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Biological, chemical and toxicological perspectives on aerial and roots of *Filago germanica* (L.) huds: Functional approaches for novel phyto-pharmaceuticals

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ABSTRACT

We investigated into the effects of methanol and dichloromethane extracts from aerial and roots of *Filago germanica* (L.) Huds (Asteraceae) on key enzymes (cholinesterases, α-glucosidase and urease), antioxidant capabilities, cytotoxic potential and secondary metabolomics profile. Total phenolic and flavonoids were determined by spectrophotometric technique and secondary metabolites composition by UHPLC-MS. Antioxidant activities were assessed employing free radical scavenging, ferric reducing power and phosphomolybdenum assays. The cell-toxicity was evaluated by MTT assay against breast (MCF-7, MDA-MB-231), cervix (CaSki) and prostate (DU-145) cancers. Overall, methanol extracts were found to have higher total bioactive contents and antioxidant potential. UHPLC-MS analysis revealed significant variation in the secondary metabolites in the methanol extracts. The most common derivatives belong to seven groups i.e. alkaloids, benzoic acids, flavones, flavonols, flavan-3-ols, terpenoids and saponins. The major polyphenolic compounds were found to be kampferol, robinin, luteolin, ferulic acid, benzoic acid and salicylic acid. All the extracts showed moderate cholinesterases inhibition, whereas methanol extracts exhibited highest urease inhibition and all extracts presented a relatively high inhibition against α-glucosidase. Similarly, all extracts showed strong to moderate cytotoxicity with IC_{50} values ranging from 53.02 to 382.7 μg/mL. Overall, results have suggested *F. germanica* to be a lead source for novel natural products.

1. Introduction

Natural products have manifested their value as an origin of molecules with therapeutic potential, and still represent an important pool for the identification of novel drug leads (Toiu et al., 2018). In a continuous effort towards the discovery of lead compounds for the management of human ailments, medicinal plants offer great scope for bioprospection. Indeed, mankind has always used plants for basic needs (food and remedy). The documentation of practices involving the use of local plants (as medicine, food, etc.) has given rise to terms such as ethnobotany and functional food plants (Mocan et al., 2017; Zengin et al., 2018). With the contemporary advances in science, therapeutic potentials of plants have gained an importance all over the world because of their pharmacological activities (antioxidant, anticancer, antimicrobials and enzyme inhibitors amongst others) and nutraceutical benefits (Krishnaiah et al., 2011). Natural products are emerging as a

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valuable alternative for replacing synthetic drugs and the recent research is more focused on benefits of phytochemicals in health-promoting effects (Shahidi and Ambigaipalan, 2015). Currently, there has been much emphasize in phytochemicals with different biological properties such as antioxidant, antimicrobial, antiviral, antimutagenic, anticancer activity, or their use in pathologies such as Alzheimer's and cardiometabolic disorders (Atanasov et al., 2015). On the basis of these mentioned facts, new plant-derived products or phytomedicines are considered as prospective materials (Llorent-Martínez et al., 2017).

The genus *Filago* belongs to the Asteraceae family and is represented by about 32,000 species around the world. The plants of this family have been used in conventional medicine as anticancer (Adebayo et al., 2010; Farombi and Owoeye, 2011; Bartolome et al., 2013), antioxidant, anti-diabetic (Chiang et al., 2004; Adebayo et al., 2010; Bartolome et al., 2013), antibacterial (Albayrak et al., 2010), antifunfgal (Bartolome et al., 2013), antiviral (Galani et al., 2010) and for treating neurological disorders (Thakur and Mengi, 2005). *F. germanica* is the common species of this genus, traditionally used for treating spots, cuts, bruises and stripes. This plant has also reported to treat sore throats, sciatica and cancers. (Hatfield, 2009). As mentioned, the plants of this family have traditional use in cancer, diabetes and neurological diseases, but *F. germanica* have not yet been reported for such biological, chemical and cytotoxicity studies. Thus, in this paper, we have specifically reported methanol and dichloromethane (DCM) extracts of *F. germanica* aerial and root parts for their *in vitro* antioxidant activity employing various protocols, highlighting their free radical scavenger potential, reducing capability and total antioxidant capacity as well as their inhibition potential against the key enzymes such as acetylcholinesterase and butrylcholinesterase (neurodegenerative disorders), α-glucosidase (diabetes) and urease (ulcer). Moreover, cytotoxic studies were also performed on breast (MCF-7, MDA-MB-231), cervix (CaSki) and prostate (DU-145) cancer cell lines. The obtained results could be valuable for designing new phyto-pharmaceuticals and functional ingredients.

2. Material and methods

2.1. Plant collection and extraction

Aerial and root parts of *F. germanica* were collected in July from the desert area in Rahim Yar Khan, Pakistan and identified by Dr. H. Waris, Taxonomist at Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur. In addition, a voucher specimen number (FG-WP-01-15-109) was deposited in the herbarium of Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan. The shade-dried aerial and roots were subjected for extraction by maceration (72 h) successively with DCM and methanol at room temperature with occasionally shaking. The resultant extracts were concentrated by Rotavapor-R20 at 35 °C.

2.2. Total bioactive contents and secondary metabolite profile

2.2.1. Total phenolic contents (TPC)

Total phenolic content assay was done by utilizing well-established Folin–Ciocalteu reagent method (Kahkonen et al., 1999). Samples (300 μL) were mixed with 1.5 mL of the 10% Folin–Ciocalteu reagent, followed by an addition of 1.2 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) solution. The test tubes were placed in dark at room temperature for 30 min before absorbance values were measured at 765 nm. Results were expressed as mg gallic acid equivalent per gram (mg GAE/g).

2.2.2. Total flavonoid contents (TFC)

Total flavonoid contents was determined with the aluminium chloride colorimetric method (Chew et al., 2009). Equal volumes of 10% aluminium chloride and 1.0 M potassium acetate (0.1 mL each) were added to 0.5 mL of extract, followed by 2.8 mL of distilled water. The solution was well mixed and incubated at room temperature for 30 min before absorbance was recorded at 435 nm. The results were expressed as mg quercetin equivalent per gram of dry weight (mg QE/g).

2.2.3. Secondary metabolite profiling

Secondary metabolites were evaluated by RP-UHPLC-MS. UHPLC of Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source was used. Column specifications were as: Agilent Zorbax Eclipse XDB-C18, narrow-bore 2.1 × 150 mm, 3.5 μm (P/N: 930990-902). Column and auto-sampler temperature were maintained at 25 °C and 4 °C, respectively. Flow rate was 0.5 mL/min. Mobile phases used were: A - 0.1% formic acid in water, B - 0.1% formic acid in acetonitrile. Injection volume was 1.0 μL. Run time was 25 min and post-run time was 5 min. Full scan MS analysis was done over a range of *m/z* 100–1000 using electrospray ion source in negative mode. Nitrogen was supplied as nebulizing and drying gas at flow rates of 25 and 600 L/hour, respectively. The drying gas temperature was 350 °C. The fragmentation voltage was optimized to 125 V. Analysis was performed with a capillary voltage of 3500 V. Data was processed with Agilent Mass Hunter Qualitative Analysis B.05.00 (Method: Metabolomics-2017- 00004.m). Identification of compounds was done from Search Database: METLIN_AM_PCDL-N−170502.cdb, with parameters as: Match tolerance: 5 ppm, Positive Ions: +H, +Na, $+NH_4$, Negative Ions: H.

2.3. Antioxidant activities

2.3.1. DPPH radical scavenging capacity (RSC)

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) assay was performed by standard method as described previously (Koleva et al., 2002). In this method, 1 mL of plant extract of different concentrations (1000–15.625 μg/mL) was added to 2 mL of DPPH solution (0.059 mg/mL methanol). Absorbance was measured at 517 nm after 30 min incubation. Data was expressed as:

$$RSC\ (\%) = 100 - \{(abs_c\text{-}abs_s) / abs_c\}$$

Abs_s = absorbance of sample, Abs_c = absorbance of control.

Ascorbic acid (AA) was used as control with IC_{50} 0.00387 mg/mL Therefore, free radical scavenging activity was also expressed as equivalent of ascorbic acid (AAEAC) using the following equation.

$$AAEAC = IC_{50}\ (Ascorbic\ acid)/\ IC_{50}\ (sample) \times 10^5$$

2.3.2. Ferric reducing antioxidant power assay (FRAP)

The reducing power assay estimates the ability of the plant extracts to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}), was performed according to standard method as reported earlier (Saeed et al., 2012). Plant samples (1000 μg/mL) were added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v), incubated for 20 min at 50 °C. After 20 min, trichloroacetic acid (2.5 mL, 10% w/v) was added. The contents were divided into two halves; equal volume of water was added in one half of 2.5 mL and then 0.5 mL of $FeCl_3$ solution (0.1% w/v) was added. The contents were incubated for 30 min at 25 °C and the absorbance was measured at 700 nm. The results were expressed as equivalent of gallic acid (mg GAE/g).

2.3.3. Phosphomolybdenum total antioxidant capacity assay (TAC)

The total antioxidant capacity of extracts was evaluated by phosphomolybdenum method (Prieto et al., 1999). Briefly, plant extract solution (0.3 mL, 1 mg/mL) was mixed with 3 mL of molybdate reagent solution, incubated at 95 °C for 90 min and the absorbance of the solution was measured at 695 nm against blank. TAC was expressed as mg GAE/g.

2.4. Enzyme assays

2.4.1. Chloinesterase inhibition assay

Chloinesterase (acetylcholinesterase and butyrylcholinesterase) inhibition activities were determined spectrophotometrically according to standard methods as reported previously (Ellman et al., 1961). Total reaction mixture in 96-well plate was 100 µL containing 60 µL of phosphate buffer (50 mM, pH 7.7), 10 µL plant sample of 10 mg/mL stock solution. Then 10 µL enzyme (0.005 units AChE or 0.5 units BChE) was added. The reaction mixture was mixed, incubated at 37 °C for 10 min and its absorbance was taken at 405 nm using Synergy HT, Biotek, USA 96-well plate reader. After that, 10 µL of 0.5 mM substrate (acetylthiocholine iodide for AChE and butyrylthiocholine chloride for BChE) and 10 µL of 0.5 mM DTNB was added to the above reaction mixture to initiate the reaction and incubated again at 37 °C for 30 min. Absorbance was again measured at 405 nm. Eserine was used as control.

The inhibition (%) was calculated as

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100$$

EZ–Fit Enzyme kinetics software was used to calculate IC_{50} values (Perrella Scientific Inc. Amherst, USA).

2.4.2. α-Glucosidase inhibition assay

100 µL reaction mixture in 96-well plate contained 70 µL of phosphate buffer (50 mM, pH 6.8), 10 µL plant sample (0.5 mM) and 10 µL of baker's yeast enzyme (0.057 units). The reaction contents were mixed, incubated for 37 °C for 10 min and its absorbance was taken at 400 nm. Reaction was initiated by adding 10 µL of substrate, *p*-nitrophenyl-D-glucopyranoside (0.5 mM) (Chapdelaine et al., 1978). Incubation was continued for further 30 min and after-read. Acarbose was used as positive control. The percent inhibition and IC_{50} values were determined as given above for cholinesterases.

2.4.3. Urease inhibition activity

The total assay mixture of 85 µL in 96 well plates contained phosphate buffer (50 mM, pH 7.0) 10 µL sample and jackbean urease enzyme (25 µL of 0.14 units). Contents were incubated at 37 °C for 5 min. After incubation, 40 µL of urea substrate (20 mM) was added and incubation continued for further 10 min. Then, 115 µL of freshly prepared phenol hypochloride reagent was added in each well and further incubated for 10 min at 37 °C for colour development. Absorbance was measured at 625 nm (Weatherburn, 1967). Kojic acid was used as control. The inhibition (%) and IC_{50} results were determined as given above for cholinesterases.

2.5. MTT cytotoxicity assay

MCF-7 and MDA-MB 231 (breast cancer), CaSki (cervical cancer) and DU-145 (prostate cancer) cell lines were used to test cytotoxicity. The breast cancer cell lines were maintained in DMEM culture medium while RPMI-1640 media was used for CaSki and DU-145 cells. Both media were supplemented with 10% FBS (foetal bovine serum) and 1% P/S (penicillin-streptomycin) environment. Cells were cultured at 37 °C in a humidified atmosphere in 5% CO_2 incubator. Cells were maintained in their respective media, seeded in 96 well plate and incubated for 24 h at 37 °C. Cells with > 80% confluency were tested with the plant extracts at a concentration range of (500–15.625 µg/mL) for 48 h. After 48 h incubation, the medium was aspirated by adding MTT solution (5 mg/mL) and incubated again for 4 h. After given duration, wells were solubilized with 100 µL DMSO per well and absorbance was recorded at a primary wavelength (570 nm) and reference wavelength (670 nm) using microplate reader Infinite@Pro-200 Tecan, Switzerland (Nemudzivhadi and Masoko, 2014). Each plate contained the sample, negative control and blank. DMSO (1%) was used as a negative control. The percentage cell viability (%) was calculated by using following formula

$$\text{Cell viability (\%)} = Abs_s - Abs_c \times 100$$

All of the experiments were carried out in triplicates and the IC_{50} values were calculated using Graph pad prism software (V7).

2.6. Statistical analysis

All the experiments were carried out in triplicates to calculate the mean values which are expressed as the mean ± standard deviation (SD). The results were analysed employing one way analysis of variance (ANOVA). Tukey's test was used for the post hoc treatment using SPSS (Statistical Package for Social Science) 24.0 for windows. The IC_{50} (half maximum concentration of the extract which cause 50% inhibition) was determined using Graph Pad Prism V7 software.

3. Results and discussion

3.1. Phytochemical composition

Aerial and root parts of *F. germanica* were extracted using methanol and DCM and the extraction yields are given in Table 1. The extracts were tested for total bioactive contents to establish their total phenolic and flavonoid contents and the results are assembled in Table 1. The highest phenolic contents were found in both aerial-MeOH (28.08 ± 0.93 GAE/g) and root-MeOH (29.95 ± 1.46 mg GAE/g) extracts. Similar results were observed for flavonoids where the methanol extracts exhibited higher total flavonoid contents as compared to DCM extracts (Table 1).

Secondary metabolite components of *F. germanica* aerial and root methanol extracts were determined using liquid chromatography mass spectrometry. The samples were run based on standard optimized chromatographic conditions. A linear gradient elution with water and methanol containing 0.1% fromic acid as the mobile phase offered the best resolution. A typical chromatogram of the extracts with mass spectrometric detection in negative ion mode exhibited complex patterns of peaks as shown in Fig. 1. The LC/MS analysis of *F. germanica* aerial methanol extract has revealed the presence of 32 different compounds and given in Table 2.

The tentatively identified compounds for *F. germaica* aerial methanol extracts (Table 2 and Fig. 1) can be classified into two main groups: flavonoids and phenolics. Among the identified twelve flavonoids, the three major flavonoids were kaempferol 3-(2G-glucosylrutinoside) (4.82%), kaempferol 4′-methyl ether 3-(2Glc-glucosylrutinoside) (3.68%) and tricetin 7-methyl ether 3′-glucoside-5′-rhamnoside (3.06%). Similarly, out of five proposed phenolic compounds, p-salicylic acid (2.49%) was the major one. The fatty acids present include 11-hydroperoxy-12, 13-epoxy-9-octadecenoic acid, 5, 8, 12-trihydroxy-9-octadecenoic acid, 12-oxo-10Z-octadecenoic acid, cis-9, 10-epoxystearic acid. Two saponins quillaic acid and spinasaponin A, two

Table 1
Extraction yields (%), total phenolic and flavonoid contents of *F. germanica* extracts.

Samples	Yields (%)	Total phenolic content (mg GAE/g)[a]	Total Flavonoid content (mg QE/g)[b]
Aerial-MeOH	11.4	28.08 ± 0.93[a]	35.55 ± 1.48[a]
Aerial-DCM	9.32	21.74 ± 2.59[b]	7.76 ± 0.42[b]
Root-MeOH	10.2	29.95 ± 1.46[c]	20.53 ± 0.66[c]
Root-DCM	8.6	22.80 ± 0.5[b]	16.76 ± 0.64[d]

[a] GAE: gallic acid equivalent.
[b] QE: quercetin equivalent. Data from three repetitions, with mean ± standard deviation; means with different superscript letters in the same column are significantly ($p < 0.05$) different.

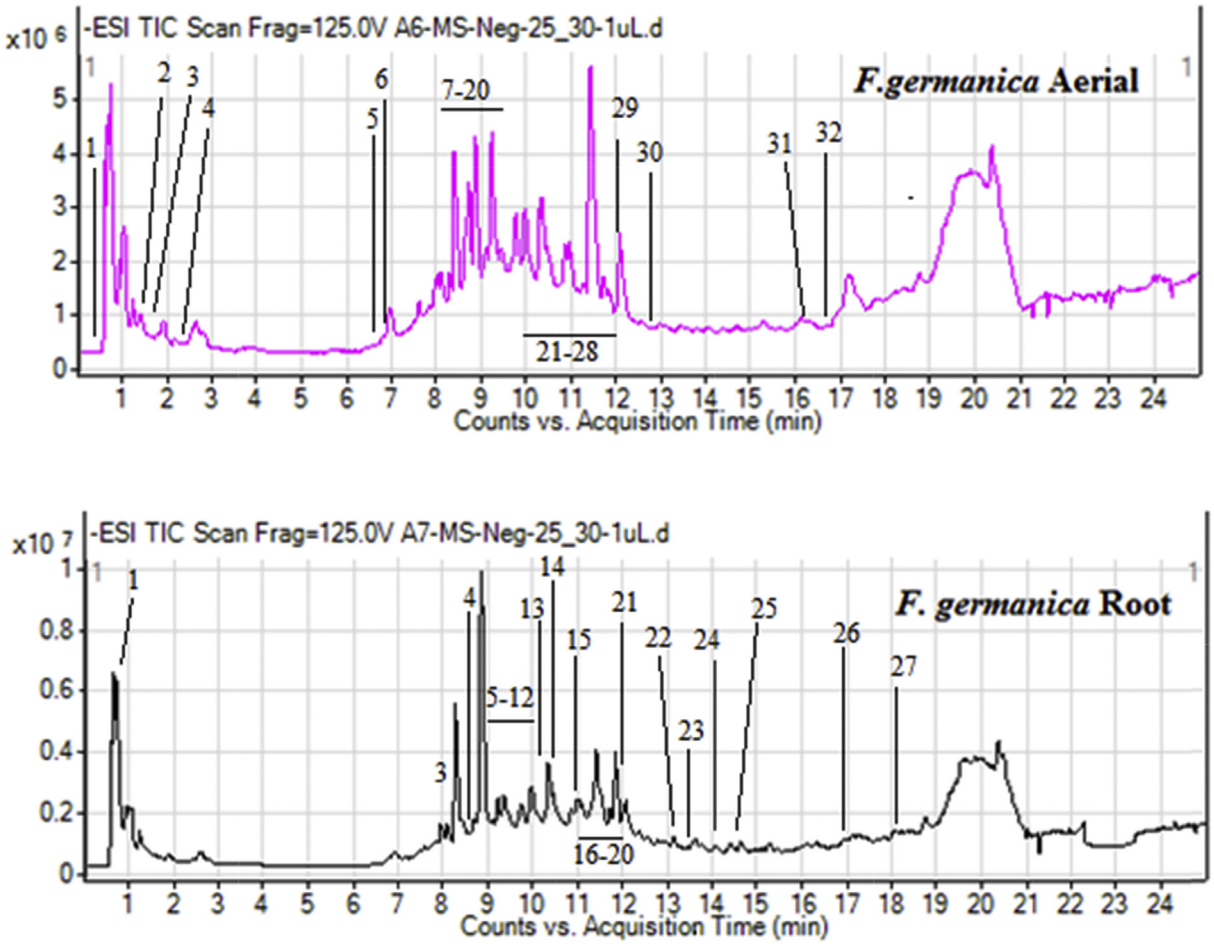

Fig. 1. Total ion chromatogram of *F. germanica* aerial and root methanol extracts.

terpenoids neryl rhamnosyl-glucoside and (6S)-dehydrovomifoliol and two alkaloids 1, 3, 9-trimethyluric acid and narciclasine were also present.

The root methanol extract was found to possess 27 secondary metabolite compounds which can be classified into four main groups: flavonoids, phenolics, alkaloids and saponis (Table 3). Out of eight flavonoids, grossamide was the major one (1.6%). In case of phenolic compounds, similar to *F. germanica* aerial methanol extract results, p-salicylic acid (3.27%) was the major one. Three alkaloids hypoxanthine, 3-O-acetylhamayne and militarinone A and three saponins betavulgaroside VI, spinacoside C and betavulgaroside IV were also present.

Overall, UHPLC-MS results showed that the aerial methanol extract contained maximum flavonoids as compared with root methanol extract, as also confirmed by the above total flavonoid content results (Table 1). *P*-salicylic acid was the major phenolic compound in both aerial and root methanol extracts. The higher amount of total bioactive contents in *F. germanica* methanol extracts (Table 1) can be linked to the higher numbers of phenolic and flavonoid compounds in these extract as confirmed by LC-MS results. The presence of phenolic, flavonoids, saponins and terpenoids in the methanol extrcats of *F. germanica* are in agreement with that of other *Filago* species (Khalfallah et al., 2017). This is the first report on the preliminary UHPLC-MS analysis of *F. germanica* aerial and root methanol extracts.

3.2. Biological evaluation

3.2.1. Antioxidant activities

Free radicals are molecules with singlet electron and are responsible for many diseases like cancer, AIDS and neurodegenerative diseases. To cope up with these disorders, the scavenging capacity of antioxidants is of quite important (Kumari and Parida, 2016). The antioxidant potential of *F. germanica* aerial and root extracts were assessed using a panoply of *in vitro* assays. The radical scavenging potential, reducing antioxidant power and total antioxidant capacity were tested using DPPH, FRAP and phosphomolybdenum assays, respectively and the results are depicted in Table 4. The results revealed the significant radical scavenging potential of the aerial-MeOH and root-MeOH extracts with mean IC_{50} values of 101.29 ± 1.05 and 172.65 ± 2.23 μg/mL, respectively. These were higher than the scavenging activities observed for aerial-DCM (IC_{50}; 415.08 ± 2.49 μg/mL) and root-DCM (IC_{50}; 294.03 ± 1.38 μg/mL) extracts. Similar to DPPH results, the aerial and root-MeOH extracts were found to be most active in FRAP assay with reducing powers of 60.58 ± 0.15 and 57.72 ± 0.42 mg GAE/g, respectively. Our findings are consistent with previous results (Pavithra and Vadivukkarasi, 2015; Sowndhararajan and Kang, 2013; Uysal et al., 2017) which confirmed that phenolic and flavonoid rich methanol extracts tend to show the strongest radical scavenging and reducing power capacities. This higher DPPH and FRAP antioxidant activities of the methanol extracts can be linked with their higher total bioactive contents (Table 1) and presence of flavonoids and phenolics as confirmed by the UHPLC-MS results (Tables 2 and 3). Previously, many researchers had also reported a highly positive correlation among total bioactive contents, radical scavenging potential and reducing capacities of different plants (Mahomoodally et al., 2018; Uysal et al., 2018; Zengin et al., 2018). The results of phosphomolybdenum assay showed that the aerial-DCM (51.19 ± 1.25 mg GAE/g) and root-DCM (57.76 ± 0.90 mg GAE/g) extracts exhibited the higher ($p < 0.05$) antioxidant potential compared to aerial-MeOH (30.15 ± 2.37 mg

Table 2
UHPLC-MS of *F. germanica* aerial methanol extract.

S. No.	RT (min)	Base Peak (m/z)	Peak height	AUC	Proposed Compounds	Compound class	Mol. formula	Mol. Mass	Vol (%)
1	0.69	209.06	390068	1215694	1,3,9-Trimethyluric acid	Alkaloid	C8 H10 N4 O3	210.07	0.45
2	1.33	147.03	78591	209721	2-Propenyl propyl disulfide	Disulfide	C6 H12 S2	148.03	0.08
3	1.79	169.01	49139	373138	2,4,6-Trihydroxybenzoic acid	Phenolic	C7 H6 O5	170.02	0.14
4	2.51	306.06	46012	558605	Narciclasine	Alkaloid	C14 H13 N O7	307.06	0.21
5	6.89	153.01	73928	839751	Patulin	Lactone	C7 H6 O4	154.02	0.31
6	6.92	263.07	139632	1208877	3-Hydroxy-4-butanolide	Lactone	C10 H16 O8	264.08	0.45
7	8.27	361.09	425215	2915210	5,7,3′-Trihydroxy-6,4′,5′-trimethoxyflavanone	Flavonoid	C18 H18 O8	362.10	1.08
8	8.3	415.16	47598	350186	Phenylethyl primeveroside	Glycoside	C19 H28 O10	416.16	0.13
9	8.39	755.20	1392569	12997800	Kaempferol 3-(2G-glucosylrutinoside)	Flavonoid	C33 H40 O20	756.21	4.82
10	8.60	739.20	559471	4453046	Robinin	Flavonoid	C33 H40 O19	740.21	1.65
11	8.70	769.22	943282	9926673	Kaempferol 4′-methyl ether 3-(2Glc-glucosylrutinoside)	Flavonoid	C34 H42 O20	770.22	3.68
12	9.05	375.10	45006	275905	2,8-Dihydroxy-3,4,9,10-tetramethoxypterocarpan	Flavonoid	C19 H20 O8	376.11	0.1
13	9.07	593.15	255768	1975624	Luteolin 7-rhamnosyl(1- > 6)galactoside	Flavonoid	C27 H30 O15	594.15	0.73
14	9.11	193.05	62293	376110	Ferulic acid	Phenolic	C10 H10 O4	194.05	0.14
15	9.16	461.24	69671	740408	Neryl rhamnosyl-glucoside	Terpene	C22 H38 O10	462.24	0.27
16	9.21	623.16	681331	8267210	Tricetin 7-methyl ether 3′-glucoside-5′-rhamnoside	Flavonoid	C28 H32 O16	624.17	3.06
17	9.40	931.25	90832	966614	Kaempferol 3-neohesperidoside-7-(2″-ferulylglucoside)	Flavonoid	C43 H48 O23	932.25	0.36
18	9.63	915.25	69044	672761	Kaempferol 3-rhamnoside-7-[6‴-ferulyglucosyl-(1- > 3)-rhamnoside]	Flavonoid	C43 H48 O22	916.26	0.25
19	9.95	137.02	796247	6715372	p-Salicylic acid	Phenolic	C7 H6 O3	138.03	2.49
20	9.98	393.08	64701	496288	9-Hydroxy-4-methoxypsoralen 9-glucoside	Glycoside	C18 H18 O10	394.09	0.18
21	10.28	739.18	62532	576489	6-Glucopyranosylprocyanidin B2	Flavonoid	C36 H36 O17	740.19	0.21
22	10.84	439.12	266758	2242117	Ginkgolide C	Flavonoid	C20 H24 O11	440.13	0.83
23	11.15	327.21	64338	811632	11-hydroperoxy-12,13-epoxy-9-octadecenoic acid	Fatty acid	C18 H32 O5	328.22	0.3
24	11.24	335.07	52994	201587	4-Caffeoyl-1,5-quinolactone	Phenolic	C16 H16 O8	336.08	0.07
25	11.55	329.23	119953	1120040	5,8,12-trihydroxy-9-octadecenoic acid	Fatty acid	C18 H34 O5	330.24	0.41
26	11.60	285.04	45271	369827	Isoscutellarein	Flavonoid	C15 H10 O6	286.04	0.14
27	11.83	955.45	45404	477332	Quillaic acid 3-[xylosyl-(1- > 3)-[galactosyl-(1- > 2)]-glucuronide]	Saponin	C47 H72 O20	956.45	0.18
28	11.83	793.43	52541	531920	Spinasaponin A	Saponin	C42 H66 O14	794.44	0.2
29	11.99	623.24	44243	508128	Grossamide	Phenolic	C36 H36 N2 O8	624.24	0.19
30	12.53	221.11	52386	442210	(6S)-dehydrovomifoliol	Terpenoid	C13 H18 O3	222.12	0.16
31	16.11	295.22	75782	842582	12-oxo-10Z-octadecenoic acid	Fatty acid	C18 H32 O3	296.23	0.31
32	16.91	297.24	57906	786598	cis-9,10-Epoxystearic acid	Fatty acid	C18 H34 O3	298.25	0.29

RT: retention time; AUC: area under curve.

Table 3
UHPLC-MS of *F. germanica* root methanol extract.

S. No.	RT(min)	Base Peak (m/z)	Peak height	AUC	Proposed Compounds	Compound class	Mol. formula	Mol. Mass	Vol (%)
1	0.67	135.03	141116	614299	Hypoxanthine	Alkaloid	C5 H4 N4 O	136.03	0.26
2	8.38	755.20	101566	793947	Kaempferol 3-(2G-glucosylrutinoside)	Flavonoid	C33 H40 O20	756.21	0.33
3	8.39	317.06	207734	1275639	5,7,2′,5′-Tetrahydroxy-6-methoxyflavanone	Flavonoid	C16 H14 O7	318.07	0.53
4	8.67	331.08	120105	790015	3′-Deoxydryopteric acid	Flavonoid	C17 H16 O7	332.09	0.33
5	9.20	393.08	179220	1219112	9-Hydroxy-4-methoxypsoralen 9-glucoside	Coumarin	C18 H18 O10	394.09	0.51
6	9.20	623.16	110512	1040962	Tricetin 7-methyl ether 3′-glucoside-5′-rhamnoside	Flavonoid	C28 H32 O16	624.17	0.44
7	9.22	328.11	145361	1073530	3-O-Acetylhamayne	Alkaloid	C18 H19 N O5	329.12	0.45
8	9.24	321.06	99197	652912	Gallocatechin-4beta-ol	Flavonoid	C15 H14 O8	322.07	0.59
9	9.72	521.27	93789	664041	Physangulide	Steroid	C28 H42 O9	522.28	0.46
10	9.94	137.02	932121	7807674	p-Salicylic acid	Phenolic	C7 H6 O3	138.03	3.27
11	9.96	353.08	119974	913864	Biflorin	Napthaquinone	C16 H18 O9	354.09	0.38
12	9.98	553.33	103372	759702	Notoginsenoside R10	Glycoside	C30 H50 O9	554.34	0.32
13	10.27	739.18	236393	2672118	6-Glucopyranosylprocyanidin B2	Flavonoid	C36 H36 O17	740.19	1.12
14	10.44	342.13	359937	2947201	N-trans-Feruloyl-4-O-methyldopamine	Cinnamic acid	C19 H21 N O5	343.14	1.24
15	10.96	465.12	302232	2343467	Silandrin	Flavonoid	C25 H22 O9	466.12	0.98
16	11.01	809.43	115286	911439	Dianoside A	Carbohydrate	C42 H66 O15	810.44	0.38
17	11.08	971.45	103669	922719	Betavulgaroside VI	Saponin	C47 H72 O21	972.45	0.39
18	11.54	329.23	108071	1091883	5,8,12-trihydroxy-9-octadecenoic acid	Fatty acid	C18 H34 O5	330.24	0.46
19	11.82	955.45	484371	4983248	Quillaic acid 3-[xylosyl-(1- > 3)-[galactosyl-(1- > 2)]-glucuronide]	Triterpenoid	C47 H72 O20	956.4	2.09
20	11.99	623.24	278373	3822455	Grossamide	Flavonoid	C36 H36 N2 O8	624.24	1.6
21	12.05	925.44	228572	2262835	Spinacoside C	Saponin	C46 H70 O19	926.45	0.95
22	13.09	647.38	136744	1471158	2a-Hydroxygypsogenin 3-O-b-D-glucoside	triterpenoid	C36 H56 O10	648.38	0.62
23	13.58	777.37	160353	2296998	Elaterinide	Cucurbitacin	C38 H54 O13	718.35	0.96
24	14.37	793.40	168571	2259931	Betavulgaroside IV	Saponin	C41 H62 O15	794.41	0.95
25	14.61	631.38	156196	2376897	oleanolic acid 3-O-beta-D-glucosiduronic acid	Triterpenoid	C36 H56 O9	632.39	1
26	16.90	297.24	104568	1404124	cis-9,10-Epoxystearic acid	Fatty acid	C18 H34 O3	298.25	0.59
27	18.04	458.25	149361	1946355	Militarinone A	Alkaloid	C26 H37 N O6	459.26	0.82

RT: retention time; AUC: area under curve.

Table 4
Antioxidant properties of *F. germanica* extracts.

Samples	%RSC (1 mg/mL)	DPPH IC_{50} (μg/mL)	AAEAC (mg AAE/g)	FRAP (mg GAE/g)	TAC (mg GAE/g)
Aerial-MeOH	84.50 ± 0.02[a]	101.29 ± 1.05[a]	38.21 ± 0.39[a]	60.58 ± 0.15[a]	30.15 ± 2.37[b]
Aerial-DCM	82.2 ± 0.17[a]	415.08 ± 2.49[b]	9.32 ± 0.05[b]	21.43 ± 0.17[b]	51.19 ± 1.25[a]
Root-MeOH	86.2 ± 0.08[a]	172.65 ± 2.23[c]	22.42 ± 0.28[c]	57.72 ± 0.42[a]	40.79 ± 2.19[d]
Root-DCM	61.01 ± 0.7[b]	294.03 ± 1.38[d]	13.15 ± 0.06[d]	30.40 ± 0.10[c]	57.76 ± 0.90[c]
Ascorbic acid	89.96 ± 1.60[a]	16.82 ± 069[e]	nt	nt	nt

Data from three repetitions, with mean ± standard deviation; means with different superscript letters in the same column are significantly ($p < 0.05$) different. RSC: radical scavenging capacity; AAEAC: Ascorbic acid equivalent anti-oxidant capacity; FRAP: ferric reducing anti-oxidant power; TAC: total antioxidant capacity; nt: not tested.

Table 5
Enzyme inhibition effects of *F. germanica* extracts.

Samples	AChE		BChE		α-Glucosidase		Urease	
	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Aerial-MeOH	14.37 ± 0.25	> [a]500	56.15 ± 0.45	283.54 ± 0.36	85.12 ± 0.82	42.65 ± 0.79	71.92 ± 0.38	242.39 ± 0.26
Aerial-DCM	12.74 ± 0.23	> [a]500	42.46 ± 0.67	> [a]500	87.46 ± 0.62	32.24 ± 0.52	15.76 ± 0.53	> [a]500
Root-MeOH	21.52 ± 0.29	> [a]500	37.62 ± 0.45	> [a]500	71.81 ± 0.39	284.37 ± 0.35	92.42 ± 0.57	87.45 ± 0.42
Root-DCM	32.68 ± 0.24	> [a]500	67.27 ± 0.86	227.56 ± 0.71	92.72 ± 2.79	26.98 ± 2.57	43.25 ± 0.46	> [a]500
Eserine	91.27 ± 1.17	0.04 ± 0.01	82.82 ± 1.09	0.85 ± 0.01	nt	nt	nt	nt
Acarbose	nt	nt	nt	nt	92.83 ± 0.18	37.45 ± 0.16	nt	nt
Kojic acid	nt	nt	nt	nt	nt	nt	98.21 ± 0.18	21.25 ± 0.15

Values are expressed as means ± S.D. of three replicates.
[a] The IC_{50} value was higher than 500 μg/mL, nt: not tested, AChE: acetylcholinesterase; BChE: butrylcholinesterase.

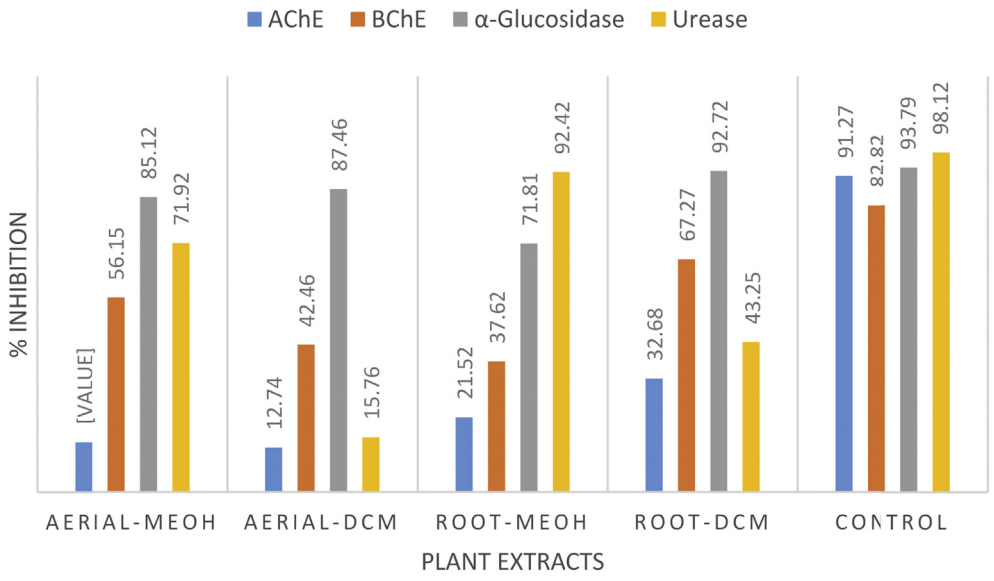

Fig. 2. Comparison of percentage enzyme inhibition (0.5 mg/mL) of *F. germanica* extracts.

GAE/g) and root-MeOH (40.79 ± 2.19 mg GAE/g) extracts. As this antioxidant assay is regarded as a total antioxidant capacity assay (both phenolics and non-phenolics), so the observed results regarding this assay can be linked to the presence of non-phenolic antioxidants like tocopherol or vitamin C in the DCM extracts. These results are in accordance with the previous studies (Albayrak et al., 2010; Liorent-Martinez et al., 2017) which had reported the higher total antioxidant capacities of DCM extracts. Similarly, some researchers have also reported a weak correlation among total bioactive contents and phosphomolybdenum assay (Niciforovic et al., 2010; Sarikurkcu et al., 2015).

3.2.2. Enzyme inhibition activities

Alzheimer's disease and diabetes mellitus are the major health problems because of the drastic increase in their prevalence. The recent research reported the prevalence of diabetes to be about 9% within adults, which is presumed to be doubled by 2025 (Waltenberger et al., 2016). Similarly, above 50 million people are also patients of Alzheimer's disease, and this figure is supposed to have an increase of almost three times until 2050 (Prince, 2015). Likewise, urease is a key target enzyme to kill *Helicobacter pylori* present in stomach and causes gastrointestinal diseases like gastritis, duodenal, peptic ulcer, and gastric cancer (Mahernia et al., 2015). Though, urease has not yet received due consideration as a pharma target in the management of kidney stone formation, its inhibition might contribute in the prevention of stone formation in obese and/or diabetic patients (Picot et al., 2017b). Hence, new efficacious treatment options have prime importance for the management of these disorders (Mao et al., 2015; Rescigno et al., 2002). Amongst most effective strategies to combat these disorders, key enzyme inhibitory theory has been the one, well accepted (Mocan et al.,

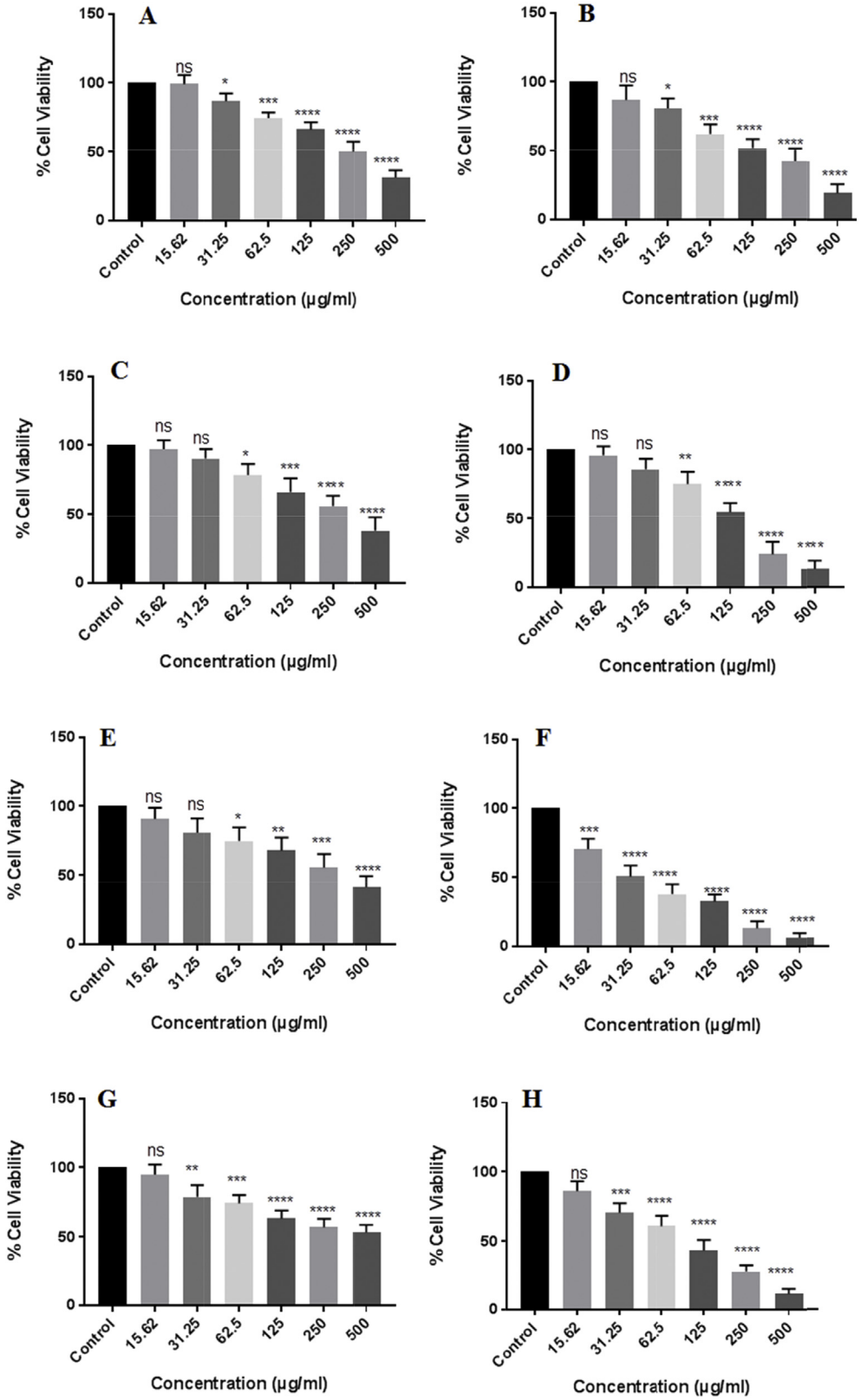

Fig. 3. Cytotoxicity of methanol and DCM extracts of *F.germanica* aerial parts. The graphs shows cytotoxic effects as (A) MCF-7 (aerial-MeOH), (B) MCF-7 (aerial-DCM), (C) MDA-MB-231 (aerial-MeOH), (D) MDA-MB-231 (aerial-DCM), (E) CaSki (aerial-MeOH), (F) CaSki (aerial-DCM), (G) DU-145 (aerial-MeOH) and (H) DU-145 (aerial-DCM). **** indicates significant difference when compared with untreated (control) cells (p value $<$ 0.05).

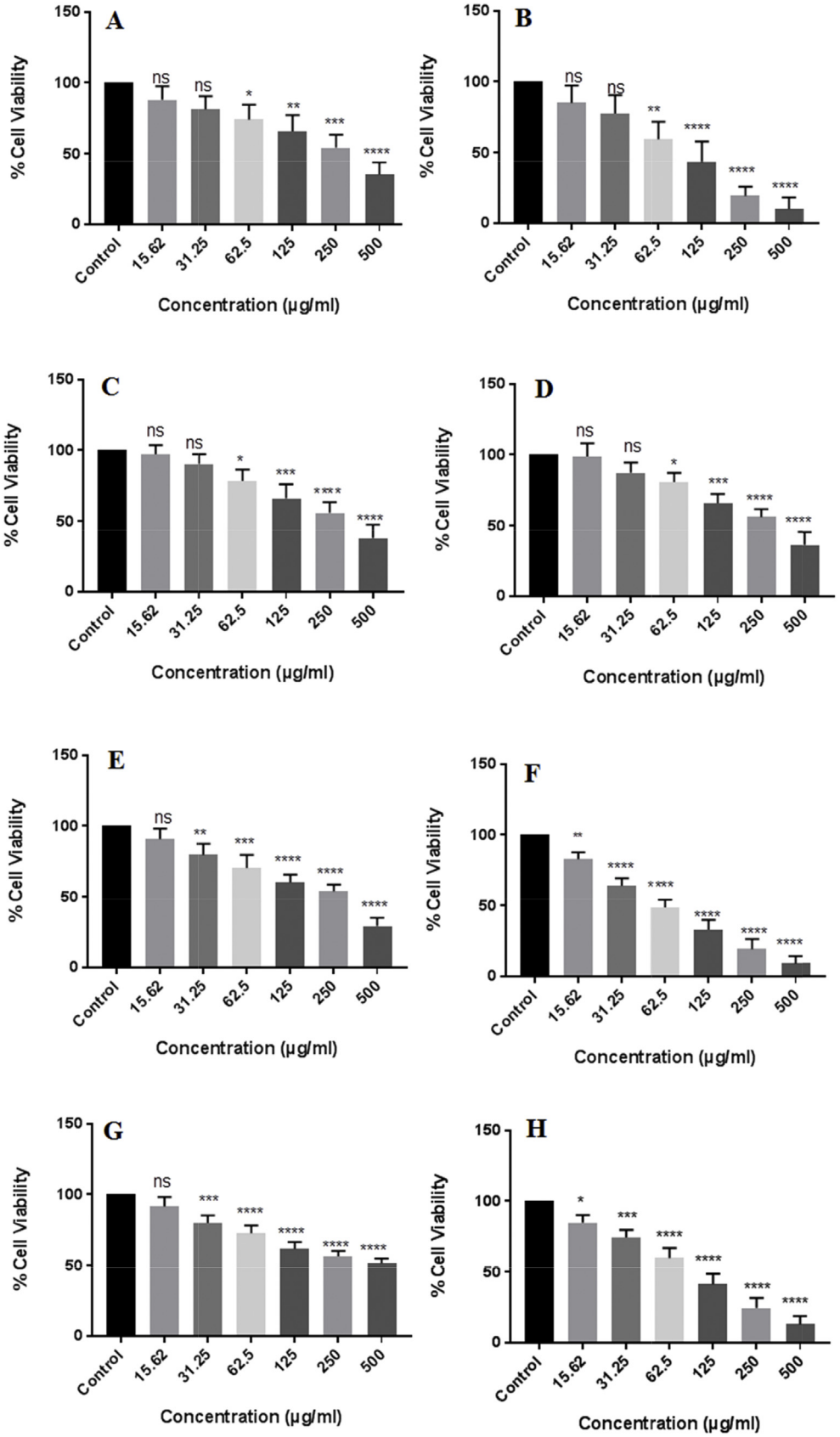

Fig. 4. Cytotoxicity of methanol and DCM extracts of *F. germanica* root parts. The graphs shows cytotoxic effects as (A) MCF-7 (root-MeOH), (B) MCF-7 (root-DCM), (C) MDA-MB-231 (root-MeOH), (D) MDA-MB-231 (root -DCM), (E) CaSki (root-MeOH), (F) CaSki (root-DCM) (G) DU-145 (root-MeOH) and (H) DU-145 (root-DCM). **** indicates significant difference when compared with untreated (control) cells (p value < 0.05).

Table 6
Cytotoxicity of *F. germanica* extracts.

Cell lines	IC_{50} value (μg/mL)			
	Aerial-MeOH	Aerial-DCM	Root-MeOH	Root-DCM
MCF-7	186.6	159.1	382.7	113.8
MDA-MB-231	169.3	169	> [a]500	192.1
CaSki	235.5	> [a]500	260.1	53.02
DU-145	114.6	192.1	> [a]500	101

Values are expressed as means ± S.D. of three replicates, value represents concentration that reduces cell viability to 50%.
[a] The IC_{50} value was higher than 500 μg/mL.

2016). Enzyme inhibitory assays have become a very prominent tool to assess the potential health benefits of herbals, dietary supplements, and nutraceuticals for the development of functional foods (Mocan et al., 2017). Therefore, the enzyme inhibition capabilities of *F. germanica* aerial and root parts, towards acetylcholinesterase, butrylcholinesterase, α-glucosidase and urease were tested, and the results are given in Table 5. A comparison of percentage enzyme inhibition of all extracts compared to positive controls is shown in Fig. 2.

All extracts were found to be least active against acetylcholinesterase enzyme with IC_{50} values more than 500 μg/mL. However, the higher butrylcholinesterase inhibition activity was observed for aerial-MeOH (IC_{50}; 283.54 ± 0.36μg/mL) and root-DCM (IC_{50}; 227.56 ± 0.71 μg/mL) extracts. For α-glucosidase inhibition, both aerial and root-DCM extracts exhibited the strongest inhibitory potential with IC_{50} values of 32.24 ± 0.52 and 26.98 ± 2.57 μg/mL, respectively. This observed higher α-glucosidase inhibition can be correlated to their higher total antioxidant capacities and the presence of non-phenolic antioxidants like tocopherol or vitamin C. Some of the previous studies had also reported the higher α-glucosidase inhibition for DCM extracts (Albayrak et al., 2010; Llorent-Martínez et al., 2017). As for urease, aerial-MeOH ($IC_{50;}$ 242.39 ± 0.26 μg/mL) and root-MeOH ($IC_{50;}$ 87.45 ± 0.42 μg/mL) extracts showed the highest inhibition which might be explained with higher amount of flavonoids found in these methanol extracts and our results are supported by some previous researchers who reported the similar results and a linear correlation between phenolic compounds and urease inhibition (Arfan et al., 2010). This observed higher urease inhibition might be due to the action of several phytochemicals acting in different ways as reported earlier (Picot et al., 2017a). Likewise, total bioactive composition and antioxidant assays findings, the methanol extracts of *F. germanica* aerial and roots exhibited the highest inhibition against all tested enzymes, which indicates a clear and strong connection for total bioactive contents, antioxidant activity and enzyme inhibition potentials.

3.2.3. In-vitro cytotoxicity

Cancer is one of the most important causes of death in many countries. During the last decades, scientists have paid more attention in discovery of new anticancer drugs. Different classes of anticancer drugs are used for cancer treatment among which cytotoxic drugs are very important (Jafarian et al., 2014). In the present study, cytotoxic potential of methanol and DCM extracts of aerial and root parts of *F. germanica* were tested against four different human cancer cells. All extracts exhibited different levels of cytotoxicity. The dose dependant effect of all extracts (500–15.62 μg/mL) on cell viability is depicted in Fig. 3 and Fig. 4. The IC_{50} values were also calculated and are shown in Table 6.

The aerial-MeOH extract was active against all four cell lines with IC_{50} values ranging from 114.6 to 186.6 μg/mL, whereas the root-MeOH extract was significantly active only for MCF-7 (IC_{50}; 382.7 μg/mL) and CaSki (IC_{50}; 260.1 μg/mL) cell lines. The aerial-DCM extract showed highest cytotoxic potential for both MCF-7 and MDA-MB-231 breast cancer cell lines. Similarly, the root-DCM extract showed the most cytotoxic potential against cervix cancer with IC_{50} value of 53.02 μg/mL. The same extract was also significant cytotoxic against MCF-7, MDA-MB-231 and DU-145 with IC_{50} values of 113.8, 192.1 and 101 μg/mL, respectively.

Overall, the aerial-MeOH and root-DCM extracts were found to be more cytotoxic towards all tested cancer cell lines. As indicated by UHPLC-MS results, the studied plant extracts contains higher numbers of flavonoids, sapnonis and terpenoids. Previously, many phytochemicals such as flavonoids, saponins (Man et al., 2010; Xu et al., 2016) and terpenoids (Huang et al., 2012; Salminen et al., 2008) have been reported for cytotoxicity against different cancer cells. Saponins and flavonoids have been also reported to be involved in the cytotoxic phenomenon as some of them are reported for their interaction with cellular membranes, thus increasing permeability and causes cell damage (Lopez et al., 2013). It is reasonable to assume that the observed cytotoxicity of these studied extracts might be, at least partly owing to the presence of these flavonoids, saponins and terpenoids. This is the first report on cytotoxicity of the methanol and DCM extracts from aerial and roots of *F. germanica* against any carcinoma cells.

4. Conclusion

The present research is the foremost effort to explore in detail phytochemical, antioxidant, enzyme inhibitory and cytotoxic potential of *F. germaica* aerial and roots. We found that the polar methanol extracts possessed more phenolic and flavonoid compounds and exhibited superior radical scavenging and reducing power, cholineseterases and urease inhibition and cytotoxicity potential. Whereas, the DCM extracts exhibited better total antioxidant capacity and α-glucosidase inhibition. The plant contains important bioactive secondary metabolites including kampferol, robinin, luteolin, isoscutellarein, salicylic acid, ferulic acid and benzoic acid. *P*-salicylic acid was the major phenol present in both aerial and root methanol extracts. Current findings suggest that *F. germanica* has good potential as a source of natural antioxidant and enzyme inhibitory compounds. Moreover, this plant can be further exploited in cancer management because of its observed anti-proliferative potency against breast, cervix and prostate cancers. However, further research is needed in order to isolate the potential bioactive compounds of this plant.

Conflicts of interest

Authors have no conflict of interest.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.016.

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