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Standardization of antioxidant activities of honey and propolis of *Tetragonula iridipennis*, a stingless bee species from Nagaland, North-East India

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Abstract
Antioxidant activities of honey and propolis of stingless bee, *Tetragonula iridipennis* from Nagaland were standardized. As honey components varies with climates and vegetations, samples were collected twice in a year during winter and summer. Propolis was taken from honey cells (cerumen) once in a year, as their hives were perennial structure. Antioxidant activities were estimated in terms of total phenolic contents, flavonoid contents, free radical (DPPH) scavenging activities and ferric reducing antioxidant power (FRAP). Highest phenolic contents (128.97±0.01mgGAE/Kg) were recorded in winter honey followed by summer honey (117.32±0.03mgGAE/Kg). In contrast, flavonoid contents were found to be maximum in propolis (75.83±0.002mgQE/Kg). DPPH radical scavenging activities were measured in terms of IC50 values, where winter honey and propolis exhibited significantly high antiradical activity when compared with standard ascorbic acid. Highest FRAP values were recorded in winter honey, followed by propolis and summer honey respectively. In fact, the results signified honey and propolis of *T. iridipennis* as reliable sources of antioxidants with adequate market potentialities.

Keywords: Tetragonula iridipennis, antioxidant, phenolics, flavonoid, DPPH, FRAP

Introduction

Stingless bees are the largest group of eusocial bees with more than 600 described species. These are spread throughout the tropical and subtropical areas (Lavinas *et al*., 2019; Thomas and Kharnaior, 2021) [22, 34]. Honey and propolis are two precious products of stingless bees with high medicinal values and have significant role in various health issues (Choudhari *et al*., 2012) [8]. Bees collected nectar and pollen as row materials from the flowering plants and converted to honey and stored in the hive. Propolis is glue-like substance produced from plant resins and bee wax, mixing with mandibular secretions and utilized for construction of beehive (Omar *et al*., 2019) [27]. However, contents of these products highly diverged depending on their entomological origin and ecological trait (Do Vale *et al*. 2018; May-Canche *et al*. 2022) [12, 25].

Tetragonula iridipennis is a common stingless bee species found in India and SriLanka (Schwarz, 1939) [30]. The species is widely distributed in Nagaland, a state of north-east India. The species can be easily identified by their robust sized penis valves, tapering towards the apex (Vijayakumar & Jayaraaj, 2014) [35]. Local communities of Nagaland do practice this bee-keeping from remote past by their traditional knowledge and products were used in traditional medicine (Das, *et al*., 2019) [11]. But no any scientific analytical studies were done so far, due to which Meliponiculture has not been picked up as a component of rural livelihood in spite of huge potentialities. In the present study, attempt has been made to standardize the antioxidant activities of honey and propolis of *T. iridipennis* from Nagaland, India. It is noteworthy to mention that the bioactive components present on stingless bee products are strongly influenced by their geographical location, climatic conditions, botanical resources and harvesting season (Silvano, *et al*., 2014; Biluca, *et al*., 2016; Rao, *et al*., 2016; Mduda, *et al*., 2023) [32, 5, 28, 26].

Antioxidant activities of a bioactive compound are based upon its capability to inhibit oxidative stresses and protect the body from various cellular ailments (Comert & Gokman, 2018) [10]. These compounds can donate an electron to neutralize the free radicals and reduce their ability to damage essential biomolecules of the cells (Fang, *et al*., 2002; Cianciosi, *et al*., 2018; Esa, *et al*., 2022) [14, 9, 13]. Antioxidant activities are mainly attributed to its Phenolic profile (Biluca da Silva *et al* 2020; Mahmood *et al* 2020) [6, 23]. According to structural conformities, phenolic compounds can be classified into two groups, - Phenolic acids and Flavonoids (having an aromatic ring and one or more hydroxyl groups). Therefore, for evaluation of antioxidant activities of honey and Propolis of *T. iridipennis*, it has been found to be essential to estimate their phenolic contents, flavonoid contents along with assessment of free radical scavenging activities and ferric reducing antioxidant power.

Materials and Methods

Samples of honey and propolis were collected freshly from three different locations of Nagaland (latitude of 25º40'N 94º07'E and longitude of 25.67ºN 94.12ºE), where summer temperature ranging from average 26±2°C and that of winter 14±2°C with annual rainfall average 2899mm. As components of stingless bee honey varies depending upon the prevailing vegetations (Santos, *et al*., 2021; Mduda, *et al*., 2023) [29], honey samples were collected twice a year during extreme of winter and summer, referred as SBHw and SBHs respectively. Propolis sample (SBP) were collected from cerumen (honey cells) only once in a year as the hive found to be perennially occurring structure. Experiments were performed in triplicate for each sample and mean values with standard deviations (SD) were taken into consideration.

Estimation of total phenolic Content

The Total Phenolic Contents were determined spectrophotometrically, following the Folin-Ciocalteu method with required modifications. For both the honey samples (SBHw and SBHs), 1ml of the aqueous extract of honey solutions (1gm/20ml) were mixed with 0.2N Folin-Ciocalteu reagent and wait for five minutes. Sodium carbonate (75%) was added to the mixture before incubation at room temperature for two hours in the dark. For methanolic extracts of propolis sample (SBP), a mixture was prepared by adding 1.5ml of Folin-Ciocalteu reagent and 1 ml of 7% Na2CO3 resulting in a total volume of 3ml with sample. The mixture was incubated for 30 minutes at 45°C. For the samples, the absorbances were measured using a UV-Vis spectrophotometer at 765nm. Gallic acid (0.2-12µg/ml) has been taken as a standard to produce a calibration curve and results were expressed as mg GAE/kg±SD.

Estimation of total flavonoid Content

The total flavonoid contents of SBHw, SBHs and SBP samples were determined spectrophotometrically using the procedure adopted by Zinkovic, *et al*., 2019. A mixture of 0.15ml of 5% sodium nitrate, 0.15ml of 10% aluminium chloride, and 1ml of 5% sodium hydroxide were added to 0.5ml of aqueous extracts of honey as well as 1mg/ml of methanolic extract of propolis. The absorbances were measured at 510nm. The total flavonoid content was calculated using a quercetin (QE) standard curve prepared from different concentrations. The results were expressed as mgQE/kg±SD.

The formula applied for calculation of both the phenolic and flavonoid contents-

$$C = \frac{cV}{m}$$

Where, C=Total phenolic/flavonoid contents expressed in mg GAE/QE per kg

c=Conc. of standards obtained from calibration curve in mg/ml

V=Volume of extract in ml

m=Mass of extract in kg

Antioxidant property assay

Antioxidant property of SBHw, SBHs and SBP were determined in terms of free radical scavenging activities of DPPH radical and Ferric Reducing Antioxidant Power (FRAP) assay.

The ability of the extracts of honey and propolis samples to scavenge DPPH free radicals were assessed by the suggested method of Stankovic, *et al*., (2014) [33], after required modifications. A stock solution of DPPH was prepared by dissolving 4mg of DPPH in 100ml of methanol, resulting in a concentration of 40µg/ml. The working solution was made by diluting the DPPH solution with methanol until an absorbance of about 0.98 ± 0.02 at 517 nm was achieved using a spectrophotometer. From this diluted solution, 1.5ml was added to various concentrations of the samples prepared from a 1mg/ml stock. These mixtures were incubated for five minutes in the dark at room temperature before measuring the absorbance. The absorbance (Abs) values were recorded, and the percentage scavenging of the DPPH radical was calculated using the formula-

DPPH inhibition (%) = Abs Control- Abs Sample/Abs Control x100

IC-50 (Half maximal inhibitory concentration) values were estimated from % scavenging activity calculated against concentrations of the samples.

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP of SBHw, SBHs and SBP was evaluated by the method recommended by Benzie and Devaki (2018) [4]. FRAP reagent was freshly prepared by mixing 300mM (millimolar) acetate buffer (pH 3.6), 10mM 2,4,6-tris(1-pyridyl)-1,3,5-triazine (TPTZ) solution in 40mM Hcl, and 20mM FeCl3 in water at a volume ratio of 10:1:1. This mixture was then heated in a water bath at 37°C for 15 minutes before use. For the test, 100µl of the sample extract (1mg/ml) was dissolved in 1ml of methanol and treated with 3ml of the working FRAP reagent. The mixture was incubated at 37°C for 4-5 minutes. After incubation, the increase in absorbance was recorded at 593 nm. A standard curve for FeSO4 was prepared using varying concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8µM. The FRAP values were obtained by comparing the absorption change in the test mixture with the standard curve of ferrous sulfate. The resultant FRAP value was expressed as mM of Fe equivalent as per ml of the sample.

Results and Discussions

Total phenolic (TPC) and flavonoid contents (TFC) estimated for winter honey (SBHw), summer honey (SBHs) and propolis (SBP) sample of *T. iridipennis* were enlisted in the Table 1 and Figure 1

Table 1: Total phenolic (TPC) and flavonoid (TFC) contents of winter honey, summer honey and propolis of *T. iridipennis* expressed as TPC (mgGAE/Kg) ±SD and TFC (mgQE/Kg) ±SD respectively.

Test	SBHw* Mean±SD	SBHs** Mean±SD	SBP*** Mean±SD	Comments
TPC	128.97±0.01	117.31±0.03	6.8±0.003	SBHw>SBHs>SBP
TFC	37.79±0.005	21.13±0.003	75.83±0.002	SBP>SBHw>SBHs

*Winter honey, **Summer honey, ***Propolis, SD=Standard deviation

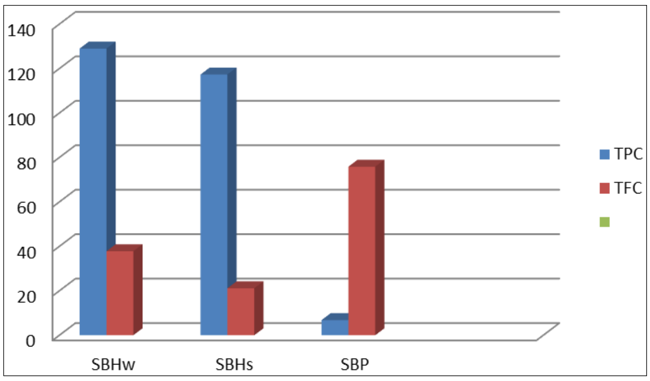

Fig. 1: Total phenolic contents (TPC) and flavonoid contents (TFC) of winter honey (SBHw), summer honey (SBHs) and propolis (SBP) of *T. iridipennis.*

where high amount of TPC were recorded in the winter and summer honey (128.97±0.01 and 117.31±0.03 mgGAE/Kg respectively) and comparatively least amount in propolis (6.8±0.003 mgGAE/Kg). In the nature, phenolic compounds are found in the form of polyphenols. Foraging bees collected these from the nectar and pollen of the flowers, converted to honey by their enzymatic actions. High phenolic contents attributed to high antioxidant properties (Thomas and Kharnaior, 2021) [34]. Esa *et al* (2022) [13] claimed that high amount of phenolic compound in honey enhanced cell proliferation, repair cell structure and hence accelerated wound healing process by its antioxidant capacities. Contrast to this, TFC found to be considerably higher (75.83±0.002 mgQE/Kg) in propolis than that of honey samples (37.79±0.005mgQE/Kg and 21.13±0.003mgQE/Kg for SBHw and SBHs respectively). Propolis is a mixture of plant resins collected from cracked barks, leaves, stems, fruits of the plants and vegetables that are naturally rich sources of flavonoids (Araújo, *et al*., 2016) [2]. Working on propolis of Brazilian stingless bee, *Melipona orbignyi*, Campos, *et al*. (2014) [7] claimed that flavonoids were the compound responsible for various biological activities of propolis including microbiostatic action and prevention of oxidative stress. Available literatures suggested that there was a strong positive correlation between antioxidant activity and presence of phenolic and flavonoid compounds (Lavinas, *et al*., 2018; Kasote, *et al*., 2019; Asem, *et al*., 2020; Zeb, 2020; Kruk, *et al*., 2022) [22, 17, 3, 36, 21]. From the results, it can be assumed that both the products of *T. iridipennis* were highly effective antioxidants. Due to their quantitative variations, exhibited differential actions at cellular level *ie*. honey acts as a promoter of cell viability, cell proliferation and thereby speed up wound recovery (Rao, *et al*., 2016; Ismail, *et al*., 2021) [28, 16], while propolis shows expertise as antimicrobial (Marcucci, 1995) [24] and anti-tumor agent (Campos, *et al*., 2014), protect the cells from infections. The results also highlighted the variations of these contents in winter and summer honey (SBHw and SBHs). In this context, Rao, *et al*., (2016) [28] and Shamsudin *et al*., (2019) [31] opined that, honey constituents greatly influenced by harvesting season, floral sources and weather.

Radical scavenging activities of SBHw, SBHs and SBP of *T. iridipennis* were assessed and IC50 values were calculated for DPPH inhibitions and placed in the table 2 and figure 2.

Table 2: DPPH free radical scavenging assays (%) of winter honey, summer honey and propolis of *T. iridipennis* at 3 different concentrations, taking ascorbic acid as standard.

Test	Concentra-tio (µg/ml)	SBHw * Mean±SD	SBHs ** Mean±SD	SBP *** Mean±SD	ASC **** Mean±SD
1	10	7.92±0.010	6.45±0.01	3.6±0.001	5.84±0.05
2	150	69.13±0.004	23.27±0.002	49.27±0.003	93.69±0.001
3	350	76.95±0.0002	45.43±0.0005	80.95±0.005	98.01±0.003

*Winter honey, **Summer honey, ***Propolis, ****Ascorbic acid.

Values of highest concentrations were depicted and IC50 (Half-maximal Inhibitory Concentration) values were calculated (Fig.2).

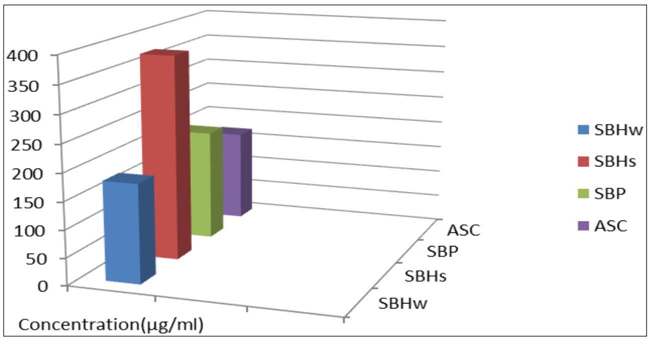

Fig2: Effective concentrations of winter honey (SBHw), summer honey (SBHs), propolis (SBP) and standard ascorbic acid (ASC) for scavenging 50% of DPPH (IC50 values).

IC50 denotes sample concentration at which 50% of DPPH radicals were able to scavenge. As IC50 is inversely proportionate to antioxidant activity (Kedare and Singh, 2011) [18], highest radical scavenging activity was recorded in winter honey (IC50:189.52±0.13µg/ml), followed by propolis (IC50:203.4±0.14 µg/ml). However, lowest (IC50: 374.1±0.07 µG/ml) activity was observed in summer honey in comparison to that of standard ascorbic acid (169.328±0.01µg/ml). Working on honey of wild stingless bees from West Amazonian Ecuador, Guerrini, *et al*., (2009) [15] claimed that these have higher DPPH inhibition capacity than that of *Apis mellifera*. Abd Jalil, *et al*. (2017) [1] opined that non-enzymatic antioxidant like phenolic compounds not only scavenge free radicals, but can inhibit their formation within the cells. It is well known that chemical components of honey fluctuate with season and vegetations (Kishore, *et al*., 2011) [20]. This may be the probable reasons for noticeable differences between winter and summer honeys in respect of their antiradical activities.

FRAP (Ferric Reducing Antioxidant Power) assays were done to measure the ability of the tested samples to reduce a ferric salt. Data presented in the table 3 and figure 3

Table 3: Ferric reducing antioxidant power (FRAP) assay of winter honey (SBHw), summer honey (SBHs) and propolis (SBP) samples expressed as Mm of FeSO4 equivalent/ml of sample.

Sl. No.	Sample ID	Absorbance at 593 nm	MmFeSO4 equivalent/ml±SD*	Comment
1	SBHw	0.276	0.804±0.004	SBHw>SBP>SBHs
2	SBHs	0.153	0.455±0.01	
3	SBP	0.178	0.52±0.004	

*Standard deviation

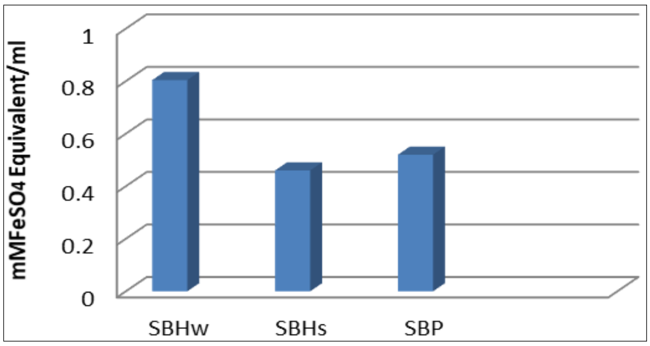

Fig3: FRAP values for winter honey (SBHw), summer honey (SBHs) and propolis (SBP).

exhibited highest Ferric reducing antioxidant power in winter honey (0.804±0.004mMFeSO4 equivalent/ml). Reducing power of propolis was as high as 0.52±0.004mMFeSO4 equivalent/ml, followed by summer honey (0.455±0.01mMFeSO4 equivalent/ml). Khalil, *et al*., (2011) [19] opined that FRAP values were influenced by presence of phenolic and flavonoid compounds.

Present study unveiled the antioxidant activities of Honey and propolis of stingless bee, *T. iridipennis* from Nagaland and open the door for commercialization of these valuable products for therapeutic as well as neutraceutical use. Variations of antioxidant activities in different samples were related to concentrations of polyphenols (Ismail, *et al*.,2021) [16] that the foraging bees collected from nature, which in turn related to vegetations and harvesting season.

Plate 1: *T. iridipennis in nature*

Plate 2: *T. iridipennis* (preserved)

Plate 3: Honey pot (ceramen**)**

Plate 4: Brood cells

Plate 5: Hive entrance

Plate 6: Arrangement of cells inside the

Conclusion

Although, products of stingless bees were utilized as traditional medicine for centuries, extensive studies were pursued on its antioxidant properties only in the last few decades due to their huge potentialities in the pharmaceutical industries. *T. iridipennis* is an Indian stingless bee species widely cultivated by local people of Nagaland with their traditional knowledge without any scientific documentation. In the present study, antioxidant activities of honey and propolis were assessed by focusing on phenolic and flavonoid contents, radical scavenging activities and ferric reducing capacities. Studies have highlighted significant antioxidant activities of both the products in variable quantities, which in turn, influenced by climatic factors, botanical sources and harvesting season.

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