Isolation and Characterization of 1,3-Dicapryloyl-2-linoleoylglycerol: A Novel Triglyceride from Berries of *Hippophae rhamnoides*

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1,3-Dicapryloyl-2-linoleoylglycerol (1), a novel triglyceride, was isolated from berries of *Hippophae rhamnoides*. The structure was elucidated on the basis of MS, 1D and 2D NMR experiments including HMQC and HMBC. The metal chelating, free radical scavenging, and lipid peroxidation inhibiting properties of the compound were also estimated with particular reference to radiation protection. In case of metal chelation and superoxide ion scavenging, 1 showed maximum inhibition at 50 μg/ml (11%) and 100 μg/ml (55%), respectively, whereas in lipid peroxidation, 1 showed maximum inhibition (57%) at 2 mg/ml as compared to quercetin as a control.

Key words *Hippophae rhamnoides*; Elaeagnaceae; 1,3-dicapryloyl-2-linoleoylglycerol; metal chelation; free radical scavenging; lipid peroxidation

Hippophae rhamnoides (Elaeagnaceae), commonly known as Seabuckthorn, grows in various regions of Asia, Europe and Northern America.[1)] It is traditionally used to treat radiation damage, burns, oral inflammation and gastric ulcers.[2—4)] Berries of *H. rhamnoides* are a rich source of vitamins (A, C, E, K), carotenes (α, β, γ), flavonoids, tannins, triterpenoids, essential amino acids and glycerides of palmitic, stearic, oleic and linoleic acids.[5—9)] Ethanolic extract of berries is found to inhibit apoptosis, DNA fragmentation and chromium-induced free radical production.[10,11)] We have also reported that the ethanolic extract of berries renders significant protection against lethal ^{60}Co gamma radiation-induced genotoxicity in both *in vivo* and *in vitro* model systems.[12,13)] Therefore, our interest lies in isolation and characterization of constituents from ethanolic extract of berries for its biological activities. In this regard, we have isolated 1,3-dicapryloyl-2-linoleoylglycerol (**1**), a novel triglyceride, from ethanolic extract of berries of *H. rhamnoides*. Triglyceride **1** was estimated for metal chelating, free radical scavenging and lipid peroxidation inhibiting activities with particular reference to radiation protection.

Results and Discussion

Ethanolic extract of berries of *H. rhamnoides* upon fractionation with chloroform followed by repeated column chromatography, provided **1** as a colorless oil. A molecular ion peak at *m/z* 607 $(M+H)^+$ in FAB-MS along with ^{1}H- and ^{13}C-NMR spectral data (Table 1) showed its molecular formula to be $C_{37}H_{66}O_6$. The presence of a strong absorption band at 1746 cm^{-1} in the IR spectrum along with signals at δ_C 173.1 (C-1″), 172.9 (C-1′, C-1‴) in ^{13}C-NMR spectrum suggested the presence of three ester carbonyls in **1**. The proton signals at δ_H 4.13 (2H, dd, H-1a, H-3a), 4.28 (2H, dd, H-

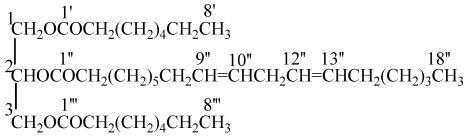

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Table 1. ^{1}H, ^{13}C and HMBC Spectral Data of **1** in $CDCl_3$ (ppm, *J* in Hz)

Atom	^{1}H (δ)	^{13}C (δ)	HMBC (H↔C)
1a, 3a	4.13, dd (*J*=14.0)	62.0	C-1′, C-1‴
1b, 3b	4.28, dd (*J*=14.0)	62.0	C-1′, C-1‴
2	5.26, m	68.8	H-1a, H-1b, H-3a, H-3b
1″	—	173.1	H-2, H-2″, H-3″
1′, 1‴	—	172.9	H-1a, H-1b, H-2′, H-3′, H-3a, H-3b, H-2‴, H-3‴
2″	2.30, t (*J*=7.6)	34.1	H-4″
2′, 2‴	2.29, t (*J*=7.6)	33.0	H-4′, H-4‴
3′, 3″, 3‴	1.59, m	25.4	H-5′
		25.9	H-5′, H-5‴
4′, 5′, 7′, 4″, 5″, 7″,	1.25, m	22.5, 22.6, 22.7,	—
15″, 17″, 4‴, 5‴, 7‴		24.8, 27.9—29.6	—
6′, 6‴	1.25, m	31.7	H-5′, H-7′, H-5‴, H-7‴
8″, 14″	2.02, m	27.1	H-6″, H-10″, H-12″, H-16″
8′, 8‴	0.86, bt	14.0	H-6′, H-6‴
11″	2.76, m	25.6	C-9″, C-10″, C-12″, C-13″
9″, 10″, 12″, 13″	5.35, m	127.8—130.1	H-8″, H-11″, H-14″
16″	1.25, m	31.8	H-15″, H-17″
18″	0.86, bt	14.1	H-16″

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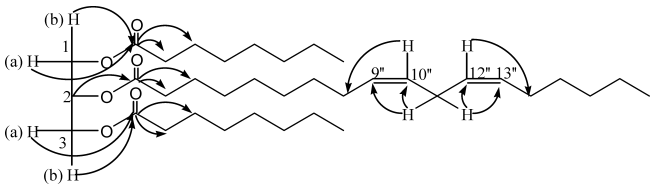

Fig. 1. Key Correlations Observed in HMBC (H↔C) Spectra of **1**

1b, H-3b) and 5.26 (1H, m, H-2), and the carbon signals at δ_C 62.0 (C-1, C-3) and 68.8 (C-2) along with MS fragments at *m/z* 263 $(C_{18}H_{31}O)^+$ and 127 $(C_8H_{15}O)^+$ confirmed that **1** is a triglyceride with C_{18} and C_8 fatty acid esters.[14] In HMBC spectrum (Table 1), the carbonyl carbon signals at δ_C 173.1 (C-1″) and 172.9 (C-1′, C-1‴) showing correlation with proton signals at δ_H 2.30 (2H, t, H-2″), 2.29 (4H, t, H-2′, H-2‴) and 1.59 (6H, m, H-3″, H-3′ H-3‴), also confirmed that **1** is a triglyceride with long chain fatty acid esters. The proton signals at δ_H 2.02 (4H, m, H-8″, H-14″) and 2.76 (2H, m, H-11″) showing correlation with carbon signals at δ_C 127.8—130.1 (C-9″, C-10″, C-12″, C-13″), confirmed the presence of two olefinic bonds in **1**. The proton signals at δ_H 5.35 (4H, m, H-9″, H-10″, H-12″, H-13″) correlating with signals at δ_C 127.8—130.1 (C-9″, C-10″, C-12″, C-13″) in the HMQC spectrum also indicated the presence of two olefinic bonds in **1**. The proton signals at δ_H 2.76 (2H, m, H-11″) correlating with carbon signals at δ_C 127.8—130.1 (C-9″, C-10″, C-12″, C-13″) along with the peaks at *m/z* 263 $(C_{18}H_{31}O)^+$ in the MS confirmed the presence of linoleic acid moiety in **1**. The position of linoleic acid moiety was confirmed on the basis of ^{13}C-NMR. The presence of only two carbon signals for three ester carbonyls at δ_C 173.1 (C-1″), 172.9 (C-1′, C-1‴) suggested **1** as a symmetrical triglyceride,[14—17] hence, confirming position of linoleic acid moiety at C-2. Saponification of **1** followed by methylation of the obtained acid upon GC-MS analysis also confirmed the presence of one linoleic and two caprylic acid moieties in **1**, confirming it as 1,3-dicapryloyl-2-linoleoylglycerol. Triglyceride **1** was analyzed for metal chelating, free radical scavenging and lipid peroxidation inhibiting activities.

Metal chelation activity of **1** was evaluated using 2,2′-bipiridyl assay. Metal chelation activity got increased with increasing concentration of **1** and maximum inhibition of iron-2,2′-bipyridyl (chromogen) complex was found to be at 50 μg/ml (11%) with respect to control. The metal chelating activity of **1** was found to be less than that of quercetin (50 μg/ml) which was used as a standard (34%) (Fig. 2).

The superoxide ion scavenging activity was evaluated using NBT reduction assay in terms of percentage inhibition of the formation of formazan crystals (chromogen). Superoxide ion scavenging activity was found to be concomitantly increasing with increasing concentration of **1** and maximal inhibition of formazan crystal formation (chromogen) was found to be at 100 μg/ml (55%) with respect to control. The superoxide ion scavenging activity of **1**, however, was found to be less than that of quercetin (100 μg/ml), which was used as a standard (99%) (Fig. 3).

Thiobarbituric Acid Reactive Substances (TBARS) assay allows measurement of the radiation-induced oxidative damage. The results of this assay are depicted in Figs. 4 and 5 which reveal that **1** inhibits radiation (200 Gy) induced lipid

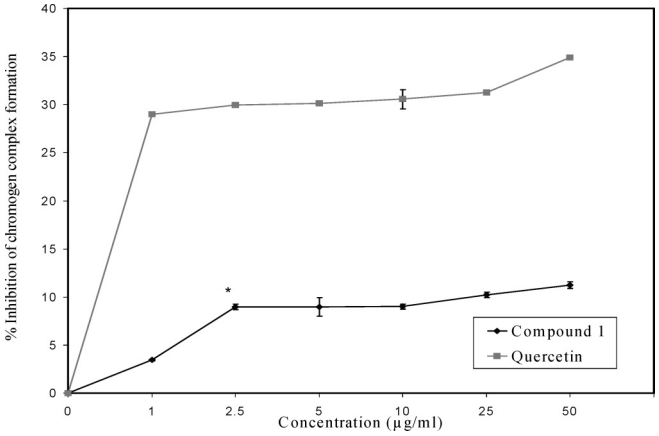

Fig. 2. Effect of Concentration of **1** on Chelation of Iron

The percentage inhibition of iron-2,2′-bipyridyl chromogen complex containing all reagents, but without the extract, was considered as 0% inhibition. ＊Maximum percentage inhibition of chromogen complex formation was observed at 50 μg/ml (11%) with respect to control (quercetin).

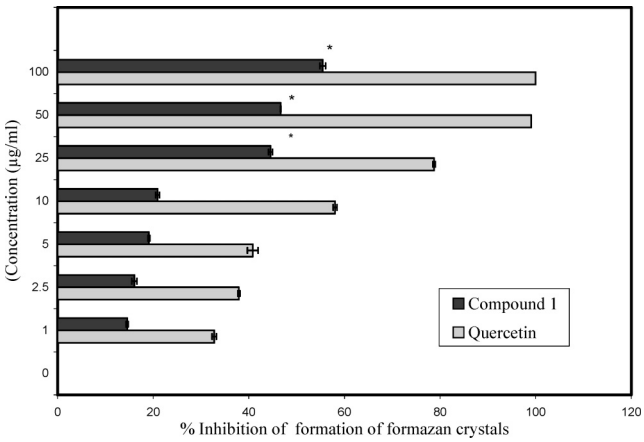

Fig. 3. Evaluation of Superoxide Ion Scavenging Activity of **1**

The % inhibition of formation of formazan crystals was evaluated in triplicate. Each experiment was repeated thrice and 0% inhibition was treated as control. The values are expressed as mean±S.D. ＊Compound **1** with respect to control (0% inhibition; $p<0.05$).

peroxidation in a dose dependent manner (0.5—2.0 mg/ml) with maximal inhibition (57%) at 2 mg/ml. This was found to be significant ($p<0.05$) as compared to control (0% inhibition). Although **1** inhibited maximal iron/ascorbate+radiation-induced lipid peroxidation upto 1 mg/ml (41%), a decrease in percentage inhibition was observed at higher doses indicating its inability to tackle iron/ascorbate-induced lipid peroxidative stress.

Experimental

General UV and IR spectra were recorded on Analytikjena Specord 200 and Perkin Elmer 1760 X FT-IR spectrophotometers, respectively. ^{1}H-(300 MHz, $CDCl_3$) and ^{13}C- (75.4 MHz, $CDCl_3$) NMR spectra were recorded on a Bruker AM-300 spectrometer. FAB-MS was recorded on JOEL JMS-Hx 110 mass spectrometer. GC-MS was performed on Shimadzu QP 2010 gas chromatograph. 2,2′-Bipyridyl, quercetin, malonaldehyde, phenazine-methanosulfate, nitrobluetetrazolium, ascorbate and thiobarbituric acid were purchased from Sigma Chemicals (St. Loius, MO, U.S.A.).

Experimental Animals Adult (6—8 weeks) Swiss albino Strain 'A' mice (25±2 g), bred locally in the animal house of the Institute of Nuclear Medicine and Allied Sciences, Delhi (India), were maintained under controlled temperature (25±2 °C), under 12 h alternating dark and light cycles in polypropylene cages. Standard food pellets (M/S Amrut Feeds Pvt. Ltd., Kolkata, India) and drinking water was provided *ad libitum*. Permission for

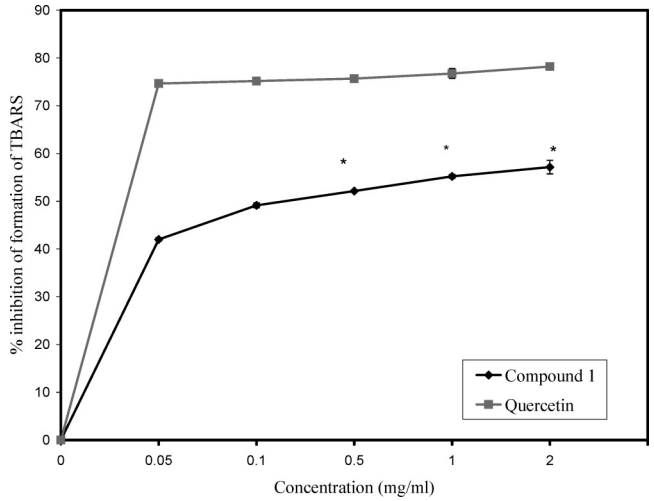

Fig. 4. Effect of **1** on Radiation Mediated Lipid Peroxidation Evaluated in Liver Homogenate of Strain 'A' Mice

Each experiment was performed in triplicate and was repeated three times and the percentage inhibition of lipid peroxidation activity is expressed as percentage inhibition of TBARS (nanomoles of MDA (malonaldehyde) formed $\times 10^6$). Lipid peroxidation in control represents 0% inhibition. * Maximal % inhibition of activity at 2 mg/ml with respect to control ($p<0.05$).

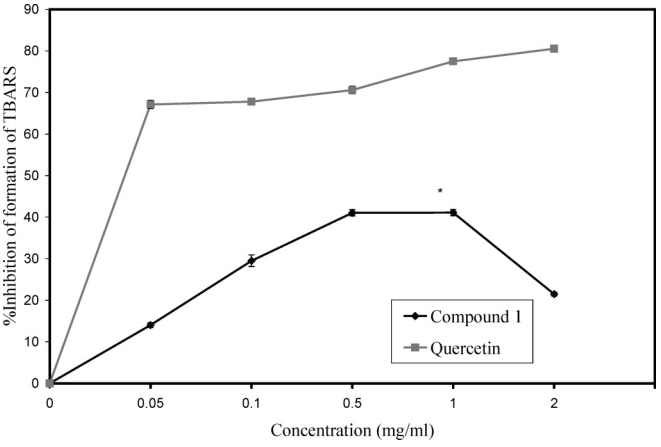

Fig. 5. Effect of **1** on Iron/Ascorbate+Radiation-Mediated Lipid Peroxidation Evaluated in Liver Homogenate of Strain 'A' Mice

Each experiment was performed in triplicate and was repeated three times and the percentage inhibition of lipid peroxidation activity is expressed as percentage inhibition of TBARS (nanomoles of MDA (malonaldehyde) formed $\times 10^6$). Lipid peroxidation in control represents 0% inhibition. * Maximal % inhibition of activity at 1 mg/ml with respect to control ($p<0.05$).

use of animals was taken from the Institutional Animals Ethics Committee (IAEC) of INMAS and all experiments were carried out strictly in accordance with the laid down institutional guidelines and keeping Indian National Science Academy (INSA) guidelines for the care and use of laboratory animals for research purposes.

Plant Material Berries of *H. rhamnoides* were collected from hilly regions of Western Himalaya. The plant was confirmed as *H. rhamnoides* by comparison with the Voucher specimen (IHBT No. 1047) kept in herbarium of Biodiversity Centre at the Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India.

Extraction and Isolation The berries of *H. rhamnoides* were shade dried for 4—6 weeks, powdered and 100 g of the powder was repeatedly extracted with ethanol at room temperature (4×200 ml). The solvent from the combined extract was evaporated under reduced pressure followed by lyophilization. The resulting dark reddish gummy residue (47 g) was fractionated successively with chloroform, ethyl acetate and acetone. The residue (5.2 g) obtained from evaporation under reduced pressure of the chloroform solubles was subjected to column chromatographic separation on silica gel (100 g) using a gradient of *n*-hexane–ethyl acetate (100 : 0—0 : 100) as an eluting solvent system resulting in thirty eight fractions (1—38). Fractions 6—13 were combined according to their profiles, yielding 1.1 g, and was further chromatographed on silica gel (40 g) using a gradient of *n*-hexane–ethyl acetate (100 : 0—0 : 100), giving a subfraction of interest. This subfraction (270 mg) was purified by preparative TLC (silica gel), in which development of plates was carried out with *n*-hexane–ethyl acetate (98 : 2). Compound **1** (68 mg) was obtained from this subfraction.

Metal Chelation Activity Metal chelation activity was determined employing 2,2′-bipyridyl assay[18] in terms of percentage inhibition of the formation of iron–bipyridyl (chromogen) complex. To a 4 ml ferric chloride solution (5 μg/ml, 0.005 N HCl), **1** (2 ml) was added in varying concentrations. The obtained mixtures were incubated at room temperature for 10 min and aliquots of 2 ml each were taken from the reaction mixture and mixed with sodium sulphite (final concentration: 0.05 M) and 2,2′-bipyridyl (0.2%). The solution was reincubated in a hot water bath (*ca.* 55 °C) for 5 min, after which the reaction tubes were cooled to room temperature and the absorbance recorded spectrophotometrically at 520 nm.

Superoxide Ion Scavenging Activity The superoxide ion quenching ability of **1** was determined using NBT reduction assay.[19] Varied concentrations of **1** were mixed with 1 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3) and 0.1 ml of phenazinemethanosulfate (186 μM). Nitrobluetetrazolium (300 μl, 300 μM) was added to the above solution and the final volume was adjusted to 3 ml. Reaction was initiated by adding 200 μl of NADH (780 μM). The whole reaction mixture was incubated at 37 °C for 90 s. The reaction was terminated by adding 1 ml of glacial acetic acid, followed by shaking with 4 ml of *n*-butanol and allowed to stand for 10 min at room temperature. The *n*-butanol layer was separated by centrifugation and the color intensity of chromogen in the *n*-butanol layer was measured at 560 nm against *n*-butanol.

Lipid Peroxidation The degree of lipid peroxidation was evaluated in terms of TBARS assay.[20] Liver homogenate (10%, 3 ml)[13] was taken in 35 mm petriplates (Tarsons, India) to which different concentrations of **1** were added and mixed gently to form a homogenous solution. Lipid peroxidation was initiated by adding 20 μl of ferric chloride and 200 μl ascorbate and subjecting it to irradiation (250 Gy). Thereafter, petridishes were incubated at 37 °C for 30 min. The homogenate (1 ml) was pipetted out for estimating lipid peroxidation levels.

1,3-Dicapryloyl-2-linoleoylglycerol (**1**): Colorless oil (*n*-hexane : ethyl acetate 98 : 2). UV (MeOH) λ_{max} nm (log ε): 205. IR ($CHCl_3$) γ_{max} cm^{-1}: 3008, 2925, 2854, 1746 (>C=O), 1642 (C=C), 1463, 1377, 1163, 723. FAB-MS *m/z* (rel. int. %): 607 $(M+H)^+$ (2), 576 (12), 548 (100), 311 (18), 263 (14), 237 (12), 154 (8), 137 (12), 127 (16). ^{1}H- and ^{13}C-NMR (300 MHz, 75.4 MHz, $CDCl_3$) see Table 1.

Saponification and Methylation of Compound 1 Compound **1** (20 mg) was stirred with 5% methanolic KOH solution (10 ml) for 4 h at r.t. This solution was evaporated under reduced pressure and the obtained residue was stirred with ether (20 ml) and water (4—5 ml). The ether layer was discarded and the aqueous layer was acidified with dil. HCl and extracted with ethyl acetate. The ethyl acetate part was dried over sodium sulphate and evaporated under reduced pressure to yield colorless oil (16.4 mg). The colorless oil was dissolved in MeOH : Toluene (60 : 40) and refluxed in a Dean Stark assembly for 8 h. After completion of the reaction, solvent from the reaction mixture was completely evaporated under reduced pressure. To the residue, water (20 ml) was added and acidified with dil. HCl and extracted with ethyl acetate. Ethyl acetate part was dried over sodium sulphate and evaporated under reduced pressure to yield the corresponding methyl esters.

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