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GC-MS Profiling and Antioxidant Activity of Bioactive constituents in Polyfloral Honey from Indigenous *Apis cerana indica* Colonies

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Abstract

Honey is a nutritionally rich sweetener produced by honeybees, contains wide spectrum of phytochemicals of known health benefits. The present study aimed to investigate the phytochemical composition and antioxidant potential of polyfloral honey produced by domesticated *Apis cerana indica* colonies also known as Indian bee from the Western Ghats, Karnataka. Ethyl acetate solvent extracted honey samples were analyzed using Gas chromatography and mass spectrometry to identify volatile and semi volatile bioactive constituents. A total of ten compounds were identified, including phytol, palmitic acid vinyl ester, and eicosanoic acid derivatives, with palmitic acid vinyl ester and octadecanoic acid derivatives acquiring prominent peak area. The presence of bioactive phytocompounds enhances the nutraceutical richness of polyfloral honey, encouraging its inclusion in an individual diet. The *in vitro* antioxidant activity, evaluated using DPPH assay, demonstrated considerable free radical scavenging ability (IC_{50} = 171.90±1.58 μg/mL). These findings support the use of *Apis cerana indica* honey as a natural dietary antioxidant source. Furthermore, extensive research is required to correlate the specific floral source to specific honey origin to use GCMS as biomarker for honey identification and authentication.

Key words : *Apis cerana indica,* GCMS analysis, Phytol, DPPH assay.

Honeybees (*Apis cerana indica*), eusocial Hymenopterans heavily rely on foraging resources like nectar and pollen. Their close mutualistic and co-evolutionary relationship with bee pasturage serves as important bioindicators of environmental quality. Nectar is the watery sugar syrup produced by floral nectaries is the primary source of energy for bees, providing carbohydrates that are converted into honey by regurgitation, digestion, enzymatic transformation and evaporation. Pollen, also known as bee bread or ambrosia provides proteins, lipids, vitamins, and minerals necessary for the growth and development of bee colony. The selective foraging behavior of honeybee results in honey's distinctive biochemical profile, infused with plant-derived bioactive constituents like fatty acids, organic acids, terpenoids, alkaloids, steroids, saponins, and polyphenols[3]. The phytocompounds in honey vary significantly between varieties, affect the honey's flavor and color, and can be used to identify the honey's origin due to their compositional differences[25]. This naturally complex composition of honey contributes to its inherent medicinal efficacy.

Natural honey is a carbohydrate rich product primarily composed of sugars and waters with other minor constituents including amino acids, proteins, minerals, vitamins, enzymes, volatile compounds and dietary antioxidants[9] consumed by mankind not only as sweetener and flavour enhancer but also as nutraceutical tonic with lot of health advantages include antimicrobial, anticancer, antidiabetic, anti-inflammatory, antitumor, anti-mutagenic, hepatoprotective, cardioprotective, wound management, gastrointestinal disorders[17, 21, 22] and in recent times it also used against COVID-19 due to its immune booster properties[1]. The positive effect of honey goes beyond its sweetness and dietary functions used as a natural food preservative to help prevent food from spoiling[15] and as an ingredient in the beverage, cosmetics, confectionary, tobacco and pharmaceutical industries[4].

The economic value and quality of honey are intrinsically linked to its floral origin and the biodiversity of foraging landscape. While often more accessible and affordable, polyfloral honeys gain significant value by reflecting their multifarious nectar sources and regional botanical diversity. This abundance of floral diversity in hotspot regions like the Western Ghats, driving polyfloral nectar collection, likely influences the quality and nutraceutical characteristics of the produced honey compared to regions with less diverse flora. Understanding the relationship between regional floral diversity, bee foraging preferences for polyfloral sources, and resultant honey quality is crucial for maximizing the economic and perceived value of polyfloral honeys, especially those from biodiverse areas such as Western Ghats[11,18]. Furthermore, conservation strategies aimed at preserving and enhancing this floral richness are essential for sustaining high-quality polyfloral honey production and supporting healthy bee populations within this ecologically important zones. Therefore, for our study particularly multifloral nectar among honey-producing bees, *Apis cerana indica* of the Western Ghats, where floral resources are notably rich was selected.

The variation in the metabolites composition of honey is directly linked with its flowering patterns of honey producing landscape[2]. To the best of our knowledge, till now there are no reports on GC-MS investigations carried out to determine the bioactive constituents of polyfloral honey of Western Ghats of Karnataka produced by local bees, *Apis cerana indica*. Keeping this in view, the present study was undertaken to identify and report the biologically active phytocompounds present in ethyl extract of polyfloral honey and their possible effects on managing oxidative stress.

Honey sample collection :

For the present study polyfloral honey sample was directly purchased from the local beekeeper having indigenous domesticated *Apis cerana indica* colonies from the Western Ghats of Karnataka during the peak honey flow period of the year 2024. Honey collected is amber in colour with unique mild flavor and strong aroma. Nutritional profile of polyfloral honey was studied previously by authors aligns well with established codex standard for high quality honey with 16% water content and 82% carbohydrates. The major carbohydrates found are 39% levulose, 33% dextrose and 2% sucrose. A small amount of proteins (0.55%), water soluble vitamins, macro and micro nutrients are also recorded which adds to the nutraceutical benefits.

Solvent extraction of honey :

Honey extract was prepared using a solvent extraction technique[14]. A 50% weight/ volume solution was prepared by dissolving 10 grams of honey in 20ml of ultra-pure Milli-Q water. The resulting mixture was homogenized for ten minutes using a vortex mixer. It was then subjected to solvent extraction using 100ml ethyl acetate in the presence of anhydrous sodium sulphate to aid in dehydration. The organic layer was concentrated to dryness under reduced pressure using a rotary evaporator. The dried extract was filtered and stored in amber glass vials at 4ºC until analysis.

GCMS analysis :

The profiling of different bioactive compounds in honey extract was done accordingly the method prescribed by (research paper) with some adjustments using a GCMS-QP2010 SE single quadrupole system. Chromatographic separation was achieved using a DB-5MS capillary column [0.25mm (i.d.) × 30m length, 0.1µm film thickness). The column oven temperature was initially set at 80ºC, held for 10 minutes, then ramped to 200ºC at a rate of 10ºC/min, and finally increased to 330ºC at a rate of rate of 20ºC/min and held for 5 minutes. The total run time was 35 minutes. The injector temperature was maintained at 280ºC, with the injection carried out in split mode at a split ratio of 10:1. Ultrapure helium gas was used as the carrier gas at a constant column flow of 1.5mL/min. The linear velocity was maintained at 45.1cm/sec, and the flow was 19.5mL/min purge flow was set at 3 mL/min.

Identification of bioactive constituents :

Mass spectrometer was operated in scan acquisition mode. The ion source temperature was set at 200ºC, and the interface temperature was maintained at 300ºC. The system operated under a solvent-cut time of 1.40 minutes. Mass spectra were recorded over an m/z range of 35-500, with a detector voltage of 0.95 kV. The scan speed was set to 1666 amu/sec, with an acquisition time of 0.30 seconds per scan. Data acquisition and analysis were conducted using GCMS Solutions 4.3 version, and compound identification was done based on mass spectra, retention time, and characteristic fragmentation patterns using the standard references listed in the NIST 20 mass spectral library.

Free radical scavenging activity by DPPH assay :

The free radical scavenging ability of polyfloral honey was evaluated using the method described by Blois[6] with slight modifications. The assay involved 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), prepared as a 0.004% solution in methanol. Different concentrations of polyfloral honey varying between 100-500 µg/mL were mixed with an equal volume of the DPPH solution. The mixtures were shaken thoroughly and incubated in the dark at room temperature for 35 minutes to allow for the reaction and color development. At the end of incubation period, the reduction in DPPH radicals was measured by recording the absorbance at 517nm using UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). A blank and ascorbic acid as a positive control were also prepared for comparison. The % of free radical scavenging activity was calculated using the formula:

$$\% \; of \; inhibition = \frac{Mean \; OD \; of \; blank - Mean \; OD \; of \; test \; samples}{Mean \; OD \; of \; blank} \times 100$$

The IC_{50} value, indicating the concentration of polyfloral honey required to inhibit 50% of DPPH radicals, was determined from the dose-response curve plotted with percentage inhibition on the Y-axis against sample concentration on the X-axis using a Microsoft Excel spreadsheet version 2010.

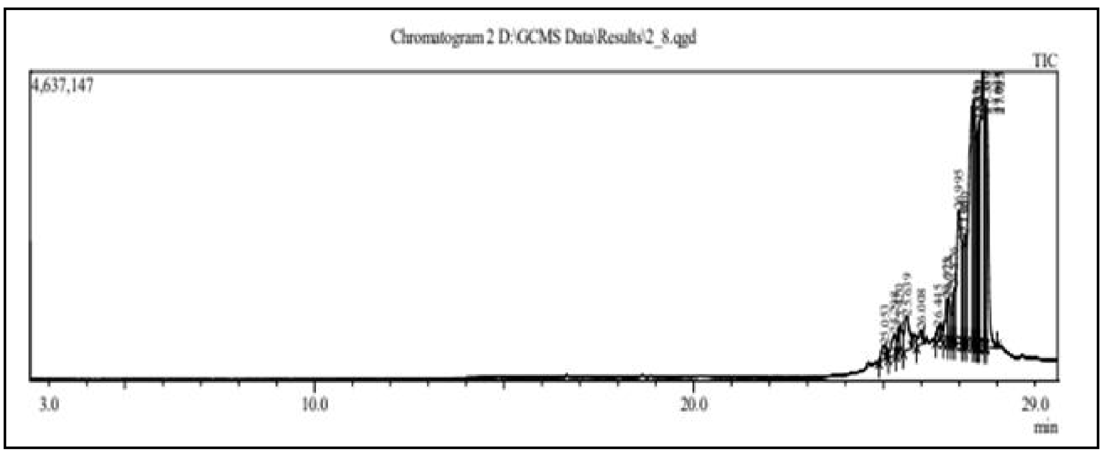

Figure 1. GCMS Chromatogram showing peaks for bioactive constituents in polyfloral honey

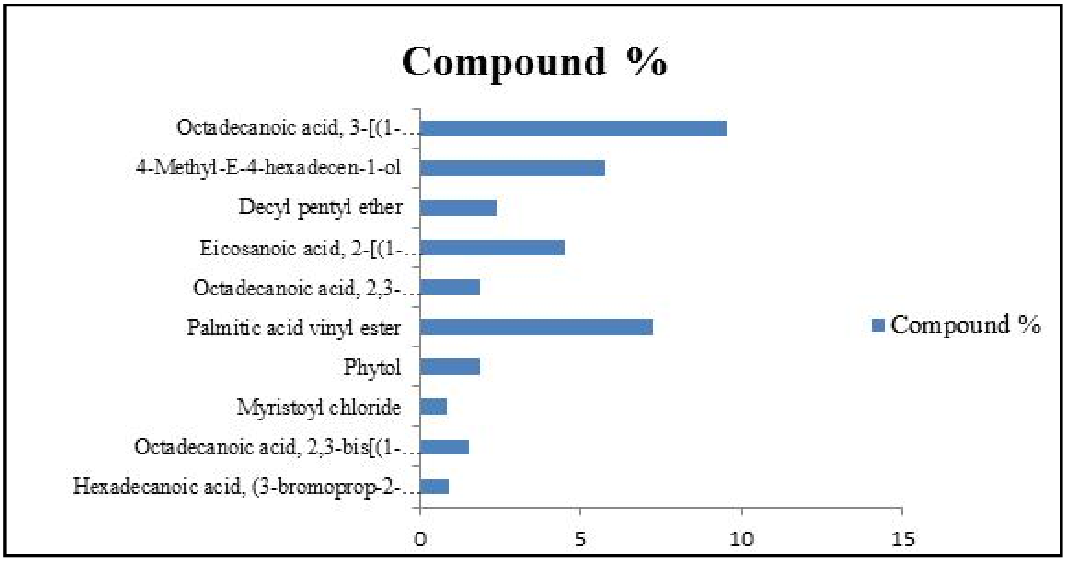

Figure 2. GCMS of polyfloral honey showing percentage of different bioactive compounds

Table-1. List of identified volatile compounds in polyfloral honey of *Apis cerana indica*

Sl. No	R.T time in min	Name of the compound	Molecular formula	Molecular weight in g/mol	Average Peak area %
01	25.05	Hexadecanoic acid, (3-bromoprop-2-ynyl) ester	$C_{19}H_{33}BrO_2$	373.4	0.91
02	25.29	Octadecanoic acid, 2,3-bis[(1-oxotetradecyl)oxy]propyl ester	$C_{49}H_{94}O_6$	779.3	1.54
03	25.42	Myristoyl chloride	$C_{14}H_{27}ClO$	246.81	0.82
04	25.64	Phytol	$C_{20}H_{40}O$	296.5	1.85
05	26.44	Palmitic acid vinyl ester	$C_{18}H_{34}O_2$	282.5	7.25
06	27.10	Octadecanoic acid, 2,3-bis(acetyloxy) propyl ester	$C_{25}H_{46}O_6$	442.6	1.87
07	27.16	Eicosanoic acid, 2-[(1-oxohexadecyl) oxy]-1-[[(1-oxohexadecyl)oxy]methyl] ethyl ester	$C_{55}H_{106}O_6$	863.4	4.50
08	27.51	Decyl pentyl ether	$C_{15}H_{32}O$	228.41	2.36
09	27.66	4-Methyl-E-4-hexadecen-1-ol	$C_{17}H_{34}O$	254.5	5.76
10	27.71	Octadecanoic acid, 3-[(1-oxohexadecyl) oxy]-2-[(1-oxotetradecyl)oxy]propyl	$C_{51}H_{98}O_6$	807.3	9.53

Table-2. Antioxidant activity of polyfloral honey

Sl. No	Concentration in µg/mL	Polyfloral honey		Std. ascorbic acid	
		% inhibition	IC_{50} value	% inhibition	IC_{50} Value
01	100	38.62±1.12	171.90 ± 1.58	78.64±3.67	39.03±0.26
02	200	52.41±1.35		84.33±2.14	
03	300	64.84±1.62		89.01±1.68	
04	400	72.11±1.95		91.57±1.11	
05	500	79.53±2.08		94.20±0.93	
The values are expressed as Mean of triplicate measurements ± Standard deviation					

Bioactive constituents found in honey are secondary metabolites produced by plants and held in nectar, and present in honey in various chemical forms adds to quality and possible health benefits of honey. The specific phytochemicals found in honey are majorly depends on the kinds of nectar source that the bee visit. Therefore the present study was undertaken to record the bioactive phytoconstituents in polyfloral honey by using gas chromatography – mass spectroscopy analytical technique. The presence of ten peaks indicating presence of ten bioactive phytoconstituents in honey with their retention time, molecular formula, molecular weight and average peak area in percentage are presented in Table 1, and Figure 1 and Figure 2.

GC-MS analysis of the honey sample shows the presence of ten distinct compounds, categorized into five major chemical classes based on their structural characteristics and functional groups. These classes include 3 simple esters, 3 triacylglycerol analogs 1 fatty acid derivative, 2 unsaturated alcohols and 1 ether.

The polyfloral honey of *Apis cerana indica* from the Western Ghat region exhibit a rich chemical composition, with Octadecanoic acid, 3-[(1-oxohexadecyl)oxy]-2-[(1-oxotetradecyl)oxy]propyl (9.53%), was found in abundant as a major compound followed by Palmitic acid vinyl ester (7.25%); 4-Methyl-E-4-hexadecen-1-ol (5.76%); Eicosanoic acid, 2-[(1-oxohexadecyl)oxy]-1-[[(1-oxohexadecyl)oxy]methyl]ethyl ester (4.50%); Decyl pentyl ether (2.36%); Octadecanoic acid, 2,3-bis(acetyloxy)propyl ester (1.87); Phytol (1.85%); Hexadecanoic acid, (3-bromoprop-2-ynyl) ester; (0.91%) and Myristoyl chloride (0.82%) were also detected. The presence of these varieties of phytocompounds supports earlier findings indicating that polyfloral honey from the Western Ghat region possesses bactericidal activity against *Bacillus* sp., *Enterococcus* sp., *Streptococcus* sp. and *S. aureus* in controlling their growth[5], highlighting its versatile applications in culinary use and medicine.

Wu *et al.* reported that presence of fatty acids and their derivatives in *Apis cerana* honey contribute to its potent free radical scavenging activity and effectively inhibited inflammatory responses *in vitro*[24]. The detection of specific fatty acid derivatives, even at low concentrations is considered to significantly influence the characteristic flavour and its botanical source attribution of honey[20]. Wilkins *et al.*[23] suggested that these compounds may serve as biomarkers of floral source[23]; the identification of this compound class in our polyfloral honey sample underscores its potential role in the honey's chemical profile and sensory properties. One such fatty acid derivative identified is Myristoyl Chloride chemically known as tetradecanoyl chloride is a colourless liquid readily dissolves in ether and is miscible with other organic solvents is primarily used as an organic synthetic raw material, surfactant, insecticide, and medication intermediary[12].

Phytol, an aromatic dietary acyclic diterpene alcohol present in polyfloral honey is a component of chlorophyll pigment, vitamin E and K to be found in fish, ruminant animals' meat, green vegetables and dairy products[19]. It directly activates PPARα in the liver, independent of its conversion to phytanic acid. This direct activation involving binding and gene upregulation, results in significant reduction in triglycerides, cholesterol, and LDL levels in animal models, demonstrating marked antidyslipidemic properties beneficial in management of type II diabetes and obesity related cardiovascular risks[16]. Adding to that, phytol is well-known for its wide range of biological activities including antimicrobial, cytotoxic, antianxiety, immune-modulating, anti-inflammatory, anti-allergic, antioxidant and antinociceptive qualities[7, 13].

Antioxidant properties of honey are linked to its levels of various phytocompounds, including flavonoids, phenolics, peptides, ascorbic acid, organic acids, carotenoids, β-carotene, lycopene, enzymes and maillard reaction products[8, 10]. These properties are of critical importance, enabling honey to act as a prophylactic agent and facilitate to cure various illnesses. In the present study free radical scavenging activity by DPPH assay exhibited concentration-dependent increase in % inhibition ranging from 38.62±1.12% at 100μg/mL to 79.53±2.08% at 500μg/mL, with a IC_{50} value of 171.90±1.58 μg/mL. In comparison standard ascorbic acid demonstrated significantly higher activity with % inhibition values between 78.64±3.67% and 94.20±0.93%, and a lower IC_{50} value of 39.03±0.26 μg/mL. These findings suggest that although free radical scavenging potency of honey is low compared to standard ascorbic acid still its effective. The findings align well with earlier observations that natural honey exhibits antioxidant effects and support its traditional use as a medicinal agent.

Each ecological niche is characterized by specific flora and regional topography, which could directly or indirectly, influences the local bee pasturage to produce nectar and pollen with specific bioactive volatile and semi-volatile phytocompounds which are species specific act as biomarker for the identification of particular honey variety and its quality assessment. In the present study, ten bioactive compounds were identified in polyfloral honey through GC-MS analysis; belong primarily to esters, triacylglycerol analogs, alcohols, ethers and fatty acid derivatives with most of them associated with managing overall metabolic health. Phytol (1.85%), an unsaturated diterpene alcohol known for antioxidant, anti-inflammatory and cytoprotective activities, contributes to the honeys radical scavenging potential.

Thus the results of the present study support the potential of polyfloral honey produced by *Apis cerana indica* colonies of Western Ghats as a source of natural compounds with pharmacological relevance. Furthermore, Future studies focusing on the isolation, characterization and quantification of these bioactive compounds are needed to fully elucidate its contribution to the honey's overall quality assessment for therapeutic use.

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