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Antioxidant Activity and Minimum Inhibitory Concentration of the Crude Methanolic Extract of *Caesalpinia pulcherrima* (L.) Swartz

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ABSTRACT

This study was conducted to determine the antioxidant and antimicrobial activity of the leaves, flowers and seeds of Caesalpinia pulcherrima L.(Swartz) methanolic extracts. The methanolic extracts were subjected to qualitative phytochemical screening, minimum inhibitory concentration (125, 250, 500 and 1000 µg/mL) and antioxidant activity by 2,2-diphenylpicrylhydrazyl assay (DPPH, 50, 100, 250, 400 and 800 µg/mL). All the extracts showed potent antioxidant activity at 800 µg/mL concentrations comparable to the ascorbic acid standard (p values: leaves=0.088, flowers=0.102, seeds=0.056). For the antimicrobial activity, the leaf extracts showed inhibition against all the selected microorganisms, Staphylococcus aureus, Pseudomonas aureginosa, Escherichia coli, Enterococcus faecalis, Streptococcus mutans, Bacillus subtilis, Aspergillus niger and Candida albicans, at 1000 µg/mL. These activities can be attributed to the phytochemicals present in the extracts including carbohydrates, reducing sugars, flavonoids, phenols, tannins, alkaloids and triterpenoids. In conclusion, all the C. pulcherrima plant parts used possess antioxidant activity while the leaves have potent antimicrobial activity against the selected microorganisms. These results can be used to utilize this plant as a potential source of new antioxidant compounds and antimicrobial agents for several infectious diseases, particularly in endodontic infections where microorganisms such as C. albicans and E. faecalisare dominant pathogens.

Keywords: *Caesalpinia pulcherrima*, minimum inhibitory concentration, DPPH, phytochemical screening

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INTRODUCTION

Plants have been utilized since ancient time for their medicinal and therapeutic values. To establish a concrete claim on their therapeutic use, these plants have become subjects of many research studies under traditional herbal medicine. Among the plants of interest is *Caesalpinia pulcherrima* (L.) Swartz, locally known in the Philippines as "*Bulaklak ng Paraiso*" or Caballero of the family Fabaceae. It is known by a variety of English common names such as peacock flower, dwarf Poinciana, Mexican bird of paradise and pride of Barbados. Considered pantropical after being introduced from tropical America, it is commonly seen in both rural and urban environs in the Philippines. Its propagation is principally by seeds, grows well in all kinds of soil, and is highly drought-resistant but reputed to be intolerant to flooding.

Folkloric medicinal and herbal benefits from different parts of *C. pulcherrima* are known in many parts of the world. The fruits, bark, flowers, fruits, leaves, seeds and stem have been observed to possess antimicrobial, antifungal antiviral, antitumor and cytotoxic properties. This plant has also been reported as an anti-inflammatory and abortifacient agent and used for the treatment of diarrhea, dysentery, bronchial and malarial infections [1]. Likewise, it is utilized for rheumatism, skin infections and ulcer [2], asthma, tumors and skin diseases [3] and as an antipyretic [4]. In the Philippines, its therapeutic use is very limited except for a report in the province of La Union as purgative and emmenagogue [5]. The leaves and flowers of *C. pulcherrima* showed antimicrobial activity against *Bacillus cereus, Micrococcus luteus,* methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* [6]. The extracts from the fresh and dried flowers also displayed strong antibacterial activity against*B. cereus, S. aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli and Enterococcus faecalis* [7]. Extractions from the dried fruits proved to have inhibitory effect on *E. coli, Proteus vulgaris, P. aeruginosa* and *S. aureus,* as well as antifungal activity against *Candida albicans, Aspergillus niger* and *Rhizopus oligosporus* [2]. Similarly, extracts from the flowers and leaves and fresh pods have shown to have antioxidant properties and claimed to be attributable to the phenolic compounds present in the plant [1,6].

The present study focuses on determining the minimum inhibitory concentration of the crude methanolic extracts of the leaves, flowers and seeds of the local variety of*C. pulcherrima* in support to its antimicrobial activity claims. The present study also explores the antioxidant properties of the aforementioned crude plant parts.

MATERIALS AND METHODS

Collection and Authentication of Plant Materials
Flowers, leaves and seeds of *C. pulcherrima* were collected from the province of Batangas, Philippines. A voucher specimen (voucher number: 16-06-578) was submitted to the National Museum for proper authentication.

Crude Methanolic Extraction
The flowers, leaves and seeds of *C. pulcherrima* were washed thoroughly, air-dried, and milled. The powdered plant samples were placed in an Erlenmeyer flask and macerated with methanol separately for 3 days. Intermittent shaking was performed on each flask on the first day and allowed to stand thereafter. The mixtures were filtered and the filtrates were concentrated *in vacuo* at a maximum temperature of 40^{o}C using a rotary evaporator. The concentrates were transferred to an evaporating dish and allowed to evaporate to dryness on a water bath. The crude methanolic extracts were collected, placed on amber bottle containers and stored for future use.

Phytochemical Screening
The methods of Mamta and Jyoti [8] were used with some modifications.

Test for Carbohydrates
Extract was treated with 2 drops of Molisch reagent. The test tube containing the mixture was inclined and concentrated sulfuric acid was added on the side of the test tube. Formation of violet ring at the junction indicated the presence of carbohydrates.

Test for Reducing Sugar
Equal amounts of Fehling's A and Fehling's B were added to the extract. The mixture was boiled and the formation of brick red precipitate indicated the presence of reducing sugars.

Test for Flavonoids
The extracts were treated with few drops of lead acetate solution. The formation of yellow colored precipitate indicated the presence of flavonoids.

Test for Phenols
A fraction of the extract was treated with 5% aqueous ferric chloride. Formation of deep blue or black color indicated the presence of phenols.

Test for Tannins
To the extracts, 1 mL of water was added. A blue to black colored solution indicated the presence of tannins.

Test for Saponins
One mL of the extracts was transferred to the test tubes and was diluted with 20 mL distilled water. The tubes were shaken vigorously by hand for 15 minutes. A foam layer on the top of the solution indicated the presence of saponins.

Test for Alkaloids
One gram of samples was taken in a conical flask and ammonia solution (3 mL) was added. The mixture was allowed to stand for a few minutes and then chloroform was added. The mixture was shaken by hand and then filtered. The chloroform was evaporated from the crude extract by a water bath and Mayer's reagent (3 mL) was added. A cream colored precipitate indicated the presence of alkaloids.

Test for Steroids
The crude plant extracts (1 mg) were taken in a test tube and dissolved with chloroform (10 mL). Concentrated sulfuric acid was then added on the side of the test tube. A red upper layer and yellow with green fluorescence lower layer indicated the presence of steroids.

Test for Triterpenoids
The crude plant extracts (5 mg) were dissolved in chloroform (2 mL) and acetic anhydride (1 mL) was added. Concentrated sulfuric acid was thenadded to the solution. Formation of reddish violet color indicated the presence of triterpenoids.

DPPH Radical Scavenging Assay
The method was adapted from Indrianingsih, *et. al.* [9]. The samples were dissolved in methanol at various concentration (50, 100, 200, 400 and 800 ug/mL) and treated with DPPH (1 mM in methanol). The mixtures were left to stand for 30 minutes at room temperature in the dark. Absorbance was measured at 517 nm usingUV-Vis spectrophotometer. The ability of the samples to scavenge the DDPH radical were calculated using the following equation:

$$\text{DPPH Scavenging Activity (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Minimum Inhibitory Concentration
The procedure was adapted from the broth dilution method of Andrews [10] with some modifications and performed in the Microbiology Laboratory of the College of Public Health, University of the Philippines Manila. Test samples were prepared using 0.5 mL of the extract with a concentration of 125 ug/mL, 250 ug/mL, 500 ug/mL and 1000 ug/mL followed by inoculation with 0.5mL of $1x10^8$ cfu/mL microbial suspension. The test samples were incubated at 37 °C for 24 hours for bacterial species while at room temperature for fungus for 72 hours. The selected microorganisms, namely, *S. aureus, P. aureginosa, E. coli, E. faecalis, S. mutans, B. subtilis, A. niger* and *C. albicans*, were bought from the Philippine National Collection of Microorganisms (PNCM). After the prescribed incubation time, the inhibitory concentrations were determined by comparing the turbidity of the solution with the negative control. Triplicate measurements were performed with extract + broth as negative control and inoculated broth + DMSO as positive control. Dimethyl sulfoxide (DMSO) was used as solvent in dissolving the extract in place of methanol due to the toxic effects of methanol on microorganisms. The negative control was also used to check the sterility of the extract.

STATISTICAL ANALYSIS
Experimental results were analyzed by SPSS 17.0 software. The data represents the mean $\pm$ SD of three parallel measurements. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test. The $p\ values < 0.05$ were regarded to be significant.

RESULTS

Phytochemical Screening
The crude methanolic extracts of *C. pulcherrima* leaves, flowers and seeds were subjected to phytochemical screening and the results are presented in Table 1.

Table 1. Results of the phytochemical analysis of the *C. pulcherrima* crude methanolic extracts

Phytochemicals Tested	Crude Methanolic Test Samples		
	C. pulcherrima Leaves	*C. pulcherrima Flowers*	*C. pulcherrima Seeds*
Carbohydrates	+	+	+
Reducing Sugars	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Tannins	+	-	-
Saponins	-	-	-
Alkaloids	+	+	-
Steroids	-	-	-
Triterpenoids	+	+	-

(+) – presence of phytochemical; (-) – absence of phytochemical

DPPH Radical Scavenging Assay

In the radical scavenging assay, the deep violet color of DPPH lightens or turns yellow upon the reaction with the samples. All the samples exhibited high percentage of radical scavenging activity. At 800 ug/mL, ascorbic acid gave the highest activity followed by *C. pulcherrima* flowers *(p=0.102)*, leaves *(p=0.088)* and then seeds *(p=0.056)*. It is also noteworthy that increasing the concentration also increases the radical scavenging activity for all samples (Table 2).

Table 2. Percentage radical scavenging activity of the *C. pulcherrima* crude methanolic extracts and standard ascorbic acid expressed as mean ± SD

Sample	% Radical Scavenging Activity				
	50 ug/mL	*100 ug/mL*	*200 ug/mL*	*400 ug/mL*	*800 ug/mL*
Ascorbic Acid	95.13 ± 0.09	97.79 ± 0.35	98.13 ± 0.33	98.77 ± 0.24	99.05 ± 0.11
C. pulcherrima leaf extract	87.86 ± 0.08	93.03 ± 0.24	95.25 ± 0.87	95.88 ± 0.53	97.15 ± 0.17*
C. pulcherrima flower extract	86.52 ± 0.82	93.84 ± 1.15	94.93 ± 1.76	95.35 ± 0.22	97.22 ± 0.47*
C. pulcherrima seed extract	49.52 ± 0.44	88.70 ± 0.90	95.49 ± 0.36	96.30 ± 0.44	96.94 ± 0.17*

**Means with asterisk are not statistically significant with ascorbic acid standard (p>0.05).*

Minimum Inhibitory Concentration

Minimum inhibitory concentration was determined in *C. pulcherrima* methanolic extracts at concentrations of 125, 250, 500 and 1000 µg/mL. The leaves and flower extracts exhibited inhibition against *S. aureus*, *P. aureginosa* and *S. mutans* at 1000 µg/mL concentration. On the other hand, the leaves and the seed extracts displayed inhibition on *B. subtilis*, *A. niger* and *C. albicans* growth at 1000 µg/mL concentration. All the selected plant parts (leaves, seeds and flowers) demonstrated inhibition against *E. coli* while only the leaves showed inhibition against *E. faecalis* at 1000 µg/mL concentration. The minimum inhibitory concentration of *C. pulcherrima* crude methanolic plant parts against selected microorganisms are summarized in Table 3.

Table 3. Minimum inhibitory concentration (ug/mL) of the three *C. pulcherrima* crude methanolic extracts against selected microorganisms

Sample	Minimum inhibitory concentration, ug/mL							
	S. aureus	***P. aeruginosa***	***S. mutans***	***B. subtilis***	***A. niger***	***C. albicans***	***E. coli***	***E. faecalis***
C. pulcherrima leaf extract	1000	1000	1000	1000	1000	1000	1000	1000
C. pulcherrima flower extract	1000	1000	1000	NI	NI	NI	1000	NI
C. pulcherrima seed extract	NI	NI	NI	1000	1000	1000	1000	NI

**NI – No inhibition*

DISCUSSION

Antioxidants are tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress [11]. One of the methods used to identify the antioxidant activity of samples is the DPPH radical scavenging assay. It is a rapid, inexpensive and simple method used in most studies of antioxidants [12]. DPPH is a stable nitrogen radical that has been widely used to measure the ability of antioxidants to inhibit pre-formed free radicals [13]. The principle is the neutralization of DPPH radicals whereby antioxidants react with purple-colored solution, free radical DPPH, and by donating hydrogen, it translates to a clear DPPH-H, wherein the degree of discoloration indicates the antioxidant potential [14]. With this method, the determination of antioxidant power is possible by measuring the decrease in absorbance at 517 nm [15].

In the present study, the three methanolic extracts of *C. pulcherrima* exhibited antioxidant activity. The activity was observed to be dose-dependent. The three extracts at 800 µg/mL showed a comparable activity to the reference standard, ascorbic acid ($p > 0.05$). Among the three extracts, the flower has the highest antiradical power followed by leaf and then seed extracts. The antioxidant activity of many phytomedicines is attributed to flavonoid and phenolic compounds. As presented in Table 1, flavonoids and phenols were present in the three extracts of *C. pulcherrima.* In addition, several studies have quantified and reported that flavonoid and phenolic compounds are abundant in *C. pulcherrima* flowers, leaves and seeds [6, 16-17]. The activities of these compounds are mainly due to the redox properties, which allow them to act as reducing agents and hydrogen atom donors [18].

Due to the prevalent antimicrobial resistant cases in the world, it is important to utilize different plants as new sources of antimicrobial agents. In this study, the minimum concentration of methanolic crude extracts of the leaves, flowers and seeds of *C. pulcherrima* required for inhibiting common microorganisms were at 1000 µg/mL concentration. Among the extracts, the leaves inhibited all the selected microorganisms, the gram positive (*S. aureus, S. mutans, B. subtilis, E. faecalis*), gram negative (*P. aureginosa, E. coli*), fungi (*C. albicans*) and molds (*A. niger*). The flower extracts did not inhibit both fungi and molds while the seeds inhibited some bacteria and both fungi and molds. The higher antimicrobial potency of the leaves of *C. pulcherrima* compared to the flower extract is also observed in microorganisms including *S. aureus, Salmonella typhi, C. albicans* and *Cryptococcus neoformans* [16]. Moreover, the leaf extract inhibited both *C. albicans* and *E. faecalis*, microorganisms commonly associated with endodontic infections [19], and thus has the potential to be used in the management of such cases.

As with the antioxidant activity, phenolic compounds, such as tannins and flavonoids, are the possible compounds responsible for the antimicrobial activities of the selected plant parts. These compounds protect the plants from microbial infection by disrupting bacterial cell envelopes, forming complexes with bacterial cell wall, thus, inactivating bacterial adhesins, enzymes and transport proteins [20]. Carbohydrates and reducing sugars are also known antimicrobials that work by disrupting the bacterial cell membrane. This results in the inactivation of bacterial enzymes and transport proteins by entering in the inner membrane. This inhibits cell respiration and growth, ensuing bacterial cell death [21]. Likewise, alkaloids and triterpenoids are potent antimicrobials that work through disruption of bacterial membrane integrity and by inhibiting nucleic acid synthesis and division of bacterial cell [22-23].

CONCLUSION

This study showed the potential of *C. pulcherrima* flower as a potent antioxidant and the leaves as a source of antimicrobial compounds. Further studies for the detection of compounds present in the extracts could lead to new antimicrobial and antioxidant agents. Moreover, these results can be utilized through formulation of different pharmaceutical preparations for various diseases, particularly in dentistry, as the leaf extract showed antimicrobial activity against *S. mutans*, *E. faecalis* and *C. albicans*, microorganisms commonly associated with dental diseases.

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