Original article

UHPLC-MS/MS-GNPS based phytochemical investigation of *Equisetum arvense* L. And evaluation of cytotoxicity against human melanoma and ovarian cancer cells

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ABSTRACT

Equisetum arvense L. is widely used as a traditional medicine for the management of inflammation and cancer. In the present study, phyto-chemical analysis of *E. arvense* was carried out and its cytotoxic potential against human melanoma (MDA-MB-435) and ovarian cancer cells (OVCAR3) was evaluated. Phyto-chemical profile of *E. arvense* methanolic extract and its fractions was established employing UHPLC-MS/MS and Global Natural Product Social molecular networking. Cytotoxic activity was evaluated using absorbance assay (CellTiter-Blue® Cell Viability Assay). Overall, 22 compounds were identified in the crude extract and polarity-based fractions of *E. arvense*. Flavonoids, flavonoid-O-glycosides and phenolic acids were found to be the major classes of phyto-chemicals. In addition, the crude extract of *E. arvense* and its fractions were found active against the tested cell lines. The highest anti-cancer activity against OVCAR3 cells was exhibited by the *n*-hexane fraction. These results indicated that *E. arvense* is rich in flavonoids and might be used for the development of anti-cancer drugs against melanoma and ovarian cancers.

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1. Introduction

Cancer is currently considered the second leading cause of mortality worldwide (Sushma et al., 2021). Cancer leads to death due to uncontrolled growth and proliferation of cells (Sakarkar and Deshmukh, 2011). Chemotherapy and radiotherapy are partially effective for inducing senescence within the tumor mass (Finkel et al., 2007). However, chemotherapeutic agents have been found to attack both healthy and cancerous cells, causing side effects such as bone marrow suppression, hair loss, and toxicities to the cardio-vascular, pulmonary, ocular and central nervous systems (Komarova and Wodarz, 2005). Plants have been used to cure cancer since ancient times (Graham et al., 2000), and many bioactive secondary metabolites have been identified, especially in the anti-tumor and anti-infection areas. In cancer chemotherapy, about 67% of the effective drugs are derived from natural products including vinblastine and vincristine from *Catharanthus roseus*, paclitaxel from *Taxus brevifolia,* and etoposide from *Podophyllum* species (Cragg et al., 2006; Gurib-Fakim, 2006). Anti-cancer agents from plants have different molecular mechanisms and have offered various targets for future anti-cancer drugs (Kinghorn et al., 2003).

E. arvense is one of the most popular species of Equisetaceae family. It is locally known as "Bonakey" in Pakistan. The plant is widely distributed in North America, North Africa, Europe, and Northern Asia (Boeing et al., 2021). Traditionally, this plant has been used for the treatment of cancer, urinary tract infections, hypertension (as a diuretic), kidney stones, brittle nails, minor wounds and burns (Al Mohammed et al., 2017; Carneiro et al., 2014; Nagai et al., 2005; Rao, 2002). Pharmacologically, it possesses anti-oxidant, anti-tumoral, anti-microbial, smooth muscle relaxant, anti-convulsant, sedative, anxiolytic, anti-nociceptive,

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anti-inflammatory, anti-diabetic, diuretic, platelet aggregation inhibitory, cytotoxic, osteoblastic response promoting, and anti-leishmanial properties (Boeing et al., 2021; Carneiro et al., 2019; Do Monte et al., 2004; Hedaya, 2017; Lemus et al., 1996;

Milovanović et al., 2007; Steinborn et al., 2018; Vieira et al., 2020). *E. arvense* has been reported to contain secondary metabolites such as flavonoids, alkaloids, phenol, phytosterols, minerals (calcium, potassium, magnesium, silica, selenium, zinc, iron etc.)
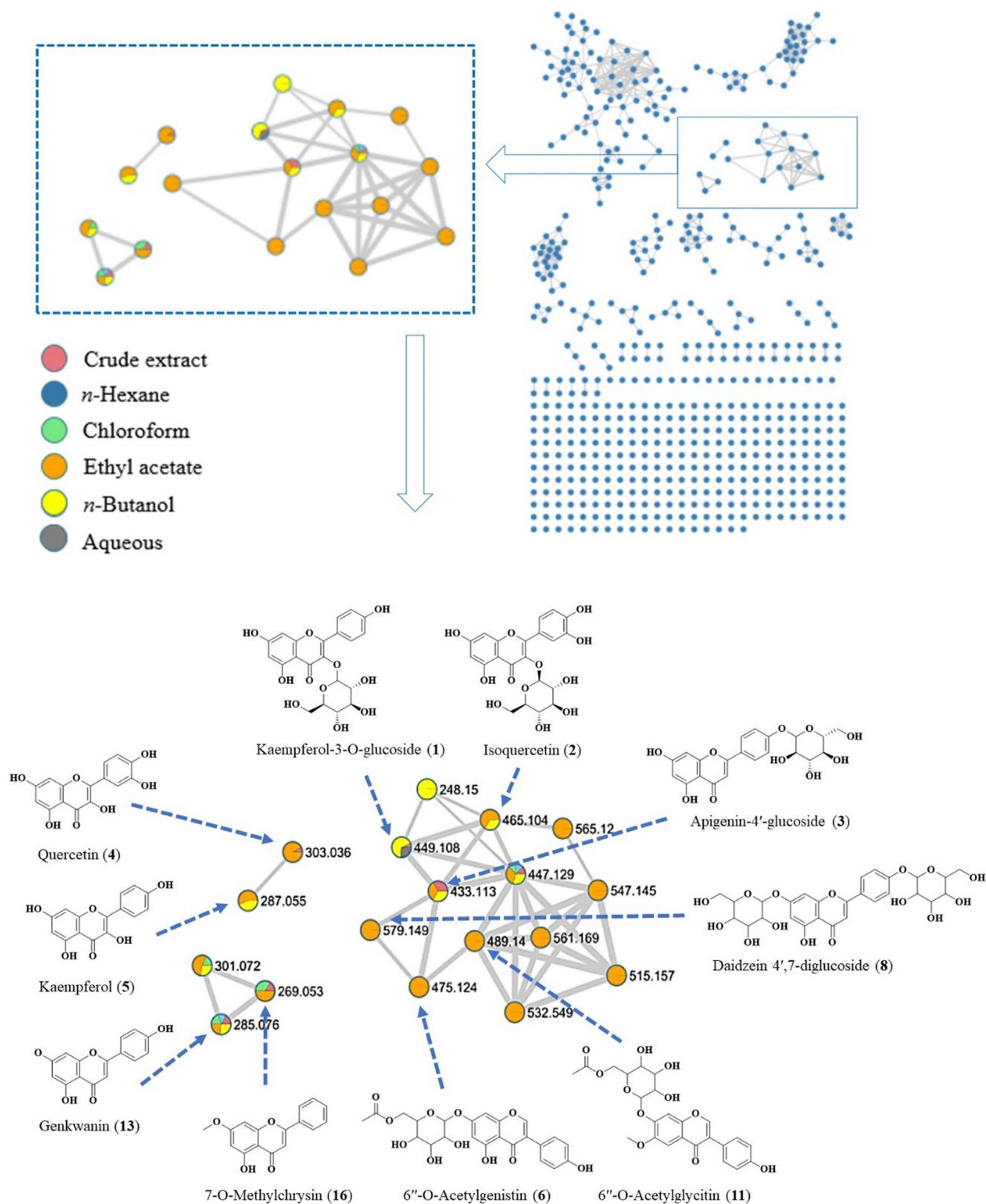

Fig. 1. Molecular networking analysis of *E. arvense* crude extract and fractions highlighting the flavonoids and their derivatives performed using LC-MS/MS technique in positive ion mode.

and vitamins (C, E, K, B_1, B_2, and B_6). Quercetin, apigenin, onitin, kaempferol, equisetumoside A, equisetumoside B, equisetumoside C isolated from *E. arvense* have shown anti-oxidative activities (Al-Snafi, 2017; Jun et al., 2001; Milovanović et al., 2007; Mimica-Dukic et al., 2008; Sandhu et al., 2010; Sola-Rabada et al., 2016).

The present study aimed to explore phytochemical profile of *E. arvense* and to evaluate its cytotoxic potential against human melanoma and ovarian cancer cells.

2. Materials and methods

2.1. Plant material

Whole plant of *Equisetum arvense* was collected from Naran (altitude of 2,450 m; latitude 34.9093° N, 73.6507° E), Khyber Pakhtunkhwa, Pakistan. Authentication was made by a plant taxonomist, Dr. Abdul Nazir (Assistant Professor), Department of Environmental Sciences, COMSATS University Islamabad (Abbottabad Campus). The voucher specimen (voucher number; CUHA-214/202) was deposited at the same institute.

2.2. Extraction and fractionation

The collected medicinal plant was garbled, washed with tap water and shade dried. The dried plant material was pulverized to obtain fine powder. The powdered plant material (11.5 Kg) was macerated with methanol (45 L) for 21 days. The same procedure was repeated twice for further 7 and 3 days, respectively, using fresh solvent. Then, the extracted material was filtered using Whatman filter paper and the filtrates were concentrated at 38 °C under reduced pressure using rotary evaporator (Büchi Rotavapor®, Switzerland) to afford a crude extract (520 g).

The polarity-based fractions of *E. arvense* were prepared by solvent–solvent extraction. For this, crude extract (500 g) was sus-

pended in distilled water and extracted successively with solvents including *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The resultant fractions were concentrated using rotary evaporator to yield the *n*-hexane (93.88 g), chloroform (14 g), ethyl acetate (8 g), *n*-butanol (118 g) and water (90.47 g) fractions.

2.3. Phytochemical analysis

2.3.1. Sample preparation for UHPLC–MS/MS analysis

The methanolic extract and its fractions of *E. arvense* were dissolved in liquid chromatography mass spectrometry grade methanol (1 mg/mL) and filtered through 0.2 μm membrane filter into HPLC autosampler glass vials.

2.3.2. UHPLC–MS/MS analysis

Metabolomics profiling of the extract and fractions was carried out using a Shimadzu (Kyoto, Japan) Nexera UHPLC system coupled with Impact II quadrupole/time-of-flight mass spectrometer (Bruker, MA, USA). The electrospray ionization technique was used to acquire the spectra in positive ion mode. For chromatographic separation, a CORTECS UHPLC C-18 column was used with particle size of 1.6 μm, internal diameter of 2.1 mm and 100 mm column length (Waters, Milford, MA, USA) with a pre-column (2.1 mm × 5 mm). The gradient program was set using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution profile was as follows: 5% B (2.50 min), 5–100% B (6 min), 100% B (2.40 min) and 5% B (3 min) and total run time 13.90 min. The oven temperature was set at 40 °C. The sample injection volume was 5 μL and the flow rate was kept at of 0.4 mL/min. Spectra were recorded with *m/z* range of 80 to 1000.

2.3.3. Molecular networking

The LC-MS/MS data of the *E. arvense* crude extract and fractions were subjected to Global Natural Product Social molecular networking analysis available online at (https://gnps.ucsd.edu/Pro-
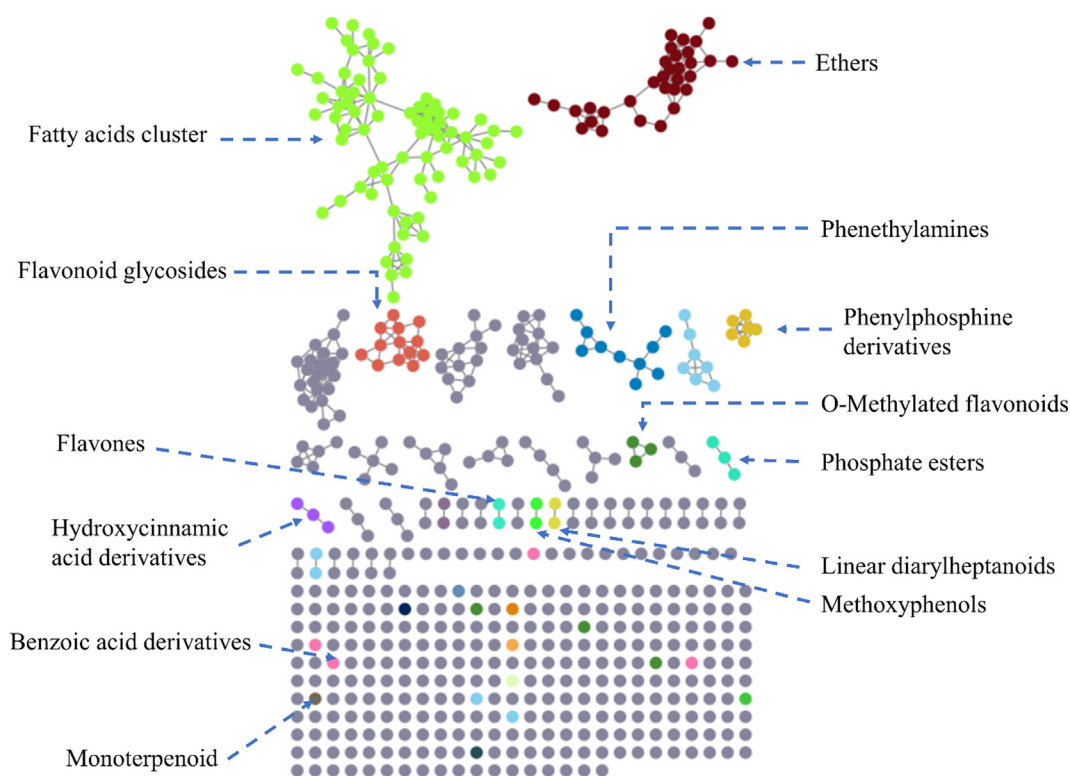

Fig. 2. Identification of major classes of chemical constituents of *E. arvense* crude extract and fractions using MolnetEnhance technique and GNPS molecular networking.

Table 1
Tandem mass-based metabolomics approach for chemical composition of *E. arvense* crude extract and fractions. Most intense fragments are in bold text.

Compound No.	RetentionTime (min)	Precursor Ion*	Fragment Ions (MS2)	Molecular Formula	Exact Mass	Dereplication Result	Distribution	Cosine
11	4.44	449.108	**287.057**, 85.029, 153.019, 97.029	$C_{21}H_{20}O_{11}$	448.101	**Kaempferol-3-*O*-glucoside**	EC, EB, EA	0.96
22	4.90	465.104	**303.051**, 85.029, 97.029, 127.04	$C_{21}H_{20}O_{12}$	464.095	**Isoquercetin**	EE, EB	0.94
33	5.14	433.113	**271.061**, 153.019, 147.045, 119.05	$C_{21}H_{20}O_{10}$	432.106	**Apigenin-4′-glucoside**	EC, EE, EB	0.97
44	5.27	303.036	**153.019**, 137.024, 229.05, 165.019	$C_{15}H_{10}O_7$	302.043	**Quercetin**	EC, EE	0.91
55	5.38	287.055	**153.02**, 121.031, 213.056, 165.019	$C_{15}H_{10}O_6$	286.048	**Kaempferol**	EE, EB	0.92
66	5.54	475.124	**271.061**, 153.019, 147.045, 127.038	$C_{23}H_{22}O_{11}$	474.116	**6″-*O*-Acetylgenistin**	EE	0.87
77	5.57	287.056	**153.019**, 135.045, 89.04, 161.025	$C_{15}H_{10}O_6$	286.048	**Luteolin**	EE, EB	0.90
88	5.63	579.149	**271.061**, 147.045, 303.051, 148.048	$C_{27}H_{30}O_{14}$	578.164	**Daidzein 4′,7-diglucoside**	EE	0.84
99	5.81	271.056	**153.019**, 91.055, 119.051, 145.03	$C_{15}H_{10}O_5$	270.053	**Apigenin**	EC, EE, EB	0.92
1010	5.91	331.082	**258.053**, 153.019, 270.053, 242.058	$C_{17}H_{14}O_7$	330.074	**Tricin**	ECh, EE	0.76
1111	6.07	489.140	**285.077**, 270.053, 242.059, 167.038	$C_{24}H_{24}O_{11}$	488.132	**6″-*O*-Acetylglycitin**	EE	0.96
1212	6.59	287.513	**167.035**, 119.05, 147.043	$C_{16}H_{14}O_5$	286.084	**Sakuranetin**	EE	0.78
1313	6.66	285.077	**242.058**, 167.034, 119.05, 225.055	$C_{16}H_{12}O_5$	284.068	**Genkwanin**	EC, EH, EB	0.90
1414	6.74	181.123	**91.052**, 79.055, 107.086, 158.971	$C_{11}H_{16}O_2$	180.115	**Dihydroactinidiolide**	EC, EH, ECh	0.90
1515	7.04	137.060	**94.042**, 79.035	$C_{10}H_{16}$	136.125	**Myrcene**	EC, EH	0.96
1616	7.56	269.053	**226.064**, 124.016, 167.036, 197.06	$C_{16}H_{12}O_4$	268.26	**7-*O*-Methylchrysin**	EC, ECh, EE	0.88
1717	8.04	279.342	**81.071**, 95.086, 109.096	$C_{18}H_{30}O_2$	278.225	**Pinolenic acid**	EC, EH, ECh, EE	0.82
1818	8.17	353.269	**81.071**, 95.086, 79.054	$C_{21}H_{36}O_4$	352.261	**Monolinolenin**	EC, EH, ECh	0.84
1199	4.29	217.098 [M + Na]⁺	**144.081**, 115.055, 103.055	$C_{11}H_{14}O_3$	194.094	**4-(*sec*-butoxy)benzoic acid**	EC, EB	0.80
2020	4.53	429.180 [M + Na]⁺	**203.054**, 369.116, 351.106, 149.061	$C_{17}H_{26}O_{11}$	406.148	**8-Acetylharpagide**	EB	0.82
2121	8.86	347.256 [M + Na]⁺	**91.055**, 81.071, 105.07, 283.164	$C_{20}H_{20}O_4$	324.136	**2′,4′-dihydroxy-4-prenyloxy chalcone**	EH	0.71
2222	4.50	177.078 [M + H-H₂O]⁺	**89.039**, 117.032, 80.993	$C_{18}H_{18}O_5$	194.058	***trans*-Ferulic acid**	EC, EH, ECh, EE, EB, EA	0.78

* Precursor Ion is a protonated molecule $[M + H]^+$, unless stated otherwise. EC, EH, ECh, EE, EB and EA represent crude methanolic extract, *n*-hexane fraction, chloroform fraction, ethyl acetate fraction, *n*-butanol fraction, aqueous fraction, respectively.

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teoSAFe/stat 6 ic/gnps-splash.jsp). The data were converted to GNPS compatible (".mzML" format) files via MSConvert package (Version 3.0.19330, Proteowizard Software Foundation, USA). The

converted ".mzML" files were uploaded on the GNPS platform using WinSCP version 5.17.6. The spectral networks were visualized with the help of Cytoscape 3.7.2 (Wang et al., 2016).
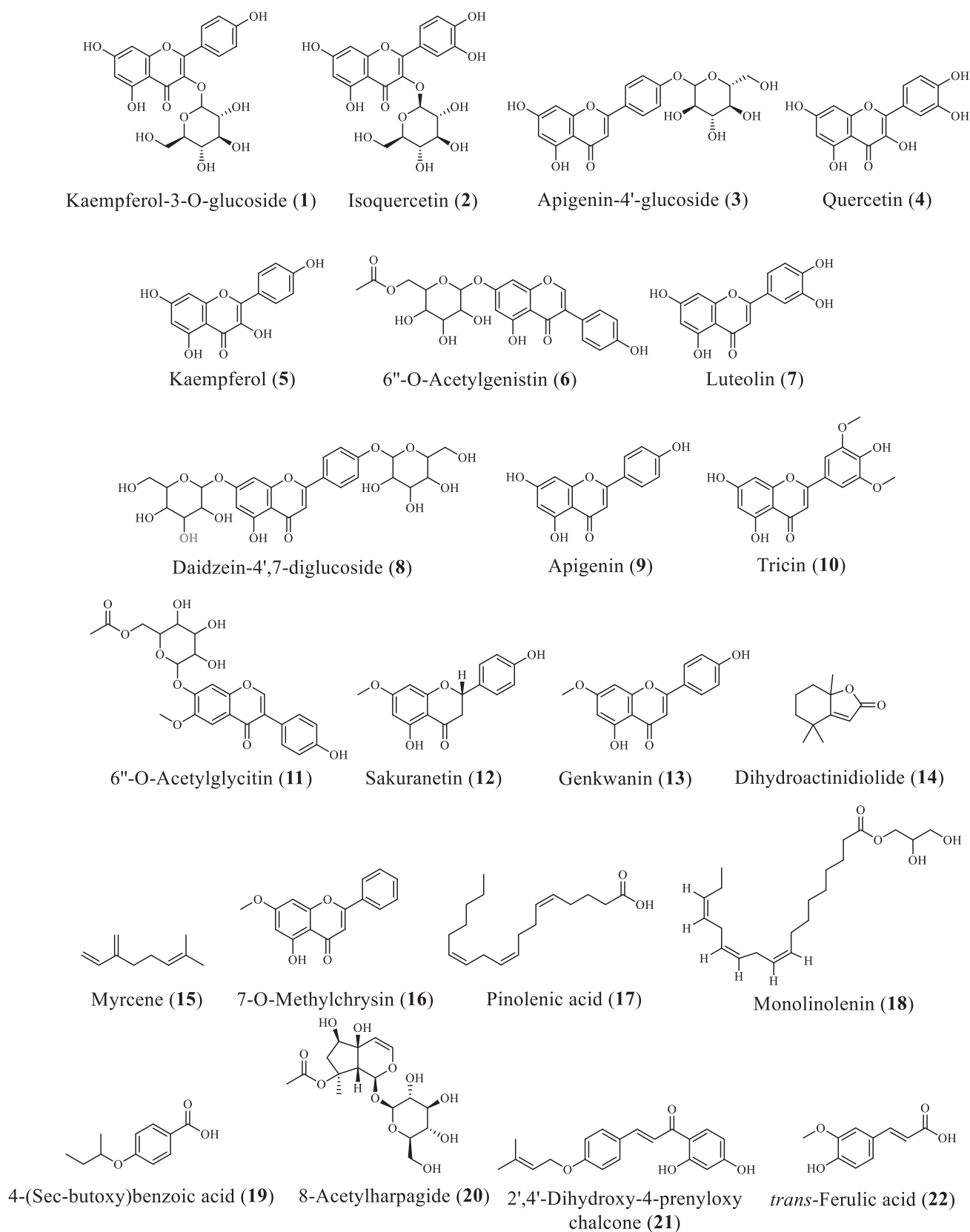
Fig. 3. The structures of constituents tentatively identified in the extract and fractions of *E. arvense* through LC-MS/MS.

2.4. Anti-cancer activity of crude extract and fractions of E. Arvense

MDA-MB-435 (melanoma) and OVCAR3 (ovarian) cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were proliferated at 37 °C in CO_2 incubator in RPMI 1640 media, supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 U/mL). Cells in the log phase of growth were trypsinized followed by washing to remove all the traces of enzymes. Cells in the concentration of 5,000 were seeded in each well of 96-well flat-bottom plate (Microtest 96®, Falcon, USA) and the cells were incubated overnight at 37 °C in CO_2 incubator. The crude extract and fractions of E. arvense (250 and 500 μg/mL) dissolved in dimethyl sulfoxide were added to corresponding wells. Dimethyl sulfoxide at the concentration of 0.05% was taken as a control. The cells were incubated with test samples and control for 72 h at 37 °C and observed for viability with an absorbance assay kit (CellTiter-Blue®, Madison, WI, USA). The % cell viability was calculated using the following formula;.

$$\%Cell\ viability = \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

3. Results

3.1. Phyto-chemical analysis

The UHPLC-ESI-MS/MS analysis, Global Natural Product Social (GNPS) molecular networking and MolnetEnhancer techniques were applied to identify the phyto-chemical composition of E. arvense extract and fractions. In total, 579 nodes and 786 edges were identified as shown in Fig. 1. The results suggested the presence of simple flavonoids, methylated flavonoids, flavonoids-O-glycosides, acylated flavonoid glycosides, chalcone, phenolic acids, iridoid glycosides and fatty acids (Fig. 2). For tentative identification of compounds, cosine score greater than 0.7 was set in the molecular networking analysis. Twenty-two compounds were noted in the crude extract and fractions of E. arvense. The bioactive ethyl acetate fraction was rich in flavonoid and their derivatives. A list of tentatively identified compounds with their spectrometric characteristics are given in Table 1. Simple non-methylated flavonoids include quercetin (**4**), kaempferol (**5**), luteolin (**7**) and apigenin (**9**); simple methylated flavonoids include tricin (**10**), sakuranetin (**12**), genkwanin (**13**), and 7-O-methylchrysin (**16**). Four flavonoid glycosides were identified as kaempferol 3-O-glucoside (**1**), isoquercetin (**2**), apigenin-4'-O-glucoside (**3**), and daidzein-4',7-diglucoside (**8**). Acylated isoflavonoid glycosides identified included 6''-O-acetylgenistin (**6**) and 6''-O-acetylglycitin (**11**), whereas trans-ferulic acid (**22**) was a phenolic acid, compound (**21**) as a chalcone derivative (2',4'-dihydroxy-4-prenyloxy chalcone), and 8-acetylharpagide (**20**) as an iridoid glycoside. Fatty acids included pinolenic acid (**17**) and monolinolenin (**18**). Myr-cene (**15**) and dihydroactinidiolide (**14**) were observed as terpenes. 4-(sec-butoxy) Benzoic acid (**19**) was identified as a derivative of benzoic acid. The structures of these compounds are presented in Fig. 3.

3.2. Cytotoxic activity

In the present study, cytotoxic activity of the crude extract and fractions of E. arvense was evaluated using the absorbance assay. From the results, it was observed that, at maximum tested concentration (500 μg/mL), all the samples were found equipotent against MDA-MB-435 cells (~95% inhibition). However, at the same concentration, chloroform fraction was more potent than the other tested samples in OVCAR3 cancer cells. The cytotoxicity test results are given in Table 2.

4. Discussion

Tandem mass spectrometry coupled with liquid chromatography (LC-MS[n]) is widely employed for separation and tentative identification of complex mixtures from botanical sources. On the other hand, GNPS molecular networking has been adopted for the determination of phyto-chemical composition and identification of chemical constituents. Such approaches were used to establish a comprehensive, and the detailed phyto-chemical composition of E. arvense. MolnetEnhancer technique was also applied to identify major classes of phyto-chemical constituents present in the extract and fractions. Twenty-two compounds were noted in the crude extract and respective fractions of E. arvense including non-methylated flavonoids, methylated flavonoids, flavonoids-O-glycosides, acylated isoflavonoid-O-glycosides, chalcone derivative, phenolic acids, iridoid glycoside, terpenes, benzoic acid derivatives and fatty acids. The bioactive ethyl acetate fraction was rich in flavonoid and their derivative. In total, four flavonoid glycosides were annotated by their MS fragmentation pattern that showed a loss of 162 Da (Bashir et al., 2021). The chemical constituents identified by LC-MS/MS analysis in E. arvense being reported for first time in current study included 6''-O-acetylgenistin (**6**), daidzein-4',7-diglucoside (**8**), tricin (**10**), 6''-O-acetylglycitin (**11**), sakuranetin (**12**), myrcene (**15**), 7-O-methylchrysin (**16**), monolinolenin (**18**), benzoic acid (**19**), 8-acetlyharpagide (**20**), 4-(sec-butoxy)benzoic acid and 2',4'-dihydroxy-4-prenyloxy chalcone (**21**). Previously reported compounds from E. arvense included kaempferol 3-O-glycoside (**1**), isoquercetin (**2**), apigenin-4'-O-glycoside (**3**), quercetin (**4**), kaempferol (**5**), luteolin (**7**), apigenin (**9**), genkwanin (**13**), dihydroactinidiolide (**14**), pinolenic acid (**17**), and trans-ferulic acid (**22**). This study has extended the spectrum of phyto-chemical profile of E. arvense.

The compounds identified in LC-MS/MS experiment are arranged in terms of retention time in Table 1. Higher number of –OH groups and the presence of sugar moieties in the flavonoids

Table 2
The % inhibition of MDA-MB-435 and OVCAR3 cancer cells proliferation after treatment with extract and fractions of E. arvense.

Extract/fraction	Inhibition of cells (%)			
	MDA-MB-435 cells		OVCAR3 cells	
	250 μg/mL	500 μg/mL	250 μg/mL	500 μg/mL
E. arvense extract	87.39	94.40	88.51	92.87
n-Hexane fraction	94.94	94.58	95.08	95.50
Chloroform fraction	86.21	94.81	87.51	93.99
Ethyl acetate fraction	95.20	94.38	84.46	93.12
n-Butanol fraction	88.94	94.71	88.44	92.87
Aqueous fraction	89.78	94.40	87.29	91.92

glycoside causes higher polarities and faster elution (shorter retention time) (Biesaga, M., 2011; Rothwell et al., 2005). In the current study, simple flavonols: quercetin (**4**) having five –OH groups eluted first at 5.27 min, while kaempferol (**5**) with four –OH groups eluted at 5.38 min. Similarly, in flavones: luteolin (**7**) having four –OH groups eluted at 5.57 min and apigenin (**9**) with three –OH groups eluted at 5.81 min. However, in case of methylated flavonoids, structural features confirmed the expected elution sequence i.e., tricin (**10**) > sakuranetin (**12**) > genkwanin (**13**) > and 7-*O*-methylchrysin (**16**). Hydroxyl group at position 5 in 6″-*O*-acetylgenistin (**6**) eluted earlier than 6″-*O*-acetylglycitin (**11**) where, the methoxy group is present at position 6 in ring A of flavonoid skeleton. Among flavonoid glycosides, kaempferol-3-*O*-glucoside (**1**), isoquercetin (**2**), apigenin-4′-*O*-glucoside (**3**), and daidzein-4′,7-diglucoside (**8**) eluted at 4.44. 4.90, 5.14 and 5.63 min, respectively.

In the current study, cytotoxic activity of the crude extract and fractions of *E. arvense* was evaluated using the absorbance assay. Treatment of cells with the extract or fractions of *E. arvense* for 72 h resulted in reduction in MDA-MB-435 and OVCAR3 cancer cells viability in comparison to dimethyl sulfoxide (control) treated cells (no cell death). These results indicated that extract and fractions can be used for the isolation and development of new anti-cancer drugs.

Natural compounds and/or their structural analogues have historically made major contributions to pharmacotherapy, especially for infectious diseases and cancer (Atanasov et al., 2021). In this study, flavonoids and phenolic acid were identified as major constituents in the LC-MS/MS analysis of the plant extract and fractions. Among the identified compounds, kaempferol-3-*O*-glucoside (**1**), isoquercetin (**2**), apigenin-4′-glucoside (**3**), quercetin (**4**), kaempferol (**5**), luteolin (**7**), apigenin (**9**), tricin (**10**), sakuranetin (**12**), genkwanin (**13**), dihydroactinidiolide (**14**), myrcene (**15**), pinolenic acid (**17**) and *trans*-ferulic acid (**22**) have been reported for anti-proliferative effects against various cancers cells including melanoma, colorectal, ovarian and breast cancer (Chen et al., 2011; Harlev et al., 2013; Stompor, 2020; Tavsan and Kayali, 2019; Tomko et al., 2020; Wang et al., 2015). So, these constituents alone or in combination with other phyto-constituents might be responsible for the cytotoxic effect of tested samples. Further work is in progress to isolate and purify the individual bioactive molecules and to explore the mechanism of these entities at molecular level.

5. Conclusions

In conclusion, LC–MS/MS analysis disclosed that *E. arvense* was rich in flavonoids, flavonoid glycosides, phenolic acids, chalcone derivative, iridoid glycoside, and fatty acids. Furthermore, crude extract and fractions of *E. arvense* were found equipotent against melanoma cancer cells with 94% inhibition. However, *n*-hexane fraction showed the strongest cytotoxic potential among crude extract and fractions in inhibiting ovarian cancer cells proliferation (95% inhibition). Isoquercetin, apigenin, tricin, genkwanin, dihydroactinidiolide and *trans*-ferulic acid identified through LC-MS/MS analysis are expected to present cytotoxic properties of the tested samples. Further research related to the isolation of active compound(s) may be carried out to develop new anti-cancer agents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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