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Comparative Evaluation of Antioxidant Activity, Phenolic Profile, and Reducing Power of Selected Medicinal Herbs Using Multiple In Vitro Assays

[1,2]Anusha Srinivas, Ramesh Chandran K[1,2] Sreeja P C[1,2] Sapna Nehra[1*]

[1]School of Basic and Applied Sciences, Nirwan University Jaipur,
[2]Innovation Centre, Mane Kancor Ingredients Private Limited, Kochi, Kerala 683573

***Corresponding Author: nehrasapna111@gmail.com**

Article

Oxidative stress a risk factor for many chronic diseases because of the excess generation of free radicals, which is why the usage of natural antioxidants is effective. This study is an exhaustive comparative review of the antioxidant properties of *Hibiscus rosa-sinensis*, *Moringa oleifera*, and *Ocimum sanctum*. Ethanolic extracts of the plants that were chosen were examined by means of an assortment of in vitro antioxidant tests, such as DPPH, ABTS, and FRAP. Radical scavenging activity has been communicated as IC_{50} values from the reduction of the concentration of DPPH, total phenolic (TP) content and total flavonoid (TF) content. The outcomes showed that *Moringa oleifera* had the highest antioxidant activity, with the lowest IC_{50} value (0.38 ± 0.02 mg/mL), followed by *Hibiscus rosa-sinensis* (0.48 ± 0.03 mg/mL) and *Ocimum sanctum* (0.60 ± 0.04 mg/mL). The results of the study prove the relevance of the phytochemical compounds as the key factors in the antioxidant efficacy of the herbs mentioned above and their potential utilization in nutraceutical and pharmaceutical formulations.

Key words: Antioxidants; IC_{50}; Phenolic compounds; DPPH; FRAP; ABTS; Medicinal plants; Oxidative stress

Introduction

Reactive oxygen species (ROS), including superoxide radicals ($O_2^{-}\bullet$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2), are generated as by-products of normal cellular metabolism. While ROS play essential roles in physiological processes such as cell signalling and immune defence, excessive production results in oxidative stress, leading to cellular damage through lipid peroxidation, protein denaturation, and DNA mutations (Anand *et al.* 2019). Cellular reactions due to the oxidation is one of the key factors to the growth of chronic diseases, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases.

Antioxidants mitigate oxidative damage by neutralizing free radicals by loss of electron, hydrogen atom transfer, or metal chelation mechanisms. Now a days we can see a paradigm shift toward natural antioxidants derived from plant sources due to their safety profile and broad-spectrum biological activities.

Medicinal herbs are rich in bioactive components like phenolic acids, flavonoids, tannins, and pigments, which contribute for antioxidant activity (Ayadi *et al.* 2016). Among these, *Moringa oleifera* is recognized for its exceptional nutritional and phytochemical composition, including large amount of quercetin, chlorogenic acid, and kaempferol (Sreelatha *et al.* 2009). *Hibiscus rosa-sinensis* contains anthocyanins, flavonoids, and chlorophyll derivatives, together provides potent radical scavenging activity. Ocimum sanctum (Tulsi), a cornerstone of traditional medicine, is known for its diverse phytoconstituents such as eugenol, rosmarinic acid, and ursolic acid (Gupta *et al.* 2012).

Despite extensive studies on individual herbs, comparative evaluation using multiple antioxidant assays is essential for a holistic understanding of their antioxidant mechanisms, as different assays reflect distinct modes of action (electron transfer vs hydrogen atom transfer) (Prior *et al.* 2005). In order to overcome this situation, the present study aims to systematically compare the radical scavenging activity of selected medicinal herbs using DPPH, ABTS, and FRAP assays and to establish correlations with phenolic and flavonoid contents.

2. Methods and Materials

2.1 Chemicals and Reagents

Reagent materials were of analytical grade and used without further purification. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), Folin–Ciocalteu reagent, gallic acid, quercetin, ferric chloride ($FeCl_3$), and TPTZ (2,4,6-tripyridyl-s-triazine) were procured from standard chemical suppliers. Ethanol was used as the extraction solvent. All solutions were prepared using distilled water, and reagents were freshly prepared prior to analysis to make sure the consistency.

2.2 Plant leaves preparations

Fresh leaves of *Hibiscus rosa-sinensis, Moringa oleifera,* and *Ocimum sanctum* were collected from local sources and were washed with distilled water to remove adhering dust and impurities. The cleaned samples were shade-dried at ambient temperature (25–30°C) for 7–10 days to preserve thermolabile phytoconstituents. The dried leaves then pulverized into fine powder using a mechanical grinder to achieve complete passage through an ASTM 60-mesh sieve to make sure constant particle size. The powdered samples were stored in airtight containers at room temperature until further use.

2.3 Plant Extracts Preparations

The extraction was done using the maceration method with ethanol as the solvent. Approximately 10 g of powdered leaves was combined with 100 mL of ethanol (1:10 w/v) in a conical flask. The mixture was subjected to continuous agitation at 150 rpm for two days at ambient conditions to accelerate the effective extraction of active phytochemical molecules. Following extraction, the extract was filtered to remove solid residues. Distilled off the solvent miscella in a waterbath at 40°C to obtain a semi-solid extract. The concentrated extracts were further dried to constant weight and stored at 4°C in airtight containers until further analysis.

2.4 Preparation of Extract Solutions

Stock solutions were prepared by dissolving the dried extracts in ethanol to get a concentration of 1 mg/mL. Serial dilutions were then carried out to prepare working concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL. These concentrations were used for all antioxidant assays.

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2.5 DPPH Radical Scavenging Assay

Effectiveness of the scavenging of free radical by the phytochemical molecule was evaluated by measuring the concentration reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) while interacting with the phytochemical molecule (Brand-Williams *et al.* 1995). 0.1mM ethanolic DPPH was made and kept in the dark to prevent photodegradation. Equal volumes (1 mL each) of DPPH solution and phytochemical compounds of interest at various dilutions were mixed and kept in the dark at ambient conditions for half an hour. Measured absorbance at 517 nm using a UV-Visible spectrophotometer after the mixture was incubated. A control containing DPPH solution without extract was used for comparison. The percentage of DPPH inhibition was calculated, and IC_{50} values (mg/mL) were deduced from the concentration-response curve that showed the amount of the compound which completely halts 50% of the free radicals.

2.6 ABTS Radical Cation Decolorization Assay

The ABTS ((2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation assay is another method was the method that was used to assess the ability of phytochemical extracts to counteract the effects of oxidation. The ABTS radical cation ($ABTS^{+}$) was produced by mixing ABTS solution and potassium persulfate (7 mM and 2.45 mM respectively) and allowing the mixture to keep in dark atmosphere about 12–16 hours. The resulted solution was diluted with ethanol to measure the absorbance at 734 nm. For the assay, ABTS solution was mixed with phytochemical extract (1 mL and 100 µL respectively), and the solution was kept under incubatio for 6 minutes. The absorbance was subsequently documented at 734 nm (Re, Roberta *et al.* 1999). The percentage inhibition was computed in a similar way to the DPPH assay.

2.7 FRAP Assay

The plant extracts' reducing power was measured using Ferric Reducing Antioxidant Power (FRAP) assay. The reagent was 10:1:1 solution made by combining 300 mM acetate buffer with 10 mM TPTZ solution 40 mM HCl, and ferric chloride solution (20 mM) (Benzie *et al.* 1996). Measurement was done by, mixing 1 mL of FRAP reagent and 100 µL plant extract and incubated at 37°C for 30 minutes and then measure the absorbance at 593 nm. The antioxidant activity was expressed as µmol Fe^{2+} equivalents per gram of extract using a standard calibration curve.

2.8 Determination of Total Phenolic Content (TPC)

The TPC of the phytochemical extracts was measured by the Folin–Ciocalteu method. Briefly, 0.5 mL of sample was reacted with 2.5 mL of diluted Folin–Ciocalteu reagent (1:10). After 5 minutes, add 2 mL sodium carbonate solution (7.5%). Kept the solution at ambient condition for half an hour and then measure the absorbance at 765 nm. Calculated the TPC using gallic acid calibration curve and communicated as mg gallic acid equivalents (GAE) per gram of extract (Singleton *et al.* 1999).

2.9 Determination of Total Flavonoid Content (TFC)

The TFC was measured by aluminium chloride colorimetric method. Mix 0.5 mL phytochemical extract and 1.5 mL of methanol and then add 0.1 mL aluminium chloride (10%) and 0.1 mL potassium acetate (1 M). Subsequently, 2.8 mL of distilled water was added to the mixture (Chang *et al.* 2002). The solution was kept at ambient conditions for half an hour, and measure absorbance at 415 nm. The flavonoid content was calculated using a quercetin standard curve and expressed as mg quercetin equivalents in one gram of extract.

2.10 Statistical Analysis

Conducted triplicate (n = 3) experiments and documented the values as mean ± standard deviation. Performed the statistical analysis by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to asses' the significance in the difference among the samples. Considered a p-value < 0.05 as statistically significant.

3. Results and Discussion

3.1 Radical Scavenging Activity

The radical scavenging activity of the selected herbal extracts was initially evaluated using the DPPH radical scavenging assay. All extracts exhibited a concentration-dependent increase in radical scavenging activity within the tested range (0.2–1.0 mg/mL). Among the investigated samples, *Moringa oleifera* demonstrated the highest antioxidant activity, as evidenced by the lowest IC_{50} value of 0.38 ± 0.02 mg/mL. Hibiscus rosa-sinensis showed moderate activity with an IC_{50} value of 0.48 ± 0.03 mg/mL, while *Ocimum sanctum* exhibited comparatively lower activity with an IC_{50} value of 0.60 ± 0.04 mg/mL

The results clearly indicate that the radical scavenging efficiency follows the order:
Moringa oleifera > *Hibiscus rosa-sinensis* > *Ocimum sanctum* (Kaur *et al.* 2001, Zheng *et al.* 2001).
Statistical analysis using one-way ANOVA revealed that the differences in IC_{50} values among the extracts were statistically significant ($p < 0.05$), confirming the variability in antioxidant potential among the selected herbs.

Table 1. DPPH Scavenging Activity of Herbal Extracts

CONCENTRATION (mg/ml)	HIBISCUS (% inhibition)	MORINGA (% inhibition)	TULSI (% inhibition)
0.2	35	45	30
0.4	50	60	40
0.6	61	70	50
0.8	70	79	60
1	76	85	66

Table 2. IC_{50} Values of Selected Herbs

HERB	IC_{50} (mg/ml)
Moringa oleifera	0.38
Hibiscus rosa-sinensis	0.48
Ocimum sanctum	0.6

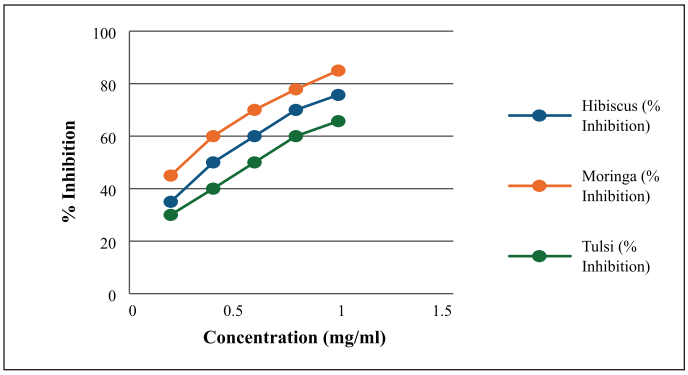

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Figure 1. DPPH scavenging activity of *Hibiscus rosa-sinensis*, *Moringa oleifera*, and *Ocimum sanctum* at various concentrations

3.2 ABTS Scavenging Activity

Antioxidant capacity of the phytochemical extracts was further evaluated using the ABTS radical cation decolorization assay. Similar to the DPPH results, all extracts exhibited significant radical scavenging activity, with Moringa oleifera showing the highest inhibition (82.1%), followed by Hibiscus rosa-sinensis (70.2%) and Ocimum sanctum (60.8%).

The ABTS assay results corroborated the findings obtained from the DPPH assay, indicating consistent antioxidant behaviour across different assay systems. The higher activity observed in Moringa oleifera suggests a greater ability to neutralize both hydrophilic and lipophilic radicals.

Table 3. ABTS Radical Scavenging Activity

HERB	ABTS (% inhibition)
Moringa oleifera	82.1
Hibiscus rosa-sinensis	70.2
Ocimum sanctum	60.8

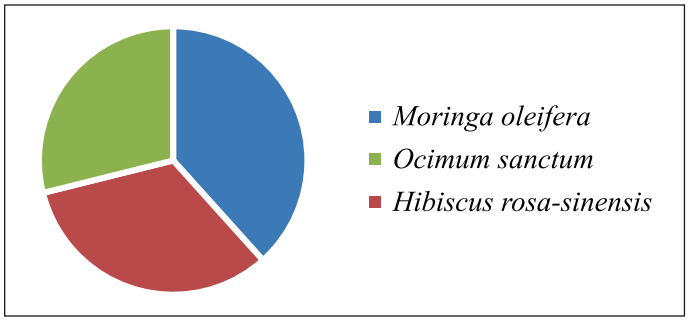

Figure 2. Comparative ABTS scavenging activity of selected herbal extracts

3.3 FRAP Assay

Reducing power of phytochemical extracts was measured by FRAP assay. The results demonstrated that all extracts possess considerable reducing capacity, with *Moringa oleifera* exhibiting the highest ferric reducing ability (650 µmol Fe^{2+}/g extract), followed by *Hibiscus rosa-sinensis* (520 µmol Fe^{2+}/g extract) and *Ocimum sanctum* (480 µmol Fe^{2+}/g extract). The FRAP results states that the extracts can donate electrons to reduce Fe^{3+} to Fe^{2+}, thereby acting as effective antioxidants.

Table 4. FRAP Assay

HERB	FRAP (µmol FE^{2+}/g extract)
Moringa oleifera	650
Hibiscus rosa-sinensis	520
Ocimum sanctum	480

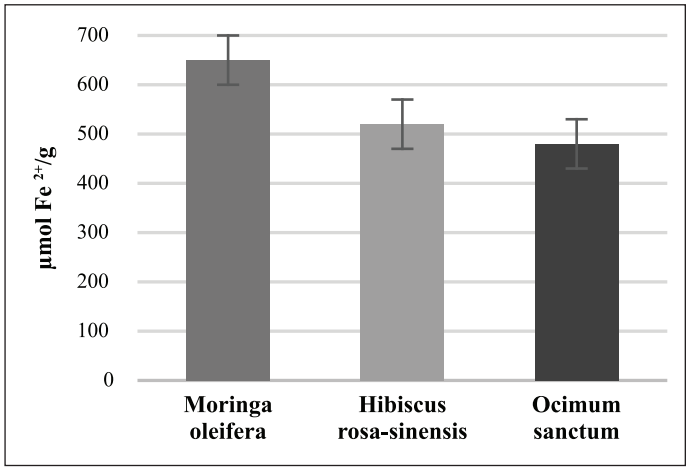

Figure 3. FRAP assay of selected herbs

3.4 Total Phenolic Content

TPC of the phytochemical extracts was evaluated by Folin–Ciocalteu method. *Moringa oleifera* showed the highest TPC (110 mg GAE/g extract), followed by *Hibiscus rosa-sinensis* (85 mg GAE/g extract) and *Ocimum sanctum* (75 mg GAE/g extract).

The higher TPC in *Moringa oleifera* is likely responsible for its higher antioxidant activity, as phenolic compounds are known to act as potent hydrogen donors and radical scavengers (Dorman *et al.* 2000).

3.5 Total Flavonoid Content

Moringa oleifera had the maximum TFC (95 mg QE/g extract), followed by *Hibiscus rosa-sinensis* (70 mg QE/g extract) and *Ocimum sanctum* (60 mg QE/g extract).

Flavonoids showed a crucial role in exhibiting radical scavenging activity through their ability to chelate metal ions and scavenge free radicals, thereby enhancing the overall antioxidant potential of the extracts (Wojdylo *et al.* 2007).

Table 5. Phytochemical Content of Herbal Extracts

HERB	TPC (mg GAE/g)	TFC (mg QE/g)
Moringa oleifera	110	95
Hibiscus rosa-sinensis	85	70
Ocimum sanctum	75	60

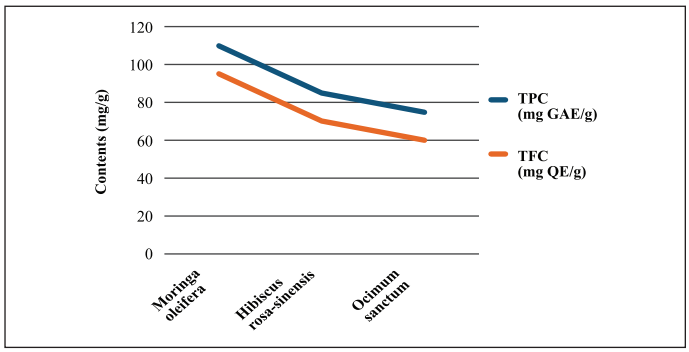

Figure 4. Relation of TPC and TFC

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3.6 Overall Comparative Analysis

A comprehensive comparison of antioxidant activity across all assays reveals a consistent trend, with *Moringa oleifera* exhibiting the highest antioxidant potential, followed by *Hibiscus rosa-sinensis* and *Ocimum sanctum* (Cai *et al.* 2004). The agreement among DPPH, ABTS, and FRAP assays highlights the reliability and robustness of the findings.

The combined results suggest that radical scavenging ability of the selected herbs is strongly influenced by their phytochemical composition, particularly phenolic and flavonoid compounds.

4. Discussion

The research was focused on the investigation of the radical scavenging capability of *Hibiscus rosa-sinensis, Moringa oleifera,* and *Ocimum sanctum*, which was performed by several in vitro methods, including DPPH, ABTS, and FRAP. These findings demonstrate that the extracts can lead to a growth increment in the antioxidant activity in a dose-dependent manner, thereby showing their ability to abolish the free radical effect. Among the tested herb species, *Moringa oleifera* exhibited the best antioxidant performance as shown by its lowest IC_{50} in the DPPH experiment and excellent results in the ABTS and FRAP methods.

The reason for the rich biological activity of *Moringa oleifera* is its higher total phenolic compounds and especially flavonoids. In fact, phenolic compounds are the strong donors of hydrogen atoms and/or electrons to free radicals, it is the same as stabilizing them interrupting the oxidative chain reactions. The high amount of phenolic molecules in *Moringa oleifera*, such as its phenolic superiority, is connected to this fact. In contrast, *Hibiscus rosa-sinensis* showed a moderate level of antioxidant capacity, which can be explained by the presence of anthocyanins and related pigments. The bioactive molecules are transported in antioxidants with the help of two kinds of mechanisms; the first one is the hydrogen transfer mechanism, while the other one is the electron transfer mechanism.

The concordance shown in the DPPH, ABTS, and FRAP assays, is their own credibility, while, whatever one analytical method might miss, the use of several is the answer that. They are all a variety of operation modes such as electron transfer and free radical neutralization and hence, they could make a thorough evaluation of antioxidant behaviour. Furthermore, there was a strong positive correlation between TPC and radical scavenging activity which indicated that phenolic molecules are the main contributors of antioxidant activity of the extracts. This discovery is concurrent with the previously published reports and with the help of phenolic compounds, they are plants that show antioxidant activity. The overall findings of this comparative study refer to the relationship of the inter-herbal antioxidants, their phytochemical properties, and the importance of these properties in defining their effects on biology.

5. Conclusion

The scientific research in this paper is established on in vitro tests to compare the antioxidant activity of *Hibiscus rosa-sinensis, Moringa oleifera,* and *Ocimum sanctum*. The distinct outcome of the tests is that *Moringa oleifera* possesses the highest antioxidant power, leaving *Hibiscus rosa-sinensis and Ocimum sanctum* next. The result points out that the radical scavenging activity of the plant extracts is strongly associated with the total concentration of phenolic compounds especially the flavonoid molecules. The direct relationship between phytochemical composition and antioxidant capacity becomes noticeable only after one considers the other compounds that are involved in the control of oxidative stress. The study's authorship of different experimental designs displays the full spectrum of the antioxidant mechanisms and makes their results much more reliable. The outcome expresses that these medicinal plants can be produced in pure natural antioxidant form especially *Moringa oleifera* which can be used in pharmaceutical, nutraceutical, and functional food industries.

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