Phytochemical and Antimicrobial Analysis of "Lupo" (*Alternantherasessilis* L.R.BR.)

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Abstract

In this study, the presence of selected phytochemicals from the aqueous, acetone, and ethanolic extracts was screened and the antimicrobial and antifungal potentials of the expressed juice of "Lupo" (*Alternanthera sessilis*) may be possible nutritional and medicinal values. The extracts were subjected to the tests for Alkaloids,Steroids, Anthraquinones, Flavonoids, and Tannins, using standard procedures in Guevara's, *A Guidebook to Plant Screening: Phytochemical and Biological,2005*. The Kirby-Bauer technique of minimum antibacterial concentration and minimum inhibitory concentration (MIC) were used in the assay. Results show that alkaloids, flavonoids, steroids, and anthraquinones, are present in all extracts of *Alternanthera sessilis*, while tannins is only in the aqueous extract. *Alternanthera sessilis* when extracted using water contains all the five phytochemicals tested, and is beneficial when mixed in food products, because it can supply alkaloids and steroids that exhibit dramatic physiological effects on the heart muscles. It can also supply natural cathartics or purgatives due to the presence of anthraquinones. Flavonoids present have antiviral, anti-fungal, anti-inflammatory and cytotoxic activities. Tannins present in aqueous extract have potential value as cytotoxic and/or antineoplastic agents, and as astringents. Results of antimicrobial and antifungal analyses show that *Alternanthera sessilis*(*Lupo*) expressed juice possess an antibacterial activity exhibiting an MIC of < 100µg/mL against *Escherichia coli* referenced strain used. There is low MIC of 252.5µg/mL against *Staphylococcus aureus* and *Candida albicans.*

Keywords: Phytochemical, Antimicrobial, Antifungal, *Alternanthera sessilis*

Greens encompass many medicinal and phytochemical effects, which are very useful in treating and preventing many diseases especially bioactive functional compounds like alkaloids, flavonoids, tannins and phenolic compounds (Sudha & Mathangi, 2013).

One of the plants that is widely used for its various nutritional and medicinal values is A*lternantherasessilis* - an aquatic plant known by several common names, including sessile joyweed and dwarf copperleaf (Hossain, Faisal, Rahman, Jahan, & Rahmatullah, 2014). In the Philippines, particularly in Iloilo it is commonly called "Lupo" that commonly grows in marshy areas and wetlands. Locally, it is being used as vegetable and is claimed by folks to lower sugar and cholesterol levels and blood pressure. Despite its abundance and medicinal values, very few consume it due to its unacceptable taste.

"Food as Medicine" is one of the basic concepts of traditional Siddha Indian Medicine. Because the greens possess medicinal properties, Tamil culture usually include greens in household recipes, *Alternantherasessilis* becomes part of the regular diet of South Indians. It is usually eaten because it can give the body a "golden luster" from which the name "Ponnankanni" is derived. It is also known to be rich in antioxidants, sterol compounds which give cooling effect to eyes and body. It also relieves neuritis, treats 96 types of eye diseases, and aids disease-free healthier life (Walter, Merish & Tamizhamuthu, 2014).

In Das, Ashok Kumar, Mastanaiah and Das (2015), the ethanolic extract of *Alternantherasessilis in* aerial parts exhibited significant hypoglycemic activity in streptozotocin induced diabetic rats. This evidence points to the possible presence of saponins which is believed to be responsible for the observed antidiabetic activity. Thus, if saponins is present in plants, it possesses activities attributed to protective action on lipid peroxidation and enhances effects on cellular antioxidant defense, thereby contributing to the protection against oxidative damage in streptozotocin induced diabetes (STZ-induced diabetes).

According to Rao, Rao, Nelson, Nagaiah, and Reddy (2011), the alcoholic and aqueous extracts of *Alternanthera sessilis* show significant reduction in blood glucose levels of STZ-induced diabetic rats and the activity of both the extracts were quite significant and encouraging. The antidiabetic activity of *A*. sessilis is attributed to the presence of triterpenoids, phytosterols and glycosides. The results further reveal that the aqueous extract is slightly less effective than alcoholic extract.

"Lupo", *A. sessilis,* has long been used as vegetable and found to be good analgesic and antihyperglycemic (Hossain et al., 2014). It exhibits antihelmintic activity (Venilla & Nivetha, 2015), antimicrobial and wound healing activities (Jalapuri, Agrawal, Patil, Chimkode, & Tripathiz, 2008), hepatoprotective and hematinic activities (Arollado & Osi, 2010), and nootropic/memory enhancing activity (Gupta & Singh, 2012). Thus, it is believed to be good for people with high blood pressure.

On the other hand, the antimicrobial drug discovery community generally believes that conserved essential genes have a great potential to yield broad-spectrum antimicrobial agents (Liu et al., 2012). The California Health Institute emphasized the importance of promoting antibiotic discovery and development to combat the growing threat to antibiotic resistance and emerging pathogens that are highly resistant to known antibiotics (Beckstrand, 2012). Since its earliest discovery, the global use of antibiotics has improved the quality and length of life for countless people.

This study aims to provide scientific proof of the presence of phytochemicals in the aqueous, ethanolic, and acetone extracts of *Alternanthera sessilis* (*Lupo*), namely: Alkaloids; steroids; anthraquinones; flavonoids; and tannins, and to confirm their antimicrobial and antifungal potential. The results will provide information to the make an Information, Education, and Communication (IEC) materials that may convince people to mixed it with other healthy food products.

Methodology

The phytochemical and microbial analyses were done last June, 2016 at the West Visayas State University Chemistry and Biology laboratories. Two replicates were used for both analyses. Three extracts (aqueous, ethanolic, and acetone) of air-dried *Alternanthera sessilis* leaves were tested for presence or absence of five secondary metabolites such as alkaloids, steroids, anthraquinones, flavonoids and tannins, while expressed juice was used for antimicrobial and antifungal testing. The plant material was taken from West Visayas State University – Lambunao Campus, where the plant was and is still being propagated for use in the other projects of the "Health and Wealth" program of the University, for which the IEC materials be used for adopted barangay.

Figure 1 shows the process in the conduct of the phytochemical analysis of "Lupo" (*Alternanthera sessilis*).

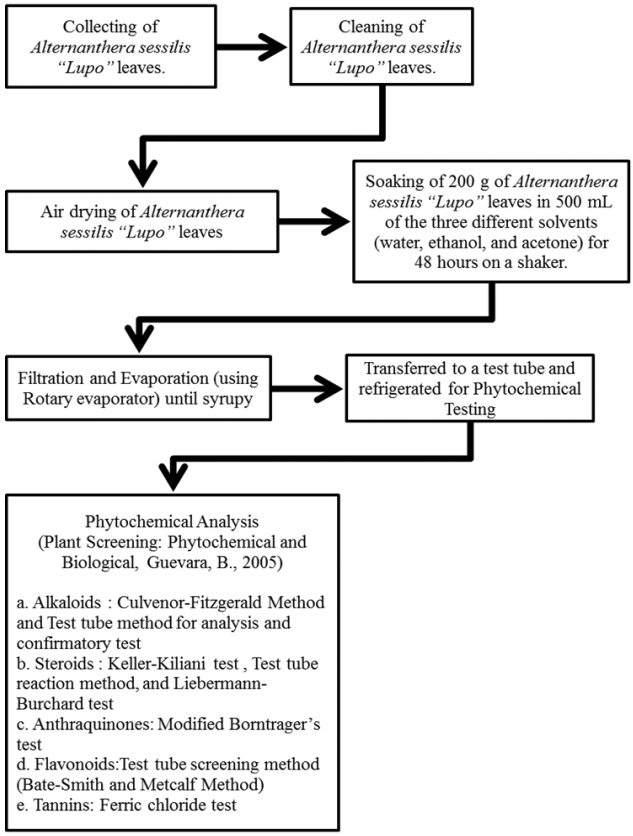

Figure 1. Flow chart of the Process in Phytochemical Analysis of *Alternanthera sessilis*

Preparation of the Extract

Alternanthera sessilis, transported from Lambunao, was washed thoroughly with clean water and was air-dried. The 200 gram air-dried sample was soaked in respective solvent in erlenmeyer flasks and placed in a shaker for 24 hours. The mixture was filtered and placed in a rotary evaporator until syrupy. The syrupy mixture was transferred to a test tube and kept in a refrigerator until analysis.

The three flasks were then placed in a bath mixer for 48 hours for thorough mixing. After 48 hours, each mixture was filtered into another flask and the corresponding solvents evaporated (rotary evaporator) until the mixture was syrupy. The extracts were transferred to three test tubes which were refrigerated with the acetone extract to avoid further evaporation inside the freezer. Portions of each extract were then analyzed in duplicates for the presence of phytochemicals: Alkaloids,Steroids, , Anthraquinones, Flavonoids, and Tannins.

Preparation of the Test Reagents for Alkaloids

Mayer's reagent. 1.4 g of Mercuric oxide was dissolved in 60 mL water. The resulting solution was then poured into a solution of 5.0 g KI in 10 mL of water. Enough water was then added to make 100 mL.

Dragendorff's reagent. Equal parts of solution A (0.85 g bismuth (III) nitrate, dissolved in a mixture of 10 mL acetic acid and 40 mL water) and solution B (8.0 g of KI, dissolved in 20 mL of water) were mixed well and stored in a dark bottle at room temperature. This solution served as the stock solution.1 mL of the stock solution was mixed with 2 mL acetic acid and 10 mL water to prepare the Dragendorff's reagent.

Test Reagent for Steroids

Iron (III) chloride reagent. 3 mL of 1% $FeCl_3$ was dissolved in 50 mL of glacial acetic acid.

Test Proper

Two replicates of each of the extract were analyzed using standard procedures: laboratory test tube method for Alkaloids analysis (Confirmatory test, and test for quaternary bases and/or amine oxide); test tube reaction methods for Steroids (Keller-Kiliani test for 2-deoxysugars); Borntrager's test tube screening method for Anthraquinones; test tube screening methods for Flavonoids (test for leucoanthocyanins: Bate-Smith and Metcalf method); and the test tube screening method for Tannins (Ferric chloride test) (Guevarra, 2005). Results of the tests were recorded and tabulated in a table.

Figure 2 shows the process in the Antimicrobial and antifungal testing of *Alternanthera sessilis* leaves expressed juice.

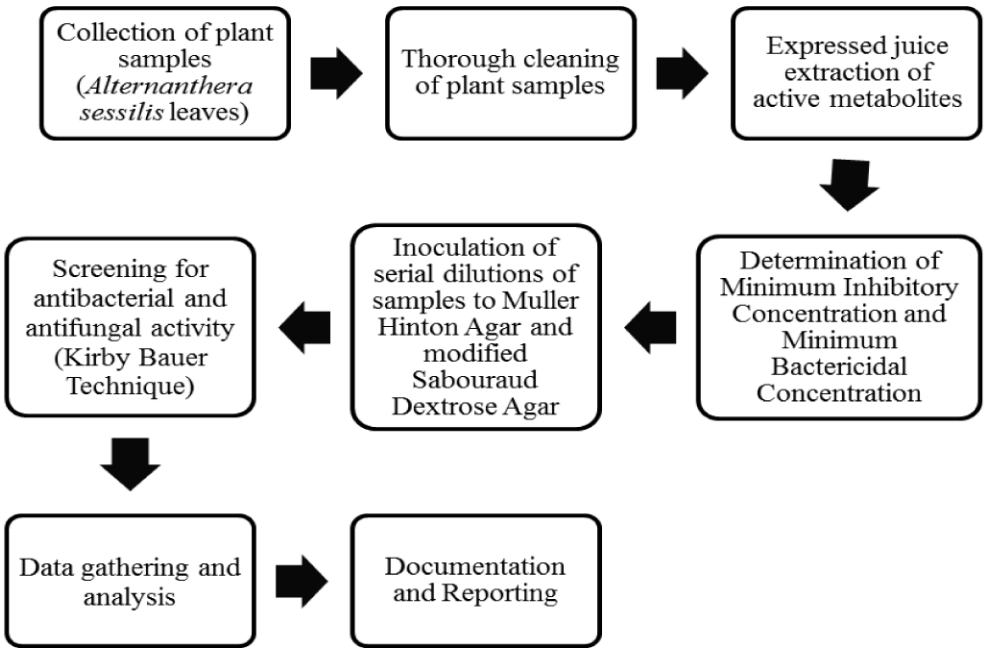

Figure 2. Flowchart of the Experimental Design for Antimicrobial and Antifungal Analysis

Culture Medium

The medium used were Mueller Hinton Agar for antibacterial testing and Sabouraud Dextrose Agar (Merck) supplemented with tetracycline for antifungal assay to inhibit the growth of other micro-organisms that may hamper the fungal activity.

Expressed Juice Extraction

Fresh clean leaves of *Alternanther asessilis* (L.) R. BR (Lupo) were cut into small pieces using a sterile stainless scissors. The cut green leaves were then pounded using a sterile mortar and pestle. To express the juice, sterile cheese cloth was used to squeeze the leaves of the plant. The juice was collected in a clean, sterile amber bottle for antimicrobial assay.

Screening for Antimicrobial Activity

Microbial strains. Standard test strains, *Escherichia coli* ATCC 25923, *Staphylococcus aureus* ATCC 25922, and *Candida albicans* were used. These control strains are very stable strains when stored properly and are recommended for quality control of antibiotic susceptibility tests (Clinical and Laboratory Standard Institute [CLSI], 2008). Furthermore, their responses would provide insight on the potential clinical efficacy of the drug under test against fungal, gram-positive and gram-negative clinical isolate.

Identification of the standard strains were confirmed using conventional procedures of gram stain, colonial morphology on selective agar, and biochemical tests. Both test bacteria were maintained and stored in nutrient agar slants and the fungal strain in Sabourauds dextrose agar slant at refrigeration temperature.

Screening for Antimicrobial Activity using the Kirby-Bauer Method

The inocula were prepared from fresh cultures of *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli* with sterile normal saline solution. The cell concentration was adjusted to 0.5 McFarland Standard or a density of 10^8cfus/mL, then swabbed onto 4-mm deep Mueller-Hinton agar (MHA, Merck) and Sabourauds Dextrose agar (SDA, Merck). Ten uL of each of the crude extracts (final disk concentration: 1000ug/mL) were dispensed onto three sterile 6-mm paper disks (Whatman). The extract disks were air-dried to evaporate the solvent before placing onto the inoculated culture plates. Commercial antibiotic standard disk containing 30 ugl tetracycline (BBL) and Ketoconazole (Jantsen) were included in the assay. Distilled water was used as negative control disk. The disks were applied in equidistant fashion on the MHA and SDA plates. The culture plates were then incubated at 35^0C to 37^0C for 18 to 24 hours and 20^0C to 25^0C for 3 to 5 days. After incubation, zones of inhibition were measured using a calliper and their antimicrobial activities were assessed.

Determination of the Minimum Inhibitory (MIC) and Minimum Bactericidal (MBC) Concentrations

Serial dilution of the active expressed extract. Ten test tubes for each active expressed juice sample were prepared for the two-fold serial dilution starting at 1,010 ug crude extract/mL in 0.025% phosphate buffer. One (1) mL of Mueller-Hinton Broth (Merck) was dispensed from tubes 2-12. One mL of 1,010 ug/mL crude extract was dispensed to tubes 1 and 2. The extract in tube 2 which was diluted with the pre-dispensed broth was mixed thoroughly and 1 mL was transferred to tube 3, continuing the same process for the rest of the tubes, and discarding 1 mL of the mixed solution from the last tube. The resulting extract concentration from tubes 1-10 were 1010 ug/mL to 1.97 ug/mL, respectively.

Dispensation of Inoculums to Extract Dilutions

1mL or an equal volume of the standardized inoculums (1.5×10^8 cfu/mL was dispensed in each of tubes 1-10 of the serially diluted extract, making the final concentration at 505 ug/mL to 0.98 ug/mL in 1-10 distilled water. Drug controls (tetracycline and ketoconazole) were included following the same procedure. All procedures were done in duplicate for all samples. The tubes were incubated at 35^0C for 24 hours and MIC was read as the lowest concentration of the active culture extract in the tube which exhibited absence of growth.

Determination of the Minimum Bactericidal Concentration

From the tubes that did not show a trace of growth vis-a-vis the negative and media controls, 10ul aliquots were spread onto drug-free Mueller Hinton Agar plates. The inoculated plates were incubated at 35^0C to 37^0C, 24 hours for bacteria. The least concentration of the test sample in the plate that showed no visible growth was taken as the Minimum Bactericidal concentration (Forbes et al., 2002).

Procedure of Data Analysis

For the antimicrobial activity, the average of the zones of inhibition was used for descriptive analysis. The mean was calculated from the replicates of each of the bacterial and fungal isolates.

Results and Discussion

Table 1 shows positive results for alkaloids, steroids, anthraquinones, and flavonoids in all three extracts of *Alternanthera sessilis*. Tannins is not present in the ethanolic and acetone extract, but present in aqueous extract as in the study of Sudha and Mathangi(2013).

Table 1

Summary of Phytochemical Analysis in the Three Solvents

	Solvent used		
	Ethanol	Acetone	Aqueous
Alkaloids	+	+	+
Steroids	+	+	+
Anthraquinones	+	+	+
Flavonoids	+	+	+
Tannins	-	-	+

Note: (+)=slight precipitation/positive on test; (-)=negative precipitation/negative on test.

Results showed presence of the five phytochemicals (Sudha & Mathangi, 2013) in the aqueous extracts, suggesting that *Alternanthera sessilis*can provide natural chemicals that have good medicinal values for diabetes (Hossain et al., 2014; Rao et al., 2011), high blood pressure (Arollado & Osi, 2010), eye diseases (Walter et al., 2014) and memory enhancement (Gupta & Singh, 2012). Since all five phytochemicals can be extracted by water, *Alternanthera sessilis* may be incorporated with food and food products to neutralize its taste to become acceptable to the younger generations while supplying consumers with antioxidants and sterol compounds which has a cooling effect on the eyes and body, thereby relieving neuritis and treating eye diseases to promote a disease-free healthier life (Walter et al., 2014).

Expressed juice yielded a blackish green color with a distinct odor and taste. Results of the antimicrobial assays suggest that great *Alternanthera sessilis* (Lupo) expressed juice has great antibacterial potential against *Escherichia coli*.

Based on conventional identification procedures, *Staphylococcus aureus* presented the following characteristic features: Gram positive cocci in clusters, B hemolysis on blood agar, salt tolerance and mannitol fermentation on Mannitol Salt Agar, catalase positive and coagulase positive on slide tests; while *Escherichia coli* was gram negative *coccobacilli*, colonies with greenish metallic sheen, blue black under transmitted light on Eosin Mythelene Blue Agar. *Candida albicans* showed. On Sabouraud's dextrose, agar colonies are white to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology shows spherical to sub spherical budding yeast-like cells or blastoconidia.

An MIC of 100 ug/mL was arbitrarily designated as a value with good potency. Analysis of the data was based on this figure.

Table 2 presents the overall activity of *Alternanthera sessile* (Lupo) expressed juice against microbial reference strains *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. Results indicate that *Alternanthera sessilis* (*Lupo*) expressed juice possessed an antimicrobial activity exhibiting an MIC of < 100 ug/mL against *Escherichia coli* referenced strain used. There is low MIC of 252.5ug/mL against *Staphylococcus aureus* and *Candida albicans*.

The control drug (tetracycline, TE) and Ketoconazole were used to validate the assay used but the potency between the expressed juice and TE and Ketoconazole cannot be compared because of purity issues and differences in chemistry and structure.

Table 2

Activity of Alternasnthera sessilis (Lupo) expressed juice against Staphylococcus aureus, Escherichia coli and Candida albicans

Microbial Isolate Reference Strain	Mean Zone of Inhibition (mm)*	Minimum Inhibitory Concentration (ug/mL)
Escherichia coli	25.3	25.7
Staphylococcus aureus	17.7	252.5
Candida albicans	16.3	252.5
Control: Tetracycline	30.0	1.0
Control: Ketoconazole	28.0	1.0

Note: *Disk diameter 6 mm; disk concentration : 1000ug

Expressed juice of plant active at 100ug/mL must have a good potency level and depending upon the general chemical nature of the compounds responsible for the activity. Subsequent purification techniques maybe decided upon. In this study, arbitrarily assigning an MIC of 100ug/mL as a good activity level for the *Alternanthera sessilis* (*Lupo*) leaves expressed juice tested indicates that it has a great potential against gram-negative bacterium, *Escherichia coli* with an MIC of 25.7ug/mL and a low MIC of 252.5ug/mL against *Staphylococcus aureus* and *Candida albicans.*

Alternanthera sessilis (*Lupo*) leaves expressed juice if developed pharmaceutically can be useful to control gram-negative bacterial infections. Many pathogens are not responding anymore to currently available antimicrobials. Indeed, there is an urgent need to search for new compounds. This study has responded to this urgent call.

Results generated by the present study are very promising and yielded antimicrobial property which could be tapped as main ingredients for new antimicrobial drugs (Liu et al., 2012). Arbitrarily designating an MIC of 100ug/mL as a good potency level for an expressed juice to be a candidate for further studies, an MIC of 25.7ug/mL against *Escherichia coli* and an MIC of 252.5ug/mL against *Staphylococcus aureus* and *Candida albicans* were determined. It is therefore recommended that further studies be done on the bioactive *Alternantherasessilis* (*Lupo*) plant until new drugs are developed (Beckstrand, 2012).

Conclusions and Recommendations

Alkaloids, steroids, anthraquinones, and flavonoids are present in minute amount in all extracts (ethanolic, acetone, and aqueous), while tannins is present in aqueous but not in ethanolic and acetone extracts of *Alternanthera sessilis*. *Alternanthera sessilis* is a good source of phytochemicals beneficial to the body and may be included in the daily diet to live a healthier life.

Alternanthera sessilis can be part of the diet because it is a source of alkaloids, steroids, anthraquinones, and flavonoids. Tannins are only found in the aqueous extract. It is therefore a valuable and readily accessible plant material which have various medicinal values.

Since *alternanthera sessilis* contain alkaloids, steroids, anthraquinones, flavonoids and tannins it has medicinal values. It can produce definite physiological action on the body, and can therefore be included into the daily diet menu for a healthy life in a natural way (Sudha&Mathangi, 2013)

Since *Alternanthera sessilis* showed antimicrobial and antifungal properties, it is recommended that further studies should be conducted to separate specific phytochemicals for the production of drugs (Liu et al., 2012; Beckstrand, 2012).

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