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RESEARCH ARTICLE - MEDICAL TECHNIQUES

Evaluating the Effect of Artemisia Aerial Parts Extracts on Candida albicans growth and Shear Bond Strength of Soft Denture Liner

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Abstract

After a period of use in the oral cavity the bond between denture base and soft denture liner weaken or fails creating a suitable environment for fungal colonization. Traditional medical plants that have antifungal agents are considered more safe and with few side effects. The objective of this study was to evaluate the effect of oil and ethanol extracts of Artemisia aerial parts on *Candida albicans* growth and shear bond strength of soft denture liner. *Candida albicans* species were isolated and diagnosed, oil and ethanol extracts of Artemisia were prepared, the effect of the extracts on *Candida albicans* were tested by agar well diffusion test and micro dilution test. Finally, 30 specimens of soft denture liner were prepared for testing shear bond strength by Universal Instron machine before and after incorporation of the extracts. The results showed that all the concentrations of oil extract inhibit the growth of *Candida albicans* except (12.5 mg/ml) and there were significant differences ($P \leq 0.05$) between all concentrations compared with Nystatin except between (100 and Nystatin) mg/ml in which there were no significant difference. In ethanol extract only the concentrations (100 and 75) mg/ml inhibited the growth of *Candida albicans* and there were significant differences between all concentrations ($P \leq 0.05$) with Nystatin, while shear bond strength was increased in ethanol extract and there were significant differences with the control ($P \leq 0.05$), but it was not affected in oil extract and there were no significant differences with the control ($P > 0.05$). Interestingly, it's concluded that oil and ethanol extracts of Artemisia aerials parts had an effect on *Candida albicans*, while shear bond strength was not affected by oil extract but increased in ethanol extract.

Keywords: Artemisia aerial parts extracts; Candida albicans; shear bond strength

1. Introduction

Soft denture lining materials are resilient materials that provide an even distribution of the functional load over the residual ridge and avoid stress concentrations at denture-bearing area [1, 2]. After a period of use in the oral cavity the bond between denture base and soft denture liner weakens or failed. Failure of the bond create a suitable environment for growth of bacterial and fungal colonization and reduce the durability of the relining material [1]. The yeast Candida is present in the oral cavity of 60-100% of denture wearers [3], these microorganisms have the ability to colonize denture surfaces and form a biofilm, essential in the progression of denture stomatitis [4]. Many fungal infections are treated with anti-fungal drugs such as Nystatin , Fluconazole, Amphotericin B and Miconazol but with the time many anti-fungal drugs become unsuccessful due to the development of resistant species in addition to its side effects, thus traditional medical plants that have antifungal agents are considered more safe and with few side effects when used according to the recommended dosage [5,6].The genus Artemisia retain to Asteraceae familly which consist of 500 species distributed through the world [7], it is an aromatic and medical plant widly used in traditional medicin for treatment of diabetes mellitus, coughs, colds and in healing of wounds, also it has antibacterial, antifungal, anti-inflammatory and antiheleminthic properties [8,9]. Oil and curde extracts of Artemisia had antimicrobial effects against a variety pathogen including Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans, and Malassezia spp [10,11]. A study reported that oil extract from the aerial parts of Artemisia had antifungal activity against Candida albicans and Saccharomyces cerevisiae var. [12].

Nomenclature	
DMSO Dimethyl Sulfonic Oxide	SDA Sabouraud Dextrose Agar Media
SD Standard Deviation	SDB Sabouraud Dextrose broth Media
SPSS Statistical Package for Social Science	P P-value
P/L Powder/ Liquid ratio	ADA American Dental Association
ASTM American Society for Testing and Material	µl Microliter
µg microgram	CLSI Clinical Laboratory Standard Institute
W Watt	CLF Colony Forming Unit

α-thujone, β-thujone, camphor and 1,8-cineole are considered the active components in Artemisia oil which had the antimicrobial activity [8]. The antifungal activity of ethanol extract of Artemisia was attributed to the presence of active antifungal components in this extract such as: scopoletin, betulinic acid, and acacetin which had antimicrobial activity against gram-positive, gram-negative bacteria and against Candida albicans [13]. The aim of this study was to evaluate the effect of oil and ethanol extracts of Artemisia aerials parts on the growth of Candida albicans and shear bond strength of soft denture liner.

2. Materials and Methods

2.1. The Period of the Study and Distribution of Samples

The period for extract and biological study continued from 20/11/2020 to 20/12/2020, while the period for the prosthetic study continued from 2/1/2021- 2/2/2021. The total dosages for biological study were 120 (5 concentrations for oil extract with 10 replicas and 5 concentrations for ethanol extract with 10 replicas, 10 replica of Dimethyl Sulfonic Oxide (DMSO) which was used as a negative control and 10 replica of Nystatin used as positive control. The extraction method and microbiological study were done in the Ministry of Science and Technology/Pollutant Treatment Center /microbiological labs. While the total samples for prosthetic study were 30 samples of self -cure soft liner (EZ-SOFT), control group, oil extract group and ethanol extract group (10 samples for each group).

2.2. Microbiological Study

2.2.1. Isolation and Examination of Candida albicans species

Candida albicans was isolated from four elderly patients aged 60 years who attended the Medical city /Laboratory division/Microbiology unite suffering from denture stomatitis, Candida genus were isolated using a sterile cotton swab that was gently rubbed over the intra oral lesions, sub cultured on Sabouraud Dextrose Agar Media SDA (OXOID, UK) and incubated at 37^0C for 48 h. After that germ tube formation, microscopic examination, grams stain, and API Candida systems were done for the diagnosis of Candida albicans species [14, 15].

2.2.2. Collecting and Extraction of the Plant

Artemisia aerial parts were bought from special local market in Baghdad, cleaned and washed to remove stones, debris and left to dry without exposure to sun light, then grinded with an electrical grinder (Brown, Germany) [16]. Fifty grams from plant powder of both ethanol and oil extract of Artemisia was placed in soxholate extractor (Cole-Parmer, USA). For ethanol extract preparation, 400 ml of ethanol 98% and 100 ml of distal water were added. While 500 ml of n-hexane were added for the preparation of oil extract then for both extracts the process continued for a period of (4h / 4 days), after that the extract evaporated by rotary evaporator (Cole-Parmer, USA) under low pressure at temperate (50-60 ^{0}C), to dry it completely the residue was placed in an oven at 25^0C then kept in a dark sterile place until used [17].

2.2.3. Agar Well Diffusion Test (Disc Diffusion Method)

Agar well diffusion test was used for testing the sensitivity of *Candida albicans* [17]. Using McFarland densitometer, the appropriate turbidity of Candida colony equal to 0.5 McFarland (1.5×10^6 CFL [Colony Forming Unit] /mL) was prepared. SDA media (20ml) was poured in a sterile petri dish (8cm diameter) after solidification, media streaked with *Candida albicans* suspension using a sterile swab in three directions for equal distribution of *Candida albicans* growth, wells with 5mm diameter were made in the media and filled with different concentrations of both extracts about 50 µl in each well by a sterilized micropipette tip, DMSO was used as a negative control and Nystatin (44 µg/mL) used as a positive control. Five concentrations for both oil and ethanol extracts (12.5 ,25 ,50, 75 ,100) mg/ml were prepared by dissolving 10g of crude extract in 100 ml of DMSO, the concentrations in each well were left for about 10min before incubation in order for the extract to diffuse into agar media. Finally, the plates were incubated at 37^0C for 48h.The anti-fungal sensitivity was assessed by measuring the diameter of inhibition zone in millimeter using a measuring ruler [18] (Fig.1-A). The experiments were in ten replicates and the average were reported.

2.2.4. Micro Dilution Test for the Determination of Minimum Fungicidal Concentration for Both Extracts

Minimum Fungicidal Concentration (MFC) was determined using the micro dilution method as recommended by CLSI (Clinical Laboratory Standard Institute) with some modifications [19]. Serial dilutions from stock solution of four extracts were prepared using low dilution [20] starting from 100% to 10% were prepared in 96-well micro titer plates in a volume of 100 µl (Fig. 1-B) using Sabouraud Dextrose both (SDB) media (OXOID,UK)[21,11] ,to test the sterility of the extract for negative growth (SDB + DMSO) only were added in a well, for sterility control (SDB + DMSO+ test extract), for positive growth (SDB + DMSO + test extract + yeast microorganism) [22].The freshly grown yeasts

were suspended in the media and the cell density adjusted to ($1.5x10^6$ cell/ml) which is equal to 0.5 McFrland standard at 530 nm wavelength using the spectrophotometric method. Then the wells were incubated at 35 ^{0}C without shaking [21]. After that a loopful of broth was removed from each well and subcultured on SDA plates and incubated at 35 ^{0}C for 48 h. Then the lowest concentrations (highest dilution) that completely inhibit the growth of yeasts will be considered as the Minimum Fungal Concentration (MFC). [11, 23].

2.3. Prosthetic Study

2.3.1. Preparation of Heat Cure Acrylic Specimens

In order to evaluate the shear bond strength between acrylic denture base and soft lining materials special acrylic block were prepared. Sixty specimens of heat cure acrylic blocks (VERACRIL, Colombia) with dimensions of 75mm length , 25 width and 5mm depth with stopper about 3mm according to ADA [24] (American Dental Association Specification 1999) were prepared using the conventional flasking, packing, curing, finishing and polishing methods [25] . Each two specimens of heat cure acrylic block were assembled leaving a space with dimensions of 25mm length x25 mm width and 3 mm depth [26,27] as in (Fig. 1-C) which was then filled with self-cure soft denture liner.

2.3.2. Preparation of Soft Denture Liner Specimens

Self-cure soft denture liner (EZ-SOFT) was used in this study. It was mixed according to manufacturer's instruction in control group (P/L ratio 1g : 1ml), while for experimental specimens the percentage of the plant extracts were 30 % for oil extract and 70% for ethanol extract which was determined by micro dilution test, these percentages were subtracted from the volume of soft liner liquid to obtain accurate P/L ratio, the extracts were mixed with the liquid part of soft lining material for 20 seconds [28] using probe sonication apparatus at 120 W and 60 KHz for complete homogeneity, then the powder was added and the mixture mixed until reaching to dough stage packed into the space between two acrylic blokes and processed according to manufacturer's instructions.

2.3.3. Shear Bond Strength Test

Universal Instron 1195 testing machine with suitable grip was used for testing shear bond strength, l00 kg load with 0.5 mm/min cross head speed was applied on the specimens to measure the shear load. The maximum load applied on the specimens until failure was recorded then the value of shear bond strength was calculated according to ASTM specification D-638m(American Society for Testing and Material and . 1986).

$$\text{Bond strength} = \frac{F}{A}\ (\text{N/mm}^2),\ \text{F= Maximum load}\ ,\ \text{A = Cross section area}$$

2.3.4. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The surface morphological changes of randomly selected specimens of each studied group were examined using a scanning electron microscope (SEM) [29], specimen for each group was placed on the SEM stag and examined. The photomicrographs of SEM were done under different power magnification). Then EDS was done in order for analysis of the elements presents in the specimens [30].

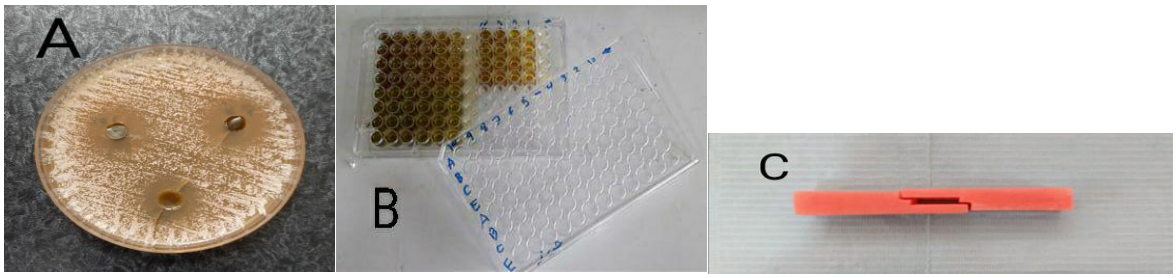

Fig. 1 (A) Effect of extract on Candida species (B) 96-well micro titter plates (C) Two heat cure acrylic blocks when assembled together

2.4. Statistical Analysis

Data were entered and analyzed using SPSS V 0.25 (IBM, U.S.A) for Windows. Descriptive statistics (frequencies, mean ± standard deviation, minimum, maximum values and with tables and graphs) and inferential statistics (Kruskal-Wallis H test, one-way ANOVA test and Pairwise Post-hoc Bonferroni test) were used. A P-value≤0.05 was considered statistically significant.

3. Results

3.1. Results of Biological Study

The concentrations (12.5, 25, 50) mg/ml in ethanol extract and the concentration 12.5 mg/ml in oil extract and DMSO (negative control) after incubation showed no inhibition zone in SDA media, so only the concentrations that inhibit the growth of *Candida albicans* were analyzed using SPSS V 0.25 for Windows as below:

The total samples were 6 concentrations: 4 for oil extract, 2 for ethanol extract, each one consists of 10 frequencies and 10 for Nystatin as described in (Table1)

Table 1 show Mean ± SD, minimum and maximum values of the groups. The highest mean value was recorded by Nystatin while the lowest mean value was recorded by the ethanol extract concentration 75 mg /ml.

Table 1 Description of study sample

Substance and concentrations (mg/ml)	Sample size	Mean ± SD*	Mini.	Max.
Oil extract of Artemisia	**40**			
25	10	11.4 ± 0.966	10	12
50	10	14.2 ± 1.03	13	15
75	10	17.1 ± 1.449	15	18
100	10	19.4 ± 0.966	18	20
Ethanol extract of Artemisia	**20**			
75	10	10.8 ± 1.03	10	12
100	10	14.2 ± 1.03	13	15
Nystatin	**10**	23.8 ± 0.632	22	24

***** Standard Deviation

3.1.1. Oil extract of Artemisia

The total samples were 40 and each group consisted of 10 frequencies compared with Nystatin (N=10). The Kruskal-Wallis H test showed that there was a statistically significant difference in inhibition zone between the different concentrations when compared with Nystatin group, test statistic; 46.500, p = 0.0001, mean rank distribution is shown in (Fig. 2).

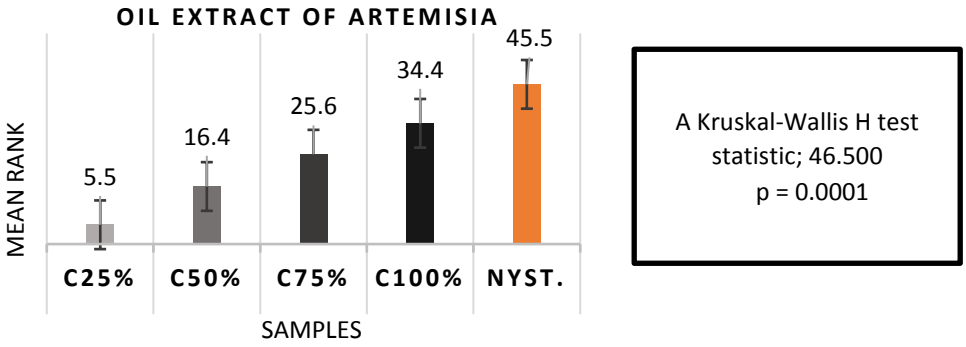

Fig. 2 Comparison of the effect of oil extract of Artemisia in different concentrations with Nystatin

In (Table 2) Post-hoc pairwise comparison indicated significant differences between all concentrations except between (25 and 50), (50 and 75), (50 and 100), (75and 100) and (100 and Nystatin) mg/ml in which there were no significant differences, even the inhibition zones increased with increasing the concentrations but still less than those obtained by Nystatin inhibition zone.

Table 2 Pairwise comparison of oil extract of Artemisia in different concentrations with Nystatin

Pairs (Concentrations(mg/ml))	Std. Error	Std. Test Statistic	Adj. Sig. *	Sig
25-50	6.436	1.694-	0.903	N.S
25-75	6.436	3.131-	0.017	N.S
25-100	6.436	4.498-	0.0001	S
25- Nystatin	6.436	6.215	0.0001	S
50-75	6.436	1.437-	1.00	N.S
50-100	6.436	2.805-	0.050	S
50- Nystatin	6.436	4.522	0.0001	S
75-100	6.436	1.367-	1.00	N.S
75- Nystatin	6.436	3.084	0.02	S
100- Nystatin	6.436	1.717	0.860	N.S

***** Post-hoc Bonferroni correction for multiple tests

3.1.2. Ethanol Extract of Artemisia

The Total samples were 20 and each group consisted of 10 frequencies compared with Nystatin (N=10). The Kruskal-Wallis H test showed that there was a statistically significant difference in the inhibition zone between the different concentrations when compared with Nystatin group, test statistic; 27.071, p = 0.0001, mean rank distribution showed in (Fig. 3).

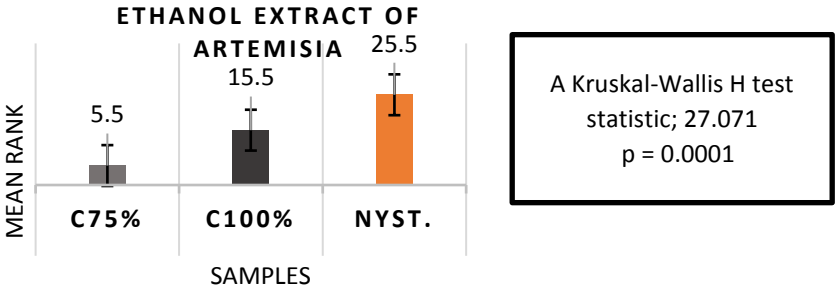

Fig. 3 Comparison of the effect of ethanol extract of Artemisia in different concentrations with Nystatin

In (Table 3) Post-hoc pairwise comparison indicated significant differences between all concentrations and with Nystatin, even the inhibition zones increased with increasing the concentrations but still less than those obtained by Nystatin inhibition zone.

Table 3 Pairwise comparison of ethanol extract of Artemisia in different concentrations with Nystatin inhibition zone

Pairs (Concentrations(mg/ml))	Std. Error	Std. Test Statistic	Adj. Sig. *	Sig.
75-100	3.844	2.601-	0.028	S
75-Nystatin	3.844	5.203	0.0001	S
100-Nystatin	3.844	2.601	0.028	S

***** Post-hoc Bonferroni correction for multiple tests

3.1.3. Micro Dilution Test

In this test the lowest concentration (highest dilution) that completely inhibit (killed) the growth of yeasts will be considered the Minimum Fungal Concentration (MFC), The results showed that the MFC for Artemisia oil was at the concentration 30% (MFC_{30}), the MFC for Artemisia alcohol at the concentration 70% (MFC_{70}) and this concentration will represent the concentration incorporated in the soft denture liner.[11,23] as shown in (Table 4, 5).

Table 4 MFC for oil extracts of Artemisia

	Control	10	20	30	40	50	60	70	80	90	100
A	-	-	-	+	+	+	+	+	+	+	+
B	-	-	-	+	+	+	+	+	+	+	+
C	-	-	-	+	+	+	+	+	+	+	+
E	-	-	-	+	+	+	+	+	+	+	+

(+) mean there were no growth of Candida species on SDA media, (-) mean there were growth of Candida species on SDA media.

Table 5 MFC for ethanol extracts of Artemisia

	Control	10	20	30	40	50	60	70	80	90	100
A	-	-	-	-	-	-	-	+	+	+	+
B	-	-	-	-	-	-	-	+	+	+	+
C	-	-	-	-	-	-	-	+	+	+	+
E	-	-	-	-	-	-	-	+	+	+	+

3.2. Results for the Prosthetic Study

3.2.1. Shear bond strength test (N\mm^2)

Three groups were enrolled and analyzed to identify their effect on shear bond strength of soft denture liner with different extracts and concentrations of Artemisia aerial part compared with the control. The total samples for this test were 30 specimens and each group consist of

10 specimens compared with the control. Table 6 shows the mean, standard deviation, minimum and maximum values of all groups. The highest mean value for shear bond strength was recorded by Artemisia alcohol group while the lowest mean value was recorded by the control group.

Table 6 Description of study sample (N= 30)

Shear bond strength test (N\mm^2) groups	Sample size	Mean ± SD*	Mini.	Max.	ANOVA
Artemisia oil	10	0.151 ± 0.024	0.119	0.198	F= 227.443, Sig. 0.00001
Artemisia alcohol	10	0.879 ± 0.124	0.686	1.071	
Controls	10	0.127 ± 0.022	0.104	0.159	

*** Standard Deviation**

One-way ANOVA test in (Table 6) showed that there was a statistically significant difference in shear bond strength test between the different groups, test statistic; F=227.443, p = 0.00001.

Figure 4 shows the distribution of mean values of shear bond strength.

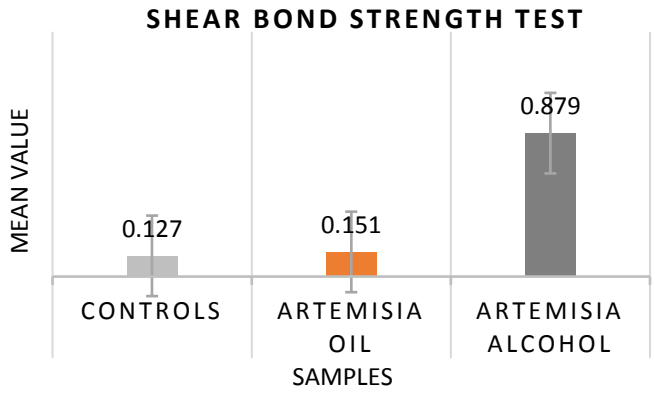

Fig. 4 Comparison of shear bond strength test means values in different groups with controls

In (Table 7) Post-hoc pairwise comparison indicated significant differences between all groups except between the control and Artemisia oil where there was no significant difference

Table 7 Pairwise comparison of mean values for shear bond strength test in different groups.

Table 7 Pairwise comparison of mean values for shear bond strength test in different groups

Pairs comparison	Std. Error	Sig. *	Sig.
Control - Artemisia oil	0.010	0.165	N.S
Control - Artemisia alcohol	0.039	0.000005	S
Artemisia oil - Artemisia alcohol	0.040	0.000007	S

* Post-hoc Games-Howell test**.**

3.2.2. Scanning Electron Microscopic (SEM) Test

SEM test under different magnifications power showed the surface of control specimen (pure soft denture liner) and experimental specimens (soft denture liner incorporated with plants extracts). (Fig. 5) show the regular surface of pure soft denture liner with magnification power (1000x and 3000x).

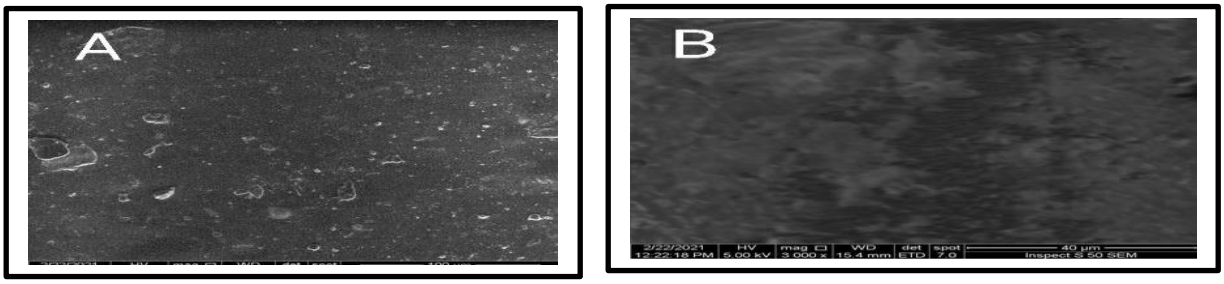

Fig. 5 photomicrographs by SEM for pure soft denture liner specimen (A) with 1000x magnification (B) with 3000x magnification

Figure 6 show a slightly irregular surface of soft denture liner when incorporated with Artemisia oil at concentration 30% with magnification power (1000x and 3000x).

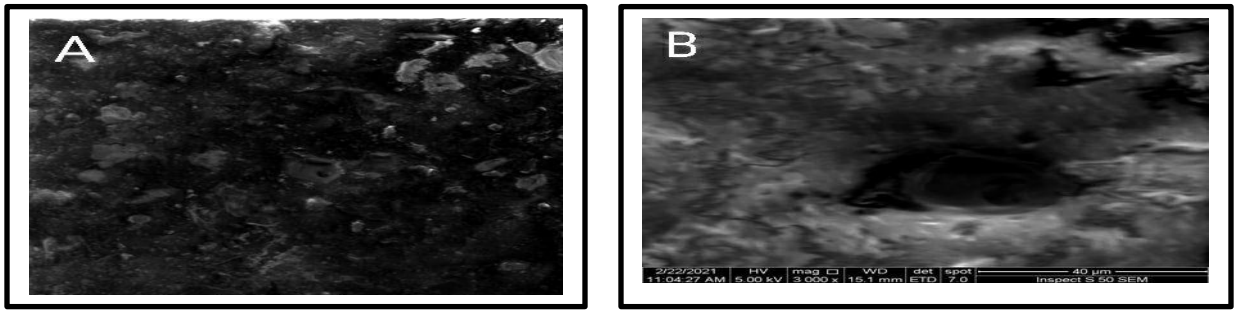

Fig. 6 photomicrographs by SEM for soft denture liner incorporated with Artemisia oil(A) with 1000x magnification (B) with 3000x magnification

Figure 7 show regular surface of the soft denture liner when incorporated with Artemisia alcohol at a concentration 70% with magnification power (1000x and 3000x).

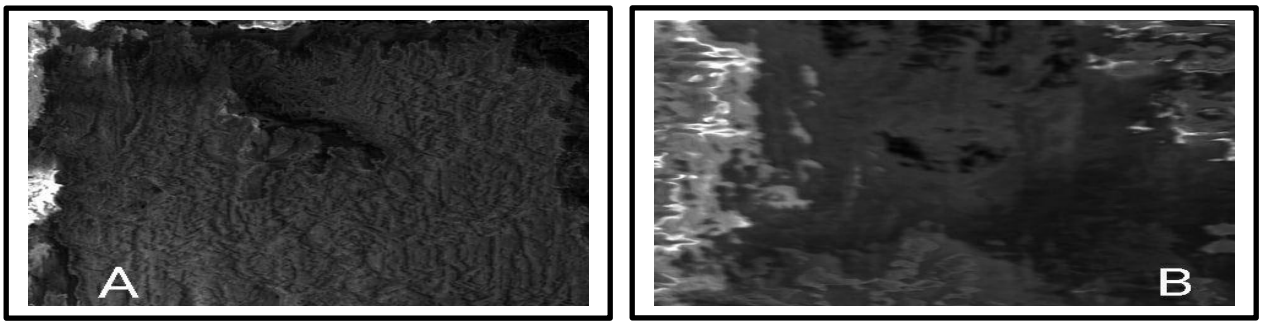

Fig. 7 photomicrographs by SEM for soft denture liner incorporated with Artemisia alcohol (A) with 1000x magnification (B) with 3000x magnification

3.2.3. Energy Dispersive Spectroscopy (EDS)

This test was used to determine the composition of soft denture liner specimen before and after incorporation of plants extracts. (Fig. 8-A) show the composition of pure soft denture liner specimen which are (C,H,O),(Fig. 8-C) show the composition of soft denture liner specimen incorporated with Artemisia oil which are (C, H, O, Ta, AL, Co, F, Cu,Si, Ni, Na, Ma, Ca, K), while (Fig. 8-B) show the composition of soft denture liner specimen incorporated with Artemisia alcohol which are (C, H, O, Cu, Co, Ni, Zn, Mg, Si, Na)

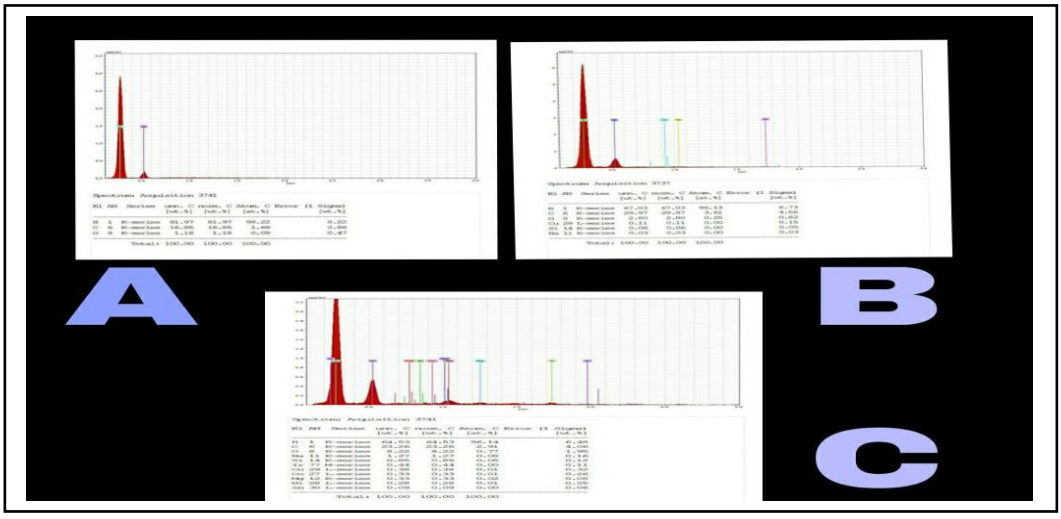

Fig. 8 EDS analysis of soft denture samples for A- Pure sample, B- Sample incorporated with ethanol extract, C- Sample incorporated with oil extract

4. Discussion

In vitro studies have shown that treatment with antifungal drugs is unsuccessful due to the development of resistant species in addition to its side effects [10,11].

In the microbiology study the anti-candida effect of Artemisia aerial parts were analyzed and it was observed that in the oil extract of Artemisia the inhibition zone was increased when the concentration increased but still in a less degree than the positive control Nystatin, the Maximum value was recorded by Nystatin while the minimum value recorded by the concentration 25 mg/ml, there were significant difference between all concentration with Nystatin except the concentration 100mg/ml was non-significant with Nystatin, however there were mathematical difference but there were no statistical difference between them, this mean that Artemisia oil had antifungal effect against Candida albican. The antifungal activity of Artemisia oil is due to the presence of active components such as (α-thujone, β-thujone, camphor and 1,8-cineole) which are considered the active components in Artemisia oil which had the antimicrobial activity as reported by [8]. This results was in agreement with other results [8,17,31] which reported that the oil extract of Artemisia had an antifungal effect against Candida albicans, also oil extract of Artemisia had antifungal activity against other pathogens and bacteria as reported by [12,32,33] , but it was in dis-agreement with [34] who discussed that the extract caused inhibition in growth of gram-positive and gram-negative bacteria but had no effect on Candida albicans.

While in Artemisia ethanol extract inhibition zone were increased when the concentration increased, the Maximum value was recorded by Nystatin while the minimum value was recorded by the concentration 75 mg/ml, meaning that the Artemisia ethanol extract has an antifungal activity against Candida albicans. The antifungal activity of Artemisia ethanol extract is due to presence active components such scopoletin, betulinic acid, and acacetin which are considered the active component that had antimicrobial activity against gram-positive, gram-negative bacteria and against Candida albicans [13], these results are in agreement with other studies [13,35,36,37], but it was in disagreement with L., Zhang [38] who showed that Artemisia extract don't have activity on Candida albicans and Aspergillus fumigatus these differences may be either due to the low concentrations used in the study which reached to 0.01 mg/ml do not possess the high concentration of flavonoid which act as an active anti-microbial component, or the extraction method used in the study does not extract the major bioactive constituents [39,40], but it was active on gram positive bacteria (Staphylococcus aureus) and on gram-negative bacteria (Acinetobact baumannii and Pseudomon aeruginosa),

The results of the prosthetic study showed that ethanol extracts groups increased the bond strength with significant difference P-value≤0.05 compared with the control group, this increase in bond strength was related to many factors such as the high concentration of the extracts materials and the extract act as a filler that caused uniform distribution in soft denture liner material[41] and this was confirmed by SEM as seen in (Fig.6 and 7) also the high concentration of the extract reduced the plasticizer in soft denture liner leading to increasing the hardness of the polymer [42]. While the group of the oil extracts had no effect on bond strength and there were no significant differences with the control group, this may be due to the specimens being examined after 24 hours storage in distal water they were not subjected to leaching out of oils to the surface and forming an oily layer that results in the decrease of bond strength [43].

There were some studies incorporating plants extracts into soft denture liner and studied their effects on shear bond strength, such as [44] which reported significant increase in shear bond strength after incorporation of Aloe Vera extracts , while another study reported that there were significant decrease in the bond strength at different time of intervals when virgin coconut oil was incorporated in soft denture liner [43]

To the best of our knowledge, there was no previous studies incorporating ethanol or oil extracts of Artemisia aerial parts into soft denture liner in order to compare with this study.

5. Conclusion

In the present study, it was concluded that oil and ethanol extracts from aerial parts of Artemisia had antifungal activity against *Candida albicans* , but in a degree less than the positive control Nystatins except the concentration 100 mg/ml in oil extract which was non-significant with Nystatin and considered as the more effective concentration in both extracts while shear bond strength increased in ethanol extract but was not affected by oil extract.

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