Middle-East Journal of Scientific Research 22 (5): 681-689, 2014
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2014.22.05.21923

Evaluation of Antibacterial Activities of Compounds Isolated from Fruits of S*ida rhombifolia linn*

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Abstract: The main objective of this study was to isolate compounds from fruit of Sida rhombifolia Linn (*S. rhombifolia*) and evaluation of their antibacterial activities against four bacterial strains namely *Staphylococcus aureus* (DSMZ346), *Escherichia coli* (KL2DSM 498), *Pseudomonas aeruginosa* (DSMZ 1117) and *Salmonella thyphimurium* (ATCC 13311) employing agar disk diffusion method. Two compounds labeled as compounds SR-2 and SR-3 were isolated by subjecting the acetone crude extract of the dried fuits to column chromatography. The structures of the compounds were characterized using spectral analyses (IR and NMR spectroscopies) and comparing with literature reports. Compound SR-2 and compound SR-3 were proposed to be 1,3-dilinoleoyl-2-oleine and n-hexacosa-11- enoic acid, respectively. Compound SR-2 was isolated for the first time from this plant and its family whereas compound SR-3 was isolated from the root of this plant. *In vitro* antibacterial activity tests indicated that compound SR-3 is more active than compound SR-2 but less active than the reference compound (Gentamicine). Further antibacterial activity tests are required using other bacterial strains to decide the fate of the isolated compounds as drug leads.

Key words: Sida rhombifolia Linn · 1,3-dilinoleoyl-2-oleine · n-hexacosa-11-enoic acid · *In vitro* antibacterial activities · Fruits of *S. rhombifolia*

INTRODUCTION

Medicinal plants have been used for centuries to treat numerous human diseases. Literature reports refer WHO reports that estimate about 70-80% of world population, most of whom are from developing countries, still relies on medicinal plants for their primary health cares [1-4]. Moreover, several drugs currently in use in modern medicin are originated from medicinal plants [5] and still there is a huge potential to discover more drugs in the future.

Ethiopia is the home of many plant species that are commonly used in disease treatment by local healers as the majority of people in Ethiopia depend on traditional medicine that involve use of plant parts of medicinal plants [6]. Some of the factors attributed for these fact are accessibility, affordability as compared to modern drugs, socio-cultural background and their effectiveness against a number of health problems [7].

S. rhombifolia is one of the family of Sida species. It is small gregarious shrub that grows in the tropical and sub-tropical regions of the world [8-10]. It has several medicinal values and the use of its different parts for treatment of human illness have been reported in severa literature reports [10-14]. For instance, there are reports that revealed the use of the plant for treatment of tuberculosis [9,15], rheumatic pain, cardiac ailments and biliary problems in children [10] and dysentery and diarrhea (its roots) [16,17] to mention some. Other medicinal uses of the different morphological parts of *S. rhombifolia* are reported in several reports [18]. In Ethiopia, the leaves of *S. rhombifolia* is used to treat skin disease, wounds, rabies and skin bleeding [19, 20]. In the study area (Jimma), the stems of *S. rhombifolia* (locally named as karaba in Afan Oromo) are used as tooth brush and its leaves and stem barks are also used for the treatment of wound [21].

There are experimental reports showing that solvent extracts of different parts of the plant to show promising biological activities. This suggests the potential of the plant to discover new drug candidates in the future. Some of the reports in this regard are mentioned in this section. The methanolic extracts of the aerial parts showed anti-inflammatory activities in animal model study [22].

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A report by Poojari *et al*. indicated chemopreventive and hepatoprotective potentials of the seed extract of *S. rhombifolia* [23]. The ethyl acetate extract of the plant has been reported to show antibacterial activities [17]. The methanolic extract of root of *S. rhombifolia* was reported to show antidiarrheal activity [24]. Screening of the extract of the aerial parts of the plant for anti-arthritic activity, such as adjuvant-induced arthritis, motor performance, mean distance travelled and histopathological study showed that the polar constituents (ethanol and aqueous extracts) of the plant *S. rhombifolia* were useful in the treatment of arthritis [25,26]. The *In vitro* anti-bacterial activity study of fruit extracts of *S. rhombifolia* was found to show significant *In vitro* antibacterial activities against different bacteria strains [27]. Our recent report also showed moderate to good *In vitro* antibacterial activities of solvent extracts of roots of *S. rhombifolia* [18].

Phytochemical investigation of *S. rhombifolia* showed the presence of compounds that belong to different classes. For instance, compounds that belong to β-phenethylamines, quinazolines and carboxylated tryptamines [28] and alkanes and stroides [29] have been reported from the roots and aerial parts of this plant species. Similarly, isolation of alkaloid constituents such as β-phenethylamine, ephedrine, ψ-ephedrine, quinazoline such as vasicine, vasicinol, vasicinone, carboxylated tryptamines such as S- (+)-Nb-methyltryptophan methyl ester, choline and betaine from aerial *S. rhombifolia* were reported by Prakash *et al*. [30]. 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 *S. rhombifolia* [31-34]. There are also reports that indicate presence of ecdysteroids and/or their glycosides in *S. rhombifolia* [35,36]. Isolation of this group compounds was reported from methanol extract of the whole 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 (http://www.answers.com/topic/pharmacology-biologic al-activities-of-tagetes). Ekramul and coworkers isolated pure compound phenyl ethyl β-D glucopyranoside from the stem of the plant *S. rhombifolia* and reported its larvicidal activity against common filaria vector [37]. We also reported isolation of n-hexacos-11-enoic acid, stigmasterol and β-sitosterol from the chloroform extract of root of this plant [18]. Otemberg and coworkers also reported the isolation of eleven secondary metabolites (sitosterol and stigmasterol, sitosterol-3-O -β-D- glucopyranoside and stigmasterol-3-O-β-Dglucopyranoside, phaeophytin, 17-ethoxypheophorbide, 13-hydroxyphaeophytin, 17-ethoxypheophorbide, 5,7-dihydroxy-4'-methoxyflavone, cryptolepinone and a salt of cryptolepine) from on 95% ethanol extract of aerial parts of *S. rhombifolia* [38]. Daucosterol was recently isolated from the n-hexane soluble fraction of methanolic extract of the stems of S. rhomboidea [39]. As mentioned above, the *In vitro* activity study of the fruit extracts of *S. rhombifolia* showed significant *In vitro* antibacterial activities [27]. The mentioned antibacterial activities could be due to the combined or individual effect of phytoconstituents present in the fruits. This needs further experimental study to confirm or reject these claims. To the best of our knowledge, there are no reports about isolation and characterization of compounds from the fruits of *S. rhombifolia*. Therefore, the present study was initiated to isolate compounds from its fruits and also to evaluate their antibacterial activities.

MATERIALS AND METHODS

Chemicals, Reference Drugs, Culture Medium and Bacterial Strains: Laboratory grade methanol, acetone, chloroform, petroleum ether, ethyl acetate and DMSO (Sigma Aldrich Chemicals Co. Ltd.) were used as solvent for gradient extractions and column elution. Silica gel (60-120 mesh size) was used for column chromatography. Standard antibiotic disc Gentamycin (10µg) was used as positive control. Mueller Hinton agar and Nutrient broth were used for the biological activity test. Pre-coated TLC (silica gel 60, F254, Merk) UV254 and 365nm (Uvitec chamber) and iodine chamber were used for visualization during the experiment. Rotary evaporator (Heidolph, UK) and horizontal shaker (Grant GLS400) were used for vacuum removal of solvents and for agitation, respectively. The bacterial strains (*Staphylococcus aureus* (DSMZ346), *Escherichia coli* (KL2DSM 498), *Pseudomonas aeruginosa* (DSMZ 1117) and *Salmonella thyphimurium* (ATCC 13311)) were obtained from the Department Biology, Jimma University. Bruker Advance 400 MHz was used for spectral (1H-NMR, 13C-NMR and DEPT-135) analysis using CDCl3 as solvent. IR spectra (KBr) data were obtained using Perkin-Elmer BX. Both NMR and IR spectal analyses were carried out at Department of Chemistry, Addis Ababa University, Ethiopia.

Collection and Preparation of Plant Material: The fruits of Sida rhombifolia Linn were collected (in December 2012) from Abba Jifar district, near Jimma town, Ethiopia. Identification of this plant was made by a botanist (Mr. Kitesa Hundara) and a specimen with voucher number Ad001 was deposited at the Herbarium of the Department of Biology, Jimma University. 1000g of fruit of the plant was collected, air-dried under shade and then powdered using a milling machine at the Department of Animal nutrition, Jimma University.

Extraction: Hundred grams of the powdered plant material was sequentially extracted with petroleum ether (least polar), chloroform, acetone and methanol (most polar) using maceration technique with constant horizontal shaking at 25°C for 72 hrs. The extract matter was filtered first using a cotton plug followed by Whatman No 1 filter paper. The filterates were concentrated using rotary evaporator. The resulting crude extracts were weighed and stored in refrigerator below 4°C [40] until used for antibacterial assays and chromatographic isolation of compounds. After comparing the antibacterial activities of the crude extracts of the above mentioned solvent systems (data not given), the acetone extract was chosen for chromatographic isolation of its constituents. Then, a bulk of the powdered material (1000 g) was subjected to extraction employing the same procedure (gradient extraction) and three solvent systems namely petroleum ether, chloroform and acetone.

Antibacterial Activity Tests and Isolation of Compounds

***In vitro* Antibacterial Activity Tess:** The bacterial strains used in this test were *S. aureus* (DSMZ346), *E. coli* (KL2DSM 498), *P. aeruginosa* (DSMZ 1117) and *S. thyphimurium* (ATCC 13311). After the preliminary antibacterial activity screening of crude extracts using a standard disc diffusion method [41] and the above bacterial strains, the acetone extract as it shown better activity than petroleum ether and chloroform extracys and also more number of components in its TLC as compared to that methanol extract, was selected for column chromatography isolation of its components. Subsequently a bulk of powdered material 1000 g was extracted utilizing the same procedure with petroleum ether, chloroform and acetone. The stock bacterial cultures were maintained at 4 °C on slants of nutrient agar. Active cultures for the experiments were prepared by transferring a loop full of bacterial cells from the stock cultures to test tubes containing 5 ml Mueller-Hinton broth that was incubated for 24 hrs at 37°C. A cell suspension of each organism equivalent for McFarland 0.5 turbidity standard was obtained by preparing 1% V/V of H2SO4 and 1% W/V BaCl2 then 95.5 ml of 1% V/V of H2SO4 mixed with 0.5 ml of 1% BaCl2W/V for comparison of the turbidity to a cell suspension of each organism in order to have a suspension containing approximately 1-2x108 CFU ml^{-1}. The bacterial suspension were spread over solid Mueller Hinton agar plates with a sterile swab. Then 6 mm diameter sterile discs (Whatmann No 3 paper) were placed on the surface of the inoculated Agar in Petri dishes and 50µl of 100 mg ml^{-1} concentration of test solutions (of the compounds) that were prepared by dissolving in DMSO) were also applied onto the discs using micropipette. After addition of test solutions on the discs, they were allowed to diffuse for 5 minutes and the plates were then kept in an incubator at 37°C for 24 hrs [41]. The antibacterial activity was evaluated by measuring the diameter of the zone of growth inhibition surrounding the discs in millimeter using ruler.

Isolation of compounds and structural elucidation of compounds: The acetone crude extract, that showed relatively superior antibacterial activity, was subjected to column chromatographic isolation using a glass column packed with 120 g of activated silica gel. The silica gel which was activated at 100°C for 1 hr was loaded into the column using petroleum ether slurry. The acetone and methanol crude extracts showed superior antibacterial activity than petroleum ether and chloroform crude extracts (data not given). Thus, the acetone crude extract was selected for column chromatography separation. The acetone crude extract 10 gm was thoroughly mixed with 15 gm of silica gel by using mortar and pestle. Finally, the acetone extract that was adsorbed onto silica gel was loaded into previously packed column. The column was eluted with petroleum ether and ethyl acetate combinations increasing polarity. (i.e. 100:0, 98:2, 96:4, 92:8, 90:10, 88:12, 86:14, 84:16, 82:18, 80:20, 70:30). 150 fractions each with volume of 20 ml were collected. Identities of fractions were examined by running TLC. The spots on the developed TLC plates were visualized under UV light (at 254 nm and 365 nm) and iodine chamber. Fractions showing similar TLC profiles (color and Rf) were combined. Two compounds (compound SR-2 and compound SR-3) were isolated from the acetone crude extract. Compound SR-2 was obtained by combining fractions 52-62 and re-running column chromatographic separation using a small column and 100% chloroform as eluent. Fractions 13-20 gave pure yellow oily compound labeled as compound SR-2. Its Rf value was determined as 0.62 in petroleum ether and ethyl

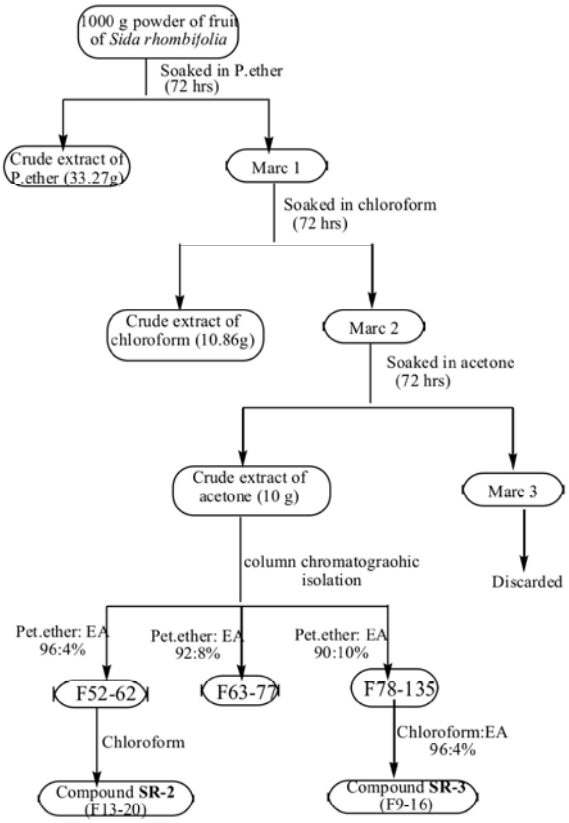

Fig. 1: Steps followed during isolation of compound SR-2 and compound SR-3.

acetate combination (94:6%). In the same manner, a small column was packed with 12 g silica gel and 96:4% chloroform and ethyl acetate for column elution of fraction 78-135 obatined from elution of the crude extract. Here, fractions 9-16 afforeded 293 mg of pure colorless crystal labeled as compound SR-3. Its Rf value was determined as 0.56 in chloroform and ethyl acetate (96:4%) (Fig. 1). The structural elucidation of the isolated compounds was doen based on their observed 1H-NMR, 13C-NMR, DEPT-135 and IR spectral data and also in comparison with reported data in the literature. All spectral analyses were carried out at the Department of Chemistry, Addis Ababa University, Ethiopia.

RESULTS AND DISCUSSION

As discussed in the introduction section, scientific investigations indicated that crude extracts from different parts of the plant including its fruits showed several types of biological activities including antibacterial activities. This suggests the potential of the plant as source of new antibacterial drugs. In this paper, we report isolation of two compounds (compound SR-2 and compound SR-3) from the fruits of *S. rhombifolia* and evaluation of their *In vitro* antibacterial activities. Their stuructures were elucidated based on the observed ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and IR spectral data and reported data in literature.

Structure Elucidation of Compound SR-2: The IR spectrum of compound SR-2 (Suplementary material 1) showed the characteristic absorption band of carbonyl group at 1738 cm^{-1}. The absence of broadband around 3300-2500 and 3650-3200 cm^{-1} confirmed that the compound has no carboxylic and an alcohol functional group, respectively. Similarly, no band at/near 2820 and 2720 cm^{-1} means the compound SR-2 has no aldehydic functional group. The absence of strong to weak bands in the range of 1600 and 1500-1430 cm^{-1} indicated that the compound has no aromatic functional group. Moreover, the compound can not be anhydride as there are no bands at 1810 and 1760 cm^{-1}. Therefore, the observed carbonyl group band at 1738 cm^{-1} indicates that compound SR-2 is most likely an ester. The observed band at 1217 cm^{-1} could be C-O stretching vibration of an ester. The strong band at 3019 cm^{-1} stands for C-H stretching vibration of an alkenes and the bands at 2928 and 2856 cm^{-1} represent the C-H stretching vibration of CH2 and CH3 groups.

In the 1H-NMR spectrum (Supplementary material 2) of compound SR-2 the peak around d5.27-5.38 (m) displayed the presence of olefinic protons of fatty acid -CH=CH-. The peak at d5.26 (m) can be attrirbuted to a tertiary proton glycerol backbone whereas the peaks at d4.14 and 4.3 could represents the protons of -CH2O- group glycerols as indicated in literature reports [42-44]. This suggests that the compound SR-2 is most likely posses triglyceride structure. However, there are four triglycerides reported in literature [42-45] (Fig. 2). Among the sbove mentioned triglycerides, compound 1,2,3-triolein (Fig. 2A) and 1,2,3-trilinolein (Fig. 2D) were rulled out as 13C-NMR peak of their C-18 is expected to be singlet. In the 13C-NMR spectral data of compound SR-2, C-18 showed two peaks at d14.11 and 14.07 (Suplementary material 3). For compound 1,3-dioleoyl-2-linolein (Fig. 2B), the integrated value for methylene protons (at d2.78) flanked between carbon-carbon double bonds (bis-allylic) was two. Whereas for 1,3-dilinoleoyl-2-oleine (Fig. 2C), the integrated value for methylene protons (at d2.78) flanked between carbon-carbon double bonds (bis-allylic) was four. In the case of compound SR-2, the observed integration was found to be the same with

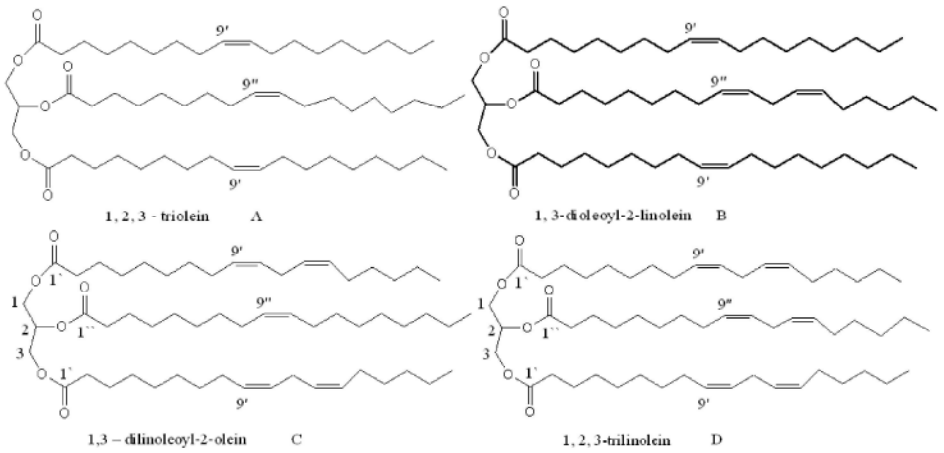

Fig. 2: The chemical structures triglycerides reported in literature [42-45].

Table 1: The observed ^{1}H-NMR data of compound SR-2 and reported data of 1, 3-dilinoleoyl-2-oleine.

H	^{1}H- NMR data of compound SR-2	^{1}H-NMR data reported for 1, 3-dilinoleoyl-2-oleine*
1a	4.14	4.12
1b	4.30	4.28
2	5.27	5.25
3a	4.15	4.12
3b	4.29	4.28
1`, 1``	-	-
2`, 2``	2.31, 2.33	2.28,2.29
3`, 3``	1.62	1.59
4`, 4``	1.27	1.25
5`, 5``	1.27	1.25
6`, 6``	1.27	1.25
7`, 7``	1.27	1.25
8`, 8``	2.05	2.02
9`, 9``	5.38	5.33
10`, 10``	5.37	5.33
11`, 11``	2.78,2.05	2.75, 2.02
12`, 12``	5.35	5.33
13`, 13``	5.35	5.33
14`, 14``	2.05	2.02
15`, 15``	1.27	1.25
16`, 16``	1.27	1.25
17`, 17``	1.27	1.25
18`, 18``	0.88, 0.90	0.86, 0.87

*Data from ref. 42

1,3-dilinoleoyl-2-oleine (Fig. 2C). Moreover, the value of carbonyl carbon obtained was similar with the value of carbonyl carbon of compound 1,3-dilinoleoyl-2-oleine reported from our lab [42]. Thus, compound SR-2 is most likely the same that of 1,3-dilinoleoyl-2-oleine (Fig. 2C). Some peaks from 1H-NMR data (Table 1) also confirm this assumption. For instance, the peak at d2.78(m) integrated for four protons and d2.05(m) represents protons of bis-allylic and allylic methylenes of polyunsaturated and unsaturated acyl chains, respectively. The proton peaks (of H-2 and H-3) at d2.33 and d1.62 indicate acyl moieties in triacylglycerols, respectively. The peak at d1.27 represents methylene envelope whereas the peak at d0.90 was integrated for nine protons and attributed to protons of methyl groups.

From ^{13}C-NMR and DEPT-135 spectrum (Suplementary materials 3 and 4) of compound SR-2, the peak at d173.28, 173.24 and 172.83 represent quaternary carbon of carbonyl carbons of ester (Table 2). The peaks in the range of d130.20-127.89 revealed C=C bonds; the peak at d68.88 indicate β-glycerol carbon (-CH) whereas the peak at d62.09 represent a-glycerol carbon (-CH2). The peak at d14.0 and 14.1 indicated presence of methyl (-CH3) groups in the structure.

The observed IR, 1H-NMR, 13C-NMR and DEPT-135 spectral data were found to be consistent with the reported data of 1,3-dilinoleoyl-2-oleine (Fig. 2C). Consequently, the chemical structure of compound SR-2 proposed to be similar to that of 1,3-dilinoleoyl-2-oleine (Fig. 2C). To the best of my knowledge, this is the first report of isolation of 1,3-dilinoleoyl-2-oleine from *S. rhombifolia* and its family.

Structure Elucidation of Compound SR-3: Compound SR-3 was isolated as colorless crystal with Rf value of 0.56 in chloroform and ethyl acetate (92:8%). The IR spectrum (Suplementary material 5) of compound SR-3 indicated a broad absorption band in the range of 3300-2500 cm^{-1} which represent carboxylic group whereas the strong band at 1708 cm^{-1} represents the presence of C=O group of carbonyl functional group. The band at 3013 cm^{-1} represents C-H stretch band of alkenes whereas the bands at 2918 and 2850 cm^{-1} indicate C-H stretching

Table 2: The observed ^{13}C-NMR and DEPT-135 data ofcompound SR-2 and reported ^{13}C-NMR data of 1,3-dilinoleoyl-2-oleine.

C No.	^{13}C-NMR data of compound SR-2	^{13}C-NMR data reported for 1,3-dilinoleoyl-2-oleine*	DEPT-135 data of compound SR-2	Remark
1	62.09	62.1	62.09	-CH_2
2	68.88	68.9	68.89	-CH
3	62.09	62.1	62.09	-CH_2
1`, 1``	173.28-172.83	173.2- 172.8	-	Quaternary
2`, 2``	34.02, 34.18	34.0, 34.2	34.04, 34.18	-CH_2
3`, 3``	31.93, 31.52	31.9, 31.5	31.93, 31.52	-CH_2
4`, 4``	22.57	22.4	22.57	-CH_2
5`, 5``	22.69	22.7	22.69	-CH_2
6`, 6``	24.83	24.8	24.83	-CH_2
7`, 7``	24.87	24.9	24.87	-CH_2
8`, 8``	27.19	27.2	27.19	-CH_2
9`, 9``	127.89- 130.20	127.8- 130.2	127.89-130.20	-CH
10`, 10``	127.89- 130.20	127.8- 130.2	127.89-130.20	-CH
11`, 11``	25.62, 27.19	25.6, 27.2	25.62, 27.19	-CH_2
12`, 12``	127.89- 130.20	127.8- 130.2	127.89-130.20	-CH
13`, 13``	127.89- 130.20	127.8- 130.2	127.89-130.20	-CH
14`, 14``	27.19	27.2	27.19	-CH_2
15`, 15``	29.04-29.70	29.0-29.7	29.04-29.70	-CH_2
16`, 16``	29.04-29.70	29.0-29.7	29.0-29.70	-CH_2
17`, 17``	29.04-29.70	29.0-29.7	29.0-29.70	-CH_2
18`, 18``	14.11-14.07	14.1-14.0	14.11-14.07	-CH_3

* Data from ref. 42, 45.

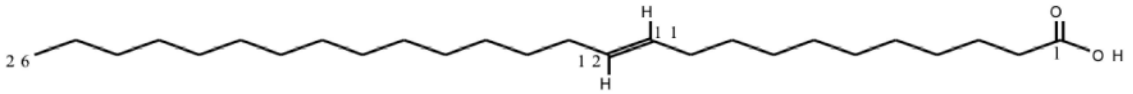

Fig. 3: The proposed structure of compound SR-3 (and the reported structure of n-hexacos-11-enoic acid) [18,46].

of methylene and methyl groups, respectively. In the ^{1}H-NMR spectrum of compound SR-3 (Supplementary material 6) the peak at d 10.83 represented to the proton of carboxylic acid; multiplets at d 5.37 and d 5.35 assigned to olefinic protons; triplets at d2.78 and 2.76 can be attributed to protons attached to carbon adjacent to the carboxylic group. The two multiplets observed at d2.35 and 2.07 can represent protons attached to a carbon that is adjacent to olefinic carbon. The peaks at d1.64, 1.33 and 1.27 can be attributed to protons of methylene groups. Triplet peak at d0.89 represents to methyl protons of an aliphatic hydrocarbon chain. Based on the observed 1H-NMR and IR spectral data and literature report [18,46], compound SR-3 could be unsaturated carboxylic acid and it is most likely n-hexacos-11-enoic acid (Fig. 3). The observed 1H-NMR data of compound SR-3 and reported data of that of *n*-hexacos-11-enoic acid are given in Table 3. The above claim was further confirmed using the spectral data obtained form were confirmed the 13C-NMR and DEPT-135 spectra (Suplemntary material 7 and 8). A peak at d180.49 indicates quaternary carbon atoms of carbonyl carbons of carboxylic group. The peaks at d130.09 (C-11) and 127.88 (C-12) indicated C=C bonds; the peaks in the rang of d34.12 - 22.70 could represent aliphatic methylene carbon (-CH2) whereas the peak at d14.08 indicates the presence of methyl (-CH3) group in the structure (i.e. C-26). As shown in Table 3, the observed 13C-NMR are consistent with that of data reported for n-hexacos-11-enoic acid [18].

Futher confirmation of the functional groups present in compound SR-3 carried out using chemical and physical tests. Treating compound SR-3 with aqueous solution of sodium bicarbonate (NaHCO3), a common reagent for qualitative test of acids, resulted in the formation of effervescence which indicates the evolution of carbon dioxide. This observation has confirmed the presence of carboxylic acid functional group in the compound. Observation of color change of potassium permanganate (KMnO4) solution from violate color to brown also confirmed the double bond in compound SR-3. A physical test using melting point measurement indicated that the observed mp (238-240 oC) of compound SR-3 to be consistent with reported mp value (240-242 oC) of n-hexacos-11-enoic acid [18] was of which is comparable to the reported mp value [46]. Therefore, from the aforementioned spectroscopic data as well as chemical

Table 3: ^{1}H-NMR, ^{13}C-NMR and DEPT-135 data of compound SR-3 along with the reported ^{1}H-NMR and 13C-NMR data of n-hexacos-11-enoic acid.

C.No	1H-NMR data of compound SR-3	^{1}H-NMR data reported for *n*-hexacos-11-enoic acid*	^{13}C -NMR data of compound SR-3	^{13}C-NMR data reported for *n*-hexacos-11-enoic acid*	DEPT-135 of compound SR-3	Nature of the carbon atoms
1	10.83(H of CO2H)	-	180.49	177.30	-	C
2	2.78, 2.76	2.77, 2.75	34.05	34.00	34.11	CH_2
3	1.64	1.65	24.67	24.70	24.67	CH_2
4	1.27	1.25	25.61	25.70	25.61	CH_2
5	1.27	1.25	29.28	29.30	29.28	CH_2
6	1.27	1.25	29.47	29.50	29.47	CH_2
7	1.27	1.25	29.63	29.70	29.63	CH_2
8	1.33	1.30	29.69	29.70	29.69	CH_2
9	1.33	1.30	29.69	29.70	29.69	CH_2
10	2.35	2.34	31.96	32.00	31.96	CH_2
11	5.37	5.39	130.09	130.10	130.09	CH
12	5.35	5.32	127.88	127.90	127.88	CH
13	2.03	2.01	31.54	31.60	31.54	CH_2
14	1.33	1.30	29.74	29.70	29.74	CH_2
15	1.33	1.30	29.69	29.70	29.69	CH_2
16	1.27	1.25	29.69	29.70	29.69	CH_2
17	1.27	1.25	29.69	29.70	29.69	CH_2
18	1.27	1.25	29.69	29.70	29.69	CH_2
19	1.27	1.25	29.69	29.70	29.69	CH_2
20	1.27	1.25	29.69	29.7	29.69	CH_2
21	1.27	1.25	29.69	29.7	29.69	CH_2
22	1.27	1.25	29.08	29.1	29.08	CH_2
23	1.27	1.25	29.05	29.0	29.05	CH_2
24	1.27	1.25	27.19	27.2	27.19	CH_2
25	1.33	1.30	22.70	22.7	22.70	CH_2
26	0.89	0.88	14.08	14.2	14.07	CH_3

*Data from ref. [18,46]

and physical tests, compound SR-3 is to be most likely n-hexacos-11-enoic acid. Isolation of this compound was also reported from our lab from the root of the same plant [18].

Summary of Spectral Data of the Isolated Compounds: Compound SR-2 (yellow oily, 1 gm): IR (KBr) νmax cm^{-1} 3019, 2856, 2400, 1738, 1466, 1217 and 771, 1H-NMR(400 MHz, CDCl3): δ0.88(t, 6H, H-18'x2), δ0.90 (t,3H, H-18"), δ1.27(m, 56H, H-(4'-7'and 15'-17) x 2 and H-(4"-7" and 15" -17"), δ 1.62 (t, 6H, H-3' x 2 and 3"), δ205 (m, 12H, H- (8',14') x 2 and H-(8",11"), δ2.31(t, 4H, H-2' x 2), δ 2.33(t, 2H, H-2"), δ 2.78 (m, 4H, H -11' x 2), δ 2.05 (m, 14H, H- 8' x 2, H-8", H-11", H-14' x 2, H- 14"), δ 4.14 (dd, 1H, H-1a, 3a), δ4.30 (dd, 1H, H-1b, 3b), δ5.27 (m, 1H, H-2), δ5.38 (m, 3H, H-9' x 2, H-9"), δ 5.37(m, 3H, H-10' x 2, H- 10"), δ 5.35 (m, 4H, H-12' x 2, H- 13' x 2); 13C-NMR(100 MHz, CDCl3): δ14.07(C-18"), δ14.11(C-18' x2), δ22.57, 22.69, 24.83, 24.87, 27.19, 29.04-29.70 (C- (4'-8' and 15'-17') x 2 and 4"-8" and 15"-17"), δ27.19C-(14', 14"), δ127.89-130.20 (C-(9',10,12',13') x 2 and C-(9", 10", 12", 13")), δ27.19 (C-(14' x 2) and C-14"), δ34.18, 34.02, 31.93, 31.52,(C-(2",2' x 2, 3' x 2 and 3"), δ173.28-172.83(C-1' x 2 and C-1"), δ62.09 (C-(1and 3), δ68.88C-2.

Compound SR-3 (colorless crystal 293 mg): mp 238-240°C; IR (KBr) νmax cm-1 721, 941, 1207, 1464, 1708, 2850, 2918 and broad band in the region 3300-2500, 1H-NMR (400 MHz, CDCl3): d0.89 (t, 3H, H-26), δ1.27(m,26H, (H-(4-6) and H- (16-24)) x 2), δ1.33,(m, 10H, (H-(8,9,14,15 and 25)x2, δ1.64 (m, H-3 x 2), δ2.78 and 2.76 (t, 2H, H-2), δ2.35 and δ2.03 (m, 4H, (H-(10 and 13) x 2), δ5.37 and δ5.35 (m, 2H, H-11 and 12), δ10.83(s, 1H, H-O). 13C-NMR (100 MHz, CDCl3): δ14.08(C-26), δ22.70 (C-25), δ24.67 (C-3), δ25.61(C-4), δ27.19 (C-24), δ29.05(C-23), δ29.08(C-22), δ29.69 (C-(8,9) and C-(15-21), δ29.74(C-14), δ31.54(C-13), δ127.88(C-12), δ130.09(C-11), δ31.96(C-10), δ29.63(C-7), δ29.47(C-6), δ29.28(C-5), δ34.05(C-2), δ180.49(C-1).

Antibacterial Test of the Isolated Compounds: *In vitro* antibacterial activity of the isolated compounds (compound SR-2 and compound SR-3) was carried out by employing agar diffussion method and four bacterial species namely *E. coli*, *S. aureus*, *P. aeruginosa* and S. typhimurium and following standard procedures reported in literature [41] (See methods and materials section). Diameter of inhibition zone was measured using ruler (Supplementary material 9) and the results are given (in mm) in Table 4.

Table 4: Inhibition zone of the isolated compounds in mm (50 µg/ml) and the reference compound (Gentamicine).

S.No	Compounds	*P. aeruginosa*	S. aureas	*S. thyphimurium*	*E. coli*
1	Compound SR-2(1, 3-dilinoleoyl-2-oleine)	9	8	-	-
2	Compound SR-3(n-hexacosa-11-enoic acid)	10	14	15	11
3	Gentamicine	20	18	20	24

The isolated compounds showed relatively lower antibacterial activities as compared to the reference compound (Gentamicine) (Table 4). Compound SR-2 is showed low antibacterial activities only against *S. aureus* and *P. aeruginosa*. The compound did not show activity against the rest of bacterial strains (*E. coli* and *S. thyphimurium*).This observation is consistent with previous reports that showed low antibacterial activities of triglycerides [47] as compared to reference drugs. Compound SR-3 showed relatively better activity than compound SR-2 and also showed growth inhibitory activities against all the bacterial strains used in the experiment. This result was consistent with the reported moderate antibacterial activity of nhexacosa-11-enoic acid [18] and also reported antibacterial activities of free fatty acids [48].

CONCLUSION

In the present study, two compounds labeled as compound S R-2 and compound SR-3 were isolated from acetone extract of the fruits of *S. rhombifolia*. The compounds were proposed to be 1,3-dilinoleoyl-2-oleine and n-hexacosa-11-enoic acid, respectively, based on spectroscopic data and information from l iterature reports. With the objective of isolation of compounds from this plant species that could show antibacterial activities, *In vitro* antibacterial activity tests were carried out using disc diffusion method and using four bacterial strains (*P. aeruginosa* and *S. aureus*, *S. thyphimurium* and *E. coli*). The results indicated that compound SR-2 or 1,3-dilinoleoyl-2-oleine showed low activity *P. aeruginosa* and *S. aureus* but no antibacterial activity against *S. thyphimurium* and *E. coli*. The other compound (compound SR-3 or n-hexacosa-11-enoic acid) was found to showed better activity than compound SR-2. But as compared to the reference compound its antbacterial activities were lower than the reference compound (Gentamicine). The results suggest that further tests are needed to evaluate activities of the compound SR-3 against other bacterial species to explore its potential as lead compound in the development of antibacterial agents.

ACKNOWLEDGEMENT

The authors thankfully acknowledge Jimma University for financial support.

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