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Characterization of Antibacterial Activity of Selected Essential Oils

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A B S T R A C T

In the present investigation, eleven essential oils (Curry Leaf, Ginger, Azowan, Betel leaf, Black cumin, *Marjoram*, *Hedychium*, *Calamus*, *Peppermint*, Cinnamon, *Basil*) were screened, in order to assess their antimicrobial activities against three bacteria (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*). Of the eleven essential oils studied, Azowan, Peppermint, Basil, Black cumin and Cinnamon oils showed strong antibacterial activity against the pathogens. The Minimum inhibitory concentration (MIC) values of the oils, ranged between 0.48-125 µl/ml. The Minimum Killing Time (MKT) of the oils varied from 0 to 60 mins, at room temperature. The oils retained their antibacterial activities, even after heat treatment (100°C for one hour), and on autoclaving which indicated the presence of barostable and thermostable components in these oils. The phenol co-efficient values of the oils fall between 0.25-0.5. The antibiotic sensitivity pattern of the pathogens had shown multiple antibiotic resistance (MAR). The activities of the oils reported to be bactericidal in nature, and were well compared to the standard antibacterial compounds.

Introduction

Many bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents and showed resistance to old and newly produced drugs. For the last few decades, multi drug resistance has become an increasing concern for both gram positive and gram negative bacteria (Brunel and Guery, 2017). The resistance of the microorganisms increased due to over and misuse of the commercial antimicrobial drugs and chemotherapeutic agents as well as a lack of new drug development by the pharmaceutical industry (Gould and Bal, 2013; Viswanathan, 2014; Michael *et al.*, 2014).

This situation has forced the researchers and academicians, to search for new antimicrobial substances from various sources, foremost are the medicinal and aromatic plants, as plant

products are biodegradable, and without any side effects to the animals and environment.

Several aromatic and medicinal plants produce essential oils (EOs). EOs are hydrophobic liquid, clear or unusually coloured, composed of complex compounds that are volatile in nature. They are characterised by strong odour and obtained from medicinal and aromatic plants part such as flowers, leaves, seeds, bark, fruits and roots (Burt, 2004). EOs have been reported to possess antifungal, antibacterial, antiviral, antioxidant, analgesic and anti- inflammatory activities (Gao *et al.,* 2011; Gilles *et al.,* 2010; Kordali *et al.,* 2005; Mourey & Canillac, 2002; Prakash *et al.,* 2011). There is an dire need for newer antimicrobial compounds, and EOs are less studied. So, this prompted us to test the antibacterial activity of some selected essential oils against the bacterial pathogens such as *E.coli, S. aureus* and *P. aeruginosa.*

Materials and Methods

Pathogens

The test pathogens [*Escherichia coli* (MTCC-1675), *Staphylococcus aureus* (MTCC-902), and *Pseudomonas aeruginosa* (MTCC-741)] were used in this study and were obtained from P.G Department of Microbiology, College of Basic Science and Humanities, OUAT, Bhubaneswar.

Essential oils

Eleven different EOs *viz.,* Curry Leaf (*Murraya koenigii*), Azowan (*Trachyspermum ammi*), Betel leaf (*Piper betel*), Black cumin (*Nigella sativa*), Peppermint (*Mentha piperita*) from Southern species, Madurai, Ginger (*Zingiber officinales*), Marjoram (*Origanum marjorana*), Hedychium (*Hedychium coronarium*), Calamus (*Acorus calamus*) from Hitesh Aromatics, Mandi, Himachal Pradesh, and Cinnamon (*Cinnamomum verum*), Basil (*Ocimum basilicum*) were procured from Flower and Fragrance development cooperation, Berhampur, Odisha, were used to assess their antimicrobial activities against the test pathogens.

Media and Chemicals

Nutrient broth (NB), Nutrient agar (NA) and Tween 80 were procured from Hi-Media Ltd. Mumbai, India, and all the media were prepared according to manufacturer's instruction. The media were supplemented with 0.75% of Tween 80 (T-80) to facilitate the miscibility of the oils.

Preliminary screening of oils by Disc Diffusion Method (DDM)

For the preliminary screening of the EOs, Disc Diffusion Method (DDM) described by Bauer *et al.,* (1996) was used, to assess their antimicrobial properties qualitatively.

Determination of the Minimum Inhibitory concentration (MIC) of the oils

Minimum Inhibitory Concentration of the oils was determined by Tube Dilution Method described by Rath *et al.,* (1999), with slight modification. In brief, NB was supplemented with 0.75% Tween 80 and was dispensed into test tubes and sterilized. By the help of sterile pipette, 0.5ml of the oil was transferred to the first test tube and mixed by vortexing for a homogenous emulsion. Further, the sample was serially diluted as two- fold dilution. From the 10^{th} test tube, 0.5ml of emulsion was discarded. 20 µl of freshly grown culture was added to all test tubes, and incubated at 37°C for 24 hrs. One tube without oil served as control. A loop full of bacterial culture was taken from each test tube, and then streaked on the agar plates. Plates were incubated at 37°C for 24 hrs. After the incubation period,

the plates were observed for the bacterial growth. No growth of the test organism at a particular concentration was determined as MIC for that oil.

Determination of the Minimum Killing Time (MKT) of the oils

The Minimum Killing Time (MKT) of the oil was described by Rath *et al.,* (2005). Overnight incubated NB cultures of microorganisms were inoculated into NB containing MIC level of the oils, and were incubated at 37°C. One tube without oil served as Control. Under aseptic conditions, one loop full of the sample from the test tubes was sub cultured on to NA plates at 0′, 2′, 5′, 10′, 15′, 30′, 1hr, 2hrs, 3hrs, 4hrs and 5hrs of interval and incubated at 37°C for 24hrs. After incubation, the plates were observed for bacterial growth. No growth on sub-culturing at the particular time, was regarded as the minimum time required to kill the bacteria by that oil.

Effect of temperature and pressure on antibacterial activity of oils

An experiment was designed to study the effect of high temperature (by treating the oils at 100°C for 1hr, in a boiling water bath) and pressure (autoclaving the oils) on antibacterial activity of the test oils by Disc Diffusion Method. Sterile filter paper discs were mounted on the surface of the pre-cultured (from 24hrs old culture, swept on NA plates) bacterial pathogens on agar plates at equal distance. Subsequently, all the EOs were heated at 100°C for 1hr and autoclaved at 121°C and 15 lbs pressure, for 20 mins.

Using a sterile pipette, EOs were loaded (at MIC level) over the sterile filter paper discs separately, and incubated at 37°C for 24 hrs. Plates were observed for zone of clearance around the discs which indicates positive antibacterial activity of the treated oils, in comparison to control oil.

Determination of the Phenol Coefficient value of the oils

Selective oils that had shown better antibacterial properties, were subjected to phenol coefficient test, in order to compare their efficiency, described by Rath and Mohapatra (2015).

Determination of drug sensitivity pattern of test pathogens

An experiment was conducted, to study the antibiogram as well as the multiple antibiotic resistances (MAR %) pattern of the test pathogens, in order to compare the antibacterial activity of the EOs with standard antibacterial drugs. The antibiogram pattern was studied following the method of Bauer *et al.,* (1996). Standard antibiotic discs, procured from Hi-Media, Mumbai were used in this study. After the incubation period, plates were observed for susceptibility (sensitivity of the organism to an antibiotic) or resistance (no zone of clearance around the disc) of the pathogen towards a specific antibiotic. The multiple antibiotic resistance % of the pathogens was determined by using the formula:

$$\text{MAR} = \frac{\text{Number of antibiotics to which the pathogen Showed resistance}}{\text{Total no. of antibiotics used}} \times 100$$

Results and Discussion

The bioactivities of eleven EOs were evaluated against three pathogens (*E.coli, S. aureus* and *P. aeruginosa*). The EOs of Azowan, Peppermint, Basil, Black cumin and Cinnamon had shown strong antibacterial activity, in terms of their zone sizes against

the test pathogens (Table-1). The Zone of Inhibition ranged from 8-32 mm. Azowan oil had shown similar results against *E.coli* and *S. aureus* (25 mm), respectively whereas, showed zone size of 30 mm against *P. aeruginosa*. The three test organisms were resistant to Curry leaf oil. Resistance was too observed in case of *E.coli* and *S. aureus* against betel leaf and Calamus oil. The oils differ in their antibacterial activities against the test pathogens, in terms of Zone of inhibition. In comparison to our observations, Mekonnen *et al.,* (2016) also reported that, *Trachyspermum schimperi* had shown higher zone sizes of 23.5 mm, 12 mm and 16 mm against *S. aureus, E.coli*, and *P. aeruginosa*, respectively.

Minimum Inhibitory Concentration (MIC) values of four oils (Betel leaf, Cinnamon, Marjoram and Peppermint) evaluated against three test pathogens, ranged from 0.48-125 µl/ml (Table-2). Lowest MIC values of 0.97 µl/ml and 0.48 µl/ml were observed in case of Cinnamon oil against *E.coli* and *S. aureus,* respectively.

Highest MIC value of 125 µl/ml was observed in case of peppermint against *S. aureus.* Oils of Betel leaf and Marjoram had shown MIC value of 62.5 µl/ml against *S. aureus* and *P. aeruginosa*, respectively, whereas Cinnamon oil showed similar MIC value of 62.5 µl/ml against *P. aeruginosa.* In comparison to our observations, Lv *et al.,* (2011) also reported lower MIC value (0.1-0.4 µl/ml) of Cinnamon oil against *S. aureus* and *E.coli*. Generally, higher MIC values observed against Gm-ve bacteria in comparison to Gram+ve bacteria could be attributable to the thick layer of lipopolysaccharide (more resistant to EOs) outer membrane covering the cell wall as compared with the gram-positive bacteria having homogenous peptidoglycan layer structure (Salton, 1953; Sikkema, D *et al.,* 1995; Hsouna *et al.,* 2011). In contrast, in our studies, we observed higher MIC values of EOs against *S. aureus* as compared to *E.coli*. The activities of the EOs were observed to be bactericidal, as no growth was recorded on subculture onto NA plates from the MIC dilution tubes. The Minimum Killing Time (MKT) of the oils, varied from 0 to 60 mins, at room temperature (Table-3). Oils of Betel leaf killed both the pathogens (*E.coli* and *S. aureus)* within 30 mins. Peppermint oil killed *E.coli* and *P. aeruginosa* within 60 mins.

The oils of Marjoram and Cinnamon killed all the three test pathogens within 0 min. Killing of these pathogens immediately by these EOs suggested that the oils cause an irreversible damage to the cellular structure of the pathogens, when they come in contact with the oil mixture (Pattnaik *et al.,* 1995; Rath *et al.,* 1999a, b, 2001, 2002 & 2005).

The tested oils had shown antibacterial activity even after treating them at 100ºC for an hour (in a boiling water bath), and autoclaving (121ºC and 15 lb pressure for 20 mins) (Table-4).

In case of Marjoram and Peppermint oil, zone size was highly decreased, when treated with high pressure and temperature as compared to normal untreated oil, against *E.coli* and *S. aureus*. Betel leaf and Cinnamon oil when treated with high temperature, the zone of inhibition was same as that of untreated oil against *E.coli* and *S. aureus,* respectively. All the oils had shown decrease in the zone size by boiling and autoclaving, against *P. aeruginosa*, but the changes were not remarkable. Observation of antibacterial activity of the tested EOs even on heat treatment and autoclaving, indicated the presence of some thermostable and barostable components in the oils. Rath *et al.,* (2001) observed decreased activity of turmeric leaf and rhizome essential oils, against these pathogens.

Table.1 Antimicrobial activities of essential oils by using Disc Diffusion Method

Sl. no.	Essential Oils	Zone of Inhibition (ZOI) in mm.		
		E.coli	***S. aureus***	***P. aeruginosa***
1.	Curry leaf	R	R	R
2.	Ginger	12	11	20
3.	Azowan	25	25	30
4.	Betel leaf	R	R	20
5.	Black cumin	12	20	32
6.	Marjoram	10	14	10
7.	Hedychium	8	R	14
8.	Calamus	R	R	10
9.	Peppermint	11	10	25
10.	Basil	24	18	23
11.	Cinnamon	24	10	20

*R- Resistant

Table.2 Minimum Inhibitory Concentration (MIC) of oils

Sl.no.	Essential Oils	MIC VALUES (µl/ml)		
		Escherichia coli	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
1.	Betel leaf	7.81	62.5	62.5
2.	Cinnamon	0.97	0.48	62.5
3.	Marjoram	31.25	62.5	62.5
4.	Peppermint	3.9	125	31.25

***R= Resistant to particular oil**

Table.3 Minimum Killing Time of essential oils against test pathogens

Sl.no.	Essential Oils	MINIMUM KILLING TIME (MKT) AT 37◦C IN MINS		
		Escherichia coli	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
1.	Betel leaf	30mins	30mins	0min
2.	Cinnamon	0min	0min	0min
3.	Marjoram	0mins	0min	0min
4.	Peppermint	60mins	0min	60mins

Table.4 The effect of temperature and pressure on antibacterial activities of the oils

Essential oils	Zones of Inhibition (in mm) at different temperatures								
	E.coli			*S. aureus*			*P. aeruginosa*		
	UT	100°C	A	UT	100°C	A	UT	100°C	A
Betel leaf	15	15	14	12	14	12	12	2	9
Cinnamon	14	15	13	13	13	14	10	9	10
Marjoram	13	7	8	11	10	9	10	10	9
Peppermint	11	8	9	18	17	16	7	9	R

UT- Untreated i. e oils were tested without heating or autoclaving, A: autoclaved oils, oils were applied at MIC level on discs.

Table.5 Phenol Co-efficient value of essential oils

Essential oils	Phenol coefficient
Betel leaf	0.25
Marjoram	0.5
Peppermint	0.5
Cinnamon	0.25

Table.6 Antibiogram pattern of the test pathogens tested by DDM of Bauer *et al.*, (1996)

PATHOGENS	ANTIBIOGRAM		
	Sensitive to	Resistant to	MAR%
Escherichia coli	Co(18), Cq(13),GEN (18), AK(19), CE (22), C(31), S(18), NF(22), Do(20)	MET, AP, NS, P, B, Lz, PB, Ax	47.05%
Staphylococcus aureus	Co(21), Cq(21), GEN(21), AK(21), CE(16), C(29), S(21), NF(24), P(23), B(15), Do(27), Ax(19)	MET, AP, NS, Lz, PB	29.41%
Pseudomonas aeruginosa	Cq(13), GEN(17), Ak(19), CE(22), C(31), S(20), NF(22), Do(19)	Co, MET, AP,NS, Ax, P, B, Lz, PB	52.94%

The values in paranthes indicates zones of inhibition in mm

They reported that the viscosity of the oil increased at higher temperature (100^0C) which could be an inhibiting factor in the diffusibility of the oils over the plates and results in smaller zones of inhibition. Das (2008) and Das *et al.*, (2009) reported the antibacterial activity of EOs of three *Ocimum spp.* and their cocktail mixture at high temperature and pressure treatment. They observed the presence of heat stable and baro stable components in these oils, as reported in our studies.

The phenol co-efficient value of the four oils tested, ranged from 0.25- 0.5 (Table-5). Highest phenol co-efficient value (0.5) was observed in case of Marjoram and Peppermint. Betel leaf and Cinnamon oil had shown lowest phenol co-efficient value of 0.25. Rath *et al.*, (2008) reported that samples with lowest MIC values had shown highest phenol coefficient values which differs from our investigation.

The antibiogram pattern of the test pathogens is presented in Table-6. From the antibiogram pattern, all the organisms were resistant to Methicillin, Amphotericin-B, Nystain, Linezolid and Polymyxin-B, whereas, the sensitivity pattern differs. The antibiotic sensitivity pattern of the pathogens showed multiple antibiotic resistance (MAR). Highest MAR% was recorded in case of *P. aeruginosa* (52.94%), followed by *E.coli* (47.05%) and *S. aureus* (29.41%). The zone of inhibition observed in case of the essential oils could be well compared with standard antibiotics. Smaller zone of inhibition by Eos with respect to antibiotics used, could be attributable to the crudeness of the active compound(s) present in the tested EOs.

Senhaji *et al.*, (2007) observed the antibacterial activity of essential oil from *Cinnamum zeylanicum* against *E.coli* 0157:H7 is through outer membrane disintegration and increasing the permeability to ATP through cytoplasmic membrane that corroborates with the findings observed in this investigation as *E.coli* was resistant to Penicillin and Bacitracin. Similarly, Rath *et al.*, (2005) also reported the anti-staphylococcal activity of Juniper and Lime essential oils against methicillin resistant *Staphylococcus aureus* (MRSA) through inhibition of cell membrane synthesis. This corroborates with our observations. Through this investigation we have placed in record, the antibacterial activity of the essential oils, is suggestive of their uses in discovery of newer antimicrobial compounds, and / or in pharmaceutical, aroma and cosmetics industries with proper scientific investigations.

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