The Journal of Phytopharmacology 2022; 11(4):255-259
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2022; 11(4): 255-259
July- August
Received: 15-07-2022
Accepted: 02-08-2022

doi: 10.31254/phyto.2022.11405

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Hepatoprotective activity of Amalakyadi Gana, a polyherbal ayurvedic formulation in paracetamol-induced hepatotoxicity in mice

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ABSTRACT

Since ancient times, Amalakyadi Gana, a polyherbal formulation of Susruta Samhita (6th century BCE), has been used for the prevention and treatment of numerous gastrointestinal diseases. This formulation consists of fruits of *Phyllanthus emblica*, *Terminalia chebula*, *Piper longum*, and the root of *Plumbago zeylanica*. The hepatoprotective efficacy of this formulation was evaluated following the acute toxicity study in mice to validate its ayurvedic uses. The hepatoprotective efficacy was assessed using paracetamol-induced hepatotoxicity in Swiss albino mice. Research drug exhibited in normalizing the PCM-dependent rise of serum liver function markers. After administration of the aqueous extract of Amalakyadi Gana, relevant blood biochemical measures showed significant ($P < 0.05$) hepatoprotective activity in a dosage-dependent manner, especially at the dose of 700 mg/kg orally in mice. When compared to the control group, significant ($p < 0.05$) histological alterations were also observed in the liver tissues. This formulation exhibited results in normalizing the liver architecture by decreasing necrotic foci along with the normal liver parenchymal structure in the research drug pre-treated groups mainly at the dose of 700 mg/kg, caused due to paracetamol toxicity. The research drug's sustained activity was comparable to that of the silymarin (200 mg/kg, *p.o.*) reference medicine. This formulation possesses significant hepatoprotective activity without any toxicity in mice.

Keywords: Amalakyadi Gana, Hepatoprotective, *Phyllanthus emblica*, *Terminalia chebula*, *Piper longum*, *Plumbago zeylanica*.

INTRODUCTION

The prevalence of liver diseases around the world contributes to higher rates of morbidity and mortality and serves as an example of the most frequent reason for new medications to fail. The danger of developing liver toxicity is widespread due to the unavoidable adverse effects demonstrated by synthetic medications, the exact prevalence of hepatotoxicity is difficult to quantify because of underreporting, challenges in detection, and insufficient observations [1]. Drug-related liver impairment accounts for about 10% of deaths, which is quite high. Acute liver failure cases that are treated with prescribed drugs account for about 50% of all cases [2]. The primary cause of many non-communicable diseases, daily exposure to different xenobiotics, has emerged as a major public health concern worldwide [3]. Foreign substances, such as environmental toxins, food additives, toxicants, viral load, and alcohol, as well as others, such as acetaminophen, ibuprofen, carbon tetrachloride, aminoglycosides, and carbon monoxide, frequently build up in the body and mainly cause liver or kidney toxicity [4]. These are the causative factors for liver damage that manifests as fibrosis and scarring (cirrhosis), which may lead to liver failure [5]. Hepatic damage is brought on by the over-the-counter antipyretic and analgesic medication paracetamol (PCM) [6]. N-acetyl-p-benzoquinone imine (NAPQI), the primary reactive metabolite of PCM, is produced when the liver enzyme cytochrome P450 is activated at high doses [7]. NAPQI lowers the glutathione concentration in the liver by up to 80 to 90 percent and then forms covalent bonds with proteins and other macromolecules to cause hepatotoxicity [8,9]. Hepatic disorders are among the most severe illnesses, and society faces difficulty in treating them. There are limited options for treating these problems with current medications. In experimental animal models, the hepatoprotective properties of numerous traditional herbal treatments are being investigated [10]. There is an increase in the usage of herbal medicines to treat various disorders. Scientific investigations have supported the use of conventional treatments for a variety of disorders.

The research drug has been selected from one of the most distinguished and most often studied treatises on Indian medicine *Susruta Samhita* of Susrutacharya during 6th century BCE, which is having the combination of four ingredients in equal amounts- fruits of *Phyllanthus emblica* L., *Terminalia chebula* Retz., *Piper longum* L., and root of *Plumbago zeylanica* L., and used in various gastrointestinal disorders [11]. *Phyllanthus emblica* is rich in ascorbic acid, gallic acid, chebulinic acid, chebulagic acid, ellagic

acid, 3-ethylgallic acid, corilagin [12], etc. and possesses cytoprotective [13], immunomodulating [13], hepatoprotective [14-16] effects. *Terminalia chebula* mainly consists of tannins (20-40%), which produce chebulic acid, D-galloyl glucose during hydrolysis [17]. Other compounds are chebulagic acid, chebulinic acid, ellagic and gallic acid [18], etc. The fruit pericarp of *T. chebula* showed cytoprotective [19] activity. The fruits of *Piper longum* mainly contain piperine, chavicine, piperidine, piperetine, a resin, (2E,4E,14Z)-N-Isobutyleicosa-2,4,14-trienamide, (2E,4E)-N-Isobutyleicosa-2,4-dienamide, (2E,4E,12Z)-N-Isobutyleicosa-2,4,12-trienamide, piperanine, pipernonaline, piperlonguminine, pellitorine, guineensine, etc.[20] Experimental and clinical study reports reveal hepatoprotective, antipyretic, CNS depressant, anti-inflammatory, analgesic, and antioxidant activities of piperine [21]. Major chemical constituents of *Plumbago zeylanica* are plumbagin, 3-chloroplumbagin, 3,3'-biplumbagin, 1,2(3)-tetrahydro-3,3'-biplumbagin, chitranone, zeylinone, isozeylinone, elliptinone, droserone, isoshinanolone, maritinone [22], etc. Crude and ethanolic extract of the root of *P. zeylanica* showed metabolic effects in rat liver [23]. Amalakyadi Gana has not yet been subjected to pharmacological evaluation for its hepatoprotective activity. Considering this fact, the present study design is planned to assess the hepatoprotective activity of Amalakyadi Gana against PCM-induced hepato-toxicity in mice.

MATERIALS AND METHODS

Collection and identification of plant parts

All the plant parts required for the preparation of the research drug in crude form were procured from the Barabazar herbal market, Kolkata. Crude plant parts were duly authenticated from the Pharmacognosy Unit, Department of Dravyaguna, Institute of Post Graduate Ayurvedic Education & Research, Kolkata (IPGAER). Voucher specimens (SVP/PG/09-12/2019) have been deposited in the Dravyaguna Museum of IPGAER for future reference.

Preparation of research drug

Fruits of Amalaki (*Phyllanthus emblica* L.), Haritaki (*Terminalia chebula* Retz.), Pippali (*Piper longum* L.), and root of Chitraka (*Plumbago zeylanica* L.) were shade dried in good condition in equal proportion and grind (40 mesh) properly. Make a decoction of the above-mentioned plant parts by adding sixteen parts of water and then boiling to reduce up to one-eighth as described in ayurveda [11,24]. The filtered extract was concentrated under reduced pressure below 50^0C through a rotary evaporator. The reduced extract was put in petri-dishes and air-dried to free water, away from sunlight. This process was repeated thrice, and lastly, a brownish-green research drug was obtained (yield 5.31%, w/w), which was kept in a refrigerator at 4^0C for further use.

Animals

Swiss albino mice weighing about 20 to 25 g of either sex were used for *in vivo* evaluation. The animals were procured from a CPCSEA-approved breeder (1828/PO/Bt/S/15/CPCSEA) in Kolkata, India, and kept under the standard environment in fixed 12 h light and dark cycles at 24 ± 2^0C in the animal house (1180/GO/Re/S/08/CPCSEA), IPGAER, Kolkata. Animals were housed in polypropylene cages and fed food, water *ad libitum*. Acclimatization of animals was done for 14 days before performing any experiments. Before experiments, the animals were fasted for 6 hours but allowed free access to water. The acute toxicity study and hepatoprotective experiments were performed using Swiss albino mice. All experimental protocols were approved (IAEC/IPGAER/519/2018) by the Institutional Animal Ethical Committee, IPGAER, Kolkata.

Animal groupings and drug administration protocol

A total of thirty-five Swiss Albino mice were taken for the present study. Initially, fifteen mice were administered normal food and water *ad libitum* for 7 days following a single dose of paracetamol (500 mg/Kg) orally. Blood and organ samples were collected from sacrificed mice at 0, 8, and 12 h to assess the acute liver injury induced by PCM. Collected samples at 0 h marked as the control group. Maximum liver injury was observed after 8 h of paracetamol administration in mice. To evaluate the protective effects of research drug from liver injury due to PCM, 20 mice were randomly grouped (4 groups, n = 5) and pretreated for 7 days with either vehicle (water), standard drug silymarin (200 mg/kg), or research drug at 500 and 700 mg/kg doses. After one week of vehicle, standard, and research drug administration, a single dose of PCM (500 mg/Kg) was administered to each group. Blood and liver samples were collected 8 h post-administration of PCM. The research drug dose was fixed based on the human dose of this formulation.

Biochemical assay

Collected blood samples drawn by cardiac puncture were centrifuged using a centrifuge machine at 1000 × g for 10 min to get serum and were stored in a freezer at −30 °C until analysis. Biochemical parameters include total serum bilirubin, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and serum alkaline phosphatase (ALP) were analyzed by using an automated serum analyzer (Micro Lab RX 50) and biochemical kits of SIEMENS, India.

Histopathological assay

The liver samples were kept in neutral buffered formalin solution at 10% v/v after being washed with normal saline. The tissues were embedded in paraffin after being fixed, cut into 5 µm sections using a microtome (rotary), then stained using the hematoxylin and eosin dye. Under an Olympus biological microscope CX23, the histological analysis of the liver slices was performed. Based on pathologic lesions in the appropriate sections stained, the degree of drug-induced liver damage was assessed. Changes in experimental histological parameters for liver tissues were rated using a scoring system employed for histopathological examinations following standard protocols [25-27]. Pyknotic nuclei, cytoplasmic vacuolation, cellular infiltration, necrosis, and sinusoidal dilatation were the chief histological abnormalities of the liver that were taken into consideration. For each group of the liver, the severity of the histological damage was assessed and categorized.

Statistics

The Student's t-test was used for statistical analysis comparing means between two groups, and the one-way ANOVA was used for statistical analysis comparing means among more than two groups. The Kruskal-Wallis test was used to analyze histopathologic data and find out how each experimental parameter was affected by each group. Using the Statistical Package of Microsoft EXCEL, all data were analyzed and represented as Mean ± SE. Statistical significance was defined when $P < 0.05$ [28].

RESULTS

The administration of the research drug in Swiss Albino mice at a dose of 2000 mg/kg body weight did not cause any deaths, as well as no signs of acute toxicity, were observed for 14 days.

PCM administration induced acute liver injury

Total serum bilirubin, AST, ALT, and ALP levels in the PCM-treated mice group were significantly ($p < 0.05$) elevated at 0, and 8 hours, but transaminase levels dropped or returned to normal after 24 hours. These findings demonstrated that 8 hours of paracetamol treatment resulted in a considerable rise in serum liver function indicators (Table 1) and significant ($p < 0.05$) changes in the histology of liver tissues (Table 2) were also observed. Mice liver samples collected after 8 hours of exposure to paracetamol treatment showed some aberrant morphological characteristics (Fig.1).

Effects of research drug in pre-treated mice exposed to PCM-induced acute liver injury

Research drug at both the doses (500 and 700 mg/kg) significantly prevented the paracetamol-dependent rise in total serum bilirubin, AST, ALT, and ALP levels when pretreated with the research drug and compared with the control (Table 1), which was also consistent with the overall histopathological changes in the liver (Fig. 1). The research drug at the dose of 700 mg/kg pretreated group exhibited significant ($p < 0.05$) reduction of liver injury by reducing the liver bio-markers and normalize liver architecture (Table 2, marked in Fig.1) when compared with control (PCM 8 h). Research drug at the dose of 500 mg/kg pretreated group also shows significant ($p < 0.05$) changes, but less than the higher dose (Table1, Table 2). The research drug exhibited dose-dependent hepato-protective activity in mice. Histological findings exhibited that in the research drug (700 mg/kg) pretreated group, there were fewer pyknotic nuclei, cytoplasmic vacuolation, focal hepatic necrosis, sinusoidal dilatation, centrilobular necrosis, perivascular and portal cell infiltration and the parenchymal structure of the liver was normal compared to the control group (PCM 8 h). Research drug at 700 mg/kg exhibited comparable activity with Silymarin (200 mg/kg) (no significant difference noted) in normalizing the PCM-dependent rise of serum liver function markers (Table 1) and liver architecture (Table 2, Fig.1).

Table 1: Effects of research drug on liver enzymes on PCM induced hepatotoxicity in mice

Biochemical parameters	Group I	Group II	Group III	Group IV
	Control (vehicle)	Silymarin at 200 mg/kg	Research drug at 500 mg/kg	Research drug at 700 mg/kg
Total Bilirubin	3.00±0.11	1.46 ± 0.14	2.26 ± 0.18	1.68 ± 0.16
AST	231.45 ± 7.18	121.82 ± 6.40	185.93 ± 7.75	127.28 ± 6.15
ALT	349.65 ± 11.54	159.87 ± 12.73	221.08 ± 14.00	164.43 ± 12.62
ALP	263.62 ± 14.75	191.1 ± 6.42	227.17 ± 7.79	196.3 ± 6.26

Significance related to control, $p < 0.05$ (ANOVA test); Values expressed are Mean ± SE (n = 5); SE= standard error

Table 2: Effect of PCM and 7 days pre-treatment with research drug and silymarin after 8 h of single-dose exposure of PCM on histopathological changes in Swiss albino mice liver.

Lesion description	PCM		Silymarin (200 mg/kg) + PCM	Research drug (500 mg/kg) + PCM	Research drug (700 mg/kg) + PCM	Kruskal-Wallis Test for assessment of organ lesions among groups
	0 h	8 h	8 h	8 h	8 h	
Pyknotic nuclei	0.26 ± 0.03	0.86 ± 0.26	0.43 ± 0.22	0.71 ± 0.60	0.51 ± 0.37	0.17
Cytoplasmic vacuolation	0.11 ± 0.05	0.71 ± 0.21	0.11 ± 0.05	0.43 ± 0.26	0.27 ± 0.19	0.11
Focal hepatic necrosis	0.00 ± 0.00	0.53 ± 0.18	0.19 ± 0.11	0.39 ± 0.28	0.23 ± 0.17	0.03
Sinusoidal dilatation	0.11 ± 0.08	0.65 ± 0.17	0.41 ± 0.25	0.55 ± 0.29	0.37 ± 0.19	0.09
Centrilobular necrosis	0.00 ± 0.00	0.67 ± 0.21	0.27 ± 0.13	0.52 ± 0.19	0.35 ± 0.11	0.01
Perivascular and portal cell infiltration	0.19 ± 0.09	0.79 ± 0.17	0.29 ± 0.18	0.59 ± 0.34	0.31 ± 0.25	0.07

The values are given as Mean ± SE; Asymptotic Significance ($p < 0.05$)

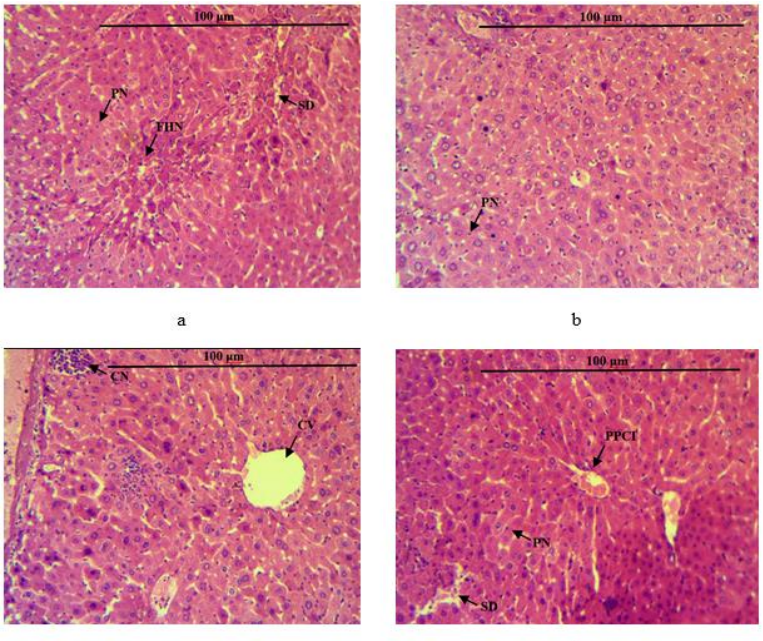

Figure 1: Histopathological examination of liver section (M × 100) done after 8 h of PCM exposure in all treated groups; (a) Microscopic analysis of mice livers only exposed to PCM; (b) The protective effect of silymarin (200 mg/kg) in pre-treated mice group exposed to PCM; (c) The protective effect of research drug (500 mg/kg) in pre-treated mice group exposed to PCM; (d) The protective effect of research drug (700 mg/kg) in pre-treated mice group exposed to PCM; PN- pyknotic nuclei, FHN- focal hepatic necrosis, SD- sinusoidal dilatation, CN- centrilobular necrosis, CV- cytoplasmic vacuolation, PPCI- perivascular and portal cell infiltration, M- magnification

DISCUSSION

Hepatic impairment is one of the main inevitable aspects of drug or xenobiotic disposition, among other drug therapy problems [29]. PCM is generally regarded as a safe medicine, but hepatotoxicity [6] and increased mortality [30] are the most prominent side effects. Numerous investigations have demonstrated that liver biomarkers had a dose- and time-dependent relationship after a single injection of PCM [31]. This present study is aimed to investigate the hepatoprotective potential of Amalakyadi Gana aqueous extract consisting of fruits of *Phyllanthus emblica* L., *Terminalia chebula* Retz., *Piper longum* L., and root of *Plumbago zeylanica* L against PCM-induced toxicity in experimental Swiss Albino mice that have not yet been tested. Glutathione is depleted as a result of PCM overdose, and the concentration of its metabolite (NAPQI) is elevated. Hepatocyte cell necrosis is caused by mitochondrial protein binding to NAPQI, which produces harmful protein adducts [32]. An increase in ALT and AST values in the presence of PCM-induced damage denotes aberrant and/or unregulated liver metabolism. According to the results of this investigation, the research drug at both doses significantly reverses these elevations as well as protects the liver architecture. There are significant limitations to the current study, even though it describes the protective benefits of Amalakyadi Gana for the first time against paracetamol-induced liver injury in mice. To characterize the effects of each derivative solely, research is required to determine the components of Amalakyadi Gana. These findings may not only advance our knowledge of potential mechanisms behind this hepatoprotective activity of the research drug but also help in the development of novel therapeutic strategies for the benefit of suffering people with liver diseases. Phenols, flavonoids, saponins, tannins, alkaloids, terpenoids, etc. are only a few of the components of Amalakyadi Gana that have been identified and many of them are reported for hepatoprotective effects [12-23]. According to some evidence, Amalakyadi Gana helps enhance the liver's normal functioning. However, additional study is needed to delve deeper into the Amalakyadi Gana- derived chemicals and identify the many molecular and transcriptional mechanisms involved in pathogenesis.

CONCLUSION

Amalakyadi Gana from the Sushruta Samhita has been shown to improve both histological and biochemical parameters in mice and to decrease acute PCM-mediated liver damage *in vivo*. Amalakyadi Gana may lessen the severity of liver damage brought on by paracetamol. It is conceivable that Amalakyadi Gana will be used in the future to treat liver toxicities brought on by varied etiology.

Acknowledgements

This research work was included in a thesis submitted by S. Ray for her M.D. (Ayurveda) degree at the West Bengal University of Health Sciences, Kolkata, India. The Director of Ayurveda, Government of West Bengal, provided the resources needed to carry out this study, which is acknowledged by the authors.

Conflict of Interest

None declared.

Financial Support

None declared.

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HOW TO CITE THIS ARTICLE
Ray S, Taraphdar AK, Gupta M. Hepatoprotective activity of Amalakyadi Gana, a polyherbal ayurvedic formulation in paracetamol-induced hepatotoxicity in mice. J Phytopharmacol 2022; 11(4):255-259. doi: 10.31254/phyto.2022.11405