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Antimicrobial activity, cytotoxicity, and phytochemical screening of *Voacanga globosa* (Blanco) Merr. leaf extract (Apocynaceae)

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ABSTRACT

Objective: To determine the antibacterial, antifungal, antiprotozoal, cytotoxic, and phytochemical properties of ethanol extracts of leaves of *Voacanga globosa* (Blanco) Merr. (*V. globosa*). **Methods:** The extracts were tested against bacteria and fungus through disc diffusion assay; against protozoa through growth curve determination, antiprotozoal and cytotoxicity assays. **Results:** The extract revealed antibacterial activities, inhibiting the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *Salmonella typhimurium*. Antifungal assay showed that it inhibited *Candida albicans*. The antiprotozoal assay against *Trichomonas vaginalis* and *Entamoeba histolytica* showed that *V. globosa* can inhibit the parasites, wherein the action can be comparable to metronidazole. With the *in situ* cell death detection kit, *Trichomonas vaginalis* and *Entamoeba histolytica* exposed to *V. globosa* leaf extract was observed to fluoresce simultaneously in red and yellow signals signifying apoptotic-like changes. Preliminary phytochemical screening revealed the chemical composition of plant extract containing alkaloids, saponins, 2-deoxysugars, and hydrolysable tannins. **Conclusions:** Thus, this study provides scientific evidence on the traditional use of *V. globosa* leaf extract in treating microbial diseases. Further, the leaf extract can possibly be used to produce alternative forms of antimicrobials.

1. Introduction

The importance of a country's diverse medicinal plants lies not only in their chemotherapeutic value in traditional medicine but also in their potential as sources of new chemical entities for drug discovery. Although the Philippines boasts of its biodiversity and rich cultural traditions of plant use, scientific understanding of medicinal plants remains largely unexplored and pharmacological investigation of the Philippine flora only gained momentum recently. For instance, Vital and Rivera characterized the antibacterial, antifungal, antiprotozoal properties of *Chromolaena odorata* and *Uncaria perrottetii* in the Philippines[1].

There is a continuous and urgent need to discover plants with antimicrobial activities with these diverse chemical structures and novel mechanisms of actions. The wide acceptance of traditional medicine as an alternative form of healthcare and the alarming increase in the incidence of new and re-emerging infectious diseases bring about the necessity to investigate these medicinal plants. Moreover, since many plants are unexamined, therapeutic results have been mixed resulting to poisoning[2]. Another concern is the development of resistance to the antibiotics in current clinical use[3].

Plant extracts have great potential as antimicrobial compound against microorganisms[4]. The medicinal value of plants lies in the bioactive compounds such as alkaloids, flavonoids, tannins, and phenolic compounds that produce a definite physiological action on the human body. The increasing use of plant extracts in the food, cosmetic, and pharmacological industries suggests that in order to extract active compounds, a systematic study of medicinal plants

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is very important[2]. Thus, this study aimed to evaluate the potential antimicrobial activities of *Voacanga globosa* (*V. globosa*), which is traditionally used in the Philippines. *V. globosa* (Blanco) Merr., known as "bayag-usa" in Filipino, belongs to Family Apocynaceae. Traditionally, it is used to treat cancer and tuberculosis which may be due to its alkaloid content, tabernaemontanine[5]. Specifically, this study determined the antibacterial, antifungal, antiprotozoal, and apoptotic effects of the plant extracts through agar diffusion assay, growth curve analysis, antiprotozoal assay, and cytotoxicity assay. This study provides scientific proof on the use of these plants which are being utilized traditionally as herbal medicines.

2. Materials and methods

2.1. Selection and collection of plant material

Leaves of *V. globosa* were collected from Bataan, Philippines and identified by the Dr. Jose Vera Santos Memorial Herbarium (Philippine University Herbarium) of the Institute of Biology, University of the Philippines. Voucher specimen of the herb was prepared and deposited at the Institute of Biology.

2.2. Preparation of extracts

Leaves of the plant were air dried for several days. The dried plant materials were ground to a coarse powder using a dry mill. For the extraction procedure, 200-500 g of powdered plant material was soaked in 95% ethanol (1:5) for 72 hours[6]. The solvent was then removed by rotary evaporation. The dried extract was stored inside the refrigerator.

2.3. Organisms and culture media

Microorganisms were obtained from the culture collections of the Microbiological Research and Services Laboratory and Molecular Protozoology Laboratory of the Natural Sciences Research Institute at the University of the Philippines Diliman. Organisms used were as follows: bacteria: *Escherichia coli* ATCC 25922 (*E. coli*), *Salmonella typhimurium* UPCC 1368 (*S. typhimurium*), *Pseudomonas aeruginosa* UPCC 1244 (*P. aeruginosa*), *Staphylococcus aureus* ATCC 6538 (*S. aureus*), *Bacillus cereus* UPCC 1281 (*B. cereus*), *Micrococcus luteus* ATCC 9341 (*M. luteus*); fungus: *Candida albicans* 2168 (*C. albicans*); protozoa: *Entamoeba histolytica* HK9 (*E. histolytica*) and *Trichomonas vaginalis* Tv1:MCP (*T. vaginalis*). Bacterial cultures were maintained on nutrient agar (NA), while *C. albicans* was maintained on Sabouraud dextrose agar (SDA). *E. histolytica* and *T. vaginalis* were grown in BI-S-33 medium[7].

2.4. Antibacterial and antifungal activity of the plant extracts

Disc diffusion assay on agar plates were used to determine the antibacterial and antifungal activities of *V. globosa* extract. Bacteria were inoculated into nutrient broth (NB), while fungus was inoculated into Sabouraud dextrose broth (SDB) at 37 ℃ for 6 hours. The turbidity of the resulting suspensions was diluted with NB and SDB to obtain a transmittance of 74.3% (absorbance of 0.132) at 600 nm. The percentage is found spectrophotometrically comparable to 0.5 McFarland turbidity standard. This level of turbidity is equivalent to approximately 1.5×10^8 CFU/mL[8]. These bacterial cultures were then inoculated on the surface of Mueller-Hinton agar (MHA) plates for bacteria and SDA for fungus. Subsequently, filter paper discs (6 mm in diameter) saturated with extracts (25 μL) were placed on the surface of each inoculated plate. Antibiotics were used as positive control (penicillin and chloramphenicol for bacteria, while nystatin for fungus), while solvent (95% ethanol) of the plant extracts as negative control[9]. The tests were carried out in triplicates. The plates were incubated at 37 ℃ for 24 hours. At the end of incubation, zones of inhibition were measured with a transparent ruler. Zones of clearing greater than 6 mm were considered susceptible to the extracts.

2.5. Antiprotozoal activity of the plant extracts

2.5.1. Growth curve analysis

Growth curves were constructed for *E. histolytica* and *T. vaginalis*. Upon reaching 80% confluence, trophozoites were detached by mixing. Trophozoites (1.5×10^5/mL) were screened in tubes with BI-S-33 medium and incubated at 37 ℃ for 170 hours[7]. Every 24 hours, the trophozoites were detached and counted to obtain the curve of growth for each time. Generation time was computed during the logarithmic phase of the parasite.

2.5.2. Antiprotozoal assay

In this assay, 1.5×10^5/mL parasites were grown in BI-S-33 medium and exposed to 0.1% *V. globosa* leaf extract to study the viability of the parasites exposed to the plant extracts for 24, 48, and 72 hours. Only three periods of time were considered for the experiment (24, 48, and 72 hours) of the parasite exposure to the plant extracts based on the growth curve constructed. Afterwards, the parasites were detached and counted in a Neubauer counting chamber and the counts were compared with those of the positive (metronidazole) and negative (95% ethanol) control[1]. Each assay was performed in triplicate.

2.5.3. Detection of apoptosis (cytotoxicity assay)

E. histolytica and *T. vaginalis* trophozoites were observed to determine the presence of apoptosis by a Tunel method. To observe apoptotic-like changes, the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics)

was used. This method allows the recognition of apoptotic nuclei in protozoal preparations by labeling the free 3′-OH termini with modified nucleotides in an enzymatic reaction (fragment end labeling). Terminal deoxynucleotidyl transferase (TdT) binds to these exposed 3′-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the polymerization of labeled nucleotides in a template-dependent manner. The trophozoites were obtained by centrifugation. They were washed, fixed and permeabilised prior to the labeling protocol.

Fluorescein labels incorporated in the nucleotide polymers were detected by fluorescence microscopy using Olympus IX51. Viable cells were stained in green by a fluorescein derivative. The apoptotic cells exhibited reddish and yellow-green fluorescence and necrotic cells were stained only in red.

2.6. Phytochemical screening

The plant extracts were submitted to the Chemical and Energy Division of the Department of Science and Technology (DOST) for chemical analysis to identify and characterize some of their composition. The tests done followed the procedure in the Laboratory Manual for the UNESCO Sponsored Workshop on the Phytochemical, Microbiological, and Pharmacological Screening of Medicinal Plants[10].

3. Results

3.1. Antibacterial and antifungal activity of V. globosa leaf extract

V. globosa leaves were subjected to ethanol and rotary evaporation to examine the antimicrobial activities. Table 1 summarized the antibacterial and antifungal activities of *V. globosa* leaf extract. The leaf extract inhibited almost all bacteria and the fungus. Inhibition of the positive control, chloramphenicol, was comparable to that of the plant extract. Further, the plant extract inhibited other microorganisms which were not inhibited by penicillin. The solvent used as negative control exerted no effect against the microorganisms which suggest the effectiveness of the plant extracts (Table 1).

3.2. Antiprotozoal activity of V. globosa leaf extract

T. vaginalis is a flagellated organism that is the most common cause of non-viral sexually transmitted infection, trichomoniasis[11]. In this study, a growth curve of *T. vaginalis* was constructed and analyzed to be able to determine the specific time when the plant extracts will be added. Based on the growth curve, the maximal growth was achieved after 72 hours of incubation that corresponded to 1.0×10^6 cells/mL. Results showed that *V. globosa* can inhibit *T. vaginalis* after 24 hours of exposure (Figure 1). The leaf extract exhibited an antiprotozoal activity comparable to that of the positive control, metronidazole.

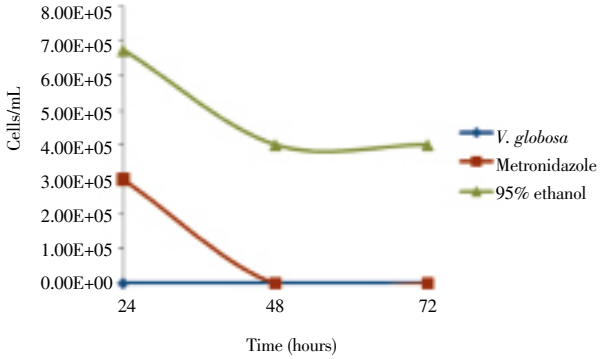

Figure 1. Effect of *V. globosa* leaf extract on *T. vaginalis* trophozoites growth *in vitro*.
Trophozoites were grown in BI-S-33 medium[7] containing plant extracts and incubated at 37 ℃ for 72 hours. Every 24 hours the trophozoites were counted in a Neubauer chamber. Each point represents the mean of triplicate determinations.

E. histolytica, on the other hand, is a common pathogenic protozoan transmitted to people via contaminated water and occasionally through food-borne route. With the growth curve analysis that was done, the maximum number of parasites was observed after 96 hours of incubation. This corresponded to a concentration of 1.0×10^6 cells/mL. After the growth curve was constructed for *E. histolytica*, the extract was also evaluated for the antiprotozoal activities. *V. globosa* leaf extract effectively inhibited the growth of the parasites (Figure 2). This result was also comparable to the effect of metronidazole, the positive control used.

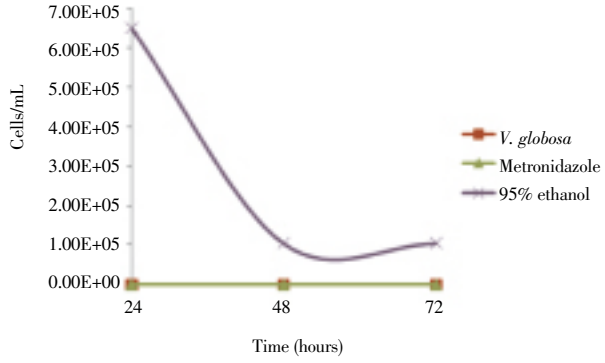

Figure 2. Effect of *V. globosa* leaf extract on *E. histolytica* trophozoites growth *in vitro*.
Trophozoites were grown in BI-S-33 medium[7] containing plant extracts and incubated at 37℃ for 72 hours. Every 24 hours the trophozoites were counted in a Neubauer chamber. Each point represents the mean of triplicate determinations.

The possibility of the solvent, ethanol, causing this observed effect was excluded since growth continued in the culture inoculated with the solvent. On the other hand,

metronidazole is a drug of choice recommended for the treatment of human trichomoniasis and amebiasis. However, potential carcinogenic, teratogenic, embryogenic effects of this drug and clinical and laboratory-based drug-resistant protozoan isolates have been reported[12].

3.3. Detection of apoptosis (cytotoxic activity of V. globosa leaf extract)

Apoptosis or programmed cell death is the most common form of eukaryotic cell death. With the kit that was used, necrotic cells fluoresce in red color, living cells fluoresce in green and apoptotic cells fluoresce in yellow green and red simultaneously[13]. *V. globosa* extract induced apoptotic-like changes to *T. vaginalis* and *E. histolytica* trophozoites after 24 hours of exposure (Figure 3a). Cells in all cases showed a clear loss of normal morphology. On the other hand, the negative control (with 95% ethanol) gave a green color (Figure 3b). This signified the presence of viable cells.

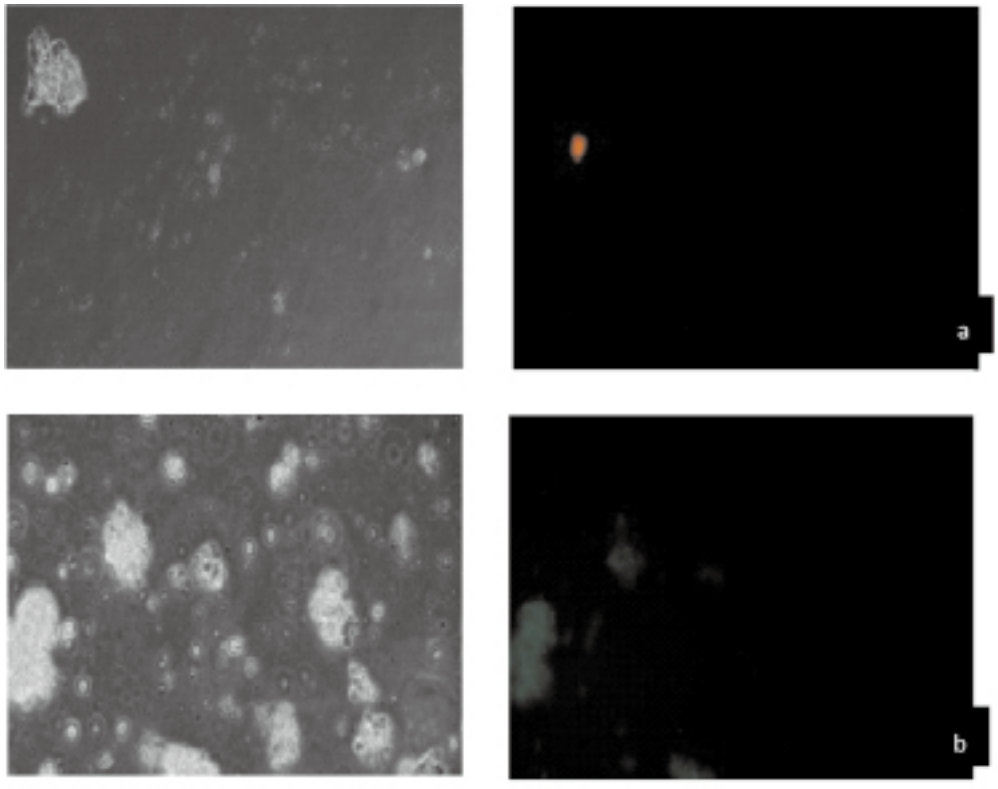

Figure 3. Apoptosis detected by Tunel method exposed to: 0.1% concentration of *V. globosa* extract against *E. histolytica* trophozoites showing yellow green and red fluorescence (a); negative control (95% ethanol) against *V. globosa* extract showing green fluorescence only (b). Photographs on the left panel are under light microscope (200×), while photographs on the right are under fluorescence microscope (200×).

Table 1
Antimicrobial activity of *V. globosa* leaf extract, positive and negative control determined by disc diffusion assay.

Test organisms	Zone of inhibition(mm)			
	V. globosa(0.1%)	Penicillin(10 μg)	Chloramphenicol(10 μg)	Nystatin
P. aeruginosa	10.7 ± 1.5	–	17.3 ± 2.5	nd
E. coli	–	–	19.3 ± 0.6	nd
B. cereus	12.7 ± 1.2	–	28.3 ± 1.2	nd
S. aureus	13.3 ± 1.2	17.0 ± 5.0	26.0 ± 2.6	nd
M. luteus	25.0 ± 0.0	25.0 ± 0.0	11.0 ± 2.6	nd
S. typhimurium	10.0 ± 1.7	–	24.0 ± 8.7	nd
C. albicans	7.0 ± 0.0	nd	nd	25.0 ± 3.6

(–): no activity; nd: not determined; values: mean of 3 replicates and expressed as mean ± SD

3.4. Phytochemical screening of V. globosa leaf extract

V. globosa leaf extract was found to contain alkaloids, saponins, 2-deoxysugars, and hydrolysable tannins. The absence of leucoanthocyanin, benzopyrone nucleus, free fatty acids, steroids, and triterpenes were also evident in the phytochemical screening.

4. Discussion

Few studies have been done on the antibacterial and antifungal properties of *V. globosa*. Due to the reported development of resistance by bacteria and fungi to various commercially available antimicrobial agents, the leaf extract of plants are potential sources of new compounds which may be developed as effective drugs against microorganisms. The use of penicillin is no longer recommended due to the potency of widespread resistance to it[3]. Further, the use of this plant may offer a new source of antifungal agent against the pathogenic *C. albicans* since this fungus is not easily inhibited by other drugs.

Antiprotozoal activity is rarely done in antimicrobial experiments. In this study, the leaf extract was tested against *T. vaginalis* and *E. histolytica*. The antiprotozoal assay revealed that the parasites were inhibited by the extract. However, the exact mechanism of inhibition is unknown, thus, cytotoxicity assay was performed. Apoptosis induced by antiparasitic drugs has also been barely studied in protozoan parasites[13]. In the method that was used, the effects are attributable to the plant extract since the kit preferentially and specifically labels DNA strand breaks generated during apoptosis. It allows the discrimination of apoptosis from necrosis and from primary DNA strand breaks induced by cytostatic drugs or irradiation. These effects might be consequences of the activation of apoptotic mechanisms that may be exclusive for microorganisms lacking mitochondria[13]. Thus, the assays performed in *V. globosa* leaf extract play an essential role in discovering various capabilities of the plant which are seldom investigated. Moreover, this discovery offers great possibilities in the discovery of new drugs.

The chemical compounds found in the leaf extracts through phytochemical screening such as alkaloids, as evidenced in this study, may bring about its antibacterial, antifungal, antiprotozoal, and cytotoxic properties. The few studies on this plant available were mainly on the chemical characterization of the plant extract[5].

Thus, all the plant extracts can inhibit gram positive and gram negative bacteria, fungus, and protozoa such as *T. vaginalis* and *E. histolytica* trophozoites. Moreover, the extracts can also induce apoptotic-like changes. This study provides scientific evidence on the traditional medicinal use of *V. globosa* in the Philippines.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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