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Original article

Comparison of antimicrobial efficacy of Propolis, *Azadirachta indica* (Neem), *Melaleuca alternifolia* (Tea tree oil), *Curcuma longa* (Turmeric) and 5% Sodium hypochlorite on *Candida albicans* biofilm formed on tooth substrate: An *In-vitro* study

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ABSTRACT:

Aim: To determine the antimicrobial efficacy of Propolis, *Azadirachta indica* (Neem), *Melaleuca alternifolia* (Tea tree oil) ,*Curcuma longa*(Turmeric) and 5% Sodium hypochlorite on *Candida albicans* biofilm.
Materials and methods: Extracted human mandibular premolars were biomechanically prepared, vertically sectioned, placed in tissue culture wells exposing the root canal surface to *C. albicans* grown on Sabouraud Dextrose Agar to form a biofilm. At the end of 2 days, all groups were treated with test solutions and control for 10 min and evaluated for Candida growth and number of colony forming units were calculated. The readings were subjected to statistical analysis using ANOVA and Tukey's post hoc test. **Results:** Sodium hypochlorite and propolis groups exhibited highest antimicrobial efficacy against *C. albicans* with no statistically significant difference. It was followed by *Melaleuca alternifolia* (Tea tree oil), Curcuma longa(turmeric) group. *A.indica* (Neem) had limited antifungal action followed by the negative control group of saline. **Conclusion:** According to the results of this study, propolis can be used as an effective antifungal agent similar to that of sodiumhypochlorite, although long-term *in vivo* studies are warranted.

KEYWORDS: Antimicrobial efficacy; *Azadirachta indica*; *Candida albicans*;*Curcuma longa*; *Melaleuca alternifolia*; propolis.

Statement of Originality of work: The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and that each author believes that the manuscript represents honest and original work.

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INTRODUCTION

The aim of non-surgical endodontic therapy is to remove pathogenic microorganisms from the root canal system, shape the system appropriately and obturate it with a suitable material[1]. An important prerequisite for achieving long-term success of root canal treatment is complete debridement and effective disinfection of the root canal system[2]. Complete disinfection however is not always achievable through instrumentation alone due to the anatomical complexities of the root canal[3]. Thus, the use of irrigants and medicaments is necessary.

Candida albicansis the most common fungus seen in the root canals, 21% in primary infections[4] and 18% in cases of retreatments[5]. Due to the biofilm formation and the physicochemical properties of the microorganisms, it can survive harsh conditions[6].

Several irrigants have been recommended for the use in non surgical endodontic procedures. Sodium hypochlorite has been the gold standard as an endodontic irrigant due to its high antimicrobial action and ability to dissolve the organic material. However, it has various deleterious effects like tissue toxicity, unpleasant taste and odour, inability to remove smear layer and fully eradicate microbes from the infected canals, staining and corrosion of instruments and allergic potential[7-9].

There has been a shift of trend towards herbal medication. Use of plant parts as folklore medicine has been trailed by traditional healers since time immemorial. Natural products have become more popular in the modern day dentistry because of advantages like fewer side effects, inexpensive, better patient tolerance and renewable nature[10].

Propolis is a resinous material that honeybees collect from various plant species and mix with wax and other substances and exhibits a wide range of biologic activities, including antimicrobial, anti-inflammatory, antioxidant, anesthetic and cytotoxic properties[11]. It is believed that flavonoids account for most of the biological activities in propolis[12].

Neem (*Azadirachtaindica*) has been one of the most versatile medicinal plants having a wide spectrum of biological activity. Neem leaf and its constituents exhibit immunomodulatory, anti-inflammatory, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarciogenic properties[13].

Tea tree oil (*Melaleucaalternifolia*) is a native Australian plant, the oil of which has many properties that favour its use in dentistry. The major component, terpenin-4-ol is responsible for its antibacterial and antifungal properties. It also holds mild solvent action, and hence could hold potential applications in root canal treatment for dissolving the necrotic pulp tissue. An *in vitro* study showed that tea tree oil might disinfect the root canal system as effective as NaOCl. Also, the toxicity of tea tree oil is lesser than NaOCl[14].

Curcuma longa (commonly called as turmeric) belonging to *Zingiberaeceae* family has been used as a traditional medicine from the ancient times[15]. Curcumin (diferuloylmethane) is the main yellow bioactive component of turmeric and has been shown to have a wide spectrum of actions like anti-inflammatory, antioxidant, antibacterial, antifungal, antiprotozoal and antiviral activities[16].

The present study was undertaken keeping in mind the side effects of sodium hypochlorite. So, this study was undertaken to have an irrigant similar in antimicrobial action to sodium hypochlorite, without having its side effects. The null hypothesis was that the herbal irrigants used in this study could be as effective as sodium hypochlorite at eliminating *Candida albicans*.

MATERIALS AND METHODS

C. ALBICANS CULTURE PREPARATION

A pure culture of *C. albicans* (MTCC 3017) was inoculated on Sabouraud Dextrose Agar (Himedia, Mumbai), incubated at 37°C overnight and adjusted to an optical density of one with sterile brainheart infusion broth.

TEST SOLUTIONS PREPARATION

Propolis (Herbal Biosolutions, Delhi) was prepared by diluting a 33% commercially available alcoholic extract using warm saline in a ratio 2:1, to form an 11% alcoholic extract[17].

Alcoholic extract of neem was prepared using 10g of fresh neem leaves powder (The Indian Neem Tree Company ,Mumbai) which was added to 100 ml of absolute ethanol(Sterling Chemicals and Alcohols Pvt. Ltd., Mumbai) [18]. The extracts were then filtered through muslin cloth for coarse residue and then through a Whatman no. 1 filter paper and kept in an air tight amber-colored container[19]. Alcoholic extract of turmeric was prepared using 99% purity turmeric powder 2gm which was added to 100ml of absolute alcohol[20].

Tea tree oil (RYM Exports, Mumbai) was used in

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the available form (2% by volume) as it already contains terpenin-4-ol as one of its constituents[21].

TOOTH SAMPLES PREPARATION

Single rooted human mandibular premolar teeth with Vertucci type I canal configuration were sectioned below the cement enamel junction to obtain a standardized tooth length of 8 mm. Teeth with caries and fractured restorations were excluded from the study. The teeth were cleaned of superficial debris, calculus, tissue tags and stored in normal saline[22].

The root canals were then instrumented using the crown down technique and rotary instruments to an apical size of ProTaper F3. A total volume of 2 ml of 5% sodium hypochlorite (Prime Dental Products Private Limited, Thane, Maharashtra) was used between each instrument during the cleaning and shaping procedure. All teeth were vertically sectioned along the mid-sagittal plane into two halves. The concave tooth surface was minimally grounded to achieve flat surface to enable placement in tissue culture wells exposing the root canal surface to *C. albicans* to form a biofilm[23]. The samples were then sterilized by ultraviolet radiation in a biosafety cabinet (Accumax India, New Delhi) and placed in the wells of tissue culture plates. The cultured yeast was inoculated in the wells containing tooth samples at 37°C for 2 days.

GROUPING AND ASSESSMENT PROTOCOL

The samples were divided into six experimental groups with 20 samples each (after vertical sectioning) and irrigated with 3 ml of each irrigant for 10 min.

- Group 1 — Propolis
- Group 2 — *A. indica*(Neem)
- Group 3 — *M.alternifolia* (Tea tree oil)
- Group 4 — *C. longa* (Turmeric)
- Group 5 — 5% Sodium hypochlorite
- Group 6 — Sterile saline (Negative control).

Sterile paper point technique was used for sampling of root canals and inoculated in Sabouraud Dextrose Agar and incubated at 37°C for 24 h in a petridish, which was then analyzed by the digital colony counter (Spectronics India, Haryana) and the readings were subjected to statistical analysis using analysis of variance and post hoc Tukey tests.

RESULTS

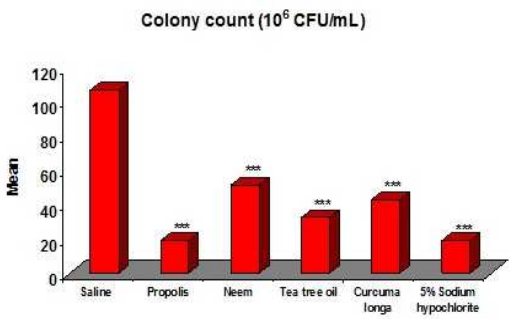
(Table 1 and figure 1)

Table 1. Mean and standard deviation showing inter group differences obtained from the readings of digital colony counter.

Groups	N	Mean	SD	Minimum	Maximum
Saline	20	106.85^{e}	4.43*	99	114
Propolis	20	19.45^{a}	3.25	14	25
Neem	20	51.70^{d}	4.19*	44	59
Tea tree oil	20	32.55^{c}	4.26*	26	40
Curcuma longa	20	43.50^{b}	3.92*	37	50
5% Sodium hypochlorite	20	19.30^{a}	3.56	12	26

p<0.001(ANOVA), *Statistically significant intergroup differences, abcde statistically signigicant difference.

Figure 1. Mean colony counts of six groups.



DISCUSSION

Candida albicans was chosen for this study as it is the most common fungus seen in the root canals, 21% in primary infections[4] and 18% in cases of retreatments[5]. Biofilm has extracellular polymeric coating around the organisms which make them survive harsh environmental conditions, thereby

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making the penetration of antimicrobial agents and irrigants difficult and thus adding to the virulence of microorganism[24]. To simulate the clinical conditions, biofilm model has been used in this study.

C. albicans has an initial period of adherence (0-2h) followed by subsequent microcolony formation (2-4 h). Dimorphic switching occurred thereafter with a transition from budding-yeast forms to filamentous pseudo- and true-hyphalforms (4-6h). Micro-colonies then become interlinked by the hyphal extensions, forming a confluent monolayer (6-8h). The complexity of the biofilm increases with time, taking on 3D architecture with spatial heterogeneity as it matured (8-48h). The biofilm after 24 and 48h consists of a mixture of yeast cells, pseudohyphae and true hyphae. Filamentous forms were the most important factor in the 3D architecture; with yeast cells located in the basal layer[24]. *C. albicans* mutants that are deficient in the production of hyphae have demonstrated an inability to form 3D biofilms. Therefore, the dimorphic switching observed in this species is a pivotal factor for biofilm formation and the pathogenic potential of *C. albicans*[25,26], which is why the 48 h biofilm model was used.

Sodium hypochlorite (NaOCl) has high antimicrobial action and ability to dissolve the organic material. In spite of its deleterious effects like tissue toxicity, unpleasant taste and odour, inability to remove smear layer and fully eradicate microbes from the infected canals, staining and corrosion of instruments and allergic potential[7-9], it has been widely recommended as an irrigant. Ayhanet al.(1999) demonstrated that sodium hypochlorite lowered colony forming units below the limit of detection after 10s in the case of *C. albicans*.[27] Also, study by Thilla S Vinothkumar et al.(2013) demonstrated that NaOCl was highly efficient in reducing the *C.albicans* in root canals[28]. These studies are in support of our study where sodium hypochlorite exhibits highest antimicrobial activity.

Propolis exhibits antimicrobial, anti-inflammatory, healing, anesthetic and cariostatic properties. According to Takaisi-Kikuni and Schilcher(1994), it prevents fungal cell division and also breaks down fungal cell wall and cytoplasm similar to the action of some antibiotics[29]. Kujumgiev et al.(1999) reported the antimicrobial action of propolis to be due to flavonoids and esters of phenolic acids[30]. In an inflammatory process there is a slight decrease of pH; thus, it would be most beneficial to use the extract of propolis of slightly acidic pH. The pH chosen for propolis was 6 based on the results of the study conducted by Ivančajić et al.(2010)[31] since the inhibitory effect of propolis was the strongest in a slightly acidic environment (pH=6). This is in support of our study as propolis effectively inhibited Candidal biofilm similar to the action of sodium hypochlorite. Ethanolic extracts of the test irrigants were used in this study as the inhibitory action of propolis is solvent dependent and ethanolic extracts have exhibited to be more active towards most microorganisms[31]. So, to standardize our test groups, all the test irrigants were prepared using ethanol as the solvent. Results of our study are in accordance with results shown by Tyagi et al.(2013)[32].

A.indica (Neem) (pH=6.8) has antimicrobial properties due to the presence of alkaloids, glycosides, saponins, flavonoids, steroids, anthraquinone and tannic acid[33]. Acidic ethanolic extract was found to be better than aqueous extract in cases of *C. albicans*,[34] therefore to standardize we prepared both our test solutions using ethanol. Bohora et al.,2010 have concluded that neem leaf extract has a significant antimicrobial effect against *C. albicans*[35]. In our study, Neem showed antimicrobial activity but it was the least among all the herbal groups taken. This difference in the result may be due to different working conditions or due to different test irrigants taken in our study.

Tea tree oil alters the permeability of *C.albicans* cells and also inhibits respiration in *C. albicans* in a dose-dependent manner[36]. Studies by D'Auria et al.(2001)[37] and Hammer et al.(2000)[38] have shown that tea tree oil inhibits the germ tubes or mycelial conversion on *C.albicans*. An *in vitro* study showed that tea tree oil might disinfect the root canal system as effective as NaOCl. Also, the toxicity of tea tree oil is lesser than NaOCl[14]. The results of our study showed that tea tree oil exhibits good antimicrobial activity but less than that of sodium hypochlorite.

Curcuma longa, a member of a ginger family possesses antiinflammatory, antioxidant, antimicrobial and anticancer activity[16]. In an *in vitro* study conducted by Neelakantan P et al.(2011), it has been shown that curcumin has significant anti bacterial activity against *E.faecalis*

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and can be used as an alternative to sodium hypochlorite for root canal irrigation[18]. A study by Nadia et al., 2004 on the antibacterial efficacy of turmeric found that ionic, resin and ethanolic fractions of turmeric are 100% effective against all tested gram positive organisms, which are resistant to most of the broad spectrum antibiotics used[39]. JhaHarit et al.(2013) also concluded that ethanolic extract is effective then aqueous extract against bacteria and fungi[20]. The antimicrobial activity of ethanolic extract was due to the presence of various type of curcumoid like substrate, which was confirmed by the TLC. Therefore, ethanolic extract of turmeric was used for our study. The results of our study showed that *Curcuma longa* exhibited good antifungal activity but it was significantly less as compared to propolis, tea tree oil and sodium hypochlorite. Saline exhibited least antifungal action as was expected.

Thus, the null hypothesis was proved to be partially right wherein propolis performed equally efficient as sodium hypochlorite. Other irrigants like tea tree oil, *Curcuma longa* and neem also exhibited antimicrobial activity.

CONCLUSION

Under the limitations of this study, it was concluded that:

1. Propolis performed equally well as sodium hypochlorite against *C. albicans* biofilm formed on extracted tooth surface.
2. Tea tree oil and *Curcuma longa* also exhibited good antimicrobial action.

Fungal inhibition potential of propolis, tea tree oil and *Curcuma longa* observed in this study opens perspectives for their use as root canal irrigant. However, preclinical and clinical trials are needed to evaluate biocompatibility, safety and their antimicrobial action especially against *Enterococcus faecalis* before they can conclusively be recommended as intracanal irrigating solutions, but *in vitro* observations of these irrigants appears promising.

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