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Research Article

IN VITRO STUDY OF ANTIBACTERIAL ACTIVITY OF AROMATIC AND MEDICINAL PLANTS ESSENTIAL OILS WITH SPECIAL REFERENCE TO CINNAMON OIL

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ABSTRACT

Antibacterial activity of 19 essential oils was screened against four test bacteria namely *Pseudomonas aeruginosa* NCIM 5029, *Staphylococcus aureus* NCIM 5021, *Salmonella typhimurium* NCIM 2501 and *Bacillus subtilis* NCIM 2063. Varying degree of antibacterial activity was recorded with the highest being depicted by *Cinnamomum zeylanicum* followed by *Cymbopogon ciatrus* and *Carum copticum*, against all bacteria. MIC of cinnamon oil was determined through agar well diffusion assay. *Staphylococcus aureus* and *B. subtilis* were most susceptible, exhibiting MIC values 0.2 and 0.4 µl oil/well respectively. Antibacterial assay of the column chromatography fractions clearly indicated that the primary compound cinnamaldehyde is responsible for major antibacterial activity. Bioautography of cinnamon oil was done to detect qualitative antibacterial activity.

Keywords: Antibacterial activity, Essential oil, Cinnamon oil, Chromatography, Bioautography

INTRODUCTION

In the present time, spread of drug resistant pathogens is a very serious problem for the successful treatment of microbial diseases[1]. Hence, plant origin herbal medicines are considered as safe alternatives of synthetic drugs. There are varied methods of medicines like Aurveda, Homeopathy and Unani, which utilize plant materials for drug production. Currently, Aurveda considered as a vital system of medicine and governed the worldwide recognition and having non-toxic substances. However, newly discovered non-antibiotic substances such as certain essential oils[2] and their constituent chemicals[3] have shown good fighting potential against drug resistant pathogens.

Essential oils are aromatic oily liquids, which are obtained from various plant parts such as flowers, buds, seeds, leaves, twigs, bark, woods, fruits and roots by steam distillation. Scientifically these oils have been proved highly potent antimicrobial agents in comparison to antibiotics. These plant essential oils are rich source of scents and used in food preservation and aromatherapy. These possess multiple antimicrobial i.e., antibacterial[4], antifungal, anticancer, antiviral, insecticidal and antioxidant properties[5,6,7]. Some essential oils such as aniseed, calms, camphor, cedar-wood, cinnamon, eucalyptus, geranium, lavender, lemon, lemongrass, lime, mint, nutmeg, rosemary, basil, vetiver and winter green are traditionally used by people in different parts of the world. Cinnamon[8], clove, rosemary and lavender oils have shown both antibacterial and antifungal properties[9].

Cinnamon (*Cinnamomum zeylanicum*) is a small evergreen tree, 10-15 meters (32.8-49.2 feet) tall, belonging to the family Lauraceae, native to Sri Lanka and South India. The flowers, which are arranged in panicles, have a greenish colour and have a distinct odour. The fruit is a purple one-centimeter berry containing a single seed. Its flavour is due to an aromatic essential oil which makes up 0.5 to 1% of its composition[10].

It contains medicinally important essential oil in leaves, fruits inner and outer bark. Much of cinnamon's bioactivity resides in its oil, which is about 90% cinnamaldehyde. It is used mainly in medicine, foods and cosmetics and is employed in aromatherapy as a rub to promote blood circulation. It also contains both anti-fungal and anti-bacterial principles that can be used to prevent food spoilage due to bacterial contamination[11,10].

In the present study we have evaluated antibacterial activity of 19 essential oils with special reference to cinnamon essential oil.

MATERIAL AND METHODS

Collection and verification of plant material

The dried plant materials were collected from local market of Bhopal, India and the fresh plant materials were collected from the farms around Bhopal. Eight essential oils namely *Pogostemon cablin* (Patchouli), *Cymbopogon ciatrus* (Lemon grass), *Citrus sinensis* (Sweet Orange), *Cedrus deodara* (Red Cedar wood), *Rosmarinus officinalis* (Rosemary), *Ocimum sanctum* (Tulsi), *Ocimum basilicum* (Basil) and *Pinus roxburghii* (Pine) were purchased directly from the perfume and aroma oil shop in Bhopal. Identification and authenticated of the plant materials and essential oils was done in the laboratory by using relevant literature.

Extraction of volatile oil by steam distillation

The dried plant materials were powdered in a grinder and subjected to steam distillation, using the Clavenger apparatus (Pyrex), as described by Harbone[12]. Distillation was done for 4 hours and the oil was drained off and dried over anhydrous sodium sulphate[13]. Fresh plant parts were chopped into pieces with cutter and used as such for steam distillation. All the oils were stored at 4°C. Essential oil of 11 plant species was extracted in the laboratory using appropriate plant material. The common name, plant part used and volatile oil content (w/w) obtained are shown in Table 1.

Table 1: Plant species and their parts used for extraction of essential oil

S. No.	Plant Species	Common Name	Plant Part	Essential oil content (%)
1)	*Syzygium aromaticum*	Clove	Floral buds	7.5
2)	*Cinnamomum zeylanicum*	Cinnamon	Stem bark	3.9
3)	*Cumin cyminum*	Cumin	Seeds	1.1
4)	*Curcuma longa*	Turmeric	Rhizome	2.8
5)	*Mentha spicata*	Peppermint	Leaves	0.6
6)	*Piper nigrum*	Black pepper	Seeds	0.2
7)	*Tagetes patula*	Marigold	Whole plant	0.5
8)	*Foeniculum vulgare*	Fennel	Seeds	2.4
9)	*Eucalyptus globules*	Eucalyptus	Leaves	0.75
10)	*Carum copticum*	Ajwain	Seeds	2.4
11)	*Elettaria cardamomum*	Cardamom	Seeds	3.4

Test organisms and preparation of inoculum

Four bacterial cultures namely *Pseudomonas aeruginosa* NCIM 5029, *Staphylococcus aureus* NCIM 5021, *Salmonella typhimurium* NCIM 2501 and *Bacillus subtilis* NCIM 2063 used in the present study were obtained from NCIM, NCL, Pune, India. Cultures were raised on Nutrient Agar Medium (Peptone-5gm, Beef Extract-3 gm, NaCl-5gm and deionized water-1L) plates. For prolonged storage the active bacterial colonies from the plate culture were suspended in glass vials containing sterile glycerol solution (16%) and kept at -20°C. Inoculums were prepared by transferring loop full of glycerol bacterial suspensions in 50 ml nutrient broth followed by 48 hours incubation in orbital shaking incubators set at 150 rpm. *Staphylococcus aureus* and *S. typhimurium* were incubated at 37°C while *Pseudomonas aeruginosa* and *Bacillus subtilis* were incubated at 30°C.

Screening of antibacterial activity

Preliminary screening of antibacterial activity of essential oils was done through agar well diffusion assay[14]. Petriplates containing Nutrient Agar Medium were inoculated aseptically with 50 µl incubated broth culture through spread plate technique and left for 30 min. Five wells of 5 mm diameter were uniformly punched in the petriplates by using sterile cork borer followed by numbering of wells. Into each well, 14 µl of dimethyl sulphoxide (DMSO) was dropped with the help of fully autoclavable micropipette (Eppendorf, Germany). Thereafter 6 µl each of essential oils were also dropped into the wells so as to make the total volume of 20 µl in each well. Before incubation plates were kept in refrigerator at 4°C for 3 h, to allow oil and DMSO to diffuse into the agar medium. Plates were finally incubated at respective temperatures for 24 hours. DMSO without oil was set as negative control. The diameter of the zone of inhibition around each well was measured in mm with the aid of zone reader scale (Himedia, India).

Determination of MIC

Minimum inhibitory concentration (MIC) was determined through agar well diffusion assay only for cinnamon oil because it produced highest antibacterial effect against all the four bacterial strains. For determination of MIC, cinnamon oil was added into the wells in the decreasing quantity from 6 µl to 0.1 µl. Volumes less than 1 µl were prepared through dilution method. Final volume in each well was adjusted to 20 µl with DMSO. Lowest volume of oil which showed inhibition zone was regarded as the MIC[15].

Comparative evaluation of cinnamon oil and cinnamaldehyde

To evaluate the effectiveness of cinnamon oil it was compared with cinnamaldehyde 90% (Oxford Chemicals, India) through agar well diffusion assay at 6 µl/well quantity.

Separation of cinnamon oil into fractions through column chromatography and antibacterial assay of these fractions

Silica gel gravity column chromatography was performed for fractionation of cinnamon oil based on increasing solvent polarity. Silica gel (60-120 mesh) slurry was made in petroleum ether 40-60°C and poured into a glass column (60 x 3 cm, ASGI, India) with sintered disc, to make an effective column of 45 x 3 cm size. Analytical grade solvents of Rankem, India were used. Ten ml of cinnamon oil was loaded onto column bed and eluted first with 400 ml petroleum ether followed by 400 ml each of ether:chloroform (9:1), ether:chloroform (7:3), ether:chloroform (3:7), chloroform, chloroform:acetone (9:1), chloroform:acetone (7:3) and lastly with acetone. Column fractions were collected separately and concentrated by using rotary vacuum evaporator, buchi type (Scientech, India) at 50°C and finally weighed.

Antibacterial potential of all the eight column fractions was determined through agar well diffusion assay against *S. aureus* at 6µl/well quantity. Some column fractions formed crystals and therefore, were dissolved in least quantity of DMSO for antibacterial assay. Cinnamaldehyde (90%) was used as positive control.

Analysis of column fractions through TLC

Silica gel TLC was performed for analysis of column fractions along with Cinnamaldehyde (90%). Silica plates of 1 mm thickness were prepared with silica gel G (Rankem, India) by using TLC apparatus (Perfit India Ltd., India). Plates were activated in an oven (Tempo, India) for 2 hours at 65°C. Eight column fractions 5 µl each were spotted with the help of Hamilton syringe (Germany) along with and Cinnamaldehyde (90%). Petroleum ether, toluene and ethyl acetate in a combination of 7:2:1 was used as developing solvent. When the solvent phase reached the top, the plate was air dried and exposed to iodine vapours in a glass chamber in dark for 30 min. The plate was then taken out and photographed[16].

Bioautography study

A bioautography technique was employed to define the active constituent cinnamaldehyde[17]. Two TLC plates (1 & 2) were loaded with 5µl cinnamon oil and run in developing solvent. Plates were dried in laminar air flow and plate 1 was used for bioautography by placing it in a sterile petriplate (15 cm diameter) and immersed completely in nutrient medium. Plate was allowed to solidify and then inoculated with *S. aureus* through spread plate method followed by incubation at 37°C for 24 hours. Plate 2 was stained with iodine vapours and visualized along with bioautography plate.

RESULTS AND DISCUSSION

The results of antibacterial activity of essential oils of nineteen aromatic plants are shown in Table-2. Among these oils, 12 were found to possess antibacterial activities. *Cymbopogon citraus*, *Cinnamomum zeylanicum* and *Carum copticum* exhibited higher level of antibacterial activity against all the bacterial strains. *Pseudomonas aeruginosa* and *S. typhimurium* showed inhibition only with these three oils. Seven oils namely, *P. cablin*, *F. vulgare, C. longa*, *P. roxburghii*, *E. cardamomum*, *C. cyminum* and *P. nigrum* did not showed any antibacterial activity. *Cedrus deodara* exhibited weak activity only against *S. aureus*. Other oils were high to moderately antibacterial. Among all the oils, *C. zeylanicum* was most active, as it illustrated highest inhibition in all the tested bacteria.

Gram +ve bacteria were more prone to inhibition by wide variety of oils as compare to gram –ve bacteria. It may be due to volatile action of essential oils and due to absence of lipo-polysaccharide layer in Gram positive bacteria that might function as an effective barrier against any incoming bio-molecule[18,19]. There might be another possibility that essential oils may successfully inhibit microbial respiration and increase the plasma membrane permeability, which results in to death of bacterial cells after massive ion leakage[20,21].

Similarly, Prabuseenivasn *et al.*[8] tested 21 essential oils in their study and found 19 oils possessed antibacterial activity. Cinnamon, clove, geranium, lemon, lime, orange and rosemary oils exhibited significant inhibitory effect. Cinnamon oil had the most potential bactericidal properties. They found that this oil showed promising inhibitory activity even at low concentration, whereas aniseed, eucalyptus and camphor oils were least active against the tested bacteria. In general, *B. subtilis* was the most susceptible. On the other hand, *K. pneumoniae* exhibited low degree of sensitivity.

Matan *et al.*[22] have reported Antimicrobial activity of *Cinnamon* bark. 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 CO_2 (40%) and low concentration of O_2 (<0.05%). *Aspergillus flavus,* which is known to produce toxins, was found to be the most resistant microorganism. Derwich *et al*. [23] revealed chemical composition and antibacterial activity of essential oils obtained from *Rosmarinus officinalis* (family *Lamiaceae*) against *E. coli, P. aeruginosa, S. aureus, K. pneuomonae, S. typhi, S. intermedius, B. subtilis, S. mutans, M. luteus,* and *P. mirabilis*.

The antimicrobial mode of action is considered to arise mainly from the potential of hydrophobic essential oils to disrupt the bacterial cell membrane and its structures, which leads to ion leakage. Essential oils are mixture of many major and minor compounds usually 20 to 50. In many oils usually two or more than two compounds are responsible for antibacterial activities but, in some single major compound is responsible for antibacterial activity.

The results of MIC of cinnamon oil as obtained through agar well diffusion assay are presented in Table -3. MIC in the present study was considered as minimum quantity of oil which showed bacterial inhibition zone through agar well diffusion assay. *Staphylococcus aureus* and *B. subtilis* were most susceptible, exhibiting MIC values 0.2 and 0.4 µl cinnamon oil per well respectively. *Pseudomonas aeruginosa* was least susceptible, with the MIC value 2 µl oil per well. The susceptibility of bacterial strains in the decreasing order was *S. aureus* > *B. subtilis* > *S. typhimurium* > *P. aeruginosa*.

Table 2: Screening of antibacterial activity of essential oils

S. No.	Essential oils (6µl)		Inhibition zone diameter (mm) in four bacterial species			
	Scientific name	Common name	*P. aeruginosa*	*S. aureus*	*S. typhimurium*	*B. subtilis*
1)	*Mentha spicata*	Peppermint	-	11.66	-	12
2)	*Pogostemon cablin*	Patchouli	-	-	-	-
3)	*Cymbopogon ciatrus*	Lemon grass	7.66	22.33	12.33	19.66
4)	*Citrus sinensis*	Sweet Orange	-	14.66	-	12.66
5)	*Cinnamomum zeylanicum*	Cinnamon	8.66	32.33	26	24.66
6)	*Syzygium aromaticum*	Clove	-	12	-	10
7)	*Cedrus deodara*	Red Cedar wood	-	6	-	-
8)	*Eucalyptus globulus*	Eucalyptus	-	16.66	-	-
9)	*Rosmarinus officinalis*	Rosemary	-	18	-	15
10)	*Ocimum sanctum*	Tulsi	-	12	-	14.33
11)	*Ocimum basilicum*	Basil	-	10.33	-	10.66
12)	*Carum copticum*	Ajwain	3	27.33	9.66	25
13)	*Tagetes patula*	Marigold	-	9.33	-	-
14)	*Foeniculum vulgare*	Fennel	-	-	-	-
15)	*Curcuma longa*	Turmeric	-	-	-	-
16)	*Pinus roxburghii*	Pine	-	-	-	-
17)	*Elettaria cardamomum*	Cardamom	-	-	-	-
18)	*Cumin cyminum*	Cumin	-	-	-	-
19)	*Piper nigrum*	Black pepper	-	-	-	-
20)	*Negative Control (DMSO)*		-	-	-	-

Values are mean inhibition zone (mm) of three replicates; '-' indicates no activity

Table 3: Determination of MIC of Cinnamon oil through agar plate well assay method

S. No.	Quantity of Cinnamon oil (µl/well)	Inhibition zone diameter (mm) in various bacterial species			
		P. aeruginosa	*S. aureus*	*S. typhimurium*	*B. subtilis*
1)	6	8.66	32.33	26	24.66
2)	5	7.33	30	24	20.33
3)	4	6	29.66	19.33	17.33
4)	3	4.33	22.33	18.33	13.66
5)	2	1.66	19.33	15.66	11
6)	1	-	12.33	6.66	8.66
7)	0.8	-	8.33	3.33	7
8)	0.6	-	3	0.66	4.33
9)	0.4	-	1.33	-	2
10)	0.2	-	1	-	-
11)	0.1	-	-	-	-

Values are mean inhibition zone (mm) of three replicates; '-' indicates no activity

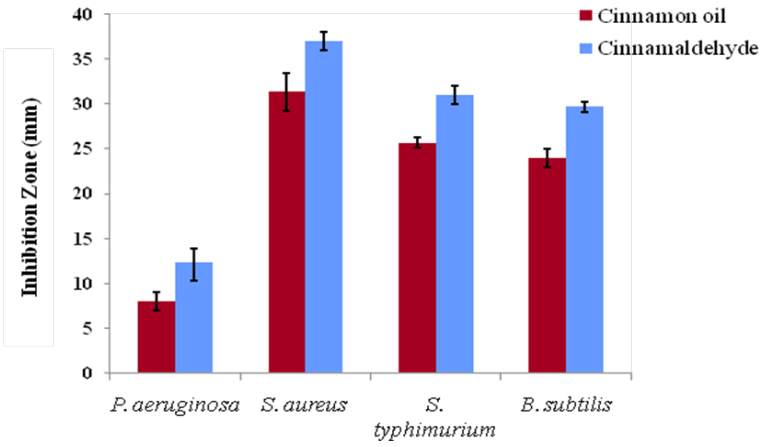

Fig. 1: Comparative evaluation of cinnamon oil and cinnamaldehyde (6µl per well)

Comparative evaluation of antibacterial activity of oil and its active compound is shown in Fig.1. Cinnamaldehyde (90%) was more antibacterial than cinnamon oil. Besides 70-80% cinnamaldehyde, cinnamon oil also contains other antibacterial compounds such as eugenol (8-14%) and cinnamic acid in minor quantity. Some other compounds present in lesser proportions are terpenic compounds, such as α- pinene and caryophyllene[17].

Table 4: Column fractions of cinnamon oil and results of their antibacterial assay (6µl/well)

S. No.	Column Fraction No.	Elution Solvent(s)	Solvent Ratio	Column fractions (wt. in gm)	Antibacterial activity (mm) of column fractions against *S. aureus*
1)	I	Ether	100%	4.28	-
2)	II	Ether : Chloroform	9:1	0.1	23.66±1.52
3)	III	Ether : Chloroform	7:3	1.24	26.66±0.57
4)	IV	Ether : Chloroform	3:7	1.98	27.0±2.51
5)	V	Chloroform	100%	0.89	30.33±1.52
6)	VI	Chloroform : Acetone	9:1	0.29	4.33±2.08
7)	VII	Chloroform : Acetone	7:3	0.19	2.0±0.57
8)	VIII	Acetone	100%	0.02	-
9)	Cinnamaldehyde				32.66±0.57

Values of antibacterial activity are mean inhibition zone (mm) of three replicates ±standard deviation ; '-' indicates no activity

Cinnamon oil was fractioned into eight fractions through silica gel column chromatography (Table-4). Ether yielded highest quantity (4.28 gm) of cinnamon oil fraction while least quantity (0.02 gm) was obtained with acetone. Except for ether and acetone fraction all fractions revealed antibacterial activity against *S. aureus*. Cinnamaldehyde used as positive control; exhibited highest activity (32.66±0.57 mm) followed by fraction V, IV, III, II, VI, and VII. As expected fraction VII exhibited least activity (2.0±0.57 mm). It was predicted that most active component cinnamaldehyde of cinnamon oil was eluted when chloroform was used with ether. By increasing polarity of eluting solvent by increasing the percentage of chloroform, antibacterial activity was also increased. Activity was decreased sequentially in more polar fractions VI and VII where cinnamaldehyde was absent. These results also indicate that besides cinnamaldehyde cinnamon oil contains other more polar compounds having mild antibacterial activity.

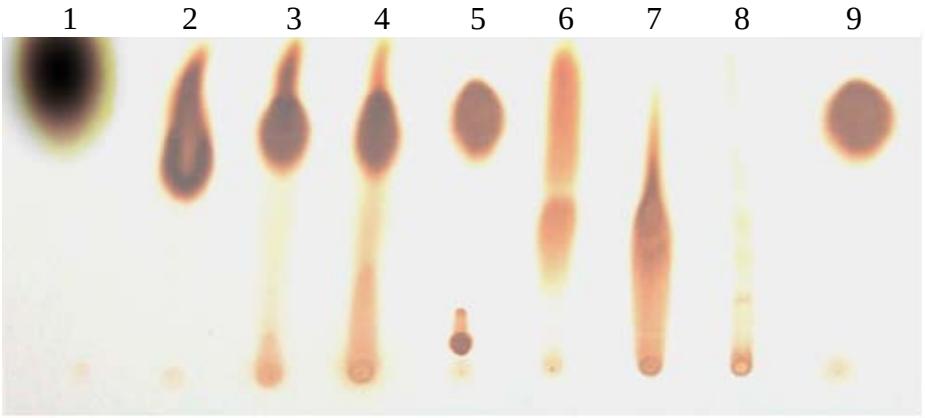

Fig. 2: TLC profile of column fractions of cinnamon oil in the order of their polarity from the most non polar at left (spot no. 1) to most polar at right (spot no. 8). Cinnamaldehyde (90%) is at spot no. 9. Plate was stained with iodine vapors for visualization of spots

The TLC profile of column fractions of cinnamon oil shown in Fig. 2 indicated presence of substantial quantity of cinnamaldehyde in fraction II, III, IV and V (corresponding spots 2, 3, 4 and 5 in TLC plate) upon comparison with cinnamaldehyde in lane 9. All the column fractions were tested for the antibacterial activity against *S. aureus* as it was the most susceptible bacterium. It is clear that fraction VI, VII and VIII lacks cinnamaldehyde. This was the reason for low degree of antibacterial activity exhibited by these fractions. It also indicated that besides cinnamaldehyde there are other compounds in cinnamon oil responsible for antibacterial activity. Among all the fractions, fraction V revealed highest activity because of its higher cinnamaldehyde content. The non-polar oily fraction I was completely inactive against bacteria due to lack of cinnamaldehyde and other polar active compounds. The antibacterial activity of the cinnamon oil is primarily attributed to its cinnamaldehyde content.

Chakraborty and Shah[24] tested the piper betel leaf extract against four different pathogenic bacteria namely *S. pyogenes, S. aureus, P. vulgaris* and *E. coli*. They also standardized TLC procedure for separation of secondary metabolite from leaf extracts.

Bioautography study revealed position of cinnamaldehyde on TLC plate. This experiment showed clear inhibition zone corresponding to cinnamaldehyde (3-phenyl-2-propenal) after separation on TLC. On silica gel thin-layer chromatograms, cinnamon essential oil was separated into two prominent bioautographic spots with different Rfs (0.74 for Cinnamaldehyde and 0.83 for non polar oily compound) which showed activity against *S. aureus*. The Rf values of inhibition zone center point (plate 1) and cinnamaldehyde (plate 2) were same (Fig. 3). This clearly proved that the primary compound responsible here for antibacterial activity of cinnamon oil is its major compound cinnamaldehyde.

Bioautography was used to detect qualitative antibacterial activity. Since this method is visual, the stability of the compounds on the plate can be easily verified[25].

Gende *et al*.[17] evaluated antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil and its main components against *Paenibacillus larvae*, and employed bioautography technique to define the active constituents of the oil.

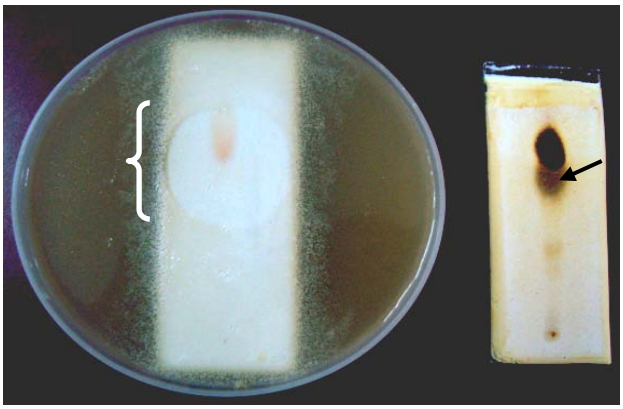

Fig. 3: The spot shown by arrow in the plate 2 at right is cinnamaldehyde stained with iodine. Large zone of inhibition observed at the corresponding position in plate 1 in bioautography.

CONCLUSION

Many essential oils exhibited antibacterial activity against two and more bacterial strains. *Cinnamomum zeylanicum* essential oil revealed most potential antibacterial activity followed by *Cymbopogon ciatrus* and *Carum copticum*. The primary component of cinnamon oil can be fractionated through column chromatography. TLC and bioautography can be employed to define the active constituents of essential oils. Cinnamon oil can be used for the development of new antibacterial and therapeutic agents after detailed investigation and trials.

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