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Original research

Evaluation of antibacterial and probiotic growth stimulatory activities of some honey types produced in Khuzestan

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A B S T R A C T

Honey has a long history of benefits for the treatment of digestive ailments. Certain honey types have well-established bioactive properties including antibacterial and prebiotic activities. This study was conducted with the aim of determining the amount of phenolic compounds and also investigating the prebiotic and antimicrobial activities of astragalus, thyme, and clover honey in Khuzestan. As a result of the phenolic content analysis among the honey samples, thyme honey showed the most prominent results in terms of the amount of phenolic compounds. Astragalus, thyme, and clover honey samples had good antibacterial activity against *Salmonella typhi* and *Listeria innocua*. Moreover, the studied types of honey have improved the stimulating effect on the growth of selective probiotic bacteria (*L. plantarum*). Thus, this study provides fundamental knowledge of honey prebiotic and antibacterial activities for the development of functional products.

Keywords: Honey; Thyme; Astragalus; Clover; Prebiotic

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1. Introduction

Honey is a by-product of the nectar of flowers and the upper aero-digestive tract of the honey bee (Eteraf-Oskouei et al., 2013; Akbari et al., 2020). The composition of the produced honey is influenced by the source of the plants that the bees feed on (Almasaudi, 2021; Gul & Pehlivan, 2018). However, glucose and fructose have been introduced as the dominant compounds in honey obtained from different sources. (Akbari et al., 2020; Almasaudi, 2021; Cheung et al., 2019). In addition to carbohydrates, honey has been proposed as a rich source of minerals, amino acids, protein, vitamins, organic acids, phenolic compounds, and enzymes. (Almasaudi, 2021; Cheung et al., 2019; Gul & Pehlivan, 2018; Mustar & Ibrahim, 2022).

Honey has attracted a lot of attention as a natural product with wide therapeutic effects, and its use in traditional medicine has a long history (Eteraf-Oskouei et al., 2013). For example, for a long time, honey has been used as a suitable product for gastrointestinal, cardiovascular, liver, and wounds (Almasaudi, 2021; Gul & Pehlivan, 2018; Eteraf-Oskouei et al., 2013). Honey has also been introduced as a natural prebiotic product to promote good digestive health. Prebiotic product by stimulating the development and promotion of the metabolic activity of microorganisms in the colon, probiotics lead to the improvement and increase of the health of the host (Mustar & Ibrahim, 2022; Schell et al., 2022). In addition, antibacterial, antiviral, and antiparasitic activity is other properties of honey that have been mentioned in several studies (Eteraf-Oskouei et al., 2013; Gul & Pehlivan, 2018). Honey has been utilized since ancient times as a traditional remedy in the treatment of bacterial infections, coughs and colds, and various infectious diseases (Almasaudi, 2021). The prevalence of infectious diseases and some claims about the effect of some natural compounds on the prevention and treatment of some diseases have attracted researchers' attention to the use of natural compounds from plant sources (Leyva-Jimenez et al., 2019). On the other hand, the adverse effects of chemicals and synthetic drugs on human health have increased the desire to use traditional and more natural methods (Gul & Pehlivan, 2018).

Consumer preference is often influenced by the sensory characteristics and different bioactive attributes of honey. Among all the chemical compounds in honey, phenolic compounds are one of the most important substances affecting their biological activity (Cheung et al., 2019). Therefore, this study was conducted with the aim of determining the total phenolic content of honey of thyme,

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astragalus, and clover honey of Khuzestan. Then the antimicrobial activity and prebiotic potential of honey were investigated.

2. Material and Methods

2.1. Materials

All solutions, chemical compounds, and culture media were prepared from Merck Company. Thyme, astragalus, and clover honey were obtained from reputable beehives in Ahvaz, and the tests to verify the characteristics and authenticity of the samples were carried out in the Faculty of Food Hygiene of the Shahid Chamran University of Ahvaz.

Pure culture of *Lactobacillus plantarum* (PTCC 1896) as an indicator of probiotic bacteria and microbial strains of *Salmonella typhi* (PTCC 1609) and *Listeria innocua* (ATCC 33090) bacteria was prepared from the Iranian Scientific and Technological Research Center in lyophilized form.

2.2. Evaluation of the phenolic content

Polyphenols in honey were measured by coloring method using Folin–Ciocalteu reagent; 2.5 g of honey was dissolved in 25 mL of distilled water and filtered by Watman filter paper. 0.5 mL of this solution was mixed with 2.5 mL of Folin–Ciocalteu reagent and kept for 5 min. Then, 2mL sodium carbonate 0.7 M was added to the solution and kept at ambient temperature in darkness for 2 h. The solution adsorption was read by spectrophotometer at a wavelength of 750 nm against the control sample. In the control sample, distilled water was used instead of the sample. The phenolic acid content was calculated in milligrams of gallic acid per kg (Kıvrak & Kıvrak, 2017; Alvarez-Suarez et al., 2010).

2.3. Evaluation of the prebiotic ability of honey

The pure cultivation of *L. plantarum* grew up to a constant growth phase in the MRS broth medium. After activation of the species, 10^6 CFU/ml was added to the MRS medium, and and different concentrations of honey (1, 3, and 5%) was added to the MRS medium. Simultaneously, MRS media (1, 3, 5%) glucose as simple sugar and MRS media with 1,3 and 5% artificial honey (33.5 g glucose, 42 g fructose, 7.5 g galactose, and 17 mm distilled water) were prepared. The *L. plantarum* was counted in these mediums at 0, 24, 48, and 72 hours (Mohan et al., 2017).

2.4. Evaluation of the antimicrobial activity of honey

The antimicrobial activity of honey against *S. typhi* and *L. innocua* pathogens was investigated. For this purpose, first, Different concentrations of honey were prepared in a TSB medium, and each tube containing 10^6 CFU/mL was incubated for 24 h at 37°C. The overnight bacterial cultures grown on Mueller-Hinton agar plates were suspended in water to obtain an optical density at 600 nm = 0.132 (corresponds to 0.5 McFarland turbidity standard) (Małgorzata et al., 2020). Then bacteria were diluted in double concentrated Mueller-Hinton broth medium to final cells concentrations of 1-3 × 10^6 CFU/ml and utilized to determine MIC. The concentration of honey (versus control without bacteria) that did not have visible turbidity was considered as MIC. Then, by cultivating concentrations equal to or less than MIC on a solid medium (Muller Hinton Agar) and incubating for 24 h, the plate without growth was considered as MBC (Hbibi et al., 2020).

2.5. Data analyzing

The results were recorded as means ± standard deviations by IBM SPSS statistic software (Version 20.0, USA). One-way ANOVA and Duncan's multi-range tests with the probability of $p < 0.05$ had been utilized to statistically analyze data.

3. Results and Discussion

3.1. Phenolic content of thyme, astragalus, and clover honey

Honey has a variety of enzymatic and non-enzymatic antioxidants (Ahmed et al., 2018). Phenolic acids (quercetin, kaempferol, galangin, naringin, luteolin, pinocembrin) and flavonoids (caffeic acid, cinnamic acid, gallic acid, coumaric, chlorogenic) are the two primary compounds affecting the antioxidant activity of honey (Dong et al., 2013; Pauliuc et al., 2020). Determining the amount of honey phenolic compounds can be a proper parameter for evaluating its quality and therapeutic potential (Krikpatrick et al., 2017). According to Table 1, there was a statistically significant difference between the three groups of honey, thyme, astragalus, and clover, in phenolic content ($p > 0.05$).

Table 1. Comparing the phenolic content of kinds of honey.

Honey	Phenolic content
Thyme	946.48 ± 12.8 [a]
Astragalus	524.6± 4.31 [b]
Clover	334.7± 21.2 [c]

Different letters indicate a significant difference in column ($p < 0.05$).

With an average of 946.48 mg GAL/g, Thyme honey has more phenolic content than astragalus and clover honey. As mentioned above, phenolic acids and flavonoids are the main factors of antioxidant activity that also affect the taste and color of honey, so that darker honey contains more phenolic compounds. Also, the plant flora of the honey breeding area has a significant effect on the amount and type of phenolic compounds (Zhu et al., 2013).

Cheung et al. (2019) investigated plant sources, seasons, and environmental factors on the content of phenolic compounds of honey samples and concluded that honey plant origin plays the most crucial role in phenolic compounds (Cheung et al., 2019). In another study, the phenolic content in 33 samples of Chinese honey was in ranged from 10.43 mg GAL/g (Acacia honey) to 149.6 mg GAL/g (Red date honey), which this difference was attributed to plant source (Dong and colleagues., 2013). Gül and Pehlivan reported that the total phenolic content of some monofloral honey types produced across Turkey ranged between 470.70 ± 7.43 and 34.37 ± 0.44 mg GAE/100 g (Gul & Pehlivan, 2018).

3.2. Prebiotic effect of thyme, astragalus, and clover honey

At this stage, the prebiotic effect of thyme, astragalus, and clover honey at concentrations of 1, 3, and 5% on *L. plantarum* and at 0, 24, 48, 72 h were investigated. The results were compared with the control sample and artificial honey.

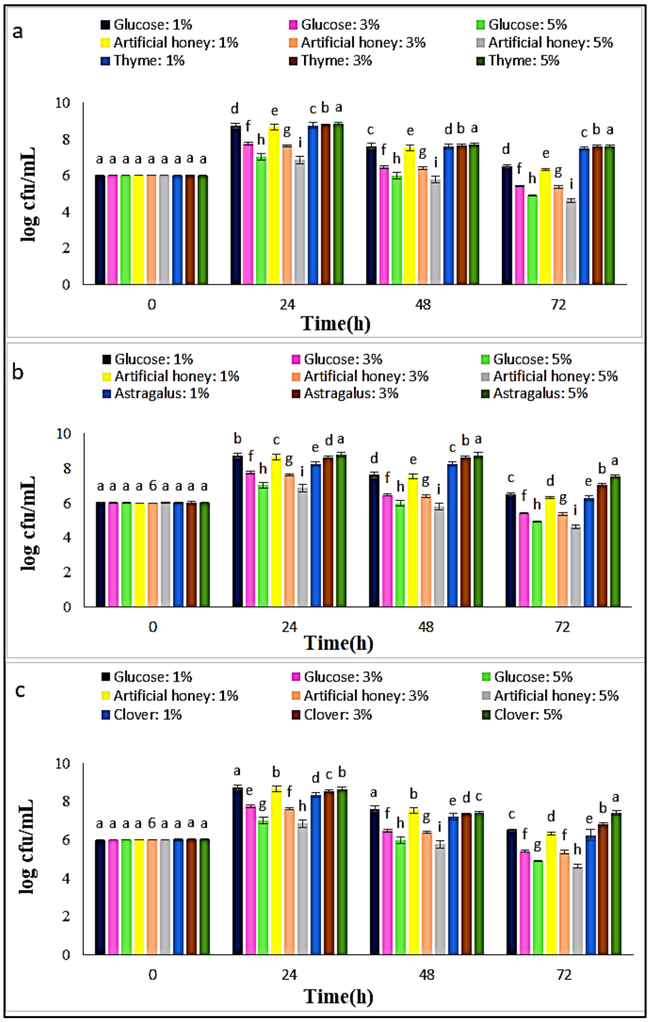

Fig. 1. Effects of thyme (a), astragalus (b), clover (c), artificial honey and glucose in different concentrations on the growth of L. plantarum. Different letters show a significant difference in the different treatments at the same time ($p < 0.05$).

In Fig. 1a, the effect of different concentrations of thyme and artificial honey (control) and glucose on the growth of probiotic bacteria at different test times was investigated. At the fermentation time of 0-24 h, a sample of 5% thyme honey showed the highest growth rate of probiotics which was significantly higher than the media containing artificial honey and glucose ($p < 0.05$). On the other hand, the growth rate of bacteria in the medium containing 1% thyme honey was significantly lower compared to the medium containing 5% thyme honey ($p < 0.05$). At fermentation time of 24-48 h, all levels of thyme, control sample (1%), and artificial honey (1%) had the highest probiotic growth and showed a significant difference with the other media ($p < 0.05$). Moreover, at this time, the media containing 5% of glucose and artificial honey had the lowest count of *L. plantarum* cell ($p < 0.05$). On the last day of storage (72 h), media containing thyme honey with concentrations of 3 and 5% showed the highest probiotic growth rate, which was significantly higher than other media ($p < 0.05$). At this time (72 h), the media containing 5% of artificial honey had the lowest probiotic growth rate ($p < 0.05$).

In Fig. 1b, the effect of astragalus and artificial honey and glucose on the growth of probiotic bacteria was investigated at different test times. At the fermentation time of 24 h, the media containing astragalus honey at variuse concentration, glucose honey (1%) and, artificial honey (1%) had the highest probiotic growth rate. Moreover, the media containing of 5% artificial honey had the lowest probiotic growth rate. At the fermentation time of 48 h, the highest and lowest probiotic growth rates were related to media containing 5% of astragalus and 5% artificial honey samples, respectively. On the last day of the fermentation (72 h), a media containing 5% showed the highest probiotic growth rate. And a media containing 5% of artificial honey had the lowest growth rate of probiotic.

In Fig. 1c, the effect of Clover and artificial honey and glucose on the growth of probiotic bacteria was surveyed. The results show that the *L. plantarum* counts increased significantly in all substrates during the fermentation time of 0-24 h ($p < 0.05$). The bacterial counts increased with increasing honey concentration. At this time, glucose (1%) and control (5%) medium had the highest and lowest effect on *L. plantarum* growth, respectively. At the fermentation time of 48 h, the highest and lowest probiotic growth rates were related to media containing 1% of glucose and and 5% of artificial honey, respectively. On the last day of the fermentation (72 h), a media containing 5% clover honey showed the highest probiotic growth rate. And a media containing 5% of artificial honey had the lowest growth rate of probiotic.

In the present study, all three honey samples showed an excellent prebiotic effect, which is probably due to their high phenolic content, which leads to stimulating the growth of probiotic bacteria. According to the results, all three honey showed the highest prebiotic properties at the concentration of 5%. On the last day of fermentation (72 h), the count of probiotic bacteria was higher in the media containing the studied types of honey compared to the media containing artificial honey and glucose. Therefore, it seems that studied types of honey support the growth and viability of the *L. plantarum*. The decreased viability of the *L. plantarum* in the medium containing glucose and artificial honey compared to the media containing honey is due to the structural complexity of honey, which allows the production of more enzymes that these enzymes are needed to metabolize food compounds. Moreover, the honey as prebiotic compound gives microorganisms the power to maintain stability and stability. Such results have also been observed in the study on date kernal and oak polysaccharides. So that viability in the medium containing prebiotics was more than glucose-containing medium (Tadayoni et al., 2014). Similarly, it was reported in Shell et al.'s study that honey supports the growth of probiotic species of *Bifidobacterium* and *Lactobacillus* (Schell et al., 2022). In another study, it was stated that the growth of *L. acidophilus* and *B. bifidum* bacteria in an MRS medium enriched with sesame honey was much higher compared to the MRS medium without honey (Das et al., 2014).

According to reports, honey contains a large amount of fructose oligosaccharides, which cannot be fermented by human digestive enzymes. The utilized of these oligosaccharides only is possible by probiotic organisms such as *Bifidobacterium* and *Lactobacillus*. Therefore, honey can improve the metabolic function of the host gut by providing suitable metabolic substrates for probiotic bacteria (Chettri & Kumari, 2020, Mustar & Ibrahim, 2022).

Table 2. MIC and MBC definition of thyme, astragalus and clover honey.

Microorganism	MBC (w/v)			MIC (w/v)		
	Clover	Astragalus	Thyme	Clover	Astragalus	Thyme
Salmonella Typhi	3.01±0.06	3.17±0.01	3.11±0.01	40	30	30
Listeria innocua	3.27±0.04	3.23±0.02	3.32±0.06	35	25	25

In general, it has been stated in extensive studies that honey contains various compounds that provide the necessary conditions for the growth of helpful probiotic microflora and at the same time prevent the growth of pathogenic bactria, which confirms the prebiotic potential of honey (Chettri & Kumari, 2020). These results confirm the findings of the present study. However, in several previous studies, it has been reported that some prebiotics, despite the stimulating effect of probiotic bacteria, can also stimulate the growth of pathogens. For example, fructooligosaccharides can enhance eubacterium biform and *Clostridium perfringens*, while some non-carbohydrate prebiotics only stimulates probiotics' growth and do not cause unwanted side effects (Liao et al., 2018).

3.3. *Antimicrobial properties of thyme, astragalus, and clover honey*

Due to the increasing resistance of pathogenic microorganisms to antibiotics, the use of natural compounds with antimicrobial potential has attracted much attention (Małgorzata et al., 2020). Honey can be one such potential therapeutic alternative with antimicrobial potential. In this study, the MIC of thyme, astragalus, and clover honey in different concentrations against *S. Typhi* and *L. innocua* showed that these honey have good antimicrobial properties (Table 2). All three honey samples showed no antimicrobial properties at concentrations less than 25% volume in the present study. MIC for thyme and astragalus honey for *S. Typhi* bacteria was 30% volume/weight concentration, and for Listeria bacteria, this amount was 25% volume/weight. However, for honey, clover MIC had a higher amount. Based on the results, thyme and astragalus honey had higher antimicrobial activity compared to clover honey. Similarly, Voidarou et al. (2011) studied the antibacterial activity of different kinds of honey against pathogenic bacteria and reported that all honey samples due to different plant origins have different antibacterial activity, and thyme honey showed the highest antimicrobial activity (Voidarou et al., 2011). Many researchers have evaluated the antibacterial activity of honey, and it was stated that natural honey has a sizeable antibacterial activity against pathogenic bacteria (Mohan et al., 2017). Małgorzata et al. (2020) observed that Polish buckwheat honey had antimicrobial activity against *S. aureus*, *E. coli*, *S. enterica* and *K. pneumoniae* (Małgorzata et al., 2020).

The honey has several mechanisms of antimicrobial activity (Schell et al., 2022). Based on previous studies honey contains a high amount of antimicrobial components like flavonoids, cinnamic acid, glucose oxidase, ascorbic acid, phenolic acids, and carotenoid derivatives (Chettri & Kumari, 2020). The amount of these honey compounds is influenced by the floral source, geographical area, and climatic conditions (Chettri & Kumari, 2020). Al-Zahrany et al. (2012) considered the differences in antibacterial and antioxidant activities of different kinds of honey related to natural changes in the origin of flowers and plants and geographical places of honey (Al-Zahrany et al., 2012). It has been stated that hydrogen peroxide content (H_2O_2), water content, acidity, and other phytochemicals are effective factors on the antibacterial activity of honey. In the meantime, hydrogen peroxide is one of the main reasons for antimicrobial activity of honey. Many studies show that hydrogen peroxide leads to the cell death of many bacterial and yeast cells through degradation of the chromosomal DNA (Chettri et al., 2020; Kıvrak & Kıvrak, 2017). On the other hand, it is stated that the metabolism of prebiotics by probiotics in the intestines can lead to the production of short-fat chain acids, acid, and various other metabolites thus inhibiting the growth of pathogens and reducing their population in the intestines (Lee et al., 2021; Mohan et al., 2017). Moreover, increasing the count of probiotics may lead to a decrease in the total level of nutrients for absorption by pathogens, thus improving the health of the digestive system and increasing resistance to pathogen infections (Mustar & Ibrahim, 2022). It has been reported in previous studies that the indigestible oligosaccharides in honey are fermented by probiotic microorganisms (*Bifidobacterium* and *Lactobacillus*) through a saccharolytic fermentation, which leads to more growth of probiotic and the inhibition of pathogenic bacteria in the human gastrointestinal (Chettri & Kumari, 2020). On the other hand, as a result of saccharolytic fermentation, a large value of energy is released with the end product, such as short-chain fatty acids and gases, all of which have inhibitory attributes against pathogens (Chettri & Kumari, 2020). It has also been reported that lactic acid bacteria (*Lactobacillus* sp.) bacteria prevent pathogens from proliferating or surviving by disintegrating the outer part of the pathogen's cell wall (Mustar & Ibrahim, 2022).

4. Conclusion

On the one hand, the resistance of pathogens, and on the other hand, the importance, acceptability, and consumption of probiotic products of natural and plant origin, cause society's attention and tendency to produce, optimize and consume these products. Honey is a product that has long been considered and studied by most researchers due to its unique properties. Thyme, astragalus, and clover honey contained a high amount of phenolic compounds, which were significantly higher in thyme honey compared to other samples. In this study, the potential of these honey samples to promote probiotic growth was elucidated at in vitro level. This could be attributed to their polyphenol content. The studied honey also had antibacterial activity against *S. Typhi* and *L. innocua*, and clover honey showed the lowest antimicrobial activity. Therefore, it can be stated that thyme, astragalus, and clover honey can be used in functional foods due to their phenolic compounds and antimicrobial and prebiotic potential.

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Conflict of interest

Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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