Phytochemical Screening, Total Phenolic Contents and Biological Evaluation of Aerial Parts of *Nepeta praetervisa*

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Summary: This study was designed to explore the phytochemical screening, total phenolic contents, radical scavenging potential and urease inhibitory activities in various fractions of the aerial parts of *Nepeta praetervisa.* Sub-fractions (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous) were prepared from the crude methanolic extract using partition chromatography. Phytochemical tests were performed and revealed the presence of various classes of secondary metabolites in various sub-fractions (Table-1). Total phenolic contents of all the fractions were determined using Folin-Ciocalteu (FC) reagent and the ethyl acetate sub-fraction was found to possess the highest level of phenolic contents (627.25 mg gallic acid equivalent (GAE)/g) as compared to the other fractions. The radical scavenging activity was determined at various concentrations ranging from 2.5 - 0.15µg /10µL by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method. At the lowest concentration level, the ethyl acetate sub-fraction showed maximum level of antioxidant activity (78%) compared to BHA used as standard. The decreasing order of activity was ethyl acetate>chloroform>aqueous>*n*-butanol>methanol>*n*-hexane. On the other hand when all these fractions were screened for urease inhibition activity using indophenols method, the ethyl acetate sub-fraction showed significant urease inhibitory activity (68 %) compared with the standard thiourea at the concentration of 50 µg /10 µL. The decreasing order of activity of various sub-fractions was ethyl acetate>chloroform>hexane>aqueous, while *n*-butanol sub- fraction was inactive.

Keywords*: Nepeta praetervisa*, Total phenolic contents, Antioxidant activity, Urease inhibition activity.

Introduction

Nepeta is the largest genus of the family Lamiaceae with about 250 species distributed mainly in south-west, central Asia, Europe, North America and North Africa [1, 2]. Out of these 30 species occur in India [3] and 58 species in Pakistan [4]. Various *Nepeta* species are used in traditional medicines e.g. as antispasmodic, diaphoretic, vulnerary, diuretic, antiasthamatic, febrifuge and as sedative agents [5-7]. The alcoholic extract of *N. cataria* revealed a biphasic effect on the behavior of chicks [8]. The aerial parts of *N. cataria* and related compounds, carvacol, thymol, nepetalactones, nepetalic acid, gerniol and citronellol were used as bath agents. These agents are effective in promoting sleep, calming nervous system, relieving mental pressure [9]. Neuropharmacological activity of essential oil from *N. sibthorpii* has also been reported [10]. Several *Nepeta* species are used for the treatment of cardiovascular complaints like angina pectoris, cardiac thrombosis, tachycardia and weakness of the heart [11-16]. The methanolic extract of *N. sibthorpii* and a fractions containing phenols and related compounds (chlorogenic acid, rutin, luteolin 7-*O*-glucoside and a luteolin derivative) showed radical-scavenging effects on DPPH radical [17]. The chemotaxonomic and ethnopharmacological importance of the genus *Nepeta* prompted us to carry out pharmacochemical studies on one of its species *N. praetervisa.* This species grows in Ziarat valley of Balochistan province of Pakistan. No pharmacological or phytochemical studies have been carried out on this plant so far.

The antioxidant inhibits the oxidation of compounds present especially in the food products. In the body antioxidant protects cells against the effect of free radical and therefore they are essential for preservation of human health. Free radicals are common outcome of normal aerobic cellular metabolism [18]. In-built antioxidant system of body plays its decisive role in prevention of any loss due to free radicals. However, imbalanced defense mechanism of antioxidants, over production or incorporation of free radicals from environment to living system leads to serious penalty leading to

neuro-degeneration. Apart from several other environmental or genetic factors, oxidative stress (OS) leading to free radical attack on neural cells contributes calamitous role to neuro-degeneration and end into a range of disorders such as Alzheimer's disease, Parkinson's disease, aging and many other neural disorders. Toxicity of free radicals contributes to proteins and DNA injury, inflammation, tissue damage and subsequent cellular apoptosis [19, 20]. In current research, antioxidants are now being looked upon as persuasive therapeutic against overproduction of ROS due to having capability to combat by neutralizing free radicals by oxidation. Diet is major source of antioxidants but medicinal herbs are catching attention to be commercial source of antioxidants at present. Much work has been devoted to explore antioxidant potentials of various extracts and secondary metabolites of isolated medicinal plants. In recent era, urease inhibiting drugs are paying attention for their potent anti-ulcer role [21]. Urease activity has been revealed as vital virulence determinant in the pathogenesis for numerous clinical circumstances, detrimental for human being and animal health and for agriculture [22, 23]. Therefore, strategies are being designed based on urease inhibition for the treatment of infection caused by urease producing bacteria.

This work has been carried out with a major aim of determination of antioxidant and urease inhibitory potentials of various fractions of *Nepeta praetervisa*.

Results and Discussion

Phenolic compounds are secondary metabolites which are synthesized in plants. They posses biological properties such as: antioxidant, antiapoptosis, anti-aging, anticarcinogen, anti-inflammation, anti-artherosclerosis, cardiovascular protection, improvement of the endothelial function, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilities [26]. The results revealed that *N. praetervisa* extract contains significant amount of phenolic compounds (Table-1**)**. Phenolic contents possess good antioxidant potential and can produce beneficial effects on human health. Phenols and related compounds are also very vital plant constituents because of their scavenging ability [24] due to the presences of hydroxyl groups. All the fractions of *N. praetervisa* showed good amount of phenolic content and ethyl acetate sub-fraction was found to contain the maximun level (627.25 mg GAE/ g of fraction).

The DPPH assay is a rapid and efficient method, widely used for the evaluation of antioxidant activity. It is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging). This test has been the most accepted model for evaluating the free radical scavenging activity of new drugs.

When a solution of DPPH is mixed with 10 µL solution of different fractions it is suggested to donate a hydrogen atom, which give rise to the reduced form (diphenylpicrylhydrazine; non radical) with the loss of this violet color (although there would be expected to be a residual pale yellow color from the group still present) [25].

The Fig. 1 shows a comparison of radical scavenging activity of various sub-fractions at different concentration levels ranging from 0.15-2.5 µg /10 µL. It was found to increase in dose dependant manner. At the lowest concentration level, the ethyl acetate sub-fraction showed maximum antioxidant activity (78%) compared to BHA used as standard. The decreasing order of activity was ethyl acetate>chloroform>aqueous>n-butanol> methanol> n-hexane. The IC_{50} values for this sub-fraction are presented in Table-1.

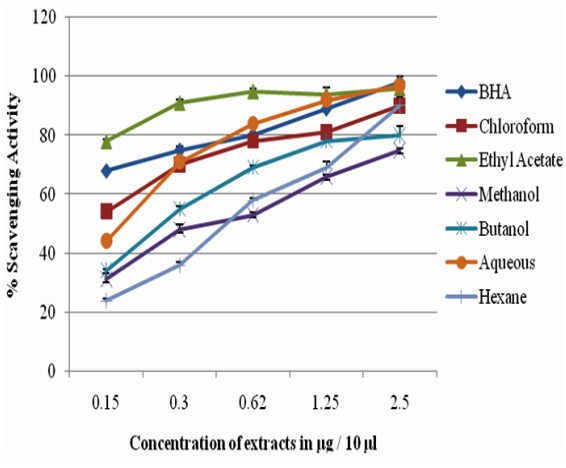

Fig. 1: % Antioxidant potential of various sub-fractions and BHA at different concentrations.

Table-1: Biological activities and phytochemical screening of *N. praetervisa* extracts.

S. No.	Extract	Phenolic Contents (mgGAE/ g fraction)	[a]DPPH Antioxidant Activity IC_{50} (µg / mL)	[b]Urease Inhibition (%inhibition)	Phytochemical investigation of *N. praetervisa* extracts						
					Saponins	*Flavanoids*	*Tannins*	*Alkaloids*	*Glycosides*	*Terpenoids*	*Reducing sugars*
1	Chloroform	44.50	180	45	+	+	-	+	+	+++	-
2	Ethylacetate	627.85	110	68	++	+++	++	++	++	++	++
3	Methanol	80.25	640	10	++	+++	++	+++	+++	+++	++
4	*n*-Butanol	57.20	300	0	+++	+++	+++	+++	+++	+	+++
5	Aqueous	7.8	260	25	+++	+	+++	+	+++	-	+++
6	*n*-Hexane	61	460	30	-	-	-	-	-	++	-

a) BHA was used as standard , IC_{50} = 54 µg /mL.
b) Thiourea was used as a standard at the concentration of 50 µg 10/ µL which showed 93% urease inhibition potential.
+ slight presence.
++ moderate presence.
+++ strong presence.

Various fractions were also screened for urease inhibitory activity and the results are illustrated in Table-1 and Fig. 2. The ethyl acetate sub-fraction showed significant urease inhibitory activity (68%) at the concentration of 50 µg /10 µL compared to the standard thiourea which showed 93% inhibition at the same level of concentration. The decreasing order of activity of various sub-fractions was ethyl acetate> chloroform> hexane> aqueous, while the *n*-butanol sub-fraction was inactive. The ethyl acetate sub-fraction can therefore be considered as a potent urease inhibitor which can be used for the treatment of ulcer.

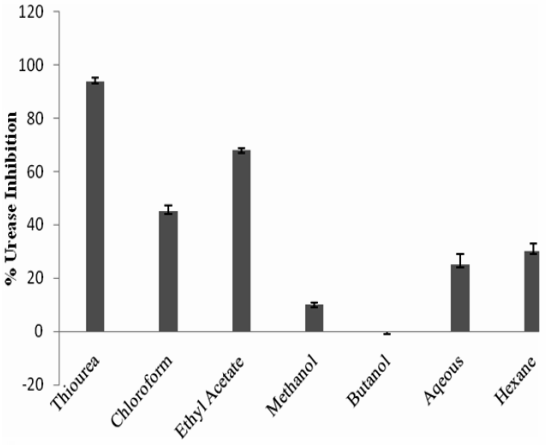

Fig. 2: % Urease inhibition of extracts and thiourea at conc 50µg /10µL.

Phytochemical screening of all the extracts was performed and is presented in Table 1**.** It can be observed that ethyl acetate sub-fraction contains highest level of phenolic and flavonoidal constituents. Terpenes and related compounds were detected in all the extracts. Alkaloids were present in methanol, *n*-butanol and aqueous fractions. Tannins and glycosides were found in ethyl acetate, *n*-butanol and aqueous sub-fractions while n-hexane was devoid of these compounds. The saponins were detected in ethyl acetate and n-butanol sub-fractions.

Experimental

Plant Material and Preparation of Extracts

The aerial parts of *Nepeta praetervisa* Rech. were collected from Ziarat valley Quetta. The whole plant (2 kg) was dried at room temperature, chopped into small pieces and extracted with nethanol(3x5L)at r.t. The combined extract was freed of solvent to obtain dark green residue (200 g) which was suspended in water (1L) and fractionated successively with n-hexane (3 x1L), chloroform (3 x 1L), ethyl acetate(3 x 1L) and n-butanol (3 x1L). These sub-fractions were evaporated on a rotary evaporator, respectively. The resulting sub-fractions were subjected to total phenolic contents, *in vitro* antioxidant, urease inhibition activities and phytochemical screenings.

Determination of Total Phenolic Contents

Total phenolic contents for each sub-fraction were evaluated by using Folin-Ciocalteu (FC) reagent according to the reported method [26], with a slight amendment. Each sub-fraction (0.1 mL) was mixed with 0.75 mL of FC reagent (formerly diluted 1000-fold using distilled water) and incubated for the period of 5-6 min at 22°C, followed by addition of 0.06% solution of Na_2CO_3. It was kept at 22°C for 90 min, absorbance was calculated at 725 nm. All tests were executed in triplicate. The phenolic contents were determined from a gallic acid standard curve.

DPPH Radical Scavenging Assay

The antioxidant activity of various sub-fractions was assessed by measurement of

scavenging ability of free radical 2, 2'- diphenyl-1-picryl hydrazyl. A 90 µl of 0.3 mM DPPH solution (in ethanol) was added to 10 µL of each of the sub-fraction and the mixture was incubated for 30 min at 37˚C.The absorbance was then measured at 517 by microtitre plate reader (Spectra max plus 384 Molecular devices USA). BHA was used as positive control.

% DPPH scavenging activity = *Ac-As/ Ac x 100*

where; *Ac = Absorbance of control (DMSO treated); As = Absorbance of sample*

Urease Inhibition Assay

Urease Inhibition activity was determined by reported method [27]. Briefly describing, to 80 µL phosphate buffer solution of pH 8.2, 10 µL Urease (Jack bean) solution was added followed by 10µL of relevant sub- fraction in concentration of 50µg 10/µL and incubated at 30°C for 15 min. Then 50 µL phenol reagent and 70 µL of alkali reagent were added. Increase in absorbance was measured after 50 min at 630 nm on microtitre plate reader (Spectramax Plus 384 Molecular Device, USA). All reactions were performed in triplicates. The standard inhibitor used in this assay was thiourea and percentage inhibition was calculated by formula:

$$\% \text{ Inhibition} = \frac{\text{OD control} - \text{OD test comp}}{\text{OD control}} \times 100$$

Phytochemical Screening of Extracts

The protocol developed by Sofowora [28], Trease and Evans [29] and Harborne [30] was used to test the presence of tannins, alkaloids, terpenoids, saponins, reducing sugars, flavonoids, and glycosides in various sub-fractions.

Determination of Saponins

Each sub-fraction (0.1g) was stirred in a test tube, the resulting foaming which persisted on warming was indicative for the presence of saponins.

Determination of Tannins

Each sub-fraction (0.1g) was stirred with 20ml distilled water and filtered. To the filtrate two drops of 5% iron (III) chloride ($FeCl_3$) were added. Blue- black or blue- green coloration was an evidence for the presence of tannins.

Determination of Alkaloids

Each sub-fraction (0.1g) was stirred with 1% hydrochloric acid on a steam bath. After filtration the filtrate was divided into two portions.One of the portion(1mL) was treated with 3 drops of Mayer's reagent.The other portion was similarly treated with Dragendorff's reagent. Both the portions were mixed and made up to 100ml with distilled water.On mixing development of turbidity indicated the presence of alkaloids.

Determination of Glycosides

Each sub-fraction (0.1 g) was taken into two different beakers. Sulphuric acid (5 mL) was added to one of the beakers(Fraction A) while 5 mL of distilled water was added to another beaker(Fraction B). Both the beakers for heated for 4 min followed by filtration.Each filtrate was treated with 10% sodium hydroxide(o.5 mL) and filtered. Each of the filtrate was heated with Fehling's solution for 3 min.The appearance of reddish brown precipitate in Fraction A and absence of such precipitates in Fraction B revealed the presence of glycosides.

Determination of Flavonoids

To each sub-fraction small pieces of magnesium ribbon were added, followed by dropwise addition of conc. HCl.Colours ranging from crimson to magenta indicated flavonones, orange to red indicated flavones, red to crimson indicated flavonols.

Determination of Terpenoids (Salkowski test)

Each sub-fraction(0.1 g) was mixed with 1 mL of chloroform and conc. H_2S0_4 (2 mL) was carefully added to form a layer. A reddish brown colouration of the interface revealed the presence of terpenoids.

Determination of Reducing sugars

Each sub-fraction was shaken with distilled water and filtered. The filtrate was boiled with drops of Fehling's solutions A and B for 3 min. An orange red coloration indicated the presence of reducing sugars.

Conclusion

The present study revealed that the ethyl acetate sub-fraction contains maximum concentration of phenolic and flavonoidal constituents. It therefore, showed potent antioxidant potential with an IC_{50} value of 110 µg/ mL. It also revealed significant urease inhibitory activity. This fraction can be used either as such for the treatment of various ailments after clinical trials, or may be subjected to further isolation studies to obtain pure bioactive secondary metabolites of potential therapeutic utility.

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