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EVALUATION OF HEPATOPROTECTIVE AND ANTIOXIDANT ACTIVITY OF *ALTERNANTHERA SESSILIS* (LINN.) ON PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT
The ethanolic extract of *Alternanthera sessilis* Linn. (Amaranthaceae) aerial parts were investigated for any *in-vitro* and *in-vivo* antioxidant and hepatoprotective effects. Ethanolic extract of *Alternanthera sessilis* (EEAS) was prepared and acute toxicity study was carried out to fix the dose. Different groups of animals were administered with paracetamol (500 mg/kg, PO, once in a day for 7 days). EEAS at the dose of 200 and 400 mg/kg/day and silymarin at 25 mg/kg/day were administered to the paracetamol treated rats for seven days. The effects of EEAS and silymarin on serum transaminases (SGOT, SGPT), alkaline phosphatase (ALP), bilirubin (Direct and Total), cholesterol (HDL and Total) and total protein were measured in the paracetamol-induced hepatotoxic rats. Further, the effects of the extract on lipid peroxidation (LPO), thaiobarbituric acids (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were estimated. EEAS at dose of 400mg/kg and silymarin produced significant ($p < 0.05$) hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, total cholesterol and *in-vivo* lipid peroxidation and significantly ($p < 0.05$) increasing the levels of GSH, SOD, CAT and HDL cholesterol than the dose of 200mg/kg. From the results, it was suggested that EEAS could protect the liver cells from paracetamol induced liver damage by its antioxidative effect on hepatocytes, hence eliminating the deleterious effects of toxic metabolites of paracetamol which is dose dependent.

KEYWORDS: *Alternanthera sessilis*, Hepatoprotective effect, Antioxidants, Paracetamol.

INTRODUCTION
Liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy production and reproduction. Because of its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target for toxicity produced by drugs, xenobiotics and oxidative stress. More than 900 drugs, toxins and herbs have been reported to cause liver injury and drugs account for 20–40% of all instances of fulminant liver failure. In the absence of reliable liver protection drugs in modern medicine, a large number of medicinal preparations are recommended for the treatment of liver disorders and quite often claimed to offer significant relief. Attempts are being made globally to get scientific evidences for these traditionally reported herbal drugs. This scenario provides a severe necessity to carry out research in the area of hepatotoxicity.[1]

Alternanthera sessilis Linn. (Amaranthaceae) is an annual or perennial prostate herb with several spreading branches, bearing short petioled simple leaves and small white flowers, found throughout the hotter part of India, ascending to an altitude of 1200m.[2] The plant spreads by seeds, which are wind and water-dispersed and by rooting at stem nodes. Young shoots and leaves are eaten as a vegetable in Southeast Asia.[3] It is a weed of rice throughout tropical regions and of other cereal crops, sugarcane and bananas. Although it is a weed, it has many utilities. The leaves are used in eye diseases, cuts, wounds and antidote to snake bite; skin diseases.[4] It is also reported about the wound healing property of *Alternanthera sessilis* Linn.[5] The degenerative and necrotic changes in the liver and kidney in Swiss mice, caused by oral administration of water extract of *Alternanthera sessilis* in high doses through histopathological test were revealed.[6]

MATERIALS AND METHODS

Plant Material

The plant was identified by the botanists of the VR College, Nellore, Andhra Pradesh. After authentication, fresh aerial parts of the young and matured plants were collected in bulk from the rural belt of Jangalakandriga, Nellore, Andhra Pradesh, India during early summer (May-June), washed, shade dried and then milled in to coarse powder by a mechanical grinder.

Preparation of plant extract (EEAS)

The powdered plant material (400gm) was defatted with petroleum ether (60-80^{0}C) and then extracted with 1.5 litre of ethanol (95%) in a Soxhlet apparatus. The solvent was removed under reduced pressure, leaving a greenish-black sticky residue (yield: 14.8% w/w with respect to dried plant material). The dried extract (EEAS) was stored in a desiccators till needed.

Chemicals Used

All the chemicals and reagents used in the study were of analytical grade. Kits (Span Diagnostics Ltd., India and Excel diagnostics Ltd., India) were used for biochemical estimations.

Animals

Studies were carried out using Wistar albino rats (150–180gm) of male sex. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than five animals per cage and maintained under standard laboratory conditions. They were allowed free access to standard dry pellet diet and water *ad libitum*. Swiss albino mice of either sex weighing between 25-30gm were acclimatized to laboratory condition for 10 days before commencement of experiment. They were also allowed free access to standard dry pellet diet and water *ad libitum*. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Acute toxicity Studies

The test was carried out as suggested by Seth *et. al.*, 1972.[7] Swiss albino mice of either sex weighing between 25-30 g were divided into different groups of six animals each. The control group received normal saline (2 ml/kg, PO). The other groups received 100, 200, 300, 400, 600, 800 mg/kg of EEAS respectively through oral route. Immediately after dosing, the animals were observed continuously for the first 4 hours for any behavioural changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any.

Hepatoprotective Activity

- ***Paracetamol-Induced Liver Damage in Rats (Acute Model)***

The test was carried out as suggested by Murugesh *et. al*., 2005.[8] Five groups each comprising of six male Wister albino rats weighing in the range of 150-180 gm was selected. Group I served as control and fed orally with normal saline 5 ml/kg daily for seven days. Group II rats were similarly treated as Group I. Group III and IV were treated with ethanolic extract 200 and 400 mg/kg/day orally for seven days, while Group V was fed Silymarin 25mg/kg as standard daily for seven days.[9] Paracetamol suspension was given by oral route in a dose of 500 mg/kg/day to all rats except rats in Group I for seven days. The biochemical parameters were determined after 18 hours of fasting of the last dose. Daily records of body weight of all groups of animals were maintained during the whole experimental period.

- ***Biochemical Studies***

After the treatment period, the animals of all groups were anaesthetized and sacrificed. Blood was drawn from heart and serum was separated for the assay of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase ALP, Bilirubin (Direct and total) and Cholesterol (Total and HDL). Analysis of SGOT, SGPT, ALP, Bilirubin (Direct and total) and Cholesterol (Total and HDL) was performed using analytical kits from Span Diagnostics Ltd., Surat, India. Serum GOT and GPT were measured according to the method of Rietman and Frankel, 1975[10], serum ALP was measured according to the method of King *et. al*., 1965[11], serum bilirubin was estimated following Malloy and Evelyn method, 1937.[12] Serum cholesterol (Total and HDL) was measured according to Warnick *et. al*., 1985.[13]

- ***$FeCl_2$-Ascorbic Acid Stimulated Lipid Peroxidation in Liver Homogenate***

The Wister albino rats weighing 150-180 gm were killed by decapitation and their liver tissues were quickly removed. A 2gm portion of liver tissue was sliced and then homogenized with 10 ml of 150 mM KCl Tris-HCl buffer (pH 7.4). The reaction mixture was composed of 0.25 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.4), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM $FeCl_2$ and 0.05 ml of various concentrations of EEAS. The products of lipid peroxidation were quantified by the formation of the thiobarbituric acid-reactive material, MDA. 1,1,3,3 Tetraethoxypropane was used as a standard for calibration of MDA.[14]

Determination of In-vivo Antioxidant Activity

After collection of blood samples the rats were sacrificed and their livers were excised, rinsed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.2) blotted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation (TBARS) by the method of Fraga *et. al*., 1981.[15] A part of homogenate after precipitating proteins was used for estimation of glutathione by the method of Ellman *et. al*., 1959.[16] The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4^{0}C. The supernatant thus obtained was used for the estimation of SOD by the method described by Kakkar *et. al*., 1984.[17] CAT activity was measured by the method of Maehly *et al*., 1954.[18]

➢ ***Determination of Thiobarbituric Acid Reactive Substances (TBARS)***

TBARS in tissues was estimated by the method of Fraga *et. al*., 1981. To 0.5 ml tissue homogenate, 0.5 ml saline and 1.0 ml 10% TCA were added, mixed well and centrifuged at 3000 rpm for 20 min. To 1.0 ml of the protein-free supernatant, 0.025 ml of thiobarbituric acid (TBA) reagent was added; the contents were mixed well and boiled for 1 h at 95^0C. The tubes were then cooled to room temperature under running water and absorbance measured at 532 nm.

➢ ***Determination of Reduced Glutathione (GSH)***

GSH was determined by the method of Ellman *et. al*., 1958. 0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml precipitating reagent (1.67 g of meta phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2.0 ml of the filtrate, 4.0 ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5, 5-dithio- bis- 2-nitrobenzoic acid) reagent were added and read at 412 nm.

➢ ***Determination of Super Oxide Dismutase (SOD)***

The activity of SOD in tissue was assayed by the method of Kakkar *et. al*., 1984. The assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1 ml phenazine methosulphate (186mM/L), 0.3 ml NBT (300 mM/L), 0.2 ml NADH (780mM/ L) and approximately diluted enzyme preparation and water in a total volume of 3 ml. After incubation at 30^0C for 90 sec, the reaction was terminated by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol.

➢ ***Determination of Catalase (CAT)***

Catalase was assayed according to the method of Maehly and Chance, 1954. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-4^0C and centrifuged at 5000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The specific activity of Catalase is expressed in terms of (mole of H_2O_2 consumed/min/mg of protein.

Statistical Analysis

Data for the *in-vitro* antioxidant activity was expressed as Mean ± SD from three separate observations. Data for hepatoprotective activity and *in-vivo* antioxidant activity were expressed as Mean ± SEM from 6 rats in each group. Hepatoprotective and *in-vivo* antioxidant activity were analysed statistically using one-way analysis of variance (ANOVA), followed by Dunnett's t-test. The minimum level of significance was fixed at $p<0.05$.

RESULTS

Acute Toxicity Studies

In acute toxicity study, it was found that the extract induced sedation and temporary postural defect at all tested doses. However, there was no mortality at any of the tested doses till the end of 14 days of observation and the dose fixed as 400mg/kg body weight.

Biochemical Studies

Rats subjected to paracetamol only, developed significant ($p<0.05$) hepatocellular damage as evident from significant increase in serum activities of GOT, GPT, ALP and bilirubin concentration as compared to normal control group, which has been used as reliable marker of hepatotoxicity (Table 1). Oral administration of EEAS with a dose of 400mg/kg exhibited significant reduction ($p<0.05$) in paracetamol-induced increase in levels of GOT, GPT, ALP and bilirubin (Total and Direct) concentration than the dose of 200mg/kg. Treatment with Silymarin (25mg/kg, PO) also reversed the hepatotoxicity significantly ($p<0.05$). Table 1 also reveals that total cholesterol level of serum of the rats treated only with paracetamol increased significantly ($p<0.05$) while HDL level decreased significantly ($p<0.05$) with respect to the control group. But, EEAS was successful in blunting this paracetamol-induced increase in serum cholesterol level and decrease in HDL level, which was comparable with the reference drug Silymarin.

Liver Weight

Table 1 also reveals that the liver weight of rats treated with paracetamol only decreased significantly ($p<0.05$), which was blunted by EEAS and Silymarin.

In-vitro Lipid Peroxidation

$FeCl_2$-ascobic acid induced *in-vitro* lipid peroxidation study revealed that EEAS had significant anti lipid peroxidation potential with IC_{50} value being 160.21 g/ml, which was comparable with the reference drug α-tocopherol (Table 2).

In-vivo Antioxidant Activity

In-vivo lipid peroxidation study reveals that rats of paracetamol treated group showed significant increase ($p<0.05$) in Malondialdehyde (MDA) when compared with rats of normal control group. EEAS and Silymarin were able to significantly blunt ($p<0.05$) this rise in MDA level. There was a marked decrease in the level of GSH and the activities of SOD and CAT in paracetamol treated group when compared with normal control group. The GSH level and activities of SOD and CAT were significantly increased ($p<0.05$) in EEAS and Silymarin treated groups (Table 3).

RESULT

Table 1: Effect of ethanolic extract of *Alternanthera sessilis* aerial parts on paracetamol-induced hepatotoxicity in rats.

Group	Treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (KA units)	Bilirubin (mg/dl)		Cholesterol (mg/dl)		Liver Weight
					Total	Direct	Total	HDL	
I	Control	46.33 ± 0.95	55.33 ± 0.67	78.00 ± 1.79	0.57 ± 0.02	0.08 ± 0.01	116.42 ± 1.90	9.98 ± 0.61	10.90 ± 0.45
II	Paracetamol Treated	126.50 ± 2.92*	117.67 ± 3.20*	153.67 ± 4.39*	5.43 ± 0.31*	0.96 ± 0.05*	175.46 ± 5.37*	1.71 ± 0.29*	7.28 ± 0.49*
III	Paracetamol + Extract (200mg/kg)	66.23 ± 2.23**	71.25 ± 2.89**	127.25 ± 2.98**	1.10 ± 4.32**	0.09 ± 0.01**	158.75 ± 3.58**	8.79 ± 0.98**	9.11 ± 0.56**
IV	Paracetamol + Extract (400mg/kg)	64.67 ± 3.00**	67.67 ± 3.24**	115.33 ± 2.11**	0.94 ± 0.05**	0.18 ± 0.03**	144.94 ± 5.64**	10.94 ± 0.98**	10.07 ± 0.50**
V	Paracetamol + Silymarin	57.00 ± 2.24**	67.00 ± 4.72**	93.33 ± 2.62**	0.91 ± 0.04**	0.26 ± 0.03**	152.28 ± 4.83**	35.42 ± 2.05**	10.25 ± 0.34**

All values are Mean ± SEM, n=6 rats in each group.

***P* < 0.05 as compared with Group I, ****P* < 0.05 as compared with Group II.**

Table: 2 Effect of ethanolic extract of *Alternanthera sessilis* aerial parts on *in-vitro* Lipid Peroxidation.

Sl. No.	Sample	Concentration (µg/ml)	% inhibition	IC_{50}
1.	Ethanolic Extract	50	17.25 ± 1.01	160.21
2.		100	28.02 ± 2.13	
3.		150	39.98 ± 2.01	
4.		200	73.46 ± 2.15	
5.	α-tocopherol			133.86

n=3, Values are Mean ± S.D.

Table: 3 Effect of ethanolic extract of *Alternanthera sessilis* aerial parts on LPO, antioxidant enzymes and GSH in liver of paracetamol -induced hepatotoxic rats *in-vivo*.

Group	Treatment	LPO[a]	SOD[b]	CAT[c]	GSH[d]
I	Control	1.69 ± 0.27	12.59 ± 0.42	64.40 ± 3.25	61.59 ± 2.11
II	Paracetamol treated	8.09 ± 0.42*	4.49 ± 0.28*	43.31 ± 1.97*	32.42 ± 0.79*
III	Paracetamol + Extract (200mg/kg)	2.32 ± 0.05**	9.01 ± 0.75**	63.25 ± 3.01**	57.44 ± 1.09**
IV	Paracetamol + Extract (400mg/kg)	3.71 ± 0.40**	9.17 ± 0.35**	58.48 ± 2.31**	54.66 ± 1.11**
V	Paracetamol + Silymarin	4.28 ± 0.30**	10.39 ± 0.37**	56.20 ± 1.92**	50.78 ± 0.95**

All values are Mean ± SEM, n=6 rats in each group,
***P < 0.05 as compared with Group I, ****P < 0.05 as compared with Group II**
a= nmole of MDA/mg of protein, b= Units/mg of protein,
c= µmole of H_2O_2 consumed/min/mg of protein, d= µg/mg of protein.

DISCUSSION

Paracetamol (Acetaminophen) is a widely used antipyretic and analgesic, produces acute liver damage if overdoses are consumed. Paracetamol is mainly metabolized in liver to excretable glucuronide and sulphate conjugates.[19-20] However, the hepatotoxicity of Paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-450,[21] to a highly reactive metabolite N-acetyl-P-benzoquinone imine (NAPQI).[22]

Paracetamol produces hepatic necrosis when ingested in very large doses. The hepatoprotective activity of the ethanolic extract was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which give a good idea about the functional state of the liver.[23] Necrosis or membrane damage releases the enzyme into circulation and therefore, it can be measured in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore, SGPT is more specific to the liver and is thus a better parameter for detecting liver injury.[24] The results demonstrate that the ethanolic extract of *Alternanthera sessilis* caused significant inhibition of SGOT and SGPT levels. Serum ALP and bilirubin levels on the other hand, are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure.[25] Our results also demonstrate that the ethanolic extract of *Alternanthera sessilis* caused significant inhibition of serum ALP and bilirubin levels. Effective control of alkaline phosphatase activity and bilirubin level points towards an early improvement in secretory mechanism of hepatic cells.

The effects of the extract on liver weights of rats are shown in Table 1. The paracetamol treated rats showed a significant loss in liver weight. But the extract administration significantly prevented this paracetamol-induced weight loss of liver in rats, which is comparable with that of standard drug administration.

Most hepatotoxic chemicals including paracetamol and alcohol damage liver by inducing, directly or indirectly, lipid Peroxidation.[26] *In-vitro* lipid peroxidation in liver homogenate can proceed in a non-enzymatic way. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+} and it has been reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. In order to clarify the mode of action of ethanolic extract, *in-vitro* lipid peroxidation experiments were carried out. According to the result obtained, the extract inhibited $FeCl_2$-ascorbic acid-stimulated lipid peroxidation in liver homogenate (Table 2).

Cells have a number of mechanisms to protect themselves from the toxic effects of ROS. SOD removes superoxide (O^{-2}) by converting it to H_2O_2, which can be rapidly converted to water by CAT.[27] In addition, a large reserve of reduced glutathione is present in hepatocytes and red blood cells for the detoxification of xenobiotics or free radicals. However, oxidative stress results in toxicity when the rate at which the ROS are

generated exceeds the cell capacity for their removal. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. MDA is one of the end products in the lipid peroxidation process.[28]

In our *in-vivo* study elevation in levels of end products of lipid peroxidation in liver of rats treated with paracetamol were observed. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage. Pre-treatment with EEAS significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of extract is due to its antioxidant effect. GSH protects cells against free radicals, peroxides and other toxic compounds and a deficiency of GSH within living organisms can lead to tissue disorder and injury. From this point of view, exogenous ethanolic extract of *Alternanthera sessilis* supplementation might provide a mean to recover reduced GSH levels and to prevent tissue disorders and injuries.

From the results it was found that after EEAS-supplementation elevated GSH level in rats with paracetamol could be blunted to normal level. Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators, enzymes such as SOD and CAT.[29] The SOD dismutases superoxide radicals O_2 - into H_2O_2 plus O_2, thus participating with other antioxidant enzymes, in the enzymatic defence against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The observed increase of SOD activity suggests that the ethanolic extract of *A. sessilis* have an efficient protective mechanism in response to ROS.

CAT is a key component of the antioxidant defence system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Administration of ethanolic extract of *Alternanthear sessilis* was found to increase the activities of catalase in paracetamol induced liver damage rats to prevent the accumulation of excessive free radicals and protects the liver from paracetamol intoxication. This ability of EEAS to protect the liver from paracetamol-induced damage might be attributed to its ability to restore the activity of antioxidative enzymes. Thus, results of these studies together with those of earlier ones, suggest that EEAS has an ability to protect the liver from paracetamol-induced damage through its direct antioxidative effect.

It is well documented that hepatocellular enzymes (SOD, CAT) serve as biomarkers of hepatocellular injury due to alcohol and drug toxicity.[30] So the studies on antioxidant enzymes (SOD, CAT) have been found to be of great importance in assessment of liver damage.

It can be summarised that EEAS was found to prevent paracetamol-induced oxidative stress and hepatic injury which is dose dependent. Since these models of hepatic damage in the rat simulate many of the features of human liver pathology, so it can be suggested that natural antioxidants and scavenging components of EEAS might be effective as plant hepatoprotectors and thus may have some obvious therapeutic implications. Therefore, it seems logical to infer that, because of its antioxidant property, EEAS might be capable of protecting the hepatic tissue from paracetamol-induced injury and inflammatory changes.

The ethanolic extract of *Alternanthera sessilis* is reported to be rich in different secondary metabolites. Presence of these metabolites in the ethanolic extract had already been examined and was confirmed through preliminary phytochemical screening also.[31] Studies have confirmed that indeed oxidative stress plays an important role in the initiation and progression of liver disease. The activity may be attributed to their protective action on lipid peroxidation and at the same time the enhancing effects on the cellular antioxidant defence contributing to the protection against oxidative damage in paracetamol-induced hepatotoxicity which had been proved to be dose dependent.

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