Original Article

Antifungal Efficacy and the Mechanical Properties of Soft Liners against *Candida albicans* after the Incorporation of Garlic and Neem: An *In vitro* Study

Seenivasan Madhan Kumar, V. Anand Kumar, Parathasarthy Natarajan, Gayathri Sreenivasan

Department of Prosthodontics, Sri Ramachandra University, Chennai, Tamil Nadu, India

Abstract

Objectives: To evaluate the *in vitro* growth inhibition of *Candida albicans*, in the soft-liner material and Shore A hardness from resin-based denture soft lining materials modified by neem or garlic incorporation.

Materials and Methods: Resin discs were prepared with poly methyl methacrylate (PMMA) and soft liners incorporated with varying concentrations of neem or garlic. For antifungal activity, resin discs were placed on agar plates inoculated with *C. albicans* and were evaluated after 2, 4, and 7 days using the streaking method. The hardness of the PMMA was evaluated with the use of Shore A at 2, 4, and 7 days. Data were statistically processed by SPSS software (IBM Company, Chicago, USA) using Kruskal–Wallis test, and *post hoc* comparisons were done using Dunn's test. $P < 0.05$ was considered statistically significant.

Results: Neem and garlic added to PMMA soft liner had an inhibitory effect on *C. albicans*. Both the neem and garlic when added showed positive results against *C. albicans* when compared to the control group. The soft liner hardness increased statistically by time but not for the different plant extract concentrations.

Conclusions: Within the limitations of this *in vitro* study, it was found that neem and garlic can be used as an additive to tissue conditioner to reduce the adherence of *C. albicans* without significantly affecting the hardness of the heat-polymerized acrylic resin.

Keywords: *Antifungal property and tissue conditioners, Candida albicans, garlic, neem*

Received : 28-09-17.
Accepted : 02-04-18.
Published : 17-05-18.

Introduction

Early in the second century AD, Galen[1] described oral *Candida* as aphthas albus, and these infections are opportunistic and called as "the disease of the diseased."[2] Denture-induced stomatitis is found in about 65% of denture wearers particularly, the patients who are immunocompromised, elderly, or the patients undergoing immunosuppressive therapy.[3] Several researchers have reported that causes are multifactorial for the denture-induced stomatitis such as lack of oral hygiene status, allergy for the denture base material, trauma from occlusion, existing fungal infections, dietary deficiency, and hematological disorders. Microporosities and roughness in the denture surface will lead to adherence of *Candida albicans* and formation of the colonies on the fitting surface of the denture.[4] These microorganisms have to be removed either by mechanical or chemical cleansing; however, in some situations, these cannot be completely removed from the denture surfaces.[5,6] Poly methyl methacrylate (PMMA) is the most common material used in the fabrication of removable complete/partial denture prosthesis. Constructing smoother tissue surface and well-polished denture may reduce the adherence of *C. albicans* cells when compared to rough surfaces of the denture.[5-7] For that, soft lining

Address for correspondence: Dr. Seenivasan Madhan Kumar, Department of Prosthodontics, Sri Ramachandra University, Room No. 8, Chennai - 600 102, Tamil Nadu, India.
E-mail: madhanshashwath@gmail.com

Access this article online

Quick Response Code:

Website: www.jispcd.org

DOI: 10.4103/jispcd.JISPCD_343_17

How to cite this article: Kumar SM, Kumar VA, Natarajan P, Sreenivasan G. Antifungal efficacy and the mechanical properties of soft liners against *Candida albicans* after the incorporation of garlic and neem: An *In vitro* study. J Int Soc Prevent Communit Dent 2018;8:212-7.

materials are applied over the tissue surfaces of the denture to provide equal distribution of the functional load during occlusion and also a cushioning effect between the hard denture base and the tissues to improve the tissue health. However, studies have reported that soft lining materials are also prone to adhesion of *C. albicans*.[8]

Denture-induced stomatitis are treated with antifungal drugs such as nystatin and fluconazole.[9] Although systemic dosage of antifungals may be effective against mucosal lesions, they are not effective for *Candida*-infested denture fitting surface.[1] Furthermore, these drugs are found to have toxic effect on human beings if it is overused. Hence, alternative antifungal agents extracted from the plants are being researched for the use of treating oral candidosis.

India is considered to be a rich emporium of medicated plants, fundamentally utilized as a part of preventive and healing medicine.[9] Neem (*Azadirachta indica*) is found to have antifungal effect and antimicrobial effect.[10-12] Garlic is another indigenous to Asian region and utilized as a topping, particularly in a large portion of the Asian cooking[13] since it acts as an antioxidant[14] (antimicrobial, antibacterial, antiviral, antifungal, and antiprotozoal properties) and also have beneficial properties on the cardiovascular and immune systems.[15,16] However, only few or no studies have been reported on the use of neem and garlic as an adjunct for the use of treating denture-induced stomatitis.

The purpose of this study was to determine whether addition of neem and garlic extracts to the soft liner would inhibit the growth of *C. albicans* and also to evaluate the hardness of the soft lining material after the incorporation of neem and garlic.

Materials and Methods

The study was carried out for 10 days in total, which includes the sterilization and the observation of the *Candida* growth in the samples and also the mechanical testing of the acrylic sample for hardness. Institutional review board no SRPOC/2017/950 was obtained for this study.

Specimen preparation

Preparation of acrylic discs

Power sampling method was carried out to find out the number of samples to be included for the study and it has been concluded that 30 samples can be selected for this pilot study; hence, 30 acrylic discs measuring 12 mm in diameter and 6 mm in thickness were prepared using PMMA according to the manufacture's instructions [Table 1].

Preparation of PEM discs

30 wax discs were fabricated measuring 12 mm in diameter and 6 mm in thickness [Figures 3-5]. Three-part dental flask was used for flasking the fabricated wax patterns using dental stone. Reverse flasking technique was followed by wax elimination. Disc-shaped mold space was created where heat-activated PMMA resin was packed and cured according to the manufacturer's instructions to obtain the acrylized discs [Figure 1].

Thirty acrylized discs were then grouped into three groups [Table 2]. These samples served as a base on which the soft liner to be tested was added later. One side of each sample was polished using tungsten carbide acrylic bur, silicon carbide papers, followed by pumice and rouge. The contradicting surface was left unpolished resembling the tissue surface of the denture.[15,17] An index of the prepared specimens was made using polyvinyl siloxane material on which soft liner material was added. All these procedures were carried out in the laminar air-flow chamber to avoid contamination (aseptic environment), and also, the discs were disinfected by exposing them in ultraviolet light for 30 min [Table 2].

Table 1: Materials used in the study

Material	Manufacturer
Heat cure poly methyl methacrylate	DPI heat cure, DPI, Dental products of India, Bombay Burmah Trading corporation, Ltd., India
Modeling wax no. 02	The Hindustan Dental Products, Hyderabad, India
Aquasil soft putty/ regular set (polyvinyl siloxane)	Dentsply
Heat cure cold mold seal	DPI heat cure, DPI, Dental products of India, Bombay Burmah Trading corporation, Ltd., India
Dental stone	
Soft liner	GC soft liner, 0104B236-0000, GC India

Figure 1: Wax patterns

Table 2: Specimen grouping of neem extract, garlic, and plain soft-liner samples without the addition of neem and garlic extract added to the tissue conditioner

Groups	Products impregnated	Number of specimens
Group A	Neem	10
Group B	Garlic	10
Control group	Plain soft liner (mechanical and microbiological testing)	5 (without neem)
Control group	Plain soft liner (mechanical and microbiological testing)	5 (without garlic)

Sterilization of the specimen

The specimens were handled carefully which includes the utilization of nonsterile nonpowdered latex gloves for all phases of investing, dewaxing, packing, and retrieval of the specimens. Duplicates were handled with stainless-steel tweezers and were put away in sterile distilled water for 6–8 days and water was changed every 24 h.

Preparation of neem and garlic powders

To obtain neem (*A. indica*) and garlic (*Allium sativum*) powder, crisp neem leaves were collected from the trees and garlic was bought from the open markets. The preparation of the powder was done at the Department of Pharmacology, Sri Ramachandra University, Porur, Chennai.

Preparation of neem extract

One kg of crisp, shade-dried leaves of *A. indica* grinded in 4 L of refined water and permitted to soak overnight. The suspension was centrifuged at 5000 rpm for 20 min and separated through a Whatman No. 1 channel paper. The supernatant liquid was permitted to dissipate in glass petri dishes under tube light to give heat and to anticipate clamminess so that no organism growth occurs. At the point when it is totally dry, the powder was gathered by scratching and was put away.[17]

Preparation of garlic extract

The peeled garlic was dried in a microoven at 55°C for 3 h, and the dried garlic was grounded to pass through a 1 mm sieve. Active ingredient of garlic was extracted using hydroalcohol in the ratio of 7:3; extracts (concentrates) were subjected to shaking at room temperature overnight at a speed of 1000 vib/min. The concentrates were separated, and the deposit was again extricated with 100 ml of solvent. This method was rehashed thrice to guarantee the entire extraction of phenolic compounds. At that point, the filtrate was lyophilized using a lyophilizer to obtain the powder.[18-20] The powders were kept separately in sterile, dry screw-capped bottles, which were stored in a dry cool place for 1 week.

Methodology

The prepared neem and garlic powders were then measured utilizing a sensitive electronic balance (weighing machine) in five focuses, i.e., 50 μg, 100 μg, 200 μg, 400 μg, and 500 μg. These powders were blended with the powder part of soft liner (GC Soft liner, 0104B236-0000, GC India) over an electronic vibrator for 10 min each for appropriate scattering of the garlic and neem powders with the soft liner powder.

The soft liner was then mixed according to the manufacturer's instructions (2.2 g of powder to 1.8 g of liquid, room temperature [20°C ± 1.08°C and 50% ±5% relative humidity]), under aseptic conditions and packed into the polyvinyl siloxane mold. The previously made heat cure acrylic discs with roughened surface were placed over the soft liner and pressed until a small amount of soft liner material was displaced. The excess soft liner was cut off using a sterile bard parker blade, and the specimens were stored in an airtight container with sterile water.

Microbiological testing

All the microbiological testing and procedures were carried out in a closed laminar flow chamber to avoid any contamination by microorganisms.

Preparation of stock solution – neem and garlic

Ten milligrams of the active extract was added to 10 ml of brain heart infusion (BHI) broth so that each ml contains 1 mg of the active ingredient of neem and garlic. Doubling dilution of the active ingredient was prepared (100%, 50%, 25%) using BHI broth and artificial saliva (Wet Mouth). To all the test tubes, 10 ml of 10^8 CFU (ATCC 90028) *C. albicans* strains were added. The tubes were incubated for 24 h at 37FC.

After 24 h, the tubes were checked for the presence of any growth by observing the turbidity, Gram staining, and by subculture on blood agar plates at 2nd, 4th, and 7th day. All the tubes which were clear, Gram stain of the broth showing no organisms, and subcultures showing no growth were recorded as no growth of any turbidity, negative for Gram staining and showed no colonies on subculturing into blood agar plates were taken as no growth or inhibition. While the tubes which were turbid, positive Gram staining and showed colonies on subculturing into blood agar plates were recorded for growth of colonies.

Mechanical testing

Shore A Durometer – the effect of incorporation of garlic and neem on the hardness property of the soft liner was evaluated by using Shore A durometer instrument after 2, 4, 7, and 10 days. For the mechanical testing purpose, specimens were prepared under control group and test group (with incorporation of neem and garlic powder).

During hardness testing, the specimen was supported by a glass slab, and indenter was allowed to penetrate the

specimen. Average of three different readings was taken as a testing value. The distance between the indenter and specimen surface was fixed by 20 mm, and contact time after penetration was 5 s. The reading was noted directly from the scale which represents the hardness value.

Analysis shore a hardness

Measurements were made according to ASTM D2240 using a digital Shore A durometer (Instrutherm, Sao Paulo, SP, and Brazil) based on a scale hardness tester (Wallace, Kingston, England). Six measurements were made on each side of all specimens.

Results

Microbiological testing

On subculturing on the agar plates, there were no colonies observed after 2nd, 4th, and 7th day with neem. However, with garlic, few colonies were observed on the 4th day after subculturing [Figure 2].

The hardness value was noted from the scale after 2nd, 4th, and 7th days [Table 3].

For comparison between the groups, Kruskal–Wallis test was used. *Post hoc* comparisons were done using Dunn's test. $P < 0.05$ was considered to be significant. For comparison within the same group for different days, Friedman's test was used. R version 3.3.0 software (IBM Company, Chicago, USA) was used for the analysis [Table 3].

Group 1 is control group, 2 and 3 being neem and garlic, respectively. On comparing the groups, there was no statistically significant change in hardness.

On day 4 and day 7, there was a statistically significant change in hardness observed between the groups.

On day 4: Group 2, i.e., neem group shows significant increase in hardness compared to the control group. The difference of hardness between the neem and garlic is not significant.

On day 7: Group 2, i.e., neem group shows significant increase in hardness compared to the control group. The difference of hardness between the neem and garlic is not significant [Figures 6 and 7].

Discussion

Dental prostheses made with acrylic (PMMA) will be coated with salivary proteins when inserted in the oral environment which in turn forms a pellicle. This pellicle alters the surface properties of PMMA and mediates subsequent adherence of microorganisms including *Candida* species in the denture surface (Edgerton and Levine, 1992). It has been reported that surface roughness

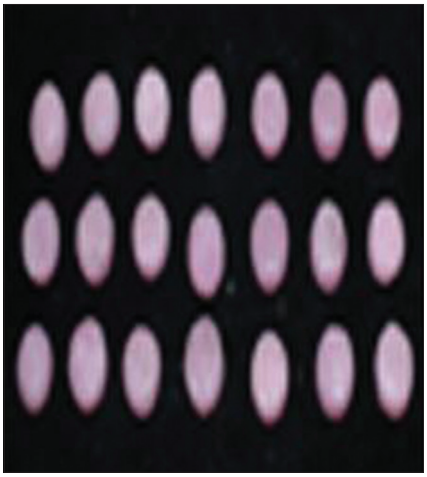

Figure 2: Finished acrylic discs

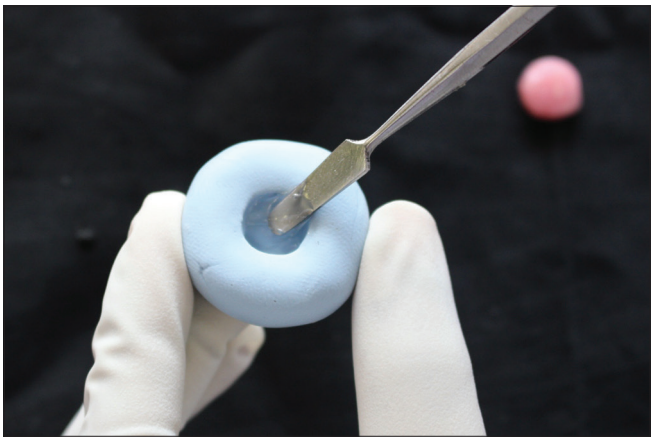

Figure 3: Addition of the soft liner to the polyvinyl siloxane index

Table 3: The hardness test value. Statistical analysis of hardness of the samples incorporated with neem, garlic, and the plain sample (without the addition of neem or garlic) measured on different days and without any microbial agents against *Candida albicans* using Kruskal-Wallis test

Serial number	Group	Day 2			Day 4			Day 7			P^b
		Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	
1	1	10	10.0	0	10.7	10.7	0	12.0	12.0	0	0.007
2	2	14	14.1	4.3	13.3	13.8	2.6	16.0	16.2	3.0	0.074
3	3	13	13.0	2.3	15.7	15.2	2.3	18.3	18.1	2.8	0.015
P^a			0.17			0.006			0.03		

SD=Standard deviation

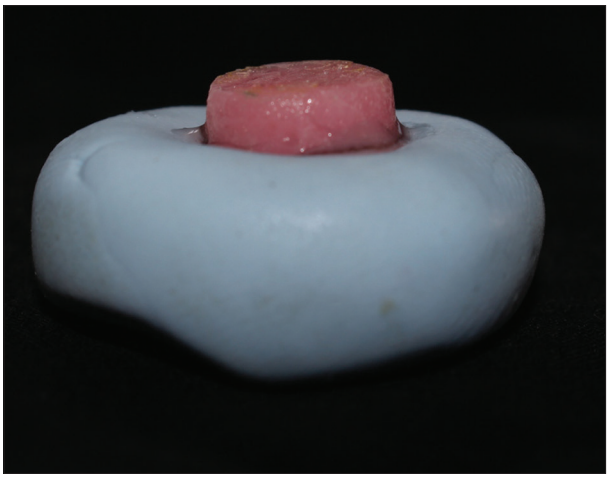

Figure 4: Pressing of the acrylic disc in the index containing soft liner

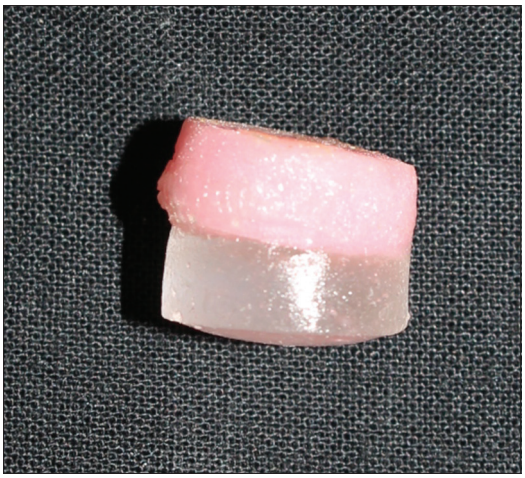

Figure 5: Finished acrylic-soft liner sample

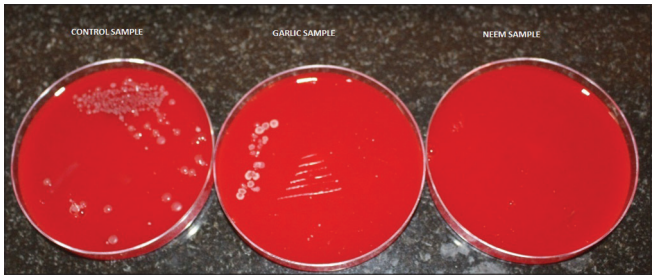

Figure 6: Culture growth observed on 4th day

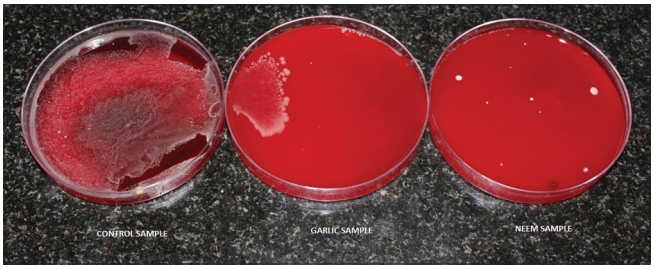

Figure 7: Culture growth observed on the 7th day

is a vital property because of its impact on microbial adhesion.[21] For this reason, if a soft-liner material incorporated with antifungal drugs, which has antifungal efficacy to be developed to interfere on the denture surface to avoid colonization and penetration by *C. albicans* in the denture.[21] However, it has been reported that antifungal drugs have some toxic effects on the patient if it is overused; hence, the plant extracts neem (*A. indica*) and garlic (*A. sativum*) which are naturally available and also possess antifungal property can be used for the same.[4,14,22-27] This leads to the microbiological investigation conducted in this study, which confirms that the released concentrations of the antifungal agents (plant extracts) from the soft lining materials were able to induce an antifungal effect against *C. albicans* on agar culture. Furthermore, the changes in any hardness of the denture base after the incorporation of the plant extracts were analyzed. The limitations of this particular study are decreased sample size and lack of oral environment, as this was an *in vitro* study. Further studies have to be done *in vivo* to confirm the results. The future prospects of the study can also include using these naturally available antifungal sources in drug delivery system.

Conclusions

Within the limits of this pilot study, it can be presumed that utilization of naturally occurring antifungal specialist agents such as neem and garlic can be utilized as an alternative for treating denture-induced stomatitis. However, further studies have to be conducted to find the pharmacological effects such as antibacterial, anticarcinogenic, and anti-inflammatory activities of the plant extracts for the use in the oral environment.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Budtz-Jörgensen E, Stenderup A, Grabowski M. An epidemiologic study of yeasts in elderly denture wearers. Community Dent Oral Epidemiol 1975;3:115-9.
2. Doddanna SJ, Patel S, Sundarrao MA, Veerabhadrappa RS. Antimicrobial activity of plant extracts on *Candida albicans*: An *in vitro* study. Indian J Dent Res 2013;24:401-5.
3. McCullough MJ, Ross BC, Reade PC. *Candida albicans*: A review of its history, taxonomy, epidemiology, virulence attributes, and methods of strain differentiation. Int J Oral Maxillofac Surg 1996;25:136-44.
4. Almas K. The antimicrobial effects of extracts of *Azadirachta indica* (neem) and *Salvadora persica* (Arak) chewing sticks. Indian J Dent Res 1999;10:23-6.
5. Verran J, Maryan CJ. Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. J Prosthet Dent 1997;77:535-9.
6. Radford DR, Challacombe SJ, Walter JD. Denture plaque and

adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med 1999;10:99-116.
7. Waltimo TM, Orstavik D, Sirén EK, Haapasalo MP. *In vitro* susceptibility of *Candida albicans* to four disinfectants and their combinations. Int Endod J 1999;32:421-9.
8. Bagg J. Essentials of Microbiology for Dental Students. Oxford publisher, Oxford, UK: Oxford University Press; 2006.
9. Sholapurkar AA, Pai KM, Rao S. Comparison of efficacy of fluconazole mouthrinse and clotrimazole mouthpaint in the treatment of oral candidiasis. Aust Dent J 2009;54:341-6.
10. Dubey S, Manisha Chaodary T, Gupta P. Comparative study of the antimicrobial efficiency of neem leaf extract, sodium hypochlorite and Biopure MTAD – An *in vitro* study. Indian J Dent Adv 2012;4:740-3.
11. Jadhav V, Shetty MM, Kalavathy N, Kumar R. Effect of 3 types of antifungal agents on hardness of 2 different commercially available tissue conditioners: An *in vitro* study. SRM J Res Dent Sci 2013;4:150-3.
12. Barua DR, Basavanna JM, Varghese RK. Efficacy of neem extract and three antimicrobial agents incorporated into tissue conditioner in inhibiting the growth of *C. albicans* and *S. mutans*. J Clin Diagn Res 2017;11:ZC97-101.
13. Jackson R, McNeil B, Taylor C, Holl G, Ruff D, Gwebu ET, *et al.* Effect of aged garlic extract on caspase-3 activity, *in vitro*. Nutr Neurosci 2002;5:287-90.
14. Benkeblia N. Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). LWT Food Sci Technol 2004;37:263-8.
15. Ali IL, Yunus N, Abu-Hassan MI. Hardness, flexural strength, and flexural modulus comparisons of three differently cured denture base systems. J Prosthodont 2008;17:545-9.
16. Kathariya M, Adhimuthu A, Kathariya R, Prasanna P, Kathariya R, Bhate K. Effect of water soluble neem metabolite (soluneem) compared to chlorhexidine on common oral bacteria: An *in vitro* study. Oral Health Dent Manage 2014;13:1086-90.
17. Katara G, Hemvani N, Chitnis S, Chitnis V, Chitnis DS. Surface disinfection by exposure to germicidal UV light. Indian J Med Microbiol 2008;26:241-2.
18. Bhanwra S, Singh J, Khosla P. Effect of *Azadirachta indica* (neem) leaf aqueous extract on paracetamol-induced liver damage in rats. Indian J Physiol Pharmacol 2000;44:64-8.
19. Iqbal S, Bhanger MI. Stabilization of sunflower oil by garlic extract during accelerated storage. Food Chem 2007;100:246-54.
20. Quirynen M, Bollen CM. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. J Clin Periodontol 1995;22:1-4.
21. Pusateri CR, Monaco EA, Edgerton M. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. Arch Oral Biol 2009;54:588-94.
22. Barua DR, Basavanna JM, Varghese RK. Efficacy of neem extract and three antimicrobial agents incorporated into tissue conditioner in inhibiting the growth of C. Albicans and S. Mutans. J Clin Diagn Res 2017;11:ZC97-10.
23. Nayak A, Nayak R, Soumyo GB, Bhat K, Kudalkar M. Evaluation of antibacterial and anticandidal efficacy of aqueous & alcoholic extract of neem (*Azadirachta indica*) – An *in vitro* study. Int J Res Ayurveda Pharm 2011;2:230-5.
24. Ackia Lekshmi NC, Sowmia N, Viveka S, Brindha RJ, Jeeva S. The inhibiting effect of *Azadirachta indica* against dental pathogens. Asian J Plant Sci Res 2012;2:6-10.
25. Prabhakar J, Senthilkumar M, Priya MS, Mahalakshmi K, Sehgal PK, Sukumaran VG, *et al.* Evaluation of antimicrobial efficacy of herbal alternatives (Triphala and green tea polyphenols), MTAD, and 5% sodium hypochlorite against *Enterococcus faecalis* biofilm formed on tooth substrate: An *in vitro* study. J Endod 2010;36:83-6.
26. Raghavendra SS, Balsaraf KD. Antifungal efficacy of *Azadirachta indica* (neem) – An *in vitro* study. Braz J Oral Sci 2014;13:242-5.
27. Hegde V, Kesaria DP. Comparative evaluation of antimicrobial activity of neem, propolis, turmeric, liquorice and sodium hypochlorite as root canal irrigants against *E. faecalis* and *C. albicans* – An *in vitro* study. Endod 2013;25:38-45.