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Simultaneous Identification of Phenolic Compound from the Honey of Stingless Bee by Using HPLC

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Simultaneous Identification of Phenolic Compound from the Honey of Stingless Bee by Using HPLC

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Abstract
Apitherapy is a branch of alternative medicine that uses honey bee products including honey, propolis, pollen, bee venom, and royal jelly. Stingless bee honey reportedly has many medicinal and therapeutic uses and excellent potency. This study aimed to identify the phenolic compounds using a fast and specific reversed-phase HPLC method in the extract of stingless bee honey. A magnetic stirrer was used for the pretreatment process of a sample with methanol at a temperature of 50°C for 40 min, followed by separation on a column size of 250 mm × 4.6 mm (5 µm) hypersil gold-C18 (Thermo Electron Corporation) with water–methanol–acetonitrile (45:40:15 v/v/v) containing acetic acid 1.0% as a mobile phase. A 254-nm wavelength was used to detect the extract. The standard retention times of the gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were found to be at 2.610, 2.875, 3.150, 5.789, and 8.983 min, respectively. The existence of gallic acid, rutin, ascorbic acid, kaempferol, and quercetin in the stingless bee honey extract was found to match according to the standard retention time. In the stingless bee honey, the retention times of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were found to be at 2.613, 2.866, 3.157, 5.790, and 8.966 min, respectively. In the stingless bee honey, the amounts of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were 1.426%, 2.533%, 16.922%, 1.851%, and 13.773%, respectively. According to the results, it is concluded that stingless bee honey is rich in phenolic acids and flavonoid compounds that have strong antioxidant properties.

Keywords: Stingless bee honey; Phenolic compounds; Gallic acid; Quercetin; Rutin; Ascorbic acid; Kaempferol; HPLC.

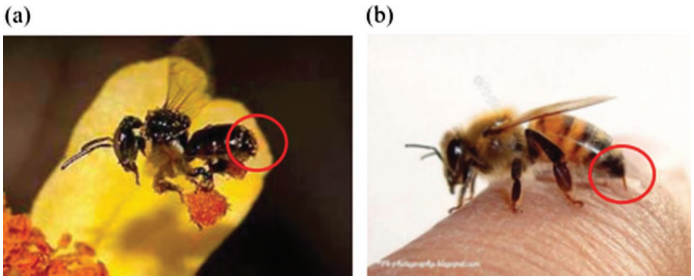
1. INTRODUCTION

The study of the antioxidant potential of phenolic extracts derived from natural products (plants and honey) is one of the hot topics in the scientific community; however, in vitro studies are the most common [1-3]. Phenolic compounds have shown promising antioxidant properties [4]. Similar to plants, honey is also used in the treatment of different diseases. The class of alternative drug treatment in which the products of the honey bee, such as pollen, honey, propolis, bee venom, and royal jelly, are used is known as apitherapy [5].

Honey is a natural sweetener and energy-rich medicinal product, and it is produced by diverse honey bee species containing stingless and stinged bees. Since ancient times, honey has had the most important place in herbal medicine, with various serviceable uses, which affect its physical and chemical composition [6]. Thousands of years ago, it has been used as a food and as a medicine. A large number of different cultures have widely used honey as a medicine for many disorders; moreover, honey was used as an agonist whereby it was mixed with some medicines to enhance their effects [7].

As compared to honeybees, stingless bees generally are smaller in size and range from 2 to 14 mm. However, the largest stingless bee is comparable to a honeybee. The morphology of stingless bees is rather different from honey bees. First, stingless bees do not possess functional sting (Figure 1) as the name implied, and hence, they do not sting. However, they have strong mandibular musculature that allows them to attack intruders by inflicting a mild bite. Some of the stingless bees may emit caustic liquid from their mouth and cause intense skin irritation. Second, the wax glands of the stingless bees are located dorsally; whereas, honeybees have a ventral position of the wax glands. Third, stingless bees are known to have reduced wing venation that may in part relate to dwarfism [8]. The honey of stingless bee, like many other types of honey, is a sugary liquid that has a superb taste and odor. Honey manufactured by a stingless bee is commonly known as "Kelulut" in Malaysia. Furthermore, Kelulut honey has higher contents of polyphenols and flavonoids in contrast to the honey produced by the *Apis* spp. [9]. A previous study demonstrated that Kelulut honey is made of mainly water, carbohydrates, amino acids,

Figure 1: Comparison of sting between (a) stingless bee and (b) honey bee.



minerals, and vitamins. The flowers of many medicinal shrubs and herbs are very small. Normal honey bees are much bigger and are not cut out to take away the nectar and pollen from these flowers. Bigger bees are mostly circulated in fruit orchards, where all sorts of chemicals and hormones are used for production. However, the small size of stingless bees have ability to take nectar from these medicinal flowers, and this increases the medicinal value of their honey; this honey is used for the preparation of different medicines. A study reported that stingless bee honey possesses distinctive and divergent phenolic and flavonoid composites that have been shown to have a vital function with regard to its antiinflammatory, antibacterial, and anti-oxidant activities of the Borneo (Sabah and Sarawak) stingless bees honey [6]. According to Rao and Colleagues, the honey of the stingless bee is also used in the treatment of glaucoma and cataracts [10]. The honey of the stingless bee also has wound healing [11] and anticancer [12] properties.

Nowadays, people rely on natural product treatments. Moreover, the honey of the stingless bee is becoming gradually more popular because of its potential part in contributing to the health of human beings. Stingless bee honey is mainly a rich source of antioxidant compounds, which act as natural antioxidants.

2. METHOD(S)

2.1. Chemicals

There were some chemicals used for analysis in this research, such as kaempferol, rutin, quercetin, gallic acid, ascorbic acid, acetonitrile, methanol, and glacial acetic acid that were purchased from Sigma-Aldrich, USA. Honey was purchased from the Kinabalu adventure supply.

2.2. Extraction

In this study, 10 g of stingless bee honey was added into a flask with 150 ml of methanol. The flask was placed onto a magnetic stirrer, specifically at 50°C for 40 min. After that, the solution was filtered with the help of filter paper. Furthermore, the filtered solution was concentrated using a rotary evaporator; then, the concentrated sample was collected and kept in a refrigerator at 4°C until the time of the experiment.

2.3. Isolation of Flavonoids from Stingless Bee Honey Using HPLC

The samples were analyzed by using the Aglient 1200 HPLC system, and a UV detector with a column size of 250 mm × 4.6 mm (5 µm) hypersil gold-C18 (Thermo Electron Corporation) was set at 254 nm. The combination of water, methanol, and acetonitrile (45/40/15, v/v/v) had been used as a mobile phase [13]. The injection volume was 10 µl, and the flow rate was set at 0.7 ml/min.

2.3.1. Preparation of the Standard and Sample Solutions

Methanol was used as a solvent for the preparation of the standard and sample solution; 10 mg of standard kaempferol, rutin, gallic acid, ascorbic acid, and quercetin were dissolved in 25 ml of the solvent. For the sample preparation, 10 mg of the honey extract was dissolved in 10 ml of solvent, the same as a standard preparation [14].

The amounts of standard kaempferol, rutin, gallic acid, ascorbic acid, and quercetin in the stingless bee honey were calculated using the following assay formula.

$$\%\text{Assay} = \frac{\textit{Sample Area}}{\textit{Standard Area}} \times \frac{\textit{Standard Weight}}{\textit{Standard Dilution}} \times \frac{\textit{Sample Dilution}}{\textit{Sample Weight}} \times \textit{Standard Purity}$$

3. RESULTS AND DISCUSSION

The identification of antioxidant compounds, were carried out because they are an important class of phytochemicals. Therefore, stingless bee honey was analyzed for the possible presence of these flavonoids. For this purpose, gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were selected. The standard retention times of the gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were found to be 2.610, 2.875, 3.150, 5.789, and 8.983 min. The presence of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol in the stingless bee honey extract was found to match according to the standard retention time. Figures 2 and 3 illustrate the comparison between the standard and stingless bee honey extract samples, and Tables 1 and 2 show the retention time, area, height, and % area of the standards and samples.

In the stingless bee honey sample, the retention times of the gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were found to be at 2.613, 2.866, 3.157, 5.790, and 8.966 min, respectively. The peak at 2.103 shows an unknown compound, and a different testing is required for the identification of this unknown compound. The amounts of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were calculated using the assay formula. In stingless bee honey, the amounts of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were 1.426%, 2.533%, 16.922%, 1.8519%, and 13.773%, respectively.

In 2017, Oliveira and Co. also identified phenolic compounds from the stingless bee honey. They identified the kaempferol, gallic acid, and quercetin from the honey of stingless bee. Flavonoids are valuable antioxidants and have relevant

Figure 2: HPLC chromatogram of the standards. (a) Gallic acid, (b) rutin, (c) ascorbic acid, (d) quercetin, and (e) kaempferol.

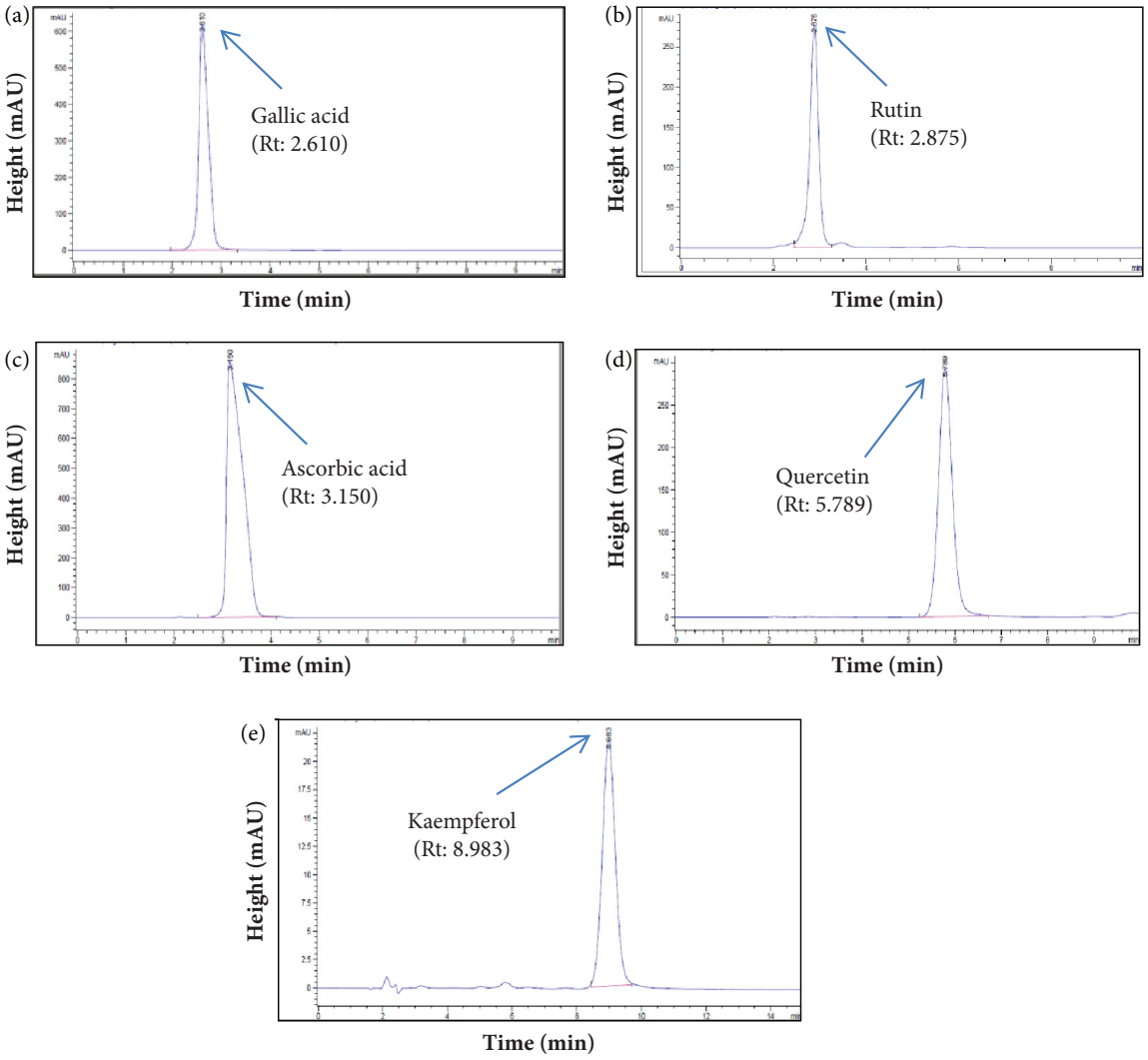


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Figure 3: HPLC chromatogram of the stingless bee honey.

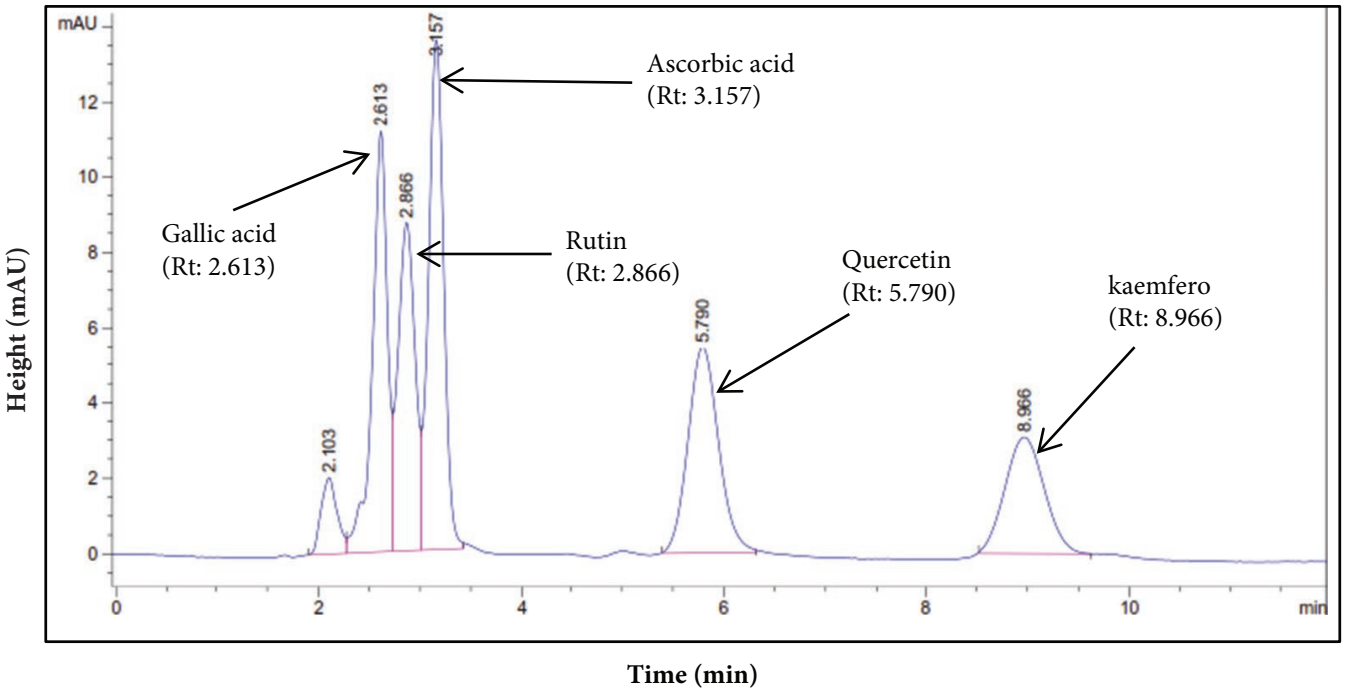

Table 1: Retention time, area, height, and % area of the standards (gallic acid, rutin, ascorbic acid, quercetin, and kaempferol).

Standards	Retention time (min)	Area (mV s)	Height (mV)	Area (%)
Gallic acid	2.610	8416.88379	619.74945	100.00
Rutin	2.875	3771.66162	277.45462	100.00
Ascorbic acid	3.150	19602.3	855.53058	100.00
Quercetin	5.789	6126.98340	292.44537	100.00
Kaempferol	8.983	611.26239	21.69806	100.00

Table 2: Retention time, area, height, and % area of standards (gallic acid, rutin, quercetin, and kaempferol) in the extract of stingless bee honey.

Standards	Retention time (min)	Area (mV s)	Height (mV)	Area (%)
Gallic acid	2.613	115.65893	11.16102	20.0463
Rutin	2.866	101.64617	8.71647	17.6176
Ascorbic acid	3.157	140.66875	13.55285	24.3811
Quercetin	5.790	113.80716	5.44963	19.7254
Kaempferol	8.966	82.70238	3.07554	14.3342

anticarcinogenic activity. The identification of flavonoids in honey suggests that they might have chemopreventive action [15]. Another work was conducted by Silva and Co., in 2013, on the extract of stingless bee honey. They identified the gallic acid from the stingless bee honey using an HPLC method [16]. Sousa and Co., in 2016, worked on the stingless bee honey and assessed the polyphenolic profile. They identified the quercetin, kaempferol, and rutin [17]. Another research was conducted on the honey of stingless bee and to identify the quercetin from the stingless bee honey [18]. Truchado and Co., in 2011, also identified the different flavonoids using the HPLC method. Along with other flavonoids, they also identified quercetin and kaempferol in the honey of stingless bees [19]. Additionally, Yazan and Co. conducted a comparative study on the stingless bee (Trigona)

honey and tualang honey extract and identified the ascorbic acid by using reversed-phase HPLC. According to their result, stingless bee honey has exhibited a higher amount of ascorbic acid compared to tualang honey [20].

CONCLUSION

In this research, the antioxidant compounds were extracted; further, these compounds were identified in the extracts of stingless bee honey by using the HPLC. The presence of these compounds in the stingless bee honey by matching the retention time of the standard with the samples' retention times and the % yields were calculated using the assay form. The results of the stingless bee honey showed the presence of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol. In the stingless bee honey, the amounts of gallic acid, rutin, ascorbic acid, quercetin, and kaempferol were 1.426%, 2.533%, 16.922%, 1.851%, and 13.773%, respectively. The results obtained from the current physicochemical analysis of stingless bee honey were similar to previous studies. However, the presence of phenolic compounds (gallic acid, rutin, ascorbic acid, quercetin, and kaempferol) in the honey of stingless bee may be useful for nutraceuticals and pharmaceutical applications. Further research is needed to unlock and understand the potential health benefits of the stingless bee honey.

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Authors' Contributions
This work was carried out with the collaboration of all authors. All authors contributed equally to this work.

Conflict of Interest
None.

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