



Phytochemical Characterization and *In Vitro* Antioxidant Evaluation of Selected Medicinal Plants with Potential Anti-Anemic Activity

Mayuri Thumar^{1*}, Trupesh Pethani²

1.B. K. Mody Government Pharmacy College, Polytechnique campus, Rajkot, Gujarat, India

2.Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India

ABSTRACT

Oxidative stress is closely associated with the pathogenesis of anemia and several chronic disorders, making natural antioxidants an important source of therapeutic agents. The present study investigated the in vitro antioxidant potential and quantitative phytochemical profile of hydroalcoholic extracts of *Spinacia oleracea*, *Withania somnifera*, *Embblica officinalis*, and *Caesalpinia bonducella*. Total phenolic and flavonoid contents were determined using Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively, while antioxidant activity was evaluated by DPPH, ABTS, nitric oxide, superoxide scavenging, reducing power, and FRAP assays. Among the tested plants, *E. officinalis* demonstrated the strongest antioxidant activity, exhibiting approximately 95.15% DPPH, 95.67% ABTS, 94.00% nitric oxide, and 95.18% superoxide radical inhibition at 500 µg/mL. *C. bonducella* showed comparable radical scavenging activity, whereas *W. somnifera* displayed moderate activity. *S. oleracea* contained the highest iron concentration (11.431 ppm) despite showing comparatively lower antioxidant activity. The findings suggest that the antioxidant efficacy of the selected plants is strongly associated with their phytochemical constituents, particularly phenolic and flavonoid compounds, supporting their potential application in the development of herbal hematinic formulations.

Keywords: Antioxidant activity; Anemia; Phenolic compounds; Herbal hematinic formulation; Medicinal plants

*Corresponding Author Email: mayuri.thumar@gmail.com

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INTRODUCTION

Reactive oxygen species (ROS), including superoxide, hydroxyl radicals, peroxy radicals, and peroxynitrite, are continuously generated during normal cellular metabolism(1). Excessive production of these free radicals results in oxidative stress, leading to lipid peroxidation, protein oxidation, DNA damage, and impairment of normal cellular functions (2). Natural antioxidants derived from medicinal plants can neutralize these reactive species and thereby protect biological systems from oxidative injury (3). Plant-derived polyphenols, flavonoids, vitamins, and other hydrophilic phytochemicals have attracted considerable attention because they effectively inhibit peroxide formation and reduce malondialdehyde production (4). Previous investigations have demonstrated that spinach extract possesses remarkable scavenging activity against superoxide, hydroxyl, and peroxy radicals, while its antioxidant constituents significantly improve ferric reducing antioxidant capacity and oxygen radical absorbance capacity in biological systems. Since antioxidants operate through multiple mechanisms, a single analytical method cannot comprehensively evaluate antioxidant capacity. Therefore, the study employed six complementary *in vitro* assays such as DPPH, ABTS, nitric oxide, superoxide radical scavenging, reducing power, and FRAP together with quantitative estimation of total phenolics and flavonoids. This multiparametric approach provides a more reliable assessment of the antioxidant potential of medicinal plant extracts. The objective of this study was to quantitatively evaluate phytochemical constituents and compare the *in vitro* antioxidant activity of hydroalcoholic extracts of four selected medicinal plants in order to identify promising candidates for future herbal hematinic formulation development.

MATERIALS AND METHOD

Plant Materials and Preparation of Extracts

Hydroalcoholic extracts of four medicinal plants *Spinacia oleracea* (leaves), *Withania somnifera* (roots), *Emblica officinalis* (fruits), and *Caesalpinia bonducella* (leaves) were used for phytochemical and antioxidant evaluation. The dried extracts were accurately weighed and dissolved in methanol to obtain stock solutions of 1 mg/mL, which were subsequently diluted to the required concentrations for each assay.

Chemicals and Reagents

Folin–Ciocalteu reagent, gallic acid, quercetin, DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, sodium nitroprusside, Griess reagent, nitro blue tetrazolium (NBT), riboflavin, EDTA, potassium ferricyanide, trichloroacetic acid, ferric chloride, TPTZ, Trolox, and analytical-grade methanol

were used throughout the study. All reagents were of analytical grade and freshly prepared before use.

Quantitative Phytochemical Analysis

Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method described by Singleton et al.(5) Gallic acid was used as the reference standard, and calibration solutions (10–60 µg/mL) were prepared in methanol. Briefly, 100 µL of plant extract or standard solution was mixed with 10 mL distilled water and 1.5 mL Folin–Ciocalteu reagent. After 5 min, 4 mL of 20% sodium carbonate solution was added, and the volume was adjusted to 25 mL with distilled water. The reaction mixture was incubated for 30 min at room temperature, and absorbance was measured at 765 nm using a Shimadzu UV-1700 UV–Visible spectrophotometer. All determinations were performed in triplicate, and results were expressed as mg gallic acid equivalents (GAE)/g of dry extract.

Determination of Total Flavonoid Content (TFC)

Total flavonoid content was estimated according to the aluminium chloride colorimetric method of Zhishen et al. (6). Quercetin served as the standard (0.05–0.5 mg/mL). An aliquot of 0.5 mL of each extract was mixed with 2 mL distilled water followed by 150 µL of 10% sodium nitrite. After 5 min, 150 µL of 10% aluminium chloride was added. Following an additional 6 min, 1 mL of 1 M sodium hydroxide and 2 mL distilled water were added, and the mixture was thoroughly vortexed. Absorbance was recorded at 510 nm against the reagent blank. Results were expressed as mg quercetin equivalents (QE)/g of dry extract, and all analyses were carried out in triplicate.

***In Vitro* Antioxidant Activity**

The antioxidant potential of the hydroalcoholic extracts was evaluated at concentrations ranging from 25–500 µg/mL using six complementary *in vitro* assays: DPPH radical scavenging, ABTS radical cation decolorization, nitric oxide scavenging, superoxide scavenging, reducing power, and ferric reducing antioxidant power (FRAP). All experiments were performed in triplicate.

DPPH Radical Scavenging Assay

DPPH radical scavenging activity was evaluated following the method of Miliauskas et al. (7) A freshly prepared DPPH solution (12.5 mg in 500 mL methanol) was protected from light. 100 µL of each extract at different concentrations was mixed with 3.9 mL of DPPH solution and incubated in the dark for 15 min. Absorbance was measured at 516 nm using methanol as blank.

Ascorbic acid was used as the positive control. Radical scavenging activity was calculated as percentage inhibition using the equation:

$$\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}$$

where A_{blank} and A_{sample} represent the absorbance of the control and test sample, respectively. IC_{50} values were determined from concentration response curves.

ABTS Radical Cation Decolorization Assay

ABTS radical scavenging activity was determined according to the method of Miller et al. (8). The ABTS radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark for 12–15 h. The working solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 100 μL of extract was added to 3 mL of ABTS radical solution, mixed thoroughly, and incubated for 6 min. Absorbance was measured spectrophotometrically, Trolox was used as the reference antioxidant, and results were expressed as percentage inhibition and IC_{50} values.

Nitric Oxide Radical Scavenging Assay

Nitric oxide scavenging activity was evaluated using the modified method of Rao (9). Different concentrations of plant extracts were prepared in methanol and mixed with 2 mL of 10 mM sodium nitroprusside in phosphate-buffered saline. The reaction mixtures were incubated for 150 min at room temperature. Following incubation, 5 mL of freshly prepared Griess reagent was added, and the chromophore formed was measured at 546 nm. Curcumin served as the standard antioxidant. Percentage inhibition and IC_{50} values were calculated for each extract.

Superoxide Radical Scavenging Assay

Superoxide radical scavenging activity was determined by the riboflavin–NBT photoreduction method described by Robak et al. (10). The reaction mixture consisted of plant extract, 100 μL riboflavin solution, 200 μL EDTA solution, 100 μL NBT solution, and phosphate buffer (50 mM, pH 7.4), with a final volume of 3 mL. The assay was conducted under monochromatic light while protecting the reaction mixture from visible light using aluminium foil. Absorbance was recorded at 590 nm against phosphate buffer, with ascorbic acid used as the positive control. Radical scavenging activity and IC_{50} values were subsequently calculated.

Reducing Power Assay

The reducing power of each extract was determined according to Oyaizu with slight modifications (11). Briefly, plant extract was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min,

followed by addition of 2.5 mL of 10% trichloroacetic acid and centrifugation at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with an equal volume of distilled water and 0.5 mL of 0.1% ferric chloride. The resulting Prussian blue complex was quantified by measuring absorbance at 700 nm. Higher absorbance indicated greater reducing capacity.

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power was estimated using the method of Benzie and Strain (12). The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ prepared in 40 mM HCl, and 20 mM ferric chloride solution. 25 μ L of plant extract was added to 700 μ L of FRAP reagent, and absorbance was measured immediately (0 min) and after 30 min at 593 nm. A calibration curve was prepared using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1–1.0 mM), and results were expressed as $\mu\text{mol Fe(II)}$ equivalents per gram of dry extract.

Statistical Analysis

All experiments were performed in triplicate ($n = 3$), and results were expressed as mean $\pm$ standard deviation (SD). Percentage radical scavenging activities were calculated using the standard inhibition formula, while IC_{50} values were obtained from concentration–response curves for each antioxidant assay.

RESULTS

Quantitative phytochemical analysis

Total phenolic and flavonoid contents

The total phenolic content (TPC) and total flavonoid content (TFC) of the hydroalcoholic extracts were quantified using gallic acid and quercetin as reference standards, respectively. The calibration curves demonstrated excellent linearity for both gallic acid and quercetin, confirming the reliability of the colorimetric methods used for quantitative estimation.

Among the four medicinal plants, *Emblica officinalis* exhibited the highest phenolic and flavonoid contents, followed by *Caesalpinia bonducella* and *Withania somnifera*, whereas *Spinacia oleracea* showed comparatively lower levels (Figure 1 & 2). The higher accumulation of phenolic and flavonoid constituents in *E. officinalis* suggests its superior antioxidant potential and supports its traditional use as a potent medicinal plant.

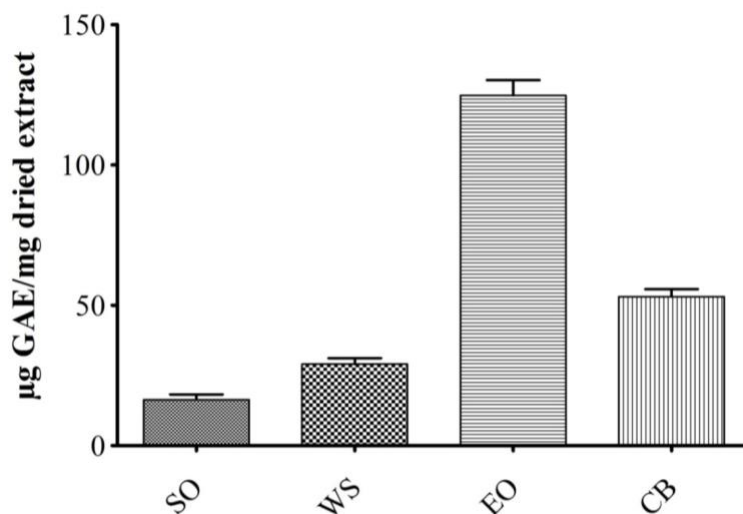


Figure 1: TPC of hydroalcoholic extract of *S. oleracea* leaves, *W. somnifera* root, *E. officinalis* fruit, and *C. bonducella* leaves. Results are expressed as mean $\pm$ SD (n = 3).

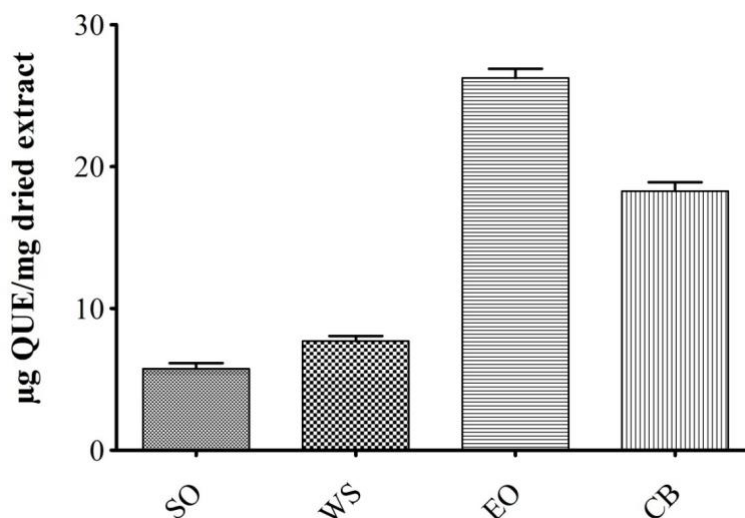


Figure 2: TFC of hydroalcoholic extract of *S. oleracea* leaves, *W. somnifera* root, *E. officinalis* fruit, and *C. bonducella* leaves. Results are expressed as mean $\pm$ SD (n = 3).

In vitro antioxidant activity

DPPH free radical scavenging activity

All extracts demonstrated concentration-dependent DPPH radical scavenging activity over the tested range (50–500 $\mu\text{g/mL}$) (Table 2). *E. officinalis* showed the strongest activity, with inhibition increasing from $56.15 \pm 3.11\%$ at 50 $\mu\text{g/mL}$ to $95.15 \pm 3.27\%$ at 500 $\mu\text{g/mL}$. *C. bonducella* also exhibited remarkable scavenging activity ($94.62 \pm 1.11\%$), followed by *W. somnifera* ($89.62 \pm 1.15\%$). *S. oleracea* displayed the lowest activity, reaching only $34.26 \pm 1.39\%$ inhibition at the highest concentration (Table 1).

Table 1: DPPH radical scavenging activity at 500 µg/mL

Plant extract	% Inhibition (Mean ± SD)
<i>Emblica officinalis</i>	95.15 ± 3.27
<i>Caesalpinia bonducella</i>	94.62 ± 1.11
<i>Withania somnifera</i>	89.62 ± 1.15
<i>Spinacia oleracea</i>	34.26 ± 1.39

Results are expressed as mean ± SD (n = 3).

ABTS radical cation scavenging activity

A similar concentration-dependent pattern was observed in the ABTS assay. The hydroalcoholic extract of *E. officinalis* exhibited the highest scavenging capacity, producing 95.67 ± 1.14% inhibition at 500 µg/mL. *C. bonducella* (93.27 ± 1.24%) and *W. somnifera* (92.00 ± 0.87%) also demonstrated substantial antioxidant activity, whereas *S. oleracea* showed comparatively weaker inhibition (46.32 ± 1.45%). These findings indicate that *E. officinalis* possesses broad-spectrum antioxidant activity against both hydrophilic and lipophilic free radicals (Table 2).

Table 2: ABTS radical scavenging activity at 500 µg/mL

Plant extract	% Inhibition (Mean ± SD)
<i>Emblica officinalis</i>	95.67 ± 1.14
<i>Caesalpinia bonducella</i>	93.27 ± 1.24
<i>Withania somnifera</i>	92.00 ± 0.87
<i>Spinacia oleracea</i>	46.32 ± 1.45

Results are expressed as mean ± SD (n = 3).

Nitric oxide radical scavenging activity

The nitric oxide scavenging assay further confirmed the antioxidant efficacy of the selected plants. *E. officinalis* produced the greatest inhibition (94.00 ± 1.23%), followed closely by *C. bonducella* (93.86 ± 1.38%). *W. somnifera* exhibited moderate activity (85.45%), whereas *S. oleracea* showed the lowest nitric oxide scavenging potential (41.33 ± 0.98%). The progressive increase in inhibition with increasing concentration demonstrates dose-dependent antioxidant behavior (Table 3).

Table 3: Nitric oxide radical scavenging activity at 500 µg/mL

Plant extract	% Inhibition (Mean ± SD)
<i>Emblica officinalis</i>	41.33 ± 0.98
<i>Caesalpinia bonducella</i>	85.45 ± 1.42
<i>Withania somnifera</i>	94.00 ± 1.23
<i>Spinacia oleracea</i>	93.86 ± 1.38

Results are expressed as mean ± SD (n = 3).

Superoxide radical scavenging activity

Superoxide radical scavenging activity increased consistently with concentration in all extracts. *E. officinalis* demonstrated the highest activity ($95.18 \pm 1.02\%$), followed by *C. bonducella* ($95.44 \pm 1.37\%$), indicating nearly equivalent efficacy between these two plants. *W. somnifera* achieved $88.88 \pm 1.25\%$ inhibition, whereas *S. oleracea* remained considerably less active ($41.04 \pm 0.38\%$). The results suggest that phenolic-rich extracts possess superior capacity to neutralize reactive oxygen species (Table 4).

Table 4: Super oxide radical scavenging activity at 500 µg/mL

Plant extract	% Inhibition (Mean $\pm$ SD)
<i>Emblica officinalis</i>	41.04 ± 0.38
<i>Caesalpinia bonducella</i>	88.88 ± 1.25
<i>Withania somnifera</i>	95.18 ± 1.02
<i>Spinacia oleracea</i>	95.44 ± 1.37

Results are expressed as mean $\pm$ SD (n = 3).

Reducing power assay

The reducing power assay demonstrated a concentration-dependent increase in absorbance for all plant extracts, indicating enhanced electron-donating ability with increasing extract concentration. The reducing capacity followed a trend comparable to the radical scavenging assays, with *E. officinalis* and *C. bonducella* exhibiting the greatest reducing potential, followed by *W. somnifera*. *S. oleracea* showed comparatively lower reducing activity throughout the concentration range (Figure 3).

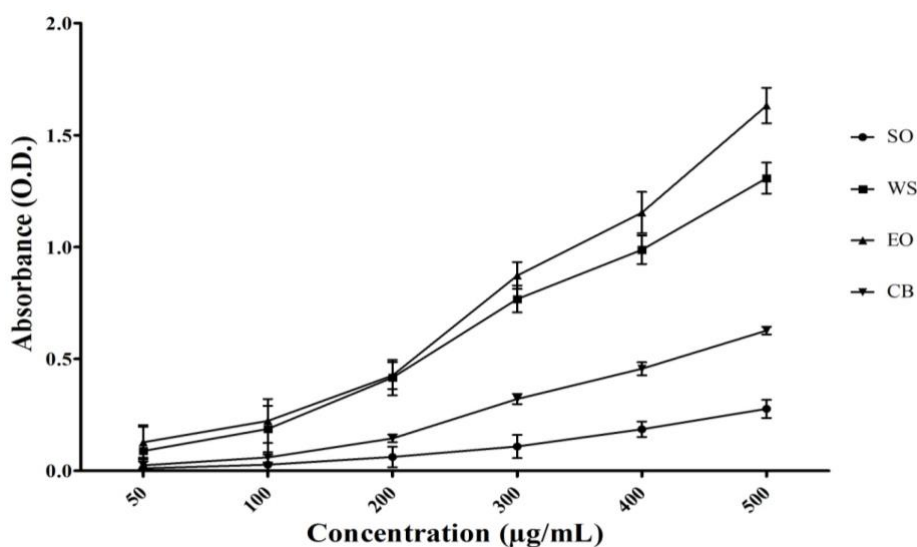


Figure 3: Reducing capacity assessment of hydroalcoholic extract of *S. oleracea* leaves, *W. somnifera* root, *E. officinalis* fruit, and *C. bonducella* leaves. Results are expressed as mean $\pm$ SD (n=3)

Ferric reducing antioxidant power (FRAP)

The FRAP assay confirmed the ferric ion reducing ability of the investigated extracts. The antioxidant capacity increased proportionally with extract concentration in all samples, and the highest ferric reducing activity was observed in *E. officinalis*, followed closely by *C. bonducella*. The FRAP profile was consistent with the results obtained from DPPH, ABTS, nitric oxide, and superoxide assays, indicating a strong correlation between reducing power and overall antioxidant activity (Figure 4).

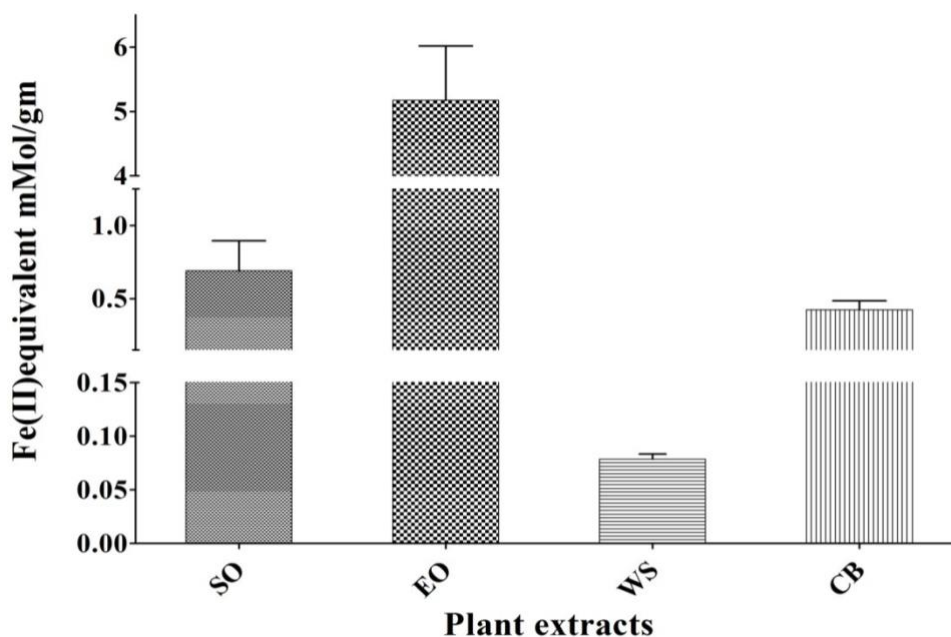


Figure 4: Fe (II) Equivalent mMol/gm hydroalcoholic extract of *S. oleracea* leaves, *W. somnifera* root, *E. officinalis* fruit, and *C. bonducella* leaves. Results are expressed as mean $\pm$ SD (n=3).

Based on the collective results of all six *in vitro* antioxidant assays, the overall antioxidant potency of the hydroalcoholic extracts followed the order: *Emblia officinalis* > *Caesalpinia bonducella* > *Withania somnifera* > *Spinacia oleracea* (Table 5)

Table 5: Comparison of antioxidant activity at 500 μ g/mL

Plant extract	DPPH	ABTS	Nitric oxide	Superoxide
<i>Spinacia oleracea</i>	34.26	46.32	41.33	41.04
<i>Withania somnifera</i>	89.62	92.00	85.45	88.88
<i>Emblia officinalis</i>	95.15	95.67	94.00	95.18
<i>Caesalpinia bonducella</i>	94.62	93.27	93.86	95.44

Values are expressed as percentage inhibition (mean of triplicate determinations).

E. officinalis consistently exhibited superior free radical scavenging and reducing activities, highlighting the complementary therapeutic value of these medicinal plants for future polyherbal hematinic formulations.

DISCUSSION

The present investigation demonstrated considerable variation in antioxidant activity among the four selected medicinal plants, indicating differences in their phytochemical composition and reducing capacities. The use of multiple antioxidant assays enabled evaluation of distinct mechanisms of radical scavenging and electron-donating ability, thereby providing a comprehensive antioxidant profile.

Phytochemical significance

Quantitative phytochemical analysis confirmed the presence of appreciable phenolic and flavonoid constituents in all hydroalcoholic extracts. Phenolic compounds are well-known hydrogen and electron donors that stabilize reactive oxygen species, while flavonoids contribute through metal chelation and inhibition of oxidative chain reactions (13). These phytochemicals are therefore considered major contributors to the observed antioxidant activity.

DPPH and ABTS radical scavenging activity

The DPPH assay evaluates the hydrogen-donating ability of antioxidants. *Embllica officinalis* exhibited the highest scavenging activity, reaching 95.15% inhibition at 500 µg/mL, followed closely by *C. bonducella* (94.62%). *Withania somnifera* showed moderate activity (89.62%), while *Spinacia oleracea* demonstrated substantially lower inhibition (34.26%). These findings indicate that amla possesses highly effective hydrogen-donating phytochemicals capable of neutralizing stable free radicals.

A similar pattern was observed in the ABTS assay, which measures both hydrophilic and lipophilic antioxidant activity. *E. officinalis* again produced the greatest inhibition (95.67%), confirming its broad-spectrum antioxidant effectiveness. *C. bonducella* and *W. somnifera* also exhibited strong ABTS scavenging activity, whereas spinach remained comparatively less active.

Nitric oxide and superoxide scavenging

Nitric oxide and superoxide radicals are biologically important reactive species involved in inflammation and oxidative cellular injury(14). The results demonstrated concentration-dependent inhibition for all extracts, with *E. officinalis* consistently producing the highest scavenging effect (94.00% NO and 95.18% superoxide inhibition at 500 µg/mL). *C. bonducella* showed nearly equivalent activity, suggesting the presence of potent antioxidant metabolites capable of suppressing reactive nitrogen and oxygen species.

Reducing power and FRAP

Both reducing power and FRAP assays assess the electron-donating ability of antioxidant molecules by reducing ferric ions to the ferrous form (15). The study demonstrated that antioxidant capacity increased with concentration in all extracts, supporting the concept that phenolic-rich plant extracts possess strong reducing potential. The consistency between radical scavenging assays and reducing assays strengthens the reliability of the overall antioxidant findings.

CONCLUSION

The present study demonstrated that all four selected medicinal plants possess measurable antioxidant activity and significant phytochemical constituents. Among them, *Emblica officinalis* exhibited the strongest antioxidant potential across DPPH, ABTS, nitric oxide, and superoxide radical scavenging assays, whereas *Spinacia oleracea* contained the highest iron concentration. The findings indicate that antioxidant activity is closely associated with phenolic and flavonoid phytochemicals rather than iron content alone. These results support the selection of *E. officinalis*, *S. oleracea*, *W. somnifera*, and *C. bonducella* as promising candidates for the development of polyherbal hematinic formulations with combined antioxidant and nutritional benefits.

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