Chemical studies on the parasitic plant *Thonningia sanguinea* Vahl†

Ama Kyeraa Thomford, ab Reda Fouad Ahmed Abdelhameedac and Koji Yamada*a

*Thonningia sanguinea* Vahl plays an important role in traditional medicine in many African cultures. A study of the *n*-hexane fraction of the whole plant of *T. sanguinea* led to the isolation of two glucocerebrosides molecular species TSC-1 and TSC-2, one β-sitosteryl-3β-β-glucopyranoside-6′-O-fatty acid ester molecular species, TSS-1, and seven known triterpenes (1–7). The ethyl acetate faction also afforded five known lignans (8–12) and one known flavanone (13). Their structures were elucidated by means of chemical and spectroscopic methods (methanolysis, NMR and mass spectrometry). Spectral analyses of the glucocerebrosides revealed mainly sphingosine-type (TSC-1) and phytosphingosine-type (TSC-2) cerebrosides, with both possessing mainly 2-hydroxy fatty acid and β-D-glucopyranosyl moieties. TSS-1 was found to be a β-sitosterol-type with fatty acid methyl esters and β-D-glucopyranosyl moieties. The classes of compounds isolated from this plant are well known for their interesting biological activities including antimicrobial, antioxidant, anticancer, antiinflammatory and analgesic. They may therefore be responsible in part or in whole for these activities, hence validating the traditional uses of the plant. To the best of our knowledge, this is the first report on the isolation of all these compounds from *T. sanguinea*.

1. Introduction

*Thonningia sanguinea* Vahl is a flowering plant in the monotypic genus *Thonningia* of the family Balanophoraceae. Synonyms for the plant include *T. angolensis*, *T. coccinea* Mangenot, *T. dubia* Hems., *T. elegans* Hems., and *T. ugandensis* Hems. It is a fleshy subterranean herb growing from an underground tuber. It is a parasitic plant growing on the terminal roots of host plants such as *Hevea brasiliensis*, *Phoenix dactylifera* and *Theobroma cacao*. The flowering stem produces a bright red or pink inflorescence containing male and female flowers. The plant grows in Tropical Africa, from Senegal to Ethiopia, south to Angola, Zambia and Tanzania. It is commonly found in rainforests, gallery forests and adjacent woodland. Commonly known in English as "ground pineapple", it is also known as "kwaebedwa" in the local Akan language of Ghana.

*Th. sanguinea* is best known for its use in traditional medicine in many African countries. In Ghanaian traditional medicine for instance, this plant is used in treatment of bronchial asthma sometimes for prophylaxis, sexually transmitted diseases and as an aphrodisiac. It is used to treat diarrhoea and worm infestation in Cote d’Ivoire and Congo. It is also mixed with *Capsicum* to produce a topical cream for treating haemorrhoids and torticollis. It is also used to treat dysentery, sore throat, skin infections, abscesses, dental caries, gingivitis, fever, malaria, heart disease, rickets, and rheumatism.2,3,4

Chemically, an aqueous and hydroalcoholic flower extract of *T. sanguinea* revealed the presence of alkaloids, catechin tannins, flavonoids, saponins, quinones and polyphenols on preliminary screening. Brevifolin carboxylic acid (BCA), gallic acid (GA) and two ellagitannins: thonningianins A (Th A) and Th B are the only four compounds reported to have been isolated from the plant. N’guessan et al. reported that both GA and BCA, isolated from *T. sanguinea* demonstrated moderate antibacterial activity against *Salmonella enteritidis*, *Salmonella typhimurium*, and *Salmonella abony* in the disc diffusion method, with significant antioxidant activity in the DPPH radical scavenging activity assay.5 GA is well known as a potent antioxidant phenolic compound, with numerous biological activities including antitumor, antimicrobial and antimelanogenic. BCA has been shown to inactivate HBsAg and inhibit hepatitis B virus replication and tumour growth.6 The ellagitannins isolated from the plant also possessed hepatoprotective actions, potent antimicrobial effects, and significant free radical scavenging activity against DPPH by ESR analysis.7 Th A effectively inhibited the proliferation of HepG-2 human hepatocellular carcinoma cells by inducing apoptosis. This was observed as an increase in the sub-G1 cell population,
DNA fragmentation, and increase in the content of reactive oxygen species. Th A was also shown to be a potent in vitro inhibitor of rat liver crude glutathione S-transferases (GSTs) and hGSTP1-1 activity.

Biological study on T. sanguinea plant extracts (in vivo and in vitro assays) indicated the prophylactic potential of the aqueous extract and its n-butanol fraction in bronchial asthma. The active agents extracted into n-butanol and may be flavonoids and/or phenolic in nature. The anticcidiotic activity of the extract against Eimeria sp. sporozoites, antibacterial effects against some multidrug resistant strains of Salmonella enterica and their effects on extended spectrum-β-Lactamases (ESBL) producing Escherichia coli and Klebsiella pneumoniae strains have been reported. The significant antimalarial effects of T. sanguinea root extracts against Plasmodium falciparum (in vitro), Plasmodium berghei and Plasmodium chabaudi (in vivo) have also been reported. The aqueous extract of T. sanguinea exhibited hepatoprotective activity towards a variety of different toxicants including galactosamine, carbon tetrachloride and aflatoxin B1. It also suppressed CYP3A2 and CYP1A2 expression at the level of transcription, protects against aflatoxin B1 acute hepatotoxicity in Fischer 344 rats and also inhibits the liver drug metabolising enzymes of rats.

The aqueous decoction of T. sanguinea has been used for more than thirty years as a mono-herbal product produced by the Centre for Plant medicine Research, Akuapem-Mampong, Ghana (CPMR) under the registered names CAMPA-T® and NINGER®. Due to the reported clinical effectiveness of these herbal formulations at the CPMR clinic, they have been approved for use as standardised herbal medicines by the Food and Drug Authority. CAMPA-T® and NINGER® are now part of the Essential Herbal Medicines List recommended by the Food and Drug Authority. CAMPA-T® and NINGER® have also been reported. The aqueous extract of Thonningia sanguinea has been approved for use as standardised herbal medicines by the Centre for Plant Medicine Research from the eastern region of Ghana in the month of January, 2015 and authenticated by the curator of their herbarium. A voucher specimen with the number CSRPM no. 140 was assigned to the sample.

2.2. Plant collection and identification
Thonningia sanguinea whole plant was collected by the staff of the Centre for Plant Medicine Research from the eastern region of Ghana in the month of January, 2015 and authenticated by the curator of their herbarium. A voucher specimen with the number CSRPM no. 140 was assigned to the sample.

2.3. Extraction and isolation
The whole plant of T. sanguinea was shade-dried for seven days and pulverised. The dried powdered plant material (3.5 kg) was extracted by cold maceration with MeOH (3 × 10 L for 3 days), followed by MeOH : CHCl3 (1 : 1; 3 × 10 L for 3 days). The filtrates were pooled together and concentrated in vacuo using the rotary evaporator. The methanol/chloroform crude extract (423 g) was dissolved in distilled water and serially partitioned between n-hexane, ethyl acetate, n-butanol and methanol (Nacalai Tesque, Inc., Kyoto, Japan). Column chromatography (CC) was performed using Sephadex LH-20 (25–100 mm, GE Healthcare UK Ltd., Buckinghamshire HP7 9NA, UK), silica gel Purasil 60 Å, 230–400 mesh (Whatman, Sanford, ME, USA) and Cosmosil 140C18 PREP silica gel 90 Å, 40–63 mesh (Nacalai Tesque, Inc., Kyoto, Japan). TLC was performed on 0.25 mm thick, precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany). Spots were developed with 5% H2SO4 : MeOH and detected by illumination under a short wavelength UV (254 nm). Analytical HPLC was performed on a Cosmosil 5C18AR-II 4.6 mm × 250 mm column (Nacalai Tesque, Inc., Kyoto, Japan) with methanol (Nacalai Tesque, Inc., Kyoto, Japan) at a flow rate of 0.8 mL min⁻¹ and Cosmosil-sugar-D 4.6 ID × 250 mm, 1 mL min⁻¹, refractive index (RI) detector using 95% acetonitrile.

Preparative HPLC was performed on a Develosil 5C18 4.6 mm × 150 mm column (Nacalai Tesque, Inc., Kyoto, Japan) using 100% MeOH as solvent, at a flow rate of 0.5 mL min⁻¹ on a Jasco DG-2080-53 Plus degasser, Jasco PU-2080 Plus pump, Jasco AS-2055 Plus auto sampler, Jasco CO-2065 Plus column oven (maintained at 35 °C) and Jasco MD-2018 Plus PDA detector (Jasco Co. Ltd., Tokyo, Japan).

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HPLC using 100% MeOH to afford compound 6 (200 mg). Fraction H-5 (330 mg) was subjected to repeated silica gel CC using CHCl₃ : MeOH (1:0-1:1) to afford seven fractions. H-5-6 (138 mg) was further chromatographed on a C₁₈ RP silica gel column (10 g) using MeOH : H₂O (8:2 - 1:0) and acetone : H₂O (1:1 - 1:0) to afford compound 7 (22 mg). Fraction H-10 (600 mg) was chromatographed on silica gel (20 g) using CHCl₃ : MeOH (9.8:0.2 - 1:1) to afford eleven sub-fractions (H-10-1-H-10-11). H-10-4 (350 mg) was further chromatographed on a C₁₈ RP silica gel column (10 g) using 100% MeOH to afford compound 1 (28 mg), TSS-1 (94 mg) and a mixture purified by preparative HPLC using 100% MeOH to afford compound 2 (40 mg) and 4 (15 mg). Fraction H-13 (2.3 g) was chromatographed on silica gel (100 g) using CHCl₃ : MeOH (9.8:0.2 - 2:8) to afford five sub-fractions H-13-1-H-13-5. H-13-4 (340 mg) was subjected to repeated silica gel CC using CHCl₃ : MeOH (9.5:0.5 - 1:1) to afford TSC-1 (30 mg), TSC-2 (45 mg) and a mixture purified by preparative HPLC using 100% MeOH to afford compound 3 (13 mg) and 5 (8 mg). The ethyl acetate fraction (70 g) was subjected to silica gel CC (240 g) using CHCl₃ : MeOH (9.8:0.2 - 3:7) and 80% acetone to afford twenty two sub-fractions (E-1-E-22). Fraction E-2 (1.08 g) was subjected to silica gel CC (10 g) using n-hexane : EtOAc (8:2 - 2:8) to give fourteen sub-fractions (E-2-1-E-2-14) which afforded compound 8 (100 mg) and 9 (200 mg). Fraction E-9-5 (500 mg) was subjected to silica gel CC (15 g) using CHCl₃ : MeOH (9.9:0.1 - 0.9:6:4) to give six sub-fractions (E-9-1-E-9-16). E-9-7 (175 mg) was subjected to repeated silica gel CC (10 g) using n-hexane : EtOAc (8:2 - 1:1) and CHCl₃ : MeOH (9.7:0.3 - 3:1) to afford compound 10 (14 mg) and 11 (23 mg). E-9-8 (168 mg) was chromatographed on a C₁₈ RP silica gel column (10 g) using MeOH : H₂O (3:7 - 4:6) to afford compound 12 (117 mg) and 13 (18 mg).

2.3.1 TSS-1. White amorphous powder. IR (hydroxyl), 1720 (carbonyl). Positive-ion FAB-MS: m/z 837, 851, 865, 879, 893, 906, 921, 935, 949, 963 and 977 [M + Na]+ series. ¹H NMR (CDCl₃) δH: 0.68-0.93 (3H, t), 1.25 (2H, s, nCH₂), 4.37 (1H, d, J = 8.0 Hz, glucose H-1). ¹³C NMR: see Spectral data.

2.3.2 TSC-1. White amorphous powder. IR (hydroxyl), 1640 and 1540 (amide). [α]D⁰₂⁵ = -31.3 (c 0.1 in MeOH). Positive-ion FAB-MS: m/z 736, 764, 778, 792, 806, 820, 834, 848 and 862 [M + Na]+ series. ¹H NMR (CD₂N₂) δH: 0.86 (6H, br. t), 4.89 (1H, d, J = 8.0 Hz, glucose H-1). ¹³C NMR: see Table 1.

2.3.3 TSC-2. White amorphous powder. IR (hydroxyl), 1640, 1540 (amide). [α]D⁰₂⁵ = +29 (c 0.1 in MeOH). Positive-ion FAB-MS: m/z 810, 824, 838, 852, 866 and 880 [M + Na]+ series. ¹H NMR (CD₂N₂) δH: 0.83 (6H, br. t), 4.93 (1H, d, J = 8.0 Hz, glucose H-1). ¹³C NMR: see Table 1.

2.3.4 Methanolation of TSC-1. TSC-1 (5 mg) was heated with 5% HCl in MeOH (0.5 mL) at 70 °C for 8 h in a sealed small-volume vial. The reaction mixture was extracted with n-hexane and the extract was concentrated in vacuo to yield a mixture of fatty acid methyl ester (FAME) products. The methanolic layer was neutralized with Ag₂CO₃, filtered, and the filtrate was concentrated in vacuo to give a mixture of long chain base (LCB) and methyl glycoside products. The mixture was further evaporated and reacted with acetic anhydride/pyridine (1:1) (0.2 mL) at 70 °C for 8 h in a sealed small-volume vial followed by evaporation in vacuo to dryness. The mixture was separated using preparative TLC to afford the LCB, LCB acetates and LCB glucoacetates. ¹H, ¹³C NMR and FAB-MS analyses were performed on the FAMES and the LCB products.

2.3.5 Methanolation of TSC-2. In the same manner as described for TSC-1, TSC-2 (5 mg) was methanolyzed and the reaction mixture was worked up to give the FAMES, LCB, LCB acetates and LCB glucoacetates. The ¹H, ¹³C NMR and FAB-MS analyses were performed on the FAMES and the LCB products.

2.3.6 FAB-MS analysis of the FAME mixture from TSC-1. Positive molecular ion peaks at 287, 315, 329, 343, 357, 371, 385, 399 and 413 [M + H]+ indicated the presence of C-16, C-18-C-25 fatty acid methyl esters in TSC-1. See Fig. 1.

2.3.7 FAB-MS analysis of the LCB products from TSC-1. Positive ion FAB-MS analysis showed molecular ion peaks at 395 [M+] (LCB) and 424 [M + H]+ (LCB) acetate) indicating the presence of a C-16 LCB, while 652 [M + Na]+ (LCB glucoacetate) indicated the presence of a C-18 LCB in TSC-1. See Fig. 1.

2.3.8 FAB-MS analysis of the FAME mixture from TSC-2. Positive molecular ion peaks at 343, 357, 371, 385, 399 and 413 [M + H]+ indicated the presence of C-20-C-25 fatty acid methyl esters in TSC-2. See Fig. 1.

2.3.9 FAB-MS analysis of the LCB products from TSC-2. Positive ion FAB-MS analysis showed molecular ion peaks at 455 [M+] (LCB acetate) and 484 [M + H]+ (LCB acetate) indicating the presence of a C-16 and C-18 LCB respectively in TSC-2. See Fig. 1.

2.3.10 Identification of the sugar moiety in TSC-1. TSC-1 (5 mg) was heated with 5% HCl in MeOH (0.5 mL) at 70 °C for 8 h in a sealed small-volume vial. The reaction mixture was extracted with CHCl₃ to remove the release fatty acid. The methanolic was neutralized with Ag₂CO₃ to give the methylated sugar followed by HPLC analysis. HPLC analysis (Cosmosil-sugar-D, 4.6 ID × 250 mm, 1 mL min⁻¹, RI detector, 95% acetonitrile) against standard glucose and galactose. TSC-1 showed a retention time identical to glucose (glucose R = 14.11 min, galactose R = 13.27 min). In the same way, the sugar moiety was identified as glucose for TSC-2 and TSS-1.

2.3.11 Determination of the absolute configuration of the glycoside moiety in TSC-1 (Tanaka et al. Method).¹⁸ The glycosidic bond in TSC-1 (2 mg, 1.1 × 10⁶ mol) was hydrolyzed by heating in 0.5 M HCl (0.1 mL) and neutralized with Amberlite IRA400. After drying in vacuo, the residue was dissolved in pyridine (0.1 mL) containing 1-valine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. A 0.1 mL solution of 0-tolyl isothiocyanate (0.5 mg) in pyridine was added to the mixture, which was heated at 60 °C for 1 h. The reaction mixture was directly analyzed by reversed-phase HPLC. The peaks at 18.68 min which were coincided with the arylsulfonyl derivative of β-glucose (β-glucose R = 19.22 min). In the same way as described for TSC-1, the absolute configuration of the glucose moiety (D-form) of TSC-2 (at R = 18.5 min) and TSS-1 (at R = 18.7 min) were also determined. See Fig. 1 and 2; Spectral data.
2.4. Spectral data

2.4.1 β-Sitosteryl-3β-D-glucopyranoside-6'-O-methyl esters (TSS-1). 1H NMR (chloroform-d, 300 MHz, TMS) δH (ppm), 0.84 (3H, terminal Me), 0.68 (3H, s, H-18), 0.84 (3H, sJ= 7.8 Hz, H-28), 0.87 (3H, s, H-26), 0.88 (3H, s, H-27), 1.01 (3H, s, H-19), 1.25 (2H, s, nCH2), 2.14 (1H, m, H-23a), 2.82 (1H, m, H-23b), 3.12 (1H, m, H-24γ), 3.67 (1H, m, H-24α), 4.94 (1H, d, J= 8.0 Hz, H-1). 13C NMR (chloroform-d, 100 MHz, TMS) δC (ppm), 25.4 (C-8), 50.3 (C-9), 36.9 (C-10), 21.2 (C-11), 39.9 (C-12), 42.5 (C-13), 56.7 (C-14), 24.8 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-21), 26.5 (C-23), 45.3 (C-24), 29.1 (C-25), 19.0 (C-26), 19.1 (C-27), 23.0 (C-28), 11.9 (C-29), 101.8 (C-7), 75.8 (C-8), 75.8 (C-10), 69.9 (C-14), 73.6 (C-2), 63.1 (C-6′), 174.7 (C-1′), 34.2 (C-2′), 24.9 (C-3′), 22.5 (C-4′) and 14.12 (terminal Me).¹⁹

2.4.2 β-Sitosteryl-3β-D-glucopyranoside-6′-O-palmitate (1). 1H NMR (chloroform-d, 300 MHz, TMS) δH (ppm), 0.84 (3H, terminal Me), 0.68 (3H, s, H-18), 0.84 (3H, sJ= 7.8 Hz, H-28), 0.87 (3H, s, H-26), 0.88 (3H, s, H-27), 1.01 (3H, s, H-19), 1.25 (2H, s, nCH2), 2.14 (1H, m, H-23a), 2.82 (1H, m, H-23b), 3.12 (1H, m, H-24γ), 3.67 (1H, m, H-24α), 4.94 (1H, d, J= 8.0 Hz, H-1). 13C NMR (chloroform-d, 100 MHz, TMS) δC (ppm), 25.4 (C-8), 50.3 (C-9), 36.9 (C-10), 21.2 (C-11), 39.9 (C-12), 42.5 (C-13), 56.7 (C-14), 24.8 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-21), 26.5 (C-23), 45.3 (C-24), 29.1 (C-25), 19.0 (C-26), 19.1 (C-27), 23.0 (C-28), 11.9 (C-29), 101.8 (C-7), 75.8 (C-8), 75.8 (C-10), 69.9 (C-14), 73.6 (C-2), 63.1 (C-6′), 174.7 (C-1′), 34.2 (C-2′), 24.9 (C-3′), 22.5 (C-4′) and 14.12 (terminal Me).¹⁹

Table 1 1H and 13C NMR spectral data of TSC-1 and TSC-2 (measured in CD3OD)²⁰

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²⁰ Spectra were acquired at 23 °C. Chemical shifts are given in δ (ppm) and are referenced to internal solvent signals for CD3OD at 7.19 (δH) and 123.5 (δC) ppm. X and y are olefinic signals (double bond location).
and 5.33 (1H, m, H-6). $^{13}$C NMR (pyridine-$d_5$, 100 MHz, TMS) $\delta_c$ (ppm), 37.4 (C-1), 30.2 (C-2), 109.5 (C-3), 42.3 (C-4), 140.9 (C-5), 121.9 (C-6), 32.1 (C-7), 32.0 (C-8), 50.3 (C-9), 36.9 (C-10), 21.2 (C-11), 39.8 (C-12), 42.3 (C-13), 56.9 (C-14), 24.5 (C-15), 28.3 (C-16), 56.9 (C-17), 12.1 (C-18), 19.4 (C-19), 40.7 (C-20), 21.4 (C-21), 138.8 (C-22), 129.4 (C-23), 51.4 (C-24), 32.1 (C-25), 21.2 (C-26), 19.1 (C-27), 25.7 (C-28) and 12.1 (C-29).$^{19}$

2.4.6 β-Stigmasterol-3β-o-glucopyranoside (5). $^1$H NMR (pyridine-$d_5$, 300 MHz, TMS) $\delta_h$ (ppm), 0.68 (3H, s, H-18), 0.88 (3H, d, $J = 6.4$ Hz, H-27), 0.89 (3H, t, $J = 7.5$ Hz, H-29), 0.92 (3H,
2.4.7 Cholesterol (6). $^1$H NMR (chloroform-$d$, 400 MHz, TMS) $\delta_{H}$ (ppm), 0.69, 1.02 (6H, s, C-18, C-19), 0.83 (6H, t, C-26, C-27), 0.93 (3H, d, $J = 6.5$ Hz, C-21), 3.55 (1H, m, H-3) and 5.38 (1H, s, H-6). $^{13}$C NMR (chloroform-$d$, 100 MHz, TMS) $\delta_{C}$ (ppm), 37.4 (C-1), 31.7 (C-2), 71.8 (C-3), 43.3 (C-4), 140.9 (C-5), 121.7 (C-6), 32.0 (C-7), 31.7 (C-8), 50.3 (C-9), 36.9 (C-10), 21.2 (C-11), 39.8 (C-12), 42.3 (C-13), 56.9 (C-14), 24.5 (C-15), 28.3 (C-16), 56.9 (C-17), 12.1 (C-18), 19.4 (C-19), 40.7 (C-20), 21.4 (C-21), 138.8 (C-22), 129.4 (C-23), 51.4 (C-24), 32.1 (C-25), 21.2 (C-26), 19.1 (C-27), 25.7 (C-28), 12.5 (C-29), 102.5 (C-30), 75.3 (C-3’, 78.1 (C-3’), 71.7 (C-4’), 78.4 (C-5’) and 62.8 (C-6’).$^{29}$

2.4.8 Betulinic acid (7). $^1$H NMR (chloroform-$d$, 400 MHz, TMS) $\delta_{H}$ (ppm), 4.93 (1H, brs, H-29b), 4.76 (1H, brs, H-29a), 3.52 (1H, m, H-18), 3.46 (1H, m, H-3), 1.78 (3H, s, H-30), 1.27 (3H, s, H-23), 1.21 (3H, s, H-24), 0.84 (3H, s, H-25), 1.04 (3H, s, H-26), 1.05 (3H, s, H-27). $^{13}$C NMR (pyridine-$d_5$, 100 MHz, TMS) $\delta_{C}$ (ppm), 39.22 (C-1), 28.24 (C-2), 78.07 (C-3), 39.48 (C-4), 55.85 (C-5), 18.73 (C-6), 34.76 (C-7), 41.05 (C-8), 50.89 (C-9), 37.55 (C-10), 21.14 (C-11), 26.05 (C-12), 38.55 (C-13), 42.79 (C-14), 30.23 (C-15), 32.83 (C-16), 56.58 (C-17), 47.73 (C-18), 49.69 (C-19), 151.29 (C-20), 29.96 (C-21), 37.46 (C-22), 28.62 (C-23), 16.32 (C-24), 16.32 (C-25), 19.42 (C-26), 14.85 (C-27), 178.86 (C-28), 109.93 (C-29) and 19.42 (C-30).$^{29}$

2.4.9 (+)-Epipinoresinol (8). $^1$H NMR (chloroform-$d$, 400 MHz, TMS) $\delta_{H}$ (ppm), 6.97–6.76 (6H, m, H-6, 6”, 3’, 3’, 2’, 2”), 3.95 (3H, s, H-5’, OMe), 3.85 (3H, s, H-5”, OMe), 3.83–3.75 (2H, m, H-9, 9’), and 3.37–3.28 (2H, m, H-8, 8’). $^{13}$C NMR (chloroform-$d$ 100 MHz, TMS) $\delta_{C}$ (ppm), 146.69, 146.39 (C-3, 3’), 145.29, 144.56 (C-4, 4’), 133.00, 130.30 (C-1, 1’), 119.17, 116.37 (C-6, 6’), 114.19 (C-5, 5’), 108.47, 108.30 (C-2, 2’), 87.71, 82.07 (C-7, 7’), 70.95, 69.67 (C-9, 9’), 55.97, 55.91 (3, 3’ – OMe) and 54.46, 50.1 (C-8, 8’).$^{29}$

2.4.10 (+)-Pinoresinol (9). $^1$H NMR (chloroform-$d$, 400 MHz, TMS) $\delta_{H}$ (ppm), 6.9 (2H, d, $J = 1.5$ Hz, H-6’, 6”), 6.78 (2H, d, $J = 8.0$ Hz, H-2’, 2”), 4.73 (2H, d, $J = 5.0$ Hz, H-7’, 7”), 4.24 (2H, dd, $J = 1.5, 8.0$ Hz, H-2’, 2”), 4.38 (2H, dd, $J = 4.0, 9.5$ Hz, H-9’), 3.80 (6H, s, H-5’, 5”, OMe) and 3.09 (2H, m, H-8, 8’). $^{13}$C NMR (chloroform-$d$ 100 MHz,
whole plant. The known compounds were identified by comparison with authentic samples or reported spectral and physical data. See Fig. 1 and 2; Spectral data.

3.1. Chemistry

3.1.1 β-sitosteryl-3β-O-glucopyranoside-6′-O-fatty acid methyl ester molecular species (TSS-1). TSS-1 (94 mg) was obtained as a white amorphous solid, and showed as a single spot on silica gel TLC plate. It exhibited a strong absorption at 3350 cm⁻¹ indicating the presence of a hydroxyl group and a band at 1720 cm⁻¹ (carbonyl) indicating a stretching of a normal aliphatic ester. The ¹H and ¹³C NMR spectra reveal characteristic signals for a β-sitosterol glucoside and long fatty acid methyl esters. A series of molecular ion peaks in the positive FAB-MS spectra confirmed the possible structure of TSS-1. See Fig. 2 and Spectral data.

The NMR spectral data of TSS-1 in CDCl₃ showed resonances of a carboxylic acid group (δc 174.8), a long methane chain centered at δt 1.25 (δc 29.1–29.9) and overlapped methyls at δt 0.68–0.85 (δc 14.1), indicating normal type terminal methyls of the fatty acids. The characteristic signals of a sitosterol skeleton were determined as follows: a methine proton at δt 3.55 (1H, m, H-5, δc 79.5) and an olefinic proton signal at δt 5.38 (1H, m, H-5, δc 140.3) were assigned as C-3 and C-5 respectively. Two angular methyl protons at δt 0.68 (3H, s) and 1.01 (3H, s), corresponding to δc 11.8 and 19.3 were assigned as C-18 and C-19 respectively. The proton signals at δt 0.87 (3H, s, δc 19.0; C-26) and 0.88 (3H, s, δc 19.8; C-27) indicated the presence of an isopropenyl group in the molecular structure. The proton signal at δt 0.84 (3H, t, J = 7.8 Hz; δc 11.9) was assigned as C-29. All other NMR assignments were in agreement with known β-sitosteryl-3β-O-glucopyranoside-6′-O-fatty acid methyl esters.²⁸ Characteristic signals indicative of a presence of a monosaccharide moiety at δt 3.38–4.53, 6H with an anomic proton signal at δt 4.37 (1H, d, J = 7.8 Hz; δc 101.2) were observed. See Fig. 1 and Spectral data.

To identify the sugar moiety in TSS-1, the methylated sugar moiety in the aqueous layer after methanolation was analysed by HPLC against standard sugars (glucose and galactose) and identified as glucose (glucose tR = 11.41 min, galactose tR = 13.27 min). The coupling constant of the anomic proton at δt 5.14 (1H, d, J = 8.0 Hz) and the chemical shift of the anomic carbon δc (101.2) confirmed the β-configuration of the glucopyranoside moiety (α-glucopyranosyl: J = 3.7 Hz; δc 98.5).²⁸ The absolute configuration of the sugar moiety was determined by the Tanaka et al. method.²⁹ Direct HPLC analysis of the reaction mixture of the sugar moiety exhibited a peak at tR = 18.7 min, which were coincided with the aryl-isothiocyanate derivative of α-glucose, confirming the absolute configuration of the sugar moiety (α-glucose tR = 19.22 min).²⁸

The positive FAB-MS spectral data showed a series of molecular ion peaks at m/z: 851, 865, 879, 893, 907, 921, 935, 949, 963 and 977 [M + Na]⁺. Therefore, TSS-1 is presumed to be a molecular species consisting of β-sitosteryl-3β-O-glucopyranoside-6′-O-fatty acid methyl ester possessing mainly a hydroxy fatty acid moiety (normal type terminal methyl groups at δc 14.2) and a β-O-glucopyranosyl moiety. The core structure of the
β-sitosterol-3β-D-glucopyranose skeleton of TSS-1 was characterized by comparison of its $^{13}$C NMR spectral data with that of known β-sitosterol-3β-D-glucopyranose-6-O-fatty acid esters. See Fig. 2 and Spectral data.

### 3.1.2 Cerebrosides from TSC-1. TSC-1 (30 mg) was obtained as a white amorphous solid, and showed as a single spot on silica gel TLC plate. Strong hydroxy (3422 cm$^{-1}$) and amide absorptions (1650, 1540 cm$^{-1}$) were observed in the IR spectrum.

The NMR spectral data of TSC-1 in CD$_3$OD showed resonances of a secondary amide proton doublet at $\delta_{H}$ 8.35 (1H, d, J = 8.8 Hz), a long methylene chain, centered at $\delta_{H}$ 1.26, ($\delta_{C}$ 29.1-29.3) and overlapped methyls at $\delta_{H}$ 0.86 ($\delta_{C}$ 13.6), indicating the presence of a sphingolipid skeleton. Characteristic signals indicative of a monosaccharide moiety at $\delta_{H}$ 3.90–4.89 (6H), with the anomic proton signal at $\delta_{H}$ 4.89 (1H, d, J = 8.0 Hz; $\delta_{C}$ 105.5) were observed. The characteristic resonances for the 2-amino-1,3,2-triol region of the hydrocarbon chain were observed at $\delta_{H}$ 4.77 (1H, m, H-2); 4.59 (1H, m, H-2'); 4.77 (1H, m, H-1b); 4.21 (1H, m, H-1a); 4.71 (1H, m, H-3) corresponding to the following $^{13}$C NMR data: $\delta_{C}$ 54.0 (C-2), 71.8 (C-2'), 70.8 (C-1), 71.8 (C-3), and an amide carbonyl signal at $\delta_{C}$ 175.0 (C-1'). See Fig. 1 and Table 1.

The presence of two disubstituted double bonds at $\delta_{C}$ 131.4 (2CH, C-4, C-5) and 129.9 (2CH) were observed. The $E$ geometry for the double bonds was supported from the characteristic chemical shift of the allylic carbons at $\delta_{C}$ 32.2, 32.3 and 34.2 (Z geometry = $\delta_{C}$ 27.0).

The positive FAB-MS spectral data showed a series of molecular ion peaks due to [M + Na]$^+$ at m/z: 736, 764, 778, 792, 806, 820, 834 and 848. TSC-1 is hence presumed to be a molecular species consisting of a sphingo-type cerebroside possessing mainly a 2-hydroxy fatty acid moiety (normal type terminal methyls at $\delta_{C}$ 13.6) and a β-D-glucopyranosyl moiety.

The sphingosine skeleton was characterized by comparison of its $^1$H and $^{13}$C NMR spectral data (Fig. 1 and Table 1) with that of known cerebroside. The relative stereochemistry of the ceramide moiety is presumed to be (2S,3R,4E,2'R) since the characteristic $^{13}$C NMR signals (C-1, 2, 3, 4, 1' and 2') in addition to the optical rotation value of $[\alpha]_{D}^{20} = -31.3$ are in good agreement with those of the sphingo-type glucocerebroside molecular species possessing a 2S,3R,4E,2'R configuration. The analysis of the methylated sugar against standard sugars (glucose and galactose) using HPLC indicated that the sugar moiety in TSC-1 was glucose (glucose $\tau_R$ = 14.11 min, galactose $\tau_R$ = 13.27 min). The coupling constant of the anomic proton at $\delta_{H}$ 4.89 (1H, d, J = 8.0 Hz) and the chemical shift of the anomic carbon $\delta_{C}$ (105.5) confirmed the β-configuration of the glucopyranoside moiety (α-glucopyranoside: J = 3.7 Hz; $\delta_{C}$ 98.5). The absolute configuration of the sugar moiety was determined using the Tanaka et al. method.

HPLC analysis of the reaction mixture exhibited a peak at $\tau_R$ = 20.5 min, which were coincided with the aryl-isothiocyanate derivative of D-glucose, confirmed the absolute configuration of the sugar moiety (L-glucose $\tau_R$ = 19.22 min).

In the positive FAB-MS spectral of TSC-2, a series of molecular ion peaks due to 810, 824, 838, 852, 866 and 880 [M + Na]$^+$ were observed. Therefore, TSC-2 is presumed to be a molecular species consisting of a phytosphingosine-type cerebroside possessing mainly a 2-hydroxy fatty acid moiety (normal methyls at $\delta_{C}$ 13.6) and a β-D-glucopyranosyl moiety.

The core structure of the phytosphingosine skeleton in TSC-2 was characterized by comparison of its $^1$H and $^{13}$C NMR spectral data (Fig. 1 and Table 1) with that of known cerebrosides. The relative stereochemistry of the ceramide moiety is presumed to be (2S,3R,4R,2'R) since the characteristic $^{13}$C NMR signals (C-1, 2, 3, 4, 1' and 2') and the optical rotation value $[\alpha]_{D}^{20} = +29.4$ are in good agreement with those of the...
phytosphingosines-type glucocerebroside molecular species possessing a 2S,3R,4R,2’R configuration.24

In the FAB-MS analyses of the methanalysis products of TSC-2, the FAME mixture showed molecular ion peaks at 343, 357, 371, 383 and 413 [M + H]+, indicating the presence of C-20–C-25 fatty acid methyl esters, possessing normal terminal methyl groups (δC 13.6). The LCB also indicated the presence of a C-16 and C-18 long chain base identified from the corresponding molecular ion peaks at of the LCB acetates at m/z 455 [M + H]+ and 484 [M + H]+ respectively. See Fig. 1 and Table 1.

3.2. Discussion

A sphingosine-type (TSC-1) and phytosphingosine-type (TSC-2) cerebrosides, with both containing mainly a 2-hydroxy fatty acids and β-D-glucopyranose moieties were isolated from the n-hexane fraction of T. sanguinea in this study. Cerebrosides and ceramides have received a lot of interest in their isolation and characterization due to their significant biological activities such as immunomodulatory antioxidant, antitumor, anti-inflammatory and antiviral.29,30

Seven triterpenes were isolated from the n-hexane fraction of plant: β-sitosterol-3β-D-glucopyranoside-O-fatty acid methyl esters molecular species TSS-1, β-sitosterol-3β-D-glucopyranoside-O-palmitate 1, β-sitosterol 2, β-sitosterol-3β-D-glucopyranoside 3, β-stigmasterol 4, β-stigmasterol-3β-D-glucopyranoside 5, cholesterol and betulinic acid 7. Biological functions of plant sterols include antihelminthic, antidiabetic, antiinflammatory, antiapoptotic, antioxygenic, antioxidant, immunomodulatory and neuroprotective in neurodegenerative disorders like Alzheimer’s disease.31,32

Betulinic acid exhibits a variety of biological and medicinal properties such as inhibition of HIV, antibacterial, antimalarial, antinflammatory, antihemimetic, antioxygenic, anti-HSV-1 and anticancer activities.33

The five lignans isolated from the ethyl acetate fraction of T. sanguinea: (+)-epipinoresinol 8, (+)-pinoresinol 9, (+)-cycloolivil 10, (+)-secoisolariciresinol 11 and (+)-isolariciresinol 12 are reported to also have antiviral, antifungal, antimicrobial antifeedant and insecticidal properties and are probably related to plant defense against various pathogens and pests. They also have significant biological activities including antitumor, antinflammatory, immunosuppression, cardiovascular, antioxidant and antiviral.34 Pinoresinol and secoisolariciresinol are mammalian lignan precursors which are converted into enterodiol (END) and enterolactone (ENL) by the intestinal microflora.22,23 These enterolignans afford protection against osteoporosis, cardiovascular diseases, liver diseases, hyperlipidemia, breast cancer, colon cancer, prostate cancer and menopause syndrome.25,37-39 The flavanone (+)-eriodictyol 13, also isolated from the ethyl acetate fraction is reported to have significant antinflammatory, anticancer, neurotrophic, and antioxidant effects.28

4. Conclusions

In summary, this paper describes the isolation of two glucocerebrosides, TSC-1 and TSC-2, one molecular species TSS-1, seven triterpenes 1–7, five lignans 8–12, and one flavanone 13, from the n-hexane and ethyl acetate fractions of whole plant of T. sanguinea. To the best of our knowledge, all the isolated compounds from T. sanguinea in this study are being reported for the first time. These compounds have a wide range of biological activities and may act individually or in synergy to produce these biological effects. They may therefore be partly, or wholly responsible for these biological actions, giving credence to the use of T. sanguinea in traditional medicine. Further work to increase the amount of isolated cerebrosides molecular species to determine the double bond location in the long chain bases as well as purify and characterize the individual pure cerebrosides is underway.

Conflicts of interest

There are no conflicts to declare.

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