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SCREENING THE PHYSICO-CHEMICAL PROPERTIES AND PROBIOTIC POTENTIAL OF THE INNATE MICROFLORA OF WILD HONEY

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Abstract– Honey, one of the nature's wonders is in limelight in the recent years for its therapeutic potential. The quality of honey is determined by its nutraceutical, chemical, physical and microbiological characteristics. In addition to physico-chemical characteristics the present study was aimed to evaluate the probiotic potential of innate microflora of the wild honey collected from the foot hills near Madurai. The quality of honey was reported to vary based on the botanical origins, handling, transportation and storage conditions. Honey extracted from the beehives was found to be acidic (pH 3.79) with moderate moisture (18.6%), low hydroxy methyl furfural (HMF) level (6.56 mg/kg) and ash content (0.46%). Assessment of nutraceutical, microbiological and physico-chemical parameters contribute to the elaboration of standards for quality control and promote the usage of wild honey for medicinal purpose. Two different bacteria were isolated from the honey samples and they were identified as *Bacillus* and *Pseudomonas*. It can be inferred that the acidic and high sugar content reduce the occurrence of bacteria in the honey. Both isolates exhibited probiotic properties such as tolerance to acid, bile salt, gastric juice, auto-aggregation potential, antibiotic resistance and the absence of haemolytic activity.

INTRODUCTION

Honey is one of the nature's wonders with a vivid therapeutic potential. Honey is collected by the bees especially *Apis* species from the nectar of blossoms or from the secretion of living parts of plants or excretions of plant sucking insects on the living parts of plants. Bees collect the viscous fluid, transform it by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen. According to the available literature, evidence of harvesting honey, dates back 10,000 years but yet little information is known about it. Literature, both folklore and scientific, have been extolling the virtues of honey: anti-microbial, anti-mutagenic, anti-oxidant, anti-inflammatory, anti-diabetic, anti-tumour etc., The outcome of scientific attention, along with the turn of consumers to natural products (phytomedicines) leads to the commercialization of honey for pharmaceutical usage. Therefore, quality measurements of honey are inevitable as it plays an important role in medicine, food analysis, environmental studies or in the exchange of goods and services. Till date no direct parameter is available to determine the exact quality of honey. The physico-chemical parameters like moisture, sucrose, hydroxymethylfurfural (HMF), trace minerals protein content and acidity illustrate the quality of the honey (Draiaia *et al.,* 2015).

In addition to physico-chemical parameters, innate microflora was reported to influence the quality and therapeutic contributions. The intrinsic properties of honey such as high sugar content, acidity, water activity, presence of organic acids, hydrogen peroxide, phenolic compounds and bee wax were reported to be responsible for the least occurrence of microbes (French *et al.,* 2005). However, honey is reported to harbor a few and a limited variety of microorganisms (Snowdon and Cliver, 1996). A high count of bacteria would represent the contamination from secondary sources

(Irulina and Fritz, 2005). The primary sources of bacterial contamination include secondary and postharvest sources. In addition, innate micro flora is reported to be responsible for the spoilage of honey, therefore regardless of their origin they require some form of laboratory identification. Hence, in this study the quality of the crude honey samples collected from the foot hills near Madurai was assessed by physio-chemical characteristics and microbial occurrence. Further probiotic potential of the innate microflora isolated from the honey sample was also analysed.

MATERIALS AND METHOD

Sample Collection

Honey was harvested from the beehives of *Apis indica* collected from the foot hills near Madurai, Tamil Nadu, India. The sample was collected by squeezing the comb, filtered and stored under aseptic conditions at 5 °C until further analyses. The honey collected for analyses is of multifloral origin.

Physicochemical properties of honey

The honey was analysed for pH, moisture, ash, sugars, electrical conductivity and hydro methyl furfural (HMF) following Association of Official Analytical Chemists (AOAC) methods (1990, 1996). The total phenol content was measured as described by Singleton *et al*. (1999). The total flavonoid content was measured by the method described by Saric *et al*. (2012) with slight modifications. Viscosity of honey was calculated using Ostwald's viscometer following the method of Akoh (1991). Trace minerals was determined using ICP-AES and ICP-MS following the method of Chua *et al.* (2012). Three replicates were maintained for all the experiments in this study unless otherwise represented.

Isolation and Identification of Bacteria

Bacteria present in the honey were isolated by spread plate technique using nutrient agar (Cappuccino and Sherman 2014). Haemolytic activity of the isolated was examined using brain heart infusion agar supplemented with sheep blood (5%). Non-haemolytic isolate was selected and identified based on morphological characteristics and their reactions to various biochemical tests as described by Bergey's manual of determinative bacteriology (Brenner *et al.,* 2005).

Screening for probiotic properties

Acid and bile salt tolerance

Probiotic microorganism must fulfil several criteria like tolerance to acid and bile salt in order to survive in the gastrointestinal tract as well as to grow in the lower intestinal tract. Therefore, in this study to determine the acid tolerance property, Cells of the isolate in tryptic soy broth incubated for 24h at 30 °C were harvested by centrifugation (10,000 rpm / 10 min), washed, re-suspended in 1 ml of sterile phosphate buffered saline (PBS) at different pH for various time intervals (0, 60, 120, 180 min). Later the mixture was transferred to fresh tryptic soy broth and incubated at 30 °C. The growth of bacteria was measured at A620 nm after 24 h of incubation and the percent survival of strain to different pH was calculated as described by Erkilla and Petaja (2000). Data were statistically analysed using analysis of variance (ANOVA).

The bile salt tolerance of the isolate was determined as per Ahire et al. (2011). Peptone water (9ml - 10%) supplemented with different concentration of bile salt (0.3 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %) was prepared and inoculated with one ml of the isolate and incubated at 37 °C. Growth of bacteria was measured (A620 nm) at different time intervals and the percent survival of the isolate was calculated.

Gastric juice tolerance

Tolerance of the isolates to gastric juice was determined as described by Fernandez *et al*. (2003) with slight modification. Mid log phase bacterial pellets were collected by centrifugation (10000 rpm for 10 minutes), washed thrice in PBS (pH: 7.3) and resuspended in the same buffer (10ml). The cell suspension was diluted (1:10) in synthetic gastric juice and then incubated at 37 °C. After incubation at different time intervals (0, 60 and 120 minutes), survival rate was measured by transferring 100µl sample on fresh nutrient broth and incubated at 37°C for 24h. The bacterial growth rate was measured at 620 nm after 24h of incubation.

Auto-aggregation and antibiotic susceptibility assay

Auto-aggregation assay was performed following the procedure described by Del Re *et al.* (2000) and Ahire *et al.* (2011) with slight modifications. Isolate were grown at 37° C for 24 h in nutrient broth. The

cells were pelleted, washed twice with PBS (pH 7.3), resuspended in the same buffer to get an absorbance (620 nm) of 0.5 as A_0. In auto-aggregation assay, the absorbance was measured (A_1) to the bacterial suspension incubated for different time intervals (0, 2, 4, 6, 8, 10 and 12 h). Auto-aggregation percentage was calculated using the following formula,

$$\text{Auto} - \text{aggrgation percentage} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

A_1 represents the absorbance at different time intervals and A_0 represents the absorbance at 0 h.

The susceptibility of the isolate to eight different antibiotics was determined using standard antibiotic discs (Hi Media, Mumbai) on the surface of Muller Hinton agar seeded with a lawn of the isolate. After 24 h incubation at 37°C the plates were observed for zone of inhibition.

RESULTS AND DISCUSSION

Phytochemical constituents and physical parameters

Phytochemical constituents and physical parameters of wild honey collected from the beehives is depicted in Table 1. The sample was acidic (pH 3.79) with moderate moisture (18.6%) and low HMF level (6.56 mg/kg). The viscosity was found to be 3.2 centipoise. Gomez-Diaz *et al*. (2009) reported that the viscosity of honey decreases as the water content increases. Free acidity, lactic acidity and total acidity of the honey sample were found to be 15.48 mEq.kg^{-1}, 15.91 mEq.kg^{-1} and 31.39 mEq.kg^{-1} respectively. The ash content and electrical conductivity was found to be 0.46% and 0.47 µS/cm respectively. Sugar is the main component of honey and it was found to be 64.93g/100g. It is evident from the results that the soluble sugars constitute nearly 95.73% of the total sugar content. The total phenolic (90.21 mg GAE/100g) and flavinoid (61.47 mg QE/100g) content was found to be within the permissible limit. Being a natural product, the physico-chemical parameters of the honey extracted assumed to vary with the samples collected from different locations. The physico-chemical values analyzed were within the acceptable international standards and indicate the freshness, flavour and stability of the product. The trace mineral composition of honey samples was analysed by ICP-AES and ICP-MS. Five major elements were detected and the most abundant mineral was found to be Potassium (915 ppm). Calcium and Phosphorus were in moderate amount (49 ppm and 43 ppm respectively). Occurrence of other elements like Mg and Fe were found to be in trace level. Naman *et al*. (2005) reported that electrical conductivity value reflect the total mineral (ash) and

Table 1. Physicochemical parameters of the honey

Parameters	Unit	Value
pH	-	3.79
Ash content	%	0.46
Moisture content	g/100g	17.27
Free acidity	Meq/kg	15.48
Lactic acidity	Meq/kg	15.91
Total acidity	Meq/kg	31.39
Viscosity	Centipoise	3.10
Ec µS/cm	0.47	
HMF	Mg/kg	4.26
Total sugar	g/100g	78.79
Reducing sugar	g/100g	71.79
Sucrose	g/100g	7.00
Phenols mg	GAE/100g	90.21±0.45
Flavonoid mg	QE/100g	61.47±0.62
Potassium	ppm	915±12
Calcium	ppm	49±1.40
Phosphorus	ppm	43±1.20
Magnesium	ppm	12±0.53
Iron	ppm	2.1±0.93

Table 2. Biochemical and Physiological response of endophytic bacteria isolated from the wild honey.

Biochemical tests	A	B
Grams staining	Gram Positive	Gram Negative
Shape	Rods	Rods
Color of the colony	Creamy White	White
Motility	Motile	Motile
Endospore	+	-
Catalase	+	+
Oxidase	variable	+
H 2 S Production	-	-
Methyl Red Test	-	-
Voges - proskauer Test	+	-
Citrate Utilization	+	+
Indole Production Test	-	-
Starch Hydrolysis	+	+
Protinase	+	+
Cellulase	+	+
Lipase	+	+
Nitrate reduction	+	-
Hemolytic Activity	-	-
Glucose	+	+
Fructose	+	+
Sucrose	+	+
Type of Siderophore	-	Hydroxamate
Putative Identification	Bacillus sp.	Pseudomonas

acid content of the sample.

Isolation and identification of bacterial strain

Two different bacteria were isolated from honey. The isolates were initially screened based on the colony morphology and growth characteristics. The isolates were subjected to biochemical analyses. The biochemical profile of the isolates is presented in Table 2. Both the isolates were rod shaped and one was found to be gram positive (*Bacillus sp.*) whereas the other was gram negative (*Peudomonas sp*.). Both the isolates responded positively to citrate utilization, starch hydrolysis and production of proteinase, lipase, and cellulase. *Pseudomonas* emit flourescence on exposure to UV light and it was assumed as *Pseudomonas flourescens*. The other isolate was identified as Bacillus based on spore forming potential and it responds positively to Voges- Proskauer and nitrate reduction test. Both isolates utilized glucose, fructose and sucrose as their carbon source. Of the two isolates, Pseudomonas alone was found to produce siderophore (hydroxomate nature). Lopez and Alippi (2007) reported the occurrence of aerobic spore-forming bacteria in honey. Siderophore production was reported to inhibit the growth of pathogenic bacteria sequestering the essential element iron, from the host environment.

Probiotic activity

In furtherance to the identification of innate microflora, the probiotic potential of the isolates was evaluated by a battery of assays. Absence of haemolytic activity and catalase production is considered as a safety prerequisite for the selection of a potential probiotic strain. Both the isolates failed to exhibit haemolytic activity on blood agar plates, which ensures its safety. Enzyme catalase production revealed that both the isolates could withstand the stress conditions generated by reactive oxygen species. The acid tolerance profile of the two isolates is depicted in the Table 3. *Pseudomonas* was found to be highly susceptible to acidic conditions compared to the other. The percent survival of the isolates *Bacillus* and *Pseudomonas* at pH 4 after three hours of incubation was found to be 89.7 and 40.00 respectively. Ahire *et al*. (2011) reported that tolerance to low pH and bile salt enables a bacterium to survive, grow and exert its action during the gastrointestinal transit. Bile salt tolerance potential of both the isolates is high and falls in close proximity with each other. Ronka *et al*. (2003) reported that tolerance to low pH and bile salt under *in vitro* conditions help to predict the survival of a strain under acidic conditions (1.5 to 4 pH) that exist in the human gut. It is evident from the results that the isolate *Bacillus* sp. showed greater resistance to gastric juice compared to *Pseudomonas* sp. Auto-aggregation potential illustrates the ability of the isolates to adhere with the epithelial cells and mucosal surface which is considered as an important property for an ideal probiotic. It is evident from the results that a linear relationship exists between incubation period and auto-aggregation potential of the isolates. Auto-aggregation potential of *Bacillus* sp at 2 and 12 hrs of incubation was found to be 21.00 and 78.33 respectively. Both the isolates were found to be resistant to amoxicillin, ampicillin, kanamycin rifampicin, streptomycin and tetracyclin but found to be susceptible to erythromycin and penicillin. The control plates maintained under identical conditions exhibited confluent growth. The basis of resistance to different antibiotics is not elucidated in the study however, early reports indicated that it may be an intrinsic property of the bacteria.

Table 3. Influence of pH on the growth and survival percentage of the isolates

Isolates	pH	Hours of incubation				% survival after 180 min
		0 min	60 min	120 min	180 min	
Bacillus sp.	1	0.05±0.01	0.01±0.003	0	0	0
	2	0.26±0.01	0.24±0.03	0.19±0.01	0.14±0.04	53.8
	3	0.61±0.04	0.6±0.02	0.51±0.06	0.44±0.03	72.1
	4	0.88±0.05	0.85±0.01	0.82±0.02	0.79±0.01	89.7
Pseudomonas						
	1	0.02±0.00	--	--	--	0
	2	0.07±0.01	0.02±0.07	--	--	0
	3	0.20±0.03	0.15±0.04	0.10±0.05	0.08±0.04	40.00
	4	0.30±0.03	0.24±0.04	0.20±0.01	0.12±0.04	40.00

Evans and Lopez (2004) postulated that the probiotic bacteria that reside within the honey are capable of inducing an equally strong immune response in the host system when ingested. Validation of nutraceutical and ethnopharmacological claims by a series of assays, the results of this study has proven the safety for use of natural unpasteurized honey collected from the beehives of *Apis indica* for the treatment of many diseases.

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