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EFFICACY TESTING OF MOUTHWASHES AND HERBAL EXTRACTS ON *CANDIDA ALBICANS* NCIM 3471

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ABSTRACT

Background: Microbial communities found in mouth are highly complex with 1000 species present and are shown to be second most complex in our body after colon. Depending upon the age, immunity and personal hygiene, microorganisms can cause various acute and chronic infections such as Candidiasis and oral thrush. The study deserves further investigation for clinical application of herbal extracts in treatment of oral fungal infections. **Objective:** To test the efficacy of commercial mouthwashes and herbal extracts on *Candida albicans* NCIM 3471. **Materials and Methods:** Commercial mouthwashes (MW) and herbal extracts (HE) were used to check their efficacy on the oral opportunistic pathogen *Candida albicans* NCIM 3471, using disc diffusion method and well diffusion method. The presence of phytochemicals was assessed by qualitative analysis and TLC was performed using silica gel coated plate. **Results:** HE of clove, garlic and tea tree oil showed better antifungal activity than other HE. Combination of ginger and garlic followed by MW3 and garlic gave better synergistic action as compared to others. The MIC of clove, garlic, and tea tree oil was found to be 18.2µg/ml, 16.6µg/ml 32.56µg/ml respectively. The extracts also showed presence of phytochemicals such as alkaloids, ketones, phenols, flavanoids, and tannins. **Conclusion:** Results of the present study suggest that herbal extracts are more efficient as compared to mouthwashes and should be brought in commercial use.

KEYWORDS: Candidiasis, Efficacy, Disc diffusion, Synergism.

INTRODUCTION

The human mouth harbors one of the most diverse microbes in human body including viruses, fungi, protozoa, archaea and bacteria. Microbial communities found in mouth are highly complex with 1000 species present and is shown to be second most complex in body after colon.[1] Oral candidiasis, oral thrush, denture-related stomatitis are infections caused by an overgrowth of most common pathogen *Candida albicans* accompanied by various symptoms including burning and painful sensation, change of taste and swallowing difficulties.[2] Risk factors that increase incidence of *Candida* infection include compromised immunity, hormonal imbalance, use of antibiotics, metabolic and nutritional disorders and poor oral hygiene.[3]

Central theme of success in human therapeutics in the 20th century was the discovery and development of antibiotic and antibacterial agents for treatment of fungal infections. However, usage of these is becoming restricted, because micro-organisms are able to develop resistance to antibiotics soon after their introduction.[4] Since plant derived essentials have a long traditional use and cover wide range of biological activities, in various studies their efficacy have been tested over range of oral bacteria and fungi.[1]

Prevention can be achieved with the use of antifungal mouthwashes because of their properties such as antiseptic, amphipathic effects, biocompatibility on oral candidiasis.[5][6] The natural antioxidants from the plants, fruits, and their waste have attracted attention due to their safety.[7]

Natural products and their derivatives (including antibiotics) represent more than 50% of all drugs in clinical use in the world. According to World Health Organization, about 80 percent of people living in developing countries rely on harvested wild plants for some part of their primary health care. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world which include use of clove, garlic, ginger, green tea, honey, pomegranate, orange, tea tree oil and curds.

Herbal formulations have been used which are harmless, non-toxic, and have strong antibacterial effects.[5] Herbal medicines can be in the form of liquid, powder or mixtures, raw/boiled. The use of herbs and spices is most ancient approach to healing, due to their

properties such as anti-oxidative, antimicrobial, anti-inflammatory and antifungal.[8] Fruits and vegetables are most popular due to their nutritional value worldwide and rich sources of beneficial antioxidants, minerals, vitamins, and fibres. The regular consumption of fresh fruits and vegetables may reduce the risk of diseases. Phenolic compounds, including their subcategory flavonoids, anthocyanins, catechins, isoflavones, lignins, phenolic acids are present in all plants and have been studied extensively in legumes, cereals, olive oil, fruits and tea. Many phenolic compounds have antioxidant properties. These compounds can be used as ingredients in cosmetics, pharmaceuticals, nutraceuticals and food.[7] Research has been carried out giving information on bioactive compounds of fruits and its waste in Bangladesh. The researchers developed a simple and rapid method for extraction of the crude extracts from plants in various solvents at different concentrations.[7] These extracts have shown important antifungal activity against *Candida albicans*.

Effectiveness of two drugs used simultaneously is greater than that of either drug used alone. The study supports these ideas and apart from classical antifungal treatments, combination therapies and preventive therapies represent new strategies for treating invasive fungal infections.

The objective of this study was to develop a simple and rapid method for extraction of bioactive compounds from herbal compounds, to find their antimicrobial activity, to check synergistic action between best resulting compounds, to detect presence of phytochemicals and to determine the minimum inhibitory concentration.

MATERIALS AND METHODS

In this *in vitro* study, standard culture of *Candida albicans* NCIM 3471 was purchased from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune, Maharashtra, India. The culture was maintained on Sabauroud's dextrose medium (HIMEDIA, India). The cells of this standard culture were grown in Sabauroud's dextrose broth and incubated at 37°C for 18-24 h on an orbital shaker. After incubation, the turbidity was observed and the cell densities were then adjusted to 0.5 McFarland standards (10^6 cfu/ml) at 530nm using spectrophotometric method. Four commercial mouthwashes MW1, MW2, MW3, and MW4 were purchased from a local medical store. The herbal compounds such as ginger, garlic, clove, green tea, tea tree oil, black cumin seeds and honey that are meant to have essential bioactive compounds were purchased from a local grocery store. Orange and pomegranate peels were obtained from local market.

Antifungal activity of commercial mouthwashes against *Candida albicans* NCIM 3471

100µl of both commercial mouthwashes and culture were mixed in microfuge tubes and incubated for 30 seconds and 60 seconds. After incubation the tubes were centrifuged at 8000rpm for 10minutes. The supernatant was then discarded and 0.5ml of sterile phosphate buffered saline (PBS) pH 7.4 was added to the pellet for washing. The centrifugation was repeated at 8000rpm for 10minutes. The resulting supernatant was discarded and 1ml of PBS was added to the pellet. 100µl of this suspension was then taken and spread on Sabauroud's agar plate. The experiment was performed in triplicates and the plates were incubated at 37°C for 48h. Similarly antifungal activity of commercial mouthwashes was checked by performing disc diffusion method.

Aqueous extraction of bioactive compounds from herbal compounds

10g of each herbal compound was weighed, dried and ground in motor and pestle. The powder was transferred to bottles containing 10ml of sterile distilled water and soaked for 48h. The solution was then taken in microfuge tubes and centrifugation was performed at 8000rpm for 10minutes. The supernatant was collected and filtered using 0.45µm membrane filters. The resulting extracts were then stored at 20°C. From this solution 100µl was taken and spread on Sabauroud agar plate and disc diffusion method was performed.

Solvent extraction of bioactive compounds from fruit peels

Peels of orange and pomegranate were dried, ground in motor and pestle. 10g of this powder was weighed and 50ml of solvent (methanol, ethanol, and acetone) was added. The extraction was done in a thermostat water bath at 40°C for 30min in a covered conical flask. Liquids extract was separated from solids by filtering it through Whatman filter paper no.1. The resulting filtrate was then collected and dried to remove moisture at 50±2°C in a water bath. Sterile paper discs were dipped in this solution and also in the solvent as control to perform disc diffusion method so as to observe the zones of inhibition.

Synergism between the herbal extracts and mouthwashes

Synergistic reaction was checked between the extracts and mouthwashes showing good antifungal activity by mixing them in equal volumes. Combinations of MW3 and MW4, MW3 and garlic, green tea and garlic, ginger and garlic and ginger, garlic and clove were prepared. To check the best synergistic reaction between the extracts and commercial mouthwashes disc diffusion method was performed.

The antifungal activity of herbal extracts of clove, garlic and tea tree oil against *Candida albicans* NCIM 3471 was evaluated in their pure form as well as in dilutions ranging from 1:2 to 1:64.

Phytochemical Screening

The herbal extracts were tested to check the presence of bioactive compounds by using following standard methods.

Test for Alkaloids

To 2ml of extract, 1.5ml of 1% HCl was added and heated in water bath, followed by addition of 6 drops of Wagner's reagent. The formation of orange precipitate indicated the presence of alkaloids in the extract.

Test for Phenols

To 2ml of extract, 5% ferric chloride solution was added. Deep blue color indicates the presence of phenols in the extract.

Test for Ketones

To 1ml of extract few drops of sodium nitroprusside solution was added. Red color indicates the presence of ketones in the extract.

Test for Flavonoids

5ml of dilute ammonia was added to test solution followed by addition of few drops of concentrated H_2SO_4. The formation of yellow color indicates the presence of flavonoids.

Test for Tannins

100µl of extract was dissolved in distilled water and to this 2ml of 5% ferric chloride was added. Blue green color formation indicates the presence of tannins.

Thin layer chromatography (TLC) of herbal extracts

TLC of Clove

TLC of clove extract was performed on a plate coated with thin layer of silica gel where clove oil was used as a standard. Solvent system used was hexane: isopropanol (2:1). The spots were visualized under UV.

TLC of Garlic

TLC of garlic extract was performed on a plate coated with thin layer of silica gel (5mg of garlic powder dissolved in 1.5ml of methanol) and alanine was used as a standard. Solvent system used was n-butanol: acetic acid: water (60:40:20). Alanine & alliins could be detected as red or pink spots, while allicins were visualized as orange spots under UV.

RESULTS

Antifungal activity of commercial mouthwashes against *Candida albicans* NCIM-3471

The antifungal activity of the commercial mouthwashes was checked against *Candida albicans* NCIM-3471 using Disc diffusion method. The diameter of the zones obtained were measured in mm. Commercial mouthwashes MW 3 and MW 2 showed maximum zones of inhibition as compared to MW 1 and MW 4 (Table - 1).

Table -1: Zone diameter of commercial mouthwashes.

Commercial Mouthwashes	Zone diameter (mm)
MW 1	8.33±0.471
MW 2	10±0.816
MW 3	11.67±0.471
MW 4	9±0.816

Determination of antifungal activity of ginger and clove extracts at various concentrations

against *Candida albicans* NCIM-3471

The antifungal activity of clove and ginger against *Candida albicans* NCIM-3471 was checked at concentrations of 5%, 10% and 15%. Concentration of 10% showed higher inhibitory effect (Table - 2).

Table 2: Zone diameter (mm) of ginger and clove.

Herbal extracts	Zone diameter (mm)		
% (v/v)	5%	10%	15%
Ginger	11.66±0.47	12.66±1.88	14.33±2.49
Clove	23±0.81	28±1.41	21.66±2.26

Determination of antifungal activity and zone diameter of Garlic, Green tea, Tea tree oil, Black cumin seeds and Honey

Antifungal activity against aqueous extracts of garlic, green tea, tea tree oil, black cumin seeds and honey was carried out at 10% and found to be as in Table - 3. Garlic extract

showed maximum antifungal activity as compared to green tea extract and tea tree oil while black cumin seeds extract, honey and curds showed no inhibitory activity on *Candida albicans* NCIM-3471.

Table-3: Zone diameter (mm) of garlic, green tea and tea tree oil.

Herbal extracts	Zone diameter (mm)
Garlic	23.25±1.57
Green tea	10.33±0.471
Tee tree oil	26.5±1.86
Black cumin seeds	00
Honey	00
Curd	00

Determination of antifungal activity and zone diameter of Pomegranate and Orange peels

The antifungal activity of fruit peels such as pomegranate and orange were checked by solvent extraction method (ethanol, methanol and acetone). The acetone extracts showed higher antifungal activity against *Candida albicans* NCIM-3471 was higher as compared to ethanol and methanol extracts.

Table-4 Zone diameter of Pomegranate and Orange peels.

Herbal extracts	Zone diameter (mm)		
	methanol	ethanol	acetone
Pomegranate	11±1.5	13±1	23.3±1.81
Orange	15.6±2.05	17.6±0.94	22±2

Figure 1 shows the comparison of effect of commercial mouthwashes and herbal extracts on *Candida albicans* NCIM-3471 and the results show that clove followed by tea tree oil and garlic have higher antifungal activity as compared to other herbal extracts and mouthwashes.

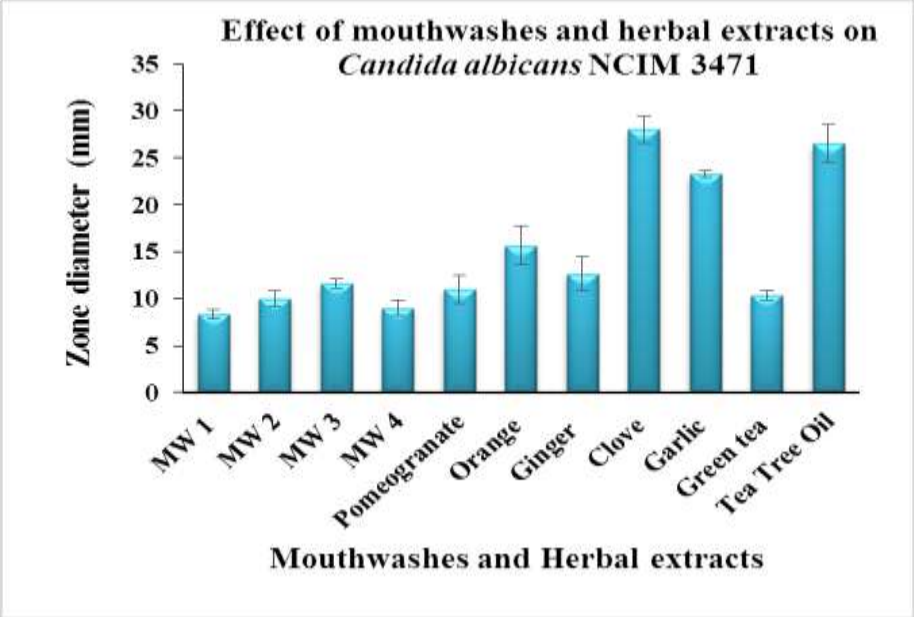

Figure-1: Comparison of zone diameter of mouthwashes and herbal extracts.

Synergistic action between commercial mouthwashes and herbal extracts

The interaction between two or more substances to produce a combined greater effect than their separate effects is called synergy. In this study synergistic action was carried out between mouthwashes and herbal extracts showing better action individually. The results obtained are shown in Figure-2.

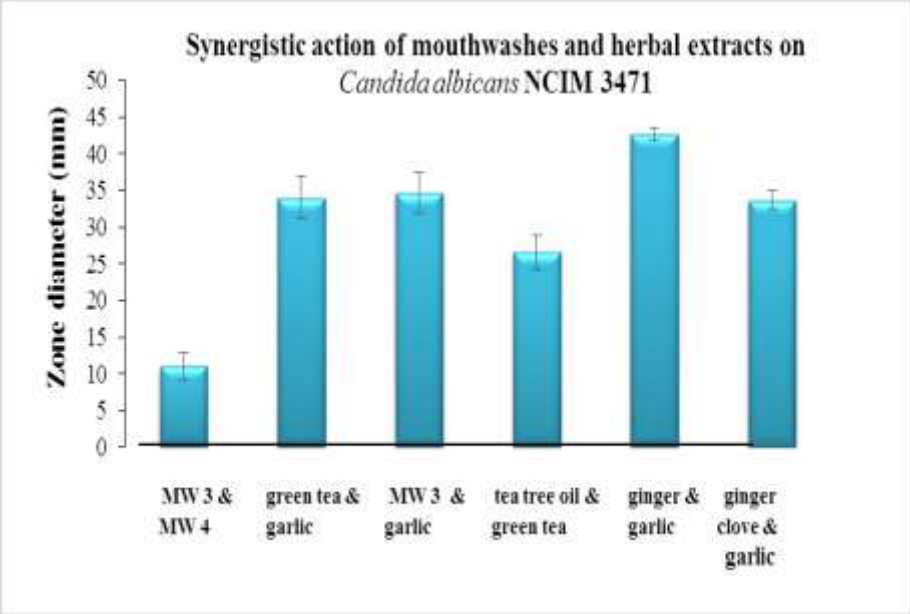

Figure-2: Zone diameter in synergism.

Minimum Inhibitory Concentration (MIC)

MIC of Garlic and Tea Tree Oil

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of antibiotics/ curing agents at which the microbial growth is inhibited. MIC values of clove, garlic and tea tree oil against *Candida albicans* NCIM 3471 were determined and the result obtained is shown in Table-5.

Table-5: MIC values of extracts against *Candida albicans* NCIM- 3471.

Herbal extracts	MIC values(µg/ml)
Clove	18.2
Garlic extract	16.6
Tea tree oil	32.5

Determination of presence of phytochemical components

Aqueous herbal extracts such as green tea, garlic, ginger, tea tree oil, and black cumin seeds were checked for the presence of bioactive constituents of plants known as plant phytochemical components such as alkaloids, tannins, flavanoids, ketones and phenolic compounds. The results obtained by qualitative analysis are shown in Table-6.

Table 6: Aqueous extracts showing presence of Phytochemicals.

Extracts	alkaloids	phenols	ketones	flavanoids	tannins
Ginger	-	-	-	-	-
Black cumin seeds	-	+	+	+	-
Green tea	+	+	+	-	+
Clove	+	+	+	+	+
Garlic	-	-	-	+	-

+ present - absent

Thin layer chromatography (TLC)

TLC is separation of compounds based on the competition of the solute and the mobile phase for binding places on the stationary phase. TLC of garlic extract was carried out and its R_f value was found out.

$$R_f \text{ value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Table-7: R_f values of clove and garlic extract

Herbal extract	R_f values
Clove	Test = 0.32 Eugenol = 0.33
Garlic	Test = 0.15 Allicin = 0.13

The Rf values of clove and garlic were found to be as represented in Table 7 indicating presence of eugenol and allicin respectively.

DISCUSSION

Mouthwashes are widely used as agents for oral hygiene and to prevent teeth and gums disorders. Natural products have been used worldwide as medicine, for 1000's of years. The commercial antifungal agents reported earlier show less efficacy against *Candida albicans* NCIM 3471. In present study antifungal activities of herbal extracts checked on *Candida albicans* NCIM-3471. In previous studies, antifungal effect of ginger and clove was checked against *S. mutans* and found to be 0.57mm and 2.02mm at 10% concentration while in present investigation the extracts showed positive results against *Candida albicans* NCIM-3471 with zone of inhibition of 12.66mm and 28mm at 10% concentration. [8] The results showed clearance of fungal growth, indicating herbal extracts are more effective than commercial mouthwashes. Previous work focused on synergism between chemical components of mouthwashes and also between herbal extracts and antibiotics.[9] In this study we demonstrated the synergistic action between herbal extracts and commercial mouthwashes, out of which MW 3/garlic and ginger/clove were more effective than the other combinations. This study is the first report where synergism was checked between mouthwashes and herbal extracts, it proves combination of extracts can be more beneficial than individual component. MIC of garlic was checked previously on *Porphyromonas gingivalis* and was found to be 16.6µl/ml and in present studies on *Candida albicans* NCIM-3471 it was found to be 16.6µl/ml.[10] Thus, there is a need of identifying antifungal agents that are more effective than the commercial agents.

CONCLUSION

The present study indicates that herbal extracts have better antifungal activity than chemical compounds in commercial mouth washes. It suggests the use of herbal extracts in order to maintain the oral hygiene

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