Comparative analysis of antioxidant, antimicrobial and analytical profile of essential oils extracted from *Cinnamomum zeylanicum* (Blume) bark and *Myristica fragrans* (Houtt) fruit.

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ABSTRACT

The objective of this study is to compare the antimicrobial activity of the essential oil from bark of *Cinnamomum zeylanicum* and fruit of *Myristica fragrans* (Houtt**).** The essential oil was extracted from fruit peels by steam distillation using Clevenger's type apparatus. The essential oil of *Cinnamomum zeylanicum* and *Myristica fragrans* was analysed by GC-MS and their antimicrobial and antioxidant activity were tested. *In vitr*o antioxidant activity as assessed by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, Superoxide assay and Reducing power assay. Their antimicrobial activity is tested against four strains of bacteria *Escherichia coli*, *Streptococcus bacillus*, *Proteus vulgaris* and *Staphylococcus aureus* Efficacy of the essential oil of *Cinnamomum zeylanicum* and *Myristica fragrans* was compared using Well Diffusion method. Ampicillin and Amphotericin-B, an anitibiotic, is used as a positive control. According to the results of the minimal inhibitory concentration (MIC) we can conclude that the essential oil of both the plant *Cinnamomum zeylanicum* and *Myristica fragrans* has antimicrobial potential against all microorganisms studied. The essential oil of *Cinnamomum zeylanicum* showed strong antimicrobial activity against *E.coli*. However, the essential oil of *Myristica fragrans* was found to have more effective antimicrobial activity showing its maximum efficacy for *S.aureus.* Present study concludes that essential oil from *Cinnamomum zeylanicum* and *Myristica fragrans* have potent antioxidant role and also have a broad spectrum antibacterial activity against human pathogens.

Keywords: *Cinnamomum zeylanicum*, *Myristica fragrans*, 2, 2'-diphenyl-1-picrylhydrazyl

1. INTRODUCTION

Antibiotic resistance has become a global concern (Westh H et al., 2004). This forced the researchers to search for new chemotherapeutic agents to fight the infections caused by drug-resistant microbes and to reduce the harm caused by antibiotics (Bocanegra-Garcı´a V et al., 2009). The spread of drug resistant microbial pathogens is one of the most severe threats to successful treatment of infectious diseases (Owlia P et al., 2010). Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava *et al.,* 1996). The use and misuse of antimicrobial agents has led to the development of resistance which is threatening their effectiveness in the treatment and prevention of bacterial infections (Fish DN and Ohlinger MJ, 2006).

E. coli strains are considered to be excellent indicators of antimicrobial resistance because they are part of the normal microbiota of people and animals, and also occur in the environment (Aarestrup FM et al., 2008). *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are two opportunistic pathogens that cause severe and life-threatening infections in immunocompromised patients (Lestari ES, 2004). *Proteus vulgaris* (*P. vulgaris*) is widespread in nature, mainly found in flora of human gastrointestinal tract. Proteus ranked third as the cause of hospital-acquired infections (Bahashwan SA et al., 2013). The organism is short rods shaped,

motile, non-sporing and chemoheterotroph bacterium with diverse mode of transmission (Herter CA and Broeck CT, 1911).

Essential oils are now a days in great demand in the market, since they have several applications as anesthetic, anodyne, antiseptic, astringent, disinfectant, fumigant, inhalant, insect repellant, arthritis, asthma, boils, bronchitis, burns, cancer, diabetes, diarrhea, diphtheria, dysentery, fever, flu, inflammation, leprosy, malaria, mastitis, sores, sore throat, worms, and wounds (Elliot WR and Jones D, 1986). Sometimes their major application were found in the soap and cosmetic manufacturing companies (Bajaj YPS, 1995). Essential oils are the natural products obtained from herbs contain secondary metabolites in the form of volatile organic compounds present in various parts of the plant, such as flowers, fruits, seeds, stems, and roots (F. Bakkali et al.,2008). These oils may have various properties such as antioxidant, insecticidal, antiviral, antibacterial and antifungal (Borges et al., 2016).

Nutmeg is the dried kernel of broadly ovoid seed of *Myristica fragrans* Houtt. (Myristicaceae), a bushy evergreen tree 10–20 m high, indigenous to India, Indonesia and Srilanka (Gils CV and Cox PA,1994).*M. fragrans* commonly known as nutmeg is widely used as spice and in alternative medicine it has been reported to used as tonic (Chopra RN et al., 1958), nervous stimulant (Ainslie W , 1979), aromatic, narcotic, astringent, hypolipidemic, antithrombotic, antifungal, antidysentric and anti inflammatory properties (Nadkarni AK, 2002).

C. zeylanicum, also known as Ceylon cinnamon (the source of its Latin name, zeylanicum) or 'true cinnamon', is indigenous to Sri Lanka. One important difference between 'true' cinnamon and the 'cassia' cinnamon is their coumarin content (Archer AW, 1988). Its branch peel without the epidermis and subereous layer is marketed as the commercial cinnamon which has been used a long in perfumery, culinary and native medicine systems (Gayoso, C.W et al., 2004 and Lopez Diaz et al., 2002).

The aim of our study is to examine the *in vitro* antioxidant and *in vitro* antimicrobial activity of essential oils from bark of *Cinnamomum zeylanicum* and fruit of *Myristica fragrans* against selected two Gram positive and two Gram-negative bacteria to justify their use as antimicrobial agents. The oil composition was also were characterized by gas-chromatography/mass spectrophotometrical analyses.

2. METHOD AND MATERIAL

2.1 Plant material

Bark of *Cinnamomum zeylanicum* (Lauraceae) and fruits of *Myristica fragrans* (Myristicaceae) were obtained from local market, Bhopal M.P. India authenticated by Dr. Zia-Ul-Hasan, Head & Botanist, Department of Botany, Safia College of Science, Bhopal (M.P.)India. Plant authentication voucher numbers obtained was ..... for *Cinnamomum zeylanicum* and *Myristica fragrans* respectively.

2.2 Extraction of plant for volatile oil (Burits and Bucar, 2000).

The essential oil extraction process was conducted using the hydro-distillation method using a modified Clevenger apparatus connected to a 4 L round-bottom, ground mouth flask. The extraction process was conducted for a period of two hours while the solution was kept boiling. Later, the hydrolate (water and oil) was collected and centrifuged for 5 minutes for the separation of the organic phase from the aqueous phase. The essential oil was then isolated with the aid of a Pasteur pipette, placed in a glass bottle and stored under refrigeration.

2.3 Determination of physical characters of the volatile oil

The yield of the volatile oils was calculated as below formula on fresh weight basis. Color, odor and taste were determined according to the Egyptian Pharmacopeia method (2005).

Collected volatile oils were measured and percentage yield for each extract was determined using formulae:

$$\% \text{ yield} = \frac{\text{Volume of volatile oil collected}}{\text{Weight of plant material used}} \times 100$$

2.4 Quantification of constituents of essential oils

For the qualitative evaluation of the essential oils, they were subjected to gas chromatography coupled with mass spectrometry (GC/MS). The operational setting were: fused silica capillary column (30 m × 0.25 mm), helium carrier gas, flow rate 1 mL per minute, varying temperature. GC peaks were identified by comparing MS fragmentation pattern and relative retention time with those of the reference compounds. Quantitative determination of a constituent was made using the calibration curve of the dose-peak area of a pure compound.

2.5 Anti-microbial activity

2.5.1 Bacterial strain

The microbial strains used in this experiment were *Staphylococcus aureus (MTCC-737), Escherichia coli (MTCC-1687), Streptococcus bacillus (MTCC-389)* and *Proteus vulgaris (MTCC-1771).* Throughout the experiment, the strains were stored under refrigeration in freezing culture medium (15 mL glycerol, 0.5 g bacteriological peptone, 0.3 of yeast extract and 0.5 g NaCl, per 100 mL of distilled water, with the final pH adjusted to 7.2–7.4).

2.5.2 Reference drugs

Ampicillin and amphotericin B (Sigma Aldrich) were used.

2.5.3 Well diffusion assay (Magaldi, S et al., 2004)

Mueller Hinton agar media was prepared according to the manufacturer's instructions. Microbial suspension of density 10^6 CFU/ml were used for inoculation on the Mueller Hinton agar media. Four wells of 6 mm diameter and 5 mm depth were prepared on the solid agar in each plate using a sterile borer. Test oil samples of various concentrations (25µl, 50µl, 75µl and 100 µl) were added. The plates were allowed to stand for 1 h at room temperature for diffusion of the oil sample and incubated at 37 °C for 24 h. After 24 h, the zones of inhibition were measured using a digital Vernier caliper. All experiments were conducted in triplicate and the mean values of the diameter of inhibition zones ± standard deviations were calculated. Ampicillin (50µl of 10 mcg/ml) was used as a positive control.

2.5.4 Minimum Inhibitory concentration (MIC) Determination (Eloff, 1998)

Briefly, different concentrations of oil sample were added to sterile broth further overnight incubated inoculums were added to each tube so that the initial concentrations of microorganism remain at 10^8 CFU/ml. Tubes were incubated for 24 h at 37 °C. After 24 h the tubes were observed for presence/ absence of microbial and fungal growth. Further, the presence of bacterial and fungal growth was confirmed by observing OD at 515 nm. The analyses were conducted in triplicate. The MIC was defined as the lowest concentration of an antimicrobial that inhibited the visible growth of a microorganism after overnight incubation

2.6 In vitro antioxidant assay

2.6.1 DPPH radical scavenging activity (Gulçin et al., 2006; Jain and Jain 2011)

The free radical scavenging activity of the oil sample was determined *in vitro* by 2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay. Different concentrations of test sample was prepared with methanol in the range from 20 to 100 mcg/ml. 2ml test sample was added to 1ml of DPPH solution and incubated the mixture in dark at room temperature for 10 min. The absorbance was measured at 515 nm by using Systronic's-2202 double beam UV-Vis spectrophotometer, against blank (methanol). The % Inhibition was calculated using following formula:

% Inhibition = [(AC 515 nm- AS 515 nm/ AC 515 nm) x 100]

2.6.2 Reducing Power Assay (Jain and Jain, 2011)

A different concentration of oil sample was prepared. 0.5 ml of sample was added with 0.5 ml of (0.2 M, pH 6.6) phosphate buffer and 0.5 ml of (0.5 ml, 1%W/V) potassium ferricyanide. Reaction mixture was incubated at 50° C for 20 min. After cooling, added 1.5 ml of trichloroacetic acid solution (10% W/V) to terminate the reaction. 0.5 ml ferric chloride (0.1% W/V) was added and absorbance was measured at 700 nm. A calibration curve was plotted between absorbance *versus* concentration. Increment in the absorbance of the reaction mixture indicates the increment in reducing capacity.

2.6.3 Superoxide Scavenging Assay (Hazra et al., 2008)

To the reaction mixture containing 0.1 ml of NBT, 0.3 ml of oil sample and 1 ml of alkaline DMSO will be added to give a final volume of 1.4 ml and the absorbance will be measured at 560 nm. Plain DMSO used as blank and reaction mixture without extract (water in place of extract) used as control. 50% inhibition of extract was calculated by plotting a graph between absorbance and concentration.

3. RESULTS

The yield of the essential oil of the bark of *Cinnamomum zeylanicum* and fruit of *Myristica fragrans* was found to be 5gm on fresh weight basis, respectively). Slight differences were noted between the two oils in their color, odor and taste Table 1.

S. No.	TEST	*Cinnamomum zeylanicum*	*Nutmeg Oil*
1	Colour	Yellowish	Pale Yellow
2	Odour	Pleasant	Peculiar delightful
3	Taste	Sweet	Bitter

Table 1: Physical Characters of essential oil of the *Cinnamomum zeylanicum* bark and of *Myristica fragrans* fruit.

3.1 GC-MS Spectra

Chromatograms of GC/MS analyses of the oils, displayed in Figs. 1 and 2 and revealed quantitative variations in the oil composition of both *Cinnamomum zeylanicum* and *Myristica fragrans*.

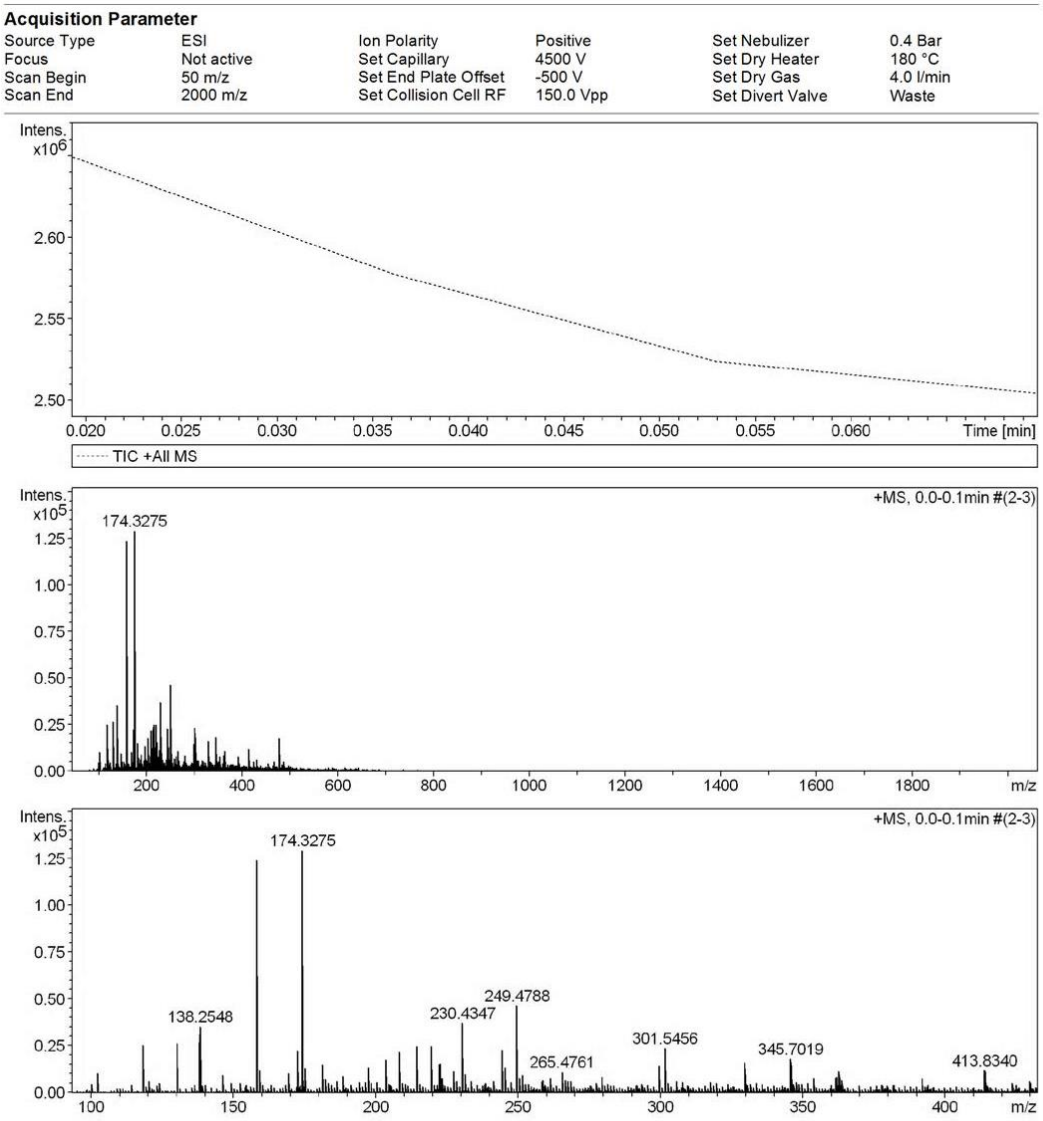


Figure:1 The chromatogram of *Cinnamomum zeylanicum* essential oil

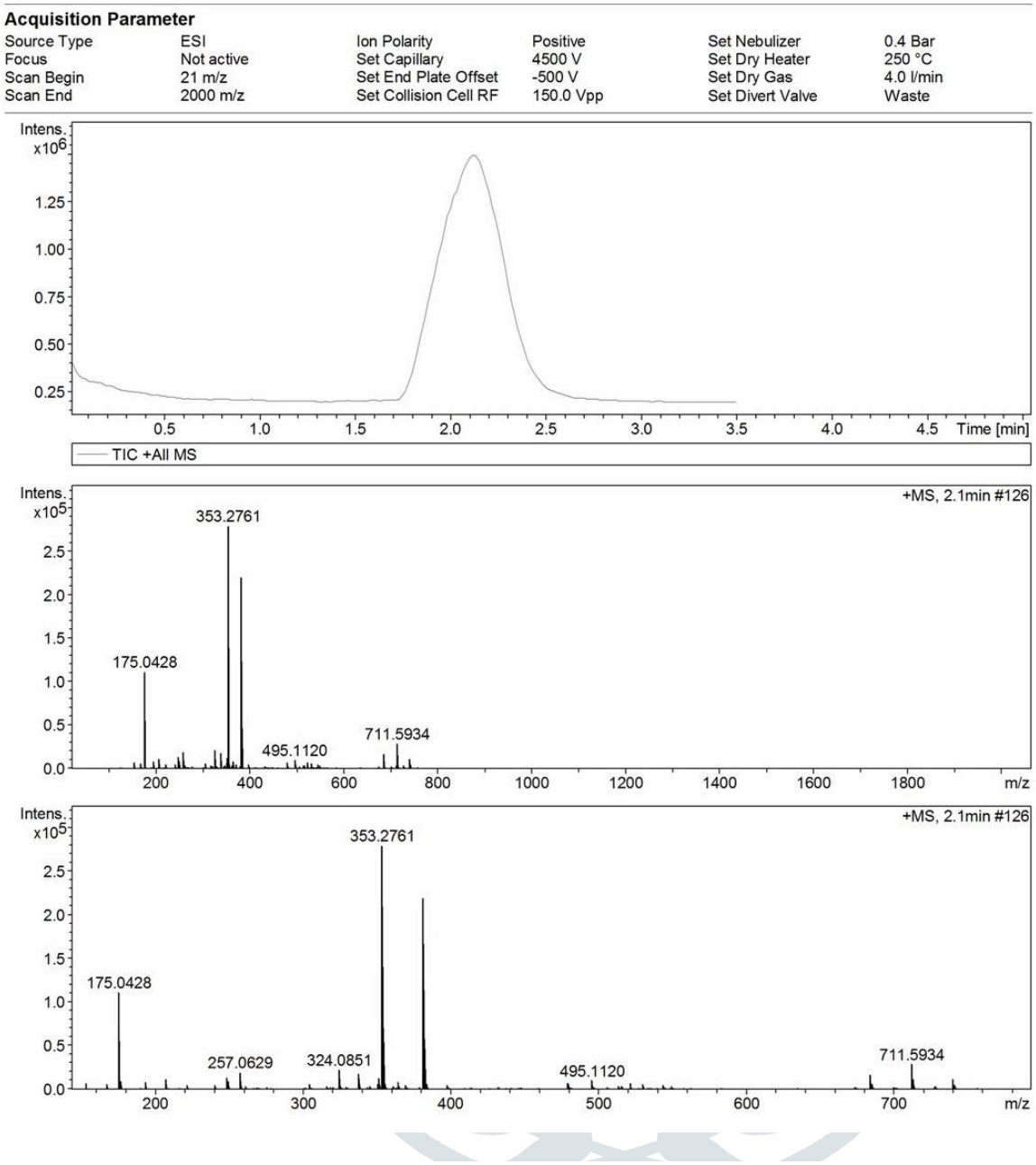


Figure 2: The chromatogram of *Cinnamomum zeylanicum* essential oil

3.2 Antimicrobial activity

The antimicrobial activity of the oil sample of *Cinnamomum zeylanicum* and *Myristica fragrans* were studied in different concentrations (25, 50, 75 and 100 µg/ml) against four pathogenic bacterial strains, two Gram-positive (*Staphylococcus aureus* MTCC-737 *and Streptococcus bacillus* (MTCC-389*)* and two Gram-negative (*Escherichia coli* MTCC 1687and *P. vulgaris* MTCC-1771). These strains have been selected for the basis of its application purpose of further studies.

Antibacterial potential of extracts were assessed in terms of zone of inhibition of bacterial and fungal growth. The results of the antibacterial are presented in Tables 2 and 3.

Table 2: Anti Bacterial Activity of essential oil of *Myristica fragrans* fruit

Name of the Organism	Zone of inhibition (mm^2) Mean±SD				
	100 µg/ml	75 µg/ml	50 µg/ml	25 µg/ml	Standard (Ampicillin) 10 µg/ml
Escherichia coli	13.24±0.297	10.95±0.902	9.64±0.096	7.89±0.531	22.78±0.860
Proteus vulgaris	11.63±0.800	10.39±1.041	9.31±1.338	8.04±0.740	26.16±0.569
Streptococcus bacillus	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	25.87±0.568
Staphylococcus aureus	14.69±0.480	10.94±0.569	10.31±0.207	9.50±0.500	31.60±0.529

Table 3: Anti Bacterial Activity of essential oil of *Cinnamomum zeylanicum* bark

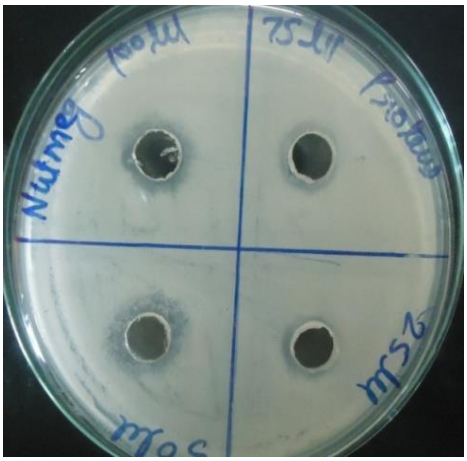
Name of the Organism	Zone of inhibition (mm^2) Mean±SD				
	100 µg/ml	75 µg/ml	50 µg/ml	25 µg/ml	Standard (Ampicillin) 10 µg/ml
Escherichia coli	15.78±0.442	13.30±0.672	11.23±0.228	9.44±0.587	22.78±0.860
Proteus vulgaris	11.01±0.625	10.70±0.701	9.63±0.415	8.63±0.650	26.16±0.569
Streptococcus bacillus	11.56±0.089	10.16±0.396	9.53±0.148	9.07±0.671	25.87±0.568
Staphylococcus aureus	15.15±0.386	13.05±0.502	11.19±0.447	10.08±0.415	31.60±0.529

Table 4:Minimum Inhibitory Concentration:

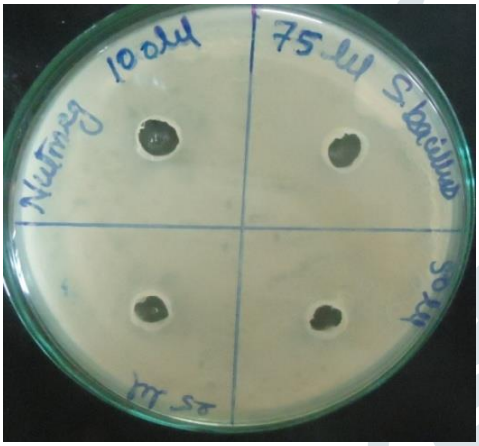
Organisms	MIC Concentration(µl/ml)	
	Nutmeg oil	**Cinnamon oil**
E. coli	3.125	0.390
P. vulgaris	1.562	1.562
S. bacillus	6.250	0.781
S. aureus	0.781	0.390

Antibacterial activity of oil of *Myristica fragrans* fruit

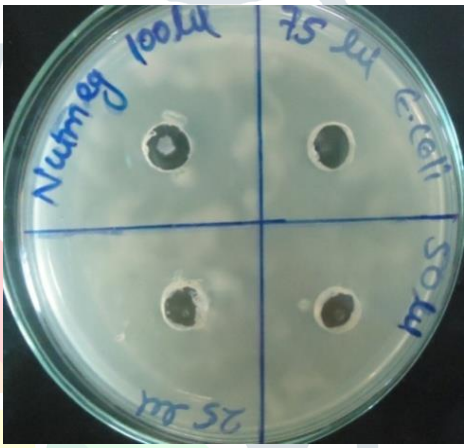
M. fragrans against *Staphylococcus aureus*



M. fragrans against *Proteus vulgaris*

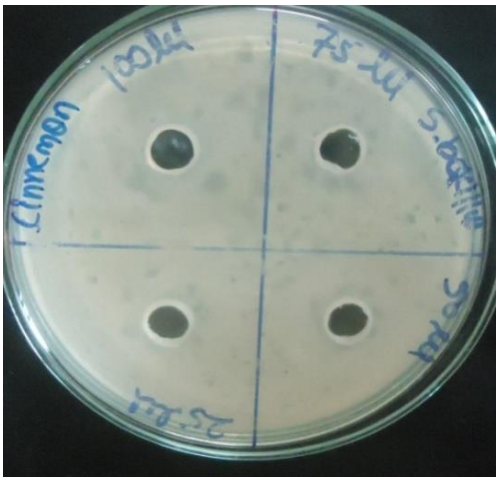


M. fragrans against *Streptococcus bacillus*

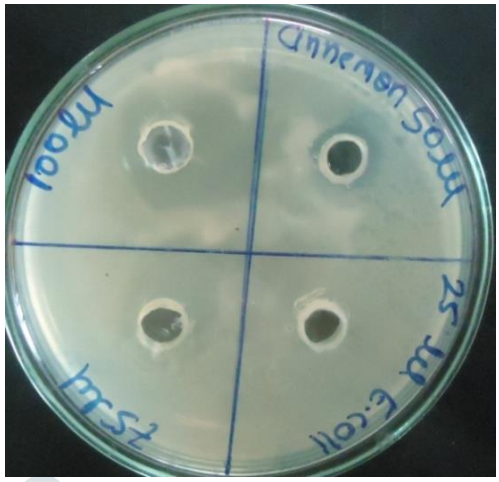


M. fragrans against *Escherichia coli*

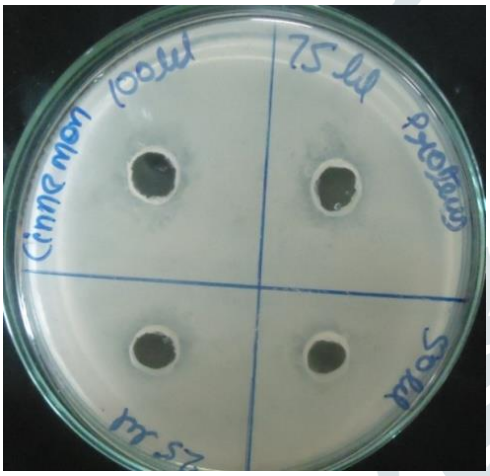
Antibacterial activity of oil of *Cinnamomum zeylanicum* bark



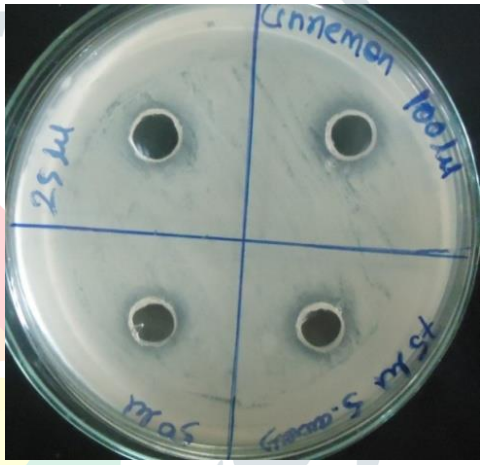
C. zeylanicum against *Streptococcus. bacillus*



C. zeylanicum against *Escherichia coli*

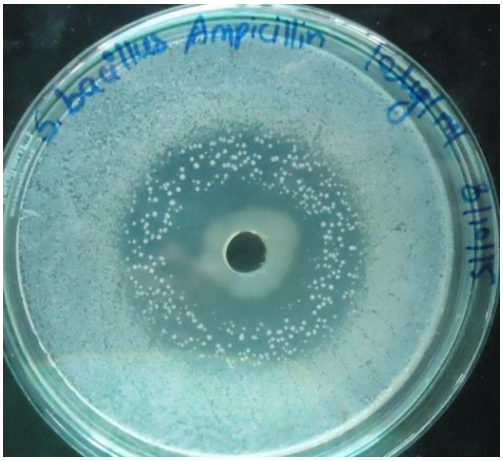


C. zeylanicum against *Proteus vulgaris*

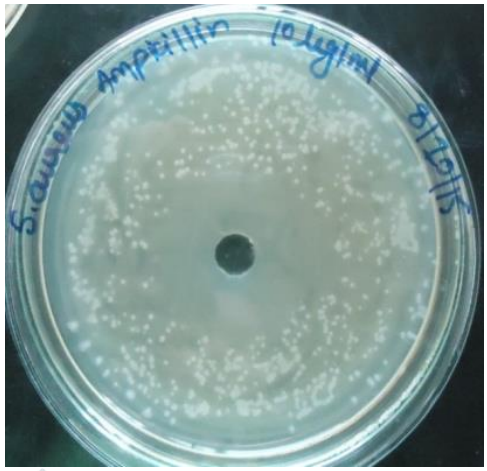


C. zeylanicum against *Stappylocccus aureus*

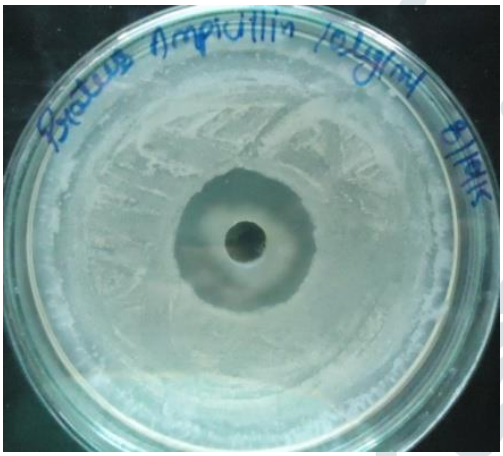
Antimicrobial Activity of Standard drug



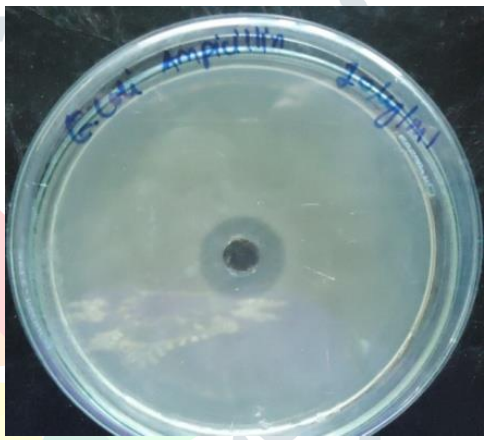
Ampicillin (10µg/ml) against *S. bacillus*



Ampicillin (10 µg/ml) against *S. aureus*



Ampicillin (10 µg/ml) against *Proteus vulgaris*



Ampicillin (10 µg/ml) against *E. coli*

3.3 Antioxidant Assay

The antioxidant activity of oil of bark of *Cinnamomum zeylanicum* (Lauraceae) & fruits of *Myristica fragrans* (Myristicaceae) was assessed by three models: DPPH assay, Superoxide scavenging assay and Reducing power assay. The antioxidant activity is related to the presence of bioactive compounds. Compounds like phenolic acids, flavonoids, tannins, etc. have high antioxidant potential. These assays give an over view of the antioxidant potential of an extract. Higher the antioxidant value more will be the bioactive compounds present in the extract. The DPPH radical scavenging activity using ascorbic acid as standard showed an increasing value of IC50, with the increase in polarity of the solvents. Essential oil of *Cinnamomum zeylanicum* (Lauraceae) showed highest quenching capacity of 34.95µg/ ml AAE while essential oil of fruits of *Myristica fragrans* (Myristicaceae) showed minimum scavenging activity of 61.51µg/ ml AAE.

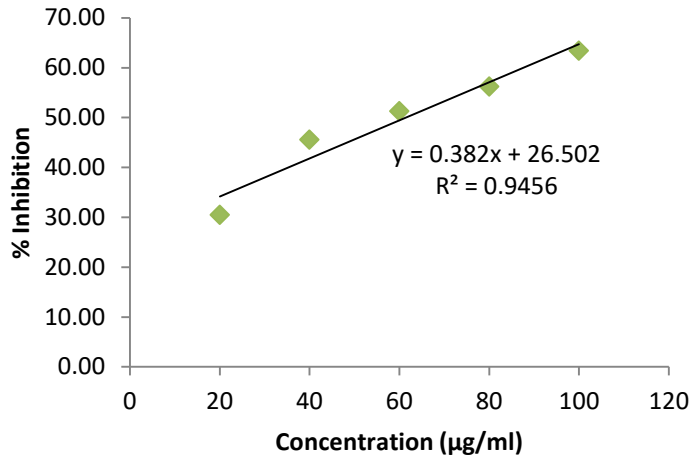


Figure 5: DPPH antioxidant activity of *Myristica fragrans*

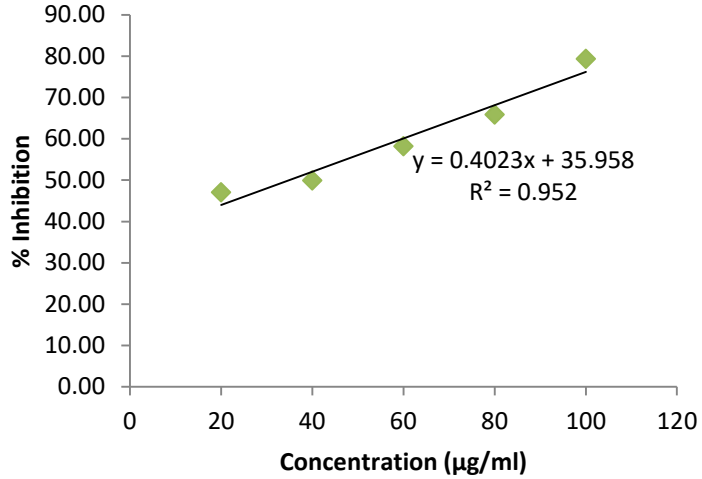


Figure 6: DPPH antioxidant activity of *Cinnamomum zeylanicum*

Superoxide anion radical is one of the strongest reactive oxygen species amongst free radicals and gets converted to other harmful ROS such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases. *Myristica fragrans* exhibited an IC50 value of 82.97µg/ml AAE, whereas the *Cinnamomum zeylanicum* showed the 50.00 µg/ml Ascorbic acid equivalent (AAE).

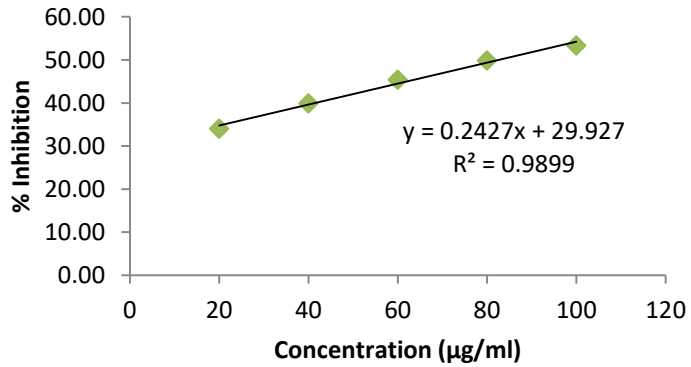


Figure 7: Superoxide anion radical scavenging activity of *Myristica fragrans*

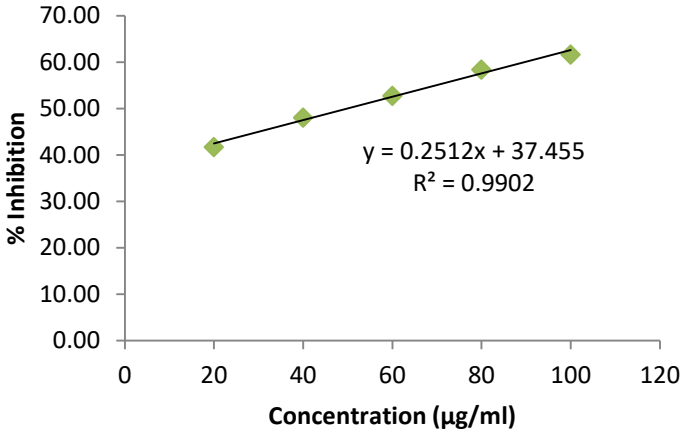


Figure 8: Superoxide anion radical scavenging activity of *Cinnamomum zeylanicum*

Reducing power assay of oil of bark of *Cinnamomum zeylanicum* (Lauraceae) & fruits of *Myristica fragrans* (Myristicaceae) was compared with standard ascorbic acid. *Cinnamomum zeylanicum* volatile oil showed the better reducing activity as compared to *Myristica fragrans* volatile oil.

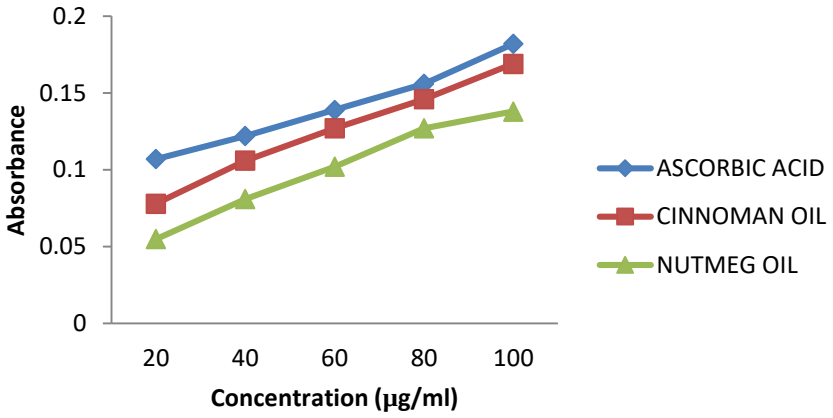


Figure 9: Reducing power assay of oil of *Cinnamomum zeylanicum* bark and *Myristica fragrans* fruits

4. DISCUSSION

Essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties (Burt, 2004, Kordali *et al.*, 2005). Some oils have been used in cancer treatment (Sylvestre *et al.*, 2006). Some other oils have been used in food preservation, aromatherapy and fragrance industries (Faid *et al.*, 1995).

Cinnamaldehyde was the predominant active compound found in cinnamon oil (Simic *et al.*, 2004 and Baratta *et al.*, 1998). Earlier studies suggested that the antibacterial activity of cinnamon oil was probably due to their major component, cinnamaldehyde and their properties could be multiple. Cinnamaldehyde is a natural antioxidant and the animal studies suggest that an extract of cinnamon bark taken orally may help prevent stomach ulcer. *Myristica fragrans* can also help with respiratory problems such as a cough from the common cold. It is often found as an ingredient in cough syrups. It is also said to be able to help with asthma (Blumenthal *et al.*, 1998).

Free radicals and other reactive oxygen species like hydroxyl radical (.OH), superoxide anion (.O2−), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) can cause oxidative damages to biological macromolecules which can lead to initiation and/or progression of various diseases. The present study has demonstrated the potential antioxidant activity in essential oil of bark of *Cinnamomum zeylanicum* (Lauraceae) & fruits of *Myristica fragrans* (Myristicaceae).

The essential oils of bark of *Cinnamomum zeylanicum* (Lauraceae) & fruits of *Myristica fragrans* (Myristicaceae) also exhibited broad spectrum antibacterial activity at the given concentrations, when compared to ampicillin as a standard. Bark of *Cinnamomum zeylanicum* was superior as antibacterial agent to that of fruits of *Myristica fragrans* against all the tested organisms. This could be justified by a higher percentage of active components in *Cinnamomum zeylanicum* compared to *Myristica fragrans*. Referring to Table 4, the MIC of the essential oil of the bark of *Cinnamomum zeylanicum* against *Staphylococcus aureus* MTCC-737 *and and Streptococcus bacillus* (MTCC-389*)* and two Gram-negative (*Escherichia coli* MTCC 1687and *P. vulgaris* MTCC-1771) recorded as (0.390 and 0.78l µl/ml) and (0.390 and 1.562 µl/ml), while the oil of *Myristica fragrans* exhibited MIC value of (0.781 and 6.250 µl/ml)and (3.125 and 1.562 µl/ml) respectively. Thus, these essential oils could be considered as potent antibacterial agents.

Therefore, these essential oils and their major component may be considered as possible sources for the development of new antimicrobial agents and may be used in synergy with currently available synthetic antibiotics or antimicrobials. The antioxidant and antimicrobial activity possessed by *Cinnamomum zeylanicum* (Lauraceae) & *Myristica fragrans* (Myristicaceae) could be helpful in preventing or slowing the progress of various oxidative stress-related diseases and infections by opportunistic pathogenic microorganisms.

5. CONCLUSION

The growing interest in natural bioactive compounds has led to conduct further studies addressing the replacement of synthetic chemical agents in the industrial sector, since natural products are less harmful to health. Gram-positive bacteria were more susceptible to the action of the essential oil than Gram-negative bacteria. The antimicrobial activity of essential oils depends on the chemical composition of the plant, which can vary according to the period of the year (Yesil-Celiktas et al., 2007). The greatest resistance of Gram-negative bacteria to the action of essential oils can occur due to the complexity of the double membrane of these microorganisms, which limits the diffusion of hydrophobic compounds through the lipopolysaccharide component (Burt, 2004; Holley and Patel, 2005).

Medicinal plants have been extensively studied for their antioxidant and free radical scavenging activity in the last few decades. Among these natural antioxidants, phenolic compounds have been shown to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical (Pratt, DE 1980).

The results of the antioxidant activity reported in the literature are difficult to compare, since it is strongly influenced by the determination method. Several methods have been described for assessing the antioxidant activity of chemicals present in essential oils and plant extracts. Some authors propose tests that rely on reducing free radicals generated *in vitro*, resulting from the antioxidant activity of substances assessed, especially the DPPH method, because it is a quick and feasible alternative (Molyneux, 2004). Regarding the antioxidant activity, the oil was effective exhibiting values close to those of the synthetic antioxidant.

The essential oils from *Cinnamomum zeylanicum & Myristica fragrans* showed varying degrees of antibacterial activity against various pathogens. From the above experiment it can be inferred that oil of *Cinnamomum zeylanicum* suggest significant growth inhibiting effects on Gram-positive (*E. coli*) and *Myristica fragrans* against Gram-negative bacteria (*S. aureus*). The efficacy of bark oil of *Cinnamomum zeylanicum* against these microorganisms may provide a scientific ground for the application of the herb in the prevention and treatment of bacterial infections caused by various pathogenic bacteria such as *Staphylococcus aureus, Proteus vulgaris, and Streptococcus bacillus* and *Escherichia col*i, which have developed resistance to antibiotics. The incorporation of this oil into the drug formulations is also recommended. The results of this study present the herb as a good candidate to explore new alternative antibacterial agents to combat pathogenic microorganisms.

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