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Cytotoxicity Evaluation and Phytochemical Investigation of *n*-hexane extract of *Centaurea glomerata* Vahl.

Fatma H. Sadek[1], Mostafa M. Abazied[1,*], Abou El-Hamd H. Mohamed[1] , Hussein A. El-Damarany[1]

[1] Chemistry Department, Faculty of Science, Aswan University

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Abstract:

Genus Centaurea, known for its diverse pharmacological properties, has been extensively studied for its essential oils (EOs) and bioactive compounds. This study investigated the dichloromethane: methanol (CH_2Cl_2: CH_3OH) (1:1 v/v) extract and the eluted *n*-hexane fraction of *Centaurea glomerata*, focusing on its volatile components and cytotoxic potential against Hepatocellular carcinoma (*HepG2*) and Breast adenocarcinoma (*MCF-7*) cell lines. Moreover, the study included isolation of three interested compounds. Cytotoxicity assays revealed that the methanol: dichloromethane (CH_2Cl_2:CH_3OH) (1:1 v/v) extract exhibited significant growth inhibition in both cancer cell lines at higher concentration of 100 µg/ml, while the isolated compounds showed limited activity against cancer cells. These findings suggested that the crude extract of dichloromethane: methanol of *Centaurea glomerata* exhibits synergistic effect, which is the interaction of two or more constituents when their combined effect is greater than the sum of the effects seen when each constituent is given alone. This effect enhances the extract efficacy against diverse cancer cells.

Keywords: Gas Chromatography; Cytotoxicity; *Centaurea glomerata*.

1- Introduction

Centaurea species have a long history of use in traditional medicine across various cultures. They are commonly employed to treat numerous ailments including diabetes, diarrhea, hypertension, microbial infections, rheumatism, and wound healing (Ceyhan Güvensen et al., 2019; Polat et al., 2022; Reda et al., 2021). For instance, in Egypt, *C. alexandrina* is used as a remedy for hyperglycemia, while in North Africa, *C. calcitrapa* is utilized for fever, eye diseases, and as a diuretic (Reda et al., 2021). Recent scientific investigations have validated many of these traditional uses, confirming that *Centaurea* extracts and EOs possess significant biological activities including antioxidant, antimicrobial, anti-inflammatory, anticancer, and enzyme inhibitory properties (Güven et al., 2005; Korga et al., 2017; Teneva et al., 2024; Zengin et al., 2016).

Corresponding author*: E-mail address: mostafa.abazied@aswu.edu.eg

Cytotoxicity refers to the detrimental effects of chemical or biological agents on cellular function and viability. In drug development, understanding cytotoxicity is crucial, as toxicity-related failures in late-stage development can be costly and time-consuming. Early screening for potential toxicities, combined with a comprehensive understanding of the biological target, compound structure, and physicochemical properties, is essential for mitigating risks and ensuring the safety and efficacy of new therapeutic agents (Gould & Templin, 2023).

Centaurea genus, belonging to family Asteraceae, is one of the largest and most taxonomically complex genera, with over six hundred species distributed globally, particularly in the Mediterranean and Western Asian regions. Many *Centaurea* species have been traditionally used in folk medicine across various cultures for their wide range of pharmacological properties. For instance, in Turkish folk medicine, *Centaurea* species have been employed to treat ailments such as stomach pain, abscesses, asthma, hemorrhoids, headaches, inflammatory conditions, urogenital disorders, and microbial infections. They are also known for their cytostatic, diuretic, antinociceptive, antipyretic, and wound-healing properties(Akkol et al., 2009; Aktumsek et al., 2011; Dumlu & Gürkan, 2006; Kilic, 2013; Koca et al., 2009; Köse et al., 2016).

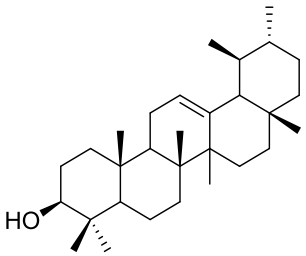
Despite the extensive use of *Centaurea* species in traditional medicine, scientific research on their essential oils and bioactive compounds remains limited. EOs, which are complex mixtures of volatile terpenes, terpenoids, and hydrocarbons, are known for their diverse potent biological activities. Depending on the plant species, growth environment, and extraction techniques, the EOs content might vary greatly. *Centaurea glomerata*, a species native to the Mediterranean region has not been extensively studied for its essential oil profile or cytotoxic potential. The extract was obtained, and its EOs were analyzed using gas chromatography-mass spectrometry (GC/MS). Additionally, three major compounds were isolated and characterized. Compound (1, 2) and CH_2Cl_2:MeOH extract (1:1v/v) cytotoxicity effects were evaluated against *HepG2* and *MCF-7* cell lines. This study elucidates the phytochemical profile and bioactive potential of *C. glomerata*, offering critical insights that advance the understanding of the *Centaurea* genus's pharmacological value within ethnobotanical and pharmaceutical research context (Garcia-Jacas et al., 2000) (Arif et al., 2010).

1. Materials and Methods

Aerial parts of *Centaurea glomerata* were collected from Burg El Arab, Alexandria, Egypt. The plant material was identified and authenticated in the herbarium unit of Faculty of Science, Aswan University, Egypt. The plant material was air dried then grounded to fine powder, soaked in CH_2Cl_2:MeOH (1:1v/v) and was eluted over a silica gel flash column chromatography, and the extract of *n*-hexane was the first fraction to be eluted.

The results of cytotoxicity evaluations were obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37°C.

2. Results and Discussion

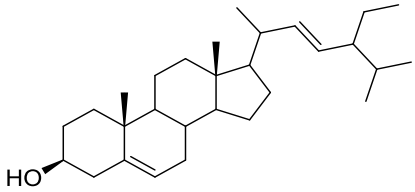


Compound (1)

Molecular Formula: $C_{30}H_{50}O$

***α*-Amyrin**

MW: 426

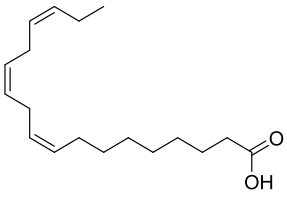


Compound (2)

Molecular Formula: $C_{29}H_{48}O$

Stigmasterol

MW: 412



Compound (3)

Molecular Formula: $C_{18}H_{34}O_2$

Linolenic acid

MW: 280

^{1}H-NMR spectrum showed peaks at downfield region of the spectrum, a doublet proton signal at $\boldsymbol{\delta_H}$ 5.14 ppm was assigned to the olefinic proton (1H, H-12), a triplet at $\boldsymbol{\delta_H}$ 3.16 ppm, suggesting for oxymethine proton (H-3). Also, the ^{1}H-NMR analysis showed the presence of eight methyl groups associated to H-23 - H-30 assigned as follows: 0.95 (3H, s, H-23), 0.74 (3H, s, H-24), 0.83 (3H, s, H-25), 0.91 (3H, m, H-26), 1.10 (3H, s, H-27), 0.76 (3H, s, H-28), 0.85 (3H, s, H-29), 0.79 (3H, d, H-30). These NMR features complied with established features of triterpenoid amyrins. It showed several signals for the other remaining protons.

^{13}C-NMR (APT) spectrum of compound (1) suggested the presence of thirty carbons, which were classified as follows: eight methyl carbon signals at [$\boldsymbol{\delta_C}$ 21.48 (C-23), 15.79 (C-24), 23.00 (C-25), 15.02 (C-26), 25.99 (C-27), 28.08 (C-28), 22.45 (C-29) and 15.80 (C-30)], nine methylene carbon signals at [$\boldsymbol{\delta_C}$ 38.8 (C-1), (C-2), 18.65 (C-6), 32.8 (C-7), 21.95 (C-11), 27.02 (C-15), (C-16), 34.18 (C-21), 73.07 (C-22). Seven methine carbon signals appeared at [$\boldsymbol{\delta_C}$ 78.74 (C-3), 55.60 (C-5), 47.80 (C-9), 121.32 (C-12), 55.08 (C-18), (C-19), 38.7(C-20)] and six quaternary carbon signals at [$\boldsymbol{\delta_C}$ 37.13 (C-4), 38.6 (C-8), 36.69 (C-10), 140.16 (C-13), 40.3(C-14), 42.1(C-17)].

^{1}H-NMR ($CDCl_3$, 400 MHz) of compound (2) showed protons signals at $\boldsymbol{\delta_H}$ 5.33, 5.14 and 5.01 for the protons H-7, H-22 and H-21 respectively, six methyl singlet signals at $\boldsymbol{\delta_H}$ 0.91, 0.69, 0.84,0.99, 0.81 and 0.53 for the protons H-15, H-16, H-26, H-27, H-28 and H-30 respectively, and $\boldsymbol{\delta_H}$ 3.50 (1H, m, H-2).

^{13}C-NMR spectrum in $CDCl_3$ of compound (2) suggested the presence of twenty nine carbons, which were classified as follows: six methyl carbon signals at [$\boldsymbol{\delta_C}$ 18.83 (C-15), 11.95(C-16), 19.01C-26), 19.3 (C-27), 19.8 (C-28 and 11.79 (C-30]. Nine methylene carbon signals were assigned at [$\boldsymbol{\delta_C}$ 36.50 (C-1), 42.22 (C-3), 29.66 (C-6), 31.88 (C-8), 39.69 (C-13), 21.08 (C-14), 26.13 (C-18), 25.43 (C-19), and 25.92 (C-29]. Eleven methine carbon signals appeared at [$\boldsymbol{\delta_C}$ 71.7 (C-2), 122.45 (C- 7), 31.76 (C-9), 56.10 (C-10), 50.19 (C-11), 57.19 (C-20), 129.27 (C-21), 138.28 (C-22), 40.51 (C-23), 31.53 (C-24) and 51.24 (C-25)]. The remaining three quaternary carbon signals were

indicated at $\boldsymbol{\delta_C}$ 140.22 for (C-4), 36.4 for (C-5 and C-12).

^{1}H-NMR spectrum of compound (3) shows some characteristic signals corresponding to the different chemical groups. ^{1}H-NMR showed peaks at 4.10-5.33 was assigned to the four olefinic hydrogens. Also, showed the presence of the CH_2 group which known as the bis-allylic group as a triplet signal at 2.74 ppm. The triplet signal appeared at $\boldsymbol{\delta_H}$ 2.26 which may be attributed to oxymethylene group, it showed peak at 1.92 ppm for the allylic CH_2 groups. The signal of the methyl group.

3.1 Cytotoxicity of Isolated Compounds and Extracts

To the best of our knowledge, the previous study of the biological properties of *Centaurea* Species shows cytotoxicity impact of chloroform extract of *C. musimomum* aerial parts on human carcinoma of the nasopharynx (*KB*) cells was assessed by (Medjroubi et al., 2005). Significant cytotoxic action was shown by the data, with growth inhibition of 89% at 10 µg/ml and 26% at 1 µg/ml. Also, non-cytotoxicity doses to healthy human intestinal epithelial cells, the crude extract of *C. ainetensis* was efficient against human colon cancer cells HCT-116 (p53+/+). (Reda et al., 2023), a sesquiterpene lactone (Salograviolide A), identified in the crude extract as bioactive compound, which inhibit the growth of colon cancer cell lines (El-Najjar et al., 2008). Human myeloid leukemia HL-60 cells were significantly cytotoxically affected by Algerianin, a novel acylated flavonoid glucoside that was isolated from *C. africana*, with an IC50 of 26.1 mM (Seghiri et al., 2009). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluate the cytotoxic activity of *n*-hexane, chloroform, and aqueous methanol extracts of the entire plant of *C. arenaria* against cervix adenocarcinoma (*HeLa*), breast adenocarcinoma (*MCF7*), and skin epidermoid carcinoma (A431) cells. At a dosage of 10 g/ml, the chloroform extract showed strong inhibitory action against tumour cell growth (above 85%) (Csapi et al., 2010).

Here, after confirming the structures of the three compounds. **Fig (1,2,3)** depicts the behavior of compound (1), compound (2) and CH_2Cl_2: methanol (1:1 v/v) extract, respectively, against liver cancer (*HepG2*) Hepatocellular carcinoma with doses (50 and 100 µg/ml). While **Fig (5,6,7)** depicts compound (1), compound (2) and CH_2Cl_2: methanol (1:1 v/v) extract, respectively, against breast cancer cells (*MCF-7*).

As shown in both **Figures (4)** and **(8)** the cytotoxicity of the CH_2Cl_2:MeOH extract, against breast cancer (*MCF-7*) and liver cancer (*HpG2*) by using SRB assay method, was effective in concentration of 100 µg/ml, this effect is attributed to the synergistic effect of the extract composition of compounds rather than the former two compounds.

a. Results of cytotoxicity assay of compounds 1,2 and extract (CH_2Cl_2:MeOH) (1:1 v/v) against liver cancer (HepG2)

Sample information:

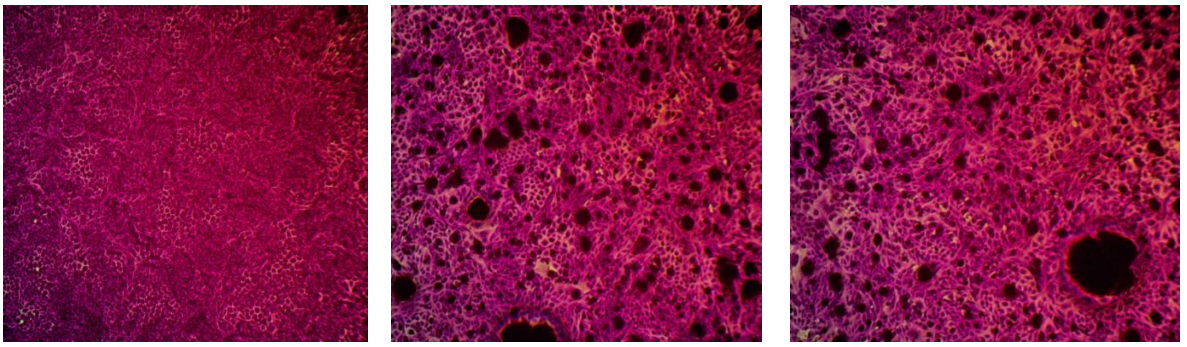
cODE	SAMPLE TYPE	CELL LINE	REQUESTED TEST
COMPOUND (1)	Pure compound		

COMPOUND (2)	Pure compound	*HepG2*: *Hepatocellular carcinoma*	SRB (quick screening concentration)
Ef	Extract		

a1. Compound (1)

Table 1: The percent of cell viability in human liver cancer cell treated with compound (1).

Conc.	**Cell viability percent**	**Conc.**	**Cell viability percent**	**Control Average**
50 µg/ml	92.99	100 µg/ml	95.84	**1.11**
50 µg/ml	93.33	100 µg/ml	96.61	
50 µg/ml	86.21	100 µg/ml	99.31	
50 µg/ml AVG	90.84	100 µg/ml AVG	97.25	
50 µg/ml STD	3.28	100 µg/ml STD	1.49	



Control of liver cancer cells | Conc. 50 µg/ml of Compound (1) against liver cancer | Conc. 100 µg/ml of Compound (1) against liver cancer

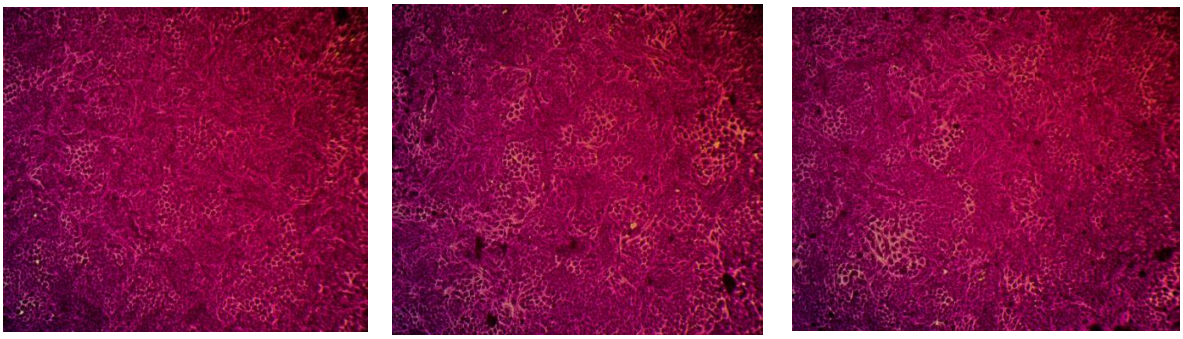
Fig. 1: Human liver cancer cells treated with doses (50 and 100 µg/ml) of compound (1).

a2. Compound (2)

Table 2: The percent of cell viability in human liver cancer cell treated with compound (2).

Conc.	**Cell Viability percent**	**Conc.**	**Cell Viability percent**	**Control Average**
50 µg/ml	102.72	100 µg/ml	101.11	1.11
50 µg/ml	100.83	100 µg/ml	99.12	

50 µg/ml	101.69	100 µg/ml	103.42	
50 µg/ml AVG	101.75	100 µg/ml AVG	101.22	
50 µg/ml STD	0.77	100 µg/ml STD	1.76	



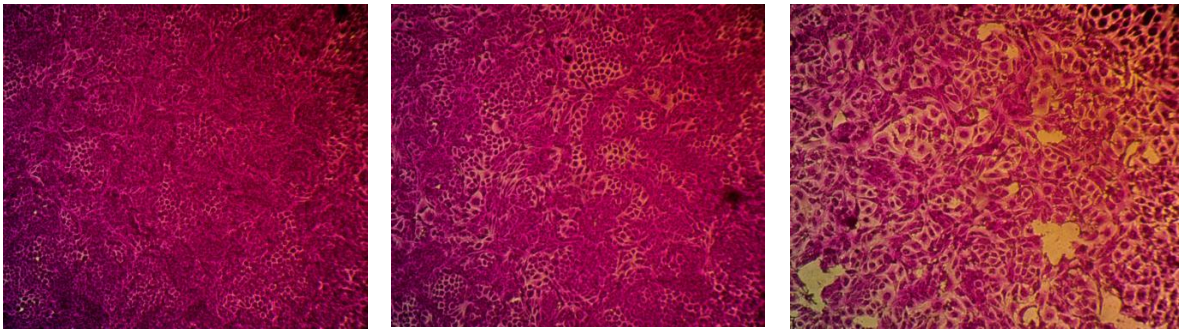
Control of liver cancer cells | Conc. 50 µg/ml of compound (2) against liver cancer | Conc. 100 µg/ml of compound (2) against liver cancer

Fig 2: Human liver cancer cells treated with doses (50 and 100 µg/ml) of compound (2)

a3. Extract (CH_2Cl_2-MeOH) (1:1 v/v) (Ef).

Table 3: The percent of cell viability in human liver cancer cell treated with (Ef)-extract (CH_2Cl_2:MeOH) (1:1 v/v).

Conc.	Cell viability percent	Conc.	Cell viability percent	Control Average
50 µg/ml	91.82	100 µg/ml	39.95	
50 µg/ml	91.54	100 µg/ml	37.27	
50 µg/ml	94.05	100 µg/ml	37.53	0.03582
50 µg/ml AVG	92.47	100 µg/ml AVG	38.25	
50 µg/ml STD	1.12	100 µg/ml STD	1.20	



Control of liver cancer cells | Conc. 50 µg/ml of extract against liver cancer | Conc. 100 µg/ml of extract against liver cancer

Fig. 3: Human Liver cancer cells treated with doses (50 and 100 µg/ml) of extract (CH_2Cl_2: MeOH) (1:1 v/v)

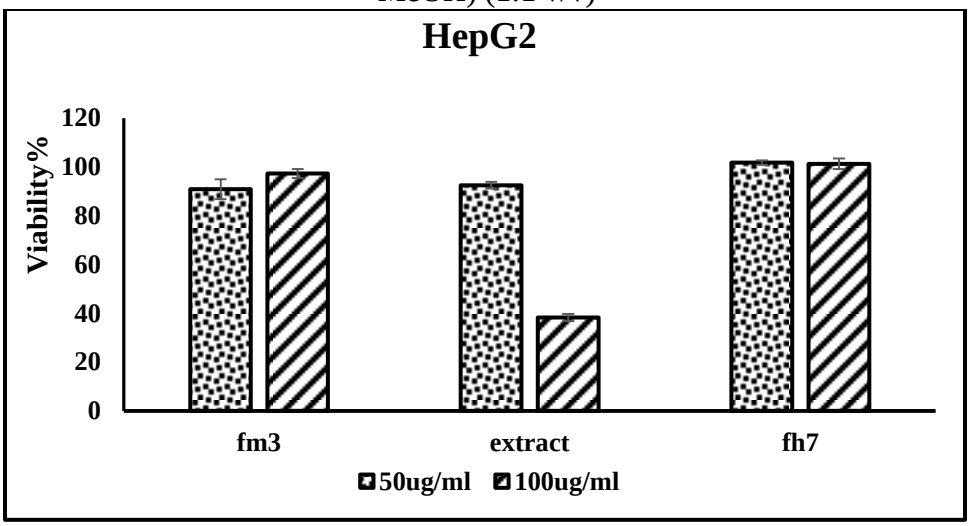


Fig. 4: Comparison between compound (1), compound (2) and extract (CH_2Cl_2:Me OH) (1:1) against liver cancer.

b. Results of cytotoxicity assay of compounds 1, 2 and extract (CH_2Cl_2:MeOH) (1:1 v/v) against Breast cancer (MCF-7).

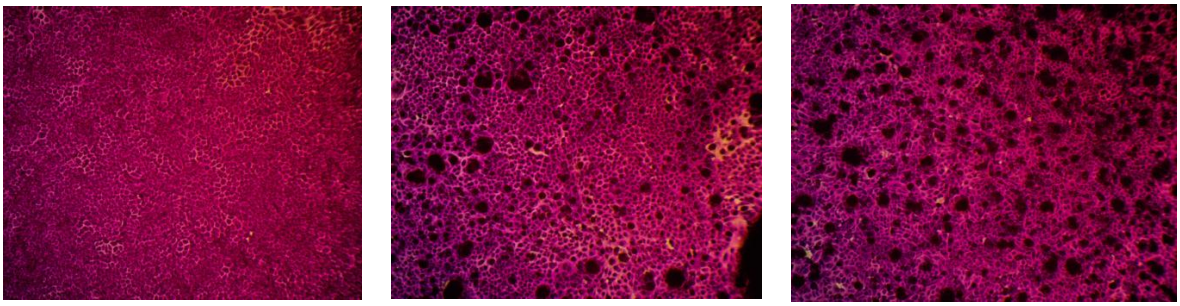
Sample information:

CODE	SAMPLE TYPE	CELL LINE	REQUESTED TEST
Compound 1	Pure compound		
Compound 2	Pure compound	*MCF-7*: Breast *Adenocarcinoma*	SRB(quick screening concentration)
Ef	Extract		

b1. Compound (1)

Table 4: The percent of cell viability in human breast cancer cell treated with compound (1).

Conc.	Cell Viability percent	Conc.	Cell Viability percent	Control Average
50µg/ml	78.75	100µg/ml	91.62	1.32
50µg/ml	82.67	100µg/ml	95.49	
50µg/ml	84.72	100µg/ml	104.50	
50 µg/ml AVG	82.05	100 µg/ml AVG	97.21	
50 µg/ml STD	2.47	100 µg/ml STD	5.40	



Control of breast cancer cells

Conc. 50 µg/ml of compound (1) against breast cancer cells

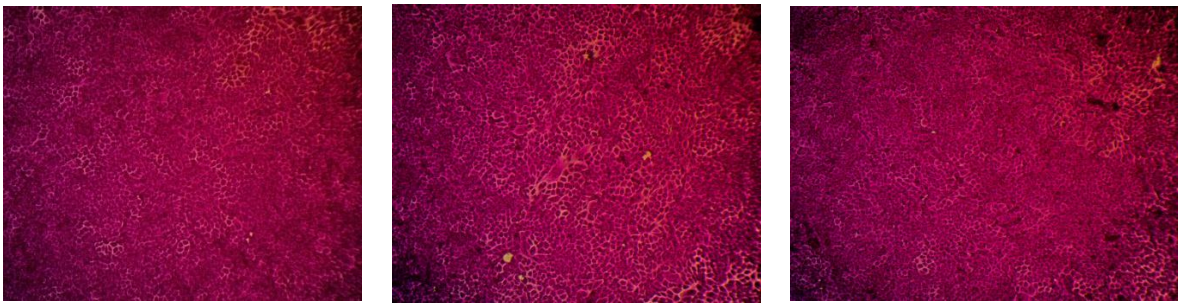
Conc. 100 µg/ml of compound (1) against breast cancer cells

Fig 5: Human breast cancer (*MCF-7*) cells treated with doses (50 and 100 µg/ml) of compound (1).

b2. Compound (2)

Table 5: The percent of cell viability in human breast cancer cell treated with compound (2) (fh7).

Conc.	Cell Viability percent	Conc.	Cell Viability percent	Control Average
50 µg/ml	97.25	100 µg/ml	92.69	**1.32**
50 µg/ml	97.72	100 µg/ml	91.03	
50 µg/ml	97.06	100 µg/ml	92.41	
50 µg/ml AVG	97.34	100 µg/ml AVG	92.04	
50 µg/ml STD	0.28	100 µg/ml STD	0.73	



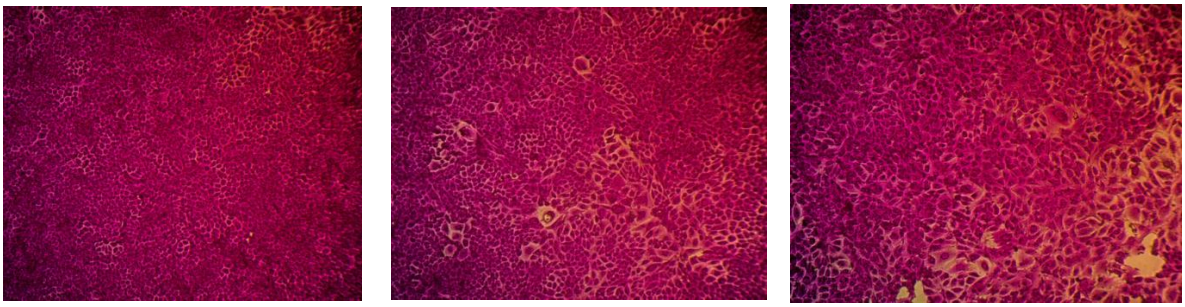
Control of breast cancer cells | Conc. 50 µg/ml of compound (2) against breast Cancer cells | Conc. 100 µg/ml of compound (2) against breast Cancer cells

Fig. 6: Human breast cancer (*MCF-7*) cells treated with doses (50 and 100 µg/ml) of compound (2).

b3. Extract (CH_2Cl_2:MeOH) (1:1 v/v).

Table 6: The percent of cell viability in human breast cancer cell treated with extract CH_2Cl_2: MeOH (1:1 v/v).

Conc.	Cell Viability percent	Conc.	Cell Viability percent	Control Average
50 µg/ml	81.85	100 µg/ml	58.06	0.03306
50 µg/ml	85.32	100 µg/ml	54.77	
50 µg/ml	84.87	100 µg/ml	56.57	
50 µg/ml AVG	84.02	100 µg/ml AVG	56.47	
50 µg/ml STD	1.54	100 µg/ml STD	1.35	



Control of breast cancer cells | Conc. 50 µg/ml of extract against breast cancer cells | Conc. 100 µg/ml of extract against breast cancer cells

Fig. 7: Human breast cancer (*MCF-7*) cells treated with doses (50 and 100 µg/ml) of extract CH_2Cl_2:MeOH (1:1 v/v)

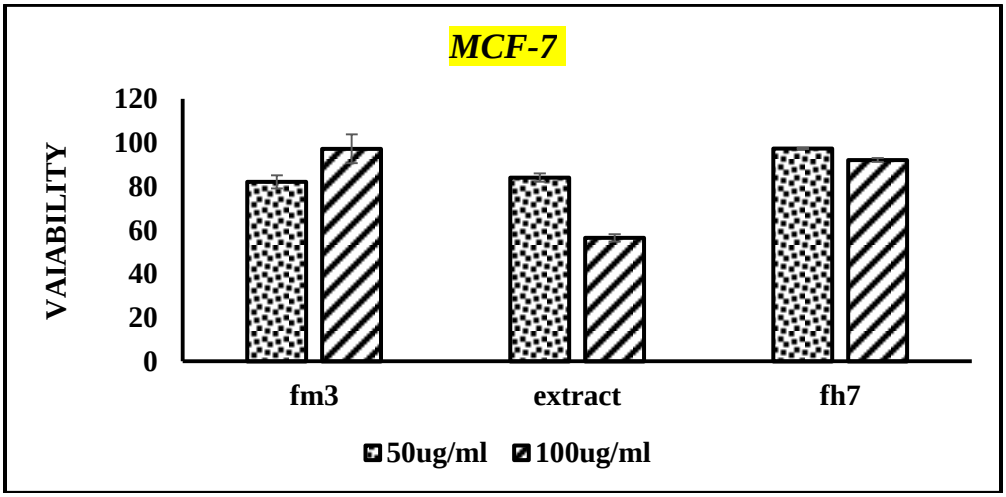


Fig. 8: Comparison between compound (1), compound (2) and extract *(*CH_2Cl_2: MeOH) (1:1) against breast cancer.

3.2 GC/MS of n-hexane fraction of Centaurea glomerata Vahl.

Centaurea is one of the largest and most diverse genera in the Asteraceae family, comprising more than 700 species distributed worldwide, with particular prevalence in the Mediterranean region, Western Asia, Eastern Anatolia, and the Balkan Peninsula (Reda et al., 2021; Teneva et al., 2024). The genus has attracted considerable scientific interest due to its rich phytochemical composition, including sesquiterpene lactones, triterpenes, flavonoids, lignans, and essential oils (Reda et al., 2021; Yıldırım et al., 2022).

In recent years, there has been growing scientific interest in characterizing the essential oils from various *Centaurea* species, as these may serve as valuable sources of bioactive compounds (Teneva et al., 2024). Studies have shown that the composition of these essential oils varies significantly among species and is influenced by genetic, geographic, climatic, and seasonal factors (Azadi & Mojab, 2017; Reda et al., 2021). Compounds such as caryophyllene oxide, germacrene D, spathulenol, and *β*-caryophyllene are frequently identified as major components in Centaurea EOs (Azadi & Mojab, 2017; Ertaş et al., 2014; Taştan et al., 2017).

Weighting 0.01g of sample (*n*-hexane extract) dissolves it in 1 ml of grade *n*-hexane takes 10 µl and dissolve in 1ml *n*-hexane. 1µl of the sample was injected automatically into the device. The gas chromatography and mass spectrophotometer used was Agilent technologies 7890A GC system, coupled with Agilent technologies 5977A-MSD mass spectrometer, this system was equipped with HP-5 GC column/Agilent (30 m 0.32 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as a carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:20 using the following temperature program: 80 ºC for 3 min; rising at 20.0ºC /min to 180 ºC and held for 1 min then rising at 4ºC / min to 220ºC and held for 1 min, rising at 20ºC/min to 250ºC and held for 5 min. Oil samples were diluted with *n*-hexane (1:10 v/v) and 1 µL of the mixtures were injected. Mass spectra were obtained by electron ionization, using a spectral range of *m/z* 50-550. Most of the compounds were identified using mass spectra **(Wiley spectral library collection and NSIT library)**.

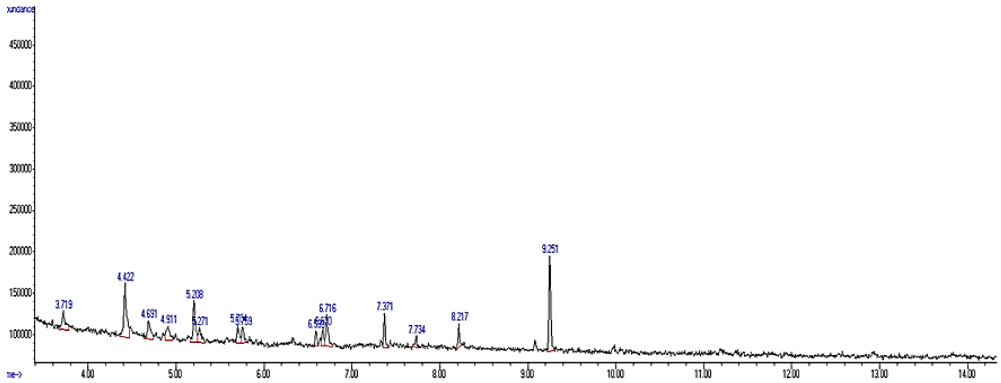
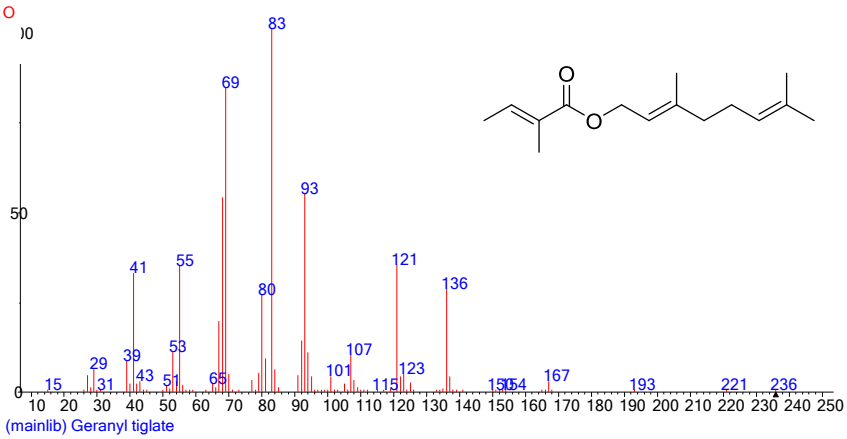


Fig. 9: GC Chromatogram for hexane extract of *Centaurea glomerata.*

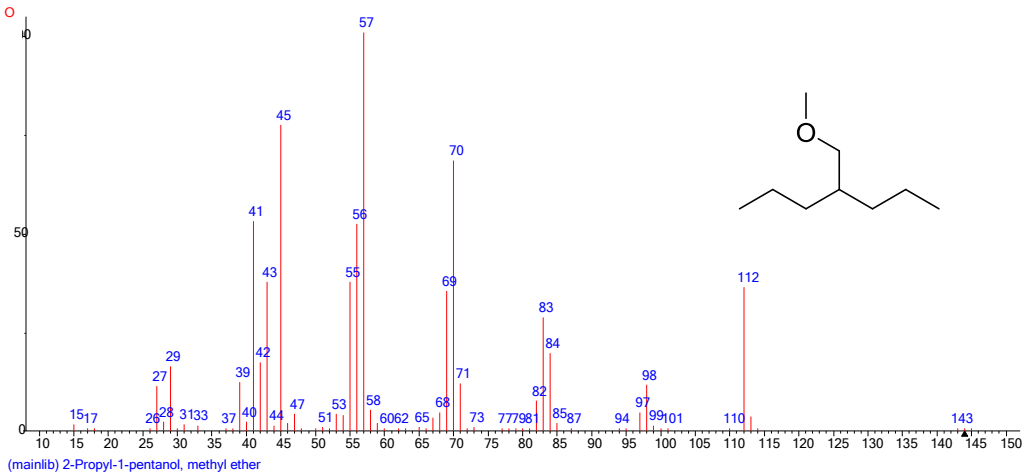
Table 7: Identified components in hexane extract of *C. glomerata* using GC-MS and their relative percentages.

No.	RT	Compound Name	M.Wt	Molcular Formula	AREA%
1	3.716	Geranyl tiglate	236	$C_{15}H_{24}O_2$	6.300
2	4.420	4-(methoxymethyl)heptane	144	$C_9H_{20}O$	15.891
3	4.689	Acetylene tetrachloride	166	$C_2H_2Cl_4$	6.259
4	4.912	3-Methyl-2-butenoic acid, 2-chlorophenyl ester	210	$C_{11}H_{11}ClO_2$	5.065
5	5.210	2,4-Dimethylheptane	128	C_9H_{20}	10.125
6	5.273	Chloroform	118	$CHCl_3$	7.801
7	5.702	2-O-dodecyl 1-O-hexyl oxalate	342	$C_{20}H_{38}O_4$	3.804
8	5.759	Bromodichloromethane	162	$CHBrCl_2$	4.221
9	6.594	Hexahydrobenzoyl chloride	146	$C_7H_{11}ClO$	2.493
10	6.715	2,2-Dichloro-1,1,1-trifluoroethane	152	$C_2HCl_2F_3$	7.885
11	7.373	5-methyltetradecane	212	$C_{15}H_{32}$	5.430
12	7.733	2,2-dichloroacetyl) 2,2-dichloroacetate	238	$C_4H_2Cl_4O_3$	2.331
13	9.249	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	$C_{14}H_{22}O$	18.636

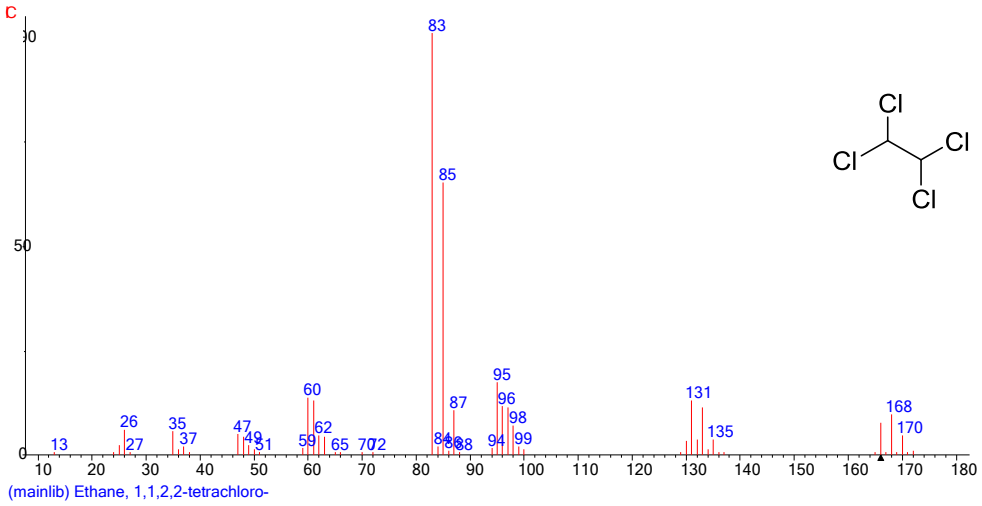
1 Geranyl tiglate



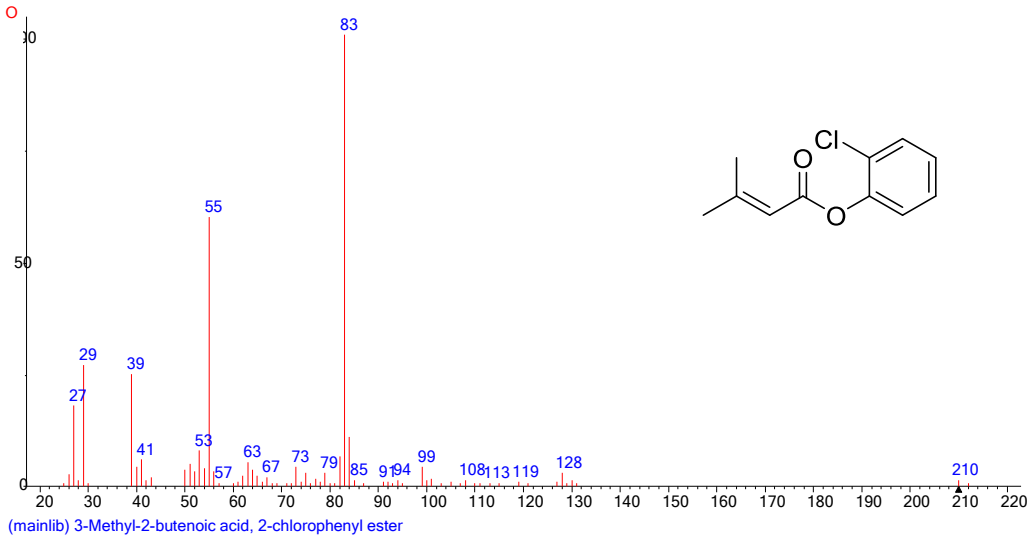
2 4-(methoxymethyl)heptane



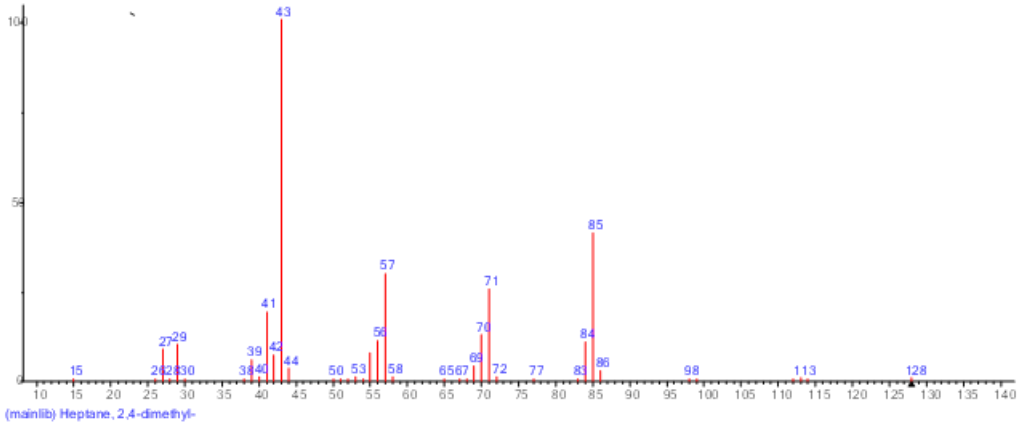
3 Acetylene tetrachloride



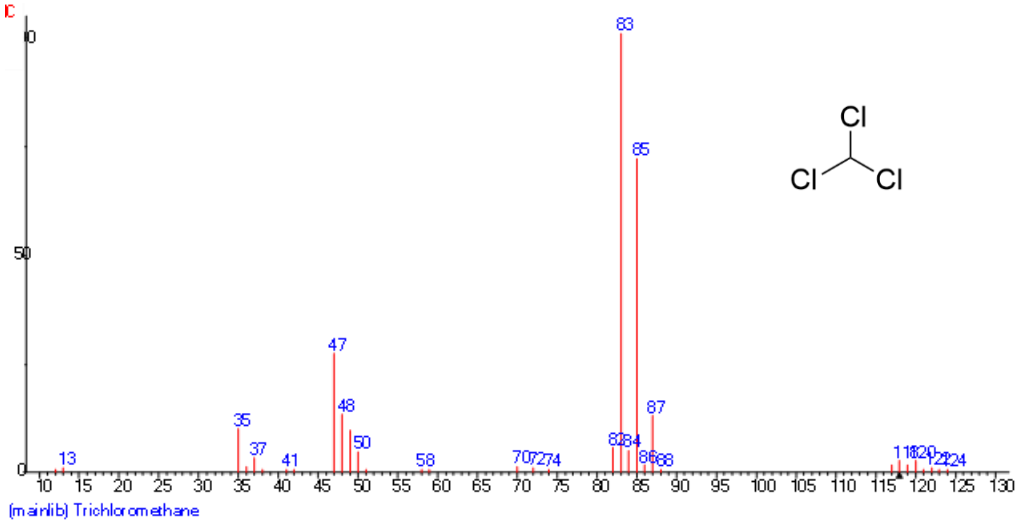
4 3-Methyl-2-butenoic acid, 2-chlorophenyl ester



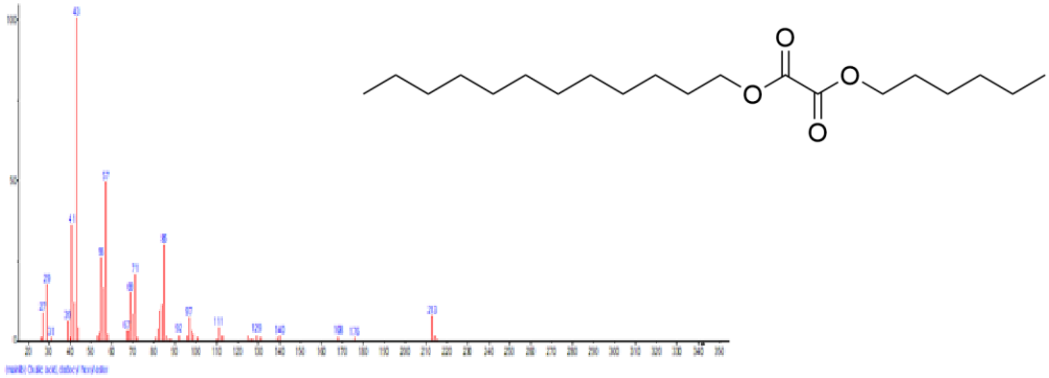
5 2,4-Dimethylheptane



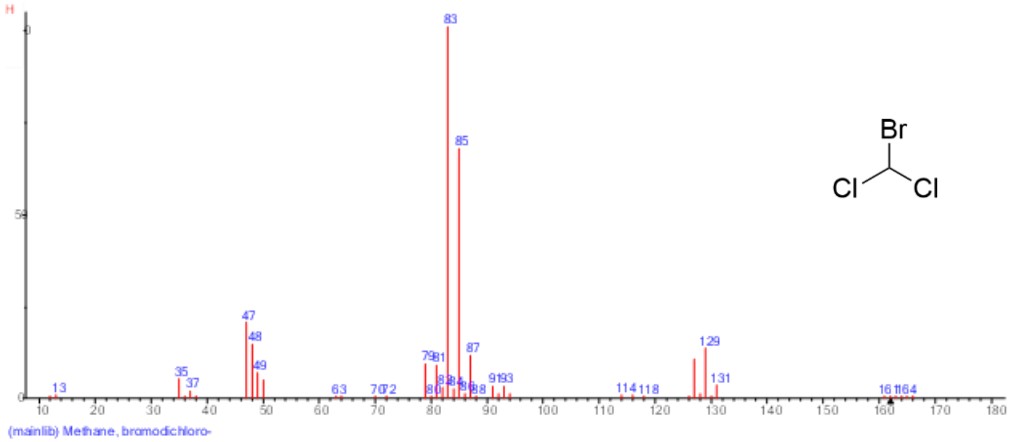
6 Chloroform



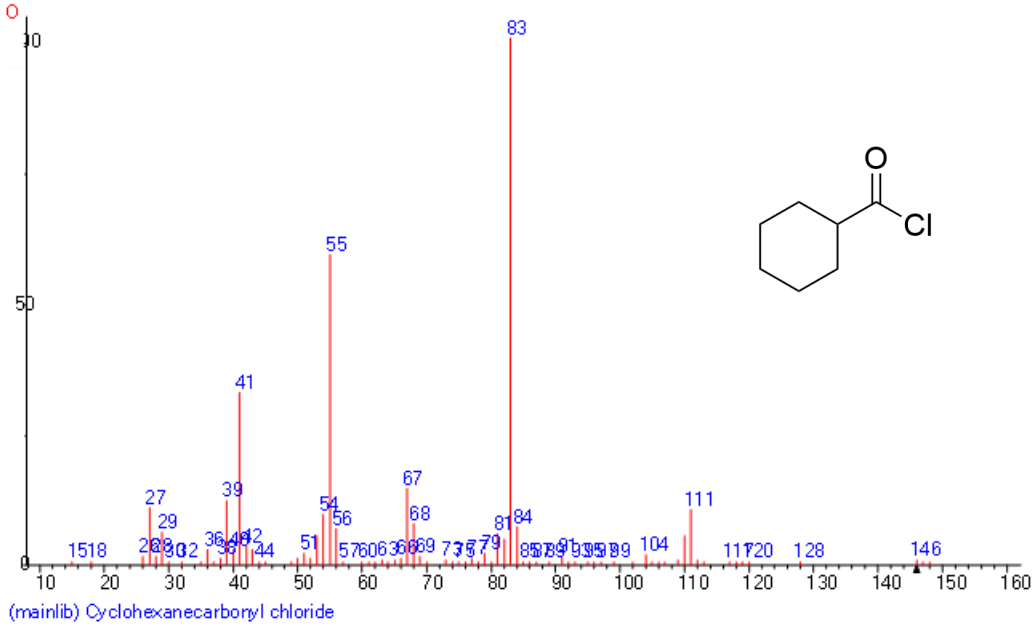
7 2-O-dodecyl 1-O-hexyl oxalate



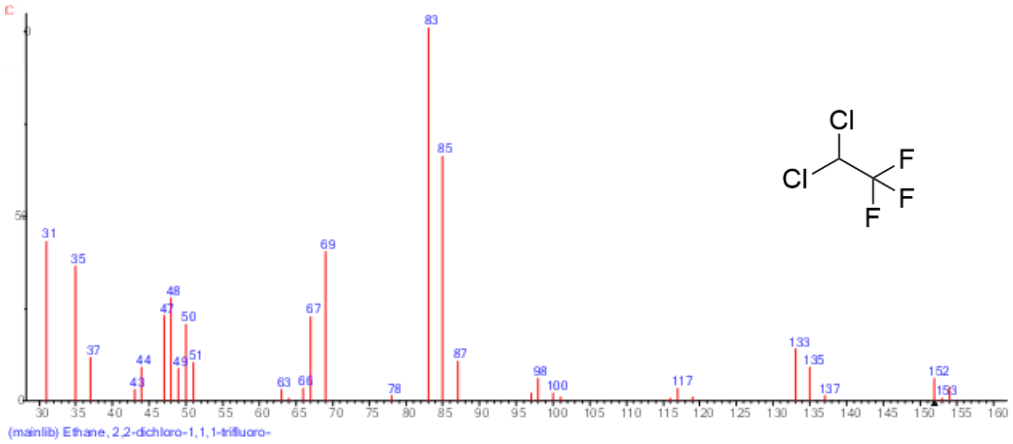
8 Bromodichloromethane



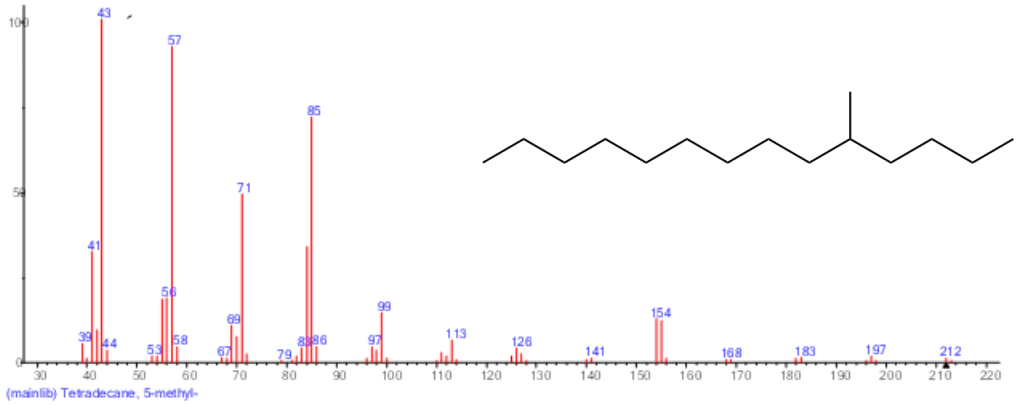
9 Hexahydrobenzoyl chloride



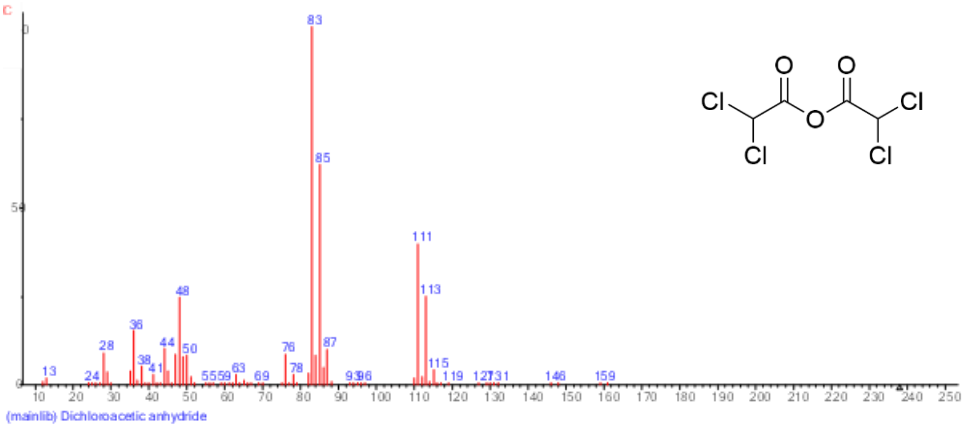
10 2,2-Dichloro-1,1,1-trifluoroethane



11 5-methyltetradecane



12 2,2-dichloroacetyl-2,2-dichloroacetate



13 Phenol, 2,4-bis(1,1-dimethylethyl)-

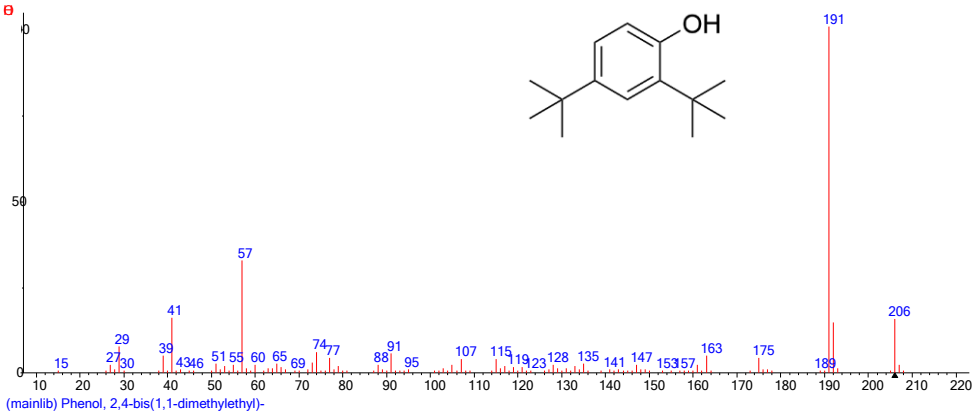


Figure 8: Mass spectrum of *n*-hexane extract of *Centaurea glomerata* Vahl.

3. Conclusion

This study highlights the phytochemical complexity and cytotoxic potential of *Centaurea glomerata* extract. The GC-MS analysis of *n*-hexane extract identified several volatile compounds, while the isolation and characterization of three major compounds provided insights

into the chemical diversity of this species. Although the isolated compounds showed limited cytotoxicity, the methanol-dichloromethane extract demonstrated significant growth inhibition in both *HepG2* and *MCF-7* cancer cell lines, particularly at higher concentrations. This suggests that the anticancer activity of *C. glomerata* may be attributed to the synergistic effects of multiple bioactive components rather than individual compounds. Future research should focus on optimizing extraction methods, exploring the mechanisms of action, and evaluating the *in vivo* efficacy of these extracts. The findings underscore the potential of *C. glomerata* as a source of novel anticancer agents and contribute to the growing body of knowledge on the medicinal properties of the *Centaurea* genus.

Conflict of interest statement:

The authors declare no conflicts of interest regarding this manuscript.

Ethical approval:

Not applicable.

Acknowledgment:

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