

Neuromodulatory and Protective Effects Induced by the Association of Herbal Extracts from *Valeriana officinalis*, *Ziziphus jujuba*, and *Humulus lupulus* with Melatonin: An Innovative Formulation for Counteracting Sleep Disorders

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Assays for Total Phenolic and Flavonoid Contents

The total phenolic content was determined by employing the methods given in the literature with some modification. The sample solution (0.25 mL) was mixed with diluted Folin–Ciocalteu reagent (1 mL, 1:9, v/v) and shaken vigorously. After 3 min, Na₂CO₃ solution (0.75 mL, 1%) was added, and the sample absorbance was read at 760 nm after a 2 h incubation at room temperature. The total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE/g extract). The total flavonoid content was determined using the AlCl₃ method. Briefly, the sample solution (1 mL) was mixed with the same volume of aluminum trichloride (2%) in methanol. Similarly, a blank was prepared by adding a sample solution (1 mL) to methanol (1 mL) without AlCl₃. The sample and blank absorbances were read at 415 nm after a 10 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. Rutin was used as a reference standard and the total flavonoid content was expressed as milligrams of rutin equivalents (mg RE/g extract) [1].

Determination of Antioxidant Effects

Antioxidant (DPPH and ABTS radical scavenging, reducing power (CUPRAC and FRAP), phosphomolybdenum, and metal chelating (ferrozine method)) [2].

For the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay: The sample solution was added to 4 mL of a 0.004% methanol solution of DPPH. The sample absorbance was read at 517 nm after a 30 min incubation at room temperature in the dark. DPPH radical scavenging activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline) 6-sulfonic acid) radical scavenging assay: Briefly, ABTS⁺ was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12–16 h in the dark at room temperature. Prior to beginning the assay, the ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.02 at 734 nm. The sample solution was added to the ABTS solution (2 mL) and mixed. The sample absorbance was read at 734 nm after a 30 min incubation at room temperature. The ABTS radical scavenging activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For the CUPRAC (cupric ion reducing activity) activity assay: The sample solution was added to a premixed reaction mixture containing CuCl₂ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM), and NH₄Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding a sample solution (0.5 mL) to a premixed reaction mixture (3 mL), without CuCl₂. Then, the sample and the blank absorbances were read at 450 nm after a 30 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For the FRAP (ferric reducing antioxidant power) activity assay: The sample solution was added to a premixed FRAP reagent (2 mL), containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1 (v/v/v). Then, the sample absorbance was read at 593 nm after a 30 min incubation at room temperature. The FRAP activity was expressed as milligrams of trolox equivalents (mg TE/g extract).

For the phosphomolybdenum method: The sample solution was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The sample absorbance was read at 695 nm after a 90 min incubation at 95 °C. The total antioxidant capacity was expressed as millimoles of trolox equivalents (mmol TE/g extract).

For the metal chelating activity assay: Briefly, the sample solution was added to FeCl₂ solution (0.05 mL, 2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Similarly, a blank was prepared by adding the sample solution (2 mL) to the FeCl₂ solution (0.05 mL, 2 mM) and water (0.2 mL), without ferrozine. Then, the sample and the blank absorbances were read at 562 nm after 10 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The metal chelating activity was expressed as milligrams of EDTA (disodium edetate) equivalents (mg EDTAE/g extract).

1. References

1. Zengin, G.; Aktumsek, A. Investigation of Antioxidant Potentials of Solvent Extracts from Different Anatomical Parts of *Asphodeline anatolica* E. Tuzlaci: An Endemic Plant to Turkey. *Afr J Tradit Complement Altern Med* 2014, 11, 481–488, doi:10.4314/ajtcam.v11i2.37.
2. Uysal, S.; Zengin, G.; Locatelli, M.; Bahadori, M.B.; Mocan, A.; Bellagamba, G.; De Luca, E.; Mollica, A.; Aktumsek, A. Cytotoxic and Enzyme Inhibitory Potential of Two *Potentilla* Species (*P. Speciosa* L. and *P. Reptans* Willd.) and Their Chemical Composition. *Front Pharmacol* 2017, 8, 290, doi:10.3389/fphar.2017.00290.