

Research Article



Evaluation of Anticancer Activity of *Sida cordifolia* L. against Aflatoxin B₁ induced Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma is the fourth common cancer globally occurring by many factors such as mycotoxins, chemicals, genetical, viral factors etc. AFB₁ is a potent mycotoxin classified in group I carcinogens by IARC found in contaminated food stuffs. The present study was aimed at evaluating the ethanolic extract of whole plant of *sida cordifolia* L against AFB₁ induced HCC. Hepatocellular carcinoma (hepatoma) was induced in wistar rats by intra peritoneal administration of AFB₁ (250µg/kg/dose) for 7 days. The effect of concurrent administration of ethanolic extract of *sida cordifolia* L. at doses of 250mg/kg and 500mg/kg were given orally was observed by estimating the serum levels of SGOT, SGPT, ALP, LDH, GGT and TP. DNA, RNA and TP were estimated from liver. Antioxidant and pro-oxidant studies were also measured. The results showed a significant decrease in serum SGOT, SGPT, ALP, LDH and GGT and increase in total proteins of groups treated with EESC. Elevated DNA and RNA found decreased with a prominent increase in total proteins in liver of EESC groups. Significant increase in antioxidants and a decrease in lipid peroxidation were also observed. The extracts at both doses (250mg/kg and 500 mg/kg b.w) shows a significant (p<0.001) reversal of altered biochemical, molecular and antioxidant parameters showing that *sida cordifolia* L. possess antioxidant and antitumor activity.

Keywords: Aflatoxin B₁, Hepatocellular carcinoma, *Sida cordifolia* L, Silymarin.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant neoplasm of hepatocytes and constitutes more than 80% of primary malignant liver neoplasms in the world.¹ It is the fourth most common cancer in world.² Global incidence of HCC accounts for 5-6% of all cancers in humans with more in males than females. Exposure to contaminated food with mycotoxins such as Aflatoxin B₁ (AFB₁), Fuminosin B₁(FB₁), T-2 toxins and some chemical carcinogens like diethyl nitrosamine, N-Nitrosobis (2-oxopropyl) amine etc are found to be risk for developing HCC. AFB₁ is a mycotoxin metabolite obtained from the *Aspergillus flavus* and *A. parasiticus* found in food stuffs, oil seeds, corn, rice and peanuts which are stored improperly.³ Aflatoxin B₁ is considered as a potent carcinogen classified by the International Agency for Research on cancer (IARC) and is a genotoxic hepatocarcinogen that cause HCC. AFB₁ is metabolized to AFB₁ 8, 9-epoxide by hepatic microsomal enzymes (CYP 450) that forms AFB₁-N7 guanine adducts leading to genetic changes that cause DNA strand breakage and oxidative damage that may lead to HCC.⁴

Sida cordifolia L is also called Indian Ephedra or Bala in ayurveda belongs to Malvaceae family. It is a perennial shrub; distributed throughout the tropical and sub-tropical plains all over India and srilanka up to an attitude of 1050m.⁵ The leaves, stems and roots are used in folk medicines treating diuresis, Inflammation, hypoglycemia.^{6,7} In ayurvedic system the plant is used for the treatment of Parkinson's disease and rheumatism.^{8,9} The preliminary phytochemical screening of *sida cordifolia* L. was reported to have flavonoids, glycosides, alkaloids, steroids and amino acids. Ephedrine

and Pseudoephedrine are the major alkaloids in the leaves and seeds of *sida cordifolia* L.¹⁰ various parts of the plant have been reported as anti-asthmatic, anti-ulcerogenic, antioxidant, hepatoprotective and CNS depressant.^{10, 11}

The present study was undertaken to evaluate anticancer activity of whole plant of *sida cordifolia* against Aflatoxin B₁ induced hepatocellular carcinoma.

MATERIALS AND METHODS

Collection and Authentication of plant

The whole plant of *sida cordifolia* L. was collected from the surrounding areas of Tirumala-Tirupathi and authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, S.V University, Tirupathi, Andhra Pradesh, India.

Preparation of extract

The parts of the plant were washed, shade dried and grounded to powder. The powder was passed through a sieve no.40 to get coarse powder. 100 grams of the powder was weighed and then subjected to soxhlet extraction using 70% ethanol (70:30) as solvent. The solvent was removed using rotary evaporator to obtain semi solid ethanol extract. The extract were dried to constant weight and used for phytochemical screening.¹²

Preliminary phytochemical screening

The extract was used for qualitative determination of phytoconstituents like alkaloids, flavonoids, glycosides, carbohydrates, saponins, amino acids, tannins and phenols.^{13, 14}



Production of AFB₁ from *Aspergillus flavus*

A toxigenic strain *Aspergillus flavus* was collected from Department of plant pathology, Regional Agricultural research station, Tirupati and the culture was maintained on PDA for further inoculation. A spore suspension of *Aspergillus flavus* @ 2.2 X 10⁵ spores in 0.01% Tween 20 was inoculated in to 250 ml conical flasks containing liquid media Yeast Extract Sucrose (YES). These cultures were incubated at 28± 1°c for 15 days. Toxin was extracted on 15 days and quantified by spectral analysis.

Extraction of toxin

Aflatoxin was extracted from the culture filtrate by solvent extraction procedure. The filtrate was defatted with n-hexane (60°-75°c) and Sodium Chloride (80 mg/ml) and this was added to it, prior to extraction of Aflatoxins with chloroform. Added half volume of chloroform to culture filtrate and chloroform extract was dried under room temperature, stored at 4° c until further analysis.

Thin Layer Chromatography (TLC)

TLC plates (Borosil) were freshly coated with silica gel –G (Qualigens) (0.75 mm thin) and activated at 110°C for 30 min and were used in Aflatoxin analysis. The dried chloroform extracts (toxin) were made up to 1 ml using Benzene: Acetonitrile (98:2). Different concentrations of the extract i.e., 2µl, 5µl, 10µl, 20µl and 30µl were spotted on the TLC plate along with the reference standards. The plates were developed in the solvent system viz., Toluene: Ethyl Acetate: Formic acid (6:3:1) and then observed on a U.V Transilluminator (365nm) to detect the presence of Aflatoxin by blue or green fluorescence. After developing the TLC plate, the illumination portion was cut and redissolves in methanol. Optical density was recorded under 220 to 500 nm against an appropriate blank using UV Spectrophotometer.

Experimental animals

Female wistar rats weighing 100-150gms were used for the study, the animals were acclimatized to a standard environmental conditions (temperature 25 ± 2° C and 12 h light/12 h dark cycle. The animals were given commercial pellet diet ad libitum and had free access to water. The experimental protocol was approved by Institutional animal Ethical Committee (Registered No. 1521/P0/a/11/CPCSEA).

Acute Toxicity Studies

Acute Toxicity Studies for ethanolic extract of *Sida cordifolia* L. (EESC) were conducted as per OCED: 423 guidelines using wistar rats. The defined doses of 5, 50, 300 mg/kg, 2000 mg/kg and 5000 mg/kg b. wt. were administered by oral route and observed for signs of toxicity for first 2 hours up to 14 days.¹⁵

AFB₁ induced HCC / experimental design

Group I Normal control received 0.5ml DMSO/ rat/ 7 days i.p

Group II Hepatoma control received a total of 7 doses of 250 µg/kg/dose¹⁶ for 7 days. The AFB₁ was dissolved in DMSO and administered i.p

Group III Standard control received silymarin at dose 100 mg/kg/b.w + AFB₁ pretreated.

Group IV Test I received ethanolic extract of *Sida cordifolia* L (EESC) at dose level (250 mg/kg/b.w/p.o.) from 7th day to 14th day +AFB₁ pretreated

Group V Test II received ethanolic extract of *Sida cordifolia* L (EESC) at dose level (500 mg/kg/b.w/p.o.) from 7th day to 14th day +AFB₁ pretreated

On 15th day, blood samples were collected through cardiac puncture after euthanizing rats. The collected blood was subjected to centrifuge and serum was used for estimation of biochemical parameters SGOT, SGPT (Reitman and Frankel method), ALP, LDH, GGT (Spectrophotometric methods) and Total Proteins (Biurets method). The livers were isolated and used for molecular DNA, RNA (spectrophotometric method) Total proteins (Lowry's method), antioxidant and pro-oxidative and histopathology studies.

RESULTS

Preliminary Phytochemical Screening

Preliminary phytochemical evaluation of whole plant of ethanolic extract of *Sida cordifolia* L. showed the presence of Alkaloids, Flavonoids, Tannins, Phenolics, Carbohydrates, Glycosides, Saponins, Steroids and Amino acids.

Acute Toxicity Studies

The procedure was followed according to OECD guidelines 423 in wistar rats. There were no signs of toxicity observed up to a dose level of 5000 mg/kg b.wt. The mortality rate was found nil and the herbal extract was found safe up to a dose level of 5000 mg/kg body weight.

Effect of EESC on Serum Biochemical Parameters

Table 1, shows a significant increase (P <0.001) in serum biochemical parameters SGOT, SGPT, ALP, GGT & LDH and decrease in serum total proteins in Aflatoxin B1 induced hepatoma bearing animals when compared to vehicle control. Treatment with ethanolic extract of *Sida cordifolia* L. (250 & 500 mg/kg b.wt) reversed the altered serum parameters compared to hepatoma induced group.

Effect of EESC on Tissue parameters

Table 2 shows a significant increase (P < 0.001) in DNA, RNA and pro-oxidant MDA levels and a significant decrease (P < 0.001) in tissue total proteins, antioxidant enzymes SOD and CAT in Aflatoxin B1 induced hepatocellular carcinoma group when compared to vehicle control. The pretreatment with EESC showed reduced levels of DNA, RNA and MDA levels and increased levels of total proteins, SOD and CAT.

Table 1: Effect of EESC on Serum biochemical parameters in Aflatoxin B1 induced Hepatocellular carcinoma

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	LDH (IU/L)	GGT (IU/L)	TP (mg/dL)
Vehicle 0.5ml/kg DMSO	94.4 ± 4.456	25.42 ± 3.37	181.8 ± 4.96	946 ± 21.9	42.9 ± 2.93	5.92 ± 0.50
Hepatoma 250µg/kg	225.9 ± 5.282	89.22 ± 2.988	358.5 ± 5.58	1738 ± 23.46	190.4 ± 4.46	3.82 ± 0.168
Silymarin 50mg/kg	151.2 ± 4.313***	37.58 ± 2.475***	255.8 ± 4.745***	869.5 ± 4.19***	50.1 ± 1.77***	5.86 ± 0.283**
EESC 250mg/kg	185.7 ± 4.503***	47.84 ± 1.956***	328.7 ± 3.851***	1066 ± 13.28***	86.54 ± 5.77***	5.36 ± 0.224*
EESC 500mg/kg	164.2 ± 3.866***	41.1 ± 3.2***	281.2 ± 4.30***	961.5 ± 10.4***	58.8 ± 5.13***	5.78 ± 0.54**

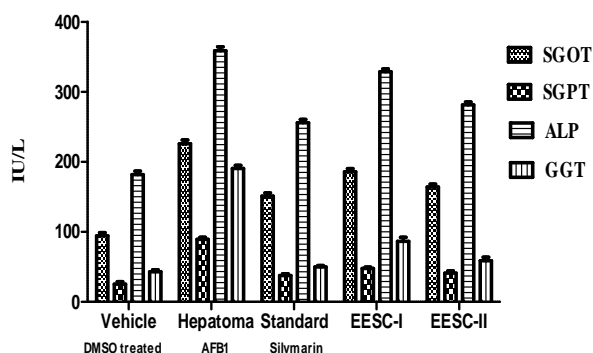
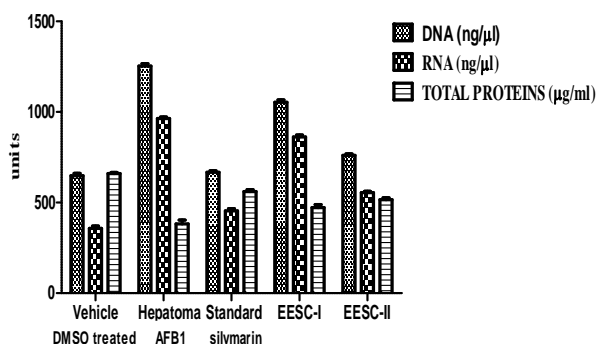
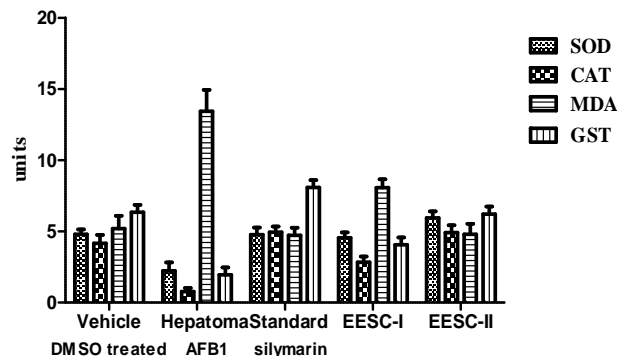
Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnetts Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05 indicates comparison with Hepatoma Control.

Table 2: Effect of EESC on Tissue Parameters in AFB1 induced Hepatocellular Carcinoma

Groups	DNA	RNA	Total Protein	SOD	CAT	MDA	GST
Vehicle 0.5ml/kg DMSO	648.4 ± 13.91	385.4 ± 12.36	660.2 ± 5.91	1.63 ± 0.34	0.36 ± 0.58	5.59 ± 0.91	0.74 ± 0.51
Hepatoma 250µg/kg	1254 ± 12.68	963.8 ± 7.83	382.2 ± 21.77	0.428 ± 0.58	0.17 ± 0.24	8.83 ± 1.5	0.35 ± 0.53
Silymarin 100mg/kg	667.6 ± 8.63***	454 ± 12.03***	561 ± 10.25***	1.365 ± 0.51**	0.346 ± 0.4***	6.32 ± 0.55***	0.68 ± 0.52***
EESC 250mg/kg	1054 ± 12.36***	862.4 ± 10.08***	472.4 ± 16.04***	0.74 ± 0.38**	0.24 ± 0.39*	7.87 ± 0.58***	0.46 ± 0.5*
EESC 500mg/kg	761 ± 7.31***	554.2 ± 7.55***	516 ± 10.31***	0.954 ± 0.44***	0.32 ± 0.51***	6.79 ± 0.73***	0.619 ± 0.49***

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnetts Multiple Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05 indicates comparison with Hepatoma Control

DNA-ng/µl; RNA-ng/µl; Total Proteins-µg/ml, SOD-U/mg protein; CAT-µmoles of H₂O₂/min/mg protein; MDA-µmoles of MDA/gm tissue/hr, GST-µmoles of thioether formed/mg protein/mi

Figure 1: Effect of EESC on serum biochemical parameters in AFB1 induced Hepatocellular Carcinoma**Figure 2:** Effect of EESC on DNA, RNA and Total Proteins in AFB1 induced Hepatocellular carcinoma**Figure 3:** Effect of EESC on Tissue parameters in AFB1 induced Hepatocellular carcinoma

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnetts Multiple Comparison Test at Significance value (P < 0.05).

Histological studies: The histological changes of different groups were shown in the Figure 4.

DISCUSSION

Liver diseases are a 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. However, we do not have 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 continues.

AFB1 produced from the YES media cultured fungi of *Aspergillus flavus* which was separated by solvent extraction method and TLC chromatography method further quantified by spectrophotometric method yielded 6.2 mg/ml. The obtained AFB1 was dissolved in DMSO at a concentration of 250 µg/kg/dose was used in inducing HCC.

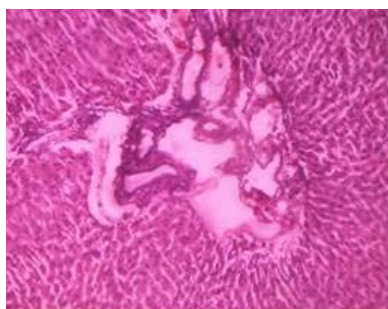
The use of rats as experimental animals for hepatoprotective activity is mainly due to the structural

homology of rat TNF and similarity in CYP 450 enzyme system.¹⁷

The present study was carried out inducing Aflatoxin B1 (AFB1) a metabolite of the mould *Aspergillus flavus*, most potent liver carcinogen for the experimental induction in rats.¹⁸ AFB1 is metabolized through CYT P450 enzymes to produce highly reactive intermediate AFB1 8, 9-epoxide along with generation of reactive oxygen species known to cause hepatocellular carcinoma.¹⁹ The metabolite forms adducts with DNA leading to activation of c-myc proto-oncogene,²⁰ and inactivation of p53 tumor suppressor gene which play an important role in tumor development.²¹



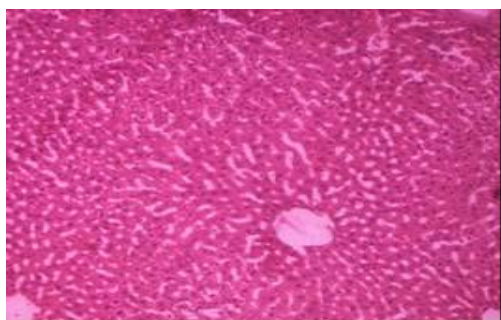
Group I showing normal architecture of hepatocytes with prominent central vein



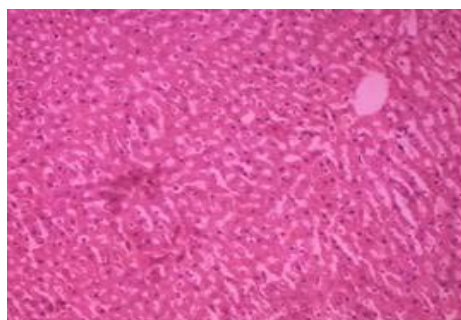
Group II showing ballooning of hepatocytes with focal necrosis and intense cytoplasmic granules



Group III showing restoration of architecture of hepatocytes with prominent central vein



Group IV showing inhibition of focal necrosis of hepatocytes with kupffer cells prominent



Group V showing restoration of architecture of necrotic hepatocytes similar to silymarin

Silymarin is naturally occurring polyphenolic flavonoid isolated from *silybum marianum* used medicinally for centuries as an herbal medicine in various liver related disorders. Silymarin offers liver protection against carcinogens through various mechanisms such as antioxidant activity,^{22,23} inhibition of lipid peroxidation,²⁴ inhibition of liver fibrosis by reducing/stopping the conversion of hepatic stellate cells into myofibroblasts,²⁵ inhibiting cyclin dependent kinases arresting abnormal cell growth, stimulation of hepatocyte regeneration through activation of ribosomal RNA polymerase initiating protein synthesis.²⁶

The assessment of liver function can be made by estimating the activities of various serum enzymes such as SGOT, SGPT, ALP, LDH and GGT. During hepatic damage, there may be increase in these enzymes levels in serum with the extent of liver damage.

SGOT & SGPT are the liver specific enzymes and are amino transferases that catalyze the inter conversion of amino acids and α-keto acids by the transfer of amino

group. These are sensitive and reliable indices for necessary hepatotoxic as well as hepatoprotective effect of various compounds and this tendency is also known to be distinct in rodents.²⁷ The elevated SGOT and SGPT levels in Aflatoxin B1 induced hepatocellular carcinoma is due to increased free radical membrane damage of liver cells and release of cytosolic enzymes into the serum which was in accordance with the report of Rocchi.²⁸ The EESC treatment at both dose levels showed a significant decline in the abnormal levels of SGOT/SGPT might be due to restoring the cell membrane stability and integrity of hepatocytes suggesting hepatoprotective activity which was shown in Figure 1.

Alkaline phosphatase is a membrane bound enzyme involved in transport of metabolites across cell membrane, protein synthesis, secretory activities and glycogen metabolism. ALP is used as a specific tumor marker in diagnosis of liver cancers.²⁹ Very high ALP levels was noticed in the serum of AFB1 induced hepatoma group may be due to the disturbance in the secretory

activity affected in the membrane permeability and produce derangement in the transport of metabolites³⁰ which was restored on treatment with ethanolic extract of *Sida cordifolia* and silymarin shown in Table 1.

γ -GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain, considered to be one of the best indicators of liver damage.³¹ It has important role in metabolism of foreign substances, cell growth and differentiation.³² A parallel increase in γ -GT activity with alkaline phosphatase activity is frequent in hepatocellular carcinoma. It is due to the tumor progression along with synthesis of γ -GT.^{33,34} The elevated level of γ -GT is predominantly reduced with EESC treatment in a dose dependent manner.

LDH is a sensitive marker in solid neoplasm.³⁵ There is a significant increase in serum LDH levels in AFB1 induced HCC rats indicating the over expression of glycolysis for survival of tumor cells which catabolizes large amounts of glucose during abnormal cell growth.^{36,37} The treatment with EESC using 250, 500mg/kg body weight and silymarin significantly decreased the levels of LDH when compared to hepatoma group presented in Table 1.

Decreased biosynthesis and secretion of protein in AFB1 induced hepatic carcinoma rats might be due to formation of Aflatoxin adducts with protein leading to denaturation of structural proteins and this is due to excessive intracellular Ca^{2+} homeostasis altering the metabolism leading to mitochondrial dysfunction of enzymes.³⁸ There is a marked rise in protein levels observed in EESC and silymarin treated rats indicating regeneration of hepatocytes stimulating the membrane attached ribosomes for protein synthesis.

The levels of DNA and RNA directly relate to the size of the tumor formed and are an indicator of tumorigenesis. However there is less elevation in RNA levels compared to DNA in AFB1 induced hepatoma groups. The decreased levels of tissue proteins in AFB1 induction due to degradation of proteins by decreased RNA polymerase synthetase.^{39,37} Animals treated with silymarin significantly increased the protein synthesis due to stimulation of ribosomal RNA leading to synthesis of proteins. The abnormal levels of DNA, RNA and Total Proteins were significantly restored upon treatment with EESC when compared to AFB1 induced group shown in Table 1 and Figure 2.

SOD initiates the conversion of superoxide radical to H_2O_2 and catalase converts H_2O_2 to H_2O . Depletion in the activities of these enzymes might be due to an enhanced free radical production inactivating the enzymes in Aflatoxin intoxicated rats.^{40, 41} This defense enzymatic activity of SOD and catalase was increased in rats treated with EESC at both dose levels and silymarin that might be due to the presence of polyphenols contributing to the antioxidant property compared to hepatoma control.

Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals.⁴²

The AFB1 treated rats significantly increased the lipid peroxidation which was measured by estimating its end product MDA levels. AFB1 might induce the generation of oxygen free radicals attack the cell membrane rich in PUFA, initiating a chain reaction leading to peroxidation of fatty acids.⁴³

Malondialdehyde is one of products of lipid peroxidation that reacts with DNA to produce MDA-DNA adducts, which have been implicated in the induction of G→T transversions and A→G transitions⁴⁴ in sequences for genetic instability is emerging as a possible direct link between oxidative stress and human cancers.^{45,46} The declined levels of lipid peroxidation in EESC and silymarin treated rats contribute to the potential inhibitors of lipid peroxidation due to the presence of polyphenols like flavonoids and phenolic compounds as they are reported to exhibit antioxidant property.

Presence of flavonoids has been reported to protect cell membranes and lipoproteins from oxidative stress induced damage by stimulating the regeneration of vit C and Vit E.^{47, 48}

Drug metabolism plays a key role in detoxification of toxins leading to inactivation of metabolites that are responsible for oxidative stress and cellular damage. GST is a phase II soluble protein found in the cytosol and microsomes of liver cells that detoxify the unstable reactive metabolites and inhibit adduct formation.⁴⁹ The balance between the production of AFB1 8, 9-epoxide and its rate of inactivation of GST-conjugation determines the susceptibility to hepatocellular carcinoma.⁵⁰ There is a significant decrease in GST levels in AFB1 treated rats indicating reduced conjugation of formed AFB1 epoxide which lead to develop HCC in rats which was significantly induced by treatment with EESC and silymarin shown in Table 2 and Figure 3.

The histological studies reveals that the liver sections treated/induced with AFB1 showed a marked focal necrosis with balloon degeneration of hepatocytes accompanied by mononuclear infiltration. Congestion of central vein with intense cytoplasmic granules were found in liver cells due to nuclear aggregation might be due to protein and DNA adduct modifications lead to ultra-structural changes when compared with normal showing normal architecture of liver cells with prominent kupffer cells.

Animals treated with silymarin showed a significant restoration of necrotic cells by maintaining the parenchymal cell membrane integrity whereas group IV and V animals treated with EESC preserved the architecture of liver cells by reducing the ballooning of cells along with prominent kupffer cells and nucleated cells exhibiting anticancer activity.

CONCLUSION

In recent times, there is an increased risk of malignancy of environmental pollution such as exposure to genotoxicity and carcinogenic chemicals. This has led to the



development of several preventive agents. In light of the continuing need for effective cancer chemo preventive agents, plants are used as important source that could produce potential chemotherapeutic agents.

In the present study, the ethanolic extract of *Sida cordifolia* L. was evaluated for anticancer activity against AFB1 induced hepatocellular cancers in wistar rats. There is a significant restoration of altered abnormal serum and tissue parameters exhibiting anticancer activity indicating protective effect of extract. The above findings are supported by the histological observations restoring the necrotic cells to normal architecture of liver.

From this it was concluded that the herbal extract of EESC at both dose levels (250 & 500mg/kg body weight) has a prominent role in showing anticancer activity and in a dose dependent manner this is due to the presence of phytochemical constituents like alkaloids, flavonoids, tannins and phenolic compounds present in it contributing to antioxidant mechanism against oxidative damage.

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