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Research Article

Determination of Antioxidant and Hepatoprotective Ability of Flavonoids of *Cichorium intybus*

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ABSTRACT
The hydroalcoholic fraction of the leaves of *Cichorium intybus* was tested for the presence of flavonoids and the hepatoprotective activity of this flavonoid containing extract was tested against hydrogen peroxide induced toxicity in HepG2 cell lines. A dose dependent increase in the viability/restoring effect was observed when hydrogen peroxide exposed HepG2 cells were treated with the different concentrations (100ng/ml- 200µg/ml) of the hydroalcoholic fraction. It was observed that in the presence of 0.5mM hydrogen peroxide cell viability was reduced from 100% to 66% and with 1mM hydrogen peroxide, it was further reduced to 19.3%. The damaging effect was restored in the presence of the hydroalcoholic fraction of *Cichorium intybus* leaf extract, in a concentration dependent manner. This study can further lead to the isolation and characterization of the flavonoids of the plant extract responsible for the hepatoprotective properties.

Keywords: - Antioxidant, Hepatoprotective, Liver cell culture, HepG2, Flavonoids, Hydrogen peroxide.

INTRODUCTION

Liver plays a major role in detoxification. Liver being the largest organ of the body contributes to about 2% of the total body weight in an average adult human. Liver is associated with most of the physiological processes, which include growth, immunity, nutrition, energy metabolism and reproduction. Synthesis and excretion of bile, albumin, prothrombin and production of the compliments, which is the major effector of the humoral branch of the immune system, occurs mainly in the liver. The detoxification of the harmful chemicals occurs in the liver[1] and so any injury to it or impairment of its function can lead to many implications on one's body system. Managing liver diseases is still a challenge to modern medicine. Oxidative stress increases when there is imbalance between reactive oxygen species (ROS) formation and their scavenging by antioxidants. Excess generation of ROS results in oxidative damage to biomolecules leading to lipid peroxidation, mutagenesis and carcinogenesis.

Flavonoids occur as a group of about 4000 naturally occurring polyphenolic compounds, found universally in foods of plant origin. These are primarily recognized as the pigments responsible for the colors of leaves, especially in autumn[2]. Flavonoids or bioflavonoid (from the Latin word flavus means yellow), also collectively known as Vitamin P and citrin, are a class of plant secondary metabolites or yellow pigments having a structure similar to that of flavones. They are usually subdivided as per their substituents into flavanols (kaempferol, quercetin), anthocyanins, flavones, flavonones and chalcones. These flavonoids show a diversified biochemical and pharmacological actions viz., antiinflammatory, antioxidant, hepatoprotective, antiallergic, antiviral, antithrombotic, antiviral and anticarcinogenic activities[3].

In China, there are three species in the genus *Cichorium*: *Cichorium intybus* L., *C. glandulosum* Boiss. Et Huet and *C. endivia* L. The another two *Cichorium* species are considered to be folk medicines used for the treatment of

Fig.1 : Leaves of Cichorium intybus.

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Table 1: Phytochemical Screening of *C.intybus* leaf extract

S.No	Constituents	Leaf extract
1	Alkaloids	+
2	Carbohydrates	+
3	Glycosides	+
4	Phenolic compounds and tannins	+
5	Flavonoids	+
6	Proteins and free amino acids	+
7	Saponins	+
8	Sterols	–
9	Acidic compounds	–
10	Mucilage	–
11	Lipids/fats	–
12	Resins	+
13	Sesquiterpene	+

liver diseases except *C. endivia* , their hepatoprotective effects were related to their antioxidant capacity as was demonstrated in previous studies [4,5,6] .

In the present study we performed the preliminary phytochemical screening, estimated the total flavonoid content of *Cichorium intybus* leaf extract and evaluated its antioxidant capability against hydrogen peroxide induced toxicity in Hep G2 cell line, which are derived from the liver tissue of a Caucasian American male with a well-differentiated hepatocellular carcinoma [7].

MATERIAL AND METHODS

Collection of Plant Material: The seeds of *Cichorium intybus* were procured from khari bawli market, Delhi, India and were sown in the botanical garden Hamdard University, New Delhi .The leaves were collected and segregated as per three sizes viz. short, medium, long. They were identified by the head, raw materials herbarium and museum, NISCAIR, New Delhi and a voucher specimen number NISCAIR/RHMD/Consult/-2010-11/1603/201 was deposited at the herbarium of national institute of science communication and information resources, New Delhi.

Preparation of Leave extract: The leaves were collected from the herbal garden in the month of January and then they were washed with distilled water and dried away from direct light, and then powdered. The powder was kept in three different closed container at 4ºC. The powdered material (500 g) was extracted three times in water-ethanol (1:1, v/v) by hot soxlation and was then concentrated under reduced pressure. The dried extract was then suspended in water-ethanol (1:1, v/v) and the volume adjusted to 500 ml. (1 g plant powder per ml). The concentrated extract was divided in 25 ml aliquots, labeled properly and kept at -20ºC for further investigation.

Determination of Total Flavonoid content in leaves: Chemical test were performed in the ethanolic extract which showed the presence of Flavonoids[8,9]. Shinoda test, Ammonia test and Zinc chloride test were positive showing the presence of flavonoids. The total flavonoid content was determined by aluminum chloride reagent method. 0.5 ml of 10mg/ml stock solution of ethanolic fraction of *Cichorium intybus* was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate aqueous solution and 2.8 ml of distilled water. Appearance of yellow colour indicates the presence of flavonoids. This solution was incubated for 30min at room temperature. The absorbance was measured immediately against the prepared blank at 415nm.Quercetin was used as a standard and the results were expressed as mg of quercetin equivalents (QE) per g of dry extract [10].

Flavonoid Content (%) of solid sample =

$$\frac{\text{Flavonoid } (\mu g/ml) * \text{ total volume of ethanol extract(ml)} * \text{Dilution factor} * 100}{\text{Sample weight (gm)}}$$

***Invitro* assay for cytotoxicity in hydroalcoholic leaf extract of *Cichorium intybus* (MTT assay)**

Cell culture and treatments: HepG2 human Caucasian hepatocyte carcinoma cells (National Centre for Cell Science (NCCS) Pune, Maharashtra, India) were routinely grown in DMEM supplemented with 10% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 lg/mL streptomycin, in a humidified atmosphere of 95% air 5% CO_2 at 37˚C. Test extract was prepared in DMSO at final concentration of 100 mg/ml and further diluted in serum free medium for cell treatments. Stock solutions of H_2O_2

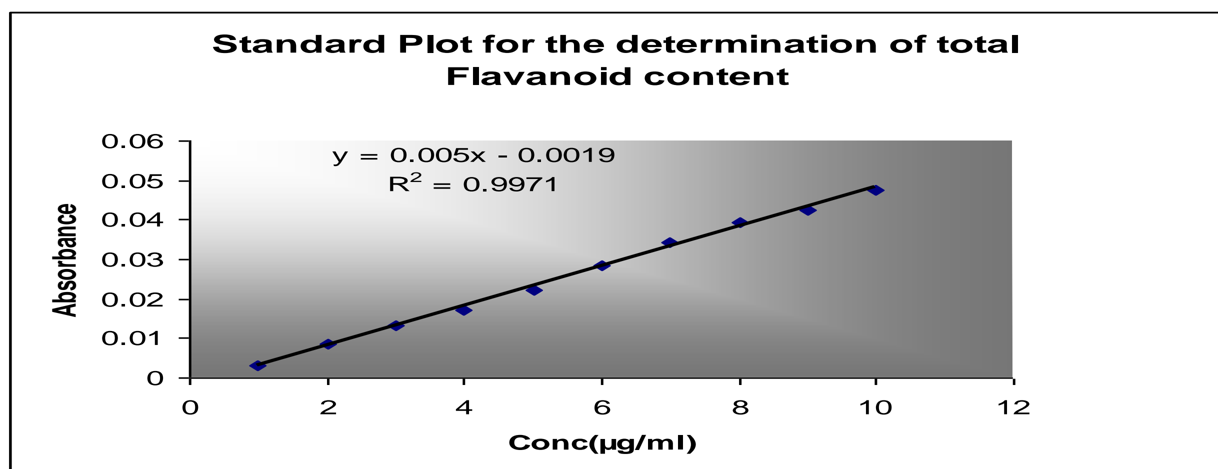

Fig. 2: Standard Plot for the determination of Total Flavonoid content .

Table 2: Result of Total Flavonoid Content in *Cichorium intybus* leaves.

S.No	Conc of Extract	Absorbance	Total Flavonoid content of QE from calibration curve(µg/ml)	Average Value (µg/ml)	Flavonoid content in the test extract (mg of Quercetin equivalent/gm of extract)
1	1 mg/ml (5mg of the extract was dissolved in 5ml of ethanol)	0.1945	39.22	39.29	39.2mg/gm
		0.1949	39.36		

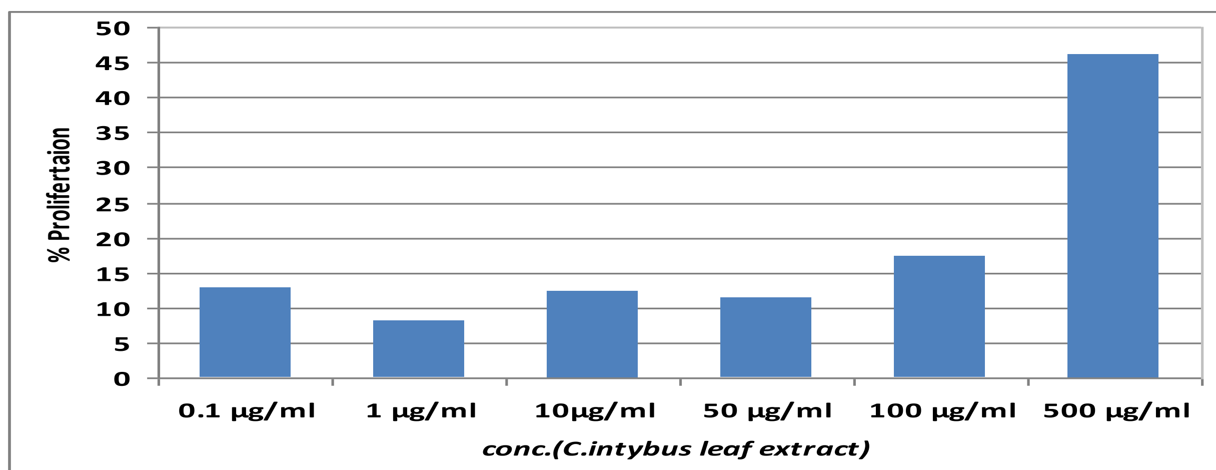

Fig 3: Graph of % cell proliferation vs. extract concentration after 48 hrs.

Table 3: %Cell Viability data with different conc. of leaf extract with 0.5mM H_2O_2 induced oxidation.

		% inhibition (w.r.t Untreated)	% viability of cells
Untreated		0.00	100
Concentration(H_2O_2)	0.5 mM	33.68	66
Concentration (*C.intybus* leaf extract)	100 ng/ml	8.51	91
	1 ug/ml	2.98	97
	50 ug/ml	2.87	97
	100 ug/ml	8.05	92
	200 ug/ml	20.87	79

were freshly prepared in serum-free DMEM. At the treatment stage, the final DMSO concentration was never higher than 0.1%.

Proliferative effect of Drug Combination on HepG2 cells: The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide, MTT-assay [11] was used to evaluate the antiproliferative activities of the drugs alone and in combination. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which are then photometrically quantified. An increase in the number of living cells results in an increase in total metabolic activity, which leads to a stronger color formation. 5000 HepG2 cells in 200 µl were incubated in 96well plate for 24 h in a CO_2 incubator. Cells were then treated with leaf extract in the concentration range of 100 ng/ml – 200 µg/ml for 48 h. After 48 h, 20 µl MTT (5mg/ml) was added to each well and incubated for 2h. Following this, media was removed and 100 µl of DMSO were added to each well in order to solubilize the formazan. OD was measured at 540 nm.

Cell proliferation was expressed as [(OD of treated cells – OD of control cells)/OD of control cells]*100

Determination of protective effect of *C.intybus* leaf extract against H_2O_2 induced damage: For the viability assays, 10,000 cells in 200 µL of complete DMEM were placed in each well of a 96 well culture plate for 24 h in a CO_2 incubator. Cells were then pre-treated with different concentration of *C. intybus* extract (100 ng/ml to 200 µg/ml) for 24 hours. Cells pre-treated with the extract were then exposed to H_2O_2, oxidative damage at final concentrations of 0.5 mM and 1 mM for a time period of 2 h. Cells treated with H_2O_2 alone served as controls. After 2 h, medium was removed and cells were replenished with fresh culture medium. Cells were allowed to recover the oxidative damage for another 24 h of incubation. The effect on cell viability/survival against H_2O_2 damage was determined by MTT assay. The results were expressed as the percent cell viability:

Percent cell viability = [OD of control cells (H_2O_2 damaged) - OD of treated cells (leaf extract pre-treated

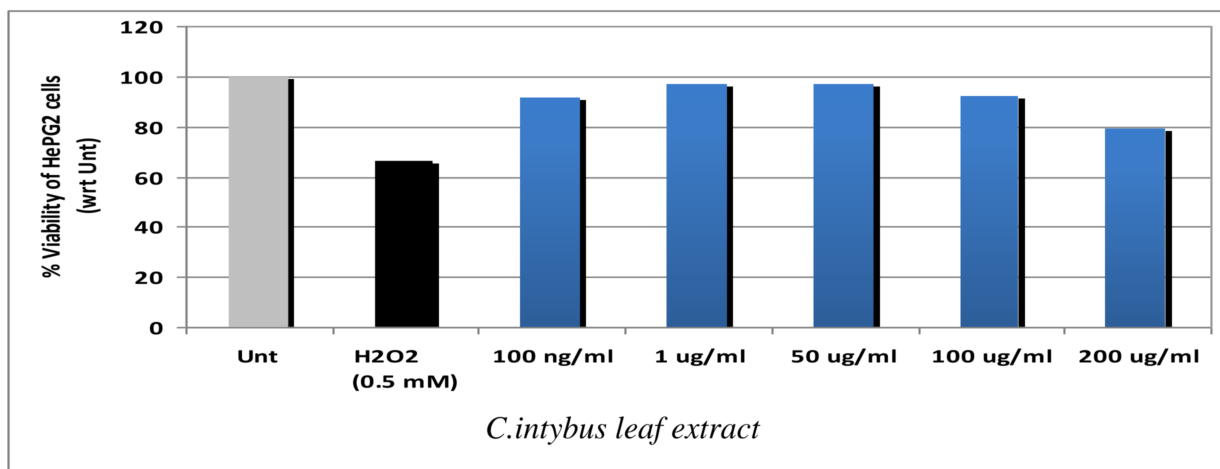

Fig. 4: Graph of % viability of HepG2 cells treated with 0.5 mM H_2O_2 with different concentrations of C.intybus leaf extract.

Table 4: %Cell Viability data with different conc. of leaf extract with 1.0 mM H_2O_2 induced oxidation.

		% inhibition (w.r.t Untreated)	% viability of cells
	Untreated	0.00	100.0
	H_2O_2 (1 mM)	80.67	19.3
Concentration(*C.intybus* leaf extract)	100 ng/ml	65.88	34.1
	1 ug/ml	73.48	26.5
	50 ug/ml	69.39	30.6
	100 ug/ml	74.05	26.0
	200 ug/ml	74.84	25.2

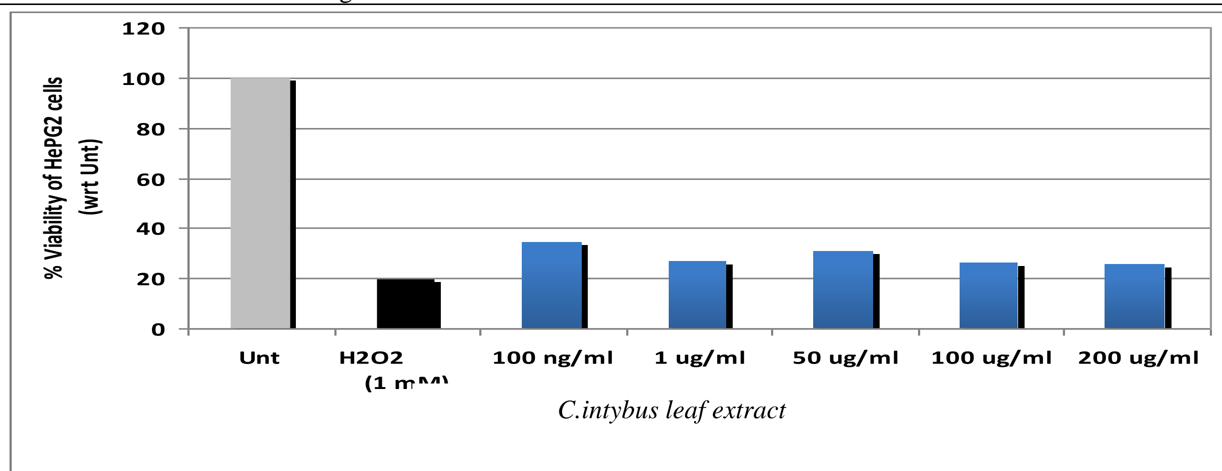

Fig 5: Graph of % viability of HepG2 cells treated with 1.0 mM H_2O_2 with different concentrations of C.intybus leaf extract.

cells exposed to H_2O_2) / OD of control cells (H_2O_2 damaged] × 100.

RESULTS AND DISCUSSIONS

Effect of test extract on cell proliferation: HepG2 cells were treated with *C.intybus* leaf extract in concentration range of 100 ng/ml – 200 ug/ml for a time period of 48 h. Till 48 h, no adverse effect on cell viability was observed. Infact, there was an increase in cell proliferation in this concentration range, which suggests that extract itself is not cytotoxic to cells. Hence for cytoprotection assays, we used this concentration range for cell treatments.

Protective effect of *C.intybus* leaf extract against H_2O_2 induced cell damage: It is well known that H_2O_2 can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell. Similarly, the organic hydrogen peroxide induces an array of cellular dysfunctions, including peroxidation of membrane lipids, glutathione and protein thiol depletion, alteration of calcium homeostasis, and DNA damage, eventually leading to cell death. In the present studies, we aimed to investigate the protective effects of *C.intybus* leaf extract on HepG2 cell viability against H_2O_2 induced damage. % cell viability data was generated by taking two different conc. of H_2O_2 0.5mM and 1mM.

DISCUSSION

A variety of chemical constituents like phenols, coumarins, monoterpenes, glycosides, alkaloids and xanthenes are reported in liver protective plants [12]. Amongst these phenolics possess a wide spectrum of biological activities such as antioxidants, anticarcinogenic, antimutagenic, as well as the ability to modify the gene expression[13, 14]. Numerous epidemiological studies confirm significant relationship between high dietary intake of flavonoids and reduction of cardiovascular and carcinogenic risk. Herbal polyphenolic compounds in the cell can function as antioxidant and antiprooxidant by scavenging reactive oxygen species via enzymatic and non-enzymatic reactions[15, 16, 17] .The flavonoids and phenolic acids are known to possess antioxidant activities due to the presence of the hydroxyl groups in their structures thus they help in playing an important role in the defence system against the oxidative damage due to endogenous free radicals[18]. The antioxidant capabilities of the phenolic compounds are important for the human body to destroy the free radicals that exist in our body. Many of the polyphenols such as flavonoids have been identified as powerful antioxidants; play a significant role in the treatment of many diseases, including liver cirrhosis [19].

Previous studies have reported some biological active constituents such as sesquiterpene lactones and phenolic compounds in *C. intybus*[20]. The sesquiterpene lactones such as lactucin and lactucopicrin were isolated from chicory and reported for its antibacterial and antimalarial activity[21].This plant is also used to treat AIDS, dysmenorrhoea, cancer, impotence, diabetes, insomnia, splenitis and tachycardia[8]**.** Inulin is used to replace fat or sugar and reduce the calories of food. It is suitable for consumption by diabetics[9] and is also used in inulin clearance test to measure glomerular filtration rate-GFR[22]. Recent pharmacological investigation of the root extract of this plant exhibited immunomodulator, antitumor and anticancer properties[23]. The root is rich in alkaloids, which forms an ingredient or adulterant in coffee. Beside roots, the deep purple flower heads yield blue dye. The flowers are also used in floral clocks by Linnaeus[24]. The antifungal activity of chicory was also reported in some of the studies[25, 26, 27, 28]. Based on the studies carried out in chicory, world wide report shows that the roots and leaves of this plant possess strong antibacterial and nematicidal effect[29]. In another species of chicory , namely *C.endivia* , it was found that from a 60% ethanol eluate extract there was considerable antioxidant potency *in vitro*[30]. Similar work on the extracts of *Cichorium intybus* and *Solanum nigrum* (constituents of Liv.52) has been reported to contain many polyphenolic compounds, mainly flavonoids, besides these tannins, the high molecular weight polyphenolic compounds, which play a protective role in plants, is also reported in *C.intybus.* The tannin content in all parts of *C.intybus* was found to be higher than the range (41-166 mg/100g) in leafy vegetables[31]. Similar studies on the effect of *Silybum marianum* and *Cichorium intybus* extracts on liver cells suggested that hepatoprotective action due to the presence of flavonoids and their antioxidant effects[32].

The Preliminary phytochemical screening of the plant extract reported the presence of Alkaloids, Carbohydrates, Phenolics, flavonoids, tannins, and proteins etc (Table 1). The total flavonoid content of the leaf extract was evaluated by the method of Woisky and Salatino, 1998. It was evaluated in terms of mg of Quercetin equivalent/ gm of the extract. It was found to be 39.2 mg Quercetin equivalent/gm of extract (Table 2, Fig.2).

The antioxidant activity of the extract may be due to the presence of polyphenolic constituents[33,34]. Based on this we evaluated the antioxidant capacity of the leaf extract in HepG2 cell lines by hydrogen peroxide induced toxicity. A concentration range of 100 ng/ml – 200 ug/ml was selected and the effect of cells proliferation in this range was determined for 48hrs. There was infact an increase in % proliferation from 12.76 $\pm$ 0.7% (0.1 ug/ml) to 46.23 $\pm$ 1.2 % (500 ug/ml), suggesting that the extract itself is non-toxic for the cells (Fig 3). The cells were intoxicated with two different conc of H_2O_2. It was observed that after 2 hr exposure of 0.5 mM hydrogen peroxide resulted in loss of cell viability from 100% to 66%. The damaging effect induced by H_2O_2 was found to be restored in the presence of *Cichorium intybus* leaf extract. The recovering effect was found to be maximum at 1µg/ml and 50µg/ml of the extract where the cell viability was restored to 97% (Table 3, Fig 4). In another set of assays, induction of cellular damage with 1mM H_2O_2 resulted in loss of cell viability from 100% to very low levels of 19.3%. With the extract, the cell viability was restored to 34.1% at 100 ng/ml (Table 4, Fig 5).

On the basis of above findings , it can be concluded that the hydroalcoholic fraction of *Cichorium intybus* has an antioxidant capacity due to the presence of Flavonoids , which can be further characterized, isolated and formulated to evaluate their individual antioxidant capacities.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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