Triterpenes and Sterols from *Sonneratia alba*

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ABSTRACT

Chemical investigation of the dichloromethane extract of *Sonneratia alba* Sm. afforded mixtures of oleanolic acid (1a) and ursolic acid (1b), α-aminocinnamate (2a) and β-aminocinnamate (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-amino cinnamate, β-amino 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. 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. *S. alba* has many commercial applications. 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. *S. alba* is also used in traditional medicine as a compress for swellings and sprains. The leaves, trunk and bark exhibits antioxidant properties, while the sepal shows antioxidant and anti-lipid peroxidation properties. 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 lup-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. 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. Moreover, the petroleum ether extract of the leaves of *S. alba* yielded oleanolic acid, betulin, betulinic acid, α-luphenolic acid, methyl gallate and 5-hydroxymethylfurfural. In another study, the CHCl₃ and aqueous soluble fractions of *S. alba* showed significant free radical scavenging activity with IC₅₀ values of 15.58 ± 0.55μg/mL and 15.06 ± 0.35μg/mL, respectively. At a concentration of 400μg/disc, the CHCl₃, CHCl₃ and aqueous soluble fractions inhibited bacterial growth with zone of inhibitions ranging from 7-9 mm, 7-10 mm and 7 mm, respectively. The CHCl₃ soluble fraction also showed cytotoxic activity with an LC₅₀ value of 7.94 ± 0.450 μg/mL. These extracts yielded lupeol, oleanolic acid, β-sitosterol, stigmasterol and sitost-4-en-3-one. 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 antymycobacterial activity with MIC values of 25 and 50 mg/mL respectively. 2,6-Dimethoxy-p-benzoquinone showed antimarial activity against *P. falciparum* with an IC₅₀ value of 3.08 mg/mL. In another study, 3,3'-di-O-methyllellagic acid from *S. alba* was reported to exhibit a stronger antioxidant activity than the ascorbic acid standard with IC₅₀ values of 11.35 and 17.64 μ g/mL, respectively. 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%\textsuperscript{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 stigmastanol (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 AND METHODS**

**General Experimental Procedure**

\( ^1H \) (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 (\( \delta \) 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\(_2\)SO\(_4\) 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\(_2\)Cl\(_2\) (10% increment) as eluents. Fifty millilitre 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 millilitre fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One millilitre 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\(_2\)Cl\(_2\) 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\(_2\)Cl\(_2\) at 10% increment. The 30% acetone in CH\(_2\)Cl\(_2\) fraction was rechromatographed (3 \( \times \)) using CH\(_3\)CN:Et\(_2\)O:CH\(_2\)Cl\(_2\) (1.5:1.5:7, \( \nu/\nu \)) 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\(_2\)Cl\(_2\) 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\(_2\)Cl\(_2\) at 10% increment. The 30% acetone in CH\(_2\)Cl\(_2\) fraction was rechromatographed (2 \( \times \)) using 15% EtOAc in petroleum ether to afford 4 (5 mg) after washing with petroleum ether. The 40% acetone in CH\(_2\)Cl\(_2\) 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\(_2\)Cl\(_2\) was rechromatographed (3 \( \times \)) using CH\(_3\)CN:Et\(_2\)O:CH\(_2\)Cl\(_2\) (1:1.8, \( \nu/\nu \)) to yield a mixture of 1a and 1b (2 mg) after trituration with petroleum ether.

**Oleanolic acid (1a):** \(^1\)HNMR (CDCl\(_3\), 500 MHz): \( \delta \) 3.20 (dd, \( J = 10.0, 4.5 \) Hz, H-3\(a\)), 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); \(^{13}CNMR (CDCl_3, 125 MHz): \delta \) 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), 15.3 (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): \( \delta \) 3.20 (dd, \( J = 10.0, 4.5 \) Hz, H-3\(a\)), 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); \(^{13}CNMR (CDCl_3, 125 MHz): \delta \) 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).
**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-'), 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).

**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.*
Stigmasterol (3b): 1H NMR (500 MHz, CDCl3): 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): 1H NMR (500 MHz, CDCl3): δ (CDCl3, 500 MHz) δ 4.68 (H-29b), 4.55 (H-29a), 3.18 (H-3), 1.68 (s, H-19), 0.96 (s, H-23), 0.78 (s, H-24), 0.83 (s, H-25), 0.94 (s, H-26), 1.06 (s, H-27), 0.91 (s, H-28), 1.68 (s, H-30).

Squalene (5): 1H NMR (500 MHz, CDCl3): 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 CH2).

RESULTS AND DISCUSSION
Silica gel chromatography of the dichloromethane extract of the fruits of S. alba afforded oleanolic acid (1a)15, urso acid (1a)6, α-aminocinnamate (2a)17, β-aminocinnamate (2b)11, β-sitosterol (3a)13, 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 urso acid (1b) from the fruit and the and the 1:3 ratio of 1a and 1b from the twigs were deduced from the intensities of the 1H NMR resonances for the olefinic protons21 at δ 5.27 for 1a and δ 5.23 for 1b and the allylic methine protons21 at δ 2.81 for 1a and δ 2.18 for 1b. The α-aminocinnamate (2a) and β-aminocinnamate (2b) in a 1:3 ratio was deduced from the intensities of the 1H NMR resonances for the olefinic protons22 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 1H NMR resonances for the olefinic protons23 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 necrosis25. Triterpene 1a was found to inhibit mouse skin tumor24 and exhibited significant anti-tumor activity against human colon carcinoma cell line HCT 1525. 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 development26. A study reported that urso acid (1b) induced apoptosis in tumor cells by activation of caspases and modulation of pathways affecting cell proliferation and migration27. 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 HCT1525 and inhibited the growth of colon cancer-initiating cells by targeting STAT329. Triterpene 1b showed anti-estrogenic effects suggesting its potential use as therapeutic agents against estrogen-dependent tumors30. It has potential therapeutic use against prostate cancer through its anti-proliferative and apoptotic effects31. 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 manner32. A study on cervical cancer cells TC-1 reported that urso acid-activated autophagy induced cytotoxicity and reduced tumor growth in a concentration-dependent manner33. 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 compound34. Compound 3a and baicalein inhibited the proliferation of MCF-7 breast cancer cells induced by PhIP35. A study reported that α-aminocinnamate (2a) and β-aminocinnamate (2b) inhibited inflammation with 50% inhibitory dose (ID₅₀) of 0.61 and 0.75 μmol/ear. Triterpene 2a and 2b also induced Epstein-Barr virus early antigen with IC₅₀ values of 401 and 405 m mole ratio/32 pmol TPA, respectively37. β-Sitosterol (3a) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells38. It was shown to be effective for the treatment of benign prostatic hyperplasia39. 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 carcinogenesis39. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake40. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells41. Stigmasterol (3b) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions42. It lowers plasma cholesterol levels, inhibits intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Wistar as well as WKY rats43. Other studies reported that stigmasterol showed cytostatic activity against Hep-2 and McCoy cells43, markedly inhibited tumour promotion in two stage carcinogenesis experiments44, exhibited antimutagenic45, topical anti-inflammatory46, antioestocortic47 and antioxidant48 activities. Lupeol (4) exhibited antimicrobial, antiviral, anticanine, and anti-inflammatory activities49. It exhibited antiurolithiatic and diuretic activity50. It prevented the formation of vesical calculi and reduced the size of the preformed stones in rats51. Squalene (5) was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis52. It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties53. A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 have anti-proliferative effects on breast cancer cells54. The preventive and therapeutic potential of squalene containing compounds on tumour promotion and regression have been reported55. A recent review on the bioactivities of squalene has been provided56.

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 steroids (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.

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