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Impact of Clove and Eucalyptus Essential Oils versus Fluoride on Resin Hardness and Selected Oral Microorganisms

Amal Abdul Latif Rashid[1], Jinan Mohammed Rashad Shihab[2], Fatima Kadhim Ghadeer[1]
[1]*College of Health and Medical Techniques, Middle Technical University, Baghdad, Iraq*
[2]*Technical Medical Institute, Middle Technical University, Baghdad, Iraq*

Abstract

The objectives of this study were to assess the effect of syzygium aromaticum (clove) and Eucalyptus essential oils on hardness of acrylic resin and selected oral pathogens (*Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans*) in comparison to calcium fluoride. 120 samples were constructed from heat cured material then divided into 2 groups, for microbial test and for hardness test, then these samples subdivided according to additive incorporation: control without additive, 2.5% eucalyptus oil, 2.5% syzygium aromaticum oil and calcium fluoride (CaF_2) with concentration (0.5%, 1.5% and 2.5%). Surface hardness test (measured via shore D tester), Agar well technique for assessing sensitivity test and ELISA reader to confirm adherence of *Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans*. Hardness mean value were increased significantly in syzygium aromaticum and eucalyptus oil groups in comparison to control group, all CaF_2 groups showed no significant differences with control. All tested groups revealed weak adherence *against Streptococcus mutans, Staphylococcus aureus* and *Candida albicans*. High inhibition zone value recorded by eucalyptus oil. While syzygium aromaticum oil and CaF_2 groups recorded no inhibition zone. Incorporation of (2.5%) for syzygium aromaticum and eucalyptus oil extracts were significantly improved hardness of heat cured resin material, while 0.5%, 1.5% and 2.5%CaF_2 showed no significant effect on hardness, both oil extracts and CaF_2 demonstrated antimicrobial and revealed weak adherence effect against *Streptococcus mutans, Staphylococcus aureus* and *Candida albicans*.

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Email: amal_dentist58@mtu.edu.iq

Introduction

There is interest in the use of plant derived oils as natural therapeutic active agents with antimicrobial properties. Oils that exhibit dual antibacterial and antifungal activity are particularly promising for oral applications, especially among acrylic wearers as space maintainer appliance. Many individuals especially pediatric immune comprised and malnutrition using acrylic appliance had experience of oral infections caused by bacteria together with fungi, necessitating agents that can effectively target both types of pathogens [1,2]. Some plant oil serves as a potential dual-action agent against oral bacteria and fungi at the same time, demonstrating the ability to inhibit microbial growth associated with acrylic appliance related to fungal infection [3,4]. This highlights its potential as a safe, natural, and more effective option for both preventive and therapeutic use.

The popularity of PMMA is based on its many advantages, that include lighter weight, biocompatible, inexpensive, and better esthetics. However, it had poor mechanical properties such as hardness , which are considered the main cause of prosthesis fractures [5], many additive had add to advance hardness and prevent microbial adherence. Research has been devoted to the development of a new process that incorporates various compounds into (PMMA) providing a new of (PMMA) that offers the strength of this compound, with the flexibility of polymer matrix [6].

Many studies focused the attention on the cultivation and production of medicinal plants because of their particular in nature.

Eucalyptus oil inevitable the presence of special mechanisms for defense and antimicrobial agents in the androgen's forms, they suppose as a potential role of antimicrobial components. The active ingredient of this plant was essentially: alkaloids, phenolic, starches, flavonoids, steroids essential oils, terpenes, and pigments [7]. Also exhibited its antimicrobial effects against fungal, bacterial, viral agents and parasitic [8].

Syzygium aromaticum was one of the more valuable spices that had been used for centuries for preservative of food also using in many medicinal purposes. The antimicrobial activities of it have been proved against several bacterial together with fungal strains [9].

Calcium fluoride occurs naturally as a mineral. Calcium fluoride dissociates into calcium cations and fluorine anions, its efficacy in prevention the dental caries [10]. Realizing fluoride's capable for preventing dental caries by inhibiting and interacting with *S. mutans* in adverse ecological action conditions this species was considered as major cause of dental caries [11]. *Staphylococcus aureus* had natural habitat both in humans' skin also in nasopharynx, could cause wide variety of infections involving soft tissues and skin infective such as angular cheilitis these bacteria colonize the implanted device causing disseminate or local damage [12]. *Candida albicans* is the most prevalent trusted source cause of candida infections such as oral stomatitis [13].

Surface hardness of material was the most important properties to assume the dense material that more resistance to wear and degradation of the surface [14].

The addition of eucalyptus oil to acrylic resin increased its hardness [15]. Syzygium aromaticum had antibacterial and antifungal properties, so it is used to disinfect polymethylmethacrylate by immersion and did not alter their properties [16].

The real-world effectiveness of fluoride addition to dental appliance among patients wearing fluoride incorporated appliance showed a reduction in caries on adjacent natural teeth, and decrease the incidence of fungal infection. however, the incorporation of fluoride did not significantly the aesthetic and comfort that lead to patient acceptance [17]. Calcium fluoride might be a better option for maintaining a smoother surface while still providing a sufficient release of fluoride ions and it most efficacy than sodium fluoride in sustained fluoride release [18,19].

Since oil plants extract(syzygium aromaticum and Eucalyptus oil) and calcium fluoride was used as antibacterial agent , there was a lack in knowledge concerning the incorporation of these oils and calcium fluoride into hardness of acrylic resin material utilized as space maintainer and their antimicrobial effect the null hypothesis of this study was the addition of these additive will improve the hardness and antimicrobial properties of resin material, for that the aim of study particularly to assess the antimicrobial effect and surface hardness of resin material utilized for construction of space maintainer incorporating with these oils and different concentration of calcium fluoride.

Material and Methods

Preparation of Samples

Samples for hardness test were designing according to ADA specification as rectangular shaped according to (ADA No.12, 1999) [20], dimensions were (65x10x2.5 mm) length, width and thickness accordingly (Figure 1A). Metal patterns were constructed by CNC to prepare samples from acrylic material, for microbiological test disc shapes of modeling wax were prepared to test the inhibition zone and adherence ability with the diameter of 6mm and thickness 1 mm (Figure 1B) [21-23].

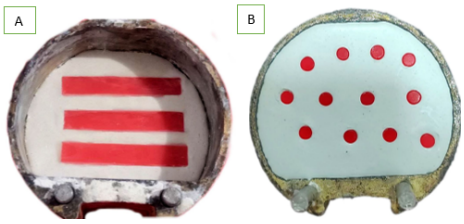

Figure 1. A - samples for hardness test; B - samples for microbiological test.

Groups

120 samples were constructed for this study for control group prepared samples without adding any additive (0%),while for the experiment samples they divided into five groups according to additive 2.5% eucalyptus oil, 2.5% syzygium oil from (Evans Medical Limited), and 0.5%, 1.5%, 2.5% calcium fluoride (Thomas Baker, India), for each group 10 samples were prepared.

Addition of Syzygium Aromaticum, Eucalyptus Oils and Calcium Fluoride into Resin

Powder and liquid of Veracril (Colombia) heat cured acrylic resin were used according to manufacturer's instruction for control group mix 15 g of powder with 10 ml of liquid and for experimental specimens used 2.5% percentage of oils of syzygium aromaticum and Eucalyptus and the calcium fluoride powder at percentages of 0.5%, 1.5%, and 2.5%. These additives oils and calcium fluoride were added to liquid of acrylic, and these percentages decreasing from the volume of the powder (polymer) of acrylic to result powder liquid accurately ratio for mixing.

Calcium fluoride (all concentrations) and the two oils were mixed 20 seconds with monomer of acrylic resin [24] via using sonication apparatus probe (60 KHz and 120 W) to become homogenic completely, then powder (polymer) were added, and the mixture was mixed in a container and stay until reached the dough stage.

Preparation of Samples

The standard flasking, packing and curing technique for molds was done procedure, finishing and polishing were done according to standardized methods [24,25].

Shore D Hardness Test

Measurement of surface hardness**:** the accuracy of Shore D hardness tester was equal to 0-100 HD (China), used to test the surface hardness of the acrylic cure samples according to ISO7619 (ASTMD 2240). Device was vertically placed over the sample which was encouragement on horizontal, rigid base. The distance from the hardness tester indenter and the specimen surface was about (5 to 12mm). The contact period from the specimen surface to the indenter was six seconds. For each specimen, approximately five hardness measurements were obtained from the scale reading value, the mean of these values was computed [26].

Identification of Oral Microbial Species

Brain Heart Infusion medium was used to culture *Candida albicans* on for 48 hours, 37°C in an incubator, germ tube formation and examination by microscopic, furthermore API Candida systems were done to identify the *Candida albicans* [27].

Streptococcus mutans was performed according to their morphological characteristics on Mitis Salivarius Bacitracin Agar medium. They were Gram positive and able to ferment sorbitol and mannitol in concentration of 1 % in Brain Heart Infusion broth [28].

Staphylococcus aureus diagnosed by Gram's stain also according to cell morphology, motility, oxidase and catalase tests, the diagnosis was confirmed by a Vitek-2 compact system [29].

Sensitivities of Oral Species to Two Oil Extracts and Different Concentrations of Calcium Fluoride

25 ml of (MHA) Muller-Hinton Agar media, pH 7.0, poured into sterile Petri dish (8cm diameter), this for bacterial species but for C. albicans species filled with SDA Sabaurad dextrose agar media then left at room temperature for 24 hours.

0.1 ml of microbial species inoculum spread to each plate, left at 25°C (20 minutes), then wells of equal size and depth were prepared in the agar by 4-5 wells in each plate, then the well was filled with (0.1 ml) of selected agents.

Plates were left for 1 hour. at room temperature then incubation at 37^{0}C for 48 hours for *Candida albicans* and for bacteria species 1 hour at room temperature, then incubated anaerobically for 24 hours at 37°C [30].

After that the inhibition zone were calculated in millimeter via using measuring ruler [31]. The experiments were accomplished 5 replicates and the average were reported. The zone of inhibition was across the diameter of each well. Complete resistance of bacteria to the tested agent was indicated when there was no zone of inhibition diameter of well=6mm.

Biofilm Formation Test

Biofilm formation test done via assay of crystal violet stain. Colonies cultures in Trypticase Soy Broth diluted in TSB with 2% w/v glucose. polystyrene microplate (Italy) of 96 well, full with ten hundred litter of dilute culture. After that incubation was done at 37◦C for 24 hours, content of wells poured down and turning over and shaking out liquid, then well had wash 3 times by Phosphate Buffered Saline ensuring not adhere any cells, wells washed and dried via air ,the levels of biomass of biofilm on the well surfaces determine with staining assay by (CV) Crystal Violet. The cell of bacteria in biofilm settled firstly by ethanol stain after that for 15 minutes with crystal violet 1% (Merck), well washed 3 times by sterile distal water, then dried by air. CV bound redaction was done by adding ethanol (95%) and the CV level in the ethanol measured using means of the Optical Density (OD), the wavelength 360 nm by spectrophotometer (USA), biofilm assay in triplicate for each isolate [32].

Results

S.E, minimum and maximum values of surface hardness test for all groups. The eucalyptus showed the high mean value for hardness test while the PMMA 2.5% calcium fluoride showed the minimum mean value for hardness test. Both groups of syzygium aromaticum and eucalyptus oil showed the hardness test value more than control group, while all groups with adding calcium fluoride showed lower value than control group. ANOVA test revealed the significant differences among groups ($P \leq 0.0001$).

Table 2 shows post hoc analysis for action of two oils and 0.5%, 1.5% and 2.5%calcium fluoride on hardness test of resin materials, the differences were significant between all groups except between control group with calcium fluoride concentrations and between calcium fluoride groups with each other.

Adherence Test

Figure 2 shows the adherence value at O.D630 detected by ELISA reader for both types of oils eucalyptus and syzygium aromaticum oil have anti biofilm activity against *Streptococcus mutans* and *Candida albicans* and prevent its adherence on acrylic sample, for *Staphylococcus aureus* had low effect on adherence, all the isolate revealed no adherence or weak adherence except *Staphylococcus aureus* with both type of oil showed adherence activity to acrylic material.

Figure 3 shows the adherence value at O.D630 detected by ELISA reader for all calcium fluoride concentrations (0.5%, 1.5%, and 2.5%) revealed no adherence or weak adherence of all isolated oral species (*Candida albicans, Staphylococcus aureus and Streptococcus mutans*).

Sensitivity Test

The maximum inhibition zone recorded by eucalyptus on the *Staphylococcus aureus* then by *Streptococcus mutans* and *Candida albicans*, while syzygium aromaticum oil recorded no inhibition zone.

Also, different concentrations of calcium fluoride recorded no inhibition zone on *Staphylococcus aureus, Streptococcus mutans* and *Candida albicans*, as seen in Figure 4.

Discussion

Microbial reservoir of acrylic resin material (utilized for construction of dentures and space maintainer) still the obstacle of this material. Many attempts were done to exhibit acrylic materials antifungal and antibacterial effect, also to advance their hardness property, such addition of plant extracts and fluoride salt to solve this problem.

The study suggested that the structure and solubility of calcium fluoride allowed for a more gradual and long-lasting release of fluoride ions, which could provide prolonged protective effects for space maintainer wearers [33].

Acrylic materials are widely used in many applications in the field of dentistry such as used for manufacture of space maintainer and dentures due to their suitable chemicals, mechanical and physiological properties [34]. The current study aimed to assess the effect of using eucalyptus and syzygium aromaticum oils and calcium fluoride on the oral microbial activity and hardness of acrylic prosthesis.

Acrylic resins material using for oral prosthesis may act as reservoirs for pathogens and had the potential to support biofilm formation [35]. Microbial growth on the prosthesis surface results from the adherence (attachment) of microbial biofilm cell and from adhesive interactions between Candida species and oral bacteria. Several studies have demonstrated an association between *Candida albicans* or other species of Candida, and some oral bacteria such as *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus mutans*, *Actinomyces viscosus* and *Fusobacterium nucleatum* [35].

Surface hardness asses density of material, more dense material more resistance to wear less adhesion to oral bacteria. The most standard and popular methods to test surface hardness was Shore D, all concentration of CaF_2 had no significant differences with control(no effect the hardness of acrylic material) this might due to that calcium fluoride particles were poorly soluble so remained dispersed physically rather than chemically integrated within polymer network ,so CaF_2 act as inert material rather than reinforcing agents, leading to no improvement in hardness and not contribute in increasing the hardness of the material [36].

Syzygium aromaticum and eucalyptus extracts likely act as natural reinforcing agents that improve the structural integrity and hardness of the acrylic material might be due to possible chemical interactions with the polymer matrix because of certain bioactive compounds in syzygium aromaticum and Eucalyptus (such as polyphenols, tannins, and essential oils) may interact with the acrylic polymer chains, leading to increased cross-linking, which enhances surface hardness [37].

syzygium aromaticum and calcium fluoride (0.5, 1.5 and 2.5) % showed no inhibition for oral species (*staph. Aureus, strep. Mutans* and *candida albicans)* this explained that calcium fluoride density was high and its molecular weight also moderately high, so these properties especially the more density effect the spreading of calcium fluoride into agar and inhibition the bacteria and fungi growth [10], syzygium aromaticum had antibacterial and antifungal properties duo to its eugenol compound also used to disinfect PMMA agree with Al-Khafaji and Mahmood [38].

In the standardized sensitivity tests (disk diffusion method), eugenol might not diffuse fully through the agar medium, result in no visible inhibition or tiny inhibition zone, even the syzygium aromaticum was active. In nature, syzygium might work synergistically with other conditions not replicated in the lab, this could enhance its activity in vivo but not in vitro [39].

Eucalyptus oil had shown to produce a larger inhibition zone in disk diffusion test in comparison to other essential oils contain eugenol, this might due to its high diffuse ability and the presence of 1,8-cineole, a compound with strong antimicrobial activity, relatively small and diffuses well through agar media. Eucalyptus oil contains bioactive compounds that exhibit antimicrobial effects against a wide range of bacteria and fungi. These properties enhance the penetration into microbial cells and increase the effectiveness in disk diffusion tests [40].

No adherence for all the specimens except staph. Aureus with eucalyptus and syzygium aromaticum due to its ability to develop antibiotic resistance and form robust biofilms [41].

Calcium fluoride recorded no adherence for all species consequently fluoride was widely used to control dental caries also to promote remineralization [42] because of fluoride characteristics like enzyme inhibition, it can inhibit bacterial enzymes such as enolase by binding to its active site, thereby disrupting energy production [43].

Conclusion

Incorporation of (2.5%) for syzygium aromaticum and eucalyptus oil extracts were significantly improved hardness of heat cured resin material, while 0.5%, 1.5% and 2.5%CaF_2 showed no significant effect on hardness, both oil extracts and CaF_2 demonstrated antimicrobial and revealed weak adherence effect against *Streptococcus Mutans, Staphylococcus Aureus* and *Candida albicans*.

Conflict of Interest

The authors declared no conflicts of interest.

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Table 1. Descriptive data for surface hardness test of groups.

Groups	N	Mean	Std.D	Std. E	Min	Max
Control	10	73.15	1.95	0.61	71.3	75
2.5% Syzygium	10	76.98	1.2	0.37	75.6	78.8
2.5% Eucalyptus	10	80.65	4.02	1.27	74.5	84.6
0.5% CaF_2	10	72.45	1.36	0.51	71	74.5
1.5% CaF_2	10	72.72	0.39	.14	72.24	73.28
2.5% CaF_2	10	71.23	0.18	0.07	71	71.5

Table 2. Multiple comparisons analysis of the study sample for the surface hardness sample.

Group	Group	Mean Difference	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	2.5% Syzygium	-3.83	0.95518	0.000	-5.7538	-1.9062
	2.5% Eucalyptus	-7.5	0.95518	0.000	-9.4238	-5.5762
	0.5 % CaF_2	-0.69762	1.05255	0.511	-2.8176	1.4223
	1.5 % CaF_2	-0.42177	1.05255	0.691	-2.5417	1.6982
	2.5 % CaF_2	1.21429	1.14166	0.293	-1.0851	3.5137
Syzygium	2.5%Eucalyptus	-3.67000	0.95518	0.000	-5.5938	-1.7462
	0.5% CaF2	4.52762	1.05255	0.000	-6.6476	-2.4077
	1.5% CaF2	-4.25177	1.05255	0.000	-6.3717	-2.1318
	2.5% CaF2	-5.74190	1.05255	0.000	-7.8619	-3.6220
Eucalyptus	0.5 % CaF2	-8.19762	1.05255	0.000	-10.3176	-6.0777
	1.5 % CaF2	-7.92177'	1.05255	0.000	-10.0417	-5.8018
	2.5 % CaF2	-9.41190'	1.05255	0.000	-11.5319	-7.2920
0.5% CaF2	1.5% CaF2	-.27585	1.14166	0.81	-2.5753	2.0236
	2.5% CaF2	1.21429	1.14166	0.293	-1.0851	3.5137
1.5% CaF2	2.5% CaF2	1.49014	1.14166	0.198	-0.8093	3.7895

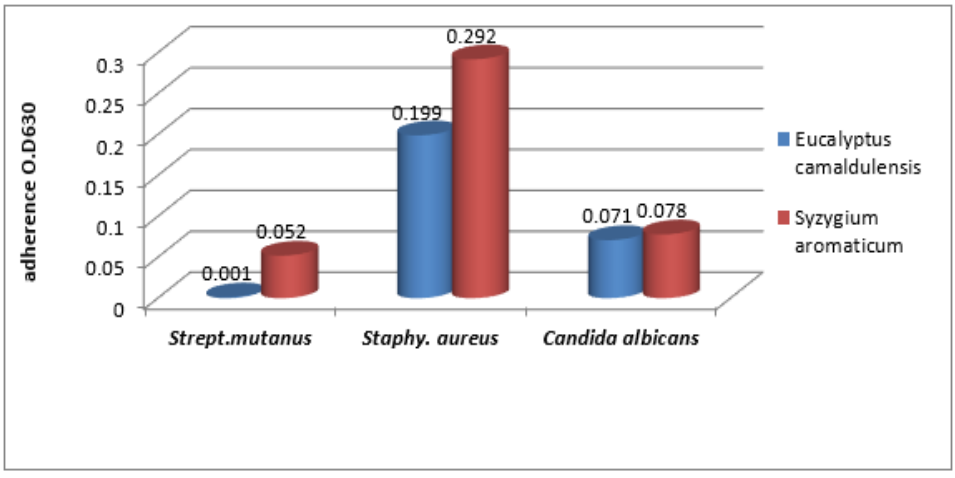

Figure 2. Adherence value at O.D630 detected by ELISA reader for eucalyptus oil and syzygium aromaticum oil.

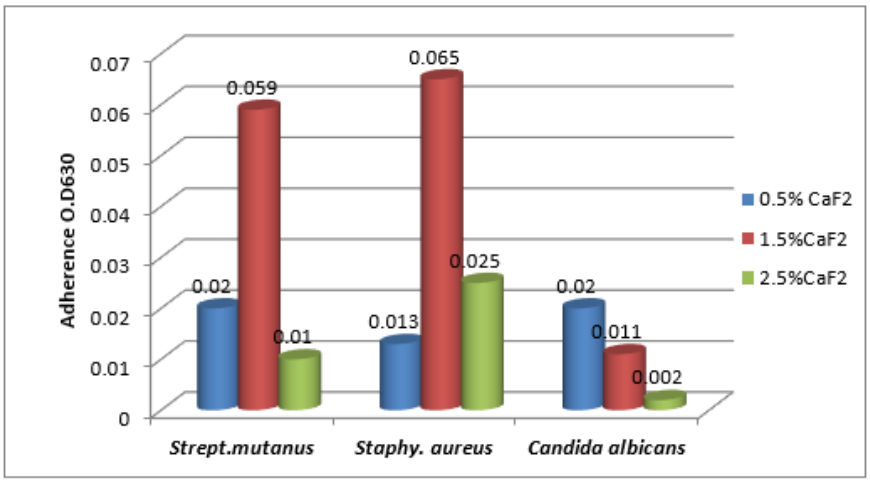

Figure 3. Adherence value at O.D630 detected by ELISA reader for CaF_2 groups.

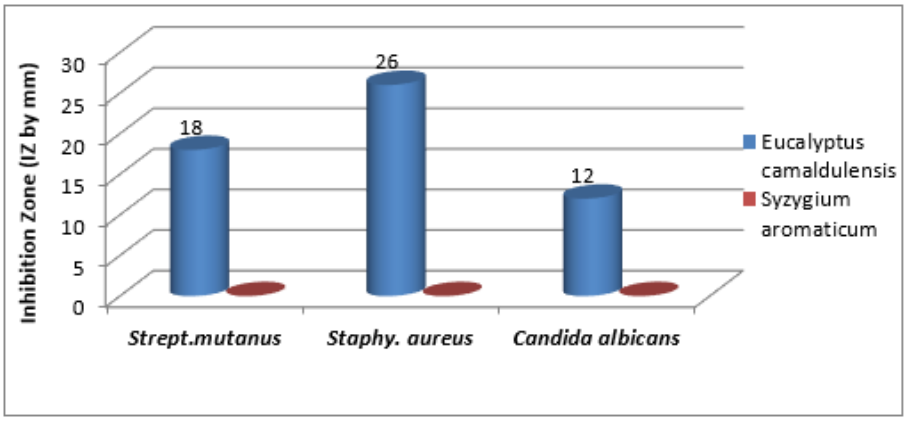

Figure 4. Inhibition zone with *Staphylococcus aureus*, *Streptococcus mutans* and *Candida albicans* by eucalyptus oil and syzygium aromaticum oil.