Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 898 - 905

Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IN VITRO ANTIMICROBIAL, INSECTICIDAL, CYTOTOXIC ACTIVITIES AND THEIR PHYTOCHEMICAL ANALYSIS OF METHANOLIC EXTRACT AND ITS FRACTIONS OF *PEUCEDANUM BELUCHISTANICUM* LEAVES

NIZAM BALOCH, ABDUL MANAN KAKAR, SAJID NABI, MASOOM YASINZAI AND DR. YASSER M.S.A. AL-KAHRAMAN

Institute of Biochemistry, University of Balochistan, Quetta, Pakistan

ABSTRACT

The aim of the present investigation deals with biological evaluation *Peucedanum Beluchistanicum leaves.* For this purpose different biological assay of methanolic extract (Crude) and its fractions that are chloroform fraction, *n*-hexane fraction, Ethyl acetate fraction, *n*-butanol fraction and aqueous fraction were carried out. The results from the agar diffusion method indicated that Crude showed maximum antibacterial activity against *Staphylococcus aureus* with the inhibition zone (28.3±0.04mm). Whereas Ethyl acetate fraction also showed strong activity against *Staphylococcus aureus* (25.2±0.9mm). On the other hand, Crude showed maximum activity against *Candida albicans* and *Candida glaberata* with % inhibition of (73±0.4%) and (68±0.2 %) respectively and Chloroform fraction showed good activity against *Candida albicans* with % inhibition of (62±0.12%). Furthermore, Crude showed maximum insecticidal % mortality against *Tribolium castaneum* with (86%) mortality whereas (80%) mortality against *Rhyzopertha dominica.* and also Chloroform fraction also showed maximum activity against *Tribolium castaneum* with (70%) mortality. Moreover Chloroform fraction showed significant Cytotoxic activity with ED_{50} value 0.85 µg//ml and Crude showed Cytotoxic activity with ED_{50} value 0.98 µg//ml. Furthermore, the phytochemical analysis of Crude and its fractions showed the presence of Alkaloids, Flavonoids, Phenols, Saponins and Diterpenes.

KEYWORDS:Antimicrobial, Insecticidal, Cytotoxic Activity, Phytochemical Analysis, *Peucedanum Beluchistanicum* Leaves

DR. YASSER M.S.A. AL-KAHRAMAN
Institute of Biochemistry, University of Balochistan, Quetta, Pakistan

INTRODUCTION

More than 500,000 plant species around the globe, only about 1-10% of this large floral diversity have been evaluated for their medicinal properties [1, 2]. Till this date only 1% of plant species in these habitats have been evaluated for their pharmaceutical properties [3].Plant resulting compounds have been, and are still, important as such or as models for medicines: 50 % of the prescription products in various countries in Europe and the US are either natural products or natural product derivatives [4, 5] . To date about 50 drugs have come from tropical plants [3]. Plants continue to be a potent source of lead compounds. Although combinatorial techniques have been used for the optimization of a number of recently approved agents, these methods have not been able to identify a *de novo* combinatorial compound[5]. Examples of successful medicines derived from natural product leads include most antibiotics, the acethyl choline esterase (ACE) inhibitors, many anticancer agents, the immuno suppressants, cyclosporine and rapamycin and the antiparasitic avermectins [6]. Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. In the developing countries bacterial infections are still the main cause of deaths [7]. The need for new antibacterial drugs is constant, because of the continuous development of antibiotically resistant strains of pathogenous bacteria such as methicillin-resistant *Staphylococcus aureus*(MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vancomycin-resistant enterococci (VRE). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority have not been adequately evaluated. Considering the vast potentiality of plants as sources of antimicrobial drugs with reference to antibacterial and antifungal agents, a systematic investigation was undertaken to screen the local flora of Balochistan for the different biological activities [8-10]. Plant specie, *Peucedanum beluchistanicum* belongs to family *Umbelliferae*; which is glabrous plant, stems many from the base. Leaves bipinnate, 20 cm long; Fruit dorsally compressed; stylopodium depressed; styles reflexed. This plant has never been reported for their pharmacological activity. Hence the present study is carried out for to study the *in vitro* antimicrobial, insecticidal, cytotoxic activity and phytochemical analysis of *Peucedanum beluchistanicum.*

MATERIALS AND METHODS

Plant material

The Leaves of *Peucedanum Beluchistanicum* were collected from District Kalat, Balochistan province, Pakistan.

Extraction and fractionation

The fresh leaves were washed, sliced and dried under shade for 15 days. The leaf 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 [9]. Thereafter, the methanol was decanted and filtered with Whatman filter paper. The filtrate was subsequently concentrated under reduced pressure at 45^{o}C in the rotatory evaporator (Stuart RE 300) and dried to constant weight (460 g) in vacuum oven (LINN high therm.) at 45^{0}C. This was a crude methanolic leaves extract (CME). The CME was then further fractionalized, where 250g of CME was suspended in 250ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for five fractions, namely, *n*-hexane fraction (NHF), chloroform fraction (CHF), Et-acetate fraction (EAF), *n*-butanol fraction (NBF) and aqueous fraction (AQF).

Biological activities

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

Preparation of the tested organisms

A) Preparation of standard bacterial suspensions

The average number of viable, *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* organisms per ml of the stock suspensions was determined by means of the surface viable counting technique [11]. About (108- 109) colony forming units per ml was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

B) Preparation of standard fungal suspensions

The fungal cultures (*Microsporum canis, Candida albicans, Aspergillus flavus, and Candida glaberata)* were maintained on Saboraud Dextrose Agar, incubated at 25ºC for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100 ml) of sterile normal saline and the suspension was maintained for further use.

Antimicrobial activity

Testing for antibacterial activity

The cup-plate agar diffusion method was used [12] to assess the antibacterial activity of the prepared extracts. 0.6 ml of standardized bacterial stock suspensions of 108 -109 colony forming units per ml was thoroughly mixed with 60 ml of sterile nutrient agar. 20 ml of the inoculated nutrient agar was distributed into sterile Petri dishes. The agar was left to set and in each of these plates, 4 cups, 10mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed. Alternate cups were filled with 0.1ml of each extract using micropipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 18 hours. Two replicates were carried out for each extract against each of the test organism. The Simultaneous addition of the respective solvents instead of extracts was carried out as controls. After incubation the diameters of the growth inhibition zones were measured, averaged and the mean values were tabulated (Table 1).

Testing for anti-fungal activity

The same method as for bacteria was followed. Instead of nutrient agar media, yeast and mould extract agar was used. The inoculated medium was incubated at 25ºC for two days for *Microsporum canis* and *Candida albicans* and three days for *Candida glaberata* and *Aspergillus flavus.*

Insecticidal activity

Crude extract and all fractions were evaluated against different insects viz., *Tribolium castaneum, Sitophilus oryzea, Callosbruchu sanalis, and Rhyzopertha dominica*. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loaded in a Petri dishes covered with the filter papers. After 24 hours, 10 test insects were placed on each plate and incubated at 27 °C for 24 hours with 50% relative humidity in growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls. Permethrin was used as a standard drug, while Permethrin, acetone and test insects were used as positive and negative controls [13-17].

The percentage mortality was calculated by the formula:

$$\text{Growth regulation (\%)} = \frac{\text{Number of insects alive in test}}{\text{Number of insects alive in control}} \times 100$$

Brine shrimp Cytotoxicity assay

The brine shrimp Cytotoxicity assay was performed by using the methodology according to the procedure described by [18].Brine shrimp (*Artemia salina*) larvae used as test organisms, were hatched at 37^{o}C in artificial seawater.

Different concentrations i.e. 1000, 100, and 10µg/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. The 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).

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. After addition of a 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). The 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. The appearance of bluish black color indicated the presence of phenols.

4. Test for Saponins

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 saponins.

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 [19-21].

RESULTS AND DISCUSSIONS

Antibacterial activity

The antibacterial activity of the methanolic extract and different fractions from the leaves of *Peucedanum Beluchistanicum* posses good antibacterial activity against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus*. Table 1 shows the zone of inhibition against different species of gram positive and gram negative bacteria. The results from the agar diffusion method [10] indicated that 100% methanolic extract (Crude) showed good activity against *Staphylococcus aureus* and *Salmonella typhi*, with the inhibition zones (28.3±0.04 mm) and (26.19±0.15). Least activity was exhibited

against *Pseudomonas aeruginosa* with the smallest inhibition zone (15.6±0.03mm). n-hexane fraction showed good activity against *Staphylococcus aureus* and *Salmonella typhi* with the inhibition zones (25.2±0.9mm) and (22.6±0.14mm) respectively. Et- acetate fraction was effective against *Staphylococcus aureus* with inhibition zone (24.12±0.2mm).Chloroform fraction showed moderate activity against *Staphylococcus aureus* with inhibition zone (16.7±0.2mm). *n*-butanol fraction showed low activity against *Bacillus subtilis* and *Staphylococcus aureus* with the inhibition zones (10.2±0.05 mm) and (14.1±0.08). *n-hexane* fraction was potent against *Staphylococcus aureus*, with the inhibition zone (14.1±0.08 mm). In general, the antimicrobial activity of the tested extract and fractions is comparable with the standard drugs, Impenum.

Table 1
Antibacterial Activity of leaves of Peucedanum Beluchistanicum

Bacterial species	Zone of Inhibition of Std. drug* (mm)	Zone of inhibition (mm)					
		Crude	n-hexane	Chloroform	Et-acetate	n-butanol	Aqueous
Bacillus subtilis	36.03±0.24	23.02±0.12	16±0.10	12.01±0.08	13.04±0.01	10.02±0.05	-
Escherichia coli	32.02±0.12	19.01±0.11	14.02±0.03	-	-	-	-
Pseudomonas aeruginosa	32.08±0.18	15.06±0.03	13.19±0.09	-	10.08±0.01	-	-
Salmonella typhi	40.12±0.02	26.19±0.15	22.06±0.14	13.04 ±0.06	16.06±0.01	12.02±0.8	10.03±0.07
Staphylococcus aureus	43.24±0.27	28.03±0.04	25.02±0.9	16.07±0.2	24.12±0.2	14.01±0.08	11.04±0.6

Impenum(10µg disc)

Antifungal activity

The antifungal activity of the methanolic extract and different fractions from leaves of *Peucedanum Beluchistanicum* posses good antifungal activity against *Microsporum canis, Candida albicans, Aspergillus flavus,* and *Candida glaberata*. Table 2. Shows % inhibition against different species of fungi compared to the standard drug (Miconazole and Amphotericin B). The result indicated that Crude showed maximum activity against *Candida albicans* and *Candida glaberata* with % inhibition of (73%±0.4) and (68%±0.24) respectively and showed least % inhibition against *Aspergillus flavus* with (12%±0.23) inhibition. Chloroform fraction showed good activity against *Candida albicans* with % inhibition of (62%±0.12) showed least % inhibition against *Aspergillus flavus* with (10 %±0.8) inhibition. *n*- Hexane fraction showed moderate % inhibition against *Candida glaberata* with (42%±0.8) inhibition. Rest of the fractions showed least activity below then (40%) mortality.

Table 2
Antifungal activity of leaves of *Peucedanum Beluchistanicum*

Fungal species	% Inhibition of Std. drug*	% inhibition					
		Crude	n-hexane	Chloroform	Et-acetate	n-butanol	Aqueous
Microsporum canis	98.14±0.22 Micanzole	63.01±0.22	32.02±0.13	51.06±0.25	22.01±0.6	-	-
Candida albicans	110.8±0.15 Micanzole	73.33±0.4	-	62.08±0.12	39.02±0.8	-	-
Candida glaberata	110.28±0.2 Micanzole	68.22±0.2	42.02.08	54.13±0.19	36.04±0.2	28.19±0.1	22.07±0.8
Aspergillus flavus	20.06±0.4 Amphotericin B	12.18±0.23	9.07±0.6	10.12±0.8	-	-	-

Percent inhibition activity, 0-39= Low (non-significant); 40-59= moderate; 60-69= Good; above 70= Significant

Insecticidal Activity

Methanolic extract and its fractions from the leaves of *Peucedanum Beluchistanicum* were evaluated for its insecticidal activity against *Tribolium castaneum, Sitophilus oryzea, Rhyzopertha dominica* and *Callosbruchus analis* Table 3. Shows the % mortality of different species of insects as compared to standard drug (Permethrin). Crude showed maximum insecticidal % mortality against *Tribolium castaneum* with (86%) mortality whereas (80%) mortality against *Rhyzopertha dominica.* Least was against *Callosbruchus analis* with 40% mortality. Chloroform fraction showed maximum activity against *Tribolium castaneum* with (70%) mortality and (60%) against *Rhyzopertha dominica*. *n*-hexane showed moderate activity with 60% mortality against *Tribolium castaneum.* Et-acetate and *n*-butanol fractions showed low insecticidal activities with % mortality less than 50%. Aqueous fraction did not show any insecticidal activity.

Name of Insects	% Mortality of Std. drug*	% Mortality					
		Crude	n-hexane	Chloroform	Et-acetate	n-butanol	Aqueous
Tribolium castaneum	100	86	60	70	40	30	-
Sitophilu soryzea	100	70	40	50	30	-	-
Rhyzopertha dominica	100	80	30	60	40	20	-
Callosobrushusanalis	100	40	20	50	-	-	-

Cytotoxic Activity

The 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 the present study, Chloroform fraction of *Peucedanum Beluchistanicum* leaves showed maximum ED_{50} values 0.85µg/ml while Crude showed significant activity with ED_{50} values of 0.98µg/ml. On the other hand Fractions n-hexane and n-butanol showed ED_{50} values of 6.3 and 21.48µg/ml respectively. AQF and Et-acetate showed the lowest activity with ED_{50} value of <100µg/ml comparatively with Standard drug.

TABLE 3

Cytotoxic Activity of Peucedanum Beluchistanicum leaves extract and its fractions

Extract/ Fractions	Number of brine shrimp	% death at doses			ED_{50} µg/ml
		1000µg/ml	100µg/ml	10µg/ml	
Crude	30	27	24	20	0.98
n-hexane	30	24	20	16	6.3
Chloroform	30	28	25	21	0.85
Et-acetate	30	18	14	10	>100
n-butanol	30	22	17	14	21.48
Aqueous	30	18	15	17	>100
DMSO(-ve)	30	-	-	-	-
Etoposid (+ve)	30	30	27	24	0.56

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 Peucedanum Beluchistanicum leaves.

S.No	Constituents/ Test	Crude	n-hexane	Chloroform	Et-acetate	n-butanol	Aqueous
(1)	Alkaloids						
a)	Hager's Test	+	-	+	-	-	-
b)	Wagner Test	+	-	+	-	-	-
(2)	Flavonoids						
c)	Lead acetate Test	+	-	+	+	+	-
d)	Alkaline Reagent Test	+	-	+	+	+	-
(3)	Tannins						
e)	$FeCl_3$ Test	+	-	+	-	+	+
4	Saponins						
f)	Foam Test/Froth Test	+	+	+	+	+	+
(5)	Diterpenes						
g)	Copper Acetate Test	+	+	+	-	+	-

(-) Absent, (+) Present

CONCLUSION

This study evaluated first time as an *in vitro* study of Antimicrobial, insecticidal, cytotoxic activities and phytochemical analysis of *Peucedanum Beluchistanicum leaves* that showed tremendous results against different pathogenic organisms. We recommend the future study on different aspects of pharmacological actions *in vitro* and *in vivo.*

ACKNOWLEDGEMENT

The Authors are thankful to the Higher Education Commission of Pakistan and University of Balochistan, Quetta, Pakistan for financial support.

REFERENCES

1. Farnsworth N. R, Soejarto D.D., Global importance of medicinal plants. In: Akerele O, Heywood V, Synge H. (Eds.), Conservation of Medicinal Plants. Cambridge University Press, Cambridge, (1991).
2. Verpoorte R., Pharmacognosy in the New Millenium: Lead finding and Biotechnology. *Journal of Pharmacy and Pharmacology,*52: 253-262 (2000).
3. Gurib-Fakim A., Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine,* 27: 1-93(2006).
4. Cordell J., Natural products in drug discovery– Creating a new wisdom. *Phytochemistry Reviews*, 1: 261-273 (2002).
5. Newman D.J, Cragg G.M, Snader K.M., Natural Products as Sources of New Drugs over the Period 1981- 2002. *Journal of Natural Products*, 66: 1022-1037(2003).
6. Harvey AL, Waterman P: The continuing contribution of biodiversity to drug discovery.*Current Opinion in Drug*

Discovery and Development,1 (1): 71-76 (1998).
7. Iwu M.M, Duncan A.R, Okunji C.O., New Antimicrobials of Plant Origin. In: Perspectives on new crops and new uses. Ed.: Janick, J., ASHS Press, Alexandria, VA, (19990.
8. Sajid Nabi., Nisar Ahmed., Muhammad Javed Khan., ZahoorBazai., Masoom Yasinzai., Yasser M.S.A. Al-Kahraman.,*In vitro* Antileishmanial, Antitumor Activities and phytochemical Studies of Methanolic Extract and its Fractions of *Juniperus Excelsa Berries*. *World Applied Sciences Journal*, 19 (10): 1495-1500 (2012).
9. Muhammad Javed Khan., Nizam.U.Baloch., Sajid Nabi., Nisar Ahmed., Zahoor Bazai., Masoom Yasinzai., Yasser. M. S. A. Al-Kahraman., Antileishmanial, cytotoxic, antioxidant activities and phytochemical analysis of *Rhazya stricta Decne* leaves extracts and its fractions.*Asian Journal of Plant Science and Research*, 2 (5):593-598 (2012).
10. Abdul Aziz Khan, Nizam.U.Baloch, Sajid Nabi, Muhammad Javed Khan, M. Sharif Jamali and Yasser. M. S. A. Al-Kahraman.,*In vitro* antimicrobial and insecticidal activity of Methanolic extract and its fractions of *Berberis Baluchistanica* roots. *World Journal of Pharmaceutical Research*, Volume 2, Issue 1, 219-226 (2012).
11. Miles A.A. and Misra S.S., Estimation of bacterial power of blood.*J.Hyg*,38:7 (1938).
12. Kavanagh F., Analytical Microbiology. F. Kavanagh (ED), VOLII, Academic press, New York, and London, P:11 (1972).
13. Abbott W.S., A method of computing effectiveness of insecticides. J*. Econ. Ent.*,18(2): 265-67(1925).
14. Atta-ur-Rahman, Choudhary M.I and William J.T., Bioassay techniques for drug development. Harward academic Publisher, 67-68 (2001).
15. Collins P.J., Resistance to grain protectants and fumigants in insect pests of stored products in Australia. In: (Ed.): H.J. Banks, E.J. Wright and K.A. Dameevski. CSIRO Stored Grain Research Laboratory, Presented in Australian Post Harvest Technical Conference, 55- 57(1998).
16. Rashid R, Farah M and Mirza M.N., Biological screening of Salvia cabulica. *Pak. J. Bot*, 41(3): 1453-1462 (2009).
17. Tabassum R., Naqvi S.N.H, M. Azmi A, Nurulain S.M and Khan M.F.,Residual effect of a neem fraction, nimolicine and an insect growth regulator, dimilin, against stored grain pest Callosobruchus analis. *Proc. Pakistan Congr. Zool*., 17: 165-170 (1997).
18. [Ahmad M.S, Hussian M, Hanif A.S, Qayyum M, Mirza B., *Chem. Bio. Drugs Design.,(71)568-576*(2008).
19. Bachaya H.A, Iqbal I, Khan M. N, Jabbar J, Gilani A.H, Din I.U.,*Int. J. Agric. Biol*., (11) 606-610 (2009).
20. Maniyar Y, Bhixavatimath P, Agashikar N.V.,*J Ayurveda Integr Med*, 1: 287-291(2010).
21. Roopashree T.S, Dang R, Rani S.R.H, Narendra C., *International Journal of Applied Research in Natural Products*, *1(3): 20-28*(2008).
22. Mayerhof E.R, Koncz-kalman R.Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redel G.P, Schell J.B, Hohn K.J.,*Embo J.*,*10*:*697-704*(1991).