Antioxidant effect and study of bioactive components of *Valeriana sisymbriifolia* and *Nardostachys jatamansii* in comparison to *Valeriana officinalis*

Mehdi Ansari Dugaheh[1], Faramarz Meisami[1], Zahra Torabian[1] and Fariba Sharififar[2*]
[1]Research center of Pharmaceutics, Department of Pharmaceutics, Kerman University of Medical Sciences, Kerman, Iran
[2]Herbal and Traditional Medicines Research Center, Department of Pharmacognosy, Kerman University of Medical Sciences, School of Pharmacy, Kerman, Iran

Abstract: The roots of *Nardostachys jatamansi* have been used as a substitute for valerian in Iranian traditions. Moreover, six species from *Valeriana* genus such as *V. sisymbriifolia* grow in Iran which has not been studied yet. We aimed to study of antioxidant effect of *Valeriana officinalis*, *Nardostachys jatamansi* and *Valeriana sisymbriifolia* and comparing their content of valerenic acid and valepotriate. Antioxidant effect was evaluated using diphenylpicrylhydrazyl (DPPH) inhibition and beta carotene-bleaching assays. Identification of valepotriates was achieved using chemical and TLC method. Qualitative and quantitative analysis of valerenic acid was performed using TLC and spectrophotometry methods. Among the tested samples, *V. Officinalis* showed the highest DPPH inhibition effect with IC_{50} value of 38mg/mL. All of the tested plants potentially inhibited beta-carotene oxidation. The calibration curve of authentic valerenic acid was linear in the range of 2-51 mg L^{-1}. The most and least amount of valepotraites was detectable in *V. officinalis* and *V. sisymbriifolia* respectively. Total valerenic acid in different plant species ranged from 0.02% in *V. sisymbriifolia* to 0.07% (w/w) in *V. Officinalis.* Our results indicated that all three tested plants contain different amount of valepotriates and valerenic acid. The highest percentage of valepotriates and valerenic acid was detectable in *V. officinalis.* Overall can conclude that *N. jatamansii* and *V. sisymbriifolia* would be a good candidate for substitutation of *V. officinalis* with noticeable antioxidant effect.

Keywords: *Nardostachys jatamansi*, phytochemistry, *Valeriana officinalis*, *Valeriana sisymbriifolia*, antioxidant.

INTRODUCTION

The genus of *Valeriana* is one of the most extensive investigated medicinal plants which belongs to Valerianaceae family and comprises about 230 species (Bos *et al.*, 2002). The roots and rhizomes of *V. officinalis* L. (valerian) have been used widely as an aid in promoting sleep, anxiolytic, antispasmodic and antiepileptic (Mills and Bone, 2000). The antidepressant, antihypertensive and anti broncho spastic effects of the plant have been reported (Miyasaka *et al*., 2006). Valerian contains various compounds including essential oil and its sesquiterpenoids (valerenic acid), epoxy iridoid esters (valepotriates) with remarkable pharmacologic activity, amino acids (GABA, tyrosine, arginine), alkaloids, phenolic acids and flavonoids (Upton *et al*., 1999). The compounds responsible for the pharmacologic activities of the plant have not been completely identified; however two main groups of valerenic acids and valepotriates would be more responsible for the plant activity. Hydrophilic valerenic acids are from sesquiterpenoids whereas hydrophobic valepotriates are from monoterpenoids (Gao and Björk, 2000). At least six species from *Valeriana* grow in mountainous regions of Iran (Moussavi-Allashlou, 2000) which have been studied fewer. *V. sisymbriifolia* is one of these species which grows in Kerman province, southeastern of Iran without any known medicinal uses. Gao and Björk (2000) reported the mean content of valepotriates in more than 25 species and varieties of *Valeriana* which ranged from 0.03 to 1.81 mg/g dry plant (Gao and Björk, 2000). In another study, the content of isovaltrate in *V. Mexicana, V. officinalis*, and *V. wallichii* has been determined 4.39, 0.528 and 0.095mg/g dry plant respectively (Hazelhoff *et al*., 1979). However determination of bioactive compounds of this species may be useful for finding new medicinal sources.

Nardostachys jatamansi Jones DC, belongs to Valerianaceae family which has been suggested in traditional medicine as sedative, anticonvulsant, anti cholinesterase and analgesic (Ahmad *et al.*, 2006; Ahmed *et al*., 2009). This plant is widely used as an antioxidant, protective in cerebral ischemia and tranquilizer (Saleem *et al*., 2003; Lyle *et al*., 2009). The plant is similar to *V. officinalis* and has been sold in Iranian traditional herbal markets (Attary) as a substitute for valerian. At the present study, the antioxidant and free radical scavenging effects of these three medicinal plants have been compared to butylated hydroxytoluene (BHT). Although the antioxidant effect *of V. officinalis* and *N. jatamansii* have been reported in preceding studies (Fiyaz *et al*., 2009; Sudati *et al*., 2009), it is for the first time this effect has been studied for *V. Sisymbriifolia*. Moreover, the

**Corresponding author*: e-mail: fsharififar@kmu.ac.ir

qualitative analysis of valepotriates and valerenic acids and quantitative analysis of valerenic acid in *V. sisymbriifolia* and three samples of *Nardostachys jatamansi* (a, b, c) have been compared to *V. officinalis*.

MATERIALS AND METHODS

Plant materials

V. officinalis was prepared from Soha Gisa Co., Iran and *V. sisymbriifolia* was gathered from the Hezar mountains, Kerman province in August 2008. The roots and rhizomes were separated and dried in shade. Three samples from *N. jatamansi* were prepared from Attary randomly. The dried plants were milled and stored in -20°C until testing.

Chemicals

Valerenic acid was purchased from Fluka. Analytical TLC plates were prepared from Merck (Germany). All solvents and the other chemicals were from analytical grade.

Antioxidant activities

DPPH scavenging activity assay

For evaluating the free radical scavenging of the plant samples, DPPH assay test was used in triplicate as described by Burits and Bucar, 2000. 30 µL of plant extract was added to 3 mL of a 0.004% methanol solution of DPPH and sonicated for 2 minutes in 25 °C. After 30 minutes incubation in room temperature, absorbance was measured at 517nm (burits and Bucar, 2000). Inhibition of DPPH free radical in percent (I %) was calculated in following way:

$I\% = (A_{blank} - A_{sample} / A_{blank}) * 100$.

Beta-carotene bleaching experiment

50 µL of each extract (10 mg/mL) was spotted on silica gel GF_{254} coated paltes (Merck, Germany) and was sprayed with reagent beta-carotene (0.1 mL linoleic acid added to 9 mg beta-carotene in 30 mL chloroform) to give an orange color background. The plates were kept at room temperature. The samples with antioxidant activity can prevent from plate discoloration. The color zone diameter of each sample was measured in comparison to BHT. The more color zone diameter indicates the more antioxidant effect (Mehta *et al*., 1994).

Microscopic analysis of plants

The microscopic identification of the plants was done using microscope (Olympus, Japan). 1-2 drop of chloral hydrate was mixed with plant powders for discoloration, heated on alcohol flame and the lam was studied by microscope.

Chemical detection of valepotriates in the plant sample

For the detection of valepotriates, 200mg of each plant sample was added to a test tube containing 5 mL of dichloromethane and was shaken for one min. After standing in the room temperature for 5 min., the extract was filtered. The filter was washed with 2 mL of the same solvent and added to filtrate. The filtrate was concentrated on water bath at 30°C to dryness and then was re-dissolved in 0.2 mL methanol. 0.1 mL of the sample was added to a mixture of acetic acid-HCl (1:1). After 15 min, the appearance of deep blue color indicated the presence of valepotriates (Petrone and Swisher, 1999).

TLC of valepotriates

TLC has been used extensively for qualitative analysis of valepotritaes as described by Gao and Björk (2000). 200mg of each plant samples was extracted with 5 mL dichloromethane. The mixture was shaken for two min., filtered and the filter also was washed with 2mL of dichloromethane. The solvent was evaporated on water bath to dryness and then was re-dissolved in 2 mL methanol.10 micro liter of the sample was spotted on a silica gel GF_{254} coated plates. The plates were developed in toluene- ethyl acetate (2.5:7.5) as mobile phase. After drying the plate, the spots were observed under UV light at 254 nm. The plates were also sprayed with the reagent of 2,4 dinitropheylhydrazine for revealing the valepotriates. After the spraying of reagent, the plate was heated at 110°C for 5-10 min (Bos *et al*., 2002).

TLC of valerenic acid

200mg of plant sample in a test tube was added to 5 mL dichloromethane and was shook for 1 min., after 5 min. standing at room temperature was filtered and the filter was washed with the same solvent. The filtrate was concentrated on water bath, re-dissolved in 2mL methanol and 10 micro liter of sample was spotted on plate silica gel GF_{254} and developed in hexane-ethyl acetate-glacial acetic acid (0.5:35:65) as a mobile phase. Valerenic acid was used as reference (2µg/mL). After drying, the plates were detected under UV light at 365nm. For the revealing the plate, it was sprayed with HCl-acetic acid (1:4) reagent and heated at 110°C for 5 min. The spots were observed in visible light (Petrone and Swisher, 1999).

Spectrophotometric method for determination of valerenic acid

Sample preparation

200mg of each plant sample was extracted with 5 mL dichloromethane and was shaken for 2 min. After 5 min standing at room temperature, the extracts were filtered. The filtrate was completely dried under N_2.1 mL dichloromethane was added to the sample and was shaken for 30 seconds in a shaker. 2 mL of NaOH, 2% was added, upper phase was separated and its pH was adjusted to 2 with HCl 25%. 1 mL of petroleum ether-diethyl ether (1:2) was added and upper phase was separated again and dried under N_2. Valerenic acid (30µg/mL) was used as positive control (Petrone and Swisher, 1999).

Calibration curve of valerenic acid

A solution of 200µg/mL of valerenic acid in methanol was prepared as stock solution and 5 different

concentrations were prepared in the range of 5-30 µg/mL. The absorbance of each sample was measured by spectrophotometer (Lambda 25, America) at 225nm in triplicate and the average of results was recorded.

Determination of valerenic acid content by spectrophotometry

Sample preparation

200 mg of each plant sample, 200µL distilled water and 200 µL from standard solution of valerenic acid (30µg/mL) were added to the test tubes and were extracted with 5 mL DCM by continuous shaking for 1 min. The samples were hold in room temperature for 5 min, filtered and completely dried under N_2. 1 mL DCM was added to each sample, was shaken for 3sec, 2 mL NaOH 2% was added and the upper phase was separated and pH was adjusted to 2 with HCl 25%. 1 mL petroleum ether- diethyl ether (1:2) was added; upper phase was separated and completely dried under N_2. After adding 2 mL methanol to dried samples, their spectrum were recorded at 200-400nm. The absorbance was measured at 225nm. The determination of total valerenic acid concentration was calculated from the calibration curve of valerenic acid (Bounthanh *et al*., 1982).

STATISTICAL ANALYSIS

The results were reported as Mean ± SD of three separated determinations and were analyzed by one-way ANOVA. The multiple comparisons between different samples were made using Post hoc of Tukey´s test. Differences with $p<0.05$ considered significant.

RESULTS

The results of DPPH assay and Beta-carotene bleaching

The obtained results show that the radical scavenging activity of tested plants was in a dose-dependent manner. The extract of *V. Officinalis* exhibited the highest percent of DPPH inhibition (72%) in comparison to *V. Sisymbriifolia* and *N. Jatamansii* (61% and 67% respectively) (fig. 1). Among the tested plants, the most inhibition was exhibited by *V. Officinali* (IC_{50}= 38mg/mL).

All of the tested plants inhibited the beta carotene oxidation effectively. This activity was comparable to BHT. The color zone diameter was 3.4, 3.5, 3.3 and 3.5 for *V. officinalis, V. sisymbriifolia, N. jatamansii* and BHT respectively.

The results of microscopic analysis of the plant

The results of microscopic analysis of *V. officinalis* show some differences with the other tested plants, The most findings show the presence of parenchyma cells with starch granules and endodermis of root with sinuous tangential walls in *V. officinalis* which were absent in the samples of *V. sisymbriifolia* and *N. jatamansi.* The presence of cortical parenchyma cells with some brown contents was specific in *V. sisymbriifolia*. For *N. jatamansi*: narrow thick wall fibers and parenchyma cells were distinguishable.

The results of chemical detection of valepotriates

Under reaction with the reagents, *V. officinalis* and *V. sisymbriifolia* induced visible deep blue to purple colors

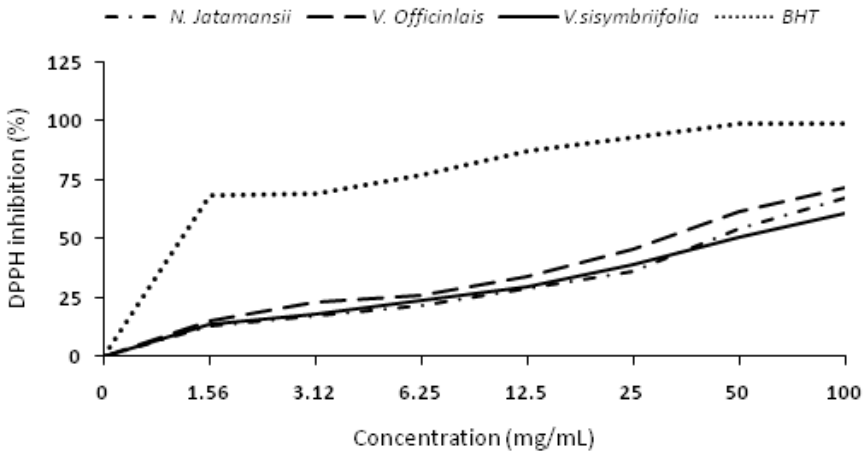

Fig. 1: Radical scavenging effect of three tested plants from Valerianaceae in comparison to BHT in DPPH assay.

while *N. jatamansi* showed pale blue color after three min.

The results of TLC of valepotriates

The results of TLC of valepotraites have shown in the fig. 2. As shown, 5 fractions were distinguishable in the *V. officinalis* and *V. sisymbriifolia* in toluene-ethyl acetate (2.5:7.5) as mobile system. Two of these spots were detectable in the three samples of *N. jatamansi* which corresponded to valtrate/isovaltrate and didrovaltrate. The information of compounds which corresponded to detected spots has given in table 1.

The results of TLC of valerenic acids

The result of TLC fingerprint of different plant samples for detecting of valerenic acids indicated strongly the presence of valerenic acid in all of the tested plants. The plants of *V. offiicnlais* and *V. sisymbriifolia* exhibited similar pattern of valerenic acids which appeared as spots in the R_f of 0.62, 0.48, 0.2 and 0.15 at 254nm. These spots corresponded to baldrinal, valerenic acid, acetoxy valerenic acid and hydroxyvalerenic acid respectively. The three samples from *N. jatamansi* only have shown the spots pertained to valerenic acid.

The results of quantitative determination of valerenic acid

The information for calibration curve of valerenic acid at 225nm are, regression equation, y = 0.0.032x-o.o53 (y is absorbance and x defines concentration) and the correlation coefficient (r) = 0.998. The results of determination of valerenic acid in various plant samples have given in the table 2. The highest and the least percentage of valerenic acid was due to *V. officinalis* and *V. sisymbriifolia* respectively (0.075% and 0.020% g/g).

DISCUSSION

Nowadays, the traditional extracts from medicinal plants have been replaced by standard extracts. The plant of *V. officinalis* is an herbaceous plant by limited distribution in Iran. *V. sisymbriifolia*, is an endemic species which grow in Hezar mountains of Kerman province of Iran without any known medicinal uses. At the present work, the antioxidant effect and the content of valepotriates and valerenic acids of three plants from Valerianaceae family has been evaluated. Our findings indicate that all of tested plants effectively scavenge the DPPH free radical. Additionally these plants potentially inhibited the beta-

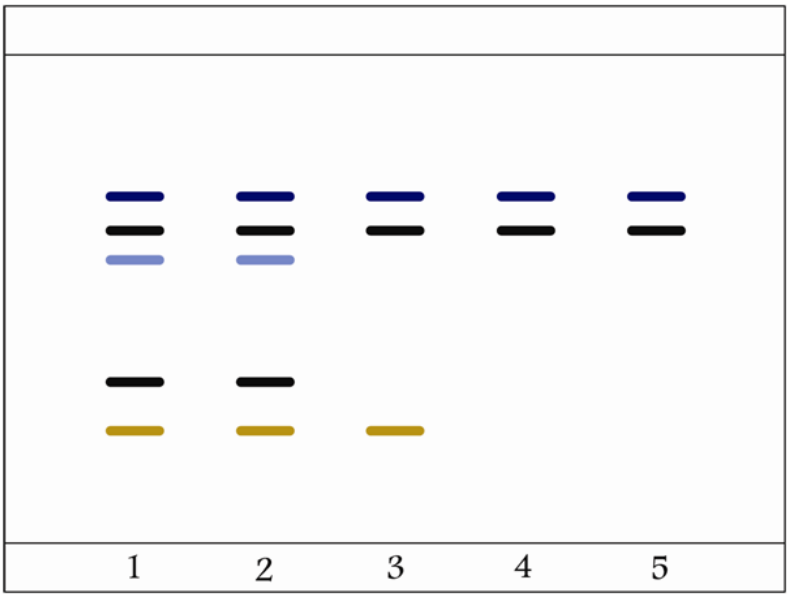

Fig. 2: The results of TLC of tested plants for valepotriate analysis.
1-*V. officinalis*, 2- *V. sisymbriifolia*, 3- *N. jatamansi* (sample a), 4- *N. jatamansi* (sample b), 5- *N. jatamansi* (sample c)

Table 1: The results of TLC of valepotriates of *V. officinalis* on silica gel GF_{254} in toluene-ethyl acetate (2.5:7.5)

No.	Compound	R_f	Color without reagent	Color with reagent	UV light (254nm)
1	Valtrate/isovaltarte	0.71	-	Deep blue	+
2	Didrovaltrate	0.64	-	Brown	-
3	Acevaltrate	0.58	-	Blue	+
4	Valtarte hydrin	0.33	-	Pale blue	-
5	Baldrinal	0.23	Yellow	Brown	+

Table 2: The results of quantitative analysis of valerenic acid in different plant samples at 225nm (g/100g dry plant)

Order	*V. officinalis*	*V. sisymbriifolia*	*N. jatamansi*, 1	*N. jatamansi*, 2	*N. jatamansi*, 3	Water	Valerenic acid
1	0.068	0.023	0.000	0.011	0.029	0.005	0.006
2	0.088	0.024	0.050	0.013	0.024	-0.009	0.007
3	0.083	0.007	0.038	0.024	0.026	0.000	0.005
4	0.062	0.026	0.036	0.023	0.045	0.005	0.006
Mean	0.075*	0.020	0.031	0.018	0.031	0.000	0.006
SD	0.014	0.010	0.007	0.006	0.011	0.008	0.000

*Significantly different from the other sample ($p<0.05$)

carotene oxidation. It is for the first time this effect has been studied for *V. Sisymbriifolia*.

Two main groups of valepotriates and valerenic acids were determined as biomarkers for standard extract of valerian (Bounthanh *et al*., 1982). The results of TLC of valepotriate indicated that the content and composition of these compounds are the same in *V. officinalis* and *V. sisymbriifolia*, but with some differences in three samples of *N. jatamansi*. These differences could be attributed to the process of plant preparation. Both of *V. officinalis* and *V. sisymbriifolia* were prepared from natural sources freshly, dried in suit condition and then stored in freezer until testing, whereas *N. jatamansi* is imported from India. Therefore its valepotriates which are unstable in the various conditions of temperature and humidity might be decomposed. Due to the cytotoxicity and mutagenicity of valepotriates and the DNA synthesis inhibition (Bounthanh *et al*., 1982; Gao and Björk, 2000), the low content of valepotriate may be desired.

The pattern of TLC of valerenic acids showed that valerenic acid is the main compound of valerenic acids. The comparison of tested plants exhibited similarity in valerenic acids pattern in two *Valeriana* species (*V. Officinalis* and *V. Sisymbriifolia*), but completely different from *N. jatamansi*. Total valerenic acid was measured quantitatively by plotting its calibration curve at 225nm at the range of 5-30 µg/mL. Each sample was measured three times and the results were presented as Mean ± SD. The regression equation and correlation coefficient of valerenic acid was determined. The spectrum of standard solutions of valerenic acid was taken at this wavelength. The highest and the least percentage of valerenic acid was due to *V. officinalis* and *V. sisymbriifolia* with 0.075% and 0.020 % valerenic acid respectively. The percentage of valerenic acid in three samples of *N. jatamansi* varied about 0.018 to 0.031%. Powdered valerian contains at least 0.04% valerenic acid in USP (USP 300). Ebrahimzadeh *et al*., (2008) reported the content of valerenic acid in different organs of *V. sisymbriifolia* from Mazandaran province (Ebrahimzadeh *et al*., 2008). As this research, the most percentage of valerenic acid was reported in the roots (0.036-0.041%) and rhizomes (0.038-0.051). The more percentage of valerenic acid in *V. sisymbriifolia* from Mazandaran in comparison to one in sample from Kerman might be due to the environmental condition such as temperature, sunny hours per day and especially rainy climate of Mazandaran which completely differs in these two regions. Our findings also indicated that among the tested samples, *V. officinalis* contains significantly the highest amount of valerenic acid ($p<0.05$), while the other samples exhibited no significant difference in valerenic acid content ($p>0.05$).

Finally our results show that *V. sisymbriifolia* and the other species of *Valeriana* and also *N, jatamansii* would be a good alternative for *V. officinalis*, especially considering the antioxidant activity of these plant species, however further studies are needed.

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