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European Journal of Experimental Biology, 2015, 5(7):26-35

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Pharmacological and molecular activity of methanolic extract of *Cichorium intybus* Linn seeds

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ABSTRACT

The oxidative damage of cellular tissue by reacting active species causes various human diseases like cardiovascular disease, cancer, aging and nephropathy. The antioxidant supplements obtained from plants are useful to cure the oxidative damage in cells. Recently, attention has focused on phytochemicals as new sources of natural antioxidant agent. The plant Cichorium intybus L., commonly denoted as chicory which belongs to family Asterac eae and geographically found in Asia and Europe. Chicory have medicinally active constituent like flavonoids, saponins tannins, alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments and unsaturated sterols. Hence, in the present study methanolic extract of Cichorium intybus Linn seeds was evaluated for antioxidant invitro method and molecular activity using RAPD electrophoresis methods

Keywords: *Cichorium intybus*, Antioxidant, DPPH.RAPD

INTRODUCTION

In present scenario the use of herbs and botanicals for the health and wealthiness increasing day by day because they offer a natural safeguard against variety of diseases. The exposure of drugs and chemicals to living organism causes toxicity. But mostly compounds are not toxic to all parts of a living organism because toxic actions of these compound are manifested in particular target organs. This concept was used for the assessment of toxic compound against their specific particular organ. Many factors determine the susceptibility of a particular organ to toxicity. These include the pharmacokinetics and metabolic fate of the compound, and the ability of target organ to respond to the toxicants. Many organs, like kidney, can metabolize chemicals to their toxic reactive intermediates. Which initiate the toxicity by binding to cellular macromolecule and produce reactive oxygen species, which causes oxidative damage of cellular structures such as membranes or nucleic acid (Godfraind 2006). Thus, it is mandatory that the human being moves towards traditional and alternative source of medicine for the health and wealthiness of human for. The plant *Cichorium intybus* L, commonly denoted as chicory which belongs to family Asteraceae and geographically found in Asia and Europe . Chicory have medicinally active substance like flavonoids,

saponins tannins alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments and unsaturated sterols.(Molan et al., 2003; Nandagopal and Kumari, 2007; Muthusamy et al., 2008; Atta et al., 2010). It has been reported that fresh leaves and seeds of chicory are contain phenols, vitamins A, C, calcium, phosphorus and potassium (Mulabagal et al., 2009). .chicory stimulates the immunity and prevents inflammation and bacterial diseases due to presence of cichoric acid (Nayeemunnisa, 2009).

Chicory has been traditionally used for the treatment of jaundice and gallstones fever, diarrhoea, (Afzal et al.,2009; Abbasi, 2009). The studies on animal have shown that chicory contain hepatoprotective and anti-diabetic activities (Saggu et al., 2014). Hence, the present research was an endeavor to evaluate anti oxidant and molecular activity of chicory.

MATERIALS AND METHODS

Plant material:

Collection and authentication of chicory seeds

The Seeds of chicory were collected from local market of Delhi and authenticated, NISCAIR New Delhi. The voucher specimen was deposited in Pharmacognosy laboratory of Vivek College of Technical Education, Bijnor.

Preparation of chicory extract:

The Seeds of chicory were dried at room temperature in shade and grounded in a mortar. The chicory powdered was extract with methanol in soxhlet apparatus

Anti oxidant activity of chicory (Invitro method)

Evaluation of antioxidant activity:

Antioxidant activity of methanolic extract of chicory was carried out using following *invitro* methods (Ali *et al*., 2008).

1, 1-Diphenyl-2-picrylhydrazyl free radical scavenging assay (DPPH) Method:

Chicory extract of different concentrations ranging from(20- 100 µg/ml) was added to the methanolic solution of DPPH and shaken. The absorbance of these solution was determined at 520 nm, and the radical scavenging activity of chicory methanolic extract was determined using following equation:

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control –OD sample) / OD control}}{\text{OD control}} \text{ X } 100$$

Hydrogen per oxide scavenging method

Hydrogen per oxide scavenging of chicory was determined by Ruch *et al*., 1989. Method. In these method aqueous solution of *chicory* in different concentration (5 µg/ml – 25µg/ml) was mixed with 2 mM, 0.6 ml solution of hydrogen peroxide in phosphate buffer . After 20 min the absorbance of hydrogen peroxide presence in these was determined at 230 nm and the percentage inhibition of hydrogen per oxide in *Cichorium intybus* and ascorbic acid were calculated using the following equation:

$$\text{\% Inhibition} = \frac{A0-A1}{A0} \text{ X } 100$$

Where A_0 denoted the absorbance of the control, and A_1 denoted the absorbance of chicory extracts or ascorbic acid.

Reducing Power method

The reducing power of *Cichory* were determined by using (Oyaizu, 1986) methods. In these method The aqueous solution of *Chicory* extracts in different concentration (10 µg/ml – 50 µg/ml) were added in 2.5 ml, 0.2 M phosphate buffer and 2.5 ml, 1 % solution of potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 min. After incubation 2.5 ml TCA (10%) was added to the reaction mixture and centrifuged for 10 min. The 2.5 ml of supernatant was added to 2.5 ml of distilled water and 0.5 ml ferric chloride (1%). The absorbance of these mixture was scaned using UV spectrophotometer at 700 nm (Schimadzu UV-Vis 1601).

Molecular marker analysis

Plant Material

The *Cichorium intybus* varieties was used in this research. The sample of *Cichorium intybus* varieties were collected from various places in the state of Uttar Pradesh. One gram of young leaves was harvested fresh for DNA isolation.

Reagents/Chemicals used

An extraction buffer consisting of 3 % CTAB (w/v), NaCl (2 M), Tris HCl pH 8.0 (100 mM) and EDTA pH 8.0 (20 mM), PVP (1.5 %), - mercaptoethanol 2.5 % (V/v) was prepared. Ribonuclease A (10 mg/ml), chloroform: Isoamylalcohol (24:1) v/v/v), Ethanol (70 %,100 %), Sodium acetate (3M) solution (pH 8.0), and TE buffer (Tris HCl, 10 Mm,pH 8.0 , 1mM EDTA; pH 8.0) were the additional solutions required.

Protocol for isolation of DNA:

Plant material was surface sterilized with sterile distilled water followed by 80% ethanol. The sample was cut into pieces of approximately 1 mm size with the sterile blade. The pre-chilled mortar and pestle was used to ground material in the presence of liquid nitrogen. The frozen powder was transferred in 8 ml of the extraction buffer (pH 8) into a 30 ml centrifuge tube. The extraction buffer and frozen powder was mixed well and incubated at temperature 65°C for 30 min. After incubation the mixture was cooled at room temperature and thereafter, equal volume of the mixture of chloroform: isoamylalcohol (24:1) was added. The mixture was centrifuged at 5000 rpm for 10 min at 4°C. The aqueous phase was transferred to a fresh tube and the DNA was precipitated by adding 0.6 volumes of ice-cold isopropanol and stored at –20°C for 30 min. Precipitated DNA was centrifuged at 9000 rpm for 10 min at 4°C. Supernatant was decanted carefully and pellet was washed with 80% ethanol. The pellet was dried at 37°C for 12 min and was dissolved in 400 ml of 1x TE. The crude DNA sample was treated with 1 ml RNase (10 mg/ml stock) for 10 min at 37°C and equal volume of the mixture of chloroform: isoamylalcohol (24:1) was added. After that it centrifuged at 8000 rpm for 10 min at 4°C. The aqueous phase was taken and 0.6 volumes of isopropanol was added. It was then kept at -20°C for 10 min. The mixture was centrifuged at 8000 rpm for 5 min at 4°C and supernatant decanted carefully. The pellet was washed with 80% ethanol twice and dried at 37°C for 10 min. Finally DNA pellet was dissolved in 50 ml of 1x TE buffer.

Quantification and restriction of DNA:

The DNA yields of the samples shall be determined using a UV-VIS spectrophotometer at 260 and 280 nm.The purity of DNA was determined by estimating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8 %. Agarose gel depending on the intensities of band when compared with lambda DNA marker (used to determine the concentration).

Optimization of RAPD and PCR reaction

For the optimization of RAPD reaction using DNA extracted from different *Cichorium intybus* varieties, Oligonucleotide primers from RPI-C series (Bangalore Genei, Bangalore ,India) and also OPC series (Operon, Technologies Inc. Almeda CA, USA) were used for amplification to standardize the PCR conditions. The reactions were carried out in a DNA thermocycler (Bio Rad). Reactions without DNA were used as negative controls. Each 25µl reaction volume contained about 1 x PCR Buffer (10mM Tris-HCL pH 8.3;50mM KCL),3mM MgCl2, 200 µM dNTP mix, 1.5U of Taq DNA polymerase(Genei, Bangalore) 0.6µM of single primer (Genei, Bangalore and Operon Technologies Inc. Almeda CA, USA), 50 ng of temptate DNA. The thermocycler was programmed for an initial denaturation step of 2 min at $94C^0$, followed by 36 cycles of 1 min at $94C^0$; 1min at $35C^0$, extension was carried out at 7 $2C^0$ for 3 minutes and final extension at $72C^0$ for 5 minutes and at last the hold temperature was of 4oC. PCR products were electrophoresed on 1.5% (W/V) agarose gels, in 1 x Tris Borate-EDTA (TBE) Buffer at 70 V for Five hour and then stained with elthidium bromide (1.0 µg/ml). Gels with amplification fragments were visualized and photographedunder UV light. Medium range DNA Ruller was used as molecular marker (Bangalore Genei, Bangalore, India) to know the size of the fragments. For each experiment there producibility of the amplification products was tested twice using similar reaction conditions at different times.

RESULTS AND DISCUSSION

***In Vitro* Antioxidant Activity**

DPPH method

The *in vitro* antioxidant activity of chicory methanolic extract was determined by DDPH method and the control for DPPH method was found to be 1.049 and detailed results of DDPH activity of ascorbic acid are shown in table 1 and

scavenging activity of ascorbic acid are shown in fig.1. The DDPH activity of chicory extract is shown in table 2 and Fig. 2.

Table: 1 DPPH activity of Ascorbic acid

S. No.	Conc. (µg/ml)	Absorbance at 520 nm	% Inhibition	IC_{50} (µg/ml)
1	20	0.5199	50.43	
2	40	0.3606	65.62	
3	60	0.2786	73.44	**38**
4	80	0.2491	76.25	
5	100	0.1670	84.08	

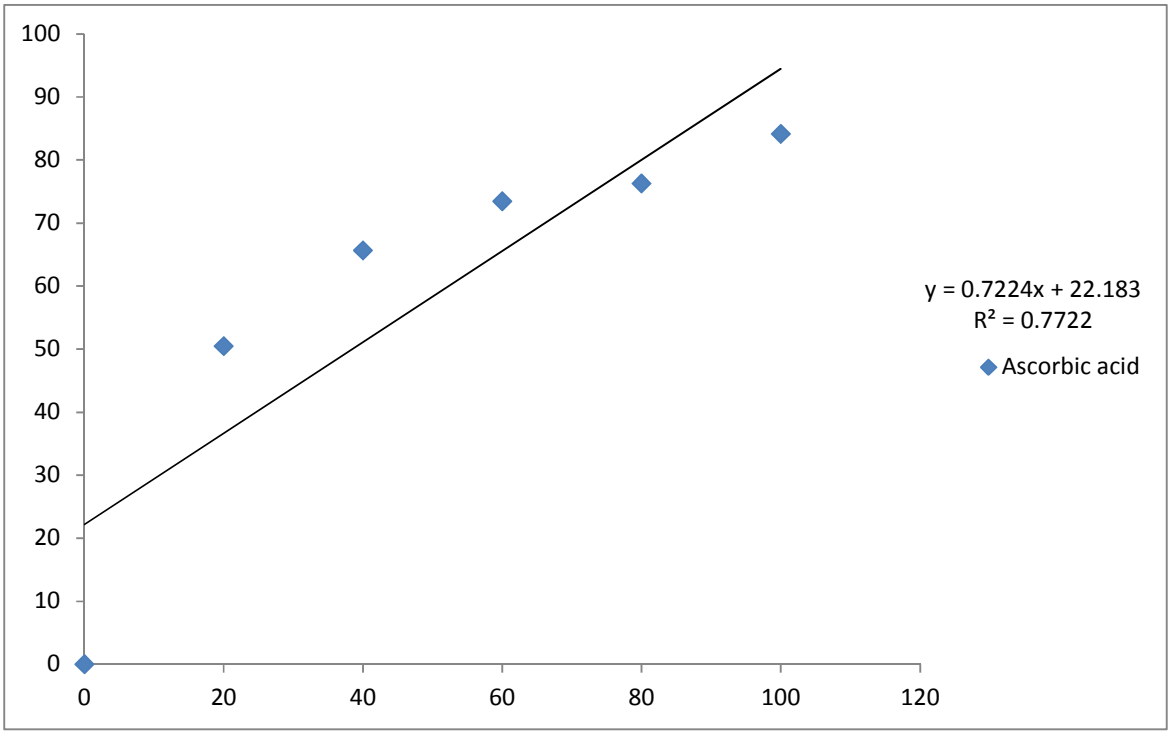

Figure 1: % scavenging activity of ascorbic acid by DPPH method

Table: 2 DPPH activity of *Cichorium intybus*

S. No.	Conc. (µg/ml)	Absorbance at 520 nm	% Inhibition	IC_{50} (µg/ml)
1	20	0.8121	22.58	
2	40	0.7468	28.8	
3	60	0.5652	46.12	**59**
4	80	0.3216	69.34	
5	100	0.1592	84.82	

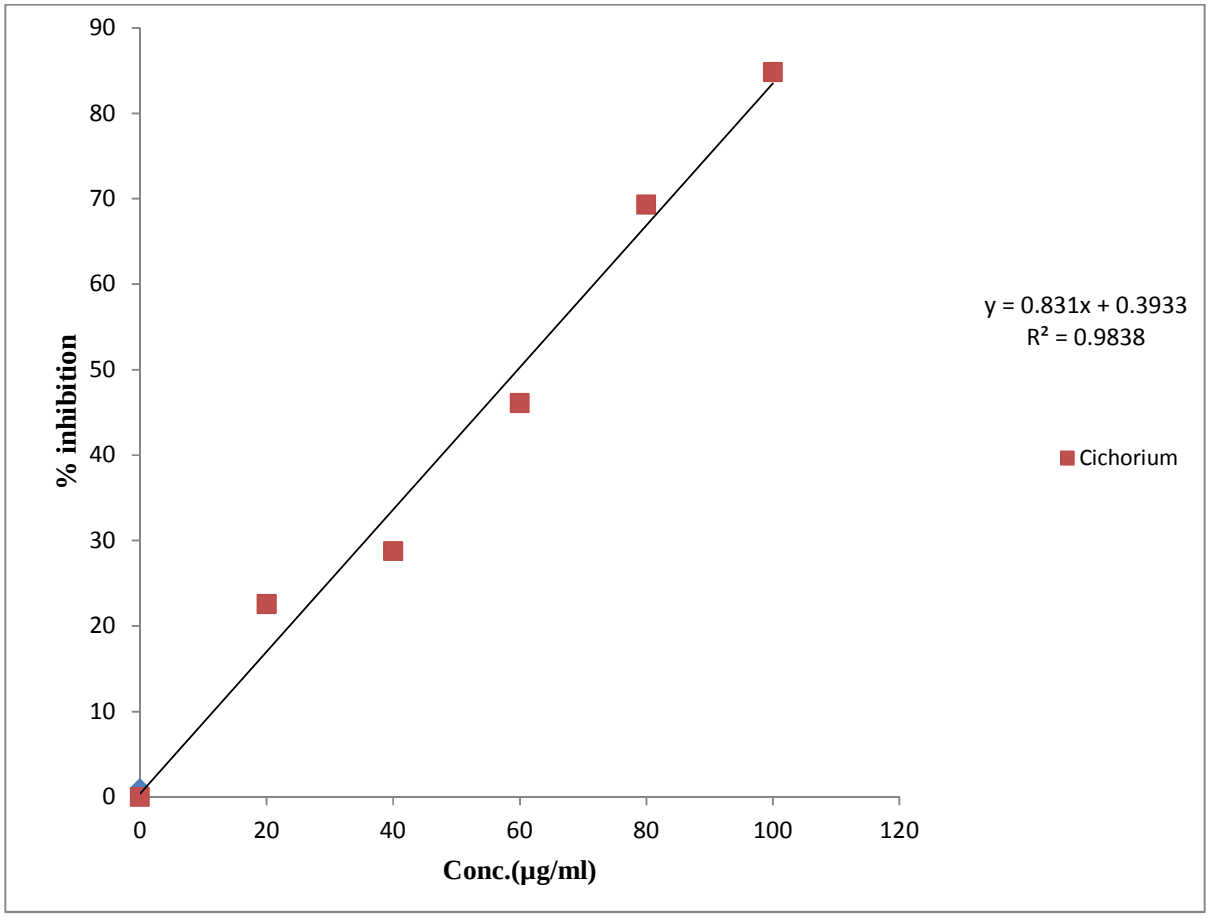

Figure 2: Percentage scavenging activity of Cichory extract by DPPH method

***In Vitro* Antioxidant Activity**

Hydrogen Peroxide Scavenging Method

The *in vitro* antioxidant activity of chicory methanolic extract was determined by hydrogen per oxide method and the control for hydrogen per oxide method was found to be 0.648 and detailed results of hydrogen per oxide activity of ascorbic acid are shown in table 3 and scavenging activity of ascorbic acid are shown in fig.3. The hydrogen per oxide activity of chicory extract are shown in table 4 and Fig. 4.

Table: 3-Percentage scavenging activity of *Ascorbic acid*

S. No.	Conc. (µg/ml)	Absorbance at 230 nm	% Inhibition	Mean± SEM	IC_{50} (µg/ml)
1	5	0.301, 0.302, 0.305	53.54, 53.39, 52.93	53.28±0.1835	
2	10	0.276, 0.280, 0.279	57.4, 56.79, 56.94	57.04±0.1835	
3	15	0.203, 0.256, 0.177	67, 60, 73	66.66±3.350	2.5
4	20	0.177, 0.221, 0.148	73, 66, 77	72.00±3.125	
5	25	0.159, 0.187, 0.121	75, 71, 81	75.66±2.906	

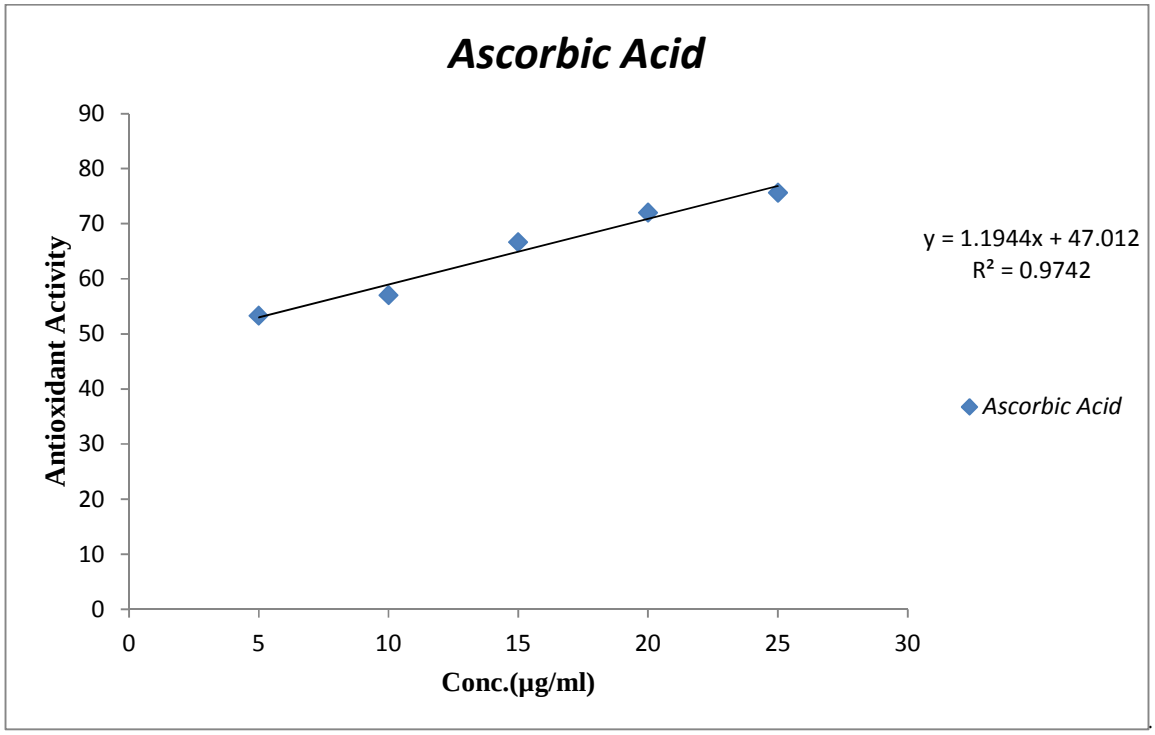

Figure 3: Percentage scavenging activity of *ascorbic acid* in Hydrogen peroxide method

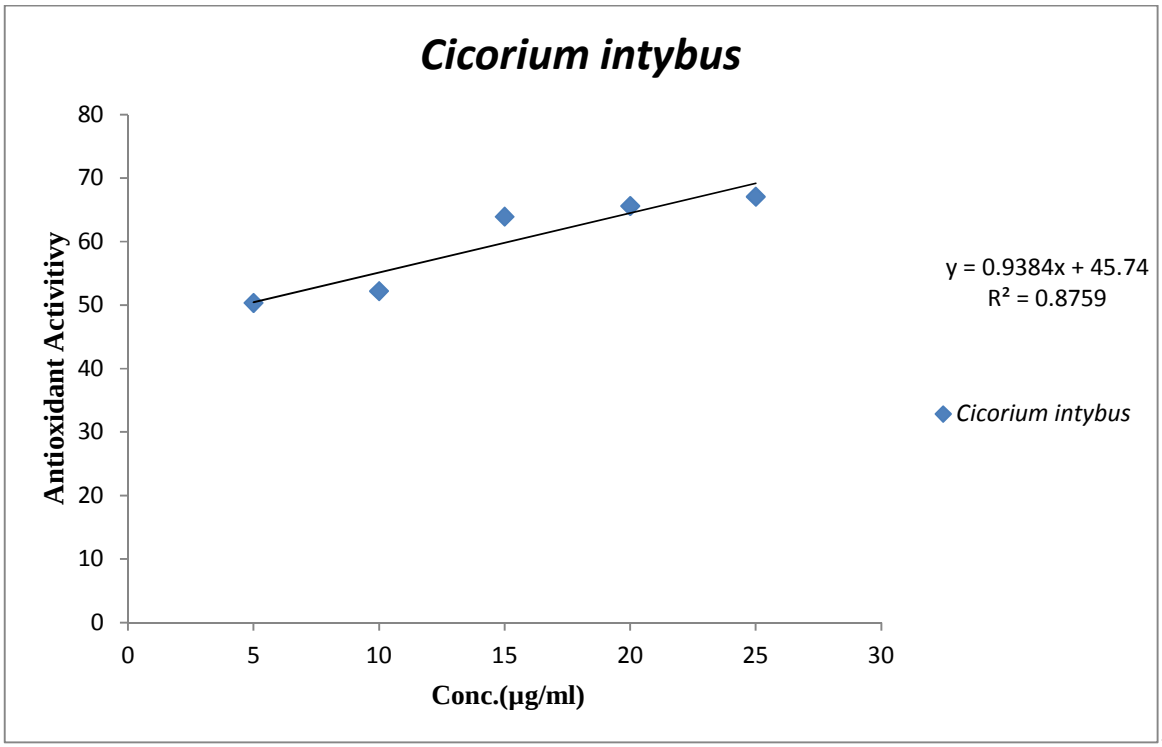

Figure 4: percentage scavenging activity of *Cichorium intybus* in Hydrogen peroxide

Table: 4-Percentage scavenging activity of sample *Cichorium intybus*

S. No.	Conc. (µg/ml)	Absorbance at 230 nm	% Inhibition	Mean± SEM	IC_{50} (µg/ml)
1	5	0.325, 0.328, 0.313	49.84, 49.38, 51.69	50.30±0.7059	
2	10	0.307, 0.325, 0.297	52.62, 49.84, 54.16	52.20±1.264	
3	15	0.214, 0.244, 0.243	66.97, 62.34, 62.5	63.93±1.517	4.54
4	20	0.211, 0.230, 0.228	67.43, 64.5, 64.81	65.58±0.9293	
5	25	0.202, 0.212, 0.226	68.82, 67.28, 65.12	67.07±1.073	

In Vitro Antioxidant Activity

Reducing Power Method

The *in vitro* antioxidant activity of methanolic extract of chicory was determined by reducing power method and detailed results of reducing power method of ascorbic acid are shown in table 5. The reducing power of chicory extract are shown in table 6.

Table: 5-Scavenging activity of *Ascorbic acid*

S. No.	Conc. (µg/ml)	Absorbance at 700 nm	Mean± SEM
1	10	0.526, 0.534, 0.529	0.529±0.002
2	20	0.565, 0.572, 0.569	0.568±0.002
3	30	0.610, 0.619, 0.618	0.615±0.002
4	40	0.650, 0.671, 0.667	0.662±0.006
5	50	0.679, 0.693, 0.693	0.688±0.004

Table: 6-Percentage scavenging activity of sample *Cichorium intybus*

S. No.	Conc. (µg/ml)	Absorbance at 700 nm	Mean± SEM
1	10	0.187, 0.204, 0.232	0.207±0.013
2	20	0.244, 0.262, 0.281	0.242±0.022
3	30	0.295, 0.309, 0.338	0.314±0.012
4	40	0.323, 0.331, 0.353	0.335±0.008
5	50	0.372, 0.384, 0.409	0.386±0.009

Molecular analysis:-

Table:1 *Number of amplified products generated by 10 arbitrary primers in 5 accessions of Cichorium Intybus*

PRIMER	BAND	MONOMORPHIC	POLYMORPHIC	%Polymorphism
OPAA-01	10	0	10	100
OPAA-02	12	2	10	83.33
OPAA-03	10	0	10	100
OPAA-04	14	0	14	100
OPAA-05	10	0	10	100
OPAA-06	8	6	2	25
OPAA-07	10	0	10	100
OPAA-08	14	0	14	100
OPAA-09	9	0	9	100
OPAA-10	8	0	8	100
OPAA-01	12	4	8	66.66
Total	**117**	**12**	**105**	

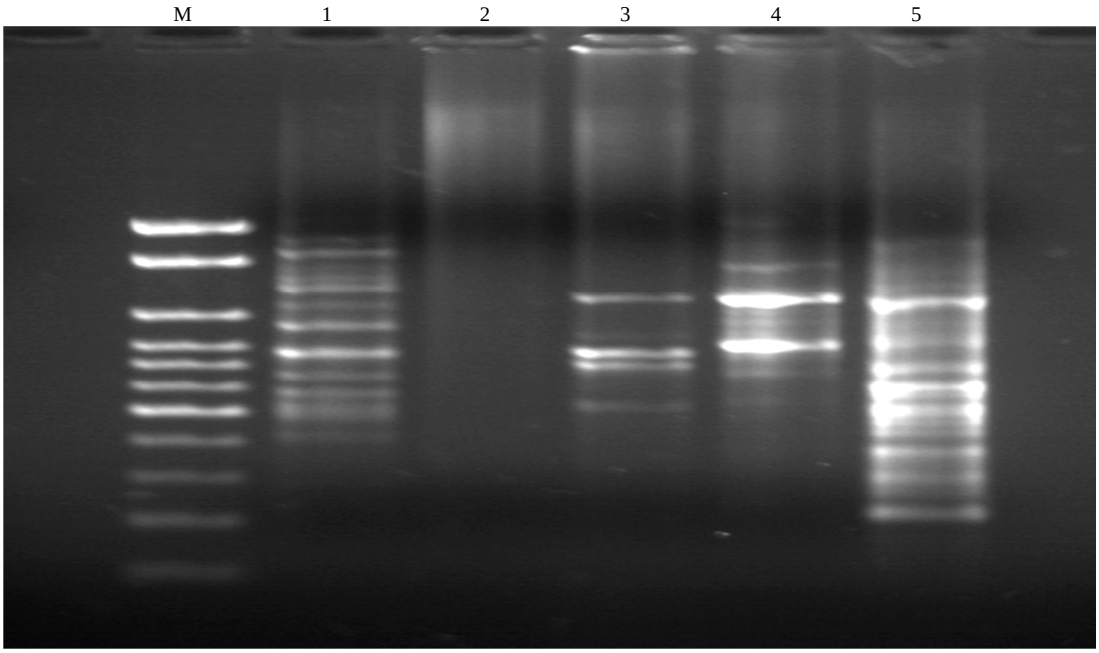

Figure-1: RAPD electrophoresis profile of *Cichorium intybus* amplified with OPAA primers
Lanes 1–5 correspond to the 5 accessions. Lane M, molecular marker 200-1500bp

Literature evidence proved that increased use of dietary antioxidants in the form of fruits and vegetables may contribute to increase the quality of life by reducing the risk of degenerative diseases associated with aging.

DPPH assay is easy, rapid and sensitive way to determine the antioxidant property of a specific compound or plant extract. DPPH method is based on the reduction of DPPH content in the presence of hydrogen donating antioxidant due to the formation of diphenyl picryl hydrozine.

Chicory extracts minimize the colour of DPPH due to the power of hydrogen donating ability. The methanolic extracts produced DPPH anion scavenging power (59µg/ml) at 100µg/ml concentration and 38µg/ml for Ascorbic acid. The above value depicted DPPH anion scavenging power of extracts. Discoloration of violet DPPH to Yellow clearly indicated the antioxidant effect of chicory extracts.

The presence of reductant in a compound is responsible for their reducing ability (Duh *et al.*, 1999), which produced antioxidative potential by breaking the free radical chain (Gordon, 1990). The methanolic extract of *Cichorium intybus* causes the reduction of the Fe^{+++} to the Fe^{++} this may be due to the presence of reactant. The reducing power of the *Cichory extract* was found to be potent and the reducing power was increased with increased in the quantity of sample.

Hydroxyl radical is highly reactive radical which attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule (Aruoma, 1999) and capable of abstracting hydrogen atoms from membrane lipids (Yen and Duh, 1994). The results indicated that the methanolic extract of *Chicory* showed concentration dependent scavenging activity against hydroxyl radical. The IC_{50} value of methanolic extract *Cichorium intybus* was found to be 4.54µg/ml against standard ascorbic acid (IC_{50} 2.50µg/ml).

Hydrogen peroxide can be toxic to cell because it may increase the hydroxyl radical in the cells (Halliwell et al., 1987). Scavenging of H_2O_2 by chicory extracts may be attributed to the presence of phenolics compound in a concentration-dependent manner. For RAPD analysis of *Cichorium intybus seeds* OPAA primers were used. Out of which most reproducible primers were used for the fingerprinting. In the present study ten RAPD primers were used for the fingerprinting, **(Table 1 and figure 1).**

All the primers used in the present study were polymorphic, number of bands produced by each primer varied from 8-14. All the bands produced in the present study were scored for the analysis. Ten primers were used in the study of RAPD markers analysis to standardization of suitable specific primers amplifying the genetic materials accessions.

Analysis of five accessions of tested samples revealed of polymorphism. The total 177 bands were scored for the 10 RAPD primers out of which 12 bands were monomorphic. In case of *C. intybus* The number of genetic loci detected with RAPD markers are much higher than detected with morphological and chemical / biochemical markers .

CONCLUSION

Finally it was concluded that the methanolic extract of *Cichorium intybus* (Chicory) is found to more effective and potent as an anti oxidant while the number of genetic loci detected with RAPD markers are much higher than detected with morphological and chemical / biochemical markers.

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