

## Full Length Research Paper

# Phytochemical and biological analyses of *Citharexylum spinosum*

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The phytochemical screening of *Citharexylum spinosum* L. aerial parts resulted in the presence of flavonoids, tannins, carbohydrates and/or glycosides, triterpenes and/or sterols and saponins. The percentage of hydrocarbons and sterols in *C. spinosum* petroleum ether extract were 99.57 and 0.3%, respectively. In petroleum ether extract, saturated fatty acids (78.76%) and unsaturated fatty acids (9.14%) were found. Chromatographic fractionation of 80% aqueous, methanol and chloroform extracts of *C. spinosum* resulted in isolation of 10 compounds;  $\beta$ -Sitosterol,  $\beta$ -Sitosterol 3-O- $\beta$ -D-glucopyranoside, Oleanolic acid, Gallic acid, Quercetin, 6-Methoxy acacetin 7-O- $\beta$ -D-glucopyranoside, Naringenin, Quercetin 3-O- $\alpha$ -L-rhamnopyranoside (Quercetrin), 1, 2, 6-tri-O-galloyl- $\beta$ -D-glucopyranoside and Rutin. The antipyretic activity of aqueous methanolic residue using Brewer's yeast-induced pyrexia in rats was significant at dose 300 mg/kg. All tested samples had no analgesic activity. The major isolated compounds were quercetin and quercetrin, their biological activities, antimicrobial and cytotoxic activities, were determined parallel to the extracts. It was found that the aqueous methanolic residue, chloroform extract, quercetin and quercetrin exerted significant antimicrobial activity. From 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay on A2780 human ovarian cell line, quercetrin showed moderate cytotoxic activity, whereas quercetin showed significant cytotoxic activity.

**Key words:** *Citharexylum spinosum*, lipoidal matter, phenolics, antipyretic, antimicrobial.

## INTRODUCTION

Family Verbenaceae includes about 100 genera and more than 3000 species. Among the largest genera of Verbenaceae is *Citharexylum* which comprises 115 species (Dahiya, 1979; Starr et al., 2006; Mohammed et al., 2014). Genus *Citharexylum* was reported to contain triterpenes, sterols, irridoids, lignan glycoside, phenolic and flavonoids.

Different species of genus *Citharexylum* are famous to have antiulcer, antihypertensive, hepatoprotective effects, immunomodulatory, antimicrobial, anti-Schistosomal, antioxidant, nephroprotective, radical scavenging, cytotoxic activities and regulating immediate type of allergic reaction (Khalifa et al., 2002; Ganapaty et al., 2010; Khan and Siddique, 2012; Kadry et al., 2013;

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Allam, 2014; Mohammed et al., 2016). Among these species is *Citharexylum spinosum* L. which is a popular ornamental tree in many tropical and subtropical regions and are known as fiddlewood. It has been used in folk medicine as diuretic, antipyretic, antiarthritic and in liver disorders (Lawrence, 1951; Turner and Wasson, 1997; Wagner et al., 1999; Starr et al., 2006).

## MATERIALS AND METHODS

### Plant material

Aerial parts (leaves and stems) of *C. spinosum* L. were collected from Zoo garden, Giza, Egypt in January, 2014. The plant was identified by Mrs. Terase Labib, senior specialist of plant taxonomy, floral and taxonomy department, El-Orman garden, Giza, Egypt. Voucher specimens are kept in the herbarium of Pharmacognosy Department, Faculty of pharmacy, Helwan University, Cairo, Egypt.

### Cell line, micro-organisms, animals, chemicals, standard materials, media and drugs

The human ovarian cell line, RPMI-1640 media was supplemented with 10% heat inactivated foetal bovine serum (FBS), L-glutamine and 5% penicillin + streptomycin, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Paracetamol, Saline (0.9%NaCl) and 20% aqueous suspension of Brewer's yeast in normal saline. All chemicals were from Sigma/Aldrich, USA.

Multidrug-resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were selected among clinical isolates obtained from Outpatient Clinics of the Research Institute of Ophthalmology (RIO) while Imipenem and Ciprofloxacin discs were purchased from Oxoid, England. Adult albino mice weighing 25 to 30 g and rats weighing 120-130 g of either sex were used in the present study. All animals were kept in a controlled environment of air and temperature with access to water and diet *ad libitum*. Anesthetic procedures and animal handling were in compliance with the ethical guidelines of Medical Ethics Committee of the National Research Centre; Polyamide S6 (50-160 µm, Fluka chemie AG, Switzerland) for column chromatography, Microcrystalline cellulose (E. Merck, Darmstadt, Germany) for column chromatography, Sephadex LH-20 (25-100µm, Pharmacia, Uppsala, Sweden) for column chromatography, Silica gel 60 F<sub>254</sub>, precoated aluminium sheets (20 x 20, 0.2mm thickness), (E. Merck, Darmstadt, Germany) for thin layer chromatography, Silica gel G 60 for column chromatography (70-230 mesh, 60 Å, E. Merck, Germany) and Whatman No.1 for paper chromatography (Whatman Ltd., Maidstone, Kent, England). Spraying reagents were done according to common methods (Smith, 1960; Stahl, 1969; Balbaa et al., 1981; Markham, 1982).

### NMR spectrometers

<sup>1</sup>H and <sup>13</sup>C NMR spectra (University of Louisiana at Monroe) were recorded at 400 and 100 MHz, respectively, in appropriate deuterated NMR solvent, on a JEOL Eclipse ECS-400 NMR spectrometer (Boston, MA, USA). For analysis and spectral processing, chemical shifts reported δ ppm values relative to TMS using Delta™ NMR Data Processing Software (JEOL Inc, MA, USA).

HP 5890 series Gas Chromatograph System with an FID/MS detector, Faculty of Agriculture, Cairo University was used for lipoidal matters analysis. We used UV lamp (Marne La Vallee, VL-215 LC, France) for visualization of spots on paper and thin layer

chromatograms to follow up the columns fractionation on columns at 254 and/or 365 nm. Hot plate (Harvard Apparatus, Kent, UK), sterile pipettes and 96 well cell culture microplate were used for pharmacological studies.

### Preliminary phytochemical screening

Air dried powdered aerial parts (leaves and stems) of *C. spinosum* L. was subjected to preliminary phytochemical screening for its constituents, according to methods mentioned in the references of Trease and Evans (1989), Evans (1996) and the British Pharmacopea (1993).

### Preparation and fractionation of lipoidal matter of *C. spinosum* L. aerial parts

The air-dried powder of *C. spinosum* L. aerial parts (90 g) was extracted with petroleum ether (b.p. 60 to 80°C) and evaporated to give residue (3 g). This residue was kept for the preparation of unsaponifiable matters (USM) and total fatty acids (TFA) according to previous studies (El-Said and Amer, 1965; British Pharmacopea, 1993). TFA and USM of *C. spinosum* L. aerial parts were subjected to methylation followed by GC-MS analysis. Tentative identification was carried out by comparison of their R<sub>f</sub>-values. The relative concentration of each constituent was calculated based on the peak area integration (Vogel, 1961).

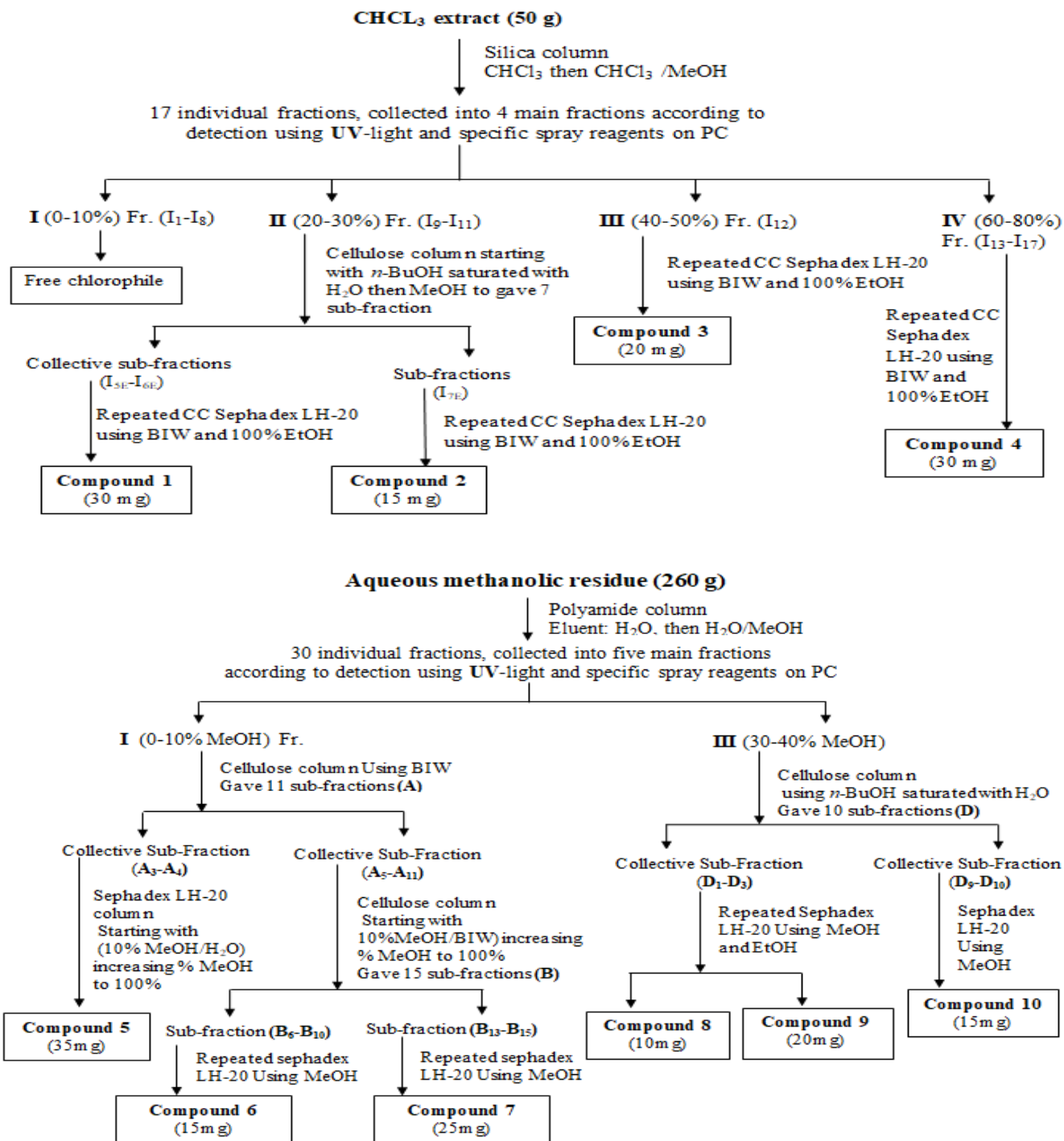
### Extraction and purification of active constituents from *C. spinosum* L. aerial parts

The air-dried ground aerial parts (1350 g) of *C. spinosum* L. were subjected to exhaustive extraction with hot 80% aqueous methanol under reflux (50°C). The extract was dried under vacuum (50°C) to give dry total extract (360 g). This dry extract was defatted by petroleum ether which resulted in 20 g of dried petroleum ether residue, and 330 g of the remaining residue was successively extracted with chloroform, under reflux at 50°C to yield 50 g of chloroform extract, 2 g of ethyl acetate extract, 10 g of *n*-butanol extract and 260 g of remaining aqueous methanolic residue.

The 2D-PC and TLC revealed that, ethyl acetate and *n*-butanol extracts had limited constituents, while concentrated in aqueous methanolic residue and chloroform extract. Fractionation, isolation and purification were performed as illustrated in Figure 1. Paper chromatography (PC) according to Mabry et al. (1970), column chromatography and thin layer chromatography (TLC) according to Stahl (1969), GC-MS conditions for unsaponifiable matters analysis and GC - MS conditions for fatty acid methyl esters analysis were performed according to Vogel (1961), mild and complete acid hydrolysis were done according to the methods described by Harborne (1984).

### Cell culture and MTT cell proliferation assay

A human ovarian cell line A2780 was incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>, 95% air and 100% relative humidity, to maintain continuous logarithmic growth. RPMI-1640 media was supplemented with 10% heat inactivated Foetal Bovine Serum (FBS), L-glutamine and 5% penicillin + streptomycin. Cells were checked for Mycoplasma, by measuring the bio-luminescence (Myc Alert sample detection kit; Lonza, Switzerland), using a multiplate reader (Synergy HT, BioTek, USA). The MTT *in vitro* cell viability colorimetric assay was used for measuring cellular proliferation, inhibitory activity and cytotoxicity of the plant samples.



**Figure 1.** Flow charts of fractionation and purification of compounds isolated from CHCl<sub>3</sub> extract and aqueous methanolic residue of *C. spinosum* aerial parts.

The colour of MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide is yellow (tetrazole), which changed to purple (reduced to formazan). When mitochondrial dehydrogenase enzymes are active therefore, reduction indicates cell viability which can be measured as optical density (OD). Cells were incubated at 37°C overnight. Final concentrations of each sample (in DMSO was filtered with Nylon 0.22 µm x 25 mm) in wells were 1, 10, 25, 50

and 100 µg/ml in 200 µl of media (DMSO 0.1%). 20 µl medium was added to each control well, and incubated for 48 h. Each concentration was tested in triplicates (n=3). MTT was added into each well. Plates were incubated for 3 h, supernatant was aspirated, and 100 µl of DMSO was added to each well. Plates were shaken for 5 min at 26°C using STUART scientific orbital shaker (Redhill, Surrey, UK) and absorbance was read on multi-

plate reader (Synergy HT, BioTek, USA). The OD of the purple formazan A<sub>570</sub> is proportional to the number of viable cells.

When the amount of formazan produced by treated cells is compared with the amount of formazan produced by untreated control cells, the strength of the drug in causing growth inhibition can be determined. Through plotting growth curves of absorbance against sample(s) concentration, thus formulation concentration causing 50% inhibition (IC<sub>50</sub>) compared to control cell growth (100%) were determined (Hansen et al., 1989). GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com) was used for analysis.

#### Determination of LD<sub>50</sub>

The alcoholic sample was dissolved in distilled water then given orally to adult albino mice in graded doses up to 4 g/kg (the maximum given dose) and the control group received the same volume of the vehicle. The percentage mortality for samples as well as the general behavior of the animal was recorded 24 h later (Armitage, 1971).

#### Estimation of analgesic activity using hot Plate Test

Two doses of 100 and 300 mg/kg body weight for chloroform and methanolic extract each and 50 mg/kg paracetamol (as standard) was administered orally to adult albino mice weighing 25 to 30 g of either sex using 25-gauge needle (Farshchi et al., 2009). Tested animal was placed on a hot plate with fixed temperature 55±0.5°C (Harvard Apparatus Ltd., Kent, UK), till the appearance of withdrawal response in terms of hind paw licking, biting or jumped off. A cut-off time to remove mouse from the plate of 30 seconds was used to minimize the tissue damage (Pini et al., 1997; Lavich et al., 2005; National committee for clinical laboratory standard (NCCLS), 1997).

#### Estimation of antipyretic activity

Aqueous methanolic residue and chloroform extract of *C. spinosum* L. aerial parts were used to evaluate their antipyretic activity using Brewer's yeast-induced pyrexia in rats as described by, Loux et al. (1972).

Fever was induced by injecting 20 ml/kg of 20% aqueous suspension of Brewer's yeast in normal saline subcutaneously. Temperature across rectum (using thermal probe Eliab thermistor thermometer) was recorded after 18 h and served as base line of elevated body temperature. The extracts samples (100 and 300 mg/kg) was administered orally, using paracetamol (50 mg/kg, orally) as reference. Control group received distilled water. Rectal temperature was determined at 1 and 2 h after test samples/reference drug administration.

#### Preparation of the plant samples for antimicrobial evaluation

The antimicrobial activity of the aqueous methanolic residue, chloroform extract, compounds 5 and 8 obtained from *C. spinosum* L. aerial parts were evaluated using the agar well diffusion method as described by Rahbar and Diba (2010). All samples were dissolved in 0.5 ml methanol. A loopful of the tested organisms was inoculated into 5.0 ml of nutrient broth and incubated at 37°C for 24 h.

50 µl of 24 h culture organism was dispensed into 5 ml broth and incubated for 2 h to standardize the culture to 10<sup>6</sup> cfu/ml. Cotton swab was immersed into standardized culture to be spread onto the

surface of, the agar plate. Sterilized 6 mm cork borer was used to punch 5 wells for the extracts. From each of the 4 extract samples, 100 µl was dispensed into the corresponding 4 wells while the fifth was used for negative control (methanol). To allow diffusion of the tested extract samples, the plates were left at room temperature for at least 1 h. Two discs of antibiotic (imipenem and ciprofloxacin) were placed as positive control. These plates were incubated at 37°C for 18 to 24 h. Zones of inhibition surrounding the wells and discs were measured to evaluate their antimicrobial activity.

## RESULTS

### Preliminary phytochemical screening, hydrocarbon, sterol and fatty acid contents in *C. spinosum*

Phytochemical screening as preliminary tests of aerial parts of *C. spinosum* revealed the presence of carbohydrate and/or glycosides, tannins, flavonoids, irridoids, unsaturated sterols and/or triterpenes, saponins and the absence of anthraquinones, volatiles, coumarins, and alkaloids or compound containing nitrogenous bases. Identification of hydrocarbons and sterols content of USM fraction was carried out by GC-MS; the conditions were adopted as mentioned. Tentative identification of hydrocarbons and sterols was carried out by, comparison of their retention times.

Quantitation was based on peak area integration. The results of USM analysis for *C. spinosum* L. are compiled in Table 1 and Figure 2. It was found that, hydrocarbons represented a higher percentage (99.57%) than that of sterols (0.30%). 6-Phenyldodecane (10.03%) and 5-Phenyldodecane (9.96%) represented the major hydrocarbons while β-Sitosterol (0.30%) represented the only sterol identified. It could be concluded that, the saturated fatty acids (78.76%) represented a higher percentage than that of unsaturated ones (9.41%). 14-methyl Pentadecanoic acid (34.8%) and Hexadecanoic acid (25.1 %) represented the major identified saturated fatty acids while 9-Octadecanoic acid (2.62 %) represented the major unsaturated fatty acid, Table 2 and Figure 3.

### Characterization and identification of isolated compounds

Air dried powdered aerial parts of the plant under investigation (1350 g) was subjected to exhaustive extraction with 80% MeOH under reflux. After drying the extract under reduced pressure, the residue was defatted by petroleum ether and the remaining residue was fractionated by chloroform, ethyl acetate and *n*-butanol under reflux (50°C), respectively. The 2D-PC analysis proved that active constituents are concentrated in the chloroform extract and aqueous methanolic residue when compared to ethyl acetate and *n*-butanol extracts.

Aqueous methanolic residue, and chloroform extract were subjected to fractionation according to the illustrated

**Table 1.** GC-MS analysis of USM of *C. spinosum* L.

Identified compound	RRT*	Percentage area
5-Phenyl decane	0.8	1.75
4-Phenyl decane	0.814	1.33
3-Phenyl decane	0.834	0.95
2-Phenyl decane	0.872	1.23
6-Phenyl undecane	0.90	3.86
5-Phenyl undecane	0.907	9.78
4-Phenyl undecane	0.917	6.84
3-Phenyl undecane	0.937	4.27
5-Phenyl dodecane	0.966	0.05
2-Phenyl undecane	0.975	7.07
p-Didecyl benzein	0.988	0.07
6-Phenyl dodecane	1	10.03
5-Phenyl dodecane	1.005	9.96
4-Phenyl dodecane	1.02	6.50
3-Phenyl dodecane	1.04	4.54
1-Nonadecene	1.057	0.04
2-Phenyl dodecane	1.07	7.53
6-Phenyl tridecane	1.09	8.57
5-Phenyl tridecane	1.1	5.08
4-Phenyl tridecane	1.11	3.56
3-Phenyl tridecane	1.13	2.47
2-Phenyl tridecane	1.16	4.24
$\beta$ – Sitosterol	1.19	0.30
Total hydrocarbon		99.57
Total sterols		0.30
Total identified Compounds		99.57
Unidentified compounds		0.13

RRT\*: Relative retention time of 6 - Phenyl dodecane with RT = 24.53 min.

Figure 1. Identification of isolated compounds are based on chemical and physical methods including  $^1\text{H}/^{13}\text{C}$  NMR and HMBC. Based on these data and by comparison with reported literature data (Haddock et al., 1982; Barakat et al., 1987; Agrawal and Bansal, 1989; Mahmoud et al., 2001; Seebacher et al., 2003; Shalaby and Bahgat, 2003; Marzouk et al., 2004; Aboutabl et al., 2008; Rahmana et al., 2009; Ahmad et al., 2010; Kamal et al., 2012; Onoja and Ndukwe, 2013; Haggag et al., 2013; Allam, 2014; Khan and Hossain, 2015; Mohammed et al., 2016) and authentic samples, the compounds identified were ten; 1;  $\beta$ - Sitosterol, 3; Oleanolic acid and 4; Gallic acid were isolated once before from genus *Citharexylum*, while 2;  $\beta$ -Sitosterol 3-O-  $\beta$  -D-glucopyranoside 5; Quercetin , 6; 6-Methoxy acacetin 7-O- $\beta$ -D-glucopyranoside, 7; Naringenin, 8; Quercetin 3-O- $\alpha$ -L-rhamnopyranoside (Quercetrin), 9; 1, 2, 6-tri-O-galloyl- $\beta$ -D-glucopyranoside, 10; Rutin were isolated for the first time from genus *Citharexylum* (Figure 4). Two major compounds (5 and 8) subjected to biological activities, their spectral data are summarized as follow:

#### Compound 5

Is a yellow amorphous powder (20 mg), with chromatographic properties:  $R_f$  values; 0.6 ( $S_1$ ), 0.4 ( $S_2$ ); brilliant yellow fluorescent spot by UV- light. It gave pale green color and orange fluorescence with  $\text{FeCl}_3$  and Naturstoff spray reagents, respectively.  $^1\text{H-NMR}$  (400 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  ppm 7.71 (1H, d,  $J=2.0$  Hz, H-2'), 7.62 (1H, dd,  $J=8.3, 2.0$  Hz, H-6'), 6.86 (1H, d,  $J=8.3$  Hz, H-5'), 6.36 (1H, d,  $J=1.8$ Hz, H-8), 6.15 (1H, d,  $J=1.8$ Hz, H-6).  $^{13}\text{C-NMR}$  PENDANT (100 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  ppm 175.99 (C-4), 164.23 (C-7), 161.17 (C-5), 156.86 (C-9), 147.34 (C-2), 146.62 (C-4'), 144.88 (C-3'), 135.90 (C-3), 122.79 (C-1'), 120.29 (C-6'), 114.85 (C-2'), 114.48 (C-5'), 103.17 (C-10), 97.85 (C-6), 93.02 (C-8).

#### Compound 8

Is an orange amorphous powder (16 mg), with chromatographic properties:  $R_f$  values; 0.39( $S_1$ ), 0.63 ( $S_2$ ) on PC; dark purple fluorescent spot under long UV-light

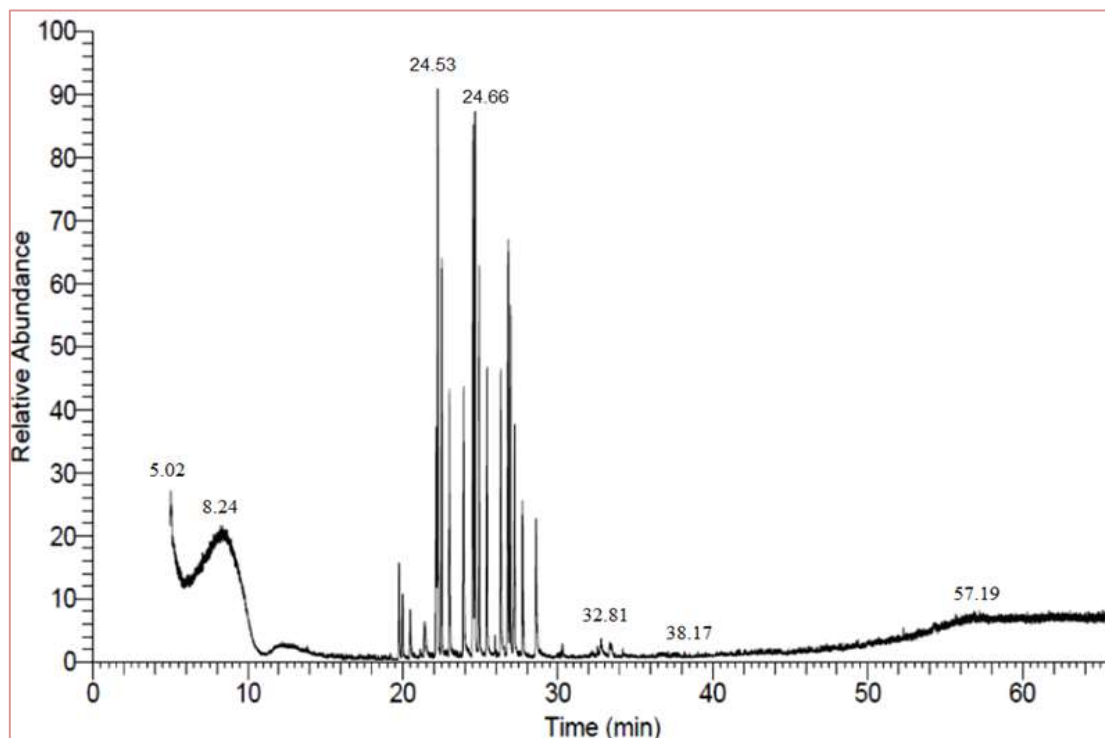


Figure 2. GC-chromatogram of USM of *C. spinosum* L.

Table 2. GC-MS analysis of fatty acids of *C. spinosum* L.

Identified compound		RRT*	Percentage area
Tetradecanoic cid	C(14:0)	0.784	3.38
14-Methyl Pentadecanoic acid	C(16:0)	1	34.8
9-oxo, Nonanoic acid	C(9:0)	1.016	2.64
Hexadecanoic acid	C(16:0)	1.042	25.1
Stearic acid	C(17:0)	1.199	5.22
Octadecanoic acid	C(18:0)	1.237	3.8
10,13-Octadecadienoic acid	C(18:2)	1.278	2.39
18-methyl nonadecanoic acid	C(20:0)	1.38	3.82
Oleic acid	C(18:1)	1.676	1.99
6-Octadecanoic acid	C(18:1)	1.817	2.41
9-Octadecanoic acid	C(18:1)	1.863	2.62
Saturated fatty acid			78.76
Unsaturated fatty acid			9.41
Unidentified compounds			11.83

RRT\*: Relative retention time of 14-Methyl Pentadecanoic acid with RT = 23.76 min.

which turned yellow fluorescence on exposure to ammonia vapors and gave a green color and orange fluorescence with  $\text{FeCl}_3$  and Naturstoff spray reagents, respectively. Complete acid hydrolysis resulted in Quercetin in organic layer and Rhamnose in aqueous layer (CoPC).  $^1\text{H-NMR}$  spectrum (400MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  ppm 7.30 (1H, d,  $J=2.2$  Hz, H-2'), 7.27 (1H, dd,  $J=2.2, 8.2$  Hz, H-6'), 6.88 (1H, d,  $J=7.7$  Hz, H-5'), 6.32

(1H, d,  $J=1.8$  Hz, H-8), 6.15 (1H, d,  $J=1.8$  Hz, H-6), 5.32 (1H, d,  $J=1.3$  Hz, H-1''), 4.19 (1H, dd,  $J=1.3, 3.2$  Hz, H-2''), 3.71 (1H, dd,  $J=3.2, 9.6$  Hz, H-3''), 3.33 (1H, m, H-5''), 3.32 (1H, m, H-4''), 0.91 (3H, d,  $J=5.94$  Hz, H-6'').  $^{13}\text{C-NMR}$  PENDANT (100 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  ppm 178.4 (C-4), 164.8 (C-7), 161.8 (C-5), 157.9 (C-2), 157.2 (C-9), 148.3 (C-4'), 145.1 (C-3'), 134.7 (C-3), 121.6 (C-1'), 121.5 (C-6'), 115.5 (C-2'), 115.0 (C-5'), 104.3 (C-10),

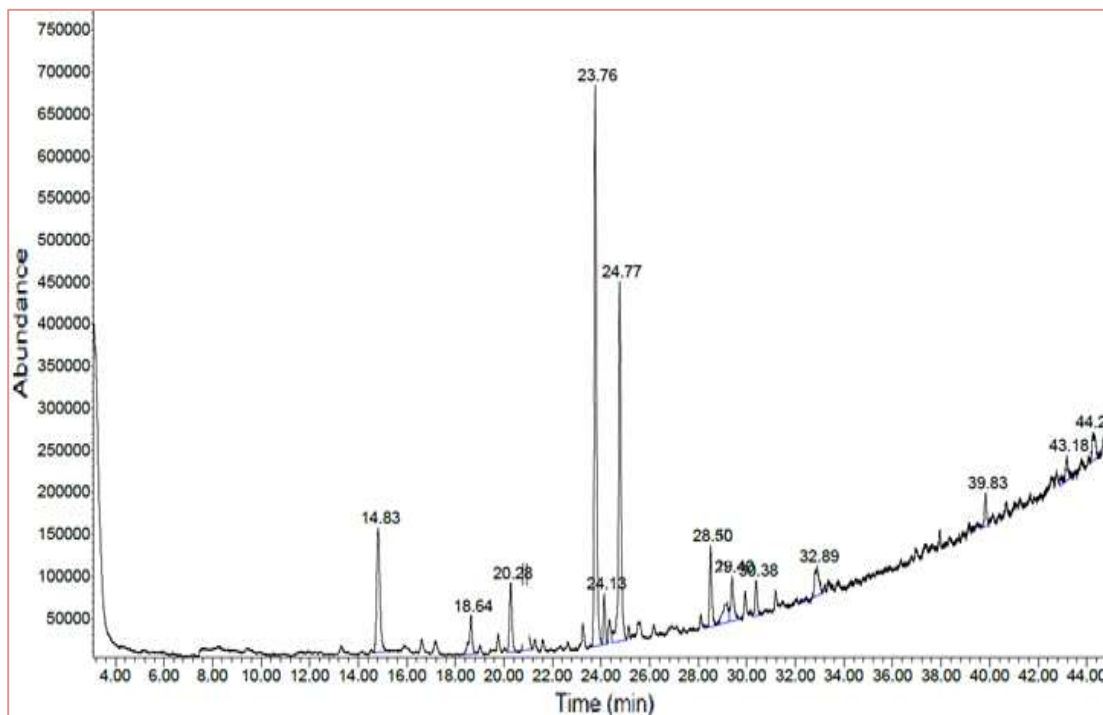


Figure 3. GC-chromatogram of fatty acids of *C. spinosum* L.

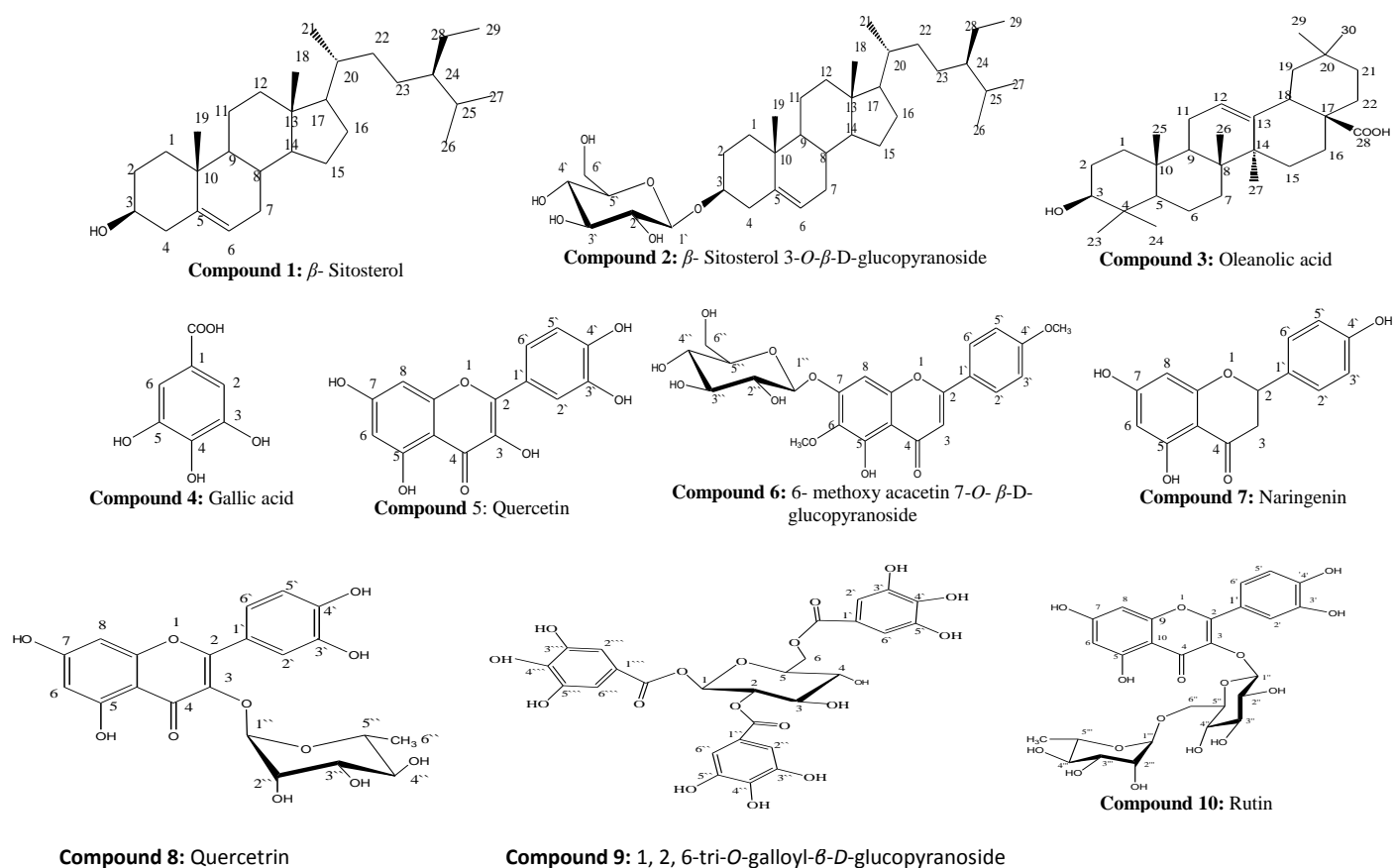


Figure 4. Isolated compounds of *C. spinosum*.

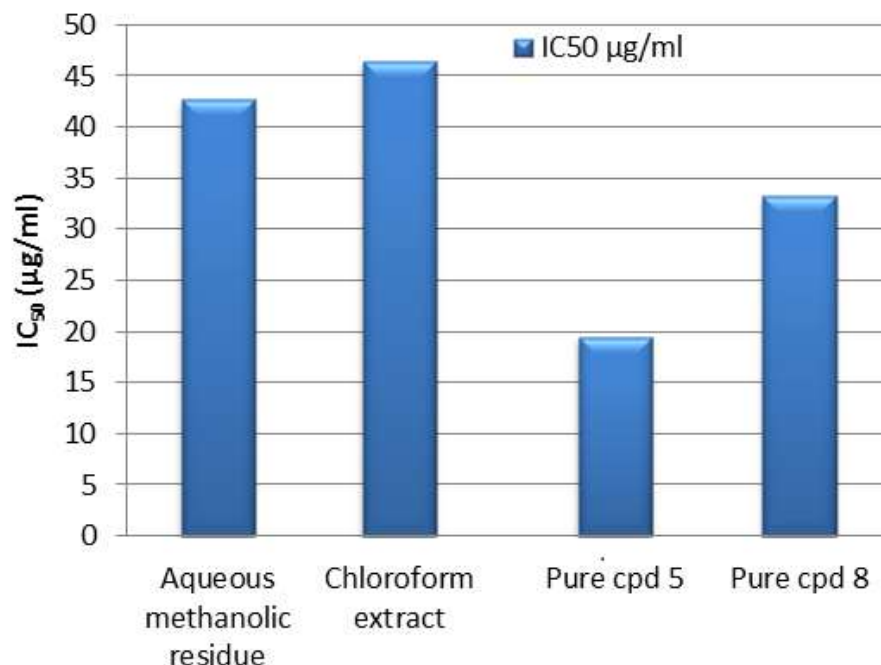


Figure 5. Cytotoxicity of the plant samples against A2780 ovarian cell line.

Table 3. Cytotoxicity of the plant samples against A2780 ovarian cell line.

Sample	IC <sub>50</sub> µg/ml
Aqueous methanolic residue	42.7
Chloroform extract	46.4
Compound 5 (Quercetin)	19.5
Compound 8 (Quercetrin)	33.2

102.2 (C-1<sup>''</sup>), 98.5 (C-6), 93.4 (C-8), 71.8 (C-4<sup>''</sup>), 70.6 (C-3<sup>''</sup>), 70.5 (C-2<sup>''</sup>), 70.5 (C-5<sup>''</sup>), 16.3 (CH<sub>3</sub>-6<sup>''</sup>).

Quercetin showed significant cytotoxic effect as IC<sub>50</sub> 19.5 µg/ml.

## Biological study

### Cytotoxic activity

Cytotoxic activity of aqueous methanolic residue, chloroform extract, and pure compounds (5 and 8) obtained from the aerial parts of *C. spinosum* were examined against A2780, a human ovarian cell line. Activity was reported in terms of an IC<sub>50</sub> (concentration in µg/ml necessary to produce 50% inhibition) (Figure 5) and (Table 3). The treatment of A2780 ovarian cell line with an aqueous methanolic residue, chloroform extract showed weak cytotoxic effect as their calculated IC<sub>50</sub> which were 42.7 µg/ml and 46.4 µg/ml, respectively.

While pure compound 8 (identified later as Quercetrin) showed moderate cytotoxic effect calculated (IC<sub>50</sub>) as 33.2 µg/ml, pure compound 5 (identified later as

### Determination of median lethal dose (LD<sub>50</sub>)

On low doses (less than 2 g/kg of total aqueous methanol extract of *C. spinosum*), it was observed that animals moved and fed normally. The behavior of mice has changed at a dose of 2 g/kg extract. Mice showed abnormal signs like fatigue, loss of appetite and mortality. The 50% of dead animals were estimated at 3 g/kg extract.

In contrast, all animals died at a dose of 4 g/kg. LD<sub>50</sub> value was calculated by-probit analysis which is 2.86 g/kg body weight.

### Analgesic and antipyretic activities

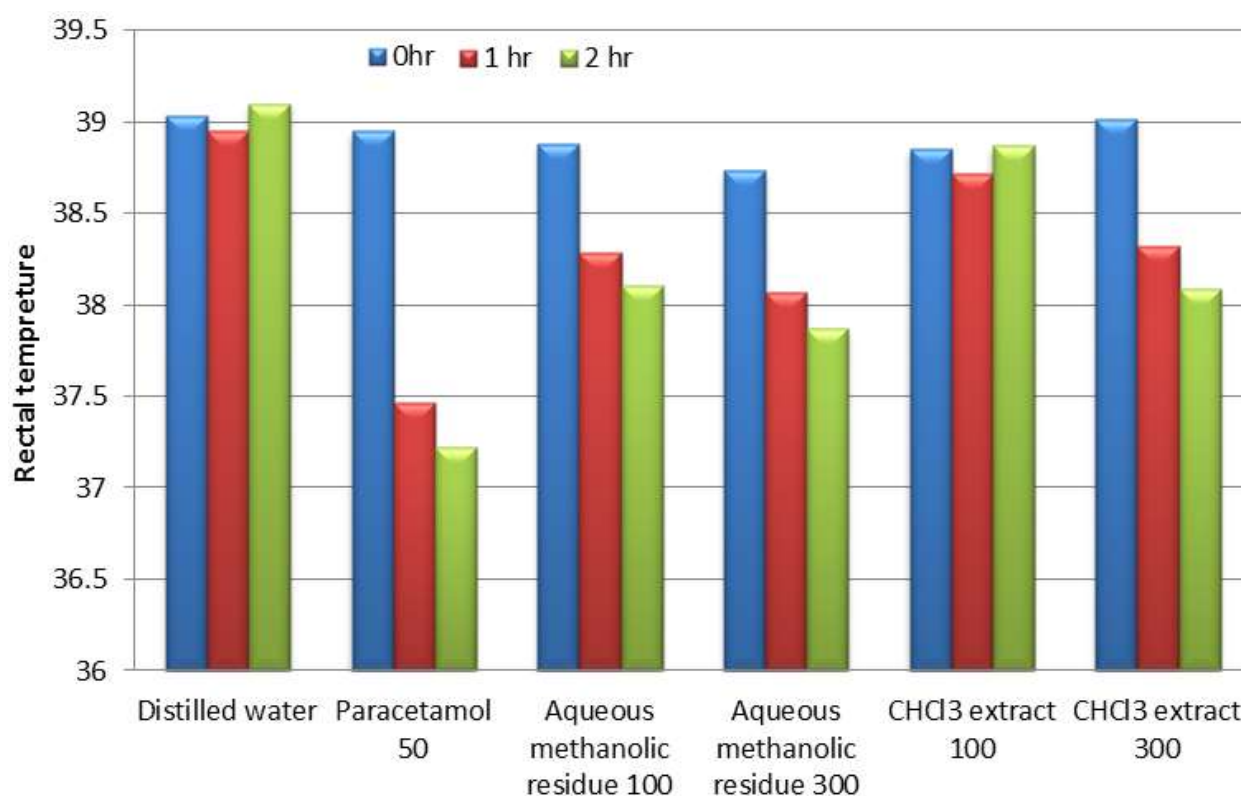
Using hot plate test, the analgesic effect of plant samples



**Table 4.** Antipyretic activity of aqueous methanolic residue and chloroform extract compared to the effect of paracetamol in yeast suspension-induced hyperthermia in rats

Treatment dose (mg/kg)	Rectal temperature (°C) after yeast injection		
	0 h	1 h	2 h
Distilled water	39.03 ± 0.2	38.95 ± 0.16	39.1 ± 0.15
Paracetamol 50	38.95 ± 0.25	37.47 ± 0.25**	37.22 ± 0.1**
Aqueous methanolic residue 100	38.88 ± 0.26	38.28 ± 0.15*	38.1 ± 0.26*
Aqueous methanolic residue 300	38.73 ± 0.28	38.07 ± 0.19*	37.87 ± 0.24**
CHCl <sub>3</sub> extract 100	38.85 ± 0.29	38.72 ± 0.12	38.87 ± 0.21
CHCl <sub>3</sub> extract 300	39.02 ± 0.26	38.32 ± 0.31	38.08 ± 0.31*

Data are represented as mean value ± S.D., n = 6. \* Significant difference when compared to untreated group at \* p < 0.05, \*\* p < 0.01.



**Figure 6.** Antipyretic activity of aqueous methanolic residue and chloroform extract compared to the effect of paracetamol and control.

was studied. All tested samples at both concentrations (100 mg/Kg) and (300 mg/Kg) showed non-significant analgesic activity as compared to paracetamol as standard and saline as control. As shown in (Table 4) and (Figure 6), aqueous methanolic residue at concentration 100 mg/kg and chloroform extract at 300 mg/kg showed moderate antipyretic activity while aqueous methanolic residue at 300 mg/kg showed significant antipyretic activity as compared to paracetamol as standard and distilled water as control.

#### Antimicrobial study

Aqueous methanolic residue of *C. spinosum* exerted marked antimicrobial activity against all tested multidrug-resistant Gram +ve and -ve bacteria. Chloroform extract exerted antimicrobial activity against the tested Gram +ve and -ve bacteria. It showed that, the activity on Gram +ve is higher than Gram -ve bacteria. Pure compound 5 exerted marked activities against the tested Gram +ve *S. aureus* and Gram -ve bacteria *E. coli*, but showed no



**Figure 7.** Antimicrobial activity of *C. spinosum* L. against Multidrug-resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* selected among clinical isolates obtained from Outpatient Clinics of Research Institute of Ophthalmology.

**Table 5.** Antimicrobial screening.

Sample	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
	(mm)	(mm)	(mm)
Mean levels of the inhibition zones			
Aqueous methanolic residue	20	20	15-17
Chloroform extract	20	15	10
Compound 5	17-19	15-17	-
Compound 8	15	10-15	-
Imipenem	25	25	10-15
Ciprofloxacin	25	25	20

antimicrobial activity against *P. aeruginosa*.

Compound 8 showed moderate antimicrobial activity against the tested Gram +ve *S. aureus* and Gram -ve bacteria *E. coli* but showed no antimicrobial activity against *P. aeruginosa*. Fortunately, multidrug-resistant *S. aureus* and *E. coli* strains showed sensitivity to all tested samples. The results of agar well diffusion method are shown in (Figure 7 and Table 5).

## DISCUSSION

A phytochemical screening of *C. spinosum* aerial parts resulted in the presence of flavonoids, tannins, carbohydrates and/or glycosides, triterpenes and/or sterols and saponins. Also, it revealed the absence of alkaloids, volatiles, anthraquinones and coumarins. The percentages of hydrocarbons and sterols in *C. spinosum* pet-ether extract were 99.57 and 0.3%, respectively. It was found that 6-phenyldodecane (10.03%) and 5-phenyldodecane (9.96%) represented the major hydrocarbons while  $\beta$ - Sitosterol (0.30 %) represented only identified sterol.

Concerning the composition of fatty acids content in

pet-ether extract, it could be concluded that the percentage of saturated fatty acids (78.76%) represented higher percentage than that of unsaturated fatty acids (9.14%). 14-methyl Pentadecanoic acid (34.8%) and hexadecanoic acid (25.1%) represented the major identified saturated fatty acids while 9-octadecanoic acid (2.62%) represented the major unsaturated fatty acid. These results are in accordance with previous studies of different species of genus *Citharexylum* (Khalifa et al., 2002; Ayers and Sneden, 2002; Shalaby and Bahgat, 2003; Balazs et al., 2006; Ganapaty et al., 2010; Allam, 2014; Mohammad et al., 2016).

Furthermore, the 80% aq. methanolic residue and chloroform extract of *C. spinosum* were purified by employing diversity of chromatographic techniques to afford ten compounds,  $\beta$ - Sitosterol Gallic acid and Oleanolic acid were isolated once before from genus *Citharexylum* (Allam, 2014; Khan and Hossain, 2015; Mohammed et al., 2016; Allam, 2017), while;  $\beta$ - Sitosterol 3-O- $\beta$ -D-glucopyranoside, 6-Methoxy acacetin 7-O- $\beta$ -D-glucopyranoside, Naringenin, 1, 2, 6-tri-O-galloyl- $\beta$ -D-glucopyranoside, Rutin were isolated for the first time from genus *Citharexylum* in addition to two major compounds (5 and 8).

According to chromatographic properties of compound 5 (R<sub>f</sub> – value), fluorescent under UV-light and change in color with FeCl<sub>3</sub> and Naturstoff reagents compound 5 was expected to be quercetin aglycone (Harborne, 1984). <sup>1</sup>H-NMR spectrum showed two characteristic aromatic spin coupling system, the first ABX of three proton resonances at δ 7.71, 7.62, 6.86 were assignable to H-2', 6' and 5' of 3', 4' dihydroxy B-ring. The second coupling system was described as typical AM system of two meta-coupled doublets at δ 6.36 and 6.15 for H-8 and H-6 of 5, 7-dihydroxylated ring- A. The absence of any signals in the aliphatic region proved the aglycone structure. <sup>13</sup>C-NMR spectrum exhibited fifteen <sup>13</sup>C resonances of the Quercetin moiety with key carbon signals of quercetin nucleus at 175.99 (C-4), 146.62 (C-4'), 144.88 (C-3'), 120.29 (C-6'), 122.79 (C-1'), 114.85 (C-2') and 114.48 (C-5') (Agrawal and Bansal, 1989).

Based on the above discussed data and in comparison with previous reported data (Agrawal and Bansal, 1989) and authentic sample, compound 5 was identified as Quercetin which is isolated for the first time from genus *Citharexylum*. The chromatographic properties of compound 8 (R<sub>f</sub>-values, fluorescence under UV-light and change in color with FeCl<sub>3</sub> and natrustoff reagents) and products of acid hydrolysis, was expected to be quercetin rhamnoside (Harborne, 1984).

<sup>1</sup>H-NMR spectrum showed two characteristic aromatic spin coupling system, the first one ABX of three proton resonances δ7.30, 7.27, 6.88 were assignable to H-2', 6' and 5' of 3', 4' dihydroxylated B-ring. The second coupling system was described as typical AM system of two meta-coupled doublets at δ 6.32 and 6.15 for H-8 and H-6, respectively of 5, 7- dihydroxylated ring-A. Concerning the sugar moiety and doublet signal at 5.32 ppm with J=1.3Hz (H-1''), doublet of doublet signal at 4.19 ppm with J=1.4, 3.2 Hz (H-2'') together with a doublet signal at 0.91with J=5.9 Hz (H-6''), were all characteristic for α-L-rhamnopyranoside moiety. In accordance with the earlier discussed data along with a comparison of the previous reported data (Agrawal and Bansal, 1989; Mahmoud et al., 2001), supporting evidence for the structure of glycoside was achieved by <sup>13</sup>C-NMR spectrum which showed the characteristic 15 <sup>13</sup>C resonance for 3-O-substituted quercetin. The sugar moiety was confirmed as rhamnose from characteristic resonance at δ ppm 102.2 and 16.3 for anomeric carbon and CH<sub>3</sub>-6'', respectively, together with the rest of carbon resonances for rhamnose carbons. Compound 8 was confirmed as Quercetin 3-O-α-L-rhamnopyranoside (Quercetrin), which is isolated for the first time from genus *Citharexylum*.

Cytotoxic activity of aqueous methanolic residue, chloroform extract and pure compounds (5 and 8) obtained from the aerial parts of *C. spinosum* L. were examined against A2780; a human ovarian cell line using MTT cell proliferation assay. It was found that, aqueous methanolic residue and chloroform extract had weak

cytotoxic activity, pure compound 8 (Quercetrin) had moderate cytotoxic activity, while pure compound 5 (Quercetin) had significant cytotoxic activity. Estimation of analgesic activity done using hot plate test showed that, aqueous methanolic residue and chloroform extract had no analgesic activity. The antipyretic activity of aqueous methanolic residue and chloroform extract were evaluated using Brewer's yeast-induced pyrexia in rats, which found that aqueous methanolic residue at 300 mg/kg had antipyretic activity, while chloroform extract had weak antipyretic activity.

In the present study, the antimicrobial activity was evaluated using agar well diffusion method. For aqueous methanolic residue of *C. spinosum*, results were almost the same against the tested Gram positive and negative bacteria while chloroform extract showed stronger antimicrobial activity against Gram positive than negative bacteria. This is in contrast to the study made by Shalaby and Bahgat (2003), who reported stronger antimicrobial activity against Gram negative bacteria and positive bacteria tested by disc diffusion method. Different species of the genus *Citharexylum* were reported to have antiulcer, antihypertensive and hepatoprotective effects, immunomodulatory, antimicrobial, anti-*Schistosoma mansoni* activities, antioxidant nephroprotective, radical scavenging, cytotoxic activities and regulating immediate type of allergic reaction (Shin et al., 2000; Khalifa et al., 2002; Shalaby and Bahgat, 2003; Bahgat et al., 2005; Khan and Siddique, 2012; Kadry et al., 2013; Allam, 2014).

## CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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