

Determination of Antioxidants by Four Different Methods in Medicinally Important Plant *Ebenus Stellata* of Balochistan

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Articles Information	Abstract
Received: 25.09.2020 Accepted: 20.11.2020 Published: 01.12.2020	The quantification of a plant, which is vital in medication bearing antioxidant activity of the North West area of the province Balochistan <i>Ebenus Stellata</i> family Fabaceae is explained in this paper. The plant composition was determined chemically through well-known four analytical tests and sensitive EMR operating techniques. The crude extract was obtained from the plant by using methanol, and the antioxidant activity of that extract was measured. Additionally, with the help of spectrophotometer, antioxidant potentials were determined through the DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) assay, Ferric ion (Fe ⁺³) reducing antioxidant power assay, Ferric (Fe ⁺³) reducing antioxidant potential (FRAP) assay, and total antioxidant capacity (TAC) of the phosphomolybdenum assay. The potency of the DPPH assay of <i>Ebenus Stellata</i> was 179 mg/g of ascorbic acid concentration. Moreover, the FRAP and TAC values were 9.2 mg and 35 mg of ascorbic acid/g of <i>Ebenus stellata</i> extract, respectively. Furthermore, RP denotes 2.6 mg/g of ascorbic acid concentration. The concerned plant showed high antioxidant activity.
Keywords: Antioxidants <i>Ebenus stellata</i> DPPH FRAP TAC Spectrophotometer	

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1. Introduction

The Flora of Pakistan is wealthy in the medicinal plant, due to its different environmental change regions. According to the general study of Pakistan, 6,000 kinds of blossoming plants are there. Among these plants, very nearly 400-600 are vital restorative species [1,2]. The majority of the plants can be used in medicines for the treatment of various illnesses and can function as common defenders [3].

The early human civilization used the *Ebenus Stellata* plant for medicinal purposes to treat different diseases. Chinese, sub-continent regions and African society confirmed the applications of *Ebenus stellata* plants for medicinal purposes. Therapeutic approaches have been made a significant advancement by separating sensitive biochemical elements from analeptic plants in the 19th century. With this breakthrough in medicine, the popular drug used for malaria treatment, i.e., cinchona alkaloid was accomplished through researchers (French Cavetto and Pelletier) who exhibited the bioremediation on the ground level [4]. The therapist's medicinal plants' efficiency is due to antioxidants found in plants; therefore, it can be utilized to cure various diseases [5].

The oxidation process (addition of oxygen and removal of hydrogen) plays a vital role in cellular metabolism. The

cells generate energy by utilizing oxygen as fundamental particles. Oxygen increases immunity in a body, which in turn fights off lethal and dangerous ailments. Different types of in-organic and organic radicals are formed, such as peroxides of hydrogen and alkyl (H₂O₂ & RO₂R, respectively) during the process of antireductionism. Besides, these free radicals' reaction with the substrate's central electron in biopolymers includes CH₂O, nucleic acid, and lipids. This disorder interferes with the functioning of the cell, which affects the immune system. Such interruption results in carcinogenesis, cardiovascular and inflammatory ailments [6]. Tissue destructions and the capability of oxidants become jiffs by antioxidants [7]. The influence of active oxidants can be reduced via the moderation of oxidative destruction of biochemical polymers [8].

Briefly, the oxidation process's delay can be reduced by retardation of concentration of oxygen found locally to make the environment for the less oxidative pathway. The active chemicals inhibit the excitation mechanism from destroying unpaired electron bearing species called free radicals, which can rapidly capture hydrogen from molecules containing hydrogen. The exploration of free radicals can be prevented by binding the d-block metallic ions, serving as the catalyst. Moreover, radical production

conservation can be stopped by the fragmentation of peroxides. They combine with catenated spreading radicals such as the peroxy and ethereal radicals to reduce hydrogen's continuous release from the peripheral chain of long-chain carboxylic acid [9].

The merging of vital force & anthropogenic antioxidants to food suppresses the harmful reduction. For example, engineered Antioxidants like butylated hydroxyanisole (BHA) also butylated hydroxytoluene (BHT) exist as human diet items regardless of their safety and toxicity [10,11]. Therefore, the requirement of the latest and valuable antioxidants remains of great importance [12]. Furthermore, Different medical notations and Epidemiological studies have shown the association of vegetables, fruits, and diseases such as immune markers, cardiovascular disease, cancer, and inflammatory diseases [13]. The primary source of antioxidants is believed to be herbs, vegetables, and fruits [14]. Hence, the healing effects of herbs are related to their antioxidants effects [15,16]. Flavonoids and aromatic alcoholic are the significant antioxidants found in plants [17]. The extracted antioxidants obtained through medically necessary plants possess vast actions and active ingredients compared to the non-reproductive part of plants [18]. So, extracts of plants are tested for their antioxidant capacity and removed antioxidants, which are more potent and less harmful to living beings [19].

This is particularly important for the individuals, Doctors, Dieticians, and health workers to detect different edible antioxidant potential and medicative plant life. Various methods can be used to find the antioxidant capacity of the extracts of the plant in the anatomical and physiological parts of the living body. In the reaction method, a test carried out for the antioxidants are divided into two main ranges: Reduced Atom Transfer assay (HAT) such as restriction for effected automated-oxidation of LDL, total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), crocin surfactant tests and transfer assay in which electron number changes (ETA) that contain the TPC assay by the Folinchemical constituent which can act as a reagent, ferric ion (Fe^{3+}) reducing antioxidant power (FRAP), Trolox equivalence antioxidant capacity (TEAC), "total antioxidant potential" copper metal complex test which can accept electrons and act as Lewis acid and the assay which is vital for the concerned phenomena is DPPH assay. Each distinct technique controls the antioxidant action (AOA) under explicit states used in this dissertation, so one should not conclude from the knowledge obtained as evidence for "total antioxidant activity".

Preferably, plant extract and its antioxidant activity are predominantly dependent on the separation process, and specific circumstances such as volume affected and solubility depended on conditions: absolute alcohol, wood spirit, ester (ethyl acetate). Simple ketone and deionized water are mostly used as a solvent. The most important thing to note is that the therapeutic remedies used in

families in situations that vary from the circumstances used in laboratories where research is conducted, subsequently, the antioxidant capacity would be changed in each [19-21].

Ebenus stellata belongs to the family Fabaceae, a vast taxon of flowering plants and extensively used for research and medicinal usage. The genus *Ebenus* belongs to this family and has more than 100 species [22]. The plant extract, which is a taxon of the Fabaceae family, has exhibited adverse effects in its group, among which *E. stellata* is a major one to have minute toxicity, which leads to its use as anticonvulsants [23]. *E. Stellata* is used in a group of medicines called anticonvulsants (antiepileptic or anticonvulsants medicines) [24].

The physical technique applied for separation and purification (extraction) might be achieved by calibrating various modified procedures like digestion, soxhlet extraction, room temperature extraction (RTE), sonication, percolation, decoction, and supercritical fluid extraction [25]. As for the determination of antioxidants, not a single achievement was reported on this pharmaceutical plant. Such an attempt will be the latest to study and examine this plant's potency for antioxidant activity. The abstract of this knowledge is to quantify the antioxidant activity by the addition of various tests of *E. Stellata* of the Fabaceae family to find the exercise of this plant as a bulk source.

The endemic plant extract antioxidant potential was determined with the connections of radical scavenging actions and non-oxidation power; the linkage between parameters was also checked. For the up to date work, a single plant species has opted. The selection of plants was based on the testing of the findings that primitive healers have used it specifically to cure different ailments. The plant was tested for antioxidant ability to test its hidden antioxidant potential.

2. Method and Material

Identification and collection of samples

The medicinal plant was identified as *Ebenus Stellata*. This plant was collected from different areas of District Zhob, Province Balochistan. Then, it was brought to the lab of the Department of Chemistry, University of Balochistan, Quetta, Pakistan for further process.

Preparation of sample extraction

The assembled samples were dried under a shade. Later, it was ground to a fine powder to increase the surface area. 5 kg of powdered sample was taken and added to the 15 Litre of methanol for a week. It was then filtered through Whatman No.1. The rotatory evaporator was operated for filtrate at low pressure and 35 °C to obtain the slightly dried CME (crude methanolic extract). A suitable environment was provided to the CME for evaporation in a pre-weight china dish without light. Once the evaporation was done, the weighted residue was 300 g. Eventually the extract was used for the detection of antioxidants.

3. Results and Discussion

Di-phenylpicryl hydrazyl (DPPH) radical

The 2, 2-diphenyl-1-picrylhydrazyl abbreviated as DPPH and is reliable for scavenging organic radical as reported in the literature. Barku, Naeema, and Yan [26-28] examined the Scavenging radicals via basic methods sequenced by the DPPH evaluate containing various methods mentioned in finding antioxidants in the CME, which is pronounced as a crude methanolic extract. The various aliquots of a mixture of CME, such as 0.2-1.0 ppm, have been made in MeOH. The suitable ready about 10 drops solution of DPPH (0.1 milli Molar = 3.943 mg/0.1 L in MeOH) was mixed with 2 ml of all the mixture 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The solvent used with the same sequence applied for the ascorbic acid solutions 0.2 to 1 mg/L under positive control of the DPPH method. For about 30 minutes, the DPPH solution against the test sample was kept in the dark. By using Ultra-Violet-Visible spectrophotometer (UV-VIS 1700, Shimadzu), the lambda max was chosen as 517 nm for absorbance spectra.

Wood sprit was utilized instead of plant extract for statistical requirement the absorbance of the control, test solutions of 1ml DPPH (0.1 milli molar + 2 ml MeOH) for the preparation of the aforementioned sample was obtained in this test solution. For blank, MeOH was used. The scavenging ability of DPPH was measured by deducing the blank absorbance from the analyte absorbance relatively. The intense organic reactive radical capturing capacity of the CME of Ebenus Stellata be the hypo-graphical signal of the mixture under reactivity.

The mathematical formulations were utilized for capturing of active DPPH by CME.

$$\% \text{age of radical capturing activity} = \frac{(\text{Abs (control)} - \text{Abs (sample)}) / \text{Abs (control)} \times 100\%}{}$$

where; As, Abs (control) = Absorbance of DPPH solution
Abs (sample) = Absorbance of the crude methanolic extract and vitamin C (ascorbic acid) solutions plus DPPH. The DPPH radical catching ability of the CME in contrast with vitamin C (ascorbic acid) is shown in (Figure 1).

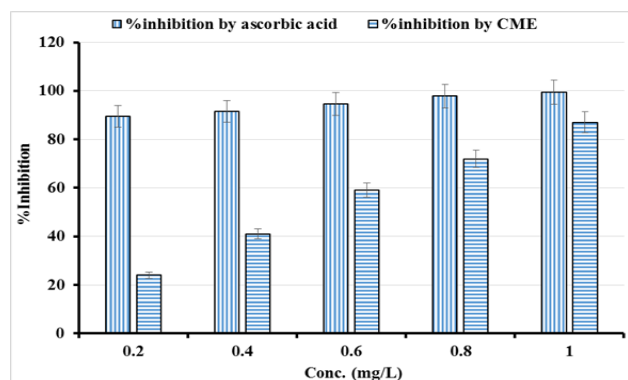


Figure 1. If you increase the concentration of CME may be gained more inhibition %.

Ferric ion (Fe⁺³) reducing antioxidant power assay

The Fe⁺³ reducing antioxidant potential of the CME (crude methanolic extract) of Ebenus Stellata was quantified by the proposed procedure of Saeed *et al.* with little change. [28]. The Stock mixture was setup by homogenization of the exact amount of extract (10 mg) of a sample plant with 0.001L of homogeneous mixture extract. Also, various aliquots were set up using extract in the series of 0.2 to 1.0 mg/ml in MeOH. The different aliquots of impure extract were coupled with an acidic buffer of phosphoric acid and salt (2.50 mL, 0.2 M, pH 6.60) and 1% Potassium ferricyanide (0.0025 L). Also, the mixture was gested for 20 minutes ranged in an H₂O containing container at 50.5-51.5 °C. After-all gestation, 2.54 ml of 10.5-11.5% trichloroacetic acid (Cl₃C-COOH) was homogenized within the sample with stable homogenization for about 1/4rth of an hour at 1450 rpm (EBA 20, Hettich Zentrifugen). Alternately, the mixture's residue was obtained and added the exact amount of deionized was utilized to detect the absorbance at 700 nano-meter. For the making of control, the usual method was utilized to eradicate mixtures. A solution containing Vitamins-C was utilized as an antioxidant. The uprising in the reacting mixture's % transmittance denotes the amplification of reducing signals of the methanolic extract of Ebenus Stellata.

Ferric (Fe⁺³) reducing antioxidant potential (FRAP) assay

The developed technique of the FRAP assay was made easy to work for the determination of RE of CME of Ebenus stellata [29]. The technique's core purpose is to reduce the high oxidative state of iron to a low oxidative state, i.e., from ferric to ferrous act as CMA in the complex, at a high acidic range of pH. The CME reduction by electrons indicating the existence of concerned chemical constituents. The UV-Vis spectrophotometer was used to take the absorbance of a complex at 583 nm. The acetic buffer solution (pH 3.65) of FRAP was made by vigorous shaking of 10mL of 23mM solution to 1.0 mL of 10.0 mM tripyridyl-triazine (TPTZ) in 40 mM HCl arranged by the mixing of 1.0mL of 20.0 mM of ferric chloride in the volumetric ratio of solutions (v/v/v: 10:1:1). For obtaining a standard curve, different concentration of ascorbic acid was made. For obtaining an accurate result, a fresh solution was used regularly. The volume of sample was opted 0.5 mL exactly of CME mixture at various aliquots (0.2-1.0 mg/mL) & 2.0 mL of FRAP reagent were homogenized with about 1.0mL of UHP water. As a result, the tube used for chemical change was incubated for 30minuts at a normal temperature (37 °C). The UV-VIS absorbance of the concerned mixture was determined at a lambda max of 593 nm. The same technique was utilized to achieve the calibration curve and obtain the standard solution of ascorbic acid. The readings were noted by using acetate buffer instead of reagent.

Values of CME absorbance and blank were computed. In this assay, the Ascorbic acid solution was used to calculate the plant extract as a reference. The values of FRAP were quantified in less than a gram of ascorbic acid/g of the C.

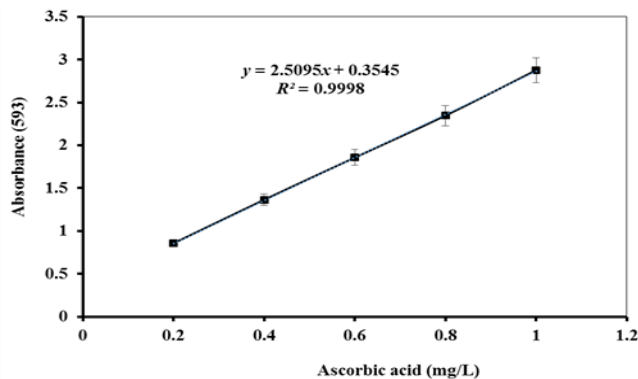


Figure 2. FRAP calibration curve.

Total antioxidant capacity: (TAC) (Phosphomolybdenum {PM} Assay)

In the following experimental work, the crude methanolic extract potency as an antioxidant of the concerned plant can be quantified. The main objective of this reaction is to make a complex of Mo, which is green in color and its chemical formula is [Mo (V)] and due to the reduction of Mo (VI) to Mo (V) in an acidic medium and this whole reaction carried out by CME. The spectrophotometric process can be assessed to determine the total antioxidant potential of the chemicals. [30]. An extract of 0.5ml of the different aliquots (0.2-1.0 mg/ml) of methanolic extract was vigorously homogenized properly with Mo-reagent (5.0 mL) and 0.60 M H₂SO₄, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture).

Maximum time was given for the incubation at 95 °C in a water bath. The room temperature was provided to the test solution. After that, the absorbance was recorded using the available device (1700, UV-VIS-Shimadzu) lambda max 695 nm against solvent, free of the analyte. For the blank formation, 0.5 ml of MeOH was homogenized with a reagent solution (5 mL) and provided a similar environment.

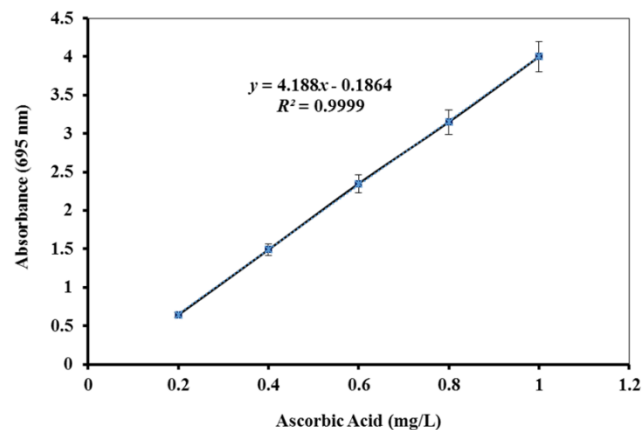


Figure 3. Calibration Curve for TAC.

For the unknown composition of the sample, the CME was used as mg equivalent of V-C for the antioxidant activity. Various concentrations of ascorbic acid (0.2, 0.4, 0.6 0.8, and 1.0 mg/ml) with MeOH are used to make the celebration curve.

The determination of the antioxidant potential of CME of the *Ebenus stellata* of Fabaceae taxon via various techniques is the main objective of this work. The plant antioxidant capacity is explicated and has an important role in treating heart diseases, age disorders, and involuntary action disease. Besides that, their role against carcinogenic actions makes them significant for living creatures [31]. The crude methanolic Extract of *Ebenus stellata* is examined for its entire natural antioxidant potential with well-known techniques. The spectrophotometers (UV-VIS 1700, Shimadzu) extensive use in the research environment essential for determining antioxidants in the Extract of the plant [32]. Inexpensive techniques having a low cost, high sensitivity, and fairly easy to use, which increases their utility [33]. As we know that through resonance and conjugation, the highly unstable substances called free radicals can be stabilized.

Radicals of DPPH were optimized and chosen as a maximum in this essay to calculate the numerous antioxidants in the Extract so-called CME of *Ebenus Stellata*. Because of the sustenance of this radical, reachable and genuine solvent can be used for this purpose. (Water, methanol and ethanol) [34].

For the measurement of lambda max, a UV-V is spectrophotometer was utilized at 517 nm for free radicals to measure its absorbance. It is easy to judge the position of the absorbance band and color change since the Hydrogen is eliminated from antioxidants by the action of free radicals present in the CME. The capability of the CME to catch the radical is observed at each standard. Furthermore, the free radical capturing ability of the methanolic Extract is calculated while evaluating it to the organic acid known as ascorbic acid, a herbal inactive oxygen species (antioxidant). The inhibition of DPPH rises in % age to 94.04% of *Ebenus Stellata* at a mixture aliquot of 1.0mg/ml of the methanolic extract mixture displaying that plant full of antioxidants.

The plant extract reducing potential is also quantified through Fe⁺³ (reducing) to Fe⁺² ions because of the presence of antioxidants in the CME as compared to vitamin C. The lambda max of potassium ferricyanide [K₃Fe(CN)₆] conversion is nicely assessed in this reduction; at first, it can accept electrons and make a [K₄Fe(CN)₆] complex owing to methanolic Extract often converted in the presence of FeCl₃ to ferric ferrocyanide. The greater amount of antioxidants in the CME of the plant depicts enhancement in the absorbance.

The antioxidant amount was determined in the methanolic extract of the plant by using an innovative form of the FRAP test. Ferric complex, which is in +3 oxidations state (colorless), is reduced to +2 oxidations state, which is bluish in the color complex due to the help

of vital antioxidants exist in the CME of the plant in a mechanism where the proton is available in less quantity. The reduction is appropriately checked via mathematical determination, the signal (absorbance) at 593 nm. The standard calibration curve is used to collect experimental data of vital antioxidants present in the methanolic Extract. The FRAP method tells us a concentration of 34 mg/g. This amount of antioxidants in *Ebenus Stellata* express that it is a rich source of natural antioxidants.

Figure 3 shows the complete result of total antioxidant capacity (TAC). The reduction degree of Mo (VI) to Mo (V) can be determined by PM assay. The complex formation reaction is quantified by this technique, which uses antioxidant, oxidant, and molybdenum as a ligand for complex formation. Also, the polar and non-polar antioxidant capacity estimation can be achieved via the TAC of the phosphomolybdenum model. At high temperature, the production of auto-oxidation incubated for a long period. Also, it gives a linear assessment of the reducing capacity of antioxidants. FRAP is a well-known test as it is maintained regardless of the concentration of free metal ions. The complex formed by phosphomolybdenum ligand having green color without free metal ion induction solution. So, its deviations can be indicated.

4. Conclusion

The Flora of Pakistan is wealthy in the medicinal plant, due to its different environmental change regions. According to the general study of Pakistan, 6,000 kinds of blossoming plants are there. The concentration of antioxidants present in the plants CME is reported in this article by using four consolidated Methods such as, DPPH, FRAP, TAC and RP assays. *Ebenus Stellata* contains multiple antioxidants.

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