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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF SEVERAL STINGLESS BEE POLLENS PROCESSED USING DIFFERENT DRYING METHODS

Farklı Kurutma Yöntemleri Kullanılarak İşlenen, Çeşitli İğnesiz Arı Polenlerinin Fitokimyasal Taraması, Antioksidan ve Anti-inflamatuvar Özellikleri

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ABSTRACT

Antioxidants play a crucial function in fighting free radicals that can harm biomolecules in the human body and damage cells, which can lead to immunological disorders including inflammation. In this research, we evaluated bee pollen extracts from six stingless bees (*Heterotrigona itama*, *Tetragonula reepeni, Tetragonula testaceitarsis, Tetragonula fuscobalteata, Tetragonula iridipennis, and Tetragonula pagdeni*) processed using different drying methods for antioxidant and anti-inflammatory activity. Each sample was divided into two specimens, where one was processed by ovendrying at 40°C and the second was chilled at 4 ºC, then extracted by ethanol. The extracts of *T. fuscobalteata* had the highest antioxidant activity when dried in an oven with an IC_{50} value of 36.47 µg/mL, while *T. reepeni* using a chiller was 41.30 mg/mL. The highest anti-inflammatory activity was for oven-dried *T. fuscobalteata* with an IC_{50} of 39.70 mg/mL, while chilled *T. reepeni* was 34.30 µg/mL. Different drying techniques can affect the antioxidant and anti-inflammatory activity of bee pollen extracts as well as their potential as food, pharmaceutical and cosmetic ingredients.

Keywords*: Antioxidant, Anti-inflammatory, Bee pollen, Drying method, Stingless bees*

ÖZ

Antioksidanlar, insan vücudundaki biyomoleküllere zarar verebilecek ve hücrelere zarar verebilecek, iltihaplanma dahil dejeneratif hastalıklara yol açabilecek serbest radikallerle savaşmada çok önemli bir işlev oynamaktadır. Bu araştırmada, farklı kurutma yöntemleriyle işlenmiş altı iğnesiz arıdan (*Heterotrigona itama*, *Tetragonula reepeni, Tetragonula testaceitarsis, Tetragonula fuscobalteata, Tetragonula iridipennis* ve *Tetragonula pagdeni*) arı poleni ekstraktlarını antioksidan ve anti-inflamatuvar olarak değerlendirilmiştir. Arı polenleri, 40ºC'de fırında kurutma yoluyla kurutulmuş ve

4ºC'de soğutularak, ardından etanol ile özümlenmiştir. Fırında *T. fuscobalteata* ekstreleri 36.47 µg/mL IC_{50} değerleri ile en yüksek antioksidan değerine sahipken, soğutma grubu *T. reepeni* 41.30 mg/mL olarak bulunmuştur. En yüksek anti-inflamatuvar etki fırına göre *T. fuscobalteata*, IC_{50} değeri 39.70 mg/mL ve soğutucuya göre *T. reepeni* 34.30 µg/mL belirlenmiştir. Bu bulgular, farklı kurutma tekniklerinin arı polenlerinin antioksidan ve anti-inflamatuvar özelliklerini etkilediğini ve gıda, ilaç ve kozmetik için güçlü bir malzemeye sahip olduğunu ileri sürmektedir.

Anahtar Kelimeler: Antioksidan, Anti-inflamatuvar, Arı poleni, Kurutma yöntemi, İğnesiz arılar

GENİŞLETİLMİŞ ÖZET

Amaç: Dejeneratif hastalıkların artan yaygınlığı, pahalı tedavi masrafları ve geleneksel ilaçların kullanımından kaynaklanan önemli yan etki riski, gıdanın ilaç olarak e konusunda halkın farkındalığını artırmıştır. Arıların yan ürünlerinden biri olan arı poleni, fenolik bileşikler ve protein, amino asitler, lipitler, makro-mikro bileşenler ve vücudun bağışıklık sistemini desteklemek için çok önemli olan diğer vitaminler gibi besinler içerdiğinden tam bir gıda olduğu düşünülmektedir. Arı poleni, büyük miktarda aktif madde içerdiğinden günlük yemek takviyeleri için alternatif bir kaynaktır. Ancak Kellut arı çiftçilerinin ürettiği arı poleni, bu arı çiftçilerinin arı polenini faydalı özelliklere sahip gıda ürünlerine nasıl dönüştüreceklerini anlamadıkları için sıklıkla israf edilmektedir. Bu çalışmanın amacı altı iğnesiz arıdan (*Heterotrigona itama, Tetragonula reepeni, Tetragonula testaceitarsis, Tetragonula fuscobalteata, Tetragonula iridipennis ve Tetragonula pagdeni*) elde edilen arı poleni ekstraktlarının farklı kurutma yöntemleri ile antioksidan ve anti-inflamatuar olarak değerlendirilmesidir.

Gereç ve Yöntem: Doğu Kalimantan Eyaleti, Samarinda şehrinin Lempake ilçesindeki iğnesiz arı çiftçileri, iğnesiz arı poleni (*H. itama, T. reepenni, T. pagdeni, T. iridipennis, T. fuscobalteata ve T. testaceitarsis*), daha sonra Haziran 2022'de toplandı.

Bulgular ve tartışma: Ekstraksiyon öncesi arı polleninin verim değeri 14,89-61,29 % (40°C'de etüvde) ve 40,91-78,44 % (4°C'de soğutucu) arasında değişmektedir. Arı poleni ekstraktının verim değeri 12,00-47,11 % (40 °C'de fırın) ve 18,18-77,59 % (4°C'de soğutucu) arasında değişmektedir. Arı polleninin besin değeri, tüm arı polen türlerinin chiller 4ºC ve fırın 40ºC yönteminde nem içeriği, kül içeriği, lipid içeriği ve protein içeriği içerdiğini göstermektedir. iğnesiz arı poleni ekstraktlarının fitokimyasal taramasının sonuçları, altı iğnesiz arıdan 40ºC etüvde kurutulan etanolik ekstraktın alkaloidler, flavonoidler, kumarinler, tanenler içerdiğini ve soğutucu 4°C kurutma yöntemi ekstraktının alkaloidler, flavonoidler, triterpenoidler, steroidler içerdiğini göstermiştir, kumarinler, saponinler, tanenler. 40°C etüvde ve 4°C chiller kurutma yöntemi kullanılarak kurutulmuş arı polleninin antioksidan ve anti-inflamatuvar aktivitesi, 4°C chiller kurutma yönteminin IC50'sinin 40°C etüvde kurutma yöntemine göre daha yüksek antioksidan ve anti-inflamatuvar aktivite değeri gösterdiğini göstermektedir. Antioksidan aktivitenin en yüksek oranı, 73,14 % 'lük bir inhibisyon yüzdesine ve 36,47 µg/mL'lik bir IC_{50} değerine sahiptir. Böylece, en düşük IC_{50} 34,30 µg/mL ve en yüksek anti-inflamatuar inhibisyon yüzdesi 85,54 % olmuştur.

Sonuç: Altı iğnesiz arı ekstresi, 4°C'de soğutucular ve 40°C'de fırınlar kullanılarak kurutuldu ve her ikisi de çeşitli fitobileşenlerin varlığı nedeniyle çeşitli antioksidan sistemlere karşı potansiyel in vitro antioksidan aktivite gösterdi. Bu aktivite aynı zamanda güçlü membran stabilize edici ve anti-inflamatuar etkiler göstermiştir. Tüm sonuçlara göre, altı arı poleni (iğnesiz arı) ekstraktının aktivitesi her iki kurutma işlemi için doza bağımlıdır. Bu nedenle gıda takviyesi, ilaç ve kozmetik kaynağı olarak kullanılmak üzere altı arı poleni müstahzarı önerilebilir.

INTRODUCTION

Several physiological processes in the body, including the activation of enzymes, the release of mediators, diapedesis, or the circulation of white blood cells through capillaries to areas of inflammation, are the body's natural protective response to injured tissue (Singh, et al. 2019). The earliest reaction to tissue injury is the acute inflammatory phase, which causes local vasodilation and increased capillary permeability, leading to fluid build-up in the affected area. If left unchecked, it might result in the progressive deterioration of tissue

or turn into a chronic phase that harms crucial body organs. Leukocyte and phagocytic infiltration into the area of inflammation can result in increased oxygen reception stimulation and the production of reactive oxygen species (ROS) like superoxide ion radicals ($O2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), hydroperoxyl ($OOH^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxy ($RO^{\bullet}$) radicals as characteristics of chronic inflammation during cell metabolism (Tutun, et al. 2021). Primary cell molecules can be affected by excessive production of ROS, which can lead to the oxidation of proteins, amino acid changes, peptide chain disintegration, enzyme inactivation, denaturation and lipid peroxidation; and changes to membrane processes, glucose autooxidation, DNA filament fragmentation, base mutation, and modifications of gene expression (Martemucci, et al. 2022). Then, it can cause cell membrane damage. The role of antioxidant and anti-inflammatory molecules is important in neutralizing free radicals that can cause cell and biomolecule damage in the body.

Bee pollen is a functional food source that has strong nutrition and therapeutic properties (Rao 2020). It contains large numbers of compounds such as phytosterols, carbohydrates, enzymes, phenolic compounds, flavonoids, nucleic acids, triterpenes, and vitamins. These chemical components have various effects, including antioxidant and anti-inflammatory activity, thus showing considerable biological potential to be studied as new drug sources. However, the antioxidant and anti-inflammatory activity of these factors influences the antioxidant and anti-inflammatory activity of bee pollen, such as chemical composition, geographical conditions, botanical origin, and treatment methods (Kalaycıoglu, et al. 2017) (Spulber, et al. 2018).

Traditional drying techniques are hardly standardized and mostly rely on pollen being exposed to hot air flow (Castagna, et al. 2020), which has a negative harmful effect on the nutritional quality of bee pollen (Dias, et al. 2016; Cinkmanis, Dimins and Mikelsone 2017; Barajas, Cortes-Rodriguez and Rodriguez-Sandova 2012). Recent studies have proposed novel techniques that operate at lower temperatures, such as 40˚C ovens (Anjos, et al. 2019); and 4˚C chillers (Rzepecka-Stojko, et al. 2014), to decrease the water content in bee-collected pollen and also to be acceptable to consumers in order to address these issues.

Hot air drying of bee pollen may lead to a decrease in the antioxidant and anti-inflammatory activity of bee pollen extracts. On the other hand, the freeze-drying technique preserves total amino acid, proline, and rutin content, and only removes the water content of bee pollen (Ranieri, et al. 2017). In addition, freeze drying supports higher levels of total phenolic, total flavonoids, and antioxidant activity (Dias, et al. 2016). In vitro, anti-inflammatory testing using bee pollen extract from *Scaptotrigona affinis postica* (stingless bee), when first reported, showed antioxidant activity in all treatments and was able to block cyclooxygenase (COX) enzyme (Lopes, et al. 2020).

The extract of stingless bee *Tetragonula sarawakensis* has antioxidant activity with an inhibitory concentration value of 39% at 100 mg/mL (Sari, et al. 2021). However, to the best of the authors' knowledge, studies of the antioxidant and anti-inflammatory activity of bee pollen extracts (*H. itama, T. reepeni, T. pagdeni, T. iridipennis, T. fuscobalteata, and T. testaceitarsis stingless bee*) from East Kalimantan, Indonesia, processed using an oven drying method at 40˚C and a chiller drying method at 4˚C, have not been reported. Thus, the present study aims to determine the antioxidant and anti-inflammatory activity and conduct phytochemicals analyses of the extracts of six stingless bee pollen processed by oven drying at 40˚C and chilling at 4˚C.

MATERIALS AND METHODS

Reagents and chemicals

Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphate buffer, potassium dihydrogen orthophosphate, sodium carbonate, sodium monohydrogen orthophosphate, ethanol, and sodium chloride were purchased from Sigma Aldrich, Germany. Distilled water was used to prepare all of the solutions, reagents, and buffers. Indomethacin, a standard non-steroidal anti-inflammatory drug, was obtained from the Faculty of Pharmacy at Mulawarman University, Indonesia.

Bee pollen material

Stingless bee pollen samples (*H. itama*, *T. reepenni*, *T. pagdeni*, *T. iridipennis*, *T. fuscobalteata*, and *T. testaceitarsis*) were collected in June 2022 from stingless bee farmers in the Lempake area, Samarinda city, East Kalimantan Province, Indonesia. Bee pollen (Fig. 1) was taken using a *stainless-steel* spoon, then put in *polyethylene*

plastic, and stored in the refrigerator for onr day before the drying process. Then, the next day, the bee pollen was separated from the propolis, weighed, and dried in an oven (Memert UN 50, Germany) at a temperature of 40ºC for 8–10 days or a chiller (Modenna SC 1303, Italy) at a temperature of 4°C for 15–22 days. Bee pollen samples were weighed to determine the yield (Eq.1). Then, they were stored in glass vials before further testing.

$$Yield\ (\%) = \frac{weight\ of\ the\ fresh\ bee\ pollen}{weight\ of\ dried\ bee\ pollen} x100 \qquad (1)$$

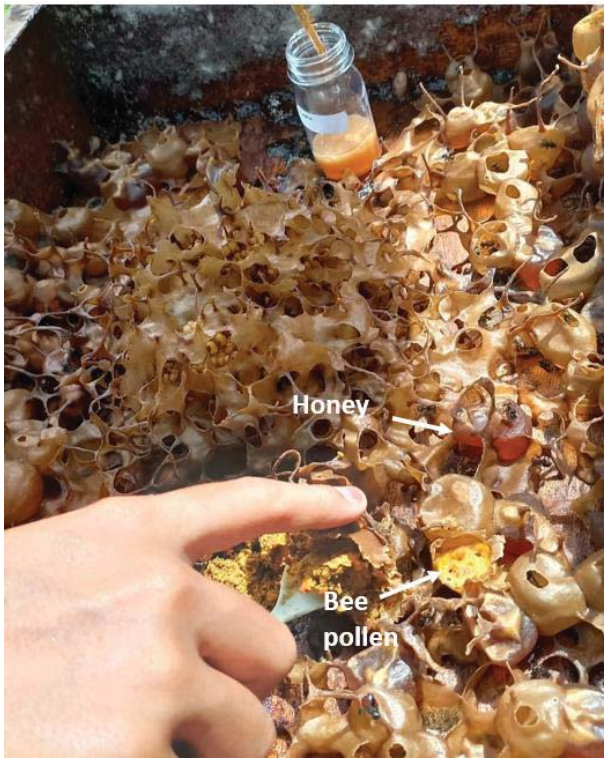

Figure 1. *H. itama* coloni (arrow: honey and bee pollen)

Blood Sample

Red blood samples of patient volunteers and healthy people were used in this study. They were collected by the Indonesian Red Cross Society (PMI). The venepuncture method was used for blood collection. Blood (5 mL) was taken from every healthy patient using a new syringe and needle, a spirit swab, and a tourniquet. The samples were collected in ethylene diamine tetra acetate (EDTA) and plain bottles and then gently inverted for mixing. All experiments were performed within 72 hours of blood collection.

Extraction

Dried bee pollen (30g dry weight) was extracted by a cold maceration (soaking) method using 96% ethanol (1:2 w/v ratios) as a solvent and shaking for 3 × 24 hours at room temperature. Then, the extract solution was filtered to get the filtrate from the solvent. The filtrate was then concentrated using a rotary vacuum evaporator to obtain a crude ethanolic extract of bee pollen. Furthermore, the crude extract was put into a sample bottle that had been weighed and put in an oven at 40ºC for 3-4 days to produce a yield of the extracted sample, as presented in Eq.

$$Yield\ (\%) = \frac{weight\ of\ the\ bee\ pollen\ extract}{weight\ of\ dried\ bee\ pollen} x100 \qquad (2)$$

Proximate Analysis

The common method for determining the water content, ash content, lipid content, and protein of six bee pollen dried was utilized to estimate the samples' proximate composition using a different approach. Measurements were done in triplicate, and results were reported on a dry basis (AOAC 2006).

Phytochemical Analysis

To identify the phytochemicals present in all extracts, qualitative chemical analysis was used to evaluate each one.

Determination of alkaloids

Four drops of Dragendorff and diluted sulphuric acid (H_2SO_4) 2 N were added to a sample (5 mL), and the mixture was agitated. A prominent orange-red precipitate indicated the presence of alkaloids. A standard alkaloid, quinine, was used as a positive control (Harbone 1987).

Determination of flavonoids

Five drops of diluted sodium hydroxide (1% NaOH) were added to 1 mL of 96% ethanol with 1 mg of bee pollen extract. Flavonoids are indicated by the appearance of a yellow fluorescence color (Kokate 2001).

Determination of triterpenoid and steroid

Liebermann-Burchard reagent enables identifying these compounds, after boiling and cooling the bee pollen extract mixture with a few drops of acetic anhydride, concentrated H_2SO_4 was added vis the

test tube's sides. At the intersection of two layers, a brown ring developed. Steroids were detected via the upper layer's shift to green, while triterpenoids were detected via the layer's deep red coloration (Harbone 1987).

Determination of coumarins

A total of 1 mg of bee pollen extract in 1 mL of ethanol extract was mixed with a few drops of 1% NaOH and then added to ethanol. The formation of a yellow color indicated the presence of coumarin (Senthilmurugan, Vasanthe and Suresh 2013).

Determination of saponins

Ten mL of distilled water was used to dilute a 1 mL sample of extract. The formation of a stable foam upon vigorously shaking of the mixture indicates the presence of saponins (Harbone 1987).

Determination of tannins

Ten mg of the extract solution was dissolved in 10 mL of ethanol 96% in a test tube and lead acetate $(CH_3COO)_2Pb$ 1% w/v was added. Tannins were declared positive if the reaction caused an extract precipitate to form (Kokate 2001).

Determination of carotenoids

One mg of bee pollen extract dissolved in 1 mL of ethanol 96 % had 5 mL of chloroform added in a test tube, this was then shaken and filtered, and then 85% w/v H_2SO_4 was added. The formation of a blue color on the surface indicated the presence of carotenoids (Senthilmurugan, Vasanthe and Suresh 2013).

Antioxidant activity

The free radical scavenging activity of bee pollen extracts was measured by 1,1-Diphenyl-2-picrylhydrazil (DPPH) according to Arung et al. (2008), with modification. Samples were prepared by adjusting the concentration of crude extracts to 100 ppm by diluting them in 98% v/v ethanol. 100 µL of each diluted sample was added to 3 mL of 0.1 mM DPPH ethanolic solution and shaken vigorously. The mixture of samples and DPPH was placed in dark conditions at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm by a UV-visible spectrophotometer. Vitamin C was used as a positive control. The percentage of DPPH scavenging effect was calculated as follows (Eq.3):

$$\%\ \text{DPPH Scavenging} = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} x100 \quad (3)$$

The inhibition concentration (IC_{50} value, mg/mL) is the level at which the figure was obtained after 50% of the DPPH radicals were scavenged, with the aid of interpolation from a linear regression analysis.

Membrane stabilization activity as an anti-inflammatory Test

The technique of stabilizing the erythrocyte membrane was used to study anti-inflammatory efficacy *in vitro* according to Oyedapo et al. (2010) with modification. The reference sample, control, and different concentrations of extract (6.25–100 µg/mL) were each combined with 1 mL of phosphate buffer, 2 mL of hyposaline, and 0.5 mL of hRBC suspension. The typical medication was indomethacin (100 µg/mL). All the assay solutions were centrifuged for 10 minutes at 5,000 rpm after 30 minutes of incubation at 37°C. A spectrophotometer operating at 560 nm was used to determine the haemoglobin concentration after the supernatant liquid was decanted. Assuming that 100% of the hemolysis produced in the control was created, the percentage of membrane hemolysis was estimated and calculated by the formula as follows (Eq. 4)

$$\text{Inhibition of hemolysis}\ \% = 100\ x\ \frac{100 - \{Abs\ of\ test\ drug - Abs\ of\ drug\ control}{\{Abs\ blood\ control\}} \quad (4)$$

For each sample, the maximal inhibitory concentration (IC50) was determined by plotting the concentration against the percentage inhibition of hemolysis.

Statistical analysis

Assays for anti-inflammatory and antioxidant activity were performed in triplicate (n = 3), and the results were presented as mean and standard deviation.

ANOVA techniques were used to perform a one-way analysis of variance. A least significant difference (LSD) test was used to identify significant differences between means. Statistics were deemed to be "statistically significant" if $p<0.05$.

RESULTS

Percentage yield of bee pollen

Table 1 shows the yield values of six types of bee pollen processed with the OD method at 40ºC and CH at 4ºC and their ethanol extracts. This table shows that the highest bee pollen extract is found in the 4˚C CH method for the bee pollen types *T. testaceitarsis* (78.44%) and *T. fuscobalteata* (77.59%). While the lowest yield value was found in the 40˚C OD method for the bee pollen extracts *T. testaceitarsis* (14.89%) and *T. testaceitarsis* (12.00%). The yield value of bee pollen before extraction ranges from 14.89–61.29% (OD at 40 ºC) and 40.91–78.44% (CH at 4ºC). While the yield value of the extract of bee pollen ranges from 12.00–47.11% (OD at 40 ºC) and 18.18–77.59% (CH at 4ºC).

Table 1. Yields of stingless bee pollen processed by oven drying and chilling, and ethanol extracts.

Species	Yield of drying bee pollen (g)		Yield of ethanol extracts of bee pollen (g)	
	Oven 40˚C	Chiller 4˚C	Oven 40˚C	Chiller 4˚C
H. itama	1.21 (61.29%)	45.9 (70.59%)	0.93 (47.11%)	6.21 (53.23%)
T. reepeni	14.12 (33.71%)	17.8 (45.51%)	10.69 (25.42%)	5.50 (43.64%)
T. pagdeni	5.51 (44.84%)	45.3 (70.86%)	3.88 (31.58%)	6.21 (22.03%)
T. iridipennis	16.69 (28.48%)	41.5 (74.22%)	14.15 (24.15%)	6.60 (18.18%)
T. fuscobalteata	16.69 (29.15%)	13.2 (40.91%)	14.13 (24.69%)	5.81 (77.59%)
T. testaceitarsis	34.01 (14.89%)	37.1 (78.44%)	27.39 (12.00%)	6.42 (46.88%)

*The percentage value of the yield produced on a dry basis is shown by the numbers in brackets.

Proximate composition

Table 2 shows the water content, ash content, lipid content, and protein content using the CH drying method, which is higher than the OD method. The highest water content is found in bee pollen *T.testaceitarsis* at 46.2%, and the lowest is in bee pollen *T.reepeni* at 9.93%. The highest ash content is in bee pollen *T.reepeni* at 1.41% and the lowest is in *T.fuscobalteata* at 0.24%. The highest lipid content, in *T.testaceitarsis,* was 7.54%, and the lowest was in bee pollen *T.fuscobalteata* at 3.37%. The highest protein content was in bee pollen *H.itama* with a value of 17.25% and the lowest was in *T.testaceitarsis* at 16.20%. With oven drying, bee pollen *T.testaceitarsis* has a higher moisture content of 10.05%, while there were higher levels of ash, lipid, and protein in *T.reepeni* bee pollen, with values of 2.37%, 7.06%, and 20.02 %, respectively. Meanwhile, the lowest values of water, ash, lipid, and protein content were found in *T. pagdeni*, *T.fuscobaltetata*, and *T.testaceitarsis.*

Table 2. Composition of the proximate value of dry bee pollen using two different methods

Bee pollen samples	Chiller 4°C				Oven 40°C			
	Water content (%)	Ash content (%)	Lipid content (%)	Protein content (%)	Water content (%)	Ash content (%)	Lipid content (%)	Protein content (%)
H. itama	41.8±0.12	1.36±1.07	4.97±0.91	17.25±0.09	32.4±1.20	1.60±1.24	4.55±0.88	8.42±0.02
T. reepeni	9.93±3.96	1.41±0.14	7.34±0.14	17.08±0.10	10.05±7.80	2.37±2.08	7.06±4.20	10.02±0.04
T. pagdeni	40.6±0.09	0.27±0.06	6.12±0.13	16.98±0.35	28.6±1.01	2.07±1.09	3.13±0.88	7.56±0.03
T. iridipennis	31.9±0.47	0.50±0.11	7.41±0.83	16.28±0.18	31.4±1.00	1.55±1.02	4.97±0.97	7.95±0.07
T.fuscobalteata	29.1±0.48	0.24±0.01	3.37±0.07	16.55±0.26	28.7±0.99	0.45±0.09	2.12±0.83	7.39±0.04
T. testaceitarsis	46.2±0.09	1.34±1.05	7.54±2.35	16.20±0.09	39.9±0.04	2.17±0.21	2.93±0.84	5.84±0.06

*Data are presented as averages from triplicate experiments ± SD

Phytochemical screening

Table 3 shows the results for phytochemical screening of stingless bee pollen extracts. The results indicate that the ethanolic extract from six stingless bees (*H. itama, T. reepeni, T. pagdeni, T. iridipennis, T. fuscobalteata*, *T. testaceitarsis*) with 40°C OD contains alkaloids, flavonoids, coumarins and tannins; and the CH dried sample extracts contain alkaloids, flavonoids, triterpenoids, steroids, coumarins, saponins, and tannins. However, the extracts from the oven at 40°C do not show saponins and alkaloids for *H. itama* and *T. reepeni*.

Table 3. Phytochemicals of pollen from six stingless bees from East Kalimantan, Indonesia

Sample	Phytochemical screening															
	Oven 40°C								Chiller 4°C							
	Al	Fl	Tr	St	Ku	Sa	Ta	Ka	Al	Fl	Tr	St	Ku	Sa	Ta	Ka
H. itama	-	+	-	-	+	-	+++	-	+	+++	+	+	++	+	+++	-
T. reepeni	-	+	-	-	++	-	++	-	+	+++	+	+	+++	+	+	-
T. pagdeni	++	++	-	-	++	-	++	-	++	++	+	+	+	+	+	-
T. iridipennis	++	+	-	-	+	-	+	-	+++	++	+	+	++	-	++	-
T.fuscobalteata	++	+++	-	-	++	-	+	-	++	+++	+	+	++	+	++	-
T.testaceintasis	+	++	-	-	+++	-	+	-	++	+++	+	-	+++	+	++	-

Description: Al = Alkaloid, Fl =Flavonoid, Tr = Triterpenoid, St = Steroid, Ku = Coumarin, Sa = Saponin, Ta = Tannin, Ka = Karotenoid (-) = no compound, (+) = compound (weak), (++) = compound (medium), (+++) = compound (strong).

Antioxidant activity

Determination of the antioxidant activity of extracts from pollen from stingless bees was carried out by examining free radical scavenging activity using DPPH assays. Figure 2 shows that the antioxidant activity of dried bee pollen using the OD 40°C method was significantly different ($p<0.05$) from the CH 4°C treatment. Bee pollen from *T. fuscobalteata* and *T. pagdeni*, had no significant differences in the 40°C OD treatment, where the inhibition values were 93.95% and 93.87%, respectively. Bee pollen from *T. testaceitarsis*, *T. iridipennis*, *T. reepeni*, and *H. itama* are all quite different, with inhibition

percentages of 43.31%, 31.52%, 83.54%, and 51.70%, respectively. Thus, all varieties of bee pollen in the CH 4˚C method were significantly different, with *T. reepenni* having the highest inhibition value (94.60%), followed by *H. Itama* (82.11%), *T. pagdeni* (73.14%), *T.fuscobalteata* (70.68%), *T.iridipennis* (58.31%), and *T.testaceitarsis* (45.70%). The vitamin C control in both drying methods was not significantly different from *T.pagdeni*, *T.fusbalteata*, and *T.reepenni*. However, it was significantly different from bee pollen from *T. testaceitarisis*, *T. iridipennis*, and *H. itama*. Table 4 shows the IC_{50} of each sample, the OD 40˚C method shows a lower IC^{50} compared to the CH 4˚C method. *T.fuscobalteata* bee pollen had a concentration of 36.47 µg/mL using the 40˚C OD technique, followed by *T. pagdeni* 47.93 µg/mL, *T. reepeni* 76.93 µg/mL, *T.itama* 92.33 µg/mL, *T. testaceitarsis* 116.83 µg/mL, and *T. iridipennis* 158.98 µg/mL. Bee pollen *T. reepeni* had a lower IC_{50} in the CH 4ºC method with a value of 41.30 µg/mL, followed by *H.itama* 53.64 µg/mL, *T.pagdeni* 60.10 µg/mL, *T.fuscobalteata* 66.72 µg/mL, and *T.iridipennis* 85.51 µg/mL. When comparing IC_{50} values, the OD 40 ˚C approach yielded a lower antioxidant activity value than the CH 4˚C method, despite the fact that, statistically, the two procedures have quite different effects on each type of bee pollen.

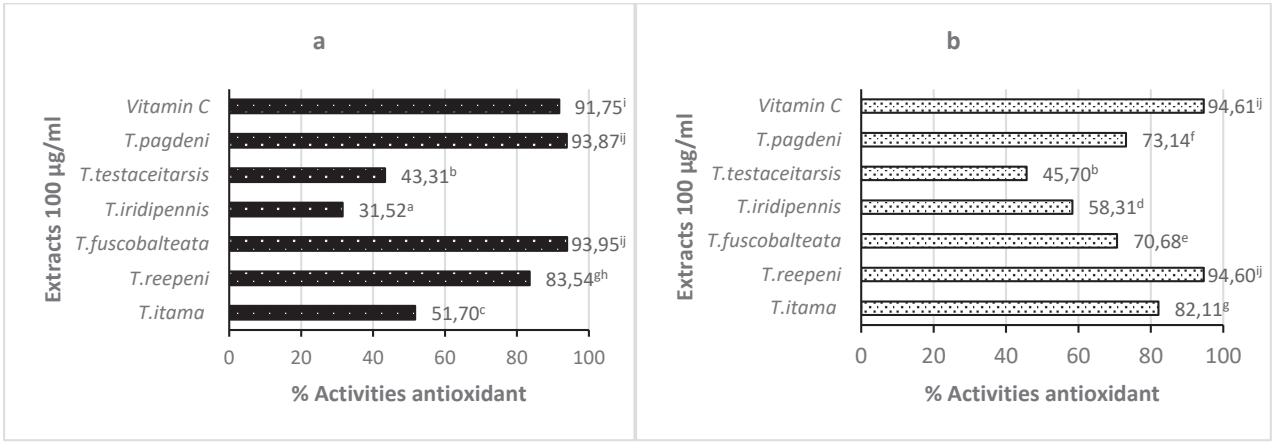

Figure 2. Antioxidant activity of dried crude extracts of bee pollen using different methods: (a) OD 40˚C and (b) CH 4˚C

Table 4. Antioxidant and Anti-inflammatory Activity of Dried Six Bee Pollen Extracts using Different Drying Methods

Species	Antioxidant activity IC_{50} (µg/mL)		Activity of anti-inflammatory IC_{50} (mg/mL)	
	Oven drying at 40°C	Chiller drying at 4 °C	Oven drying at 40°C	Chiller drying at 4 °C
T. itama	92.33^{j}±0.768	53.64^{d}±2.777	56.40^{f}±7.182	44.10^{d}±0.178
T. reepeni	76.93^{g}±5.942	41.30^{b}±4.155	54.13^{ef}±1.272	34.30^{c}±0.179
T. pagdeni	47.93^{c}±0.712	60.10^{e}±0.540	54.90^{e}±8.997	54.76^{f}±4.983
T. iridipennis	158.98^{l}±1.832	85.51^{h}±6.843	78.35^{h}±0.844	115.23^{j}±2.721
T. fuscobalteata	36.47^{b}±0.708	66.72^{f}±4.026	39.70^{c}±0.850	68.25^{g}±0.542
T. testaceitarsis	116.83^{k}±1.804	102.24^{j}±1.569	53.99^{e}±0.823	78.53^{hi}±2.268
Vitamin C	4.75^{a}±0.099	-	-	-
Indomethacin	-	-	23.16^{a}±2.391	-

Membrane stabilization profile (Anti-inflammatory activity)

Figure 3 shows the *in vitro* membrane stabilization of bee pollen extracts from six stingless bees (*H. itama T.reepeni, T.pagdeni, T.iridipennis, T.fuscobalteata*, and *T.testaceitarsis*) using OD 40ºC and CH 4ºC methods, and then indomethacin (standard drug) on human sickle erythrocytes exposed to hyposaline solution (induced lysis). The results show that the anti-inflammatory activity of the six types of bee pollen was significantly different ($p<0.05$) in the two

drying methods. For method OD 40˚C on bee pollen *T.itama* it was 81.36%, for *T.fuscobalteata* 76.24%, for *T. pagdeni* of 72.64%, and for *T. reepenni* of 71.68%, significantly different from *T. testaceitarisis* at 58.28%, *T. iridipennis* at 43.50% and indomethacin as treatment controls. While the CH 4˚C method for bee pollen *T. itama* at 85.85%, *T. reepenni* at 86.54%, and indomethacin were not significantly different compared to bee pollen *T. testaceitarisis* at 61.86%, and *T. iridipennis* at 46.80%, which was significantly different from *T. pagdeni* at 73.83% and *T. fuscobalteata* at 74.62%. Table 4 shows the anti-inflammatory activity of six bee pollen extracts with half the maximum concentration (IC_{50}). *T. fuscobalteata* bee pollen extract using the OD 40ºC method showed the lowest IC_{50} value of 39.70 µg/mL at a dose of 100 µg/mL, followed by *T. testaceitarisis* at 53.99 µg/mL, *T. reepeni* at 54.13 µg/mL, *T. Itama* at 56.40 µg/mL, and *T. iridipennis* at 78.35 µg/mL. Whereas in the CH 4˚C method, the lowest stabilizing membrane was found in bee pollen *T. reepeni* with an IC_{50} value of 34.30 µg/mL at a dose of 100 µg/mL, followed by *H. itama* at 44.10 µg/mL. *T.pagdeni* at 54.76 µg/mL, *T. fuscobalteata* at 68.25 µg/mL, *T. testaceitarissis* at 78.53 µg/mL, and *T. iridipennis* at 115 µg/mL. The absorbance results for indomethacin membrane stabilization show that the lowest positive control at a dose of 100 µg /mL was 23.16 µg/mL, with an inhibition percentage of 88.34%.

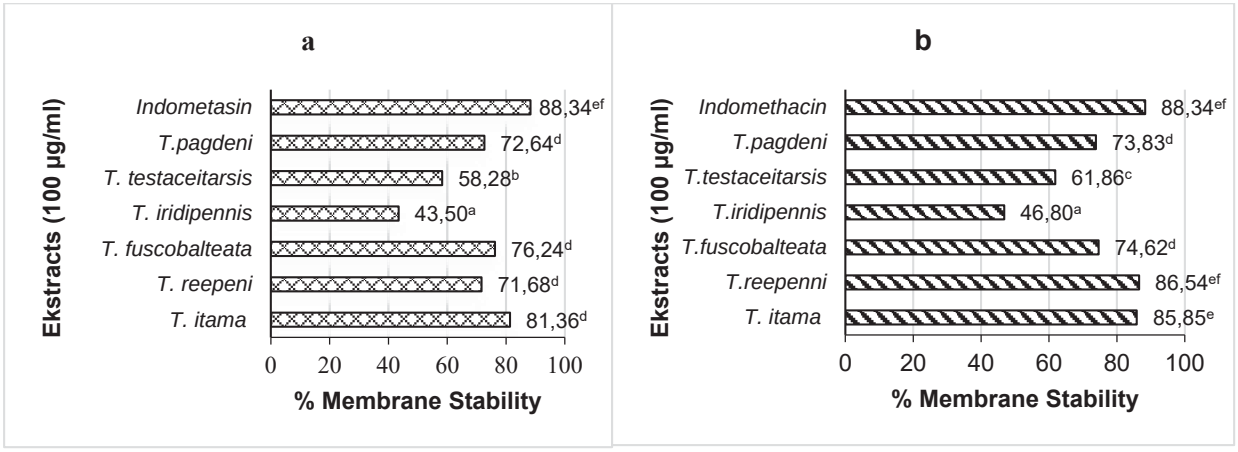

Figure 3. Effect of different dryer methods, (a) oven 40ºC, and (b) chiller 4ºC, on the stabilization membrane of crude extracts of bee pollen.

DISCUSSION

Percentage yield of bee pollen

All extracts in Table 1 were used to evaluate their phytochemical content and antioxidant activity on free radicals as well as anti-inflammatory activity on red blood cell membrane stabilization. The CH method at 4°C gave a higher yield value than the 40°C OD method, both before and after the extraction of bee pollen. This result is higher than that of Arung et al. (2021), who reported that the yield of bee pollen extract without the drying process ranged from 30.2–67.7%. The high yield value in this study is due to differences in the processing methods carried out before and after extraction, resulting in different residual water content (Ranieri, et al. 2017).

Proximate composition

The results show that six types of kelulut bee pollen contained nutritional value, such as moisture content, ash content, fat content, and protein content, when stored at 4°C and 40°C. The nutritional content of the chiller 4ºC method with all types of bee pollen showed the highest value compared to the oven 40˚C method. The chiller 4ºC method uses a low temperature, where the cooling process occurs due to the release of heat in the bee pollen into the cooling room environment, and the release of heat from the cooling room environment to the outside of the cooling system until it reaches a certain desired temperature so that the bee pollen is dry and the nutritional content is little changed. In the

drying process with the chiller 4°C method, there is also no discernible heat exchange (heat to change the temperature without changing the phase), so the temperature of the drying room with the chiller method of 4°C can be used**.** In chiller drying, the water content component was between 9.93–46.20%, ash content 0.24–1.36%, lipid content 3.37–7.54%, and protein 16.20–17.25%; while for the oven method, water content was 10.05–39.9%, ash content 0.45–2.37%; lipid 2.12–7.06%, and 5.84–10.02% for protein. This is almost the same as in (Campos, et al. 2015), who found that protein and lipid range from 10–40% and 1–13%, respectively. The nutritional range of bee pollen originating from East Kalimantan in Indonesia has so far not been identified by the authors. However, in Brazil and Colombia, the nutritional composition of bee pollen was almost the same, namely in the range of 16.1–32.1% for protein (Almeida-Muradian, et al. 2005) and 2.8-9.7% for lipids (Fuenmayor, et al. 2014)..

Phytochemical screening

The results showe that alkaloids, flavonoids, coumarins, and tannins were found in high concentrations in bee pollen extracts. This may be due to their diuretic role and early phytochemical analysis revealed the existence of antioxidant components or other bioactive chemicals that may explain bee pollen use in treating inflammatory diseases. The difference in the number of phytochemicals produced may be influenced by the drying factor, where the temperature can maintain the color and quality of the dried food product. The differences between treatments could depend on different residual water content, which negatively affected the content of flavonoid compounds (Ranieri, et al. 2017). Thus, each phytochemical will show its potential for biological action, such as flavonoids playing a role in antioxidant potential (Ranieri, et al. 2017), and alkaloids being important in other antimicrobial, analgesic, and antispasmodic processes (Hassan, Akmal and Khan 2020).

A recent study reported that the main compound in bee pollen, benzoic acid, might enhance its antibacterial, antifungal and antioxidant properties (Kahraman, et al. 2022). The presence of phytochemicals in extracts of bee pollen dried by the OD method at 40°C and the CH 4°C drying method might show novel biological behaviour that increases the possibility of discovering new compounds such as antioxidants and anti-inflammatories.

Antioxidant activity

Antioxidant testing of six dried bee pollen extracts from stingless bees with OD 40°C and CH 4°C methods showed significant differences. The lowest IC_{50} value was found in the OD 40°C method, followed by the CH 4°C method, for *T. reepeni* bee pollen. It is suspected that the OD 40°C method can remove the water content contained in bee pollen, which might affect the antioxidant levels in *T. repeeni* bee pollen. Minarti et al. (2022) reported that the method of drying bee pollen at an oven temperature of 60ºC can affect its chemical content. Additionally, Isik and Doymaz (2018) found that drying bee pollen at 40ºC might preserve well its quality sensory attributes, and physicochemical properties. The findings of an Anova test on Vitamin C IC_{50} values were substantially different from those of six bee pollen crude extracts. This is assumed to be a result of the six bee pollen extracts having varying antioxidant capabilities due to the various drying techniques. Antioxidant activity depends on the pollen content of various sites (Kocot, et al. 2018) (Saral, et al. 2019).

In this study, six types of bee pollen were used, which means that each type will show a different antioxidant capacity. The use of a cold temperature method is reported to be able to maintain the biological capacity of phytochemical compounds contained in bee pollen. In contrast, using heat and a temperature of 40°C will reduce the effectiveness of the compounds in bee pollen extracts. Even though hot air drying is one of the most popular techniques for preserving bee pollen quality, it can negatively impact on the food value of bee pollen (Cinkmanis, Dimins and Mikelsone 2017).

Bee pollen contains phytochemical compounds such as flavonoids, phenolic acids, phytoalexins, and vitamins, and these compounds are sensitive to heat, so that bee pollen is easily damaged (Tutun, et al. 2021). The findings of IC_{50} calculations indicate that the extent of free radical inhibition by the extract decreases with decreasing test concentration. The IC_{50} values indicate having ery strong antioxidant activity if the IC_{50} value is less than 50 ppm (Sukandar, et al. 2017).

The percentage of free radical scavenging and IC_{50} in all bee pollen extracts was significantly different to the IC_{50} of vitamin C. It is suspected that vitamin C is easily soluble, while bee pollen extract is rather difficult to dissolve, so that the compounds available in bee pollen extract may not dissolve completely.

The free radical scavenging capacity of the six bee pollen extracts will increase depending on the concentration of the treatment. In the DPPH assay, the ability of crude extracts of the six bee pollens examined to act as hydrogen atoms or electron donors in the transformation of DPPH• to the reduced form of DPPH-H was investigated. All samples assessed were able to reduce purple stable radical DPPH to yellow DPPH. The in vitro test method with DPPH (Kocot, et al. 2018) (Özcan, et al. 2019) increased the antioxidant activity compared to the other methods (A. O. Lopes, et al. 2020)

In addition, this study also used ethanol as a solvent for bee pollen extract. Ethanol extract showed DPPH radical scavenging activity of 93.60% compared to other solvents (Mohammad, Mahmud-Ab-Rashid and Zawawi 2021). Thus, the relationship of percentage inhibition of the six crude extracts of bee pollen to the positive control of 100 µg/mL gave a high active capability comparable to that of the positive control.

Membrane stabilization profile (Anti-inflammatory activity)

Stabilization of blood membranes (erythrocytes) has been used as a method to determine anti-inflammatory activity in vitro. Anti-inflammatory activity in the OD 40˚C and CH 4ºC methods showed significantly different effects (P<0.05) on the six bee pollen extracts. Figures 2a and 2b show the results of percentage tests on the activity of stabilizing the erythrocyte membrane of extracts of *T.reepeni* (CH 4˚C) and *T.fuscobalteata* (OD 40˚C), which had the lowest IC_{50} values of 34.30 µg/mL and 39.70 µg/mL compared to the other bee pollens. And these values were higher than the positive control (Indomethacin), which had an IC50 value of 23.16 µg/mL. The relationship between the percentage inhibition of the six crude extracts of bee pollen and the positive control of 100 µg/ml showed a high active capability comparable to that of the positive control. So the six crude bee pollen ethanol extracts may be able to inhibit completely at a concentration of 100 ppm in the CH 4ºC and OD 40ºC methods. These results indicate that the membrane stabilization activity of bee pollen extracts using the OD 40 ˚C drying method was significantly different compared to extracts using the CH 4˚C method. This is presumably because the drying method with a low temperature and long time can maintain the characteristics and stability of bee pollen. (Ranieri, et al. 2017) explain that the use of a low temperature and a long time can prevent a Maillard reaction, which in this case may affect anti-inflammatory activity. To the best of the author's knowledge, the anti-inflammatory activity of six varieties of bee pollen from East Kalimantan (*H. itama*, *T. reepeni*, *T. pagdeni*, *T. iridipennis*, *T. fuscobalteata*, *T. testaceitarsis*) has not previously been reported. Both the oven at 40ºC and the chiller at 4ºC drying methods demonstrate the presence of powerful flavonoids, which are expected to play a significant role in stabilizing red blood cells and are related to the results of phytochemical studies on the six bee pollen extracts (inflammation) as presented in Table 3. According to research by Denisow and Denisow-Pietrzyk (2016), quercetin, a type of flavonoid with anti-inflammatory characteristics, can inhibit the metabolism of arachidonic acid in bee pollen. When arachidonic acid levels drop, the acid level lowers the production of proinflammatory prostaglandins and has an anti-inflammatory impact. Thus, the effect is good for local pain elimination and the prevention of platelet aggregation after bee pollen administration. The alkaloid content in six bee pollen extracts with different drying methods also showed an influence on the inflammatory process, because alkaloids are secondary metabolites derived from the primary metabolites of amino acids, especially amino acids, through biosynthetic pathways (Bai, et al. 2021).

Alkaloids can act on leukocytes, neutrophils, and endothelial cells to block inflammation at the cellular level. In addition, coumarin content also affects the anti-inflammatory properties of cells, so that phytoconstituents such as umbelliferone, scopoletin, columbiatnetin, visniadin, marmin, and many others, derived from the coumarin nucleus, were found to have anti-inflammatory and antioxidant activity (Bansal, Sethi and Bansal 2012).

As a strong antioxidant, the coumarin nucleus has potential as a candidate for the development of anti-inflammatory drugs. This is in line with the results of testing the antioxidant activity of six bee pollen extracts with different drying methods showing a high percentage of free radical inhibition at 94.60% and a low IC_{50} of 36.47 µg/mL (Table 2). Bee pollen extract shows a high antioxidant capacity, suggesting it is able to handle excess ROS that can cause imbalance processes, such as cell damage that causes inflammatory diseases and others. The highest percentage of anti-inflammatory inhibition was 85.54% and the lowest IC_{50} was 34.30 µg/mL. This means that the value of the highest percentage

of erythrocyte membrane stabilization of the six bee pollen extracts is close to the value of indomethacin as a positive control.

Conclusion

With the presence of diverse phytoconstituents, both an oven at 40ºC and a chiller at 4ºC drying methods for six stingless bee extracts had potential for *in vitro* antioxidant activity in different antioxidant systems. Furthermore, this activity demonstrated strong membrane stabilization as an anti-inflammatory action. All of the findings presented indicate that the activity of both drying methods for six bee pollen (stingless bee) extracts is dosage-dependent. Thus, the six bee pollen extracts have potential as antioxidants and anti-inflammatory and can be recommended for use as a source of food supplements, drugs, and possibly cosmetics.

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Declaration of interest: The authors declare that there is no conflict of interests.

Ethics: The Research was conducted in *in vitro* and not with animals or human.

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