AN IN VITRO STUDY OF ANTIOXIDANT CAPACITY AND Radical Scavenging Effect of Spinacia oleracea Leaf Extract

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ABSTRACT

Objective: The study was carried out to evaluate the preliminary phytochemical screening and antioxidant activity of ethanolic extract of Spinacia oleracea (SO).

Methods: The leaves of SO were shade dried, and the extract was prepared using solvent ethanol by Soxhlet extraction method. The preliminary phytochemical screening was carried out on the leaf extract of the plant. The total phenolic content and total flavonoids were estimated using Folin-Ciocalteu’s and aluminum chloride reagents, respectively. Antioxidant activities were studied using 1,1-diphenyl-2-picrylhydrazyl, nitric oxide, hydrogen radical, lipid peroxidation, and phosphomolybdenum radical scavenging assays.

Results: The preliminary phytochemical analysis revealed the presence of bioactive constituents such as phenols, alkaloids, flavonoids, saponins, and glycosides. As SO is a rich source of different bioactive components, it contains a considerable amount of flavonoids and phenols. The different antioxidant assays proved that spinach is one of the best antioxidants with its ability to scavenge different radicals that generate oxidative stress.

Conclusion: The observed activity may be associated with bioactive components such as phenols and flavonoids present in the leaf extracts and could have greater importance as nootropic plant in oxidative stress-related degenerative diseases such as Alzheimer and dementia.

Keywords: Spinacia oleracea, Antioxidant activity, 1,1-Diphenyl-2-picrylhydrazyl, Saponins, Flavonoids, Phenols, Anti-Alzheimer activity.

INTRODUCTION

The oldest form of health care known to humanity is the use of herbs as medicine. It has been used in all cultures throughout the history. In ancient time, tribal people methodically collected information on herbs and developed well-defined herbal pharmacopeias. There is physical evidence of the use of herbal remedies. In the 20th century, much of the pharmacopeia of scientific medicine was derived from the herbal knowledge of the native people. Plants or part of a plant that has been converted into phytomedicines using simple processes involving harvesting, drying, and storage is known as "herbal drug." In addition to the definition, it also includes other crude products derived from plants, which no longer show any organismic structure, such as essential oils, fatty oils, resins, and gums [1,2].

The substances that neutralize free radicals or their actions are known as "antioxidants." Every cell is having adequate protective mechanisms to fight against any harmful effects of free radicals such as glutathione peroxidase, glutathione reductase, thioredoxin, thiols, and disulfide bonding. These are buffering systems in every cell. Vitamin E and C, carotenoids, flavonoids, and related polyphenols are essential nutrient which is responsible for preventing the propagation of free radical reactions in all cell membranes in the human body. Nowadays, the demand for natural drug products which produces antioxidants is increasing day by day.

“Oxidative stress” is a concept that defines the relationship between free radical and a disease. The generation of prooxidants in the form of reactive oxygen species and reactive nitrogen species which are present in healthy human body when gets exposed to adverse physicochemical, environmental, or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, and overnutrition the favorable conditions for prooxidants occurs by shifting the delicate balance between prooxidants and antioxidative mechanism in body which further results in the formation of "oxidative stress.”

Natural compounds which are derived from dietary sources provide a large number of antioxidants. Tea is also rich sources of antioxidants. Catechin is one of the most active constituents present in it which acts as a potent antioxidant. Apart from the dietary sources, a number of Indian medicinal plants are also a rich source of antioxidants. [3,4] Superoxide dismutase, catalase, glutathione peroxidase, or non-enzymatic compounds, such as uric acid, bilirubin, albumin, and metallothioneins, are the enzymes but known for their endogenous antioxidant activity. Exogenous antioxidants came into existence when the endogenous factors were not able to control oxidative stress causing agents. Examples of exogenous antioxidants are Vitamin E, Vitamin C, β-carotene, Vitamin K, flavonoids, mineral Se, Vitamin D, and Vitamin K2 [5].

Plants are one of the most important sources of medicines. The medicinal plants are rich in secondary metabolites (which are potential sources of drugs) and essential oils of therapeutic importance. The important advantages claimed for the therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective, and their easy availability. Spinacia oleracea Linn. (SO) is a plant having medicinal property native to central and southwestern Asia. It is cultivated for the sake of its succulent leaves and was introduced in Europe in the 15th century. It is the favorite food among Indians in the winter season [6].

SO is plant known as spinach is one of the rich sources of nutrients. It is widely cultivated all over the world having different pharmacological activities. Therefore, the aim of the present study is to determine different phytoconstituents of SO and its in vitro antioxidant activity to correlate its medicinal or pharmacological activity due to the presence of abundant phytoconstituents [7].

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MATERIALS AND METHODS

Instrument
UV Spectrophotometer JASCO V-730.

Software: Spectra manager.
Version 2.13.00.

Collection of plant material
The fresh leaves of SO were collected from different regions of Pune (Maharashtra, India). The plant material was authenticated by the Botanical Survey of India, Pune.

Preparation of ethanolic extracts
The leaves of SO were washed under running tap water. The leaves were shade dried, and then, they were minced using a mixer grinder. 50 g of coarse powder was weighed for the Soxhlet extraction procedure. 50 g extract was placed in a thimble of muslin cloth. The thimble was mounted on a round bottom flask which was placed on heating mantle. Around 250 ml solvent, i.e., ethanol was added to the thimble to complete one and half manual cycle of Soxhlet. Condenser was placed over the Soxhlet extractor, and soxhletion process was started at 55°C. Until complete discoloration of the solvent in the syphon tube, i.e., up to seven syphon cycles, the process was carried out. The extract was removed, and the solvent was evaporated with the help of rotary evaporator. A semisolid extract obtained was weighed, and the percentage yield was calculated.

Methods
Phytochemical screening
The ethanolic leaf extract was tested for the presence of phytoconstituents such as flavonoids, alkaloids, glycosides, phenolics, and saponins using various tests [8].

Determination of total phenolic content
Principle
The Folin–Ciocalteu’s assay has been used as a measure of total phenolic content. The mechanism is based on oxidation-reduction reaction. In the original Folin–Ciocalteu’s assay, the carbonate is used for pH adjustment and the end point of the reaction was attained after 120 min at room temperature [9-11].

Procedure
It has been reported by a number of methods [9-13], but the procedure was referred from reference [12,13]. Folin–Ciocalteu’s reagent was used for the estimation of phenolic content. 1 ml Folin reagent and 0.2 ml extract concentration were added and kept at room temperature for 5 min, and then, 0.8 ml of 2% sodium carbonate was added to it. Then, volume was made up by distilled water up to 10 ml and kept for incubation at room temperature for 2 h, and the absorbance was taken at 760 nm against blank as ethanol. Gallic acid was used a standard. Total phenolic content was expressed in μg of gallic acid equivalent of dry plant material [12,13].

Total flavonoid content
Principle
Total flavonoid content was determined by aluminum chloride (AlCl₃) colorimetric method. In this method, AlCl₃ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid-labile complexes with the orthohydroxyl groups in the A-or B-ring of flavonoids [14].

Procedure
2% AlCl₃ hexahydrate (6H₂O) was prepared (2 g in 100 ml ethanol). Aliquots of 1.5 ml of each extract dilutions were added into an equal volume of 2% AlCl₃-H₂O. The mixture mixed properly and absorbance was taken at 367 nm after 10 min of incubation at room temperature. Quercetin was used as a standard for calibration curve. Linear equation was plotted. Flavonoid content expressed in mg of quercetin equivalent by dry weight of spinach [15].

In vitro antioxidant activity
1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity
Principle
DPPH assay performed with some modifications in Brand-Williams protocols. Delocalization of spare electron over the molecule gives stability to DPPH radical, and thus, it prevents dimer formation. This radical is used in DPPH radical scavenging capacity assay to quantify the ability of antioxidants to quench the DPPH radical. When the radical form reduced to its non-radical form stable organic nitrogen-centered free radical, then the dark purple color of DPPH gets lost to a pink color which when reduced to its non-radical form by antioxidants which becomes colorless. In model system for the investigating the scavenging activities of several natural compounds, DPPH radical is widely used. When the DPPH radical is scavenged, the absorbance decreases at wavelength 517 nm with a change in color of the reaction mixture from purple to yellow [16,17].

Procedure
The stock solution of DPPH was prepared by dissolving 8 mg in 100 ml ethanol. The extract dilutions were prepared from the 1000 ppm solution in series of volumetric flasks to obtain appropriate range. 1 ml from the extract dilutions was added to the previously prepared volumetric flasks containing 1 ml of DPPH into each and kept it for 20 min at the dark place. 2 ml of ethanol was added into it after 20 min. It was mixed properly by vortexing it. The absorbance was measured at 517 nm against ethanol. Quercetin was used as a standard. The percentage of inhibition was calculated against blank.

\[ I\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100 \]

Where \( A_{\text{blank}} \) is the absorbance of control reaction (containing all reagents except the test compound) [18,19].

Nitric oxide scavenging assay
Principle
It is a diazotization reaction. Sodium nitroprusside generates nitric oxide in phosphate buffer at physiological pH which interacts with oxygen to produce nitrite ions which were measured by using Griess reaction reagent at wavelength 540 nm.

Procedure
Phosphate buffer (pH 7.8) was prepared. 10 mM sodium nitroprusside was dissolved in phosphate buffer. 3 ml of the above solution was added to 2 ml of extract and reference (quercetin) with different concentrations. Incubated the resulting solution at 25°C for 1 h. Similar procedure was repeated for blank sample which served as control. To 5 ml of the incubated mixture, add 0.5 ml of Griess reagent. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and coupling with α-naphthyl ethylenediamine was measured at 540 nm. Activity is measured in the terms of percentage inhibition [20].

Reducing power assay
Principle
The total antioxidant activity can be quantified by the ferric reducing antioxidant power assay. The flavonoids and phenolic acids possess strong antioxidant activity due to which they form the complex with metal atoms, particularly iron and copper. The method is based on the principle of increase in the absorbance of the reaction mixtures, as absorbance increases, it indicates an increase in antioxidant activity. The antioxidant compound present in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid, and ferric chloride, which was measured at 700 nm by UV-spectrophotometer.
Hydroxyl radical scavenging activity

**Principle**

Hydroxyl radical scavenging assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of plant sample. In this assay, quantification of the 2-deoxyribose degradation product is done, by its condensation with thiobarbituric acid. The reaction carried out between ascorbic acid-iron-ethylenediaminetetraacetic acid (EDTA) model which is responsible for the formation of hydroxyl radical. The reaction was performed in two conditions: In the presence of EDTA to determine OH scavenging capacity and in the absence of EDTA to determine its metal chelation property [22].

**Procedure**

Reaction mixture [Phosphate buffer [pH 7.4, 100 µl], extract concentration [500 µl], 1.04 mM EDTA [200 µl], 1:1 v/v Ferric Chloride [200 µl], 1.0 mM hydrogen peroxide [100 µl], and mM ascorbic acid [100 µl]] was prepared and incubated for 1 h at 37°C. Then, 1 ml 2.8% trichloroacetic acid and 1% thiobarbituric acid were added to the mixture. The mixture was heated at 100°C for about 15 min and then cooled. After cooling, absorbance was taken at 532 nm. Thiourea was used as standard as it is a good scavenger of hydroxyl radical [23-25].

Lipid peroxidation inhibition assay

**Principle**

The assessment of lipid peroxidation involves thiobarbituric acid reactivity. The thiobarbituric acid involved for boiling the test sample and free radicals were produced by FeSO₄ (10% in distilled water, v/v) was added to the 0.1 ml of extract, and the volume was made up to 1 ml by distilled water. 0.05 ml FeSO₄ (0.07M) was incubated at 37°C for 30 min to induce lipid peroxidation. Then, 1.5 ml acetic acid (20%) and 1.5 ml thiobarbituric acid (8%) were added to the above mixture. The mixture was vortexed for 5 min and then heated at 95°C for 1 h. Cooled and 1 ml butanol was added to it. Then, it was centrifuged at 3000 rpm for 10 min. Pink-colored product shows maximum absorbance at 532 nm when two molecules of thiobarbituric acid react with one molecule of malondialdehyde and this reaction is called thiobarbituric acid assay [26].

**Procedure**

Egg yolk homogenate was prepared and was used as a lipid source, and free radicals were produced by FeSO₄, 0.5 ml egg yolk homogenate (10% in distilled water, v/v) was added to the 0.1 ml of extract, and the volume was made up to 1 ml by distilled water. 0.05 ml FeSO₄ (0.07M) was incubated at 37°C for 30 min to induce lipid peroxidation. Then, 1.5 ml acetic acid (20%) and 1.5 ml thiobarbituric acid (8%) were added to the above mixture. The mixture was vortexed for 5 min and then heated at 95°C for 1 h. Cooled and 1 ml butanol was added to it. Then, it was centrifuged at 3000 rpm for 10 min. Pink-colored product obtained showed maximum absorbance at 532 nm. The same procedure was repeated for ascorbic acid which was used as a standard [27].

Phosphomolybdenum reduction assay

**Principle**

The assay is based on the reduction of Mo (VI) to Mo (V) by the methanol extract of leaves of SO and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1 ml of various concentrations (5-150 µg/ml) of extract was added to 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. Greenish-blue color was produced. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer. Ascorbic acid was used as a reference compound [28].

**Procedure**

1 ml of various concentrations of extract added with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer. Ascorbic acid was used as a standard [29].

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening carried out on the ethanolic extract of SO revealed the presence of various bioactive ingredients such as alkaloids, glycosides, saponins, phenols, and flavonoids. It is documented that these phytoconstituents may have several pharmacological activities (Table 1).

**Total phenolic content**

The total phenolic content of the ethanolic extracts of SO was measured by using Folin-Ciocalteu reagent, and the results were expressed in terms of mg of gallic acid equivalent (the standard curve equation y = 0.093x - 0.0403, R² = 0.995). Phenolic compounds may contribute directly to antioxidant action. The total phenolic content found was 11.25% of “gallic acid equivalent” per mg extract in ethanol.

**The total flavonoid content**

The total flavonoid content of the extracts was expressed in terms of quercetin equivalent (the standard curve equation y = 0.0998x - 0.0236, R² = 0.9871). The total flavonoid content of extract was 3.51% “quercetin equivalent” per mg plant extract.

**In vitro antioxidant activity**

**DPPH radical scavenging activity**

DPPH assay of ethanolic extract of SO revealed the presence of antioxidant potential. The percentage of inhibition was observed in all the concentrations of the plant extract. The free radicals were trapped and scavenged by the plant extract in a concentration-dependent manner. The percentage inhibition of scavenging activities, i.e., inhibitory concentration (IC₅₀) value of ethanol extract of SO for DPPH shown at 50 μg/ml concentration. Polyphenolic compounds in the plant are responsible for showing the antioxidant activity (Table 2).

It indicates that amount of elements contributing to DPPH radical scavenging activity is equal to 31.11% calculated in terms of quercetin.

### Table 1: Phytochemical tests and results of the ethanolic leaf extract of Spinacia oleracea

<table>
<thead>
<tr>
<th>S. No</th>
<th>Phytoconstituents</th>
<th>Chemical reagents</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Mayer's reagent</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dragendorf's reagent</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hager's reagent</td>
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<td></td>
<td></td>
<td>Wagner's reagent</td>
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<tr>
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<td>Legal's test</td>
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<td>Keller–Kiliani test</td>
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<td></td>
<td></td>
<td>Liebermann's test</td>
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<tr>
<td>2</td>
<td>Glycoside</td>
<td>Bajet test</td>
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<tr>
<td></td>
<td></td>
<td>Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Saponins</td>
<td>Roam test</td>
<td>+</td>
</tr>
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<td></td>
<td></td>
<td>Hemolysis test</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Flavonoids</td>
<td>Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Phenolic components</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*+Represents that compound is present, –represents that compound is absent*
Nitric oxide scavenging assay
Ethanolic extract of SO caused a moderate dose-dependent inhibition of nitric oxide with an IC₅₀ of 60 µg/ml which gave 56.66% inhibition. Quercetin was used as a reference compound, and its IC₅₀ value is 0.2 µg/ml (Table 3).

Reducing power assay
Fe³⁺ was transformed to Fe²⁺ in the presence of an ethanolic extract of SO and the reference compound quercetin to measure the reductive capability. At 10 µg/ml, the absorbances of the plant extract and quercetin were almost the same. The results of reducing power assay are provided in Table 4. In the assay, the absorbance is increased with increasing the concentration of the extracts indicating the presence of constituents with reducing power (Table 4).

Hydroxyl radical scavenging assay
Hydroxyl radical-mediated deoxyribose degradation in Fe³⁺-EDTA-ascorbic acid-H₂O₂ reaction mixture was done in this assay. The results are shown in Table 5. The IC₅₀ values of the ethanolic extract of SO for scavenging hydroxyl radicals are 250 µg/ml in the presence of EDTA with maximum inhibition at a concentration of 2000 µg/ml and IC₅₀, i.e., IC₅₀ value of standard 100 µg/ml in the presence and absence of EDTA with maximum inhibition at a concentration of 500 µg/ml (Table 5).

The results revealed that the amount of elements contributing to hydroxyl radical scavenging activity is equal to 35.39% calculated in terms of thiourea.

Lipid peroxidation inhibition assay
The result of anti-lipoperoxidation free radicals of the ethanolic extract of SO to prevent peroxidation showed that trapping potential for LPO radicals with IC₅₀, i.e., IC₅₀ is shown by concentration 1500 µg/ml. Oxidative stress in cells and tissues can be best checked by its lipid peroxidation assay, a well-established mechanism both in plants and animals (Table 6).

Phosphomolybdenum reduction assay
The absorbance of SO extract was found to be 0.1624 at a concentration of 100 µg/ml and 0.2124 at 150 µg/ml. The reducing power calculated by this method indicates that the content of reducing agents is 28.05% in terms of ascorbic acid (Table 7).

Table 2: DPPH scavenging activity of extract and quercetin

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of ethanolic extract of SO (µg/ml)</th>
<th>% inhibition by extract*</th>
<th>Concentration of quercetin (µg/ml)</th>
<th>% inhibition by quercetin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>47.83</td>
<td>5</td>
<td>47.28</td>
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<tr>
<td>2</td>
<td>50</td>
<td>56.71</td>
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<td>49.76</td>
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<tr>
<td>6</td>
<td>90</td>
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<td>30</td>
<td>61.06</td>
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<tr>
<td>7</td>
<td>100</td>
<td>60.55</td>
<td>35</td>
<td>65.01</td>
</tr>
</tbody>
</table>

*Average of two readings. SO: Spinacia oleracea, DPPH: 1,1-Diphenyl-2-picrylhydrazyl

Table 3: Nitric oxide scavenging activity of extract and quercetin

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of ethanolic extract of Spinacia oleracea (µg/ml)</th>
<th>% inhibition by extract*</th>
<th>Concentration of quercetin (µg/ml)</th>
<th>% inhibition by quercetin*</th>
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<td>3</td>
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<td>1</td>
<td>63.21786</td>
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<tr>
<td>4</td>
<td>80</td>
<td>71.97491</td>
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<td>80.92962</td>
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<tr>
<td>5</td>
<td>100</td>
<td>93.35472</td>
<td>10</td>
<td>94.16308</td>
</tr>
</tbody>
</table>

*Average of two readings

Table 4: Observations of reducing power assay

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of ethanolic extract of Spinacia oleracea (µg/ml)</th>
<th>Absorbance of ethanolic extract of Spinacia oleracea at 700 nm*</th>
<th>Concentration of quercetin (µg/ml)</th>
<th>Absorbance of quercetin at 700 nm*</th>
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<tr>
<td>1</td>
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<td>0.0880</td>
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*Average of two readings

Table 5: Observations after addition of EDTA and without addition of EDTA

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<thead>
<tr>
<th>S. No.</th>
<th>Concentration of thiourea (µg/ml)</th>
<th>% inhibition after addition of EDTA</th>
<th>% inhibition without addition of EDTA</th>
<th>Concentration of extract of Spinacia oleracea (µg/ml)</th>
<th>% inhibition after addition of EDTA</th>
<th>% inhibition without addition of EDTA</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>100</td>
<td>54.26</td>
<td>47.27</td>
<td>750</td>
<td>87.83</td>
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<td>2</td>
<td>200</td>
<td>76.38</td>
<td>56.39</td>
<td>1000</td>
<td>92.68</td>
<td>95.46</td>
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<tr>
<td>3</td>
<td>300</td>
<td>81.59</td>
<td>97.40</td>
<td>1500</td>
<td>98.75</td>
<td>98.06</td>
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<tr>
<td>4</td>
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<td>98.49</td>
<td>98.49</td>
<td>2000</td>
<td>99.56</td>
<td>99.64</td>
</tr>
</tbody>
</table>

*Average of two readings. EDTA: Ethylenediaminetetraacetic acid
CONCLUSION

Preliminary phytochemical investigation of the ethanolic extract of SO revealed the presence of saponins, flavonoids, tannins, triterpenoids, and alkaloids. The systematic literature collection, pertaining to this investigation, indicates that the plant phenolics constitute major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is worthwhile to determine their total amount in the plants chosen for the study. The content of total phenolics in the extracts was determined using the Folin–Ciocalteau assay and quantified as gallic acid equivalents. The total phenolic content was found to be 11.25 % of “gallic acid equivalent” per mg extract in ethanol. The total flavonoid content was determined by incorporating the AICl method using quercetin as the standard. The total flavonoid content was found to be 3.51 % “quercetin equivalent” per mg plant extract in ethanol. There is strong evidence, indicating that flavonoids are capable of modulating cellular and molecular processes involved in memory and learning. Flavonoids have been shown to modulate critical neuronal signaling pathways involved in the processes of memory and, therefore, are likely to affect synaptic plasticity and long-term potentiation mechanisms. Various antioxidant assays based on colorimetric reactions were performed, for example, DPPH assay, nitric oxide scavenging assay, reducing power assay, hydroxyl radical scavenging assay, lipid peroxidation assay, and phosphomolybdenum reduction assay. The results confirm the antioxidant potential of extract.

AUTHORS’ CONTRIBUTIONS

All the authors have contributed equally to the manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest.

REFERENCES