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Antibacterial Activity of Honey Samples on Methicillin Resistant *Staphylococcus Aureus* (MRSA) Isolated From Human Conjunctiva

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Abstract: *Honey exhibits antimicrobial activities against different types of bacteria in world wide. This study aims to analysis the antimicrobial effects of three types of honey (named as S1, S2 &S3) obtained from different places of Theni district, South India against methicillin resistant Staphylococcus aureus isolated from human conjunctiva. Three honey samples are subjected to antimicrobial activity with different dilutions (0%), (25%), (50%), (75%) and (100%) against MRSA method. In 100% dilution of S1 sample showed maximum zone of inhibition (14±0.0mm) was observed. Three honey samples were extracted with various solvents such as ethanol, methanol and ethyl acetate and antimicrobial activities of honey extractions were analyzed. Methanol extract of S1 sample showed maximum zone of inhibition (14±0.0mm) was observed. The MIC of methanol extract of S1 sample showed better bacteriostatic activity at 3.12%. From this study we found S1 sample have better antibacterial activity than S2 and S3 sample against MRSA isolated from human conjunctiva.*

Keywords: *conjunctiva, MRSA, honey, disc diffusion method, MIC*

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I. INTRODUCTION

Structures of eye are damaged by ophthalmic infections, which can lead to vision loss and even blindness if left untreated [(1)]. Bacterial conjunctivitis is the most common ocular infection seen by primary care physicians worldwide, which is self-limiting and largely presents as an acute infection [(2,3)]. In order to efficiently eliminate a pathogen treatment should be rapidly and highly effective and to minimize the risks of developing antibiotic resistance. Bacterial resistance development to specific antibiotics is an important consideration for clinicians treating ocular infections. Bacterial resistance has been emerging worldwide, likely due to widespread and inappropriate dosing of broad-spectrum antibiotics for systemic infections, exacerbated by inadequate compliance to full treatment duration[(4)]. As a result of its multiple drug resistance and its increasing prevalence, MRSA is a serious cause of morbidity and mortality worldwide [(5)]. Antibacterial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens emerge and spread, the effectiveness of the antibiotics is reduced. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to ocular vision, and for all kinds of antibiotics, including the major last-resort drugs, the frequencies of resistance are increasing worldwide [(6,7)]. Therefore, alternative antimicrobial strategies are urgently required, and thus this situation has led to a re-evaluation of the ancient therapeutic remedies, such as plants and plant-based products, including honey [(8-10)].

Honey is a sweet and flavorful of natural product, which has high nutrition value and beneficial effects on human health, with antioxidant, antimicrobial properties and wound healing effects[(11)].Honey composed of carbohydrates approximately 82.4% and in which includes 38.5% fructose, 31.0% glucose and 12.9% from carbohydrates consisting of maltose, sucrose and other sugars [(12,13)]. Honey was used by humans is traced to some 8000 years ago as depicted by Stone Age paintings [(14)]. Aristotle (384-322 BC) described pale honey as being ‘‘good for sore eyes and wounds” [(15,13)].

The composition of honey varies from one honey to another depending on several factors. A major factor is the floral region, as the nectar from different plants which contain different compositions of the main sugars and trace elements. These compositions are influenced by climatic conditions, environment surrounding the plant and the soil type [(16)]. The bactericidal effect of honey is reported to be dependent on concentration of honey used and the nature of the bacteria [(17,18)].

Considering the fact that there might be some specific constituents which may be contributing to the antimicrobial behaviour, it was decided to carry out the studies using different solvents. In this present study, we

assessed the antibacterial activity of various honey samples obtained from various places of Theni district, South India against methicillin resistant *Staphylococcus aureus* (MRSA) isolated from Human conjunctiva.

II.MATERIALS AND METHODS

2.1.Bacterial Sample

Methicillin resistant *Staphylococcus aureus* was isolated from the conjunctiva of patients who attending the Sankara eye care hospital, Trichy district, South India. The bacterial slants were incubated overnight at 37^0C. 0.5 McFarland density of bacterial culture was adjusted in normal saline (85%) using densitometer to achieve the final concentration $1X10^8$ cfu/ml of test organism individually.

2.2.Honey Sample

Honey samples were obtained from local bee keepers in three different places of Theni district in the month of October 2016 and stored at room temperature. The identification of honey was performed by the bee hunters based on their geographical hunting area and floral availability at the location of bee hives. Samples were named as Honey sample 1(S1), Honey sample 2(S2) and Honey sample 3(S3) and these samples were collected from Megamalai, Agamalai and Cumbum respectively.

2.3.Antimicrobial activity of honey samples against methicillin resistant *Staphylococcus aureus* [19]

Different concentrations of each honey constituting, 25% v/v, 50%v/v, 75% v/v and 100% v/v were made in sterile distilled water. These preparations were done by dissolving the respective volumes: 250µl, 500µl, 750µl of each honey into corresponding volumes of sterile distilled water to give a 1ml preparation. For 100%v/v, 1000µl of honey was taken in a test tube without adding distilled water. Filter paper discs were prepared by the method of Cheesbrough (2004). The discs were impregnated with the different concentrations of each honey. Then discs were placed over the Muller Hinton agar plates inoculated with Methicillin resistant *Staphylococcus aureus* and incubated for 24 h at 37 ºC. Triplicates were maintained and the mean values ± standard error was calculated.

2.4. Preparation of honey extracts using various organic solvent [21]

The active compounds of honey were extracted with various organic solvents such as methanol, ethanol and ethyl acetate.10 g of each honey was taken in a test tube and 25 mL of methanol was added. Later, the solution was mixed well by vortexing and centrifuged at 3000 rpm for 10 min at 25◦C. The supernatant was collected from each test tube and transferred to stopper test tube by filtrations. The resulting supernatant was evaporated to dryness with rotary evaporator and reconstituted with 10 ml of 1% dimethyl sulphoxide (DMSO) and mixed well by vortexing. Same procedure was followed for three honey samples with ethanol and ethyl acetate.

2.5. Disc diffusion method [21]

In vitro antibacterial activity of honey extracts of methanol, ethanol, and ethyl acetate was evaluated using the disc diffusion method. The discs were impregnated with the different concentration. The disc diffusion technique was employed as previously described by Bauer et al. (1966). Filter paper discs were impregnated with different concentration (100µg, 200µg, 300µg and 400µg) of honey extracts and air-dried. A sterile cotton swab was dipped into the standardized MRSA bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates. The plates were allowed for 3 to 5 min to dry. Thereafter, all discs were placed on the plates and pressed gently to ensure complete contact with the agar. A distance of at least 15 mm was maintained from the edges of the plates to prevent overlapping of inhibition zones. 15 min following placement of the discs, the plates were incubated for 24 h at 37°C. They were then examined and the diameter of the zone of inhibition was measured in mm. The experiment was repeated in triplicates for MRSA isolate. Discs loaded with 1% DMSO served as control. Triplicates were maintained and the mean values ± standard error was calculated.

2.6. MIC determination [22]

The minimum inhibitory concentration of the honeys was determined using broth tube dilution method .Briefly, ten sterile test tubes were placed in rack, labeled each 1 through 8. Honey control tube (HC) and growth control tube (GC) were used as a quality control. 1 ml of freshly prepared sterilized nutrient broth was added to each tube and cooled. Then 1 ml of undiluted honey solution 100 % was added to test tube number 1 and HC with a sterile micropipette and tips. Then twofold serial dilution was performed by transferring 1 ml undiluted honey into the second tube with separate sterile micropipette and tips and vortexed for homogenization. After a through mixing, 1 ml was transferred with another sterile micropipette from tube 2 and tube 3. These procedures continued until eighth tube with a dilution of 1:128 (1,1/2,1/4,1/8,1/16,1/32,1/64,1/128) was reached and finally 1 ml was taken and discarded from tube 8. The GC

tube received no honey was served as a growth control while the HC tube received no bacterial inoculums was served as a honey(sterility) control.

Except the HC tube, each tube was inoculated with 1 ml of MRSA culture of respective prepared organism. Tubes were then incubated at 37 °C for 24 h and observed by visual inspections for the presence and absence of growth (turbidity).

III. RESULTS

Three types of honey samples were taken and diluted in various concentrations (0%, 25%, 50%, 75% and 100%) were subjected to antimicrobial activity against MRSA. S1, S2 and S3 are not form zone of inhibition in 0% concentration against MRSA. In 25% concentration of S1 honey sample (11±0.7 mm) zone of inhibition was observed and S2 honey sample showed (4±0.0 mm) zone of inhibition.

In 50% concentration of S1 honey sample showed (12±0.6 mm) and S2 honey sample showed (5.5±0.7 mm) and S3 honey sample showed (4.5±0.6 mm) zone of inhibition. In 75% concentration of S1 honey sample (12.5±0.0 mm) zone of inhibition were observed and S2 honey sample showed (06±0.5 mm) and S3 honey sample showed (05±0.0 mm) zone of inhibition were observed.

In 100% concentration of S1 honey sample (14.0±0.0mm) zone of inhibition were observed and S2 honey sample showed (06.5±0.0 mm) and S3 honey sample showed (5.5±0.0 mm) zone of inhibition were observed. The results were showed in Table :1

Table 1:-Antimicrobial activity of honey samples with different dilutions against MRSA

HONEY SAMPLES	CONCENTRATION OF HONEY (%V/V)				
	0%	25%	50%	75%	100%
S1	-	11±0.7	12±0.6	12.5±0	14±0
S2	-	4±0	5.5±0.7	6±0.5	6.5±0
S3	-	-	4.5±0.6	5±0	5.5±0

Honey samples S1, S2 and S3 were used to extracts by using various solvents as Ethyl acetate, ethanol, and methanol in various concentration 100µg, 200µg, 300µg and 400µg. In S1 sample 100µg of ethylacetate extracts showed (8±0.3 mm), 100µg of methanol extracts showed (9±0.6 mm), ethanol and control were not formed zone of inhibition. 200µg of ethylacetate extracts showed (8.2±0.2 mm), 200µg of ethanol and methanol extracts showed (8±0.0 mm) and (10.5±0 mm) respectively, zone of inhibition were not formed in control. 300µg of ethylacetate extracts showed (8.5±0.0 mm), 300µg of ethanol formed (8.3±0.3 mm) zone of inhibition and methanol extracts showed (12.5±0.4 mm).

Zone of inhibition were not formed in control. 400µg of ethylacetate extracts showed (9±0.3 mm), 400µg of ethanol formed (10±0.0 mm) zone of inhibition and methanol extracts showed (14±0.0 mm). Zone of inhibition were not formed in control.

In S2 sample 100µg of ethanol extracts showed (4±0.0 mm), 100µg of methanol, ethylacetate and control extract was not formed zone of inhibition. 200µg of ethylacetate extracts showed (5.5±0.0 mm), 200µg of ethanol showed (4.0±0.0 mm) and methanol extracts showed (07±0.0 mm), zone of inhibition were not formed in control.

300µg of ethylacetate extracts showed (5.5±0.2 mm), 300µg of ethanol formed (4.5±0.3 mm) zone of inhibition and methanol extracts showed (09±0.0 mm). Zone of inhibition were not formed in control. 400µg of ethylacetate extracts showed (06.0±0.0 mm), 400µg of ethanol formed (05±0.0 mm) zone of inhibition and methanol extracts showed (7.5±0.0 mm). Zone of inhibition were not formed in control.

In S3 sample 100µg and 200µg of ethylacetate, ethanol, methanol and control extracts are not formed zone of inhibition. 300µg of ethylacetate showed (06±0.0 mm), 300µg of ethanol showed (4±0.0mm) zone of inhibition. 300µg of methanol and control extracts are not formed zone of inhibition. 400µg of ethylacetate extracts showed (6.5±0.0 mm), 400µg of ethanol formed (4.5±0.0 mm) zone of inhibition and methanol extracts showed (7.5±0.0 mm). Zone of inhibition were not formed in control. The results were showed in Table: 2

In S1 sample Ethanol extracts showed (6.25%) of Minimum inhibitory concentrations, (3.12%) Minimum inhibitory concentrations in methanol extracts, Ethyl acetate extracts were showed (12.5 %) minimum inhibitory concentration. In S2 sample ethanol, methanol and ethylacetate extracts were showed (25%) of minimum inhibitory concentrations. In S3 sample ethanol and ethyl acetate extracts showed (50%) of minimum inhibitory concentrations and (25%) of minimum inhibitory concentrations were showed in methanol extracts. The results were showed in Table: 3

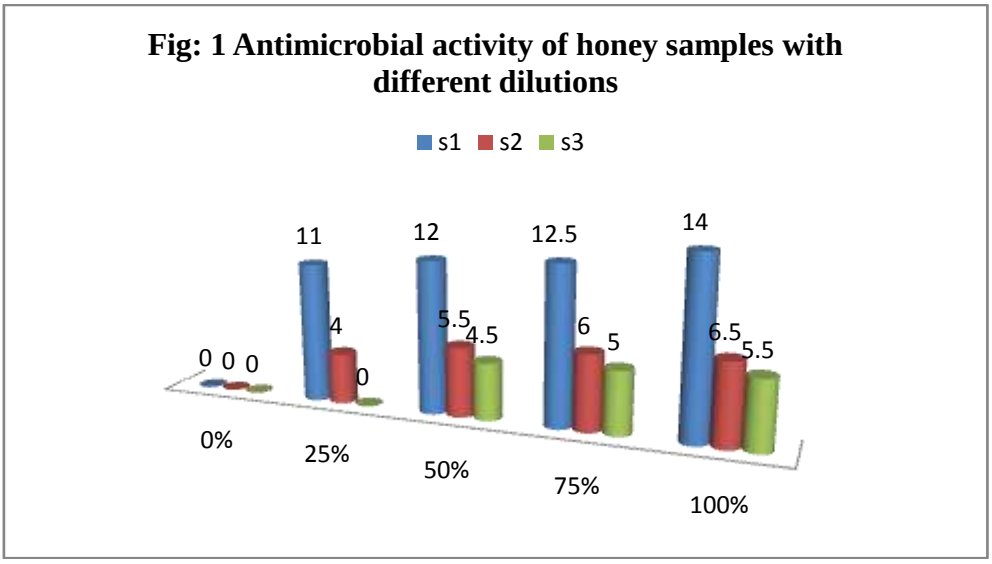
Table 2:-Antimicrobial activity of various extracts of honey samples by disc diffusion method

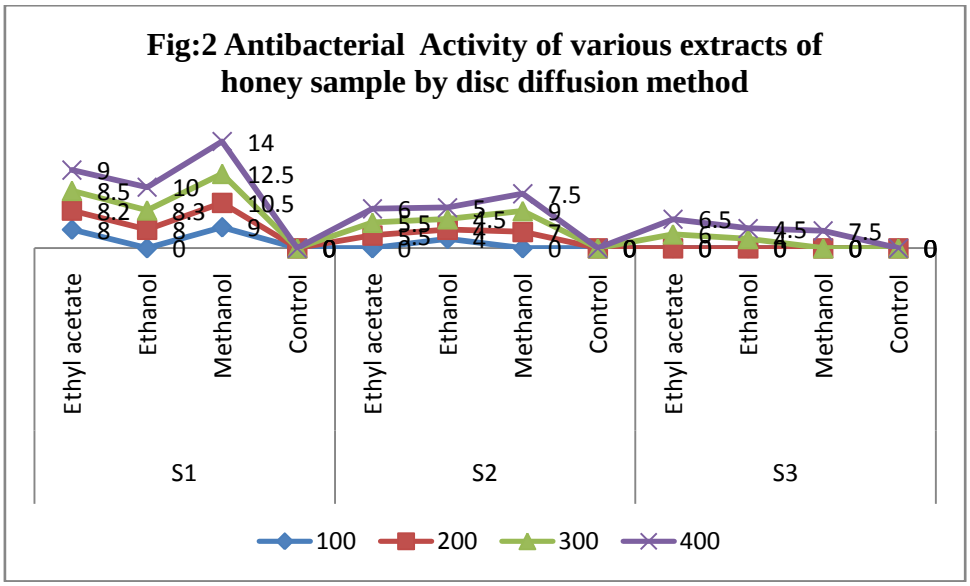
HONEY SAMPLES	EXTRACTS	CONCENTRATION OF HONEY (ZONE OF INHIBITION(mm)			
		100µg	200µg	300µg	400µg
S1	ETHYL ACETATE	8±0.3	8.2 ±0.2	8.5±0	9±0.3
	ETHANOL	-	8±0	8.3±0.3	10±0
	METHANOL	9±0.2	10.5±0	12.5±0.4	14±0
	CONTROL	-	-	-	-
S2	ETHYL ACETATE	-	5.5±0	5.5±0.2	6±0
	ETHANOL	4±0	4±0	4.5±0.3	5±0
	METHANOL	-	7±0	9±0	7.5±0
	CONTROL	-	-	-	-
S3	ETHYL ACETATE	-	-	6±0	6.5±0
	ETHANOL	-	-	4±0	4.5±0
	METHANOL	-	-	-	7.5±0
	CONTROL	-	-	-	-

Table 3:-Minimum inhibitory concentration (MIC) of various extracts of honey samples

SAMPLES	EXTRACTS	DILUTIONS								MIC% V/V
		1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
S1	ETHANOL	-	-	-	-	-	+	+	+	6.25
	METHANOL	-	-	-	-	-	-	+	+	3.12
	ETHYL ACETATE	-	-	-	-	+	+	+	+	12.5
S2	ETHANOL	-	-	-	+	+	+	+	+	25
	METHANOL	-	-	-	+	+	+	+	+	25
	ETHYL ACETATE	-	-	-	+	+	+	+	+	25
S3	ETHANOL	-	-	+	+	+	+	+	+	50
	METHANOL	-	-	-	+	+	+	+	+	25
	ETHYL ACETATE	-	-	+	+	+	+	+	+	50

\- Absence of bacterial growth + Presence of bacterial growth





IV. DISCUSSION

Three honey samples (S1, S2 and S3) are subjected to antimicrobial activity with different dilutions (0%), (25%), (50%), (75%) and (100%) against MRSA method (Fig 1). According to the result of S1, S2 and S3 sample, zone of inhibition was not observed in (0%) dilution. In (25%) S1 honey sample showed maximum inhibition (11±0.7mm), minimum zone of inhibition (4±0.7 mm) were observed in S2 honey sample while in S3 honey sample, zone of inhibition were not formed.

Honey concentration increases, the antibacterial effects of the honey sample on the organisms also increases [23]. The inhibition zones observed against *Klebsiella spp., Bacillus spp., Pseudomonas spp., Salmonella spp. and Shigella spp.* Showed proportionality with the increase in concentration of the honey samples from 50% to 100% (w/v) [24].

In 50% of honey sample dilution, S1 sample showed maximum zone of inhibition (12±0.6mm) when S3 showed minimum zone of inhibition (11±0.7mm) while moderate level of zone of inhibition (5.5±0.7mm) were observed in S3 sample.

In 75% of honey sample dilution, S1 sample showed maximum zone of inhibition (12±0.6mm) when S3 showed minimum zone of inhibition (05±0.0mm) while moderate level of zone of inhibition (5±0.0mm) were observed in S3 sample.

In 100% of honey sample dilution, S1 sample showed maximum zone of inhibition (14±0.6mm) when S3 showed minimum zone of inhibition (6.5±0.0mm) while moderate level of zone of inhibition (5.5±0mm) were observed in S3 sample.

Three honey samples S1, S2 and S3 are applied to analysis antimicrobial activity(Fig 2). These three honey samples are extracted with ethylacetate, ethanol, methanol and control. Among these ,in S1 sample 400 µg of methanol extracts showed maximum zone of inhibition (14±0.0mm), 400 µg of ethanol extracts showed zone of inhibition (10±0.0mm), 400 µg of ethyl acetate extracts showed zone of inhibition (9±0.3mm) were observed. In control absence of zone of inhibition was observed.

In S2 sample 300 µg of methanol extracts showed maximum zone of inhibition (9±0.6mm) when S2 showed minimum zone of inhibition (4.0±0.0mm) while moderate level zone of inhibition (7.5±0mm) were observed. In control absence of zone of inhibition was observed.

In S3 100 µg and 200 µg of ethylacetate, ethanol and methanol are not formed the zone of inhibition. While control was not formed the zone of inhibition in all concentration of dilution maximum zone of inhibition was (7.5±0.0mm) in 400 µg of methanol extract. 400 µg of ethanol extract showed moderate level when 300 µg of ethanol extract showed minimum level of antibacterial activity. The inhibitory activity against test microorganisms is of interest because these organisms cause infection. The methanol extracts showed highest activity on test organism as compared to ethanol and ethyl acetate. This may be due to better solubility and polarity of the active components in methanol compared to ethanol and ethyl acetate [21].

Honey samples S1, S2 and S3 were subjected to extract with ethanol, methanol and ethylacetate to dilute with various dilution factors 1, ½, ¼, 1/8, 1/16, 1/32, 1/64, 1/128.

According to the results, 1/64 and 1/128 were showed the presence of minimum inhibitory concentration in all extracts of S1, S2 and S3 honey samples while dilution 1 and ½ were showed that absence

of minimum inhibitory concentration but S2 and S3 extracted samples were showed the presence of minimum inhibitory concentration in the dilution factors of 1/8 and 1/16.

Among three extracted samples S1, S2 and S3. Ethanol and ethyl acetate extracts were showed maximum 50% of MIC while ethanol, methanol and ethyl acetate extracts of S2 and methanol extract of S3 were showed that moderate level of MIC 25% but S1 sample were showed low level of minimum inhibitory concentration in methanol extract 3.12% of MIC. When ethyl acetate showed 12.5 % and ethanol showed 6.25% of minimum inhibitory concentration.

Antibacterial activity of honey may be because of the ability of honey to kill microorganisms has been attributed to its high acidic nature (pH being 3.2-4.5), hydrogen peroxide concentration, high osmotic effect, and its phytochemical nature, i.e. its content of tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, flavonides, streptomycin, sulfathiazole, trepens, benzyl alcohol and benzoic acids [25,26,27]. Similar studies have been reported in the form of antibacterial activity of honey against *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Streptococcus pyogenus, Salmonella flexneri* and *Salmonella typhi* [25,28,29].

V.CONCLUSION

The present study concluded that honey has both bacteriostatic as well as bactericidal activity against MRSA activity. Honey samples of methanol extracts resulted in a broad spectrum of antibacterial activity. The study showed that honey, a kin to antibiotics, possesses certain organisms sensitive to it and provides alternative therapy against multi drug resistant bacteria. Therefore, there is need to characterize the active components of honey extracts and encourage to investigate possible benefits of the use of honey among therapies in the treatment of bacterial infections.

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