ORIGINAL RESEARCH PAPER

Volume-8 | Issue-5 | May-2019 | PRINT ISSN No 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

PHYTOCHEMICAL INVESTIGATION AND HEPATOPROTECTIVE EFFECT OF *AEGLE MARMELOS* IN PARACETAMOL INTOXICATED RATS

Pharmacology

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ABSTRACT

Objective: To evaluate the hepatoprotective effect of Aegle marmelos (AM) on paracetamol induced hepatotoxicity and to study the phytochemical composition by GCMS analysis.

Methods: The rats were administered with ethanolic leaf extract of AM (200mg/kg b.wt) prior to paracetamol (2g/kg b.wt) for 21 days.Animals were sacrificed using ether, blood & liver were collected.Biochemical estimation and histopathological analysis were performed.

Results: The pre-treatment with A. marmelos in paracetamol intoxicated rats has shown improvement in the levels of oxidative stress parameters and there was significant reduction of serum marker levels. Extensive hepatic necrosis was evident in paracetamol treated rats, whereas no necrosis observed in AM treated rats.

22 constituents were eluted from AM extract. n-Hexadecenoic acid and phytol were the major compounds.

Conclusion: The results obtained in the present study suggest that ethanolic extract of AM possess significant hepatoprotective activity against paracetamol induced hepatotoxicity.

KEYWORDS

Hepatotoxicity, *Aegle marmelos*

INTRODUCTION

Nature has been a source of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources since they are potent source of chemical entities for the development of drugs. Medicinal herbs are largely used either directly as home remedies or indirectly as modern medicines. [1] In the present days, people are attracted more towards herbal medicines and their consumption. Good patient compliance, better therapeutic effect, cost-effectiveness and less toxicity, could be the reasons for choosing a drug from natural source. Herbal drugs or their extracts are prescribed widely, even when their biologically active compounds are unknown. [2] In the absence of effective drugs for the treatment of liver diseases, natural remedies from medicinal plants are considered to be effective and safe alternative treatment for hepatotoxicity. Herbal medicines are found to be intensely beneficial against liver disorders as they promote the process of healing and regeneration of the liver cells with lesser side effects. Since several decades, plants (whole or parts) have been employed for treating hepatotoxicity due to their antioxidant properties. [3] Keeping all these facts in mind, the present study was undertaken to investigate leaf extract of *Aegle marmelos* for its hepatoprotective effect in paracetamol induced hepatotoxicity.

MATERIALS AND METHODS:

Drugs and chemicals:

Ethanol (99.9%), formaldehyde and ether were purchased from Essarkay Chemicals & Equipment Centre Mangalore. Other chemicals and reagents of the analytical grade for the biochemical estimations were purchased from Sri Durga Laboratory, Mangalore.

Collection of leaves of *Aegle marmelos* (AM) and Preparation of ethanolic plant extracts

The leaves of AM collected from the local areas of Kasaragod district, Kerala in the month of February. The plant material was identified and authenticated by Dr. Neoline J Pinto, Head of Botany Department, St. Agnes College, Mangalore.

The fresh leaves were dried under shade and then powdered with a mechanical grinder to obtain coarse powder. The powder was then subjected to extraction in a Soxhlet apparatus for 72 hours using 90% of ethanol. The extract was concentrated using rotary evaporator and stored in the refrigerator at 4°C for further use. The percentage yield of the extract was 10%.

Experimental animal selection and Grouping

Wistar albino rats of either sex, weighing between 150-250 g, were procured from the animal house of K. S Hegde Medical Academy. They were housed in polypropylene cages with paddy husk bedding at room temperature. The animals were fed with standard pellet diet and water *ad libitum*. During the study period, the rats were kept in separate cages and they were marked for the purpose of identification. Maintenance of animals were done based on the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

The experimental protocols and procedures employed in this study were approved by the Institutional Animal Ethics Committee of K. S Hegde Medical Academy.

The grouping of the animals was as follows.

Group I - as normal control.

Group II -rats were administered with single dose of paracetamol (2g/kg b.wt)

Group III -AM extract (200mg/kg b.w)for 21 days +single dose of paracetamol (2g/kg b.wt) on 21st day .

Group IV- Silymarin (50 mg /kg b.wt.) for 21 days + 2g paracetamol /kg b.wt. on 21st day

Collection of Samples for biochemical estimation and histopathological analysis

At the end of paracetamol/extract treatment period, all the animals were sacrificed next day. Biochemical and histopathological studies were conducted. Blood was collected from each rat by cardiac puncture and transferred to a plain tube, allowed to clot for 30 minutes and serum was separated by centrifugation at 3000 rpm for 15 minutes. The serum was used for the estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin. The liver was dissected, rinsed with water and a part of it was transferred to 10% formalin and used for histopathological procedures. Another portion of it was used for the preparation of homogenate which was used for biochemical analysis of glutathione peroxidase (GPx), and reduced glutathione (GSH)

Preparation of Liver Homogenate

1 g of the liver was cut into small pieces, suspended in 0.1M Tris-HCl buffer, pH 7.5, and homogenized using Potter-Elvejhem apparatus. The resultant homogenate (10% w/v) was centrifuged at 700 g for 10 minutes to remove the unbroken cells and cell debris. The supernatant was collected and used for the estimation of GSH, GPx

Histopathological Analysis: The sections of rat liver tissue were

prepared and stained by haematoxylin and eosin for histopathological study as per standard protocol. [4]

Phytochemical Analysis:

1 g of sample was extracted in 100 ml of diethyl ether using Soxhlet apparatus. The extract was concentrated to dryness and analyzed for composition by using Gas Chromatography-Mass Spectrometry (GC-MS).Briefly, column oven temperature and injector temperature was fixed at 70°C , 250°C respectively. Carrier gas was Helium with 99.9995% purity. Column used was DB-5ms Agilent, length and diameter was 30.0m ,0.25mm respectively.

Statistical analysis

Statistical analysis was carried out using SPSS ver. 17 software. One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests were used. p <0.05 was considered as statistically significant. All values were expressed as mean ± standard deviation.

RESULTS

Table 1: Effect of AM extract on various biochemical parameters and antioxidant status in paracetamol induced toxicity

Parameters	Control	PCM (2g/kgb.w)	AM(200mg/kg)+ PCM(2g/kg b.w)	Silymarin (50mg/kg) +PCM(2g/kg)
Serum total bilirubin (mg/dL)	0.53±0.191	4.26±0.41a ***	1.40±0.71b ***	0.81±0.09 c ***
Serum AST (IU/L)	115 ±2.8	268 ±41.1a ***	116.51±4.08b ***	97.15±1.89c ***
Serum ALT (IU/L)	39±1.2	55±1.6a ***	38.57±2.31b ***	43.64±1.30c ***
Liver GSH (µmoles of GSH/g tissue)	2.033±0.174	0.17±0.04 a **	1.86±0.04b ***	1.75±0.06c ***
Tissue GPx (µmoles GSH oxidised/ min/g tissue)	21.15 ±2.0	7.6±1.2a ***	20.86±10.85b *	23.99±5.02c **

* p <0.05, ** p <0.01,***p< 0.001
a- (PCM 2g/kg) vs Control, b- (PCM 2g/kg) vs AM(200mg/kg)+PCM(2g/kg), c- (PCM 2g/kg) vs Silymarin(50mg/kg)+PCM(2g/kg)

ALT= alanine transaminase, AST=aspartate transaminase, GSH= glutathione, GPx= glutathione peroxidase

Effects of paracetamol with or without plant extracts on histological structure of the liver

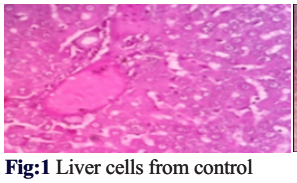

Fig:1 Liver cells from control group showed the normal architecture of cell with preserved cytoplasm and nucleus.

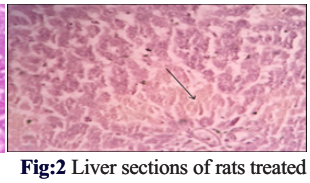

Fig:2 Liver sections of rats treated with paracetamol (2g/kg bw) displaying extensive necrosis in hepatocytes. (H & E stain; (40X)

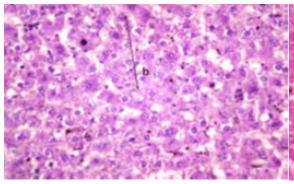

Fig:3 Liver sections of rats treated with AM (200mg/kg bw/) + paracetamol 2g/kg b. wt displaying presence of lymphocytic infiltration, no necrosis is seen. (H & E stain, (40X)

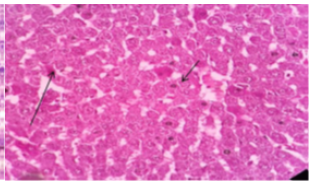

Fig:4 Liver sections of rats treated with silymarin (50mg/kg) + paracetamol (2g/kg) displaying presence of diffuse feathery degeneration in hepatocytes. (H & E stain; (40X)

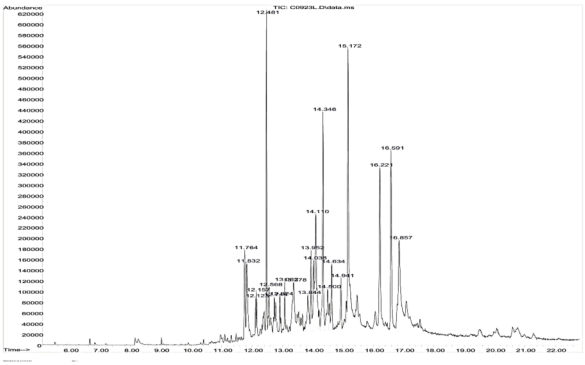

Fig: 5 GLC Chromatogram of AM leaf extract.

Table :2 Phytochemicals identified in AM leaf extract using GC-MS analysis.

Peak	RT	% Area	Name	Match
2	11.830	5.14	Niacinamide	95
3	12.124	1.17	Caryophyllene	99
4	12.155	1.17	cis-.beta.-Farnesene (E)	97
5	12.481	7.80	Phenol, 2,4-bis(1,1-dimethylethyl)	96
6	12.568	1.10	Naphthalene, 1,2,3,4,4a,5,6,8a-oct ahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	98
8	12.925	0.87	9-Octadecene, (E)-	91
10	13.375	5.34	Bis(2-ethylhexyl) phthalate	83
18	14.939	1.90	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	99
19	15.170	13.52	n-Hexadecanoic acid	99
21	16.590	7.95	Phytol	99
22	16.859	8.38	9,12-Octadecadienoic acid (Z,Z)-	95

DISCUSSION:

Paracetamol can be taken as a classic model for drug-induced liver injury and has a clear dose-dependency in both animals and humans. Clinical evidence of excessive consumption of paracetamol in humans, as well as experimental evidence in animal studies, were well documented. [5]

Reactive metabolites such as N-acetyl -p- benzoquinone imine (NAPQI) generated through Phase I and Phase II reactions cause depletion of GSH. In phase II pathways (detoxification), the majority of the drug is metabolized into stable compounds by glucuronidation and sulphation. Around 5-10% of the drug is converted into toxic quinone imine by *P*-450 metabolism. When phase II pathways become saturated, as GSH gets exhausted, causing inadequate detoxification. The NAPQI binds to enzymes, nucleic acids, lipids and other cell constituents lead to secondary toxic hepatocellular damage. 6

The earliest biochemical markers of hepatocellular damage are elevations in ALT and AST. ALT is found in high concentrations in the liver. It leaks into the extracellular space and enters the blood whenever there is a liver injury. Elevation in serum ALT remains the clinical biochemistry gold standard for drug-induced liver injury (DILI). Increase in serum AST level is significantly less specific than that in serum ALT as it is more ubiquitously present in extrahepatic organs. The elevated levels of serum AST/ALT indicate acute hepatic necrosis, cholestasis etc., resulting from tissue damage. [7]

The determination of serum bilirubin is an index for assessing hepatic function. When liver cells are damaged, decreased uptake, decreased conjugation and reduced excretion of bilirubin cause the build-up of bilirubin in the blood and extracellular fluid resulting in hyperbilirubinemia. [8]

In present study, serum ALT, AST and total bilirubin were increased significantly in paracetamol intoxicated rats compared to control. This was prevented by AM leaf extract as in case of silymarin. The serum ALT levels were same or close to control values in AM leaf extract and silymarin treated rats. However, silymarin was more effective in reducing the serum AST level than AM leaf extract.

AM leaf extract was also effective in maintaining serum bilirubin

within or close to normal reference level in rats treated with paracetamol. The effects were comparable to that of silymarin.

Similar results have been reported with respect to AM extracts in other studies. Jayachandra *et al*, noted a significant reduction in the levels of serum ALT, AST and total bilirubin when carbon tetrachloride (CCl4) intoxicated rats were administered with leaf extracts of *A. marmelos*. [9]

Oxidative stress has been implicated in hepatotoxicity due to a large dose of paracetamol. This could be due to accumulation of unconjugated NAPQI, and its capability to binds to proteins and subcellular structures to induce rapid cell death leading to liver failure. The major anti-oxidants present in the body are GSH, GPx. Depletion of GSH level eventually leads to enhanced lipid peroxidation. Reducing level of GSH in the liver mitochondria is an important indicator of liver damage. Hydrogen peroxide and lipid peroxides, formed by the action of free radicals on membrane lipids, are decomposed by GPx using glutathione as the hydrogen donor. [10] The antioxidant enzymes levels may decrease because of their inactivation by ROS when these are produced in excess.

The liver GSH contents in rats treated with plant extract with paracetamol was significantly higher than those in rats given only paracetamol. The plant extracts were more effective in increasing GSH content in paracetamol toxicity as the levels were much closer to control. AM leaf extract was a little more effective than silymarin.

The alterations in antioxidants caused by AM leaf extracts in present study are in agreement with those reported in other studies. Ramamurthy et al, in 2015 observed a significant decrease in liver GSH, and GPx in rats intoxicated with Staphylococcus aureus. With the treatment of alcoholic extract of air-dried leaves, the levels of liver GSH and GPx were significantly improved and similar to that observed with the use of silymarin [11]

Both AM leaf extract as well as silymarin, prevented to a great extent the histopathological abnormalities observed in paracetamol intoxicated rats. Only focal lymphocytic infiltration around portal tract and diffuse hepatocyte swelling in rats given *AM* leaf extracts prior to paracetamol.(Fig:3) However extensive necrosis of the liver cell has been observed when rats were treated single dose of Paracetamol.(fig:2)

These protective effects were similar to those of silymarin. On administration of silymarin and paracetamol, some feathery degeneration was present in hepatocytes.

Other studies have also reported similar protective effects of AM extracts. In the study by Sastry et al demonstrated regenerative effect and milder degree of vacuolation in the hepatocytes of rats treated with 400mg AM extract/kg body weight before paracetamol administration. [12]

n-Hexadecenoic acid was the major component of AM extract (13.52%). It has been shown to inhibit phospholipase A_2 and thus have an anti-inflammatory effect. [13] Phytol and 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione were two other compounds found. Phytol exhibits anti-inflammatory, antibacterial effects. It also has antioxidant action as it scavenges free radicals. 9,12-Octadecadienoic acid (Z, Z) has anti-inflammatory and hepatoprotective action. [14] The actions of the above constituents in the *AM* leaf extract explains their hepatoprotective effects observed in the present study.

CONCLUSION:

The biochemical ,histopathological and phytochemical findings suggested that ethanolic extract of *A. marmelos* leaf extract possess significant hepatoprotective activity against paracetamol induced hepatotoxicity.

ACKNOWLEDGEMENT:

The authors would like to thank NITTE(Deemed to be university) for allowing us to carrying out this study.

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