Dynamic Comparison of Free Radical Scavenging Abilities of *Hypericum Perforatum L.*, *Herba Verbenae Officinalis*, and *Valeriana Officinalis L.* Extracts

Yi-ming Li[1], Yan-Ru Liu[2,3], Bing-Kun Xiao[2], Jian-Yun Yang[2], Rong-Qing Huang[2*]

[1]Anhui Medical University,Hefei 230032, P. R.China;
[2]Beijing Institute of Radiation Medicine, Haidian District, Beijing 100850, P.R. China;
[3]The School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenhe District, Shenyang 110016, P.R. China

ABSTRACT

Objectives: The purpose of this research was to compare the dynamic antioxidant activities and the free radical scavenging abilities of three botanical supplements, *Hypericum perforatum L*. (HPL), *Herba Verbena Officinalis* (HVO), and *Valeriana officinalis L*. (VO), which have been reported to effectively treat menopause symptoms. **Methods:** The antioxidant activities of the three supplements were determined by the ferric reducing ability of plasma (FRAP) assay. In addition, their free radical scavenging abilities were studied by ftheir interactions with the stable radicals of 2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). **Results:** The results indicated that the HPL ethanolic extract exhibited greater antioxidant activity (1.2688 ± 0.01 mmol Trolox/g) than the HVO ethanolic extract (1.1686 ± 0.043 mmol Trolox/g) and the VO ethanolic extract (0.2579 ± 0.0031 mmol Trolox/g) as compared to Nilestriol (0.0026 ± 0.0012), a positive control agent in the experiment. Moreover, the HPL extract showed remarkable free radical scavenging activity against ABTS•+, and the HVO extract was the most potent against DPPH•. **Conclusion:** These activities may be attributed to the total flavonoid or phenolic acid contents of among these extracts.

Key words: ABTS•+, DPPH•, *Hypericum perforatum L., Herba Verbena Officinalis,* Menopause Oxidative Stress, *Valeriana officinalis L.*

INTRODUCTION

Menopause generally occurs in women between 45 and 55 years of age. This permanent cessation of menses causes ovarian dysfunction and accelerates the decline of endogenous hormones, leading to many psychopathological reactions, such as hot flashes, arteriosclerosis, skin aging, insomnia, depression, etc. Previously, the link between oxidative stress and ovarian hormone secretion has been well documented. Synthetic hormones taken by women to restore natural ovarian hormone levels have been regarded as a "double-edged sword," since they increase the risk of cardiovascular diseases and breast cancer.[1-3] As an alternative therapy, some plant extracts (phytoestrogens) have been confirmed as powerful radical scavengers not only *in vitro* but also in rodents and human trials.[4,5] Recently, the correlation between female metabolism disorders, especially those causing undesirable menopause symptoms, and oxidative stress has been reported; in addition, there has been an increased interest in phytotherapy due to the risk of hormone therapy for menopause symptoms.[4] *Hypericum perforatum L.* (HPL).[6-9][Abdali, 2010 #104; van Die, 2009 #108; Al-Akoum, 2009 #106; Sloley, 2000 #82] *Herba Verbenae Officinalis* (HVO),[10] and *Valeriana officinalis L.* (VO)[11-13] are three traditional herbal supplements that have been used for many years in female metabolic disorder treatments and have been shown to improve

***Corresponding author:**
Mr. Rongqing Huang
Beijing institute of Raiation Medicine,
No.27, Taiping Road, Beijing,
Beijing 100850, PR China, Beijing-China ,
E-mail: rqhuang@aliyun.com

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women's health in experimental and clinical studies. Redox reactions *in vitro* and *in vivo* primarily involve the transfer of electrons and hydrogen atoms.[14] The synthetic stable molecules 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)[15] have been used to investigate the kinetics of radical scavenging reactions.[15-18] The reaction between antioxidants and DPPH free radicals (DPPH•) has a kinetic feature and is widely used to assess the ability of antioxidants to transfer labile protons to radicals.[15] The blue-green free radical cation of ABTS ($ABTS^{\bullet+}$), which is used to assess the ability of antioxidants to receive electrons from radicals, has also been extensively used to characterize antioxidants in solution.[17,18] The purpose of this study was to investigate the dynamic antioxidant activities and the free radical scavenging abilities of HPL, HVO, and VO extracts compared with the positive control Nilestriol, (3-(Cyclopentyloxy)-17alpha-ethinyl-1, 3, 5(10)-estratrien-16alpha, 17beta-diol), an orally available estrogen-type drug that has been often used in hormone replacement therapy to treat long-term side effects due to menopause symptoms.[19] Based on our previous methods,[20] the antioxidant activities of the three extracts and Nilestriol were determined by the ferric reducing ability of plasma (FRAP) assay. The free radical scavenging process was monitored by measuring absorbance as a function of time of reactions of each extract with the radicals $ABTS^{\bullet+}$ and DPPH•. The results of this study will determine whether these plant ethanolic extracts have the capacity to combat oxidative stress to treat undesirable menopause symptoms.

MATERIALS AND METHODS

Chemicals and Materials

Nilestriol (97% pure) and the stable free-radical scavengers ABTS (95% pure) and DPPH (95% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The total antioxidant capacity assay kit was purchased from Beyotime (Jiangsu, China). The standards rutin and gallic acid, and other chemicals and reagents (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Extracts preparation

Entire herbs of HPL, aerial parts of HVO, and roots/rhizomes of VO were collected from Shanxi, Hubei, and Gansu provinces in China, respectively. The crude plants were washed thoroughly with distilled water, dried under shade, and ground into powders that could pass through a sieve of pore size 0.5 mm. Each dry powder (100 g) was extracted with 1000 mL of 95% ethanol, and this process was repeated three times at room temperature(Each 1 g of extract was equivalent to 100 mg of dry herb).Then, the ethanolic extracts were filtered, and the filtrates were combined. Finally, ethanol was removed by vacuum distillation using a rotary evaporator at a reduced pressure and no more than 50°C, and the extracts were subsequently lyophilized and stored at -20°C until use.[21]

Botanical composition analysis

According to preclinical and clinical trials, flavonoids and phenolic acid are the major phytochemical compound types used for menopausal symptom relief treatment. Therefore, the total flavonoid and phenolic contents were determined via a UV-2501PC UV-Vis Recording Spectrophotometer (SHIMADZU, Japan). The total flavonoid content was determined by an aluminium chloride colorimetric method.[22] The absorbance was determined at 403 nm. The total flavonoid content was expressed as mg of rutin equivalents (RE)/g of dry plant powder (DP) that was calculated by the calibration curve equation: $y=24.5x + 0.0137$ ($r=0.9998$), where y and x represent absorbance and RE, respectively (the standard rutin concentration ranged from 2 to 24 μg/mL). The total phenolic acid content was determined using the Folin-Ciocalteu method.[23] The absorbance was measured at 500 nm. The standard curve of the method was $y=2.5757x - 0.006$ ($r=0.9990$), where y is absorbance and x is gallic acid equivalents (GAE; the standard gallic acid concentration ranged from 25 to 250 μg/mL). The total phenolic acid content was expressed as mg of GAE/g of DP based on the above calibration curve equation.

Antioxidant activity assay

To measure the total antioxidant activity of Nilestriol (positive control), HPL, HVO, and VO extracts, the FRAP assay, developed by Benzie *et al.* was used. Standard solutions of $FeSO_4 \bullet 7H_2O$ (0.625, 1.25, 2.5, 5, and 10 mM) were prepared in deionized water. Each extract was diluted 2:98 (v/v) in 2,3,5-triphenyl-tetrazolium chloride (TPTZ), and the experimental procedure followed that of Benzie *et al.*[24] The FRAP was defined as the concentration of $FeSO_4$ that provided an antioxidant activity equivalent to 1 mM of the substance under investigation, and the antioxidant activity was given in units of mmol Trolox/g of extract.

Dynamic process of scavenging ABTS•+

A stock solution of $ABTS^{\bullet+}$ was prepared according to the

procedure of Re *et al.*,[25] with some minor modifications. The ABTS$^{\bullet+}$ solution was produced by incubating 2.8 mM potassium persulfate with 7 mM ABTS in water for 12 to 16 h. The solution was kept in the dark until use, and it was used within 24 h. The stock solution was diluted 125-fold with phosphate-buffered saline (PBS, 5 mM sodium phosphate, pH 7.2) to obtain a standard solution that contained approximately 36 μM ABTS$^{\bullet+}$ and 18 μM ABTS. The HPL, HVO, and VO extracts and control reagents were added as described, and the reaction was followed by monitoring the change in the absorbances of ABTS and ABTS$^{\bullet+}$ by a Cary 50 spectrophotometer (Varian) using Cary WinUV software.

Determining the dynamic change of ABTS•+ and ABTS

The absorbance of the reactions between ABTS$^{\bullet+}$ and HPL, HVO, or VO extract was measured and compared with the PBS control (5 mM, pH 7.2). After reading the initial absorbance, each extract was diluted 2:98 (v/v) in ABTS$^{\bullet+}$ stock solution. (The final concentrations of HPL, HVO, and VO extracts were 30 μg /mL). The reaction of each extract with the standard ABTS$^{\bullet+}$/ABTS solution was followed by measuring the absorbance at 415 nm and 340 nm using absorbance scanning on a Cary 50 spectrophotometer (Varian) using Cary WinUV software. The concentrations of ABTS and ABTS$^{\bullet+}$ were calculated using the following equations: $\varepsilon_{340} = 4.8 \times 10^4/M/cm$ and $\varepsilon_{415} = 3.6 \times 10^4/M/cm$, respectively.[18] The initial reaction rates of ABTS$^{\bullet+}$ consumption for 0 to 600 s were determined.

DPPH radical scavenging assays

A working solution of DPPH$^{\bullet}$ was prepared using a 0.1 mM DPPH solution in methanol, and the solution was kept in the dark before use. The proton transfer reactions between each extract (HPL, HVO, and VO) and DPPH$^{\bullet}$ were monitored in real time by a Cary 50 (Varian) spectrophotometer using Cary WinUV software. The DPPH$^{\bullet}$ concentration was calculated using the following equation: ε_{515}=11,240/M/cm.[26] The reaction between DPPH$^{\bullet}$ and each extract was observed by measuring the absorbance from 260 to 700 nm of a reaction mixture of extract in DPPH$^{\bullet}$ solution (5:95 (v/v)). Each extract or control reagent (5 mM PBS, pH 7.2) was added as described for each experiment, and the reaction was followed by monitoring the change in the absorbance of DPPH$^{\bullet}$ at 515 nm. Time-resolved absorption spectra were collected with times ranging from 0 to 600 s.

Table 1: Total phenolic acid and flavonoid contents of the dry plant extracts (n = 3).

Plant extract	Total Flavonoid (mg RE/g DP)	Total Phenolic acid (mg GAE/g DP)
HPL	86.8 ± 3.4	47.3 ± 1.4
HVO	14.9 ± 0.3	80.7 ± 2.6
VO	4.2 ± 0.3	22.8 ± 0.8

Total flavonoid content is expressed as mg of rutin equivalents (RE) per g of dry plant powder (DP). Total phenolic acid content is expressed as mg of gallic acid equivalents (GAE) per g of DP. Data are represented as means ± standard error (SE) of three replicates.

RESULTS

Botanical composition analysis

The contents of total flavonoids and total phenolic acid among HPL, HVO, and VO extracts are listed in Table 1 Clearly, HPL had a greater total flavonoid content among these extracts, while VO contained the least. On the other hand, the highest content of total phenolic acid was observed in HVO, followed by HPL and VO.

Antioxidant activity determination

The FRAP values of HPL, HVO, and VO extracts compared to Nilestriol were determined in order to evaluate their antioxidant activities. Their reducing abilities were measured spectrophotometrically by their absorbance at 593 nm, and the data were summarized. According to the standard reaction curve, the mean antioxidant activities (represented by the FRAP values) of Nilestriol, HPL, HVO, and VO extracts were 0.0026±0.0012, 1.27±0.010, 1.17±0.043, and 0.258±0.0031 mmol Trolox/g (±SE), respectively. Thus, HPL, HVO, and VO extracts had a low but significant level of antioxidant activity.

Reaction between ABTS•+ and HPL, HVO, and VO extracts

The kinetics process is important for the free radical scavenging activity determination of Nilestriol, HPL, HVO, and VO extracts; thus, ABTS•+ was used to evaluate the antioxidant capacities.[27] In our experiments, the ABTS•+ concentration was significantly reduced by each extract studied (Figure 1A,1B,1C,1D). The spectra were collected from 290 nm to 900 nm as a function of time, and they revealed a decrease in the ABTS•+-specific absorbance at 415 nm and a corresponding increase in the ABTS-specific absorbance at 340 nm. ABTS•+ alone and Nilestriol exhibited a minor amount of auto-scavenging activity (Figure 1E); however, the HPL, HVO, and VO extracts significantly increased this activity in a time-dependent

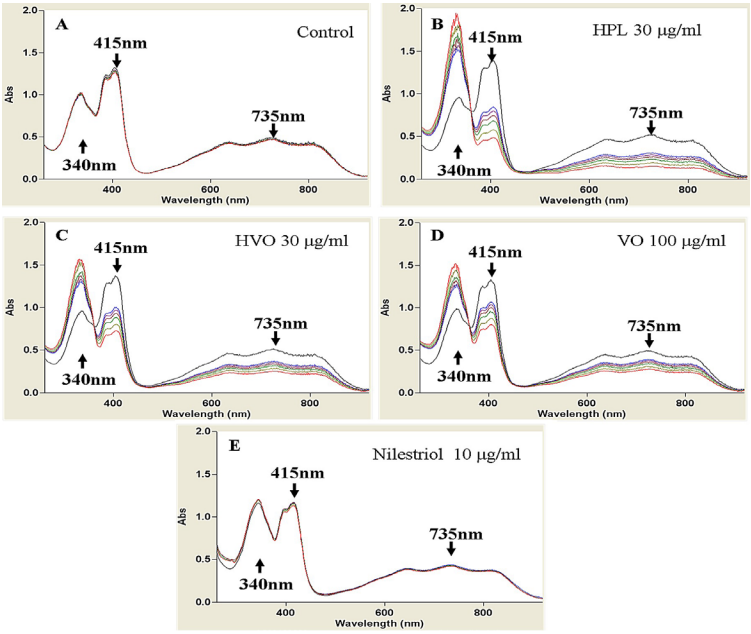

Figure 1: Reactions between ABTS•+ and PBS control (A), HPL (B), HVO (C), VO (D) extracts, and Nilestriol (E).

The control and extracts were added to the ABTS•+ solution. The final concentrations of HPL and HVO were 30 μg/ml, and that of VO was 100 μg/ml. The absorption spectra at 340, 415, and 735 nm were read at regular time intervals from 0 s to 600 s.

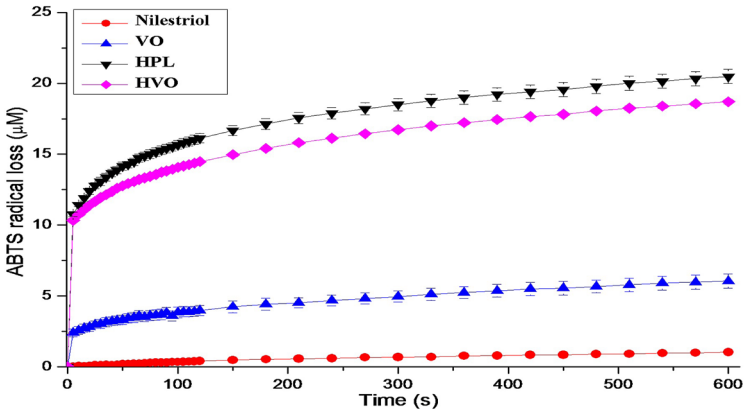

Figure 2: The ABTS•+ dynamic scavenging activities of HPL, HVO, and VO extracts and Nilestriol.

The final concentrations of HPL, HVO, and VO were 30 μg/ml, while that of Nilestriol was 10 μg/ml. Reactions between the standard ABTS•+/ABTS solution and HPL, HVO, or VO extracts or Nilestriol were followed by reading the absorbances at 415 nm and 340 nm. The initial reaction rate and the end of ABTS•+ consumption during the initial rapid reaction phase were estimated as shown in the figure.

manner. Thus, HPL, HVO, and VO extracts have a slow but stable level of dynamic ABTS•+ scavenging activity.

Dynamic process of ABTS formation and ABTS•+ loss

To determine the process of ABTS formation and ABTS•+ loss, the total concentrations of ABTS•+ and ABTS were calculated. Upon addition of each extract to the ABTS•+ solution, the dark green free radicals were converted into the colorless compound ABTS. The amounts of increased ABTS and decreased ABTS•+ were quantified by measuring the increase in the absorbance at 340 nm (ε340 =4.8 × 104/M/cm) and the decrease in absorbance at 415 nm (ε415=3.6 × 104/M/cm). As shown in (Figure 2), ABTS formation and ABTS•+ consumption by Nilestriol, HPL, HVO, and VO extracts occurred in a time-dependent manner.

Reaction between DPPH• and HPL, HVO, and VO extracts

The typical sets of full-band scanning that were recorded after mixing 0.1 mM DPPH• with Nilestriol (0.12 mg/mL), HPL, HVO, and VO extract solutions (0.25 mg/mL, respectively). The reactions were completed within 600 s (Figure 3A, 3B, 3C, 3D, 3E). DPPH• was significantly reduced by HPL, HVO, and VO extracts. The spectra were collected from 260 nm to 800 nm as a function of time, and they revealed a decrease in the DPPH•- specific

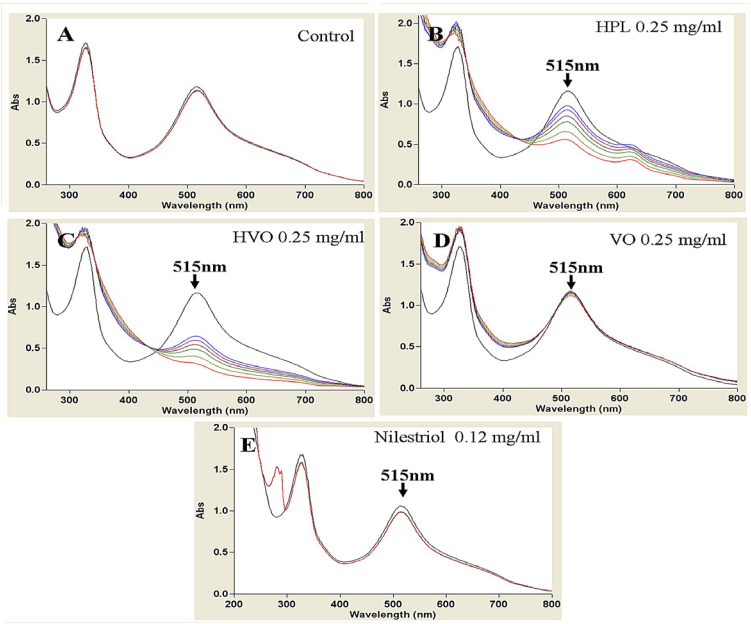

Figure 3: Reactions between DPPH• and PBS control (A), HPL (B), HVO (C), VO (D) extracts and Nilestriol (E).

The extracts or control reagents were added to the DPPH• solution to give an extract final concentration of 0.25 mg/ml and a Nilestriol final concentration of 0.12 mg/ml. The absorption spectra at 515 nm were read at regular time intervals from 0 s to 600 s..

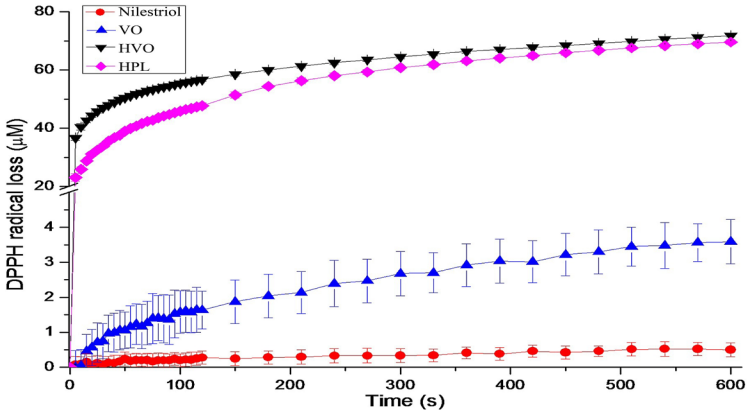

Figure 4: The DPPH• dynamic scavenging activities of HPL, HVO, and VO extracts and Nilestriol.

The final concentrations of HPL, HVO, and VO were 0.25 mg/ml and that of Nilestriol was 0.12 mg/ml. The reactions of each extract with the standard DPPH•/DPPH solution were followed by measuring the absorbance at 515 nm. The initial reaction rate and the end of DPPH• consumption during the initial rapid reaction phase were estimated as shown in the figure.

absorbance at 515 nm. The time profiles of these changes are shown in (Figure 3A, 3B, 3C, 3D, 3E). Thus, HPL, HVO, and VO extracts have a slow but stable dynamic DPPH• scavenging activity.

Dynamic process of DPPH• loss

To identify the process of DPPH• loss, the DPPH• concentration was calculated. Nilestriol, HPL, HVO, and VO extracts were reacted with DPPH•, which converted the red radicals into colorless compounds. The amount of the reduction of DPPH• was quantified by measuring the increase in the absorbance at 515 nm (ε515=11,240/M/cm). As shown in (Figure 4), DPPH• consumption occurred in a time-dependent manner.

DISCUSSION

The botanical supplements HPL, HVO, and VO have been shown to demonstrate antioxidant and free radical scavenging abilities.[28] Previous research has only investigated the dynamic parameters of their free radical scavenging activities with respect to hydroxyl radical scavenging, so new data are needed for other radicals. VO is widely used for the treatment of anxiety and unrest.[29,30] One in vivo study has demonstrated the free radical oxidation of valerian extract, suggesting that it could be modulated

by psychotropic drugs.[31] Arecent study examined the antioxidant activities of terpenoids from valerian oil using DPPH• scavenging activity and FRAP assays. The results indicated that terpenoid structures scavenged DPPH• and increased the ferric reducing capacity in a concentration-dependent manner.[32] These reports suggest that free radical scavenging is an important physiological function of HPL, HVO, and VO extracts; however, the kinetic process of radical scavenging has not yet been documented.The main findings of our study were that HPL, HVO, and VO extracts all show potent antioxidant activity and dynamic scavenging activities of $ABTS^{\cdot}+$ and DPPH·. Our current results suggest the following: (1) rapid and stable radical scavenging capacity is an important characteristic of HPL, HVO, and VO extracts, (2) the scavenging activity of HPL, HVO, and VO extracts is time-dependent, and (3) the $ABTS^{\cdot}+$ and DPPH· scavenging ability of these extracts gradually increased during the time course of the reaction, (4)HPL indicated a remarkably high total flavonoid content when compared to HVO and VO, which showed a negative correlation to ABTS•+ scavenging activities. Meanwhile, the total phenolic acid content of HVO was twice and four times greater than HPL and VO, respectively, which highly correlated with its DPPH• scavenging activities. Therefore, these data support the hypothesis that HPL, HVO, and VO extracts may prevent reactive oxygen species-related injuries by scavenging free radicals in the body.

CONCLUSION

According to the assays of ABTS•+ and DPPH•, we found that HPL, HVO, and VO extracts had a slow but stable level of dynamic ABTS•+ and DPPH• scavenging activity. These activities may be attributed to the total flavonoid or phenolic acid contents of among these extracts.

CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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