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IN VITRO ANTIMICROBIAL, ANTITUMOR AND CYTOTOXIC ACTIVITIES OF METHANOLIC EXTRACT AND ITS FRACTIONS OF *ACANTHOLIMON LONGISCAPUM*

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ABSTRACT

The aim of the present investigation deals with biological evaluation *Acantholimon longiscapum* 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 (31.18±0.01mm). On the other hand, Crude showed maximum activity against *Candida albicans* and *Candida glaberata* with % inhibition of (76.10%±0.02%) and (82.02%±0.11%) respectively. On the other hand, Crude also showed tremendous Antitumor activity with % inhibition 85.23% as comparable to the standard drug.

Key words: Antimicrobial, Antitumor, Cytotoxic, *Acantholimon longiscapum* leaves.

INTRODUCTION

In the recent years, research on medicinal plants has attracted a lot of attentions globally. Large body of evidence has accumulated to demonstrate the promising potential of Medicinal Plants used in various traditional, complementary and alternate systems of treatment of human diseases. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, etc, which have been found *in vitro* to have antimicrobial properties (Dahanukar *et al*., 2000), (Cowan, 1999).

Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by the physicians; several are already being tested in humans. Scientists realize that the effective life span of any antibiotic is limited, so new sources especially plant sources are also being investigated. Second the public is becoming increasingly aware of the problems with the over prescription and misuse of traditional antibiotics. In addition many people are interested in having more autonomy over their medical care. A multitude of plants compounds (often of unreliable purity) is readily available over the counter from herbal suppliers and national food stores and the self medication with these substances is a common practice to certain extent (Cowan, 1999).

Plant species *Acantholimon longiscapum* belongs to family *Plumbaginaceae* and is found in Balochistan province. This plant has never been evaluated for its pharmacological activities. So, in continuation of our previous work (Sajid *et al*., 2012), (Muhammad *et al*., 2012), (Abdul *et al*., 2012) 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

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fractions from the "*Acantholimon longiscapum*" plant species in bioassays such as (a) Antimicrobial activity (b) Antitumor activity and (c) Cytotoxic activity

MATERIALS AND METHODS

Plant material

Leaves of *Acantholimon longiscapum* was collected from Isplingi-Quetta, 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 (2 kg in 6L) for 72hours. 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. The filtrate was subsequently concentrated under reduced pressure at 45°C in rotatory evaporator (Stuart RE 300) and dried to constant weight (400 g) in vacuum oven (LINN high therm) at 45°C. This was crude methanolic leaves extract. The Crude was than further fractionalized, where 200 g of Crude was suspended in 2500ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for five fractions, namely, *n*-hexane, Chloroform, Et-acetate, *n*-butanol and Aqueous fractions.

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 (Miles and Misra, 1938). 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 (Kavanagh, 1972) 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 were 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 extracts 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. Simultaneously 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.*

Crown Gall Tumor Inhibition (Potato Disc) Assay

Antitumor potato disc assay was performed for *Acantholimon longiscapum* leaves by using *Agrobacterium tumefaciens* (At- 10). *Acantholimon longiscapum* leaves extract and its fractions were tested for *in vitro* antitumor activities reported as (Ahmad *et al*., 2008).

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 1000µg/ml final concentration. One drop (10µl) 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 numbers of tumors in vehicle control (DMSO) were 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.

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

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 (*Artemia salina*) larvae used as test organisms, were hatched at 37°C in artificial sea water. Different concentrations i.e. 1000, 100, and 10 µg/ml (control) of CME and five fractions, namely, *n*-hexane, Chloroform, Et-acetate, *n*-butanol and Aqueous fractions. 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°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).

RESULTS

Antibacterial activity

The antibacterial activity of the methanolic extract and different fractions from leaves of *Acantholimon longiscapum* possess good antibacterial activity against *Bacillus subtilis*., *Escherichia coli*., *Pseudomonas aeruginosa*., *Salmonella typhi*., *and Staphylococcus aureus*. Table 1 shows the zone of inhibition against different species of gram positive and gram negative bacteria.

Antifungal activity

The antifungal activity of the methanolic extract and different fractions from leaves of *Acantholimon longiscapum* possess 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).

Antitumor Activity

The antitumor activities of *Acantholimon longiscapum* leaves extract and its fractions showed good and moderate levels of tumor inhibition. Table 2 shows % inhibition of Tumors**.**

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 (Mayerhof *et al*., 1991).

Table 1. Antibacterial Activity of Crude and its Fractions of *Acantholimon longiscapum* leaves

Bacterial species	Zone of Inhibition of Std. drug* (mm)	Zone of inhibition (mm)					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Bacillus subtilis	36.06±0.03	28.02±0.02	14.55±0.12	23.08±0.01	18.02±0.12	15.09±0.01	13.55±0.06
Escherichia coli	35.11±0.02	19.22±0.07	12.03±0.01	15.52±0.08	10.06±0.04	-	-
Pseudomonas aeruginosa	32.01±0.09	15.04±0.03	-	13.48±0.06	-	-	-
Salmonella typhi	40.12±0.01	27.35±0.02	17.02±0.09	24.04±0.01	13.08±0.01	-	-
Staphylococcus aureus	43.22±0.08	31.18±0.01	18.52±0.01	28.04±0.10	16.02±0.14	20.12±0.09	14.46±0.09

* imipenem (10µg disc)

Table 2. Antifungal activity of Crude and its Fractions of *Acantholimon longiscapum* leaves

Fungal species	% Inhibition of Std. drug*	% of inhibition					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Microsporum canis	98.04%±0.02 Miconazole	60.02%±0.06	31.49%±0.07	54.06%±0.02	42.32%±0.09	30.42%±0.11	26.02%±0.14
Candida albicans	110.08%±0.02 Miconazole	76.10%±0.02	42.28%±0.09	68.01%±0.03	46.25%±0.10	40.01%±0.03	36.21%±0.04
Candida glaberata	110.25%±0.06 Miconazole	82.02%±0.11	62.42%±0.01	75.18%±0.04	40.10%±0.07	43.01%±0.21	32.02%±0.01
Aspergillus flavus	20.12%±0.06 Amphotericin B	11.031±0.03	07.03%±0.09	10.06%±0.01	-	-	-

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

Table 3 Cytotoxic Activity of *Acantholimon longiscapum leaves* 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	30	27	24	0.56
CCF	30	28	25	21	0.85
CAF	30	30	27	24	0.56
AQF	30	24	20	17	3.67
DMSO(-ve)	30	-	-	-	
Etoposid (+ve)	30	30	27	24	0.56

Table 4. Antitumor activity of Crude and its Fractions of *Acantholimon longiscapum* leaves

Extract/ Fractions	Average number of tumors [a] ± SE	% inhibition of Tumors [b, c]
Crude	1.5±0.10	85.23
***n*-hexane**	-	-
Chloroform	2.1±0.04	66.12
***Et*-acetate**	-	-
***n*-butanol**	5.2±0.04	30.28
Aqueous	-	-
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.

DISCUSSION

The results from the agar diffusion method indicated that 100% methanolic extract showed maximum activity against *Staphylococcus aureus*, with the inhibition zone (31.18±0.01 mm). Good activity of Crude was exhibited against both *Bacillus subtilis* and *Salmonella typhi* with the inhibition zones (28.02±0.02) and (27.35±0.02) respectively. Least activity was exhibited against *Pseudomonas aeruginosa* with the smallest inhibition zone (15.04±0.03mm). Chloroform fraction showed strong activity against *Staphylococcus aureus* with (28.04±0.04mm) zone inhibition and good activity against both *Bacillus subtilis* and *Salmonella typhi* with the inhibition zones (23.08±0.01) and (24.04±0.10) respectively. *n*-butanol fraction showed good activity against *Staphylococcus aureus* with (20.12±0.09mm) zone inhibition . *Et*-acetate fraction showed moderate activity against *Bacillus subtilis* with the inhibition zone (18.02±0.12mm). *n*-hexane showed moderate activity against *Staphylococcus aureus* and *Salmonella typhi* with the inhibition zones (18.52±0.01mm) and (17.02±0.09mm) respectively. Aqueous fraction showed least activity amongst the fractions. The antimicrobial activity of the tested extract and fractions is comparable with the standard drugs, imipenem.

The result indicated that Crude showed maximum activity against *Candida glaberata* and *Candida albicans* with % inhibition of (82.02%±0.11) and (76.10 %±0.02) respectively and showed least % inhibition against *Aspergillus flavus* with (11.01%±0.03) inhibition. Chloroform fraction showed good activity against *Candida glaberata* and *Candida albicans* with % inhibition of (75.18%±0.04) and (68.01%±0.03) respectively and showed least % inhibition against *Aspergillus flavus* with (10.06±0.01) inhibition. *n*-hexane

fraction showed good % inhibition against *Candida glaberata* with (62.42%±0.01) inhibition. Et-acetate, *n*-butanol and Aqueous fractions showed moderate and low % inhibition against all fungal species except *Aspergillus flavus*.

Crude of *Acantholimon longiscapum* leaves showed 85.23% inhibition which is significant level of tumor inhibition that is comparable to Standard drug Vincristine (100 % tumor inhibition) and Chloroform fraction showed moderate level of tumor inhibition with 66.12% inhibition.

In present Study, CME and CAF of *Acantholimon longiscapum* leaves showed ED_{50} values 0.56 µg/ml while CCF showed significant activity with ED_{50} value 0.85 µg/ml. AQF showed the lowest activity with ED_{50} value of 3.67 µg/ml comparatively with Standard drug.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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