Australian Journal of Crop Science AJCS

AJCS 10(8): 1207-1212 (2016) ISSN:1835-2707
DOI: 10.21475/ajcs.2016.10.08.p7903

Up-regulation of wheat nitric oxide synthase gene in response to *Zataria multiflora* essential oil dispersion

Mohammadreza Raisi-Nafchi[1], Gholamreza Kavoosi[1]*, Seyed Mehdi Nassiri[2]

[1]Institute of Biotechnology, Shiraz University, Shiraz, 71441-65186, Islamic Republic of Iran
[2]Department of Biosystems Engineering, Shiraz University, Shiraz, 71441-65186, Islamic Republic of Iran

***Corresponding author: ghkavoosi@shirazu.ac.ir**

Abstract

Overproduction of nitric oxide (NO) results in nitrative stress, in turn causing peroxidation of lipids, oxidation of protein and damage to nucleic acids among many others. Presumbly, a simple mode to suppression of nitrative stress is found to be a natural antioxidant from aromatic plants. *Zataria multiflora* essential oil (ZO) is known for its potent antioxidant activities. Highly insoluble in water, ZO can be easily degraded by light, oxygen, high temperature and extreme pH. Dispersion of ZO into polyvinylpyrrolidone (PVP) might enhance its stability and self-life while storage and applications. The present research assesses *in vitro* antioxidant function of PVP/ZO dispersion versus nitrite and nitric oxide (NO). Followed by this efficiency of the PVP/ZO dispersion on the production of nitric oxide synthase (NOS) mRNAs in the wheat seedlings in hydroponic condition was evaluated. PVP and PVP/ZO dispersions characterized with negative zeta-potential was a non-Newtonian shear-thickening fluid. PVP/ZO was fraught with effective in vitro nitrite (IC_{50}, 160 µg/mL) and NO (IC_{50}, 168 µg/mL) scavenging activities. The result indicated that PVP/ZO dispersion up-regulated NOS (3.5 folds, at 30 µg/mL) mRNA production while down-regulated it at higher concentration. Hence, once applied at low concentration PVP/ZO can be promising as plant modulator for plant growth regulation.

Keywords: PVP, Zataria oil, dispersion, Nitric oxide, Nitric oxide synthase.

Introduction

Nitric oxide (NO) is found to be a signaling molecule generated upon oxidation of L-arginine and involved in diverse physiological process. As a signaling molecule, NO is involved in various physiological functions such as regulation of plant growth (Bethke et al., 2006), oxidative damage (Neill et al., 2002), metal toxicity (Saxena; Shekhawar, 2013), drought stress (Garcia-Mata; Lamattina, 2001), cold stress (Zhao et al., 2009) and salinity stress (Misra et al., 2011) and plant-microbe interaction (Romero-Puertas et al., 2004). Nonetheless, given the concentration and action site, overproduction of NO results in nitrative stress, which causing peroxidation of lipids, oxidation of protein, damage to nucleic acids, suppression of enzymes and programmed cell death (Sharma et al., 2012). Resistance to nitrative damage has been correlated with an increase in the antioxidant capacity of cells or effective scavenging of NO. Driven from aromatic plants, natural antioxidant can be a simple and accessible way to suppression of nitrative stress and its worse outcomes.

One of these promising medicinal plants with strong antioxidant function is *Zataria multiflora. Z. multiflora* is a thyme-like plant in Lamiaceae family, has several functional features including antioxidative and anti-inflammatory (Kavoosi et al., 2012). In this context, the Zataria essential oil (ZO) has played a crucial role in pharmaceutical and in the food industries. ZO is one of the ten promising essential oils with potent antioxidant function, which at very low concentrations (10-20 µg/mL, depends on the chemical composition) introduced strong antioxidant function (Kavoosi; Rabiei, 2015). Nonetheless, ZO insoluble in water and can be easily degraded by light, oxygen, high temperature and extreme pH. Thus, in order to enhance the stability of ZO along processing, storage and application, a promise and th applicable protection system is required. Incorporation of ZO into dispersions of different types of polymer with mild operating conditions is a simple way to formulate ZO. Polymers including polyvinylpyrrolidone (PVP) are characterized with high potential for binding to polyphenol compounds such as polyphenol-brearing essential oil. PVP applied in the granulation of several multivitamins and drugs. It rapidly dissolves in water and many organic solvents to form a clear solution and has also been applied as a stabilizer in emulsions, dispersions and suspensions (Sun et al., 2013). PVP with this promising character is useful polymer for preparation essential oil dispersions. In the ongoing study, accordingly, PVP/ZO dispersions were prepared by incorporation different concentrations of ZO into PVP solutions. The study was conducted in three steps: We primarily evaluated the physical (zeta-potential, particle size) and rheological (viscosity) properties of PVP/ZO dispersions. Next, the in vitro antioxidant function of PVP/ZO dispersions versus nitrite and NO were examined. Then, the efficiency of PVP/ZO dispersion on the production of nitric oxide synthase (NOS) mRNA in the wheat seedling in hypotonic condition was investigated.

Results and Discussion

Zeta-potential and particle size of dispersion

The zeta-potential and particle size of PVP was estimated to be 3.8 mV and 34 nm, respectively. PVP/ZO100 showed

Table 1. Zeta-potential and particle sizes of polyvinylpyrrolidone (PVP) dispersion incorporated with different concentrations of Zataria oil (ZO).

Dispersion	Zeta potential (-mV)	Particle size (nm)
PVP	3.8 ± 0.4^{e}	34 ± 3^{f}
PVP/ZO20	4.2 ± 0.3^{de}	47 ± 4^{e}
PVP/ZO40	5.0 ± 0.5^{cd}	60 ± 4^{d}
PVP/ZO60	5.7 ± 0.3^{bc}	74 ± 5^{c}
PVP/ZO80	6.4 ± 0.5^{ab}	93 ± 5^{b}
PVP/ZO100	7.0 ± 0.6^{a}	115 ± 7^{a}

The zata-potential (surface charge) and hydrodynamic particle sizes were determined using a Brookhaven instruments corporation 90 Plus zeta-sizer as reported in materials and methods. The values are expressed as means ± standard deviation for three independent experiments. Mean values with different letters within a column are significantly different by Duncan test at ($p < 0.05$). An increase in the ZO content, caused a significant increase in the zeta-potential and particle sizes of PVP dispersions.

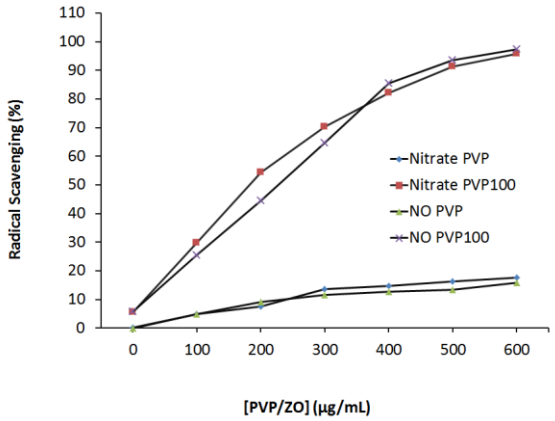

Fig 1. Nitrite and nitric oxide (NO) scavenging activity of poly vinylpyrrolidone (PVP) dispersion incorporated with 100 mg of Zataria oil (ZO) per gram of PVP powder (PVP/ZO100). Nitrite and NO concentration were detected using Greiss reagents in the absence and presence of PVP or PVP/ZO100. PVP did not nitrite and NO scavenging effects, while PVP/ZO100 dispersions displayed a concentration dependent NO and nitrite scavenging activity.

Table 2. Viscosity (mPa.s) of polyvinylpyrrolidone (PVP) dispersion incorporated with different concentration of Zataria oil (ZO).

	Shear rate (s^{-1})			
Dispersion	1	2	5	10
PVP	1 ± 0.3^{d}	4 ± 1.0^{c}	12 ± 1.2^{b}	18 ± 1.5a
PVP/ZO20	1 ± 0.4^{d}	4 ± 1.1^{c}	12 ± 1.5^{b}	18 ± 1.7^{a}
PVP/ZO40	1 ± 0.5^{d}	4 ± 1.3^{c}	12 ± 1.4^{b}	18 ± 1.8^{a}
PVP/ZO60	1 ± 0.6^{d}	4 ± 1.0^{c}	12 ± 1.5^{b}	18 ± 1.8^{a}
PVP/ZO80	1 ± 0.4^{d}	4 ± 1.2^{c}	12 ± 1.7^{b}	18 ± 1.6^{a}
PVP/ZO100	$1 \pm .05^{d}$	4 ± 1.3^{c}	12 ± 1.6^{b}	18 ± 1.5^{a}

The apparent viscosities (mPa.s) of the dispersions were quantified through the use of Brookfield Viscometer (spindle 02) at spindle rotational speed of 1, 2, 5 and 10 $s^{-1.}$ The values are expressed as means ± standard deviation for three independent experiments. Mean values with different letters within a row are significantly different by Duncan test at ($p < 0.05$). The viscosity of PVP dispersions did not affect by an increase in ZO content. The viscosity of PVP dispersions increased by increasing rotational speed of spindle, therefore, PVP and PVP/ZO dispersions were non-Newtonian shear-thickening fluid.

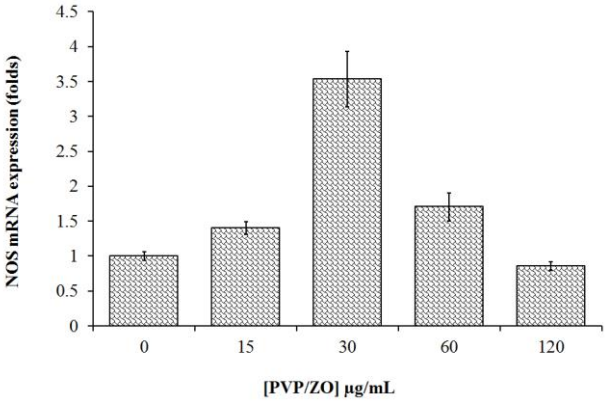

Fig 2. Expression of nitric oxide synthase (NOS) mRNA in response of polyvinylprrolidone (PVP) dispersion incorporated with 100 mg of Zataria oil (ZO) per gram of PVP powder (PVP/ZO100). PVP did not any effects on the NOS mRNA production (data not shown), while PVP/ZO100 dispersions displayed a concentration dependent NOS mRNA generation.

Table 3. Primers used for real-time PCR analysis.

Genes name	Orientation	Sense 5′-3′ sequence	Product length (bp)
18srRNA	Forward	CGCTCCTACCGATTGAATGG	127
	Reverse	CCTTGTTACGACTTCTGCTTCC	
NOS	Forward	ACAGATTTGAATTGCATTGGCGA	188
	Reverse	GCAGATTTCCCAACATTTGCTGA	

Primer design (in form of exon junction) was carried out using Allele ID 7 software. Forwaed and reverse primer sequences for the internal control 18S rRNA and nitric oxide synthase (NOS) genes along product length are shown.

largest zeta-potential and particle size. Thus, at high concentrations ZO significantly improved zeta-potential and particle size of PVP dispersion (Table 1). ZO substantially elevated the zeta-potential and particle size of PVP dispersion, which is as per the results of Sanchez-Gonzalez et al. (2011) on the hydroxypropylmethylcellulose (HPMC) emulsion incorporated with bergamot oil and lemon oil and the results of Bonilla et al. (2012) on chitosan emulsion incorporated with basil and thyme oil. ZO (due to hydroxyl group) have negative zeta-potential; rationally the negative zeta-potential of PVP/ZO must be elevated that is line with our experimental results. The increase in the zeta-potential by the addition of ZO could be related to the interaction of hydrophobic sites (isopropyl or methyl) of thymol, charvacrol, *p*-cymene or γ-terpinene with hydrophobic sites of PVP via van der Waals interactions. These effects insert slight new negative charge on the PVP chain. The increase of the zeta-potential of PVP dispersions also could be related to saturation of PVP chain by ZO. By this effects PVP chain coated by ZO, so all parts of PVP chain become negative (El-Houssiny et al., 2012). In PVP dispersion, interaction between PVP chains via van der Waals interactions made a particle with average particle size of 34 nm. In PVP/ZO dispersion hydrophobic sites (methyl and isopropyl) of thymol, charvacrol, *p*-cymene or γ-terpinene can interact with hydrophobic site of PVP through van der Waals attractive force, so the average particle sizes of PVP increases (Guzey; Mc Clements, 2007). The rise in the particle size of PVP dispersion in the presence of ZO can be served as a great suggestion for PVP and ZO interaction probably through hydrogen binding. Accordingly, PVP/ZO100 dispersion had the largest zeta-potential and the largest particle size.

Viscosity behavior

The viscosity of PVP dispersions dealt with in the present research was elevated by increasing rotational speed of the spindle in the analyzed speed ranges. The PVP and PVP/ZO dispersions taken here were non-Newtonian shear-thickening fluid (Table 2). ZO did not affect viscosity of PVP dispersions evaluated in the ongoing study, which is in line with the results of Peng and Li (2014) on the chitosan dispersion incorporated with lemon, thyme and cinnamon oils. Sanchez-Gonzalez et al. (2011) reported that by increasing the bergamot and lemon oil content, a loss was found in the viscosity of chitosan dispersion, which is opposing with present results. The physical properties of the dispersed particle, including the average particle size, polydispersity, zeta-potential and even particle shape greatly will influence the rheological properties of dispersions (Mc Clements, 2007). The viscosity of PVP modified as a function of shear rate. PVP showed non-Newtonian shear thickening flow behavior. For a shear thick fluid, on the increase in the shear rate, the particle start to physically collide with each other so the particle-particle interactions elevated, causes a shear thickening effect (Mc Clements, 2007). Accordingly, the authors suggested a high interaction force between PVP particles at high shear rate.

Nitric oxide and nitrite scavenging effect

PVP/ZO100 dispersions displayed a concentration dependent NO and nitrite scavenging function with IC_{50} values of 168 and 160 µg/mL, respectively (Figure 1). Sodium nitropruside (SNP) acts as a drug by releasing NO which is converted to nitrite. Nitrite reacts with Griess reagents (sulfanilamide, HCl, N-(1-naphthyl)-ethylenediamine) to generate a pink-red color complex. In the presence of NO or nitrite scavenger (antioxidant compounds) the production of this complex is disrupted (Tarpey et al., 2004). Although NO is essential in bactericidal functions, NO over production serves as cytotoxic molecules. NO can also react with superoxide anion to form peroxynitrite. Peroxynitrite is a powerful oxidant and nitrating agent, which can damage protein, DNA and lipid molecules. Damage to biological macromolecules can result in physiological dysfunction (Bohm et al., 2010). Nitrite binds cytochrome C oxidase, in competition with oxygen. This event elicits intracellular signaling events, including the diversion of oxygen to generation of reactive oxygen species (ROS) with potentially damaging effects and oxidative stress (Mittler et al., 2004). The NO and nitrite scavenging function of PVP/ZO observed in our study imply the beneficial role of these products for reducing damage in biological membrane and also may protect tissues versus oxidative damage by NO and peroxynitrite.

NOS expression

We further examined the stimulatory effects of PVP/ZO dispersion on the production/generation of NOS mRNA. PVP/ZO up-regulated NOS mRNA production at 30 µg/mL (3.5 folds of control), while at higher concentration down-regulated it (Figure 2). In plants species, NO involved in many physiological processes, including pathogen infection (Mur et al., 2013), stimulation of seed (Beligni; Lamattina, 2000), germination of pullen (Sirova et al., 2011), floral resolution (He et al., 2004), senesencene (Jasid et al., 2009), stomatal closure (Neill et al., 2008) and root development (Pagnussat et al., 2003). Thus, NO is a signaling molecule that participates at physiological concentrations in a diverse regulatory plant pathway. In plants NO may produce by several pathway, including nitrate reductase (Planchet; Kaiser, 2006), nitrite:NO reductase (Moreau et al., 2010), NOS like enzyme (Prochazkova et al., 2014), xanthine oxidoreductase (Wang et al., 2010) and polyamine oxidase (Wimalasekera et al., 2011). Depending of the concentration and site of actions NO could serves be either as cytoprotectant and/or cytotoxic. NO at low concentrations played as cytoprotective by; (1) elimination excess amounts of nitrites from plant cells, (2) scavenging of reactive oxygen species, (3) regulation of antioxidant enzymes like, superoxide dismutase, ascorbic peroxidase and catalases (Yang et al., 2014). In relatively high concentrations, NO

functions as a cytotoxin and mimics stress factor in inducing membrane injury, damage to protein and nucleic acid, which eventually results in apoptosis and production of superoxide with adverse effects on respiration rate, photosynthesis electron transport chain and root/shoot development (Yang et al., 2014). Our experimental results indicated PVP/ZO had NO scavenging (at 168 µg/mL) and NOS up-regulatory (3.5 folds at 30 µg/ml) function. Thus, PVA/ZO at low concentration had potent NOS up-regulatory effects, but at higher concentrations had NO scavenging effects. Nonetheless, more study required to investigate the expression of other enzyme such as; nitrate reductase, nitrite:NO reductase, xanthine oxidoreductase and polyamine oxidase and their effects on the NO production.

Materials and Methods

Chemicals

Polyvinylpyrrolidone or polyvidon (catologe No: 107443) were purchased from Merck (Darmstadt, germany). Sulfanilamide, N-(1-naphthyl)-ethylenediamine, sodium nitroproside and sodium nitrite were purchased from Sigma-Aldrich chemical Co. (Saito Louis, Mo, USA).

Plant materials

The aerial parts of ZM were obtained from wild plants in the mountains of Fars province, Iran. The plant leaves were separated from the stem and were dried in the shade for 72 h. The air-dried leaves were hydro-distilled in distilled water for 3 h using an all-glass Clevenger-type apparatus (Herbal Exir Co., Mashhad, Iran). The obtained ZMEO was dehydrated over anhydrous sodium sulfate and stored at 4 ℃ until analyzed by GC-MS. Gas chromatography – mass spectrometry (GC-MS) analysis of ZO was carried out by using of Agilent gas chromatograph (Santa Clara, CA 95051 USA) equipped with silica capillary HP-5MS column (30 m × 0.25 mm i.d.; thickness 0.25 µm) coupled with a 5975-C mass spectrometer. Retention indices (RI) were determined using retention times (RT) of n-alkanes (C_8-C_{25}) that were injected after essential oil under the same chromatographic conditions. The compounds were identified by comparison of retention indices (RI) with those reported in the literature and by comparison of their mass spectra with the Wiley GC/MS Library published data (Kavoosi; Rabiei, 2015). GC-MS analysis indicated that the main component of ZO were thymol, carvacrol, *p*-cymene, and γ-terpinene (Kavoosi; Rabiei, 2015). The density of ZO calculated by digital balance (Acculab, Sartorius group, Germany) and the average was reported 996 mg/mL. Therefore, each µL of ZO is approximately equal to 1 mg. ZO were dissolved in one volume of tween 20; hence, the final concentration of emulsified ZO was taken as 500 mg/mL that was applied for preparation of PVP/ZO dispersions. Tween/ZO was a Newtonian fluid with negative zeta-potential (-8 mV) and 65 nm average particle size diameter.

Preparation of dispersion

To preparation PVP dispersion one gram of PVP dissolved in 80 mL of distilled water under continuous stirring at 40 ℃ until a homogenous solution was obtained. The homogenous PVP solution sonicated using an ultrasonic (Bandlin, Germany) at 140 W for 5 min at the temperature 30 ℃. To preparation of PVP/ZO dispersions different concentrations of ZO (20, 40, 60, 80 and 100 mg/g of PVP) were added to the dispersions and mixed under mechanical stirring at 800 RPM for 12 h at 40 ℃. The PVP dispersions divided into six groups named; PVP, PVP/ZO20, PVP/ZO40, PVP/ZO60, PVP/ZO80 and PVP/ZO100. As an emulsifier, glycerol (400 mg/g PVP) was added to all dispersions and mixed under mechanical stirring at 800 RPM at 40 ℃. At the end, distilled water was added until the final volume was reached to 100 mL under mechanical stirring at 800 RPM at 40 ℃. The final dispersions were stored at 4 ℃ until further experiments. Before physical tests, the dispersions were equilibrated at room temperature (25-27 ℃) for 3 h to reach a constant temperature. The dispersions were stable for at least 100 days as we checked by visual observation through testing phase separation.

Zeta-potential of particle

The dispersions were diluted to a final concentration of 50 µg/mL by deionized water. The electrophoretic mobility of particles in the dispersion was determined by Phase Analysis Light Scattering (PALS) technique using a Brookhaven instruments corporation 90 Plus zeta-sizer (New York, 11742, USA). The Bi-PALS zeta potential analyzer software provided an average of electrophoretic mobility and a measure of zeta-potential using smolouchewsky model.

Effective hydrodynamic diameter of particle

The dispersions were diluted to a final concentration of 50 µg/mL of deionized water. The effective hydrodynamic diameter of particles was determined based on the principle of dynamic light scattering (DLS) by using a Brookhaven instrument corporation 90 Plus particle size analyzer. The Bi-9000 particle sizing software provided an average effective hydrodynamic diameter of the particles.

Apparent viscosity

The dispersions were decanted into a 200 mL glass cylinder (80 mm × 70 mm) and left on an anti-vibration bench with a flat level for 10 min to equilibration. The apparent viscosities (mPa.s) of the dispersions were quantified through the use of Brookfield viscometer (DV-II + PRO digital viscometer, Meddleboro, USA, spindle 2) at spindle rotational speed of 1, 2, 5 and 10 $s^{-1.}$ The software of Brookfield instrument directly provided the apparent viscosity of the dispersions.

Nitric oxide scavenging assay

Twenty microliters of different concentrations of PVP or PVP/ZO100 dispersions were incubated with 0.5 mL of sodium nitropuroside (20 µg/mL in 100 mM sodium citrate pH 5) at 37 ºC for 2 h. After incubation, 0.5 mL of Griess reagent was added and the absorbance (A) was read at 540 nm using Unico 2100 spectrophotometer (New Jersey, USA). The percentage of NO scavenging was calculated by using the following formula: $[(A_{control} - A_{test}) / A_{control}] \times 100$. $A_{control}$ = absorbance of Griess reagent in the presence of sodium nitropuroside and A_{test} = absorbance of Griess reagent in the presence of sodium nitropuroside and PVP or PVP/ZO. The equations were obtained from the graphs plotting the NO scavenging percentage versus different concentrations of PVP or PVP/ZO dispersions were applied to calculate IC_{50} (Tarpey et al., 2004).

Nitrite scavenging assay

Twenty microliters of different concentrations of PVP or PVP/ZO100 dispersions were incubated with 0.5 mL of sodium nitrite (10 µg/mL in 100 mM sodium citrate pH 5) at 37 ºC for 2 h. After incubation, 0.5 mL of Griess reagent was added and the absorbance (A) was read at 540 nm using Unico 2100 spectrophotometer (New Jersey). The percentage of nitrite scavenging was calculated as follows: $[(A_{control} - A_{test}) / A_{control}] \times 100$. $A_{control}$ = absorbance of Griess reagent in the presence of sodium nitrite and A_{test} = absorbance of Griess reagent in the presence of sodium nitrite and PVP or PVA/ZO. The equations were obtained from the graphs plotting the nitrite scavenging percentage versus different concentrations of PVP or PVP/ZO dispersions were applied to calculate IC_{50} (Tarpey et al., 2004).

Plant growth conditions

Seeds of *Triticum aestivum* L. cv. Shiraz were surface-sterilized with sodium hypochlorite (5%) and planted in 1 kg pot under hydroponic conditions. Wheat seedlings received tap water at greenhouse conditions with about 60% relative humidity for two week until the size of leaves reached to about 10 cm. Diurnal cycles at each temperature (27/24) were set at 16 h day and 8 h night cycle. At this state, the wheat seedling was treated with 50 mL of different concentrations (15-500 µg/mL) of PVP/ZO dispersion diluted in tap water. The equal concentrations (15-500 µg/mL) of PVP dispersion were applied as control. Treatment was continued for three days. 24 h after treatment, young wheat leaves were harvested and frozen in liquid nitrogen and then stored at -70 ºC until use.

RNA preparation

Total RNA was extracted from 100 mg of leaf matter using RNX-Plus buffer (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. Briefly, leaf samples were grounded in liquid nitrogen with a mortar. Then 1 mL of RNX-Plus extraction buffer was added to ground powder in an RNase-free microtube, mixed thoroughly and left for 5 min at room temperature and then 200 µL of chloroform was added and mixed gently. This mixture was centrifuged at 10000 g for 15 min at 4 ºC, and the supernatant precipitated with an equal volume of isopropanol for 15 min on ice in a new tube. The resulting pellet was washed using 75 % ethanol, dried for 5 min at room temperature and re-suspended in 50 µL of RNase-free water. Quantification of total RNA was performed with a Nano Drop ND 1000 spectrophotometer at 260 nm (Thermo Fisher Scientific, Wilmington, DE, USA). The integrity RNA was checked by visual observation of 28S rRNA and 18S rRNA bands on an agarose gel electrophoresis before real-time PCR analysis.

DNase treatment and cDNA synthesis

DNase treatment was carried out using a Fermentas DNase Kit (Fermentas, Hanover, MD, Germany) using the manufacturer's protocol and again the integrity of total RNA was checked by electrophoresis in agarose gel. 5 µg of DNase-treated RNA was converted to cDNA with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas) using the manufacturer's protocol in a 20 µL final volume.

Primer design and real-time quantitative PCR analysis

The primer was designed using Allele ID 7 software (Premier Biosoft Intl, Palo Alto, CA, USA) for NOS based on the aligned nucleotide file. The wheat 18S rRNA (AJ272181) gene was applied as internal control for data normalization (Table 3). Primers for the PCR reactions were designed to have a melting temperature of about 55 °C and to give a PCR product between 100 and 200 bp. Real-time PCR was performed using a line Gene K Thermal cycler (Bioer Technology Co, Hangzhou, China). The cDNA samples were diluted 1:5 by using nuclease-free water, and 5 µL of cDNA was applied for real-time PCR. The final volume for relative real-time PCR was 20 µL containing 4 pmol of each primers, 5 µL (diluted) of the first-strand cDNA and 1x SYBR Premix Ex Taq TM Π (Takara, Japan). A melting curve was run after the PCR cycles followed by heating from 50 to 95 °C. The proper control reaction was carried out without the reverse transcriptase treatment. For each sample, the subsequent real-time PCR reactions were performed at twice under identical conditions.

Data normalization and quantitative PCR verification

For real-time data analysis, the relative expression of the target gene in each sample was compared with the control sample (corresponding to control plants) and was determined with the delta-delta Ct method. The Ct for each sample was calculated using the Line-gene K software (fqdpcr ver. 4.2.00), where Ct refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification. In this analysis method, the relative expression of the target gene in the control sample was equal to one (2^0) by definition (Larionov et al., 2005).

Statistical analysis

All data are presented from at least four independent experiments and expressed as the mean ± standard deviations. The significant differences between treatments were analyzed by Duncan test at $P<0.05$ by using statistical package for the social sciences (SPSS, Abaus Concepts, Berkeley, CA) software version 16.

Conclusion

In light of all foregoing results, PVP dispersion was a non-Newtonian shear-thickening fluid with negative zeta-potential and nanoscale particle sizes. ZO leads a significant rise in zeta-potential and particle size of PVP dispersion. PVP/ZO dispersion had effective nitrite and NO scavenging in a dose dependent manner with IC_{50} of 160 and 168 µg/mL, respectively. The potent in vitro antioxidant function of PVP/ZO dispersion implies that such products can effectively be applied as a promising antioxidant versus synthetic antioxidant to reduce oxidative stress and oxidative damages in the plants. PVP/ZO also up-regulated NOS mRNA 3.5 folds at 30 µg/mL. Thus, these products at low concentration can be applied as plant modulator for regulation of plant growth. Nonetheless, more specialized invetigations are required to investigation other enzymes in the NO producing networks. On the other hand, this study was focused on wheat seedling in the unstressed conditions. There is a urgent need to more study on the other plant on the stressed and non-stressed conditions. It will be very promising if PVP/ZO dispersion diminished oxidative stress of plants in response to abiotic and biotic stress. Thus, it is better to be focused on

natural antioxidant function on plant oxidative stress resistance in stressed plants in forthcoming investigations.

Acknowledgments

This work was supported by the financial support from Shiraz University (grant No. 88-GRAGRST-108). The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

References

Beligni MV, Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta. 210: 215-221.

Bethke PC, Libourel IGL, Reinöhl V, Jones RL (2006) Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. Planta. 223:805–812.

Bohm FMLZ, Ferrarese MDLL, Zanardo DIL, Magalhaes JR, Ferrarese-Filho O (2010) Nitric oxide affecting root growth, lignification and related enzymes in soybean seedlings. Acta Physiol Plant. 32: 1039-1046.

Bonilla J, Atares L, Vargas M, Chiralt A (2012) Effect of essential oils and homogenization conditions on properties of chitosan-based films. Food Hydrocolloid. 26: 9-16.

El-Houssiny AS, Ward AAM, Mansour SH, Abd-El-Messieh SL (2012) Biodegradable blends based on polyvinyl pyrrolidone for insulation purposes. J Appl Polym Sci. 124:3879-3891.

Garcia-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses versus drought stress. Plant Physiol. 126:1196-1204.

Guzey D, Mc Clements DJ (2007) Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by β-lactoglobulin-pectin complexes. J Agri Food Chem. 55: 475-485.

He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Fiorani F (2004). Nitric oxide represses the Arabidopsis floral transition. Science. 305:1968-1971.

Jasid S, Galatro A, Villordo JJ, Puntarulo S, Simontacchi M (2009) Role of nitric oxide in soybean cotyledon senescence. Plant Sci. 176:662-668.

Kavoosi G, Rabiei F (2015) *Z. Multiflora*: chemical and biological diversity in the essential oil. J Essent Oil Res. 27: 428-436.

Kavoosi G, Teixeira da Silva JA, Saharkhiz MJ (2012) Inhibitory effects of *Z. multiflora* essential oil and its main components on nitric oxide and hydrogen peroxide production in lipopolysaccharide-stimulated macrophages. J Pharm Pharmacol. 64: 1492-1500.

Larionov A, Krause A, Miller W (2005). A standard curve based method for relative real time PCR data processing. BMC Bioinformatic. 6:62-70.

Mc Clements DJ (2007) Critical review of techniques and methodologies for characterization of emulsion stability. Crit Rev Food Sci Nutr. 47: 611-649.

Misra AN, Misra M, Singh R (2011) Nitric oxide ameliorates stress responses in plants. Plant Soil Environ. 57:95–100.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci. 9: 490-498.

Moreau M, Lindermayr C, Durner J, Klessig DF (2010) NO synthesis and signaling in plants–where do we stand?. Physiol Plant. 138: 372-383.

Mur LA, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Gupta KJ (2013) Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plant. 5: 52-62.

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008) Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot. 59:165–176.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot. 53:1237-1247.

Peng Y, Li Y (2014) combined effects of two kinds of essential oils on physical, mechanical and structural properties of chitosan films. Food Hydrocolloid. 36: 287-293.

Planchet E, Kaiser WM (2006) Nitric oxide production in plants: facts and fictions. Plant Signal Behav. 1:46-51.

Pagnussat GC, Lanteri ML, Lamattina L (2003) Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol. 132: 1241-1248.

Prochazkova, D, Haisel D, Pavlíkova D (2014) Nitric oxide biosynthesis in plants–the short overview. Plant Soil Environ. 60:129-134.

Romero-Puertas MC, Perazzolli M, Zago ED, Delledonne M (2004) Nitric oxide signalling functions in plant–pathogen interactions. Cell Microbiol. 6:795-803.

Sanchez-Gonzalez L, Chiralt A, Gonzalez-Martinez C, Chafer M (2011) Effect of essential oils on properties of film forming emulsions and films based on hydroxypropylmethylcellulose and chitosan. J. Food Eng. 105: 246-253.

Saxena I, Shekhawat GS (2013) Nitric oxide (NO) in alleviation of heavy metal induced phytotoxicity and its role in protein nitration. Nitric Oxide. 32:13–20.

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012:1-25.

Sirova J, Sedlarova M, Piterkova J, Luhova L, Petrivalsky M (2011) The role of nitric oxide in the germination of plant seeds and pollen. Plant Science. 181:560-572.

Sun W, Araci Z, Inayathullah M, Manickam S, Zhang X, Bruce MA, Butte MJ (2013) Polyvinylpyrrolidone microneedles enable delivery of intact proteins for diagnostic and therapeutic applications. Acta Biomater. 9:7767-7774.

Tarpey MM, Wink DA, Grisham MB (2004) Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am J Physiol Regul Integr Comp Physiol. 286: R431-R444.

Wang BL, Tang XY, Cheng LY, Zhang AZ, Zhang WH, Zhang FS, Shen JB (2010) Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin. New Phytologist. 187:1112-1123.

Wimalasekera R, Villar C, Begum T, Scherer GF (2011) Copper amine oxidase1 (cuao1) of *Arabidopsis thaliana* contributes to abscisic acid-and polyamine-induced nitric oxide biosynthesis and abscisic acid signal transduction. Molecular Plant. 4:663-678.

Yang F, Ding F, Duan XH, Zhang J, Li XN, Yang YL (2014) ROS generation and proline metabolism in calli of halophyte Nitraria tangutorum Bobr. to sodium nitroprusside treatment. Protoplasma. 251:71–80

Zhao MG, Chen L, Zhang LL, Zhang WH (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing resistance in Arabidopsis. Plant Physiol. 151:755-767.