Indonesian Journal of Pharmacy

VOL 35 (3) 2024: 409–424 | RESEARCH ARTICLE

Phytochemical, Nutraceutical and Pharmacological Aspects of the Philippine native *Acalypha angatensis* Blanco, Fl. Filip.

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Article Info

Submitted: 03-10-2023
Revised: 15-05-2024
Accepted: 17-05-2024

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ABSTRACT

Acalypha is a valuable genus that has interesting pharmaceutical applications. This study investigated the phytochemical composition and thin layer chromatography (TLC) profile, as well as the antioxidant and antibacterial activities of the ethanolic leaf extract of *Acalypha angatensis* Blanco, Fl. Filip. Qualitative phytochemical screening revealed the presence of tannins, phenolics, flavonoids, saponins, steroids, and triterpenoids. Nutrient analysis showed that potassium and calcium contents were at 1.499 g/100g and 1.38 g/100g, respectively. The determination of the total phenolic content (TPC) showed that this species has an average of 282.24 ± 3.56 mg GAE/g and a total flavonoid content (TFC) of 36.24 ± 3.77 mg QE/g of dried extract. These values are higher than other *Acalypha* species that have already been studied. Antioxidant activity, measured using the DPPH antioxidant assay, showed a concentration-dependent radical scavenging effect, with an IC_{50} of 34.02 µg/mL. The ethanolic extract exhibited substantial antibacterial activity against the gram-positive bacteria, *Enterococcus faecium* (NCTC 12204) and MRSA (ATCC 33592), with a minimum inhibitory concentration (MIC) of 4 mg/mL. Higher concentrations (>20 mg/mL) were needed for inhibition of the gram-negative strains (*Klebsiella pneumoniae* and *Enterobacter cloacae)*. These results suggest that *A. angatensis* has good antioxidant and antibacterial activities against gram positive bacteria, supporting its use in traditional medicine. This species has the potential to be a raw material for pharmaceutical and nutraceutical products.

Keywords: *Acalypha angatensis*, Antibacterial, Antioxidant, TLC profile, Phytochemicals

INTRODUCTION

The genus *Acalypha,* the fourth largest genus under the family Euphorbiaceae (Seebaluck *et al*., 2015), are described as being herbaceous to large shrubs or small trees (Sagun *et al*., 2010). The species *Acalypha angatensis* grows in thickets mainly found at medium elevation and ascending to 1500m (Pelser *et al*., 2018), in secondary forests or riversides (Sagun *et al*., 2010). This species is native to Taiwan and to the Philippines, distributed in the latter across Luzon and Mindanao (Pelser *et al*., 2018). The epithet *angatensis* pertains to the type locality of Angat, Bulacan Province, Philippines (Sagun *et al*., 2010). In the Cordillera region, *A. angatensis* is traditionally used for medicinal purposes, i.e. fungal infections (Cordillera Administrative Region–Tuklas Lunas Development Consortium Project 3, n.d.). However, there are no studies that have been done to validate these claims, as well as discover other bioactivities of this native plant. Research on the medicinal properties of the other species of *Acalypha* are available. The review by Seebaluck and co-authors (2015) provides a good overview of the ethnopharmacology and phytochemistry of this genus. Traditional practices used this genus for the treatment and management of wounds, infections, dysentery, respiratory problems such as bronchitis and asthma, diabetes, jaundice, and liver inflammation. Most of the cited traditional uses are

from African countries, while a few studies were documented in Asia, including China and Thailand. All parts of the plant, including the leaves, stems, and roots, are used to prepare decoctions and mixtures that effectively address a wide range of ailments. Some notable pharmacological researches include its promising antioxidant potential, with high DNA protection activity against photolysed oxidative damage and marginal toxicity (Rajkumar *et al*., 2010), and its analgesic and antimalarial activities (Udobang *et al*., 2010). In the study of Kim and colleagues (2020), *A. australis* was the most effective anti-inflammatory among different Chinese herbal plants and was associated with the regulation of inflammatory responses, inhibiting NF-kB activation in macrophages.

In the Philippines, *Acalypha canescens* Benth. or Maraotong (Ilocano) is traditionally used to treat dysentery and skin conditions, and to manage insomnia and rheumatic complaints (Stuart, 2017). It is also used as a laxative, cathartic, expectorant, anthelmintic, and emolient for snake bites. Meanwhile, *Acalypha caturus* Blume or Ambugtunong (Bisaya) is a food source, and its leaves are used to hasten wound healing, particularly in the Mountain Province (Stuart, 2023). Both are native to the Philippines.

Exploring new antioxidant and antibacterial leads remains a thriving area of investigation, particularly in the context of nutraceutical and pharmaceutical research. Antioxidants provide protection against reactive oxygen species (ROS) commonly associated with chronic diseases, such as diabetes, cancer, and inflammation (Sarangarajan *et al*., 2017). Moreover, natural antioxidants play a crucial role in preventing the rancidity caused by the oxidation of unsaturated fats, stabilizing food colors, and participating in the chemoprevention of various diseases (Pires *et al*., 2020). Antimicrobial compounds naturally produced by plants, including *A. angatensis*, may be applied for food processing, preventing spoilage and proliferation of pathogenic microorganisms (Gutiérrez-del Río *et al*., 2021; Huang *et al*., 2022). This plant may also contribute to the mitigation of antibiotic resistance. Other species were documented to possess antibacterial properties. As mentioned by Begum and co-authors (2021), the World Health Organization (WHO) has acknowledged antibiotic resistance as one of the most important public health issues to date. For example, more than 50% of the bacterial isolates from water, soil, and vegetables grown in gardens watered with polluted surface waters of Metro Manila, Philippines, have antibiotic resistance (Vital *et al*., 2018).

Because of the increased focus of researchers on the search for viable alternatives to the management of infections and chronic diseases, plants and plant products have been formulated to address this. Chinese and Ayurvedic formulations are proof that many of our plant biodiversity are good materials for formulating herbal preparations not only against infection, but also for other human illnesses. Like many native plants of the Philippines, no published work has been found on the basic chemical profile and bioactivities of *A. angatensis.* Hence, the present study provides the first report on the TLC profile and pharmacologic properties of this species, specifically its antioxidant and antibacterial activities against antibiotic-resistant strains. This study aimed to document and evaluate the health benefits of *A. angatensis* as a possible source of pharmaceutical or nutraceutical products on the market.

MATERIALS AND METHODS

Plant materials

The plant samples of *A. angatensis* were collected from barangays Balakbak and Cuba, Kapangan, Benguet province, Philippines. The collected plants were deposited at the University of the Philippines Baguio Herbarium, Department of Biology, College of Science, University of the Philippines Baguio, with accession number UPBH-2024-EUPHORB-002-4. The leaves were carefully cleaned, washed, and oven dried for an average of three days. After drying, these were homogenized to a fine powder and stored securely in sealed plastic containers.

Preparation of the extract

Crude leaf extracts were prepared by placing 50 g of the powdered leaf sample in beakers and adding 500 mL of 80% ethanol. The powdered leaves were macerated and occasionally stirred for 48 h, then filtered using Whatman No. 1 into an Erlenmeyer flask. The ethanolic extract was concentrated under reduced pressure at 50℃ in a rotary evaporator, then dried using the Biotage V10-Touch solvent evaporation system at 48℃. The dried ethanolic leaf extract was stored in the refrigerator until further analysis.

Nutrient and Proximate Analysis

Oven-dried leaf samples were sent for nutrient analysis at the Department of Science and Technology - Food and Nutrition Research Institute

(DOST-FNRI). The Association of Official Agricultural Chemists (AOAC) methods AOAC 999.10 and AOAC 985.35 were used for sodium, potassium, iron, calcium, and zinc analysis. All assays were performed with two replicates. Meanwhile, the Department of Science and Technology - Cordillera Administrative Region - Regional Standards and Testing Laboratory (DOST-CAR-RSTL) performed the proximate analysis using the following methods: gravimetric method for ash test (AOAC 942.05), oven method for moisture analysis (AOAC 934.01), Soxhlet extraction for crude fat analysis (AOAC 920.39), and Kjeldahl method for crude protein analysis (AOAC 981.10, in accordance with the VELP Scientifica UDK 139 and DKL 20 manuals). Tests for carbohydrate and energy in kcal were calculated based on the Philippine Food Composition Tables of the 1997 FNRI, DOST Handbook.

Phytochemical Screening

The phytochemical contents of the ethanolic leaf extract were determined using standard colorimetric methods as preliminary qualitative analysis. All methods were conducted in triplicates.

Test for Tannins (Gelatin test)

Gelatin-salt solution was prepared by mixing 1% gelatin solution and 10% sodium chloride (NaCl). One milliliter ethanolic leaf extract was placed in a test tube and four drops of the gelatin-salt solution were added. Precipitate forms when tannins are present (Ben *et al*., 2013; Shaikh & Patil, 2020).

Test for Phenolics (Ferric chloride test)

In a test tube, 1 mL of the ethanolic leaf extract and four drops of 10% ferric chloride solution were mixed. A dark-green or bluish-black color indicates the presence of phenolics (Shaikh & Patil, 2020; Vimalkumar *et al*., 2014).

Tests for Flavonoids

Flavonoids were detected using the two common procedures below.

Lead acetate test

One milliliter of the ethanolic leaf extract and three drops of 10% lead acetate were mixed in a test tube. The formation of a yellow precipitate confirms the presence of flavonoids (Shaikh & Patil, 2020; Vimalkumar *et al*., 2014).

Shinoda's test

Ethanolic leaf extracts were dried and dissolved in 10 mL distilled water and in 5 mL ethanol (95%). Both solutions were filtered but only the sample in ethanol was heated in a water bath. Three magnesium ribbon fragments were then added to 1 mL of the sample solutions. A few drops of HCl were also added. The formation of pink to crimson color is an indication of the presence of flavonol glycosides, while an intense cherry red color indicates the presence of flavanones, and an orange-red color indicates the presence of flavonols (Bijekar *et al*., 2015; Gul *et al*., 2017; Shaikh & Patil, 2020).

Tests for Saponins (Foam test)

Two milliliters of ethanolic leaf extract were added to 5 mL distilled water in a test tube. The formation of a foam that lasts for 10 min confirms the presence of saponins (Ben *et al*., 2013).

Test for Alkaloids

The presence of alkaloids was detected using four procedures: Mayer's test, Wagner's test, Dragendorff's test and Hager's test (Ben *et al*., 2013; Shaikh & Patil, 2020; Vimalkumar *et al*., 2014).

Test for Steroids and Triterpenoids (Liebermann-Burchard Test)

One gram of the dried ethanolic leaf extract was dissolved in 1 mL of chloroform and 1 mL of acetic anhydride. Concentrated H_2SO_4 (1-2 drops) was added along the side of the test tube. A range of colors including blue to green, violet, and red confirmed the presence of steroids and triterpenoids (Shaikh & Patil, 2020; Jagessar, 2017).

Test for Cardiac Glycosides

Keller-Kiliani Test

A solution of 1.5 mL glacial acetic acid with one drop of 5% ferric chloride was prepared. The solution was added to 1.5 mL of the ethanolic leaf extract, and then 1 mL concentrated sulfuric acid was added carefully along the sides of the test tube. The presence of cardiac glycosides is indicated by the appearance of a brown ring between the layers (Gul *et al*., 2017; Shaikh & Patil, 2020).

Kedde's Test for Unsaturated Lactone Ring

Four milliliters of the ethanolic leaf extract were dried and then dissolved in 1-2 mL of methanol. Alcoholic KOH (1-2 mL) and 1% alcoholic 3,5-dinitrobenzene (3-4 drops) were added, after which the solution was heated. A violet color indicates the presence of an unsaturated lactone ring (Shaikh & Patil, 2020; Jagessar, 2017).

In general, the presence of cardiac glycoside is confirmed when the extract is positive for Keller-Kiliani, Kedde's, and Liebermann-Burchard tests.

Quantification of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Dried ethanolic leaf extracts were dissolved in distilled water at concentrations of 2 mg/mL and 1 mg/mL for the quantification of TPC and TFC, respectively.

Total Phenolic Content (TPC)

The TPC of *A. angatensis* ethanolic leaf extract was determined following the Folin-Ciocalteu method by Al-Dhabi and colleagues (2017). A sterile 2-mL microtube was prepared, where 100 µL of the diluted leaf extract (2 mg/mL), 150 µL Folin & Ciocalteu's phenol reagent (Sigma-Aldrich, USA), and 1 mL distilled water were mixed. The mixture was vortexed for 1 min, then 600 µL sodium carbonate (10% w/v) was added. The mixture was again vortexed for 1 min, then incubated in the dark at room temperature for 2 h. The absorbance of the sample at 760 nm wavelength was determined using the BMG Labtech FLUOstar Omega microplate reader. Gallic acid standard solution was also prepared, and a reference curve was generated. All were done in triplicate. The resulting TPC values were expressed as Gallic acid equivalents (mg GAE/g of dried extract).

Total Flavonoid Content (TFC)

The TFC of *A. angatensis* ethanolic leaf extract was determined using an aluminum chloride assay (Alnajar *et al*., 2012). A sterile 2-mL microtube was prepared, where 200 µL of the diluted leaf extract (1 mg/mL), 600 µL of 95% ethanol, 40 µL of 1 M potassium acetate, 40 µL of aluminum chloride, and 1.12 mL of distilled water were mixed. The mixture was incubated at room temperature for 30 min. The absorbance of the sample at 415 nm wavelength was determined using the BMG Labtech FLUOstar Omega microplate reader. A quercetin standard solution was also prepared, and a reference curve was generated. All were done in triplicate. The resulting TFC values were expressed as Quercetin equivalents (mg QE/g of dried extract).

Antioxidant activity assay

DPPH assay was employed to determine the antioxidant activity of the *A. angatensis* ethanolic leaf extract (Masuku *et al*., 2020). The dried ethanolic extract was dissolved in distilled water at a concentration of 400 µg/mL and serially diluted to 12.5 µg/mL. The same concentration of ascorbic acid was prepared for the positive control. A 0.051 mM DPPH solution in methanol was also prepared in an amber reagent bottle.

In a 96-well microplate, 100 µL of each leaf extract and of ascorbic acid (12.5 to 400 µg/mL) were dispensed in separate wells in triplicate. Methanol was used as the negative control. In each well, 100 µL of DPPH solution was added, and the microplate was incubated at ambient temperature for 30 min. The absorbance at wavelength 517 nm was determined using the BMG Labtech FLUOstar Omega microplate reader with 200 µL of Methanol as the blank. DPPH radical scavenging activity was determined by computing the percentage inhibition using the formula:

$$\% \, Inhibition = 1-\left(\frac{Abs_{\,sample}}{Abs_{\,control}}\right)\times 100$$

where, Abs_{sample} is the absorbance of the sample, and $Abs_{control}$ is the absorbance of the negative control.

Antioxidant activity of *A. angatensis* ethanolic leaf extract in terms of IC_{50}, or the sample concentration required to inhibit 50% of the DPPH free radicals, was also calculated based on linear regression plots.

Thin-layer chromatography profiling

The dried ethanolic leaf extract of *A. angatensis* was re-dissolved in 80% ethanol. Henceforth, the procedure for TLC profiling was done according to the protocol of Punzalan and Villaseñor (2019). A mixture of n-hexane (Duksan Pure Chemicals, South Korea) and ethyl acetate (Scharlau ACS Basic, Spain/European Union) was prepared to produce the mobile phase of 70% hexane:30% ethyl acetate which was selected for profiling as it yielded the best resolution of the extract components. A small amount of the solvent system was used for initial saturation of the developing chamber, and the setup was covered and left to stand for at least 5 min. TLC plates of approximately 4.5 cm by 9 cm from aluminum sheets of TLC silica gel (Merck TLC Silica gel 60 F_{254}) were lightly drawn on with lines corresponding to the origin line at the bottom and the solvent front at the top. In an incubator (Tryte Technologies Electrothermal Thermostatic Incubator TNP-9082-11), a marked plate was heated at 55°C for 20 min and was carefully spotted with the sample on the origin line, with the use of a capillary tube (Kimble Chase 34500-99 Borosilicate Glass Melting Point Capillary Tube).

Following chamber saturation, the spotted TLC plate was placed inside the chamber, and the setup was covered to allow the developing solvent to travel up to the solvent front of the plate.

The chromatogram was then brought out of the chamber, and left to air dry for 5 min.

The developed plate was viewed under UV light (Analytik Jena UVP UVGL-58, Analytik Jena UVP Chromato-Vue® Cabinet C-10) at long (365 nm) and short (254 nm) wavelengths, and the spots were marked with a pencil. Afterwards, vanillin-sulfuric acid reagent (4 g of vanillin in 25 mL of concentrated sulfuric acid) was sprayed evenly onto the chromatogram surface of the plate, which was then heated at 110℃ until coloration on the chromatogram was observed, and the spots were marked.

To calculate the retention factor (R_f) values, the following formula was used:

$$R_f = \frac{\text{distance traveled by the sample component from the origin}}{\text{distance traveled by the solvent from the origin}}$$

Antibacterial Assay

The ethanolic leaf extract of *A. angatensis* was tested for its antibacterial activity against pathogens with antibiotic resistance genes. *Klebsiella pneumoniae* (NCTC 13440), methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33592), *Enterococcus faecium* (NCTC 12204), and *Enterobacter cloacae* (ATCC BAA-2341) were used. These pathogens have the following antibiotic resistance mechanisms: MecA positive, Metallo-beta-lactamase positive, VanA-type glycopeptide resistance, BlaKPC positive, respectively.

Stock cultures of all the pathogens are maintained at 4°C in glycerol solution. Prior to the bioassay, cells from the glycerol stock were inoculated into test tubes of cation-adjusted Mueller-Hinton Broth (CAMHB) and incubated overnight at 37°C. The bacterial suspensions were adjusted to an optical density equivalent to 0.5 McFarland Standard. The minimum inhibitory concentration (MIC) of *A. angatensis* ethanolic leaf extract against the test pathogens was determined by employing the broth microdilution method and the resazurin microtiter test (Sarker *et al*., 2007; Clinical and Laboratory Standards Institute, 2014; Elshikh *et al*., 2016).

The dried ethanolic leaf extract was prepared at concentrations of 40, 32, 24, 20, 16, 12, 8, 4, and 2 mg/mL in 2% dimethyl sulfoxide (DMSO). Ciprofloxacin, as the positive control, was prepared at a concentration of 200 ppm in sterile distilled water.

In a 96-well microplate, well components were dispensed accordingly, and each well type had triplicate wells. Sample wells contained 50 µL bacteria and 50 µL leaf extract (final well concentrations of 20, 16, 12, 10, 8, 6, 4, 2, and 1 mg/mL). Sample blank wells contained 50 µL CAMHB and 50 µL leaf extract (final well concentrations of 20, 16, 12, 10, 8, 6, 4, 2, and 1 mg/mL). Positive control wells contained 50 µL bacteria and 50 µL ciprofloxacin (a final well concentration of 100 ppm). Positive blank wells contained 50 µL CAMHB and 50 µL ciprofloxacin (a final well concentration of 100 ppm). Negative control wells contained 50 µL bacteria and 50 µL CAMHB. Sterility control wells contained 100 µL CAMHB. Each well has a total of 100 µL final volume. The microplate was sealed and incubated at 37°C for 22 h.

Thereafter, 20 µL of 300 ppm resazurin was dispensed into each well. The microplate was re-incubated for 15 min at room temperature. Bacterial growth is indicated by the reduction of resazurin and a color change from blue to pink. The fluorescence (in RFU or relative fluorescence units) of the samples was measured using a BMG Labtech FLUOstar Omega microplate reader with 544 nm excitation and 590 nm emission filters and gain at 700 nm. Percentage inhibition was calculated using the formula:

$$\%\ Inhibition = \left[1 - \frac{RFU_{sample} - RFU_{blank}}{RFU_{neg\ ctrl} - RFU_{sterlility\ ctrl}}\right] \times 100\%$$

The concentration that inhibited the growth of 99% of the pathogen was recorded as the MIC.

The contents of the microplate wells were inoculated into nutrient broth and incubated for 24 h at 37℃ for the determination of the minimum bactericidal concentration (MBC). Turbidity in the broth medium indicates bacterial growth.

RESULTS AND DISCUSSION

Phytochemical and Nutritional analysis

Qualitative phytochemical screening was performed on the ethanolic leaf extract to determine the general bioactive constituents of *A. angatensis.* The extract contains various secondary metabolites including tannins, polyphenols, flavonoids, saponins, steroids and triterpenoids (Table I). Similar to our results, polyphenols, flavonoids, and saponins were detected in *A. hispida, A. racemosa, A. marginata, A. indica,* and *A. wilkesiana* leaf extracts (Dada *et al*., 2019; Chandra *et al*., 2012; Iniaghe *et al*., 2009). Phytosterols were also detected in *A. wilkesiana* leaf extracts (Madziga *et al*., 2010).

Table I. Summary of results of phytochemical screening of *A. angatensis* leaf extract

Test	Positive Result	Result
Tannins		
Gelatin test	white precipitate	+
Polyphenols		
Ferric chloride test	dark green/bluish-black color	+
Flavonoids		
Lead acetate test	yellow precipitate	+
Shinoda's test	red color	+
Saponins		
Foam test	foam persists for 10 min	+
Alkaloids		
Mayer's test	creamy white/yellow precipitate	-
Wagner's test	brown/reddish precipitate	-
Dragendorff's Test	red/reddish-brown precipitate	-
Hager's Test	creamy white/yellow precipitate	-
Steroids and Triterpenoids		
Liebermann Burchard test*	colors ranging from blue to green, violet and red	+
Cardiac Glycosides		
Keller-Killani test	brown ring between layers	-
Kedde's test	disappearing violet color	-
Liebermann Burchard test	colors ranging from blue to green, violet and red	+

*test was also conducted under tests for cardiac glycosides

Table II. Nutrient analysis and proximate composition of *A. angatensis* leaves

Analyte	Concentration in g/100 g	Reference Methods
Sodium, g	0.002	AOAC 999.10 (Modified)
Potassium, g	1.499	AOAC 999.10 (Modified)
Iron, g	0.010	AOAC 985.35 (Modified)
Calcium, g	1.380	AOAC 985.35 (Modified)
Zinc, g	0.002	AOAC 985.35 (Modified)
Proximate composition		
Ash, % w/w	7.29	AOAC 942.05
Moisture, % w/w	7.73	AOAC 934.01
Crude Fat, % w/w	0.75	AOAC 920.39
Crude Protein, % w/w	23.03	AOAC 981.10
Carbohydrate, % w/w	63.03	FNRI, DOST Handbook
Energy, kcal	358	FNRI, DOST Handbook

Tannins and alkaloids may or may not be present in some studies. *A. indica*, *A. manniana*, and *A. wilkesiana* leaf extracts were positive for tannins (Teklani & Perera, 2016; Awe *et al*., 2013; Noumedem *et al*., 2013; Chandra *et al*., 2012), while this was absent in some *Acalypha* spp. (Iniaghe *et al*., 2009).

Lastly, alkaloids and cardiac glycosides were absent in *A. angatensis* but present in other *Acalypha* spp. (Awe *et al*., 2013; Madziga *et al*., 2010; Chandra *et al*., 2012; Noumedem *et al*., 2013).

Dried leaf samples were subjected to nutrient analysis. Table II presents a summary of the results, showing that the dried leaf samples had relatively high potassium (K) and calcium (Ca) contents, measuring 1.499 g/100g and 1.38 g/100g, respectively. Additionally, the samples exhibited low sodium (Na) content and moderately low levels of iron (Fe) and zinc (Zn). Relatively high K and Ca with low Na levels were also detected in *A. wilkesiana* leaf extracts and powdered leaves (Kingsley *et al*., 2013). A similar trend is also

observed on *A. wilkesiana* and *A. hispida* leaf samples by Okonwu & Ahunanya (2020). In another study, Ca and K was detected as the highest elements in *A. indica* powdered leaf and ethanolic leaf extract, respectively (Refilda *et al*., 2021). High amount of potassium with low sodium levels in leaves has a potential for diuretic drug since the effects of sodium can be countered by potassium (Kingsley *et al.*, 2013). Sodium, potassium, and calcium regulate the body's fluid balance while iron and zinc are important enzyme co-factors (Madziga *et al.*, 2010).

The result of proximate analysis on the amounts of ash, moisture, crude fat, crude protein, carbohydrates, and energy in the *A. angatensis* leaves (Table II). The ash content reflects the amount of mineral elements present in the leaves (Kingsley *et al*., 2013). The recorded ash content (7.29%) for *A. angatensis* leaves is lower than the previously reported values for *A. indica, A. wilkesiana, A. hispida, A. racemosa,* and *A. marginata* leaves which ranges from 10.32% to 15.68% (Nazri *et al*., 2016; Kingsley *et al*., 2013; Iniaghe *et al*., 2009). The moisture content, on the other hand, is an index of water content that may affect the stability and susceptibility of the leaves to microorganisms (Kingsley *et al*., 2013; Nazri *et al*., 2016). The moisture content (7.73%) of *A. angatensis* leaves is also lower than those of other *Acalypha* spp. (9.49% to 12.0%) (Nazri *et al*., 2016; Kingsley *et al*., 2013; Iniaghe *et al*., 2009). A lower moisture content contributes to better storage and a longer shelf life of the plant sample (Nazri *et al*., 2016).

The crude fat (0.75%) of *A. angatensis* leaves is lower than the reported values for other *Acalypha* spp. with values as low as 2.20% and as high as 6.30% (Nazri *et al*., 2016; Kingsley *et al*., 2013; Iniaghe *et al*., 2009). Dietary fats could absorb and retain flavors from food thus increasing palatability (Iniaghe *et al.*, 2009). Meanwhile, the crude protein (23.03%) of *A. angatensis* leaves is comparable to that of *A. indica* (23.98%) (Nazri *et al*., 2016), and is higher when compared to those of *A. wilkesiana*, *A. hispida, A. racemosa,* and *A. marginata* (11.84% - 18.15%) (Kingsley *et al*., 2013; Iniaghe *et al*., 2009). Proteins are important in many biological functions, such as enzymatic activities and immune support functions (Kingsley *et al*., 2013).

The analysis showed that the composition of *A. angatensis* leaves has a high carbohydrate content at 63.03%. This value is also higher when compared with those of *A. indica* (51.10%) (Nazri *et al*., 2016), *A. hispida* (48.48%), *A. racemosa* (45.26%), and *A. marginata* (38.24%) (Iniaghe *et al*., 2009). Lastly, the computed energy was at 358 kcal, which relates to the availability of transferable energy in the leaves (Kingsley *et al*., 2013). Overall, nutrient and proximate analysis data of the *A. angatensis* leaves will aid in its standardization.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Standard calibration curves of gallic acid (R^2 = 0.994) and quercetin (R^2 = 0.9788) were generated and used to quantify the TPC and TFC values of *A. angatensis* ethanolic leaf extract (Table III). Phenolics and flavonoids are known pharmaceutical active ingredients (Açıkgöz, 2020). In this study, we present the first report on the TPC and TFC of *A. angatensis* ethanolic leaf extract. The TPC of *A. angatensis* leaf extract at 282.24 ± 3.56 mg GAE/g is higher than those previously reported TPC values for other *Acalypha* species. Anokwuru and colleagues (2011) recorded TPC values ranging from 58.7 to 137.7 mg GAE/g for *A. wilkesiena* leaf extract, while Alaribe and colleagues (2021) reported values from 0.17 to 0.29 mg GAE/g for *A. fimbriata*. The two studies used different extraction solvents (i.e., methanol, acetone, ethyl acetate, ethanol), hence the range of TPC values.

Comparing the results of this study with published data, the TFC of *A. angatensis* leaf extract is higher than that of *A. wilkesiana* and *A. fimbriata*. *A. wilkesiana* has a TFC ranging from 34.1 to 40.5 mg QE/g (Anokwuru *et al*., 2011), while *A. fimbriata* has a TFC ranging from 0.13-0.40 mg QE/g (Alaribe *et al*., 2021). It has previously been observed that the presence of phenolics and flavonoids in plants, as mentioned by Açıkgöz (2020), is greatly affected by genetics, environmental factors, season, plant parts, and extraction conditions.

Antioxidant Activity

The oxidation of free radicals is known to cause cellular damage contributing to many degenerative diseases (Onocha *et al*., 2011). Antioxidants are substances that inhibit these oxidative mechanisms by scavenging free radicals. In this study, the DPPH assay method was used to assess the antioxidant properties of *A. angatensis* ethanolic leaf extract (Table III). DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical that accepts electrons from antioxidants, resulting in its reduction and change in color from purple to yellow (Gholivand & Piryaei, 2012).

Table III. Total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity of *Acalypha angatensis* leaf extract (n = 3).

	TPC (mg GAE/g) ± SD	**TFC (mg QE/g) ± SD**	**DPPH % inhibition**	**IC_{50} (µg/mL) ± SD**
A. angatensis leaf extract	282.24 ± 3.56	36.24 ± 3.77	85.79%	34.02 ± 0.54
Ascorbic acid	--	--	94.10%	23.31 ± 0.90

This scavenging activity of antioxidants is also indicated by a decreased absorption of the DPPH at 517 nm (Prasad *et al*., 2009).

The DPPH radical scavenging activity of *A. angatensis* ethanolic leaf extract was found to be concentration-dependent, which peaked at 100 g/mL with 85.79% inhibition and stabilized at higher concentrations. Literature shows that the DPPH radical scavenging activity of *A. angatensis* leaf extract is stronger compared to those of other *Acalypha* species. Anokwuru and co-authors (2011) reported that the DPPH scavenging activity of *A. wilkesiana* ranged from 70.17% to 85.65% inhibition using different extraction solvents. At 100 µg/mL, the methanolic extract of *A. fruticosa* showed 52.75% inhibition (Mothana *et al*., 2010).

IC_{50} values show that the scavenging activity of *A. angatensis* ethanolic leaf extract (34.02 µg/mL) is weaker than that of the standard ascorbic acid (23.31 µg/mL). When compared with other *Acalypha* species, the scavenging activity of *A. angatensis* extract is better than that of *A. fimbriata* extract, with an IC_{50} ranging from 61.03 to 82.24 µg/mL (Alaribe *et al*., 2021), but weaker than that of *A. manniana* extract, with an IC_{50} ranging from 3.34 to 4.80 µg/mL (Noumedem *et al*., 2013).

The observed antioxidant activity may be due to the presence of phenolics and flavonoids in the extract. Phenolics are good scavengers of free radicals because of their ability to donate hydrogen and thus largely contribute to the antioxidant properties of plant extracts (Noumedem *et al*., 2013; Alaribe *et al*., 2021). Flavonoids are also known to be strong antioxidants because of their phenolic hydroxyl groups (Anokwuru *et al*., 2011).

Thin Layer Chromatography (TLC) Profiling

Profiling of *A. angatensis* ethanolic leaf extract by TLC was conducted for chemical characterization. The developing solvent system of 70% hexane:30% ethyl acetate yielded the best resolution of the components, hence selected for profiling. The chromatograms in Figure 1 show the TLC profile of the extract under the two selected wavelengths of UV light and after post-derivatization with vanillin-sulfuric acid, displaying 10 spots under UV at 365 nm (long UV), 6 under UV at 254 nm (short UV), and 9 using vanillin-sulfuric acid spray.

Under long UV, most of the spots fluoresced red, two of which had lighter intensities, while two spots exhibited pink fluorescence. The spots under short UV were fluorescence-quenching and seen as dark bands. Application of vanillin-sulfuric acid reagent and subsequent heating at 110°C resulted in the visualization of spots of various coloration: different hues of violet, and combinations of gray with violet and with brown.

The TLC profile of *A. angatensis* ethanolic leaf extract is summarized in Table IV. Most of the bands appeared at UV_{365} and after staining with vanillin-sulfuric acid. Some spots from two of the three visualization methods generated the same R_f values such as those with values of 0.61, 0.79, and 0.93 under both UV_{365} and vanillin spray application, and bands with R_f = 0.09 and 0.67 at both wavelengths of UV light. Across all detection methods, a total of three sets of spots were calculated to have the same R_f values of 0.39, 0.50, and 0.76.

Thin-layer chromatography is one of the basic tools used to identify and characterize the chemical components of an extract, hence it is useful in providing the profile of a plant or herb (Braz *et al*., 2012; Medic-Šaric *et al*., 2008). Coupled with bioassays, TLC can be employed to rapidly screen botanicals and medicinal plants for their potential pharmacological properties, such as antimicrobial, free radical scavenging, and enzyme-inhibiting activities (Kowalska & Sajewicz, 2022).

The retention factor (R_f) value obtained in TLC can be used to describe and characterize a compound by comparing this value to that of a known or reference (Kumar *et al*., 2013). Therefore, the R_f values of the chromatogram bands in the ethanolic leaf extract of *A. angatensis* can help in the process of identifying the components of the extract.

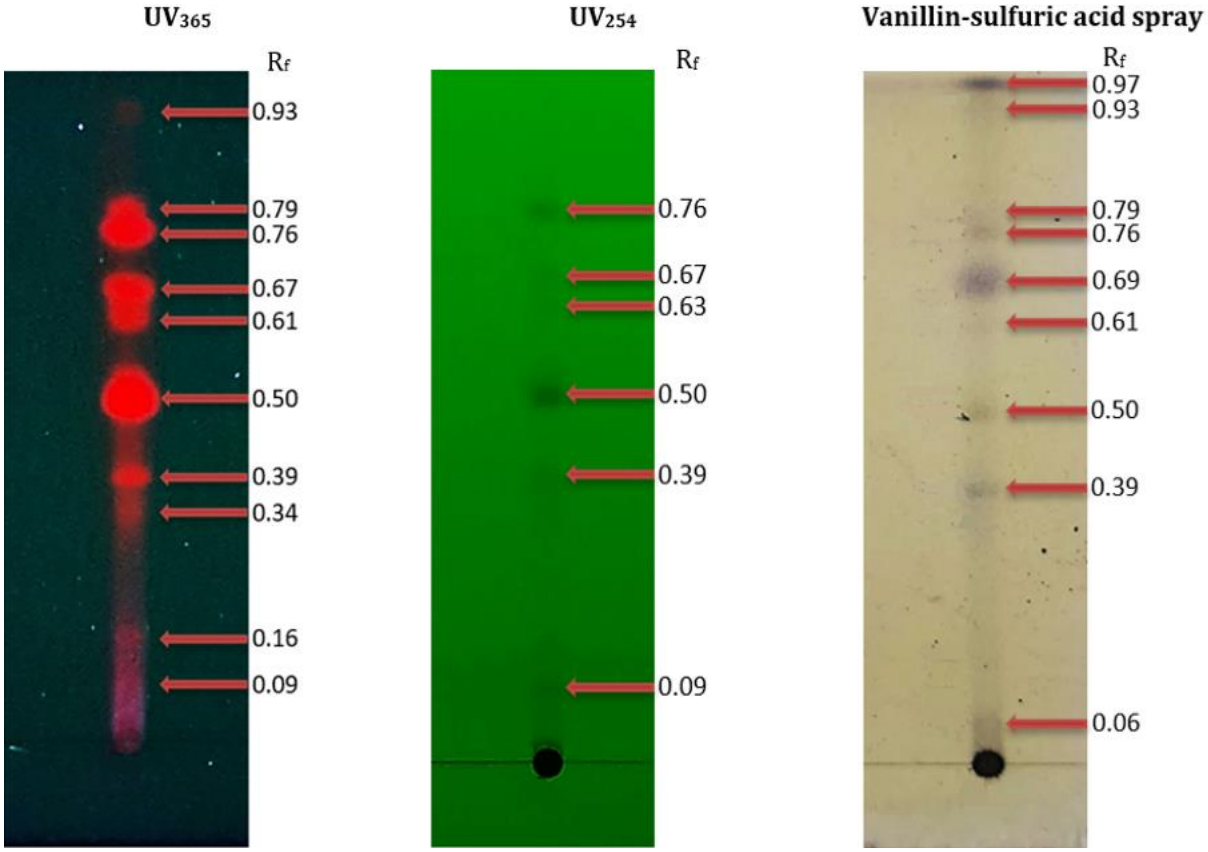

Figure 1. TLC chromatograms of *Acalypha angatensis* ethanolic leaf extract visualized under UV at 365 nm, UV at 254 nm and after spraying with vanillin-sulfuric acid. Mobile phase: hexane: ethyl acetate (7: 3 v/v).

For instance, some R_f values (0.06, 0.76, 0.93, and 0.97) of the constituents in *A. angatensis* leaf extract are comparable to those of some identified classes of compounds (0.06 (tannins), 0.76 (flavonoids), 0.93 (terpenes), and 0.97 (terpenes)) in *Acalypha integrifolia* extracts using hexane:ethyl acetate as the developing solvent (Seebaluck-Sandoram *et al*., 2018). These spots having identical or similar R_f values suggest that these may belong to the same class of compounds (Kumar *et al*., 2013).

The TLC profile of a plant extract can also give insight on the polarity of its components (Talukdar *et al*., 2010; Kumar *et al*., 2013). Determining the R_f values can be used to assess the relative polarity of the constituents, which can help in improving the selection of the most suitable solvent system to separate the component phytochemicals and isolate active compounds from the extract (Bhardwaj *et al*., 2016; Sharma & Paliwal, 2013). The mobile phase system of 70% hexane:30% ethyl acetate gave the best resolution of the phytochemicals in *A. angatensis* ethanolic leaf extract, indicating that this solvent system best separates the components.

In the current study, the TLC data based on visualization supports the results of the phytochemical screening done. The presence of phenolics in the leaf extract of *A. angatensis* may be indicated by the profile from all three methods of detection. The red and the pink fluorescence of the bands under UV_{365} are among the colors exhibited by phenolics under UV light, as a result of their strong absorption of UV light owing to their aromatic structure (Harborne, 1998; Martin-Puzon *et al*., 2015). On plates of silica gel with fluorescence indicators viewed under UV at 253 nm, phenolics may be seen as dark spots against a fluorescent background (Harborne, 1998). The appearance of dark spots in the chromatograms of *A. angatensis* leaf extract under UV_{254} may thus confirm the occurrence of phenolic compounds.

Table IV. Summary of *Acalypha angatensis* ethanolic leaf extract TLC profile.

R_f value	UV_{365} nm (10 spots)	UV_{254} nm (6 spots)	Vanillin spray (9 spots)
0.06	-	-	Violet-gray
0.09	Pink	Quenching	-
0.16	Pink	-	-
0.34	Faint red	-	-
0.39	Red	Quenching	Violet-gray
0.50	Red	Quenching	Gray-brown
0.61	Red	-	Light violet
0.63	-	Quenching	-
0.67	Red	Quenching	-
0.69	-	-	Dark violet
0.76	Red	Quenching	Violet-gray
0.79	Red	-	Light violet
0.93	Faint red	-	Light violet
0.97	-	-	Dark violet

Furthermore, violet-colored bands appearing after the application of vanillin-sulfuric acid to the chromatogram might suggest likewise as it has been previously shown that phenolics displayed violet coloration using the same detection method (Napiroon *et al*., 2017).

Flavonoids cause fluorescence quenching; hence their detection is usually done under UV at 254 nm (Medic-Šaric *et al*., 2008). Therefore, their occurrence in *A. angatensis* leaf extract may be corroborated by the observation of dark bands in the chromatogram visualized at UV_{254}. Meanwhile, the presence of tannins in the sample might be supported by the observation of violet spots in post-derivatization with vanillin-sulfuric acid, a similar result for the same method conducted on tannic acid (Sharma *et al*., 1998). Lastly, it has also been determined that saponins give violet to violet blue coloration when sprayed with vanillin-sulfuric acid (Anandakumar *et al*., 2009; Karthika *et al*., 2014; Senguttuvan & Paulsamy, 2014), which suggests that violet spots in the chromatogram of *A. angatensis* leaf extract might correspond to its saponin component.

Antibacterial Activity

The antibacterial activity of *A. angatensis* leaf extract against pathogens with antibiotic resistances was determined. The extract was observed to be more effective against the gram-positive bacteria *E. faecium* and MRSA with known MIC values (Table V). While there were no MICs recorded for *A. angatensis* leaf extract against the gram-negative pathogens, its inhibition reached 87.10% at 16 mg/mL against *E. cloacae*, and 95.24% at 20 mg/mL against *K. pneumoniae*. Ciprofloxacin at 100 ppm resulted in 99% inhibition of all the pathogens. Bacterial growth was observed when samples from the microplate sample wells were reinoculated in nutrient broth and incubated for another 24 h. Therefore, the activity is non-bacteriostatic at these assay concentrations.

There is little published data on the antibacterial activity of *Acalypha* extracts against pathogens with antibiotic resistance genes. In addition, no previous study has investigated the antibacterial bioactivity of *A. angatensis.* In this paper, we present the first report on the antibacterial activity of *A. angatensis* leaf extract against pathogens with antibiotic resistance genes.

The ethanolic leaf extract of *A. angatensis* was found to have inhibitory activity against vancomycin-resistant *E. faecium* and MRSA, with MICs recorded at 4 mg/mL. Gutierrez-Lugo and colleagues (2002) purified three cycloartane-type triterpene compounds from *A. communis* extract, which they found to be active against vancomycin-resistant *E. faecium*: 16α-hydroxymollic has a MIC of 8 μg/mL, 15α-hydroxymollic has a MIC of 32 μg/mL, and 7β,16β-dihydroxy-1,23-dideoxyjessic acids has a MIC of 8 μg/mL. 16α-hydroxymollic was also found effective against methicillin-resistant *S. aureus*, with a higher MIC of 64 μg/mL (Gutierrez-Lugo *et al*., 2002). It is thus not far-fetched that similar compounds capable of inhibiting vancomycin-resistant *Enterococcus* are found in *A. angatensis* leaf extract.

Prior reports on the antibacterial property of *Acalypha* extracts show activity against *S. aureus*

resistant to antibiotics. Haruna and co-authors (2013) reported that *A. wilkesiana* methanol leaf extract was effective against *S. aureus* strains ATCC 25923 (resistant to ampicillin, cloxacillin, erythromycin, gentamicin, penicillin, and streptomycin) and ATCC 55620 (resistant to ampicillin, chloramphenicol, cloxacillin, erythromycin, gentamicin, penicillin, streptomycin, and tetracycline) with MICs of 25 and 50 mg/mL, respectively. The same extract was observed to inhibit the growth of *K. pneumoniae* ATCC 700603 (resistant to amoxicillin, cotrimoxazole, nitrofurantoin, nalidixic acid, and co-amoxiclav) with a recorded MIC of 50 mg/mL (Haruna *et al*., 2013). While it is important to note that the *S. aureus* used were of different strains, it is remarkable that the MIC recorded for MRSA in the present study is significantly lower. Despite this, it is notable that the genus *Acalypha* possesses compounds capable of controlling multi-drug resistant strains of *S. aureus.*

In a separate study by Noumedem and colleagues (2013), *A. manniana* methanol leaf extract was found active against *S. aureus* ATCC 25922, with a MIC of 0.25 mg/mL and an MBC of 4.09 mg/mL. The same extract inhibited the growth of *K. pneumoniae* ATCC 13883 with an MIC of 0.51 mg/mL and an MBC of 4.09 mg/mL (Noumedem *et al*., 2013). These reported MICs of *A. manniana* extract for *S. aureus* and *K. pneumoniae* are significantly lower than the values obtained in the present study; however, the previous study used strains of test pathogens that lack antibiotic resistance genes.

Several studies have recognized the critical role played by flavonoids, polyphenols, saponins, tannins, and alkaloids in the antimicrobial activity displayed by extracts of *Acalypha* (Alade & Irobi, 1993; Gotep *et al*., 2010; Anokwuru *et al*., 2014; Zahidin *et al*., 2017). Previous studies have attributed the antimicrobial properties of *A. hispida* and *A. wilkesiana* to the tannins geraniin, corilagin, and gallic acid found in each of the extracts (Gotep *et al*., 2010; Adesina *et al*., 2000). More recent evidence confirms the aforementioned: geraniin, corilagin, quadrangularic acid M, and shikimic acid isolated from *A. wilkesiana* extract was reported to show bactericidal activity against extended-spectrum beta-lactamase-producing *K. pneumoniae* (700603) (Anokwuru *et al*., 2014). These findings suggest that the antibacterial activity of *A. angatensis* leaf extract may be possibly attributed to tannins, polyphenols, flavonoids, and saponins in the extract. The effects of other chemical constituents cannot be discounted, however. Therefore, further studies with more focus on the isolation and identification of the many phytochemical components are recommended. With this, the actual mechanisms of action that explain bioactivity of the extract against antibiotic resistant strains can be elaborated.

CONCLUSION

This study highlighted the phytochemical characteristics, unique TLC profile and pharmacologic properties of *A. angatensis* leaf extract. The results show that the plant extract is rich in tannins, polyphenols, flavonoids, saponins, steroids and triterpenoids. Also, the ethanolic extract had good antioxidant properties and antibacterial activities against gram positive antibiotic-resistant *E. faecium* and methicillin-resistant *S. aureus.* With these characteristics, folkloric use of this species for treatment of infections is supported. It can be concluded that the plant is an attractive and promising source of raw material for the development of nutraceutical and pharmaceutical products. Further work is necessary to purify and elucidate the phytochemical compounds in this species.

ACKNOWLEDGEMENT

The authors are grateful for the support provided by the Department of Science and Technology-Philippine Council for Health Research and Development. We also thank Dr. Liezel M. Magtoto for the identification of our plant sample.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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