INTRODUCTION

Asteraceae is a plant family having about 25000 to 30000 species and more than 1000 genera. Most of its species are used as sources of rubber, medicines, edible oils, vegetables and pesticides etc. Out of 1000 genera, some are most famous as ornamental plants. *Aster, Inula, Xanthium, Eupatorium, Carpesium, Saussurea* and *Taraxacum* are some genera having medicinal importance. Sesquiterpenoids are natural products found abundantly in plants of this family. Phytochemical studies reported the presence of 1000 natural eudesmanoids along with many different oxygenation and cleavage patterns.

Different phytochemical, pharmacological and synthetic studies conducted on eudesmane-type sesquiterpenoids revealed the use of these plants in the treatment of bacterial, fungal and neoplastic diseases. These compounds are also proved in plant growth regulator activities [1]. Two new triterpenoids, 18alpha, 19beta-20(30)-taraxacan-3beta, 21alpha-dio (chichoridol) and 17-epi-methyl-6-hydroxyangolensate (intybusolidol) are obtained from methanolic extract of seeds of *Citrus intybus*.

The water extract of *Citrus intybus* has an antioxidant effect on LDL and inhibits the production of thioarbituric acid reactive substance and the degradation of fatty acids in LDL [2]. Aqueous, ethanolic and ethyl acetate extracts of *Citrus intybus* have antibacterial activity. The activity of ethyl acetate proved to be the most among all. Aqueous extract has activity against *Agrobacterium radiobacter* species, *Tumefaciens, Erwinia carotovora, Pseudomonas fluorescens* and *P. aeruginosa* [3]. Ethanolic extract of *Citrus intybus* (CLE) has hypoglycemic and hypolipidemic properties. This has been used widely in India as a traditional treatment for diabetes mellitus [4]. *Cichorium* B and C are present in *Citrus intybus* which are two new benzisoschromenes [5].

RESULTS

Phytochemical analysis of crude extract (CLE) indicated that it highly contains saponins, along with tannins, cardiac glycosides, terpenes and sterols (Table 1). Effects of CLE on serum enzyme showed that the serum ALP, SGOT, SGPT and TB levels in normal control group were very close to values of vehicle control group. This indicated that DMSO had least effects on serum enzymes level in albino rats. The levels of serum marker enzymes in intoxicated group were very high in comparison to normal control group. It represented that Nimesulide clearly produced toxicity in rats. However, there was remarkable reduction in serum enzymes in standard control group (Table 2). It showed that there was no any effect of DMSO on serum enzyme levels. Crude extract of *Citrus intybus* prevented the elevation of serum enzyme markers in all three doses. Statistical analysis indicated that aqueous ethanolic extract of *Citrus intybus* leaves significantly (P < 0.001) reduced serum enzyme markers at dose level of 200 and 300 mg/kg p.o while CLE 100 mg/kg p.o reduced the enzymes with significance level of P < 0.01.

Similarly, Histopathological examination of the liver tissue (figure 5) from Nimesulide treated animals revealed that it had produced profound ballooning degeneration, inflammation, apoptotic cells, fibrosis and congestion especially in sinusoids. Pretreatment with Silymarin, CLE (100 mg/kg po), CLE (200 mg/kg po) and CLE (300 mg/kg po) reduced the inflammation and degenerative changes.

DISCUSSION

Hepatoprotective activity of any substance can be found by assessing the level of serum hepatic markers (ALP, SGOT, SGPT and TB). When hepatocytes are damaged then hepatic enzymes are leaked into serum. Thus level of enzymes in serum is increased [6]. Different chemicals and drugs damage the hepatocytes which results into leakage of enzymes (ALT and AST) in blood while animals pretreated with crude extract of different plants prevent the elevation of enzyme levels. Serum enzyme level returns to normal when hepatic cells are regenerated [7].

Nimesulide was used to induce hepatic cell damages in albino rats. It significantly (P < 0.001) raised the level of hepatic enzymes (ALP, SGOT, SGPT and TB). Histopathological analysis of photomicrographs of intoxicated control group represented that it produced marked inflammation in hepatocytes along with apoptosis, fibrosis and large areas of ballooning-degeneration as shown in figure 5c. The mechanism by which Nimesulide damaged the cell is that it impaired the synthesis of ATP from mitochondria along with disorganization of their nitro group. Moreover, it damaged the cells by causing a metabolic block with target proteins, by oxidative-reductive pressure, immune-mediated interactions, interference with hepatobiliary transportation and by mitochondrial injury [8].
Phytochemical analysis indicated that C.I.E is enriched with saponins, tannins, glycosides, terpenes and sterol. Tannins are responsible for hepatoprotective activity [9]. Saponins (sialosaponins) prevent peroxidation of lipids by scavenging toxic agents [10]. Saponins present in plant extract might have role in scavenging of free radicals produced in liver by metabolism of Nimesulide. Flavonoids, glycosides and triterpines found in different plant species have anti-oxidative potential and prevent hepatocellular damage. The plant has anti-oxidative and anti-inflammatory properties. Its hepatoprotective action is due to presence of natural anti-oxidants [11].

It is acting an antioxidant due to presence of both prooxidant and biological antioxidant constituents [12]. Cichorium intybus has potential to prevent nitrosamine induced oxidative damage of hepatocytes [13]. Proposed mechanism of hepatoprotective action of C.I.E is that it might scavenge free toxic species in liver. Hepatoprotective studies claimed that different plants possess different active chemical constituents which scavenge free radicals [14].

Hepatotoxic agents like CCl4 and Paracetamol produce histopathological changes (steatosis and fibrosis) in hepatocytes [15]. Hepatocellular necrosis, fibrosis and lymphocyte infiltration was observed in photomicrographs of rat livers [16]. Photomicrographs of liver slides of randomly selected rats of experimental control groups showed that less hepatic damage occurred in hepatocytes. C.I.E generated fewer score of hepatocellular damages (ballooning-degeneration, apoptosis, inflammation and fibrosis) as shown in figures 5e, 5f and 5g in comparison of intoxicated control group (figure 5c).

**MATERIALS AND METHODS**

The approval of this study (Ref. No. 1560/Pharm) was taken from the Board of the Advanced Study and Research (BASAR), the Islamia University, Bahawalpur and the Institutional Ethical Committee, Faculty of Pharmacy and Alternative Medicine, the Islamia University, Bahawalpur.

**Plant Material**

Green fresh leaves of plant were collected from local fields of Sahiwal division. Plant material was then identified by the botanist and specimen was preserved in the herbarium vide Voucher No.CI-LE-04-12-045 at the Faculty of Pharmacy and Alternative medicine, the Islamia University of Bahawalpur, Pakistan.

**Preparation of Extract**

Plant material was properly washed with water and dried properly. Completely dried material was then ground to coarse powder by using electric grinder (National, Japan). 1000 g of ground powder was macerated in 70% aqueous ethanol for five days. Soaked material was thoroughly stirred thrice daily. At the end of 5th day of maceration, it was filtered through muslin cloth and then through Whatmann filters paper No. 1. Residue was again macerated to obtain more filtrate. This was repeated thrice and filtrate obtained after three soakings was evaporated by using rotary evaporator at 30-40°C. In the end, thick, viscous, semisolid paste of dark brown color was obtained. The paste obtained was weighed out to find percentage yield. The extract obtained was 212 g and percentage yield calculated was 21.2%. The extract was packed in air tight container and labeled as C.I.E. It was then put into refrigerator for future use [17].

**Pharmacological Materials**

Diagnostics kits (ALP, SGOT, SGPT and TB), Ethanol, Formalin, Xylene, Paraffin Wax, Eosin, Hematoxylin, Canada balsam and Nimesulide. All the chemicals of analytical grade were purchased from Merck, Human-Germany and Nimesulide was donated by Sami Pharmaceuticals, Pakistan upon request. Silmoran was purchased from Abbott Laboratories, Pakistan. Ketamine and Dizepam were purchased from local Pharmacy.

**Phytochemical Analysis**

Different secondary metabolites are present in plant materials which exhibit various pharmacological activities [18]. Crude extracts were subjected to phytochemical analysis for identification of alkaloids, cardiac glycosides, steroids, tannins, and saponins. Following methods were used for analysis.

**Tests for Saponins**

**Foam test:** 500 mg of crude extract was dissolved in boiling water in test tube. Then it was cooled down and vigorously shaken to produce the forth [19]. Presence of forth indicated the saponins.

**Tests for Tannins**

**Ferric chloride test:** Extract was dissolved in 10 ml of distilled water and then filtered. 1% aqueous or alcoholic FeCl3 was added in filtrate which produced intense green, purple, blue or black colour which indicated the tannins.

**Iodine test:** Extract was treated with dilute iodine solution. Formation of transient red colour indicated the presence of tannins.

**Nitric acid test:** extract was treated with dilute nitric acid and the formation of reddish to yellow colour indicated the presence of tannins.

**Gelatin test:** 0.5 g of extract was mixed with 1% gelatin solution containing 10% NaCl. Formation of white precipitates indicated the presence of saponins [20].

**Test for Alkaloids**

500-600 mg of crude extract was treated with 8 ml of 1% HCl, warmed on water bath and then filtered and divided in to four test tubes.

**Hager's test:** 2 ml of filtrate was mixed with few drops of Hager's reagent (saturated aqueous solution of picric acid). Appearance of turbidity or yellow precipitates indicated the presence of alkaloids.

**Wagner's test:** 2 ml of filtrate was mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitates indicated the presence of alkaloids.

**Dragendorff's test:** 2 ml of filtrate was mixed with Dragendorff's reagent. Appearance of turbidity or precipitates indicated the presence of alkaloids.

**Mayer's test:** 2 ml of filtrate was mixed with Mayer's reagent. Appearance of turbidity or precipitates indicated the presence of alkaloids [21].

**Tests for Glycosides**

**Keller-Kiliiani Test:** Took extract solution in test tube and added few drops of FeCl3 in it. Concentrated CH3COOH and concentrated H2SO4 were added carefully along the wall of test tube. Reddish brown coloration at the junction of both layers and bluish green color at the upper layer indicated the presence of glycosides.

**Tests for terpenes and Sterols**

**Libermann-Burchard Test:** 30 ml of crude extract was added in petroleum ether. Petroleum ether was evaporated to get dry residue. Residue was then extracted with 20 ml of chloroform and the chloroform layer was then treated with anhydrous sodium sulphate. 0.5 ml of acetic anhydride was mixed with 5 ml of chloroform layer. Then two drops of concentrated H2SO4 was added which gave green, blue and pink to purple colors. Green to pink color indicated the presence of sterols while pink to purple colors is proof of presence of triterpenes [22].

**Experimental Animals**

Sprague-Dawley albino rats of both sexes weighing 180-200 g were used in this study. All animals were kept in Polycarbonate cages of size 47x34x18 cm3 in animal house of Faculty of Pharmacy and Alternative Medicine. They were provided standard temperature (25 ± 2°C) and humidity (50-55 %) along with exposure of 12:12 hours light and dark cycle till end of study. Before initiation of experiments, the rats were acclimatized for one week and provided with free excess of water and food.
Induction of Hepatotoxicity

Nimesulide was used to induce hepatotoxicity in albino rats. Nimesulide was solubilized in Dimethyl sulfoxide (DMSO) and administered orally on daily basis. Although this is very effective NSAID yet it is associated with severe adverse effects like hepato-biliary, cutaneous and gastrointestinal system. Acute hepatitis, fulminant hepatic failure, cholestatic liver injury, multiple enterocolic perforations and end stage renal failure with Nimesulide intake have been reported in various case reports of hepatotoxicity. Even fatal hepatic failure leading to withdrawal of drug in various countries but this is still in practice in some developing countries [8].

Hepatoprotective Activity

For evaluation of hepatoprotective activity of crude extract of Cichorium intybus, Albino Sprague-Dawley rats of both sexes weighing 180-200 g were divided into seven groups with seven animals in each group. Group-I received normal saline at dose of 5ml/kg p.o. once daily. Group-II was given DMSO at dose of 5ml/kg p.o. Group-III received Nimesulide 100 mg/kg p.o. for seven days to produce hepatotoxicity. Group IV was Standard Control given Silymarin alone for first eight days at dose of 25 mg/kg p.o. and then along with Nimesulide (100 mg/kg p.o.) for further seven days. Group IV-VI was given crude extract alone at dose of 100, 200 and 300 mg/kg p.o. respectively for first eight days and then Nimesulide in dose of 100 mg/kg p.o. along with plant extracts to study the hepatotoxicity for further seven days. 24 hours after the last treatment dose, the animals were given anesthesia by administration of diazepam (5 mg/kg i.p.) and ketamine (50 mg/kg i.p.). Animals were dissected and 3ml of blood was taken by cardiac puncture from each rat. Serum was collected by centrifugation of each sample of blood and then levels of serum enzymes were monitored by using diagnostic kits.

Histopathology

Diazepam was injected in dose 5 mg/kg i.p. to induce hypnosis before induction of anesthesia. Then Ketamine (50 mg/ kg i.p) was injected to induce anesthesia. After that rats were dissected and livers were preserved in 10 % formalin. Liver sections were dehydrated in ethanol, cleared in xylene and then fixed in paraffin. 4-5 µm sections were cut to prepare slides and hematoxylin and eosin dye was used for staining slides [16].

Table 1: Phytochemical constituents of Cichorium intybus (CLE)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Phytochemical Tests</th>
<th>Phytochemical Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saponins</td>
<td>+ve</td>
</tr>
<tr>
<td>2</td>
<td>Foam Test +</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Haemolysis Test</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>Tannins</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Iodine Test</td>
<td>-ve</td>
</tr>
<tr>
<td>6</td>
<td>Ferric Chloride Test</td>
<td>+ve</td>
</tr>
<tr>
<td>7</td>
<td>Nitric Acid Test</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>Gelatin Test</td>
<td>+ve</td>
</tr>
<tr>
<td>9</td>
<td>Alkaloids</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Hager’s Test</td>
<td>-ve</td>
</tr>
<tr>
<td>11</td>
<td>Wagner’s Test</td>
<td>+ve</td>
</tr>
<tr>
<td>12</td>
<td>Mayer’s Test</td>
<td>-ve</td>
</tr>
<tr>
<td>13</td>
<td>Dragendorf Test</td>
<td>-ve</td>
</tr>
<tr>
<td>14</td>
<td>Cardiac glycosides</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Keller Killani Test</td>
<td>+ve</td>
</tr>
<tr>
<td>16</td>
<td>Terpenes and sterols</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Libermann-Burchard test</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Note: (+) and (-) signs repot the relative presence and absence of constituents in ClE.

Table 2: Effects of different doses of Cichorium intybus extract (CLE) on ALP, SGOT, SGPT and TB level in Nimesulide intoxicated albino rats

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment Group</th>
<th>Level of ALP (IU/L)</th>
<th>Level of SGOT (IU/L)</th>
<th>Level of SGPT (IU/L)</th>
<th>Level of TB (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Control</td>
<td>220.77 ± 15.56</td>
<td>112.24 ± 5.27</td>
<td>51.60 ± 4.35</td>
<td>0.85 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle Control</td>
<td>219.17 ± 15.92</td>
<td>108.61 ± 4.22</td>
<td>51.04 ± 4.35</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>Intoxicated Control</td>
<td>889.01 ± 24.71***</td>
<td>223.29 ± 7.57***</td>
<td>115.57 ± 5.67***</td>
<td>3.60 ± 0.16***</td>
</tr>
<tr>
<td>4</td>
<td>Standard Control</td>
<td>260.16 ± 17.91</td>
<td>116.69 ± 5.76</td>
<td>58.03 ± 3.34</td>
<td>0.95 ± 0.15</td>
</tr>
<tr>
<td>5</td>
<td>CLE 100 mg/kg</td>
<td>565.89 ± 27.33***</td>
<td>193.69 ± 3.72***</td>
<td>93.50 ± 2.98***</td>
<td>2.91 ± 0.13***</td>
</tr>
<tr>
<td>6</td>
<td>CLE 200 mg/kg</td>
<td>453.26 ± 20.05***</td>
<td>145.86 ± 4.37***</td>
<td>78.84 ± 2.86***</td>
<td>2.85 ± 0.15***</td>
</tr>
<tr>
<td>7</td>
<td>CLE 300 mg/kg</td>
<td>399.43 ± 16.47***</td>
<td>165.10 ± 4.29***</td>
<td>88.01 ± 2.40***</td>
<td>2.52 ± 0.15***</td>
</tr>
</tbody>
</table>

P-values: *** ≤ 0.001 vs. vehicle control, **=0.05, *<0.05, "<0.01, ""<0.001 vs. intoxicated control

[Values are mean ± SE from 7 animals in each group]

Fig. 1: Effect of different doses of CLE extract on ALP level in Nimesulid intoxicated albino rats.
Fig. 2: Effect of different doses of CLE extract on SGOT level in Nimesulid intoxicated albino rats.

Fig. 3: Effect of different doses of CLE extract on SGPT level in Nimesulid intoxicated albino rats.

Fig. 4: Effect of different doses of CLE extract on TB level in Nimesulid intoxicated albino rats.
Statistical Analysis of Results

Results were expressed as Mean ± SEM (n=7). Student t test was applied. P values were considered as P > 0.05 non-significant (ns), and P < 0.05 as significant.

CONCLUSION

On the basis of results it is concluded that aqueous-ethanolic extract of Cichorium intybus (Ci.E) fresh dried leaves has major role in preventing Nimesulide induced hepatocellular damage in albino rats. There was marked reduction in level of four liver markers ALP, SGOT, SGPT and TB by the use of extract in 300 mg/kg as compared to other two doses.

The present investigations strongly strengthen the use of Cichorium intybus as hepatoprotective plant because it was scientifically proved that the plant is a potential source of useful drug due to the presence of phytochemical constituents. Histopathological studies of experimental control groups clearly supported the hepatoprotective functions of crude extract (Ci.E) because of decreased destructive pattern of hepatocytes. So it can be used for the treatment of hepatic diseases and also exploited for the use in pharmaceutical industries. However, further studies are required to isolate the active principle form the crude extract for proper drug development.

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