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Bioactive metabolite profiles and antimicrobial activity of ethanolic extracts from *Muntingia calabura* L. leaves and stems

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ABSTRACT

Objective: To determine the bioactive phytochemicals and antimicrobial activity of leaf and stem ethanolic extracts from *Muntingia calabura* L. (*M. calabura*).

Methods: Dried leaves and stems of *M. calabura* were extracted with 95% ethanol. The antibacterial and antifungal activities of the extracts were examined using the disc diffusion assay. The minimum inhibitory concentration (MIC) of each extract showing antimicrobial activity was determined. The dried extracts were subjected to phytochemical screening to determine the presence of bioactive components. Total phenolic and flavonoid contents were also determined by the Folin–Ciocalteu method and the aluminum chloride method, respectively.

Results: Varying degrees of antimicrobial activity were exhibited by the leaf and stem extracts against *Pseudomonas aeruginosa* (*P. aeruginosa*), *Salmonella typhimurium*, *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis*, and *Candida albicans* (*C. albicans*), with minimal activity against *Escherichia coli*. Based on the MIC, the extracts showed the highest activity against *C. albicans*, *S. aureus* and *P. aeruginosa*. Phytochemical screening revealed the presence of sterols, flavonoids, alkaloids, saponins, glycosides and tannins in the leaf extract; however, no triterpenes were detected. In the stem extract, triterpenes were detected along with relative amounts of flavonoids, saponins, glycosides and tannins. Alkaloids and sterols were absent in the stem extract.

Conclusions: *M. calabura* leaf and stem ethanol extracts are potential sources of antibacterial agents against *P. aeruginosa* and *S. aureus*. This study reports for the first time the high degree of antifungal activity of *M. calabura* ethanolic extract, especially against *C. albicans*.

1. Introduction

Traditional medicine encompasses multiple indigenous traditions around the world. In Western and other developed nations, its usage is often in conjunction with, and complementary to, modern medicine. As a testament to its persistence, over 80% of the world's population is known to still utilize traditional medicine for primary healthcare. Most are in underdeveloped countries, many of which are situated in biodiversity hotspots in Southeast Asia, Africa, Central and Southern America among others. Plants are the single largest source for traditional medicines [1]. They account for over 25% of new drugs tested for clinical use [2]. Newman and Cragg noted that in the field of cancer research, almost 80% of new chemical entities discovered were derived from natural sources, or semi-synthetic modifications thereof [3].

Secondary metabolites are the sources of plant natural products used in medicine. These compounds are broadly divided into phenolics which include polyphenols, flavonoids,

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tannins and quinones known for their potent antioxidant, cytotoxic and antimicrobial activities [4–7]; alkaloids which are cytotoxic and have a wide range of physiological effects [8–10]; glycosides; terpenes and other volatile compounds, most of which are endogenously utilized as plant defense compounds [11]. However, the under-utilization of plants remains a challenge for developing drugs. The use of plant-derived compounds and their derivatives is well-established in other medicinal applications, most notably in chemotherapeutic agents [12,13]. Moreover, antimicrobial agents derived from plant metabolites have increasingly gained attention in the past few years [7]. The emergence of multi-drug resistant bacteria and the rise of infectious diseases have led to the reevaluation of the use of antibiotic agents in treatment, and novel solutions such as the use of plant secondary metabolites as resistance-modifying agents. In addition, there is a marked decline in the development of new classes of antibiotics since 1960. These factors underscore the importance of searching for alternative sources of antimicrobial agents from plants [14,15].

Muntingia calabura L. (*M. calabura*) is a shrub introduced from Tropical America to Southeast Asia. It is well-adapted in areas where it is introduced, often growing as roadside trees [1,16]. This plant species is heavily indigenized in most localities. Its leaves are distinctively lanceolate in shape, with margins irregularly serrate. The plant flowers throughout the year; its fruits are berries which turn red when mature with lenticularly shaped seeds [17].

Its leaves, stems and roots have been documented to have traditional medicinal usage in various modes of applications. In Peru, its leaves and bark are used as antiseptics, and to treat swelling in the lower extremities. Leaf decoctions are also popular treatments in South America to reduce gastric ulcers. In the Philippines, the flowers are used to treat headache and for relief of incipient colds. Its roots are also used as emmenagogues in Malaysia and Vietnam, although in these countries, *M. calabura* is considered as a neglected species [1].

A recent review revealed only thirty published studies on *M. calabura*, which focused mostly on leaf ethanolic and methanolic extracts [1,18]. In consonance with its traditional usage, researchers have identified several bioactive properties of the plant. Anti-inflammatory, anti-nociceptive [18–20] and antioxidant activity of the leaves [19], cytotoxicity against leukemia cell lines of the roots [21], and antimicrobial activity of the leaves [22] of *M. calabura* have been observed and demonstrated.

This paper presented the antimicrobial activity of the crude ethanolic extracts from the leaves and stems of *M. calabura* against the following bacteria: *Escherichia coli* (*E. coli*), *Salmonella typhimurium* (*S. typhimurium*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Bacillus subtilis* (*B. subtilis*); and the fungus *Candida albicans* (*C. albicans*). The phytochemicals of the leaf and stem extracts which are responsible for the bioactive properties are also reported.

2. Materials and methods

2.1. Plant material

M. calabura leaves and stems were collected in April–June, 2015 from Paniqui, Tarlac, Philippines. Leaf and stem specimens were identified and authenticated at the Jose Vera Santos Memorial Herbarium (PUH) at the Institute of Biology, University of the Philippines, Diliman, Quezon City, Philippines. Samples were then laid and air-dried for two weeks before pulverization.

2.2. Preparation of crude extracts

Leaves and stems weighting 100 g each were immersed in 95% ethanol at a ratio of 1:10 (w/v) for 72 h. The mixtures were then decanted and filtered. The filtrate was concentrated under reduced pressure using a rotary evaporator (Laborota 4001, Heidolph) with the temperature set at 40 °C. The crude leaf and stem extracts were then air-dried for 14 days. After air-drying, extracts were reconstituted in 95% ethanol, and filtered through a Whatman No. 1 filter paper for further bioassays.

2.3. Phytochemical screening

Ethanolic extracts derived from the leaves and stems of *M. calabura* were subjected to phytochemical screening for the presence of tannins, flavonoids, alkaloids, sterols, triterpenes, saponins, and glycosides following standardized methods [23].

2.4. Estimation of total phenolic and flavonoid contents

Total phenolic content of the ethanolic extracts was determined by the modified Folin–Ciocalteu method [24]. A total of 10 mL Folin–Ciocalteu reagent and 200 μL of Na_2CO_3 (2%, w/v) were added to 100 μL of plant extract solution (1 mg/mL). Then, the resulting mixture was incubated at 45 °C with shaking at 120 r/min for 15 min. The absorbances of the samples were measured at 765 nm using a UV-Vis spectrophotometer. Results were expressed as mg gallic acid equivalent (GAE)/g plant extract. The same procedure was used for making a standard curve of gallic acid with a concentration range of 0–100 μg/mL.

Total flavonoid content was determined using the modified aluminum chloride method [24]. One milliliter of plant extract solution (1 mg/mL) was mixed with 3 mL ethanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 mol/L potassium acetate, and 5.6 mL of distilled water and kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm using a UV-Vis spectrophotometer. The total flavonoid content was computed from a calibration curve made with rutin as standard (0–200 μg/mL in ethanol). The concentration of total flavonoids was then expressed as mg rutin equivalents/g crude extract.

2.5. Antimicrobial assay

Potential antibacterial and antifungal activities of the plant extracts were examined using the disc diffusion assay. Organisms used to test the antimicrobial activity of the plant extracts were *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *B. subtilis* (bacteria) and *C. albicans* (fungus). Microbial suspensions in 0.1% peptone at 0.5 McFarland were inoculated on nutrient agar plates for bacterial samples, and glucose yeast peptone agar plates for fungal samples. For each agar plate, three equidistant wells, one for each replicate, were drilled using a cork borer. Then 200 μL of each plant extract (10 mg/mL) was aspirated into each well. In addition to the plant extracts, ethanol

diluted in 0.1% peptone water was prepared as negative control. The plates inoculated with microbial samples, and supplied with extracts, were then incubated at 37 °C and observed after 24 h. The average diameters of the zones of inhibition of the extracts against the tested organisms were then measured in order to evaluate the antimicrobial activity.

2.6. Minimum inhibitory concentration (MIC)

The MIC of each extract showing antibacterial and/or antifungal activity was determined following the protocol described by the Clinical and Laboratory Standards Institute [25]. The MIC is defined as the lowest concentration of the crude extract that does not permit any visible microbial growth. The MIC of each extract was determined by dilution of each bacterial and fungal strain in liquid culture medium, measuring the optical density after 24 h of incubation, and then visually inspecting the turbidity of each inoculated well.

3. Results

3.1. Phytochemical screening

The phytochemical profiles of the leaf and stem ethanolic extracts of *M. calabura* are presented in Table 1. The leaf ethanolic extract was found to contain a greater number of secondary metabolite classes when compared to the stem ethanolic extract. Sterols, flavonoids, alkaloids, saponins, glycosides, and tannins were detected in the leaf extract while triterpenes were not detected. In the stem ethanolic extract, triterpenes, a class of volatile metabolites, were however, detected. Alkaloids and sterols were not detected in the stem extract.

Table 1
Phytochemical profiles of leaf and stem ethanolic extracts of *M. calabura*.

Constituents	Leaf extracts	Stem extracts
Sterols	+	–
Triterpenes	–	++
Flavonoids	+++	++
Alkaloids	+	–
Saponins	+++	+++
Glycosides	++	+++
Tannins	++	+++

+: Constituents present in little amounts; ++: Constituents present in moderate amounts; +++: Constituents present in abundance; –: Constituents not detected.

3.2. Total phenolic and total flavonoid contents

The total phenolic content of *M. calabura* was (75.7 ± 5.4) mg GAE/g crude extract for the leaf extract and (91.5 ± 6.4) mg GAE/g crude extract for the stem extract. On the other hand, the total flavonoid content was (112.8 ± 6.6) mg rutin equivalent/g crude extract for the leaf extract and (55.3 ± 7.5) mg rutin equivalent/g crude extract for the stem extract.

3.3. Antimicrobial activity

Both stem and leaf extracts demonstrated varying degrees of antimicrobial activity against *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *B. subtilis*, and *C. albicans*, while minimal activity was observed against *E. coli* based on the results of the disc diffusion assay (Table 2). Furthermore, MIC values in the range of 0.625–10.000 mg/mL were obtained for *P. aeruginosa* (2.5 mg/mL for leaf and stem ethanolic extracts), *S. aureus* (1.25 mg/mL for leaf and stem ethanolic extracts), and *C. albicans* (2.5 mg/mL for the stem extract). The MIC values against *S. typhimurium* and *B. subtilis* were both higher than the maximum value at the range tested (10 mg/mL) for both extracts. The MIC value of the leaf ethanolic extracts against *C. albicans* may be 0.625 mg/mL, or lower than the concentration range tested.

Table 2
Diameter of zones of inhibition for *M. calabura* leaf and stem ethanolic extracts against bacterial and fungal strains from University of the Philippines Natural Sciences Research Institute Culture Collection.

Organisms	Average inhibition zone diameter (mm)		MIC (mg/mL)	
	Leaf extract	Stem extract	Leaf extract	Stem extract
E. coli	12.3	10.0	–	–
P. aeruginosa	20.0	15.7	2.500	2.500
S. typhimurium	19.0	19.0	> 10.000	> 10.000
S. aureus	37.7	24.7	1.250	1.250
B. subtilis	17.0	16.0	> 10.000	> 10.000
C. albicans	18.7	19.0	0.625[a]	2.500

[a]: MIC may be 0.625 mg/mL or lower.

4. Discussion

Phytochemical analysis of *M. calabura* revealed the presence of sterols, flavonoids, alkaloids, saponins, glycosides, and tannins in the leaf ethanolic extract and triterpenes in the stem ethanolic extract. These plant secondary metabolites have been reported to exhibit medicinal and physiological activities.

Our study provides significant additional information on the antimicrobial activity of this plant. There are very few studies on the antimicrobial properties of *M. calabura* [18,22]. The results of our study indicate that *M. calabura* is an alternative source of antibacterial agents against *P. aeruginosa* and *S. aureus*. The high degree of activity against *C. albicans* is one of the significant findings that have not been reported in other previously published studies. The antifungal activity of *M. calabura* is indeed promising, thus would warrant further investigations through the inclusion of more isolates of fungal species. It has been known that the occurrence of pathogenic fungi may have exerted selective or evolutionary pressure on plants to produce antifungal compounds as a first-line defense system [26,27].

The divergent bioactivities of plants belonging to the same species may be due to a difference in phytochemical profiles across geographical regions [28,29]. It is known that variations in the growing conditions are major contributors to the differences in secondary metabolite profile. Different stressors may emphasize the production of one metabolite over another in response to the various needs of the plant. Variations in altitude, carbon dioxide levels, insect and pathogenic presence are all known to affect the composition of the metabolite constituents [30]. A slight variation in the secondary metabolite profile might be expected for *M. calabura* plants grown in the Philippines.

Polyphenols, especially the flavonoids are typically implicated in antimicrobial activities. In one study [22], four types of flavonoids (three flavones and one chalcone) isolated from *M. calabura* leaf ethyl acetate extracts demonstrated antimicrobial activity against methicillin-sensitive *S. aureus* and methicillin-resistant *S. aureus*, but not against *P. aeruginosa*, *B. subtilis*, *Bacillus cereus*, and *E. coli*. Following the MIC results of this study, it is interesting to note that the leaf and stem crude ethanolic extracts exhibited inhibitory activity against *P. aeruginosa* and *C. albicans*, which have not been previously reported even for fractionated extracts. Antimicrobial assays to determine the effects of *M. calabura* extracts on multi-drug resistant bacteria are currently underway.

In conclusion, *M. calabura* leaf and stem ethanolic extracts can serve as new and alternative sources of antibacterial agents against *P. aeruginosa* and *S. aureus*. This paper is the first report on the high degree of antifungal activity of *M. calabura* leaf and stem extracts against *C. albicans*.

Conflict of interest statement

We declare that we have no conflict of interest.

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