ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Chemical Constituents And Antioxidant Activity Of Ethanolic Extract Of Propolis From Malaysian Stingless Bee Geniotrigona thoracica Species.

Nurul Hakimah Mohd Salim[1], Eshaifol Azam Omar[1], Wan Adnan Wan Omar[1], and Rafeezul Mohamed[2]*.

[1]Integrative Medicine Cluster,
[2]Regenerative Medicine Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia.

ABSTRACT

Propolis is a complex resinous mixture collected by bees from the buds and exudates from variety of plants. Its chemical compounds are strongly depending on the geographical area and specificity of the plant that are taken by the bees. This research is aimed to investigate the chemical constituents and the antioxidant properties in vitro of Malaysian stingless bee *Geniotrigona thoracica* (*G. thoracica*) species. The collected propolis were extracted with 80% ethanol. The ethanolic extract of propolis (EEP) were dried and investigated their chemical constituents by high performance liquid chromatography (HPLC). Total phenolic compounds (TPC) were measured by Folin-Ciocalteu, and total flavonoid contents (TFC) were also determined by the $AICl_3$ colorimetric method with a microplate reader. The antioxidant properties were measured by DPPH scavenging receptor assay. The compounds identified by HPLC were Caffeic acid, *p*-coumaric acid, Myricetin, Quercetin, Naringenin, Hesperitin, Kaempferol and Baicaline. TPC of EEP from G. thoracica was 221.569 ± 0.02 mg GAE/g whereby the TFC was 214.56 ± 0.05 mg QE/g. EC50 value with DPPH assay of *G. thoracica* was 48.3 ± 0.2 µg/ml. In conclusion, compounds of phenolic acid and flavonoid are the main constituents in EEP of *G. thoracica* which may contribute to their antioxidant properties. The TPC, TFC and antioxidant activity of EPP from *G. thoracica* were comparable and superior to propolis from some countries.

Keywords: Ethanolic extract of propolis, *Geniotrigona thoracica*, Total phenolic content, Total flavonoid contents, Antioxidant

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Propolis or known as bee glue is a sticky material that are used by stingless bees to protect their hive from invaders or any adverse weather condition. It is collected by stingless bees from the buds and exudates from variety of plants [1-4]. Generally, propolis consist of 10% essential oil, 5% pollen, 30% wax, 50% resin and other organic compounds [5]. The composition of propolis usually contain variety of chemical compounds such as polyphenols (flavonoids, phenolic acids and their esters) and their composition are depending on the plant vegetation, geographical area, bee species, season and collection time [6]. Its chemical composition influences the different biological and pharmacological activities of the propolis [7]. Propolis has been shown to have wide range of biological activities such as antibacterial [8], antifungal [9], anti-inflammatory [10], antioxidant [11], antiviral [12] and immunomodulatory activities [13].

Moreover, the presence of polyphenol in propolis can help in treatment various diseases such as cancer, aging and cardiovascular diseases due to its antioxidant activity [6]. According to Nenadis et al. flavonoid and phenolic acid are the major classes of phenolic compound that were extensively reported in relation to their antioxidant activity [14]. The antioxidant play an important role in eliminating free radicals by donating hydrogen or as singlet oxygen quencher and metal ion chelators [6,15]. Currently, wide arrays of method such as gas chromatography mass spectrometry and high-performance liquid chromatography are been used in analysing the compound in propolis extract [16-18]. Previous studies showed that the chemical composition of Brazilian propolis are differ from Europe, Asia and North American [19-21].

In Malaysia, approximately 32 species of stingless bee had been verified and documented [22]. However, limited studies have been carried out on the chemical constituents and antioxidant properties of propolis from Malaysian stingless bee. Therefore, the current study was performed to determine the phytochemical content, chemical profiles which included total phenolic content (TPC) total flavonoid content (TFC) and antioxidant properties of Malaysian stingless bee, *Geniotrigona thoracica* (*G. thoracica*) species.

MATERIALS AND METHODS

Chemicals

Ethanol, methanol and aluminium chloride were purchased from Fisher Scientific, USA. Folin-Ciocalteau reagent were purchased from Merck, Germany. Sodium carbonate and gallic acid were obtained from Biobasic Canada. Quercetin and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma Aldrich, USA.

Samples collection

The propolis of Malaysian stingless bee, *G. thoracica* was collected from Syamille Agrofarm and Resort, Kuala Kangsar, Perak. The stingless bee's species was identified by Entomology Section, Malaysian Agriculture and Research Development Institute (MARDI), Serdang, Malaysia.

Samples preparation

The propolis sample was prepared according to protocol from Ibrahim et al. with some modifications. Propolis was kept at -80°C and ground into powder formed. The ethanolic extract of propolis (EEP) were prepared by soaking propolis extract in 80% of ethanol and macerated for three days at room temperature. Then, EEP was evaporated off by using water rotavapor to obtain dry extract. The dry extract then was proceeded to freeze drying to obtain the powder form of EEP. The powder formed of EEP was kept in -20°C for further analysis.

HPLC analysis of EEP from *G. thoracica*

The standard stock solution of Caffeic acid, *p*-Coumaric acid, Quercetin, Naringenin, Hesperetin, Kaempferol, Baicaline and Myricetin were prepared by dissolving accurately weighed standard in methanol to give the concentration of 15 ppm. All standard solutions were stored in freezer at -20°C prior to analysis. There were two mobile phases that were used in the chromatography. Mobile phase A consist of HPLC-grade

methanol: acetonitrile: deionised water (40:5:55) and 0.1% formic acid meanwhile for mobile phase B; methanol: acetonitrile: deionised water (80:5:15) and 0.1% formic acid. The solvents were prepared according to their ratio and were filtered through 0.22 µm filter (Pall Gelman, Sigma Aldrich, USA) followed by 5 minutes sonication to degases the solvents prior to analysis. HPLC analysis was carried out at room temperature using High Performance Liquid Chromatography Pro-Star 401 Varian model (Agilent Technologies, USA) with Eclipse Plus C18 column (150 mm X 4.6 mm, 5 µm particle size) (Agilent, USA). The sample injection volume was 10 µl and the wavelength of the detector was operated at 300 nm.

Determination Total Phenolic content (TPC)

The TPC in EEP of *G. thoracica* was determined based on modified spectrophotometrical method [23] with Folin-Ciocalteu reagent was carried out and added into 96 well microplate. The gallic acid is used as standard in this assay because it response comparable to most other phenolics on a mass basic [24]. Briefly, 4 mg of EEP was dissolved in 1 ml methanol as a stock. Then, 10 µl of stock solution was added into 96 well plates. 50 µl of the Folin-Ciocalteu reagent which previously diluted 1:10 was then added into stock solution and mixed well. Following incubation for 5 minutes, the mixture was added with 40 µl of 7.5% of sodium carbonate (Na_2CO_3) solution (final volume V_f = 100 µl). Then, the reaction mixture was incubated for another 30 minutes at room temperature with minimal light exposure and subsequently the absorbance of the mixture was measured by the spectrophotometer at λ=760 nm. Gallic acid was used to calculate standard curve (3.125-100 µg/ml, r^2 = 0.993). The total phenolic content was expressed in milligram of gallic acid equivalent (GAE) in 1 gram of EEP.

Determination of Total Flavonoid content (TFC)

The TFC in EEP of *G. thoracica* was determined by using a modified spectrophotometrical method from Chua et al. with 2% $AlCl_3$ was utilised [25]. EEP stock solution was prepared by dissolving 4 mg EEP in 1 ml methanol. 100 µl of the 2% aluminium chloride ($AlCl_3$) were mixed well with 100 µl of EEP stock solution in 96 wells plate (final volume V_f = 200 µl). Following 10 minutes incubation at room temperature, the absorbance of the reaction mixture was measured by spectrophotometer at λ=415 nm. Quercetin was used as a standard to calculate standard curve (3.125-100 µg/ml, r^2 = 0.999). The total flavonoid content was expressed in milligram of quercetin equivalent (QE) in 1 gram of EEP.

Determination of radical scavenging activity test using DPPH assay

The antioxidant properties of EEP from *G. thoracica* was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. A few concentrations of EEP (0.0125, 0.025, 0.05, 0.2 and 0.4 mg/ml) were dissolved in 1 ml methanol. DPPH solution (200 µM) was freshly prepared by dissolving 3.94 mg of DPPH in 50 ml of methanol. A total of 200 µl of methanolic solution of DPPT was mixed well with the 50 µl of EEP solution which previously added in 96 well plate (final volume = 250 µl per well). The reaction mixture was incubated for 30 minutes in the dark condition and the absorbance of the reaction mixture was measured by spectrophotometer at λ = 517nm. A trolox (3.125-100 µg/ml) was used as a standard. The percentage of inhibition was calculated according to the formula below [25]:

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The antioxidant capacity of the sample was express as EC50, where it showed the concentration of a sample that are needed to reduce 50% of DPPH.

Statistical analysis

The chemical analysis was carried out in triplicate. Mean ± standard deviation was calculated for TPC, TFC and antioxidant. The correlation was carried using SPSS software version 19.0. Significant differences were statistically considered at the level of $P < 0.01$.

ISSN: 0975-8585

RESULTS AND DISCUSSION

Non-volatile compounds in EEP of G. thoracica

The variation of propolis's chemical composition and biological properties showed by each species of stingless bee depend on the specific local vegetation at the site of collection [6]. Our propolis samples from *G. thoracica* were obtained from 12-acre Syamille Agrofarm in Kuala Kangsar, Perak. This farm is surrounding by dipterocarp forest which consist of wide array of flora ranging from *Cocos nullifera*, *Antigonan leptopus*, *Cuphea hyssofolia*, *Averrheo bilimbi*, *Citrus microcarpa*, *Durio zibethinus L* and *Syzygium* spp. In addition, variety of flower species grow around Syamille Agrofarm may influence the composition of flavonoid and phenolic compounds found in propolis of *G. thoracica* which may have therapeutic effects on various diseases. HPLC analysis of non-volatile compounds in EEP of *G. thoracica* showed the presence of eight peaks with retention time that correlates with the retention time of the standards. The identity of these peaks was obtained by comparing their retention times with those of standards and the coefficient of variation was below 4% [26]. Peak 1 indicates the presence of Caffeic acid (t_R =2.11), peak 2 is *p*-Coumaric acid (t_R =2.84), peak 3 shows Myricetin (t_R =4.49), peak 4 indicates Quercetin (t_R =7.35), peak 5 is Naringenin (t_R =8.24), peak 6 is Hesperitin (t_R =9.51), peak 7 indicates Kaempferol (t_R =12.73) and peak 7 is Baicaline (t_R =15.03). Full HPLC chromatogram of non-volatile compounds in EEP of *G. thoracica* is shown in Figure 1.

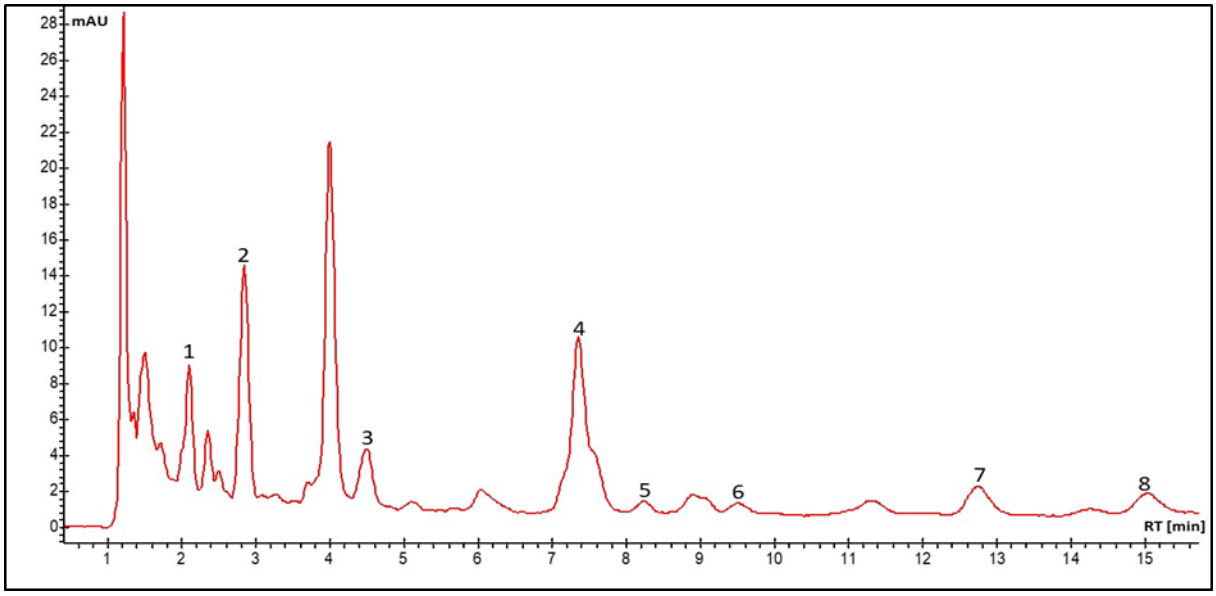

Figure 1: HPLC chromatogram of EEP from G. thoracica.

Caffeic acid and *p*-Coumaric acid are phenolic acid compound whereby Quercetin, Myricetin, Naringenin, Hesperitin, Kaempherol and Baicaline are flavonoid compound [27-29]. Flavonoid is the main group of the phenolic compound which contributed for antioxidant properties in propolis. Phenolic compound may exert antioxidant effects by responding towards oxidative stress in human body to maintain a balance between oxidant and antioxidant substance [30,31]. Thin layer chromatography analysis on the methanol extract of propolis collected from Besut, located at the east coast of Malaysia showed the presence of terpenoids, flavonoids and essential oil. However, no steroids, saponins and coumarins were detected [32]. Moreover, Bufalo et al. reported that Caffeic acid and *p*-Coumaric acid were identified in ethanolic extract of Chinese propolis [33]. Meanwhile, the chemical compounds identified in Chilean propolis samples were Caffeic acid, Kaempferol, *p*-Coumaric acid, Pinobaksin, Quercetin and Vanillin [34]. In addition, Caffeic acid, *p*-Coumaric acid and Quercetin were identified in EEP from the United States of America, Argentina, Australia, Bulgaria, South Africa and Hungary [6].

TPC, TFC and antioxidant activity in EEP of *G. thoracica*

Table 1: Chemical analysis of EEP from *G. thoracica*

TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (µg/ml) (EC50)
221.57 ± 0.02	214.56 ± 0.05	48.3 ± 0.2

*Result as given in mean and standard deviation.

The TPC and TFC compound in EEP of *G. thoracica* were determined and shown in Table 1. The TPC of EEP from *G. thoracica* was 221.569 ± 0.02 mg GAE/g, which was higher compared to methanol extract of propolis of *G. thoracica* from Besut, Terengganu (0.0291 mg GAE/g) [32]. In comparison to TPC of other propolis, TPC of EEP from our *G. thoracica* are superior to Algerian propolis (1.71 to 53.51 mg GAE/g) [35], Bolivian propolis (43.0 ± 0.3 to 176.0 ± 4.8 mg CAE/g) [36] and Brazilian propolis (31.88 ± 0.61 to 204 ± 3.80) [37]. However, TPC of EEP from *G. thoracica* are inferior to Argentina propolis (587 ± 20 to 593 ± 15 mg GAE/g) [38]. Moreover, TPC of EEP from *G. thoracica* are within the range of TPC in Chinese propolis (145.54 + 75.89 to 233.98 + 70.84 mg GAE/g) [39]. Furthermore, TFC measured in EEP of *G. thoracica* was 214.56 ± 0.05 mg QE/g (Table 1). Similarly, TFC of EEP of *G. thoracica* was higher than TFC of methanolic extract of propolis of same species from Besut, Terengganu (0.0615 mg QE/g) [32]. The TFC of EEP from *G. thoracica* also superior than Algerian propolis (1.25 to 49.46 mg QE/g) [35], Chinese propolis (124.92 + 79.74 to 126.23 + 78.46 mg QE/g) [39], Argentina propolis (165 ± 12 to 185 ± 15 mg QE/g) [38] and Bolivian propolis (5.5 ± 0.6 to 57.1 ± 2.8 mg QE/g) [36]. Finally, our finding also revealed that TPC was higher compared to TFC in EEP of *G. thoracica* and this result was supported by previous studies that showed phenolic content is higher in ethanol extract of propolis [40,41]. The different of TPC and TFC may possibly linked with the floral species, specific foraging activities and diets of bees species [42,43]. Furthermore, the polarity of solved utilised in extraction of propolis also influenced TPC and TFC value [44]. High content of phenolic and flavonoid constituents in polar solvents contribute to the high concentration of these compounds detected in the extraction [45]. The used of 80% ethanol in propolis extraction in this study also gave superior results of TPC and TFC value of EEP from *G. thoracica* compared to propolis from other countries.

The antioxidant test was carried out to verify the antioxidant potential of EEP from *G. thoracica* by using DPPH scavenging method. The scavenging activity of DPPH free radical was represent as EC50, the required concentration to reduce 50% of DPPH from initial concentration [46]. A lower EC50 value correspond better with the higher DPPH radical scavenging capacity as it required lesser amount of extract to reduce 50% initial concentration of DPPH [46]. Based on our study, EC50 of EEP from *G. thoracica* was 48.3 ± 0.2 µg/ml (Table 1). Previous study by Ibrahim et al. showed that the concentration of methanol extract of propolis from *G. thoracica* required to reduced 50% of DPHH was 6.25 - 800 µg/ml [32]. Furthermore, the EC50 value of EEP from *G. thoracica* also within the range of Bolivian propolis (4.54 to 48.27 µg/ml) [36] and Brazilian propolis (21.50 to 78.77 µg/ml) [37], but it superior than Chinese propolis that reported by Wang et al. which ranged from 15.49 ± 70.59 to 28.69 ± 71.52 µg/ml [39].

CONCLUSION

The phenolic acid and flavonoids compounds are main constituents of EEP from *G. thoracica*. The TPC, TFC and antioxidant capacity of EEP from *G. thoracica* were comparable and sometimes superior to propolis from other countries.

ACKNOWLEDGEMENT

This study was supported by a research university individual (RUI) grant (1001/PCIPPT/811279) from Universiti Sains Malaysia.

REFERENCES

[1] Bankova VS, Solange LC, Marcuci MC. Apidologie 2000; 31: 3-15.
[2] Bankova VS, Popova M, Trusheva B. Nat Prod Comm 2006; 1: 1023-1028.
[3] Rai MK, Cordell GA, Martinez JL, Marinoff M, Rastrelli L. Medicinal plants: Biodiversity and drugs Science Publisher, 2012, ISBN: 978-1-57808-793-8.

[4] Daleprene JB and Abdalla DS. Evid-Based Complement Alter Med 2013; Article ID 175135, 8 pages.
[5] Uzel A, Sorkun K, Oncag O, Cogulu D, Gencay O, Salih B. Microbiol Res 2005; 160: 189-195.
[6] Kumazawa S, Hamasaka T, Nakayawa T. Food Chem 2004; 329-339.
[7] Bankova V. J Apiproduct and Apimedical Sci 2009; 1: 23-28.
[8] Oliveira A, Franca H, Kuster R, Teixeira L, Rocha L. J Venomous Animal And Toxin Incl Trop Dis 2010; 16: 121-130.
[9] Ota C, Unterkircher C, Fantinato V, Shimizu MT. Mycoses 2001; 44: 375-378.
[10] Naito Y, Yasumuro M, Kondou K, Ohara N. Phyther Res 2007; 21: 452-456.
[11] Kurek-Gorecka A, Rzepecka-Stojko A, Gorecki MM, Stojko J, Sosada M, Swierczek-Zieba GG. Molecules 2014; 74: 5901-5921.
[12] Schnitzler P, Neuner A, Nolkemper S, Zundel C, Nowack H, Sensch KH, Reichling J. Phyther Res 2010; 24: 890-902.
[13] Chan GCF, Cheung KW, Sze DMY. Clin Rev Allergy and Immunol 2013; 45: 678-697.
[14] Nenadis N, Wang LF, Tsimidou M, Zhang HY. J Agric Food Chem 2004; 52: 4669-4674.
[15] Okawa M, Kinjo J, Nohara J, Ono M. Biol Pharm Bull 2001; 24: 1202-1205.
[16] Christov R, Bankova V. J Chromatogr 1992; 623: 182-185.
[17] Pereira AS, Pinto AC, Cardoso NJ, de Aquino Neto FR, de Souza Ramos MF, et al. J High Resolut Chromatogr 1998; 21: 396-400.
[18] Maciejewicz W, Daniewski M, Bal K, Markowski W. Chromatogr 2001; 53: 343-346.
[19] Marcucci MC and Bankova V. Current Topic in Pytochem 1999; 2: 115-123.
[20] Tazawa S, Warashina T, Noro T. Nat Med (In Japanese) 2000; 54: 306-313.
[21] Fujimoto T, Nakamura J, Matsuka M. Honeybee Sci (In Japanese) 2001; 22: 9-16.
[22] Norowi MH, Sajap AS, Rosliza J, Fahimie MJ, Suri R. Retrieved from http://www.niaes.affrc.go.jp/sinfo/sympo/h22/1109/paper_04.pdf.
[23] Freira KRL, Lins ACS, Dorea MC, Santos FAR, Camara CA, Silva TMS. Molecules 2012; 17: 1652-1664.
[24] Waterhouse AL. Current protocols in food analytical chemistery (unit II.I, pp. II.I.I-II.I.8) 2001. New York, NY: John Wiley & Sons.
[25] Chua LS, Rahaman NLA, Adnan LA, Eddie Tan TT. Methods in Chemistry 2013; 1-8.
[26] Pietta PG, Gardana C, Pietta AM. Fitoterapia 2002; 73: S7-S20.
[27] Nardini M, D'Aquino M, Tomassi G, Gentilli V, Di Felice M, Scaccini C. Free Rad Biol Med 1995; 19: 541-552.
[28] Zhang LY, Cosma G, Gardner H, Shi X, Castranova V. America J Physiol and Cell Physiol 2000; 279: 954-960.
[29] Benkovic V, Kopjar N, Horvat Knezevic A, Dikic D, Basic I, Ramic S, et al. Biol and Pharma Bull 2008; 31: 1778-1785.
[30] Materska M, Perucka I. J Agric Food Chem 2005; 53: 1750-1756.
[31] Siddhuraju P. Food Chem 2006; 99: 149-157.
[32] Ibrahim N, Nurul Farah Shakila MN, Muhammad Muslim MR, Abdul Jamil Z,Zhari I, Khamsah Suryati M. Malay J Analy Sci 2016; 20: 413-422.
[33] Bufalo MC, Fereira I, Costa G, Francisco V, Liberal J, Cruz MT, Lopes MC, Batista MT, Sforcin JM. J Ethnopharmacol 2013; 149: 84-92.
[34] Castro C, Mura F, Valuenzuela G, Figueroa C, Salinas R, et al. Food Res Inter 2014; 64: 873-879.
[35] Mouhoubi-Tafinine Z, Ouchemoukh S, Tamendjari A. Ind Crops Prod 2016; 88: 85-90.
[36] Nina N, Quispe C, Jimenez-Aspee F, Theoduloz C, Gimenez A, Schmeda-Hirschmann G. J Sci Food Agric 2016; 96: 2142-2153.
[37] Bittencourt MLF, Ribeiro PR, Franco RLP, Hilhorst HWM, Castro RD, Fernandez LG. Food Res Int 2015; 76: 449-457.
[38] Salas AL, Alberto MR, Zampini IC, et al. Phytomedicine 2016; 23: 27-31.
[39] Wang K, Zhang J, Ping S, et al. J Ethnopharmacol 2014; 155: 300-311.
[40] Miguel MG, Nunes S, Dandlen SA, Cavaco MA, Antunes MD. Food Sci Technol 2014; 34: 16-23.
[41] Sun C, Wu Z, Wang Z, Zhang H. Evid-Based Complement Altern Med 2015; Article ID 595393: 9 pages.
[42] Carpes ST, Begnini R, Alencar SM, Masson ML. Cienca e Agrotecnologia 2007; 31: 1818-1825.
[43] Marghitas LA, Stanciu OG, Dezmirean DS, Bobis O, et al. Food Chem 2009; 115: 878-883.
[44] Nurdianah Harif F, Mohd Gahimee J, Rosliza J, Wan Adnan WO. J Apicultural Res 2017; 56: 130-135.
[45] Stankovic MS. Kragujevac J of Sci 2011; 33: 63-72.
[46] Pratami DK, Mun'im A, Sahlan M, Sundowo A. Pharmacog J 2018; 10: 128-135.