Research Article

In vitro Antileishmanial, Cytotoxic, Antioxidant activities and Their Phytochemical Analysis on Methanolic Extract and it is Fractions of *Perotis hordeiformis* leaves

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ABSTRACT

The aim of the present investigation deals with biological evaluation of *Perotis hordeiformis*leaves. For these purpose different biological assays of crude methanolic extract (CME) and its fractions that are chloroform fraction, n-hexane fraction, acetone fraction and aqueous fraction were carried out. The results of CME showed maximum Antileishmanial activity with IC_{50}29.67 µg/ml, moreover CME also showed significant Cytotoxic activity with ED_{50} value 2.38 µg/ml. Antioxidant analysis of CME determined the maximum antioxidant activity with IC_{50} value 31.90 µg/ml. Furthermore, the phytochemical analysis of CME and its fractions showed the presence of Alkaloids, Flavonoids, Phenols, Saponins and Diterpenes. The extract and fractions were also appreciating for further biological investigations in future.

Keywords: Antileishmanial, Antioxidant, Brine shrimp Cytotoxicity, *Perotis hordeiformis,* Phytochemical analysis.

INTRODUCTION

Humans have used plants as therapeutic agents from pre-historic times.[1] Since then, out of estimated 250,000 flowering plant species in the world[2], 15% have been evaluated phytochemically and only 6% have been screened for biological activity.[3] While a relatively small portion of all plants have been used for medicinal purposes, their importance should not be undermined as almost 65% of the world's population has incorporated them into their primary modality of health care.[4] Moreover, the use of medicinal plants has brought a number of clear-cut benefits including (i) isolation of compounds that nowadays are used as drugs (e.g., digoxin, morphine, taxol); (ii) synthesis of new compounds possessing higher activity and/or lower toxicity than the parent compounds found in medicinal plants (e.g., metformin, verapamil, and amiodarone which are based, respectively, on galegine, podophyllotoxin, and khellin); (iii) application of the agents from plants as tools for pharmacological research (e.g., mescaline, yohimbine); and, finally, (iv) using the whole plant or its parts as an approved herbal remedy (e.g., echinacea, garlic, ginkgo biloba, St. John's wort, and many others).[5]

The important point is that ethno-medical information could facilitate the discovery of new drugs by providing a preliminary list of most promising candidate plants for further investigation. Besides shortening the pipeline to drug discovery, using the plants as a starting point has another advantage of potentially reducing the toxic side effects, since active compounds from the plants used by humans are likely to be safer than those with no history of human use.[6]

Perotis hordeiformis belongs to family *Poaceae* is an Annual or short-lived perennial. Mainly found in sandy places, along seashores. Guangdong, Hebei, Jiangsu, Yunnan [India, Indonesia, Malaysia, Nepal, Myanmar, Pakistan, Sri Lanka, Thailand].

This species is very close to Perotis indica and is sometimes included within it. No single character by itself is reliable for separating the two, but the combination of characters given in the key will usually suffice. Hence, in continuation of our previous work[7-14], the present study is carried out to study the *in vitro* Antileishmanial, Cytotoxic, Antioxidant and their phytochemical analysis of *Perotis hordeiformis* leaves.

MATERIALS AND METHODS

Plant material

The leaves of *Perotis hordeiformis* was collected from Soorab, Balochistan province, Pakistan. The plant was identified by taxonomist Prof. Dr. Mudassir.Asrar.Zaidi, University of Balochistan, Quetta, Pakistan.

Extraction and fractionation

Fresh leaves were washed, sliced and dried under shade for 30 days. The leaves extract was prepared in analytical grade methanol (2 kg in 6L) for 72hours. Then the methanol was removed and residue was immersed in methanol for further seven days. Thereafter, the methanol was decanted and filtered with Whatman filter paper. The filtrate was subsequently concentrated under reduced pressure at 45°C in rotatory evaporator (Stuart RE 300) and dried to constant weight (300 g) in vacuum oven (LINN high therm) at 45°C. This was crude methanolic leaves extract. (CME) The CME was than further fractionalized, where 200g of CME was suspended in 300 ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for four fractions, namely, *n*-hexane fraction (NHF), chloroform fraction (CHF), acetone fraction (ACE), and aqueous fraction (AQF).

Biological activities

Following biological activities were performed on the extract and its fractions.

Antileishmanial assay

Culture of parasites

L. major promastigotes were isolated from a patient with Cutaneous leishmaniasis from (Bolan Medical complex), Quetta, Pakistan. The promastigotes were grown in NNN medium and then cultured in 199 medium supplemented with 10% fetal bovine serum x (FBS) (PAA laboratories Gmbh).

Samples preparation

25, 50, 250 and 500µg/ml concentrations of CME and its fractions were prepared for *in-vitro* studies. The extracts were dissolved in DMSO and diluted in 199 medium containing 10% F.B.S. the final volume was adjusted to 2000 µl with 199 medium, for each well a 24 well micro plate in all experiments. The final concentration of DMSO was0.5% (v/v) as this concentration will not affect the parasite growth rate, mobility morphology.[15] 100 *L. major* parasites were transformed into each well. After hemocytometer counting, promastigotes were suspended to yield $1x10^6$ cell/ml in each well, as reference drug. Amphotericin B was prepared in sterile DMSO at 20 µg/ml concentration. The highest concentration of DMSO and 199 medium were also used for control groups. Micro plates were incubated at 24°C. The numbers of parasites were counted with a hemocytometer under a high microscope after 6, 12, 24, 48 hours. All the *in-vitro* experiments were run in triplicate and the results were expressed as a % inhibition in parasite numbers. The drug concentration required for 50% inhibition *in-vitro* (IC50) was calculate with parametric statistical procedure (Finney probitic analysis program) with the associated with 95% confidence interval.[16]

Brine Shrimp Cytotoxicity Assay

The brine shrimp Cytotoxicity assay was performed by using the methodology according to the procedure described by.[17] Brine shrimp (*Artemia salina*) larvae used as test organisms, were hatched at 37 °C in artificial sea water. Different concentrations i.e. 1000, 100, and 10 µg/ml (control) of CME, NHF, CCF, ACF and AQF were in methanol and used against brine shrimp larvae. The death rate of these larvae was observed against all concentration of different fractions. For this purpose, 0.5ml sample of each and every fraction was taken in 20ml vial, solvent from each vial was evaporated followed by addition of 2ml of artificial sea water, 30 shrimps were transferred into each vial, final volume was adjusted to 5ml by artificial sea water and kept under florescence light at 25°C for 24 hours. Test was performed in triplicate after this, deaths were counted, and percentage survival was counted with ED_{50} values were determined by (Finney Computer program).

Antioxidant Assay

The free radical scavenging activity was measured by using 2, 2-diphenyl-1-picryl-hydrzyl (DPPH) assay. DPPH radical assay radical assay was performed according to the procedure described by.[18] DPPH solution was prepared by dissolving 3.2mg in 100ml of 82% of methanol. A volume of 2800µl of DPPH solution was added to glass vials followed by addition of 200 µl of CME, leading to the final concentration of 100, 50, 25, 10 and 5 µg/ml (negative control), mixture were shaken well and incubated in dark at 25°C for 1 hour. Absorbance was measured at 517nm using spectrophometer (Pharma Spec 1700 Shimadzu). Ascorbic acid (AsA) was used as positive control. Each test was measured according to formula and IC_{50} were calculated by graphical method. Same procedure was then repeated with other fractions such as (NHF), (CCF), (ACF) and (AQF),

(%) scavenging effect = [(AC-AS)/ AS] x 100

Where; "AC" is the absorbance of negative control and "AS" is the absorbance of Test Sample.

Phytochemical Analysis

Test for alkaloids

a) Hager's test

1g of ACF was dissolved in 10ml of distilled water followed by filtration. Then 1g of picric acid was prepared by dissolving in 10ml of distilled water. By adding few drops of picric acid in ACF solution. Appearance of yellow precipitates confirmed the presence of alkaloids.

b) Wagner's test

1g of CME was dissolved in 10ml of distilled water followed by filtration. Then the filtrate was treated with Wagner's reagent **(**Iodine in potassium iodide**).** Formation of brown reddish precipitates confirmed the presence of alkaloids.

Test for flavonoids

a) Lead acetate test

1g of CCF was diluted in 10ml of distilled water, followed by filtration. Few drops of lead acetate were added in the filtrate. Appearance of yellow precipitates indicated the presence of flavonoids.

b) Alkaline reagent test

1g of CCF was diluted in 50ml of distilled water followed by filtration. Then 1g of NaOH was diluted in 10ml of distilled to form NaOH solution. Then the filtrate was mixed and shaken with NaOH solution. A yellow colored appeared. Then few drops of HCL were added in the solution. The yellow color of solution turned into colorless solution, indicating the presence of flavonoids.

Test for phenols

a) $FeCl_3$ test

1g ACF of is diluted in 10ml of distilled water followed by filtration. Then in the filtrate few drops of $FeCl_3$ solution were added. Appearance of bluish black color indicated the presence of phenols.

Test for Saponins

a) Frothing test/ foam test

1g of crude extract is diluted with 4ml of distilled water with constant shaking for 10 minutes in a graduated cylinder. Formation of 1cm layer of foam confirmed the presence of saponin.

Test for Diterpenes

a) Copper acetate test

1g gram of CME was diluted in 10ml of distilled water followed by filtration. Few drops of copper acetate solution were added in filtration. Emerald green color confirmed the presence of diterpenes.[19-21]

RESULTS AND DISCUSSION

Crude Methanolic Extract (CME) of *Perotis hordeiformis* leaves were prepared and partitioned into four fractions i.e. CCF, ACF, NHF and AQF. The plant crude extract their partitions were evaluated for their biological activities Antileishmanial, Brine shrimp Cytotoxicity and Antioxidant activities.

Antileishmanial Activity

In- vitro Antileishmanial effect of *Perotis hordeiformis* leaves. The extract and its fractions showed good inhibition activity against the promastigotes of *L. major*. Table (1) show the IC_{50} of extract and fractions ranged between 29.67 to <100µg/ml. CME was found to be more active amongst the fractions. CME showed the highest Antileishmanial activity with IC_{50}29.67 µg/ml. CAF showed good activity with IC_{50}46.51 µg/ml. rest of the factions were less active with IC_{50}<100µg/ml DMSO and 199 culture controls were found to be inactive in all experiments. The reference drug Amphotericin B. was found to have 100% inhibitions after 48 hours with IC_{50} 21.64µg/ml.

Table1: *In-vitro* efficacy of *Perotis hordeiformis* leaves extract and its fractions

Extracts/ Fraction	Doses (µg/ml)	Number of Promastigotes (1x 10^4)	% inhibition	(IC_{50})µg/ml
CME	25	53	47	29.67
	50	42	58	
	250	30	70	
	500	20	80	
CCF	25	80	20	<100
	50	78	22	
	250	67	33	
	500	51	49	
CAF	25	55	45	46.51
	50	48	52	
	250	40	60	
	500	35	65	
NHF	25	66	34	<100
	50	60	40	
	250	52	48	
	500	40	60	
AQF	25	-	-	<100
	50	-	-	
	250	-	-	
	500	-	-	
DMSO(-ve)	25	100	-	
	50	100	-	
	250	100	-	
	500	100	-	
Standard Drug Amphotericin B	25	50	50	21.64
	50	25	75	
	250	12	88	
	500	0	100	

Table 2: *In-vitro* efficacy of *Perotis hordeiformis* leaves extract and its fractions

Extract/ Fractions	Number of brine shrimp	% death at doses			ED_{50} µg/ml
		1000µg/ml	100µg/ml	10µg/ml	
CME	30	30	25	21	2.38
CCF	30	20	17	14	21.50
CAF	30	27	23	19	2.04
NHF	30	22	18	15	11.47
AQF	30	18	14	10	<100
DMSO(-ve)	30	-	-	-	-
Etoposid (+ve)	30	30	27	24	0.56

Table 3: DPPH scavenging antioxidant activities of CME and its Fractions of *Perotis hordeiformis*leaves

Extract/Fractions	100 µg/ml	50 µg/ml	25 µg/ml	10 µg/ml	5 µg/ml	IC_{50} µg/ml
CME	77.32	70.09	34.53	22.10	12.76	31.90
CCF	50.01	31.04	19.10	-	-	93.14
CAF	70.20	62.09	30.58	18.05	10.59	41.42
NHF	-	-	-	-	-	<100
AQF	-	-	-	-	-	<100
ASA	95.04	94.80	90.02	86.40	43.60	5.5

Table 4: Phytochemical analysis of CME and its Fractions of *Perotis hordeiformis* leaves

Constituents/Test	CME	CCF	CAF	NHF	AQF
Alkaloids					
Hager's Test	-	+	+	+	+
Wagner's Test	+	+	+	-	-
Flavonoids					
Lead acetate Test	+	+	+	+	-
Alkaline Reagent Test	+	-	+	-	-
Phenols					
$FeCl_3$ Test	+	-	+	-	-
Saponins					
Foam Test/Froth Test	+	+	+	+	+
Diterpenes					
Copper acetate Test	+	-	+	-	-

(-) Absent, (+) Present

Cytotoxic Activity

Brine shrimp cytotoxicity assay has been considered as prescribing assay for anti-microbial, anti-fungal, insecticidal and anti-parasitological activities. Brine shrimp assay in suggested to be a convenient probe for the pharmacological activities in Plant Extracts [22]. In present study, CME of *Perotis hordeiformis* leaves showed ED_{50} values 2.38 µg/ml while CAF showed significant activity with ED_{50} values of 2.04 µg/ml. On the other hand Fractions NHF and CCF showed good activities with ED_{50} values of 11.47 and 21.50 µg/ml respectively. AQF showed the lowest activity with ED_{50} value of <100 µg/ml comparatively with Standard drug.

Antioxidant activity

DPPH free radical scavenging assay was used to evaluate antioxidant potential of our samples. CME as well as its fractions showed effective free radical scavenging activity as determined by DPPH assay. The results of free radical scavenging are given in table (3). CME has showed maximum antioxidant activity with the IC_{50} value of 31.90 µg/ml. On the other hand CAF showed good antioxidant activity with IC_{50} value of 41.42 µg/ml. Other fractions; CCF has IC_{50} value of 93.14 µg/ml. respectively. While NHF and AQF showed lowest Free radical scavenging activity and have IC_{50}<100 µg/ml. CME has excellent free radical scavenging with IC_{50} 31.90 µg/ml which is comparable to Ascorbic acid. Phytochemical assay of the CME shows that it has high concentrations of Phenols which are known to

be potent antioxidant. *Perotis hordeiformis* leaves have excellent pharmacological importance and it should be investigated further for Isolation, Purification and Characterization of valuable compounds.

Preliminary phytochemical Analysis

Phytochemical analysis showed the presence of Alkaloids, Flavonoids, saponins, Phenols and Diterpenes.

Whereas terpenoids and cardiac glycoside were completely absent.

CONCLUSION

In conclusion, the results of CME showed maximum Antileishmanial activity with IC_{50} 29.67 µg/ml, moreover CME also showed significant Cytotoxic activity with ED_{50} value 2.38 µg/ml. Antioxidant analysis of CME determined the maximum antioxidant activity with IC_{50} value 31.90 µg/ml. This may be due to the phytoconstituents present in *Perotis hordeiformis*, so this preliminary study confirms that the methanolic leaves extract and its fraction may have active compounds in higher amount, therefore plant should significant activity towards pathogens

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