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RESEARCH PAPER

Chemical and Mineral Composition of the Mono-floral Pollen of Honeybees (*Apis mellifera*) in Ethiopia

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Abstract

This investigation aimed to investigate the chemical and mineral composition of pollen collected from Oromia, Ethiopia. The moisture level of analyzed pollen ranged from 10.3 ± 1.3% (*Sesame indicum*) to 17.3 ± 0.5% (*Eucalyptus globules*), the ash concentration ranged from 1.7 ± 0.3% (*G. scabra*) to 3.0 ± 0.5% (*Brassica carinata*), the protein content ranged from 16.3 ± 0.5% (*G. scabra*) to 24.9 ± 5.6% (*Eucalyptus globules*), the total dietary fiber ranged from 1.4 ± 0.7% (*E.* globules) to 2.6 ± 0.85% (*B. carinata*) and crude carbohydrate ranged from 54.1 ± 5.2 % (*E. globules*) to 69.1 ± 1.0% (*G. scabra*). Potassium and magnesium were the most prevalent minerals in bee-collected pollen samples. *B. carinata* pollen has the greatest calcium (Ca) (2321.3 ± 608.78 mg/kg) and magnesium (Mg) (1024.8 ± 19.9 mg/kg) concentrations compared to others. *E. globules* pollen had the greatest levels of potassium (K) (10596.9 ± 1610.1 mg/kg) and sodium (Na) (380.9 ± 95.9 mg/kg). Lead, a toxic element, was not detected in pollen samples from the study's site. Because of its botanical and geographic origins, bee pollen has a diverse nutritional composition. Results indicated that pollen is a useful food supplement for human nutrition due to its greater concentrations of essential components.

Introduction

Bee pollen is one of the products of bees and is frequently referred to as the most complete food. Bee pollen contains at least 200 biologically active compounds that may have therapeutic benefits (Kurek-Górecka et al., 2020; Thakur and Nanda, 2020). Pollen contains about 2 - 60% of proteins, 13 - 55% of carbohydrates, and 1 - 20% of lipids (Ares et al., 2022). Moreover, bee pollen comprises various minerals such as sodium, potassium, magnesium, calcium, phosphorus, iron, copper, and zinc that support different physiological activities in honeybees as well as in humans (Zhang et al., 2022). Apart from its nutritional value, bee pollen is composed of considerable amounts of polyphenolic compounds, primarily flavonoids, which may act as strong antioxidants (Thakur & Nanda, 2020). They possess diverse biological properties such as antioxidant, anti-aging, anti-carcinogen, anti-inflammatory, cardioprotective, and improved endothelial function (Matuszewska et al., 2021).

Because it includes substances that are beneficial to human health, bee pollen is categorized as a functional food. This means that it can be added to food products to boost their nutritional content and bioactive content. Additionally, bee pollen can improve an animal's development, reproduction, and immunity; it is suggested as a feeding supplement for livestock (Al-Kahtani et al., 2021). Therefore, bee pollen has gained increasing research attention worldwide (Thakur & Nanda, 2020). However, the nutritional composition of pollen varies widely depending on floral types; geographic origin, climate, and soil type (Morais et al., 2011).

Promoting bee-collected pollen as a dietary supplement for the enhancement of human health requires identifying the most important pollen source plants and evaluating the quality of their pollen, particularly in light of the growing interest in bee-collected pollen as a nutritional and api-therapeutic substance. Ethiopia is equipped with a high population density of *Apis mellifera* and a diverse range of flora, which provide ideal conditions for year-round pollen harvesting (Gratzer et al., 2021). As a result, the country has enormous potential for producing large amounts of high-quality pollen. Concerning Ethiopia's central highlands, cultivated crops, associated weeds, and a high density of honey bee colonies all contribute to an impressive amount of pollen collection that can be

turned into food supplements for both domestic and foreign markets. Thus, describing the nutritional characteristics encourages the use of pollen as a dietary supplement both domestically and abroad. Although bee pollen is a rich source of bioactive and nutritious substances, and Ethiopia has significant potential for high-quality pollen production, little research has been conducted on the characteristics of monofloral pollen. Therefore, this study is intended to examine the chemical and mineral properties of pollen collected from various floral sources in Oromia for further application in food or health.

Material and Methods

Sampling of the pollen

The pollen samples were collected from the central parts of Ethiopia representing highlands, midland, and lowland areas. The specific areas were: Holeta (9°03’26.19” N, 38°33’22.45” E, altitude 2370 m), Menagesha Forest (38°34’30” 8°57’0” N, 38°31’30”, altitude 2924 m), Gedo (9°00’59.12” N, 37°26’58.19” E, altitude 2515 m) and Bako (9°06’59.23” N, 37°03’23.02” E, altitude 1670 m). In each apiary, twelve colonies of *Apis mellifera* honey bees were equalized to ensure that they were at the same strength for pollen collection. The colonies were arranged in 3 frames of equal strength (honey, pollen, sealed brood), and a 1:1 sugar syrup was used for feeding (Oztokmak et al., 2023). The colony strength of each selected colony was estimated by observing the number of frames covered with bees, honey, brood, and pollen. The colony entrances were equipped with 16% efficient pollen traps, which removed pollen loads from workers' corbicula over 24 hours. Pollen traps were harvested twice a week.

Preparation of sample

The pollen loads were cleaned to remove impurities, weighed on a balance for the total weight and moisture, and classified using a set of sieves with different-sized meshes (0.5, 1.0, 2.0, and greater than 2.0 mm). Pollen of the same botanical origin was then isolated from the pooled samples based on visual appearance and color. Further confirmation of the botanical origin and monoflorality was achieved through melissopalynological analysis. The grinding process typically begins with drying the bee pollen to reduce moisture content. Bee pollen was then dried until the mass stabilized (humidity of 9 - 12%). Once dried, the pollen is weighed and placed into the grinder. After grinding, the pollen must be mixed thoroughly to achieve homogeneity using a vortex mixer. The mixing duration should be sufficient to ensure that all particles are evenly distributed, typically around 5-10 minutes.

Following that, the samples were then kept in the dark at -20 °C until laboratory analysis for 1 to 2 months (Ghosh et al., 2020).

Identification of botanical origin of the pollen loads

Pollen load samples (2 g) were mixed with water to disaggregate the pollen grains from the surrounding material, facilitating their separation and analysis. They were rinsed, centrifuged, and mounted in glycerin jelly after being properly homogenized, following the protocol outlined by Louveaux et al. (1978). Pollen sediments were placed on microscope slides with glycerin jelly. Types of pollen were determined by comparing them to slides from the Holeta Bee Research Center's pollen reference collection (Figure 1). According to Ares et al. (2022), the predominant taxon in the composition of the sample that was collected is well-defined if it is more than 80%. When these criteria are not met, the pollen is classified as multifloral. (Escuredo et al., 2012).

Characterization of pollen chemical composition

Moisture content

The moisture content of pollen was examined using AOAC procedures (AOAC, 1995) in oven (BioBase).

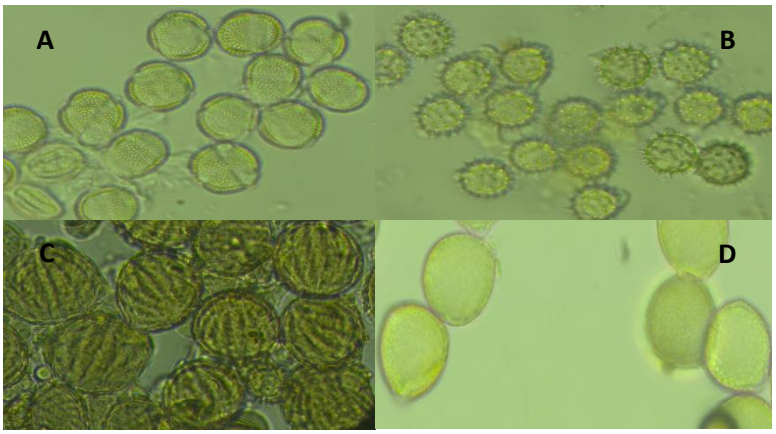

Figure 1: Photographs of pollen types analyzed. A, *Brassica carinata*, B, *Guizotia scabra*, C, *Sesamum indicum,* D, *Trifolium* spp.

The crucibles' weight was recorded using an analytical balance (W_1), and in every dry crucible (W_2), 5 g of pollen samples were measured and then dried for 3 hours at 105°C in an oven. After 3 hours, the crucibles were taken out of the oven and allowed to cool in desiccators. Once they had cooled, the weight of the sample and the crucibles was recorded (W_3). After the sample-containing crucibles were taken out of the oven to dry, their weight was recorded until it was constant. At last, the moisture content was estimated after the last constant measurement was obtained.

$$\text{Moisture } \% = \left(\frac{\text{W2} - \text{W3}}{\text{W2} - \text{W1}}\right) \times 100$$

Where;
W_1= Weight of crucible
W_2=Weight of the sample with crucible
W_3 = Final weight with crucible

Ash content determination

The ash content was assessed in the Muffle furnace (BioBase JKKZ.5.12GJ, Shandong, China) following Thiex et al. (2012) protocol. About 2.5 g of pollen samples were measured into each crucible. The samples were burned on a hot plate under a fume hood until the smoke stopped, and then they were placed in a Muffle furnace and heated to 550°C for 5 hours. The weight of the crucibles was measured after they were cooled in a desiccator. The total amount of ash was determined following the formula from AOAC (2000).

$$\text{Ash(wet basis)}\% = \frac{\text{M}_{\text{Ash}}}{\text{M}_{\text{Wet}}} \times 100$$

Crude protein determination

For characterizing the total protein content, 1 g of the samples was digested in a macro Kjeldahl flask containing 4 g of the catalytic mixture (1:3 $CuSO_4$ and K_2SO_4) and 20 mL of concentrated H_2SO_4 (95 - 97%) in the presence of a catalyst (potassium sulfate, copper sulfate) until the solution turned clear and blue-green in color. The resulting ammonia was collected in a boric acid solution and distilled, after which it was neutralized with 90 mL (30%) NaOH. To determine the total protein, the nitrogen values were multiplied by a conversion factor of 6.25 (Roulston et al., 2000).

$$\text{Nitrogen}\% = \frac{\text{V HCl} \times \text{N HCl} \times 14.0 \times 100}{1000 \times \text{Wo}}$$

$$\text{Protein}\% = 6.25 \times \text{Nitrogen}\%$$

Where;
V = Amount of HCl (in milliliters) used to reach the titration's endpoint
N = Molarity (M) of the HCl
Wo = Weight of the sample based on dry matter
14 = Molecular weight of atomic nitrogen
6.25 = Conversion factor

Total dietary fiber determination

For determining the fiber content, 2 g of milled pollen, defatted in petroleum ether, were heat digested with a solution of H_2SO_4 0.113 M, and subsequently, with a solution of NaOH 0.313 M, for 30 minutes in each digestion. After neutralizing the residue with hot water, washing was performed with 20 mL of ethanol and 10 mL of ethyl ether. The residue was then incinerated at 550°C in an oven and the fibers were quantified by gravimetry (AOAC, 2000).

Carbohydrate concentration of the pollen

The carbohydrate content of bee pollen was determined by calculation using different methods (AOAC, 2000).

$$\text{Total Carbohydrate (\%)} = 100 - \%\,(\text{Moiture} + \text{Protein} + \text{Total dietary fiber} + \text{Ash})$$

Since it is not feasible to incorporate crude fat content data, it is omitted from the formula of carbohydrate calculations. Although bee pollen is supposed to have a very low-fat content (less than 5%), the formula used to calculate the carbohydrate content lumps fat content in the proximate composition into the carbohydrate fraction, which causes the carbohydrate content to be overestimated.

Mineral analysis of pollen

The mineral content of pollen samples was evaluated by flame atomic absorption spectrometer and flame photometer instruments (Elico, CL-378, India). By dilution of stock solutions (1000 mg/L) of each element, standard solutions of trace elements have been prepared for calibration. Fresh serial dilutions of every element under examination had been prepared. To reduce the impact of the organic matrix, sample digestion with microwave assistance was done before analysis. Briefly, 0.5 g of the pollen sample was digested with 2 mL H_2O_2 (30% v/v) and 4 mL HNO_3 (65% v/v). A blank control was digested similarly. The initial 500 W was used for the digesting program, which ramped up for one minute and was maintained for four minutes. The second phase started at 1000 W, increased for 5 minutes, and then had a 5-minute hold period. The third phase started at 1400 W of power and ramped up for 5 minutes, with a 10-minute pause time in between dilution steps (up to a ten-fold dilution) was carried out before analysis. Ca, Mg, and Pb studies were carried out using flame atomic absorption spectroscopy (AAS). To analyze Na and K, flame atomic emission spectroscopy was used. The calibration standards were prepared by diluting the standards with a 2% solution of nitric acid in ultrapure water. Calibration curves were constructed by plotting the signal on the y-axis (analyte peak area) against the analyte concentration on the x-axis. Quantification of mineral elements (Ca, K, Mg, Na,) was carried out using a calibration curve that covered a range of concentrations from 10 to 150 mg/L (10, 25, 50, 75, and 150 mg/L). The graphs obtained in all the

calibration curves were straight lines, with the coefficient of the determination values (R^2) higher than 0.99. Using the appropriate fuel and oxidant combination, each trace mineral element was measured at its wavelength using cathode lamps (AOAC, 1990).

Data Analysis

The obtained data are presented as mean values ± standard error (SE), calculated from measurements taken in triplicate. Data analysis was performed using SPSS software (version 20), and mean differences were assessed with Tukey's multiple range test at a significance level of $P < 0.05$.

Results and Discussion

Chemical composition

Bee pollen collected by honey bees is considered a healthy food with a wide range of nutritional and therapeutic properties. The results of the analysis of pollen from different plant types are presented in Table 1.

Moisture content

The current study found that the moisture content of *Eucalyptus globules* (17.3 ± 0.5%) pollen samples was significantly different ($P < 0.05$) from others, whereas *Sesamum indicum* pollen has the lowest (10.3 ± 1.3%). The moisture content detected in this study is higher than that of Romanian pollen samples, which ranges from 3.0 to 11.9% (Oroian et al., 2022). However, these findings were lower than those of an earlier study conducted in Ethiopia by Addi et al. (2017), who observed a moisture content of 19.3 - 25.0% of bee-collected pollen in Southwest Ethiopia. Based on pollen composition and the standardization of the analytical method, these results showed that the moisture content of the current study was within the permissible ranges of 20 - 30% moisture (Campos et al., 2008). The study found that the moisture content of dried pollen should range between 6 to 8% to maintain the quality and stability of pollen (Campos et al., 2008). Fresh pollen has a higher nutritious and biological value than dried pollen collected by bees. Its high water content also makes it a perfect culture medium for microorganisms. The excessive moisture content detected in some pollen samples could be related to insufficient pollen drying or storage conditions. Bee-collected pollen should be harvested daily and stored in a nitrogen atmosphere to preserve its high quality until consumption (Campos et al., 2010).

Ash content

B. carinata pollen had significantly ($P < 0.05$) higher ash content (3.0 ± 0.5%) than other pollen types (Table 1). From the current study finding, the lowest ash content was recorded for *Guizotia scabra* pollen samples (1.7 ± 0.3%). These results are consistent with findings by Almeida-Muradian et al. (2005), who reported that pollen collected by bees in Romania had an ash content of 2.2%, and Oroian et al. (2022), who documented ash concentrations ranging from 2.29% to 4.02%. However, Addi et al. (2017) discovered a lower ash concentration in pollen from forests in Southwest Ethiopia for *Guizotia* spp. (1.4 ± 0.1%) and *Planatago lanceolatum* (1.3 ± 0.3%). In the case of *Brassica* spp. Yang et al. (2013) also reported a similar ash percentage, 3.53 ± 0.10%. In China, bee pollen from *Brassica* spp. is commonly used as a natural dietary supplement and as a herbal remedy that increases the body's resilience to illnesses, such as cancer (Wu et al., 2007). According to Velásquez et al. (2017), pollen collected by bees from *Brassica* spp. exhibited antibacterial activity against harmful pathogens. Almeida-Muradian et al. (2005) suggested a 2-6% ash percentage for dry pollen pellets internationally. According to Herbert and Shimanuki (1978), pollen pellets usually incorporate 2 to 4% ash by dry weight. Ash content is a quality indicator that is dependent on the kind of soil, the plant's ability to accumulate minerals, and its botanical origin (Carpes et al., 2009).

Protein content

The usefulness of bee pollen obtained from different floral origins for bees and humans can be assessed based on its protein, amino acid, and fatty acid compositions. The current study found that protein content varied significantly among pollen samples, ranging from 16.3 ± 0.5 g/100 g (*G. scabra*) to 24.9 ± 5.6 g/100 g (*E. globules*). The protein content of *Eucalyptus*

Table 1: Proximate composition of pollen (average ± SE)

Pollen type	Proximate composition				
	Moisture content (%)	Ash content (%)	Protein (%)	Crude fiber (%)	Carbohydrate (%)
Sesamum indicum	**10.3±1.3^{b}**	2.2 ±0.1^{bc}	21.4±0.3^{ab}	2.2±0.4^{a}	64.0±1.5^{b}
Guizotia scabra	10.5±1.2^{b}	**1.7 ±0.3^{c}**	**16.3±0.5^{c}**	2.4±1.3^{a}	**69.1±1.0^{a}**
***Plantago* spp.**	12.5±2.0^{b}	2.6 ±0.1^{ab}	19.7±1.4^{bc}	1.4±0.7^{b}	63.9±0.7^{b}
Eucalyptus globules	**17.3± 0.5^{a}**	2.3±0.6^{bc}	**24.9±5.6^{a}**	**1.40±0.7^{b}**	**54.1±5.2^{c}**
Brassica carinata	12.4± 2.8^{b}	**3.0±0.5^{a}**	19.4± 0.5^{bc}	**2.6±0.9^{a}**	64.7±0.9^{b}

The lowest and highest values of the corresponding parameters are indicated by bold numbers. There is no significant difference ($P < 0.05$) between means with the same letters in the same column

globules was found significantly different ($P < 0.05$) from other pollen types. The results align with the findings published by Addi et al. (2017), who reported 15.0 - 27.1 g/100 g. The protein content of monofloral bee pollen varies significantly between countries despite coming from distinct botanical sources. According to Taha et al. (2019), the protein content of *Brassica* pollen has a nutritious value of 18.9% in Saudi Arabia and (27.27 ± 0.72) in China. Spulber et al. (2020) revealed that *Brassica* spp pollen is an important source of protein, even though the highest protein content was recorded for *E. globules* in the current study. Furthermore, Alshallash et al. (2023) observed a greater protein content of 30% for *Eucalyptus* spp. The results of the current study are also in line with the guidelines provided by Bogdanov (2004) and Campos et al. (2008), who set the protein content of bee-collected pollen dry weight at 10 to 40 g/100 g. Proteins were the second most abundant component in pollen. The amount of crude protein in pollen varies greatly depending on the type of plant the pollen comes from. The primary feature that determines the quality of honey bee-collected pollen is its protein content (Somerville, 2001).

E. globules pollen is regarded as a superfood with a variety of nutritional and medicinal benefits. Flavonoids, alkaloids, tannins, and propanoids are just a few of the phytochemical compounds that can be found in abundance in the leaves, stems, and roots of *E. globulus* (Dixit et al., 2012). Alshallash et al. (2023) observed the highest DPPH scavenging activity, while Araújo et al. (2017) reported a significant inhibitory effect of pollen from *Eucalyptus* spp. *Eucalyptus* spp. has been used as an antibiotic, to support liver and renal function, or just to give the body an extra boost of vitamins and nutrients (Campos et al., 2021). According to the current findings, the protein content of *S. indicum* and *E. globules* pollen is categorized as average. According to Hernández-Monzón et al. (2019), sesame (*Sesamum indicum* L.) is regarded as a superfood because it includes approximately 15 essential amino acids, 80% polyunsaturated fats, and minerals like bioavailable calcium, iron, and zinc that aid in the digestion of carbohydrates, proteins, and fats. Protein levels in pollen samples vary greatly, which could be influenced by floral type, geographical location, and condition of storage (Negrao & Orsi, 2018). Proteins are highly valued in the food industry not only for their role in producing amino acids essential for human growth and nutrition but also for their numerous functional properties in food systems and their ability to enhance nutritional value.

Total dietary fiber

B. carinata pollen had significantly ($P<0.05$) the highest value of total dietary fiber (2.6 ± 0.9%) than *Plantago* spp (1.4 ± 0.7) and *E. globules* (1.40 ± 0.66%). The results presented here agree with the findings made by Kostic et al. (2015), who found an average total dietary fiber of 2.7 ± 1.2 % in Serbia, and Bogdanov (2016) found that the total dietary fiber of pollen ranged between 0.3 and 20%. Our findings are further confirmed also by Hassan's (2011) study, which found a total dietary fiber content of 1.37% in the palm pollen grain. Furthermore, the total dietary fiber in the current investigation fits within the range recommended by Campos et al. (2008) (1 - 13%). The variance in crude fiber composition could be attributed to different plant species. Starch and insoluble polysaccharides such as cellulose, pectin, cellulose, and sporopollenin make up crude fiber (Bogdanov, 2016). The primary components of bee pollen are cellulose and callose, which make it a useful source of fiber for food. Dietary fiber, also referred to as roughage, is the portion of food that stays intact in the stomach and small intestine and adds no nutritional value to the food but is vital to human health.

Carbohydrate content

Carbohydrate content ranged from 54.1 ± 5.2 (*E. globules*) to 69.1 ± 1.0 g/100 g (*G. scabra).* In the present study, the carbohydrate content of *G. scabra* was significantly different ($P < 0.05$) from other pollen types. A similar conclusion was reached by Spulber et al. (2020), who noted that *Plantago lanceolata* has the highest concentration of macronutrients (carbohydrates). The values observed in the current study are closely comparable to those reported by Nogueira et al. (2012) (67.8 - 73.2 g/100 g) and Yang et al. (2013) (59.4 - 75.7 g/100 g). However, these results were greater than Campos et al. (2008), who reported 13 to 55%. Thakur and Nanda, (2020) summarized in their review that pollen consists of 54.2% (18.5-84.3%) carbohydrates on average. Carbohydrates in pollen vary

Table 2: The mineral content (average ± SE) of pollen

Pollen type	Mineral content			
	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)
Sesamum indicum	339.2±1.1^{b}	7231.4±310.3^{bc}	234.1 ±3.7^{cd}	152.4±6.4^{b}
Guizotia scabra	1998.3±1123.1^{ab}	4470.0±1196.8^{cd}	555.0±421.0^{bcd}	139.04±98.8^{b}
***Plantago* spp.**	577.4±5.0^{b}	8277.6±57.5^{ab}	199.1±0.5^{d}	81.6±2.1^{b}
***Trifolium* spp.**	2202.4±869.4^{a}	4180.3±421.3^{d}	1227.1±12.6a	123.3±19.1^{b}
Brassica carinata	2321.3±608.8^{a}	7396.3±3336.8^{bc}	1024.8±19.9^{ab}	280.7±133.2^{ab}
Eucalyptus globules	2123.5±404.3^{ab}	10596.9±1610.1^{a}	863.4±242.4^{abc}	380.9 ±95.9^{a}

There is no significant difference ($P < 0.05$) between means that have the same letters in the same column

based on the botanical and geographic origins. The main carbohydrates included are fructose, glucose, and sucrose (more than 90% of the total carbohydrate content) (Bertoncelj et al., 2018). Pollen is primarily composed of carbohydrates, which are an essential source of energy and nutrition.

Mineral content

Table 2 illustrates the mineral content of pollen collected by honey bees. In this study, the calcium (Ca) concentration of pollen obtained from honey bees ranged from 339.2 ± 1.1 mg/kg (*S. indicum*) to 2321.3 ± 608.8 mg/kg (*B. carinata*). In comparison to this finding, Addi et al. (2017) found a lower calcium concentration (160 to 435 mg/kg) in pollen collected from several plant species. The calcium concentrations of *G. scabra*, *Trifolium* spp, and *P. lanceolata* are 379.0 ± 0.8 mg/kg, 232.1 ± 51.0 mg/kg, and 214.5 ± 5.2 mg/kg, respectively, according to Addi et al. (2017). However, the calcium content observed in the current study was higher than the values reported by Asmae et al. (2021), which ranged from 2.2 ± 1.0 mg/kg to 22.7 ± 2.6 mg/kg in Moroccan monofloral pollen samples. Furthermore, the Ca amount found is within the recognized ranges of 200 - 3000 mg/kg based on the composition of pollen and standardization of analytical methods (Campos et al., 2008). Ca is involved in both root development and plant physiological processes (Amadou et al., 2022). It is also responsible for the water balance in extracellular and intracellular media and the depolarization of cellular membranes.

In all samples, potassium (K) was the most prevalent element. The maximum potassium concentration (10596.9 ± 1610.1 mg/kg) was found in *E. globules* pollen, whereas *Trifolium* spp pollen had the lowest (4180.3 ± 421.3 mg/kg). At $P < 0.05$, the K concentration of *E. globules* pollen is significantly different from other pollens. In a similar study, Addi et al. (2017) revealed that the concentration of K in pollen samples from several plant species ranged from 0.9 to 592.3 mg/kg. Furthermore, the K concentration in the current investigation was within the acceptable ranges of 4000 - 20000 mg/kg based on the composition of pollen and analytical method standardization (Campos et al., 2008). Asmae et al. (2021) also found a similar average of K in Moroccan monofloral bee pollen, ranging from 485.4 ± 9.3 to 4594.3 ± 18.3 mg/kg. In plant nutrition, potassium is a crucial element involved in several processes, including enzyme activation, photosynthesis, and water absorption. In addition, potassium is essential for nerve impulse transmission, protein synthesis, lipid metabolism, muscle contraction, and maintaining fluid and electrolyte balance in animals and humans.

Trifolium spp pollen contains a significantly greater concentration of magnesium (Mg) (1227.1 ± 12.6 mg/kg) than other pollen species, whereas *Plantago* spp pollen contains less Mg (199.1 ± 0.5) mg/kg. The Mg concentration range in this study agrees with the findings of Aldgini et al. (2019), who reported Mg content of 641.4 to 1575.2 mg/kg. In a related study conducted in Romania, the amounts of magnesium ranged from 702 to 965 mg/kg (Harmanescu et al., 2007). The magnesium concentration of pollen collected by bees ranged from 68.7 ± 5.3 to 793.4 ± 13.6 mg/kg, according to comparable data found by Asmae et al. (2021). The current study's Mg concentration was similarly within the acceptable ranges of 200 - 3000 ppm based on the composition of pollen and analytical method standardization (Campos et al., 2008). Magnesium (Mg) is an essential element for human as well as plant physiology. It is present in many enzymes and involved in the structure of proteins, lipids, and carbohydrates. According to Graikou et al. (2011), bee pollen from *Trifolium* spp is high in phenolic acids and flavonoids. This mixture has been shown to stimulate cellular antioxidant systems by other natural products and to exhibit the observed free radical scavenging activity on HFL-1 human fetal lung embryonic fibroblasts.

Except for *B. carinata*, the sodium (Na) concentration in *E. globules* (380.89 ± 95.85 mg/kg) is statistically significantly higher than that of other pollen types. These results agree with those of Addi et al. (2017), who discovered that bee pollen collected from several plant species had a Na concentration ranging from 4.8 - 610 mg/kg. The same authors previously reported a Na concentration of *G. scabra* (405.8 ± 0.3 mg/kg), which is comparable to the current study. The results of this investigation aligned with those of Asmae et al. (2021), who showed that sodium levels in Moroccan bee-collected pollen samples ranged from 91.9 ± 0.6 mg/kg to 397.2 ± 4.1 mg/kg. Because bee pollen is a significant source of minerals, it has great nutritional value and is used extensively in Ethiopian food.

Hemolymph osmotic pressure and intracellular and intercellular fluids are regulated by a mixture of calcium (Ca), phosphorus (P), and magnesium (Mg) (Matuszewska et al., 2021). The composition and mineral variation in pollen can be influenced by factors such as soil type, climate, geographical origin, and botanical species, as plants accumulate varying amounts of mineral salts (Liolios et al., 2019; Taha, 2019). Other parameters, such as the season of collection (Taha et al., 2019) and pollen load storage (Human & Nicolson, 2006), influenced the mineral content.

Lead (Pb) was not found in all pollen types sampled from the study area. Asmae et al. (2021) also reported that Moroccan monofloral bee pollen was free of lead (Pb). The lead level of the pollen collected by bees cannot exceed 50 µg/100 g, hence these values are within allowable limits for pollen quality (Campos et al., 2008). Lead is a toxic metal that poses a serious risk to human health. This component is considered to be one of the main causes of pollution in the environment.

Because bee pollen is a naturally occurring source of nutrients, consuming it is highly recommended as a

dietary supplement. However, because it may contain heavy metals, pesticides, bacterial and fungal toxins, and allergic reactions, there are some risks involved (Dinkov & Stratev, 2016). According to Mauriello et al. (2017), lactic acid bacteria, yeasts, molds, total viable count, and Enterobacteriaceae which thrive at moderate temperatures may cause microbial deterioration in bee pollen due to unsanitary manufacturing and storage circumstances. Additionally, individual pollen grains are collected from wind-pollinated weeds and trees as well as insect-pollinated plants, which may cause allergic reactions due to accidental intake of airborne pollens. This is why allergic reactions, including anaphylaxis, are typically immediate IgE-mediated hypersensitivity responses observed after pollen intake (Choi et al., 2015). According to McNamara and Pien (2019), consuming pollen can reduce the threshold for mast cell degranulation during exercise because it increases gastrointestinal permeability or osmotic effects. As a result, people who consume pollen can experience exercise-induced anaphylaxis. It is advised that these people skip exercise for 4-6 hours after consuming pollen. Considering these factors, it can be concluded that the main causes of bee pollen allergies include its combination with airborne allergens, fungi reacting to allergic compounds, pesticide contamination, and exercise following ingestion.

Conclusion

The Chemical and mineral contents of monofloral pollen obtained from the Oromia region were evaluated in this study. The best bee-collected pollen for animal or human food supplements can be found with the help of the periodic compilation of pollen nutrition data. According to the data we obtained, the average moisture content, ash content, total dietary fiber, protein, and carbohydrate concentrations of pollen samples are 12.3± 2.8%, 2.2 ± 0.6%, 2.1 ± 1.0%, 19.6 ± 3.6%, and 64.1 ± 5.5 %, respectively. *E. globules* pollen has the maximum moisture and protein content, but the smallest total dietary fiber and carbohydrate content. *G. scabra* has the highest carbohydrate content, but the lowest protein and moisture level. Regarding the mineral composition, potassium, and magnesium were found to be prominent in all bee-collected pollen samples, while lead was not traceable in any of them. It is concluded that because pollen has a nutritional composition that meets human needs, it can be used to supplement food for humans. These data can be utilized to guide healthcare recommendations and consumer decisions in addition to assisting beekeepers in producing pollen from bees. The certification and standardization of bee-collected pollen produced in Ethiopia will be a future endeavor to improve bee pollen knowledge to promote its application in the food industry.

Ethical Statement

There are no ethical issues with the publication of this article.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Teferi Damto: investigation, methodology, data curation, formal analysis, writing-original draft, and writing-review and editing. Meseret Gemeda: conceptualization, data curation, investigation, methodology, and writing-review and editing. Dheressa Kebaba: investigation data curation, methodology, formal analysis, and writing review, and editing.

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REVIEW PAPER

A Novel Approach on Propolis Extraction: Supercritical Carbon Dioxide Extraction, Advantages and Disadvantages

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Abstract

Propolis is a unique bee product rich in bioactive compounds. The natural structure of propolis is resinous and waxy, which makes it indigestable for humans. Extraction process is a neccessity in order to obtain bioactive compounds of propolis. Ethanolic maceration is one of the most employed methods for propolis extraction. However, this method has some shortcomings, such as solvent residue, dark coloration, and a lenghty process. To eliminate these shortcomings, new and environmentally friendly technologies are also employed. Supercritical carbon dioxide extraction is one such method. This method has been increasingly employed in recent years. This review highlights properties, advantages, and disadvantages of supercritical carbon dioxide extraction.

Introduction

Propolis is a bee product produced by bees using various secretions from different plant species, combined with their own secretions and wax. Propolis is mainly produced to strengthen weak points such as holes and cracks in the hive, defend against invaders and maintain a constant temperature in the hive (Devequi-Nunes et al., 2018).

Propolis has various functional properties, especially antioxidant and antimicrobial activities due to the numerous polyphenols in its structure. Propolis' chemical composition is the key factor of determining its biological activity. The quantity and diversity of polyphenols contained in propolis affects its bioactivity and provides different functional properties (Ghisalberti, 1979).

Propolis is not suitable for human consumption in its raw form, making the extraction process essential to benefit from its rich composition. Therefore, it is important that the bioactive components remain undamaged during extraction. Ethanol or methanol solutions are widely used in propolis extraction. According to the existing literature, a 70-80% ethanol solution is reported to dissolve the majority of bioactive components. In their study, Margeretha et al., (2012) extracted propolis with ethanol and water. They reported that the amount of extract obtained from propolis is associated with its wax content. According to their findings, extraction yields were between 50% and 70% when ethanol was used as a solvent, while the extraction yields were approximately 10% when water was used.

The extraction process for propolis and other natural products typically consists of four steps. Extraction is completed with the stages of entering the solid matrix of the solvent, dissolving the components in solvent, separating the extract from the solid matrices and collection of soluble substances. Factors affecting extraction efficiency are the characteristics of the solvent used, particle size of the raw material, temperature and duration of the process (Zhang et al., 2018). Extraction is a separation technique that involves isolating a desired compound from a matrix. It can be described as the process of removing a soluble substance from an insoluble residue, whether liquid or solid, by using a liquid solvent. Thus, it is a solvation process that relies on mass transfer phenomena (Herrero et al., 2010; Ahmad et al., 2019).

In the context of natural matrices, extraction techniques play a fundamental role in isolating desired compounds. The selection of an appropriate solvent is critical for this process. Ethanol, methanol, ethyl ether, chloroform, and acetonitrile are usually used as solvents. Traditional ethanolic maceration is commonly used for extraction of propolis. But, this method has

several disadvantages, including lengthy processing times, solvent residues, the presence of beewax in the final product, and the dark coloration of the extracts. This method also offers some advantages such as simplicity, cost-effectiveness, and suitability for small- to medium-scale operations (Machado et al., 2016; Reis et al., 2020; Sun et al., 2022).

According to Brazilian propolis regulations, propolis must contain a maximum of 25% wax (w/w), 8% moisture (w/w), and a minimum of 0.5% flavonoid content (w/w) (Anonymous, 2001). In Turkish propolis regulations, propolis must contain at least 40% balsam (w/w), a maximum of 8% moisture (w/w), a minimum of 10% total phenolics (w/w), 3% total flavonoids (w/w), and a maximum of 50% wax (w/w) (TFL, 2024).

To address the previously mentioned shortcomings, researchers have explored new extraction methods. Consequently, ultrasound-assisted extraction, microwave-assisted extraction, and supercritical carbon dioxide extraction have been utilized for propolis extraction. This review focused on the characteristics of supercritical carbon dioxide extraction, advantages and disadvantages of this method in the context of propolis extraction.

Supercritical Carbon Dioxide Extraction

Supercritical refers to the state of a substance where it acts as a non-condensing, single-phase fluid, occuring when the substance exceeds its critical temperature and pressure. In this supercritical state, the substance exhibits unique physicochemical properties, such as high density, intermediate diffusivity, and low viscosity and surface tension, combining characteristics of both gases and liquids (Amaral et al., 2017). Supercritical fluid posesses a density similar to that of a liquid, while its viscosity and diffusivity are comparable to those of a gas. Thus, a supercritical fluid can function as a solvent with properties similar to a liquid, and it offers enhanced mass transfer kinetics (Temelli et al., 2012).

Supercritical fluid extraction is a process that utilizes a supercritical fluid to isolate desired compounds from a matrix. This technique has been extensively applied for extraction bioactive substances from natural products (Biscaia & Ferreira, 2009). The method leverages pressure and temperature to enhance the extraction efficiency. Additionally, low viscosity and high diffusivity of supercritical fluids, make the process is notably swift (Yuan et al., 2019).

Supercritical fluid extraction is increasingly recognized as a viable alternative to traditional methods. Solvents in the supercritical phase exhibit unique properties that enhance their ability to extract substances. The high density of these fluids provides them with strong solvating capabilities, while their high diffusivity and low viscosity enable efficient penetration into solid matrices. Choosing the appropriate supercritical fluid is crucial for process development, with a variety of compounds available as solvents. However, carbon dioxide is often preferred in separation systems for its safety and cost-effectiveness, despite alternatives like ethylene, methane, nitrogen, xenon, and fluorocarbons (Ahmad et al., 2019).

The critical point of carbon dioxide (CO_2) was first identified by Andrews in 1869. Its initial use as a solvent occurred in Russia and the USA during the 1960s. By 1993, 42 distinct oils were commercially extracted using CO_2. Supercritical carbon dioxide is widely favored among supercritical fluids due to its non-toxicity, non-flammability, non-corrosiveness, non-explosiveness, cost-effectiveness, and low critical pressure and temperature (73.8 bar and 31.0°C). CO_2 is easily affordable and readily available in high purity. It is known to minimally alter bioactive compounds, preserving their therapeutic and functional properties. Supercritical carbon dioxide is a preferred alternative to organic solvents as it is, capable of dissolving lipophilic substances, and easily separable from end products. Another advantage is that CO_2 is gaseous at ambient temperature and pressure, simplifying compound recovery and results in solvent-free extracts (Joana Gil-Chávez et al., 2013). Furthermore, CO_2 is eco-friendly and classiffied as “Generally Recognized As Safe” (GRAS) by both the FDA (U.S. Food and Drug Administration) and EFSA (European Food Safety Authority) (Ahmad et al., 2019). The non-polar nature of CO_2 allows non-polar components to exhibit higher solubility than polar ones of similar molecular weight. Larger molecular size reduces solubility in supercritical fluids. Hence, non-polar solutes with low molecular weight and high vapor pressure are more soluble in supercritical carbon dioxide under low-density conditions, whereas solubilizing larger, slightly polar, and less volatile solutes requires higher densities. This allows for high selectivity by adjusting temperature and pressure, a key advantage of supercritical carbon dioxide extraction technology, often reducing the need for further refining. When the target compound is polar, the polarity of the supercritical solvent can be enhanced by adding a polar cosolvent. The cosolvent interacts with the solute through hydrogen bonding, charge-transfer complex formation, and dipole-dipole interactions, as well as with the solvent, thereby increasing the solvent mixture density and improving solubility. Ethanol, a solvent classified as GRAS, is the preferred cosolvent for food applications. Numerous research groups have employed this gradient method, maintaining a high modifier composition to enable supercritical fluid chromatography separation of polar compounds (Paviani et al., 2010; Ahmad et al., 2019).

The easiest way to separate a supercritical fluid from a solution is by changing the pressure. Since the critical temperature of CO_2 is close to room temperature, it can be separated from the solution by altering the pressure while keeping the temperature constant.

In the supercritical carbon dioxide extraction the cosolvent plays a vital role in improving solubility and efficiency. Short-chain alcohols, such as ethanol and methanol, are commonly preferred due to their effectiveness (Tirado et al., 2018). Other polar cosolvents including acetonitrile, acetone, water, ethyl ether, and dichloromethane are also utilized (Salleh, 2012). Supercritical CO_2, being non-polar, benefits from the addition of cosolvents to enhance polarity and imrove extraction efficiency. This approach also allows for operation at lower pressures with reduces the required amount of supercritical solvent, offering economic advantages. Ethanol is particularly advantageous as a cosolvent in supercritical extraction due to its non-toxic nature (Salleh, 2012; Pimentel-Moral et al., 2019).

Previous Studies with Supercritical Carbon Dioxide Extraction

The data indicated that optimal extraction conditions, yielding higher amounts of the target compounds kaempferol and formononetin, as well as the greatest total polyphenols and antioxidant activity, were attained using a 4% cosolvent. The presence of ethanol alongside supercritical CO_2 increased the extraction of total phenolics from red propolis by up to 57%. Additionally, the antioxidant capacity of the extracts improved by 70%. This enhancement is likely due to the increased polarity of the solvent and ethanol's ability to expand the extraction surface area within the natural solid matrix (Souza et al., 2018; Reis et al., 2020).

According to Biscaia and Ferreira (2009), both single-step and two-step procedures were applied for extraction. The single-step process used a fixed pressure and temperature, and required ethanol as a cosolvent. In the two-step process, two different pressure values were used. During the first step, carried out at an average pressure of 100–150 bar, soluble components such as wax and essential oils were separated. In the second step, the pressure was increased to 250–300 bar allowing for the isolation of components responsible for the antioxidant and antimicrobial properties of propolis, such as phenolic acids and flavonoids.

Machado et al., (2015) investigated optimal conditions for the supercritical fluid extraction of Brazilian green propolis. They explored cosolvent ratios of 1-2%, temperatures of 40-50°C, and pressures of 250-350-400 bar. The study was evaluated in terms of total phenolic, total flavonoid, antioxidant capacity, Artepillin C, and p-coumaric acid. The best results were obtained at 50°C, 350 bar, and 1% ethanol. In addition, their findings showed that supercritical extraction reduced total phenolic and total flavonoid values, while increasing the amounts of Artepillin C and p-coumaric acid.

Stahl et al. (1988) extracted raw propolis using supercritical CO_2 at 600 bar and 40°C, separating wax and retaining insoluble flavonoids. Catchpole et al. (2004) utilized supercritical carbon dioxide both as an antisolvent to precipitate high molecular mass components and as a solvent to extract ethanol and soluble components from ethanolic propolis extracts. Lee et al. (2007) obtained highly pure 3,5-diprenyl-4-hydroxycinnamic acid from Brazilian propolis by extracting with supercritical carbon dioxide modified with ethanol as a cosolvent, followed by column chromatography. Chen et al. (2009) found that a supercritical carbon dioxide extract containing 41.2% (wt) 3,5-diprenyl-4-hydroxycinnamic acid effectively inhibited the growth of human colo-205 cancer cells, despite its relatively low yield compared extraction with ethyl acetate using a Soxhlet apparatus. Paviani et al. (2010) explored the supercritical fluid extraction of a dried ethanolic extract from Brazilian propolis, focusing on component fractionation. They reported greater selectivity at lower solvent densities, highlighting significant differences in the phenolic content between the extracts and raffinates.

In a study, conducted by Monroy et al. (2022), following extraction, the ethanolic and hydroalcoholic extracts were fractionated using supercritical CO_2 as an antisolvent at a constant temperature of 50°C. The process involved four incremental pressures across a series of separators operating at 200, 100, and 80 bar, concluding with atmospheric pressure (1.013 bar). The method was evaluated based on extraction yield, total phenols, total flavonoids, antioxidant activity, and color. The results indicated that pressure impacted both the yield and phenolic compound concentration, with the most effective fractionation occurring in the first and second separators. Notably, all extracts exhibited potent antioxidant activity.

Machado et al. (2016) compared total phenolic compounds and total flavonoids obtained via both ethanolic extraction and supercritical carbon dioxide extraction. Their findings demonstrated that ethanolic extraction is generally more efficient for total phenolic compounds and total flavonoids. On the other hand, supercritical carbon dioxide extraction proved more effective for isolating specific compounds, such as p-coumaric acid and Artepillin C.

Fractionation of ethanolic propolis extracts with supercritical carbon dioxide yielded 11 to 18% returns, with minor differences in Artepillin C composition compared to the original ethanolic extracts. However, the chemical profiles of the four markers were distinctly different from those of the ethanolic extracts. The selectivity of supercritical carbon dioxide was evident in the chemical profile changes of the extracts, which varied with temperature and pressure. This suggests that higher temperatures and pressures than those applied in this study might result in extracts with increased yields and higher marker concentrations, especially Artepillin C (Reis et al., 2020).

Advantages of Supercritical Carbon Dioxide Extraction

Supercritical fluid extraction is regarded as a technological breakthrough, particularly for high-value products, due to its low-temperature operation, efficient solvent usage, recyclability, reduced energy requirements, and enhanced product quality owing to the absence of solvent residue. Versatility of supercritical fluid extraction lies in its selective nature, achieved by precisely controlling temperature and pressure during the extraction process. Consequently, supercritical fluid extraction is increasingly viewed as a viable option for the pharmaceutical, fine chemical, and food industries (Paviani et al., 2013).

Supercritical CO_2 particularly stands out due to its lower critical temperature and pressure (31°C and 74 bar) compared to other supercritical solvents, making it advantageous for extracting thermosensitive compounds (Novak et al., 2014). Moreover, supercritical fluid extraction preserves the chemical integrity of the extracted substances, including their antioxidant capacity, owing to the use of low temperatures (Machado et al., 2019).

The environmentally friendly properties of supercritical CO_2 provide a key incentive for substituting organic solvents. In the event of accidental release, supercritical carbon dioxide poses no environmental hazard due to its non-toxic and safe nature. The use of non-flammable supercritical carbon dioxide as a solvent significantly lowers the risk of explosions reactions, especially those involving highly reactive substances. The superior heat transfer capability of supercritical carbon dioxide ensures effective temperature management, preventing hot spots or runaway reactions in highly exothermic processes (Ahmad et al., 2019; Paviani et al., 2010).

Gas-liquid catalyzed chemical reactions are typically diffusion-controlled. This limitation can be minimized by removing the gas-liquid interface and enhancing diffusivity with supercritical carbon dioxide, thereby increasing reaction rates by reducing mass transfer barriers (Pereda et al., 2005).

The activity and selectivity of porous catalysts are affected by adsorption/desorption and pore transport. In conventional gas or liquid reaction media, one of these factors is typically favorable while the other is not. Traditional media often make it challenging to achieve desired fluid properties such as gas-like transport, liquid-like solvent power, and heat capacity, which are essential for optimal system performance and enhancing the stability of porous catalysts in supercritical reaction media. Supercritical carbon dioxide addresses these challenges by providing adjustable fluid properties, such as diffusivity and viscosity, through changes in pressure or temperature. These properties enhance catalyst activity, product selectivity, and the stability of porous catalysts. Additionally, supercritical carbon dioxide aids in the penetration of reactants into the porous structure of the catalyst (Zhang et al., 2014).

In certain chemical reactions, carbonaceous byproducts can lead to catalyst deactivation through coke formation, which accumulating on both the internal and external surfaces of the catalyst. Supercritical carbon dioxide helps mitigate this issue by removing and transporting these materials due to its high diffusivity, thereby increasing the catalyst's lifetime and facilitating its regeneration. Separating products from traditional solvents is often laborious and energy-intensive. In contrast, within a supercritical carbon dioxide reaction medium, products can be easily separated by merely reducing the CO_2 pressure. The acceleration of reaction rates and simplification of product separation enable the use of smaller continuous reactors compared to traditional ones with equivalent performance. This advantage is enhances process safety and reduces the spatial requirements of chemical plants (Baiker, 1999; Reverchon & De Marco, 2006).

Disadvantages of Supercritical Carbon Dioxide Extraction

Supercritical CO_2 extraction is a well-established method for extracting propolis (Banskota et al., 2001). However, its widespread use in propolis processing is limited due to its low yield of flavonoids, high costs, significant energy consumption, and inefficient raw material usage.

Some drawbacks of supercritical fluid extraction compared to traditional liquid solvents for separation processes include the requirement for high pressure, the complexity of recycling measures to lower energy costs, and the significant capital investment in equipment (Sun et al., 2022).

Conclusion

In the context of extraction, bioactive compounds are the most important substances derived from natural resources. Propolis is one of the most valuable bee products, containing numerous biactive compounds, such as polyphenols, flavonoids, and terpenes. The primary problem with propolis' consumption is its poor digestability in humans. Consequently, researchers have been exploring effective extraction techniques for years.

Extraction efficiency has traditionally been the primary focus. However, with increasing environmental concerns, new methods have been developed. From this perspective, supercritical carbon dioxide extraction is applied for propolis extraction due to its non-toxic properties for both humans and the environment. Additionally, supercritical carbon dioxide extraction offers many advantages, including cost-effectiveness, reduced energy requirements, recyclability, and the absence of solvent residue. Moreover, it is one of the best methods tfor obtaining specific compounds.

There are two significant disadvantages of supercritical carbon dioxide extraction. Firstly, due to its

Table 1: Some studies using supercritical carbon dioxide extraction on propolis extraction

Materials from	Cosolvent	Process Conditions	Authors
Italy	-	w: 32 g t: 30 min f: 2 L/min P: room conditions	De Zordi et al., 2014
Brazil	EtOH	w: 5 g t: 118 min f: 1.65 g/min P: 250 bar T: 50°C	Monroy et al., 2017
Brazil	EtOH	w:- t:- f: 1 mL/min P: 20 Mpa T: 54.85°C	Wu et al., 2009
Brazil	EtOH	w:- t:- f: - P: 20-15-10 Mpa T: 60°C	Wang et al., 2003
Indonesia	-	w: 50 g t: 240 min f: 6.59-23.41 g/min P: 150 bar T: 50°C	Fachri et al., 2020
Brazil	EtOH	w: 2.5 g t: 20 min f: P: 200-300-400 bar T: 40-50-60°C	Saito et al., 2021
Commercial Samples	EtOH	w:- t:- f: - P: 250-300 bar T: 59.85°C	Catchpole et al., 2004
Taiwan	EtOH	w: 10 g t:- f: 10 mL/min P: 13.8-27.6 Mpa T: 34.85-59.85°C	C. R. Chen et al., 2009
Türkiye	EtOH	w: 10 g t: 150 min f: 6 g/min P: 150-250-350 bar T: 50°C	Sonverdi et al., 2024
Brazil	EtOH	w: 7.5 g t: 150 min f: 6 g/min P: 350 bar T: 50°C	Dantas Silva et al., 2017

w: weight of propolis; t: time; f: flow rate; P: pressure; T: temperature

non-polar nature, carbon dioxide is not effective for extracting polar compounds. To address this shortcoming, the use of ethanol as a cosolvent is essential. The second disadvantage is high invesment costs. However, this disadvantage can be compensated by the production of high-value-added products.

Ethical Statement

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Conflict of Interest

The author declare that there is no conflict of interest.

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