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Research Article

SUPPRESSION OF PARACETAMOL TOXICITY BY ANTIOXIDANT PRINCIPLES OF *HYBANTHUS ENNEASPERMUS* (L.) F. MUELL. IN MICE BLOOD AND LIVER

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ABSTRACT

The present study was designed to investigate the hepatoprotective activity of the ethanolic extract of *Hybanthus enneaspermus.* The ethanolic extract was evaluated for its hepatoprotective activity. Anti-oxidant activity of ethanolic extract was determined by the enzyme assays CAT, SOD, GST, GR and LPO in mice. The blood serum and liver markers and histopathology of liver were studied to prove its hepatoprotective effect. The paracetamol toxicity tested in mice has elicited more of serum transaminases, alkaline phophatases & reduced the content of liver glutathione, elevation of lipid peroxidation and antioxidant enzymes. However the plant extract collected form *HYBANTHUS ENNEASPERMUS* leaves has restored the normal activities of enzymes by serving one of the plant products as an antioxidant to mice serum and liver. our results were also conducted that due to depletion of lipid peroxidation in blood & liver the plant product showed protection to hepatocyte by depleting the necrotic cells in the liver.

Keywords: *Hybanthus enneaspermus*, Hepatoprotective effect, Antioxidant role, Paracetamol.

INTRODUCTION

In eukaryotic organisms organ like the liver generate reactive oxygen species, (ROS) that induce oxidative tissue damage. These ROS, which react with cell membranes and thus induce lipid peroxidation or cause inflammation, have been implicated as important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer [1, 2, 3]. A major defense mechanism is the antioxidant enzymes, which convert active oxygen molecules into non-toxic compounds [4, 5].

Liver diseases remain as one of the serious health problems. However as on today, we do not have satisfactory liver protective drugs in allopathic medical practice for serious liver disorders. Herbal drugs play a role in the management of various liver disorders most of which speed up the natural healing processes of the liver. Numerous medicinal plants and their formulations are used for liver disorders in ethno medical practice as well as traditional system of medicine in India. More than fifteen thousand of these plants are evaluated for their hepatoprotective action in light of modern medicine [6].

In our study to observe the liver function retention a plant which is nearly to our forests selected and worked on its role on the protection of liver tissue. The selected plant, of *Hybanthus enneaspermus (L.)F.Muelle liver (*Family: *Violaceae)* is a perennial herb.10cm high, globorous or hairy often with woody torches found in warmer parts of India. Leaves are alternate, simple, clustered, rarely opposite, linear to lanceolate and 1-5cm long[7]. It is popularly known Ratnapurus (Hindi), Orithal thamarai (Tamil) and Ratna Purusha (Telugu). Local name is Madanamast and common name is spade flower. An infusion of the plant extract is given in case of cholera. It has been reported to have anti-inflammatory[8], antitussive[9], antiplasmodial[10], anticonvulsant[11], aphrodisiac, and free radical scavenging activity[11].Also it has diuretic and demulcent properties. Now it is being used in number of herbal formulations prescribed for gonorrhea and urinary infections (Wealth of India, 1959).

However, no work has been reported on the hepatoprotective properties of this plant. Keeping this in view, the present study has been undertaken to investigate hepatoprotective activity and antioxidant role of the ethanolic extract (EEHE) of *Hybanthus enneaspermus (L.)F.Muell* on paracetamol induced liver damage in mice.

MATERIALS AND METHODS

Plant materials

The plant, *Hybanthus enneapermus,* leaves were collected from the surroundings of Sri Venkateswara University campus, Tirupati, Andhra Pradesh, India and identified by comparison with a voucher specimen deposited in the herbarium of Department of Botany, of our University campus.

Extraction

The collected plant leaves were air-dried in the shade and powdered. About 100g of powdered leaves was soaked in 500ml of absolute ethanol for more than six hours and the extracts were decanted and then concentrated in-vacuo to yield dense residues. The samples were transferred to glass vials and stored in refrigerator for further studies[20].

Experimental animals

The male albino mice weighing about 25±5.0gms and 3 months old were purchased from Sri Venkateswara Enterprises, Bangalore, India. The animals were grouped and housed in polyacrylic cages with not more than six animals per cage and maintained under standard laboratory conditions. The animals were fed with standard pellet diet with fresh water *ad libitum*. All the animals were allowed to acclimatize to laboratory condition for a week before commencement of an experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Drugs and chemicals

SGOT, SGPT, ALP kits, Thiobarbituric acid (TBA), Nitro blue seven tetrazolium chloride (NBT), reduced Glutathione (GSH) were purchased from Genei, Bangalore, India, and the rest of the chemicals utilized were of analytical grade.

Paracetamol-induced hepatotoxicity in mice (Acute treatment)

The male mice divided into control, paracetamol and plant product treatments were allowed for seven days of treatment and the experiments were made as follows for each group.

Group I: control mice (Saline 1ml/ kg bw, ip), Group II: toxic control treated with paracetamol alone (300mg/kgbw in saline, ip), Group III: treated with ethanolic *Hybanthus enneaspermus* extract alone (100mg kg bw in Carboxy methyl cellulose, orally), Group IV: treated with ethanolic *Hybanthus enneaspermus* extract alone (200mg kg bw in CMC, orally), Group V are treated with ethanolic *Hybanthus enneaspermus* extract (100mg) and Paracetamol (300mg), Group VI are treated with ethanolic *Hybanthus enneaspermus* extract (200mg) and Paracetamol (300mg) respectively. Paracetamol administration was observed for 48 hrs interval. Remaining treatments duration was for about 7 days. On the eighth day mice were sacrificed by decapitation.

Biochemical studies

The blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500rpm at 300°C for 15 min and utilized for the estimation of various biochemical parameters namely SGOT, SGPT [12], SALP[13]. After collection of blood samples the mice of different groups were sacrificed and their livers were excised immediately and washed in ice cold normal saline, followed by 50 mM Tris-HCl buffer (pH 8.0), and then the tissue was homogenized and centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for assaying antioxidant enzymes and liver marker enzymes. For lipid peroxidation, 10% tissue homogenate was prepared in 1.15% KCl and for estimating total sulfhydryl content the tissue was homogenized in 0.2 M EDTA.

Serum hepatospecific markers

The assays for SGOT/AST were conducted by the method of King (1965a). To 1 mL of substrate 0.2 mL of enzyme was added and the mixture was incubated at 37°C for 1 hr. To the control tubes the enzyme was added after the reaction, and it was arrested immediately by the addition of 1 mL of DNPH reagent. The tubes were kept at room temperature for 30 min. Then 5 mL of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The colour developed was read against the blank at 540 nm using a colorimeter. The enzyme activity was expressed as units/L (One unit corresponds to enzyme that liberates one micro mole of pyruvate/min).Similar to above the SGPT assays were also conducted and the readings were read at 540nm.

The activity of alkaline phosphatase was assayed by the method of King (1965b). The incubation mixture containing 1.5 mL buffer, 1 mL substrate, 0.1 mL magnesium chloride were preincubated at 37°C for 10 min. Then 0.1 mL of enzyme was added and incubated at 37°C for the 15 min. The reaction was arrested by the addition of 1 mL of Folin-phenol reagent. The control tubes received enzyme after the addition of Folin -phenol reagent. Then 1 mL of sodium carbonate was added and the tubes were incubated at 37°C for 10 min. The colour developed was read at 640 nm in a colorimeter. Standards and blank were treated in a similar manner. The activity was expressed in units/L (One unit corresponds to enzyme that liberates one micro mole of phenol/min/mg protein under incubation conditions).

Estimation of superoxide dismutase activity (E.C.1.15.1.1)

Superoxide dismutase was measured according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine transition by the enzyme. To 0.5ml of the homogenate 2ml of carbonate buffer and 0.5mL of 0.6mM epinephrine was added. Epinephrine was the last component to be added and the adrenochrome formed in the next 4 min was recorded at 470 nm in spectrophotometer. SOD activity was expressed in units/min/mg protein (One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of epinephrine auto-oxidation).

Estimation of catalase activity (E.C.1.11.1.6)

Catalase assay was carried out by the method of Aebi (1984). The decomposition of H_2O_2 was followed directly by measuring the decrease in absorbance at 240 nm. To 0.5 mL of the tissue homogenate 1.5 mL of phosphate buffer was added. Then one mL of H_2O_2 was added and change in absorbance was recorded after every 15 seconds up to one min. The activity of catalase was expressed as µmoles of H_2O_2 utilized/min/mg protein.

Estimation of Glutathione peroxidase activity, (E.C.1.11.1.9)

Assay of glutathione peroxidase was carried out by continuous monitoring of NADPH oxidation in a recycling assay as described by Wendel (1981). Total GPx was measured by using cumene hydro peroxide as a substrate. The reaction mixture contained 0.1 mL of phosphate buffer, 0.1 mL glutathione reductase, 0.1 mL reduced glutathione and 0.1 mL mµ/nm of NADPH. To this 0.5 mL of homogenate was added and incubated at 37°C for 10 min. The reaction was initiated by the addition of 100 µL of cumene hydro peroxide. The linear decrease in absorption was recorded at 340 nm. The spontaneous reaction was assayed without enzyme and was subtracted from the samples. Activity of GPx was expressed as µmoles of GSH oxidized/min/ mg protein[21].

Estimation of glutathione reductase activity (E.C.1.6.4.2)

Glutathione reductase was assayed by the method of Staal et al., (1969). The reaction mixture containing 1 mL of phosphate buffer, 0.5 mL of each of EDTA, and glutathione oxidized and 0.2 mL of NADPH was made up to 3 mL with distilled water. After the addition of 0.1 mL of tissue homogenate, the change in optical density at 340 nm was monitored for every 30 seconds for 2 min. The enzyme activity was expressed as µmoles of GSH formed/min/mg protein.

Estimation of glutathione-s-transferase activity, (E.C.2.5.1.18)

Glutathione-s-transferase activity was determined by measuring the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitro benzene (CDNB) as a substrate by the method of Habig et al., (1974). The reaction mixture contained 2.7 mL phosphate buffer, of 0.1M 100nm CDNB and 0.1 mL of the tissue homogenate, was incubated at 37°C for 10 min. The reaction was initiated by the addition of 0.1 mL of 100Mm glutathione. The reaction was monitored spectrophotometrically for increase in absorbance at 340 nm. Measuring and subtracting the rate in the absence of enzyme was made correction for the spontaneous reaction. The enzyme activity is expressed as units/min/mg protein.

Estimation of lipid peroxidation

Lipid peroxidation in tissues was carried out by the method of Okhawa et al., (1979). The assay mixture contained 0.1 mL of 10% tissue homogenate (prepared in 1.15% KCl), 0.2 mL of %SDS and 1.5 mL of mMTBA. The mixture was finally made up to 4 mL with distilled water and boiled at 95°C for 1 hr. After cooling, 1 mL of distilled water and 5 mL of n-butanol: pyridine mixture were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. Then the absorbance of the organic layer was measured at 532 nm. Amount of lipid peroxidation is expressed as nmoles of MDA produced/mg protein.

Histopathological study

Small pieces of liver tissues in each group were collected and kept in 10% neutral buffered formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6µm in thickness were cut and stained with hematoxylin and eosin (H&E). These sections were examined photo microscopically for necrosis, steatosis and fatty changes of hepatic cells.

Statistical analysis

The experimental results were expressed as the Mean ± SE for six animals in each group. The biochemical parameters were analyzed statistically using one-way analysis of variance ANOVA, followed by Dunnett's multiple range test (DMRT). P value of < 0.01 was considered as statistically significant for the analyzed data.

RESULTS

The effects of paracetamol and EEHE on serum and liver transaminases, and alkaline phosphatases, were studied in mice and their results are summarized in Table-1. Administration of paracetamol (300mg/kg; body weight), after 48 hours of intoxication resulted in a significant ($P<0.01$) elevation of transnminases and phosphatases than control group. On administration of EEHE (Group V to IV) (Table-1) the levels of these enzymes were found to almost normal and also less than control group. The effects of EEHE on mice liver lipid peroxidation and enzymic antioxidants namely SOD, CAT, GST, GPx, GR levels shown (Table-2). The levels of TBARS considerably increased in mice treated with paracetamol as compared to the normal mice. Treatment with EEHE (100 mg and 200 mg/kg/day) has resulted in a significant decrease ($P<0.01$) in levels of TBARS and brought them near to normal level (Table 2). Significant decreases in the activities of enzymic antioxidants (SOD, GST, GR, CAT, and GPx) were noted

after single administration of paracetamol. Upon administration of EEHE, the activities of enzymic antioxidants were significantly (P<0.01) reversed to near normal (Table2). Histopathological studies of mice liver tissue from Group I showed normal architecture, normal hepatic central vein and hepatocytes with centrally placed nuclei (Fig1**).** Histological analysis of liver tissues of mice treated with paracetamol alone showed severe condition of central vein, hyperplastic nuclei, more granularity of the cytoplasm, cloudy swelling changes (Fig2). Liver tissues of mice treated with *Hybanthus enneaspermus* alone showed hepatocytes with centrally placed prominent nucleus, sinusoids and with central vein (Fig3). Liver tissues of mice treated with *Hybanthus enneaspermus* extract and paracetamol mixture showed infiltration, recovered sinusoids space and increased size of hepatocytes (Fig 4).

Table 1: Effect of extract of *Hybanthus enneaspermus* on serum and liver markers of control and experimental mice against Paracetamol toxicity

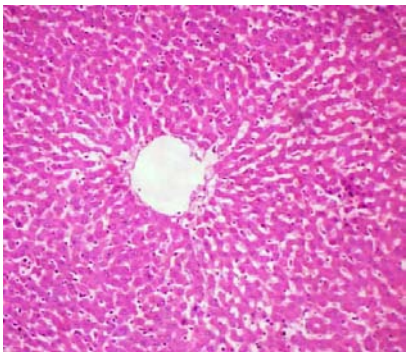
Groups [G1 – G7]	SERUM			LIVER		
	AST	ALT	ALP	AST	ALT	ALP
Control	52.63±0.42^{f}	81.63±0.38^{f}	132.53±0.42^{f}	24.29±0.32^{f}	110.27±0.34^{c}	111.38±0.42^{f}
Paracetamal[300mg]	91.52±0.33^{a}	172.42±0.39^{a}	316.57±0.36^{a}	79.60±0.34^{a}	120.64±0.37^{a}	308.57±0.34^{a}
EEHE[100mg]	45.37±0.28^{e}	97.42±0.26^{d}	116.50±0.41^{e}	31.60±0.40^{e}	113.51±0.40^{b}	115.44±0.36^{e}
EEHE[200mg]	42.24±0.39^{e}	89.44±0.36^{e}	115.62±0.38^{e}	20.37±0.43^{g}	109.50±0.33^{c}	111.62±0.29^{f}
P+EEHE[100mg]	82.43±0.41^{b}	154.57±0.50^{b}	288.65±0.36^{b}	74.418±0.40^{b}	118.40±0.42^{a}	250.51±0.31^{b}
P+EEHE[200mg]	79.46±0.29^{c}	132.59±0.39^{c}	260.6±0.34^{c}	70.33±0.40^{c}	112.39±0.43^{b}	220.41±0.36^{c}

Values are expressed as Mean ± SE by Duncan's multiple range test (DMRT). Means having same subscripts in each colum do not differ significantly at 0.01 levels by Duncan's Multiple range Test (DMRT). p<0.01
Note: Activity observed in units/L

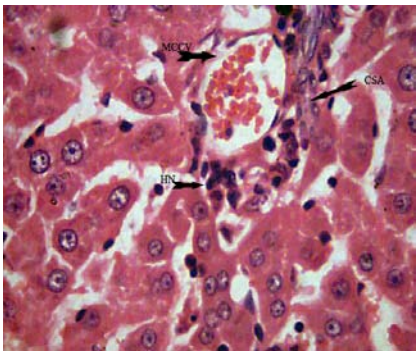
Table 2: Effect of extract of *Hybanthus enneaspermus* on enzymic antioxidants in liver of control and experimental mice against Paracetamol toxicity

Groups	CAT	SOD	GPX	GR	GST	LPO
Control	48.22±0.38^{a}	19.06±0.36^{a}	0.156±0.03^{b}	4.72±0.34^{a}	39.47±0.33^{a}	42.39±0.41^{d}
Paracetamol[300mg]	26.80±0.20^{d}	9.39±0.19^{d}	0.135±0.08^{c}	1.38±0.44^{d}	19.55±0.30^{e}	61.43±0.41^{a}
EEHE[100mg]	41.88±0.34^{b}	14.34±0.44^{b}	0.146±0.09^{a}	3.49±0.39^{b}	30.52±0.33^{c}	36.71±0.40^{e}
EEHE[200mg]	44.84±0.25^{b}	15.56±0.38^{b}	0.154±0.04^{b}	3.62±0.29^{b}	32.64±0.44^{b}	39.48±0.37^{d}
P+EEHE[100mg]	35.99±0.13^{c}	10.41±0.43^{c}	0.136±0.04^{c}	2.66±0.30^{c}	21.71±0.45^{d}	55.59±0.36^{b}
P+EEHE[200mg]	40.93±0.24^{b}	11.68±0.33^{c}	0.144±0.04^{a}	3.53±0.39^{b}	27.59±0.34^{c}	49.45±0.38^{c}

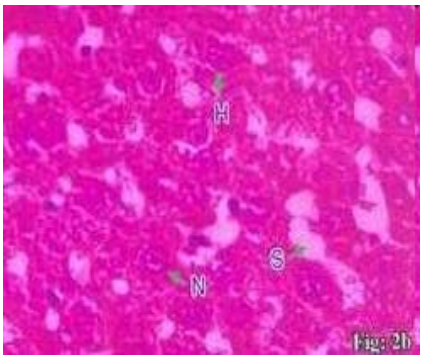
Values are expressed as Mean ± SE by Duncan's multiple range test (DMRT). Means having same subscripts in each column don't differ significantly at 0.01 level by Duncan's Multiple range Test (DMRT). p<0.01
Note: µ moles of activity observed per min per mg of protein



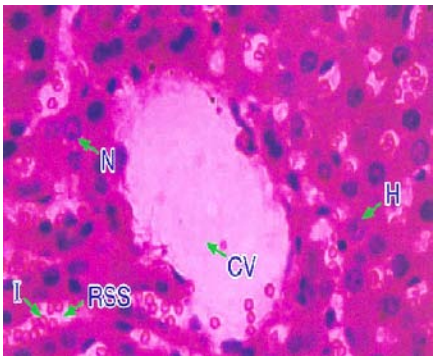
CONTROL LIVER
Section of liver revealing normal architecture, normal hepatic central vein, hepatocytes with nuclei, sinusoidal spaces.



PARACETAMOL TREATED
Section of liver showing severe condition of central vein, hyperplastic nuclei, more granularity of the cytoplasm, cloudy swelling changes



EEHE ALONE
Section of liver revealing normal architecture, normal hepatic central vein, hepatocytes with nuclei, sinusoidal spaces.



ACTIVEFRACTION+PARACETAMOL
Section of liver showing infiltration, recovered Sinusoids space (RSS) and increased of hepatocytes

Fig. 1: Histopathology of section cuttings of liver with different treatments

DISCUSSION

Paracetamol (acetaminophen) is a commonly and widely used analgesic and antipyretic agent. An excessive dose of acetaminophen causes acute failure by depleting the normal levels of hepatic glutathione, after formation of acetaminophen adducts[14]. So glutathione protects hepatocytes by combining with the reactive metabolite of paracetamol thus preventing their covalent binding to liver proteins[15]. In living systems, liver is considered to be highly sensitive to toxic agents. The study of different enzyme activities of liver such as SGOT, SGPT and SALP have been found to be of great value in the assessment of clinical and experimental liver damage[16]. In the present investigation it was observed that the animals treated with acetaminophen resulted in significant hepatotoxicity as shown by the elevated levels of serum aminotransferases alkaline phosphatases. These elevations in the enzyme levels directly reflect in hepatic structural variations. The rise in the SGOT is usually accompanied by an elevation in the levels of SGPT, which play a vital role in the conversion of amino acids to keto acids[17]. To reduce these toxic activities antidotes like acetylcystiene for paracetamol toxicity shall be used. In our study the pretreatment with EEHE, both at the dose of 100mg/kgbw and 200mg/kgbw, significantly attenuated these enzyme activities. The regularization of serum enzyme activities by EEHE suggests that the plant products present in HE are able to serve as antidotes to reduce the damage to the hepatocytes so as to protect the membrane integrity against acetaminophen induced leakage of marker enzymes into the circulation. The above changes can also be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. In addition to AST Serum ALP on the other hand is also related to hepatic cell damage. Increase in serum level of ALP is due to increased synthesis in presence of increasing biliary pressure due to paracetamol[18]. Effective control of alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell was observed under the treatment of HE.

Lipid peroxidation has been postulated to the destructive process of liver injury due to acetaminophen administration. In the present study the elevation in the levels of end products of lipid peroxidation in the liver of mice treated with paracetamol was observed. The increase in malondialdehyde (MDA) levels in liver has suggested enhanced lipid peroxidation and that lead to tissue damage, and failure of antioxidant defense mechanisms in the prevention of formation of excessive free radicals. Treatment of EEHE to mice has significantly reversed these changes in liver. Hence it may be possible that the mechanism of hepatoprotection by EEHE is due to its antioxidant effect on liver. As mentioned above in addition to formation of adduct of GSH to various molecules and antidote GSH also functions on the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST [19]. Further our present study has revealed that the decreased level of GST has been associated with an enhanced lipid peroxidation in paracetamol-treated mice. Administration of EEHE to mice has significantly normalized the level of GST activity in a dose-dependent manner. Acetylcystiene is antidote for hepatotoxicity.

In all organisms the enzymic antioxidant defense system is a natural phenomenon of protection against lipid peroxidation. SOD, CAT and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage [22]. In the present study, it was observed that the EEHE significantly increased the hepatic SOD activity in paracetamol induced liver damage in mice. This show EEHE can reduce reactive free radicals that might lessen oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme. Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and in the liver. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals[23]. Therefore the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of EEHE increased the activities of CAT in paracetamol-induced liver damage in mice to prevent the accumulation of excessive free radicals and protected the liver from paracetamol intoxication. The histopathological observations in paracetamol-treated mice showed severe necrosis, with disappearance of nuclei. This could be due to the formation of highly reactive radicals because of oxidative threat caused by paracetamol. All these changes were very much reduced histopathologically in mice treated with EEHE. Based on the above results, it could be concluded that EEHE exerts significant hepatoprotection against paracetamol-induced toxicity.

CONCLUSION

The present investigation indicates that EEHE exert significant protection against paracetamol-induced toxicity by its ability to ameliorate the lipid peroxidation through the free radicals scavenging activity, which enhanced the levels of antioxidant defense system. Our study also showed that pretreatments with EEHE doses of 100mg/kg and 200mg/kg with paracetamol (300mg/kg) combination treatments have same effect nearer to control mice. Therefore EEHE appears to be useful in the attenuation of paracetamol induced lipid peroxidation and showed more prominent effect. The extract showed significant activity against paracetamol induced liver damage in mice when compared with EEHE and paracetamol combination treatment. Further investigation is underway to determine the exact phytoconstituents in the extracts that are responsible for its hepatoprotective effect.

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