Effects of *Cichorium intybus* on serum oxidative stress, liver and kidney volume, and cyclin B1 and Bcl-2 levels in the brains of rats with ethanol induced damage

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Abstract: We investigated the effects of an aqueous root extract of *Cichorium intybus* on Bcl-2 and cyclin B1 levels in the brain, kidney and liver volumes and changes of serum total antioxidant status (TAS) and total oxidant status (TOS) levels in ethanol induced damage in rats. The rats were divided into five groups: non-treated controls (C), maltodextrin in tap water treated (MD), 6.4% ethanol in tap water treated (ET), *Cichorium intybus* + maltodextrin in tap water treated (C1+MD), and *Cichorium intybus* + 6.4% ethanol in tap water treated (C1+ET). Rats in the C1+MD and C1+ET groups were treated with 200 mg/kg water extract of *Cichorium intybus*. Chronic ethanol administration significantly increased cyclin B1 and decreased Bcl-2 levels in the brain and significantly decreased TAS values, increased TOS values of serum and significantly decreased kidney volume in the ET group. There was no significant difference in the liver volume or liver cell count. Our data revealed that ethanol administration induces an overexpression of cyclin B1 and decreases levels of Bcl-2 in rat brains and induced oxidative stress in the blood. *C. intybus* treatment possessed a partial amelioration effect on cyclin B1 levels and TAS values.

**Key words:** Bcl-2; Brain; *Cichorium intybus*; Cyclin B1; Ethanol; Oxidative stress.

Introduction

Alcoholism is a serious medical, economic and social problem (1). The liver is a vital organ due to its role in the detoxification of various substances (2). The metabolism of ethanol occurs primarily in liver (3) as well as in the brain (4). Therefore, the liver is a prime recipient of the harmful effects of alcohol (5).

Alcohol toxicity is associated with an increase in the formation of free radicals and the progression of oxidative stress (6). Other organs including the brain are also affected by alcohol (5). For example, ethanol exposure causes brain damage (7). Alcohol also causes various types of neuronal damage; it decreases neurogenesis in animals (8), prolongs the cell cycle and may induce neuronal death in cultured cells (9, 10). Cyclin B1, a protein that plays an important role in the management of the cell cycle from the G2 to M phases (11), may also play a significant role in neuronal survival (12).

Alcohol is also reported to increase apoptotic neuronal death (9). It was suggested that Bcl-2, an anti-apoptotic protein (13), protects neurons from ethanol neurotoxicity (14). Chronic alcohol consumption causes a decrease in Bcl-2 (13) but the effects of ethanol on cyclin B1 levels in the brain are unknown.

Recent studies have focused on finding effective plants for the treatment of diseases related to alcoholism (15). Medicinal plants provide molecules which can be candidates for the treatment of various diseases and conditions. The plants are also valued to flavour foods, giving the food a dual role, i.e. flavour and bioactive compounds (16-21). *Cichorium intybus* L. (commonly known as chicory) was cultivated as a medicinal plant in ancient Egypt (22). The root of the *C. intybus* is used as coffee, a substitute or food ingredient after processing and the aerial parts are consumed in salads (23). Different parts of the *C. intybus* are used in traditional medicines worldwide (24). The phytochemicals are present throughout the whole plant, but are primarily found in the roots (25). *C. intybus* roots have anti-diabetic (26), anti-oxidative (27), anti-inflammatory (22, 28) and antihepatotoxic effects (29). As well, the roots are used as hepatoprotective agents (30). Various studies have reported that *C. intybus* possesses significant hepatoprotective properties against carbon tetrachloride-induced liver damage (31) and hepatocellular damage (29). *C. intybus* is reported to have hepatoprotective effects on isoniazid induced hepatotoxicity (32) and ameliorates the oxidative stress and hepatic injury induced by 4-tert-octylphenol (33). In light of these studies, the hepatoprotective properties of *C. intybus* are well known, however there is no study concerning the effects of *C. intybus* on ethanol induced damage of the liver kidney, brain or blood. Therefore, the aim of this study is to evaluate the protective effects of the aqueous root extract of *C. intybus* on the volumes of the liver and kidney, Bcl-2 and cyclin B1 levels of the brain, total antioxidant status (TAS) and total oxidant status (TOS)
levels of serum and oxidative stress index (OSI) in ethanol administered rats.

Materials and Methods

Plant material

*C. intybus* plant was collected from the Van Yuzuncu Yil University campus, (38°34’23.1” N, 43°17’17.8” E) Van, Turkey, during autumn 2014. Following identification in the Faculty of Science, Department of Biology, a voucher specimen was prepared with a code number VANF163742.

Preparation of extract

*C. intybus* roots were dried for one month, crushed and sieved with a mesh size of 1mm. The powder of the plant was suspended in distilled water. It was kept in a 70°C water bath for 50 minutes while stirring frequently. The prepared mixture was left to cool for 10 minutes. Aqueous extract of the plant was obtained after filtering through filter paper.

Animals and treatments

Two-months-old male Wistar albino rats were obtained from the Van Yuzuncu Yil University Animal Experiments Unit. Animals were kept in a 12-hour dark-light period in a room with a constant temperature (21°C). Food and ethanol solution or maltodextrin solution or tap water were available *ad libitum*. The experimental protocol was approved by the Ethics Committee of Van Yuzuncu Yil University.

Rats were randomly divided into five groups (n=7). (1) Intake of tap water for 18 days (Control), (2) Intake of maltodextrin for 18 days (MD), (3) Chronic intake of ethanol for 18 days (ET), (4) Ethanol intake plus C. intybus treated for 18 days (CI+ET), (5) Maltodextrin intake plus C. intybus treated for 18 days (CI+MD). Ethanol was aMDinistered in drinking water at a concentration of 6.4 % v/v for 18 days. Control animals received tap water for 18 days. Rats in the ET and CI+ET groups received 6.4% ethanol (Sigma-Aldrich Co., St. Louis, MO) in tap water. Rats in the MD and CI+MD groups received maltodextrin in tap water (equivalent to ethanol derived calories). Maltodextrin was added to the tap water to provide a similar caloric intake to groups (34) which did not consume alcohol. The C. intybus water extract (200 mg/kg) (32) was aMDinistrated daily to the rats by gastric gavage in the CI+ET and CI+ MD groups for 18 days. Blood, brain, liver and kidney tissues were obtained from rats under general anaesthesia with 15 mg/kg xylazine and 50 mg/kg ketamine.

Histopathological analysis

The liver and kidney organs were treated with a 10% formaldehyde solution for 72 hours then embedded within paraffin. Paraffin sections of the liver and kidney (5 μm) were stained with hematoxylin and eosin. Investigations were performed under a light microscope. For photographs AxioVision 3.1 Zeiss axioplan 2 imaging (Germany, Göttingen) were used. For evaluation of stereological analysis, a modified method of the Cavalieri principle was used (an unbiased counting frame used during the numerical analysis at x40 magnification (35). Then total tissue volume rates were calculated via pointed ruler provided in Shtereom 1.5 version software (36, 37). Volume (mm³) was estimated using the following formula:

\[ \text{Volume: } t \times \text{ap} \times \Sigma P \]

where \( t \) is section thickness, \( \text{ap} \) is the area of each point on the point-counting grid, and \( \Sigma P \) is the total number of points hitting the related area (35).

Measurement of serum TAS, TOS and determination of OSI

Serum TAS (38) and TOS (39) were determined spectrophotometrically using kits (Rel Assay Diagnostics kit; Mega Tip, Gaziantep, Turkey). OSI is defined as the ratio of TAS to TOS value and calculated with the formula (40):

\[ \text{OSI (arbitrary unit)} = \frac{[\text{TOS (μmol H}_2\text{O}_2 \text{ Eq/L)]}}{\text{TAS (μmol Trolox Eq/L)}} \times 100 \]

Western blot analysis

Tissues obtained from the brain were homogenized by steel beads in a NP-40 lysis buffer (2 mM Tris–Cl pH 7.5, 150 mM NaCl, 10% glycerol and 0.2% NP-40 plus a protease inhibitor cocktail). Following centrifugation (Centric 200R) at 14.000 RPM for 10 min at 4°C, the final supernatant was used as total protein. Protein quantities were measured with the Bradford method (41). All samples equated to the same total protein amount by using a lysis buffer as a diluent. Then, 5x loading buffer was added to the upper layer of the obtained supernatant. The mixture was boiled at 100°C for 15 min. All cellular proteins were electrophorized on 4–12% SDS-PAGE acrylamide gels and transferred onto polyvinylidene fluoride membranes (PVDF). They were incubated for 1 hour in tris-buffered saline and 5% Tween (TBS-T) containing 5% skim milk powder (Sigma). The incubation of membranes was conducted overnight at 4°C with primary antibodies against β Tubulin (ProteinTech, 1:1000), Bcl-2 (ProteinTech, 1:1000) and cyclin B1 (ProteinTech 1:1000) washed in TBS-T and incubated for 1 hour at room temperature with a corresponding horseradish peroxidase (HRP) conjugated anti-rabbit antibody (Santa Cruz Biotechnology, 1:5000). Development of the membranes was performed with the SuperSignal West Pico HRP substrate kit (Pierce) and each membrane was filmed with a chemiluminescent imaging system (Chemidoc MP, Biorad). Image J analysis software was used to determine the integrated intensity of individual bands (42).

Statistical analysis

SPSS for Windows was used as the statistical analysis program. Kruskal Wallis and Tukey tests were used for statistical analysis. Values are expressed as median. The number of repeats in the groups is 7.

Results

Histopathological results

Sinusoids of normal width and regular hepatocyte columns were observed in the microscopic assessment of the liver tissues of the control group (Figure 1a). Normal orientation of hepatocyte columns was found to be degenerated and formed irregular cell formations in the liver tissues of the ET group. In addition, the sinusoid
Figure 1. (a) Vena centralis (VC), hepatocyte columns (thick arrow) and sinusoid structure (thin arrow) in the liver of control group (hematoxylin and eosin, x40). (b) Vena centralis (VC), irregular hepatocyte columns (thick arrow), narrowed sinusoid structure without continuity (thin arrow) and hepatocyte bearing degenerative features and its nucleus (dashed arrow) in ET group (hematoxylin and eosin, x40). (c) Vena centralis (VC), hepatocyte columns with normal appearance (thick arrow) and sinusoid structure (thin arrow) in the liver of CI+ET group (hematoxylin and eosin, x40). (d) General appearance of kidney tissue of control group. Glomerule (G), Bowman capsule (B) and tubule (T) structures are visibly normal in structure (hematoxylin and eosin, x20). (e) General appearance of kidney tissue of ET group. Glomerule (G), narrowed Bowman capsule (B) and nucleus of epithelial cells of degenerated tubule epithelial cells (thin arrow) structures are visible (hematoxylin and eosin, x20). (f) General appearance of CI+ET group. Glomerule(G), Bowman capsule (B) and tubule (T) are normal in structure (hematoxylin and eosin, x20).

Table 1: Descriptive statistics and results of comparisons among groups for kidney volumes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Median</th>
<th>Mean</th>
<th>St. Dev</th>
<th>Minimum</th>
<th>Maximum</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.52a</td>
<td>18.99</td>
<td>1.69</td>
<td>16.09</td>
<td>20.81</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>98.78b</td>
<td>158.88</td>
<td>67.51</td>
<td>62.20</td>
<td>207.78</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>122.87b</td>
<td>151.90</td>
<td>21.16</td>
<td>83.37</td>
<td>136.36</td>
<td>0.033</td>
</tr>
<tr>
<td>CI+DM</td>
<td>204.54a</td>
<td>204.71</td>
<td>20.08</td>
<td>180.79</td>
<td>228.94</td>
<td></td>
</tr>
<tr>
<td>CI+ET</td>
<td>102.60b</td>
<td>113.31</td>
<td>58.51</td>
<td>56.23</td>
<td>202.36</td>
<td></td>
</tr>
</tbody>
</table>

C = control, DM = dextrinated maltose treated group, ET = ethanol treated group. CI+DM = Cichorium intybus + dextrinated maltose treated group, CI+ET = Cichorium intybus + ethanol treated group. Different letters indicate significant differences between groups (p<0.05).

Structure was significantly narrowed and lost continuity in some regions. In this group, vacuolar spaces in hepatocytes, and degeneration of hepatocyte cells and nucleus in some areas were observed (Figure 1b). In the CI+ET group, the liver was normal in appearance under the microscope with regular hepatocyte cords. In addition, sinusoidal structures were within normal width and normal properties (Figure 1c).

Stereological evaluation of tissues showed that the total tissue volume of livers in the CI+ET group was similar to the control group. When these two groups were compared to the ET group, total tissue volume of the liver in the ethanol group was found to be higher without statistical significance. When groups were assessed according to the stereological cell count of the liver, the highest cell number was observed in the CI+ET group. However, this increase was not found to be statistically significant.

In the kidneys of the control group, the microscopic view showed the Bowman capsule with normal width and regular tubular epithelium (Figure 1d). In the kidneys of the ET group, the Bowman capsule width was lower and the structure and ordering of tubule epithelial cells were deteriorated (Figure 1e). In the CI+ET group the Bowman capsule was found to be normal in width and tubule epithelial cells were regular and normal in structure (Figure 1f).

The kidney volumes of ET, CI+ET and MD groups were significantly decreased compared to the control. The kidney volume in the CI+MD group was found to be similar to the control (p<0.05, Table 1).

Oxidative stress parameters

TAS values decreased significantly in all groups compared to the control (p<0.001, Table 2). Compared to the ET group, TAS values were significantly increased in the CI+ET group (p<0.001, Table 2). On the other hand, TOS values were significantly increased in all groups compared to the control (p<0.001, Table 2). Consequently, OSI values were found to be higher in all groups compared to the control (p<0.001).

Western results

According to western analysis the expression level of Bcl-2 protein in the brain was significantly decreased in the ET, CI+ET and CI+MD groups compared to the control group (p<0.001). Compared to the ET and CI+ET groups the expression level of Bcl-2 protein in the CI+MD group shows a significant increase in the brain (p<0.001). Bcl-2 levels of the control and MD groups were found to be similar (Table 3, Figures 2a, b).

The expression level of cyclin B1 protein in the brain was significantly increased in ET, MD, CI+ET, and CI+MD groups compared to the control (p<0.001) (Table 4, Figures 2c, d). Compared to the ET group

Table 2: TAS, TOS and OSI results among groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>TAS</th>
<th>TOS</th>
<th>OSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.815</td>
<td>6.540</td>
<td>0.016</td>
</tr>
<tr>
<td>ET</td>
<td>2.020</td>
<td>19.600</td>
<td>0.101</td>
</tr>
<tr>
<td>DM</td>
<td>2.070</td>
<td>24.000</td>
<td>0.109</td>
</tr>
<tr>
<td>CI+DM</td>
<td>2.213</td>
<td>35.734</td>
<td>0.174</td>
</tr>
<tr>
<td>CI+ET</td>
<td>2.375</td>
<td>35.200</td>
<td>0.148</td>
</tr>
</tbody>
</table>

C = control, DM = dextrinated maltose treated group, ET = ethanol treated group. CI+DM = Cichorium intybus + dextrinated maltose treated group, CI+ET = Cichorium intybus + ethanol treated group. Different letters indicate significant differences between groups (p<0.001). Values are expressed as median.
Cichorium intybus L.

### Table 3: Effects of dextrinated maltose, ethanol and/or C. intybus on Bcl-2 levels in brain tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bcl-2/Beta tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.846</td>
</tr>
<tr>
<td>ET</td>
<td>9.730</td>
</tr>
<tr>
<td>DM</td>
<td>29.985</td>
</tr>
<tr>
<td>CI+DM</td>
<td>19.985</td>
</tr>
<tr>
<td>CI+ET</td>
<td>10.711</td>
</tr>
</tbody>
</table>

C = control, DM = dextrinated maltose treated group, ET = ethanol treated group. CI+DM = Cichorium intybus + dextrinated maltose treated group, CI+ET = Cichorium intybus + ethanol treated group. Different letters indicate significant differences between groups (p<0.001). Values are expressed as median.

### Table 4: Effects of dextrinated maltose, ethanol and/or C. intybus on cyclin B1 levels in brain tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cyclin B1/Beta tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.985</td>
</tr>
<tr>
<td>DM</td>
<td>7.352</td>
</tr>
<tr>
<td>ET</td>
<td>43.254</td>
</tr>
<tr>
<td>CI+DM</td>
<td>19.647</td>
</tr>
<tr>
<td>CI+ET</td>
<td>27.736</td>
</tr>
</tbody>
</table>

C = control, DM = dextrinated maltose treated group, ET = ethanol treated group. CI+DM = Cichorium intybus + dextrinated maltose treated group, CI+ET = Cichorium intybus + ethanol treated group. Different letters indicate significant differences between groups (p<0.001). Values are expressed as median.

Discussion

We investigated the effects of *C. intybus* on the volumes of the liver and kidney in ethanol induced damage in rats. We found hepatocyte and sinusoid pathologies. Alcohol consumption induces oxidative stress in the liver and other tissues and leads to lipid peroxidation, suggesting that this situation is a complex process (43). Depending on the amount and duration of alcohol consumption, deterioration of liver tissue, accumulation of fat in hepatocytes, widespread inflammation, swelling and necrosis in hepatocytes as well as within the necrosis foci and neutrophil accumulation was reported (44). If alcohol consumption continues, extensive necrosis, inflammation and fibrosis are seen (44).

Acute tubular necrosis and renal tubule dysfunction have been shown to occur in the kidney due to alcohol consumption (45). Similarly, in the present study we observed similar pathologies in the tubule cells. We also observed that the nucleus of tubular epithelial cells showed localization close to lumen. We observed that the appearance of the CI + ET group was close to the control group. These results showed that *C. intybus* attenuated the negative histopathological changes of alcohol in the liver and kidney tissues due to its hepatoprotective properties.

We investigated the effects of *C. intybus* on serum TAS and TOS levels and OSI values on ethanol induced damage in rats. In the present study, it can be concluded that *C. intybus* aMDinistration attenuated the negative changes of ethanol on TAS levels in serum. Consistent with our results, it was reported that the water extract of *Cichorium intybus* L. showed a remarkable antioxidative effect (on low density lipoprotein in vitro) (27). However, *C. intybus* and/or ethanol aMDinistration increased the TOS and OSI values. Results suggest that such aMDinistration exerted an oxidative stress.

We also investigated the cyclin B1 levels in the brain. We found that chronic ethanol aMDinistration significantly increased cyclin B1 levels in the brain in the ET group compared to all other groups. *C. intybus* treatment significantly decreased the cyclin B1 levels compared to the ET group. Cyclin B1 is a member of the cyclin family which consists of cyclin A, B, D, E and others (12). The accumulation of cyclin B1, which is promoted by the N-methyl-D-aspartate receptors (NM-DARs) stimulation in neurodegenerative diseases, led to neuronal apoptotic death (46). Additionally, it was also suggested that aberrant overexpression of cyclin B1 leads to non-specific cell death (47). In the present study, we showed that chronic ethanol aMDinistration increased the cyclin B1 levels in the brain. Since cyclin B1 overexpression in the brain may lead to neuronal death, in this study, it may be suggested that *C. intybus* treatment may prevent cell death by decreasing cyclin B1 expression.

Chronic alcohol exposure causes oxidative stress, especially in brain cells (13). It was reported that oxidative stress plays an important role in apoptosis (48). Overexpression of Bcl-2, which is a protein that inhibits apoptosis in the central nervous system can hinder apoptosis in the neurons which respond to nerve growth factor (49). We also investigated the Bcl-2 levels in the brains of rats. We found that ethanol aMDinistration significantly decreased the Bcl-2 levels compared to all other groups. Consistent with our results, the expression of the antiapoptotic protein Bcl-2 in the brain was found...
to be significantly decreased in the alcohol treated rats (13). In the present study, *C. intybus* did not change the ethanol induced decrease in Bcl-2 levels.

In conclusion, chronic alcohol consumption leads to overexpression of cyclin B1. *C. intybus* treatment significantly decreased the cyclin B1 levels and increased the TAS levels compared to the ET group. Since we used only a 200mg/kg dose of *C. intybus* extract, more studies are required to determine whether different doses of *C. intybus* alter the effects of chronic ethanol intake, especially on liver and oxidative stress parameters.

**Acknowledgements**

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**Conflict of interest**

None declared.

**References**