

COMPARISON OF TOTAL FLAVONOID, PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY IN LEAF AND SEED EXTRACTS FROM WHITE HOLY BASIL (*Ocimum sanctum*)¹Neha Mahajan, ²Jagtar Singh and ³Shweta Sinha

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ABSTRACT: *Ocimum sanctum*, commonly known as the white holy basil herb belonging to *Lamiaceae* family is one of the oldest and popular medicinal plant rich in various phytonutrients and antioxidants. In this study, the comparative evaluation of flavonoids, phenolic content, and antioxidant capacity was carried out in methanolic extract prepared from *O. sanctum* leaves and seeds. The TAC, TPC, and the TFC were measured by ammonium molybdate, Folin-Ciocalteu and aluminum chloride method respectively. Antioxidant activity was also determined by using DPPH and FRAP assay. In response to the above assays, TACs of *O. sanctum* leaf and seed extracts were 25-248 and 0.011-0.109 µg AAE/10 mg of extract respectively. The TFC assay showed that leaf extract of *O. sanctum* (14-225 µg QE/10mg extract) had higher flavonoid content than the seed extract (0.009-0.119 µg QE/10 mg extract) and the TPC assay in the leaf extract (4.49-9.31 µg GAE/mg extract) was higher than those present in seed (4.10-9.05 µg GAE/mg extract). In DPPH assay, % inhibition in *O. sanctum* leaf extract was determined in the range 18-76% while in seed extract it was 6-29% and in FRAP assay, leaf extract displayed reducing power in range 0.48-5.50 µg FSE /mg extract while in seed extract it was 0.16-5.46 µg FSE /mg extract. It was observed that *O. sanctum* leaf extract had high total phenolic and flavonoid content in addition to antioxidant capacity as compared to its seed extract.

Key Words: *Ocimum sanctum*; flavonoid content; phenolic content; antioxidant activity; DPPH assay; FRAP assay

Abbreviations: TAC: Total Antioxidant Capacity TPC: Total Phenolic Content TFC: Total Flavonoid Content DPPH: 2, 2-diphenyl-1-picrylhydrazyl FRAP: Ferric Reducing/Antioxidant Power

INTRODUCTION

The free radicals are chemical species that are produced through frequent physiological and biochemical metabolism in the human body as byproduct (Halliwell *et al.*, 1990; Young & Woodside, 2001). Chemically reactive oxygen species (ROS) and free radicals such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydroxyl radical (OH[•]) *etc.* are capable of tissue damage and loss of function in a number of tissues and organs (Verma *et al.*, 2009). They can also damage biologically relevant molecules such as DNA, proteins, carbohydrates and lipids (Young & Woodside, 2001). These radicals lead to oxidative stress in the human body, which has major role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer and in the aging process (Fang *et al.*, 2002; Freidovich, 1999). Moreover, our body can neutralize mild oxidative stress by increasing antioxidant defense mechanism but severe oxidative stress increases free radicals that can lead to cellular injury and death (Shukla *et al.*, 2012). This scenario leads the researchers to quest for different sources of antioxidant globally. Although, there are many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), they are volatile and easily decompose at high temperatures that may results in liver damage and carcinogenesis (Barlow *et al.*, 1990). Therefore, the search for natural food antioxidants increases which are present in medicinal and dietary plants and that might help to prevent the oxidative damage (Gardner *et al.*, 2000; Youdim *et al.*, 1994). Previous researches have shown that in plants, phenolics are the major antioxidants to prevent the oxidation of the susceptible substrate (Maestri *et al.*, 2006).

Plant phenolics includes diterpenes (carnosol and carnosic acid), flavonoids (quercetin and catechin), phenolic acids (gallic, protochatechuic, caffeic, and rosmarinic acid) and volatile oils (eugenol, carvacrol and thymol) (Brewer *et al.*, 2011). Various herbs of family *Lamiaceae* have been used to screen antioxidant capacities in them such as *Ocimum sanctum* (holy basil), *Mentha piperta* (peppermint), *Origanum vulgare* (oregano), *Salvia officinalis* (sage), and *Thymus vulgaris* (thyme) (Dragland *et al.*, 2003; Yi *et al.*, 2010). Among them *O. sanctum* is a herb that is native to India and other tropical areas of Asia. Besides adding flavour and distinctive aroma to food, it is a clinically proven cancer fighter, neuropathy healer, and anti-microbial (Baritoux *et al.*, 1991; Chipault *et al.*, 1956; Risch *et al.*, 1997). It is also an excellent source of the β -carotene and is found to be the key component in the prevention of free radical damage (Patil *et al.*, 2011). Phenolic compounds such as eugenol, cirsilineol, isothymusin, isothymonin, rosmarinic acid are present in it along with orientin and vicenin, which have been proved as good antioxidant compounds (Vrinda *et al.*, 2001).

The objective of the present study was to compare the total flavonoid content (TFC), total phenolic content (TPC) and total antioxidant capacity (TAC) of white holy basil (*O. sanctum*) by three different antioxidant-testing assays including DPPH and FRAP in extracts prepared from its leaves and seeds.

MATERIALS AND METHODS

Chemicals

The chemicals used in the study included: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, USA), methanol, potassium ferricyanide, ferric chloride, ascorbic acid, sulphuric acid, sodium sulphate, ammonium molybdate, 2,4,6- tripyridyl-s-triazine (TPTZ), hydrochloric acid, trichloroacetic acid, sodium acetate, glacial acetic acid (HiMedia, Mumbai, India).

Collection, harvesting and drying of samples

White variety of herb *O. sanctum* (Figure 1) was taken from local areas in Chandigarh. Fresh leaves and seeds were used for sample preparation. Seeds were ground into fine powder in an electronic grinder while approximately 10g of leaves were washed and then used for extraction. The pre-washed leaves were dried at room temperature for 3 days and then ground into fine powder in an electronic grinder.

Sample extraction

Ten grams of each powdered sample prepared from leaves and seeds were extracted for 2 h at room temperature in 100 ml of 80% methanol with agitation on orbital shaker at 150 rpm. The mixture was then filtered with Whatman no. 1 filter paper. Filtrates were collected, dried in oven at 45°C and stored at 4°C for further antioxidant analysis.

CHEMICAL ANALYSIS

Estimation of total flavonoids content (TFC)

Aluminum chloride method was used to determine TFC in samples. TFC of the extracts was determined according to the method given by Chang *et al.* (2002). Stock (14 mg/ml) for each sample was used to make different concentrations (14-210 μ g/ml). Three hundred microliter of 50% of methanol was added to 100 μ l of the prepared concentrations. Thereafter, 20 μ l of 10% aluminum chloride and 20 μ l of 1M potassium acetate was added in each eppendorf and final volume was made upto 1ml with distilled water. Then the mixture was vortexed thoroughly and after incubation for 30 min at room temperature, absorbance was measured at 415 nm against a blank using 50% methanol. The results were expressed as μ g of Quercetin Equivalent (QE)/10 mg extract.

Estimation of total phenolic content (TPC)

The TPC of the extracts was determined according to the method given by Singleton and Rossi (1965). Stock (3 mg/ml) for each sample was used to prepare different concentrations (3-45 μ g/ml) and to each concentration, 500 μ l of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and 400 μ l of sodium carbonate (7.5% w/v) were added. Thereafter, the mixture was vortexed thoroughly and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 765 nm against a blank using 50% methanol. The results were expressed in μ g Gallic Acid Equivalent (GAE)/ mg of extract.

Estimation of total antioxidant capacity (TAC)

The TAC was estimated by molybdate assay (Prieto *et al.*, 1999). This assay measures the absorbance at 695nm when Mo (VI) is reduced to Mo (V) by the sample. Stock of each sample (14 mg/ml) was used to prepare different concentrations (14-210 μ g/ml). To this, 1ml of TAC reagent (3.24 ml sulphuric acid + 0.397g sodium sulphate + 0.494g ammonium molybdate, volume made upto 100 ml) was added. Thereafter, the mixture was vortexed thoroughly and after incubation for 90 min at 95°C, the absorbance was measured at 695 nm against blank using distilled water. The results were expressed as μ g Ascorbic Acid Equivalent (AAE)/10 mg extract.

2, 2- Diphenyl-1-Picrylhydrazyl (DPPH) assay

Free radical scavenging activity of extract was measured by the method of Krings & Berger (2001). The hydrogen atom or electron donating ability of the extracts was measured from the bleaching of the purple-coloured methanol solution of DPPH. 50% methanol was used as blank and standard free radical scavenger; trolox was used as a positive control.

The negative control contained 600 μ l of 0.1 mM DPPH and 300 μ l of 50% methanol without sample. Stock of each sample (3 mg/ml) was used to prepare different concentrations (30-300 μ g/ml) and 600 μ l of DPPH was added. Then, the reaction mixture was incubated for 20 min and the absorbance was measured at 517nm.

The free radical scavenging activity was calculated according to the following equation (Yen and Duh, 1994):

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

Where, A_0 - absorbance of the negative control

A_s - absorbance of the sample.

Ferric Reducing/Antioxidant Power method (FRAP Assay)

FRAP of extract was measured by the method given by Benzie & Strain (1996). Reagents for FRAP assay include 300 mM/l acetate buffer (pH 3.6), 10 mM/l TPTZ in 40 mM HCl/l, 20 mM/l FeCl_3 anhydrous. The working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ solution and FeCl_3 anhydrous in the ratio of 10:1:1. Prepared FRAP solution was incubated at 37°C for 15 min prior to the experiment. Stock (3 mg/ml) of each sample was used to prepare different concentrations (3-45 μ g/ml). Then, 1ml of the FRAP reagent was mixed with these concentrations and incubated for 30 min in the dark. The absorbance of the reaction mixture was measured at 593nm to monitor the ability of sample to reduce ferric ions to ferrous form. Results were expressed as μ g Ferrous Sulphate Equivalents (FSE)/mg of extract.

Statistical Analysis

All analyses were conducted in triplicates and expressed as mean values. All statistical analysis was conducted using Microsoft excel, 2007. T-test was used to determine the significant differences among means. Differences at $p < 0.05$ were considered to be significant.

RESULTS

Total antioxidant and flavonoid content assay

TAC and TFC assay values of *O. sanctum* leaf and seed extracts are presented in Table 1. TAC of *O. sanctum* leaf extract was found to be higher (25-248 μ g Ascorbic Acid Equivalents (AAE)/10 mg of extract) than the seed extract (0.011-0.109 μ g AAE/10 mg of extract) (Figure 2). The TFC in terms of Quercetin Equivalent (QE) revealed that leaf extract of *O. sanctum* (14-225 μ g QE/10 mg extract) had higher flavonoid content than the seed extract (0.009-0.119 μ g QE/10 mg extract) (Figure 3).



Figure 1: A photograph of an experimental plant *O. sanctum*.

Total phenolic content assay

TPC measured by Folin-Ciocalteu method in terms of μg of gallic acid equivalent (GAE)/mg of extract displayed that phenolic content in the leaf extract of *O. sanctum* (4.49-9.31 μg GAE/mg extract) was higher than those present in its seed extract (4.10-9.05 μg GAE/mg extract) (Table 2; Figure 4).

DPPH free radical scavenging assay

Methanolic extracts were screened for their possible antioxidant activity by DPPH scavenging assay (Table 2). In DPPH assay, percentage inhibition in *O. sanctum* leaf extract was in range of 18-76% while in seed extract it was 6-29% (Figure 5). IC50 value of *O. sanctum* leaf extract was 10.81 while 23.1 in case of seed extract. The higher antioxidant activity is reflected with lower IC50 value.

Ferric Reducing/Antioxidant Power assay

Extracts of *O. sanctum* seeds and leaves were subjected to FRAP assay. Results were expressed in terms of μg ferrous sulphate equivalents (FSE) /mg extract. In case of *O. sanctum* leaf extract, results were in range 0.48-5.50 μg FSE /mg extract while in case of seed extract it was 0.16-5.46 μg FSE /mg extract (Table 2). Antioxidant activity was high in leaf extract as compared to extract prepared from its seeds in FRAP assay (Figure 6).

Table 1. Total antioxidant capacity (TAC) and total flavonoid content (TFC) in *O. sanctum* leaf and seed extract.

Assays performed	Concentration ($\mu\text{g}/\text{ml}$)	<i>O. sanctum</i> (leaf extract)	<i>O. sanctum</i> (seed extract)
TAC (μg AAE/10 mg extract)	14	25.70 \pm 0.714	0.011 \pm 0.0012
	42	39.03\pm0.412	0.014\pm0.0006
	70	64.97\pm0.710	0.029\pm0.0015
	98	143.2 \pm 0.411	0.062 \pm 0.0004
	126	164.6 \pm 0.810	0.083 \pm 0.0407
	154	189.9\pm0.710	0.086\pm0.0014
	182	225.3 \pm 0.819	0.099 \pm 0.0018
	210	248.7\pm0.409	0.109\pm0.0453
TFC (μg QE/10 mg extract)	14	14.75\pm1.79	0.009\pm0.001
	42	53.54\pm1.89	0.022\pm0.000
	70	84.96\pm1.43	0.036\pm0.002
	98	105.43\pm2.17	0.058\pm0.000
	126	136.37\pm2.86	0.088\pm0.002
	154	157.31\pm1.48	0.094\pm0.002
	182	189.92\pm0.71	0.108\pm0.001
	210	255.61\pm1.4	0.119\pm0.001

Experiments were performed in triplicate and expressed as mean \pm SD. Values with bold letters are significantly different ($p < 0.05$) and vice-versa.

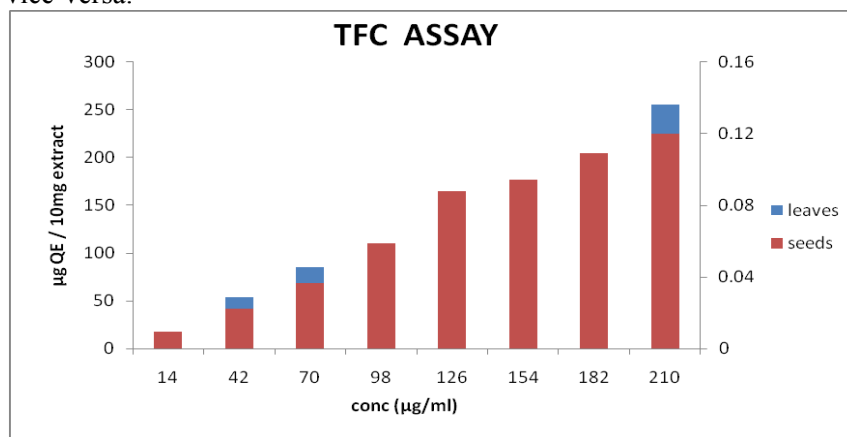


Figure 2: Total flavonoid content of extracts (14 mg/ml) from seeds (red) and leaves (blue) of *O. sanctum* was measured at 415nm with different concentrations (14-210 $\mu\text{g}/\text{ml}$) using the colorimetric assay. Each value represents mean SD of three experiments.

Table 2. TPC, DPPH and FRAP assay in *O. sanctum* leaf and seed extract.

Assays performed	Concentration (µg/ml)	<i>O. sanctum</i> (leaf extract)	<i>O. sanctum</i> (seed extract)
TPC (µg GAE/mg extract)	12	4.49±0.439	4.10±0.168
	15	5.33±0.333	5.03±0.130
	17	6.05±0.349	5.61±0.137
	21	6.57±0.189	6.38±0.587
	24	8.66±0.335	8.13±0.205
	27	9.31±0.201	9.05±0.252
DPPH (% inhibition)	2	18.20±0.55	6.14±0.83
	4	31.03±0.36	8.74±0.34
	6	32.48±0.47	15.11±0.16
	8	35.93±0.54	19.76±0.50
	10	36.65±0.18	21.09±0.33
	12	76.34±0.57	29.84±0.09
FRAP (µg FSE/mg extract)	0.5	0.48±0.012	0.160±0.025
	1.5	1.47±0.016	1.46±0.064
	2.5	3.33±0.050	2.70±0.016
	3.5	3.73±0.130	3.30±0.047
	4.5	4.64±0.035	4.37±0.097
	5.5	5.50±0.026	5.46±0.120

Experiments were performed in triplicate and expressed as mean ± SD. Values with bold letters are significantly different (p<0.05) and vice-versa.

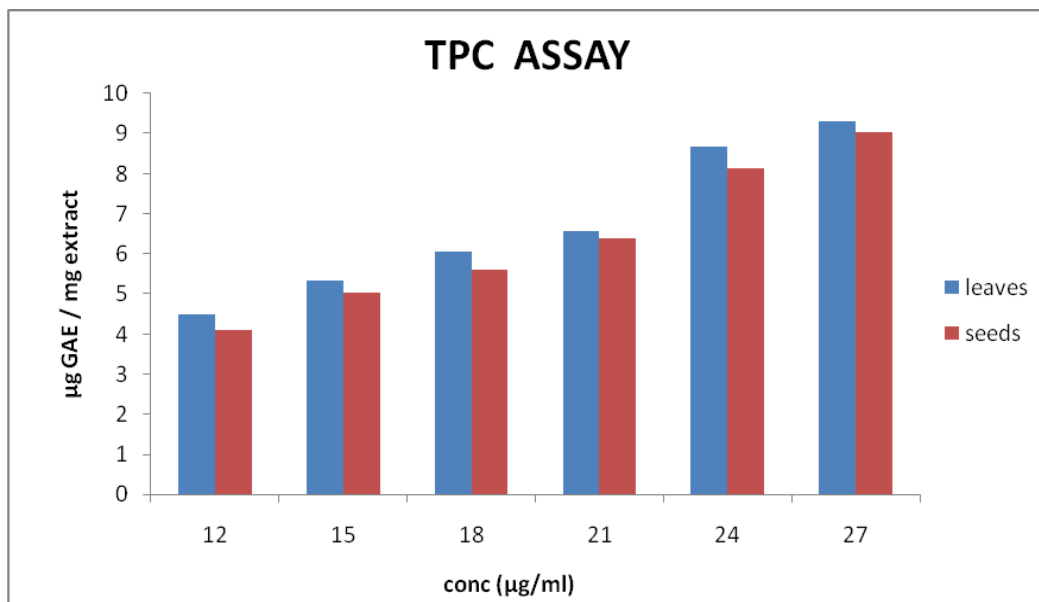


Figure 3: Total phenolic content of extracts (3 mg/ml) from seeds and leaves of *O. sanctum* was measured at 765 nm with different concentrations (3-45 µg/ml) using the colorimetric assay. Each value represents mean SD of three experiments.

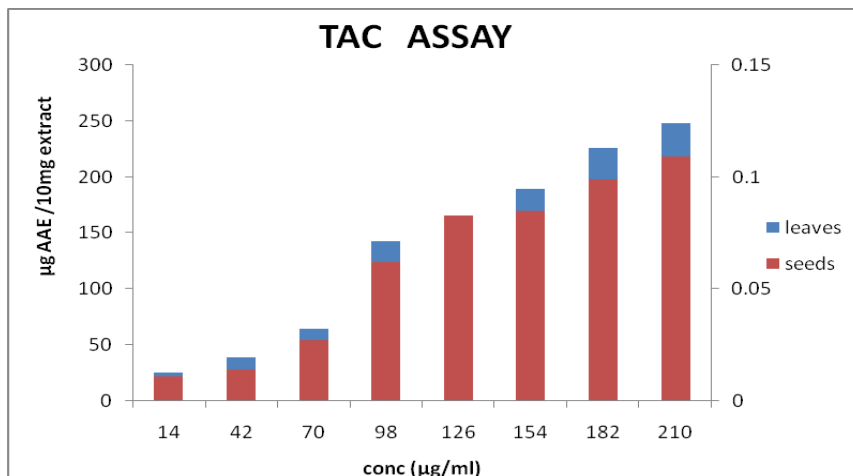


Figure 4: Total antioxidant capacity of extracts (14 mg/ml) from seeds and leaves of *O. sanctum* was measured at 695nm with different concentrations (14-210 µg/ml) using phosphomolybdenum complex formation method. Each value represents mean SD of three experiments.

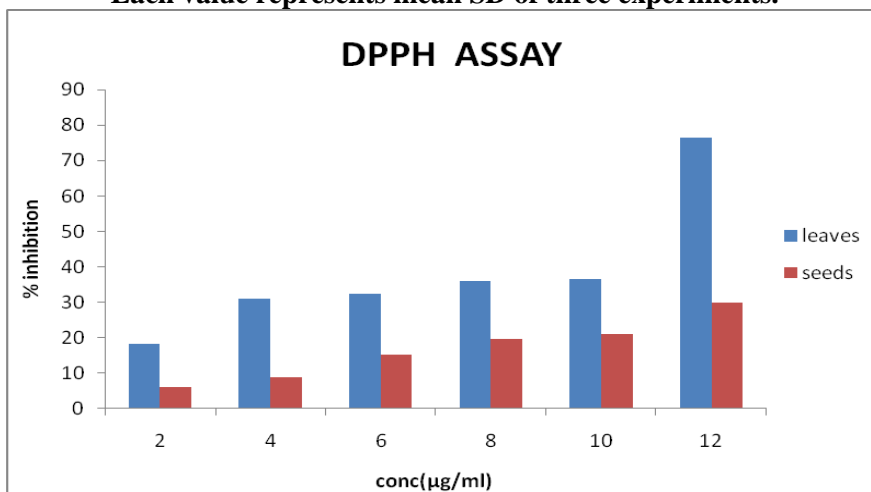


Figure 5: Antioxidant activity of extracts (3 mg/ml) from seeds and leaves of *O. sanctum* was measured at 517nm as % scavenging of DPPH radical with different concentrations (30-300 µg/ml). Each value represents mean SD of three experiments.

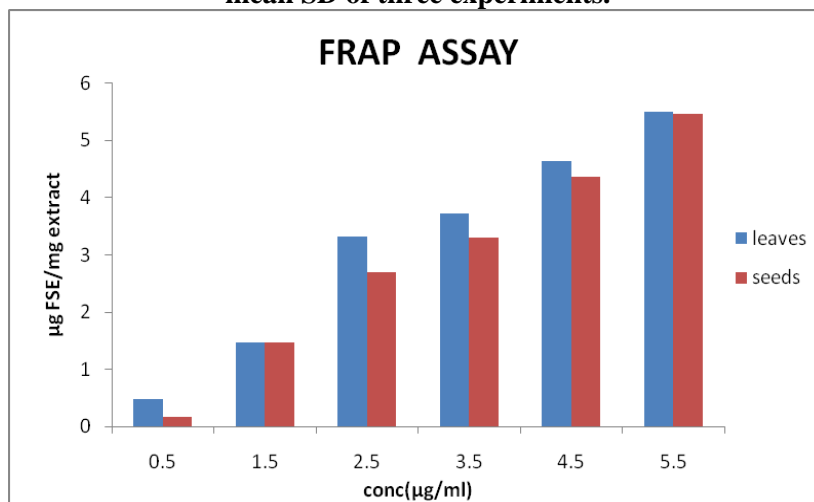


Figure 6: Ferric Reducing/Antioxidant Power assay of extracts (3 mg/ml) from seeds and leaves of *O. sanctum* measured at 593 nm with different concentrations (3-45 µg/ml) using ferric reducing ability of samples. Each value represents mean SD of three experiments.

DISCUSSION

Total antioxidant and flavonoid content assay of *O. sanctum* leaf and seed extracts were higher than the present study done by using 70% ethanolic extract. The *O. sanctum* leaf and seed extracts were having 14.16 mg/g catechin equivalents of flavonoid contents (Venuprasad *et al.*, 2013) and using 80% methanolic extract, the TFC was 98.71 ± 1.144 mg QE/g dry weight (DW) (Padmaja and Srinivasulu, 2013). Among other herbs of Lamiaceae such as *Salvia officinalis*, TFC was measured by using oven (45°C) with a maximum in the air dried plants (0.923 mg of QE/g of DM) (Hamrouni-Sellami *et al.*, 2013) and methanolic extract of *Thymus vulgaris* leaves was 1.71 mg QE/g DW (Hossain *et al.*, 2013).

Total phenolic content (TPC) was in contrast to the observations reported by Sarfraz *et al.* (2011), in which TPC estimated in *Ocimum sp.* leaf, seed extracts were 3.97 ± 0.14 to 5.67 ± 0.77 and 5.67 ± 0.77 mg GAE/g DW respectively, which exhibited that seed extract, had higher phenolic content than the leaf extract. In other studies also, very high total phenolic content in aqueous extracts of *O. sanctum* leaves were reported. Leaf extract prepared in water showed 90.4 ± 4.5 mg GAE/g of polyphenols (Suanarunsawat *et al.*, 2011) while 70% ethanol leaf extract revealed 148.9 mg/g GAE of polyphenols (Venuprasad *et al.*, 2013). Methanolic extract of *O. sanctum* leaves was found to have 58.06 ± 0.245 mg GAE/g of extract (Padmaja and Srinivasulu, 2013), 3.0 mg/ml of TPC (Hakkim *et al.*, 2007) and Juliani and Simon (2002) reported total phenolic content equal to 51.5 mg GAE/g DW. Among other herbs of the Lamiaceae family such as *Thymus vulgaris* and *Salvia officinalis*, TPC determined was 2.13 ± 0.11 and 1.34 ± 0.09 mg of GAE/g of fresh weight respectively (Zheng *et al.*, 2001) which was lower than the TPC value of holy basil determined in the present study.

In DPPH free radical scavenging assay, observations found in the present study were in contrast to Sarfraz *et al.* (2011), in which total antioxidant activity of *O. sanctum* seed and leaf extract displayed 84.59 and 79.39% inhibition activities respectively. Other studies found that the IC₅₀ in leaf extract was 395 ± 16.2 µg/ml (Venuprasad *et al.*, 2013), 0.46 mg/ml (Hakkim *et al.*, 2007) and 20.6 µg/ml (Juntachote and Berghofer, 2005). Among other herbs of Lamiaceae family such as *Thymus vulgaris*, 43.95% inhibition activity was reported (Hussein *et al.*, 2011). Further, in ethanolic extract of *Salvia officinalis*, it was 39.75% inhibition activity at concentration of 10 µg/ml (Rasmy *et al.*, 2012).

For Ferric Reducing/Antioxidant Power assay, leaf extract of *O. sanctum* in 80% methanol manifested 0.009 ± 0.001 µM ferric reducing ability (Padmaja and Srinivasulu, 2013) and in 76% ethanol, it was 40.46 ± 2.16 mg Trolox equivalent/g DW and 15.98 ± 0.85 mg vitamin C equivalent/g DW (Wangcharoen and Morasuk, 2007). Juliani and Simon (2002) reported the antioxidant activity of *O. sanctum* by using ABTS and FRAP assays. For Trolox equivalent antioxidant activity (TEAC) with ABTS assay, it was 297 µM Trolox/g DW while in FRAP assay it was 420 µM AA/g DW.

CONCLUSIONS

Free radicals and ROS generate oxidative stress in human body, which results in an increased risk for many diseases. However, antioxidants were found to be extremely beneficial in reducing their production. They may directly react with the chemically active radicals to destroy them or indirectly decrease their production by inhibiting the free radical generating enzymes. They can also enhance the antioxidant defense mechanism of the body by expressing other antioxidant enzymes. Many researches have been conducted for identifying new antioxidants from natural sources. The present study was designed to investigate antioxidant activity of white holy basil (*O. sanctum*) leaf and seed extracts and it can be concluded that leaf extract had high phenolic and flavanoid content as well as good antioxidant capacity as compared to its seed extract. The results from the present study can be used as preliminary test for purification of active compounds from *O. sanctum* for future study.

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