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In-vitro antimicrobial, insecticidal, antitumor, antioxidant activities and their phytochemical estimation of methanolic extract and its fractions of *Nepeta praetervisa* leaves

Yasser MSA Al-Kahraman[1]*, Nizam Baloch[1], Abdul Manan Kakar[1]and Sajid Nabi[1]

*Corresponding author:

Yasser MSA Al-Kahraman

[1]Institute of Biochemistry, University of Balochistan, Quetta, Pakistan.

Abstract

The aim of the present investigation deals with biological evaluation *Nepeta praetervisa 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. Furthermore, Crude showed maximum insecticidal % mortality against both *Triboliumcastaneum and Sitophilusoryzea*with (80%) mortality. On the other hand, Crude also showed tremendous Antitumor activity with % inhibition 85.23% as comparable to the standard drug. Chloroform fraction showed maximum DPPH free radical scavenging activity with the IC_{50} value of 19.66μg/ml.Furthermore, the phytochemical estimation of Crude and its fractions showed the presence of Alkaloids, Flavonoids, Phenols, tannins and Diterpenes.

Keywords: Antimicrobial, Insecticidal, Antitumor, Antioxidant, phytochemical estimation, *Nepeta praetervisa*leaves.

Introduction

Plants can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works in both mixture of traditional medicine and single active compounds are very important. Where the active molecule cannot be synthesized economically, the product must be obtained from the cultivation of plant material. About 121 (45 tropical and 76 subtropical) major plant drugs have been identified for which no synthetic one is currently available. The scientific study of traditional medicines, derivation of drugs through bio-prospecting and systematic conservation of the concerned medicinal plants are thus of great importance [1].

Nepeta is a genus of annual or perennial herbs; it belongs to the Lamiaceae family, which includes approximately 250 species. These plants are localized to central and southern Europe, Asia, the Middle East, northern Africa, and to tropical mountains in Africa[2, 3]. Nepeta species are used in the traditional medicine of many countries and have a large ethnobotanical effect: diuretic, diaphoretic, vulnerary, antitussive, antispasmodic, antiasthmatic, tonic, febrifuge, emmenagogue and carminative [2, 4].

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 [5]. Thus, only limited work has been done in the medicinal flora of Balochistan province. So the present work has been carried out to study the *in vitro* antimicrobial and insecticidal activity of *Nepeta praetervisa.*

In continuation of our previous work [6-8] the aim of this study was to screen for medicinal plant 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) Antimicrobial activity (b) Insecticidal activity (c) antitumor activity (d) Antioxidant and (e) their phytochemical Estimation.

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Material and Methods

Plant material

The leaves of *Nepeta praetervisa*was collected from Soorab, 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 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. The Crude was than further fractionalized, where 250g of Crude was suspended in 250ml 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*(ATCC6059*)*, *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC 7221), *Salmonella typhi* (ATCC 700931), *Staphylococcus aureus* (ATCC7221)organisms per ml of the stock suspensions was determined by means of the surface viable counting technique [9]. 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 (*Microsporumcanis*(ATCC 36299)*, Candida albicans*(ATCC 90028*), Aspergillusflavus*(ATCC 16883) *and Candida glaberata*(ATCC 90030)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 [10] 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 *Microsporumcanis*and *Candida albicans*and three days for *Candida glaberata*and *Aspergillusflavus.*

Insecticidal activity

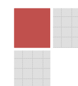
Crude extract and all fractions were evaluated against different insects viz., Triboliumcastaneum, Sitophilusoryzea, Callosbruchusanalis, and Rhyzoperthadominica. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loaded in a Petri dishes covered with the filter papers. After 24 hours, 10 test insects were placed in 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 [11-15].
The percentage mortality was calculated by the formula:

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

Crown Gall Tumor Inhibition (Potato Disc) Assay

Antitumor potato disc assay was performed for Nepeta praetervisaleaves by using Agrobacterium tumefaciens(At- 10). Nepeta praetervisaleavesextract and its fractions were tested for in vitro antitumor activities. Reported as [16].

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 % I2, 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.

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

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 [17]. 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 200μl of Crude, leading to the final concentration of 100, 50, 25, 10 and 5μg/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 *n*-hexane, Chloroform, Et-acetate, *n*-butanol and Aqueous fractions.

(%) scavenging effect = [(AC-AS)/ AS] x 100

Where; "AC" is the absorbance of negative control and "AS" is the absorbance of Test Sample.

Phytochemical Study

Phytochemical Screening

Phytochemical screening for major bioactive constituents like alkaloids, phenolics, flavonoids and tannins were determined by using standard phytochemical methods [18, 19].

Determination of Total Phenolics

Total phenolic content of methanolic extract of *Nepeta praetervisa 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 [20]. 200μl of sample (1mg/ml) was added to 100μl diluted (1:10) FolinCiocalteu reagent and equilibrated for few minutes. Then 800μl 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

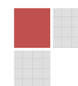
Total flavonoid content was determined by aluminum chloride colorimetric method [21]. This method is based on the formation of a complex flavonoid-aluminium, having the absorbance maximum at 430 nm. 0.5 ml of plant extract (1mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 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 430 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 as such 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 [22, 23].

Tannins Estimation

The tannin content in samples was estimated by the method of Price and Butler [24]. 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_2PO_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

Antibacterial activity

The antibacterial activity of the methanolic extract and different fractions from leaves of *Nepeta praetervisa 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. 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, Impenum.

Table 1.Antibacterial Activity of Crude and its Fractions of *Nepeta praetervisa*leave

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

*Impenum(10µg disc)

Antifungal activity

The antifungal activity of the methanolic extract and different fractions from leaves of *Nepeta praetervisa* possess good antifungal activity against *Microsporumcanis, 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 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 *Aspergillusflavus*.

Insecticidal Activity

Methanolic extract and its fractions from leaves of *Nepeta praetervisa* were evaluated for its insecticidal activity against *Triboliumcastaneum, Sitophilusoryzea, Rhyzoperthadominica* and *Callosbruchusanalis.* (Table 3) Shows the % mortality of different species of insects as compared to standard drug (Permethrin). Crude showed maximum insecticidal % mortality against *Triboliumcastaneum* and *Sitophilusoryzea* with (80%) mortality whereas (70%) mortality against *Callosbruchusanalis.* Least was against *Rhyzoperthadominica* with 60% mortality. Chloroform fraction also showed good activity against both *Triboliumcastaneum* and *Sitophilusoryzea* with (70%) mortality whereas (60%) mortality against *Callosbruchusanalis*. *n*-hexane, Et-acetate, *n*-butanol and Aqueous fractions showed low insecticidal activities with % mortality less than 50%.

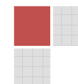


Table 2. Antifungal activity of Crude and its Fractions of *Nepeta praetervisa* leaves

Fungal species	% Inhibition of Std. drug*	% 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
Aspergillusfla vus	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. Insecticidal activityof Crude and its Fractions of *Nepeta praetervisa*leaves

Name of Insects	% Mortality of Std. drug*	% Mortality					
		Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Triboliumcastaneum	100	80	40	70	20	-	-
Sitophilusoryzea	100	80	40	70	40	30	30
Rhyzoperthadominica	100	60	20	50	30	-	-
Callosobrushusanalis	100	70	40	60	40	30	20

Antitumor Activity

The antitumor activities of *Nepeta praetervisa* leaves extract and its fractions showed good and moderate levels of tumor inhibition. Crude of *Nepeta praetervisa* 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

Table 4. Antitumor activity of Crude and its Fractions of *Nepeta praetervisa* 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.

Antioxidant activity

DPPH free radical scavenging assay was used to evaluate antioxidant potential of our samples. Crude 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). Chloroform fraction showed maximum antioxidant activity with the IC_{50} value of 19.66µg/ml. On the other hand, crude showed good antioxidant activity with IC_{50} value of 22.73µg/ml. Other fractions; n-butanolhas IC_{50} value of 67.63µg/ml. While n-hexane and Et-acetate showed lowest Free radical scavenging activity and have IC_{50}<100µg/ml. Chloroform fraction has excellent free radical scavenging with IC_{50}19.66µg/ml which is comparable to Ascorbic acid. Phytochemical assay of the Chloroform fraction shows that it has high concentrations of Phenols which are known to be potent

antioxidant. *Nepeta praetervisa* leaves have excellent pharmacological importance and it should be investigated further for Isolation, Purification and Characterization of valuable compounds.

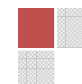


Table 5. DPPH scavenging antioxidant activities of Crude and its Fractions of *Nepeta praetervisa* leaves

Extract/ Fractions	100 µg/ml	50 µg/ml	25 µg/ml	10 µg/ml	5 µg/ml	IC_{50} µg/ml
Crude	72.02	71.11	60.04	30.11	14.06	22.73
n-hexane	49.10	24.02	-	-	-	<100
Chloroform	80.09	75.13	64.01	32.18	16.01	19.66
Et-acetate	40.02	-	-	-	-	<100
n-butanol	55.02	45.11	30.01	-	-	67.63
Aqueous	-	-	-	-	-	-
ASA	95.04	94.79	90.02	86.5	44.01	5.5

Table 6. Quantitative estimation of phytoconstituents present in methanol extract and it is fractions of *Nepeta praetervisa* leaves.

Phytoconstituents	Quantity (mg/g plant extract and it is fractions)
Alkaloids	17.01±0.06
Phenolics	22.1±0.01
Flavonoids	20.01±0.10
Tannins	18.30±0.14
Diterpenes	15.10±0.08

Preliminary Phytochemical Screening

Phytochemical analysis showed the presence of Alkaloids, Flavonoids, Tannins, Phenols and Diterpenes. Whereas terpenoids and cardiac glycoside were completely absent (Table 6).

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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