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Antileishmanial, cytotoxic, antioxidant activities and phytochemical analysis of *Rhazya stricta Decne* leaves extracts and its fractions

Muhammad Javed Khan[1], Nizam.U.Baloch[1], Sajid Nabi[1], Nisar Ahmed[1], Zahoor Bazai[2], Masoom Yasinzai[1] and Yasser. M. S. A. Al-Kahraman[1*]

[1]*Institute of Biochemistry, University of Balochistan, Quetta, Pakistan.*
[2]*Botany Department, University of Balochistan, Quetta, Pakistan*

ABSTRACT

The aim of the present investigation deals with biological evaluation of Rhazya stricta Decne 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 ED_{50} 14.93 µg//ml, while the ACE showed significant Cytotoxic activity with ED_{50} value 1.54 µg//ml. Antioxidant analysis of CME determined the IC_{50} values 36.599 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 investigations in future.

Keywords: *Antileishmanial*, Brine shrimp Cytotoxicity, Antioxidant, *Rhazya stricta Decne* leaves

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INTRODUCTION

Balochistan province of Pakistan, possesses rich medicinal herbal resources, but has not been evaluated scientifically, four indigenous medicinal plants namely *(Berberis baluchistanica, seriphidium quetenase, Iphino aucheri* and *Ferula costata*) showed potential antibacterial activity [1]. In Balochistan Province a large number of medicinal plants have historically been used to treat a wide range of diseases [2]. Today, thousands of plants, traditionally used as a medicine, are being explored for their antimicrobial activities and chemical components [3]. The American society of microbiology has recommended the development of novel antibacterial agents because of the emergence of multi-drug resistant bacterial pathogens [4]. *Rhazya* genus of an *apocynacae* comprises of two species namely *Rhazya stricta Dencne* and *Rhazya orientlis* [5]. It has wide range of applications and biological importance in Pakistan traditional ethno-medicine. *Rhazya stricta* has been used in indigenous medicinal drugs to cure different types of ailments in Pakistan, India, Afghanistan, Saudi Arabia, Qatar, United Arab Emirates (UAE), Iran and Iraq [6]. The leaves, flowers and fruits of *Rhazya stricta* are also used to treat cancer [7]. Crude ethanolic extract of *Rhazya stricta* has led good results regarding anti-bacterial lipo-oxygenase and acetyl choline sterase activities [8]. *Rhazya stricta* has also been widely used in Arabian folk medicines for the treatment of diabetes mellitus, inflammatory conditions, and helminthiasis [9]. In addition to alkaloids, isolation of some non-alkaloidal compounds has also been reported from *Rhazya stricta* plant [10]. Leishmaniasis is considered one of the serious tropical diseases in many countries. It is caused by protozoa, *Leishmania.* Three major clinical manifestation of leishmaniasis are recognized: visceral, cutaneous and Muco-cutaneous leishmaniasis [11]. Growing number of AIDS cases have brought increase attention towards these diseases in developed countries [12]. Present drugs which are widely used not only show side effects but also have drug resistance and treatment failure specially in immuno-

comprromised patients who often fail respond or are prone to relapse [13]. This disease is becoming more epidemic in many parts of Pakistan, specially Balochistan and Sindh provinces are vulnerable to *Cutaneous leishmaniasis*. On the other hand, Reactive Oxygen Species (ROS) are produced during cell metabolism in both normal and pathological conditions. Many diseases are caused by (ROS) such as Parkinson's disease, Cancer, Cirrhosis, Arthritis, Asthma and Coronary heart diseases [14]. Destruction of Biomolecules can be controlled by Antioxidants [15]. Antioxidants are capable to prevent or delay oxidative processes by inhibiting the initiation or propagation of an oxidative chain reaction. They maintain the nutritional quality and increase shelf life of food and also is used to prevent many oxidative-stress related diseases [16]. Two major synthetic antioxidants are used such as 2,3-ter-butyl-4-methoxyphenol (BHA) and 2,6-di-ter-butyl-4-methylphenol (BHT) but problem of these synthetic antioxidants are that they have caused undesired health effects [17]. Thus there is still need for development of new drugs. The antileishmanial, antioxidant, and Cytotoxicity activities of the *Rhazya stricta* species in this study up to this time not been investigated. We now present the results of antileishmanial activities of *Rhazya stricta*. According to literature review previously the crude extract and its fractions of *Rhazya stricta decne* had not been reported as an antileishmanial agent.

MATERIALS AND METHODS

Plant material

The leaves of *Rhazya stricta Decne* was collected from District Nushki, 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 15 days. The leaves 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. There after, 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 (460 g) in vacuum oven (LINN high therm) at 45°C. This was crude methanolic leaves 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 (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 *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 [18]. 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* (IC_{50}) was calculate with parametric statistical procedure (Finney probitic analysis program) with the associated with 95% confidence interval [19].

Brine shrimp Cytotoxicity assay

The brine shrimp Cytotoxicity assay was performed by using the methodology according to the procedure described by [20]. Brine shrimp (*Artemia salina*) larvae used as test organisms, were hatched at 37 ºC 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 25^0C 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 [21]. 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 25ºC 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

1. 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.

2. Test for flavonoids

c) 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.

d)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.

3. Test for phenols

e) $Fecl_3$ 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.

4. Test for saponin

f) 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. [22-24]

5. Test for Diterepenes

g) 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.

RESULTS AND DISCUSSION

Crude Methanolic Extract (CME) of *Rhazya stricta decne* 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.

Antileshminial Activity

In- vitro Antileishmanial effect of *Rhazya stricta decne* leaves. The extract and its fractions showed good inhibition activity against the promastigotes of *L. major* even with a concentration of 25µg/ml. most of extract and its fractions had an inhibition higher then (50%). Table (1) show the IC_{50} of extract and fractions ranged between 14.93 to 82.81 µg/ml. CME was found to be more active than fractions. CME showed the highest antileishmanial activity of 14.93 % at 25 µg/ml. The AQF was the weakest one showing 15 % inhibition at 500 µg/ml. The CCF showed good activity 61 % inhibition at 25 µg/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.

Table1. *In-vitro* efficacy of *Rhazya stricta decne* leaves extract and its fractions.
% Inhibition of death *L. major parasite*

Extracts/ Fraction	Doses (µg/ml)	Number of Promastigotes (x 10^4)	% inhibition	(IC_{50})µg/ml
CME	25 50 250 500	35 30 08 0	65 70 92 100	14.93
NHF	25 50 250 500	73 50 38 22	27 50 62 78	82.81
CCF	25 50 250 500	39 33 12 3	61 67 88 97	16.53
ACF	25 50 250 500	55 51 47 39	45 49 53 61	72.90
AQF	25 50 250 500	90 90 87 85	10 10 13 15	>100

Cytotoxic Activity

Brine shrimp cytotoxity 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 [25]. In present study, CME of *Rhazya stricta decne* leaves showed ED_{50} values 2.04 µg/ml while ACF showed significant activity with ED_{50} values of 1.54 µg/ml. On the other hand Fractions NHF and CCF showed good activities with ED_{50} values of 1.44 and 3.65 µg/ml respectively. AQF showed the lowest activity with ED_{50} value of >100 µg/ml comparatively with Standard drug.

Table (2) *In-vitro* cytotoxic bioassay of *Rhazya stricta decne* leaves extract and its fractions

Extract/ Fractions	Number of Brine Shrimps	% deaths at doses			ED_{50}
		1000µg/ml	100µg/ml	10µg/ml	
CME	30	27	23	19	2.04
NHF	30	24	21	18	1.44
CCF	30	26	20	18	3.65
ACF	30	28	25	20	1.54
AQF	30	17	12	08	>100
DMSO –ve	30	0	0	0	0
Etoposide +ve (Standard Drug)	30	30	27	24	0.56

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 36.59 µg/ml. On the other hand ACF showed good antioxidant activity with IC_{50} value of 42.15 µg/ml. Other fractions; NHF has IC_{50} value of 55.7 µg/ml. respectively. While CCF and AQF showed lowest Free radical scavenging activity and have IC_{50} >100 µg/ml. CME has excellent free radical scavenging with IC_{50} 36.59 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. *Rhazya stricta decne* leaves have excellent pharmacological importance and it should be investigated further for Isolation, Purification and Characterization of valuable compounds.

Table 3:- DPPH scavenging antioxidant activities of CME and its Fractions of *Rhazya stricta decne* leaves.

Extract/ Fractions	100 µg/ml	50 µg/ml	25 µg/ml	10 µg/ml	5 µg/ml	IC_{50} µg/ml
CME	81.2	61.12	35.55	22.82	14.41	36.59
NHF	74.13	50.43	24.61	16.71	8.29	55.7
CCF	54.24	36.68	-	-	-	>100
ACF	79.47	58.25	33.78	20.61	12.89	42.15
AQF	31.28	12.48	-	-	-	>100
ASA	95.05	94.81	90.01	86.3	44.7	5.4

Preliminary phytochemical Analysis

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

Table 4:- Phytochemical analysis of CME and its Fractions of *Rhazya stricta decne* leaves.

S.No	Constituents/ Test	CME	NHF	CCF	ACE	AQF
(1)	**Alkaloids**					
a)	Hagers 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	+	+	+	+	-

(-) Absent, (+) Present

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