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Antibacterial Activity and Concentration Dependent Modulation of Angiogenesis of the Saponins of *Schefflera luzoniensis.*

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ABSTRACT

The antibacterial and angiogenic potentials of the saponin-containing *n*-butanol extract and fractions of *Schefflera luzoniensis* were assessed using paper disc-diffusion, MABA and chicken chorioallantoic membrane assays. Moderately strong zone of inhibitions and a 50µg/mL minimum inhibitory concentration were observed for the *n*-butanol sub-extract and saponin-containing fractions against representative Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus epidermidis* and *Staphylococcus aureus*). Fraction two and five showed activity versus *Mycobacterium tuberculosis* $H_{37}Rv$ based on the results of MABA (MIC_{50} = 64 µg/ml). The *n*-butanol sub-extract induced angiogenesis at 10 µg/mL concentration as it promoted blood vessel proliferation in chicken chorioallantoic membranes, while higher concentrations resulted to suppressed neovascularization. Following the OECD 425 guidelines for acute oral toxicity determination, the *n*-butanol sub-extract was found non-toxic up to 2000 mg/kg body weight of Swiss mice. HPLC-MS profiling of the antibacterial fraction five revealed the presence of two known antibacterial oleanene glycosides, namely, scheffoleoside A (**1**) and F (**2**) as the major saponin glycoside constituents. Thus, the Philippine endemic plant *S. luzoniensis* is a promising source of saponins with potential antibacterial, wound healing and cytotoxic properties.

Keywords: Saponins, antibacterial, angiogenesis, *Schefflera luzoniensis*, oleanene glycosides, scheffoleoside A, scheffoleoside F.

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INTRODUCTION

Growing concerns on potential problems such as multi-drug resistance have awakened research efforts to identify new and better antibiotic alternatives. Novel strategies in the discovery of new antimicrobial leads have been carried out, such as development of compounds to treat infectious diseases or to improve animal growth including dietary use of probiotics, prebiotics [1], organic acids [2] and medicinal herbs [3]. On the other hand, pathways related to angiogenesis direct the proliferation of new capillaries during various physiological and pathologic stages that play significant roles in tumor growth, metastasis, embryonic development, wound healing and arteriosclerosis [4-5]. Angiogenic cessation is a promising approach to inhibit tumor growth, invasion and metastasis.

Saponins are glycoside-rich compounds whose chemical structures are composed of a fat-soluble triterpenoidal or steroidal nucleus (aglycone) linked to one or more sugar residues. Triterpenoidal saponins abounds in soybean, alfalfa and Quillaja [6] while steroidal saponins are predominant in Yucca, tomato and oats [7]. Among their noted biological effects are haemolytic activity and antibacterial activity [8-9].

Plant species of the genus *Schefflera* (Araliaceae) includes about 200 species that are widely distributed in various tropical regions. Pharmacological studies have shown the extracts of these plants to possess anti-inflammatory, antifungal, anticancer, antiviral, antibacterial, hypoglycemic, insect repellent properties [10], wound healing activity, bacteriostatic actions in tuberculosis models [11] and analgesic activities. Previous phytochemical studies revealed triterpenoidal saponins, steroidal glycosides and organic acids as main constituents of *Schefflera* species. *Schefflera luzoniensis* Merr is an endemic small shrub abundant in the lowland to medium forests of Mount Banahaw, Southern Luzon, Philippines [12]. The samples used in this study were acquired from Mount Buhi in the Province of Camarines Sur, Philippines. Interestingly, studies related to its pharmacological potentials especially antibacterial and angiogenic properties have not been reported in the literature. As part of our research efforts to discover biologically active phytochemicals from Philippine medicinal plants [13-18], we investigated the antibacterial and angiogenic activities of the saponin-containing *n*-butanol sub-extract and fractions of *S. luzoniensis*.

MATERIALS AND METHODS

Plant Material

Leaves of *S. luzoniensis* were collected in Mount Buhi, Camarines Sur, Philippines in May, 2013 and authenticated by Mr. Wilfredo F. Vendivil of the Philippine National Museum (PNM) in Intramuros, Manila. A voucher specimen (PNM007034) was deposited in the herbarium of PNM for reference.

Extraction and fractionation

The air-dried leaves of *S. luzoniensis* were ground on a Wiley mill with fine mesh, extracted with distilled methanol (9.9 L) and concentrated under reduced pressure to afford a green syrupy extract. The crude methanolic extract (101.7 g) was suspended (with sonication) in water and extracted with solvents of increasing polarity (petroleum ether, dichloromethane and *n*-butanol) to afford three sub-extracts from which the *n*-butanol sub-extract was found to contain most of the saponin compounds. The *n*-butanol sub-extract (22.0 g) was mixed with Celite 545 and placed on top of a silica gel column (Merck Art 7734). The column was eluted with gradient additions of methanol in ethyl acetate (10 % increment) to afford five fractions.

Phytochemical screening for saponins

To test for the presence of saponins, thin-layer chromatograms developed in 7:3 ethyl acetate-methanol of the *n*-butanol sub-extract and its fractions were sprayed with α-naphthol-sulfuric acid (for glycosides), vanillin-sulfuric acid (for triterpenes & unsaturated sterols) and Liebermann-Burchard reagent (for steroids). In addition, a froth test was also conducted.

Liquid chromatographic-electrospray ionization mass spectrometric analysis

One milliliter each of the saponin-containing fractions of *S. luzoniensis* was subjected to LC-ESIMS on a TSQ 7000 Thermo Finnigan mass spectrometer fitted with a positive mode electrospray source. Reversed phase-high performance liquid chromatography (RP-HPLC) was performed on an HP Agilent 1100 LC using a LunaC18 (2 mm x 150 mm) column. Solvent A was MilliQ water while solvent B was UV grade methanol with 10 percent gradient. Flow rate use of 0.2 ml/min was set.

Biological Assays

Acute oral toxicity determination

Acute toxicity of the aqueous solution of the *n*-butanol sub-extract was carried out using acute toxicity method as described in OECD 425 (Organization for Economic Cooperation and Development) guidelines [19]. The extract was found to be non-toxic and safe up to a dose of 2000 mg/kg body weight.

Antibacterial Assays

Paper-disc diffusion assay and minimum inhibitory concentration (MIC) determination

Three strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus cereus* ATCC 11778) and three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* USTCMS 1040) were used in the antibacterial assay [20]. A 1000µg/ml concentration of the *n*-butanol sub-extract and saponin-containing fractions were incorporated in the Mueller-Hinton agar. Concentrations of 100, 50, 25, and 12.5µg/ml were prepared via serial dilution on a microtiter plate. Antibacterial activity was examined using a sterilized Costar 3596 96-well cell culture cluster flat bottom with lid microtiter plate reader after incubation at 37 ^{o}C for 24 hours. The minimum inhibitory concentration is defined as the lowest concentration of the test samplesat which no growth or turbidity is observed.

Microplate Alamar Blue Assay (MABA)

One milligram each of the *n*-butanol sub-extract and saponin-containing fractions were assessed for activity against *Mycobacterium tuberculosis* H_{37}Rv using a colorimetric Microplate Alamar Blue assay (MABA) [21].

Chicken-chorioallantoic membrane (CAM) assay

The *n*-butanol sub-extract was diluted via serial dilution with ethanol to prepare 10, 100 and 1,000 µg/ml of sample concentrations. Retinoic acid was used as negative control (2µg/ml) and ethanol as positive control. Samples (30 µl) were applied on the sterilized six millimeter paper discs using a micropipettor. A total of six groups and three zero-day old fertilized chicken eggs were assigned for each group. Eggs were incubated for eight days at 37 ^{o}C. On the 8th day, the assay was continued on a laminar flow hood under sterile conditions. The paper discs containing the samples were placed inside the eggs and incubated further for two days. Afterwhich, the chorioallantoic membranes (CAM) were collected and examined for branching of blood vessels using a BestScope BLM-320 dissecting microscope (40x magnification). Quantification was carried out using an AngioQuant software, an automated image analysis for angiogenesis [22].

RESULTS AND DISCUSSION

The *n*-butanol sub-extract and fractions of *S. luzoniensis* was positive in general for the presence of steroidal and/or terpenoidal saponins using reaction thin-layer chromatography (α-naphthol, vanillin-sulfuric acid and Liebermann-Burchard as spray reagents) and froth tests. The *n*-butanol extract was found to be safe and non-toxic up to 2000 mg/kg body weight of Swiss mice. Abnormalities were not observed in the organs through analysis of gross necropsy results.

Antibacterial assay of the *n*-butanol sub-extract and five fractions using paper disc-diffusion method showed positive activity against Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus epidermidis* and *Staphylococcus aureus*) (Table 1). Using the broth dilution microplate method, a minimum inhibitory concentration (MIC) was observed at 50µg/ml test concentration against both *Staphylococcus species*. A Mueller-Hinton broth with the test organism *Staphylococcus epidermidis* served as positive control. No activity versus *Mycobacterium tuberculosis* $H_{37}Rv$ was noted for all test samples except fraction two and five which exhibited an $MIC_{50}$of 64 µg/mL based on the results of the colorimetric Microplate Alamar Blue assay (Table 2). Using Single Factor Analysis of Variance with Tukey's Honestly Significant Difference (HSD), the *S. luzoniensis n*-butanol sub-extract at 10µg/mL concentration showed significant increased blood vessel formation (vascularization) thus inducing angiogenesis in vivo (Table 3). For comparison, ethanol was used as positive reference compound. At higher concentrations, the 1000 and 100 µg/mL test extract solutions suppressed blood vessel formation when compared to the negative, anti-angiogenic control, retinoic acid.

Table 1: Antibacterial activity of the *n*-butanol extract and fractions of *S. luzoniensis*.

Test Sample	Test Organism					
	Bacillus cereus	*Klebsiella pneumoniae*	*Escherichia coli*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*
n-butanol extract	11.6	-	-	-	14.3	-
Fraction 1	-	-	-	-	12.0	-
Fraction 2	-	-	-	-	10.0	-
Fraction 3	-	-	-	11.6	12.0	-
Fraction 4	-	-	-	12.0	11.3	-
Fraction 5	-	-	-	11.6	10.6	-

Table 2: Antituberculosis activity of the *n*-butanol extract and fractions of *S. luzoniensis*.

Test Sample	% Inhibition at 64 µg/ml	MABA MIC_{50} (µg/ml)
n-butanol extract	3	>64
Fraction 1	43	>64
Fraction 2	51	64
Fraction 3	27	>64
Fraction 4	29	>64
Fraction 5	51	64
Rifampin	99%	0.098

Table 3: Mean Number of Blood Vessel Branch Points of *S. luzoniensis* at different concentrations.

Test Sample	Mean No. of Blood Vessels
1000 µg/mL	75.9
100 µg/mL	24.5
10µg/mL	445.6
Retinoic acid[a]	32.4
Ethanol[b]	187.1
Untreated	80.4

[a]negative control; [b]positive control.

Chemical profiling of fraction 5 which showed activity against *S. aureus*, *S. epidermis* and *M. tuberculosis* $H_{37}Rv$ was carried out using reversed-phase HPLC-ESIMS. A major peak exhibiting a dehydrogenated molecular ion [M-H] at *m/z* 957.5055 corroborating possibly to the two known diastereomeric oleanene glycosides, scheffoleoside A (**1**) and F (**2**) (Figure 1) was detected.

Both saponins have been isolated from *S. octophylla* [23]. *S. octophylla* has been reported to treat dermatitis, diabetes, cough, cataract, hypertension, memory impairment and wound [11]. The wound-healing property may be attributed to the angiopromoting effects of the saponins, especially, the oleanene glycosides.

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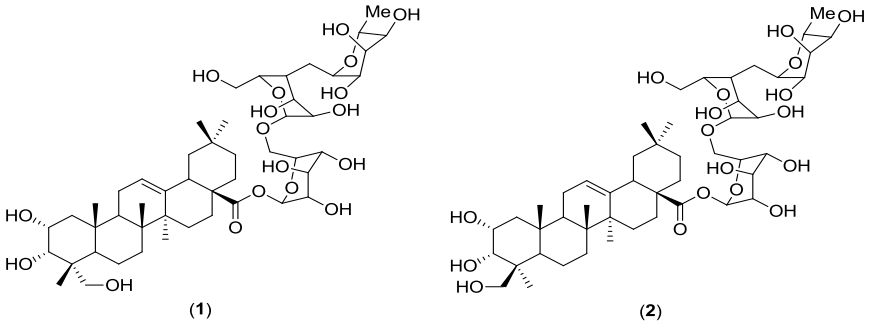

Figure 1: Structure of scheffoleoside A (1) and F (2).

CONCLUSION

In conclusion, the *n*-butanol sub-extract and fractions of *Schefflera luzoniensis* was found to contain steroidal and/or terpenoidal saponins based on the phytochemical screening test results. The saponin extract and fractions possessed antibacterial activity against Gram-positive bacteria with two fractions showing activity against *Mycobacterium tuberculosis* $H_{37}Rv$. The saponins were also observed to possess modulatory effects on angiogenesis by concentration-dependent induction or suppression of vascularization in chicken-chorioallantoic membrane models.

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