Evaluation of the Synergistic Effect of *Daucus carota* Honey and Essential Oils against *Candida albicans*

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ABSTRACT

The resistance of pathogenic fungal strains to the commonly used antifungal has necessitated a search for novel types of antifungal agents. The main objective of this study was to investigate the antifungal activities of essential oils (EOs) of medicinal plants (*Mentha pelugium Eugenia caryophyllata, Pelargonium graveolens*) and wild carrot *(Daucus carota*) honey when used jointly by the determination of MIC (Minimum Inhibitory Concentration) against *Candida albicans*. The result of our study indicated that the essential oils and honey are efficient against the tested strain. Honey MIC value was 6% (vol/vol), whereas the MIC values of EOs were 0.4µl/ml for *Pelargonium graveolens* and *Mentha peligium* EOs (vol/vol) and 0.33 µl/ml for *Eugenia caryophylatta* EO. When honey and EOs are used jointly, we noticed a decrease of the MIC values which is may be due to their synergistic effect. These preliminary results suggest that honey and EOs could be used together to manage superficial fungal infections.

I. Introduction:

Candida species are the most common pathogens fungal responsible for the majority of human infections ranging from localized superficial to systemic candidiasis [1]. This species are considered important pathogens due to their versatility and ability to survive in various anatomical sites. *Candida albicans* is the predominant cause of invasive fungal infections and represents a serious public health challenge with increasing medical and economic importance due to the high mortality rates and increased costs of care and duration of hospitalization. In recent years there is an increase incidence of drug resistant pathogens and the toxicity of existing antifungal compounds [2].Hence, there is a great demand for novel antifungal agents, justifying the intense search for new drugs that are more effective and less toxic than those already in use [3].This situation has drawn attention towards the antimicrobial activity of natural products [2].This products have been used for thousands of years in folk medicine for several purposes. They are both fundamental sources of new chemical diversity and integral components of today's pharmaceutical compendium [4]. 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 and its antimicrobial properties have been extensively reviewed [5]. The essential oils from many plants are known to possess antibacterial and antifungal activities [6].The aim of this study was to evaluate the synergistic action between honey and essential oils against *Candida albicans* and to investigate their joint potential use as an alternative for the treatment of infectious diseases.

II. Materials and methods

A. 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 2014.

B. Physicochemical analysis of honey sample

Moisture in honey was determined in a refractometer (Jena 181282, Carl Zeiss, Oberkochen, Germany), and the pH of the honey solution was measured by a pH meter (CG840 Schott, Gerate GmbH, Hamburg, Germany). HMF content was measured according to the method of White [7] and was based on the determination of UV absorbance of HMF at 284 nm. The results are expressed in milligrams per kilogram (mg/Kg).

- *HPLC Analysis for sugars of honey sample*

Sugars and organic acids were analyzed by a Hewlet-Packard (1090) liquid chromatograph equipped with a photodiode array detector (PDA) and a Waters 410 differential refractometer (Milipore Corp., Milford, MA) connected in series. Data were processed using a Hewlet-Packard 85-B computing system and a Beckman Analogue Interface Module 406 with Gold V.711 software. Isocratic separation of the compounds was carried out at a flow rate of 0.4 mL/min on a stainless steel Ion-300 column (300 mm ¥ 7.8 mm, 10 mm) containing a cation-exchange polymer in the ionic hydrogen form, combined with an IonGuard GC801 precolumn (Interaction, San Jose, CA). Filtered (0.22 mm nylon) and degassed 0.0085 mol/L H2SO4 solution was used as the mobile phase. Both columns were maintained at 23C. Samples were dissolved in mobile phase, filtered through a micro-filter (politetrafilvoretileno [PTFE] or Teflon, 4 mm, 0.22 mm) and 20 mL (50% of total sample volume before filtration) was injected. The post column effluent was introduced in sequence into the PDA detector (scanning range 210–300 nm; 1.2 nm resolution) and a refractive index detector (sensitivity setting 16x, [8].

C. Essential oils extraction

The aerial plant parts (leaves and flowers, 30 g) of *Mentha pelugium* 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 EOs were dried over anhydrous sodium sulfate and stored in the dark at 2- 4°C. The yield of the essential oils was 1.56% and 7.45% (v/w) for *M. pelugium* and *E. caryophyllata* respectively. Except *Geranium (Pelargonium graveolens)* EO which was purchased from Algeria, the plants were obtained from a local store during the year 2014.

D. Gas chromatography-mass spectrometry (GC-MS):

GC analysis was carried out using a Shimadzu 2010 Plus gas chromatograph coupled to a Shimadzu QP2010 Ultra mass selective detector. The separation was performed by means of a Restek Rxi-5MS capillary column, 60 m lenght, 0.25 mm i.d. and a 0.25 µm phase thickness. The split mode was used. The oven program was as follows: Initial temperature was 60 ^{o}C for 2 min, which was increased to 240 ^{o}C at 3 ^{o}C min^{-1}, 250 ^{o}C was maintained for 4 min. Helium (99.999%) was used as carrier gas with a constant flow-rate of 1 mL min^{-1}. Detection was carried out in electronic impact mode (EI); ionization voltage was fixed to 70 eV. Scan mode (40-450 *m/z*) was used for mass acquisition. The volatile compounds were identified by comparison of their retention indices (relative to C7-C30 alkane standards), and matching mass spectral data with those held in FFNSC1.2 and W9N11 library of mass spectra and literature comparison [9].

E. Evaluation of the antifungal activity:

- *Fungal strain and inoculum standardization*

Candida albicans was kindly provided by a medical analyses laboratory it was isolated from a vaginal sample. Prior to the experiment the strain was maintained by subculture in the specific media; the inoculum suspension was obtained by taking five colonies from 48hours cultures. The colonies were suspended in 5 ml of sterile saline (0.85% NaCl) and shacked for 15 seconds. The density was adjusted to the turbidity of a 0.5 McFarland Standard (equivalent to 1×10^8 cfu/mL).

- *Minimum Inhibitory Concentration Measurement (MIC)*

The MIC of honey and essential oils have been determined, separately, using an agar incorporation technique method. Honey was added in increasing quantities (v/v) into media for a final volume of 5 ml. Essential oils were incorporated into Mueller-Hinton media. The mixture was shacked moderately and poured into plates, then standard inoculum of 0.5 McFarland of fungal strain was inoculated and the plates were incubated at 32°C for 48 hours. The MIC was determined based on the lowest concentration of honey and essential oils that inhibited the growth of tested organism.

F. Minimum Synergistic Inhibitory Concentration Measurement (MSIC)

To determine the minimum synergistic inhibitory concentration, volumes of honey were mixed with volumes of essential oils lower than the MIC values determined in the first step and then incorporated into Mueller-Hinton media. The mixture was shacked moderately and poured into plates, then standard inoculums of 0.5 McFarland of the fugal strain were inoculated and the plates were incubated at 32°C for 48 hours.

j. Statistical analysis

Isobolographic analysis was carried out using Statistica® 7software to measure the synergistic antifungal action of honey and EOs against the tested fungal.

III. Result and discussion:

Table 1 summarizes the physicochemical values of *Daucus carota* honey

TABLE 1. Values of physicochemical properties of *Daucus carota* honey

Honey	pH	Water content	HMF (mg/Kg)	Glucose (mg/g)	Maltose (mg/g)	Saccharose (mg/g)	Fructose (mg/g)
Daucus carota	4.62	16.4	13.98	326.26	37.16	11.67	406,49

The pH value of our sample *Daucus carota* honey was 4.62 which confirmed that this variety was acidic in nature. The acidity of the honey may be due to the presence of organic acids such as gluconic acid and also due to phosphate and chloride ions [10]. The pH acid of our honey was consistent with the results reported by various studies Kamboj et al [11]; Nayik and Nanda [12]; Shobham et al [13].This low pH inhibits the growth of microorganisms and influences the texture and stability of the honey sample [14].

Water is the second largest constituent of honey. The moisture content is one of the most important characteristics influencing physical properties of honey such as viscosity and crystallization, as well as other parameters: color, flavor, taste, specific gravity, solubility and conservation. Moisture content of honey is a limiting factor in determination of its quality, stability and spoilage resistance against yeast

fermentation. The higher moisture content is the higher probability of honey fermentation during storage [15].

The moisture content of our honey sample was below 20% maximum value allowed by Codex standards (Table 01). Such results were also observed by Omafuvbe and Akanbi [16] Nayik and Nanda [12]; Shobham et al [13].

Tosi et al [17] reported that hydroxymethylfurfural (HMF) as a quality parameter to check the honey freshness and high temperature processing. The higher value of HMF indicates overheating during processing, prolonged storage or adulteration with invert sugar. Our honey sample showed an HMF level lower than the limit (40 mg/kg), recommended by the Codex Alimentarius [18]

The glucose content in our honey was lower than the fructose content which indicated the natural feeding of honey colonies and confirmed the high quality of studied type of honey. These obtained results supported the previous several studies on different honey types (Buba et al., 2013[19]; EL-Metwally, 2015[20]; El Sohaimy et al 2015[15]).

Saccharose content is important to detect heavy sugar feeding of the bees or adulteration by direct addition of saccharose. According to some studies, the amount of sucrose has been used to discriminate the adulteration of honey samples by sugar syrups. For example, supplementary feeding of honey bees with sucrose syrup caused a higher sucrose level in honey. It comprises a little over 1% of the composition of honey [21].This seems to be the case of our honey.

The main compounds identified in the tested essential oils are showed in Table 02

TABLE 2. Chemical composition of essential oils (RI-retention index)

Sample Code	Name of Compound	Area (%)	RI
Eugenia caryophyllata			
1	*α* –Pinene	0.27	944
2	Benzaldehyde	0.97	971
3	Trimethylbenzene	0.45	1003
4	Eucalyptol	0.42	1041
5	Benzenepropanol	0.88	1170
6	Endo-Borneol	0.13	1175
7	Benzylidene malonaldehyde	0.37	1229
8	Benzylidene acetaldehyde	57.75	1279
9	Bornyl acetate	0.25	1294
10	Eugenol	2.34	1365
11	*E*-caryophyllene	0.17	1434
12	Alloaromadendrene	7.99	1762
13	*β* –Cedrene	0.63	1772

14	Pentacosane	4.03	2506
15	10, 12-Tricosadiynoic acid methyl ester	23.33	2609
Mentha pulegium			
1	p-Menthan-3-one	5.32	1163
2	p-Menth-4(8)-en-3-one	23.81	1249
3	Cinnamaldehyde	60.39	1278
4	p-Beritone	7.16	1352
5	Pentocosene	3.32	2500
Pelargonium graveolens			
1	Benzene 1,3,5 trimethyl	13.86	1002
2	Menthone	19.00	1161
3	Isomenthone	20.20	1172
4	Citronellol	30.81	1229
5	Citronellyl formate	16.13	1276

In our study we found that the major compounds of *Eugenia caryophyllata* essential oil are Benzylidene acetaldehyde 57.75%, 10, 12-Tricosadiynoic acid methyl ester 23.33%, Alloaromadendrene 7.99%, Pentacosane 4.03%, and Eugenol 2.34%. The chemical composition of our essential oil is different from result of other studies which showed that the major component of clove essential oil is usually eugenol, β- caryophyllene, α-humulene, caryophyllene oxide and eugenylacetate respectively, although different in concentration [22, 23-24]. The chemical composition of essential oils depends on climatic, seasonal and geographic conditions, harvest period and distillation technique [25].

The result of GC-Ms analysis showed that the major compounds of *Mentha pulegium* essential oil are cinnamaldehyde 60.39%, *p*-Menth-4(8)-en-3-on 23.81%, and p-Beritone 7.16%. In a study done by Nickavar and Fatemeh [26] they showed that 18 constituents were identified in the oil of *M. pulegium* and pulegone (48.7 %) and menthone (26.8 %) were found to be the main constituents. Other study done by Ainane et al [27] indicated that piperitone (31.27%) and piperitenone (22.98%) are the major compounds of the essential oil of *Mentha pulegium* grown in the region of Settat Morocco.

Citronellol, isomenthone, menthone and citronellyl formate are the most chemical compounds of our geranium essential oil. The main results of a study done by Mnif et al [28] showed that *P. graveolens* essential oil was characterized by the predominance of two compounds: citronellol and geraniol with respective amounts of 27.53 and 25.85 %.

Bigoset al [29] found in their study that citronellol 26.7% and geraniol 13.4% representing the major compounds of essential oil of geranium.

- *Antifungal effect of Daucus carota honey and essential oils*

Honey and all the essential oils were effective against the tested strain (*Candida albicans*) and the MIC values varied widely depending of the natural products. The MIC value of honey was 6%.The MIC values of EOs varied widely depending on the botanical origin. The EO from *E. caryophylata* was the most effective one with a MIC value of 0.3µl/ml as shown in the Table 03.

TABLE 3. MIC values of *Daucus carota* honey and essential oils against *Candida albicans*

	MIC values			
	Honey %	**Essential oils µl/ml**		
Substances	*Daucus carota* honey	*Geranium*	*Eugenia caryophylatta*	*Mentha pelugium*
Candida albicans	6%	0.4	0.33	0.4

The antifungal activity of honey is thought to be attributed to the high concentration of sugars and low content of water [30]. Several factors may influence this activity. These factors include its physico-chemical properties, botanical and entomological origin. In addition, there are a great variety of components, including phenolic acids, flavonoids and other biomolecules, in different honeys. Biological activities of honey is mainly attributed to the phenolic compounds [31]. The antimicrobial action of these compounds has been related to their ability to denature proteins, being generally classified as surface active agents [32]. The antifungal activity of honey against *Candida albicans* has been reported in many studies.

Estevinho et al [31] found in their study that lavender honey inhibited the growth of pathogenic yeasts *Candida albicans, Candida krusei, and Cryptococcus neoformans.*

In a study done by Anyanwu [32] he was evaluated the antifungal activity of honey samples against *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Microsporum gypseum, Candida albicans*, and *Saccharomyces* sp. The obtained results revealed that the honey samples showed varying levels of inhibitory activity at various concentrations against the tested fungi *M. gypseum* was the most sensitive of all the studied fungal, while *C. albicans* was the least sensitive.

Other study done by Al Zahrani et al [33] they evaluated the antimicrobial potency of four varieties of honey from different botanical and geographical origins (Manuka, Acacia, Lavender, Wild carrot) they found that all honey samples were effective against *Candida albicans.*

The result of a study done in Australia by Irish et al [34] indicated that four varieties of honey have an antifungal effect against *Candida* species (*C. albicans*, *C. glabrata* and *C. dubliniensis)*.

In a study done Koc et al [35] they evaluated the ability of honey samples from different floral sources to inhibit the growth of four yeast strains (*Candida albicans, C. krusei, C. glabrata* and *Trichosoporon* spp.). The result of this study indicated that all of the tested yeast strains were inhibited by honeys.

The result of our study (Table 03) indicated that all the tested essential oils were active against *Candida albicans*, this activity is attributed to the presence of small terpenoid and phenolic compounds [36]. It is generally assumed that the mechanisms by which the constituents of essential oils inhibit the growth of micro-organisms may be partially dependent on their hydrophobicity. It enables them to embed in the cell wall, damage the lipid layer of the cell membrane and mitochondria, impair enzyme systems and exhibit side effects on various proteins . Some of them inhibit microbial growth by causing also a global arrest in protein synthesis or inducing cytoplasm coagulation [06]. Various studies showed that essential oils have antifungal activity against *Candida albicans* .

Abdulaziz et al [1] found that both essential oils of *Rosemarium officinalis* and *Thymus vulgaris* are active against the selected fluconazole resistant *C. albicans* isolates.

Hammer et al [37] have investigated the antimicrobial activity of a large number of essential oils against a diverse range of organisms comprising Gram positive and Gram negative bacteria and yeast, they found that the essential oils of *Eugenia caryophyllata (clove oil), Pelargonium graveolens(Geranium oil) and Mentha piperita* have an antifungal effect against *Candida albicans.*

Budzyńska et al [38] found that essential oils of *Eugenia caryophyllata, Pelargonium graveolens and Mentha piperita* have an antifungal effect against *Candida* Spp (*C. albicans* strains (ATCC 10231, ATCC 90028) and 50 clinical isolates: *C. albicans* (n = 20), *C. glabrata* (n = 13), *C. kru sei* (n = 6), *C. parapsilosi*s (n = 5), C. *tropicalis* (n = 6)).

The result of a study done by Bhat et al [39] reveled that the essential oil of *Eugenia caryophylatta* had antifungal activity against the oral isolates of candida species (*Candida albican, Candida glabrata, Candida tropicalis*)

Ahmad et al [40] found in their study that Eugenol and methyleugenol the major compounds in the essential oils of many aromatic plants, such as clove (*Eugenia caryophyllata*) have antifungal effect against 64 fluconazole sensitive and 34 fluconazole resistant clinical Candida.

In other study done by Pinto et al [3] they found that essential oil of *Eugenia caryophyllata* and their major compounds eugenol showed a broad spectrum of activity against a variety of pathogenic yeasts (*Candida albicans, Candida Krusei, Candida tropicalis Candida glabrata, Candida parapsilosis*) and filamentous fungi (*Epidermophyton floccosum ,Trichophyton rubrum,Trichophyton mentagrophytes, Microsporum canis, Microsporum gypseum, A. flavus , A. fumigatus, A. niger*) including fungi with decreased susceptibility to fluconazole.

In a study done by Matsuzaki et al [41] they confirmed the antifungal activities against *C. albicans* of seven essential oils from aromatic plants, lemongrass (*Cymbopogon citrates*), eucalyptus (*Eucalyptus globules*), tea tree (*Melaleuca alternifolia*), peppermint (*Mentha piperita*), sweet marjoram (*Origanum majorana*), geranium (*Pelagonium graveolens*) and rosemary (*Rosmarinus officinalis*).

Adding EOs to honey resulted in a significant decrease in the MIC of EOs and honey.

Isobolographic representations show a synergistic action between honey and the different varieties of EOs in term of antifungal activity (Figures 1, 2, 3).

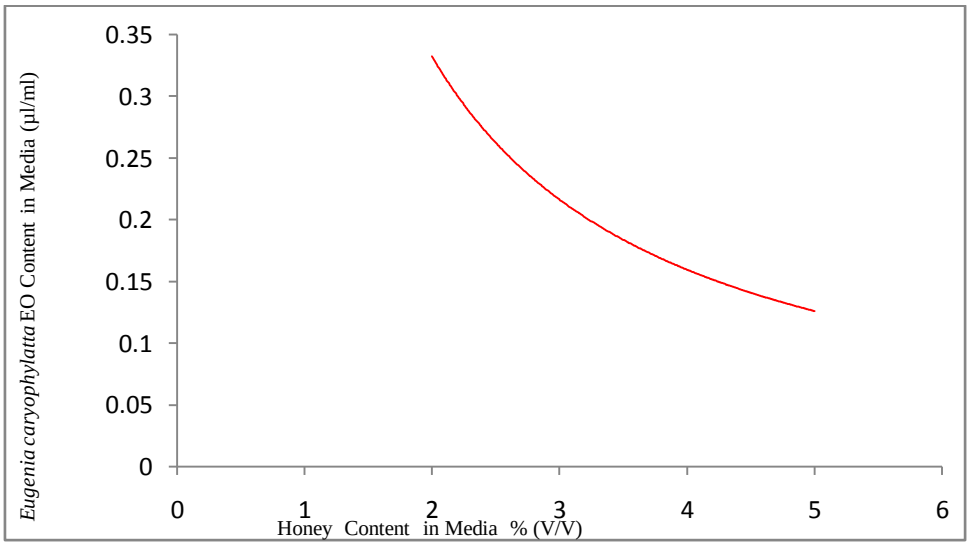

Fig 1: Isobologram representing synergistic effect of *Eugenia caryophylatta* essential oil and *Daucus carota* honey against *Candida albicans.*

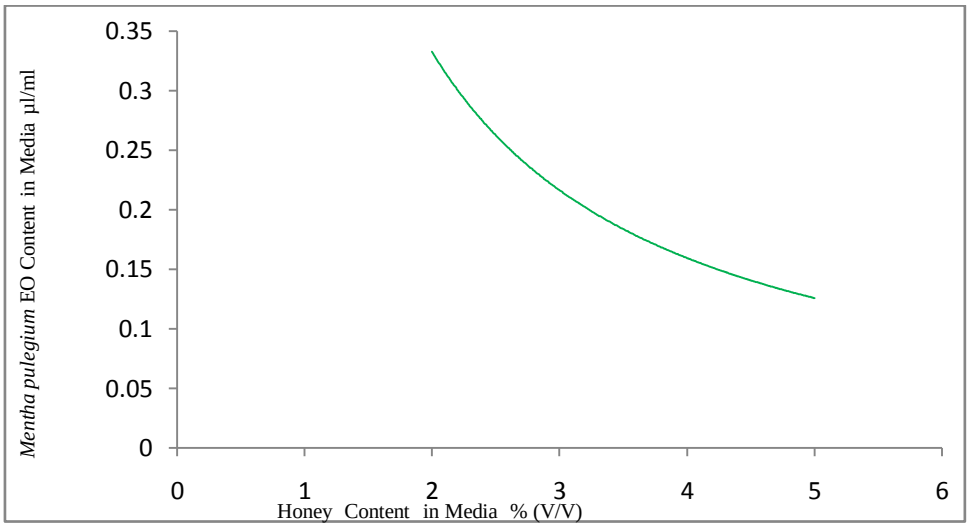

Fig 2: Isobologram representing synergistic effect of *Mentha pulegium* essential oil and *Daucus carota* honey against *Candida abicans.*

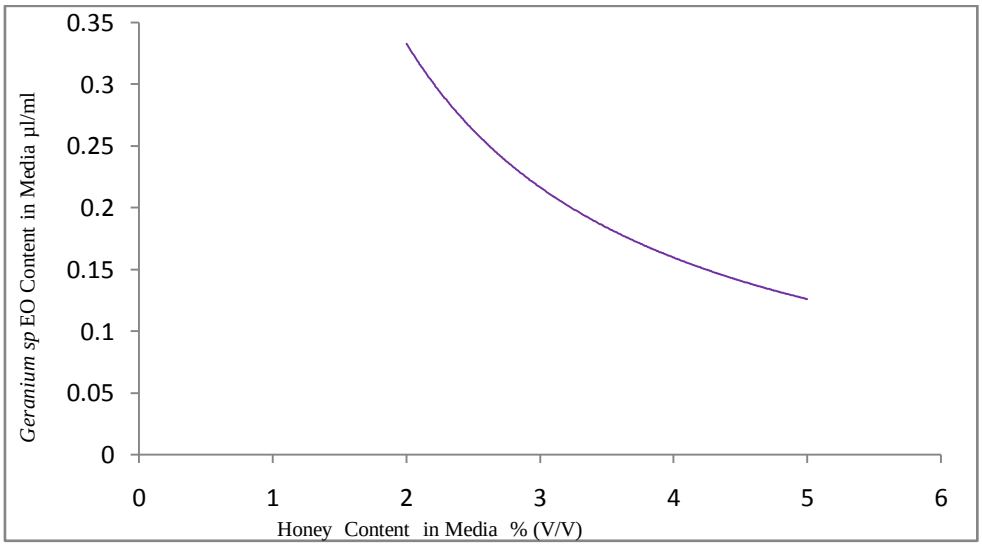

Fig 3: Isobologram representing synergistic effect of *Geranium sp* essential oil and *Daucus carota* honey against *Candida albicans*

The synergistic effect of honey and other natural compounds against bacteria and fungi has been reported by many studies .Azahrani et al [5] found in their research that *Daucus carota* honey and five essential oils types (*Thymus fontanesii*, *Thymus vulgaris,Origanum vulgaris, Eugenia caryophyllata* and *Geraniuma*) have a synergistic antifungal effect against two fungi strains *Aspergillus niger* and *Aspergillus flavus.*

The result of a study done by Boukraa et al [42] indicate that five varieties of honey from different botanical origins :Manuka honey (*Leptospermum scoparium*) Acacia honey (*Acacia*),wild carrot honey (*Daucuscarota* L),Berringa honey (*Leptospermum polygalifolium*) Sidr honey (*Ziziphus zizyphus*) and essential oils extract from four medicinal plants *Thymus vulgaris,Thymus fontanesii Origanum vulgare* and *Eugenia caryophyllata* have a synergetic antibacterial effect against *Pseudomonas aeruginosa* ATCC 27853.

The result of a research done by Khosravi-Darani et al [43] showed that three kinds of honey of Iran and alcoholic extract of mint and zataria, as well as extract and starch of ginger have a synergistic antimicrobial action against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans.*

The result of a study done by Abdellah et al [44] showed that *Daucus carota* honey and the powder of *Thymus ciliatus* acted synergistically against three pathogenic bacteria, namely *Staphylococcus aureus* OxaR ATCC 43300, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

In a study done by Boukraa et al [45] they found that honey and starch have a synergistic action against *Candida albicans.*

Another study done by Boukraa et al [46] they demonstrated that honey and starch have a synergistic action against *Pseudomonas aeruginosa.*

Boukraa and Amara [47] found that three varieties of honey and starch have a synergistic effect against *Staphylococcus aureus* and *Escherichia coli.*

Boukraa [48] indicates that four varieties of honey from different botanical origins and royal jelly have a synergistic antibacterial effect against *Pseudomonas aeruginosa* ATCC 27853.

IV. Conclusion

The extensive use of antifungal chemicals in medical area has led to the selection of resistant fungal strains. So, to overcome this problem, it is necessary to find out alternative medicines that could be efficient and safe for use. The re-emergence of using natural antifungal compounds is not new. Honey and EOs are natural products which may be used jointly to boost their antifungal action against pathogenic fungi.

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