INTRODUCTION

Sugar beet, Beta vulgaris L. is native to the coasts of Mediterranean, extensively cultivated in Europe, America and many parts of India. In Egypt, sugar beet was cultivated on 1982 in an area of 16943 feddan increased to 235259 at 2008 with a mean production of 50700 tons sugar [1]. Aqueous and ethanolic extracts of beet roots have been reported to possess free radical scavenging activity, reducing the radical cations and phase II enzyme-inducing activities in murine hepatoma cell in vitro study [2]. There are some reports indicating the potential hepatoprotective, antioxidant and anti-inflammatory activity of B. vulgaris. Duke [3] and Agrawal [4] in their investigation dealing with -induced hepatic injury in rats, found that ethanolic extract of B. vulgaris given at doses of 1000, 2000, and 4000 mg/kg b. wt not only prevented the rise in serum level of ALT and ALP but also improved serum lipid profile in a dose-dependent manner. Ethanolic extract effects were attributed to phenolic amides. Murakami et al. [5] isolated hypoglycemic saponins from the roots of sugar beet. They succeeded in isolating nine triterpenes aminoglycosides called beta vulgaroside with a unique substituent acetal type which was found to show potent hypoglycemic activity.

Ranju [6] showed the effect of ethanol extract of B. vulgaris on CCl4 induced hepatotoxicity in rats. Rats induced with CCl4 showed a significant reduction in levels of protein and a significant increase in the levels of bilirubin and also the activities of AST, ALT and ALP. Treatment with an alcoholic extract of beet for a period of 10 d significantly restores the levels of the liver function similar to that of silymarin [7]. Histological profile of the animals treated with alcoholic extract exhibited significant liver protection against the toxicant as evident by the presence of normal hepatic cords, the absence of necrosis and lesser fatty infiltration [6].

The aim of the present work is to evaluate the role of leaves extract of sugar beet plant as a hepatoprotective agent. Sugar beet leaves is considered as an agriculture waste, so if it has this effect will be an added value to the plant.

MATERIALS AND METHODS

Chemicals and reagents

The assay kits used for biochemical measurements of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), ALP (EC 3.1.3.1), GGT (EC 2.3.2.2), Reduced Glutathione (GSH), Malondialdehyde (MDA), Nitric oxide (NO), total protein, albumin, and bilirubin were purchased from Bio diagnostic Company, Dokki, Giza, Egypt. All other chemicals used were of analytical reagent grade.

Plant materials and extractions

Seeds of sugar beet were obtained from the Department of Sugar Crops Ministry of Agricultural Giza, Egypt. The seeds were sown directly on 25 October 2011 in rows 60 cm apart and one meter in between plants in the Experimental Farm, of Agriculture Faculty Cairo University, Giza. All seeds germinated after 7 to 10 d from sowing. The leaves were collected, cleaned, oven dried, grounded and then kept at room temperature until extracted. The leaves were extracted with increasing polarity of solvent as follows, pet ether, chloroform, ethyl acetate and methanol. All these extracts were subjected to analysis for polyphenol content and free radical scavenging activity.

For isolation of active substances, air-dried leaves of sugar beet (1 kg) were extracted with 70% ethanol several times. The combined alcoholic extracts were concentrated under reduced pressure at 45 °C to yield 52g residue. The crude alcoholic extract was dissolved in hot water, left overnight, filtered and was successively partitioned with chloroform then n-butanol. The butanol part was evaporated till solvent-free to give (4.5g). The butanol part was fractionated by loading on polyamide column using 80% methanol, in fractions of 50 ml, then monitored on TLC. Similar fractions were combined, then evaporated. Four main substances were obtained, detected by UV light 366 nm. Each substance was then passed through small Sephadex LH20 column, eluted with 50% methanol, then fractionated by TLC plates to check its purity and crystalized from MeOH.

Determination of polyphenolic content

Total soluble phenolics were determined using Folin-Ciocalteau reagent by the method of Slinkard and Singleton [8]. The amount of total phenolic compounds is calculated as mg of gallic acid equivalents from the calibration curve of gallic acid. The phenolic acids found in the alcoholic extract of leaves of sugar beet were determined by HPLC adopting the method described by Leonardis et al. [9]. The HPLC system used a HP 1100 chromatograph [Agilent...
Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, quaternary pump and a diode array detector. The measurements were integrated by Chemstation chromatographic software interfaced to a personal computer. The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm I.D., 5 µm, USA).

**Free radical scavenging activity by DPPH**

The model of scavenging the stable DPPH radical is widely used to evaluate antioxidant activity in a relatively short time compared to other methods. The method adopted was that of Shimada et al. [10].

**In vivo study**

**Animals**

Adult male healthy Wistar strain albino rats weighing 150-200g were obtained from the Animal House, National Research Centre, Dokki, Egypt. Animals were fed a standard diet (control diet; consists of vitamin mixture 1%, mineral mixture 4%, corn oil 10%, sucrose 20%, cellulose 0.2%, casein (95% pure) 10.5%, and starch 54.3%). Water ad libitum. Animals were acclimatized to the laboratory condition for one week before starting the experiment. Temperature through the housing was adjusted to 24°C with relative humidity 65 ± 5% and 12/12 hr of light/dark cycles. The experimental work on rats was performed with the approval of the Animal Care & Experimental Committee, National Research Centre, Giza, Egypt. Approval No 134 2010.

**Acute oral toxicity experiment**

Fifteen rats of 200 ± 10g were subdivided into three subgroups (5 rat each) and received one oral dose of 500, 1000, and 1500 mg/kg b.w. of leaves extracts as mg/kg b.w. No. of dead animals were counted along 15 d. The mortality rate and LC50 were monitored.

**Hepatoprotective experimental design**

Healthy male rats were divided into five groups, eight/rats each. Group 1 served as normal control rats. Group 2 was received beet leaves alcoholic extract (500 mg/kg b.w) administered orally. Group 3 was CCl4 treated rats. 0.5 ml CCl4/kg b.w. diluted to 1:9 (v/v) in olive oil which was injected intraperitoneally [4]. Group 4 was injected with CCl4 and treated with beet leaves extract (500 mg/kg b.w) administered orally at the same time and for the same duration [11]. Group 5 was injected with CCl4 and treated with legalon drug (silymarin) as reference herbal drug, orally at dose 100 mg/kg b.w [12]. The administration regime was twice per week for six consecutive weeks.

**Blood samples**

Animals were sacrificed under anesthesia and blood sample were withdrawn from the retro-orbital venous plexus in clean and dry test tubes. Blood left 10 min to clot and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant serum was collected and stored at –80°C until analysis.

**Liver homogenate**

The liver was excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissue was then kept at-80°C until analysis. At experiment time, a portion of liver was weighed and homogenized with saline (0.9% NaCl) [1: 9 w/v] using glass homogenizer at 4°C. The homogenates were centrifuged at 3000 rpm for 10 min at 4°C and the clear supernatant was used for further determination of liver function enzymes, oxidative stress markers and hepatic total protein and bilirubin.

**Biochemical studies**

MDA as a product of polyunsaturated fatty acid oxidation was determined by the method of Buege and Aust [13]. Its concentration was calculated using the extinction coefficient value 1.56 x 10^4 M^-1 cm^-1 and was read at 535 nm. NO was estimated by the method of Nishikimi et al. [14] where the increase in NADH oxidation was measured at 560 nm using its molar extinction coefficient 6.22 x 10^3 M^-1 cm^-1. NO was assayed by the method of Mosshage et al. [15]. The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. Hepatic aspartate and ALT were measured by the method of Gella et al. [16] where the transfer of an amino group from aspartate or alanine formed oxalacetate or pyruvate, respectively and the developed color was measured at 520 nm. Hepatic alkaline phosphatase was catalyzed in an alkaline medium; the phosphate group transferred from 4-nitrophosphate to 2-amino-2-methyl-propanol CAMP and liberated 4-nitrophenol. The developed color was measured at 510 nm [17].

**Histopathological examination**

Small pieces of liver tissues in each group were collected in 10% neutral buffered formalin. The tissues were processed and embedded in paraffin wax. Sections of 5µm thickness were cut and stained with eosin. The sections were examined microscopically for the evaluation of histopathological changes [20].

**Statistical analysis**

Analysis of data was carried out by one-way analysis of variance (ANOVA) Costat computer software program accompanied with the least significant difference between means at P≤0.05.

**RESULTS AND DISCUSSION**

**Polyphenolic content**

A major and great part of phytochemicals which is constantly synthesized by plants for development of drugs and for the defensive purpose are polyphenolic in nature. These polyphenols comprise antioxidant character that is produced as a defensive mechanism to prevent tissues destruction caused by highly reactive chemical species, which are formed from various biochemical reactions [7]. It is also considered as a major class of natural antioxidants. In the present study, polyphenols were determined using Folin-Ciocalteu method.

Table 1 compiles the polyphenolic content in different beet leaves extracts which reveals that alcohol extract possesses the higher polyphenolic content.

**Table 1: Total polyphenolic in sugar beet leaves extracts**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Phenolic content ug/gallic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet. ether</td>
<td>0.66 ± 0.12^a</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.31 ± 0.15^a</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.59 ± 0.06^b</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.63 ± 0.17^a</td>
</tr>
</tbody>
</table>

Data are means ± SD., Values are not sharing superscripts letters differ significantly at p<0.05.

**Phenolic acid content**

Phenolic acids were determined using HPLC, gallic acid was the highest, amounted to 344 ug/ml followed by ferulic 89.7 while a small amount of other acids were found (Table 2).

**Table 2: HPLC determination of phenolic acids in B. vulgaris leaves alcoholic extract**

<table>
<thead>
<tr>
<th>Phenolic acid</th>
<th>Catechin</th>
<th>Gallic</th>
<th>Coffeic</th>
<th>Vanellic</th>
<th>Coumaric</th>
<th>Ferutic</th>
<th>Cinnamic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.2</td>
<td>3.79</td>
<td>1.25</td>
<td>0.46</td>
<td>39.7</td>
<td>3.54</td>
<td></td>
</tr>
</tbody>
</table>
Isolation and structure elucidation

Alcohol extract (52g) was dissolved in hot water, filtered then successively extracted with chloroform (which was discarded) then with butanol to give 5.4g. The butanol fraction was subjected to fractionation with paper chromatography on Whatman 3MM with butanol-acetic acid-water 4:2:1 (v/v) as eluant. Four major bands were detected under UV light with the following Rf values, 1.9, 2.5, 3.3 and 3.4. All the bands were cut off, eluted with 70% alcohol, evaporated and were subjected to Sephadex LH-20 column, eluted with 50% MeOH. The pure compounds (fig. 1) were subjected to H-1NMR, which reveals the following identification:

**Compound 1**

Compound I was obtained as a yellow amorphous powder, with Rf value 0.34 (BAW 4:2:1), and appeared as a yellow spot on PC with purple under UV light, which showed bright yellow color when fumed with ammonia vapours. 1H NMR (500 MHz, MeOD) of compound 1 indicated with presence of quercetin moiety appeared at 6.18 (1H, d, J= 2.0 Hz, H-6), 6.39 (1H, d= 1.9 Hz, H-8), 6.88 (1H, d= 8.3 Hz, H-5), 7.62 (1H, dd, J=8.3, 2.1Hz, H-6), 7.74 (1H, d, J=2.1Hz, H-2). Identification of the compound was further confirmed by co-chromatography with the authentic sample of the aglycone quercetin as well as comparing the spectral data with those reported for quercetin (C15H10O7).

**Compound 2**

Compound 2 was obtained as a brownish yellow amorphous powder, with Rf value 0.19 (BAW 4:2:1), and appeared as a yellow spot on PC with purple color under UV light, and also showed bright yellow color when fumed with ammonia vapours. Complete acid hydrolysis yielded quercetin aglycone and galactose as sugar moiety in which both of them were co-chromatographed with authentic samples. 1H NMR (Me OD, 500 MHz): 6.12 (1H, d, J= 1.9 Hz, H-6), 6.30 (1H, d= 1.9 Hz, H-8), 6.85 (1H, d= 8.0 Hz, H-5), 7.57 (1H, dd, J= 2.0, 7.5Hz, H-6), 7.82 (1H, d, J= 2.0 Hz, H-2), 5.94 (1H, d, J=7.6 Hz, H-100), 3.82 (1H, m, H-200), 3.74 (1H, m, H-300), 3.85 (1H, m, H-400), 3.45 (1H, m, H-500), 3.58 (1H, dd, J=11.0 and 7.0 Hz, H-60a), 3.65 (1H, dd, J= 11.0 and 4.0 Hz, H-60b).

**Compound 3**

Compound 3 was isolated as a yellow amorphous powder, with Rf value 2.5 (BAW 4:2:1). It has a purple color under UV light turning to bright yellow when fumed with ammonia vapours. Complete acid hydrolysis yielded quercetin aglycone and glucose as sugar moiety in which both of them were co-chromatographed with authentic samples. H-NMR (500MHz, MeOD) spectrum indicated the presence of quercetin moiety appeared at δ 7.70 (1H, d, H-2), J= 1.9 Hz), 7.58 (1H, dd, H-6', J=8.5Hz, 1HδO), 6.86 (1H, d, H-5), J=8.5 Hz), 6.36 (1H, d, H-8, J= 2.2Hz), 6.17 (1H, d, H-6, J= 2.2Hz), 5.22 (1H, d, H-1'), J=7.3 Hz), 3.85-3.30 (6H, sugar protons). Quercetin 3-O-glucoside is the 3-glucoside of the flavonol quercetin, a type of polyphenols. Polyphenols are plant secondary metabolites with a molecular formula of C21H20O12 hence synergistic effects were reported [7]. The alcoholic extract of leaves proved to have the highest total polyphenolic content with minor ones may play a significant role in the antioxidant activity and therefore synergistic effects were reported [7]. The alcoholic extract of leaves proved to have the highest total polyphenolic content with higher scavenging activity.

### Table 3: Radical scavenging activities (% DPPH inhibition)

<table>
<thead>
<tr>
<th>Sugar beet leaves Extract</th>
<th>Sample concentration µg/ml</th>
<th>IC50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.25</td>
<td>2.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.80±1.34*</td>
<td>15.23±2.32*</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.26±0.39*</td>
<td>5.15±0.65*</td>
</tr>
<tr>
<td>Pet-ether</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6.16±0.67*</td>
<td>11.86±1.64*</td>
</tr>
</tbody>
</table>

Data are means ± SD of eight rats. Unshared letters between brackets are significant value between groups

Hepatoprotective effect of sugar beet leaves extract

Six parameters were assayed AST, ALT, ALP, GGT, tissue protein, albumin and bilirubin to explore the status of these parameters in different rat groups. Control rats when administered the alcoholic beet leaves extract, no significant changes in the values of the six parameters were observed. This means that beet leaves extract is safe enough and did not alter these parameters. The diseased or intoxicated rats (CCl4 group) show drastic changes in correspondence to each parameter when compared with control. As shown in table 4 CCl4 developed severe hepatic damage, evidenced by marked elevation of the parameters studied when compared with control.
Table 4: Ameliorative effect of alcoholic extract of sugar beet leaves on liver enzymes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT u/ml</th>
<th>% of change</th>
<th>AST ul/ml</th>
<th>% of change</th>
<th>AIP</th>
<th>% of change</th>
<th>GGT</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.76±2.1</td>
<td>0.00</td>
<td>39.39±1.7</td>
<td>0.00</td>
<td>7</td>
<td>83.93±6.23</td>
<td>0.00</td>
<td>258±0.26</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>125.62±8.5</td>
<td>1.07</td>
<td>60.20±1.7</td>
<td>2.37</td>
<td>118.85±3.73</td>
<td>50.58</td>
<td>5.0±0.46</td>
<td>94.57</td>
</tr>
<tr>
<td>beet</td>
<td>45.60±0.66</td>
<td>4.20</td>
<td>42.79±0.97</td>
<td>8.63</td>
<td>83.82±2.46</td>
<td>9.40</td>
<td>3.6±0.26</td>
<td>17.8</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;+beet</td>
<td>48.50±8.63</td>
<td>10.83</td>
<td>49.80±3.6</td>
<td>26.43</td>
<td>92.40±0.77</td>
<td>17.07</td>
<td>3.5±0.31</td>
<td>37.2</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;+silymarin</td>
<td>3.67±0.63</td>
<td>0.845±0.24</td>
<td>312.81±12.2</td>
<td>24.78±6.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is a mean of 8 rats±S. D. Statistical analysis is carried out by one-way analysis of variance (ANOVA) followed by Duncan’s test, using SPSS (SPSS 17.0 for windows). Values are not sharing superscripts letters (a, b, c, d) differ significantly at p<0.05. Percentage of change = (treatment-control)/control x 100.

Table 5: Effect of beet extract on total protein, albumin, and bilirubin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T. P g/dl</th>
<th>Albumin %</th>
<th>Bilirubin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.12±0.15</td>
<td>5.05±0.23</td>
<td>0.91±0.07</td>
</tr>
<tr>
<td>beet extract</td>
<td>7.29±0.39</td>
<td>5.01±0.33</td>
<td>0.92±0.04</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6.14±0.31</td>
<td>4.26±0.11</td>
<td>2.12±0.14</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;+beet extract</td>
<td>7.31±0.55</td>
<td>5.02±0.09</td>
<td>1.00±0.06</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;+silymarin</td>
<td>43.66±0.52</td>
<td>5.02±0.09</td>
<td>2.88±0.13</td>
</tr>
</tbody>
</table>

Each value is a mean of 8 rats±S. D. Statistical analysis is carried out by one-way analysis of variance (ANOVA) followed by Duncan’s test, using SPSS (SPSS 17.0 for windows). Values are not sharing superscripts letters (a, b, c, d) differ significantly at p<0.05.

Toxicated animal group by CCl<sub>4</sub> caused significant changes in the levels of total protein; albumin and bilirubin amounted to 6.14, 4.26 and 2.12 mg/dl respectively. These parameters were restored to nearly values in control when treated with an alcoholic extract of B. vulgaris leaves, the change in total protein concentration might be due to the imbalances between the rate of protein synthesis and the rate of its degradation in the liver.

The same pattern was obtained with albumin. When diseased rats were treated with beet extract, total protein and albumin restored to that levels of healthy control rats. Dealing with bilirubin, diseased rats possessed high level than that of control, which returned to nearly that of control meaning that alcoholic extract of B. vulgaris leaves has the ability to protect and treat intoxicated liver. In this concern, Kim et al. [7] reported the occurrence of two flavones glycosides from B. vulgaris, vitisin 7-O-β-D glucopyranoside, and vitexin 2-O-β-D-glucopyranoside. Their hepatoprotective activity was assessed by measuring their effects on the release of ALT from the primary cultures of rat hepatocyte injured by CCl<sub>4</sub>. In addition, the flavones c-glycoside vitexin is known to possess an inhibitory activity on TNFα [22].

Hayes [23] reported that one of the most indicators for liver damage and function is the increase in the activities of transaminases, in the serum. According to [24], the development of toxic liver injury follows a two-staged course; the first phase characterized by initiation of the injury and may involve direct interaction with a toxicant, which may exhibit dose dependency. In contrast, the second phase is characterized by the progress of the injury in an independent toxicant fashion that is dominated by secondary events, which is holding true with the results in the present investigation.

Table 6: Effect of B. vulgaris on hepatic antioxidant hepatic antioxidant enzymes levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>Normal control treated with B. vulgaris</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt; treated with B. vulgaris</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt; treated with Acetylcysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>2.00±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.28±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.65±0.26&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA</td>
<td>2.30±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1±0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.80±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NO</td>
<td>4.3±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.21±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.35±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means±SD of eight rats in each group. Data are expressed as μg/mg protein for glutathione, nitric oxide and MDA. Total protein is expressed as mg/gram of liver tissue. Unshared letters between groups are the significance value at p ≤ 0.05.
The chemical part of this investigation revealed the occurrence of higher polyphenolic content and higher radical scavenging activity for the alcoholic extract. The fractionation of this extract indicates the occurrence of phenolic acids from which Gallic acid was higher polyphenolic content and higher radical scavenging activity. It is interesting that B. vulgaris leaves are considered as a waste product to the industry of sugar and hence can be added value to the production of sugar beet in Egypt.

CONCLUSION

Based on our results, we suggest that beet leaves extract showed a remarkable anti-hepatotoxic activity against carbon tetrachloride induced hepatic damage, by ameliorating all liver enzymes, antioxidant enzymes which confirmed by histopathological examination.

ACKNOWLEDGMENT

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

REFERENCES


