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Isolation of Aliphatic Carbonyl Hydrocarbons and in-vitro Antioxidant Activity of Silybum Marianum Seeds

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Abstract

Silybum marianum (milk thistle) seeds and other parts of this medicinal plant have been used traditionally for centuries in the treatment of liver and several other diseases. Purified extracts made from the fruits of Silybum marianum (SM) chiefly contains silymarin and are available commercially. Silymarin is used as a dietary supplement and is one of the widely used alternative medicines. The present study was conducted to investigate the in- vitro antioxidant potential of SM seed extracts by DPPH method and to isolate the phytoconstituents present in ethyl acetate extract. Phytochemical investigation of the seeds of SM yielded eight aliphatic carbonyl hydrocarbons. The chemical structure of all the phytoconstituents were elucidated on the basis of spectral data analysis. Antioxidant activity of methanolic seed extract was found to be better than the ethyl acetate as well as standard ascorbic acid which could be due to high flavonoid content. Thus result of this study support use of silymarin as a dietary natural antioxidant supplement in drug and food industry to prevent free radical related diseases.

Keywords: Silybum marianum, Silymarin, Antioxidant, DPPH

Introduction

Silybum marianum (L) Gaertn. Syn Carduus marianus (L) is commonly known as milk thistle or holy thistle[1]. It is an erect, stout, annual herbaceous plant that belongs to family Asteraceae (Compositae). Milk thistle grows in paddocks along road sides, wastes grounds and is distributed from sea to the sub mountain regions till 1100 m of attitude.[2] Seeds of Silybum marianum are useful in controlling haemorrhage and also used for the treatment of jaundice and calculi of liver and gall-bladder.[3] Silybum marianum (SM) has been studied extensively and reported to have multiple pharmacological actions such as anti-inflammatory, antibacterial, hepatoprotectant, anticancer, antioxidant etc .[4,5]

SM fruits are rich in phenolic compounds such as flavonoid complex, silymarin. Silymarin consists of a large number of flavolignans including silybin (or silybinin), isosilybin, silydianin and silychristin[6] and is the major constituent of standardized extract. Seeds also contain taxifolin, quercetin, betaine with silybonol proteins, fixed oil and free fatty acids, which may contribute to the health giving effects of milk thistle seeds.[7]

The aim of the present study was to isolate and characterize the structure of the phytoconstituents present in the ethyl acetate extract of SM seeds and to study the free radical scavenging activity by DPPH method.

Materials and Method

General: The melting points were recorded using a Perfit melting point apparatus and are uncorrected. IR spectra were recorded in KBr pellet on Win IR FTS 135 instrument. ^{1}H NMR spectra were recorded in $CDCl_3$ on Bruker DRX 300 NMR

spectrophotometer using TMS as an internal reference. Chemical shift values are expressed as δ ppm. FAB mass spectra were scanned at 70eV on a Jeol D-300 instrument. Column chromatography for isolation of chemical constituents was performed on silica gel (60-120 mesh, Merck). Purity of the isolated compounds was checked by thin layer chromatography on pre- coated silica gel G plates using iodine vapors as a locating reagent.

Plant material

Silybum marianum (L) Geartn. seeds were purchased from local market of Delhi and authenticated by the taxonomist, Faculty of Science, Jamia Hamdard, New Delhi. A voucher specimen was deposited in the herbarium of Faculty of Pharmacy, Jamia Hamdard, New Delhi for future reference.

Preparation of extract:

The dried powdered seeds (1 Kg) were extracted with ethanol by cold percolation for two weeks. The alcoholic extract was dried under reduced pressure to obtain a dark brown viscous mass (110 gm, % yield; 11%). it was fractionated into ethyl acetate (80 gms) and methanol (30 gm) soluble portions

Isolation of phytoconstituents:

The viscous mass of ethyl acetate extract was adsorbed on silica gel (60-120 mesh) to prepare the slurry. It was dried in air, loaded on to the silica gel column prepared in petroleum ether and then the column eluted successively in order of increasing polarity with petroleum ether, petroleum ether-chloroform (9:1, 8:2, 1:1 v/v), chloroform and chloroform-methanol (99:1 v/v). The fractions collected were subjected to TLC and fractions having same R_f values were combined together. The isolated compounds were re-crystallized in suitable organic solvents to obtain the pure compounds.

18- hydroxyl 6-oxo icosanoic acid (1)

Elution of column with petroleum ether-chloroform (9:1) gave colorless crystals of 1 which was recrystallized from hexane-benzene (1:1), 450 mg, R_f; 0.35 (petroleum ether: chloroform, 1:1), m.p. 68-69 ^{0}C; IR (KBr): 3649, 2360, 1780, 1540, 1025, 751 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, t, CH_3), 1.26 (16H, brs, 8XCH_2), 1.41 (4H, m, 2XCH_2), 1.56 (6H, m, 3XCH_2), 2.0 (1H, s, OH), 2.31 (2H, t, C$\underline{H}_2$COOH), 2.50 (4H, t, C$\underline{H}_2$-CO-C$\underline{H}_2$), 3.55 (1H, m, CH), 10.8 (1H, s, COOH), FABMS m/z (rel. int); 342 (M^+] $C_{20}H_{38}O_4$ (21.5%), 314 (13.5%), 228 (14%), 129 (58%), 59 (100%).

Icosanoic acid pentyl ester (2)

Elution of column with petroleum ether-chloroform (9:1) afforded colorless needle like crystals of 2 which was recrystallized from benzene. 680 mg, R_f; 0.61 (benzene: ethyl acetate, 1:1), m.p. 35-36 ^{0}C; IR (KBr): 2918, 2850, 1738, 1508, 1036, 746 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.89 (6H, t, 2xCH_3), 1.25 (32H, brs, 16XCH_2), 1.40 (4H, m, 2XC$\underline{H}_2$-CH3), 1.59 (2H, m, C$\underline{H}_2$$CH_2$OCO), 1.65 (2H, m, $COCH_2$C$\underline{H}_2$), 2.26 (2H, t, C$\underline{H}_2$CO), 4. 09 (2H, t, C$\underline{H}_2$-OCO), FABMS m/z (rel. int); 382 (M^+] $C_{25}H_{50}O_2$ (6.5%), 352 (30%), 338 (42.2%), 310 (26%), 295 (6%), 267 (11.2%), 156(11.20%), 142 (11.3%), 87 (100%), 54 (30%).

n-Heptadecanoic acid(3)

Elution of column with petroleum ether-chloroform (8:2) furnished a colorless crystalline compound 3, recrystallized from benzene, 350 mg, R_f; 0.29 (chloroform: ethyl acetate, 1:1), m.p. 52 ^{0}C; spectra was comparable with an authentic sample; 272 (M^+] $C_{17}H_{34}O_2$.

25-oxo nonacosanoic acid (4)

Elution of column with petroleum ether-chloroform (8:2) produced colorless crystalline flakes of 4, recrystallized from acetone. 475 mg, R_f; 0.73 (chloroform: ethyl acetate, 1:1), m.p. 48-49 ^{0}C; IR (KBr): 2918, 2850, 1701, 1652, 1559, 1508 1066, 782 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.91 (3H, t, CH_3), 1.28 (38H, brs, 19XCH_2), 1.32 (2H, m, CH_3C$\underline{H}_2$), 1.65 (6H, m, 3xC$\underline{H}_2$$CH_2$CO), 2.34 (2H, t, C$\underline{H}_2$COOH), 2. 39 (4H, t, C$\underline{H}_2$-CO), FABMS m/z (rel. int); 452 (M^+] $C_{29}H_{56}O_3$ (6.2%), 367 (8.4%), 311 (19%), 284 (21.2%), 256 (19.7%), 85 (10%), 56 (27%).

Pentanoic acid hetpadecyl ester (5):

Elution of column with petroleum ether-chloroform (1:1) afforded colorless crystals of 5 which was recrystallized from chloroform. 550 mg, R_f; 0.37 (benzene: ethyl acetate, 6:4), m.p. 47-48 ^{0}C; IR (KBr): 2919, 2851, 2360, 1712, 1468, 1168, 1038 723 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.90 (6H, t, 2xCH_3), 1.26 (26H, brs, 13XCH_2), 1.60 (4H, m, 2XC$\underline{H_2}$-CH_3), 1.62 (2H, m, C$\underline{H_2}CH_2OCO$), 1.65 (2H, m, $COOCH_2C\underline{H_2}$), 2.31 (2H, t, C$\underline{H_2}COO$), 4.14 (2H, t, COO-C$\underline{H_2}$), FABMS m/z (rel. int); 340 (M^+] $C_{22}H_{44}O_2$ (4.8%), 312 (7.2%), 284 (37.8%), 256 (75%), 213 (10.5%), 157(6.0%), 143 (3.3%),57 (76.%).

Tricosan 5-one (6)

A yellow crystalline compound 6 was isolated by elution of column with petroleum ether-chloroform (1:1) that was recrystallized in chloroform. 450mg, R_f; 0.37 (benzene: ethyl acetate, 6:4), m.p. 54-55 ^{0}C; IR (KBr): 2918, 2850, 2340, 1703, 1463, 1038, 789, 668 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.88 (6H, t, 2xCH_3), 1.25 (28H, brs, 14XCH_2), 1.36 (4H, m, 2XC$\underline{H_2}$-CH_3), 1.63 (4H, m, C$\underline{H_2}CH_2CO$), 2.71 (4H, t, C$\underline{H_2}$CO), FABMS m/z (rel. int); 338 (M^+] $C_{23}H_{46}O$ (13.6%), 310 (17.0%), 282 (29.9%), 254 (11.8%), 184(9.6%), 128 (43.3%) ,73 (100 %), 43 (78 %).

5-hydroxy pentanoic acid henicosylester (7)

Elution of column with 100% chloroform furnished white amorphous powder of 7 that was recrystallized from ethylacetate. 375 mg, R_f; 0.47 (benzene: ethyl acetate, 1:1), m.p. 58-59 ^{0}C; IR (KBr): 3384, 2916, 2850, 2361, 1730, 1469, 1100, 1045 785 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.88 (3H, t, CH_3), 1.25 (34H, brs, 17XCH_2), 1.49 (2H, m, C$\underline{H_2}CH_2$OH 1.60 (2H, m, C$\underline{H_2}$-CH_3), 1.62 (2H, m, C$\underline{H_2}CH_2COO$), 1.64 (2H, m, $COOCH_2C\underline{H_2}$), 2.03 (1H, s, OH), 2.35 (2H, t, C$\underline{H_2}$COO), 3.53 (2H, m, C$\underline{H_2}$OH), 4.22 (2H, t, COO-C$\underline{H_2}$), FABMS m/z (rel. int); 412 (M^+] $C_{26}H_{52}O_3$ (4.0%), 384 (7.2%), 339 (17.2%), 311 (15.8%), 265 (43%), 133 (13.5%), 73(9.0%), 55 (67.%).

Nonadecanoic acid butyl ester (8)

Elution of column with chloroform-methanol (99:1) furnished colorless crystals of 8 which was recrystallized from ethylacetate. 400 mg, R_f; 0.52 (ethylacetate: chloroform, 1:1), m.p. 32-33 ^{0}C; IR (KBr): 2919, 2851, 2360, 1712, 1468, 1168, 1038 723 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.88 (6H, t, 2xCH_3), 1.25 (28H, brs, 14XCH_2), 1.30 (4H, m, 2XC$\underline{H_2}$-CH_3), 1.57 (2H, m, C$\underline{H_2}CH_2OCO$), 1.62 (2H, m, $OCOCH_2C\underline{H_2}$), 2.21 (2H, t, OCOC$\underline{H_2}$), 4.19 (2H, t, C$\underline{H_2}$OCO), FABMS m/z (rel. int); 354 (M^+] $C_{23}H_{46}O_2$ (3.7%), 261 (9.2%), 237 (10.8%), 134 (11.3%), 73 (81%), 55 (45%).

Determination of antioxidant activity by DPPH- scavenging assay

The free radical scavenging activity of the ethyl acetate extract (EAE) and methanolic extract (ME) of SM seeds and of standard solution (ascorbic acid) were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method as per reported method.[8] The assay mixture contained 2 mL of 1.0 mM DPPH radical solution prepared in methanol and 1 mL of standard or extract solution of different concentrations (10-100 μg/mL). The solution was rapidly mixed and incubated in dark at 37 ^{0}C for 20 min. The decrease in absorbance of each solution was measured at 517 nm using UV/Vis spectrophotomer. Ascorbic acid, a well known antioxidant was used as positive control while DPPH radical solution with 1 mL methanol was taken as blank. The percent (%) radical scavenging was calculated by the following formula:

$$\% \text{ free radical scavenging activity} = \frac{[\underline{A_c} - \underline{A_s}]}{A_c} \times 100$$

Where A_c= Absorbance of control at 517 nm and

A_s= Absorbance of sample

Results and Discussion

Phytochemical investigation of the ethyl acetate extract yielded eight aliphatic carbonyl hydrocarbons. All isolated compounds showed characteristic IR absorption band at 1701-1780 cm^{-1} due to the presence of carbonyl group (C=O) in their structure. Compound **1** and **7** displayed additional absorbance bands in IR

spectrum at 3649 and 3384 cm^{-1} respectively, indicating them to be alcohol. On the basis of FAB mass and ^{1}HNMR spectra, molecular peak of the compounds was determined and molecular formulae were obtained. Identity of compounds was further established by mass fragmentation patterns which showed cluster of peaks and the corresponding peaks of each cluster were 14 (CH_2) mass units apart confirming saturated hydrocarbon nature of the phytoconstituents. Compound no **2** and **8** gave distinct fragments at 87 (100%) and 73 (81%) that are accounted for pentyl and butyl alcohol fragments obtained from the cleavage of ester linkage. Similarly fragmentation pattern of compound **5** and **7** also indicated them to be esters in which the alcohol portion is the predominant portion of the molecule. ^{1}H NMR spectral data further confirmed the presence of long chain of hydrocarbon along with one or more carbonyl groups. A three protons triplet around δ0.9 in all ^{1}H NMR spectra is ascribed to methyl protons that indicates free terminal primary methyl group in all compounds. Compound number **2**, **6** and **9** gave peak for six protons and was due to presence of two terminal primary methyl groups. Signal of methylene protons adjacent to carbonyl group appeared downfield as a triplet. The other methylene protons appeared between δ 1.25- 4.22. On the basis of these spectral studies isolated compounds were characterized and are presented in figure 1.

DPPH free radical scavenging activity is an easy and widely used method for testing in- vitro antioxidant activity of natural compounds or a plant extracts[9]. The scavenging effect of different concentration of EAE and ME of SM seeds on the DPPH free radical was compared with standard antioxidant, Ascorbic acid. The results are expressed as % inhibition and are shown in Table 1. ME showed a dose dependent scavenging activity, and was found to be better than the EAE as well as ascorbic acid. The antioxidant activity of EAE was found to be comparable with ascorbic acid. It was not surprising that ME showed higher scavenging activity at all tested concentrations than the standard ascorbic acid that is due to the presence of silymarin and other flavonoids in the plant material. The free radical scavenging property of SM is therefore, one of the mechanisms by which this plant is effective as a traditional medicine against variety of diseases.

Conclusion

Our results demonstrated that SM seeds have powerful antioxidant activity and therefore it can be concluded that regular consumption of the seeds of SM can be beneficial in preventing free radical related diseases. We also isolated and characterized few long chain hydrocarbon and their derivatives for the first time from the ethyl acetate fraction of SM seeds.

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Figures and Tables

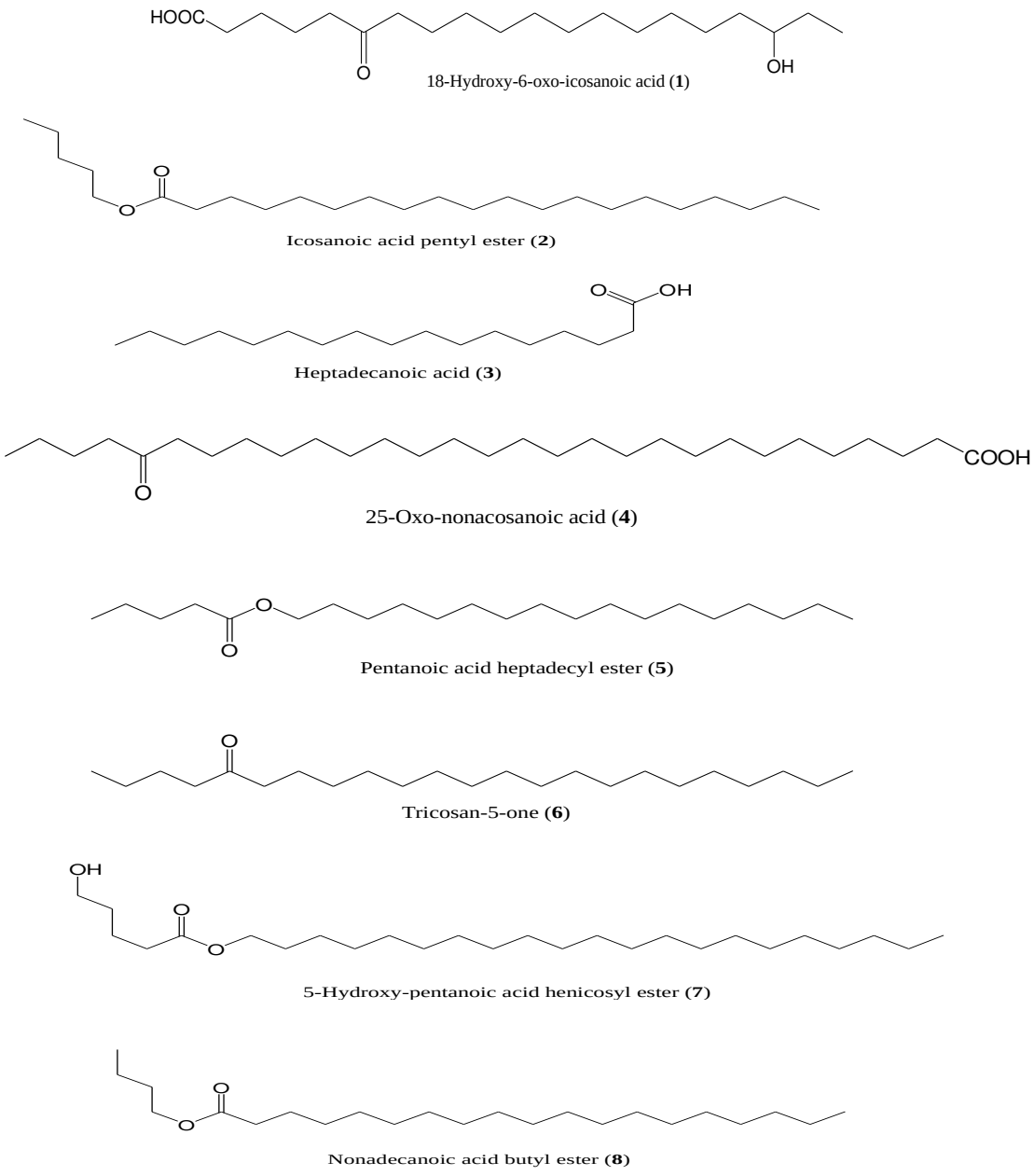

Figure 1: Chemical structure of isolated compounds (1-8) from the seeds of Silybum marianum

Table 1: Percentage inhibition of DPPH free radical by EAE and ME of Silybum marianum/ Ascorbic acid at 517nm

Concentration (µg/mL)	% inhibition of DPPH		
	EAE	ME	Ascorbic acid
10	13.5±1.23	33.1±1.47	21.58±0.12
20	76.34±2.2	95.34±3.25	89.68±0.35
50	85.54±2.9	99.1±1.6	90.71±1.23
100	91.23±.3.1	99.7±.1.3	92.32±2.05

Values are mean ± SD, n=3