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Anticancer Mechanism of the Non-polar Extract from *Echium angustifolium* Mill. Aerial Parts in Relation to Its Chemical Content

Hanan M. El-Tantawy, Ahmed R. Hassan*, Hala E. Taha

Medicinal and Aromatic Plants Department, Desert Research Center, El-Matariya 11753, Cairo, Egypt

Abstract

Our previous findings elucidated that the hydroethanolic extract of *Echium angustifolium* Mill. aerial parts and its defatted (polar) fraction supported by bio-guided fractionation possessed potential anticancer and antioxidant activities, as well as other documented plant therapeutic uses. Consequently, the present study aimed to evaluate the hexane (non-polar) extract of *E. angustifolium* aerial parts for *in vitro* anticancer, antioxidant, and anti-inflammatory activities accompanied by characterization of its bioactive constituents using GC-MS and LC-ESI-MS techniques in order to gain a deeper understanding of this medicinal plant that could be developed as a phytomedicine. This extract exhibited high inhibition against HCT116 (IC_{50} = 12 µg/mL) & HEPG2 (IC_{50} = 18 µg/mL) cancer cell lines, with antioxidant and anti-inflammatory potentials. GC-MS of the analyzed extract led to identifying 24 volatile constituents, among them, ethyl esters of palmitic acid and linoleic acid were the most abundant oxygenated compounds. Meanwhile, 10 nonvolatile components were annotated by LC-ESI-MS comprising five phenolic acid derivatives, two lignans, echimidine, acetylshikonin, and quinic acid. Moreover, some of these phytoconstituents were identified in this plant species for the first time. These results justify using non-polar *E. angustifolium* extract as a possible source of anticancer, antioxidant, and anti-inflammatory agents, which can be better confirmed by further *in vivo* studies.

Keywords: *Echium angustifolium*; Non-polar Extract; Biological Activity; Chemical Profile; GC-MS; LC-ESI-MS

1. Introduction

During carcinogenesis, normal cells undergo a series of changes such as altered proliferative capacity, metastatic potential, and invasiveness. Among the various molecules and factors that have been proposed to be involved in the etiology of cancers, cyclooxygenase-2 (COX-2) and oxidative stress are associated with several types of cancers. Firstly, excess reactive oxygen species (ROS) are produced over a long time, and thus significant damage may occur to cell structure and functions, then that damage may induce mutations and neoplastic transformation leading to progression of inflammation, and finally causing cancer initiation [1-3]. Therefore, anticancer agents have demonstrated properties to inhibit the proliferation of cancer cells and induce apoptotic cell death [4] through varieties of mechanisms including their antioxidant or anti-inflammatory characteristics [5, 6]. It is thought that plant-derived compounds such as polyphenols in a person's diet can improve health and reduce the risk of cancers by being natural antioxidants or anti-inflammatory agents [4, 7]. So, it become of interest to search for natural anticancer agents of plant origin.

Genus *Echium* L., Boraginaceae family, comprises 67 species, which are distributed in many regions around the world. The medicinal use of the *Echium* species can be referred many years ago in the Mediterranean countries. Various reports from folk medicine listed the importance of *Echium* plants as a phytomedicine due to their antioxidant, anti-inflammatory, sedative and anxiolytic effects [8]. *Echium angustifolium* Mill. is a wild perennial plant used for grazing and medical purposes in Northwestern of Egypt [9]. Some studies reported the anticancer potential for *E. angustifolium* extracts and pure compounds [10, 11], and antioxidant activity [11, 12]. The plant was noticed to be biologically active owing to the existence of different classes of metabolites such as pyrrolizidine alkaloids

*Corresponding author e-mail: drarahdrc@gmail.com
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(echimidine) [13], lignans [10, 11], spermidine phenolic acid derivatives, phenolic acids [11], and unsaturated fatty acids (linoleic, linoleic, and stearidonic acids) [14].

Our previous results which revealed the potential anticancer activity of the total (80% EtOH) and the defatted extracts from *Echium angustifolium* aerial parts supported by bio-guided fractionation against liver and colon cancer cell lines [11]. In our course for a better knowledge of this medicinal plant, it is of great interest to subsequently characterize the chemical profile and bioactivities of hexane (non-polar) *E. angustifolium* extract as well. Therefore, the aim of this study was to assess the effect of the non-polar plant extract against colorectal (HCT116) and hepatocellular (HEPG2) cancer cell lines in relation to its *in vitro* antioxidant (DPPH & ABTS assays) and anti-inflammatory (cyclooxygenase (COX) inhibition assay) potentials. Additionally, utilizing GC-MS and LC-ESI-MS techniques, bioactive compounds in that fraction should be identified.

2. Experimental

Plant Material

In April 2017, the aerial parts of the plant *Echium angustifolium* Mill. were collected from the Agiba region of Mersa Matruh Governorate on Egypt's Northwestern coast. Prof. Dr. Azza El-Hadidy, Professor of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Egypt, authenticated the plant sample and Its voucher specimen (Ea-2017-A) was deposited in the Herbarium of Cairo University, Faculty of Science.

Extraction and Fractionation

E. angustifolium aerial parts were air-dried and then ground to afford 2 kg of the plant powder. This powder was extracted with 80% ethanol *via* room temperature maceration (3 × 6L, each 48 hours). The extracts were combined and concentrated under vacuum at 45 °C until dry, yielding 146 g of the total extract. The hydro-ethanolic extract was previously exposed to *in vitro* anticancer experiment. Then, the total extract was fractionated using liquid-liquid fractionation by suspending the dried extract in 70% MeOH (600 mL) in a separating funnel and partition with *n*-hexane, giving 33.8 g of *n*-hexane fraction (henceforth, non-polar extract) and 105 g of the defatted fraction (polar extract) [11]. Then, the non-polar *E. angustifolium* extract was tested *in vitro* for anticancer, antioxidant, and anti-inflammatory effects. Furthermore, the phytoconstituents in this non-polar extract were identified *via* GC-MS and LC-ESI-MS.

In Vitro Anticancer Activity

The colon (HCT116) and liver (HEPG2) human cancer cell lines were obtained in frozen state under liquid nitrogen (-180 ºC) from the American Type Culture Collection. The cancer cell lines were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt. The cells were suspended in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) in presence of 1% antibiotic antimycotic mixture (10.000 U/mL K-penicillin, 10.000 U/mL streptomycin sulphate and 25 µg/mL amphotericin B) and 1% L-glutamine (all purchased from Lonza, Belgium). The *in vitro* anticancer activity of non-polar extract of *E. angustifolium* aerial parts was studied using the method previously reported by [15]. Cells were plated in 96-multiwell plate (10^4 cells /well) for 24 hours before treatment with the evaluated extract. Different concentrations of the tested extract (0, 5, 12.5, 25 and 50 µg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the investigated extract for 48 hours at 37 ºC and atmosphere of 5% CO_2. Thereafter, the cells were fixed, washed and stained with sulforhodamine B stain (SRB). Excess stain was washed with acetic acid and attached stain was recovered Tris-EDTA buffer Color intensity was measured in an ELISA reader. The relation between surviving fraction and extract concentration is plotted to get the survival curve of each tumor cell line after the examined extract was added. The IC_{50} value of the tested extract (µg/mL) is expressed as the mean value of data points ± SD (n = 3).

In Vitro Antioxidant Activity

The antioxidant activity of the non-polar extract from *E. angustifolium* aerial parts was inspected using *in vitro* DPPH and ABTS methods.

DPPH (2, 2'-diphenyl-1-picryl-hydrazyl hydrate) Free Radical Assay:

Seven concentrations of trolox standard were prepared from the stock solution (1000 µM) in methanol including 5, 10, 20, 40, 50, 60, and 80 µM. Solution of 0.1 mg/mL of the non-polar extract in methanol was also prepared. Then, 100 µL of freshly prepared DPPH reagent (0.1 % in methanol) were added to 100 µL of the tested extract in 96 wells plate (n = 6). Then, the reaction was incubated in dark at room temperature for 20 min [16, 17]. The resulting decrease in DPPH color intensity was recorded using microplate reader FluoStar Omega (BMG LABTECH, Germany) at 540 nm. Data are represented as means ± SD according to the following equation and expressed as µM TE/ mg extract using the linear regression equation extracted

from the linear dose-inhibition calibration curve of Trolox.

Percent inhibition = [(Average absorbance of blank – Average absorbance of the test) /Average absorbance of blank] × 100

ABTS (2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonate) Assay:

The tested extract (0.1 mg/mL) and trolox stock solution of 1 mM in methanol were prepared, and subsequently eight serial dilutions from Trolox were prepared in the concentrations of 700, 600, 500, 400, 300, 200, 100 and 50 µM. Thereafter, 192 mg of ABTS were dissolved in 50 mL volumetric flask with distilled water. 1 mL from this solution was added to 17 µL of 140 mM potassium persulphate and the mixture was left for 24 hours in the dark. Then, 1 mL of the reaction mixture was diluted with methanol to the volume of 50 mL to afford the freshly prepared ABTS reagent. 190 µL from this reagent were mixed with 10 µL of the investigated extract in 96 wells plate (n = 4). Finally, the reaction was incubated for 120 min at room temperature in dark and the reduction in intensity of ABTS color was examined by microplate reader FluoStar Omega at 734 nm [18]. Data are represented as means ± SD (µM TE/ mg extract) according to the previously mentioned equation.

In Vitro Anti-infammatory Activity (In Vitro Cyclooxygenase (COX) Inhibition Assay):

The ability of the tested non-polar extract to inhibit the conversion of Arachidonic Acid (AA) to prostaglandin H2 (PGH2) by ovine COX-1 and human recombinant COX-2 was determined using an enzyme immunoassay (EIA) (kit catalogue number 560131, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The inhibitory assays were performed in the presence of non-polar extract/anti-inflammatory drug (Celecoxib) with various concentrations (0, 0.039, 0.156, 0.625 and 2.5 µg/mL) on COX-2, and at serial concentrations (0, 5, 12.5, 25, 50 and 100 µg/mL) on COX-1, which dissolved in DMSO. Briefly, to a series of supplied reaction buffer solutions (950 µL, 0.1 M Tris-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 µL) enzyme in the presence of heme (10 µL). Then, 20 µL from each concentration of test extract/standard drug were added to get a final volume of 1 mL. These solutions were incubated at 37 °C for 10 min, after which 10 mL of AA (100 mM) solution were added and the COX reaction was stopped by the addition of 50 µL of 1 M HCl after 2 min. PGF2α, produced from PGH2 by reduction with stannous chloride was measured by enzyme immunoassay. Thereafter, the plate is washed to remove any unbound reagents and then Elman's reagent (which contains the substrate to acetylcholine esterase) is added to the well. The product of this enzymatic reaction produces a distinct yellow color. The intensity of this color was determined spectrophotometric at 410 nm [19]. The anti-inflammatory effect of the examined extract was obtained by calculating percent inhibition by the comparison of extract treated to various control incubations. The concentration of the tested extract causing 50% inhibition (IC_{50}, µg/mL) was calculated from the concentration-inhibition response curve (triplicate determinations).

GC-MS Analysis of the Non-polar Plant Extract

GC-MS analysis of the non-polar extract from *E. angustifolium* aerial parts were carried out using Trace GC-ISQ mass spectrometer (Thermo Scintific, Austin, TX, USA) with a direct capillary column TG-5MS of 30 m length, 0.25 µm internal diameter and 0.25 µm film thickness. Sample was injected under the following conditions: column oven temperature was initially held at 50 °C, then increased by 5 °C/min to 250 °C (hold for 2 min), and then increased to the final temperature 300 °C/min by 30 °C and hold for 2 min. The injector and MS transfer line temperatures were kept at 270, 260 °C respectively; Helium was used as a carrier gas a constant flow rate of 1 mL/min. The solvent delay was 4 min and diluted sample of 1 µL was injected automatically using autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50-650 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their relative retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral databases [20].

LC-ESI-MS/MS Analysis of the Non-polar Plant Extract

The chemical profile for the non-polar extract of *E. angustifolium* aerial parts was also achieved by LC-ESI-MS on an Acquity-UPLC™ system (Waters, Milford, USA) equipped with BEH-C18 column, with 2.1 × 50 mm dimensions and a 1.7 µm particle size. The mobile phase consisted of H_2O as solvent (A) and CH_3CN as solvent (B), each containing 0.1% of HCOOH. The gradient program was carried out using a gradient of solvent B in solvent A over 32 min at a flow rate of 0.2 mL/min as follows: 0–2 min (10% of solvent B), 2–5 min (30% B), 5–15 min (70% B), 15–22 min (80% B), 22–26 min (80% B), 26–29 min (100% B), 29–32 min (10% B). The ionization parameters were as follows: source temperature 150 °C, cone voltage 30 eV، capillary voltage 3 kV, dissolution temperature 400 °C, cone gas flow 50 L h^{-1}, and dissolution gas flow 600 L h^{-1}.

Mass spectra of the extract's compounds were detected by XEVO TQD triple quadrupole mass spectrometer equipped with ESI detector within the *m/z* range of 100 and 1000 for both positive (ESI^+) and negative (ESI^-) ionization modes [21]. Data acquisition and processing were performed using the Masslynx 4.1.

3. Results and Discussion

On the basis of our previous study, which represented the fractionation of the total extract (80% EtOH) of *E. angustifolium* aerial parts by partition chromatography using *n*-hexane to furnish defatted (polar) and hexane (non-polar) extracts. After that, *in vitro* anticancer activity was investigated only for the total extract and the polar fraction [11]. Consequently, examination of the non-polar fraction as anticancer besides its antioxidant and anti-inflammatory properties were studied in the current work, accompanied by identification of its chemical contents using GC-MS and LC-ESI-MS.

In Vitro Anticancer Activity

The IC_{50} of the total (80% EtOH) extract of *E. angustifolium* plant against liver (HEPG2) and colon (HCT116) cancer cell lines were previously estimated as 22 and 15 µg/mL, respectively [11]. Herein, the non-polar extract under research inhibited the proliferation of both HEPG2 and HCT116 cancer cells with IC_{50} values of 18 and 12 µg/mL compared to doxorubicin had IC_{50} of 4.57 and 3.73 µg/mL, respectively (Table 1). Different *E. angustifolium* extracts and their isolated compounds have previously been shown to have *in vitro* anticancer effects towards breast (MCF7), colon (HCT116), and liver (HEPG2) cancer cell lines [10, 11]. These reports, combined with the above-mentioned findings, validated *E. angustifolium* plant extracts' remarkable anticancer potential.

LC/MS analysis of the non-polar extract that will be elaborated later revealed the presence of anticancer phytochemicals such as phenolic acid derivatives having hydroxyl group substitutions, which also have antioxidant and anti-inflammatory activities [22-24]. Moreover, GC/MS technique that will be also discussed later detected a variety of fatty acids and their derivatives, such as palmetic and linoleic acids, which are known for their anti-inflammatory properties [25, 26], implying that they could be used as anticancer agents. As a result, it's possible that the anticancer effect of *E. angustifolium* non-polar extract against liver and colon cell lines is attributable to the presence of fatty acids and phenolic acid derivatives.

In Vitro Antioxidant Activity

Antioxidant activities of some extracts from *E. angustifolium* aerial parts have previously been demonstrated [11, 27, 28], so it grabbed our attention to assess antioxidant power of the non-polar extract that could be act as an anticancer agent. The non-polar extract induced a moderate antioxidant effect by two *in vitro* techniques (DPPH and ABTS). Trolox equivalent antioxidant capacity was determined, which yielded values of 131.05 and 1407.24 µM TE/ mg extract using DPPH and ABTS tests, respectively (Table 2). This extract was proven to include a number of well-known antioxidants, including cinnamic acid derivatives [29], and acetylshikonin [30]. Despite this, antagonism between substances in the plant mixture could explain the mild effect [31].

In Vitro Anti-infammatory Activity (In Vitro Cyclooxygenase Inhibition Assay)

The non-polar extract from *E. angustifolium* aerial parts and the conventional drug celecoxib have been evaluated for their inhibitory activity of COX-1 and COX-2 isozymes using an ovine-COX-1/COX-2 assay kit. Assuming that COX-2 enzyme is expressed by a variety of cancerous cells and is linked to carcinogenesis [32], accordingly, the cyclooxygenase inhibitors effect for the non-polar extract was assessed as a potential anti-inflammatory mechanism for cancer cell suppression. The non-polar extract inhibited the COX-2 enzyme with IC_{50}= 0.089 µg/mL, indicating that the extract may have anti-inflammatory properties that are greater than celecoxib, which has an IC_{50} of 0.31 µg/mL (Table 2).

The selective inhibition of COX-2 is a great achievement in the search for powerful anti-inflammatory drugs with fewer side effects. So, when the inhibition effects of our non-polar extract and celecoxib on the COX-1 enzyme were compared, it was noticed that celecoxib, with an IC_{50}= 95.7 µg/mL and a selectivity index (SI) of 308.71, was still highly selective than the non-polar extract, with an IC_{50} of 12.5 µg/mL and a selectivity index of 140.44. The non-polar extract was found to include a variety of phytochemicals, including oleic, palmitic, and linoleic acids, as well as phenolic compounds, which help to control inflammation by inhibiting the COX-2 enzyme [33, 34]. Finally, these data suggest that non-polar extract of *E. angustifolium* aerial parts, which has a possible COX-2 enzyme selectivity, may have anti-cancer capabilities due to its COX-2 inhibitory anti-inflammatory activity.

Table 1: *In vitro* anticancer activity of *E. angustifolium* aerial parts extracts against HEPG2 and HCT116 cancer cell lines

Sample/Doxorubicin	IC_{50} (µg/mL)[a] Mean ± SD	
	HEPG2	HCT116
Total extract (80% EtOH)	22 ± 0.6	15 ± 1.1
Non-polar extract	18 ± 0.5	12 ± 1.4
Doxorubicin (positive control)	4.57 ± 0.5	3.73 ± 0.6

[a] IC_{50} value is the concentration of the tested extract (µg/mL) that decreased the number of viable cells by 50%.

Table 2: *In vitro* antioxidant and anti-inflammatory activities of the non-polar extract from *E. angustifolium* aerial parts

Sample	Antioxidant activity Mean ± SD		Anti-inflammatory activity		
	µM TE/ mg extract		IC_{50} (µg/mL)[a] Mean ± SD		SI[b]
	DPPH	ABTS	COX-1	COX-2	
Non-polar extract	131.05 ± 11.08	1407.24 ± 85.36	12.5 ± 0.2	0.089 ± 0.001	140.44
Celecoxib[c]	-	-	95.7 ± 0.1	0.31 ± 0.01	308.71

[a] IC_{50} value is the extract concentration required to produce 50% inhibition of COX-1 or COX-2 for means of two determinations and deviation from the mean is < 10% of the mean value.
[b] Selectivity index IC_{50} (COX-1)/IC_{50} (COX-2).
[c] Standard selective COX-2 inhibitor

GC-MS Analysis of the Non-polar Plant Extract

24 compounds were identified in the non-polar extract of *E. angustifolium* aerial parts through GC-MS (Figure 1 & Table 3). These identified components consisted mainly of a high relative percentage of 17 non-oxygenated hydrocarbons (71.33%) in addition to 7 oxygenated compounds represented 28.04%. The non-oxygenated aromatic hydrocarbons were a combination of phenyl-substituted acyclic alkanes (ranging from C_{10} to C_{13}) with major compounds; 5-phenyldodecane (13.7%) and 5-phenylundecane (9.92%). The oxygenated compounds included two fatty acids (palmitic acid and oleic acid), four fatty acid esters (palmitic acid methyl ester, palmitic acid ethyl ester, linoleic acid ethyl ester, and stearic acid ethyl ester), and a sesquiterpene lactone (tetraneurin-A-diol). Among them, the most prevalent constituents were palmitic acid ethyl ester (11.32%) and linoleic acid ethyl ester (7.37%).

LC-ESI-MS/MS Analysis of the Non-polar Plant Extract

LC-ESI-MS chemical profile of the non-polar extract produced from *Echium angustifolium* aerial parts revealed the tentative annotation of ten components (peaks **1**, **3-11**, and **13**) along with two unknown nitrogenous compounds (peaks **2** and **12**) on the basis of MS data, and by comparing their molecular ion peaks with the reported data. The typical base peak ion chromatogram (BPC) with positive and negative ionization modes of the analysed fraction was shown in Figure 2. The suggested compounds and their basic structures were summarized in Table 4, and depicted in Figure 3, respectively. The ESI-MS spectrum of each identified peak in BPC of the nonpolar fraction exhibited a characteristic adduct ion $[M+H]^+$ in positive ion mode and $[M-H]^-$ in negative mode.

The peaks labeled as; **1**, **2**, **5**, **6**, **8**, and **11** were inferred to be phenolic acid derivatives, among which peak **5** showed pseudo-molecular ion peak at *m/z* 438 $[M+H]^+$ as well as a fragment ion at *m/z* 146 $[M+H-292]^+$ in accordance with the loss of two coumaroyl moieties. Based on that information and by comparing with our previous findings [11], this compound was found to be dicoumaroyl spermidine [35]. Peaks **2** and **8** displayed the same mass spectra in both ionization modes, indicating a molecule with a molecular mass of 208, which we previously suggested as dimethylcaffeic acid [11, 36]. Similarly, the rest peaks that were categorized as phenolic acids (**1**, **6**, and **11**) were tentatively identified as methoxy cinnamic acid [37, 38], rosmarinic acid [15, 39], and caffeic acid [21, 36], respectively as shown in Table 4. Moreover, peak **10** presented $[M+H]^+$ and $[M-H]^-$ at *m/z* 193 and 191, respectively, suggesting that this compound was recognized as quinic acid [15, 40]. Peak **4** had *m/z* 398 $[M+H]^+$, indicating that it is related to echimidine [13, 41]. Meanwhile, peaks **9**, **7**, and **13** depicted $[M-H]^-$ at *m/z* 329, 357, and 721, and by referring to literature they were tentatively annotated as acetylshikonin [42, 43], pinoresinol [39], and trigonotin C [44], respectively.

A literature survey revealed that caffeic acid, dimethylcaffeic acid, echimidine, dicoumaroyl spermidine, rosmarinic acid, acetylshikonin, and pinoresinol have been previously identified from *E. angustifolium* [11, 13, 39, 45]. Additionally, quinic acid was also found in other *Echium* species (*Echium humile*) [40]. However, methoxy cinnamic acid and

trigonotin C were observed for the first time in this genus.

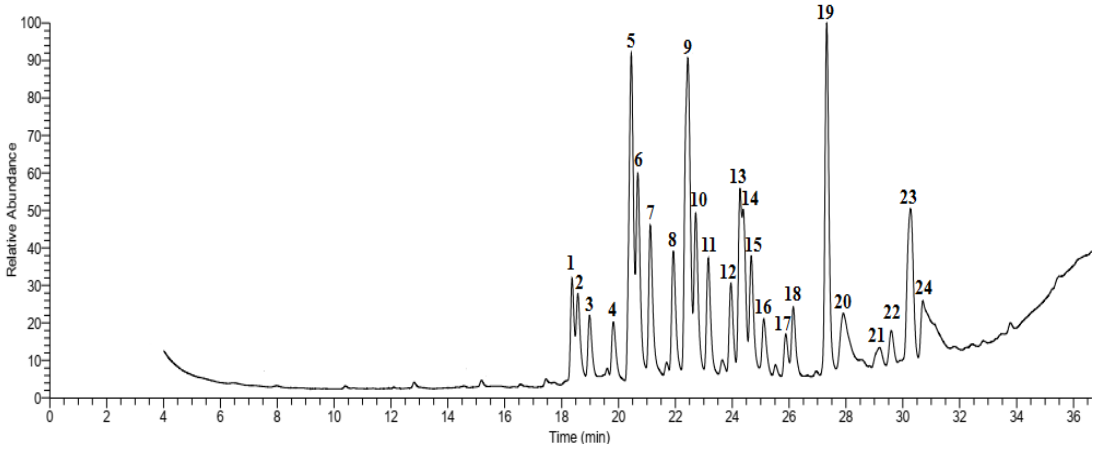

Figure 1: GC-MS chromatogram of the non-polar extract from *E. angustifolium* aerial parts

Table 3: Phytocomponents identified in the non-polar extract of *E. angustifolium* aerial parts by GC-MS.

	RT (min)	Name of the compound	Molecular formula	MW	Peak area (%)
1	18.37	5-phenyldecane	$C_{16}H_{26}$	218	2.66
2	18.58	4-phenyldecane	$C_{16}H_{26}$	218	1.76
3	18.98	3-phenyldecane	$C_{16}H_{26}$	218	1.97
4	19.81	2-phenyldecane	$C_{16}H_{26}$	218	1.81
5	20.45	5-phenylundecane	$C_{17}H_{28}$	232	9.92
6	20.68	4-phenylundecane	$C_{17}H_{28}$	232	4.29
7	21.11	3-phenylundecane	$C_{17}H_{28}$	232	4.55
8	21.93	2-phenylundecane	$C_{17}H_{28}$	232	3.86
9	22.44	5-phenyldodecane	$C_{18}H_{30}$	246	13.70
10	22.71	4-phenyldodecane	$C_{18}H_{30}$	246	3.87
11	23.15	3-phenyldodecane	$C_{18}H_{30}$	246	3.61
12	23.95	2-phenyldodecane	$C_{18}H_{30}$	246	2.94
13	24.28	6-phenyltridecane	$C_{19}H_{32}$	260	5.83
14	24.39	5-phenyltridecane	$C_{19}H_{32}$	260	4.48
15	24.67	4-phenyltridecane	$C_{19}H_{32}$	260	3.17
16	25.11	3-phenyltridecane	$C_{19}H_{32}$	260	1.64
17	25.88	2-phenyltridecane	$C_{19}H_{32}$	260	1.27
18	26.15	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	270	1.94
19	27.32	Palmitic acid ethyl ester	$C_{18}H_{36}O_2$	284	11.32
20	27.89	Palmitic acid	$C_{16}H_{32}O_2$	256	1.94
21	29.18	Oleic Acid	$C_{18}H_{34}O_2$	282	1.22
22	29.59	Tetraneurin-A-diol	$C_{15}H_{20}O_5$	280	1.21
23	30.27	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308	7.37
24	30.69	Stearic acid ethyl ester	$C_{20}H_{40}O_2$	312	3.04

Total identified compounds 99.37%
Non-oxygenated hydrocarbons 71.33%
Oxygenated compounds 28.04% including fatty acid derivatives (26.83%)

RT = Retention Time, MW= Molecular Weight

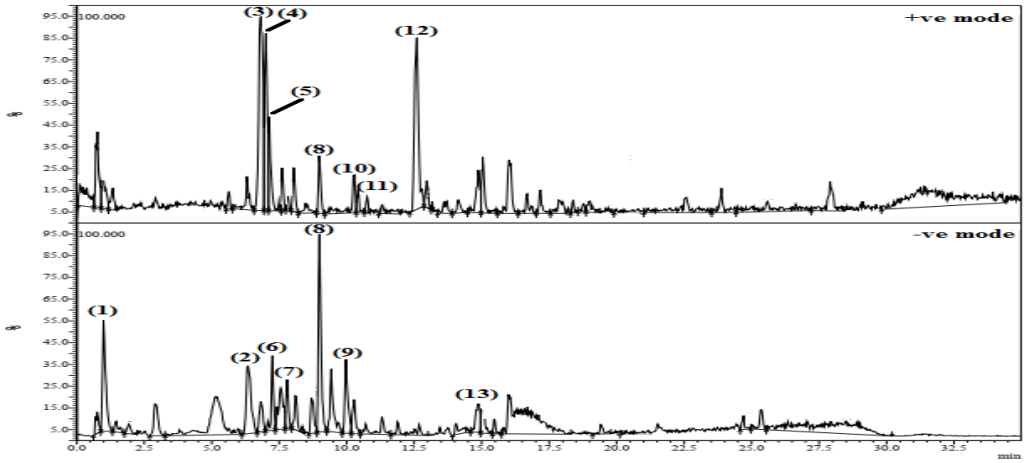

Figure 2: LC-ESI-MS base peak chromatogram of the non-polar extract from *E. angustifolium* aerial parts

Table 4: The suggested Phytochemicals in the non-polar extract of *E. angustifolium* aerial parts by LC-ESI-MS analysis in both negative and positive ionization modes

Identified peaks	RT (min)	$(M-H)^-$ *m/z*	$(M+H)^+$ *m/z*	Tentative identification	References
1	0.99	177	-	Methoxy cinnamic acid	[37, 38]
2	6.32	207	209	Dimethylcaffeic acid	[11, 36]
3	6.82	-	446	Unknown nitrogenous compound	[13]
4	7.00	-	398	Echimidine	[13, 41]
5	7.13	-	438	Dicoumaroyl spermidine	[11, 35]
6	7.63	359	-	Rosmarinic acid	[15, 39]
7	7.79	357	-	Pinoresinol	[39]
8	8.99	207	209	Dimethylcaffeic acid isomer	[11, 36]
9	9.97	329	-	Acetylshikonin	[42, 43]
10	10.27	193	191	Quinic acid	[15, 40]
11	10.42	-	181	Caffeic acid	[21, 36]
12	12.59	-	274	Unknown nitrogenous compound	[13]
13	14.88	721	-	Trigonotin C	[44]

RT = Retention Time

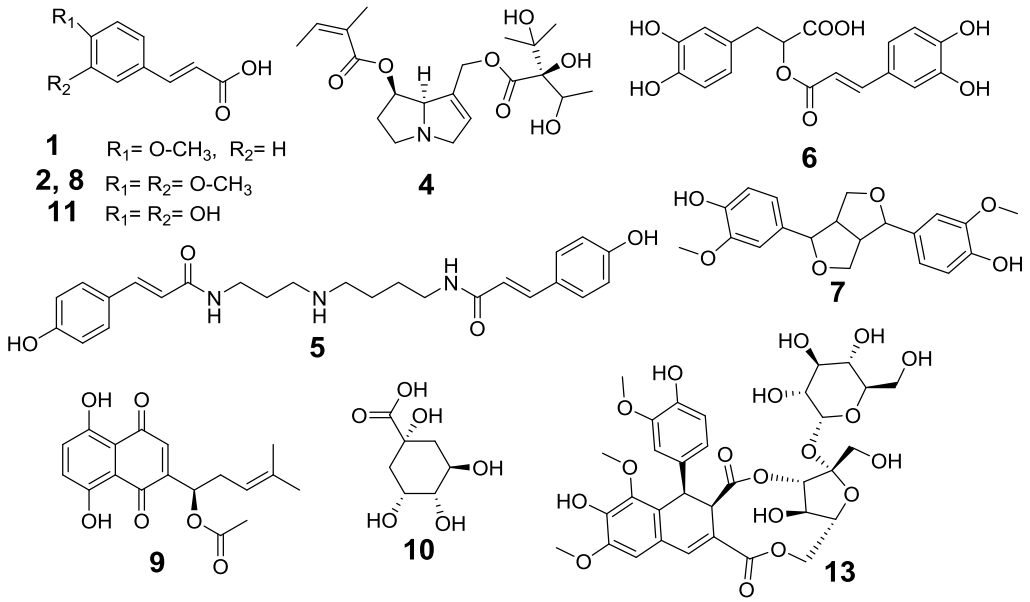

Figure 3: Chemical structures of the tentatively identified compounds in the non-polar fraction of *E. angustifolium* aerial parts by LC-ESI-MS analysis

4. Conclusion

In summary, the non-polar extract of *E. angustifolium* aerial parts showed promising *in vitro* anticancer, antioxidant and anti-inflammatory activities. Additionally, the phytoconstituents of this plant were identified by GC-MS and LC-ESI-MS and by reviewing the published data, quinic acid, methoxy cinnamic acid, and trigonotin C weren't previously found in *E. angustifolium*. Based on the foregoing information, the current research sought to determine the mechanism by which the non-polar extract induces an anticancer impact. So, it can be concluded that the occurrence of cinnamic acid derivatives and fatty acid derivatives in the non-polar extract could be responsible for its potential effect on cancer cells through their antioxidant and anti-inflammatory actions.

5. Conflicts of Interest

There are no conflicts to declare.

6. Acknowledgments

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7. List of Abbreviations

Abbreviation	Full term
E. angustifolium	*Echium angustifolium*
GC-MS	Gas chromatography-mass spectrometry
LC-ESI-MS	Liquid chromatography-electrospray ionization-mass spectrometry
BPC	Base peak ion chromatogram
HCT116	Human colorectal carcinoma cell line
HEPG2	Human hepatocellular carcinoma cell line
MCF7	Human breast carcinoma cell line
IC_{50}	Half maximal inhibitory concentration
ROS	Reactive oxygen species
DPPH	2, 2'-diphenyl-1-picryl-hydrazyl hydrate
ABTS	2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonate
ELISA	Enzyme-linkide immunosorbent assay
EDTA	Ethylene diamine tetra acetate
SRB	Sulforhodamine B stain
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
AA	Archidonic acid
PGH2	Prostaglandin H2
PGF2α	Prostaglandin F2 alpha
EIA	enzyme immunoassay
± SD	Standard deviation
SI	Selectivity index
µM TE/g	Micro molar trolox equivalents per gram
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
MeOH	Methanol

8. References

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