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Evaluation of Antioxidant Activity and Characterization of Indian Rock Bee Mead

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Mead is an alcoholic beverage made from honey. The honey is a natural product which consists of complex mixture of sugars, minerals, proteins, vitamins, organic acids, phenolic acid, flavonoids and antioxidant properties. Mead retains most of the nutritional qualities and antioxidant properties of honey. The present investigation on the evaluation of antioxidant activity and characterization of Indian rock bee mead was carried out with an aim to analyse polyphenol, flavonoids, antioxidant properties and flavour compounds of mead produced during ageing. *In vitro* antioxidant activity of honey and wine have been examined by FRAP and DPPH methods. Total phenolic content, total flavonoids content and FRAP antioxidant activity had increased up to 6th month and thereafter slight decrease was noticed even under favourable storage condition (15º C). The DPPH free radical scavenging activity, was slightly decreased after 3rd month of ageing. Twenty two volatile compounds (C6 compounds, alcohols, ethyl esters, volatile fatty acids, acetate, aldehyde and silicon) and four sugar compounds were identified in mead using GC-MS. The results confirmed that the mead production enhances the flavour and antioxidant properties of honey.

Key words: Mead, Total Phenolic Content, Total Flavonoids, Antioxidant Activity, GC-MS, Flavour.

Mead is one of the world's oldest alcoholic beverages, containing 8-18% (v/v) of ethanol, which results from the alcoholic fermentation of diluted honey carried out by yeast. Though mead is the oldest fermented product being used by man yet it is difficult to find it commercially[1], since mead producers face several problems, like delayed and arrested fermentation, production of off-flavours by the yeast and lack of uniformity of the final product.

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Honey is a natural product, a highly concentrated solution of a complex mixture of sugars. It also contains small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals. The components in honey responsible for its antioxidative effect are flavonoids, phenolic acids, ascorbic acid, catalase, peroxidase and carotenoids[2]. Rock bees (*Apis dorsata*) are giant bees found all over India in sub-mountainous regions up to altitude of 2700 m. This honey has higher amount of enzymes, amino acids and minerals than *Apis cerana* and *Apis mellifera* honey.

Over the last few decades health effects of honey and wine consumption have been studied in depth. Special attention has been focused on protection against cancer and cardiovascular

disease. Generally, it is established that an oxidation process is involved in the initial development steps of these diseases. It is well known that natural polyphenols possess such physical and chemical properties that contribute to a proper and efficient protection from oxidation of important biomolecules such as lipids, proteins and nucleic acids[3]. Honey and wines are an excellent source of dietary polyphenols. However, not all phenolic compounds display the same antioxidant activity and the phenolic composition of wine can be strongly affected. It is probably during ageing, whether in the barrel or in the bottle, that the greatest number of polymerization and condensation reactions occur, notably modifying the composition of the wine[4] and its quality attributes.

Aroma compounds play an important role in the quality of wine because those compounds produce an effect on sensory senses. The aroma of the wine consists of 600 to 800 aroma compounds. The total content of aroma compounds in wine accounts to approximately 0.8g/L to 1.2 g/L[5]. To understand the chemical compounds in wine that showed sensory characteristics, it is necessary to obtain some information regarding both volatile composition and sensory properties[6]. GC-MS is an important analysis technique for minor volatile components to the aroma of the wine [7]

Mead is a heath care product produced in large quantities in all advanced countries, but yet it is difficult to find it as a commercial product in India. Although many advances in the developments of mead have been made over the last few years, particularly in terms of enhancing the aroma and antioxidant properties of wine, there is still scope for future development. The objective of the present work was to evaluate the antioxidant activity and characterization of mead to understand the antioxidant and flavour compounds of mead.

MATERIALS AND METHODS

Honey

In the present study, rock bee honey was obtained from a local bee keeper at north-east region of Dindugal district, Tamil Nadu. The mead samples were obtained from department of Agricultural Microbiology, TNAU, Coimbatore, Tamil Nadu.

Total Phenolic content

Phenolic compounds in honey were estimated by a spectrophotometric determination with a modified Folin-Ciocalteu method[8]. TPC of each wine sample was determined using the Folin-Ciocalteu colorimetric method[9].

Total Flavonoid content

The total flavonoid content of the honey and mead was determined using a modified colorimetric method[10].

In-vitro Antioxidant activities of honey and mead

Ferric reducing antioxidant power

FRAP was performed according to a modified method described by[11]. FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. FRAP assay was performed as detailed below using a Varian Spectrophotometer. About 3 mL of freshly prepared FRAP reagent (redox indicator) was mixed with 100 µL of distilled water and served as blank. In another cuvette, same amount of FRAP reagent was taken and added with 200 µL properly diluted honey (0.1g/mL) was mixed with 1.5 mL of FRAP reagent. In case of mead sample100 µL was taken. Then, the reaction mixture was incubated at 37° C for 4 min. Absorbance readings at 593 nm were taken from one min till completion of reaction i.e., up to 8 min rather than the early reports of 4 min. FRAP reagent was pre-warmed at 37°C and always freshly prepared by mixing 10 volume of 300 mM/L acetate buffer, pH 3.6 with 1 volume of 10 mmoL 2,4,6-tris (1-pyridyl) -5-triazine (TPTZ) solution in 40 mM/L HCl with 1 volume of 20 mM $FeCl_3.6H_2O$. A calibration curve was prepared, using an aqueous solution of ferrous sulphate $FeSO_4.7H_2O$ (100, 200, 400, 600 and 1000 µM/L). FRAP values were expressed as micromoles of ferrous equivalent [µM Fe (II)] per 100gram of honey.

DPPH free radical-scavenging activity

This method is based on the reduction of the free radical DPPH. For honey sample, the determination was based on the method proposed by[12]. Properly diluted honey solutions (0.2 g/mL) 1 mL were mixed with 2.7 mL of methanolic solution containing DPPH radicals (0.024 mg/mL). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at

517 nm. The Radical-Scavenging Activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = [(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution.

The free radical scavenging activities of mead samples was assayed using a stable DPPH, following a standard method[13]. For mead sample, the reaction takes place when 1mL of DPPH (0.1mM solution of DPPH in methanol) was mixed with 3mL of the mead sample at room temperature. After a reaction time of 60 min, absorbance values at 517 nm were measured.

GC-MS analysis

In order to identify the volatile compounds, the mead was analysed directly without any previous treatment. Chromatographic analyses were performed using thermo trace GC ultra equipped with DB 35 mass spectrometer. The analytes were separated using a capillary standard non-polar column (30 m x 0.25 mm i.d., 0.25µm film thickness). The temperature of the injector and detector was set to 250º C. The oven temperature was held at 50° C for 5 min, then programmed to run from 50° C to 260° C at 10º C / min , and then held at 260º C for 30 min. Helium was used as the carrier gas at 125kPa with a flow of 1Ml/ min. Injection of 1µL was made in the splitless mode.

Statistical analysis

The data generated from the experiments were statistically analysed as per the procedure suggested by Gomez and Gomez[14].

RESULTS AND DISCUSSION

Total polyphenol and flavonoid content

TPC of the honey and mead sample was investigated using the modified Folin-Ciocalteu method. Phenolics are present in all plants, they are also found in honey, which received considerable attention because of their potential antioxidant activity. Their content depends on the geographical and botanical origin of honey; also, darker types have been found to have a higher content than the light coloured honey types. In the present investigation total phenolic and flavonoid contents were analysed in honey and mead upto one year (Figure 1). In general TPC was increased during the ageing process. The average content of TP (68.20 mg GAE/100g honey) obtained from our honey is similar with the TPC of Tualang honey[15]. Perez[16] reported that wine constitutes a dynamic system in continuous evolution, in which numerous reactions involving polymerization and condensation take place between its phenolic compounds during the ageing process.

Flavonoids have a unique position among the natural substances used by man for therapeutic purposes. The pharmacological importance of phenolic compounds include their use as components in anti-inflammatory, antihepatotoxic, antitumor, leucodermic, antipsoriasis, antibacterial and antiviral preparations. As in total phenolic content, similar increase pattern was determined in total flavonoid content. This was expected because flavonoids are one of the compounds that fall into the group of phenolics or polyphenols. The increase of total flavonoid content (11.60 CEQ/ 100 of honey; 11.85 CEQ/100mL in 6 month aged mead) was not as high as that of total phenolic content. The phenol and flavonoid contents increased upto 6th month after that slight decrease was noticed.

FRAP and DPPH free radical scavenging activity

FRAP and DPPH antioxidant activity were analysed in honey and mead upto one year (Fig 2). In this assay, the antioxidant activity was determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron[11]. The reductive capacity of the mead evaluated in FRAP assay showed higher indices in mead aged for 6 months. Sanza[17] observed that the greater transfer of ellagitannins from the wood to the wine, during the early stages of ageing. These compounds are responsible for the oxidation mechanism in wine and it is possible that they increase its reductive potential.

DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants[18]. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. DPPH free radical scavenging activity was tend to slightly decrease after 3rd month of ageing. Since the antioxidant

mechanism is mediated by electron transfer. Perez[16] also observed that the DPPH activity reduced during ageing process. From these results, it is clear that the studies on effect of ageing on the antioxidant capacity of the wines are not an easy task and it is mainly based on the mechanism involved in the methods that are practically used.

GC-MS

The GC-MS analysis of mead showed the presence of 26 compounds belonging to seven group of volatile compounds and one sugar compound (Table 1; Fig 3). In this study, we have quantified 2 C6 compounds, 3 alcohols, 4 sugars, 3 ethyl esters, 7 volatile fatty acids, one acetate, one aldehyde, 4 silicon and one other compound. The volatile compounds are derived from honey, yeast strain fermentation and vinification process. Many of these volatile compounds are commonly found in all types of wine. Figure 4, shows the percent area covered for volatile compounds and sugars present in mead. The largest group of volatile compound present in mead is alcohol, accounting to 26.23%, followed by volatile fatty acids (23.18%) and sugars (21.18%). Higher alcohol and esters

Table 1. Volatile composition of mead, area covered and functions of different compounds

Compounds	Area covered (%)	Functions
C_6 compounds[2]		
4H-Pyran-4-one	2.36	Aroma compound
3-Deoxy-d-mannoic lactone	1.49	-
Alcohols[3]		
Phenyl ethyl alcohol	15.92	Pleasant floral odour, Antimicrobial activity
Glycerine	6.57	Sweetness
Benzene ethanol	2.64	Pleasant floral odour, Antimicrobial activity
Sugars[4]		
3-o-methyl-d-glucose	16.52	Sugar
D-Fructose	2.91	Sugar
D-Glucose	1.00	Sugar
D-Allose Ethyl esters[3]	0.75	Sugar
Ethyl-a-d-glucopyranoside	2.00	-
Ethyl hydrogen succinate	0.76	Aroma compounds
4-Ethyl benzoic acid	0.71	Antiseptic (treatment of fungal skin diseases), used in the production of phenol
Volatile fatty acids[7]		
Sorbic acid	12.94	-
9-octadecanoic acid	2.68	Fatty acids, aroma compound
2-Butenoic acid	1.35	Fragrant
Ester-1,3,5(10)-trien-17a-ol	1.10	Fatty acid, aroma compound
9-Hexadecanoic acid	0.77	-
Dodecanoic acid	0.74	Fatty acids, aroma compound
2-Pentenoic acid	0.62	Volatile esters
Acetates[1]		
Diethoxy methyl acetate	3.40	Fragrant
Aldehyde[1]		
2-furancarboxy aldehyde	9.84	Whose content determined by aging (increased during aging), vinous sensory
Silicon[4]		
Cyclohexasiloxane	4.92	The best anti aging product
Cyclooctasiloxane	1.06	Aroma compound
Cyclopentasiloxane	1.06	Used in cosmetics, beauty products, conditioner
Cycloheptasiloxane	0.98	Flavouring compound
Other[1]		
Desulphosingrin	0.83	Aroma of buckwheat

produced during alcoholic fermentation, play an important role in the flavour of wine. The most abundant compound in mead was phenyl ethyl alcohol (15.92%) which was in accordance with the earlier literatures[7]. These compounds play an important role in flavour and also have antimicrobial activity. Ethyl esters are one of the most important groups of aroma compounds in wine, and their concentrations depend on several factors, such as yeast strain, fermentation temperature, aeration and sugar content. These compounds contribute positively to the overall wine quality, and most of them have a mature flavour and fruity aroma that contribute to the "fruity" and "floral" sensory properties of wines[19]. Soufleros[20] reported that the short chain fatty acids are minor compounds in wine and the odour may be as strong as that of acetic acid; therefore, these acids can contribute significantly to the aroma of wines and spirits. 2-furancarboxy aldehyde (9.84%) was present in mead and is the main determinant of the age of wine, whose content increased during the ageing process. In mead 3.4% acetates were found in small amount which were in accordance with Perestrelo[19] the acetates are formed by the reaction of acetyl CoA with higher alcohol, which are formed through the degradation of amino acids or carbohydrates. The best anti ageing compound present in mead was Cyclohexasiloxane (4.92%).

Scientific studies related to antioxidant activity and flavour compound of mead is very rare. In this study, we observed the increased total phenolic, flavonoids content and antioxidant activity during ageing. The study will bring out a new value added honey product in India which will be both an alcoholic beverage of Indian origin and an excellent health tonic cum medicine. This opens a wide opportunity in market and will benefit both wild honey collectors and bee keepers.

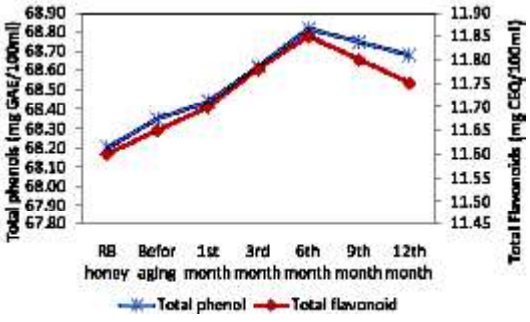

Fig. 1. Total phenol and flavonoid content of rock bee honey and mead

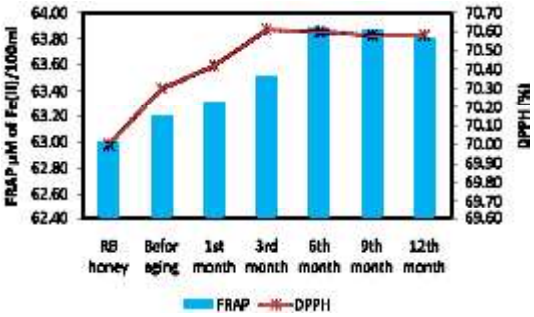

Fig. 2. Evolution of FRAP and DPPH antioxidant activity of rock bee honey and mead

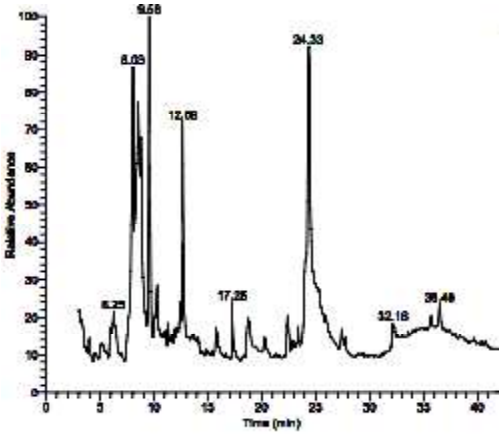

Fig. 3. GC-MS chromatogram of volatile compounds in mead

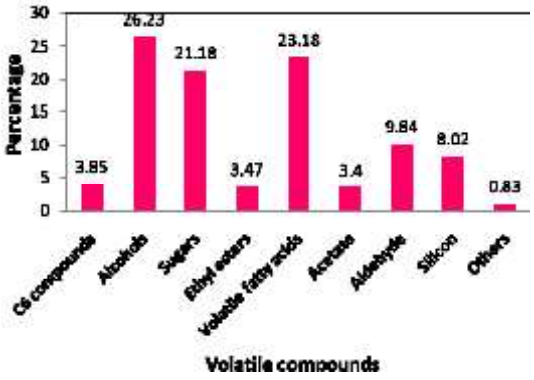

Fig. 4. Percent area covered for volatile compound families and sugars persent in mead

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