Available online on www.ijcpr.com
International Journal of Current Pharmaceutical Review and Research; 6(6); 256-261

ISSN: 0976 822X

Research Article

Triterpenes and Sterols from *Sonneratia alba*

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Available Online:23rd October, 2015

ABSTRACT
Chemical investigation of the dichloromethane extract of *Sonneratia alba* Sm. afforded mixtures of oleanolic acid (**1a**) and ursolic acid (**1b**), α-amyrin cinnamate (**2a**) and β-amyrin cinnamate (**2b**), and β-sitosterol (**3a**) and stigmasterol (**3b**) from the fruit; lupeol (**4**), and mixtures of **1a** and **1b**, and **3a** and **3b** from the twigs; and **1b** and squalene (**5**) from the leaves. The structures of **1-5** were identified by comparison of their NMR data with literature data.

***Keywords*:** *Sonneratia alba*, oleanolic acid, ursolic acid, α-amyrin cinnamate, β-amyrin cinnamate, β-sitosterol, stigmasterol, lupeol, squalene

INTRODUCTION

Sonneratia alba J.E. Sm., of the family Sonneratiaceae (APG Lythraceae), is commonly known as mangrove apple, and is considered as the most widespread of all mangrove trees, abundant in East Africa, Southeast Asia, Northern Australia, Borneo, and the Pacific Islands[1]. It is a medium-sized tree, found in low-intertidal zones, growing along seashores and at the mouth of tidal creeks on sandy, rocky or muddy soils, and also on coral terraces[2,3]. *S. alba* has many commercial applications[4]. It is widely used for firewood and construction, often timbered for making ribs for boats, house materials and flooring, and bridges and wharfs. The pneumatophores of this species are used to produce cork and floats. In India and Indonesia, the fruit is used to make a beverage. In other Malay regions, the fruit is eaten ripe, while the leaves are eaten either raw or cooked[5]. *S. alba* is also used in traditional medicine as a compress for swellings and sprains[4]. The leaves, trunk and bark exhibits antioxidant properties[6], while the sepal shows antioxidant and anti-lipid peroxidation properties[7]. A number of studies have been conducted on the chemical constituents and biological activities of *S. alba.* 3β-Hydroxy-lup-9(11),12-diene,28-oic acid, lupeol and lupan-3β-ol which were isolated from the bark of *S. alba* exhibited antibacterial activity against the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and *Streptococcus mutans* ATCC 25175, with minimum inhibitory concentrations ranging from 15-33 to 35-55 μg/mL, respectively[8]. Furthermore, lupan-3β-ol and lupeol isolated from the bark of *S. alba* displays antibacterial activity against Gram-positive bacteria, *S. aureus* and *S. mutans* with MIC values of 94.1 and 120; and 35.2 and 22.6 mg/mL, respectively[9]. Moreover, the petroleum ether extract of the leaves of *S. alba* yielded oleanolic acid, betulin, betulinic acid, alphitolic acid, methyl gallate and 5-hydroxymethylfurfural[10]. In another study, the $CHCl_3$ and aqueous soluble fractions of *S. alba* showed significant free radical scavenging activity with IC_{50} values of 15.58 ± 0.55μg/mL and 15.06 ± 0.35μg/mL, respectively. At a concentration of 400μg/disc, the CCl_4, $CHCl_3$ and aqueous soluble fractions inhibited bacterial growth with zone of inhibitions ranging from 7-9 mm, 7-10 mm and 7 mm, respectively. The CCl_4 soluble fraction also showed cytotoxic activity with an LC_{50} value of 7.94 ± 0.450 μg/mL. These extracts yielded lupeol, oleanolic acid, β-sitosterol, stigmasterol and sitost-4-en-3-one[11]. Lupeol, oleanolic acid, betulinic acid, 2,6-dimethoxy-*p*-benzoquinone, stigmasterol and β-sitosterol were isolated from the twigs of *S. alba*. Lupeol and betulinic acid exhibited antimycobacterial activity with MIC values of 25 and 50 mg/mL respectively. 2,6-Dimethoxy-p-benzoquinone showed antimalarial activity against *P. falciparum* with an IC_{50} value of 3.08 mg/mL[12]. In another study, 3,3'-di-O-methylellagic acid from *S. alba* was reported to exhibit a stronger antioxidant activity than the ascorbic acid standard with IC_{50}values of 11.35 and 17.64 μ g/mL, respectively[13]. Moreover, the gamma linolenic

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acid (GLA) percentage in the leaves of *S. alba* was reported as 36.20%, while in the stem it was 11%[14]. In this study, the dichloromethane extracts of the different parts of *S. alba* afforded mixtures of oleanolic acid (**1a**) and ursolic acid (**1b**) in a 3:1 ratio, α-amyrin cinnamate (**2a**) and β-amyrin cinnamate (**2b**) in a 1:3 ratio, and β-sitosterol (**3a**) and stigmasterol (**3b**) in a 3:1 ratio from the fruit; lupeol (**4**), and mixtures of **1a** and **1b** in a 1:3 ratio, and **3a** and **3b** in a 3:1 ratio from the twigs; and **1b** and squalene (**5**) from the leaves. The structures of **1**-**5** are presented in Fig. 1. To the best of our knowledge this is the first report on the isolation of **1b**, **2a**, **2b**, and **5** from *S. alba.*

MATERIALS AD METHODS

General Experimental Procedure

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were acquired in $CDCl_3$ on a 500 MHz Agilent DD2 NMR spectrometer with referencing to solvent signals (δ 7.26 and 77.0 ppm). Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Sample Collection

Samples of fruits, leaves and twigs of *Sonneratia alba* were collected from the De La Salle University – Bro Alfred Shields FSC Ocean Research (SHORE) Center in Matuod, Lian, Batangas in April 2014. The sample was authenticated by one of the authors (EHM) and deposited at De La Salle University Herbarium with voucher specimen #924.

General Isolation Procedure

A glass column 18 inches in height and 1.0 inch internal diameter was packed with silica gel. The crude extracts were fractionated by silica gel chromatography using increasing proportions of acetone in CH_2Cl_2 (10% increment) as eluents. Fifty milliliter fractions were collected. All fractions were monitored by thin layer chromatography. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Two milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation of the chemical constituents of the fruits

The freeze-dried fruits of *S. alba* (140.0 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (0.70 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 20% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 5% EtOAc in petroleum ether to afford a mixture of **2a** and **2b** (4 mg) after washing with petroleum ether. The 30% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using 15% EtOAc in petroleum ether to afford a mixture of **3a** and **3b** (5 mg) after washing with petroleum ether. The 60% to 80% acetone in CH_2Cl_2 fractions were combined and rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1.5:1.5:7, v/v) to yield a mixture of **1a** and **1b** (3 mg) after trituration with petroleum ether.

Isolation of the chemical constituents of the twigs

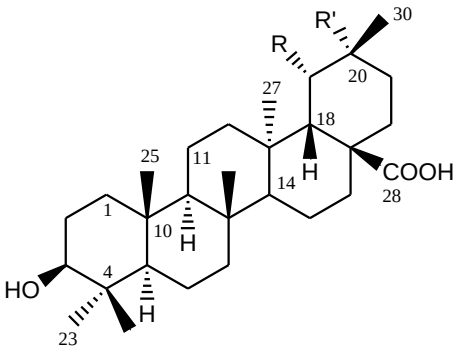
The air-dried twigs of *S. alba* (113.4 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (0.5 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 30% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using 15% EtOAc in petroleum ether to afford **4** (5 mg) after washing with petroleum ether. The 40% acetone in CH_2Cl_2 was rechromatographed using 15% EtOAc in petroleum ether to afford a mixture of **3a** and **3b** (3 mg) after washing with petroleum ether. The 70% acetone in CH_2Cl_2 was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) to yield a mixture of **1a** and **1b** (2 mg) after trituration with petroleum ether.

Isolation of the chemical constituents of the leaves

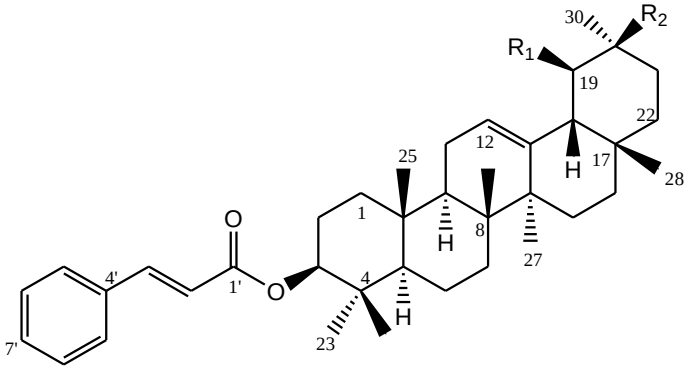
The air-dried leaves of *S. alba* (309.5 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The solvent was evaporated under vacuum to afford a crude extract (11 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (2 ×) using petroleum ether to afford **5** (10 mg). The 40% acetone in CH_2Cl_2 was rechromatographed using 15% EtOAc in petroleum ether to afford a mixture of **3a** and **3b** (3 mg) after washing with petroleum ether. The 70% acetone in CH_2Cl_2 was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) to yield **1b** (5 mg) after trituration with petroleum ether.

***Oleanolic acid* (1a):** ^{1}HNMR ($CDCl_3$, 500 MHz): δ 3.20 (dd, J = 10.0, 4.5 Hz, H-3α), 5.27 (t, *J* = 3.5 Hz, H-12), 2.81 (dd, *J* = 4.2, 13.8 Hz, H-18), 1.15 (s, Me-27), 0.97 (s, Me-23), 0.91 (s, Me-25), 0.92 (d, J = 6.5 Hz, Me-30), 0.75 (s, Me-26), 0.76 (s, Me-24), 0.88 (J = 6.5 Hz, Me-29); ^{13}CNMR ($CDCl_3$, 125 MHz): δ 38.4 (C-1), 27.2 (C-2), 79.0 (C-3), 38.4 (C-4), 55.2 (C-5), 18.3 (C-6), 32.6 (C-7), 39.3 (C-8), 47.6 (C-9), 37.1 (C-10), 23.0 (C-11), 122.5 (C-12), 143.7 (C-13), 41.6 (C-14), 27.7 (C-15), 23.4 (C-16), 46.5 (C-17), 41.1 (C-18), 45.9 (C-19), 30.7 (C-20), 23.6 (C-21), 32.4 (C-22), 28.1 (C-23), 15.5 (C-24), 15.33 (C-25), 17.0 (C-26), 25.9 (C-27), 182.3 (C-28), 33.1 (C-29), 23.8 (C-30).

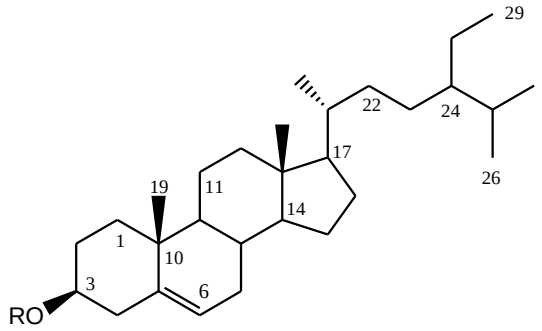
***Ursolic acid* (1b):** ^{1}HNMR ($CDCl_3$, 500 MHz): δ 3.20 (dd, J = 10.0, 4.5 Hz, H-3α), 5.23 (t, *J* = 3.5 Hz, H-12), 2.18 (1H, d, *J* =11.4 Hz, H-18), 1.07 (s, Me-23), 0.81 (s, Me-24), 0.94 (s, Me-25), 0.86 (s, Me-26), 1.20 (s, Me-27), 0.80 (d, J = 6.5 Hz, Me-29), 0.91 (d, J = 6.5 Hz, Me-30); ^{13}CNMR ($CDCl_3$, 125 MHz): δ 38.5 (C-1), 27.2 (C-2), 79.0 (C-3), 38.5 (C-4), 55.2 (C-5), 18.3 (C-6), 33.1 (C-7), 39.5 (C-8), 47.5 (C-9), 37.1 (C-10), 23.6 (C-11), 125.8 (C-12), 138.0 (C-13), 42.0 (C-14), 29.4 (C-15), 23.4 (C-16), 47.9 (C-17), 52.5 (C-18), 30.7 (C-19), 30.6 (C-20), 27.2 (C-21), 37.0 (C-22), 24.2 (C-23), 15.4 (C-24), 15.6 (C-25), 17.0 (C-26), 24.2 (C-27), 177.5 (C-28), 22.7 (C-29), 24.1 (C-30).



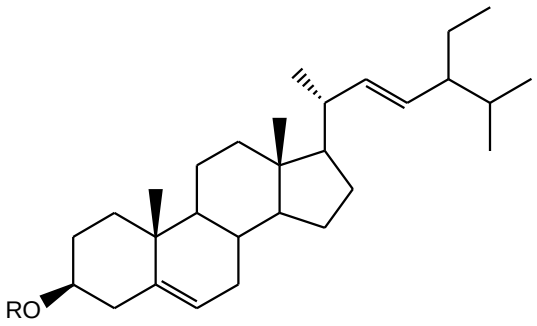
1a R = H, R' = CH_3
1b R = CH_3, R' = H



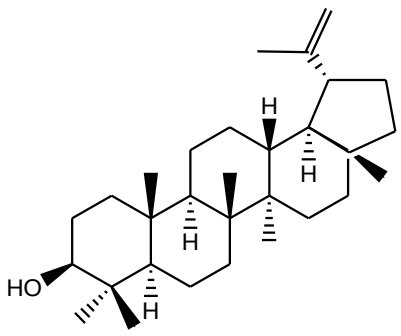
2a R_1 = CH_3, R_2 = H
2b R_1 = H, R_2 = CH_3



3a R = H



3b



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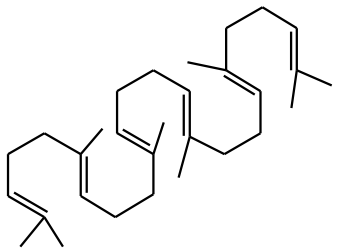

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Fig. 1. Chemical structures of oleanolic acid (**1a**), ursolic acid (**1b**), α-amyrin cinnamate (**2a**), β-amyrin cinnamate (**2b**), β-sitosterol (**3a**), stigmasterol (**3b**), lupeol (**4**), and squalene (**5**) from *Sonneratia alba*.

α-Amyrin cinnamate (**2a**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.65 (dd, *J* = 6.0, 10.1 Hz, H-3), 5.13 (t, *J* = 3.8 Hz, H-12), 0.92 (s, Me-23), 0.95 (s, Me-24), 1.01 (s, Me-25), 1.03 (s, Me-26), 1.08 (s, Me-27), 0.80 (s, Me-28), 0.80 (d, *J* = 5.9 Hz, Me-29), 0.92 (d, *J* = 5.9 Hz, Me-30), 7.67 (d, *J* = 16.0 Hz, H-2'), 6.44 (d, *J* = 16.0 Hz, H-3'), 7.53 (H-5', H-9'), 7.38 (H-6', H-8'), 7.38 (H-7').

β-Amyrin cinnamate (**2b**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.63 (t, *J* = 6.0 Hz, H-3), 5.17 (t, *J* = 3.7 Hz, H-12), 0.90 (s, Me-23), 0.93 (s, Me-24), 0.97 (s, Me-25), 0.96 (s, Me-26), 1.13 (s, Me-27), 0.82 (s, Me-28), 0.86 (s, Me-29), 0.85 (s, Me-30), 7.65 (d, *J* = 16.0 Hz, H-2'), 6.43 (d, *J* = 16.0 Hz, H-3'), 7.53 (H-5', H-9'), 7.37 (H-6', H-8'), 7.37 (H-7').

β-Sitosterol (**3a**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.50 (m, H-3), 5.35 (d, 4.8, H-5), 0.66 (s, Me-18), 0.99 (s, Me-19), 0.93 (d, 6.6, Me-21), 0.84 (d, *J* = 6.6, Me-26), 0.83 (d, *J* = 6.0, Me-27), 0.86 (t, *J* = 6.0, Me-29).

Stigmasterol (**3b**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.50 (m, H-3), 5.33 (d, *J* = 4.8, H-5), 0.68 (s, Me-18), 0.99 (s, Me-19), 1.01 (d, *J* = 6.6, Me-21), 5.13 (dd, *J* = 8.4, 15.6 Hz, H-22), 5.00 (dd, *J* = 8.4, 15.0 Hz, H-23), 0.84 (d, *J* = 6.6 Hz, Me-26), 0.83 (d, *J* = 6.0 Hz, Me-27), 0.80 (t, *J* = 6.0 Hz, Me-29).

Lupeol (**4**): ^{1}H NMR (500 MHz, $CDCl_3$): δ ($CDCl_3$, 500 MHz) δ 4.68 (H-29b), 4.55 (H-29a), 3.18 (H-3), 1.68 (s, H_3-30), 0.96 (s, H_3-23), 0.78 (s, H_3-24), 0.83 (s, H_3-25), 0.94 (s, H_3-26), 1.06 (s, H_3-27), 0.91 (s, H_3-28), 1.68 (s, H_3-30).

Squalene (**5**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.08-5.13 (6H. =CH), 1.58 (18H, allylic Me, *cis*), 1.66 (6H, allylic Me, *trans*), 1.94-2.07 (20H, allylic CH_2).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of the fruits of *S. alba* afforded oleanolic acid (**1a**)[15], ursolic acid (**1a**)[16], α-amyrin cinnamate (**2a**)[17], β-amyrin cinnamate (**2b**)[17], β-sitosterol (**3a**)[18], stigmasterol (**3b**)[18], lupeol (**4**)[19], and squalene (**5**)[20]. The structures of **1**-**5** were identified by comparison of their NMR data with literature data. The 3:1 ratio of oleanolic acid (**1a**) and ursolic acid (**1b**) from the fruit and the and the 1:3 ratio of **1a** and **1b** from the twigs were deduced from the intensity of the ^{1}H NMR resonances for the olefinic protons[21] at δ 5.27 for **1a** and δ 5.23 for **1b** and the allylic methine protons[21] at δ 2.81 for **1a** and δ 2.18 for **1b**. The α-amyrin cinnamate (**2a**) and β-amyrin cinnamate (**2b**) in a 1:3 ratio was deduced from the intensities of the ^{1}H NMR resonances for the olefinic protons[17] at δ 5.13 for **1a** and δ 5.17 for **1b**. The 3:1 ratio of the mixture of β-sitosterol (**3a**) and stigmasterol (**3b**) from the fruit and twigs was deduced from the intensities of the ^{1}H NMR resonances for the olefinic protons[22] of **3a** at δ 5.33 and **3b** at δ 5.33, 5.13 and 5.00. Although bioassays were not conducted on the isolated compounds (**1**-**5**), there were previous studies that reported on their biological activities. Oleanolic acid (**1a**) was found to be anti-mutagenic and anti-tumor, inhibiting proliferation of gastric, colon, and liver cancer cells by inducing apoptosis and necrosis[23]. Triterpene **1a** was found to inhibit mouse skin tumor[24] and exhibited significant anti-tumor activity against human colon carcinoma cell line HCT 15[25]. A recent study identified **1a** as an anti-tumor compound able to suppress aerobic glycolysis in MCF-7 breast cancer cells by inducing a metabolic switch in the PKM2 to PKM1 ratio which is important in cancer development[26]. A study reported that ursolic acid (**1b**) induced apoptosis in tumor cells by activation of caspases and modulation of pathways affecting cell proliferation and migration[27]. It also decreased proliferation and induced apoptosis in gastric cancer cell line BGC-803 and hepatocellular cancer cell H22 xenograft, both *in vivo* and *in vitro*[28]. Triterpene **1b** exhibited anti-tumor activity against human colon carcinoma cell line HCT15[25] and inhibited the growth of colon cancer-initiating cells by targeting STAT3[29]. Triterpene **1b** showed anti-estrogenic effects suggesting its potential use as therapeutic agents against estrogen-dependent tumors[30]. It has potential therapeutic use against prostate cancer through its anti-proliferative and apoptotic effects[31]. A recent study reported that **1b** inhibited cell growth and proliferation of Jurkat leukemic T-cells, inhibiting PMA/PHA induced IL-2 and TNF-α production in a concentration and time dependent manner[32]. A study on cervical cancer cells TC-1 reported that ursolic acid-activated autophagy induced cytotoxicity and reduced tumor growth in a concentration-dependent manner[33]. Another study evaluated the antitumor activities of **1b** on U87MG brain cancer cells and found that both G1-phase arrest and autophagy were induced by the compound[34]. Compound **3a** and baicalein inhibited the proliferation of MCF-7 breast cancer cells induced by PhIP[35]. A study reported that α-amyrin cinnamate (**2a**) and β-amyrin cinnamate (**2b**) inhibited inflammation with 50% inhibitory dose (ID_{50}) of 0.61 and 0.75 μmol/ear. Triterpenes **2a** and **2b** also induced Epstein-Barr virus early antigen with IC_{50} values of 401 and 405 mole ratio/32 pmol TPA, respectively[17]. β-Sitosterol (**3a**) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells[36]. It was shown to be effective for the treatment of benign prostatic hyperplasia[37]. It was also reported to attenuate β-catenin and PCNA expression, as well as quench radical *in vitro*, making it a potential anticancer drug for colon carcinogenesis[38]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake[39]. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells[40]. Stigmasterol (**3b**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions[41]. It lowers plasma cholesterol levels, inhibits intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Winstar as well as WKY rats[42]. Other studies reported that stigmasterol showed cytostatic activity against Hep-2 and McCoy cells[43], markedly inhibited tumour promotion in two stage carcinogenesis experiments[44], exhibited antimutagenic[45], topical anti-inflammatory[46], antiosteoarthritic[47] and antioxidant[48] activities. Lupeol (**4**) exhibited antimicrobial, antiviral, anticancer, and anti-inflammatory activities[49]. It exhibited antiurolithiatic and diuretic activity[50]. It prevented the formation of *vesical calculi* and reduced the size of the preformed stones in rats[51]. Squalene (**5**) was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis[52]. It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties[53]. A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 have anti-proliferative effects on breast cancer cells[54]. The preventive and therapeutic potential of squalene containing compounds on tumour promotion and regression have been reported[55]. A recent review on the bioactivities of squalene has been provided[56].

CONCLUSION

S. alba is used in traditional medicine as a compress for swellings and sprains, hence it has anti-inflammatory activity. The leaves, trunk and bark were reported to exhibit antioxidant properties, while the sepal showed antioxidant and anti-lipid peroxidation properties. The triterpenes (**1a-1b, 4-6)** and sterols (**3a** and **3b**) which were obtained from the different parts of *S. alba* were reported to exhibit anti-oxidant and anticancer properties. Furthermore, **2a**, **2b**, **3b** and **5** were reported to exhibit anti-inflammatory properties. Thus, the anti-inflammatory and anti-oxidant properties of *S. alba* may be partly attributed to the synergistic effects of **1-5** which were obtained from the different parts of the mangrove.

ACKNOWLEDGEMENT

A research grant from the De La Salle University Science Foundation through the University Research Coordination Office is gratefully acknowledged.

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