Evaluation of anti-oxidative, antimicrobial and anti-diabetic potential of *Adiantum venustum* and identification of its phytochemicals through GC-MS

Javerya Hamid, Dildar Ahmed* and Abdul Waheed
Department of Chemistry, Forman Christian College, Lahore, Pakistan

Abstract: The present study aimed to explore antioxidant, antimicrobial and anti-diabetic properties of the fern *Adiantum venustum*. The TPC (total phenolic content) of methanolic extract of the plant was 247.95±0.0007µg of Gallic acid equivalents per gram of dried extract (mg GAE/g). The highest TPC was in n-butanolic fraction, which was 981.45±0.1562mg GAE/g. Hexane fraction showed lowest TPC (256.95±0.0420mg GAE/g). Ethyl acetate fraction exhibited highest total flavonoid content (TFC), i.e., 62.0±0.050mg of Rutin equivalents per gram of sample. DPPH (1,1-diphenyl-2- picryl hydrazyl) radical scavenging activity of the plant was significant. n-Butanolic fraction was most potent with IC_{50} being 1.06mg/mL. The IC_{50} of methanolic extract was 1.50mg/mL, that of aqueous fraction was 2.51 mg/mL, and that of chloroform fraction was 2.65mg/mL. Antibacterial potential of the fern was tested against two Gram-positive bacterial strains, *Pseudomonas aeruginosa* and *Escherichia coli* and two Gram-negative bacterial strains, *Bacillus subtilis* and *Staphylococcus aureus*. n-Butanolic fraction showed highest zone of inhibition (ZOI, 25.13±1.237 mm) against *P. aeruginosa*. Ethyl acetate fraction was most active (ZOI, 15.75±1.060 mm) against *S. aureus*. Against *E. coli*, aqueous and n-butanolic fractions were most active (ZOI, 14.75±0.353 and 14.50±0.707mm, respectively). Minimum inhibitory concentration (MIC) of methanolic extract against *B. subtilis* was as low as 1.98mg/mL. The hexane fraction was least toxic against all the fractions. The plant displayed significant alpha-amylase inhibitory activity. Chloroform fraction was most effective with lowest IC_{50} (1.10mg/mL) followed by ethyl acetate fraction (1.92mg/mL). *A. venustum* is rich in phenolics and has substantial antioxidant, antimicrobial and alpha-amylase inhibitory potential.

Keywords: *Adiantum venustum*, antioxidant, antimicrobial, anti-diabetic, phenolics, flavonoids, GC-MS.

INTRODUCTION

Adiantum (family Adiantaceae/ Pteridaceae) is a large genus comprising about 200 species of ferns (Pan *et al*., 2011). They are distributed around the world from hot tropic regions to cold temperate zones. The genus is known to have a variety of ethno-pharmacological activities including antitumor, antiulcer, antihypoglycemic, antimicrobial, antiviral, anti-pyretic, diuretic and expectorant (Sing *et al*., 2013; Sher *et al*. 2011). Brahmachari *et al*., 2003). They are also used to treat jaundice, cough, fever, headache and muscular pain (Lone *et al*., 2015; Razaq *et al*., 2010). From various Adiantum plants, many types of natural products have been isolated, which include triterpenoids, sterols, flavonoids, phenolics and many other phytochemicals (Pan *et al*., 2011). Common species of Adiantum found in Pakistan include *A. capillus-veneris*, *A. caudatum*, *A. venustum* and *A. trapeziforme* (Iltaf *et al*., 2012).

A. venustum (full name *Adiantum venustum* D. Don) is commonly called evergreen maidenhair or hardy maidenhair. In Pakistan, it is found in Chitral, Swat, Murree, Hazara and Kashmir and is also found in Afghanistan, Bhutan, China, India, Nepal and Tibet, and (Hassler, 2016). *A. venustum* has rich ethnomedicine. It is used to treat snake and scorpion stings. Its fronds are considered to be astringent, diuretic and expectorant and a paste prepared from its rhizomes is used to treat cuts and wounds (Amjad *et al*., 2015; Haq, 2012). Decoction of leaves is used to treat cough, stomach pain and urinary disorders (Yousuf *et al*., 2012). The plant is also used to treat chronic tumors and to prevent hair loss, and is also used as a tonic (Wani *et al*., 2016).

Plants, which have been used to cure diseases since the time immemorail, contain natural products with a wide range of bioactivities including antioxidant, anti-diabetic, and antimicrobial. Natural antioxidants are required to combat oxidative stress, which is caused by over production of free radicals in the body. It is a major cause of a number of hazardous therapeutic disorders inculdidng cancer, cardiovascular dieases and diabetes (Ahmed *et al*., 2013). Although, there are a number of anti-hyperglycemic drugs are available, their side effects and high cost demand safer and more affordale alternatives. About 800 plant species are thought to have hypoglycemic activity (Arif *et al*., 2014), which are required to be explored to find better remedies for the ailment.

Increasing resistance of microorganisms against existing antibiotics is another problem of grave concern (Ntei-Kang *et al*., 2013). Plants being rich in various types of

**Corresponding author:* e-mail: dildarahmed@gmail.com

chemical substances have great prospects for more effective and safer antimicrobial agents. They can provide common people with affordable and easily accessible therapeutic remedies for different types of infections.

Taking into account the rich folkloric application of the plant, we planned to investigate *Adiantum venustum* for its antioxidant, anti-diabetic and antimicrobial properties. Consequently, methanolic extract of the plant and its sub-fractions in various solvents were studied for their possible therapeutic potential.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and α-amylase were purchased from MP Biomedicals, France; methanol, dimethyl sulfoxide (DMSO), sodium hydroxide, sodium nitrite, aluminium chloride, rutin, Folin-Ciocalteu reagent, anhydrous sodium carbonate, starch, and Mueller-Hinton agar were purchased from Merck, Germany; Gallic acid was purchased from Scharlau, Switzerland. GC-MS analysis of the plant samples was carried out on Agilent Technologies GCMS 7890 A)/5975C.

Collection and preparation of plant material

The plant material was collected from hills near Abbottabad (Pakistan) in March 2012. The plant was identified by the taxonomist Dr. Zaheeruddin Khan of Government College University, Lahore, were a specimen of the plant is kept under voucher No. GC.Herb.Bot.2990. The plant was cleaned, washed, air- dried and pulverized to powder and stored at 25°C. For extraction, the powdered plant material (50g) was soaked in methanol and allowed to stand for 15 days with occasional shaking. The extract was filtered off and solid residue was recovered for repeated maceration. Filtrates were pooled and the solvent was evaporated on a rotary evaporator to obtain dried methanolic extract (MeOH, 15g). A part of the methanolic extract (10g) was fractionated successively into solvents with increasing polarity to obtain hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), n-butanolic (n-BuOH), and residual aqueous fraction with yields (%) as 6.574, 5.244, 2.704, 1.264, 4.08, respectively.

Test microorganisms

Standard strains of common pathogenic microorganisms used for antimicrobial study were *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538). The first two are Gram-positive while the latter two Gram-negative. The strains were maintained in Mueller-Hinton agar (MHA) slants at 4°C. Microorganisms were obtained from Pharmagen, Lahore, Pakistan.

Study of antioxidant properties

Total phenolic content (TPC)

Total phenolic contents were evaluated by a standard protocol (Slinkard *et al*., 1977). Stock solution of Gallic acid standard (5000 mg/L) was prepared by dissolving 0.5 g Gallic acid in 10mL methanol in a 100-mL volumetric flask, making the volume up to the mark by adding distilled water. Solutions of different concentrations (50 mg/L, 100mg/L, 150mg/L, 250mg/L, 500mg/L) were prepared by diluting (1mL, 2mL, 3mL, 5mL, 10mL, respectively) stock solution with distilled water making the final volume as 100mL. Sodium carbonate solution was prepared by adding 200g anhydrous sodium carbonate in 800mL distilled water and the solution was boiled for a minute and allowed to stand as such for 24h. Then, a few crystals of sodium carbonate were added and the solution was filtered. The filtrate was poured in 1-L volumetric flask and distilled water was added up to the mark. In a test tube, 40 µL standard/plant extract/fraction (1 mg in 5mL methanol), 3.16mL distilled water and 200 µL Folin-Ciocalteu reagent was taken. After mixing, it was incubated for 8 min. Then, 600µL sodium carbonate solution was mixed and the mixture was incubated at 40°C for 30 min. Blank was prepared by taking methanol (without plant extract). Absorbance was recorded at 765 nm. Total phenolic contents were expressed as µg Gallic acid equivalent/g of dried plant sample, which was calculated using the formula,

$C = c\ (mg/mL) \times [V\ (mL) / M\ (g)]$

Where, C=total phenolic content (mg/g), c= concentration established from calibration curve (GAE, µg/mL), V= total volume of the sample in test tube (i.e., 40µL + 3.16mL + 200µL + 600 µL=4mL), M = total mass of the sample in the test tube in grams (4mL volume contained 0.0008 g plant sample).

Total flavonoid content (TFC)

Total flavonoid content of each sample was determined using a scheme (Sahreen *et al*., 2010). Rutin was used as a standard, and its calibration curve was drawn using concentrations ranging from 100mg/L to 700mg/L (100mL stock solution was prepared by thoroughly dissolving 0.5g rutin in 50mL methanol in a 100-mL volumetric flask, and making the volume up to the mark with distilled water). To 300µL of a plant extract/ fraction (1 mg in 5mL methanol) or standard, 3.4mL 30% aqueous methanol and 150µL 0.5M $NaNO_2$ solution was added. After an interval of 5min, 150µL 0.3 M $AlCl_3$ solution was mixed, and the mixture was again kept on incubation for 5 min. After that, 1mL 1M NaOH solution was added. Blank was prepared by replacing $AlCl_3$ with distilled water. The absorbance was measured at 506nm. Total flavonoid contents were expressed as µg Rutin equivalent/g of dried plant sample, which was calculated using the formula,

$C = c\ (mg/mL) \times [V\ (mL) / M\ (g)]$

where C = total flavonoid contents (mg/g), c = concentration established from calibration curve (RE, µg/mL), V = total volume of the sample in test tube (i.e.,

300 µL + 3.4mL + 150 µL + 150µL + 1mL = 5mL), M = total mass of the sample in test tube in grams (4mL volume contained 0.001 g plant sample).

DPPH radical scavenging activity

DPPH radical scavenging activity was measured using a standard method (Brand–Williams *et al*., 1995). Stock solution of DPPH was prepared by dissolving 12 mg DPPH in methanol to make 50mL solution. Then absorbance of 0.98 (±0.02) at 517 nm was achieved by adding methanol to the stock solution in order to get working solution of DPPH radical. Ascorbic acid was used as a standard. Different concentrations (7mg/mL, 6 mg/mL, 5mg/mL, 4mg/mL, 3mg/mL, 2mg/mL, 1mg/mL, and 0.05mg/mL) of ascorbic acid (standard), plant extract and fractions were prepared in methanol. Absorbance was measured at 517 nm by taking 3mL working solution of DPPH mixed with 100µL standard or plant extract/fractions (7mg/mL) in test tube and allowed to stand for a period of 30 min. All other concentrations were assessed for the antioxidant assay in the same manner. For blank, 3mL methanol was added in 100µL standard/plant extract or fractions instead of DPPH solution. Control was prepared by adding 3mL DPPH working solution to 100 µL methanol. The DPPH radical scavenging activity was calculated by the formula given below:

%Radical Scavenging Activity = $[1- (A_{sample} /A_{control})] \times 100$

Where, A_{sample} and $A_{control}$ are absorbance of sample and control, respectively.

Antibacterial activity

Zones of inhibition

The antibacterial activity was determined by disc diffusion method (Ervin *et al*., 1974). In 100-mm sterilized plates, 21mL autoclaved (121°C for 15min) Mueller- Hinton agar was added. For bacterial culture, autoclaved seed agar (100mL at 50°C) was taken in a flask. Inoculum was prepared by adding 3mL 0.9% w/v Normal Saline in culture slants containing bacterial culture. It was then shaken gently (in circling motion) so that maximum culture was transferred into Normal Saline, containing bacterial culture. Here turbidity was adjusted (by adding saline water) according to 0.5% McFarland standard. This inoculum was poured into seed agar. Immediately after the plates got solidified, 4mL seed agar was added. In this way, 25mL standard volume of medium in 100-mm plate was attained. In order for agar to set, the plates were refrigerated for 1h. Holes were dug into it (by 8mm puncture) and carefully evacuated. Samples (40mg/mL each) were added into the holes, and kept for incubation for 24 h at 30-35°C.

Evaluation of minimum inhibitory concentrations (MICs)

Minimum inhibitory concentration was determined by agar diffusion assay following a previously reported method (Nostro *et al*., 2000). With the stock of 30 mg/mL of a plant extract/fractions, 1%-5% incremental dilutions were prepared. For 1% dilution plate, 0.2mL (30mg/mL) plant extract/fraction was added into 19.8mL single strength MHA. For 2% dilution plate, 0.4mL (30mg/mL) plant extract/fraction was added into 19.6mL MHA. In this way, 5 dilution plates were prepared for each plant extract/fraction. With the help of a multi-point inoculator (containing the inoculum), all bacterial cultures were transferred into the plates at one time. Three control plates were also prepared: (a) a plate containing Mueller-Hinton agar and the inoculum. This was to check the sustainability of the cultures, (b) a plate containing only agar, and (c) a plate containing agar with plant extract. All plates were then incubated for 24h and the plates were observed next day. The lowest dilution that completely inhibited the growth of the culture was the minimum inhibitory concentration.

Evaluation of alpha-amylase inhibitory activity

The enzyme alpha-amylase inhibitory activity was determined by method described by Nickavar *et al*. (2009). To prepare 20mM sodium phosphate buffer, 0.356 g Na_2HPO_4, 0.24g NaH_2PO_4 and 0.0392g NaCl were taken in a 100-mL volumetric flask and dissolved in distilled water making the volume up to the mark. The pH was maintained at 6.9 with 1M NaOH or 1M phosphoric acid. To 100mL buffer solution, 0.001 g alpha-amylase was mixed to make 0.5unit/mL enzyme solution. Fresh starch solution (0.5% w/v) was prepared by boiling 0.125 g starch in 25mL deionized water for 15min. The coloring reagent was prepared by mixing reagents A and B. The reagent A consisted of 20mL of 96mM 3,5-dinitrosalicilic acid (0.438g dissolved in 20mL distilled water), and reagent B was made by mixing 8mL of 2M NaOH and 12 g of sodium potassium tartrate tetrahydrate followed by heating at 50-70°C with constant stirring. Distilled water (12mL) was added. Acarbose was used as standard. Serial dilutions of standard or plant extract/fraction (4mg/mL, 3mg/mL, 2mg/mL, 1mg/mL, 0.50mg/mL, 0.25mg/mL and 0.05mg/mL) were prepared in DMSO. Three wrapped test tubes were taken for control, blank and sample. In the first (sample) and second (blank) test tubes, 0.5mL standard or plant extract/fraction (4mg/mL) and 0.5mL enzyme solution were added. In the third (control) test tube, 0.5mL DMSO and 0.5mL enzyme solution was taken. All three test tubes were incubated for 30 min at 25°C. Then, 1mL starch solution was added in first and third test tubes, and 1mL coloring reagent was added in the second test tube. Again all test tubes were incubated for 3 min at 25°C. After that, 1mL coloring reagent was added in first and the third test tubes while 1mL starch solution was added in the second one. All test tubes were heated in the water bath at 85°C for 15 min. Test tubes were then allowed to cool down. In each test tube, 9mL distilled water was added and absorbance was measured at 540 nm on a UV-visible spectrophotometer. In this way

all other concentrations were examined. Percent alpha-amylase inhibitory activity of a sample was calculated using the formula given below:

%Alpha-amylase inhibitory activity = [1- (A_{sample} /$A_{control}$)] x 100

Where A_{sample} and $A_{control}$ are absorbance of sample and control, respectively.

GC-MS protocol

The GC-MS used to identify the chemical constituents was Agilent Technologies GCMS (7890 A) equipped with an HP-5 MS column; sample (1.0µL) was injected manually in the splitless mode. MS Agilent Technologies (5975C) system working at 70 eV; oven temperature programmed from ambient to 310°C at the rate of 10°/min; inlet temperature 230°C; mass scan range 50-800 amu; NIST 05 spectral library was used interpret spectra.

RESULTS

Antioxidant properties

Total phenolic and flavonoid contents

Total phenolic (TPC) and flavonoid (TFC) contents were measured in terms of standard Gallic acid (GAE) and Rutin (RE) equivalent, respectively. The results are depicted in fig 1. The standard curve equation for Gallic acid was y = 0.000x – 0.015, r^2 = 0.999 and that for Rutin was y = 0.0001x + 0.1, r^2 = 0.965.

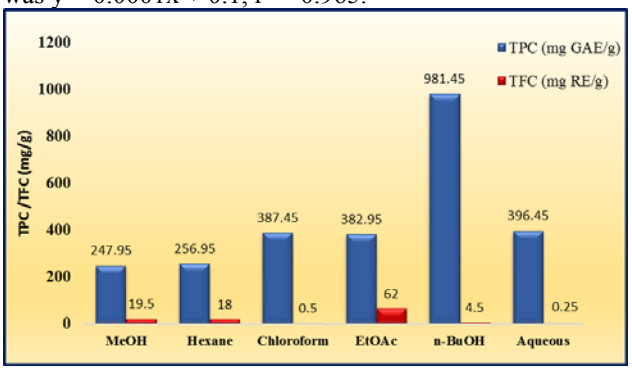

Fig. 1: Total phenolic (TPC) and flavonoid (TFC) contents (mg/g) of methanolic extract (MeOH) of *Adiantum venustum* and its hexane, chloroform, ethyl acetate (EtOAc), n-butanolic (n-BuOH) and aqueous fractions.

Total phenolic contents ranged from 247.95±0.0007 to 981.45±0.1562mg GAE/g. The methanolic extract showed total phenolic content as 247.95±0.0007mg GAE/g. The highest phenolic content was present in n-butanolic fraction, i.e., 981.45±0.1562mg GAE/g. The aqueous, chloroform and ethyl acetate fractions also showed significant contents, i.e., 396.45±0.0056, 387.45±0.0360 and 382.95±0.0420mg GAE/g, respectively. However, hexane showed the lowest TPC (256.95±0.0420 mg GAE/g).

Total flavonoid contents were 62.0±0.050 to 0.25±0.000 mg RE/g. Polar factions, n-butanolic (04.5±0.028µg RE/g) and aqueous (0.25±0.000 mg RE/g) fractions, did not show significant results except ethyl acetate fraction that exhibited the highest value, i.e., 62.0±0.050mg RE/g. The hexane also showed significant TFC (18.0±0.000mg RE/g).

DPPH radical scavenging activity

The DPPH is a radical which changes its color from violet to pale yellow by taking an electron or H radical from antioxidant substances, and, thus, provides a viable method to study the ability of a substance to scavenge free radicals. This is perhaps the most common method used to analyze antioxidant activity of both natural and synthetic substances. In the present study, methanolic extract of *A. venustum* and its fractions in different solvents were subjected to this assay and the results are displayed in table 1. Ascorbic acid (vitamin C) was used as a standard. The extract and fractions exhibited antioxidant activity in a dose dependent manner. The IC_{50} values of MeOH extract and hexane, $CHCl_3$, EtOAc, n-BuOH and aqueous fractions were (in mg/mL) 1.50, 2.61, 2.51, 1.90, 1.06 and 2.65, respectively. Among the fractions, n-BuOH was, thus, most potent followed by MeOH. Aqueous and $CHCl_3$ fractions showed lowest efficacy. The results suggest that the polar fractions contained molecules with functionalities capable to scavenge free radicals.

Antibacterial activity

Antibacterial activity was tested against four standard bacterial strains. Two Gram-positive were *Pseudomonas aeruginosa* and *Escherichia coli*, while two Gram-negative were *Bacillus subtilis* and *Staphylococcus aureus*. Antibacterial activities of the methanolic extract of *A. venustum* and its fractions were determined in terms of zones of inhibition (ZOI) and MICs, and the results are illustrated in tables 2 and 3. The MeOH extract and all of its fractions were highly active against *P. aeruginosa* where n-butanolic showed the highest zone of inhibition, which was 25.13±1.237mm. Chloroform, ethyl acetate and aqueous fractions exhibited almost equal activity (ZOI around 21mm). Hexane fraction showed the lowest antibacterial activity with ZOI 17.60±0.884mm. In case of *S. aureus*, ethyl acetate fraction was most active (ZOI 15.75±1.060mm). Against *E. coli*, aqueous and n-BuOH fractions were most active (ZOI 14.75±0.353 and 14.50±0.707mm, respectively). Aqueous fraction exhibited highest toxicity against *B. subtilis* (ZOI 17.00±0.707mm). Notably, the $CHCl_3$ fraction that showed considerable efficacy against *P. aeruginosa* and *B. subtilis*, was completely ineffective against *S. aureus*. The hexane and EtOAc fractions were least potent except against *P. aeruginosa*. *P. aeruginosa* was most susceptible among all the strains used in the study. Plant sample showed better efficacy against this microbe as compared to amoxicillin (table 2).

Table 1: Percentage free radical scavenging activity of methanolic extract of *Adiantum venustum* and its fractions at different concentrations in DPPH assay.

Conc. (mg/mL)	%Activity						
	Ascorbic acid	MeOH	Hexane	$CHCl_3$	EtOAc	n-BuOH	Aqueous
7.00	100.70±0.707	92.20±1.210	59.50±0.029	65.90±1.230	96.60±0.212	90.60±0.121	87.60±0.000
6.00	99.40±0.000	85.90±0.132	45.60±0.404	49.80±0.808	86.30±0.303	90.10±0.707	82.40±0.353
5.00	96.30±0.353	81.90±0.002	39.10±0.152	45.20±2.112	75.20±0.402	89.60±0.000	64.20±0.176
4.00	95.10±0.176	79.80±0.712	37.10±0.809	43.60±0.707	66.10±0.881	88.20±0.404	62.70±2.113
3.00	94.30±0.353	75.00±0.202	35.70±0.303	41.50±1.716	63.90±1.101	87.60±0.201	57.30±1.707
2.00	91.90±0.121	65.40±2.113	30.50±0.707	29.90±1.202	56.80±0.301	86.30±1.212	36.80±0.909
1.00	92.20±1.414	38.80±0.419	19.60±1.707	20.60±0.104	32.30±2.121	85.60±1.414	24.10±0.303
0.05	90.20±0.176	10.60±0.302	07.80±1.212	06.21±0.909	12.90±1.767	10.00±0.353	11.90±0.707
IC_{50}	----	1.50	2.61	2.51	1.90	1.06	2.65

Table 2: Antibacterial activities in terms of mean zones of inhibition of methanolic extract of *Adiantum venustum* and its fractions against various bacterial strains in comparison with two standard antibiotic drugs. (concentration of each of the drugs and samples was 40 mg/mL)

Bacterial strains	Mean zones of inhibition (mm)							
	Amoxicillin	Cefixime	MeOH	Hexane	$CHCl_3$	EtOAc	n-BuOH	Aqueous
Pseudomonas aeruginosa	16.75±0.170	33.75±1.768	20.25±0.353	17.60±0.884	21.50±1.768	21.25±2.474	25.13±1.237	21.50±1.786
Escherichia coli	38.75±1.767	40.25±1.767	13.00±0.707	10.00±0.000	11.75±0.353	12.00±1.414	14.50±0.707	14.75±0.353
Staphylococcus aureus	23.25±1.060	50.00±4.242	14.25±0.353	13.75±1.060	----	15.75±1.060	13.75±1.060	13.75±1.060
Bacillus subtilis	43.25±1.060	38.50±2.121	16.75±0.353	10.50±0.707	16.00±1.414	12.25±1.060	14.25±0.353	17.00±0.707

Table 3: Minimum Inhibitory Concentrations (MICs) of methanolic extracts of *Adiantum venustum* and its fractions against different bacterial strains

Bacterial Strains	MIC of extract/fractions (mg/mL)					
	MeOH	Hexane	$CHCl_3$	EtOAc	n-BuOH	Aqueous
Pseudomonas aeruginosa	3.63	4.29	3.30	3.30	3.30	3.30
Escherichia coli	3.96	4.95	3.96	3.30	3.96	3.30
Staphylococcus aureus	3.63	4.62	3.30	3.30	3.96	3.30
Bacillus subtilis	1.98	4.95	3.96	3.30	3.96	3.96

Table 4: Percentage inhibition of alpha-amylase by methanolic extract of *Adiantum venustum* and its fractions at different concentrations.

Conc. (mg/mL)	% Inhibitory activity					
	Acarbose	MeOH	Hexane	$CHCl_3$	EtOAc	n-BuOH
4.00	79.70±1.414	60.50±0.043	90.90±0.001	70.10±0.851	84.20±1.101	55.60±1.411
3.00	74.20±1.060	59.90±1.006	78.60±0.414	66.40±0.143	81.10±0.414	44.60±0.770
2.00	60.90±0.353	54.30±0.707	41.90±1.060	65.10±0.770	63.30±0.890	39.30±0.001
1.00	57.80±0.000	43.50±1.414	37.80±0.007	51.30±0.019	65.60±0.101	27.40±0.667
0.50	36.10±0.707	25.60±1.060	24.60±2.121	34.20±1.110	48.30±0.067	25.00±0.000
0.25	34.70±0.003	18.20±0.353	13.20±1.414	32.90±0.353	36.40±1.101	23.70±1.414
0.05	28.60±0.212	12.50±0.007	15.80±0.129	29.10±1.112	20.30±2.121	22.80±0.707
IC_{50}	1.11	1.22	3.62	1.10	1.92	3.84

The minimum concentration required to inhibit the growth of these bacteria (MIC) ranged from 1.98 to 4.95 mg/mL (table 3). All the fractions were lethal to *P. aeruginosa* at a concentration of 3.30mg/mL except hexane which was needed in much higher dose to kill this microorganism. Remarkably, the MeOH extract was active against *B. subtilis* at concentration as low as 1.98 mg/mL. The hexane fraction was least toxic against all the fractions. The chemical compounds with antibacterial activities in this plant are, thus, mostly polar in nature.

Alpha-amylase inhibitory activity

Alpha-amylase is an enzyme that converts starch into maltose and other simple reducing sugars. In the present

Table 5: GC-MS data of methanolic extract of *Adiantum venustum* and its fractions, provided with retention time (min) and %age of the detected compounds.

Sr. No.	Retention time (min)	%Age of total	Compound name	Molecular formula
Methanolic fxtract				
1.	21.565	8.546	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
2.	23.442	1.317	Octadecanoic acid	$C_{18}H_{36}O_2$
3.	28.264	4.443	Beta-Sitosterol	$C_{29}H_{50}O$
4.	21.215	1.183	Pentadecanoic acid-14-methyl-methylester	$C_{17}H_{34}O_2$
Hexane fraction				
1.	21.571	1.818	n-Hexadecanoic acid (Palmitic acid)	
2.	23.442	0.556	Octadecanoic acid	$C_{18}H_{36}O_2$
3.	21.209	0.222	Pentadecanoic acid-14-methyl-methylester	$C_{17}H_{34}O_2$
Chloroform fraction				
1.	21.565	1.334	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
2.	5.057	1.763	Benzene-1,3-dimethyl	C_8H_{10}
3.	5.714	0.986	p-Xylene	C_8H_{10}
Ethyl acetate fraction				
1.	21.559	0.977	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
n-Butanolic fraction				
1.	21.559	0.502	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
2.	5.508	1.987	n-Butyl ether	$C_8H_{18}O$
3.	6.717	1.202	2,4-bis(1,1-dimethylethyl)-phenol	$C_{14}H_{22}O$
Aqueous fraction				
1.	21.565	42.355	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
2.	28.271	6.348	Beta-Sitosterol	$C_{29}H_{50}O$
3.	6.408	17.599	Oxime-methoxy-phenyl	$C_8H_9NO_2$
4.	8.160	16.817	Glycerin	$C_3H_8O_3$

study alpha-amylase inhibitory activity of methanolic extract of fern *A. venustum* and its fractions were evaluated and the results are shown in table 4. The plant displayed remarkable potential to inhibit the enzyme. The inhibitory effect was dose dependent as inhibition of enzyme decreases with decrease in concentration of extract or fraction (table 4).

At higher concentrations, hexane fraction was most potent followed by ethyl acetate and then chloroform. However, chloroform fraction displayed minimum IC_{50} (1.10 mg/mL) followed by ethyl acetate fraction (1.92mg/mL).

In the nutshell, less polar fractions (hexane, chloroform and ethyl acetate) were stronger inhibitor than polar ones. Aqueous fraction did not show any activity, while n-butanolic was only poorly active. Thus, less polar fractions, especially chloroform and ethyl acetate, have compounds that have the ability to inhibit activity of alpha-amylase, and, therefore, can be used to manage diabetes type II (Ahmed *et al*., 2014).

GC-MS analysis

GC-MS (gas chromatography mass spectrometry) analysis showed that *Adiantum venustum* possess fatty acids, sterols, glycerin, oximes and phenolic compounds. Fatty acids that are present in this plant species are n-hexadecanoic acid (palmitic acid), pentadecanoic acid and octadecanoic acid. Palmitic acid was the most abundant fatty acid present in all plant samples. Octadecanoic acid and pentadecanoic acid were present only in methanolic extract and hexane fraction. Beta-sitosterol was present only in aqueous fraction. Aqueous fraction contained oxime-methoxy-phenyl and glycerin. Chloroform fraction contained benzene-1,3-dimethyl (m-Xylene) and benzene-1,4-dimethyl (p-Xylene). The n-butanolic fraction contained n-butyl ether and 2,4-bis(1,1-dimethylethyl)-phenol. Table 5 provides the summary of GC-MS analysis of methanolic extract of *A. venustum* and its fractions.

STATISTICAL ANALYSIS

The assays carried out to determine activities were conducted at least thrice and the data were subjected to one-way analysis of variance (ANOVA) and results were expressed as mean ± SD.

DISCUSSION

Literature showed *A. venustum t*o be used as a remedy for a number of therapeutic disorders (Pan *et al*., 2011). The present study was executed to examine its antioxidant,

antibacterial and anti-diabetic properties. GC-MS analysis was carried out to identify chemical compounds in the plant.

The DPPH scavenging property can be correlated with total phenolic contents (TPC) and total flavonoid content (TFC), although other classes of compounds also possess free radical scavenging potential. The n-BuOH fraction that had the highest TPC, readily scavenged the DPPH radical with lowest IC_{50} value (1.06mg). The ethyl acetate fraction, which showed highest TFC, also exhibited considerable potency to inhibit the DPPH radical with IC_{50} as 1.90mg. The flavonoids and polyphenols present in this species (Pan *et al*., 2011) may be responsible for its significant scavenging activity. Furthermore, GC-MS analysis revealed the presence of n-hexadecanoic acid in it which is known to possess antioxidant property (Rajeswari *et al*., 2012).

The present study confirmed the ethno-medicinal repute of this plant to possess antimicrobial activity. It exhibit significant results in inhibiting bacterial species specially *Pseudomonas aeruginosa*. As the results show, the methanolic extract and its fractions inhibited the growth of *Pseudomonas aeruginosa* more effectively than standard antibiotic amoxicillin. Therefore, an aqueous decoction or extract of this plant should be effective against infections caused by this microbe. In general, polar fractions proved to be more lethal towards test microbes than their non-polar counterparts. The inhibitory property might be due to the presence of compounds like 2,4-bis(1,1-dimethylethyl)-phenol in n-butanolic fraction, and presence of beta-sitosterol in aqueous fraction. Both of these compounds are reported to exhibit antibacterial property (Velmurugen *et al*., 2012; Murugesan *et al*., 2011). The plant also has a variety of triterpenoids which are known to display antimicrobial potential (Pan *et al*., 2011). The plant has also been found to be potent in inhibiting alpha-amylase activity in the present *in vitro* analysis. The inhibitory action was dose dependent and less polar fractions, chloroform and ethyl acetate, exhibited significant activity. These fractions should therefore be explored for remedies for this ailment. The GC-MS analysis gives a view of the volatile substances present in the plant. n-Hexadecanoic acid is abundantly present in *A. venustum* and upon fractionation most of it appeared in aqueous fraction.

CONCLUSION

Methanolic extract of *Adiantum venustum* and its fractions in different solvents showed significant antioxidant, antimicrobial and anti-diabetic properties. Antioxidant compounds were concentrated in comparatively polar fractions while alpha-amylase inhibitory substances in nonpolar fractions. Remarkably, significant antimicrobial activity of the plant against *Pseudomonas aeruginosa* may provide potential leads, upon further activity-guided phytochemical analysis, for new antibiotic drugs.

ACKNOWLEDGEMENTS

The authors are grateful to management and staff of Pharmagen, Lahore, for their support in antimicrobial analysis of the plant samples.

REFERENCES

Ahmed D, Younas S and Mughal QMA (2014). Study of alpha amylase and urease inhibitory activities of *Melilotus indicus* (Linn). *Pak. J. Pharm. Sci.*, **27**(1): 57-61.

Ahmed M, Khan MI, Khan MR, Muhammad N, Khan AU and Khan RA (2013). Role of medicinal plants in oxidative stress and cancer. *Sci. Rep.*, **2**: 641.

Amjad MS, Arshad M and Qureshi R (2015). Ethnobotanical inventory and folk uses of indigenous plants from Pir Nasoora National Park, Azad Jammu and Kashmir. *Asian Pac. J. Trop. Biomed.,* **5**(3): 234-241.

Arif T, Sharma B, Gahlaut A, Kumar V and Dabur R (2014). Anti-diabetic agents from medicinal plants: A Review. *Chem. Biol. Lett.,* **1**(1): 1-13.

Brahmachari G, Mondal S, Chatterjee D and Brahmachari AK (2003). Phychemical and biological activities of *Adiantum* species. *J. Sci. Ind. Res.,* **62**: 1119-1130.

Brand-Williams W, Cuvelier ME and Berset C (1995). Use of free radical method to evaluate antioxidant activity. *Lebensm. Wisse. Technol.*, **28**: 25-30.

Ervin FR and Bullock WE (1974). Simple Assay for Clindamycin in the Presence of Amino glycosides. *Antimicrob. Agents Chemother.*, **6**(6): 831-835.

Haq F (2012). The ethno botanical uses of medicinal plants of Allai Valley, Western Himalaya Pakistan. *Int. J. Plant Res.*, **2**(1): 21-34.

Hassler M (2016). World Ferns: Checklist of Ferns and Lycophytes of the World (version Mar 2016). In: Species 2000 & ITIS Catalogue of Life, 27th June 2016. Available at: http://www.catalogueoflife.org/col/details/species/id/5f95e304a5dcf0e11b2ffcf4739ebe88/source/tree (last accessed on 28 July 2016).

Iltaf S, Khan Z and Riaz N (2012). A Contribution to the taxonomic study of fern flora of Punjab, Pakistan. *Pak. J. Bot.*, **44**: 315-322.

Lone PA, Bhardwaj AK1 and Bahar FA (2015). Study of indigenous/traditional medicinal plant knowledge- an endeavourtowards new drug discovery. *Afr. J. Tradit. Complement. Altern. Med.*, **12**(2): 73-95.

Murugesan S, Vijayakumar R and Panneerselvam A (2011). Evaluation of phytochemical constituents from the leaves of *Memecylon umbellatum* Burm. f. *Res. J. Pharm. Boil. Chem. Sci.*, **2**(4): 1145-1152.

Nickavar B and Yousefian N (2009). Inhibitory effects of six *Allium* species on α-amylase enzyme activity. *Iran. J. Pharm. Res.*, **8**(1): 53-57.

Nostro A, Germano MP, D'Angelo V, Marino A and Cannatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plants antimicrobial activity. *Lett. Appl. Microbiol.*, **30**(5): 379-384.

Ntie-Kang F, Mbah JA, Mbaze LM, Lifongo LL, Scharfe M, Hanna JN, Cho-Ngwa F, Onguene PN, Owono LCO, Megnassan E, Sippl W and Efange SM (2013). CamMedNP: Building the Cameroonian 3D structural natural products database for virtual screening. *BMC Complementary and Altern. Med.*, **13**: 88.

Pan C, Chen YG, Ma XY, Jiang JH, He F and Zhang Y (2011). Phytochemical constituents and Pharmacological Activities of plants from the Genus Adiantum: A Review. *Trop. J. Pharm. Res.*, **10**(5): 681-692.

Rajeswari G, Murugan M and Mohan VR (2012). GC-MS analysis of bioactive components of *Hugonia mystax L. Res. J. Pharm. Boil. Chem. Sci.*, **3**(4): 301-308.

Razaq A, Rashid A, Ali H, Ahmad H and Islam M (2010). Ethnomedicinal potential of plants of Changa valley district Shangla, Pakistan. *Pak. J. Bot.,* **42**(5): 3463-3475.

Sahreen S, Khan MR and Khan RA (2010). Evaluation of antioxidant activities of various solvent extracts of Carissa opaca fruits. *Food Chem.*, **122**(4): 1205-1211.

Sher Z, Khan Z and Hussain F (2011). Ethnobotanical study of some plants of Chagharzai valley, district Buner, Pakistan. *Pak. J. Bot.*, **43**(3): 1445-1452.

Sing S, Khatoon S, Singh H, Behera SK, Khare PB and Rawat AKS (2013). A report on pharmacognostical evaluation of four Adiantum species, Pteridophyta, for their authentication and quality control. *Braz. J. Pharmacog*., **23**(2): 207-216.

Slinkard K and Singleton VL (1977). Total Phenol Analysis: Automation and Comparison with Manual Methods. *Am. J. Enol. Vitic.,* **28**: 49-55.

Velmurugen S, Babu MM, Punitha SMJ, Viji VT and Citarasu T (2012). Screening and characterization of antiviral compounds from *Psidium guajuva* Linn root bark against white spot syndrome virus. *Indian J. Nat. Prod. Resour.*, **3**(4): 208-214.

Wani M H, Shah MY and Naqshi AR (2016). Medicinal Ferns of Kashmir, India. *Int. J. Bioassays*, **5**: 4677-4685.

Yousuf J, Verma RK and Dar H (2012). Traditional plant based therapy among rural communities of some villages of Baramulla district (Jammu and Kashmir). *J. Phytolog*., **4**(5): 46-49.