

Research Article



Comparative Antioxidant Potential of Leaves and Fruit Extracts of *Terminalia bellerica* Roxb from Himachal Pradesh.

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ABSTRACT

Terminalia bellerica Roxb is a well-known medicinal plant and used in Ayurvedic herbal formulation to treat various ailments. In the current study, we correlated the phenolic and flavonoids contents with antioxidant potential of fruits and leaves of *Terminalia bellerica* from Himachal Pradesh. Quantitative phytochemical analysis of total phenolic and flavonoids content in ethanolic extract of fruit and leaves of *Terminalia bellerica* was done by Folin-Ciocalteu assay and aluminium chloride assay respectively. Analysis of antioxidant activity was done by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power assay (FRAP), Nitric oxide assay (NO) and Total antioxidant activity. Phytochemicals screening analysis showed the presence of all the phytoconstituents such as alkaloids, phenolics, tannins, flavonoids, carbohydrates, glycosides, phytosterols, phytosteroids, saponins, proteins except amino acid. Total phenolic content (TPC) is more (177 ± 0.19532 mg/g gallic acid equivalents) as compare to leafy part (169 ± 0.39 mg/g gallic acid equivalents) whereas total flavonoids (TFC) is more in leafy part (79.79 ± 0.73 mg/g rutin equivalents) as compare to fruit part of plant (74.23 ± 1.04 mg/g rutin equivalents) respectively. DPPH activity of ethanolic extract of fruits (IC_{50} - $5.90 \mu\text{g/ml}$) was more than that of leaves (IC_{50} - $6.60 \mu\text{g/ml}$). FRAP activity of fruits (IC_{50} - $6.50 \mu\text{M Fe (II)}$ equivalents) was more than that of leaves (IC_{50} - $9.69 \mu\text{M Fe (II)}$ equivalents). Nitric oxide (NO) scavenging activity of leaves (IC_{50} - $65.321/\text{ml}$) was higher than that of fruits (IC_{50} - $156.94 \mu\text{g/ml}$). Total antioxidant activity of fruits (IC_{50} - $96.978 \mu\text{g/ml}$) was more than that of leaves (IC_{50} - $105.83 \mu\text{g/ml}$). The results of this study showed that ethanolic extract of leaves and fruits are equally rich in phenolic and flavonoids and possess comparative antioxidant potential. Therefore, leaves could be used as a source of natural antioxidant in food and pharmaceutical industry in addition to fruits of *T. bellerica*.

Keywords: *Terminalia bellerica*, DPPH, FRAP, NO, IC_{50} , Antioxidants.

INTRODUCTION

Many Indian medicinal plants are considered potential sources of antioxidant compounds. Natural products are known to play important roles in both drug discovery and chemical biology. In recent years, the use of natural antioxidants has been promoted because of concerns on the safety against synthetic drugs.¹ About 80% of the population in the third world countries relies on traditional plant based medicines for their primary health care needs. Natural products and related drugs are used to treat 87% of different human diseases.² About 25% of the prescribed drugs in the world are prepared from a variety of plant materials as leaves, stems, roots, bark etc.³ However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compound as well as their mode of action.⁴

Free radicals are an indispensable part of all biological processes.⁵ Antioxidants act by reducing or inhibiting chain reactions of oxidative processes by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves.⁶ Due to their safety and high nutritional and therapeutic effects, natural antioxidants present in food and other biological materials have fascinated a great deal of interest.⁷ Use of synthetic antioxidants has been eliminated from many

food products as they require extensive and expensive testing and has to fulfill safety standards. The increasing importance in the search for natural alternatives of synthetic antioxidants has led to the evaluation of antioxidants in a number of plant sources.⁸

Terminalia is a genus of large trees (upto 40 m high) of the flowering plant family *Combretaceae*, comprising around 200 species distributed across humid, semi-humid, tropical regions of the world. Approximately 24 different species of *Terminalia* have already been reported from various states and Union Territories of India.⁹ *Terminalia bellerica* Roxb commonly known as Bahera is found in deciduous forests throughout the greater part of Madhya Pradesh, Uttar Pradesh, Punjab, Himachal Pradesh, Maharashtra states of India except the dry region of Western India. It is an integral part of Ayurvedic laxative formulation, Triphala used in treatment of common cold, pharyngitis and constipation.¹⁰⁻¹² The extract of *T. bellerica* has been reported to exhibit a variety of biological activities and pharmacological effects including anti-malarial, anti-bacterial, anti-HIV, anti-fungal, anti-mutagenic, and antioxidant effects.¹³

Therefore, present study was undertaken to evaluate phenolic and flavonoid contents in ethanolic extract of fruits and leaves and their correlation with antioxidant



activity, so that leaves can be replaced with fruits as a source of natural antioxidant.

MATERIALS AND METHODS

Collection of plant materials

The plant materials for the current study were collected from Himachal Pradesh (30°22'40"- 33°12'40" N to 75°45'55"- 79°04'20" E). The fruits and leaves sample of *Terminalia bellerica* were collected in the month of October from the Kangra (altitude 850 meters, temperature 25-30°C) district of Himachal Pradesh, India. The collected sample were thoroughly washed with running tap water followed by distilled water. Further, leaves and fruits sample were surface sterilized with 0.1% mercuric chloride (HgCl₂) followed by 70% ethanol, and finally with sterilized water. The plant materials were dried in a hot air oven at 40°C, until dried completely and powdered using electric grinder mixer. The powdered plant materials were stored in air tight bottles in dark until use.

Chemicals and Reagents

Aluminum chloride, Ascorbic acid, 2,2'- diphenyl-2-picrylhydrazyl (DPPH), Sodium nitrite (NaNO₂), 2,4,6-tripyrindyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co., U.S.A. Ferric chloride, Folin-Ciocalteu reagent, Gallic acid and Rutin were procured from Loba Chemie Pvt. Ltd, Mumbai, India. All the chemicals and reagents used in this study were of analytical grade.

Preparation of leaves and fruit extract

The dried powder of leaves and fruits sample (10 g) of *Terminalia bellerica* were defatted with petroleum ether (60-80°C) using cold maceration process. The defatted fruit and leaves sample (10 g) were mixed with 100 ml ethanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 7 days to ensure complete extraction. The extracts were filtered through Whatmann No. 1 filter paper and then centrifuged at 4000g for 5 min. The solvent phase was collected and evaporated at 40°C. The dried crude extracts were stored at 4 °C in air tight bottles until use.

Qualitative phytochemical analysis

The ethanolic extract of leaves and fruits of *T. bellerica* were analyzed for the presence of various phytoconstituents such as phenolics, flavonoids, tannin, saponins, alkaloids, glycosides, phytosterols and carbohydrate previously described by method.¹⁴⁻¹⁵

Quantitative estimation of Total Phenols

The total phenolic content of ethanolic extract of fruits and leaves were determined by using Folin-Ciocalteu reagent method.¹⁶ Total phenolic content was calculated from calibration curve of gallic acid (5-100 µg/ml) and expressed in terms of gallic acid equivalents (GAE) per gram of extract. The total content of phenolic compounds

in plant extracts in gallic acid equivalents (GAE) was calculated using the following equation

$$C = (c \times V)/m$$

Where 'C' is total content of phenolic compounds in mg/g plant extract in GAE, 'c' is the concentration of gallic acid estimated from the calibration curve (mg/ml), 'V' is the volume of extract in ml and m is the weight of crude plant extract in gram.

Quantification of total flavonoid Content

Total flavonoid content were quantified in ethanolic extract of fruits and leaves of *T. bellerica* by using aluminium chloride (AlCl₃) method.¹⁷ The flavonoid content was calculated from standard curve of rutin (5-100 µg/ml) and expressed as rutin equivalents (RE) per gram of extract. The total content of flavonoid content in extract were expressed as rutin equivalents and can be calculated by the following equation:

$$C=(c \times V)/m$$

Where 'C' is total content of flavonoid compounds in mg/g plant extract in rutin equivalent; 'c' is the concentration of rutin calculated from the calibration curve in mg/ml, 'V' is the volume of extract in ml, and 'm' is the weight of crude plant extract in gram.

In-vitro antioxidant activity

DPPH radical scavenging assay

DPPH radical scavenging activity of the crude extract was measured according to previously described method.¹⁸ Ascorbic acid was used as standard. DPPH radical scavenging activity was calculated from the following equation-

$$\text{DPPH radical scavenging activity (\%)} = A_c - A_s / A_c \times 100.$$

Where A_s - absorbance of the test sample, A_c -absorbance control reaction

Ferric Reducing Antioxidant Power (FRAP) assay

The reducing power of crude extract was measured using the method.¹⁹ Ascorbic acid was used as standard. The antioxidant capacity of extract/standard was calculated from the linear calibration curve of FeSO₄ (2.5-20 µM) and expressed as µmol FeSO₄ equivalents per gram of extract.

Nitric oxide (NO) scavenging assay

Nitric oxide scavenging assay of crude extract were performed by the method.²⁰ Ascorbic acid was used as standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

$$\% \text{ inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the samples and standard.

Total antioxidant activity

Total antioxidant activity of the extracts was measured by the phosphomolybdenum method described by Prieto.²¹ Ascorbic acid was used as reference standard. The antioxidant activity is expressed as the number of equivalents of ascorbic acid (AAE).

Statistical analysis

The analyzed data are expressed as the mean $\pm$ SD of three measurements. Inhibition concentration (IC_{50}) and total phenolic and antioxidant were determined by linear regression analysis method. The correlation coefficients between studies parameters were demonstrated by linear regression analysis.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

The amount of phytoconstituents varies in different medicinal plants and even in different parts of the medicinal plants. Phytoconstituents such as phenolics and flavonoids are powerful antioxidant and have been used in various therapeutics such as diabetes, cardiovascular disorders etc. In the present study, ethanolic extract of fruits and leaves of *T. bellerica* showed the presence of alkaloids, phenolics, tannins, flavonoids, carbohydrates, glycosides, phytosterols, phytosteroids, saponins, and proteins. Individual amino acids were undetectable in both leaves and fruit extracts. (Table-1).

Our results support previous finding that *Terminalia bellerica* possess phenols, flavonoids Glycosides, Saponins, Tannins are present in *Terminalia bellerica*.¹³ Ram found that flavonoids, tannins, steroids, cardiac glycosides and alkaloids by Wagner's reagent were found in maximum amount and triterpenes are absent.²²

Quantitative estimation of total phenolics and flavonoids content

The phenolic compounds are one of the most effective antioxidative constituent that contributes to the antioxidant activity in medicinal and food plants and it's important to quantify the TPC and TFC. Total phenolic content of the ethanolic extract of leaves and fruit of *T. bellerica* was calculated from standard curve of gallic acid ($y = 0.023x - 0.0054$; $R^2=0.9995$), whereas the estimation of flavonoid content of the ethanolic extract of leaves and fruit of *T. bellerica* was calculated from standard curve of rutin ($y = 0.0048x - 0.0062$; $R^2=0.9905$). There was increase in total phenolics and flavonoids with increase in concentration of extracts (5-100 mg). Ethanolic extract of fruits possess higher amount of phenolic content (177 ± 0.19532) mg/g gallic acid equivalents) as compared to that of leaves (169 ± 0.39 mg/g gallic acid equivalents), whereas ethanolic extract of leaves (79.79 ± 0.73 mg/g

rutin equivalents) had higher amount of total flavonoid content as compared to that of fruits (74.23 ± 1.04 mg/g rutin equivalents).

These results showed that fruits possess higher amount of phenolic content as compared to that of leaves, however higher phenolic content was not always accomplished with high amount of flavonoids²³ as in our results leaves possess slight higher amount of flavonoids as compared to that of fruits.

In the previous study showed that methanolic extract of leaves of *T. bellerica* had 259.28mg GAE/g of phenolic content, likewise, the TFC were 16.15 mg Q/g extract²³ whereas it is also found that methanolic extract of fruits of *T. bellerica* possess 278.50 ± 19.28 mg/gm GAE of phenolic content as compared to TFC 30.17 ± 1.63 .²⁴

In-vitro antioxidant activity

Different methods have been used to evaluate the antioxidant characteristics, but no single method alone can provide the proper antioxidant property of the extracts. Therefore, it has been recommended to compare to compare the antioxidant potential by different methods.²⁵⁻²⁶ Both the extracts exhibited good antioxidant potential comparing with that of standard ascorbic acid. Ethanolic extract of fruits showed more (Table 1) antioxidant capacity (DPPH, FRAP and total antioxidant activity) as compared to that of leaves. On the other hand, extract of leaves showed more (IC_{50} -65.321/ml) nitric oxide scavenging activity as compared to that of fruits (Figure-2). IC_{50} value (half maximal inhibitory concentration) indicates how much of a particular drug or other substance is required to inhibit a given biological process. IC_{50} value comparison of different antioxidant assay showed that both leaves and fruits have more antioxidant potential as compared to that of ascorbic acid. Arya demonstrated good scavenging activity in methanolic crude extract of leaves of *T. bellerica*.²³ Our results are similar like that Chowdhari showed that methanolic extract of fruit of *T. bellerica* showing the good total antioxidant capacity increased concomitantly with polyphenol content of the plant extracts analyzed. In previous study methanol and aqueous methanol extracts showed good antioxidant activities, also had the highest amount of polyphenols.²⁷ Ethyl acetate and chloroform extracts, which showed antioxidant activities due to their phenolic content. Petroleum ether extract showed the lowest total phenolic content and the lowest antioxidant activity. This study provides a definitive report about the free radical scavenging capacity of *T. bellerica*, since the antioxidant activity of a drug may depend on the free radical scavenging activity.²⁸

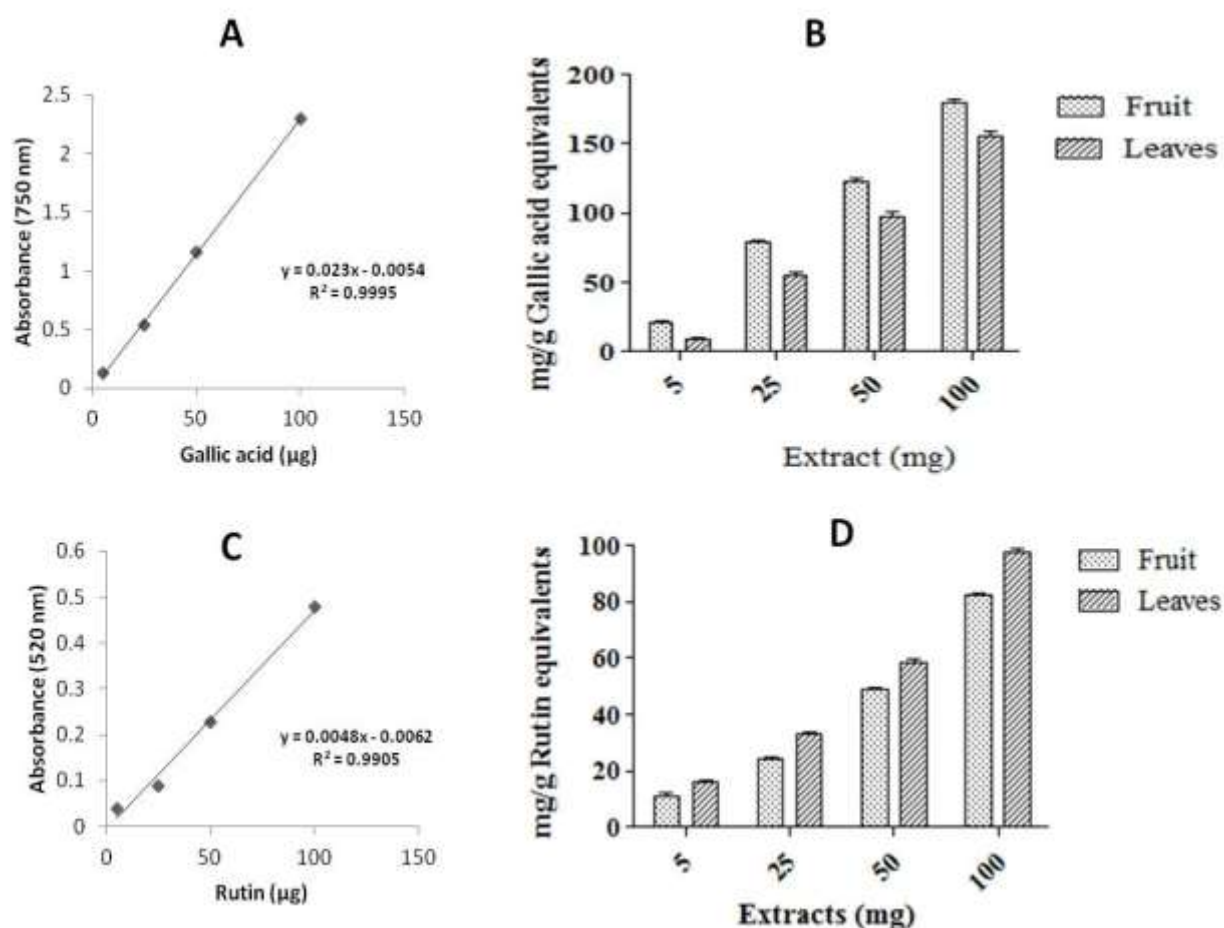


Figure 1: Quantitative estimation of total phenolic and flavonoid content of ethanolic extract of leaves and fruit of *T. bellerica*: A) Standard curve of Gallic acid (GAE). B) Total phenolic content expressed as Gallic acid equivalents per gram extract. C) Standard curve of Rutin D) Total flavonoid content expressed as Rutin equivalents per gram extract.

Table 1: Phytochemical constituents of ethanolic extract of leaves and bark of *T. bellerica*.

S. No.	Phytoconstituents	Tests	Fruits	Leaves
1.	Alkaloids	Dragendroff's test	+	+
		Hager test	+	+
2.	Phenolics and Tannins	Ferric chloride test	+	+
		Gelatin test	+	+
3.	Phytosteroids	Liebermann-Burchard's test	+	+
4.	Phytosterol	Salkowski reaction test	+	+
5.	Saponin	Foam test	+	+
6.	Carbohydrates	Bradford test	+	+
		Fehling test	+	+
7.	Glycosides	Borntrager test	+	+
8.	Proteins/aminoacid	Millon test	+	+
		Ninhydrin test	-	-
9.	Flavonoids	Lead acetate test	+	+

'+' indicates presence and '-' indicates absence of phytoconstituents.

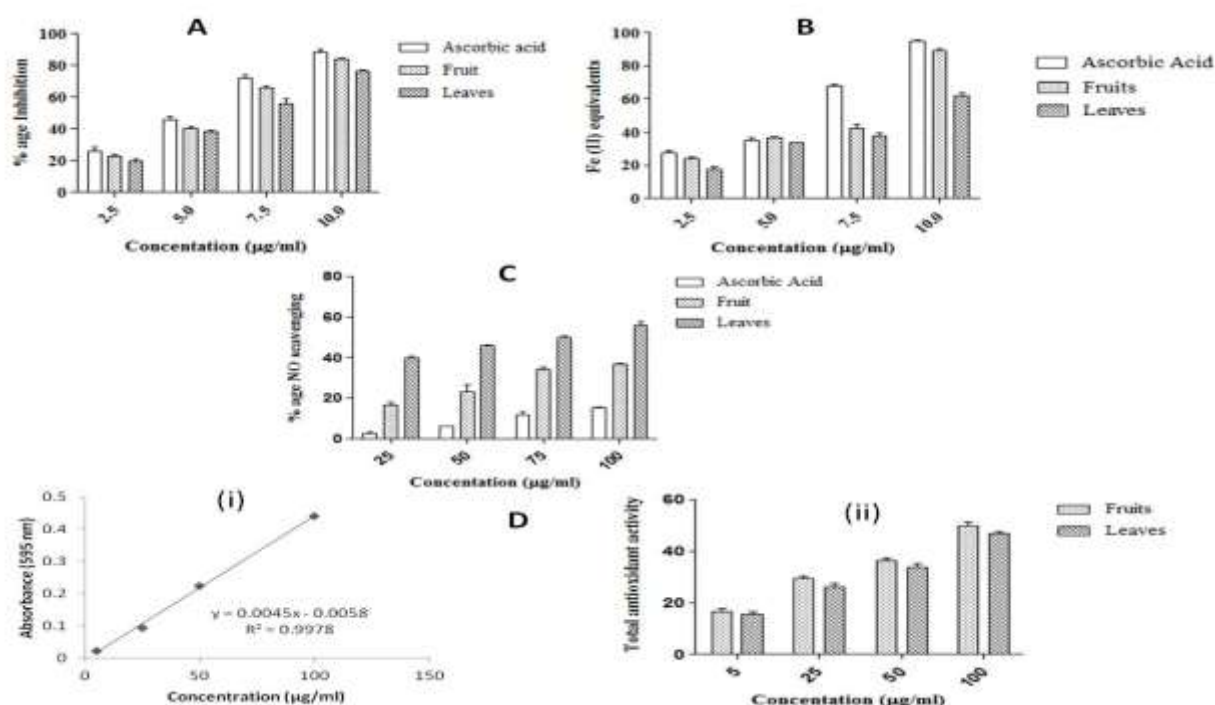


Figure 2: Different methods to evaluate antioxidant potential of ethanolic extract of leaves and fruits of *T. bellerica*: A) DPPH radical scavenging activity B) Ferric reducing antioxidant power C) Nitric oxide scavenging activity D) Total antioxidant activity.

Table-2: Half maximal inhibitory concentration (IC_{50}) of ethanolic extract of fruits and leaves using different antioxidant assays.

S. No.	Antioxidant assay	Half maximal inhibitory concentration (IC_{50}) (μ g/ml)		
		Standard Ascorbic acid	Fruits	Leaves
1	DPPH	4.38	5.90	6.60
2	FRAP	5.58	6.50	9.69
3	NO	294.36	156.94	65.321
4	Total antioxidant activity	-	96.978	105.83

DPPH (2,2-diphenyl-1-picrylhydrazyl) in μ g/ml, Ferric Reducing Antioxidant Power assay (FRAP) in μ M, Nitric oxide assay (NO) in μ g/ml and Total antioxidant activity in μ g/ml. Lower the value of IC_{50} , more is antioxidant capacity.

Table 3: Correlation between phenolics and flavonoids with antioxidant activities of leaves and fruits extract of *T. bellerica*

Antioxidant Assays	Correlation coefficient (R^2)			
	Total phenolic content		Total flavonoid content	
	Fruit	Leaves	Fruit	Leaves
DPPH radical scavenging activity	0.98	0.99	0.89	0.93
Ferric Reducing Antioxidant Power (FRAP) assay	0.84	0.95	0.97	0.97
Nitric oxide scavenging activity	0.93	0.99	0.78	0.93
Total antioxidant activity	0.99	0.99	0.95	0.95

Correlation of total phenolics and flavonoids content with antioxidant activity

It has been found that fruits and leaves contain antioxidant potential along with vitamins.²⁹ Several

studies have shown that the amount of phenolics in medicinal plants is directly correlated with antioxidant activity. Phenolic compounds possess redox property due to which they act as reducing agents, singlet oxygen quenchers, metal ion chelators and hydrogen donors.³⁰

Several workers have reported the relationships between phenolic content and the antioxidant activity of various plants. Some workers found positive correlation between the phenolic content and the antioxidant activity, while others found no such relationship. A strong relationship between total phenolics and antioxidant activity has been observed in previous study.³¹⁻³⁵ In contrast, no correlation between phenolic content and antioxidant activity was observed in previous study.³⁶⁻³⁷ Some researcher also found the moderate correlation between TPC and antioxidant activity, whereas it is also found strong correlation between TPC and antioxidant activity of leaves of *Terminalia bellerica*.³⁸ Our results also showed that total phenolics and flavonoids were directly correlated with antioxidant capacity of ethanolic extract of both leaves and fruits of *T. bellerica* as shown in table 2.

CONCLUSION

The results from this study support previous findings that fruit extract of *T. bellerica* possess higher antioxidative activities. Total phenolic contents presented as GAE were also highest in fruit extracts, which confirmed that phenolic compounds play an important role in exhibiting anti oxidative activity. The fruit and leafy parts of plants are considered rich in polyphenols and flavonoids, which contributes to their antioxidant capacity. Leaves of *T. bellerica* could be used as alternate source of antioxidative phytochemicals.

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