Medicinal & Aromatic Plants
ISSN: 2167-0412

Gaweesh et al., Med Aromat Plants 2015, 4:1
DOI: 10.4172/2167-0412.1000180

Research Article Open Access

Phytoconstituents, Cytotoxic, Antioxidant and Hepatoprotective Activities of the Aerial Parts of *Lycium shawii* R. Growing in Egypt

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Abstract

The fruit and decoction of *Lycium shawii* Roem and Schult (Solonaceae) aerial parts are used to prevent from liver damage in folk medicine. In the present study biological activities and phytochemical study of aerial parts of *Lycium shawii* R. Family Solanaceae were evaluated. Methylene chloride fraction exhibits potent inhibition of cell growth against MCF7 cancer cell. Ethyl acetate fraction has the highest radical scavenging activity as determined by DPPH assay. Ethyl acetate fraction showed the highest hepatoprotective activity against CCL_4-induced hepatotoxicity compared to standard silymarin. Phytochemical study of bio-active fractions (Methylene chloride and Ethyl acetate) of *L. Shawii* R. resulted in isolation and structural determination of 5 compounds; 5,9-Heneicosadienoic acid methyl ester, *β*-Sitosterol, Atropine, Trans-Ferulic acid and 5- Hydroxy Ferulic acid.

Keywords: *Lycium shawii*; Cytotoxic; Hepatoprotective; Antioxidant; Phytoconstituents

Introduction

Genus solanum, known as nightshade plant is an economically important genus from which the family name (Solanaceae) was derived. Family Solanaceae consists of approximately 98 genera and 2,700 species [1], with a great diversity of habitats, morphology and ecology. The family is rich in alkaloids, amides, peptides, flavonoids, coumarins, lignans, terpenoids, sterols, and steroids, organic acids and their derivatives, polysaccharides, carotenoids, nutrients, and essential oils [2].

Lycium (Boxthorn) is a genus of family Solanaceae, comprises about 90 species of thorny shrubs [3], they are xerophytic plants distributed worldwide and has its natural range among all temperate and tropical continents, with centers of diversity in southern South America, Southern Africa and Southwestern North America. In Egypt; the genus is represented by three species distributed in the Arabian Desert (east of the Nile),Western desert the Red Sea coastal region, Gebel Elba, surrounding mountains and the South east corner of Egypt at the Sudan frontier [3]. Genus *Lycium* was reported to have antidiabetic [4,5], anticancer [6] and antioxidant [7] activities. In folk medicine, Decoction of aerial parts is used as a laxative and a diuretic and fruit known to cure jaundice and protects liver from damage.

Breast, liver and colorectal cancers were recorded to be the most common cancers in Egypt according to NCI (Egyptian National Cancer Institute) reports. Cytotoxic substances of natural origin have attracted the attention of researches as potential antitumor preparations that promote human health without recognizable side effects. Free radicals contribute to more than one hundred disorder in humans including cancer, hepatitis, atherosclerosis, artheritis and gastritis [8]. Antioxidant supplements are used to reduce oxidative damage from free radicals and active oxygen species. Synthetic antioxidants such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA) and trolox are widely used as antioxidants in the pharmaceutical and food industry. However, they have shown to have toxic and/or mutagenic effects [9], because of their toxicity, the use of natural antioxidants receives a lot of attention nowadays because they are less toxic [10]. Phenolic compounds, mainly tannins and flavonoids, stand out as the major group of natural antioxidants acting as efficient scavengers of free radicals and interrupt oxidative chain reactions [11]. Hepatoprotective effects of various natural compounds were proven to be directly related to their antioxidant activities by prevention of the pro-oxidant effect of bile acids [12] and reduction of lipid peroxidation [13].

Phytochemical studies concerning *Lycium* species had attracted the attention of many authors. The recent surge of interest in the chemistry of the genus resulted in isolation and identification of compounds of various classes of alkaloids, flavonoids, coumarins, sterols, and steroids, organic acids and their derivatives, polysaccharides, carotenoids, nutrients, and essential oils [14].

Reported studies concerning *Lycium shawii* R. were limited to evaluation of the antioxidant activity using DPPH radical scavenging assay [15], thus the present study aimed to evaluate cytotoxic, *in vitro* antioxidant activity and *in-vivo* hepatoprotective activity of the hydro-alcoholic fraction of *Lycium shawii* R., and the fraction thereof and phytochemical studying of bio-active fractions.

Experimental

General

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury, Bruker AM 400 and Jeol Ex-270 instrument with a 300/75 MHz, 400/100 MHz and 500/125 MHz using CDCl3, DMSO or CD3OD solution with tetramethylsilane as internal standard. Mass spectra were determined using a Finnigan FINNIGAN SSQ 7000 mass spectrometer.

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Received November 29, 2014; **Accepted** February 20, 2015; **Published** March 02, 2015

Citation: Gaweesh A, Sengab AENB, El-Hefnawy HM, Osman SM, Abdou AM (2015) Phytoconstituents, Cytotoxic, Antioxidant and Hepatoprotective Activities of the Aerial Parts of *Lycium shawii* R. Growing in Egypt. Med Aromat Plants 4: 180. doi:10.4172/2167-0412.1000180

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Plant material and extraction

Aerial parts (leaves and stem) of *L. shawii* were collected from Suez (Egypt) in March 2010. The taxonomical features were kindly confirmed by Agriculture Research Center, Cairo, Egypt. A voucher specimen (LS#0310) was kept in the Department of Pharmacognosy, Faculty of Pharmacy, October 6 University. The plant material was washed together with tap water, dried in shade at ambient temperature for 8 days (1.6 kg). The examined plant was powdered in an electric mill and then defatted by *n*-hexane (3 L × 3 times). The solvent was filtered and cake was percolated in ethanol 70%. Aqueous ethanol was evaporated under reduced pressure till dryness (160 g). 150 g of hydro-alcoholic extract was suspended in 500 ml double distilled water and sonicated for 30 min. Suspension was fractionated using solvent–solvent extraction with *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol respectively. The fractions were concentrated by rotary evaporator (Sineco Technology Co. Ltd, Shanghai, China) under vacuum till dryness and the yield of fractionion was determined. All the dried fractions were preserved in the refrigerator until further use.

Materials for *in vitro* study of cytotoxic activity

Three cancer cells, were obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection and maintained at Tumor Biology Department, National Cancer Institute, Cairo, Egypt:

- HepG2 (human hepatocellular carcinoma cell line).

- MCF7 (human breast adenocarcinoma cell line).

- HCT116 (human colon tumor cell line).

Animals

Adult mature Wistar strain male albino rats (140 ± 10 g) were purchased from animal house of National Organization for Drug Control and research (NODCAR). Rats were housed at the animal facility of Faculty of Pharmacy, October 6 University, for one week prior to the experimentation and were allowed free access to standard pellet diet and tap water. Animals were housed under suitable laboratory conditions, with 12 h light/dark cycles at an ambient temperature of 22° C ± 2°C and a humidity of 65-70%. The principles of laboratory animal care were followed throughout the duration of experiment and instructions were followed regarding injection and other treatment of experiment.

Materials for Chromatography

Precoated silica plates 60 F254 (20 × 20) was Purchased from E. Merk, Germany, silica for column (60-220) Purchased from E. Merk, Germany, sephadex LH-20 Purchased from Sigma Co., USA and Diaion HP20 Purchased from Sigma Co., USA.

Chemicals

Silymarin was purchased from CID Co., Giza, Egypt. Carbon tetrachloride, (E. Merck (I) Ltd., Bombay), DPPH, Rutin (Sigma-Aldrich Co., USA.), and olive oil was purchased from local market in Cairo, Egypt. All other chemicals and solvents used were purchased from local firms in Egypt and were of the highest purity grade available.

Kits

Biodiagnostic Co., Cairo, Egypt

Cytotoxic activity

Plant fractions were screened for their cytotoxic activity by SRB assay as described by Skehan et al. [16] against HepG2, MCF7, HCT116 cancer cells. The experimental surviving fractions of blank sample (cells without any of tested samples) were found to equal 1 for all of the three cell lines. The relation between the surviving fraction and the different concentrations of the tested samples were plotted to get the survival curve of each tumor cell. The IC_{50} is defined as the concentration sufficient to produce 50% inhibition of the cell growth.

Statistical analysis

The experiment was done in triplicate and the results were given as mean ± standard deviation (SD) (Figure 1).

Results and Discussion (1)

The results revealed that *n*-hexane fraction exhibits potent inhibition of the cell growth of HCT116 cancer cells with IC_{50} value of 7.8 ± 0.197 µg/ml while methylene chloride fraction exhibits potent inhibition of the cell growth of MCF7 cancer cells with IC_{50} value of 11 ± 0.195 µg/ml. *n*-butanol fraction showed IC_{50} value of 9 ± 0.088 µg/ml for HePG2 cancer cells. It seems that sterols interact with the biomemberane by forming a complex with cholesterols. Flavonoids in general are non-selective inhibitors of many enzymes and other proteins. Under normal physiological conditions, the polyphenols dissociate to negatively charged phenolate ions which can interact with positively charged functional groups of different enzymes and proteins in the cell through ionic and hydrogen bonds, which dramatically inhibit their 3D structures and inconsequence their functions [17].

Radical scavenging activity

Plant fractions were screened in-vitro using DPPH radical photometric assay in a process guided by its discoloration in triplicate method described by Carlos et al. [11]. The reaction mixture for different plant fractions consisted of 2 ml of 0.125 mM DPPH-ethanol solution, 1.8 ml of 0.05 M Tris-HCl buffer (PH 7.4) and 0.2 ml of each tested fraction concentration. The absorbance was measured at 516 nm at room temperature immediately after adding the various fraction concentration. Analysis were performed using ethanol to reset the spectrophotometer. Absorbance was compared to a negative control consisting of DPPH hydroalcoholic solution for each fraction or standard. The antioxidant activity was calculated from equation:

$$\%AA=1-(Abs_{sample}-Abs_{blank}/Abs_{negative\ control})\times 100$$

then plotted versus the concentrations of plant fraction or standard. IC_{50} values calculated from the curve for each concentrations of fractions or standard. The IC_{50} is defined as the concentration sufficient to cause 50% decrease of the maximum AA estimated in 100%.

Statistical analysis

All results were expressed as mean ± SD (Figure 2).

Results and Discussion (2)

The results revealed that the ethyl acetate extract exhibits potent scavenging activity with IC_{50} value of 55.4 ± 3.48 µg mL^{-1}. According to De Melo et al. [18], IC_{50} of ethyl acetate is considered a potent antioxidant activity compared with IC_{50} of standard Rutin IC_{50}=24.03 ± 0.198 µg mL^{-1}. Some studies have suggested that fractions or compounds that exhibit activity against the DPPH free radical can be considered as primary antioxidants [19,20].

In vivo hepatoprotective study

Determination of LD_{50}: LD_{50} of aerial parts of *L. shawii* R. was

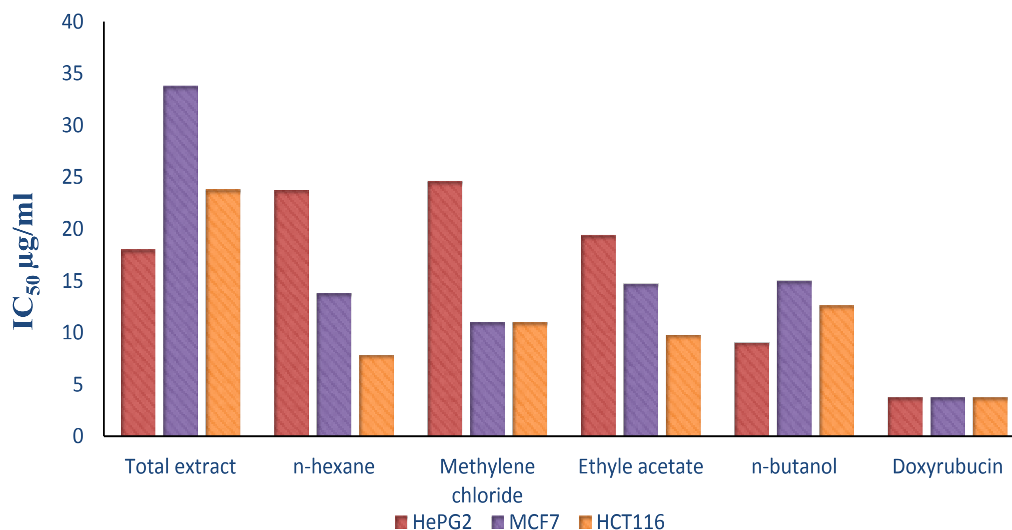

Figure 1: IC_{50} of different fractions of *L. shawii* R., against different cancer cells as determined by SRB assay.

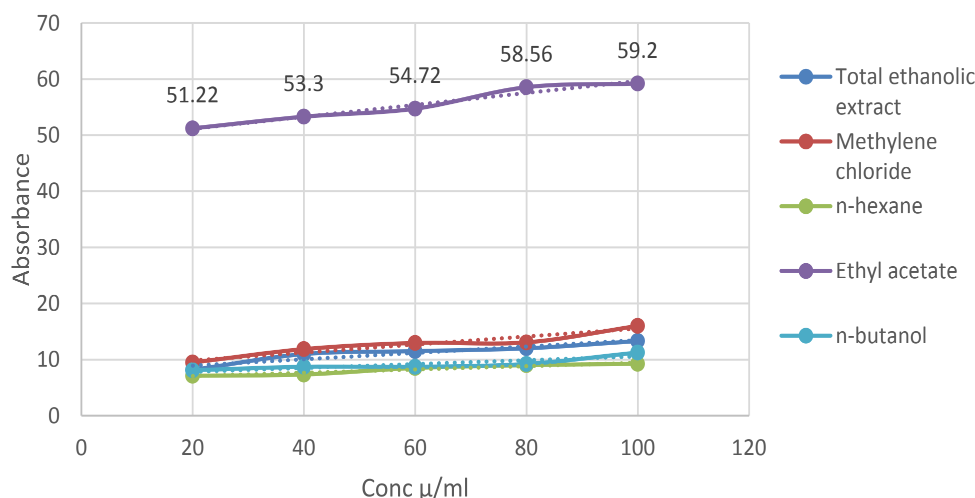

Figure 2: The scavenging capacity of different aerial parts fractions of *L. shawii* R. screened *in vitro* using DPPH radical spectrophotometric assay.

determined according to Kurber [21]. Dose level of 1, 2, 3, 4 and 5 g/kg body weight were chosen as dose level that would be expected to allow the identification of dose producing evident toxicity.

CCl_4-induced hepatotoxicity: The liver injury was induced by CCl_4 according to methods described previously by Shibayama and Yoshitake et al. [22-24]. Liver damage was induced in rats with a 1:1 (v:v) mixture of CCl_4 and olive oil, administered subcutaneously at a dose of 0.5 ml/kg body weight. Rats were divided into 8 groups and treatment schedule was as followed: Normal group and CCl_4 group, rats remain under normal conditions. Silymarin group, rats received 200 mg/kg p.o. once daily of silymarin, other groups received hydro-alcoholic fraction (300 mg/kg, p.o), *n*-hexane (30 mg/kg, p.o), methylene chloride (45 mg/kg, p.o), ethyl acetate (15 mg/kg, p.o) and *n*-butanol (15 mg/kg, p.o), respectively according to their percentage of fractionation for 7 days before the injection of CCl_4. At the 8th day all groups except normal group were subjected to hepatotoxicity by CCl_4.

Assay of hepatoprotective activity: All animals were anaesthetized using ether, then blood was withdrawn from the retro orbital plexus after 24 h from CCl_4 injection, centrifuged at 3000 rpm at 4°C for 10 min and the serum was thus separated. Various biochemical parameters i.e., aspartate amino transferase (AST), alanine amino transferase (ALT) were estimated using kits according to the methods described by Reitman and Frankel [25].

Statistical analysis

Values were expressed as mean ± SD (n=7-9). Statistical difference between groups were computed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. The level of significance was accepted at $p<0.05$.

Results and Discussion (3)

The pretreatment with ethyl acetate extract (15 mg/kg, po, 7 days) resulted in significant decrease in serum levels of AST and ALT compared to levels of CCL4-pretreated group. The potent

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hepatoprotective activity of ethyl acetate fraction of the aerial parts of *L. shawii* R. may be attributed to its high content of phenolic compounds [15]. Hepatoprotective effects of various natural compounds were proved to be related to their antioxidant activities [13,26] (Table 1).

Histological investigation

Parts of the isolated hepatic tissue were fixed in 10% formalin solution and then dehydrated in ascending grades of alcohol and embedded in paraffin. Four micron-thickness sections were taken, stained with hematoxylin and eosin solutions and examined under light microscope. The specimens were examined blindly by a single pathologist without knowledge of the groups [27] (Figure 3).

Phytochemical study

The phytochemical study was carried out for the different aerial parts fractions using the standard procedures to qualify the components. The phytochemical screening was carried out for the components such as volatile substance Wagner et al. carbohydrates [28], proteins and amino acid [28,29], saponins, alkaloids [28], flavonoids [30], anthraquinones, cardiac glycosides [30], sterols, terpenoids, triterpenoids [28], coumarins [29], tannins [31] and cyanogens [32].

Phytochemical screening

Extraction and phytochemical screening of different aerial parts of *L. shawii* R. yielded 165 g total hydroalcoholic extract. Fractionation of hydroalcoholic extract yielded 10 %, 15 %, 5 %, and 5%for *n*-hexane fraction (HF-LSFE), methylene chloride fraction (MeF-LSFE), ethyl acetate fraction (EF-LSFE) and *n*-butanol fraction (BF-LSFE), respectively. The results of phytochemical screening for the different solvents fractions of *L. shawii* showed that carbohydrates have been detected in all the fractions of *L. shawii*. The phytosterols, terpenes and Coumarins were present in *n*-hexane and methylene chloride fractions. Alkaloids were present in methylene chlorideand *n*-butanol fractions. Cardiac glycosides, anthraquinones and saponins were absent in all plant fractions. Preliminary phytochemical screening revealed that flavonoids and tannins were present in ethyl acetate and *n*-butanol fractions with different ratios which were reported to possess antioxidant and hepatoprotective activity [33].

Treatment	AST (U/L)	ALT (U/L)
Normal	45.3 ± 7.06	32.7 ± 4.61
Negative control (CCl_4)	$326.8 \pm 7.2^{5*}$	$186.6 \pm 6.^{1*}$
Positive control (Silymarin)	$91.7 \pm 4.^{34*}\#$	$46.87 \pm 7.01^{*}\#$
Total ethanolic extract	$116.9 \pm 4.8^{2*}\#$	$76.93 \pm 7.^{7*}\#$
n-hexane	$135.7 \pm 3.8^{6*}\#$	$97.97 \pm 8.76^{6*}\#$
Methylene chloride	$107.8 \pm 5.6^{7*}\#$	$67.13 \pm 7.08^{3*}\#$
Ethyl acetate	$80.95 \pm 6.5^{1*\#}@$	$28.23 \pm 5.07^{8*\#}@$
n-Butanol	$122.4 \pm 14.0^{2*}\#$	$72 \pm 7.0^{6*}\#$

*Significant different from normal group, #Significant different from CCl_4 group, @Significant different from Silymarin group.

Table 1: Effect of different fractions of *L. shawii* aerial parts on serum biochemical parameters in CCl_4-induced hepatotoxicity rats (values are mean ± SD of 7 rats).

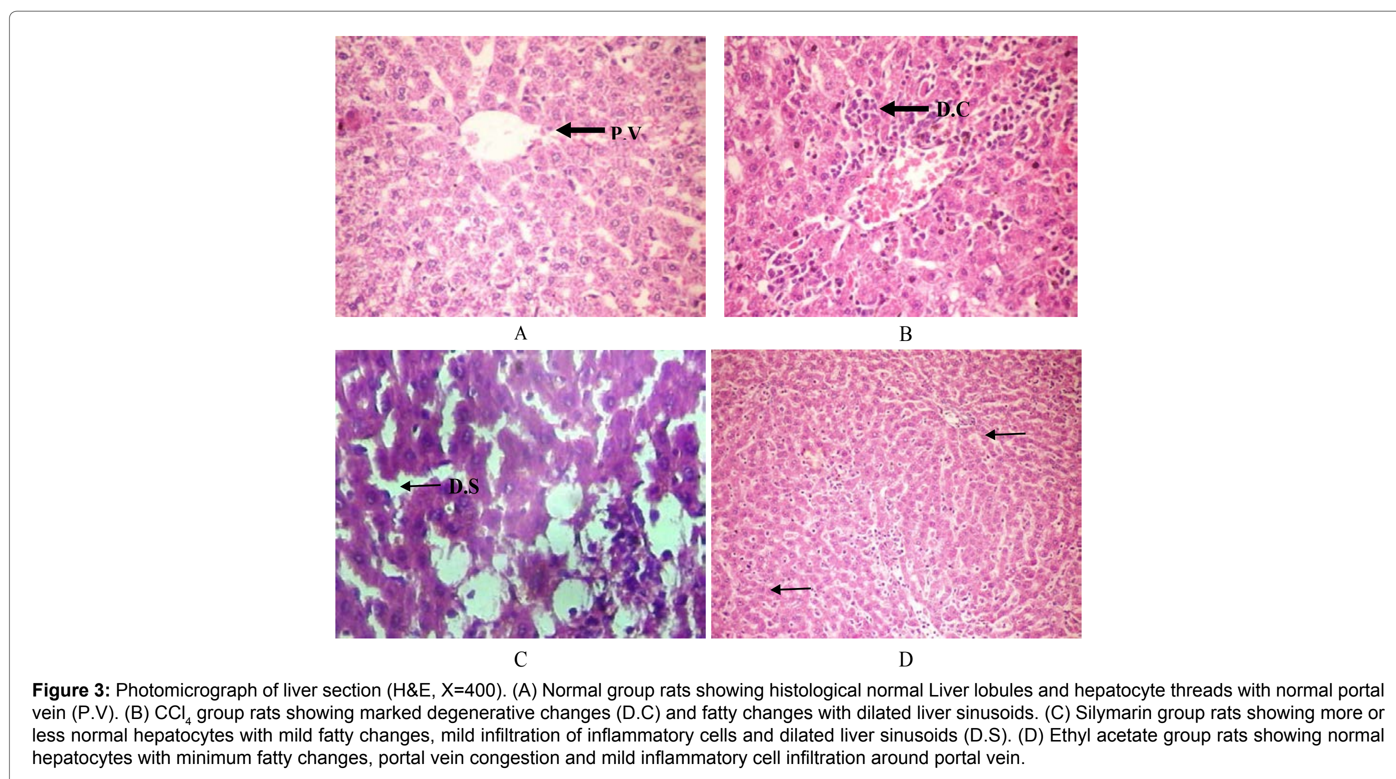

Figure 3: Photomicrograph of liver section (H&E, X=400). (A) Normal group rats showing histological normal Liver lobules and hepatocyte threads with normal portal vein (P.V). (B) CCl_4 group rats showing marked degenerative changes (D.C) and fatty changes with dilated liver sinusoids. (C) Silymarin group rats showing more or less normal hepatocytes with mild fatty changes, mild infiltration of inflammatory cells and dilated liver sinusoids (D.S). (D) Ethyl acetate group rats showing normal hepatocytes with minimum fatty changes, portal vein congestion and mild inflammatory cell infiltration around portal vein.

Thin layer chromatography

Detection of steriods, terpenoids, cardiac glycosides, cyanogens, saponins, flavonoids, tannins and coumarins was carried according to methods described by Harborne and Wagner et al. [34,35].

Isolation of major components of methylene chloride fraction

15 g of methylene chloride fraction were chromatographed on silica gel (60-220) column (5 × 80 cm, 600 g), gradient elution was used starting with 100% *n*-hexane with increasing the polarity by methylene chloride at a rate of 25% increase till 100% methylene chloride, then the polarity increased by MeOH in a rate of 10% till 100% MeOH. Fractions (250 ml each) were collected, monitored and similar fractions were pooled together to yield seven collective fractions. Fractions were evaporated till dryness under reduced pressure and weighted.

Fraction I (0.46 g), eluted with *n*-hexane, showed one major spot with blue colour under UV_{356} nm that becomes brown after spraying with p-anisaldehyde reagent, indicating phytosterol nature. The fraction was purified on Sephadex LH-20 using methylene chloride 100% to yield compound 1.

Fraction II (1.5 g), eluted with 25:75 hexane/methylene chloride showed 1 major spot. This fraction was purified by re-chromatography on silica gel column (2 × 40 cm, 60 g) using 50:50 hexane/methylene chloride for elution to yield compound compound 2.

Fraction IV (2.5 g), eluted with methylene chloride showed 1 major spot. The fraction was chromatographed on diaion HP-20 column (3 × 60 cm, 100 g). The column was washed with 0.5 liters of distilled water, then eluted with MeOH and acetone, respectively. Subfractions were evaporated under reduced pressure and weighed. Subfraction II (MeOH) was eluted with 0.5 liter 0.5 M NH_4OH to yield 3 subfractions (150 ml for each fraction). Subfractions were evaporated till dryness and weighed. Subfraction II (0.3 g) was purified by re-chromatography on sephadex LH-20 column (1 × 10 cm, 15 g) using 50% MeOH for elution to yield compound 3.

Fraction V (1.4 g), eluted with 90:10 CH_2Cl_2: MeOH showed 3 spots. The spot at R_f 0.4 appeared major with blue colour under UV365 nm. Half gram of this fraction was re-chromatographed using MPLC column (2 × 10 cm, 20 gm) and eluted with 8:2 hexane/ethyl acetate to yield compound 4.

Isolation of major components of ethyl acetate fraction

8 g of ethyl acetate fraction was chromatographed on silica gel column (3 × 80 cm, 400 g). The elution process started with methylene chloride with increasing the polarity by MeOH in a rate of 10% till reaching 100% methanol as an eluent. Finally MeOH: NH_3 (10%) to ensure complete elution. Fractions (100 ml each) were collected, monitored and similar fractions were pooled together. Each fraction was evaporated till dryness under reduced pressure and weighted.

Fraction III (2 g), eluted with methylene chloride/MeOH (50:50) was further chromatographed over silica gel column (2 × 60 cm, 80

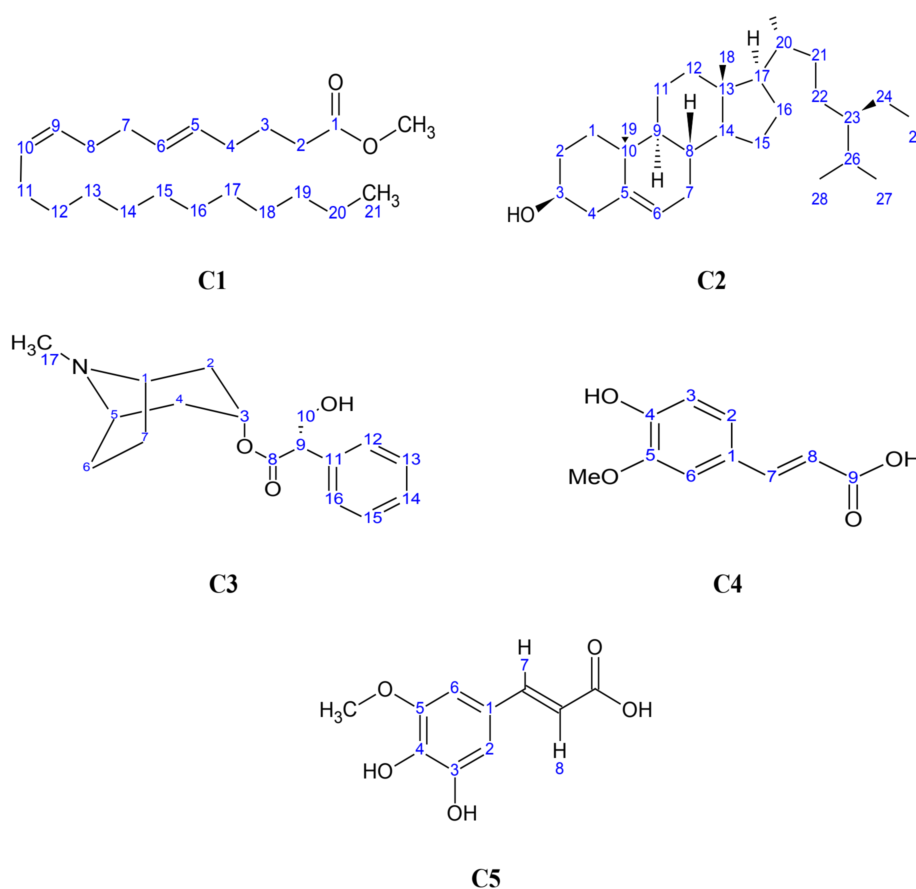

Figure 4: Structures of compounds C1-C5.

g) with methylene chloride: MeOH (50:50 v/v). Subfractions (25 ml of each) were collected and monitored by TLC and similar fraction pooled together.

Sub-fractionII (0.52 g) exhibits two major spots on TLC under short UV. Further purification was carried on sephadex LH-20 (1 × 30 cm, 25 g) eluted with MeOH: H_2O (50:50) v/v to yield compound 5.

Characterization of Isolated Compounds

The isolated compounds were elucidated by, ^{1}H-NMR, ^{13}C-NMR, The analytical data were as follow:

5,9-Heneicosadienoic acid methyl ester (C_1)

^{1}H-NMR (DMSO, 300 MHz) δ 5.33 (2H, m, H_5, H_6, H_9, H_{10}), 3.57 (3H, s, OCH_3), 2.28 (2H, t, *J*=6, 9 Hz, H_2), 2.01 (2H, d, *J*=3 Hz, H_{16}, H_{19}), 1.5 (2H, t, 6, 9Hz, H_3), 1.2-1.4 (22H, m), 0.85 (3H, t, *J*=6, 6 Hz, CH_3); ^{13}C-NMR (DMSO, 75 MHz) δ 173 (CO), 130 (C6), 128 (C10), 121 (C5,9), 51 (OCH_3), 21-31 (11C), 14 (CH_3).

β-Sitosterol (C_2)

^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.36 (1H, t, *J*=6 Hz, H_5, H_6), 3.53 (1H, m, H_3), 1.03 (3H, s, H_{19}), 0.93 (3H, d, *J*=6 Hz), 0.87 (3H, t, *J*=6, 9 Hz, H_{27}), 0.83 (3H, d, *J*=9 Hz, H_{28}), 0.79 (3H, d, *J*=9 Hz, H_{25}), 0.68 (3H, s, H_{18}); MS m/z (% relative intensity): 414 [M+] (97.13%), 396 (53.2%), 381 (39%), 329 (56.2%), 303 (57.4%), 255 (55.6%), 213 (60.4%), 187 (26.1%), 173 (32.8%), 145 (89.2%), 133 (74.2%), 119 (53.7%), 107 (100%).

Atropine (C_3)

^{1}H-NMR (DMSO, 400 MHz) δ 7.7 (4H, m, H_{12}, H_{13}, H_{14}, H_{15}, H_{16}), 5.41 (1H, m, H_3), 4.1 (2H, q, *J*=3.2, 4 Hz, H_{10a}, H_{9a}), 3.5 (2H, d, *J*=8 Hz, H_{10b}, H_{9b}), 3.13 (2H, m, H_1, H_5), 2.66 (s, N-CH_3), 2.33 (2H, d, *J*=16 Hz, H_2, H_4), 2.01 (2H, t, *J*=8 Hz, H_{7b}, H_{6b}), 1.63 (2H, d, *J*=8 Hz, H7a, H6a).

Trans-ferulic acid (C_4)

^{1}H-NMR (CD_3OD, 400 MHz) δ 7.53 (1H, d, *J*=15.88 Hz, H_7), 7.19 (1H, d, *J*=1.93, H_6), 7.11 (1H, dd, *J*=1.93, 8.23 Hz, H_2), 6.8 (1H, d, *J*=8.23 Hz, H_3), 6.5 (1H, d, *J*=15.88 Hz, H_8), 3.95 (3H, s, OCH_3); ^{13}C-NMR (CD_3OD, 100 MHz) δ 174 (CO), 149 (C-5), 147 (C4), 141 (C*β*), 125 (C-1), 122 (C2), 118 (C3), 115 (Cα), 111 (C-6), 56.45 (OCH_3).

5-Hydroxy ferulic acid (C_5)

^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.61(1H, d, *J*=16 Hz, H_7), 6.92 (1H, s, H_2) 6.85 (1H, s, H_6), 6.29 (1H, d, *J*=16 Hz, H_8), 3.91 (3H, s, OCH_3); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 171 (CO), 147.39 (C3), 144.00 (C7), 139 (C5), 134 (C4), 126 (C1), 116 (C8), 108 (C6), 104 (C2), 56.52 (OCH_3) (Figure 4).

Conclusion

n-hexane and methylene chloride fraction of *L. shawii* R. aerial parts possessed a potent cytotoxic activity against HCT116 and MCF7 cancer cells respectively as compared to Doxorubucin, while ethyle acetate fraction showed the most potent antioxidant activity as compared to standard Rutin, in addition to the potent hepatoprotective activity against CCl_4-induced hepatotoxicity, compared to standard silymarin. The chromatographic study of the bioactive methylene chloride fraction resulted in isolation and identification of 4 compounds: 5,9-Heneicosdienoic acid methyl ester, *β*-Sitosterol, Atropine and trans-Ferulic acid. Chromatographic study of the bioactive ethyl acetate fraction resulted in isolation and identification of 1 compound: 5-hydroxy Ferulic acid.

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