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Chemical Composition and Cytotoxic Activity of *Centaurea scoparea* Sieb against Four Human Cell Lines

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Abstract
The aim of this work was to investigate the lipoidal matter composition of the petroleum ether extract of *Centaurea scoparea* Sieb, to determine the total flavonoidal content of the ethanolic extract and to investigate the cytotoxic activity of the total ethanolic and the sesquiterpene lactone extracts against four human cell lines, Hela (cervical carcinoma cell line), Hep-G_2 (liver carcinoama cell line), HCT 116 (colon carcinoma cell line) and MCF_7 (breast carcinoma cell line) using SBR assay. GLC analysis of the plant resulted in the identification of some fatty acids that were unrecognized formerly. Palmitic acid as the main saturated constituent (14.7%) and oleic acid as the main unsaturated acid (11.8%). Hydrocarbons ranging from C_5 - C_{27} in addition to phytol, citronellol epoxide, 1- eicosanol and 2-nonadecanol were also identified for the first time. Total flavonoidal content of the aerial part of the plant was detected as 41.2 ± 1.7 mg RU / g dry powder. Concerning cytotoxicity tests, the cells were treated with Doxorubicin as the positive control. The plant extracts were applied to the selected cell lines at four increasing doses. Sesquiterpene lactone extract showed lesser IC_{50} values than the total alcoholic extract. The highest cytotoxic activity were shown against MCF_7 cell line with IC_{50} = 8.03± 0.48µg / ml. On the other hand, the total ethanolic extract was mostly active against HCT 116 with IC_{50} = 14.3 1.34±µg / ml.

Key Words:
Centaurea scoparea, Asteraceae, chemical composition, cytotoxicity.

INTRODUCTION

Centaurea L. is a large genus which comprised of several species many of which are used in folk medicine [1]. It is one of the biggest genera of family Asteraceae as this genus with over 400 to 700 species and 199 taxons [2, 3]. Various *Centaurea* species have certain biological activities and were used as anti- inflammatory [4], antibacterial [5, 6], as diuretic and mild astraingent [7] antihepatotoxic [8], antioxidant [9, 10], cytotoxic / cytostatic [11, 12].

Several bioactive compounds have been isolated and purified from different species of *Centaurea*. These isolates include, but are not limited to, sesquiterpene lactones [10, 13], lignans [10], volatile constituents [14], essential oils [15], aglycone flavanoids [1, 9], flavanoid C-glycosides and other biologically active constituents [16].

Centaurea scoparea Sieb is a medicinal plant grown in Egypt and has two verities green that is localized to Red sea area and grey- conescent that is localized in Siant Catherine region [17]. Previous investigation of *Centaurea scoparea* (*Phaepappus scoparius* Sieb) [18] showed that it contains phenolic compounds as arctigenin, matairesinol, ω-hydroxypropioguaiacon and vanillin.

Flavonoidal content investigation of the plant [18, 19] revealed that the majority of them related to flavonoidal aglycones as apigenin, luteolin, salvigenin, kaempferol, hispidulin and cirsimaritin. Latter, a flavonoidal glycoside and other flavones were isolated [20] from the other species located in the Red Sea area. Also, sesquiterpene lactones of the guianolide type both chlorinated and non-chlorinated as cynaropicrin, diain, janerin and deacyclocynaropicrin [18]. Chlorohyssopifolin E and acroptilin [19] were been isolated. Lipoidal matter investigation of the plant previously revealed the presence of some fatty acids many of them were unidentified together with unsaponifiable matter [19] that still need more clear investigation. Thus, one of the main aims of this work was to investigate the lipoidal matter content of the plant with nearly complete recognition of its content together with assaying the total flavonoidal content that its individual compounds were previously isolated and identified adequately. Also to invitro test the plant cytotoxicity against four common human cancer cell lines. Cytotoxicity tests depended upon the idea that sesquiterpene lactones are known as potent cytotoxic drugs, thus sesquiterpene lactone extract was tested together with the total alcoholic extract of the plant. Comparing IC_{50} results of both extracts with Doxorubicin the potent cytotoxic drug hopping that this may be a safe source of anticancer drug.

MATERIAL AND METHODS

General experimental procedure

The plant was collected from the flowering plants grown in Siant Catherine, Sian, Egypt in April 2003 and was identified by Prof. Dr. N. El- Hadidy, Department of Botany, Cairo University, Cairo, Egypt. Voucher specimen was kept in Herbarium, National Research Center, Giza, Egypt. The aerial parts of the plant were air dried and ground into fine powder. The air-dried powder was kept for lipoid matter analysis, sesquiterpene lactone extraction and total alcoholic extract chemical and biological investigations.

All solvents and Chemicals were purchased from Sigma Chemical Co. (St., Louis, USA). Rutin (purity>98%) was obtained from Sigma-Aldrich. Solvent evaporation and concentration was performed on Rotavapor Heidolphvv 2000l and determination of flavonoidal content using

Spectrophotometer (Optima SP-300, Japan). GLC analysis was performed on Shimadzu 5000 GC/MS apparatus oven temperature 50°Cfor 0 min., ramp3^{0}C /min to 270^{0}C, carrier gas was Helium, solvent delay= 5 min., injection temperature= 250° C, PEGA 10% Column 30.0m x250µm. TLC analysis was done on precoated silica gel 60 F254 plates(Germany).

Cytotoxicity assays were measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise). Germany

4.2. Isolation of Lipids [21]

Aerial pats of *C. scoparea* Sieb (200 g) was defatted with petroleum ether (40-60^{O}C) till exhaustion. The solvent was stripped off under reduced pressure so as to yield the lipoidal matter residue (1.2 g).The lipoidal matter was subjected to saponification for subsequent investigation of both unsaponifiable and saponifiable fractions.

4.3. Preparation of unsaponifiable matter and fatty acids

The light petroleum extract (0.5 g) was refluxed with alcoholic potassium hydroxide (10%) for 2 h, after stripping off ethanol and dilution with water, the unsaponifiable matter was extracted with ether. The residue left after evaporation of chloroform was weighed (6.89 g) and kept for further investigation. Samples of the unsaponifiable fraction were subjected to GLC analysis.

4.4.Preparation of fatty acids methyl ester

The soapy aqueous layer was acidified with 10% hydrochloric acid and the liberated fatty acids were extracted with ether. The brownish fatty residue left after evaporation of ether was weighed (0.1g) and kept for further study. Total fatty acids were subjected to methylation [22].

The fatty acid residue (0.3g) was subjected to saponification and methylation for subsequent investigation by gas chromatography.

4.5.Sesquiterpene lactone extraction

The air dried powdered plant material (100 g) was extracted successively with light petroleum: ether: methanol (1:1:1) mixture at room temperature for 3 times. The resultant extract was concentrated under reduced pressure at 30^{0}C. The residue was dissolved in five fold its quantity of methanol, cooled down to 15^{0}C before being filtered through a piece of linene to remove hydrocarbons. TLC analysis of residue on silica gel F percolated plates showed six spots using ether : $CHCl_3$ (8:2) as solvent system and anisaldehyde/ H_2So_4 as spray reagent. The greenish brown residue (2.1 g) was kept in refrigerator for cytotoxic investigations.

4.6.Preparation of total ethanolic extract

The air dried powdered plant material (100 g) was extracted with ethanol (95%) at room temperature till exhaustion. Ethanolic extract was evaporated under reduced pressure and the residue (9.2 g) was kept in refrigerator for determination of total flavonoidal content and also investigation of the cytotoxic activity against the four human cell lines. TLC analysis of residue on silica gel F percolated plates showed eight spots using $CHCl_3$: MeOH (9:1) as solvent system and anisaldehyde/ H_2So_4 as spray reagent.

4.7.Determination of total flavonoid concentration in the alcoholic extract

The content of flavonoids in the total ethanolic extract of *C. scoparea* was determined using spectrophotometric method [23]. The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax= 415 nm. The mean value of absorbance of three readings was determined. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

4.8.Determination of cytotoxicity

Human tumor cell lines

Non small human colon carcinoma (HCT116), epitheloid cervixcarcinoma (Hela), human hepatocellular liver carcinoma (HepG2), human breast carcinoma (MCF-7) cell lines were obtained in frozen state under liquid nitrogen (-180ºC) from the American Type Culture Collection. The tumor cell lines were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt.

Culture media

The cells were suspended in RPMI 1640 medium (SIGMA ALDRICH) supplemented with 10% fetal calf serum (SIGMA, USA) in presence 1% antibiotic antimycotic mixture (10.000 U/ml K-penicillin, 10.000µg/ml streptomycin sulphate and 25 µg/ml amphotericin B) and 1% L-glutamine (all purchased from Lonza ,Belgium).

Assay method for cytotoxic activity

The cytotoxicity against HCT 116, Hela, Hep-G2 and MCF-7 cells were tested in the National Cancer Institute, according to the SRB (Sulforhodamine B) assay (3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyltetrazolium bromide) method by Skehan et al [24], Adriamycin® (Doxorubicin) 10 mg vials Pharmacia, Sweden) was used as the reference drug. Briefly, cells were seeded in 96-multiwell plates at densities of 10^4cells/well in a fresh media and incubated under normal growth condition for approximately 24 h before treatment with the tested sample to allow attachment of cells to the wall of the plate. Then, 200 µl aliquot of serial dilution with DMSO (100%) of alcoholic extract and sesquiterpene extract(0, 1,2.5, 5 and 10µg/ml) were added and the plates were incubated for 24, 48 and 72 h at 37°C in a humidified incubator containing 5% CO_2 in air.

Control cells were treated with vehicle alone. Three wells were prepared for each individual dose. Following 24, 48 and 72 h treatment, cells were fixed, washed and stained with Sulforhodamine B stain (Sigma, USA). Colour intensity was measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise ,Germany).The relation between the mean of surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified plant extract. Negative control was treated with the vehicle (0.1%

DMSO) used for diluting the tested drug. Doxorubicin (1.0 µg/ml) was used as the positive control. Also, determination of IC_{50} of Doxorubicin for all the tested cell lines was also so as done for direct comparison with the tested extracts.

The experiment was performed triplicate with DMSO at 1% and Doxorubicin as negative and positive controls respectively.

4.9.Statistical analysis

All the aforementioned experiments were conducted in triplicate trials. Data were expressed as mean ± standard deviation (SD). Data were analyzed by using one-way ANOVA followed by Duncan's multiple range tests using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

GLC of the fatty acid methylesters resulted in the identification of twelve fatty acids both saturated and unsaturated ones. The results are listed in Table 1. The majority of them were saturated fatty acids within which palmitic acid was the main constituent (14.7%). Unsaturated fatty acids represents nearly 26.3% of the main fatty acid content within them oleic acid is the main constituent (11.8%). The other unsaturated fatty acids were detected as linoleic acid, 14- octadecenoic acid and 11-octadecenoic acid. The latter two were been identified for the first time. Among the saturated fatty acids, stearic acid, undecanoic acid, heneicosanoic acid and heptacosanoic acid were identified in this plant for the first time.

Concerning with the unsaponifiable lipoidal fraction, GLC separation and investigation revealed the presence of hydrocarbons ranging from C_5-C_{27}. Other compounds include acyclic diterpene alcohol namely phytol that was represented by a relatively high concentration (11.5 %) in addition to some diterpenes, oxygenated diterpenes together with sterols and triterpenes. Some constituents as Citronellol epoxide (R or S), the acyclic diterpene epoxide was identified by a concentration 4.1%.

Determination of the total flavonoidal content of the plant may help to give a clear idea about this plant as these compounds possess abroad spectrum of chemical and biological activities depending on their antioxidant activities. *C. scoparea* showed flavonoidal total content of 41.2± 1.7 mg rutin equivalent/g of dry sample.

These flavonoids were been previously isolated and nearly identified adequately.

Upon testing the cytotoxic activities, ethanolic and sesquiterpene extracts exhibited promising activities against the tested four human cell lines, Hela, Hep-G2, HCT-116 and MCF-7. Despite of having no antineoplastic activity against Hela cells, sesuiterpene lactone extract showed higher cytotoxicity than ethanolic extract against the tested cell lines.This was expressed by lower C_{50} values of the ethanolic extract as shown from Table 1 &2. Sesquiterpene lactone extract exhibited the highest cytotoxicity against MCF-7 with IC_{50} = 8.03µg/ ml followed by Hep-G2 and finally HCT 116 with IC_{50} = 9.3 and 12.8µg/ ml. The results are shown in Fig. 1

Concerning the ethanolic extract, it showed the highest cytotoxic activity against HCT-116 cell lines with IC_{50} = 14.3 µg/ ml. IC_{50} values against the other tested cell lines obey the following order, Hep-G2, MCF-7 and finally Hela cells with IC_{50} = 15.1, 16.6 and 27.5µg/ ml respectively. The results are shown in Fig. 2.

Beside being the positive control, Doxorubicin was tested in the concomitant experience for its IC_{50} values against the four tested cell lines. The cytotoxicity was the highest against Hela cells followed by MCF-7 then Hep-G2 and finally HCT-116 with IC_{50} = 3.68, 4.13, 5.18 and 5.3µg/ ml respectively.

Table 1: GLC analysis of the Unsaponifiable Matter of the petroleum ether extract of *Centaurea scoparea* Sieb.

Identified compound	Retention time (min)	Percent composition
Pentane	7.35	9.5
1- Hexanol, 2 ethyl	16.67	70
Tetradecane	37.55	4.3
Nonadecane	41.5	6.1
Hexadecane	42.1	4.5
Pentadecane	43.41	11.4
Tridecane	44.3	10.3
Heptacosane	45.29	4.2
Eicosane	45.51	3.5
Citronellol epoxide (R or S)	50.61	4.1
1-Eicosanol	51.92	3.3
2- Hexadecanol	58.44	2.5
2- Nonadecanol	59.13	5.1
Phytol	59.42	11.5
Oxirane, tetradecyl	59.43	4.5
Stigmasterol	59.48	1.34
β-Sitosterol	59.49	7.36
β-amyrin	59.52	3.2

Table 2: GLC analysis of the Saponifiable Matter of the petroleum ether extract of *Centaurea scoparea* Sieb.

Identified Compound	Retention time (min)	Percentage Composition
Tridecanoic acid	46.47	13.6
Hexadecanoic acid (palmitic acid)	53.4	14.7
Undecanoic acid	54.72	8.3
Pentadecanoic acid	54.72	5.3
Octadecanoic acid (stearic acid)	56.98	14.3
11- Octadecenoic acid	57.02	5.2
9,12- Octadecadienoic acid (linoleic acid)	58.86	3.2
9- Octadecenoic acid (oleic acid)	59.01	11.8
14- Octadecenoic acid	61.02	6.1
Heneicosanoic acid	62.57	7.6
Eicosanoic acid (arachidic acid)	63.1	3.2
Heptadecanoic acid	65.55	6.7

Table 3: Cytotoxic activity of crude alcoholic extract of *Centaurea scoparea* Sieb against cultured different cell lines in vitro.

Surviving Fraction of Human cell lines	Extract Conc. µg/ml					IC_{50}
	0.00	5.00	12.5	25	50	
Hela	1.00	0.863± 0.08	0.732± 0.04	0.526± 0.04	0.242 ±0.03	27.5± 2.77
Hep-G2	1.00	0.784± 0.05	0.565± 0.08	0.231± 0.03	0.213± 0.03	15.1± 1.47
MCF-7	1.00	0.659± 0.07	0.561± 0.03	0.386± 0.06	0.256± 0.01	16.6± 1.55
HCT-116	1.00	0.714± 0.08	0.532± 0.07	0.305± 0.04	0.279± 0.01	14.3± 1.34

All data are presented as values ± SD (µg/mL) from independent experiments performed in triplicate as SBR assay and Doxorubicin was used as the positive control.

Table 4: Cytotoxic activity of sesquiterpene lactone extract of *Centaurea scoparea* Sieb against cultured different cell lines in vitro.

Surviving Fraction of Human cell lines	Extract Conc. µg/ml					IC_{50}
	0.00	5.00	12.5	25	50	
Hela	1.00	0.984 ±0.03	0.789 ±0.04	0.632 ±0.04	0.574 ±0.03	------
Hep-G2	1.00	0.706 ±0.04	0.361 ±0.06	0.212 ±0.01	0.286 ±0.01	9.3 ±0.58
MCF-7	1.00	0.538 ±0.07	0.439 ±0.06	0.265 ±0.01	0.327 ±0.01	8.03 ±0.48
HCT-116	1.00	0.636 ±0.03	0.506 ±0.0	0.422 ±0.03	0.409 ±0.02	12.8 ±1.18

All data are presented as values ± SD (µg/mL) from independent experiments performed in triplicate as SBR assay and Doxorubicin was used as the positive control.

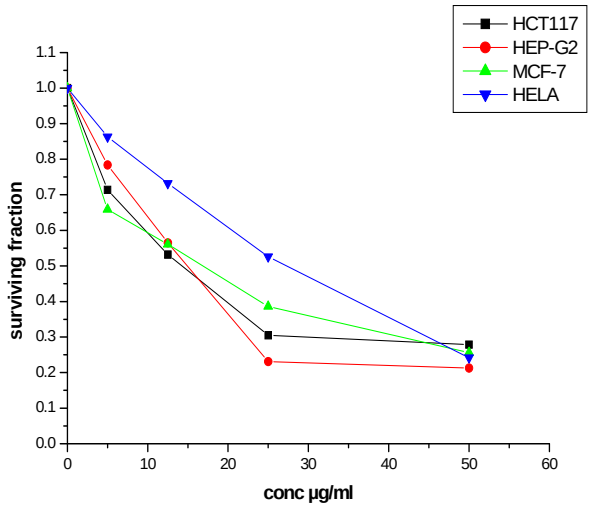

Fig. 1: Cytotoxic activity of total ethanolic extract

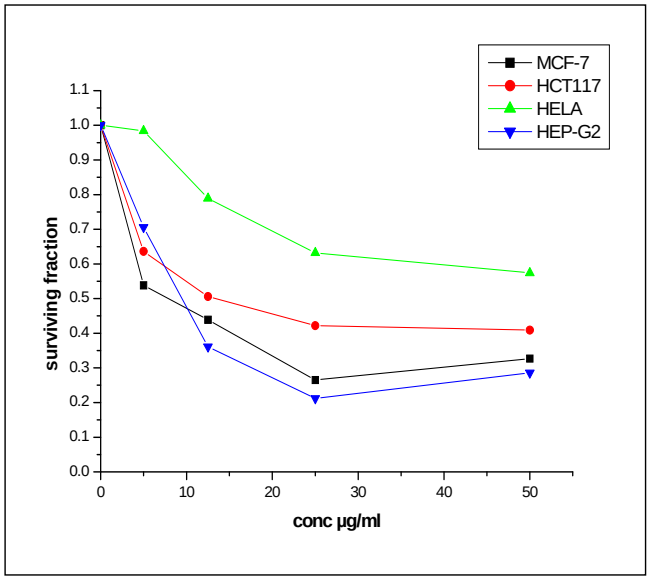

Fig. 2: Cytotoxic activity of sesquiterpene lactone extract

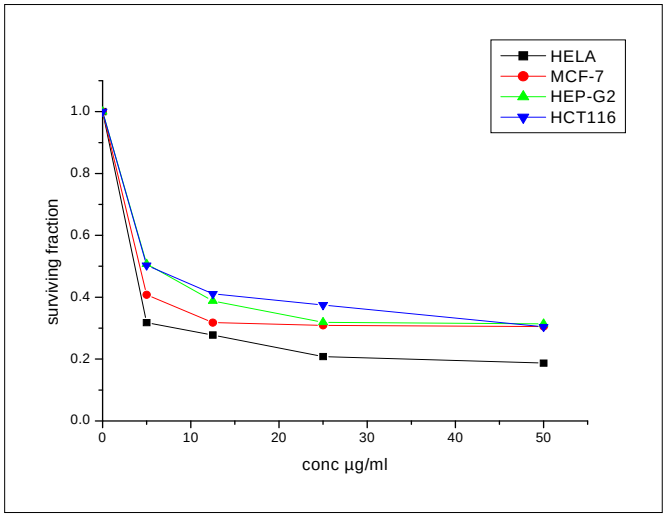

Figure 3: Cytotoxicity of Doxorubicin against four human cell lines

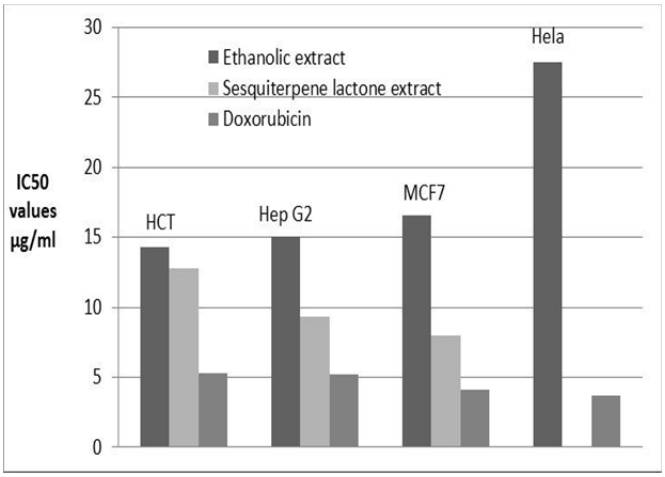

Fig. 4: Comparison between the mean IC_{50} values of the ethanolic and sesquiterpene lactone extracts of *C. scoparea* Sieb and Doxorubicin against HCT 116, Hep-G2, MCF-7 and Hela cell lines

DISCUSSION

The role of natural products as a source for remedies has been recognized since ancient time [25]. These natural plants help to supply new anticancer drugs related to different groups and have new mechanisms of action. *C. scoparea* related to such drugs that are proven for their use in folk medicine in Egypt. Investigation of the fatty acid composition of the plant resulted in identification of some fatty acids that were not identified previously as stearic acid, undecanoic acid, tridecanoic acid, heptacosanoic acid, 14-octadecanoic acid and 11- octadecenoic acid. All together with oleic, palmitic, linoleic, oleic and linolenic acids are important fatty acids. Thus the results suggested that C. scoparea could be used as a potential source for saturated and unsaturated fatty acids in food industry, cosmetics and pharmaceutical preparations. Also, the unsaponifiable fraction of the lipids was found to contain compounds related to diterpene alcohols as 1-eicosanol (4-.1%) and phytol (11.5%), the acyclic diterpene alcohol that is found in plants and algae and can be used as a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1. It is a naturally occurring bioactive secondary metabolite proven to have a potent antimycobacterial agent [26]. Phytol was not previously isolated from this plant.

In addition, oxirane tetradecyl and the diterpene epoxide, citronellol epoxide (S or R) were identified for the first time in this work with a concentration 4.5 and 4.1% respectively.

Determination of the total flavonoidal content by 41.2 mg RU/g of dry sample referred to the idea that this ethanolic extract possess antioxidant activity. This was supported by many researches that relates the antioxidant activity to the flavonoidal content generally in plants and even in *Centaurea species* [27].

In accordance with the plant cytotoxicity, the two extracts exhibited potent activity against all the tested cell lines except with Hela cell line that was resistant to the sesquiterpene lactone extract. Sensitivity to cancerous cell lines varied according to cell types. According to Suffness and Pezzuto, 1990 extracts with IC_{50}values 30<µg/ml were considered active and promising for the search for new anticancer agents [28]. Thus on determining the 50% inhibitory concentration (IC_{50}), extracts were considered active and promising. The most potent one was sesquiterpene lactone extract against MCF-7 human cell line as its IC_{50} was 8.03µg/ml that nearly represents double the doxorubicin IC_{50} against the same cell line. From another point of view, the cytotoxicity of the alcoholic extract of *C. scoparea* may be mainly due to its flavonoidal content [20]. However, the cytotoxicity of the sesquiterpene lactone extract of the plant could be attributed to its high content of guaianolide and chlorinated guianolide sesquiterpene lactones.

In conclusion, this study highlight the importance of *Centaurea scoparea* Sieb extracts as potent cytotoxic drugs of medicinal plant origin and also as a rich source of both saturated and unsaturated fatty acids and some lipoidal compounds. The mechanism by which extracts exhibited such antineoplastic activity still unclear.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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