“STUDY OF MEDICINALLY USEFUL ANTIMICROBIAL PHYTOCHEMICAL CONSTITUENTS IN SELECTED MEMBERS OF ANNONACEAE”

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Plants have been an inseparable entity of human existence and since time immemorial human being has made use of plants in different ways and also used plants as a source of medicine. The universal role of plants in the treatment of diseases has been established by their employment in all important systems of medicine. Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millennium BC.

Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. Natural products have been an integral part of the ancient traditional medicine systems, e.g. Chinese, Ayurvedic and Egyptian (Sarker & Nahar, 2007). Over the years they have assumed a very central stage in modern civilization as natural source of chemotherapy as well as amongst scientist in search for alternative sources of drugs. About 3.4 billion people in the developing world depend on plant-based traditional medicines. This represents about 88 per cent of the world’s inhabitants, who rely mainly on traditional medicine for their primary health care. According to the World Health
Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as phytochemicals (‘phyto-‘ from Greek - phyto meaning ‘plant’) or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests (Abo et al., 1991; Liu, 2004; Nweze et al., 2004; Doughari et al., 2009).

Due to ineffectiveness of most drugs as a result of microbial resistance to available agents most especially in developing countries, more patients are seen in medical centers than ever. The intractable problem of antimicrobial resistance has led to the resurgence of interest in herbal products as sources of novel compounds to suppress or possibly eradicate the ever increasing problems of emergence of newer diseases thought to be brought under control. In view of this, it is therefore very important to search for effective but of low cost and reliable traditional therapeutic agents, hence also the abuse of drugs for ailment is in high increase which motivated drug resistant organisms. The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethno pharmacology but the practice dates back since antiquity. Ethno pharmacology has been the mainstay of traditional medicines the entire world and currently is being integrated into mainstream medicine. Different catalogues including De Materia Medica, Historia Plantarum, Species
Plantarum have been variously published in attempt to provide scientific information on the medicinal uses of plants. The types of plants and methods of application vary from locality to locality with 80% of rural dwellers relying on them as means of treating various diseases. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich *et al.*, 2004). Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhoea, and tuberculosis as well as increase in resistance by several bacteria to various antibiotics and the increasing cost of prescription drugs, for the maintenance of personal health (Levy, 1998; Van den Bogaard *et al.*, 2000; Smolinski *et al.*, 2003). Unfortunately, rapid explosion in human population has made it almost impossible for modern health facilities to meet health demands all over the world, thus putting more demands on the use of natural herbal health remedies. Current problems associated with the use of antibiotics, increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as methicillin resistant *Staphylococcus aureus*, *Helicobacter pylori*, and MDR *Klebsiella pneumonia* has revived the interest in plants with antimicrobial properties (Voravuthikunchai & Kitpipit, 2003). In addition, the increase in cases of opportunistic infections and the advent of Acquired Immune Deficiency Syndrome (AIDS) patients and individuals on immunosuppressive chemotherapy, toxicity of many antifungal and antiviral drugs has imposed pressure on
the scientific community and pharmaceutical companies to search alternative and novel
drug sources (Doughari, 2011)

**Review of Literature:**

India has a valuable heritage of herbal remedies for various ailments. In India vast populations of rural people still depend to a greater extent on the indigenous systems of medicines (Singh V., 2001) Of late there are reports of the multi drug resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs (Ahmad I. et al). The prevalence of multidrug resistance of many microbes has revealed exploration of alternative antimicrobial agents. Antibiotics are sometimes associated with side effects (Cunha, 2001). Medicinal plants have become the focus of intense study in terms of validation of their traditional uses through determination of their actual pharmacological effects. Synthetic drugs are not only expensive but have more side effects. (Kachwaha et al., 2012). There are some advantages reported from using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature. (Vermani K., 2002) Plants have a limitless ability to synthesize aromatic substances mainly secondary metabolites (Mallikharjuna et al 2007). Though a vast amount of resources have been utilized, still lots of them remain to be brought to the mainstream of pharmaceutical research, (Doughari, 2011)

**Antimicrobial activity:**

Phytoconstituents employed by plants to protect them against pathogenic insects, bacteria, fungi or protozoa have found applications in human medicine (Nascimento et al., 2000). Plants can also exert either bacteriostatic or bactericidal activity ob microbes.
The volatile gas phase of combinations of *Cinnamon* oil and clove oil showed good potential to inhibit growth of spoilage fungi, yeast and bacteria normally found on IMF (Intermediate Moisture Foods) when combined with a modified atmosphere comprising a high concentration of CO2 (40%) and low concentration of O2 (<0.05%) (Jakhetia *et al*, 2010). *A. flavus*, which is known to produce toxins, was found to be the most resistant microorganism. It is worthy of note that antimicrobial activity results of the same plant part tested most of the time varied from researcher to researcher. This is possible because concentration of plant constituents of the same plant organ can vary from one geographical location to another depending on the age of the plant, differences in topographical factors, the nutrient concentrations of the soil, extraction method as well as method used for antimicrobial study. It is therefore important that scientific protocols be clearly identified and adequately followed and reported.

Due to the above standing reports of the varied uses of the plants in the pharmaceutical scenario, it was hence decided to carry out a project that would study the antimicrobial and phytochemical aspects of plants of a particular family and it was hence suggested to involve the family Annonacea in the study.

It was suggested in the project that a brief phytochemical investigation of the methanolic, aqueous and petroleum ether extracts of the selected members of Annonaceae would be done in the project. The following members were taken for the analysis: *Uvaria narum*, *Annona glabra*, *Artabotrys odoratissimus*, and *Cananga odorata*.

The members of Annonaceae are shrubs, small trees or woody climbers; branches glabrous or pubescent; bark and leaves sometimes aromatic. Leaves alternate, sometimes distichous, exstipulate, simple, entire, pinnately nerved; petioles very short. Inflorescence
cymes, umbels or of solitary flowers, terminal or axillary, sometimes extra axillary or from old wood; bracts and bracteoles present, often caducous. Flowers bisexual, actinomorphic; pedicels often jointed in middle or near base. Sepals 3, free or slightly connate at base, valvate or slightly imbricate. Petals 6 in two whorls of 3 each, similar to dissimilar, free or slightly connate, imbricate or valvate, rarely saccate at base, fleshy to membranous. Stamens many, spiral on a prominent receptacle; filaments short or absent; connectives often produced beyond anther lobes, generally oblong, truncate; anthers adnate, sessile or subsessile, 2-4 loculed; locules sublateral, extrorse. Carpels many, rarely few or 1, free or rarely connate with distinct stigmas, pilose or glabrous; ovules 1-many, anatropous, basal or parietal; styles short or absent; stigmas clavate or capitate. Fruit an aggregate of sessile or stalked, distinct or rarely united, dehiscent or indehiscent, woody or fleshy fruitlets; seeds ovoid or ellipsoid, large, glabrous with copious ruminate endosperm; embryo minute.

**Annona glabra** L., Sp. PI

Small tree, 5 - 6 m high; branchlets glabraous; bark grey outside and red within, cracked. Leaves distichous, oblong or elliptic-ovate, cuneate or rounded at base, acute at apex, 6 - 20 x 3 - 8 cm, thin-coriaceous, glabrous, green above paler beneath; lateral nerves 8-10 pairs; petioles up to 2 cm long. Flowers on extra-axillary tubercles, solitary or paired, up to 2cm across, creamy yellow, fragrant; pedicel 1.5-2cm long, glabrous, recurved at anthesis. Sepals 3, ovate, 3 x 3 mm, ovate orbicular, acute, glabrous. Petals 3+3, creamy yellow, broadly ovate, thick and fleshy; outer ones larger, obtuse, 1.5 -2 x 1-1.5cm, base inflexed and some what subcordate, margins thickened towards apex; the inner smaller, up to 1.7x1.2cm, often with reddish blotches within. Stamens numerous, 3mm long;
filaments short; connectives prolonged, slightly convex at apex, concealing anthers. Carpels many, free, 1-ovuled. Fruits subglobose or ovoid, 7-5cm across, smooth, yellow to orange when ripe; seeds 13-15mm long, ovoid, obovoid or oblong, often strongly margined, rounded at ends, smooth, embedded in yellow pulp.

Fl. & Fr.: Jan.-April.

Local name: Kattatha Distribution: Kerala: ALP, KTM

Habitat: Cultivated, also became naturalized along the backwaters

Geographical: Native of Tropical America and West Indies

*Artabotrys hexapetalus* (L. f.)

Scandent shrubs; young branches slightly puberulous. Leaves elliptic-oblung, acute at base, acuminate at apex, 6-15 x 2-4.5 cm, thinly coriaceous, glabrous; lateral nerves 6-18 pairs; petiole 5-8mm long. Flowers solitary or paired on terminal or leaf-opposed hooked peduncles, ca 2 cm across, drooping, fragrant; pedicels 8-12mm long, pubescent. Sepals 3, ovate, acute and reflexed at tip, 5-7x5-6cm, pubescent. Petals 3+3, ovate-lanceolate, spreading from clawed saccate coherent base, greenish yellow, glabrous; outer ones 2-3x0.5-0.8mm; the inner slightly smaller. Stamens many; anthers beaked concealing anther lobes. Carpels many, sickle shaped, pubescent or glabrous; style curved, ovules 2; stigma blunt. Fruitlets 14-20, ovoid, apiculate, yellow when mature, 3-4 cm long, glabrous; seeds brown, deeply grooved on one side, smooth.

Fl.: & Fr.: Almost throughout the year.

Local names: Manoranjini, Madanapoo
Habitat: Grown as garden plant, also naturalized

*Cananga odorata* (Lam.) Hook. f. & Thoms

Trees, up to 15m tall; young branchlets puberulous; bark grey, smooth. Leaves oblong to broadly elliptic, obtuse, acute or truncate at base, often unequal, acuminate at apex, 15-21x5-7.5 cm, glabrescent; lateral nerves 7-9 pairs; petioles 1-2 cm long, grooved. Flowers few, pendulous, in axillary peduncled cymes, often from old branches, yellowish green, fragrant; bracts subulate, caducous, 3-angled, 1 - 2 mm long; peduncles woody, to 1.8 cm long, pubescent; pedicels 3-3.5 cm long, pubescent. Sepals 3, shortly connate at base, ovate, acute, reflexed at apex, ca 4-6 x 3-5 mm, pubescent, valvate in bud. Petals 3+3, oblong-lanceolate, acute, 3-6x0.5-1.5cm, a purple blotch at the base inside, grey-pubescent; the inner ones shorter and narrower. Receptacle sub-convex, pubescent. Stamens many, ca 3.5mm long; connectives broadly appended, acute, concealing anthers. Carpels many, oblong, 2.5-3 mm long, glabrous except at base; ovules several; styles short, slender. Fruitlets ovoid or globose, rounded at both ends, 1.5 - 2.5 x ca 1.5 cm; seeds 6 -12, transversely compressed, pale brown with pitted surface; stalks 1 - 2 cm long.

Fl.: & Fr.: Almost throughout the year.

Local names: Langilangi, Kanangamaram

Habitat: Grown as ornamental plant

Distribution

Kerala: All districts
*Uvaria narum* (Dunal)

Woody, climbing shrub; branchlets stellate-tomentose when young, glabrous when old. Leaves oblong-lanceolate or elliptic, subobtuse to cuneate at base, acuminate at apex, 8 - 15 x 3 - 6 cm, coriaceous, glabrous, brownish beneath when dry; lateral nerves 8-14 pairs; petioles 2.5 - 5 mm long. Flower solitary, extra-axillary, leaf-opposed or terminal, ca 3 cm across; pedicel 2 - 3 cm long; bracts oblong, 1 - 3 mm long, glabrous, caducous. Sepals 3, orbicular or ovate, obtuse, reflexed at apex, ca 5 mm, minutely tomentose outside, puberulous inside. Petals 6(-8) in 2 series, sub equal, connate at base, orbicular or ovate, inflexed at apex, 1 - 2 x 0.8 - 1.5 cm, minutely tomentose, flesh-coloured. Receptacle slightly raised, ca 8 mm across. Stamens numerous, all fertile, ca 1.7 x 0.7 mm; connectives broad, truncate. Carpels many, oblong, ca 4 mm long, scarlet; ovules many; stigmas broad. Fruitlets many, ovoid-oblong or cylindric, obtuse at both ends, scarlet, slightly constricted when dry; stalk 2.5-5 cm, seeds 4-6, 1-seriate, ca 1 cm, ovoid, plano-convex, smooth, chestnut-brown.

Fl. & Fr.: Throught the year

Local names: Kooril, Narumpanal

Habitat: In the plains and deciduous forests at lower altitudes

Distribution: Kerala: All districts Geographical; South India and Sri Lanka
Chapter 2.

Materials and Methods:

As stated before the plants that were decided to be used were Uvaria narum, Cananga odorata, Artabotrys hexapetalous and Annona glabra. The plants of Uvaria narum were collected from Ulloor, Amalanagar of Trichur district in Kerala. The materials of Annona glabra were collected from the coastal areas of Kumarakam, while Cananga odorata were collected from the Kottayam district while Artabotrys hexapetalous were collected from the S.B.College, Changanacherry, All the plant materials were authenticated by Expert Botanist Dr.V.T. Antony of S.B. College Changanacherry. A voucher specimen of all the plants has been deposited in the herbarium of department of P.G.studies in S.B.College, Changanacherry. The leaves of the plants were washed thoroughly in cold normal water and dried in shade at 28±2°C. The dried leaves were then taken and powdered carefully in a dry grinder to make sure that it did not get overheated. The dried barks were taken and for some the dried seeds were also taken.

Extraction from the plant is an empirical exercise in which different solvents are utilized under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process depends on the most appropriate assay. Once extracted from the plant, the bioactive component then has to be separated from the co extractives. Further purification steps may involve simple crystallization of the compound from the crude extract, further solvent partition of the co extractives or chromatographic methods.
in order to fractionate the compounds based on their acidity, polarity or molecular size. Final purification, to provide compounds of suitable purity for such structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation, or distillation. (Doughori, 2011)

20gm of the powdered materials were taken and extracted at room temperature 25±2°C on the rotary shaker for around 48 hrs in three different mediums of petroleum ether, methanol and distilled water. In some cases Soxhelet extraction was also done and has been specifically mentioned in the respective cases. The resulting mixture was then filtered and the filtrate further subjected to centrifugation. The obtained filtrate was the required plant extract. The methanol and petroleum ether extracts were evaporated to dryness and the aqueous extract was kept on water bath at around 50°C and evaporated to dryness. The evaporated weight of the extract was noted. The extracts were stored in dry glass containers for further evaluation.

**Antibacterial Screening:**

No single method is sufficient to study the bioactivity of phytochemicals from a given plant. An appropriate assay is required to first screen for the presence of the source material, to purify and subsequently identify the compounds therein. Assay methods vary depending on what bioactivity is targeted and these may include antimicrobial, antimalarial, anticancer, seed germination, and mammalian toxicity activities. The assay method however should be as simple, specific, and rapid as possible.
Test organisms:

The antimicrobial activity was individually tested against Gram-positive Bacteria i.e. *Staphylococcus aureus*, and Gram negative bacteria *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, *Serratia marcescens* and *Proteus vulgaris*. All the test strains were obtained from the P.G. and Research Department of Botany and Biotechnology, S.B. College, Changanacherry, and maintained on Mueller Hinton Agar (Merck.), and was subculture every two weeks.

Culture medium and inoculums:

The stock cultures of microorganisms used in this study were maintained on Nutrient Agar slants at 4°C. Inoculum was prepared by suspending a loopful of bacterial cultures into 10mL of nutrient broth and was incubated at 37°C for 24 hrs. 10^6 cfu/ml bacterial cells with O.D.1 were taken to study antibacterial activity.

Antibacterial activity assay:

The disc diffusion method was adopted to test the antibacterial activity. The disc diffusion method was used to determine the growth inhibition of bacteria by the plant extract according to Kirby Baehr’s method of checking sensitivity (Baehr’s method, 1966). Paper discs containing different concentration of dissolved plant extracts were used to check the antibacterial activity. The discs were prepared by punching the Whatmann’s No.1. filter paper (5mm in diameter), sterilized in glass bottles and autoclaving at 121°C for 15 min. 100mg. of the dried extract was dissolved in 1ml. of the respective solvents (i.e. Petroleum ether, and Methanol) to make the concentration 10µg /µl of the respective pure solvent an drawing out the required amount in µl. The discs
were loaded with three concentrations of the extract i.e. 5 µl (50µg), 10µl(100µg) and 20µl(200µg) from the above prepared stock of the extract and evaporated to dryness. The pure solvents of Petroleum Ether and Methanol were taken as the negative control. The antibiotic discs of the standard drug Chloramphenicol (30µg) and Amikacin (30 µg) were used as the positive control to compare the results of experimental plant.

**Culture medium:**

The Mueller-Hinton Nutrient agar medium (Merck.) was sterilized in a flask and cooled to 50-55°C, and poured into autoclaved and sterilized petriplates, where 20mL was poured into each petriplates, which was then swirled first in clockwise and then in anticlockwise direction to distribute the medium homogenously. The medium was then allowed to solidify in the room temperature. The prepared bacterial inoculums were inoculated on the solidified nutrient agar plates by using autoclaved sterile cotton swabs that had been dipped in the diluted suspension of the organism, as stated above. The discs were then aseptically placed evenly on the surface of the inoculation and gently pressed down with the forceps to ensure complete contact with the inoculated solid agar medium. Plates were also set for the positive control (antibiotic discs) as well as for the negative control (Pure solvents). The plates were finally incubated at 37° for 18-24 hrs. The plates were examined after 24hrs for a clear zone of inhibition. All experiments were done in triplicates and all measurements were taken in mm. Standard deviation was calculated for every measurement and recorded to understand the variation in the individual results.
**Preliminary Phytochemical Analysis:**

All the sample extracts were analyzed for the presence of various phytoconstituents as flavonoids, alkaloids, glycosides, steroids, saponins, and tannins according to the standard methods used by RNS Yadav* and Munin Agarwala (Yadav and Agarwal, 2011)

**Test for phenols and tannins**

Crude extract was mixed with 2ml of 2% solution of FeCl3. A blue-green or black coloration indicated the presence of phenols and tannins.

**Test for flavonoids**

**Shinoda test**

Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

**Alkaline reagent test**

Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicated the presence of flavonoids.
Test for saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for glycosides

Liebermann’s test

Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H₂SO₄ was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

Salkowski’s test

Crude extract was mixed with 2ml of chloroform. Then 2ml of concentrated H₂SO₄ was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

Keller-kilani test

Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of FeCl₃. The mixture was then poured into another test tube containing 2ml of concentrated H₂SO₄. A brown ring at the interphase indicated the presence of cardiac Glycosides.
Test for steroid

Crude extract was mixed with 2ml of chloroform and concentrated H$_2$SO$_4$ was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids.

Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H2SO4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

Test for terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H2SO4 was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

Test for alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer’s And Wagner’s reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Phytochemical screening using HPTLC method of screening:

Phytochemical screening using HPTLC was done at Tropical garden research institute, Palode Thiruvananthapuram, Kerala. An HPTLC system (CAMAG, Switzerland) made of a Linomat V sample applicator, a CAMAG twin trough plate development Chamber, CAMAG TLC Scanner 3 and WinCATS Software 4.03 was used for the analysis of these extracts. The aim of the HPTLC analysis was to obtain a fingerprint of the aqueous,
methanol and Petroleum ether extracts of plants that showed the maximum antimicrobial activity.

**Antifungal activities:**

1. **Collection of samples**

   Plant samples showing disease symptoms were collected from different gardens located in Alleppey district of Kerala state.

2. **Preparation of culture medium for isolation of fungus**

   Potato dextrose agar (PDA) was used for the isolation of fungi from naturally infected plant samples. 200 g of fresh potato was used for preparing one litre of medium. It was washed thoroughly in tap water, peeled and cut into thin slices, boiled in 500ml of distilled water for 20 min. and filtered through a double layer of muslin cloth. 20 g dextrose was dissolved in the potato extract. 20 g of agar powder was separately boiled in 500 ml of distilled water till the agar powder dissolved completely. It was added to the expressate, which was then made up to one litre with distilled water and sterilized at 121°C for 15 min. The sterilized and cooled medium was poured to 90 mm sterile Petri plates.

3. **Isolation from diseased tissue:**

   Symptomatic samples collected from different localities were washed thoroughly with tap water. The isolations were made from diseased pods following tissue segment method (Rangaswami, 1972). For this, pieces of infected tissue were taken with sterile scalpel from the advancing margin of lesion and cut into small pieces. These pieces were surface
sterilized with 0.1 percent mercuric chloride (HgCl₂) solution for about one min. and washed in three changes of sterile distilled water. The surface sterilized pieces of diseased tissue were then quickly passed over the flame of a spirit lamp and inoculated on PDA plates. The plates were then incubated at a temperature of 28±2°C. Axenic culture of the pathogen was obtained by single hyphal tip method and stock cultures were maintained on PDA slants.

4. Identification of fungi

Isolated fungal colonies were characterized based on the colony morphology, mycelial and conidial characters.

5. In vitro evaluation of plant extracts

The effect of plant extracts on the mycelial growth of *Fusarium* sp. was studied. The relative efficacy of different solvent extracts of *Uvaria narum* on the *in vitro* growth of *Fusarium* was evaluated in the laboratory by poisoned food technique (Nene, 1971).

Stock solutions of 10000 ppm concentration were prepared in different solvents. Adequate volume of stock solution was incorporated into PDA medium, cooled to 45°C to get the final concentration. The medium was gently shaken to ensure proper and uniform distribution of the extract. Fifteen ml of the poisoned medium was then poured into each of the sterile plate. After solidification of the medium, each plate was inoculated centrally with mycelial disc of 5 mm diameter cut from the periphery of 5-day-old culture of *Fusarium* sp. growing on PDAA medium. The plates were incubated at room temperature (28 ± 2°C) for five days. Plates amended with solvents alone and sterile distilled water served as control. Radial growth of the fungus in each plate was
recorded on third and fifth day of inoculation by measuring the diameter of the colony in
two directions at right angles to each other. The average of the two measurements was
taken as the colony diameter. The per cent inhibition of growth was calculated using the
formula given by Vincent (1927),

\[
I = \frac{C - T}{C} \times 100
\]

I - Inhibition of fungal growth

C - Mycelial growth in control

T - Mycelial growth in treatment
Chapter 3

Preliminary Phytochemical Analysis of the selected members of Annonacea and the HPTLC screening of the three extracts of *Uvaria narum*

The three sample extracts of *U. narum*, of *Canaga odorata, Annona glabra* and *Artabotrys odoratissimus* were analyzed for the presence of various phytoconstituents as flavonoids, alkaloids, glycosides, steroids, saponins, and tannins according to the standard methods used by Chandrabhan Seniya et al (Chandrabhan, 2011). The result of the screening has been shown in Table 2.

**Phytochemical screening using HPTLC method of screening:**

Phytochemical screening using HPTLC was done at Tropical garden research institute, Palode Thiruvananthapuram, Kerala. An HPTLC system (CAMAG, Switzerland) made of a Linomat V sample applicator, a CAMAG twin trough plate development Chamber, CAMAG TLC Scanner 3 and WinCATS Software 4.03 was used for the analysis of these extracts. The aim of the HPTLC analysis was to obtain a fingerprint of the aqueous, methanol and Petroleum ether extracts of leaf of *Uvaria narum*.

Section 3.1: **Phytochemical analysis of Bioactive compounds in three solvent extracts of *Uvaria narum***

Phytochemical studies revealed the presence of various secondary metabolites and were almost in accordance with the earlier studies on the same plant. (Joji Reddy *et al*, 2012), and the results have been documented in Table 2. One striking point was that even though the aqueous extract showed the presence of saponins, tannins, triterpenoids and steroids it
showed absolutely no antimicrobial activity in this study. The methanolic extract that showed an Intermediate Bioactivity against bacterial pathogens, indicated the presence of alkaloids, cardiac glycosides, condensed tannins, triterpenoids and steroids. The most bioactive extract among the three extracts, the Petroleum ether extract contained triterpenoids and steroids among its important constituents that perhaps gave it its immense antimicrobial activity against harmful human pathogens. (Trease, 2011).

Qualitative estimation of secondary metabolites from the plant parts used:

Table 1. Phytochemical analysis of three extracts of Uvaria narum

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Section 3.2 Preliminary Phytochemical Analysis of the leaves and barks of Canaga odorata and Annona glabra

The studies on the extracts of Cananga odorata and Annona glabra were conducted to determine their antimicrobial properties. Much literature is not available on Cananga odorata though some work it seems to have been done on Annona glabra.

As mentioned before, Cananga odorata belonging to family Annonaceae is a moderately sized tree growing to a size of 20m or so. It bears extremely fragrant flowers and fruits come under the category of etaerio of berries. The antimicrobial studies have gained a worldwide acceptance at many levels and several plants are being subjected to these antibacterial studies bringing us closer and closer to the idea that plants are the most reliable sources of natural drugs that can be competent and reliable enough to fight the
war against bacteria. As part of the project it was decided to consider the above-mentioned plants as a subject for antimicrobial studies.

The leaves and stem barks of *Cananga odorata* were collected from Kottayam district and that of *Annona glabra* were collected from Kumarakam area where they have been reported to be in abundance. A voucher specimen of the plant has been deposited in the herbarium of department of P.G.studies in S.B.College, Changanacherry. The leaves of the plants were washed thoroughly in cold normal water and dried in shade at 28±2°C.after which they were powdered carefully in a dry grinder .20mg of the powdered material was taken and extracted at room temperature 25±2°C on the rotary shaker for around 48 hrs in three different mediums of petroleum ether, methanol and distilled water. The extracts were then prepared as stated in the previous chapter of materials and methods. The extracts were stored in dry glass containers for further evaluation.

Table 2.Phytochemical analysis of three extracts of Leaves of *Cananga odorata*

<table>
<thead>
<tr>
<th></th>
<th>Saponins</th>
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Table 3. Phytochemical analysis of three extracts of Bark of *Cananga odorata*

<table>
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Table 4. Phytochemical analysis of three extracts of Leaves of *Annona glabra*

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Table 5. Phytochemical analysis of three extracts of Barks of *Annona glabra*

<table>
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</table>

Section 3.3  **Preliminary Phytochemical Analysis of the leaves barks and seeds of *Artabotrys odoratissimus***

As mentioned before, *Artabotrys odoratissimus*, a straggler member of Family Annonaceae was collected from the botanical garden of SB college, Changanacherry. The plant bears beautiful and extremely fragrant flowers that bear etaerio of achenes. A voucher specimen of the plant has been deposited in the herbarium of department of P.G.studies in S.B.College, Changanacherry. The leaves and the bark of the plants were
washed thoroughly in cold normal water and dried in shade at 28±2°C after which they were powdered carefully in a dry grinder. 20mg of the powdered material was taken and extracted at room temperature 25±2°C on the rotary shaker for around 48 hrs in three different mediums of petroleum ether, methanol and distilled water. The extracts were then prepared as stated in the previous chapter of materials and methods. The extracts were stored in dry glass containers for further evaluation.

The phytochemical investigation showed the following revelations:

Table 3. Phytochemical analysis of three extracts of Bark of *Artabotrys odoratissimus*

<table>
<thead>
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Table 3. Phytochemical analysis of three extracts of Leaves of *Artabotrys odoratissimus*

<table>
<thead>
<tr>
<th></th>
<th>Saponins</th>
<th>Steroids</th>
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Table 4. Phytochemical analysis of three extracts of Seeds of *Artabotrys odoratissimus*

<table>
<thead>
<tr>
<th></th>
<th>Saponins</th>
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</tbody>
</table>
The HPTLC analysis of *Uvaria narum* was taken, as the Petroleum ether and methanol extract of this plant leaves showed the maximum bioactivity against a range of bacteria. The fingerprints were taken at 254nm, 360nm and 584 nm gave the fingerprint of petroleum ether extract, in methanol and the water extract at can be further used to identify the original material. The Petroleum ether extract showed 11 compounds at 580nm, methanolic fraction showed 14 compounds and aqueous extract showed 14 compounds at 580 nm, after the plates were derivatised with anisaldehyde and sprayed with concentrated sulphuric acid.

The HPTLC graph at the above said wavelengths for each of the extracts ie Petroleum ether, Aqueous Extract and Methanol Extract has been taken and presented in the following pages.
Peak display of hexane extract scanned at 580nm

Derivatised plate of hexane extract in the white light
hexane extract scanned at 254 nm

Developed plate of the hexane extract scanned at 254nm
Peak display of hexane extract scanned at 366nm

Developed plate of hexane extract scanned at 366nm.
Peak display of methanol extract at 580nm

Derivarised plate of methanol extract visualized at the white light
Peak display of methanol extract at 366nm
Peak display of methanol extract at 254nm

Developed plate of methanol extract visualized at 254nm
Peak display of water extract scanned at 580nm

Derivatised plate of water extract at 580nm
Chapter 4

“Comparative analysis of antimicrobial activities of the three extracts of the Leaves and Barks of some selected members of Annonaceae”

Section 4.1

Plants have a limitless ability to synthesize aromatic substances mainly secondary metabolites (Mallikharjuna et al 2007). Though a vast amount of resources have been utilized, still lots of them remain to be brought to the mainstream of pharmaceutical research. One such underexplored and under exploited plant is *Uvaria narum* (Dunal) Wall, that is mostly confined to the lower altitude areas of Western Ghat Ranges that includes Kerala and lower foothills of Western ghats in Karnataka. A host of ethnobotanical uses have been reported for the plant. Roots & leaves have been reported to be used in intermittent fever, biliousness, jaundice, bowel diseases and eczema (Khare C.P.,2007)

*Uvaria narum* has been kept in the family Annonaceae, It has been reported to be containing acetogenins, (Hishama et al 1991 )that are a characteristic feature of family Annonaceae and it also includes the stereoisomers  Glutinone, Glutinol, Taraxerol, Beta sitosterol, benzyl benzoate, and patchoulenone.(Virinder R. et al,1995). Uvariamicin-I, II and III are the novel acetogenins being isolated from the bark of Uvaria. (Hishama et al, 1990). The Antibacterial and antioxidant activity of the root bark of T has been reported in earlier journals( Subrahmany et al,2011) and antimicrobial activity of the leaf extract has also been established (Reddy,2012). Since, ethnombotanically this plant has been used for the treatment of bowel diseases, jaundice and in eczema, it may imply about its
suggested antimicrobial effect against some isolated human pathogens. This study aimed at understanding the phytochemical properties of this plant and evaluating the antimicrobial properties of its aqueous methanolic and petroleum ether extracts. An HPTLC of the three extracts were also conducted to obtain the number of compounds in each of them.

**Materials and methods:**

The plant was collected from Ulloor, Amalanagar of Trichur district in Kerala and was authenticated by Expert Botanist Dr. V.T. Antony of S.B. College Changanacherry. A voucher specimen of the plant has been deposited in the herbarium of department of P.G. studies in S.B. College, Changanacherry. The leaves of the plants were washed thoroughly in cold normal water and dried in shade at 28±2°C after which they were powdered carefully in a dry grinder. 20mg of the powdered material was taken and extracted at room temperature 25±2°C on the rotary shaker for around 48 hrs in three different mediums of petroleum ether, methanol and distilled water. The extracts were then prepared as stated in the previous chapter of materials and methods. The extracts were stored in dry glass containers for further evaluation.

**Antibacterial Screening:**

**Test organisms:**

The antimicrobial activity was individually tested against Gram-positive Bacteria i.e. *Staphylococcus aureus*, and Gram negative bacteria *Escherichia coli, Klebsiella pneumonia, Enterobacter aerogenes, Serratia marascaens* and *Proteus vulgaris*. All the test strains were obtained from the P.G. and Research Department of Botany and
Biotechnology, S.B.College, Changanacherry, and maintained on Mueller Hinton Agar (Merck.), and was subcultured every two weeks.

**Culture medium and inoculums:**

The stock cultures of microorganisms used in this study were maintained on Nutrient Agar slants at 4°C. Inoculum was prepared by suspending a loopful of bacterial cultures into 10mL of nutrient broth and was incubated at 37°C for 24 hrs. 10^6 cfu /ml. bacterial cells with O.D.1 were taken to study antibacterial activity.

**Antibacterial activity assay:** The disc diffusion method was adopted to test the antibacterial activity. The disc diffusion method was used to determine the growth inhibition of bacteria by the plant extract according to Kirby Baeur’s method of checking sensitivity (Baeur’s method, 1966). Methods used for testing the antibacterial sensitivity of the plant extracts has been discussed in detail in the previous chapter of materials and methods. The paper discs were loaded with three concentrations of the extract i.e. 5 µl (50µg), 10µl(100µg) and 20µl(200µg) from the above prepared stock of the extract and evaporated to dryness. The pure solvents of Petroleum Ether, distilled water and Methanol were taken as the negative control. The antibiotic discs of the standard drug Chloramphenicol (30µg) and Amikacin (30 µg) were used as the positive control to compare the results of experimental plant.

**Culture medium:**

The culture medium was prepared as mentioned in the previous chapter of materials and methods. All experiments were done in triplicates and all measurements were taken in
mm. Standard deviation was calculated for every measurement and recorded to understand the variation in the individual results.

**Results :**

**Antimicrobial analysis:** The bioactivity of the extracts was determined by observing and measuring the clear zone of inhibition that appeared around each disc loaded with the extract. The results have been shown in Table 1.1 along with those extracts and concentration that showed maximum diameter of the clear zone of inhibition. The components derived in Petroleum Ether extracts showed the maximum activity while Methanolic extract showed intermediate activity while distilled water extract showed absolutely no bioactivity. The sensitivity of the antibiotic discs as positive control and the pure solvents at 200µl as negative controls have been shown in Table 1.2

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>50µg</th>
<th>100µg</th>
<th>200µg</th>
<th>50µg</th>
<th>100µg</th>
<th>200µg</th>
<th>50µg</th>
<th>100µg</th>
<th>200µg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em></td>
<td>18±0.1</td>
<td>20±0.57</td>
<td>22±0.4</td>
<td>18±0.4</td>
<td>16±0.1</td>
<td>27±0.5</td>
<td>-</td>
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</tr>
<tr>
<td><em>Escherichia</em></td>
<td>21.5±0.4</td>
<td>34±0.10</td>
<td>38±0.4</td>
<td>18±0.4</td>
<td>19±0.1</td>
<td>20±0.5</td>
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<tr>
<td><em>Klebsiella</em></td>
<td>20±0.1</td>
<td>20±0.4</td>
<td>24±0.4</td>
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<td>-</td>
<td>19±0.10</td>
<td>-</td>
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<tr>
<td><em>Enterobacter</em></td>
<td>20±0.5</td>
<td>20±0.8</td>
<td>30±0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Serratia</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15±0.10</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Proteus</em></td>
<td>14±0.6</td>
<td>25±0.8</td>
<td>30±0.8</td>
<td>16±0.4</td>
<td>17±0.4</td>
<td>18±0.45</td>
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</tbody>
</table>

- The results are the mean values of triplicate tests measured in two directions after 18-24 hrs at 37°C.
- AE= Aqueous extract ; ME= Methanol Extracts;  Pet EE= Petroleum ether Extracts.
- SD of the results have also been calculated.
Mean of the Inhibition Zone as shown by different antibiotics * (Positive control) and different solvents** (Negative Control).

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amikacin</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>17±0.48</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>17.5±0.75</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>22.05±0.07</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>27.5±0.68</td>
</tr>
<tr>
<td>Serratia marascens</td>
<td>20.5±0.78</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>30±0.01</td>
</tr>
</tbody>
</table>

- Antibiotics discs taken at a concentration of 30 µg /m
- PE= Petroleum ether; ME= Methanol ; DW= Distilled water . Amount used=200µl

**Discussion:**

In this study, both the methanol and the petroleum ether extracts of *Uvaria narum* showed considerable antimicrobial activity against the harmful human pathogens, and on an average Petroleum ether extract was found to be the most active among the three extracts in its activity against the bacteria. The HPTLC screening too showed the presence of around 11 compounds in PE extracts. In fact the activities at times were found to be higher than the standard antibiotics as Chloramphenicol and Amikacin (30µg each) screened under similar conditions. As per the standard table of sensitivity test (Sharma Kanika, 2011), a zone with a diameter above 10mm is considered as intermediately sensitive while one above 15mm is said to be sensitive and the zone below 10 is to be marked as resistant.
Graph 3.1

Comparative effect of U. narum leaf extracts on different bacteria at 50μg conc.

Graph 3.2

Comparative effect of U. narum leaf extract on different bacteria at 100μg
Graph 3.3

(Comparative effect of *U. narum* leaf extract on different bacteria at 200µg)

Graph 3.4

(Comparative effect of the two antibiotics on different bacteria)
Section 4.2 Antimicrobial activities of the stem bark and leaves of *Canaga odorata* and *Annona glabra*

The studies on the extracts of Cananga odorata and Annona glabra were conducted to determine their antimicrobial properties. Much literature is not available on Cananga odorata though some work it seems to have been done on Annona glabra.

As mentioned before, Cananga odorata belonging to family Annonaceae is a moderately sized tree growing to a size of 20m or so. It bears extremely fragrant flowers and fruits come under the category of etaerio of berries. The antimicrobial studies have gained a worldwide acceptance at many levels and several plants are being subjected to these antibacterial studies bringing us closer and closer to the idea that plants are the most reliable sources of natural drugs that can be competent and reliable enough to fight the war against bacteria. As part of the project it was decided to consider the above-mentioned plants as a subject for antimicrobial studies.

The leaves and stem barks of *Canaga odorata* were collected from Kottayam district and that of *Annona glabra* were collected from Kumarakam area where they have been reported to be in abundance. A voucher specimen of the plant has been deposited in the herbarium of department of P.G.studies in S.B.College, Changanacherry. The leaves of the plants were washed thoroughly in cold normal water and dried in shade at 28±2°C after which they were powdered carefully in a dry grinder. 20mg of the powdered material was taken and extracted at room temperature 25±2°C on the rotary shaker for around 48 hrs in three different mediums of petroleum ether, methanol and distilled water. The extracts were then prepared as stated in the previous chapter of
materials and methods. The extracts were stored in dry glass containers for further evaluation.

**Antibacterial Screening:**

**Test organisms:**

The antimicrobial activity was individually tested against Gram-positive Bacteria i.e. *Staphylococcus aureus*, and Gram negative bacteria *Escherichia coli, Klebsiella pneumonia, and Serratia marcescens*. All the test strains were obtained from the P.G. and Research Department of Botany and Biotechnology, S.B.College, Changanacherry, and maintained on Mueller Hinton Agar (Merck.), and was subcultured every two weeks.

**Culture medium and inoculums:**

The stock cultures of microorganisms used in this study were maintained on Nutrient Agar slants at 4°C. Inoculum was prepared by suspending a loopful of bacterial cultures into 10mL of nutrient broth and was incubated at 37°C for 24 hrs. $10^6$ cfu /ml. bacterial cells with O.D.1 were taken to study antibacterial activity.

**Antibacterial activity assay:**

The disc diffusion method was adopted to test the antibacterial activity. The disc diffusion method was used to determine the growth inhibition of bacteria by the plant extract according to Kirby Baeur’s method of checking sensitivity (Baeur’s method, 1966). Methods used for testing the antibacterial sensitivity of the plant extracts have been discussed in detail in the previous chapter of materials and methods. The paper discs were loaded with three concentrations of the extract i.e.20 µl (200µg), 40µl (400µg) and
60µl (600µg) from the above prepared stock of the extract and evaporated to dryness. In case of *Annona glabra* for both leaf and stem, it was seen that the concentrations of the extract taken at lower levels of 400 and 600 µg failed to yield any results and hence it was needed to take a concentration at a higher level. Hence, an amount of 1gm, 2gm, and 4gm were taken to test the sensitivity of the extracts in three mediums against the bacteria. Pure solvents of Petroleum ether, distilled water and Methanol were taken as the negative control. The antibiotic discs of the standard drug Chloramphenicol (30µg) and Amikacin (30 µg) were used as the positive control to compare the results of experimental plant.

**Culture medium:**

The culture medium was prepared as mentioned in the previous chapter of materials and methods. All experiments were done in triplicates and all measurements were taken in mm. Standard deviation was calculated for every measurement and recorded to understand the variation in the individual results.

**Results:**

**Antimicrobial analysis:** The bioactivity of the extracts was determined by observing and measuring the clear zone of inhibition that appeared around each disc loaded with the extract. The results have been shown in Table 1.1 along with those extracts and concentration that showed maximum diameter of the clear zone of inhibition. In case of *Cananga*, the extracts derived in PE and water showed no activity for both the leaf and the bark extracts, while the ones derived in methanol extracts showed the maximum activity. For Cananga the derivation at 200µg to 600µg was enough for the bacteria to
respond. At this concentration, in case of leaf, Serratia responded to the extract while no other bacteria taken for study showed any response. But in case of the bark the methanolic extract alone showed the response to the bacteria taken for study, while the extracts of PE and water showed absolutely no response. The sensitivity of the antibiotic discs as positive control and the pure solvents at 200µl as negative controls have been shown in Table 1.2

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Zone of inhibition</th>
<th>Methanol</th>
<th>Water</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pet Ether</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200ugm 400ugm 600ugm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serratia</td>
<td>- - -</td>
<td>20±1.2</td>
<td>22±1.2</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>- - -</td>
<td>16±1.3</td>
<td>17±0.8</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>- - -</td>
<td>10±1.6</td>
<td>11±0.9</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Zone of inhibition</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pet Ether</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200ugm 400ugm 600ugm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serratia</td>
<td>- - -</td>
<td>13±1.3</td>
<td>15±0.9</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>- - -</td>
<td>16±1.3</td>
<td>17±0.8</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>- - -</td>
<td>10±1.6</td>
<td>11±0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pet Ether</td>
</tr>
<tr>
<td></td>
<td>1gm 2gm 4gm</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>- - -</td>
</tr>
<tr>
<td>Serratia</td>
<td>- - -</td>
</tr>
</tbody>
</table>
Mean of inhibition zone as shown by the crude extracts of bark of (Annona glabra)

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Pet Ether</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1gm</td>
<td>2gm</td>
<td>4gm</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>12±0.4</td>
<td>11±0.8</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serratia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean of inhibition Zone of Antibiotic discs against bacteria

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Ciproflaxin</th>
<th>Gentamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>32±0.3</td>
<td>19.5±0.7</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>17±0.48</td>
<td>17±0.48</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>17±0.48</td>
<td>17±0.48</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>17±0.48</td>
<td>17±0.48</td>
</tr>
<tr>
<td>Serratia</td>
<td>17±0.48</td>
<td>17±0.48</td>
</tr>
<tr>
<td>Proteus</td>
<td>17±0.48</td>
<td>17±0.48</td>
</tr>
</tbody>
</table>

Discussion:

In this study, only the methanolic extracts of the bark of Cananga odorata showed an extremely convincing result against all the three bacteria studied. The petroleum ether extracts of both the leaves and bark of Cananga odorata showed absolutely no result against the human pathogens, maybe the concentration at a higher level needs to be studied, which was not done in this case.
Chapter 5

Discussion

The plants are having special ability to synthesize aromatic substances mainly secondary metabolites which are cleverly used by plants as defensive molecules against predation by microorganisms, insects and herbivores. Some of these defensive molecules give plants their medicinal values which are appreciated by human beings. Traditional medicine, like orthodox medicine has its own methods and techniques of application which however aims at healing diseases. (Wurochekke et al, 2008). Early publications have pointed out that relatively little of the world’s plant biodiversity has been extensively screened for bioactivity and that very little of the estimated microbial biodiversity has been available for screening. (Dougheri et al, 2007). With the growing realization that the chemical diversity of the natural products is a better match to that of the synthetic drugs and also with the emergence of multi drug resistant pathogen, (Feher and Schmidt,2003) , the interest in drug discovery from natural chemical compounds seem to be increasing again (Galm and Shen,2007)

In this study, both the methanol and the petroleum ether extracts of *Uvaria narum* showed considerable antimicrobial activity against the harmful human pathogens, and on an average Petroleum ether extract was found to be the most active among the three extracts in its activity against the bacteria. The HPTLC screening too showed the presence of around 11 compounds in PE extracts. In fact the activities at times were found to be higher than the standard antibiotics as Chloramphenicol and Amikacin (30µg each) screened under similar conditions. As per the standard table of sensitivity test (Sharma Kanika,2011), a zone with a diameter above 10mm is considered as intermediately sensitive while one above 15mm is said to be sensitive and the zone below 10 is to be marked as resistant.

This study showed that the Petroleum ether extracts exhibited a very good inhibitory activity against five out of six bacteria that were taken (22mm-38mm inhibition zone at 200 µg ) and its activity against *Escherichia coli* that causes important gastrointestinal disorders has been really effective and was much higher than the antibiotics
Chloramphenicol and Amikacin. While *Klebsiella pneumonia* and *Proteus vulgaris* were competent enough with the antibiotics, *Serratia* showed absolutely no sensitivity to Petroleum ether extracts in any of its triplicates.

The effect of the Methanolic extract was the most profound in *Staphylococcus aureus*, (which is an important agent of Skin Infection) with the extracts showing the maximum activity at 200 µg with an inhibition zone of 27mm. This could be a possible reason to explain its popular use in traditional medicines against skin ailments. *Serratia* showed sensitivity to the methanolic extract alone (15mm inhibition zone at 200 µg). The methanolic extract showed inhibition to all other bacteria but in a lesser amount than the Petroleum ether extract.

Taking the result from the phytochemical analysis, it can be ascertained that the hydrophobic compounds of steroidal nature present in the Petroleum ether extract must have conferred the antimicrobial property to the extract. (Joji Reddy et al, 2012).

In case of *Cananga odorata*, the methanolic extracts of both leaves and bark showed a high level of activity microbial activity but were at a slightly higher concentration than the *Uvarium* extracts. The Aqueous and the Petroleum ether extracts showed very negligible activity and it could be that these extracts might have showed a better activity had the concentration of the extracts been taken at a higher level.

In *Annona glabra*, the leaves and the bark extracts showed a good antimicrobial property against the bacteria that were taken, albeit, at a much higher concentration than was expected. In fact all the three extracts of aqueous, petroleum ether and methanol of both leaves and bark showed a good antimicrobial activity.

The phytochemical analysis of *Cananga* showed a good amount of presence of secondary metabolites that must have rendered it, its antimicrobial properties. Almost all the metabolites tested were found to be present except the phenols. The petroleum ether extracts showed the minimum presence of the secondary metabolites.

The phytochemical analysis of *Annona glabra* too was supporting in nature and that too showed the presence of secondary metabolites. While *Artabotrys hexapetalous* showed
the presence of a vast range of secondary metabolites in all its extracts and also showed the concomitant presence of phenols in its Bark.

**Conclusion:**

Vast literature of the chosen plants from this family is not available which means this plant has not been brought as yet to the mainstream of research. From the above experiments it can be seen that the methanolic and Petroleum ether extract of *Uvaria narum* showed extensive antimicrobial activity against considerable bacteria specially against E.coli. and methanolic extract against *Staphylococcus aureus*. The preliminary phytochemical screening of the plant revealed the presence of several phytochemical compounds in as Cardiac glycosides, Tannins, Triterpenoids and Steroids in its methanolic extract and Triterpenoid and Steroid in sufficient amount in its Petroleum ether extract that must have conferred its antimicrobial property. It’s been a highly encouraging study as the positive results have shown that further studies should be conducted on this plant to derive the lead molecules that have actually conferred this antimicrobial property on *Uvaria narum*. (Trease, 2011).
Antibiotic discs (Positive control) showing activity against Serratia

Antibiotic discs (Positive control) showing activity against Proteus

Antibiotic discs (Positive control) showing activity against Klebsiella

Negative control of H₂O, CH₃OH and PE showing no activity against Proteus
U. narum (Leaf) CH₃OH. against Enterobacter

U. narum (Leaf) CH₃OH. against Proteus

U. narum (Leaf) PE against Proteus
Bark (A. glabra) CH$_3$OH activity against Proteus (A. glabra) CH$_3$OH activity against Staphylococcus

A. glabra (Bark) CH$_3$OH activity against E. Coli A. glabra (Bark) H$_2$O activity against Klebsiella

A. glabra (Bark) CH$_3$OH activity against Klebsiella A. glabra (Leaf) CH$_3$OH activity against (Proteus)
Anti fungal effect of the Three Extracts of leaves of *Uvaria narum* on the Plant Fungus “Fusarium”

Since it was suggested to study the antifungal aspect of *Uvaria* as well, it was decided that an antifungal study would also be conducted on the leaf to study its bioactivity against *Fusarium colletotrichum*, specially the variety that very significantly affected the garden beans type.

The Fusarium species was isolated as per the norms written in the introduction part. The hyphae was allowed to be established on the potatoe dextrose medium and then was recultured on slants to be maintained for the subsequent uses.

Around 1000mg of the substance was taken and dissolved in 1ml. of the desired extracts and then they were thoroughly stirred to dissolve it. Then the different concentrations of the extract were taken and mixed in the petridishes along with the PDA medium. This method is called Poison food Technique.

The results were in good correlation to those shown by these extracts in the case of antibacterial studies where the Petroleum ether showed the maximum inhibition of the fungal hyphae. This is in comparison to the control that got filled with the fungal mycelium.

The results have been given in the tables which show that the *Uvaria narum* extracts in petroleum ether could give the maximum inhibition to the growth of the fungal hyphae.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatments (500microgm/500microltr)</th>
<th>Radial growth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether extract</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>Methanol extract</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous extract</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Inhibition zone of the mycelium
Fusarium spores

Pestalotia spores
Thievalopsis

Curvularia


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