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Evaluation of Antibacterial Activities of Compounds Isolated From *Sida rhombifolia* Linn. (*Malvaceae*)

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Abstract

The main objective of this study was to isolate compounds from roots of *Sida rhombifolia* and subsequently evaluate their antibacterial activities. Crude gradient extracts were obtained from three solvents (petroleum ether, chloroform and methanol) with increasing solvent polarity using cold maceration technique. The *in vitro* antibacterial activity evaluation of gradient extracts and isolated compounds was done on four different pathogenic bacterial strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium*) using agar disc diffusion technique. The results showed that antibacterial activities were comparable to each other. But their activities were relatively weaker as compared to that of the reference compound (ciprofloxacin). Among the three crude extracts, the chloroform extract was subjected to column chromatographic separation that led to isolation of SRL-1, SRL-2 and SRL-3. The chemical structures of the compounds were found to be *n*-hexacos-11-enoic acid, stigmasterol and β-sitosterol, respectively, based on physical properties and spectroscopic (IR and NMR) data as well as literature reports. The observed antibacterial activities of the crude extracts and the isolated compounds could justify the traditional use of the plant for the treatment of different bacterial infections. Thus, further test is recommended on large number of bacterial strains to decide the potentials of the compounds as candidates in development of antibacterial drugs.

Keywords: *Sida rhombifolia*; Extraction; Isolation; Antibacterial activity; *n*-Hexacos-11-enoic acid; Stigmasterol; β-Sitosterol

Introduction

Use of natural products for curing wide variety of human and domestic animal diseases has a long history that goes to human civilization. These products have been used as good sources of many modern drugs for treatment of several human diseases such as cardiovascular, cancer, malaria, mental diseases, etc. Most of these modern drugs have been obtained or discovered from medicinal plants [1-7]. Such drugs have been discovered after observing the medicinal use of a particular plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by herbalists, and subsequent isolation of bioactive compounds from the plant or part of the plant that was traditionally used for treatment of different human illnesses [8,9]. All these facts indicate that medicinal plants still have an immense potential as sources of modern drugs.

Sida rhombifolia is one of the 200 species in *Sida*. It grows in tropical and warm regions, and distributed throughout the tropics [10,11]. *Sida rhombifolia Linn* is known for its wide range of medicinal uses. For instance, it is used for treating stings and bites of scorpion, snake and wasp (its flowers), skin diseases and sores (its stem), treat stomach disorders, stomach pain, digestion problem (its roots), malaria, flatulence, diarrhea (its root decocted), dysentery (roots), irritable bowel syndrome, gastritis, enteritis, hemorrhoids (its roots and leaves), diabetes (its leaves), chicken pox, blood cleaning, fatigue [11,12], headache and migraine headache (its fruits), eye problems, conjunctivitis, toothaches (roots), fever, gum infection, swelling, tonic, wounds (root and leaves) [13], ophthalmia and swelling (its leaves), cuts and wounds (its leaves) are some of the example to mention [14-16]. In Ethiopia, *Sida rhombifolia* is also widely used by herbalists to treat different human diseases. These include use of its leaves to treat skin disease, wounds and inflammations [17], rabies and skin bleeding [18]. In Jimma area, it is locally known as *karaba*, and its stems are used as tooth brush and the leaves and stem barks are used for the treatment of wound [19].

There are reports on scientific studies on evaluation of biological activities of extracts from different morphological parts of *Sida rhombifolia*. *In vitro* antibacterial activity test of aqueous-methanol extract of the whole part of *Sida rhombifolia* showed effective antibacterial activities [11]. Methanolic extract of its fruit was found to show significant *in vitro* antibacterial activities against several bacteria species [20]. Reports also indicated the ability of leaf extract to ameliorate GM-induced nephrotoxicity and renal dysfunction as demonstrated from the results of animal model study [21]. In another report, it has been discussed that methanolic extract of its aerial part showed anti-inflammatory activity in animal model study [22]. Ethanol and aqueous extracts of aerial parts of the plant were also reported to be useful in the treatment of arthritis [23]. A recent report from experimental result of Ranjan et al. [24] also showed significant anti diarrheal activity of methanolic extract of *Sida rhombifolia*. An *in vitro* study using ethanol extract of roots, stems, leaves, and whole plant showed antioxidant activities. This indicated that *Sida rhombifolia* could be promising a source of natural antioxidants [25,26]. Similar results were also reported recently using root and stem extracts on animal models [27]. Aqueous extract of leaves was administered to hyperbilirubinemic rats, and showed potential of this plant as source new drugs for hyperbilirubinemic subjects [28]. A study carried out, in Bangladesh, indicated that ethyl acetate extract of its leaves showed potent cytotoxicity that was comparable to reference standard, gallic acid, and weak antibacterial activity against both Gram-positive and Gram-negative test organisms [29]. Recent reports, on the other hand, indicated that aqueous-methanol extract of *Sida rhombifolia* showed

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significant antibacterial activity (against pathogenic bacteria involved in diarrhea) [16]. Antidiabetic properties of aqueous extract leaves of *Sida rhombifolia* using normal and streptozotocin-induced diabetic rats was also reported recently. The extract showed good hypoglycemic and hypolipidemic effects. The results were claimed to provide scientific evidence in favor of the traditional use of *Sida rhombifolia* leaves for the treatment of diabetes mellitus [30]. A report by Poojari et al. indicated chemo preventive and hepatoprotective potentials of seed extract as demonstrated by its effect in rats of diethylnitrosamine-hepatocellular preneoplastic foci and carbon tetrachloride-induced hepatotoxicity [31]. Ethyl acetate and aqueous extracts of *Sida rhombifolia* was also reported to show marked antibacterial activity (against *K. pneumonia, S. aureus,* and *S. mutans*) and significant antifungal activity (against *A. niger, C. albicans* and *M. gypseum*) [32].

The results obtained from biological activity tests of crude extracts of *Sida rhombifolia* have initiated researchers to carryout isolation and characterization of compounds from the plant (or its parts) which subsequently subjected to biological activity tests in order to evaluate their potential as leads in the drug discovery processes for treating human diseases. Phytochemical analyses the fruits, roots, leaves and stem of *Sida rhombifolia* revealed the presence of saponins, tannins, amino acids, fatty acids, sterolic compounds, alkaloids, terpenoids, carbohydrates, lignans, glycosides, phenolics, steroids and flavonoids. Notably, both tannins and phenolics have been reported to possess antibacterial activities [27,33,34].

The presence of ecdysteroids and/or their glycosides in *Sida rhombifolia* was reported by Yang- Hong et al. [35] and Jadhav et al. [36]. Isolation of this group compounds was reported from methanol extract of the whole plant parts of the plant. The ecdysteroids were 20-hydroxyecdysone-3-β-D-glucopyranoside, 20-Hydroxyecdysone, pterosterone-3-β-D-glucopyranoside, ecodysone, ecdysone-3-β-D-glucopyranoside, ecdysone and 20-hydroxy- (25-acetyl) ecdysone-3-β-D-glucopyranoside [37]. The detection or characterization of 20-Hydroxyecdysone from *Sida rhombifolia* using HPLC method was previously reported by Jadhav et al. [38]. Soxhlet extraction of stem of *Sida rhombifolia* with methanol, followed by solvent-solvent partitioning using chloroform, petroleum ether and ethyl acetate gave single glycoside known as phenyl ethyl-β-D-glucopyranoside. The report indicated isolation of this compound from *Sida rhombifolia* for the first time. *In vitro* antibacterial activity test of the compound showed that it is effective against several bacteria strain [39]. The compound was also found to show larvicidal activity against common filarial vector [29]. Daucosterol was recently isolated from the *n*-hexane soluble fraction of methanolic extract of the stems of S. rhomboidea [40]. The authors also reported antimicrobial and antioxidant activities of n-hexane, carbon tetrachloride and dichloromethane soluble fractions of the methanol extract. The results indicated that the dichloromethane and carbon tetrachloride soluble fractions showed moderate inhibitory activity to microbial growth while the n-hexane fraction showed highest cytotoxicity. The dichloromethane soluble fraction also revealed potent antioxidant activity [40]. Isolation and characterization of alkaloid constituents such as β-phenethylamine, ephedrine, ψ-ephedrine, quinazoline such as vasicine, vasicinol, vasicinone, carboxylated tryptamines such as S- (+)-N_b-methyltryptophan methyl ester, choline and betaine from aerial *Sida rhombifolia* were reported by Prakash et al. [41]. Sterols (β-sitosterol, stigmasterol, campesterol, stigmasterol, spinasterol and cholesterol), *n*-alkanes (e.g., nonacosane and hentriacontane) and *n*-alcohols were also identified/reported from dried whole and aerial parts of *Sida rhombifolia* [37,42-44]. As discussed above, crude extracts of different part of *Sida rhombifolia* including its roots showed effective antibacterial activities [11,24,45]. However, there are no reports on the evaluation of antibacterial activities of compounds isolated from its roots. Thus, in our study efforts were made to isolate and characterize compounds from the root parts of *Sida rhombifolia Linn*, and to evaluate their antibacterial activities.

Methods and Materials

Chemicals and apparatus

General laboratory grade solvents such as petroleum ether, chloroform, ethyl acetate, acetone and methanol (Purchased from supplied by Sigma Aldrich Chemicals Co. Ltd) and distilled water for extraction and column elution. Silica gel (60-120 mm mesh size) and TLC (silica gel, UV-254) pre-coated on aluminum sheets were used for chromatographic analyses. Compound spots on TLC plates were detected using UV (uvitec chamber) and iodine vapor. Evaporation of solvent was carried out using a rotary evaporator (Heidolph, UK) and HY-5A Manoeuvre style vibrator (Rotary shaker) were used for extraction. A standard antibiotic disc (ciprofloxacin, 5 µg) and culture medium (Mueller Hinton agar, nutrient broth) were used for the antibacterial activity test. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 were recorded using Bruker Advance 400 MHz spectrometer. $CDCl_3$ was used as a solvent in all spectroscopic analysis. Infrared (IR) spectra (KBr) were obtained from Perkin-Elmer BX infrared spectrometer (400-4000 cm^{-1}). Melting point apparatus (Griffin) was used for melting point determination. All spectroscopic analysis were carried out at the Department of chemistry, Addis Ababa University.

Collection of plant material and extraction

Sida rhombifolia Linn was collected in November 2011 from Sokoru District, Jimma Zone and South-western Ethiopia. The collected plant material was dried at room temperature without exposing it to direct sun light. The dried material was then milled using a mechanical grinder. Botanical identification was made by Dr Remesh (a botanist) and a specimen is deposited (voucher number BA013) in the Herbarium of Department of Biology, Jimma University. 100 g of powdered of root of was successively extracted using maceration technique in three solvent systems (petroleum ether, chloroform and methanol) for 72 hrs in each solvent. Each solution was filtered using cotton and Whatmann filter paper No. 3. Each of the filtrate was concentrated at reduced pressure using rotary evaporator, and was subjected to antibacterial activity test. After comparing the antibacterial activities of the crude extracts of the above mentioned solvent systems, the chloroform extract was chosen for chromatographic isolation of its constituents. Then a bulk of the powdered material (900 g) was subjected to extraction employing the same procedure, and two solvent systems (petroleum ether and chloroform).

Evaluation of antibacterial activity

Test organisms: *Staphylococcus aureus* ATCC25903, *Escherichia coli* ATCC25722, *Pseudomonas aeruginosa* DSMZ1117 and *Salmonella thyphimurium* ATCC13311 were used for antibacterial activity tests. All are standard strains obtained from the Department of Boilogy, Jimma University.

Preparation of test solutions and antimicrobial assay using disk diffusion method: Test solutions were prepared by dissolving 100 mg of each of the crude extracts in 1 ml of dimethyl sulfoxide (DMSO) to achieve final stock concentration of 100 mg/ml solution of test sample. A cell suspension of each organism was freshly prepared by tranfering isolated colonies seleced from 24 hrs agar plate in to a broth and a sespention terbidity was adjusted to a 0.5 McFarland turbidity

standard (1×10^8 CFU/mL) in sterile saline solution. The solution was then diluted 1:20 to yield 5×10^5 CFU/mL [46]. The bacterial suspension (5×10^5 CFU/mL) was spread over the 90 mm Petri dishes containing Mueller Hinton agar using a sterile cotton swab. Then six mm diameter sterile discs (Whatmann No 3 paper) were placed on the surface of the inoculated Agar in Petri dishes, and 50 µl of each test solutions were applied onto the discs. After addition of test solutions on the discs, the extract was allowed to diffuse for 5 minutes and the plates were then kept in an incubator at 37°C for 24 hrs [47]. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs in millimeter with ruler. Ciprofloxacin, which is a broad spectrum antibiotic, was used for comparison. Similar procedures were used for evaluation of antibacterial activities of the pure compounds.

Isolation and characterization of compounds: The crude chloroform extract of roots of *Sida rhombifolia* was subjected to column chromatography (CC) that was packed with silica gel to isolate compounds. A glass column was packed with 100 g silica gel slurry dissolved in petroleum ether. The crude material was adsorbed onto dry of silica gel. Then the solvent was allowed to evaporate, and the dry sample adsorbed to the silica gel was applied into the column that was already packed with silica gel. TLC analyses of the crude material gave good separation of pigments on TLC plate in solvent system that was composed of petroleum ether and ethyl acetate mixture. Therefore, the mixture was used in different combinations with increasing polarity (in the ratio 100:0, 98:2, 96:4, 94:6, 92:8, 90:10%) to elute the column. A total of 123 fractions each with 40 ml were collected. Solvents were removed from the fractions under reduced pressure using rotary evaporator. The developed spots on TLC plates were visualized under UV light at 254 and 365 nm and then by exposure to iodine vapor. The fractions that showed the same TLC development profiles (color and R_f) were combined and concentrated to dryness under reduced pressure using rotary evaporator. The structures of the compounds were elucidated based on combined spectral data which include Infra Red, Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR and DEPT-135) spectra data and melting point values as well as comparison of these data with data in literature. All spectroscopic analysis were carried out at Department of chemistry, Addis Ababa University.

Results and Discussion

Scientific investigations also indicated that extracts from different parts of the plant including its roots showed several types of biological activities including antibacterial activities suggesting the potential of the plant as source of new antibacterial drugs. In this work, extraction and isolation of compounds from roots of *Sida rhombifolia*, and evaluation of antibacterial activities of compounds isolated from roots of *Sida rhombifolia*.

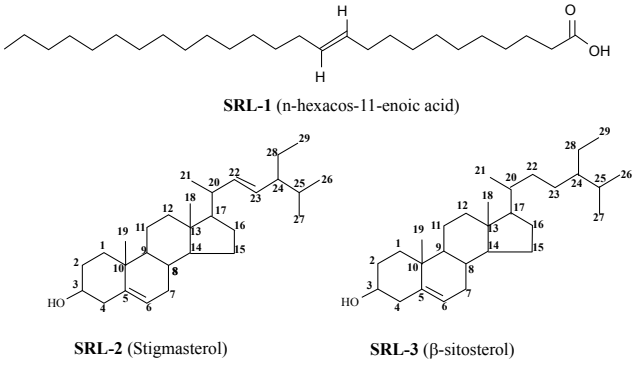
Evaluation of antibacterial activities crude extracts and isolation compounds from roots of *Sida rhombifolia*

For preliminary antibacterial activity tests, 100 g of plant material (roots) was subjected to gradient extractions using maceration technique in three solvent systems; namely petroleum ether chloroform and methanol. Removal of solvents from filtrates resulted in 1.62 g, 2.04 g and 1.9 g of crude material from petroleum ether, chloroform and methanol extracts, respectively. The extracts were subjected to antibacterial activity tests using four bacterial species (*Staphylococcus aureus* ATCC25903, *Escherichia coli* ATCC25722, *Pseudomonas aeruginosa* DSMZ1117 and *Salmonella thyphimurium* ATCC13311) following the procedures described above. The anibacterial activity tests were carried out in duplicate for each crude extract against each of the the test bacterial species, and the results are given as average mean ± standard deviation of the duplicates observed inhibition zone (in mm) (Table 1).

Though their antibacterial activities were relatively lower than that of the reference compound (ciprofloxacin), the three crude extracts showed significant activities against all the bacterial species. The results are consistent with previous reports describing antibacterial activities of methanolic root extract *Sida rhombifolia* [24] and traditional use roots of this plant to treat bacterial infections or wounds [13]. Close observation of the data (Table 1) indicated that the activity of the methanol extract is relatively (slightly) higher than chloroform and petroleum ether extracts against *E. coli* and *S. typhimurium* (Table 1). In the case of chloroform extract, it was more active against *E. coli* (17 ± 0) and *S. aureus* (18 ± 1) but less active than against *P. aeruginosa* (13.8 ± 0) and *S. typhimurium* (14.5 ± 1.5). The activities of petroleum ether extracts were generally lower as compared to that of methanolic and chloroform extracts. Though the methanolic extract showed relatively superior antibacterial activities as compared to the chloroform and petroleum ether extracts in the preliminary antibacterial activity tests, the chloroform extract (the next more active extract) (Table 1) was selected for further analysis, i.e., for isolation of compounds using column chromatography due to the complexity of TLC profile of the methanol extract. Thus, a 900 g of the powdered plant material (root) was macerated by petroleum ether and chloroform using gradient extraction technique. 14.5 g of crude extract was obtained from chloroform extract. Then 8.0 g of the crude extract was adsorbed onto 10 g of silica gel that subsequently loaded into glass column packed with 100 g of silica gel. The column was eluted with petroleum ether and ethyl acetate mixture in different combination with increasing polarity (in the ratio 100:0, 98:2, 96:4, 94:6, 92:8, 90:10%). A total of 123 fractions each with 40 ml were collected. Some of fractions were combined based similarity of their TLC profiles. Thus, fractions 24-25 were combine to affored 0.2 g of compound (labelled as SRL-1); fractions 45-47 were also combine to give 0.26 g of pure product (labelled as SRL-2). Similarly, fractions 49-51 were also combined to affored 0.18 g of pure compound that is labelled as SRL-3.

Characterization of the isolated compounds from roots of *Sida rhombifolia*

The three pure compounds (SRL-1, SRL-2 and SRL-3) from the chloroform extract of the root of roots of *Sida rhombifolia* were characterized to be *n*-hexacos-11-enoic acid, stigmasterol and β-sitosterol, respectively (Figure 1). The compounds were characterized using spectroscopic techniques (NMR and IR spectroscopic



SRL-1 (n-hexacos-11-enoic acid)

SRL-2 (Stigmasterol) **SRL-3** (β-sitosterol)

Figure 1: The proposed structure of compounds (SRL-1, SR-2 and SR-3) isolated from roots of *Sida rhombifolia*.

Extracts	Bacterial species			
	E. coli	*S. aureus*	*P. aeruginosa*	*S. typhimurium*
PE extract	16 ± 1	16 ± 2	10 ± 0	16.5 ± 0.5
Chl. extract	17 ± 0	18 ± 1	13.8 ± 0	14.5 ± 1.5
Met. extract	20 ± 0	15 ± 2	16 ± 0	17.5 ± 2
Ciprofloxacin	35	32	28	30

PE: petroleum ether; Chl.: chloroform; Met.: methanol extract

Table 1: Bacterial inhibition zones (in mm) of 50 mg/ml of crude extracts of roots of *Sida rhombifolia*.

techniques). The structural elucidation was done by comparing the observed spectral and melting point data with the reported data of these compounds in the literature.

Structure elucidation of SRL-1

Compound SRL-1 was obtained colorless crystal with R_f value of 0.6 (petroleum ether and ethyl acetate, 80:20%). Its IR (KBr) spectra displayed characteristics absorption band for hydroxyl group of carboxylic acid (3407 cm^{-1}) carbonyl group of carboxylic acid (1705 cm^{-1}), and long aliphatic chain (731 cm^{-1}). The strong band at 3017 cm^{-1} represents C-H stretch of alkenes whereas the bands at 2925 and 2833 cm^{-1} indicate C-H stretching of methylene and methyl groups, respectively (Supplementary material 1). Thus, compound is probably an aliphatic acid. The ^{1}H-NMR spectrum of SRL-1 (Supplementary material 2) showed the presence of olefinic group at δ5.38 and 5.34 assigned to H-11 and H-12 respectively. Two doublets at δ2.82 and 2.79 where accounted to C-2 methylene protons adjacent to a carboxylic group. Two multiplets at δ2.36 and 2.08 both integrated for two protons can be assigned to protons attached to C-10 and C-13, respectively, which is adjacent to olefinic carbons whereas a triplet at δ0.91 was ascribed to protons at C-24. The remaining methylene protons resonated at δ1.65 (2H), 1.33 (10 H) and 1.27 (26-H). The ^{13}C-NMR and DEPT-135 data of SRL-1 (Supplementary material 3) presented important signals for carboxylic carbon at 179.8 (C-1), vinylic carbons at 130 (C-11) and 128.2 (C-12), for methyl carbon at 14.2 (C-26) and for methylene carbons between 34.01 and 22.71. The DEPT-135 spectrum (Supplementary material 4) was also consistent with the ^{13}C-NMR data. The absence signals between δ5.32-2.82 (in ^{1}H-NMR spectrum) and between δ128-34.01 (^{13}C-NMR spectrum) ruled out the existence of any carbinol carbon in the molecule. The observed IR and NMR data were found to be consistent with the reported data of *n*-hexacos-11-enoic acid. Moreover, the observed mp value (236-237°C) was comparable to mp value (240-242°C) reported for *n*-hexacos-11-enoic acid [48]. Thus, based on this observation, the chemical structure of SRL-1 was proposed to be identical with that of *n*-hexacos-11-enoic acid (Figure 1). The observed NMR data of SRL-1 and the reported data for *n*-hexacos-11-enoic acid [48], are given in (Table 2).

Structure elucidation of SRL-2

This compound was obtained as a white powder with R_f value of 0.33 (petroleum ether and ethyl acetate, 80:20%). IR (KBr) spectrum of SRL-2 has no a doublet band at/near 2850 and 2750 cm^{-1} that indicated the compound has no aldehyde functional group. The absence of strong band in the range or around 1700-1800 cm^{-1} also confirmed that the compound has no carbonyl group. The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicated that the compound has no aromatic functional group. On the other hand, the observed stretching band at 3429 cm^{-1} indicates the presence of hydroxyl functional group. The strong band at 3007 cm^{-1} represents C-H stretch of alkenes whereas the bands at 2930 and 2858 cm^{-1} indicated C-H stretching of methylene and methyl groups. The observed IR data suggested that the compound could be an alcohol possessing a C=C double bond in its chain (Supplementary material 5). In the ^{1}H-NMR spectrum of SRL -2, the peaks at δ0.70, 0.71 0.82, 0.86, 1.03 and 1.27 indicated the presence of protons of six methyl (-CH_3) groups whereas the peak at δ3.55 indicated presence of protons of a carbon attached to oxygen (hydroxyl group). The peaks at δ5.06, 5.14 and 5.38 indicated the presence of olefinic protons in SRL-2 (Supplementary material 6). The ^{13}C-NMR showed signals at 140.7, 121.7 and 138.3, 129.2 ppm which are assigned to C-5, C-6 and C-22, C-23 double bonds, respectively. The δ value at 71.8 ppm is due to C-3 β-hydroxyl group (Supplementary material 7). Totally ^{13}C-NMR and DEPT-135 (Supplementary material 8) spectra showed 29 and 26 signals, respectively, that can be assigned to six methyl, nine methylene, eleven methane and three quaternary carbon atoms. The observed IR and NMR data were found to be consistent with the reported data of stigmasterol [49-51]. Moreover, the observed melting point of SRL-2 (169-171°C) was found to be in good agreement with the reported melting point of stigmasterol (i.e. 176°C) [52]. Thus, based on these observations, the chemical structure of SRL-2 was proposed to be identical with that of the stigmasterol (Figure 1). The NMR data of SRL-2 and reported data of stigmasterol are given in (Table 3).

Structure elucidation of SRL-3

This compound was obtained as a colorless needle-like solid with R_f value of 0.30 (petroleum ether and ethyl acetate, 80:20%). Analysis of IR (KBr) spectrum of SRL-3 showed absence of a doublet band at/near 2850 and 2750 cm^{-1} indicated that the compound has no aldehyde functional group. The absence of strong band (s) around (or in the range of 1700-1800 cm^{-1}) confirmed that the compound has no carbonyl group. The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicated that the compound has no aromatic functional group. Thus, the observed stretching band at δ3432 cm^{-1} indicated the presence of hydroxyl functional group. The strong band at δ3007 cm^{-1} represents C-H stretch of alkenes whereas the bands at δ2930 and δ2855 cm^{-1} indicate C-H stretching of methylene and methyl groups. The data indicated the compound is an alcohol with C=C bond in its chain (Supplementary material 9). In the ^{1}H-NMR spectrum of SRL-3, the peaks at δ0.70, 0.71 0.80, 0.82, 0.95 and 1.03 indicated presence of protons of six methyl (-CH_3) groups whereas the peak at δ3.55 indicated presence of protons attached to hydroxyl group carbon. The peak at δ5.37 indicates presence of olefinic protons in the structure (Supplementary material 10). The ^{13}C-NMR (Supplementary material 11) has shown signals at δ140.8 and 121.7 that can be assigned to C-5, C-6 indicating C-C stretch of olefines. The value at δ71.8 is can be attributed to C-3 β-hydroxyl group. Totally, the ^{1}H-NMR and ^{13}C-NMR data showed 29 and 26 signals, respectively, for six methyl, nine methylene, eleven methane and three quaternary carbon atoms. The observed IR and NMR data were found to be consistent with the reported data of β-sitosterol [49,51,26]. The observed melting point (136-138°C) was also found to be in good agreement with reported melting point of β-sitosterol (i.e., 133°C) [51-53]. Thus, based on similarities of spectral and melting point data, the chemical structure of SRL-3 was proposed to be identical with that of β-sitosterol (Figure 1) [54]. The NMR data of SRL-3 and that of β-sitosterol are given in (Table 4).

Antibacterial test of the isolated compounds

In vitro tests were carried out to evaluate antibacterial activities of the isolated compounds (SRL-1, SRL-2 and SRL-3) using Agar diffusion method and four bacterial species: *E. coli, S. aureus, P. aeruginosa* and *S. typhimurium*. The activities of the compounds were expressed in terms of growth inhibition zones (given in mm). The growth inhibitory activities of the compounds are given in (Table 5).

Similar to that of the crude extracts, the antibacterial activities of the isolated compounds were lower than that of the reference drug

Carbon	^{13}C-NMR data of SRL-1	Reported ^{13}C-NMR data of *n*-hexacos-11-enoic acid*	^{1}H-NMR data of SRL-1	Reported ^{1}H-NMR data of *n*-hexacos-11-enoic acid*	DEPT-135 data of SRL-1	Nature of carbon
1	179.8	177.3	-	-	-	C
2	34.0	34.01	2.8 2, 2.79	2.77, 2.75	34.0	CH_2
3	24.6	24.7	1.65	1.65	24.6	CH_2
4	25.2	25.7	1.27	1.25	25.6	CH_2
5	29.3	29.3	1.27	1.25	29.3	CH_2
6	29.5	29.5	1.27	1.25	29.6	CH_2
7	29.7	29.7	1.27	1.25	29.7	CH_2
8	29.7	29.7	1.33	1.3	29.7	CH_2
9	29.7	29.7	1.33	1.3	29.7	CH_2
10	31.9	32.0	2.36	2.34	31.9	CH_2
11	130	130.1	5.38	5.39	130.0	CH
12	128.2	127.9	5.34	5.32	127.9	CH
13	31.5	31.6	2.01	2.01	31.9	CH_2
14	29.7	29.7	1.33	1.3	29.7	CH_2
15	29.7	29.7	1.33	1.3	29.7	CH_2
16	29.7	29.7	1.27	1.25	29.7	CH_2
17	29.7	29.7	1.27	1.25	29.7	CH_2
18	29.7	29.7	1.27	1.25	29.7	CH_2
19	29.7	29.7	1.27	1.25	29.7	CH_2
20	29.7	29.7	1.27	1.25	29.7	CH_2
21	29.7	29.7	1.27	1.25	29.7	CH_2
22	29.1	29.1	1.27	1.25	29.1	CH_2
23	29.0	29.0	1.27	1.25	29.0	CH_2
24	27.2	27.2	1.27	1.25	27.2	CH_2
25	22.7	22.7	1.33	1.3	22.7	CH_2
26	14.2	14.2	0.91	0.88	14.1	CH_3

*Data from Surendra et al.

Table 2: ^{13}C-NMR, DEPT-135 and ^{1}H-NMR data of SRL-1 along with the corresponding reported ^{13}C-NMR and ^{1}H-NMR data of *n*-hexacos-11-enoic acid.

C. No.	^{13}C-NMR data of SRL-2	Reported ^{13}C-NMR data of stigmasterol*	DEPT-135 data of SRL-2	^{1}H-NMR data of SRL-2	Reported ^{1}H-NMR data of stigmasterol*	Nature of the carbon
1	37.2	37.5	37.2			CH_2
2	31.6	31.8	31.6			CH_2
3	71.8	71.9	71.8	3.55	3.45	CH
4	42.2	42.2	42.3			CH_2
5	140.7	140.9	-			C
6	121.7	121.7	121.7	5.38	5.33	CH
7	31.9	31.9	31.9			CH_2
8	31.9	32.2	31.9			CH
9	50.1	50.3	50.1			CH
10	36.5	36.6	-			C
11	21.0	21.0	21.0			CH_2
12	39.7	39.7	39.7			CH_2
13	42.3	42.5	-			C
14	56.7	57.0	56.7			CH
15	24.3	24.4	24.4			CH_2
16	29.7	28.9	28.2			CH_2
17	56.0	56.0	55.9			CH
18	12.2	12.4	12.2	0.70	0.68	CH_3
19	19.4	19.4	19.4	1.03	0.97	CH_3
20	40.5	40.5	40.5			CH
21	21.1	21.1	21.1	1.20	1.01	CH^3
22	138.3	138.4	138.3	5.36	5.12	CH
23	129.2	129.4	129.2	5.06	4.98	CH
24	51.2	51.3	51.2			CH
25	39.7	32.0	39.7			CH
26	19.0	19.0	19.0	0.83	0.86	CH_3
27	21.2	21.2	21.2	0.71	0.71	CH_3
28	25.4	25.4	25.4			CH_2
29	12.0	12.0	11.8	0.82	0.78	CH_3

*Data from Sammia et al.

Table 3: ^{13}C-NMR, DEPT and ^{1}H data of SRL-2 in comparison with reported data of stigmasterol.

C. No.	^{13}C-NMR data of SRL-3	Reported ^{13}C-NMR data of β-sitosterol*	^{1}H-NMR data of SRL-3	Reported ^{1}H-NMR data of β-sitosterol*	Nature of carbon
1	37.2	37.3			CH_2
2	31.6	31.6			CH_2
3	71.8	71.8	3.5	3.45	CH
4	42.2	42.2			CH_2
5	140.7	140.8			C
6	121.7	121.7	5.37	5.33	CH
7	31.9	31.9			CH_2
8	31.9	31.9			CH
9	51.2	51.2			CH
10	36.5	36.5			C
11	21.1	21.1			CH_2
12	39.7	39.8			CH_2
13	42.3	42.3			C
14	56.7	56.8			CH
15	24.3	24.3			CH^2
16	28.2	28.2			CH^2
17	56	56.7			CH
18	12.0	12.0	0.70	0.68	CH_3
19	19.4	19.4	0.95	0.97	CH_3
20	36.5	36.5			CH
21	18.8	18.9	1.03	1.01	CH_3
22	33.9	33.9			CH_2
23	26.0	26.2			CH_2
24	45.8	45.8			CH
25	29.2	29.1			CH
26	19.8	19.8	0.82	0.86	CH_3
27	19.4	19.4	0.71	0.71	CH_3
28	23.0	23.0			CH_2
29	12.2	12.2	0.80	0.78	CH_3

*Data from Sammia et al.

Table 4: ^{13}C-NMR, DEPT-135 and ^{1}H-NMR data of SRL-3 in comparison with reported data of β-sitosterol.

S. No	Compounds	*E. coli*	*S. aureus*	*P. aeruginosa*	*S. typhimurium*
1	SRL-1(*n*-hexacos-11-enoic acid)	13	12	8	11
2	SRL-2 (Stigmasterol)	14	12.5	9.5	13
3	SRL-3 (β-sitosterol)	13.5	11	8.5	12
	Cipro floxacin	35	32	28	30

Table 5: Inhibition zone in mm of the isolated compounds using 50 mg/L of their solutions.

(ciprofloxacin) against all bacterial species used in the experiment, and their growth inhibition values were also comparable to each other (Table 5). Moreover, the compounds showed the least antibacterial activities against *P. aeruginosa* (Table 5 *vs.* Table 1). As shown above (Table 5), SRL-1 (*n*-hexacos-11-enoic acid) showed comparable antibacterial activity with that of SRL-2 and SRL-3. However, there are no reports in literature to compare and contrast our result. The antibacterial activities of SRL-2 (stigmasterol) were found to be moderate as compared to compared to the reference drug (ciprofloxacin). This observation is in good agreement with previous reports that showed low antimicrobial (or antibacterial) activities of stigmasterol against Acetobacter sp., *E. coli*, *S. aureus*, and *Streptococcus sp, P. aeruginosa*) [50,54,55]. On the other hand, antibacterial activity of SRL-3 (β-sitosterol) was also found to be consistent with literature reports that discuss low/moderate antibacterial activity of β-sitosterol against several bacterial species that include *S. aureus, E. coli*, and *P. aeruginosa* [56-60].

Summary of Spectral Data of the Isolated Compounds

SRL-1 (colorless crystal, 0.2 g), mp 236-237°C; IR (KBr) V_{max} cm^{-1} 731, 967, 1285, 1459, 1705, 2833, 2925, 3017, 3407, ^{1}HNMR (400 MHz, $CDCl_3$): δ0.91 (3H, H-26), δ1.27 (26H, 2×CH_2), δ1.33 (10H, 5×CH_2), δ1.65 (2H, H-3), δ2.0 (2H, H-10), δ207 (2H, H-13), δ2.36 (1H, H-10 δ2.7 (2H, H-2), ^{13}CNMR δ2.5.34 (1H, H-11), δ5.38 (1H, H-12), (400 Hz $CDCl_3$): δ14 (C-26), δ22.7 (C-25), δ24.6 (C-3), δ25.2 (C-4), δ27.2 (C-24), δ29.0 (C-23), δ29.1 (C-22), δ29.3 (C-5), δ29.5 (C-6), δ29.7 (C-11) δ31.54 (C-13), δ31.9 (C-10), δ37.2 (C-10), δ34.0 (C-26), δ128.2 (C-12), δ130.2 (C-11).

SRL-2 (White powder, 2.6 g), mp 169-171°C; IR V_{max} cm^{-1} (KBr), 959, 1048, 1377, 1466, 1715, 2858, 2930, 3007, 3429, (^{1}H-NMR (400 MHz, $CDCl_3$): δ0.7 (3H, H-18), δ0.71 (3H, H-27), δ0.82 (3H, H-29), δ1.03 (3H, H-21), δ3.55 (1H, H-3), δ5.06 (1H, H-23), δ5.36 (1H, H-22) δ5.38 (3H, H, H-6), ^{13}CNMR, (400 Hz $CDCl_3$): δ12 (C-29), δ12.2 (C-18), δ19.03 (C-26), δ19.4 (C-19), δ21 (C-11), δ21.1 (C-21), δ21.2 (C-27), δ24.3 (C-15), δ25.4 (C-28), δ29.7 (C-16) δ31.6 (C-2), δ31.9 (C-7, C-8), δ36.5 (C-10), δ37.2 (C-10), δ39.7 (C-12, C-25), δ40.5 (C-20), δ42.2 (C-4), δ42.3 (C-13), δ50.1 (C-9), δ51.2 (C-24), δ56.0 (C-17), δ56.7 (C-14), δ71.8 (C-3), δ127.1 (C-6), δ129.2 (C-23), δ138.3 (C-22), δ140.7 (C-5).

SRL-3 (colorless needle 0.18 g), mp 136-138°C ; IR V_{max} cm^{-1} (KBr), 943, 1055, 1388, 1633, 1710, 2855, 2930, 3007, 3432, ^{1}HNMR (400 MHz, $CDCl_3$): δ0.7 (3H, H-18), δ0.71 (3H, H-27), δ0.7 δ0.80 (3H, H-27), 7 δ0.82 (3H, H-26), δ0.95 (3H, H-29), δ1.03 (3H, H-21), δ3.5 (1H, H-3), δ5.37 (1H, H-22), ^{13}CNMR, (400 Hz $CDCl_3$): δ12 (C-18), δ12.2 (C-29), δ18.8 (C-21), δ19.4 (C-27), δ19.8 (C-26), δ21.1 (C-11), δ23.0 (C-28), δ24.3 (C-15), δ26.0 (C-23) δ28.2 (C-16), δ31.9 (C-7, C-8), δ33.9 (C-22), δ42.2 (C-4), δ42.3 (C-13), δ45.8 (C-9), δ50.1 (C-24), δ56.0 (C-17), δ56.7 (C-14), δ71.8 (C-3), δ127.1 (C-9), δ140.7 (C-5).

Conclusions

In conclusion, three compounds (SRL-1, SRL-2 and SRL-3) were isolated from the crude acetone extract. The identities of the compounds were determined to be *n*-hexacos-11-enoic acid, stigmasterol and β-sitosterol, respectively, based on physical properties and spectroscopic (IR and NMR) data as well as literature reports. The isolation of SRL-1 (*n*-hexacos-11-enoic acid) is reported for the first time from *Sida rhombifolia*. *In vitro* test results showed that the antibacterial activities of the isolated compounds were found to be lower than the reference compound (Ciprofloxacin). When compared to each other, the antibacterial activities of the compounds were comparable to each other. But the activities of the compounds were relatively generally lowest against *P. aeruginosa*. The results were also consistent with that of the crude extracts. The observed antibacterial activities of the crude extract and the isolated compounds could justify the traditional use of the plant for the treatment of different bacterial infections. Thus, further test is recommended on large number of bacterial strains to decide their potential as candidates in development of antibacterial drugs.

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