Evaluation of hepatoprotective effects of Aegle *marmelos* alone in combination with Trigonella *foenum* against acetaminophen-induced hepatotoxicity in Wistar rats

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Abstract

The present study aimed to assess the hepatoprotective properties of the fruit extract of Aegle marmelos (L.) Correa of the Rutaceae family. Furthermore, the study evaluated whether the hepatoprotective activities of A. marmelos were enhanced in combination with fenugreek. The Wistar rats used in the study were randomly split into seven groups: (i) normal control, (ii) acetaminophen group, (iii) silymarin group, (iv) extract foe 100 mg/kg body weight, (v) extract at 200 mg/kg, (vi) extract at 400 mg/kg, and (vii) extract at 100 mg/kg with fenugreek.The hepatotoxicity was induced by the oral administration of acetaminophen at 50 mg/kg for seven days. The animals were treated 30 minutes before acetaminophen administration and then for five more days. At the end of this period, animals were sacrificed, and serum samples were collected to determine levels of alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase. The increase in serum biochemical markers was significant in the acetaminophen group compared with the normal control group. Treatment with A. marmelos and the toxicities associated with acetaminophen were effectively mitigated in a dose-dependent fashion; the 400 mg/kg dose showed the best hepatoprotective effect of all doses analyzed. The extract-400 group outcomes were equatable to those found in the silymarin group. The low dose of A. marmelos was further combined with the support of fenugreek to investigate the enhancement of its hepatoprotective effects. The combined administration enhanced the effects of fenugreek, making the combination group results comparable to those of the high dose A. marmelos.

Keywords: *Aegle marmelos*, Marmelosin, Silymarin, Anti-inflammatory, Hepatotoxicity.

INTRODUCTION

The human body's largest and most vital organ is the liver. It is the primary location of haematopoiesis during the intrauterine era and begins to develop in the human embryo during

the third and fourth week of life[1]. Located under the diaphragm in the upper right corner of the abdomen, the healthy human liver weighs roughly 1500g.

The human liver is made up of four lobes that are uneven in shape and are connected to two reddish-brown blood veins called the hepatic artery and the hepatic portal vein. The liver accounts for around 2.4% of the total body weight. Heapatic lobes contain both parenchymal and nonparenchymal cells[2]. The hepatic sinusoid mostly contains two types of nonparenchymal cells: kuffer cells and sinusoidal endothelial cells. In the process of making bile, the liver performs essential processing. Hepatocytes in the liver generate bile, which is a combination of water and salts, as well as cholesterol and bilirubin. Bile is released from duodenal cell lines after digestion by means of a hormone called cholecystokinin[3]. Metabolic processing of carbohydrates, lipids, and proteins, as well as the conversion of these macromolecules into biologically active materials and the excretion of other potentially harmful chemicals, are primarily the functions of this organ.

Epidemiology

Indian population

There are over 10 lakh new cases of liver cirrhosis detected each year in India. Liver disorders rank among India's top 10 killers, according to the World Health Organisation. As of May 2014, the most recent data released by the World Health Organisation indicated that 2.44 percent of all fatalities in India were attributed to liver illness[4].

Worldwide affected population

On a daily or occasional basis, chronic liver disease affects around 29 million Europeans and 30 million Americans. Liver cirrhosis was the leading cause of death for over 170,000 Europeans in 2013. In 2010, 156,900 women and 336,400 men lost their lives to alcoholic liver disease (ALD), a condition characterised by cirrhosis of the liver[5]. Numerous studies have shown that dietary and lifestyle variables have contributed to liver cancer's rise to the position of top cancer killer globally. Liver cancer was responsible for an estimated 788,000 deaths in 2015, according to the World Health Organisation[6]. Over 5,000 liver transplants are performed each year in Europe alone, contributing to a global total of over 25,000 transplants performed in 2014.

Drug induced hepatotoxicity

The primary goal of medicinal digestion is to facilitate the release of a medicine with a lower polarity by means of the progressive formation of more polar metabolites. 5 The next step is for the kidneys to excrete the solvent component in water. The polar metabolites are typically delivered during Phase I digestion by oxidation, decrease, or hydrolysis by CYP450 catalysts[7]. Phase II digestion, which involves glucuronidation or sulphation, makes them water-soluble and available for discharge. 5 However, we can't ignore the fact that

articulation of protein-related properties, in addition to changes in normal and pathologic blood flow to the liver, may impact the activities of hepatic medication processing catalysts[8]. It is possible for a drug or its metabolite to lawfully cause hepatotoxicity by penetrating nearby vulnerable hepatocytes. 5 The two most well-studied medications that include this ingredient are acetaminophen and paracetamol. Direct hepatotoxicity of paracetamol is dose-dependent and can occur after exposure to either a very large single dose or a very large combination dose of the drug[9]. The presence of a toxic CYP2E1-derived metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which can cause hepatocyte injury, can also reach subcellular organelles and trigger apoptosis or putrefaction. 5 Partially linked hepatotoxicity can manifest in a variety of ways, and some drugs are also suitable for this purpose[10].

After taking bromfenac, cyclophosphamide (which damages hepatic sinusoidal cells directly), or methotrexate, hepatocyte rot might occur, causing direct hepatotoxicity. 5 Cocaine, phencyclidine, and niacin can all trigger ischaemic rot. Amiodarone can trigger steatohepatitis[11].

The use of some plants and the consumption of different fruits have played fundamental roles in human health care. Approximately 80% of the world's population has employed traditional medicine for health care, which is based predominantly on plant materials[12].

Aegle marmelos generally known as Bael is an underappreciated fruit native to the Indian subcontinent, which includes Nepal, India, Sri Lanka, Bangladesh, and Thailand. It is a medium-sized deciduous tree belonging to the Rosaceae family. It is a subtropical plant that can adapt to a wide range of habitats and can be grown worldwide[13]. Bael is reported to have number of coumarins, alkaloids, steroids, and essential oils. Root and fruits contain coumarins such as scoparone, scopoletin, umbellliferone, marmesin and skimming. Fruits in addition contain xanthotoxol, imperatorin and alloimperatorin and alkaloids like aegeline and marmelline[14].

Fenugreek (Trigonella foenum-graecum), a member of the fabaceae family of plants, is highly effective in the treatment of diabetes, atherosclerosis, liver disease, cardiovascular disease, and kidney disease. In addition, the important chemicals found in abundance in fenugreek seed have a major impact on the treatment and management of disease via the regulation of a wide range of biological functions. Because of its high antioxidant content, fenugreek seed may be able to prevent pathogenesis by scavenging free radicals[15]. A comprehensive protective impact on the cumulative effect of cyclophosphamide and l-buthionine-SR-sulfoximine was observed when the activity of all enzymes were restored by pre-treatment of the herbal extract, demonstrating the therapeutic significance of fenugreek. The key components found in abundance in fenugreek seed have important roles in biology. Galactomannan, sapogenin, trigonelline, and 4-hydroxyisoleucine are the active components of fenugreek seed, which have anti-diabetic and potential breast cancer therapeutic roles[16].
Hence present study was planned to evaluate hepao-protective activity of Aegle *marmelos* fruit in combination of Trigonella *foenum* seed using vivo models.

MATERIAL AND METHODS:

Selection, identification and authentication of plant material

The fresh fruits were sourced from the Varanasi district in the month when they were naturally occurring in the UP East region, and a botanist verified their authenticity. The botanical specimens were authenticated and taxonomically identified by the Botanical Survey of India in Pune, Maharashtra, India, and then placed in a herbarium.

After being carefully rinsed with water to eliminate any dust, the Aegle marmelos fruits were left to dry naturally in the shade. Following drying, the plant components were ground into a coarse powder using a mechanical mixer. They were then stored in an airtight container for future research. A fine powder was made from fenugreek seeds that were bought from a nearby market, after which they were cleaned of any debris[17].

Extraction of plant material

Based upon the extensive literature survey, we decide to use the alcoholic solvent such as ethanol for extraction of phytoconstituent from powder of *Aegle marmelos* leaves and *Trigonella foenum* seeds for further study.

Extraction of Aegle marmelos fruit:

The fresh fruit of *Aegle marmelos* were dried and ground into powder at room temperature. Three hundred grams (300 g) of powdered plant material was weighed and extracted with 1.5 L of mixture of ethanol for 72h at about 64ºC using the Soxhlet extractor apparatus. After filtration, the extract *of Aegle marmelos* was concentrated under low pressure and 40ºC temperature on a rotary evaporator[18]. The *Aegle marmelos* extract was stored in a refrigerator at 4°C until the time of use.

Extraction of fenugreek seed:

This study uses hot soxhlet extraction apparatus, First, fenugreek seeds were ground using a mixer and using a weighing scale, 100 grams were taken and put into a parchment paper and then extracted with 350 ml 95% ethanol using a Soxhlet. The extraction process was done until the solution color in the circulator became clear[19]. The sample extract was then concentrated using rotary evaporator and stored in a refrigerator at 4°C until the time of use.

Figure 1: Soxhlet extraction of Aegle marmelos fruit & Trigonella *Trigonella foenum* seeds

Drugs and chemicals

The necessary chemicals utilized during this research work have been Acetominophen, Formalin, Diagnostic kits and Silymarin.

Equipment

Soxhlet apparatus, Autoanalyser, Digital Balance, Desiccator, Hot Air oven.

Preliminary Phytochemical Screening of Extracts

The plant's extracts were tested for phytochemical components using a conventional qualitative chemical examination, which helped to identify the various phytoconstituents present in the plant. By using well-established methods, we were able to ascertain the presence or absence of several phytochemical components, including alkaloids, carbohydrates, glycosides, saponins, amino acids, proteins, tannins, phenolics, steroids, and fixed oils[20].

Experimental animals

We obtained male and female albino rats weighing 150-200g from the Animal House at Saraswati Higher Education and Technical College of Pharmacy in Gahni, Ayar, Varansi. A room temperature of 25 ± 1°C and a 12-hour light/dark cycle were the perfect circumstances

in which the animals were maintained. A normal mouse pellet diet was given to the animals, and they were allowed unrestricted access to water[21]. The relative humidity ranged from 44-56%. The rats should not eat anything for at least one hour before the test.

Acute Toxicity Test:

Acute toxicity study of the extract was performed in overnight fasted albino mice by following fixed dose method as per OECD guidelines No.423. Mortality & toxic symptoms in the treated animals were observed continuously for the first 3 h after dosing, periodically during the first 24 h and then daily observation for a total period of 14 days[22].

Swiss albino mice were tested for acute oral toxicity with an ethanol extract of the dried fruit from *A. marmelos* & extract of the dried seed from *T. foenum* at dose 300, 600, 1200 & 2000 mg/kg. Over the course of 24 hours, the animals were carefully watched for signs of death or other adverse effects[23]. This included respiration, sedation, posture, lethargy, diarrhoea, skin colour, hair condition, and coma, among other criteria.

EXPERIMENTAL DESIGN

Assessment of hepatoprotective activity

Acetominophen-induced hepatotoxicity in rats

All animal experiments performed in accordance with the guidelines of Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India. The Institutional Animal Ethics Committee (IAEC) approved the animal experimentation protocols.

The experiment will be perform according to the modified procedures described previously 42 rats were randomly divided into 7 groups and treated with normal saline, graded doses of ethanolic extract of A. marmelos, T. foenum and silymarin for five days. Group A (Control group) received normal saline (NS) only (10 mL/kg, p.o.). Group B received Paracetamol (50 mg/kg, i.p.), Groups C, D and E received ethanolic extract of A. marmelos 100 200 mg/kg p.o.and 400 mg/kg p.o and Group F received combine dose of ethanolic extract of A.marmelos and T. foenum (100 mg/kg, p.o.). Group F received Silymarin {standard drug}, (100 mg/kg, p.o.), respectively, plus Paracetamol (50 mg/kg, i.p.)[24].

Table 1: Experimental design

Groups	**Treatments**
Group-A	Normal saline (NS) only (10 mL/kg, p.o.)
Group-B	Paracetamol (50 mg/kg, i.p.)

Group-C	Paracetamol (50 mg/kg, i.p.) plus 100 mg/kg, p.o.)A. marmelos extracts
Group-D	Paracetamol (50 mg/kg, i.p.) plus 200 mg/kg, p.o.)A. marmelos extracts
Group-E	Paracetamol (50 mg/kg, i.p.) plus 400 mg/kg, p.o.) A. marmelos extracts
Group-F	Paracetamol (50 mg/kg, i.p.) plus 100 mg/kg, A. marmelos extracts plus 100 mg/kg T. foenum p.o.) extracts
Group-G	Paracetamol (50 mg/kg, i.p.) plus 100 mg/kg, p.o.)Silymarin (Standard drug)

Rats from various groups were sacrificed on day 6 of the treatment regimen after being sedated with light ether. This was done twenty-four (24) hours following the last treatment. The blood was drawn from the retro-orbital plexus and placed in a vial without anticoagulants. The separated serum sample was centrifuged at 5,000 rpm for 10 minutes, which was then used for biochemical parameters analysis using standard diagnostic kits. The liver was immediately taken out and washed with ice-cold saline[25]. The blood and liver samples were assessed for their biochemical as well as histological observation.

RESULTS

Phytochemical Analysis of A. marmelos fruit extract:

The results of the preliminary phytochemical evaluation of the A. marmelos extracts are shown in Table 1. The presence of phenols, flavonoids, tannins, terpenoids, coumarin glycoside, alkaloids, carbohydrates and proteins was detected.

Table 2: Phytochemical analysis of *A. marmelos* fruit extract

Sr. No.	Phytochemical Compounds	Method	Observation	Inference
1.	Alkaloid	Mayer's test	Cream color precipitate at bottom	Present
2.	Terpenoids test	Salkowski test	Reddish brown coloration at interface	Present
3.	Proteins	Biuret test	Violet color	Present
4.	Carbohydrates	Molish test	Violet ring at junction	Present
5.	Tannins test	Ferric chloride	Dark green coloration	Present
6.	Flavonoid	Sulphuric acid test	yellow colored precipitate.	Present
7.	Coumarin glycoside	Alkali test	Blue-green fluorescence	Present

Phytochemical Analysis of Fenugreek seed extract:

The results of the preliminary phytochemical evaluation of the T. foenum extracts are shown in Table 1. The presence of phenols, flavonoids, tannins, terpenoids, coumarin glycoside, alkaloids, carbohydrates and proteins was detected.

Table 3: Phytochemical analysis of *T. foenum* seed extract

Sr. No.	Phytochemical Compounds	Method	Observation	Inference
1.	Alkaloid	Mayer's test	Cream color precipitate at bottom	Present
2.	Saponin	Foam test		Present
3.	Tannins test	Ferric chloride	Dark green coloration	Present
4.	Flavonoid	Sulphuric acid test	yellow colored precipitate.	Present
5.	Phenolic	Fecl3 test	Dark green colouration	Present
6.	Triterpenoid	Salkowski's test	Reddish brown colouration	Present
7.	Glycoside	Keller-Killiani Test	Brown colour ring formation	Present
8.	Steroid	Liebermann-Burchard Test	Blue –green colouration	Present

Acute toxicity (LD_{50}) study:

At doses of 300, 600, 1200, and 2000 mg/kg, an ethanol extract of the dried fruit from A. marmelos was administered orally to Swiss albino mice to assess acute oral toxicity. The mice's behaviour and physiological activity were recorded throughout the trial, which lasted for 14 days, and the results should show that the extract is not harmful at a dose of 1200 mg/kg. Mice died after receiving a test dose of 2000 mg/kg of the ethanolic extract of A. marmelos.

Table 4: LD_{50} determination of A. marmelos fruit extract

Group	Species	Dose mg/kg	No. of Animals	Mortality	LD50 mg/kg
Group-I	Swiss albino mice	300	6	0/6	≥ 2000 mg/kg
Group-II		600	6	0/6	
Group-III		1200	6	0/6	
Group-IV		2000	6	2/6	

Oral administration of 300, 600, 1200, and 2000 mg/kg of an ethanol extract of the dried seed from T. foenum to Swiss albino mice was used to evaluate acute oral toxicity. Results should demonstrate that the extract is safe at a dosage of 1200 mg/kg based on the behavioural and physiological data obtained throughout the 14-day experiment. A lethal dose of 2000 mg/kg of T. foenum ethanolic extract was administered to mice.

Table 5: LD_{50} determination of T. foenum seed extract

Group	Species	Dose mg/kg	No. of Animals	Mortality	LD50 mg/kg
Group-I	Swiss albino mice	300	6	0/6	≥ 2000 mg/kg
Group-II		600	6	0/6	
Group-III		1200	6	0/6	
Group-IV		2000	6	3/6	

Pharmacological Evaluation:

Effect of Aegle marmelos treatment on serum biochemical parameters

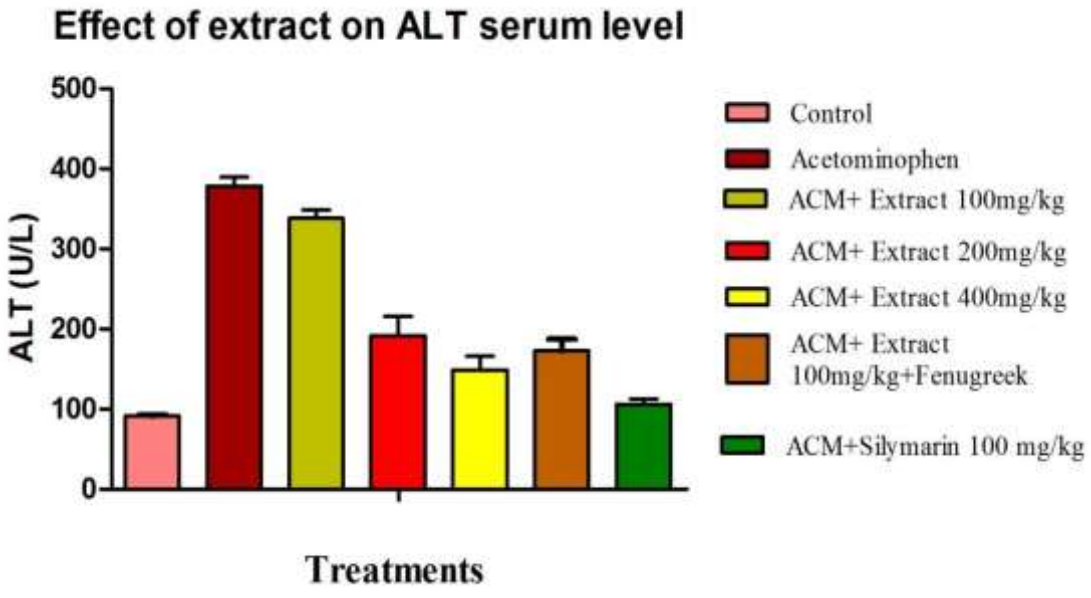
The measurement of serum hepatic enzymes allows one to assess the kind and extent of hepatocellular injury. Increasing blood hepatic enzymes (AST, ALT, and ALP) following ACP treatment demonstrated liver injury. In the ACP group, AST, ALT, and ALP levels were noticeably higher than in the normal control group ($p < 0.001$). In contrast to the ACP group, those given 200 mg/kg of silymarin or 400 mg/kg of A. marmelos saw a decrease in elevated blood enzymes. Since the ACP group and the extract-100 mg/kg dosage group did not differ significantly in enzyme levels, this 100 mg/kg dose of A. marmelos proved ineffective. When 100 mg/kg of A. marmelos and 100 mg/kg of T. foenum were given jointly, the levels of AST, ALT, and ALP were significantly lower ($p < 0.001$) compared to the ACP group. This indicates that the addition of T. foenum increased the efficacy of the 100 mg/kg dose of A. marmelos, which was already quite low. When comparing the silymarin group to the A. marmelos group, we also discovered the same thing. The significantly greater enzyme levels in the extract-100mg/kg group compared to the silymarin group [AST ($p < 0.001$), ALT ($p < 0.001$), and ALP ($p < 0.001$) revealed that the low dose of A. marmelos (100 mg/kg) alone was ineffective. Table 5 shows that the group that got 100 mg/kg of T. foenum + A. marmelos extract showed no change compared to the silymarin group. Various A. marmelos treatment groups showed a dose-dependent reduction in enzyme levels.

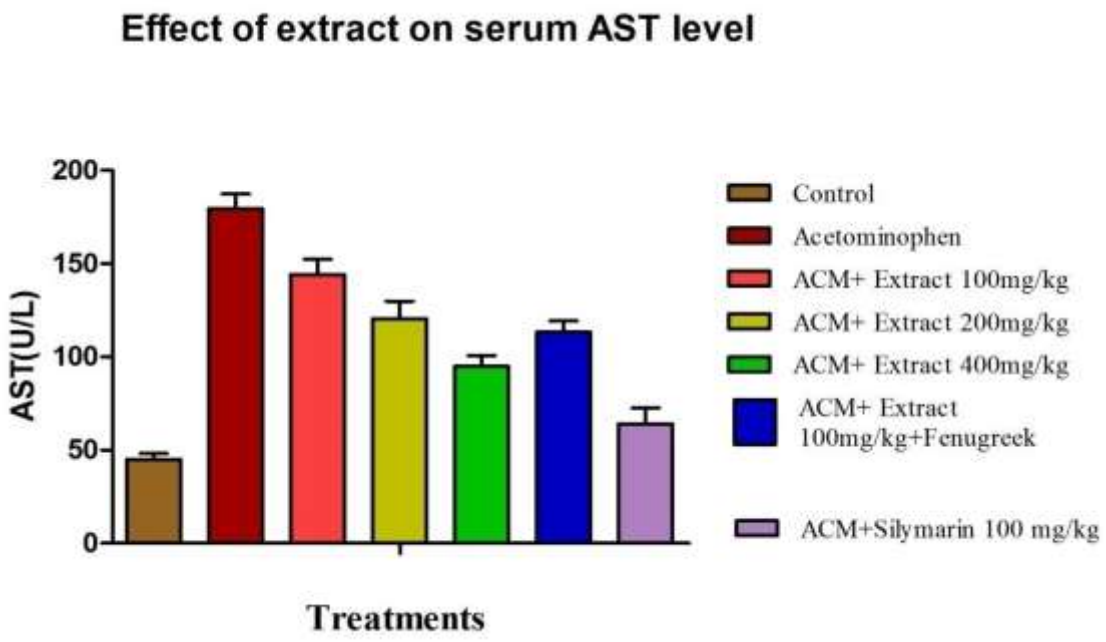
Table 6: Effect of Aegle marmelos on ACM-induced alterations in serum ALT, AST and ALP content.

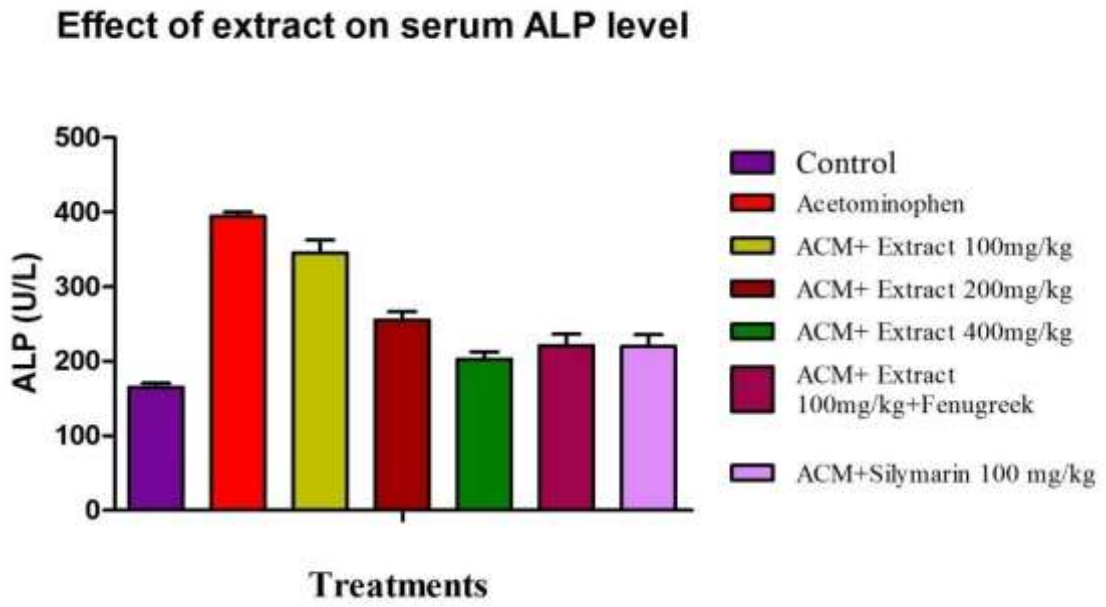
Groups	ALT (U/l)	AST (U/l)	ALP (U/l)
Group-A	91.25±1.480	45.16±3.16	165.07±5.38
Group-B	378.01±11.43	179.48±7.86	394.56±5.73
Group-C	338.09±10.62	144.02±08.35	345.81±17.09
Group-D	191.33±24.36	120.38±9.60	255.24±11.17
Group-E	148.90±16.94	94.92±5.80	202.59±10.03
Group-F	172.78±14.44	113.32±5.88	221.09±15.42
Group-G	105.92±6.72	64.05±8.75	219.77±15.95

Data presented as mean±SEM (n = 6)

There was a non-significant decrease in serum enzymes in the treatment group that received 400 mg of extract compared to the group that received 200 mg of extract. In comparison to the extract-100 group, the enzyme levels were substantially lower in the extract-200 and extract-400 groups. There was no statistically significant change in the enzyme levels between the extract-200 and extract-400 groups and the extract-100 + T. foenum group (Table 6).







Histopathological Examination:

Histological examination of PCM-induced liver damage showed central venous constriction, hepatocellular enlargement, and necrosis, indicating severe hepatotoxicity. In the healthy control group, as shown in Fig. 5, the liver parenchyma and hepatocytes have normal architecture, with separate hepatic cords and a central vein. Hepatic damage was less severe in the A. marmelos (400 mg/kg) group compared to the PCM group. Extract-100 and its combination with fenugreek further confirmed its hepatoprotective activity by reducing vascular distortion and lymphocyte infiltration.

Figure 2: Histological appearance in tratment groups.

DISCUSSION

The main cells in the liver that facilitate a variety of metabolic processes are hepatocytes. A metabolite problem results from any distortion in this organ. It is possible to harm the liver with an excessive amount of paracetamol. Free radical production and oxidative processes appear to have a relationship with acetaminophen-induced hepatotoxicity[26]. So, it stands to reason that substances or extracts with antioxidant and free radical scavenging capabilities could shield the liver from acetaminophen's harmful effects. The fact that antioxidant activity and hepatoprotective effects can work together to prevent hepatocellular damage from

starting and progressing lends credence to this theory[27]. This study examined the effects of co-administration of fenugreek and the hepatoprotective capability of A. marmelos fruit extract after it had been phytochemically standardised.

Additionally, there are other important considerations within the chemically induced hepatotoxic mechanism that pertain to the inflammatory processes brought about by paracetamol[28]. There is some evidence that these inflammatory processes play a role in the production of reactive oxygen species (ROS) and nitric oxide (NO), two molecules that can impact the liver's ability to heal or sustain injury. Therefore, it is plausible to assume that extracts with anti-inflammatory characteristics may also have a hepatoprotective impact.
Multiple sources confirm that A. marmelos included a high concentration of phenolic chemicals in its early phytochemical investigations.

One of the most common ways that researchers mimic liver damage is by using paracetamol, which is notorious for causing hepatic necrosis[29]. Because the hepatocytes' transport ability is impaired during liver injury, the serum levels of the cellular enzymes ALT, AST, and ALP rise. When the serum AST level rises, it means that the cell membranes at the sinusoidal surface of the liver are damaged and transport function is interrupted. An increase in serum ALT, AST, and ALP levels after acetominophen treatment was also noted in this investigation, which could indicate hepatic injury[30]. Furthermore, during the pretreatment phase, the increased serum enzymes can be reduced through the dose-dependent protective action of A. marmelos and silymarin on hepatocellular membranes against the harmful effects of ACM. This protective action can be achieved either alone or in combination with fenugreek.

Inflammatory cells are activated and mobilised due to direct oxidative damage. The subsequent release of inflammatory mediators, such as cytokines, damages the liver. Liver cirrhosis is associated with an increase in the activity of TNF-α, a multifunctional cytokine that has multiple immunologic and metabolic consequences[31]. Hepatic encephalopathy, heightened susceptibility to infections, and cirrhosis-associated hyperdynamic circulation are among the consequences that are often thought to be caused by it[32]. The inflammatory cytokines, including pro-inflammatory cytokines like TNF-α and Transforming growth factor-β and anti-inflammatory cytokines like IL-10, contribute to the worsening of liver injury, according to experimental findings from animal models and human observations[33]. Animal studies on alcoholic liver disease, viral or autoimmune hepatitis, and other liver diseases have all shown that IL-10 is beneficial. In our work, pretreatment with A. marmelos and silymarin significantly decreased the elevated levels of TNF-α generated in acetaminophen-induced liver damage[34]. It is yet unknown if the hepatoprotective mechanism displayed by A. marmelos involves a decrease in anti-inflammatory cytokine levels, since IL-10 levels did not decrease[35]. Before we can determine the exact mechanism(s) responsible for the hepatoprotective properties of A. marmelos, more research is needed to understand the underlying mechanism.

CONCLUSION

Aegle marmelos fruit extract has hepatoprotective properties, according to the research summary. The presence of coumarins (such as marmelosin), flavonoids, tannins, and elevated amounts of rutin in the extracts suggests that the hepatoprotective benefits of A. marmelos fruit may be attributed to its antioxidant and anti-inflammatory qualities. In the group that received PCM, the levels of anti-inflammatory cytokines were considerably elevated after treatment with A. marmelos. Research has shown that A. marmelos fruit extract is just as effective as silymarin in protecting the liver. Combining A. marmelos fruit extract with fenugreek increased its hepatoprotective effects. Fenugreek seeds and A. marmelos fruit extract may have a synergistic effect, increasing the bioavailability of the latter through enhanced absorption or decreased metabolism of its paraphyletic components. Accordingly, in light of our study's findings, additional thorough investigations are required to determine A. marmelos's pharmacologically active components and to uncover other potential hepatoprotective modes of action.

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