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Hepatoprotective effects from the leaf extracts of *Brassica juncea* in CCl_4 induced rat model

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ABSTRACT

Brassica juncea is a cruciferous cormophyte medicinal plant. Despite its popular usage, no study has been published concerning its hepatoprotective activity till date. This study was designed to evaluate the effects of Brassica juncea leaf extracts on carbon tetra chloride (CCl_4) induced Wistar albino rat model. The animals were divided into 7 groups (Normal, Positive control, Standard, Pet ether, Chloroform, Ethyl acetate and Ethanol). Liver marker enzymes were assayed in serum. Samples of livers were observed under microscope for the histopathological changes. Serum levels of alanine amino transferase (ALT), Aspartate amino transferase (AST), alkaline phospahtase (ALP) and bilirubin level were significantly increased in positively control group. Among different extracts groups, ethanolic extract and pet ether group indicates maximum inhibition of necrosis shows reduction of ALT, AST ALP and total bilirubin give highly significant results $p<0.0001$. Ethyl acetate did not show any significant result. In conclusion the pet ether and ethanolic leaf extract of Brassica juncea could be a better drug of choice as a hepatoprotective plant source for the liver patients.

Key words: Hepatoprotectivity, *Brassica juncea*, CCl_4, Liver markers.

INTRODUCTION

The Liver is very important organ of our human system having various multifunctional activities like metabolism and excretion. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [1, 2]. It has great potential to detoxicate toxic substances and then produced the useful ones [3]. Health problems have been widely reported due to long term ingestion of chemicals from various

environmental sources which lead to hepatitis, cirrhosis and alcoholic liver diseases. Thus liver diseases are some of the fatal disease in the world today [4]. Hepatotoxicity may be caused by thousands of synthetics chemicals, drugs, bacteria, fungi, plants and animals toxicants [5]. Treatment of diseases associated with the liver is very vital, and must be done with importance and extensive care. There are few conventional drugs that can stimulate liver function and offer hepatic protection or help in the regeneration of hepatic cells but they showed the hepatotoxicity at particular dose[6]. Today the modern drugs are larger in their number than the natural ones to treat various diseases. Modern sciences are now beginning to accept the use of standardized plant extracts for the treatment in various disorders. It is estimated that out of 25,000 to 500,000 species of plants only 1 to 2% of the terrestrial plants have been reasonably well investigated for hepatoprotectivity. In India, there are few numbers of medicinal plants and their formulations which are used to treat this disorder. Because of this fact, efforts are being made to find suitable curative agents for the treatment of liver diseases in natural plants products [7].

The family Brassicaceae (=cruciferae) consists of 350 genera and about 3500 species and includes several genera like *Camelina*, *Crambe*, *Sinapis*, *Thlaspi* and *Brassica*. The genus Brassica is the most important one within the tribe Brassiceae, which includes some crops and species of great worldwide economic importance such as *Brassica juncea* L, *Brassica oleraea* L. *Brassica napus* L and *Brassica rapa* L. The same species can be utilized for several uses according to different forms or types. The genus is categorized into oilseed, forage, condiment and vegetable crops by using their buds, inflorescences, leaves, roots, seeds and stems [8].

Indian mustard (*B. juncea* L. Czern and Coss) popularly known as rai, raya and laha is one of the most important oil seed crops of the country and its occupies considerably large acreage among the brassica group of oil seed crops. India stands first both in acreage and production of rapeseed and mustard in Asia. In India, mustard and rape seed are being grown largely in states like Uttar Pradesh, Rajasthan, Haryana, Assam, Gujarat, Punjab, west Bengal, Madhya Pradesh and also some states of south region [9]. Brassica juncea is erecting much branched 3’ to 6’ high annual plant with slender and tapering root. The stem branches from the axil of the fourth or fifth leaf upward. Lower leaves petioled, green, sometimes with a whitish bloom, ovate to obovate, variously lobed with toothed, scalloped or frilled edges, lyrate-pinnatisect, with 1-2 lobes or leaflets on each side and a larger sparsely sectose, terminal lobe; upper leaves sub entire, short petioled, 30-60 mm long, 2-3.5 mm wide, the fruit is siliqua. The pods are bilocular with a false septum between two halve [9]. Pharmacological activities of *Brassica juncea* has reported by various researchers. Leaf extract of *Brassica juncea* has studied to treat diabetic cataract [10], antioxidant activity both *in vitro* and *in vivo* [11], antinociceptive, anti-hyperglycemic activity [12] and hematological studies [13]. Leaf extracts of *Brassica juncea* significantly prevented the development of insulin resistance in rats fed fructose-enriched diet [14]. Anticancer activity has reported on isolation of new compound from leaf extract of *Brassica juncea* [15]. The pharmacological effects of mustard oil have a great deal of interest. The essential oil of *B juncea* has very high application value and can be used to suppress the growth of microorganism in seafood, such as *Helicobacter pyheri* and *Vibrio parahaemolyticus*. It also shows inhibitory effects on growth of bacteria that cause food poisoning and fungi. The oil exhibits significant inhibitory activities against *Aspergillus niger*, *A. flavus*, *Trichoderma viride*, *Candida albicans*,

C. utilis, C tropicalis, Cryptococcus neoformans, Trichosporon mucoides, Trichophyton tonsrans and Geotrichum capitatum. Moreover the oil shows inhibitory effect on tumor cells and is effective in anti-platelet and anti cancer. Considering there is a lot of pharmacological activities have been reported till now because of this plant is very common in use. Till date there is no hepatoprotectivity study has been reported on *Brassica juncea*. With the broad aim, our present study was to investigate the hepatoprotective activity of leaf extracts of *Brassica juncea* on liver after Carbon tetra chloride ($CCl_{4)}$ induction in rat model.

MATERIALS AND METHODS

Drugs and Chemicals

All the drugs used in this study were of pharmaceutical grade. CCl_4 was purchased from (Merck, Darmstadt, Germany). Olive oil (Roberts Laboratories Limited, Belton, England). Ursodiol (Himedia Laboratories Pvt Ltd, Mumbai, India). All other reagents and chemicals used in the study were of analytical grade.

Plant Material

Leaves of *Brassica juncea* were collected in the month of September 2010 from Botanical garden, Punjab University, Chandigarh, India. The specimen plant (NISCAIR/RHMD/Consult/-2010-11/678/276) was identified with the help of literature and authenticated by Dr. H. B. Singh, Scientist F & Head, Raw Materials Herbarium and Museum, N.I.S.C.A.I.R, New Delhi, India. The fresh plant material were cleaned with distilled water to dry at 35º-40ºC for 10 days and, pulverized in electric grinder and the powder was passed through sieve No.60 and used for further extraction.

Preparation of extracts

The dried powdered of leaves (2kg) was successively Soxhlet extracted using petroleum ether (60-80^o), chloroform, ethyl acetate, ethanol, chloroform, acetone, for 72 h each. Crude aqueous extract of these leaves were prepared separately by maceration for 24 hrs. The last trace of solvent is removed by reduced pressure distillation and then vacuum dried. A dark semi solid mass was obtained. It was stored below 4ºC until further used. When needed, the extract was suspended/dissolved in desired solvent and used. The extracts were concentrated by performing the qualitative chemical tests to determine the presence of alkaloids, anthracine, glycosides, sterols, phenolic compounds, flavonoids and saponins, respectively.

Animals

Adult male Wistar albino rats weighing about 180-200g were used with the approval of the institute animal ethics committee (MMCP/IEC/10/59 Reg no. 828/ac/04/CPCSEA-Reg. Dt. 16.06.2004). The animals were housed under standard conditions of temperature (24±28°C) and relative humidity (60-70%) with a 12:12 light-dark cycle. The animals were fed with standard pellet diet (Lipton India, Ltd) and water a*d-libitum*

Experimental Design

Hepatoprotectivity [16]

Hepatotoxicity was induced by CCl_4 (1.5ml/kg body weight in olive oil, injected intraperitoneal) on day 2nd and 3rd in a group 2 to 7 according to the method described by Carbonari *et al*. The extracts dose was selected on the basis of preliminary studies. Extracts were suspended in 0.2 ml of 2% w/v carboxy methyl cellulose with 2.0% Tween 80 and administered orally (500 mg/kg) to rats. Ursodiol (150mg/kg) was given to standard group.

The animals were assigned to eight groups for 7 days model, each group consisting of 8 rats.

Group 1-Normal control treated with vehicle only (saline water) for 7 days.
Group 2-Positive control treated with vehicle only (saline water) for 7 days followed by CCl_4 on day 5.
Group 3-Treated with Urosidol (150 mg/ kg, p.o.) for 7 days followed by CCl_4 on day 7.
Group 4-Treated with Pet ether extract (500mg/kg, p.o) for 7 days followed by CCl_4 on day 7.
Group 5- Treated with chloroform extract (500mg/kg, p.o) for 7 days followed by CCl_4 on day 7.
Group 6-Treated with ethyl acetate extract (500mg/kg, p.o) for 7 days followed by CCl_4 on day 7.
Group 7- Treated ethanol extract (500mg/kg, p.o) daily for 7 days followed by CCl_4 on day 7.

Blood collection and biochemical assays

Approximately twenty four hours after the intraperitoneal administration of CCl_4, blood sample was collected through carotid artery and collected in previously labeled centrifuging tubes and allowed to clot for 30 min at room temperature before being centrifuged at 5000 rpm for 10 minutes [17]. The separated serum was used for the estimation of some biochemical parameters like Alanine aminotransferase (ALT/SGPT), Aspartate aminotransferase (AST/SGOT), ALP and Total bilirubin level.

Histochemical studies

A portion of liver tissue in each group of rats was selected and fixed in 10% formalin diluted water and processed for paraffin embedding. Sections were stained with hematoxylin and eosin and observed under microscope [18].

Statistically analysis

Data were expressed as mean ± SEM of six observations and statistically assessed by two ways analysis of variance and the group means were compared student's *t*-test on statistically software program, SYSTAT 10.6. A probability of $p<0.05$, $p<0.01$ and $p<0.001$ were considered as significant.

RESULTS

The preliminary phytochemical analysis of the crude extracts (pet ether, ethyl acetate, chloroform and ethanol) of *Brassica juncea* indicated the presence of flavonoids, tannins, alkaloids, phenolic compounds, volatile oils and terpenoids as shown in table 1. The TLC studies carried out showed the green and yellow colour spots except pet ether not showed green colour (absence of terpenoids) as figure 1. The administration of CCL_4 induced acute liver damage which was well indicated (Table-2) by increased ALT, AST, ALP and TBL when compared with

normal control group. The highly significant ($p<0.0001$) reduction was observed in all the parameters in the ethanolic extract group and pet ether group in comparison with positive control treated group. Ethyl acetate extract treated group did not show any significant effect in any of these parameters. Among these, ethanolic extracts shows maximum percentage reduction in ALT (45.69%), AST (44.55%), ALP (39%) and TBL (77.17%) after administration of CCl_4. Though chloroform extract group also shows significant results but not the similar reduction in these parameters as in pet ether and ethanolic group. These results suggested the possibility of the ethanolic extract to give very good protection against liver injury upon CCl_4 induction within 72 hrs.

Histochemical studies

Histopathological studies also provided supportive evidence for the biochemical analysis. Normal control group showed a normal liver architecture, hepatocytes very well arranged, central and portal veins without alterations figure 2(a). The livers of rats treated with CCl_4 for 5 days showed total loss of hepatic architecture with extensive accumulation of connective tissue resulting in formation of continuous fibrotic septa, nodules of regeneration, fatty changes, noticeable alterations in the central vein, hepatic necrosis, vacuolization, congestion of sinusoids, kupffer cell hyperplasia, crowding of central vein, pronounced inflammation from portal to portal tract bridges and apoptosis compared to the normal control (figure 2b). Oral administration of urosodiol shows recovery of hepatic architecture with uniform central vein dilation and precentral hepatitis with lymphomonocytes surrounding the vein. Some little foci were observed in portal triaditis. Only central zone hepatitis as figure 2c. Oral extract of pet ether and ethyl acetate shows chronic active hepatitis around the portal tracts with inflammatory bridges, congestion of sinusoids, crowding of central vein and scattered fatty changes as figure 2d &2f. Chloroform extract treated group shows widespread cell degeneration, both hydropic and fatty changes. There are thick inflammatory bridges between portal tracts with parenchymal collapse. Inflammation around portal tracts includes some loss of lamina limitans figure 2e. among these plant extract, treatment with ethanolic extract returned the injured live to quite normal. Less pronounced destruction of the liver architecture without fibrosis and minimal inflammation. Only peripheral zonal fatty changes were observed as figure 2g.

Table 1. Qualitative Phytochemical analysis of extracts of *Brassica juncea* leaf

Phytochemical constituents	Leaf extract
Alkaloids	+
Saponins	-
Glycosides	-
Tannins & Phenolic Compounds	+
Carbohydrates	-
Triterpenoids	+
Proteins and Amino acids	-
Fixed Oils Fats	-
Flavonoids	+
Volatile oils	+
(+) Present, (-) Absent	

Table 2: Effect of *Brassica juncea* leaf extracts on the activity of serum enzymes in rats at 8th day.

Treatment Groups	Dose	SGPT U/L (%)	SGOT U/L (%)	ALP U/L (%)	Total bilirubin (%)
Group-1 (Normal)	-	68.2 ± 13.48 (47.27)	163.02±28.83 (43.94)	183.20±18.40 (37.64%)	0.36±2.23 (96.14)
Group-2 (Positive Control)	1.5 (ml/kg)	129.28±8.73	290.84±10.23	293.81±7.30	9.33±4.23
Group-3 (Standard)	150 (mg/kg)	62.3±13.19*** (51.81)	164.72±18.19*** (43.36)	189.19±2.40*** (35.60)	0.58±1.24*** (93.78)
Group-4 (Pet ether)	500 (mg/kg)	94.27±10.23*** (27.08)	202.33±24.34*** (30.43)	265.73±13.77 ** (9.55)	5.34±2.25 * (42.76)
Group-5 (Chloroform)	500 (mg/kg)	112.33±12.42* (13.11)	243.40±29.41** (16.31)	245.49±19.77 *** (16.44)	7.03±2.05 ns (24.65)
Group-6 (Ethyl acetate)	500 (mg/kg)	118.31±18.49 ns (8.48)	269.27±36.79 ns (7.41)	267.41±21.43* (8.98)	7.53±1.05 ns (19.29)
Group-7 (Ethanol)	500 (mg/kg)	70.20±11.43*** (45.69)	161.24±22.14*** (44.56)	179.12±25.17*** (39)	2.13±2.05** (77.17)

Figure 1: TLC picture showing the constituents of *Brassica juncea* extracts.

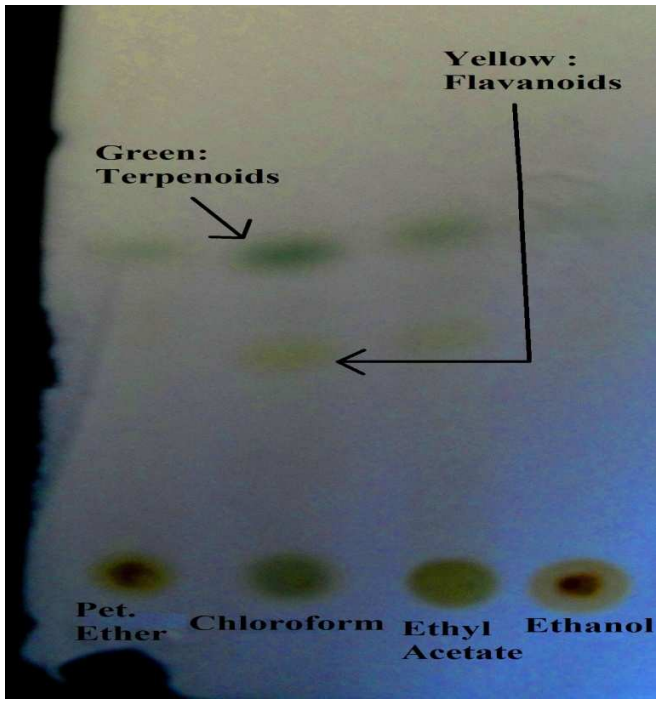


Figure 2 Photomicrograph of the Histopathology of liver with haemotoxylin and eosin (H & E) (magnification 100x).

Figure 2(a) Group 1 (Normal)

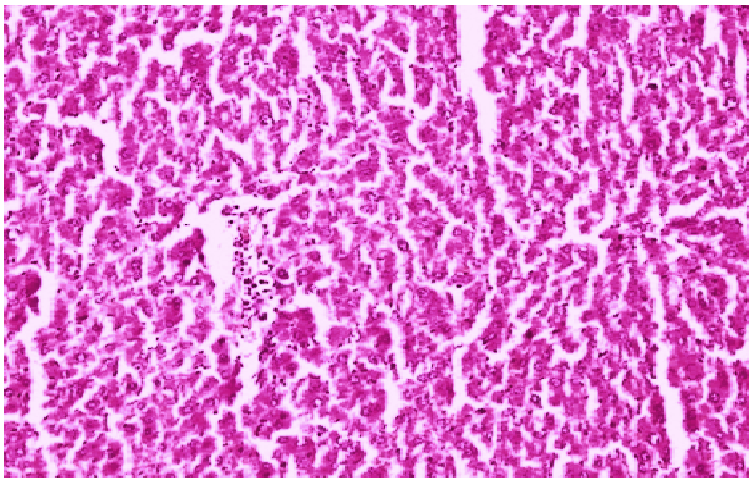


Figure 2 (b) Group 2 (Positive Control)

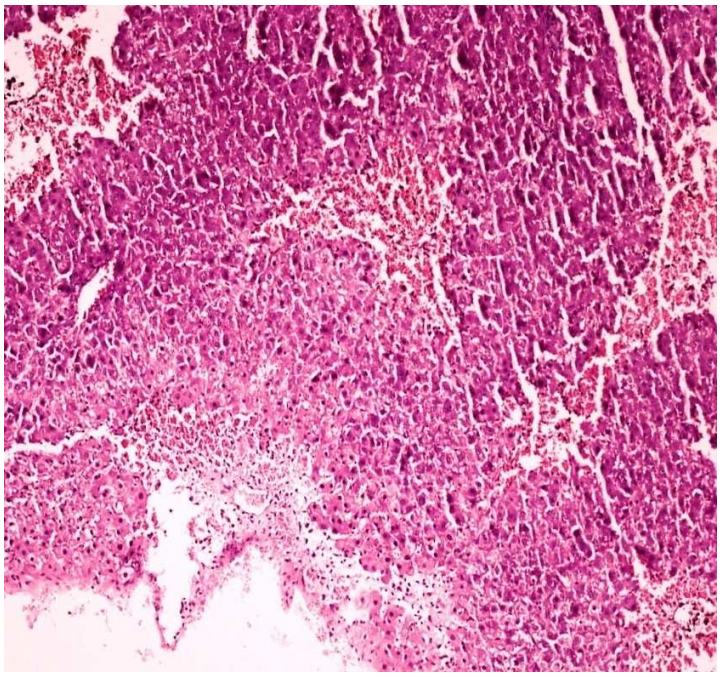


Figure 2(C) Group 3 (Standard)

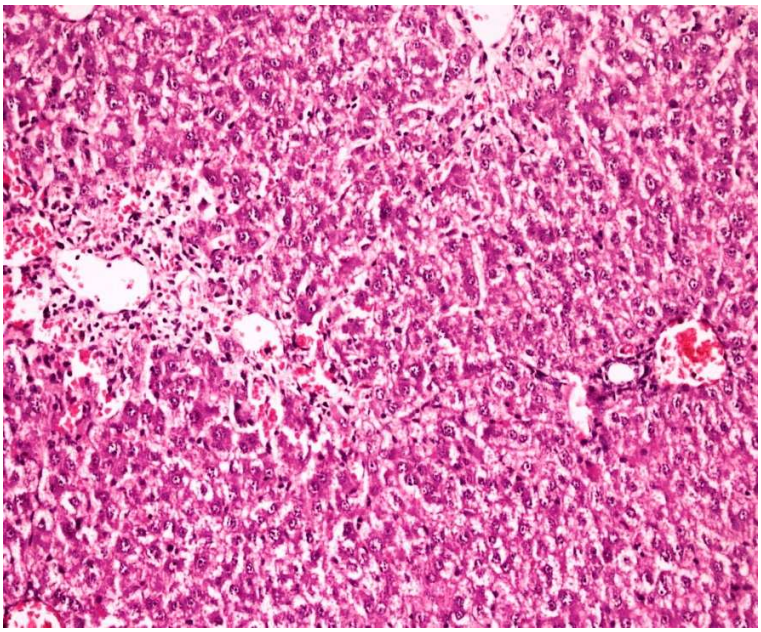


Figure 2(d) Group 4 (Pet ether)

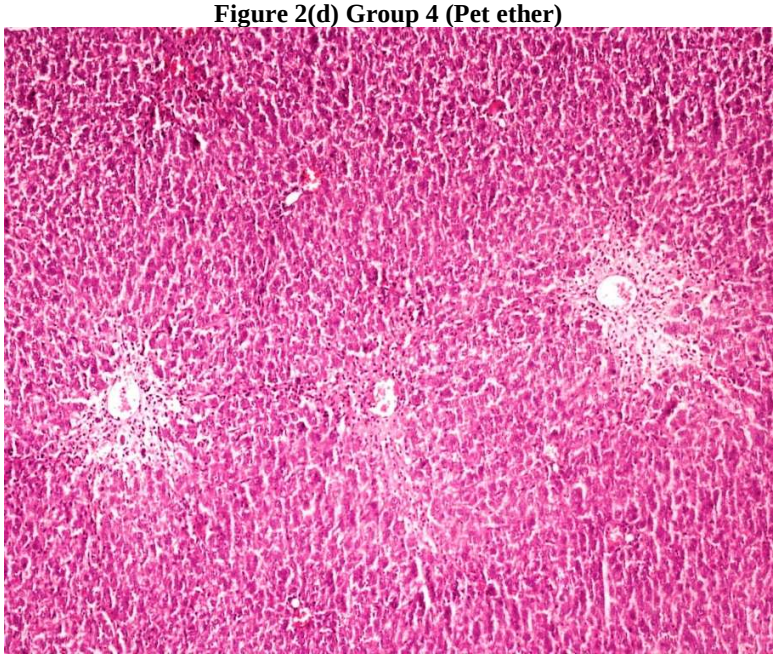


Figure 2(e) Group 5 (Chloroform)

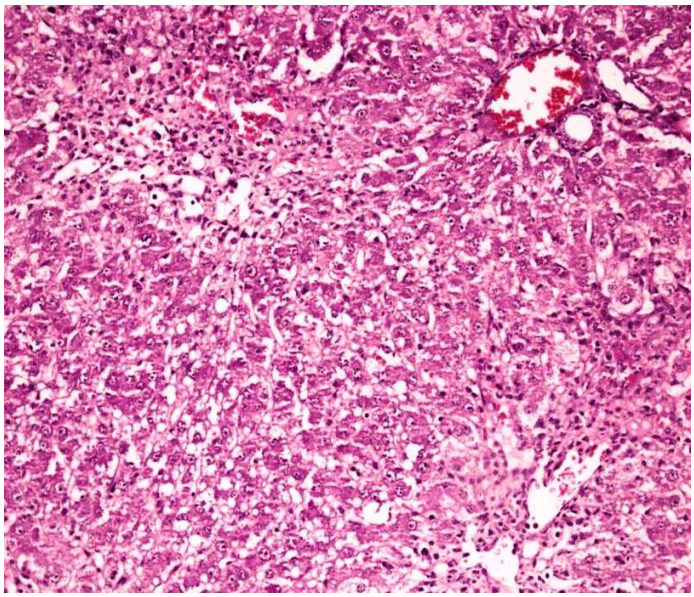


Figure 2(f) Group 6 (Ethyl acetate)

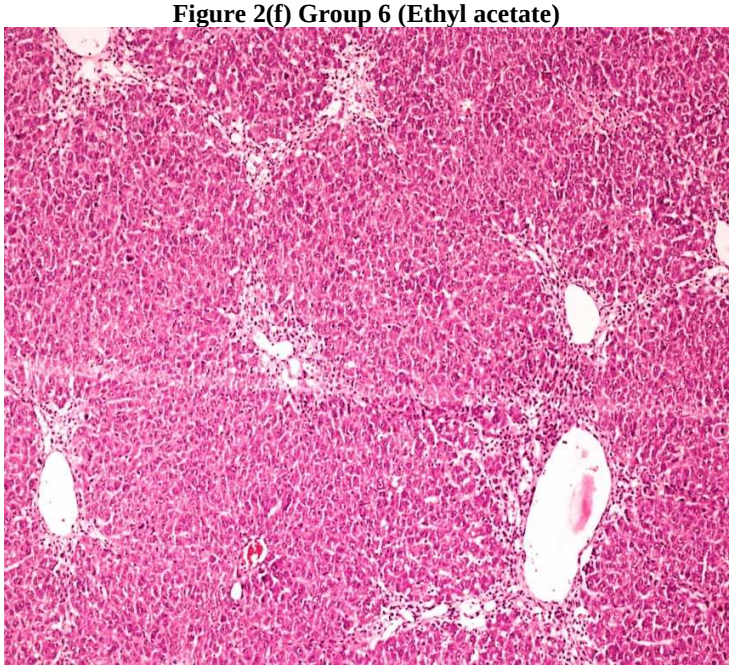
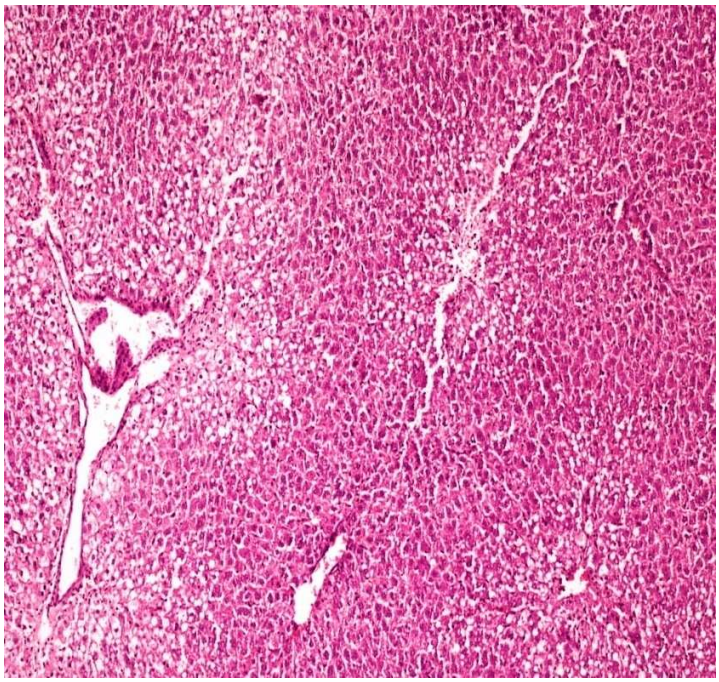


Figure 2(g) Group 7 (Ethanol)



DISCUSSION

Reactive oxygen species (ROS) are causative factors of degenerative diseases, including some hepatopathies. Liver is an important organ actively involved in metabolic functions and is a frequent target of number of toxicants. Carbon tetra chloride has been widely used for inducing experimental hepatic damage due to free radical formation during its metabolism by hepatic microsome, leading to lipid peroxidation, and consequently, liver damage. The resulting hepatic injury is characterized by leakage of cellular enzymes into blood stream and by necrosis and fibrosis [19]. It is selected as hepatotoxicant in inducing injury to the liver as it is known to cause hepatotoxicity in man and experimental animals when take overdose. Popularity of herbal remedies is increasing globally and at least one quarter of patients with liver diseases use ethno-botanicals [20]. Many medicinal plants provide relief of symptoms comparable to that of conventional medicinal agents. Since time immemorial, mankind has made the use of plants in the treatment of various ailments. Many formulations containing herbal extract are sold in the Indian market for liver disorders [21]. Therefore the present study was aimed at evaluating the scientific basis for the traditional use of *Brassica juncea* using invivo experimental model.

Several studies shown that α-tocopherol (Vitamin E) and silymarin are potent antioxidant that could protect the liver against CCl_4 hepatotoxicity indicating that oxidative stress could play a pivotal role in CCl_4 hepatic injury [22]. Assessment of liver damage can be made by estimating the activities of serum ALT, AST, ALP, TB which are enzymes and proteins originally present in higher concentration in cytoplasm. The parameters used to confirm the effects of the herbal medicinal plants in this study were biochemical and histological. The elevated levels of these entire marker enzymes observed in the CCl_4 treated group 2 rats in this present study corresponded to the extensive liver damage induced by toxins, the tendency of these marker

enzymes to return towards a near normalcy in group 3, 4 and group 7 (urosodil and ethanolic extract) treated rats was a clear manifestation of hepatoprotective effect of *brassica juncea*. The protective effect of *brassica juncea* is also proved by histopathological examination of livers of rats treated with ethanolic extracts prior to CCl_4 administration which were almost normal in structure with slight changes. Although the exact mechanisms behind this protection are uncertain, many theories have been proposed. In the last decades, special attention has been paid towards edible plants, especially those that are rich in secondary metabolites and now days, there is an increasing interest in the antioxidant activity of such phytochemical present in diet. Recent reports suggest that cruciferous vegetables act as a good source of natural antioxidants due to the high levels of carotenoids, tocopherols and ascorbic acid and strong epidemiological evidence shows that these compounds may help to protect the human body against damage by reactive oxygen damage by reactive oxygen species. In addition to carotenoids, tocopherols and ascorbic acid, most of the anti-oxidative effect related to plant intake in mainly due to presence of phenolic compounds. However we can assume that the protective factor in this study is due to presence of alkaloids, flavonoids, saponins, tannin and terpenoids. The probable mechanism is mediated by their higher quantity of flavonoids or terpenoids in ethanolic extract or by their combination via antioxidant and free radicals scavenging activities [23]. Flavonoids, phenolic acids and some terpenoids have been reported to possess antioxidant activities by different mechanisms [24].The second probable mechanism is due to effective blocking of oxidative stress and cytokines production. We can believe that in this study, ethanolic extract protected against lipopolysaccaride induced liver damage through decreasing the level of TNF- and IL6 and prevented cytotoxic effect of oxygen free radicals and cytokines [25]. However, further studies will be required on molecular level and isolation of active constituents to substantiate this effect. In last conclusion represent the present study demonstrated that ethanolic and pet ether extract in comparison with other extracts groups of *Brassica juncea* has better hepatoprotective effect in CCl_4 induced liver damage. However, it is necessary to determine other parameters such as oxidative stress markers and molecular biology assays to confirm our findings. Also, we need to isolate and purify the active of this plant and to determine its mechanism of action.

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