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LC-MS/MS profiling phytochemical content of *Echinophora chrysantha* (Apiaceae) and antiproliferative, antioxidant activity

Abstract

This study was conducted to determine the antioxidant and antiproliferative activity and phytoconstituents of the hydroalcoholic extract of *Echinophora chrysantha* (Apiaceae) (EC). LC-MS/MS was used for the identification of non-volatile secondary metabolites. With the LC-MS/MS-based analysis of EC extract, thirty-three non-volatile metabolites were identified belonging to the flavonoid and organic acid categories. The extract showed excellent antiproliferative activity against HT-29 (IC_{50} =4.07±0.2μg/ml) and HeLa (1.41±0.1μg/ml) with low cytotoxicity toward normal cell lines with IC_{50} values ranging from 91.24±4.0 to 118.03±3.1μg/ml, inspected through the MTT method. The plant extract showed a high antioxidant activity of IC_{50}=11.52±1.83μg/ml in DPPH and 63.58±4.05µg TE (Trolox equivalent) g^{-1} in reducing power assay. Identification of metabolites and evaluation of the bioactivity of EC also supports the traditional usage of this plant and its medicinal value.

Keywords: *Echinophora chrysantha,* antioxidant, antiproliferative activity

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Introduction

Plant secondary metabolites, such as terpenes, phenolics, steroids, and alkaloids, are produced by plants for defense, reproduction, antifungal and antibacterial actions, and attracting or repelling insects. Because of their diverse biological functions, these metabolites serve as a basis or model for many pharmaceutical raw materials. One of the most crucial steps for assessing plant secondary metabolites as raw materials for drug discovery is subjecting an herbal extract to various biological activities and mimicking the ethnopharmacological use. Therefore, this process supports the known therapeutic benefit of plants and their ubiquitous use in traditional practices. Many human ailments are treated using plant-based alternative medicines, and due to their nutritional benefits, numerous wild plants are used as food. *Echinophora* species are edible and used in folk medicine as an appetite enhancer, digestive regulator, and pain reliever in colds in Turkey.[1] *Echinophora* genus represented only 6 species, three of them including *E. chrysantha*, endemic to the flora of Turkey.[2] Previous studies about this plant conducted are more confined to the determination of essential oil content.[3-6] However, flavonoids, polyacetylenes, monoterpenoid glycosides, and prenylated coumarin derivatives were reported in the *Echinophora* genus.[7] The chemical content and bioactivity of the extract of EC were not performed extensively. The purpose of this study was to identify the chemical composition of *Echinophora chrysantha* extract based on LC-MS/MS analysis and to evaluate the antiproliferative and antioxidant characteristics of the extract.

Materials and methods

Plant materials

E. chrysantha samples were collected from Pöske Mountain, 8th-kilometer Erzincan-Kelkit Road, southern slopes in June 2021 during the inflorescence period. The samples were authenticated by Prof. Dr. Ali Kandemir (Erzincan Binali Yıldırım University, Faculty of Art and Science, Department of Biology). A voucher specimen was deposited at EBYU herbarium (AKSIT-2021-12). The aerial parts of plant material were dried in shadow for the extraction process.

Preparation of extracts

The air-dried aerial parts of *E. chrysantha* were well-grounded using a lab mill. 10 g of fine powdered material was macerated overnight via 500 ml of hydroalcoholic solution (70%). The extraction process was repeated in triplicate. The plant residue was filtered off and the solutions were combined and then the ethanol was evaporated from the azeotropic mixture. The water was lyophilized to give a dark yellow amorphous solid. The extract was kept at +4°C in dark bottles till used.

LC-MS/MS quantification of phenolic compounds

A quantitative assessment of phytochemicals was carried out using a Shimadzu-Nexera model HPLC equipped with an autosampler (SIL-30AC), column oven (CTO-10ASVP), binary pumps (LC-30AD), degasser (DGU- 20A3R) and mass detector (LCMS-8040) following the validated and previously published method.[8] The chromatographic

separation was achieved on an Agilent Poroshell 120 EC-C18 (150 mm x 2.1 mm x 2.7 µm) column. The column oven temperature was fixed at 40°C. Eluent A (water + 5 mM ammonium formate + 0.1 formic acid) and Eluent B (methanol + 5 mM ammonium formate + 0.1 formic acid) were used for elution in gradient mode. The gradient elution profile employed was 20–100% B (0–25 min), 100–100% B (25–35 min), and 20–100% B. (35-45 min). Additionally, the injection volume and solvent flow rate were adjusted to 5 µl and 0.5 ml/min, respectively. The tandem mass spectrometer outfitted with an ESI source was used for the mass spectrometric detection. LabSolutions software was used to acquire and process UPLC-ESI-MS/MS data (Shimadzu). The phytochemicals were quantified using the multiple reaction monitoring (MRM) technique. Based on the screening of certain precursor phytochemical-to-fragment ion transitions, the MRM approach was developed to specifically detect and quantify phytochemicals. To produce the best possible fragmentation and the most amount of the intended product ions being transmitted, the collision energies (CE) were optimized. The MS operating parameters were as follows: 15 L/min for drying gas (N_2), 3 L/min for nebulizing gas (N_2), 250°C for the DL, and 400°C for the heat block, and 350°C for the interface.

Antioxidant activity assay

DPPH radical scavenging ability

The free radical scavenging activity of samples was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Shimada et al. with slight modification.[9] Briefly, 0.26 mM solution of DPPH in ethanol was prepared and 1 ml of this solution was added to a 3 ml sample solution containing 10, 20, 40, and 80 µg/ml test material. The mixture was vortexed and allowed to stand at room temperature for 30 min in dark. Then the absorbance was measured at 517 nm in a spectrophotometer. The DPPH radical scavenging ability was calculated using the following equation: DPPH· Scavenging Activity (%) = $[(A_0-A_1)/A_0]x100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample. All tests were conducted in triplicate. The experimental data were expressed as mean ± standard deviation.

Ferric reducing power assay

The reducing power of the sample was determined according to the method of Oyaizu (1986) with slight modification.[10] The phosphate buffer (0.2 M, pH 6.6) added to 100 µl of sample solution up to 1.25 ml and added 1.25 ml $[K_3Fe(CN)_6]$ (1%). The mixture was incubated at 50°C in a water bath for 20 min and allowed to cool at room temperature, and 1.25 ml trichloro acetic acid (TCA) (10%) and 0.25 ml $FeCl_3$ (0.1%) were added. The absorbance of the final mixture was measured at 700 nm. The absorbance of samples converted to mmol Trolox equivalent activity/g extract using a calibration curve obtained various Trolox concentrations (10-100 µmol/l). The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations.

Estimation of total phenolic content

The total phenolic content of the extract was assessed spectrophotometrically using the Folin-Ciocalteu reagent.[11] The extract and standard were prepared as stock solutions at a concentration of 1 mg/ml, and 100 µl of these solutions were diluted with 4.5 ml of distilled water. Following the addition of 300 µl of a 2% Na_2CO_3 solution, 100 µl of Folin-Ciocalteu reagent was added to the mixture and it was left at room temperature for 10 minutes. After vigorous vortex stirring, this mixture was incubated for 120 minutes at room temperature. After incubation, the absorbance of the mixture at 760 nm was measured using a spectrophotometer. The calibration curve (y=0.117x-0.011) was obtained with different concentrations of gallic acid (1, 5, 10, 25, 50, 100, 250, 400, and 800 µg/ml) used for calculations and the results were expressed as mg gallic acid equivalent phenolic substance/g extract.

Estimation of total flavonoid content

The total flavonoid content of the extract was determined by the spectrophotometric method using aluminum chloride.[12] Stock solutions of the extracts and standards to be tested were prepared at 1 mg/ml in methanol. 100 µl of the prepared stock solutions were taken into the tubes and methanol was added to the final volume of up to 4.8 ml. Then, 100 µl of 1 M NH_4CH_3COO solution and 100 µl of 10% $AlCl_3$ solution were added to the mixture and vortexed, and the resulting reaction mixture was incubated for 45 minutes under room conditions. After the incubation period, the absorbance of the mixture was measured at 415 nm. Using the calibration curve drawn with different concentrations of quercetin (1, 5, 10, 25, 50, 100, 250, 400, and 800 µg/ml), the total amount of flavonoids in the extract was given as mg quercetin equivalent/g extract.

Antiproliferative activity

Cancer cell lines and cell culture

In this study, A172 (ATCC, CRL-1620) and C6 (ATCC, CCL-107) brain cancer cell lines, HeLa (ATCC, CCL-2) and A2780 (RRID, CVCL-0134) gynecological cancer cell lines, SW620 (ATCC, CCL-227) and HT29 (ATCC, HTB-38) colon cancer cell lines, and Beas2B (ATCC, CRL-9609), RPE (ATCC, CRL-4000), and HSF (ATCC, CRL-7449) normal cell lines, lung, retinal, and skin, respectively, were used. All cell preparation procedures were carried out in a sterile environment in a laminar cabinet. The cell lines were used after they were confluent at 37°C, 5% CO_2 conditions in the supplemented DMEM medium containing 10% FBS and 2% PenStrep solution. Measuring plates were seeded with 10,000 cells per well. After 16 hours of pre-incubation, test extracts were added, and measurements were performed after 24 hours of incubation.

Antiproliferative activity assay

MTT test was used to measure the effects of the extracts on cell proliferation and NCI-60 survival parameter values. This test protocol was applied after the incubation of test extracts and cancer cell lines for 24 hours. The results were reported as % cell inhibition and the optical density of the solvent (DMSO) treated cells was assumed to be 100%. Accordingly, the % inhibition was calculated according to the formula [1-(A test substance/A solvent control)×100. The MTT method was used on cells with increasing concentrations of each test extract (1.96, 3.91, 7.81, 15.63, 31.25, 62.5, and 125.0 µg/ml) over a specified range to determine the IC_{50} concentrations of test extracts. It was analyzed by using a logarithmic function on the logarithmic curve prepared from the absorbance after the following formulas were used for the measurement of NCI-60 survival parameters (GI_{50}, TGI, and LC_{50}); Cell proliferation: [(Ti-Tz)/(C-Tz)]x100 if Ti>/=Tz (cytocytic effect) or [(Ti-Tz)/Tz]x100 if Ti<Tz (cytocidal or cytotoxic effect) (Tz; zero point, C; control growth, Ti; inhibition by test substance). GI_{50}: Concentration value that reduces growth by 50% ([(Ti-Tz)/(C-Tz)]x100=50), TGI: Concentration value that reduces growth by 100% (Ti=Tz), LC_{50}: concentration value that by 50% kill cells in the medium ([(Ti-Tz)/Tz]x100=-50).[13,14]

Cytotoxicity test

Whether the test extracts to be tested are cell cytotoxic or cytostatic was determined by the LDH method. An increase in the number of

cells that die during the incubation period, depending on the extracts tested, will result in an increase in LDH in the culture supernatant. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is found in most cells and is stable. For this purpose, the LDH cell cytotoxicity kit was used according to the manufacturer's procedure. Briefly, the change in the amount of formazan formed because of LDH enzyme activity was measured and evaluated according to the formula below; % Cytotoxicity = [(Substance Absorbance - Low Control / High Control - Low Control) x 100].

Results and discussion

LC-MS/MS determination of phenolic content of EC extract

A review of the literature discloses no studies on the application of tandem mass spectrometry and electrospray ionization to liquid chromatography for performing quantitative investigations of the phenolic composition of EC extract. Therefore, 53 phenolic and flavonoid components in the hydroalcoholic extract of EC were accurately analyzed for quantitative purposes using an LC-MS/MS system. It was favored in the current study because the negative ionization pattern of phenolics and flavonoids was more sensitive and selective. The flavonoids, catechins, phenolic acids, and phenolic aldehydes made up the 33 chemicals that were identified as the corresponding fragment ions by the appropriate molecular ions, MS/MS fragments, associated collision energy for these fragments, and validation parameters were given in Supplementary Materials Table S1, and the quantified results for EC extracts were displayed in Table 1.

Table 1 Phenolic content determined by LC-MS/MS of *E. chrysantha* extract (mg/g extract)

#	Analyte				
1	Vanillin	1.95	28	Ferulic acid	nd
2	Daidzin	<LOD	29	Salicylic acid	0.03
3	Piceid	<LOD	30	Luteolin-7-O-glucoside	0.08
4	Coumarin	nd	31	Quercetin-3-O-glucuronide	0.02
5	Hesperidin	11.07	32	Quercetin-3-O-glucoside	29.41
6	Quinic acid	44.17	33	Rutin	31.48
7	Fumaric acid	1.83	34	Genistein	<LOD
8	Aconitic acid	0.93	35	o-Coumaric acid	<LOD
9	Gallic acid	nd	36	Ellagic acid	nd
10	Protocatechuic acid	0.09	37	Rosmarinic acid	nd
11	Gentisic acid	nd	38	Fisetin	nd
12	Epigallocatechin	<LOD	39	Apigenin-7-*O*-Glucoside	nd
13	Protocatechuic aldehyde	0.02	40	Quercetin-3-*O*-rhamnoside	0.1
14	Catechin	<LOD	41	Kaempferol-3-*O*-glucoside	0.52
15	Chlorogenic acid	15.85	42	Kaempferol-3-*O*-rutinoside	1.13
16	Tannic acid	nd	43	Daidzein	<LOD
17	4-hydroxy benzoic acid	nd	44	Genistein	nd
18	Epigallocatechin gallate	<LOD	45	Quercetin	0.11
19	Cynarin	nd	46	Luteolin	0.01
20	Vanillic acid	nd	47	Hesperetin	nd
21	Epicatechin	<LOD	48	Naringenin	0.01
22	Caffeic acid	0.08	49	Kaempferol	nd
23	Syringic acid	<LOD	50	Apigenin	0.03
24	Syringic aldehyde	nd	51	Amentoflavone	nd
25	Epicatechin gallate	<LOD	52	Acacetin	nd
26	*p*-Coumaric acid	0.28	53	Chrysin	0.01
27	Sinapic acid	<LOD			

The flavonoids hesperidin, luteolin-7-*O*-glucoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, rutin, quercetin, luteolin, naringenin, apigenin, and chrysin were detected and quantified; however, fisetin, apigenin-7-*O*-glucoside, genistein, hesperetin, kaempferol, amentoflavone, and acacetin were not found in EC extract. Furthermore, eleven organic acids (salicylic acid, quinic acid, fumaric acid, aconitic acid, *o*-coumaric acid, protocatechuic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, sinapic acid) were characterized in EC extract. Rutin, quercetin-3-*O*-glucoside, and hesperidin were found to be the most abundant flavonoids (31.48, 29.41, 11.05 mg/g extract) (Table 1; Figure 1, 2). Besides, quinic acid and chlorogenic acid (44.17, 15.85 mg/g extract) were found to be the most plentiful organic acid in EC extract (Table 1). The isoflavonoid derivatives, daidzin, genistein, and daidzein were not detected. Despite some research being found to evaluate the volatile profiling of EC, no attempt has been made to chemical characterization of hydroalcoholic extract. This is the first report on the organic acid and flavonoid content of EC extract.

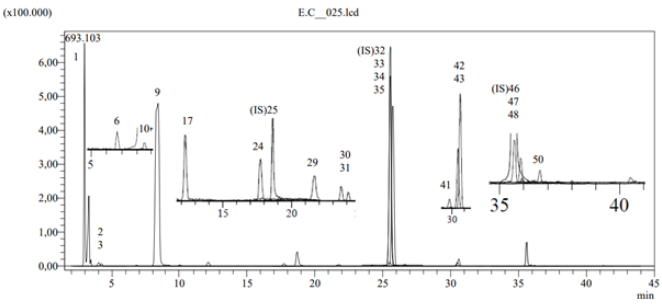

Figure 1 LC-MS/MS MRM chromatogram of hydroalcoholic extract of *Echinophora chrysantha*.

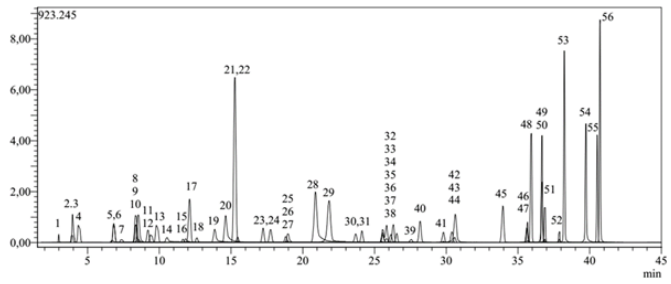

Figure 2 LC-MS/MS MRM chromatogram of standards.

Antioxidant activity of EC extract

The determination of total phenolic (TPC) and flavonoid content (TFC) as well as DPPH and reducing power activity can be conducted efficiently and rapidly using spectrophotometric techniques. In this study, the TPC and TFC of EC extract were calculated as 73.11±1.73 mg GAE (gallic acid equivalent) and 71.54±2.78 mg QE (quercetin equivalent) per gram extract, so close to each other. Also, the extract was found as a good radical scavenger by 11.52±1.83 µg/ml IC_{50} value while positive control has 8.25±1.16 µg/ml. It was found that the extract had a reducing power of 63.58±4.05 g TE (Trolox equivalent), which reflects the capacity to reduce Fe^{3+} to Fe^{2+} (Table 2).

The antioxidant potential of the hydroalcoholic extract of EC has not been considered and assessed in any study up to today. So, the antioxidant activity and total phenolic and flavonoid content of EC extract were examined in the current study. Our finding of antioxidant activity predicated on the DPPH and TPC and TFC confirms earlier findings over several *Echinophora* species that were reported previously.[15-17]

Evaluation of the antiproliferative activity of the extract

Due to the ever-changing mutational burdens of cancers, many anticancer drugs in modern chemotherapy still do not exhibit the desired therapeutic properties. In addition, the advantages of use are decreasing day by day due to the resistance mechanisms and undesirable side effects against chemotherapeutic agents for various reasons. For this reason, research on the development of new anticancer drugs that can be used in cancer treatment continues intensively. In this context, the MTT test was performed to measure the effects of EC extract on cell proliferation and IC_{50} values. Accordingly, when the lethal concentration (LC_{50}) values of the extract on Beas2B, RPE, and HSF control cells were examined, it is seen that the EC extract has a lower LC_{50} value than the control anticancer drug, 5FU, indicating that the extract does not have undesirable toxicity. As stated in the NCI-60 screening methodology, high LC_{50} values indicate that the cytotoxic effects of the test substances are less, and it is desirable.

The growth inhibitory effect (GI_{50}) and TGI (total growth-inhibitory) are other indicators used to evaluate the cell proliferation pattern of the tested extract. The low GI_{50} and TGI values mean the cytostatic effects of the test materials are more, and this is also a desirable situation. The GI_{50} values of the extract for Beas2B, HSF, and RPE control cells (1.80-2.02 µg/ml) were higher than 5FU (1.39-1.43 µg/ml). Furthermore, the TGI value of the extract in SW260 was found to be much lower than 5-FU, however, they were found to be comparable to 5-FU in A172 and A7280. The EC extract was found good total growth inhibitor for HeLa and HT29 by 1.36±0.2 and 4.07±0.3 µg/ml, respectively. The results showed that the extract has no cytostatic effect against only C6 cell lines (132.30±3.4 while 5-FU has 39.87±1.5). Considering the Total Growth Inhibition (TGI) and IC_{50} values of the extract for control, the extract was not toxic against normal cells. The extract of EC shows lower TGI and higher IC_{50} values than 5-FU in Beas2B, RPE, and HSF normal cells. It can be concluded that the toxicity of the extracts was within safe limits. When the above-mentioned cell proliferation parameters are evaluated together, the tested extract has a wide range of chemotherapeutic uses that allow safe regulation of their use doses. When the antiproliferative effect of extracts on glioblastoma (A172 and C6), gynecological (HeLa and A2780), and colon (SW620 and HT29) cancer cell lines is examined, the EC extract contains some phytochemicals that affect the different cell proliferation pathways in different cell lines. The fact that the extract has low GI_{50} values and high LC_{50} ratios against cancer cell lines shows that it was a suitable candidate for advanced pharmacological tests However, when considering the effects of the extract on the normal cell lines Beas2B, RPE, and HSF, it can be concluded that both GI_{50}, TGI, and LC_{50} values, as well as IC_{50} values, were within the desired limits (Table 3). This finding indicates that the tested extract was cancer-specific.

Cytotoxic activity of extracts

The cytotoxic properties of extracts can be determined by analyzing how they affect membrane integrity. With the aid of a kit, the activity of cytoplasmic lactate dehydrogenase (LDH), which leaks from the compromised plasma membrane to the environment, is assessed. The level of leakage that might result in indirect membrane damage is indicated by LDH activity. In this study, the extract's ability to cause cytotoxicity was evaluated using IC_{50} concentrations. As a result, the extract was determined to be effective in the MTT proliferation test, which revealed cytotoxicity values against normal cell lines (Beas2B, RPE, and HSF) at IC_{50} ranging between about 11.7 and 12.6% (Table 4). When the findings of the MTT proliferation test and the LDH cytotoxicity test were considered collectively, we strongly recommended the usage in future clinical investigations on EC extract, which has a potent antiproliferative effect against cancer cells and reduced cytotoxicity toward normal cells.

Table 2 Antioxidant activity of *Echinophora chrysantha* extract

Sample & Standard	DPPH IC_{50} (µg/ml)	Total phenolic (mg GAE/g extract)	Total flavonoid (mg QE/g extract)	Reducing power (µg TE/g extract)
E.C	11.52±1.83	73.11±1.73	71.54±2.78	63.58±4.05
Trolox	8.25±1.16	-	-	-

Table 3 Antiproliferative activity of *Echinophora chrysantha* extract

Cell lines	*Echinophora chrysantha* extract				5-Fluorouracil			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC50	IC_{50}
A172	2.34	43.02±2.2	>1000	83.68±3.1	1.29	48.77±2.0	386.42±8.5	49.94±1.9
A2780	1.11	46.64±1.8	>1000	70.67±2.2	1.59	47.12±1.9	391.24±10.1	53.41±2.7

Citation: Akşit Z, Akşit H, Şimşek S, et al. LC-MS/MS profiling phytochemical content of *Echinophora chrysantha* (Apiaceae) and antiproliferative, antioxidant activity. *Pharm Pharmacol Int J.* 2022;10(5):190–194. DOI: 10.15406/ppij.2022.10.00384

Table Continued...

Cell lines	*Echinophora chrysantha* extract				5-Fluorouracil			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC50	IC_{50}
SW620	1.23	19.06±1.1	>1000	46.99±1.4	1.29	35.36±1.9	411.54±9.2	43.18±2.1
HT29	1.07	4.07±0.3	>1000	4.07±0.2	1.54	55.37±3.6	423.82±8.3	65.30±2.1
C6	2.24	132.30±3.4	>1000	91.97±3.1	1.39	39.87±1.5	348.65±9.1	46.11±2.4
HeLa	1	1.36±0.2	>1000	1.41±0.1	1.27	37.18±1.4	393.06±9.5	32.74±1.7
RPE	2.02	>1000	>1000	118.03±3.1	1.43	33.08±1.8	417.72±10.8	34.62±1.7
Beas2B	1.8	133.32±4.3	>1000	91.24±4.0	1.41	30.64±2.2	357.86±7.8	32.43±2.0
HSF	2.15	>1000	>1000	114.38±3.5	1.39	39.87±1.5	348.65±9.1	46.11±2.4

Table 4 Cytotoxicity of *Echinophora chrysantha* extract at IC_{50} concentrations against the cell lines

	Beas2B	RPE	HSF	A172	C6	HeLa	A2780	SW620	HT29
EC	11.8±0.9	12.5±1.0	12.1±1.0	15.1±1.2	14.8±1.3	15.2±1.3	15.1±1.3	15.2±1.3	14.0±1.3
5FU	10.1±1.0	10.7±1.0	10.9±1.1	10.8±1.0	11.5±1.1	11.9±1.1	10.1±1.0	11.0±1.3	10.9±1.2

*Percent cytotoxicity was noted as mean values ± SDs of three independent measures.

Conclusion

In conclusion, LC-MS/MS-based analysis of the hydroalcoholic extract of *Echinophora chrysantha* resulted in the identification of thirty-three phytochemicals, and the extract displays a considerable level of antioxidant in DPPH and antiproliferative activity against especially HT-29 and HeLa cell lines. The findings confirm the traditional use of *E. chrysantha* and can be used to guide future pharmacological and phytochemical studies.

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None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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