Antifungal Activity of Some Plant Extracts

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ABSTRACT
Background Due to the increase in the microorganism resistance to most of conventional antifungal drug, studies have encouraged to discover novel treatments for infections caused by Candida species. Hence, alternative therapies are crucial and the use of herbal medicines seems being a promising solution for the candidiasis treatment. **Materials and Methods** the antifungal effect of some medicinal plant extracts (peppermint and pomegranate) was confirmed by some studies. But there was still a lack of knowledge associated with 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 25mg/ml) from them were taken, tested candida albicans was used to evaluate these extracts on its sensitivity, viability counts, and minimum inhibitory concentration (MIC) (in vitro)**. Results** showed that all concentration of pomegranate showed zone of inhibition, there were highly significant differences between concentrations except between (10, 15) and (20, 25), 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 highly significant differences with Negative control (Distilled water) $P<0.05$, but there were no significant difference $P>0.05$with positive control (Nystatin) and with each other. **Conclusions** All plant extracts used in this study had antifungal effect and influence on candida albicans. For this reason can be used as disinfectants, mouth wash or soaking solutions and as an alternate to drug in the treatment of denture stomatitis.

Keywords: Candida Albicans, pomegranate extract, peppermint extract, microbiota in the oral cavity, antifungal drugs.

Introduction

Candida albicans is a commensal organism of the mucosal microbiota in the oral cavity, also the most inspected virulence attributes in mucosal infections and disseminated infections affecting susceptible individual. (Calderone and Fonzi, 2001, Mimica-Dukic et al, 2003, Tayel and El-Tras, 2010, Alsaidy et al, 2014). Increasing the microorganism resistance to the most of conventional antifungal drugs has encouraged studies to discover new treatments for the infections caused by candida species. (Harris, 2002, Cesar de souza Vasconcelos et al, 2003, Budzynska et al, 2011), usually, the plants present numerous of bioactive compounds that can be potent antimicro-

bial agents against Candida albicans. (Koo et al, 2000, Samet et al, 2007, Madugula et al, 2017). The antifungal activity of the extract isolated from peels of Pomegranate against Candida albicans has been related predominantly to the presence of punicalagin which considered the main component of this plant. (Bagamboula et al, 2004, Duraipandiyan et al, 2006, Pereira et al, 2006, Jurenka, 2008, Endo et al, 2010, Hofling et al, 2010). Furthermore, the pomegranate peels extract causes severe damage to the C. albicans yeasts cellular structure, interfere with the fungal growth/development, and thus preventing tissue invasion. (Prashanth et al, 2001, Anibal et al, 2013, Bakkiyaraj et al, 2013). The major components of Peppermint (ex: - menthol and menthone) were well known for their inhibition effect on the yeast growth. (Bennis et al, 2004, Bulad et al, 2004, Samber et al, 2015, Lima, 2017, Desam et al, 2017). 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

Preparation of medicinal plant extracts used in this study (peppermint and pomegranate) was done in Ministry of Science and Technology/Ibn Albeatar Center. The fresh leaves of peppermint and pericarp of pomegranate were collected, The extract powder was prepared from aqueous extract of leaves, and pericarp of previously mentioned plants by using the freeze dried method (Lyophilization) through the use of lyophilizer machine (Kassab-Bashi, 2014), Then evaporated by vacuum rotary evaporator at 55 ºC. (Abdollahzadeh et al, 2011), the crud extracts were weighed and dissolved in a distilled water to calculate the concentrations needed for different tests. The prepared medicinal plant extracts were kept at (25 ± 1) ºC in dry containers. (Kassab-Bashi, 2014).

Isolation and Identification of C. albicans

The C. albicans was isolated and identified according to the culture characteristic, microscopic appearance, germ tube formation and (API) candida system. (Marsh et al. 2013, Abdollahzadeh et al, 2011).

Fabrication of acrylic resin specimens used to evaluation viable count of C. albicans Preparation of Specimens

The 96 specimens were fabricated in a form of square metal shaped pattern with dimensions (10x10x2.3 mm) length, width, thickness respectively. (Chladek et al, 2011), 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. (Craig, 1997).

In vitro experiments

Antimicrobial sensitivity

Well diffusion method (agar diffusion technique) was used for assessment of antimicrobial activity of the studied extracts. (AL-Mizraqch et al, 2010). In Sabouraud dextrose agar medium (OXOID, UK) Candida were cultured at 37 for 24 hours. After 24 hr, the colonies were suspended in tubes which contain 5ml of brain heart infusion

(BHI) broth; the final concentration of cells was 106 CFU/ml (Colony Forming Unit/ml). Each tube was adjusted to match 0.5 McFarland scale (1.5 x 108 CFU/ml). Wells (6mm diameter) were punched in the agar and filled with 50 µl of each plant extract, Nystatin as positive control and distilled water as negative control. Five concentrations (5, 10, 15, 20, and 25mg/ml) of each plant extract were prepared. All plates were incubated at 37 ºc for 24 hr; the antimicrobial activity was assessed by measuring the diameter of the inhibition zone. (AL-Mizraqch et al, 2010), all experiments were accomplished in eight replicate and the results reported as an averages.

Testing procedure to determine the effect of plant extracts (pomegranate and peppermint) on viable count of C. albicans

For examining the antimicrobial activity of the plant extracts, Candida albicans was diluted in 0.9% NaCl. , a yeast suspension of approximately 107 CFU/ml which equal to 0.5 McFarland standards was prepared using a McFarland densitometer, in a tube containing 9.9 ml of Sabouraud dextrose broth each specimen was placed, into which were dispensed 100 µl of the yeast suspension. The final concentration of cells was 105 CFU/ml, after 24 hour of incubation at 37◦C, 100µL of each mixture was transferred to 9.9ml of 0.9% NaCl and tenfold dilution was achieved .From the second dilution 100µl was taken and spread on Sabouraud dextrose Agar and incubated aerobically in incubator for 24hr at 37ºC.This dilution was taken because it showed a countable range of CFU according to Sutton, 2011. The viable counts of all plates were calculated and statistically analyzed, and the material AFE (Antifungal efficacy) was calculated according to Chladek et al, 2011 using the following equation:

AFE [%]= (VC-Vt)/Vc x 1oo%

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

Figure (1): (A) McFarland densitometer, (B) Placement of specimen in the broth, (C) serial dilution.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The Minimum Inhibitory Concentration was determined by the broth dilution method (Jareemit et al, 2017), the plant extracts were diluted at the volume of 1 mL of broth 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, then 1 mL of C. albicans suspension was added. A positive control group was used Nystatin 23mg/mL and negative control group was

used SDB at volume of 2 mL. All tubes were incubated at 37ºC for 24 hours. Then, the yeast colonies were observed to get the MIC and MFC value. The MIC defined as the lowest concentration that inhibit the visible growth of the yeast (Ana et al, 2017). The MFC was defined as the lowest concentration cultivated in plate with SDA in which growth was less than (3 CFU) (Espinel-Ingroff et al, 2002).

Statistical Methods

The data were statistically analyzed by software computer program SPSS version 23 to get descriptive statistics , the inferential statistics were used in order to accept or reject the statistical hypothesis which include analysis of variations (ANOVA) test with multi comparison , and least significant difference (LSD) test.

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 C. albicans to different concentration of medicinal extracts, in vitro Pomegranate extract

The inhibition zone of different concentrations of pomegranate with ANOVA test were shown in the figure (2), here were highly significant difference between them.

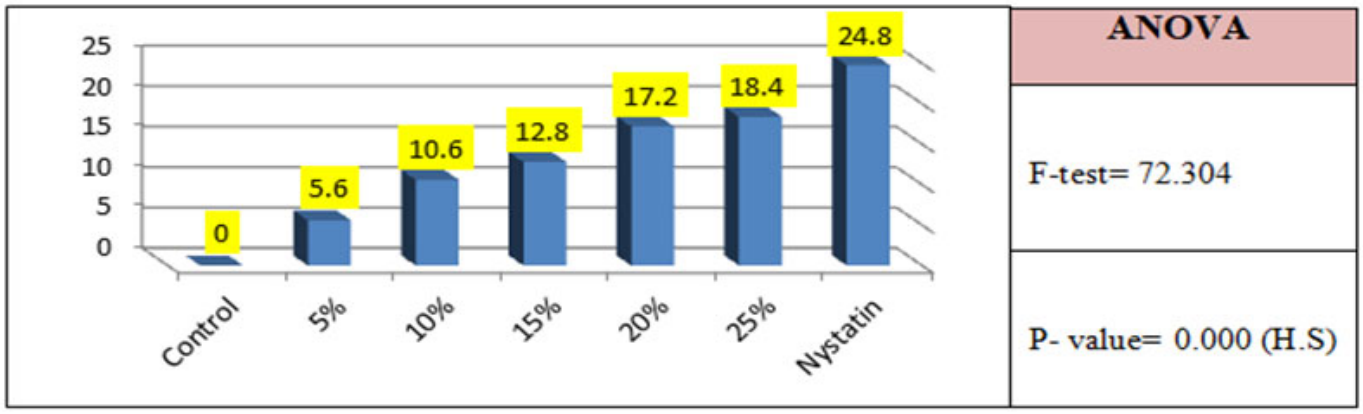

Figure (2): Distribution of inhibition zone of pomegranate.

Table (1): LSD of inhibition zone Concentration of pomegranate.

	5%	10%	15%	20%	25%	Nystatin
Control	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01
5%	-	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01
10%	-	-	N.S *P*=0.123	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01
15%	-	-	-	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01
20%	-	-	-	-	N.S *P*=1.38358	H.S *P*<0.01
25%	-	-	-	-	-	H.S *P*<0.01

\In Table (1) 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%).

Peppermint extract
For each concentrations of peppermint of (5%, 10%, 15%, 20%, and 25%) recorded zero zone of inhibition.

Effect of medicinal extracts on the viability count of candida albicans, in vitro
All experimental groups (5%, 10%, 15%, 20% and 25% for both extracts) showed a lower mean values than control group, the AFE of the pomegranate extract group was (87.2% , 89.3%, 92.1%, 92.5%, and 96.6%) respectively, while for peppermint extract group the AFC was (82%, 86.2%, 89.7%, 94.2% and 95%) respectively. Figure (3) showed the bar chart distribution of viability count of different concentration of pomegranate and peppermint with negative and positive control with ANOVA test, the viability count were varying with concentration of pomegranate and peppermint. 25% of pomegranate and peppermint showed the Maximum reduction of candida count (3.4) and (5) respectively, while 5% recorded the Minimum Reduction for both extracts which was equal to (13) and (18.2) respectively. Table (2) show the LSD between groups there were highly significant differences between Negative control with all concentration, But there were no significant difference between Nystatin and all concentrations, also there were no significant differences of all concentrations with peppermint and with each other. Figure (4) showed the bar chart distribution of minimum inhibitory concentrations of pomegranate and peppermint extracts with ANOVA test , there were highly significant differences between all groups $P<0.01$. In table (3) there were highly significant difference between Nystatin and all concentrations of both extracts except (Nystatin with 100%peppermint) 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%), (25% with 20%) and (20% with 15% pomegranate) there were non-significant difference $P>0.05$.

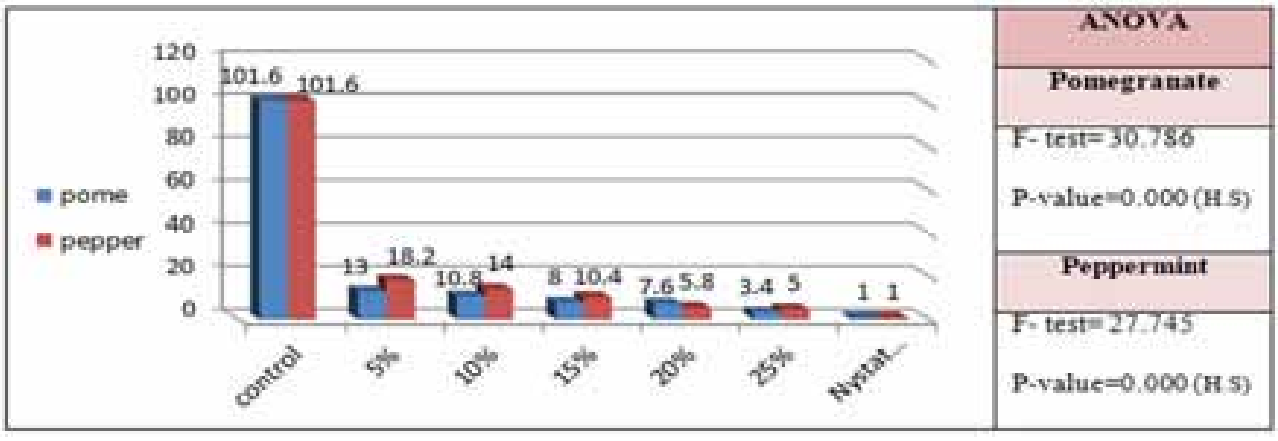

Figure (3): Distribution of viability count Concentration of pomegranate and peppermint.

Table (2): LSD of viability count of pomegranate concentration.

	5%	10%	15%	20%	25%	Nystatin
Control	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01	H.S *P*<0.01
5%	-	N.S *P*>0.05	N.S *P*>0.05	N.S *P*>0.05	N.S *P*>0.05	N.S *P*>0.05
10%	-	-	N.S	N.S *P*>0.05	N.S *P*>0.05	N.S *P*>0.05
15%	-	-	-	N.S *P*>0.05	N.S *P*>0.05	N.S *P*>0.05
20%	-	-	-	-	N.S *P*>0.05	N.S *P*>0.05
25%	-	-	-	-	-	N.S *P*>0.05

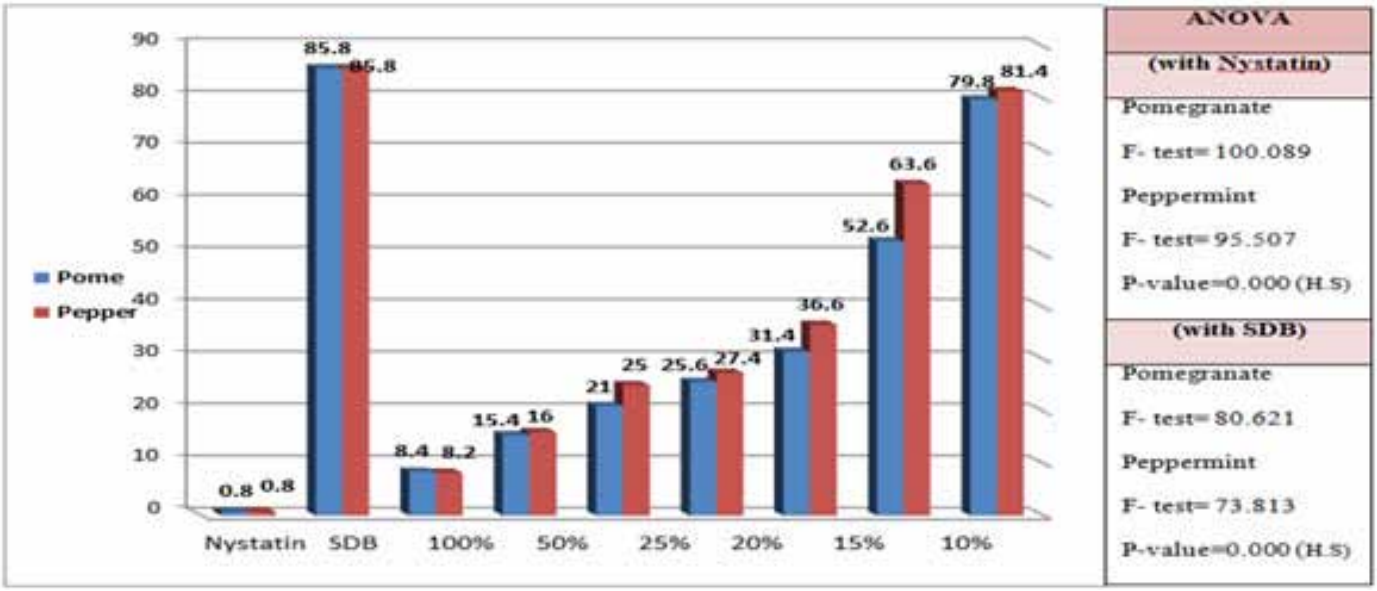

Figure (4): Distribution of minimum inhibitory concentrations of pomegranate and peppermint extracts.

Table (3): LSD of minimum inhibitory concentration of pomegranate and peppermint extracts with Nystatin.

	100%	50%	25%	20%	15%	10%	5%
Nystatin	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01
100%	-	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01
50%	-	-	N.S	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01
25%	-	-	-	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01
20%	-	-	-	-	(pomegranate) N.S P>0.05	H.S P<0.01	H.S P<0.01
15%	-	-	-	-	-	H.S P<0.01	H.S P<0.01
10%	-	-	-	-	-	-	H.S P<0.01

In table (4) there were highly significant difference between SDB with all concentrations of both extracts 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%), (25% with 20% and 15% pomegranate) and (20% with 15%) there were non-significant difference P>0.05.

Table (4): LSD of minimum inhibitory concentration of pomegranate and peppermint extracts with SDB.

	100%	50%	25%	20%	15%	10%	5%
SDB	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	N.S P>0.05
100%	-	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01
50%	-	-	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01	H.S P<0.01
25%	-	-	-	N.S P>0.05	H.S P<0.01	H.S P<0.01	H.S P<0.01
20%	-	-	-	-	N.S P>0.05	H.S P<0.01	H.S P<0.01
15%	-	-	-	-	-	H.S P<0.01	H.S P<0.01
10%	-	-	-	-	-	-	H.S P<0.01

Discussion

Plant extracts were used for long time to inhibit fungal growth, many studies conformed this effectiveness because the constituents of the extracts inhibit and/or cause damage to yeast, candida albicans growth and development. (César de Souza Vasconcelos et al, 2003,Tayel and El-Tras, 2010, Alsaidy et al,. 2014). In present study pomegranate and peppermint extract were tested for their effect on C. albicans. Extracts were prepared by freeze dried (lyophilization). (Kassab-Bashi, 2014), with preparation of pomegranate extract, the difficulty of it was increasing in density so the extract was followed by evaporation by vacuum rotary evaporator at 55 oC until extract powder was obtained. This is attributed to texture of pomegranate extract which is viscous in nature, lyophilizer machine was useful. Sensitivities of C. albicans 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, this indicate that chemical constituents of pomegranate 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 (Jurenka, 2008). These results were in agreement with other studies that concluded that the fruit peel of Punica granatum was effective for inhibiting Candida albicans growth. (Tayel and El -Tras, 2010, AL-Mizraqch et al, 2010, Madugula et al, 2017). The results were also in accordance with the report of César de Souza Vasconcelos et al., 2003, Duraipandiyan et al 2006, and with those of Pereira et al., 2006 who assessed the minimum inhibitory concentrations of adherence of pomegranate extract against S. mitis, S. mutans, S. sanguis, and C. albicans, but in contrast with Abdollahzadeh et al, 2011 that show there was no effect on C. 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 in agreement with that of Mimica-Dukic et al, 2003 and AL-Mizraqch et al, 2010, who mention to the lack of the inhibitory effect of the water extracts of peppermint but disagree with other result (Alsaidy et al, 2014) that showed there were inhibition zone. The diameter of the inhibition zone will depend on the ability of the test substance to diffuse uniformly through the agar medium (Bagamboula et al, 2004). The variant in inhibitory effectiveness of water extracts was attributed to the chemical composition of the plant and its effective compounds and concentration, as well as their solubility in water or organic solvents during the extraction process, duration, and timing of plant collecting and other factors. (Alsaidy et al, 2014). Viability count of C. 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 pomegranate With control (D.W) and even with each other's in reduction of candida count. conclusions of previous studies indicated that the chemical constituents of pomegranate have antifungal effect (Höfling et al, 2010). These results were in accordance with the results of Prashanth et al, 2001 who found that extracts of pomegranate (Punica granatum Linn) has strong antimicrobial activity. Also different extracts of pomegranate give good antibacterial activity against different bacterial strains. (AL-Mizraqch et al, 2010). In actual fact, when we performed the chromate-graphic screening of extract, the main compounds identified were ellagic acid derivatives and ellagitannins, such as punicalin, it has been found that punicalagin, pedunculagin, telimagrandin and galagildilacton have important antimicrobial activity against Candida albicans cellular structure (Anibal et al, 2013, Bakkiyaraj et

al, 2013). Hence, the main compound involved in anti-Candida action exist of the Pomegranate fruit peels seems to be the punicalagin which was detected by present study and other studies .(Endo et al, 2010, Bakkiyaraj et al, 2013, García-Villalba et al, 2015), this was in agreement with (Prashnath et al, 2001, AL-Mizraqch et al, 2010), 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 C. albicans. For peppermint the counts of viability were decreased with highly significant difference with control, this result in agreement with (Lima, 2017, Desam et al, 2017) which concluded that peppermint (Mentha piperita L.) oils show antibacterial and antifungal action against gram positive and gram negative bacteria, in addition to yeast and fungi, typically 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 peppermint and its main constituents (Menthol, and Menthone) on the structure and function of membrane integrity may be a result of reducing ergosterol levels that eventually cause cell death rendering mint fungicidal. (Samber et al, 2015)

Conclusions

Within the limitations of this study, it can be concluded that:
-Pomegranate and peppermint extract can be used as an effective natural herb against C. albicans when used in disinfectant form.
-Pomegranate and peppermint as a denture disinfectants used in 25% results in reduction of C. albicans cells count.

Conflict of interest

We are the author's (Taisir Khaleel Ismael and Amal Abdul Latif), state that the manuscript for this paper is original, and it has not been published previously (or part of MSc. dessertation) and is not under consideration for publication elsewhere, and that the final version has been seen and approved by all authors.

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