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Phytochemical analysis and quantification of flavonoid content of ethyl acetate fraction from 'Alugbati' (*Basella rubra* L.) leaves

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Abstract

Basella rubra L. (Alugbati) is a branching, herbaceous vine with heart-shaped leaves widely grown in Asia, with the leaves used as a herb in soups and salads. Several studies have highlighted this plant's nutritional and biological benefits, which contain various phytochemical substances, including flavonoids. Flavonoids are polyphenolic compounds known to scavenge free radicals through specific mechanisms. This study aimed to extract and quantify flavonoids from the leaves of *Basella rubra* L. to emphasize its potential benefits as a food or nutraceutical. The dried leaves were pulverized and macerated with 99% methanol before partitioning with ethyl acetate and water. The fractions were tested qualitatively and then analyzed using Fourier Transform Infrared Spectroscopy (FTIR) to detect the presence of flavonoids, followed by thin layer chromatographic (TLC) analysis to confirm the presence of flavonoids. A total flavonoid content (TFC) test was performed to quantify the extracted flavonoids. The positive results from the qualitative tests and the subsequent FTIR spectra reflecting the constituent bonds and functional groups validated the presence of flavonoids in the plant fractions. The aqueous and ethyl acetate fractions yielded a total flavonoid content of 2.71 ± 0.01 and 9.61 ± 0.04 mg QE/g extract, respectively. The plant's physical and environmental background, the extraction process used, and the plant part used are all potential contributors to its flavonoid content. To maximize its nutritional and therapeutic potential, further research into other plant organs, and performing other extraction and purifying procedures, is recommended.

Keywords: Phytochemical; Flavonoid; Ethyl Acetate; *Basella rubra;* Leaves

1. Introduction

The genus *Basella*, locally called *Alugbati*, is a branched, herbaceous plant with cordate leaves. This plant has been documented in two species, each with a different stem color: violet (*Basella rubra* L.) and green (*Basella alba* L.) [1]. Because it is often cultivated in Asian countries, it has grown in popularity for food and medicine. The plant contains

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various nutrients and minerals, such as vitamins A and C, calcium, and magnesium [2]. Numerous studies have also shown that the plant has been used as an alternative medicine for various ailments [3, 4, 5]. Phytochemical examination of several plant extracts suggested the presence of medicinal compounds, including carotenoids, saponins, and flavonoids [5]. Flavonoids are polyphenolic compounds that scavenge free radicals through several mechanisms, including chelation, inhibition of radical-producing enzymes, and hydrogen or electron transfer through their hydroxyl groups. It is responsible for the diverse properties of flavonoids, including antioxidant, hepatoprotective, anti-inflammatory, and chemotherapeutic effects [6, 7, 8]. These factors prompted the researchers to fractionate a crude extract from the leaves of *Basella rubra* L. to obtain flavonoids, which will be subsequently validated through qualitative testing and FTIR analysis before being evaluated for total flavonoid content (TFC).

2. Materials and Methods

Methanol (MW = 32.04 g/mol, ≥99% purity) and ethyl acetate (MW = 88.10 g/mol, ≥99.5% purity) were obtained from Thermo Scientific Chemicals (Waltham, Massachusetts, USA). Unless noted otherwise, all materials and reagents used are of analytical grade.

2.1. Plant Authentication and Collection

Mature leaves of *Basella rubra* L. were collected from a vacant farm lot in San Carlos, Pangasinan, in February 2024. Subsequently, a plant sample was forwarded to the Far Eastern University Herbarium for verification.

2.2. Preparation of Crude Methanolic Extract

The leaves were adequately cleaned with distilled water to remove adhering dirt before being air-dried for 10 days in the shade and pulverized with a cutting mill (Wiley™ Model 4, Thomas Scientific, USA). About 49.33 g of dried leaves were macerated in 99% methanol for 36 hours in a steel percolator and filtered through cellulose paper (Whatman® Grade 1, Sigma-Aldrich, USA). The filtered extract was concentrated using a rotary evaporator (RV 10™, IKA®, Malaysia) at 45 °C, resulting in a green, syrupy product. The crude methanolic extract was placed in a tight amber bottle at a cold temperature until used. The percentage yield was computed using the following formula, where CME and DL represent the weights of the crude methanolic extract and dried leaves, respectively.

$$\%\ yield = \left(\frac{CME}{DL}\right) x\ 100$$

2.3. Fractionation of Crude Ethanolic Extract

After weighing, the crude extract was placed in a beaker with 75 mL of distilled water. The suspended extract was partitioned with 75 mL of ethyl acetate in a 250 mL separatory funnel, set on an iron stand, and separated until a distinct layer formed between the two solvents. The aqueous solution was gradually released into one beaker, then the ethyl acetate fraction into another. The procedure was repeated three times, and both solutions were collected for further concentration. The pool of ethyl acetate fraction was concentrated using a rotary evaporator at the same temperature, and the aqueous fraction was lyophilized (FSF-18N, Faithful Instrument Co. Ltd., China). The percentage yield was computed using the following formula, where W_2 is the product's weight after fractionation, and W_1 is the weight of the crude methanolic extract before fractionation.

$$\%\ yield = \left(\frac{W_2}{W_1}\right) x\ 100$$

2.4. Qualitative Phytochemical Analysis

The plant fractions were divided into labeled test tubes and qualitatively tested for flavonoids using the following tests:

2.4.1. Bate-Smith and Metcalf's Test

2 mL of extract was treated with 0.5 mL of concentrated hydrochloric acid (HCl) and observed for the appearance of a violet or strong red color.

2.4.2. Lead Acetate Test

2 mL of extract was treated with 0.5 mL of 10% lead acetate and observed for the presence of a yellow color.

2.4.3. Shinoda's Test

2 mL of extract was treated with a small piece of magnesium foil and 0.5 mL of concentrated HCl then observed for a pink or cherry red color.

2.4.4. Sodium Hydroxide (NaOH) Test

Five drops of 2 N sodium hydroxide (NaOH) were added to the extract. An intense yellow color develops, which disappears upon adding a few drops of 0.1 N HCl.

2.4.5. Wilstatter Test or Cyanidin Test

2 mL of extract was mixed with three pieces of magnesium turnings and 0.5 mL of concentrated HCl, then observed for the formation of a purple color.

2.5. Fourier Transform Infrared Spectrophotometer (FTIR) Analysis

The IR spectra were acquired using an FTIR spectrophotometer (IRSpirit-T™, Shimadzu, Philippines). A small amount of each sample was placed on the ATR crystal surface, and the IR spectrum was recorded. The spectrum was measured by collecting 20 scans from a wavenumber range of 4000 cm^{-1} to 500 cm^{-1}, with a resolution of 4 cm^{-1}. The spectrum was analyzed with LabSolutions™ IR software, and the results were given as percentage (%) transmittance.

2.6. Thin Layer Chromatography (TLC)

TLC analysis was performed using modified procedures from Galib et al. [9]. The set-up consisted of commercially available, silica gel-coated aluminum plates with fluorescent indicator F_{254} pre-sized to 1 cm by 6 cm (Merck® Silica Gel 60, Sigma-Aldrich, USA), and a mobile phase system made up of ethyl acetate, toluene, and formic acid in a 4:4:1 ratio. Using a capillary tube, each sample was applied to the TLC plate as a single spot. The strip or plate is then positioned with the spotted end immersed into the mobile phase system, ensuring the samples are not submerged. As the mobile phase flows to the opposite end, the components of each sample separate at different distances. The plate was removed once the mobile phase reached the upper end. The TLC plates are examined under UV light at 366 nm, where flavonoids and polyphenols are expected to exhibit fluorescence as violet bands. The procedure will be repeated twice, with both plates viewed under visible light, but the latter sprayed with a mixture of $K_2Fe(CN)_6$ and $FeCl_3$. The retention factor (Rf) needed to identify the compounds present in each sample is calculated by dividing the distance traveled by the compound by that of the mobile phase. The samples were compared to a positive control of 1 milligram of quercetin dissolved per milliliter of methanol (1 mg/mL).

2.7. Total Flavonoid Content (TFC)

TFC analysis was conducted using modified protocols from Shraim et al. [10]. In separate 10 mL volumetric flasks, 2 mL of each plant fraction was mixed with 5 mL of methanol before immediately adding 0.5 mL of 5% sodium nitrite ($NaNO_2$). After 10 minutes of incubation, 0.5 mL of 10% aluminum chloride ($AlCl_3$) was incorporated into each volumetric flask. After another 10 minutes of incubation, 3 mL of 2 M sodium hydroxide (NaOH) was added, followed by methanol to bring the volume to 10 mL. The resulting mixtures were incubated for 30 minutes before being measured for absorbance at 510 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (LAMBDA 365+, PerkinElmer®, USA). The standard curve was generated using a standard quercetin solution (0 mcg/mL to 50 mcg/mL) and the same procedure. TFC values were calculated as milligrams of quercetin equivalents per gram (mg QE/g) of dry fraction.

3. Results and Discussion

3.1. Percentage Yield of Ethyl Acetate Fraction

11.28 g of crude extract was extracted from 49.33 g of dried leaves, yielding 22.87%. The crude extract was partitioned with ethyl acetate and water, and the weight and percentage yields are shown in Table 1.

Table 1 Fractions obtained from methanolic crude extracts of *Basella rubra* L leaves

Fraction	Weight (g)	Yield (%)
Aqueous	1.002	8.88
Ethyl Acetate	1.912	16.95

3.2. Qualitative Phytochemical Analysis

Table 2 shows the findings of phytochemical tests conducted to detect the presence of flavonoids.

Table 2 Qualitative testing to indicate the presence of flavonoids in the obtained fractions

Test	Fraction	
	Aqueous	Ethyl Acetate
Bate-Smith and Metcalf's	+	+
Lead Acetate	+	+
Shinoda's	+	+
Sodium Hydroxide (NaOH)	+	+
Wilstatter or Cyanidin	+	+

(+) present; (-) absent

Both fractions yielded positive results in all tests, indicating the presence of flavonoids. Numerous researchers have already studied the relationship between solvent polarity and the amount of polar compounds extracted from plants, such as flavonoids [11, 12, 13, 14, 15]. Flavonoids are polyphenolic compounds that are relatively polar due to their several hydroxyl groups [14]. As a result, polar solvents such as ethyl acetate are expected to extract more flavonoids. Ethyl acetate is a solvent with an intermediate polarity that is relatively polar due to the presence of pi and lone pair electrons [13, 16]. Due to its intermediate polarity, it can extract a broader range of polar and nonpolar flavonoids [11]. Conversely, water is a highly polar solvent, resulting in reduced solubility and subsequent extraction of organic compounds that are generally lipophilic and nonpolar [17].

3.3. Results of FTIR Analysis

Figure 1 illustrates the FTIR analysis for the ethyl acetate fraction from *Basella rubra* L. leaves, which are interpreted in Table 3.

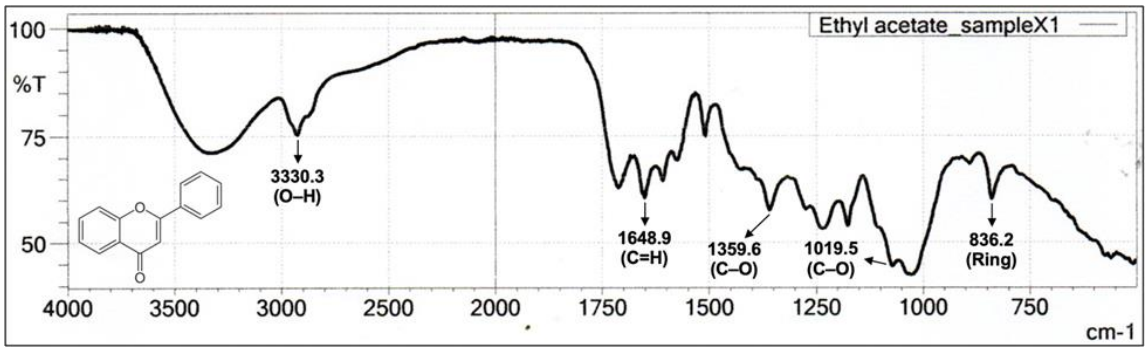

Figure 1 FTIR spectra of the ethyl acetate fraction from *Basella rubra* L. leaves

Table 3 Results of FTIR analysis of *Basella rubra* L. leaf ethyl acetate fraction

Peak Number	Wavenumber (cm^{-1})	Chemical Bond
1	836.2	Ring
2	1019.5	C–O
3	1359.6	C–O
4	1648.9	C=H
5	3330.3	O–H

FTIR analysis predicted the chemical bonds or functional groups in the ethyl acetate fraction from *Basella rubra* L. leaves. The findings from the qualitative tests were supported by the FTIR results, which showed a broad peak at 3330.3 cm^{-1}, as well as sharp peaks at 836.2 cm^{-1}, 1019.5 cm^{-1}, 1359.6 cm^{-1}, and 1648.9 cm^{-1}, indicating that distinct chemical bonds commonly found in flavonoids were present. The results were similar to the investigation of Sravan Kumar et al. [18] into the antioxidant activity of the defatted extracts from both *Basella* species.

3.4. Results of TLC Analysis

Figure 2 displays the chromatogram for the ethyl acetate and aqueous fractions of *Basella rubra* L. leaves, which are interpreted in Table 4.

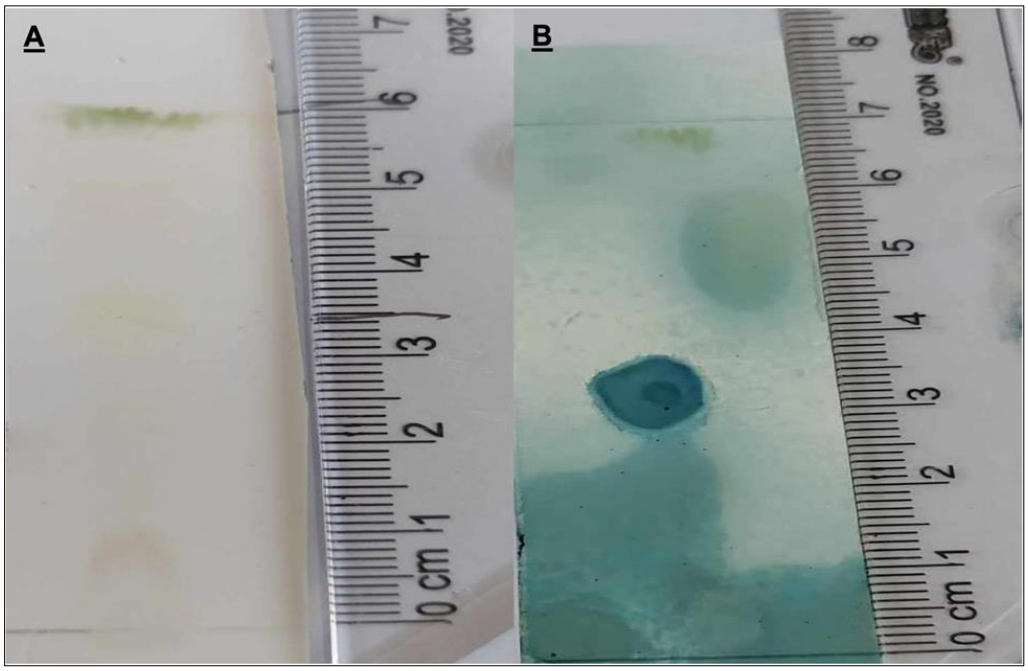

Figure 2 Chromatograms of leaf fractions of *Basella rubra* L. (A) Ethyl acetate fraction, (B) Aqueous fraction

Table 4 Results of TLC analysis of leaf fractions of *Basella rubra* L

Fraction	R_f Value
Aqueous	0.54 0.61
Ethyl Acetate	0.46 0.48

As reported by Gwatidzo et al. [19], flavonol glycosides may exhibit an orange-yellow band, biflavonols can show a yellow-green band, while blue bands may be due to certain types of deoxyisoflavones, flavanones, and anthocyanidins. The chromatograms from the TLC analysis revealed similar results, with yellow, green, orange, and blue spots indicating flavonoids. Among the two fractions, the ethyl acetate fraction produced R_f values of 0.46 and 0.48, similar to quercetin (R_f = 0.43), suggesting a more significant quantity or a higher purity of flavonoids. The R_f values for the ethyl acetate fraction are almost identical to those published by Sharma et al. [20] for quercetin (R_f = 0.49).

3.5. Quantification of Flavonoid Content

Table 5 shows the total flavonoid content of the two fractions from the crude leaf methanolic extract of *Basella rubra* L. TLC analysis indicates that the ethyl acetate fraction contains more flavonoids, as verified by TFC results. The aqueous fraction yields 2.71 ± 0.01 mg QE/g of extract, whereas the ethyl acetate fraction yields 9.61 ± 0.04 mg QE/g. Although the values are similar to the various cultivars investigated by Zhang et al. [5], the current results show a comparatively low quantity of flavonoids. This could be attributed to multiple factors, such as physical injuries, environmental

conditions, and specific hormones, all of which can influence the gene expression responsible for flavonoid biosynthesis in plants [21]. The extraction process may have also contributed to the low amount of flavonoids extracted. Traditional procedures, such as maceration and partition, are still employed today because they are less expensive. Still, they have various drawbacks, including the requirement for large quantities of solvents and lengthier extraction times, which result in a small amount of compounds. Furthermore, the low amount of compounds extracted from traditional methods necessitates additional purification, resulting in a significantly smaller amount of pure compounds, making the process time-consuming and inefficient. Hence, newer extraction methods are suggested to improve flavonoid extraction. Also, flavonoid analysis using chromatographic methods coupled with mass spectroscopy (MS) and nuclear magnetic resonance (NMR) is required to confirm the purity of extracted compounds. Lastly, the flavonoid amount may vary depending on which plant part was extracted. According to current studies, the flavonoid content of different plant parts varies depending on the plant. Nurshalina et al. [22] described the stems of *Physalis minima* L. as the ones that contain the most flavonoids. Phuyal et al. [23] found that the bark of *Zanthoxylum armatum* DC has the highest flavonoid content. Very few studies compare flavonoid concentration in different *Basella* plant parts, necessitating further research.

Table 5 Quantification of flavonoid content in leaf fractions of *Basella rubra* L

Fraction	Total Flavonoid Content (mg QE/g)
Aqueous	2.71 ± 0.01
Ethyl Acetate	9.61 ± 0.04

4. Conclusions

This study focuses on the leaves of *Basella rubra* L., which are often used as natural food and potentially include nutritional and medicinal components. Previous studies have demonstrated that flavonoids are present in *Basella* species. As a result, the researchers focused on extracting and quantifying the flavonoid content. This confirmation of flavonoid content lays the stage for further investigation into the plant's numerous biomedical properties. It is also clear that the impure fraction extracted using traditional methods contained a significant amount of flavonoids, implying that a higher concentration could be extracted if other plant parts and alternative extraction and purification processes were utilized, maximizing its nutritional and medicinal benefits.

Compliance with ethical standards

Disclosure of conflict of interest

The authors disclose that no direct or indirect conflicts of interest.

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