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Original Article

Effect of Different Concentrations of Some Medicinal Plant Extracts on Sensitivity and Viability Count of Candida Albicans (As In Vitro Study)

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Abstract

The antifungal effect of some medicinal plant extracts (peppermint and pomegranate) was confirmed by some studies. However, there was still a lack of knowledge regarding the specificity of these extracts on sensitivity and viability counts of candida albicans, so for this reason this study was conducted.

Water extract (peppermint and pomegranate) was prepared, different concentration (5, 10, 15, 20, and 25 mg/ml) from them were taken, tested candida albicans was used to evaluate these extracts on its sensitivity and viability counts (in vitro).

Result showed that all concentration of pomegranate showed zone of inhibition, the zone were increased when concentration of extract was increased, but peppermint revealed zero zone of inhibition on Sabouraud dextrose agar medium.

All concentrations of both extracts were effect in reducing viability counts of candida albicans with statistically significant differences with control $P<0.05$, 25% pomegranate extract and 20% peppermint recorded the maximum mean value in reduction the number of candida albicans.

The differences were not significant ($P>0.05$) for all concentrations of pomegranate and peppermint with each other's except for 25% concentration there was significant difference $P<0.05$.

Introduction

The fungus Candida albicans is a commensal organism of the mucosal microbiota in the oral cavity, gastrointestinal and genitourinary tracts, presenting ability to switch reversibly between yeast, pseudohyphal, and hyphal growth forms. This polymorphism is one of the most investigated virulence attributes in mucosal infections and disseminated infections affecting susceptible individuals [1].

The candidiasis treatments involve administration of antifungical agents. However, the use of these agents can cause side effects and lead to develop microbial resistance, being related in the presence of a small number of tolerant fungal cells, since the drugs decrease of the ergo sterol synthesis in the cell membrane [2].

The increase in the microorganism resistance to conventional antifungal drugs has encouraged studies to discover new treatments for infections caused by Candida species. Therefore, alternative therapies for the candidiasis treatment are crucial and the use of herbal medicines seems being a promising solution [3, 4]. Usually, the plants present numerous bioactive compounds that may be potent antimicrobial agents against C. albicans [5, 6].

The antifungal activity of the extract and compounds isolated from peels of Pomegranate against C. albicans has been associated predominantly to the presence of punicalagin, considered the main component of this plant [7].

In addition, the pomegranate peels extract causes serious damage to the C. albicans yeasts cellular structure, interfering on fungal growth/development, and consequently preventing tissue invasion [8].

Peppermint and its major components (ex: menthol and menthone) were well known for their inhibitory effect on the growth of yeast [9, 10].

In this study an attempt to study the antifungal effect of different concentrations of (peppermint and pomegranate) on sensitivity and viability counts of candida albicans.

Materials and Methods

Cultivation and preparation of water extract

The medicinal plants that used in this study includes: peppermint and pomegranate were bought from a public market in Baghdad. The extract was done in Ministry of Science and Technology/Ibn Albeatar Center. The fresh leaves of peppermint were collected, washed gently with water, then 200 gram mixed with 1000 ml distilled water, ground or milled with blender, then each plant extract placed in shaker for 8 hr ,after that The extracts were filtered with clean gauze to separate the solid particles from liquid extracts. The same procedure was used with fruit pericarp of pomegranate. The extract powder was prepared from aqueous extract of leaves, and pericarp of previously mentioned plants using the freeze-dried method (Lyophilization) by using lyophilizer machine [11]. The filtrate was collected and evaporated by vacuum rotary evaporator at 55 ^{o}C until crud extract powder was obtained[12].The crud extract was weighed and dissolved in distilled water to calculate the concentrations needed for different experiments. All the prepared M.P.E.s was kept at (25 ± 1) ºC in clean, dry containers [11].

Isolation and Identification of candida albicans

Candida albicans was isolated from patient with symptoms of denture stomatitis attending to the college of dentistry/Baghdad University; this was done by using sterile cotton swab and gentle rubbing of the intraoral lesions, then C. albicans was identified according to culture characteristic, microscopic appearance, germ tube formation and API candida system [13].

Antimicrobial sensitivity

Well diffusion method (agar diffusion technique) was used for assessment of antimicrobial activity of all subjected extracts [14].

Candida were cultured in Sabouraud dextrose agar medium (OXOID, U.K) at 37 for 24 hours. After 24 hr, the colonies were suspended in tubes containing 5 ml of brain heart infusion (BHI) broth. The final concentration of cells was 10^{6} CFU/ml (Colony Forming Unit/ml). The cell suspension in each tube was adjusted to match 0.5 McFarland scale (1.5 x 10^{8} CFU/ml). Wells (6 mm diameter) were punched in the agar and filled with 50 µl of each crude extract, Nystatin as positive control and distilled water as negative control. Five concentrations (5, 10, 15, 20, and 25 mg/ml) of each extract were prepared. All plates were incubated at 37 ºc for 24 hour; the antimicrobial activity was assessed by measuring the diameter of the inhibition zone. [14].

All experiments were accomplished in five replicate and the results reported are averages. MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration). The MIC was determined by the broth dilution method [15]. The plant extracts were diluted by at a concentration of 100 mg/mL, 50 mg/mL, 25 mg/mL, 20 mg/mL, 15 mg/mL, 10 mg/mL and 5 mg/mL respectively, at the volume of 1 mL. Then 1 mL of C. albicans suspension was added. A positive control group was used Nystatin 23 mg/mL and negative control group was used SDB 2 mL. These tubes were incubated at 37 ºC temperature for 24 hours. After that, the yeast colonies were observed to get the MIC and MFC value.

The MIC was defined as the lowest concentration that inhibits the visible growth of the yeast [16, 17]. The MFC was considered as the lowest concentration cultivated in plate with SDA in which growth was less than 3 CFU [18].

Fabrication of acrylic resin specimens used to estimate viable count of C. albicans

Preparation of Specimens

96 specimens were fabricated in the form of square metal shaped pattern with dimensions (10 x10 x 2.3 mm) length, width, thickness respectively [19]. Pink heat cured acrylic resin (Vertex, Netherlands) was mixed according to the manufacturer's instruction (3:1) by volume. Then the conventional flasking, packing, finishing and polishing procedures were followed in the preparation of the specimens [20], as seen in figure (1).

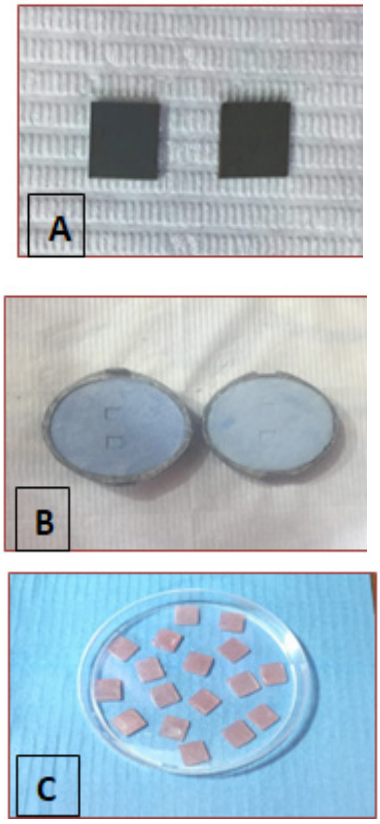

Figure 1: (A) metal patterns (B) mould preparation(C) acrylic specimens for viable count test.

Distribution of sample

Total sample were 96 samples were divided into three groups according to immersion:- group 1 (positive and negative control) 16 specimens were immersed in Nystatin 23 mg/mL and distilled water for 8 hour, group 2 (pomegranate) 40 specimens and group 3 (peppermint) 40 specimens were immersed for 8 hour in each extracts. Group 2 and group 3 were subdivided into 5 subgroup according to concentration of each extract (5, 10, 15, 20, and 25 mg/ml).

Evaluating the effect of plant extracts (pomegranate and peppermint) on viable count of C. albicans

To examine the antimicrobial activity of the plant extracts, C. albicans was diluted in 0.9% NaCl, and a yeast suspension of approximately 10^7 CFU/ml (0.5 McFarland standards) was prepared using a McFarland densitometer (Fig.2 A). Each specimen was placed in a tube containing 9.9 ml of Sabouraud dextrose broth, into which were dispensed 100 µl of the yeast suspension (Fig.2 B). The final concentration of cells was 10^5 CFU/ml .After 24 hours of incubation at 37 ◦ C, 100 µL of each mixture was transferred to 9.9 ml of NaCl (0.9%) and tenfold dilution was performed (Fig.2 C). From the second dilution, 100 µl was taken and spread on Sabouraud dextrose Agar and incubated aerobically for 24 hrs at 37º C.This diluation was taken, cause it showed a countable range of CFU according to [12] .The viable counts of all plates were calculated and statistically analyzed, and the material AFE (Antifungal efficacy) was calculated according to [19] using the following equation:

AFE [%]

Where Vc was the number of viable fungal colonies of the control specimen and Vt was the number of viable fungal colonies of the test specimen.

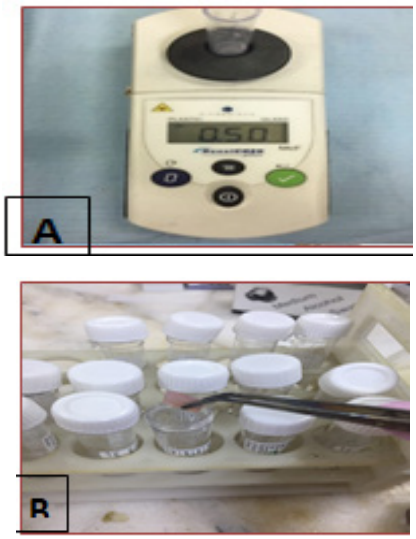

Figure 2: (A) McFarland densitometer, (B) Placement of specimen in the broth, (C) serial dilution.

Results

Medicinal extracts produced from this method, through dissolving 80 gram of extract powder in one liter of distilled water .the final weight of extract 6.6 gram of extract for peppermint. While for pomegranate 8 gram of extract were obtained. Sensitivity of candida albicans to different concentration of medicinal extracts, in vitro.

Pomegranate extract

The inhibition zone of different concentrations of pomegranate were shown in the figure (3).

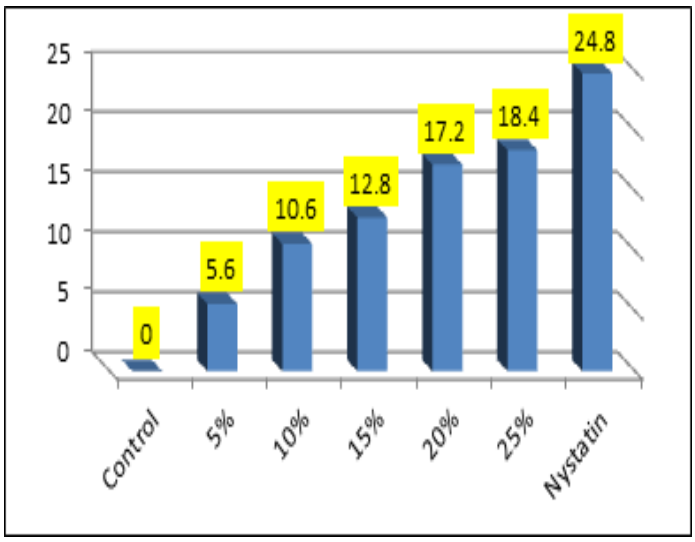

Figure 3: Distribution of inhibition zone of pomegranate.

Table 1: ANOVA test of inhibition zone between group of pomegranate, there was highly significant differences between these groups (P<0.01).

	F-test	P-value	Sig
Between groups	72.304	0.000	HS

In Table(2) show the LSD of inhibition zone of pomegranate group , there were highly significant differences between groups, but there were no significant difference between (10% with 15%) and (20% with 25%) .

Table 2: LSD of inhibition zone Concentration of pomegranate.

	Mean Difference		SE	P-value
Control	5%	-5.60000-*	1.38358	.000
	10%	-10.60000-*	1.38358	.000
	15%	-12.80000-*	1.38358	.000
	20%	-17.20000-*	1.38358	.000
	25%	-18.40000-*	1.38358	.000
	Nystatin	-24.80000-*	1.38358	.000
5%	10%	-5.00000-*	1.38358	.001
	15%	-7.20000-*	1.38358	.000
	20%	-11.60000-*	1.38358	.000
	25%	-12.80000-*	1.38358	.000
	Nystatin	-19.20000-*	1.38358	.000
10%	15%	-2.20000	1.38358	.123
	20%	-6.60000-*	1.38358	.000
	25%	-7.80000-*	1.38358	.000
	Nystatin	-14.20000-*	1.38358	.000
15%	20%	-4.40000-*	1.38358	.004
	25%	-5.60000-*	1.38358	.000
	Nystatin	-12.00000-*	1.38358	.000
20%	25%	-1.20000	1.38358	.393
	Nystatin	-7.60000-*	1.38358	.000
25%	Nystatin	6.40000*	1.38358	.000

Peppermint extract

For each concentrations of peppermint of (5%, 10%, 15%, 20%, and 25%) recorded zero zone of inhibition as shown in the figure (4 A).

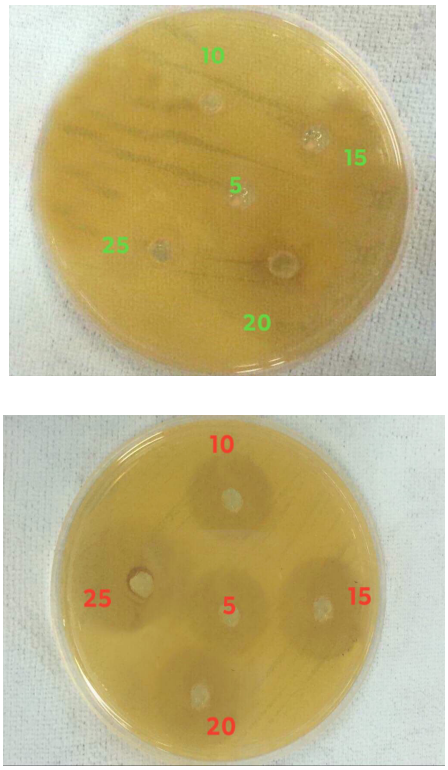

Figure 4: (A) No inhibition zone for peppermint, (B) Inhibition zone for pomegranate.

Effect of medicinal extracts on the viability count of candida albicans, in vitro

Figure (5) showed the bar chart distribution of viability count of different concentration of pomegranate and peppermint with negative and positive control, the viability count were varying with concentration of pomegranate and peppermint.

25% of pomegranate showed the Max. reduction of candida count (3.4), while 5% recorded the Min. Reduction which was equal to (13), while for peppermint 25% showed the Max. reduction of candida count (5), while 5% recorded the Min. Reduction which was equal to (18.2).

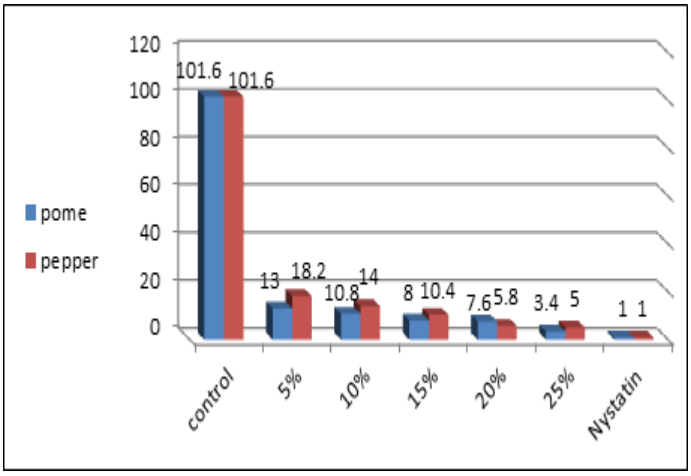

Figure 5: Distribution of viability count Concentration of pomegranate and peppermint.

Table (3) show ANOVA test of viability count of pomegranate and peppermint extracts, there was highly significant differences between groups (P<0.01).

Table 3: ANOVA between groups of viability count of pomegranate Concentration.

	F-test	P-value	Sig
Between groups of pomegranate	30.786	0.000	HS
Between groups of peppermint	27.745	0.000	HS

Table (4) show the LSD between groups there were highly significant differences between Negative controls with all concentration, But there were no significant difference between Nystatin and all concentrations, also there were no significant differences of all concentrations with each other.

Table 4: LSD of viability count of pomegranate concentration.

		Pome			Pepper		
		Mean Difference	**Std. Error**	**Sig.**	**Mean Difference**	**Std. Error**	**Sig.**
Control	**5%**	88.60000*	9.14362	.000	83.40000*	9.51840	.000
	10%	90.80000*	9.14362	.000	87.60000*	9.51840	.000
	15%	93.60000*	9.14362	.000	91.20000*	9.51840	.000
	20%	94.00000*	9.14362	.000	95.80000*	9.51840	.000
	25%	98.20000*	9.14362	.000	96.60000*	9.51840	.000
	Nystatin	100.60000*	9.14362	.000	100.60000*	9.51840	.000
5%	**10%**	2.20000	9.14362	.812	4.20000	9.51840	.662
	15%	5.00000	9.14362	.589	7.80000	9.51840	.419
	20%	5.40000	9.14362	.560	12.40000	9.51840	.203
	25%	9.60000	9.14362	.303	13.20000	9.51840	.176
	Nystatin	12.00000	9.14362	.200	17.20000	9.51840	.082
10%	**15%**	2.80000	9.14362	.762	3.60000	9.51840	.708
	20%	3.20000	9.14362	.729	8.20000	9.51840	.396
	25%	7.40000	9.14362	.425	9.00000	9.51840	.352
	Nystatin	9.80000	9.14362	.293	13.00000	9.51840	.183
15%	**20%**	.40000	9.14362	.965	4.60000	9.51840	.633
	25%	4.60000	9.14362	.619	5.40000	9.51840	.575
	Nystatin	7.00000	9.14362	.450	9.40000	9.51840	.332
20%	**25%**	4.20000	9.14362	.650	.80000	9.51840	.934
	Nystatin	6.60000	9.14362	.476	4.80000	9.51840	.618
25%	**Nystatin**	2.40000	9.14362	.795	4.00000	9.51840	.678

Figure (6) showed the bar chart distribution of minimum inhibitory concentrations of pomegranate and peppermint extracts.

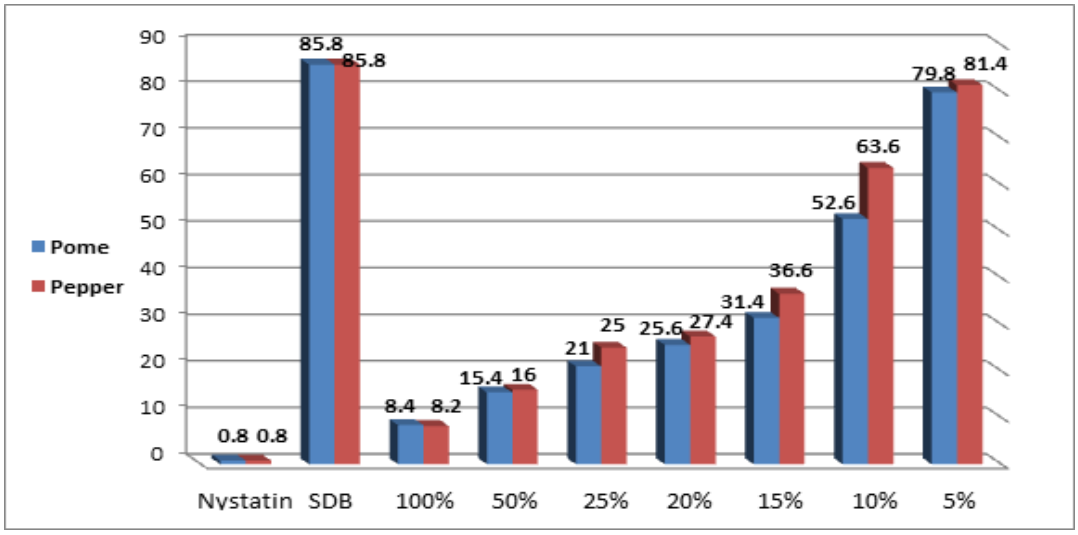

Figure 6: Distribution of minimum inhibitory concentrations of pomegranate and peppermint extracts.

Table 5: ANOVA between groups of MIC of pomegranate and peppermint Concentration.

Pomegranate & peppermint with Nystatin			
	F-test	**P-value**	**Sig**
Between groups of pome.	100.089	0.000	HS
Between groups of pepper.	95.507	0.000	HS
Pomegranate & peppermint with SDB			
	F-test	**P-value**	
Between groups of pome.	80.621	0.000	HS
Between groups of pepper.	73.813	0.000	HS

Table (5) show ANOVA test of minimum inhibitory concentrations of pomegranate and peppermint extracts with Nystatin and with SDB, there was highly significant differences between all groups (P<0.01).

In table (6) there were highly significant difference between Nystatin and all concentrations except (Nystatin with 100%) there were non-significant difference P>0.05, also there were highly significant difference of all concentrations with each other except between (100% with 50%), (50% with 25% and 20%), (25% with 20% and 15%) and (20% with 15%) there were non-significant difference P>0.05.

Table 6: LSD of minimum inhibitory concentration of pomegranate and peppermint extracts with Nystatin

		Pomegranate			**Peppermint**		
		Mean Difference	**Std. Error**	**Sig.**	**Mean Difference**	**Std. Error**	**Sig.**
Nystatin	100%	-7.60000-*	3.63249	.044	-7.40000	3.99124	.073
	50%	-14.60000-*	3.63249	.000	-15.20000-*	3.99124	.001
	25%	-20.20000-*	3.63249	.000	-24.20000-*	3.99124	.000
	20%	-24.80000-*	3.63249	.000	-26.60000-*	3.99124	.000
	15%	-30.60000-*	3.63249	.000	-35.80000-*	3.99124	.000
	10%	-51.80000-*	3.63249	.000	-62.80000-*	3.99124	.000
	5%	-79.00000-*	3.63249	.000	-80.60000-*	3.99124	.000
100%	50%	-7.00000	3.63249	.063	-7.80000	3.99124	.059
	25%	-12.60000-*	3.63249	.002	-16.80000-*	3.99124	.000
	20%	-17.20000-*	3.63249	.000	-19.20000-*	3.99124	.000
	15%	-23.00000-*	3.63249	.000	-28.40000-*	3.99124	.000
	10%	-44.20000-*	3.63249	.000	-55.40000-*	3.99124	.000
	5%	-71.40000-*	3.63249	.000	-73.20000-*	3.99124	.000
50%	25%	-5.60000	3.63249	.133	-9.00000-*	3.99124	.031
	20%	-10.20000-*	3.63249	.008	-11.40000-*	3.99124	.007
	15%	-16.00000-*	3.63249	.000	-20.60000-*	3.99124	.000
	10%	-37.20000-*	3.63249	.000	-47.60000-*	3.99124	.000
	5%	-64.40000-*	3.63249	.000	-65.40000-*	3.99124	.000
25%	20%	-4.60000	3.63249	.215	-2.40000	3.99124	.552
	15%	-10.40000-*	3.63249	.007	-11.60000-*	3.99124	.007
	10%	-31.60000-*	3.63249	.000	-38.60000-*	3.99124	.000
	5%	-58.80000-*	3.63249	.000	-56.40000-*	3.99124	.000
20%	15%	-5.80000	3.63249	.120	-9.20000-*	3.99124	.028
	10%	-27.00000-*	3.63249	.000	-36.20000-*	3.99124	.000
	5%	-54.20000-*	3.63249	.000	-54.00000-*	3.99124	.000
15%	10%	-21.20000-*	3.63249	.000	-27.00000-*	3.99124	.000
	5%	-48.40000-*	3.63249	.000	-44.80000-*	3.99124	.000
10%	5%	-27.20000-*	3.63249	.000	-17.80000-*	3.99124	.000

In table (7) there were highly significant difference between SDB with all concentrations except (SDB with 5%) there were non-significant difference P>0.05, also there were highly significant difference of all concentrations with each other except between (100% with 50%), (50% with 25% and 20%), (25% with 20% and 15%) and (20% with 15%) there were non-significant difference P>0.05.

Table 7: LSD of minimum inhibitory concentration of pomegranate and peppermint extracts with SDB.

		Pomegranate			Peppermint		
		Mean Difference	**Std. Error**	**Sig.**	**Mean Difference**	**Std. Error**	**Sig.**
SDB	100%	77.40000*	4.64435	.000	77.60000*	4.93001	.000
	50%	70.40000*	4.64435	.000	69.80000*	4.93001	.000
	25%	64.80000*	4.64435	.000	60.80000*	4.93001	.000
	20%	60.20000*	4.64435	.000	58.40000*	4.93001	.000
	15%	54.40000*	4.64435	.000	49.20000*	4.93001	.000
	10%	33.20000*	4.64435	.000	22.20000*	4.93001	.000
	5%	6.00000	4.64435	.206	4.40000	4.93001	.379
100%	50%	-7.00000	4.64435	.142	-7.80000	4.93001	.123
	25%	-12.60000-*	4.64435	.011	-16.80000-*	4.93001	.002
	20%	-17.20000-*	4.64435	.001	-19.20000-*	4.93001	.000
	15%	-23.00000-*	4.64435	.000	-28.40000-*	4.93001	.000
	10%	-44.20000-*	4.64435	.000	-55.40000-*	4.93001	.000
	5%	-71.40000-*	4.64435	.000	-73.20000-*	4.93001	.000
50%	25%	-5.60000	4.64435	.237	-9.00000	4.93001	.077
	20%	-10.20000-*	4.64435	.035	-11.40000-*	4.93001	.027
	15%	-16.00000-*	4.64435	.002	-20.60000-*	4.93001	.000
	10%	-37.20000-*	4.64435	.000	-47.60000-*	4.93001	.000
	5%	-64.40000-*	4.64435	.000	-65.40000-*	4.93001	.000
25%	20%	-4.60000	4.64435	.329	-2.40000	4.93001	.630
	15%	-10.40000-*	4.64435	.032	-11.60000-*	4.93001	.025
	10%	-31.60000-*	4.64435	.000	-38.60000-*	4.93001	.000
	5%	-4.60000	4.64435	.329	-56.40000-*	4.93001	.000
20%	15%	-5.80000	4.64435	.221	-9.20000	4.93001	.071
	10%	-27.00000-*	4.64435	.000	-36.20000-*	4.93001	.000
	5%	-54.20000-*	4.64435	.000	-54.00000-*	4.93001	.000
15%	10%	-21.20000-*	4.64435	.000	-27.00000-*	4.93001	.000
	5%	-48.40000-*	4.64435	.000	-44.80000-*	4.93001	.000
10%	5%	-27.20000-*	4.64435	.000	-17.80000-*	4.93001	.001

Discussion

Medicinal extracts were used for long time to inhibit fungal effect, many studies confirmed this effectiveness because extracts constituents inhibit and/or cause damage to yeast, candida albicans growth and development. [21, 22, 23, 24].

In present study pomegranate and peppermint extract were tested for their effect on candida albican. Extracts were prepared by freeze-dried (lyophilization). [11], with preparation of pomegranate extract, the difficulty of it was increase in density so the extract was followed by evaporation by vacuum rotary evaporator at 55 ^{o}C until crud extract powder was obtained. This is attributed to texture of pomegranate extract which is viscous in nature lyophilizer machine was useful.

Sensitivities of candida albican to different concentrations of both extracts were studies following Agar Well Technique. Pomegranate extract inhibit the growth of candida albicans, the zone of inhibition was found to increase when the concentration of pomegranate extract increased, and this indicate that chemical constituent of pome. Extract have antifungal effects, tannin which was detected by GC analysis by present study in addition to other constituents such as punicalagins and gallic acid [25],These result were in agreement with other studies that concluded that the fruit peel of Punica granatum was effective for inhibiting Candida albicans growth [21,22,26]. The results were also in accordance with the report of Vasconcelos et al [23], Duraipandiyan et al [18], and with those of Pereira (9), who assessed the minimum inhibitory concentrations of adherence of Punica granatum Linn extract against S. mitis, S. mutans, S. sanguis, and C. albicans. [27], but in contrast with Sh. Abdollahzadeh [28] and Singh's study[29], that show there was no effect on candida albicans.

For peppermint extract there were no inhibition zone in all concentration this was due to peppermint extract were difficult to diffuse in the media. This result was agree with that of Mimica [30] who mention to the lack of the inhibitory effectiveness of the water extracts of peppermint .and disagree with other result [31] that show there were inhibition zone. The diameter of the zone of inhibition will depend on the ability of the test substance to diffuse uniformly through the agar medium [32].

The variation in inhibitory effectiveness of water extracts was attributable for chemical composition of the plant, and its effective compounds and concentration, as well as solubility in water or organic solvents during the extraction process, duration, and timing of plant harvesting and other factors [31].

Effect in viability count of candida albicans were studied because it gives scientific result in comparison with sensitivity method, result showed there were highly significant difference $P<0.01$ between all concentration of pome. With control (D.W) and even with each other's in reduction of candida count. Conclusions of previous studies indicate that the chemical constituents of pomegranate have antifungal effect [33]. These results were in agreement with the results of [31] who found that extracts of Punica granatum has strong antimicrobial activity. In addition different extracts of Punica granatum give good antibacterial activity against different bacterial strains. [34]

In fact, when we performed the chromatographic screening of extract, the main compounds identified were ellagic acid derivatives and ellagitannins, such as punicalin. Recently, it has been found that punicalagin, pedunculagin, telimagrandin and galagildilacton have important antimicrobial action against C. albicans yeast cellular structure [35, 36]. Thus, the main compound involved in anti-Candida action present of the P. granatum fruit peels seems to be the punicalagin which was detected by present study and other studies [37, 35, 38]. this was in agreement with[34,35], this study that showed different concentrations have effect on both sensitivity and in reduction of viability counts because tannin constituent in extract that revealed by GC analysis in Ibn Al-beatar Center ,which have antifungal activity with candida albican.

For peppermint the counts of viability were decreased with highly significant difference with control, this result in agreement with [39,40] which concluded that Mentha piperita L. essential oils show significant antibacterial and antifungal activity against gram positive and gram negative bacteria, as well as yeast and fungi, mostly because menthol and menthone are main chemical constituents.

The GC analysis of peppermint has Menthol, and Menthone constituents that had also effect on fungi. The effect of mint and its main constituents (Menthol, and Menthone) on the structural and functional aspects of membrane integrity may arise from reducing ergosterol levels that ultimately cause cell death rendering mint fungicidal [41].

All plant extracts used in this study had antifungal effect on candida albicans. For this reason can be used as disinfectants, mouth wash or denture soaking solutions and as an alternative to drug solutions in the treatment of denture stomatitis. After further testing, it will be able to be clinically applied in microorganisms-related infectious diseases, such as DS.

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