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Full Length Research Paper

Antimicrobial activity, cytotoxicity and phytochemical screening of *Ficus septica* Burm and *Sterculia foetida* L. leaf extracts

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Ethanol extracts of leaves of *Ficus septica* Burm and *Sterculia foetida* L. were examined for their antibacterial, antifungal, antiprotozoal, and cytotoxic properties. To determine these activities, the extracts were tested against bacteria and fungus through disc diffusion assay; against protozoa through growth curve determination, antiprotozoal and cytotoxicity assays. The extracts revealed antibacterial activities, inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli*. Antifungal assay for *F. septica* extract showed that it inhibited *Candida albicans*. The antiprotozoal assay against *Trichomonas vaginalis* showed that *F. septica* can reduce the number of parasites. Moreover, antiprotozoal assays against *Entamoeba histolytica* revealed that *F. septica* and *S. foetida* can inhibit the growth of the parasites, wherein the action can be comparable to metronidazole. With the *in situ* cell death detection kit, *T. vaginalis* exposed to *F. septica* and *E. histolytica* exposed to *F. septica* and *S. foetida* were observed to fluoresce in red surrounded by a yellow signal signifying apoptotic-like changes. Preliminary phytochemical screening revealed the chemical composition of *F. septica* extracts containing alkaloids, quaternary base, tannins, 2-deoxysugars, and benzopyrone nucleus, while *S. foetida* possessing tannins, 2-deoxysugars, leucoanthocyanin, and benzopyrone nucleus. Thus, these plant extracts can possibly be used to produce alternative forms of antimicrobials.

Key words: Leaf extract, antibacterial, antifungal, antiprotozoal, cytotoxic, phytochemical screening, *Ficus septica*, *Sterculia foetida*.

INTRODUCTION

Medicinal plants are natural sources of compounds that can be used against many diseases today (Kubmarawa et al., 2007). Since a variety of plants grow in every conceivable place, having access to them would require only previous knowledge of their location and certain unique characteristics, such as a plant's habit of growth. As such, plants can be obtained easily. This aspect would be vital in discovering medicinal plants with high biological activity, low toxicity and which are acquired at a low price (Calzada et al., 2007). Various studies have been done which utilized plants in investigating possible antimicrobial drugs and in discovering the different medicinal properties of plants, although, these studies are not enough to cover the world's biodiversity and the traditional use of medicinal plants.

The purpose of this study was to discover the therapeutic ability of some plants found in the Philippines with an end goal of providing cheaper nature-based alternative medicine to the public in the midst of high-priced medicine produced by pharmaceutical companies. The public faces major threats from various pathogenic organisms. A study by Zheng and Wu (2007) proved that

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most people in developing countries resort to local traditional medicine due to lack of doctors in their communities and their financial incapability in purchasing market-based medicine. Hence, extensive research on the use of cheaper plant-based therapy is imperative nowadays. Through this study, the importance of the plant biodiversity in the country was also highlighted as a result of the dependence on them for medicinal purposes.

Ficus septica (Family Moraceae) and *Sterculia foetida* L. (Family: Sterculiaceae) are plants with possible medicinal uses found in the Philippines. In this study, various pathogenic organisms of public importance were tested against leaf extracts of these plants. To assess the efficacy of these plants, disc diffusion assay was performed on the bacterial and fungal test organisms, while growth curve analysis, antiprotozoal and cytotoxicity assays were done on the protozoan parasites. Antiprotozoal and cytotoxicity assays are rarely done in antimicrobial experiments, thus, this study provided information on the activity of the plant extracts against a broad range of microorganisms. Furthermore, the plant extracts were subjected to preliminary phytochemical screening to analyze the possible antimicrobial compounds they contain. The study provides scientific evidence on the possible use of these plants to produce alternative forms of medicine.

MATERIALS AND METHODS

Collection and identification of plant materials

Ficus septica and *Sterculia foetida* were gathered from the University of the Philippines, Diliman, Quezon City, Philippines. The plants were identified by the Dr. Jose Vera Santos Memorial Herbarium (Philippine University Herbarium) at the Institute of Biology, University of the Philippines. Voucher specimens of the herbs were prepared and deposited at the Institute of Biology. Prior consent was obtained and authorized by the corresponding agencies of the government. The fieldwork and data collection were conducted in accordance with the institutional, national, and international principles and guidelines of plant use and conservation of biodiversity.

Preparation of plant extracts

Plant samples were air-dried and ground to a coarse powder using a dry mill. Then, the powdered leaf was soaked in 95% ethanol (1:5) for 72 hours. The solvent was removed under vacuum using a rotary evaporator (Vital and Rivera, 2009).

Microorganisms and culture media

Microorganisms were obtained from the culture collections of the Microbiological Research and Services Laboratory and the Molecular Protozoology Laboratory of the Natural Sciences Research Institute at the University of the Philippines-Diliman. Organisms were as follows: bacteria: *Escherichia coli* UPCC 1195, *Pseudomonas aeruginosa* UPCC 1244, *Staphylococcus aureus* UPCC 1143, *Bacillus cereus* UPCC 1281; fungus: *Candida albicans* UPCC 2168. Bacterial cultures were maintained on nutrient agar (NA). *C. albicans* was maintained on Sabouraud dextrose agar (SDA). Protozoans used in the study were *Trichomonas vaginalis* DSHC 2021 and *Entamoeba histolytica* HK-9. T. vaginalis and E. histolytica were grown in BI-S-33 medium (Diamond et al., 1978).

Antibacterial and antifungal activities of the plant extracts

Disc diffusion assay on agar plates were used to determine the antibacterial and antifungal activities of the extracts. Bacteria were inoculated into nutrient broth (NB), while fungi were inoculated into Sabouraud dextrose broth (SDB) at 37^{o}C 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 (Rojas et al., 2006). Then, these bacterial cultures were inoculated on the surface of Mueller-Hinton agar (MHA) plates for bacteria and SDA for fungi. Subsequently, filter paper discs (6 mm in diameter) saturated with extracts (25 µl) dissolved in water were placed on the surface of each inoculated plate. Antibiotics were used as positive control (ampicillin and gentamicin for bacteria, while nystatin for fungi), while solvent (95% ethanol) of the plant extracts as negative control. The plates were incubated at 37°C for 24 h. At the end of incubation, zones of inhibition were measured. All tests were done in triplicates.

Antiprotozoal activity of the plant extracts

To analyze the antiprotozoal activity of the plant extracts, growth curve determination and the antiprotozoal assay were performed. The protocol for this assay was patterned from that of Moon et al. (2006) and Perez-Arriaga et al. (2006). First, growth curves of *E. histolytica* and *T. vaginalis* were constructed by diluting them in BI-S-33 medium to give a final count of 1 x 10^{6} cells/mL. Then, the cultures were incubated at 37°C for 120 h (T. vaginalis) and 35.5°C for 312 hours (*E. histolytica*). Every 24 h, the cells were detached and counted to obtain the growth curve for each time. In the antiprotozoal assay, the same concentration of cells were grown in the same culture medium and exposed to 10% concentration of the plant extracts. 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 (Fournet et al., 1994). Each assay was performed in triplicate.

Detection of apoptosis (Cytotoxicity assay)

T. vaginalis and *E. histolytica* 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 *T. vaginalis* and *B. hominis* preparations by labeling the free 3'-OH termini with modified nucleotides in an enzymatic reaction (fragment end labeling). Fluorescein labels incorporated in the nucleotide polymers were detected by fluorescence microscopy. Viable cells were stained in yellow-green by a fluorescein derivative. The apoptotic cells exhibited reddish and yellow-green fluorescence and necrotic cells were stained only in red.

Data analysis

All assays were done in triplicates. Values obtained were expressed as means ± standard deviation.

Table 1. Antimicrobial activity (mm) of the plant extracts, positive and negative controls on bacteria and fungus determined by disc diffusion assay.

Test extracts and control	Zone of inhibition (mm)				
	E. coli	***B. cereus***	***S. aureus***	***P. aeruginosa***	***C. albicans***
S. foetida	19.50 ± 1.80	-	19.00 ± 2.00	-	-
F. septica	13.00 ± 1.00	-	13.83 ± 4.01	-	17.67 ± 1.53
Ampicillin	16.83 ± 6.93	-	16.50 ± 3.04	-	nd
Gentamicin	33.17 ± 1.04	20.33 ± 3.88	32.00 ± 1.73	20.00 ± 0.00	nd
Ethanol	-	-	-	-	-
Nystatin	nd	nd	nd	nd	14.33 ± 0.58

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

Phytochemical screening

The plant extracts were submitted to the Chemical and Mineral 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 (1986).

RESULTS AND DISCUSSION

Antibacterial and antifungal activities of the plant extracts

F. septica and *S. foetida* showed varying levels of antibacterial properties (Table 1). All test plants were able to inhibit two bacteria under study, namely, *E. coli* and *S. aureus.* On the other hand, *B. cereus* and *P. aeruginosa* were not inhibited by the plant extracts. Of the plants tested, *S. foetida* induced the highest zone of inhibition, with computed microbial indices of 1.44 and 1.38 for *E. coli* and S*. aureus*, respectively. For *F. septica*, the microbial index computed for *E. coli* is 0.625, while for *S. aureus*; the computed microbial index is 0.729. The inhibition of the positive control, ampicillin, was comparable to those of the plant extracts. The solvent used as negative control exerted no effect against the microorganisms which suggest the effectiveness of the plant extracts (Table 1).

In the evaluation of the antifungal property of the plant extracts, only *F. septica* extract greatly inhibited *C. albicans* (Table 1). The computed microbial index for *C. albicans* is 1.208. C. albicans is an opportunistic yeast that can cause vaginal, oral, and lung infections. 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. There was no inhibition observed in the other plant extract which suggest that higher concentrations of the extract or other parts of the plants may be used. Few studies have been done on the antibacterial and antifungal properties of these plants. In fact, a study was performed on other species of *Ficus* that tested them against bacteria and fungi (Mandal et al., 2000; Nair and Chanda, 2006; Kubmarawa et al., 2007). Moreover, the use of S. *foetida* as an antimicrobial agent is still unexplored in scientific research. This study pioneered research work regarding the antimicrobial properties of these plant extracts. Due to the reported development of resistance by bacteria and fungi to various commercially available antimicrobial agents, the leaf extracts of these plants are potential sources of new compounds which may be developed as effective drugs against microorganisms if specific chemical components can be isolated and purified. The use of ampicillin is no longer recommended due to the potency of widespread resistance to it (Ertürk et al., 2006).

Growth curve analysis and antiprotozoal assay

T. vaginalis is a flagellated organism that is the most common cause of non-viral sexually transmitted infection, trichomoniasis (Rein and Müller, 1990). 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. It can be observed that the maximal growth was achieved after 72 h of incubation that corresponded to 1.75×10^6 cells/mL. Based on the growth curve constructed, the antiprotozoal assay was done. Results showed that *F. septica* was the extract with the most pronounced effect on *T. vaginalis* (Figure 1). The leaf extract exhibited an antimicrobial activity (comparable to that of the positive control, metronidazole.

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 h of incubation. This corresponded to a concentration of 1.2×10^6 cells/mL. After the growth curve was constructed for *E. histolytica*, the extracts were evaluated for their antiprotozoal activities. *F. septica* and *S. foetida* leaf extracts effectively inhibited the growth of the parasites (Figures 2 and 3). This result was comparable to the effect of metronidazole, the positive control used.

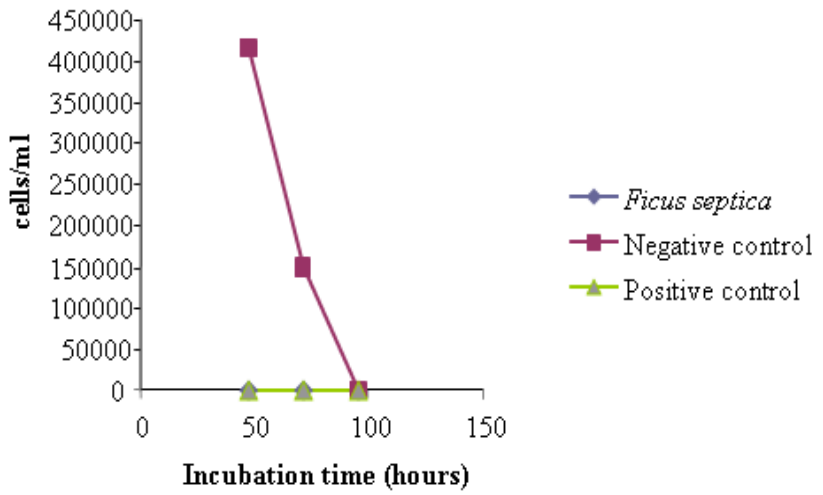

Figure 1. Growth of *T. vaginalis* when exposed to *F. septica* leaf extracts, positive and negative controls. Cells were grown in BI-S-33 culture medium (Diamond et al., 1978) and incubated at 37°C. Counting was done during the 48th, 72nd and 96th hour of incubation. *F. septica* and the positive control, metronidazole, had the same growth inhibition activity, thus, showing the same line of growth.

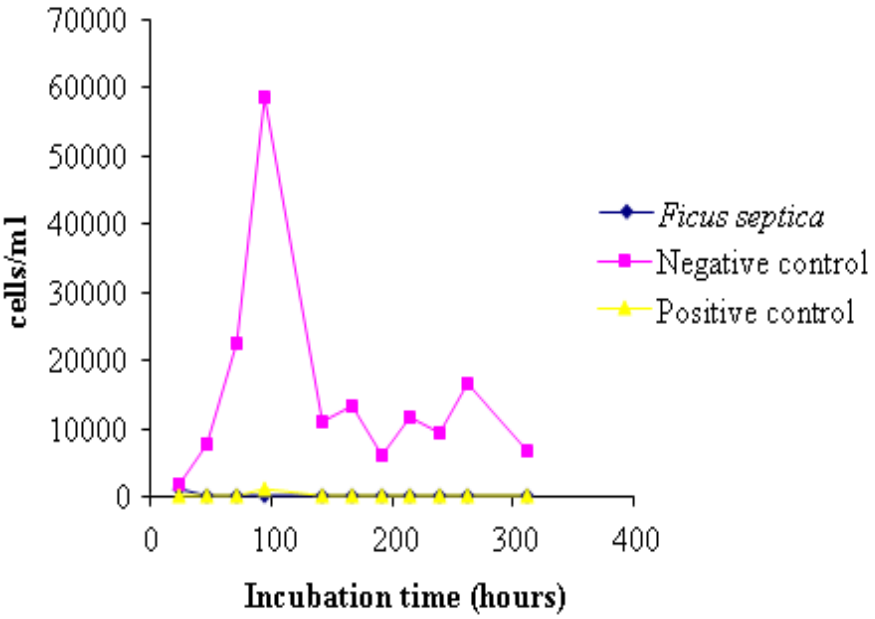

Figure 2. Growth of *E. histolytica* when exposed to *F. septica* leaf extracts, positive and negative controls. Cells were grown in BI-S-33 culture medium (Diamond et al., 1978) and incubated at 35.5°C. Counting was done from the 24th hour until the 312th h of incubation. *F. septica* and the positive control, metronidazole, had a similar growth inhibition activity, thus, showing the same line of growth

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, for the positive control, metronidazole lysed and killed the cells after 24 h of incubation. Metronidazole is a drug of choice recommended for the treatment of human trichomoniasis. However, potential carcinogenic, teratogenic, embryogenic effects of this drug and clinical and laboratory-based drug-resistant protozoan isolates have been reported (Calzada et al., 2007).

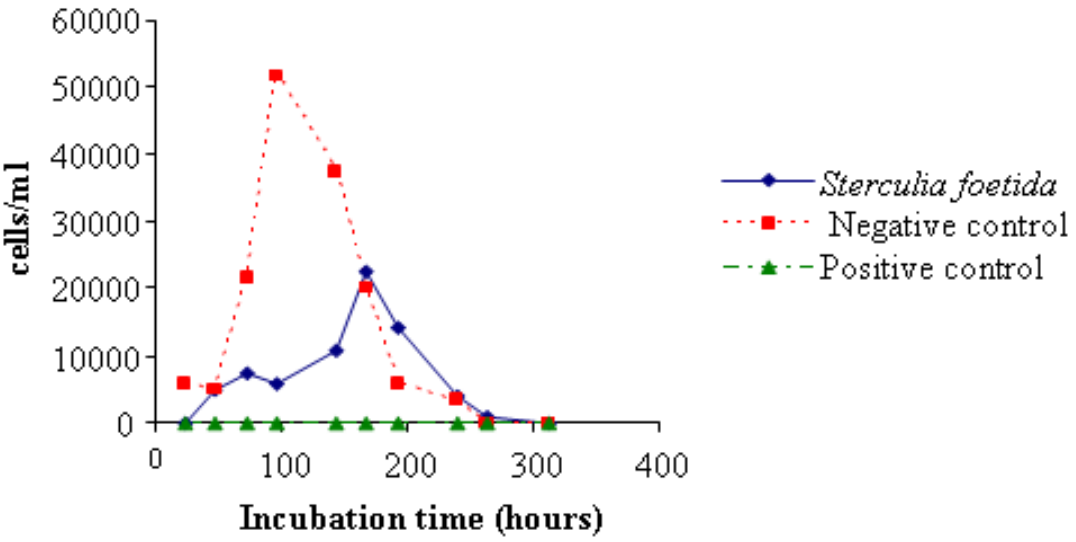

Figure 3. Growth of *E. histolytica* when exposed to *S. foetida* leaf extracts, positive and negative controls. Cells were grown in BI-S-33 culture medium (Diamond et al., 1978) and incubated at 35.5°C. Counting was done from the 24th hour until the 312th h of incubation.

Detection of apoptosis (Cytotoxicity assay)

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 yellow green and apoptotic cells fluoresce in yellow green and red simultaneously (Perez-Arriaga et al., 2006). *F. septica* extract induced apoptotic-like changes to *T. vaginalis* trophozoites after 72 h exposure. Moreover, red surrounded by yellow signals were also observed in *F. septica* and *S. foetida* against *E. histolytica* (Figure 4). Cells in all cases showed a clear loss of normal morphology. On the other hand, the negative control (with ethanol) and the control without any extract or solvent gave a green color. This signified the presence of viable cells.

Apoptosis induced by antiparasitic drugs has been barely studied in protozoan parasites (Perez-Arriaga et al., 2006). In the method that was used, the effects are attributable to the plant extracts 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 (Perez-Arriaga et al., 2006). Antiprotozoal and cytotoxicity assays are rarely done in antimicrobial studies. Thus, the assays performed in these two plants play an essential role in discovering various capabilities of these plants which are seldom investigated. Moreover, this discovery offers great possibilities in the discovery of new drugs.

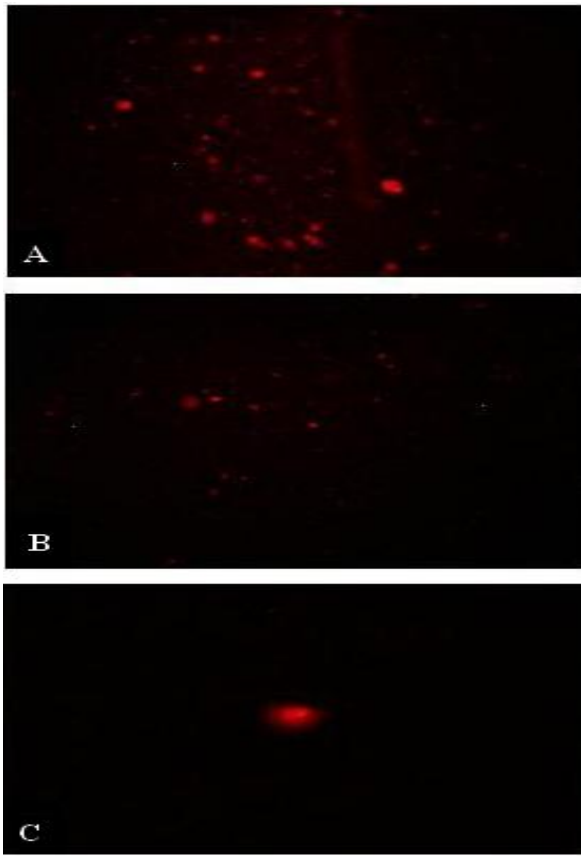

Figure 4. Apoptosis detected by Tunel method exposed to plant extracts: *F. septica* against *E. histolytica* (A); *F. septica* against *T. vaginalis* (B); and *S. foetida* against *E. histolytica* (C) showing red fluorescence. Photographs are under fluorescence microscope (400x).

Phytochemical screening

Chemical tests showed the presence of alkaloids, quaternary base, tannins, 2-deoxysugars, and benzopyrone nucleus in *F. septica* extract. A study performed by Damu et al. (2005) found eight alkaloids from the methanol extracts of the stems of *F. septica*. Another experiment was the extraction of phenanthroindolizidine alkaloid and antofine from *F. septica* leaves (Baumgartner et al., 1990). On the other hand, chemical analysis of *S. foetida* leaf extract revealed the presence of tannins, 2-deoxysugars, leucoanthocyanin, and benzopyrone nucleus. An investigation of another plant part, the roots, resulted to the presence of lupeol, n-triacontanol, beta-sitosterol, stigmasterol and beta-sitosterol-3-O-Beta-D-glucopyranoside. The seeds contain sterculic acid triglyceride. This triglyceride was known to have effects on proliferation of smooth muscles in rabbits (Mujumdar et al., 2000). The few studies available were mainly on the chemical characterization of the plant extracts. The chemical compounds found in the leaf extracts of these plants, as evidenced in this study, may bring about their antibacterial, antifungal, antiprotozoal, and cytotoxic properties.

Thus, all the plant extracts can inhibit Gram positive and Gram negative bacteria and can reduce the number of *T. vaginalis* and *E. histolytica*. Moreover, the extracts can also induce apoptotic-like changes. *F. septica* can inhibit the growth of almost all microorganisms tested, making it a more potent plant extract than *S. foetida*. These plants are potential substitutes for drugs being used today such as, ampicillin, which has been known to be evaded by resistant bacteria. The knowledge that these plants exhibit vital antimicrobial properties in selected organisms offers a lot to the research world in the search for more drugs that are easily accessible, widely affordable, and highly effective.

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