Evaluation of Addition of Plant Fixed Oil Extracts (Flax, Rosemary) as Antifungal on Color Stability of a Heat Cured Soft Lining Material

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ABSTRACT

Introduction: Loss of softness, increase hardness and colonization of acrylic soft liner denture base material by C. albicans are significantly inevitable clinical problems, therefore enhancement of softness and giving antifungal activity to soft liner material to lengthen the life span of it are recommended.

Aims: This study were conducted to evaluate the addition of plant fixed oils extract (flax and /or Rosemary) as antifungal and their effect on color stability of heat cured acrylic soft liner material cured by two different cycles.

Materials and Methods: Total number of (112) samples have been prepared in this study. Samples were divided into two main groups, according to the method of curing into short and long cycles. Each main group has been sub divided into four sub-groups, according to the type of additive materials { Flax, Rosemary, mixed (Flax and Rosemary), and control group}.

Color stability and antifungal tests were applied on each of two main groups (short and long cycle). The color stability test was done at two different periods of immersion in distilled water (two and thirty days); except for antifungal tests were done after (two, seven and thirty) days of immersion in distilled water.

Results: The results showed that acceptable levels of color change (ΔE) in vitro were obtained after oils addition to soft liner (vertex). The soft liner with oil showed antifungal activity to some extent.

Conclusions: Conclusions revealed that the addition of Flax oil and Rosemary oil showed antifungal activity to some extent, with no effect on the acceptable range of the physical properties (color).

Introduction:

Denture soft lining materials are usually used in prosthodontics to provide a cushioning layer on the fitting surface of a complete denture. The material absorbs some of the masticatory energy and reduces the energy transmitted to the underlying tissues [1].

One of the more serious problems is the colonization of soft liner surface by C. albicans [2].The adherence of C. albicans to polymers such as denture acrylic resins or soft lining materials is the first step in colonization, yielding to the development of pathogenesis and eventually causing infection [3].

Acrylic resin liners are frequently affected by water sorption and a loss of chemical components, which can alter their stiffness [4]. The success of the soft lining materials depends partly on their color stability, and softness upon time [5].

A resilient liner with antifungal activity can be of great advantage for patients with a high risk of denture stomatitis [6]. Candida can readily colonize and actually invade soft denture liner. Particularly, in the case for patients whose denture cleansing regimens are poor. In such instances antifungal agents may be considered as an adjunct in the management strategy, therefore, there have been attempts to incorporate antifungal agents directly into the soft liner material [7].

Plants extracts have traditionally been used in folk medicine, as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts [8]. Oils are concentrated, hydrophobic liquid containing volatile aromatic compounds from plants. They possess a wide spectrum of pharmacological activities. The antimicrobial effects of oils have been documented and used in herbal medicine in many countries [9].

MATERIALS AND METHODS

Short and long Cycles Groups:

Fifty six samples cured by short curing cycle according to (ADA Specification No. 12, 2009)[10], samples cured for 90 minutes in 70°C followed by 30 minutes in 100°C, and fifty six samples cured by long curing cycle (9 hours in 73°C) using thermostatically controlled curing unit. The color test was done after two days and thirty days intervals and for antifungal tests that were done at 2, 7, 30, day's intervals respectively. These groups were sub divided into four sub-groups according to the type of additive materials. The sub groups as follows:

a.Flax oil as additive group (FS), and (FL).
b.Rosemary oil as additive group (RS), and (RL).
c.Equal mixture of Flax and Rosemary oils as additive group (FRS), and (FRL)..
d.Control without additives group (CS), and (CL).

Preparation of the samples:

Dental flasks with dental stone (Elite, Zhermack SPA, Bovazecchino, Italy) as investment material was used with the hard plastic foils (Imprelon, Scheu Dental- GmbH) of different thicknesses and shapes

(ADA Specification No. 12, 2009) using computerized numerical control machine to produce molds of production of different samples used in the study. For color stability a model of 30 mm length, 15mm width and 3mm thickness, while for antifungal tests, a disc prepared according to International Standards Organization [11], 5 mm in diameter were aseptically fabricated. The surface area of each specimen was 55 mm2 [12,13].

Denture soft lining material (Vertex) samples have been prepared according to manufacturer instruction with powder: liquid ratio of 3gm: 2.5ml. For samples with oils addition, the plant oil extracts (Flax and Rosemary) were mixed with monomer (14) at concentration of 5% per volume by an adjustable micropipette (DragonLab, China) with a ratio of 125µL for each 2.5ml monomer.

After complete curing, the samples were removed from their moulds, and stored in containers with non-ionized distilled water for each specific test at 37°C temperature for two days, and thirty days immersion time until tests were performed.

Color Stability:

Sample's shape and size was described previously, at two time intervals; two days and 30 day after curing samples and immersion in non ionized distilled water, sample of all groups were assessed for its color stability. A digital image scanner (Epson, China) was used for scanning and obtaining samples images, each sample was scanned at 600dpi, and the resultant jpg image used for color assessment by image processing tool of Matlab 2010 software to obtain L*a*b values for each sample image.

A special syntax script was programmed and added to image processing tool in order to export the L*a*b* values for all pixel of the image, and then the average L*a*b* values were recorded automatically into Microsoft Excel file. The L*a*b* values for each sample were calculated two times after two days (L1*a1*b*1), and after thirty days (L2*a2*b*2). The (CIE L*a*b*) color difference metrics were used for the color stability analysis of the samples in this study. The total color change (ΔE) of each sample was calculated. For each sample for color evaluation using the equation below:

$$\Delta E = [(L^*2-L^*1)2 + (a^*2-a^*1)2 + (b^*2-b^*1)2]1/2$$ [15].

If (ΔE=0) no color difference was detected after exposing the sample to the testing environment. A (ΔE=3.7) or less was considered to be clinically acceptable in vitro, while (ΔE=6.8) was considered to be clinically acceptable in vivo study [16, 17].

Antifungal Tests:

To evaluate antifungal activity of extracted oils (Flax and Rosemary), and antifungal potentials of modified denture soft lining materials after addition of oils, two different tests were used. *C. albicans*, the target fungus, was isolated and incubated for testing purposes.

***C. albicans* Isolation and Identification:**

C. albicans was obtained by taking swabs from ten volunteers wearing old dentures attending Department of Prosthodonotics at College of Dentistry / University of Mosul. The collected swabs cultured on Sabouraud's dextrose agar and incubated at 37°C for 24 hours.

To identify C. albicans after incubation and to select the pathogenic strain according to prototype ATTC strain of *C. albicans* (No. 10231, American Type Tissue Culture) from the collected ten swabs, the following tests were done:

1. Culture morphological features assessment for *C. albicans* colonies, it should be creamy to white, flat or domed, and have a dry glistering or waxy surface [18].
2. *C. albicans* takes gram positive stain. It appears under light microscope as spherical to oval budding cells (3-6 nm) in the yeast or the blastospore form [19].
3. [18,20]showed the ability of pathogenic *C. albicans* to form germ tube, by incubating a loop full Candida in 0.5 ml serum for 3 hours at 37°C. A wet film reveals the presence of filamentous out growth, germ tube from which *C. albicans* can be readily differentiated from other species.
4. The isolated fungus was also identified by *C. albicans* API kit which is a standardized system for Candida species identification to ensure that the isolated fungi met the ATTC 10231 strain for C. albicans (American Type Tissue Culture No. 10231,).

Disc Diffusion Susceptibility Test of C. albicans for Different Antifungal Agents and Oil Extracts:

Candida albicans susceptibility to different antifungal agents compared with oil extracts (Flax and/or Rosemary) was investigated using disc diffusion method. A sterile filter paper discs Wattman No.1 was prepared and immersed in 15:l of two antifungal solutions (Nystatin 100000 IU and Fluconazole 3mg/ml). Another set (two set for Flax and Rosemary) of filter paper discs were prepared and immersed into 15:l of Flax oil and Rosemary oil respectively [21].

A loopfull from fresh *C. albicans* culture was taken and inoculated in 5 ml of nutrient broth and incubated for 24 hours at 37°C, a swab from the broth was cultured on Mulluer Hinton agar, one disk of each antifungal solutions and two oil extracts (Flax and/or Rosemary) discs (one disc for each) were placed on the surface of the culture as shown in figure (1), and then the culture was incubated for 24 hours at 37°C, Five duplicates were done for each disc. The inhibition zones were measured using ruler, the average values then calculated [22, 23].

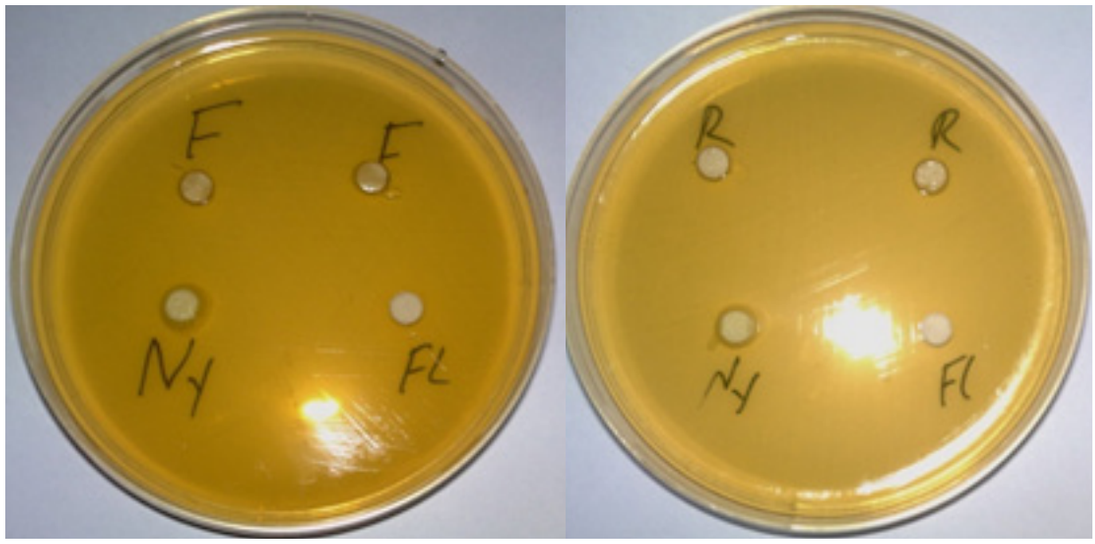

Figure (1). Disc diffusion Susceptibility tests of Candida albicans, A: Flax oils and control. B: Rosemary oil and control.

Modified Denture Soft Lining Material Antifungal Activity Assessment by Standard Colony Counting Test:

Samples from each sub-group for both short and long curing cycles were investigated for its antifungal potentials using standard colony counting test. Modified denture soft lining material samples with additives and control without additives were prepared according to (ISO, 1992) standards with total surface area of 55mm2, as it described previously. This test was repeated over three periods: two, seven, and thirty days after curing. *C. albicans* broth culture was prepared by taking a loop-full from fresh *C. albicans* culture then inoculated into 5 ml of nutrient broth and incubated for 24 hrs at 37°C, then compare with tube of McFarland No. 3.

Each of described disc specimens were immersed in a test tube containing 4.5 ml of a sterilized nutrient broth then it inoculated with 0.5 ml of *C. albicans* broth culture of McFarland No. 3 (as mentioned above), another 24 hours incubation was done at 37°C for the test tubes containing disc specimens. After incubation, the broth was removed with a sterile pipette. The specimen discs were rinsed 5 times with sterile distilled water to remove the loosely adherent *C. albicans*. Then the discs were carefully placed in sterile test tubes that contained sterile saline and placed over digital electric shaker (DragonLab, China) for 60 minutes. Dilution by taking 0.5ml from the supernatant added to 4.5ml of nutrient broth, and then 100µL of each diluted supernatant was placed by using glass spreader on Petri dish plates that contained Sabouraud's dextrose agar. The plates were returned to the incubator at 37°C for 24 hours. Colony formation was then counted after incubation [12, 13]. This method is accepted by ATTC 10231 (American Type Tissue Culture) and it was implemented by many authors: [24, 21, 23,13, 25].

Statistical Analysis:

Statistical analysis was made using SPSS 19 computer software; One way analysis of variance ANOVA followed by Duncan's multiple range tests were used to compare between groups.

Results and discussion:

Color Stability of Soft Lining Material:

L*A*B* Means and Standard Deviation:

The color stability of four different groups of soft liner cured by short cycle and long cycle after storing in distilled water at different periods of time (two and thirty days) was evaluated. Means and standard deviation of (L*A*B*) values for the four groups of short cycle at two and thirty days periods were shown in figure (2). While for long cycle at two and thirty days periods were shown in figure (3).

Measuring Color Changes According to the (CIE L*A*B*) Color system:

To measure color changes of denture soft lining ma-

Iraqi Dental Journal Volume:34 Issue:2; 2012

terials, color change (ΔE) comparison between short cycle sub-groups (control group with other sub-groups). All sub-groups of short cycle group showed an accepted (ΔE) value in vitro after modification of denture soft lining materials, as shown in table (1) for two days and thirty days period

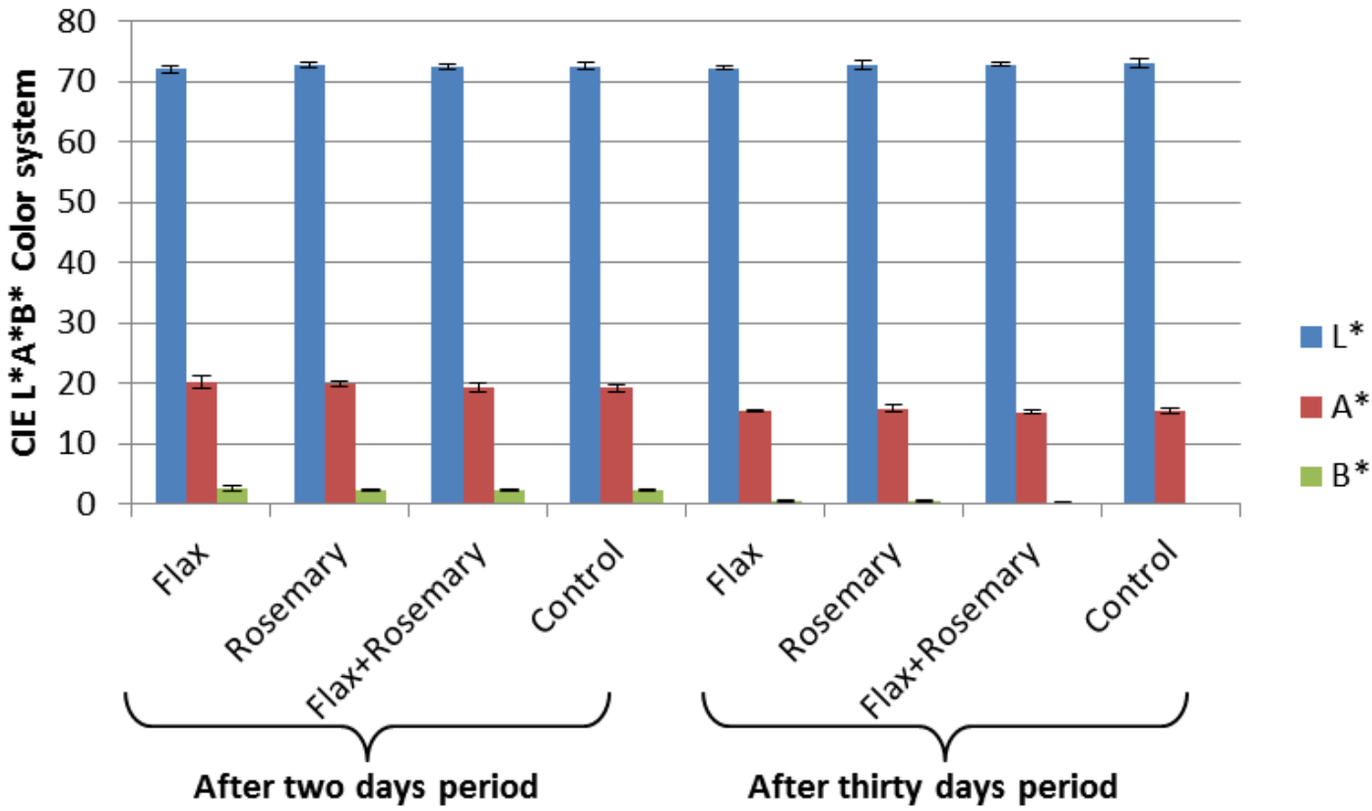

Figure (2). Mean and ± standard deviation for (L*A*B*) values for short cycle groups at two and at thirty days periods.

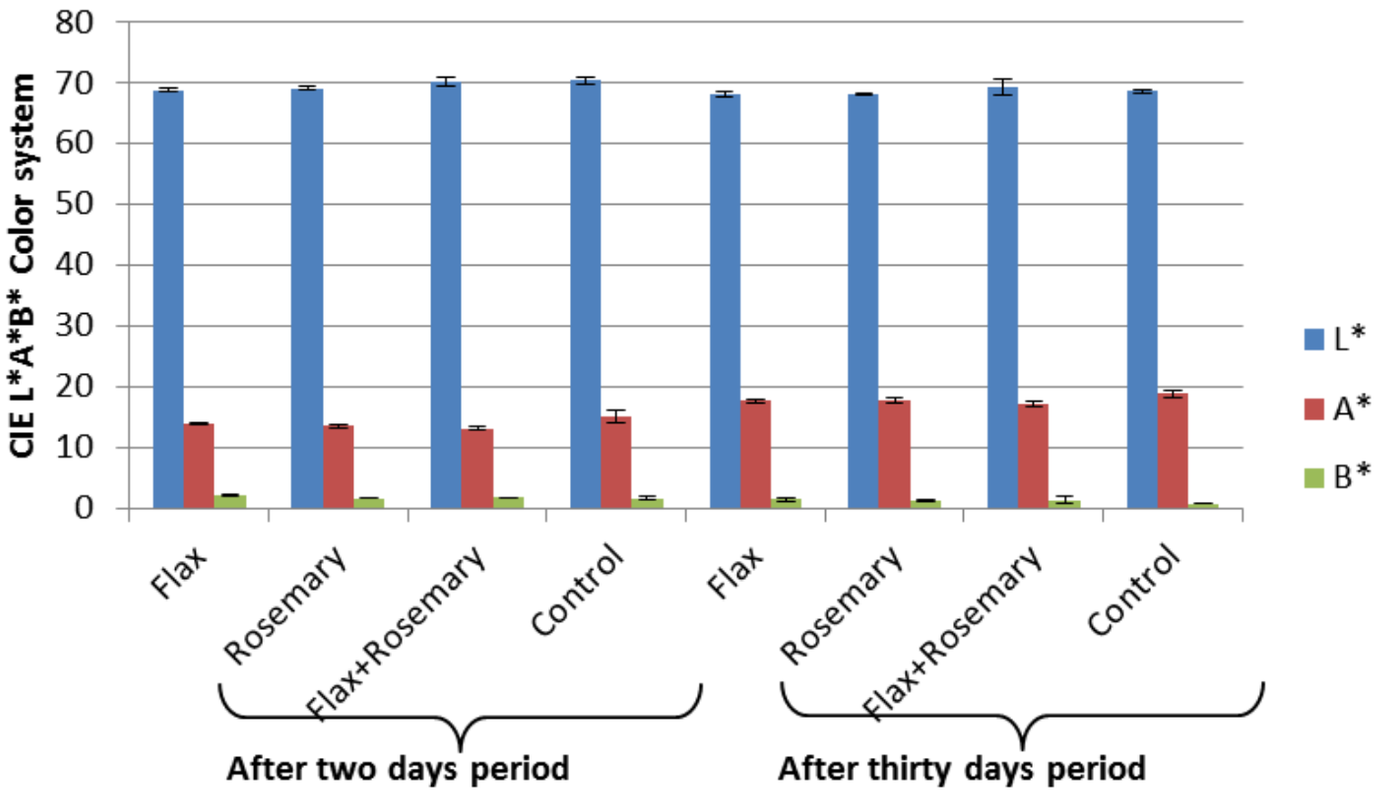

Figure (3). Mean and ± standard deviation for (L*A*B*) values for long cycle groups at two and at thirty days periods.

For long cycle groups, a comparison between its sub-groups (control group with other sub-groups) was made. All sub-groups of long cycle group showed an accepted (ΔE) value in vitro after modification of denture soft lining materials, as shown in table (1) for two days and thirty days period.

Table (1). Color changes in (CIE L*A*B*) color system between short cycle and long cycle groups at two, and thirty days period.

Sub-group	ΔE	In vitro	ΔE	In vitro
	Short cycle(Two days)		Short cycle(30 days)	
FS vs CS	1.120577	Acceptable	0.840081	Acceptable
RS vs CS	0.678493	Acceptable	0.553109	Acceptable
FRS vs CS	0.152886	Acceptable	0.2986	Acceptable
	Long cycle(Two days)		Long cycle(30 days)	
FL vs CL	1.899686	Acceptable	1.440598	Acceptable
RL vs CL	1.942214	Acceptable	1.362461	Acceptable
FRL vs CL	1.869191	Acceptable	2.014417	Acceptable

(ΔE) = 0 no color change; (ΔE) ≤ 3.7 accepted in vitro color change.

To find color changes after immersion of samples for thirty days another comparison were done between same sub-group at two days period and thirty days periods. All sub-groups for both short and long cycle groups were shown an in vitro not acceptable color changes (even for control group); while it showed an acceptable color changes in vivo. Short cycle and long cycle color changes described in table (2).

Table (2). Color changes in (CIE L*A*B*) color system for short and long cycle groups comparing between two days and thirty days periods.

Sub-group	ΔE	In vitro	In vivo	Sub-group	ΔE	In vitro	In vivo
	Short cycle				Long cycle		
FS vs FS	5.177563	Not acceptable	Acceptable	FL vs FL	3.873428	Not acceptable	Acceptable
RS vs RS	4.511494	Not acceptable	Acceptable	RL vs RL	4.376261	Not acceptable	Acceptable
FRS vs FRS	4.578319	Not acceptable	Acceptable	FRL vs FRL	4.063143	Not acceptable	Acceptable
CS vs CS	4.353215	Not acceptable	Acceptable	CL vs CL	4.321842	Not acceptable	Acceptable

(ΔE) = 0 no color change; (ΔE) ≤ 3.7 accepted in vitro color change; (ΔE) ≤ 6.8 accepted in vivo color change.

Antifungal Activity Results:

Disc Diffusion Test Results: *C. albicans* susceptibility to different antifungal agents (Nytatin and Fluconazole) compared with oil extracts (Flax and Rosemary) was investigated using disk diffusion method on Mulluer Hinton agar. Four groups (flax oil, rosemary oil, Nystatin, Fluconazole; respectively) were subjected to disc diffusion test each group with five duplicates. Inhibition zones were shown in figure (4). One way ANOVA test comparing the means of inhibition zones for each group was shown in table (3); there were significant differences between groups at $p \leq 0.05$.

Table (3). One way ANOVA, test for disc diffusion antifungal activity.

	Summation of Squares	Degree of freedom	Mean Square	F	Significance
Between Groups	923.800	3	307.933	68.430	0.000
Within Groups	72.000	16	4.500		
Total	995.800	19			

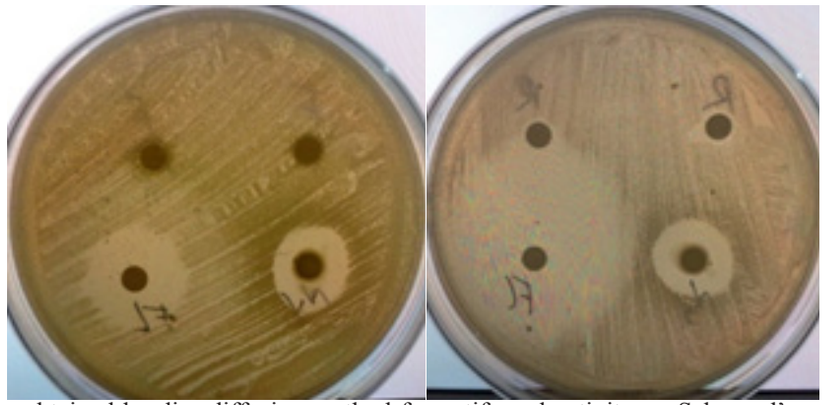

Figure (4) Inhibition zones obtained by disc diffusion method for antifungal activity on Sabaroud's agar media. A. for Flax oil compared with Fluconazole and Nystatin, B. for Rosemary oil compared with Fluconazole and Nystatin.

Iraqi Dental Journal Volume:34 Issue:2; 2012

Inhibition zone means measured in (mm) with its standard deviation and Duncan's multiple range are shown in figure (5) showing significant differences between groups.

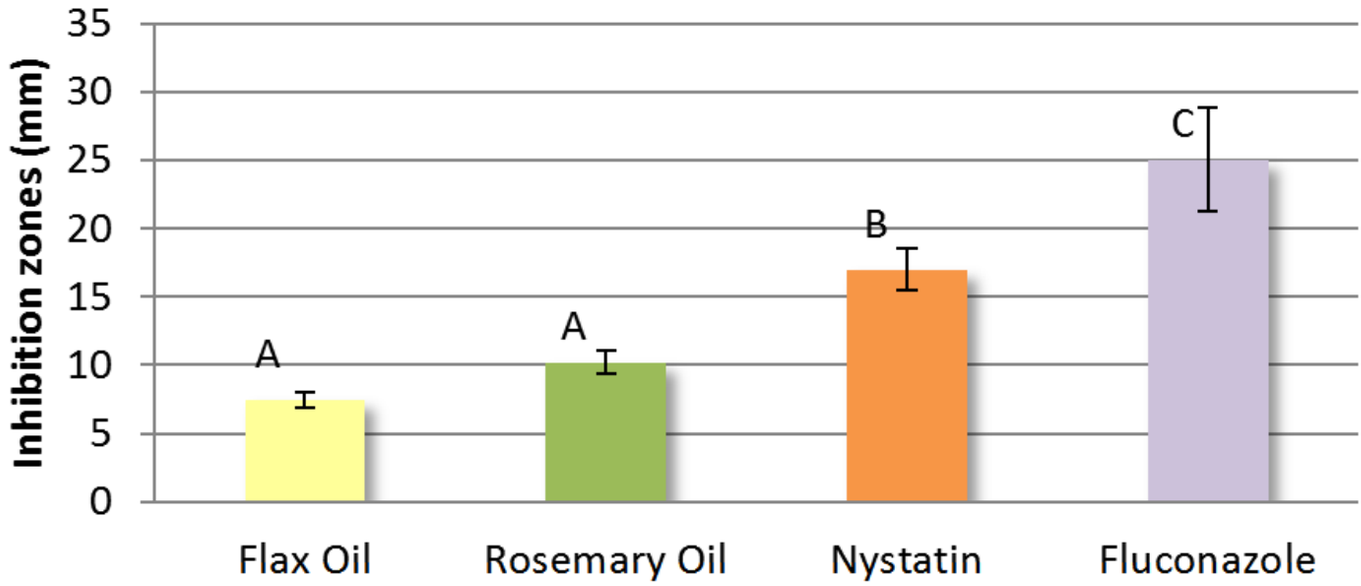

Figure (5). Inhibition zone means in (mm) for disc diffusion antifungal test.
Different letters means significant differences for Duncan's multiple range tests.

Standard Colony Count Test Results:

Denture soft lining materials samples from each sub-group for both short and long curing cycles were investigated for its antifungal potentials using standard colony counting test. Standard colony tests were repeated for two, seven, and thirty days for both short and long cycle.

(Figure 6 and 7) showed trend line for colony counts for both short and long cycle sub-groups over the three mentioned period.

Short cycle sub-groups results for two; seven and thirty days were shown in figure (8), (9) and (10) respectively.

Long cycle sub-groups for two, seven and thirty days were shown in figure (11), (12) and (13) respectively.

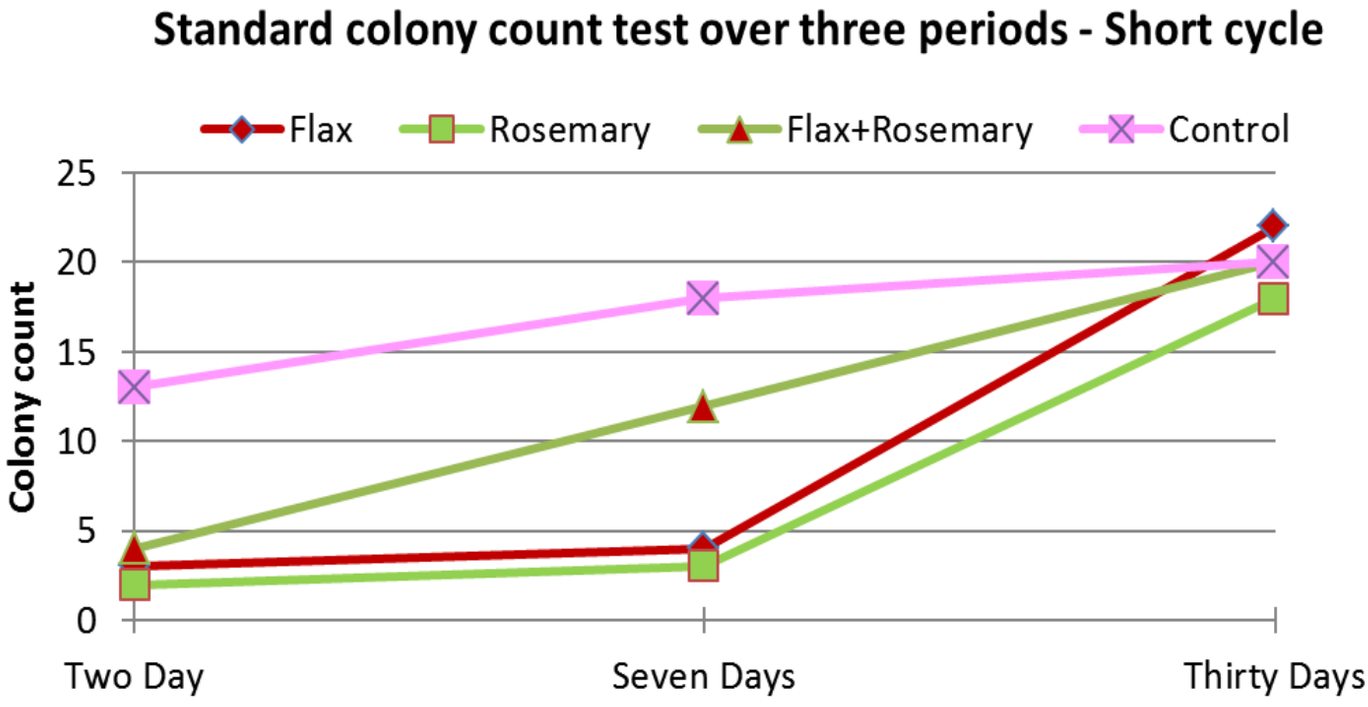

Figure (6). Standard colony count test trend line over three periods of immersion (two, seven, and thirty days) for short cycle.

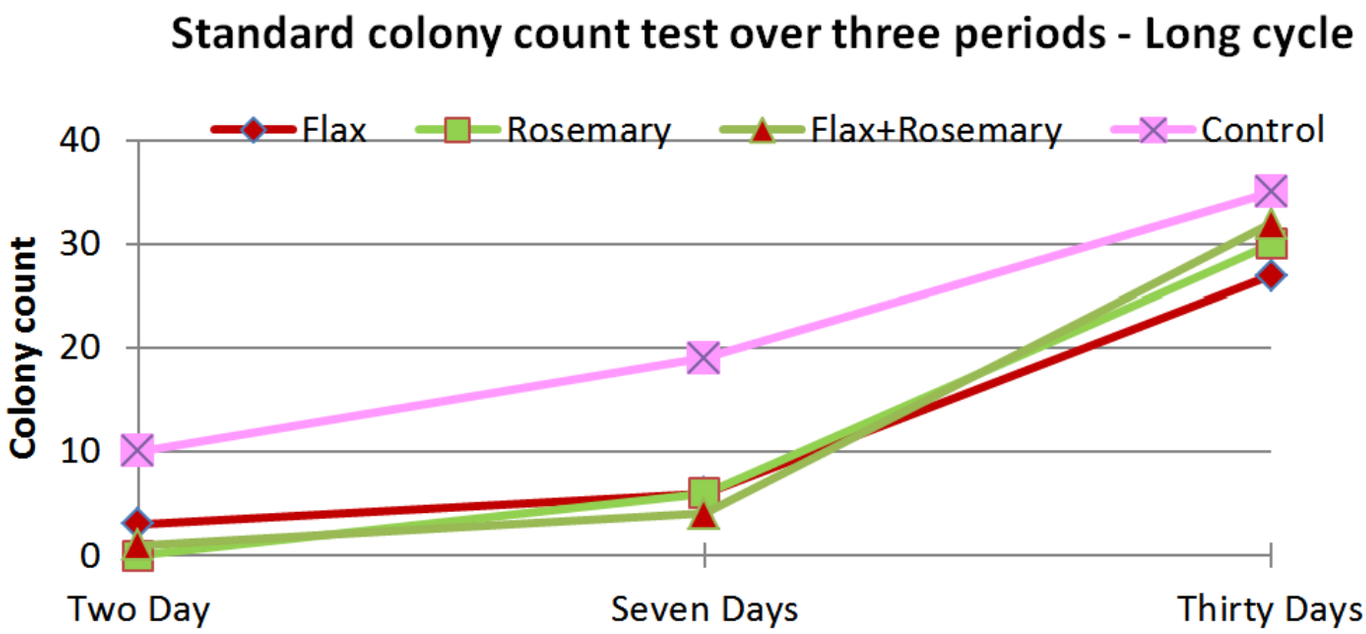

Figure (7). Standard colony count test trend line over three periods of immersion (two, seven, and thirty days) for long cycle.

Figure (8). Standard colony count test for short cycle at two days period.

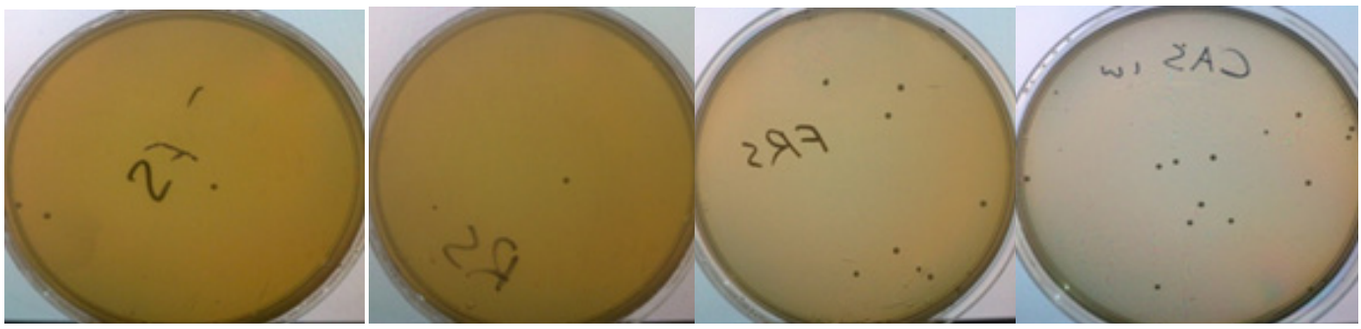

Figure (9). Standard colony count test for short cycle at seven days period.

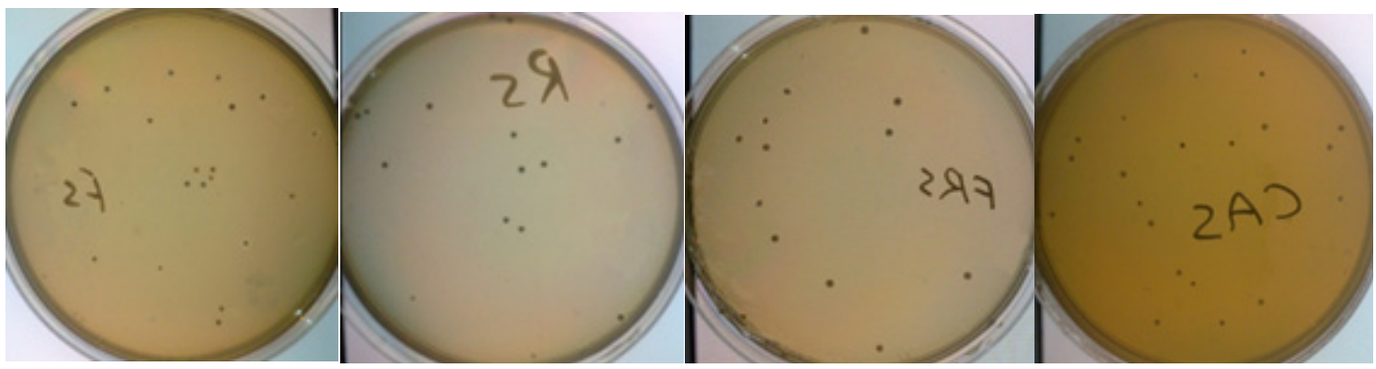

Figure (10). Standard colony count test for short cycle at thirty days period.

Iraqi Dental Journal Volume:34 Issue:2; 2012

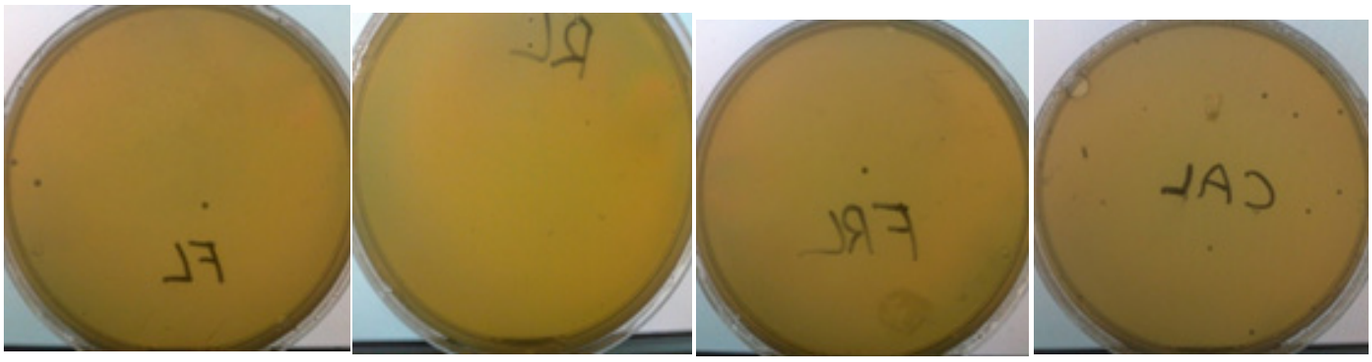

Figure (11). Standard colony count test for long cycle at two days period.

Figure (12). Standard colony count test for long cycle at seven days period.

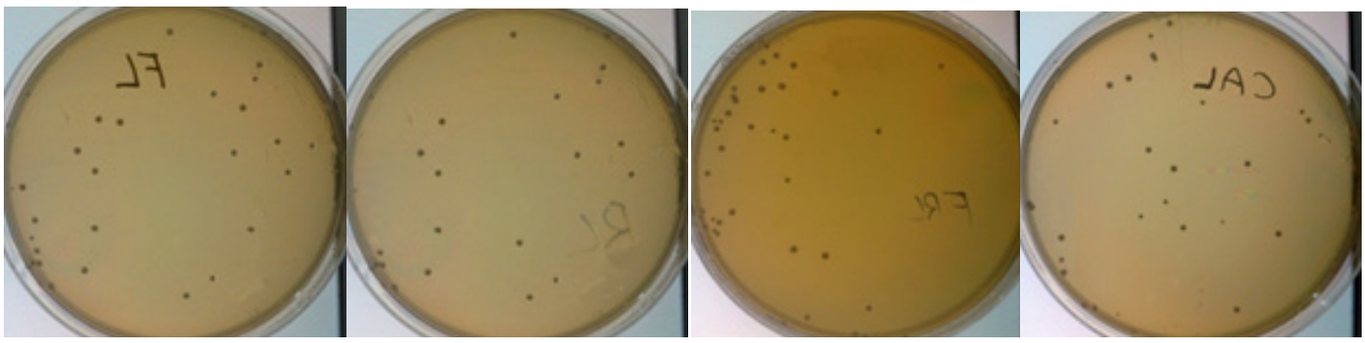

Figure (13). Standard colony count test for long cycle at thirty days period.

Discussion:

Color Stability of Soft Lining Material:

The levels of color change (ΔE) have been evaluated for short cycle groups stored at distilled water for two and thirty days by comparing control group against other three groups as in table (1) and the result was acceptable in vitro and for long cycle groups as in table (2) also the result was acceptable in vitro.

In order to check the effect of immersion time for both short and long cycle caparison was done between two and thirty day, result for short and long cycle groups was acceptable in vivo but not in vitro.

This result can be explained by the chemical structure of soft denture liners. Since, vertex is a polymethyl/ethyl methacrylate with a plasticizer, dibutyl phthalate. Polymethyl/ethyl methacrylate polymer is hydrophilic, attracting water soluble dyes to the surface of the lining material as a result of electrostatic charges [5]. It was stated that plasticized acrylic resin soft liners had high solubility and sorption [26]. In the acrylic resin material, this occurs due to the higher plasticizer solubilisation and consequently higher water absorption [27]. [28] said that the presence of plasticizer in the liner composition increases chain stretching of small organic molecules in the polymer, making diffusion of staining solutions easier. Staining agents may also penetrate the spaces created by leaching of the plasticizer.

Antifungal Activity:

The antifungal activity result inhibition zone of Flax oil, Rosemary oil, nystatin and fluconazole short and Duncan's multiple range tests of it present in figure (5), with fluconazole had the greater inhibition zone followed by nystatin then by rosemary and flax respectively.

The standard colony count result for short and long cycle groups at different immersion time (two,

seven and thirty days) present in figure (7 and 8), showed that in short cycle, the rosemary had the best antifungal followed by flax oil group, this at two and seven days and at thirty days flax oil group followed by mixed then by rosemary groups, while for long cycle rosemary group had the best antifungal followed by mixed group, this is at two and seven days, but for thirty days the flax group had best antifungal activity then followed by rosemary.

The antifungal effects of Rosemary essential oil can be attributed to the Monoterpens combination [29].

Flax oil contains Phenolic acids which are among the phytochemicals (plant chemicals) found abundantly in plants. They appear to have antioxidant, anticancer and antimicrobial activities. Flax contains about 8 to 10 gm of total phenolic acids per kilogram (kg) of flax. Flax contains about 35–70 milligrams (mg) of flavonoids/100 g [30]. Rosemary is credited as antimicrobial (diterpenes). Gram-negative bacteria such as Staphylococcus aureus and S. epidermidis have been found to be more susceptible to rosemary oil than other Gram-negative bacteria such as Escherichia coli and Pseudomonas aeruginosa [31]. The Rosemary oil is very effective against drug-resistant mutants of bacteria and fungi and that it has greater efficacy against fungus than bacteria [32].

Conclusions:

The addition of oils to soft liner results in antifungal activity last within one month and acceptable levels of color change (ΔE) in vitro obtained after oils addition to soft liner (vertex).

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