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HEPATOPROTECTIVE ACTIVITY OF CAESALPINIA BONDUCELLA PLANT EXTRACTS IN AZATHIOPRINE INDUCED HEPATOTOXICITY IN MICE

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ABSTRACT

The present study has been designed to achieve the following aims and objectives.To evaluate the hepatoprotective activity of Caesalpinia bonducella on Azathioprine induced oxidative stress in mice. On the basis of our findings, it may be worthy to suggest that *Caesalpinia bonducella* has antioxidant activity against Azathioprine induced oxidative stress in mice by decreasing the oxidative stress biomarkers serum AST, serum ALT in liver *Caesalpinia bonducella* has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver tissue in standard and test extracts treated mice. *Caesalpinia bonducella* has hepatoprotective effect against Azathioprine induced toxicity in liver by observing the histopathological changes in mice liver tissue. In this study, using ascorbic acid as a standard, at 100mg/kg shows the ALT levels is 139.1 ± 1.28***when compared with standard,(EECB) - low - dose-100mg/kg shows ALT levels 157.9± 0.71** and EECB high dose-200mg/kg shows ALT is 145.7± 0.55***. Based on these results, 200mg of EECB shows good anti oxidant activity.

Key words: *Caesalpinia bonducella,* Azathioprine etc.

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INTRODUCTION

HEPATOTOXICITY

The liver is the largest gland in the human body. It is reddish-brown organ of unequal size and shape. It lies below the diaphragm in the abdominal-pelvic region of the abdomen. This organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production and detoxification. Its primary role is to control the flow and safety of substances absorbed from the digestive system before distribution of these substances into the systemic circulatory system[1].

The liver is divided into four lobes: Right, left, caudate and quadrate. Right and left lobes are the largest, while the caudate and quadrate are smaller and located posteriorly. A human liver normally weighs 1.44-1.66kg (3.2-3.7Ib). It is connected to two large blood vessels, one called the hepatic artery and one called the portal vein[2].

Hepatotoxicity may be predictable or unpredictable. Predictable reactions typically are dose related and occur which are exposed shortly after some threshold for toxicity is reached. Chemicals such as carbon tetrachloride, phosphorus, and chloroform fairly predictable hepatotoxins that are no longer used as drugs. Unpredictable hepatotoxic reactions occur without warning, are unrelated to dose, and have variable latency periods, ranging from a few days to 12 months[13,14].

Liver injury is defined as an alanine aminotransferase (ALT) level of more than three times the upper limit of the normal range, an alkaline phosphatase (ALP) level of more than twice the upper limit of normal, or a total bilirubin (TB) level of more than twice the upper limit of normal if associated with any elevation of the alanine aminotransferase or alkaline phosphatase level. Liver injury is further characterized as hepatocellular when there is a predominant initial elevation of the alanine aminotransferase level or as cholestatic when there

is a predominant initial elevation of the alkaline phosphatase level a mixed pattern comprises elevations of both the alanine aminotransferase and alkaline phosphatase levels. Recognizing the pattern of liver injury helps to categorize it, since drugs tend to create injury predominantly in one or another pattern.

Hepatocyte disruption: Covalent binding of the drug to intracellular proteins can cause a decrease in ATP levels, leading to actin disruption. Disassembly of actin fibrils at the surface of the hepatocyte causes blebs and rupture of the membrane.

Transport proteins disruption: Drugs that affect transport proteins at the canalicular membrane can interrupt bile flow. Loss of villous processes and interruption of transport pumps such as multidrug resistance associated protein 3 prevent the excretion of bilirubin, causing cholestasis.

Activation of Cytolytic T-cell: Covalent binding of a drug to the P-450 enzyme acts as an immunogen, activating T cells and cytokines and stimulating a multifaceted immune response.

Apoptosis of hepatocytes: Activation of the apoptotic pathways by the tumor necrosis factor-alpha receptor of Fas may trigger the cascade of intercellular caspases, which results in programmed cell death.

Mitochondrial disruption: Some drugs inhibit mitochondrial function by a dual effect on both beta-oxidation energy productions by inhibiting the synthesis of nicotinamide adenine dinucleotide and flavin adenine dinucleotide, resulting in decreased ATP production.

Bile duct injury: Toxic metabolites excreted in bile may cause injury to the bile duct epithelium.

Liver injury due to carbon tetrachloride in rats was first reported in1936 and has been widely and successfully used by many investigators. Carbon tetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl3O-,a reactive oxidative free radical, which initiates lipid peroxidation. Administration of a single dose of CCl4 to a rat produces, within 24 h, a centrilobular necrosis and fatty changes. The development of necrosis is associated with leakage of hepatic enzymes into serum.[22]

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$CCl_4 \rightarrow CCl_3O^- + O^-$

Thioacetamide interferes with the movement of RNA from the nucleus to cytoplasm which may cause membrane injury. A metabolite of thioacetamide (perhaps s-oxide) is responsible for hepatic injury. Thioacetamide reduce the number of viable hepatocytes as well as rate of oxygen consumption. It also decreases the volume of bile and its content i.e.bile salts, cholic acid and deoxycholic acid. Dose of thioacetamide: 100mg/kg, S.C.[23]

AZA is an important drug used in the therapy of autoimmune disorders and in preventing graft rejection. The nitro- conjugated double bond of imidazole ring of AZA is a Michael acceptor.AZA is cleaved in vitro to 6-MP non enzymatically by a nucleophilic attack of sulfhydryl groups primarily GSH, on the β carbon in the activated double bond. AZA toxicity to rat hepatocytes was preceded by depletion of GSH. Prior GSH depletion enhanced toxicity, while supplemental GSH was protective. In hepatocytes GSH is consumed during metabolism of AZA to 6-MP. The mechanism of AZA toxicity to hepatocytes involves depletion of GSH leading to mitochondrial injury with profound depletion of ATP and cell death by necrosis. Lipid peroxidations as well as altered levels of some endogenous scavengers are taken as indirect in vivo reliable ind

Initial Mechanisms of Toxicity Direct Cell Stress, Direct Mitochondrial Impairment and Specific Immune Reactions

Drug metabolites or less frequently parent drugs cause direct cell stress, target mitochondrial function, or trigger specific immune reactions. The drug metabolizing enzyme system responsible for the creation of hepatotoxic reactive metabolites is the polymorphic cytochrome P450 (CYP450) family that mediates oxidative phase-I drug metabolism. However, conjugative phase- II metabolism may also result in hepatotoxic metabolites, e.g. acyl glucuronides are well known to cause DILI. This Reactive metabolites can exert cell stress initially through a wide range of mechanisms including depletion of glutathione (GSH), or binding to enzymes, lipids, nucleic acids and other cell structures, they may also specifically inhibit other hepatocellular functions such as the apical (canalicular) bile salt efflux pump (BSEP, ABCB11 gene), which causes the intracellular accumulation of substrates may leads to secondary toxic hepatocyte

damage. In case of initial targeting of mitochondria, reactive metabolites or parent drugs uncouple or inhibit the mitochondrial respiratory chain causing ATP depletion and increased concentrations of reactive oxygen species (ROS), inhibit β-oxidation leading to steatosis(e.g.after intramitochondrial accumulation of amiodarone).[32]

METERIALS & METHODS

Collection and Authentification of Plant Material

The leaves of caesalpinia bonducella is collected and authenticated by Dr K Madhava Chetty, Department Of Botany, Sri Venkateswara University, Tirupati.

Extraction of Plant Material

The leaves are grinded in to a coarse powder with the help of suitable grinder.

Cold Extraction (Ethanol Extraction)[40,41]

In this work the cold extraction process was done with the help of ethanol. About 200gms of powdered material was taken in a clean, flat bottomed glass container and soaked in 750 ml of ethanol. The container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

Evaporation of Solvent(drying procedure)

The filtrates (ethanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vaccum dissecator for 7 days[42].

% Yield value of Ethanol Extract from Aerial Parts of *c.bonducella*Plant.

Powder taken for extraction = 200gm

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Weight of the empty china dish = 50.0gm

Weight of the china dish with extract = 90 gm

Weight of the extract obtained = (70 -50.0) gm

= 20

% yield of ethanol extract = (weight of extract)/(powder taken for extraction) × 100

= 20 /200 ×100 = 10%.

Preliminaryphytochemicalscreening[43,44]

Freshly prepared leaf extract of plant were tested for the presence of phytochemical constituents by using reported methods.

Acute toxicity studies[45]

The Acute oral toxicity test of the extracts was determined prior to the experimentation on animals according to the OECD (Organization for Economic Co-operation and Development) guidelines no 423. Female Albino Swiss mice (25-35 g) were taken for the study and dosed once with 2000 mg/kg. The treated animals were monitored for 14 days to observe general clinical signs and symptoms as well as mortality. No mortality was observed till the end of the study revealing the 2000 mg/kg dose to be safe. Thus, 1/10 and 1/20 doses of 2000 mg/kg i.e. 200 mg/kg and 100 mg/kg were chosen for subsequent experimentation.

Induction procedure[46, 47, 48]**:-**

Induction of oxidative stress:-

50mg/kg. of Azathioprine solution was given through i.p route to all the group of animals and the samples were collected from the animals through retro-orbital plexus root and the liver, kidney bio marker parameters were estimated like SGOT, SGPT.

Experimental design:-

The animals were assigned to five groups, each group containing six mice:

Group I: mice were orally administered with normal saline (1.2ml/day) for 21days as the normal control.

Group II: mice were administered with Azathioprine (50mg/kg. I.P)(single dose)

Group III: mice treated with *Caesalpinia Bonducella* (100mg/kg) by oral for 21days. administered with Azathioprine (50mg/kg. I.P)(single dose)

Group IV: mice treated with *Caesalpinia Bonducella* (200mg/kg) by oral for 21days. administered with Azathioprine (50mg/kg. I.P)(single dose)

Group V: group-II mice treated with ascorbic acid (100mg/kg) by oral for 21days. administered with Azathioprine (50mg/kg. I.P)(single dose)

Collection of blood samples and liver[16-18]:

Blood samples were collected from all the groups of animals 24hours after the 21st day of treatment through puncture of retro orbital plexus and were centrifuged at 3000 revolutions per minute (RPM) for 15 minutes. Serum was separated and stored at-20ºc and used for estimating SGOT, SGPT, levels. mice were killed by over anesthesia. A midline abdominal incision is made to open up the abdominal cavity and access the liver. The liver are removed rapidly and washed with saline. Then fixed quickly in formaldehyde. The liver was homogenized in 0.25 M cold sucrose solution and centrifuged at 5000 rpm for five minutes. The supernatant which is store at -20^{0}C used for the quantitative estimation of superoxide dismutase within 48hours by using UV spectrophotometry.

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Estimation of biochemical parameters:-

The following are the biochemical parameters estimated to evaluate the effect of the test materials against the experimentally induced oxidative stress in mice. They are SOD, ALT (SGPT), AST (SGOT).

Estimation of Superoxide Dismutase (SOD)[41,42]

Superoxide dismutases are the enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen.

The enzyme superoxide dismutase (SOD) was determined in erythrocytes using photo oxidation method, which is briefly described below.

Principle: In this assay free radicals are generated by photo-oxidation of o-dianisidine sensitized by riboflavin. The photo oxidation of O-dianisidine involves a complex series of free radical chain reactions involving the superoxide anion ($O_2^{\bullet -}$) as the propagating series (Figure 5.1). A general free radical scavenging compound has a inhibitory effect on this reaction leading to a decrease in the oxidized dianisidine measurable by UV/visible spectrophotometer. In contrast, any compound which specifically scavenges $O_2^{\bullet -}$ will remove the $O_2^{\bullet -}$ from step 3 and 4 in Figure 5.1 thus increasing the amount of oxidized dianisidine and hence will have an augmentary effect in this reaction. This assay can thus be used to determine whether a compound is a general, free radical or a scavenger specific for the super oxide anion. A substance with no free radical scavenging activity.

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RESULTS AND DISCUSSION

IN VIVO STUDIES

Evaluation of antioxidant activity of *Caesalpinia Bonducella* (EECB) using azathioprine induced oxidative stress in mice

SUPEROXIDE DISMUTASE:

Superoxide dismutase is class of enzyme that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide. It is an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide dismutase activity was estimated in tissue homogenate with help of pure bovine superoxide dismutase standard. The values were shown in below table, and figure.

Table 4: Standard graph values of superoxide dismutase

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SOD(µU)	Absorbance
0	0
1000	0.105
3000	0.231
6000	0.435
9000	0.632
12000	0.816
15000	1.012

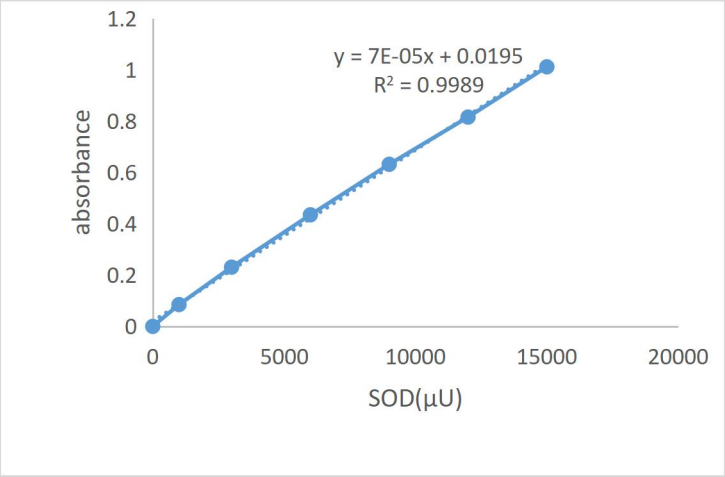

Figure 3: Standard graph of superoxide dismutase

Table 5: Superoxide dismutase levels in liver tissue homogenate

S.no	Group	Treatment	SOD(U/mg) in liver

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1	I	Normal group (saline)-1.2ml/day	8.75±0.17
2	II	Toxic control (AZP)-50mg/kg	1.58±0.05
3	III	(EECB) low dose-100mg/kg	4.75±0.08**
4	IV	EECB high dose-200mg/kg	6.57±0.071**
5	V	Standard ascorbic acid-100mg/kg	7.12±0.41***

All the values are expressed as mean ±SD (n=6); ** indicates p<0.001, *** indicates p<0.0001 vs toxic control.

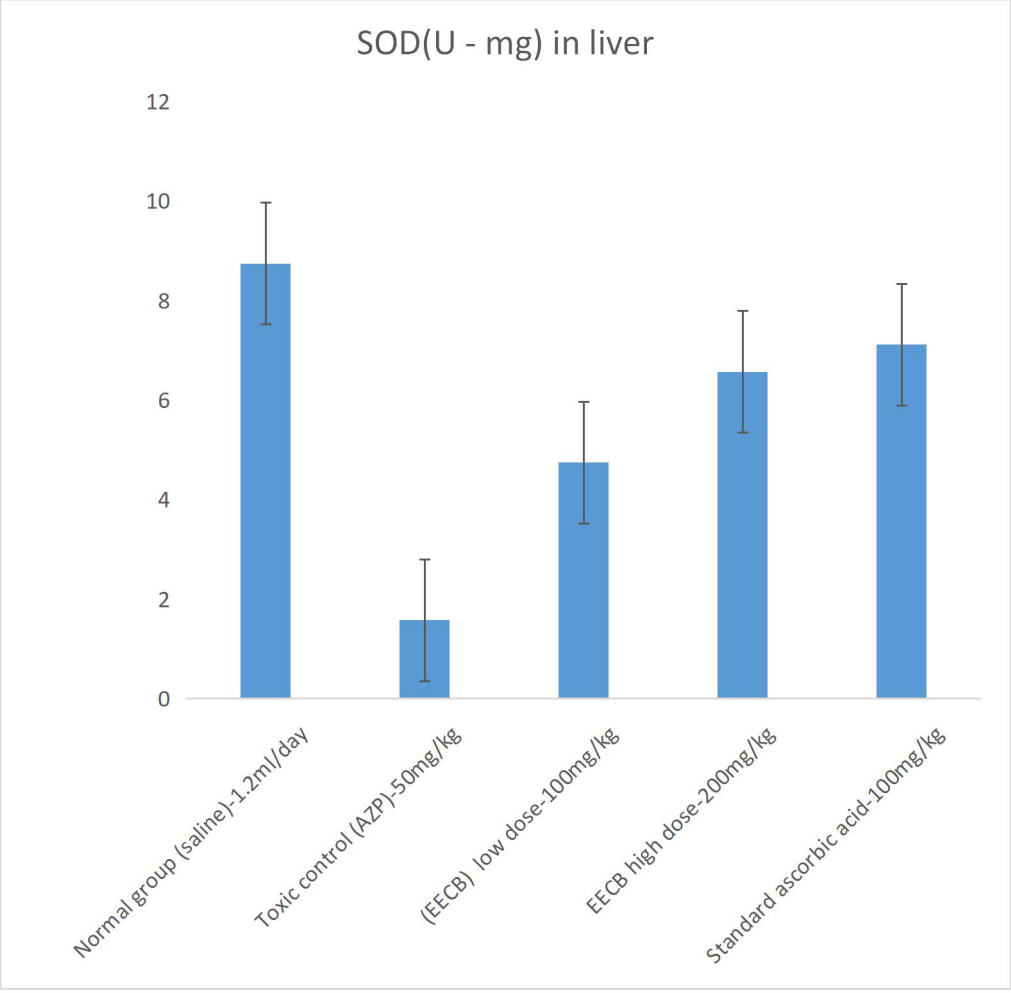

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Figure 4: Effect of EECB on superoxide dismutase levels in liver tissue homogenate in mice treated with AZP.

In this study, we found that 50mg/kg dose of azathioprine causes significant ($p<0.001$) decrease in superoxide dismutase levels. This reduction indicates that oxidative stress and toxicity is produced with azathioprine. Post treatment with EECB at the dose of 100mg/kg and 200mg/kg after a 50mg/kg dose of azathioprine administration, shown a significant ($p<0.001$, $p<0.0001$) dose dependent increase in levels compared to toxic control group.

SERUM ALANINE AMINOTRANSFERASE (ALT):

Table 6: Effects of test compound on serum ALT levels in mice treated with azathioprine

S.no	Group	Treatment	ALT (IU/L)
1	I	Normal group (saline)-1.2ml/day	136.65± 1.28
2	II	Toxic control (AZP)-50mg/kg	195.7± 1.5
3	III	(EECB) low dose-100mg/kg	157.9± 0.71**
4	IV	EECB high dose-200mg/kg	145.7± 0.55***
5	V	Standard ascorbic acid-100mg/kg	139.1 ± 1.28***

All the values of mean ±SD; (n= 6), ** indicates $p<0.001$, *** indicates $p<0.0001$ vs toxic control.

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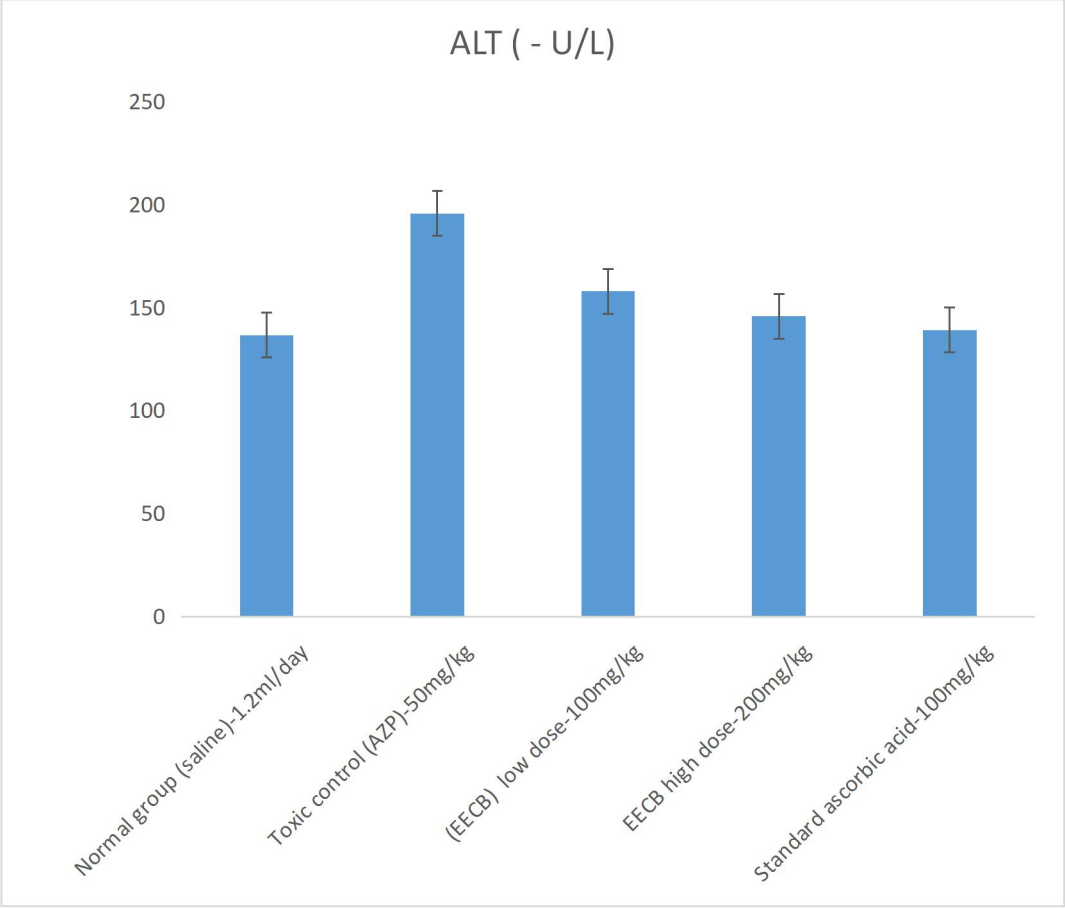
Azathioprine and test compound effects on ALT in mice from various groups shown in following figure. Measurements of ALT levels in AZP intoxicated female albino mice, and treated mice with EECB indicate the effect of treatment. The normal control group ALT level show 136.65± 1.28IU/L. After AZP treatment, the ALT level is 195.7± 1.5IU/L. This AZP treated group ALT level was increased compared to the normal control group in 21days. After 21days treatment, the test compound low dose ALT level was (157.9± 0.71IU/L) decreased compared to the toxic control group has shown significance (**p<0.001) and at high dose ALT level was (145.7± 0.55IU/L) decreased compared to the toxic control group has shown significance (***p<0.0001). On treatment standard ascorbic acid serum ALT level 139.1 ± 1.28, has shown significant (***p<0.0001).



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SERUM ASPARTATE AMINOTRANSFERASE (AST):

Table 7: Effects of test compound on serum AST levels in mice treated with azathioprine

S.no	Group	Treatment	AST(IU/L)
1	I	Normal group (saline)-1.2ml/day	139.7±1.43
2	II	Toxic control (AZP)-50mg/kg	227.15±1.49
3	III	(EECB) low dose-100mg/kg	159.0± 1.31***
4	IV	EECB high dose-200mg/kg	143.7± 0.92***
5	V	Standard ascorbic acid-100mg/kg	140.3± 1.06***

All the values of mean ±SD; n= 6, *** indicates $p<0.0001$ vs toxic control.

The above table shows the effect of test compound on serum AST levels in mice intoxicated with AZP. After 21days, the normal control group shows the AST level is 139.7± 1.43IU/L. In AZP control group level is 227.15± 1.49IU/L, increased compared to the normal group. Treatment with EECB at low dose AST level was (159.0±1.31IU/L) decreased compared to the toxic control group has shown significance (***$p<0.0001$) and at high dose AST level was (143.7± 0.92IU/L) decreased compared to the toxic control group has shown significance (***$p<0.0001$). On treatment standard ascorbic acid serum AST level 140.3±1.06, has shown significant (***$p<0.0001$).

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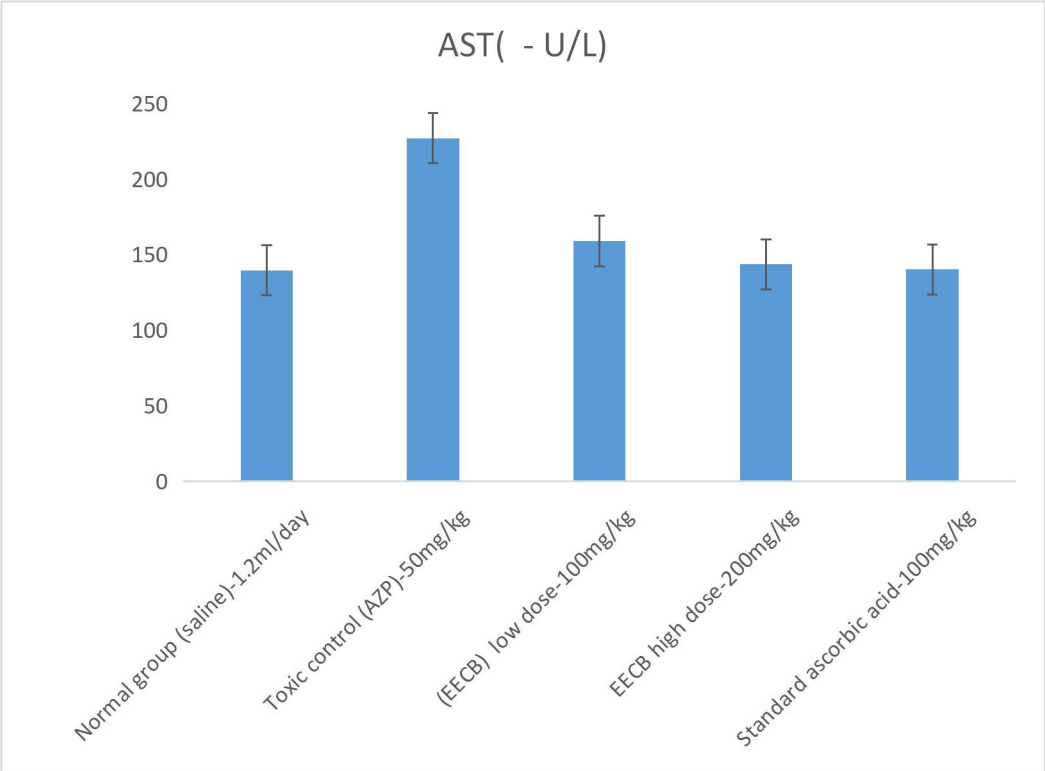

Figure 6: Effects of EECB on serum AST levels in mice treated with azathioprine

DISCUSSION

% Yield of the *Caesalpinia bonducella*is found to be **10.0**

Superoxide dismutase is class of enzyme that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide. It is an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide dismutase activity was estimated in tissue homogenate with help of pure bovine superoxide dismutase standard

In this study, we found that 50mg/kg dose of azathioprine causes significant ($p<0.001$) decrease in superoxide dismutase levels. This reduction indicates that oxidative stress and toxicity is produced with azathioprine. Treated with EECB at the dose of 100mg/kg and 200mg/kg after a 50mg/kg dose of azathioprine administration, shown a significant ($p<0.001$, $p<0.0001$) dose dependent increase in levels compared to toxic control group.

Azathioprine and test compound effects on ALT in mice from various groups shown in following figure. Measurements of ALT levels in AZP intoxicated female albino mice, and treated mice with EECB indicate the effect of treatment. The normal control group ALT level show 136.65± 1.28IU/L. After AZP treatment, the ALT level is 195.7± 1.5IU/L. This AZP treated group ALT level was increased compared to the normal control group in 21days. After 21days treatment, the test compound low dose ALT level was (157.9± 0.71IU/L) decreased compared to the toxic control group has shown significance (**$p<0.001$) and at high dose ALT level was (145.7± 0.55IU/L) decreased compared to the toxic control group has shown significance (***$p<0.0001$). On treatment standard ascorbic acid serum ALT level 139.1 ± 1.28, has shown significant (***$p<0.0001$).

the effect of test compound on serum AST levels in mice intoxicated with AZP. After 21days, the normal control group shows the AST level is 139.7± 1.43IU/L. In AZP control group level is 227.15± 1.49IU/L, increased compared to the normal group. Treatment with EECB at low dose AST level was (159.0±1.31IU/L) decreased compared to the toxic control group has shown significance (***$p<0.0001$) and at high dose AST level was (143.7± 0.92IU/L) decreased compared to the toxic control group has shown significance (***$p<0.0001$). On treatment standard ascorbic acid serum AST level 140.3±1.06, has shown significant (***$p<0.0001$).

the estimation of serum bilirubin also also proved that the higher doses of extract has hepatoprotective activity.

Effect of ethanolic extract of CB on histopathological examination of mice liver in azathioprine-induced hepatotoxicity. (a) Group 1 (normal): Showing normal histology of mice liver. (b) Group 2 (toxic control): N-Focal Necrosis, PTI-Extensive portal triad inflammation, CVC-

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Central vein congestion. (c) Group 3 (low dose): CVC-Central vein congestion, RH-Regenerating hepatocytes. (d) Group 4 (high dose): MCD-Mild central vein dilation, VMI-Very mild inflammation (e) group 5 (standard): nearly normal liver cells observed.

CONCLUSION

On the basis of our findings, it may be worthy to suggest that

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- *Caesalpinia bonducella* has antioxidant activity against Azathioprine induced oxidative stress in mices by decreasing the oxidative stress biomarkers serum AST, serum ALT and serum bilirubin in liver
- *Caesalpinia bonducella* has antioxidant effect, elevated by measuring antioxidant enzymes. There is increase in superoxide dismutase in liver tissue in Azathioprine induced oxidative stress in mices.
- *Caesalpinia bonducella* has hepatoprotective effect against Azathioprine induced toxicity in liver by observing the histopathological changes in mice liver tissue.

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