Evaluation of Antioxidant Activity, Total Phenolic and Flavonoids Contents of *Orthosiphon stamineus*, *Teucrium polium*, and *Berberis vulgaris* Decoctions

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Abstract

Original Article

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Introduction: In recent decades, there has been a great deal of attention toward the beneficial effects of herbs in prevention and treatment of different diseases. Phytochemicals, flavonoids, phenolic compounds, and antioxidants are the most common words in this era. Some of the herbs due to their long history of usage are more interesting to be examined and found out their possible medicinal capabilities which in this study antioxidant activity, total phenolic and flavonoids contents of *Orthosiphon stamineus*, *Teucrium polium*, and *Berberis vulgaris* were analyzed.
Methods: Decoction of the herbs were prepared and analyzed. Total Phenolic Content (TPC) were evaluated with Folin-Ciocalteu's phenol reagent. Colorimetric Aluminum Chloride method was used for flavonoid determination and the antioxidant activities of the extracts were determined on their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, based on a modified method of Brand Williams.
Results: Based on the obtained results *B. vulgaris* showed the highest level of TPC, TFC and antioxidant activity followed by *O. stamineus*, *T. polium*. No degradation of analyzed activities was found after mixing all these decoctions.
Conclusion: According to the present study, results of TPC, TFC, and DPPH scavenging activities showed possible beneficial effects of studies herbs and their mixed ones due to their components which might be used in prevention, suppression or treatment of NCDs.
Keywords: *Orthosiphon stamineus, Teucrium polium, Berberis vulgaris*

Introduction

Plants have been used for many purposes including health and medical aims for several thousands of years. One of the most notable of their characteristics is their antioxidant capabilities. Herbs could be considered as one of the main sources of antioxidants. In fact, many anti-inflammatory plant products have remarkable antioxidant effects, either as general antioxidants or free radical scavengers or by reducing redox imbalance following glutathione depletion[1]. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation reactions can produce free radicals[2]. Free radical production occurs constantly in all cells as part of normal cellular functions. However, high free radical levels from endogenous or exogenous sources might be a risk factor for many diseases such as cancer. In turn, these free radicals can start a series of chain reactions that can damage cells. Antioxidants terminate this process by preventing the formation of radicals by either scavenging them or by promoting their decomposition[3] and in this regard, they could stop the initiation stage of cancer. The study of antioxidant use in cancer treatment is a rapidly evolving area. Antioxidants have been extensively studied for their ability to prevent cancer in humans in the last decades[4]. Significant laboratory evidence from chemical, cell culture, and animal studies show that antioxidants may reduce or possibly avert the development of cancer[5].

Herbs also contain many kinds of phytochemicals. The term “phytochemicals” refers to a wide variety of compounds made by plants but is mainly used to describe those compounds that may affect human health[6]. Phytochemicals are non-nutritive plant chemicals that

have protective or disease preventive properties. They are nonessential nutrients, meaning that they are not required by the human body for sustaining life. It is well known that plant produces these chemicals to protect themselves but recent research demonstrates that they can also protect humans against diseases[7,8].

There are more than a thousand known phytochemicals. Some of the well-known phytochemicals are lycopene in tomatoes, isoflavones in soy and flavonoids in fruits. There are many phytochemicals and each works differently. So far, they have been identified with antioxidant[9], hormonal action[10], enzymes stimulation[11], DNA replication interfering[12,13], anti-bacterial effect[14,15], and physical action capabilities[16]. Most phytochemicals have antioxidant activity, protect our cells against oxidative damage, and reduce the risk of developing certain types of cancer. Phytochemicals with antioxidant activity: Allyl sulfides (onions, leeks, garlic), carotenoids (fruits, carrots), flavonoids (fruits, vegetables), polyphenols (tea, grapes)[17–19].

Natural phenolic compounds might have a significant role in prevention and treatment of non-communicable diseases (NCD). Flavonoids, phenolic acids, tannins, curcuminoids, stilbenes, coumarins, lignans, quinones are some of the phenolic compounds present in pharmaceutical herbs and dietary plants[20]. Due to their antioxidant properties and remarkable effects and potential in the prevention of various oxidative stress-related diseases such as cancer, plant polyphenols, have been vastly considered in many studies[21].

Large cohort studies like Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) suggested that the flavonoid quercetin may prevent renal cell cancer among male smokers[22]. A network of case-control studies showed that total flavonoids, flavanones, and flavonols were inversely related to oral and laryngeal cancers.

In addition, laryngeal cancer was inversely related to flavanols, whereas flavanones were inversely related to esophageal cancer. They also found a reduced risk of colorectal cancer with a high consumption of anthocyanidins, flavonols, flavones, and isoflavones. Inverse associations with breast, ovarian and renal cancer were found with flavones and flavonols as well[23]. The Finnish Mobile Clinic Health Examination Survey (FMCHES)[24], and ATBC found inverse associations between dietary flavonoids with lung cancer risk[25]. In a prospective study, it has been shown that lung cancer is related to low levels of retinol, α-tocopherol, β-carotene, lycopene, β-cryptoxanthin[26].

In this study three different herbs which have been extensively used as either medicinal use or cosine usage. *Berberis vulgaris L.* (Barberry) grows in Asia and Europe; the plant is well known in Iran and has been used extensively as a medicinal plant in traditional medicine. Barberry has played a prominent role in herbal healing for more than 2,500 years[27]. Traditionally it has been used as antimalarial, antirheumatic, antiseptic, astringent bitter tonic, depurative, diuretic, dysmenorrhea, purgative, sedative, and liver tune-up[28–30].

Figure 1- Flower, fruit and dried fruit of *Berberis vulgaris* in Iran (*Left to right adapted from Wikimedia/Free.serpico, shabnamak.ir, flickr.com***).**

The second studied herb in this study was *Teucrium polium L.* or *Teucrium capitatum L.* (*Labiatae*), known popularly as Golden/Felty germander, which is a sub-shrub and herb native to the Mediterranean region and the Middle East. Both flowers and its leaves are used in cooking and for medicinal purposes, particularly for the treatment of stomach ailments. An infusion of the leaves and flowers of the plant is consumed as a refreshing beverage. This infusion is also used for liver ailments, gastrointestinal diseases, fevers, colds, diarrhea, stomach pains and fevers [31].

Figure 2- Different types of *Teucrium polium* (Left to right adapted from arrowheadalpines.com, perso.numericable.fr, fitoapteka.org**).**

T. polium has been long used in Iran commonly used as decoctions or infusions for its diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive analgesic, antibacterial, and antidiabetic effects[32–34].

The last studied herb was *Orthosiphon stamineus* Benth or Cat's Whiskers (family: *Lamiaceae*) or Misai Kucing (Malay for “Cat's Whiskers”), which is commonly used as Java Tea. It is a medicinal plant, native in South East

Asia (Malaysia, Indonesia, and Thailand) and some part of Tropical Australia[35].

This plant is best used for treating the ailments or problems of kidney and bladder due to its mild diuretic action.

It is used as a remedy for kidney stone and nephritis. It has been used for rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhea, syphilis, urinary tract and renal diseases, gallstone, eruptive fever, influenza, hepatitis, jaundice, and biliary lithiasis[36–38].

Figure 3- Leaves and different flowers of *Orthosiphon stamineus* (Left to right adapted from flickr.com, newikis.com, davesgarden.com**)**

Materials and Methods

The high quality of dried Iranian barberry was purchased from Iranian local market. Dried *T. polium* was purchased and imported from certified herbal marketing in Tehran/Iran. *O. stamineus* was obtained by University Agriculture Park of UPM. In order to make a decoction of the herbs, a common method with small modification was carried out[39]. First, dried herbs were weighed and washed 3 times with tap water. Then the washed herbs were put into a 10-liter beaker. For each 100g of dried herbs, 4000 ml of distilled water was added. Then the mixture was heated up to 70˚C to decrease the water content to 1000 ml through evaporation. After these steps, the residues were filtered. The liquids were cooled and kept in the fridge at 4˚C in clean bottles. The procedure has been illustrated in Figure 4.

Figure 4- Protocol of preparation of herbal decoctions

Determination of total phenolic content (TPC)

TFC were evaluated with Folin-Ciocalteu's phenol reagent[40,41]. Five ml of the decoction was mixed with 5 ml Folin-Ciocalteu reagent previously diluted with water (1:9 v/v). After 5 minutes, 4 ml of 7% Na_2CO_3 solution was added and thoroughly mix with a vortex mixer for 5 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the Shimadzu UV visible spectrophotometer (UV-1650 PC, Japan). All experiments were conducted 3 times and readings were obtained in triplicates. Samples of extract were evaluated at a final concentration of 0.1 mg/ml. The total phenolic content was expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: y=0.608x+0.5057, R^2=0.9365, where y was the absorbance x was the concentration. The procedure has been illustrated in Figure 5.

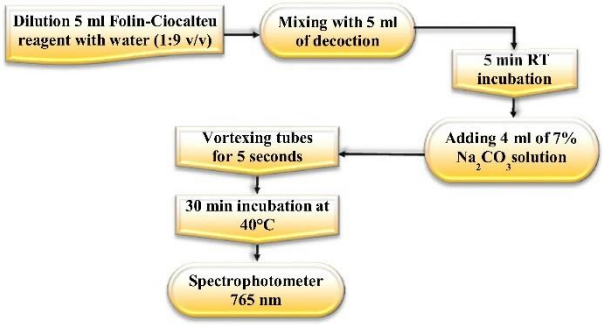

Figure 5- Protocol of total phenolic determination (RT: Room temperature)

Determination of total flavonoid content (TFC)

Colorimetric Aluminum Chloride method was used for flavonoid determination[40]. Half a ml of each plant decoctions was separately mixed with 0.5 ml of 2% Aluminum Chloride. After one-hour incubation at room temperature, the absorbance was measured at 420 nm using Shimadzu UV visible spectrophotometer (UV-1650 PC, Japan). A yellow color indicated the presence of flavonoids. All experiments were conducted 3 times and readings were obtained in triplicates. Extract samples were evaluated at a final concentration of 0.1 mg/ml. TFC were calculated as quercetin equivalents (mg/g) using the following equation based on the calibration curve: y=0.2048x-0.2731, R^2=0.9851, where y was the absorbance x was the concentration. The procedure has been illustrated in Figure 6.

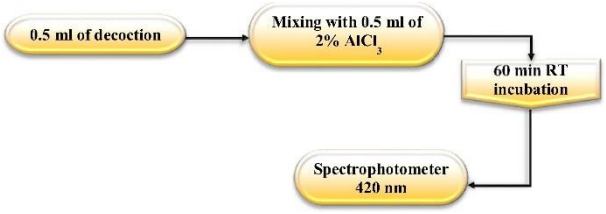

Figure 6- Protocol of determination of total flavonoids (RT: Room temperature)

Assay of DPPH Scavenging Activity

The antioxidant activities of the extracts were determined on their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, based on a modified method of Brand Williams and co-workers (1995) with different concentrations (0.025-0.5 µg/ml) of each extract

were added at an equal volume to methanolic solution of DPPH (100µM)[42]. The mixture was allowed to react at room temperature in the dark for 30 minutes. The absorbance value was read at 518 nm using an ELISA plate reader (ChroMate® Microplate Reader, USA). Butylated hydroxyanisole (BHA), rutin and α-tocopherol were used as positive control for synthetic and natural antioxidant respectively. Three replicates were made for each test sample. The percentage antioxidant activity was calculated by using the following equation:

$$\%\ \text{Antioxidant activity} = \frac{OD\ \ Control - OD\ \ Sample}{OD\ \ Control} \times 100$$

Antioxidant activity was reported as IC_{50}, define by the concentration of samples required (mg/ml) to scavenge 50% of the free radicals. All experiments were conducted 3 times and readings were obtained in triplicates. The procedure has been illustrated in Figure 7.

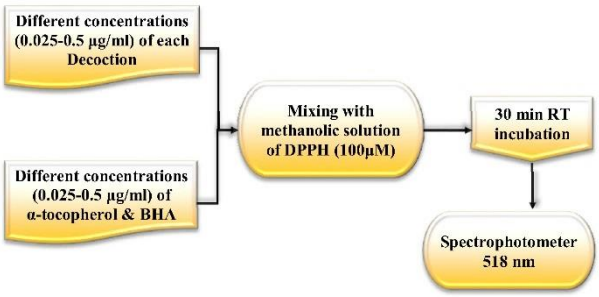

Figure 7- Protocol for determination of assay of dpph scavenging activity (RT: Room temperature**)**

Statistical analysis

Data were expressed as Means±SEM by using IBM SPSS Statistics Software (V.22, Chicago, IL). Statistical differences between different decoctions were determined using one way repeated measures analysis of variance (ANOVA) followed by Dunkan's multiple range post hoc test[43]. Differences between decoctions were considered significantly different when the P value was less than 0.05.

Results

TPC, TFC, and DPPH scavenging activities of different decoctions have been demonstrated in Figures 8-10. As Figure 8 shows, *B. vulgaris* decoction have the significant highest amount of phenolic content as compared to others, followed by the synergistic, *O. stamineus*, and *T. polium* decoction ($p<0.05$). *B. vulgaris* decoction almost had three and two times more phenolic content as compared to *T. polium* and *O. stamineus* decoction respectively.

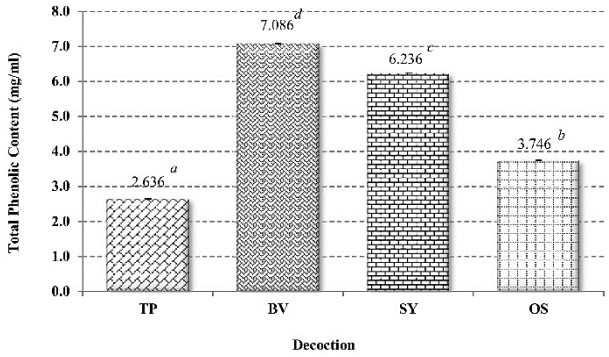

Figure 8- Comparison of the total phenolic content of different decoctions

[abcd] Values with the different superscripts are significantly different at $p<0.05$ based on one-way ANOVA, Duncan's post hoc test.
TP: *T. polium*, OS: *O. stamineus*, BV: *B. vulgaris*, SY: Synergistic

Nearly similar to TPC results, as Figure 9 shows, *B. vulgaris* decoction have the significant highest amount of TFC as compared to others, followed by *O. stamineus*, the synergistic, and *T. polium* decoction ($p<0.05$).
B. vulgaris decoction almost had five and two times more flavonoids content as compared to *T. polium*, and both *O. stamineus* and the synergistic decoction respectively.

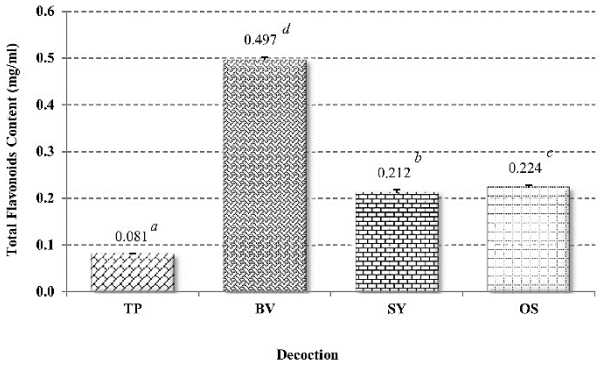

Figure 9- Comparison of the total flavonoids content of different herbal decoctions

[abcd] Values with the different superscripts are significantly different at $p<0.05$ based on one-way ANOVA, Duncan's post hoc test.
TP: *T. polium*, OS: *O. stamineus*, BV: *B. vulgaris*, SY: Synergistic

DPPH Scavenging Activities

As Table 1 and Figure 10 shows, all the decoctions had similar antioxidant activity at 20µg/ml concentration.
By increasing the concentration their scavenging activity increased differently. *B. vulgaris* showed significantly higher antioxidant activity as compared to another decoction ($p<0.05$). *T. polium* and *O. stamineus* showed similar activity and significantly lesser than both *B. vulgaris* and synergistic decoction ($p<0.05$). No significant difference was observed between 80 and 160 µg/ml concentration ($p>0.05$).

IC_{50} results also showed significantly lower amount for α-tocopherol, rutin, and BHA. *B. vulgaris* and synergistic decoction showed better activity as compared to other decoctions ($p<0.05$).

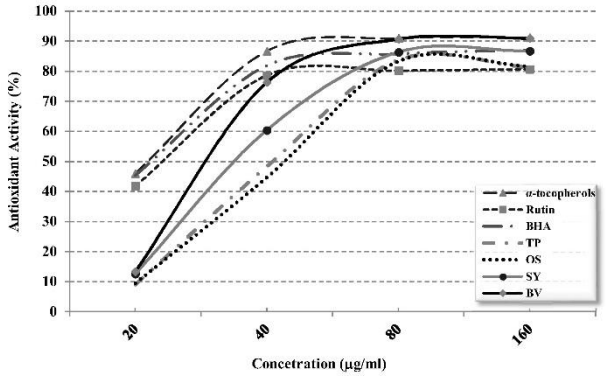

Figure 10- Antioxidant activity of different decoctions compared to standards based on dpph antioxidant scavenging

[abc] Values with the different superscripts are significantly different at $p<0.05$ based on one-way ANOVA, Duncan's post hoc test.
BHA: Butylated hydroxyanisole, TP: *T. polium*, OS: *O. stamineus*, BV: *B. vulgaris*, SY: Synergistic

Table 1- Comparison of the DPPH scavenging activity of different decoctions with standards

Substance	20	40	80	160	IC_{50}
	Mean±SEM (µg/ml)				
α-Tocopherols	46.0±0.4 [c]	86.6±1.9 [c]	91.6±1.2 [b]	91.2±3.5 [b]	22.4±0.8 [a]
Rutin	41.9±0.3 [c]	78.7±1.3 [c]	80.2±1.7 [a]	80.8±1.0 [a]	24.5±0.5 [a]
BHA	45.41±0.4 [c]	81.9±1.2 [c]	85.8±0.4 [b]	87.0±5.5 [a]	22.5±0.6 [a]
TP	9.1±0.8 [a]	48.4±1.7 [a]	83.7±0.5 [a]	81.2±1.4 [a]	41.5±0.7 [c]
OS	9.5±0.6 [a]	44.7±6.6 [a]	83.3±1.6 [a]	81.5±1.7 [a]	45.2±0.8 [c]
SY	12.6±0.4 [b]	60.4±1.6 [b]	86.3±0.3 [b]	86.7±1.1 [b]	34.4±0.9 [b]
BV	13.4±0.6 [b]	76.2±0.4 [c]	90.6±2.2 [b]	90.9±0.6 [b]	31.2±0.5 [b]

[abc] Values in the same column with the different superscripts are significantly different at $p<0.05$ based on one-way ANOVA, Duncan's post hoc test. The IC_{50} value is defined as the number of decoctions or standards, which need to decrease the initial DPPH radical concentration, by 50%.
BHA: Butylated hydroxyanisole, TP: *T. polium*, OS: *O. stamineus*, BV: *B. vulgaris*, SY: Synergistic

Discussion

Unlike most modern drugs, which they are made by special and certain amount of elements and chemicals, complementary drugs like herbal do not usually have the same contents. Any kind of herbal drugs is influenced by many factors. Genetic, pre-harvest (season of harvest, soil, type of water) and post-harvest factors (storage, temperature, moisture) can affect total phenolic, flavonoid, and antioxidant capacity as well as other elements and compounds[44,45]. Apart from mentioned factors, different preparation methods have different effects on herbal drugs too. Based on the present results, *B. vulgaris* decoction had a significantly higher amount of phenolic content as compared to others. After *B. vulgaris* the synergistic, showed significantly higher TPC level than *O. stamineus*, which had significantly higher TPC than *T. polium* decoction. *B. vulgaris* decoction almost had three and two times more phenolic content as compared to *T. polium* and *O. stamineus* decoction respectively. All these three herbs have shown different amount of TPC based on their preparation and their source[35,46–50]. Although TFC showed similar pattern to TPC which was expected, DPPH scavenging activity revealed that amount of TPC and TPC might not be necessarily counted as antioxidant activity. As *T. polium* with lower level of both TPC and TFC as compared to other decoctions, but with similar antioxidant activity to *O. stamineus*, it showed that there should be a compound in *T. polium* which has high level of antioxidant activity. Almost similar to total phenolic content results, *B. vulgaris* decoction has the significantly highest amount of flavonoids content as compared to others ($p<0.05$), followed by *O. stamineus*, the synergistic, and *T. polium* decoction. *B. vulgaris* decoction had about five and two times more flavonoids content as compared to *T. polium*, and both *O. stamineus* and the synergistic decoction respectively. Based on the present study, all the decoctions had similar antioxidant activity at 20 mg/ml concentration. By increasing the concentration, their scavenging activity increased differentiation. *B. vulgaris* showed significantly higher antioxidant activity as compared to other decoction, which this pattern was similar to previous studies[49,51]. *T. polium*, and *O.*

stamineus showed similar activity and significantly lesser than both *B. vulgaris* and synergistic decoction. As the present study showed both *T. polium*, and *O. stamineus* have shown high levels of scavenging activity in previous studies[35,52–55]. No significant difference was observed between 80 and 160 µg/ml concentration. IC_{50} results also showed a significantly lower amount for α-tocopherol, rutin, and BHA. *B. vulgaris* and synergistic decoction showed better activity as compared to other decoctions. Many studies on different herbs and fruits have shown anti-tumor activities with higher scavenging activity[56,57].

Overall, results of TPC, TFC, and DPPH scavenging activities showed possible beneficial effects of studies herbs and their mixed ones due to their components which might be used in prevention, suppression or treatment of NCDs.

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