The antioxidant, general toxicity and insecticidal activities of *Nepeta scrophularioides* Rech. f. extracts in different developmental stages

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Abstract: The essence of the present study is to focus on the antioxidant, general toxicity and insecticidal properties of the extracts of *Nepeta scrophularioides* Rech.f. during different developmental (vegetative, flowering, post-flowering) stages. The samples were subjected to screening for their possible antioxidant activities by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The extractsof the flowering and the post-flowering stages showed higher antioxidant activity than those from the vegetative stage. The MeOH extracts of *N. scrophularioides* in different development stages were tested for cytotoxicity by brine shrimp toxicity assay. The result obtained for the bio assay was found to be significantly lethality. Among the samples, the extracts of flowering stage were found to be the most active with a LC_{50} value of 0.078 mg/mL. All three extracts showed significant insecticidal activity at the concentration of 20 mg/mL dose of test sample after 24 h. The extracts of vegetative and post-flowering were the most potent samples.

Keywords: Antioxidant; general toxicity; insecticidal; *Nepeta scrophularioides*.

INTRODUCTION

The genus *Nepeta* (Lamiaceae) contains more than 280 species that are distributed over a large part of the worlds specially in central and southern Europe, and west, central and southern Asia (Tepe*et al.,* 2007). The Iranian flora comprises from 67 species of *Nepeta*. In many countries *Nepeta* species are used as traditional medicine (Ghannadi*et al.,* 2003). *Nepeta scrophularioides* Rech. f. is an endemic species in Iran that has a biological and pharmacological properties and used as a medicinal plant. It was found that plant foods and products posses variety of biologically active compounds and they contain different kinds of biological activity including antioxidant, allelopathic, antimicrobial and bio-regulatory properties (Bakkali *et al.,* 2008).

Free radicals oxidize the bimolecular (e.g., lipid protein, amino acids, and DNA) that injured cells and death them (Zhang *et al.,* 2006). Antioxidants, may be inhibit or delay the oxidation of oxidizable substrate in a chain reaction. So, It is clear to be very key role in prevention of some diseases (Tepe *et al.,* 2007). It has been known that most of natural substances occurring in higher plants have antioxidant activities. Natural antioxidants are not only interesting for consumers and used a lot as food additive (Neffati *et al.,* 2009; Wannes *et al.,* 2010). They are also very important to good industry too (Zhang *et al.,* 2006).

The variation in bioactivities might be attributed to three major factors: genetic, developmental stages (ontogeny) (Msaada *et al.,* 2009b) and environmental factors (geographic position, growing region, climatic condition) (Msaada *et al.,* 2009b; Wannes *et al.,* 2010). These factors usually cause complex variation patterns in the phytochemical agents and their activities (Msaada *et al.,* 2009b).

The present study supports that brine shrimp bioassay is simple, reliable and convenient method for assessment of bioactivity of medicinal plants and extends the support for further research. *Artemia salina* can be used in a laboratory bioassay in order to determine the toxicity by the estimation of the medium lethality concentration LC_{50} (Meyer *et al.,* 1982). Which have been reported for a series of toxins and plant extracts (Lewan *et al.,* 1992).

The bioactivity of extract of *Nepeta scrophularioides* Rech. f. has not been explored yet. The purpose of this study is to investigate the antioxidant, general toxicity and insecticidal properties of *Nepeta scrophularioides* extract in the different phonological stages.

MATERIALS AND METHODS

Plant material and extraction procedure

The plant material (aerial parts) used for the present study was collected during the vegetative (June), the flowering (July) and the post-flowering (August 2013) phases from "Mishow mountains" in eastern Azerbaijan in Iran at an altitude of 1800 m. The identity of the plants was confirmed by anatomical examination in comparison with the herbarium specimens (Voucher nos. Tbz-FPh 703) retained in the school of pharmacy, Tabriz University of Medical Science, Iran. The dried and ground aerial parts of *N. scrophularioides* (100 g) were Soxhlet-extracted, successively, with methanol (MeOH) (1.2 L each). This

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extract were concentrated using a rotary evaporator (IKA/ RE300 model) at a maximum temperature of 45°C.

DPPH· free radical-scavenging assay

The hydrogen atoms or electrons donation ability of the essential oils were determined spectrophotometrically by bleaching of purple coloured methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) as a reagent (Takao *et al.,* 1994). DPPH, molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Sigma-Aldrich (Germany). DPPH (4 mg) was dissolved in $CHCl_3$ (50 mL) to obtain a concentration of 80 µg/mL.

Qualitative assay

The extracts of vegetative, flowering and post flowering stages were applied on a TLC plate and sprayed with DPPH solution, using an atomizer. It was allowed to develop for 30 min. The colour changes (purple to yellow) were noted.

Quantitative assay

The extracts of plants were dissolved in MeOH to obtain a concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0039, 0.0019, 0.0009, 0.00048 mg/mL. Diluted solutions were mixed with DPPH (1 mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance of samples and blank (without sample) was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the standard (Quercetin) from Sigma-Aldrich (Germany). Percent inhibition of the free radical DPPH (I%) was calculated in the following way: I%=[(AB-AA)/AB]×100, where AB: absorbance of blank; AA: absorbance of test samples. Concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against test sample concentrations (Takao *et al.,* 1994).

Brine shrimp lethality assay

The method of Meyer *et al.* (1982) was adopted to study the general toxicity of the extracts. The brine shrimp eggs (was purchased from Biology laboratory of Tabriz university) were hatched in a conical flask containing brine shrimp medium (300 mL), the flasks were well aerated with the aid of an air pump (Apparatus) and kept in a water bath at 29-30°C, a bright light was left on, and the nauplii hatched within 48 h. The stock solution of each extract (5 mg/mL) was serially diluted ten-times, solution of each concentration (1 mL) was transferred into clean sterile universal vials with a pipette, and aerated seawater (34g sea salt/1 L distilled water) (9 mL) was added. About 10 nauplii were transferred into each vial with a pipette. A check count was performed. The number alive after 24 h was noted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 s of observation. The experiment was carried out in triplicate and the average values were noted. The controls used were DMSO (Sigma-Aldrich), normal saline, and podophyllotoxin (3 µg/mL). Abbots formula was used to correct the values, i.e., *P=Pi-C/1-C*, where *Pi* is observed mortality, *P* denotes the observed non-zero mortality rate and *C* represents the mortality rate of the DMSO control.

Insecticidal assay

Adults of *Acanthoscelides obtectus* were collected from a laboratory culture. *Acanthoscelides obtectus* was reared in glass containers containing 0.5 kg of the bean. All insect species were reared at 27±2°C, 12% moisture content in continuous darkness for about 3 weeks without exposing to insecticides. Adults used in the experiments were 1-3 weeks old and of mixed sex.

Different fractions of methanolic extract were dissolved in MeOH to obtain concentrations of 20, 10 and 5 mg/mL. The filter paper (9 cm of diameter) received 1 mL of these extracts, and was placed on a Petri dish (9 cm of diameter). The control was treated with pure methanol. After the solvent evaporation, 10 adults of *Acanthoscelides obtectus* were placed in each Petri dish, maintained at 27±0.5°C, 12% moisture content and a 12 h photo phase. The experimental design was completely randomized, with three replicates. Insect mortality was evaluated after 4, 8, 24 and 48 h of exposure to impregnated filter paper (Sigma). The procedure used was that described by Loschiavo *et al.* (1963) and modified by Freedman *et al.* (1982). Beetle responses to treated discs versus control discs were converted to "percentage of mortality".

STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Analysis of variance was performed by ANOVA procedures (SPSS 16.0 for Windows). The significant differences between individual means were determined by Tukey post hoc tests ρ values inferior to 0.05 were regarded as significant.

RESULTS

Antioxidant activity

The model system of scavenging DPPH free radicals is a sample method to evaluate the antioxidant activity of antioxidants. DPPH is a stable free radical which can readily experience reduction in the presence of an antioxidant (Neffati *et al.,* 2009). This model is based on the ability of constituents present in plant extract to donate an electron to a free DPPH· radical and the reaction may be followed by the colour changes (Kraujalis *et al.,* 2011). The reduction ability of DPPH radicals formation was determined by the decrease in its absorbance at 517nm induced by antioxidants (Bounatirou *et al.,* 2007).

In the qualitative DPPH assay, all of the MeOH extracts showed low levels of anti oxidant property evident from faint with spots against a pink background on the TLC plate. In the stable free radical form DPPH is purple and when in contact with anti oxidant compounds, it becomes yellow. This resulting decolourisation is stoichiometric with respect to the concentration of anti oxidant (Choi *et al.,* 2002).

In the quantitative assay (table 1), the extracts of the flowering and the post – flowering stages showed higher activity than those of the vegetative stage statistically significant differences ($\rho<0.05$). Quercetin is known as a natural free-radical scavenger, was used as a positive control. The DPPH radical-scavenging activities of extracts were lower than that of quercitin. The IC_{50} value of the MeOH extracts were determined to be 0.132±0.011 mg/mL (vegetative stage), 0.062±0.008 mg/mL(flowering stages) and 0.097±0.014 mg/mL (post-flowering stage) in contrast of 0.0037±0.0005 mg/mL for quercetin. Among the samples, the MeOH extract of flowering stage was found to be the most active, with a IC_{50} value of 0.062±0.018 mg/mL that exhibited low reducing power, whereas the vegetative oil sample was showed the lowest DPPH radical – scavenging value and the highest reducing power (table 1).

General toxicity

The brine shrimp lethality bio assay represents a rapid, inexpensive and simple bio assay for testing plant extract's bio activity which in most cases correlates reasonably well with cytotoxic and anti tumor properties (Hasan *et al.,* 2006). This assay is considered a useful tool for preliminary assessment of toxicity and for the isolation of bioactive compounds from plant extracts. After enumerating the number of shrimps surviving after 24 h the percentage inhibition was evaluated. All samples show positive results in this assay, but not necessary all extracts or compounds that show a positive result in this assay are cytotoxic. And LC_{50} value of < 1 mg/mL is considered to be significant, and the lower the value the higher is the toxicity of the test sample.

The MeOH extracts of *N. scrophularioides* in different development stages were tested for cytotoxicity by brine shrimp toxicity assay. All three extracts of *N. scrophularioides* showed high level of toxicity toward brine shrimp (LC_{50} value were in the range of 0.078 to 0.505 mg/mL). High levels of toxicity were observed with the extracts of flowering and post flowering stages with a LC_{50} value of 0.078± 0.008 and 0.156± 0.011 mg/mL respectively (table 1).

Table 1: Antioxidant activity and general toxicity of *N. scrophularioides* MeOH extracts during different developmental stages.

Assay	mg/mL			
	Vegetative stage	Flowering stage	Post-flowering stage	Quercetin podophyllotoxin
Antioxidant activity (IC_{50})	a	c	b	d
	0.132± 0.011	0.062± 0.008	0.097± 0.014	0.0037± 0.0005
General toxicity (LC_{50})	a	b	c	d
	0.505± 0.019	0.078± 0.008	0.156± 0.011	0.0027± 0.0008

Values with letters (a - d) are significantly different at $\rho<0.05$.

Table 2: Percent mortality of MeOH extracts of *N. scrophularioides* at concentration of 5, 10 and 20 mg/mL after 4, 8, 24 and 48 h.

Hours	Conc. mg/mL	MeOH extracts			Control
		Vegetative	Flowering	Post-flowering	
		% mortality			
4	5	-	-	-	-
	10	-	-	-	-
	20	63	43	36	-
8	5	-	-	-	-
	10	26	-	-	-
	20	87	71	77	-
24	5	16	-	-	-
	10	43	-	-	-
	20	100	80	100	-
48	5	20	13	-	-
	10	53	26	23	-
	20	100	80	100	-

Insecticidal Activity

This is the first report on the insecticidal property of the MeOH extract of *N. scrophularioides*. The MeOH extracts showed significant insecticidal activity in different developmental stages and it was concentration dependent (table 2). At the concentration of 20 mg/mL, all of samples were very toxic after 24 h. However, no significant insecticidal activity was observed at 10 and 5 mg/mL dose of test sample. Among the samples, the highest percent mortality was observed in vegetative stage. At the concentration of 20 mg/mL, the percent mortality rates were %100, %100 and %80 after 24 h in vegetative, post-flowering and flowering respectively (table 2).

DISCUSSION

Antioxidant activity

The evaluation of the antioxidant activity showed that exracts were relatively low antioxidants. This might be attributed to major sesquiterpenes components in extracts composition. The studies established *Nepeta* species contain mono terpenssesquiterpens, syclopentanoidiridoids derivatives and Nepetalactones (Cigremis *et al.,* 2010). The our previously researches on phytochemical of *N. scrophularioides* essential oil established that sesquiterpenes components (%63,%81 and 78% for vegetative, post-flowering and flowering respectively) were the most representative group (Javazi, 2012). Also, the evaluation of the antioxidant activity showed that essential oils were relatively poor antioxidants (Javazi, 2012).These results are in agreement with Bounatirou *et al.* (2007); Neffati*et al.* (2009) and Zhang *et al.* (2006) studies on the oil composition contained major sesquiterpenes components showed low antioxidants activity. This is also in agreement with the finding of Kraujulis *et al.* (2011) where it was reported that extracts of *Nepeta* species possessed medium antioxidant power.

All extracts showed antioxidant activity, but statistically significant differences were observed in comparison to the developmental stage criteria. During the maturity of plant, there were changes in compounds. These changes could be as a result of chemical modifications in terpenes in effect of temperature and light – initiated oxidative process (Bounatirou *et al.,* 2007). The antioxidant and other biological activities may vary, based on the variations in the chemical composition (Msaada *et al.,* 2009a, 2009b).

General toxicity

The result obtained for the bio assay was found to be significantly lethality. The studies on brine shrimp lethality bioassay of *N. scrophularioides* essential oil exhibited significant general toxicity, also (Javazi, 2012). The significant lethality of brine shrimp due to extracts of *N. scrophularioides* is an indicative of the presence of potent cytotoxic components which warrants further investigation.

Insecticidal activity

The MeOH extracts showed significant insecticidal activity in different developmental stages and it was concentration dependent (table 2). As known, the extracts of the genus *Nepeta* plants compose of phenolic compounds, especially terpenoids and phenolic acids (Cigremis *et al.,* 2010). Based on the literature data, terpenoid compounds especially monoterpenoids and sesquiterpenoids might be responsible for the insecticidal activity (Afshar *et al.,* 2011; Takao *et al.,* 1994). The studies on insecticidal activity of *N. scrophularioides* essential oil against of *Oryzeaphillus mercator* showed high mortyality as the dose of essential oils and exposure period increased (Javazi, 2012).

CONCLUSION

The evaluation of bioactivity of *Nepeta scrophularioides* indicated that MeOH extract were relatively poor antioxidants. It might be resulted from the presence of sesquiterpenes components in essential oils composition. Whereas, all three extracts showed significant general toxicity and insecticidal properties. In conclusion, the MeOH extract of *N. scrophularioides* during phenological stages have shown great potential as botanical pesticides. This is the first study of its kind that establishes the medicinal value of *N. scrophularioides* on scientific basis. It also provides the basis for further analysis and purification of the compounds active against pests to be exploited in the development of alternative pesticides.

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