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Evaluation of Antioxidant Activity in Leaves of *Eichhornia crassipes* in Different Fractions of Hydroethanolic Extract

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Abstract: The plant *Eichhornia crassipes* is the worst aquatic weed. This analysis was attributed to discern the presence of the antioxidant property of *E. crassipes* leaves. The hydroethanolic extract, fractionated with different non-polar (hexane and chloroform) and polar (ethyl acetate, n-Butanol, and aqueous) solvents, was used for the analysis. The total phenolic content (372±0.76 mg GAE g^{-1} dry weight) and flavonoid content (452.6±0.65 mg RE g^{-1} dry weight) were highest in the ethyl acetate extract. Both phenolic and flavonoid content showed a strong correlation with the antioxidants. The ethyl acetate fractions showed the highest antioxidant activity, DPPH:187.97±0.08µg/ml; H_2O_2:91.76±0.03 mg AAE/gm; FRAP: 151±0.23 µmol Fe^{2+} /gm; Metal Chelating Activity: 224.08 ± 0.13µg/ml; SOD:176.91±0.14µg/ml. The results showed ethyl acetate extract exhibited strong reducing power and total antioxidant activity better than the standards.

Keywords: antioxidants; *E.crassipes*; ethyl acetate; free radicals; herbal plants; leaves.

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1. Introduction

Free radicals are toxic and cause damage to the cells through oxidative stress and lead to the development of several diseases. Both normal cell metabolism and external sources such as pollution, toxicants, and radiation lead to the generation of an unpaired electron atom or group of atoms and are chemically reactive. The initiation of free radicals leads to detrimental effects such as lipid peroxidation, protein damage, DNA damage, and loss of enzyme activity [1,2,3]. The protective efficacy of antioxidants against several diseases caused by free radicals has drawn attention to herbal therapy [4]. Plants are considered biochemical manufacturers since they can biosynthesize a wide range of natural compounds. These natural compounds are the primary and secondary metabolites. E.crassipes (Mart.) Solms, commonly called water hyacinths (Pontederiaceae), the awful aquatic weed [5]. It grows rapidly and covers the entire water body [6]. It is an abundant source of chemicals in oxidative antioxidant enzymes with analeptic properties. It can be used as a valuable source of biomass [7]. Anaerobic fermentation can convert methane, a natural gas [8,9]. It is possible to grow the plant for use in wastewater treatment and include it in a system that collects biomass for use in fuel production [10]. This biomass has a positive environmental impact and poses no threat to the survival of plants that produce food, feed, or fiber because it is a waste product of wastewater treatment [11]. When combined with dirt, cow manure, and wood ashes, withered water hyacinths can yield usable

compost in about two months. Compost or green manure made from decomposed water hyacinth can also be applied to deficient soils[12]. As a result, the plant may be harvested and used to make various beneficial goods necessary for both plants and animals to survive. The objective was to determine the natural chemical constituents and antioxidant role of *E.crassipes* to be used as a potential drug.

2. Materials and Methods

In this study, all analytical grade chemicals will be procured and used from Himedia, Sigma-Aldrich, and E-Merck Company (India).

2.1. Collection of plant samples

The *E. crassipes* have been chosen for their medicinal properties and procured in the summer months from Chekhla, Sanand, Ahmedabad district, Gujarat, India. The collected fresh green leaves were washed with water. The leaves were dried and ground, and stored in airtight containers. In the Patanjali Research Centre, Haridwar, the plant was identified and authenticated (Authentication no: 2626).

2.1.1. Preparation of extract.

The powder was macerated with hydroethanol for 72h. The hydroethanolic crude extract was fractionated with the non-polar (Hexane and Chloroform) and polar (Ethyl acetate, n-Butanol, and aqueous) solvents successively to yield different fractions. The extracts for further use were dried and stored at 4°C.

2.2. Quantitative physiochemical assays.

2.2.1. Ascertainment of total phenolic content.

The Folin–Ciocalteu's method was followed to estimate the phenolic content [13]. Added to the sample (1mg/ml) was 500 µl of the previously diluted Folin- Ciocalteu reagent (1:1). It was then incubated for 5min, to the mixture 1ml of 20% sodium carbonate, and 12.5 ml DW was put in and mixed. It was left 30min to 2h in the dark, and observation of the sample was taken at 720nm. TPC was estimated as mg gallic acid/100g as a standard compound. The gallic acid dissolved in methanol in the range 10-50 mg/ml to prepare the standard curve. To calculate the TPC, the following formula was used.

$$T=CV/M$$

Where: T=Total phenolic concentration; C=Gallic acid concentration obtained from the calibration curve (mg/ml); V=Volume of extract (ml); M=Weight of leaf extract

2.2.2. Ascertainment of total flavonoid content.

Total flavonoid content was estimated as per the standard method described by Chang *et al*. [14]. A volume 5% of 1ml sodium nitrite solution was added into the sample solution (1mg/ml), then added 150µl of 10% $AlCl_3$ and 1M of NaOH and vortexed. The yellow color indicated the flavonoids, and absorbance was recorded at 415nm. The standard solution (quercetin dissolved in methanol) was prepared in the range 10-100 mg/ml to prepare the

standard curve. The following formula was used to determine the total flavonoid content. The TFC was indicated in mg of quercetin equivalents per gram dry weight.

$$T=CV/M$$

Where: T=Total flavonoid concentration; C=Concentration of quercetin from calibration curve (mg/ml); V=Volume of extract (ml); M=Weight of plant extract

2.3. Antioxidant assays.

2.3.DPPH radical scavenging activity

As described by Blois [15], the extract's antioxidant properties and DPPH free radical scavenging activity was determined. The 1ml of varying concentrations of each extract (10 to 500µg/ml) was mixed to equal volume to the 2ml stock solution of methanolic DPPH (1mM). The mixture of 2ml methanol and 2ml DPPH was used as a control and left 30min for incubation, absorbance was recorded at 517nm, and percentage inhibition was calculated and compared with the standard. Ascorbic acid ranging from 100-500 µg/ml in solution was used as standard. The absorbance recorded was used to calculate the percentage inhibition was calculated using the following formula.

$$\text{Inhibition of DPPH activity (\%)} = Ac- As/ Ac\times100$$

Where the absorbance of the control (Ac) and sample (As)

2.3.2. H_2O_2 scavenging activity.

The hydrogen peroxide scavenging activity was estimated as per the protocol by Ruch *et al*. [16]. The phosphate buffer (pH 7.4) was used to prepare the stock solution of hydrogen peroxide (40 mM). To 0.6ml of hydrogen peroxide was added, various doses of EC leaves were extracted (10-500 µg/ml) and incubated for 10min in the dark. At 230nm, the absorbance was recorded against a blank solution, and ascorbic acid was taken as a positive control in the reaction. The inhibition of hydrogen peroxide by the extract was calculated using the given formula.

$$\text{Inhibition of Hydrogen peroxide activity (\%)} = Ac- As/ Ac\times100$$

Where Ac and As are the absorbances of the control and sample.

2.3.3. Ferric reducing antioxidant power assay (FRAP).

FRAP is assessed following the methodology of Benzie & Strain [17]. Fresh FRAP was prepared by adding 3.1 gm of sodium acetate buffer in 10 ml glacial acetic acid, and volume was made up to 1L using double distilled water having pH 3.6 and 4°C, solution of 10mM of TPTZ in 40 mM of HCl and 20 mM of ferric chloride in H_2O. By mixing 50 ml acetate buffer, 5 ml TPTZ, and 5 ml of $FeCl_3$ fresh working solution was prepared. The 2 ml of the stock solution was prepared, and different concentrations (10-500 µg/ml) were added and left for 30 min; absorbance was recorded at 593nm, and reducing capacity was determined. The extract's antioxidant power was expressed as µmol Fe^{2+}/gm, and determinations were made in triplicates.

2.3.4. Metal chelating activity.

The metal chelating activity was estimated by taking100ml of plant extract of different concentrations (10-500µg/ml) and mixing it with 50µl of 2mM ferrous chloride and 200µl of 5mM ferrozine solution [18]. The absorbance was measured at 562 nm after it had been completely mixed and incubated for 10 min at room temperature in the dark. The 100 µl EDTA (10 mg in 1ml DMSO) was used as standard. The percentage inhibition of the complex formation of ferrozine-Fe^{2+} was calculated using the following formula.

Inhibition of Metal Chelating activity (%) = Ac-As/Ac×100

As=absorbance of samples containing Fe^{2+} Ferrozine complex mixed with different concentrations of extract; Ac=absorbance of Fe^{2+} Ferrozine complex in the absence of extract. Ascorbic acid was used as the positive control.

2.3.5. Superoxide radical scavenging activity.

The methodology of Nishikimi *et al*., [19] was used to evaluate the superoxide radical extract activity. The reaction solution was prepared by adding NBT (1M) in phosphate buffer (100mM) and NADH (1m) in 100 mM phosphate buffer having pH 7.4. Then 1ml NBT and 1ml solution from NADH was added. The reaction commenced with adding 100µl of PMS (60µm in phosphate buffer pH 7.4 in 100mM) solution in the plant with varying concentrations (10-500µg/ml). Incubate for 15min, absorbance at 530 nm was read and compared to the standard ascorbic acid in 50mM phosphate buffer. The enzyme required to inhibit NBT reduction by 50% is expressed as the unit of SOD. The values were calculated using the given formula.

Inhibition of Superoxide radical activity (%) = Ac- As/ Ac×100

Where Ac and As are the absorbances of the control and sample.

2.4. Statistical analysis.

The mean ± standard error mean was used to express the data. Utilizing the statistical program GraphPad Prism, the IC50 values were calculated using the linear regression analysis. Correlation among the antioxidant properties versus total phenolic/flavonoid contents was determined by plotting IC_{50} ($\mu g ml^{-1}$) against TPC and TFC ($mg g^{-1}$).

3. Results and Discussion

Phytochemicals present in the plant dissolve in different solvents, so non-polar to polar solvents were selected for the study.

3.1. Quantitative analysis.

Compositions of phytochemicals in plants include phenolic and flavonoids, which are antioxidants, excellent scavengers of harmful free radicals, and reactive oxygen species [20,21]. Spectrophotometrically, the total phenolic and flavonoid content were measured using gallic acid and rutin as the base for calibration curve plotting [Figure 1]. The non-polar and polar solvents were widely used for phenolic extraction. The properties of extracting solvents significantly influenced the overall quality of the determined phenolics. The complete phenolic and flavonoid content of fractions of the *E. crassipes* hydroethanolic leaf extracts is given in

(Table1). In the ethyl acetate fraction, maximum phenolic (372±0.76 mg GAE g^{-1}dry weight) and flavonoid content (452.6±0.65 mg RE g^{-1}dry weight) was observed.

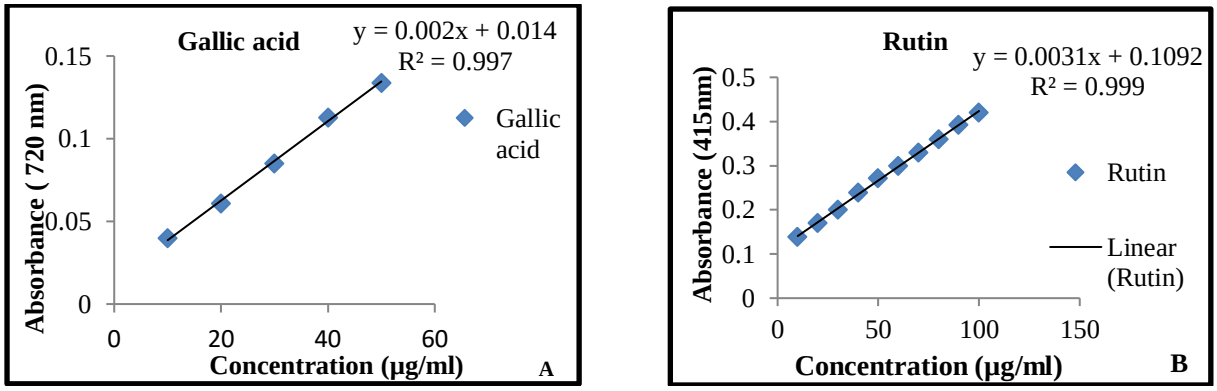

Figure 1. Estimation of Total (a) Phenolic Content; (b) Flavonoid Content.

Table 1. Total Phenolic and Flavonoid content in the different fractions of the hydroethanolic extract of leaves of *E.crassipes*

Extracts of *E. crassipes* (Leaf) in different fractions of hydroethanolic extract	**Total Phenolic Content (mg GAE g^{-1} dry weight)**	**Total Flavanoid content (mg RE g^{-1} dry weight)**
Hexane	110±1	57.2±0.51
Chloroform	173.2±0.57	97.2±0.86
Ethyl acetate	372±0.76	452.6±0.65
n-Butanol	320±1	230±0.85
Aqueous	290±1	354.6±1.02

3.2. Antioxidant activity.

Antioxidants play a significant part in helping to prevent and scavenge radicals. Using spectrophotometric methods, the antioxidant capacity of various extracts is calculated [Table 2]. The plant's maximum reduction potential was measured in the ethyl acetate fraction of the *E. crassipes* leaf. Free radicals are produced spontaneously as products of metabolic processes in biological systems, and they can cause considerable damage to tissues and macromolecules, leading to various serious clinical consequences. The high total phenolic and flavonoid content in the leaf extracts of *E.crassipes* can be utilized to measure antioxidant activity. Flavonoids are a wide category of phenolics that play a vital part in the antioxidant capacity of plants [22]. Flavonoids are an important element of any medicine and play a vital role in treating various disorders. They are used as raw ingredients to create innovative and effective medications in pharmaceutical sciences [23]. The hydroxyl group in the phenols and the free OH groups in flavonoids are responsible for the radical scavenging activity.

Table 2. Antioxidant activity in the fractions of hydroethanolic extract of *E. crassipes* leaves in different solvents ranging from non-polar to polar.

Samples	**DPPH (µg/ml)**	**FRAP (µmol Fe^{2+}/gm)**	**H_2O_2 (mgAAE/gm)**	**SOD (µg/ml)**	**Metal chelating activity (µg/ml)**
Standard	**67.06**±0.22	-	60.48±0.10	47.8±0.14	12.97±0.33
Hexane	383.9±0.08	19.24±0.06	337.75±0.05	433.69±0.23	412.27±0.14
Chloroform	340.62±0.12	25.18±0.17	279.13±0.03	357.5±0.20	331.6±0.13
Ethyl acetate	187.97±0.08	151±0.23	91.76±0.03	176.91±14	224.08±0.13
n-Butanol	260±0.08	45.18±0.13	136.33±0.04	325.54±0.14	386.66±0.09
Aqueous	216.8±0.15	31±0.23	105.58±0.12	197.96±0.20	260.11±0.21

3.2.1. DPPH.

DPPH is a commonly used free radical for detecting the scavenging potential of the antioxidants present in an extract. The less a compound's IC50 value, the greater its radical

scavenging behavior. The antioxidant capacity of extracts is inversely related to the IC50 estimate [24]. The increase in sample concentration increases the % inhibition. Therefore, this plant's ethyl acetate extract has the greatest capacity to scavenge radical DPPH as contrasted with other extracts (Table 2). The value of IC50 ranged from 383.9±0.08 to 187.97±0.08 and indicates that the proportion of ethyl acetate has the lowest IC50 value of 187.97±0.08 (r=0.988) relative to the values obtained for normal ascorbic acid is 67.06±0.22 (r=0.979) [Figure 2]. The ability of the extracts to scavenge DPPH was used to gauge their free radical scavenging activity because of the extracts' odd number of electrons. This test is crucial for learning how reactive organic compounds are with stable free radicals [25]. The phenols and flavonoids correlate well with almost all the antioxidants (Table 2) due to their structural dissimilarities [23,26]. The lesser IC_{50} values indicate strong antioxidants and an increased concentration of phenols and flavonoids. The phytocompounds antioxidant action is mostly owing to their redox characteristics, which aid in the adsorption and neutralization of free radicals as well as the decomposition of peroxides [27]. The antioxidant's capacity to donate electrons or hydrogen helps to stabilize the DPPH molecule, making it a stable diamagnetic molecule.

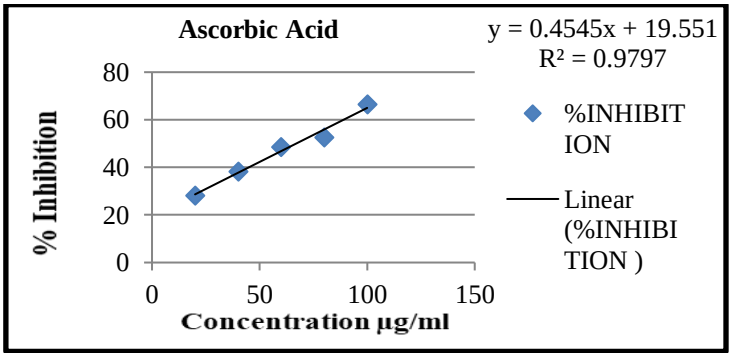

Figure 2. Standard graph curve for estimation of DPPH scavenging activity.

3.2.2. FRAP.

The FRAP assay requires a single-electron reduction of the complex light-yellow Fe (TPTZ)2 (III) by sample antioxidants to the complex Fe (TPTZ)2(II) blue at acidic pH. An extremely blue color suggests a reduction of the sample components.

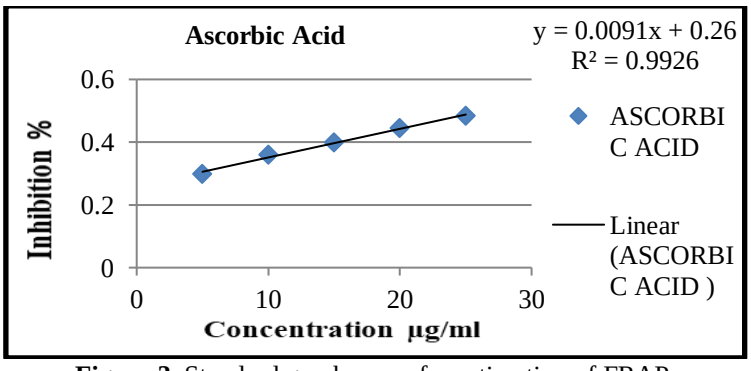

Figure 3. Standard graph curve for estimation of FRAP.

FRAP concentrations ranged from 19.24±0.06 to 151±0.23 µmol Fe^{2+}/gm for the different extracts. The largest FRAP values were derived from ethyl acetate fraction 151±0.23 µmol Fe^{2+}/gm (r=0.992). The FRAP approach relied on antioxidants' ability to limit the absorption of Fe^{3+} ion complexes and TPTZ due to the production of Fe^{2+} ions. The FRAP value was calculated by comparing the changes in absorbance values in the sample mixture

with those obtained from increased Fe3+ concentrations [Figure 3]. In general, the presence of compounds with reducing capabilities is linked to the presence of compounds that operate by breaking free radical chains by donating hydrogen atoms [28].

3.2.3. SOD.

The enzyme preparations of leaves of *E. crassipes* were evaluated for their superoxide dismutase (SOD) activity. The superoxide radical is first produced in cellular oxidation reactions, and its results are magnified as it creates free radicals and oxidizing agents that destroy cells and effectively initiate lipid peroxidation [29]. The fraction of leaf extract of ethyl acetate showed the lowest IC50 value, 176.91±14 µg/ml, and the maximum was exhibited by hexane at 433.69±0.23 µg/ml. The higher activities were observed in non-polar solvents and lowest in polar solvents comparable to the commercial ascorbic acid with an IC50 value of 47.8±0.14 µg/ml (R^2=0.995) [Figure 4]. The superoxide radical is a major biological source of reactive oxygen species; despite being a weak oxidant, it generates singlet oxygen and potent, deadly hydroxyl radicals, which cause oxidative stress. The extract's ability to effectively scavenge for superoxide radicals was demonstrated by our finding's, and its correlation with total flavonoid content suggested that it may have antioxidant potential [30,31].

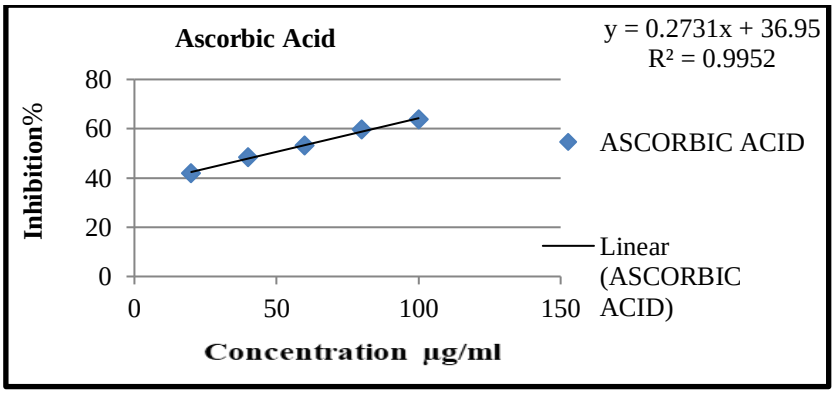

Figure 4. Standard graph curve estimation for estimation of superoxide radicals.

3.2.4. H_2O_2.

The ability of the H_2O_2 to penetrate the biological membrane and its nature to form hydroxyl radicals in the cells makes it toxic. The phenolic contents in the extracts neutralize H_2O_2 to water by donating electrons. The extract thus possesses antioxidant components, which are good electron donors and speeds up the conversion of hydrogen peroxide (H_2O_2) to water (H_2O). The different leaf extract fractions showed scavenging activity compared to the commercial ascorbic acid with an IC50 value of 60.48±0.10 mgAAE/gm (R^2=0.997) [Figure 5]. The H_2O_2 scavenging activity of ethyl acetate extract possesses high anti-scavenging activity with a low IC50 value of 91.76±0.03 mgAAE/gm. The hexane fraction demonstrated the lowest inhibition relative to control at a concentration of 337.75±0.05 mgAAE/gm. Hydrogen peroxide is a mild oxidizing agent that directly inactivates a few enzymes by oxidizing their crucial thiol (-SH) groups. The H_2O_2 is rapidly degraded into oxygen, and water, which can form hydroxyl radicals (•OH), causing DNA damage and triggering lipid peroxidation. Hydrogen peroxide was effectively scavenged by the ethyl acetate fraction, which may have been caused by the presence of phenolic groups that could transfer electrons to hydrogen peroxide, neutralizing it into water [32].

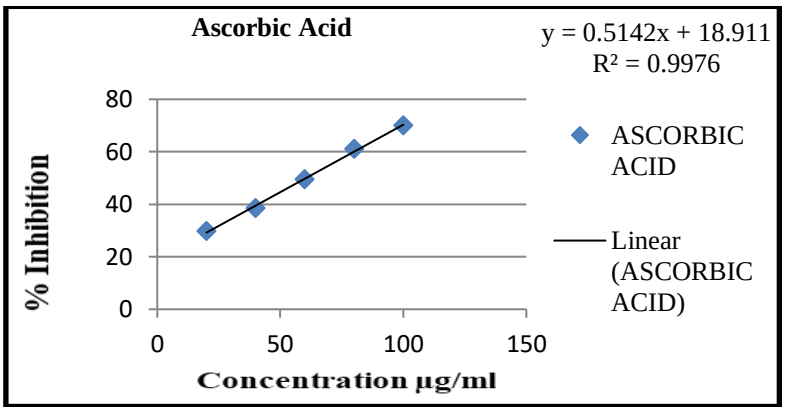

Figure 5. The standard graph curve for estimation of H_2O_2 scavenging activity.

3.2.5. Metal chelating.

The ferrous ion (Fe^{2+}) chelating activity of the analyzed fractions was calculated by rendering the ferrozine complex with iron (II) that can be spectrophotometrically calculated at 562 nm absorbance. Polyphenolic compounds fight to form this complex with ferrozine, and absorbance decreases. The findings have been presented as a percentage of ferrozine inhibition Fe^{2+}complex creation. Thus, through binding with Fe (II), polyphenolic compounds do not cause them to act as pro-oxidants and thus contribute to an indirect defense mechanism. The findings indicate that all the fractions analyzed exhibited different chelating activities. Ethyl acetate chelated more iron with IC50 values of 224.08±0.13, and hexane demonstrated minimal with IC50 values of 412.27±0.14. However, the extracts were less efficient compared to the commercial chelator EDTA [Figure 6]. The correlation between total flavonoid and Fe-chelating ability is high, with an R^2 value of 0.743.

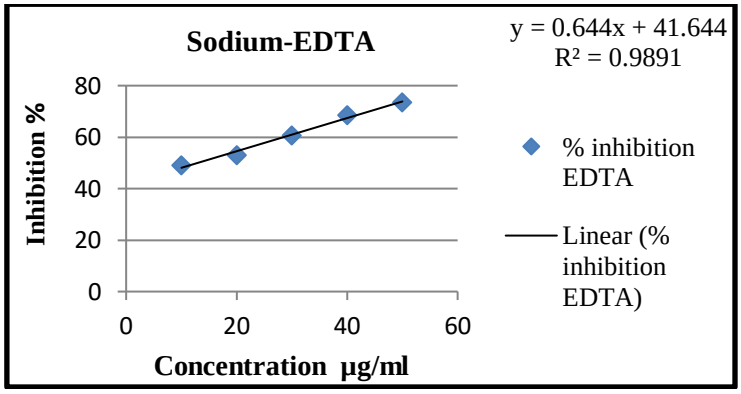

Figure 6. The standard graph curve for estimation of metal chelating activity.

3.3. Correlation between antioxidant assays by correlation analysis.

The correlation coefficients for TPC and TFC with the DPPH, FRAP, H_2O_2, SOD, and Metal chelating activity assays are shown in Table 3. The overall phenolic content with DPPH (R^2= 0.917), H_2O_2 (R^2=0.877), and SOD (R^2=0.734) showed a strong correlation with almost all the antioxidant assays. Furthermore, the overall flavonoid content is strongly associated with most of the antioxidant assays. Total flavonoids had a close interaction with DPPH (R^2=0.957), H_2O_2 (0.940), SOD (0.941), and Metal chelating behavior (0.743) as well as a positive association with TPC, and TFC (R^2=0.824). The DPPH radical scavenging evaluation demonstrated a close association between flavonoids and phenolics. It can be because many other molecules, including carotenoids, tocopherol, phenols, and vitamin C, also contribute to

antioxidant function [23]. The dissimilarity in the correlation coefficient between various antioxidant methods suggests that the overall antioxidant function cannot be measured with a single assay [26]. The metal-chelating ability could protect cells from oxidative damage caused by metal-catalyzed breakdown processes. The concentration of active chemicals, phenol, and flavonoid in certain extracts in this investigation is directly related to chelator action. The chelating activity was seen in certain extracts with significant phenol and flavonoid content.

Table 3. Correlation of antioxidant activity against phenolic and flavonoid content. Each value in the table is represented as Mean ± SEM (n = 3). * Indicates significance at P < 0.001.

Correlation	DPPH	FRAP	H_2O_2	SOD	Metal chelating activity
TPC	0.917	0.534	0.877	0.734	0.448
TFC	0.957	0.602	0.940	0.941	0.743

4. Conclusions

According to this study, the plant extract has phytochemical components that can donate hydrogen to a free radical in order to scavenge any possible damage. The high concentration of antioxidants in the leaves of *E.crassipes* is an indication that this troublesome weed can be used to isolate the active ingredients for the study of pharmacognostic activity. A greater degree of antioxidant activity is found in the ethyl acetate extract of *E. crassipes* leaves than in the other extracts. The current work can help complement information to define, authenticate, and standardize bioactive metabolites to standardize their herbal formulations.

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Conflicts of Interest

The authors declare no conflict of interest.

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