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ANTIOXIDANT, ANTITUMOR ACTIVITIES AND PHYTOCHEMICAL INVESTIGATION OF *HEDERA NEPALENSIS* K.KOCH, AN IMPORTANT MEDICINAL PLANT FROM PAKISTAN

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Abstract

Hedera nepalensis is a ground-creeping evergreen woody plant growing mainly in the Himalayas and Kashmir. This plant is frequently used in folk medicines for the treatment of various ailments. The present research focused on the pharmacological evaluation and phytochemical analysis of crude methanolic extract (CME) and three fractions, n-hexane (n-HF), ethyl acetate (EAF) and aqueous (AQF). The biological assays used for this study included DPPH free radical scavenging assay, DNA protection assay and potato disc antitumor assay. Maximum antioxidant activities with IC_{50} values of 9.834 ppm and 14.22 ppm were shown by EAF and AQF, respectively. Crude methanolic extract (CME) and the fractions also exhibited significant DNA protection activity in $^{\bullet}OH$ induced DNA damage assay, at all the concentrations tested. Both EAF and AQF showed well-defined tumor inhibition in the potato disc antitumor assay, with the lowest IC_{50} values shown by EAF and AQF (less than 1 ppm). Phytochemical analysis showed the presence of flavonoids, steroids, tannins, terpenoids and cardiac glycosides in the crude extract and its fractions. The present study demonstrated that EAF and AQF of *Hedera nepalensis* have potent antioxidant and antitumor activity with the presence of effective phytochemicals.

Introduction

Hedera is a genus of 15 species belong to the family Araliaceae, which includes about 70 genera and 700 species of flowering plants. Though the taxonomy and phylogenetic relationships of *Hedera* species remain problematic, the genus is well known for its economical importance (Ackerfield & Wen, 2002). The species selected in the present research work is *H. nepalensis*, locally known as Arbambal (Shah & Khan, 2006). Traditionally, the plant is largely used in folk medicine, where its different parts, along with the extract, are used for the treatment of various diseases. Studies have shown that the leaves and berries are stimulating, diaphoretic, cathartic, and used to treat indolent ulcers and abscesses. A decoction of the leaves is effective against lice (Qureshi *et al*., 2007). *Hedera nepalensis* has hypoglycemic properties and is found to be effective against fever, pulmonary infections and rheumatism (Shah & Khan, 2006). Hamayun *et al*., (2006) reported its anticancer properties as well. Inayatullah *et al*., (2007) screened the crude methanolic extract of *H. nepalensis* (leaves + stem) for different biological activities such as brine shrimp cytotoxic activity, potato disc antitumor activity and phytotoxic activity. This plant has also been evaluated for its antifungal activity by Xue *et al.,* (2010).

In view of the medicinal importance of *H. nepalensis*, based on traditional knowledge and surveyed literature, the present study focused on the fractionation procedure, antioxidant activities and phytochemical analysis of the crude methanolic extract and fractions. Following the work of Inayatullah *et al.,* (2007), the potato disc antitumor activity was also conducted on the fractions of *H. nepalensis* to find out how the different phytochemicals are separated into different fractions.

Materials and Methods

Hedera nepalensis was collected from Ayubia, pipeline track (near Muree Pakistan), during July 2008 and was identified by Dr. Mir Ajab Khan (taxonomist, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan). A voucher specimen (herbarium number HMP460) and deposited in the herbarium collection.

Extraction and fractionation procedure: Fresh aerial parts of the plant were collected from the field, rinsed with distilled water, cut into small pieces and shade dried at room temperature. Extraction from the aerial part was carried out following a maceration procedure. A total of 671 g of plant material was ground in 3.0 l of methanol using a kitchen blender. The poorly homogenized mixture was kept for four weeks at room temperature (25°C ± 2°C) in an extraction bottle. After four weeks, the mixture was filtered by squeezing the plant material in gauze cloth before filtering with Whatman #1 filter paper. The filtrate was then concentrated in a rotary evaporator at 45°C under low pressure and dried to a constant weight of 15.0 g. This crude methanolic extract (CME) was subjected to different biological assays for pre-screening of potential pharmacological activities. CME was also investigated for the presence of some important phytochemicals, including steroids, alkaloids, cardiac glycosides and saponins. The crude methanolic extract (15 g) was then suspended in 250 ml distilled H_2O. This aqueous suspension was then fractionated by solvent-solvent extraction, first with n-hexane and then with ethyl acetate, using a separating funnel (Pyrex, England). Three fractions, namely n-hexane fraction (n-HF), ethyl acetate fraction (EAF) and aqueous fraction (AQF), were obtained. All the fractions were concentrated in a rotary evaporator and dried to a constant weight in a vacuum oven at 45ºC. These fractions were then subjected to the same biological assays and phytochemical analysis used with the CME. The extracts were kept at 4ºC when not in use.

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Bioassays

Procedures for the selected bench top bioassays are given below in detail.

1. Antioxidant assays: In order to evaluate the antioxidant potentials, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and DNA protection assay were used.

a. DPPH free radical scavenging assay: The DPPH assay was performed according to the procedure described by Kulisic *et al.*, (2004) and modified by Obeid *et al*., (2005). In a glass vial, 2800 µl of 0.1 mM DPPH solution (in 82% methanol) was mixed with 200 µl of test sample (in methanol). This stock solution was serially diluted with methanol to obtain final concentrations of 100 ppm, 50 ppm, 25 ppm, 10 ppm, 5 ppm, 2 ppm and 1 ppm. Each concentration was assayed in triplicate. The vials were capped, shaken well and kept in the dark at 37ºC for one hour. After incubation, the change in colour (from deep-violet to light-yellow) of DPPH free radical was then measured by taking the absorbance of the reaction mixtures at 517 nm on a UV/Visible spectrophotometer (DAD Agilent 8453). A mixture of 2800 µl of 82% methanol and 200 µl of methanol was used as a blank while ascorbic acid was used as a positive control. The percentage scavenging of DPPH free radical for each concentration of test sample was calculated by using the following formula:

$$\% \text{ Scavenging} = [(A-B)/A] \times 100$$

where
A = Absorbance of negative control
B = Absorbance of test sample
IC_{50} values were calculated by a table curve using 2D v4 (AISN software)

b. DNA protection assay: Antioxidant and pro-oxidant activities of CME and its fractions were evaluated by conducting a DNA protection assay according to the method reported by Tian & Hua (2005). Plasmid DNA (pBR322 Fermentas) with a concentration of 0.5 µg/3 µl was treated with three different concentrations of plant extracts (30 ppm, 300 ppm and 3000 ppm dissolved in methanol) in the final reaction volume of 15 µl. Fenton reaction was induced by addition of 30% H_2O_2 (4 µl) and 2 mM $FeSO_4$ (3 µl) into the reaction mixture. Four controls (untreated DNA, DNA treated with 2 mM $FeSO_4$, DNA treated with 30% H_2O_2, DNA treated with 2 mM $FeSO_4$ and 30% H_2O_2) were run simultaneously. Each mixture was incubated at 37ºC for one hour. After incubation, 3 µl of bromophenol blue (loading dye) was added to each reaction mixture, the samples were loaded on a 1% agarose gel containing TBE (Tris, boric acid, EDTA) buffer and ethidium bromide, and visualized with Doc-IT (VWR). Evaluations of antioxidant or pro-oxidant effects on DNA were based on the increase or loss percentage of a super-coiled monomer, compared with the control value. To avoid the effects of photo excitation of samples, experiments were done in the dark.

2. Antitumor potato disc assay: CME and its fractions were screened for their antitumor activities by using the antitumor potato disc assay as reported by McLaughlin & Rogers (1998). A 48-hour-old bacterial culture of *Agrobacterium tumefaciens* (At 10) was used in this experiment. Inocula with five concentrations of test samples (1000, 100, 10, 5 and 1 ppm) were prepared with 1.5 ml autoclaved distilled water, 2.0 ml of bacterial culture (1 x 10^8 CFU/ml) and 0.5 ml sample solution in DMSO. A negative control was prepared by replacing the sample solution with 0.5 ml DMSO. Red-skinned potatoes were surface sterilized using a 0.1% $HgCl_2$ solution. Potato discs of 8 mm x 4 mm were prepared. Autoclaved plain agar (1.5%) was poured in small petri plates and allowed to solidify. Ten discs were placed on the agar surface of each plate, and then 50 µl of inoculum was poured on the surface of each disc. The plates were sealed with Parafilm and incubated at 28°C in dark. The experiment was carried out in triplicate. After 21 days of incubation, potato discs were stained with Lugol's solution (10% KI, 5% I_2), and tumors were counted. Percentage tumor inhibition was calculated with the following formula:

$$\text{Percentage inhibition} = (1- Ns/Nc) \times 100$$

where
Ns = Average number of tumors in sample
Nc = Average number of tumors in negative control
More than 20% tumor inhibition was considered significant (McLaughlin & Rogers, 1998).

Phytochemical analysis: The crude methanolic extract of *H. nepalensis* and its fractions were qualitatively analyzed for the presence or absence of certain phytochemicals by using standard methods of analysis (Harborne, 1993; Sofowara, 1993; Trease & Evans, 2002; Edeoga *et al.,* 2005; Parekh & Sumitra, 2007) as described by Parekh and Sumitra, (2007). For alkaloids, 400 mg plant material was mixed with 20 ml methanol and filtered. A mixture of 2 ml filtrate + 1% HCl + was steamed and filtered, and 1 ml filtrate + 6 drops of Mayer's reagent, Wagner's reagent or Dragendorff reagent was checked for precipitates. A creamish precipitate, a brownish-red precipitate or an orange precipitate indicated the presence of alkaloids using the respective reagents. The presence of carbohydrates was determined using Bradford's and Fehling's tests. In Bradford's test, 3 ml of filtrate + 2 ml of Bradford's reagent were mixed, heated for 7-12 minutes in a water bath, and a red precipitate indicated the presence of disaccharides. In Fehling's test, 0.5 g of plant material was dissolved in 5 ml distilled water and filtered, 1 ml each of Fehling's A and B were mixed, boiled for 2 minutes on a spirit lamp, 1 ml of filtrate was added, and the mixture was boiled again for 1 minute. A red precipitate indicated the presence of carbohydrates (reducing sugars). For tannins, 100 mg of sample was dissolved in 5 ml distilled water and filtered, and 2 ml filtrate + 2 ml $FeCL_3$ was checked for a blue-black precipitate that indicated the presence of tannins. The presence of phlobatannins was determined by mixing 0.5 g plant material in 5 ml distilled water, boiling and filtering, and adding add 1% aq. HCl to the filtrate; a red precipitate indicated the presence of phlobatannins. For

saponins, the frothing test and emulsion test were used. In the frothing test (0.5 ml filtrate + 5 ml of distilled water, shaken well), frothing persistence indicated the presence of saponins. In the emulsion test, 0.1 g plant material in 5 ml distilled water was filtered, 2 ml filtrate + few drops of olive oil were shaken and checked for the presence of an emulsion, which indicated the presence of saponins. For cardiac glycosides, the Keller-Kiliani test was used (2 ml filtrate + 1 ml glacial acetic acid + $FeCl_3$ + concentrated H_2SO_4); a green-blue color indicted the presence of cardiac glycosides. For the identification of steroids, the Liebermann-Burchard reaction was used. For this 100 mg plant materials in 5 ml chloroform were mixed and filtered, then 2 ml filtrate + 2 ml acetic anhydride + concentrated H_2SO_4 was checked for color. Steroids appeared as green to pink and terpenoids as pink to purple. For flavonoids, 100 mg plant material in 5 ml ethanol was mixed and filtered. A 2 ml filtrate + concentrated HCl + magnesium ribbon will produce pink-tomato red color if flavonoids are present.

The presence or absence of these phytochemicals is represented in Table 3 using plus (+) and minus (-) signs respectively, as displayed by Parekh & Sumitra, (2007) and Usman *et al*., (2007).

Results and Discussion

Bench top bioassays have proved to be good tools for biological evaluation of plant extracts. According to Jerry *et al.,* (1998) crude botanical extracts are comprised of very effective mixtures of bioactive compounds, and it is quite possible to sort out which activities are due to which components with the help of simple bioassay procedures and various separation techniques. Antioxidant activity of *H. nepalensis* crude extract and its fractions was determined by using two assays. These assays showed dose-dependent free radical scavenging capacity and a protective effect on DNA cleavage. DPPH assay is considered to be a simple, convenient and rapid method for screening of plant extract/compounds for their antioxidant potential by measuring the reducing ability of tested compounds. All three fractions were tested for their DPPH scavenging activity. A well-known antioxidant, ascorbic acid, was used as positive control. The lowest IC_{50} values were observed for EAF (9.83) and AQF (14.2). DPPH scavenging patterns for CME and its three fractions, along with IC_{50} values, are presented in Table 1.

Table 1. DPPH free radical scavenging activities of CME and its fractions along with IC_{50} values.

No.	Test samples	% Scavenging at different concentrations (ppm)							IC_{50} (ppm)
		100	50	25	10	5	2	1	
1.	CME	80.4	71.8	35.2	0	0	0	0	35.29
2.	n-HF	58	23	23	0	0	0	0	89
3.	EAF	82	82	81	59	24	4	0	9.834
4.	AQF	81	80	81	30	10	0.4	0	14.22
5.	Asc. acid	95.04	94.8	90.0	86.4	44.7	17.6	1.77	7.02

CME = crude methanolic extract, n-HF = n-hexane fraction, EAF = ethyl acetate fraction, AQF = aqueous fraction, Asc. Acid = ascorbic acid

Antioxidant effects of CME and its fractions were also investigated *in vitro* by using a free radical-induced oxidative plasmid (pBR322) DNA break system. All the fractions, along with the crude extract, exhibited significant DNA protection activity, while no obvious DNA damage was observed in any of the test samples as presented in Fig. 1(a, b). Despite its low DPPH free radical scavenging activity, n-HF exhibited pronounced DNA protection activity, indicating that while n-HF may not possess free radical scavenging potential, it might have some effect on the Fenton reaction. The nature of DNA protection is ambiguous because there are several potential inhibition pathways. The antioxidants can inhibit the reaction by reacting directly with H_2O_2 or reacting with intermediates formed from enzymes and H_2O_2 (Martinez *et al.,* 2001). DNA protection was variable for all three concentrations of the extract and fractions tested. Previous research indicated that the leaves of *H. nepalensis* have anticancer properties (Hamayun *et al*., 2006). Inayatullah *et al*., (2007) examined the crude methanolic extract of *H. nepalensis* (leaves + stem), so we decided to check all the fractions for antitumor activity. Our results showed significant tumor inhibition. The n-HF and EAF exhibited 95% and 90% tumor inhibition at 1000 ppm, respectively. Tumor inhibition was observed in a concentration-dependent mode: tumor inhibition increased with increase in concentration of the test samples (Table 2). Unlike the fractions, the crude extract did not exhibit a pronounced level of tumor inhibition, which might be due to low concentration or the antagonistic effects of the compounds present in the crude extract. The effect of crude extract of *H. nepalensis* on viability of *A. tumefaciens* was found to be quite insignificant (Inayatullah *et al.,* 2007), indicating that the extract/fractions are not involved in killing the bacterium (*A. tumefaciens*) that causes tumors, but rather inhibit tumors by other means.

Table 2. Inhibition of tumor formation by CME and its fractions along with IC_{50} values.

No.	Extract/Fraction	% Inhibition at different concentrations (ppm)					IC_{50} (ppm)
		1000	100	10	5	1	
1.	CME	67	63	60	60	36	2.96
2.	n-HF	90	78	66	58	45	3.6
3.	EAF	95	90	80	85	61	<1 ppm
4.	AQF	84	85	71	74	66	<1 ppm

CME = crude methanolic extract, n-HF = n-hexane fraction, EAF = ethyl acetate fraction, AQF = aqueous fraction

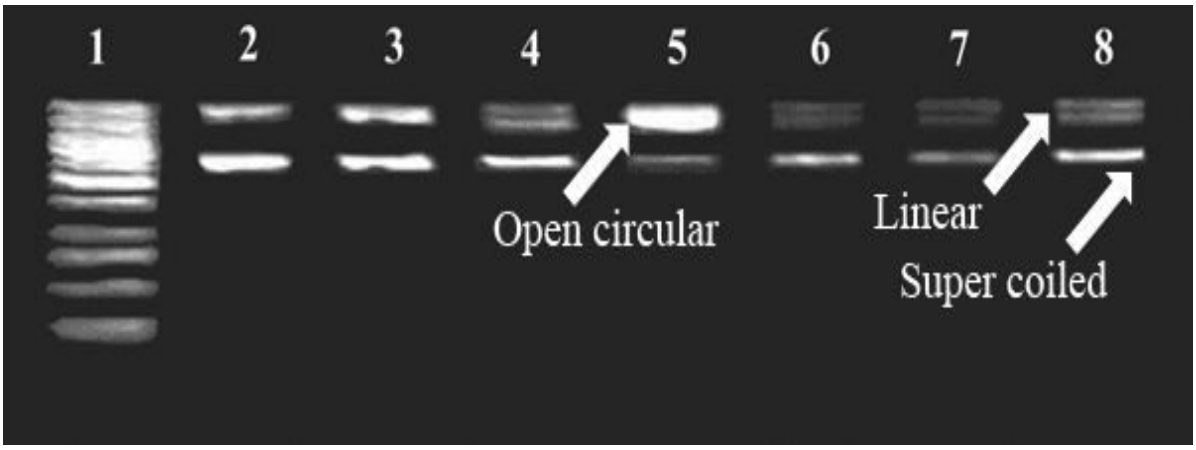

Fig. 1(a). DNA protection assay with three different concentrations of CME
1. 1 kb DNA Ladder, 2. Plasmid DNA (pBR322), 3. Plasmid DNA treated with 2 mM $FeSO_4$, 4. Plasmid DNA treated with 30% H_2O_2, 5. Plasmid DNA treated with 2 mM $FeSO_4$ + 30% H_2O_2, 6. CME (1000 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 7. CME (100 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 8. CME (10 ppm) + plasmid DNA treated with $FeSO_4$+H_2O_2.

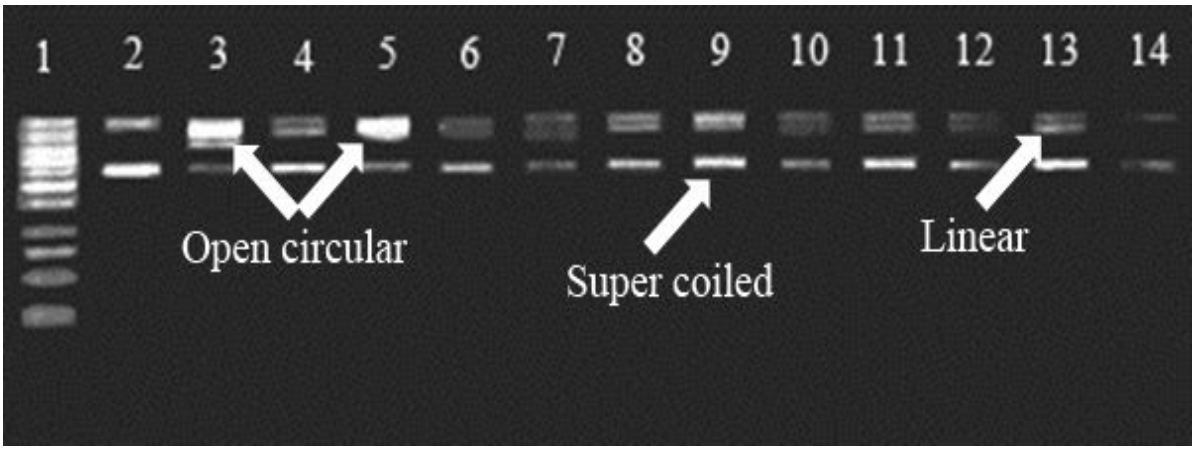

Fig. 1(b). DNA protection assay with all the fractions of CME at three different concentrations
1. 1 kb DNA ladder, 2. Plasmid DNA (pBR322), 3. Plasmid DNA treated with 2 mM $FeSO_4$, 4. Plasmid DNA treated with 30% H_2O_2, 5. Plasmid DNA treated with 2 mM $FeSO_4$ + 30% H_2O_2, 6. n-HF (1000 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 7. n-HF (100 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 8. n-HF (10 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 9. EAF (1000 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 10. EAF (100 ppm) plasmid DNA treated with $FeSO_4$ + H_2O_2, 11. EAF (10 ppm) plasmid DNA treated with $FeSO_4$ + H_2O_2, 12. AQF (1000 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 13. AQF (100 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2, 14. AQF (10 ppm) + plasmid DNA treated with $FeSO_4$ + H_2O_2.

The preliminary phytochemical studies demonstrated the presence of alkaloids, flavonoids, steroids, tannins and terpenoids it varying concentrations. Terpenoids were detected at high concentrations in all the extract/fractions except n-HF, as shown in Table 3. Tannins were found at moderate concentrations, while phlobatannins were completely absent. Terpenoids, flavonoids, alkaloids and tannins are considered to possess high antioxidant activities, which prevent or can be used in the treatment of many diseases, including cancer (Madhuri & Pandey, 2009). Therefore, the presence of appreciable to moderate amounts of these phytochemicals can be correlated with the possible significant medicinal potential of the plant.

Conclusion

Crude methanolic extract of *Hedera nepalensis* and its ethyl acetate and aqueous fractions showed significant antioxidant activity in a DPPH free radical scavenging activity assay and DNA protection assay. The results for the potato disc antitumor assay showed that this plant has highly potent antitumor agents. Further isolation and purification of bioactive compounds from EAF and AQF in the future may reveal the presence of potent novel antioxidants and anticancer agents from *H. nepalensis*.

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Table 3. Phytochemical analysis of CME and its fractions.

No.	Constituents/test	Extract/fractions			
		CME	n-HF	EAF	AQF
1.	Alkaloids				
	Dragendorff's	-	-	-	-
	Mayer's	++	-	++	-
	Wagner's	+	-	++	-
2.	Carbohydrates				
	Bradford's	+	+	-	-
	Fehling's	+	+	+	+
3.	Flavonoids				
	Harborne	++	-	+	+
4.	Steroids				
	Liebermann-Burchard reaction	++	++	-	-
5.	Saponins				
	Frothing Test	-	-	+	+
	Emulsion Test	-	+	+	-
6.	Tannins				
	$FeCl_3$ Test	++	+	-	-
7.	Phlobatannins	-	-	-	-
8.	Terpenoids	+++	-	+++	+++
9.	Cardiac Glycosides				
	Keller-Kiliani Test	+	-	++	++

Key: CME = Crude methanolic extract, n-HF = n-hexane fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, +++ = Good amount, ++ = Moderate amount, + = Trace amount, - = Completely absent

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