ImmunoAnalysis, 2023, 3, 9
doi:10.34172/ia.2023.09
https://ia.tbzmed.ac.ir/

Original Article

Bioactivity Assays of *Eryngium thyrsoideum*; Focusing on Cytotoxic Effects, Antioxidant Activity, and Antimalarial Properties

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ARTICLE INFO

Article History:
Received: June 19, 2023
Accepted: June 20, 2023
ePublished: July 15, 2023

Keywords:
Eryngium, Extraction, Essential oil, Bioactivity, Antimalaria

Abstract

Background: The genus *Eryngium*, *Apiaceae* is widely distributed in the world and used in traditional medicine for different therapeutic purposes. *Eryngium thyrsoideum* was investigated due to a lack of phytochemical studies on the probable bioactivities of its compounds.

Methods: The aerial parts of the plant were extracted using n-hexane, dichloromethane, and methanol (MeOH) by the soxhlet method. The MeOH extract was exposed to C18 Sep-Pak fractionation by a step gradient of MeOH-H_2O. The essential oil was obtained by the Clevenger apparatus. Free-radical scavenging activity of the extracts and fractions was assessed using the DPPH-scavenging activity method. Total flavonoid content was determined by Aluminum chloride colorimetric and phenolic content was determined by using the Folin-Ciocalteu reagent method. α-Glucosidase inhibitory and antimalarial activity of extracts and essential oil and cytotoxic activity of essential oils was also established in this study.

Results: The 20% solid phase extraction fraction demonstrated high antioxidant activity (0.13 ± 0.01 mg/mL) and also indicated the most total flavonoid and total phenolic contents (474.20 ± 23.11 and 27.90 ± 0.55 mg/100 g) respectively, in comparison to other fractions. We observed a moderate β-Hematin Formation Assay inhibitory effect of the essential oil (10.37 ± 0.02 mg/mL) and DCM extract (7.64 ± 0.01 mg/mL). Low levels of α-glucosidase inhibition were observed in n-hexane extract (9.78 ± 1.1%) and essential oil of aerial parts (2.7 ± 1.1%). Cytotoxic activity for the essential oils is confirmed by representing respectively IC_{50} with 2.51 mcg/mL and 1.07 mcg/mL in 24 and 48 hours in MCF-7 cell lines.

Conclusion: The presence of phenolic compounds and flavonoids in MeOH extract and fractions is effective on the antioxidant potency of *E. thyrsoideum*. Essential oil of this plant presented noticeable effects on human breast cancer cell lines.

Introduction

As the richest genus of the *Apiaceae* family, *Eryngium* is widely distributed with about 250 species in the whole world, especially in Eurasia, south America, central America, and North Africa. From the point of taxonomical view, the *Eryngium* genus is the most complex group of plants in the carrot family.[1-3] Like numerous plants in the *Apiaceae* family, some species from *Eryngium*, have been used locally as remedial plants in traditional medicine like *Eryngium campestre*, *E. serbicum,* and *E. foetidum*.[4,5]

These plants are well-known in the traditional medicine of many countries. Different parts of these plants are used in the treatment of tumors, kidney disease, high blood pressure, skin disorders, and are used as diuretics.[3] Besides, an infusion of aerial parts or roots of these plants is used to treat tapeworms and venereal diseases. Also, these infusions are used to reduce headaches and body pain and to improve digestive diseases.[6]

In various studies, *E. caucasicum, E. billardieri, and E. thyrsoideum* have been shown to be effective in reducing blood sugar and increasing insulin in addition to anti-inflammatory and antioxidant impacts in diabetic mice.[7-9] In addition, aerial parts of *E. cymosum* were safe in rats and lowered blood glucose levels in postprandial and fasting state.[10] The extracts of *E. caucasicum* and *E. caeruleum* can reduce blood sugar levels by inhibiting the α-glucosidase enzyme, which is similar to the effect of acarbose in reducing blood sugar.[11,12] It should be mentioned that *E. foetidum* is used as an anti-malarial plant in the folk medicine of Eastern French Guiana, as well.[13]

Regarding the other bioactivity effects of the *Eryngium*

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genius, we decided to study one plant from this genus. *E. thyrsoideum* which grows in the north-west of Iran was selected as our plant in this investigation. Referring to the Flora of Iran, Mozaffarian et al[14] This plant is a perennial herb, 20-80 cm tall, and a non-branched with spiny leaves, and unlike many of its relatives, it does not turn blue-violet during the growing season and stays light-green.

The chemotaxonomic and bioactivity potential of *Eryngium* genius and the lack of studies about this plant interested us to evaluate *in vitro* antidiabetic, cytotoxic, antimalarial, and antioxidant activity of the *E. thyrsoideum* extractions and essential oil in this study. Moreover, the total flavonoid and phenolic contents of aerial part extractions of the *E. thyrsoideum* were determined in this report.

Methods and Materials

Chemicals

Methanol (MeOH) and dichloromethane were purchased from Samchun, South Korea, and Mojallali Chemicals, Iran. N-hexane, acetone, sodium hydroxide, acarbose, quercetin, and 5,5′-Dithiobis, 2-nitrobenzoic acid (DTNB) provided from Merck, Germany. Hematin, gallic acid, sodium carbonate (Na_2CO_3), Rutin flavonoid, aluminum chloride, chloroquine diphosphate, Folin-Ciocalteu reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were gained from Sigma-Aldrich, Germany. The human breast cancer cell lines (MCF-7 cells) were obtained from the Pasture Institute of Iran, Tehran, Iran. Analytical grade of materials used in this study.

Plant material

Aerial parts of *E. thyrsoideum* was harvested from the Alavian dam region, near Maragheh, East Azerbaijan province, Iran. The plant was identified in Tabriz University of medical sciences, the Herbarium of Pharmacy faculty with herbarium code TBZ-FPH – 4046.

Extracting and separating fractions

The aerial parts of the plant were dried and powdered, then extracted using soxhlet apparatus, first, n-hexane solvent was used for extraction, and after that dichloromethane, and MeOH was used in order, to get an extract from the same plant material.

The MeOH extract (2 g) was separated into fractions with solid-phase-extraction (SPE) with Sep-Pak cartridge (C18, 35 cc, 10 g) through a step grade of MeOH-water gradient. We obtained 200 mL of each 10:90, 20:80, 40:60, 60:40, 80:20, and 100:0 fractions. Concentrating and drying of all SPE fractions was done with rotary evaporator apparatus (Heidolph, Germany), and the maximum temperature was 40 °C.

Essential oil isolation

One hundred grams of leaves, flowers, seeds, stems and spiny sepals of the plant were kept at room temperature to be dried and milled and submitted for 2 hours in a Clevenger tool and using n-hexane (2 mL) as a collecting agent. The volatile oil *of E. thyrsoideum* was yellow. Essential oil is separated from solvent by using sodium sulfate anhydrous. Then the solvent was eliminated and the obtained oil was kept in closed vials at – 4 °C for further analysis.

Antioxidant activity

The antioxidant activity of the extractions, fractions, and volatile oil was evaluated with the use of an adapted DPPH assay test.[15] DPPH was prepared with 0.08 mg/mL concentration in chloroform and 2 mL of this solution was added into separated dilutions of extracts, fractions, and solvent as a negative control. Next, we wait until the solution was homogenous at room temperature and was kept in a light-protected place for 30 minutes to allow the reaction to happen. This process was repeated for quercetin as a positive control.[16] At last, the absorbance of samples was determined at 517 nm by UV/Visible Spectrophotometer (Shimadzu, Japan). DPPH reduction percentages were determined by using the following equation:

$$I(\%) = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} \times 100$$

Finally, $RC_{50\ was}$ deduced from the dose-response equation.

MTT assay

Evaluation of the cytotoxic activity of essential oils was evaluated by the MTT assay method. MCF-7 (human breast cancer cell line) cells were seeded with RPMI 1640 culture medium, by adding in, penicillin-streptomycin as antibiotics and 10% fetal bovine serum, at 5×10^3 per well, individually at 37 °C for 24 hours in 96-well plates and incubated. After this time, the previous medium was changed with the new one which was containing essential oil (10, 50, 100, 200, 300, 400, 500 µg/mL). After 24 and 48 hours (two groups) exposure of cells at 37 °C to various concentrations of the essential oil, the medium was substituted with a new medium containing 0.5 mg/mL MTT. After additional 4 hours incubation, the medium was removed and the Dimethyl sulfoxide was added to dissolve the remaining formazan crystals. The absorbance of MTT was measured at 570 nm. The below equation was used to calculate relative cell viability (%):

$$\text{Cell viability}\ (\%) = \frac{OD\ samples}{OD\ control} \times 100$$

We compared the activity of volatile oil with the absorbance of wells containing the essential oils and, the absorbance of wells in the absence of extracts used as negative control and positive control was doxorubicin. To calculate IC_{50}, dose-response curves were depicted using Microsoft Excel.[17-19]

In vitro β-hematin formation assay

Anti-malarial potency of extracts and essential oil assessed by the heme biocrystallization method defined by Heshmati Afshar et al.[20] This technique was employed in the assay of cell-free β-hematin formation. Various samples of the extracts based on their concentration from 0.4 to 2 mg/mL in dimethyl Sulfoxide were diluted with 100 µL of hematin which is mixed with 0.1 M NaOH, 10 µM HCl and 10 mM oleic acid. The proper volume for initiation of the reaction is 1000 µL, and was adjusted by using buffer (sodium acetate, pH = 5), and the microtubes were incubated for 24 h at 37 °C by continuous shaking. Following incubation, the samples were precipitated by centrifuging 12 000 rpm for 10 minutes, and the resulting hemozoin residues were eliminated repeatedly in 2.5% (w/v) SDS (sodium dodecyl sulfate) in phosphate-buffered saline. The sediments were then washed 4-6 times in sodium bicarbonate (0.1M, pH = 9.0) until the liquid was clear. Next, the upper liquid was removed and the hemozoin sediment was suspended again, in 1 mL of NaOH (0.1 M). Final samples absorbance was read at 400 nm using a UV/Visible Spectrophotometer (Shimadzu, Japan). Chloroquine is used as a positive control. Final results reported as the rate of inhibition (I%) of heme crystallization, which was determined by the following equation:

$$I(\%) = \frac{Abs\,blank - Abs\,sample}{Abs\,blank} \times 100$$

Total flavonoids content determination

The flavonoid content was measured by the aluminum chloride colorimetric test.[21] First, 0.5 mL of each MeOH/Water fraction and MeOH extract (1 mg/mL) was distinctly mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of MeOH and lastly with 2.8 mL of purified water. Then stayed at 25°C for 30 minutes, the appearance of yellow color indicated the existence of flavonoids in the solution. The absorbance was quantified with a UV-Vis spectrophotometer (Shimadzu, Japan) at 510 nm. We used rutin as a standard to calculate the content of flavonoids by preparing different concentrations of that (0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125 mg/mL) from a calibration curve.

Determination of total phenolic content

The Folin-Ciocalteu reagent method was selected to evaluate the total phenolic compounds in MeOH extract and MeOH/water fractions,[22] In this experiment, 1 mL of MeOH/water fractions and MeOH extract (1 mg/mL) were combined with 200 µL of Folin-Ciocalteu reagent and left to react for 5 minutes. Afterward, 1 mL of sodium carbonate aqueous solution (2% w/v) was added to the reagent and samples and allowed to stay for 30 minutes, during which phenolic compounds were extracted and measured by calorimetry at 750 nm. To create a standard curve, first acetone: water mixture (60:50, v/v) was prepared and gallic acid was added to the solution to reach 5, 10, 15, 25, 50, 75, and 100 mg/L concentrations. Gallic acid was used as an equivalent standard (mg/g of dry mass) to determine the phenolic compound content of the extracts and fractions.

Inhibition of α-glucosidase

The α-glucosidase inhibition assay was conducted as the technique outlined in the reference.[23]

To prepare the reaction mixture, 1 mL of the extracts, MeOH fractions, and essential oil with 500 mg/mL concentration in DMSO was combined with 69 mL of sodium phosphate buffer (pH 7.0, 0.1 M) and 40 µL of α-glucosidase sample solution (1 U/mL). Incubation was initiated for 15 minutes at 37 °C, and after that, continued with mixing the 40 µL of 5 mM p-nitrophenyl α-D-glucopyranoside as a substrate solution. The reaction continued at 37 °C for 30 minutes and was terminated by the addition of 150 mL of 0.1 M Na_2CO_3. UV-absorbance was read at 405 nm to measure the percentage of inhibition using the following equation. The positive control used in this assay was acarbose.

$$\%inhibition = \frac{Abs\,405(Control) - Abs\,405(sample)}{Abs\,405(Control)} \times 100$$

To establish the negative control, the enzyme-produced p-nitrophenol was measured for absorption at 405 nm in the absence of the extract. Inhibition potency of the extracts and fractions on α-glucosidase was assessed to determine their IC_{50} values.

Statistical analysis

Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses. Also, T-test was used to compare and validate the mean of the data sets. Significance was set at $P < 0.05$.

Results and Discussion

Clevenger apparatus gave an odorous light yellow oil with a count of 1.1% W/W in order for *E. thyrsoideum* according to the dry mass. Due to its low essential oil content, this plant is among the poor-oil plants. Determination of the phenolic content of MeOH extract showed that fraction 20% of MeOH extract had higher phenolic contents of 474.20 ± 23.11 mg/100 g and the same extract represented the highest flavonoid content of 27.90 ± 0.55 mg/100 g (Table 1).

The determination of flavonoid and phenolic compound contents is crucial as the biological activities of many plant extracts and attributed to their antioxidant activity, primarily because of the presence of flavonoids.[24] According to the references, the total phenolic content and DPPH inhibition activity of the extracts are related.[25] Phenolic contents of extracts are responsible for the reduction of free radicals and exhibit chelating properties.[26] Our present study reveals that the total flavonoid and phenolic contents of the 20% fraction were higher in comparison to the other samples, responsible

for their free radical scavenging activity. Then, the antioxidant activity of *E. thyrsoideum* extracts can be qualified the flavonoids.

We assessed the free-radical scavenging activity of the extracts, SPE fractions, and essential oil using the DPPH assay of free-radical scavenging (Table 1). The MeOH extract and 20% solid phase extraction fraction demonstrated high potency antioxidant activity at 0.19 ± 0.01 mg/mL and 0.13 ± 0.01 respectively. In contrast, the n-hexane extract, 0.61 ± 0.01 mg/mL, and the 100% MeOH SPE fraction, 0.57 ± 0.02 mg/mL exhibited the lowest activity. Quercetin flavonoid is used as a control in this method.

To test the anti-malarial activity of the volatile oil and extracts of *E. thyrsoideum*, we used the *in vitro* β-hematin formation assay. We observed a moderate inhibitory effect of the essential oil 10.37 ± 0.02 mg/mL and DCM extract 7.64 ± 0.01 mg/mL (Table 2). On heme biocrystallization properties. This result provides valuable information for the further development of anti-malarial therapies based on *E. thyrsoideum* extracts. Additional studies are necessary to evaluate the potential of these extracts against malaria and other related diseases.

Inhibition of α-glucosidase activities of the extracts and essential oil was evaluated using 5 mg/mL of the diverse extracts of this plant. Low levels of α-glucosidase inhibition were observed in n-hexane extract at 9.78 ± 1.1% and essential oil of aerial parts at 2.7 ± 1.1%. The results were associated with those of acarbose inhibition at 41.9% (Table 2).

These findings suggest that the three obtained extracts and the essential oil of the plant have limited efficacy as α-glucosidase inhibitors. Further studies are necessary to investigate the potential of these plant extracts and the essential oil in lowering blood sugar levels and to evaluate their potential antidiabetic activities using other methods.

We evaluated the antitumor activity of the essential oil using the MTT assay on MCF-7 cells. The IC_{50} values, at 24 and 48 hours, were 2.51 mcg/mL and 1.07 mcg/mL, respectively (Table 3). The essential oil exhibited moderately high levels of cytotoxicity to inhibit the MCF-7 cell line, as evidenced by its IC_{50} values (Figures 1, 2).

The findings of this study are in line with previous research indicating the potential of essential oils derived from various *Eryngium* species for biological activity.[27] Specifically, the essential oil derived from aerial parts showed strong cytotoxic effects against cancer cells and this may be valuable to operate further studies on the composition of this essential oil. In comparison to other plants of the *Eryngium* genus, this plant demonstrated rather high cytotoxic activity.[28] Nevertheless, additional studies are necessary to elucidate the mechanisms underlying these effects and to evaluate their efficacy in vivo.

Conclusion

The 20% SPE fraction obtained from the MeOH extract of the aerial parts of *E. thyrsoideum* showed high potential as a source for developed phytochemical studies because of its strong antioxidant activity and high content of total phenolic and flavonoid compounds. More studies are recommended to elucidate the bioactive compounds present in this fraction. Additionally, the essential oil

Table 2. Antimalarial activity and α-glucosidase inhibition potential of *Eryngium thyrsoideum* extracts and essential oil

Sample	β-Hematin formation assay IC_{50} (mg/mL)	α-Glucosidase inhibition (%)
n-Hexane extract	NI*	9.78 ± 1.1
DCM extract	7.64 ± 0.01	NI
MeOH extract	NI	NI
Essential oil	10.37 ± 0.02	2.7 ± 0.9
Chloroquine (positive control)	0.47 ± 0.01	-
Acarbose (positive control)	-	41.9 ± 0.7

* No inhibitory activity.

Table 3. Cytotoxicity activity of *Eryngium thyrsoideum* essential oil

Sample	MTT assay (MCF7 24 h) (µg/mL)	MTT assay (MCF7 48 h) (µg/mL)
Essential oil	2.51 ± 0.61	1.07 ± 0.14
Doxorubicin	0.41 ± 1.20	0.27 ± 0.11

Table 1. Total phenolic and total flavonoid contents, and antioxidant potential of the essential oil, extracts and respective fractions of *E. thyrsoideum*.

Sample	Total flavonoids content (mg/100 g)	Total phenolic content (mg/100 g)	Antioxidant activity (RC_{50}) (mg/mL)
DCM extract*	-	-	0.53 ± 0.05
MeOH extract	39.01 ± 0.02	1218.80 ± 10.28	0.19 ± 0.01
n-Hexane extract*	-	-	0.61 ± 0.01
10% MeOH/Water fraction	1.30 ± 0.12	20.60 ± 0.73	0.32 ± 0.13
20% MeOH/Water fraction	27.90 ± 0.55	474.20 ± 23.11	0.13 ± 0.02
40% MeOH/Water fraction	9.50 ± 0.07	199.20 ± 3.87	0.21 ± 0.01
60% MeOH/Water fraction	4.70 ± 0.12	77.70 ± 3.81	0.39 ± 0.04
80% MeOH/Water fraction	0.80 ± 0.04	37.40 ± 0.75	0.42 ± 0.02
100% MeOH fraction	1.10 ± 0.10	117.60 ± 5.18	0.57 ± 0.02
Quercetin (positive control)	-	-	3.11×10^{-5}

* Total flavonoid and total phenolic content assay, just operated on MeOH extracts and MeOH/water fractions.

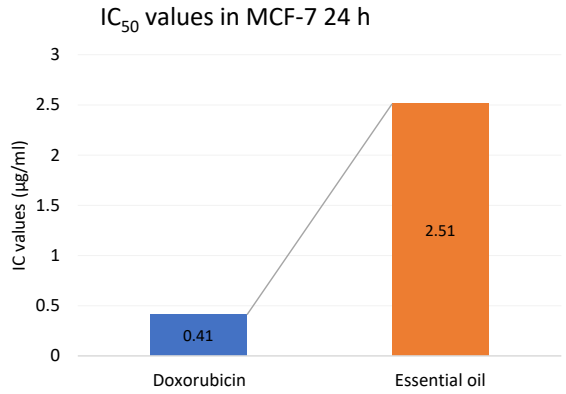

Figure 1. IC_{50} values of essential oil and positive control in 24 h

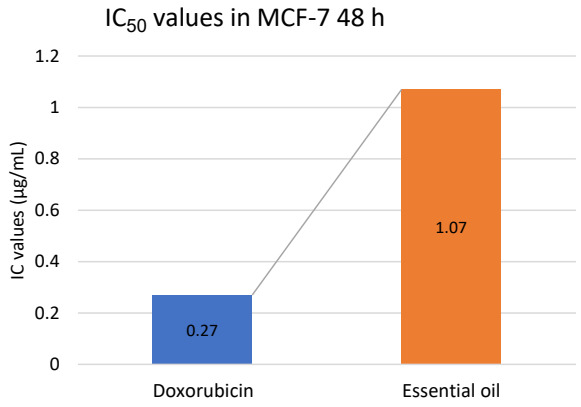

Figure 2. IC_{50} values of essential oil and positive control in 48 h

of the plant exhibited strong cytotoxicity and moderate antimalarial activity, making it a valuable subject for future research. Extracts and essential oil did not show any valuable anti-malarial and α-glucosidase inhibitory activities. Another method should be operated and other pathways should be reviewed.

Acknowledgments

The results reported in this paper were part of a student (Ph.D.) thesis, registered in Tabriz University of Medical Sciences (thesis no. 61088). This study was funded with grants provided by the faculty of pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran (Grant No.61088).

Authors' Contribution

Conceptualization: Abbas Delazar, Parina Asgharian.
Methodology: Abbas Delazar.
Software: Keyvan Sardari.
Validation: Parina asgharian.
Formal analysis: Keyvan Sardari, Solmaz Asnaashari.
Investigation: Keyvan Sardari.
Resources: Abbas Delazar.
Data curation: Parina Asgharian, Solmaz Asnaashari.
Formal analysis: Parina Asgharian, Keyvan Sardari.
Project administration: Abbas Delazar, Parina Asgharian.
Funding acquisition: Abbas Delazar.
Visualization: Keyvan Sardari.
Supervision: Abbas Delazar, Parina Asgharian.
Writing–original draft: Keyvan Sardari.
Writing–review & editing: Abbas Delazar, Parina Asgharian.

Competing Interests

The authors declare that there is no conflict of interest.

Ethical Approval

Ethical approval for this study was taken from Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1397.304).

Funding

Research granted with Tabriz University of Medical Sciences.

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