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EVALUATION OF HEPATOPROTECTIVE EFFECT OF *AVERRHOA CARAMBOLA* L. LEAVES ON ANTI-TUBERCULAR DRUGS INDUCED HEPATOTOXICITY

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ABSTRACT

The aim of the present study is to evaluate the hepatoprotective effect of ethanolic extract of AverrhoacarambolaL.leaves on anti-tubercular drugs induced hepatotoxicity. Hepatotoxicity is induced in albino rats by combination of anti-tubercular drugs such as H (27 mg/kg p.o.), R (40 mg/kg p.o.), Z (66 mg/kg p.o.) and E (53 mg/kg) for 35 days. Silymarin (100 mg/kg p.o.) is taken as the standard drug. Ethanolic extract of Averrhoa carambola L. 250mg/kg and 500 mg/kg administration orally for 35 days with one hour prior administration of anti-tubercular drugs. Among 250 & 500 mg/kg, 500mg/kg showed significant reduction in serum liver marker enzymes like AST, ALT, ALP, total bilirubin and total cholesterol, whereas increase in the levels of total HDL, when compared to control. Invivo antioxidant studies, we found a significant increase in the levels of SOD, CAT, GSH, GPx and GRx whereas marked decrease in the levels of lipid peroxidation levels as compared to control. Histopathological examination of the liver sections treated with ethanolic extract of Averrhoa carambola L. 250 mg/kg and 500 mg/kg showed significant reduction in liver cell necrosis and inflammation in the centrilobular region compared to hepatic control. The above results suggest that Averrhoa carambola L. leaves showed protective effect on anti-tubercular drugs induced hepatotoxicity.

KEYWORDS : *Averrhoa carambola L. leaves, anti-tubercular drugs, hepatotoxicity, liver marker enzymes, and invivo antioxidant parameters.*

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INTRODUCTION

The liver is central to the metabolism of virtually every foreign substance including anti-tubercular drugs. Isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) are first line drugs of the directly observed treatment short course (DOTS) strategy for control of tuberculosis (TB) developed by world health organization (WHO) and all these drugs have been found to be potentially hepatotoxic[1]. Hepatotoxicity as injury to the liver that is allied with diminished liver function. It has a considerable impact on health

because many of the hepatic reactions induced by pharmaceutical preparations can be very severe. Drug induced hepatotoxicity is a common adverse effect with currently used anti-TB drugs and they potentiated by multiple drug regimen[2].

Nowadays, herbal drugs are fast emerging trend as alternative therapy or supplementation with clinically proven drugs in a variety of pathophysiological states. Thus, hepatoprotective agents (herbal drugs) used in combination with anti–tubercular drugs as supplementation may result effective in protect too against liver dysfunction caused by anti-tubercular drugs that may lead to decrease morbidity and mortality rates.

Averrhoa carambola L. (Family: Oxalidaceae) commonly known as star fruit. It is a native of south East Asia and cultivated in some parts of India. It has been widely used in ayurveda, preparation of its fruit and leaves are used to skin diseases, pruritis, worm infestations, diarrhea, vomiting, hemorrhoids, intermittent fever, over-perspiration and general debility[3]. It is also used in traditional medicines in countries like China, Phillipines and Brazil for various ailments[4]. The present study is undertaken to evaluate the hepatoprotective effect of ethanolic extract of *Averrhoa carambola* L. (EAC) leaves on anti-tubercular drugs induced hepatotoxicity.

MATERIAL AND METHODS

Preparation of plant material and extract

Averrhoa carambola L. leaves were collected from tirumala hills, identified and authenticated by Dr.K.Madhava chetty, Assistant Professor, Department of Botany, S.V.University, Tirupati. The fresh leaves were washed, dried and powdered; the powdered material is passing through a 40 mesh sieve to get fine powder. The coarsely powdered leaves were extracted with ethanol as solvent by soxhlet apparatus. Appearance of colorless solvent in the siphon tube was taken as the end-point of extraction. The extract was concentrated to ¾ of its original volume by distillation.

Acute toxicity studies

Acute toxicity studies were performed for EAC according to OECD guidelines 423[5]. 10 mice were selected for the study and oral administration of EAC at a dose of 100, 1000, 2000, 4000 mg/kg given at 48 hrs interval simultaneously. In this acute toxic study, observed animals for any changes in consumption of food, water; body weight; behavioural changes and mortality rates.

Animals

Healthy adult albino rat species of wistar strain weighing 150 gm–250 gm were used for the study and they were purchased form Invivo biosciences, Bangalore. The animals were housed in clean metabolic cages, maintained in controlled temperature (22±3°c) and light cycle (12 hour light and 12 hour dark). They were fed with standard pellet diet and water *ad libitum*. The protocol is approved by the Institutional animal ethical committee of Krishna Teja Pharmacy College (1521/PO/a/11/CPCSEA).

Study protocol

Hepatotoxicity is induced by using H-Isoniazid (27 mg/kg, p.o), R-Rifampicin (40 mg/kg, p.o), Z-Pyrazinamide (66 mg/kg, p.o) and E-Ethambutol(53 mg/kg,p.o) were dissolved in normal saline, administration for 35 days and Silymarin (100 mg/kg, p.o) is used as the standard. The animal oral doses of anti–tubercular drugs were extrapolated from daily human dose using the conversion table based on body surface area[6].

Experimental procedure

Experimental animals were randomly divided in to 5 groups, each group containing 6 animals and the treatment schedule for 35 days as follows.

Group I: Control (Normal saline 1ml/kg, p.o)

Group II: Hepatic control (anti – tubercular drugs – HRZE, p.o.)

Group III: Silymarin (100mg/kg, p.o)+one hour prior administration of anti– tubercular drugs

Group IV: EAC (250 gm/kg, p.o)+one hour prior administration of anti- tubercular drugs

Group V: EAC (500 gm/kg, p.o) + one hour prior administration of anti- tubercular drugs

On 36th day, animals were sacrificed under ether anaesthesia and blood samples were

collected from cardio-vascular puncture. After collecting the blood in eppendroff tubes kept aside for 1 hour at room temperature and the serum is separated by centrifugation at 2000 RPM for 15 mins and stored until analyzed for liver marker enzymes. On the same day, liver is removed and stored in 10% formalin solution for the estimation of invivo antioxidant parameters; and processing for histopathological studies.

Estimation of Biochemical Parameters

AST and ALT were estimated by Reitman and Frankel method, ALP was estimated by kind king's method. Total Bilirubin, total cholesterol were estimated by Jendrassik and Grofs method and CHOD/POD method respectively.

Estimation of In-Vivo Antioxidant Parameters

Superoxide dismutase (SOD)

SOD activity is determined by the inhibition of auto catalyzed adrenochrome formation in the presence of homogenate at 480 nm. The reaction mixture contained 150µl of homogenate, 1.8 ml of carbonate buffer (30 mM, pH 10.2), and 0.7 ml of distilled water and 400µl of epinephrine (45 mM). Auto oxidation of epinephrine to adrenochrome is performed in a control tube without the homogenate. Activity is expressed as µmoles/min/mg protein[7].

Catalase (CAT)

The catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 is recorded at 254 nm. The reaction mixture contained 2.6 ml of 25 mM potassium phosphate buffer pH 7.0 and 0.1ml of tissue homogenate and is incubated at 37˚C for 15 min and the reaction is started with the addition of 0.1 ml of 10 mM H_2O_2. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve is used for the calculation of enzyme activity. One unit of catalase activity is defined as the amount of enzyme causing the decomposition of µmol H_2O_2/mg protein/min at pH 7.0 at 25°C[8].

Glutathione peroxidase (GPx)

The reaction mixture consisted of 0.2 ml of 0.4 M tris buffer, 0.1 ml of sodium azide, 0.1 ml of hydrogen peroxide, 0.2 ml of glutathione and 0.2 ml of supernatant. Incubated at 37°C for 10 min. The reaction is arrested by the addition of 10% TCA and the absorbance is measured at 340 nm. Results were expressed in µmol/min/mg protein[9].

Glutathione reductase (GRx)

The reaction mixture contained 2.1 ml of 0.25 mM, potassium phosphate buffer pH 7.6, 0.1 ml of 0.001 M NADPH, 0.2 ml of 0.0165 M oxidized glutathione, 0.1 ml of BSA (10 mg/ml). The reaction is started by the addition of 0.02 ml of tissue homogenate with mixing and the decrease in the absorbance at 340 nm is measured for 3 min against a blank. Glutathione reductase activity is expressed as µmol NADPH oxidized /min/mg protein at 30˚C[10].

Reduced glutathione (GSH)

The method is based on the reaction of reduced glutathione with dithionitrobenzoic acid (DTNB) to give a compound that absorbs at 412 nm. Briefly after centrifugation, 0.5 ml of supernatant is taken and mixed with 2.0 ml of 0.3 mol/L di-sodium hydrogen phosphate (Na_2HPO_4) solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml, 1% sodium citrate) is added and the absorbance is measured immediately after mixing at 412 nm. Results were expressed in mM GSH/min/mg protein[11].

Lipid peroxidation (TBARS)

For TBARS, 1.0 ml of tissue homogenate (Tris-HCl buffer, pH 7.4) is mixed with 2.0 ml of TBA-TCA-HCl reagent (Thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA mixed in 1:1:1 ratio). The resultant solution is placed in water bath for 15 min., cooled and centrifuged at 1000 rpm at room temperature for 10 min. The absorbance of the clear supernatant is measured against reference blank at 535 nm. The results were expressed as nM/min/mg tissue protein[12].

Histopathological studies

Livers from rats were fixed in 10% neutral formalin solution, dehydrated in graded alcohol and embedded in paraffin. Fine sections obtained were mounted on glass slides and counter-stained with Hematoxylin Eosin (H&E) for light microscopic analyses.

Statistical analysis

The results are presented as Mean ± S.E.M (n=6 in each group). Analyses were performed using One-way ANOVA followed by Tukey posthoc for the difference between the control and treatment groups.

RESULTS

On acute toxicity studies

The EAC was found to be safe since no animal died even at the dose of 4000mg/kg when administered orally and the animals did not show any gross behavioral changes.

On Biochemical Parameters

Animals treated with anti-tubercular drugs showed a significantly increased (P< 0.05) in AST, ALT, ALP, total bilirubin and total cholesterol while HDL levels significantly decreased (P< 0.05) compared to control. Pretreatment with Silymarin (100mg/kg), EAC 250 mg/kg and 500 mg/kg showed significantly decreased liver marker enzymes whereas significantly increased HDL levels when compared to hepatic control. The results are presented in the table 1.

Table 1: **Effect of EAC on serum AST, ALT, ALP, TB, Total cholestrol and HDL on anti -tubercular drugs induced hepatotoxicity in rats**

Groups	Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TB (IU/L)	Total cholesterol (mg/dl)	HDL (mg/dl)
Group-I	Control	122.67± 6.38	63 ±3.02	182 ±6.27	0.06 ±0.01	83.5±6.3	33.8±2.1
Gropu-II	Anti-TB Drugs (Hepatic control)	386.50 ± 14.60#	639.33 ±17.96 #	315.83 ±7.46 #	0.39 ±0.01#	131.3±16.2#	17.4±1.9
Group-III	Silymarin (100 mg/kg)	166.67 ±2.90***	136.33 ±5.76*	138 ±9.65***	0.08 ±0.02**	88.7±5.9***	33.6± 2.4***
Group-IV	EAC (250mg/kg)	301.16 ±8.94*	244.33 ±9.64*	216.83 ±6.96*	0.14±0.01*	125.6±10.5**	22.5±1.8**
Group-V	EAC (500mg/kg)	199.17 ±7.73**	166.67 ±5.98***	173.66 ±4.63*	0.06±0.05**	113.1±13.1**	31.7±3.1**

Data are expressed as Mean ± S.E.M (n=6), One-way ANOVA Tukey posthoc;
#p≤0.05 vs. Control (Group I); *p≤0.05 vs. Hepatic control (Group II);
**p≤0.01 vs. Hepatic control (Group II); ***p≤0.001 vs. Hepatic control (Group II).

Invivo – Antioxidant parameters

Oral administration of anti-tubercular drugs resulted in decreased SOD, CAT, GPx, GRx, GSH and increased TBARS levels when compared to control group. Animals pretreatment with Silymarin (100mg/kg), EAC 250mg/kg and 500mg/kg significantly increased SOD, CAT, GPx, GRx, GSH and decreased TBARS levels when compared to hepatic control. In this, EAC 500mg/kg showed a significant effect in enzymatic & non-enzymatic levels. The results are presented in table 2.

Table 2: Effect of EAC on antioxidant parameters on anti -tubercular drugs induced hepatotoxicity in rats

Group	Treatment	SOD (µmoles/min /mg)	CAT (µmol/mg/min)	GPx (µmol/mg/min)	GRx (µmol/mg/min)	TABRS (nM/min /mg)	GSH (mM/min /mg)
Group I	Control	3.55±0.26	34.99 ±0.98	28.99 ±0.64	30.81 ±0.94	34.95 ±1.98	2.47 ±0.12

Group II	**Anti-TB Drugs (Hepatic Control)**	1.62 ±0.66#	15.49 ±0.57#	12.64 ±0.66#	18.34 ±0.31#	88.29 ±5.88#	0.74 ±0.06#
Group III	**Silymarin (100ml/kg)**	3.71 ±0.19***	33.89 ±0.08**	29.91 ±0.82**	30.86 ±0.44***	33.89 ±3.23***	2.27 ±0.09**
Group IV	**EAC (250mg/kg)**	2.28 ±0.33*	25.22 ±0.95**	21.43 ±0.15*	23.77 ±0.59**	48.32 ±2.34*	1.32 ±0.07*
Group V	**EAC (500mg/kg)**	2.88 ±0.81**	31.99 ±0.34**	26.28 ±0.39***	29.46 ±0.35**	37.28 ±3.32*	1.99± 0.04**

Data are expressed as Mean ± S.E.M (n=6), One-way ANOVA Tukey posthoc;
#p≤0.05 vs. Control (Group I); *p≤0.05 vs. Hepatic control (Group II);
**p≤0.01 vs. Hepatic control (Group II); ***p≤0.001 vs. Hepatic control (Group II).

Histopathological studies:

Hepatocytes of control group showed a normal lobular architecture of liver (fig.1). In anti-tubercular drugs treated group the hepatocytes showed cell necrosis and inflammation in the centrilobular region of the liver with portal triaditis (fig.2). Pretreatment with Silymarin (100mg/kg), EAC 250mg/kg and 500mg/kg groups of liver showed minimal inflammation with moderate portal triaditis and their lobular structure was normal (fig 3-5), showing that EAC have significant hepatoprotective activity.

HISTOPATHOLOGICAL SLIDES

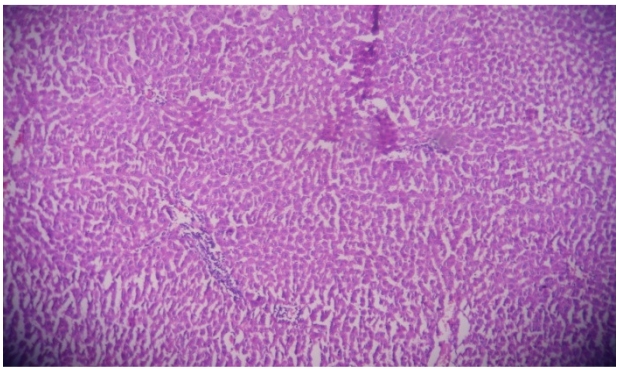

Figure – 1: Control (Normal saline 1 ml/kg)–Hepatocytes showed a normal lobular architecture of the liver.

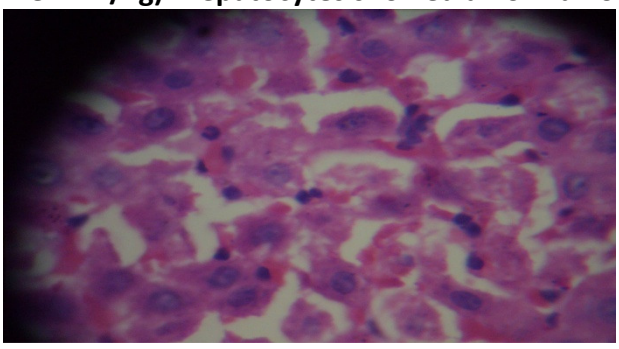

Figure – 2: Hepatic control (anti-TB drugs HRZE) – Hepatocytes showed liver cell necrosis & inflammation observed in the centrilobular region with portal triaditis

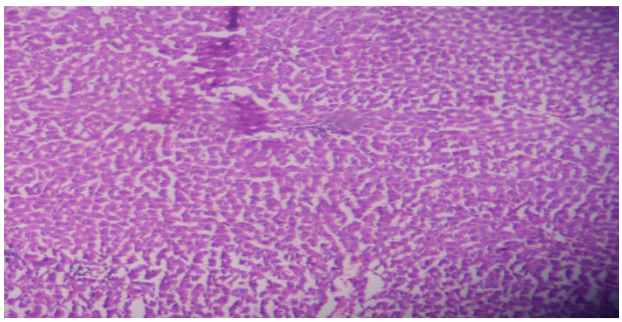

Figure – 3: Standard (silymarin-100 mg/kg) – Hepatocytes showed normal lobular architecture of the liver

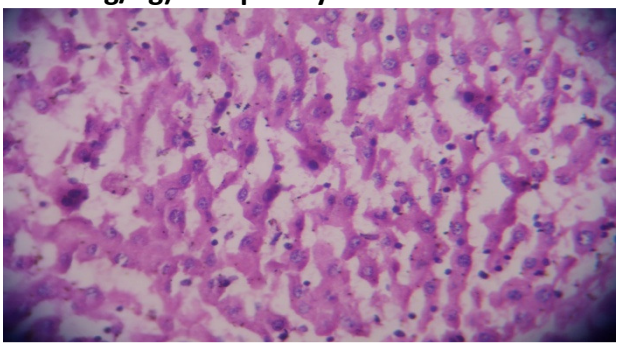

Fig 4: Hepatocytes of the EAC (250mg/kg) pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

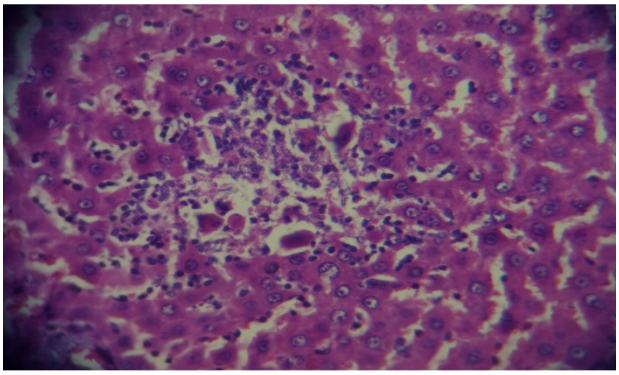

Fig 5: Hepatocytes of the EAC (500mg/kg) pretreated group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal.

DISCUSSION

Tuberculosis is a leading health problem worldwide particularly in developing countries. As per WHO (World Health Organization) estimate, a million people globally develop active TB and 1.7 million die of it annually. In India, it is estimated that nearly 2 million people develop active disease every year and about 0.5 million die from it[13]. Anti-tubercular drugs like isoniazid, rifampicin, pyrazinamide and ethambutol are the first choice of drugs for therapy of TB. Anti-tubercular drugs are individually hepatotoxic and given in combination their toxic effect is enhanced[6]. Exact mechanism of anti-tubercular drugs induced hepatotoxicity is not clear. Isoniazid is metabolized by acetylation gives acetyl isoniazid and them hydrolyzed in to acetyl hydrazine and isonicotinic acid. Acetyl hydrazine is either hydrolyzed in hydrazine or acetylated into diacetylhydrazine. Hydrazine & acetyl hydrazine are the toxic metabolites of isoniazid & them known to cause irreversible cellular damage[14]. Rifampicin, a cyt P450 enzyme inducer, in combined with isoniazid, induces the hydrolysis pathway of isoniazid metabolism. There by quickly isoniazid is converted into hepatotoxic metabolites[15] Pyrazinamide, in combination with isoniazid & rifampicin appears to be associated with an increased incidence of hepatotoxicity. Individually hepatotoxicity is not observed for ethambutol.

In this study the results suggest that the statistically significant variations in liver marker enzymes in hepatic control group indicate that hepatic damage is produced by anti-tubercular durgs. Following treatment with EAC (250 mg/kg&500mg/kg) and silymarin (100mg/kg), all the parameters restored to the normal levels.

AST is a cytosolic enzyme present in the liver, and it is released only from liver. ALT is a mitochadrial enzyme released from heart, liver, skeletal muscles & Kidney. In hepatic control animals these AST and ALT levels are elevated might be due to hepatocyte injured/dies, and they can leak through the liver cell membrane into the circulation. Pretreatment of EAC significantly decreased the plasma levels of AST and ALT the effect were observed depend on the dose.

In our study, the elevated ALP levels were observed in the hepatic control animals. It may be due to defective excretion or by increased production of ALP by hepatic parenchymal cells/duct cells. Pretreatment of EAC 250 mg/kg and 500 mg/kg significantly decreased the levels of ALP marker enzyme in the serum. The increased total bilirubin is observed in hepatic control animals. Estimation of bilirubin, metabolic product of the breakdown of heme is one of the better liver functional tests. Pretreatment of EAC significantly decreased the levels of bilirubin in the serum might be by inhibiting cyt P450 liver enzyme responsible for metabolism of bilirubin.

The total cholesterol levels were higher in anti-tubercular drugs administration. The higher cholesterol levels in the liver might to due to increased uptake of LDL from the blood by the tissue uptake[16]. Pretreatment of EAC may be responsible for decreased synthesis of cholesterol by inhibiting the enzyme responsible for the synthesis of cholesterol. This effect may be responsible for the improvement in the serum HDL levels. Thus, the hepatoprotective action of EAC may be mediated through decreased AST, ALT, ALP, total bilirubin, cholesterol synthesis, and increased HDL levels.

Enzymatic antioxidants mechanisms play an important role in the elimination of free radicals (ROS). SOD and CAT are endogenous enzymatic antioxidants present in all oxygen metabolizing cells involve in clearance of superoxide and hydrogen peroxide. The suppression of SOD and CAT activity as a result of hepatic damage is reported[17]. Similar findings were observed in our study in hepatic control animals. The oral administration of EAC significantly recovered SOD & CAT towards normal in a dose dependent manner. GSH is the endogenous non-enzymatic antioxidants in our body system and it is protective against chemically induced hepatic damage and oxidative stress[18]. Depleted GSH level was observed in anti-tubercular drugs induced hepatic damage in rats. It is confirmed that from the present study EAC dose dependently and significantly restored hepatic GSH content. Anti-tubercular drugs administration decreased GPx and GR_X levels. EAC dose dependently increased the levels of GPx and GR_X. The results of the histopathological studies also support the results of biochemical parameters.

Lipid peroxidation is a phenomenon involved in peroxidative loss at unsaturated lipids, thus bringing about cellular lipid degradation and membrane disordering. TBARS levels as a marker of oxidative ROS results in lipid peroxidation and subsequently increase in TBARS levels. Increased Lipid Peroxidation causes degradation of cellular macromolecules leading to tissue damage[19]. A marked increase in the concentration of TBARS in anti-tubercular drugs indicated that enhanced lipid peroxidation leading to tissue injury and failure of the antioxidant defense mechanisms to prevent over production of ROS. EAC showed ability to prevent anti-tubercular drugs induced elevation of TBARS level, suggesting that EAC inhibited hepatic lipid peroxidation. This implies that reduction in free radicals production and subsequent decrease in damage to the hepatocellular membrane. The hepatoprotective effect of EAC may be due to presence of preliminary phytochemical constituents like flavonoids, steroids and triterpenoids, tannins and glycosides which might have scavenged the free radical offering hepatoprtoection.

CONCLUSION

This study showed that EAC showed protective action against anti-tubercular drugs induced hepatotoxicity. The hepatoprotective role of EAC might be due to its antioxidant potential mechanism suggesting that the extract of plant may be useful to prevent the oxidative stress induced damage.

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