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In vitro Anti-candida Activity of Different Saudi Honeys and Honey Mixed with Taifi Rose Oil

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***Candida albicans* is a common human yeast that infect several epithelial tissues including vagina. The increase of drug-resisting *C. albicans* encouraged the researchers to find alternative treatment. Honey medical signatures such as bactericidal, antifungal and anti-candida made it a possible candidate for disease treatment. In addition, rose essential oil possesses a wide range of biochemical activities in folkloric medicine including anti-microbial activities. The present research utilizes honey alone or in conjunction with Taifi rose (*Rosa damascena*) oil as anti-candida agent to treat *vaginal candidiasis*. Three local monoforal honeys from different flower sources and/or geographic origins were tested with four concentrations (50, 80 and 95%), while two concentrations of the Taifi rose oil (1 and 2%). Anti-candida activity of honey alone or in conjunction with Taifi rose oil was determined as well as phenolic and flavonoids contents were determined. Also, GC-MS analysis of volatile oils and alkaloids were evaluated. The results of this study indicated that acidity is within the allowed range for commercialization and long-lasting storage. All honeys tested inhibited completely the *C. albicans* growth at concentrations 80% and 95% either incubation after 48 or 72 h. Also, only Markh and Manuka honeys were completely inhibited *C.albicans* growth at 50% concentration. Also, *C.albicans* growth inhibited completely at 2% Taifi rose oil after the incubation periods of 48 and 72 h. The phenolic compounds and flavonoids were analysed by mass spectrometry analysis which revealed the Markh honey showed the presence of gallic acid and quercetin that proved to have antifungal activity. It could be concluded that mixed Markh honey and Taifi rose oil treatment was capable to inhibit *C.albicans* growth completely. Further research is required to determine the anti-candida activity of the mixture of Markh honey and Taifi rose oil in the human body as a new therapeutic drug to treat *vaginal candidiasis*.**

Keywords: Phenols, flavonoids, *vaginal candidiasis*.

Candida albicans is among the most common human yeast that adheres and colonizes epithelial tissues of the mucosal membranes including the oral, gastrointestinal tract, bladder and genitalia especially vagina (Rodrigues *et al*., 2019). *Candida* infection was raised dramatically in terms of severity mainly due to the increase of disorder incidences - such as cancer and AIDS-,

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chemotherapy, etc.(WHO, 2014 and Darvishi *et al*. 2015). The incidence of fungal infections by *Candida* spp. is increasing in both the community and hospital environments. *C.albicans* causes over 50% of oral infections, while as high as > 90% of vaginal candidiasis (Irish *et al*. 2006; Bahadoran *et al*., 2010 and Cortegiani *et al*., 2018).*Vaginal candidiasis* is common in Saudi Arabia in which 70.2% is yeast vaginitis caused by *C. albicans*(Al-Aali 2013). Overgrowth of yeast can result from pregnancy, high-dose oral estrogen use, contraceptives tablets, over usage of broad-spectrum antibiotics, uncontrolled diabetes, corticosteroids and obesity (Darvishi *et al*. 2015 and Yokoyama *et al*., 2019). The increase of drug-resisting *C.albicans* encouraged the researchers to find alternative treatment.

Over thousands of years, the old Egyptian, Greek and Muslims have used honey as a folkloric medicine for many diseases (Hegazi, 2012). Honey has biological activity as enhance immune response (Hegazi *et al*., 2015 and 2017a), potential antibacterial activity (Hegazi *et al*.,2014a, 2014b and 2017b), antitumor and antioxidant((Hegazi *et al*.,2014c) and antioxidant (Hörmet-Öz *et al*. 2009). However, the differences between honey types are due to maturation of the honey itself inside the honey bees and the derivation from different kinds of flowersources (Michalkiewicz *et al*. 2008). Moreover, seasonal climatic, geographic origin, harvesting time, storage and processing conditions all play significant roles of honey bioactive effects (Michalkiewicz *et al*. 2008). In Arab cultures, honey is extremely used for both nutrition and therapy, however, there is a limited number of research investigations on the antimicrobial activity of honey published in Arabian region (Hegazi 2011 and Hegazi *et al*.,2017b).

Other alternative suggestion for candida treatment is the rose oil. Rosaspecies members of the Rosacea family are important decorative plants with pink flowersand have beenreferred to as the "King of Flowers" and the "Sambal of Love" (Mahboubi 2016). *Rosa damascena* is one of the most important Rosa specieswhoseproducts arerose concrete,rose water, rose oil, rose absolute, and dried petals (Abdel-Hameed *et al*. 2013). These products have long been used incosmetics, perfumes, medicinal purposes and food industries (Mahboubi 2016). Rose essential oil in addition to its perfuming effects was reported to possess a wide range of biochemical activities in folkloric medicine such as hypnotic,analgesic, antispasmodic, anticonvulsant, anti-inflammatory, anti-oxidants and anti-microbial activities(Ulusoy *et al*. 2009). Taifi rose (Ward Taifi, e.g., *Rosa damascena trigintipetala* Dieck) is one of the most important commercially propagatedrose in Taif, Saudi Arabia. Taifi rose essential oil is very expensive and is known for its good perfumery applications and the use in cosmetic products (Hagag *et al*. 2014).

Up to our knowledge, no research on the influence of Taifi rose oil alone or in conjunction with honey as anti-microbial agent was reported. In this study, the anti-candidia effects of different kinds of Saudi honey in addition to Taifi rose essential oil was investigated individually and in combination.

MATERIALS AND METHODS

Microorganism and culture conditions

One local *C.albicans* isolate used in this study was kindly provided by the Microbiology Unit, East Jeddah Hospital, Jeddah, Saudi Arabia. The *C. albicans* isolate was previously identified byVITEK microbial identification system. The isolate was originally cultured on Sabouraud dextrorse agar (SDA) media (Oxoid, UK) and incubated at 37°C for 48-72 h. One single colony was subcultured in three replicates on Nutrient Broth (NB) (Himedia). Rate of candida growth was evaluated in suspension cultures by spectrophotometer at OD600 and diluted to 1.0 OD by nutrient broth media (NB).

Preparation of honey and Taifi rose's essential oil samples

Four kinds of monoforal honey fromdifferent flower source and/or geographic origin, were used. Three of them (Markh, Qatad and Sider) were obtained from a commercial Al Nahl Al Gawal Aprifarm (AlGuthami Foundation, Jeddah, Saudi Arabia), while the fourth is Manuka honey (New Zealand). All honey types were stored in sterile bottles in the dark at 4°C temperature until used. Four different concentrations (50, 80 and 95%) of honey were used in addition to the control (nutrient broth media without honey). Dilutions were prepared just before use. Essential oil extracted from Taifi rose(*Rosa damascena*

trigintipetala) was kindly provided by farms of Taif rose. Two different concentrations of the Taifi rose oil (1 and 2%) were used in addition to the control (nutrient broth media without oil).

Physiochemical analysis of honey

The physiochemical analysis of honey samples (Hegazi *et al*., 2018).Thephysicochemical properties in terms of glucose, fructose, and sucrose contents and parameters of identity and quality of local honeys in terms of moisture content and acidity. TheCodex Alimentarius Committee (SASO, 1990) and Hegazi *et al*., 2018) permitted a maximum value in commercialized honey.

Estimation of anti-candida activity

A freshly grown *C.albicans* suspension was cultured in Falcon tubes (50 ml) with different honey (50, 80 and 95%) or Taifi rose oil (1 and 2%) dilutions in nutrient broth (Himedia) in three replicates. Based on the results, an extra culture of *C. albicans* was prepared to include the lowest concentration of a selected local honey that showed the highest anti-candida activity mixed with Taifi rose oil at the low concentration (1%). All tubes were incubated at 37°C for 48 and 72 h incubation periods with continuous shaking at 150 rpm. For control, microbe-free media was used. Each broth culture (100 µl) was serially diluted by sterile water to the dilution factors 10^2, 10^3 or 10^4. Then, cultures were spread into SDA agar plates and incubated at 37°C for 72 h. The colony forming unit (CFU)was measured by open CFU 3.9.0 software (Geissmann 2013).

Determination of total phenols and flavonoids in Markh honey

For the determination of free phenols and flavonoids, the Markh honey sample was diluted 10 times with (80%) ethanol solution. For determination of total phenols and flavonoids, the honey samples were further diluted 10 times by 2M HCl and heated at 95°C for 30 min to liberate the ether binding phenols and flavonoids. The total phenols were determined by Lowry method (Lowry *et al*. 1951) , while the total flavonoids were determined in both extracts by the aluminum chloride method(Akbay *et al*. 2003; Barker 2019)

The phenolic compounds and flavonoids were detected in hydrolysate solution of honey by mass spectrometry using Xivo TQD mass unit: Waters, after electrospray ionization (ESI) using daughter scan mode and negative ion detection [M-H].The capillary and cone volts were 2.7 KV and 30 V, respectively, while the desolvation gas flow was 600 L/h. The MW and daughter ions for the different compounds were detected. Gallic acid has MW of 170, while daughter ions of 150.6, 125 and 97. Caffeic acid has MW of 180, while daughter ions of 161, 107.1 and 88.7. Vanillic acid has MW of 182, while daughter ions of 59.2, 67.1, 78.8, 93.0, 106.4 and 120.8. Quercetin has MW o f302, while daughter ions of 273, 178.9 and 151.

GC-MS analysis of volatile oils and alkaloids

The analysis was performed using Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C, then increased by 5°C/min to 230°C and held for 2 min, then increased again to the final temperature of 290°C by 30°C/min and held for 2 min. The injector and MS transfer line temperatures were kept at 250 and 260°C, respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database.

RESULTS

General description of the different honey types used in the present study is shown in Figure 1. It included the physicochemical properties in terms of glucose, fructose, and sucrose contents and parameters of identity and quality of local honeys in terms of moisture content and acidity. The results indicated that contents of glucose and fructose are almost the same in the three used local honey. Sucrose content was much less in the three types of honey especially Qatad. Moisture content is almost similar for the three types of honey averaging ~15%. Acidity was much higher in Markh (40 meq/kg) as compared with the other two types of honey (Figure 1).

As shown in Table 1and Figure 2, all four

honey types completely inhibited the *C.albicans* growth at both concentrations 95% and 80% after either incubation period, e.g., 48 or 72 h. On the other hands, Markh and Manuka honey continued to completely inhibit *C. albicans* growth at 50% concentration, while Qatad and Sider honey gave counted growth at both incubation periods of 48 and 72 h (Figure 2). Much heavier growth was shown for the latter two types of honey as compared with Markh and Manuka honey at 50% concentration. Overall, Sider seemed to have the least anti-candida effects, while Manuka had the highest effects at both incubation periods as compared to the other types of honey. The data of Taifi rose oil indicated that 2% had completely inhibited *C.albicans* growth at the incubation periods of 48 and 72 h, while 1% concentration caused a declined *C.albicans* growth (Table 2). Based on the above-mentioned results, Markh was selected for further analysis in order to detect the accumulative effects of mixing honey at either 30 or 50% with rose oil at 1% for either incubation

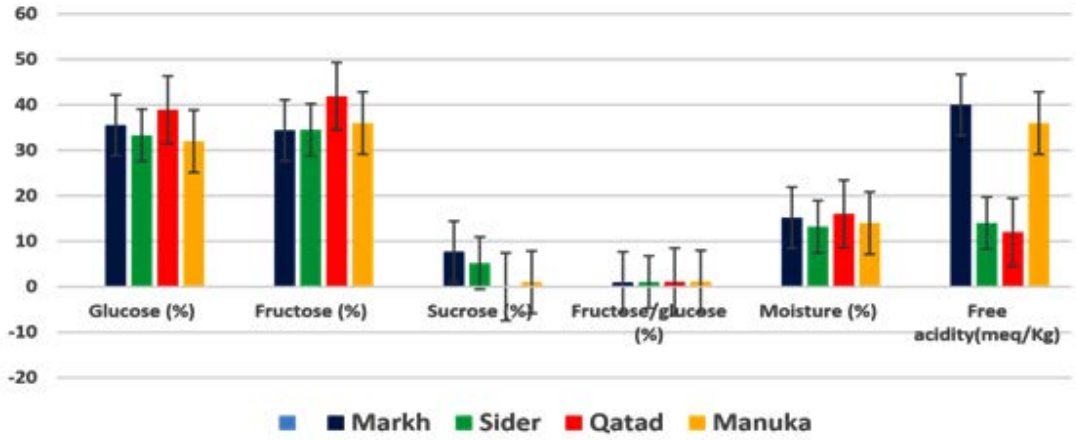

Fig. 1. Physiochemical properties of Examined honey samples

Table 1. Influence of different kinds of honey dilutions (at 95, 80, 50 and 30%) on *in vitro* growth of *C.albicans*

	48 h				72 h			
	95%	80%	50%	30%	95%	80%	50%	30%
Manuka	0	0	0	256	0	0	0	258
Markh	0	0	0	799	0	0	0	642
Qatad	0	0	225	N/A*	0	0	82	N/A
Sider	0	0	1879	N/A	0	0	1989	N/A
Candida Control		27900				128400		

Table 2. Influence of Markh honey and combinations with Taifi rose oil (at 1 and 2%) on *in vitro* growth of *C.albicans*

Time	48 h		72 h	
Concentration	1%	2%	1%	2%
Taifi rose oil	1.81	0	2.25	0
Mixed treatment (50% Markh + 1% rose oil)	0	0		
Candida Control	27900		128400	

period of 48 or 72 h, respectively. The results indicated that the two mixed honey/oil treatments were capable to completely inhibit *C.albicans* growth (Table 2).

The analysis of phenols showed the presence of 0.1 % free phenols and 0.96% of total phenols (free and ether bind phenols). The mass spectrometry analysis for detection of phenolic compounds and flavonoids showed the presence of caffeic acid, gallic acid and quercetin (Figure 3). The mass spectrum of gallic acid clearly showed

the [M-H] ion at m/z 169 and all daughter ions of 150.6, 125 and 97 were detected (Figure 2a). The mass spectrum of caffeic acid clearly showed the [M-H] ion at m/z 178.9 and all daughter ions 161, 107.1 and 88.7 were detected (Figure 2b). The mass spectrum of quercetin showed the [M-H] ion at m/z 301.1 and all daughter ions of 273, 178.9 and 151were detected (Figure 2c).

DISCUSSION

The mechanism of the anti-candida effect of honey is not completely understood, however, several honey characteristics have been proposed. They include acidity and osmolarity due to high sugar content up to a certain threshold (Hegazi *et al*. 2017; Zam *et al*. 2018 and Hegazi *et al*., 2018). In this study, Markh honey had the highest free acidity as compared with the other two kinds of local honey (Table 1), thus, had the highest anti-candida effect (Table 2). Free acidity is an important parameter related to the deterioration of honey. Then, we speculate that Markh is potent to more storage periods. Free acidity is characterized by the presence of organic acids in equilibrium with several other elements such as lactone, internal esters and some inorganic ions such as phosphates, sulfates and chlorides (Moreira *et al*. 2010 and Hegazi *et al*. 2017and Hegazi *et al*., 2018). The free acidity in Markh honey was 40. The Codex Alimentarius Committee on Sugars stated that values higher than 50 may be indicative of fermentation of sugars into organic acids especially in monoforal honeys (Fett *et al*. 2015). Therefore, many organic acids have influence on honeys' free acidity (SOS, 1999, Alves *et al*. 2013; Tornuk *et al*. 2013; Hegazi *et al*. 2018).

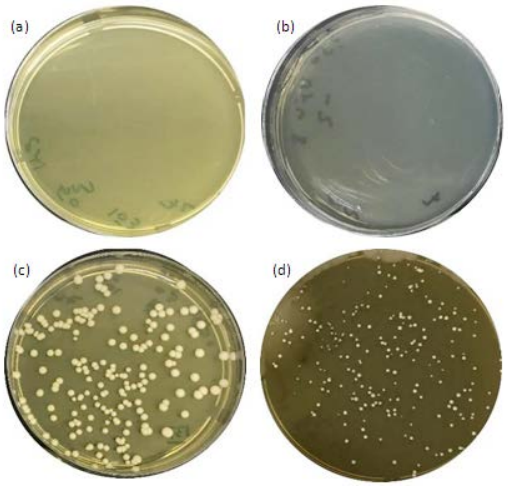

Fig. 2. *C.albicans* growth at 103 CFU for 48 h incubation on Saboraud Dextrouse agar medium with 50% concentration of honey. (a) = Manuka honey, (b) = Markh honey, (c) = Qatad honey, (d) = Sider honey

The antifungal activity of honey also relies on several other factors such as activity of glucose oxidase to retard ripening of nectar, hence, reduce the level of hydrogen peroxide (AL-Waili *et al*. 2013; Hegazi 2011; Hegazi *et al*. 2017; Zam *et al*. 2018). Moreover, geographic origin, harvest season and flower source contribute to antibacterial mechanism of honey Hegazi *et al*. 2017; Zam *et al*. 2018). In the current study, four different kinds of honey from different flower source in different geographic origins were studied. Markh and Qatad are monofloural types of honey from *Leptadenia pyrotechnica* and *Astragalus spinosus* flowers, respectively, growing in Tihama, Madinah Almonawarah region, Saudi Arabia. They have ambergris and light ambergris colours and are grown at spring and autumn seasons, respectively. Sider is also a monofloural honey from *Ziziphus nimmularia* flowers growing in Fakhrah region in Saudi Arabia. It has an ambergris colour and is grown at autumn season. The fourth kind of honey is Manuka New Zealand honey, from the flowers of native Manuka tree. Manuka has a light ambergris colour.

Prior results indicated that the flower source of Markh honey (*Leptadenia pyrotechnica*) has components such as phytols, cardenoids, flavonoids, alkaloids, steroidal glycosides and terpenes (Ghaneian *et al*. 2015; Verma *et al*. 2014). Phytol (3,7,11,15-tetramethylhexadec-2-EN-1-OL) is an important member of branched chain unsaturated terpene, and is a product of chlorophyll metabolism in plants. Interestingly, phytol can inhibit microbes (Ghaneian *et al*. 2015). Manuka honey has been also shown to contain high levels of methylglyoxal (MGO) produced by the non-enzymatic conversion of dihydroxyacetone that presents at high concentrations in the nectar of *Leptospermum scoparium* flowers. Reports indicate that neutralization of MGO in Manuka honey abolished the antimicrobial activity of the honey against *Staphylococcus aureus*, but did not abolish the antimicrobial activity against *Escherichia coli*

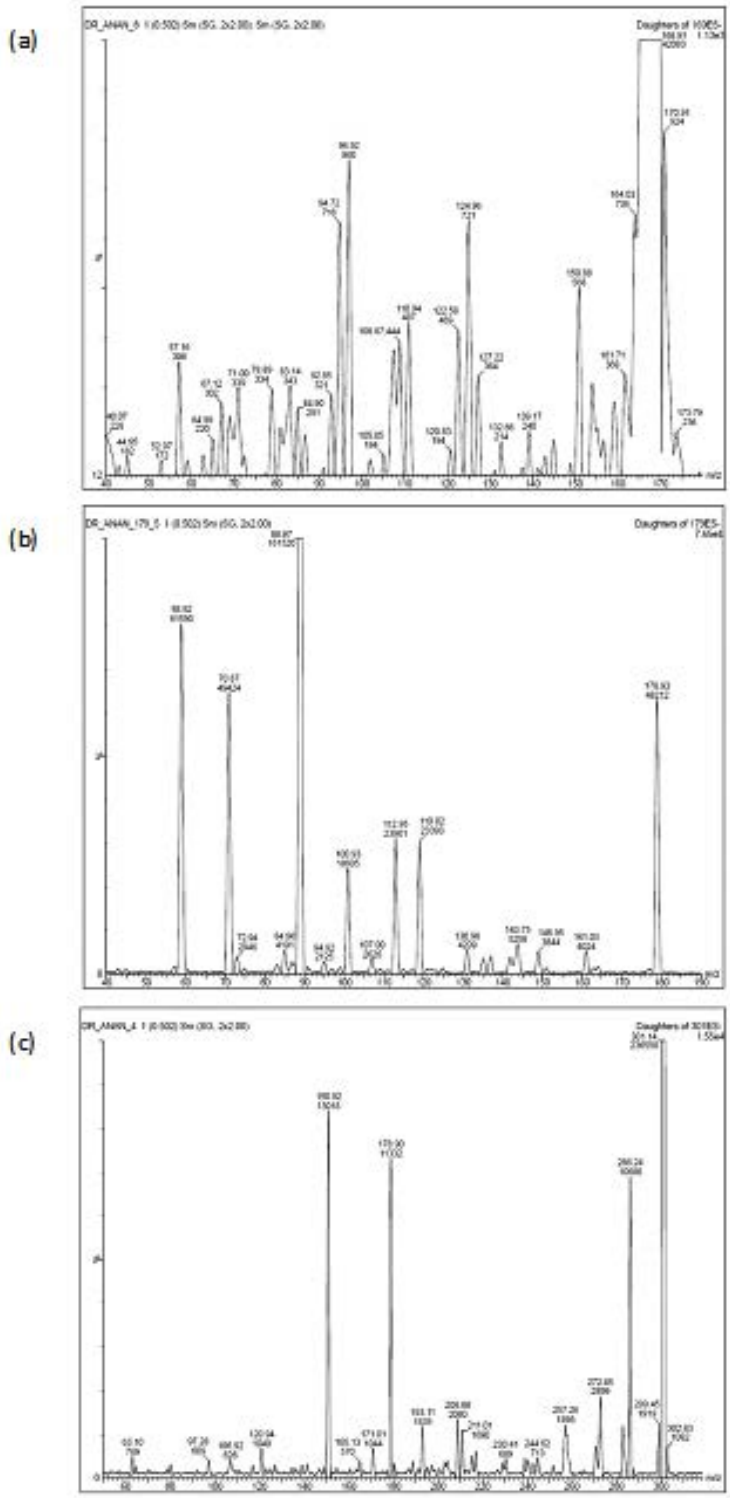

Fig. 3. The mass spectra of gallic acid (a), caffeic acid (b) and quercetin (c) showing the [M-H] ion of gallic at m/z 169, caffeic at m/z 168.9 and quercetin at m/z 301.1 in addition to the daughter ions for each compound

and *Pseudomonas aeruginosa*(Kwakman *et al*. 2011; McLoone *et al*. 2016; Matzen *et al*. 2018). The authors concluded that MGO is not fully responsible for Manuka honey's non-peroxide antimicrobial activity and that other components, possibly polyphenols, may be responsible.

In contrary to our results, Hegazi and others indicated that Qatad honey has the highest antibacterial activity on *Klebsiella pneumoniae* as compared with other Saudi honey (Hegazi *et al*. 2017). However, the latter study was concentrated on the anti-bacterial, rather than anti-candida, influence. Al-Waili *et al*. (2013) indicated that the minimum inhibitory concentration to inhibit candida growth is 70%. They also indicated that the rate of growth after 72 h was similar to that after 24 h when candida was cultured on the same honey concentration. Other reports indicated that honey at 80% could completely inhibit the candida growth when incubated for 2-6 hour (Khosravi-Darani *et al*. 2013). Our results recommend the further use of either 30 or 50% in treating vaginitis. Higher concentration might be sticky enough to prevent penetration of the honey within the tissues, thus, retards the anti-candida effect.

The detected phenolic compounds are among those of the aerial part of *Leptadenia pyrotechnica* plant (Khasawneh *et al*. 2011). The quercetin is a flavonoid exists in the form of quercetin-3-â-D-glucoside in the plant (Moustafa *et al*.2009). This compound is well known as an antioxidant and antimicrobial agent. Quercetin also showed antifungal activity against *Cryptococcus* spp. (Oliveira *et al*. 2016). It was the most active flavonoid tested against dermatophytes (Bitencourt *et al*. 2014). The alcoholic extract of *Sebastiania commersoniana* exhibited significant antifungal activity against the dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The active components in this extract include quercetin and gallic acid that are among flavonoid and phenolic compounds (Hnatyszyn *et al*. 2007). Among the tested phenolic compounds against *Candida* sp., gallic acid showed the highest MIC value (Alves *et al*. 2014). The GC/MS analysis of volatile oils and alkaloids showed absence of any volatile oil compounds, while the alkaloid 5-Heptyl-2-pyrrolidinone was detected at retention time 6.2 min. Pyrrolidin derivatives have been extensively studied as antifungal compounds (Bharose and Gajera 2018; Moradi *et al*. 2013). Interestingly, previous investigations of the chemical composition of Manuka honey also showed the presence of gallic acid, caffeic acid, and quercetin (Alvarez-Suarez *et al*. 2014).. Therefore, the antimicrobial activity of Manuka honey can also be attributed to the presence of these compounds.

In the present study, the possible accumulative impact of Taifi rose oil mixed with Markh honey on the candida growth, rather than the separate impact of either treatment (1% rose oil or 30% Markh honey), was suggested. Prior reports showed that honey and ginger extract hada more significant inhibition of *Candida albicans* growth (Khosravi-Darani *et al*. 2013). Darvishi *et al*. (2015) also indicated that honey mixed by yogurt is more effective on treating vaginal candidiasis than clotrimazole vaginal cream. The present study its kind to provide a substantial *in vitro* investigation of the anti-candida effects of Markh honey and Taifi rose oil, either separately and mixed. Further research is required to determine the anti-candida activity of the mixture when treating vaginal candidiasis in the human body. Successful results might suggest the use of the new mixture as a therapeutic drug for patients with vaginal candidiasis.

CONCLUSION

From the current results, it is concluded that a substantial *in vitro* investigation of the anti-candida effects of Markh honey and Taifi rose oil, either separately or mixed,indicates the possible use as complementary anti-candida agent.

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