

Hepatoprotective Study on *Aegle marmelos* Leaves Extract against *Staphylococcus aureus* Intoxicated Albino Rats

V. Ramamurthy* and R. Gowri

Department of Biochemistry, Marudupandiyar College, Vallam Post, Thanjavur, 613 403, Tamil Nadu, India

ABSTRACT

Objectives: The Liver is the largest organ in the body carrying out most of the biochemical synthesis and secretory functions. Living in a world of inadequately controlled environment, pollution and expanding therapy with potent drugs, it is continuously exposed to variety of xenobiotics and therapeutic agents resulting in its structural or functional damage. In an attempt to establish its pharmacological potential, we studied the Hepatoprotective activity of alcohol extract of *Aegle marmelos* obtained via extraction of air-dried leaves.

Methods: The rats were administered the ethanol extract (dose range of 100 mg/kg) orally for 15 days. The animals were randomly divided in to four groups, each containing three animals. They were subjected to the intraperitoneal injection of bacterial suspension at a dose of 5×10^6 CFU/0.1 mL once in every three days. Then the animals were treated with the alcoholic extract of *Aegle marmelos* daily for 15 days in physiological saline at concentration of 100mg/kg of body weight. The Silymerin (25mg/kg) was used as the standard control.

Results: At a dose of 100 mg/kg, the extract produced no sign of toxicity in rats. The extract exhibited significant ($p < 0.05$) dose-dependent Hepatoprotective activity for the *Staphylococcus aureus* induced model. In alcoholic extract of treated groups there was statistical significant decrease in the levels of serum bilirubin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase (SALP) as compared to the hepatotoxic group. In the liver by a significant reduction in the activity levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase and also reduced glutathione content were observed in the liver of *S. aureus* intoxicated rats over the control.

Conclusion: The alcoholic extracts of the leaves of *Aegle marmelos* possess Hepatoprotective activity, Anti stress, anti oxidant properties that may be due to cytoprotective mechanism.

Address for Correspondence

Department of
Biochemistry,
Marudupandiyar
College, Vallam Post,
Thanjavur, 613 403,
Tamil Nadu, India.

E-mail:

v.ramamoorthy07@gmail.com

These results support the ethno medical uses of the plant in the treatment of liver.

Keywords: Hepatoprotective activity, *Aegle marmelos*, Hepatotoxicity, *Staphylococcus aureus*, Enzyme activity, Biochemical studies.

INTRODUCTION

Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles¹. Liver functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. Additionally, it also handles the metabolism and excretion of drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating those².

During the recent past, there is a perception that, a parallel increase exists between bacterial infections and disorders of human health, posing a serious threat to public. Among different types of bacteria, *Staphylococcus aureus* is one of the hospital-borne pathogen which is implicated in hospital and community-acquired diseases. It is anaerobic opportunistic gram positive pathogen capable of causing pathology in virtually every tissue of the host. *S. aureus* is generally considered as non-invasive extracellular pathogen that damages the host cells at least in part in two ways, firstly either by adhering to the extracellular matrix of the cells and/or secondly by invading and persisting in the cells thereby interrupting signaling mechanisms. The persisting nature of the *S. aureus* suggests that it has ability to overcome host defense mechanisms and more over, colonization properties of

bacteria could explain its chronic effects including osteomyelitis and mastitis. Emergence of antibiotic (methicilin and vancomycin) resistant strains even becomes a huge threat to public health. Many studies indicated that *S. aureus* affects almost all mammalian hosts and causes a range of diseases including skin infection, nasal colonization, sepsis, renal failure, arthritis and endocarditis³.

Liver injury is a common feature of bacterial toxemia during sepsis condition which leads to the development of severe shock and multiple organ failure. Klintman *et al.*⁴ suggests that liver injury caused by *S. aureus* mediates Fas lignad, activated by reactive oxygen intermediates. Earlier it has been suggested that *S. aureus* has ability to induce free radicals in Swiss albino mice⁵. Nevertheless, it has been suggested that reactive oxygen species is also one of the factors associated with apoptosis of hepatocytes, studies related to the *S. aureus* infection and pro and antioxidant status in the liver tissue is poorly understood.

Liver diseases are serious health problem. In the absence of reliable liver protective drugs in allopathic medical practices, herbs play a role in the management of various liver disorders. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices and in traditional system of medicine in India. There is no satisfactory remedy for serious liver disease; most of the herbal drugs speed up the natural healing process of liver. So the search for

effective hepatoprotective drug continues⁶. Several Indian medicinal plants have been extensively used in the Indian traditional system of medicine for the management of liver disorder. Some of these plants have been already reported to strong antioxidant activity⁷⁻¹².

Aegle marmelos Correa (family Rutaceae) appears to be relevant and this plant is available in India, Bangladesh, Myanmar, Burma and Sri Lanka. The leaves, roots, bark, seeds and fruits are edible and have medicinal values. Its distribution is mainly within the sub-Himalayan forests, in dry hilly places ascending to 4,000 feet. It is called "Shivadume", the tree of Shiva. In Hindu mythology leaves and wood of *Aegle marmelos* are used to worship Lord Shiva. The medicinal properties of this plant have been described in the Ayurveda. In fact, as per Charaka (1500 B.C) no drug has been longer or better known or appreciated by the inhabitants of India than the Bael. Hindus also believe that goddess Lakshmi resides in Bael leaves. It is therefore widely cultivated and commonly found in the vicinity of temples¹³.

Aegle marmelos belongs to the family Rutaceae, commonly called as Bael (English), Vilvam (Tamil) and is found throughout India. Bael is a medium sized deciduous tree bearing strong axillary thorns and leaves with 3 or 5 leaflets. Bael leaves are extremely useful for treating diabetes, jaundice, cholera and asthma. Bael leaves are made into a poultice and used in the treatments of ophthalmic. Bael leaf poultice is applied to inflammations—with black pepper for edema, constipation and jaundice.

The decoction of the leaf and root bark is useful in intermittent fever, hypochondriasis, melancholia and palpitation of the heart¹⁴. The leaves and bark have been used in medicated enema. The leaves are also used in diabetes mellitus. The greatest

medicinal value, however, has been attributed to its fruit¹⁵ and the unripe fruit is said to be an excellent remedy for diarrhoea and is especially useful in chronic diarrhoeas¹⁵. The effectiveness of *A. marmelos* fruit in diarrhoea and dysentery has resulted in its entry into the British Pharmacopoeia. Moreover, Chopra¹⁶ has appropriately stated that "No drug has been longer and better known nor more appreciated by the inhabitants of India than the Bael fruit." Charaka has described this plant as a Rasayana. Therefore, in the present study was aimed to investigate whether injection of *S. aureus* induces oxidative stress and if so, *Aegle marmelos* leaf reduces the *S. aureus* -induced oxidative stress in the liver of rats.

MATERIALS AND METHODS

For the present study, the mature green leaves of *Aegle marmelos* belongs to family Fabaceae were collected from in and around area of Pattukkottai, Thanjavur District, Tamil Nadu, South India. The plant was identified with the help of manual of Tamil Nadu and Karnatic flora¹⁷⁻¹⁸ with standard references¹⁹.

Preparation of plant extract

The *Aegle marmelos* was collected, washed, cut into small pieces and dried at room temperature (28±1°C) for two weeks and made into powder for further analysis. Extraction is a process, to separate or isolate the secondary metabolites from plant material. It is basically two types i.e. heat and cold extraction. Heat extraction has some advantage over cold extraction like time consistency and also no contamination by microbes. An apparatus called soxhlet did heat extraction. 100g of the plant leaf powder were packed into the thimble of a soxhlet apparatus. The ratio of the plant powder and solvents were maintained at 1:4.

Bacterial strain

The test bacterial clinical isolate, *S. aureus* was collected from the Department of Biochemistry, Marudupandiyar College, Thanjavur. The preliminary confirmation and phenotypic studies were performed according to standard protocols by using gram staining and biochemical parameters including coagulase test and screened by growing on Baird-Parker selective Agar (Hi Media, India). After confirmation studies, the bacterial culture was grown in tryptic broth and incubated over night. The bacterial culture was then centrifuged at 10,000 rpm for 20 min and the pellet was resuspended and washed with sterile phosphate buffer saline (PBS). The absorbance was measured at 620 nm using a UV-spectrophotometer (Schimadzu) and the viable bacterial count was adjusted to approximately 1.0×10^9 colony forming units (CFU)/mL, which corresponds to an optical density of 1.6. Serial dilution was performed with PBS to get a final concentration $5 \times 10^6/0.1$ ml of bacterial suspension⁵.

Experimental design

The animals were randomly divided in to four groups, each containing three animals. Four groups (Group I, Group II, Group III and Group IV) of rats, three rats in each group were taken. Group-I: Served as normal, which received, feed and water only. Group-II: Animals in group 2, received single intraperitoneal injection of bacterial suspension at a dose of 5×10^6 CFU/0.1 mL once in every three days. Group-III: Single intraperitoneal injection of bacterial suspension at a dose of 5×10^6 CFU/0.1 mL once in every three days. Then the animals were treated with the alcoholic extract of *Aegle marmelos* daily for 15 days in physiological saline at concentration of 100mg/kg of body weight. Group-IV: Single intraperitoneal injection of bacterial suspension at a dose of 5×10^6 CFU /0.1

mL once in every three days. Then the animals were treated with Silymerin for 15 days at concentration of 25 mg/kg of body weight. The injection dose of bacteria and plant extract dose was based on earlier reports³. The experimental period for the present study was 15 days. After 15th days of herbal treatments, the animals were fasted for 12hours after the last dose of drug treatment and were scarified cervical decapitation under mild chloroform anesthesia. The blood was collected for serum separation. The organs were excised and they were washed in ice-cold saline until homogenized. Liver 10%, homogenate was prepared in 0.1ml Tris HCl buffer P^H 7.4. The serum separated by the centrifugation process and was used for following estimation.

Biochemical parameters

Biochemical parameters like serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) by the methods of Reitman & Frankel²⁰, alkaline phosphatase²¹, total bilirubin²² and protein²³ were analyzed. Reduced glutathione (GSH) was estimated using DTNB²⁴. The blood glutathione was estimated by the method of Beutler *et al.*²⁵. The catalase was determined by the methods of Aebi²⁶. The activity of superoxide dismutase was assayed by the method of Woolliams²⁷. The concentration of Thiobarbutiric acid reactive substances (TBARS) was measured in liver using the method of Ohkawa *et al.*²⁸.

RESULTS

The treatment with the extract did not decrease water and food consumption rats. The body weight of the rats treated with alcoholic extract once a day during 15 days (sub-acute treatment) did not show any significant change when compared with the control group, although had a tendency to

decrease body weight (100 mg/kg). This decrease can be associated with the decrease of liver weight at the dose of 100 mg/kg in comparison with the control group without any concomitant alteration in the activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Estimation of the serum activity of total bilirubin, protein, reduced glutathione, TBARS, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase is one of the most widely used means of measuring hepatocellular injury (Table 1).

Antioxidant activity of *Aegle marmelos* leaves extract was studied by LPO method and dose 100mg/kg produced significant antioxidant activity as shown in Table 1. The maximum antioxidant activity was exhibited at dose 100mg/kg in alcoholic extract of *Aegle marmelos*. The results of the present study suggests that co-administration of extract of *Aegle marmelos* ameliorates antioxidant status in *S. aureus* induced oxidative stress in the liver of rats as evidenced by decrease in the lipid peroxidation products and increase in the activity levels of antioxidant enzyme and reduced glutathione levels. There are changes in the level of GSH and TBARS in the liver homogenate of normal and experimental rats. The activity of GSH was observed to changes significantly in *S. aureus* intoxicated rats. This indicated that *Aegle marmelos* improved the enzymatic antioxidant status in rat liver since it is known that a marked increase in SOD and CAT activity can offer first line protection against the damaging effects of superoxide radicals in the liver.

DISCUSSION

The present study was carried out to evaluate the hepatoprotective activity of *Aegle marmelos* against *S. aureus* induced hepatocellular degenerative in albino rats.

The effectiveness of this medicinal plant was screened by assessing biochemical changes of different groups of experimental animals. *Aegle marmelos* possessed very high levels of alkaloids and flavonoids and are employed in medicinal uses. The plants studied here can be seen as a potential source of useful drugs. The results of biochemical parameters revealed the elevation of enzyme level in *S. aureus* treated group, indicating that *S. aureus* induces damage to the liver (Table 1). Liver tissue rich in both transaminases increased in acute hepatic diseases SGPT, which is slightly elevated by cardiac necrosis is a more specific indicator of liver disease^{6,29}. A significant reduction ($P < 0.005$) was observed in SGPT, SGOT, ALP, total bilirubin and protein levels in the groups treated with silymarin and extract of *A. marmelos*. The results confirmed that the enzyme levels were almost restored to the normal levels^{10,12}.

The present study was observed that *Aegle marmelos* has a significant hepatoprotective effect in *S. aureus* intoxicated rats that hepatocellular degenerative and necrotic changes are slight without advanced fibrosis and cirrhotic process in treated group. However, Ramamurthy and Raveendran¹¹ found that plants can prevent liver fibrosis and cirrhosis, suggesting that the medicinal plants protect liver against fibrosis possibly through immunomodulator and antioxidant activities. Ramamurthy and Abarna² observed that *Phyllanthus niruri* has a significant hepatoprotective effect in *S. aureus* intoxicated rats that hepatocellular degenerative and necrotic changes.

Liver is the most important and main part of the animal body. It is highly affected primarily by toxic agents and that is why the above-mentioned parameters have been found to be of great importance in the assessment of liver damage. The abnormal

high level of serum ALT, AST, ALP and bilirubin observed. In our study (Table 1) are the consequences of *Staphylococcus aureus* induced liver dysfunction and denotes the damage to the hepatic cells. Treatment with *Aegle marmelos* reduced the enhanced level of serum ALT, AST, ALP and bilirubin, which seem to offer the protection and maintain the functional integrity of hepatic cells.

Liver is the plays an important role in the protein synthesis. It is considerably affected when there is a disturbance in protein metabolism. The site-specific oxidative damage of some of the susceptible amino acids of proteins is now recorded as the major cause of metabolic dysfunctions during pathogenesis³⁰. Decrease in serum bilirubin after treatment with the extract in liver damage indicated the effectiveness of the extract in normal functional status of the liver. In the present study the lowered level of total proteins and bilirubin recorded in blood sample of *Staphylococcus aureus* treated rats reveals the severity of hepatotoxicity. In the *Aegle marmelos* treated group, the protein and bilirubin level of animal was almost normal. This result is support by stimulations of protein synthesis have been advanced as a contributory hepatoprotective mechanism, which accelerates the regeneration process and the protection of liver cell¹.

The results of the present study suggests that the administration of extract of *Aegle marmelos* ameliorates antioxidant status in *S. aureus* induced oxidative stress in the liver of rats as evidenced by decrease in the lipid peroxidation products and increase in the activity levels of antioxidant enzyme and reduced glutathione levels. Liver sepsis is a serious ongoing problem all over the world and *S. aureus* is also one of the major contributors of liver sepsis and consequently liver injury. It is well known that *S. aureus* produces a broad array of

virulence determinants which may be structural components of the bacterial cell envelope and adhesins or toxic enzymes, which are secreted extracellularly³¹. These virulence determinants are believed to mediate pathogenesis of bacteria including sepsis⁴. Studies of Klintman⁴ also reported that Fas ligand is one of the virulence factors responsible for liver injury caused by *S. aureus*. Further, studies of Weglarczyk *et al.*³² also suggested that *S. aureus* infection leads to release of reactive oxygen free radicals which is important for the activation of CD95 (Fas) - Fas ligand interactions thereby leads to apoptosis of monocytes. Thus, it is apparent that *S. aureus* induced effects are complex and at least in part mediates oxidative stress³³.

In general a balance exists between the generation of lipid peroxidation products viz., reactive oxygen species (ROS) and the level of endogenous antioxidants during physiological conditions which serve to protect tissue from oxidative damage. Disruption of this balance, either through increased production of ROS or decreased levels of antioxidants, results in a condition referred to as “oxidative stress”. Thus, evaluation of lipid peroxidation, antioxidant enzyme status of reduced glutathione content in biological tissue has been always used as markers for tissue injury and oxidative stress. Lipid peroxidation can cause changes in membrane fluidity, permeability and increase the rate of protein degradation, which eventually lead to cell lysis. It is well acknowledged that free radical scavengers such as SOD, CAT and GSH and metabolism regulatory enzymes such as GSH-Px, GR and GST can protect the cellular system from deleterious effect of free radicals³⁴. SOD, as a first line defense antioxidant enzyme plays an important role in the dismutation of superoxide and thereby leads to hydrogen peroxide which is eventually neutralized by GPx and catalase.

In the present study, there was a significant increase in the lipid peroxidation products with a significant decrease in the activity levels of antioxidant enzymes such as SOD and catalase, antioxidant content reduced glutathione in the liver of rats intoxicated with *S. aureus*. The decrease in the activity levels of SOD and catalase indicates accumulation of superoxide ions and also hydrogen peroxide ions, which might lead to an observed increase in the lipid peroxidation products in the liver of rats intoxicated with *S. aureus*.

ALT and AST are the specific markers to assess hepatocellular damage leading to liver cell necrosis. In present study ALT and AST activities were assessed as it is the more specific index of liver cell damage. High level of SGOT indicates liver damage such as due to cellular damage. SGPT catalyses the conversion of alanine to pyruvate and glutamate is released in a similar manner. Therefore SGPT is more specific to the liver and a better parameter for detecting liver damage¹. In the present study *Staphylococcus aureus* injection significantly increased serum ALT and AST indicating induction of hepatic damage. The extracts of *Aegle marmelos* at the dose of 100 mg/kg decreased the levels of both SGOT and SGPT. In the present investigation, it was observed that serum SGOT, SGPT and ALT levels were significantly reduced in animals receiving *A. marmelos* and Silymarin than those given *S. aureus* alone indicating that the degree of hepatic cell damage was lesser magnitude in treated groups.

CONCLUSION

Historically, *Aegle marmelos* (Bael) has been used for the number of ethnobotanical purposes. At present *Aegle marmelos* has become an important source of medicine for curing various human and animal diseases. Apart from exploring

possibilities to prepare standardized drugs by using different plant parts of *Aegle marmelos*. In conclusion, the results of present study demonstrate that extracts of *A. marmelos* has potent hepatoprotective activity against *Staphylococcus aureus* induced liver damaged rats. The extract is non-toxic even at relatively high concentrations. The hepatoprotective activity is probably due to the presence of flavanoids.

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Table 1. Effect of *Aegle marmelos* extracts on some biochemical and serum marker enzyme parameters in *Staphylococcus aureus* intoxicated albino rats

Parameters	Control	<i>Staphylococcus aureus</i> treated group	<i>Aegle marmelos</i> treated group	Silymarin (25 mg/kg) treated group
Bilirubin (mg/dl)	0.89 ± 0.25	2.60 ± 0.22	1.08 ± 0.23	0.98 ± 0.20
Protein (g/dl)	7.15 ± 0.29	5.78 ± 0.28	6.59 ± 0.35	6.95 ± 0.68
TBARS (n moles/ml)	2.75 ± 0.28	5.80 ± 0.33	3.10 ± 0.22	2.85 ± 0.35
GSH (μ mole/g of tissue)	8.65 ± 0.22	4.45 ± 0.26	8.12 ± 0.35	8.42 ± 0.26
SGOT (IU/L)	131 ± 0.33	195 ± 0.43	155 ± 0.98	139 ± 0.29
SGPT (IU/L)	45 ± 0.25	113 ± 0.22	55 ± 0.45	50 ± 0.36
ALP (IU/L)	125 ± 0.16	275 ± 0.65	165 ± 0.86	130 ± 0.18

Table 2. Effect of *Aegle marmelos* on Antioxidant status in the liver of *S. aureus* intoxicated rats

S. No	Treatment group	GSH (μ mole/g of tissue)	TBARS (n moles/ml)	LPO (μg/mg/protein)	SOD (μ mole/g of tissue)	Catalase (μ mole/g of tissue)
1	Control	9.50 ± 1.07	2.50 ± 2.04	0.46 ± 0.04	21.5 ± 0.05	10.2 ± 0.60
2	<i>S. aureus</i> treated groups	5.01 ± 0.02	4.90 ± 2.82	0.85 ± 0.02	13.7 ± 0.29	5.25 ± 2.91
3	<i>Aegle marmelos</i> treated groups	8.09 ± 5.06	3.82 ± 4.60	0.51 ± 0.04	19.8 ± 0.54	8.75 ± 1.25
4	Silymarin (25 mg/kg)	8.52 ± 0.06	2.85 ± 2.05	0.49 ± 0.05	20.2 ± 0.07	9.80 ± 1.45