make the final volume of tubes by adding 2.8 ml of distilled water. Incubate these tubes to 37^{o}C for 30 min. The observation of reaction mixture was measured at 415nm. Quercetin was used for plotting calibration curve with its various concentrations.

Antibacterial activity –

Disc diffusion method-to study the antimicrobial activity of plant extracts, agar diffusion method was used against different microorganisms. In this method the pure culture of microorganism is sub cultured nutrient broth and is kept for incubation at 37^{o}C and the stock culture is revived. For preparation of discs (6 mm) Whatman's filter paper was used. These disc were left to dry under laminar air flow in different plant extracts overnight. Gentamycin in concentration 0.1 mg/ml was used as standard to check antimicrobial activity. For negative control the discs were prepared in respective solvent. To check the antimicrobial activity the media used is Muller Hilton agar. The test organisms were spread over the solidified Muller Hilton agar plate. Then the discs already prepared in different extracts were positioned on MH Agar media. . Then these plates were kept in microbiological incubator at 37^{o}C for 24 hours. Antimicrobial activity was interpreted by the size of diameter of zone of inhibition i.e the clear area around the disc

For Micropropagation-

Media preparation-MS (Murashige and Skoog) Media containing 3% (w/v) sucrose was prepared in autoclaved distilled water. Plant growth hormones like 2, 4-D and BA was added in media for callus induction. The pH of media was 5.8 using 0.1N NaOH and 0.1 N HCl. After this, agar (gelling

material) was added in the concentration of 0.8% (w/v). This media was then autoclaved at 121^{o}C temperature, 15psig pressure for 15 minutes.

Washing of explants- For micropropagation branches of bael were taken and then leaves were removed. The nodal segment was washed with water thoroughly for 15 minutes. These nodal explants were further washed with teepol solution for 5 minutes and then washed with distilled water. These washed nodal explants were kept in laminar flow to provide aseptic conditions. For the surface sterilization nodal explants were treated with 0.1% $MgCl_2$ solution for 5 minutes. Then it is washed with sterile distilled water for 3-4 times.

Inoculation of explants in media for callus induction- For callus induction MS media was supplemented with combination of two growth hormones i.e. 2,4-D (1.5 mg/l) + BA (0.5 mg/l). Under laminar air flow the media was solidified and the washed explants were inoculated in the media. These culture tubes were kept at particular photoperiod for callus induction.

Media for shoot generation- After about 40 days the green colored callus was induced from the nodal explant. This callus was then sub cultured in new media having different concentrations of growth hormone. For shoot generation, MS media was supplemented with BA (1.5 mg/l).

Thin layer chromatography-

Preparation of TLC plates- To perform TLC the Silica Gel G was used as solid phase. Slurry of silica gel was prepared using distilled water so that it can be easily poured over the glass plate. 20cm * 5 cm glass slides were taken and the slurry was poured over the plates. The prepared plates were allowed to set or dry at room temperature. Then the dried plates were put into hot air over for 1 hr at 110^{o} C so that plates get activated. Mark a line with pencil 1 cm above the bottom of the plate and make a spot of extract with the help of small capillary tube.

Preparation of mobile phase - Three different solvents were used to make the mobile phase. Ethyl acetate, toluene and formic acid were taken in ratio 12:36:5 respectively. Solution was kept in TLC chamber to saturate it.

Sample spotted TLC plates were put in chamber and it was allowed to run 2/3 rd of the plate height. Plates were kept at room temperature to dry. Then these plates were observed under transeluminator. Different bands were observed and the distance travelled by solvent and sample was noted.

High performance liquid chromatography – Out of all the extracts, four extracts are having significant number of compounds. These four extracts are methanol and chloroform extract of leaf and bark. These extracts were sent in CDRI, Lucknow.

Chapter 6

Result and Discussion

In extraction of plant part i.e. leaves and bark, different amounts of crude extracts were obtained. It was observed that all the extracts in different solvents are having different yield of crud extract. The amount of crude extract of plant part in different solvents is shown in Table 1. In all the extracts the percentage extractive values were calculated using the following formula-

$$\text{Percent Extracts} = \frac{\text{Weight of dried extract}}{\text{Weight of leaf material}} \times 100$$

Table 1: Amount of crude extract of leaves and bark in different solvents.

Sample	S.No.	Solvent	Amount of crude extract (gms.)/10 gms	Percent extracts (%)
Leaf	1.	Petroleum ether	0.140 gms	1.40
	2.	Water	2.70 gms	27.0
	3.	Alcohol	1.208 gms	12.0
	4.	Chloroform	0.341 gms	3.41
Bark	5.	Petroleum ether	0.261 gms	2.61
	6.	Water	0.114 gms	1.14
	7.	Alcohol	0.180 gms	1.80
	8.	Chloroform	0.256 gms	2.56

From Table 1, it is clear that out of all the extracts, leaf in distilled water had given maximum yield (2.70 gms) of crude extract. For leaf extraction distilled water is the better solvent. After water, alcohol is good solvent but petroleum ether had given less amount (0.140 gms) of extract. For bark extraction petroleum ether (0.261 gms) and chloroform (0.256 gms) are good solvents as they had given much amount of extract than alcohol (0.180 gms) and water (0.114 gms). From this it is clear those leaves and bark contain certain compounds which can be extracted using specific solvent system.

Shailesh *et al.*, (2013) also found that in case of leaf, the water extract has high extraction percentage as compare to the chloroform extract.

Leaf and bark extracts in different solvent i.e. petroleum ether, distilled water, methanol and chloroform were checked for the presence of phytochemicals. Phytochemical tests performed indicated the presence of different classes of secondary metabolites like alkaloids, saponins, flavonoids and reducing sugars etc. It shows that for the isolation of specific secondary metabolite specific solvent should be used. Plants are an important source of active natural products and these products differ widely in their structures, biological properties and mechanisms of actions.

Test for alkaloids- All the extracts were tested for presence of alkaloids. The observation and inference of all the extracts are shown in Table 2.

Table 2: Chemical tests to check the presence of alkaloids in different extract of Aegle *marmelos.*

Plant part	Sample No.	Solvent used for extraction	Observation for Wagner test	Inference	Observation for Dragendorff test	Inference
LEAF	1.	Petroleum ether	Red brown ppts	Present	Yellowish ppts	present
	2.	Distilled water	Red brown ppts	Present	Yellowish ppts	present
	3.	Methanol	Red brown ppts	Present	Yellowish ppts	present
	4.	Chloroform	Red brown ppts	Present	Yellowish ppts	Present
BARK	5.	Petroleum ether	Red brown ppts	Present	Yellowish ppts	Present
	6.	Distilled water	Red brown ppts	Present	Yellowish ppts	Present
	7.	Methanol	Red brown ppts	Present	Yellowish ppts	present
	8.	Chloroform	red brown ppts	Present	Yellowish ppts	Present

From Table 2, it is clear that all the extracts are having alkaloids. Alkaloid test was performed using two different reagents and these reagents were Wagner's reagent and Dragendorff's reagent. Wagner's reagent gave red brown colored precipitates which indicate the presence of alkaloids. Dragendorff test gives yellowish precipitates on reaction with alkaloids present in sample. Using these reagents all the extracts was tested and it was observed that all the extracts are having alkaloids

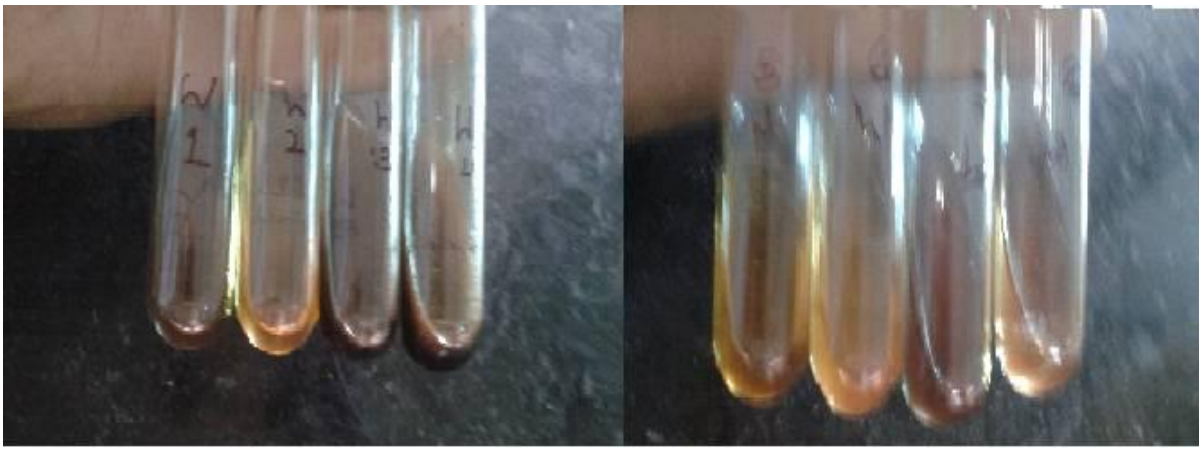

Fig. 5 Test to check the presence of alkaloids (Wagner test) (Tube 1, 2, 3, 4, 5, 6, 7 and 8 are leaf extract in petroleum ether, water, methanol, chloroform and bark extract in petroleum ether, water, methanol, chloroform respectively).

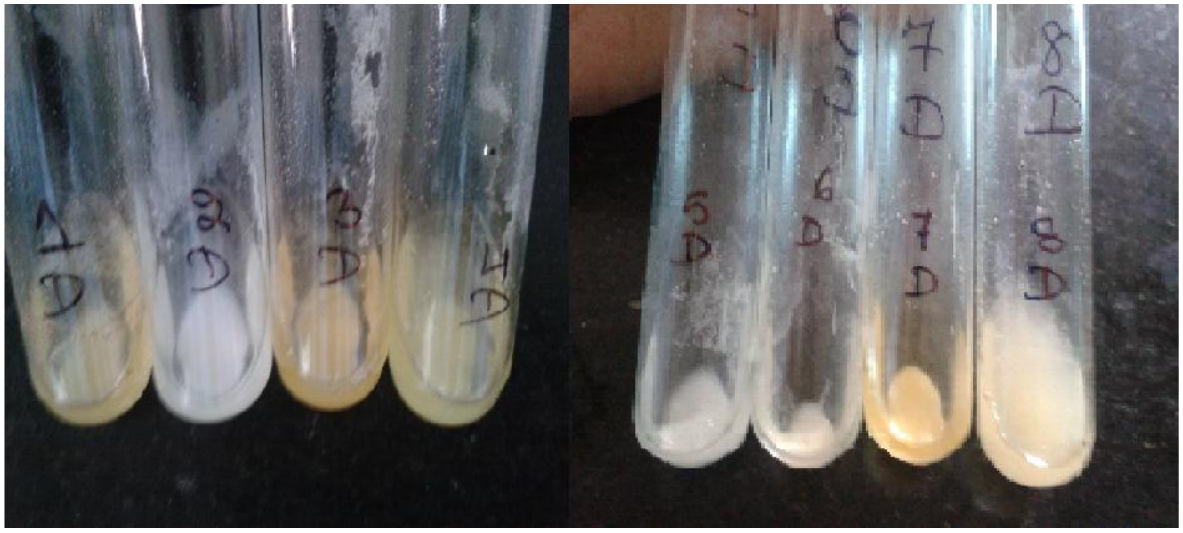

Fig. 6 Test to check the presence of alkaloids (Dragendorff test). (Tube 1, 2, 3, 4, 5, 6, 7 and 8 are leaf extract in petroleum ether, water, methanol, chloroform and bark extract in petroleum ether, water, methanol, chloroform respectively).

Mariya *et al.*, (2013) also found that water, methanol and chloroform extract of leaves are having alkaloids.

Test for phenolic compounds- All the leaf and bark extracts were tested for the presence of phenolic compounds. The observations and inference are shown in Table 3.

Table 3: Chemical tests to check phenolic compounds in different extract of Aegle *marmelos.*

Plant part	S No.	Solvent for extraction	Observation	Inference
Leaves	1.	Petroleum ether	Green color	Present
	2.	Distilled water	Green color	Present
	3.	Methanol	Green color	Present
	4.	Chloroform	Green color	Present
Bark	5.	Petroleum ether	No green color	Absent
	6.	Distilled water	No green color	Absent
	7.	Methanol	Green color	Present
	8.	Chloroform	Green color	Present

From Table 3 it is clear that phenolic compounds were present in some extracts but phenols were absent in other extracts. In case of leaves, the phenolic compounds were observed in petroleum ether, water, methanol and chloroform extract. Similarly in case of bark, phenols were observed in methanol and chloroform extract. In petroleum ether and water extract of bark phenols were not observed.

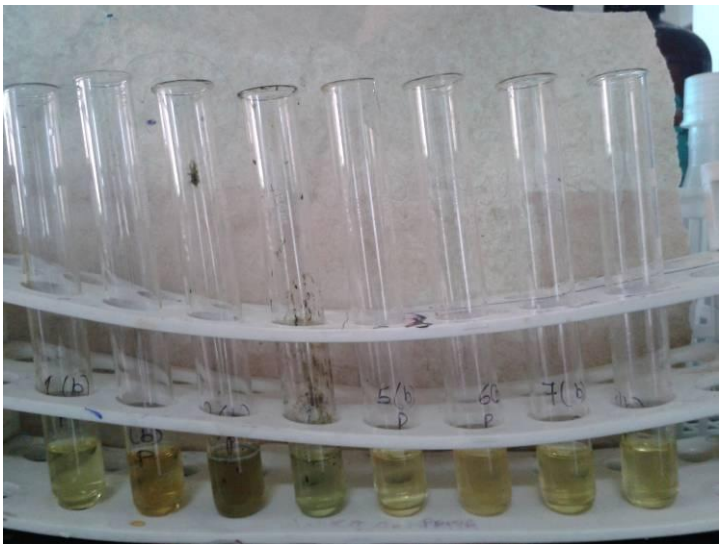

Fig. 7 Test to check the presence of phenolic content in plant extracts (Tube1, 2, 3, 4, 5, 6, 7, 8 are leaf extract in petroleum ether, water, methanol, chloroform and bark extract in petroleum ether, water, methanol, chloroform respectively).

Saroj *et al.*, (2011) also found that leaf extract in petroleum ether, methanol and chloroform contain phenolic compounds. **Shailesh *et al.*, (2013)** found that phenols are present in water extract of bael. **Mariya *et al*., (2013)** also observe the same results for water, methanol and chloroform extract of leaves.

Test of saponins- All the extracts of leaf and bark were tested to check the presence of saponins. Saponins forms a layer of foam on shaking continuously. The observations and inference of saponins is shown in Table 4.

Table 4: Chemicals test to check the presence of saponins in plant extracts.

Plant part	S No.	Solvent used for extraction	Observation	Inference
Leaf	1.	Petroleum ether	No foam formation	Absent
	2.	Distilled water	Foam formation	Present
	3.	Methanol	Foam formation	Present
	4.	Chloroform	No foam formation	Absent
Bark	5.	Petroleum ether	Foam formation	Present
	6.	Distilled water	Foam formation	Present
	7.	Methanol	Foam formation	Present
	8.	Chloroform	Foam formation	Present

From Table 4, it is clear that saponins are present in most of the extracts but absent in petroleum ether and chloroform extract of leaf. In case of bark saponins were observed in all the extracts i.e. petroleum ether, water, methanol and chloroform extract.

Saroj *et al.*, (2011) also observed the same result for leaf extracts. They observed that saponins are absent in petroleum ether and chloroform extract of leaves and saponins are present in water and methanol extract of leaf.

Fig 8- Test to check the presence of saponins in leaf extracts. (Tube 1, 2, 3, 4 are leaf extract in petroleum ether, water, methanol, chloroform respectively).

Fig 9- Test to check the presence of saponins in Bark extracts. (Tube5, 6, 7 and 8 are bark extract in petroleum ether, water, methanol, chloroform respectively.)

Test of reducing sugars- All the leaf and bark extracts were tested to check the presence of reducing sugars. For this Fehling solution A and B were used. The observation and inference for reducing sugars is shown in Table 5.

Table 5: Chemical test to check the presence of reducing sugars in extracts.

Plant part	S No	Solvent used for extraction	Observation	Inference
Leaf	1.	Petroleum ether	No precipitate	Absent
	2.	Distilled water	Brick red precipitate	Present
	3.	Methanol	Brick red precipitate	Present
	4.	Chloroform	No ppts	Absent
Bark	5.	Petroleum ether	Brick red precipitate	Present
	6.	Distilled water	Brick red precipitate	Present
	7.	Methanol	Brick red precipitate	Present
	8.	Chloroform	Brick red precipitate	Present

From Table 5, it is clear that reducing sugars are present in petroleum ether and water extract of leaves but reducing sugars were absent in methanol and chloroform extract of leaf. Reducing sugars are observed in all the four extracts of bark. Layer of foam was observed in petroleum ether, water, methanol and chloroform extract of bark.

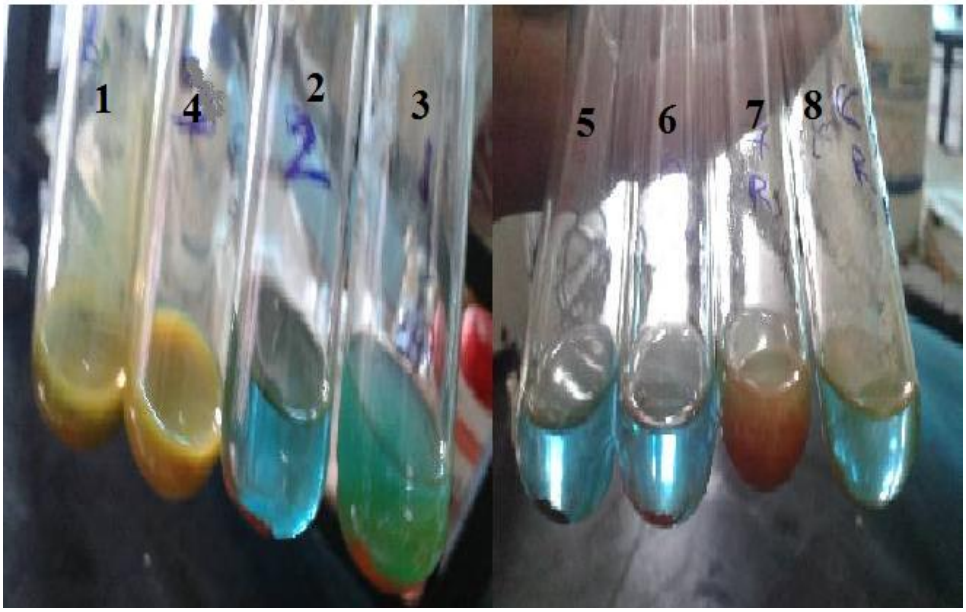

Fig. 10 Test to check the presence of reducing sugars. (Tube 1, 2, 3, 4, 5, 6, 7, 8 are leaf extract in petroleum ether, water, methanol, chloroform and bark extract in petroleum ether, water, methanol, chloroform respectively).

Vanitha *et al*., (2012) had also observed the same results for leaf extracts. They observed that reducing sugars are present in water and methanol extract of leaf and petroleum ether. But reducing sugars are absent in petroleum ether and chloroform extract.

Phenolic and Flavonoid content –

Antioxidant activity is mainly shown because of the presence of phenolic contents in plant extracts. Total phenolic content was checked in leaf and bark extract with different solvents like petroleum ether, distilled water, methanol and chloroform. The absorbance of unknown samples were taken and calibrated in standaerd curve to check phenolic content in samples. Standard graph of gallic acid is shown in Fig 11. Standard graph of gallic acid is having regression value, R^2= 0.996, y= 0.012x + 0.002. The amount of phenols in leaf and bark extract is shown in Table 6. In methanol extract of leaf the amount of phenol is maximum i.e. 66.4 mg/grams of dried leaves. In chloroform extract of leaf minimum amount of phenols was observed i.e. 16 mg/grams of dried leaves. In case of bark, methanol extract is having high amount (54.8 mg/g) of phenols. In bark, petroleum ether was having minimum amount (12.8 mg/g) of phenols. **Tupe *et al*., (2012)** also found that total phenolic content in methanolic extract is 63.01 mg GAE/ gm.

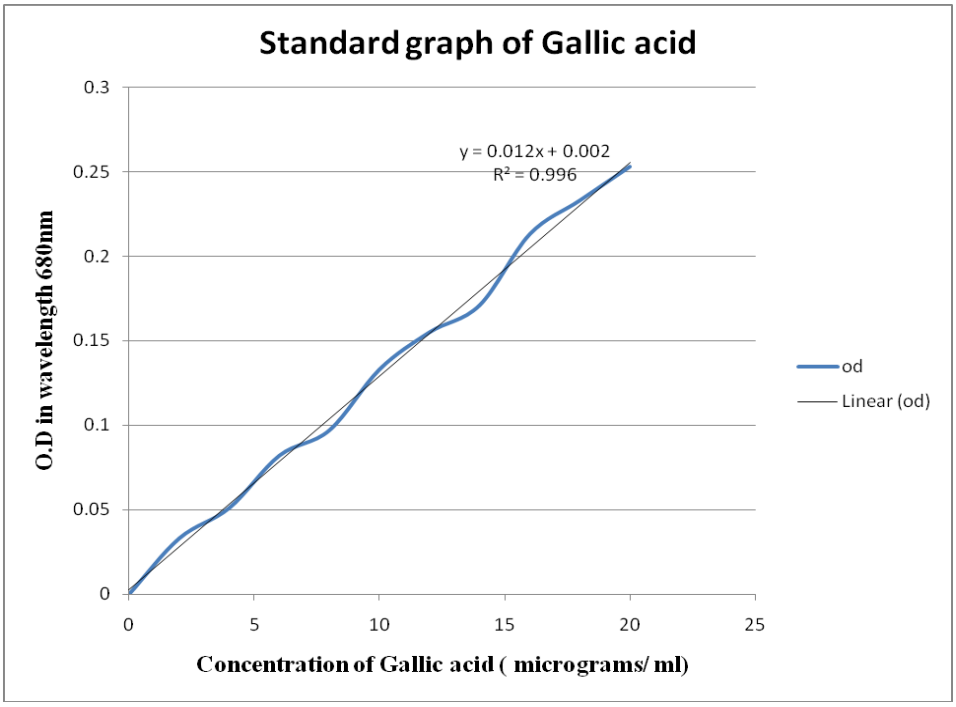

Fig. 11 Gallic acid standard curve for estimation of phenolic content

Table 6: Total phenolic and flavonoid content of different extracts of bael plant

Plant Part	S. No.	Solvent used	Total phenolic compound (TPC) mg/g	Total flavonoid compound (TFC) mg/g
Leaf	1.	Petrolem ether	32	77
	2.	Distilled water	48	7
	3.	Methanol	66.4	98
	4.	Chloroform	16	241
Bark	5.	Petrolem ether	12.8	1.5
	6.	Distilled water	30.4	5
	7.	Methanol	54.8	55.5
	8.	Chloroform	26.4	73.5

Total flavonoid content estimation-

Flavonoids are having hydroxyl groups that are responsible for radical scavenging activity in plants. Standard graph for flavonoid content is shown in Fig 12.

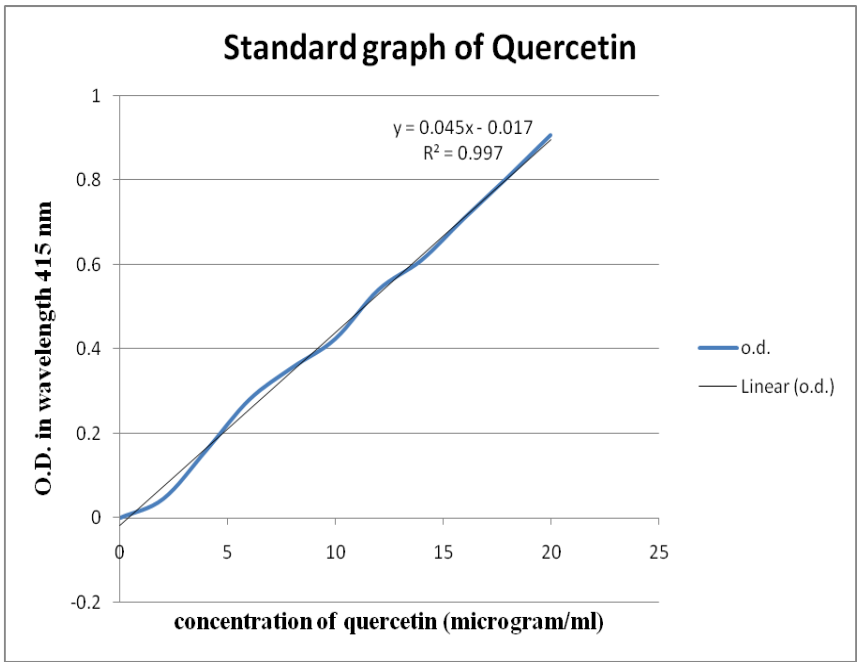

Fig. 12 Quercetin standard curve for estimation of flavonoid contents.

By calibrating the O.D of unknown samples in standard graph of flavonoids the amount of flavonoids in unknown samples was calculated. It was found that chloroform extract of laves was having maximum amount (241 mg/g) of flavonoids. Minimum amount of flavonoids was found to be in petroleum ether extract (1.5 mg/g) of bark. High amount of flavonoids was found in leaf extracts. In bark extract flavonoids were present in less amount as compare to the leaf extract. Overall, the extracts are having high amount of flavonoids as compare to the phenolic compounds.

Antimicrobial activity

The antimicrobial activity of petroleum ether, distiller water, methanol and chloroform extract of leaf and bark was studied by disc diffusion method. Test organisms for antimicrobial activity are *E. coli, Bacillus cereus, Bacillus subtilis, Staphylococcus pneumonia.* The activity of extract against a particular microorganism is calculated by the diameter of zone of inhibition. Higher diameter of zone of inhibition means the extract is more effective against that microorganism. No zone of inhibition means the microorganism to be tested is resistant against that extract. Gentamycin is an antibiotic which is used as standard to check the antimicrobial activity of plant extracts. Effect of different plant extracts on *E. coli* is shown in Fig 14.

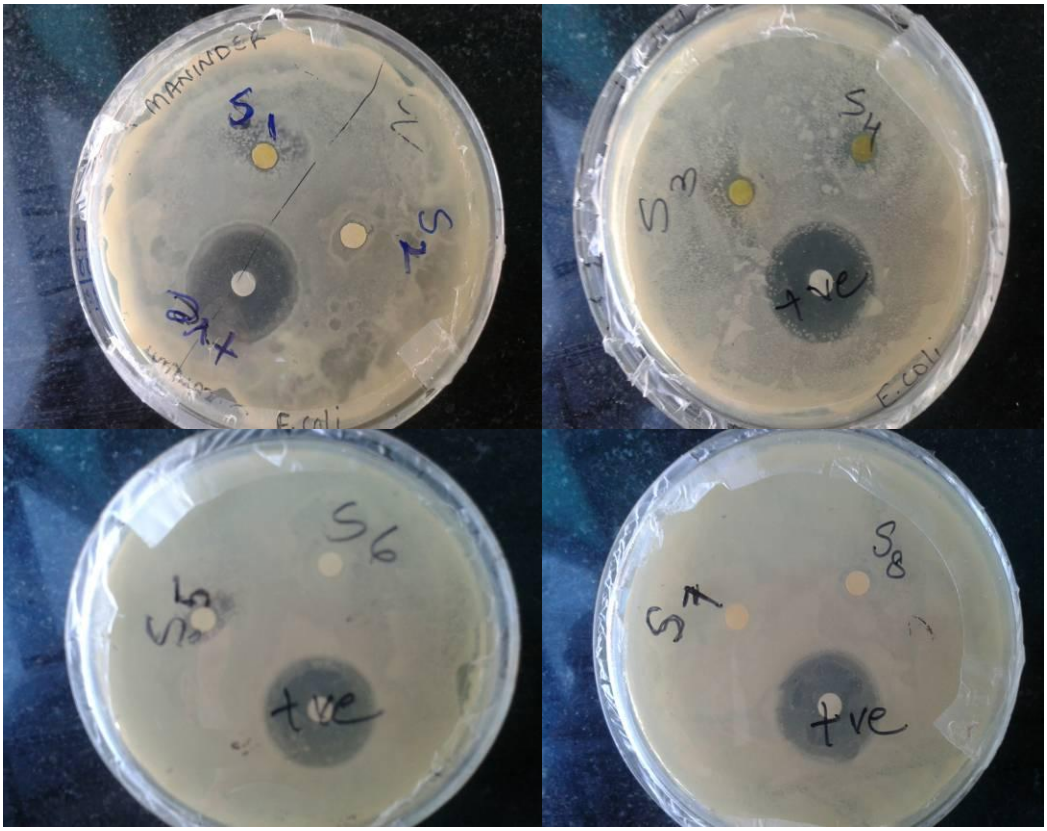

Fig. 13 Effect of leaf and bark extract on *E. coli* (S1-lesf+petroleum ether, S2- leaf+water,S3 – leaf + methanol, S4 – leaf +chloroform, S5- bark+ petroleum ether, S6- bark + water , S7 – bark+ methanol, S8- bark +chloroform).

From Fig 13 it is clear that maximum activity against *E. coli* is shown by bark extract in petroleum ether and chloroform solvents. There was no activity in water and methanol extract of bark. Leaf extract in petroleum ether, water, methanol and chloroform had shown the antimicrobial effect. In case of petroleum ether and chloroform extract the zone of inhibition was found to be 10 mm. In Gentamycin the zone of inhibition was having diameter of 26mm. When compared with the standard, the petroleum ether and chloroform extracts had shown 38.46 % inhibition. Whereas the water and methanol extracts had not shown any activity against *E. coli*.

Table 7: Effect of different extracts on *E. coli* and *Bacillus subtilis* bacteria

Plant part	S. No.	Solvent for extraction	Diameter of zone of inhibition in *E. coli* (in mm)	Percentage inhibition	Diameter of zone of inhibition in *Bacillus subtilis* (in mm)	Percentage inhibition
Leaf	1.	petroleum ether	8 mm	30.76%	11 mm	44 %
	2.	Distilled water	9 mm	34.61%	8 mm	32 %
	3.	Methanol	8 mm	30.76	8 mm	32 %
	4.	Chloroform	7.5 mm	28.84%	11 mm	44 %
Bark	5.	petroleum ether	10mm	38.46%	-----	----
	6.	Distilled water	-----	-----	-----	-----
	7.	Methanol	-----	------	-----	------
	8.	chloroform	10 mm	38.46%	8 mm	32 %
Gentamycin			26mm		25 mm	

Antimicrobial activity against *Bacillus subtilis* was checked using same method. It was found that petroleum ether and chloroform extract of leaves had shown maximum activity against *Bacillus subtilis*. The diameter of zone of inhibition in case of petroleum ether and chloroform extract of leaves was 11 mm. The diameter of zone of inhibition in standard was 25 mm. When compared with standard the petroleum ether and chloroform extracts shown 44% antimicrobial activity against *Bacillus subtilis.* But in case of bark, only the chloroform extract had shown antimicrobial activity against *Bacillus subtilis.* Chloroform extract of bark had shown 32% antimicrobial activity against *Bacillus subtilis.* *Bacillus subtilis* had shown resistance against petroleum ether, methanol and chloroform extract of bael.

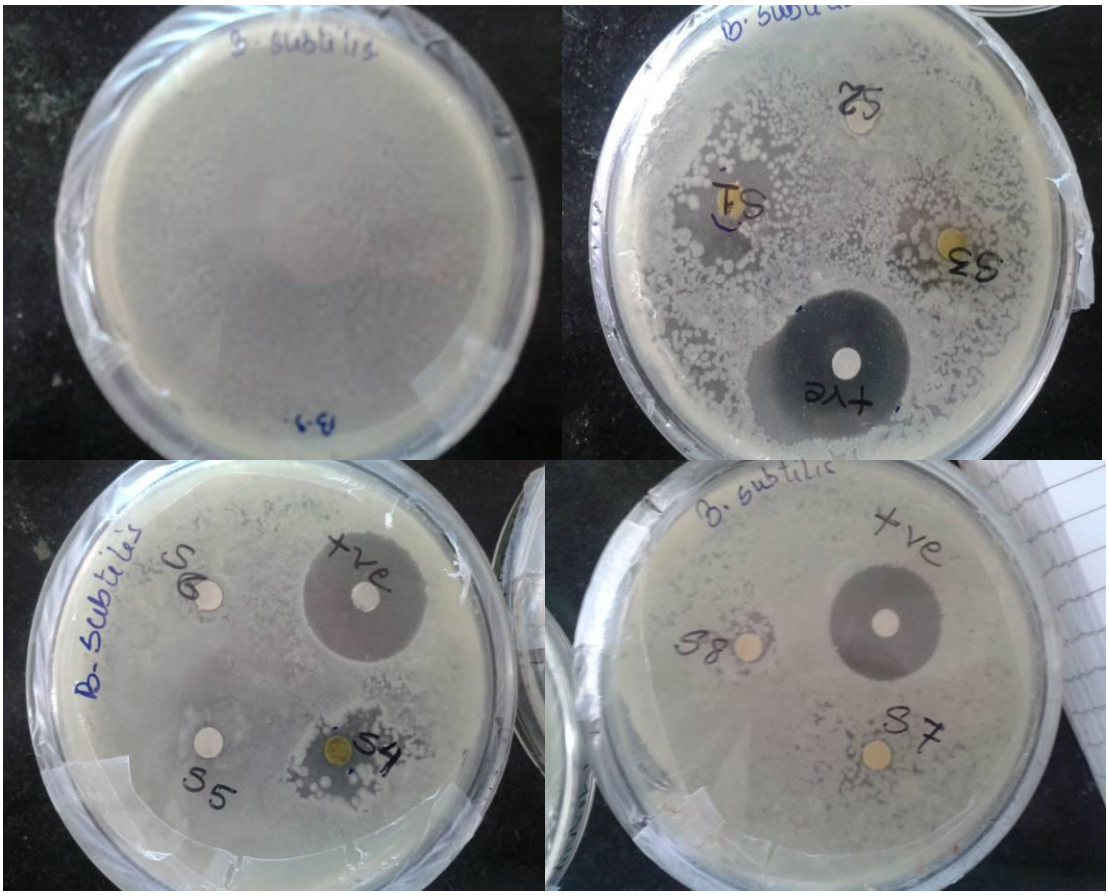

Fig. 14 Effect of leaf and bark extract on *Bacillus subtilis*(S1-lesf+petroleum ether, S2- leaf+water,S3 – leaf + methanol, S4 – leaf +chloroform, S5- bark+ petroleum ether, S6- bark + water , S7 – bark+ methanol, S8- bark +chloroform).

Antimicrobial activity against *Staphylococcus pneumonia* was checked using disc diffusion method. It was found that maximum activity against *Staphylococcus pneumonia* was shown by the petroleum ether extract of leaf. The zone of inhibition of leaf extract in petroleum ether was 15 mm. This zone of inhibition was compared with the standard and it was found that the extract had shown 71.42 % antimicrobial activity against *Staphylococcus pneumonia*. Leaf extract in methanol and bark extract in petroleum ether had shown minimum activity against *Staphylococcus pneumonia* . In both the cases the zone of inhibition was having diameter 8mm. leaf in methanol and bark in petroleum ether had shown 38.09% antimicrobial activity. *Staphylococcus pneumonia* was found to be resistant against water extract of leaves and bark. No zone of inhibition was observed in water extract of leaves and bark.

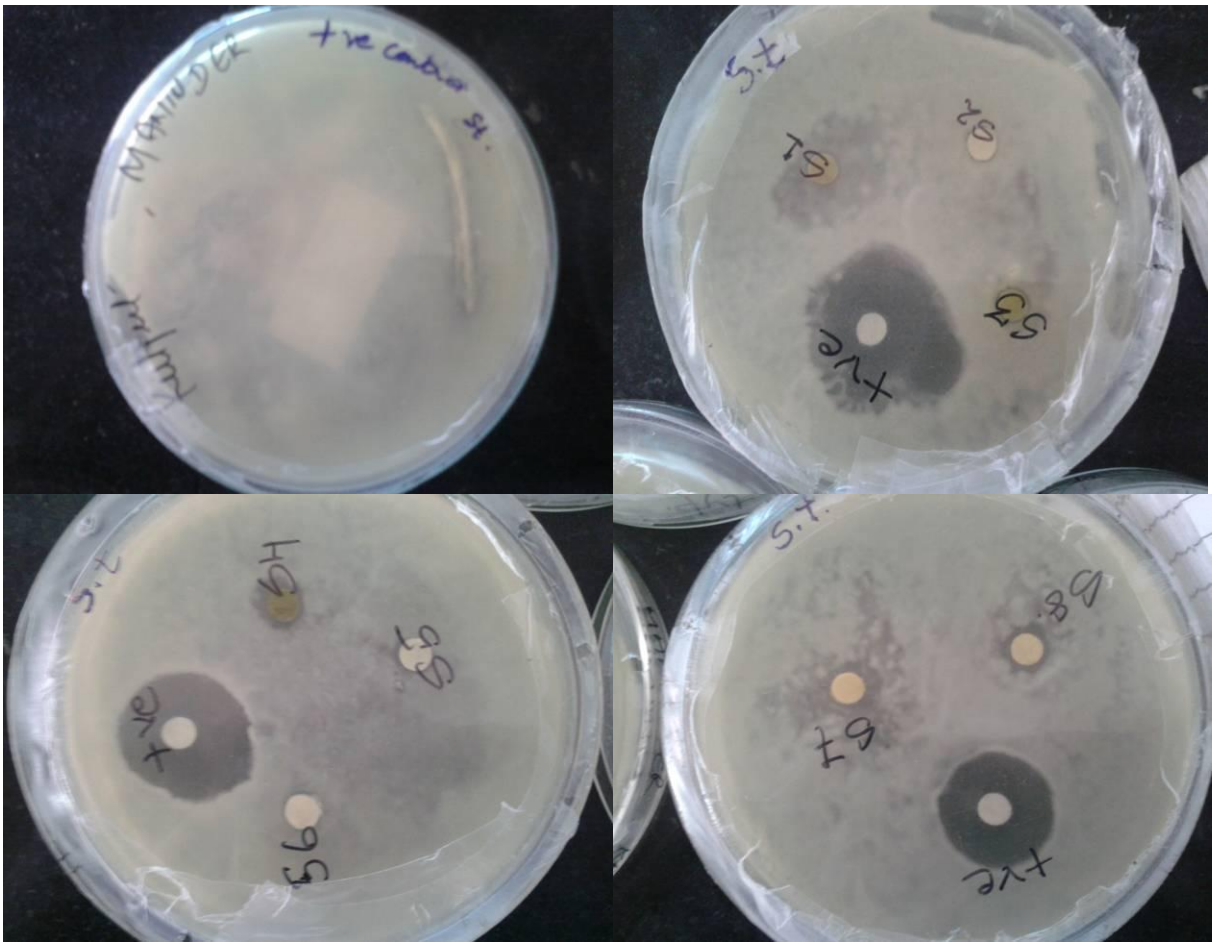

Fig. 15 Effect of leaf and bark extract on *Staphylococcus pneumonia* (S1-lesf+petroleum ether, S2-leaf+water,S3 – leaf + methanol, S4 – leaf +chloroform, S5- bark+ petroleum ether, S6- bark + water , S7 – bark+ methanol, S8- bark +chloroform

Table 8: Effect of different extracts on *Staphylococcus pneumonia* and *Bacillus cereus*

Plant part	S. No.	Solvent for extraction	Diameter of zone of inhibition in *Staphylococcus pneumonia* (in mm)	Percentage inhibition	Diameter of zone of inhibition in *Bacillus cereus* (in mm)	Percentage inhibition (%)
Leaf	1.	petroleum ether	15mm	71.42%	9 mm	37.5
	2.	Distilled water	-----	-----	11 mm	45.83
	3.	Methanol	8 mm	38.09%	-----	-----
	4.	Chloroform	9 mm	42.85%	13 mm	54.16
Bark	5.	petroleum ether	8 mm	38.09%	15 mm	62.5
	6.	Distilled water	-----	----	12 mm	50
	7.	Methanol	12 mm	57.14%	16 mm	66.66
	8.	Chloroform	9 mm	42.85%	8mm	33.33
Gentamycin			21 mm		24 mm	

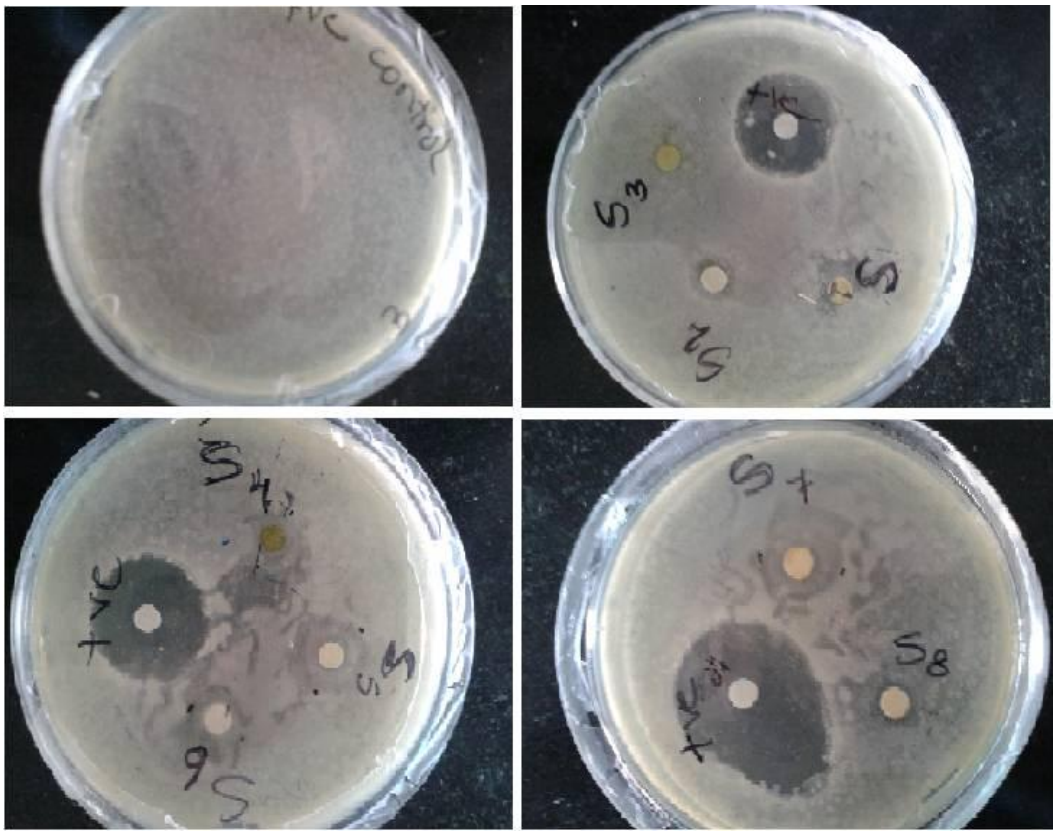

Fig. 16 Effect of leaf and bark extract on *Bacillus cereus* (S1-leaf+petroleum ether, S2- leaf+water,S3 – leaf + methanol, S4 – leaf +chloroform, S5- bark+ petroleum ether, S6- bark + water , S7 – bark+ methanol, S8- bark +chloroform

Antimicrobial activity was checked against the microorganism *Bacillus cereus*. Maximum zone of inhibition was observed in case of methanol extract of bark with the diameter of zone of inhibition 16 mm. When compared with the standard having zone of inhibition 24 mm it was found that the bark extract in methanol had shown 66.66% inhibition. Bark extracted in chloroform had shown minimum activity against *Bacillus cereus* with zone of inhibition f 8mm. when compared with the standard the extract was found to be having 33.33% inhibition. Methanol extract of leaves had not shown any zone of inhibition. It indicates that *Bacillus cereus* is resistant against methanol extract of leaves. All the bark extracts in petroleum ether, water, methanol and chloroform had shown antimicrobial activity.

From this, it is clear that maximum activity against *E. coli* is shown by the petroleum ether and chloroform extract of bark. For *Bacillus subtilis* maximum activity was observed by petroleum ether and chloroform extract of leaves. For *Staphylococcus pneumonia*, leaves extracted in petroleum ether had shown more activity. For *Bacillus cereus*, bark extracted in methanol had shown maximum activity.

Micropropagation-

After about 40 days of inoculation, green colored callus was observed and this callus was used for the purpose of sub culturing. The callus induced from axillary nodal explant is shown in Fig 17.

Fig. 17 Formation of green colored callus from nodal explant.

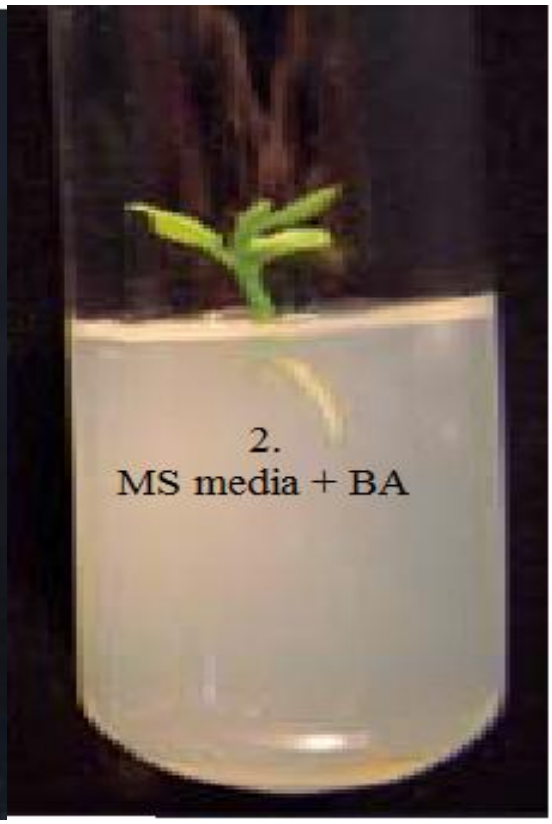

Fig. 18 formation of shoots from callus

This callus was further sub cultured in MS media having benzlyaminopurine (BA). BA is used to initiate shoot formation from the callus. After 4 weeks of subculturing, shoots were observed. In Fig 18, shoots can be seen growing on MS media having BA in appropriate concentration.

TLC Analysis-

When seen under UV different bands were observed and the distance travelled by the solvent and bands of sample was noted. Then Rf value of all the extracts was calculated. The Rf value of all the extracts is shown in Table 9.

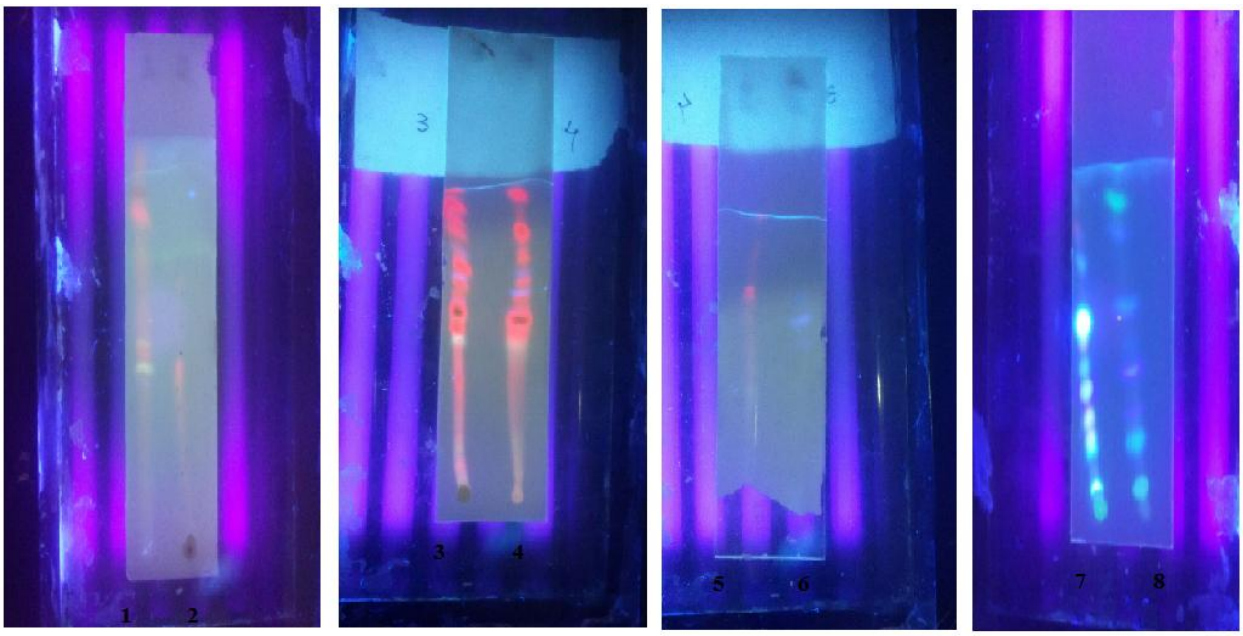

Fig. 19 TLC analysis of different extracts of *Aegle marmelos.* (1-leaf+petroleum ether, 2- leaf+ water, 3 – leaf + methanol, 4 – leaf +chloroform, 5- bark+ petroleum ether, 6- bark + water , 7 – bark+ methanol, 8- bark +chloroform).

From Fig. 19 it is clear that samples 3, 4, 7 and 8 are having more compounds as compare to 1, 2, 3 and 4. In petroleum ether and water extract of leaf and bark, less number of bands was observed. In methanol and chloroform extract of leaf and bark, more number of bands was observed. The Rf values of these extracts were compared with the Rf values of standard and these extracts were analyzed for the presence of different compounds.

Rf values of the extracts were calculated using the formula-

$$\text{Rf value} = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

Table 9: Rf values found in different extracts of *Aegle marmelos* and compounds present in extracts.

Plant part used	Sample no.	Solvent used	Rf values	Name of the compound present in extract
Leaves	1.	Petroleum ether	0.44, 0.77	Apigenin, 3Hydroxyflavone
	2.	Distilled water	0.56,0.67,0.76	Ferulic acid, 6 -Hydroxyflavone, 3-Hydroxyflavone
	3.	Methanol	0.51,0.56,0.62,0.65,0.77,0.88	Kaempferol, Ferulic acid, Chrysin, Galangin, 3-Hydroxyflavone, Flavone
	4.	Chloroform	0.46,0.55,0.62,0.65,0.67,0.88	7-Hydroxyflavone,o/p Coumaric acid, Chrysin, Galangin, 6-Hydroxyflavone, Flavone
Bark	5.	Petroleum ether	0.77, 0.88	3-Hydroxyflavone, Flavone
	6.	Distilled water	0.55	o/p Coumaric acid
	7.	Methanol	0.23,0.39,0.46,0.56,0.88	Morin, Quercetin, 7-Hydroxyflavone, Ferulic acid, Flavone
	8.	Chloroform	0.23,0.55,0.77, 0.88	Morin, o/p Coumaric acid, 3-Hydroxyflavone, Flavone

When these Rf values were compared with the standard it was found that the extracts contain different compounds like morin, Quercetin, apigenin, caffeic acid, Quercetin, apigenin, hydroxyflavone, dihydroxyflavone, coumaric acid, galangin, flavones etc. The compounds present in different extracts are shown in Table. 9. From Table 9, it is clear that sample 1(leaf in petroleum ether) contain apigenin and 3 hydroxyflavone. Sample 2(leaf in distilled water) contain ferulic acid, 3 hydroxyflavone and 6 hydroxyflavone. Sample 3 contain six compounds i.e. Kaempferol, ferulic acid, Chrysin, galangin, 3 hydroxyflavone, flavone. Sample 4 (leaf in chloroform) contain 7Hydroxyflavone,

o/p Coumaric acid**,** Chrysin, Galangin, 6Hydroxyflavone and Flavone. Sample 5 (bark in petroleum ether) contain 3Hydroxyflavone and Flavone only. Sample 6 (bark in water) is having o-Coumaric acid and p-coumaric acid. Sample 7 (bark in methanol) contain Morin, Quercetin, 7-Hydroxyflavone, Ferulic acid and Flavone. Sample 8 (bark in chloroform) is having Morin, ortho and para coumaric acid, 3Hydroxyflavone and Flavone.

It is clear that leaf extraction in methanol and chloroform is showing presence of many compounds. After these the bark extraction in methanol is giving many compounds as compare to bark in chloroform. For both leaf and bark methanol is giving good range of compounds. Te samples having good range of compounds were further used for HPLC analysis.

High performance liquid chromatography-

TLC of all the extracts gave an idea about the presence of phytochemicals present in the leaf and bark extracts. HPLC analysis of four extracts having more significant compounds was done. These four extracts were leaf extract in methanol and chloroform and bark extracts in methanol and chloroform. The standards used for this analysis were- Marmelosin, Marmesin, Quercetin and Kaempferol. HPLC graphs are shown in Fig. 20(leaf in methanol), Fig 21 (leaf in chloroform), Fig. 22(bark in methanol) and Fig. 23(bark in chloroform).

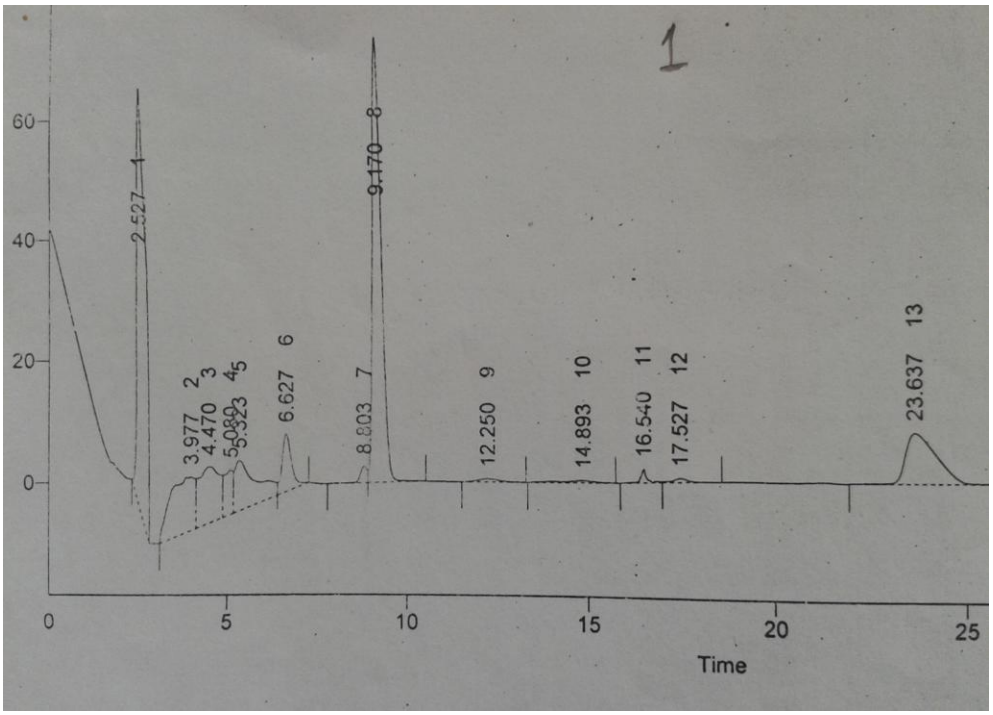

Fig. 20 HPLC analysis of leaf extract in methanol

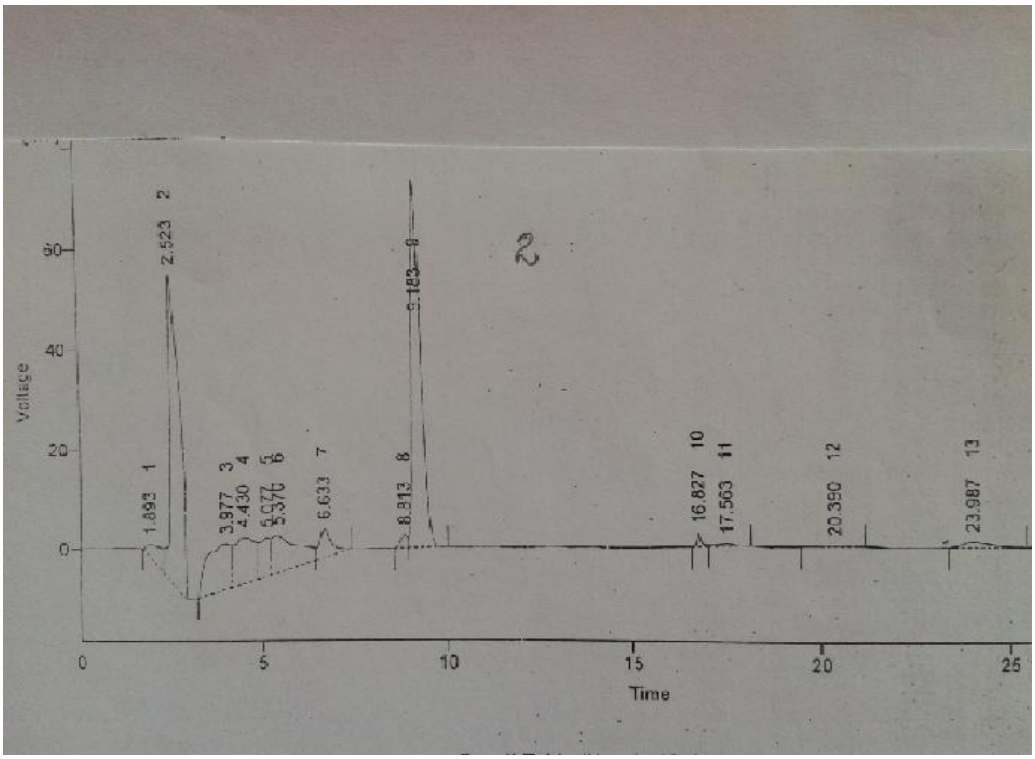

Fig 21 HPLC analysis of leaf extract in chloroform

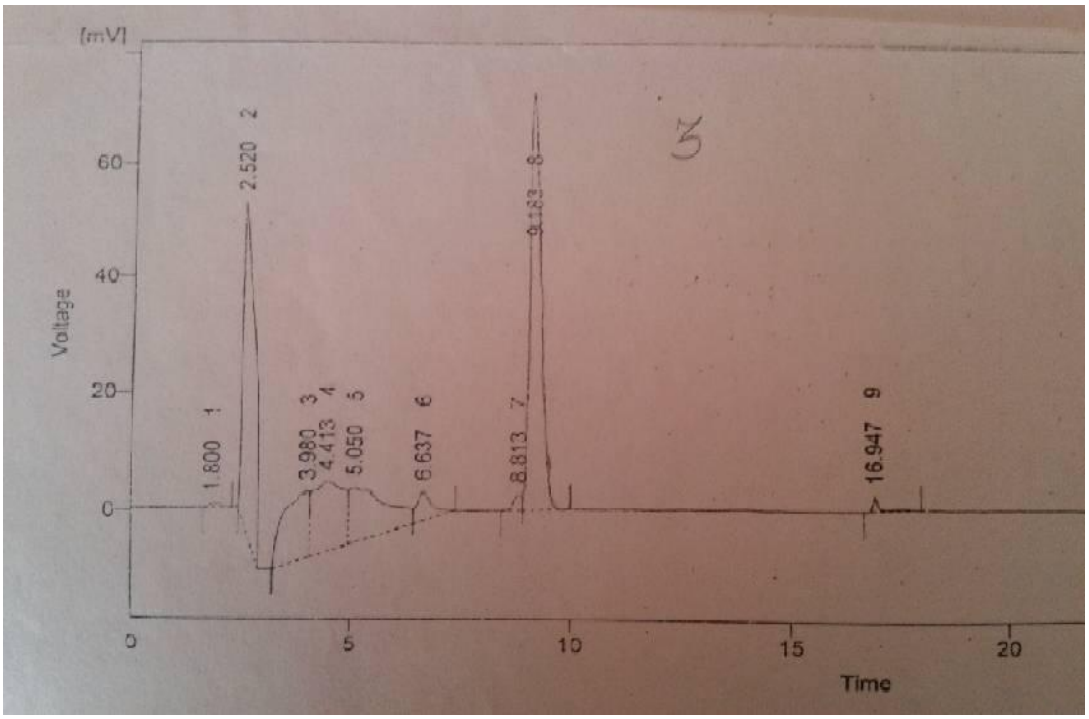

Fig. 22 HPLC analysis of bark extract in methanol

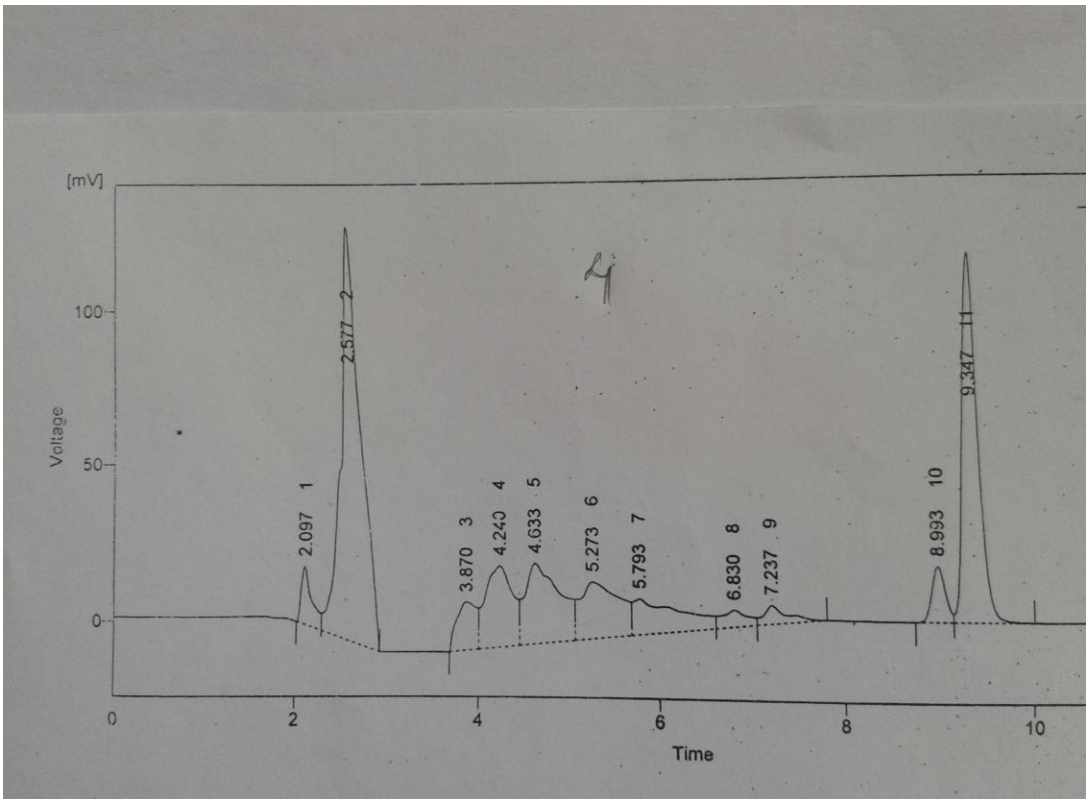

Fig. 23 HPLC analysis of bark extract in chloroform.

From Figs. ,20,21,22,23 it is clear that all the four extracts are having Marmelosin, Marmesin, Quercetin and Kaempferol. The amount of these extracts in sample is shown in Table 10.

Table 10: Amount of Marmelosin, Marmesin, Quercetin and Kaempferol in samples.

Sample No.	Plant part/solvent used	Marmelosin (mg/g)	Marmesin (mg/g)	Quercetin (mg/g)	Kaempferol (mg/g)
1.	Leaf / Methanol	67.5	80.0	40.2	11.5
2.	Leaf / Chloroform	65.5	78.2	20.4	10.5
3.	Bark / Methanol	46.5	50.1	15.7	8.5
4.	Bark / Chloroform	42.0	48.7	12.6	7.5

From Table 10, it is clear that Marmelosin is present in all the four extracts. Maximum amount of Marmelosin is observed in methanol extract of leaf (67.5mg/g). In case of leaf, methanol is having more Marmelosin as compared to the chloroform extract. In case of bark also the methanol extract (46.5 mg/g) is having high yield of Marmelosin then the chloroform extract. Marmesin is also present in all the four extracts but higher amount of Marmesin is observed in methanol extract of leaf. Methanol extract yield higher Marmesin (80 mg/g) as compare to the chloroform extract (78.2mh/g). Similarly, methanol extract of bark yield higher (50.1 mg/g) Marmesin as compare to chloroform extract (48.7 mg/g). Quercetin is present in high amount in Methanol extract of leaf (40.2 mg/g) and lowest mount was observed in bark extract in chloroform (12.6 mg/g). Kaempferol is also present in all the four extracts. Maximum amount was observed in methanol extract of leaf (11.5 mg/g) and lowest amount was observed in chloroform extract of bark (7.5 mg/g). It indicates that methanol extract of leaf had given maximum yield of compounds. So for extraction purpose methanol is best solvent. There were some peaks also observed in HPLC graphs. These can be considered as unknown compound.

Umadevi *et al*., (2012) had also observed the same results in case of alcoholic extract of leaves of bael. But instead of Kaempferol they found Ursolic acid in the alcoholic extract of leaves.

CHAPTER 7

CONCLUSION

Aegle marmelos contains different classes of secondary metabolites and these metabolites are further used as herbal and ayurvedic medicines. For the chemical characterization the selection of solvent should be done carefully because there are some solvents in which the phytochemical test shows positive result and same extract shows negative result in other solvent. It was found that the bioactive compounds isolated from plants are used in almost 25% of prescribed drugs. From the study on bael tree it is clear that bael has became the major source of medicine for curing various diseases in humans and animals. In conservation of medicinal plants and reintroduction to nature's endangered species micropropagation played an important role. The medicinal plants which are aromatic and of medicinal use need to be multiplied in short span of time and establishment in their natural habitat. The conventional methods for plant regeneration are slow and it was found that the seeds thus produced are no much viable so researchers are now trying to increase yield of secondary metabolites, production and to make the plant resistant to adverse environmental conditions.

The results obtained from the phytochemical analysis had shown similarities with the literature. But in quantitative analysis of plant extract, differences in amount of TPC & TFC were observed. These differences may be due to change in geographical region. In anitimicrobial activity, the extracts had shown their inhibitory effect. But some extracts had shown no antimicrobial activity. In TLC there were some bands for which there is no standard available to know the name of these compounds. So further studies can be carried to know about the unknown compounds present in bands. Similarly in HPLC of the extracts, it was observed that there are some peaks for which no standard compound has been identified. So further studies can be done to know about the unknown compounds shown by the peaks of HPLC graph. By doing this we will be having a wide range of bioactive compounds. Further these bioactive compounds can be isolated and used in preparation of traditional medicines.

CHAPTER 8

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