African Journal of Pharmacy and Pharmacology Vol. 5(20), pp. 2323-2329, 29 November, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.641
ISSN 1996-0816 © 2011 Academic Journals

Full Length Research Paper

Biological screening of *Viola betonicifolia* Smith whole plant

Naveed Muhammad* and Muhammad Saeed

Department of Pharmacy, University of Peshawar, Peshawar, Pakistan.

Accepted 1 November, 2011

Crude methanolic extract as well as subsequent solvent fractions of *Viola betonicifolia* (VB) whole plant were tested for various *in-vitro* biological activities, including nematicidal, antioxidant, larvecidal, phytotoxic and cytotoxic. All extracts were also tested for their total flavonoid and phenolic contents. A dose dependent effect was observed against both nematodes. Ethyl acetate fraction was highly effective against *Meloidogyne incognita*, followed by chloroform and methanolic extract while the highest mortality of *Meloidogyne javanica* was observed against ethyl acetate followed by chloroform and methanolic extract with 45, 43 and 31% mortality, respectively. The antioxidant activity was tested using 1, 1-diphenyl 1-2-picryl-hydrazyl (DPPH) scavenging and reducing power assay for all extracts. The chloroform fraction showed highly significant antioxidant activity followed by ethyl acetate and methanolic extract. The maximum larvecidal effect against *Aedes aegypti* was observed for chloroform fraction followed by ethyl acetate fraction. In case of phytotoxic activity, butanol fraction was most effective followed by ethyl acetate fraction. Significant results were found by aqueous fraction with LC_{50} 46 µg/ml and chloroform with LC_{50} 56 µg/m against brine shrimp. Phytochemical studies indicated that ethyl acetate fraction was rich of flavonoid and phenolic contents followed by chloroform and methanolic extract. It was concluded that the ethyl acetate and chloroform fractions are the most significant sources of antihelmintic, antioxidant, larvecidal, phytotoxic and cytotoxic compounds.

Key words: Nematicidal, antioxidant, larvecidal, phytotoxic and cytotoxic.

INTRODUCTION

Viola betonicifolia Smith belongs to family Violaceace. Locally, it is known as banafsha. It is a perennial herb of 8 to 20 cm in height. The stem of the plant is absent and leaves are triangular or obtuse and petiole is longer than lamina. Roots are slender, unbranched and rhizome is short. *V. betonicifolia* is available in various countries of the word like Pakistan, India, Nepal, Sri lanka, China, Malaysia and Australia (flora of Pakistan). In Pakistan, it is available in Swat, Hazara and Dir. Traditionally it is used as antipyretic, astringent, diaphoretic, anticancer, purgative, anti-epileptic, anti-anxiety and antitussive (Hamayun, 2005). It is also used in sinusitis, skin, blood disorders, pharyngitis (Bhatt and Negi, 2006), kidney diseases, pneumonia and bronchitis. Flowers are used in lung troubles, cough and boil (Husain et al., 2008). In continuation of our research work on Pakistani medicinal plants (Saeed et al., 2010; Saeed et al., 2010, 2011; ur Rahman et al., 2011), we investigated *V. betonicifolia* for various biological activities. However, in this piece of research work, we tested the crude methanolic extract as well as subsequent solvent fraction of the whole plant of *V. betonicifolia* for nematicidal, antioxidant, larvecidal, phytotoxic and cytotoxic activities.

*Corresponding author. E-mail: drnaveedrph@gmail.com.

METERIALS AND METHODS

Plant and extraction

Whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtonkhawa in April, 2010. Plant specimen was identified by Professor Dr. Muhammad Ibrar, Department of Botany, University of Peshawar and specimen was deposited in the herbarium under voucher number 6410/Bot. The collected whole plant (12 kg) was air dried and powdered. The powdered was extracted by maceration with methanol at room temperature for 14 days with

occasional shaking. The methanolic extract was filtered and concentrated by rotary evaporator at low temperature (45℃). The methanolic extract was dissolved in distilled water and further fractioned with chloroform, *n*-hexane, ethyl acetate, butanol and aqueous fractions. The crude extract and its subsequent fractions were screened for phytochemical study and *in-vitro* biological studies.

Nematicidal activity

Culture preparation

Fresh egg masses of *Meloidogyne javanica* and *Meloidogyne incognita,* collected from stock culture maintained on tomato (*Lycopersicon esculentum*) root tissues were kept in water for egg hatching. The eggs suspension were poured on a cotton-wool filter paper and incubated at 28 ± 2℃ to obtain freshly h atched juveniles. Juveniles collected within 48 h were used (Haq et al., 2010; Nisar et al., 2011).

Mortality test

Crude extracts were dissolved in water (passed through Whatman filter paper No.1) to make dilutions of 2, 1 and 0.5%. Experiments were performed under laboratory conditions at 28 ± 2°C. Glass tubes 15 cm long and 8 cm were taken for bioassay, and 3 ml were taken from all dilutions in each tube. The required amounts of nematode suspension (100 freshly hatched second stage juveniles 3 ml suspension) were poured into tubes, to each of which equal amount of plant extract had already been poured. Distilled water with nematode larvae was taken as control. The dead nematodes were observed under stereoscopic binocular microscope after 24 and 48 h and percentage mortality was calculated. Nematodes were considered dead if they did not move when probed with a fine needle (Djian et al., 1991).

DPPH free radical scavenging assay

25 mg of each sample was taken and dissolved in distilled methanol and was diluted up to 50 ml. From this stock solution, various concentrations of 20, 40, 60, 80, 250 and 500 µg/ml were prepared by dilution method. 5ml of each solution was taken in a test tube and 1 ml of 0.001 M of DPPH solution was added to it. All these solutions were kept in the dark for 30 min. Also, 5 ml methanol was taken and 1 ml of DPPH solution was added, for the control solution. At the end of incubation period, the mixtures were examined for the antioxidant activity using Optima UV-Visible spectrophotometer (Agilent Technologies) at wavelength of 517 nm (Kato et al., 1988). The experiments were performed with triplicate readings. DPPH (%) was determined using the following formula:

$$\text{DPPH (\%)} = \frac{\text{Control abs - Extract abs}}{\text{Control}} \times 100$$

Reducing power assay

For reducing power assay, extract solution (2 ml), phosphate buffer (2 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2 ml, 10 mg/ml) were mixed, and then incubated at 50℃ for 20 min. Tri chloroacetic acid (2 ml, 100 mg/L) was added to the mixture. A volume of 2 ml from each of the aforementioned mixtures was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride in a test tube. After 10 min reaction, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated a high reducing power. Butylated hydroxytoluene (BHT) was used as reference standard (Amarowicz et al., 2004).

Larvecidal activity

Larvae were hatched following the recommended methods and the mature Larvae were transferred into the test solution with pasture pipette into a test tube (20 larvae/solution). All the extracts were dissolved in respective solvents and then 10, 50, 100, 200, 400 and 500 ug/L samples were transferred to solution containing larvae. Mortality of each extract was determined after 24 h exposure at 28℃ following recommended protocol (Jawale et al., 2010). The LC_{50} were calculated using GhraphPad prism 5 software.

Phytotoxicity

The phytotoxic activity of crude and all subsequent solvent fractions of VB were evaluated using *Lamna minor* plant (Atta-ur-Rahman, 1991). 15 mg of respective extract was dissolved in 1.5 ml of respective solvent and from this solution 5, 50 and 500 µl were transfer to the flask (3 flasks for each concentration). This concentration was equivalent to 10, 100 and 1000 µg/ml, respectively. The solvent was allowed to evaporate overnight under sterilized condition in laminar flow. 20 ml of E. medium was added to each flask. Other flasks (3 for each) were supplemented with E. medium and standard drug (Atrazine) served as negative and positive control. To each flask ten plants with 2 to 3 fronds were transferred and kept in all the flasks under about 12 h day light conditions. Plants were observed daily and on the seventh day the numbers of fronds were counted. The percentage of growth inhibition was recorded with reference to the negative control using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{(100- Number of fronds in test sample)}}{\text{Number of fronds in negative control}} \times 100$$

Cytotoxicity

About 20 mg of respective extract was dissolved in 2ml of respective solvent, and from this solution 5, 50 and 500 µl were transfer to vials (3 vials/concentration). This concentration was equivalent to 10, 100 and 1000 µg/ml, respectively. The solvent was allowed to evaporate overnight. 5 ml with seawater water solution (38 g/L) were added to each vial. After 36 h of hatching and maturation of larvae as nauplii, 10 larvae were transferred to each vial using a Pasteur pipette. The vials were then placed at room temperature (25 to 27℃) under illumination. Other vi als were supplemented with brine solution served as positive controls (Atta-ur-Rahman, 1991).

Total phenolic contents

About 2 g of all tested extract were treated with about 20 ml of Folin-Denis reagent, 30 ml of 20% Na_2CO_3 and diluted by a factor 100 with distilled water (Woisky and Salatino, 1998). The resulting mixture was kept as such at room temperature for 30 min. It was then filtered and the absorbance was measured at 770 nm against the blank using Spectronic 20D (Milton Roy). The total phenol content of each sample was calculated by comparing with a standard curve of tannic acid used as blank.

Table 1. Nematicidal activity of *V. betonicifolia* against *M. incognita.*

Plant	Mortality observed against different concentration (%)						Control
	24 h			48 h			
	2	1	0.5	2	1	0.5	
Butanol	29	16	7	43	28	11	2
Hexane	18	10	5	36	23	16	1
Aqueous	14	6	1	27	19	10	2
Methanolic	37	24	18	60	45	29	1
Ethyl acetate	40	30	24	77	62	42	1
Chloroform	38	29	20	71	59	39	1

Table 2. Nematicidal activity of *V. betonicifolia* against *M. javanica*.

Plant	Mortality observed against different concentration (%)						Control
	24 h			48 h			
	2	1	0.5	2	1	0.5	
Butanol	27	14	9	42	19	14	1
Hexane	20	11	7	38	16	10	1
Aqueous	12	9	4	20	11	6	2
Methanolic	31	23	15	58	39	27	2
Ethyl acetate	45	22	16	65	56	40	2
Chloroform	43	21	18	68	57	38	2

Total flavonoid determination

Aluminum chloride colorimetric method was used for flavonoids determination. Each extract (0.5 ml of 1:10 g/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm (Slinkard and Singleton, 1977).The calibration curve was prepared by preparing quercetin solutions at 12.5 to 100 µg/ml concentrations in methanol.

Statistical analysis

All assays were carried out in triplicates and results were expressed as mean ± SEM. The IC_{50} values were calculated using the GraphPad Prism 5 software.

RESULTS

The crude methanolic extracts as well as the subsequent solvent fractions were tested in three concentrations, that is, 0.5, 1 and 2% against *M. incognita* and *M. javanica* as shown in Tables 1 and 2. A dose dependent effect was observed against both nematodes. Ethyl acetate fraction was highly effective against *M. incognita* and showed 40% mortality at 2% dose after 24 h followed by chloroform and methanolic extract with 38 and 37% mortality after 24 h, while at the 2% concentration, the percentage mortality of ethyl acetate, chloroform and methanol was 77, 71 and 60, respectively after 48 h. The remaining solvent fractions also showed nematicidal effects at all doses in comparison with control treatment. The highest mortality of *M. javanica* was observed against ethyl acetate followed by chloroform and methanolic extract with percentage mortality of 45, 43 and 31, respectively. All the extracts showed dose dependent and time dependent activity. The percentage of DPPH free radical scavenging effects are shown in Table 3; all the samples were tested for their antioxidant activity at different concentrations, that is, 20, 40, 60, 80, 250 and 500 ppm. A dose dependant DPPH scavenging effect was observed for all samples. The chloroform fraction showed maximum activity followed by ethyl acetate and methanolic extract. The IC_{50} values were calculated for all observations using Prism 5 software. The chloroform and ethyl acetate fractions also proved to be effective antioxidant in reducing power assay as shown in Figure 1.

The larvecidal activity of all extracts are presented in Table 4, the extracts were tested in various concentrations, that is 10, 50, 100, 200 and 500 ppm. The excellent mortality was observed against chloroform fraction, followed by ethyl acetate. The phytotoxic activity of *V. betonicifolia* whole plant against *Lemna minor* is shown in Table 5. The extracts were tested in three various concentrations (10, 100 and 1000 ppm). The highest phytotoxic effect was shown by butanol fraction, followed

Table 3. Percentage of DPPH scavenging activity of *V. betonicifolia*.

Solution (ppm)	Scavenging activity (%)						
	M	Ch	EA	B	H	W	BHT
Control	2.04 ± 0.02	0.77 ± 0.00	0.72 ± 0.07	0.73 ± 0.02	0.75 ± 0.06	O.76 ± 0.09	
20	0.50 ± 1.34	2.34 ± 1.56	2.67 ± 1.76	0.89 ± 0.99	1.24 ± 1.92	0.33 ± 1.54	10.11 ± 0.00
40	5.27 ± 2.36	6.45 ± 0.88	5.25 ± 3.23	1.413 ± 0.81	1.94 ± 1.86	14.93 ± 2.65	33.76 ± 0.02
60	9.26 ± 1.36	12.45 ± 0.95	11.56 ±3.12	8.618 ± 1.34	9.43 ± 1.87	12.28 ± 0.96	56.90 ± 0.08
80	25.44 ±1.87	30.34 ± 0.82	31.35 ±1.87	21.46 ± 0.43	13.73 ± 2.13	15.38 ± 0.89	70.23 ± 0.00
250	62.15 ± 0.74	69.91 ± 0.18	70.34 ±2.12	28.53 ± 2.14	16.36 ± 2.13	15.60 ± 1.34	81.23 ± 0.01
500	68.35 ± 2.14	81.34 ± 1.54	80.87 ±2.15	38.04 ± 1.54	34.53 ± 2.76	30.4 ± 2.65	92.45 ± 1.98
IC_{50}	110 ppm	80 ppm	82 ppm	176 ppm	500 ppm	496 ppm	60 ppm

Values are mean ± SEM (n = 3); M, methanolic; H, hexane; Ch, chloroform; EA, ethyl acetate; B, Butanol; W, aqueous.

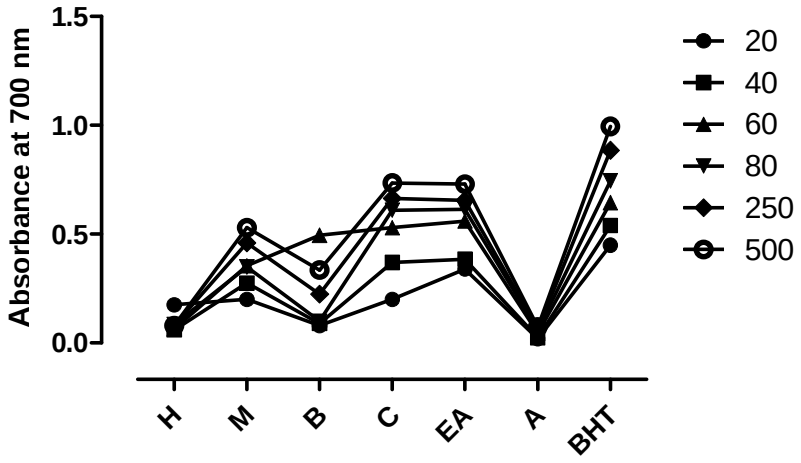

Figure 1. Reducing power assay of various extracts of *V. betonicifolia* (M, methanolic; H, hexane; C, chloroform; EA, ethyl acetate; B, butanol; A, aqueous).

Table 4. Larvecidal effect of *V. betonicifolia* against *A. aegypti*.

Extract	Percent mortality					LC_{50} (µg/ml)
	10 ppm	100 ppm	200 ppm	400 ppm	600 ppm	
Hexane	10.1 ± 1.34	12.33 ± 0.92	14.23 ± 1.22	14.21 ± 2.52	14.34 ± 1.32	397
Methanol	30.21 ± 1.02	38.98 ± 2.53	45.78 ± 1.74	53.15 ± 1.07	60.28 ± 2.06	61.3
Butanol	8.23 ± 2.02	12.67 ± 1.12	20.28 ± 1.54	25.87 ± 0.02	26.56 ± 2.05	291
Aqueous	6.34 ± 1.52	6.89 ± 1.67	7.34 ± 0.99	7.98 ± 2.13	8.21 ± 3.14	-
Chloroform	50.23 ± 0.82	65.23 ± 2.09	70.34 ± 2.76	79.56 ± 2.09	87.98 ± 2.87	13.03
Ethyl acetate	45.34 ± 1.22	59.23 ± 2.98	68.56 ± 1.02	75.87 ± 3.10	86.12 ± 3.25	16

Values are mean ± SEM (n = 3).

by ethyl acetate fraction. The percentage of mortality of all extracts against brine shrimp are presented in Table 6. Significant results were found by aqueous fraction with LC_{50} 46 µg/ml and chloroform with LC_{50} 56 µg/ml.

The uppermost quantity of flavonoid and phenolic contents were found in ethyl acetate followed by chloroform and methanolic extract while no flavonoid was observed in *n*-hexane fraction as shown in Figures 2 and 3.

Table 5. Phytotoxic activity of *V. betonicifolia*.

Name	Number of fronds	Percent growth inhibition		
		10 ppm	100 ppm	1000 ppm
		GI (%)	GI (%)	GI (%)
Control	30	-	-	-
Hexane	30	26.23 ± 1.45	30.36 ± 2.68	53.02 ± 2.02
Methanol	30	30.65 ± 2.96	43.09 ± 2.01	66.08 ± 0.96
Butanol	30	60.35 ± 1.78	66.07 ± 1.45	83 ± 2.16
Aqueous	30	26.47 ± 1.09	50.47 ± 2.91	66.48 ± 0.95
Chloroform	30	23 ± 1.73	33.66 ± 1.76	50.98 ± 1.04
Ethyl acetate	30	33.34 ± 0.93	60.38 ± 2.06	73.72 ± 1.90

Values are mean ± SEM (n = 3).

Table 6. Brine shrimp cytotoxic effect of *V. betonicifolia*.

Name	Number shrimps	Percent mortality			LC_{50} (µg/ml)
		10 ppm	100 ppm	1000 ppm	
Hexane	30	30.34 ± 1.45	50.23 ± 1.98	90 ± 1.09	60.08
Methanol	30	10.34 ± 2.12	10.23 ± 0.87	100.23 ± 1.45	375.12
Butanol	30	2.44 ± 2.45	20.18 ± 1.67	30.45 ± 1.38	-
Aqueous	30	30.67 ± 1.45	60.27 ± 2.01	80.29 ± 1.49	46.34
Chloroform	30	3.33 ± 1.82	40.20 ± 2.03	100.37 ± 1.02	56.93
Ethyl acetate	30	10.44 ± 1.82	60 ± 1.82	80.23 ± 1.54	175.04
Etoposide					7.4625

Values are mean ± SEM (n = 3).

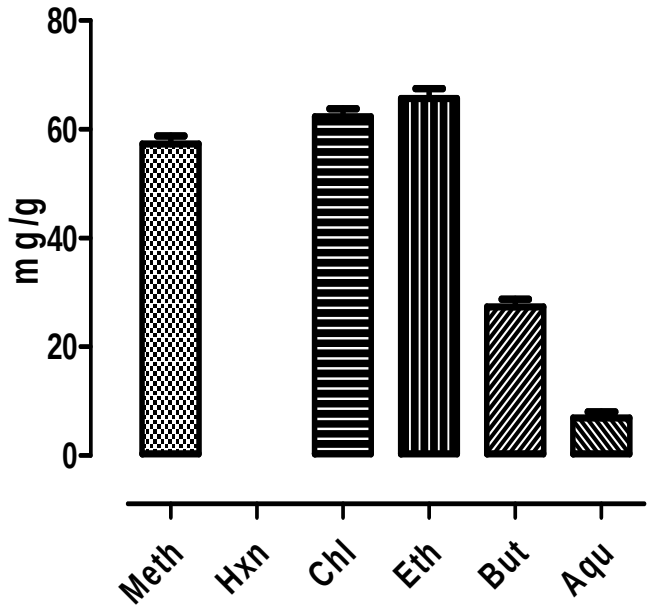

Figure 2. Total flavonoids in methanolic and subsequent solvent factions of *V. betonicifolia* (Meth, methanolic; Hxn, hexane; Chl, chloroform, Eth, ethyl acetate, But, butanol and Aqu, Aqueous).

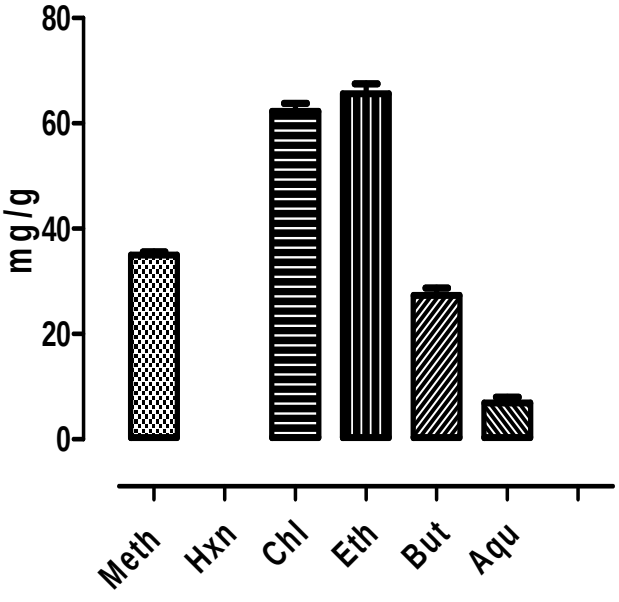

Figure 3. Total phenolic contents in methanolic and subsequent solvent factions of *V. betonicifolia* (Meth = Methanolic, Hxn = Hexane, Chl = Chloroform, Eth = Ethyl acetate, But = Butanol and Aqu = Aqueous).

DISCUSSION

Nematicidal effects

Almost all types of plants are affected by plant parasitic nematodes. These pathogens destroy fiber crops, horticultural and food crops due to which the production of crops is decreasing (Haq et al., 2010). The *M. incognita and M. javanica* are well known root-knot nematodes affecting a large number of plants. The larvae of these pathogens infecting plant roots by forming a gall at roots and demolishing the production and quality of plants (Eisenback and Triantaphyllou, 1991). These plant pathogens are mostly controlled using chemical nematicids which greatly reduced the attack of these pathogens and killing them, but due to high price, non availability at right time and a source of environmental pollution, the research for natural nematicids is of need in agriculture. The ethyl acetate, chloroform and methanolic extracts of *V. betonicifolia* whole plant are effective in controlling the pathogenicity of these plant parasites.

Antioxidant effects

The antioxidant activity was quantified by two methods, that is, DPPH scavenging and reducing power assay. In both tests, the chloroform and ethyl acetate fractions were found to be good antioxidant. The decreasing order of DPPH radical scavenging ability of samples are chloroform > ethyl acetate > methanol > ethyl acetate > butanol > water > n-hexane fractions. The experimental scavenging activities of these extracts against the DPPH system might be due to such compounds which posses' antioxidant activity. The LC_{50} values of DPPH radicals scavenging for the chloroform, ethyl acetate and methanol were 80, 82 and 110 ug/m, respectively, while the *n*-hexane fraction had an LC_{50} 500 ug/ml as shown in Table 3. The free radical DPPH scavenging activities of all extracts were less than BHT. In the reducing power assay, the higher the absorbance the higher will the antioxidant effect be. In this assay, the presence of reductants, result in the reduction of ferric cyanide complex to the ferrous form by donating an electron. A dose dependent increase was observed for all fractions; however, the sequence for reducing power was chloroform > ethyl acetate > methanol > butanol > n-hexane > aqueous fraction as shown in Figure 1.

Larvecidal effects

Dengue fever is measured a health problem throughout the world, and especially in tropical countries where favorable environmental conditions are responsible for the propagation of vectors *Aedes aegypti* (Jawale et al., 2010). The dengue fever could be controlled by reducing the production of this mosquito. Among the tested samples of *V. betonicifolia* solvent extracts, the chloroform extract was effective against this vector with LC_{50} 13.03 µg/ml, followed by ethyl acetate and methanolic extract having LC_{50} 16.00 and 61.30 µg/ml.

Phytotoxic effects

The outstanding phytotoxic effects were observed against butanol fraction with 83% inhibition while the ethyl acetate produces 73% growth inhibition. There was no complete inhibition by any fraction as shown in Table 5. All fractions are weak phytotoxic beside the aforementioned fraction which have the phytotoxic potential.

Cytotoxic effects

The cytotoxic effect of all the fractions was compared with standard cytotoxic drug (etoposide). It is interesting to note that except n-hexane and aqueous fraction, all samples were not effective cytotoxic at concentration of 10 µg/ml, while at 1000 µg/ml concentration, 100% cytotoxic effect was shown by methanolic and chloroform fraction. The LC_{50} was calculated for all the tested samples as given in Table 6. The top most effective cytotoxic fraction was aqueous fraction followed by chloroform and hexane with LC_{50} values 46.34, 56.93 and 60.08 µg/ml, respectively.

Total flavonoid and phenolic contests

The flavonoid contents were quantified in methanol (39 mg/g), chloroform (63.89 mg/g), ethyl acetate (65.36 mg/g), butanol (28 mg/g) and aqueous (6.86 mg/g) as shown in Figure 2, while the phenolic contents in methanol (34 mg/g), chloroform (62.00 mg/g), ethyl acetate (64.13 mg/g), butanol (28.32 mg/g) and aqueous (6.46 mg/g) as shown in Figure 3. The ethyl acetate and chloroform fractions are a good source of these phytochemicals.

According to a research the phenolic compound are responsible for nematicidal activities of *Meloidogyne* species (Mahajan et al., 1992). The chloroform and ethyl acetate fractions are effective against our tested plant pathogens and these extracts are rich source of polyphenolic compounds. Therefore, the nematicidal activity of our tested samples might be due to the presence of the polyphenolic constituents. The chloroform and ethyl acetate fractions are also good antioxidant and this activity could also be attributed to the presence of the polyphenolic constituents. Several researchers proved that there is good relationship between the antioxidant activity and phenolic contents while other found no strong relationship between phenolic contents and antioxidant

action (Velioglu et al., 1998; Ismail et al., 2004). In this study there was good relation between these phyto-chemicals and antioxidant effects.

It is very interesting that the plant extracts, and specially, the chloroform and ethyl acetate fractions are effecttive nematicidal and phytotoxic. There is a strong relationship between these two activities, because both activities are helpful in increasing the quality of plants and production of crops. These extracts are effective against weeds which are harmful for plants, such as crops, fiber plants, food and vegetables, and also, effective nematicidal against root-knot nematodes; therefore, these extracts are a good source for plants protection against weeds and nematodes.

ACKNOWLEDGEMENT

We are thankful to Higher Education Commission (HEC) of Pakistan for providing scholarship for this study.

REFERENCES

Amarowicz R, Pegg R, Rahimi-Moghaddam P, Barl B, Weil J (2004) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem., 84: 551-562.

Atta-ur-Rahman (1991) Studies in Natural Products Chemistry: Bench-Top Bioassay for the Discovery of Bioactive Natural Products an update. Elsevier Science Publishers, B.V., Netherland, p. 9.

Bhatt V, Negi G (2006). Ethnomedicinal plant resources of Jaunsari tribe of Garhwal Himalaya, Uttaranchal. India J. Trad. Knowl., 5: 331-335.

Djian C, Pijarowski L, Ponchet M, Arpin N, Favre-Bonvin J (1991) Acetic acid: a selective nematicidal metabolite from culture filtrates of Paecilomyces lilacinus (Thom) Samson and Trichoderma longibrachiatum Rifai. Nematologica, 371: 101-112.

Eisenback JD, Triantaphyllou HH (1991) Root-knot nematodes: Meloidogyne species and races. Man. Agric. Nematol. pp. 191-274.

Flora of Pakistan. http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=200014334.

Hamayun M (2005) Ethnobotanical profile of Utror and Gabral valleys, district Swat, Pakistan. . Ethnobotanical Leaflets, (1): 9.

Haq MZ, Ahmad M, Akhter M (2010) Nematicidal activity of selected flora of Pakistan. Pak. J. Bot., 42: 2119-2123.

Husain SZ, Malik RN, Javaid M, Bibi S (2008) Ethonobotanical properties and uses of medicinal plants of Morgah biodiversity park, Rawalpindi. Pak. J. Bot., 40: 1897-1911.

Ismail A, Marjan ZM, Foong CW (2004) Total antioxidant activity and phenolic content in selected vegetables. Food Chem. 87, 581-586.

Jawale C, Kirdak R, Dama L (2010) Larvicidal activity of Cestrum nocturnum on Aedes aegypti. Bang. J. Pharmacol., 5: 39-40.

Kato K, Terao S, Shimamoto N, Hirata M (1988) Studies on scavengers of active oxygen species. 1. Synthesis and biological activity of 2-O-alkylascorbic acids. J. Med. Chem., 31: 793-798.

Mahajan R, Kaur D, Bajaj K (1992) Nematicidal activity of phenolic compounds against Meloidogyne incognita. Nematologia Med., 20: 217-219.

Nisar M, Kaleem WA, Qayum M, Marwat IK, Zia-ul-Haq M, ALI I, Choudhary M (2011) Biological screening of Zizyphus oxyphylla edgew stem. Pak. J. Bot., 43: 311-317.

Saeed M, Khan H, Khan MA, Khan F, Khan SA, Muhammad N (2010) Quantification of various Metals and cytotoxic profile of aerial parts of Polygonatum verticillatum. Pak. J. Bot., 42: 3995-4002.

Saeed M, Muhammad N, Khan H (2011) Assessment of Heavy Metal Content of Branded Pakistani Herbal Products. Trop. J. Pharm. Res., 10: 499-506.

Saeed M, Muhammad, N, Khan H (2010) Analysis of Toxic Heavy Metals in Branded Pakistani Herbal Products. J. Chem. Soc. Pak., 32: 471.

Slinkard K, Singleton VL (1977) Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic., 28: 49.

ur Rahman S, Ismail M, Muhammad N, Ali F, Chishti KA, Imran M (2011) Evaluation of the stem bark of Pistacia integerrima Stew ex Brandis for its antimicrobial and phytotoxic activities. Afr. J. Pharm. Pharmacol., 5: 1170-1174.

Velioglu Y, Mazza G, Gao L, Oomah B (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem., 46: 4113-4117.

Woisky RG, Salatino A (1998) Analysis of propolis: some parameters and procedures for chemical quality control. J. Apicult. Res., 37: 99-105.