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ORIGINAL ARTICLE

In vitro Antioxidant Potential of *Euclea crispa* (Thunb.) Leaf Extracts

Chella Perumal Palanisamy, Devaki Kanakasabapathy[1], Anofi Omotayo Tom Ashafa

Phytomedicine and Phytopharmacology Research Group, Department of Plant Sciences, University of the Free State, QwaQwa Campus, Phuthaditjhaba 9866, South Africa, [1]Department of Biochemistry, Karpagam Academy of Higher Education, Coimbatore, Tamil Nadu, India

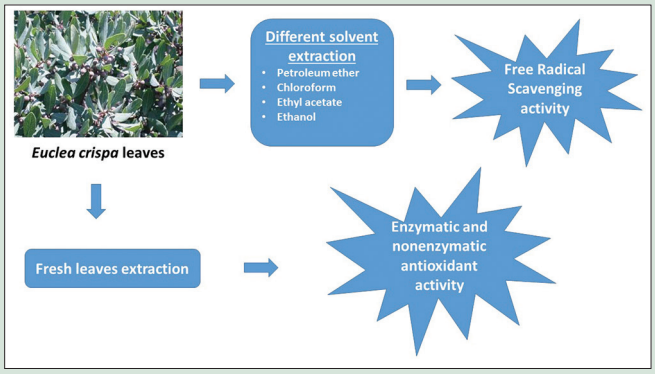
ABSTRACT

Background: *Euclea crispa* is a South African medicinal plant belonging to the family Ebenaceae. **Objectives:** The objective of this study was to analyze the *in vitro* antioxidant activity of different extracts of *E. crispa* leaves. **Materials and Methods**: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, reducing power assay, ferric reducing antioxidant power (FRAP) assay, hydroxyl scavenging assay, and nitric oxide scavenging assay were used to analyze free-radical scavenging activity. The superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), total reduced glutathione (TRG), and estimation of vitamin C assays were carried out to analyze the enzymatic and nonenzymatic antioxidants on a fresh leaf of *E. crispa*. **Results:** The DPPH radical scavenging assay (135.4 ± 0.7 μg/ml), hydroxyl scavenging assay (183.6 ± 0.9 μg/ml), and nitric oxide scavenging assay (146.2 ± 1.3 μg/ml) showed the significant half maximal inhibitory concentration (IC_{50}) values in ethanolic extract when compared to the ethyl acetate, chloroform, and petroleum ether extract of *E. crispa* leaves. Further, the ethanolic extract exhibited good reducing power assay and FRAP assay showed (the maximum absorption of 0.79 and 0.68 at 500 μg/ml, respectively) when compared to other solvent extracts. The fresh *E. crispa* leaves possess high content of enzymatic and nonenzymatic antioxidants such as SOD (41.3 ± 0.34 units/mg protein), CAT (124 ± 0.54 μmole of H_2O_2 consumed/min/mg protein), GPX (261.2 ± 0.42 μg of glutathione oxidized/min/mg protein), TRG (42.3 ± 0.16 μg/mg protein), and estimation of vitamin C (185 ± 0.39 μg/mg) assays. **Conclusion:** Based on the results obtained from this study, it can be concluded that the *E. crispa* leaves can be used for the preparation of antioxidative therapeutic agents. However, further studies are necessary to substantiate the current findings.

Key words: *Euclea crispa* leaves, enzymatic, free-radical scavenging activity, nonenzymatic antioxidant activity, solvent extractions

SUMMARY

- *E. crispa* leaf extracts have a variety of secondary metabolites, particularly alkaloids, steroids, flavonoids, tannins, phenols, glycosides, saponins, and terpenoids.
- The ethanolic extract of *E. crispa* leaves possess significant free radical scavenging activity when compared with ethyl acetate, chloroform, and petroleum ether extract
- The fresh *E. crispa* leaves displayed high content of enzymatic and nonenzymatic antioxidants.



Abbreviations Used: DPPH: 2, 2-diphenyl-1-picrylhydrazyl, FRAP: Ferric reducing antioxidant power, SOD - Superoxide dismutase, CAT: Catalase, GPX: Glutathione peroxidase, TRG: Total reduced glutathione, ROS: Reactive oxygen species, DNA: Deoxyribonucleic acid, EDTA: Ethylenediaminetetraacetic acid, TCA: Trichloroacetic acid, TBA: Thiobarbituric acid, NED: Naphthyl ethylenediamine dihydrochloride, IC50: Half maximal inhibitory concentration.

Correspondence:
Dr. Anofi Omotayo Tom Ashafa,
Department of Plant Sciences, University of the Free State, Qwaqwa Campus,
Phuthaditjhaba 9866, South Africa.
E-mail: ashafaaot@ufs.ac.za
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INTRODUCTION

Reactive oxygen species (ROS), such as singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide, are frequently generated as byproducts of biological reactions or from exogenous factors. Every molecule of ROS leads to oxidative damaging effects on living cells including DNA if excess ROS are not eliminated by the antioxidant system.[1] ROS plays a major role in the formation of chronic and degenerative diseases including cancer, autoimmune, inflammatory, cardiovascular, neurodegenerative diseases, and aging. Radical scavenging antioxidants are mainly significant in protecting cells from the injury of free radical.[2] Thus, antioxidants with free-radical scavenging activities may have enormous significance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated.[3]

Recent investigations have shown that varieties of bioactive substances are present in medicinal plants which are widely used in the prevention and management of various diseases. The demand for natural food constituents has resulted in broad research on naturally occurring antioxidants which can deactivate highly reactive free radicals.[4] In this respect, flavonoids, alkaloids, terpenoids, and phenolic compounds which are usually found in medicinal plants have been reported to have high antioxidant activity as well as multiple biological effects.[5] Currently, the over-the-counter synthetic antioxidants might be unsafe if used over a prolonged period and its toxicity has also been criticized. It is generally assumed that frequent use of plant-derived phytochemicals

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may contribute to shift the stability in the direction of sufficient antioxidant status. As a result, attention to natural antioxidants, in particular, those from plant origin has deeply amplified in recent years.[6]

Euclea crispa is an afrotropical plant species, commonly known as the blue guarri (Eng.); bloughwarrie (Afr.); motlhaletsogane (Setswana); and iDungamuzi, umGwali (isiZulu). It is a hardy and evergreen plant that usually forms a dense stand of shrubs or grows to tree size. It is widespread and common in the interior regions of Southern Africa.[7] This plant is used conventionally against a wide range of ailments such as gonorrhea, leprosy, scabies, diarrhea, and wound infections and previous reports have shown that the plant possessed antibacterial and antifungal activities.[8] Therefore, the main aim of this study is to evaluate *in vitro* antioxidant potential of *E. crispa* leaves extracts since this aspect of medicinal potential of this valuable plant have not been reported in any scientific literature.

MATERIALS AND METHODS

Plant collection

Fresh leaves of *E. crispa* were collected from Qwaqwa campus, University of the Free State, South Africa during April 2017 and identified by Prof. AOT Ashafa. The plant sample was authenticated at University of the Free State herbarium with herbarium collection of Taylor and Van Wyk, 1994 with reference number: 6404000-400. Collected plant leaves were washed under running tap water to remove contaminants and foliar debris, air dried, powdered, and stored in airtight container at 4°C for further studies.

Preparation of extract

Using exhaustive extraction procedure, the powdered plant material 100 g each was continuously soaked with petroleum ether, chloroform, ethyl acetate, and ethanol. All flasks containing the plant material and solvents were kept on the shaker (LabconPlatform Shaker, PTY, Durban, South Africa) for 72 h at room temperature. The extracts were collected, filtered using Whatman No: 1 filter paper, and concentrated to dryness using rotary evaporator (Cole-Palmer, South Africa) set at 40°C. The dried extracts were stored at 4°C until further use.

Phytochemical screening

The preliminary phytochemical screening[9] was performed on petroleum ether, chloroform, ethyl acetate, and ethanol extracts of *E. crispa* leaves.

Free radical scavenging activities

The free-radical scavenging activities of different extracts of *E. crispa* leaves were determined using various *in vitro* assays such as 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay, nitric oxide radical scavenging assay, hydroxyl radical scavenging assay, reducing power assay, and ferric reducing antioxidant power (FRAP) assay.

2, 2-diphenyl-1-picryl-hydrazyl radical scavenging assay

The DPPH radical scavenging activity of *E. crispa* leaf extracts was analyzed using the standard method.[10] In brief, the reaction mixture contained 100 μM DPPH in methanol, various concentrations (100–500 μg/ml) of the extracts, and incubated for 30 min at room temperature. The decrease in the absorbance was measured at 517 nm. The scavenging activity was calculated as a percentage of the radical reduction. All tests were performed in triplicate.

Nitric oxide radical scavenging assay

The nitric oxide was generated by sodium nitroprusside and measured.[11] The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4), and various doses (100–500 μg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25°C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then, 1 ml of naphthyl ethylenediamine dihydrochloride (NED) (0.1% w/v) was added, and the mixture was incubated for another 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed triplicate.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of *E. crispa* leaf extracts was measured.[12] All the solutions were freshly prepared. To 1 ml reaction mixture contained, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); ethylenediaminetetraacetic acid (EDTA) (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM); and various concentrations (100-500 μg/ml) of the test sample. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml of 2.8% trichloroacetic acid (TCA), then 1 ml 1% aqueous TBA was added, and the mixture was incubated at 90°C for 15 min for the odor to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed in triplicates. Percentage of inhibition was evaluated by comparing the test and blank solutions.

Reducing power assay

The reducing power capacities of the extracts were assessed using the modified method of Oyaizu.[13] Various concentrations (100–500 ug/ml) of the extracts (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to end the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature, and the absorbance was measured at 700 nm against a suitable blank solution. All tests were performed in triplicates. A higher absorbance of the reaction mixture indicated greater reducing power.

Ferric reducing antioxidant power reducing power assay

Ferric reducing power of the extracts were determined using FRAP assay.[14] In brief, the stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mMHCl, 20 mM$FeCl_3$, $6H_2O$, and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 μl) was mixed with 90 μl of water and 30 μl of test sample/ethanol/distilled and water/standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 min, and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2}+) form. The absorption at 540 nm was recorded. The calibration curve was plotted with absorbance at 595 nm vs concentration of ferrous sulfate in the range of 0.1 mM ethanol solutions. The concentrations of $FeSO_4$ were, in turn, plotted against the concentration of standard antioxidants.

Enzymatic and nonenzymatic antioxidants assays

Extract preparation

The 5g of fresh *E. crispa* leaves were ground with 10 ml of 50% ethanol in a prechilled mortar and pestle, and the extracts were centrifuged at 10,000 g at 4°C for 10 min. The supernatant was obtained and used within 4 h for various enzymatic and nonenzymatic antioxidants assays.

Superoxide dismutase assay

The superoxide dismutase (SOD) assay was carried out using the standard method.[15] In brief, 1.4 ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-methionine, 0.04 ml of 1% (v/v) triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride, and 0.1 ml of 50 mM EDTA) was added to 100 µl of the sample and incubated at 30°C for 5 min. A volume of 80 µl of 50 µM riboflavin was added, and the tubes were exposed for 10 min to 200 W-Philips fluorescent lamps. After the exposure time, 1 ml of Griess reagent was added and the absorbance was calculated at 543 nm.

Catalase assay

In the catalase (CAT) assay,[16] the enzyme extract (0.5 ml) was added to the reaction mixture containing 1 ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O, and incubated for various time period (0, 30, 60, 90 s, respectively). The reaction was completed by the addition of 2 ml of acid reagent. To the control, the enzyme was added after the addition of acid reagent. All tubes were heated at 50°C for 10 min, and the absorbance was calculated at 610 nm.

Glutathione peroxidase assay

The glutathione peroxidase (GPX) assay was performed using the standard method[17] with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer, 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water, and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 s, respectively. The reaction was completed with the addition of 0.5 ml of 10% TCA and after centrifugation, 2 ml of the supernatant was added to 3 ml of phosphate buffer, and 1 ml of DTNB reagent. The color developed was read at 412 nm and the enzyme activity was calculated.

Estimation of total reduced glutathione

The amount of reduced glutathione in the samples was estimated using the method of Boyne and Ellman.[18] A volume of 1 ml sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA, and 30 g NaCl dissolved in 100-ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% trisodium citrate). The absorbance was read at 412 nm, and the enzyme activity is expressed regarding µg of glutathione utilized/min/mg protein.

Estimation of Vitamin C

The estimation of ascorbic acid (Vitamin C) was carried out using the prescribed method.[19] In brief, the assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent, and 1–2 drops of thiourea. After incubation at 37°C for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulfuric acid and the absorbance was calculated at 540 nm.

Statistical analysis

The results obtained were expressed as the mean ± standard deviation. The statistical comparisons among the groups were performed using statistical package program (SPSS 10.0, IBM, Armonk, New York, United States).

Table 1: Qualitative analysis of secondary metabolites present in different solvent extracts of *Euclea crispa* leaves

Phytochemical constituents	Solvents			
	Petroleum ether	Chloroform	Ethyl acetate	Ethanol
Alkaloids	-	-	+	+
Steroids	-	+	-	+
Flavonoids	-	+	-	+
Tannins/phenols	-	+	+	+
Aminoacids and proteins	-	+	+	+
Sugars	-	-	-	-
Glycosides	+	+	+	+
Saponins	+	+	+	-
Terpenoids	+	-	-	-

+: Presence of secondary metabolites; -: Absence of secondary metabolites

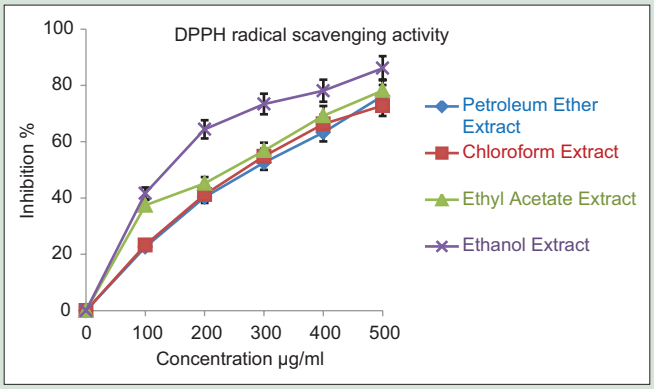

Figure 1: 2, 2-diphenyl-1-picryl-hydrazyl radical scavenging activity of different solvent extracts from *Euclea crispa* leaves

RESULTS

The phytochemical screening results are illustrated in Table 1. These results indicated that *E. crispa* leaf extracts have a variety of secondary metabolites, particularly alkaloids, steroids, flavonoids, tannins, phenols, glycosides, saponins, and terpenoids.

The dose-response DPPH radical scavenging activity of various extracts is shown in Figure 1. With regard to the estimated IC_{50} values of all the extracts of *E. crispa* leaves displayed significant DPPH radical quenching property [Table 2]. Among the extracts, the most active was ethanolic extract (135.4 ± 0.7 µg/mL), followed by ethyl acetate (238.8 ± 0.4 µg/mL), chloroform (265.3 ± 1.2 µg/mL), and petroleum ether (276.5 ± 0.8 µg/mL) extracts. The result of nitric oxide scavenging activity of the extracts is shown in Figure 2. Among the extracts, the ethanolic extract of *E. crispa* leaves showed superior IC_{50} value of 146.2 ± 1.3 µg/mL when compared to other extracts of ethyl acetate (241.6 ± 0.9 µg/mL), chloroform (226.2 ± 1.6 µg/mL), and petroleum ether (316.4 ± 0.4 µg/mL). The results of hydroxyl radical scavenging assay obtained from the current study are illustrated in Figure 3. In this assay, the ethanol extract of *E. crispa* leaves showed better IC_{50} values of 183.6 ± 0.9 µg/ml when compared to ethyl acetate (209.3 ± 1.3 µg/mL), chloroform (238.2 ± 0.5 µg/mL), and petroleum ether (271.5 ± 1.3 µg/mL) extracts. In reducing power assay, for the observation of reducing capacity, "Fe3+– Fe2 + transportation" in the presence of extracts were determined. These results are illustrated in

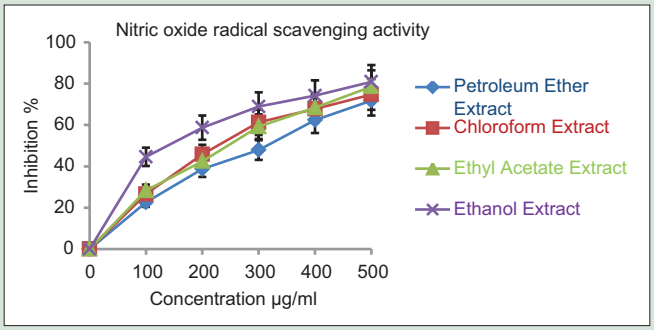

Figure 2: Nitric oxide radical scavenging activity of different extracts from *Euclea crispa* leaves

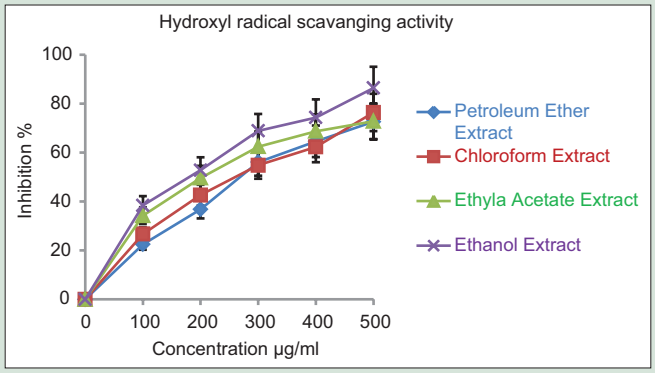

Figure 3: Hydroxyl radical scavenging activity of different extracts from *Euclea crispa* leaves

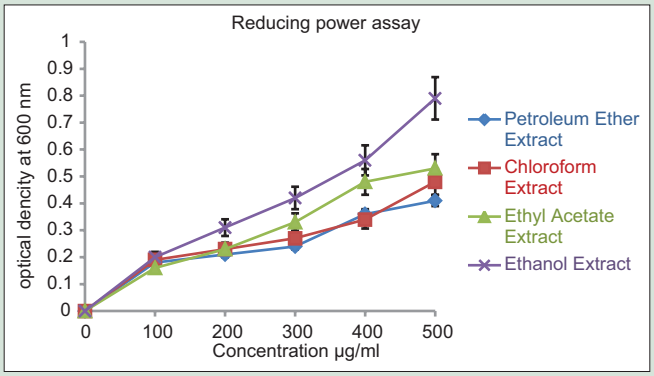

Figure 4: Reducing power assay

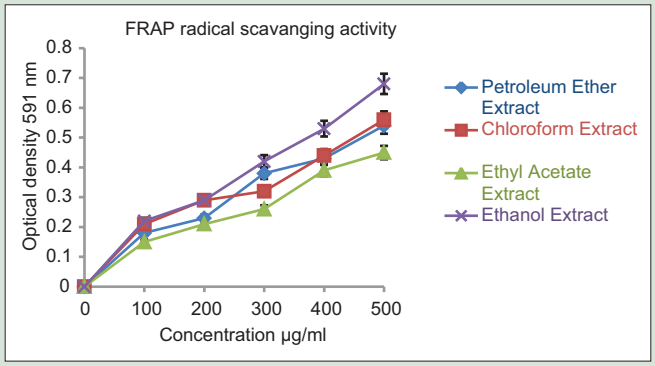

Figure 5: Ferric reducing antioxidant power radical scavenging assay

Table 2: Free-radical scavenging capacity of different solvent extracts of *Euclea crispa* leaves

Extracts	IC_{50} values (µg/ml)		
	DPPH radical scavenging assay	Nitric oxide radical scavenging assay	Hydroxyl radical scavenging assay
Petroleum ether	276.5±0.8	316.4±0.4	271.5±1.3
Chloroform	265.3±1.2	226.2±1.6	238.2±0.5
Ethyl acetate	238.8±0.4	241.6±0.9	209.3±1.3
Ethanol	135.4±0.7	146.2±1.3	183.6±0.9

DPPH: 2, 2- diphenyl-1-picryl-hydrazyl; IC_{50}: Inhibitory concentration

Figure 4. The reducing capacity of extracts at 500 µg/ml concentration, the absorbance of petroleum ether extract was 0.41, chloroform extract was 0.48, ethyl acetate was 0.53, and ethanol extract was 0.79. It indicates that ethanol extract has high reducing power activity than other extracts. FRAP assay showed that, at 500 µg/ml of the absorbance [Figure 5] of petroleum ether extract 0.54, chloroform extract 0.56, ethyl acetate 0.45, and ethanol extract 0.68. The ethanol extract of *E. crispa* leaves has higher reducing power activity than petroleum ether, chloroform, and ethyl acetate extracts.

The fresh *E. crispa leaves* having high content of enzymatic and nonenzymatic antioxidants such as SOD (41.3 ± 0.34 units/mg protein), CAT (124 ± 0.54 µmole of H_2O_2 consumed/min/mg protein), GPX (261.2 ± 0.42 µg of glutathione oxidized/min/mg protein), total reduced glutathione (TRG) (42.3 ± 0.16 µg/mg protein), and estimation of vitamin C (185 ± 0.39 µg/mg protein) assays are showed in Table 3.

DISCUSSION

The varieties of phytochemical components are present in medicinal plants which are possessing beneficial importance in medical sciences, and they are commonly associated with various pharmacological activities of natural products. In this study, the ethanolic extract of *E. crispa* exhibited a variety of secondary metabolites such as alkaloids, steroids, flavonoids tannins, phenols, and glycosides which are known to be responsible for the antioxidant, antimicrobial, antidiabetic, and anticancer activities of the plant.[20]

Free radicals are the major cause of various chronic and degenerative diseases in the living systems. The vast amounts of synthetic molecules are available for free-radical scavenging activity but adverse side effects are associated with these compounds.[21] An alternative solution for this persistent global problem is to consume the naturally available antioxidants from medicinal plants because they have been reported to possessed lower side effects and comparatively safe.[22] Most frequently, the DPPH radical scavenging assay, nitric oxide radical scavenging assay, hydroxyl radical scavenging assay, reducing power assay, and FRAP reducing power assay are preferred for determining antioxidant activity of the plant. In this study, free-radical scavenging activity was observed to be concentration dependent and confirmed with the previous reports on several other plant species. Among other solvent extracts of *E. crispa* leaves, ethanolic extract was found to exhibit the highest scavenging activities and this could be attributed to the fact that the ethanolic extract of *E. crispa* possess a higher antioxidant molecules such as alkaloids, steroids, flavonoids tannins, phenols, and glycosides as evidenced using phytochemical screening [Table 1]. According to Perumal *et al.*, 2012,[23] Rad *et al.*, 2013,[24] and Owaisi *et al.*, 2014,[25] the most active antioxidant

Table 3: Levels of Enzymatic and nonenzymatic antioxidants present in fresh leaf sample of *Euclea crispa*

Parameters	Values
SOD (units/mg protein)	41.3±0.34
CAT (μmole of H_2O_2 consumed/min/mg protein)	124±0.54
GPX (μg of glutathione oxidized/min/mg protein)	261.2±0.42
TRG (μg/mg protein)	42.3±0.16
Estimation of vitamin C (μg/mg protein)	185±0.39

SOD: Superoxide dismutase; GPX: Glutathione peroxidase; TRG: Total reduced glutathione; CAT: Catalase

activity was observed in ethanol extract due to the presence of more phytochemical constituents. Further, more polar solvent of ethanol can often extract antioxidant compounds in higher quantities. Overall, the high-polarity solvent was very effective in extracting more antioxidant compounds when compared to an intermediate polar solvents and nonpolar solvents.

Antioxidants are substances that neutralize free radicals or their actions. Nature has endowed each cell with adequate protective mechanisms against any harmful effects of free radicals as follows: SOD, GPX, glutathione reductase, thioredoxin, thiols, and disulfide bonding are buffering systems in every cell. α-Tocopherol (Vitamin E) is an essential nutrient which functions as a chain-breaking antioxidant which prevents the propagation of free-radical reactions in all cell membranes in the human body. Ascorbic acid (vitamin C) is also part of the normal protecting mechanism. Other nonenzymatic antioxidants include carotenoids, flavonoids and related polyphenols, α-lipoic acid, and glutathione .[26] In this study, the fresh *E. crispa* leaves possess SOD, CAT, GPX, TRG, and vitamin C in higher quantity [Table 3] and this could be evidenced that, the *E. crispa* leaves might have potential enzymatic and nonenzymatic antioxidant agents.

CONCLUSION

In this study, the photochemical components of alkaloids, steroids, flavonoids, tannins, phenols, glycosides, saponins, and terpenoids were present in the different solvent extracts of *E. crispa*. The *in vitro* free-radical scavenging assays such as DPPH radical scavenging assay, nitric oxide radical scavenging, hydroxyl radical scavenging, reducing power assay, and FRAP reducing scavenging assay possess moderate scavenging activities. The fresh leaves of *E. crispa* possess superior enzymatic and nonenzymatic antioxidant activities. Therefore, based on the results, it can be concluded that the *E. crispa* leaves may hold an enormous resource of pharmaceutical properties.

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Conflicts of interest

There are no conflicts of interest.

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