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Original Article

Antioxidant, Antimicrobial and Phytochemical Analysis of *Cichorium intybus* Seeds Extract and Various Organic Fractions

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Abstract

This study was carried out to evaluate the antimicrobial and antioxidant effectiveness of methanolic extract and different fractions (*n*-butanol, ethyl acetate, chloroform and *n*-hexane) of *C.intybus* seeds. The antimicrobial activity was determined by the disc diffusion method and minimum inhibitory concentration (MIC) against a panel of microorganisms (four bacterial strains, *i.e. P. multocida, E. coli, B. subtilis* and *S. aureus* and three fungal strains, *i.e* A. *flavus, A. niger and R. solani)*. The results indicated that seeds extract and fractions of *C. intybus* showed moderate activity as antibacterial agent. While Antifungal activity of *C. intybus* seeds extract/fractions was very low against A. *flavus* and *A. niger* while mild against *R. solani.* The *C.intybus* seeds extract/fractions contained appreciable levels of total phenolic contents (50.8-285 GAE mg/100g of Dry plant matter) and total flavonoid contents (43.3-150 CE mg/100g of Dry plant matter). The *C. intybus* seed extract/fractions also exhibited good DPPH radical scavenging activity, with IC_{50} ranging from 21.28-72.14 µg/mL. Of the *C .intybus* seeds solvent extract/fractions tested, 100% methanolic extract and ethylacetate fraction exhibited the maximum antioxidant activity. The results of the present investigation demonstrated significant ($p < 0.01$) variations in the antioxidant and antimicrobial activities of *C. intybus* seeds solvent extract/fractions.

Keywords: *C.intybus;* Antimicrobial activity; Resazurin; *A. flavus;* Minimum inhibitory concentration.

Introduction

The medicinal plants have immensely contributed to health needs of humans throughout their existence. Microorganisms cause a number of deleterious diseases in man, animal and plants. Synthetic drugs and antibiotics do not completely cure these diseases because the microorganisms develop resistance against these compounds. Therefore, work is going on the extraction of anti-infectional compounds including bioactive from natural sources like plants and animals. The antimicrobial compounds show broad-spectrum bioactivity against infection causing agents such as fungi, bacteria, protozoon, viruses and yeasts (1).

The preservative effect of many plants and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues.

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Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity.

Therefore, it is of great interest to carry out a screening of the plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterisation of their constituents. The experimental plant Chicory (*Cichorium intybus* L.) belongs to family Asteraceae and it is a small aromatic biennial or perennial herb. It grows as a wild plant on roadsides in its native Europe, and in North America and Australia, where it has become naturalized. It is variously used as a tonic and appetite stimulant, and as a treatment for gallstones, gastro-enteritis, sinus problems cuts and brusises. Chicory is well known for its toxicity to internal parasites.

Heimler *et al*. (2) reported the conventionally and biodynamically grown chicory (*Cichorium intybus)* for its polyphenol content and antiradical activity. HPLC/DAD/Ms analysis identified five hydroxycinnamic acids and eight flavonoids (quercetin, kaempferol, luteolin and apigenin glycosides) *in c.intybus* plant. Norbeak *et al*. (3) reported the anthocyanins from flowers of *Cichorium intybus*.

Experimental

Plant material

Seeds of the selected medicinal plant *Cichorium intybus* were purchased from the local market of Faisalabad and identified from the Department of botany University of Agricultural Faisalabad, Pakistan.

Preparation of extract and fractions

Three Kg seeds of *C. intybus* were powdered and stored in the clean container. In the weighed amount of powdered seeds the measured amount of 100% methanol (2 × 15 L) was added and kept for 4-5 days at room temperature. The solvent was then removed using rotary evaporator. Extract (300 g) became viscous, was dried on water bath and then stored at – 4 ^{0}C. The process was repeated three times with intervals of four days. The methanolic extract was dissolved in distilled water and fractionation was done using different polarity based solvents and obtained successively *n*-hexane (125 g), chloroform (60 g) ethylacetate (55 g), and *n*-butanol (40 g) fractions.

Phytochemical analysis

Phytochemical screening of the extracts was carried out according to the methods described by Trease and Evans (4) for the detection of active components like saponins, tannins, alkaloids, steroids, flavonoids, Anthraquinones.

Antimicrobial assay of plant extracts and different fractions

Microbial strains

The methanolic extract and its different fractions were individually tested against a panel of microorganisms, including four bacteria, *Escherichia coli, Bacillus subtilis*, *Staphylococcus aureus* and *Pasturella multocida* and three pathogenic fungi, *Aspergillus niger, Aspergillus flavus* and *Rhizopus solani.* The pure bacterial and fungal strains were obtained from the Biological Division of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The purity and identity of the strains were verified by the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28°C using potato dextrose agar (Oxoid).

Disc diffusion method

The antimicrobial activity of the *C. intybus* seeds extract and its different fractions was determined by the disc diffusion method (5). The discs (6 mm in diameter) were impregnated with 10 mg/mL extract/fractions (100 µL/disc) placed on the inoculated agar. Extract/fractions were dissolved in 10% sterile dimethyl sulfoxide. Rifampcin (100 µL/disc) (Oxoid) and Fluconazole (100 µL/disc) (Oxoid) were used as positive control for bacteria and fungi, respectively. Disc without samples was used as a negative control. Antimicrobial activity was evaluated by measuring the inhibition zone.

Resazurin microtitre-plate assay

The minimum inhibitory concentration (MIC) of the extract and fractions was evaluated by a modified resazurin microtitre-plate assay as reported by Sarker *et al*. (6) with modification. Briefly, a volume of 100 µL of extracts/fractions solutions in 10% dimethyl sulfoxide (DMSO, v/v) was transferred into the first row of the 96 well plates. To all other wells, 50 µL of nutrient broth and muller hinton broth for bacteria and fungi respectively were added. Two-fold serial dilutions were performed using a multichannel pipette such that each well had 50 µL of the test material in serially descending concentrations. To each well 10 µL of resazurin indicator solution (prepared by dissolving 270 mg resazurin tablet in 40 mL of sterile distilled water) were added. Finally, 10 µL of bacterial/fungal suspension were added to each well. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each plate had a set of controls: a column with a broad spectrum antibiotics as positive control, a column with all solutions with the exception of the test samples, a column with all solutions with the exception of the bacterial/fungal solution adding 10 µL of broths instead and a column with 10% DMSO (v/v) solution as a negative control. The plates were prepared in triplicate, and incubated at 37°C for 24 h and 28°C for 48 h for bacteria and fungi respectively. The absorbance was measured at 620 nm by micro quant for fungus and at 500 nm for bacteria. The color change was then assessed visually. The growth was indicated by color changes from purple to pink or colorless. The lowest concentration at which color change appeared was taken as the MIC value.

Antioxidant activity

Determination of total phenolic contents (TPC)

Amount of Total phenolic contents were determined using Folin Ciocalteu reagent process (7). 1 milligram of extract was dissolved with 0.5 ml of 50 time diluted folin reagent and 7.5 mL of deionized water. Mixture was placed at room temperature for 10 min. 1.5 mL of 10% Na_2CO_3 (w/v) was added to the mixture. Heated the mixture on water bath for 20 min at 40°C. It was cooled and the absorbance was noted at 755 nm. Amounts of total phenolics were calculated using calibration curve for gallic acid (10-100 ppm). The consequences were expressed as gallic acid equivalent per dry matter. Sample was analyzed thrice and results averaged.

Determination of total flavonoid contents (TFC)

Spectrophotometric method following a previously reported method was used to measure TFC (8). In a few words, plant extract of each material (1mL containing 0.1 mg / mL) was placed in a 10 mL volumetric flask, then added distilled water 5 ml and 0.3 mL of 5% $NaNO_2$ was added to each volumetric flask initially; after 5 min., 0.6 mL of 10% $AlCl_3$ was added. After another 5 min, 2 mL of 1 M NaOH was added and volume made up with distilled water. Then solution was mixed. At 510 nm absorbance of the reaction mixture was taken using a spectrophotometer. TFC were evaluated as catechin equivalents (g/100 g of dry plant matter). Three readings were taken for each sample and results were averaged.

DPPH radical scavenging assay

The antioxidant activity of the samples was assessed through their ability of scavenging 2, 2_-diphenyl-1-picrylhydrazyl stable radicals (DPPH). The DPPH assay was performed as described by Mimica-Dukic *et al.* (9). The samples (from 10 to 500 µg mL-1) were mixed with 1 mL of 90 µM DPPH solution and made up with 95% methanol, to a final volume of 4 mL. Synthetic antioxidant, BHT was used as control. After 1 h incubation period at room temperature, the absorbance was recorded at 515 nm. Percent radical scavenging concentration was calculated using the following formula:

$$\text{Radical Scavenging (\%)} = 100 \times (\text{Ablank} - \text{Asample} / \text{Ablank})$$

Here, Ablank is the absorbance of the control (containing all reagents except the test essential oil/compounds), and Asample is the absorbance of the test samples. Extract/fractions concentration providing 50% inhibition (IC_{50}) was calculated from a graph plotting percentage inhibition against extract/fraction concentration.

Table 1. Phytochemical constituents of *C. intybus* seeds in methanol and water extracts.

Phytochemicals	Inference	
	Extracts of *Cichorium intybus* seeds	
	Methanolic extract	*Water extract*
Alkaloids	+	+
Flavonoids	+	+
Tannins	+	+
Steroids	+	+
Saponins	+	+
Anthraquinones	+	+

Key = +, Present

Statistical analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of difference $p \leq 0.01$ was considered to denote a statistically significance All data were presented as mean values ± standard deviation (SD).

Results and Discussion

Phytochemical analysis

Phytochemical components such as alkaloids, flavonoids, saponins, tannins, steroids, anthraquinone were determined in *Cichorium intybus* seeds and results are presented in Table 1. Phytochemical analysis revealed that important chemicals like alkaloids, flavonoids, tannins, saponins, anthraquinons, steroids and terpenoids were present in *Cichorium intybus* seeds. The presence of flavonoids and Tannins in *Cichorium intybus* seeds extract are in agreement with previously reported results of Nandagopal and Ranjitha (10) and Muthusamy *et al*. (11).

Antimicrobial activity of Cichorium intybus seeds

The antimicrobial activity of the methanolic extract and fractions from *C. intybus* seeds against a panel of food-borne and pathogenic microorganisms were assessed. The results are presented in Table 2. The extract and fractions exhibited considerable antimicrobial activity against all the strains tested. The results from the disc diffusion method, followed by measurement of minimum inhibitory concentration (MIC), indicated that 100% methanolic extract showed good activity against *E. coli* and *P. multocida ,* showing the inhibition zones (18.7 and 15.5 mm) and the lowest MIC values (80.4 and 140 mg/mL), respectively. Least activity was exhibited against *A. niger* and *B. subtilis*, with the smallest inhibition zones (12.3 and 12 mm) and the highest MIC values (182 and 198 mg/mL). Ethylacetate fraction showed strong activity against *E. coli* and *S. aureus* with inhibition zones (22.5 and 21 mm) and the lowest MIC values (50.3 and 60.2 mg/mL), respectively. Least activity was exihibited against *A. flavus* and *A. niger* with inhibition zones (12.5 and 11.5 mm) and the highest MIC values (180 and 205 mg/mL), respectively. Chloroform fraction showed good activity against *S.aureus* and *E. coli,* showing the inhibition zones (17 and 16.5 mm) and the lowest MIC values (110 and 125 mg/mL), respectively. Least activity was exhibited against *B. subtilis* and *P. multocida*, with the smallest inhibition zones (12 and 10 mm) and the highest MIC values (198 and 215 mg/mL). *n*-butanol fraction showed good activity against *P. multocida* and *S.* aureus*,* showing the inhibition zones (21.5 and 18 mm) and the lowest MIC values (60.1 and 90.2 mg/mL), respectively. Least activity was exhibited against *A. flavus* and *A. niger*, with the smallest inhibition zones (13 and 11 mm) and the highest MIC values (178 and 210 mg/mL). *n*-hexane fraction showed good activity against *S. aureus* and *E.coli,* showing the inhibition zones (18 and 15.2 mm) and the lowest MIC values (90.2 and 140 mg/mL), respectively. Least activity was exhibited against *A. flavus* and *B. subtilis*, with the smallest inhibition zones (12

Table 2. Antimicrobial activity in terms of inhibition zones and minimum inhibitory concentration of methanolic extract and different fractions of *C. intybus* seeds against selected bacterial and fungal strains[a].

Tested Microorganisms	Methanolic extract and different fractions						
	Methanol 100%	**Ethylacetate**	**Chloroform**	***n*-butanol**	***n*-hexane**	**Fluconazole**	
Diameter of inhibition zone[b]							
Bacillus subtilis	12.0±1.63[a]	17.2±2.21[d]	12±1.41[ab`]	15.5±1.25[c]	10±1.47[b]	18.50±1.73[a]	------
Pasturella. multocida	15.5±1.25[a]	17.7±2.09[d]	10.0±1.47[ab]	21.5±1.91[c]	13.5±2.06[b]	27.5±1.65[c]	------
Staphylococcus aureus	14.5±1.00[b]	21.0±2.58[c]	17.0±1.58[b]	18.0±1.63[a]	18.0±1.82[c]	23.4±0.866[a]	------
Escherichia coli	18.7±0.21[a]	22.5±0.41[e]	16.5±0.25[b]	16.0±1.41[c]	15.2±1.40[e]	25.5±1.65[c]	------
Aspergillus niger	12.3±2.21[cd]	11.5±1.00[a]	11.5±1.26[de]	11.0±0.81[e]	13.0±0.81[b]	------	14.4±0.32[c]
Aspergillus flavus	12.5±1.29[c]	12.5±1.73[b]	12.5±0.97[d]	13.0±1.41[ab]	12.0±0.81[c]	------	24.8±0.76[a]
Rhizopus solani	13.0±1.82[b]	14.2±1.25[b]	12.5±1.25[c]	14.7±1.89[c]	[a] 12.5±0.95	------	18.7±0.98[c]
Minimum inhibitory concentration (MIC)[c]							
Bacillus subtilis	198±1.32	110±7.34	198±6.30	140±5.52	5.11 215±	80.4±6.68	------
Pasturella. multocida	140±1.36	90.2±4.10	215±7.26	60.1±3.71	178±8.81	8.32±3.77	------
Staphylococcus aureus	160±5.50	60.2±2.65	110±1.74	90.2±1.82	90.2±9.30	41.2±3.83	------
Escherichia coli	80.4±1.26	50.3±2.14	125±7.70	125±1.31	140±8.16	23.5±1.0	------
Aspergillus niger	182±1.38	205±7.42	205±0.90	210±4.75	178±1.29	------	160.2±6.71
Aspergillus flavus `	180±1.80	180±7.42	180±0.90	178±4.75	198±1.28	------	33.4±2.52
Rhizopus solani	178±4.08	160±3.83	180±3.81	140±2.52	180±8.52	------	80.4±1.74

[a]Values are mean ± SD of three separate experiments.
[b]Diameter of inhibition zone (mm) including disc diameter of 6 mm.
[c]Minimum inhibitory concentration, MIC (mg/mL).
Letters in superscript show the significance of the results against single strain.

and 10 mm) and the highest MIC values (198 and 215 mg/mL). In general, the antimicrobial activity of the tested extracts and fractions is comparable with the standard drugs, rifampcin and fluconazole. The results indicated that plant extract and fractions showed poor antifungal activity against *A. niger and A. flavus*. Mild antifungal activity was shown against *R. solani.*

Petrovic *et al*. (12) reported the antibacterial activity of root extracts of *C.intybus* against *E. coli, S. aureus, B. subtilis and P. multocida* strains where all the tested extracts showed antibacterial activity, the ethyl acetate extract being the most active one. These results are in agreement with our analysis where ethylacetate fraction of *C. intybus* seeds was being most potent against *E. coli* and *S. aureus*. Overall *C.intybus* seeds showed mild antibacterial and poor antifungal activity. Nandagopal and Ranjitha (10*)* reported the antimicrobial activity of *Cichorium Intybus* and experimentally prove that the whole plant contains a number of medicinally important compounds such as inulin, esculin, volatile compounds (monoterpenes and sesquiterpenes), coumarins, flavonoids and vitamins. Root extracts showed more inhibitory action on *Bacillus subtilis, Staphylococcus aureus* and S*almonella typhi* than *Micrococcus luteus* and *Escherichia coli*. Some of the organic compounds were detected in the extracts and fractions of *C.intybus* seeds include alkaloids, flavonoids, saponins, tannins, steroids, anthraquinone (Table 1). These compounds have previously been reported to have antimicrobial activities recorded against these organisms. Plant chemicals are thought to have the potentiality of useful drugs if properly harnessed (13-15). The *C.intybus* seeds extract/ fractions were found to be poor against fungal strains which mean they may not be useful for treatment of fungal infections. The cell wall components of bacteria are quite different from those of fungi. While the cell wall of bacteria are either made up of acetyl muramic acid (AMA) or acetyl glucose amine (AGA) fungal cell wall is made up of fungal cellulose. This may explain the reason for the differences in their

Table 3. Antioxidant activities of *C. intybus* seeds methanolic extract and various fractions[a].

Antioxidant assays	Methanolic extract and various fractions					
	Methanol	**Chloroform**	**Ethylacetate**	***n*-butanol**	***n*-hexane**	**BHT**
Total phenolic contents[b] (mg/100 g of Dry plant matter)	285 ± 4.92	170 ± 5.91	210 ± 4.15	120±3.21	50.8 ± 2.44	—
Total flavonoid contents[c] (mg/100 g of Dry plant matter)	150 ± 2.81	113 ± 3.61	132 ± 4.37	90±3.57	43.3 ± 1.84	—
DPPH, IC_{50} (µg/mL)	21.28 ± 1.65	38.25 ± 1.69	29.42 ± 2.55	50.21 ± 1.81	72.14 ± 2.42	19.5 ± 0.95

[a]Values are mean ± SD of three separate experiments.
[b]Total phenolic contents expressed as gallic acid equivalent.
[c]Total flavonoid contents expressed as catechin equivalent.

susceptibility to the plant extract/fractions in this experiment. Therefore we would like to state that constituents of this plant seeds may serve as a source of drugs useful in treatment of bacterial infections.

Antioxidant activity

The total phenolic contents (TPC) and total flavonoid contents (TFC) of *C. intybus* seed extract/fractions are presented in Table 3. The amounts of TPC and TFC extracted from *C. intybus* seeds in different solvent systems were in the ranges 50.8-285 GAE (mg/100 g of Dry plant matter) and 43.3-150 CE (mg/100 g of Dry plant matter), respectively. Generally the significant difference ($p < 0.01$) was observed among different solvent systems and fractions. 100% Methanolic extract of the *C. intybus* seeds showed the highest TPC and TFC, 285 and 150 mg/100 g, respectively. These differences in the amount of TPC and TFC may be due to varied efficiency of the extracting solvents to dissolve endogenous compounds. The ability of different solvents to extract TPC and TFC was of the order: 100% methanol > ethylacetate > chloroform > *n*-butanol > *n*-hexane. Methanol is efficient and the most widely used to extract antioxidative components including phenolic acids and other phenolic components (16). Although, chloroform and ethyl acetate also extracted reasonable amounts of TPC and TFC, however, due to comparatively lower their polarity, were less effective. Conversely, *n*-hexane being non-polar in nature was the least effective for the extraction of phenolics. Akroum *et al*. (17) reported different phytochemicals in *C. intybus* as sterols, phenolic acids and volatile oils, so it's concluded that antioxidant activity of this plant may be due to these constituents which are also present in *C. intybus* seeds extracts as shown in Table 1.

As the concentration of phenolic compounds or degree of hydroxylation of the phenolic compounds increases, DPPH scavenging activity increases, hence does the antioxidant activity. *C. intybus* seeds extracts and fractions showed excellent radical scavenging activity, with IC_{50} (the extract concentration providing 50% of inhibition) values of 21.28–72.14 µg/mL. When compared with the synthetic antioxidant BHT (IC_{50}=19.5 µg/mL), both extracts and fractions offered slightly lower antioxidant activity. *n*-hexane fraction showed maximum IC_{50} value and its antioxidant was lowered as compared to other extract and fractions. Akroum *et al*. (17) reported the IC_{50} 15 > 1000 of *C.intybus* leaves methanolic extract. These results are in accord with our results where IC_{50} also in range of these results.

Conclusion

The methanolic extract and ethylacetate fraction of seeds exhibited good antioxidant activity. The various fractions of *C. intybus* showed moderate activity as antibacterial agent while antifungal activity of *C. intybus* extract/ fractions was very low against A. *flavus* and *A. niger* while mild against *R. solani*. A compaeison among the results leads us to the conclusion that constituents of this plant extract may serve as a source of drugs useful in the chemotherapy of some infections caused by bacteria and also as an antioxidant agent.

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