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Antimicrobial efficacy of essential oils of clove, neem and cinnamon on Streptococcus mutants and lactobacillus acidophilus in comparison with 0.2% chlorhexidine – An *in vitro* study

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Abstract:
Background*:**Dental caries is the most common, chronic infectious disease impacting around 3.5 billion people around the world according to Global Burden of diseases (2019). In-vitro studies of essential oils of neem, clove and cinnamon and their antimicrobial efficacy against S.mutans and L.acidophilus at different concentrations will help develop Phytochemical drugs with better synergistic effect as an adjunct to maintaining good oral hygiene.*
Materials and Methods*: Authenticated commercially available pure clove oil, neem oil and cinnamon oil were purchased and diluted to obtain 1%, 5%, and 10% concentrations. The ATCC (American Type Culture Collection) strains of S.mutans (25175), and L. acidophilus (4356) was procured, the bacterial cultures were maintained on BHI (Brain Heart Infusion) agar and sheep blood agar slants respectively, with periodic subculturing and stored at 4° C. Antimicrobial efficacy was assessed using the agar well diffusion method. 0.2% chlorhexidine was used as the positive control and DMSO (Dimethyl sulfoxide) as a negative control.*
Results*: The essential oils of clove, neem and cinnamon showed antimicrobial efficacy against S.mutans and L. acidophilus at different concentrations. The cinnamon oil at 10% concentration showed a 22.54±0.69 (Mean±SD) zone of inhibition againstL. acidophilus similar to 0.2% Chlorhexidine. The antimicrobial efficacy of all three essential oils increased as there was an increase in concentration.*
Conclusion:*The cinnamon essential oil had overall superior antimicrobial activity against both bacteria as compared to the other two essential oils, clove and neem. Cinnamon oil showed a mean zone of inhibition similar to 0.2% chlorhexidine against L. acidophilus at 10% concentration.*
Key Word*: Clove oil, Neem oil, Cinnamon oil, Streptococcus mutans, Lactobacillus acidophilus.*

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I. Introduction

Oral health is very important for general health since it affects a person's ability to consume food, communicate, and live life to the fullest. Oral health has its implications on systemic conditions like the cardiovascular system, diabetes-related gum disease, and respiratory illnesses [1]. The prevalence of oral disorders among chronic diseases is high and expensive treatment costs make them significant social and economic issues. The human oral cavity contains more than 700 different bacterial species [2]. The oral microbiota of different people, however, may differ significantly. Sometimes, the defence mechanism against the invasion of foreign germs is assisted by microbial interactions within the oral cavity. The oral health of an individual may suffer negatively due to these invading microbes. Oral illnesses such as dental caries, periodontal disease, and mucosal and systemic diseases are influenced by unbalanced microbial flora [3]. Over the course of several decades, dental caries has spread over the globe to become one of the most widespread chronic infectious oral diseases. The oral microbiota and dental plaque both are thought to be primarily composed of *Streptococcus mutans* a gram-positive coccus, facultative anaerobe and *Lactobacillus acidophilus* is a rod-shaped, anaerobic gram-positive microorganism [3,4].Caries and periodontal diseases can be prevented by brushing teeth often and implementing a variety of other oral hygiene practices, even though the creation of biofilm in the oral environment is a normal and continuous process. Due to the inadequacy of mechanical means of plaque removal, patients and the general population may seek additional benefits from chemotherapeutic antiplaque medications [5].

Since the dawn of human history, essential oils made from plants and herbs have been utilized medicinally [9]. There are several biological characteristics of essential oils, including larvicidal activity,

analgesic, anti-inflammatory, antifungal, and antioxidant capabilities[10,11]. Utilizing different essential oils that include a wide range of phytochemicals might inhibit or limit bacterial development. The antibacterial, antibiotic, and antiviral activities of neem (*Azadirachta Indica)*are well-known in the medicinal plant world. The main components of neem oil in terms of phytochemistry are oleic acid, linoleic acid, hexadecanoic acid and alkaloids like *nimbin, tannins* and *nimbidin* [12]. As spices, clove (*Syzygium aromaticum*) and cinnamon oil (*Cinnamomum verum*), are used to improve the flavour of food. Their fragrant, carminative, antibacterial and antioxidant qualities are well established [13]. Due to their widespread availability, low cost, biodegradability, and safety, essential oils are widely used [14,16].

A thorough analysis of these essential oils provides the opportunity to examine their potential for use as antibacterial agents against the most common microorganisms responsible for tooth decay and gum disease. In light of this, the current study aims to compare the antibacterial effects of essential oils of neem, clove, and cinnamon at different concentrations with those of 0.2% chlorhexidine against Streptococcus mutans and Lactobacillus acidophilus.

II. Material And Methods

The in-vitro study was carried out to test the antibacterial activity of clove oil, neem oil and cinnamon oil against streptococcus mutans and lactobacillus acidophilus. The protocol of the study was approved by the institutional ethical committee (RP No: 46/2020). The study was carried out in an integrated collaborative biopharmaceutical laboratory which is recognized by the department of scientific and industrial research (DSIR) Government of India.

Bacterial Strain and Inoculum

We obtained strains of Streptococcus mutans (25175) and Lactobacillus acidophilus (4356), both of which are available from the American Type Culture Collection (ATCC). The bacterial cultures were maintained on Brain heart infusion agar and on sheep blood agar culture slants, respectively, by performing periodic subculturing while being kept at a temperature of 4 degrees Celsius. The cell suspensions of the cultures were adjusted to the concentration of 1-2 x 10^6 cells per millilitre. The bacterial strains were then plated on Soyabean casein-digested agar.

Chemicals and reagents

Commercially available pure authenticated clove oil, neem oil and cinnamon oil were purchased. The essential oils purchased were examined for their purity and then dilution was carried out. The essential oils were diluted using 5% tween 20 solution. The concentrations prepared were 1%, 5% and 10% for all the three essential oils. All other chemicals used were of the highest purity available from commercial sources.

Dilution of the essential oils

The essential oils were diluted using 5% tween 20 solution. 990µl of tween 20 was diluted with 10µl of each essential oil separately to obtain 1% concentration. 950µl of tween 20 was diluted with 50µl of each essential oil separately to obtain 5% concentration. 900µl of tween 20 was diluted with 100µl of each essential oil separately to obtain 10% concentration.

Soyabean casein digested agar plates preparation

The Soyabean casein digested agar was prepared using the manufacturer's instruction (HIMEDIA Soyabean Casein Digest Medium – M1838-500G; LOT: 0000413018; EXP: 2014-23). 35.7 grams of powder was added to 1000 ml distilled water after which it was boiled to ensure complete dissolution of the medium. The media was autoclaved at 15lbs pressure (118- 121°C respectively) for 15 minutes.

Assessment of mean zone of inhibition for clove oil, neem oil and cinnamon oil making use of agar well diffusion method

The antimicrobial characteristics of clove, neem and cinnamon oil were evaluated by making use of the agar well-diffusion method. For this, the prepared Soyabean casein digested agar was poured into Petri plates and allowed to cool in a laminar airflow unit under aseptic conditions.

10µl of the standardized bacterial suspension of *S.mutans* and *L.acidophilus* was dispensed and spread over the agar Petri plates. 5mm wells were then dug out from each Petri plate of the set agar. The wells were filled with Samples (clove, neem and cinnamon of 1%, 5%, 10% each) (25µl) and Positive control 0.2% Chlorhexidine (25µl), Negative control Dimethyl sulphoxide (DMSO) (25µl). The treated plates with *S.mutans* and *L.acidophilus* were incubated at 37°C for 24hrs.

The zone of inhibition (ZOI) surrounding the samples were measured by taking diameters from 3 different points after 24 hours. All the experiments were done in triplicates sets (Figure 12-17). The zone of

inhibition was computed by subtracting 5mm as the diameter of the testing sample from the average values obtained.

Statistical analysis

The statistical analysis was done using SPSS software version 24 (IBM, Chicago, USA). Quantitative data is presented as mean and standard deviation.

- The comparison of mean zone of inhibition of different group of agents was done using one-way ANOVA with Turkey's Post-hoc Test.
- The statistical significance was fixed at 0.05.

III. Result

The antimicrobial efficacy of all the samples and their concentrations was determined by the well diffusion method. The level of antimicrobial activity is determined by the size of the inhibition zone shown by the essential oil.

Mean inhibition zone for *S.mutans*

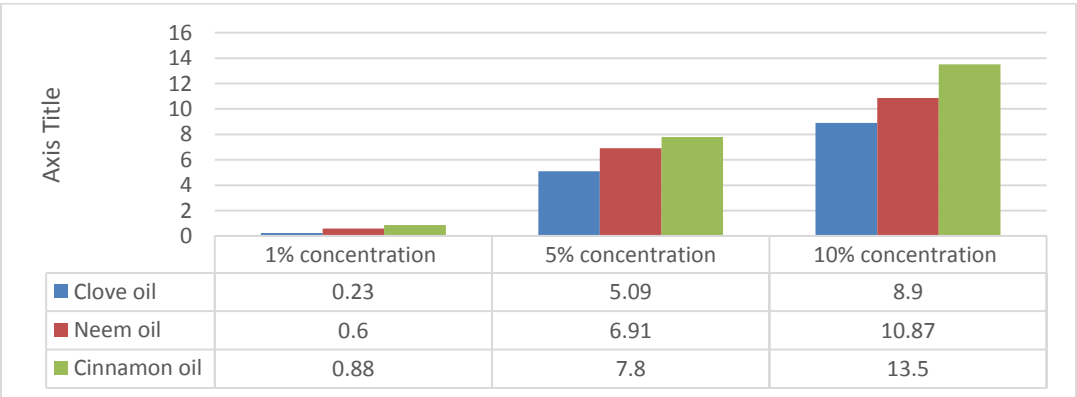
The mean zone of inhibition for *S.mutans* against all the agents is given in Table 1. The mean inhibition zone increased as the concentrations of the essential oils increased. Cinnamon oil at 10% concentration showed 13.50±3.10 (mean±SD) which was the highest inhibition zone among three essential oils.

Table no 1: The Mean zone of inhibition for *Streptococcus mutans* against Clove, neem and cinnamon oils at 1%, 5% and 10% concentrations in comparison with Chlorhexidine 0.2%.

Extract / Standard	Group	1% concentration	5 % concentration	10 % concentration
Clove oil	G1	0.23±0.10	5.09±0.65	8.90±0.56
Neem oil	G2	0.60±0.00	6.91±1.24	10.87±0.60
Cinnamon oil	G3	0.88±0.14	7.80±0.54	13.50±3.10
0.2% Chlorhexidine	G4	23.40±1.16	23.03±0.51	23.42±0.38
* Statistical inference		F value: 3462.10 df : 3 p value: 0.001	F value: 777.10 df : 3 p value: 0.001	F value: 115.23 df : 3 p value: 0.001
Post hoc analysis		G1 vs G2 – 0.55 G1 vs G3 – 0.62 G1 vs G4 – 0.00 G2 vs G3 – 0.99 G2 vs G4 – 0.00 G3 vs G4 – 0.00	G1 vs G2 – 0.96 G1 vs G3 – 0.25 G1 vs G4 – 0.00 G2 vs G3 – 0.10 G2 vs G4 – 0.00 G3 vs G4 – 0.00	G1 vs G2 – 0.23 G1 vs G3 – 0.15 G1 vs G4 – 0.00 G2 vs G3 – 0.26 G2 vs G4 – 0.00 G3 vs G4 – 0.00

*** one way ANOVA with Post hoc Statistical analysis**

Table 1: Mean inhibition zone for S.mutans against clove, neem and cinnamon oils at different concentrations



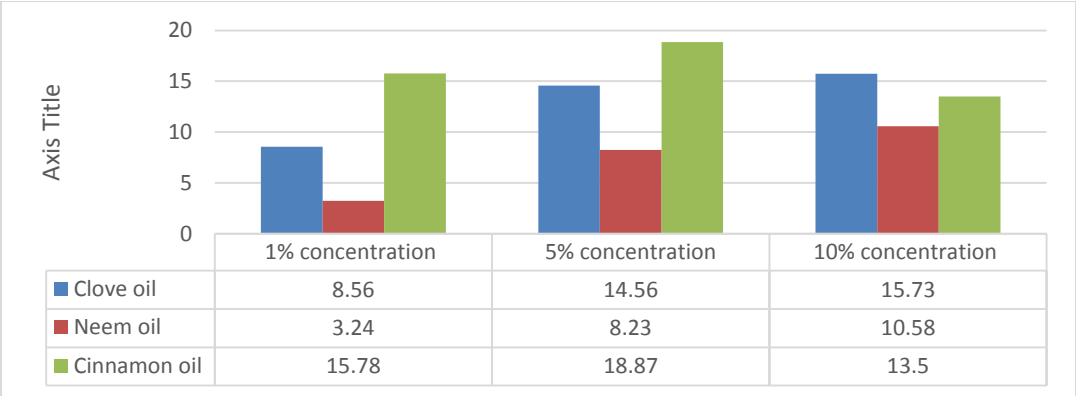
Mean inhibition zone for *L.acidophilus*

The mean zone of inhibition for *L.acidophilus* against clove, neem, cinnamon oil and 0.2% chlorhexidine is given in Table 2. The mean inhibition zone increased as the concentrations of the essential oils increased. The 0.2% chlorhexidine showed the highest mean zone of inhibition. Cinnamon oil at 10% concentration showed mean zone of inhibition (22.54±0.69) similar to 0.2% chlorhexidine (23.78±0.64) the Tukey's post hoc test revealed that there is no statistically significant difference between both the groups.

Table 2: Mean inhibition zone for L.acidophilus against clove, neem and cinnamon oils at different concentrations

Extract / Standard	1% concentration	5 % concentration	10 % concentration
Clove oil	8.56±0.83	14.56±1.06	15.73±1.28
Neem oil	3.24±0.41	8.23±0.54	10.58±0.92
Cinnamon oil	15.78±1.01	18.87±1.04	22.54±0.69
0.2% Chlorhexidine	24.33±0.75	24.73±0.59	23.78±0.64
* Statistical inference	F value: 542.05 df : 3 p value: 0.00	F value: 413.40 df : 3 p value: 0.00	F value: 65.21 df : 3 p value: 0.00
Post hoc analysis	G1 vs G2 – 0.01 G1 vs G3 – 0.01 G1 vs G4 – 0.00 G2 vs G3 – 0.03 G2 vs G4 – 0.00 G3 vs G4 – 0.00	G1 vs G2 – 0.00 G1 vs G3 – 0.00 G1 vs G4 – 0.00 G2 vs G3 – 0.00 G2 vs G4 – 0.00 G3 vs G4 – 0.00	G1 vs G2 – 0.00 G1 vs G3 – 0.00 G1 vs G4 – 0.00 G2 vs G3 – 0.01 G2 vs G4 – 0.00 G3 vs G4 – 0.40

*** one way ANOVA with Post hoc Statistical analysis**



IV. Discussion

Oral health in its entirety is best achieved with regular hygiene practices and making use of different oral hygiene aids. Over the period of time, there are different oral hygiene aids available in the market. There are a variety of toothbrushes available, some of which use motorized technology. Additionally, there are new-age mouthwashes available that combine different genre chemicals and bioactive agents to work effectively and address different problems.

The incorporation of organic compounds in conventional medical and dental treatments has been ascribed the name "phytotherapeutics" or "ethnopharmacology," and it is well-known that these treatments have a number of advantages over traditional pharmaceuticals [14]. The versatility of medicinal plants' health-promoting functionalities, cost-effectiveness, availability, and minimal adverse effects related to the drugs derived from these plants continue to pique the interest of researchers. This interest has been sustained by the fact that medicinal plants are readily available. Essential oils are well-known for the antibacterial, antioxidant, antifungal, and antiviral qualities that they possess. Essential oils are basically procured from natural resources and contain a wide variety of chemical compounds that are known for their therapeutic potential.

Cinnamon oil showed antimicrobial efficacy against *Streptococcus mutans and Lactobacillus acidophilus.* The antimicrobial properties of cinnamon oil is well established and proved in the study conducted

by Prabuseenivasan S *et al* (15), where seven essential oils were selected to determine the minimum inhibitory concentration against six bacterial species and cinnamon oil showed promising antimicrobial activity even at a lower concentration. Which was in accordance to this study.

In contrast to this investigation, the research carried out by Rajini Kanth *et al*(16)found that cinnamon essential oil had a smaller mean zone of inhibition in comparison to clove oil. This may be due to the difference in the methodology of the study, the bacterial culture was obtained by decayed tooth and the culture of the bacteria did not have differentiation among different species.

The study conducted by M. Fadli *et al* (17) evaluated the antimicrobial activity of the combination of essential oil and traditional antibiotics. The synergetic effects of the combination of essential oils and antibiotics were promising increasing the antimicrobial activity of the drug and reducing the minimum effective dose. This can contribute towards lowering the toxic side effects and treatment costs. The evaluation of the synergistic effects must be done both in vitro and in vivo since they can vary depending on the active ingredients in the essential oils, their composition, and the length of exposure. Studies on the mechanisms of action of essential oils have not yet been extensively established.

V. Conclusion

The cinnamon essential oil had overall superior antimicrobial activity against both bacteria as compared to the other two essential oils, clove and neem. Cinnamon oil showed a mean zone of inhibition similar to 0.2% chlorhexidine against *L.acidophilus* at 10% concentration.

STRENGTHS AND LIMITATIONS

The highlights of the present study are

- The invitro observations were made in well-monitored strict lab conditions.
- This was the first of its kind study, where three different essential oils were evaluated for antimicrobial activity at three different concentrations (1%, 5%, 10%) in comparison with 0.2% chlorhexidine against *Streptococcus mutans* and *Lactobacillus acidophilus.*

Limitations

- Because it is an in-vitro study, it is not possible to reproduce biological variables seen in the human body.
- The diversity in drug distribution to infection sites within the body may vary in comparison to in-vitro conditions.

Recommendation

- Many other essential oils can be tested for antimicrobial efficiency and can be compared with the three mentioned in this study
- Antimicrobial efficacy can be assessed further in in-vivo conditions for the formulation of drugs

The combination effects and synergistic effects of different essential oils can be studied further.

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