

Specific and nonspecific characteristics of the leaf extract of *Blumea balsamifera* originated from East Java, Indonesia

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Abstract: *Blumea balsamifera* extract has been shown to have biological activities such as anti-fungal, anti-tumor, radical scavenger, anti-obesity, hepatoprotective, antioxidant, anti-microbial, anti-inflammatory, antiplasmodial, and wound healing. The quality and efficacy of *B. balsamifera* extract are determined by its specific and nonspecific characteristics. This study explored the characteristics of *B. balsamifera* extract through a series of standardization processes. The dried leaves of *B. balsamifera* were powdered and macerated with 70% ethanol. After the solvent evaporation, a thick extract was obtained and stored in a refrigerator. The specific characteristics tested were organoleptic evaluation, total phenolic content (Folin-Ciocalteu method), total flavonoid content (aluminium chloride colorimetric method), and phytochemical screening using thin layer chromatography. The nonspecific characteristics tested were extraction yield, moisture content, total ash content, and acid-insoluble ash. The results of the study showed that the extraction process resulted in a yield of 13.4%. The extract had a thick and sticky appearance, a dark brown colour, a characteristic aroma, and a bitter taste. The moisture content, total ash content, acid-insoluble ash content, total phenolic content, and total flavonoid content of the extract were found to be 8.33 ± 0.57 %ml/g, 11.63 ± 0.86 %g/g, 2.26 ± 0.12 %g/g, 43.86 ± 0.89 mg GAE/g extract and 25.48 ± 0.42 mg QE/g extract, respectively. Analysis using thin layer chromatography demonstrated that the extract included phenolics, flavonoids, terpenoids, and steroids.

Keywords: *Blumea balsamifera*, extract, flavonoids, phenolics, standardization.

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INTRODUCTION

Plant extracts are utilized in traditional medicine because they are freely accessible and have higher safety and efficacy than single-compound medications. However, due to differences in the chemical compositions and efficacies of herbal extracts, standardization helps ensure their quality, consistency, and safety [1]. Plant extract standardization should ideally depend on particular primary components that are feasible to examine, with their quantity

signifying the extract's efficacy [2]. Many key components have failed to reflect the efficacy of plant extracts in real-world conditions, and many therapeutic markers are difficult to detect and quantify due to their low levels [3]. As a result, selecting appropriate chemical ingredients for standardization of plant extracts is critical. In general, herbal plant extracts outperform conventional medicines in various ways, such as being easier to use and less expensive, requiring less effort and time, and sometimes being more effective than a single drug.

A medicinal plant's pharmacological activity is generated by the presence of phytochemicals produced by the plant's secondary metabolism, such as phenolics, flavonoids, steroids, saponins, terpenoids, and alkaloids [4]. *Blumea balsamifera* (L.) DC. belongs to Asteraceae family, has been shown to exhibit pharmacological activities, such as antifungal [5], anticancer [6,7], antidiabetic [8,9], antioxidant [10], anti-microbial [11], anti-inflammatory [12], antiplasmodial [13], and wound healing [14]. In Southeast Asia and China, it is also used as a carminative drug (preventing flatulence), vermifuge (anthelmintic), diaphoretic and expectorant [9]. *B. balsamifera* has more than 100 phytochemical compounds, including monoterpenes, sesquiterpenes, diterpenes, phenolics, flavonoids, tannins, organic acids, blumeatin, esters, alcohols, dihydroflavones and sterols [15].

Plant extracts are primarily examined for physiochemical characteristics and phytoconstituent identification by analytical procedures with standardization. However, there are many factors that can affect the quality of the *B. balsamifera* extract, one of which is environmental factors where the plant is grown. Therefore, standardization for specific and nonspecific characteristics of *B. balsamifera* extract, especially in the Bondowoso regency, East Java, Indonesia, is needed to ensure safety, efficacy, and quality. This study was aimed to examine the characteristics of *B. balsamifera* extract through a series of standardization processes. We tested the specific characteristics of the extract including organoleptic evaluation, total phenolic content, total flavonoid content, and phytochemical screening using thin layer chromatography. The nonspecific characteristics were also tested including extraction yield, moisture content, total ash content, and acid insoluble ash.

MATERIALS AND METHODS

Materials

B. balsamifera leaves were collected in Sempol Village, Bondowoso Regency, East Java. The plant was identified at the Bali Botanic Garden, Indonesian Institute of Science. Ethanol 96% (food grade) was purchased from PT. Brataco. Toluene and acetone were purchased from Smart Lab (analytical grade). acetic acid, sodium carbonate, n-hexane, ethanol, gallic acid, DMSO, aluminum chloride, and Foline

Ciocalteu were purchased from Merck (analytical grade). Gallic acid, quercetin, eugenol, stigmasterol and quinine were purchased from Sigma-Aldrich (analytical grade).

Extraction Process

Fresh leaves of *B. balsamifera* was washed thoroughly with running water, drained for 24 hours at room temperature, and dried in an oven at 50°C for 3 days. Dried leaves of *B. balsamifera* were powdered using blender. One kg of powdered leaves of *B. balsamifera* was put into a glass jar and added 10 L of ethanol (70% v/v). The maceration process was carried out for 24 hours, with several times of stirring. The mixture was filtered and the macerate is poured into a large holding bottle. This maceration process was carried out 3 times and the collected macerate was then concentrated using a rotary evaporator (IKA RV 8 V) at 50°C followed by evaporation on a water bath. Furthermore, the thick extract obtained was put in a dark bottle and stored in the refrigerator.

Organoleptic Evaluation

The extract was evaluated terms of shape, smell, colour, and taste using the five senses [16]. The evaluation was conducted by an expert.

Measurement of Moisture Content

Determination of the moisture content of the extract is carried out using the azeotropic or toluene distillation [17]. It was performed by adding 5 g of extract in the flask filled with 200 ml of water-saturated toluene. The flask was heated until the toluene began to boil and the distillation rate was adjusted at approximately 2-4 drops per second, until most of the water has distilled off. The receiving tube was cooled to room temperature. The volume of water suspended in the receiving tube was read carefully. Moisture content was calculated in % w/w.

Measurement of Total Ash Content

Two grams of the extract was weighed in a porcelain crucible that had been ignited and tared. The sample was ignited slowly in the muffle furnace until the temperature reached 800°C. The crucible was then cooled and weighed. The total ash content was calculated against the weight of the extract and expressed in % w/w [17].

Measurement of Acid insoluble ash content

The ash obtained from the determination of the total ash content was mixed with 25 ml of dilute hydrochloric acid for 5 minutes. The acid-insoluble portion was collected, filtered through an ashless filter paper, washed with hot water, and ignited in a muffle furnace at 800°C to a constant weight. The acid insoluble ash content was calculated against the weight of the test material and expressed in %w/w [17].

Phytochemical Screening Using Thin Layer Chromatography (TLC)

The phytochemical screening of *B. balsamifera* extract was performed by thin layer chromatography (TLC) using F₂₅₄ silica gel plates as the stationary phase. The mobile phase used was toluene: acetone (10 ml: 10 ml + 3 drops of acetic acid). The test solution was made by diluting 1 gram of extract in 10 ml of ethanol to make a concentration of 10%. The solution of 0.1% of gallic acid, quercetin, eugenol, stigmasterol, and quinine were used as references. The spray reagents of FeCl₃, AlCl₃, vanillin-sulfuric acid, Liebermann Burchard, and Dragendorff were used to detect the existence of phenolics, flavonoids, terpenoids, steroids, and alkaloids, respectively.

Measurement of total phenolic content (TPC)

The total phenolic content (TPC) of the extract was measured using the Folin-Ciocalteu colorimetric method [18]. A total of 1 ml of the extract solution was mixed with 5 ml of Folin-Ciocalteu reagent (7.5% v/v in water). The mixture was vortexed and left for 8 minutes at room temperature. Afterwards, 4 ml of NaOH solution (1% w/v, in water) was added to the mixture. The mixture was incubated for 1 hour. The absorbance of the solution was measured using UV-Vis spectrophotometer at a wavelength of 730 nm against blank. Quantification was carried out with a standard curve of gallic acid (5-100 µg/ml). The results were expressed in %gallic acid. The total phenolic content was calculated using the equation below:

$$\text{TPC} = (\text{C} \times \text{V} \times \text{DF})/\text{M} \quad (1)$$

Where TPC: total phenolic content (mg GAE/g extract), C: total phenolic concentration (µg/ml), V: volume of the extract solution (ml), DF: dilution factor, M: mass of the extract (g).

Measurement of total flavonoid content (TFC)

Measurement of the total flavonoid content (TFC) from plant extracts was determined using the aluminium chloride colorimetric method [19]. The extract solution was prepared by mixing 0.2 g of extract with ethanol, stirring for 30 minutes, filtering and adjusting the volume of the filtrate to 25 ml. A total of 0.50 ml of the extract solution was mixed with 1.5 ml of 95% ethanol followed by the addition of 0.10 ml of 10% aluminium chloride, 0.10 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The mixture was incubated at 27°C for 30 minutes and its absorbance was measured spectrophotometrically at 415 nm. The concentration of total flavonoid was calculated based on the quercetin linear regression equation (25-100 µg/ml). The TFC were calculated using the equation below:

$$\text{TFC} = (\text{C} \times \text{V} \times \text{DF})/\text{M} \quad (2)$$

Where TFC: total flavonoid content (mg QE/g extract), C: total flavonoid concentration (µg/ml), V: volume of the extract solution (ml), DF: dilution factor, M: mass of the extract (g).

RESULTS AND DISCUSSION

Characteristics of BBE.

A plant may have a variation in term of chemical constituents if it grows in different geographical and environmental conditions. So that uniformity is needed in terms of making herbal medicines to ensure the quality, efficacy, and formulation of herbal medicinal ingredients in accordance with established standards [20]. Standardization is a process of measuring the quality of herbal medicines and is an important factor in determining the quality and purity of the final product [21]. The purpose of standardization is to create herbal plant extracts whose quality, efficacy, and safety are guaranteed, scientifically tested, and extensively used, both for self-medication and in formal health services [22]. Standardization has the advantage of not only ensuring the quality, efficacy, and dependability of the phytomedicine due to more homogeneous, stable active components, but also increasing the yield during the extraction process [23]. The standardization of medicinal plant extracts is extensive and comprehensive. This involves the process of establishing a set of inherited standards or features, constant parameters, quality and quantity values. Plant extracts are essentially examined for physiochemical

characteristics and phytoconstituent identification by analytical procedures with chemical characterization. The quality of herbal medicines is typically influenced by a number of factors, such as: (1) herbal medicines are typically mixtures of many compounds; (2) the active compounds are frequently unknown; (3) selective analytical methods or reference compounds may not be commercially available; (4) plant materials differ chemically and naturally; and (5) different sources and quality of raw materials [24].

The use of medicinal plants necessarily requires standardization in order to ensure the quality, efficacy, and safety of medicinal raw materials in accordance with the requirements specified in the Indonesian Herbal Pharmacopoeia, *Materia Medika Indonesia*, and the Regulation of the Minister of Health of the Republic of Indonesia. Specific and non-specific characteristics are included in standardization. Specific characteristics are a set of properties that focus on substances that have pharmacological effects, such as organoleptic, microscopic, soluble compounds in specific solvents, and identification of marker compounds. Non-specific characteristics are properties that focus on material stability and safety, including water content, ash content, loss of drying, acid insoluble ash, and etc. [17].



Figure 1. *Blumea balsamifera* (L.) DC.

Plant determination

The determination of plant is a specific measurement conducted to confirm the identity of plant and ensure that no errors occur when using a plant as an herbal medication. *B. balsamifera* plant (Figure 1) was obtained from Sempol Village, Bondowoso Regency, East Java, on May 20 2020. The determination test of this plant was carried out by an expert Botanist at Bali Botanic Garden, Indonesian Institute of Science (Identification number: B-192/IPH.7/AP/ VII/2020). The results of the determination test confirmed that the plants used in this research was *Blumea balsamifera* (L.) DC from Compositae family.

Organoleptic evaluation

The organoleptic test aims to provide an initial introduction to simplicia and extracts using the five senses by describing shape, colour, smell and taste [17]. According to Table 1, BBE is described as a thick and sticky extract with a dark brown colour, distinctive aroma, and very bitter taste. The organoleptic test in this investigation provided the same description as the information provided by Indonesian Herbal Pharmacopoeia, particularly for BBE. In addition, Eriadi and Alfiah also reported the results of the BBE organoleptic test, namely that the extract had a description of a viscous extract, brown colour, characteristic odour, and bitter taste [25].

Total phenolic content (TPC)

Determination of TPC was carried out by the Folin-Ciocalteu method with gallic acid as a standard. The linear regression equation $y = 0.0076x - 0.0097$ with $r = 0.9926$ is obtained from the gallic acid concentration vs absorbance plot (Figure 2A). Based on the calculation using the linear regression equation and equation 1, the TPC of BBE was 43.86 ± 0.89 mg GAE/g extract (Table 1).

Table 1. Specific characteristics of *B. balsamifera* extract.

Parameter tested	Experiment	Indonesian Herbal Pharmacopoeia Standard
Determination	<i>Blumea balsamifera</i> (L.) DC.	<i>Blumea balsamifera</i> (L.) DC.
Organoleptic	Thick and sticky extract, dark brown color, characteristic aroma, bitter taste	Viscous extract, dark brown color, characteristic aroma, slightly bitter
Total phenolic content (mg GAE/g extract)	$43.86 \pm 0.89^*$	-
Total flavonoid content (mg QE/g extract)	$25.48 \pm 0.42^*$	Not less than 1.31%

*Values are expressed in mean \pm standard deviation (n=3). GAE: gallic acid equivalent; QE: quercetin equivalent.

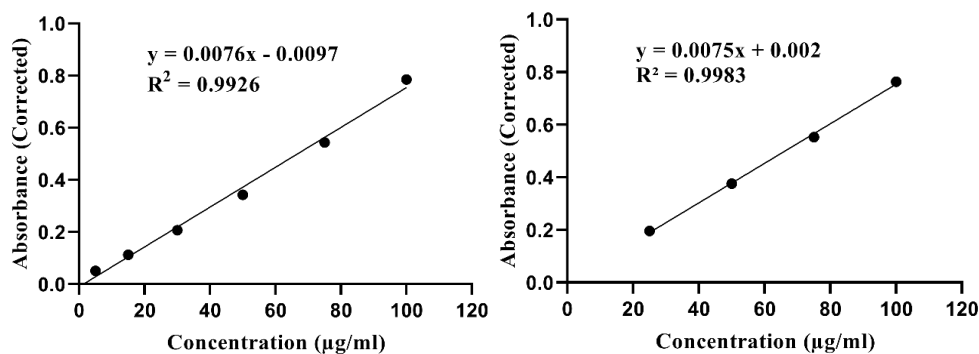


Figure 2. The linear regression curves of (A) gallic acid and (B) quercetin

Total flavonoid content (TFC)

In this study, the determination of total flavonoid content was carried out by the AlCl_3 colorimetric method with quercetin as a standard. The linear regression equation obtained is $y = 0.0075x + 0.002$ with $r = 0.9983$ (Figure 2B). Based on calculation using the linear regression equation and equation 2, the TFC of BBE was 25.48 ± 0.42 mg QE/g extract. When compared to the total flavonoid content of BBE at Indonesian Herbal Pharmacopoeia, the total flavonoid content of BBE in this study was in accordance with the standard. Other researchers have reported the TPC and TFC of BBE. For example, Dao et al. reported that BBE had a TPC of 5.65 ± 0.20 mg GAE/100 mg of extract [26] and Thach et al. showed that the TFC of BBE was 23.34 ± 0.67 mg catechin/g of extract [27].

Thin layer chromatography (TLC) profile

In the phytochemical screening of plant extracts, the characterization of the active ingredients plays an important role in terms of tracing their pharmacological activity. This can be done using thin layer chromatography (TLC). This approach is fast and efficient, combining sensitivity and simplicity with minimal cost. Analysis of the bioactive compounds contained in BBE was carried out qualitatively using the TLC method. The group of compounds tested in this study including phenolics, flavonoids, terpenoids, steroids, and alkaloids with standard compounds for each group namely gallic acid, quercetin, eugenol, stigmaterol, and quinine, respectively. Toluene:acetone (8 ml:2 ml + 3 drops of acetic acid) was used as a mobile phase. The chromatogram of and BBE is shown in Figure 3 and the summary of the TLC profile is revealed in Table 2.

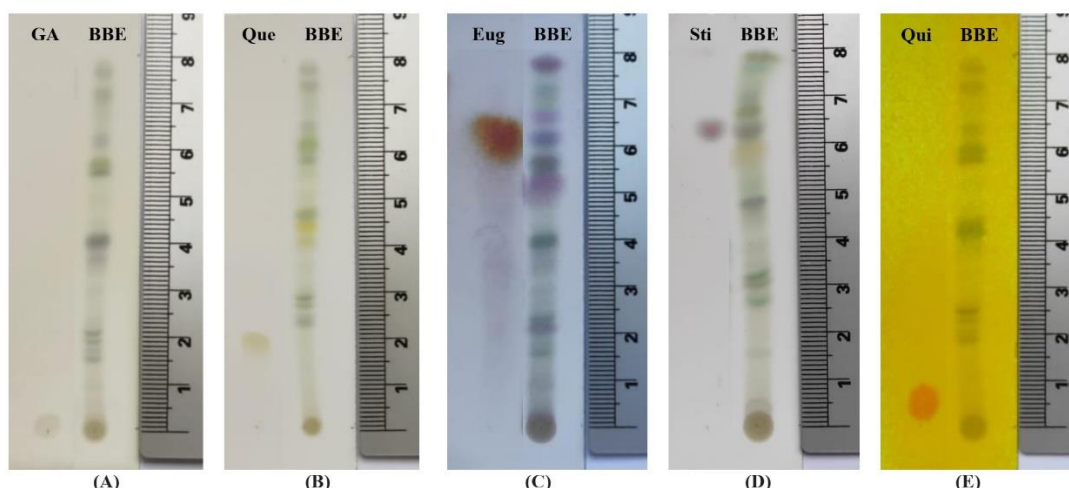


Figure 3. The chromatogram of BBE which was observed under visible light after being sprayed with spray reagents. The mobile phase used was toluene:acetone (8 ml:2 ml + 3 drops of acetic acid). Spray reagents used include (A): FeCl_3 ; (B) AlCl_3 ; (C): vanillin-sulfuric acid; (D): Liebermann-burchard; and (E): Dragendorff. TLC: thin layer chromatography; GAc: gallic acid; Que: quercetin; Eug: eugenol; Sti: stigmaterol; Qui: quinine; BBE: *B. balsamifera* extract.

Table 2. Thin layer chromatography profile of *B. balsamifera* extract

Spot number	Rf	Spraying Reagent					Predictable group of compounds
		FeCl ₃	AlCl ₃	Vanillin-sulfuric acid	Liebermann burchard	Dragendorff	
1	0.0	-	-	-	-	-	-
2	1.6	Grey	-	Green	Green	Grey	Steroid
3	2.0	Grey	Grey	Bluish grey	Brown	Grey	Phenolic
4	2.3	Grey	Grey	Bluish grey	Green	-	Steroid
5	3.0	-	-	Grey	-	-	-
6	3.3	-	Yellow	-	-	-	Flavonoid
7	4.0	Grey	Yellow	Green	Grey	Green	Phenolic
8	5.3	-	-	Violet	Yellowish brown	-	Terpenoid
9	5.6	Yellow	Grey	Green	Brown	Grey	Steroid
10	6.0	Grey	Yellowish green	Blue violet	-	Grey	Phenolic
11	6.8	-	Grey	Blue violet	-	-	Terpenoid
12	7.2	Grey	Grey	Green	Green	Grey	Steroid
13	8.0	Grey	Grey	Violet	Yellowish	Grey	Terpenoid

The stationary phase used are F254 silica gel plates. The mobile phase used was toluene:acetone (8 ml:2 ml + 3 drops of acetic acid). All bands appeared on the plates were observed under visible light.

Based on Table 2, the presence of phenolics was detected at Rf values of 2.0, 4.0, and 6.0 (spot no. 3, 7, and 10, respectively) which was indicated by the appearance of gray-coloured spots after being sprayed with FeCl₃ (Figure 3A). The presence of flavonoids was detected at Rf value of 3.3 (spot no. 6) by the appearance of yellow spots after being sprayed with AlCl₃ (Figure 3B). The Rf values of 5.3, 6.8, and 8.0 (spot no. 8, 11, and 13, respectively) indicated the presence of terpenoids as indicated by the violet and blue violet-coloured spots after being sprayed with vanillin-sulfuric acid (Figure 3C). The presence of steroids was detected at Rf values of 1.6, 2.3, 5.6, and 7.2 (spot no. 2, 4, 9, and 12, respectively) which were indicated by green and brown-coloured spots after being sprayed with Liebermann burchard (Figure 3D). No alkaloids were apparent on the TLC plate, proving that the concentration of alkaloids in BBE was extremely low (Figure 3E). In previous studies, BBE was reported to contain flavonoids, phenolics, saponins, tannins, and steroids. The presence of alkaloids was not detected [28]. Furthermore, Masyudi et al. reported that BBE contains flavonoids, phenolics, tannins, steroids, and saponins.

While the presence of alkaloids and terpenoids was not found [29].

Extraction Yield

Table 3. Nonspecific characteristics of *B. balsamifera* extract

Parameter tested	Experiment	Indonesian Herbal Pharmacopoeia Standard
Extraction Yield (%)	13.4	Not less than 10.6%
Moisture content (% ml/g)	8.33±0.57*	No more than 14%
Total ash content (% g/g)	11.63±0.86*	No more than 6.7%
Acid insoluble ash (% g/g)	2.26±0.12*	No more than 2.5%

*Values are expressed in mean ± standard deviation (n=3).

Extraction yield is a nonspecific parameter showing the total amount of components that can be extracted by a certain solvent. Several

factors can affect the yield, including: type of solvent, ratio of sample to solvent, extraction temperature, sample particle size, and extraction time [30,31]. In this study, extraction was carried out on each 600 g of dry leaves powder using 70% ethanol. The extraction process produced 80.2 g of BBE. Based on the calculation, the extraction yield of BBE was 13.4%. The extraction yield of BBE in this study is in accordance with the extraction yield of BBE in the Indonesian Herbal Pharmacopoeia (Table 3).

Moisture Content

Determination of moisture content in a plant extract aims to avoid fungal growth in the extract and to maintain the quality of the extract [32]. Ideally, the moisture content of the extract should not exceed 10%. If the moisture content is too high or above 10%, it can cause microbial growth which ultimately affects the stability of the extract [16]. In this study, the determination of the moisture content of the BBE extract was carried out by the toluene distillation method. Based on the result depicted in Table 3, the moisture content of BBE is 8.33 ± 0.57 %ml/g. This result indicates that BBE have met the quality requirement for moisture content. When compared to the moisture content value at the Indonesian Herbal Pharmacopoeia, the moisture content value of BBE in this investigation is lower, indicating that this value is acceptable.

Total Ash Content

Ash is an inorganic substance produced by the combustion of organic matter, the amount of which varies depending on the type of material and the combustion process. The ash content of a material indicates the quantity of minerals it contains [32]. Determination of the ash content of the extract aims to determine the internal mineral content in the extract [33]. In this study, the ash content was determined by burning 2 g of the BBE in a muffle furnace at 550°C until a constant weight was obtained. As a result, the total ash content of BBE was 11.63 ± 0.86 %g/g, respectively (Table 3). This result indicates that the inorganic mineral content in the extract is very high. The ash content value in this study was greater than that in the Indonesian Herbal Pharmacopoeia which stated that the ash content of BBE was no more than 6.7%. This was probably caused by the natural conditions where the plants grow, the process of washing the fresh leaves, and the process of burning the extracts using a muffle furnace.

Acid insoluble ash content

Determination of acid insoluble ash content aims to determine the levels of external minerals contained in the extract, especially sand (silica) and other impurities that are still present when the plant samples are processed [33]. The acid insoluble ash content of the BBE in this study was 2.26 ± 0.13 %g/g. In Indonesian Herbal Pharmacopoeia, it is stated that the acid insoluble ash content of BBE is no more than 2.5% (Table 3). This indicates that the acid insoluble ash content of the BBE in this study complies with Indonesian Herbal Pharmacopoeia standard.

CONCLUSION

In this study, the characterization of *B. balsamifera* extract was performed. The extraction process produced a yield of 13.4%. Organoleptic evaluation revealed that the extract had a thick and sticky appearance, a dark brown color, a characteristic aroma, and a bitter taste. The moisture content, total ash content, acid-insoluble ash content, total phenolic content, and total flavonoid content of the extract were found to be 8.33 ± 0.57 %ml/g, 11.63 ± 0.86 %g/g, 2.26 ± 0.12 %g/g, 43.86 ± 0.89 mg GAE/g extract and 25.48 ± 0.42 mg QE/g extract, respectively. Phytochemical screening using thin layer chromatography revealed that the extract contained phenolics, flavonoids, terpenoids, and steroids.

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