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Research Article

IN VITRO ANTILEISHMANIAL, CYTOTOXIC ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *NEPETA PRAETERVISA* LEAVES EXTRACT AND ITS FRACTIONS

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ABSTRACT

Objective: This study sought to give scientific basis to *Nepeta praetervisa* already used for traditional purpose whose Antileishmanial and Cytotoxic activities has not been evaluated.

Methods: For this purpose different biological assay (Antileishmanial assay and Brine shrimp Cytotoxicity assay) of Crude Methanolic Extract (CME) and its fractions: chloroform fraction (CCF), acetone fraction (CAF) and aqueous fraction (AQF) were carried out.

Results: The result of CAF showed significant antileishmanial activity with IC_{50} value 24.41 µg/ml and CME result showed moderate activity with IC_{50} value 49.07 µg/ml comparatively with standard drug Amphotericin B. AQF showed lowest activity with IC_{50} value <100 µg/ml. On the other hand, the results of CME showed maximum brine shrimp cytotoxic activity with ED_{50} value 0.60 µg/ml and CAF showed maximum activity with ED_{50} value 0.56 µg/ml.

Conclusion: Phytochemical analysis showed the presence of Carbohydrate, Tannins, Phenols, Alkaloids, Flavonoids, Diterpenes, Quinones, Cardiac glycosides, Terpenoids, Triterpenoids, Coumarins and Acids which makes this plant biologically active.

Keywords: Antileishmanial, Cytotoxicity (Brine shrimp), Phytochemical Analysis, *Nepeta praetervisa*

INTRODUCTION

Nepeta is a genus of annual or perennial herbs; it belongs to the *Lamiaceae* family, which includes approximately 250 species. Baluchistan is blessed with diverse flora and fauna due to diverse ecological condition [1, 2], a series of papers on medicinal plants of Pakistan are included some information on plants of Baluchistan [3]. Nepeta species are used in the traditional medicine of many countries and have a large ethno botanical effect: diuretic, diaphoretic, vulnerary, antitussive, antispasmodic, antiasthmatic, tonic, febrifuge, emmenagogue and carminative [4, 5]. *Nepeta praetervisa* in Brahui language known as Simsok, is 40cm tall, perennial herb, with erect stem and crenate leaves. Tea of *Nepeta praetervisa* is given for cold and used as a cure of pneumonia [6].

This plant has never been evaluated for its pharmacological activities. So, in continuation of our previous work [7-9] the aim of this study was to screen for medicinal leaves extracts of this province that could be useful for the development of new tools for the control of infectious diseases. While pursuing this goal, we initiated a systematic evaluation of extracts and fractions from the "*Nepeta praetervisa* " plant species in bioassays such as (a) Antileishmanial (b) Cytotoxic activity (c) and their phytochemical analysis

MATERIALS AND METHODS

Plant material

Nepeta praetervisa were collected from District Kalat, Balochistan province, Pakistan.

Extraction and Fractionation

Fresh leaves were washed, sliced and dried under shade for 30 days at room temperature. The dried material was ground to fine powder using a mechanical grinder (*IKA®MF 10 basic micro fine grinder drive).* The leaves extract was prepared in analytical grade methanol (3 kg in 6L) for 72 hours. Then the methanol was removed and residue was immersed in methanol for further five days. Thereafter, the methanol was decanted and filtered with Whatman filter paper No 1. The filtrate was subsequently concentrated under reduced pressure at 40 ^{0}C in rotatory evaporator (*Stuart RE 300*) and dried to constant weight (460 g) in vacuum oven (*LINN high therm*) at 45 ^{0}C. This was crude methanolic leaves extract (CME). The CME was than further fractionized, where 250g of CME was suspended in 300 ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for three fractions, namely, chloroform fraction (CCF), Acetone fraction (CAF) 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 infected patient 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

Different concentrations 25, 50, 250 and 500 µg/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 was 0.5% (v/v) as this concentration will not affect the parasite growth rate, mobility morphology [10]. 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 ^{0}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 calculated with parametric statistical procedure (Finney probitic analysis program) with the associated with 95% confidence interval [11].

Brine shrimp Cytotoxicity assay

The brine shrimp Cytotoxicity assay was performed by using the methodology according to the procedure described by [12]. Brine

shrimp (*Artemia salina*) larvae used as test organisms, were hatched at 37 ^{0}C in artificial sea water. Different concentrations i.e. 1000, 100, and 10 µg/ml (control) of CME, 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 ^{0}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) [11].

Phytochemical Analysis

Chemical tests were carried out on methanolic extract and their fractions using procedures to identify the phytochemicals as described by Sofowara, Trease and Evans and Harborne [13-16].

Test for Carbohydrates

To 2ml of extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Purple color formation indicated the presence of carbohydrates.

Test for Tannins

To 1ml of extract, 2ml of 5% ferric chloride was added. Formation of greenish black color indicated the presence of tannins.

Test for Saponins

To 2ml of extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicated the presence of saponins.

Test for Flavonoids

5ml of dilute ammonia solution was added to a portion of the aqueous filtrate of extract followed by addition of concentrated Sulphuric acid. Appearance of yellow coloration indicated the presence of flavonoids.

Test for Alkaloids

To 2ml of extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color indicated the presence of alkaloids.

Test for Anthocyanin and Betacyanin

To 2ml of extract, 1ml of 2N sodium hydroxide was added and heated for 5minutes at 100ºC. Formation of yellow color indicated the presence of betacyanin.

Test for Quinones

To 1ml of extract, 1ml of concentrated sulphuric acid was added. Formation of red color indicated the presence of quinones.

Test for Glycosides

To 2ml of extract, 3ml of chloroform and 10% ammonia solution was added. Pink color formation indicated the presence of glycosides.

Test for Cardiac glycosides

To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Brown ring formation at the interface indicated the presence of cardiac glycosides.

Test for Terpenoids

To 0.5ml of extract, 2ml of chloroform was added and concentrated sulphuric acid was added carefully. Red brown color formation at the interface indicated the presence of terpenoids.

Test for Triterpenoids

To 1.5ml of extract, 1ml of Libemann–Buchard Reagent (acetic anhydride + concentrated sulphuric acid) was added. Formation of blue green color indicated the presence of triterpenoids.

Test for Phenols

To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of green color indicated the presence of phenols.

Test for Coumarins

To 1 ml of extract, 1ml of 10% Sodium hydroxide was added. Formation of yellow color indicated the presence of coumarins.

Test for Acids

1ml of extract was treated with sodium bicarbonate solution. Presence of effervescence indicated the presence of acids.

RESULTS

Crude Methanolic Extract (CME) of *Nepeta praetervisa* leaves were prepared and partitioned into three fractions i.e. CCF, CAF and AQF. The plant crude extract their partitions were evaluated for their biological activities Antileishmanial and Brine shrimp Cytotoxicity.

Antileishmanial Activity

In vitro Antileishmanial effect of *Nepeta praetervisa leaves* as shown in **Table 1.**

Table 1: Antileishmanial activity of *Nepeta praetervisa leaves* extract and its fractions

Extracts/ Fraction	Doses (µg/ml)	Number of Promastigotes (1x 10^4)	% inhibition	(IC_{50})µg/ml
CME	25	61	39	
	50	46	54	49.07
	250	32	68	
	500	22	78	
CCF	25	68	32	
	50	53	47	97.23
	250	41	59	
	500	32	68	
CAF	25	51	49	
	50	38	62	24.41
	250	20	80	
	500	14	86	
AQF	25	88	12	
	50	79	21	<100
	250	68	32	
	500	60	40	
DMSO(-ve)	25	100	-	
	50	100	-	
	250	100	-	
	500	100	-	

IC_{50} was calculated with parametric statistical procedure (Finney probitic analysis program)

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 [17]. Cytotoxic Activity of *Nepeta praetervisa leaves* extract and its fractions as shown in **Table 2**.

Preliminary Phytochemical Screening

Phytochemical analysis showed the presence of Carbohydrate, Tannins, Phenols, Alkaloids, Flavonoids, Diterpenes, Quinones, Cardiac glycosides, Terpenoids, Triterpenoids, Coumarins and Acids. Whereas Anthocyanin and Betacyanin and Glycosides were completely absent (Table 3).

Table 2: Cytotoxic Activity of *Nepeta praetervisa leaves* extract and its fractions

Extract/ Fractions	Number of brine shrimp	% death at doses			ED_{50}
		1000µg/ml	100µg/ml	10µg/ml	µg/ml
CME	30	28	25	22	0.60
CCF	30	21	16	10	74.9
CAF	30	30	27	24	0.56
AQF	30	18	15	12	<100
DMSO(-ve)	30	-	-	-	
Etoposid (+ve)	30	30	27	24	0.56

ED_{50} values were determined by (Finney Computer program)

Table 3: Phytochemical Analysis of *Nepeta praetervisa* leaves extract and its fractions

Phytochemical Test	CME	CCF	CAF	AQF
Carbohydrate test	+	-	+	-
Tannins test	+	-	+	-
Saponin test	+	+	+	+
Flavonoid test	+	+	+	-
Alkaloid test	+	-	+	+
Anthocyanin and Betacyanin test	-	-	-	-
Quinones	+	-	+	-
Glycosides test	-	-	-	-
Cardiac glycosides test	+	-	-	-
Terpenoids test	+	+	+	-
Triterpenoids	+	+	+	-
Phenols	+	+	+	-
Coumarins	+	-	+	-
Acids	+	+	+	-

DISCUSSION

Antileishmanial Activity

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%). IC_{50} of extract and fractions ranged between 24.41 to >100 µg/ml. CAF was found to be more active than fractions. CAF showed the highest Antileishmanial activity with IC_{50} value 24.41 µg/ml. The AQF was the weakest one showing IC_{50} value >100µg/ml. The CME showed good activity with IC_{50} value 49.07 µg/ml. DMSO and 199 culture controls were found to be inactive in all experiments. The reference drug Amphotericin B showed IC_{50} value 7.5 µg/ml after 48 hours.

Cytotoxic Activity

In present study, CME of *Nepeta praetervisa* leaves showed significant result with ED_{50} values 0.60 µg/ml while CAF showed maximum activity with ED_{50} value of 0.56 µg/ml. AQF showed the lowest activity with ED_{50} value of <100 µg/ml comparatively with Standard drug.

CONCLUSION

In conclusion, the Crude Acetone Fraction (CAF) of *Nepeta praetervisa* leaves showed significant antileishmanial activity with IC_{50} value 24.41 µg/ml with reference to standard drug. Whereas, Crude Methanolic Extract (CME) showed maximum brine shrimp cytotoxic activity with ED_{50} value 0.60 µg/ml with reference to standard drug. This may be due to the phytoconstituents present in *Nepeta praetervisa*, 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.

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