ORIGINAL ARTICLE

Total antioxidant capacity using ferric reducing antioxidant power and 2, 2-diphenyl-1 picryl hydrazyl methods and phenolic composition of fresh and dried drumstick (*Moringa oleifera*) leaves

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Context: Dietary and life-style transitions have instigated accumulation of free radicals in our body causing oxidative stress, which is etiological in Non Communicable Diseases. Antioxidants are the means to counter oxidative stress. Thus, comprehensive knowledge of potential sources of antioxidants is vital. **Aim:** To assess the total antioxidant capacity (TAC) using ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays and phenolic composition of fresh and dried drumstick leaves from Western India. **Settings and Design:** This was an experimental study conducted under laboratory settings. Samples were procured from three different parts of the city of Vadodara, Western India. **Materials and Methods:** Fresh, oven-dried, and air-dried form of leaves were quantified for total phenol content (TPC) (Folin Ciocalteau assay), individual phenols (high performance liquid chromatography). Reducing property and free radical inhibiting property were quantified to understand the TAC using FRAP and DPPH assays respectively. **Statistical Tests:** Regression analyses were used for TPC and FRAP calculations. **Results:** The TPC ranged from 141.59 mgGAE/100 g to 185.32 mgGAE/100g; FRAP levels ranged from 0.49 mg/g to 0.7 mg/g, and DPPH ranged from 4.5% to 12.99% among raw and blanched Drumstick leaves sample in fresh, air-dried, and oven-dried form. High Pressure Liquid Chromatography analysis revealed the presence of gallic acid, epigallocatechingallate, chlorogenic acid. **Conclusion:** The underutilised drumstick leaves have strong antioxidant capacity attributable to its phenolic composition. Thus, they are cheap and accessible source of antioxidants for medicinal and commercial purposes.

Key words: Antioxidants, drumstick leaves, ferric reducing antioxidant power, *Moringa oleifera*, total antioxidant capacity, 2,2-diphenyl-1-picrylhydrazyl

INTRODUCTION

Prevailing life-style and fast food paradigm have caused oxidative stress and rising prevalence of non-communicable diseases globally.[1,2] Fruits and vegetables are concentrated source of antioxidants and five servings/day is the recommended dietary intake.[3] However, as per the National Family Health Survey-III (2007) of India only12.7% people consume fruits daily, attributable to the dietary alterations.[4] Similar transitions have been observed worldwide. Approximately, 16 million (1.0%) Disabled Average Life Years and 1.7 million (2.8%) of deaths worldwide are ascribed to low fruit and vegetable consumption.[5]

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Highly productive agricultural land is increasingly being used for urban development due to population pressure and increasing urbanization. Over-dependence on a few plant species exacerbates many acute difficulties faced by communities in the areas of food security, nutrition, and health.[6] Global food security and economic growth now depend on a declining number of plant species. This has placed the future supply of food and rural incomes at risk.[7] There is an urgent need of evidential science/evidence based studies on region specific underutilised plants, which maybe potential sources of antioxidants and nutrition to the rising population to manage morbidities ascribed to under-nutrition and NCDs.

Earlier studies on *Moringa oleifera* leaves have revealed their role in combating micronutrient malnutrition. These leaves are an excellent source of highly bioavailable beta-carotene as shown by animal trials and validation on human population by incorporating recipes of these dehydrated leaves into large scale national feeding programmes such as Integrated Child Development Scheme and the Mid-day Meal

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programme of India.[8,9] The purpose of this study was to assess total antioxidant capacity (TAC) using the modern techniques such as ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays along with polyphenol profile by HPLC of fresh, air-and oven-dried forms of drumstick leaves to identify its antioxidant potential.

MATERIALS AND METHODS

Sampling

M. oleifera leaves were collected from three different zones of the Vadodara city. Leaves were washed, blanched and used for analysis in fresh, oven-dried or shade-dried form.

Pre-preparation

For fresh leaves sample, leaves were grinded and made into paste in an electric mixer, the paste was used for analysis of Phenols, DPPH, and FRAP. The oven-dried sample was prepared by drying the leaves in an electric oven at 70°C until two consecutive weights were the same. For shade-dried sample, the leaves were dried on filter paper in shade on laboratory platforms for 2-3 days until two consecutive weights were obtained.

The oven-dried and shade-dried leaves were then powdered using an electric mixer and used further for sample preparation for FRAP, DPPH, and total phenol content (TPC) assays. All samples extracts were then prepared in solvent (80:20 methanol water).

Polyphenol profiling

Quantitative and qualitative estimation of polyphenols was performed to develop a polyphenol profile for each sample. Quantitative estimation of total phenol was carried out using spectrophotometric technique based on Folin-Ciocalteau assay.[10] The products of the reaction have blue colour and were read at 765 nm.[11] The advantage of using Folin-Ciocalteau method is that gives fairly equivalent response to different phenols, but it also responds to sulfur dioxide and sugar and is applicable to hydrophilic antioxidants.[12] For qualitative estimation of phenols HPLC analysis was selected. Standardization of the assay was carried out by preparing standard curve using 0, 50, 100, 150, 200, 250 mg/l solutions of gallic acid in methanol: Water (80:50, v/v). The standard curve plotted for various concentrations have been discussed in Figure 1. Calculation was carried out through regression analysis and the equation used was: Y = 0.008x + 0.103. Since 10 g sample is the requirement of the assay, resultant values were for 10 g sample. They were then calculated and expressed as mg/100 g weight gallic acid equivalents.

The HPLC analysis was carried out at Food Division, National Institute of Nutrition, Hyderabad. Samples

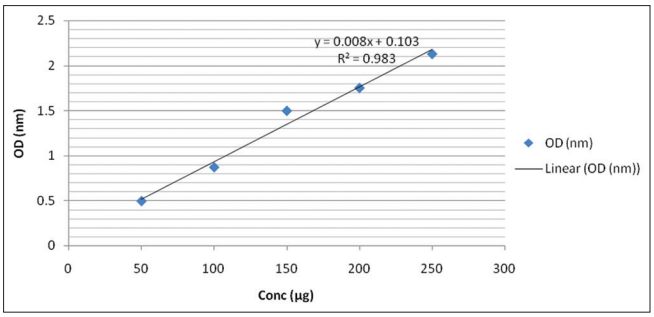

Figure 1: Standard curve for total phenol content (Folin assay)

were analysed for 21 polyphenol compounds for which standards were available. Shimadzu LC-6A model (Shimadzu, Tokyo, Japan) was used for conducting the liquid chromatography. It was fitted with a Waters í-Bondapack (Waters Corp., Milford, MA): Dionex-Acclaim C18 column (100 µM × 2.1 µM × 2.2 µM) and an SCL-6A system controller. 20 µL sample loop injection system was used. Detection was done by a Ultra-Violet visible spectrophotometer SPD-6AV set at a sensitivity of 0.04 AUFS and a wavelength of 280 nm.

Elution

At a flow rate of 0.47 mL/min.

Mobile Phase

Pump A having 10% methanol and 90% buffer; Pump B having 70% methonal and 30% buffer (Buffer used was a 50 ml sodium-dihydrogen phosphate–dihyrate buffer with H_3PO_4, pH of the buffer was maintained at 3.3). The compounds were quantified using a Shimadzu C-R4A Chromatopak data processor at a chart speed of 2.5 mm/min.

Quantification of antioxidant potential

Single antioxidant mechanism does not give an overview of antioxidant potential of the compound, hence reducing power and radical inhibiting property were analysed by FRAP and DPPH.

Sample preparation for FRAP and DPPH

Dissolve 1 g of dried sample in solvent (80:20 methanol water). Shake it for 30 min in magnetic shaker or water-shaker bath. And add 20 ml of solvent to the Supernatant. Again shake it for 30 min. Centrifuge and separate supernatant. Make volume upto 50 ml with the help of the solvent.

Estimation of reducing property of antioxidants

Ferric Reducing Antioxidant Potential (FRAP) method is based on the reduction of a ferric Tripyridal Triazine (TPTZ) complex to its ferrous, coloured form in the presence of antioxidant. The FRAP assay directly measures antioxidants with a reduction potential below the reduction potential of the Fe^{3+}/Fe^{2+} couple.[13] The FRAP assay was performed as described by Benzie and Strain (1996).[14] Standardisation

of the assay was done by preparing standard curve using 0.2-1.2 µg/ml solutions of Trolox in water. The standard curve plotted for various concentrations is depicted in Figure 2. Calculation was done through regression analysis. Equation used was: y = 0.6173x + 0.2264. Mean ± SD was calculated for each sample. The resultant values were expressed as µmol/g Trolox equivalent.

Estimation of free-radical inhibiting property of antioxidants

This assay was performed as described by Brand and William *et al*, 1995.[15] The reaction of DPPH is monitored by the decrease of the absorbance of its radical at 517 nm. Standard series of 1-4 µg concentrations of Gallic Acid was taken and volume was made up to 1ml with methanol. Thereafter, the samples were treated with the same procedure.

Calculation was done using the formula:

$$\%\text{Inhibition} = \{(\text{Optical Density}_{\text{Control}} - \text{Optical Density}_{\text{Sample}})/\text{Optical Density}_{\text{Control}}\} \times 100$$

Mean ± SD was calculated for each sample. The resultant values were expressed as percentage radical inhibiting property.

IC_{50} was calculated and expressed as mg sample/mg DPPH.

RESULTS

TPC

Results for TPC of fresh, air and oven dried drumstick leaf samples ranged from 141.59 mgGAE/100 g to 185.32 mgGAE/100 g [Table 1]. Moisture content of leaves was recorded as 83%. The TPC of fresh to oven-dried drumstick leaves was about 36% higher.

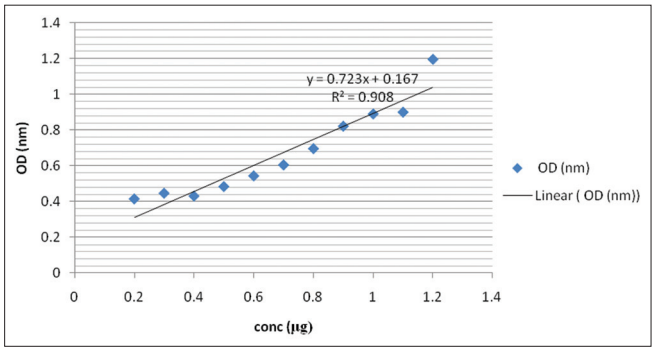

Figure 2: Standard curve for ferric reducing antioxidant power assay

Individual Polyphenol

HPLC analysis for quantification of individual polyphenol revealed the presence of gallic acid, 3,4-hydroxy benzoic acid, chlorogenic acid, P-coumaric acid, and various Flavonoids like epigallocatechingallate, apigenin, querecetin-3-beta D Gluoside, apigenin-7-O neohesperidose and luteolin were found in both air-and oven-dried samples. Epigallocatechin and Quercetrin-3-D Galactoside were found only in oven-dried form. Kaemperfrol, and gallocatechin were found only in air-dried form. Figures 3 and 4 describes the HPLC analysis chromatograms and Table 2 gives detailed analysis of air-dried and oven-dried samples.

TAC of *M. oleifera* Leaves

Free radical reducing power as assessed by FRAP for drumstickleaves (fresh, air-dried and oven-dried form) ranged from 0.49 mmol/g to 0.7 mmol/g and free radical inhibiting capacity as assessed by DPPH ranged from 4.5% to 12.9%. Air-dried *M. oleifera* leaves exhibited highest reducing power (FRAP) and free radical inhibiting power (DPPH) as compared to fresh and oven-dried form of drumstick leaves. IC_{50} value (mg sample/mg DPPH) for fresh sample was 0.77, for air-dried was 0.822 and for oven-dried was 0.83.

DISCUSSION

Phenolic compounds are secondary metabolites, associated with flavour and colour characteristics of fruits and vegetables. Accumulation of free radicals formed in the body due to environmental and biological factors causes oxidative stress, which disturbs normal redox state and may also trigger chronic diseases; the potent antioxidant and health promoting properties of polyphenols have won them considerable attention.[16] Phenols have been classified into various classes such as Flavonoids (quercetin), phenolic acids (chlorogenic acid) and proanthocyanidins and anthocyanins. Phenolic compounds exhibit a wide range of physiological properties such as protective and vasodilatory effects.[17-20]

Table 1: Results of the phenolic compounds and total antioxidant activity of drumstick (*Moringa oleifera*) leaves sample of this study

Drumstick (*M. oleifera*) leaves	TPC (mgGAE/100 g)	Phenolic composition	Antioxidant capacity	
			FRAP (mmol/g)	DPPH (% inhibition)
Fresh leaves	141.59±95.3	Gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, Epigallocatechingallate, lutelion, apigenin, Querecetin-3-Beta D gluoside, apigenin-7-O-neohesperidoside, P-Coumaric acid, hesperitin	0.49±0.2	4.50±0.45
Air-dried leaves	158.82±59.8		0.70±0.3	12.99±5.0
Oven-dried leaves	185.32±30.2		0.50±0.2	9.54±5.2
True retention %	22.52		16.6	8

TPC – Total phenol content; FRAP – Ferric reducing antioxidant power; DPPH – 2,2-diphenyl-1-picrylhydrazyl; *M. oleifera* – *Moringa oleifera*

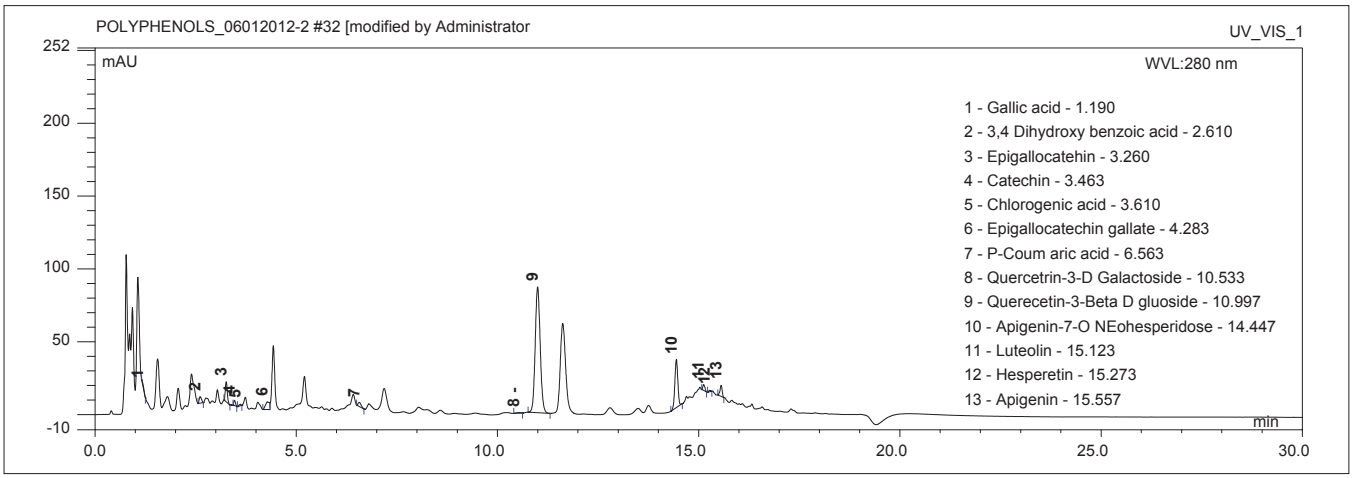

Figure 3: Chromatogram for oven dried drumstick (*Moringa oleifera*) leaves

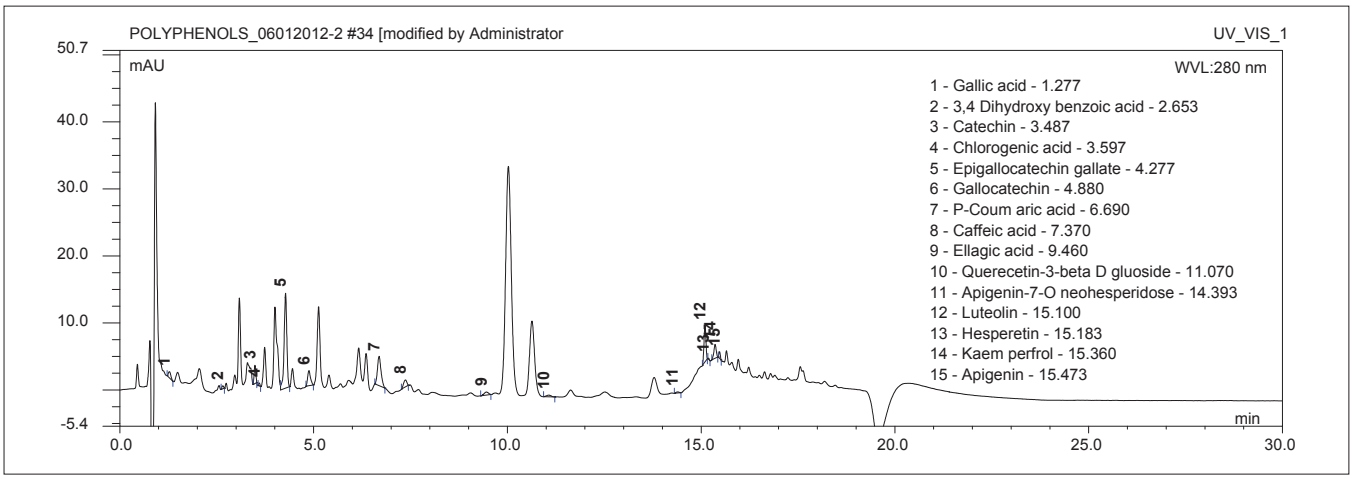

Figure 4: Chromatogram for air-dried *Moringa oleifera* leaves

Table 2: Details of the HPLC analysis for ovendried and airdried *Moringa oleifera* sample

Phenolic composition	Retention time (min)	
	Oven dried sample	Air dried sample
Name of the phenol		
Gallic acid	1.19	1.28
3,4-dihydroxy benzoic acid	2.61	2.65
Epigallocatehin	3.26	-
Catechin	3.46	3.49
Chlorogenic acid	3.61	3.60
Epigallocatechingallate	4.28	4.28
P-coumaric acid	6.56	6.69
Quercetrin-3-D galactoside	10.53	-
Querecetin-3-beta D glucoside	11.00	11.07
Apigenin-7-O neohesperidose	14.45	14.39
Luteolin	15.12	15.10
Hesperetin	15.27	15.18
Kaemperfrol	-	15.36
Apigenin	-	15.47
Gallocatechin	-	4.88
Caffeic acid	-	7.37
Ellagic acid	-	9.46

HPLC – High pressure liquid chromatography

Among fruits and vegetables, phenols are the major source of antioxidants.[21]

Our values obtained for TPC lie well within the range observed by various other investigators, who observed TPC content of drumstick leaves to be about 260 mgGAE/100 g[22] and 33.82 mg/g to 336.95 mg/g GAE dry extract.[23] This variation could be due to varietal differences and sample acquisition methods. Presence of kaempferol and quercetin[24,25] alkaloids, flavonoids, anthocyanins, proanthocyanidins and cinnamates have been reported in some Indian varieties of drumstick leaves.[25] The major bioactive compound among polyphenols was found to be kaempferol (flavanoid) in our oven-dried sample (4.491 mg/100 g) and in other studies as freeze-dried sample (104.7-225.4 mg/100 g),[24] ethyl acetate extract (49.7 mg/100 g),[24] strikingly this was not obtained in oven-dried sample of the study [Table 3]. Quercetin has been recorded to inhibit oxidative modification of Low Density Lipoprotein[26,27] and cytotoxicity of low-density lipoprotein.[28] Quercetin has also been observed to significantly decrease H_2O_2 induced intracellular Reactive Oxygen Species elevation. These secondary generated ROS activates mitochondria permeability transition induction, which plays an important role in ischemia/hypoxia induced apoptotic in cardiomyocytes *in vitro*.[29] Flavonoids also inhibit cyclooxygenase, leading to lower platelet aggregation and reduced thrombotic tendencies.[28] These cardio-protective actions of Flavonoids, especially, quercetin can be hypothesised to be manifested by *M. oleifera* leaves as our oven-dried sample contains 26.06 mg quercetin/100 g. Epigallocatechin (0.83 mg/100 g in our oven-dried sample) show anti-cancer properties and protect cells against damage induced by exposure to lead.[30] Naturally, occurring polyphenols have been found to have reducing effect on platelet aggregation.[29]

Studies have shown phenol rich diets reduce oxidative DNA damage, oxidative stress.[31] Phenolics present in *Moringa* leaf extracts are good electron donors and could terminate the radical chain reaction by converting free radicals to more stable products.[21] The scavenging effects of phenolics (flavonoids) such as Quercetin (observed in the samples) and rutin on superoxide radicals have been established by the Electron Spin Resonance method also.[32]

A comparison of dry *Moringa* leaf powder FRA*P* values with commonly consumed herbs reveal that air-dried *Moringa* has FRA*P* values higher than basil, oregano and stevia but lower than mint [Table 4]. Oregano is an expensive Italian herb, steiva exhibits anti-diabetic properties and is also expensive. Thus, *Moringa* leaves provide cheaper alternative. Antioxidant capacity (FRA*P* values) of *moringa* leaves when compared to that of the commonly consumed vegetables such ascapsicum, spinach, cauliflower, cabbage and onion clearly reveals high reducing potential [Table 5]. IC_{50} value (mg sample/mg DPPH) for fresh sample was 0.77, for air-dried was 0.822 and for oven-dried was 0.83. Lower the IC_{50} value, stronger the antioxidant potential. From the values obtained we can conclude fresh drumstick leaves have better radical inhibiting potential. The strong antioxidant activity (as assessed through FRAP and DPPH assays) as exhibited by the samples can be postulated as the synergistic effect of the various polyphenols present. Moreover, in our on-going study, TPC and their respective FRA*P* values have shown a strong correlation value of 0.764. This is in agreement with other studies that have observed similar linear correlation coefficient

Table 3: Comparison of polyphenols composition of *Moringa oleifera* with other studies

Phenol group	Name of the phenolic acid	Air dried-dl (Mg/100 g)	Oven dried-dl (Mg/100 g)	Other studies[24] (µg/G)
Hydroxybenzoicacids and derivatives	Gallic acid	0.806	0.669	534.4
	3,4-dihydroxy benzoic acid	0.109	0.144	
	Chlorogenic acid	0.256	1.031	488.5
	Gallocatechin	0.013	(-)	
Hydroxycinnamic acids and derivatives	P-Coumaric acid	0.54	0.3521	
	Caffeic acid	(-)	(-)	
	Ferulic acid	(-)	(-)	128.2
Flavonoids	Lutelion	0.003	0.003	
	Epigallocatechin	(-)	0.837	
	Epigallocatechingallate	0.079	0.043	
	Apigenin	0.002	0.002	
	Hesperidin	0.001	(-)	
	Hesperetin	(-)	0.00058	
	Kaemperfrol	4.491	(-)	497.6
	Catechin	(-)	(-)	
	Querecetin-3-beta D gluoside	0.00083	0.326	807
	Apigenin-7-O-neohesperidoside	0.0002	0.141	
	Quercetin-3 dgalactoside	(-)	26.065	

Table 4: Comparison of dry *moringa* leaf powder ferric reducing antioxidant power values with commonly consumed herbs

Name	FRAP value (mmol/100g)
Basil	19.9
Stevia	36.4
Oven dried *moringa* (present study)	49.5
Oregano	63.2
Air dried *moringa* (present study)	70.9
Mint	116.4

FRAP – Ferric reducing antioxidant power

Table 5: Comparison of drumstick leaf ferric reducing antioxidant power values with commonly consumed vegetables

GLV	FRAP value (mmol/100 g)
Air-dried *M. oleifera*[a]	70.99
Fresh *M. oleifera*[a]	49.15
Capsicum[b]	2.46
Spinach[b]	0.98
Onion[b]	0.67
Broccoli[b]	0.58
Raddish[b]	0.4
Tomato[b]	0.31
Cauliflower[b]	0.23
Cabbage[b]	

[a]present study; [b](Halvorsen *et al.*, 2002); FRAP – Ferric reducing antioxidant power; GLV – Green leafy vegetables; *M. oleifera* – *Moringa oleifera*

values-0.7919,[33] 0.86,[34] 0.982[35] and concluded that phenols are major contributor to antioxidant capacity.

CONCLUSION

M. oleifera leaves have exhibited high phenol content, rich polyphenol profile and strong antioxidant capacity. When compared to the commonly consumed vegetables and expenisve herbs like oregano, it has come out as more potent antioxidant. The antioxidant potential is attributed to its polyphenol content. These phenols also provide myriad protective actions. These leaves endow with the added benefits of nutritional antioxidants (β-carotene and vitamin-C). These can be consumed as tea, vegetable, side-dishes like chutney, soups, etc. Further human intervention trials are warranted as to study the beneficial effects on humans, for knowledge about molecular basis of these effects animal and cell model studies are required.

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