Chapter- 6

Studies on antibacterial activity of different extracts of Naringi crenulata and Aegle marmelos and the identification of the active principle
6. STUDIES ON ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS OF NARINGI CRENULATA AND AEGLE MARMELOS AND THE IDENTIFICATION OF THE ACTIVE PRINCIPLE

6.1 INTRODUCTION

Health is wealth according to an old saying. The WHO has defined health as not only the state of complete physical, mental and social well-being but also freedom from diseases or any infirmities. The overall health status of any population may serve as an index of its prosperity and welfare. The earth as it is today has turned out to be a hostile environment to live and it is a sad fact that 16% of the world population is challenged with serious health care problems. Protection against various diseases is therefore imperative. India has the distinction of hosting about 16% (>1 billion) of the world population and records 20% of world's mortality and 22% morbidity. These staggering figures call for measures towards health care.

Infectious diseases are still persisting as major health problem and nearly about 11 million children die each year. The worst affected are the developing countries, which accounts for nearly 99% of the mortality due to infectious diseases (Dipika Sur et al., 2004; Nongkynrih et al., 2004). In India 7 million infectious disease deaths (of which, two million deaths are observed mostly in children and young adults every year) account for 70% of infectious disease deaths and 13% of all deaths world wide (www.prcdc.org). The most frequently reported diseases are Leptospirosis, acute dysentery, typhoid fever and acute hepatitis (Jacob John, 2004). Such negative health trend calls for a renewed interest in prevention and treatment of infectious diseases. Though plenty of antibiotics are readily available, increasing incidence of resistance to antibiotics particularly in gram-negative bacteria (Alonso et al., 2000; Sader et al., 2002) has urged the researchers to look for viable and sustainable alternatives. It is evident that the Indian people have a tremendous passion for medicinal plants (over 3000 plants have been used indigenously) and use them
for a wide range of health related applications from a common cold to memory improvement and treatment of poisonous snake bites to a cure for muscular distrophy and the enhancement of body’s general immunity (Pearce and Purushothaman, 1992). But on the other hand, it is observed that the plant biodiversity has not been conserved and protected and also less explored against new diseases (www.nri.org).

The alternate approach to drug discovery is through the medicinal plants. Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products for the prevention and cure of different human diseases. It is documented that 80% of the world population has faith in traditional medicine, particularly plant drugs for their primary health care. India is a gold mine of well-recorded and traditionally well-practiced knowledge of herbal medicine. This country is perhaps the largest producer of medicinal herbs and is rightly called the ‘botanical garden’ of the world. India officially recognizes over 3000 plants for their medicinal value. It is generally estimated that over 6000 plants in India are in use in traditional, folk and herbal medicine representing about 75% of the medicinal needs of the Third world countries (Rajashekaran, 2002). In 1991, 42 new agents were introduced to medical practice of which 16 were natural products or were derived from natural products. Similarly in 1992, 4 new chemical entities were introduced and among them were natural products or their derivatives.

Medicinal plants also have the following benefits and aid in drug discovery.

1. Plants are sources of wide variety of novel drug molecules (chemical discovery). Example: Alkaloids, terpenoids, lignans, glycosides etc.

2. Natural products serve as lead molecules for the development of many popular drugs.
3. Herbal drugs are having lesser side effects than the other classes of synthetic drugs.

4. Possibility of multidrug / target therapy.

Moreover, the potential for developing antimicrobials into medicines appears rewarding from both the perspective of drug development and phytomedicines. Many workers have used plant-derived antibiotics against the human pathogens. Cipadessa baccifera. Miq, a bushy shrub distributed in North Circas, Deccan and Western Ghats is used to treat dysentery, skin itch, malaria and burns (Jiangsu, 1977), Clausena dentate (Wild), a small tree distributed in North Circas, Hills of Ganjam, Malabar, Anamalais, Pulneys etc., is used against kidney pains (Armando, 2003), while Clausena excavata is familiar for its antimycobacterial and antifungal activities (Sunthitikawinsakul et al., 2003).
6.2 REVIEW OF LITERATURE

Increase in the infectious diseases coupled with emergence of new diseases and resistance of microbes to potential antibiotics have warranted the scientists to look for new curatives, which are efficient as well as environment friendly. Plants have always been rich alternative sources, which have not been efficiently explored.

The use of plants as antimicrobial from wide range of higher plants from time to time has been experimented by several earlier workers (Cowan et al., 1999; Samy and Ignacimuthu, 2000). The choice of botanicals is more appropriate due to its efficiency and economy and also because they are free from side effects. To cite examples, Harpagophytum procumbens is against rheumatism (Chrubasik et al., 1996), Clausena dentata against cancer, muscular pain, malarial fever, ulcer and liver disorders (Rastogi and Mehrotra, 1984). Valeriana officinalis (Valerian) is used as sedative.

The stem extract of Fibraurea chloroleuca showed the greatest activity over both gram positive and gram negative bacteria, Candida spp and dermatophytic fungi (Mohtar et al., 1998), while the extract from 8 medicinal plants of Trinidad was found to be highly effective against Staphylococcus aureus (Chariandy et al., 1995). Substantial inhibitory action of Thymus vulgaris against Candida albicans and Escherichia coli and of Vetiveria zizanioides against Staphylococcus aureus (Hammer et al., 1999) has been reported. Petroleum ether and alcohol extracts of aerial parts of Argemone mexicana showed high antimicrobial activity on Bacillus subtilis and Escherichia coli (Sangameswaran et al., 2004). Essential oils of Melia dubia exhibited bacteriostatic and fungistatic activities against Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Fusarium oxysporum and Candida albicans (Nagalakshmi et al., 2001). Dodonaea viscosa leaf extract at 12.5mg/ml exhibited antimicrobial activities against Bacillus subtilis,
*Pseudomonas aeruginosa, Staphylococcus aureus* and particularly on *Candida albicans* (Kpemissi et al., 2003).

Novel terpenes extracted from *Buddleja globosa* were found to possess antifungal activity against *Trichophyton rubrum, Trichophyton interdigitale* and *Epidermophyton floccosum*. Six triterpenoids having a lupane and oleane isolated from the leaves of *Licania heteromorpha*, Bentham var. *heteromorpha* were found to be active against gram-positive bacteria and yeast (Braca et al., 2000). Evaluation of the antibacterial and anti-inflammatory properties of *Dodonaea viscosa, Rumex nervosus* and *Rumex abyssinicus* showed that it was active against *Staphylococcus aureus* and coxsackie virus B3 and influenza A virus (Getie et al., 2003).

Crude methanolic extract of *Caulerpa lentilifera, Caulerpa racemosa, Caulerpa microphysa* and *Caulerpa sertularioides* yields two secondary metabolites caulerpin and phytol. Caulerpin showed moderate antibacterial activity against 8 species of bacteria isolated from algal surface (Vairappan, 2004).

Antimicrobial drugs currently used in medicinal practices for treating various diseases often cause serious side effects such as immunosuppression of the host and development of resistance. Medicinal and aromatic plants and their essences are rich in antibacterial compounds, which could be used to combat bacterial diseases (Abramowize, 1990, Rajendhran et al., 1998; Samy et al., 1998; Meera et al., 1999). The volatile substances of aromatic plants and essential oils are extensively used medicinally in Ayurveda and aromatherapy. These have a great variety of biodynamic actions (Larrondo et al., 1995; Perez et al., 1999) and the antibacterial effect of volatile substances of medicinal plants has been studied by several authors (Agnihotri and Vaidya, 1996; Rajendharan et al., 1998; Hashem and Saleh, 1999; Dorman and Deans, 2000).

Acetone and methanol extracts of *Anisomeles indica, Anisomeles malabrica, Blumea lacera* and *Melia azadirachta* showed antibacterial activity
against *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcesens* and *Staphylococcus aureus* (Ramasamy and Manoharan, 2004).


Ramana et al., (2003), carried out the preliminary phytochemical analysis of *Eupatorium odoratum*. They observed the presence of glycosides, flavonoids, steroids, saponins and tannins in this plant. The petroleum ether and butanol extract of *Eupatorium odoratum* showed antifungal activity against *Aspergillus niger*, *Aspergillus flavus* and *Colletotrichum gloeosporioides*. Methanol extract showed activity against *Macrophomina phaseolina*.

Four pentacyclic triterpenes were isolated from *Combretum imberbe* Engl. & Diels and *Terminalia stuhlmannii* Engl. & Diels. Several of these compounds had antibacterial activity against *Mycobacterium fortuitum* and *Staphylococcus aureus* (Katerere et al., 2003).

Petroleum ether extracts from rhizome of *Acorus calamus* Linn showed antibacterial activity against four organisms namely *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Rani et al., 2003).
A preliminary antibacterial screening of 82 Indian medicinal plants traditionally used in medicines was carried out by Ahmad et al., (1998). Aqueous, hexane and alcoholic extracts of each plant were tested against *Bacillus subtilis* ATCC 6051, *Proteus vulgaris* ATCC6380, *Salmonella typhimurium* ATCC23654, *Pseudomonas aeruginosa* ATCC25619, *Escherichia coli* K-12 and *Staphylococcus aureus*. Out of 82 plants, 56 exhibited antibacterial activity against one or more test pathogens. Extract of five plants showed strong and broad-spectrum activity. Alcoholic extracts showed greater activity and alcoholic extracts of *Emblica officinalis*, *Terminalia chebula*, *Terminalia belerica*, *Plumbago zeylanica* and *Holarrhena antidysenterica* showed activity against test bacteria.

Three extracts of *Pergularia daemia* were examined for antibacterial activity against six bacterial strains viz., *Shigella dysenteriae*, *Salmonella typhi*, *Escherichia coli*, *Vibrio cholera*, *Staphylococcus aureus* and *Bacillus cereus*. Methanolic extract was active against *Salmonella typhi*, *Vibrio cholera*, *Staphylococcus aureus* and *Bacillus cereus* (Rajasekara Pandian et al., 2005).

A large segment of the world's population relies on traditional remedies to treat a plethora of diseases. Medicinal herbs and herbal extracts are an indispensable part of the traditional medicine practiced all over the world due to low costs, easy access and ancestral experience (Marini-Bettolo, 1980).

Sohni et al., (1995) reported the antiamoebic effect of a crude formulation against *Entamoeba histolytica*. Ethanol extract of five medicinal plants viz., *Boerrhaeia diffusa*, *Barberis aristata*, *Tinospora cardifolia*, *Terminalia chebula* and *Zingiber officinale* was used for this study.

Both crude and acetone extracts of *Punica granatum*, *Tamarindus indica*, *Garcinia gummigutta*, *Averhoea carambola* and *Spondias pinnata* were active against *Salmonella typhi*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas sp*,
and *Bacillus* sp. The crude extract was found to be more active than acetone extract (Bibitha Babu et al., 2002).

Elizabeth, (2002) has showed antibacterial activity of *Balanites roxburghii* on certain human pathogenic microorganisms.

Chavan *et al.*, (2005) have reported bacteriostatic activity of *Ricinus communis* leaves extract against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumoniae*.

A new naturally occurring sterol, compound 5 and 6 known as stigmasterols were isolated from the fruits of *Ailanthus altissima* by repeated column chromatography and RP-HPLC. In addition, the 95% ethanol extract and compounds from the fruits of *Ailanthus altissima* were assayed for *in vitro* antibacterial activity (Zhao *et al.*, 2005).

Chloroform, ethanol and aqueous extracts of the roots of *Withania somnifera* were investigated for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhi*. Among various extracts, maximum antibacterial activity was exhibited by ethanol extract followed by chloroform extract and then aqueous extract, which had no activity. The good bactericidal activity is due to the presence of voithanolides, which makes it an alternative medicine in treating infections caused by the above microorganisms (Poonkothai *et al.*, 2005).

Usha *et al.*, (2005) have investigated the alcoholic extract of *Cassia auriculata* leaves, which exhibited potential antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The ethanolic extract of *Bougainvilleae spectabilis* Linn showed antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella sp* and *Escherichia coli* (Kothari *et al.*, 2006).

Fazeli *et al.*, (2007) investigated antimicrobial effects of two spices (*Rhus coriaria* L. *sumac*) and *Zataria multiflora* Boiss. (avishan-e shirazi or zaatar) used in Iranian traditional medicine against some pathogenic food-
borne bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, *Proteus vulgaris* and *Shigella flexneri*. They observed that the hydroalcoholic extract of the above said spices showed antibacterial activity against almost all the bacterial strains. Sumac showed better activity against the tested bacteria compared to avishan-e shirazi and inhibited *Bacillus cereus* and *Staphylococcus aureus*. 
6.3 MATERIALS AND METHODS

6.3.1 Chemicals and glasswares

The chemicals, glasswares and equipments used for experiments and their sources of supply are presented below:

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Sources of supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Benzene</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Chloroform</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Ethanol</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Hexane</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Methanol</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>LOBA CHEMIE, Mumbai</td>
</tr>
<tr>
<td>Silica gel (60-120 mesh size)</td>
<td>Sisco Research Laboratory, Mumbai</td>
</tr>
<tr>
<td>Peptone broth</td>
<td>Hi media Laboratories Pvt. Ltd, Mumbai</td>
</tr>
<tr>
<td>Mueller Hinton agar</td>
<td>Hi media Laboratories Pvt. Ltd, Mumbai</td>
</tr>
<tr>
<td>Readymade TLC plates</td>
<td>Sisco Research Laboratory, Mumbai</td>
</tr>
</tbody>
</table>

**Glasswares**

<table>
<thead>
<tr>
<th>Glasswares</th>
<th>Sources of supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petridishes (90mm dia)</td>
<td>Borosil Glassworks Ltd, Mumbai</td>
</tr>
<tr>
<td>Erlenmeyer’s flasks (100, 250 and 500ml)</td>
<td>Borosil Glassworks Ltd, Mumbai</td>
</tr>
<tr>
<td>Beakers (50, 150, 250, 500 and 1000ml)</td>
<td>Borosil Glassworks Ltd, Mumbai</td>
</tr>
<tr>
<td>Measuring cylinders (25, 100, 500ml)</td>
<td>Borosil Glassworks Ltd, Mumbai</td>
</tr>
<tr>
<td>Glass column</td>
<td>Borosil Glassworks Ltd, Mumbai</td>
</tr>
</tbody>
</table>

**Equipments**

<table>
<thead>
<tr>
<th>Equipments</th>
<th>Sources of supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micropipettes (100, 200 and</td>
<td>Nichipet EX, Japan</td>
</tr>
</tbody>
</table>

217
The glasswares were initially cleaned with detergent and then immersed in chromic acid cleaning solution overnight. They were washed thoroughly with tap water and rinsed with distilled water and later they were dried in a hot air oven. The glasswares used in the study were autoclaved at 15lbs pressure (121°C) for 20 minutes.

### 6.3.2 Media used

#### 6.3.2.1 Peptone water pH 7.2±0.2 (at 25°C)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gram/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>10.0g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0g</td>
</tr>
</tbody>
</table>
6.3.2.2 Mueller- Hinton Agar pH 7.3±0.2 (at 25°C)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gram/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion from Casein acid</td>
<td>- 300.0g</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>- 17.50g</td>
</tr>
<tr>
<td>Starch</td>
<td>- 1.50g</td>
</tr>
<tr>
<td>Agar</td>
<td>- 17.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>- 1000.0ml</td>
</tr>
</tbody>
</table>

38.0g of MHA was dissolved in 1000ml of distilled water

6.3.3 Plant material

The leaves of *Naringi crenulata* and *Aegle marmelos* were collected from the Kerala Forest Research Institute, Peechi, Thrissur District, Kerala.

6.3.4 Pathogen source

*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi* and *Staphylococcus aureus* were obtained from the Department of Biotechnology, Muthayammal College of Arts and Science, Rasipuram, Tamil nadu.

6.3.5 Phytochemical extraction and antimicrobial assay

The recent interest in the plant kingdom as a potential source of new drugs, envisage alternate strategies for the fractionation of plant extracts rather than on a particular class of compound, since not all the chemical compounds elaborated by plants are of equal interest to the pharmacognosist. The so-called active principles are frequently alkaloids or glycosides and these, therefore, deserve special attention. Other groups such as carbohydrates, fats and proteins are of dietetic importance, and many such as starches and gums are used in pharmacy but lack any marked pharmacological actions. Other substances such as calcium oxalate, silica, lignin and coloring matters, may be assistance in the identification of drugs and the detection of adulteration. The
present approach is based on bioactivity guided fractionation and isolation of active compounds.

6.3.5.1 Extraction of plant material:

The leaves of *Naringi crenulata* and *Aegle marmelos* were collected, shade dried and powdered. 300g of powdered leaves were extracted successively using non-polar to polar solvents viz, petroleum ether, benzene, chloroform, acetone, isopropanol, ethanol, methanol and aqueous in a Soxhlet apparatus for 18 hours (Plate 6.3.1). After extraction they were filtered through Whatmann filter paper, the filtrate was collected and concentrated with rotary evaporator to remove the solvent content. These extracts were then poured into petridishes and kept over a water bath for about half an hour to yield powdered form of extract. These extracts were transferred to a clean tight container and used for further analysis.

Plate 6.3.1

![Extraction Of Leaf Powder In Soxhlet Apparatus](image)
6.3.5.2 Preparation of extract discs

5% of each extract was weighed and dissolved in its respective solvent. Sterile discs of 6 mm diameter were soaked in these extracts for 18 hours and were completely dried to remove the solvents under laminar airflow. Separate controls were prepared.

6.3.5.3 Inoculum preparation

25 ml of peptone broth was added to conical flasks and autoclaved. They were then cooled to room temperature. A loop full of culture was retrieved from the pathogen slants and inoculated in separate conical flasks containing peptone broth. The cultures were incubated in a shaker for 8 hours at 37°C and it is considered as the ‘starter culture’.

6.3.5.4 Disc diffusion assay (Kirby and Bauer method (1966))

Sterile Mueller –Hinton agar plates were prepared. The optical density of the inoculum was adjusted and the desired amount was added to the plates. A lawn was made using a sterile spreader. The discs were placed equidistant from each other and incubated overnight at 37°C. The zones of inhibition were measured using a ruler and the average was taken from the mean of triplicates.

6.3.6 Fractionation of plant material

In the present study, the isopropanol extracts of *Naringi crenulata* was chromatographed on silica gel column using the following solvents.

6.3.6.1 Fractions of *Naringi crenulata*

Packing material with mesh size: SiO₂ (60-120 mesh size)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Solvent combination with ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>Hexane: ethanol (9:1)</td>
</tr>
</tbody>
</table>

Different fractions were collected. Thin layer chromatography was also done to identify the number of compounds.
6.3.6.2 Thin Layer Chromatography

Thin layer chromatography of the isopropanol extract as well as the fractions of *Naringi crenulata* was done using the following solvents.

<table>
<thead>
<tr>
<th>Extract/fraction</th>
<th>Solvent ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>Hexane: Ethanol (9:1)</td>
</tr>
<tr>
<td>F1- F6</td>
<td>Hexane: Ethanol (9:1)</td>
</tr>
</tbody>
</table>

Six fractions (F1- F6) were obtained. Fraction 6 was rechromatographed for isolation of pure compound.

6.3.6 Purification of the compound

The fraction was further purified by separating through a column chromatography. The mobile phase was selected by using TLC plate. A small amount of extract was spotted in TLC plate and the sample was eluted using different percentage of ethyl acetate: hexane as solvents. For this, 10%, 20% and 50% Ethyl acetate: Hexane combinations were tried. The most suitable mobile phase was found to be 20% Ethyl acetate: Hexane to view the distinct spot in TLC.

6.3.6.1 Separation by column chromatography

6.3.6.1.1 Preparation of Admixture: 30ml of hexane and twice the amount of silica gel (24g, 60-120 mesh) was added to 12gm of extract and it was uniformly mixed by using a pestle and mortar.

6.3.6.1.2 Column packing: 120gm of silica gel was taken in a suitable column (dia 5cm X 50cm height) and packed very carefully without air bubbles using hexane as solvent. The admixture was added at the top of the stationary phase and started separation of compounds by eluting with solvent gradually with increasing order of polarity (0- 100% Ethyl acetate in Hexane). Pure fraction obtained in 5% Ethyl acetate: Hexane. All the fractions were collected.
separately, pooled and solvent removed under reduced pressure using rotary evaporator.

6.3.7 Characterization and structure elucidation of isolated compound

Isolated compound was further checked by TLC and characterized by $^1$H, $^{13}$C NMR and Mass spectroscopic techniques. Chemical structure of the compound was elucidated.
6.4 OBSERVATIONS

The present study aimed at identifying the antimicrobial activity of different solvent extracts from the leaves of *Naringi crenulata* and *Aegle marmelos* against human pathogens as well as the identification of the active principle present in the leaves of *Naringi crenulata*. The results are discussed in detail.

6.4.1 Effect of different extracts on pathogens.

Table 6.4.1: Effect of different extracts of *Naringi crenulata* on bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Petroleum ether</th>
<th>Benzene form</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Isopropanol</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>7</td>
<td>0</td>
<td>9</td>
<td>12</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>13</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>7</td>
<td>0</td>
<td>9</td>
<td>12</td>
<td>10</td>
<td>15</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>14</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>10</td>
<td>0</td>
<td>7</td>
<td>12</td>
<td>14</td>
<td>11</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

6.4.1.1 Antibacterial activity of *Naringi crenulata*

In general, among the 8 extracts, isopropanol, methanol and ethanol extracts showed better inhibition (Table 6.4.1). Isopropanol extract showed a high degree of inhibition against all the six pathogens, the growth of *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus aureus* were inhibited up to 14 mm. Methanol extract showed better inhibition against *Klebsiella pneumoniae* (15 mm), *Bacillus subtilis* and *Escherichia coli* (13 mm). The benzene and aqueous extracts had no effect on all the 6 pathogens.
The inhibition in the growth of *Bacillus subtilis* and *Klebsiella pneumoniae* was observed in all the extracts except benzene and aqueous. The inhibition observed from trace was upto 14mm for *Bacillus subtilis* and 15mm for *Klebsiella pneumoniae*. The petroleum ether, chloroform and acetone extract showed moderate inhibition. *Salmonella typhi* showed resistance against Petroleum ether extract, whereas *Escherichia coli* was resistant to chloroform and acetone extract (Fig 6.4.1 and Plate 6.4.1).

**Fig: 6.4.1 Antibacterial activity of Naringi crenulata**
Plate 6.4.1 Antibacterial activity of different extracts of *Naringi crenulata* on bacterial pathogens (A-L)

**A:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Bacillus subtilis*

**B:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Bacillus subtilis*

**C:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Escherichia coli*

**D:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Escherichia coli*

**Legend:**

P- petroleum ether  M- methanol  
C- chloroform  E- ethanol  
A- acetone  I- Isopropanol  
Aq- Aqueous  Be- Benzene
Plate 6.4.1: Antibacterial activity of different extracts of *Naringi crenulata* on bacterial pathogens (A-L)

A: Antibacterial activity of pet. ether, chloroform, acetone and aqueous extracts of *Naringi crenulata* on *Bacillus subtilis*

B: Antibacterial activity of methanol, ethanol, isoprop. and benzene extracts of *Naringi crenulata* on *Bacillus subtilis*

C: Antibacterial activity of pet. ether, chloroform, acetone and aqueous extracts of *Naringi crenulata* on *E.coli*

D: Antibacterial activity of methanol, ethanol, isoprop. and benzene extracts of *Naringi crenulata* on *E.coli*
Plate 6.4.1 Antibacterial activity of different extracts of *Naringi crenulata* on bacterial pathogens

**E:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Klebsiella pneumoniae*

**F:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Klebsiella pneumoniae*

**G:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Proteus vulgaris*

**H:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Proteus vulgaris*

<table>
<thead>
<tr>
<th>P</th>
<th>petroleum ether</th>
<th>M</th>
<th>methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>chloroform</td>
<td>E</td>
<td>ethanol</td>
</tr>
<tr>
<td>A</td>
<td>acetone</td>
<td>I</td>
<td>isopropanol</td>
</tr>
<tr>
<td>Aq</td>
<td>Aqueous</td>
<td>Be</td>
<td>Benzene</td>
</tr>
</tbody>
</table>
E: Antibacterial activity of pet. ether, chloroform, acetone and aqueous extracts of Naringi crenulata on K. pneumoniae

F: Antibacterial activity of methanol, ethanol, isoprop and benzene extracts of Naringi crenulata on K. pneumoniae

G: Antibacterial activity of pet. ether, chloroform, acetone and aqueous extracts of Naringi crenulata on P. vulgaris

H: Antibacterial activity of methanol, ethanol, isoprop. and benzene extracts of Naringi crenulata on P. vulgaris
Plate 6.4.1 Antibacterial activity of different extracts of *Naringi crenulata* on bacterial pathogens

**I:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Staphylooccus aureus*

**J:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Staphylooccus aureus*

**K:** Antibacterial activity of Petroleum Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *Salmonella typhi*

**L:** Antibacterial activity of methanol, ethanol, isopropanol and benzene extracts of *Naringi crenulata* on *Salmonella typhi*

P- petroleum ether     M- methanol
C- chloroform          E- ethanol
A- acetone             I- isopropanol
Aq- Aqueous            Be- Benzene
I: Antibacterial activity of Pet. Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *S. aureus*

J: Antibacterial activity of methanol, ethanol, isoprop. and benzene extracts of *Naringi crenulata* on *S. aureus*

K: Antibacterial activity of Pet. Ether, Chloroform, Acetone and Aqueous extracts of *Naringi crenulata* on *S. typhi*

L: Antibacterial activity of methanol, ethanol, isoprop and benzene extracts of *Naringi crenulata* on *S. typhi*
6.4.1.2 Antibacterial activity of Aegle marmelos

Table 6.4.2: Effect of Different solvents of Aegle marmelos on bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Petroleum ether</th>
<th>Benzene</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Isopropanol</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>13</td>
<td>21</td>
<td>11</td>
<td>12</td>
<td>0</td>
<td>13</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>14</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>11</td>
<td>14</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>15</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

The benzene, methanol and ethanol extracts were superior over others (Table 6.4.2). The benzene extract inhibited the growth of Bacillus subtilis, Klebsiella pneumoniae and Staphylococcus aureus and their zone of inhibition was measured as 21 mm, 14 mm and 11 mm respectively. The other organisms showed moderate inhibition (8 mm) towards benzene extract. The methanol extract inhibited the growth of every organism tested. It showed high degree of inhibition against Escherichia coli (19 mm) followed by Klebsiella pneumoniae, Staphylococcus aureus and Salmonella typhi (15 mm and 14 mm respectively). Ethanol extract inhibited the growth of Escherichia coli, Bacillus subtilis and Salmonella typhi and their zone of inhibition ranges from 16 mm, 15 mm and 13 mm respectively. Aqueous extract inhibited the growth of Klebsiella pneumoniae (15 mm) and Bacillus subtilis (13 mm). Salmonella typhi showed resistance to the aqueous extract (Fig 6.4.2 and Plate 6.4.2).
Fig. 6.4.2 Effect of different solvents of *Aegle marmelos* on bacterial strains
Plate 6.4.2 Antibacterial activity of different extracts of *Aegle marmelos* on bacterial pathogens

**A:** Antibacterial activity of different extracts of *Aegle marmelos* on *Bacillus subtilis*

**B:** Antibacterial activity of different extracts of *Aegle marmelos* on *Klebsiella pneumoniae*

P- Petroleum ether  M- Methanol
C- Chloroform  E- Ethanol
A- Acetone  I- Isopropanol
Aq- Aqueous  Be- Benzene
Plate 6.4.2: Antibacterial activity of different extracts of *Aegle marmelos* on bacterial pathogens

A: Antibacterial activity of different extracts of *Aegle marmelos* on *B. subtilis*

B: Antibacterial activity of different extracts of *Aegle marmelos* on *K. pneumoniae*
Plate 6.4.2 Antibacterial activity of different extracts of *Aegle marmelos* on bacterial pathogens

**C:** Antibacterial activity of different extracts of *Aegle marmelos* on *Escherichia coli*

**D:** Antibacterial activity of different extracts of *Aegle marmelos* on *Proteus vulgaris*

P- Petroleum ether    M- Methanol
C- Chloroform         E- Ethanol
A- Acetone            I- Isopropanol
Aq- Aqueous           Be- Benzene
C: Antibacterial activity of different extracts of *Aegle marmelos* on *E. coli*

D: Antibacterial activity of different extracts of *Aegle marmelos* on *P. vulgaris*
Plate 6.4.2 Antibacterial activity of different extracts of *Aegle marmelos* on bacterial pathogens

**E:** Antibacterial activity of different extracts of *Aegle marmelos* on *Staphylooccus aureus*

**F:** Antibacterial activity of different extracts of *Aegle marmelos* on *Salmonella typhi*

P- Petroleum ether  M- Methanol  
C- Chloroform       E- Ethanol     
A- Acetone          I- Isopropanol  
Aq- Aqueous         Be- Benzene
E: Antibacterial activity of different extracts of *Aegle marmelos* on *S. aureus*

F: Antibacterial activity of different extracts of *Aegle marmelos* on *S. typhi*
6.4.2 Bioassay of fractions against human pathogens.

In continuation of the previous experiment, the active extract (isopropanol) was fractionated and the activity was tested against the human pathogens (Table 6.4.3). After separation using column chromatography, six fractions (F1- F6) were obtained. Among the six fractions, F6 was found to be most effective against four pathogens (Fig 6.4.3 and Plate 6.4.4). The inhibition zone was to the extent of 20 mm to 14 mm with *Bacillus subtilis* (20 mm) being the most susceptible one followed by *Salmonella typhi* (17 mm), *Klebsiella pneumoniae* and *Proteus vulgaris* (14mm each).

Plate 6.4.3

Separation Of Active
Compound By Column
Chromatography
Table 6.4.3: Effect Of Isopropanol Fractions of *Naringi crenulata* on Bacterial Strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Zone of Inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction 1</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0</td>
</tr>
</tbody>
</table>

Fig: 6.4.3 Effect of isopropanol fractions of *Naringi crenulata* on bacterial strains
Plate 6.4.4 Antibacterial activity of 6 fractions of isopropanol extract of
Naringi crenulata on bacterial pathogens

A: Antibacterial activity of 6 fractions of isopropanol extract of Naringi crenulata
on Bacillus subtilis

B: Antibacterial activity of 6 fractions of isopropanol extract of Naringi crenulata
on Escherichia coli

P- Petroleum ether  M- Methanol
C- Chloroform     E- Ethanol
A- Acetone        I- Isopropanol
Aq- Aqueous       Be- Benzene
Plate 6.4.4: Antibacterial activity of 6 fractions of isopropanol extract of *N. crenulata* on bacterial pathogens

A: Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *B. subtilis*

B: Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *E. coli*
Plate 6.4.4 Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on bacterial pathogens

**C:** Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *Klebsiella pneumoniae*

**D:** Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *Proteus vulgaris*

**E:** Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *Staphylococcus aureus*

**F:** Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *Salmonella typhi*

- P- Petroleum ether
- C- Chloroform
- A- Acetone
- Aq- Aqueous
- M- Methanol
- E- Ethanol
- I- Isopropanol
- Be- Benzene
C: Antibacterial activity of 6 fractions of isopropanol extract of *Narinoi crenulata* on *K.

D: Antibacterial activity of 6 fractions of isopropanol extract of *Narinoi crenulata* on *P. vulgaris*

E: Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *S. aureus*

F: Antibacterial activity of 6 fractions of isopropanol extract of *Naringi crenulata* on *S. typhi*
Plate 6.4.5: Thin Layer Chromatography of fraction 6 of isopropanol extract of *Naringi crenulata*. Compound was indicated by arrow
6.4.3 Separation and Purification of The Bioactive Principle

In the present study, isopropanol extract of *Naringi crenulata* was fractionated in the SiO₂ column (60-120 mesh size) using different solvent combinations.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Solvent used</th>
<th>Number of fractions eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>Hexane: Ethanol</td>
<td>6</td>
</tr>
</tbody>
</table>

6.4.4 Purification, Characterization and Structure Elucidation of Isolated Compound.

Among the six fractions (F1- F6) obtained after column chromatography, sixth fraction (F6) was further purified by rechromatography using combination of Ethyl acetate: Hexane as solvent. Isolated compound was further checked by TLC (Plate 6.4.5). The compound was observed as a fluorescent spot and was further characterized by ¹H, ¹³C NMR and Mass spectroscopic techniques. Chemical structure of the compound was elucidated and discussed in Table (6.4.4 and 6.4.5) and possible structure of the compound is given below.

Plate 6.4.5

Thin Layer Chromatography of fraction 6 of isopropanol extract of *Naringi crenulata*. Compound was indicated by arrow.
Table 6.4.4: Proton NMR Spectral Data Analysis $^1$H NMR, 200MHz, CDCl$_3$

<table>
<thead>
<tr>
<th>No</th>
<th>$\delta$, ppm</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1, 2</td>
<td>5.10- 5.36</td>
<td>-CH=CH-, m</td>
</tr>
<tr>
<td>H-5, 10</td>
<td>7.26- 7.52</td>
<td>Ar-H, m</td>
</tr>
<tr>
<td>H- 7</td>
<td>4.05</td>
<td>HO-C=C-H, m</td>
</tr>
<tr>
<td>H-8</td>
<td>2.33</td>
<td>-CH$_2$-, m</td>
</tr>
<tr>
<td>H- 13, 14</td>
<td>0.94- 1.47</td>
<td>2- CH$_3$, S</td>
</tr>
</tbody>
</table>

m- multiplet, Ar- aromatic, S- singlet

Table 6.4.5: $^{13}$C NMR, 200 MHz, CDCl$_3$

<table>
<thead>
<tr>
<th>No</th>
<th>$\delta$, ppm</th>
<th>Group</th>
</tr>
</thead>
</table>
| C- 1, 2, 4, 5, 6, 9, 10, 11 | 119.05- 146.77 | Ar-C, -CH=CH$_2$C-  
                              |               | O               |
| C-3     | 174.41          | C=O             |
| C-7     | 81.58           | CH$_2$C-        
                              |               | O               |
| C-8     | 23.54           | CH$_2$          |
| C-12    | 74.54           | HO-C            |
                              |               | CH$_3$         |
| C-13, 14 | 14.94      | 2- CH$_3$       |
Mass: 230(M/Z)

STRUCTURE OF MARMESIN

Marmesin molecular formula: $\text{C}_{14}\text{H}_{14}\text{O}_{4}$
Data: RMK4346.D01
Mass Peak #: 34  Ret. Time: 11.250
Scan #: 1111
Base Peak: 42.85 (75642)
Medicinal plants are important therapeutic aid for various ailments. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century (Zaika, 1975). In India, from ancient times, different part of medicinal plants has been used to cure specific ailments. Today, there is widespread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects. Natural antimicrobials can be derived from plants, animal tissues, or microorganisms (Gordon and David, 2001). The shortcoming of the drugs available today propels the discovery of new pharmacotherapeutic agents in medicinal plants (Cordell, 1993). To determine the potential and promote the use of herbal medicine, it is essential to intensify the study of medicinal plants that find place in folklore (Awadh Ali et al., 2001 and Nair et al., 2005).

Antimicrobial drugs currently used in medicinal practices for treating various diseases often cause serious side effects such as immunosuppression of the host and development of resistance. Medicinal and aromatic plants and their essences rich in antibacterial compounds could be an alternate way to combat bacterial diseases (Abramowize, 1990; Samy et al., 1998; Meera et al., 1999; Ramasamy and Manoharan, 2004). In order to reduce the impact of allopathic drugs natural and herbal usage is ideal with out side effects.

Several investigators have reported antimicrobial activity of many medicinal plants like Argemone mexicana (Sangameswaran et al., 2004), Sand dune species (Kpemissi et al., 2003), Dodoneae viscose, Rumex nervosus, Rumex abyssinicus (Getie et al., 2003), African combretaceae (Katerere et al., 2003) and Pergularia daemia (Rajasekara Pandian et al., 2005).

6.5.1 Antibacterial activity of Naringi crenulata and Aegle marmelos

In the present investigation, antimicrobial activity of two plant extracts against six microbial species was recorded. Considerable antimicrobial activity
was detected in isopropanol, methanol and ethanol extracts of *Naringi crenulata* and benzene, methanol and ethanol extract of *Aegle marmelos*.

In general, isopropanol, methanol and ethanol extract showed better inhibition for all the six pathogens. The highest degree of inhibition was found against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhi*. Sangameswaran *et al.*, (2004), have observed that alcohol extracts of aerial parts of *Argemone mexicana* showed high antimicrobial activity on *Bacillus subtilis* and *Escherichia coli*. Bandyopadhyay *et al.*, (2005) reported antibacterial activity of methanolic extracts of leaves of *Camellia sinensis* (L.) on *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Shigella spp*, *Salmonella spp*, *Bacillus spp*, *Klebsiella spp* and *Pseudomonas aeruginosa*.

Suryani *et al.*, (2007) found that the methanol extract of *Centipeda minima* exhibited antimicrobial activity against *Enterobacter aerogenes*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Yersinia enterocolitica* and *Shigella sonnei*. The greatest inhibition zone was found against *Klebsiella pneumoniae*. This result is in concordance with the present study.

Several workers (Martin, 1995; Paz *et al.*, 1995; Vlientinck *et al.*, 1995) have generally reported that water extract of plants do not have much activity against bacteria. Eloff (1998) reported that methanol was more effective solvent for plant extraction than ethanol, n- hexane and water. Similarly, in our study the isopropanol and methanol extracts exhibited higher activity followed by ethanol, petroleum ether, chloroform and acetone extracts. The water extract did not show antibacterial activity. Durmaz *et al.*, (2006) reported similar observation in *Allium vineale*, *Chaerophyllum macropodum* and *Prangos ferulacea*.

Indrayan *et al.*, (2002) reported the antibacterial activity of aqueous and organic extracts of *Ceasalpinia sappan* against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi* as well as *Staphylococcus aureus*. In the present study, all the six pathogens showed resistance against benzene
and aqueous extracts of *Naringi crenulata*. Petroleum ether and chloroform extracts showed moderate activity against these pathogens.

*Bacillus subtilis* showed more susceptibility to the benzene extract of leaves of *Aegle marmelos* in the present study. The methanol extract was found to be superior to other extracts. It showed activity against every pathogen tested followed by ethanol and aqueous extracts. Burade *et al.*, (2005) has reported similar results in *Piper betle*, where methanol as well as ethanol extracts showed stronger and broad-spectrum antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi, Proteus vulgaris* and *Shigella flexneri*. The same author has reported antibacterial activity of ethanol and methanol extracts of *Tamarindus indica* leaves against the above said pathogens (Burade *et al.*, 2005a). *Salmonella typhi* showed resistance to the aqueous extract of *Aegle marmelos*. Except *Bacillus subtilis* and *Staphylococcus aureus*, all the other organisms showed resistance to acetone extract. Isopropanol extract has no antibacterial activity.

Isopropanol extract of *Naringi crenulata* showed highest activity in all the test bacteria, indicating that it contained the active compound. Hence it was further purified by column chromatography using SiO$_2$ column. Six different fractions were obtained and all these fractions were collected separately. The antibacterial activity of these fractions was performed against the six pathogens. Except *Escherichia coli* and *Staphylococcus aureus*, all the other organisms showed strong activity against fraction 6 (F6) at 5% followed by fractions 5, 4 and traces of activity by fraction 2 and 3. Fraction 1 has no effect. The fraction 6 was selected for further studies.

**6.5.2 Purification, Characterization and Structure Elucidation of Isolated Compound.**

Fraction 6 was further purified by rechromatography using combination of Ethyl acetate: Hexane as solvent. Isolated compound was further checked by
TLC and characterized by $^1$H, $^{13}$C NMR and Mass spectroscopic techniques. Chemical structure of the compound was elucidated and the possible structure of the compound was assigned as Marmesin (C$_{14}$H$_{14}$O$_4$).

Goswami et al., (2005) has reported Marmesin as a linear dihydro furanocoumarin. It was first isolated from the bark of *Aegle marmelos* by Chatterjee and Mitra, (1949). Furanocoumarins are found to possess dermal photosensitizing activity (Musago and Rodighiero, 1962). They have received much attention on account of their ability to perform cycloaddition reactions with DNA during irradiation with UV light (Zarbska, 1994; Moor and Gasparro, 1996; Brown, 2001), a property has given rise to wide ranging photochemotherapeutic applications (Miolo et al., 1989). These coumarins have also found their way in the treatment of human immunodeficiency disease (North et al., 1993).

Uwaifo, (1984) reported the mutagenic property of Marmesin. He isolated coumarins from three Nigerian medicinal plants, namely, *Afraegle paniculata, Clausena anisata,* and *Azadirachta indica* and they were screened on 6 Ames tester strains (TA92, TA94, TA97, TA98, TA100, TA102). Marmesin was mutagenic in all tester strains except TA94 and TA102.

Afek et al., (1995), has reported the role of marmesin in celery resistance to pathogens during storage. He observed that marmesin has greater antifungal activity.

From the literature, it was revealed that Marmelosin is the therapeutically active compound of *Aegle marmelos*. It has been isolated as a colourless crystalline compound (Dixit and Dutt, 1932). It acts as a laxative and diuretic (Nadkarni, 1954; Ambasta, 1986).