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Research Article

Identification of Phenolic Compounds from Honey obtained from Theni District, Tamilnadu, India

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Abstract

Honey is a sweet and flavorful, natural product, which is consumed for its high nutritive value and for its effects on human health, with antioxidant, bacteriostatic, anti-inflammatory and antimicrobial properties, as well as wound and sunburn-healing effects. Phenolic compounds are an important group of active compounds in honey. The aim of the present study was to identify and characterize the phenolic compounds from Megamalai, Theni District. In the present study, these phenolic compounds were extracted and recovered, with solid phase extraction procedure. The recovered fractions, from five gram of honey, yielded a dry residue of 51±0.5mg. Nearly every gram of honey yielded a residue of 10mg. These extracts were identified and quantified, with HPLC, GC-MS and FTIR. A total of five different phenolic acids were identified, with the most abundant being gallic acid, rutin, followed by caffeic acid, ferulic acid and quercetin. These phenolic compounds containing good antioxidant and antibacterial potential. Overall, The study confirms that the honey sample from Megamalai, Theni District is a good source of phenolic acids and flavonoids. It is recommended that further studies should bring out the antimicrobial properties of these phenolic compounds and other hidden antimicrobial and antioxidant properties from this honey.

Keywords: Honey, Phenolic compounds, HPLC, GC-MS, FTIR

INTRODUCTION

Honey is a sweet and flavorful, natural product, which is consumed for its high nutritive value and for its effects on human health, with antioxidant, bacteriostatic, anti-inflammatory and antimicrobial properties, as well as wound and sunburn-healing effects[1]. The composition of honey varies from one honey to another, depending on several factors. A major factor is the floral source, as the nectar from different plants will contain different compositions of the main sugars and trace elements. These compositions are influenced by soil type, climatic conditions (seasons) and the environment surrounding the plant [2]. The bioactive compounds, in honey, present a valuable supplement for healthy population [3]. Many scientific studies have demonstrated the many virtues of honey, based on their properties (antibacterial, antifungal and antiviral), more nutritionally than at the therapeutic level.

The identification and quantification of phytochemicals is necessary in determining the potential

health benefits of honey. Al-Mamary reported these health-promoting bioactive components and health benefits [4]. Phenolic phytochemicals are the secondary metabolites of plants. It has been previously determined that the bioactive phytochemicals, in honey, that account for the honey's antioxidant capacity, are from the flavonoid and phenolic acid groups.

In order to retrieve these compounds, an extraction process is necessary. Many research studies have used Solid Phase Extraction (SPE) to eliminate all other components, except the bioactive compounds of interest. In an experiment, on the isolation and identification of phenolic acids in Malaysian honey, the honey samples were absorbed onto preconditioned C18 column cartridges [5] .

Each solvent, used in the extraction procedure, had varied concentration of different phytochemical constituents. The recovery of chemical components was best in the case of ethanol [6]. Ethanolic extract of propolis showed greater amount of tannins and glycosides whose presence promoted the antibacterial and antioxidative properties of Propolis. Water extract of bee pollen contained maximum amount of saponins, flavonoids [7]. The ultimate goal of both the above methods was to keep the phenolic and flavonoid compounds on the column while eluting any polar compounds present, like sugar.

This method does not differentiate between phenolic compounds and further analysis is needed in order to identify the different phenolic phytochemicals. In a study that analyzed total phenolics in Burkina honey, the total phenolics observed, ranged from 32.59 to 114.75 mg/100g of honey [8]. The total content of phenolic compounds, depending on the botanical source of honey and collection region, varies from 46.0 to 753.0 μg/g [9].

These differences can be explained, by the different geographical locations and floral sources of the honey samples. Values, from other studies show similar variance between total phenolics. One test, used *in vitro* conditions to test antioxidant activity of honey samples, using guinea pig liver homogenate[10]**.** This technique had never been used before for the honey analysis and has not been repeated since, even though it showed a direct, positive correlation between the total phenolic content and the antioxidant capacity.

High Performance Liquid Chromatography (HPLC) has been commonly used to quantify compounds. In the case of honey, these are the most commonly used flavonoid and phenolic compounds, but some research analysed the carbohydrate components as well. HPLC, performed with Mass Spectrometry (MS) is used to quantify and tentatively identify the compounds in a sample of honey. With the help of HPLC/MS, specific, single compounds can be quantified and identified whereas the previous tests did not [11]. The isolated volatiles were analyzed by GC-MS and FTIR technique, which led to the identification of bioactive compounds in honey, which are of great importance [12].

Materials and methods:

Sources of honey sample

Honey sample was purchased, from local bee keeper, Megamalai, Theni District in the month of October 2016 and stored at room temperature. The identification was performed by the bee hunters, based on their geographical hunting area and floral availability at the location of bee hives.

Extraction of phenolic compounds [13]

Solid Phase Extraction (SPE) was used for the extraction of phenolic compounds. Five g of raw honey was dissolved, in 50 ml of acidified, deionised water (pH 2.0, achieved with trifluoracetic acid) and the phenolics were absorbed into preconditioned isolute C18 column (Supelco, Supelclean LC-18, 0.5 g, India). The cartridges were preconditioned by sequentially passing, three ml, each of methanol and acidified water (pH 2.0), at a dropwise flow rate. Ten ml of the aqueous honey solution was passed through the preconditioned cartridges, at a dropwise flow rate, to ensure an efficient adsorption of phenolic compounds. The adsorbed phenolics were then eluted from the cartridges, by passing 1.5 ml of 90% (v/v) methanol/water solution at a drop-wise flow rate. Extraction was repeated three times. Methanol extracts of honey phenolic compounds were stored in a refrigerator and identified and quantified by HPLC.

Identification and quantification of phenolic compounds using High Performance Liquid Chromatography (HPLC) [13]

The analysis of honey extracts was performed employing, by the Shimadzu HPLC system, with a UV detector. Honey phenolic compounds were separated, on a LiChro-CART RP-18 analytical column, 150 × 4.0 mm i.d. (E Merck, New Delhi, India), packed with C18 stationary phase, particle size 5 μm. The linear gradient was used at a flow rate of 0.5 ml/min. The time of HPLC run was over 35 min. The binary mobile phase consisted of a solvent A (ultra pure water with 0.1% of phosphoric acid) and solvent B (pure methanol with 0.1% of phosphoric acid). Elution from the column was achieved with the following gradient: 0 min to 10 min B increased from 35% to 55%; 10–25 min B increased to 62%; 25–30 min B increased to 85% and kept constant till 35 min. The UV detector was operating at a wavelength of 280 nm. The identification of phenolic compounds was performed by comparing the retention time of analytes and reference compounds. Phenolic acids (gallic acid, chlorogenic acid, caffeic acid, coniferic acid, ferulic acid and trans-cinnamic acid) and flavonoids (rutin, quercetin, kaempferol, apigenin, myricetin, narigenin, and hesperitin), commonly found in honey samples, were used as reference compounds. All reagents were obtained from Sigma (New Delhi, India).

Characterization of phenolic compounds using Gas Chromatography-Mass Spectrometry (GC-MS) [14]

GC-MS technique involves the characterization of phenolic components in a test sample. In this analysis, a capillary column was used. Inert gas helium was used as carrier gas. The components of a compound were evaporated, in the injector of GC equipment and segregated in the column by adsorption and absorption technique with a suitable temperature of oven, at about 325^0C, controlled by software. The GC column was heated in the oven and each and every component of the test compound was eluted from the column and the time, termed as Retention Time (RT), was noted. The eluted components were tested in the mass detector. The spectrum of the unknown components was stored in the NIST library and thus the name, molecular weight and the structure of the components of the test compound were ascertained. It is not possible, to make an accurate identification of molecules, by gas chromatography or mass spectrometry alone. The mass spectrometry normally requires a very pure sample while gas chromatography, using a traditional detector, detects multiple molecules that happen to take the same amount of time, to travel through the column and have the same retention time, which results in two or more molecules to co elute. Therefore, while identifying mass spectrum appears at a characteristic retention time, in a GC-MS analysis, it typically tends to increased certainty that analytic of interest was in the sample.

Fourier-Transform Infrared Spectroscopy (FT-IR) analysis [15]

ATR mode,l FT-IR spectrometer was used, for the analysis of phenolic extracts of honey. The compounds, acquired through SPE, were subjected to FT-IR. The sample was individually milled with potassium bromide (KBr), to form a very fine powder. The powder was then compressed into a thin pellet, which can be analyzed. The spectrum was recorded, using ATR technique, for measurement.

Results and Discussion:

Phenolic compounds are an important group of active compounds in honey. These compounds disrupt the bacterium cell wall, interfering with the ATP pool and altering its membrane potential, resulting in the bacterium's death [16]. Flavonoids are also an important group of active compounds and their action results in the inhibition of DNA, RNA and proteins synthesis of bacteria and by altering its membrane permeabilization[17].

Solid phase extraction

In present study, these phenolic compounds were extracted and recovered, with solid phase extraction procedure. The recovered fractions, from five gram of honey, yielded a dry residue of 51±0.5mg. Nearly every gram of honey yielded a residue of 10mg. These extracts were identified and quantified, with HPLC, GC-MS and FTIR. Solid phase extraction is a simple technique, using inexpensive disposable extraction columns and it provides many advantages such as reduction of solvent consumption and high recoveries of the analytes [14].

Identification and quantification of phenolic compound using HPLC (280nm)

Phenolic acids (gallic acid, chlorogenic acid, caffeic acid, coniferic acid, ferulic acid and trans-cinnamic acid) and flavonoids (rutin, quercetin, kaempferol, apigenin, myricetin, narigenin, and hesperitin) commonly found in honey samples, were used as reference compounds for the identification of the unknown peaks. The phenolic extractions were subjected to the HPLC analysis and the chromatogram was compared with HPLC reports of reference phenolic compounds.

According to the results of the sample (0.36mg/gm), gallic acid was observed at 8.908 retension time, rutin (0.23 mg/gm) was observed at 10.90 retension time, 0.10mg/gm of caffeic acid was observed at 14.700 retension time, 0.09mg/gm of ferulic acid was observed at 17.517 retension time and 0.13mg/gm of quercetin was observed at 19.933 retension time. Area and height of the peaks are tabulated in Fig: 1 and Table: 1. HPLC, which is the method of choice for food phenolic analysis, was used in this study for the identification of honey phenolics. The eluted compounds were detected at 280 nm because much of the phenolic compounds were reasonably high absorbance, at this value[18]. According to the present results of HPLC techniques, gallic acid was the dominant compound when compared to rutin, caffeic acid, ferulic acid and quercetin.

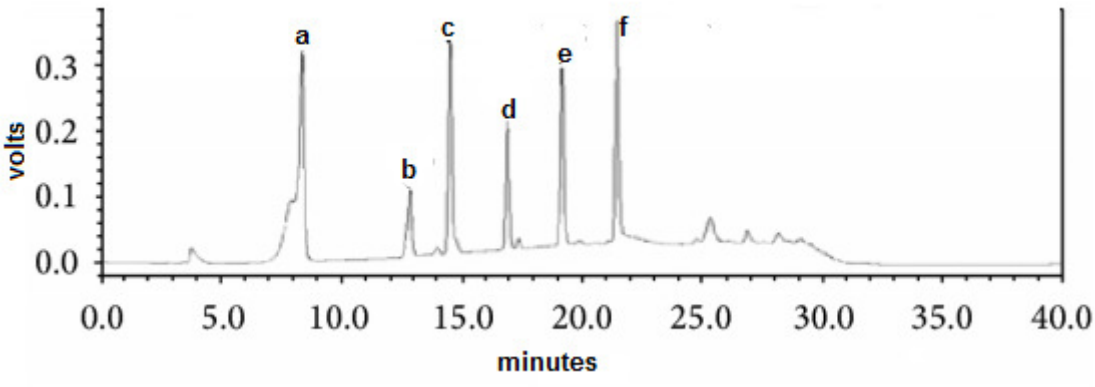
GC-MS analysis of phenolic extraction

Combined GC-MS analysis, for the phenolic extract from honey sample also provided the mass spectra for gallic acid, rutin, caffeic acid, ferulic acid and quercetin and it was confirmed by direct comparison with an authentic standard. These reports represented 3,4,5 - trihdroxybenzoic acid (Gallic acid), quercetin – 3 – O – rutinoside (Rutin), 3,4 – dihydroxycinnamic acid (caffeic acid), 4- Hydroxy – 3- methoxycinnamic acid (ferulic acid) and 2- (3,4 – dihyroxyphenyl)-3,5,7 – trihydroxy – 4H – chrome- - one (Quercetin), at the retention time of 9.217, 10.520, 12.340, 13.154 & 14.009 respectively. Molecular formulae of gallic acid, caffeic acid, rutin, quercetin & ferulic acid were $C_7H_6O_5$, $C_9H_8O_4$, $C_{27}H_{30}O_{16}$, $C_{15}H_{10}O_7$ & $C_{10}H_{10}O_4$ respectively. Molecular weights of gallic acid (170.12g//mol), rutin (610.521g/mol), caffeic acid (180.16g/mol), ferulic acid (194.186g/mol) and quercertin (02.238g/mol) ,were also obtained. The results were showed in Figure: 2 and Table: 2.

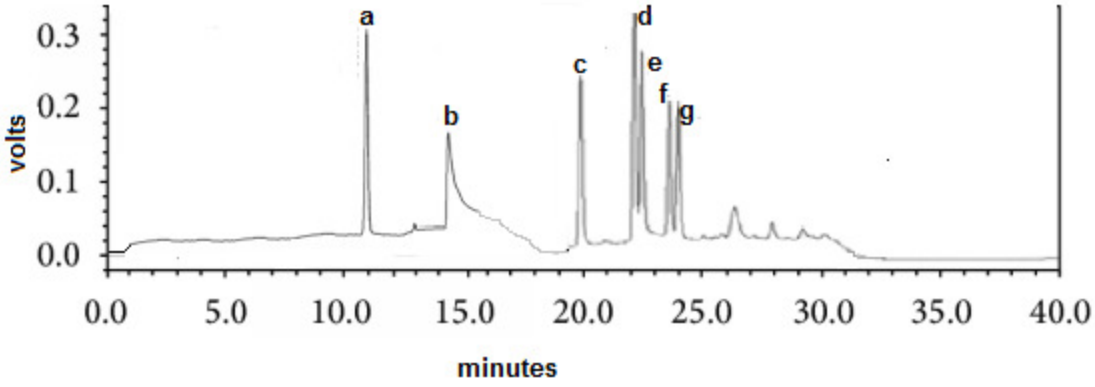
FTIR analysis of phenolic extraction

FTIR analysis of phenolic compounds was shown in Figure 3. In the present study, it was observed that bands, at 3366 cm^{-1} and at 3273 cm^{-1}, were due to stretching vibrations of hydroxyl groups; the bands at 2924 cm^{-1} & 2883 cm^{-1} related to stretching vibrations of CH_2 and CH_3 groups; the band at 2130 cm^{-1} was due to stretching vibrations of C=O group; the band at 1640 cm^{-1} was related to stretching vibrations of C=C group; the bands at 1457 cm^{-1} and 1252 cm^{-1} were due to stretching vibrations of CH_2 and CH_3; the band at 1086 cm^{-1} was related to stretching vibrations of C-O- group and bands at 945 cm^{-1} & 878 cm^{-1} were not identified. Renata Nunes Oliveira concluded that the presence of these groups indicated phenolic compounds in plant extracts [15].

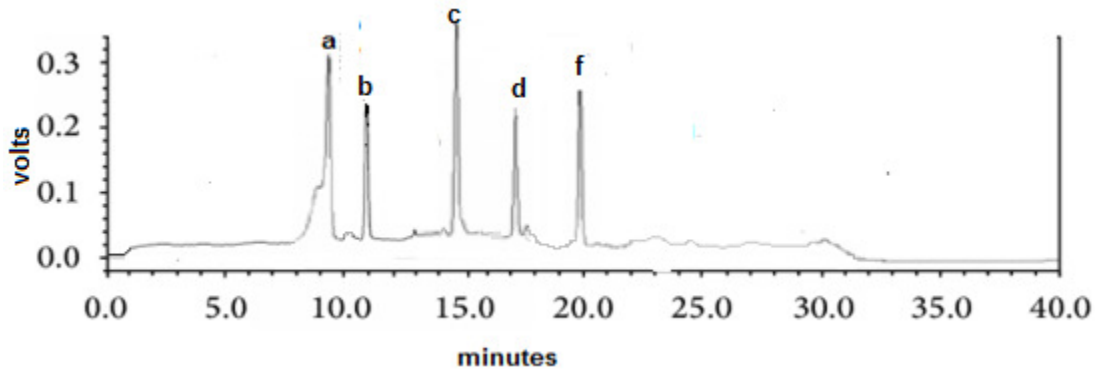
Fig:-1 HPLC chromatogram of phenolic compounds



Phenolic acid standards a) gallic acid, b) chlorogenic acid, c) caffeic acid, d) ferulic acid, e) coniferic acid, f) trans-cinnamic acid



Flavonoid standards a) rutin b) myricetin c) quercetin d) narigenin e) hesperitin f) kaemferol g) apigenin



Honey sample 1 a) gallic acid b) rutin c) caffeic acid d) ferulic acid e) quercetin

Table:-1 HPLC chromatogram of phenolic compounds from honey sample

Retention time	Area	Area %	Height	Height %	Concentration (mg/l)	Name
8.908	72946	75.6	7453	98	0.36	Gallic acid
10.900	5316	5.5	5	0.065	0.23	Rutin
14.700	2904	3	6	0.078	0.10	Caffeic acid
17.517	4622	4.7	138	1.81	0.09	Ferulic acid
19.933	10700	11	2	0.026	0.13	Quercetin

Fig:- 2 GC-MS Chromotogram of phenolic compounds in honey sample

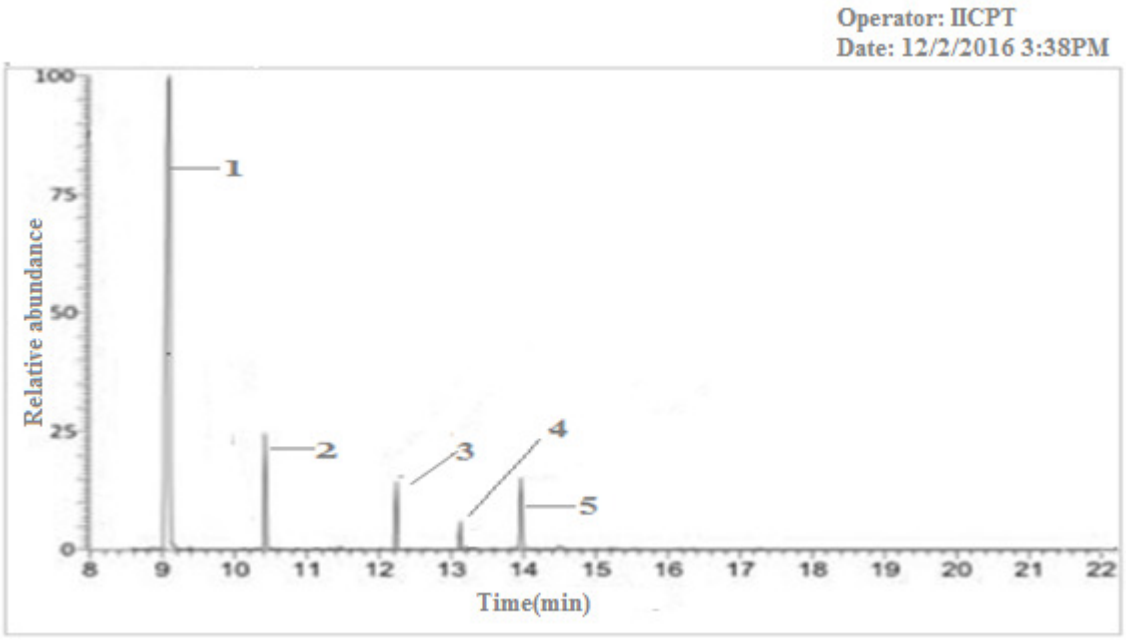
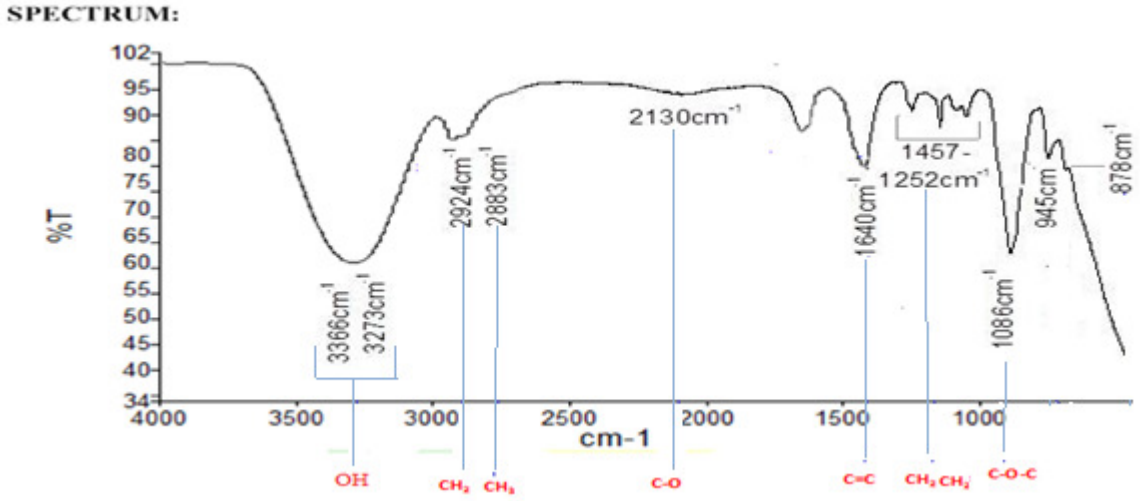


Table:- 2 GC-MS chromotogram of phenolic compounds in honey sample

S.no	Rt	Name of the compound	Common Name	Molecular formula	Molecular Weight
1	9.217	3,4,5-trihydroxybenzoic acid	Gallic acid	$C_7H_6O_5$	170.12 g/mol
2	10.520	quercetin-3-O-rutinoside	Rutin	$C_{27}H_{30}O_{16}$	610.521 g/mol
3	12.340	3,4-Dihydroxycinnamic acid	Caffeic acid	$C_9H_8O_4$	180.16 g/mol
4	13.154	4-Hydroxy-3-methoxycinnamic acid	Ferulic acid	$C_{10}H_{10}O_4$	194.186 g/mol
5	14.009	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	Quercertin	$C_{15}H_{10}O_7$	302.238 g/mol

Fig 3:- FTIR analysis of phenolic extraction from honey sample



Conclusion:

This is the first study to identify the phenolic compounds in honey sample from Megamalai, Theni district, Tamil Nadu, India. Gallic acid and rutin were the most abundant phenolic compounds among all the phenolic compounds, followed by caffeic acid, ferulic acid and quercetin. These phenolic compounds containing good antioxidant and antibacterial potential. Overall, our study confirms that the honey sample from Megamalai, Theni District is good sources of phenolic acids and flavonoids. It is recommended that further studies should bring out the antimicrobial properties of these phenolic compounds and other hidden bioactive properties from this honey.

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