

Research Article

Phytochemical and Antimicrobial Studies of Medicinal Plant *Piper longum* Linn.

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Available online: 1st June 2014

ABSTRACT

The plant *Piper longum* Linn. was screened for secondary metabolites and anti microbial activity. Standardization of the solvent system and quantities required to get maximum separation of individual secondary metabolites was done. The methodology used showed that excellent separation of compounds was obtained. The various extracts of the fruit showed the presence of, 6 Essential Oils, 3 bitter principles, 13 arbutin, 7 steroids, 4 Coumarins, 4 alkaloids, and 4 sterols. The development of such fingerprint for the fruits of *Piper longum* Linn is useful in differentiating the species from the adulterant and also acts as biomarker for this plant in the Pharmaceutical industry. The in vitro antibacterial activity was performed by Agar ditch, Agar disc diffusion and Agar well diffusion method. The phytochemical extracts obtained after confirmation with HPTLC were examined for anti microbial activity against the pathogens infecting the respiratory tract i.e. *Staphylococcus aureus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*. In addition powder was tested for its minimum inhibitory concentration and further solvent fractions were tested for the zone of inhibition. *Klebsiella pneumonia* was the most resistant bacteria, while *Staphylococcus aureus* was the most susceptible.

Key words: *Piper longum* Linn., HPTLC, anti-bacterial activity, secondary metabolites.

INTRODUCTION

Plants play a vital role in the existence and survival of man. Besides being a source of food and shelter they provide a tremendous reservoir of various chemical substances with potential therapeutic ¹. The plant kingdom harbours an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Furthermore the active components of herbal remedies have the advantage of being combined with many other substances that appear inactive. However these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components ². Recent approach is the utility of natural products as sources of novel structures of therapeutic value ³.

Antibiotics resistance has become a global concern⁴. Multiple antibiotic resistance micro organisms are emerging and constituting major public health problem^{5,6}. Due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced the scientist to search for new anti microbial substances from various sources like medicinal plants. Ethno botanical record suggests that the plants are the sleeping giants of pharmaceutical industry⁷. The screening of plants extracts and plant products for antimicrobial activity has shown the higher plant represent a potential source of novel prototype⁸.

Numerous studies have identified compounds within herbal plants that are antibiotics ⁹. Traditional healing systems use plants as a source of new antibiotics ¹⁰. These

results indicate the need for further research in to traditional health systems ¹¹ the need of the hour is to screen medicinal plants for promising biological activity. The current study was oriented towards phytochemical screening of *Piper longum* and also screening for its antimicrobial activity against pathogens of the respiratory tract. It has been observed that the plant is used in many ayurvedic preparation. Hence study was carried out to extract the secondary metabolite responsible for the medicinal property and determine as to which component is responsible for the activity against the pathogens of the respiratory tract.

MATERIAL AND METHODS

The present study was concentrated on the preliminary screening, qualitative screening of metabolites and antibacterial activity from the fruits of *Piper longum* Linn. The fruits of the plant *Piper longum* Linn. were collected in the month of May - June from Sindhudurg district, Maharashtra, India. They were identified and authenticated at Blater's Herbarium, St Xavier's College, Mumbai. The fruits were air dried pulverized and used for analysis.

Preliminary screening of secondary metabolites: The shade dried plant material was powdered using mixer grinder and subjected to soxhlet extraction with petroleum ether, chloroform, 95% ethanol and distilled water for 18 h in the order of increasing polarity of solvents. The condensed extracts were used for preliminary screening of

Table-1: Preliminary screening of secondary metabolites from extract of fruits of *Piper longum* Linn.

| Sr. No. | Secondary metabolites | Test | Inference |
|---------|-----------------------|------------------------|-----------|
| 1. | Alkaloids | Dragendorff' test | ++ |
| | | Wagner's test | ++ |
| | | Mayer's test | ++ |
| 2. | Cardiac Glycosides | Molisch test | +++ |
| 3. | Saponins | Frothing test | + |
| 4. | Steroids | Shinoda test | + |
| 5. | Anthraquinones | Mg- acetate test | + |
| 6. | Flavanoid | magnesium ribbon test | ++ |
| 7. | Tannins | FeCl ₃ test | - |

Table 2: Effect of extracted solvents on various microorganisms by agar ditch method.

| Sr.No. | Extracts | <i>Staphylococcus aureus</i> | <i>Streptococcus pyogenes</i> | <i>Klebsiella pneumoniae</i> |
|--------|--------------------|------------------------------|-------------------------------|------------------------------|
| 1. | Pet-ether extract | +++ | ++ | - |
| 2. | Methanolic extract | +++ | ++ | - |
| 3. | Hot water extract | + | + | - |
| 4. | Cold water extract | ++ | + | + |

(+ Indicates Inhibition - Indicates No Inhibition)

Table 3: Effect of extracted phytochemicals on various microorganisms by agar ditch method.

| Sr.No. | Phytochemical Compound | <i>Staphylococcus aureus</i> | <i>Streptococcus pyogenes</i> | <i>Klebsiella pneumoniae</i> |
|--------|------------------------|------------------------------|-------------------------------|------------------------------|
| 1. | Essential oils | +++ | +++ | + |
| 2. | Sterols | ++ | ++ | - |
| 3. | Steroids | +++ | +++ | + |
| 4. | Cardiac-glycosides | +++ | +++ | + |
| 5. | Anthraglycosides | ++ | + | + |
| 6. | Flavonoids | + | - | - |
| 7. | Alkaloids | ++ | + | - |
| 8. | Anthraquinones | + | - | - |

(+ Indicates Inhibition - Indicates No Inhibition)

Table 4: Minimum inhibitory concentration(MIC)in accordance to test microorganisms by gradient plate technique.

| Sr.No. | Parameters | <i>Staphylococcus aureus</i> | <i>Streptococcus pyogenes</i> | <i>Klebsiella pneumoniae</i> |
|--------|----------------------------------|------------------------------|-------------------------------|------------------------------|
| 1. | Length of Growth (mm) | 39 | 41 | 60 |
| 2. | Total Length of Streak (mm) | 75 | 75 | 75 |
| 3. | Minimum Inhibitory Concentration | 5.2 | 5.47 | 8 |

phytochemicals such as alkaloids (Wagner, Mayers and Dragendorff's tests),

flavonoids (Shinoda and NaOH tests), cardiac glycosides (Keller-Kiliani, conc.H₂S₀₄ tests), saponins (foam and haemolysis tests), sterols (Lieberman-Burchard and Salkowski tests) and tannins (gelatin test) were carried out^{12,13}

Separation of secondary metabolites by HPTLC

Chromatographic conditions: Chromatography was performed on silica gel 60 F254 HPTLC plates (10 x 20 cm; 0.25 mm layer thickness). Samples and standard compounds were applied to the layer as 8 mm wide bands positioned 10 mm from the bottom of the plate, using an automated TLC applicator Linomat V (Camag, Muttenx, Switzerland) with nitrogen flow providing delivery from the syringe at a speed of 10 s/μL.

HPTLC study

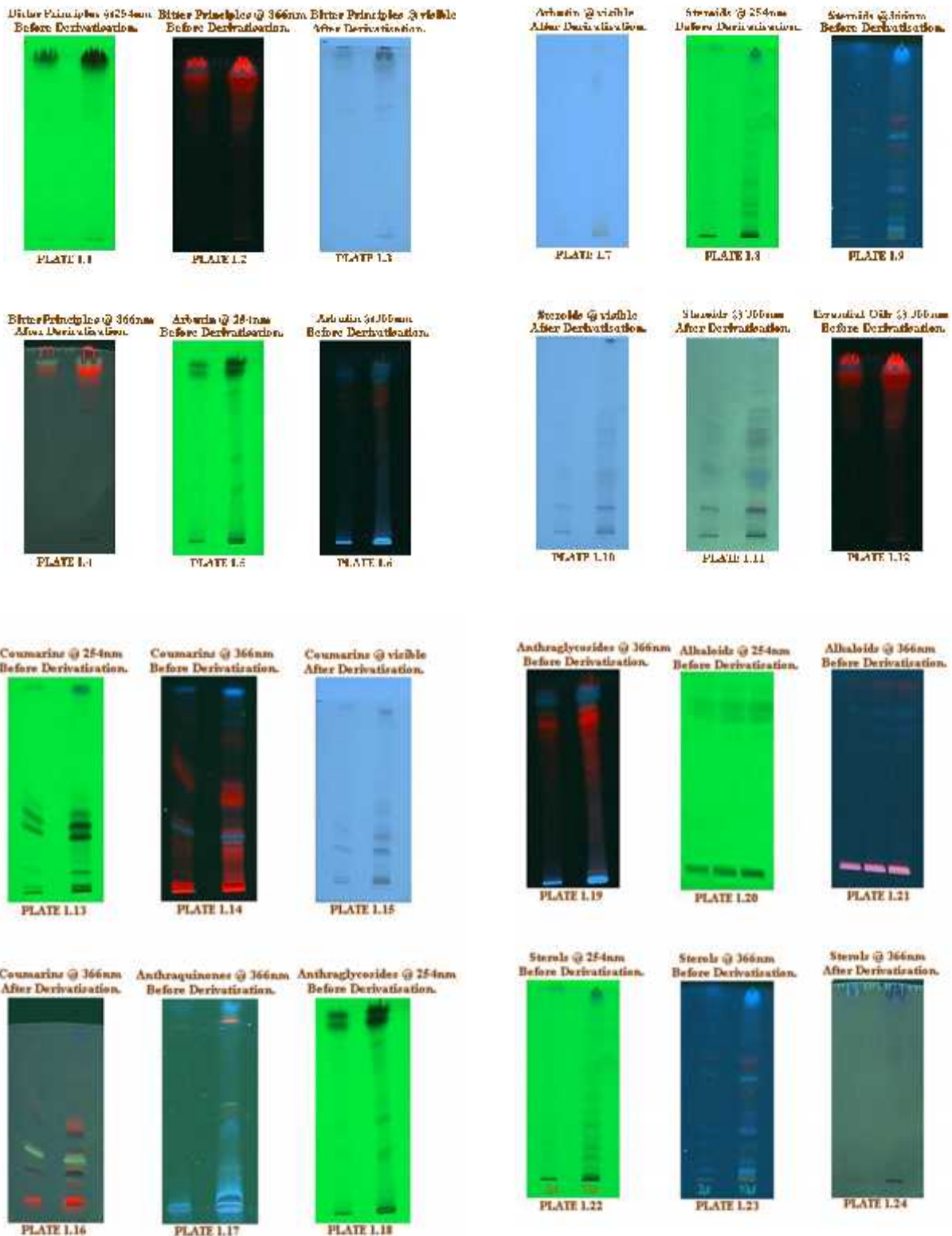
Anthra-glycosides, arbutin, bitter principles: Powdered material (1 g) was extracted by heating on a water bath for

15 minutes with 5 mL methanol. 2 μL and 10 μL of the filtrate were applied to the chromatogram. The bitter principles spots were separated using solvent system: 5% acetic acid and anisaldehyde H₂SO₄ as spray reagent¹³.

Alkaloids: The powdered material was wet with a half diluted NH₄OH and lixiviated with EtOAc for 24 h at RT. The organic phase is separated from the acidified filtrate and basified with NH₄OH (pH 11- 12).It was extracted with chloroform (3X), condensed by evaporation and used for chromatography. The alkaloid spots were separated using the solvent mixture chloroform and methanol (15:1). The colour and R_f values of the separated alkaloids were recorded both under ultraviolet (UV 254 nm) and visible light after spraying with Dragendorff s reagent¹³.

Saponins: Two grams of powdered material was extracted with 10 mL 70% EtOH by refluxing for 10 min. The filtrate was condensed, enriched with saturated n-BuOH, and thoroughly mixed. The butanol was retained, condensed and used for chromatography. The saponins were

Plate 1.1-1.24: HPTLC of Individual Components of Piper Longum Linn.



separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and Rf values of these spots were recorded by exposing chromatogram to the iodine vapours¹³ Essential oils, coumarins, phenols carboxylic acids and valepotriates: Powdered material (1 g) was extracted by

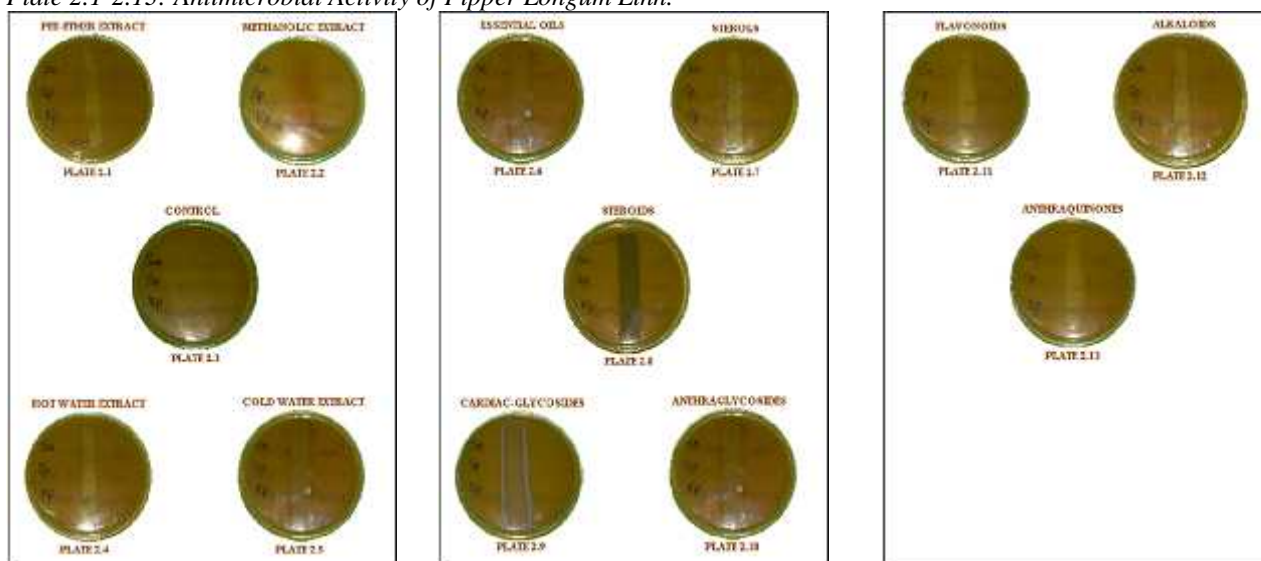
heating under reflux for 15 minutes with DCM. The filtrate was evaporated to dryness and the residue was dissolved in 1 mL toluene, 2 µL and 10 µL of the filtrate were applied to the chromatogram. Best solvent system used was toluene: ethyl acetate (9.3:0.7) and 5% vanillin in H₂SO₄ as spray reagent¹³

Table 5: Inhibition zones (IZ) in mm in accordance to test microorganisms by agar disc diffusion method.

| Sr. No. | Extracts | <i>Staphylococcus aureus</i> | <i>Streptococcus pyogenes</i> | <i>Klebsiella pneumoniae</i> |
|---------|--------------------|------------------------------|-------------------------------|------------------------------|
| 1. | Alcoholic Extract | 9 | 8 | 6 |
| 2. | Chloroform Extract | 7 | 7 | 5 |
| 3. | Aqueous Extract | 6 | 6 | 5 |
| 4. | Ampicillin | 16 | 16 | 9 |
| 5. | Chloro-m-phenicol | 12 | 11 | 8 |

Table 6: Inhibition zones (IZ) in mm in accordance to test microorganisms by agar well diffusion method.

| Sr. No. | Extracts | <i>Staphylococcus aureus</i> | <i>Streptococcus pyogenes</i> | <i>Klebsiella pneumoniae</i> |
|---------|--------------------|------------------------------|-------------------------------|------------------------------|
| 1. | Alcoholic Extract | 9 | 7 | 6 |
| 2. | Chloroform Extract | 8 | 6 | 5 |
| 3. | Aqueous Extract | 6 | 6 | 4 |
| 4. | Ampicillin | 14 | 14 | 12 |
| 5. | Chloro-m-phenicol | 12 | 12 | 8 |

Plate 2.1-2.13: Antimicrobial Activity of *Piper Longum* Linn.

Steroids: Air dried plant powder was extracted with pet ether (40-60 °C). Hot methanol was added to the green coloured extract which was obtained. This was then evaporated to reduce the volume. The steroids spots were separated using solvent mixture: DCM:diethyl ether: methanol: water (7.7:1.5:0.8:0.12) and methanolic H₂SO₄ as spray reagent¹³.

Terols: Two grams of powdered material was extracted with 10 mL methanol in water bath (80°C /15 min). The condensed filtrate was used for chromatography. The sterols were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and R_f values of these spots were recorded under visible light after spraying the plates with anisaldehyde-sulphuric acid reagent on heating at 100 °C for 6 min¹¹.

Antimicrobial Screening: For Aqueous extraction, 10 g of air dried powder was added to distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume ¼ of the original volume¹⁴. It was then

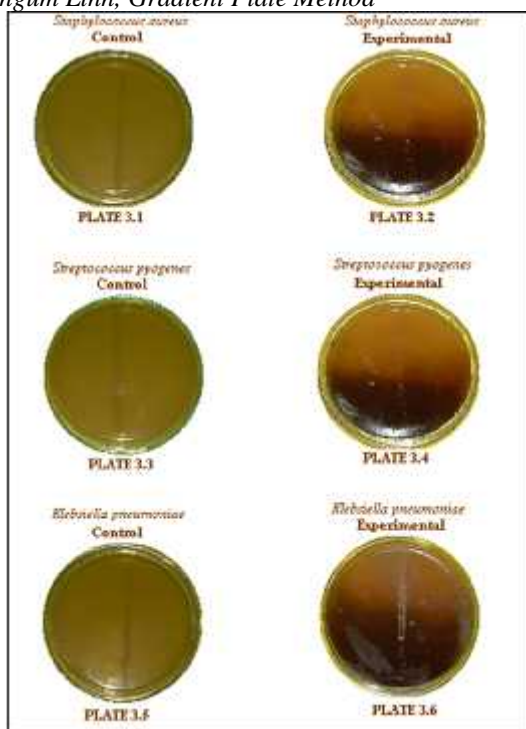
autoclaved at 121 °C and at 15lbs pressure and stored at 4 °C.

For solvent extraction, 10g of air dried powder was taken in 100 ml of organic solvent (Methanol or Ethanol and Chloroform) in a conical flask plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24h the supernatant was collected and the solvent was evaporated to make the final volume ¼ of the original volume¹⁴ and stored at 4 °C in air tight bottles.

Microorganisms: In vitro antimicrobial activity was examined for aqueous, alcoholic and chloroform extract and the various extract of individual secondary metabolites which were confirmed by HPTLC. Microorganisms were obtained from Department of Microbiology, Institute of Science, Mumbai. Microorganisms were maintained at 4 °C on nutrient agar slants. Among the 3 microorganisms investigated, the 2 gram positive were *Staphylococcus aureus*, *Streptococcus pyogenes* and 1 gram negative bacteria was *Klebsiella pneumoniae*.

Antimicrobial Assay: The antimicrobial assay was performed by three different methods viz Agar Ditch Method¹⁵, Agar Disc Diffusion Method¹⁶ and Agar Well Diffusion Method¹⁵ for aqueous and solvent (Chloroform and Alcohol) extract. The molten Mueller Hinton Agar (Hi

Plate 3.1-3.6 : Antimicrobial Activity of *Piper Longum* Linn, Gradient Plate Method



Media) was inoculated with 100 μ l of inoculum (1×10^8 Cfu) and poured in sterile petri plates. For Agar Disc diffusion Method, the disc (0.7 cm) was saturated with 100 μ l of the test compound and allowed to dry and was introduced on the upper layer of the seeded Agar plate. For Agar Well Diffusion method, a well was prepared in the plates with the help of a cork-borer (0.85 cm). 100 μ l of the test compound was introduced into the well. The plates were incubated overnight at 37 °C. Microbial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain controls were maintained where pure solvents were used instead of extract. The result was obtained by measuring the zone diameter. The experiment was conducted in triplicate and the mean values are represented. The results were compared with the standard antimicrobics Chloramphenicol (10 μ g/ml) and Ampicillin (10 μ g/ml). Gradient plate was carried out by method of Hunt and Sandham¹⁷.

RESULTS

Natural products are the main sources of bioactive molecules and have played a major role in discovery of lead compounds for the development of drugs for treatment of human diseases¹⁸. The current study was oriented towards the screening of *Piper longum* Linn. for secondary metabolites. Preliminary Phytochemical analysis revealed the presence of alkaloids, steroids, flavanoids, anthraquinones, glycosides, cardiac glycosides, and essential oils (Table 1). HPTLC screened showed the presence of different types of bitter principles, anthraquinone saponins, coumarins, phenolcarboxylic acids and steroids in addition to the compounds detected by preliminary screening. Finger printing was carried out

for petroleum ether extract and methanolic extract, the separation was found best in petroleum ether extract as compared to methanolic extract.

HPTLC Screening: HPTLC screening shows presence of Anthraglycosides, Alkaloids, Cardiac glycosides, Bitter Principles, Flavonoids, Steroids, Anthraquinones, Saponins, Essential oils, Coumarins and Phenolcarboxylic acids and Sterols. (Plate no: 1.1-1.24)

Bitter principles: The HPTLC chromatogram can be observed best at 366nm. The major compounds separated was seen at Rf =0.81, Rf =0.85 and Rf =0.87.

Arbutin: The HPTLC chromatogram can be best observed under UV (254nm). The sample concentration (10 μ l) is sufficient to generate the compounds. Spectra were observed at different Rf (0.29, 0.28, 0.43, 0.46, 0.81, 0.83, 0.17, 0.19, 0.55, 0.71, 0.74, 0.73, 0.77).

Steroids: The HPTLC chromatogram can be observed best at any wavelength (254nm, 366nm). Spectra were observed at different Rf (0.19, 0.39, 0.51, 0.59, 0.64, 0.71, 0.82).

Essential oils: A wide variety of essential oils were found to be present and best observed at 366nm.

Coumarins & phenolcarboxylic acids: Number of coumarins and phenolcarboxylic acids was found to be present in the plants. Compounds with Rf =0.17, Rf =0.25, Rf =0.29, and Rf =0.30 was observed to be best separated at 550nm, post derivatization.

Anthraglycosides: Anthraglycosides was found to be best separated with optimum sample application of 10 μ l at 366nm. There is no need for derivatization since compounds are seen best separated before derivatization. Best solvent system - Chloroform: Methanol (9:1).

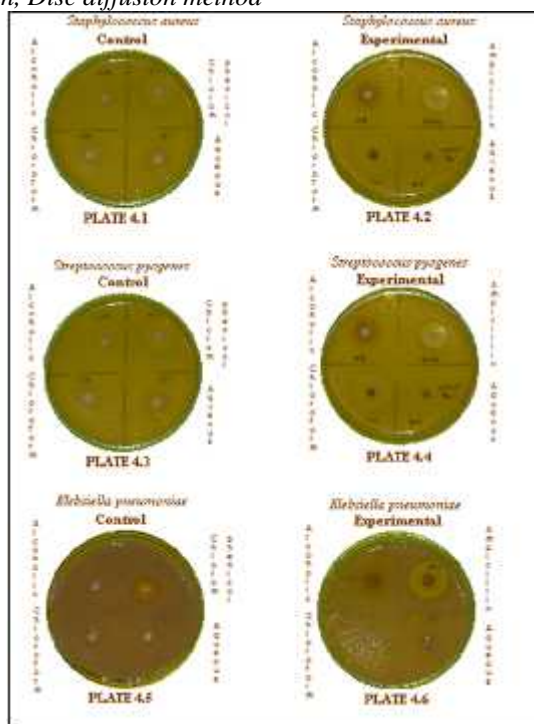
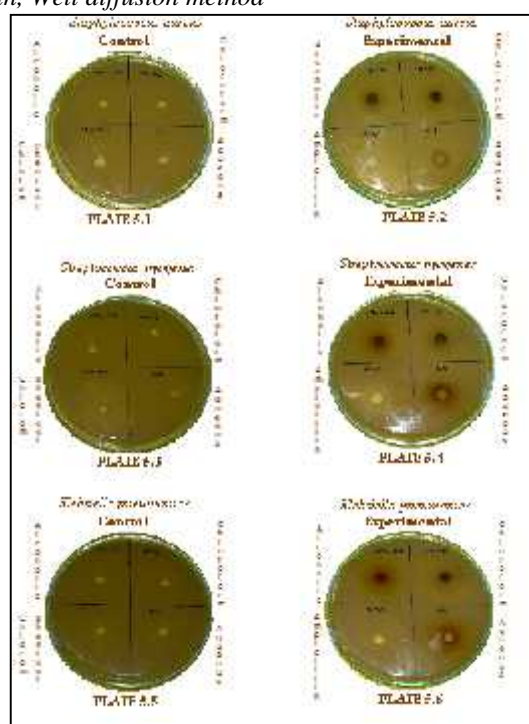
Alkaloids: Alkaloids were found to be best observed at 254nm with optimum sample application of 10 μ l. Spectra was observed at different Rf (0.64, 0.71, 0.76, 0.83).

Sterols: Sterols were found to be best observed at 366nm with optimum sample application of 10 μ l. The best compounds seen to be separated were at Rf = 0.18, Rf =0.26, Rf =0.31 and Rf = 0.65

DISCUSSION

Phytochemical Studies: Natural products are the main sources of bioactive molecules and have played a major role in discovery of lead compounds. The curative properties of medicinal plants are due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols *etc.* The preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development¹⁹. A vast array of secondary metabolites was found to be present in the plant under study. The various extract of the fruit showed the presence of 9 cardiac Glycosides, 6 Essential Oils, 3 bitter principles, 13 arbutin, 7 steroids, 4 coumarins, 4 alkaloids and 4 sterols.

Bitter principles: 3 bitter principles have been separated. The HPTLC chromatogram can be observed best at 366nm. Since the compound is highly polar in nature it can be seen just below the solvent front. Due its nature of polarity the chromatogram can further be used for

Plate 4.1-4.6: Antimicrobial Activity of *Piper Longum* Linn, Disc diffusion methodPlate 5.1-5.6: Antimicrobial Activity of *Piper Longum* Linn, Well diffusion method

preparative HPTLC. However there is still need for improvements and modification in the process of extraction in order to optimize the concentration of separation of bands and also need for the use of different solvent system to separate the structurally similar compounds. Bitter principles are reported to have Gastro protective effects²⁰.

Arbutin: 13 types of different arbutin bands have been separated. The HPTLC chromatogram can be best observed under UV (254nm). The sample concentration (10 μ l) is sufficient to generate the compounds. However there is still need for improvement and modification in the process of extraction in order to optimize the concentration of separation of bands and also for the use of different solvent system to separate the structurally similar compounds. Arbutin is also an inhibitor of melanin formation and is use in some skin-lightening It is used as an anti-infective for the urinary system as well as a diuretic. Arbutin (2.5, 12.5, or 50 μ g/mL) incubated for four days weakly inhibited the growth of human colon carcinoma HCT-15 cells²¹

Steroids: Seven types of steroids have been separated. The HPTLC chromatogram can be observed best at any wavelength (254nm, 366nm). The sample concentration 2 μ l is found to be more than suitable as compared to 10 μ l. There is no need for derivatization since compounds are seen best separated before derivatization. Extraction procedure for sample extraction is highly suitable for optimize the concentration of separation of bands. Best solvent system: Dichloromethane: Diethyl ether: Methanol: Water (7.7:1.5:0.8:0.12).

Essential oils: A wide variety of essential oils were found to be present and best observed at 366nm. The sample

concentration (10 μ l) chosen was found to be best for separation of components. The extraction procedure is also suitable to extract essential oils. There is no specific need for derivatization, as they are seen to be best separated before derivatization. Best solvent system: Toluene: Ethyl acetate (9.3: 0.7). Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries²² Volatile compounds from plants, especially essential oils have been demonstrated to possess potent antifungal, antibacterial, insecticidal and nematocidal activity²³⁻²⁷.

Coumarins & phenol carboxylic acids: Number of coumarins and phenol carboxylic acids was found to be present in the plants. The HPTLC chromatogram was best observed at 366nm with optimum sample application of 10 μ l. Derivatization is necessary since some compounds cannot be seen before derivatization. Compounds best separated at 550nm, post derivatization. The coumarins are of great interest due to their biological properties. In particular, their physiological, bacteriostatic and anti-tumour activity makes these compounds attractive for further backbone derivatization and screening as novel therapeutic agents²⁸.

Anthraglycosides: Anthraglycosides was found to be best separated with optimum sample application of 10 μ l at 366nm. There is no need for derivatization since compounds are seen best separated before derivatization. Best solvent system - Chloroform: Methanol (9:1).Anthraglycosides have been used for the treatment of hepatitis and symptoms of the reduction in leucocytes²⁹

Alkaloids: Alkaloids were found to be best observed at 254nm with optimum sample application of 10 μ l. Best solvent system- Toluene: Ethyl acetate: Diethyl amine

(7:2:1). There is no need for derivatization since compounds are seen best separated before derivatization. Plant alkaloids usually have profound physiological actions in humans with nervous system effects being the most prominent³⁰.

Sterols: Sterols were found to be best observed at 366nm with optimum sample application of 10 μ l. The best compounds seen to be separated were at 366nm, before derivatization. Need for improvements in derivatization agent and solvent system for best possible separation. Solvent system: Toluene: Diethyl ether: Ethanol: Acetic acid (4.8:4.0:4.0:0.5). Phytosterol and stanol (or Phytosterol) consumption reduces intestinal cholesterol absorption, leading to decreased blood LDL-cholesterol levels and lowered cardiovascular disease risk. It also exhibits inhibitory actions on lung, stomach, as well as ovarian and breast cancer³¹.

Anti Microbial Studies: The presence of anti microbial substance in the plants is well established³². Plants have provided a source of inspiration novel drugs compounds a plants derived medicine has made significant contribution towards human health. Successive isolation of botanical compounds from plant materials is largely dependent on the type of solvent used in the extraction procedure. The traditional use primarily water as solvent but we found in the study the plant extract by alcohol provided more consistent antimicrobial activity as compared to those extracted by water.

Agar Ditch Method: Phytochemical screening of crude extracts reveals the presence of various phytochemicals like steroids, essential oils, cardiac glycosides, anthraglycosides, flavonoids, anthraquinones, alkaloids and sterols. These bioactive compounds are reported to be responsible for antimicrobial activities in plants³³⁻³⁴. The results from Agar Ditch Method showed that the maximum inhibition was due to cardiac glycosides, anthraglycosides, sterols, steroids and essential oils. Mild inhibition was also observed by alkaloids, anthraquinones, flavonoids [Table 3] (Plate no:2.1-2.5). Similar results have been reported for alkaloids³⁵, flavanoids³⁶ and phenolic acid³⁷. It was observed that flavanoids were active on *Staphylococcus aureus*.³⁸⁻³⁹ Cold water extract is more effective than hot water extract whereas both pet-ether and methanolic extracts are effective [Table 2].

These extracts even showed a high antibacterial activity against gram-negative bacteria *Klebsiella pneumoniae*. Similar reports have been observed by Stoyanova A et al⁴⁰. Although some paper reports that mono and sesquiterpene hydrocarbons do not show any or only very weak antimicrobial effects against bacteria, fungi and yeast⁴¹⁻⁴². We have found a high activity of these groups of compounds. The extracts in this work exhibited a broad-spectrum activity. However, gram-positive bacteria have been shown to be more susceptible than gram negative ones. Bacteria are prokaryotes with thin cell wall and relatively simple genetic system, which enhance easy penetration of bioactive substances, leading to manipulation of genetic system as a result of bioactive interruption. Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of

antimicrobials as does the gram-negative bacteria⁴³. The structural composition and arrangement of the cell wall of gram-negative bacteria is such that does not easily permit the penetration of the bioactive compounds⁴⁴. Gram-positive bacteria are of a single layer, whereas the gram-negative cell wall as multi-layered structure⁴⁵. Alternatively, the passage of the active compounds through the gram-negative cell may inhibited. It is thought that observed differences may result from the doses used in this study. In addition microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains⁴⁶⁻⁴⁷. It may be also noted that some compounds do not shows antimicrobial activity, this may be because the active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed⁴⁸. Lack of activity can thus only be proven by using large doses⁴⁹. Alternatively, if the active principles are present in high enough quantities, there could be other constituents exerting an antagonistic effects or negating the positive effects of the bioactive agents⁵⁰. With no antimicrobial activity, extracts may be active against other bacterial species, which were not tested⁵¹.

Gradient Plate Technique: Gradient Plate Technique helped to study the Minimum Inhibitory Concentration of the plant material. The MIC for *Staphylococcus aureus* and *Streptococcus pyogenes* was found to be 5.2 and 5.47 respectively, which shows that the powder of *Piper longum* is active enough in inhibiting the *Staphylococcus aureus* strain and *Streptococcus pyogenes* strain against the respiratory infections, whereas MIC of *Klebsiella pneumoniae* (Plate no:3.1- 3.6) was found to be 8 which shows that to the infection of *Klebsiella pneumoniae* strain one has to increase dosage intake of *Piper* powder [Table 4].

Agar disc diffusion method and Agar well diffusion method: Two different methods were utilized to study the effects of various extracts on the three microorganisms. **Agar disc diffusion method:** In *Staphylococcus aureus* it was observed that Inhibition Zone (IZ) of all the three extracts were lesser than the standard antibiotic Ampicillin and Chloro-m-phenicol. Similar results were observed for *Streptococcus pyogenes* where Inhibition Zone (IZ) of extracts] were also lesser than the standard antibiotic Ampicillin and Chloro-m-phenicol. And in case of *Klebsiella pneumoniae* it was observed that Inhibition Zone (IZ) of chloroform extract was similar to that of aqueous extract whereas for alcoholic extract it was which is lesser as compared to the standard antibiotic Ampicillin and Chloro-m-phenicol [Table 5], (Plate no:4.1- 4.6)

Agar well diffusion method: In *Staphylococcus aureus* it was observed that Inhibition Zone (IZ) of all the three extracts were lesser than the standard antibiotic Ampicillin and Chloro-m-phenicol. Similar results were observed for *Klebsiella pneumoniae* where Inhibition Zone (IZ) of extracts were also lesser than the standard antibiotic Ampicillin and Chloro-m-phenicol. And in case of *Streptococcus pyogenes* it was observed that Inhibition Zone (IZ) of chloroform extract was similar to that of

aqueous extract whereas for alcoholic extract it was lesser as compared to the standard antibiotic Ampicillin and Chloro-m-phenicol [Table 6, [Plate no:5.1- 5.6]. All the three extracts showed lesser zone of inhibition than the standard antibiotic used. Alcoholic extract have generally found to possess a strong antimicrobial activity, similar results have been observed for *Propolis*⁵². Alcoholic extract of *Hydrastis canadensis* commonly known as golden seal have been found to have a broad-spectrum antimicrobial activity against *Staphylococcus species*, *E.coli* and *Pseudomonas aeruginosa*⁵³. Though these extracts gave minimum zone of inhibition they can be preferred as therapeutic agents because of these their natural origin and because of less possible side effects. These results show that organic extracts particularly alcoholic exhibited better antibacterial principles either polar or non-polar are effectively extracted only through the organic solvent medium⁵⁴. The antibacterial activity of plant extracts was not likely to be due to any one main active constituent but to the combined action of additional other compounds⁵⁴⁻⁵⁵. This can also be seen in our results in agar ditch method. The aqueous extracts appear to have less antibacterial activity than any of the organic extracts. This is interesting in that the traditional method of treating a bacterial infection was by administering a decoction of the plant or apart there by boiling it in water, whereas according to our results an organic solvent is better, hence, this may be more beneficial.

CONCLUSION

Phytochemical Studies: The present study revealed that the plant *Piper longum* is a good source of secondary metabolites. Preliminary screening shows the presence of alkaloids, steroids, sterols, cardiac-glycosides, essential oils, Anthraglycosides, anthraquinones, coumarins, Arbutin, Flavonoids and bitter principles. HPTLC analysis revealed the exact quantity and solvent system required for better separation of these compounds. The plant can be used to discover bioactive products that may serve leads for the development of the new pharmaceuticals that address hither to unmet therapeutic needs.

Antimicrobial Studies: The present study revealed that the plant *Piper longum* is a good source of secondary metabolites. Preliminary screening shows the presence of alkaloids, steroids, sterols, cardiac-glycosides, essential oils, anthraglycosides, anthraquinones, coumarins, arbutin, flavonoids and bitter principles. HPTLC analysis revealed the exact quantity and solvent system required for better separation of these compounds. The study revealed potential antimicrobial activity of the herbal preparation obtained from *Piper longum* fruit. The validation of potential antimicrobial activity has been validated against known pathogenic microorganisms such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*. The potential of the preparation was tested against standard antibiotics. Among the different extracts evaluated Alcoholic extract of *Piper longum* fruit exhibited higher potency as compared to other extracts against the test organisms. *Klebsiella pneumoniae* was found to be the most resistant among the three

pathogens of respiratory tract. The extract of *Piper longum* is active against both gram positive and gram negative organisms, thus the bioactive components present in *Piper longum* (i.e; cardiac-glycosides, anthraglycosides, steroids and essential oil) fruits can be a broad spectrum antibiotic. It has been established that extract of *Piper longum* possess a significant antimicrobial activity to the pathogens of respiratory tract with a broad-spectrum result.

REFERENCES

- Lewis W.H and Elvin-Lewis M.P: Medicinal plants as source of new therapeutics. Ann. Mo. Bot. Gard. 1995; 82:16-20.
- Shariff, Z.U.: Modern Herbal Therapy for Common Ailments. Nature Pharmacy Series vol.1. Spectrum Books Ltd., Ibadan, Nigeria in Association with Safari Books (Export) Ltd. UK. 2001; pp9-84.
- David J. Newman and Gordon M. Cragg: Natural Products as Sources of New Drugs over the 30 Years from 1981 to 2010; *J. Nat. Prod.*, 2012, 75 (3), pp 311–335
- Westh, H., Zinn, C.S., Rosdahl, V.T., Sarisa Study Group: An International multicenter study of Antimicrobial consumption in *Staphylococcus aureus* isolates from 15 hospitals in 14 countries. Microbial Drug Resistance, 2004; 10: 169-176.
- Appelbaum P.C. Antimicrobial resistance in *Streptococcus pneumoniae* an overview. Clinical Infect Dis., 1992; 15: 17-83.
- Huovinen P, Sundstrom I, Swedberg G and Skold O; Trimethoprim and Sulfonamide resistance. Antimicrobial and Chemotherapy, 1995; 39 (2): 279-289.
- Hamburger M and Hostettmann K; Bioactivity in plants: The link between phytochemistry and medicine. Phytochemist, 1991; 30 (2): 3864-3874.
- Afolayan, A.J.; Extracts from the shoots of *Arctotis Artotoides* inhibit the growth of bacteria and fungi. Pharm. Biol. , 2003;41:22-25.
- Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Castaldo cobianchi, R., Vuotto, M.L., Ferrara, L., Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia, 2000; 71: 110-116.
- Okpekon, T., Yolou, S., Gleye, C., Roblot, F., Loiseau, P., Bories, C., Grellier, F., Frappier, F., Lawrens, A., Hocquemiller, R; Anti parasitic activities of medicinal plants used in Ivory coast. J. Ethnopharmacol., 2004; 90:91-97.
- Romero, C.D., Chopin, S.F., Buck, G., Martinez, E., Garcia, M., Bixby, L., ; Antibacterial properties of common herbal remedies of the south west. J ethnopharmacol., 2005; 99:253-257.
- Harborne J.B. Phytochemical methods. 3rd Edition London: Chapman and Hall, 1998.
- Wagner R and Bladt S, Plant Drug Analysis, A Thin Layer Chromatography Atlas, 2nd Ed., Springer, Berlin, 1996.
- Parekh J, Nair R and Chanda S; Preliminary screening of some folklore medicinal plants from western India

- for Potential antimicrobial activity. *Indian J. Pharmacol.*, 2005; 37: 408-409.
15. Perez C, Paul M and Bazerque P; An Antibiotic assay by the agar well diffusion method. *Acta. Bio. Med. Exp.* 1990; 15: 113-115.
 16. Bauer A.W, Kirby W.M.M, Sherris J.C and Turck M; Antibiotic susceptibility testing by standard single disk method. *Am. J.Clin. Pathol.* 1996; 45: 493-496.
 17. Hunt D.E and Sandham H.J; Improved Agar gradient plate technique *Applied Microbiology*. ASM, 1969; Vol 17, No: 2 pp 329-330.
 18. Newman DJ, Cragg GM; Natural products as sources of new drugs over the last 25 years. *J Natprod*, 2007; 70:461-77
 19. Saraf Aparna; Phytochemical and Antimicrobial Studies of Medicinal Plant *Costus Speciosus* (Koen.) E-Journal of Chemistry 2010, 7(S1), S405-S413
 20. Yujiro Niiho ,Takashi Yamazaki, Yoshijiro Nakajima, Toshinori Yamamoto, Hidehiro Ando, Yasuaki Hirai, Kazuo Toriizuka, Yoshiteru Ida, Gastroprotective effects of bitter principles isolated from Gentian root and Swertia herb on experimentally-induced gastric lesions in rats; *Journal of Natural Medicines*; January 2006, Volume 60, Issue 1, pp 82-88
 21. Chemical Information Review Document for Arbutin [497-76-7] and Extracts from *Arctostaphylos uva-ursi* ;2006; National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, U.S Department of Health and Human Services Research Triangle Park, NC. <http://ntp.niehs.nih.gov/>
 22. Rios J.L, Recio M.C; Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*.2005; 100:80–84
 23. Amvam Zollo, P.H., L. Biyiti, F. Tchoumboungang, C. Menut, G. Lamaty and P.H. Bouchet, Chemical composition and antifungal activity of International Rules for Seed Testing. International thirteen essential oils from aromatic plants of Cameroon. *Flavour and Fragrance J.*; 1998; 13: 107-114.
 24. Stevic, T.; Savikin, K.; Ristic, M.; Zdunic, G.; Jankovic, T.; Krivokuca, D. and Vulic, T.: Composition and antimicrobial activity of the essential oil of the leaves of black currant (*Ribes nigrum* L.) cultivar Cacanska crna. *J. Serb. Chem. Soc* 2010; 75 (1): 35- 43.
 25. Nguefack, J., I. Somda, C.N. Mortensen P.H. Amvam Zollo. Evaluation of five essential oils from aromatic plants of Cameroon for controlling seed-borne bacteria of rice (*Oryza sativa* L.). *Seed Sci. Technol.*; 2005; 33: 397-407.
 26. Nguefack, J., S.K. Nguikwie, D. Fotio, B. Dongmo, V. Leth, A.E. Nkengfack and P.H. Amvam Zollo; Fungicidal potential of essential oils and fractions from *Cymbopogon citratus*, *Ocimum gratissimum* infection of rice by *Helminthosporium oryzae* and *Thymus vulgaris* to control *Alternaria padwickii* -and *Bipolaris oryzae*, two seed-borne fungi of rice (*Oryza sativa* L.). *J. Essential Oil Res.*; 2007; 19: 581-587
 27. Oka, Y., S. Nacar, E. Putievsky, U. Ravid, Z. Yaniv and Y. Spiegel. Nematicidal activity of essential oils and their components against the root-knot and its role in paddy seed spoilage. *Phytopathology*; 2000; 90: 710-715.
 28. Weber US, Steffen B, Siegers CP. Antitumour-Activities of Coumarin, 7-Hydroxycoumarin and its Glucuronide in Several Human Tumour Cell Lines. *Res Commun Mol Pathol Pharmacol* 1998; 99: 193-206.
 29. L. Chen, F.-Q. Yang, T.-Y. Zhang, Y.-S. Han, S.-W. Chen, Fenxiceshi Xuebao; *J. Instrum. Anal.*; 2000; 19 (No. 4) 60.
 30. Andrew Pengelly; *The Constituents of Medicinal Plants*, 2004 Publishers Allen & Unwin, Australia
 31. T A Woyengo, V R Ramprasath and P J H Jones: Anticancer effects of phytosterols *European Journal of Clinical Nutrition*, 2009; 63, 813–820
 32. Srinivasan, D., Nathan, S., Suresh, T., Perumalsamy, O.; Antimicrobial Activity Of Certain Indian Medical Plants used in folkloric medicine. *J. Ethnopharmacol.* 2001; 74:217-220.
 33. Ghoshal S, Krishna, Prasad B.N and Lakshmi V; Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. *J. Ethnopharmacol.* 1996; 50: 167- 70.
 34. Iwu M.W, Duncan A.R and Okunji C.O; New antimicrobials of plant origin. In: *Perspectives on new crops and new uses*. J. Janick (ed). ASHS Press, Alexandria, V.A.1999. pp 457 – 462.
 35. Vanbeek T.A, Verpoorte R and Vendson A.B; Antimicrobial active alkaloids from *Tabernaemontana chippli*. *Journal of Natural products*.1985; 48: 400-423.
 36. Watcher G.A, Hoggmann J, Furbacher T, Blake M.E and Timmermann B.A ; Antimicrobial and antifungal flavanones from *Eysenhardita textana*. *Phytochemistry*.1999; 52: 1469-1471.
 37. Fernandez M.A, Garcia M.D and Sanes H.T: Antimicrobial activity of the phenolic acids fractions of *Scrophularia frutescens* and *Scrophularia sambucifolia*. *Journal of Ethanopharmacology*. 1996; 53 : 11-14
 38. Anonymous; *The Wealth of India – Raw Materials*. 1:1959; CSIR. New Delhi. India.
 39. Asolkar L.V, Kakkar K.K and Chakre O.J; 1992: *Glossary of Indian Medicinal Plants with active principles Part-I* Publication and information directorate (CSIR). Dr. K.S. Krishnan Marg. New Delhi, India.
 40. Stoyanova A, Denkova Z, Nenov N, Slavchev A, Jirovetz L, Buchbauer G, Lien H.N, Schmidt E and Geissler M ; C₂H₂F₄-Oleoresins of Black pepper (*Piper Nigrum* L.) and Ginger (*Zingiber officinale* (L.) *Rosc.*) from Vietnam: Antimicrobial testings, Gas Chromatographic Analysis and Olfactoric Evaluation. *EJEAFCh* (Electronic Journal of Environmental, Agricultural and Food Chemistry), 2006; ISSN: 1579-4377, 5(5), pp. 1615-1623.

41. Griffin S, Grant W, Markham J and Leach D: Flavour Fragr. J.1999; 14, 323-332.
42. Suppakul P, Miltz J, Sonneveld K and Bigger St.W; J. Agric. Food Chem.2003; 51 (11), 3197-3207.
43. Lambert P.A; Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J. Appl. Microbiol.2002; 92: 465-545.
44. Russell A.D; Antibiotic and biocide resistance in bacteria: Introduction. J. Appl. Microbiol symposium supplement,2002; 92: 15-35.
45. Yao J and Moellering R; Antibacterial agents. In: Manual of Clinical Microbiology, Murray P, Baron E, Pfaller M, Tenover F, Tenover F, Tenover F, Tenover F (Eds).ASM. Washington DC, 1995; pp. 1281-1290.
46. Cetin T.E and Gurler N; Bakterilerilerin antibiyotiklere duyarlılık deneyinin yapılması. KuKem Dergisi, 1989;12: 2-5.
47. Ogundare A.O, Adetuyi F.C and Akinyosoye F.A; Antimicrobial activities of *Vernonia tenoreana*, African Journal of biotechnology, 2006; Vol.5 (18), pp. 1663-1668.
48. Taylor J.L.S, Rabe T, McGraw L.J, Jager A.K and van Staden J; Towards the scientific validation of traditional medicinal plants. Plant Growth Regul. 2001; 34: 23-37.
49. Fransworth N.R; Biological approaches to the screening and evaluation of natural products. In Rasoanaivo P, Ratsimamanga-Urverg S (Eds) Biological Evaluation of Plants with Reference to the Malagasy Flora, Madagascar,1993; pp.35-43.
50. Jager A.K, Hutchings A and van Staden J; Screening of Zulu medicinal plants for prostaglandin synthesis inhibitors. J. Ethnopharmacol.1996; 52: 95-100.
51. Shale T.L, Strik W.A and van Staden J; Screening of plants used by southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin synthesis inhibitors and uterine relaxing activity. J. Ethnopharmacol.1999; 64: 9-14.
52. Rhajaoui M, Qumzil H, Faid M, Lyagoubi M, Elyachioui M and Benjouad A ; Antibacterial Activity of A Moroccan *Propolis* Extracts Science Letters,2001; 3(3).
53. Scazzocchio F, Cometa M.F, Tomassini L and Palmery M; Antibacterial Activity of *Hydrastis Canadensis* Extract and its Major Isolated Alkaloids Planta Med, 2001; 67: 561-564.
54. Essawi T and Srour M; Screening some Palestinian medicinal plants for antibacterial activity. Journal of Ethnopharmacology.2000; 70: 343-349.
55. Bai D (1990): Traditional Chinese Material: A respect and prospect. Planta Medica. 56: 502.