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Microbiological Analysis, Biochemical Composition and Antibacterial Activity of Crude Honey against Multiple Drug Resistant uropathogens

Tasneem Pota and Aruna K*

Department of Microbiology, Wilson College, Mumbai-400007, India

ABSTRACT

Crude Honey sample obtained from district Gharwal, Uttarakhand was studied by carrying out microbiological analysis, antibacterial activity, total reducing sugar content, total protein content and HPTLC analysis. The countof aerobic mesophilic bacteria, yeast and mold was less than 50 cfu/g and 12 cfu/g respectively. Food borne pathogens like Coliforms, *Salmonella, Shigella and Clostridium* species were absent in the honey sample. Undiluted, 75, 50 and 10% (w/v) dilutions of honey sample were tested against 9 antibiotic resistant uropathogens and 4 gastrointestinal tract pathogens to determine its antibacterial activity. Minimum concentration of 10% honey was effective to inhibit 85% of tested organisms, which emphasizes its medicinal property. Total reducing sugar content of the honey sample was 75gm % and protein content was 2.4 mg/g. Sugar profile analyzed by HPTLC confirmed the presence of Glucose, Fructose and Maltose in the honey sample. The exceptional antibacterial activity of honey against clinical bacterial isolates signifies the importance of honey in clinical practice against bacterial infection.

Keywords: Honey, uropathogens, antibacterial activity, reducing sugar, HPTLC.

Corresponding author

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INTRODUCTION

The extended use of antibiotics in clinical practice has been the direct cause of the development of multiple antibiotic resistances among bacteria causing human infection [1]. To combat such bacterial resistance to antibiotic, scientific efforts have been made to study and develop new compounds to be used beyond conventional antibiotic therapy; the antibacterial activity of honey against different lethal bacteria has been broadly studied and reported [2-8].

Honey is a naturally assimilated carbohydrate product obtained from an insect belonging to the genus Apis, order Hymenoptera, locally known as honeybee. It is a supersaturated solution of sugars, of which 84% is a mixture of fructose and glucose as the major constituents, with sucrose, enzymes (glucose oxidase, catalase, phosphatases, invertase), proteins, vitamins (ascorbic acid, niacin, pyridoxine etc.), amino acids, organic acids (gluconic acid, acetic acid, etc.), lipids, volatile chemicals, phenolic acids, flavonoids, lysozyme, carotenoid like substances and minerals as the minor constituents [9-11]. The composition and antioxidant activity of honey depends on the floral source visited by the honeybees, environmental factors, processing and storage conditions [12-13]. Honey of the same plant species can vary due to seasonal climatic variations or to a different geographical origin [14]. However, honey represents the oldest traditional medicines in the treatment of respiratory ailment, gastrointestinal infection and various other diseases [15]. It has been used efficiently as a dressing for wounds, burns and skin ulcers, in treatment of gastric ulceration, bacterial gastroenteritis and bronchitis in children and healing of diabetic ulcers. [16-20].

Honey has been reported to maintain moist wound environment that promotes healing, its high viscosity provides a protective barrier to prevent infection, low acidity (3.2 and 4.5) inhibits growth of several bacterial pathogens, low level of hydrogen peroxide (H_2O_2) release help in tissue repairing and non-peroxide phytochemical components like methylglyoxal (MGO) contribute to the antibacterial activity of honey [11, 21- 24]. Honey is proposed to be successful alternative to conventional antibiotics whose use as a traditional remedy for bacterial infections dates back to ancient times [11, 25].

Considering the growing numbers of organisms resistant to present antibiotics and the evidence that honey could be used as an alternative, the current study focuses on *in vitro* antibacterial activity of a honey sample against antibiotic resistant uropathogens and gastrointestinal tract infection causing bacteria and also discusses its microbiological and biochemical characteristics.

MATERIALS AND METHODS

Honey sample

Honey sample harvested during the month of July 2011, was collected from a village Uchhola, Bangerpati near the hills of the Rudraprayag, located in Garhwal, Uttarakhand. The honey sample was provided by Satvik foundation, Mumbai. Upon receipt, honey sample was

stored in sterile airtight container at 4ºC till use. Before microbiological experiments, the honey sample was brought to room temperature in a water bath.

Media used

Media used for the study was purchased from Hi-media, Mumbai, India. For cultivation of test organisms Luria Bertanii agar was used. Antibacterial activity was carried out by using Mueller and Hinton agar. Microbial analysis of the honey sample was checked using Potato dextrose agar (PDA) for yeast and mold count and Glucose yeast extract (GYE) agar for aerobic mesophilic bacterial count. Deoxycholate Citrate agar was used for detection of *Salmonella and Shigella species* and Reinforced Clostridium medium for *Clostridium* detection. Violet Red Bile Agar (VRBA) was utilized for enumeration of coliforms.

Bacterial strains used

A total of 9 uropathogens collected from urinary tract infection cases, from tertiary care hospitals situated in South Mumbai and 4 gastro intestinal tract infection causing bacteria like *Salmonella typhi, Salmonella paratyphi A*, *Salmonella paratyphi B* and *Shigella spp.* obtained from this microbiology laboratory were used in the current study.

Chemicals and instruments

DNSA and Bovine Serum Albumin used were obtained from LobaChemie Ltd and FolinCiocalteau reagent from SRL Laboratories. Distilled water used was obtained from a double distillation unit of Borosil Ltd. Colorimeter(Hans 161) was used for absorbance measurements.

Bacteriological analysis

Microbial counts

Pour plate technique was performed using 1 mL of 1:1 (w/v) diluted sample of honey to gain bacterial count at 30^0C for 24hrs and yeast and molds count at 30^0C for 48hrs on Glucose Yeast Extract agar and Potato dextrose agar respectively. Microbial counts were expressed as colony forming units/gm. of sample [26].

Bacterial Detection

0.1mL of 10% honey sample was spread on Deoxycholate citrate agar plate for detection of *Salmonella and Shigella species* and incubated at 37ºC for 24 hrs. One ml of 10% honey sample was inoculated at the base of the Bijou bottles (25 ml) and filled to the brim with Differential Reinforced Clostridium medium (DRCM) broth to check the presence of *Clostridium species* which were incubated at 37°C for 7 days. The bottles were checked for turbidity and blackening. Total coliform count of honey was done using standard Violet Red Bile agar (VRBA), 1mL each of 1:10 and 1:100 dilutions of honey sample were added into the melted and

cooled VRBA butts and poured into sterile petri plates which were incubated at 37ºC for 24hrs after solidification.

Antimicrobial susceptibility of uropathogens

A total of 9 urine isolates were selected for the current study. They were identified as *Citrobacterdiversus, Proteus mirabilis, Pseudomonas aeruginosa* strain 1,*Enterobacteraerogenes*,*Citrobacteramalonaticus*, *Pseudomonas aeruginosa* strain 2, *Escherichia coli* strain 1,*Klebsiellapneumoniae* and *Escherichia coli* strain 2. Antibiotic sensitivity test of these pathogens was carried out using Kirby Bauer methodso as to obtain Antibiogram pattern in our earlier studies [27].

Assay of Antibacterial activity

Antibacterial activity of honey for the bacterial isolates has been determined by agar well diffusion assay [28]. Test cultures were grown on Luria Bertani agar slants and culture densities were prepared using sterile phosphate buffer saline pH 7.2. Culture density was adjusted to 0.12-0.14 O.D. at 530nm. Each test culture (0.4mL) was mixed with molten cooled sterile Mueller and Hinton agar butt (20mL) and was poured into 9cm Petri plates. Wells of diameter 8mm were bored in the agar plates. 50µL of undiluted, 10%, 50% and 75% (w/v) of honey prepared in sterile distilled water were added to each well. The plates were incubated for 24 hours at 37ºC. Results were interpreted by measuring the size of the zone diameter of inhibition (ZDI) surrounding the wells on the agar plates.

Biochemical analysis

Total reducing sugar and total protein content

The total reducing sugar was determined by 3, 5 Di-nitrosalicylic acid (DNSA) method. In principle the reducing sugar reduces DNSA to 3-amino-5-nitro salicyclic acid resulting in the formation of a reddish orange colouration which is measured colorimetrically at 540nm [29]. 0.01% and 0.02% of honey sample was used for reducing sugar analysis. Folin Lowry method was employed for determining the protein content [30]. 0.5% and 0.25% of honey sample was used for protein content analysis.

Detection of Sugar by HPTLC (High Performance Thin Layer Chromatography

5µL of the honey sample (10%) and 5µL of the standard sugars (1µg/µL) were applied on silica gel TLC plates (Merck, Darmstadt, Germany). Sample application was done by CAMAG Linomat 5 auto sampler at a speed of 15 uL/sec. Solvent system was Acetone: Water (1:1, v/v). Spots were revealed by dipping the plate into Phosphomolybdic acid reagent followed by drying the plate and heating at 110° C for 10 mins, till colour develops. Scanning was done by CAMAG TLC Scanner 3 at a speed of 100mm/s and data resolution of 100 µm/step. Wavelength used was 580 nm. Sugars in honey sample were identified by comparing with the R_f values of

standard sugars. Standards used were Glucose, Fructose, Sucrose, Maltose, Xylose and Lactose. The HPTLC was performed by ANCHROM HPTLC, Mumbai, India.

RESULTS AND DISCUSSION

Microbial count

In our study the aerobic mesophilic bacterial count of honey sample obtained was 50 cfu/g (Table 1). This value was recorded within the range of present industry experience where the bacterial levels of finished honeys range from 1 to 5000 cfu/g, although viable counts of finished products reported in informal industry ranges between 10^4 and 10^5cfu/g and viable counts in raw honey sample can also reach 10^4–10^5cfu/g [10].According to published data, the value of total aerobic mesophilic bacterial count for honey can range from 0 to several thousand per gram. This variation in bacterial counts may be due to the type of sample (raw, finished or retailed), the freshness of the honey, the time of harvest and the analytical techniques used [10]. Viable bacterial count from 3 to 9500 cfu/g with mean value of 227cfu/g from various samples of commercial honey obtained from different location of France has been reported [31]. The study of honey samples from retail outlet in Japan reported a mean aerobic count of 83 cfu/g [32]. The findings of the present study are in resonance with the above.

Table 1. Microbial count of honey sample

Sample	Media	Yeast & Mold count cfu/g	Media	Bacterial count cfu/g
Honey	Potato Dextrose Agar	12	Glucose Yeast Extract Agar	50

In our study, yeasts and molds count was 8 to 15 cfu/g with an average mean of 12 cfu/g (Table 1). There are a few reports that quantify the levels of molds and yeasts in honey samples. In French honey samples the mean count of yeast and moldwas 254cfu/g with a range from zero to 2500 cfu/g [33]. In Italian honey sample the mold count was found to range from 1 to 43 cfu/g [34]. An occurrence of yeast and mold counts ranging from 0 to $1.5x10^5$cfu/g in industrial and domestic production of honey samples had been recorded [35].

Hence, a minimal load of yeasts, molds and bacteria in the crude honey sample obtained from Gharwal district, implies good quality and longer shelf life of the sample.

Bacterial Detection

Our study showed absence of *Salmonella, Shigella, E. coli* and *Clostridium species* in the honey sample. All these organisms are food borne pathogens, causative agents of many gastrointestinal tract infections. It was reported that microorganisms which are associated with bees like, *Bacillus, E. coli, Enterobacter, Klebsiella, Proteus* and *Clostridium* which have been found in honey [10]. Our results are in consent with few studies, where *Clostridium species*was found to be absent. Presence of *C. botulinum* in the 3% of industrialized and domestichoney

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samples has been reported in one of the studies, but total coliforms, *Salmonella and Shigella species* were not found in any sample [33-37].

Antibacterial activity

The antibacterial activity of honey sample against all test pathogens in terms of zone diameter of inhibition (ZDI) is reported in Table 2& 3. All the pathogenic test organism showed inhibition at the lowest concentration, i.e. at 10%, except for *E. aerogenes* and *K. pneumoniae* which were found to be inhibited at 75% concentration of honey while undiluted concentration of honey inhibited *P. aeruginosa* (strain 1) and *S. paratyphi B*. More than 85% of the isolates studied showed a progressive increase in inhibition at higher concentration of honey. Maximum inhibition was acquired with undiluted honey.

Table 2. Antibacterial activity of honey against uropathogens

Name of the Uropathogen	Diameter of inhibition zone (mm)				Sensitive towards antibiotics	Intermediate Towards antibiotics	Resistant towards antibiotics
	Honey concentration (w/v)						
	UD	75%	50%	10%			
Citrobacter. diversus	37	35	33	22	AS, BA, CH	-	CF, PC, RC, CI, TE, ZN, GM, AK, GF, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Citrobacter. amalonaticus	46	32	18	15	AS, CH, GM, AK, GF	ZN	BA, CF, PC, RC, CI, TE, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Enterobacter. aerogenes	13	12	-	-	AS, CH	RC	BA, CF, PC, CI, TE, ZN, GM, AK, GF, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Escherichia.coli (strain 1)	45	30	20	18	AS, CH, AK, GF	ZN	BA, CF, PC, RC, CI, TE, GM, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Escherichia.coli (strain 2)	45	27	20	20	OX, AS, BA, CH, TE, GM, AK, GF		CF, PC, RC, CI, ZN, TT, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Klebsiella. pneumoniae	13	13	-	-	CH	PC	AS, BA, CF, RC, CI, TE, ZN, GM, AK, GF, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Pseudomonas.aerugi nosa (strain 1)	14	-	-	-	RC		AS, BA, CF, PC, CH, CI, TE, ZN, GM, AK, GF, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Pseudomonas.aerugi nosa (strain 2)	32	28	31	22			AS, BA, CF, PC, CH, RC, CI, TE, ZN, GM, AK, GF, TT, OX, RP, ZX, CB, NA, NX, AG, CU, CP, FG, PB
Proteus. mirabilis	44	29	29	12	AK, LOM, NET, FG, RC, GM, ZN, PF, NX, CP, CFM	ZX, CF, SPX	NA, RP, AS, CPO, CZX

Diameter of inhibition zone (mm), *including size of well (8mm)

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Key:

TT	Ticarcillin/Clavulanic acid	OX	Oxytetracycline
RP	Ceftriaxone	ZX	Cefepime
CB	Cefuroxime	NA	Naladixic acid
NX	Norfloxacin	AG	Amoxycillin/Clavulanic acid
CU	Cefadroxil	CP	Cefoperazone
FG	Ceftazidime	PB	Polymixin b
AS	Ampicillin	BA	Co-trimaxazole
CF	Cefotaxime	PC	Piperacillin
CH	Chloramphenicol	RC	Ciprofloxacin
CI	Ceftizoxime	TE	Tetracycline
ZN	Ofloxacin	GM	Gentamicin
AK	Amikacin	GF	Gatifoxacin
CPO	Cefpodoxime	CZX	Ceftizomine
LOM	Lomefloxacin	NET	Netillin
PF	Pefloxacin	CFM	Cefixime
SPX	Sparfloxacin		

Table 3. Antibacterial activity of honey against gastro intestinal tract infection causing bacteria

Organisms (GIT Bacteria)	Diameter of inhibition zone (mm)			
	Honey concentration (w/v)			
	Undiluted	75%	50%	10%
Shigella spp	31	31	26	20
Salmonella. typhi	18	36	33	30
Salmonella. typhi para A	19	42	35	28
Salmonella. typhi para B	15	-	-	-

Diameter of inhibition zone (mm) *including size of well (8mm)

Among these cultures, 8 uropathogens were found to be resistant to more than 16 antibiotics (Table 2). All the 9 uropathogens were found to be multiple drug resistant including 3rd generation Cephalosporins (Ceftazidime,Cefotaxime and Ceftriaxone) [27]. The isolates *Citrobacterdiversus, Proteus mirabilis, Enterobacteraerogenes*,*Citrobacteramalonaticus*, *Escherichia coli* strain 1,and *Escherichia coli* strain 2 were Extended Spectrum β-Lactamase (ESBL) and isolates *Klebsiellapneumoniae, Pseudomonas aeruginosa* strain 1, *Pseudomonas aeruginosa* strain 2 were *Metallo*β-Lactamase (*MBL*) producers [27]. β-lactam antibiotics are generally used in the treatment of Urinary tract infections and Gastro intestinal tract infections. Extended spectrum β-lactamases (ESBLs) are capable of hydrolyzing oxyimino-cephalosporins in addition to β-lactam antibiotics and are inhibited by β-lactamase inhibitors [38]. Metallo-β-lactamases (MBLs) are bacterial zinc enzymes that are able to hydrolyze most β-lactam antibiotics [39-40]. But with honey a significant inhibition was observed for all these organisms which were causative agents of Urinary tract and Gastro intestinal tract infections, emphasizing its antimicrobial activity. These results confirm the traditional belief of attributing medicinal properties to honey. The dataalso confirms with the antibiotic susceptible and resistant isolates of *S. aureus*, *S. epidermidis*, *Enterococcusfaecium*, *E. coli, P. aeruginosa, E. cloacae*, and *Klebsiellaoxytoca* which were killed within 24 h by 10%-40% (v/v) honey [44].Research has also

been conducted on manuka (*Leptospermumscoparium*) honey, which is effective against broad range of microorganisms including multi-drug resistant strains, and human pathogens like *Escherichia coli, Enterobacteraerogenes, Salmonella typhimurium, S. aureus,* and *Campylobacter* spp. [21, 41-43]. One of the results reported*Salmonella typhi*,*E.coli and P.aeruginosa* have minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of honey in the range of 0.625-5.00 mg/mL, and ZDI for the isolates ranged 6.94-37.94 mm, respectively [45]. Another study revealed honey as an antimicrobial agent against *Pseudomonasaeruginosa*which was found to be sensitive to honey at a minimum inhibitory concentration of 20% [46]. Honey at 60% concentration, was bactericidal for *P.aeruginosa* and bacteriostatic for *S.aureus* and *Klebsiella* spp. [47]. The concentration of honey for inhibition of *E. coli* was 6.5 % and for *P. aeruginosa* the value was 7.5 % [48]. The findings of the present study are in resonance with the above.

The antibacterial activity of the honey samples can be assigned to the varied levels of hydrogen peroxide, glucose oxidase and catalase. Non peroxide factors may also contribute to the antimicrobial properties of honey such as lysozyme, phenolic acids and flavonoids along with high osmotic pressure, low water activity, low pH and low redox potential [23, 49].

Reducing sugar content and total protein content

The reducing sugar content of crude honey sample was found to be 75gm % (Table 4). Reducing sugars, which include glucose and fructose, are the major constituents of honey [50]. The results obtained matched approximately with the values obtained for reducing sugars in Indian honey, 45.3% to 66.7% [51]. Honey sugars are formed by the action of several enzymes on nectar sucrose. The result is a complex mixture made up of 70% monosaccharides and 10-15% disaccharides composed of glucose and fructose [52]. In almost all honey types, fructose predominates, glucose being the second main sugar. Bureau of Indian Standards (BIS) in its specification for honey has prescribed a minimum level of 65% total reducing sugars. In the current study the protein content (mg/g of honey) in the sample was obtained to be 2.4 mg/g, which was determined using the bovine serum albumin (BSA) as standard (Table 4). The protein content in Indian honey was comparable to that found in Brazilian honey samples where it varied from 0.12 to 2.24 mg/g [53]. Relatively higher protein values ranging from 3.7mg/g to 9.4mg/g have been reported for Algerian honey samples [54]. The protein content of honey is normally less than 5mg/ml [14]. The protein content of the honey is dependent upon the type of nectar, and the type of flora available. It can also be attributed to the enzymes in the nectar or introduced by the bees.

Table 4: Biochemical analysis of honey sample

Sample	Protein content* (mg/g)	Reducing sugar content# gm %
Honey	2.4 mg/g	75 gm %

*By Folin Lowry method #By DNSA method

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Table 5. HPTLC Analysis

Sample	Rf value
Glucose	0.28
Fructose	0.30
Maltose	0.11
Sucrose	0.17
Lactose	0.08
Xylose	0.47
Honey Sample	0.10
	0.20
	0.28
	0.31
	0.32

HPTLC Analysis (High Performance Thin Layer Chromatography)

By comparing the Rf value of the honey sample with the Rf value of the standard sugars, by HPTLC (Table 5), it was found that the crude honey sample consists of Glucose, Fructose, and Maltose.Rf values were also close to the values of Sucrose. The data indicates that the majority of soluble sugars present in honey sample are reducing sugars. In other studies fructose, glucose, sucrose, maltose, turanose, isomaltose, melezitose[55-56] and trehalose [55]were identified and quantified. Apart from sucrose, other disaccharides identified were maltose and isomaltose [55]. The monosaccharides glucose and fructose were the predominant sugars, which confirm that the honey sample is genuine [55].

CONCLUSION

The current study testifies the honey obtained from Gharwal, Uttarakhand to be effective against the drug resistant organisms, emphasizing its importance in medicine. The development of resistant organisms against currently used antibiotics has led to the need of using alternative methods of treatments. The microbiological and some biochemical characteristics of honey were determined to provide information on their level and prevalence depending on the source. The honey sample also showed absence of food borne pathogenic bacteria. Our study indicated that the crude honey sample was approximately equal in all aspects as the processed ones. Honey being non-toxic, non-allergic, inexpensive and possessing antibacterial activity can be recommended as an effective alternate. The work was undertaken with the aim of assisting the tribal people of Rudraprayag, Gharwal in marketing the crude honey samples, and hence boosting entrepreneurship amongst them.

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