

Research Article

## Antioxidant potential and polyphenols analysis of medicinal herb *Ocimum tenuiflorum* (Shyama Tulsi)

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**Abstract**

*Ocimum tenuiflorum* considered the queen of herbs, is a clinically proven medicinal plant. *O. tenuiflorum* has two variants: *Shyama Tulsi* and *Rama Tulsi*. Yet, no clear record of polyphenols in Shyama Tulsi is available in the literature. So, the present study aimed to analyse the antioxidant potential and polyphenolic content of *O. tenuiflorum* (Shyama Tulsi). The leaves were procured from the Department of Genetics and Plant Breeding, College of Agriculture at Chaudhary Charan Singh Haryana Agriculture University (CCS HAU), Hisar, India and extracted with 50 % methanol (OtM) and sequentially fractionated with n-hexane (OtH), ethyl acetate (OtE) and n-butanol (OtB). Total phenolic and flavonoid content was determined using the Folin-Ciocalteu Colorimetric method; and the aluminum chloride using the Colorimetric method. The antioxidant potential was evaluated through DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (Ferric reducing antioxidant power) assay, using ascorbic acid as a positive control. The study revealed that total phenolic and flavonoid content were highest in methanol extract, i.e. 863.63±0.17 mg GAE/g, 310.16±0.25 mg QE/g, followed by n-butanol, ethyl acetate and n-hexane. The antioxidant potential was highest in the n-butanol fraction compared to all other fractions. HR-LCMS profiling of OtB and OtE showed the presence of different polyphenols like 6-C-Galactosylluteolin, quercitrin, apigenin 4'-O-glucoside, caffeic acid, myricitrin, kaempferol and their derivatives. The presence of different polyphenols and the higher antioxidant activity of this medicinal herb make it an alternative source for treating various ailments and human health problems.

**Keywords:** Antioxidant assay, Extract, HR-LCMS (High-Resolution-Liquid Chromatography-Mass Spectrometry), *Ocimum tenuiflorum*, Shyama Tulsi, Flavonoid, Phenolic

**INTRODUCTION**

Reactive oxygen species (ROS) are natural byproducts formed by cellular activity and include free radicals such as hydroxyl radicals, superoxides, and singlet

oxygen. These ROS are essential for the progression of biological processes like cellular proliferation, differentiation, apoptosis and intracellular signaling (Ron, 2017; Di Meo *et al.*, 2016) and the overproduction due to stress, lifestyles, pollution and exposure to UV

(Ultraviolet) radiation. Excessive production of these reactive species is linked with degradation of biological components such as proteins, lipids and nucleic acids, leading to tissue injury and damage to health conditions (Zhang *et al.*, 2016). A large number of pathological conditions, such as rheumatoid arthritis, ischemia, cancer, diabetes, metabolic disorders, are linked with oxidative and chronic stress (Chaudhary *et al.*, 2020; and Di Meo *et al.*, 2016). To manage increased oxidative stress in both healthy and pathological conditions, antioxidants are used more frequently and produced exogenously from outside (Plants) or endogenously through enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Patekar *et al.*, 2013). Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are dangerous to human health and show carcinogenic effects (Kahl *et al.*, 1996). So, the research related to natural antioxidant has gained importance (Lobo *et al.*, 2010). Polyphenols, flavonoids and essential oils are secondary metabolites derived from aromatic and medicinal plants that serve as reducing agents, free radical scavengers, inhibitors of oxidative enzymes, prevent rancidity caused by lipid oxidation as well as the onset of diseases connected with oxidative stress (Baier and Dietz, 2005; Frankel, 1995; Ghasemzadeh *et al.*, 2011; and Chaudhary *et al.*, 2020).

In Ayurveda, Siddha, Unani and other traditional systems, around 35000 medicinal plants are listed for their therapeutic effects (Panchal *et al.*, 2019). Holy basil or *Ocimum sanctum/Ocimum tenuiflorum* is a well-known popular plant used for medicinal purposes, also known as Tulsi. This aromatic perennial plant of the Lamiaceae family is indigenous to the Indian subcontinent and listed in Charaka Samhita (an Indian traditional medicine scripture) and has two varieties: light green leaves Rama/ Lakshmi Tulsi and purple leaves or dark stem Shyama / Krishna Tulsi (*Ocimum tenuiflorum*). Shyama Tulsi has more antioxidant properties than Rama Tulsi (Parajuli-Bara, 2023). Mondal *et al.* (2009) reported that chemical constituents of both varieties are similar, whereas Upadhyay *et al.* (2015) and Singh *et al.* (2018) documented some other metabolites with higher gene expression and concentration in Shyama Tulsi. These metabolites are attributed to higher antioxidant, anti-cancerous, anti-inflammatory, anti-fungal, antiseptic, analgesic, antidiabetics, radioprotective and anti-stress properties (Singh and Chaudhuri, 2018; Kumar *et al.*, 2022; Priya and Peddha, 2023)

The leaves of *Ocimum* contain various compounds such as eugenol, methyl eugenol, carvacrol, eugenol methyl ether, rosmarinic acid and apigenin (Gupta *et al.*, 2002). The extraction of phytochemicals is a challenging process as the polarity of solvents affects the antioxidant potential and its compounds. A well-known

approach, i.e., sequential fractionation, is used to get maximum antioxidants from herbs. This method was used by Chaudhary *et al.* (2020) to study the leaves of *Ocimum sanctum* and get maximum polyphenols and flavonoids. However, other studies are also available for the maximum extraction of polyphenols and flavonoids through sequential fractionation in *Ocimum sanctum*, but not reported in Rama or Shyama Tulsi (Chaudhary *et al.*, 2020; and Ao *et al.*, 2008). To date, no clear record of antioxidant activity of Shyama Tulsi through sequential fractionation methods is available in the literature. So, the present study aimed to study the antioxidant potential and phytochemicals of *O.tenuiflorum* (Shyama Tulsi) through the sequential fractionation method.

## MATERIALS AND METHODS

### Chemicals

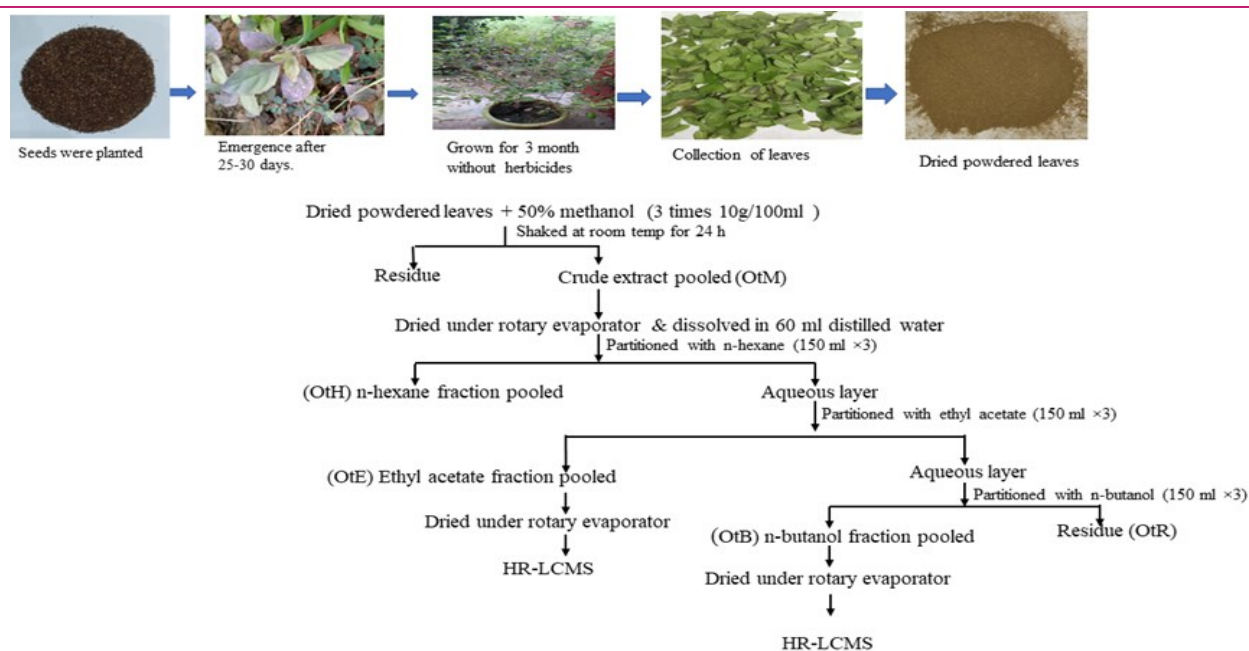
DPPH (2,2-Diphenyl-1-picrylhydrazyl), Ascorbic acid, Aluminium Chloride, trichloroacetic acid (TCA), ferric chloride, Folin–Ciocalteu reagent, ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), methanol, n-hexane, ethyl acetate and n-butanol were of analytical grade and procured from Merck (Mumbai, India). The ultrapure water was prepared using the Milli-Q System (Merck, Bombay, India).

### Plant material

*Ocimum tenuiflorum* (Shyama Tulsi) seeds of genotype HS01 were procured from the Department of Genetics and Plant Breeding, College of Agriculture at Chaudhary Charan Singh Haryana Agriculture University (CCS HAU), Hisar, India. Small-sized seeds were planted in seed pots (Chaudhary *et al.*, 2020). To accelerate germination, the soil was kept moist. After 25-30 days, emergence of seedlings took place. When germination was greater than 85%, seedlings were transferred to earthen pots. Plants were grown without pesticides for three months, and young leaves were collected, cleaned with clean water and stored for two days at room temperature. Leaves were kept for seven days at 45°C in an oven to maintain a constant dry weight (Chaudhary *et al.*, 2020)

### Preparation of extract and fractionation

Coarsely powdered 30 g of dry leaves were extracted thrice with 50% methanol (1g/10ml) for 24 hours at room temperature in a shaker and pooled. After passing through double-layered muslin cloth, the extract was centrifuged at 3000g for 5 min (Chaudhary *et al.*, 2020). The crude dried extract of *O. tenuiflorum* in methanol solvent (OtM) was obtained (26.41%) after solvent evaporation at 40°C in a rotary evaporator. After that, crude extract was suspended in 60 ml of dis-



**Fig. 1.** Flow chart for extraction of leaves of *Shyama Tulsi*

tilled water and partitioned with n-hexane (150 ml x3), ethyl acetate (150 ml x3) and n-butanol (150 ml x3) to obtain *O.tenuiflorum* hexane (OtH), *O.tenuiflorum* ethyl acetate (OtE), *O.tenuiflorum* butanol (OtB), and *O.tenuiflorum* residue (Otr) fractions (Fig 1).

#### Determination of total polyphenolic content

The extract aliquots were combined with 2 ml of 2% sodium carbonate to evaluate the total phenolic content of the preparations. After two minutes, 100 µl of 1 N Folin-Ciocalteu was added and absorbance was read spectrophotometrically at 750 nm after 30 min (Dua *et al.*, 2013). Gallic acid was used to prepare a standard curve (0-100 nmoles) and phenolic content was determined as mg gallic acid equivalent (GAE).

#### Determination of total flavonoid content

For the estimation of flavonoid content of the sample, an aliquot of the extract was diluted to form a volume 0.5 ml; 30 µl of NaNO<sub>2</sub> (5%) was added and thoroughly mixed (Dua *et al.*, 2013). After 5 min, 200 µl of NaOH (1N) was mixed and read at 510 nm. Flavonoids were reported as mg quercetin equivalents (QE), and a standard curve was prepared using quercetin (0-100 nmoles).

#### In vitro determination of antioxidant activity 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH solution (50 × 10<sup>-5</sup> M) was mixed in methanol with 1 ml of extracts prepared with various solvents (Brand-Williams *et al.*, 1995). After 15 min of incubation, absorbance was observed at 517 nm [(Ab - Aa)/Ab] × 100 was used to calculate the decrease in the

percentage of DPPH free radical scavenging activity where Ab was the optical density (OD) of control without extract; Aa was OD with extract in the reaction mixture. The amount of extract (IC<sub>50</sub>) that scavenged 50% of free radicals was calculated.

#### 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) cation radical scavenging activity

As reported in the literature (Re *et al.*, 1999), the cation radical Scavenging activity was determined using the solution 7mM ABTS prepared after mixing 2.45mM ammonium persulfate and left overnight in the dark to obtain cation radicals. Various extracts were mixed with an adequately diluted solution, and a reduction in absorbance at 734 nm was recorded. IC<sub>50</sub> and scavenging activities were determined.

#### Ferric Metal Ion Reducing Power (FRAP) metal ion-reducing property

The FRAP reagent (0.2 M phosphate buffer, 1% potassium ferricyanide, 0.1% ferric chloride) was prepared to determine the metal ion (Fe (III)) reducing property (Gupta and Panday 2014). The extract fraction was combined with the FRAP reagent, incubated at 50°C for 20 minutes and the absorbance at 700 nm was measured. The IC<sub>50</sub> value and antioxidant activity were calculated.

#### High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS) analysis

HR-LCMS) was performed on a Q-TOF mass spectrometer to confirm the presence of active constituents in the leaf extract of *Shyama Tulsi* (Anand *et al.*, 2022). The HiP sampler, binary gradient solvent pump,

column compartment and quadrupole time of flight mass spectrometer (MS Q-TOF) with dual Agilent Jet Stream Electrospray (AJS ES) ion sources comprised the liquid chromatographic system. 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) were used as solvents. A 0.300 mL/min flow rate was used and MS (Mass Spectrometry) detection was performed in MS Q-TOF. Compounds were identified via their mass spectra and their unique mass fragmentation patterns.

### Statistical analysis

The results were shown as mean  $\pm$  SD. The software SPSS/Window (SPSS 16.0) was used to calculate the IC<sub>50</sub> value. To analyze the differences in IC<sub>50</sub> values of various fractions and the relationship between IC<sub>50</sub>, polyphenolic and flavonoid content, one-way ANOVA test and Tukey's test ( $p < 0.05$ ) were used.

## RESULTS AND DISCUSSION

### Extraction, total polyphenolic and flavonoid content

The percentage yield from 50% methanolic leaf extract of Shyama tulsi (*O.tenuiflorum*) was 26.41% (Table 1). 50 % methanol extract of Shyama Tulsi leaves was subjected to differential and sequential fractionation with n-hexane, n-ethyl acetate, n-butanol and observed 0.30-6.67% yield, i.e. 1.13 to 25.25% extraction of total soluble components in methanol extract. The maximum amount of soluble components, 25.25% was extracted with n-butanol fraction, whereas 8.85% in ethyl acetate fraction and very small amount of 0.30% was extracted with n-hexane. This showed that a very small percentage of methanol soluble components were non-polar and were extracted with n-hexane. Similarly, Borah *et al.* (2018); and Chaudhary *et al.* (2020) reported 8% and 19.27% extraction yield in methanolic extract of *O.sanctum* leaves, respectively, lower than the current study.

Total polyphenolic content (TPC) with different fractions was determined by using Folin-ciocalteu and measured as Gallic acid equivalent (GAE), with values ranging from butanol to hexane fraction, i.e. 288.82 $\pm$ 0.45 mg/g to 85.60 $\pm$ 0.19 mg/g gallic acid equivalent respectively

(Table 1). Amin *et al.* (2013) illustrated TPC in the ethanolic extract of *O. sanctum* leaves was 279.05 $\pm$ 0.73 mg/g of gallic acid equivalent and Wangcharoen *et al.* (2007) documented TPC in the ethanolic extract of Shyama and Rama Tulsi was 19.46 $\pm$ 1.97 to 12.60 $\pm$ 1.02 mg GAE/g respectively. While Ishwar Pathak and Muna Niraula (2019) stated TPC content in methanol extract 80.21  $\pm$  0.89 mg GAE/g. Similarly, TPC with methanolic extract of *O. tenuiflorum* was 114.34 mg GAE/g, as reported by Vastrad *et al.* (2015). Chaudhary *et al.* (2020) revealed TPC in OsM was 87.13  $\pm$  4.6mg GAE/g. In the present study, the TPC of methanolic extract in *O.sanctum* were 863.63 $\pm$ 0.17 mg GAE/g, which was higher than reported literature studies while TPC content in *O.sanctum* with butanol solvent i.e. OsB fraction was 212.26  $\pm$  6.3 mg GAE/g, while in the present study, it was 288.82 $\pm$ 0.45 GAE/g with butanol fraction. Ishwar Pathak and Muna Niraula (2019); and Chaudhary *et al.* (2020) reported TPC content with hexane fraction of *O.sanctum* was 60.55  $\pm$  0.25, 52.68  $\pm$  1.8 mg GAE/g respectively, which was lower than the present study. The present findings showed higher TPC in Shyama Tulsi compared to previous studies and varied in the following order: OtM>OtB>OtE>OtH>OtR.

Total flavonoid content (TFC) was measured as quercetin equivalents varies from methanol 310.16 $\pm$ 0.25 to 56.89 $\pm$ 0.18 mg QE/g hexane fraction (Table 1). Results indicated that TFC was highest in methanol extract and lowest in residual fraction. Vastrad *et al.* (2015) observed that the TFC content of methanolic extract of *O.tenuiflorum* was 96.34 mg QE/g of dry extract. Pathak and Niraula (2019); and Chaudhary *et al.*, (2020) reported TFC content with methanol fraction was 67.11  $\pm$  0.43 and 221.97  $\pm$  4.6 mg QE/g, while in hexane fraction was 16.91  $\pm$  0.56, 32.86  $\pm$  2.2mg QE/g of dry extract respectively. In present study, TFC content with all fractions was greater than previously reported by Chaudhary *et al.* (2020) in *O.sanctum*. Overall, the data depicted that TFC content, TPC, and % yield were higher when compared with previous studies, and maximum polyphenols and flavonoids were extracted in butanol fraction followed by ethyl acetate

**Table 1.** Total phenolics, flavonoid and extraction yield of methanol and soluble components of Shyama Tulsi

Fraction	Phenolic mg GAE/g	Flavonoid mg QE/g	Extraction yield (%)	% Extraction of soluble components from OtM
OtM	863.63 $\pm$ 0.17 <sup>a</sup>	310.16 $\pm$ 0.25 <sup>a</sup>	26.41 $\pm$ 0.56 <sup>e</sup>	100
OtH	85.60 $\pm$ 0.19 <sup>d</sup>	56.89 $\pm$ 0.18 <sup>d</sup>	0.30 $\pm$ 0.12 <sup>a</sup>	1.13
OtE	269.98 $\pm$ 0.40 <sup>c</sup>	91.22 $\pm$ 0.26 <sup>c</sup>	2.26 $\pm$ 0.46 <sup>b</sup>	8.85
OtB	288.82 $\pm$ 0.45 <sup>b</sup>	101.11 $\pm$ 0.24 <sup>b</sup>	6.67 $\pm$ 0.54 <sup>c</sup>	25.25
OtR	75.25 $\pm$ 0.49 <sup>e</sup>	26.96 $\pm$ 0.10 <sup>e</sup>	14.15 $\pm$ 0.60 <sup>d</sup>	53.57

Each value in the table is represented as mean  $\pm$ SD (n=3). Value in the same column followed by a different later (a-e) are significantly different ( $p < 0.05$ ). OtM (50% methanol), OtH (n-hexane fraction), OtE (Ethyl acetate fraction), OtB (n-butanol fraction), OtR (Residue fraction)

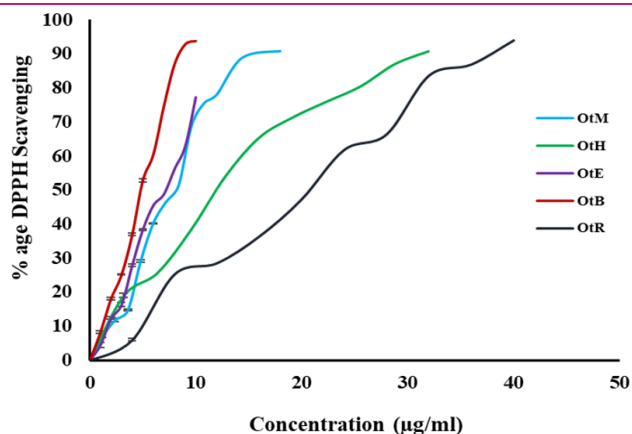
fraction. Compared to water and other solvents, methanol is a better solvent for extracting polar polyphenols and flavonoids (Kchaou *et al.*, 2013). Leaf extracts of *Ocimum tenuiflorum* in hexane, acetone and methanol had been examined (Naik *et al.*, 2015) but differential extraction was not reported previously (Rabeta *et al.*, 2013). However, Chaudhary *et al.*, (2020) showed differential extraction and concentration of polyphenols and flavonoids from *ocimum sanctum* leaves. The activity of polyphenols in various solvents may also be influenced by varied amounts and composition of phytochemicals (Agarwal *et al.*, 2017; and Saeed *et al.*, 2012). However, Shyama Tulsi has more antioxidant potential than green leaves (Parajuli-Bara 2023). In addition, a different assay was performed to determine the antioxidant potential of Shyama Tulsi extract and their ability to scavenge various types of free radicals or decrease in metal ions property.

#### **In vitro antioxidant activity**

To evaluate antioxidant potential of Shyama Tulsi leaf extract and its fractions in various solvents (50% methanol, n-hexane, ethyl acetate and n-butanol), three distinct methods i.e. DPPH, ABTS and FRAP were used. The results were shown (Fig.2 a-c). The  $IC_{50}$  values were calculated to compare its effectiveness with the standard and among themselves (Table 2).

#### **DPPH radical scavenging activity**

The analysis of antioxidant activity of different fractions through DPPH free radical scavenging potential (Fig.2a) was in following order: OtB>OtE>OtM>OtH>OtR. At a concentration of  $4.8 \pm 0.23$   $\mu\text{g/ml}$  butanol fraction exhibits 50% DPPH scavenging activity (Table 2) lowered than ethyl acetate and methanol fraction i.e  $6.85 \pm 0.51$  and  $8.5 \pm 0.24$   $\mu\text{g/ml}$  respectively. While  $IC_{50}$  value with standard ascorbic acid under identical condition was  $4.2 \pm 0.24$   $\mu\text{g/ml}$ . The result indicated that n-butanol fraction has more antioxidant potential compared to other fractions.  $IC_{50}$  value with ethyl acetate fraction in *O.sanctum* reported by Chaudhary *et al.*, 2020 was higher than current study. This may be due to more essential oils in our extract, which contributes to decreased  $IC_{50}$  value of DPPH



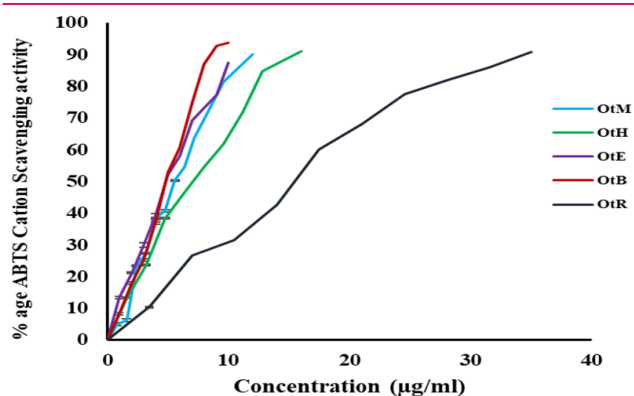
**Fig 2. (a) DPPH free radical scavenging activity of different fraction**

assay. Fadila *et al.* (2017; and Chaudhary *et al.* (2020) observed DPPH free radical scavenging activity in methanol fraction with an  $IC_{50}$  value 12  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ , respectively, while Toppo *et al.* (2019) documented  $IC_{50}$  value 40.43  $\mu\text{g/ml}$  with methanolic leaf extract of *O.sanctum*. Parajuli-Bara (2023) reported  $IC_{50}$  value for DPPH free radical scavenging activity in ethanolic extract of Shyama Tulsi was 8.83  $\mu\text{g/ml}$  while in green leaves 10.41  $\mu\text{g/ml}$ . In the present study  $IC_{50}$  value with methanolic extract was 8.5  $\mu\text{g/ml}$  lower than previously reported (Fadila *et al.*, 2017; and Chaudhary *et al.*, 2020). Chaudhary *et al.* (2020) stated OsH exhibits higher  $IC_{50}$  value compared to the current observation. All the previously reported studies showed higher  $IC_{50}$  value than current study which depict higher antioxidant potential in Shyama Tulsi. This may be due to more active constituents that could transmit hydrogen atom to free radical and lower the  $IC_{50}$  value. Both n-butanol and ethyl acetate were able to extract active antioxidants from holy basil leaves and they both had a significant amount of free radical scavenging capacity, however n-butanol exhibited better efficiency. Polyphenols extracted in hexane fraction showed higher  $IC_{50}$  value and was found ineffective in scavenging DPPH free radicals, which may be due to the formation of active part complexed with non-polar components (Chaudhary *et al.*, 2020).

**Table 2.** Radical scavenging activities of Shyama Tulsi fractions

Extract/fraction	DPPH assay $IC_{50}$ ( $\mu\text{g/ml}$ )	ABTS assay $IC_{50}$ ( $\mu\text{g/ml}$ )	FRAP assay $IC_{50}$ ( $\mu\text{g/ml}$ )
St. Ascorbic acid	$4.2 \pm 0.24^d$	$3.8 \pm 0.26^e$	$38 \pm 0.002^c$
OtM	$8.5 \pm 0.24^c$	$5.6 \pm 0.34^c$	$22.5 \pm 0.002^d$
OtH	$12 \pm 0.52^b$	$6.2 \pm 0.16^b$	$48 \pm 0.003^b$
OtE	$6.85 \pm 0.51^c$	$4.8 \pm 0.46^d$	$20 \pm 0.004^e$
OtB	$4.8 \pm 0.23^d$	$2.4 \pm 0.33^f$	$14.5 \pm 0.004^f$
OtR	$21 \pm 0.35^a$	$15.5 \pm 0.44^a$	$150 \pm 0.002^a$

Each value in the table is represented as mean  $\pm$  SD (n = 3). Values in the same column followed by a different letter (a-e) are significantly different ( $p < 0.05$ ). OtM (50% methanol), OtH (n-hexane fraction), OtE (Ethyl acetate fraction), OtB (n-butanol fraction), OtR (Residue fraction)



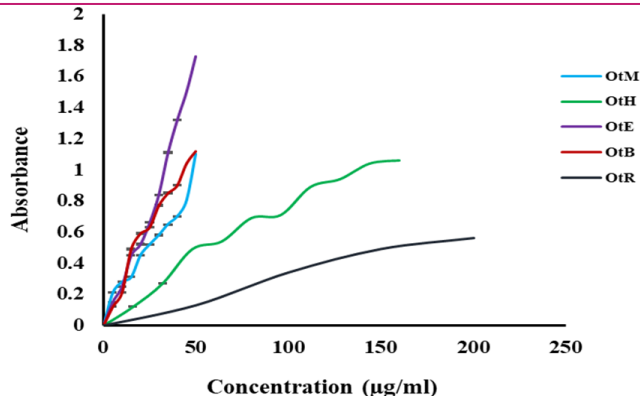
**Fig. 2. (b)** ABTS cation radical scavenging activity of different fraction

**ABTS radical scavenging activity**

The organic cation radical activity of an extract was measured by ABTS assay. Results of the present study showed OtB fraction has higher ABTS radical scavenging activity than all other fractions (Fig. 2b). The ABTS cation radical scavenging activity was in order as OtB>>OtE>OtM>OtH>OtR. OtB fraction has a greater ability to scavenge cation radicals than ascorbic acid and a lower value of IC<sub>50</sub> indicated the presence of well-organized organic cation scavengers (Table 2). OtE fraction had more antioxidant potential than leaves of *O.sanctum* reported by Chaudhary *et al.*, 2020 and may be due to some active constituents in our extract. While Toppo *et al.* (2019); Basak *et al.* (2014); Chaudhary *et al.* (2020) illustrated higher IC<sub>50</sub> values with methanolic extracts of *O.sanctum* as 53.5µg/ml, 131µg/ml, 9.2 µg/ml respectively. The present study showed that Shyama Tulsi extract had more ability to scavenge cation radicals than the reported literature.

**FRAP metal ion reducing property**

FRAP assay was used to detect total antioxidant ability of any extract. In the present study, metal ion reducing property followed the OtB>OtE>OtM>OtH>OtR trend. IC<sub>50</sub> value of OtB fraction was 14.5±0.004 µg/ml much lower than hexane (48±0.003µg/ml), and methanol fraction (22.5±0.002 µg/ml) (Table 2; Fig.2c). OtB and OtE showed a linear increase in activity upto 20±0.004 µg/ml, while in another fraction increased upto 150±0.002 µg/ml (Fig.2c). The present study showed OtB and OtE fractions had lower IC<sub>50</sub> values indicated presence of powerful reducing agents. Agarwal *et al.* (2017) stated Ferric reducing activities i.e. 77.05±2.67 in ethyl acetate extract, while in butanol fraction,



**Fig. 3. (c)** FRAP metal ion reducing property of different fractions

75.84±4.85 µg/ml were higher than the present study. Antioxidant efficiency may depend on the presence of phenolic, flavonoids, terpenes, and essential oils and the chemical structure of these compounds or their binding to other molecules such as lipids and carbohydrates. It may be enhanced by differential extraction methods using solvents with various polarities (Agarwal *et al.*, 2017; Bariyah *et al.*, 2013; and Chaudhary *et al.*, 2020).

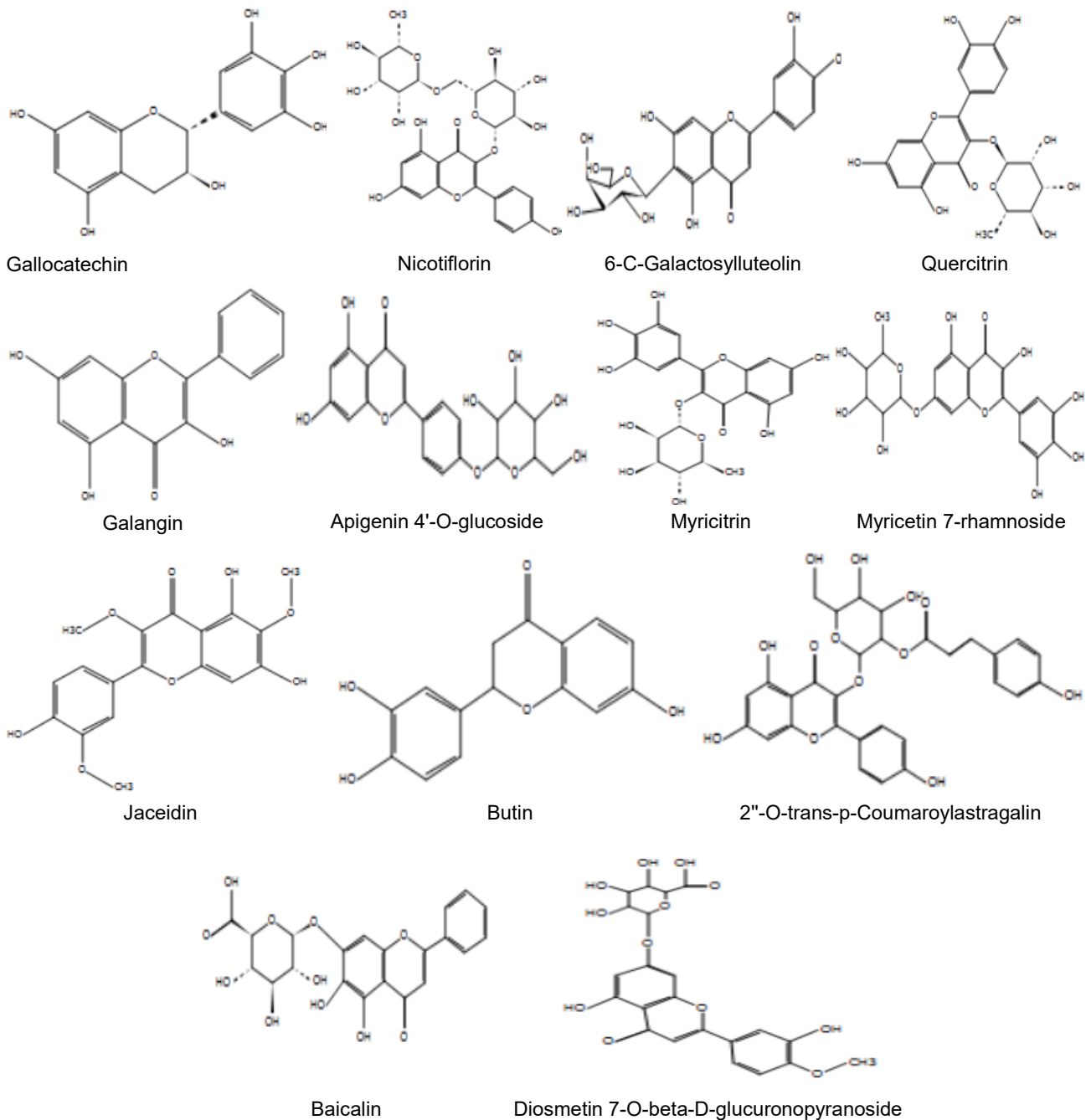
**Correlation of antioxidant activity with polyphenols/flavonoids**

To understand the co-relation between antioxidant potential and polyphenols/ flavonoid content of various fractions, data was analyzed statistically using SPSS 16.0 software. Total polyphenolic content (TPC) was positively and significantly linked with IC<sub>50</sub> values determined by DPPH scavenging activity with r>0.879, ABTS cation radical scavenging capacity with r>0.801 or metal ion reducing property with r>0.967 (Table 3). The correlation between total flavonoid content (TFC) and IC<sub>50</sub> value for the FRAP metal ion reducing assay, DPPH scavenging activity and ABTS cation scavenging ability are r>0.824, r>0.846 and r>0.914, respectively. The present observations supported the fact that polyphenols and flavonoids contributed to antioxidant capacity of the extract since antioxidant activities were positively correlated with TPC and TFC (Sangeeta and Varunda,2016; Chaudhary *et al.*, 2020). Phenolic and flavonoid compounds may efficiently remove free radicals or exchange H<sup>+</sup> quickly directly, contributing to the antioxidant property (Renen *et al.*, 2010). Polyphenols influence antioxidant properties by their π electron in the benzene ring, while flavonoids impart due to the

**Table 3.** Correlation between phenolic and flavonoid content of Shyama Tulsi and the IC<sub>50</sub> values of antioxidant activities

ASSAY	Phenolics Correlation (r)	Flavonoid Correlation (r)
IC <sub>50</sub> of DPPH radical scavenging ability	0.879	0.846
IC <sub>50</sub> of ABTS radical scavenging ability	0.801	0.914
IC <sub>50</sub> of FRAP ion reducing capacity	0.967	0.824

Each value in the table is represented as mean ± SD (n=3)



**Fig. 4.** Structure of identified polyphenolic flavonoid compound

keto group at C4 or hydroxyl groups at rings A and C (Ali and Neda 2011).

*In vivo* and *in vitro* studies, the beneficial properties of polyphenols and flavonoids are documented. It is reported that polyphenols restrain mutagenesis and carcinogenesis in humans (Tewari *et al.*, 2015). While *in vitro*, flavonoids inhibited lipid peroxidation (Catherine Rice-Evans 2001), oxidation of low-density lipoproteins (LDL) and prevent cellular damage associated with oxidative stress (Gate *et al.*, 1999; Pacheco *et al.*, 2009 and Pisoschi *et al.*, 2015). Further research is going on to study the protective behaviour of different polyphenols in various diseases caused by oxidative stress.

#### Phytochemical analysis

Polyphenols are the natural benefits of human beings, and there is a continuous need to explore sources of polyphenols. To date, no study has reported the extraction of polyphenols with the fractionation method in Shyama Tuls. To analyze the polyphenols and flavonoids in detail in the best extracts of the present study, OtE or OtB fractions were subjected to HR-LCMS (Fig. 3). Hexane fraction was not proceeded for HR-LCMS due to its lower antioxidant potential. In the present study, total 26 polyphenols (Table 4) and 18 flavonoids were identified i.e. 6-C-Galactosylluteolin, Quercitrin, Isorientin, Kaempferol 3-glucuronide, Jaceidin, Genis-

**Table 4.** Identification of polyphenolic compound in Shyama Tulsi

Fraction	MW	Compound detected	Molecular formula
OtE	594	Kaempferol 4'-glucoside 7- rhamnoside	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>
	462	Kaempferol 3-glucuronide, Luteolin 7-O-glucuronide	C <sub>21</sub> H <sub>18</sub> O <sub>12</sub>
	448	isorientin,6-C-galactosylluteolin, Quercitrin	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>
	432	Genistin, Apigenin 4'-O-glucoside	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>
	464	Myricitrin, Myricetin 7-rhamnoside	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>
	288	2-(4-Methyl-1,3-pentadienyl) anthraquinone	C <sub>20</sub> H <sub>16</sub> O <sub>2</sub>
	360	Jaceidin	C <sub>18</sub> H <sub>16</sub> O <sub>8</sub>
	358	Dihydro caffeic acid 3-O-glucuronide	C <sub>15</sub> H <sub>18</sub> O <sub>10</sub>
	286	Kaempferol	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>
	272	Butin	C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>
	194	Isoferulic acid	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>
	180	Caffeic acid, 2-Hydroxy-3-(4- hydroxyphenyl) propenoic acid	C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>
	162	3-Hydroxycoumarin	C <sub>9</sub> H <sub>6</sub> O <sub>3</sub>
	594	2''-O-trans-p-Coumaroylastragalin	C <sub>30</sub> H <sub>26</sub> O <sub>13</sub>
	OtB	594	Nicotiflorin, Kaempferol 4'-glucoside 7-rhamnoside
448		6-C-galactosylluteolin, Quercitrin	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>
462		Kaempferol 3-glucuronide	C <sub>21</sub> H <sub>18</sub> O <sub>12</sub>
446		Baicalin	C <sub>21</sub> H <sub>18</sub> O <sub>11</sub>
476		Diosmetin 7-O-beta-D-glucuronopyranoside	C <sub>22</sub> H <sub>20</sub> O <sub>12</sub>
306		Gallocatechin	C <sub>15</sub> H <sub>14</sub> O <sub>7</sub>
270		Galangin	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>
162		Umbelliferone	C <sub>9</sub> H <sub>6</sub> O <sub>3</sub>

tin, Myricetin 7-Rhamnoside, Butin, Apigenin 4'-O-glucoside, Kaempferol, Myricitrin, Luteolin 7-O-glucuronide, 2''-O-trans-p-Coumaroylastragalin, Nicotiflorin, Gallocatechin, Galangin, Baicalin, Diosmetin 7-O-beta-D-glucuronopyranoside shown in (Fig. 3). A numbers of active polyphenolics constituents such as quercetin, orientin, luteolin, apigenin, caffeic acid, chlorogenic acid, kaempferol and genistein has been reported by Chaudhary *et al.* (2020); Baliga *et al.* (2013); Upadhyay *et al.* (2015); Ullah *et al.* (2022); and Mousavi *et al.* (2018). But in the present study, in addition to the previously identified compounds were some other active polyphenolics, flavonoid compounds identified in ethyl acetate and butanol fraction as 6-C-Galactosylluteolin, Quercitrin, Jaceidin, Myricetin 7-rhamnoside, Butin, 2''-O-trans-p-Coumaroylastragalin, Apigenin 4'-O-glucoside, Nicotiflorin, Gallocatechin, Galangin, Baicalin. These results justified the higher antioxidant activity of Shyama Tulsi as compared to previously reported studies (Chaudhary *et al.*, 2020). Many biological properties of various flavonoids are reported as antibacterial, antiviral, anti-inflammatory, anticancer, antiallergic activities, protection of lipids against oxidative stress, radioprotective and enhanced DNA repair in addition to antioxidant activity (Uma Devi *et al.*, 1999; Yao *et al.*, 2004; Montoro *et al.*, 2005; Shashank and Abhay, 2013; Satyamitra *et al.*, 2014). Moreover, preclinical research demonstrated that Tulsi and its

phytochemicals (Oleanolic acid, Ursolic acid, apigenin, Rosmarinic acid,  $\beta$  caryophyllene, Eugenol, Linalool, Luteolin, Myretenal, Carnosic acid and  $\beta$ -sitosterol) increased antioxidant activity, altered gene expression, caused apoptosis, inhibited angiogenesis, metastasis, suppress chemically induced skin, liver, oral and lung cancer (Naik *et al.*, 2015; and Baliga *et al.*, 2013). Further, these polyphenols and flavonoids can be studied for their beneficial effect in various diseases.

## Conclusion

Shyama Tulsi (*O. tenuiflorum*) leaves sequentially extracted with 50% methanol, hexane, ethyl acetate, and butanol were evaluated for their antioxidant properties or phytochemicals. The present study revealed higher phenolic and flavonoid content, indicating the natural antioxidant nature of Shyama Tulsi, signifying its medicinal importance and that it may be useful in protection against oxidative stress. The higher antioxidant potential may be due to the polyphenolic compounds such as Quercitrin, 6-C-Galactosylluteolin, Nicotiflorin, Apigenin 4'-O-glucoside, caffeic acid, Genistin, Myricitrin detected in the present study by HR-LCMS. Due to this medicinal herb's higher antioxidant potential and diverse polyphenols, it can also be used as a substitute for synthesizing new medicines.



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## Conflict of interests

The authors declare that they have no conflict of interest.

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