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In vitro Synergistic Action of Honey and Essential Oils against Two *Species of Aspergillus:* A Preliminary Study

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Authors' contributions

Author LB designed the study, performed the statistical analysis, wrote the protocol, and revised the manuscript. Author FA performed the laboratory work and wrote the first draft. Authors HAA and BB revised and approved the protocol. Author SMH managed the literature search. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

In order to evaluate the synergistic action of essential oils (EO) on the antifungal activity of honey, a comparative method of adding honey with and without EO to culture media was used. One variety of honey and five EO types were used to determine the minimum inhibitory concentration (MIC) against two clinical isolates of microfungi; namely *Aspergillus niger* and *Aspergillus flavus*. In a second step, lower concentrations of honey than the MIC were mixed with a set of sub-MIC of EO and then added to media to determine the minimum synergistic inhibitory concentration (MSIC). The MIC of honey without EO was 47% (v/v) against *A. niger* and 50% (v/v) against *A. flavus*. The MIC of EO varied strongly from one variety to another one with that of *Eugenia caryophylata* being the most effective against the tested *Aspergillus* species. When the EO was mixed

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with honey, a MIC drop was observed with each variety of EO. Isobolographic representation shows a synergistic action between honey and all EO types against the tested fungi. Further research studies are needed to elucidate and optimize the effective combination of these natural products in clinical practice.

Keywords: Antifungal; honey; essential oils; synergy; aspergillus.

1. INTRODUCTION

Most of human mycoses are caused by opportunistic fungi [1,2] which produce illness by taking advantage of debilitated or immuno-compromised hosts [3]. The usual route of *Aspergillus* infection is inhalation, but it sometimes follows local tissue invasion, through surgical wounds or contaminated intravenous solutions [4]. Though over 180 *Aspergillus* species are known, only four are commonly associated with invasive infection in humans. These species include *A. fumigatus, A. flavus, A. terreus and A. niger* [5,6]. In ophthalmology, brown or black debris may be seen in infections caused by *A. niger* [7]. A recent study in China has reported that *Aspergillus* isolates represented 17.3% in fungal ocular pathology [8]. *Aspergillus* can cause chronic sphenoid sinusitis, as well as the usually fatal intracranial invasive aspergillosis originating in the sphenoid sinus in healthy individuals [9,10]. Agricultural workers, especially in the third world, whose eyes are injured with subsequent contamination by organic matter, may develop corneal infections by *Aspergillus* species [11-13]. Trauma to nails may lead to infection (onychomycosis) by environmental, especially soil, fungi. Onychomycoses by non dermatophytic molds are considered uncommon although *Aspergillus* species are known to cause this disease worldwide [14,15]. The ear is another location of *Aspergillus* infections in the healthy individual. Local lesions in both the external and middle ear, as well as in postoperative cavities, can create favorable conditions for fungal growth and subsequent otomycosis caused by *A. flavus, A. fumigatus* and *A. niger* [16-20]. Nair e*t al*. reported that among mycotic infections, the incidence of *A. flavus* is higher in diabetic foot patients. The last two decades have witnessed a dramatic rise in the incidence of life threatening fungal infections [21]. The challenge has been to develop effective strategies for the treatment of fungal diseases, considering the increase in opportunistic fungal infections in human immunodeficiency virus-positive patients and in others who are immune-compromised due to cancer chemotherapy and the indiscriminate use of antibiotics. The majority of clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Additionally, in recent years public pressure to reduce the use of synthetic fungicides in agriculture has increased. Concerns have been raised about both the environmental impact and the potential health risk related to the use of these compounds [22]. Natural products, either as pure compounds such as honey or as standardised plant extracts, provide useful opportunities for new drug leads because of the matched less availability of chemical diversity. Honey is a natural product that has been used for its antifungal activity [23] and its antimicrobial properties have been extensively reviewed [24]. A survey of literature reveals that there are many essential oils (EO) which possess antifungal activity [25-32]. The aim of this study was to evaluate the synergistic action between honey and EO against two fungi and to investigate their joint potential use as an alternative for the treatment of superficial aspergilloses.

2. MATERIALS AND METHODS

2.1 Honey Sample

Monofloral honey sample (wild carrot honey: *Daucus carota* L) was directly provided by a local beekeeper from Chlef in western Algeria during the year 2010.

2.2 Essential Oils Samples

The aerial plant parts (leaves and flowers, 30 g) of *Thymus fontanesii*, *Thymus vulgaris* and the flower buds of clove (*Eugenia caryophyllata*) were dried at room temperature, hydro-distilled for 3 h using a Clevenger type apparatus (British Pharmacopoeia, 1998). The EO were dried over anhydrous sodium sulfate and stored in the dark at 2-4°C. The yield of the essential oils was 1.56% (v/w), 2.39% (v/w), and 7.45% (v/w) for *T. vulgaris*, *T. fontanesii* and *E. caryophyllata* respectively. Except *Origanum vulgaris* and *Geranium* EO which were purchased from Turkey and Algeria respectively, the plants were obtained from a local store during the year 2012.

2.3 Fungal Strains and Inoculum Standardization

A. niger was kindly provided by the "Institut Pasteur d'Alger" whereas *A. flavus* was isolated from contaminated food at the Institute of Veterinary Sciences (Tiaret, Algeria). Pure cultures were maintained in Sabouraud agar (SA). Stock suspensions of fungi were prepared from sporulating 7-day-old cultures grown on SA at 28°C. Colonies were covered with 5 ml sterile distilled water and the surface scraped with a sterile loop. The mixture of conidia and hyphal fragments was filtered through an 8 mm sterile filter and collected in a sterile tube. This procedure removed the majority of the hyphae, producing inocula composed mainly of spores. Turbidity of the final inocula was adjusted to 0.5 McFarland Standard (equivalent to 1-5 x 10^6 spore/mL) using sterile saline.

2.4 Minimum Inhibitory Concentration (MIC) Measurement

Concentrations of honey between 40% and 50% (v/v) were incorporated into SA to test their efficiency against *A. niger* and *A. flavus*. The final volume of honey and media in each plate was 5 ml. To evaluate the effect of each EO against the tested fungi, dilutions between 1/100 and 1/1000 have been made with Tween 80. The final volume of EO solutions and media in each plate was 5 ml. The plates were inoculated and incubated at 25°C for 5 days. The MIC was determined by finding the plates with the lowest concentration of honey or EO on which the strain did not grow. All MIC values are expressed in percentage (v/v). In a second step, concentrations of honey less than the MIC were added to sub-MIC concentrations of EO, which were then incorporated into media to determine the minimum additive inhibitory concentration against the tested strains. Similarly, the final volume in each plate was 5 mL. Plates were inoculated and incubated at 25°C for 5 days and carried out in triplicate.

2.4 Statistical Analysis

Isobolographic analysis [33] was carried out using Statistica® 7 software to measure the additive antimicrobial action of honey and each EO against the tested fungi.

3. RESULTS AND DISCUSSION

Honey and all EO types were effective against the tested strains. The MIC of honey was 47% and 50% against *A. niger* and *A. flavus,* respectively. The MIC of EO varied widely depending on the botanical origin. The EO from *E. caryophylata* was the most effective against both *Aspergillus* species with a MIC of 0.01% whereas that from *T. vulgaris* was the least effective one with a MIC of 0.05% (Table 1). Adding EO to honey resulted in a significant decrease in the MIC of EO and honey. Each EO variety, except for *Geranium* with *A. flavus*, registered a decrease in MIC when added to sub-MICs of honey. Isobolographic representations show an over-additive action (synergism) between honey and the different tested EO in terms of antifungal activity (Figs. 1 and 2). This later is called synergism [33].

Table 1. MIC values of honey and EO against *A. niger* and *A. flavus*

	MIC % (*A. niger*)	MIC % (*A. flavus*)
Thymus vulgaris	0.05	0.05
Thymus fontanisii	0.03	0.05
Origanum vulgare	0.025	0.05
Geranium	0.03	0.05
Eugenia caryophyllata	0.01	0.01
Honey	47	50

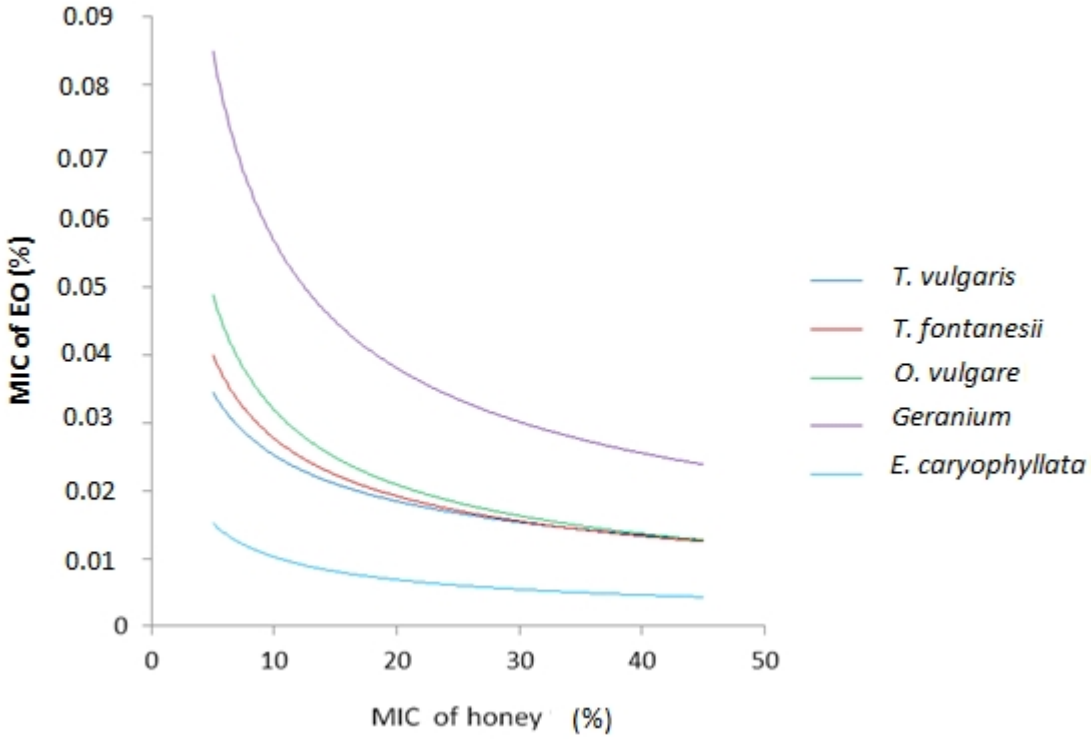

Fig. 1. Isobologram representing synergistic effect of wild carrot honey and essential oils (EO) from the tested plant species against *Aspergillus flavus*

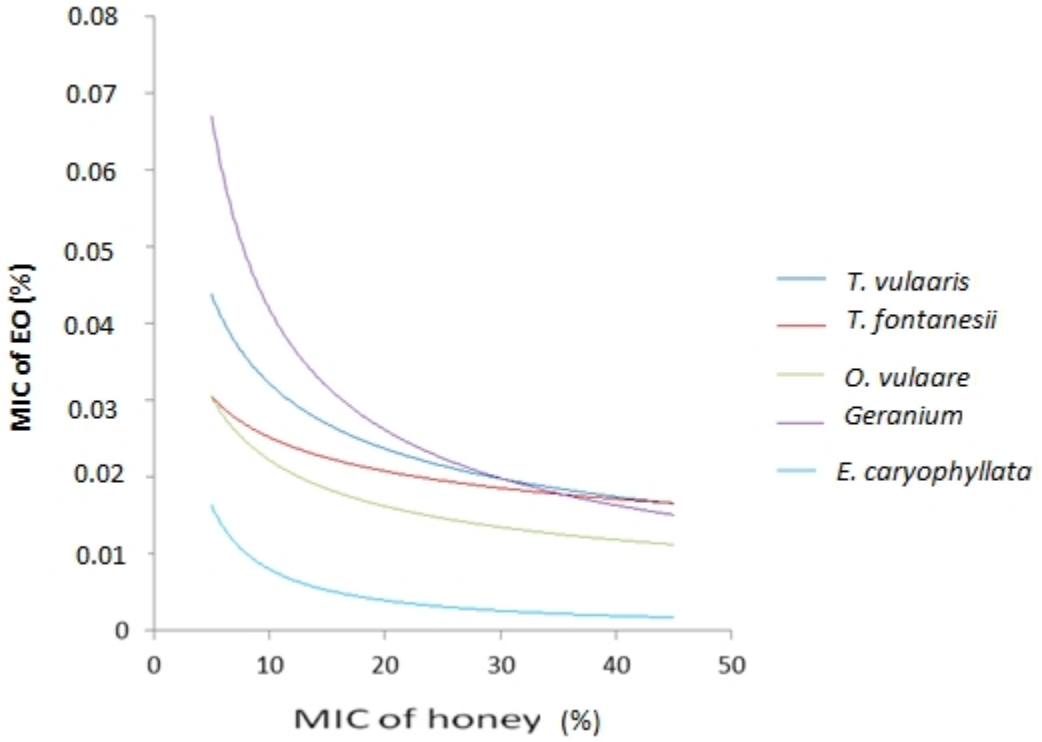

Fig. 2. Isobologram representing synergistic effect of wild carrot honey and essential oils (EO) from the tested plant species against *Aspergillus niger.*

A. niger and *A. flavus* could be implicated in ophthalmic and some cutaneous disorders [4-6]. Their resistance to conventional antifungals is a common problem [34,35] which physicians are continually seeking alternatives to overcome. Honey and EO are among the natural products that are widely used to treat infections that resist to conventional drugs [29,32,36]. The antifungal effect of EO has been extensively discussed [37-40]. Our results show that adding EO to honey acted synergistically against the tested fungi. Further research studies are needed to elucidate and optimise the effective combination of these natural products in clinical practice. The current prevalence of antifungal-resistant species has led to a re-evaluation of the therapeutic use of ancient remedies, including honey and EO. With the increased availability of licensed health care products containing honey, clinical use is expected to increase and further evidence will become available. Honey seems to have the potential to clear infection as well as to be an effective prophylactic agent that may contribute to reducing the risks of cross-infection. EO and their components as well as honey are gaining increasing interest because of their relatively safe status, wide acceptance by consumers, and their exploitation for potential multi-purpose functional use [41]. EO are composed of many different volatile compounds. It is difficult to correlate the fungitoxic activity to a single compound or classes of compounds. It seems that the antifungal and antimicrobial effects of EO and honey are the result of many compounds acting synergistically [42]. Neither of these two natural products has an adverse effect on tissues, so they can safely be used on fungal infections and inserted into cavities, including diabetic foot, to clear infection.

4. CONCLUSION

The re-emergence of using natural antifungal compounds is not new. Herbal, botanical and other nutrients found or isolated from natural sources have been used for centuries prior to what we now call conventional medicine. The ability to research, document and publish the effectiveness of these natural alternatives using the same scientific criteria demanded by the scientific community is new. Many physicians, who, before, would not think about even suggesting anything other than a prescription medication, are interested in reading the new literature and suggesting alternative protocols. As aromatic plants, herbs and spices have been used for ages both as flavouring agents and as preservatives of food, they may be effective sources of biodegradable fungi-toxicants without harmful side effects. Honey and EO may be used jointly to boost their antifungal action against opportunistic fungi becoming more and more resistant to conventional drugs.

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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