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journal homepage: www.elsevier.com/locate/sajbAntioxidative effect of Iranian *Pulicaria gnaphalodes* L. extracts in soybean oilAbolfazl Kamkar ^{a,*}, Mohammad Reza Shamse Ardekani ^b, Nabi Shariatifar ^c, Ali Misagi ^a, Amir Sasan Mozaffari Nejad ^{d,e}, Amir Hossein Jamshidi ^f^a Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran^b Department of Pharmacognosy, University of Medical Science Tehran, Tehran, Iran^c Department of Food Hygiene, University of Tehran, Tehran, Iran^d Center for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad, Andhra Pradesh, India^e Member of Young Researchers Club, Islamic Azad University, Garmsar Branch, Garmsar, Iran^f Food and Drug Deputy, IRI Ministry of Health and Medical Education, Tehran, Iran

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ABSTRACT

This study was aimed to evaluate antioxidative activities of the ethanol, methanol and water extracts of *Pulicaria gnaphalodes* in vegetable oil during the storage period. Different concentrations (0, 200, 400 and 800 ppm) of ethanol, methanol and water extracts and beta-hydroxy toluene (BHT; 100, 200 ppm) were added to soybean oil and incubated for 35 days at 65 °C. Peroxide values (PVs) and thiobarbituric acid-reactive substance (TBARS) levels were measured every week during the period of the study. Moreover, antioxidant capacities of the extracts were determined using DPPH and β -carotene–linoleic acid methods. Values were compared among groups in each incubation time points using ANOVA. Results showed that DPPH and β -carotene–linoleic acid assay findings on the *P. gnaphalodes* extracts were comparable to those found on BHT. Moreover, during incubation time, *P. gnaphalodes* extracts lowered PVs and TBARS levels when compared to the control ($p < 0.001$). In this respect, water extract was more potent than the ethanol and methanol extracts. It seems that water extract of *P. gnaphalodes* is a potent antioxidant which makes it a potential antioxidant for oil and oily products during storage.

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1. Introduction

Medicinal plants used in traditional medicine and healing are among natural antioxidant sources. Plants are rich sources of phenolic compounds such as flavonoids, tannins and anthocyanins, which are the most important natural antioxidants (Shariatifar et al., 2012). Specially crude extracts of fruit, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and therefore improve the quality and nutritional value of food (Alezandro et al., 2011). Many plant species indicate the presence of antioxidant constituents in their tissues. The importance of antioxidant compounds derived from plant material in health and protection against disease is confirmed. In addition, antioxidants raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving towards functional food with specific health effects (Hirasa and Takemasa, 1998).

Reactive oxygen species or ROSs are species such as superoxide, hydrogen peroxide, and hydroxyl radical which are associated with cell damage and play a key role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer,

and cardiovascular disease (Halliwell, 1997). Thus, recent studies have investigated the potential of herbal products as antioxidants against various diseases induced by free radicals (Hou et al., 2003). In addition, it has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Pietta, 2000).

Pulicaria species are generally known under the name “kak kosh” and “Shabang” in Iran and are commonly used as herbal tea, flavoring agent, and medicinal plants. *Pulicaria gnaphalodes* (Vent.) Boiss. is a persistent plant of about 10–30 cm high, with gold-yellow flowers, which grow on sandy, stony places in the Arabian Peninsula (Saudi Arabia), Afghanistan, Iran, Turkestan and western Tibet (Mozaffarian, 1996). Razavi Khorasan Province in Iran is the original source of this plant. There are no prior reports on the antioxidant activities of *P. gnaphalodes*. Therefore, the present study is the first evaluation of in vitro antioxidative properties of Iranian *P. gnaphalodes* ethanol, methanol and water extracts on the lipid peroxidation of soybean oil during storage.

2. Materials and methods

2.1. Sample and reagents

Aerial parts of *P. gnaphalodes* L. were collected from Razavi Khorasan Province (Iran) during the flowering period in summer 2010. A voucher

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specimen for this plant was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Tehran University, Tehran, Iran. The plants were dried in a dark place at room temperature. Dried leaves were powdered using an electrical device and stored at refrigerator (4 °C) until use. Refined, bleached and deodorized soybean oil without any antioxidants was taken from the manufacturing company (Behshahr, Oil Factory, Tehran, Iran).

2.2. Preparing extracts

In the present study, three types of extracts (ethanol, methanol and water) were used. Ethanol and methanol extracts were prepared according to the method of Shyamala et al. (2005) with some modifications. Briefly, 10 g of powdered leaves of *P. gnaphalodes* was extracted in Soxhlet apparatus with 100 ml of absolute ethanol (HPLC grade) until the extraction solvent became colorless and for preparing methanol extract, 10 g of powdered leaves was extracted with 200 ml of absolute methanol (HPLC grade, K20192408 Merck) solvents in a shaking incubator at 25 ± 1 °C for 24 h. The extraction was repeated twice at the same condition. Water extract was prepared by means of a percolator. In this regard, leaf samples were extracted with distilled water in a percolator apparatus until the extracted water became colorless. All extracts were filtered and evaporated to dryness in vacuum. The crude extracts were kept in a closed dark glass bottle and stored at 4 °C until use.

2.3. Phytochemical screening of the extracts

Phytochemical screening using thin layer chromatography (TLC) was carried out on *P. gnaphalodes* extracts. The ethanolic, methanolic and water extracts were subjected to TLC examination for group determination of the secondary materials. Modified Dragendroff's reagent according to the method of Harborne (1984) for alkaloids, ferric chloride reagent according to the method of Odebiyi and Sofowora (1978) for phenolics, Naturstoff reagent according to the method of Schnitzler et al. (2007) for flavonoids and vanillin/sulfuric acid reagent (Wagner and Bladt, 1996) for terpenoids were used. Solvent systems for developing of ready coated analytical TLC plates (Merck) were selected according to the method of Wagner and Bladt (1996).

2.4. In vitro antioxidant activity

2.4.1. DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay was done using the stable radical DPPH as a reagent according to the method of Burits and Bucar (2000). Briefly, 50 µl of the extracts (various concentrations) were added to 5 ml of the DPPH solution (0.004% methanol solution). After 30 min incubation at room temperature, the absorbance was read against pure methanol at 517 nm. The radical-scavenging activities of the samples were calculated as percentage of inhibition according to the following equation: $I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$; Where A_{blank} is the absorbance of the control (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition percentage against extract concentration using PHARM/PCS-version 4. All tests were done in triplicate. Values (mean \pm SD) of the extracts were compared with those of BHT using student's *t*-test. A *p*-value less than 0.05 were statistically considered significant.

2.4.2. β -Carotene–linoleic acid assay

Antioxidant capacity was determined by measuring the inhibition of volatile organic compounds and the conjugated diene hydroperoxides

arising from linoleic acid oxidation according to the method of Dapkevicius et al. (1998). In this regard, stock solution of β -carotene–linoleic acid mixture was prepared as follows: 0.5 mg β -carotene (Merck, K15555836) was dissolved in 1 ml of chloroform (HPLC grade) and then 25 µl linoleic acid (Sigma, L1376-500MG) and 200 mg Tween 40 (Merck, 8 22185) were added. After the evaporation of chloroform, 100 ml of oxygen saturated distilled water was added with vigorous shaking. Then, 2500 µl aliquots were dispensed into the test tubes, 350 µl of the extract (2 g/l) was added and the emulsion system incubated for 48 h at room temperature. The same procedure was performed for both BHT (as positive control) and blank. In turn, absorbance spectra of the mixtures were obtained at 490 nm. The degradation rate of β -carotene was calculated by first-order kinetics:

$$[\ln(A_0/A_t)]/t = \text{degradation rate (dr) of sample, blank sample, control sample}$$

where A_0 = absorbance of the sample – absorbance of the control sample – absorbance of blank sample at time 0 (absorbance was read immediately after the addition of alkaloids solutions). A_t = absorbance of the sample – absorbance of the control sample – absorbance of blank sample at time t = 48 h of incubation at room temperature.

Antioxidant activity was expressed as the percent of inhibition relative to the control using the equation:

$$AA\% = \frac{\text{dr control sample} - \text{dr sample}}{\text{dr control sample}} \times 100.$$

Afterward, Antioxidative capacities of the extracts were compared with those of BHT and blank. Further, all inhibition percentages were compared using with 95% confident interval.

2.5. Antioxidative activities of the extracts in Soybean oil

Antioxidative effects of the extracts on lipid peroxidation were evaluated in soybean oil according to the method of Duh (1999) with some modifications. Each sample (50 ml) was transferred to a series of capped glass test tubes. Then, *P. gnaphalodes* extracts (0, 200, 400, and 800 ppm) and BHT (100, 200 ppm) were added to the test tubes and put in a dark oven at 65 °C. The stability of oil to oxidation was evaluated each week over a 5-week period by analyzing the peroxide values (PVs) and TBARS levels. Peroxide values was measured by AOCS cd 8-53 Official Method (AOCS, 1990). For this purpose, a known weight of oil sample (3 g) was dissolved in glacial acetic acid (30 ml) and chloroform (20 ml). Then saturated KI solution (1 ml) was added. The mixture was kept in the dark for 15 min. After adding of distilled water (50 ml), mixture was titrated against sodium thiosulfate (0.02 N) using starch as an indicator. A blank titration was paralleled to treatment and the PVs (mEq of oxygen/kg) were calculated using the following formula: peroxide value = $1000 S \times N/W$. In this formula, *S* is the volume of sodium thiosulfate solution (blank corrected) in ml; *N* is the normality of sodium thiosulfate solution (0.02 N) and *W* is the weight of oil sample (gram).

Thiobarbituric acid-reactive substances (TBARS) were determined weekly, using the method of AOCS (1998). This procedure allows the direct determination of TBARS in oils and fats without preliminary isolation of secondary oxidation products. Oil sample (50–200 mg) was solubilized in 10 ml of 1-butanol, mixed with 10 ml of 0.2% TBA in 1-butanol, incubated 2 h in a 95 °C water bath and cooled for 10 min under tap water. The absorbance was measured at 532 nm against a corresponding blank (reaction with all the reagents and treatments except the oil). The standard curve was determined by the TBARS reaction of a series of aliquots (0.1–1 ml) of 0.2 mm 1, 1, 3, 3-tetra-ethoxypropane (Merck, S4258497) prepared in 1-butanol. The results were expressed as µmol malonaldehyde (MDA)/g of oil ($n=3$).

2.6. Statistical analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were then analyzed by SPSS software program version 16 (1-way ANOVA, Tooki). Mean values and pooled standard error of the mean (SEM) were calculated.

3. Results

3.1. Extract compositions

Phytochemical study showed that flavonoid, terpenoid and phenolic compounds were the major components of the methanol and water extracts.

3.2. Free radical-scavenging activity

Table 1 shows IC50 values for the three extracts in DPPH assay. Results indicated that increase in extract concentration resulted in the increase of free radical-scavenging activity. Interestingly, free radical-scavenging activities of the extracts were comparable to the BHT. In this regard, anti radical activity of the water extract was significantly more than the other two extracts and similar to BHT ($p < 0.05$).

3.3. Inhibitory effect of extracts on lipid per oxidation

Fig. 1 shows inhibition on lipid per oxidation in response to extracts. Ethanol, methanol and water extracts effectively inhibited the linoleic acid oxidation as much as 84.49%, 75.40%, and 91.67%, respectively. In this regard, with the same concentration, water extract showed higher inhibition (91.67%) compared to the BHT (88.88%) and to the control (6.55%).

3.4. Effects of extracts on the oxidative parameters of soybean oil

Fig. 2 shows the PVs (Fig. 2a) and TBARS levels (Fig. 2b) during the storage of soybean oil over a 35-day period in the presence of various concentrations of *P. gnaphalodes* water extract. The initial PVs and TBARS levels in the oil were 2 mEq/kg and 5 $\mu\text{mol/kg}$, respectively.

We have shown that water extract reduced PVs at different concentrations (200, 400, 800 ppm) and incubation time points compared to the control (0 ppm) (Fig. 2a). However, in all samples PVs showed a trend to increase from the beginning of the storage period to the end of experiment. For the control oil sample the gradual increase in PVs from day 7 to end of the storage period was significantly ($p < 0.001$) higher than the treated samples and BHT; moreover, 400 ppm of water extract effectively decreased PV values (as much as BHT in 100 ppm) and also, 800 ppm of this extract made more deduction in PV values than those of BHT in 200 ppm ($p < 0.05$).

Fig. 2b shows that the values of TBARS in both BHT and water extract (200, 400, 800 ppm) supplemented oil samples were lower than the control during days 7 until the end of the storage period ($p < 0.05$). Accordingly, 400 ppm of the water extract showed potency as effective as BHT (200 ppm) ($p < 0.05$).

Table 1

In vitro antioxidant activities of, *P. gnaphalodes* water, methanol and ethanol extracts and BHT in DPPH assay. Values (mean water extract SD) were expressed as IC50.

Sample	DPPH ($\mu\text{g/ml}$)
Water extract	5 ± 0.25
Methanol extract	9.1 ± 0.5
Ethanol extract	8.8 ± 0.3
BHT	4.9 ± 0.2

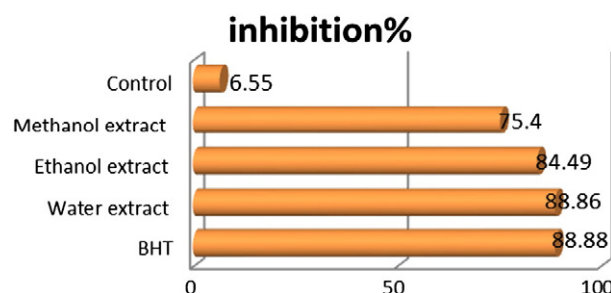


Fig. 1. Antioxidant activity of *P. gnaphalodes* extracts defined as inhibition percentage through β -carotene–linoleic acid assay.

The PVs and TBARS level of the soybean oil supplemented with ethanol extracts (0, 200, 400 and 800 ppm) and BHT (100, 200 ppm) were presented in Fig. 3. PVs of the oil sample containing 800 ppm of ethanol extract were significantly lower than the higher concentration of BHT ($p < 0.05$). In this respect, we came to know that the PVs of the oil supplemented with ethanol extract (400 ppm) and BHT (100 ppm) during days 7 until the end of storage period were comparable ($p > 0.05$). Furthermore, TBARS values of oil supplemented with ethanol extract and BHT were similar (Fig. 3b). Fig. 4 shows the effect of the *P. gnaphalodes* methanol extracts (0, 200, 400, 800 ppm) and BHT (100, 200 ppm) on the PVs and TBARS levels of soybean oil over a 35-day storage period. In this respect, we found out that the PVs of oil supplemented with all concentrations of the methanol extract and BHT were higher than the control over the 35 days of storage ($p < 0.001$). Furthermore, all concentrations except 800 ppm showed less potency than the BHT from day 7 until the end of experiment ($p < 0.05$). Interestingly, the decrease in TBARS values of the oil supplemented with the methanol and ethanol extracts was in the same line ($p > 0.05$). 35-day incubation at 65 °C. Values were expressed as mean \pm SD of the three experiments in three separate experiments.

4. Discussion

Many plant substances reduce the risk of illnesses in human. There are still many plants which have various medicinal values but still not explored and used. Plants contain many novel compounds with medicinal

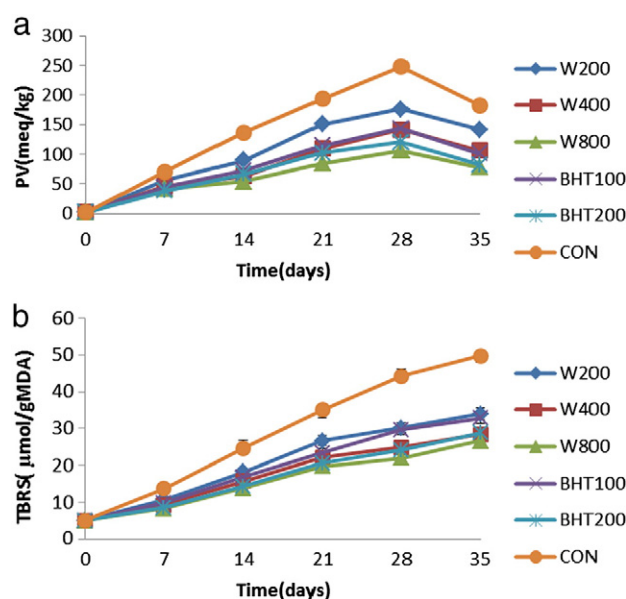


Fig. 2. Effect of *P. gnaphalodes* water extract and BHT on PVs (a) and TBARS levels (b) of soybean oil over a 35-day incubation at 65 °C. Values were expressed as mean \pm SD of the three experiments in three separate experiments.

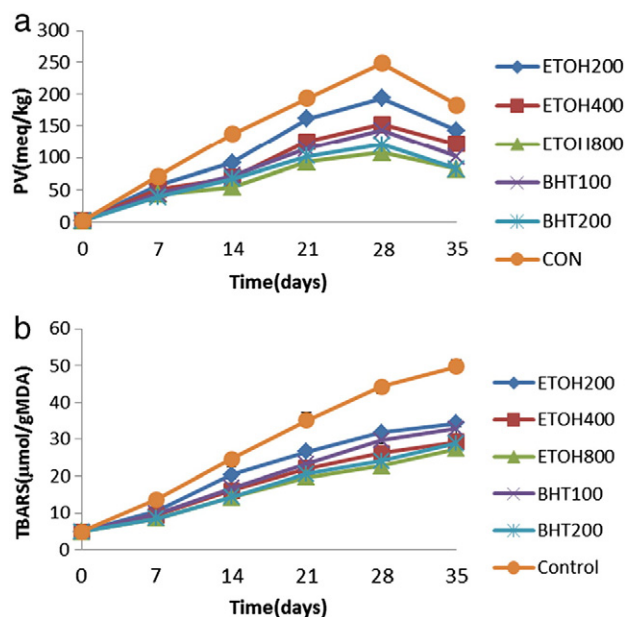


Fig. 3. Effect of *P. gnaphalodes* ethanol extract and BHT on PVs (a) and TBARS values (b) of soybean oil over a 35-day incubation at 65 °C. Values were expressed as mean \pm SD of the three experiments in three separate experiments.

values which need scientific exploration. The antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Kamkar et al., 2010b; Pietta, 2000). Free radicals are common outcome of normal aerobic cellular metabolism (Kamkar, 2009; Rao and Purohit, 2011). Free radicals cause decrease in membrane fluidity, loss of enzyme receptor activity and damage to membrane protein leading to death (Yehuda et al., 2002). Free radicals cause many human diseases like cancer, Alzheimer's disease, cardiovascular disease, kidney disease, fibrosis, diabetes, rheumatoid arthritis, epilepsy and degradation of essential fatty acids (Halliwell, 1997). Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Accordingly, antioxidants may have some benefits in the treatment of above disorders (Kamkar et al., 2012;

Sarma et al., 2010). As extracts of *P. gnaphalodes* showed comparable antioxidant activity to BHT, this plant could be considered for the treatment of the disease associated with free radicals.

In the present research, under the experimental conditions described, the three selected samples exhibited antioxidant activity and their abilities in inhibiting lipid oxidation were different according to the type of extracts. The present study, the decrease in DPPH radical-scavenging activity due to the water (IC₅₀: 5 μ g/ml), ethanol (IC₅₀: 8.8 μ g/ml) and methanol (IC₅₀: 9.1 μ g/ml) extracts of *P. gnaphalodes* was higher than those reported by Conforti et al. (2008) on the hydroalcoholic extract of Mediterranean dietary plants (17.92 μ g/ml), Kawpoomhae et al. (2010) on the water extract of *Mangifera indica* leaf (5.57 μ g/ml) and other water and organic extracts of the famous medicinal plants (Ismail and Hong, 2002; Shariffar et al., 2010; Tosun et al., 2009).

In spite of our findings about the decrease in the values of β -carotene-linoleic acid on both water and organic extracts, other researchers did not show such decrease and in some cases, they showed that BHT was more potent than the plant extracts (Tosun et al., 2009; Wu et al., 2009). These characteristics of the water, ethanol and methanol extracts of the *P. gnaphalodes* can be attributed to their phenolics, flavonoids and terpenoid constituents. These compounds have been shown in our phytochemical analysis that was similar to Ali et al.'s (1999) work on the chemical constituents of this plant in Pakistan. In this regard, Maizura et al. (2011) showed a significant and positive high Pearson's correlations (linear correlation) between antioxidant activity and phenolic contents of the plant extracts. Yuting et al. (1990) as well as Robak and Gryglewski (1988) discussed flavonoids as a group of naturally occurring benzo-g-pyrone derivatives which are able to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals. Moreover, Farombi et al. (2003) reported a potent antioxidant activity for terpenoids, so the difference in the antioxidative properties of plants can be due to their secondary metabolites. In this regard, Duh (1999) discussed that the presence and synergism of different anti-oxidants in an extract will determine the antioxidative properties of a specific extract. In this study, we showed that water, ethanol and methanol extracts of *P. gnaphalodes* are able to inhibit both primary and secondary oxidation of soybean oil during storage. While PVs and TBARS levels of the soybean oil in the control group showed a rapid increase after 35 days of incubation, a slight increase was shown in the supplemented oil samples with water, ethanol and methanol extracts.

Antioxidative effects of various plant extracts in vegetable oil have been reported, so far. For example, Anwar et al. (2007) compared antioxidative effects of various plant extracts with BHT and BHA (synthetic antioxidant) in sunflower oil. They showed that BHT and BHA made higher inhibition to primary oxidation of oil than the extract and also, Sikwese and Duodu (2007) compared antioxidative effects of sorghum crude phenolic extract with TBHQ (synthetic antioxidant) in sunflower oil in the presence of ferric ions. They showed that TBHQ made a higher inhibitory effect on primary oxidation of the oil than the extract, nevertheless their abilities for inhibiting the secondary oxidation were similar. Duh (1999) compared antioxidative activities among water extract of Harng Jyur (*Chrysanthemum morifolium* Ramat) varieties, tocopherol and butylated hydroxyanisole (BHA) in soy bean oil emulsion by measuring both primary and secondary oxidations. He showed a higher inhibition in both primary and secondary oxidations for the water extract that was in agreement with our results in this study. Such results have attributed to the presence of several antioxidants with a range of solubilities.

In line with our findings, Monfared et al. (2011) showed that free radical-scavenging activities of the ether and water extracts *Urtica dioica* L. (post and pre flowering) were comparable with BHT and also, they showed that the antioxidant effect of *U. dioica* L. extract concentrations above 200 ppm on heated sunflower oil was higher than the BHT. This feature can be due to the presence of water soluble active ingredients such as flavonoids in polar extract of plants

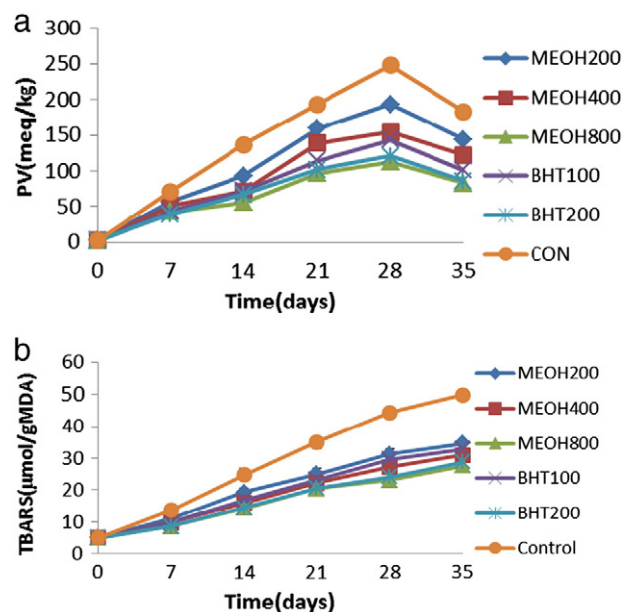


Fig. 4. Effect of *P. gnaphalodes* methanol extract and BHT on PVs (a) and TBARS values (b) of soybean oil over a 35-day incubation at 65 °C. Values were expressed as mean \pm SD of the three experiments in three separate experiments.

(Arumugam et al., 2006; Rahmat et al., 2003). Such pattern was shown by Kamkar et al. (2010a) on both water and methanol extracts of *Mentha pulegium* compared to BHT when added to sunflower oil emulsion. Marinova and Yanishlieva (1997) reported that ethanol extract of *Satureja hortensis* L. had strong antioxidative action during oxidation of sunflower oil at 100 °C by blowing air through the samples.

5. Conclusion

We have shown that *P. gnaphalodes* extract can be considered as an antioxidant to the edible oils. Water, ethanol and methanol extracts showed antioxidative potency in a descending order when added to soybean oil. In this study, the protective effect of the *P. gnaphalodes* extracts was comparable with widely used synthetic antioxidant BHT. Thus, water soluble components of *P. gnaphalodes* could be prepared and added to the commercial vegetable oils as natural antioxidant and suitable alternative for synthetic antioxidants such as BHT.

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