e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

IN VITRO ANTILEISHMANIAL, CYTOTOXIC, ANTIOXIDANT ACTIVITIES AND PHYTOCHEMICAL ANALYSIS OF *BERBERISBALUCHISTANICA* ROOTSEXTRACTS AND ITS FRACTIONS

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ABSTRACT

The aim of the present investigation deals with biological evaluation of *Berberis baluchistanica* roots. 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 CCF showed maximum Antileishmanial activity with IC_{50}36.84 µg/ml, moreover CCF also showed significant Cytotoxic activity with ED_{50} value 0.92 µg/ml. Antioxidant analysis of CME determined the maximum antioxidant activity with IC_{50} value7.03 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.

Key words: Antileishmanial, Brine shrimp Cytotoxicity, Antioxidant, Phytochemical analysis, *Berberis baluchistanica* roots.

INTRODUCTION

Indigenous knowledge that the natural medicine is totally safe and free from side effects is untrue, although the side effects of phytotherapeutic agents are fewer as compared with synthetic drugs. Over past two decades, there is an increased interest in herbal medicines worldwide as therapeutic agents (Calixto JB**,** 2000). Local ethno-botanical uses of different plants previously have been used to treat urinary problems, gastrointestinal, skin infections and respiratory diseases (Corrêa MP, 1932; Cruz CN, 1979**;** Plantas, 1989; Oliveira P and Kisue G**,** 1989; Silva I, 1995; Newall *et al*., 1996 and Biazzi ES, 1996).

The huge chemical multiplicity and a wide range of bioactivity of plants have led to the development of hundreds of pharmaceutical drugs. Research in several countries including Colombia, Argentina, Brazil, Mexico, and Bolivia have shown that many plants show activity against *Leishmania* (Fournet *et al*., 1992; Torres- Santos EC *et al*..1999; Delorenzi*et al*.2001; Ferreira *et al.* 2002; Salvador*et al*.2002).Extracts obtained frommedicinal plants offer new prospect to obtain novel compounds that are active against protozoans. On the other hand, damage of biomolecules can be controlled by Antioxidants (Howell JC, 1986). Antioxidants inhibit the initiation or promulgation of oxidative chain reaction. Major commercially available antioxidants are 2, 3-ter-butyl-4-methoxyphenol (BHA) and 2, 6-di-ter-butyl-4-methylphenol (BHT) but these synthetic antioxidants have shown negative effects on human health (Namiki M, 1990).

Balochistan is province which contains a vast majority of medicinal plants which are biologically active against different microorganisms (Sajid *et al*., 2012; Muhammad *et al.*, 2012; Abdul *et al*., 2012). *Berberis baluchistanica* (Family *Berberidaceae*), which in local language "Brahvi" known as zarch, found in Kalat District, Balochistan province. *Berberis baluchistanica* is a wild plant used as fodder for grazing animals this plant is also used as medicine by local people. They used the roots decoction for the cure of internal injuries and

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ophthalmic problems, beside this it is also used for the removal of kidney stones, as it contains berberine which is known to be useful for a number of diseases[19]. *Berberis baluchistanica* have been previously evaluated antimicrobial and insecticidal activity (Zaidi, 2011) which leads to the availability of this plant for different biological assays. As from the literature review we come to know that previously this plant has never been reported as an Antileishmanial, antioxidant and antitumor activities. So, present study is the evaluation of crude and fractions of *Berberis baluchistanica* roots as an Antileishmanial, antioxidant, antitumor activities and their phytochemical analysis.

MATERIALS AND METHODS

Plant material

The roots of *Berberis baluchistanica* was collected from District Kalat, Balochistan province, Pakistan.

Extraction and fractionation

Fresh roots were washed, sliced and dried under shade for 15 days. The roots extract was prepared in analytical grade methanol (3 kg in 8L) 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^{o}C in rotatory evaporator (Stuart RE 300) and dried to constant weight (460 g) in vacuum oven (LINN high therm) at 45^{o}C. This was crude methanolic roots extract (CME). The CME was than further fractionalized, where 250g of CME was suspended in 250ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for four fractions, namely, *n*-hexane fraction (NHF), chloroform fraction (CCF), acetone fraction (ACF), and aqueous fraction (AQF).

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 *invitro* 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 was 0.5% (v/v) as this concentration will not affect the parasite growth rate, mobility morphology (Zhai *et al*,.1999).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^{o}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* (IC_{50}) was calculate with parametric statistical procedure (Finney probitic analysis program) with the associated with 95% confidence interval (Finney DG, 1971).

Brine shrimp Cytotoxicity assay

The brine shrimp Cytotoxicity assay was performed by using the methodology according to the procedure described by (Ahmad *et al.* 2008).Brine shrimp (*Artemiasalina*) larvae used as test organisms, were hatched at 37^{o}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^{o}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 (Obeid *et al.* 2005).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 200µl of CME, leading to the final concentration of 100, 50, 25, 10 and 5µg/ml (negative control), mixture were shaken well and incubated in dark at 25^{o}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

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 yellowprecipitates confirmed the presence of alkaloids.

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

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.

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

$FeCl_3$ 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

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 saponins.

Test for Diterpenes

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 (Bachaya *et al*., 2009; Maniyar *et al*., 2010; Roopashree *et al*., 2008).

Table 1. *In-vitro* Antileishmanial efficacy of *Berberis baluchistanica* roots extract and its fractions

% Inhibition of death *L. major parasite*

Extracts/ Fraction	Doses (µg/ml)	Survival % Promastigotes (1x 10^4)	% inhibition	(IC_{50})µg/ml
CME	25	78	22	67.72
	50	66	34	
	250	58	42	
	500	49	51	
NHF	25	90	10	>100
	50	82	18	
	250	79	21	
	500	70	30	
CCF	25	62	38	36.84
	50	53	47	
	250	47	53	
	500	33	67	
CAF	25	90	10	>100
	50	83	17	
	250	74	26	
	500	69	31	

AQF	25 50 250 500	100 100 100 100	- - - -	- - - -
DMSO(-ve)	25 50 250 500	100 100 100 100	- - - -	- - - -
Standard Drug Amphotercin B	25 50 250 500	50 25 12 0	50 75 88 100	21.64

Table 2. Cytotoxic Activity of *Berberis baluchistanica* roots 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	26	22	17	4.49
NHF	30	22	17	12	39.03
CCF	30	29	26	22	0.92
CAF	30	25	21	18	2.25
AQF	30	20	15	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 *Berberis baluchistanica* roots

Extract/ Fractions	100 µg/ml	50 µg/ml	25 µg/ml	10 µg/ml	5 µg/ml	IC_{50} µg/ml
CME	91.04	86.13	80.24	72.54	38.31	7.03
NHF	70.10	62.45	40.20	21.19	10.07	37.94
CCF	82.13	64.71	49.02	24.1	12.22	27.64
CAF	-	-	-	-	-	-
AQF	35.25	26.02	-	-	-	>100
ASA	95.04	94.79	90.02	86.5	44.01	5.5

Table 4. Phytochemical analysis of CME and its Fractions of *Berberis baluchistanica* roots.

S.No	Constituents/ Test	CME	NHF	CCF	ACF	AQF
(1)	**Alkaloids**					
a)	Hager's Test	+	+	+	+	-
b)	Wagner Test	+	+	+	+	-
(2)	**Flavonoids**					
c)	Lead acetate Test	+	-	+	-	-
d)	Alkaline Reagent Test	+	-	+	-	-
(3)	**Phenols**					
e)	$FeCl_3$ Test	+	+	+	-	-
(4)	**Saponins**					
f)	Foam Test/Froth Test	+	+	+	+	+
(5)	**Diterpenes**					
g)	Copper Acetate Test	-	+	+	-	-

RESULTS

Crude Methanolic Extract (CME) of *Berberis baluchistanica* roots 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 *Berberis baluchistanica* roots as shown in Table.1

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 (Mayerhof*et al.*1991). Cytotoxic Activity *Berberis baluchistanica* roots extract and its fractions as shown in Table 2.

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).

Preliminary phytochemical Analysis

Phytochemical analysis showed the presence of Alkaloids, Flavonoids, Saponins, Phenols and Diterpenes. Whereas terpenoids and cardiac glycoside were completely absent.

DISCUSSION

Antileishmanial Activity

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 36.84 to <100µg/ml. CCF was found to be more active amongst the fractions. CCF showed the highest Antileishmanial activity with IC_{50} 36.84µg/ml. CME showed good activity with IC_{50} 67.84µg/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 Amphotercin B. was found to have 100% inhibitions after 48 hours with IC_{50} 21.64µg/ml.

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 [27].In present study, CME of *Berberis baluchistanica* roots showed ED_{50} values 4.49µg/ml, while CCF and CAF showed maximum activity with ED_{50} values of 0.92µg/ml and 2.25µg/ml respectively. On the other hand Fractions NHF and AQF showed lowest activities with ED_{50} values of 39.03µg/ml and >100µg/ml respectively comparative to the Standard drug.

Antioxidant activity

CME has showed maximum antioxidant activity with the IC_{50} value of 7.03µg/ml. On the other hand CCF showed good antioxidant activity with IC_{50} value of 27.64µg/ml. Other fractions; NHF has IC_{50} value of 37.4µg/ml. While AQF showed lowest Free radical scavenging activity and have IC_{50}>100µg/ml. CME has excellent free radical scavenging with IC_{50} 7.03µg/ml which is comparable to Ascorbic acid. Phytochemical assay of the CME and CCF shows that it has high concentrations of Phenols which are known to be potent antioxidant which was not present in CAF and AQF fraction. *Berberis baluchistanica* roots have excellent pharmacological importance and it should be investigated further for Isolation, Purification and Characterization of valuable compounds.

CONCLUSION

In conclusion, the Crude Chloroform Fraction (CCF) showed the highest Antileishmanial activity with IC_{50} 36.84µg/ml with reference to standard drug. Whereas, Crude Chloroform Fraction (CCF) showed maximum brine shrimp cytotoxic activity with ED_{50} values of 0.92µg/ml with reference to standard drug. This may be due to the phytoconstituents present in *Berberis baluchistanica* roots, 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.

Conflict of interest statement

We declare that we have no conflict of interest.

ACKNOWLEDGEMENT

The Authors are thankful to the University of Balochistan, Quetta, Pakistan for providing the facilities and also Mir Shah Ali Bugti for providing the plant.

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