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ANTIMICROBIAL, ANTIOXIDANT AND ANTICANCER ACTIVITIES OF *STRYCHNOS LUCIDA* R. BR.

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Abstract

Backgroud: *Strychnos lucida* R. Br. (Loganiaceae), a well-known indigenous medicine in Timor Leste, has been used for the treatment of ailments such as malaria, diarrhoea, fever, hypertension, cancer, diabetes mellitus and skin infections. Its pharmacological activity has never been reported. The aim of this study was to determine the biological activities of *S. lucida*, including antimicrobial, antioxidant and anticancer activities.

Materials and methods: The stem, stem bark, twig and leaves of *S. lucida* were extracted by non-polar (hexane) and polar (ethyl acetate and methanol) solvents. Antimicrobial activity of the plant extracts against 29 microorganisms was evaluated using the agar dilution method and antioxidant properties were determined using DPPH and SOD assays. Anticancer activity was investigated against HepG2, HuCCA-1, A549 and MOLT-3 cell lines using the MTT and XTT assays.

Results: It was found that the hexane and ethyl acetate extracts of *S. lucida* selectively inhibited the growth of Gram positive bacteria (*Bacillus subtilis* ATCC 6633, *Bacillus cereus* and *Streptococcus pyogenes*) with MICs range of 32-128 μg/mL. Antioxidant activities, radical and superoxide scavenging properties, were observed for ethyl acetate and methanol extracts of *S. lucida*. Particularly, the ethyl acetate extracts selectively showed inhibitory activity against MOLT-3 cells. Notably, the plant extracts showed the relationship between antimicrobial, antioxidant and anticancer activities.

Conclusion: The findings support the medicinal usage of *S. lucida*.

Key words: *Strychnos lucida*; antimicrobial activity; antioxidant activity; anticancer activity

Introduction

Medicinal plants have a long history of use as traditional medicine or folk remedy for the treatment of ailments. Most people in rural area or developing countries still rely on plant-derived preparations for primary health care (Pal and Shukla, 2003; Ramawat et al., 2009). *Strychnos lucida* R. Br. (*Strychnos ligustrina*) is a tree commonly known in Timor Leste as Ai baku moruk, in Indonesia as Bidara laut or Kayu songga, and in Thailand as Phayaa mue lek. This tree belongs to the Loganiaceae family consisting of more than 300 species grown all over the world (Rajesh et al., 2012). *S. lucida* is grown in the dry to wet mountain regions in South East Asian countries including Timor Leste.

Most Timorese people recognize this tree as a traditional medicine serving as an alternative for treating malaria, diarrhoea, inflammatory fever, hypertension, cancer, skin infections, diabetes mellitus as well as antidote for snake bite. Traditionally, people would prepare this folk remedy by scrapping its stem bark into powder, mixing with hot water, then allowing the sediments to settle at the bottom of the cup before drinking. In the western part of Timor Island (East Nusa Tenggara–Indonesia), *S. lucida* is also commercially available, particularly, its bark for making "songga cup" and sold for remedial purposes.

Phytochemical study of the bark of *S. lucida* was previously reported (Itoh et al., 2006). The plant species contained a variety of compounds such as brucine, brucine *N*-Oxide, loganic acid, loganin, ligustrinoside, *β*-colubrine, diaboline, 11-methoxydiaboline, secoxyloganin, sweroside, picconioside I, sylvestroside I, vanillic acid 4-*O*-*β*-D-glucoside, syringaresinol 4-*O*-*β*-D-glucoside, chlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, adenosine and 4-*O*-(3,5-dimethoxy-4-hydroxybenzoyl) quinic acid. Although numerous compounds have already been identified, the biological evaluation of *S. lucida* has never been reported.

Medicinal plants are of prime importance in the treatment and prevention of diseases. The scientific basis of which is required for possible use of herbal drugs in the treatment of diseases such as cancer, infections and those related with oxidative damage (Tamokou et al., 2013). This study thus reports the antimicrobial, antioxidant and cytotoxic activities of *S. lucida*.

Materials and Methods

General

Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker AVANCE 300 operating at 300 MHz. Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Reagents for cell culture and assays were of analytical grade: RPMI-1640 (Rosewell Park Memorial Institute medium), Ham's/F12 (Nutrient mixture F-12), DMEM (Dulbecco's Modified Eagle's Medium) and FBS (fetal bovine serum)

from Hyclone laboratories (USA); HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), L-glutamine, penicillin-streptomycin, sodium pyruvate, glucose, *α*-tocopherol, DPPH (2,2-diphenyl-1-picrylhydrazyl), MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), nitroblue tetrazolium (NBT) salt, L-methionine, riboflavin, Triton-100, superoxide dismutase (SOD) from bovine erythrocytes, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt) from Sigma-Aldrich (USA), DMSO (dimethyl sulfoxide, 99.9%) from Merck, and gentamicin sulfate from Government Pharmaceutical Organization (Thailand).

Plant Materials

Four parts of *S. lucida* (stem, stem bark, twig and leaves) were collected from the town of Soibada, Manatuto District–Timor Leste. The specimens were identified by the Department of Forestry–Ministry of Agriculture with the tag number (MAPDNFP 00281).

Extraction

The dried powders of *S. lucida*; stem (350 g), stem bark (150 g), twig (150 g) and leaf (50 g) were separately extracted with 1 L of hexane (3x5 days), they were filtered and the respective filtrate was evaporated *in vacuo* to provide hexane extracts of stem (0.4 g), stem bark (0.85 g), twig (3.67 g) and leaf (3.93 g). Similarly, the sequential extract of *S. lucida* were performed using 750 mL of ethyl acetate to give ethyl acetate extracts of stem (0.23 g), stem bark (1.74 g), twig (2.39 g) and leaf (0.6g). Finally, the four parts of *S. lucida* were extracted using 750 mL of methanol to yield methanol extracts of stem (8.0 g), stem bark (16.27 g), twig (14.08 g) and leaf (6.91 g). The ^{1}H-NMR (CD_3OD) and IR (Nujol) spectra of the plant extracts were recorded.

Bioactivity Tests
Antimicrobial Assay

The plant extracts were evaluated for antimicrobial activity using the agar dilution method (Baron et al., 1994). The extract was dissolved in DMSO (99.9%), and mixed with 1 mL of Müeller Hinton Broth (MHB). The DMSO (0.5%) as a control was parallel tested in this study. Each of the tested extracts was then transferred to Müeller Hinton Agar (MHA) solution to give final concentrations of 4-256 µg/mL. All microorganisms were initially cultured in MHB at 37°C overnight and were diluted with 0.9% normal saline to adjust the inoculum density to $1x10^{8}$cell/mL. The microorganisms were inoculated onto the extract-agar plates and incubated at 37°C for 24-48 h. The growth inhibition of microorganisms was analyzed. Twenty-nine microorganisms were used for the assay, including the Gram negative bacteria**,** *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Serratia marcescens* ATCC 8100, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas stutzeri* ATCC 17587, *Shewanella putrefaciens* ATCC 8071, *Achromobacter xylosoxidans* ATCC 27061, and clinical strains included *Shigella dysenteriae*, *Salmonella enteritidis*, *Morganella morganii*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Plesiomonas shigelloides*; the Gram positive bacteria tested include *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* ATCC 33186, *Micrococcus luteus* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, and clinical strains included *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus cereus*, *methicillin-resistant Staphylococcus aureus*, and the diploid fungus (yeast) were *Candida albicans* ATCC 90028 and *Saccharomyces cerevisiae* ATCC 2601. The clinical strains were obtained from the Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technolgy, Mahidol University, Thailand.

Antioxidant Assay

The plant extracts (dissolved in DMSO) were investigated for their antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide dismutase (SOD) assays.

DPPH, a stable purple color solution reacted with the antioxidant compounds to yield light-yellow color product of 1,1-diphenyl-2-picrylhydrazine which was measured by spectrophotometer (Yen and Hsieh, 1997). The reaction was initiated by adding 1 mL of 0.1 mM DPPH solution (dissolved in methanol) to the tested plant extract, and *α*-tocopheral (a positive control) with the final concentration of 300 µg/mL. The reaction was incubated in a dark room for 30 min, and the absorbance was recorded at 517 nm using UV-Visible spectrophotometer (UV-1610, Shimadzu). The percentage of radical scavenging activity (RSA) was calculated according to the following equation:

$$\text{RSA (\%)} = \left(1 - \frac{Abs._{sample}}{Abs._{control}}\right) \times 100 \quad (1)$$

where $Abs._{control}$ is the absorbance of the control reaction, and $Abs._{sample}$ is the absorbance of the tested compound.

SOD activity was determined using SOD assay (Piacham et al., 2003). The assay was performed by adding 1 mL of stock solution containing 27 mL of HEPES buffer (50 mM, pH 7.8), 1.5 mL of L-methionine (30 mg/mL), 1 mL of NBT (1.41 mg/mL) and 750 µL of Triton X-100 (1 %wt) to the plant extract. The photochemical reaction was carried out by adding 10 µL of riboflavin (44 mg/mL) to the tested extracts (final concentration of 300 µg/mL), followed by illumination under a Philips Classic Tone lamp (60 W) in a light box for 7 min. The absorbance of the reaction was measured at 550 nm using UV-Vis spectrophotometer. The percentage of SOD activity was calculated using Eq. (1). The SOD enzyme was used as a control.

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Cytotoxic Assay

Anticancer activity of the plant extracts was studied (Tengchaisri et al., 1998) against four human cancer cell lines including hepatocellular carcinoma (HepG2), cholangiocarcinoma (HuCCA-1), lung carcinoma (A549) and T-lymphoblast (MOLT-3, acute lymphoblastic leukemia) cell lines.

The assay was performed using the cell lines suspended in RPMI-1640 containing 10% FBS, which were seeded at a density of 5×10^3 - 2×10^4 cells per well in a 96-well plate (Costar No. 3599, USA) and then incubated at 37°C under a humidified atmosphere with 95% air and 5% CO_2 for 24 h. An equal volume of additional medium containing either serial dilutions of the tested compound (and a positive control) or a negative control (DMSO), was added to the desired final concentrations. The plates were further incubated for 48 h. Cell viability was determined by staining with MTT for adherent cell (A549, HuCCA-1 and HepG2 cells), and with XTT for suspended cell (MOLT-3 cells). The plates were read on a micro-plate reader (Molecular Devices, USA). The absorbance was recorded at 550 nm. IC_{50} values were deduced as the compound or drug concentration that provided 50% cell growth inhibition.

The tested cells; HuCCA-1 and A549 cells were grown in Hamm's/F12 medium containing L-glutamine (2 mM) supplemented with 100 U/mL penicillin-streptomycin and FBS (10%); MOLT-3 cells were grown in RPMI-1640 medium containing L-glutamine (2 mM), 100 U/mL penicillin-streptomycin, sodium pyruvate, glucose and 10% FBS; and HepG2 cells were grown in DMEM medium containing 100 U/ mL penicillin-streptomycin and 10% FBS.

Results

Chemical Components of the Plant Extracts

The plant extracts showed their IR absorptions of OH (hydroxyl) group at 3300-3407 cm^{-1}, and of CO (carbonyl) group at 1711-1749 cm^{-1} while the ^1H NMR spectra showed proton chemical shifts at δ 0.8-2.8, 3.4-4.4, 4.6-5.5, and 6.2-7.8 ppm. It was found that non-polar hexane extracts of the four parts of *S. lucida* contained a mixture of triterpenoids such as *β*-sitosterol. Components of polar ethyl acetate plant extracts were phenolic compounds and triterpenes. The most polar methanol extracts constituted mainly phenolics and aromatic compounds.

Antimicrobial Activity

The results of the antimicrobial assay as indicated in Table 1 showed that the hexane extracts (stem bark and twig) and the ethyl acetate extract (twig) were active against *B. subtilis* ATCC 6633, *B. cereus* and *S. pyogenes* with MICs range of 32-128 µg/mL. However, the growth inhibitions of the other 26 microorganisms were not observed.

Table 1: Antimicrobial activity (MIC) of *S. lucida.*

Plant extracts	Microorganism[a]	MIC (µg/mL)
Hexane (stem bark)	*B. subtilis* ATCC 6633	32
	B. cereus	64
	S. pyogenes	32
Hexane (twig)	*B. subtilis* ATCC 6633	32
	B. cereus	64
	S. pyogenes	64
Ethyl acetate (twig)	*B. subtilis* ATCC 6633	64
	B. cereus	64
	S. pyogenes	128
Ampicillin	S. *typhimurium* ATCC 13311	-[b]
	P. stutzeri ATCC 17587	
	C. diphtheriae NCTC 10356	
	S. aureus ATCC 29213	
	S. aureus ATCC 25923	
	S. epidermidis ATCC 12228	
	M. luteus ATCC 10240	
	B. subtilis ATCC 6633	
	S. pyogenes	
	L. monocytogenes	
	P. shigelloides	

MIC: Minimum inhibitory concentration was the lowest concentration that inhibited the growth of microorganisms. [a]Growth inhibitions of the other 26 microorganisms were not observed.

[b]Ampicillin at 10 µg/mL was used as a control of antibacterial testing system; it showed 100 % inhibition against the listed microorganisms.

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Antioxidant Activity

As indicated in Table 2, the ethyl acetate and methanol extracts of *S. lucida* displayed antioxidant potency (RSA) with IC_{50} ranges of 73.07 – 224.20 µg/mL and 50.63 – 143.13 µg/mL, respectively. All hexane extracts were found to be inactive antioxidants.

The SOD activity (Table 3) of the methanol extracts had IC_{50} ranges of 15.21 – 50.00 µg/mL while of the ethyl acetate extracts showed the IC_{50} of 40.26 – 126.00 µg/mL. In addition, the hexane extract of the stem bark and that of the twig exhibited SOD activity with the IC_{50} of 93.31 and 112.84 µg/mL, respectively. However, the hexane extract of the stem and that of the leaf did not show any antioxidant effect.

Table 2: Radical scavenging activity (IC_{50}) of *S. lucida*.

Plant extract	IC_{50} (µg/mL)[a]		
	Hexane	Ethyl acetate	Methanol
Stem	-[b]	224.20	143.13
Stem bark	-[b]	66.12	60.76
Twig	-[b]	73.07	70.40
Leaf	-[b]	154.00	50.63

[a]*α*-Tocopherol was used as a control, IC_{50} was 8.63µg/mL.
[b]At 300 µg/mL, all hexane extracts showed 2.58, 46.50, 26.13 and 46.39% inhibitions, respectively. A compound with <50% inhibition was indicated as an inactive antioxidant.

Table 3: Superoxide dismutase activity (IC_{50}) of *S. lucida*.

Plant extract	IC_{50} (µg/mL)[a]		
	Hexane	Ethyl acetate	Methanol
Stem	-[b]	71.16	50.00
Stem bark	93.31	53.46	15.21
Twig	112.84	40.26	25.52
Leaf	-[b]	126.00	21.56

[a]SOD enzyme was used as a control, IC_{50} was 0.18 µg/mL.
[b]Inhibition <50% at 300 µg/mL was indicated as an inactive antioxidant.

Anticancer Activity

As shown in Table 4, the hexane and methanol extracts displayed anticancer activity at 30 µg/mL toward the tested cancer cells, except for the ethyl acetate extracts which showed inhibitory effect against HuCCA-1, A549 and MOLT-3 cells. Anticancer activity was also assayed at 10 µg/mL as depicted in Table 5, mostly less than 10% cell growth inhibitions were noted.

Table 4: Cytotoxic activity of *S. lucida* extracts at 30 µg/mL.

Plant extract	Cytotoxicity (%)[a,b]			
	HepG2	HuCCA-1	A549	MOLT-3
Hexane				
Stem	3	18	3	17
Stem bark	0	9	4	37
Twig	1	20	3	19
Leaf	1	14	6	26
Ethyl acetate				
Stem	0	20	16	32
Stem bark	0	10	5	70
Twig	0	8	5	68
Leaf	0	5	3	86
Methanol				
Stem	2	30	6	10
Stem bark	5	20	11	4
Twig	0	20	5	10
Leaf	0	10	8	4
Doxorubicin[c]	0.26±0.040	0.49±0.063	0.16±0.028	-
Etoposide[c]	20.97±0.590	-	-	0.03±0.003

[a]The assays were performed in triplicate.
[b]Cytotoxicity <50% indicated as an inactive compound.

[c]Doxorubicin and etoposide were used as the reference drugs, and their cytotoxicities were expressed as IC_{50} (µg/mL).

Table 5: Cytotoxic activity of *S. lucida* extracts at 10 µg/mL.

Plant extract	Cytotoxicity (%)[a,b]			
	HepG2	HuCCA-1	A549	MOLT-3
Hexane				
Stem	2	6	0	3
Stem bark	0	9	0	6
Twig	3	13	0	5
Leaf	1	6	2	4
Ethyl acetate				
Stem	0	4	4	4
Stem bark	0	2	1	13
Twig	0	0	0	16
Leaf	0	0	0	23
Methanol				
Stem	1	4	6	3
Stem bark	0	7	3	2
Twig	1	3	1	0
Leaf	0	1	8	0
Doxorubicin[c]	0.26±0.040	0.49±0.063	0.16±0.028	-
Etoposide[c]	20.97±0.590	-	-	0.03±0.003

[a]The assays were performed in triplicate.
[b]Cytotoxicity <50% indicated as an inactive compound.
[c]Doxorubicin and etoposide were used as the reference drugs, and their cytotoxicities were expressed as IC_{50} (µg/mL).

Discussion

The biological study of *S. lucida* showed that the plant extracts with non-polar (hexane) and polar (ethyl acetate) solvents exerted antimicrobial, antioxidant and anticancer activities. Bioactive hexane extracts and ethyl acetate extracts selectively inhibited the growth of the Gram positive bacteria: *B. subtilis* ATCC 6633, *B. cereus* and *S. pyogenes*. Relatively higher antimicrobial activity against *B. subtilis* ATCC 6633 and *S. pyogenes* was noted for the hexane extracts (stem bark and twig) when compared with the ethyl acetate extracts (twig). So far, the antibacterial activity of *S. lucida* has not been reported in literature. However, the extracts of *S. lucida* had been documented to exhibit strong antimalarial activity against *Plasmodium falciparum* and *Babesia gibsoni* (Murnigsih et al., 2005). Other *Strychnos* species have however been shown to display antimicrobial activity. For example, *S. potatorum* exhibited inhibitory action against Gram positive bacteria such as *S. aureus*, and Gram negative bacteria like *Proteus vulgaris, S. typhimurium* and *Vibrio cholerae* (Mallikharjuna and Seetharam, 2009). *S. wallichiana* was also reported to have displayed antimicrobial activity against *E. coli, S. aureus, B. subtilis, Aspergillus niger* and *Mucor* spp. (Mallikharjuna et al., 2010). In addition, *S. nux-vomica* exerted antimicrobial activity against *S. aureus, S. typhimurium, K. pnemoniae, A. flavus* and *A. niger* (Gnanavel et al., 2012).

Strong radical scavenging activity was found in the polar solvent extracts with the methanol extract displaying higher activity than the ethyl acetate extract. The highest activity of the methanol extract was observed for the leaf (IC_{50} of 50.63 µg/mL) while for the ethyl acetate extract, the highest activity was observed for the stem bark (IC_{50} of 66.12 µg/mL) of the plant species. This could be due to phenolic containing components as indicated by the IR spectra with very strong OH group absorptions at 3397, 3401, 3407 cm^{-1} and ^{1}H NMR spectra showing aromatics protons at δ 6.2-7.8 ppm. The hexane extracts of *S. lucida* were shown to be inactive antioxidants, indicated by <50% RSA at 300 µg/mL. Similarly, stronger SOD activity was noted for methanol extracts comparing to ethyl acetate extracts. The highest SOD activity of methanol extracts was found in the stem bark (IC_{50} of 15.21 µg/mL), while of the ethyl acetate extracts was noted for twigs (IC_{50} of 40.26 µg/mL) of the *S. lucida*. However, weak SOD activity was seen in the hexane extracts (stem bark and twig) containing triterpenoids and fatty ester compounds. Antioxidant activities of *S. lucida* were not previously reported. Many plant species possessed antioxidant potency, for example, lignan glucosides of *S. vanprukii* displayed stronger antioxidant effect than ascorbic acid (Thongphasuk et al., 2004), and *S. nux-vomica* had SOD activity (Vijayakumar et al., 2009). In addition, *S. henningsii* (Oyedemi et al., 2010) and *S. mitis* (Adamu et al., 2014) were shown to exert antioxidant activity. Interestingly, only the ethyl acetate extracts (30 µg/mL) of stem bark, twig and leaf of *S. lucida* showed selective anticancer activity against MOLT-3 cells with 70, 68 and 86% inhibition, respectively. This is the first report on the anticancer activity of *S. lucida*. This study reveals the relationship between antioxidant and anticancer activities, and between antimicrobial and antioxidant activities as well as antimicrobial and anticancer activities. Particularly, these relationships were observed in the ethyl acetate extracts containing phenolics and triterpenoids. In fact, antioxidants could reduce the risk of cancers, aging, atherosclerosis and other oxidants-induced diseases. Thus, antioxidants can protect living organisms from oxidative damage caused by reactive oxygen species (Li et al., 2007). Medicinal plants with antioxidant and anticancer (Li et al., 2007; Charoensin, 2014); and with anticancer and antimicrobial (Tamokou et al., 2013) activities have been reported. In addition, a variety of medicinal plants have been documented to exert dual actions as antimicrobials and antioxidants (Prachayasittikul et al., 2008, 2009, 2010, 2012; Treeratanapiboon et al., 2011).

In conclusion, *S. lucida* was hereby reported for the first time as antibacterial, antioxidant and anticancer. This finding provides scientific justification for the use of *S. lucida* as traditional medicine by the Timorese people.

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