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ORIGINAL ARTICLE **OPEN ACCESS**

Synergistic and Antagonistic effects of essential oil extracts against *Escherichia coli, Pseudomonas spp., Staphylococcus spp. and Candida sp*

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ABSTRACT
In the present study, clove buds, peppermint and lemongrass leaves were used for extraction of their essential oils to check their antimicrobial properties against microorganisms including Escherichia coli , Pseudomonas spp., Staphylococcus spp. and Candida spp. The activity of each extract was checked against microorganisms and then combinations of the three extracts were made in the ratio- 1:1 , 1:2 , 2:1 and 1:1:1 to study their synergistic or antagonistic effect against the microorganisms and compared with their individual activities. E.coli is found to be the most susceptible organism to all three essential oils whereas Candida was least susceptible. These combinations showed moderate activity against Pseudomonas spp. and Staphylococcus spp. Therefore, the essential oils show more potent antibacterial activity than antifungal activity.
Key words: *Essential oils, ratios, synergistic effect, antagonistic effect, micro-organisms, combination.*

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INTRODUCTION

Essential oils (EOs) or aromatic plant essence are volatile and fragrant substances with an oily consistency typically produced by plants synthesized by all plant organs i.e. buds, flower, leaves, stem, seed, fruit, root, wood or bark, and are stored in secretory cells, cavities, canals and epidermic cells. The most widely used flowers are: *Jasminum spp., Rosa spp., Viola spp., Lavandula spp., S.aromaticum L.* Leaves are: *Thymus vulgaris, Eucalyptus spp., Lippia graveolens, Ocimumbasilicum, Salvia rosmarinus*[5].

EOs have been long recognised for their antibacterial, antifungal, insecticidal, antioxidant properties and are widely used in medicine. They are complex mixtures of volatile constituents biosynthesized by plants which contains more than 20 components at different concentration, which mainly include two biosynthetically related groups [1]. These main groups include terpenes and terpenoids and aromatic and aliphatic constituents, all characterised by low molecular weight. The use of combinations of Eos and their isolated components are thus new approaches to increase the efficacy of Eos in food and medicine taking advantage of their synergistic and additive effects [2].

Clove (*Syzygium aromaticum)*is considered as one of the most valuable spice and is mainly processed into clove oil. It has diverse uses in pharmaceutical and medicinal field due to its antioxidant, antibacterial, and anesthetic properties[3]. Clove oil is richin volatile compounds and antioxidants such as eugenol, beta-caryophyllene and alpha-humulene (10-40%)[4]. Eugenol is the major constituent(70-90%) in the aromatic oil extracted from cloves. In vitro, it has been shown to have antibacterial, antifungal and antioxidant activities.

Mentha piperita (peppermint) is a medicinally important plant that belongs to the family Labiate. Peppermint is a non-herbaceous plant and its leaves contain about 0.5-4% volatile oil that is composed of 50-78% free menthol, monoterpene, menthofurane and traces of Jasmine(0.15%) to improve the oils quality remarkably. Peppermint oil and menthol are bacteriostatic for a wide range of organisms. It is also found to have antiviral and antifungal activities[6]. These oils are often used for their flavour and their therapeutic or odoriferous properties, in a wide selection of products such as foods, medicine and

cosmetics. Peppermint is an excellent source of antioxidants like vitamin C and beta- carotene, which is converted to vitamin A in the body[7].
Lemongrass (*Cymbopogon citratus)* essential oil comes from the lemongrass plant , which grows in tropical and sub tropical parts of the world. The oil is made with different chemical compounds like citronellal, grenayl acetate,neral etc. It has been used in traditional medicine for pain relief, stomach problems, and fevers. It shows antioxidant, anti-inflammatory and antifungal properties. It exhibits broad spectrum fungi toxicity in some post-harvest fungi. It also inhibits several bacteria known to have deleterious effects when consumed in food [8].
Essential oils contain 250 chemical constituents, and they commonly comprise of substance mixes which have oxygen, carbon and hydrogen as their main components. It mainly comprise of two groups: **Volatile part**: Essential oil contains 90% of oil by weight, consisting of thermoterpenes and hydrocarbons and esters and **Non- volatile residue**: This contain 5-10% of the oil which contains mainly hydrocarbons, fatty acids, wax and flavonoids.
Synergism: The interaction between two or more substances that gives a greater combined effect than their individual effect is called synergism.
Antagonism: The interaction between two or more substances which gives a lesser combined effect than their individual effect is called antagonism.
The interaction between the components of essential oils may lead to synergistic or antagonistic effects. Essential oils show antimicrobial, antifungal and antioxidant activities. They are also used for pain management and skincare.

MATERIAL AND METHODS

Collection of plant material:
Around 200 grams of dried clove buds, 500 grams of fresh lemongrass leaves and 500 grams of fresh peppermint leaves were purchased from a local spice shop in Pune, Maharashtra, India.
Collection of microbial cultures:
Strains of *Escherichia coli, Staphylococcus spp., Pseudomonas spp*and *Candida spp* were taken from the Biotechnology laboratory of Abeda Inamdar Senior College and sub-cultured.
EXTRACTION OF ESSENTIALOILS:
Clove oil extraction procedure:
200 grams of dried clove buds were crushed using mortar and pestle.250 ml ethanol was added as a solvent to round bottom flask that is attached to a soxhlet extractor and condenser. The crushed clove buds were loaded into thimble, which is placed inside the soxhlet extractor. The solvent was heated using isomantle(electric heater) to boiling temperature(>78°C) for evaporation passing through the apparatus to condenser. After completion of the process, the solvent(ethanol) was evaporated using a rotatory evaporator, giving a small yield of extracted clove bud oil (about 5-10ml) in the glass bottom flask
The extracted oil was then stored in amber coloured bottle in dark[10].
Peppermint oil extraction procedure:
PMEO was extracted in two batches using 250 grams of sample per batch.
250g of fresh peppermint leaves were weighed, washed and cleaned and cut into small pieces. Then the mass was fed into distillation flask. The steam was allowed to pass through the pores of peppermint bed and extract the volatile oil with it. This extraction was carried out until whole of the volatile oil was extracted by using 250ml distilled water to generate steam in the heater. In the flask, two layers are formed after the condensed vapours are settled. The oil is separated from the flask by density difference principle [9]. The same procedure was repeated twice for batch 2 and 3.Total yield of oil was collected in amber coloured bottle and stored in dark .
Lemongrass oil extraction procedure:
LEO was extracted in two batches using 250 grams of sample per batch.
250g of fresh lemongrass leaves were weighed, washed and cleaned and cut into small pieces. The mass was into distillation flask. After preparing the bed, the outlet of the column is connected to water cooler and the outlet of the condenser is connected to the collecting flask. The steam was passed into the column from the bottom. This extraction is carried out until whole of the volatile oil is extracted by using 250ml distilled water to generate steam in the heater. In the flask, two layers were formed after the condensed vapours are settled. Watery layer remains on lower side while oily layer floats on the water. The oil was separated from the flask by density difference principle[9].
Escherichia coli, Pseudomonas spp. ,Staphylococcus spp. and *Candida spp* were sub cultured on selective media by streak plate method . Incubated at 37 °C for 24 hours. After obtaining growth, isolated colony was inoculated in nutrient broth for further use.

ANTIMICROBIAL ACTIVITIES

Antimicrobial activities were checked using two methods:

Procedure of Agar well diffusion method:

0.1 ml of log phase culture of each micro-organism was spread onto 4 plates each. Two wells of 6mm diameter were made in each plate using a flame sterilized cork borer. 0.1 ml(100microliter) of all three essential oil extracts were loaded in one well for each micro-organism and controlwas loaded into another by using sterile micro-pipette tips.Tween-80 was used as control.Plates were kept at 4°C for pre-diffusion for 15-20 minutes. After pre-diffusion, each plate was incubated at 37°C for 24 hours.After 24 hours, zone of inhibition was checked and measured

Procedure for Agar disc diffusion method:

250ml nutrient agar media was prepared in 500ml flask and autoclaved. After autoclaving, media was poured in 12 sterile petri plates, allowed to cool down and solidified. 0.1 ml of enrichment culture of each micro-organism was spread onto 4 plates each using spread plate technique. Two discs made of 6mm diameter were dipped into all three essential oil extracts and were placed in the petri plate of each micro-organism. Similarly, disc for control was also placed with the help of sterile forceps.Tween-80 was used as control. The plates were incubated at 37°C for 24 hours. After 24 hours zone of inhibition was checked and measured.

Standard plates : Plate for each micro-organism was prepared and individual activity of extracts of all three extracts was checked against them.

Combinations: Three combinations were used,1:1, 1:1:1, 1:2 and 2:1 concentration where total volume was equal to 1.2 ml. and their activity was checked against the micro-organisms used.

RESULT

Three oils with four different combinations (1:1 , 1:2, 2:1 , 1:1:1) were analysed for their synergistic or antagonistic effect against the microorganisms used. The individual effect of clove essential oil showed a maximum zone of inhibition against *C. spp*(13mm) as compared to control, followed by *S. spp.(*11mm) as compared to control, *P. spp.*(9mm) as compared to control and *E.coli* (8mm) as compared to control. Lemongrass essential oil showed a greater zone of inhibition in *E.coli* (11mm)as compared to others. Peppermint essential oil showed a greater zone of inhibition in *E.coli* (10mm) as compared to *S. spp.*(7mm).It showed no effect against *P.spp* and *C. spp.*

Combinations:

1:1- The combination C:L shows a synergistic effect against *E.coli* with a zone of inhibition of 15mm, while it showed an antagonistic effect against all other micro-organisms. Other combinations, L:M showed antagonistic effect against all micro-organisms used. C:M shows antagonistic effect against *E.coli, P.spp.* and *C.spp*, whereas it shows synergistic effect against *S.spp..*

1:1:1 – The combination of L:M:C showed highest synergistic effect against *E.coli* with a zone of inhibition of 18mm. It showed antagonistic effect in all other micro-organisms used.

1:2– A greater synergistic effect was observed in E.coli by L:M, and C:L, while they showed antagonistic effects in other micro-organisms. In C:M it shows synergistic effect against *S.spp.,* while it shows antagonistic effect against all other micro-organisms used.

2:1–The combination C:M and C:L showed a greater synergistic effect in S.spp. followed by E.coli, while they showed antagonistic effects in C.spp followed by P.spp.. The combination L:M shows antagonistic effect against all micro-organisms used.

DISCUSSION AND CONCLUSION

According to previous studies, The CEO's, MEO's and LEO's antioxidant and antibacterial activities have encouraged their application in meat, poultry and seafood, vegetables, dairy products, and edible coating films in the food industry. Even though CEO's, MEO's and LEO's is widely consumed and applied, there are still potential areas for investigation like need to define the roles of the main components in the various biological activities for potential application in the treatment of different diseases. In addition, whether there is synergy or antagonism among these components was also not determined [4].

According to our study,*E.coli* is found to be susceptible for all 3 combinations, these combinations of essential oils can be therapeutically used for all its infections. As these oils are natural they don't have any side effects hence provide a safer alternative for synthetic drugs and antibiotics. It can be concluded that Clove essential oil shows greater antimicrobial effects as compared to lemongrass and peppermint essential oils. (C>L>M). The combination of clove and lemongrass essential oils(C:L) in the ratio 1:1, 1:2 shows the most synergistic effect against *E.coli* while an antagonistic effect in other micro-organisms. 2:1 ratio shows greater synergistic effect against *E.coli* and *S. spp.* while antagonistic in others.

By analysing the results, it can be concluded that Clove essential oil shows greater antimicrobial effects as compared to lemongrass and peppermint essential oils.(C>L>M). The combination of clove and lemongrass essential oils(C:L) in the ratio 1:1, 1:2 shows the most synergistic effect against *E.coli* while an antagonistic effect in other micro-organisms. 2:1 ratio shows greater synergistic effect against *E.coli* and *S. spp.*while antagonistic in others.

The combination of lemongrass and peppermint essential oil(L:M) in all the ratios was found to be antagonistic in all micro-organisms used.

The combination of clove and peppermint essential oil (C:M) shows antagonistic effect in the ratio 1:1, 1:2, 2:1 against *E.coli, P.spp., C.spp.,* whereas it shows synergistic effect against *S.spp.*in the ratio 1:1 and 1:2, while antagonistic effect in the ratio 2:1.

The combination of all three essential oils (L:M:C) in the ratio 1:1:1 shows maximum synergistic effects against *E.coli.*

*E.*coli is the most susceptible micro-organism to all three essential oils whereas C. spp was found to be the least susceptible; therefore the essential oils show more potent antibacterial activity than antifungal activity.

Table 1. Total extraction yield

No.	Material	Weight (grams)	Volume of solvent(ml)	Running time (hours)	Temperature (°C)	Total Yield of oils (ml)
1.	Clove	200	300	48	80	5
2.	Peppermint	500	600	3	100	1.2
3.	Lemongrass	500	600	3	100	1.5

Table 2. Activity of essential oils

Essential oil extract	Zone of inhibition (mm)			
	E.coli	*P.spp.*	*S.spp.*	*C.spp*
Clove	8	9	11	13
Peppermint	10	0	7	0
lemongrass	11	6	7	8

Combinations:- L: Lemongrass extract
M: Peppermint; C: Clove extract

Table 3. Activity of standard combinations 1:1 Ratio

Essential oil extract	Zone of inhibition (mm)			
	E.coli	*P. spp.*	*S. spp.*	*C. spp*
L:M	7	0	0	3
C:M	8	8	9	6
C:L	15	0	2	1
L:M:C	18	3	4	1

A. 1:2 total volume =1.2 ml

Table 4. Activity of standard combinations 1:2 Ratio

Essential oil extract	Zone of inhibition (mm)			
	E.coli	*P.spp.*	*S.spp.*	*C.spp*
L:M	6	1	1	3
C:M	4	1	1	0
C:L	8	4	2	0

C.2:1 total volume= 1.2 ml

Table 5. Activity of standard combinations 2:1 Ratio

Essential oil extract	Zone of inhibition (mm)			
	E.coli	*P.spp.*	*S.spp.*	*C.spp*
L:M	7	0	0	0
C:M	4	5	10	1
C:L	9	3	11	0

Lemongrass extract activity

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