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In vitro anti-oxidant, anti-microbial and anti-HIV-1 reverse transcriptase activities and isolation of bergenin from *Shorea obtusa* Wall. ex Blume

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Abstract

Several *Shorea* species have been reported to have pharmaceutical properties with associated potential for use in drug discovery. While ethnobotanical surveys have investigated the medicinal use of the trunk of *Shorea obtusa* Wall. Ex Blume, there are no studies covering the tree's bioactive properties. In this study, bark and wood extracts of *S. obtusa* were investigated for total phenolic (TPC) and total flavonoid (TFC) contents, anti-oxidants, anti-microbial activity and anti-HIV-1 reverse transcriptase (RT) activities. The bark extract contained (mean ± SD) 429 ± 0.41 mg gallic acid equivalents/g TPC and 12.24 ± 0.02 mg quercetin equivalents/g TFC. Compared to the wood extract, the bark extract had greater anti-oxidant activity and half maximal inhibitory concentration of 2,2-diphenyl-1-picrylhydrazyl radical scavenging (400.40 ± 13.72 µg/mL), superoxide radical scavenging (629.48 ± 20.48 µg/mL) and ferric reducing anti-oxidant power (403.48 ± 3.96 trolox equivalent/g extract). Thus, the bark extract can be considered a potential source to develop anti-microbial and HIV-1 RT agents. The bark extract had great potential anti-microbial activity against four species of microbes but not against *Micrococcus luteus*. Specifically, the bark extract showed better activity against *Escherichia coli* and *Bacillus subtillis* strains than standard drugs and had an inhibitory effect against HIV1-RT (88.13 ± 1.80%). In addition, bergenin was purified and isolated from the bark using recrystallization and was then identified using nuclear magnetic resonance spectroscopy and mass spectrometry. Overall, the results suggested that *S. obtusa* extract could provide a promising natural source for the development of novel plant-based agents with anti-oxidant, anti-microbial and anti-HIV activity.

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Introduction

It has been known for centuries that medicinal plants provide a rich resource for natural drug research and development (Pan et al., 2013). Secondary metabolites in medicinal plants have been reported to have pharmacological properties that are beneficial (Chivian et al., 1993). Furthermore, due to their various pharmacological activities, medicinal plants have limited side effects, less toxicity and a lower cost than standard drugs (Citarasu, 2012; Simon and Prince, 2016). Thus, there is considerable interest in using natural sources such as *S. obtusa* to obtain alternative novel drugs.

Reactive oxygen species, such as superoxide and the hydroxyl, alkoxyl, peroxyl and nitric oxide radicals, play an important role in oxidative stress as they can cause damage to cellular structures and are related to various infections (Foley et al., 2016), inflammation (Mittal et al., 2014), and diseases, including cancer (Schumacker, 2015), cardiovascular (Panth et al., 2016), Parkinson's (Puspita et al., 2017), ocular (Nita and Grzybowski, 2016), and acquired immune deficiency syndrome (AIDs), according to Couret and Chang (2016). To protect against oxidative damage, such diseases are often treated with anti-oxidants and plants provide a rich source of natural anti-oxidants, such as flavonoids, phenols, lignins, tannins and terpenoids, which display pharmacological properties (Heitzman et al., 2005; Xu et al., 2017; Lourenço et al., 2019). Based on such literature, medicinal plants are commonly used to treat diseases due to their therapeutic properties and powerful anti-oxidant activity.

Infectious diseases caused by microorganisms pose serious problems worldwide, whereby antibiotic resistance has become increasingly apparent in human pathogenic microorganisms in recent years (Manandhar et al., 2017). For example, Chandra et al. (2017) reported the reduced effectiveness of antibiotics due to genetic changes in microorganisms. Another disadvantage of antibiotics is the side effects presented in human patients, such as upset stomach, vomiting and diarrhea (associated with cephalosporins, macrolides, penicillins and tetracyclines), rash and allergic reactions (associated with cephalosporins and penicillins), sensitivity to sunlight (associated with by tetracyclines), nervousness, tremors and seizures (associated with by quinolones), disruption of auditory function (associated with aminoglycosides), damage to kidneys (associated with aminoglycosides and polypeptides) and the liver (associated with rifampin) according to Demain and Sanchez (2009). Therefore, the development of novel anti-microbial agents that lack such adverse effects is urgently needed. Various medicinal plants have been reported to have anti-microbial activity due to their secondary metabolites, including alkaloids, flavonoids, phenols, steroids and terpenoids (Duraipandiyan et al., 2006; Demain and Sanchez, 2009; Djeussi et al., 2013). Therefore, increasing attention has been given to medicinal plants as a potential anti-microbial source for synthesizing novel drugs.

It is known that human immunodeficiency virus type 1 (HIV-1) causes AIDs and that the virally encoded enzyme reverse transcriptase (RT) converts the viral RNA genome into pro-viral DNA (Hu and Hughes, 2012). Since reverse transcription is an essential step in HIV-1 infection (Jonckheere et al., 2000), HIV-1 RT is a key enzymatic target to inhibit propagation in host cells. However, toxicity and complex development hinder the development of new drugs against HIV (Sarafianos et al., 2009; Silprasit et al., 2011). The current HIV-1 RT inhibitors, such as nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), are known to have adverse side effects and resistance profiles (Rai et al., 2018). Studies have suggested that medicinal plants are a potential source to develop safe and inexpensive drugs with potential metabolites for HIV-1 RT inhibitors, such as flavonoids from *Rhus succedanea* (Lin et al., 1997), garcisaterpenes A and C from *Garcinia speciosa* (Rukachaisirikul, 2003) and nitidine from *Toddalia asiatica* (Tan et al., 1991).

Shorea species have been reported as potential plants with anti-oxidant and anti-bacterial activities (Ragini et al., 2011; Subramanian et al., 2013; Daud et al., 2014; Suganya et al., 2014). Specifically, *Shorea obtusa* Wall. Ex Blume is a species of flowering plant in the family Dipterocarpaceae, which is widely distributed in Myanmar, Thailand, Cambodia, Laos and Vietnam. Other common names include Burmese sal, Siamese sal and thitya; it is widely known as 'teng' in Thailand (Pupatanapong et al., 1987). This deciduous tropical tree dominates dry dipterocarp forests and the bark of this tree is commonly used for its astringent and anticoagulant properties to help reduce bleeding (Pupatanapong et al., 1987). In terms of the biological activity of *S. obtusa*, the root extract has an anti-bacterial effect against *Mycobacterium smegmatis* at 250 µg/mL (Chea et al., 2007). However, there are currently no studies showing the effects of its bark and wood on microorganisms nor on the chemical profiles of extracts. Due to the scant data on the biological potential of *S. obtusa,* its biological activity should be further investigated to identify the major components. Therefore, this study focused on the total phenolic and flavonoid contents and the anti-oxidant, anti-microbial and anti-HIV1 activities and the identification of the bioactive compounds of extracts from the bark and wood of *S. obtusa*.

Materials and Methods

General chemicals and materials

The following chemicals were obtained and used as is without further purification unless otherwise noted: methanol (analytical grade; Merck, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH; Merck, USA), tris-HCl buffer (Tris; Amresco, USA), nitroblue tetrazolium (NBT; Sigma-Aldrich, China), *b*-nicotinamide adenine dinucleotide (NADH; Sigma, Germany), phenazine methosulfate (PMS; Sigma, Ukraine) sodium nitroprusside (SNP; Himedia, India), sulfanilamide (Carlo Erba, France), phosphoric acid (Macron Fine Chemicals, China), naphthaylethylenediamine hydrochloride (AppliChem Panreac, Germany), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ; Fluka, Switzerland), ferric chloride (Chem-supply, Australia), Folin-Ciocalteu (Merck, USA), sodium carbonate (Merck, Germany), gallic acid (Merck, USA), aluminum trichloride (Univar, New Zealand) and quercetin acid (Sigma-Aldrich, Germany). Whatman® grade 1 filtration paper was obtained from Sigma Aldrich (USA). The analysis used a Büchi Rotavapor® R-210 (Mumbai, India), a T60-visible spectrophotometer (PG Instruments, UK), a nuclear magnetic resonance instrument (NMR; Bruker Avance III HD 400 and a 100 MHz NMR spectrometer; USA) and a mass spectrometer (MS; microtof-Q III, Bruker, USA).

Plant material and extract preparation

The bark and wood of *S. obtusa* were collected from Chainat province, central Thailand, in March 2015. The voucher specimen (numbered PCERU_SO1) of the plant was identified and deposited in the Department of Botany, Kasetsart University, Bangkok, Thailand. Dried powdered bark (6.80 kg) and wood (4.00 kg) were macerated in methanol for 7 d. The methanol solution was filtered and evaporated under reduced pressure to obtain the bark extract (375.73 g; 5.52% of yield) and wood extract (233.63 g; 5.83% of yield), which were then stored at -20°C until biological activity analysis.

Quantification of phenolic and flavonoid compounds

Total phenolic content

Determination of the total phenolic content in the methanol extract was measured as described by Folin and Ciocalteu (1927). An amount (1.25 mL) of 0.2 mM Folin-Ciocalteu reagent was mixed with 0.25 mL of sample (bark and wood methanol extracts at 1 mg/mL). This mixture was left to stand at room temperature for 5 min, followed by the addition of 1 mL Na_2CO_3 (75g/L). The mixture was incubated in the dark for 60 min at room temperature. The absorbance was measured at 765 nm. The total phenolic content, expressed as gallic acid (0–300 mg/L), was calculated based on the calibration curve using the following equation: $y = 0.0014x + 0.0034$ with the coefficient of determination (R^2) = 0.9993. The results were expressed as milligrams of gallic acid equivalents (GAE)/L of dry mass.

Total flavonoids content

The total flavonoids content was determined based on the method reported by Arvouet-Grand et al. (1994). An amount (1.5 mL) of sample (bark and wood methanol extracts at 1 mg/mL) was mixed with 1.5 mL of 2% $AlCl_3$ in methanol. After 15 min, the absorbance was read at 415 nm. The total flavonoid contents, expressed as quercetin (0–75 mg/L), was calculated based on the calibration curve using the following equation $y = 0.0393x + 0.0722$ with $R^2 = 0.9932$. The results were expressed as milligrams of quercetin equivalents (QE)/kg of dry mass.

Anti-oxidant activity

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The scavenging activity was studied using the DPPH free radical, as described by Blois (1958) with some modifications. Bark (31.25–1,000 µg/mL) and wood (625–5,000 µg/mL) extracts (in 1.5 mL methanol) were mixed with 0.2 mM methanolic DPPH solution (1.5 mL). After an incubation period of 30 min at 25°C, the absorbance was read at 520 nm.

Superoxide radical scavenging activity

Superoxide radical scavenging was generated by the NADH-phenazine methosulfate (PMS) system, according to a procedure described by Kuo et al. (2001). The reaction mixture contained bark (62.5–1,000 µg/mL) and wood (100–5,000 µg/mL) extracts (in 1 mL methanol), 936 µM NADH (1 mL), and 300 µM NBT (1 mL). After incubation at room temperature for 10 min, the reaction was initiated by adding 120 µM PMS (1 mL). The reaction mixture was incubated at ambient temperature for 5 min; then, the absorbance (560 nm) of each sample was compared against blank samples.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was modified by the Griess reaction, according to the method by Suksungworn

et al. (2020). Sodium nitroprusside (10 mmol/L) in phosphate buffer saline (1.25 mL) and bark (12.5–200 µg/mL) and wood (50–400 µg/mL) extracts (in 0.25 mL methanol) were mixed, then incubated at 25°C for 150 min. After incubation, Griess reagent (0.5 mL), (prepared from 10 mL, consisting of 100 mg of sulfanilamide, 10 mL of phosphoric acid, and 10 mg of *N*-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the formed chromophore was measured at 546 nm.

Ferric reducing anti-oxidant power activity

The method evaluated using the modified method from Gan et al. (2010). Both extracts at 1 mg/mL (0.15 mL) were added to 2.85 mL of reagent (prepared from 300 mM acetate buffer (100 mL), 10 mM TPTZ solution (10 mL), and 20 mM $FeCl_3$ (10 mL). After 30 min, the absorbance was read at 593 nm. The concentration of the ferric reducing anti-oxidant power (FRAP) content, expressed as trolox (0–500 mg/L), was calculated based on the calibration curve using the following equation: $y = 0.0079x + 0.3955$, with $R^2 = 0.9952$. The results were expressed as milligrams trolox equivalent (TE)/g extract.

Anti-microbial activity

The extracts were tested against resistant microbial strains, three Gram-positive (*Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633), one Gram-negative (*Escherichia coli* ATCC 25922), and one fungus (*Candida albicans* ATCC 10231). The microtiter plates were incubated with Muller Hinton agar at 37°C for 24 hr for the bacteria and with Sabouraud dextrose agar at 30°C for 48 hr for the fungus; then, they were measured using a Multiread 400 Microplate Reader (BIOCHROM, France). Microbe suspensions were adjusted to McFarland No. 0.5 (1×10^8 colony forming units (CFU)/mL) and diluted to contain 5×10^5 CFU/mL. The suspensions were compared to the density of the McFarland No. 0.5 standard then measured against a white background with contrasting black lines. For the sample, the plant extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in the range 0.25–20 µg/mL. Then, the suspension (100 µL) and extract (100 µL) were mixed well in Muller Hinton broth and Sabouraud dextrose broth in 96-well microplates. A sterility control and growth control were compared for each strain. After incubation at 37°C for 24 hr for the bacteria and at 30°C for 48 hr for the fungus, the lowest concentration of extract displaying no visible growth by the naked eye was considered as the minimum inhibitory concentration (MIC).

Anti-HIV-1 Reverse Transcriptase activity

The inhibition assay was determined according to the fluorescence method described by Silprasit et al. (2011). A sample (2 µL) of 30 ng/µL purified recombinant HIV-1 RT was added to the well of a 96-well plate; then 2 µL of extract at1 µg/mL was added to the well (RT_{Sample}). In the control reaction ($RT_{Control}$) and the control of the blank reaction (RT_{Bank}), 2 µL of Tris and 5 µL of ethylenediaminetetraacetic acid (EDTA; 0.2 M) were used. The plate was mixed before adding 4 µL of primer/template polymerization buffer into all wells. Reactions were started by incubation at 37°C for 10 min. After 10 min, the reaction was stopped by the addition of 5 µL of 0.2 M EDTA. The fluorescence was measured at 502 nm of excitation and 523 nm of emission wavelength using a fluorescence microplate reader. Nevirapine was used as the positive control. The percentage of inhibitory effect on HIV-1 RT activity was determined using Equation 1:

$$\%Relative\ inhibition = \frac{(RT_{Control} - RT_{Bank}) - (RT_{Sample} - RT_{Bank})}{(RT_{Control} - RT_{Bank})} \times 100 \quad (1)$$

Isolation and purification of bergenin

The bark extract of *S. obtusa* was evaporated to obtain methanol extract (375.73 g). The bark extract was separated into layers using chloroform ($CHCl_3$; 3 × 500 mL). The $CHCl_3$ layer was collected and evaporated to obtain $CHCl_3$ extract (16.98 g; 0.25% yield). The residue from the separation of $CHCl_3$ was further separated with ethyl acetate (EtOAc; 3 × 500 mL). The EtOAc layer was collected and evaporated to produce the EtOAc extract (44.67 g; 0.67% yield). The precipitate obtained from the EtOAc extract was crystallized in a mixture of $CHCl_3$ and Me_2CO to yield white precipitate (1.85 g). After purification by recrystallization, the structure of the white precipitate, denoted as compound 1, was analyzed using NMR and MS.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6.01 software (San Diego, CA, USA). The unpaired t test was used to compare the statistical significance of treatments, with *p*-values lower than 0.05 considered statistically significant. Results were presented as mean ± SD.

Results and Discussion

Quantification of phenolic and flavonoid compounds

The results of total phenolic (TPC) and total flavonoid content (TFC) of the bark and wood extracts are shown in Fig. 1. The TPC was obtained to colorize Folin and Ciocalteu's reagent, which was expressed as gallic acid equivalents. The TPC of the bark extract (429 ± 0.41 mg GAE/g) was significantly higher than for the wood extract (251 mg GAE/g). The TFC of the bark extract (12.24 ± 0.02 mg QE/g) was also higher compared to that of the wood extract (1.59 ± 0.14 mg QE/g). A similar trend was also noted in the flavonoid content between both extracts. Phenolic and flavonoid compounds commonly found in plants have been reported to exhibit anti-oxidant, anti-bacterial, anti-inflammatory, anti-HIV, and anti-cancer activities (García-Lafuente et al., 2009; Ghasemzadeh and Ghasemzade et al., 2011; Dezsi, et al., 2015; Mohammed et al., 2015).

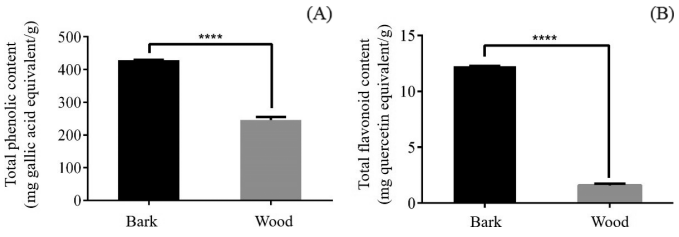

Fig. 1 Total phenolic (A) and total flavonoid contents (B) of *Shorea obtusa* extracts, where each value represents mean ± SD (n = 3) and **** = $p < 0.0001$

Anti-oxidant activities

The Anti-oxidant activities of the extracts from the bark and wood were determined using DPPH, nitric oxide (NO) radical scavenging, superoxide (SO) radical scavenging and FRAP activities (Table 1). The result of DPPH activity, which commonly are used to determine free radical scavenging activity, showed that the half maximal inhibitory concentration (IC_{50}) value of bark extract (400.40 ± 13.72 µg/mL) was higher than that of the wood extract (2,709.24 ± 2.65 µg/mL). The violet color of free radical scavenging from DPPH changed to a yellow color with the addition of extracts. Sodium nitroprusside in buffered saline was used to analyze scavenging of the nitric oxide radical and the wood extract had higher NO radical scavenging (IC_{50} = 44.87 ± 4.92 µg/mL) than the bark extract. A color change can be obtained by reacting Griess reagent with oxygen to produce nitrite ions (Suksungworn and Duangsrisai, 2021). The IC_{50} values of the bark and wood extracts were 629.48 ± 20.48 µg/mL and 5,533.70 ± 492.61 µg/mL, respectively. The FRAP activity is based on the reduction of a complex containing ferric iron and TPTZ into a ferrous colored substance (Gan et al., 2010). The results revealed that the bark extract had higher FRAP activity (403.48 ± 3.96 mg TE/g extract) than the wood extract (69.51 ± 0.70 mg TE/g extract). The data (Table 1) indicated that bark extract had the greater antioxidant potential and greater anti-oxidant activities than the wood extract, except for NO radical scavenging activity. This was consistent with Khan et al. (2012) who suggested that the TPC and TFC of *Launaea procumbens* extract had no correlation with NO radical scavenging activity. In addition, the scavenging activity increased when the concentration of extract increased. Furthermore, it has been suggested that the level of anti-oxidant activity was affected by the phenolic and flavonoid compositions, depending on the structure and chemical functional groups of the extract (Bendary et al., 2013; Duarte et al, 2014). Numerous works have reported the potential anti-oxidant properties of various *Shorea* species, including *S. tumbuggaia* (Ragini et al., 2011), *S. macroptera* (Nazri et al., 2012), *S. roxburghii* (Subramanian et al., 2013; Subramaniana et al., 2015), *S. leprosula* (Sudrajat et al., 2016), *S. robusta* (Suganya et al., 2014) and *S. kunstleri* (Daud, et al., 2014). To investigate the anti-oxidant potential of *S. obtusa* extracts, the current study investigated different activities, consisting of DPPH, NO and SO radical scavenging and FRAP activity. The stronger anti-oxidant activity of the bark extract might have been due to the greater content of phenols and flavonoids compared to the wood extract. From this, it can be suggested that the free radical scavenging capacity was correlated with the contents of phenols and flavonoids, which are

Table 1 Anti-oxidant activities of *Shorea obtusa* extracts, DPPH, nitric oxide (NO), superoxide (SO) radical scavenging, and FRAP activities

Treatment	IC_{50} value (µg/mL)			FRAP (mg TE/g extract)
	DPPH radical scavenging activity	NO radical scavenging activity	SO radical scavenging activity	
Bark extract	400.40±13.72	64.25±6.31	629.48±20.48	403.48±3.96
Wood extract	2,709.24±2.65	44.87±4.92	5,533.70±492.61	69.51±0.70
p-value summary	**	*	****	****

DPPH = 2,2-diphenyl-1-picrylhydrazyl; NO = nitric oxide; SO = superoxide; FRAP = ferric reducing anti-oxidant power; IC_{50} = half maximal inhibitory concentration; TE = trolox equivalent.
Each value represents mean ± SD (n = 3) * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$.

the most effective anti-oxidants (Chandini et al., 2008; Saddiqe et al., 2010). Similar to other reports, the current results revealed a positive relationship between the total phenolic and flavonoid contents and the anti-oxidant ability of extracts from medicinal plants (Kang et al., 2010; Jing et al., 2015; Aryal et al., 2019). Due to their strong redox properties, phenolic compounds play a role in neutralizing and absorbing free radicals, quenching singlet and triplet oxygen, and chelating metal (Catherine et al., 1996; Abdelwahab et al., 2009). Therefore, plant extracts with higher levels of total phenolic and flavonoid contents would exhibit higher anti-oxidant activity. However, the chemical constituents and biological activities of the extracts need to be further evaluated to obtain more complete results.

Anti-microbial activity

The bark extract had slightly higher anti-oxidant activity comparison to the wood extract. Subsequently, the anti-microbial effect of the *S. obtusa* bark extract was investigated against five common microbacteria, consisting of three that are Gram-positive (*S. aureus*, *M. luteus*, *Bacillus subtillis*), one that is Gram-negative (*E. coli*) and one fungus (*Candida albicans*), as shown in Table 2. The results revealed activity against all the microbes, except *M. luteus* (Table 2) with the highest anti-microbial activity against *E. coli*. Compared to commercial drugs, the bark extract of *S. obtusa* had higher potential against *E. coli* (MIC = 1 µg/mL, compared to ampicillin at 10 µg/mL), *B. subtillis* strains (MIC at 4 µg/mL, compared to gentamicin at 10 µg/mL), *S. aureus* strains (MIC at 2 µg/mL; compared to penicillin G at 1 µg/mL), and *C. albicans* (MIC at 20 µg/mL, compared to nystatin at 1 µg/mL). As mentioned, previous studies have reported anti-microbial activity of various *Shorea* species, such as *S. kunstleri*, *S. leprosula*, *S. macroptera* and *S. tolura*. For example, the methanol extract of *S. kunstleri* had activity against *C. albicans* and *S. aureus* at MIC values of 40 and 80 µg/mL (Daud et al., 2014). Interestingly, the current study of anti-microbial activity produced better MIC values of 20 and 2 µg/mL against *C. albicans* and *S. aureus*, respectively. In the other studies, *S. leprosula* extract induced inhibition against *S. aureus* and *E. coli* (Sudrajat et al., 2016), and *S. tolura* extract displayed anti-microbial activity against *E. coli*, *S. aureus* and *L. monocytogenes* (Jutaporn et al., 2011). In addirion, oligomers from the stem bark of *S. macroptera* inhibited the growth of *S. uberis* and *B. subtilis* (Nazri et al., 2012). These results confirmed that *Shorea* species have potential as anti-microbial agents against microorganisms. The root extract of *S. obtusa* at 250 µg/mL had an anti-bacterial effect against *Mycobacterium smegmatis* (Chea et al., 2007). The activity of the bark of *S. obtusa* against some microbes could suggest that there is relationship between anti-microbial activity and phenolic and flavonoid compounds. The anti-bacterial activity of phenolic compounds and flavonoids from plant extracts have been reported previously (Weston et al., 1991; Zheng et al., 1996; Afolayan and Meyer, 1997; Fang et al., 2008). Baydar et al. (2004) confirmed that phenolic compounds are the most bioactive compounds against microorganisms. Phenolic and flavonoid compounds are related to anti-microbial activity, such as inhibition of DNA and RNA synthesis (Mori et al., 1987; Cuhsnie and Lambert, 2005), formation of complexes via linkage with proteins and bacterial membrane (Zongo et al., 2011), reduction of membrane fluidity of bacterial cells (Tsuchiya and Iinuma, 2000), damage to the lipid bilayer of the bacterial membrane (Ikigai et al., 1993) and inhibition of the bacterial respiratory electron transport chain (Haraguchi et al., 1998). Some reports suggested that the position of the OH group in aromatic compounds can also increase anti-microbial activity (Cueva et al., 2010; Gyawali and Ibrahim, 2014); for example, the long aliphatic chain methoxy group substituted in position 2 of the emodine structure increased antibacterial properties (Kemegne et al., 2017). For benzophenanthridine alkaloids, the methoxy groups at positions 7, 8 and 9 also increased antimicrobial activity (Tavares et al., 2014). In flavonoids, the hydroxyl group at position 5 in the A ring, position 4 in the B ring and the methoxyl group at positions 3 and 8 in the A ring have been associated with high inhibition against *E. coli* (Wu et al., 2013).

Anti-HIV-1 reverse transcriptase activity

The activity of the bark extract of *S. obtusa* had significant HIV-1 RT inhibitory activity (88.13%) compared with standard

Table 2 Anti-microbial activity of bark extract of *Shorea obtusa* against *Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtillis*, *Escherichia coli* and *Candida albicans* in minimum inhibitory concentrations compared with standard drugs

	MIC value (µg/mL)				
	S. aureus ATCC 25923	*M. luteus* ATCC 9341	*B. subtillis* ATCC 6633	*E. coli* ATCC 25922	*C. albicans* ATCC 1023
Methanol extract	2.00	inactive	4.00	1.00	20.00
Positive control	Penicillin G1	Ampicillin 10	Gentamicin 10	Ampicillin 10	Nystatin 1

MIC = minimum inhibitory concentration.
inactive = >20 µg/mL

nevirapine (98.91% at 300 µM), as shown in Table 3. Other studies reported that many plant extracts containing high contents of anti-oxidants were correlated with good HIV-1 RT inhibition (Eldeen et al., 2005; Kapewangolo et al., 2016). Oxidative stress is caused from the accumulation of reactive oxygen species and is linked to the stimulation of HIV-1, which can result in AIDS (Pace and Leaf, 1995; Gil et al., 2003; Gil del Valle et al., 2013). In both the current work and other published reports that studied the chemical diversity and anti-HIV-1 RT inhibitory effect of medicinal plants, the anti-RT inhibitors from these plants had several functions. The pharmacological properties of these plants were attributed to their various active compounds, such as phenols and flavonoids against HIV-1 RT (Chinsembu, 2019; Kaur et al., 2020). Further investigation of the flavone compounds for their anti-HIV activity revealed that the hydroxy groups at C_5 and C_7 and the C_2–C_3 double bond were the most active. It was confirmed that the hydroxyl and halogen substituent groups in the B-ring led to increased toxicity or decreased activity of flavonoids or both in general (Hu et al., 1994).

Isolation and purification of bergenin

Based on biological activity results, the bark of *S. obtusa* had higher levels of TPC and TFC and anti-oxidant activity. Therefore, the bark extract offers a potential and more effective source of anti-microbial and anti-HIV RT activities compared to standard drugs. Further isolation and purification of the bark were performed to obtain the pure compound bergenin. The methanol bark extract was separated in layers using $CHCl_3$ and EtOAc. The EtOAc extract was purified using recrystallization in a mixture of $CHCl_3$ and Me_2CO to produce a white precipitate (compound 1), which was then identified using NMR and MS analysis. Comparison of the ^{1}H-NMR and ^{13}C-NMR data with the literature is provided in Table 4. The ^{1}H-NMR spectra revealed a signal for one aromatic proton at δ 6.98 (1H, s) and a signal for methoxy protons at δ 3.76 (3H, s). Due to waster suppression, the protons at δ 3.54–3.59 and 3.65 could not identify the splitting patterns. In the ^{13}C-NMR spectrum, signals observed at δ 61.3 and 163.7 corresponded to the carbonyl and methoxy groups, while the five peaks of methine carbons in the glucose ring appeared at δ 70.9, 72.3, 73.9, 80.0 and 81.9. By matching with the HR-MS (APCI mode) results, the mass at m/z 328.0780 correlated with $C_{14}H_{16}O_9$ and was comparable to the calculated mass at m/z 328.0794. Therefore, compound 1 was confirmed as bergenin, as shown in Fig. 2. The isolated compound was identified using ^{1}H, ^{13}C-NMR and mass spectrometry and confirmed as the same deuterated solvent (DMSO-d_6) based on comparison between the NMR data and reported literature. From the literature reviews on anti-microbial activity, bergenin has anti-microbial activity (Silva et al., 2009; Nazir et al., 2011; Raj et al., 2012). Furthermore, the bactericidal effect of bergenin inhibited all tested Gram-negative bacteria, suggesting that bergenin could be able to cross the complex and multilayered lipopolysaccharide cell walls of Gram-negative bacterial strains (Nyemb et al., 2018). The current findings were similar to those in other reports that bergenin from the bark of *S obtusa* had greater inhibition of *E. coli* than the positive control. To further investigate its activity, bergenin and derivatives were isolated from the

Table 3 HIV1-reverse transcriptase inhibitory activities of bark extract of *Shorea obtusa* at 1 µg/mL and positive control (nevirapince)

Treatment	% Relative inhibition
Bark extract	88.13±1.80
Nevirapine	98.91±0.66
p-Value summary	***

Each value represents mean ± SD (n = 3); *** = $p < 0.001$).

Table 4 ^{1}H NMR and ^{13}C NMR data (DMSO-d_6, 400 MHz for ^{1}H and 100 MHz for ^{13}C) of compound 1 and bergenin

Structure number	^{1}H spectrum of bergenin (Yusoff et al., 2016)	^{1}H spectrum of compound 1	^{13}C spectrum of bergenin (Yusoff et al., 2016)	^{13}C spectrum of compound 1
2			163.8	163.7
3			118.5	118.3
4	6.99 (s, 1H)	6.98 (s, 1H)	109.9	109.7
5			151.4	151.2
6			141.0	140.9
7			148.5	148.3
8			116.4	116.2
9	4.98 (d, J = 10.5 Hz, 1H)	4.95 (d, J = 10.5 Hz, 1H)	72.5	72.3
11	3.58 (t, J = 8.1 Hz, 1H)	3.54-3.59 (m)	82.2	81.9
12	3.22 (ddd, J = 8.1 Hz, 5.1 Hz, 1H)	3.19 (dd, J = 18.8, 9.5 Hz, 1H)	71.1	70.9
13	3.67 (ddd, J = 5.4Hz, 3.3Hz, 5.4Hz, 1H)	3.65	74.1	73.9
14	4.00 (dd, J = 10.2Hz, 9.6Hz, 1H)	3.98 (dd, J = 10.4, 9.5 Hz, 1H)	80.2	80.0
15			60.3	60.1
16	3.83 (dd, J = 10.8Hz, 1H)	3.84 (dd, J = 11.7, 1.8 Hz, 2H)	61.5	61.3
OCH_3	3.77 (s, 3H)	3.76 (s, 3H)		

NMR = nuclear magnetic resonance; DMSO = dimethyl sulfoxide; OCH_3 = methoxy group

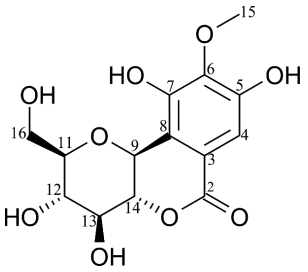

Fig. 2 Structure of bergenin

methanolic extract of the aerial parts of *Ardisia japonica* and showed moderate *in vitro* anti-HIV activity (Piacente et al., 1996). However, the synergistic actions of various compounds should be responsible for therapeutic efficacy rather than the bergenin alone (Ma et al., 2009). Although the chemical nature of the extract was not further analyzed, its active components appeared to be mainly high polar substances, which have been shown as potent inhibitors of different enveloped HIV (Marchetti et al., 1996; Lee et al., 1999).

It is noteworthy that the current report is the first on the anti-oxidant and anti-HIV properties and isolation of bergenin from *S. obtusa*. Based on the current results, the bark of *S. obtusa* has promising medicinal properties, such as anti-oxidant, anti-microbial and anti-HIV activity. Thus, the use of *S. obtusa* extract as a natural source for future pharmaceutical and medicinal development should be investigated further.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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