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Preliminary Investigation on Antimycotic Synergism of Raw Honey and Essential Oil of Thyme (*Thymus vulgaris* L.)

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Abstract

Aims: Several natural products have exhibited synergistic activity against microorganisms. In this study we investigated the existence of synergistic antifungal effect between Thyme (*Thymus vulgaris* L.) essential oil and different varieties of raw honey from Algeria.

Methodology: To evaluate antimycotic effects of mixtures of raw honey (RH) and *Thymus vulgaris* oil (TVO) using agar disc diffusion assay. The antimycotic capacity was determined against two pathogenic yeasts clinical isolates *Candida albicans* and *Rhodotorula mucilaginosa*.

Results: The results indicated that the essential oil of TVO and raw honey are efficient against the tested yeasts. The diameters of inhibition Zone (DIZ) values were all between 8.26-9.5 mm for the RH and between 8.56-10.3 mm for the TVO. RH and TVO interacted synergistically to inhibit *Candida albicans* and *Rhodotorula mucilaginosa*.

Conclusion: These results revealed that combinations of TVO with RH can be used for the development of potent and novel antifungal agents.

Keywords: Synergism; Antimycotic activity; Honey; *T. vulgaris*

Introduction

Infectious diseases accounts for high proportion of health problems in the developing countries including Algeria. The incidence of fungal infections is increasing in community and hospital environments [1]. Pathogenic fungi like *Candida albicans* (*C. albicans*) and *Rhodotorula mucilaginosa* (*R. mucilaginosa*) are commonly encountered strains associated with a wide range of conditions including kidney transplant infection [2], oral thrush and skin infection [3]. Over the last few years, the indiscriminate use of various antifungal therapies has led to the problem of multi-drug resistance. The phenomenon of drug resistance has raised interest in substances of natural origin as a therapeutic alternative. Recent years have witnessed that there is a revival of interest in drug discovery from medicinal plants for the maintenance of health in all parts of the world [4]. Research into natural products has demonstrated significant progress in the discovery of new compounds with antifungal activity. Essential oils from aromatic plants have been shown to have antifungal activities [5]. The composition of the essential oil depends on plant type, geographical location and collection season [6]. The genus Thymus has numerous species and varieties and their essential oil composition have been studied earlier [7-10]. The antifungal activity exhibited by Thymus genus essential oil has been demonstrated by several researchers [11-13]. Honey is a natural product that is used for its antifungal activity [14]. The antifungal activity of honey has been known since the 19th century. Various types of honeys have been shown to have antifungal activity, in vitro, against the following yeasts species (*C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. kefyr*, *C. glabrata*, and *C. dubliniensis*) [15]. Combination therapy can be used to expand the antifungal spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antifungal capacity. The purpose of this study was to evaluate the antifungal capacity of *Thymus vulgaris* oil and natural honey when used in association against *C. albicans* and *R. mucilaginosa*.

Materials and Methods

Extraction of essential oil

The plant powders (100 g) were subjected to hydrodistillation process for 3 h. After distillation, the oil obtained was isolated from water and dried over anhydrous sodium sulphate Na_2SO_4 [16]. The essential oil obtained was stored at +4°C until further tests.

Honey samples

Six Raw Honey (RH) produced in different regions of Algeria. The samples were taken directly from the containers that the beekeepers use for the storage of RH. All samples were collected in their original packages and were transferred to the laboratory and kept at 4–5°C until testing.

Yeast isolates and inoculum preparation

Cultures of yeasts were supplied by Microbiology Laboratory, Institute of Veterinary Sciences, University Ibn-Khaldoun, Algeria. Species included *C. albicans* and *R. mucilaginosa*. The isolates were identified by the standard biochemical methods .The yeasts isolates were subcultured on to Sabouraud Dextrose Agar (SDA, bio Merieux, Marcy-l'Etoile, France) and were incubated at 37°C for 48 h. Colonies

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from 48 h cultures were suspended in 5 ml of a sterile-saline solution. The count of yeasts was adjusted to yield 1.5×10^8 CFU/ml using the standard McFarland counting method [17].

Antimycotic assay using disc diffusion method

All the experiments were performed under sterile conditions

Antimycotic effect of TVO: The essential oil was diluted to give five different concentrations (6.25, 12.5, 25, 50 and 100 µl/ml) in 10% DMSO. The antimycotic activity of different concentrations of TVO was evaluated by the diffusion method [18]. Briefly, the oil was adsorbed on sterile paper discs (5 µL per Whatman disc of 8 mm diameter) and placed on the surface of the medium previously inoculated with a suspension of yeast. The plates were 48 h incubated at 37°C. The presence of zones of inhibition around each of disc after the period of incubation was regarded as the presence of antimycotic action while the absence of any measurable zone of inhibition was interpreted as absence of antimycotic action. DMSO was used as the negative control. Values are given as mean and Standard Deviation (SD) of tests performed in triplicate.

Antimycotic effect of RH: Antimycotic activity was carried out for RH using disc-diffusion method [18]. Petri plates were prepared with 20 ml of sterile SDA. The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. The tests were conducted at five different concentrations of the RH (6.25, 12.5, 25, 50 and 100 µl/ml) per disc). The plates were incubated for 48 h at 37°C. Zones of inhibition were recorded in millimeters and the experiment was repeated twice. The controls were set up with equivalent quantities of water.

Antimycotic Synergism of RH and TVO: After determination of DIZ of TVO and RH, various concentrations of RH and TVO below their DIZ were prepared. Mixtures of TVO and RH were prepared by mixing various concentrations of TVO with various concentrations of RH. These mixtures were tested against two yeasts described above to identify whether there was synergism between TVO and RH. Synergism was identified when the DIZ of RH in combination with TVO was higher than the DIZ of RH or TVO alone.

Results and Discussion

The results of the DIZ assays for both the essential oil and the tested RH were listed in Tables 1 and 2. The antimycotic activity of TVO and RH was confirmed by determining the DIZ (mm) against *C. albicans* and *R. mucilaginosa*.

The DIZ produced by the RH against *R. mucilaginosa* and *C. albicans* ranged from 8.25 to 9.5 and 0 mm respectively (Tables 1 and 2). The DIZ obtained also varies at different concentration used.

The essential oil also inhibited the growth of two pathogenic yeasts. The inhibitory zones ranged from 8.56 ± 0.05 to 10.3 ± 0.18 mm (Tables 1 and 2). The combination effects of antifungal activity of RH and TVO are summarized in Tables 3 and 4.

The DIZ values were between 8.3 ± 0.32 to 24 ± 0.26 mm (Tables 3 and 4). The highest DIZ obtained was by 100 µl (RH2-TVO; 24 mm). While the lowest DIZ obtained for 50 µl concentration was (RH3-TVO; 8.3 mm). Results indicate a considerable antifungal activity of TVO and RH.

Bee products play a dominant role in the discovery and development of drugs in the treatment of animal and human diseases. In medicine, bee products have been researched for their antibacterial, antifungal, antiviral, anticancer and antioxidant properties [19-22]. Particularly, the essential oil and honey that possess antimicrobial activities has

Concentrations (µl/ml)	Diameter of inhibition zone (mm) for raw honey						Diameter of inhibition zone (mm) for TVO
	RH1	RH2	RH3	RH4	RH5	RH6	
100	ND	ND	ND	ND	ND	ND	9.13 ± 0.2
50	ND	ND	ND	ND	ND	ND	9 ± 0.36
25	ND	ND	ND	ND	ND	ND	9 ± 0.17
12.5	ND	ND	ND	ND	ND	ND	10 ± 0.17
6.25	ND	ND	ND	ND	ND	ND	9.7 ± 0.81

ND: not detected

Table 1: Diameter of inhibition zone (mm) of RH and TVO on the growth of *C. albicans*.

Concentrations (µl/ml)	Diameter of inhibition zone (mm) for raw honey						Diameter of inhibition zone (mm) for TVO
	RH1	RH2	RH3	RH4	RH5	RH6	
100	9.5	8.3	9.5	8.6	8.25	8.5	10.3 ± 0.18
50	9.2	ND	9	ND	8.26	ND	9.13 ± 0.75
25	8.9	ND	8.7	ND	8.3	ND	9.1 ± 0.26
12.5	8.6	ND	8.4	ND	ND	ND	8.8 ± 0.26
6.25	ND	ND	ND	ND	ND	ND	8.56 ± 0.05

Table 2: Diameter of inhibition zone (mm) of RH and TVO on the growth of *R. mucilaginosa*.

Concentrations (µl/ml)	Diameter of inhibition zone (mm) when combination used C. albicans					
	RH1-TVO	RH2- TVO	RH3-TVO	RH4-TVO	RH5-TVO	RH6-TVO
100	24 ± 0.26	20 ± 0.43	19.6 ± 0.8	17.6 ± 0.15	19.6 ± 0.15	22.3 ± 0.8
50	16.3 ± 0.15	14.6 ± 0.58	15.6 ± 0.11	19.3 ± 0.1	21 ± 0.2	20.6 ± 0.3
25	15.3 ± 0.05	16.3 ± 0.11	16.6 ± 0.11	15.6 ± 0.05	19 ± 0.1	20.6 ± 0.25
12.5	15.3 ± 0.05	16.6 ± 0.05	16.6 ± 0.05	15 ± 0.17	19.3 ± 0.05	17.6 ± 0.25
6.25	16 ± 0.17	12.6 ± 0.4	15 ± 0	19.3 ± 0.2	18 ± 0.2	18.6 ± 0.2
DMSO	-	-		-		-

Table 3: Synergistic effect between RH and TVO on the growth of *C. albicans*.

Concentrations (µl/ml)	Diameter of inhibition zone (mm) when combination used *R. mucilaginosa*					
	RH1-TVO	RH2-TVO	RH3-TVO	RH4-TVO	RH5-TVO	RH6-TVO
100	16.6 ± 0.05	25 ± 0.00	15.6 ± 0.05	16.3 ± 0.11	16.6 ± 0.15	17.6 ± 0.3
50	18 ± 0.17	14 ± 0.23	8.3 ± 0.32	15.6 ± 0.05	13 ± 0.43	19.6 ± 0.72
25	19.6 ± 0.15	18 ± 0.43	16 ± 0.2	19.3 ± 0.32	15.6 ± 0.11	21.6 ± 0.47
12.5	14.3 ± 0.6	17.3 ± 0.11	15.3 ± 0.15	17 ± 0.1	17.3 ± 0.05	23.6 ± 0.40
6.25	15 ± 0.62	17 ± 0.1	15 ± 0.1	19 ± 0.17	16.6 ± 0.15	21.3 ± 0.35
DMSO	-	-	-	-		-

Table 4: Synergistic effect between RH and TVO on the growth of *R. mucilaginosa.*

been the subject of many scientific reports. Nevertheless, up to now, there are no literature data on the antifungal effect of combined application between honey and *T. vulgaris* essential oils. Many factors influence the antifungal activity of honey. The chemical composition of honey demonstrates considerable geographic differences. Previous studies have demonstrated that essential oils have wide antimicrobial spectra against yeast [23-25]. The essential oil from *T. vulgaris* showed a high content of oxygenated monoterpenes (56.53%) and low contents of monoterpene hydrocarbons (28.69%), sesquiterpene hydrocarbons (5.04%) and oxygenated sesquiterpenes (1.84%) [26]. Antimicrobial activity of *T. vulgaris* can be due to the high level of phenolic compounds (thymol and carvacrol) that are highly studied for its inhibitory properties against a wide range of bacteria [11]. Antimicrobial activity of essential oils depends on their chemical composition, which is determined by the genotype and influenced by environmental and agronomic conditions [26,27]. Other factors may be also responsible for this variability; the most important are the harvest period and the method of preservation and extraction [28].

The antimycotic properties of essential oils and their constituents have been documented extensively. For example, the antimycotic activity of *Thymus vulgaris* essential oil against *C. albicans* was also described by Pozzatti et al. [29] who analyzed the antimycotic activity of several essential oils against *C. albicans*. According to Sikkema, Bont and Poolman [30], essential oils accumulate in the cytoplasmic membrane and cause damage such as loss of function of selective barrier. The research on combination is very limited, and few studies have been reported. The secondary metabolites from plants are good sources for combination therapy. Our results indicated that RH collected from Algeria had a potent antimycotic activity against *C. albicans* and *R. mucilaginosa*, and showed synergistic properties when they were mixed with TVO. In the present study, TVO plus RH of combination showed synergism against *C. albicans* and *R. mucilaginosa*. Further, the results of these in vitro trials provide evidence that TVO in combination with RH could make clinically relevant synergy against *C. albicans* and *R. mucilaginosa*

Conclusion

In conclusion, RH-TVO mixtures were found to have more antimycotic effect than the use of honeys or TVO individually. Further studies are necessary to elucidate the mechanism of action of the synergistic combinations reported here.

Conflict of Interests

The authors declare that they have no conflict of interests.

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