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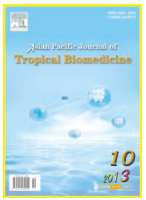

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In vitro activity of natural honey alone and in combination with curcuma starch against *Rhodotorula mucilaginosa* in correlation with bioactive compounds and diastase activity

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PEER REVIEW

Peer reviewer

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Comments

The authors reported synergistic interaction between two natural non-toxic agents, honey and curcuma, against *R. mucilaginosa*. The current findings are suggestive to prepare treatment regimen from the components of these two agents for *in vivo* application in order to combat antifungal resistance.

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ABSTRACT

Objective: To evaluate the *in vitro* activity and synergism of the combinations of natural honey and curcuma starch against *Rhodotorula mucilaginosa* in correlation with total phenolic, flavonoid contents, and diastase activity.

Methods: The Folin-Ciocalteu test was used to determine the total polyphenols content and the flavonoid content was analyzed using by the aluminum chloride method. The antifungal activity of the natural honey, determined by an agar well diffusion assay and agar incorporation method.

Results: Total phenolic content varied from (63.93±0.11) to (95.36±6.08) mg GAE/100 g honey as gallic acid equivalent. Total flavonoids content varied from (5.41±0.04) to (9.94±0.54) mg CE/100 g. Diastase activity values were between (7.3±2.8) and (26±2.8). The zone inhibition diameter for the six honey samples without starch ranged between 6 and 20 mm. When starch was mixed with honey and then added to well, a zone inhibition increase diameter 7 and 21 mm. The percentage increase was noticed with each variety and it ranged between 5% and 62.5%. The minimal inhibitory concentrations for the six varieties of honey without starch against *Rhodotorula mucilaginosa* ranged between 28% and 36% (v/v). When starch was incubated with honey and then added to media, a minimal inhibitory concentration drop has been noticed with each variety. It ranged between 6.66 % and 20% (w/v). No significant correlation was established between diastase activity and bioactive compounds.

Conclusions: The mixture of curcuma starch and honey could lead to the development of new combination antibiotics against *Rhodotorula* infections

KEYWORDS

Bioactive Compounds, Diastase number, *Rhodotorula mucilaginosa*, Honey, Curcuma starch

1. Introduction

Fungi have emerged over the past two decades as major causes of human infections, especially among immunocompromised hosts, having an enormous impact on morbidity and mortality[1,2]. Antifungal resistance, increasing costs, hospitalization time, and treatment difficulties of *Rhodotorula* infections are worldwide problems. Therefore, novel fungal therapies for effective management of *Rhodotorula* infection are urgently required. The use of natural products with therapeutic properties is as ancient as human civilization, and for a long time, mineral, plant and animal products were the main sources of drugs[3]. Honey is a natural product

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that is used for its antifungal activity[4,5]. Honey is one of the most complex mixtures of carbohydrates and other smaller components produced in nature. Sugars are the main constituents of honey, comprising about 95% of honey dry weight. The diastatic activity (α-amylase) in honey is considered a quality factor. Alpha-amylase in honey is also affected by storage time and temperature. Alpha-amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose. Diastase activity is expressed as the diastase number in Schade units and is defined as follows: one diastase unit corresponds to the enzyme activity of 1 g of honey, which can hydrolyse 0.01 g of starch in 1 h at 40 °C[6]. Therefore, it is expected that adding starch, which is the substrate of the diastase, to honey will subsequently increase the antimicrobial effect of honey[7]. Polyphenols are another important group of compounds with respect to appearance and functional properties. About 56 to 500 mg/kg total polyphenols were found in different honey types, depending on the honey type[8,9]. Recently, it was found that the bioactive compounds of honey consisted of flavonoids and phenolic acid. The flavonoid content can vary between 2 and 46 mg/kg of honey and was higher in samples produced during dry season with high temperatures[10]. The polyphenols are responsible for the antimicrobial properties of honey. The inhibitory effects of polyphenols for α-amylases have attracted great interest among researchers[11,12]. Novel combinations of honey and curcuma starch may provide a new therapeutic option for *Rhodotorula mucilaginosa* (*R. mucilaginosa*) infections. The aim of this study was to evaluate the *in vitro* antifungal activity of honey alone and in combination with curcuma starch against *R. mucilaginosa* and their correlation with bioactive compounds (total polyphenol and total flavonoid contents) and diastase number (α-amylase).

2. Materials and methods

2.1. Honey samples

The total numbers of 6 honey samples were obtained from Algeria [Tiaret, 3 samples (H3, H4 H6); Saida, 1 sample (H1); Relizane, 1 sample (H5) and Mascara, 1 sample (H2)]. All samples were transferred to the laboratory, stored in amber flasks, and kept at 4 °C until analysis.

2.2. Preparation of the stock starch solution

The stock starch solution was prepared by dissolving 0.5 g of dried soluble starch in deionised water in a volumetric flask. After heating and stirring the solution for approximately 10 min, starch was completely dissolved, and the volumetric flask was filled with deionized water to the mark.

2.3. Culture media and inoculum standardization

R. mucilaginosa were maintained on Sabouraud dextrose agar (SDA) (Merck, Germany) at 4 °C, and sub cultures were performed prior to each experiment in the same medium for 48 h at 35 °C. Turbidity standard and preparation of inocula: Stock fungal inoculum suspensions were prepared in sterile saline from 48 h cultures on SDA at 35 °C. Each suspension was adjusted visually to 0.5 McFarland turbidity standard. Dilutions of these suspensions were subcultured on SDA to determine the number of UFC/mL. The adjusted inoculum was (1×10^6-5×10^6 UFC/mL).

2.4. Physicochemical analyses

All physicochemical tests were performed in triplicate.

2.4.1. Bioactive compounds

2.4.1.1. Total flavonoids content

The total flavonoid content was determined using the aluminium chloride assay according to Amaral *et al*[13]. A 10 μL volume of a 10% (v/v) honey solution was added to the wells of a 96 well plate; then 30 μL of a 2.5% sodium nitrite, 20 μL of 2.5% aluminium chloride solutions and then 100 μL of a 2% sodium hydroxide solution were sequentially added. The samples were mixed well and the absorbance at 450 nm was measured. Total flavonoid content was expressed as mg of (+)-catechin equivalents (mg CE/kg of honey).

2.4.1.2. Total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method[14]. About 30 μL of honey solution (0.1 g/mL) was mixed with 2.37 mL of milli Q water and 150 μl of 0.2 N Folin-Ciocalteu reagent. The solution was thoroughly mixed by vortexing and incubated for 2 min at ambient temperature. A total of 450 μL of sodium carbonate solution (0.2 g/mL) was added to the reaction mixture and further incubated for 2 h at ambient temperature. The absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content was determined by comparing with a standard curve prepared using gallic acid (0-200 mg/L). The results were mean±SD and expressed as milligrams of gallic acid equivalents (mg GAE)/100 g of honey.

2.4.2. Diastase activity (diastase number)

The diastase activity was measured using the Phadebas method for α-amylase. Phadebas is a synthetic reagent which produces a blue colour when it is hydrolysed by the diastase. The absorbance at 620 nm was directly proportional to the diastase activity in the honey sample. Results were expressed in Schade units per gram of honey, defined as that amount of the enzyme which would convert 0.01 g of starch into the prescribed end point in 1 h at 40 °C under test conditions[6].

2.5. Test assays for antifungal activity

2.5.1. Agar well diffusion assay

Honeys were screened for their antifungal activity, according to the agar well diffusion method proposed by the NCCLS[15]. Briefly, solid medium diffusion procedure using wells in dishes was used to determine the anti-yeast activity of the honey. Petri plates (90 mm) were prepared by pouring 20 mL of SDA and allowed to solidify; 0.1 mL of the *R. mucilaginosa* suspensions were poured and uniformly spread. After inoculum absorption by agar, wells were made using sterile glass tubes (6 mm diameter) which were filled with. About 20 μL of test honey was added to each well. Plates were incubated at 35 ℃ for 48 h. A diffusion control of starch was used. Second step a mixture of starch-honey was prepared and incubated for 1 h at 35 ℃. After inoculation, 6 mm diameter wells were cut into the surface of the agar using a sterile cork borer. About 20 μL of mixture (honey and starch) were added to each well. Zones of inhibition were measured using a vernier caliper. The diameter of zones, including the diameter of the well, was recorded. Bioassay was performed in duplicate and repeated twice. The results were expressed in terms of the diameter of the inhibition zones: <5.5 mm, inactive; 5.5-9.0 mm, very low activity; 9.0-12.0 mm, low activity; 12.0-15.0 mm, average activity; and >15.0 mm, high activity.

2.5.2. MICs and minimum additive inhibitory concentrations (MAICs) determinations testing

Increased concentrations (1%-30% v/v) were incorporated into media to test their efficiency against *R. mucilaginosa*. Each plate with final volume of honey and media of 5 mL was inoculated and incubated at 35 ℃ for 48 h. The MICs was determined by finding the plates with the lowest concentration of honey on which the strain would not grow. All MICs values were expressed in % (v/v). After determination of MICs of honey, various concentrations of honey below their MICs were prepared. Mixtures of honey and starch were prepared by mixing various concentrations of honey with various concentrations of starch. To evaluate the effect of starch on the antifungal action of honey, 1% starch solution was prepared using sterile water. Different volumes from the stock solution were added to a range of honey concentrations lower than the MICs. The same volume of starch solution that has given inhibition with honey was added alone to media as control to check whether or not starch alone has an inhibition effect against *R. mucilaginosa*. An equivalent volume of water was added to honey instead of starch solution to confirm that additive inhibition was not due to the dilution of honey. The final volume in each plate was 5 mL. Starch content in media ranged between 1% and 8% (w/v). Honey and starch as well as honey and water were incubated for 48 h at 35 ℃ before being incorporated into media. Plates were inoculated and incubated at 35 ℃ for 48 h. Bioassay was performed in duplicate and repeated twice[16].

2.6. Statistical analysis

Each honey was analyzed in triplicate. Results are shown as mean values and standard deviation. Correlations were established using Pearson's correlation coefficient (r) in bivariate linear correlations ($P<0.01$). All statistical analyses were performed with the Statistica 7.0 software for Windows.

3. Results

3.1. Physicochemical parameters

Figure 1 shows the mean±SD, maximum and minimum values of the physicochemical parameters analysed. The honey samples analyzed in the present work showed a range of values, between 26 and 7.3 Schade units. One sample (H5) showed values below 8 Schade units (Figure 1).

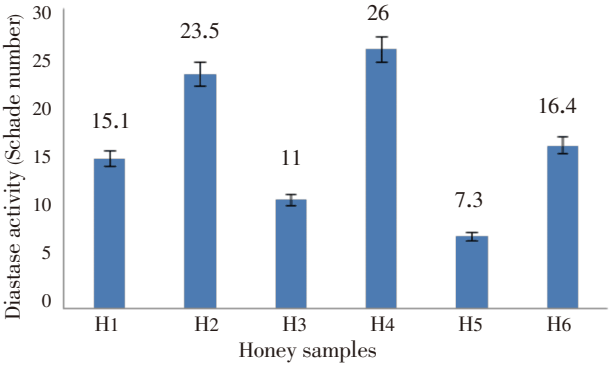

Figure 1. Diastase activity of the honey samples (mean±SD; n=3).

The explanation for the low content of diastatic activity found in this one sample could be accounted for an inadequate processing or storage conditions. The phenolic concentrations of all honey varieties were estimated as mg of GAE/100 g honey. The total polyphenol content ranged from (63.93±0.11) to (95.36±6.08) mg GAE/100 g honey and the total flavonoid content varied from (5.41±0.04) to (9.94±0.54) mg CE/100 g honey (Figures 2 and 3).

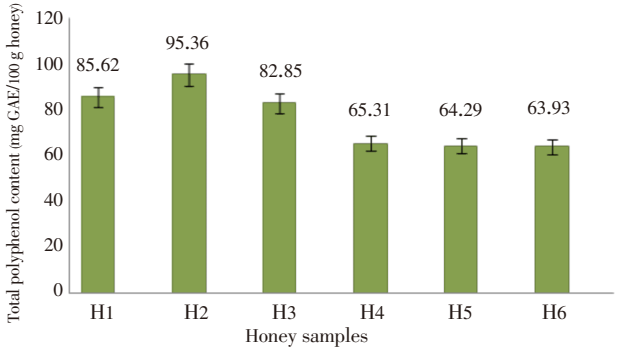

Figure 2. Ttotal phenolic content of six honey samples (mean±SD; n=3).

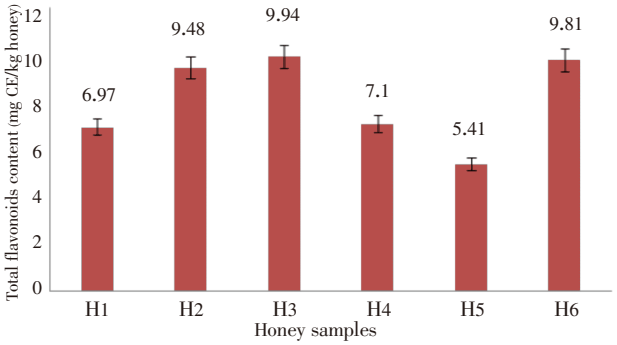

Figure 3. Total flavonoid content of six honey samples (mean±SD; n=3).

3.2. Agar well diffusion assay

Initial screening with the agar well diffusion assay demonstrated that all tested honeys alone and in combination with curcuma starch had antimycotic action against *R. mucilaginosa*. The antimycotic action of honey samples decreased in the following order: H5 >H4>H3>H2>H6>H1 (Table 1).

Table 1
Effects of honey and curcuma starch alone and in combination on *R. mucilaginosa* at 35 °C for 2-day incubation.

Samples	Honey only		Honey and starch			
	ZID (mm*)	MIC %	ZIID (mm*)	IP %	MAIC %	MIC drop %
H1	20	28	21	5.00	26	7.14
H2	14	30	16	14.28	24	20.00
H3	8	36	13	62.50	32	11.11
H4	10	34	11	10.00	28	17.64
H5	6	30	7	16.66	26	13.33
H6	18	30	20	11.11	28	6.66

ZID: zone inhibition diameter; ZIID: zone inhibition increase diameter; IP: increase percentage; MIC: minimum inhibitory concentration; MIAC: minimum additive inhibitory concentration. *: <5.5 mm, inactive; 5.5-9.0 mm, very low activity; 9.0-12.0 mm, low activity; 12.0-15.0 mm, average activity; and >15.0 mm, high activity.

No zone of inhibition was determined with starch alone. Without starch, the zone inhibition diameter of the six varieties ranged between 6 and 20 mm. When starch was incubated with honey and added to well, the zone inhibition increase diameter of the six varieties ranged between 7 and 21 mm which represented an increase percentage between 5% and 16.66%.

3.3. Determination of MIC and MAICs

Without starch, the MICs of tested honeys against *R. mucilaginosa* was variable [28%-36% (v/v)]. When starch was incubated with honey and added to media, the MAICs of the five varieties ranged between 24%-32% (v/v) which represented an MIC drop between 6.66%-20% (w/v) (Table 1).

4. Discussion

Rhodotorula species are emerging opportunistic pathogens, particularly in immunocompromised patients. The management of *Rhodotorula* infections faces a number of problems including limited number of effective antifungal drugs, toxicity of the available antifungal drugs, resistance of *Rhodotorula* to commonly used antifungal drugs, relapse of *Rhodotorula* infections and the high cost of antifungal drugs[17,18]. The difficulties associated with the management of *Rhodotorula* infections necessitate the discovery of new antifungal agents in order to increase the spectrum of activity against *Rhodotorula* and combat strains expressing resistance to the available antifungal drugs. In recent years, interest in the application of honey in the treatment of infectious diseases has notably increased. Although several *in vitro* studies have demonstrated the antibacterial activity of honey[19,20], limited numbers of studies have examined the activity of honey against fungi. In a recent study, the antimycotic activities of Slovenian honeys toward five types of fungi (*Aureobasidium pullulans*, *Candida parapsilosis*, *Candida tropicalis*, *Cladosporium cladosporioides* and *Rhodotorula mucilaginosa*) inhibited only at honey concentrations greater than 50%[21].

Khosravi *et al.* reported that honey had antifungal activity against *Candida* species such as *Candida albicans* (*C. albicans*), *Candida parapsilosis*, *Candida tropicalis*, *Candida kefyr*, *Candida glabrata*, and *Candida dubliniensis*[22]. DeMera and Angert reported that honeys from different phytogeographic regions varied in their ability to inhibit the growth of yeasts, suggesting that botanical origin plays an important role in influencing the antifungal activity[23]. Honey produced from Algeria flora has the potential for therapeutic use, firstly because a number of floral sources produce active honey[24,25]. The antifungal effects of Algerian honey toward *C. albicans* and *R. mucilaginosa* were evaluated *in vitro*. In a recent study, Ahmed *et al.* found that the MICs of four varieties of honey without ginger starch against *C. albicans* was 38% and 42%, respectively[26]. When starch was incubated with honey and then added to media, the MICs for *C. albicans* were 32% and 36%. Diastase is a natural enzyme of honey. Its level depends upon geographic and floral origins of the product, as well as on its freshness[27]. The α-amylase present in honey hydrolyzed the starch chains to randomly produce dextrin and maltose. This increased the osmotic effect of the media, which consequently increased the antifungal activity[28]. Molan has studied sugar syrups of the same water activity as honey and found them to be less effective than honey at inhibiting microbial growth *in vitro*[29]. It was found that solutions of high osmolarity, such as honey, glucose, and sugar pastes, inhibit microbial growth because the sugar molecules tie up water molecules so that bacteria have insufficient water to grow[30]. The phenolic constitutes an

important group of compounds for the appearance and for the functional properties of honey. They are recognised as having high scientific and therapeutic interest[31]. In recent years, a large number of studies have been done on the antifungal activity of phenolic compounds of natural origin. Phenolics compounds of honey have been shown to exhibit antifungal activities[21]. Also, flavonoids are abundant class of natural phenolic compounds with several biological activities. Koc *et al*. assessed the *in vitro* antifungal activity of four Turkish honey samples from different botanical origin against *Trichosporon* spp. and *Candida* spp. and concluded that multifloral honey had the highest antifungal activity and it probably contains the highest total phenolic content[32]. The total phenolic contents vary between different honey samples depending on the geographical location of the different floral sources, such as Algeria, Libya and Turkey[33-35]. Polyphenols and their derivates have been reported to exhibit inhibitory activity against α-amylase. Lo Piparo *et al*. investigated the interactions between flavonoids and human α-amylase in order to understand the molecular requirement for enzyme inhibition[36]. No significant correlation was established between diastase activity and total phenolic contents (r=0.24).

In the present study, natural honey and curcuma starch of combination showed synergism against *R. mucilaginosa*. Thus, the mixture of natural honey and curcuma could lead to the development of new combination antibiotics against *R. mucilaginosa* infection. Further studies are warranted to explore the mechanism behind the antifungal activity of natural honey and curcuma starch.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The development of antifungal resistance among pathogenic fungus prompted the need of preparation of cost effective treatment regimen with non-antibiotic drugs, such as honey and curcuma, against *R. mucilaginosa*, and the current attempt to determine natural therapeutics is scientific and timely.

Research frontiers

The synergistic interaction between two natural agents (non-toxic) against *R. mucilaginosa* may be helpful for humankind, particularly in this part of the globe, in combating antifungal resistance.

Related reports

Several authors from different parts of the world explored time to time the antifungal role of natural agents against resistant fungal pathogens in order to search the natural sources of non-antibiotic drugs, which are cost effective and development of resistance against.

Innovations and breakthroughs

The tested agents may be considered in clinical use, but their dose determination (alone and in combination) is mandatory in order to get results in therapy.

Applications

The two agents used in the current study showed synergistic activity against *R. mucilaginosa*, and hence they can be applied against MDR *R. mucilaginosa* as effective therapeutic agents.

Peer review

The authors reported synergistic interaction between two natural non-toxic agents, honey and curcuma, against *R. mucilaginosa*. The current findings are suggestive to prepare treatment regimen from the components of these two agents for *in vivo* application in order to combat antifungal resistance.

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