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Identification of phenolic compounds and evaluation of antioxidant, antimicrobial and cytotoxic effects of the endemic *Achillea multifida*

Turgut Taşkın[a], Özlem B Özakpınar[b], Burçak Gürbüz[c], Fikriye Uras[b], Ümran S Gürer[c] & Leyla Bitiş[a*]

[a]Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, 34668, Turkey; [b]Department of Biochemistry, Faculty of Pharmacy, Marmara University, 34668, Turkey; [c]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Marmara University, Turkey

E-mails: turguttaskin@marmara.edu.tr,ozlemozakpinar@gmail.com,burcakgurbuz@windowslive.com, furas@marmara.edu.tr, umran.gurer@superonline.com, lbitis@yahoo.com, leylabitis@marmara.edu.tr

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The aim of this study was to evaluate for the first time total phenolic content, antioxidant, antimicrobial and cytotoxic effects of heptane, chloroform and methanol extracts from *Achillea multifida* (DC.) Boiss. (Compositae) aerial parts followed by identification of phenolic compounds in the methanol extract, which showed the highest total amount of phenolics, antioxidant and antibacterial activity. The phenolic compounds in methanol extract were identified for the first time by ESI-Q-TOF LC/MS. The phenolic compounds were identified as: chlorogenic acid; quercetin hexoside; luteolin-7-*O* glucoside; dicaffeoyl quinic acid; luteolin. The chloroform extract showed strong activity and selectivity against HeLa, HT-29 and MCF-7 cell lines. The results indicated that low concentrations (100 µg/mL and 50 µg/mL) of chloroform and methanol extracts had no toxic effects on the NIH/3T3 cells. Therefore, the methanol extract from *A. multifida* aerial parts may be a natural source of antioxidants and antibacterial and seems to have potential application in both medicine and the food industry. In additon, the chloroform extract might be a potential anti-cancer agent.

Keywords: *A. multifida,* Antioxidant, Cytotoxicity, Antimicrobial, ESI-Q-TOF LC/MS

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In the recent past there has been much interest in the antioxidant potential of plant-derived foods. This is because antioxidants contained in plants or plant extracts are important for human health. Antioxidants contained in plant extracts are coming to be of interest to the both medicine and food industry[1]. *Achillea* L. (Compositae) comprises 42 species in Turkey, out of which 23 taxa are native to Turkey[2]. The species of *Achillea* are known in Anatolia as Civan perçemi and Pireotu[2]. These species have pharmaceutical useful properties and are used in cosmetics and fragrances[3]. *Achillea* species are important in Turkish folk medicine for matters such as stomachache, hemorrhoids and inflammation[4]. The aerial parts of *Achillea* species are widely used in herbal teas for spasmolytic effects[5]. The species of this genus contain essential oils, phenolic compounds, sesquiterpene lactones and ionone glucosides[6]. Phenolic compounds as a group are thought of as some of the most important antioxidant and antimicrobial active agents present in this species[7]. The total amount of phenolic compounds is related to the antioxidant and antimicrobial potential.

The antimicrobial and antioxidant properties of different *Achillea* species are of special importance[8]. Researchers have been trying to develop new broad-spectrum antibiotics against the infections conditions brought about by bacteria, viruses, fungi and parasites. Extended and careless use of these broad-spectrum antibiotics has led to widespread drug resistance. There is a consequent increased reliance on non-synthetic antimicrobial agents in food additives to treat infectious diseases. Mankind has used plants for centuries. Many of these are still used today in traditional medicine. The screening of these traditional plant extracts is a primary source of potentially useful molecules in the fight against infectious diseases[9].

Cancers are another target of these investigations. Lung, colorectal and stomach cancer are among the most widely found worldwide for both sexes[10]. Plants have been used for a long time in the treatment of cancer and they are the origin of some of the more powerful anticancer agents such as vinblastine,

*Corresponding author

etoposide, teniposide, vincristine and paclitaxel. Noting and checking the sources of new and approved cancer drugs shows that 60% of these drugs are natural compounds or else derivatives thereof[11].

Achillea multifida (DC.) Boiss. is an endemic species of Turkish Flora. Our study focused on this particular plant native to Uludağ Mountain in Bursa, Western Turkey. It is a 10-30 cm tall aromatic perennial, locally known as "ebülmülk or civanperçemi''[12].

We focused on this plant as there have been no other studies (excluding the composition of essential oils and antimicrobial activities of capitulum and essential oils of plant[9,12]) to the best of our team's knowledge. The first objective of this particular study was to evaluate the antioxidant, antimicrobial and cytotoxic effects of heptane, chloroform and methanol extracts from *A. multifida* aerial parts. Subsequently, we tried to identify the phenolic compounds in the methanolic extract, which showed the highest total amount of phenolics, antioxidant and antibacterial activity. We used electrospray-quadrupole-time of flight-mass spectrometry (ESI-Q-TOF LC/MS).

Methodology

Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) and butylated hydroxytoluene was sourced from Fluka. 2,4,6-tripyridyl-s-triazine, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), ethylenediaminetetraacetic acid disodium salt dihydrate, Folin Ciocalteu's phenol reagent, gallic acid, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt, chlorogenic acid, luteolin-7-*O* glucoside, luteolin and ascorbic acid were obtained from Sigma Chemical Co. All other reagents were of laboratory grade.

Collection of plant material

Samples of *Achillea multifida* were collected from Uludağ Mountain in Bursa, Turkey, in August 2009. This species was identified by Dr Ertan Tuzlacı. The herbarium code number of this species is MARE 11719.

Preparation of the extracts

The aerial parts of these plant were treated to extract heptane, chloroform and methanol by maceration at room temperature until was achieved at a colourless solution. The heptane, chloroform and methanol extracts were filtered and evaporated using a rotary evaporator. The extracts were decanted into vials and put into refrigeration.

Q-TOF LC/MS analyses

Electrospray ionization-quadrupole-time of flight-mass spectrometry (ESI-Q-TOF LC/MS). (Agilent 6530, CA, United States) instrument, in the negative ion mode, (model G6530B, Agilent Technologies, USA) leading to the appearance of the protonated forms of the compounds under study, $[M-H]^-$ ions, was performed. The mobile phases used were water with trifluoroacetic acid (TFA) (0.1%) (Phase A) and acetonitrile with trifluoroacetic acid (0.1%) (Phase B), and the solvent gradient changed in the following pattern: 0 min. 5% B; 1 min. 5% B; 20 min. 30% B; 25 min. 60% B; 28 min. 60% B; 33 min. 95% B; 35 min. 95% B; 40 min. 5% B. The injection volume was 20 μL. The mass range scanned was m/z 100 to 1000. According to this, the optimume values of ESI-Q-TOF-LC/MS parameters in negative mode were: capillary voltage, 3500 V; drying gas temperature, 350°C; drying gas flow, 8 L/min; nebulizing gas pressure, 2 bar; and end-plate offset, −500 V. The accurate mass data for the molecular ions were processed using the Mass Hunter Workstation Software Version (B.O600 Build 6.0.633.10).

2,2-diphenyl-1-picryl-hydrazil (DPPH) scavenging activity

The DPPH radical scavenging activity extracts from *A. multifida* aerial parts were measured by the DPPH method proposed by Wei *et al*. and Aarland *et al*.[13,14] According to the results of scanning the spectrum obtained in this study, DPPH exhibited a strong absorption band (λ_{max}) at 517 nm. A solution of DPPH in methanol (0.1 mM) was prepared and 3.9 mL of this solution was added to 0.1 mL of extracts in solution across a variety of concentrations. After thirty minutes, the absorbance value was read at 517 nm. A lower absorbance showed a higher occurance of DPPH radical scavenging activity. The IC_{50} value is inversely correlated to antioxidant ability of extracts. A lower IC_{50} value reveals higher antioxidant activity.

$ABTS^+$ radical cation scavenging activity

The $ABTS^+$ assay was performed according to the method developed by Aarland *et al*. and Re *et al*.[14,15] This assay is based on the formation of the free radical cation $ABTS^{\bullet+}$ by reaction of ABTS aqueous solution (7mM) with $K_2S_2O_8$ (2.45 mM), at room temperature, under darkness, for 12–16 hrs. According to the results of scanning the spectrum

obtained in this study, $ABTS^+$ showed a strong absorption band (λ_{max}) at 734 nm.

This stock solution was diluted with methanol to an absorbance of 0.700 ± 0.020 at 734 nm. The reaction mixture comprised 3.96 mL of $ABTS^+$ solution and 0.04 mL of the extracts at a variety of concentrations. After 6 min, the absorbance value was read off at 734 nm.

Metal chelating capacity

The ferrous ions (Fe^{2+}) chelating assay was performed according to the method developed by Dinis *et al.*[16] and modified slightly. The extracts and standard (EDTA) (200µL) were added to 50 µL of $FeCl_2$ (2 mM) solution. The reaction was initiated by the addition 200 µL of ferrozine (5 mM), left standing at ambient temperature for 5 min. Then, methanol was added to this mixture until a final volume of 4 mL was achieved. According to the results of scanning the spectrum obtained in this study, the absorbance of the this mixture showed a strong absorption band (λ_{max}) at 562 nm. The mixture was left standing at ambient temperature for a further 10 min. Then, the absorbance was measured at 562 nm.

Ferric reducing antioxidant/power capacity (FRAP)

The ferric reducing power capacity assay was performed according to the method developed by Benzie & Strain[17] and modified slightly. An FRAP working solution was prepared afresh each time: 0.3 M acetate buffer (*p*H=3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl and 0.02 M $FeCl_3.6H_2O$ were mixed in 10:1:1 (v/v/v) and kept away from light. The mixture was incubated at 37 °C for 30 min. away from light. Then 0.2 mL of extract or standard solution were added to 3.8 mL FRAP working solution. After 4 min., the absorbance was measured at 593 nm. A solution of $FeSO_4.7H_2O$ was used for calibration. The ferric reducing power activity of the extracts was calculated from the linear calibration curve. Results were expressed as µM $FeSO_4$ equivalents per milligram of extracts. The calibration equation for $FeSO_4$ was absorbance= 1.668x -0.116 (R^2= 0.9982).

Determination of total phenolic compounds

The amount of total phenolic compounds in the *A. multifida* extracts were determined according to the method of Slinkard, Singleton and Mendoza-Espinoza *et al.*[18,19] 0.1 mL of extract solution was diluted with distilled water (4.6 mL). 0.1 mL of Folin-Ciocalteu reagent (diluted 1:3, v/v) was added. Then, 3 mL of Na_2CO_3 (2.0 %) were added and the mixture was left standing at ambient temperature for 2 hrs. The absorbance value was read at 760 nm. Results were expressed as milligrams of total phenolics per gram extract (mg GAE/g extract). The calibration equation for gallic acid was absorbance = 18.78x – 0.0031 (R^2 = 0.9855).

Cell culture

Human colon carcinoma cell line HT-29 (ATCC, HTB-38), breast cancer cell line MCF-7 (ATCC, HTB-38), human cervical carcinoma cell line HeLa (ATCC, CCL-2) and mouse embryonic fibroblast cell line NIH/3T3 (ATCC, CRL-1658) were used in this study for cytotoxicity experiments. The cells were maintained in DMEM, supplemented with 10% fetal bovine serum, 2 mM L- glutamine and 100 units/ml penicillin-streptomycin. The cells were incubated in a humidified 5% CO_2 incubator at 37 °C. Extracts were dissolved in dimethyl sulfoxide (DMSO).

Cytotoxicity

The cytotoxic activity of the heptane, chloroform and methanol extracts from *A. multifida* aerial parts were assayed using the MTT(3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) protocol. In short, the cells were seeded at a density of 1×10^4 cells/mL density in 96-well plates and allowed to congeal for 24 hrs in a CO_2 incubator at 37 °C. After the incubation, the extracts were added to the cells up to a final concentration of 100 µg/ mL. An untreated group was used as a negative control. Then the plates were incubated at 37 °C, in 5% CO_2, humidified atmosphere for 24 hrs. After an incubation period, the medium was aspirated and the cells washed in PBS. The medium was replaced with 100 µL fresh medium and 10 µL MTT solution (5 mg/mL) was added to each well. After 4 hrs of incubation, 100 µL permeabilization buffer was added, to solubilize the formazan crystals for 18 hrs. Absorbance was then determined at 540 nm by an ELISA plate reader. The percentage viability was calculated as:

% Viability = [OD of treated cells/OD of control cells] × 100[20]

Agar well diffusion method

Mueller Hinton agar and Mueller Hinton broth for the bacteria, Sabouraud dextrose agar and Sabouraud

dextrose broth for the yeasts were used as media. Microorganism suspensions were prepared using 18h broth culture of each bacteria and 24 hrs culture of yeast strains and adjusted to a turbidity equivalent to a 0.5 Mc Farland standard. The medium (15mL) was melted, then cooled to 55 ^{o}C and after that the microorganism (0.1mL) was inoculated in the suspension. The inoculated agar was poured onto the assay plate. Four wells, each 6 mm in diameter, were cut out of the agar after it had solidified. Then 50 µL of the heptane, chloroform and methanol extracts were placed into each well. The solvents of the extracts, meropenem (10 µg) and fluconazole (100 µg) were tested in the same way as the control. All the culture plates were incubated at 35 °C 18-24 hrs for bacteria and 48 hrs for *Candida* species. After incubation, the diameter of zone inhibition was read off in millimeters. All tests were performed three times and the average of the results calculated[21,22].

Determination of minimum inhibitory concentrations (MICs) of extracts from *A. multifida* by broth microdilution method

Determination of MIC for bacteria

The minimum inhibitory concentration (MIC) was assayed by using microbroth dilution method as recommended by Clinical Laboratory Standards Institute (CLSI). Cation-adjusted Mueller Hinton broth (BBL) was used as the test medium. The inoculum was prepared using a 4-6 hrs broth culture of each bacteria and adjusted to a turbidity equivalent to a 0.5 Mc Farland standard. After that, it was diluted in broth media to give a final concentration of 5 x 10^5cfu/mL. Sterile microtitration plates with 96 U-bottom wells were employed. Serial two-fold dilutions of each extract (100 µL) were prepared in a culture medium (100 µL). Each well of a microtitration plate was inoculated with 100 µL of 5×10^5 cfu/ml bacteria suspension. The growth control wells, which contained 100 µL of sterile extract-free medium were included. The antibacterial agent meropenem (256 - 0.125 µg/mL) was used as a positive control and tested by the same method. The plates were covered to prevent drying and incubated without agitation at 35±2 °C for 16 - 20 hrs in an ambient air incubator within 15 min of adding the inoculum. The lowest concentration of the extract that showed complete inhibition of visible growth was seen to be MIC[23,24,25].

Determination of MIC for yeasts

Antifungal activity against yeasts was assayed by the broth microdilution method under CLSI recommendations. RPMI-1640 (Sigma-Aldrich) supplemented with L-glutamine and buffered to *p*H 7 with 0.165 M morpholinepropanesulfonic acid (MOPS, Sigma) was used as the test medium. Firstly, all of the microorganisms were subcultured at least twice in Sabouraud dextrose agar to ensure viability and purity. The inoculum was prepared using a 24 hrs culture of yeast strains adjusted to a turbidity equivalent to a 0.5-2.5 x 10^3 cfu/mL in a sterile saline solution (0.85%). Sterile plastic, disposable, microtitration plates with 96 U-bottom wells were used. The microtitration plate was filled with 200 µL of culture medium containing the final extract concentration and made two-fold serial dilutions in a culture medium by transferring 100 µL of diluted extract suspension into each well. Each well was inoculated with 100 µL of 0.5-2.5 x 10^3 cfu/ml yeast suspension. The growth control well (extract-free medium only) was included in the results. Fluconazole (64 mg/mL to 0.125 mg/mL) as a positive control was tested in the same way as the antifungal agent. The microtitration plates were incubated without agitation at 35 °C in an ambient atmosphere for 48 hrs. The lowest concentration that resulted in a visible decrease of turbidity compared with that of growth-control wells is definied as the MICs of the extracts[26,27,28,29].

Statistical analysis

All data are the average of analyses in triplicate. The data were recorded as mean ± standard deviation and analyzed by the Graphpad Prism 5 Demo.

Results

Identification of phenolic compounds

The methanol extract was prepared by maceration and analysed using a ESI-Q-TOF LC/MS in negative ionization modes to identify the phenolic compounds. The phenolic compounds were identified by reference to the standard retention time, fragment ions (*m/z*) present, and other literary references on the subject. A total of 5 phenolic compounds were identified, as summarised in Table 1, including the retention time, molecular weight and formula, experimental and calculated *m/z*, and fragments.

Chlorogenic acid showed an $[M-H]^-$ ion at *m/z* 353.0913 and fragment ions, with one *m/z* 191 for

Table 1—Phenolic compounds in methanol extract of the *A. Multifida*

Compounds	RT (min)	M. weight	Molecular formula	m/z experimental	m/z calculated	Ref./Std.	Fragment ions (m/z)
Negative mode							
Chlorogenic acid	10.99	354.31	$C_{16}H_{18}O_9$	353.0913	353.0878	Std.	191,179
Quercetin hexoside	15.39	464.38	$C_{21}H_{20}O_{12}$	463.0921	463.0955	Ref.	301
Luteolin-7-O glucoside	17.02	448.37	$C_{21}H_{20}O_{11}$	447.0933	447.0876	Std.	285
Dicaffeoyl quinic acid	19.10	516.45	$C_{25}H_{24}O_{12}$	515.1051	515.1042	Ref.	353, 173
Luteolin	23.60	286.24	$C_{15}H_{10}O_6$	285.0368	285.0405	Std.	175,151,133

Ref: Reference; Std: Standards

quinic through the loss of a caffeoyl, and the other at *m/z* 179 for caffeic acid through the loss of a quinic[30].

Quercetin hexoside revealed an $[M-H]^-$ ion at *m/z* 463.0921 and fragment ions at *m/z* 301 representing quercetin aglycone through the absence of a hexoside moiety (463-162)[30]. Trifluoroacetic acid (TFA) was used as the mobile phase adducted to quercetin hexoside. Because the $[M-H]^-$ value was observed to be *m/z* 577 (463+114).

Luteolin-7-*O* glucoside showed an $[M-H]^-$ value at *m/z* 447.0933 and the product ion at *m/z* 285 representing luteolin aglycone through the absence of a glucoside moiety (447-162)[31].

Dicaffeoyl quinic acid showed an $[M-H]^-$ ion at *m/z* 515.1051 and by product ions at *m/z* 353 and 173. These were obtained due to a sequential loss of caffeoyl [M-H-caffeoyl] and quinic [M-H-caffeoyl-quinic] moiteies[30].

Luteolin showed an $[M-H]^-$ value at *m/z* 285.0368 and the product ions at *m/z* 175 [M-H-110], 151 [M-H-134] and 133 [M-H-152][32] (Fig. 1).

Total phenolics and antioxidant activity

The free radical scavenging capacity of heptane, chloroform and methanol extracts of the plant was measured by DPPH assay. Butylated hydroxytoluene and ascorbic acid were used as standards. The DPPH radical scavenging activitiy of heptane, chloroform and methanol extracts from the plant are shown in Table 2. The DPPH radical scavenging capacity of the heptane, chloroform and methanol extracts and standards showed the following order: ascorbic acid (IC_{50}:0.13±0.005 mg/mL)>BHT (IC_{50}:0.37±0.01 mg/mL)>methanol extract (IC_{50}: 1.69±0.04 mg/mL)>chloroform extract (IC_{50}: 44.16±0.3 mg/mL)>heptane extract (IC_{50}: 145.9±0.1 mg/mL). The methanol extract had the highest free radical scavenging activity. A linear relationship was detected between free radical scavenging activity and the amount of total phenolic compounds (R^2: 0.8172; Y= 0.6775 x-52.26).

The metal chelating activity of heptane, chloroform and methanol extracts was performed according to the method developed by Dinis *et al.*[16] and modified slightly. The percentages of ferrous ion chelating capacity of µg/µL concentration heptane, chloroform and methanol extracts from *A. multifida* aerial parts and EDTA are shown in Table 2. The metal chelating activity of the extracts and the standard compounds at the µg/µL concentration are exhibited in the following order: EDTA (95.78±0.2%)> methanol extract (9.67±0.8 %) >chloroform extract (4.58±0.5 %) >heptane extract (1.68±0.6 %). The methanol extract showed the highest metal chelating capacity. A linear relationship was detected between metal chelating capacity and the amount of total phenolic compounds (R^2: 0.9743; Y= 20.18 x-2.813).

An ABTS radical cation assay was used for the antioxidant capacity of plant extracts[33]. The ABTS radical cation scavenging capacity of heptane, chloroform and methanol extracts was measured according to the method of Aarland *et al.* and Re *et al.*[14,15] Ascorbic acid was used as standard. The ABTS radical cation scavenging activity of heptane, chloroform and methanol extracts are shown in Table 2. The ABTS radical cation scavenging effects of the heptane, chloroform, methanol extracts and standards are in the following order; ascorbic acid (IC_{50}: 0.19±0.006 mg/mL)>methanol extract (IC_{50}: 1.68±0.08 mg/mL)>chloroform extract (IC_{50}: 2.87±0.01 mg/mL)>heptane extract (IC_{50}: 35.78±0.2 mg/mL). The methanol extract of the plant showed the highest ABTS radical cation scavenging capacity. A linear relationship was detected between ABTS radical cation scavenging activity and the amount of total phenolic compounds (R^2: 0.9520; Y= 0.3684 x+25.87).

Ferric reducing antioxidant/power (FRAP) activity of heptane, chloroform and methanol extracts

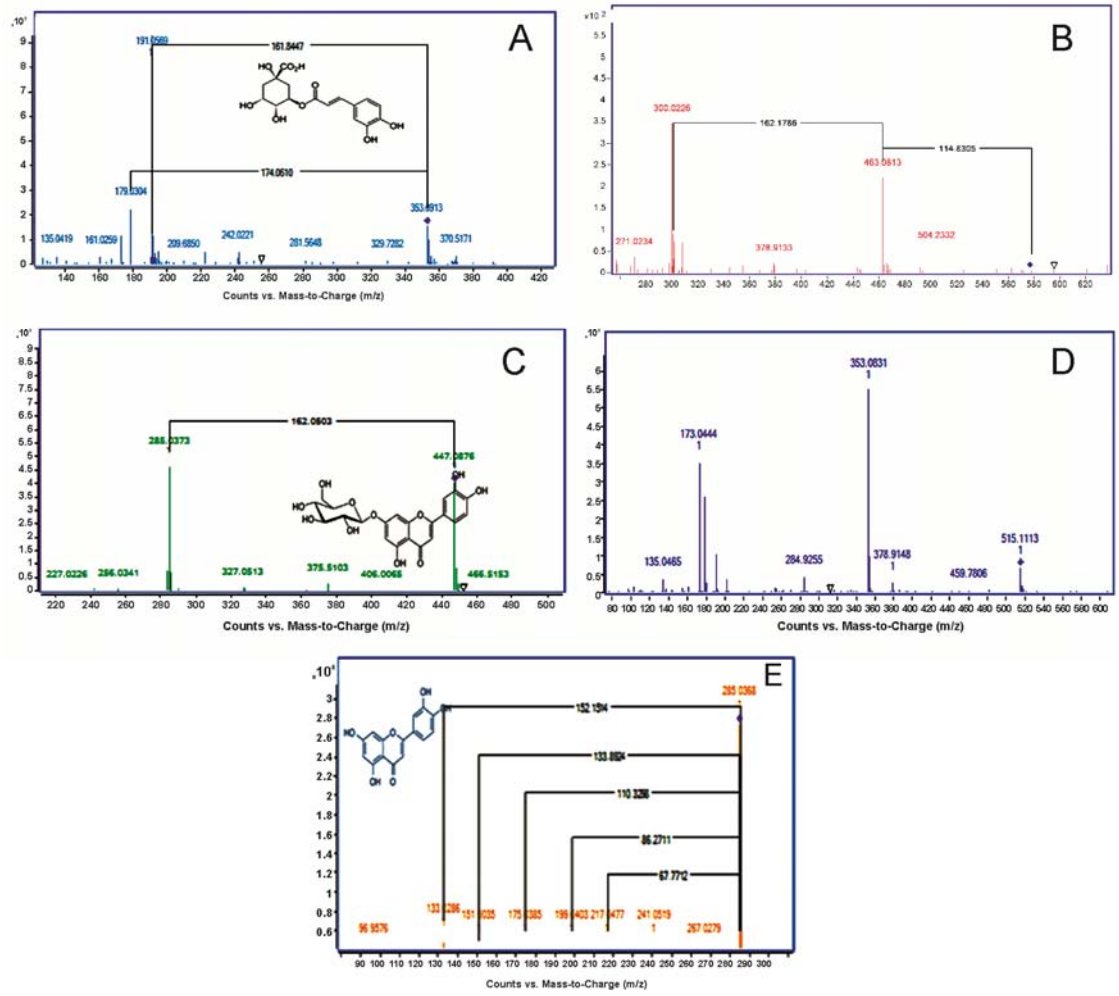

Fig. 1—MS/MS spectra of phenolic compounds identified for the first time in methanolic extract of *A. multifida* aerial parts. A: chlorogenic acid; B: quercetin hexoside; C: luteolin-7-*O*-glucoside; D: dicaffeoyl quinic acid; E: luteolin

Table 2—Metal chelating, DPPH radical scavenging, ABTS radical cation scavenging, ferric reducing antioxidant power activities and the amount of total phenolic compounds (PC) (as gallic acid equivalents) of heptane, chloroform and methanol extracts from *A. multifida* aerial parts.

Extracts/ Standarts	Metal chelating activities (%)	DPPH (IC_{50}: mg/mL)	ABTS (IC_{50}: mg/mL)	FRAP (μMFeSO$_4$/0.2mg extract	PC (mg/g extract)
Heptane	1.68±0.6 ^{a,c,d}	145.9±0.1^{a,b,c,d,e}	35.78±0.2 $^{a, b,c,d,}$	0.11±0.0003 ^{a,b,c,d}	13±0.01 ^{a,b,c}
Chloroform	4.58±0.5 ^{b,c,d}	44.16±0.3 ^{b,c,d,e}	2.87±0.01 ^{b,c,d,}	0.21±0.002 ^{b,c,d}	35.5±0.7 ^{b,c}
Methanol	9.67±0.8 ^{c,d}	1.69±0.04 ^{c,d,e}	1.68±0.08 ^{c,}	0.65±0.002 ^{c,d}	58±0.01 ^{c}
EDTA	95.78± 0.2 ^{d}	-	-	-	-
BHT	-	0.37± 0.01^{d}	-	-	-
Ascorbic acid	-	0.13±0.005^{e}	0.19 ± 0.006^{d}	2.15±0.001 ^{d}	-

These values were the mean values of three replicates ± standard deviation. Different superscript letters in each column exhibit significant differences in mean values at P< 0.05 according to Tukey's multiple comparison test.

have shown in Table 2. Ascorbic acid was used as a standard. The ferric reducing power effects of the extracts and standard are in the following order: ascorbic acid (2.15±0.001 μMFeSO4/0.2mg extract)>metanol extract (0.65±0.002 μMFeSO4/0.2mg extract)>chloroform extract (0.21±0.002 μMFeSO4/0.2mg extract)>heptane extract (0.11±0.0003 μMFeSO4/0.2 mg extract). The methanol extract of plant exhibited the highest ferric reducing antioxidant power. The linear relationship was detected between the ferric reducing antioxidant/power capacity and the amount of total phenolic compounds (R^2: 0.8833; Y= 0.0135 x-0.2167).

The total phenolic compounds in the extracts were determined from the regression equation of calibration curve (Y = 18.78x – 0.0031 (R^2 = 0.9855) and expressed in gallic acid equivalents (GAE). The amount of total phenolic compounds in heptane, chloroform, methanol extracts of the plant are shown in Table 2. Total phenolic compounds in heptane, chloroform, methanol extracts are exhibited in the following order: methanol extract (58.00±0.01 mg/g extract)>chloroform extract (35.5±0.7 mg/g extract)>heptane extract (13.00±0.01 mg/g extract). The methanol extract of plant had the highest amount of total phenolic compounds.

Cytotoxic activity

The cytotoxic activity of heptane, chloroform and methanol extracts from *A. multifida* aerial parts was determined on cancer (Human colon adenocarcinoma cancer cell line, HT29; Human cervix adenocarcinoma cancer cell line, HeLa; Human breast adenocarcinoma cancer cell line, MC-7) and normal (mouse fibroblast cell line, NIH/3T3) cells. The results are summarized in Table 3.

The chloroform extract showed a strong cytotoxic effect and selectivity activity against HeLa (67.60±2.01%) tumour cell line and moderate activity against HT-29 (58.03±5.95%) and MCF-7 (45.35±5.05%) cell lines. The heptane extract exhibited moderate activity against HeLa (49.79±1.29%) tumour cell line but this extract showed some activity against HT-29 (26.13±13.94%) and MCF-7 (13.29±8.10%) cell lines. The methanol extract of the plant exhibited little activity against HeLa (13.08±8.6%) and MCF-7 (5.85±2.87%) cell lines but no activity against HT-29 (-20.53±5.7%).

The significance of the difference between means was determined by Tukey's Multiple Comparison Test ($P < 0.05$) using the Graphpad Prism 5 Demo (Table 3).

Low concentrations (100 µg/mL and 50 µg/mL) of heptane, chloroform and methanol extracts had no toxic effects on the NIH/3T3 cells. Higher concentrations (200 µg/mL) of chloroform extract showed some toxic effects on NIH/ 3T3 cells but the same concentration of heptane and methanol extracts had no toxic effect on NIH/3T3 cells. 100 µg/mL concentration of the chloroform extract showed strong activity and selectivity activity against HeLa tumour cell lines and moderate activity against HT-29 and MCF-7 cell lines but a 100 µg/mL and 50 µg/mL concentration of this extract had no toxic effect on normal cells (NIH/3T3). Therefore, after examining the toxic effects on different normal cell lines of chloroform extract we conclude that it might be of interest to the pharmaceutical industry, as an anticancerogenic agent (Table 4).

The chloroform extract contain more nonpolar compounds (particularly methoxylated flavones and sesquiterpene lactones) than the heptane or methanol extracts. These compounds are known to exhibit strong cytotoxic activity. Thus, the chloroform extract of *A.multifida* exhibited the highest cytotoxic activity. 200 µg/mL, 100 µg/mL and 50 µg/mL concentrations of methanol extract had no toxic effect on normal cells (NIH/3T3). The methanol extract of *A.multifida* aerial parts are potential novel sources for plant-derived pharmaceutical manufacturing (particularly antioxidant and antimicrobial effective) in the future.

Antimicrobial activity

In this study, the heptane, chloroform and methanol extracts of *A. multifida* showed antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *S.epidermidis* ATCC 12228. In contrast, we did not find any effect on gram negative bacteria. The methanol extract showed antibacterial activity against *Staphylococcus aureus* ATCC 25923, *S.epidermidis* ATCC 12228, *E.coli* ATCC 25922 and *P. vulgaris* ATCC 13315. None of the extracts showed antimicrobial activity against *P. aeruginosa*

Table 3—*In vitro* cytotoxic effects of heptane, chloroform and methanol extracts from *A. multifida* aerial parts on human MCF-7, HeLa and HT-29 cancer cell lines.

Extracts (100 µg/mL)	MCF-7 (breast)	HeLa (cervical	HT-29 (colon)
	Cell death (%)		
Heptane	13.29±8.10[a]	49.79±1.29[a]	26.13±13.94[a]
Chloroform	45.35±5.05[b]	67.60±2.01[b]	58.03±5.95[b]
Methanol	5.85±2.87[c]	13.08±8.6[c]	-20.53±5.7[c]

These values were the mean values of three replicates ± standard deviation. Different superscript letters in each column exhibit significant differences in mean values at P< 0.05 according to Tukey's multiple comparison test.

Table 4—The effects on proliferation of NIH/3T3 cells of heptane, chloroform and methanol extracts from *A. multifida* aerial parts

Concentration (µg/mL)	Heptane	Chloroform	Methanol
	Cell growth (%)		
200 µg/mL	143.29	32.15	112.89
100 µg/mL	123.83	67.34	109.19
50 µg/mL	121.62	100.74	110.64

These values were the mean values of three replicates.

Table 5—Antimicrobial activity of heptane, chloroform and methanol extracts of *A. multifida*

	Heptane extract		Methanol extract		Chloroform extract		Meropenem (10 µg)	
	Inhibition zone diameter (mm)	MIC (µg/ml)	Inhibition zone diameter (mm)	MIC (µg/ml)	Inhibition zone diameter (mm)	MIC (µg/ml)	Inhibition zone diameter (mm)	MIC (µg/ml)
Bacteria								
Staphylococcus aureus ATCC 25923	7	15 625	11	31 250	6	7812.5	32	< 0.125
Staphylococcus epidermidis ATCC 12228	4	250 000	11	31 250	7	7812.5	38	< 0.125
Escherichia coli ATCC 25922	-		2	31 250	-		27	< 0.125
Proteus vulgaris ATCC 13315	-		2	62 500	-		24	16
Klebsiella pneumoniae ATCC 4352	-		-		-		22	< 0.125
Pseudomonas aeruginosa ATCC 27853	-		-		-		23	< 0.125
Yeasts				Fluconazole (100 µg)				
Candida albicans ATCC 90028	-		-		-		22	640
Candida glabrata ATCC 90030	-		-		-		16	40
Candida guilliermondii KUEN 998	-		-		-		19	10
Candida tropicalis KUEN 1021	-		-		-		14	40
Candida parapsilosis ATCC 90018	-		-		-		36	10
Candida krusei ATCC 6258	-		-		-		9	160

MIC: Minimum Inhibitory Concentration; (-): No inhibition

ATCC 27853 and *K. pneumoniae* ATCC 4352. The heptane, chloroform and methanol extracts were found to have no effect against the standard *Candida* species. Solvents used in this experiment did not inhibit the growth of any of the bacteria nor *Candida* species. The results of *in vitro* antimicrobial activities of heptane, chloroform and methanol extracts of *A. multifida* with MIC values compared to antibiotic and antifungal agents are given in Table 5.

Discussion

Phenolic compounds are currently of interest owing to their antioxidant, antimicrobial and possible anticarcinogenic activities[8,34,35,36]. Concerning the bioactivity of *Achillea* species, recent studies have reported antimicrobial, anti-inflammatory, antispasmodic, antiulcer and antioxidant activities of its polar extracts[37,38,39,40,41]. Some articles have identified phenolic compounds of *Achillea* species as worth further study[8,42].

To the best of our knowledge, there are only two reports on the antimicrobial potential of *A. multifida*[9,12]. The first report concerned the composition of the water-distilled essential oil of *A.multifida.* This was analysed using GC and GC/MS. Fifty-eight compounds from this plant were identified in this report, especially α-thujone, β-thujone, sabinene, and camphor. The essential oil of *A.multifida* showed inhibitory activity against *E.aerogenes* (MIC: 62.5 µg/mL), *P.aeruginosa* (MIC:125 µg/mL) and *C.albicans* (MIC: 62.5 µg/mL)[12]. Also, in the second report, *A.multifida* flower heads were examined in terms of their antifungal and antibacterial activity of their n-hexane, chloroform and methanol extracts[9]. In this study, the chloroform extract of *A.multifida* showed moderate activity against *S. aureus* (ATCC 6538/P) and *S. epidermidis* (MIC=50 µg/mL, respectively). In addition, this report extract indicated antibacterial activity against hemorrhagic *E.coli* (MIC=100 µg/mL). All of the extracts were found inactive against *C. albicans*.

However, in our current study, antimicrobial activity of heptane, chloroform and methanol extracts from *A.multifida* aerial parts were investigated for *Staphylococcus aureus*, *S.epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Candida* species. The methanol extract of *A.multifida* showed antibacterial activity against *S.aureus* ATCC 25923, *S.epidermidis* ATCC 12228, *E.coli* ATCC 25922 and *P.vulgaris* ATCC 13315.

To the best of our belief, there have been no studies of the antioxidant, antimicrobial (excluding essential oil and capitulum of this plant), cytotoxic effects of heptane, chloroform and methanol extracts from *A. multifida* aerial parts and phenolic compounds in methanolic extract. Hence, in our report, the heptane, chloroform and methanol extracts of *A.multifida* were tested using different methods for antioxidant, antimicrobial and cytotoxic activity.

As a result of this study, the methanol extract of plant showed the highest total amount of phenolic, antioxidant and antibacterial activity. The phenolic compounds in methanolic extract were identified by ESI-Q-TOF LC/MS.

The chloroform extract showed the highest cytotoxic activity. No bioactive compounds in chloroform extract were identified in this study. In further studies, cytotoxic compounds will be isolated (column chromatography, thin layer chromatography, chromatotron) and identified (UV, IR, NMR) from chloroform extract. These identified compound(s) or crude extracts may be of interest as antitumour agents in future studies.

Conclusion

The methanol extract showed stronger antioxidant and antibacterial activity than the chloroform and heptane extracts. The phenolic compounds in methanolic extract were identified as chlorogenic acid, quercetin hexoside, luteolin-7-*O* glucoside, dicaffeoyl quinic acid, luteolin. The results obtained showed that phenolic compounds contribute to the antioxidant and antibacterial activity of the methanol extract. The chloroform extract of *A.multifida* showed the highest cytotoxic activity. 100 µg/mL and 50 µg/mL concentration of chloroform and methanol extracts had no toxic effect on the normal cell line of NIH/3T3. Thus, the methanol extract from *A. multifida* aerial parts may be a worthy natural antioxidant and antibacterial source and to be applicable in both medicine and the food industry. In addition, the chloroform extract might be a potential source of anti-cancer agent(s).

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