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Original Article

Comparing the Antioxidant and Cytotoxicity Potentials of Some Medicinal Plants of Lamiaceae Family

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ABSTRACT

The research investigated the antioxidant and cytotoxicity potentials of four medicinal plants from the Lamiaceae family, including *Marrubium vulgare* L., *Vitex agnus-castus* L., *Phlomis olivieri* Benth., and *Nepeta ucranica* L. The study evaluated the extracts' cytotoxicity by analyzing the mitotic index in onion root tip meristematic regions and cell viability in green algae *Dunaliella salina*. The findings indicated that *P. olivieri* and *V. agnus-castus* exhibited more significant antioxidant potential, but compared to the other, *N. ucranica* showed the lowest potential. The mitotic indices in the onion root tip's meristematic region and *vulgar* aqueous extract decreased with some increase in the extract concentrations of all studied plants. Additionally, the number of cells in various mitotic phases decreased, whatever cells in interphase increased in the treatment, and *D. salina* experienced a decrease in the number of cells with an increase in extract concentration. The study also observed a decline in the number of cells in the algal suspension with growth in extract concentration, particularly when exposed to *V. agnus-castus* extract. However, medicinal plants with high antioxidant potential are used in prevention, and various treatments with caution are advised due to their observed cytotoxic effects.

INTRODUCTION

As a valuable medicinal plant, the Lamiaceae family includes several notable species, including *Phlomis olivieri*, a flowering species and a member of the mint family (Lamiaceae) indigenous to Iran. In Flora Iranica, the genus Phlomis, consists of 17 species, which P. olivieri is unique to Iran, and even researchers have found compounds such as flavonoids, iridoids, and phenylpropanoid glycosides have been identified through phytochemical studies on different species of this genus (Sarkhil *et al*., 2003). *Marrubium vulgare*, another remarkable plant from this family, is native to Europe, North Africa, and Southwest Central Asia. This perennial plant with gray leaves typically grows at a height of 25-45 cm which contains a diverse exhibition of polyphenols and flavonoids, along with compounds like apigenin, ursolic acid, beta-sitosterol, lithospermon, pectin, and ascorbic acid (Acimovic *et al*., 2020). Moreover, the perennial plant of *Nepata ucranica* contains 225 species and a flowering plant from the mint family can reach a height of 60 cm (Sharma et al., 2021). Another member of the mint family is *Vitex agnus-castus*, a flowering plant with the ability to expand in areas with saline water, can grow to a height of 1 to 5 m, and its fruit contains a combination of iridoids, flavonoids, and compounds akin to sex hormones (Schellenberg, 2001).

The green alga *Dunaliella salina* is a unicellular organism first observed by Michel Felix Dunal in salt production ponds in southern France in 1838 and was officially named by Teodoresco in 1905. One application of biotechnology involves using saline environments to produce beta-carotene (Oren, 2005). The onion (Allium cepa L.) is diploid and possesses 16 chromosomes (Trushin *et al*., 2013) which is one of the most suitable plants for detecting effective toxic substances in plant genetics. The root meristem contains a high ratio of actively dividing cells (Leme and Marin-Marales, 2009). The purpose of this research was to determine the antioxidant potential of the medicinal plant extract of the Lamiaceae family, analyze its total reduction potential, determine its cytotoxic potentials by using the alga *D. salina*, check and

determine the mitotic index of edible onion plant root cells.

MATERIAL AND METHODS

While *V. agnus-castus* plant was acquired from Fasā Research Station in Fars Province during its flowering period, the plants of *M. vulgare*, *P. olivieri*, and *N. ucranica* were gathered from Shiraz Hasan Ābād Research Center during their flowering phase. The samples were authenticated in collaboration with the Herbarium of Shiraz University and the Agricultural Research Center of Fars Province. Then, the amount of each sample of the plants cleaned, dried in the shade, and stored in a freezer was ground into powder and utilized accordingly. The antioxidant potential was assessed using the DPPH method, following the protocol established by Brand-Williams et al. (Brand et al., 1995). Instead of a microplate reader method for determining antioxidant potential, a spectrophotometric device was utilized in the present study. To prepare a 0.4 mM DPPH solution in methanol, 1.5 mg of DPPH powder was first weighed and placed into an Erlenmeyer flask with an aluminum cover, and then 10 ml 99% methanol was added to the flask. The absorbance of the prepared DPPH solution at a wavelength of 515 nm was measured at 2.2 ± 0.05. The experiment was conducted in a 96-well plate with the following materials append to each well, except three wells. Initially, 80 µl 99% methanol was added to each well, followed by 0, 2, 4, 6, 8, 10, and 12 µl of the respective plant extracts to different wells. Afterward, the total volume was arrived at 120 µl in each well by adding varying amounts of methanol, and then 100 µl of the 0.4 mM DPPH solution was append to all wells. Additionally, 100 µl 99% methanol and 100 µl of the 0.4 mM DPPH solution were added to 3 wells of the 96-well plate. The Polar Star Omega BMG LABTECH device manufactured in Germany was calibrated using methanol at a wavelength of 515 nm in the experiment utilized. Subsequently, the absorptions of both the control and sample solutions were recorded at this specified wavelength. It is necessary to mention that at the onset of the experiments, the initial absorption of DPPH in the wells was approximately 1.1±0.05. Trolox suspension was used to construct the standard curve. Notably, volumes 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µl of a 1-mM Trolox blend were utilized instead of the extracts. Therefore, each experiment lasted 30 min and was repeated three times, and then the average absorption for each concentration was determined to plot the curves. The free radical inhibition percentage was computed using the following formula:

Radical inhibition percentage = [(sample absorption - control absorption) / control absorption] × 100

To determine the IC50, the absorption of DPPH in the presence of various concentrations of each extract was utilized to establish a linear equation.

The total reduction potential was assessed using the Folin-Ciocalteu method as performed by Ainsworth *et al*., 2007, with modifications in the current study. A 10% Folin reagent compound was ready by combining 1 ml of Folin reagent with 9 ml of distilled water. Also, sodium bicarbonate was obtained by dissolving 3.7 g of sodium bicarbonate powder in 50 ml of distilled water and stirring until clear. Furthermore, 2.5 mM gallic acid solution was gained by dissolving 4 mg of gallic acid powder in 10 ml of 95% methanol to construct the standard curve. The experimental procedure took place in a 96-well plate with the following materials added to each well: Initially, 36 µl of Folin reagent solution was dispensed followed by the addition of 0, 20, 40, 60, and 80 µl of the desired plant extracts to the Folin solution in each well. Later, its volume changed to 54 µl in each well by adding varying amounts of methanol. After 5 minutes, 146 µl of alkaline sodium bicarbonate solution was introduced to each well, bringing the total solution volume of all wells to 200 µl. To zero the ELISA reader at a wavelength of 765 nm, a solution containing 36 µl of Folin reagent, 146 µl of alkaline sodium bicarbonate solution, and methanol was utilized, resulting in a final volume of 200 µl. In this experiment, the previously described method was employed to construct the standard curve, utilizing 2.5 mM gallic acid solution in volumes of 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 µl instead of the plant extracts with a final volume of 200 µl. Experiments were conducted in triplicate, and the average absorption values were utilized to generate both the sample and standard curves for each concentration. Methylene blue dye, possessing alkaline properties, was utilized to stain the chromosomal content of edible onion root cells. The solution was prepared

by gradually adding 100 ml of 95% ethyl alcohol to 1.5 g of methylene blue powder, ensuring thorough stirring while adding ethyl alcohol. Hence, 30 ml of the methylene blue solution was combined with 100 ml of distilled water containing 1 ml of 10% potassium hydroxide solution. Following this, the solution was filtered and employed for chromosome staining.

When preparing medium-sized edible onions, their thin outer skins were removed. To initiate rooting, the onions were positioned in small glasses filled with water, ensuring that their ends were in contact with the water. Subsequently, the glasses were relocated to a dark environment. Within 2-3 days, the roots had achieved the desired growth of approximately 1.5 cm. Following this, some onions were transferred to glasses containing specific concentrations of the extracts, while others were retained in the same glasses with water, serving as the control group. After 24 hours, the extracts' effect was assessed on the mitotic index. Initially, the root tips were trimmed to a length of 3 mm and then immersed in a warm hydrochloric acid solution (70°C) for 1 min. The fixing process of the roots was performed simultaneously to denature the proteins in the cells of the root tips. Next, the roots were thoroughly rinsed with distilled water multiple times, and excess water was blotted from the roots using a tissue. At this phase, the root tips were stained by adding methylene blue to the fixed roots, and then the roots were left in the dye solution at room temperature for 1 min. Each stained root was placed and the cover glass delicately at an angle of 45° on the slide. While preventing the cover glass from slipping with the thumb, the end of a bayonet needle gently pressed on the cover glass to spread the stained root on the slide uniformly. A total of 1000 cells were counted to assess the impacts of the extracts on the stages of mitotic division and determine the percentage of the mitotic index, which this experiment conducted in 3 repetitions. The mitotic index was calculated by dividing the number of cells by the total number of cells multiplied by 100.

The algal culture medium was prepared according to the specifications outlined in Table 1 (Ben-Amotz, 1993). In the next step, the necessary compounds were added to 500 ml of distilled water and then brought to a final volume of 1 L after complete dissolution. Eventually, the culture pH medium was adjusted to 7.5 and was sterilized using an autoclave device for 20 min. Algal cultivation involved adding 1 ml of algal suspension to 30 ml of the culture medium. Cells were utilized to investigate the cytotoxic potentials of the medicinal plants once they reached the end of the logarithmic growth phase. At first, 100 µl of algal suspension containing approximately 10^5 cells were dispensed into each well of the 96-well plate.

In the next step, the various volumes (0, 10, 15, 20, 40, 60, and 100 µl) of extracts were added to the algal suspension. The new volume reached 200 µl by adding different amounts of the culture medium in each well. The 96-well plate was exposed to fluorescent light with an intensity of 2000 lux over 24 hours. At the end of the incubation period, amount of 10 µl of Lugol solution was added to each well to immobilize the algae.

Table1 Composition and their final concentration in one liter of *D. salina* algae culture medium

Names of used compounds	Final concentration of compounds in the growth medium	
$MnCl_2$	7	
EDTA	5	
$FeCl_3$	2	
$ZnCl^2$	1	Micromolar
$CuCl_2$	1	
$CoCl_2$	1	
$(NH_4)_6Mo\text{-}O_{24}$	1	
$NaHCO_3$	50	
$MgSO_4$	5	
KNO_3	0.75	Millimolar
KH_2PO_4	0.2	
$CaCl_2$	0.2	
NaCl	2	Molar

Careful pipetting was performed at this stage to ensure uniform distribution of the Lugol solution and cells. Subsequently, 40 µl of the culture medium was added to 10 µl of the algal suspension, and 10 µl of this mixture was transferred to the hemocytometer slide. The cytotoxic effects of the extracts on *D. salina*, the number of cells per milliliter of the initial suspension was determined using the following formula:

Number of algal cells per milliliter of the initial suspension = Total counted cells × (number of squares/dilution factor) × 10000

All data were analyzed using the SPSS 24 and Excel 2016 statistical programs with a two-way ANOVA test. Duncan's post hoc tests were employed at a significance level of α=0.05 to detect differences within and between groups.

RESULTS AND DISCUSSION

Antioxidant Potential and Total Reduction Potential

In the presence of the Trolox antioxidant, a reduction in the DPPH free radical led to a decrease in light absorption at the wavelength of 515 nm. As the Trolox concentration increased, the reduction in the DPPH radical and light absorption enhanced. Similarly, with increasing extract concentrations, the amount of light absorption decreased. This reduction in light absorption exhibited a linear relationship with the concentration of the extracts within the range of concentrations used. Examination of the slopes of the lines revealed that among the four studied medicinal plants, *P. olivieri* and *N. ucranica* exhibited the highest and lowest DPPH radical reduction potentials, respectively. The inhibition percentages of the DPPH free radical by different concentrations of the extracts are presented in Table 2. The results showed that the free radical inhibition percentage increased when the extract concentration enhanced. The most significant effect was associated with the medicinal plants of *P. olivieri* and *V. agnus-castus*, while the lowest outcome was related to the medicinal plant of *N. ucranica*.

The relationship between antioxidant potential and IC50 showed clear opposition. The *P. olivieri* extract exhibited the lowest IC50, requiring only 0.58 mg/ml to reduce 50% of DPPH free radical, while *N. ucranica* with the lowest antioxidant potential needed 2.15 mg/ml to achieve the same effect. A statistically significant difference was observed between the antioxidant potential and IC50 values of plants studied at the significance level of α=0.05. The phenolic compounds amount was measured in mg of gallic acid equivalent per gram of the extract dry weight. A statistically significant difference (α=0.05) in the quantity of phenolic combinations was consented to among the studied plants (Table 3).

Table 2 DPPH free radical inhibition percentage with different concentrations of medicinal plant extracts

Plant / Extract (µg)	*Nepeta ucranica*	*Marrobium vulgare*	*Vitex agnus-castus*	*Phlomis olivieri*
0	0 a	0 a	0 a	0 a
20	0 a	9 a	14 b	2 a
40	1 a	11 a	18 b	24 b
60	7 b	15 b	39 c	48 c
80	10 b	29 c	54 d	60 d
100	21 c	38 d	69 e	73 e
120	27 d	48 e	73 f	90 f
Mean	9.42 *	21.42 **	38.14 ***	42.42 ***

Table 3 Antioxidant potential in terms of micromole equivalent of trolox per gram if plant dry weight, amount of phenolic compounds in mg if gallic acid equivalent per gram of plant dry weight and IC50 in mg of plant dry weight per ml of extract in four medicinal plants

Plant	Trolox equivalent (µmol TE/g DW)	IC50 (mg DW/ml)
Phlomis olivieri	798.33 ± 46.31 a	0.58 ± 0.008 a
Vitex agnus-castus	514 ± 7.76 b	0.77 ± 0.01b
Marrobium vulgare	272.66 ± 19.76 c	1.28 ± 0.07 c
Nepeta ucranica	121.33 ± 11.25 d	2.15± 0.22 d

Mitotic Index of Edible Onion Plant Root Cells

In this research, the examination of extract impacts on the number of cells in distinct stages of division revealed that out of 1000 cells in the control group, 900 cells were in the interphase stage, while 45, 35, 10, and 10 cells were in the prophase, metaphase, anaphase, and telophase stages, respectively. Also, with increasing concentrations of the plant extracts, the number of cells in the four stages of mitosis decreased, and the number of cells in interphase increased, which indicated the extracts' potential to inhibit mitotic division, with M. vulgare showing the most impressive reaction. The mitotic index in the control group was 10%. The lowest mitotic index was stated in the aqueous extracts of *M. vulgare* and *V. agnus-castus* at the concentration of 100 mg/ml, respectively, indicating their high allelopathic potentials compared to the other two plants. No statistically significant differences were apperceived in any of the studied groups at the concentration of 0-25 mg of dry weight in 1 ml at the level of $\alpha = 0.05$ (Table 4).

Cytotoxic potentials of medicinal plants of the Lamiaceae family gained using the alga *D. salina.* The cytotoxic potentials of the extracts were assessed using the alga *D. salina*. Initial cell counting revealed that the number of cells at the end of the logarithmic growth phase ranged from approximately 1.1 to 1.2 million cells per milliliter of algal suspension. Upon exposure to the plant extracts, the number of algal cells decreased with a more pronounced reduction marked at higher extract concentrations in most cases. As shown in Table 5, the diminution in the number of cells in the presence of various concentrations of the extracts is statistically significant in most cases. However, in terms of the average effect of different concentrations, no significant difference was observed between the plants except for *M. vulgare.*

In this analysis, the antioxidant potentials of the mint family four plants, were investigated. The methanolic extracts of *P. olivieri* and *N. ucranica* showed the highest 90% and the lowest 27% DPPH radical inhibition percentage, respectively, at a concentration of 120 µg. For the methanolic extract of *P. olivieri*, the mean percentage of DPPH free radical removal, IC50 value, and antioxidant potential in terms of µmol Trolex per gram of dry weight were measured at 42.42%, 0.58 mg/ml, and 798.33 µmol, respectively. The antioxidant activities of the methanolic extract of *P. olivieri* at different concentrations were assessed using the DPPH method, yielding an IC50 value of 1.417 µg/l.

Table 4 Effect of different concentration of aqueous extracts of medicinal plants in mg/ml on mitosis cells of edible onion

The stages of mitosis		Interphase	Prophase	Metaphase	Anaphase	Telophase	% Cells in mitosis
Plants	Concentrations						
Marrobium vulgare	0	900	45	35	10	10	10 a
	25	924	40	21	7	8	7.6 a
	50	968	10	15	4	3	3.2 b
	100	993	5	3	0	0	0.8 a
Vitex agnus-castus	0	900	45	35	10	10	10 a
	25	923	39	25	7	6	7.7 a
	50	964	14	18	2	2	3.6 b
	100	989	5	6	0	0	1.1 b
Phlomis olivieri	0	900	45	35	10	10	10 a
	25	919	40	25	8	8	8.1 a
	50	970	14	13	3	0	3 b
	100	981	10	6	2	1	1.9 b
Nepeta ucranica	0	900	45	35	10	10	10 a
	25	927	40	23	5	5	7.3 b
	50	964	16	19	1	0	3.6 b

roots

Table 5 Comparison of the number of *D. salina* green per milliliter of algae cells algae suspension in the presence of different concentrations of the extract in term of micrograms dry weight per microliter

Plant / Extract µg DW /µl	*Marrobium vulgare*	*Vitex agnus-castus*	*Phlomis olivieri*	*Nepeta ucranica*
0	1.2 ± 0.14 a	1.1 ± 0.14 a	1.1 ± 0.14 a	1.2 ± 0.14 a
10	0.75 ± 0.11 b	0.58 ± 0.8 b	0.67 ± 0.12 b	0.78 ± 0.08 b
15	0.58 ± 0.3 c	0.41 ± 0.05 c	0.58 ± 0.21 b	0.71 ± 0.1 b
20	0.75 ± 0.1 b	0.44 ± 0.05 c	0.44 ± 0.04 c	0.51 ± 0.22 c
40	0.57 ± 0.2 c	0.43 ± 0.04 c	0.42 ± 0.04 c	0.39 ± 0.09 d
60	0.38 ± 0.1 d	0.43 ± 0.04 c	0.38 ± 0.15 e	0.25 ± 0.07 d
80	0.048 ± 0.003 e	0.22 ± 0.01 e	0.28 ± 0.001 f	0.15 ± 0.08 g
100	0.012 ± 0.002 f	0.001 ± 0.0003 f	0.03 ± 0.003 g	0.004 ± 0.0 h
Mean	0.529 ± 0.11*	0.45 ± 0.14 **	0.49 ± 0.08 **	0.49 ± 0.09 **

As the concentration of the methanolic extract of this plant increased from 1.6 to 100 µg of dry weight/ml, the percentage of DPPH radical inhibition significantly increased. These results indicated that *P. olivieri* exhibited high antioxidant activity (Firuzi *et al*., 2009). In our study, the mean percentage of DPPH free radical removal, IC50 value, and antioxidant potential in terms of µmol of Trolex per dry weight of the methanolic extract of *V. agnus-castus* were measured at 38.14%, 0.77 mg/ml, and 514 µmol, respectively.

In 2013, Rashed conducted research on the antioxidant activities of different extracts of *V. agnus-castus*. The free radical removal percentage by its methanolic extract was 61.33%. In his research, the average DPPH free radical removal ratio, IC50 value, and antioxidant potential in µmol Trolex in dry weight (g) of the methanolic extract of *M. vulgare* were measured at 21.42%, 1.28 mg/ml, and 272.66 µmol, respectively. In a study conducted by Stanković (2011) on the concentration of phenolic compounds and antioxidant activity, it was shown that the percentage of radical inhibition varied from 27.26 to 89.78%. Concentrations of 1 µg to 1 mg of dry weight demonstrated that the response of DPPH radical to the methanolic extract of *M. vulgare* was linear, indicating that increasing the extract concentration decreased the DPPH radical absorption. In a separate study, Aslantürk et al. (2013) aimed to investigate the antioxidant and anticancer activities of different extracts of *V. agnus-castus*. The results of their research showed that the percentage of free radical removal varied between 11.66% and 79.77% as the concentration increased from 10 to 300 µg/ml, respectively. The IC50 value was determined at 83.47 µg/ml, the mean DPPH free radical removal percentage, IC50 value, and antioxidant potential in µmol Trolex in dry weight (g) of the methanolic extract of N. ucranica at 27%, 2.15 mg/ml and 121 µmol to was measured, respectively.

In 2018, Nestorović et al. investigated the biological activities of several *Nepeta* species. The consequences of their research revealed that the antioxidant potential measured in moles of Trolox equivalent per wet weight varied from 0.768 to 0.870 across the three studied species. These findings underscore the mint family's significance as a valuable source of natural antioxidants. Stankovic (2011) demonstrated the variation in phenolic compound content within a species of Marrobium plant, ranging from 18.72 to 54.77 mg of gallic acid per gram of plant dry weight through an investigation of antioxidant activity and phenolic compound quantification. In 2007, Wojdyto *et al.* examined the antioxidant activities and phenolic compound levels of 32 medicinal plants. Their results indicated that the amount of phenolic compounds in *M. vulgare* was 3.86 mg of gallic acid per 100 g of dry plant weight. Besides, Firuzi *et al*. (2009) reported the amount of phenolic compounds to be 4.6 mg of catechin per gram of dry weight of the M. vulgare plant. In 2012, Latoui et al. reported the total phenolic compound content in the methanolic extract of *V. agnus-castus*to was 46.85 mg of gallic acid per plant dry weight. The quality of phenolic compounds in *P. olivieri* was estimated in the research conducted by Firouzi and co-workers in 2010. Their findings revealed that the amount of phenolic compounds in the mentioned plant was 9 mg of catechin per gram of dry weight of the plant. The amount of phenolic compounds in the methanolic extract of *P. olivieri* was 6.85 mg of gallic acid per dry weight of the plant (Moin *et al*.,

2012). These results highlight the varying amounts of phenolic compounds in mint family plants, which serve as important sources of natural antioxidants. The differences in the reported results are likely attributed to the plants' growing locations and the methods of extraction.

The results indicated that the mitotic index percentage decreased as the concentration of the studied plant extracts in the medium of the edible onion root increased. The highest and lowest percentages of the mitotic index were observed in the control treatment (10%) and the aqueous extract of *M. vulgare* at a concentration of 100 mg of dry weight per milliliter. Additionally, the results demonstrated that as the mitotic index of onion root tip cells decreased, more cells ceased to divide in the interphase, thereby losing the possibility of entering the mitosis phase. Alkaloid phenol and flavonoid compounds are known as plant toxins, which cause changes in the divided spindle by binding to the tubulin of microtubules, thus disrupting and halting mitotic cell division in mammalian cells (Ye *et al*., 1998). The cytotoxic effects of some medicinal plants on the mitotic index of meristem cells and onion root growth were determined, in 2017. The researchers reported that the aqueous extract of the studied plants had impressive impacts on the mitotic index and root growth (Mohsenzadeh *et al*., 2017). The significant reduction of the mitotic index compared to the control group was caused by the reaction of a chemical agent with biological macromolecules and consequent interference with cell rise and development (Campos *et al*., 2008). The concentration of the studied plant extracts had a detrimental effect on the activity of the alga *D. salina*, indicating the cytotoxic effects of the aqueous extracts on the green algae. Mohsenzadeh and co-workers (2017) also investigated the cytotoxic effects of 4 types of medicinal plants on the growth of *Dunaliella* green algae, finding that the increasing concentration of the plant extracts reduced the number of *Dunaliella* green algae.

CONCLUSION

This investigation examined the antioxidant and cytotoxic properties of four species of medicinal plants belonging to the mint family (including *V. agnus-castus*, *M. vulgare*, *P. olivieri*, and *N. ucranica*). These plants have significant medicinal value and are recommended for cultivation by farmers, pharmaceutical companies, and agricultural enterprises. Although traditionally pasture-based, they can also be cultivated on farms successfully. As a result, there is potential for further advancement in the science and technology related to these medicinal plants based on the discoveries outlined in this publication. Besides, the authors have disclosed no conflicts of interest.

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