IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 19, Issue 10 Ser.12 (October. 2020), PP 45-56
www.iosrjournals.org

Evaluation of antimicrobial efficacy of Cocos nucifera husk extract, Azadirachta indica extract and Morinda citrifolia extract against Enterococcus faecalis, Staphylococcus aureus and Candida albicans: An invitro study

Dr. Parvathy V[1], Dr. Elizabeth Prabha James[2], Dr.Jayasree S[3], Dr.Durga N[4], Dr.Vidya KG[5], Dr.Muhammed Abdul Rahman TV[6]

1. Assistant Professor, Department of Conservative Dentistry, Government dental college, Kozhikode
2. Associate Professor, Department of Conservative Dentistry, Government dental college, Kozhikode
3. Professor, Department of Conservative Dentistry, Government dental college, Kozhikode
4. Postgraduate student, Department of Microbiology, Government medical college, Kozhikode
5. Assistant Professor, Department of Conservative Dentistry, Government dental college, Kozhikode
6.Assistant Professor, Department of Conservative Dentistry, Educare institute of dental sciences, Malappuram

Abstract:
Background and objectives: *The constant increase in antibiotic resistant strains and side-effects caused by synthetic drugs have prompted researchers to pursue herbal alternatives for root canal irrigation.The objective of this in vitro study is to evaluate and compare the antimicrobial activity of Coconut husk extract, Azadirachta indica extract, Morinda citrifolia extract and sodium hypochlorite against Enterococcus faecalis, Staphylococcus aureus and Candida albicans in a tooth biofilm model.*
Materials and methods: *300 single rooted teeth extracted due to orthodontic reasons were selected for the study. The root canals were then cleaned and shaped using the crown down technique and ProTaper rotary instruments to an apical size of F3, 1mm short of the apical foramen. 100 teeth were inoculated with E. faecalis, 100 teeth with S.aureus and remaining 100 teeth with C.albicans. At the end of 6th week of incubation, the samples inoculated with E.faecalis and S.aureus were divided into five experimental groups(coconut husk extract, morinda citrifolia, azadirachta indica, sodium hypochlorite,sterile saline) with 20 samples each and irrigated with 3ml of each test irrigant for 10 minutes. Similar procedure was followed for samples inoculated with Candida albicans after 48 hours of incubation. The quantitative analysis was performed by counting the colony forming units of E.faecalis,S.aureus and C.albicans using digital colony counter. The data collected was subjected to analysis of variance (ANOVA) and post hoc Tukey's tests.*
Results: *There wassignificantly less CFUs of E.faecalis in the samples treated with NaOCl, followed by AI, MC, CHE, saline. There was significantly less CFUs of S.aureus adhering to the samples treated with NaOCl , followed by CHE, MC, AI, saline. There was significantly less C.albicans were found adhering to the samples treated with NaOCl , followed by AI, CHE, MC, saline.*
Conclusions: *AI is particularly effective against C. albicans in root canal infections with no statistically significant difference against sodium hypochlorite. AI also had better antimicrobial action against E.faecalis than CHE and MC. CHE showed slightly better reduction of S.aureus colonies than AI and MC . In vivo data may be helpful in determining the real potential usefulness of these herbal extracts for the treatment of root canal infections.*

Keywords: *Antimicrobial efficacy, Coconut husk extract, Morinda citrifolia, Azadirachta indica, sodium hypochlorite, sterile saline, Enterococcus faecalis, Staphylococcus aureus, Candida albicans*

Date of Submission: 15-10-2020 Date of Acceptance: 31-10-2020

I. Introduction

Endodontic infections are polymicrobial in nature dominated by obligate anaerobic bacteria[1]. *Enterococcus faecalis (*E. faecalis)is a gram positive facultative anaerobe found in 4-40% of primary endodontic infections. It is one of the main bacteria associated with chronic apical periodontitis in failed root canal treatments.[2]Studies have investigated its occurrence in root filled teeth with the prevalence ranging from 24 to 77%. [3] E. faecalis has the ability to bind to dentin, invade dentinal tubules, and survive starvation by genetic polymorphism. It possesses a number of virulence factors, including lytic enzymes, cytolysin, aggregation systems, pheromones, and lipoteichoic acid. It can also suppress the action of lymphocytes.[4]*Staphylococcus*

aureus(S.aureus) is another bacterium also found in postendodontic treatment lesions. Most of these bacteria have developed resistance to antimicrobial drugs and it can survive long periods of dehydration and resist changes in temperature and dehydration.[2]*Candida albicans*(C.albicans) is the most common fungus seen in the root canals, 21% in primary infections and 18% in cases of retreatments.[5] C.albicans can survive harsh conditions due to biofilm formation and the physicochemical properties of the microorganisms help them to modify according to the prevailing environmental and nutritional conditions. [6] Biofilm helps in resisting the destruction of the fungus by making them thousand times more resistant to phagocytosis, antibodies and antimicrobial agents. This is attributed to the protective barrier provided by the extracellular matrix. [7]

Elimination of microbes from the root canals can be achieved with mechanical preparation combined with use of antimicrobial irrigants [8]. NaOCl is the "gold standard" of root canal irrigants due to its efficacy against pathogenic organisms and pulp degeneration in endodontic treatment, However, it is not only irritant to the periapical tissues, but also inherently possesses certain disadvantages such as staining of instruments, burning of surrounding tissues,[9] unpleasant taste and odor, high toxicity,[10] corrosive to instruments, reduction in elastic modulus and flexural strength of dentin. [11]

Due to constant increase in the antibiotic resistant strains and the side effects caused by synthetic drugs, [12] the trend has now shifted to the age-old herbal products. India is a country, rich in medicinal plants. Studies have shown the promising role of herbal irrigants as root canal irrigants. The major advantages of herbal irrigants include safety, ease of availability, increased shelf-life, cost effectiveness and lack of microbial resistance so far. [18]

Among the many herbal products that are being used in dentistry, *Cocos nucifera* is one such herb that has not been explored yet. *Cocos nucifera* (family Arecaceae) commonly known as coconut is an important fruit crop in tropical countries. The beneficial medicinal effects of *C. nucifera* including the antibacterial activity result from the secondary products present in the plant, although it is usually not attributed to a single compound but a combination of the metabolites. Phytochemical screening of *C. nucifera.* has reported that this plant material is rich in alkaloids, flavonoids, catechin, and epicatechin together with condensed tannins, which confers on its potent antimicrobial properties.[13]

Azadirachta indica (Neem) is one of the most versatile medicinal plants. It is the most commonly used traditional plant of India for household remedies against various human ailments from antiquity. It elaborates a vast array of chemically diverse and structurally complex biologically active compounds. The phytochemical constituents present in *neem* are nimbidin, nimbin, nimbolide, Azadirachtin, gallic acid, epicatechin, catechin, and margolone. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties.[14]

Morinda citrifolia is a fruit-bearing tree in the coffee family Rubiaceae.It is commonly known as "Indian mulberry" or "noni" plant. It is indigenous to tropical countries and is considered as an important folk medicine. The fruit juice has a wide range of therapeutic effects. [14] The fruit contains polysaccharides, scopoletin, vitamins and minerals. *Morinda citrifolia* juice (MCJ) has antibacterial, antiviral, antifungal, anti-tumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects.[15]

Several studies using a tooth model for determining the antimicrobial efficacy based on the agar disk diffusion method have been published, but few *in vitro* studies have been done using biofilm models that closely resemble clinical situations. Furthermore, very few studies have been carried out utilizing Cocos nucifera husk extract as an irrigant.The objective of this *in vitro* study is to evaluate and compare the antimicrobial activity of Coconut husk extract, Azadirachta indica extract, Morinda citrifolia extract and sodium hypochlorite against Enterococcus faecalis, Staphylococcus aureus and Candida albicans in a tooth biofilm model.

II. Materials and methods

Preparation of the test solutions

Preparation of Coconut husk extract – The fibrous husk of coconut shells were collected from the local coconut growers in Kozhikode, India. It was washed with distilled water to remove dirt, cut into smaller pieces and air dried for 21 days. The dried husk fiber was then blended using an electric blender. One hundred grams of the plant powder was extracted in a Soxhlet apparatus with 500 ml of ethanol and the solvent filtered by Whatman no.1 filter paper and concentrated using a rotor-evaporator.[13]

Preparation of Azadirachta indica extract-Mature plants of Azadirachta indica was collected from the local growers in Kozhikode city. Fresh leaves procured were washed thoroughly and dried in shade. The completely dried leaves were coarsely powdered and 50g was used for successive extraction in 100ml ethanol for 10 minutes with shaking in between. The crude extract was then be filtered through muslin followed by Whatman no.1 filter paper. The ethanol was evaporated and concentrated using rotary vaporizer and stored in amber colored bottles.[14]

Preparation of Morinda citrifolia extract - Fresh fruits of M.citrifolia plant were collected from local growers in Kozhikode city and dried at room temperature for 2-3 days. The air-dried fruits will be kept at 40°C in hot air

oven for 24 hrs and ground into powder .and mixed in a ratio of 1:5 with solvent ethanol. The extraction was carried out in a shaker water bath at 40°C for 48 hrs. The extract was filtered through Whatman no: 1 filter paper and concentrated to dryness using a rotary vaporiser.[14]
150mg of the prepared extracts was dissolved in 10ml of DMSO for use as stock solution.

Preparation of tooth samples

300 human mandibular premolar teeth with Vertucci Class I root canal configuration and fully formed apicesextracted for orthodontic reasons were selected for the study. Teeth that were previously endodontically treated, teeth with previous coronal restorations/caries /Fracture/ curved roots/ calcified canals/ any wasting diseases /developmental anomalies were excluded from the study. Digital radiographs of selected teeth were taken in various angulations to determine the number and morphology of the canals.

The specimens were cleaned of superficial debris, calculus and soft tissue tags lightly by means of an ultrasonic scaler. They were then disinfected with 5.25% sodium hypochlorite (NaOCl) for 30 minutes and stored at 4^0C in 0.9% physiological saline. [15] The specimens were sectioned perpendicular to the long axis of the teeth from cementoenamel junction (CEJ) with a diamond disc (Dent. Abrasive Italica) in conjunction with physiological saline irrigation to obtain a standardized tooth length of 14 mm. Canals were evaluated for apical patency and radiographs were taken to check for single canal.

The root canals were then cleaned and shaped using the crown down technique and ProTaper rotary instruments to an apical size of F3, 1mm short of the apical foramen. 1 ml of 5.25% NaOCl was used between each instrument during the cleaning and shaping procedures. [16] The samples were then irrigated with 17% ethylenediaminetetraacetic acid (EDTA), 5.25% NaOCl and 30ml distilled water for 10 minutes each to remove the smear layer.[17] The apical foramen of all roots were sealed with a temporary filling material (Cavit,3M,ESPE,Germany) and the root surfaces were coated with two layers of nail polish.

Each root specimen was then placed individually in glass bottles with numbering on each section and autoclaved twice with a 24-hour interim period at 121^0 C at 15 psi for 20 minutes to sterilize the root canals (Unique clave C-79, Confident). To verify the sterilization process, a No. 25 sterile paper point was inserted in the individual canal of randomly chosen one out of every five root specimens. These paper points were transported in 1.5 ml of sterile saline in individual sterile glass test tubes, vortexed, cultured and confirmed for zero colony forming units (CFU) [19]. After confirming sterility of root canals, microbial inoculation in each root section was done.

All procedures will be performed under strict aseptic conditions.

Antimicrobial efficacy of test irrigants against E.Faecalis and S.aureus

Preparation of inoculum

A pure culture of E. Faecalis (ATCC 29212) (HiMedia Mumbai) and S.aureus (ATCC 6538) (HiMedia Mumbai) will be inoculated separately on Blood agar plates [Himedia, Mumbai] and incubated at 37 ^{0} C overnight. The turbidity was adjusted to 0.5 McFarland standard to obtain a cell density of 1.5×10^8 cells/ml with sterile Brain-Heart Infusion broth.

Microbial inoculation of tooth specimens

A suspension with a haze of 0.5 McFarlandwas added on 300µl centrifuge tubes in such a way that it submerged the covered roots except the negative control ones and stirred well. Samples were incubated at 37°C for 6 weeks. The mixtures were refreshed with newly prepared 0.5 McFarland E.faecalis / S.aureus suspensions every alternate day to avoid nutrition depletion and accumulation of toxic end products. A sample was taken from the canal space of each specimen with a sterile no 25 paper point and inoculated onto Blood agar plates [Himedia, Mumbai] and incubated at 37°C for 24 hours to check for cell viability and purity of culture.

Grouping and assessment protocol

At the end of 6^{th} week of incubation, the samples were divided into five experimental groups with 20 samples each and irrigated with 3ml of test irrigant.

- Group A- 3 ml of Coconut Husk extract
- Group B- 3 ml of Morinda citrifolia extract
- Group C- 3 ml of Azadirachta indica (Neem) extract
- Group D- 3 ml of 5.25% Sodium hypochlorite
- Group E- 3 ml of Sterile saline.

The solutions were delivered into the root canals 2mm short of the working length with sterile plastic syringes and 27 gauge needles until the root canals and plastic tubes were totally filled with them and left in the canals for 10 minutes. All specimens were flushed with 30ml of sterile saline to prevent potential carry-over of

the irrigants. A sterile no.25 paper point will be used to collect the fluid from the canal carefully taking care not to touch the outer surface of the canals. The points will be transferred to sterile tubes containing 1ml of sterile saline solution. After vortexing, the tubes for 15 seconds, 100 µl of the contents will be removed from the tubes and placed in a petridish containing Blood Agar plates and incubated at 37°C for 24 hours. [19,20] The quantitative analysis was performed by counting the E.faecalis and S.aureus colony forming units using digital colony counter. The data collected was subjected to analysis of variance (ANOVA) and *post hoc* Tukey's tests.[1]

Antimicrobial efficacy of test irrigants against C.albicans

Preparation of inoculum

Strains of of *C. albicans*ATCC 10231 (Himedia, Mumbai) were inoculated on Sabouraud Dextrose Agar (Himedia, Mumbai) and incubated at 37°C for 48-72 hours and adjusted to an optical density of one.[16]

Microbial inoculation of tooth specimens

A suspension with a haze of 0.5 McFarlandwas added on 300µl centrifuge tubes in such a way that it submerged the covered roots except the negative control ones and stirred well. Samples were incubated at 37°C for 48 hours. The mixtures were refreshed with newly prepared 0.5 McFarland C.albicans suspensions every 24 hours. Following incubation for 48 hours,10 µl will be taken from each tube and planted on Sabouraud Dextrose Agar as a reproduction control. The presence of C.albicans in the root canals was verified at 48 hours.

Grouping and assessment protocol

The samples were divided into five experimental groups with 20 samples each and irrigated with 3ml of test irrigant.

- Group A- 3 ml of Coconut husk extract
- Group B- 3 ml of Morinda citrifolia extract
- Group C- 3 ml of Azadirachta indica (neem) extract
- Group D- 3 ml of 5.25% Sodium hypochlorite
- Group E- 3 ml of Sterile saline.

The solutions were delivered into the root canals 2mm short of the working length with sterile plastic syringes and 27 gauge needles until the root canals and plastic tubes were totally filled with them and left for 10 minutes. All specimens were flushed with 30ml of sterile saline to prevent potential carry-over of the irrigants. A sterile no.25 paper point will be used to collect the fluid from the canal carefully taking care not to touch the outer surface of the canals. The point was transferred to sterile tubes containing 1ml of sterile saline solution. After vortexing, the tubes for 15 seconds, 100 µl of the contents was removed from the tubes and placed in a petridish containing Sabouraud Agar plates and incubated at 37°C for 48 hours.[20]The plates were then analyzed by the digital colony counter and the number of CFUs of Candida albicans was counted and recorded. The data wassubjected to statistical analysis using analysis of variance and *post hoc* Tukey tests.[16]

PLAN OF ANALYSIS

The number of CFU per ml of culture was determined. The effect of each test agent on the biofilm was determined by calculating the percentage kill of viable bacteria following treatment with the test agent. The percentage kill for each test agent was calculated by the formula

1- [Average CFU(test agent) / Average CFU(negative control)] x 100

Data was entered in excel sheet and analysis done with SPSS software (Chicago,IL, USA). Statistical test such as ANOVA and post hoc analysis was performed.Significance was determined at $p < 0.05$.

III. Results

Readings of microbial count obtained from digital colony counter after irrigation with respective irrigant were as follows:

Table no 1. Mean and standard deviation showing inter group differences from the readings of digital colony counter for Enterococcus faecalis.

E.faecalis					
	Frequency	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**
Coconut husk extract	20	74.15	3.19992	70	81
Morinda citrifolia	20	62.7	2.57723	58	68

Azardirachta indica	20	50.25	2.3814	46	54
Sodium hypochlorite	20	0.3	0.47016	0	1
Sterile saline	20	92.65	2.27746	89	97
Total	*100*	*56.01*	*31.39726*	*0*	*97*
Test	ANOVA				
p-value	**<0.001**				
Inference	There exists significant difference between groups				

Figure 1. The histogram depicting the bacterial count for different treatment groups is shown in Figure 1 . Using one way ANOVA with multiple comparison, significantly less bacteria were found adhering to the samples treated with NaOCl, followed by AI, MC, CHE,saline. There were conspicuously high numbers of bacteria on dentin when saline was used.

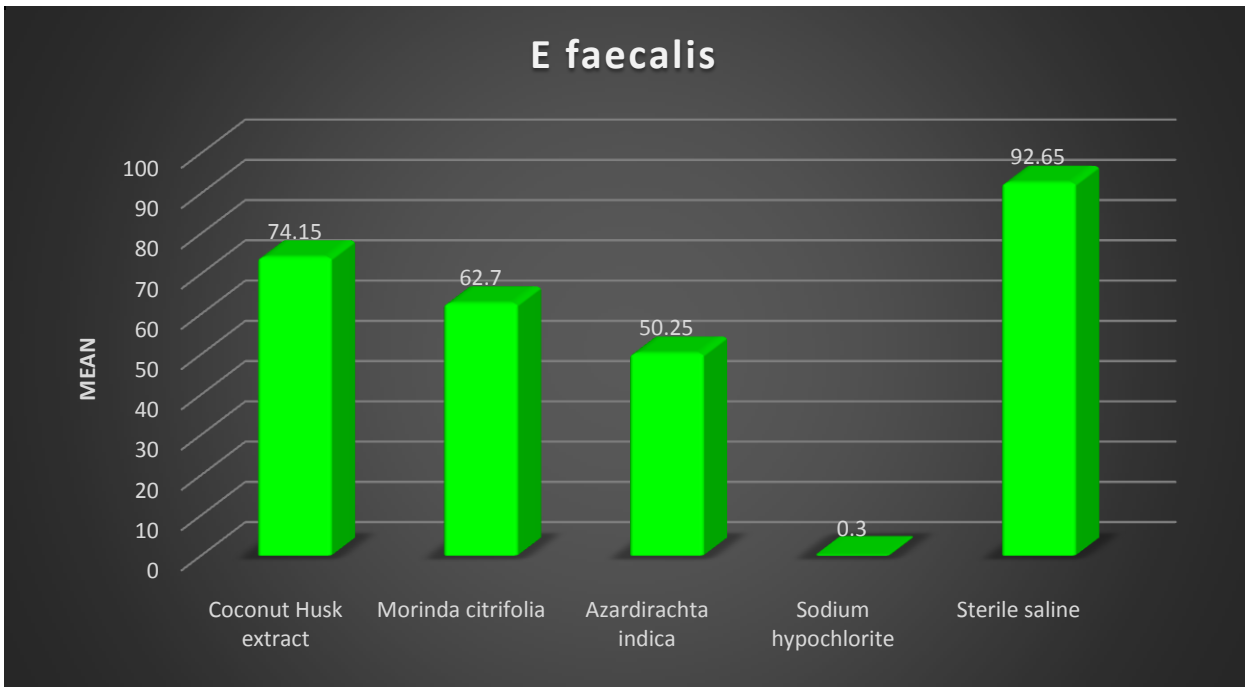


Table 2. Post hoc analysis (E.faecalis)			
Tukey HSD			
(I) Group	(J) Group	Mean Difference (I-J)	p-value
Coconut husk extract	Morinda citrifolia	11.45000*	**<0.001**
	Azardirachta indica	23.90000*	**<0.001**
	Sodium hypochlorite	73.85000*	**<0.001**
	Sterile saline	-18.50000*	**<0.001**
Morinda citrifolia	Coconut husk extract	-11.45000*	**<0.001**
	Azardirachta indica	12.45000*	**<0.001**

	Sodium hypochlorite	62.40000*	<**0.001**
	Sterile saline	-29.95000*	<**0.001**
Azardirachta indica	Coconut husk extract	-23.90000*	<**0.001**
	Morinda citrifolia	-12.45000*	<**0.001**
	Sodium hypochlorite	49.95000*	<**0.001**
	Sterile saline	-42.40000*	<**0.001**
Sodium hypochlorite	Coconut husk extract	-73.85000*	<**0.001**
	Morinda citrifolia	-62.40000*	<**0.001**
	Azardirachta indica	-49.95000*	<**0.001**
	Sterile saline	-92.35000*	<**0.001**
Sterile saline	Coconut husk extract	18.50000*	<**0.001**
	Morinda citrifolia	29.95000*	<**0.001**
	Azardirachta indica	42.40000*	<**0.001**
	Sodium hypochlorite	92.35000*	<**0.001**
* The mean difference is significant at the 0.05 level.			

The results were statistically significant.

Table 3. Mean and standard deviation showing inter group differences from the readings of digital colony counter for Staphylococcus aureus

S.aureus					
	Frequency	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**
Coconut husk extract	20	42.25	6.06001	26	52
Morinda citrifolia	20	46.8	3.62157	38	52
Azardirachta indica	20	48.85	3.99045	42	57
Sodium hypochlorite	20	0.25	0.44426	0	1
Sterile saline	20	90.45	4.22368	82	98
Total	*100*	*45.72*	*29.02771*	*0*	*98*
Test	ANOVA				
p-value	**<0.001**				
Inference	There exists significant difference between groups				

The histogram depicting the bacterial count for different treatment groups is shown in Figure 2. Using one way ANOVA with multiple comparison, significantly less bacteria were found adhering to the samples treated with NaOCL , followed by CHE, MC, AI, saline. There were conspicuously high numbers of bacteria on dentin when saline was used.

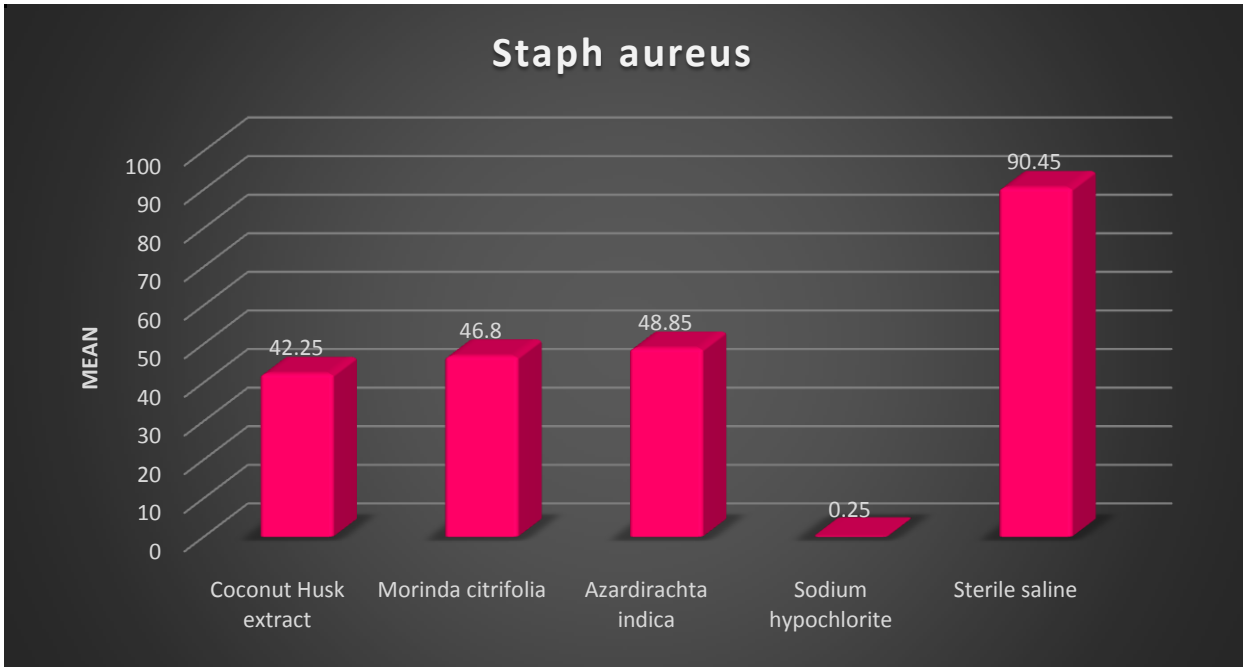


Table 4. Post hoc analysis (S.aureus)

Tukey HSD			
(I) Group	(J) Group	Mean Difference (I-J)	p-value
Coconut husk extract	Morinda citrifolia	-4.55000*	**0.006**
	Azardirachta indica	-6.60000*	**<0.001**
	Sodium hypochlorite	42.00000*	**<0.001**
	Sterile saline	-48.20000*	**<0.001**
Morinda citrifolia	Coconut husk extract	4.55000*	**0.006**
	Azardirachta indica	-2.05	**0.512**
	Sodium hypochlorite	46.55000*	**<0.001**
	Sterile saline	-43.65000*	**<0.001**
Azardirachta indica	Coconut husk extract	6.60000*	**<0.001**
	Morinda citrifolia	2.05	**0.512**
	Sodium hypochlorite	48.60000*	**<0.001**
	Sterile saline	-41.60000*	**<0.001**
Sodium hypochlorite	Coconut husk extract	-42.00000*	**<0.001**
	Morinda citrifolia	-46.55000*	**<0.001**
	Azardirachta indica	-48.60000*	**<0.001**
	Sterile saline	-90.20000*	**<0.001**

Sterile saline	Coconut husk extract	48.20000*	**<0.001**
	Morinda citrifolia	43.65000*	**<0.001**
	Azardirachta indica	41.60000*	**<0.001**
	Sodium hypochlorite	90.20000*	**<0.001**
* The mean difference is significant at the 0.05 level.			

No statistical difference was seen between MC and AI.

Table 5. Mean and standard deviation showing inter group differences from the readings of digital colony counter for Candida albicans

C.albicans					
	Frequency	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**
Coconut husk extract	20	90.2	5.46376	81	98
Morinda citrifolia	20	92.5	3.74868	82	98
Azardirachta indica	20	3.5	0.51299	3	4
Sodium hypochlorite	20	0.35	0.48936	0	1
Sterile saline	20	136.55	8.45717	112	148
Total	*100*	*64.62*	*54.27572*	*0*	*148*
Test	ANOVA				
p-value	**<0.001**				
Inference	There exists significant difference between groups				

Figure 3. The histogram depicting the bacterial count for different treatment groups. Using one way ANOVA with multiple comparison, significantly less C.albicans were found adhering to the samples treated with NaOCl , followed by AI, CHE, MC, saline. Saline (negative control) had least antimicrobial activity as expected.

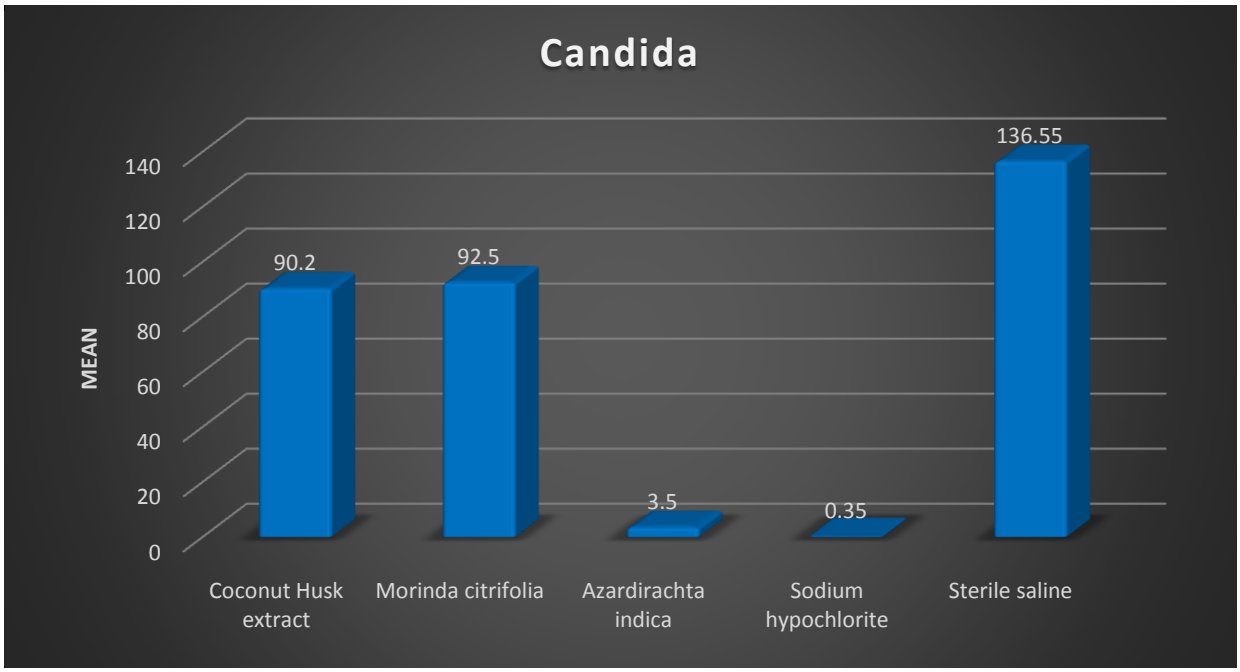


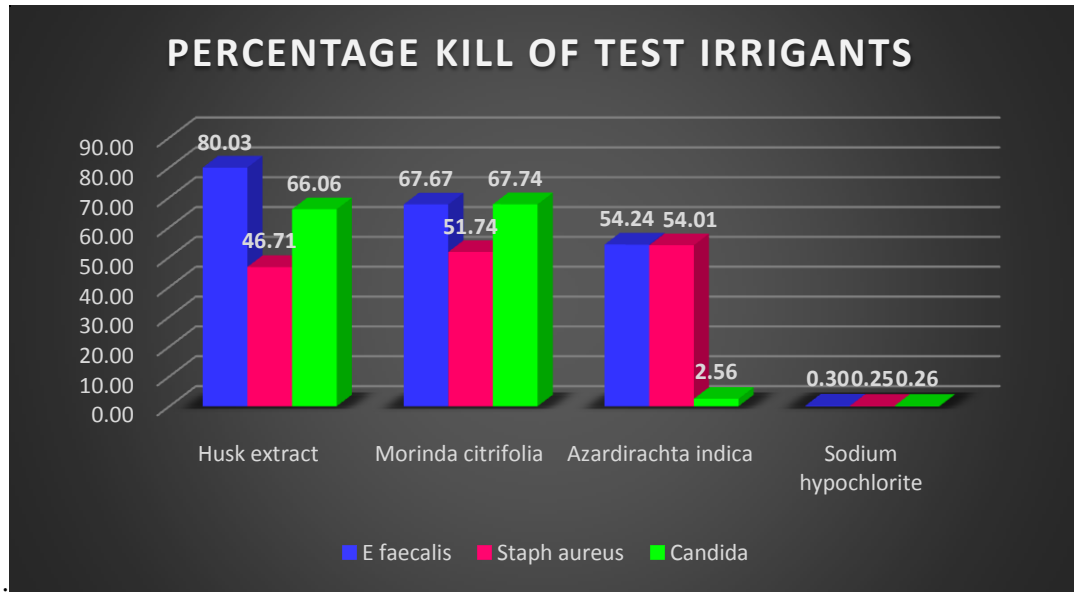
Table 6. Post hoc analysis – C.albicans			
Tukey HSD			
(I) Group	(J) Group	Mean Difference (I-J)	p-value
Coconut husk extract	Morinda citrifolia	-2.3	**0.558**
	Azardirachta indica	86.70000*	**<0.001**
	Sodium hypochlorite	89.85000*	**<0.001**
	Sterile saline	-46.35000*	**<0.001**
Morinda citrifolia	Coconut husk extract	2.3	**0.558**

	Azardirachta indica	89.00000*	<**0.001**
	Sodium hypochlorite	92.15000*	<**0.001**
	Sterile saline	-44.05000*	<**0.001**
Azardirachta indica	Coconut husk extract	-86.70000*	<**0.001**
	Morinda citrifolia	-89.00000*	<**0.001**
	Sodium hypochlorite	3.15	**0.242**
	Sterile saline	-133.05000*	<**0.001**
Sodium hypochlorite	Coconut husk extract	-89.85000*	<**0.001**
	Morinda citrifolia	-92.15000*	<**0.001**
	Azardirachta indica	-3.15	**0.242**
	Sterile saline	-136.20000*	<**0.001**
Sterile saline	Coconut husk extract	46.35000*	<**0.001**
	Morinda citrifolia	44.05000*	<**0.001**
	Azardirachta indica	133.05000*	<**0.001**
	Sodium hypochlorite	136.20000*	<**0.001**
* The mean difference is significant at the 0.05 level.			

There was no statistical difference seen between CHE and MC. No statistical difference was also seen between AI and NaOCl.

Table 7. PERCENTAGE KILL OF TEST IRRIGANTS			
	E faecalis	**S. aureus**	**C.albicans**
Coconut Husk extract	80.03	46.71	66.06
Morinda citrifolia	67.67	51.74	67.74
Azardirachta indica	54.24	54.01	2.56
Sodium hypochlorite	0.30	0.25	0.26

Figure 4 The histogram depicts the percentage kill for different treatment groups against E.faecalis, S.aureus, C.albicans



IV. Discussion

This study was conducted on E. *faecalis*, S.*aureus* and *C. albicans* as these microorganisms are commonly encountered in recalcitrant endodontic infections.

E. faecalis is the most common microorganism isolated from apical periodontitis after treatment.[1] This anaerobic gram-positive coccus has several virulence factors that make it resistant to antimicrobial agents. Among these factors are the ability to endure food restrictions and poor diet for long periods of time, adhesion to the canal walls, invasion into the dentinal tubules, changing the host defenses, having lytic enzymes, resistance to common medications, and the ability to form solid biofilms. This anaerobic gram-positive coccus has several virulence factors that make it resistant to antimicrobial agents. *Staphylococcus aureus* are also encountered in failed endodontic treatment cases, though less commonly than E.faecalis. They are Gram-positive and facultative anaerobic cocci that can survive in harsh conditions for long periods and resist changes

in temperature and dehydration. Most of these bacteria have been able to develop resistance to antimicrobial drugs.

With the increasing growth of E. faecalis biofilm, the biofilm structure becomes calcified and, as a consequence, the removal of this mature and mineralized biofilm through conventional methods becomes more difficult, ultimately leading to resistant root canal infections. Kishen et al[21]demonstrated that after 4 weeks of incubation of *E. faecalis*, bacterial cells completely cover the dentin surface and after 6 weeks, mature biofilms which have a very coherent structure together with symptoms of mineralization, are formed. Previous studies also revealed that the resistance of *E. faecalis* biofilm against antibacterial agents is affected by the increase in incubation time (biofilm age) and the physiological status of cells .[22]Hence,a 6 week biofilm model was used in this study.

Candia albicans has an initial period of adherence (0-2 h) followed by subsequent microcolony formation (2-4 h). Dimorphic switching occurred thereafter with a transition from budding-yeast forms to filamentous pseudo- and true-hyphal forms (4-6 h). Micro-colonies then become interlinked by the hyphal extensions, forming a confluent monolayer (6-8 h). The complexity of the biofilm increases with time, taking on 3D architecture with spatial heterogeneity as it matured (8-48 h). The biofilm after 24 and 48 h 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.[5] *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*,[16] which is why the 48 h biofilm model was used.

The success of an endodontic treatment depends on effective disinfection and complete sealing of root canal. The irrigants that are currently used in the field of endodontics have certain limitations, so the quest for an ideal root canal irrigant continues.

Sodium hypochlorite has known to be cytotoxic to tissues and a need for replacement with a more biocompatible irrigant is necessitated.[11]Pharmacological studies acknowledged the value of medicinal plants as a potential source of bioactive compounds.The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far. [18]Coconut husk extract,Morinda citrifolia and Azadirachta indica have a broad range of antimicrobial activity and have been suggested as natural endodontic irrigating solutions.

The exact mechanism by which the active components of the plant materials contribute to the antibacterial activity is not known. One of the mechanisms suggested is the hydrophobic activity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from the bacterial cells or the exit of critical molecules and ions will lead l- though aqueous extracts of C. nucifera husk fiber are popularly used for the treatment of diarrhea and arthritis, pharmacological in- vestigation of their beneficial or adverse biological effects is still very preliminary. Recent data from our group have shown that the aqueous extract of the husk fiber of C. nucifera, typical A variety, popularly known as “olho de cravo”, has antibacterial and antiviral (4), antitumoral (5) and antileish- manial properties (6). This extract also ex- hibited in vivo and in vitro analgesic and free radical-scavenging properties (7). Prelimi- nary study by Kirszberg et al. (5) has sug- gested that the efficacy of the antitumoral activity of C. nucifera, typical A variety, could be extended to leukemia cells having a multidrug-resistant phenotype. Al- though aqueous extracts of C. nucifera husk fiber are popularly used for the treatment of diarrhea and arthritis, pharmacological in- vestigation of their beneficial or adverse biological effects is still very preliminary. Recent data from our group have shown that the aqueous extract of the husk fiber of C. nucifera, typical A variety, popularly known as “olho de cravo”, has antibacterial and antiviral (4), antitumoral (5) and antileish- manial properties (6). This extract also ex- hibited in vivo and in vitro analgesic and free radical-scavenging properties (7). Prelimi- nary study by Kirszberg et al. (5) has sug- gested that the efficacy of the antitumoral activity of C. nucifera, typical A variety, could be extended to leukemia cells having a multidrug-resistant phenotype.

Coconut husk extracts have been widely used for treatment of diarrhea and arthritis but pharmacological investigation of beneficial or adverse biological effects is still very preliminary Coconut husk extracts have shown to have antibacterial, antiviral, antitumoral, antileishmanial properties. The extract also exhibited invivo and invitro analgesic and free radical-scavenging properties.Brushing the teeth with fibrous husk of Cocos nucifera is a common oral hygiene practice among people of rural areas of South India. However, the probable antimicrobial properties of this plant material against common root canal pathogens have not been proved scientifically. The phytochemical screening of C. nucifera has reported that this plant material is rich in alkaloids, flavonoids, catechins, and epicatechin, together with condensed tannins, which confer on it potent antimicrobial properties. These compounds inhibited in vitro Streptococcus mutansand other bacteria and microorganisms.[23,24]

The phytochemical screening of C. nucifera has reported that this plant material is rich in alkaloids, 坂avonoids, catechins, and epicatechin, together with condensed tannins, to this they have also noted other secondary metabolites like proteins, steroids, and carbohydrates. Studies on the antimicrobial properties of tea have revealed that components in tea, particularly monomeric polyphenols, especially simple catechins such as epicatechin, epicatechin gallate, and epigallocatechin gallate, are responsible for these biologi contains catechin and epicatechin, it can be contemplated that these components must be the primary constituents that confer the antimicrobial effects.

Morinda citrifolia, commercially known as noni has a broad range of therapeutic effects including antibacterial, antifungal, antiviral, antitumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects.[2] The beneficial antimicrobial effects may be the result of acubin, L-asperuloside, alizarin, scopoletin and other anthraquinones. It has antimicrobial action against *Pseudomonas aeruginosa, Proteus morgaii, S. aureus, Bacillus subtilis, E. coli, Salmonella, Shigella, E. faecalis* and *C. albicans.*Banerjee *et al*.showed that MCJ had anticandidal activity *in vitro*.[27]Jayaraman *et al*showed that MCJ showed no significant activity against *C. albicans*.[28] This might be due to the fact stated by Jainkittivong *et al* who showed that in case of *M. citrifolia* fruit longer the contact time, higher is the inhibitory effect.[29]The contact time found to be effective was 45 min, but in our study we took 10 min to standardize the procedure and simulate a clinical situation.

Azadirachta indica has antimicrobial activity due to the presence of active constituents such as nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone, and cyclictrisulphide.[14]These active constituents uncouples mitochondrial oxidative phosphorylation; thus, inhibiting the respiratory chain.This results in its anti-adherence activity by altering the bacterial adhesion and the ability of the microorganism to colonize thereby causing maximum reduction in adherence of E. faecalis to dentin.[30] Bohora *et al*. have concluded that neem leaf extract has a significant antimicrobial effect against *C. albicans*.[31] This is in accordance with our study. Acidic ethanolic extract was found to be better than aqueous extract in cases of *C. albicans*. Neem has a wide spectrum of antimicrobial action; against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis, M. pyogenes, Streptococcus mutans* and *Enterococcus faecalis*. Ethanol extract is most effective against *E. faecalis, E. coli, Proteus mirabilis*. Furthermore, effective against certain human fungi including *Trychophyton, Epidermophyton, Microsporum, Trichosporon, Geotricum* and *Candida.* [32]

Until date, no study has been conducted comparing the herbal irrigants used in this study with sodium hypochlorite in a biofilm model against E.*faecalis*, S.*aureus*, *C. albicans* and therefore, this study holds ground for future research. There was a reduction in microbial count after using the herbal irrigants but not to the same extent as sodium hypochlorite. According to the results of the study, AI is particularly effective against *C. albicans* in root canal infections and had better antimicrobial action against E.faecalis than CHE and MC. CHE showed slightly better reduction of S.aureus colonies than AI and MC . This was in accordance with studiesconducted by Tyagi et al.[16]The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far.[14,16]

V. Conclusion

Within the limitations of our study, we conclude that Coconut husk extract, Morinda citrifolia and Azadirachta indica have some amount of antimicrobial actionagainst E. faecalis, S.aureus and C. albicansowingto the great potential of bioactive compounds and are useful for rationalizing their use as endodontic irrigants. Sodium hypochlorite showed highest antimicrobial activity against all of the microorganisms used in the study. AI is particularly effective against *C. albicans* in root canal infections and had better antimicrobial action against E.faecalis than CHE and MC. CHE showed slightly better reduction of S.aureus colonies than AI and MC . In vivo data may be helpful in determining the real potential usefulness of theseherbal extracts for the treatment of root canal infections.Regarding the metabolic and physiological changes in the structure ofbiofilms over time, further studies with longer incubation times and longer contact time of irrigants with dentin and use of varying concentrations of herbal irrigants are needed in order to better simulate clinical conditions in root canal treatment.

References

[1]. Siqueira JF Jr., Rôças IN. Diversity of endodontic microbiota revisited. J Dent Res 2009; 88:969-81
[2]. Kandaswamy D, Venkateshbabu N, Gogulnath D, Kindo AJ. Dentinal tubule disinfection with 2% chlorhexidine gel, propolis, Morinda citrifolia juice, 2% povidone iodine, and calcium hydroxide. Int Endod J 2010;43:419-23.
[3]. Rocas IN, Siqueira JF Jr, Santos KR. Association of Enterococcus faecalis with different forms of periradicular diseases. J Endod. 2004;30(5):315–320.
[4]. Shingare P, Chaugule V. Comparative evaluation of antimicrobial activity of miswak, propolis, sodium hypochlorite and saline as root canal irrigants by microbial culturing and quantification in chronically exposed primary teeth. Germs 2011;1:12-21
[5]. Baumgartner JC, Watts CM, Xia T. Occurrence of Candida albicans in infections of endodontic origin. J Endod 2000;26:695-8.
[6]. Baillie GS, Douglas LJ. Role of dimorphism in the development of Candida albicans biofilms. J Med Microbiol 1999;48:671-9.

[7]. Waltimo TM, Haapasalo M, Zehnder M, Meyer J. Clinical aspects related to endodontic yeast infections. Endod Top 2004;9:66-78.
[8]. Ayhan H, Sultan N, Cirak M, Ruhi MZ, Bodur H. Antimicrobial effects of various endodontic irrigants on selected microorganisms. Int Endod J.1999;32(2):99–102.
[9]. Oncag O, Hosgor M, Hilmioglu S, Zekioglu O, Eronat C, Burhanoglu D. Comparison of antibacterial and toxic effects of various root canal irrigants. Int Endod J. 2003;36(6):423–432.
[10]. Spangberg L, Engström B, Langeland K. Biologic effects of dental materials. 3. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. Oral Surg Oral Med Oral Pathol 1973; 36:856-71.
[11]. Mohammadi Z. Sodium hypochlorite in endodontics: An update review. Int Dent J 2008; 58:329-41.
[12]. Sim TP, Knowles JC, NG YL, Shelton J, Gulabivala K. Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. Int Endod J 2001; 34:120-32.
[13]. Noopur Kaushik,Usha Rehani, Abhay Agarwal, Mayur Kaushik, and Vivek Adlakha. Antimicrobial Efficacy of Endodontic Irrigants against Enterococcus Faecalis and Escherichia Coli: An in vitro study. Int J Clin Pediatr Dent. 2013 Sep-Dec; 6(3): 178–182.
[14]. Rajesh Podar, Gaurav P Kulkarni, Shifali S Dadu, Shraddha Singh, Shishir H Singh. In vivo antimicrobial efficacy of 6% Morinda citrifolia, Azadirachta indica, and 3% sodium hypochlorite as root canal irrigants.European journal of dentistry 2015(4):529-534.
[15]. Noopur Kaushik, Usha Rehani, Abhay Agarwal, Mayur Kaushik, and Vivek Adlakha. Antimicrobial Efficacy of Endodontic Irrigants against Enterococcus Faecalis and Escherichia Coli: An in vitro study. International journal of clinical pediatric dentistry 20136(3):178-182.
[16]. Shashi Prabha Tyagi, Dakshita Joy Sinha, Paridhi Garg, Udai Pratap Singh, Chandrakar Chaman Mishra, and Rajni Nagpal.Comparison of antimicrobial efficacy of propolis, Morinda citrifolia, Azadirachta indica (Neem) and 5% sodium hypochlorite on Candida albicans biofilm formed on tooth substrate: An in-vitro study. JCD. 2013(16):532-535.
[17]. Paridhi Garg, Shashi Prabha Tyagi, Dakshita Joy Sinha, Udai Pratap Singh, Vibha Malik, Edgar Richard Maccune. Comparison of antimicrobial efficacy of propolis, Morinda citrifolia, Azadirachta indica, triphala, green tea polyphenols and 5.25% sodium hypochlorite against Enterococcus faecalis biofilm.Saudi endodontic J. 2014(4):122-125.
[18]. Comparative evaluation of antimicrobial activity of herbal extracts with 5.25% sodium hypochlorite against multispecies dental biofilm. Saudi endodontic J. 2016(6):71-76.
[19]. Manikandan Ravindranathan,Mithra N Hegde, Sucetha kumari. Antimicrobial assay of combination surfactant regimen on vancomycin -resistant Enterococcus faecalis:An invitro direct contact test. Dental Research J.2018 Nov-Dec:5(6):397-403.
[20]. E Kalyoncuoglu, E Sen Tunc, S Ozer, C Keskin, K. Bilgin,A Bbirinci. Evaluation of antifungal efficacy of QMix 2in1as a final irrigant: An in vitro study. Nigerian Journal of clinical practice. 2016:19(6):807.
[21]. Kishen A, George S, Kumar R. Enterococcus faecalis-mediated biomineralized biofilm formation on root canal dentine in vitro J. Biomed Mater Res A. 2006;77(2):406–415.
[22]. Xiao J, Koo H. Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by Streptococcus mutans in biofilms. J Appl Microbiol. 2010;108(6):2103–2113.
[23]. Koschek PR, Alviano, Gattass CR. The husk fiber of Cocos nucifera (Palmae) is a source of antineoplastic activity. Braz J Med Biol Res. 2007; 40:1339–43.
[24]. Jose M, Cyriac MB, Pai V, Varghese I, Shantaram M. Antimicrobial properties of Cocos nucifera (coconut) husk: An extrapolation to oral health.Journal of Natural Science, Biology, and Medicine, 01 Jul 2014, 5(2):359-364.
[25]. Jayaraman SK, Manoharan MS, Illanchezian S. Antibacterial, antifungal and tumour cell suppression potential of *Morinda citrifolia* fruit extracts. Int J Integr Biol. 2008;3:44–9.
[26]. Banerjee S, Johnson AD, Csiszar K, Wansley DL, McGeady P. An extract of *Morinda citrifolia* interferes with the serum-induced formation of *filamentous s*tructures in *Candida albicans and* inhibits germination of *Aspergillus nidulans*. Am J Chin Med. 2006;34:503–9.
[27]. Jainkittivong A, Butsarakamruha T, Langlais RP. Antifungal activity of Morinda citrifolia fruit extract against Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108:394–8.
[28]. Polaquini SR, Svidzinski TI, Kemmelmeier C, Gasparetto A. Effect of aqueous extract from neem (Azadirachta indica A. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans. Arch Oral Biol. 2006;51:482–90.
[29]. Bohora A, Hedge V, Kokate S. Comparison of the antibacterial efficiency of neem leaf extract and 2% sodium hypochlorite against E. faecalis, C. albicans and mixed culture — An in vitro study. Endodontology. 2010;22(1):8–12.
[30]. Nayak A, Nayak RN, Soumya GB, Bhat K, Kudalkar M. Evaluation of antibacterial and anticandidal efficacy of aqueous and alcoholic extract of neem (Azadirachta indica): An in vitro study. IJRAP. 2011;2:230–5.

Dr. Parvathy V, et. al. "Evaluation of antimicrobial efficacy of Cocos nucifera husk extract, Azadirachta indica extract and Morinda citrifolia extract against Enterococcus faecalis, Staphylococcus aureus and Candida albicans: An invitro study." *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 19(10), 2020, pp. 45-56.