CIENT PERIODIQUE

Synergistic Antimicrobial Effect of Egyptian Honey With Antibiotics and Its Capability to Restore MRSA Sensitive to Oxacillin

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Abstract

Since some types of honey interact synergistically at sub-lethal {sub minimum inhibitory concentration (MIC)} concentrations with antibiotics, the work tested this activity of six Egyptian honey brands; three of potent (fennel, 2 of anise & black seed) as well as three of fair antimicrobial activity (multi-floral) against two G-ve and two G+ve multi drug resistant bacterial strains [*E. coli AUMC B-243, Klebsiella pneumonia AUMC B-257* and *Strept. agalactiae AUMC B-253* and methicillin resistant *Staphylococcus aureus* (MRSA)] using agar dilution method. The four bacterial strains had high resistance index (RI) ranging from 0.18 up to 0.73. All tested honey brands had different potencies of antimicrobial activity against all bacterial strains with MICs values (6-15%), where the best overall MIC was with the use of anise & black seed II (8.5%) followed by fennel (9.5%). Either of potent or fair antimicrobial honey brands showed synergistic action with different antimicrobial agents against MRSA with synergistic index (SI) ranged from 0.17 up to 0.39, where the best overall SI of each honey brand against the tested antimicrobial agents was with the use of anise & black seed I (0.39) followed by fennel (0.37). None of the tested honey types showed

bactericidal action and all had bacteriostatic activity but the interesting wonderful observation as that all tested Egyptian honey brands (either with of potent or fair activity) restored MRSA (methicillin resistant *Staphylococcus aureus*) sensitive to Oxacillin being MESSA (methicillin sensitive *Staphylococcus aureus*). The study concluded that all six tested honey either of potent or fair antimicrobial activity honey brands had variable synergistic activity with antibiotics against MRSA and restored it sensitive to Oxacillin.

Introduction

The emergence of drug-resistant bacteria is one of the most important global health crises [1] and searching for effective natural safe antimicrobials is of global concern [2]. Honey proved to have a potent natural *in vitro* antimicrobial activity either produced by *Apis mellifera* [3-6] or stingless *Trigona spp.* Bee [7-9].

Synergism of this activity when be combined with other antimicrobial agents is fully studied with either Newzeland Manuka honey [10-16], Egyptian sesame, eucalyptus [17], sidr [18], Saudiansidr, sommor [19], Indian dabur & patanjali [20] or Malaysian trigona honey [21]. Consequently,the use of antimicrobial agent-phytochemical combinations that will neutralize the resistance mechanism, enabling the drug to still be effective against resistant microbes [22].

The present work aimed to evaluate the possible the synergistic interaction of some Egyptian honey brands with common antibiotics against bacterial strains. Since methicillin resistant *Staphylococcus aureus* (MRSA) was classified by the World Health Organization (WHO) as one of twelve priority pathogens that threaten human health [23], the study was designed to examinecapability of the tested Egyptian honey types to restore a clinical stubborn multidrug resistant MRSA strain (harboring both mecA & icaA genes) sensitive to Oxacillin which is sensitive to all β-lactam antibiotics.

Material and Methods

Honey Samples: The study was conducted with six different honey brands (through the preliminary work as three of them of potent antimicrobial activity; one of fennel and two of anise & black seed, while the other three were multi-floral having fair activity)were obtained from apiaries reared in different geographic locations in Egypt and kept in dark bottles away from direct sun light or any source of heat.

Bacterial Strains: Three reference strains (*E. coli AUMC B-243*, *Klebsiella pneumonia AUMC B-257* and *Strept. Agalactiae AUMC B-253*) Kindly provided from Assiut University Mycological center (AUMC) as well as a clinical MRSA strain harboring both *mecA* and *icaA* genes obtained from author's previous works originated form bovine subclinical mastitis [3] were used.

Antimicrobial Sensitivity Testing: The four bacterial strains (two Gram's positive and two Gram's negative) were tested against 11 antimicrobial agents [Oxacillin (OX) 1μg, Ampicillin /Sulbactam (SAM) (20μg/disc - 10/10μg), Cefotaxime (CTX) 30μg, Ciprofloxacin (CIP) 5μg, Vancomycin (VA) 30μg, Rifamycin (RF) 30μg, Doxycycline (DO) 30μg, Gentamicin (CN) 10μg, Oxytetracycline (T) 30μg, Penicillin (P) 10μg and Trimethoprim - Sulfamethoxazole (SXT) (25μg - 1.25/23.75μg); Bioanalyse, Turkey] using disc diffusion

sensitivity method according to Kirby-Bauer as described in the guidelines of the National Committee for Laboratory Standards [24]. For Oxacillin inhibition zones Ø around the disc were measured after 24 and 48 h using the following breakpoints: susceptible ≥ 13mm and resistance ≤ 11mm [25]. Antimicrobial resistance index (RI) of each tested bacterial strain was calculated.

Honey Minimum Inhibitory Concentration (MIC) Determination: Agar dilution method was done as each honey brand was dissolved in sterile deionized water to prepare a stock solution of 20% (v/v) honey immediately before use. Further dilutions were prepared by adding honey and sterile deionized water to sterile 10-ml volumes of molten double-strength nutrient agar (Oxoid) at 50°C and pouring immediately to produce a range of plates containing honey at 2% (v/v) intervals between 2 and 20% (v/v). Plates were dried at 37°C for 15 min before use. Undiluted overnight broth cultures of MRSA, *E. coli AUMC B-243*, *Klebsiella pneumonia AUMC B-257* and *Strept. Agalactiae AUMC B-253* was inoculated onto dried honey-containing plates as 0.3μl spots. Plates were incubated at 37°C for 24 h before visual assessment. Any visible bacterial growth against any honey concentration, higher concentration was tried till reached complete growth inhibition. Two replicate plates were used at each concentration of honey and the experiment was repeated at least twice [26].

Bactericidal and Bacteriostatic Study: It was adopted for all six honey brands against the above referred MRSA strain as follows; 50% (v/v) in deionized distilled water of each six honeys brand were dispensed into sterile tubes. Then 0.1ml MRSA strain containing $5x10^5$ was inoculated into those tubes and incubated for 24 h at 37°C. This concentration resulted in the complete inhibition of bacterial growth. In order to verify whether the honey has a bacteriostatic/bactericidal, 1ml was added to 9ml broth without honey and incubated for 24, 48 and 72 h.; 0.1ml of those cultures was placed on nutrient agar (free of honey) for 24 h to check for signs of bacterial growth. If growth had occurred the honey type was considered as bacteriostatic and bactericidal when inhibition of growth persisted [27].

Honey Antibiotics Synergism Study Against MRSA: Synergy assay was based on previously described procedures [28], where the above mentioned MRSA strain was used. The synergistic action of six types of honeys with the mentioned antimicrobial agents was assayed by the disc diffusion method on Mueller-Hinton agar. After determination of MIC of honey, various concentrations of honey, 1% below their MIC were prepared (which permitted bacterial growth). Plates containing sub-inhibitory concentrations of honeys, as well as control plates without honey, were seeded with MRSA ($5x10^5$ CFU/ml) and the previously antimicrobial discs were placed onto the seeded plates. Plates were incubated 24 h at 35°C. The effect of honey was measured by the relation of diameters of the growth inhibition zones around the discs in the presence of honey to the diameters of the growth inhibition zones around the discs without the presence of honeys.

The maximized antimicrobial activity of different antibiotics by adding honey with subMIC and synergistic index (SI) is calculated as:-

$$SI = \frac{A - B}{C}$$

A: inhibition zones Ø of sub MIC honey with antimicrobial agent.

B: inhibition zones Ø of antimicrobial agent only.

C: break point of inhibition zones Ø as reference [25].

Results & Discussion

Honey has been increasingly recognized as a potential therapeutic agent [29] due to its antibacterial activity as the most investigated biological property by the action of phytochemical contents [30] that differ geographically or seasonally [29]. So, the present work investigated 6 Egyptian honey brands for different views of their antimicrobial activity against multidrug resistant bacterial strains. The tested strains had RI 0.18 - 73 (Table 1), as high index values since RI just ≥0.2 is considered high resistant strain [31] that might be originated from environments with misuse of antibiotics where resistance developed and spread [32]. Against these resistant bacterial strains, all tested Egyptian honey brands had potent antimicrobial activity with MIC values 6-15% (Fig. 1), where the least MIC (the most potent antibacterial action) appeared with the use of Anise and Black seed II (6%) followed by fennel (8%) against either *MRSA* or *Klebsiella pneumoniae* and the least activity was related to the three multi-floral honey brands (15%) against *E. coli* as shown in (Fig. 1). Moreover, the least overall MIC (most potent) against all tested bacterial strains as of that of anise and black seed II (8.5%) followed by fennel honey (9.5%). Anise and black seed lack documentary, while the antimicrobial activity of Egyptian fennel honey has been documented against MRSA [3] or different G +ve and G -ve bacterial strains [33-35].

Table 1: Antimicrobial study of the tested strains

Bacterial strains CTX		Ø zone of inhibition of the tested bacterial strains against tested antibiotics (mm)										RI
		P	CIP	VA	SXT	RF	SAM	CN	T	OX	DO	
G + ve	*Strept. agalactiae*	15	23	27	22	8	17	18	16	11	8	17	0.73
	MRSA	14	23	31	23	30	35	20	18	40	11	36	0.36
G - ve	*E. coli*	8	8	26	20	8	15	8	21	25	8	30	0.55
	Klebsiella pneumoniae	14	40	31	25	22	28	34	30	38	8	40	0.18

Underlined: resistant results RI: resistance index
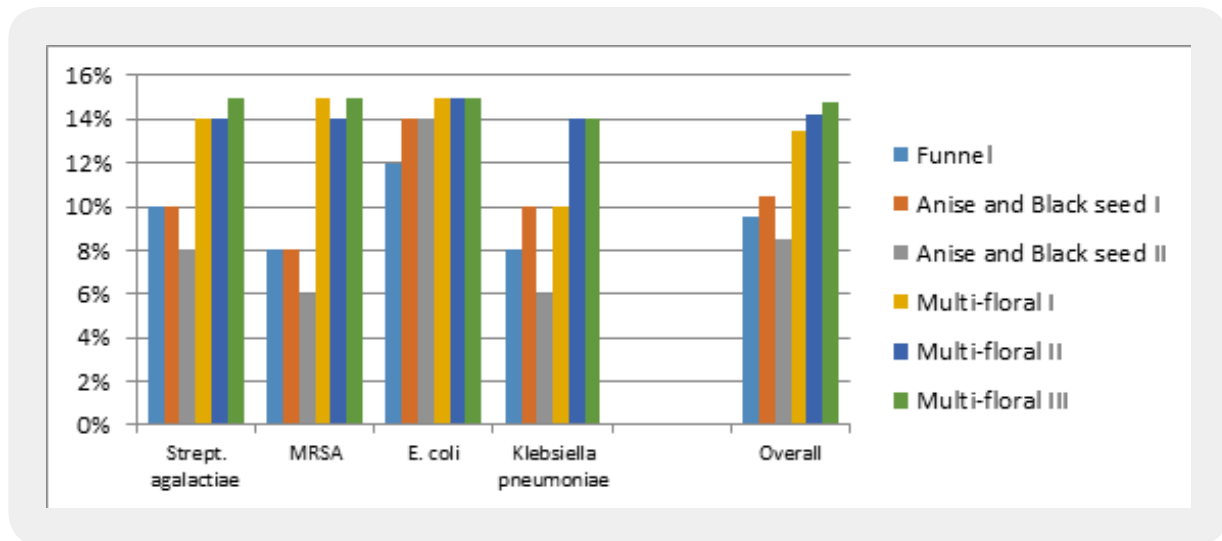
Figure 1: *Minimum inhibitory concentration (MIC) of different honey brands against multidrug resistant bacterial strains*

About the bactericidal study, neither potent nor fair antimicrobial honey brands had bactericidal action, but all of them showed bacteriostatic activity against the tested MRSA strain. The interesting promising observed result was that all tested honey (either with potent or fair antimicrobial activity) restored MRSA sensitive to oxacillin being MESSA which is sensitive to all β-lactam antibiotics penicillin derivatives, cephalosporins, monobactams, carbapenems and carbacephems [25]. (Table 2 & Fig 2,3) As documentary studies, both potent antimicrobial Saudian honey types (Sidr and *Nigella sativa*) had only bacteriostatic activity not as manuka honey which is widely documented as the most antimicrobial honey [27]. Synergistic susceptibility pattern of several microbes towards the natural antimicrobials and their combinations is indicated by significant decline in MIC or sub-lethal [18].

Table 2: *Synergistic effect of honey (↑) and the antimicrobial agents against MRSA*

| Antimicrobial agents	BP	Antimicrobial disc only	Tested honey brand					
			Funnel	Anise and Black seed I	Anise and Black seed II	Multifloral I	Multifloral II	Multifloral III
			Ø zone of inhibition of the tested antimicrobial agents(mm)					
CTX	23	14	26 ↑	21↑	32↑	14	16↑	18↑
P	29	23	26↑	28↑	28↑	24↑	24↑	32↑
CIP	21	31	40↑	45↑	33↑	34↑	33↑	40↑
VA	12	23	30↑	28↑	26↑	29↑	24↑	32↑
SXT	16	30	33↑	30↑	30↑	35↑	35↑	35↑
RF	20	35	42↑	45↑	38↑	40↑	38↑	40↑

SAM	29	20	30↑	33↑	28↑	30↑	25↑	30↑
CN	15	18	24↑	26↑	28↑	24↑	22↑	18
T	19	40	45↑	46↑	44↑	40↑	40↑	42↑
OX	13	11	18↑	19↑	18↑	16↑	16↑	16↑
DO	19	36	45↑	45↑	45↑	38↑	42↑	40↑
MRSA bacteriostatic action			√	√	√	√	√	√
MRSA bactericidal action			---	---	---	---	---	---

Underlined: resistant results (↑): positive synergism
B P: Breakpoint of Ø inhibition zone sensitivity [25]
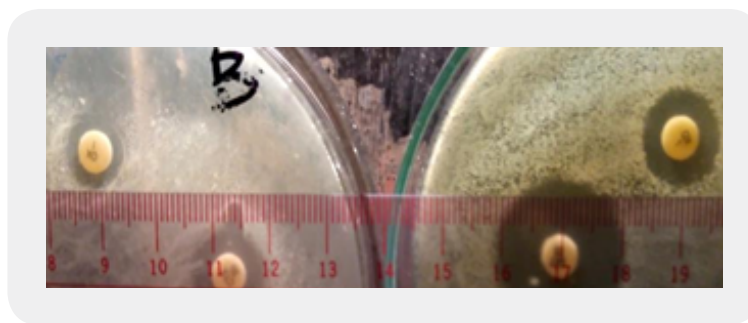
*Figure 2: Multifloral I (honey of fair antimicrobial action)*
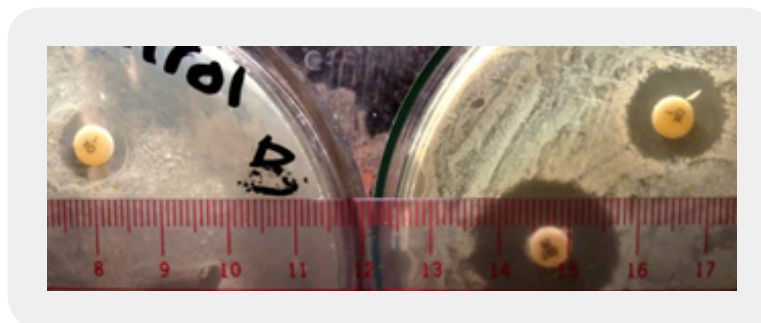
Figure 3: Anise and black seed II (honey of potent antimicrobial action) restored MRSA sensitive to Oxacillin.

Against MRSA, all examined honey brands (both with potent and fair antimicrobial activity) showed synergistic action with the tested antibiotics (Table 3, Fig 4 & 5). Moreover, the tested strain which was resistant against Cefotaxime (CTX), still resistant with sub-MIC of four honey types but only with fennel or anise and black seed II it showed sensitive activity (Fig 4). Also, against penicillin (P) still resistant except with multifloral III, while against Ampicillin /Sulbactam (SAM) appeared to still resistant only with Anise and Black seed II and restored sensitive with other honey types (Table 2). It is obvious that both potent and fair antimicrobial honey brands had variable synergistic activity with SI of each honey brand against each antimicrobial agent (0.17 - 0.39), where the three potent antimicrobial honey types had high SI more than

the other fair types, but with the highest overall SI values as with the use of anise & black seed I (0.39) followed by fennel (0.37) as shown in table (3). Against MRSA, Manuka honey showed synergistic action with linezolid [14], Oxacillin [10,13] and rifampicin [11]. Moreover, it showed active synergism with ciprofloxacin, ceftazidime, and tobramycin against *P. aeruginosa* [16]. Against *S. aureus,* Malysian trigona honey with ampicillin [21], Saudi sidr and sommor honey with Ofloxacin, Pipracillin, Amoxicillin plus Clavulanic acid or Sulphamethoxazole plus trimethoprim [19], or multifroral Algrian honey with Amoxicillin plus Clavulanic acid, Gentamicin, and trimethoprim plus Sulfamethoxazole [36] proved good synergistic actions. Also Egyptian sesame and eucalyptus with Ciprofloxacin, Cifotaxim and Tobramycin against different *Clostridium Spp.* [17] or Egyptian undefined floral honey with Ceftazidime, Cefoperazone, Cefoxitin and Imipenem against *Strept.pyogenes* [37] showed promised synergistic activity. Manuka honey interacts synergistically at sub-lethal (sub MIC) concentrations with Oxacillin, restoring the drug's effectiveness against MRSA [10,13].

Table 3: *Synergistic index (SI) of the tested honey brands with antimicrobial agents against MRSA.*

Antimicrobial agents	SI values of the tested honey brand					
	Funnel	Anise and Black seed I	Anise and Black seed II	Multifloral I	Multifloral II	Multifloral III
CTX	0.52	0.30	0.78	0.00	0.09	0.17
P	0.10	0.17	0.17	0.03	0.03	0.31
CIP	0.43	0.67	0.10	0.14	0.10	0.43
VA	0.58	0.42	0.25	0.50	0.08	0.75
SXT	0.19	0.00	0.00	0.31	0.31	0.31
RF	0.35	0.50	0.15	0.25	0.15	0.25
SAM	0.34	0.45	0.28	0.34	0.17	0.34
CN	0.40	0.53	0.67	0.40	0.27	0.00
T	0.26	0.32	0.21	0.00	0.00	0.11
OX	0.46	0.54	0.46	0.31	0.31	0.31
DO	0.47	0.47	0.47	0.11	0.32	0.21
Overall SI	0.37	0.39	0.32	0.22	0.17	0.29
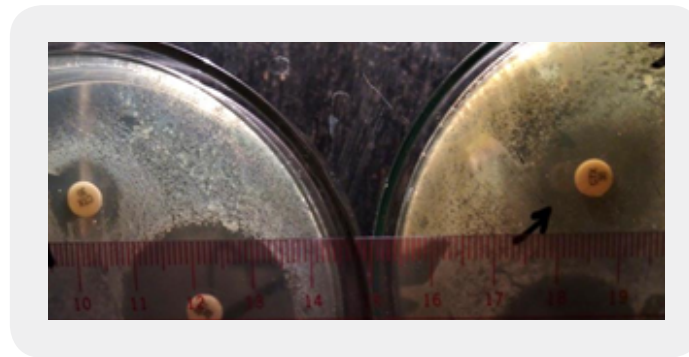
Figure 4: Synergistic action of anise and black seed II
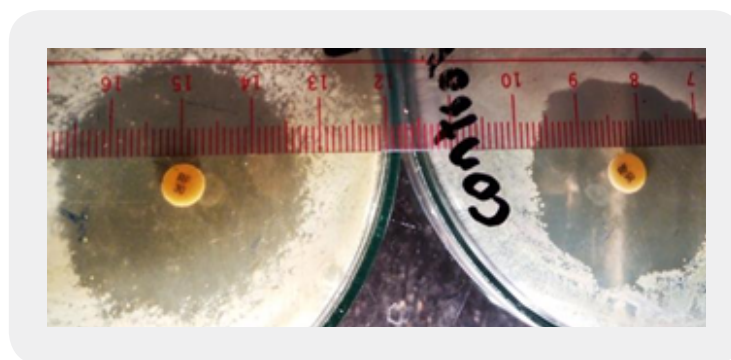
Figure 5: Synergistic action of anise and black seed II honey with CTX against MRSA. Honey with RF against MRSA

β-lactam antibiotics (e.g. Oxacillin) target enzyme penicillin-binding proteins (PBPs), the enzyme encoded *SCCmec* including *mecA* that conferring resistance to the entire class of β-lactam drugs [38] - the gene which is regulated by *mecR1* gene [39] - prevents peptidoglycan synthesis, this explain the role of *mecA* in Oxacillin resistance [40]. The restore MRSA Oxacillin resistance (being MESSA) is due to the down regulation of *mecR1* affecting *mecA* through regulation of *arlRS* gene which is necessary for MRSA adhesion, biofilm formation, and virulence [41].

It is documented that the activated *ArlR* recognizes a 20-bp imperfect inverted repeat sequence in the *ica* operon, which is involved in intercellular adhesion polysaccharide production [41]. The over expression of *spx* in *arlRS* gene [42] could be affected by Manuka honey [10] restoring Oxacillin resistance in MRSA. Manuka honey affects (down regulation) MRSA accessory gene regulator genes (*agrB, agrC, agrD*) responsible for virulence and biofilm formation and *cidB* responsible for bacterial cell division [43]. The reversion of Oxacillin resistance to Oxacillin susceptibility owing to a reduced expression of *mecR1* caused by Manuka honey [10] may have resulted from a down-regulation of *agr*. Reversal of oxacillin resistance in MRSA not only achieved by Manuka honey [10,13,44] but also it has been reported using some medicinal extracts (green tea, red sage or Chinese liquorice) [45]; owing to the affected *mecA*gene, MRSA (either from human origin or bovine mastitic milk) missed *mecA* gene using PCR testing after exposure to sidr honey by the combinations of different phytochemicals with antibiotics, since after exposure of MRSA

to different Sidr honey, mecA gene was absent, this indicates that sidr honey has an inhibitory effect on *mecA* gene of MRSA [46]. Otherwise different Sidr honey brands inhibited *coa* (coagulase) and *spa* (staph binding protein) genes of MRSA (originated from catfish) responsible for some of its virulence but did not affect *mecA* gene responsible for Oxacillin resistance [18], the issue which needs more investigation using the same honey brands against MRSA of different origins. Despite of that all the tested honey brands were bacteriostatic, it is of great interest that all tested Egyptian honey brands restored MRSA sensitive to Oxacillin regardless to its mechanism.

Conclusion

The study concluded that the tested Egyptian honey brands have good antimicrobial activity against multidrug resistant bacterial strains. All these honey types (either of potent or fair antimicrobial action) showed synergistic action with antimicrobial agents against MRSA and restored it sensitive to Oxacillin. Subsequently, the study recommended the use of honey either solo or together with the effective antimicrobial agents since it has direct action against MRSA over its MIC or even with sub MIC, it results synergism of antimicrobial action.

Conflicts of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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