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The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in swiss albino mice

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Abstract
Annona squamosa is a multipurpose tree with edible fruits and is a source of medicinal and industrial products. The alkaloids present in *Annona squamosa*, a medicinal plant has proved to have antioxidant activity. The present study has been designed to evaluate the hepatoprotective effect of custard apple *(Annona squamosa)* in Diethylnitrosamine (DEN) induced swiss albino mice. Analysis of selected biochemical parameters (Total protein, Glutamyl Oxaloacetate Transaminase (GOT), Glutamyl Pyruvate Transaminase (GPT), Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), Alpha Fetoprotein (AFP), Total and Direct bilirubin) in serum and tissue and also histopathological studies in liver are carried out in control and experimental mice. The levels of GOT, GPT, ALP, Total and Direct Bilirubin (both in serum and tissue), ACP, AFP (only in serum) are increased, and it is decreased in DEN induced along with *Annona squamosa* extract groups. But total proteins are found to decrease in DEN induced mice and increase in DEN induced along with *Annona squamosa* extract groups. Histopathology also confirms the hepato protective effect of *Annona squamosa.* So, our study has been recommended the herbal treatment of hepatoprotective effect using this plant for effective remedial. Further characterization and purification of the individual component in this plant is also suggested in formulating the strategy of treatment.

Keywords: *Annona squamosa*, Diethylnitrosamine, Biochemical analysis and Herbal therapy.

INTRODUCTION

Drugs are an important cause on liver injury. Drug-induced liver injury is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies (Michael *et al.*, 2005). The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8% - 30%) compaired to that in advanced countries (2% - 3%) with a similar dose schedule (Sharma 2004). The use of medicinal plants to treat human diseases has been performed for millenniums. Nowadays, it is known that 80% of the world population have already taken medicinal plants and 30% are prescribed by physicians (De Silva OC *et al.*, 2003).

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The curative properties of drugs are due to the presence of complex chemical substances of varied composition in one or more parts of these plants. These plant metabolites in one, according to their composition, are grouped as alkaloids, glycosides, corticosteroids, essential oils, etc. Presence of alkaloids (Mukhlesur RM *et al.*, 2005 and Farrell PHJ *et al.*, 1975), terpinoids (Farrell PHJ *et al.*, 1975), glycosides (Farrell PHJ *et al.*, 1975), flavanoids (Kotkar HM *et al.*, 2002), corticosteroids, essential oils has been reported in the plant *Annona squamosa*. The plant is described as anticytotoxic (Mukhlesur RM *et al.*, 2005 and Farrell PHJ *et al.*, 1975), antimicrobial (Kotkar HM *et al.*, 2002), antioxidant (Shirwaikar A *et al.*, 2004 and Kumar CD *et al.*, 2004), antipesticidal (Parthasarathi BVV *et al.*, 2005), antiheadlice (Tiangda CH *et al.*, 2000), anti HIV effects (Wu YC *et al.*, 1996) and vaso relaxant effects (Morita H *et al.*, 2006).

This plant *Annona squamosa* is commonly called custard apple in English and Sharifa in Hindi and sitaphalam in Telugu in India. ***Annona squamosa*** is a multipurpose tree with edible fruits and is a source of medicinal and industrial products and it possesses

potent bioactive principles in all it parts. Various phytochemical, pharmacological, antibacterial and antiovulatory studies have already been carried out with seed extraction. Ayurvedic practitioners use an extract from the stem and leaves as an indigenous uterotomic drug (Gupta RK *et al.*, 2005). The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, ruinting, worm infestation, constipation, hemorrhage, dysuria, fever, thirst, malignant tumors and ulcers and also as an abortifacient (Shirwaikar A *et al.*, 2004). The alkaloids present in *Annona squamosa*, a medicinal plant has proved to have antioxidant activity (Shirwaikar A *et al.*, 2004 and Kumar CD *et al.*, 2004). Aporphine alkaloids, terpine derivatives, glycoside and a novel diazepine, squamolone are isolated from this plant. An ethanolic extract of the leaves and stem is reported to have anti cancer activity (Farrell PHJ *et al.*, 1975). Annotemoyin-1, Annotemoyin-2, Squamocin and Cholesteryl glucopyranoside are isolated from the seeds of A*nnona squamosa*. These compounds and plant extracts has been showed remarkable antimicrobial and cytotoxic activities (Mukhlesur RM *et al.*, 2005). Flavonoid isolated from aqueous extract of *Annona squamosa* has been showed antimicrobial activity (Kotkar HM *et al.*, 2002). Bullatacin is one such compound that possessed antitumoral and pesticidal activity *invivo* (Ahmmadsahib KI *et al.*, 1993). *Annona squamosa* is said to show varied medicinal effects, including insecticidal, anti-ovulatory and abortifacient (Damaseeno DC *et al.*, 2002). So far there are no reports of clear biochemical and histopathological results indicating the hepatoprotective effect of *Annona squamosa* inducing DEN. So the present study has been designed to evaluate the hepatoprotective effect of custard apple *(Annona squamosa)* in Diethylnitrosamine (DEN) induced swiss albino mice. This would help to plan the strategy of treatment of hepatic problems using this plant.

MATERIALS AND METHODS

Preparation of leaf extract

Annona squamosa cultivation is most extensive in India and the fruit is exceedingly popular and abundant in markets. The plant material is collected, shade dried and powdered. 50 g of the dried powder has taken and mixed with 300 ml of anhydrate ethanol and kept at room temperature for 36 hours and the final extract has been taken using the soxlet apparatus (Kumar CD *et al.*, 2004).

Experimental induction of Liver injury in swiss albino mice

Selection of animals

Swiss albino mice weighing 30 to 40 g are purchased from Tamilnadu Veterinary and Animal Sciences University, Hyderabad. These mice are reared in animal house which has a well ventilated and lighted environment and also has all the facilities like racks, cages, washing and sterilization. 24 swiss albino mice weighing about 30 to 40 g are used as experimental animals. The animals are acclimatized in laboratory condition and randomly selected and also divided into 4 groups (Group I, II, III and IV), thus each group contains 6 animals. These mice are fed with normal rodent pellet diet for one month.

Treatment with Diethylnitrosamine (DEN)

A single 200 mg/Kg dose of Diethylnitrosamine (DEN) induces both extensive ethylation of liver DNA and cell necrosis, resulting in the generation of initiated hepatocytes eventually leading to hepatocellular carcinoma. The carcinogen has been administered at a standard dose of 200mg/Kg body weight, as an intra peritonial injection (Solt D *et al.*, 1976).

Group I

These groups of mice are served as control and these animals are fed with normal diet without any treatment.

Group II

The second groups are pre-treated with ethanolic extract of *Annona squamosa* at a concentration of about 5 g/Kg of body weight for 30 days.

Group III

The third group animals are treated as test animals. These animals are induced with DEN at a concentration of about 100 mg/Kg of body weight (dosage-two times, via Intra peritonial) to induce Liver injury and animals are pretreated with ethanolic extract of *Annona squamosa* about 5 g/Kg of body weight for 30 days.

Group IV

The fourth group animals are used as experimental animals. These animals are induced with liver damage by administrating DEN at a concentration of about 100 mg/Kg of body weight for two dosages has been given to animals via Intra peritonial (IP).

The grouping of animals is shown in Table 1 *[Supplementary data]*.

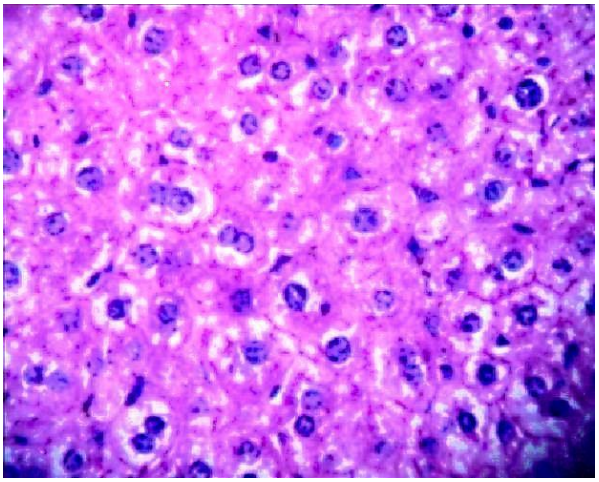

Figure 1: Normal diet animals: It shows a normal hepatic cell with well preserved cytoplasm, prominent nucleus and central vein.

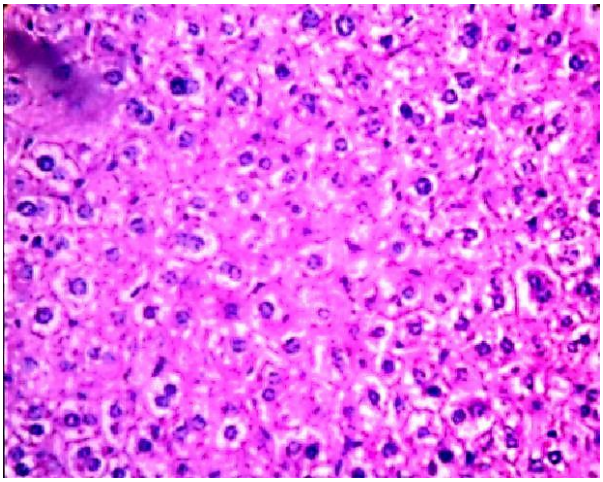

Figure 2: ***Annona squamosa*** **treated mice's**: It shows a normal lobular pattern with minimum pooling of blood in the sinusoidal spaces.

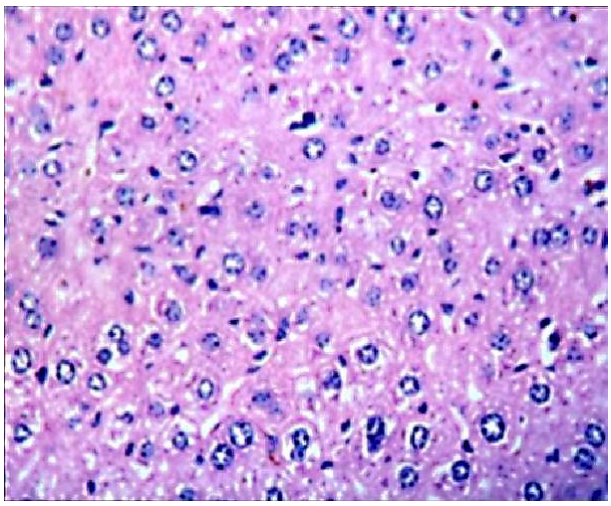

Figure 3: ***Annona squamosa*** **treated along with the induction of DEN**: It shows a recovered pattern of necrosis and blood pooling of sinusoidal space.

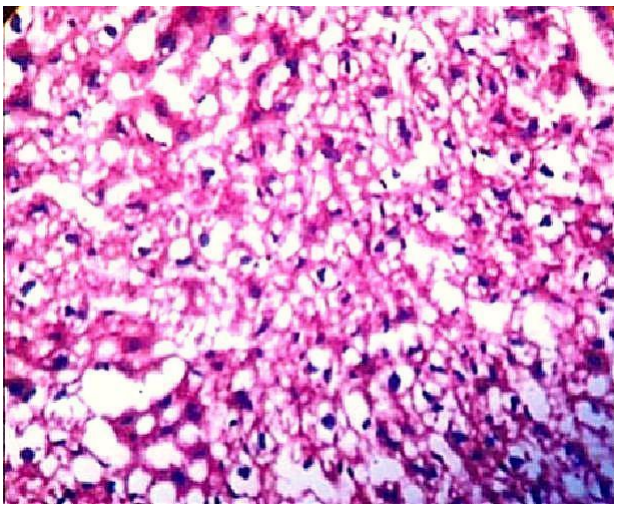

Figure 4: **Induction of DEN:** It reveals centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central venual.

Biochemical studies

After the treatment of the animals for 30 days duration, the animals are fasted overnight and weighed. Mice are sacrificed under chloroform anesthesia. They are bled by cardiac puncture and blood has been collected using insulin syringes. The obtained blood is allowed to coagulate and the serum is separated. Liver is quickly excised off, washed in saline, blotted dry and stored at 4°C. A known weight of liver tissue is homogenized using 0.1M phosphate buffer, pH 7.4; it is then centrifuged and used for various biochemical studies. Both the serum and liver tissue samples are subjected to further biochemical and tissue analysis.

Analysis of selected biochemical parameters

Following biochemical analysis parameters are analyzed in serum liver tissue.

Total protein level in both serum and liver tissue

Many plasma proteins, including albumin, fibrinogen and most globulins are formed in the liver. The plasma proteins level may be decreased; it causes some liver diseases and its dysfunction. The total proteins are estimated by Lowry's method (Lowry OH *et al.*, 1951 and Varley H *et al.*, 1991).

Serum and tissue Glutamyl Oxalo acetate Transaminase (GOT)

An enzyme that is normally present in liver and heart cells. Serum GOT (SGOT) is released into blood when the liver or heart is damaged. The blood SGOT levels are thus elevated with liver damage. Some medications

can also raise SGOT levels. SGOT is also called Aspartate aminotransferase (AST) (Walker PG *et al.*, 1972 and Varley H *et al.*, 1991).

Serum and tissue Glutamyl Pyruvate Transaminase (GPT)

Serum GPT (SGPT) is an enzyme that is normally present in liver and heart cells. It is released into blood when the liver or heart is damaged. The blood SGPT levels are thus elevated with liver damage. It is also called Alanine aminotransferase (ALT) (Walker PG *et al.*, 1972 and Varley H *et al.*, 1991).

Serum and tissue Alkaline Phosphatase (ALP)

It is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The alkaline phosphatase of normal serum in adults appears to be mainly derived from the liver. Increase in alkaline phosphatase activity occurs mainly in disease of liver and biliary tract (Walker K *et al.*, 1974 and Varley H *et al.*, 1991).

Serum Acid Phosphatase (ACP)

The determination of phosphatases can be helpful in monitoring any therapeutic response (Walker K *et al.*, 1974 and Varley H *et al.*, 1991).

Alpha Fetoprotein (AFP)

AFP can be particularly useful in early identification of liver injury and an aggressive tumors associated with hepatocellular carcinoma (HCC). Increased serum levels in adults are also seen in acute hepatitis, colitis and ataxia telangiectasia. The AFP level was estimated using ELISA kit method.

Bilirubin level in both serum and liver tissue

When the liver's function is impaired or biliary drainage blocked some of the conjugated bilirubin leaks out of the hepatocytes and appears in the urine. The presence of this conjugated bilirubin in the urine can be tested for clinically, and is reported as an increase in urine bilirubin (Varley H *et al.*, 1991).

Histopathological studies in liver

Animals are sacrificed to remove the liver. The liver has fixed in Bouin's solution for 12 hours, and then embedded in paraffin using conventional methods (Galighor AE *et al.*, 1976), cut into 5micrometre thick sections and stained using haematoxylin-eosin dye. The sections are then observed under microscope for degeneration, fatty changes, necrotic changes and evidence of hepatotoxicity if any. Results of the histopathological studies are shown in the Figures (Fig. 1 - Fig. 4).

Statistical analysis

Data are expressed as Mean, Median and standard Deviation. The significance of the difference between the means of the tests and control studies are established by applying the student's 't' test for independent samples.

RESULTS AND DISCUSSION

The results of the biochemical analysis are shown in Table 2 *[Supplementary data]*. The levels of GOT,GPT, ALP, Total and Direct Bilirubin (Both in serum and tissue), ACP, AFP (Only in serum) are increased in DEN induced mice and it is decreased in DEN induced along with *Annona squamosa* extract groups, but it still remains higher when compared to group I. Its level is decreased in group II when compared to group I. But only the amount of serum and tissue protein are decreased in DEN induced mice and it is increased in DEN induced along with *Annona squamosa* extract groups, but it still remains lower when compared to group I. Its level in group II is slightly increased when compared to group I.

Histopathological studies in liver of swiss albino mice

Group I: The liver sections of control animal are showed the normal hepatic cells with well-preserved cytoplasm, prominent nucleus and central vein (Fig. 1). Group II: The histological pattern of the liver of mice has pretreated with *Annona squamosa* extract shows a normal lobular pattern with minimum pooling of blood in the sinusoidal spaces (Fig. 2). Group III: The histopathological pattern of the liver of mice has pretreated with *Annona squamosa* extract along with DEN shows a minimal inflammation with moderate portal triaditis and their lobular architecture is normal (Fig. 3). Group IV: The liver section of DEN induced mice has revealed the centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central venual (Fig. 4).

Thus the results confirm the hepato protective effect of *Annona squamosa.* Based on the above findings from biochemical and histological studies, the plant, *Annona squamosa* is strongly recommended in the herbal treatment of hepatic problems for effective remedy. Further characterization and purification of the individual component in this plant is suggested in formulating the strategy of treatment.

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