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DETERMINATION OF SOME BIOCHEMICALS, PHYTOCHEMICALS AND ANTIOXIDANT PROPERTIES OF DIFFERENT PARTS OF *Cichorium intybus* L.: A COMPARATIVE STUDY

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ABSTRACT

The biochemical, phytochemical and antioxidant composition of root, stem, leaves and seeds of *Cichorium intybus* L was determined. A statistically significant difference ($p>0.05$) was observed among different parts of *Cichorium intybus* regarding the biochemical, phytochemical and antioxidant composition. The leaves were found to possess comparatively higher values of total sugars non-reducing sugars water soluble proteins total flavonoids, total phenolic acids and total antioxidants. On the other hand, seeds were found to possess comparatively higher contents of reducing sugars, salt soluble proteins and saponins. The phytochemical screening confirmed the presence of tannins, saponins, flavonoids, terpenoids, cardiac glycosides and anthocyanins in each part of the plant. The antioxidant potential of methanolic extracts of different parts was evaluated in terms of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity and reducing power. The leaf extract was found to show comparatively low value of IC_{50} for DPPH inhibition and high reducing power. Due to good biochemical, phytochemical and antioxidant composition, *Cichorium intybus* leaves would be valuable candidate in pharmaceutical formulations and play an important role in improving the human, livestock and poultry health by participating in the antioxidant defence system against endogenous free radicals.

Key words: Antioxidant Composition, Biochemical Analysis, *Cichorium intybus*, Phytochmical Screening, DPPH radical scavenging capacity.

INTRODUCTION

Cichorium intybus, commonly known as chicory, belongs to family Asteraceae and widely distributed in Asia and Europe (Bais and Ravishankar, 2001). All parts of this plant possess great medicinal importance due to the presence of a number of medicinally important compounds such as alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterols, flavonoids, saponins and tannins (Molan *et al*., 2003; Nandagopal and Kumari, 2007; Muthusamy *et al*., 2008; Atta *et al*., 2010). *C. intybus* has been traditionally used for the treatment of fever, diarrhoea, jaundice and gallstones (Afzal *et al*., 2009; Abbasi, 2009). The studies on rats have shown that *C. intybus* possesses anti-hepatotoxic and anti-diabetic activities (Ahmad *et al*., 2003; Pushparaj *et al*. 2007). It has been also reported that *C. intybus* possesses anti-bacterial (Petrovic *et al*. 2004; Nandagopal and Kumari, 2007), anti-inflamatory (Cavin *et al*., 2005; Maniyan *et al*. 2012), hyperglycaemic (Delzenne *et al*. 2005) and anti-ulcerogenic activities (Rifat-uz-Zaman *et al*., 2006). Moreover, *C. intybus* has been found to be a useful biomonitor of heavy metals such as Pb, Zn, Cu, and Cd (Aksoy, 2008).

C. intybus has been used as animal fodder and forage for livestock in many parts of the world. Forage chicory was used to produce a large quantity of high quality feed in the warm season under favourable conditions. Animal performance on chicory is similar to that on legumes and superior to grass-based pastures. It has been reported that grazing chicory results in reduction of some internal parasites in livestock (Tzamaloukas *et al*., 2006; Heckendorn *et al*., 2007) and, therefore, has potential to reduce the use of anthelmintics.

Phytochemicals, the plant-derived non-nutritive compounds, are one of the different types of the dietary factors which play an important role in various functions of the human body. A large number of natural compounds present in food materials have been reported to possess antioxidant properties due to the presence of hydroxyl groups in their chemical structure. The antioxidants are the synthetic as well as naturally occurring compounds that prevent the oxidative damage to the most important macromolecules such as lipids, proteins and nucleic acids present in human body as well as in food materials by scavenging the free radicals produced in various biochemical processes (Shui and Leong, 2004). The free radicals are the reactive oxygen species such as super oxide ($O_2^{-}\cdot$), hydroxyl (·OH) and peroxide (·OOH) radicals which are produced due to oxidative stress. These free radicals react with lipids, proteins and nucleic acids and cause stimulation of apoptosis which leads to various neurological, cardiovascular and some other physiological disorders (Ferrari 2000; Uttara, *et al*. 2009). Bioflavonoids,

phenolic acids, ascorbic acid and tocopherols are well known subclass of phytochemical compounds which possess antioxidant properties and are used for the treatment of cancer (Shahidi, 2000; Bergman *et al*., 2001; Barnes, 2001).

A careful review of literature have shown that little data are available on the phytochemical and antioxidant properties of various parts of *C. intybus* cultivated in Pakistan. Therefore, the present study was planed to investigate the biochemical, photochemical and antioxidant composition of various parts of *C. intybus* cultivated in irrigated regions of Pakistan.

MATERIALS AND METHODS

Sampling: The whole plant of *C. intybus* was collected directly from the field of Bait Hazara, District Dera Ghazi Khan, Punjab, Pakistan and transported to the laboratory. The root, stem and leaves were separated manually with sharp knife, washed with distilled water and dried under shade. The seeds of *C. intybus* were purchased from local market and dried under shade to remove the moisture. All parts of the plant were grinded using electric grinder and stored in air tight jars for further analysis.

Biochemical Analysis: The sugars and free amino acid contents of different parts of *C. intybus* were extracted and estimated by the method as described by Shad *et al*., (2009). The water soluble proteins in different parts of *C. intybus* were extracted by soaking ground plant material (1g) in distilled water for 4 hour. The residue obtained after filtration was soaked in 0.5 M ammonium sulphate solution for the extraction of salt soluble proteins. The water soluble and salt soluble protein fractions were determined by Biuret method (Plummer, 1979).

Phytochemical Analysis: Chemical tests for the screening of certain phytochemical compounds were performed on the aqueous and ethanolic extracts of different parts of *C. intybus* using standard procedures (Horborne, 1973; Trease and Evans, 1989; Sofoware, 1993) as described below:

Tannins: Each sample (0.25 g) was boiled in distilled water (10 ml) and then filtered. Few drops of 0.1% ferric chloride solution were added to the filtrate and the change in colour was observed. The appearance of brownish green or a blue-black colour confirmed the presence of tannins.

Saponins: Each sample (1g) was boiled in distilled water (20 mL) in a water bath and filtered. The filtrate (5ml) was mixed with distilled water (2.5 mL) and shaken vigorously until a stable persistent froth. The frothing was mixed with olive oil (2 drops) and shaken vigorously. The formation of emulsion indicated the presence of saponins.

Flavonoids: Few drops of 1% aluminium solution were added to a portion of ethanolic extract of each sample. A yellow coloration of the solution indicated the presence of flavonoids.

***Terpenoids*:** Ethanolic extract of each sample (5 mL) was mixed in chloroform (2 mL) followed by the careful addition of conc. H_2SO_4 (3 mL) with side wall of the test tube. A reddish brown ring observed at the junction of two layers indicated the presence of terpenoids.

***Cardiac glycosides*:** Ethanolic extract (5 mL) of each sample was treated with glacial acetic acid (2 mL) and aqueous solution of $FeCl_3$ (1 drop) followed by careful addition of conc. H_2SO_4 (1 mL) was added carefully with side wall of the test tube. A brown ring observed at the interface indicated the presence of cardiac glycosides.

Quantitative Analysis of Phytochemicals:

Tannins Content: The tannins content from each part of *C. intybus* were extracted in 20 % aqueous methanol and estimated by the method as described by Fagbemi *et al*. (2005).

Saponins Content: Saponins in different parts of *C. intybus* were determined by the method as described by Anhawange *et al*. (2004). The flour (5 g) was mixed with 75% ethanol (100 mL) and agitated with magnetic stirrer at 55 ˚C on a heating plate for 12 hour. The volume of the resulting ethanolic extract was reduced to about 40 mL under vacuum. The extract was taken in a 250 mL separating funnel and saponins were separated from other organic compounds with diethyl ether (20 mL). The process of purification continued until a transparent aqueous extract was obtained. The pH of the aqueous extract was adjusted to 4.5 by the addition of NaCl (4 g) followed by the successive extraction of saponins with 60 and 30 mL of n-butanol. The butanolic extract was washed twice with NaCl solution (10 mL) and evaporated to dryness. The residue obtained was weighed and saponins were calculated as g/100 g dry weight.

Total Flavonoids Content: The total flavonoids (TF) in different parts of *C. intybus* were extracted by soaking a known weight of each sample (1 g) in aqueous methanol (70% v/v) for 24 hour at room temperature. The TF content in methanolic extract obtained after filteration was estimated by $AlCl_3$ method described by Michalaska *et al*., (2007). An aliquot (1 mL) of methanolic extract was treated with 5% $NaNO_3$ (1 mL). After 6 min 10% $AlCl_3$ (1 mL) was added and volume was made up to 25 ml with 50% ethanol. The solution was allowed to stand at room temperature for 15 min and absorbance was recorded at 510 nm using UV-visible Spectrophotometer (Janeway, 6405). The flavonoids content was calculated as catechin equivalent (g/100 g dry weight) from the calibration curve ($R^2 = 0.9929$).

Total Phenolic Acids Content: Total phenolic acids (TPA) in different parts of *C. intybus* were extracted by soaking each sample (1 g) in 70% (v/v) aqueous methanol (50 mL) for 24 hour at room temperature. TPA content in methanolic extract of each part of *C. intybus* was estimated by the method described by Shad *et al*. (2012a). The methanolic extract (1 mL) was mixed with Folin-Ciocalteu reagent (0.1 mL). After 3 min 2% Na_2CO_3 solution (3 mL) and distilled water (6 mL) were added and mixed well. The reaction mixture was allowed to stand for 30 min and absorbance was measured at 765 nm. TPA content was calculated as mg gallic acid equivalent/100 g extract applying the linear regression equation obtained from a calibration curve ($R^2 = 0.996$).

Antioxidant Analysis

Total Antioxidant Content: The total antioxidants (TAO) content in methanolic extracts of each part of *C. intybus* was determined by method as described by Shad *et al*. (2012a). Methanolic extract (1 ml) was mixed with 40µM methanolic solution (3 mL) of stable 2, 2-dipheny-1-picrylyhydrazyl (DPPH) radical. The solution was allowed to stand for 30 minutes at room temperature and absorbance was recorded at 517 nm. The amount of TAO was calculated as mg Trolox and ascorbic acid equivalent/100 g of extract applying the linear regression equation obtained from a calibration curve ($R^2 = 0.9827$).

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Capacity: The free radical scavenging activity of each sample was determined by following the stable DPPH method as described by Shad *et al*. (2012b). For the estimation of DPPH radical scavenging activity, different concentrations of extracts such as (0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL) were prepared and each sample (1 mL) was mixed with 40µM stable $DPPH^{\cdot}$ solution (3 mL) and the decrease in absorbance was recorded at 517 nm after 30 minutes. The percent inhibition of DPPH radical was calculated as:

$$DPPH \cdot inhibition \quad (\%) = 100 \times Abs_0 - Abs_{30} \div Abs_0$$

where Abs_0 is the absorbance of the control and Abs_{30} is the absorbance of the sample after 30 min. IC_{50} values (mg/mL) were calculated from the linear response curve of percent inhibition of $DPPH^{\cdot}$ at various concentrations of extracts.

Reducing Power: The reducing power of the samples was estimated using the method described by Shad *et al*. (2012b). An aliquot (2.5 mL) of each methanolic extract (10 mg/100 mL) was mixed with 0.2 M phosphate buffer of pH 6.6 (2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50 ºC for 20 minutes followed by the addition of 10% trichloroacetic acid (2.5 mL). The mixture was centrifuged at 3000 × g for 10 min at room temperature. The supernatant was diluted with distilled water (5 mL) followed by the addition of 0.1% ferric chloride (1 mL). Absorbance was measured at 700 nm and the reducing power was expressed in terms of increase in absorbance. Trolox and ascorbic acid (0.01% each) were used as standard antioxidants to compare the results.

Statistical Analysis: The results were expressed as means ± standard deviation of three parallel replicates. The data were statistically analyzed by one way variance analysis (ANOVA) and the means with significant difference at 95% confidence level ($p < 0.05$) were separated in to subsets using Tuky's multiple range test. All the statistical tests were performed on the statistical software (SPSS version 12.0).

RESULTS AND DISCUSSION

Table 1 presents the biochemical composition of different parts *C. intybus*. Total sugars, reducing sugars and non-reducing sugars content ranged from 2.03±0.02 to 4.50±0.37, 0.13±0.02 to 0.44±0.10 and 1.89±0.04 to 4.27±0.37 g/100 g dry weight respectively. The water soluble protein, salt soluble protein and free amino acids content ranged from 5.57±0.58 to14.13±1.50, 6.81±0.51 to 7.94±0.30 and 1.23±0.07 to 8.46±0.24 g/100 g dry weight respectively. A statistically significant difference ($p < 0.05$) was observed among various parts of *C. intybus* regarding sugars free amino acids and water soluble protein contents. Leaves were found to be comparatively high in total sugars and non-reducing sugar content while the seeds were found to be high in reducing sugar content. The leaves were also found to posses higher values of free amino acids and water soluble protein content. The roots were found to contain lower amounts of each of the studied biochemical parameters except that of salt soluble protein content.

The phytochemicl screening of different parts *C. intybus* showed the presence of tannins, saponins, flavonoids, terpenoids, cardiac glycosides and anthocyanins in each part (Table 2). The results of quantitative analysis of phytochemicals are shown in Table 3. Tannins and saponins content of different parts of *C. intybus* ranged from 0.66±0.02 to 1.51±0.03 and 0.16±0.08 to 0.77±0.27 g/100g dry weight (Table 3). The TF and TPA content of different parts of *C. intybus* ranged from 0.05±0.03 to 0.10±0.02 and 0.47±0.07 to 2.52±0.26 g/100g dry weight respectively. A statistically significant difference ($p < 0.05$) was observed in phytochemical content of different parts of *C. intybus*. The roots were found to possess comparatively higher content of tannins but low TPA content. Seeds were found to be high in saponins content while leaves were found to possess comparatively high TF and TPA content.

Tannins, the high molecular weight polyphenolic compounds found naturally in various

plants, have been found to play a protective role in plants against micro-organisms, insects, unfavourable climatic conditions and damage by animals. On the other hand, tannins can form multiple hydrogen bonds with carboxylic groups of dietary proteins and proteolytic enzymes in the gastrointestinal tract which leads to reduced digestibility of proteins and finally the retardation of animal growth (Reed, 1995). Tannins present in *C. intybus* have been reported to enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes (Muthusamy *et al*., 2008). The tannins content in all parts of *C. intybus* was found to be higher than the range (41-166 mg/100g) reported by Kumari, *et al*. (2004) in leafy vegetables.

Saponins are the glycosidic compounds found in most of the plants, possess a bitter taste and foaming properties. Saponins have been found to possess anti-carcinogenic and antifungal activity (Rao and Sung, 1995). On the other hand, these exert hemolytic effect on red blood cells and cause inflammation and paralysis of gastro-intestinal tract (Osagie and Eka, 1998). In previous studies on mice, the median lethal dose (LD_{50}) of saponins has been reported to be 200 mg/Kg body weight (Diwan *et al*., 2000).

Polyphenolic compounds have attained great attraction from investigators because of their antiviral, antifungal, antibiotic, antitumor, anti inflammatory, antimutagenic and antioxidant activities (Wang *et al*., 2003; Innocenti *et al*., 2005; Mares *et al*., 2005; Milala *et al.,* 2009). The flavonoids and phenolic acids are known to possess antioxidant activities due to the presence of hydroxyl groups in their structures and their contribution to defence system against the oxidative damage due to endogenous free radicals is extremely important (Miranda and Buhler, 2002; Boskou, 2006).

The Trolox equivalent and ascorbic acid equivalent total antioxidant (TAO) content of different parts of *C. intybus* ranged from 48.33±0.58 to 56.87±1.03 and 64.78±4.11 to 72.37±7.26 mg/100 g dry weight respectively (Fig. 1). The statistical analysis of the results showed a significant difference ($p < 0.05$) among different parts of *C. intybus* regarding the TAO content. Leaves were found to possess comparatively high TAO content.

The ferric ion reducing powers of methanolic extracts of different parts of *C. intybus* are shown in Fig. 2. The statistical analysis of results showed a significant difference ($p < 0.05$) in the reducing abilities of different parts of *C. intybus.* Leaves were found to possess comparatively higher value of reducing power ability. However, the reducing powers of all parts of *C. intybus* were found to be low as compared to those of Trolox and ascorbic acid taken as standard antioxidants.

The DPPH· scavenging capacities of methanolic extracts of different parts of *C. intybus* in terms of percent inhibition of DPPH· are shown in Fig. 3A and 3B. IC_{50} value is the inhibitory concentration of the antioxidant at which 50% inhibition of DPPH radical occurs. A statistically significant difference ($p < 0.05$) was observed in the DPPH radical scavenging capacities of different parts of *C. intybus*. Leaves were found to possess comparatively good free radical scavenging capacity due to higher DPPH radical inhibition and lower IC_{50} value. However, all parts of *C. intybus* showed lower percentage of DPPH radical inhibition and higher IC_{50} values as compared to those of Trolox and ascorbic acid taken as standard antioxidants.

The oxidative stress caused by free radicals is delayed or even prevented by a special class of substances called as antioxidants. These compounds possess hydrogen donating abilities and thus exert their antioxidant effect by breaking the free radical chain (Ferrari 2000; Barnes, 2001; Lobo *et al*., 2010). DPPH radical is frequently used free radical to determine the antioxidant ability of some natural compounds. Due to their hydrogen donating abilities, the antioxidant compounds can inhibit the DPPH radical. The DPPH radical scavenging capacity of the antioxidant compounds may be measured as the degree of discolouration of DPPH· solution after inhibition by these compounds.

C. intybus has been found to have great medicinal importance due to the presence of phenolic compounds. The results show that all of the studied parts of *C. intybus* are good source of phenolic compounds. Due to high TF and TPA content, the leaves have been found to possess comparatively good reducing power and DPPH radical scavenging capacity. The root has been found to show comparatively low antioxidant activities due to low content of TF and TPA.

Table 1. Biochemical composition (g/100g dry weight) of different parts of *C. Intybus*.

	Sugars			Proteins		Free Amino Acids
	Total	Reducing	Non-Reducing	Water Soluble	Salt Soluble	
Root	2.03±0.02^{c*}	0.13±0.02^{b}	1.89±0.04^{c}	5.57±0.58^{b}	7.94±0.30^{a}	1.23±0.07^{d}
Stem	2.12±0.10^{c}	0.24±0.10^{b}	2.06±0.11^{c}	9.43±1.77^{b}	6.85±0.38^{a}	5.98±0.31^{b}
Leaves	4.50±0.37^{a}	0.23±0.01^{b}	4.27±0.37^{a}	14.13±1.50^{a}	6.91±0.53^{a}	8.46±0.24^{a}
Seed	3.05±0.06^{b}	0.44±0.10^{a}	2.61±0.06^{b}	8.35±1.82^{b}	6.81±0.51^{a}	2.03±0.05^{c}

*Means ± SD of (three parallel replicates) followed by different letters in each column are significantly different at confidence level $p \leq 0.05$ using Tuky's Multiple range test.

In conclusion, all parts of *C. intybus* contain considerable amounts of phytochemicals and are good source of antioxidants. However, methanolic extracts of *C. intybus* leaves possess comparatively higher amounts of TF, AA, TPA and TAO and also show high reducing power and free radical scavenging capacity. It may be suggested that *C. intybus* leaves, due to their good phytochemical and antioxidant composition, would play an important role in antioxidant defence system against endogenous free radicals and thus improving the human health.

Table 2. Screening of some phytochemicals in different parts of *C. intybus*

	Tannins	Saponins	Flavonoids	Terpenoids	Cardiac glycosides	Anthocyanins
Root	+	+	+	+	+	+
Stem	+	+	+	+	+	+
Leaves	+	+	+	+	+	+
Seed	+	+	+	+	+	+

Table 3. Phytochemical composition (g/100 g dry weight) of different parts of *C. Intybus*

	Tannins	**Saponins**	**Total Flavonoids**	**Total Phenolic Acids**
Root	1.51±0.03[a*]	0.59±0.06[ab]	0.05±0.03[a]	0.47±0.07[b]
Stem	1.32±0.04[b]	0.26±0.01[bc]	0.08±0.03[a]	2.09±0.21[a]
Leaves	0.66±0.02[d]	0.16±0.08[c]	0.10±0.02[a]	2.52±0.26[a]
Seed	0.89±0.02[c]	0.77±0.27[a]	0.06±0.01[a]	0.51±0.12[b]

*Means ± SD (three parallel replicates) followed by different letters in each column are significantly different at confidence level $p \leq 0.05$ using Tuky's Multiple range test.

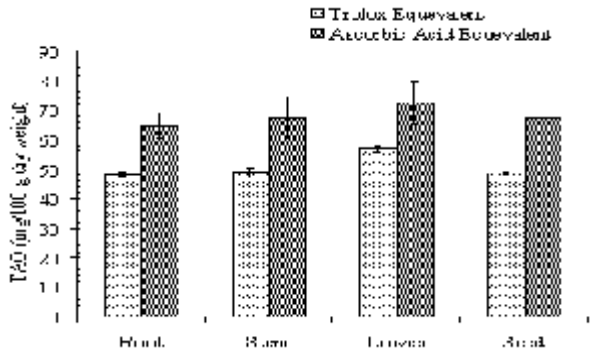

Fig. 1. Total antioxidant content (ascorbic acid as well as Trolox equivalent) of methanolic extracts of different parts of *C. intybus*

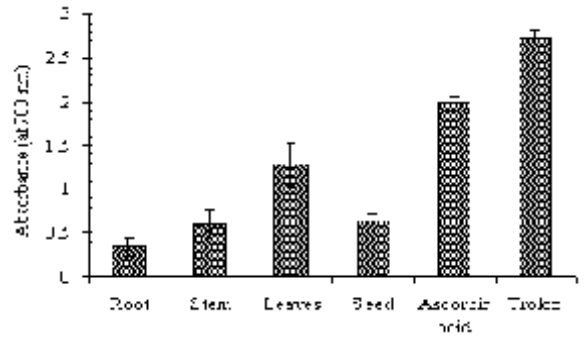

Fig. 2. Reducing power (absorbance at 700 nm) of methanolic extracts of different parts of *C. intybus* as compared to that of ascorbic acid and Trolox taken as standard antioxidants

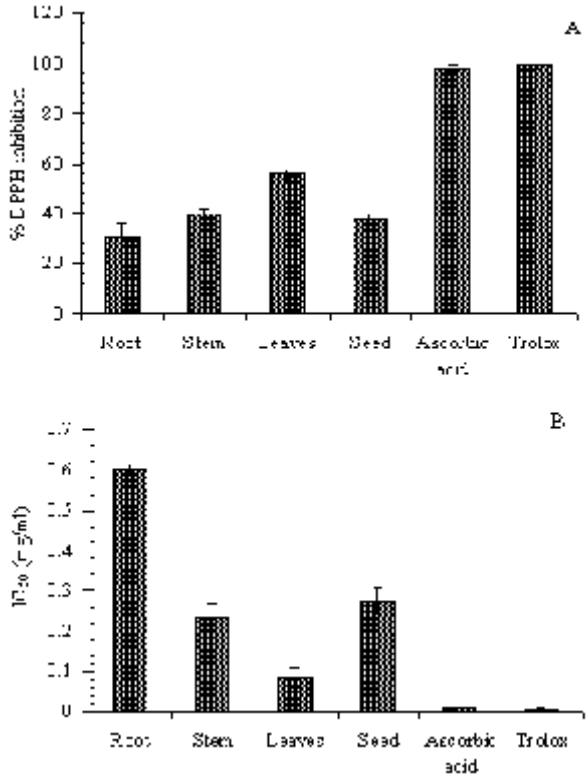

Fig. 3. (A) Free radical scavenging capacity (B) IC_{50} values of methanolic extracts of different parts of *C. intybus* as compared to those of ascorbic acid and Trolox taken as standard antioxidants

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