The Beneficial Effect Of Trifolium Flower Extracts On Paracetamol-Intoxicated Male Rats
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Abstract

Background:
Acetaminophen known as paracetamol (P) overdose can cause severe hepatotoxicity and even liver failure and hepatic centrilobular necrosis in experimental animals and humans.

Material and methods:
The male rats (n=36) were allocated into 6 groups (each group n=6 rats). Group I was kept as control. All animals in groups II-VI were given paracetamol at 2 g/kg bw by gastric gavage on days 3 post Trifolium alixanderanum (T alixanderanum) flower extracts (TEs) or N-acetylcystiene (NAC) treatments. Group III, IV and V were treated for three days by hexane extract (THE + P), ethanol extract (TEE + P) and water extract (TWE + P). Group VI received 100 mg/kg bw of antidote N-acetylcystiene (NAC + P).

Results:
Paracetamol induced a significant rise in Liver weight and hepatosomatic index, serum aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphatase (ALP), total bilirubin (T bili), liver lipid peroxides (MDA+ 4-HDNE) with a reduction of liver glutathione (GSH), glutathione peroxidase (GSHpx) and superoxide dismutase (SOD) enzymes activities. The plant extracts showed a remarkable hepatoprotective and antioxidant activity against paracetamol induced hepatotoxicity as judged from the serum marker enzymes and antioxidant levels in liver tissues.

Conclusion:
The present investigation indicated that paracetamol damaged liver cells and TEs prevented this damage when compared with control group. Trifolium flower hexane extract was the most effective superior to TEE, TWE and NAC.

Key Words: Paracetamol, T alixanderanum flower extracts, NAC, hepatosomatic index, ASAT, ALAT, ALP, GSH, MDA, Rattus rattus.

Introduction
Many herbal, medicinal and pharmaceutical plants and their extracts are widely studied by many researchers. Plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and hepatoprotective activity (DeFeuids et al., 2003, Banskota et al., 2000 and Takeoka and Dao, 2003) Liver is a key
organ regulating homeostasis within the body by various functions.

Liver injury caused by toxic chemicals and certain drugs has been recognized as a toxicological problem. Hepatotoxicity is one of very common aliment resulting into serious debilities ranging from severe metabolic disorders to even mortality (Patel et al., 2008).

Liver is the main organ which regulates many important metabolic functions. Hepatic injury is directly associated with these altered metabolic functions (Mitra et al., 1998). In past, several studies have been carried out to examine the effect of plants used traditionally by herbalists to support normal liver function and treat diseases of liver. So, various experimental evidences have confirmed the efficacy of plants such as *Silybum marrium* (milk thistle), *Curcuma longa* (turmeric) (Luper, 1999), *Nymphaea stellata* (Bhandarkar et al., 2004). In spite of significant advances in medicinal plant research and rapid strides in modern medicine, we still are in need for more precise, safe and effective treatment of liver disorders (Oliveria et al., 2005).

Paracetamol is a widely used over-the-counter drug for analgesic and antipyretic effects. Its use in overdose (suicidal or accidental) or with chronic alcohol abuse causes fulminant liver failure (Prescott, 2000 and Gyamlani and Parikh, 2002). Paracetamol induced hepatic failure is the second leading cause of liver transplantation (Lee et al., 2004). In the initial phases of toxicity acetaminophen is metabolically activated by cytochrome P450 enzymes to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) that depletes GSH (Boelsterli and Lim, 2007).

N-acetylcysteine (NAC), a cysteine prodrug, has shown promise in numerous pathological conditions involving oxidative stress ((Vosters and Neve, 2002 and Kamboj et al.,2006a). As a sulphhydryl donor NAC contributes to the regeneration of glutathione and by directly acting as a free radical scavenger (Aydin et al., 2002). Various studies have shown that NAC administration has a beneficial effect against oxidative stress in neurodegenerative diseases (Pocernich et al., 2001 and Kamboj et al., 2008).

Presently, N-acetylcysteine is the recommended clinical treatment for patients in danger of acetaminophen overdose-related hepatic toxicity. The mechanism of action of NAC is to increase glutathione levels in the hepatic cytosol and mitochondria thus detoxify the highly reactive and cytotoxic NAPQI formed via cytochromeP-450 2E1 (Grypioti, 2006). Other mechanisms, including improvement of tissue oxygen delivery and antioxidation activity, have also been identified for N-acetylcysteine (Harrison et al., 1991).

*Trifolium alexandrinum;* Egyptian clover is also known as berseem clover. Egyptian clover is a most extensively planted crop and a major forage fed to livestock as hay, silage and pasture. Some *Trifolium* species such as *Trifolium repens* Lin., *Trifolium arvense* Lin., *Trifolium pratense* Lin. are used as expectorant, analgesic, antiseptic and tonic (Sabudak et al., 2009); also may be used for menopausal hot flashes (Booth et al., 2006); the pathological
states associated with free radical overproduction e.g. diabetes (Mauri et al., 1999 and Amer et al., 2004) and possible anticarcinogenic implications (Nikkhah, 2012). Lin et al. (2004) found approximately 20 flavonoid glycoside malonates in the flower extract of red clover (Trifolium pratense L.). A recent study reported that red clover extract was composed of 35.54% isoflavones, 1.11% flavonoids, 0.06% pterocarps, ≤ 0.03% coumarins, and ≤ 0.03% tyramine. Daidzein, genistein, formononetin, biochanin A, coumestrol and naringenin (Booth et al., 2006). However no reports are available regarding the hepatoprotective activity of Trifolium alexandrinum Linn. flower extract. Keeping this fact in view the present study was undertaken to investigate the antioxidant and hepatoprotective activity of Trifolium alexandrinum Linn. extracts, against paracetamol induced hepatic damage in albino rat

**Material and methods**

N-acetylcystiene and other chemicals were purchased from Sigma, St. Louis, MO, USA.

**Trifolium extracts:**

The plant flower samples were collected at flowering stage from local region during May. Extract of shade dried flowers were used. Five hundred grams dried flowers were soaked in 2 liters hexane at room temperature for 48 hours and filtered. The filtrate was dried at 30° c till concentrated to dark brownish semisolid residue. The supernatant was soaked in 2 liters ethanol at room temperature for 48 hours and filtered. The filtrate was dried at 30° c till concentrated to brownish semisolid residue. The supernatant was soaked in 2 liters distilled water at room temperature for 48 hours and filtered. The filtrate was dried at 80° c till concentrated to pale brownish semisolid residue.

Hexane and ethanol extracts were dissolved in a small amount of ethanol and diluted with water to adjust the concentration to 200mg/ ml.

Thirty six albino rats weighing between 150 to 180g were used. They were kept under constant environmental and nutritional conditions throughout the experiment. The animals were allowed free feeding with a standard diet and drinking water ad libitum.

Animals were randomized and divided into six groups (I-VI) of six animals in each group. Group I was served as untreated control. Group II rats were treated with a single dose of paracetamol (2g/kg bw; Wagh, 2010) 24 hours before sacrificing. Group III - V were treated with 200mg /kg bw of the flowers extracts (THE+P, TEE+P and TWE+P respectively). Group VI was treated with the NAC (100 mg/kg bw). All treatments were used orally daily for three consecutive days. On the 3rd day, one h after the last doses of TEs and NAC, paracetamol suspension was given by oral route..The biochemical parameters were estimated after 24h following the paracetamol dose.

**Sample collection:**

On 4th day, animals of all groups were sacrificed after overnight fasting; twenty four hours after paracetamol treatment. Blood sample
of each group was collected separately into sterilized dry centrifuge tubes and allowed to coagulate for 30 min at 37°C. After centrifugation, sera were collected in vials and frozen until analysis. Liver tissue samples were collected for estimation of antioxidants and lipid peroxidation end product parameters and kept at –20°C till analysis.

**Biochemical studies:**

The clear serum obtained after centrifugation was used for the estimation of serum ASAT, ALAT (Schumann et al., 2002); ALP (Tietz, 1982); and serum total bilirubin (Schlebusch et al., 1995).

Liver GSH contents, SOD activity were determined by the methods of Prince and Loose (1969) and Nishikimi et al. (1972) respectively. Hepatic GSHpx was measured according to the method of Ammerman, et. al. (1980) and Lipid peroxidation in the different tissues was estimated by colorimetric assay of malondialdehyde (MDA) + 4- hydroxynonenal (4- HDNE) as described by Esterbauer et. al. (1991), using kits from Wak-Chem Medical GMBH, Germany.

**Statistical Analysis:** All data were analyzed using one-way analysis of variance (ANOVA) with SPSS statistical software (SPSS, Inc., Chicago, IL). Pairwise comparisons between groups were performed using post hoc test. Significance was considered at p ≤0.05. Data are presented as mean ± standard deviation of the mean (SD).

**Results**

Liver weight and hepatosomatic index were significantly increased in paracetamol-intoxicated group. These variances were returned back nearly to normal levels when rats treated with TEs or NAC for 3 days before paracetamol toxicity (table 1).

The concentration of some liver enzymes in serum were measured in rats undergoing treatments with paracetamol alone as well as those supplemented with TEs and NAC and in control rats. Enzymes assayed were ALAT, ASAT and ALP. Paracetamol administration produced significant increases (p < 0.05) in ASAT; ALAT and ALP compared to the control group.

There were significant decreases (p<0.05) in ASAT; ALAT and ALP in the paracetamol + TEs or NAC compared to the paracetamol group.

As shown in table 1, total bilirubin increased significantly in paracetamol treated animals when compared with control group. TEs and NAC treatments three days before paracetamol toxicity succeeded in returning this elevation back nearly to normal level.
Table (1): Body weight, liver weight, relative liver weight and liver function tests in different treated and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>P</th>
<th>THE+P</th>
<th>TEE+P</th>
<th>TWE+P</th>
<th>NAC+P</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver weight(g)</td>
<td>6.914 ± 0.559</td>
<td>10.429 ± 0.845</td>
<td>8.003 ± 0.588</td>
<td>8.468 ± 0.697</td>
<td>9.002 ± 0.924</td>
<td>8.658 ± 0.787</td>
</tr>
<tr>
<td>Hepatosomatic index</td>
<td>4.13 ± 0.26</td>
<td>6.30 ± 0.70</td>
<td>4.85 ± 0.44</td>
<td>5.09 ± 0.35</td>
<td>5.42 ± 0.41</td>
<td>5.15 ± 0.34</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>41.61 ± 2.50</td>
<td>126.72 ± 4.42</td>
<td>48.41 ± 2.56</td>
<td>51.88 ± 2.81</td>
<td>55.23 ± 3.59</td>
<td>44.15 ± 3.10</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>25.92 ± 1.54</td>
<td>67.77 ± 4.31</td>
<td>31.26 ± 2.01</td>
<td>33.76 ± 3.78</td>
<td>34.62 ± 4.30</td>
<td>30.58 ± 2.73</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>127.49 ± 5.89</td>
<td>279.34 ± 7.32</td>
<td>138.57 ± 6.02</td>
<td>149.45 ± 7.78</td>
<td>153.60 ± 5.19</td>
<td>148.66 ± 5.14</td>
</tr>
<tr>
<td>T bili.(mg/dl)</td>
<td>0.17 ± 0.01</td>
<td>0.28 ± 0.06</td>
<td>0.19± 0.01</td>
<td>0.19± 0.02</td>
<td>0.20± 0.02</td>
<td>0.18 ± 0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group.

a p< 0.05 compared to the control, b p> 0.05 compared to paracetamol.

Table (2): Some liver antioxidants and (MDA)+ 4-HNE in different treated and control groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>P</th>
<th>THE+P</th>
<th>TEE+P</th>
<th>TWE+P</th>
<th>NAC+P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (µmol/g)</td>
<td>8.165± 0.663</td>
<td>1.938± 0.275</td>
<td>6.846± 0.461</td>
<td>6.136± 0.481</td>
<td>5.998± 0.624</td>
<td>8.096± 0.872</td>
</tr>
<tr>
<td>GSHpx (U/g)</td>
<td>594.99± 63.89</td>
<td>329.39± 37.91</td>
<td>473.42± 41.40</td>
<td>412.93± 63.51</td>
<td>393.12± 23.16</td>
<td>446.94± 38.27</td>
</tr>
<tr>
<td>SOD (U/ 100g tissue)</td>
<td>2934.83± 432.22</td>
<td>1328.33± 95.83</td>
<td>2704.17± 570.34</td>
<td>2333.00± 407.56</td>
<td>2316.00± 424.94</td>
<td>2731.33± 385.55</td>
</tr>
<tr>
<td>MDA+ 4-HNE (µmol/g)</td>
<td>84.29± 4.10</td>
<td>133.55± 6.03</td>
<td>95.45± 5.88</td>
<td>93.81± 7.01</td>
<td>94.53± 7.37</td>
<td>89.58± 3.25</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each group.

a p< 0.05 compared to the control, b p> 0.05 compared to paracetamol.

Discussion

Drugs in common use can cause toxic effects on the liver. Hepatotoxicity produced by overdosing of paracetamol may serve as a model for toxic drug to the liver.

The liver has the fundamental role of deactivating all substances produced by toxic drugs (Larrey, 2003). However, to do so it needs vitaminic hepato-protective substances, such as those contained in phytherapeutic products that medicine has known for thousands of years: *Silybum marianum*, *Taraxacum officinale*, *Smilax aspera*, *Cynara scolymus*, *Salvia officinalis*, *Agropyrum repens*, *Hyssopus officinalis*, *Matricaria camomilla*, *Aloe species*, etc… They are extremely effective on many
degenerative or toxic processes for the liver. It has been reported that *Trifolium* contains flavonoids, triterpenoids and steroids (Booth *et al.*, 2006). A number of scientific reports indicated certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties (El-Hossary *et al.*, 2000, DeFeudis *et al.*, 2003 and Kumar *et al.*, 2011). Presence of those compounds in TEs may be responsible for the protective effect on paracetamol-induced liver damage in rats.

It has been suggested that high dose of paracetamol causes increased production of free radicals, which may initiate lipid peroxidation of hepatic cell membrane, mitochondrial dysfunction, hepatocellular death and ultimately increase in liver weight (Murugaian *et al.*, 2008). In this study, liver weight and hepatosomatic index increased significantly. However, Holt and Ju (2009) suggested that increased liver weight and increased serum bilirubin levels both of which are useful indicators of paracetamol induced hepatocellular damage.

In the present investigation, paracetamol administration resulted in elevated activities of ASAT, ALAT and ALP in serum against their respective control values. Similarly, serum total bilirubin level was also found to be increased significantly as a result of paracetamol toxicity. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Larrey, 2003). Hepatocellular necrosis leads to high level of serum markers in the blood, among these, aspartate transaminase, alanine transaminase represents 90% of total enzyme and high level of alanine transaminase in the blood is better index of liver injury (Ekam and Ebong, 2007). Increase in liver enzymes in serum following paracetamol administration has earlier been reported (Hardman *et al.*, 1996 and Yapar *et al.*, 2007). The elevation of the ASAT, ALAT and ALP values might be due to release of the enzymes from disrupted hepatic cells either due to necrosis or altered membrane permeability due to toxic effects of paracetamol. Similar elevations of these values in the paracetamol treated animals confirmed the findings of Bhaumik and Sharma (2002) in rabbits, Ekam and Ebong, (2007) in rats and Kanbur *et al.* (2009) in mice. The cumulative oxidative damage is likely one of the mechanisms producing the hepatotoxic effects of paracetamol administration in this study. The elevated levels of enzymes are decreased to near normal levels due to three days pre-treatment of *Trifolium* flower extracts indicated that it offered protection by preserving the structural integrity of the hepatocellular membrane against paracetamol. The molecular mechanism by which the TEs and NAC reduce liver damage maybe due to their antioxidant ability to maintain liver cell integrity even in the presence of a hepatotoxic agent such as paracetamol.

Serum bilirubin is one of the most sensitive tests employed in the diagnosis of hepatic diseases. The increase in total bilirubin in this study may be attributed to the inability of the damaged hepatic parenchymal cells to excrete the dye at normal rates (Bhaumik and Sharma, 2002). Administration of *Trifolium*
flower extracts decreased the level of serum total bilirubin suggesting that it offered protection. Many plant feeding and plant extractions were used in many other studies to decrease the levels of liver enzymes and bilirubin in paracetamol toxicity e.g, Plumbago zeylanica (Kanchana and Sadiq, 2011), Clitoria ternatea (Sarumathy et al., 2011) and oyster mushroom (Sumy et al., 2011).

Hepatic lipid peroxidation (LPO), expressed as MDA+ 4-HDNE, increased significantly in paracetamol toxicity. While, the activities of protective enzymes such as GSHpx and SOD and glutathione content in liver tissue were lowered after paracetamol administration. Enhanced LPO and reduced activities of GSHpx and SOD are indications of generation of free radical stress as a mark of hepatic damage due to paracetamol toxicity. Marked reductions in the activities of these free radical scavenging enzymes, GSHpx, associated with paracetamol toxicity were significantly reversed to normal on oral feeding of Trifolium flowers extracts as manner of the antilipid peroxidative ability to oxidative stress. (Boelsterli and Lim, 2007). Under conditions of NAPQI formation following toxic paracetamol high doses, GSH concentrations may be very low in the centrilobular cells, and the major peroxide detoxification enzyme, GSH peroxidase, which functions very inefficiently under conditions of GSH depletion (Hinson et al., 2004), is expected to be inhibited.

Trifolium flower hexane extract had the most protective effect followed by Trifolium flower ethanol extract against paracetamol toxicity. The protective activity of Trifolium flower water extract against the hepatotoxicity induced by paracetamol was the lowest in the present study. These results may be attributed to natural antioxidants in plants are usually fat soluble e.g vitamin E, A and polyphenols.

Conclusion:

It could be concluded that the biochemical alterations induced by administration of paracetamol; were improved under the effect of the used Trifolium flowers extract in a variable degrees. The most efficient was THE followed by TEE followed by NAC and TWE.

Further investigations into the appropriate isolation, characterization and concentration of the active constituents in Trifolium alixandrinum flowers extract are deemed necessary for elucidating their antihepatotoxic activity against paracetamol hepatotoxicity in rats.

The present study also suggests that concepts of traditional medicine have the potential to be transposed successfully in the context of modern medical interventions such as liver damage or toxicity.

References


Pocernich CB, Cardin AL, Racine CL, Lauterback CM and Butterfield DA (2001): Glutathione elevation and its protective role in acrolein-induced protein damage in synaptosomal membranes: relevance to brain lipid peroxidation in


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