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DETECTION OF PHENOLIC COMPOUNDS IN STINGLESS BEE (*Tetragonula biroi Friese*) PROPOLIS AND FIVE TREE SOURCES USING TANDEM LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT – Tandem liquid chromatography-mass spectrometry (LC-MS) was used to identify possible phenolic compounds in propolis of stingless bees, *Tetragonula biroi* Friese and exudates from five tree species that are utilized by T. biroi as propolis source namely avocado (*Persea americana* Mill), jackfruit (*Artocarpus heterophyllus* Lam), mango (*Mangifera indica* L.), pili (*Canarium ovatum* Engl), and rambutan (*Nephelium lappaceum* L.).

The results strongly suggest the presence of two phenolic compounds namely Artepillin C and pinobanksin-3-O-hexanoate in the propolis and in all the plant extracts.

Identification of phenolic compounds present in propolis is necessary in determining its plant source, biological properties, and therapeutic activity.

Keywords: Tetragonula biroi, propolis, flavonoids, phenolic compounds, LC-MS

INTRODUCTION

Stingless bees, Tetragonula biroi Freise, are excellent producers of propolis; a mixture of beeswax and resins from plant buds, leaves, and exudates; which they use to build their hives and protect them from harsh conditions. The composition of propolis is very complex and variable which depends on its botanical and geographical origin (Marcucci 1995). The chemical variability in different types of propolis is due to the different plant source of the bees, where they get these chemical compounds for propolis production (Bankova 2005). The Philippines is a good source for propolis due to its very diverse flora.

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At least 200 compounds were already identified in different propolis samples which include fatty and phenolic acids and esters, substituted phenolic esters, flavonoids such as flavanones, flavones, and flavonols, terpenes, β-steroids, aromatic aldehydes and alcohols, sesquiterpenes, naphthalene and stilbene derivatives (Walker and Crane 1987; Greenaway *et al.* 1991; Bankova *et al.* 1995; Marcucci 1995), caffeic, ferulic and cumaric acids (Prytzyk *et al.* 2003; Bankova *et al.* 2000).

In recent years, propolis gained so much attention due to its beneficial biological and pharmacological activities. Results of studies indicated that these effects could be ascribed to polyphenols, an important group of secondary plant compounds. The composition of different kinds of propolis has been published but those of T. biroi, both locally and internationally, are limited. Propolis is gaining popularity in the world market as source of bioactive compounds. However, the lack of phytochemical profiling limit the progress of utilizing propolis in the pharmaceutical industry.

This study identified the possible phenolic compounds present in the T. biroi propolis and various plants utilized by stingless bees as propolis source, namely avocado (*Persea americana* Mill), jackfruit (*Artocarpus heterophyllus* Lam), mango (*Mangifera indica* L.), pili (*Canarium ovatum* Engl), and rambutan (*Nephelium lappaceum* L.). The tandem liquid chromatography - mass spectrometry (LC-MS) was used in identifying the compounds..

MATERIALS AND METHODS

Sample Collection

Propolis samples were collected from the colonies of *T. biroi* at the University of the Philippines Los Baños (UPLB) Bee Program apiary located at the Institute of Biological Sciences, UPLB. Propolis was scraped from the coconut shell chambers of stingless bees using a hive tool. Resinous exudates were collected from the phloem layer, by cutting the bark of the trees (plant sources) in the vicinity of the colonies until the said layer was reached.. The samples were air-dried and stored at 4 °C until their processing.

Extraction of Phenolic Compounds from Propolis

Extracts from propolis samples were obtained using the method of Bankova *et al.* (1992). Ten (10) grams of propolis were cut into approximately 0.5 x 0.5 cm pieces and extracted with 100.0 mL of boiling methanol for two hours under reflux. The mixture was filtered twice using Whatman #41 filter paper. About 20.0 mL of water was added to the filtered extract and the aqueous layer was extracted three times with diethyl ether. The crude extract was dried over sodium sulfate and the solvent was evaporated to dryness.

Extraction of Phenolic Compounds from Resinous Exudates

Eight to 10 grams of tree buds were extracted with 20 mL acetone thrice. The resulting solution was evaporated to dryness until a brown residue appeared, which was then dissolved in 10 mL of methanol and diluted with 5 mL of water. The diluted sample was extracted with 15 mL petroleum ether thrice and then with 10 mL diethyl ether. The ether extracts were combined and dried using sodium sulfate. The resulting mixture was filtered, and then heated until only 10 mL of the solution was left.

Sample Preparation for LC-MS Analysis

The extracts (40 μL each) were filtered through a 0.22 μm filter and then diluted 100-fold with methanol. Ten microliters of the resulting solution was injected in the HPLC system.

LC-MS Analysis

The chromatographic system used was a Shimadzu Liquid Chromatograph Mass Spectrometer-Ion-Trap-Time-of-Flight (LCMS-IT-TOF) instrument. An Imtakt Unison UK-C18 symmetry column (75 x 2 mm) was used for the separation at a flow rate of 1.2 mL/min. The column was maintained at 30 °C and the flow rate split 5:1 before electrospray ionization (ESI) source. The separation was performed by means of a linear gradient elution (eluent A, 2% acetonitrile with 0.1 % formic acid; eluent B, 95% acetonitrile with 0.1% formic acid). The gradient was as follows: 20% B for 3 min, 20 – 30% B in 2 min, 30 – 40% B in 15 min, 40 – 60 % B in 10 min, 60 -90% B in 10 min and 90% B for 5 min.

Chromatographic data were acquired in the 200 – 450 nm range and were integrated at 254 nm. Mass spectrometer was operated in negative and positive full-scan mode in the range 100 – 1000 Da. The capillary voltage was set to 3.0 kV, the cone voltage to 20 V, the source temperature to 130 °C and the desolvating temperature to 350 °C.

The identities of most compounds were determined from the chromatographic data combined with mass spectrometry. Data were acquired by MassLynx 4.0 software (Micromass UK Limited) with Quan-Optimize option for fragmentation study.

RESULTS AND DISCUSSION

Identification of Possible Phenolic Compounds by LC/MS-IT-TOF

The LCMS-IT-TOF has been designed to full exploitation of sensitivity and selectivity. The ion transport to the TOF analyzer is optimized and the capability of the ion trap is redefined. The ion trap is used to focus ions before ejection into the TOF as well as supporting MS analysis with effective precursor ion selection capabilities (resolution > 1,000 at 1,000 m/z) (Cuyckens and Claeys 2004).

The HPLC chromatograms of the propolis and resinous exudate extracts at 254 nm are shown in Figures 1 to 6. Due to the unavailability of standards, the possible phenolic compounds were identified using the chromatographic and spectrophotometric data of the phenolic compounds from the study of Gardana *et al.* (2007). Mass Spectrophotometric (MS) analysis was performed to ascertain the molar mass of the compounds under investigation. From the molecular masses, it was possible to select possible candidates. Among these, the specific one was established on the basis of its ion products. MS was carried out in both positive and negative full scan mode. Table 1 shows the chromatographic and spectrophotometric data including the list of the possible phenolic compounds in the propolis and resinous exudates used in the study.

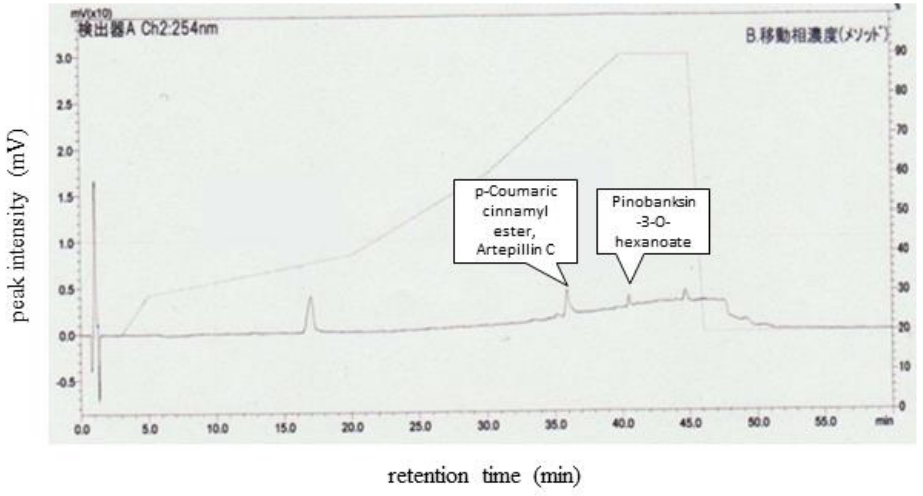

Figure 1. HPLC chromatogram of *Tetragonula biroi* propolis extract measured at 254 nm.

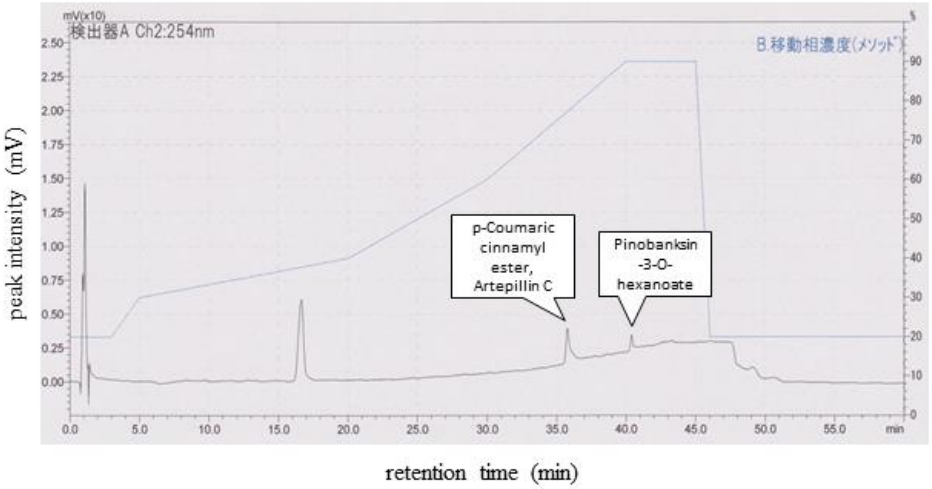

Figure 2. HPLC chromatogram of *P. americana* extract measured at 254 nm.

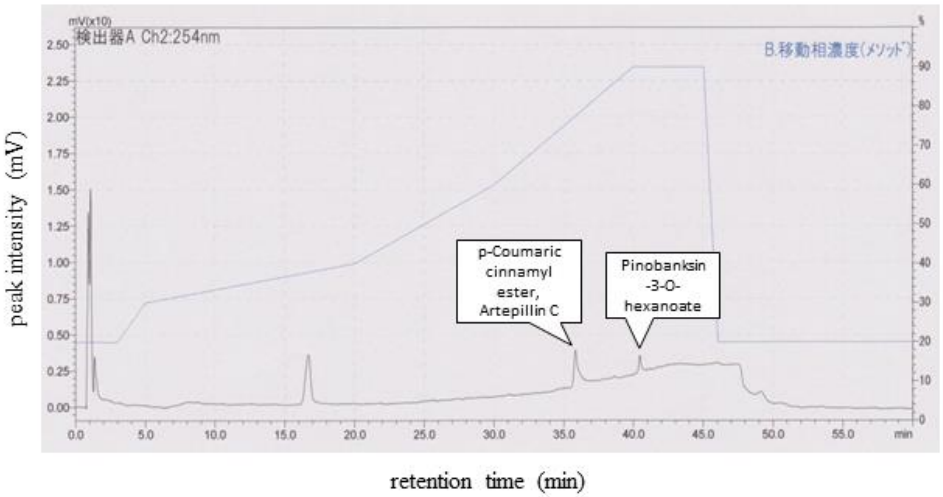

Figure 3. HPLC chromatogram of *A. heterophyllus* extract measured at 254 nm.

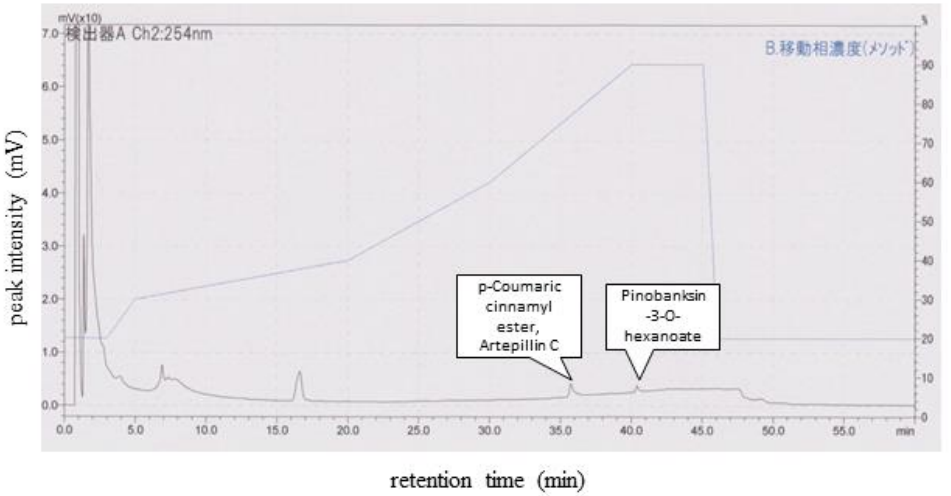

Figure 4. HPLC chromatogram of *M. indica* extract measured at 254 nm.

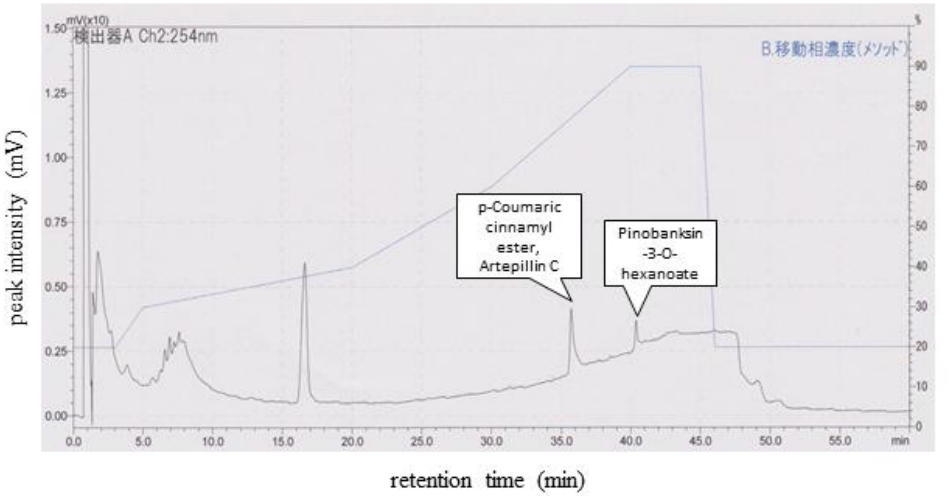

Figure 5. HPLC chromatogram of *C. ovatum* extract measured at 254 nm.

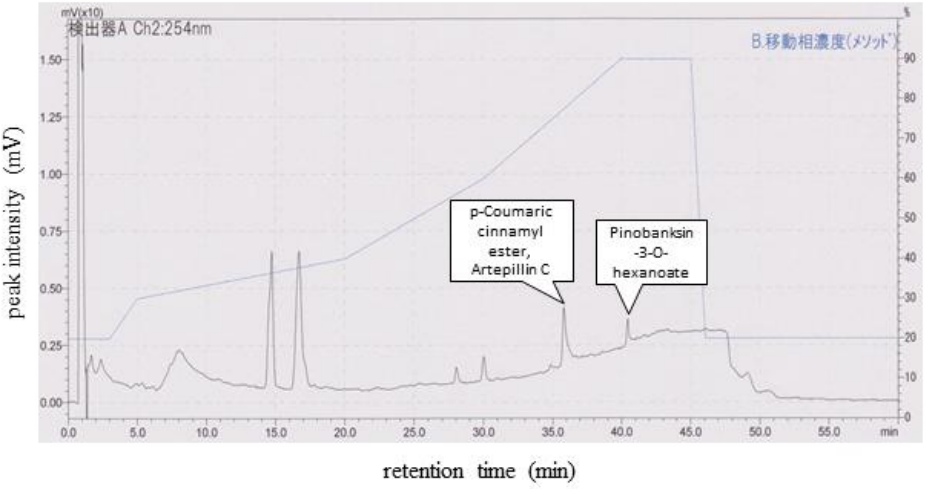

Figure 6. HPLC chromatogram of *N. lappaceum* extract measured at 254 nm.

Table 1. Summary of the possible flavonoids and phenolic compounds identified in the extracts of propolis and resinous exudates. Compounds were identified on the basis of their retention times (RT), molecular mass and mass of their ion products ($(M+H)^+$ and $(M-H)^-$).

Retention Time (min)	Possible Compound	Molecular Mass (g/mol)	$(M+H)^+$ (g/mol)	$(M-H)^-$ (g/mol)
Stingless Bee Propolis				
35.05	*p*-Coumaric cinnamyl ester	280.1099	281.1178	-
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
Persea americana extract (avocado)				
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
Artocarpus heterophyllus extract (jackfruit)				
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
Mangifera indica extract (mango)				
6.5	Quercetin	302.0427	303.0505	301.0348
35.7	Pinobanksin-3-O-(butyrate or isobutyrate)	342.1103	-	341.1025
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
39.8	3-Prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid	364.1675	365.1753	-
Canarium ovatum extract (pili)				
7.15	Pinobanksin-5-methyl-ether	286.0841	287.092	-
35.7	Pinobanksin-3-O-(butyrate or	342.1103	343.1182	-

	isobutyrate)			
36	Pinobanksin-3-O-pentenoate	354.1103	355.1182	-
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
Nephelium lappaceum extract (rambutan)				
15.95	Quercetin-7-methyl-X-methyl-ether	330.074	331.0818	-
29.2	Pinobanksin-3-O-propionate	328.0947	-	327.0869
35.7	Pinobanksin-3-O-(butyrate or isobutyrate)	342.1103	343.1182	341.1025
36	Pinobanksin-3-O-pentenoate	354.1103	355.1182	353.1025
36.3	Artepillin C	300.1725	301.1804	-
39.5	Pinobanksin-3-O-hexanoate	370.1416	371.1495	-
39.8	3-Prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid	364.1675	365.1753	-

Only two of the three phenolic compounds in the propolis extract of *T. biroi*: 4-hydroxy-2,5-diprenyl-cinnamic acid (artepillin C) and pinobanksin-3-O-hexanoate were detected in the plant exudate extracts. These compounds may have been utilized by stingless bees, because of their abundance in all tree exudates samples.

The propolis extract was also characterized by the presence of *p*-coumaric cinnamyl ester, which was not present in any of the five exudate extracts analyzed in this study. Possibly, this compound may came from other plant sources present in the vicinity of the colonies. Some of the compounds or peaks were not identified due to the lack of standards, which restricted the identification of compounds only to those present in the data of Gardana et al. (2007,) Masking and co-elution may have also occurred in the chromatographic process, which may have hindered the identification of the isolated compounds.

Other factors may have affected the results of the study. First, the accessibility of the plants from the beehive of the propolis sample – the nearer the plant is from the beehive the higher the probability that the bees will utilize it. Second, the time when the exudates were collected – there are seasons when the plant buds are coming out from the tree, which exposes more exudates available for the bees to utilize for propolis making. Third, the weather – during rainy season the exudates are washed off from the tree leaving few traces only, which can cause minimal amount of phenolic compounds that can be incorporated in the propolis. Geographical location and type of vegetation of bees are other known factors that affect the amount of phenolic compounds in propolis (Marcucci 1995).

The compounds identified in propolis and exudate extracts have potential applications in the medical and therapeutic fields. Remarkably, artepillin C is known to have various pharmacological activities. It was demonstrated to possess apoptosis-inducing (Matsuno *et al.* 1997), antioxidant (Hayashi *et al.* 1999), anti-carcinogenic (Kimoto *et al.* 2001), and anti-inflammatory properties (Paulino *et al.* 2008). It was also highly cytotoxic to a variety of malignant human and murine solid tumor cell lines *in vitro* (Konishi *et al.* 2005). Moreover, artepillin C was found to inhibit the growth of transplanted solid and mouse tumors including that of malignant melanoma, in athymic and thymic mice, respectively (Kimoto *et al.* 1998).

Even though not all the peaks were identified due to the limitations and factors stated above, the results strongly suggest that the phenolic composition of the propolis is associated to the species of plants in the vegetation area from which the bees collect the exudates. Therefore, the botanical origin of propolis can be established. With this knowledge, the beekeepers will learn the importance of the plants surrounding their apiary.

It will greatly contribute to the production of good quality propolis for use as raw materials in the medical and therapeutic fields.

CONCLUSION

The possible phenolic compounds in *T. biroi* propolis and in five tree exudate extracts utilized by stingless bees as propolis source were identified using tandem liquid chromatography - mass spectrometry (LC-MS). It was found that majority of the phenolic compounds identified in the exudate extracts were similar to the compounds found in the propolis extract. Therefore, the phenolic composition of propolis is strongly associated to the species of plants in the vegetation area from which the bees collect the exudates. This knowledge can greatly contribute to the production of good quality propolis for use as raw materials in the medical and therapeutic fields.

STATEMENT OF AUTHORSHIP

Ma. Desiree Belina-Aldemita, as lead author, prepared the draft and finalized the writing of this article for publication. She was involved in the interpretation of LC-MS results. She is a project staff of the UPLB Bee Program.

Jasmin A. Lechuga and John Marty C. Mateo are former BS Chemistry students who conducted their theses under the UPLB Bee Program. They were involved in the preparation of the propolis and plant exudate extracts that were analyzed for LC-MS.

Jose Rene L. Micor is the thesis adviser of the two students. He was also involved in the preparation of the draft and finalization of the manuscript. He is a project staff of the UPLB Bee Program.

Cleofas R. Cervancia is the coordinator of the UPLB Bee Program and co-thesis adviser of the two students. She was also involved in the preparation of the draft and finalization of the manuscript.

Amy Fradejas-Hizon is a Professor from the Kyushu University, Japan. She conducted the LC-MS analysis of propolis and plant exudate extracts. She was also involved in the interpretation of the LC-MS results.

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