Alternative & Integrative Medicine

Aamer, et al., Altern Integ Med 2014, 3:4
DOI: 10.4172/2327-5162.1000171

Research Article Open Access

Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of Honey and Bee Propolis against Multi-Drug Resistant (MDR) Staphylococcus sp. Isolated from Bovine Clinical Mastitis

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Rec date: Jun 25, 2014, **Acc date**: Sep 19, 2014, **Pub date:** Sep 21, 2014

Abstract

With the emergence of antibiotic-resistant *Staph. sp.,* search for antimicrobial agents other than antibiotic is of great concern. The study aimed to determine both MIC and MBC of different honey samples against these strains. The study was conducted with 64 different *Staph sp.* isolated from bovine mastitis and tested *in vitro* against 11 antimicrobial agents. The most MDR strains (19) were tested *in vitro* against six honey batches; marjoram, cotton, two fennel samples and two different trefoil samples as well as against 10% propolis-fennel honey mixture. Both MIC and MBC of the tested honey samples against every tested strain were determined. Propolis-fennel honey mixture showed the lowest both MIC AND MBC values against all *Staph sp.* all over the study with highly significant differences, while against different *Staph sp.,* also it had the lowest MIC and MBC values against *S. intermedius* followed by *S. aureus*. The study revealed that among the different *Staph. sp., S. aureus* was the most sensitive species to the honey antimicrobial action with highly significant differences. The study concluded that all tested *Staph. sp.* –despite of being MDR- were sensitive to the antimicrobial activity of all tested honeys where *S. aureus* was the most sensitive one, while adding 10% propolis powder would maximize its antimicrobial activity significantly.

Keywords: MIC; MBC; Apitherapy; Antimicrobial; Staphylococcus; Mastitis

Introduction

As the traditional knowledge about the use of natural products or substances should be scientifically investigated [1] and the antimicrobial application requires safe preparations, knowledge of the composition of antibacterial factors and standardized antibacterial activity [2], the *in vitro* study of honey therapeutic action is of great necessity for its applicability. Honey possesses therapeutic potential and its antimicrobial activity is widely documented as a large number of *in vitro* studies of MIC and MBC confirmed its broad-pectrum antimicrobial properties either in solo use [3-6] or in combination with other agents as royal jelly [7], bee propolis [8], ginger starch [9], garlic extract [1] or rifampicin [10] even on MDR such as *S. aureus* methicillin resistant (MRSA) [11] or vancomycin-resistant enterococci (VRE) [12]. Propolis extract also proved to possess antimicrobial activity [13-17] Moreover, subinhibitory concentration of honey in combination with oxacillin restored oxacillin susceptibility to MRSA [11]. The present work aimed to investigate the *in vitro* MICs and MBCs of different honey batches and propolis powder against different MDR *Staph. spp.* isolated from bovine clinical mastitis.

Material and Methods

Bacterial isolation

Out of 101 milk samples from clinical mastitic cows through a previous work for the same author [18], 64 *Staph. sp.* strains were recovered and be the baseline of the present study where the most MDR strains (no 19) as *Staph aureus* (6), *Staph intermedius* (3), *Staph saprophyticus* and *Staph epidermedis* (5 for each)were tested against all honey patches.

Antimicrobial sensitivity testing

All these 64 isolated *Staph. sp.* strains were tested against 11 antimicrobial agents [Oxacillin (OX) 1 µg, Ampicilin (AM) 10 µg, Cefotaxime (CTX) 30 µg, Doxycycline (DO) 30 µg, Enrofloxacin (ENR) 5 µg, Gentamicin (CN) 10 µg, Lincomycin (L) 2 µg, Oxytetracycline (T) 30 µg, Penicillin (P) 10 µ, Trimethoprim – Sulflamethaxzole (SXT) 25 µg and Cloxacillin (CX) 10 µg]* to determine the MDR strains using disc diffusion sensitivity method according to Kirby-Bauer as described in the guidelines of the National Committee for Laboratory Standards (NCCLS) [19]. For Oxacillin inhibition zones around the disc were measured after 24 and 48 h using the following breakpoints: susceptible (S) ≥ 18 mm; resistance (R) ≤ 17 mm [20].

Honey batches

Six row full strength different unprocessed honey batches were used in the study; A (marjoram), B (cotton), C (fennel-1)**, D (fennel-2)**, E (trefoil-1)** and F (trefoil-2)** as well as G (10% propolis- Fennel honey mixture) as 10% w/v bee propolis powder*** in fennel honey. To study the synergistic action and to detect the sole antimicrobial action of propolis, 50 mg propolis powder (the added amount in propolis honey mixture) was tested plain for its MIC and MBC against all tested strains.

Determination of MIC

Three to six strains of the most MDR strains from each species were chosen for the *in vitro* MIC and MBC study. Honey batches were investigated for their MIC and MBC against the chosen isolated *Staph. sp.* strains where 1 ml of the tested honey was used in bifold dilution method [21] with series of 6 tubes containing 1 ml of Mueller Hinton broth (Accumix – Verna, India) to achieve final dilutions of 50, 25, 12.5, 6.25, 3.12 and 1.62% v/v. Standard bacterial inoculums (5×10^5) of the chosen isolated *Staph. spp.* were inoculated into all 6 dilutions post thorough honey mix. The inoculated tubes were overnight incubated at 37°C. The highest dilution of the tested honey to inhibit growth (no turbidity in the tube) was considered as the MIC value of this honey batch against the tested bacterial species.

Determination of MBC

From all tubes showed no visible signs of growth/turbidity (MIC and higher dilutions), loopfuls were inoculated onto sterile Mueller Hinton agar (Accumix – Verna, India) plates by streak plate method. The plates were then overnight incubated at 37°C.. The least concentration that did not show any growth of tested organisms was considered as the MBC value of the tested honey against the tested bacterial species.

Statistical analysis

Mean values, standard deviation (SD) and ANOVA analysis were adopted by means of PASW V.18 (2010, spss Inc, Chicago, Illinois, USA). Results were considered statistically significant when P>0.05 and highly significant when P>0.01.

*Antibiotic sensitivity discs were purchased from Bioanalyse - Turkey.

**Fennel or Trefoil 1 and 2: honey batches were collected from two different pasture locations.

***Chinese bee propolis provided kindly from Plant Protection Research Institute (PPRI)- Assiut unit.

Results

The present study was conducted with 64 *Staph. sp.* strains isolated from bovine mastitis, where the most MDR strains which showed MDR pattern > 6 antimicrobials were chosen and be prepared for MIC and MBC study as shown in Table 1. Against *Staph. sp.,* all tested strains - which showed at least 6 MDR pattern - were sensitive to all tested honey batches with MICs ranged from 20.83% (trefoil-2) up to 33.33% (fennel-2) (Figure 1) and MBCs from 37.92% (cotton) up to 45.83 % v/v (for both fennel-1 and trefoil-1) (Figure 2). However, 10% propolis fennel honey mixture showed the most favorable results as the lowest both MIC and MBC (13.96% and 28.26 % v/v respectively) with highly significant differences p>0.01 (Figures 1 and 2).

Propolis powder alone gave no any bacterial inhibition. *S. aureus* showed the lowest MIC (13.3%) and MBC (27.1%) v/v with highly significant differences P>0.01 (Figures 3 and 4) among all tested *Staph. sp.* By the statistical analysis for the antibacterial activity of different honey batches against different *Staph. sp.*, it was found that propolis honey mixture had the lowest MIC value against both coagulase positive *Staph. sp.* (*S. intermedius and S. aureus*) all over the present study as 6.2% and 7.25% v/v respectively with highly significant differences P>0.01 (Figure 5), while MBC values were 12.5 and 14.58% respectively (Figure 6).

Isolates	Antimicrobial testing		Honey tested strains	
	MDR			
	No.	≥5 antimicrobials	No.	MDR pattern
S. aureus	35	30	6	9 antimicrobials
S. intermedius	9	5	3	(6-7) antimicrobials
S. saprophyticus	11	8	5	(7-9) antimicrobials
S. epidermidis	9	8	5	8 antimicrobials
Total	64	51	19	

Table 1: *Staph. sp.* isolated from bovine clinical mastitis and MDR pattern of the honey tested strains.

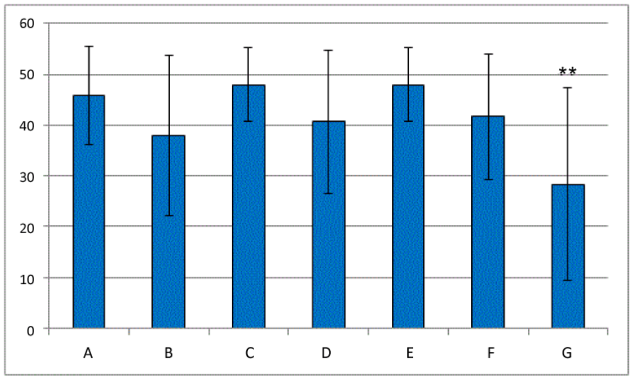

Figure 1: MIC values of different honey batches against *Staph. Sp.*

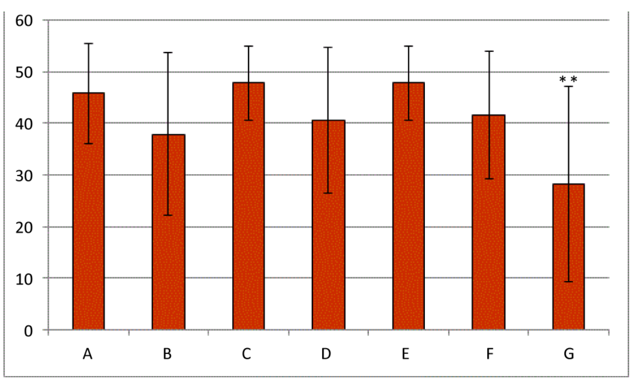

Figure 2: MBC values of different honey batches against *Staph. Sp.*

Citation: Aamer AA, Abdul-Hafeez MM, Sayed SM (2014) Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of Honey and Bee Propolis against Multi-Drug Resistant (MDR) Staphylococcus sp. Isolated from Bovine Clinical Mastitis. Altern Integ Med 3: 171. doi:10.4172/2327-5162.1000171

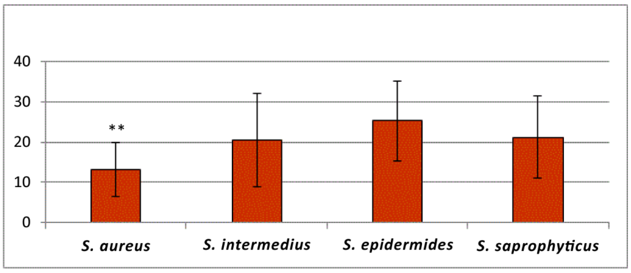

Figure 3: MIC values of honey against different *Staph. Sp.*

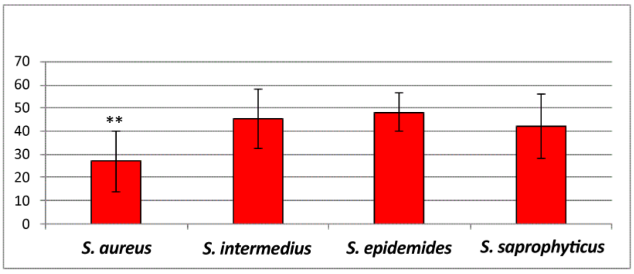

Figure 4: MBC values of honey against different *Staph. Sp*

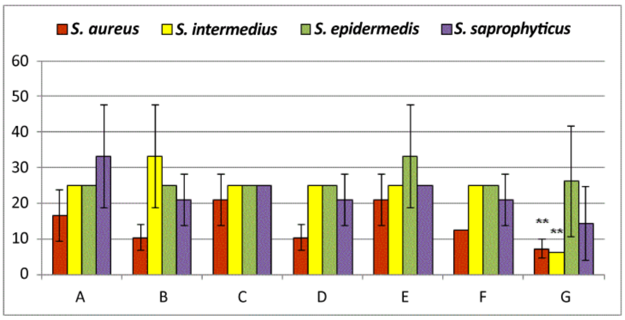

Figure 5: MIC values of different honey batches against different *Staph. Sp.*

Discussion

Veterinary apitherapy nowadays is documented either in dairy [22,23] or broiler [24] farms rather than in immunomodulation performance [25]. Concerning to apitherapeutic antimicrobial activity, it is widely documented as mentioned in the above premise. MRSA contribute the most predominant isolated species from bovine mastitis milk [18] and is widespread pathogen. It is of great concern for human public health hazard threatens transmission among dairy farm workers or their environments [26]. The emergence of antibiotic-resistant bacteria leads to the re-examination of earlier remedies such as honey [7] or propolis [27]. The antibacterial potency differences among different studied honey samples could be attributed to the natural variations in floral sources of nectar and the different geographical locations since honey micro components possess physicochemical and phytochemical characteristics resulting in its potency that differs associated with botanical and geographical origins [28].

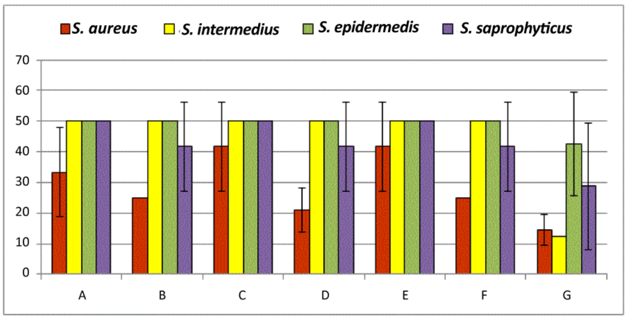

Figure 6: MBC values of different honey batches against different *Staph. Sp*

Different honey samples of different botanical or geographical origins; Egyptian honey had MIC and MBC values as 12.5 and 50% v/v [29], Malysian honey as 5% and 6.25% w/v [6], UK Manuka honey had MIC as 6% w/v [11] and Ethiopian honey as 6.25% w/v [3]. Honey antimicrobial action involves several mechanisms but mainly the presence of bacteriostatic and bactericidal action is due to production of hydrogen peroxide [30]. H2O2 alone may not be sufficient to the full activity [31], since it is in conjunction with other unknown honey components produce bacterial cytotoxic effects and DNA degradation. The concentration of polyphenols and H2O2 in different honeys may be of critical importance for bacterial cell survival [32]. Another mechanism of honey antimicrobial activity may be due to its lysosomal contents [33] or micro components as polyphenols, phenolic acids and flavonoids [34] or due to increase in cytokine release [35]. On the other hand, the mechanism of propolis antimicrobial activity is more complex and might be attributed to the synergistic activity between its various potent biological ingredients [8] that more than 300 compounds mainly phenolics and flavonoids [36]. It was found that propolis affects bacterial cytoplasmic membrane, and it inhibits motility, enzyme activity, cell division, and protein synthesis through inhibition of RNA-polymerase which can explain partially the synergism of propolis with drugs [37]. Moreover, galagin and caffeic acid derived[38] Since the synergistic action might be detected when the MIC of the combination of both studied antimicrobial agents is lower than the MIC of each alone [8], the present study was designed to test the added propolis powder (50 mg) alone where did not inhibit the tested *Staph. sp.* The present study chose Egyptian fennel honey for propolis mixture as our previous studies [25,29] recommendations. Although fennel showed low results for both MIC and MBC through the present study, its antimicrobial action was maximized giving highly significant difference (P>0.01) when propolis be added 10% w/v. The synergy of honey antimicrobial activity when be added to another antimicrobial was fully studied [1,7-10] and for propolis, the added flavonoids and phenolic acids - have antibacterial, antifungal and antiviral properties [39]- might maximize the action of these micro components present in honey resulting in synergy of its antimicrobial action. Fortunately, *S. aureus* (either MRSA or methicillin sensitive) which is the most predominant and virulent pathogen was the most sensitive *Staph. sp.* to honey antimicrobial action with highly significant. It is documented and proved that S.

aureus was the most sensitive species to the antimicrobial activity of honey among all tested bacterial species studied [1,2,40].

Conclusion

It was concluded that all tested MDR *Staph. sp.* were sensitive to the antimicrobial activity all tested honey samples, where S. aureus was the most sensitive one among the four tested *Staph. sp.* It was concluded that adding 10% w/v propolis powder to the chosen honey patch would maximize its antimicrobial activity with highly significant difference. The promising results encourage the utilization of propolis extract in combination with the chosen honey patch for treatment of subclinical bovine mastitis to achieve the synergistic antimicrobial action.

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