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# Experimental study and modeling on supercritical $CO_2$ extraction of Indonesian raw propolis using response surface method: Influence of pressure, temperature and $CO_2$ mass flowrate on extraction yield

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ABSTRACT

It is well-known that propolis shows potential as an antioxidant. A common method to extract propolis is organic solvent extraction which has drawbacks such as long extraction times and solvent residues. To overcome this, the SC-$CO_2$ extraction method was applied in this work. The milled raw propolis was fed into a vessel. The extraction consists of two stages, static (60 min) and dynamic (240 min). During the extraction, the process variables (temperature, pressure and $CO_2$ flow rate) were set to constant. When the extraction time was completed, the liquid product was prepared for analysis using HPLC. This work provides the amount of propolis wax was 7.02 wt% and the highest yield of propolis extract was 14.4 wt%. The effect of the variables on the yield was experimentally investigated and modeled using response surface method approach. The extract containing bioactive compounds such as galangin and CAPE was proved to have 24.77 μg/mL of $IC_{50}$ which is closer to $IC_{50}$ of ascorbic acid.



## 1. Introduction

Propolis is well known in traditional medicine of many countries (Popova et al., 2010). Krell (1996) reported that the composition of propolis is structured by resins (45–55%), waxes and fatty acids (25–35%), essential oils (5%), pollen (5%), and other organics and minerals (5%). More than 200 chemical compounds have been found in propolis such as flavonoids, terpenoids, aromatic acids, fatty acids, esters, phenols, aldehydes and ketones (Graikou et al., 2016; Piccinelli et al., 2013). According to its constituents, the propolis can be categorized into two groups. The first group is of Brazilian propolis type which contains a large amount of *p*-coumaric acid and its derivatives. The second group is the European propolis type which is rich in flavonoids (Kumazawa et al., 2002, 2004).

Even the composition of propolis varies according to geographic region, the constituents such as *p*-coumaric acid, galangin, chrysin, caffeic acid, cinnamic acid, ferulic acid, and caffeic acid phenyl ester (CAPE) are found as major extractable constituents in propolis. Due to its content, propolis provides the role of antibacterial, antibiotic, antiviral, anti-inflammatory and even antioxidant compounds (Popova et al., 2010; Graikou et al., 2016; Al-Ghamdi et al., 2017).

Commonly, in Europe, propolis is collected from honeybee species named *Apis* sp. On the contrary, Asian *Apis* does not produce propolis. In the meantime, a stingless bee species called *Trigona* sp. produces more propolis and less honey

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(Fatoni et al., 2008). In Indonesia, *Trigona* sp. is found in several islands such as Java, Kalimantan, Sulawesi, Bali and Lombok (Hasan et al., 2014; Rasmussen and Cameron, 2010). Fatoni et al. (2008) and Hasan et al. (2014) found a high-level concentration of flavonoid in Indonesian propolis from *Trigona* sp. Further, the chemical composition of Indonesian propolis from *Trigona* sp. is yet to be reported.

Propolis cannot be directly consumed as a crude material. It must be extracted with suitable solvents, to separate the unwanted material such as wax, protecting the bioactive compounds. The common extraction method is conventional solvent extraction using an organic solvent. The most commonly used solvent is ethanol, though organic solvents (including propylene glycol, methanol, ethyl acetate, chloroform, and n-hexane, water and olive oil) have been explored as well.

Cunha et al. (2004) performed the extraction of six samples of green propolis from the Southeastern region of Brazil using maceration and Soxhlet method in ethanol. The results showed the Soxhlet method obtained a higher yield of propolis extracts (57.65 wt%) than maceration (40.43 wt%). The highest phenolic content was 13.34 wt%. Some compounds such as caffeic acid, ferulic acid, *p*-coumaric acid, and cinnamic acid derivatives were recognized in the extract. de Funari et al. (2007) also performed the Soxhlet extraction of propolis from the Southeastern of Brazil using ethanol and methanol. This work gave 53.73 wt% methanolic extract propolis (MEP) which was higher than 38.34 wt% ethanolic extract propolis (EEP). The methanolic extract found 7.39 wt% of the phenolic content and 2.64 wt% of the flavonoid content. Both MEP and EEP consisted of artepillin C (DHCA), *p*-coumaric acid, cinnamic acid, chlorogenic acid, caffeic acid, kaempferol, kaempferide, and isosakuranetin. The water was also employed by Chen et al. (2007) to extract flavonoids and phenolic acids from Brazillian propolis using hot-pressurized fluid extraction in presence of 29% natural surfactant. The water-soluble extract contained naringenin, quercetin, kaempferol, pinocembrin, galangin, chrysin, acacetin, CAPE, caffeic acid, ferulic acid, *p*-coumaric acid, and trans-cinnamic acid. Pujirahayu et al. (2014) used the maceration method with ethanol, VCO, olive oil and propylene glycol as solvents to extract Indonesian propolis from Southeastern Sulawesi. Even the extract composition was not mentioned, the extract was reported containing flavonoids and phenolic acids. The highest yield was 18.33 wt% in ethanol extraction. The VCO, olive oil and propylene glycol gave a yield of 14.22 wt%, 14.06 wt%, and 15.88 wt%. Additional reports on solvent extraction of propolis are briefly given in Table 1.

In fact, the solvent extraction method has major drawbacks such as time-consuming and solvent residues in the extract. Specifically, the ethanolic extract of propolis (EEP) contains significant amounts of wax which are an inedible and allergenic matter (You et al., 2002).

To overcome the weaknesses of solvent extraction, supercritical $CO_2$ (SC-$CO_2$) extraction is proposed. It is well-known that SC-$CO_2$ extraction is an attractive and powerful method. The solvent is easily and quickly separated from the product (del Valle, 2015; Machado et al., 2015; Reverchon and De Marco, 2006; Subroto et al., 2017). The extraction of propolis by SC-$CO_2$ is a promising alternative technique to achieve high-quality extracts (De Melo et al., 2014; Knez et al., 2014; del Valle, 2015; Duba and Fiori, 2015).

Some research focusing on SC-$CO_2$ extraction of propolis has been published. Biscaia and Ferreira (2009) reported a 12% yield of solubilized propolis was achieved during one step stage extraction. The yield decreased to 8.9% when two-step stages extraction was performed. De Zordi et al. (2014) performed the SC-$CO_2$ to extract polyphenols from Italian propolis and to study the effect of extraction variables on yield. The chemical composition of the extract was also observed. Some compounds such as apigenin, CAPE, caffeic acid derivatives, chrysin, galangin, *p*-coumaric acid derivatives, pinobanksin, pinobanksin derivatives, pinocembrin, quercetin and quercetin derivatives were identified in extract composition. At optimum conditions of 317 bar, 40 °C and 4 h, the extraction yield was 2.5 wt% higher than the yield of 55.8 wt% achieved at 130 bar. The other SC-$CO_2$ extraction of propolis from Brazilian green propolis was published by Machado et al. (2015). This work stated that 2.972% of the accumulated extract contained 79.67% total phenol.

A different feedstock, ethanol extracted propolis, was used by Paviani et al. (2010a,b) to achieve 39.5 wt%. In the interim, a different type of propolis, a poplar-type, was also used to extract some bioactive compounds such as *p*-coumaric acid, pinobanksin, chrysin, pinocembrin, pinostrobin, and galangin. The highest yield of 10.28 wt% was produced at optimum condition ($T = 60$ °C, $P = 300$ bar). This condition also provided the highest amount of *p*-coumaric acid (1.52 μg/mg), pinobanksin (1.55 μg/mg), pinocembrin (47.24 μg/mg), and galangin (10.25 μg/mg). The highest yield of chrysin (2.03 μg/mg) and pinostrobin (79.56 μg/mg) was achieved in different condition ($P = 337$ bar, $T = 50$ °C)(Kuś et al., 2018).

More studies on supercritical carbon dioxide extraction of propolis are presented in Table 2.

Further, supercritical extraction of propolis from Indonesian *Trigona* sp. has never been published. This work reports supercritical extraction of propolis studying on design approach, characterization and antioxidant activity.

## 2. Materials and methods

### 2.1. Materials

Raw propolis from *Trigona* sp. was obtained and used directly from Balai Penelitian Teknologi Hasil Hutan Bukan Kayu (Research Institute for Non-Timber Forest Products), Mataram, Lombok, Indonesia. Raw propolis was collected in the period of April–September of 2017–2018. Food-grade liquid carbon dioxide (purity 99.99%) was supplied in a cylinder tube by PT Inter Gas Mandiri (Cikarang, Indonesia). Methanol (purity ≥ 99.9%), ethanol (purity ≥ 99.5%), acetic acid (purity ≥ 99.7%), ascorbic acid (purity ≥ 99%), and DPPH solution were purchased from Sigma–Aldrich Singapore. Myricetin (purity ≥ 96%), pinobanksin (purity ≥ 95%), kaempferol (purity ≥ 90%), and chrysin (purity ≥ 99.0%), were bought from Sigma–Aldrich Singapore. Quercetin (purity ≥ 95%), ferulic acid (European Pharmacopoeia (EP) Reference Standard) and caffeic acid phenyl ester (purity ≥ 98%) were purchased from Sigma–Aldrich Belgium. Galangin (analytical standard) was purchased from Supelco Sigma–Aldrich Belgium. Caffeic acid (purity ≥ 98%), *p*-coumaric acid (purity ≥ 95%), and cinnamic acid (purity ≥ 99%) were bought from Acros Organics Belgium.

**Table 1 – Propolis extraction using solvent extraction.**

| $w_{propolis}$, g | Solvent | Method | Condition | Extracted compounds | Yield, wt% | Ref. |
|---|---|---|---|---|---|---|
| 20 | Ethanol absolute 400 mL | Soxhlet | $T = 60\,^{\circ}C$, $t = 24$ h | Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid derivatives | 57.65 | Cunha et al. (2004) |
| 20 | Ethanol absolute 100 mL | Maceration | $T$ = not available (n/a), $t = 480$ h | Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid derivatives | 40.43 | Cunha et al. (2004) |
| 90 | Ethanol 190 mL | Maceration | $T$ = n/a, $t = 2160$ h | Artepillin C (DHCA), *p*-coumaric acid, cinnamic acid, chlorogenic acid, caffeic acid, kaempferol, kaempferide, isosakuranetin | 38.34 | de Funari et al. (2007) |
| 10 | Methanol 150 mL | Soxhlet | $t = 8$ h | Artepillin C, *p*-coumaric acid, cinnamic acid, chlorogenic acid, caffeic acid, kaempferol, kaempferide, isosakuranetin | 53.73 | de Funari et al. (2007) |
| 100 | $H_20$ | Hot-pressurized | $T = 120\,^{\circ}C$, $P = 4.5$ bar, | Naringenin, quercetin, kaempferol, pinocembrin, galangin, chrysin, acacetin, CAPE, caffeic acid, ferulic acid, *p*-coumaric acid, trans-cinnamic acid. | 35.32 mg/mL | Chen et al. (2007) |
| 25 | Ethanol 70% | Solvent | $T = 40\,^{\circ}C$, $t = 168$ h | Flavonoid, phenolic acid | 18.33 | Pujirahayu et al. (2014) |
| 25 | Olive oil | Solvent | $T = 40\,^{\circ}C$, $t = 168$ h | Flavonoid, phenolic acid | 14.06 | Pujirahayu et al. (2014) |
| 25 | VCO | Solvent | $T = 40\,^{\circ}C$, $t = 168$ h | Flavonoid, phenolic acid | 14.22 | Pujirahayu et al. (2014) |
| 25 | Propylene glycol | | $T = 40\,^{\circ}C$, $t = 168$ h | Flavonoid, phenolic acid | 15.88 | Pujirahayu et al. (2014) |
| 5 | $H_2O$ 150 mL | Soxhlet | $T = 60\,^{\circ}C$, $t = 6$ h | n/a | 14.3 | Biscaia and Ferreira (2009) |
| 5 | Ethanol 150 mL | Soxhlet | $T = 60\,^{\circ}C$, $t = 6$ h | n/a | 60 | Biscaia and Ferreira (2009) |
| 5 | Ethyl acetate 150 mL | Soxhlet | $T = 60\,^{\circ}C$, $t = 6$ h | n/a | 59.7 | Biscaia and Ferreira (2009) |
| 5 | $CHCl_3$ 150 mL | Soxhlet | $T = 60\,^{\circ}C$, $t = 6$ h | n/a | 73 | Biscaia and Ferreira (2009) |
| 5 | n-Hexane | Soxhlet | $T = 60\,^{\circ}C$, $t = 6$ h | n/a | 17 | Biscaia and Ferreira (2009) |
| 3 | Ethanol 10 mL | solvent | $T$ = room temperature (RT), $t = 24$ h | Artepillin C, 3-prenyl-4-hydroxycinnamic acid (PHCA), *p*-coumaric acid, kaempferide | 39.5 | Paviani et al. (2012) |
| 1 | Ethyl acetate 120 mL | Soxhlet | $t = 16$ h | artepillin C | 55.6 | Chen et al. (2009) |
| 25 | Methanol 250 mL | Solvent | $T = 60\,^{\circ}C$, $t = 1$ h | Caffeic acid, coumaric acid, ferulic acid, cinnamic acid, quercetin, pinobanksin, apigenin, chrysin, pinocembrin, kaempferol, kaempferide | n/a | Sulaiman et al. (2011) |
| 10 | $H_2O$ 100 mL | Solvent | $T = RT$, $t = 5$ h | Caffeic acid, trans *p*-coumaric acid, ferulic acid | Total phenol (1.6 mg/mL GAE) | Kubiliene et al. (2015) |
| 10 | Ethanol 70% 100 mL | Solvent | $T = RT$, $t = 5$ h | Caffeic acid, trans *p*-coumaric acid, ferulic acid, naringenin, kaempferol, galangin | 12.7 mg/mL GAE | Kubiliene et al. (2015) |
| 2 | Ethanol 15 mL | Solvent | $T = 70\,^{\circ}C$, $t = 0.5$ h | Artepillin C, *p*-coumaric acid | n/a | Machado et al. (2016) |
| 0.5 | Ethanol 4 mL | Solvent | $T = RT$, $t = 0.21$ h | vanillin, *p*-coumaric acid, ferulic acid, chrysin, galangin, caffeic acid phenethyl ester (CAPE) | n/a | Jerković et al. (2016) |
| 2 | Ethanol 15 mL | Solvent | $T = 70\,^{\circ}C$, $t = 0.21$ h | Artepillin C, *p*-coumaric acid | n/a | Machado et al. (2016) |
| 0.5 | Ethanol 4 mL | Solvent | $T = RT$, $t = 0.21$ h | Vanillin, *p*-coumaric acid, ferulic acid, chrysin, galangin, caffeic acid phenethyl ester (CAPE) | n/a | Jerković et al. (2016) |

**Table 2 – Propolis extraction using SC-$CO_2$.**

| $w_{propolis}$,g | Condition | Extracted compounds | Yield, wt% | Ref. |
|---|---|---|---|---|
| 33 | P = (139–346) bar, T = (30–70) °C | Naringenin, quercetin, kaempferol, isorhamnetin, pinocembrin, CAPE, galangin, chrysin, acacetin. | 9.6 (P = 277 bar, T = 45 °C) | You et al. (2002) |
| 20 | P = (100–200) bar, T = (30–50) °C, t = (3–5) h | DHCA, cinnamic acid | 12 (P = 250 bar, T = 40 °C, $CO_2$ = 5 g/min) | Biscaia and Ferreira (2009) |
| 20 | P = (100–200) bar, T = (30–50) °C, t = (3–5) h | DHCA, cinnamic acid, *p*-coumaric acid | 8.9 (P = 250 bar, T = 40 °C, two stage process) | Biscaia and Ferreira (2009) |
| 10 | P = 207 bar, T = 50 °C | Artepillin C, cinnamic acid derivatives | 0.6 (P = 207 bar, T = 50 °C) 2.6 mg/g of artepillin C | Chen et al. (2009) |
| 5 (2 g EEP) | P = (150–250) bar, T = (20–50) °C | DHCA, PHCA, *p*-coumaric acid, kaempferide | 7.3 (P = 250 bar, T = 50 °C) | Paviani et al. (2012) |
| 7.5 | T = 40 °C, P = 100 bar, $CO_2$ flux = 6 g/min | Artepillin C, *p*-coumaric acid | 2.972 | Machado et al. (2015) |
| 32 | P = (82–320) bar, T = (31–50) °C, t = (1.5–6.5) h | Apigenin, CAPE, caffeic acid derivatives, galangin, galangin derivative, chrysin, cinnamic acid derivatives, pinobanksin, pinobanksin derivatives, pinocembrin, quercetin, quercetin derivatives | 14.3 (P = 317 bar, T = 45 °C,) | De Zordi et al. (2014) |
| 50 g poplar | P = (80–340) bar, T = (35–65) °C, t = 60 min, $CO_2$ = 2 kg/h | *p*-Coumaric acid, pinobanksin, chrysin, pinocembrin, galangin, pinostrobin | 10.28 (P = 300 bar, T = 60 °C) | Kuś et al. (2018) |
| 60 g EEP | P = (90–300) bar, $CO_2$ = 0.1667 g/s | Benzophenones | 38 wt% of benzophenones (P = 300 bar, T = 40 °C, t = 3 h) | Fianco et al. (2018) |
| 5 g EEP | P = (150–350) bar, T = (20–50) °C, t = (1–2) h | *p*-coumaric acid, artepillin C, PHCA, kaempferide | 13.07 (P = 350 bar, T = 60 °C) | Paviani et al. (2010a,b) |

## 2.2. Methods

### 2.2.1. Characterization of raw material (propolis)

Raw propolis was kept in cold storage at −20 °C for further treatment. This raw propolis was pulverized in the grinder and sieved to get 14–20 of mesh. This sieved raw propolis were used in all the experiments.

The AOAC Official Method 934.01 was performed to determine the moisture content. Raw propolis (5 g) were fed into an oven at 105 °C for 1 h to release moisture content. Afterward, the sample was cooled down in a desiccator and re-weighed. This step was repeated until the weighing of the sample is constant. The water content was calculated using Eq. (1).

$$\text{Moisture} \quad (\%) = \left[1 - \frac{w_{\text{raw propolis}}}{w_{\text{dried raw propolis}}}\right] \times 100 \qquad (1)$$

To calculate the wax content, 750 mL of methanol was poured into beaker glass containing 250 g of raw propolis. The raw propolis-methanol mixture, further, was stored overnight in cold storage at -20 °C. Afterward, the wax was separated from the mixture. The wax content was computed using Eq. (2) (Yeo et al., 2015).

$$\text{Wax} \quad (\%) = \left[\frac{w_{\text{wax}}}{w_{\text{raw propolis}}}\right] \times 100 \qquad (2)$$

The Soxhlet extraction was performed to evaluate the chemical constituent of raw propolis. The 14–20 of the mesh of raw propolis (5 g) were put in the extractor flask containing 150 mL of ethanol (PA grade) and heated to a fixed temperature of 60 °C for 10 h. Subsequently, the extract was filtered and analyzed using HPLC to identify the chemical constituent.

### 2.2.2. Supercritical extraction

A modified supercritical fluid apparatus which was used in the previous studies (Salea et al., 2017; Subroto et al., 2017), was performed to conduct the supercritical carbon dioxide (SC-$CO_2$) extraction. Fig. 1 provides the experimental apparatus layout.

50 g of cold raw propolis was mechanically milled and fed into an extractor vessel. The liquid $CO_2$ (food grade) was transferred into the vessel using a high-pressure pump (Thar, USA). The SC-$CO_2$ extraction was carried out using a supercritical extractor with a $CO_2$ cycle system. During the process, the vessel pressure was kept constant and controlled by a back-pressure regulator (Tescom, USA). Temperatures in the extractor and separator vessel were maintained in the range of (33–67) °C using a heat exchanger (Lab. Companion, USA).

Extraction was performed in two stages, starting with the static extraction, and followed by the dynamic extraction. In the static extraction, 50 g of raw propolis and 50 g of $CO_2$ were fed into extractor vessel. The vessel was set to 50 °C and 150 bar. All static extraction was run at this condition for 60 min. This step was intended to withdraw the wax from the raw propolis matrices. The dynamic extraction time was set to 240 min and fixed for all dynamic extraction. During the dynamic extraction, process variables such as pressure 165.91–334.09 bar, temperature 33–18–66.82 °C, and $CO_2$ flowrate 6.59–23.41 g/min were varied following central composite design. In this study, 20 runs were performed and the center point was measured six times. The liquid extract was collected after the extraction time (240 min) was completed.

### 2.2.3. HPLC analysis

The liquid product was analyzed by HPLC (Waters Alliance) using a C-18 column to determine the constituent of propolis extract. To identify the extract composition, some compounds such as myricetin, pinobanksin, quercetin, kaempferol, galangin, chrysin, ferulic acid, *p*-coumaric acid, caffeic acid, cinnamic acid, and caffeic acid phenyl ester were used as the external standard solution. The retention times were used to compare the peaks of extracts and external standard solutions. The spiking method was also occupied to confirm the

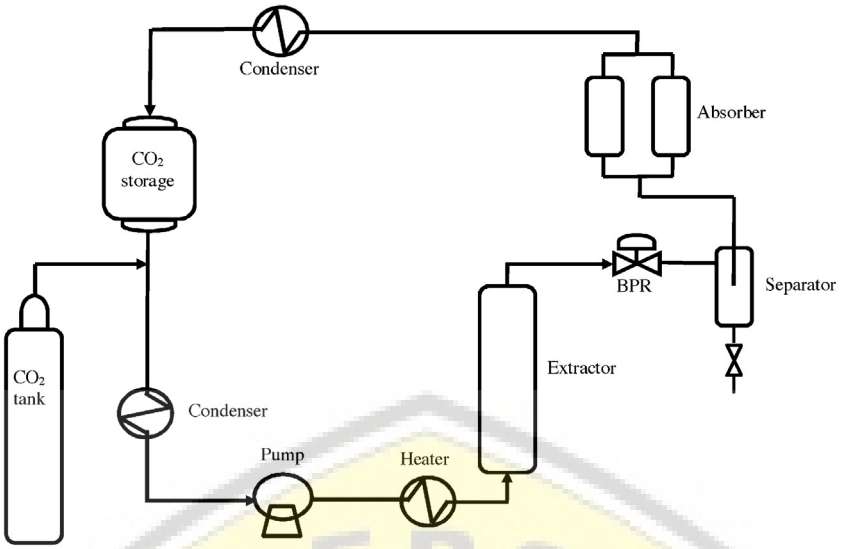


**Fig. 1 – Supercritical $CO_2$ extraction apparatus layout 1.**

peaks. To quantify the extracts, a set of concentrations of the external standard solution was injected into the HPLC system to draw calibration curves. These curves were used to calculate the extract concentration.

The mobile phase was 1.0% aqueous acetic acid (v/v) (A) and methanol (B) in the gradient mode. The gradient model was applied at 33 ◦C. For details, the gradient composition were as follows: 15%–40% (B) at 0–30 min; 40%–55% (B) at 30–65 min, 55%–62% (B) at 65–70 min, 100% (B) at 70–85 min with flow rate of 1.0 mL/min. The injection volume was 5 μL.

#### 2.2.4. *Definition of yield and statistical modeling*

The yield of propolis extract ($Y_{ext}$) is defined on a wt% basis and determined from Eq. (3).

$$Y_{ext}\ (wt\%) = \frac{w_{ext}}{w_{raw\ propolis}} \times 100\% \quad (3)$$

Statistical modeling using Design Expert 10 software (Stat-Ease) was applied to investigate the effect of parameters to extract yield. The model of extract yield was formulated in Eq. (4).

$$Y_{ext} = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3}\sum_{j=1}^{3} b_{ij} x_i x_j \quad (4)$$

The parameters including temperature, pressure and $CO_2$ flow rate are represented by the indices 1–3. The regression coefficients were calculated by statistical analyses of the data. The significance of factors was checked by their *p*-value in the ANOVA analyses. A significant factor was confirmed if the *p*-value was lower than 0.01. Further, the significant factors were needed to model the data, whereas, the insignificant factors were removed using backward elimination.

#### 2.2.5. *Antioxidant activity*

To check antioxidant activity, the DPPH (2, 2-diphenyl-1-picrylhydrazyl) method was mainly performed following a previous study (Machado et al., 2015) with modification. The extract obtained in optimum condition was reacted with 2,2-diphenyl-1-picrylhydrazyl. The extract was diluted and prepared in triplicates. The 1 mL of DPPH solution (0.1 mM) in methanol was added to a tube containing 1 mL of extract (50–300 μg/mL). The mixture, further, were incubated in dark room for 30 min at room temperature. Using spectrophotometer, the decreasing of the radical power of DPPH was determined by the value of absorbance. Absorbance itself was read in 517 nm. The same procedure was applied to a control solution consisting of 1 mL of methanol and 0.1 mM of DPPH solution. As a positive control, ascorbic acid was used.

The antioxidant activity was shown as the sequestration of free radicals expressed as the percentage of inhibition in the radical and mathematically calculated following Eq. (5). The $IC_{50}$ value is the needed concentration of extract to sequestrate 50% of DPPH radical. This value was obtained from the line of the linear regression equation based on the relationship between concentrations of extracts and its percentages of radical DPPH inhibition.

$$\%\,\text{inhibition} = \left(1 - \frac{A}{A_o}\right) \times 100 \quad (5)$$

A is an absorbance at 517 nm of the sample solution and $A_o$ is an absorbance at 517 nm of control solution.

## 3. Results

### 3.1. *Characterization of raw material (propolis)*

The characterization study on Indonesian *Trigona* sp. raw propolis was performed in this work. The moisture content, wax and chemical composition were checked in triplicates. The results indicate that raw propolis containing moisture of 10.3 wt% in average which is higher than the previous study reported by Dias et al. (2012) (3.4–5.4 wt%), Biscaia and Ferreira (2009) (6 wt%), and Machado et al. (2016) (7.03–9.16 wt%). In the interim, the wax content is 6.7 wt% which is lower than the findings of Biscaia and Ferreira (2009) (16.1 wt%) and Dias et al. (2012) (4.8–16 wt%).

Many reports inform that chemical constituent of raw propolis mostly consists of flavonoid, phenolic acids, and its derivatives. In this work, the chemical constituents of raw propolis were determined by Soxhlet extraction and the liquid extract was analyzed using HPLC. The result identifies that the Indonesian *Trigona* sp. raw propolis contains phenolic acids such as caffeic acid (3.6 wt%), ferulic acid (1.4 wt%), *p*-coumaric acid (28.7 wt%), and cinnamic acid (3.2 wt%). Sur-

**Table 3 – The extracted compound of propolis on different propolis origin and bee by Soxhlet method.**

| Propolis origin | Propolis bee | Solvent | Condition | Extracted compounds | Ref. |
|---|---|---|---|---|---|
| The Southeastern region of Brazil | *Apis mellifera* | Ethanol absolute 400 mL | $T = 60\,^{\circ}C$, $t = 24$ h | Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid derivatives | Cunha et al. (2004) |
| Cabréuva, Brazil | *Apis mellifera* | Methanol 150 mL | $t = 8$ h | Artepillin C, *p*-coumaric acid, cinnamic acid, chlorogenic acid, caffeic acid, kaempferol, kaempferide, isosakuranetin | de Funari et al. (2007) |
| South of Brazil | n/a | n/a | $T = 60\,^{\circ}C$, $t = 6$ h | Artepillin C, 2,2-dimetyl-6-carboxietenyl-2H-1-benzopirane, cinnamic acid, *p*-coumaric acid, caffeic acid, cafeoilquínic acid, kaempferol, kaempferide | Biscaia and Ferreira (2009) |
| Brazil | n/a | Ethyl acetate 120 mL | $t = 16$ h | Artepillin C | Chen et al. (2009) |
| Mataram, Indonesia | *Trigona* sp. | Ethanol | $T = 60\,^{\circ}C$, $t = 10$ h | Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, CAPE, quercetin, kaempferol galangin, chrysin, and pinobanksin | This work |

prisingly, the most important phenolic acid derivatives such as CAPE (2.4 wt%) and flavonoids such as quercetin (1.8 wt%), kaempferol (11.2 wt%), galangin (6.3 wt%), chrysin (2.4 wt%) and pinobanksin (1.7 wt%) were also found in raw propolis. These findings show the differences between Indonesian raw propolis and Brazillian, European, Mediterranean and Saudi Arabian raw propolis. The Brazillian raw propolis mostly contains hydrocinnamic acid derivatives and artepillin C (Biscaia and Ferreira, 2009). European raw propolis mainly contains flavonoids (pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, and galangin) (Bankova et al., 2002). Mediterranean raw propolis is rich with terpenes such as isocupressic acid, pimaric, imbricatoloic acid, agathadiol, totarol, 13-epi-torulosal, communic acid, 13-epi-cupressic acid, abietic acid and ferruginolon (Graikou et al., 2016). Saudi Arabian raw propolis is also rich with diterpenes such as diterpene propsiadin and diterpene psiadin (Almutairi et al., 2014). These differences can be definitely caused by the origin of propolis, the season when the propolis is collected, the kind of bee species and the feed of bee. Table 3 informs the application of Soxhlet method on different propolis origin and bee. It shows that the propolis origin and bee may play a role in propolis extraction.

### 3.2. *Effect of process conditions to extraction yield*

In this work, all supercritical extractions were performed in two stages. The first stage was static extraction which was also intended as a pre-extraction process to pull out the wax. This step was conducted at 150 bar, 50 °C and 50 g $CO_2$ for 60 min. This static condition was determined by directing preliminary research called the dewaxing process. The waxes were collected every 30 min for 60 min. This static extraction time was prolonged to 80 min and checked every 10 min to know whether the wax still remained. The result confirmed all waxes were removed from raw propolis after 60 min. In average, the 3.51 g (7.02 wt%) of propolis wax were successfully separated in this step. This value is lower than Krell (1996) who reported the raw propolis contained 25–30 wt%. However,

**Table 4 – Overview of experiments for the supercritical extraction of raw propolis.**

| Run | $T_{dynamic}$, °C | $P_{dynamic}$, bar | *m* $CO_2$, g/min | $w_{ext}$, g | $Y_{ext}$, wt% |
|---|---|---|---|---|---|
| 1 | 66.82 | 250 | 15 | 4.02 | 8.04 |
| 2 | 40 | 200 | 20 | 3.38 | 6.76 |
| 3 | 50 | 165.91 | 15 | 3.06 | 6.12 |
| 4 | 50 | 334.09 | 15 | 5.72 | 11.44 |
| 5 | 50 | 250 | 15 | 6.88 | 13.76 |
| 6 | 60 | 300 | 10 | 3.84 | 7.68 |
| 7 | 50 | 250 | 6.59 | 2.82 | 5.64 |
| 8 | 40 | 300 | 20 | 3.84 | 7.68 |
| 9 | 50 | 250 | 15 | 6.66 | 13.32 |
| 10 | 50 | 250 | 15 | 7.04 | 14.08 |
| 11 | 50 | 250 | 15 | 6.14 | 12.28 |
| 12 | 50 | 250 | 23.41 | 5.88 | 11.76 |
| 13 | 60 | 200 | 10 | 3.26 | 6.52 |
| 14 | 40 | 200 | 10 | 2.56 | 5.12 |
| 15 | 40 | 300 | 10 | 3.32 | 6.64 |
| 16 | 60 | 300 | 20 | 4.14 | 8.28 |
| 17 | 50 | 250 | 15 | 7.2 | 14.4 |
| 18 | 33.18 | 250 | 15 | 3.54 | 7.08 |
| 19 | 60 | 200 | 20 | 3.56 | 7.12 |
| 20 | 50 | 250 | 15 | 6.36 | 12.72 |

Negri et al. (2000) confirmed that Brazilian Propolis contained wax in range of 2.3–16.4 wt% and Cvek et al. (2007) found the wax of Croatian Propolis in range of 8.75–14.13 wt%. These strongly indicate that the wax content also varies depending on the origin of propolis.

Since the static extraction has removed the wax, the extraction was continued to the second stage. The second stage was a dynamic extraction. In this stage, the effect of process conditions on the extraction yield was investigated. The 20 experiments were run using a central composite design. Three independent variables, the temperature (40–60 °C), pressure (200–300 bar) and $CO_2$ flow rate (10–20 g/min), were explored and the extract was taken as the dependent variables. The results are provided in Table 4.

The center point of the central composite design was measured six times (run 5, 9, 10, 11, 17 and 20). The extraction yield was found to be on average 6.71 g (13.43 wt%). Experimen-

**Table 5 – Analysis variance of extract yield model.**

| Source | Sum of squares | df | Mean square | F value | *p*-value, Prob > *F* |
|---|---|---|---|---|---|
| Model | 179.42 | 6 | 29.90 | 23.59 | <0.0001 (significant) |
| *T* | 1.84 | 1 | 1.84 | 1.45 | 0.2496 |
| *P* | 13.76 | 1 | 13.76 | 10.85 | 0.0058 |
| *m* | 14.71 | 1 | 14.71 | 11.60 | 0.0047 |
| $(T)^2$ | 76.14 | 1 | 76.14 | 60.06 | <0.0001 |
| $(P)^2$ | 50.25 | 1 | 50.25 | 39.64 | <0.0001 |
| $(m)^2$ | 51.78 | 1 | 51.78 | 40.85 | <0.0001 |
| Residual | 16.16 | 10 | 1.62 | | |
| Lack of fit | 13.17 | 8 | 1.65 | 2.49 | 0.1654 (not significant) |

df: degree of freedom; *T*: temperature (°C); *P*: pressure (bar); *m*: mass flowrate of $CO_2$.

tally, the highest extract was about 7.2 g (14.4 wt%) and was achieved at 50 °C, 250 bar, the $CO_2$ flow rate of 15 g/min using a raw propolis intake of 50 g and dynamic time of 240 min. This finding is higher than Krell (1996) who reported raw propolis containing 10 wt% of the oil and Biscaia and Ferreira (2009) who attained 8.9 wt% Brazillian propolis extract from two stages supercritical extraction process.

### 3.3. *Statistical modeling*

In order to observe the significance of process variables on the yield, variance analysis was performed. If the *p*-value is less than 0.01, the process variable shows a significant effect. The result of variance analysis is given in Table 5.

Table 5 indicates that $CO_2$ flowrate and pressure show a significant effect on yield whereas temperature is slightly important than $CO_2$ flowrate and pressure. This fits with some reports stated that both pressure and $CO_2$ flowrate play more important than any involved variables in supercritical extraction (Reverchon and De Marco, 2006; Duba and Fiori, 2015; Salea et al., 2017).

The extraction yield was also statistically modeled and empirically formulated as a function of the independent variables (temperature *T*, pressure *P*, and $CO_2$ flow rate *m*). These variables were significant and give an effect on the extraction yield ($Y_{ext}$). The model is valid in a range of the temperature (40–60 °C), pressure (200–300 bar) and $CO_2$ flow rate (10–20 g/min). The best model involving quadratic and interaction expression for yield (wt%) was successfully developed and shown in Eq. (6).

$$Y_{ext.} = -117.714 + 2.335(T) + 0.393(P) + 2.482(m) - 0.023(T^2) - .469 \times 10^{-4}(P^2) - 0.076(m^2) \quad (6)$$

which *T* refers to temperature, *P* refers to pressure, and *m* refers to the $CO_2$ flow rate. The *R*-squared of the model is 0.9159 that indicates the model fits the experimental data. It also shows that the model can gratify 91.59% of the variability in the extraction process. A good agreement between the model and the experimental data was observed, as is shown in the parity plot provided in Fig. 2.

To describe the effects of the process conditions on the extract yield, the model predictions for yield for $T = 40$ °C and $T = 60$ °C are given in Fig. 3. At higher temperatures, the extract yield is slightly higher than at lower temperatures. It can be explained that as temperature increased from 40 to 60 °C, the solubility of the extract was also increased. This is in line

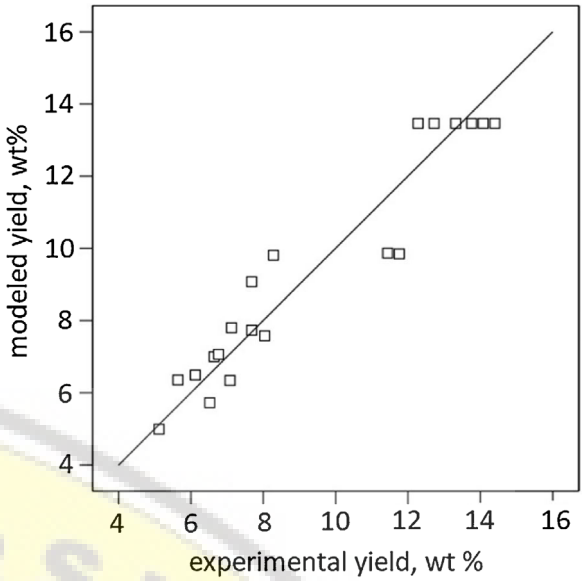


**Fig. 2 – Parity plot between the modeled and experimental mass of extracted oil.**

with Salgın and Salgın (2006) who concluded that the higher temperature process can improve the solubility of extract in supercritical extraction.

Fig. 3 describes the influence of pressure and $CO_2$ flow rate on propolis yield. At constant temperature, increasing pressure will lead to higher yield. The density of supercritical $CO_2$ increases proportionally with pressure. It subsequently controls the power of the solvent and the solubility of extract in supercritical $CO_2$ extraction. In higher density condition, the interaction between molecules and supercritical $CO_2$ is also higher, enhancing the solubility of extract. This is in accordance with Reverchon and De Marco (2006) who reported that the increasing pressure accelerates the solubility of oil.

However, in Fig. 3, since the pressure increased toward 250 bar, the extract yield declines. It is well known that high pressure contributes to improving the yield, but the pressure above 275 may play a role in the process due to the properties change in higher pressure. This gives increasing the mass transfer resistance, restricting the diffusion of supercritical $CO_2$ into molecules. Thus, it consequently inhibits the solubility.

Fig. 3 also shows the optimum point for the effect of pressure on the yield extract as well as the effect of the $CO_2$ flow rate. At the beginning of the extraction process, the convective mass transfer gives influence on the solid material (raw propolis)–supercritical $CO_2$ interaction, providing sufficient contact time between cells and supercritical $CO_2$. Thus, it allows for solvent saturation. Further, the solvent saturation is attained, the diffusion fully controls the extraction. In this period, the solubility of the extract is higher, resulting in a high yield. This is in agreement with both Rodrigues et al. (2008) and Monroy et al. (2017) who reported that the high $CO_2$ flow rate enhanced the yield. It can reduce the mass transfer resistance, saturating the supercritical $CO_2$. Thus, equilibrium is reached and the maximum yield is achieved. However, as seen in Fig. 3, the higher the $CO_2$ flow rate above 15 g/min slightly decreases the yield. As the system is rich with supercritical $CO_2$, the equilibrium deviates. It causes the solvent leaving the system unsaturated. The advantage of increasing the flow rate occurs up to the moment when the intraparticle resistance becomes the dominant mass transfer resistance. At this point,

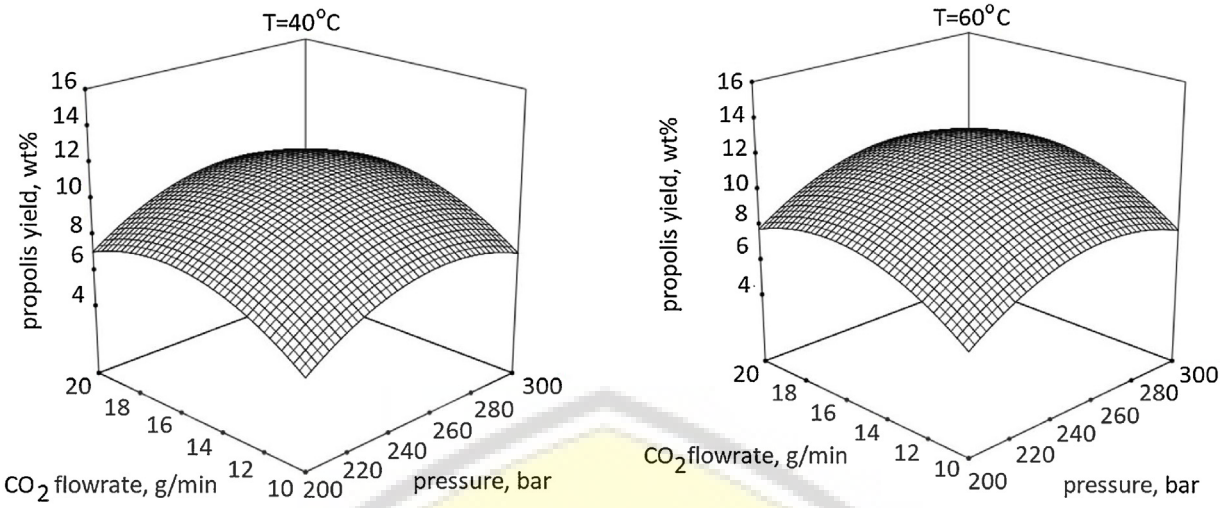


**Fig. 3 – Modeled yield of propolis versus pressure and $CO_2$ flow rate for two temperatures (*left* 40 °C; *right* 60 °C).**

further increments on the flow rate become useless, because the solutes removal is being governed by the rate of diffusion inside the particles, toward their surface.

In a high flow rate, the mass transfer resistance stays diminishing until the supercritical $CO_2$ is saturated. Further, the increase of the flow rate will lead the solvent exits the extractor unsaturated in spite of the high mass transfer rate. However, the optimum flow rate is a function of the solvent-solute interaction and process variables such as the geometry of the extractor, the temperature, and the pressure.

### 3.4. *Extract composition*

The content of propolis varies among regions. The composition is highly influenced by the regions where the propolis is produced. Among those compounds, flavonoids, phenolic acids and terpenes are major constituents that control the properties of propolis (Kumazawa et al., 2004; Watanabe et al., 2011).

In this work, Indonesian propolis composition was successfully determined by HPLC. Some bioactive compounds such as flavonoids (myricetin, pinobanksin, quercetin, kaempferol, galangin, chrysin), phenolic acids (ferulic acid, *p*-coumaric acid, caffeic acid, cinnamic acid) and ester (caffeic acid phenyl ester) were identified as seen in chromatogram profile in Fig. 4. This profile indicates that *p*-coumaric acid from the phenolic acid group, kaempferol and chrysin are clearly present in the extract of Indonesian propolis. The quantity of each chemical constituent is tabulated in Table 6 which is also indicates that the performance of supercritical extraction is better than the Soxhlet method. This is shown by extraction yield. The supercritical extraction provides a higher yield than the Soxhlet method.

The comparison of extract constituent between Indonesian propolis and another propolis origin briefly is presented in Table 7. It briefly informs that the Indonesian propolis composition is slightly similar with Taiwanese and Italian propolis. In the meantime, the Indonesian propolis also contains *p*-coumaric acid and cinnamic acid which are mostly available in Brazilian Propolis.

In the interim, some reports on identifying propolis chemical characteristics have been published. Biscaia and Ferreira (2009) found phenolic acids such as caffeic acid, *p*-coumaric acid, cinnamic acid and its derivatives including Artepillin C in the extract of Brazillian propolis. De Zordi et al. (2014) reported that Italian propolis contained flavonoids such as apigenin, chrysin, galangin, pinobanksin, pinocembrin, quercetin and phenolic acids such as caffeic acid including caffeic acid phenyl methyl ester and *p*-coumaric acid. Ahn et al. (2004) confirmed that Korean propolis consists of caffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, pinobanksin 5-methyl ether, apigenin, kaempferol, pinobanksin, cinnamylideneacetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, phenethyl caffeate, cinnamyl caffeate, and tectochrysin. Furthermore, Uruguayan propolis comprised pinobanksin, chrysin, galangin, izalpinin, kaempferol, *p*-coumaric acid, caffeic acid and phenolic esters (Kumazawa et al., 2002; Kumazawa et al., 2004).

**Table 6 – The chemical constituent profile.**

| No. | Chemical constituent | Raw propolis (sample) (wt%)[a] | Propolis extract[b] (wt%) |
|---|---|---|---|
| 1 | Caffeic acid | 3.6 | 4.4 |
| 2 | Ferulic acid | 1.4 | 2.1 |
| 3 | *p*-Coumaric acid | 28.7 | 36.8 |
| 4 | CAPE | 2.4 | 2.8 |
| 5 | Cinnamic acid | 3.2 | 3.8 |
| 6 | Quercetin | 1.8 | 3.2 |
| 7 | Kaempferol | 11.2 | 13.2 |
| 8 | Galangin | 6.3 | 8.3 |
| 9 | Myricetin | – | 1.3 |
| 10 | Pinobanksin | 1.7 | 8.8 |
| 11 | Chrysin | 2.4 | 12.7 |

[a] Soxhlet extraction.
[b] Supercritical extraction.

Nevertheless, Graikou et al. (2016) reported Mediterranean propolis was composed of diterpene compounds such as isocupressic acid, imbricatoloic acid, communic acid, abietic acid, and flavonoids such as chrysin, galangin, pinobanksin, and pinocembrin. It is unique in composition. It shows the combination of European propolis which is rich in flavonoids and Mediterranean propolis which has diterpenes as major compounds. In the interim, Jerković et al. (2016) identified caffeic acid, *p*-coumaric acid, ferulic acid, chrysin, galangin, vanillin, and caffeic acid phenethyl ester (CAPE) in the Mediterranean (Croatian) propolis.

The different result was informed by Al-Ghamdi et al. (2017) who reported Yemen Propolis consisted of triterpenoids, n-

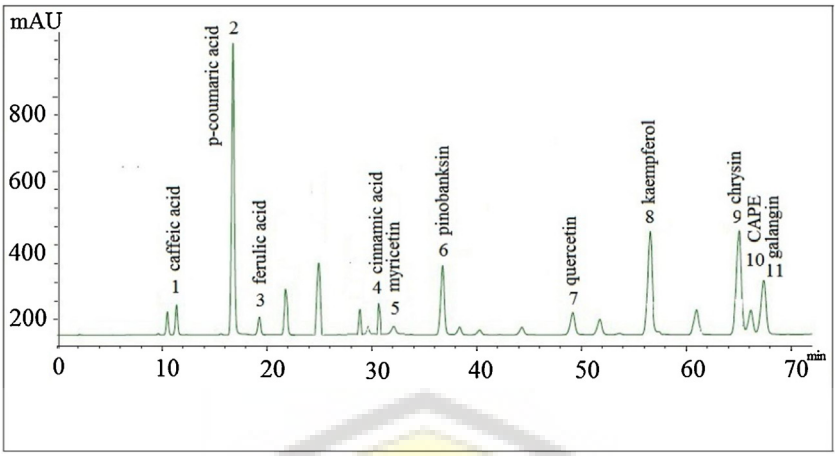


**Fig. 4 – Chromatogram profile of propolis.**

**Table 7 – The extracted compound of propolis on different propolis origin and bee by supercritical $CO_2$ extraction.**

| Propolis origin | Propolis bee | Condition | Extracted compounds | Ref. |
|---|---|---|---|---|
| Taiwan | n/a | P = (139–346) bar, T = (30–70) °C | Naringenin, quercetin, kaempferol, isorhamnetin, pinocembrin, CAPE, galangin, chrysin, acacetin. | You et al. (2002) |
| South of Brazil | n/a | P = (100–200) bar, T = (30–50) °C, t = (3–5) h | Artepillin C, cinnamic acid, *p*-coumaric acid | Biscaia and Ferreira (2009) |
| Brazil | n/a | P = 207 bar, T = 50 °C | Artepillin C, cinnamic acid derivatives | Chen et al. (2009) |
| South of the Paraná state, Brazil | n/a | T = 40 °C, P = 100 bar, $CO_2$ flux = 6 g/min | Artepillin C, *p*-coumaric acid | Machado et al. (2015) |
| Propolis origin | Propolis bee | Condition | Extracted compounds | Ref. |
| Belluno, Italy | n/a | P = (82–320) bar, T = (31–50) °C, t = (1.5–6.5) h | Apigenin, CAPE, caffeic acid derivatives, galangin, galangin derivative, chrysin, cinnamic acid derivatives, pinobanksin, pinobanksin derivatives, pinocembrin, quercetin, quercetin derivatives | De Zordi et al. (2014) |
| Mataram, Indonesia | *Trigona* sp. | T = 50 °C, P = 250 bar, $CO_2$ = 15 g/min | Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, CAPE, quercetin, kaempferol galangin, chrysin, pinobanksin, and myricetin | This work |

alkenes, n-alkanes, n-alkanoic acids, long-chain wax esters, n-alkanols, and methyl n-alkanoates. As seen in Fig. 4, due to the chemical composition, the Indonesian propolis extract from *Trigona* sp. has a similar constituent with Korean and European propolis. The Indonesian propolis extract accommodates both flavonoids which are mostly found in European propolis and phenolic acid which is commonly contained in Brazillian and Korean propolis. These reports including this finding strongly conclude that the propolis origins play an important role in the propolis composition.

### 3.5. *Anti-oxidant activity*

To check the antioxidant activity of extract, DPPH test was conducted. The result implies that propolis extract has a radical scavenging activity. Due to its composition (Fig. 4), the propolis extract gives a strong influence on its antioxidant activity. The antioxidant activity of propolis extract is supported by the presence of flavonoids and their esters such as galangin and caffeic acid phenethyl ester (CAPE). These compounds are reported as active molecules acting as the powerful antioxidant (Russo et al., 2002; You et al., 2002). However, some phenolic acid compounds in propolis such as caffeic acid, ferulic acid, cinnamic acid, and *p*-coumaric acid are stated to play a role in the antioxidant activity of propolis (Bankova et al., 1999; Hegazi et al., 2000).

Some studies have informed that free radicals can be scavenged by the components of propolis extract. This valuable information is also proven in this work that shows that the antioxidant activity of extract is high and powerful. It is indicated by the value of $IC_{50}$. The $IC_{50}$ (inhibitory concentration 50%) value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. The $IC_{50}$ value is inversely proportional to the antioxidant activity. Surprisingly, the calculated $IC_{50}$ value in this work is 24.77 μg/mL. This value is closer to the $IC_{50}$ of ascorbic acid (19.96 μg/mL). This finding strongly implies that propolis extract from the supercritical extraction of propolis has promising antioxidant quality.

## 4. Conclusion

This work concludes that supercritical $CO_2$ extraction performed in two stages process has high potentials to improve the quality of propolis extract. This work pointed the optimum condition giving 14.4 wt% yield has been detected at 50 °C, 250 bar, the $CO_2$ flow rate of 15 g/min using raw propolis intake of 50 g and dynamic time of 240 min.

The propolis extract has been successfully confirmed to contain bioactive compounds i.e. galangin, *p*-coumaric acid and caffeic acid phenyl ester. The antioxidant activity has been checked and unexpectedly gives very good results showing the $IC_{50}$ value of 24.77 μg/mL which is closer to the $IC_{50}$ value of ascorbic acid.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## References

Ahn, M.R., Kumazawa, S., Hamasaka, T., Bang, K.S., Nakayama, T., 2004. Antioxidant activity and constituents of propolis collected in various areas of Korea. J. Agric. Food Chem. 52, 7286–7292, http://dx.doi.org/10.1021/jf048726s.

Al-Ghamdi, A.A., Bayaqoob, N.I.M., Rushdi, A.I., Alattal, Y., Simoneit, B.R.T., El-Mubarak, A.H., Al-Mutlaq, K.F., 2017. Chemical compositions and characteristics of organic compounds in propolis from Yemen. Saudi J. Biol. Sci. 24, 1094–1103, http://dx.doi.org/10.1016/j.sjbs.2016.12.012.

Almutairi, S., Edrada-Ebel, R., Fearnley, J., Igoli, J.O., Alotaibi, W., Clements, C.J., Gray, A.I., Watson, D.G., 2014. Isolation of diterpenes and flavonoids from a new type of propolis from Saudi Arabia. Phytochem. Lett. 10, 160–163, http://dx.doi.org/10.1016/j.phytol.2014.08.022.

Bankova, V., Christov, R., Popov, S., Marcucci, M., Tsvetkova, I., Kujumgiev, A., 1999. Antibacterial activity of essential oils from Brazilian propolis. Fitoterapia 70, 190–193, http://dx.doi.org/10.1016/S0367-326X(98)00045-8.

Bankova, V., Popova, M., Bogdanov, S., Sabatini, A., 2002. Chemical Composition of European Propolis: Expected and Unexpected Results.

Biscaia, D., Ferreira, S.R.S., 2009. Propolis extracts obtained by low pressure methods and supercritical fluid extraction. J. Supercrit. Fluids 51, 17–23, http://dx.doi.org/10.1016/j.supflu.2009.07.011.

Chen, C.R., Lee, Y.N., Chang, C.M.J., Lee, M.R., Wei, I.C., 2007. Hot-pressurized fluid extraction of flavonoids and phenolic acids from Brazilian propolis and their cytotoxic assay in vitro. J. Chinese Inst. Chem. Eng. 38, 191–196, http://dx.doi.org/10.1016/j.jcice.2007.04.004.

Chen, C.R., Lee, Y.N., Lee, M.R., Chang, C.M.J., 2009. Supercritical fluids extraction of cinnamic acid derivatives from Brazilian propolis and the effect on growth inhibition of colon cancer cells. J. Taiwan Inst. Chem. Eng. 40, 130–135, http://dx.doi.org/10.1016/j.jtice.2008.07.014.

Cunha, I.B.S., Sawaya, A.C.H.F., Caetano, F.M., Shimizu, M.T., Marcucci, M.C., Drezza, F.T., Povia, G.S., Carvalho, P.D.O., 2004. Factors that influence the yield and composition of Brazilian propolis extracts. J. Braz. Chem. Soc. 15, 964–970, http://dx.doi.org/10.1590/S0103-50532004000600026.

Cvek, J., Medić-Šarić, M., Jasprica, I., Zubčić, S., Vitali, D., Mornar, A., Vedrina-Dragojević, I., Tomić, S., 2007. Optimisation of an extraction procedure and chemical characterisation of Croatian propolis tinctures. Phytochem. Anal. 18, 451–459, http://dx.doi.org/10.1002/pca.1001.

de Funari, C.S., de Oliveira Ferro, V., Mathor, M.B., 2007. Analysis of propolis from *Baccharis dracunculifolia* DC (Compositae) and its effects on mouse fibroblasts. J. Ethnopharmacol. 111, 206–212, http://dx.doi.org/10.1016/j.jep.2006.11.032.

De Melo, M.M.R., Silvestre, A.J.D., Silva, C.M., 2014. Supercritical fluid extraction of vegetable matrices: applications, trends and future perspectives of a convincing green technology. J. Supercrit. Fluids 92, 115–176, http://dx.doi.org/10.1016/j.supflu.2014.04.007.

De Zordi, N., Cortesi, A., Kikic, I., Moneghini, M., Solinas, D., Innocenti, G., Portolan, A., Baratto, G., Dall'Acqua, S., 2014. The supercritical carbon dioxide extraction of polyphenols from propolis: a central composite design approach. J. Supercrit. Fluids 95, 491–498, http://dx.doi.org/10.1016/j.supflu.2014.10.006.

del Valle, J.M., 2015. Extraction of natural compounds using supercritical $CO_2$: going from the laboratory to the industrial application. J. Supercrit. Fluids 96, 180–199, http://dx.doi.org/10.1016/j.supflu.2014.10.001.

Dias, L.G., Paula, A., Estevinho, L.M., 2012. Comparative study of different Portuguese samples of propolis: pollinic, sensorial, physicochemical, microbiological characterization and antibacterial activity 50, 4246–4253, http://dx.doi.org/10.1016/j.fct.2012.08.056.

Duba, K.S., Fiori, L., 2015. Supercritical $CO_2$ extraction of grape seed oil: effect of process parameters on the extraction kinetics. J. Supercrit. Fluids 98, 33–43, http://dx.doi.org/10.1016/j.supflu.2014.12.021.

Fatoni, A., Artika, I.M., Hasan, A.E.Z., Kuswandi, 2008. Antibacterial activity of propolis produced by *Trigona* spp. against *Campylobacter* spp. Hayati J. Biosci. 15, 161–164, http://dx.doi.org/10.4308/hjb.15.4.161.

Fianco, A.L., Lucas, A.M., Fasolo, D., Almeida, R.N., Pippi, B., Güez, C.M., Fuentefria, A., Vargas, R.M.F., Teixeira, H.F., Von Poser, G., Cassel, E., 2018. Polyprenylated benzophenone-enriched extracts obtained using SC-$CO_2$ from the dry ethanolic extract of Brazilian red propolis. Sep. Sci. Technol. 53, 1–8, http://dx.doi.org/10.1080/01496395.2018.1424202.

Graikou, K., Popova, M., Gortzi, O., Bankova, V., Chinou, I., 2016. Characterization and biological evaluation of selected Mediterranean propolis samples. Is it a new type? LWT Food Sci. Technol. 65, 261–267, http://dx.doi.org/10.1016/j.lwt.2015.08.025.

Hasan, A.E.Z., Mangunwidjaja, D., Sunarti, T.C., Suparno, O., Setiyono, A., 2014. Investigating the antioxidant and anticytotoxic activities of propolis collected from five regions of Indonesia and their abilities to induce apoptosis. Emirates J. Food Agric. 26, 390–398, http://dx.doi.org/10.9755/ejfa.v26i5.16549.

Hegazi, A.G., Abd El Hady, F.K., Abd Allah, F.A., 2000. Chemical composition and antimicrobial activity of European propolis. Z. Naturforsch. C. 55, 70–75.

Jerković, I., Marijanović, Z., Kuś, P.M., Tuberoso, C.I.G., 2016. Comprehensive study of Mediterranean (Croatian) propolis peculiarity: headspace, volatiles, anti-varroa-treatment residue, phenolics, and antioxidant properties. Chem. Biodivers. 13, 210–218, http://dx.doi.org/10.1002/cbdv.201500053.

Knez, Markočič, E., Leitgeb, M., Primožič, M., Knez Hrnčič, M., Škerget, M., 2014. Industrial applications of supercritical fluids: a review. Energy 77, 235–243, http://dx.doi.org/10.1016/j.energy.2014.07.044.

Krell, R., 1996. Value-added Products from Beekeeping Table of Contents By. Fao Agriculture Services Bulletin.

Kubiliene, L., Laugaliene, V., Pavilonis, A., Maruska, A., Majiene, D., Barcauskaite, K., Kubilius, R., Kasparaviciene, G., Savickas,

A., 2015. Alternative preparation of propolis extracts: comparison of their composition and biological activities. BMC Complement. Altern. Med. 15, 1–7, http://dx.doi.org/10.1186/s12906-015-0677-5.

Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chem. 84, 329–339, http://dx.doi.org/10.1016/S0308-8146(03)00216-4.

Kumazawa, S., Hayashi, K., Kajiya, K., Ishii, T., Hamasaka, T., Nakayama, T., 2002. Studies of the constituents of Uruguayan propolis. J. Agric. Food Chem. 50, 4777–4782, http://dx.doi.org/10.1021/jf020279y.

Kuś, P., Jerković, I., Jakovljević, M., Jokić, S., 2018. Extraction of bioactive phenolics from black poplar (*Populus nigra* L.) buds by supercritical $CO_2$ and its optimization by response surface methodology. J. Pharm. Biomed. Anal. 152, 128–136, http://dx.doi.org/10.1016/j.jpba.2018.01.046.

Machado, B.A.S., De Abreu Barreto, G., Costa, A.S., Costa, S.S., Silva, R.P.D., Da Silva, D.F., Brandao, H.N., Da Rocha, J.L.C., Nunes, S.B., Umsza-Guez, M.A., Padilha, F.F., 2015. Determination of parameters for the supercritical extraction of antioxidant compounds from green propolis using carbon dioxide and ethanol as co-solvent. PLOS ONE 10, 1–26, http://dx.doi.org/10.1371/journal.pone.0134489.

Machado, B.A.S., Silva, R.P.D., Barreto, G.D.A., Costa, S.S., Da Silva, D.F., Brandão, H.N., Da Rocha, J.L.C., Dellagostin, O.A., Henriques, J.A.P., Umsza-Guez, M.A., Padilha, F.F., 2016. Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PLOS ONE 11, http://dx.doi.org/10.1371/journal.pone.0145954.

Monroy, Y.M., Rodrigues, R.A.F., Rodrigues, M.V.N., Sant'Ana, A.S., Silva, B.S., Cabral, F.A., 2017. Brazilian green propolis extracts obtained by conventional processes and by processes at high pressure with supercritical carbon dioxide, ethanol and water. J. Supercrit. Fluids 130, 189–197, http://dx.doi.org/10.1016/j.supflu.2017.08.006.

Negri, G., Marcucci, M.C., Salatino, A., Salatino, M.L.F., 2000. Comb and propolis waxes from Brazil (States of São Paulo and Paraná). J. Braz. Chem. Soc. 11, 453–457, http://dx.doi.org/10.1590/S0103-50532000000500004.

Paviani, et al., 2010a. Supercritical carbon dioxide selectivity to fractionate phenolic compounds from the dry ethanolic extract of propolis. J. Food Process Eng. 33, 15–27, http://dx.doi.org/10.1111/j.1745-4530.2008.00256.x.

Paviani, L.C., Dariva, C., Marcucci, M.C., Cabral, F.A., 2010b. Supercritical carbon dioxide selectivity to fractionate phenolic compounds from the dry ethanolic extract of propolis. J. Food Process Eng. 33, 15–27, http://dx.doi.org/10.1111/j.1745-4530.2008.00256.x.

Paviani, L.C., Saito, E., Dariva, C., Marcucci, M.C., Sánchez-Camargo, A.P., Cabral, F.A., 2012. Supercritical CO 2 extraction of raw propolis and its dry ethanolic extract. Brazilian J. Chem. Eng. 29, 243–251, http://dx.doi.org/10.1590/S0104-66322012000200005.

Piccinelli, A.L., Mencherini, T., Celano, R., Mouhoubi, Z., Tamendjari, A., Aquino, R.P., Rastrelli, L., 2013. Chemical composition and antioxidant activity of Algerian propolis. J. Agric. Food Chem. 61, 5080–5088, http://dx.doi.org/10.1021/jf400779w.

Popova, M.P., Graikou, K., Chinou, I., Bankova, V.S., 2010. GC-MS profiling of diterpene compounds in mediterranean propolis from Greece. J. Agric. Food Chem. 58, 3167–3176, http://dx.doi.org/10.1021/jf903841k.

Pujirahayu, N., Ritonga, H., Uslinawaty, Z., 2014. Properties and flavonoids content in propolis of some extraction method of raw propolis. Int. J. Pharm. Pharm. Sci. 6, 338–340.

Rasmussen, C., Cameron, S.A., 2010. Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. Biol. J. Linn. Soc. 99, 206–232, http://dx.doi.org/10.1111/j.1095-8312.2009.01341.x.

Reverchon, E., De Marco, I., 2006. Supercritical fluid extraction and fractionation of natural matter. J. Supercrit. Fluids 38, 146–166, http://dx.doi.org/10.1016/j.supflu.2006.03.020.

Rodrigues, R.F., Tashima, A.K., Pereira, R.M.S., Mohamed, R.S., Cabral, F.A., 2008. Coumarin solubility and extraction from emburana (*Torresea cearensis*) seeds with supercritical carbon dioxide. J. Supercrit. Fluids 43, 375–382, http://dx.doi.org/10.1016/j.supflu.2007.07.014.

Russo, A., Longo, R., Vanella, A., 2002. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia 73, 21–29, http://dx.doi.org/10.1016/S0367-326X(02)00187-9.

Salea, R., Veriansyah, B., Tjandrawinata, R.R., 2017. Optimization and scale-up process for supercritical fluids extraction of ginger oil from *Zingiber officinale* var. Amarum. J. Supercrit. Fluids 120, 285–294, http://dx.doi.org/10.1016/j.supflu.2016.05.035.

Salgın, S., Salgın, U., 2006. Supercritical fluid extraction of walnut kernel oil. Eur. J. Lipid Sci. Technol. 108, 577–582, http://dx.doi.org/10.1002/ejlt.200600046.

Subroto, E., Widjojokusumo, E., Veriansyah, B., Tjandrawinata, R.R., 2017. Supercritical $CO_2$ extraction of candlenut oil: process optimization using Taguchi orthogonal array and physicochemical properties of the oil. J. Food Sci. Technol. 54, 1286–1292, http://dx.doi.org/10.1007/s13197-017-2542-7.

Sulaiman, G.M., Sammarrae, K.W.A., Ad'hiah, A.H., Zucchetti, M., Frapolli, R., Bello, E., Erba, E., D'Incalci, M., Bagnati, R., 2011. Chemical characterization of iraqi propolis samples and assessing their antioxidant potentials. Food Chem. Toxicol. 49, 2415–2421, http://dx.doi.org/10.1016/j.fct.2011.06.060.

Watanabe, M.A.E., Amarante, M.K., Conti, B.J., Sforcin, J.M., 2011. Cytotoxic constituents of propolis inducing anticancer effects: a review. J. Pharm. Pharmacol. 63, 1378–1386, http://dx.doi.org/10.1111/j.2042-7158.2011.01331.x.

Yeo, K.L., Leo, C.P., Chan, D.J.C., 2015. Ultrasonic enhancement on propolis extraction at varied ph and alcohol content. J. Food Process Eng. 38, 562–570, http://dx.doi.org/10.1111/jfpe.12186.

You, G.S., Lin, S.C., Chen, C.R., Tsai, W.C., Chang, C.J., 2002. Supercritical carbon dioxide extraction enhances flavonoids in water-soluble propolis. J. Chinese Inst. Chem. Eng. 33, 233–241.