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Original Article

A Quantitative Analysis of *in vitro* Antioxidant Capacity and Antioxidant Enzymes in *Bacopa monnieri* (L.) Wettst

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ABSTRACT

Background: *Bacopa monnieri* (L.) Wettst is an herbal plant used predominantly in Ayurvedic medicine. Though this plant has diverse pharmacological properties it is pivotal to acknowledge its total antioxidant capacity and enzymatic quantification which are not well established. Therefore, the principal objective of this study is to elucidate the antioxidant capacity in five different solvent extracts including ethanol, acetone, chloroform, petroleum ether and aqueous, further to quantify chlorophyll and iron content and the antioxidant enzymes of *B. monnieri* fresh stem extracts. **Materials and Methods:** The comparative identification of the antioxidant capacities of five different extracts ethanol, acetone, chloroform, petroleum ether and aqueous of *B. monnieri* stem was investigated through phosphomolybdate assay (Total antioxidant capacity). The fresh stem extracts were quantified for chlorophyll and iron content using the acetone method and the thiocyanate method respectively, furthermore quantified antioxidant enzymes are Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (Px), Ascorbate Oxidase (AO) and Polyphenol Oxidase (PPO). **Results:** The results showed the fresh stem extract has a total chlorophyll content of 0.2720±0.01 mg/g and the iron content is greatly obtained in distilled water than in 30% sulphuric acid. The ethanol extract exhibited greater antioxidant capacity with the IC_{50} value of 16±0.3 µg/mL. The enzymatic capacity of SOD, CAT, Px, AO and PPO resulted in 10.83±0.003 units of fresh weight, 41.03±0.004, 2.044±0.003, 56.15±0.003 and 57.21±0.003-unit mg^{-1} protein min^{-1} respectively. **Conclusion:** The results indicate that *B. monnieri* may serve a strong prophylactic action against ROS-related diseases with its remarkable enzymatic defense system and antioxidant capacity.

Keywords: *Bacopa monnieri*, Chlorophyll, Enzymatic antioxidants, Iron, Phosphomolybdate, Plant extract.

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INTRODUCTION

In ancient ayurvedic medicine, *Bacopa monnieri* (L.) Wettst (bhrami) is typically known as 'Medhya rasayanas pertains to the family of Scrophulariaceae. The genus Bacopa has more than 100 species varieties.[1,2] It can be cultivated up to the altitude of 4500 ft from sea level with an abundant presence of water.[1,3] It is predominantly known for its medicinal role in treating neurological disorders where the entire plant is used for its medicinal values.[1,3,4] It is also used to treat neurological problems including depression, anxiety, psychosis, epilepsy, cardiovascular ailments, pulmonary diseases and inflammatory conditions such as asthma arthritis, etc.,[1-4] The major phytocomponent responsible for its medicinal property in *B. monnieri* is saponins, a structural analog of closely associated compounds known as bacosides additionally alkaloids and sterols are also involved.[3] It is also extensively known for its anti-inflammatory, anti-lipid peroxidative, antipyretic, sedative, antimicrobial properties and wound healing.[3,4]

Reactive Oxygen Species (ROS) is the cluster of highly reactive biological components containing oxygen produced by normal physiological cellular processes. They consist of free radicals such as Hydroxyl radical (OH^+), Superoxide radical (O_2^+), Nitric Oxide radical (NO^+), Lipid peroxyl radical (LOO^+), Hydroperoxyl radical (HOO) and Non-free radicals such as Hydrogen peroxide (H_2O_2), Singlet oxygen (IO_2), Hypochlorous acid (HOCl) and ozone (O_3).[5] The low to moderate action of ROS is highly essential for normal metabolism. Extreme secretion of ROS can lead to cellular DNA damage that further causes detrimental health illnesses and precedes its progression.[6] Antioxidants are the

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biological substance that acts as the defense system for the damage caused to cellular mechanisms by ROS and can able to inhibit the oxidation of ROS. The antioxidant mechanism was generally categorized into enzymatic defense system involving Superoxide Dismutase (SOD), Glutathione Reductase (GR), Glutathione Peroxidase (GPx) and Catalase (CAT) and nonenzymatic defense system involving glutathione, Vitamin C and E, uric acid and β-carotene could able to synergistically neutralize the effect caused by the detrimental oxygen species.[5,7]

The free radical scavenging activity, hydroxyl radical scavenging activity and reducing power assay from various extracts including hexane, water, methanol, ethyl acetate and acetone.[7,8] Furthermore, several other studies have reported the phytoconstituents quantification including total flavonoid, phenol, saponin, etc., from distinct solvent extracts.[9] This study has focused on the estimation of chlorophyll content, iron content in fresh stem and Total Antioxidant Capacity (TAC) in 5 distinct Soxhlet extracts ethanol, chloroform, acetone, petroleum and aqueous, followed by the activity of antioxidant enzymes such as SOD, CAT, Peroxidase (Px), Ascorbate Oxidase (AO) and Polyphenol Oxidase (PPO) in the fresh stem extract of *B. monnieri*.

MATERIALS AND METHODS

Sample collection

B. monnieri fresh leaves are grown and collected in Coimbatore, India. The plants were authenticated botanically in the Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore (BSI/SRC/5/23/2022/118).

Preparation of plant extract

The preparation of the plant enzyme extract sample begins by weighing approximately 1 g of fresh leaves, which are then pestled with 5 mL of saline buffer (pH 7.8). The homogenates are spun at 15,000 g for 20 min at 4°C. The liquid phase (enzyme extract) is collected in a pre-chilled fresh tube. The above step is repeated to collect additional supernatants. The combined supernatants are stored on ice and the fresh stem extract is used for enzymatic assays.[10]

Quantification of chlorophyll

The chlorophyll mass of fresh stem extract was determined. The 1 g fresh leaves were washed and ground with 20 mL of 80% acetone. The homogenized sample was centrifuged at 4°C at 5000 rpm for 5 min to collect the upper liquid phase. Until the debris became colorless, the above step was performed again. Using acetone as a blank, the sample was read at 663 and 645 nm in a UV spectrophotometer. The following formulae were used for calculating the chlorophyll a, chlorophyll b and total chlorophyll content.[11]

Chlorophyll a (mg/g): 12.7 (A663)-2.69(A645).

Chlorophyll b (mg/g): 22.9 (A645)-4.68 (A663).

Total chlorophyll (mg/g): 20.2 (A645)+8.02 (A663).

Quantification of iron content

The quantity of iron was assessed using the thiocyanate procedure. The 500 µL of fresh stem extract was used. 100 µg/mL ferrous ammonium sulfate (Stock standard) was used as a standard dissolved by adding a few drops of 30% Sulphuric acid to 100 mL with demineralized water. 1 in 10 dilutions of stock was used as a working standard. The working standard ranged from 0.2 to 1 mL in different reaction containers and the volume was diluted to 1 mL using demineralized water. Then, the reaction solution contains 1 mL of 30% sulphuric, 1 mL of potassium per sulphate and 0.5 mL of potassium thiocyanate. Using a UV spectrophotometer the absorbance was read at 540 nm.[12]

Determination of phosphomolybdate assay (Total Antioxidant Capacity TAC)

The TAC of the dry stem soxhet extract using solvents including ethanol, acetone, aqueous, chloroform and petroleum ether was quantified. The 1.1 mL of reaction mixture composition containing 0.1 mL of dry stem extract was combined with 1 mL of reaction mixture containing 0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mm ammonium molybdate-the reaction mixture without a sample served as the blank. The test solution was capped, mixed thoroughly and heated in a 95°C thermal block for 90 min. Then, allow the mixture to cool at Room Temperature (RT). The Optical Density (OD) was obtained at 765 nm against a blank utilizing a spectrophotometer.[13] The antioxidant capacity was estimated using the below formula:

$$\text{Antioxidant capacity } \% = [(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100$$

Determination of enzymatic antioxidant assays

Determination of superoxide dismutase antioxidant enzyme

The SOD enzyme activity was evaluated by preparing 1.4 mL of the test solution composition was prepared as stated by Das *et al.*, 2000. The reaction solution was kept for 10 min under a 200 W fluorescent lamp. After exposure, 1 mL of Griess reagent was added. The OD of the color developed was read at 543 nm.[13]

Determination of catalase enzyme activity

The CAT enzyme activity was evaluated with minor modifications. The reaction capacity is composed of 500 µL of fresh stem extract, 2 mL of 50 mM PBS and 500 µL of 10 mM H_2O_2. The OD was assessed for 2 min intervals every 30 sec. The extinction coefficient is 39.4 mM^{-1} cm^{-1}). The CAT activity was expressed regarding µmoles of H_2O_2 consumed/min/mg protein.[14]

Determination of peroxidase activity

Preparing the reaction solution for the evaluation of the Px activity.[15] The OD was read at 430 nm for 2 min every 30 sec. The extinction coefficient of oxidized pyrogallol was used to calculate the Px activity.

Determination of ascorbate oxidase

The AO activity was assessed by weighing the fresh leaves at about 1 g and homogenizing per chilled mortar and pestle with PBS pH 6.5, then centrifuging the homogenate for 15 min at 3000 g 50°C. The Top liquid phase served as an enzyme source. The reaction solution contained 3 mL and 1 ml of substrate solution and enzyme source respectively. The change in absorbance was read using a UV spectrophotometer at 265 nm every 30 sec for about 5 min.[16]

Determination of polyphenol oxidase

The PPO activity was examined with minor modifications.[16] The reaction mixture consists of a 1:2 ratio of fresh stem enzyme extract and sterile water mixed thoroughly. 1 mL of catechol solution (0.4 mg/mL) was added to the above solution. The solution was mixed immediately. The enzyme activity was evaluated at the absorbance of 490 nm for 1 min.

Statistical analysis

All the experiments were executed in triplicates, representing the results in mean±SEM. The statistical significance of the antioxidant activity of the extracts was evaluated with one-way ANOVA between the groups followed by Duncan's multiple range test (p<0.05) in Statistical Package of Social Science (SPSS) software version 11.5.

RESULTS

Estimation of chlorophyll content

The acetone method was used to assess the chlorophyll content in the fresh stem of *B. monnieri*. The quantity of chlorophyll A and B present were evaluated as 0.1826±0.03 mg/g and 0.0902±0.01 mg/g respectively. The total chlorophyll content of the stem was calculated as 0.2720±0.01 mg/g. The results were depicted graphically in Figure 1.

Estimation of iron content

The iron mass of *B. monnieri* stem was quantified by the thiocyanate method using two different solvents such as 30% sulphuric acid and distilled water. The iron content estimated with distilled water exhibited a 51.6±0.2% higher concentration of iron content than 30% sulphuric acid. The graphical representation of iron content is given in Figure 2.

Determination of phosphomolybdate assay (Total Antioxidant Capacity TAC)

The phosphomolybdate assay evaluates both water and fat-soluble antioxidants by reducing the electrons to molybdenum ions. The extracts derived from *B. monnieri* stem exhibited various degrees of antioxidant capacity based on the dose. The TAC of the extract decreases in the following order ethanol>aqueous>acetone> chloroform>petroleum ether. The ethanol extract produced the highest antioxidant ability, with an IC_{50} value of 16±0.3 µg/ml, which is statistically equivalent to standard ascorbic acid. It was followed by 39.5±0.003 µg/ml, 57.2±0.004 µg/ml, 216.60±0.003 µg/ml, 247.27±0.002 µg/ml of the aqueous, ethanol, chloroform, and petroleum ether, respectively. The values were observed to be

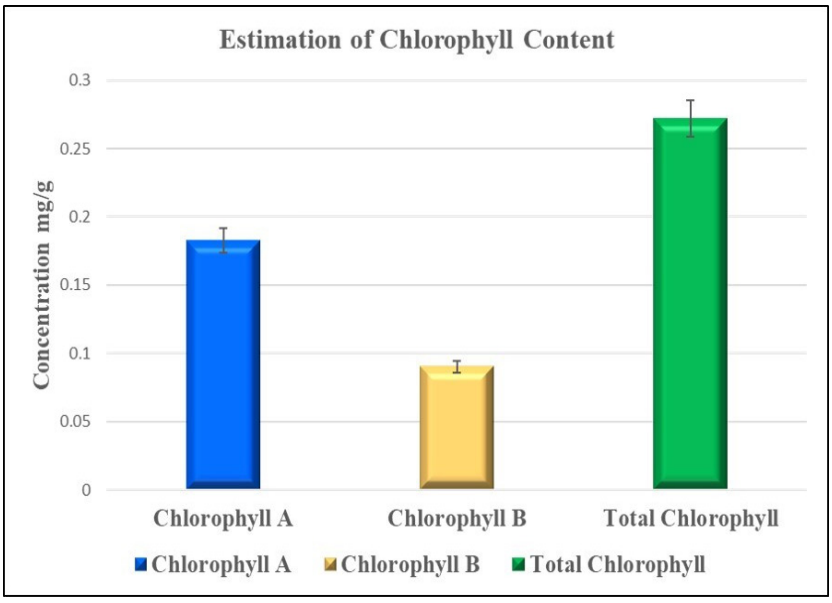

Figure 1: Quantity of chlorophyll of *B. monnieri* fresh stem extract. The bars sharing the values are represented in mean±SEM.

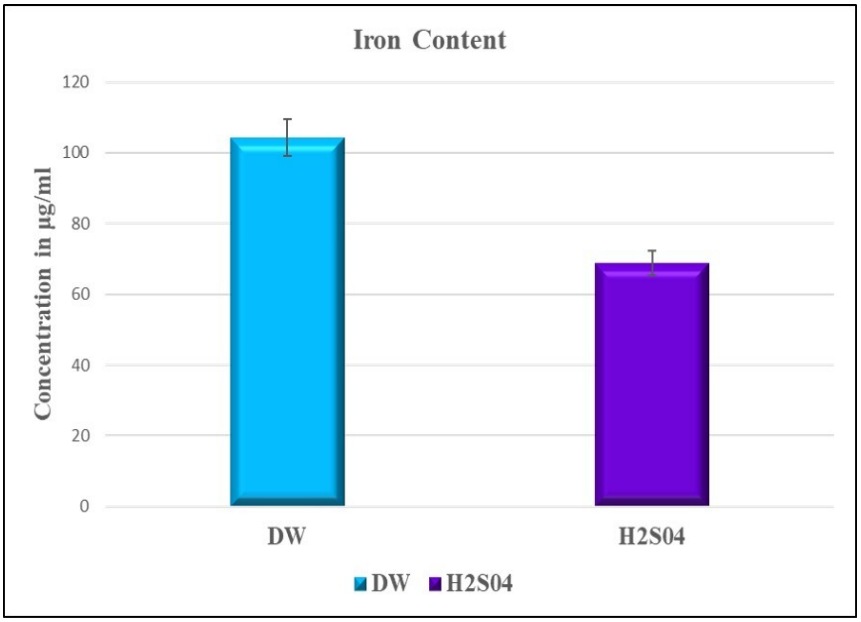

Figure 2: Quantity of iron content *B. monnieri* fresh stem extract iron in distilled water and Sulphuric acid. The bars sharing the values are represented in mean±SEM.

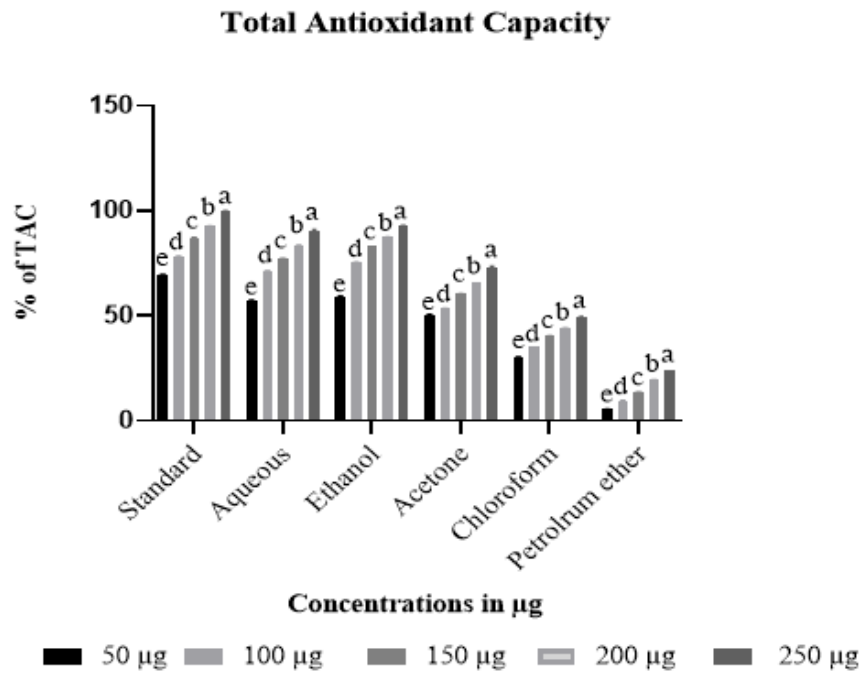

Figure 3: Total antioxidant capacity of *B. monnieri* stem extract in differential solvent-Aqueous, Ethanol, Acetone, Chloroform and Petroleum ether (Bars sharing the same alphabets are statistically significant, $p<0.05$).

statistically significant ($p<0.05$). The graphical representation of the TAC assay results is given in Figure 3.

Determination of enzymatic antioxidants

SOD molecules were generally defined as the primary defense mechanism of aerobic cells, extracellular matrix and the major source of peroxides. The molecules of SOD convert the SOD anion into H_2O_2 and molecular oxygen. The degradation of free radicals per unit in the fresh stem samples of *B. monnieri* was 10.83±0.003 Unit fresh weight.

CAT is one of the principal antioxidant enzymes, that plays a protective role by transforming H_2O_2 into water and oxygen. The CAT activity gradual decline was observed and the degradation

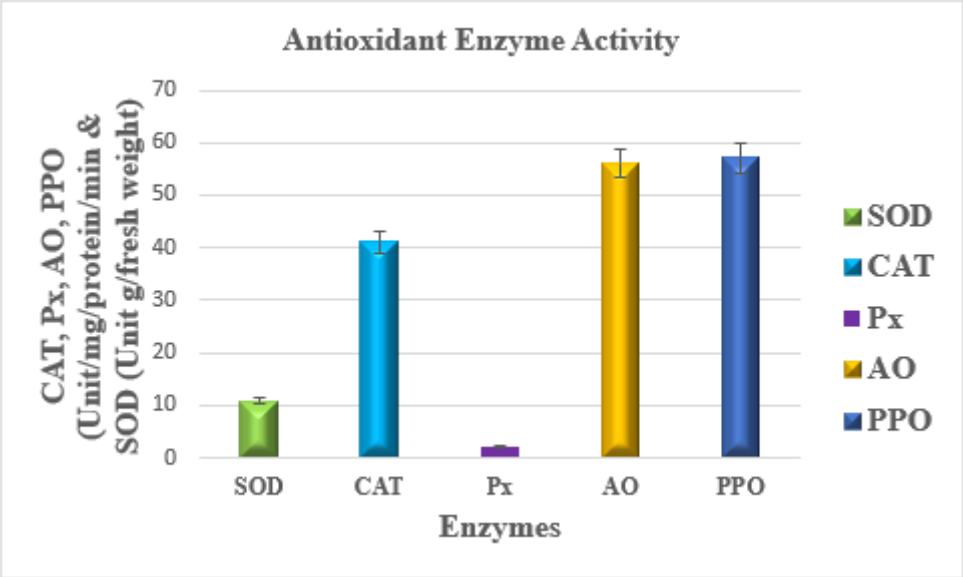

Figure 4: Comparative quantity of antioxidant enzymes of *B. monnieri* fresh stem extract. The bars sharing the values are represented in mean±SEM.

of H_2O_2 radicals per unit in the fresh stem samples of *B. monnieri* was 41.03±0.04-unit mg^{-1} protein min^{-1}.

Px activity was determined through the pyrogallol oxidation protocol, where the amount of pyrogallol oxidation determines the unit of enzyme activity into purpurogallin. A steady increase in the Px activity was observed and it exhibited oxidation of 2.044±0.003 units mg^{-1} protein min^{-1}.

AO is a multicopper oxidase enzyme that oxidizes the ascorbic acid into monodehydroascorbate and catalyzes the oxidation of other substrates upon dioxygen. A decrease in AO is correlated with an increase in ascorbic acid. This study observed a steady reduction in the AO and exhibited 56.15±0.03-unit mg^{-1} protein min^{-1} oxidation.

PPO catalyzes monophenolase activity upon hydroxylation and diphenolase activity upon oxidation. When catechol served as a substrate, the predominant inactivation of PPO was observed within the incubation period of 3 min and exhibited an oxidation capacity of 57.21±0.03-unit mg^{-1} protein min^{-1}. The estimated quantity of antioxidant enzymes evaluated in this study is comparatively given in Figure 4.

DISCUSSION

Plants go through enormous stressful conditions throughout their lifecycle that result in increased production of ROS, which may lead to the depletion of essential nutrients. The versatile effects result from the oxidative burst. The plants develop a balanced system to combat the effects caused by excess production of ROS by developing the intrinsic enzymatic defense system such as SOD, CAT, Px, etc., The chlorophyll content is the major indicator of chloroplast content, oxygen production and carbon sequestration. Generally, the chloroplast is considered an antioxidant compound and is the mandatory pigment for the photosynthesis mechanism and leaves have greater chloroplast content than stems.[17,18] The concentration of primary pigment chlorophyll a is two to three times greater than accessory pigment chlorophyll b. Most of the reports have revealed the chlorophyll content in leaves including *Allamanda blanchetti*, which has 4.998 mg/g, *Catharanthus roseus* has 6.413 mg/g, etc.[19] The chlorophyll content in leaves of *B. monnieri* is reported as 0.89 mg/g under control conditions.[20] This study has revealed the chlorophyll content in the stem as 0.2720±0.01 mg/g. Iron content is a plant that is highly rich in hemoglobin, involved in chlorophyll synthesis and can enhance the cognitive and neurological activities in the human body through increased transportation of oxygen and electrons. *B. monnieri* has 0.51 parts per million (ppm) of iron.[21] This study has reported the quantification of iron through two solvents including distilled water and sulphuric acid, the iron content estimated with distilled water exhibited a 51.6% higher concentration of iron content than 30% sulphuric acid where 104.25 µg/mL and 68.75 µg/mL were estimated in distilled water and sulphuric acid respectively. The iron content in *B. monnieri* was found to be less when compared to iron-rich traditional therapeutic plants such as *Moringa oleifera*.[22]

Many reports have analyzed the *in vitro* antioxidant assay and assessed the scavenging capacity of *B. monnieri* leaf. This study has revealed the TAC of the stem in five distinct solvent extracts of dried stem extract of *B. monnieri* where ethanol extract exhibited the greater antioxidant activity as reported by most of the reports;[8,23] with the IC_{50} value of 16±0.3 µg/mL.[24] All the extracts showed a distinct degree of antioxidant activity in a dose-dependent manner.[25] The TAC of the extract decreases in the following order ethanol>aqueous>acetone>chloroform>petroleum ether.

Methanol and aqueous extract of *B. monnieri* have shown the maximum Superoxide Anion Radical scavenging activity (SARC) of about 65.68% and 62.34% of inhibition respectively.[4] Petroleum ether and hexane plant extract has shown SARC activity of about 56.67% and 54.18% respectively. Furthermore, the reports have revealed that the methanol extract of *B. monnieri* has exposed the concentration-dependent SARC activity at 25 µg/mL and exhibits the action of 80 mU/mL SOD.[7] Followed by, some studies showed that ethanol extract of B. monnieri has exhibited dose-dependent SARC activity, with an IC_{50} value of 22.92 µg/mL.[6,7] In this study, the fresh stem extract exhibited the SOD activity of 10.83±0.003 Unit g/fresh weight. The reduced activity may be due to the influence of extraction techniques and solvents that are used for extraction protocols.[26-28]

CAT is the first enzyme known for its antioxidant activity. It is capable of degrading H_2O_2 promptly without utilizing cellular energy.[29] In this study, CAT activity has a gradual decline in activity concerning the duration and exhibited the activity of 41.03±0.004-unit mg^{-1} protein min^{-1} under optimum conditions. The Px converts H_2O_2 into water and oxygen. The CAT and Px activity has been reported to be increased during drought and stressful conditions that are expected to provide drought and stress tolerance capacity to *B. monnieri*.[29] Another study has reported that gradual and shock heat stress has steadily increased the Px activity as the total higher Px activity of 35.19 µmol min^{-1} g^{-1} dry weight.[30] This study has reported Px activity of 2.044±0.003-unit mg^{-1} protein min^{-1} under controlled conditions.

PPO is a copper-containing oxidative enzyme involved in oxidizing orthodiphenolic elements into quinones. The oxidation mechanism is important in wound response the action involved as the browning of damaged parts of tissues, secondary metabolites production such as flavonoids and tannins and one of the major metabolic regulators. *B. monnieri* has exhibited a predominant reduction in PPO activity exposed to the oxidation capacity of 57.21±0.003 -unit mg^{-1} protein min^{-1}. The reports show *Solanum tuberosum* L., *Malus domestica* and *Oryza sativa* have the greatest browning of damaged tissue.[31] AO is a multiple copper oxidase that converts ascorbate to L-dehydroascorbic acid and has an indispensable role in stress conditions, plant growth and development.[32] As there were no earlier reports on the quantification of AO this study quantified the *B. monnieri* has exhibited a predominant reduction in AO activity exposed to the oxidation capacity of 56.15±0.003 unit mg^{-1} protein min^{-1}. However, the quantification of axillary enzymatic antioxidants such as GR, Thioredoxin Reductase and GPx will be beneficial since they also have a mandatory role in boosting the defense system in plants to mitigate the oxidative damage caused by free radicals.

CONCLUSION

In recent years, the deliberate importance of antioxidant enzymes has risen since the plant ecosystem faces various stressful conditions that initiate the production of deleterious ROS. At lower concentrations, the ROS functions as a signaling molecule and at greater concentrations, they are harmful to the plants and may also cause to death of the plants. A defense system with an ameliorative mechanism to combat stressful conditions is essential to maintain the normal physiology, functioning and cellular metabolism of plants. Hence, *B. monnieri* has optimal enzymatic activity under controlled conditions and it possesses an antioxidant scavenging capacity thus it serves as a strong ROS scavenger and could influence in combating ROS interfering diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SOD: Superoxide dismutase; **CAT:** Catalase; **Px:** Peroxidase; **AO:** Ascorbate oxidase; **AO:** Ascorbate oxidase; **PPO:** Polyphenol oxidase; **H_2O_2:** Hydrogen peroxide; **IO_2:** Singlet oxygen; **HOCl:** Hypochlorous acid; **O_3:** Ozone; **ROS:** Reactive Oxygen Species; **GR:** Glutathione reductase; **GPx:** Glutathione peroxidase; **TAC:** Total Antioxidant capacity; **RT:** Room temperature; **OD:** Optical density; **PPM:** Parts per million; **SARC:** Superoxide Anion Radical Scavenging activity; **SPSS:** Statistical Package of Social Science.

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