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EVALUATION OF ANTIBACTERIAL ACTIVITY AND ADDITIVE EFFECT BETWEEN CASEIN AND TWO TYPES OF ALGERIAN HONEY

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Abstract. To demonstrate the antibacterial properties in vitro of two types of honey against *Escherichia.coli* and the study of the synergistic effect of the combination of honey and casein. Tow honeys (Euphorbia; Multifloral) were used alone and then combined with casein to evaluate their antibacterial activities against *E. coli* isolated from human urinal, mastitis cow's milk and avian diarrhea. The results in vitro of antibacterial activity with Multifloral honey samples were at two concentrations (30% and 50% v/v) against *Escherichia.coli* from (avian, cow isolates) and human isolates respectively; however, the results of antibacterial activity with Euphorbia honey samples at (20%30% and 50% v/v) concentrations against mastitis cow's milk, avian and human isolates respectively. The FIC indexof combination between casein and Euphorbia honey were 1.25, 1.16, 1.5 (Human, avian, cow) isolates respectively. But the avian and cow isolates FIC index of combination between Multifloral honey and casein was both 1.16, and 1.40 for human isolates. The FIC index values indicate an additive effect. As a conclusion; the CMI of Euphorbia honey against *E. coli* begins at20%, whereas the CMI of Multifloral honey starts with a concentration of 30%. Moreover, the combination of honey and casein has an additive effect.

Keywords: *Honey, casein, antibacterial activity, additive effect.*

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1. Introduction

Antibiotic resistance is a phenomenon as old as the advent of antibiotics. The development and spread of resistance to currently available antibiotics is a global concern (Chanda & Rakholiya, 2011). This type of bacterial resistance to the antimicrobial agents pose a very serious threat to public health, and for all kinds of antibiotics including the major last –resort drugs, the frequencies of resistance are increasing worldwide (Levy & Marshall, 2004; Mandal *et al*., 2009). Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey (Mandal *et al*., 2010a, 2010b). The natural ingredients of honey show different activities against various micro-organisms. The antimicrobial properties of honey have been well documented. An alternative medicine branch, called apitherapy, has been developed in recent years, it was argued that the combined treatment with honey and some plants showed enormous synergism effect against bacterial species comparing to their pure extracts (Patel *et al*., 2011). On the other hand, the casein peptide has antibacterial activity against lactobacilli, Gram-positive, and Gram-negative bacteria (Lahov & Regelsond 1996). However, for the sake of knowledge, there is no study about incorporating the combination of honey and casein peptide.

Therefore, the aim of this study was to investigate antibacterial activities of different honey samples collected from two different Algerian regions, and the antibacterial activity of casein and their combination effect against *Escherichia coli*.

2. Materials and methods

1. Honey samples

Two types of honey were used, named Euphorbia honey (El Bayadh region which is located in the South West -Algeria) and Multifloral honey (Tissimsilt region in the North West - Algeria). Honeys were kept in bottles away from sunlight.

2. Physical characteristics

A pH meter was used to measure the pH of 10% (w/v) solution of honey prepared in milli-Q water. (Bogdanov, 2009). EC (Electrical conductivity) was measured using a conductiviting meter and a 20% (w/v) solution of honey was suspended in milli-Q water. The optical density (OD) was measured as reported in the literature; one gram of honey was diluted with 9ml of distilled water and centrifuged for 10 min at 3000g. The absorbance of the filtrate supernatant was measured at 530 nm against distilled water as a blank using spectrophometer (Bogdanov *et al*., 1999, Wakhle, 1997) respectively.

3. Casein

Antibacterial activity of casein (Casein powder 1Kg.Prod. 22544.292 Batch n°: 0502384. VWR PROLAB. France) powder was determined by Muller-Hinton agar well diffusion assay.

4. Bacterial isolates

Bacterial strains used in this study; *Escherichia coli* isolated from human urinal infection. The bacterial strains of mastitis cow's milk isolated and identified at the Microbiological laboratory, Veterinary Science Institute -University Ibn Khaldoun-Tiaret –Algeria and the last bacterial strain isolated from avian diarrhea identified in the same laboratory.

5. Well diffusion assay for antibacterial activity

Antibacterial activity of various concentrations (5%; 10%; 15%; 20%; 30%; 50%; 70%) of honey samples was determined by Muller –Hinton agar well diffusion assay. Bacterial isolates were inoculated in 10 mL nutrient broth and placed overnight in shaking incubate at 37°C.

Four wells (6 mm diameter) were made in each Muller-Hinton agar plate by using distal end of sterile Pasteur pipette. Before making wells, each bacterial suspension (10^8 colony forming unit (cfu)/mL) was spread on single agar plate with sterile cotton swap. One hundred micro liter of each honey sample was deposited into a separate well on the Muller-Hinton agar plate. These petri plates were incubated aerobically at 37 °C for 24 h in an incubator. The diameter of zone of inhibition around the outer surface of well was measured (Barry & Thorsberry, 1985).The same technique was done with casein. Casein was deposited into a separate well on Muller-Hinton agar plate.

6. Determination of minimum inhibitory concentration (MIC)

MIC is generally defined as the lowest concentration of a given antimicrobial that prevents growth of a microorganism after a specified incubation period (Vigil *et al*., 2005). In this study MIC was calculated using agar dilution and broth dilution methods. MIC was determined in this work as the minimal honey concentration where *Escherichia. coli* growth was not visually observed. The antibacterial activity was expressed as the mean diameter of inhibition zone (mm) produced by honey (Ali et al., 2017).

7. Synergistic testing

The fractional inhibitory concentration (FIC) index was used to quantify the synergistic interactions between honey and casein against Escherichia coli. The antimicrobial assays were performed using casein in combination of the Euphorbia, Multifloral honeys. *Escherichia coli* cultures were grown in the presence of one of the two honeys with the following concentrations: 10%; 20%; 30% and in combination with casein powder, with concentrations ranging from 1/4×MIC to 1/2×MIC.

These experiments were conducted in the same manner as for the MIC determination in the susceptibility testing.

The FIC index was calculated with the following formulas:

FIC honeys = MIC of honey in combination/MIC of honey alone.

FIC casein = MIC of casein in combination/ MIC of casein alone

FIC index = FIC honey+ FIC casein. Where FIC index values of less than 0.5 indicated synergy, 0.5–0.75 indicated partial synergy, 0.76–1 indicated an additive effect, and >2 indicated antagonism.(White *et al*., 1996, Issam *et al*., 2015)

3. Results

In the examined samples; the Euphorbia and Multifloral honey samples showed the lowest EC (0.011 and 0.014 ms/cm) respectively. The pH values of two honey samples were measured and the obtained results confirmed that, all tested samples were acidic (pH 4.3) (Table 1). The high acidity of honey correlates with the fermentation of sugars present in the honey into organic acid, which is responsible for two important characteristics of honey: flavor and stability against microbial spoilage (Bogdanov *et al.,* 2008). EC is one of the good criterions of the botanical origin of honey. This measurement depends on the ash and acid content of honey. The higher ash and acid content, the higher the resulting conductivity is.

Table 1. Physicochemical characteristics of Multifloral and Euphorbia honeys

Settings	Multifloral honey	Euphorbia honey
Density(g/ml)	1.413	1.379
PH	4,3	4,3
Acidity	3	3
Electonic Conductivity EC (ms/cm)	0.011	0.014

The results in vitro of antibacterial activity of Multifloral honey samples (Table 2) at two concentrations were (30% and 50% v/v) against *Escherichia.coli* from (avian, cow isolates) and human isolate respectively; But the results of antibacterial activity of Euphorbia honey samples were shown in (Table 3) at (20%30% and 50% v/v) concentrations against cow, avian and human isolates respectively.

Table 2. Minimal inhibitory concentration (MIC) of Multifloral honey on *Escherichia. Coli* of human, avian and cows isolates

Multifloral honey's Concentrations (v/v)	*MIC* diameter of (Human isolates) (mm)	*MIC* diameter (Avian isolates) (mm)	MIC diameter of (Cow isolates) (mm)
5%	6	6	6
10%	6	6	6
15%	6	6	6
20%	6	6	6
30%	6	13	14
40%	6	15	15
50%	14	16	15
70%	15	16	16

Table 3. Minimal inhibitory concentration (MIC) of Euphorbia honey on *Escherichia. Coli* of human, avian and cows isolates

Euphorbia honey's Concentrations (v/v)	MIC diameter of (Humain's isolates) (mm)	MIC diameter of (Avian's isolates) (mm)	MIC diameter of (Cow's isolates) (mm)
5%	6	6	6
10%	6	6	6
15%	6	6	6
20%	6	6	14
30%	6	13	15
40%	6	14	15
50%	14	15	16
70%	17	17	16

The result of combination effect between honeys and casein were demonstrated in (Table 4); for the human isolate at CMI of honey 50% and 25% for casein, of each Euphorbia and Multifloral honeys. Therefore, CMI of combination effect at 20% for both honeys and 50% of casein against (Avian and cows isolates).

Table 4. Minimal inhibitory concentration (CMI) combination of Euphorbia honey, Multifloral honey and casein on *Escherichia. Coli* of human, avian and cows isolates

Honey's concentrations /casein'sconcentrations (v/v)	CMI of Casein pure (mm)	CMI of Casein and Euphorbia honey (mm)	CMI of Casein+ Multifloral honey (mm)
Human's isolates 50% H+25% C	16	13	12
Avian'sisolates 20% H+50% C	14	12	13
Cow 's isolates 20% H+50% C	15	12	13

H: Honey's concentration ; C: Casein's concentration

The results of FIC index of combination of the casein and Euphorbia honey were 1.25, 1.16, 1.5 (Human, avian, cow) isolates respectively. But the FIC index of combination of Multifloral honey and casein were 1.16 for both of Avian and cow isolates, 1.40 for human isolate. The FIC index values indicate an additive effect.

4. Discussion

Electrical conductivity (EC):

EC is a good criterion of the botanical origin of honey and it is determined in routine honey control instead of the ash content (Adenekan *et al*., 2010). This measurement depends on the ash and acid content of honey; the higher ash and acid content, the higher the resulting conductivity.

PH:

The pH values of honey samples were acidic (pH 4.3) (Table 1). The pH values of two tested types of honey samples were close to those previously reported in Indian (Saxena, 2010). The high acidity of honey correlates with the fermentation of sugars present in the honey into organic acid, it might also indicate that the honey samples have high content of minerals (EL-Metwally, 2015).

Antibacterial effect of honey:

This study showed the antibacterial potential of Algerian honeys against *E. coli*. All honey samples displayed antibacterial activity; however the potential of each honey sample at different concentration varied against the tested bacterial strain. Euphorbia honey at 20% v/v, 30% and 50% (cow's, avian's, human's isolates respectively) concentrations showed antibacterial effect, while Multifloral honey at 30% and 50% v/v concentrations had the same effect. These findings are in accordance with (Alqurashi *et al*., 2013) who compared the Sider and Mountain honeys from Saudi Arabia against gram- negative bacteria and found that tested honey samples had inhibitory effect at 40–80% concentrations against the bacteria used in his study. Honey composition and biological activity differ according to its botanical origin and geographical location (Alzahrani, 2012). In many studies antibacterial properties of honey have been reported against pathogenic bacteria(*Bacillus cereus, Enterobacteraerogenes, Escherichia coli, Klebsiella pneumonia, Micrococcus luteus, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes* (Al-Nahari *et al.,* 2015; 7. Noori *et al*., 2013; Deng *et al.*, 2018; Hegazi *et al*., 2017; Rani *et al*., 2017; Wasihun & Kasa, 2016). MIC values for *E. coli* were observed high for the tested honey samples. The reasons for less susceptibility of *E. coli* to tested honeys could be the low permeability of its cell wall, resistance, and mutation (Wasihun & Kasa, 2016).

Antibacterial effect of casein:

There are some casein-derived antimicrobial that have been researched. One such of them is casocidin-I was proved to be antibacterial against *Escherichia coli* and *Staphylococcus carnosus* (Zucht et al., 1995). Another antimicrobial casein peptide is isracidin. It is bovine α S1 –casein. It has antibacterial activity against lactobacilli, Gram-positive, and Gram-negative bacteria (Lahov & Regelsond, 1996). The same authors declared that various Gram-positive bacteria were affected by a concentration between 0.1 and 1 mg/mL.

Furthermore, (Birkemo *et al*., 2008) showed that isracidin was effective against *E. coli* DPC6053 with a minimum inhibitory concentration (MIC) of 0.2 mg/mL. However, this casein derived has mostly been tested in vivo as immunoprotectants for animal infections against such bacteria as *L. monocytogenes and Staphylococcus aureus* (Birkemo *et al*., 2008).

The results revealed that the combination of honey with casein showed great additive antibacterial effect against *Escherichia coli* also.

5. Conclusion

In conclusion, the MIC of Euphorbia honey is at 20%; MIC of Multifloral honey is at 30%. These honeys have the same pH and acidity (4.3; 3) respectively, but EC; Density are different: (0.011; 0.413) to Multifloral honey; (0.014; 0.379) to Euphorbia honey respectively.

There is an additive effect between casein and honeys, that's mean the antimicrobial effect of each one used separately against *Escherichia coli.*(FIC index values are between 0.76-1).

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