

Leonotis nepetifolia Protects against Acetaminophen-Induced Hepatotoxicity: Histological Studies and the Role of Antioxidant Enzymes

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Abstract

Aim of the study: High dose acetaminophen (APAP) increases the risk of liver injury caused by oxidative stress due to accumulation of reactive species. Although N-acetyl cysteine is the standard antidote used to treat acute APAP-induced liver failure, we proposed that known antioxidant phytochemicals in *Leonotis nepetifolia* extracts would protect against APAP-induced hepatic injury by modulating the activities of antioxidant enzymes.

Materials and methods: Methanol and aqueous extracts of *L. nepetifolia* were orally administered in doses ranging (250 mg/kg to 1000 mg/kg) as pre- and post-treatment with high dose APAP (550 mg/kg) to Swiss albino mice. Twenty-four hours after the final dose, animals were euthanized and blood and liver collected for liver enzymes (ALT and AST), histological assessment and antioxidant enzyme assays.

Results: Methanol and aqueous extracts as pre-treatment and post-treatment protected against hepatic injury. Extracts abrogated the 14-fold and 4-fold APAP-induced increases in ALT and AST respectively, including histopathological damage ($p < 0.05$). Extracts reversed APAP-induced 4-fold and 14-fold increases in GR and SOD activities respectively ($p < 0.05$). Additionally, extracts reversed APAP-induced decline in GPx activity; particularly the aqueous extract as pre-treatment increased GPx activity up to 2.2-fold over saline-treated controls ($p < 0.05$).

Conclusions: Extracts, as pre-treatment and post-treatment, prevented APAP-induced hepatic injury by modulating the activities of antioxidant enzymes. Of particular interest, is the reversal of APAP-induced decrease in GPx activity and increase in SOD activity? Extract-induced increase in GPx activity would facilitate the scavenging of hydroperoxide and peroxide reactive species generated by high dose APAP. We propose that antioxidant phytochemicals in *L. nepetifolia* may be acting as free radical scavengers which results in reduced SOD activity.

Keywords: *L. nepetifolia*; Reductase; Catalase

Abbreviations: APAP: Acetaminophen; ALT: Alanine Amino transaminase; AST: Aspartate Aminotransferase; GR: Glutathione Reductase; GPx: Glutathione Peroxidase; SOD: Superoxide Dismutase; CAT: Catalase; NAPQI: N-acetyl-p-benzoquinoneimine; CYP2E1: Cytochrome 2E1.

Introduction

The liver is the major organ responsible for the metabolism of xenobiotics and it is susceptible to drug-induced injury, including drug overdose and deleterious drug-drug interactions. Acetaminophen (APAP) is widely used as an over-the-counter analgesic and antipyretic. It is the drug most commonly associated with drug-induced hepatic injury requiring hospitalization, making it a serious public health concern. Persons at risk for hepatic APAP injury include patients prescribed high daily doses (more than 4 g/day) on a long-term basis [1], as well as those who intentionally or accidentally overdose [2-4].

At therapeutic doses APAP is primarily detoxified by Phase 2 enzymes to inactive conjugates with minute amounts being metabolized by CYP2E1 to the short-lived, but highly reactive, NAPQI. NAPQI is efficiently detoxified by combining with reduced glutathione to form an inactive conjugate. However, at high doses Phase 2 conjugation pathways become saturated and more APAP is converted to NAPQI, depleting glutathione stores and causing accumulation of this highly reactive species. Although the precise mechanism of APAP-induced hepatotoxicity is not fully understood, it is proposed that NAPQI-induced oxidative stress culminates in cellular necrosis and lipid peroxidation [5].

Although N-acetyl cysteine (NAC) is the standard antidote routinely used in the clinical setting for the management of APAP overdose, herbal remedies are becoming increasingly popular as

“natural” remedies to treat a wide range of liver disorders [6-8]. *Leonotis nepetifolia*, commonly called Shandilay in Trinidad, is among the most commonly used herbs on the island [9]; it is an upright dicotyledonous plant, growing up to one meter in height, with square-shaped stems, soft serrated heart-shaped leaves and a globose cluster of flowers at the apex [10].

Several compounds such as diterpenes, glycosidic iridoids, stigmaterol, flavonoids and tannins have been isolated from the leaves of *L. nepetifolia* [11-13]. Additionally, acteoside, martinoside and lavandulipolioside have also been isolated in the stems and these compounds have been shown to possess potent antioxidant activity [14].

In this study we used high dose APAP to induce liver injury to investigate the hepatoprotective properties of methanol and aqueous extracts *L. nepetifolia* before (pre-treatment) and after (post-treatment) hepatic insult. We proposed that both extracts would prevent liver damage by abolishing the expected APAP-induced increase in liver enzymes, whilst maintaining liver morphology. We also proposed that *L. nepetifolia* extracts would modulate the activities of antioxidant enzymes (catalase (CAT), glutathione peroxidase (GPx), glutathione

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reductase (GR) and superoxide dismutase (SOD)) in the presence of toxic APAP to facilitate hepatoprotection.

Materials and Methods

Reagents and drug

Methanol (JT Baker), HEPES buffer, EGTA, Sucrose, Tris-HCL (Sigma-Aldrich), Trichloromethane (Reidel-deHaën), N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Ethylene glycol tetraacetic acid, NaCl (AnalaR), acetaminophen (Sigma-Aldrich).

Preparation of plant extracts

Fresh plant material was obtained from two sites in North Trinidad. The botanist at the National Herbarium, University of the West Indies, St Augustine assigned the voucher number *TRIN 36510* to the specimen supplied. Collected plant material was thoroughly washed, oven-dried at 40°C to constant weight and mill-ground. For the methanol extract, milled plant material was exhaustively extracted with solvent, vacuum filtered and rotary evaporated to dryness at 45°C. The gummy product was weighted 50 mg/ml stock for dosing regimen. The aqueous extract was prepared by infusing the milled plant material in normal saline at 80°C for one hour at a weigh of 50 mg/ml. After cooling the mixture was vacuum filtered and the filtrate used.

Animals

Swiss Albino male mice (25-35 g) were obtained from the School of Veterinary Medicine Animal House at UWI. following institutional ethical approval. Animals were housed at constant room temperature (25 ± 2°C), maximum of 3 animals per cage, with free access to food and water and handled according to International Guidelines [15]. APAP and plant extract preparations were orally administered and 5-10 animals were used per treatment group.

Determination of non-lethal APAP dose

A stock APAP suspension (30 mg/ml) was prepared in warmed 0.9% saline solution. Increasing doses of APAP (200 to 600 mg/kg) were orally administered and gross behaviour was observed and survival rates noted over a 24-hour period. For surviving animals blood was collected by cardiac puncture (for ALT/AST assays) and liver harvested for histological assessment and antioxidant enzyme assay.

Pre-treatment and post-treatment experimental study designs

Pre-treatment animals received by oral gavage, either 250, 500, 750 or 1000 mg/kg plant extract (aqueous or methanol) daily over three days followed by toxic non-lethal dose of APAP administered two hours following the final plant extract dose. Post-treatment design [16], animals were administered 250, 500, 750 or 1000 mg/kg plant extract (aqueous or methanol) one (1) hour after dosing with 550 mg/kg APAP. For both treatments, animals were euthanized at 24 hours after the last dose by cervical dislocation following which blood and liver were collected. Control animals received orally saline at the same time points.

Assays for liver enzymes

Blood was allowed to clot for about 30 minutes at ambient temperature, centrifuged at 10,000 rpm for 3-5 minutes, serum collected and separated for ALT and AST assays using the Vitros V250 chemistry analyzer.

Histological assessment of hepatotoxicity

Livers were removed (the bile duct carefully dissected) and thoroughly washed in cold 0.9% saline. The left lateral lobe was fixed in 10% formol-saline solution, processed in paraffin following standardized technique for histological assessment using the haematoxylin and eosin staining technique [17]. The grading scheme for hepatocellular damage was adapted from Ref. [18]: Grade 0=No Lesions; Grade ½=individual necrotic cells at 1st layer (Zone I); Grade 1=necrotic cells at 2nd or 3rd layers; Grade 2=necrotic cells extending 3-cell layers (Zone II); Grade 3=necrotic cells extending from one central vein to the next (Zone III). The histopathological assessment was conducted by an independent anatomical pathologist blinded to treatment.

Determination of antioxidant enzyme activities [19]

Superoxide Dismutase (SOD) assay: Total superoxide dismutase activity was measured using a kit Cayman Chemicals (Michigan, USA). Briefly, tissue was homogenized in cold 16.8 mM HEPES (pH 7.2) containing 1mM EGTA, 210 mM mannitol and 70 mM sucrose. Twenty microliters of supernatant containing 40 µg of protein was used in the assay. The reaction was initiated by adding xanthine oxidase, incubated at room temperature for 20 minutes and absorbance measured at 450 nm.

Catalase (CAT) assay: Catalase activity was measured using a kit Cayman Chemicals (Michigan, USA). Briefly, tissue was homogenized in cold 50 mM phosphate buffer (pH 7.0) containing 1mM EDTA. Twenty microliters of supernatant containing 40 µg of protein was used in the assay. The reaction was initiated by adding hydrogen peroxide (H₂O₂), which is converted by catalase into water and molecular oxygen, incubated for 20 minutes at room temperature and absorbance read at 540 nm.

Glutathione Peroxidase (GPx) assay: Cellular glutathione peroxidase activity was measured using a kit supplied by Cayman Chemicals (Michigan, USA). Briefly, tissue was homogenized in cold 50 mM Tris-HCL buffer (pH 7.6) containing 5 mM EDTA and 1 mM DTT. Twenty microliters containing 40 µg of protein was used in the assay. Glutathione peroxidase oxidizes NADPH to NADP and the reaction is measured at 340 nm.

Glutathione Reductase (GR) assay: Glutathione reductase activity was measured using a kit Cayman Chemicals (Michigan, USA). Briefly, tissue was homogenized in cold 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Twenty microliters containing 40 µg of protein was used in the assay, and the reaction was initiated with 50 µl GR NADPH and absorbance read at 340 nm.

Statistical analysis

All data are expressed as mean ± standard error, and were subjected to normality test. After determining normality, one-way ANOVA was used to determine statistical differences in ALT, AST, histological grade, CAT, SOD, GPx and GR activities between treatment groups. Kruskal- Wallis test was used for non-parametric data. Following these tests, Scheffé Test was performed. Significance was determined at $p < 0.05$.

Results

Determination of toxic non-lethal APAP dose

Groups of mice (n=6) were orally administered increasing doses of APAP ranging from 200 to 600 mg/kg, and observed for 24 hours.

At 600 mg/kg, 5 out of 6 animals died, and the next lowest dose (550 mg/kg) was used as the toxic non-lethal dose. Compared with saline-treated controls, this dose caused a significant 14-fold and 4-fold increase in ALT and AST respectively (Table 1; Figures 1a and 1b). On histopathological examination this dose produced widespread centrilobular necrosis (characterized by karyorrhexis and karyolysis), with visibly enlarged hepatocytes extending to portal regions accompanied by loss of cellular arrangement and enlarged sinusoidal regions (Table 2 and Figure 2b). This dose of APAP induced a 14-fold and 4-fold increase in SOD and GR activities respectively, with no effect on CAT activity. However, GPx activity decreased to about 35% of saline-treated control levels, $p < 0.01$ (Tables 3 and 4).

Pre-treatment and post-treatment effects on serum liver enzymes

Pre-treatment with all doses of methanol extract significantly reduced the 14-fold APAP-induced increase in ALT (F (6, 33)=18.48, $p < 0.05$) (Table 1). Additionally, ALT levels in saline controls and animals pre-treated with the lower doses of methanol extracts were statistically similar, except at higher doses. Although the higher extract doses 750 mg/kg and 1000 mg/kg produced a 3 to 4-fold increase in ALT, this was significantly lower than the increase seen with APAP alone. With regards to effect on AST levels, pre-treatment with all doses of the methanol extract abolished the significant APAP-induced increase (F (6, 33)=8.44, $p < 0.05$).

Similarly, the aqueous extract as pre-treatment abrogated the APAP-induced increase in both ALT (F (6, 29)=17.45, $p < 0.05$) and AST (F (6, 29)=10.35, $p < 0.05$). As with the methanol extract, ALT levels between saline controls and animals pre-treated with the lower doses were similar. Although ALT levels at 1000 mg/kg dose were elevated it was significantly lower than that seen with APAP alone. The trend of increasing ALT with increasing doses of extracts as pre-treatment suggests that the lower doses may be providing greater hepatoprotection.

All doses of the methanol extract as post-treatment abolished the APAP-induced increase in both ALT (F (6, 36)=39.75, $p < 0.01$) and AST (F (6, 36)=16.57, $p < 0.01$). Although animals given 750 mg/kg and 1000 mg/kg aqueous extract as post-treatment had a 2- to 3-fold increase in ALT levels compared with saline controls, $p < 0.05$, these increases were significantly lower than the 14-fold APAP-induced increase, $p < 0.05$. For the aqueous extract, post-treatment similarly abrogated the increases in both ALT (F (6, 30)=16.63, $p < 0.05$) and AST (F (6, 30)=12.83, $p < 0.01$).

Pre-treatment and post-treatment effects of extracts on hepatic histopathology

Pre-treatment with methanol extract at 250, 500 and 750 mg/kg significantly reduced the APAP-induced hepatic damage (F (6, 33)=4.89, $p < 0.05$), Table 2 and Figures 2a-2c. However, at 1000 mg/kg hepatic injury was observed to a similar extent as APAP-alone treated animals, $p = 0.12$ (Figure 2d). All pre-treatment doses of the aqueous extract abolished the APAP-induced hepatic damage (F (6, 29)=7.95, $p < 0.05$); although there was marginal increase in hepatic damage seen with increasing extract doses (Figures 4a-4d).

At 500, 750 and 1000 mg/kg the methanol extract prevented the APAP-induced pathological damage as post-treatment (F (6, 36)=9.82, $p < 0.05$) (Figures 3b-3d). However, at 250 mg/kg the methanol extract (Figure 3a) did not prevent the significant APAP-induced hepatic damage, with significantly higher histological scores compared with

saline controls, $p = 0.02$. All doses of the aqueous extract, except 750 mg/kg, at post-treatment had no effect on preventing the APAP-induced liver damage (F (6, 33)=7.82, $p < 0.05$ with an effect size (η^2) of 58.7%) (Figures 5a-5d).

Pre-treatment and post-treatment effects of extracts on antioxidant enzymes

Pre-treatment with 250 mg/kg and 500 mg/kg methanol extract had no significant effect on SOD activity compared with saline-treated controls; but at 750 mg/kg and 1000 mg/kg enzyme activity increased 2.4-fold and 4.0-fold respectively (Table 3). However, these levels were significantly lower than the 14-fold increase in SOD activity induced by APAP (F (6, 34)=165.70, $p < 0.05$) (Table 3). Similarly, pre-treatment with all doses of the aqueous extract abolished the APAP-induced increase in SOD activity (F (6, 29)=243.21, $p < 0.05$), despite a 2.3-fold increase in activity at 500 mg/kg (Table 4). Post-treatment with methanol extracts at 250 mg/kg, 500 mg/kg and 1000 mg/kg caused a significant 25% to 50% decrease in SOD activity compared with saline-treated controls, with a moderate 2.8- fold at 750 mg/kg dose (Table 3). However, these changes were significantly lower than the 14-fold APAP-induced increase (F (6, 36)=319.30, $p < 0.05$). Similarly, the 500 mg/kg, 750 mg/kg and 1000 mg/kg doses of the aqueous extract as post-treatment caused a decrease in SOD activity by 38% to 81% of

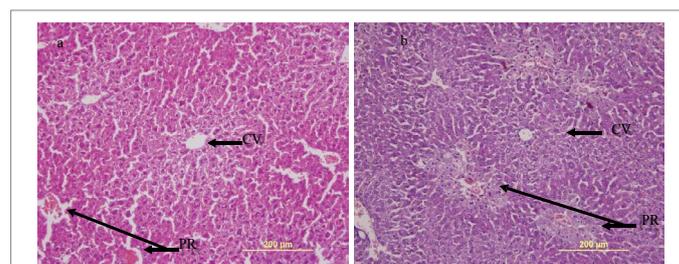


Figure 1: Photomicrographs of Haematoxylin & Eosin stained sections at magnification 20X of mouse liver fed with: a: normal saline treatment (Grade=0.0) arrows indicates normal arrangement of hepatocytes around the central vein (CV) and portal region (PR) and b: 550 mg/kg acetaminophen treatment (Grade 3.0) with derangement of hepatocytes around the around the portal regions (PR) extending for more than 3 cell layers to central vein (CV).

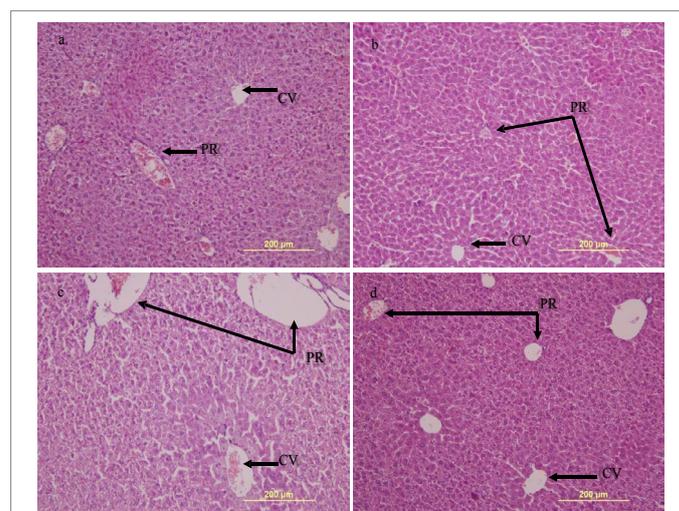


Figure 2: Photomicrographs of Haematoxylin & Eosin stained sections at magnification 20X of mouse liver fed a pre-treatment of *L. nepetifolia* methanol leaf extract at a dose of: a: 250 mg/kg (Grade 0.0); b: 500 mg/kg (Grade 0.0); c: 750 mg/kg (Grade 0.75) and d: 1000 mg/kg (Grade 0.0), arrows indicated the location of the central vein CV and portal region PR.

Treatment	Dose (mg/kg)	ALT (IU/L)		AST (IU/L)	
Control	Saline	69.67 ± 4.47		301.50 ± 30.61	
Acetaminophen	550	976.33 ± 128.24♦		1307.83 ± 225.47♦	
		Methanol extract	Aqueous extract	Methanol extract	Aqueous extract
Pre-treatment	250	65.90 ± 18.26*	58.75 ± 5.66*	213.40 ± 37.69*	275.50 ± 29.37*
	500	59.90 ± 10.41*	47.00 ± 4.81*	240.10 ± 28.82*	284.00 ± 92.03*
	750	218.25 ± 87.77* !	51.00 ± 19.80*	382.58 ± 130.42*	150.50 ± 50.07*
	1000	204.75 ± 99.41* !	307.50 ± 126.95* !	501.17 ± 162.13* !	265.67 ± 121.07*
Post-treatment	250	82.40 ± 16.73*	65.90 ± 18.25*	183.00 ± 19.36*	213.40 ± 37.68*
	500	148.83 ± 19.03*	59.90 ± 10.41*	334.83 ± 17.68*	240.10 ± 28.82*
	750	79.38 ± 7.18*	218.25 ± 87.76*	288.63 ± 9.32*	382.58 ± 130.42*
	1000	71.33 ± 9.16 *	204.75 ± 99.41*	227.67 ± 43.35*	501.16 ± 162.13* !

Table 1: ALT/AST. Serum ALT and AST concentrations (U/L) in mice pre-and post-treated with *L. nepetifolia* methanol or aqueous leaf extracts against acetaminophen-induced toxicity (♦ p<0.05, APAP-treated alone compared to normal saline control; *p<0.05, APAP-treated alone compared to extract treated and !p<0.05, normal saline control compared to extract treated).

Treatment	Dose (mg/kg)	Histological grade	
Control	Saline	0.00 ± 0.00	
Acetaminophen	550	2.50 ± 0.23 ♦	
		Methanol extract	Aqueous extract
Pre-treatment	250	0.33 ± 0.33*	0.00 ± 0.00*
	500	0.46 ± 0.33*	0.00 ± 0.00*
	750	0.80 ± 0.80*	0.86 ± 0.72*
	1000	1.30 ± 0.73	1.08 ± 0.60*
Post-treatment	250	1.80 ± 0.73	1.17 ± 0.48*
	500	0.50 ± 0.50*	2.50 ± 0.92!
	750	0.50 ± 0.16*	0.40 ± 0.24*
	1000	0.00 ± 0.00*	2.08 ± 0.37!

Table 2: Histological Grade. Histological Grading in mice pre- and post-treated with *L. nepetifolia* methanol or aqueous leaf extracts against acetaminophen-induced toxicity (♦ p<0.05, APAP-treated alone compared to normal saline control; *p<0.05, APAP-treated alone compared to extract treated and !p<0.05, normal saline control compared to extract treated).

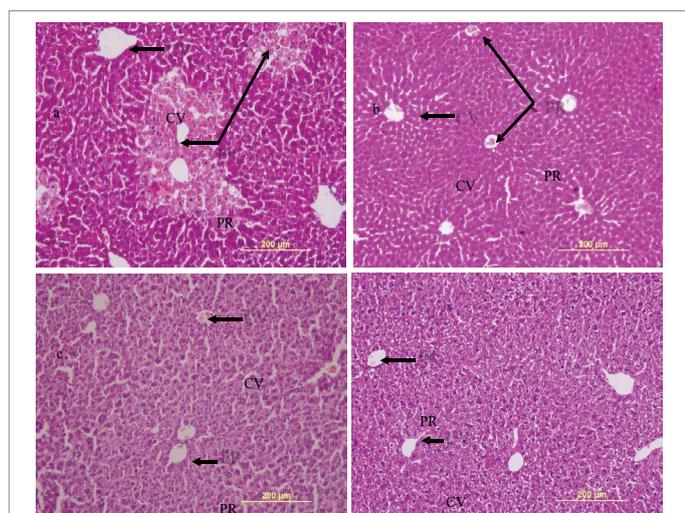


Figure 3: Photomicrographs of Haematoxylin & Eosin stained sections at magnification 20X of mouse liver fed a post-treatment of *L. nepetifolia* methanol leaf extract at a dose of: a: 250 mg/kg (Grade 3.0); b: 500 mg/kg (Grade 0.0); c: 750 mg/kg (Grade 0.0) and d: 1000 mg/kg (Grade 0.0), arrows indicated the location of the central vein CV and portal region PR.

saline-treated animals (Table 4) and abolished the significant APAP-induced increase in enzyme activity (F (6, 33)=318.50, p<0.05).

Pre-treatment with methanol extract increased GPx activity between 6% and 59% compared with saline-treated controls (Table 3). However, all doses significantly reversed the APAP-induced decrease in GPx activity (F (6, 28)=12.19, p<0.01). Similarly, all doses of the aqueous extract as pre-treatment caused an increase (between 1.4-fold to 2.2-fold) in GPx activity compared to the saline-treated controls and reversed the APAP-induced decline in enzyme activity (F (6, 28)=12.19 (p<0.01) with an effect size (η²) of 72.3%.

Post-treatment with methanol extract increased GPx activity between 8% and 50% compared with saline-treated controls (Table 3). However, all doses significantly abrogated the APAP-induced decline in enzyme activity (F (6, 35)=9.14, p<0.05) with an effect size (η²) of 61.7%. Similarly, the aqueous extract as post-treatment increased GPx activity between 30% and 82% over saline-treated controls (Table 4), and significantly abolished the APAP-induced decrease in enzyme activity (F (6, 32)=15.37, p<0.01) with an effect size (η²) of 73.3%.

Pre-treatment with methanol extract abrogated the 4-fold APAP-induced elevation in GR activity (F (6, 33)=22.99, p<0.05), although enzyme activity across the dose range increased by up to 38% over the saline-treated controls. Similarly, pre-treatment with all doses of aqueous extracts abolished the significant APAP-induced increase in GR activity F (6, 28)=19.79 (p<0.01) with an effect size (η²) of 80.9%, although there was an increase in enzyme activity between 35% and 72% over saline-treated controls.

All doses of the methanol extract as post-treatment abolished the significant APAP-induced increase in GR activity. Despite the moderate elevation of GR activity between 52% and 78% over the dose range over the saline-treated controls, this was significantly lower than the 4-fold increase seen with APAP alone (F (6, 35)=20.25, p<0.05). Similarly, all doses of the aqueous extract as post-treatment abrogated the significant 4-fold increase in enzyme activity by APAP (F (6, 28)=19.79, p<0.01). However, all doses also caused significant increases in enzyme activity between 72% and 126% over saline-treated controls (F (6, 32)=14.66, p<0.01).

Although high-dose APAP caused a slight decrease in CAT activity, this was statistically insignificant (p>0.05). For the methanol extract, except for the post-treatment dose of 250 mg/kg where there was a non-significant decrease, all pre-treatment and post-treatment

Treatment	Dose (mg/kg)	CAT (nmol/min/ml)	SOD (U/mL)	GPx (nmol/min/ml)	GR (nmol/min/ml)
Control	Saline	1979.84 ± 111.71	19.78 ± 1.23	49.66 ± 5.47	45.51 ± 4.87
Acetaminophen	550	1817.80 ± 63.65	282.45 ± 14.26♦	17.09 ± 3.53♦	180.69 ± 17.08♦
Pre-treatment	250	1909.17 ± 70.05	20.94 ± 2.09*	72.58 ± 11.67*	52.97 ± 4.69*
	500	2059.92 ± 102.49	29.98 ± 6.13*	78.95 ± 7.69*	61.12 ± 7.80*
	750	1984.45 ± 54.77	48.10 ± 10.41*	75.13 ± 5.48*	46.55 ± 5.44*
	1000	1982.53 ± 132.17	78.17 ± 4.82* !	52.76 ± 8.97*	62.83 ± 9.16*
Post-treatment	250	1734.87 ± 194.32	5.01 ± 1.25*	53.49 ± 5.55*	80.79 ± 11.40*
	500	2151.37 ± 97.29	6.94 ± 0.45*	73.22 ± 6.31*	69.33 ± 6.27*
	750	2079.30 ± 99.74	55.87 ± 3.33*	64.50 ± 5.83*	68.98 ± 6.04*
	1000	1951.33 ± 110.04	11.33 ± 1.39*	74.29 ± 8.80*	80.23 ± 6.28*

Table 3: Antioxidant enzymes (Methanol extract). Antioxidant enzymes in mice pre- and post- treated with *L. nepetifolia* leaf extract against acetaminophen-induced toxicity (♦ p<0.05, APAP-treated alone compared to normal saline control; *p<0.05, APAP-treated alone compared to extract treated and !p<0.05, normal saline control compared to extract treated).

Treatment	Dose (mg/kg)	CAT (nmol/min/ml)	SOD (U/mL)	GPx (nmol/min/ml)	GR (nmol/min/ml)
Control	Saline	1979.84 ± 111.71	19.78 ± 1.23	49.66 ± 5.47	45.51 ± 4.87
Acetaminophen	550	1817.80 ± 63.65	282.45 ± 14.26♦	17.09 ± 3.53♦	180.69 ± 17.08♦
Pre-treatment	250	1725.25 ± 212.47	16.56 ± 4.63*	108.84 ± 20.70 *	61.34 ± 4.75*
	500	1849.52 ± 107.52	45.50 ± 2.97*	108.24 ± 15.60*	76.41 ± 6.74*
	750	1788.84 ± 131.53	22.51 ± 3.52*	70.04 ± 11.33*	78.32 ± 7.52*
	1000	1980.18 ± 112.13	20.28 ± 2.07*	47.75 ± 3.58*	77.68 ± 7.29*
Post-treatment	250	1976.76 ± 151.32	22.47 ± 1.52*	47.66 ± 3.93*	94.10 ± 12.07*
	500	1750.03 ± 164.38	16.14 ± 0.97*	66.22 ± 4.32*	97.80 ± 10.38*
	750	1821.51 ± 49.89	10.41 ± 0.92*	90.42 ± 4.68*	78.44 ± 12.10*
	1000	1824.85 ± 174.19	7.64 ± 1.28* !	64.73 ± 7.96*	102.87 ± 7.52*

Table 4: Antioxidant enzymes (Aqueous extract). Antioxidant enzymes in mice pre- and post-treated with *L. nepetifolia* leaf extracts against acetaminophen-induced toxicity (♦ p<0.05, APAP-treated alone compared to normal saline control; *p<0.05, APAP-treated alone compared to extract treated and !p<0.05, normal saline control compared to extract treated).

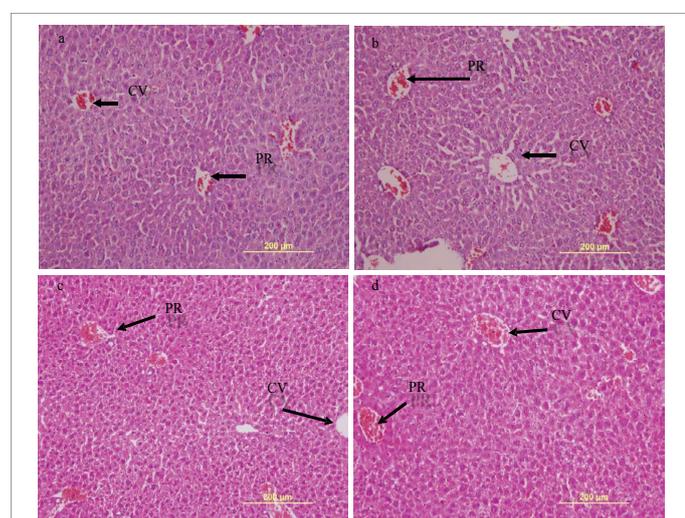


Figure 4: Photomicrographs of Haematoxylin & Eosin stained sections at magnification 20X of mouse liver fed a pre-treatment of *L. nepetifolia* aqueous leaf extract at a dose of: a: 250 mg/kg (Grade 0.0); b: 500 mg/kg (Grade 3.0); c: 750 mg/kg (Grade 0.0) and d: 1000 mg/kg (Grade 0.0), arrows indicated the location of the central vein CV and portal region PR.

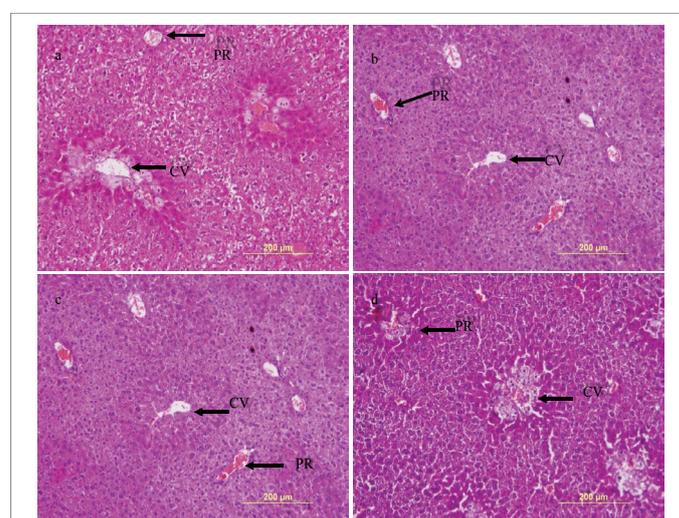


Figure 5: Photomicrographs of Haematoxylin & Eosin stained sections at magnification 20X of mouse liver fed a post-treatment of *L. nepetifolia* aqueous leaf extract at a dose of: a: 250 mg/kg (Grade 2.0); b: 500 mg/kg (Grade 0.0); c: 750 mg/kg (Grade 0.0) and d: 1000 mg/kg (Grade 2.0), arrows indicated the location of the central vein CV and portal region PR.

doses produced a non-significant increase in hepatic catalase activity over APAP alone (Tables 3 and 4). However, only the post-treatment methanol extract at 500 mg/kg produced a significant increase in enzyme activity (p=0.02). For the aqueous extract, none of the treatments produced significant changes in enzyme activity compared to APAP-alone treatment.

Discussion

The results of our study showed that both pre-treatment and post-treatment with aqueous and methanol extracts of *Leonotis nepetifolia* prevented the APAP-induced hepatotoxicity in Swiss albino mice. This was demonstrated by the extracts' ability to abolish the dramatic APAP-induced rise in liver enzymes (ALT and AST) and maintained

normal hepatic morphology. A recent study by Ref. [20] showed that the aqueous extract *L. nepetifolia*, given as chronic post-treatment, protected against CCl₄-induced hepatic damage in rats in a similar manner. Additionally, several studies have shown that the methanol and aqueous extracts of some medicinal plants protect against APAP-induced hepatotoxicity by abrogating the drug-induced increase in liver enzymes and maintaining normal liver morphology [21-24]. Several studies have identified antioxidant phytochemicals, such as polyphenols and flavonoids, which were suggested to be responsible for the medicinal plants' protective properties against APAP-induced hepatic injury [25-28].

Few studies have attempted to determine the direct effect of these hepatoprotective herbal extracts on of antioxidant enzyme activity. We hypothesized that known antioxidant compounds present in *L. nepetifolia* may be modulating the activity of antioxidant enzymes and therefore play an important role in protecting the liver against APAP-induced injury. The study by Ref. [20] also investigated the post-treatment effects of aqueous *L. nepetifolia* extract on the activities of GPx, CAT and SOD in the CCl₄-induced model of hepatic injury. These researchers showed that the plant extract had a dose-dependent effect to reverse the CCl₄-induced suppression in the activities of these antioxidant enzymes.

Glutathione plays a critical role in maintaining cellular redox balance, and high doses of APAP cause significant depletion this endogenous tripeptide antioxidant with accumulation of reactive oxygen species (ROS) leading to cellular injury and necrosis, with subsequent protein binding and lipid peroxidation [5]. To maintain this redox balance, glutathione is cycled between its reduced (GSH) and oxidized (GSSG) forms under the influence of GR and GPx. From our results, the ratio between GPx and GR in hepatic tissue of untreated control animals was approximately 1:1.

High-dose APAP caused a significant shift in the ratio in activities of these antioxidant enzymes from 1:1 to 1:10; with GPx activity falling to about 35% of saline-treated controls, with a dramatic 4-fold rise in GR activity. The cellular levels of the reduced form are critical, as it

readily binds ROS to reduce oxidative stress. This is supported by our results where increased GR activity suggests a cellular response to generate more reduced glutathione that would scavenge increasing concentrations of NAPQI produced by the metabolism of high dose APAP.

The APAP-induced attenuation in GPx activity would cause an accumulation of hydroperoxides and peroxide radicals that subsequently leads to an increase in SOD activity to remove these reactive species. However, the compensatory increase in SOD activity would not have been sufficient to prevent damage caused by these reactive species. We also observed an insignificant decline in CAT activity in the presence of high dose APAP. In their study which investigated the hepatoprotective effects of *Baccharis trimera* in rats, [29] showed that APAP at 835 mg/kg increased SOD activity, whilst suppressing the activities of both GR and GPx.

In our study, pre-treatment and post-treatment with both methanol and aqueous extracts reversed the APAP-induced suppression of GPx activity; and notably the pre-treatment aqueous extract produced between 1.4 fold to 2.2 fold increase in activity compared with saline-treated controls. These results hold the key to understanding a possible mechanism by which *L. nepetifolia* extracts may be providing hepatoprotection against APAP insult (Figure 6). Increasing GPx activity would increase the liver's capacity to remove toxic cell-damaging hydroperoxides and peroxide radicals, thus preventing injury [30]. It may be possible that this increased activity is due to transcriptional up-regulation of the gene and/or post-translational modification of the protein (enzyme) in the pre-treatment model. But, it is noteworthy that this effect is also observed in the post-treatment model even after the liver was exposed to damaging reactive species before the administration of *L. nepetifolia* extracts; this points to post-translational modification of already formed enzyme.

Although the study by Ref. [20] showed that *L. nepetifolia* reversed the CCl₄-induced decline in GPx, enzyme activity just returned to control levels at the highest dose of 300 mg/kg. In another study, an extract of *B. trimera* extract did not reverse the APAP-induced diminution of GPx activity [29].

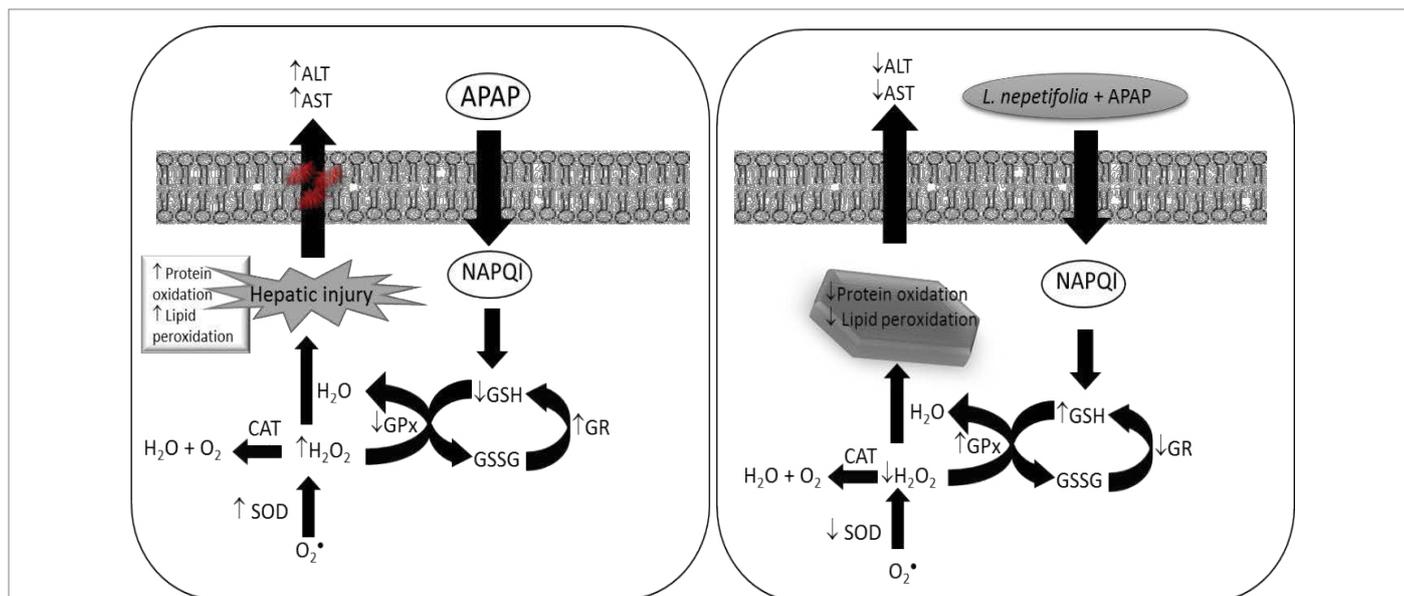


Figure 6: Illustration showing the protective effect of *L. nepetifolia* in acetaminophen-induced liver toxicity. Adapted from Padua Bda et al. in comparison to NS controls, APAP toxicity causes oxidative stress with decreased GSH levels, GPx enzyme activity and increased SOD activity resulting in elevation of H₂O₂ specifically by elevated serum ALT and AST. Administration of *L. nepetifolia* maintains oxidative balance during APAP treatment. By increasing GSH levels, GPx activity and reducing SOD activity with maintained oxidative balance and lowered serum ALT and AST cellular integrity was maintained.

All doses of both extracts as pre-treatment and post-treatment caused a moderate increase in GR activity (up to 2-fold increase in aqueous post-treatment); however, they prevented the significant 4-fold increase seen with APAP alone. Although we did not measure hepatic glutathione levels, these results suggest that there was a reduced requirement to generate this endogenous antioxidant as other mechanisms would have been at play to scavenge reactive species, such as increased GPx activity. Despite the perturbations in absolute GPx and GR activities, it should be noted that the ratio of these enzymes were close to 1:1 as seen in saline-treated controls. It may be possible that antioxidant compounds in *L. nepetifolia* may be triggering a cellular response to maintain the redox cycling of glutathione; as opposed to the 1:10 ratio seen in the livers of animals treated with APAP alone.

Pre- and post-treatment with both extracts abrogated the APAP-induced elevation of SOD activity. Although the enzymes levels for these treatments ranged between 25% decrease to 4-fold increase over saline controls, which was significantly lower than the 14-fold increase seen with high dose APAP. These results suggest that there is a reduced requirement to scavenge hydroperoxides and peroxide radicals in the presence of the extracts, which may be due to increased GPx activity or direct antioxidant effects of phytochemicals. In a study conducted by Ref. [31] which looked at the extract's anti-inflammatory effects and found that some possible active isolates included stigmaterols, diterpenes, labdanes and leonotinin which may also be providing the antioxidant capability.

Conclusions

Leonotis nepetifolia possesses a significant potential for hepatoprotection against APAP-induced toxicity possibly directly through phytochemicals contained in the extract. The extract components may be providing an up-regulation of genes during transcription of proteins (enzymes) to reduce the demand for increased GR activity during APAP toxicity. This also suggests the possible modification to augment activity of endogenous antioxidant enzymes for more efficient removal of pro-oxidants during toxic insult. Our findings therefore suggest that aqueous and methanol extracts of *L. nepetifolia* may be useful in the treatment of acetaminophen overdose.

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