Annals of Biology **41** (1) : 8-14, 2025 **http://agribioj.com** **Impact Score : 0.32 (Scopus)**

Antimicrobial Properties of Different Extracts of Honey

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(Received: June 10, 2024; Accepted: July 27, 2024)

ABSTRACT

The present study was aimed at analyzing the antimicrobial and anti-helminthic activity of different extracts of honey which was carried out via *in vitro* methods. Three extracts of honey (ethanolic, methanolic and water) were prepared and tested for antibacterial (*Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella enteric*) and antifungal (*Candida albicans* and *Saccharomyces cerevisiae*) activities by disc diffusion and broth dilution methods. The anti-helminthic (*amphistomes*) activities were assessed via bioassay method under *in vitro* conditions. The positive controls used were ampicillin (antibacterial), amphotericin B (antifungal) and albendazole (anti-helminthic). The antimicrobial activities were determined after 24 h of incubation at 37°C for bacteria and 28°C for yeasts by measuring the zones of inhibitions in millimeter and by broth dilution methods. Worm motility inhibition assay was employed for the evaluation of anti-helminthic activity of honey. Results showed that among all the extracts used, methanolic extract of honey was found to be the most effective against antimicrobial properties. It was also observed that honey showed more effectiveness towards Gram-negative as compared to the Gram-positive bacteria, and none of the extract was found to be effective against *amphistome*. The positive controls showed efficient action against all the microbes used and at a fixed concentration. The biological activities observed for honey demonstrate that in the era of antibiotic resistance, the natural product like bee products can be used for manufacturing drugs with very low side effects. However, it requires further studies on isolation of bioactive constituents.

Key words: Antibacterial, antifungal, anti-helminthic, honey, *in vitro*

INTRODUCTION

Bacterial resistance towards antibiotics has been increasing drastically, which necessitates the discovery of alternative and complementary medicines in the form of natural products as therapeutic agents. Among the natural products, medicinal plants as well as honeybee products impart very crucial role in possessing pharmacologically active bio constituents. The use of medicinal plants as antimicrobial agents against pathogenic microorganisms abounds in literature (Tyagi *et al*., 2016). One of the well-known bee products 'honeys' was used since Ayurveda as antimicrobial agent. Honey also known as 'liquid gold' is produced by honeybees from the nectar of plants and is economically the most important as well as the most well-known product of the bee hive. It is considered as a prebiotic food, affecting the microbiota and well-being of humans (Miguel *et al*., 2017).

It was the only energy rich food available to primitive man, so it is speculated that honey was one of the main environmental factors contributing to accelerated human brain evolution (Didaras *et al*., 2020). It's a product incomparable to anything else in terms of nourishment and medicinal properties. Its biological properties like antimicrobial (Stagos *et al*., 2018; Anand *et al*., 2019; Tsavea and Mossialos, 2019; Rana, 2021; Rana and Kumar, 2022; Rana and Parmar, 2022; Rana *et al*., 2022a and b) are attributed due to physical and chemical factors like high sugar content and low water content and acidity which prevent microbial growth (Albaridi, 2019; Hossain *et al*., 2022). Moreover, honey on dilution produces hydrogen peroxide due to activation of an enzyme called glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide (Brudzynski, 2020) and some other chemical compounds such as methylglyoxal, 3-phenyllactic acid (PLA), bee defensin, Major Royal Jelly Proteins (MRJPs) and bacteriocins (Nolan *et al*., 2019). It also exhibits anti-inflammatory, wound healing (Nolan *et al*., 2019) antioxidative and anticancer properties

(Ahmed *et al*., 2017; Afrin *et al*., 2019; Waheed *et al*., 2019). The present studies embodied results of investigations undertaken to evaluate honey for its antimicrobial and anti-helminthic activities.

MATERIALS AND METHODS

Honey was collected directly from the honeycombs by standard extraction procedures. It was diluted to required concentrations in distilled water and filtered through 0.22μ PTFE membrane for sterilization.

Helminths (Amphistomes): *Gastrothylax crumenifer,* were obtained from large intestine of sheep/goat procured from local slaughterhouse. Microorganisms such as bacteria (*Staphylococcus aureus:* MTCC No-1144, *Staphylococcus epidermidis:* MTCC No-9040, *Streptococcus pneumoniae:* MTCC No-2672, *Salmonella enterica:* MTCC No-3231*, E. coli:* MTCC No-2314*, Bacillus subtilis*: MTCC No-2435*, pseudomonas* MTCC No-3465 and fungi (*Candida albicans* (Yeast): MTCC No- 4748, *Saccharomyces cerevisiae* (Yeast): MTCC No-3090) were procured from IMTECH (Institute of Microbial Technology) Sector-39, Chandigarh, India. The organisms were maintained in suitable/respective media (agar plates at 4°C). The strains were checked biochemically prior to usage.

Worm motility inhibition assay was employed for the evaluation of anti-helminthic activity of honey under *in vitro* conditions at three different concentrations (100, 300 and 500 mg/ml) of honey. Mature amphistome worms (*Gastrothylax crumenifer*) were collected from the large intestine of sheep/goat procured from local slaughterhouse. The worms were washed in phosphate buffered saline (PBS pH 7.2) and then suspended in PBS. Albendazole dissolved in 1% DMSO and diluted in PBS at concentrations of 5, 10 and 15 μg/ml and PBS alone served as positive and negative control, respectively. There were three replicates for each treatment concentration. Ten vigorously motile worms were placed in each Petri dish containing test solutions and observations were made at 15, 30, 60 and 120 min intervals for cessation of motility by gross visual motility of worms as index for anti-helminthic activity. After exposure to different treatments, the worms were put in lukewarm PBS for 30 min for the confirmation of their mortality.

The microbial inoculums were prepared by growing their culture in nutrient broth overnight. Bacteria were incubated at 37°C and fungi at 25°C. After incubation, cells were harvested by centrifugation at 8000 g for 10 min and supernatant was discarded while pellet was washed and suspended in phosphate buffer saline (PBS). Optical density (OD) was then measured at 600 nm. Viable counts were determined by making serial dilutions and by spread plating on nutrient agar followed by incubation at 37°C and counting CFU 24 h later.

RESULTS AND DISCUSSION

Antibacterial activity of honey was evaluated by using ethanolic, methanolic and water extracts. For this the selected organisms were initially nonpathogenic Gram (+ve) and Gram (-ve) bacteria viz., *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pneumoniae.* Thereafter putative pathogenic Gram (+ve) bacteria viz., *Staphylococcus aureus, Staphylococcus epidermidis* and Gram (-ve) bacteria viz., *Salmonella enterica* were screened for seeing inhibitory activity of honey by disc diffusion method and broth dilution method. The stock solutions were made at a concentration of 300 mg/ml. These were serially diluted to obtain the concentration of 300, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.562 mg/ml. Agar plates were made and 25-50 μl of each organism was uniformly spread on the plates. Fresh inoculum 24-48 h prior to start of the experiment was prepared. The 25 μl of all the above-mentioned concentrations was applied on separate agar plates and incubated at their respective growth conditions. After 24 - 48 h clear zones of inhibition of culture growth around the discs having honey were measured (Tables 1 to 7). The effectiveness of bee products was also compared with standard antibiotic as positive controls, such as ampicillin (antibacterial), amphotericin B (antifungal) and albendazole (anti-helminths).

The values observed for ethanolic extract of honey against Gram (+ve) bacteria such as *S. epidermidis* ranged from 6.78±0.39 m at 300 mg/ml. For *S. aureus* the values ranged from 7.00±0.91-8.10±0.42 mm at concentrations ranging from 200-300 mg/ml; for *S. pneumonia* the range was 6.209±0.62-8.05±0.85 mm and

Table 1. Antimicrobial activity of ethanolic extract of honey against Gram (+ve) bacteria

Ethanolic extract		Gram (+ve) bacteria			
		B. subtilis	*S. epidermidis*	*S. aureus*	*S. pneumoniae*
S. No.	(mg/ml)	Zones of inhibition (mm)			
1.	25-50	NI	NI	NI	NI
2.	100	NI	NI	NI	6.209±0.62*
3.	200	NI	NI	7.00±0.91	6.88±0.18
4.	300	NI	6.78±0.39	8.10±0.42	8.05±0.85

NI–No inhibition and ZOI–Zone of inhibition.

no inhibitions were observed with ethanolic extract of honey against *B. subtilis* (Table 1). The antimicrobial activity observed by using ethanolic extracts of honey against Gram (-ve) bacteria such as *E. coli* varied from 10.05±1.32 - 22.2±1.37 mm at concentrations from 100-300 mg/ml, the values observed for *S. enterica* varied from 9.5±1.02-12.5±2.01 mm at 200-300 mg/ml of the ethanolic extract of honey. *P. aeruginosa* was not affected by any of the concentrations of honey ranging from 25-300 mg/ml (Table 2). The antimicrobial activity observed by using ethanolic extract of honey against *C. albicans* was found to vary from 11.15±1.18-16.70±1.63 mm at concentrations ranging from 100-300 mg/ml. Below 100 mg/ml, no inhibition zones were observed for *C. albicans.* Further, the results indicated that *S. cerevisiae* was not affected by any of the concentrations (25-300 mg/ml) of honey used against the yeast (Table 3).

Table 2. Antimicrobial activity of ethanolic extract of honey against Gram (-ve) bacteria

Ethanolic extract		Gram (-ve) bacteria		
		E. coli	*P. aeruginosa*	*S. enterica*
S. No.	(mg/ml)	Zones of inhibition (mm)		
1.	25	NI	NI	NI
2.	50	NI	NI	NI
3.	100	10.05±1.32*	NI	NI
4.	200	15.525±0.86	NI	9.5±1.02
5.	300	22.2±1.37	NI	12.5±2.01

NI – No inhibition and ZOI – Zone of inhibition.

The values observed for methanolic extract of honey for Gram (+ve) bacteria such as *S. epidermidis* were from 6.68±0.45-13.43±2.17 mm and for *S. aureus* 7.88±0.48-11.80±0.57 mm at concentration ranging from 100-300 mg/ml, in case of *S. pneumoniae* 6.00±0.18 - 10.55±0.105 mm response was observed for concentrations of 50-300 mg/ml and for *B. subtilis* the zones of inhibition varied from 6.58±0.30 - 8.05±1.58 mm at 200-300 mg/ml of methanolic extract of honey (Table 4).

The antimicrobial activity observed by using methanolic extract of honey against Gram (-ve) bacteria such as *E. coli* varied from 10.2±0.92-36.6±1.25 mm at range of concentrations 50-300 mg/ml. Below 50 mg/ml there were no inhibition zones against *E. coli.* The values observed for *P. aeruginosa* varied from 8.6±0.96-9.9±0.98 mm at 200-300 mg/ml range of concentrations of methanolic extract of honey. The zones of inhibition observed against *S. enterica* varied from 6.8±1.02-12.2±1.88 mm at 50-300 mg/ml

Table 3. Antimicrobial activity of ethanolic, methanolic and water extracts of honey against yeast.

Honey extracts		*C. albicans*			*S. cerevisiae*		
		MEP	EEP	WEP	MEP	EEP	WEP
S. No.	(mg/ml)	Zones of inhibition (mm)					
1.	25	NI	NI	NI	NI	NI	NI
2.	50	10.28±1.20*	NI	NI	NI	NI	NI
3.	100	17.03±0.79	11.15±1.18	NI	NI	NI	NI
4.	200	19.08±0.65	14.20±2.27	10.20±0.83	NI	NI	NI
5.	300	22.50±1.74	16.70±1.63	13.83±1.16	NI	NI	NI

NI–No inhibition and ZOI–Zone of inhibition.

Table 4. Antimicrobial activity of methanolic extract of honey against Gram (+ve) bacteria

		Gram (+ve) bacteria			
Methanolic extract		*B. subtilis*	*S. epidermidis*	*S. aureus*	*S. pneumoniae*
S. No.	(mg/ml)	Zones of inhibition (mm)			
1.	25	NI	NI	NI	NI
2.	50	NI	NI	NI	6.00±0.18*
3.	100	NI	6.68±0.45	7.88±0.48	7.109±0.02
4.	200	6.58±0.30	7.70±1.18	9.10±0.42	8.18±0.99
5.	300	8.05±1.58	13.43±2.17	11.80±0.57	10.55±0.105

NI – No inhibition and ZOI – Zone of inhibition.

Table 5. Antimicrobial activity of ethanolic extract of honey against Gram (-ve) bacteria

		Gram (-ve) bacteria		
Methanolic extract		*E. coli*	*P. aeruginosa*	*S. enterica*
S. No.	(mg/ml)	Zones of inhibition (mm)		
1.	25	NI	NI	NI
2.	50	10.2±0.92*	NI	6.8±1.02
3.	100	16.575±0.59	NI	8.9±1.09
4.	200	26.3±1.16	8.6±0.96	10.0±2.01
5.	300	36.6±1.25	9.9±0.98	12.2±1.88

NI–No inhibition and ZOI–Zone of inhibition.

concentration of methanolic extract of honey. From the results, it could be concluded that *E. coli* was most sensitive and *P. aeruginosa* was found to be the least sensitive against methanolic extract of honey (Table 5).

The antimicrobial activity observed by using methanolic extract of honey against *C. albicans* varied from 10.28±1.20-22.50±1.74 mm at concentrations from 50-300 mg/ml. No antimicrobial activity was observed below 50 mg/ml. It was also observed that *S. cerevisiae* was not affected by any of the concentrations ranging from 25-300 mg/ml methanolic extract of honey (Table 6).

It was observed that the water extract of honey was not much effective against both Gram (+ve) as well as Gram (-ve) bacteria used in the present study (Table 7). Among Gram (-ve) bacteria, only *E. coli* was inhibited by the water extract of honey and the values observed ranged from 8.095±0.98 mm at 300 mg/ml concentration. The antimicrobial activity observed by using water extract of honey against *C. albicans* was found to vary from 10.20±0.83 - 13.83±1.16 mm at concentrations ranging from 200-300 mg/ml. Below 200 mg/ml, no inhibition zones were observed for *C. albicans.* From the results, it was also observed

Table 7. Antimicrobial activity of water extract of honey against Gram (-ve) bacteria

		Gram (-ve) bacteria		
Water extract		*E. coli*	*P. aeruginosa*	*S. enterica*
S. No.	(mg/ml)	Zones of inhibition (mm)		
1.	25	NI	NI	NI
2.	50	NI	NI	NI
3.	100	NI	NI	NI
4.	200	NI	NI	NI
5.	300	8.095±0.98	NI	NI

NI–No inhibition and ZOI–Zone of inhibition.

Table 6. Antimicrobial activity of water extract of honey against Gram (+ve) bacteria

		Gram (+ve) bacteria			
Water extract		*B. subtilis*	*S. epidermidis*	*S. aureus*	*S. pneumoniae*
S. No.	(mg/ml)	Zones of inhibition (mm)			
1.	25	NI	NI	NI	NI
2.	50	NI	NI	NI	NI
3.	100	NI	NI	NI	NI
4.	200	NI	NI	NI	NI
5.	300	NI	NI	NI	NI

NI–No inhibition and ZOI–Zone of inhibition.

that *S. cerevisiae* was not affected by any of the concentrations (25-300 mg/ml) of honey used against yeasts. For honey, results obtained against the microorganisms tested were best with methanolic extracts as the zones of inhibition were highest for methanolic extract and least for water extract. Further, the results also indicated that honey was more effective against the Gram (-ve) as compared to the Gram (+ve) bacteria.

The antibacterial activity of honey against clinical isolates of *S. aureus, E. coli,* and *P. aeruginosa* were also studied previously (Wadi, 2022). Their results demonstrated the potential inhibitory effect of honey tested for the isolates and confirmed its antimicrobial as well as wound-healing activity. The healing property of honey was due to its antibacterial activity, its high viscosity and enzymatic production of hydrogen peroxide. Honey was also reported to show antifungal activities (Kunat-Budzynska *et al*., 2023) and from their studies it was also concluded that the component responsible for antifungal activities in honey was not sugar. The anti-fungal effect of honey against *C. albicans, C. tropicalis* and *S. cerevisiae* was also studied previously (Kolayli *et al*., 2020) by using different honey samples obtained from different botanical origin.

McLoone *et al.* (2016) investigated the antimicrobial properties of honey from all around the world against skin relevant microbes. A plethora of *in vitro* studies revealed that all honeys had potent microbicidal activity. Laboratory studies have demonstrated that honey is effective against several human pathogens, including *E. coli, E. aerogenes, S. typhimurium, S. aureus*, Methicillin-resistant *S. aureus* (MRSA), haemolytic *Streptococci* and vancomycin resistant *Enterococci* (Rani *et al.,* 2017; Kolayli *et al*., 2020).

The antimicrobial properties observed for above mentioned bee products could be due to cell wall lyses and plasma membrane degradation, which leads to a loss of potassium ions and the damage, caused provoking cell autolysis (Combarros-Fuertes *et al*., 2020). Quercetin, which is also found in honey, increases membrane permeability, and dissipates its potential, leading the bacteria to lose their capacity to synthesis ATP, their membrane transport and motility (Memariani *et al*., 2019).

For determining the inhibitory concentrations of different extract of honey on the growth of Gram (+ve) and Gram (-ve) microorganism, experiments were done with broth dilution method. Organisms were grown in presence of honey at concentrations ranging from 3-60 mg/ml. Growth of Gram (+ve) and Gram (-ve) non-pathogenic bacteria viz., *B. subtilis, E. coli, P. aeruginosa* and *S. pneumonia* was measured at late log phase. Then pathogenic Gram (+ve) bacteria viz., *S. aureus, S. epidermidis* and Gram (-ve) bacteria viz., *S. enterica* were screened separately for the inhibitory activity of honeybee products by broth dilution assay. Growth of each organism was measured at late log phase by taking O. D. at 600 nm (Table 8).

Honeybee products have multiple medicinal properties. The present study was undertaken to evaluate anti helminthic activity of different extracts of honey by Petri dish method (Aggarwal *et al*., 2016), in comparison with a standard drug Albendazole, against amphistome (*Gastrothylax crumenifer)* parasitizing the large intestine of sheep/goat through *in vitro* studies by the worm motility inhibition assay.

The methanolic extract of honey was used for this study as it was observed to be the most effective for microorganisms tested during the *in vitro* study. Mortality was observed after every 15, 30, 60 and 120 min in the entire test group (Table 9). The honey at the highest tested concentration (500 mg/ml) after

Table 8. Optical density observed against Gram (+ve) and Gram (-ve) bacteria against yeast with methanolic extract of honey

Honey	Gram (+ve) bacteria				Gram (-ve) bacteria		
Conc. (mg/ml)	*B. subtilis*	*S. epidermidis*	*S. aureus*	*S. pneumoniae*	*E. coli*	*P. aeruginosa*	*S. enterica*
Control	1.32	1.72	1.64	1.65	1.67	1.52	1.62
3	1.20	1.56	1.48	1.56	1.54	1.32	1.50
7.5	1.12	1.40	1.34	1.44	1.41	1.21	1.42
15	1.00	1.32	1.11	1.38	1.29	1.09	1.31
30	0.82	1.20	0.98	1.22	1.16	0.96	1.26
60	0.62	0.99	0.66	1.10	1.10	0.75	1.08

Table 9. Antihelminthic activity of methanolic extract of honey, positive control (Albendazole) and negative control (Normal saline)

	Concentrations	15 min	30 min	60 min	120 min
Methanolic extract	100 mg/ml	9	9	8	7
	300 mg/ml	8	9	8	8
	500 mg/ml	9	9	9	8
Positive control (Albendazole)	5 µg/ml	8	6	2	0
	10 µg/ml	8	5	2	1
	15 µg/ml	8	5	3	0
Negative control	(Normal saline only)	9	8	6	4

completion of 120 min of the experiment did not give any more mortality than the negative control (3 and 4 live amphistome, respectively) and was therefore not effective in controlling the parasite. The positive control using Albendazole, however, at much lower concentration (5, 10 and 15 µg/ml) was able to arrest the parasite almost completely at the end of the experiment. Results, therefore, suggested that honey was not potent antihelminthic agent and is not suitable for application against amphistome; *G. crumenifer*.

CONCLUSION

Application of honey for determination of antimicrobial activity revealed that antimicrobial activity was much higher in Gram (–ve) organisms and on pathogenic yeast *C. albicans* with both methanolic as well as for ethanolic extract of honey as compared to Gram (+ve) organisms. With methanolic extract of honey, highest inhibitory activity was observed for *E. coli.* Water extract of honey was not much effective on organisms as compared to other extracts of honey.

Amphistomes (*Gastrothylax crumenifer)* obtained from the gut of sheep/goat were taken as test organism. Honey, at all concentrations used in the present study, did not show any effect different from the negative control on the mortality of amphistome. The positive control using Albendazole was very effective even at much lower concentrations.

ACKNOWLEDGEMENTS

The authors thank the Department of Biosciences (UIBT) and UCRD, Chandigarh University for all stages of this research work.

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