



Received on 18 August 2018; received in revised form, 28 December 2018; accepted, 17 January 2019; published 01 December 2019

## HPLC ANALYSIS OF FLAVONOIDS FROM PROPOLIS OF DIFFERENT HONEYBEE SPECIES IN SELECTED LOCATIONS OF BANGALORE

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### Keywords:

Bee keeping,  
Propolis, Flavonoids, HPLC,  
Bioactive compounds, Ecosystem

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**ABSTRACT:** Propolis is a natural resinous substance with a wide range of biological properties, synthesized by bees from bud exudates of some plants. Bees make use of propolis as a building material and to defend the hive. The chemical composition of propolis varies depending on vegetation and species having plethora of therapeutic activities. To detect compounds present in propolis an efficient, precise, and reliable HPLC method was used for quantification which offers advantages in terms of speed and accuracy. This method allowed eliciting the chromatographic fingerprint by comparing the retention time and molecular weight of commercially available standards that are known to be present in propolis such as p-coumaric acid, ferulic acid, epicatechin, gallic acid, caffeic acid and quercetin in different samples. The bioactive compounds present in varying amount has anti-cancer, anti-proliferative, antioxidant, antimicrobial, anti-inflammatory, cardioprotective and hepato protective properties. The main aim of the study is to analyze the flavonoids in propolis taken from areas surrounded by moderate vegetation of different honeybee species. The interesting results provide a basis to promote bee keeping in cities having limited vegetation as it helps not only in ecosystem sustenance, but also a source of propolis to benefit mankind.

**INTRODUCTION:** Honeybees provide a wide range of benefits to humans through its marketable primary products like honey, bee-pollen, beebread and royal jelly mainly as food supplements, propolis and bee venom as treatment in different diseases due to their high biological activity. Propolis is a resinous product collected by honey bees from various plant sources like leaf buds and barks mixed with secreted beeswax.

The final product propolis is used as a sealant to seal cracks in the walls of the hive, preserve moisture and primarily to prevent the decomposition of animals and insects killed by bees after intrusion into the hive. The honeycomb is believed to be the most sterile natural environment and propolis functions as the natural immune system of the beehive. The fact that this structure with full of food and organisms can be kept free of disease and infection is quite remarkable <sup>1,2</sup>.

The variations in the chemical composition and consequently in the biological activity of propolis are associated with its type and geographic origin. Although propolis is a complex mixture, its biological activities are reported due to the

<p><b>QUICK RESPONSE CODE</b></p> 	<p><b>DOI:</b> 10.13040/IJPSR.0975-8232.10(12).5423-29</p> <hr/> <p>This article can be accessed online on <a href="http://www.ijpsr.com">www.ijpsr.com</a></p> <hr/> <p>DOI link: <a href="http://dx.doi.org/10.13040/IJPSR.0975-8232.10(12).5423-29">http://dx.doi.org/10.13040/IJPSR.0975-8232.10(12).5423-29</a></p>
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presence of the flavonoids, phenolic acids and ethers mainly obtained from plant-derived substances<sup>3</sup>. Propolis has a long history of being used in traditional medicine dating back at least to 300 BC and has been reported to have a broad spectrum of biological activities including anti-inflammatory, antibiotic, antifungal and anticancer activities<sup>4, 5, 6, 7</sup>, antimicrobial, antibacterial, antiviral<sup>8, 9, 10</sup>, antioxidant<sup>11</sup>, antitumor and immune-modulator<sup>12, 13, 14</sup>. Indian stingless bees had capability of pronouncing apoptosis against different cancer cell lines at various concentrations.

The main aim of the study is to analyze propolis of different honeybee species for the presence of flavonoids from apiaries surrounded by moderate vegetation. The relationship between the flavonoids and propolis biological effects reveal the importance of quantifying these constituents in propolis. HPLC method has been reported to quantify compounds in complex biological matrices.

#### MATERIALS AND METHODS:

**Collection of Sample:** Propolis samples belonging to *Apis mellifera*, *Apis cerana* and *Tetragonula iridipennis* were collected directly from the apiary centers situated in different locations of Bangalore surrounded by moderate vegetation (L-1 Dodabalapur, L-2 Kanakapura and L-3 Devanahalli). The samples were cleaned, packed in plastic bags, weighed and stored at  $-10^{\circ}\text{C}$  until the moment of the analysis.

**Extraction of Samples:** Samples were extracted with 70% ethanol in the ratio of 1:20 (w/v). The solution was kept in shaker incubator for 10 days in the dark and filtered. The filter cake was mixed with 70% ethanol and it was kept overnight in the refrigerator for removal of wax and used to determine flavonoid content.

Further the filtrate was lyophilized and the extracts were diluted with 5 ml of methanol (HPLC grade) in 10 ml volumetric flasks, subjected to sonication for 10 minutes and filled to volume with HPLC grade water. The samples were filtered through a  $0.45\ \mu\text{m}$  filter before analysis<sup>15</sup> and used for HPLC analysis. The samples of *Apis mellifera*, *Apis cerana* and *Tetragonula iridipennis* were denoted as SK1, SK2 and SK3 respectively.

**Determination of Total Flavonoid (TFC):** TFC content was determined by Aluminium Chloride Colorimetric method<sup>16</sup>. The assay was applied with some modifications and quercetin was used as standard. 1 ml of extract was mixed with 4 ml distilled water and 0.3 ml of 5%  $\text{NaNO}_2$  and kept for five minutes, after that 0.3 ml of 10%  $\text{AlCl}_3$  was added to the mixture and after 1 minute 2 ml of 1M NaOH was added and the total volume of mixture was brought to 10 ml with the addition of distilled water. The absorbance was monitored at 510 nm and the experiments were run in triplicates and calculated using the formula,

$$\text{mg QE / g dry extract mass} = \frac{[(\text{Absorbance of sample} - \text{Absorbance of blank}) - \text{Absorbance of blank}] / (\text{slope} \times \text{Dilution Factor})}$$

**HPLC: Chromatographic Apparatus and Analytical Conditions:** Samples were run on a Shimadzu LC- Prominence 20AT liquid chromatography with analytical column (C18 column  $250\ \text{mm} \times 4.6\ \text{mm}$ ,  $5\ \mu\text{m}$  particle size). A binary mobile phase of acetonitrile (60%) and (40%) of water was employed at a flow rate of 0.6 ml/min. Linear gradient was performed starting with 30% of acetonitrile to 100% for 30 min. All propolis extracts solution was prepared in acetonitrile at  $1000\ \mu\text{g ml}^{-1}$  and  $5\ \mu\text{l}$  and were injected through a loop system. Samples and standards were filtered through membranes with porosity of  $0.22\ \mu\text{m}$  and injected in triplicate for all the three samples.

Three different HPLC systems used in this study and were referred to as System A, B, and C for different standards. HPLC analysis was conducted using the following reference standards p-coumaric acid, ferulic acid, epicatechin, gallic acid, caffeic acid and quercetin. The identification was confirmed by direct comparison of the retention times and spectra acquired in the same analytical conditions.

#### RESULTS:

**HPLC System A: Standard Caffeic Acid, p-Coumaric Acid and Ferulic Acid:** HPLC conditions were optimized for caffeic acid, p-coumaric acid and ferulic acid analysis using  $250\ \text{mm} \times 4.6\ \text{mm}$  C18 column wherein Caffeic acid, p-coumaric acid and Ferulic acid eluted at 6.717,

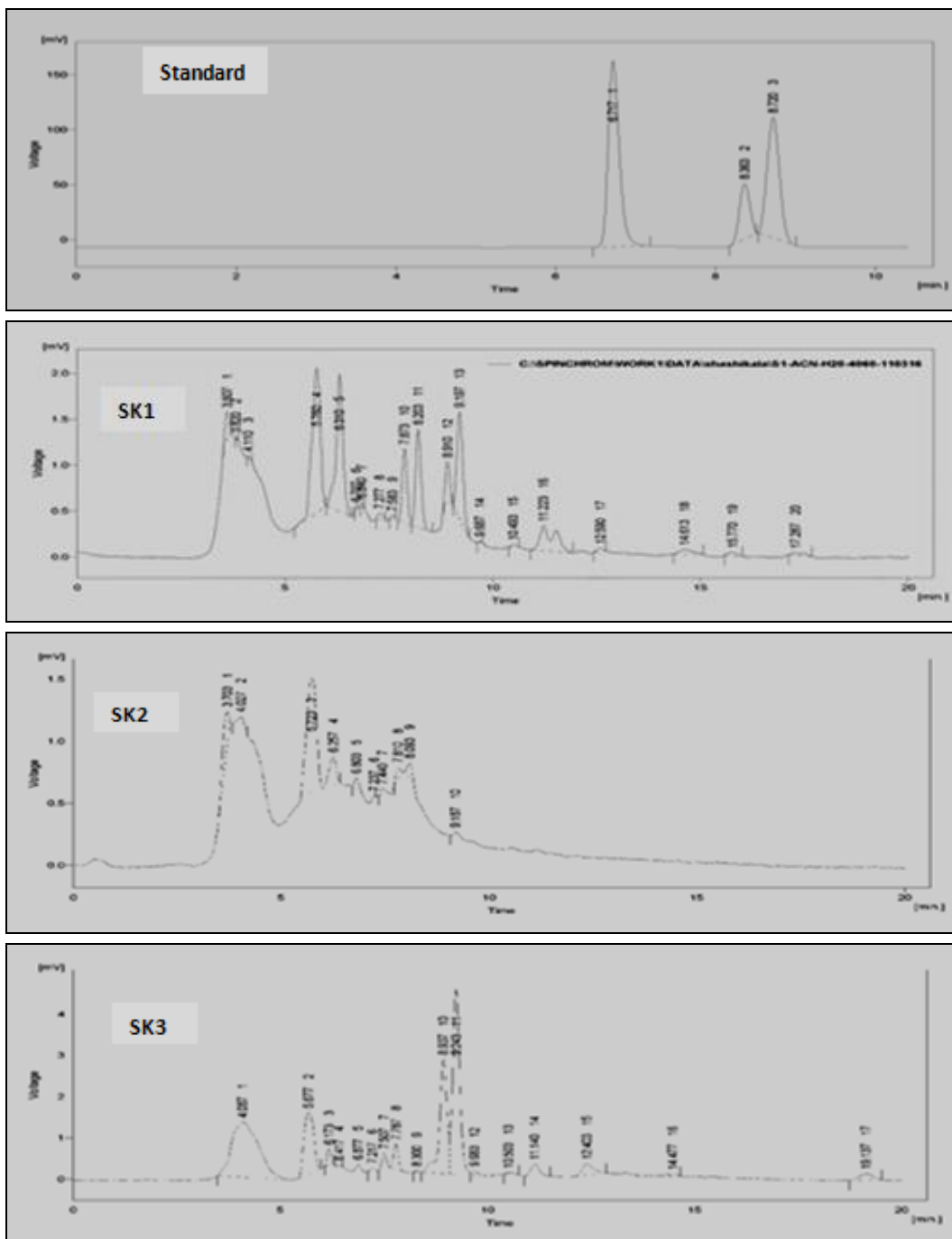
8.363 and 8.72 min respectively. In SK1 propolis total caffeic acid and p-coumaric acid content was 0.0042 and 0.22245 µg/g respectively, in SK2 total caffeic acid content observed was 0.0004 µg/g and p-coumaric acid was not found in the extract.

In SK3 total caffeic acid and p-coumaric acid content was 0.012 and 0.0052 µg/g respectively. Ferulic acid was not found in all the three samples. HPLC chromatograms of standards and test extracts are shown in **Fig. 1**.

**TABLE 1: FLAVONOID CONTENT IN PROPOLIS OF APIS MELLIFERA, APIS CERANA AND TETRAGONULA IRRIDEPENNIS AT DIFFERENT LOCATION**

Species	Location	Flavonoid (mg QE /g)
<i>Apis mellifera</i> SK1	L1	3.2 ± 0.16
	L2	3.01 ± 0.15
	L3	3.3 ± 0.16
<i>Apis cerana</i> SK2	L1	2.01 ± 0.10
	L2	1.89 ± 0.09
	L3	1.99 ± 0.09
<i>Tetragonula iridipennis</i> SK3	L1	2.8 ± 0.14
	L2	2.65 ± 0.13
	L3	2.42 ± 0.121

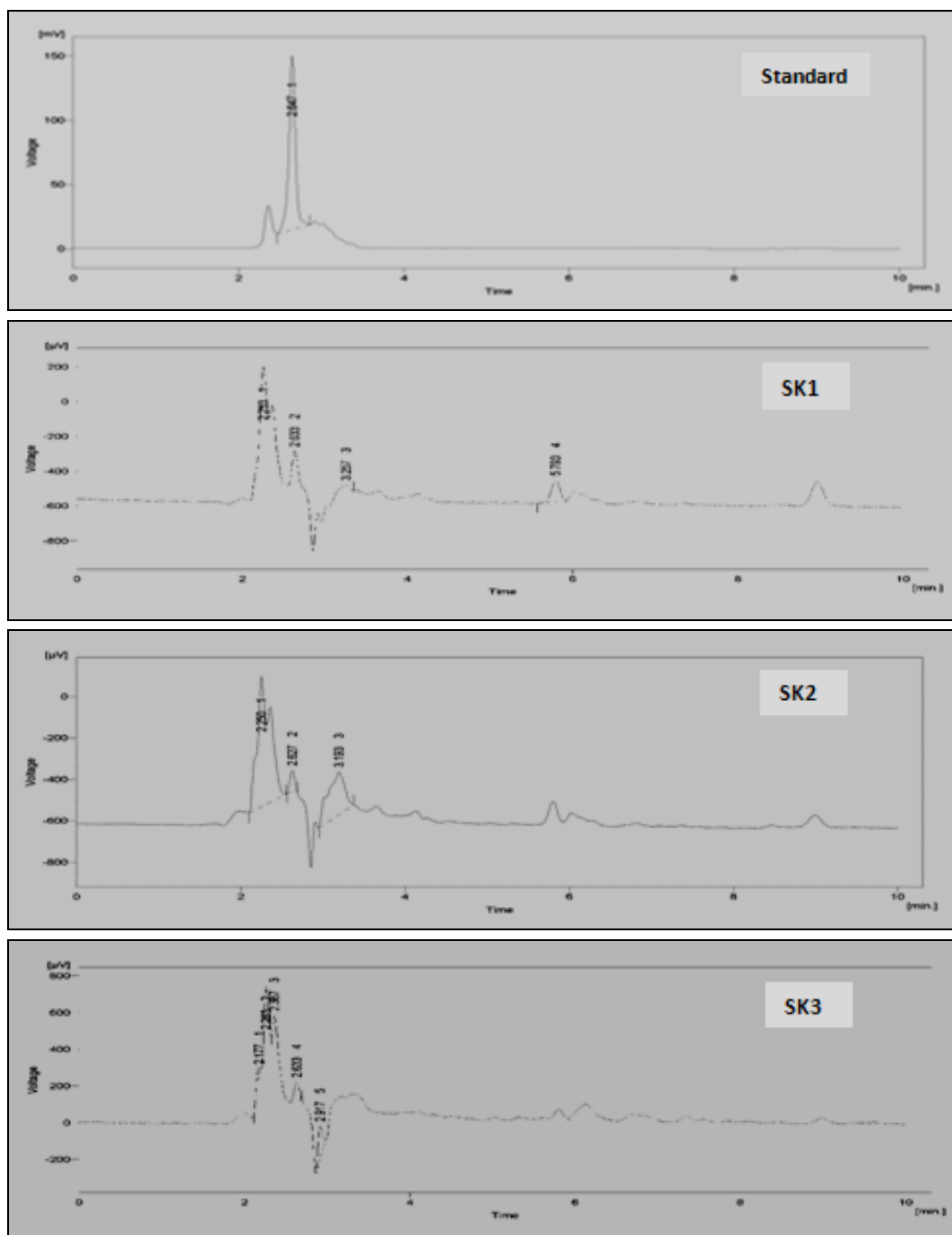
(L-1 Dodabalapur, L-2 Kanakapura and L-3 Devanahalli)



**FIG 1: HPLC CHROMATOGRAM OF STANDARD, CAFFEIC ACID, P COURAMIC ACID, FERRULIC ACID AND SAMPLE SK1, SK2, SK3**

**HPLC System B: Standard Epicatechin:** HPLC conditions were optimized for epicatechin analysis using 250 mm × 4.6 mm C18 column wherein epicatechin eluted at 2.647 min. In SK1 the total

epicatechin content was found to be is 0.0049 μg/g in SK2 it was 0.0025 μg/g and in SK3 it was observed as 0.0028 μg/g. HPLC chromatograms of standards and test extracts are shown in **Fig. 2**.



**FIG 2: HPLC CHROMATOGRAM OF STANDARD EPICATECHIN AND SAMPLE SK1, SK2, SK3**

**HPLC System C: Standard Quercetin and Gallic Acid:** HPLC conditions were optimized for quercetin and gallic acid analysis using 250 mm × 4.6 mm C18 column wherein quercetin and gallic acid eluted at 8.547 and 4 min respectively. In SK1 the total quercetin and gallic acid content was

found to be 0.680 and 0.060 μg/g respectively. In SK2 the total quercetin content was observed as 0.013 and gallic acid was not found. In SK3 quercetin was 0.097 μg/g and gallic acid 0.060 μg/g. HPLC spectrum of standards and test extracts are shown in **Fig. 3**.

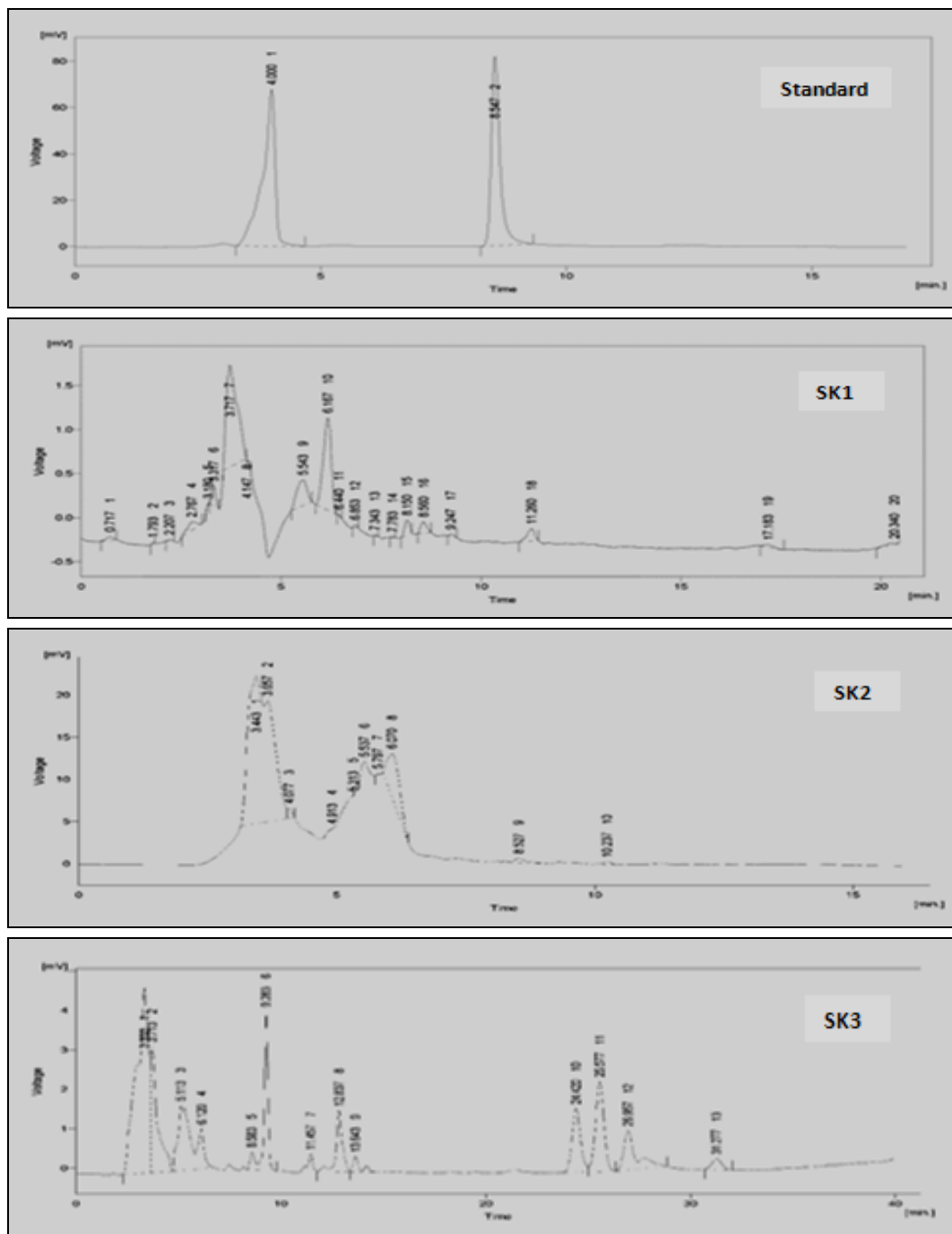


FIG. 3: HPLC CHROMATOGRAM OF STANDARD QUERCETIN, GALLIC ACID AND SAMPLE SK1, SK2, SK3

**DISCUSSION:** Flavonoids which are considered to be the important biochemical component in propolis, is derived from plant resins responsible for a variety of biological activity were found to range between 1.89 mg - 3.01 mg/g in areas with moderate vegetation.

In *Apis mellifera* the flavonoid was found to be 3.2mg/g, 3.01mg and 3.3mg/g in L1, L2 and L3 respectively. In *Apis cerana* the flavonoid content in L1, L2 and L3 the values range between 1.89 - 2.01 mg/g. In *Tetragonula iridipennis* the flavonoid

content in locations from L1 to L3 was found to be, 2.8 mg/g, 2.65 mg/g and 2.42 mg/g respectively.

The HPLC analysis of the propolis revealed presence of flavonoids. All samples showed the presence of bioactive compounds irrespective of the location. The compounds present were caffeic acid, p-coumaric acid, epicatechin, quercetin and gallic acid. HPLC results revealed that SK1 and SK3 contains caffeic acid, p-couramicacid, quercetin, gallic acid and epicatetin. However SK2 showed the presence of only caffeic acid, quercetin

and epicatechin. Ferulic acid was absent in all the samples.

Caffeic acid is well known for its anti-cancer, anti-proliferative, sedative, decongestant and hepato-protective actions<sup>17, 18</sup> and p-coumaric acid was well documented for its antioxidant behaviour<sup>19</sup>. Quercetin is known to have multifaceted biological and therapeutic effects including antioxidative, anticancer, antimicrobial, anti-inflammatory, cardioprotective and hepatoprotective activities<sup>20</sup>. Gallic acid is a phenolic compounds obtained from plants possess many potential therapeutic properties including anti-cancer, anti-microbial, anti-inflammatory and induction of cell death in cancer cells<sup>21</sup>. Epicatechin has the ability to lower blood glucose levels<sup>22</sup>. All samples were found to be biologically active which may be either anti-cancerous, antioxidant or anti-inflammatory in nature.

Our results matched with Brazilian green propolis which was rich in phenylpropanoids having cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid and their derivatives<sup>23</sup>.

**CONCLUSION:** The presence of flavonoids in propolis is the key compounds responsible for biological activity irrespective of variation in chemical composition, vegetation and geographical location. The interesting results provide a basis to promote beekeeping in metro cities to sustain environment for biodiversity in the era of technology and to explore propolis for welfare of mankind.

**FUTURE SCOPE:** Further investigations are required for quality control and standardization for the purpose of industrial application to promote drug designing.

**ACKNOWLEDGEMENT:** The authors are thankful to Maharani Lakshmi Ammanni College for Women, Bangalore University and Skanda Life Sciences, Bangalore for technical assistance.

**CONFLICTS OF INTEREST:** The authors declare no conflicts of interest.

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**How to cite this article:**

Shashikala A, Harini BP and Reddy MS: HPLC analysis of flavonoids from propolis of different honeybee species in selected locations of Bangalore. *Int J Pharm Sci & Res* 2019; 10(12): 5423-29. doi: 10.13040/IJPSR.0975-8232.10(12).5423-29.

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