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BIOLOGICAL ACTIVITIES OF *SCHEFFLERA LEUCANTHA*

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Abstract

This study investigated various biological activities of the ethanolic extract of dried ground leaves of *Schefflera leucantha* Viguier (Araliaceae). The extract possessed very low cytotoxicity to brine-shrimp with the LC_{50} of 4,111.15µg/ml; the significant antioxidant activity on DPPH with the EC_{50} of 71.90µg/ml; the inhibitory activity on mushroom tyrosinase with the IC_{50} of 10.53mg/ml using the dopachrome microplate-assay. The extract of 5-20mg/ml range in the agar dilution assay were active against various pathogenic microbial (11 species, 11 strains), with the minimum inhibitory concentration (MIC) of 5mg/ml against *Clostridium spp.*; MIC=10mg/ml against enteropathogens as *Bacteroides spp., Enterococcus faecalis* ATCC 29212, *Lactobacillus spp., Peptococcus spp.* and *Streptococcus mutans*; MIC=10mg/ml against a pneumonia causing bacteria *Klebsiella pneumoniae* and a dermatopathogen as *Propionibacterium acnes*; MIC=20mg/ml against dermatopathogens as *Staphylococcus aureus* ATCC 6538, S*treptococcus spp.* and *Candida albicans* ATCC 90028. TLC fingerprints of the specific extracts from the leaf powder exhibited zones of steroids-terpenes and flavonoids. HPLC fingerprint of the flavonoid extract was performed.

Key words: *Schefflera leucantha,* Antioxidant, Antityrosinase, Antimicrobial activity, Phytochemistry

Introduction

Schefflera leucantha Viguier, ARALIACEAE, locally known in Thailand as 'Hanuman Prasankai' is a 1-2m shrub, highly branched, cultivated elsewhere in the moist open field. The finger-like leaf composed of 5-6 compound leaves, oblanceolate-subulate, glabrous, 1.5-2cm broad, 5-10 cm long, with a 3-9cm long petiole. The white flowers are small, inflorescence. The yellow fruits are dense aggregate. The infusion of this plant is said to relieve a cold, allergies, asthma, chronic cough and respiratory tract infection. The whole plant is also used as analgesics, antipeptic and to promote blood circulation. The leaves are used for wound-healing, inflammation, cough, bronchitis, carcinogenic and removal of blood clot in the brain.

In Thailand and China the plant is widely used as an antiasthmatic. It was reported that the active bronchodilator principle is a mixture of saponins (Pancharoen *et al.*, 1994), which showed no sign of toxicity at the dose of 1000mg/kg orally (Witthawaskul et al., 2003). Aqueous extract of *S. leucantha* has been reported having hypoglycemic activity in rats (Satayavivad et al., 1996).

In the present study, we investigated various biological activities of the crude ethanol leaf extract of *S. leucantha* to provide new fields for the utilization study of the plant. TLC fingerprints and HPLC fingerprint of the leaf powder were performed for future references.

Materials and Methods

Plant material

Provided fresh leaves of *S. leucantha* were collected in November 2003 from Nakhon Rachasima province, Thailand. The plant was identified and authenticated by the Research Officer (Botany). The sample was dried in a hot air oven at 40-50^{o}C, and then pulverized into powder. The authentic plant harbarium of voucher specimen no. LRS-0111 has been kept at the Lamtakhong Plant Research Station, TISTR, where the plant are also cultivated.

Preparation of crude ethanol extract

The leaf powder was 3 times repeatedly macerated with fresh 95%ethanol in a percolator, then filtered through the cotton plug. The combined filtrate was evaporated to dryness under reduced pressure at 40-50°C. The resulting crude ethanol extract was then stored at 4^{o}C until used.

Cytotoxicity to brine-shrimp

Modified method of Solis *et al.* (1992) was used to determine the inhibitory activity on *Artemia sp.* in a 96-well micro-plate. 100µl of the crude ethanol extract solution in 0.25%Tween80-artificial seawater was added into each well containing 5 newly hatched brine shrimps in 100µl artificial seawater, then incubated at room temperature for 24h. All samples were repeated in 6 wells to make overall tested organisms of 30 for each. Counted the dead organisms under a binocular microscope (4x). Plot %Lethality *vs* Log concentration. Substituted y = 50 in the resulted linear equation to obtain the x value. The antilog x was then the LC_{50} (conc. of 50%lethality) value (Ballantyne et al., 1995). Thymol and kojic acid were used as reference standards.

Antioxidant activity
Scavenging of DPPH radical

Modified method of Hatano et al. (1989) was used to measure the free radical scavenging activity of the crude ethanol extract on DPPH (2,2-diphenyl-1-picrylhydrazyl from Sigma, Germany) which is a stable radical. In a 96-well micro-plate, added 100µl of the extract solutions (in absolute ethanol) into each well containing 100µl of 0.06mM DPPH in ethanol, mixed well, and measured the absorbance at 517nm exactly at 30min by a micro-plate reader (TECAN, Sunrise remote). All samples were run in triplicate. Determined %scavenging of test samples as follows:

$$\%\text{Scavenging} = \left(\frac{C - (A - B)}{C} \right) \times 100$$

Where A, B and C represent the absorbances of DPPH in the resulted reaction mixture, the blank, and the control respectively. Plot %Scavenging *vs* Log concentration. Substituted y = 50 in the resulted linear equation to obtain the x value. The antilog x was then the EC_{50} (conc. of 50%scavenging) value (Ballantyne *et al.*, 1995). BHT, BHA and vitamin C were used as reference standards.

Anti-tyrosinase assay

20%ethanol extract derived from the crude ethanol extract, was assayed for enzyme tyrosinase inhibition by the dopachrome method modified from Iida *et al.* (1995), using L-Dopa (Sigma Chemical) as a substrate. In a 96-well micro-plate, mixed 150µl of 0.02M sodium phostphate buffer (pH 6.8) with 50µl of the sample solution and 50µl of mushroom tyrosinase solution (314.8U/ml, Fluka). Pre-incubated the mixture at 25°C for 10min before adding 50µl of 0.34mM L-Dopa, and then incubated at 25°C for another 2min. Measured the absorbance at 492nm of the biosynthesized red dopachrome by a micro-plate reader (TECAN, Sunrise remote). All samples were run in triplicate. The absorbance differences before and after the incubation were used to calculate the percentage inhibition of tyrosinase as follows:

$$\%\text{Tyrosinase inhibition} = \left(\frac{(A - B) - (C - D)}{(A - B)} \right) \times 100$$

Where the absorbance difference A represents the control (L-Dopa mixed with enzyme in buffer); B represents the blank (L-Dopa in buffer); C represents the reaction mixture; and D represents the blank of C (L-Dopa mixed with test sample in buffer). Plot %Tyrosinase inhibition *vs* Log concentration. Substituted y = 50 in the resulted linear equation to obtain the x value. The antilog x was then the IC_{50} (conc. of 50%inhibition) value (Ballantyne et al., 1995). Kojic acid, a well-known tyrosinase inhibitor, was used as the reference standard.

Anti-microbial assay

The activities against various pathogenic microorganisms were screened using the agar dilution method (Washington and Sutter, 1980). The test microorganisms were maintained on specific assay media as Mueller Hinton Agar (MHA; Difco Laboratories) for aerobes; WC Agar (Wilkins and Chalgren, 1976) for anaerobes; and Saboraud Dextrose Agar (SDA; Difco Laboratories) for yeasts. Inoculums derived from the cultures were incubated at 37°C in corresponding broths (18-24h for aerobes in MHB; 48h for anaerobes in WC Broth; 48h for yeast in SDB). A suspension of each isolates was prepared in a fresh inoculum's medium and adjusted to McFarland 0.5 turbidity standard. Prepared dilution plates (0.5-20mg/ml) of the crude ethanol extract by adding appropriate volumes of the stock solution (0.8g/ml in acetone) into the assay medium. Spot inoculated the dilution plates with the isolates suspensions, and then incubated at 37°C (overnight for aerobes; 3 days for anaerobes; 48h for yeast). Minimum inhibitory concentrations (MICs) of the extract were determined.

TLC fingerprints

Thin-layer chromatography (TLC) of 2 different extracts containing either steroids-terpenes or flavonoids from the leaf powder were performed on 0.25mm thick TLC plates (Merck Silica gel 60 F_{254}-precoated) using suitable developing solvent systems, and special detection reagents (Merck, 1980; Wagner and Bladt, 1996). No alkaloid could be extracted from the leaf powder. These chemical groups were chosen to perform the reference TLC fingerprints because they have been the easily extracted groups possessing various bilogical activities.

1. TLC fingerprint of the steroids and terpenes. The steroids-terpenes extract was prepared by stirring 1g of the leaf powder with hexane (3x50ml) for 30min, filtered, evaporated the filtrate to dryness under reduced pressure, and then dissolved in 1ml chloroform. Applied 3µl of the extract onto a TLC plate and perform chromatography with a suitable solvent system to a distance of 10cm. Sprayed the developed plate with vanillin-sulfuric acid reagent, then heated until the spots attain maximum colour intensity of the existing steroids-terpenes compared to ref. std. β-sitosterol (Sigma, USA) in chloroform (1:1 w/v). Figured the chromatogram by a scanner.

2. TLC fingerprint of the flavonoids. The flavonoids extract was prepared by stirring 0.5g of the leaf powder with 5ml methanol on a dry block heat bath (60°C, 5min), allowed to cool, filtered, evaporated the filtrate to dryness under reduced pressure. Dissolved the dried extract in 1ml methanol, then applied 3µl onto a TLC plate and

perform chromatography to 10cm with a suitable solvent system. Sprayed the developed plate with natural products-polyethylene glycol (NP/PEG) reagent, and detected fluorescing zones of the existing flavonoids under UV-365nm compared to ref. std. rutin (Fluka, Switzerland) in methanol (1:1 w/v). Figured the UV-365nm chromatogram by a digital camera (Keter KT-1000F, Taiwan).

HPLC fingerprint

A methanolic extract containing flavonoids was prepared by shaking 1g of the leaf powder with 50ml of methanol at 1,500rpm for 2min, filtered through a Whatman paper no.41 into a volumetric flask and made up to 50ml with methanol. Filtered the extract through a 0.45μ nylon syringe filter membrane before subjected to binary gradient RP-18, 30°C, 1ml/min flow rate, HPLC analysis with 270nm UV detector. Solvent A was water with 0.2%TFA+10%methanol, and solvent B was acetronitrile with 0.3%TFA. Standard addition of rutin (Merck, Germany) and quercetin (Fluka, Switzerland) was applied to HPLC chromatogram.

Results

The extraction process yielded 9.01% of the crude ethanol leaf extract of *S. leucantha*. The biological activities of the extract exhibited the computerized graphical calculated LC_{50} of 4,111.15μg/ml on brine shrimp cytotoxicity, EC_{50} of 71.90μg/ml on DPPH radical scavenging. The 20%ethanol extract derived from the crude extract gave the IC_{50} of 10.53mg/ml on mushroom tyrosinase inhibition (Tables 1- 4).

The agar dilution method assay indicated that the crude extract was active against various representative disease causing microorganisms (11 species, 11 strains) within the concentration range of 5-20mg/ml. The minimum inhibitory concentrations (MICs) were 5mg/ml against *Clostridium spp.*; 10mg/ml against enteropathogens as *Bacteroides spp., Enterococcus faecalis* ATCC 29212*, Lactobacillus spp., Peptococcus spp.* and *Streptococcus mutans*; 10mg/ml against a pneumonia causing bacteria *Klebsiella pneumoniae* and a dermatopathogen as *Propionibacterium acnes*; and 20mg/ml against dermatopathogens as *Staphylococcus aureus* ATCC 6538, S*treptococcus spp.* and *Candida albicans* ATCC 90028 (Table 5).

TLC fingerprints of the specific extracts from *S. leucantha* leaf presented a specific pattern of steroids-terpenes developed in dichloromethane-ethylacetate-formic acid (60:5:1); and a specific pattern of flavonoids developed in ethyl acetate-formic acid-acetic acid-water (100:11:11:26) as shown in Table 6, Figure 1.

RP-HPLC fingerprint of the flavonoid extract from *S. leucantha* leaf, under a suitable 40min-program linear gradient at 270nm, was shown in Figure 2.

Table 1: Biological effects of *S. leucantha* crude ethanol leaf extract, compared to reference standards

Compound	Inhibitory effect on brine shrimp (LC_{50})	DPPH radical scavenging effect (EC_{50})	Anti-tyrosinase effect (IC_{50})
Crude ethanol extract	4,111.15μg/ml	71.90μg/ml	-
20%ethanol extract	-	-	10.53mg/ml
Kojic acid	16.68μg/ml	-	0.0023mg/ml
Thymol	13.59μg/ml	-	-
BHT	-	4.21μg/ml	-
BHA	-	4.23μg/ml	-
Vitamin C	-	1.22μg/ml	-

Table 2: Inhibitory effect on brine-shrimp of *S. leucantha* crude ethanol leaf extract

Compound	Concentration (µg/ml)	Log concentration	% Lethality	LC_{50} (µg/ml)
Crude ethanol leaf extract	2,000	3.3010	0	4111.15
	5,000	3.6990	60	
	8,000	3.9031	100	
Kojic acid	1	0	0	16.68
	10	1	16.67	
	100	2	100	
Thymol	1	0	0	13.59
	10	1	30	
	100	2	100	

Table 3: *In vitro* DPPH radical scavenging effect of *S. leucantha* crude ethanol leaf extract

Compound	Concentration (µg/ml)	Log concentration	% Scavenging	EC_{50} (µg/ml)
Crude ethanol leaf extract	10	1.0000	27.30	71.90µg/ml
	50	1.6990	34.57	
	100	2.0000	40.50	
	500	2.6990	93.03	
	1,000	3.0000	100	
BHT	0.25	-0.6021	18.69	4.21µg/ml
	0.50	-03010	23.53	
	1.25	0.0969	27.68	
	5	0.6990	57.44	
	50	1.6990	79.58	
BHA	0.25	-0.6021	9.53	4.23µg/ml
	0.50	-0.3010	14.62	
	1.25	0.0969	27.31	
	2.50	0.3979	46.15	
	25	1.3979	76.92	
Vitamin C	0.25	-0.6021	22.77	1.22µg/ml
	0.50	-0.3010	25.25	
	2.50	0.3979	67.33	
	3.75	0.5740	81.19	
	25	1.3979	95.54	

Table 4: Inhibitory effect on mushroom tyrosinase of *S. leucantha* 20%ethanol leaf extract derived from the crude ethanol extract

Compound	Concentration (mg/ml)	Log concentration	% Tyrosinase inhibition	IC_{50} (mg/ml)
20%ethanol leaf extract	8	0.9031	22.86	10.53
	10	1.0000	48.57	
	12	1.0792	60.00	
	14	1.1461	78.79	
	16.5	1.2175	88.57	
Kojic acid	$1.42x10^{-5}$	-4.8477	2.38	0.0023
	$7.1x10^{-4}$	-3.1487	25.40	
	$1.42x10^{-3}$	-2.8477	38.89	
	$1.42x10^{-2}$	-1.8477	78.57	
	$7.1x10^{-2}$	-1.1487	88.10	

Table 5: Minimal inhibitory concentrations (MICs) of *S. leucantha* crude ethanol leaf extract on various pathogenic microorganisms

Cultured strains	MIC (mg/ml)
Aerobes	
Gram negative aerobic/microaerophilic rods and cocci	
Pseudomonas aeruginosa ATCC 27853	>20
Pseudomonas vulgaris	>20
Gram negative, facultative anaerobic rods	
Escherichia coli ATCC 25922	>20
Salmonella choleraesuis subsp. *choleraesuis* ATCC10708	>20
Salmonella typhimurium ATCC 13311	>20
Salmonella velterans	>20
Shigella dysenteriae D 2137	>20
Klebsiella pneumoniae	10
Gram positive cocci	
Enterococcus faecalis ATCC 29212	10
Staphylococcus aureus ATCC 6538	20
Staphylococcus aureus ATCC 25923	>20
Staphylococcus epidermidis ATCC 14990	>20
Streptococcus spp.	20
Anaerobes	
Gram negative non-sporing rods	
Bacteroides spp.	10
Gram positive non-sporing rods	
Lactobacillus spp.	10
Propionibacterium acnes	10
Gram positive spore-forming rods	
Clostridium spp.	5
Gram positive cocci	
Peptococcus spp.	10
Streptococcus mutans	10
Yeast	
Candida albicans ATCC 10231	>20
Candida albicans ATCC 90028	20

Table 6: hR_f values of chief constituents detected on TLC fingerprints of 2 different extracts from the leaf powder of *S. leucantha* containing either steroids-terpenes or flavonoids

Zone	Steroids-terpenes		Flavonoids	
	hR_f value	visible colour	hR_f value	UV-365nm fluorescence
1	21-22	blue	5-12	pale blue
2	23-24	blue	17-20	pale green
3	36-38	pale green	20-22	pale orange
4	41-44	pale green	20-25	pale green
5	49-52	violet	25-28	pale orange
6	52-55	purple	31-33	pale orange
7	67-69	green	37-40	pale yellow
8	71-73	purple	45-50	blue-green
9	88-92	violet	50-51	pale orange
10	93-96	gray	55-58	pale blue
11			60-64	pale orange
12			65-68	green
13			68-73	pale yellow

Discussion

The concentration of 50%activity of the *S. leucantha* leaf extracts were calculated from the computerized linear equations as followed: $y = 164x - 542.69$ on brine shrimp cytotoxicity; $y = 40.784x - 25.659$ on DPPH radical scavenging; and $y = 164x - 542.69$ on tyrosinase inhibition. Where x was obtained by substituting $y = 50$, the antilog x gave the value of either the LC_{50}, EC_{50} or IC_{50}, respectively. The mild brine shrimp inhibition could be correlated with the traditional uses of squeezed extract of fresh leaves against skin cancer, breast cancer and cancer of the cervix. Further study against cancer cell lines should be performed.

The DPPH radical scavenging activity (approx.17.08, 17 and 58.94 times less effective than BHT, BHA and vitamin C respectively), suggested the leaves as a source of antioxidants and be effective in diseases caused by overproduction of radicals.

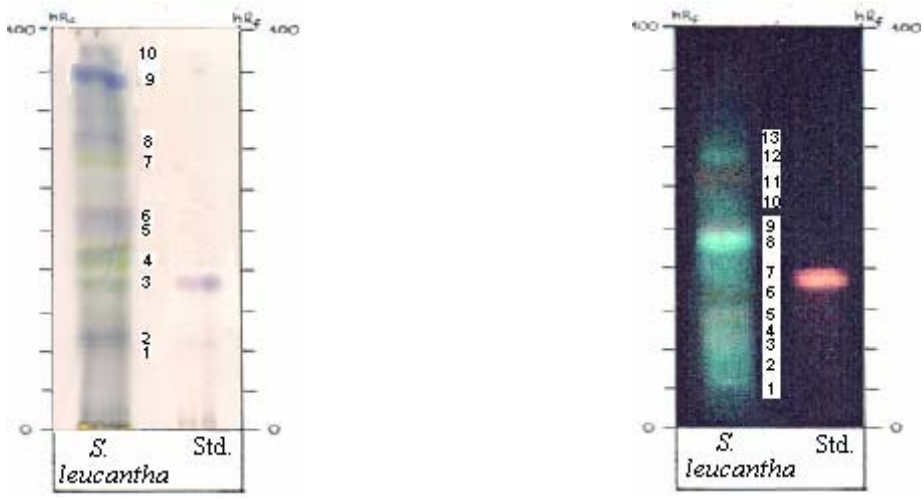
The anti-tyrosinase activity (approx.4578 times less effective than standard kojic acid) meant there were some melanin biosynthesis inhibitors in the 20%ethanol extract.

The *in vitro* anti-microbial activity against various pathogens coincided with the traditional uses to relieve respiratory tract infection.

Zoning patterns of steroids-terpenes and flavonoids on the TLC fingerprints, as well as the RP-HPLC fingerprint of the flavonoid extract, were specific enough to be references for future identification of the *S. leucantha* leaf powder.

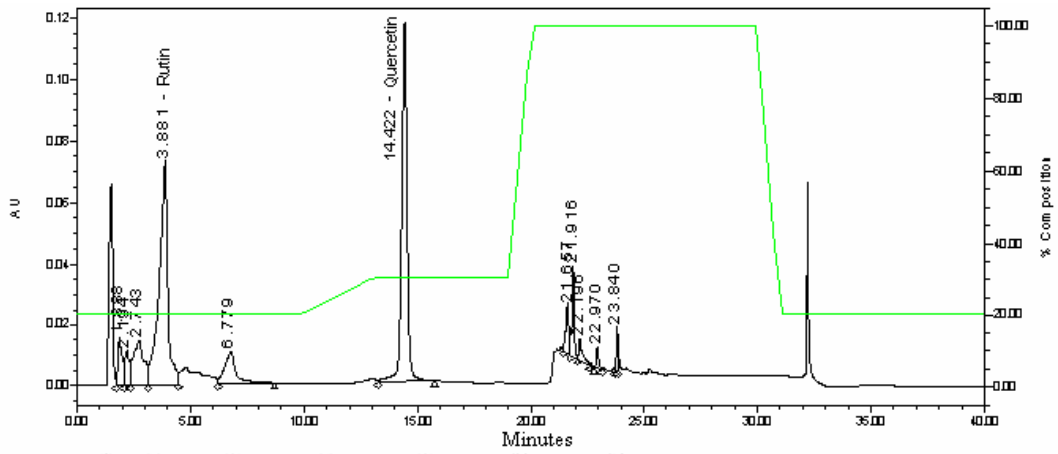
Conclusion

These results revealed that the leaves of *S. leucantha* had some values for further utilization study, either to be developed as an anti-cancer or a source of antioxidants, anti-hyperpigmentation agents, and antibiotics.



a) Steroids-terpenes compared to ß-sitosterol b) Flavonoids compared to rutin

Figure 1: TLC fingerprints of 2 different extracts from the leaf powder of *S. leucantha* developed in suitable solvent systems
(a) steroids-terpenes in dichloromethane-ethylacetate-formic acid (60:5:1),
(b) flavonoids in ethyl acetate-formic acid-acetic acid-water (100:11:11:26).



		RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
	1	1.888	186446	3.31	15658	4.96
	2	2.194	156090	2.77	11167	3.54
	3	2.743	477307	8.48	14597	4.62
Rutin	4	3.881	1921780	34.14	73102	23.16
	5	6.779	362846	6.45	10507	3.33
Quercetin	6	14.422	1978697	35.15	116130	36.79
	7	21.657	153704	2.73	16455	5.21
	8	21.916	206926	3.68	29375	9.31
	9	22.196	79835	1.42	7016	2.22
	10	22.970	48983	0.87	7216	2.29
	11	23.840	56398	1.00	14438	4.57

Pump Gradient Table

	Time (min)	Flow (ml/min)	%A	%B	Curve
1		1.00	80.0	20.0	
2	10.00	1.00	80.0	20.0	6
3	13.00	1.00	70.0	30.0	6
4	19.00	1.00	70.0	30.0	6
5	20.00	1.00	0.0	100.0	6
6	30.00	1.00	0.0	100.0	6
7	31.00	1.00	80.0	20.0	6
8	40.00	1.00	80.0	20.0	6

Figure 2: RP-HPLC fingerprint of the flavonoid extract from the leaf powder of *S. leucantha* showing polar and non-polar peaks compared with reference standard peaks of rutin (3.881min RT) and quercetin (14.422min RT) at 270nm.

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