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**COMPARATIVE ANALYSIS OF PHYTOCHEMICALS AND ANTIOXIDANT
ACTIVITIES OF FRUIT AND LEAVES OF *TERMINALIA CHEBULA* FROM
HIMACHAL PRADESH**

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ABSTRACT

The present study was aimed to quantify the total phenolic, flavonoid and antioxidant activity in ethanolic extract of fruit and leaves of *Terminalia chebula* from Himachal Pradesh and evaluate the phytoconstituents and antioxidant potential. Qualitative phytochemicals analysis of ethanolic extract of fruit and leaves revealed the presence of phenols, tannins, carbohydrates, glycosides, saponin, phytosteroids and flavonoids. Phenolic content of ethanolic extract of fruit (242.3±5.09 mg/g gallic acid equivalents) was higher than that of leaves (162.4±4.31mg/g gallic acid equivalents); whereas ethanolic extract of leaves (157.75 ± 3.88 mg/g rutin equivalents) possess higher amount of flavonoids content as compared to that of fruits (126.45±5.58 mg/g rutin equivalents). DPPH activity of ethanolic extract of fruits (IC₅₀-6.5 µg/ml) was more than that of leaves (IC₅₀-7.034 µg/ml). FRAP activity of fruit extracts (IC₅₀- 0.061µM Fe (II) equivalents) was more than that of leaves (IC₅₀-0.066µM Fe (II) equivalents). Nitric oxide (NO) scavenging activity of fruits (IC₅₀-108.47µg/ml) was higher than that of leaves (IC₅₀-147µg/ml). The total antioxidant activity of fruit (32.064 mg/g ascorbic acid equivalents) was more as compared to that of leaves (50.43 mg/g ascorbic acid equivalents). The present study showed that fruits of *T. chebula* can be used as a source of antioxidants in food and pharmaceutical industries.

**Keywords: *Terminalia chebula*, Total Phenolic content, Total Flavonoid content, DPPH,
NO, FRAP and Total antioxidant activity**

INTRODUCTION

Herbal medicines are in great demand because of their wide biological and medicinal activities. The traditional Indian system of Ayurveda and Siddha medicines support the importance of medicinal plants to treat diseases [1]. World Health Organization (W.H.O) reported that herbal medicines support about 75-80% of the whole population because of, better compatibility with the human body and fewer side effects. In recent times, research has been focused worldwide on medicinal plants. The natural pharmaceuticals are getting intense importance and popularity as safe, efficacious and cost effective medicines with extraordinary benefits due to combination of medicinal ingredients with vitamins and minerals [2]. Antioxidant-based drugs/formulations have been designed for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer during the last three decades [3]. Therefore, natural sources of antioxidants have gained more attention in the modern day research. Many Indian medicinal plants are considered potential sources of antioxidant compounds *Terminalia chebula*, *T. bellerica*, *T. muelleri*, and *Phyllanthus emblica*, all of which have antioxidant activity, showed high content of

phenolics like gallic acid [4,5]. *Terminalia chebula*, which is a member of Combretaceae family, enjoys the prime place among medicinal plants not only in India, but also in other countries of Asia and Africa subcontinent. It is one of top listed plant in Ayurvedic materia medica for the treatment of asthma, bleeding piles, sore throat, vomiting and gout [6]. It is used in Thai traditional medicine as a carminative, astringent and expectorant [7]. The 'Triphala', a herbal preparation of 'three fruits' from *T. chebula*, *T. bellerica*, *E. officinalis*, is used as laxative in chronic constipation, detoxifying agent of the colon, food digestive problems (poor digestion and assimilation) and rejuvenator of the body [8]. Triphala is also known to stimulate appetite and is useful in treating cancer and detoxification. Triphala is considered as the most versatile of all herbal formulations and is prescribed as a cardi tonic and for Candida infection [9]. Therefore, the present study was designed to evaluate phenolic and flavonoid contents in leaves and fruit pulp of *T. chebula* and correlation between phytochemistry and antioxidant activity.

MATERIALS AND METHODS

Collection of plant material

Fruit and leaves of *T. chebula* were collected in the month of August from Kangra, Himachal Pradesh, India. The fruit and leaves were washed twice with tap water distilled water followed by surface sterilization with 70% ethanol for 2-3 minutes. Surface sterilized plant material was dried followed in hot air oven at 40°C. The dried fruit and leaves were homogenized to fine powder using electric grinder and then stored in air tight bottles in dark until use.

Chemicals and Reagents

Ascorbic acid, aluminum chloride, 2,2'-diphenyl-2-picrylhydrazyl (DPPH), Sodium nitrite (NaNO₂), 2,4,6-tripyridyl-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.

Extract Preparation

The dried powder (10 g) of fruit and leaves of *T. chebula* were defatted with petroleum ether (60-80°C) and then extracted with 100 ml ethanol on a rotary shaker at 120 rpm for 72 h. After complete extraction, the solvent was centrifuged at 4000 rpm for 5 min. Supernatant was collected and then allowed

to evaporate. The dried extracts were stored at 4 °C in air tight bottles.

Qualitative analysis of phytochemicals in *T. chebula* extracts

Crude extracts of leaves and fruit were tested for the presence of various secondary metabolites such as phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate by using methods described [10,11]. For the detection of alkaloids and glycosides, 50mg of extract was dissolved in 5 ml of dilute HCl and then filtered. The filtrate was used for the detection of alkaloids and glycosides; whereas for detection of phenolics, tannins, phytosterols, phytosteroids, carbohydrate, flavonoids, proteins and amino acids, 50 mg of extract was dissolved in 5 ml of distilled water and then filtered. The filtrate was used for detection of phenolics, tannins, phytosterols, phytosteroids, carbohydrate, flavonoids, proteins and amino acids.

Dragendorff's Test for alkaloids:

500 µl of Dragendorff's reagent was added by the side of the test tube containing 1ml extract. Formation of orange or orange reddish precipitate indicated the presence of alkaloids.

Hager's Test for alkaloids: 500µl of Hager reagent was added to 1ml of extract.

Formation of yellow precipitate indicated the presence of alkaloids.

Borntrager test for glycosides:

2ml of filtrate was dissolved in 3 ml of chloroform and then shake it well. Then 1ml of ammonium solution was added. Formation of pink coloration indicated the presence of glycosides

Ferric chloride test for phenolics:

30µl of ferric chloride solution was added to the 1ml of extract. Appearance of blue color indicates the presence of hydrolysable tannins, while the green color indicates the presence of condensed tannins.

Gelatin test for tannins:

50 µl of gelatin was added to 1ml of extract. Formation of white precipitates indicated the presence of tannins.

Liebermann-Burchard test for phytosteroids:

1ml of extract was dissolved in 2 ml of acetic anhydride and heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the sides of the test tube. Formation of brown ring at the junction indicated the presence of phytosteroids.

Salkowski test for phytosterol:

2 ml of extract was added to 2ml of chloroform. After 2 minutes 20 µl of H₂SO₄ was added along the sides of the test tube.

Red brown color at the interface indicated the presence of phytosterol.

Foam test for saponins

10 mg of extract was dissolved in 20 ml of water. The solution was shaken vigorously and observed for a stable persistent froth for 15 minutes. Presence of froth at the surface indicated the presence of saponin.

Barfoed's test for Carbohydrates:

1ml of Barfoed reagent was added to 1ml of extract followed by mixing & incubating in a boiling water bath for 5 min. Reactions were cooled and then 30µl phosphomolybdic acid was added, until the solution becomes clear. A deep blue color indicates the presence of monosaccharide and light blue color indicates the presence of disaccharide.

Fehling test: 1ml of extract was added to 1ml of freshly prepared Fehling solution and heated in a water bath. Brick red precipitates indicated the presence of carbohydrates.

Million tests for Protein:

20 µl of million reagent was added to the test tube containing 1ml of extract and then heated in water bath for 10 minutes. The samples were cooled and 10µl of 1% sodium nitrite solution. Red color indicated the presence of proteins.

Quantification of secondary metabolites:

Determination of total phenolic contents:

The total phenolic content in the ethanolic extract of leaves and fruits was estimated as described [12]. Gallic acid was used as standard. Total phenolic content of *T. chebula* was calculated from calibration curve of gallic acid (5-100 µg/ml) and expressed as mg gallic acid equivalents /gm weight of the extract.

Quantification of total flavonoid Content:

The total flavonoid content in ethanolic extract of fruits and leaves of *T. chebula* were determined by using aluminium chloride (AlCl₃) method as described [13]. Rutin was used as standard. The total content of flavonoid was calculated from calibration curve of rutin (5-100 µg/ml) and expressed as mg rutin equivalents/gm of weight of extract.

In vitro antioxidant assays:

Extracts of fruits and leaves of *T. chebula* were dissolved at a concentration of 1mg/ml and then diluted in order to prepare different concentrations for antioxidant assays. Ascorbic acid was used for comparative analysis in all assays.

DPPH radical scavenging assay

The DPPH radical scavenging assay of ethanolic extract of *T. chebula* extract was performed as described by Shah et al [14]. In this method, 900 µl of 0.1 mM DPPH

solution (prepared in ethanol) was mixed with 100 µl of ethanolic extract of fruits or leaves ranging from 2.5-20 µg/mL. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank.

Nitric oxide radical scavenging assay:

The ability of ethanolic extract of fruits and leaves of *T. chebula* to scavenge nitric oxide radical was determined as described [15]. In this method, 1 ml of different concentrations of fruits or leaves extracts were mixed with 2 ml of 10 mM sodium nitroprusside and incubated for 2.5 h. Then 1 ml of 0.33% sulphanic acid was added to 150 µl of reaction mixture and allowed to stand for 5 minutes. After 5 minutes, 1 ml of 0.1% 1-naphthylethylenediamine was added and absorbance of chromophore formed was read at 590 nm after incubation of 30 minutes. IC₅₀ values and percent inhibition by various concentrations of extract were calculated by comparing the absorbance values of control and test compounds.

Ferric Reducing Antioxidant Power (FRAP) assay

The ability of ethanolic extract of fruits and leaves to reduce ferric ions was measured using the method described by Benzie and Strain. Ascorbic acid was used as positive

reference standard. The antioxidant capacity of extract was calculated from the linear calibration curve of FeSO₄ (2.5-20 μM) and expressed as μmol of FeSO₄ equivalents per gram of extract.

Total antioxidant activity: Total antioxidant activity in the ethanolic extract of fruits and leaves were quantified by phosphomolybdate method [16]. Ascorbic acid is used as a standard.

Statistical analysis:

Each sample was analyzed individually in duplication and the results are expressed as the mean value (n = 2) ± Standard Deviation. The correlation coefficients between studies parameters were demonstrated by linear regression analysis.

RESULTS AND DISCUSSIONS

Table-1-Phytochemical constituents of ethanolic extract of leaves and fruits of *T. chebula*

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

“+” sign indicates the presence, whereas “-” sign showed the absence of phytochemicals.

Phenolics are rich antioxidants due to their capability to donate electrons as well as the effectiveness of stabilizing radical

Antioxidants have gained immense importance in the treatment of several metabolic diseases, such as diabetes mellitus, arthritis, cancer, ageing, liver disorder etc. Scientific communities all over the world have intended to investigate the medicinal properties of plants for their potent antioxidant properties [17, 18]. Therefore, the present study was conducted to evaluate and compare the antioxidant potential of fruits and leaves of *T. chebula*.

Phytochemical analysis of ethanolic extract of fruits and leaves of *T. chebula* showed the presence of alkaloids, phenolics, tannins, saponins, flavanoids, and phytosterols. In contrast, proteins and free amino acids were absent (Table 1).

intermediates in the prevention of oxidation at cellular and physiological level [19]. Phenolic compounds and polyphenols are the

most abundant in plant kingdom. Fruits of *T. chebula* have been used in food and pharmaceutical preparations. In contrast, leaves have not been explored as a substitute of fruits and compared with leaves for medicinally important phytoconstituents. Therefore, we compared the presence of phytoconstituents in leaves and fruits and their antioxidant potential.

The Folin-Ciocalteu method is one of the oldest methods developed to determine the content of total phenols [20]. Ethanolic extract of fruits possess higher amount of phenolics (242.3 ± 5.09 mg/g gallic acid equivalents) as compared to leaves (162.4 ± 4.31 mg/g gallic acid equivalents (Figure 1) showed that methanolic extract of fruits of *T. chebula* had 144 mg GAE/g of phenolic content. In contrast to our study, Kathirvel *et al* [21] found that methanolic extract of leaves of *T. chebula* possess 97.62 mg/gm GAE of phenolic content. Eshwarappa *et al* [22] concluded that ethanolic extract of *T. chebula* leaves possess 538 ± 1.4 mg of GAE/g. Ethanolic extract of leaves (157.75 ± 3.88 mg/g rutin equivalents) possess higher amount of total flavanoid content as compared to fruits (126.45 ± 5.58

mg/g rutin equivalents). It was observed that the phenolic content of the *T. chebula* extracts varies as shown by various research groups. This may be due to various factors such as soil composition, age and vegetation cycle stage, quality, quantity and composition of extracted product [23, 24].

***In vitro* antioxidant activity:**

Leaves and fruits extract exhibited good antioxidant activities in comparison to ascorbic acid. However, ethanolic extract of fruits showed more antioxidant capacity (DPPH, Nitric oxide scavenging, FRAP and total antioxidant activity) as compared to that of leaves (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. Chang *et al* [25] showed that also found increase antioxidant activity in leaves and fruit of *T. chebula*. However in our findings, fruits were richer source of antioxidant in comparison to that of leaves (Table-2).

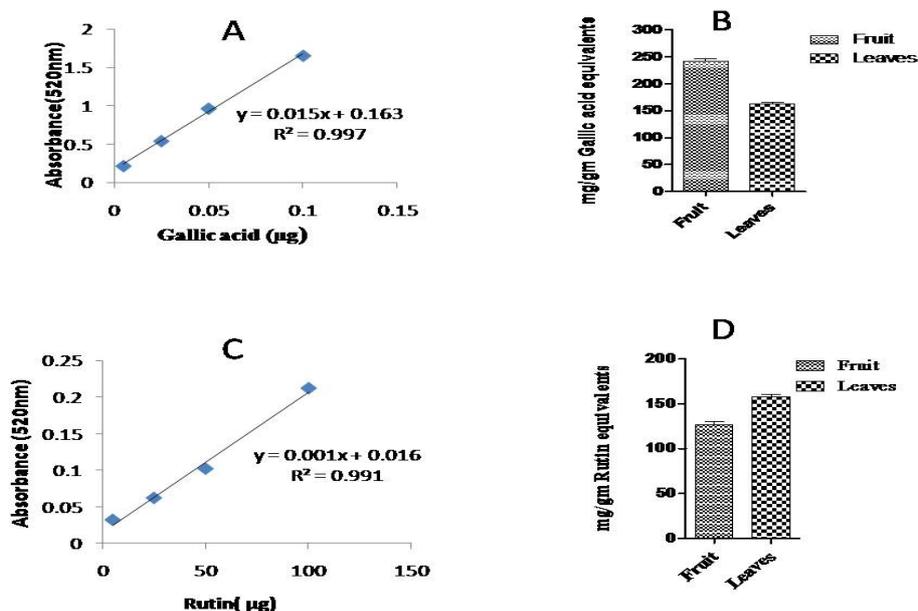


Figure-1: Quantification of total phenolics and flavanoids in fruits and leaves extract of *T. chebula*. A) Standard curve of Gallic acid (0-150 µg); B) Comparative phenolic content of ethanolic extract of leaves and fruits represented as mg/gm gallic acid equivalents; C) Standard curve of rutin (0-150 µg); and D) Comparative flavanoid content of ethanolic extract of leaves and fruit represented as mg/gm gallic acid equivalents. Values are expressed as mean ± standard deviation (n=3)

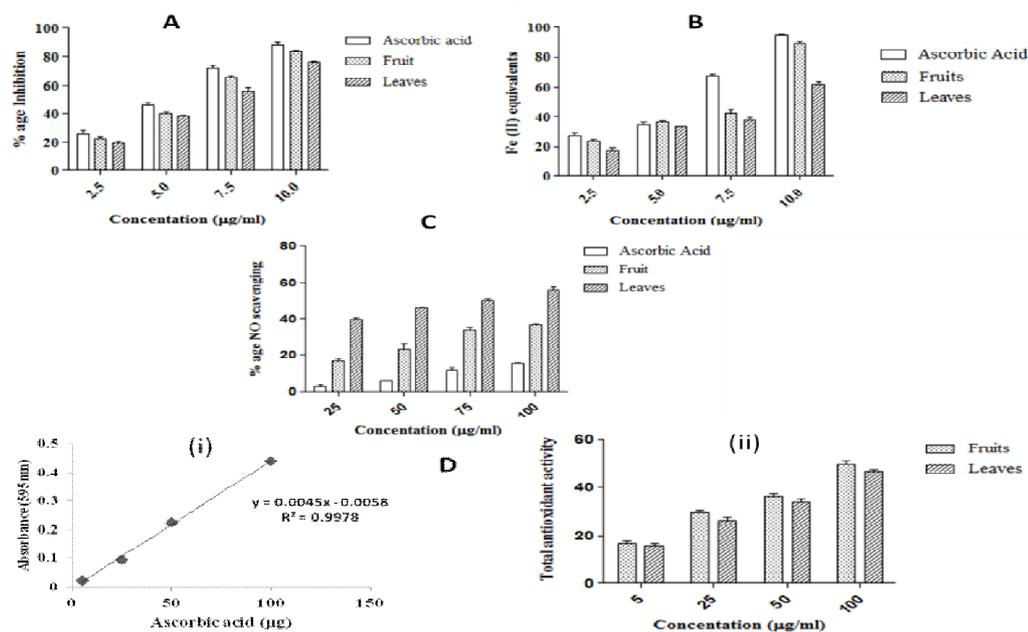


Figure-2: Antioxidant assays of fruits and leaves extract of *T. chebula*. A) DPPH assay; B) FRAP assay; C) Nitric oxide scavenging activity; D) Total antioxidant activity. (i) Standard curve of ascorbic acid (ii) Total antioxidant activity. Values were expressed as mean ± standard deviation (n=3).

Table-2 Half maximal inhibitory concentration (IC₅₀) of ethanolic extracts of fruits and leaves

Sr. No.	Antioxidant assay	Half maximal inhibitory concentration (IC ₅₀)		
		Standard Ascorbic acid	Fruits	Leaves
1	DPPH	8.99	6.55	7.034
2	FRAP	1.82	0.060	0.066
3	NO	166.6	108.47	147.327
4	Total antioxidant activity	-	32.064	50.43

DPPH (2,2-diphenyl-1-picrylhydrazyl) in µg/ml, Ferric Reducing Antioxidant Power assay (FRAP) in µM, Nitric oxide assay (NO) in µg/ml and Total antioxidant activity in mg/ml. Lower the value of IC₅₀, more is antioxidant capacity.

Correlation of total phenolic content and flavonoid content with antioxidant activity

Higher plants have the capacity to produce large number of organic phytochemical known as secondary metabolites. Phenolics are the good source of natural antioxidants and. *Terminalia chebula* fruit extracts showed higher phenolics and high antioxidant activity as compared to leaves. However, leaves showed higher amount of flavonoid as compared to that of fruits. Several studies have shown that the antioxidant potential is attributed to the polyphenolic compounds in medicinal plants. Chang *et al* [25], Katalinic *et al* [26], Petridis

et al [27] showed strong relationship between total phenolic content and antioxidant activity, whereas Hazra *et al* [28] showed strong correlation between flavonoid contents with its antioxidant capacity. However several studies have been reported in which there is no correlation between phenolics / flavonoid content with its antioxidant activity Kahkonen *et al* [29], Sengul *et al* [30]. The present study showed that there is a direct correlation between the phenolic and antioxidant activity (Table-3)

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

Antioxidant Assays	Correlation coefficient (R ²)			
	Total phenolic content		Total flavonoid content	
	Fruit	Leaves	Fruit	Leaves
DPPH radical scavenging activity	0.92	0.83	0.97	0.98
Ferric reducing antioxidant power (FRAP) assay	0.92	0.83	0.95	0.99
Nitric oxide scavenging activity	0.93	0.97	0.95	0.88
Total antioxidant activity	0.97	0.90	0.95	0.83

CONCLUSION

The results from this study support previous findings that fruit extract of *T.chebula* indicated high antioxidative activities. Total phenolic contents presented as GAE were also the highest among in the fruit extract, which confirmed that phenolic compounds

play an important role in exhibiting antioxidant activity. The fruit and leafy parts of plants are considered rich in polyphenols and flavonoids, which contributes to their antioxidant capacity. The antioxidant property of phenolic compounds is attributed

to their ability to absorb and neutralize free radicals.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article

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