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Comparison of Total Phenolic Content and Antioxidant Activity of Indonesian Propolis Extracted with Various Solvents

(Perbandingan Kandungan Fenolik Total dan Aktivitas Antioksidan Propolis Indonesia yang Diekstraksi dengan Berbagai Pelarut)

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Abstract: Propolis is a widely used medicine that may be found in both its pure form and when combined with other natural ingredients in over-the-counter preparations, cosmetics, and health foods. Some customers develop alcohol sensitivity after using ethanol, which is a common solvent to extract propolis. As propolis solvents, various Natural Deep Eutectic Solvents (NaDES) were examined in this study. Choline chloride, propylene glycol, glycerol, citric acid, and 1,2-propanediol were combined in molar ratios to create some of the NaDES that were employed. Propolis and solvents were used in a kinetic maceration ratio for the extraction process. The Folin-Ciocalteu method was used to measure the total phenolic content (TPC), and the DPPH assay was used to measure antioxidant activity. In ethanol and NaDES solvents, the TPC of propolis extract ranged from 136.52±27.9 to 365.8±20.54 mgGAE/g. The values for antioxidant activity were 45.94 to 183.76 ppm. The propolis extract with the lowest IC_{50} and the highest TPC content was choline chloride-glycerol (CCG). It was discovered that the optimum NaDES solvent for extracting propolis was CCG-based. NaDES may be a potential solvent for use in Indonesian propolis, both as an extraction medium and as a formulation element.
Keywords: Antioxidant, extraction, Indonesian propolis, NaDES, total phenolic

Abstrak: Propolis telah banyak digunakan di seluruh dunia, tersedia dalam bentuk tunggal atau dikombinasikan dengan produk lainnya dalam bentuk obat bebas, obat tradisional, komsetik, maupun suplemen makanan. Etanol merupakan pelarut umum yang digunakan untuk mengekstrak propolis, meninggalkan efek *aftertaste* yang kuat dan menyebabkan intoleransi alkohol bagi sebagian konsumen. Dalam penelitian ini, berbagai pelarut *Natural Deep Eutectic Solvent* (NaDES) digunakan sebagai pelarut propolis. Beberapa komponen NaDES yang digunakan dengan komposisi antara lain kolin klorida, propilen glikol, gliserol, asam sitrat, dan 1,2-propanediol dengan perbandingan rasio molar. Ekstraksi dilakukan dengan maserasi kinetik (1:5 m/V). Kandungan total fenolik (TPC) ditentukan dengan metode Folin-Ciocalteu, dan aktivitas antioksidan ditentukan dengan uji DPPH. TPC ekstrak propolis dalam pelarut etanol dan NaDES berkisar antara 136,52±27,9 hingga 365,8±20,54 mgGAE/g. Nilai aktivitas antioksidan berkisar antara 45,94 hingga 183,76 ppm. Ekstrak propolis kolin klorida-gliserol memiliki kandungan TPC tertinggi dan aktivitas antioksidan dengan IC_{50} terendah. Diperoleh hasil bahwa pelarut NaDES berbasis CCG adalah yang terbaik untuk ekstraksi propolis. NaDES dapat menjadi pelarut yang prospektif untuk digunakan pada propolis Indonesia, baik sebagai media ekstraksi maupun komponen formulasi.

Kata kunci: Antioksidan, ekstraksi, NaDES, propolis Indonesia, total fenolik

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INTRODUCTION

PROPOLIS is made from resin that bees collect from flowers, shoots, and plant exudates[1]. The bees transport the resin to the beehive, where it is combined with beeswax, the β -glycosidase enzyme found in saliva, and other ingredients to create raw propolis [2]. Propolis has been widely used since ancient times because it has many health benefits including antibacterial, anti-inflammatory, antiviral, antioxidant, antiprotozoal, antitumor, anesthetic, anticancer, antiseptic, antimutagenic and antihepatotoxic[3]. Propolis has high antioxidant activity due to its phenolic content, according to previous research[4]. The compounds found in propolis vary greatly depending on the biodiversity of the environment surrounding the beehive[5].

There are two types of bees that produce propolis, sting honeybee and stingless bee. Numerous studies have shown multiple biological activities of the propolis produced by *Apis mellifera* honeybees and stingless bees[6]. The propolis collected in South Sulawesi, Indonesia, from the stingless bee species *Tetragonula sapiens* served as the primary focus of this investigation. Compounds such as cycloartenol, ambonic acid, mangiferonic acid, ambolic acid, sesamin, curcumin, 8-epi-helenalin, and kushenol F were found in the propolis that was collected from stingless bees in Indonesia[4,7].

To be able to get propolis' biological properties, an extraction process must be carried out because raw propolis cannot be directly used or consumed because it still contains a lot of impurities[8]. Therefore, it is necessary to have an extraction technique to obtain bioactive compounds in propolis to be utilized into various products. Using solvents, extracting phytochemical components is the first step in removing undesirable contaminants and isolating beneficial molecules to produce phytoproducts. Organic solvents, which were once widely employed in natural product extraction are now used in enormous quantities, resulting in higher extraction costs. Organic solvents are relatively hazardous and have the potential to pollute the air, contributing to global warming. Ethanol is an effective solvent to separate propolis bioactive compounds. However, ethanol has disadvantages such as leaving a strong taste, limited application in the cosmetic and pharmaceutical industries, and the presence of alcohol intolerance for some consumers. Therefore, other alternative solvents are needed for propolis extraction[9].

The organics solvent has limitations, which green solvents may be able to overcome. They were relatively safe and environmentally friendly[10]. Green solvents have several advantages, including being simple to prepare (using environmentally friendly materials and techniques), less harmful, low energy, recyclable, and biodegradable[11]. One of the alternative solvents proposed to replace conventional ethanol solvents was Natural Deep Eutectic Solvent (NaDES)[12]. A combination of two or more NaDES components, such as amino acids, organic acids, sugars, or choline derivatives, that, in some compositions, have a high melting point decrease and become liquid at room temperature[13]. NaDES has several benefits over conventional solvents, including biodegradability, non-toxicity, non-volatility, high extraction capability, reasonable cost, and ease of preparation[14]. In this study, several NaDES solvents were compared with ethanol solvents in various combinations. The propolis extract's phenolic compound concentrations and antioxidant capacity were the factors examined.

MATERIALS AND METHODS

MATERIALS. 2,2-Diphenyl- 1-picrylhydrazyl (DPPH), choline chloride (≥98%), propylene glycol (≥99.5%), glycerol (≥99.5%), and citric acid, Foline-Ciocalteu, and sodium carbonate were provided from Sigma Chemical Co. (Sigma Aldrich, Singapore). Gallic acid, methanol, ethanol 96%, and 1,2-propanediol were purchased from Merck Co. (Darmstadt, Germany).

Sample of Propolis. The *Tetragonula sapiens* stingless beehive was collected from the Luwu Utara district of South Sulawesi Province, Indonesia, and used as the source material for propolis. The types of propolis were a mixed of honeypot inside the beehive and from outside the honeypot. The beehives were cleaned up from its honey, bee pollen, and bee bread. The fresh raw material was kept in the freezer -5 ºC until it was extracted.

METHODS. NaDES Preparation. NaDES components were prepared as shown in Table 1 with a composition of molar ratio. Each NaDES composition were mixed using a magnetic stirrer (300 rpm) with a temperature of 70 ºC until reach a homogeneous and transparent solution. Then the viscous liquid of NaDES obtained was used for propolis extraction media[9].

Propolis Extraction. A total of 50 g of frozen propolis was grinded until it reached a smaller particle size. Then fresh raw propolis was homogenized with 250 mL of 1:5 NaDES solvent. Extraction was carried out by kinetic maceration for 8 hours at room temperature with constant stirring at 300 rpm. After obtaining the extract, it was filtered using Whatman Grade 1 filter paper that had a pore size of 11µm and a Buchner funnel. The ethanolic propolis extract

Table 1. NaDES composition.

NaDES component	Molar ratio	Code
Choline chloride, glycerol, and water	1:2:1	CCGW
Choline chloride and glycerol	1:2	CCG
Choline chloride and propylene glycol	1:2	CCPG
Citric acid and 1,2-propanediol	1:4	CAPD

was also made by using the same methods, with ratio propolis sample and ethanol 96% was 1:5.

Determination of Total Phenolic Content. A series concentration of standard solution of gallic acid were made starting from 0-250 g/mL in methanol. Each of the standard solutions was pipetted into a test tube at a volume of 0.5 mL. Follin's reagent (1:10) was then added in 5 mL, vortexed, and left to stand for 5 minutes. 4 mL of 1M Na_2CO_3 was then added, and the mixture was vortexed. The mixture was then given 15 minutes to stand at room temperature before being measured in a UV-Vis Bell M-90 spectrophotometer (Bel Engineering, Italy) at a wavelength of 765 nm. The equation $Y = ax + b$ and R^2 closed to 1 was determined by measuring the calibration curve of standards (gallic acid), where Y is the yield of GAE (total phenolic content) and X is the absorbance of gallic acid or samples. Every determination was made in triplicate[4].

Antioxidant with DPPH Assay. Quantitative analysis to determine antioxidant activity was carried out using DPPH reagent[4]. The series concentration of each propolis extract sample were made by dissolving the sample in methanol to varies concentration. The sample was then vortexed to ensure an even distribution. The sample solution was then mixed with 2 mL of DPPH 0.2 mM solution. The solution was then vortexed and incubated in the dark for 30 minutes at 37 ºC. The absorbance was then measured using a spectrophotometer with a 517 nm wavelength. The antioxidant activity of the sample was determined by calculating the percentage of DPPH absorption inhibition with Equation 1 and the magnitude of the DPPH radical absorption inhibition:

$$\%\text{DPPH}scavening = \left[\frac{A_{\text{blanko}} - A_{\text{sampel}}}{A_{\text{blanko}}}\right] \times 100\% \qquad (1)$$

Where A_{sample} was the absorbance of propolis sample and A_{blank} was the absorbance of ethanol added with DPPH. The color of DPPH will change from purple to clear yellowish with the addition of antioxidants. The sample concentration that inhibit DPPH bu 50% was calculated and expressed as an IC_{50} value (ppm). All determinations were presented in mean ± SD, carried out in triplicate.

Phytochemical Analysis with LC-MS/MS. The phytochemical compound in propolis was analyzed by using LC-MS/MS methods described in the previous research with modification[4]. Five miligrams of ethanolic extract of propolis were mixed with 1 mL of methanol and filtered through a 0.2 μm PTFE membrane. Each of the propolis NaDES extracts was also passed through a 0.2 μm PTFE barrier. At IPB University's Advanced Research Laboratory, the analytical LC-MS/MS tests were done with a Thermo Scientific UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (Thermo Fisher Scientific, Waltham, MA, USA). The temperature of the column Accucore C18, 100 x 2.1 mm, 1.5 μm (ThermoScientific) was set to 30°C. The rate of flow was 0.20 mL per minute. The HPLC tests were done with a linear gradient solvent system made up of A:B (0.1% formic acid in H_2O: 0.1% formic acid in acetonitrile) as follows: t= 0-1 min, 5% B; t= 1-25min, 5-95% B; t= 25-28 min, 95%B; t= 28-30 min, 5% B. The amount injected was 2.0 μL. Under positive electrospray, the MSE function ESI ionization type was used to get the data. The range of acquisition was 100 to 1500 m/z. Data acquisition and data analysis use mzcloud dan chemspider.

RESULTS AND DISCUSSION

The Characteristics of the Propolis Extract. As depicted in Figure 1, the color characteristics of propolis extract. Extraction of propolis using ethanol solvent produces a dark brown extract with a fine precipitate (Figure 1A). In CCGW solvent (Figure 1B) the extract was light brown, in CCG solvent (Figure 1C) the extract was dark brown, in CCPG solvent (Figure1D) the extract was brown but there were still many undissolved propolis particles, in CAPD solvent (Figure IE) the extract was yellowish brown. Propolis was more soluble in ethanol than other solvents because the polarity of ethanol can attract compounds with a wide variety of polarities.

After the extraction process, the samples were left overnight at room temperature to precipitate solid particles for easier filtration process. The filtration was done with Whatman Grade 1 filter paper with a pore size of 11 μm. The duration of the filtration process for the entire sample varies depending on the solvent used. NaDES solvent has a high viscosity so that the filtration process lasts longer than the samples extracted with ethanol solvents. After the filtration process, the extract volume was obtained as listed in Table 2. V_o is the volume of the extract before the filtration process while V_T is the volume of the extract obtained after

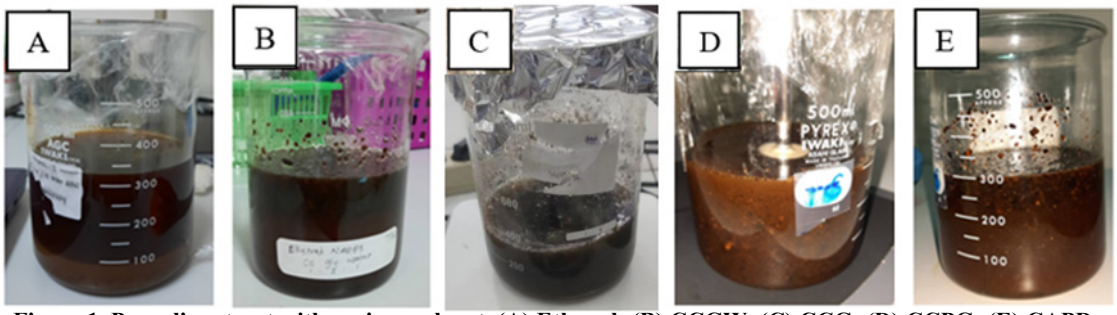


Figure 1. Propolis extract with various solvent. (A) Ethanol; (B) CCGW; (C) CCG; (D) CCPG; (E) CAPD.

the filtration process. It was found that ethanol solvent gave the greatest gain.

Propolis ranges in color from yellow to green to red to dark brown, depending on the plant the bee consumes[15]. *Tetragonula sapiens* beehives were dark brown in color. When fresh, raw propolis was hard like wax, but when warm, it softened and became very sticky[2]. The raw propolis material was frozen before extraction so that it would be simple to crush into smaller particles. By doing this, the particles' surface will be increased, making the extraction process easier. Propolis extraction was generally carried out with organic solvents such as ethanol, methanol, or chloroform. The best solvent used to extract propolis on a commercial scale so far was ethanol[16].

Ethanol is one of the most widely used solvents in propolis extraction because it is considered economical and easy to obtain. This method is suitable for obtaining propolis extracts with low wax content and high total phenolic content.[17] Propolis extraction with ethanol solvent resulted in a higher yield than water or several solvents because the wax content in propolis was more soluble in ethanol[18,19]. However, ethanol solvents have disadvantages such as leaving a strong taste, limited application in the pharmaceutical cosmetic industry, or alcohol intolerance for consumers. Therefore, the development of propolis extraction using an effective non-ethanol solvent was urgently needed[9].

A natural deep eutectic solvent was also developed for propolis extraction (NaDES). Deep eutectic solvent (DES) is a mixture of two or more solid organic or inorganic compounds that, at optimal temperature and stirring time, melt and form a stable eutectic compound. DES compounds are linked by hydrogen bonds and Van der Waals bonds[20]. The most popular form of DES uses choline chloride as the quaternary ammonium salt and urea, sugar poly-alcohols, organic acids, and phenolic compounds as hydrogen bond donors[21].

When compared with conventional solvents, the use of NaDES has several advantages, including the following: it is biodegradable, non-toxic, non-volatile, has a high extraction capability, is relatively inexpensive, and is easy to prepare[22]. Meanwhile, the weakness of NaDES was its high viscosity (>100 cP) at room temperature due to its hydrogen bonding so that NaDES solvent is difficult to apply on an industrial scale. This is usually overcome by adding water to the solvent mixture. However, the addition of water must be done carefully because water can break the hydrogen bonds so that NaDES can lose its eutectic properties.

Table 2. Propolis extract volume obtained.

Solvent	V_0 (mL)	V_T (mL)	Extract Gain (%)
Ethanol	250	210	84
CCGW	250	171	68.4
CCG	250	168	67.2
CCPG	250	177	70.8
CAPD	250	161	64.4

Several NaDES solvents that have been used for propolis extraction were choline chloride, propylene glycol, and citric acid[9,23]. Choline chloride was a quaternary ammonium salt compound, classified as GRAS (Generally Regarded as Safe), and widely used as a dietary supplement[24]. Propylene glycol is propanediol, a clear, colorless, and hygroscopic liquid composed of propane with hydroxyl groups replacing the hydrogens at positions 1 and 2. Propylene glycol is an organic solvent and diluent that is used in pharmaceuticals and a variety of other industrial applications. The Food and Drug Administration (FDA) has designated propylene glycol as a "generally recognized safe" food additive. Citric acid occurs naturally in many plants, animal tissues, and physiological fluids. Citric acid can be found in small amounts in a variety of fruits and vegetables, but it is abundant in citrus fruits, particularly lemons and limes[25,26]. Citric acid compounds are polar and their combination with propylene glycol is quite effective for extracting flavones and flavonol compounds[9]. However, previous studies have investigated the NaDES solvent and it is known that the CCPG, CCGW and CAPD solvents have polarities that are not much different from 70% ethanol[9,23].

Total Phenolic Contents Analysis. The calibration curve equation obtained when measuring the gallic acid standard curve was y=0.0055x+0.011. Because the sample solution of propolis NaDES extract was too concentrated, it was diluted 100 times, except for the CAPD EP sample, it was diluted 10 times. Table 3 displays the results of the total phenolic content of propolis extract.

The stingless bees collect plant resins in order to make propolis (cerumen and geopropolis). Propolis contain phenolic compound as secondary metabolites that are widely found in plants[3]. Secondary metabolites function as signaling compounds, attract pollinating agents for seed dispersal and protect plants from oxidants and ultraviolet radiation. Phenolic compounds can fight oxidative stress which can cause several metabolic disorders and various diseases, such as obesity, diabetes, and cardiovascular disease[27]. Phenolic compounds have properties as hydrogen or electron donor agents so that they have potential as antioxidants. The content of flavonoids and total phenolics related to propolis' biological activity can be used to calculate the phytochemical content of propolis[28].

The Antioxidant Activity of Propolis Extract. Propolis extract can fight free radicals. Table 4 showed the results of the antioxidant test done on five samples of propolis extract. The antioxidant power of propolis extract with CCG solvent was stronger than with ethanol solvent (EEP). It is important to note that, as shown in Table 3, propolis has protective properties because it contains phenolics[29]. It is thought that the antioxidant action of its compounds will help lower the risk of diseases like cancer and heart disease.

In multiple systems, caffeic acid phenethyl ester (CAPE), one of the phenolic components of propolis, can inhibit the production of reactive oxygen species[30]. Previous study compared the antioxidant activity of propolis from 14 different countries and regions. It was discovered that propolis with potent antioxidant properties contains antioxidant compounds such as kaempferol and phenethyl caffeine[31].

Three new solvents in propolis extraction namely NaDES CCPG, CCGW, and CAPD have lower antioxidant activity than extracts with ethanol and CCG solvents. However, in this study, it was found that low antioxidant activity indicated that the extraction performance was also lower than ethanol and CCG solvents.

The number and position of the hydroxyl groups in phenolic compounds can influence their ability to act as radical scavengers. The number and position of hydroxyl groups in phenolic acid compounds are directly related to their ability to scavenge free radicals[32]. When there are fewer than four phenolic hydroxyl groups on the benzene ring, phenolic acid's antioxidant activity is proportional to the number of hydroxyl groups. It is possible that in the NaDES sample there are only a few phenolic hydroxyl compounds so that they have low antioxidant activity. Another possible cause is the difference in the extraction technique used, where in this study homogenization was used while in previous studies ultrasonication was used. However, CCG solvent was quite prospective among other NaDES solvents because it has relatively higher TPC and antioxidant activity.

Phytochemical Analysis with LC-MS/MS. In Figures 2 and 3, we exhibit the results of the UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS analysis used to identify the individual chemical components of the propolis extracts CCG EP and EEP.

The primary compounds of CCG EP and EEP were identified using UHPLC Tandem HRMS analysis,

Table 3. Total phenolic content of propolis extract using various solvent.

Sample	TPC (mgGAE/g)
Ethanolic Extract Propolis (EEP)	352.52±5.12
CCGW Extract Propolis (CCGW EP)	136.52±27.9
CCG Extract Propolis (CCG EP)	365.8±20.54
CCPG Extract Propolis (CCPG EP)	293.03±91.09
CAPD Extract Propolis (CAPD EP)	278.51±0.45

Data were presented in mean±SD, n=3.

Table 4. Antioxidant activity of propolis extract with various solvent.

Sample	IC_{50} (ppm)
EEP	69.96
CCGW EP	183.76
CCG EP	45.94
CCPG EP	128.88
CAPD EP	135.99

which involved comparing the RT, molecular weight, and MS fragmentation patterns with the literature database in Mzcloud and Chemspider.The primary compounds of CCG EP and EEP were identified using UHPLC Tandem HRMS analysis, which involved comparing the RT, molecular weight, and MS fragmentation patterns with the literature database in Mzcloud and Chemspider. There was a total of 63 compounds identified in CCG EP and 39 compounds in EEP. Some of the compounds above have an important role in inhibiting free radicals as antioxidant compounds as resumed in Table 5.

The results of the study reported that putative compounds in CCG EP and EEP were quite different. CCG EP were shown more compound identified than EEP. DL-isoleucine[33], an aliphatic protein amino acids, and L-phenylalanine[34], an aromatic amino acids, which were contained in CCG EP have antioxidant activities. Kaempferol is a class of flavonoids, namely flavonols which are found in propolis. Kaempferol is thought to have antioxidant-like, neuroprotective activity that is used globally as a sedative and has been shown to have a calming effect[35]. This is the same as the previous study conducted by Falcao et al which contained amino acids and kaempferol compounds in Portuguese propolis extract[36]. Kynurenic acid[37] and Cinnamodial[38], a sesquiterpene, has antioxidant activity. Of the 40 compounds in EEP, there are major compounds that have a role as antioxidants, namely DL-Carnitine[39], DL-Stachydrine[40], Betaine[41], D-(+)-Proline[42], Kynurenic acid[37], and L-isoleucine[33].

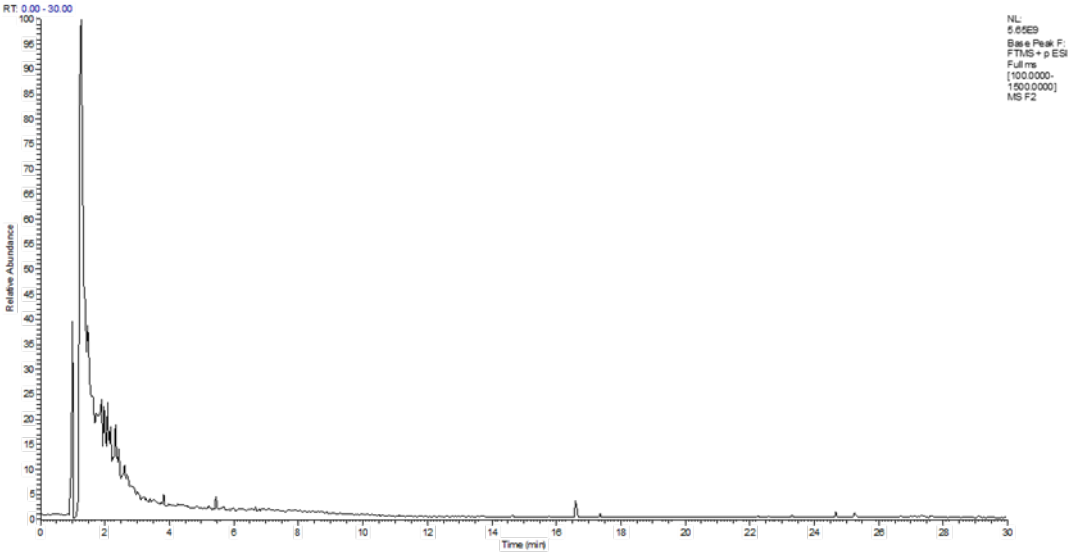


Figure 2. UHPLC vanquish tandem Q exactive plus orbitrap HRMS pofile of CCG EP.

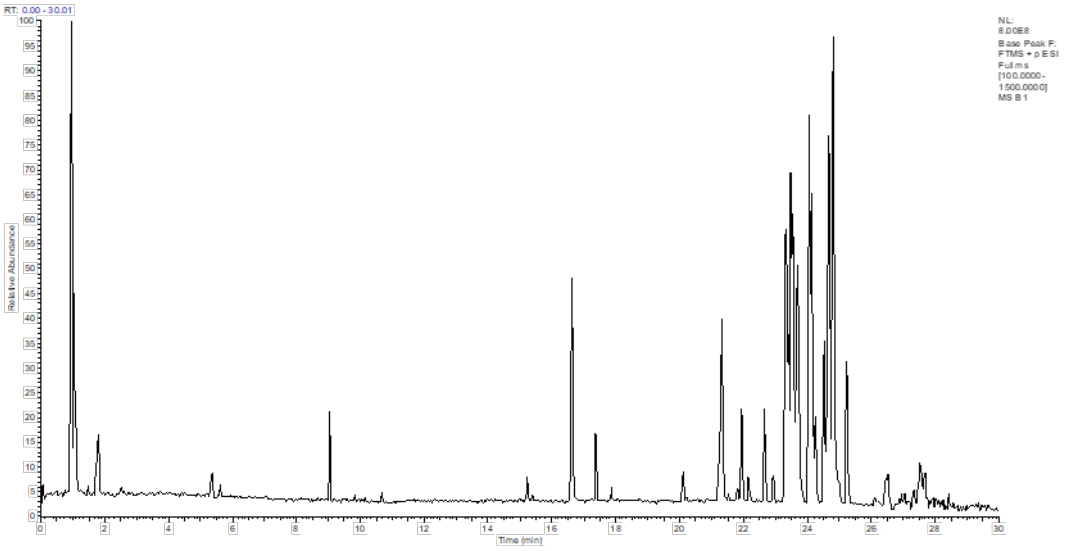
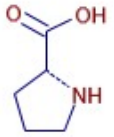
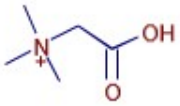
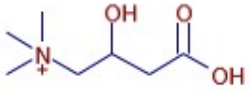
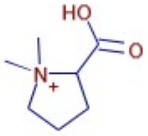
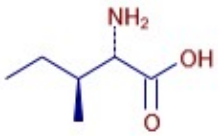
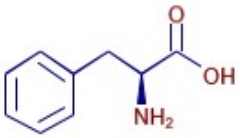
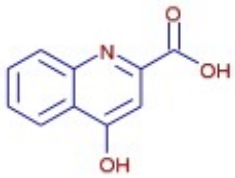
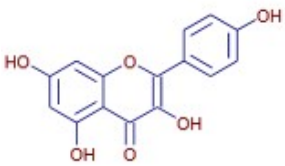
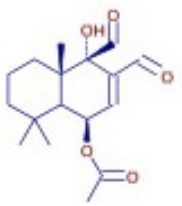


Figure 3. UHPLC vanquish tandem Q exactive plus orbitrap HRMS profile of EEP.

Table 5. Identification of chemical compounds in propolis extract.

Rt (min)	Formula	Compound	Structure	Molecular weight	CCG EP	EEP
1.08	$C_5H_9NO_2$	D-(+)-Proline		115.06339	Not identified	Identified
1.084	$C_5H_{11}NO_2$	Betaine		117.07906	Not identified	Identified
1.107	$C_7H_{15}NO_3$	DL-Carnitine		161.1049	Not identified	Identified
1.127	$C_7H_{13}NO_2$	DL-Stachydrine		143.09448	Not identified	Identified
1.62	$C_6H_{13}NO_2$	L-isoleucine		131.0946	Identified	Identified
2.43	$C_9H_{11}NO_2$	L-phenyl alanine		165.0790	Identified	Not identified
5.46	$C_{10}H_7NO_3$	Kynurenic acid		189.0426	Identified	Identified
12.92	$C_{15}H_{10}O_6$	Kaempferol		286.0477	Identified	Not identified
16.63	$C_{17}H_{25}O_5$	Cinnamodial		308.1624	Identified	Not identified

CONCLUSION

The TPC of stingless bee *Tetragonula sapiens* propolis extract in ethanol and NaDES solvents ranged from 136.52±27.9 to 365.8±20.54 mgGAE/g. The levels of antioxidant activity were measured between 45.94 and 183.76 ppm. Propolis extract in the form of choline chloride-glycerol (CCG) had the lowest IC_{50} and the highest TPC concentration and strong antioxidant activity. It was determined that the use of a CCG-based NaDES solvent for the extraction of Indonesian propolis holds a lot of promise. It has potential as an extraction media and a formulation component in Indonesian propolis.

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