Research Article

In vitro Antileishmanial, Antioxidant, Antitumor Activities and Phytochemical Estimation of *Peucedanum Beluchistanicum* Leaves Extracts and its Fractions

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ABSTRACT

This study sought to give scientific basis to *Peucedanum beluchistanicum* already used for traditional purpose whose Antileishmanial, Antioxidant, Antitumor activities has not been evaluated. Different biological assays i.e. Antileishmanial, Antioxidant, Crown gall tumor inhibition (potato disc) assay and phytochemical estimation of Crude methanolic extract (CME) and its fractions that are, *n*-hexane fraction (NHF), chloroform fraction (CHF), acetone fraction (ACE), and aqueous fraction (AQF) were used. CME showed maximum Antileishmanial activity with IC_{50} value 32.65µg/ml as comparable to other fractions. Moreover, with the presence of high concentration of phenols in CME, showed significant antioxidant activity with IC_{50} value 12.48µg/ml with respect to Ascorbic acid. On the other hand, CME also showed tremendous antitumor activity with % inhibition 78.65% as compared to the standard drug. Furthermore, the phytochemical estimation of CME and its fractions showed the presence of Alkaloids, Flavonoids, Phenols, Tannins and Diterpenes. Further pharmacological studies are necessary to evaluate this plant.

Keywords: Antileishmanial, Antioxidant, Antitumor Activities, Phytochemical Analysis, *Peucedanum beluchistanicum* Leaves.

INTRODUCTION

Medicinal plants consist of components of therapeutic values and have been used as remedies for human diseases since long. Recently, due to the resistance of pathogens against the available antibiotics and the recognition of traditional medicine as an alternative form of health care has reopened the research domain for the biological activities of medicinal plants [1]. Medicinal plants being an important natural resource and possess potential for being safe drugs, can play an important role in assuaging human health by contributing herbal medicines.

It has been widely observed in developing countries that, the use of traditional medicines are common to the maintenance of health [2]. In developing countries, for the treatment of minor ailments, and cost for personal health maintenance, herbal medicines have become more popular [3]. In addition, the use of medicinal plants in developed societies has been recognized which can be seen by the extraction and development of several drugs and chemotherapeutics from plants and traditionally used herbal remedies [4]. Today about 1500 species of medicinal plants are being used in many countries including Albania, Bulgaria, Croatia, France, Germany, Hungary, Spain, Turkey and the United Kingdom [5].

Plant species, *Peucedanum beluchistanicum* belongs to family *Umbelliferae*; which is a glabrous plant, stems many from the base. Leaves are bipinnate, 20 cm long; fruit dorsally compressed; stylopodium depressed; styles reflexed. This plant has never been reported for its pharmacological activity. Hence, in continuation of our previous work [6-8], the present study is carried out to study the *in vitro* Antileishmanial, antioxidant and antitumor activities and phytochemical estimation of *Peucedanum beluchistanicum.*

MATERIALS AND METHODS

Plant Material

The leaves of *Peucedanum beluchistanicum* were collected from District Kalat, Balochistan province, 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 72 hours. Then, the methanol was removed and residue was immersed in methanol for a 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 (460 g) in vacuum oven (LINN high therm) at 45°C. This was crude methanolic leaves extract (CME). The CME was then further fractionalized, where 250 g of CME was suspended in 250 ml 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).

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.

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 was used 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 [9]. 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 number of parasites was counted with a hemocytometer under a high microscope after 6, 12, 24 and 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 at 95% confidence interval[10].

Antioxidant Assay

The free radical scavenging activity was measured by using 2, 2-diphenyl-1-picryl-hydrzyl (DPPH) assay. DPPH radical assay was performed according to the procedure described by [11]. DPPH solution was prepared by dissolving 3.2 mg in 100 ml of 82% of methanol. A volume of 2800 µl 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 spectrophotometer (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.

Crown gall tumor inhibition (potato disc) assay:

Antitumor potato disc assay was performed for *Peucedanum beluchistanicum* leaves by using *Agrobacterium tumefaciens* (At- 10). *Peucedanum beluchistanicum* extract and its fractions were tested for *in vitro* antitumor activities.[12]

Preparation of potato discs:

Fresh, red and disease free potato tubers were surface sterilized by soaking in 0.1% HgCl solution in water for 1 minute. A core cylinder of tissue was removed from tuber by means of sterilized cork borer. 2 cm end of each tissue cylinder was discarded and remainder was cut into discs of uniform thickness by a special aseptic cutter.

Preparation of Agar Plates and Treatment

These potato discs were then transferred to petri plates each containing 25 ml of 1.5 % agar (1.5 g agar/100 ml distilled water). Five potato discs were placed on each plate and three plates were used for each test sample along with same number of plates for vehicle control (DMSO) and reference drug (Vincristine). As a stock solution, 10 mg of each compound was dissolved in 1 ml of DMSO in separate test tubes. Then 0.5 ml of stock (10 mg/ml) of the test sample was added to 2 ml of a broth culture of *Agrobacterium tumefaciens* (At-10, a 48 hours culture containing $5×10^9$ cells/ml) and 2.5 ml of autoclaved distilled water to make 1000 µg/ml final concentration. One drop (10 µl) was drawn from these test tubes using a sterile pipette and it was used to inoculate each potato disc, spreading it over the disc surface. The process starting from the cutting of the potatoes to the inoculation was completed in 30 minutes in order to avoid contamination. The lids of the petri plates were taped down with parafilm to minimize moisture loss.

Incubation and Analysis

The petri plates were incubated at 28°C for 21 days and the number of tumors was counted with the aid of dissecting microscope after staining with Lugol's solution (5 % I_2, 10 % KI in distilled water). The number of tumors in vehicle control (DMSO) was used as a reference for activity. The results were derived from the number of tumors on test discs versus those on the vehicle control disc. Percentage tumor inhibition was calculated by using formula as shown below. Twenty percent or more inhibition was considered as significant activity.

$$\% \text{ tumor inhibition} = \frac{\text{(Number of tumors in sample)}}{\text{(Number of tumors in control)}} \times 100$$

Phytochemical Screening

Phytochemical screening for major bioactive constituents like alkaloids, phenolics, flavonoids and tannin were determined by using standard phytochemical methods[13, 14].

Determination of Total Phenolics

Total phenolic content of methanolic extract of *Peucedanum beluchistanicum* leaves was determined by FolinCiocalteu method. Phenolic content was expressed as Gallic acid equivalents (GAE mg/g dry weight of extract) and the values were presented as mean ±SD of triplicate analysis with slight modifications[15]. 200µl of sample (1mg/ml) was added to 100 µl diluted (1:10) Folin Ciocalteu reagent and equilibrated for few minutes. Then 800 µl of 2.5 % aqueous Na_2CO_3 was added and mixture was allowed to stand for 60 minutes at room temperature with intermittent shaking. The absorbance of the blue color solution was measured at 765 nm on UV visible spectrophotometer (Shimadzu UVPC-1700 (Japan)). Gallic acid (50 mg %) was used as standard. The absorbance of solution was compared with Gallic acid calibration curve.

Determination of Total Flavonoids

Total flavonoid content was determined by aluminum chloride colorimetric method[16]. This method is based on the formation of a complex flavonoid-aluminium, having the absorbance maximum at 435 nm. 0.5 ml of plant extract (1mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$ and 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 15 minutes, the absorbance of the reaction mixture was measured at 435 nm on UV-visible spectrophotometer (Shimadzu UVPC-1700 (Japan)). Total flavonoid contents of leaves sample were expressed as rutin equivalents (RE mg /g dry weight of extract) through the calibration curve with rutin as standard.

Alkaloid Estimation

2.5g of the plant powder was extracted using 100ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25ml. Concentrated ammonium chloride was added stepwise for precipitation. The whole solution was kept in such a manner so that precipitate should settle down. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [17, 18].

Tannins Estimation

The tannin content in samples was estimated by the method of Price and Butler [19]. Different aliquots of sample were taken and final volume to 3 ml was adjusted by distilled water. The samples after vortexing were mixed with 1ml of 0.016M $K_3Fe(CN)_6$, followed by 1 ml of 0.02M $FeCl_3$ in 0.10 M HCl. Vortexing was repeated and the tubes were kept as such for 15 min. 5 ml of stabilizer (3:1:1 ratio of water, H_3PO_4 and 1% gum Arabic) was added followed by revortexing. Absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001M Gallic acid.

RESULTS AND DISCUSSION

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

Antileishmanial Activity

Peucedanum beluchistanicum leaves' 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 32.65 to <100 µg/ml. CME was found to be more active amongst the fractions. CME showed the highest Antileishmanial activity with IC_{50} 32.65 µg/ml. CCF showed good activity with IC_{50} 50.63µg/ml. Rest of the fractions 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} 21.64 µg/ml.

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 (2). CME has showed maximum antioxidant activity with the IC_{50} value of 12.48µg/ml. On the other hand, CCF showed good antioxidant activity with IC_{50} value of 17.45µg/ml. Other fractions; NHF has IC_{50} value of 22.90 µg/ml. AQF showed lowest Free radical scavenging activity and has IC_{50} >100 µg/ml. CME has excellent free radical scavenging with IC_{50} 12.48µg/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. *Peucedanum beluchistanicum* leaves have excellent pharmacological importance and it should be investigated further for Isolation, Purification and Characterization of valuable compounds.

Antitumor Activity

It is evidence from table (3) that *Peucedanum beluchistanicum* leaves extract and its fractions showed good and moderate levels of tumor inhibition. CME of *Peucedanum beluchistanicum* leaves showed 78.65% inhibition and CCF showed moderate level of tumor inhibition with 63.52% inhibition. CME have significant level of tumor inhibition that is comparable to Standard drug Vincristine (100 % tumor inhibition).

Table 1: *In-vitro* Antileishmanial efficacy of *Peucedanum beluchistanicum* leaves extract and its fractions. % Inhibition of death of *L. major parasite*

Extracts/ Fraction	Doses (µg/ml)	Survival % Promastigotes (1x 10^4)	% inhibition	(IC_{50})µg/ml
CME	25 50 250 500	55 42 31 22	22 34 42 51	32.65
NHF	25 50 250 500	79 70 61 55	10 18 21 30	<100
CCF	25 50 250 500	60 47 33 28	38 47 53 67	50.63
CAF	25 50 250 500	68 58 49 39	10 17 26 31	<100
AQF	25 50 250 500	100 100 100 100	- - - -	- - - -
DMSO(-ve)	25 50 250 500	100 100 100 100	- - - -	- - - -
Standard Drug Amphotericin B	25 50 250 500	50 25 12 0	50 75 88 100	21.64

Table 2: DPPH scavenging antioxidant activities of CME and its Fractions of *Peucedanum beluchistanicum* leaves

Extract/ Fractions	100 µg/ml	50 µg/ml	25 µg/ml	10 µg/ml	5 µg/ml	IC_{50} µg/ml
CME	87.05	77.02	62.14	46.10	32.01	12.48
NHF	76.10	61.25	50.02	39.03	24.52	22.90
CCF	78.20	68.10	58.01	40.20	28.13	17.45
CAF	60.01	40.22	25.02	-	-	-
AQF	-	-	-	-	-	>100
ASA	95.04	94.79	90.02	86.5	44.01	5.5

Table 3: Antitumor activity of CME and its Fractions of *Peucedanum beluchistanicum* leaves.

Extract/ Fractions	Average number of tumors [a] ± SE	% inhibition of Tumors [b, c]
CME	1.5±0.20	78.65
NHF	5.5±0.76	29.30
CCF	2.9±0.80	63.52
CAF	8.6±0.20	18.55
AQF	-	-
Vincristine Std. drug	0.0±0.0	100
Vehicle Control	8.4±0.92	-

[a]) Potato disc antitumor assay, Concentration:1000µg/ml in DMSO. [b]) More than 20% tumor inhibition is significant. [c]) Data represents mean value of 15 replicates.

Quantitative Analysis of Phytoconstituents

Phytochemical analysis showed that the Methanolic leaves extract of *Peucedanum beluchistanicum* are a rich source of Flavonoids, phenols which may be responsible for the Antileishmanial, Antitumor and Antioxidant activities.

Table 4: Quantitative estimation of phytoconstituents present in methanol extract and it is fractions of *Peucedanum beluchistanicum* leaves.

Phytoconstituents	Quantity (mg/g plant extract and it is fractions)
Alkoloids	16.18±0.06
Phenolics	20.55±0.06
Flavonoids	22.82±0.01
Tanins	18.67±0.08
Diterpenes	13.48±0.06

CONCLUSION

We conclude from the present investigation that among the extract and its fractions of *Peucedanum beluchistanicum* leaves, Methanolic Extract (CME) showed tremendous Antileishmanial, Antioxidant and Antitumor activities which may be due to its phytoconstituents, 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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