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In vitro Antibacterial and *in vivo* Brine Shrimp Lethal Active Compounds Isolated from the Leaves of *Saurauia roxburghii*

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ABSTRACT
Seven triterpenoids namely, ursolic acid (1) cis-3-O-p-hydroxycinnamoyl ursolic acid (2), trans-3-O-p-hydroxycinnamoyl ursolic acid (3), oleanolic acid (4), corosolic acid (5), maslinic acid (6) and β-amyrin (7) were isolated from the leaves of *Saurauia roxburghii* based on spectroscopic indication. Compounds 2, 3 and 4 were isolated from this plant for the first time. The LC_{50} for crude ethanol, n-hexane, chloroform, ethyl-acetate; column fractions H-13 of the crude n-hexane, C-19 of the crude chloroform and three pure compounds 1, 3 and 4 were found to be 12.59, 14.79, 14.06, 11.75, 10.96, 4.37, 6.92 and 11.88 μg mL^{-1}, respectively. The crude ethanol extract exhibited good; n-hexane, chloroform, ethyl-acetate extracts exhibited moderate; five column fractions (H-13, C-15, C-19, E-18 and E-24) exhibited low to mild and three pure compounds (1, 3 and 4) presented moderate antibacterial activity against maximum of the tested bacteria.

Key words: Triterpenoids, cis-3-O-p-hydroxycinnamoyl ursolic acid, trans-3-O-p-hydroxy-cinnamoyl ursolic acid, oleanolic acid, antimicrobial and cytotoxic activity

INTRODUCTION

Since ancient times, researchers have isolated many secondary metabolites from the medicinal plants. Different medicinal plants have traditionally been used to treat the various diseases like tumor or cancer treatment (Lee *et al*., 2007). The pharmacological screenings of plants is a significant mean for exposure the new, harmless and effective drugs. About 80% of human use herbal drug for their primary health care at least once in their life (Bhakuni *et al*., 1974; Farnsworth *et al*., 1985). Herbal medicines have a vital role in the prevention and treatment of various diseases, which are available and reasonably inexpensive (Sundaram *et al*., 2011). Medicinal plants through the multiplicities of their chemical constituents are important for the discovery of new secondary metabolites against tumors and other cancers. More than 60% of commercial anticancer drugs have been used in the United State of America, which were isolated from natural origin (Stevigny *et al*., 2005; Newman and Cragg, 2007).

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at higher doses and toxicology is simply pharmacology at lower doses (Bernhoft *et al*., 2010). Brine shrimp lethality bioassay (Meyer *et al*., 1982; McLaughlin *et al*., 1998) is a rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin. By this method, crude extracts, fractions as well as the pure compounds can be tested for their

bioactivity. In this method, *in vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products.

Saurauia roxburghii (Common name-Singkrang, Sing khau, Bon posola, PannikomariPahariKadam or BholaKadam), is synonymous to *Saurauja roxburghii*, *Ternstroemia serrata*, Family-Actinidiaceae, Synonyms-Dilleniaceae, Saurauiaceae and is an evergreen tree and important medicinal plant originating from Bangladesh. The species grows throughout Vietnam, Nepal, India, Myanmar, Thailand, Cambodia, Laos, China (including Taiwan), Japan and Malaysia and the species grows less frequently in the greater districts of Sylhet, Chittagong and Chittagong Hill Tracts in Bangladesh (Uddin, 2006; Rahman *et al*., 2007). Former phytochemical research ensued the isolation of ursolic acid, 24-hydroxy corosolic acid, corosolic acid, maslinic acid and 3b,7b, 24-trihydroxy-urs-12-en-28-oic acid (Mazumder *et al*., 2011), β-amyrin (Chowdhury *et al*., 2012) and two steroids, stigmasterol and β-sitosterol (Ahmed *et al*., 2013). To date, there have been no elaborate biological studies on antibacterial and cytotoxic activity from *Saurauia roxburghii*. The stems and leaves of the plants are extensively used as herbal medicines for a large number of severe diseases (Uddin, 2006; Rizwana *et al*., 2010) like asthma, bronchitis, hepatitis B, ulcers, fever, gout, eczema, pilesand Central Nerves System (CNS) depression. In view of its many applications, this study sought to isolate and evaluate the antibacterial and cytotoxic active pure compounds from the leaves of *Saurauia roxburghii*.

MATERIALS AND METHODS

Collection of plant material: Fresh leaves of the *Saurauia roxburghii* were collected from the hillsides of the University of Chittagong, Bangladesh and were taxonomically identified by Sardar Nasir Uddin, Senior Scientific Officer, Bangladesh National Herbarium (BNH), Dhaka, Bangladesh. A voucher specimen (DACB 32567) that contains the identification characteristics of the plant has been reserved in BNH for future reference.

Extraction and isolation: Approximately 1.0 kg of the dry ground samples was stored in a clean RB flask (5 L) and waterlogged in 3 L of ethanol. The flask was then sealed with aluminium foil and retained for seven days accompanying random trembling and rousing. The filtrate was separated from the mixture through the use of filter paper and then evaporated in 40-50°C under reduced pressure by the use of Buchii Rotary Evaporator from which a sticky concentrate crude ethanol extract (180 g) was obtained. The crude ethanol extract (30 g) was partitioned with n-hexane, chloroform and finally with ethyl acetate. The resulting evaporation of solvents gave 7.0 g of n-hexane, 5.0 g of chloroform, 2.5 g of ethyl acetate extracts and 12.0 g of aqueous soluble materials. Continual chromatographic parting and purifying of the crude ethanol and chloroform extracts of the leaves of *S. roxburghii* presented seven triterpenoids namely, cis-3-O-p-hydroxycinnamoyl ursolic acid (1), trans-3-O-p-hydroxycinnamoyl ursolic acid (2), ursolic acid (3), oleanolic acid (4), corosolic acid (5), maslinic acid (6) and β-amyrin (7). The configurations of these isolated pure compounds were elucidated by spectroscopic analysis.

Ursolic acid (1): White amorphous powder. IR (KBr) υ_{max}: 3446.79, 3101.54, 2926.01, 2854.65, 1734.01, 1653.00, 1458.18, 1375.25, 1163.08, 1035.77 and 815.89. ^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD) δ: 5.18 (1H, brs, H_{12}), 3.13 (1H, dd,10.5, 5.5 Hz, H_3), 2.14 (1H, d, 11.5 Hz, H_{18}), 0.88 (3H, d, 5.5 Hz, H_{29}), 0.81 (3H, d, 6.5 Hz, H_{30}), 1.05 (3H, s, H_{23}), 1.04 (3H, s, H_{27}), 0.92 (3H, s, H_{26}), 0.90 (3H, s, H_{24}) and 0.77 (3H, s, H_{25}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ: 39.3 (CH_2, C_1), 27.3 (CH_2, C_2), 79.3 (CH, C_3), 39.7 (Cq, C_4), 55.9 (CH, C_5), 18.9 (CH_2, C_6), 33.7 (CH_2, C_7), 40.1 (Cq, C_8), 49.5 (CH, C_9), 37.5 (Cq, C_{10}), 23.8 (CH_2, C_{11}), 126.1 (CH, C_{12}), 138.8 (Cq, C_{13}), 42.6 (Cq, C_{14}), 28.6 (CH_2, C_{15}), 24.8 (CH_2, C_{16}), 49.7 (Cq, C_{17}), 53.5 (CH, C_{18}), 39.5 (CH_2, C_{19}), 39.3 (Cq, C_{20}), 31.2 (CH_2, C_{21}), 37.4 (CH_2, C_{22}), 28.5 (CH_3, C_{23}), 16.1 (CH_3, C_{24}), 15.9 (CH_3, C_{25}), 17.4 (CH_3, C_{26}), 24.0 (CH_3, C_{27}), 181.3 (Cq, C_{28}), 17.5 (CH_3, C_{29}), 21.6 (CH_3, C_{30}).

Cis-3-O-p-hydroxycinnamoyl ursolic acid (2): Colorless powder. IR (KBr) υ_{max} : 3446.79, 2924.09, 2852.72, 1730.34, 1690.0, 1490.34, 1350.0, 1258.23, 1168.86 and 815.89. ^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD) δ: 7.62 (2H, d, 8.5 Hz, $H_{2'', 6''}$), 6.76 (2H, d, 8.5 Hz, $H_{3'', 5''}$), 6.80 (1H, d,12.8 Hz, $H_{3'}$), 5.80 (1H, d, 12.8 Hz, $H_{2'}$), 5.28 (1H, t, H_{12}), 4.57 (1H, d,10.0 Hz $H_{3α}$), 2.16 (1H, d, 11.5 Hz, H_{18}), 0.90 (3H, d, 5.5 Hz, H_{29}), 0.82 (3H, d, 6.5 Hz, H_{30}), 1.21 (3H, s, H_{23}), 1.07 (3H, s, H_{27}), 0.99 (3H, s, H_{26}), 0.91 (3H, s, H_{24}) and 0.79 (3H, s, H_{25}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ: 39.3 (CH_2, C_1), 27.3 (CH_2, C_2), 81.10 (CH, C_3), 39.7 (Cq, C_4), 55.9 (CH, C_5), 18.9 (CH_2, C_6), 33.7 (CH_2, C_7), 40.1 (Cq, C_8), 49.5 (CH, C_9), 37.5 (Cq, C_{10}), 22.18 (CH_2, C_{11}), 124.5 (CH, C_{12}), 138.5 (Cq, C_{13}), 42.6 (Cq, C_{14}), 28.6 (CH_2, C_{15}), 24.8 (CH_2, C_{16}), 46.01 (Cq, C_{17}), 53.5 (CH, C_{18}), 39.5 (CH_2, C_{19}), 39.3 (Cq, C_{20}), 31.2 (CH_2, C_{21}), 37.4 (CH_2, C_{22}), 28.03(CH_3, C_{23}), 16.1 (CH_3, C_{24}), 15.9 (CH_3, C_{25}), 17.4 (CH_3, C_{26}), 24.0 (CH_3, C_{27}), 181.3 (Cq, C_{28}), 17.5 (CH_3, C_{29}), 21.6 (CH_3, C_{30}),167.71 (Cq, $C_{1'}$), 119.70 (CH, C_2), 144.38 (CH, $C_{3'}$), 128.89 (Cq, $C_{1''}$), 133.91 (CH, $C_{2'', 6''}$), 116.2 (CH, $C_{3'', 5''}$), 157.75 (Cq, $C_{4''}$).

Trans-3-O-p-hydroxycinnamoyl ursolic acid (3): Colorless powder. IR (KBr) υ_{max}: 3446.79, 2924.09, 2852.72, 1730.34, 1690.0, 1490.34, 1350.0, 1258.23, 1168.86 and 815.89.

^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD) δ: 7.60 (1H, d, 16.0 Hz, $H_{3'}$), 6.78 (2H, d, 8.5 Hz, $H_{3'', 5''}$), 7.38 (2H, d, 8.5 Hz, $H_{2'', 6''}$), 6.29 (1H, d, 16.0 Hz, $H_{2'}$), 5.21 (1H, t, H_{12}), 4.50 (1H,

d,10.0 Hz $H_{3\alpha}$), 2.16 (1H, d, 11.5 Hz, H_{18}), 0.90 (3H, d, 5.5 Hz, H_{29}), 0.82 (3H, d, 6.5 Hz, H_{30}), 1.21 (3H, s, H_{23}), 1.07 (3H, s, H_{27}), 0.99 (3H, s, H_{26}), 0.91 (3H, s, H_{24}) and 0.79 (3H, s, H_{25}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ: 39.3 (CH_2, C_1), 27·3 (CH_2, C_2), 81.10 (CH, C_3), 39.7 (Cq, C_4), 55.9 (CH, C_5), 18.9 (CH_2, C_6), 33.7 (CH_2, C_7), 40.1 (Cq, C_8), 49.5 (CH, C_9), 37.5 (Cq, C_{10}), 22.18 (CH_2, C_{11}), 124.5 (CH, C_{12}), 138.5 (Cq, C_{13}), 42.6 (Cq, C_{14}), 28.6 (CH_2, C_{15}), 24.8 (CH_2, C_{16}), 46.01 (Cq, C_{17}), 53.5 (CH, C_{18}), 39.5 (CH_2, C_{19}), 39.3 (Cq, C_{20}), 31.2 (CH_2, C_{21}), 37.4 (CH_2, C_{22}), 28.03 (CH_3, C_{23}), 16.1 (CH_3, C_{24}), 15.9 (CH_3, C_{25}), 17.4 (CH_3, C_{26}), 24.0 (CH_3, C_{27}), 181.3 (Cq, C_{28}), 17.5 (CH_3, C_{29}), 21.6 (CH_3, C_{30}), 168.34 (Cq, $C_{1'}$), 118.23 (CH, $C_{2'}$), 144.98 (CH, $C_{3'}$), 128.56 (Cq, $C_{1''}$), 130.81 (CH, $C_{2'', 6''}$), 117.8 (CH, $C_{3'', 5''}$), 158.35 (Cq, $C_{4''}$).

Oleanolic acid (4): White amorphous powder. IR (KBr) υ_{max}: 3298.28, 3122.75, 2920.23, 2852.72, 1716.65, 1653.00, 1458.18, 1375.25 1188.15, 1035.77 and 827.46. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.06 (1H, brs, H_{12}), 3.52 (1H, m, H_3), 3.14 (1H, dd, 8.0, 8.0 Hz, H_{18}), 1.01 (3H, s, H_{27}), 0.95 (3H, s, H_{23}), 0.92 (3H, s, H_{26}), 0.89 (3H, s, H_{24}), 0.85 (3H, s, H_{30}), 0.81(3H, s, H_{29}) and 0.73 (3H, s, H_{25}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ: 39.3 (CH_2, C_1), 21.5 (CH_2, C_2), 79.3 (CH, C_3), 39.7 (Cq, C4), 55.9 (CH, C_5), 18.9 (CH_2, C_6), 33.7 (CH_2, C_7), 40.1 (Cq, C_8), 49.5 (CH, C_9), 37.5 (Cq, C_{10}), 23.8 (CH_2, C_{11}), 124.1 (CH, C_{12}), 143.5 (Cq, C_{13}), 42.6 (Cq, C_{14}), 28.6 (CH_2, C_{15}), 24.8 (CH_2, C_{16}), 48.2(Cq, C_{17}), 41.9 (CH, C_{18}), 48.4 (CH_2, C_{19}), 31.3 (Cq, C_{20}), 39.2 (CH_2, C_{21}), 37.4 (CH_2, C_{22}), 28.5 (CH_3, C_{23}), 16.1 (CH_3, C_{24}), 15.9 (CH_3, C_{25}), 17.4 (CH_3, C_{26}), 26.5 (CH_3, C_{27}), 181.9 (Cq, C_{28}), 33.1 (CH_3, C_{29}), 24.0 (CH_3, C_{30}).

2*α*, 3β-dihydroxy-urs-12-en-28-oic acid or corosolic acid (5): White amorphous powder. IR (KBr) υ_{max}: 3396.64, 2924.09, 2852.72, 1701.22, 1660.00, 1458.18, 1375.25, 1168.86, 1049.28 and 813.96. ^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD) δ: 5.25 (1H, like t, H_{12}), 3.65 (1H, ddd, 10.4, 9.8, 4.5 Hz, H_2), 2.95 (1H, d, 9.5 Hz, H_3), 2.22 (1H, d, 11.0 Hz, H_{18}), 0.96 (3H, d, 6.0 Hz, H_{30}), 0.87 (3H, d, 6.5 Hz, H_{29}), 1.12 (3H, s, H_{27}), 1.03 (3H, s, H_{23}), 1.01 (3H, s, H_{25}), 0.84 (3H, s, H_{24}) and 0.82 (3H, s, H_{26}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ : 47.1 (CH_2, C_1), 69.1 (CH, C_2), 83.9 (CH, C_3), 39.7 (Cq, C_4), 55.9 (CH, C_5), 18.9 (CH_2, C_6), 33.6 (CH_2, C_7), 39.6 (Cq, C_8), 47.3 (CH, C_9), 38.7 (Cq, C_{10}), 24.0 (CH_2, C_{11}), 125.9 (CH, C_{12}), 138.3 (Cq, C_{13}), 42.1 (Cq, C_{14}), 28.6 (CH_2, C_{15}), 24.7 (CH_2, C_{16}), 48.2 (Cq, C_{17}), 53.5 (CH, C_{18}), 40.2 (CH_2, C_{19}), 39.9 (Cq, C_{20}), 31.2 (CH_2, C_{21}), 37.4 (CH_2, C_{22}), 29.0 (CH_3, C_{23}), 17.2 (CH_3, C_{24}), 17.4 (CH_3, C_{25}), 17.38 (CH_3, C_{26}), 17.2 (CH_3, C_{27}), 181.1 (Cq, C_{28}), 17.4 (CH_3, C_{29}), 21.4 (CH_3, C_{30}).

2*α*, 3β-dihydroxy-olea-12-en-28-oic acid or maslinic acid (6): White amorphous powder. IR (KBr) υ_{max}: 3446.79, 2924.09, 2852.72, 1701.22, 1653.00, 1458.18, 1375.25, 1166.93, 1055.06 and 813.96. ^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD) δ: 5.23 (1H, like t, H_{12}), 3.60 (1H, m, H_2), 2.89 (1H, d, 10.0 Hz, H_3), 2.82 (1H, dd, 10.0, 2.0 Hz, H_{18}), 1.14 (3H, s, H_{27}), 0.99 (3H, s, H_{23}), 0.98 (3H, s, H_{25}), 0.92 (3H, s, H_{30}), 0.89 (3H, s, H_{29}), 0.82 (3H, s, H_{24}) and 0.78 (3H, s, H_{26}). ^{13}C-NMR (125 MHz, $CDCl_3$+CD_3OD) δ: 40.06 (CH_2, C_1), 67.18 (CH, C_2), 78.29 (CH, C_3), 37.49 (Cq, C_4), 48.39 (CH, C_5), 17.48 (CH_2, C_6), 31.24 (CH_2, C_7), 39.27 (Cq, C_8), 48.18 (CH, C_9), 37.42 (Cq, C_{10}), 21.55 (CH_2, C_{11}), 122.14 (CH, C_{12}), 144.01 (Cq, C_{13}), 42.64 (Cq, C_{14}), 27.26 (CH_2, C_{15}), 24.01 (CH_2, C_{16}), 48.64 (Cq, C_{17}), 39.69 (CH, C_{18}), 42.64 (CH_2, C_{19}), 28.59 (Cq, C_{20}), 33.65 (CH_2, C_{21}), 31.24 (CH_2, C_{22}), 28.50 (CH_3, C_{23}), 23.84 (CH_3, C_{24}), 16.11 (CH_3, C_{25}),17.38 (CH_3, C_{26}), 26.1 (CH_3, C_{27}), 182.17 (Cq, C_{28}), 33.1 (CH_3, C_{29}), 24.78 (CH_3, C_{30}).

β-amyrin (7): The NMR spectrum was identical to that of an authentic sample (Falodun *et al*., 2009; Chowdhury *et al*., 2012).

Bioassays

***In vitro* antibacterial bioassays:** The antibacterial activity of the test samples was determined by the disc diffusion method (Bauer *et al*., 1966; Barry, 1991; Ahmed *et al*., 2010). The samples were dissolved separately in a specific volume of chloroform and applied to sterile discs at a concentration of 500 μg $disc^{-1}$ for the crude ethanol, n-hexane, chloroform and ethyl-acetate extracts; 400 μg $disc^{-1}$ for five column fractions (H-13, C-15, C-19, E-18 and E-24) of various extracts and 300 μg $disc^{-1}$ for pure compounds 1, 3 and 4 for their antibacterial assays and standard disc of kanamycin (30 μg $disc^{-1}$) were shown in Table 1.

***In vivo* brine shrimp lethality bioassay:** The cytotoxic activity of the test samples was executed as previously designated (Meyer *et al*., 1982; McLaughlin *et al*., 1998). The experimental samples of crude extracts and column fractions were liquefied in dimethyl sulfoxide (DMSO) and sequential dilution were prepared as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 μg mL^{-1}. In contrast, the tested pure compounds were liquefied in DMSO and successive dilution were prepared as 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.3906 μg mL^{-1}. Each experimental solution was then poured into test tubes containing 10 shrimps with 5 mL of simulated saline water and incubated at room temperature for one day. After the time interval, the middle Lethal Concentration (LC_{50}) of the experimental samples was achieved from a plot of the percentage of shrimps killed against the logarithm of the sample concentration. Vincristine Sulfate (VS) was used as a positive control in this bio-assay to compare the cytotoxic activity of the test samples. For negative controls, DMSO (100 μL) was used for each of the three pre-marked test tubes containing same volume and number of simulated seawater

Table 1: *In vitro* antibacterial activity of *Saurauia roxburghii* extracts, column fractions, pure compounds and kanamycin

Name of the bacteria	500 (µg disc^{-1})				400 (µg disc^{-1})								
	Et	He	Ch	EA	H-13	C-15	C-19	E-18	E-24	Com-1	Com-3	Com-4	Kanamycin
Gram positive													
B. cereus	16±1	10±1	13±1	8±2	8±1	9±1	10±1	9±1	12±1	11±1	14±1	13±1	34±1
B. megaterium	15±1	-	13±1	-	-	7±1	9±1	-	-	8±1	9±1	14±1	34±1
B. subtilis	16±2	12±1	14±1	10±1	9±1	9±2	8±1	8±1	12±1	12±1	16±1	15±1	32±2
S. aureus	18±2	11±1	14±1	8±1	8±1	8±1	10±1	12±1	10±1	12±1	14±1	16±1	35±1
B. polymyxa	16±1	10±1	-	8±1	10±1	-	-	-	-	14±1	12±1	15±1	33±1
S. pneumoniae	16±1	12±1	9±2	-	-	-	-	-	-	12±1	8±1	16±1	34±1
M. tuberculosis	22±1	13±1	20±2	9±2	10±2	10±2	12±2	9±1	12±1	20±2	16±2	20±2	31±2
Gram negative													
E. coli	18±2	13±2	16±1	12±1	14±2	10±1	8±1	7±1	14±2	12±1	15±1	12±1	35±1
Klebsiella sp.	14±1	9±1	12±1	7±1	10±1	-	-	-	-	9±1	15±2	13±1	32±2
Proteus sp.	14±1	8±2	12±1	8±1	-	-	-	-	-	-	11±1	14±1	30±3
S. typhi	15±1	-	13±2	10±2	-	-	-	-	-	13±1	-	-	33±1
S. sonnei	14±1	7±2	15±1	7±1	10±1	10±1	13±1	7±1	12±1	-	12±1	13±1	34±1
P. Aureus	18±2	9±1	16±1	10±1	-	7±1	8±1	14±2	10±1	12±1	-	16±1	34±1
V. cholerae	14±1	-	13±1	-	-	9±1	8±2	-	-	8±1	10±1	12±1	31±2

Et: Ethanol extract, He: n-hexane extract, Ch: Chloroform, EA: Ethyl acetate extract, column fractions (H-13, C-15, C-19, E-18 and E-24) of various extract and three pure compounds (1, 3, 4), NA: No activity

Table 2: LC_{50} data for various extracts, column fractions, three pure compounds of *Saurauia roxburghii* and vincristine sulfate

Samples	LC_{50} (µg mL^{-1})
VS	0.32
Ethanol	12.59
n-hexane	14.79
Chloroform	14.06
Ethyl-acetate	15.47
H-13	11.75
C-19	10.96
Compound-1	4.37
Compound-3	6.92
Compound-4	11.88

VS: Vincristine sulphate (Std.), column fractions H-13 and C-19 of n-Hexane and chloroform extract, respectively

and shrimp nauplii, respectively. If shrimps in the tubes showed rapid mortality, then the test was considered unacceptable as the nauplii died for some reasons other than toxicity of the compounds.

After 24 h, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. For each extract, three samples were prepared for each of the bioassays. LC_{50} was calculated as Mean±SD (n = 3) and shown in Table 2.

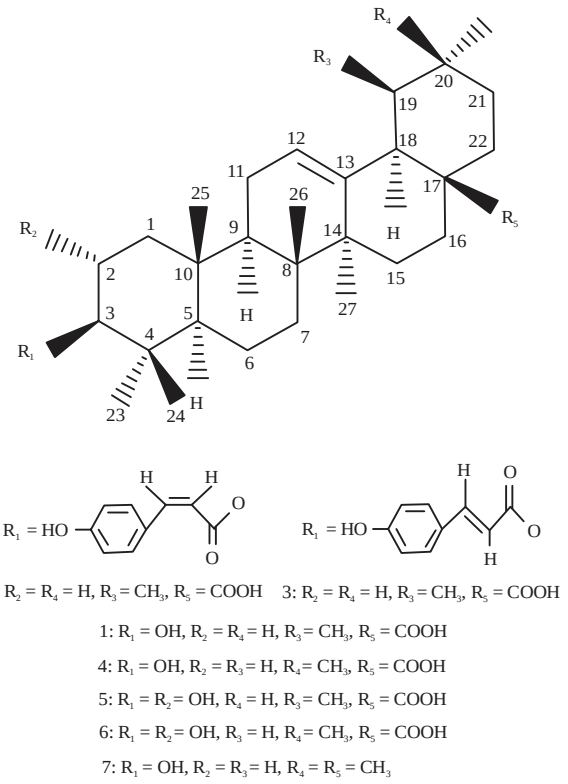

Fig. 1: Secondary metabolites isolated from *Saurauia roxburghii*

RESULTS AND DISCUSSION

Characterization of isolated compounds: The structures of isolated pure compounds are shown in Fig. 1. Ursolic acid (1) was obtained as amorphous powder and also determined by using the data of IR spectrum and hydroxyl (-OH) group was observed at 3446.79 cm^{-1}, =CO and -OH of carboxylic group at 1701.0 and 3101.54 cm^{-1}, respectively and trisubstituted double bond at 1690 and 820 cm^{-1} (Taketa *et al*., 2004). In ^{1}H NMR spectrum five methyl high field singlets at 1.05, 1.04, 0.92, 0.90 and 0.77 presented five corner methyl groups in the molecular and another two high field proton signals at δ 0.8 (3 H, d, 5.5 Hz, H-29) and 0.81 (3 H, d, 6.5 Hz, H-30) typically designated that there were two methyl groups substituted on ring. The signals at δ 126.1, 138.8 ppm of ^{13}C NMR and the signal at δ 5.18 (br s) ppm of ^{1}H NMR

suggests that this compound was 12-unsaturated pentacyclic triterpenoid, one of the olefinic carbons was a quaternary carbon (C-13). The signal at δ 79.3 ppm of ^{13}C NMR was correlated ^{1}H NMR signal at δ 3.13 (1H, dd, 10.5, 5.5 Hz) indicated a ring carbon was substituted by a germinal proton (-OH) at C-3 position. one carboxylic acid group also confirmed by ^{13}C NMR signal at δ 181.3 ppm. The signal of H-18 permitted the distinction between the oleane (δ 3.1-3.3 ppm) and ursane (δ 2.1-2.6 ppm) skeletons in the proton NMR spectrum (Falodun *et al*., 2009). Moreover, ^{13}C NMR spectrum by reason of C-18 to C-22 recommended that it was a derivative of urs-12-en (Seebacher *et al*., 2003; Ozgen *et al*., 2011). All these spectral data indicated the structure of compound 1 to be 3β-hydroxy-urs-12-en-28-oic acid known as ursolic acid. A survey of the literature exhibited compound 1 to be identical to that of a triterpenoid that was earlier reported from the plant *Ilex paraguariensis* (Taketa *et al*., 2004), *Vaccinium macrocarpon* (Huang *et al*., 2009), *Malus domestica* (Frighetto *et al*., 2008) and *Thymus sipyleus* (Ozgen *et al*., 2011).

Cis-3-O-p-hydroxycinnamoyl ursolic acid (2) was found as a colorless powder. The IR spectrum presented its characteristics -OH group at 3446.79 cm^{-1}, the conjugated ester at 1730.34 cm^{-1} along with C-O (1258.23 and 1168.86 cm^{-1}), trisubstituted double bond (1690.0, 1490.34 and 815.89 cm^{-1}) and hydrocarbon C-H absorptions (2924.09, 2852.72 and 1350.0 cm^{-1}). The ^{1}H and ^{13}C NMR data of compound 2, there are existed a ursolic acid moiety, corresponding with ursolic acid. ^{1}H NMR spectrum of compound 2 presented five methyl singlets at *δ* 1.21, 1.07, 0.99, 0.91, 0.79 along with two doublet proton signals at δ 0.90 (d, 5.5 Hz) and 0.82 (d, 6.5 Hz) for H-29 and H-30, respectively confirmed its ursane skeletons (Seebacher *et al*., 2003; Ozgen *et al*., 2011). The down field signals at δ 7.62 (2H, d, 8.5 Hz, H-2", 6") and 6.76 (2H, d, 8.5 Hz, H-3", 5") showed the presence of 1,4-disubstituted benzene fragment and two others down field signals confirmed its double bond conjugated proton in the aromatic ring at δ 6.80 (1H, d, 12.8 Hz, H-3') and 5.80 (1H, d, 12.8 Hz, H-2'). The ^{13}C NMR data also confirmed its existence triterpenoid moiety by compared with ursolic acid (1) (Sang *et al*., 2001). The additional nine carbon signals, which were all in the low field, were read as aromatic carbons. The chemical shift of a methyl group (C-23) at 28.0 ppm was assigned to ring A. The presence of a double bond in compound 2 was observed at 124.5 (C-12) and 22.2 (C-11) and the distinguishing -COOH group leads to move the quaternary C-17 at 46.0 ppm. The down field shift at 81.1 ppm approving the position of ester at C-3. Consequently, the p-hydroxycinnamic acid was connected at C-3 by the O-C ester bond. The coupling constants (J = 12.8 Hz) allocate the *α* or axial configuration to 3-H. The two olefinic protons at C-2 (δ H_a 5.8 ppm) and C-3 (δ H_b 6.8 ppm) exhibited vicinal coupling with each other. The coupling constant for H_a and H_b showed that the nature of double bond was cis (Jahan *et al*., 2001). The C-2"/6" (δ 7.62) protons showed couplings with protons at C-3"/C-5" (δ 6.76). This confirmed that the α, β-unsaturated ester and the hydroxyl group are 1,4-disubstituted to the phenyl residue. Therefore, compound 2 was recognized as cis-3-O-p-hydroxy-cinnamoyl ursolic acid and previously reported from *Tripetaleia paniculata* (Yasue *et al*., 1973), *Mimusops elengi* (Jahan *et al*., 2001) and Cranberry fruit (*Vaccinium macrocarpon*) (Murphy *et al*., 2003; Huang *et al*., 2009; Kondo *et al*., 2011). The corresponding trans isomer 3 was also obtained from the same plant as a colorless powder. The proton spectrum at δ 7.60 (1H, d, 16.0 Hz, H-3') and 6.78 (2H, d, 8.5 Hz, H-3", 5") showed the same 1,4-disubstituted benzene fragment. But the down field signals at δ 7.38 (2H, d, 8.5 Hz, H-2", 6") and 6.29 (1H, d, 16.0 Hz, H-2') were shifted from the cis isomer. The coupling constant of J = 16.0 Hz was distinguished as a trans-olefinic protons. The carbon spectral data of compound 3 were closely similar to compound 2. The equivalent carbon signal at C-2" and C-6" in the cis-p-hydroxycinnamate moiety (133.9 ppm) seemed a little downfield from the corresponding trans isomer (130.9 ppm). This is likely due to its spatial proximity to C1' (167.71 ppm) in the cis isomer. This compound previously isolated in the wood from *Tripetaleia paniculata* (Yasue *et al*., 1973), *Mimusops elengi* (Jahan *et al*., 2001) and Cranberry fruit (*Vaccinium macrocarpon*) (Murphy *et al*., 2003; Huang *et al*., 2009; Kondo *et al*., 2011) as trans-3-O-p-hydroxycinnamoyl ursolic acid.

Compound 4 was identified as oleanolic acid by assessment with ^{1}H and ^{13}CNMR spectra of compound 1 and its NMR data were analogous to ursolic acid except the environment around the dimethyl groups in the E-ring; while two methyl signals in the ursolic acid split into the doublets, they were both singlet (Seebacher *et al*., 2003; Falodun *et al*., 2009) and the presence of a germinal proton to the hydroxyl group (-OH) was observed as multiplate at δ 3.52 and corresponding H-18 proton was observed at δ 3.14 (dd, 8.0, 8.0 Hz) and a distinctive olefinic proton observed at δ 5.06. All these spectral data indicated the structure of compound 4 to be 3β-hydroxy-olea-12-en-28-oic acid known as oleanolic acid. This compound was previously isolated and reported from *Tripterospermum lanceolatum* (Lin *et al*., 1987), *Vitex negundo* (Noel and Dayrit, 2005), *Solanum xanthocarpum* (Bhatt, 2011) and *Orthosiphon stamineus*.

Compound 5 was identified as 2α-hydroxyursolic acid (Corosolic acid) by using the data of IR and NMR spectra. In IR spectrum, this compound showed the absorption band of hydroxyl group at 3396.64 cm^{-1}, carbonyl group at 1718.58 cm^{-1} along with C-O (1258.23 and 1168.86 cm^{-1}) and hydrocarbon C-H absorptions (2924, 2852 and 1350 cm^{-1}) (Kojima and Ogura, 1986). Compound 5 exposed the existence of five methyl signals at 1.12, 1.03, 1.01, 0.84 and 0.82; two methyl doublets at δ 0.87 and 0.96 in the proton NMR spectrum. This spectrum also showed that a trisubstituted olefinic proton was resonating at δ 5.25. The carbonyl group resonating at δ 181.1 in the ^{13}C NMR spectral data of 5 proposed the presence of an acid functional group and its

location was recognized at C-28. It also indicated that the two oxymethine groups at δ 2.95 (1H, d, 9.5 Hz, H-3) and 3.65 (1H, ddd, 10.4, 9.8, 4.5 Hz, H-2) are adjacent to each other. Their chemical shifts and J coupling constant were typical with the appearance of α and β-OH at C-2 and C-3, respectively (Kojima and Ogura, 1989; Seebacher *et al*., 2003). These data were also confirmed by the presence of the resonance at 67.2 ppm (C-2) and 78.3 ppm (C-3) in the ^{13}C NMR spectrum. A survey of the literature showed this compound to be identical to that of a triterpenoid as corosolic acid, that was previously reported from plant *Prunella vulgaris* (Kojima and Ogura, 1986, 1989), *Vitex negundo* (Noel and Dayrit, 2005) and from this plant (Mazumder *et al*., 2011). Compound 6 was an olean-12-ene derivative by the analysis of C-12, C-13 and 28-COOH signals at δ 122.1, 144.0 and 182.2, respectively in the ^{13}C NMR spectrum. The proton spectrum showed seven tertiary methylic group signals and a triplet at δ 5.23 (3.4 Hz) due to H-12, which are the main differences between compounds 3 and 4. The carbinolic region of the ^{1}H NMR spectrum was also defined by two signals at δ 2.89 (1H, d, 9.6 Hz) and 3.60 (1H, ddd, 10.4, 9.8 and 4.5 Hz) due to the existence of one α-OH at C-2 and one β-OH at C-3, This compound was confirmed by the resonances of the ^{13}C NMR spectrum as previously described in 5. Therefore, compound 6 was identified as 2α, 3β-dihydroxy-olea-12-en-28-oic acid or maslinic acid, also previously isolated from *Vitex negundo* (Noel and Dayrit, 2005), *Orthosiphon stamineus* and from this plant (Mazumder *et al*., 2011).

β-amyrin (7) also reported from this plants in our previous work (Chowdhury *et al*., 2012) and *Vitex negundo* (Noel and Dayrit, 2005).

Bioassays: The crude ethanol, n-hexane, chloroform and ethyl-acetate extracts and five column fractions (H-13, C-15, C-19, E-18 and E-24) of various extracts and pure compounds 1, 3 and 4 were tested for antibacterial activity against a number of both gram-positive and gram-negative bacteria. Standard disc of kanamycin (30 μg disc^{-1}) was used for comparison purpose. The zone of inhibition produced by the crude ethanol extract was found to be 8-22 mm and n-hexane, chloroform, ethyl-acetate extracts were found to be 7-20 mm at a concentration of 500 μg disc^{-1}. Similarly, five column fractions (H-13, C-15, C-19, E-18 and E-24) of various extracts yielded zones of inhibition 7-14 mm at a concentration of 400 μg disc^{-1} and three pure compounds 1, 3 and 4 yielded zones of inhibition 8-20 mm (Table 1) at a concentration of 300 μg disc^{-1}.

Further the crude ethanol extract exhibited good antibacterial activity against most of the test organisms and n-hexane, chloroform, ethyl-acetate extracts exhibited moderate antibacterial activity against the test organisms. The five column fractions of exhibits low to mild and the three pure compounds exhibited moderate antibacterial activity against most of the test organisms (Table 1).

Brine shrimp lethality bioassay was used to test the cytotoxic activity of the crude extracts, column fraction and isolated pure compounds. Table 2 shows the results of brine shrimp lethality testing after 24 h of exposure to the samples and the positive control. The LC_{50} for crude ethanol, n-hexane, chloroform, ethyl-acetate; column fractions H-13 of the crude n-hexane, C-19 of the crude chloroform and tested compounds 1, 3 and 4 were found to be 12.59, 14.79, 14.06, 11.75, 10.96, 4.37, 6.92, 11.88 μg mL^{-1}, respectively. In contrast with vincristine sulfate (positive control), it proves that most of the test materials were lethal to brine shrimp nauplii. These constructive results suggest that it may contain antitumor or pesticide compounds.

CONCLUSION

It is concluded that seven triterpenoids were isolated from *Saurauia roxburghii* based on spectroscopic evidence among them cis-3-O-p-hydroxycinnamoyl ursolic acid, trans-3-O-p-hydroxycinnamoyl ursolic acid and oleanolic acid were isolated for the first time from this plant. The crude extracts and isolated pure compounds exposed the potential antibacterial and cytotoxic activities which may provide support for some of the uses in ethnomedicine.

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