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HPTLC Fingerprinting Coupled with Chemometric Analysis for Evaluation of Different Extraction Methods on Stingless Bee's Propolis

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Propolis is a natural remedy used in folk medicine and believed to have a broad spectrum of biologically active with therapeutic properties. Propolis consists of several different materials collected by the bees including resin, wax, latex etc and used to protect their hive from predator and microorganism. In order to harness its therapeutic benefits, extraction protocol must be optimized. This work aims to use chemometrics approach to evaluate the differences of the chemical fingerprint of propolis extracts obtained from different extraction methods. Propolis was extracted by maceration, sonication and Soxhlet in 70% and 95% ethanol. High-performance thin-layer chromatography (HPTLC) coupled with chemometric were employed to evaluate the chemical profile through their chemical fingerprints. HPTLC chromatogram of propolis revealed a complex chemical fingerprint in each different extraction methods viewed under 254 nm and 366 nm. There were eleven and ten of unknown compounds detected in all extraction methods viewed under 254 nm and 366 nm, respectively. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) grouped the propolis extracts into three different clusters based on percentages of ethanol, different extraction methods and the intensities of unknown compounds present in each sample. The finding shows that extraction methods significantly produced different chemical profiles which presumably affect their biological properties.

Keywords: stingless bee, propolis, extraction methods, HPTLC, PCA, HCA

INTRODUCTION

Propolis is a natural remedy used in folk medicine and believed to have a broad spectrum of biologically active with therapeutic properties. Propolis made up by mixing insect secretion, saliva, and wax with plant resin (Lavinas et al., 2018) and consists of several different materials collected by the bees including resin, wax, latex etc and used to protect their hive from predator and microorganism (Bankova et al., 2014; Bertrams et al., 2013). It is important to highlight the fact that raw propolis is not suitable for applications in food technology, pharmaceutical or cosmetic industries due to high content of impurities that need to be

removed (Galeotti et al. 2018). It is interesting to note that these contaminants can be removed by extraction, which enables secondary metabolites or bioactive compounds to be separated from raw materials. Taken together, there are several factors that affect the extraction process such as method extraction, the choice of solvent, the solvent concentration, the extraction time and the temperature (Zhang et al., 2018; Pandey et al., 2014). In general, the samples can be extracted by various extraction methods using conventional and non-conventional methods. Conventional or traditional methods are more environmentally friendly while non-conventional methods have been suggested to enhance the extraction process. The chemical compounds contained in propolis are susceptible to various factors such as the botanical origin (Salatino et al., 2005), the geographical area (Bankova et al., 2014; Annisava et al., 2019), the bee species (Araújo et al., 2016; Nafi et al., 2019) and the harvest time (Bankova et al., 2002). Therefore, the chemical composition of propolis can vary even in the same apiary due to the different preferences of plant resources. Taken together, the previous results confirmed that the methods and solvents used for extraction also affected the chemical composition presence (Mulyati et al., 2020; Georgieva et al., 2019; Kasote et al., 2019; Pujirahayu et al., 2019; Zin et al., 2019). To date, well defined high-tech tools have been developed to produce accurate and precise results in the screening and characterization of chemical compounds. High Performance Thin Layer Chromatography (HPTLC) is a tool for separating chemical compounds in our samples achieved on high-performance layers with detection and data acquisition using an advanced work station either for qualitative analysis or for quantitative analysis. Because of its simplicity, low cost, and the ability to analyse up to twenty samples under identical conditions, HPTLC has been recognized as a the tools to perform fingerprint analysis (Milojković Opsenica et al., 2016) and become quality control tool (Mallick & Dighe, 2014). HPTLC is a robust, fast, and efficient tool for quantitative analysis of compounds (De Silva et al., 2017). Obviously, the HPTLC datasets mostly contain complex and large amounts of data and are difficult to understand and classify by human vision. To solve this problem, it is important to use chemometric analysis to interpret the data.

Chemometrics is a tool that uses mathematical and statistical methods to analyse chemical data either quantitatively or qualitatively (Otto, 2016). Two main chemometric tools that are normally used in analysing natural product samples are Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). PCA is a mathematical technique that reduces the dimensionality of large data sets to a small set by maintaining the variability of the data sets in the main component (PC) (Jollife and Cadima, 2016). Typically, most of the two PCs were used, namely PC1 and PC2, as these two PCs contributed to higher variance in certain data sets. The PC1 is perpendicular to PC2. If the factors were difficult to interpret after analysing the component, the varimax rotation was applied. The varimax rotation is an orthogonal rotation used to facilitate interpretation by reducing the variability of the main component known as the varimax factor (VFs) (Abdi & Williams, 2010). In other words, the purpose of the rotation is to improve the interpretation of the component, which makes the results easier to interpret because of the original variables and the loading may not show a clear pattern. The Varimax rotation was applied to the PCs when the eigenvalue was greater than 1. The distribution of the samples was illustrated in the score plot and in the biplot. The biplot shows the PC evaluation of the score plot (samples) and the loading factor (variables). The loading factor showed the variables that influenced the PCs and between 1 and 1. Variables with loading factor values close to -1 and 1 have a strong influence on the component, while loading factor close to 0 indicates that the variable has a weak influence on the PC component (Granato et al., 2018). In HPTLC, there are several information about the sample that can be analysed using PCA, for example R_f value (evaluation of sample polarity toward mobile phase, spectral properties (absorbance, fluorescence) and selective derivatization using different reagent (getting information on functional groups) (Morlock et al., 2014).

HCA's data is presented in a tree structure called a dendogram diagram. Each node of the tree is represented as a cluster by making horizontal cuts (dotted line) through the tree structure at a selected height (Randriamihamison et al., 2020). The dendogram consisted of two axes, with one axis shown as the distance (dissimilarity or similarity) of the cluster, while another axis is shown as samples or clusters. Samples within the same cluster suspecting to have similar properties, while samples that are distributed in different clusters might have different properties. The clades (branches) that have the same height are similar to each other, while the clades with different heights

are different. The greater the height, the more different they are. In summary, it can be said that the HCA provides a clear explanation of the similarity or dissimilarity of the samples using a dendogram.

This research examines the emerging role of application of different extraction methods used on propolis stingless bee in the context of HPTLC fingerprinting in order to harness its therapeutic benefits. In this study, we utilized PCA analysis on R_f value data set in two different wavelengths (254 nm and 366 nm) to evaluate the differences between samples. For this study we used propolis produced by species of *Heterotrigona itama*. This species is the most common domesticated species in Malaysia and produces a large amount of propolis all year round.

MATERIALS AND METHODS

Reagents and Materials

All the chemicals and reagents used in this experiment were analytical grade. Ethanol, benzene, ethyl acetate, acetic acid and methanol were purchased from Merck Sdn. Bhd, Selangor, Malaysia, while TLC silica gel 60 F_{254} aluminium sheets were purchased from Merck Millipore, Darmstadt, Germany.

Methods

Collection of samples

The *Heterotrigona itama's* propolis was collected from Apiary, Universiti Sultan Zainal Abidin, Besut Campus. Any impurities such as leaves or insects that stick at propolis were cleaned. The propolis samples were frozen at -80 ºC and ground thoroughly to powder using a waring blender. This process must be done quickly to avoid raw propolis become sticky when exposed to heat in the wearing blender. Then, the propolis powder were kept in -80 ºC for further analysis.

Preparation of Extracts

Maceration

Approximately 21 grams of propolis were extracted in 70 mL of 70% and 95% ethanol. Propolis samples were macerated for 1, 3, 5 and 7 days at room temperature. The solutions were filtered using filter paper Whatman™ (3mm) and evaporated using rotary evaporator (Heidolph Instruments GmbH 5 & Co. KG, German) under vacuum pressure at 45°C. Propolis extracts were kept in 4°C for prior analysis in order to preserve bioactive compound in the propolis. The extraction procedure used as shown in Table 1.

Sonication

Approximately 21 grams of propolis were extracted in 70 mL of 70% and 95% ethanol.

Table 2: The extraction by maceration

No	Solvent-type extraction-time	Label
1	ethanol 70% - maceration - 1 day	70M-1d
2	ethanol 70% - maceration - 3 day	70M-3d
3	ethanol 70% - maceration - 5 day	70M-5d
4	ethanol 70% - maceration - 7 day	70M-7d
5	ethanol 95% - maceration - 1 day	95M-1d
6	ethanol 95% - maceration - 1 day	95M-3d
7	ethanol 95% - maceration - 1 day	95M-5d
8	ethanol 95% - maceration - 1 day	95M-7d

The samples were sonicated at 10, 30, 60 and 120 minutes using ultrasonic bath (Jeio Tech UC-10) at 37°C. Furthermore, the solutions were then filtered through filter paper Whatman™ (3mm). The filtrates were then concentrated under vacuum pressure using a rotary evaporator (Heidolph Instruments GmbH 5 & Co. KG, German) at 45°C and kept in 4°C until further analysis. The extraction procedure used as shown in Table 2.

Table 2: The extraction by sonication

No	Solvent-type extraction-time	Label
1	ethanol 70%-sonication-10 minutes	70S-10m
2	ethanol 70%-sonication-30 minutes	70S-30m
3	ethanol 70%-sonication-60 minutes	70S-60m
4	ethanol 70%-sonication-120 minutes	70S-120m
5	ethanol 95%-sonication-10 minutes	95S-10m

6	ethanol 95%-sonication-30 minutes	95S-30m
7	ethanol 95%-sonication-60 minutes	95S-60m
8	ethanol 95%-sonication-120 minutes	95S-120m

Soxhlet

Approximately 5 grams of propolis were extracted in 150 mL of 70% and 95% ethanol. Propolis samples were subjected to 2, 4, 6 and 8 hours of Soxhlet extraction (Soxhlet extractor M-Top, Korean South). Then, the solutions were filtered through filter paper Whatman™ (3mm). The filtrates were then concentrated using a rotary evaporator (Heidolph Instruments GmbH 5 & Co. KG, German) under vacuum pressure at 45°C and kept at 4°C prior analysis in order to preserve bioactive compounds in the propolis. The extracts were labelled as 70SH-2h (ethanol 70% - Soxhlet - 2 hours), 70SH-4h (ethanol 70% - Soxhlet - 4 hours), 70SH-6h (ethanol 70% - Soxhlet - 6 hours), 70SH-8h (ethanol 70% - Soxhlet - 8 hours), 95SH-2h (ethanol 95% - Soxhlet - 2 hours), 95SH-4h (ethanol 95% - Soxhlet - 4 hours), 95SH-6h (ethanol 95% - Soxhlet - 6 hours), and 95SH-8h (ethanol 95% - Soxhlet - 8 hours).

Table 3: The extraction by Soxhlet

No	Solvent-type extraction-time	Label
1	ethanol 70% - Soxhlet - 2 hours	70SH-2h
2	ethanol 70% - Soxhlet - 4 hours	70SH-4h
3	ethanol 70% - Soxhlet - 6 hours	70SH-6h
4	ethanol 70% - Soxhlet - 8 hours	70SH-8h
5	ethanol 95% - Soxhlet - 2 hours	95SH-2h
6	ethanol 70% - Soxhlet - 4 hours	95SH-4h
7	ethanol 70% - Soxhlet - 6 hours	95SH-6h
8	ethanol 70% - Soxhlet - 8 hours	95SH-8h

HPTLC Separation of ethanolic propolis extract

The HPTLC analysis was performed by CAMAG HPTLC system (Muttenz, Switzerland) following the methodology of Azemin et al (2018). Accurately weighed 30 mg propolis extracts and dissolved in 1 mL methanol, sonicated for 20 minutes at 37°C and centrifuged for 10 minutes at 10000 rpm. A volume of 8 µL of propolis extract were spotted by CAMAG Automatic TLC Sampler 4 (ATS4) (Merck, Germany) on 20 cm x 10 cm TLC pre-coated glass plates (silica gel 60 F_{254}, layer thickness of 0.2 mm) with syringe size 25 µL and under a stream of nitrogen gas (N_2). The ethanolic propolis extract was loaded with a band length for 10.0 mm, distance from the bottom of the plate for 8.0 mm and distance between tracks was 18.5 mm. Development was carried out in mobile phase of benzene: ethyl acetate: methanol: acetic acid (24: 5.6: 4.85: 2.6 v/v/v/v) in the twin trough chamber by Automatic Developing Chamber 2 (ADC2, CAMAG). The tank was saturated for 30 minutes with a saturation pad in 25 mL of solvent system for the purpose of an even distribution of solvent vapor in the chamber. Then, the pre-conditioning was set to 10 minutes with migration distance 85.0 mm and after development the drying time was set for 5 minutes. Note that, developing chamber must be kept away from direct sunlight and heat to prevent the vaporization of solvent system which perhaps may lead to decrease of their concentration and lastly will affect the separation of compounds (Lade et al., 2014). The plate was visualised under 254 nm and 366 nm using CAMAG Visualizer equipped with CAMAG WinCats Software. After that, the plate was placed in the scanning stage by using CAMAG TLC Scanner. The scanner was set at 20 mm/s speed with slit dimension micro 8.00 x 0.20 mm macro. The retention factors (R_f) and percentages area were calculated by the WinCats software for chemometric purposes. The retardation factor (R_f value) was measured as below:

R_f value = Distance traveled by substance / Distance traveled by solvent front

Chemometric Analysis

HPTLC datasets were established by scanning the developed TLC plates by using CAMAG TLC Scanner under 254 nm and 366 nm. The scanner was set at speed 20 mm/s with slit dimension micro 8.00 x 0.20 mm, macro. The retention factors (R_f) and percentages area were calculated by the WinCats software for chemometric purposes. All HPTLC samples were subjected to unsupervised pattern recognition by Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), which analysed by XLSTAT Pro 2014

(Addinsoft, Paris, France), an add-in software program for Microsoft Excel 2010.

RESULTS

HPTLC Screening of propolis extracted by different extraction methods

HPTLC is a rapid and robust tool to screen chemical compounds in propolis. A study by Tang et al (2014) used thin layer chromatographic discriminate Chinese propolis. Similarly, Morlock et al. (2014), utilised HPTLC fingerprints combined with multivariate analysis of propolis from different countries. In this study, HPTLC analysis was employed in order to analyse various extraction conditions and solvent types (24 samples) simultaneously under identical conditions. There were several bands detected at these two wavelengths. Our results demonstrated that, there were eleven bands with different intensities were visualised under 254 nm specifically at R_f 0.25, 0.32, 0.39, 0.40, 0.44, 0.49, 0.55, 0.60, 0.63, 0.68 and 0.73 as shown in Figure 1(a). To be specific, the prominent bands at R_f 0.40, 0.55 and 0.66 are visualised in all extraction methods. Interestingly, the bands at R_f 0.55 and 0.60 displayed higher intensities in propolis extracted in 95% ethanol compared to propolis extracted in 70% ethanol suggesting that these two bands have higher concentration in propolis extracted in 95% ethanol. The HPTLC chromatogram profile of propolis extracted in 95 % ethanol and 70% ethanol were distinguished by the presence of R_f at 0.25, 0.32, 0.63, 0.68 and 0.70. In particular, the bands at R_f 0.25, 0.32 and 0.63 only appeared in propolis extracted in 70% of ethanol, while bands at higher R_f values namely 0.68 and 0.70 were only detected in 95% ethanol. Significantly, only propolis extracted in 70% of ethanol by maceration at 5 days (70M-5d: labelled as M3) and 7 days (70M-7d: labelled as M4) exhibited specific bands at R_f 0.39 and 0.49 conforming that propolis macerated more than 5 days to 7 days in 70% ethanol revealed a different HPTLC profile and contained two unique bands which vary from other extraction methods.

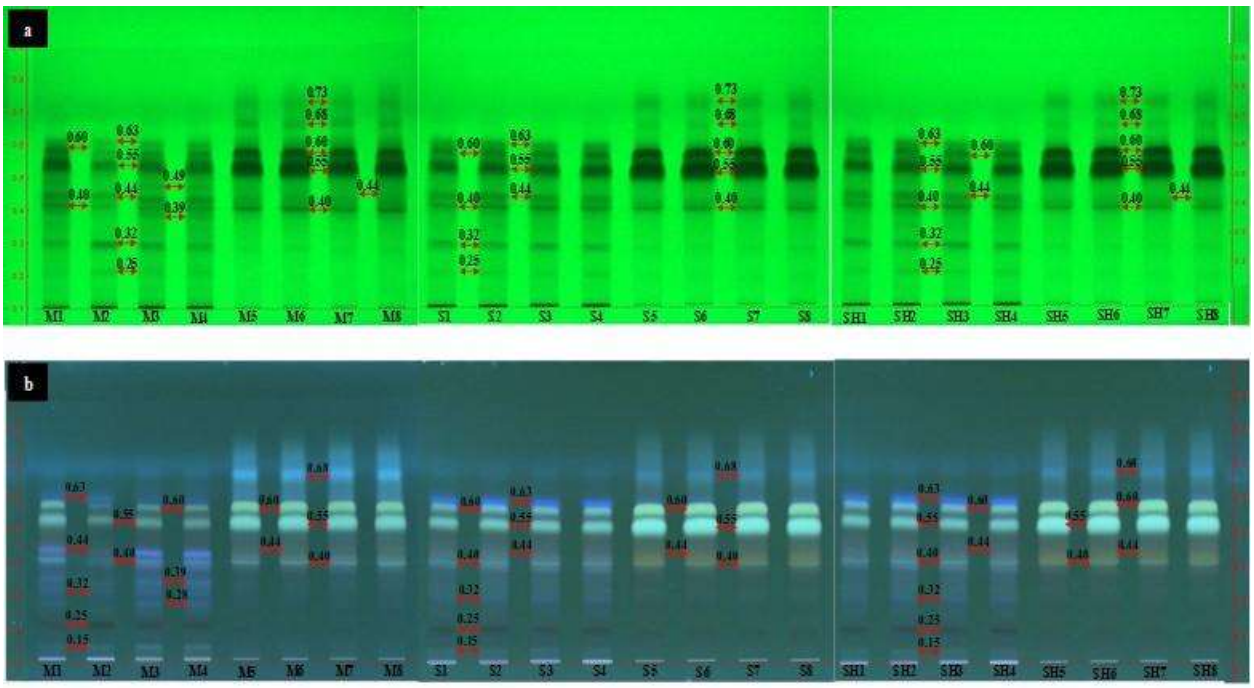

Figure 1. HPTLC chromatogram with R_f values of propolis extracted by different extraction methods viewed under UV light a) 254 nm b) 366 nm

Note : Maceration (M1: 70M-1d, M2: 70M-3d, M3:70M-5d, M4: 70M-7d, M5: 95M-1d, M6: 95M-3d, M7: 95M-5d, M8: 95M-7d) ; Sonication (S1: 70S-10m, S2: 70S-30m, S3: 70S-60m, S4: 70S-120m, S5: 95S-10m, S6: 95S-30m, S7: 95S-60m, S8: 95S-120m) ; Soxhlet (SH1: 70SH-2h, SH2: 70SH-4h, SH3: 70SH-6h, SH4: 70SH-8h, SH5: 95SH-2h, SH6: 95SH-4h, SH7: 95SH-6h, SH8: 95SH-8h)

Along with that, there were also eleven bands were observed under 366 nm yielded from HPTLC chromatograms of propolis extracted by maceration, sonication and Soxhlet in 95% ethanol and 70% ethanol at R_f 0.15, 0.25, 0.28, 0.32, 0.39, 0.40, 0.44, 0.55, 0.60, 0.63 and 0.68 as illustrated by Figure 1(b). The bands at R_f 0.40, 0.44, 0.55 and 0.60 were shown in all extraction methods. Interestingly, the band at R_f 0.40 appeared as blue colour in propolis extracted in 70% ethanol, while demonstrated as yellow colour in propolis extracted in 95% ethanol. On the other hand, the prominent bands were observed at R_f values 0.55 and 0.60 with yellow colour, where these bands detected with higher intensities in propolis extracted in 95% ethanol compared to 70% ethanol. This finding at least hinted that the application of different percentages of ethanol influenced the presence of different unknown compounds and their concentration in propolis at R_f 0.40, 0.55 and 0.60. Apart from that, the bands with blue colour at R_f 0.15, 0.25, 0.32 and 0.63 presented only in propolis extracted in 70% ethanol. Meanwhile, the band at R_f 0.68 with blue colour appeared only in propolis extracted in 95% ethanol. The results confirm that there were different unknown compounds detected with the use of different percentages of ethanol. In the same manner visualized under 254 nm, propolis extracted by maceration at (70M-5d: labelled as M3) and 7 days (70M-7d: labelled as M4) revealed two specific bands at R_f 0.28 (blue) and 0.39 (blue). To sum up, these unknown compounds only present in propolis by maceration in 70% ethanol after macerated more than 5 days. It is also interesting to assume that these two unknown compounds at R_f 0.28 and 0.39 might be polar and heat labile compounds because they appeared at lower R_f values and extracted by maceration which only kept at room temperature and do not involve heat or high temperature during the extraction process.

Chemometric analysis of HPTLC fingerprint

Principal Component Analysis

254 nm

The total variance of the data set at 254 nm explained from the first two components was 44.05% with 30.38% from VF1 and 13.67% from VF2. Figure 2(a) gave the details on the PCA score plot on extraction methods of stingless bee's propolis by maceration, sonication and soxhlet in 70% and 95% ethanol. The propolis extracted by maceration (70M-1d, 70M-3d, 70M-5d and 70M-7d), sonication (70S-60m, 95S-10m, 95S-30m, 95S-60m and 95S-120m) and Soxhlet (70SH-2h, 70SH-6h and 70SH-8h) were assigned along VF1 and the 70M-5d and 70M-7d gave the strong factor toward VF1. The loading values demonstrated that sample grouped in VF1 according to their R_f values at 0.15, 0.25, 0.32, 0.39, 0.40, and 0.49 as illustrated by Figure 2(b) and the most influence variables along VF1 were 0.39 and 0.49. On the other hand, the maceration (95M-1d, 95M-3d, 95M-5d and 95M-7d), sonication (70S-10m, 70S-30m and 70S-120m) and Soxhlet (70SH-4h, 95SH-2h, 95SH-4h, 95SH-6h and 95SH-8h) were contributed along VF2. The maceration (95M-1d, 95M-3d, 95M-5d and 95M-7d) and Soxhlet (95SH-2h, 95SH-4h, 95SH-6h and 95SH-8h) gave the strong factor along VF2. On the other hand, the VF2 was explained according to the R_f values at 0.17, 0.44, 0.55, 0.60 and 0.73 as shown by Figure 2(b). The variables that gave high corresponding to the VF2 was at R_f 0.73.

The total variance in VF4 also gave at higher percentages of 28.43%, which means there were variables that influenced the PC's model. The factor that gave strong factor in VF4 was propolis extracted in 95% ethanol by sonication in 95% ethanol (95S-10m, 95S-30m, 95S-60m and 95S-120m) and the bands at R_f 0.55 and 0.60 were variables that exhibit the strong loading on VF4 direction (with red circle in Figure 3(b)). The evidence from this study points towards the idea of first, maceration at 5 (70M-5d) and 7 days (70M-7d) located farther then other ethanolic extract displayed special bands at R_f 0.39 and 0.49 considered they have unique profiling than other ethanolic propolis extracts represent in GII. As mentioned above these samples gave strong influence along PC1 suggesting that extraction method by maceration at 5 days and 7 days seems to be interesting methods in extracting unknown compounds which are absent in other extraction methods. Second, propolis extracted in 70% ethanol might (GIII) share comparable profiling with each other. Lastly, the propolis extracted in 95 % ethanol by maceration and Soxhlet (GI) might present a different profile with sonication in 95% ethanol (GIV). Overall, a clear separation between percentages of ethanol used

was revealed along the axis of VF1 and VF2 assuming that different percentages of ethanol gave the significant role influenced the separation along PCA's component at 254 nm.

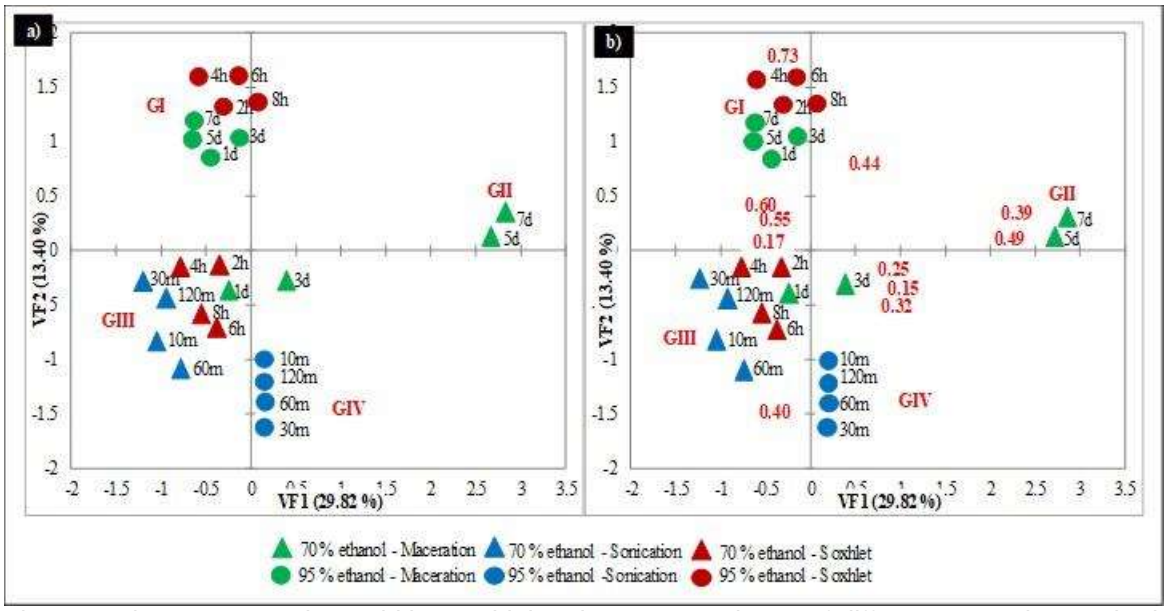

Figure 2. a) PCA score plot and b) PCA biplot along VF1 and VF2 of different extraction methods of stingless bee's propolis by maceration, sonication and soxhlet at 254 nm.

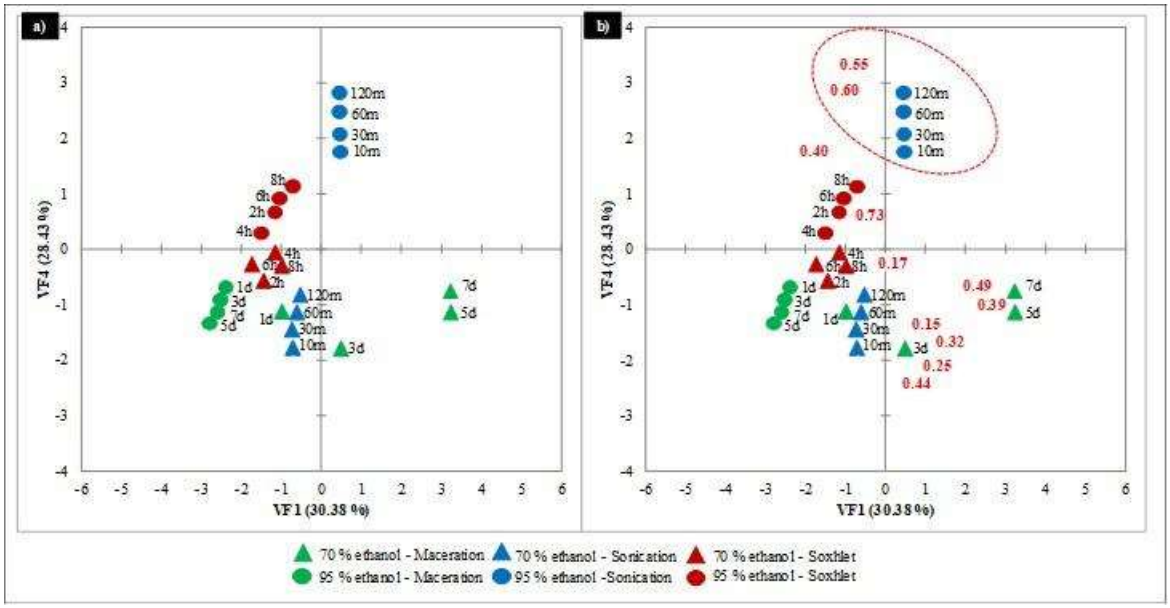

Figure 3. a) PCA score plot and b) PCA biplot along VF1 and VF4 of different extraction methods of stingless bee's propolis by maceration, sonication and Soxhlet at 254 nm.

366 nm

Figure 4(a) shows a representation of the PCA score of stingless bee's propolis extracted from maceration, sonication and Soxhlet in 70% and 95% ethanol at 366 nm. The PCA result stated that 64.29% of the total variance from VF1 was 41.52% and VF2 was 22.76%. Macerations (70M-1d, 70M-3d, 70M-5d and 70M-7d) and sonication (70S-10m, 70S-30m, 70S-60m and 70S-120m) were combined along VF1. On the other hand, the VF2 consisted of maceration (95M-1d, 95M-3d, 95M-5d

and 95M-7d), sonication (95S-10m, 95S-30m, 95S-60m and 95S-120m) and Soxhlet (70SH-2h, 70SH -4h, 70SH-6h, 70SH-8h, 95SH-2h, 95SH-4h, 95SH-6h, and 95SH-8h) as shown in Figure 4(a). Based on the factor assessment, the maceration (70M-1d, 70M-3d, 70M-5d, 70M-7d) gave the strong factor along VF1, while the Soxhlet (70SH-4h, 70SH-6h, 70SH-8h, 95SH-2h) 95SH-4h, 95SH-6h and 95SH-8h) gave the strong factor in VF2. The loading diagram showed (see Figure 4(b)) that bands at R_f 0.25, 0.32 and 0.39 gave the most positive influence along VF1, while VF2 due to the presence of a bands at R_f 0.55 and 0.60 was detected. The PCA score plot diagram at 366 nm showed that the propolis of the stingless bee's extracted by different extraction methods was divided into four different groups. This result showed that propolis extracted in 70% ethanol showed a clear distinction as they separated into different groups, especially GI (Soxhlet), GIII (maceration) and GIV (sonication). That being said, propolis, extracted by maceration and Soxhlet in 95% ethanol in GI were close together, suggested that they may have a similar profile. It was pointed out that the sonication (GII) is separate from these two methods, provided that the sonication exhibits unequal profiling with Soxhlet and maceration. On the other hand, only maceration gave an apparently different profile extracting between 70% and 95% ethanol as they were further apart. Taken together, these results would seem to suggest that the choice of extraction methods influenced the separation of PCA's component at 366 nm.

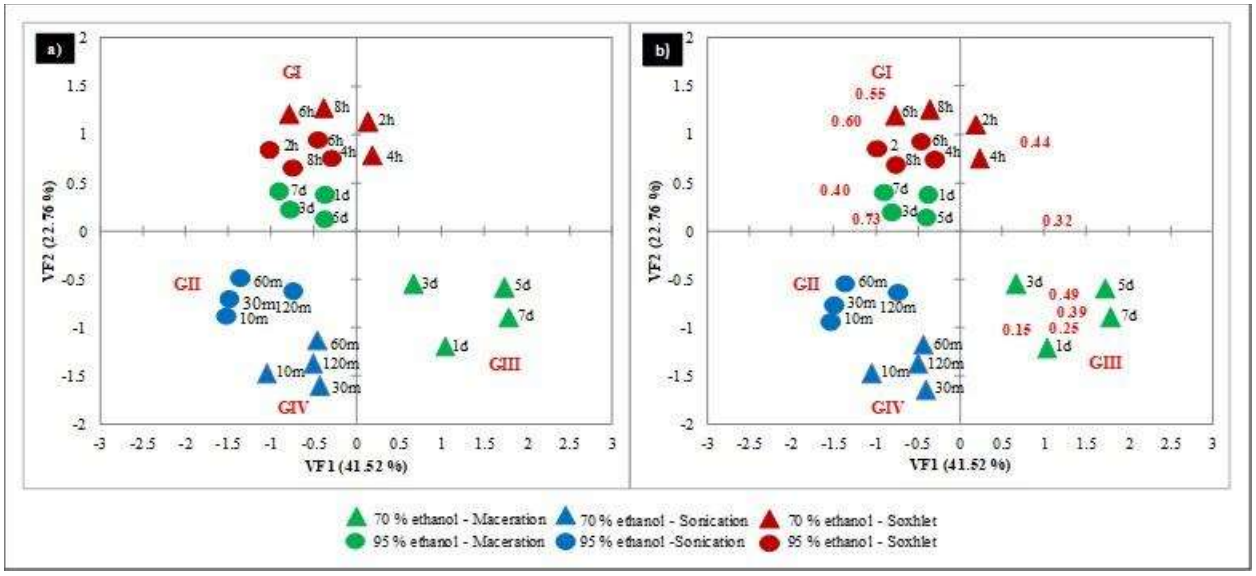

Figure 4a) PCA score plot and b) PCA biplot along VF1 and VF2 of different extraction methods of stingless bee's propolis by maceration, sonication and Soxhlet at 366 nm.

Hierarchical clustering analysis (HCA)

254 nm

The HCA dendogram of propolis, extracted using various extraction methods illustrated into three clusters (cluster I, cluster II, and cluster III), as shown in Figure 5(a). The present of unknown compound at different R*f* values which influences the clustering of propolis extracted by different extraction were simplified in profile plot as shown in Figure5(b). Interestingly, the clustering result of propolis extracted by various extraction methods at 254 nm was consistent with the grouping in the PCA's model (Figure 2(a)), with cluster I corresponding to group II and group III, which consisted of propolis extracted in 70% by maceration (70M-1d, 70M-3d, 70M-5d and 70M-7d), sonication (70S-10m, 70S-30m, 70S-60m and 70S-120m) and Soxhlet (70SH-2h, 70SH- 4h, 70SH) -6h and 70SH-8h), while Cluster II and Cluster III corresponded to Group I and Group IV, respectively, consisted of propolis extracted in 95% ethanol. In details, the propolis extracted by maceration, sonication and soxhlet in 70% ethanol, was assigned to cluster I. In particular, Cluster I was clustered based on the presence of bands at R_f 0.15, 0.17, 0.32, 0.39 and 0.49. These bands were absent in other clusters, suggesting that these zones were responsible for distinguishing between the propolis extracted in 70% ethanol and

95% ethanol. To be precise, the bands at R_f 0.39 and 0.49 have only been shown in 70M-5d and 70M-7d in cluster I indicating that, ethanolic propolis macerated more than 5 days - 7 days exhibit special unknown compounds. Based on the result of the cluster analysis in Cluster I, the similarities between ethanolic propolis within this cluster was they contained lower intensive bands at R_f 0.55 and 0.60, while higher intensive band at R_f 0.44. Cluster II was grouped from propolis extracted by maceration (95M-1d, 95M-3d, 95M-5d and 95M-7d) and soxhlet (95SH-2h, 95SH-4h, 95SH-6h and 95SH-8h). In fact, propolis grouped into this cluster based on the presence of band at R_f 0.73. In particular, the maceration and the soxhlet were separated into different clades within Cluster II due to different intensity of the band at R_f 0.73. Specifically, soxhlet has a higher intensive band compared to the maceration. Meanwhile, Cluster III consisted of sonication (95S-10m, 95S-30m, 95S-60m, and 95S-120m). In contrast, propolis extracted by sonication in 95% ethanol was clustered differently with another two methods due to the lack of a band at R_f 0.44. Although the propolis extracted by sonication in 95% ethanol had fewer separate bands, the bands present in these samples showed higher intensive bands at R_f 0.40, 0.55 and 0.60 compared to the samples in Custer I and II. Together these results provide important insights on classification of ethanolic propolis extract in which 95% ethanol and 70% ethanol were differentiated into different clusters. To put it together, the percentages of ethanol mostly influenced the separation of HPTLC profile at 254 nm.

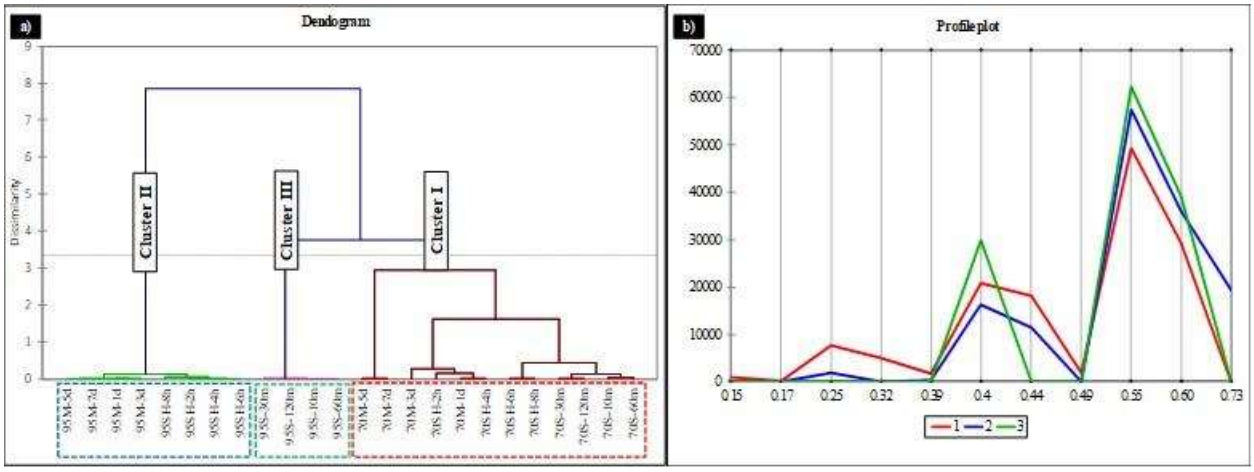

Figure 5. a) HCA of dendogram. Vertical-axis indicate the dissimilarity value and horizontal-axis shows the cluster; and b) Profile plot of propolis extraction of stingless bee by different extraction methods at 254 nm which consisted of three clusters; Cluster I (red), Cluster II (blue) and Cluster III (green). Vertical-axis indicate the intensity of the peak and horizontal-axis shows the R_f value.

366 nm

Figure 6a) demonstrated information on the cluster dendogram of stingless bee propolis extracted by maceration, sonication and soxhlet in 70% and 95% ethanol. The HCA dendogram of 366 nm HPTLC data sets of propolis extracted from various extraction methods illustrated three different clusters which were Cluster I, Cluster II, and Cluster III, as shown in Figure 6(a). The results of HCA at 366 nm were also complementary to the PCA's model (Figure 4). In detail, cluster I was aligned with group I and group II, while cluster II and cluster III corresponded to group III and group IV, respectively. Cluster I was classified by propolis extracted by maceration (70M-1d, 70M-3d, 95M-1d, 95M-3d, 95M-5d and 95M-7d), sonication (95S-10m, 95S-30m, 95S-60m and 95S-120 m) and soxhlet (70SH-2h, 70SH-4h, 70SH-6h, 70SH-8h, 95SH-2h, 95SH-4h, 95SH-6h and 95SH-8h). The main criteria of Cluster I that make up them together due to the presence of higher intensive bands at R_f 0.55 and 0.60. Cluster II was grouped from propolis extracted by maceration in 70% ethanol (70M-5d and 70M-7d) and categorized based on the presence of bands at R_f 0.15, 0.25, 0.32, 0.39, and 0.49. Interestingly, these bands were absent in other clusters. In addition, the samples within Cluster II grouped together due to the presence of lower intensive bands at R_f 0.44 and 0.55. On the other hand, propolis extracted by sonication in 70% ethanol (70S-10m, 70S-30m, 70S-60m and 70S-120m) was grouped into cluster III. They were compiled based on the presence of

a lower intensity bands at R_f 0.44, 0.55 and 0.60. In particular, the main difference between propolis extracted in 70% ethanol and 95% ethanol was the presence of high intensity bands at R_f 0.55 and 0.60 in 95% ethanol compared to 70% ethanol. In addition, the propolis extracted in 70% ethanol by maceration and Soxhlet were grouped differently with sonication due to the presence of bands at R_f 0.25 and 0.32 that were absent in sonication. In contrast, a clear difference was found in propolis extracted in 95% ethanol by the presence of a band at R_f 0.44. This band was only revealed in maceration and Soxhlet. The presence of an unknown compound at different R_f values, which influences the propolis cluster formation extracted by different extraction, has been simplified in the profile diagram (Figure6 (b)). In short, from the result of the cluster analysis at 366 nm, the distinction between different extraction methods of propolis was clustered based on the presence and intensities of unknown compounds at different R_f values.

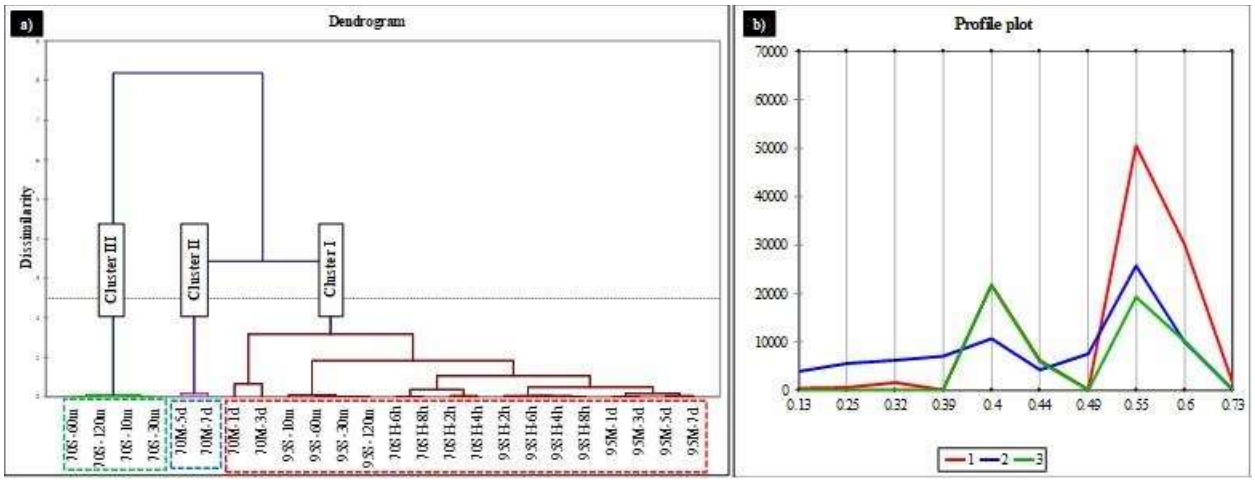

Figure 6. a) HCA of dendogram. Vertical-axis indicate the dissimilarity value and horizontal-axis shows the cluster; and b) Profile plot of propolis extraction of stingless bee by different extraction methods at 366 nm which consisted of three clusters; Cluster I (red), Cluster II (blue) and Cluster III (green). Vertical-axis indicates the intensity of the peak and horizontal-axis shows the R_f value.

DISCUSSION

HPTLC analysis can be used for both qualitative and quantitative analysis. In this study, the qualitative analysis was performed to verify the HPTLC profiling of the propolis extracted by different extraction methods. The chemical composition of propolis influenced by vegetation, the season of propolis produce and the geographical area (Bankova et al., 2014). A mixture of benzene: ethyl acetate: methanol: acetic acid in a ratio of 24: 5.6: 4.85: 2.6 (v/v/v/v) was used as a mobile phase. At first, the solvent system used was toluene: ethyl acetate: formic acid (8: 2: 0.1 v/v/v) as described by Ibrahim et al. (2016). This solvent system was chosen because the sampling location was in the same geographical area, assuming that they might have a similar HPTLC profiling and chemical composition. However, the bands were not well separated, as earlier findings suspecting that, due to different seasons during sampling and different extraction methods. Therefore, the toluene was substituted to benzene and methanol was added to improve the separation, as described by Crabtree et al. (1967). One of the propolis compositions is resin and as reported by Stahl (1969), benzene used as a solvent system to separate balm and resin. The use of benzene and methanol in this study significantly affected the separation of the unknown compound, particularly at R_f 0.55 and 0.60, which occurred with higher intensive bands. Besides that, the formic acid changed to acetic acid to improve band shape and separation as well. Detection of the presence of unknown compounds were established by observing the colour detected on the TLC plate after viewing under UV light (254 nm and 366 nm). The dark bands on a fluorescence background at 254 nm indicated the presence of unknown compounds contained with conjugated double bond and aromatic structure (Bladt, 2009). Apparently, the yellow, orange and blue colour viewed under 366 nm represent a flavonoid compound group (Stahl, 1969). The different colour appeared might be due to different classes of

flavonoid present in the propolis. Recent studies by Guzelmeric et al (2017) and Bertrams et al. (2013) demonstrated that the orange and yellow zones belonged to the flavonol and flavone. Meanwhile, blue zones correspond to flavanone, bioflavonoid and flavonol. In line with that, Wagner et al (1984) proposed that, flavonol with two adjacent OH groups in B-ring tend to exhibit orange colour zones while flavonol with single adjacent OH group in B-ring illustrated yellow colour zones after viewed under UV light at 366 nm. In accordance with previous studies, the main compounds found in Malaysian *Heterotrigona itama's* propolis extracted in ethanol including phenolic acids, flavonoids, naphthoquinones, triterpenes and phytosterol detected by ultra-high performance liquid chromatography with quadruple time-of-flight mass spectrometry (UHPLC-Q-TOF / MS) (Zhao et al., 2017) and propolis from India reported to have polyphenols, sugars, terpenes and steroids (Kasote et al., 2019). In summary, phenol, flavonoid, and terpenes are the main group of compounds in stingless propolis bees. Taking into account, the separation of the unknown compound by the stationary phase (TLC plate) is influenced by various factors such as physical properties, molecular structure and functional groups in the samples (Lade et al., 2014). In addition, the chemical composition of propolis is influenced by the bud excretions from plant sources, the climate and the geographical origin (Chewchinda & Vongsak, 2019). In particular, the bands on the TLC plates of propolis, extracted by maceration, sonication and soxhlet at 254 nm, gave general information about the presence of a conjugated double-band and aromatic structure (Bladt, 2009). Most organic compounds contain these properties. Hence, the TLC plates viewed at 366 nm were more prominent in the chemometric analysis by PCA and HCA in this study. However, there was a limit of detection while scanning by the scanner which may not be able to detect all of the bands displayed on the plate due to the less intensive bands detected. In detail, the classification of propolis extracted by various extraction methods by PCA and HCA also can be revealed by the presence of colour of bands on the TLC plate after viewing under UV light 366 nm. By PCA, the loading values showed that samples were grouped along VF1 according to their R_f values at 0.25, 0.32 and 0.39, which appeared as blue colour bands, while VF2 according to R_f 0.55 and 0.60 was declared as yellow colour zones (Figure 4). Apart from that, the classification by HCA (Figure 6) highlighted that the ethanolic propolis extract was grouped in Cluster I due to the presence of bands at R_f 0.55 (yellow colour) and 0.60 (yellow colour). Meanwhile, Cluster II was categorized based on the bands at R_f 0.15, 0.25, 0.32, 0.39 and 0.49 with all these bands appearing as blue colour bands. On the other hand, Cluster III due to the presence of R_f at 0.44, 0.55 and 0.60 consists of both yellow and blue colour bands. As suggested by Guzelmeric et al. (2017) and Bertrams et al. (2013) the yellow zones corresponded to flavonol and flavone, blue zones to flavonol and flavanone. The results of this study at least suggest that the propolis extracted in 70% ethanol contains flavonol and flavanone, while the propolis extracted in 95% ethanol contains flavonol and flavone, based on the colour detection at 366 nm. Specifically, flavonoid is divided into two groups, derived from aglycones and glycoside. The examples of flavonoid aglycones are flavones, flavonol, isoflavone, flavonones, dihydroflavonol, chalcones, and aurones, while the flavonoid glycosides are flavonol, flavones, and flavonones. The flavonoid glycoside is a polar flavonoid and increases its solubility in aqueous alcohol. Based on the extraction rules, the polar solvent extract attracted polar compounds, while less polar compound attracted less polar compound. In this study, 70% ethanol is an aqueous alcohol and suspected that 70% ethanol could extract flavonoid from glycosides.

CONCLUSION

Chemical fingerprinting analysis by HPTLC indicates that propolis extracted in 70% ethanol consists of more polar compounds. Meanwhile less polar compounds were extracted in 95% ethanol. The chemometric analysis by PCA and HCA found that the use of different percentages of solvent, extraction methods and extraction times affect the chemical composition significantly. Findings from this study showed that the choice of extraction solvent, procedure and duration of extraction is important to obtain desired extracts.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NBMZ performed the experiments and drafted the manuscript. KSM designed the experiment, reviewed and approved the manuscript and carried out final editing of this paper. AA, and ZMR facilitated the chemometric analysis. All authors read and approved the final version.

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