



ISSN Print 2231 – 3648
 Online 2231 – 3656

Available Online at: www.ijpir.com

HEPATOCELLULAR CARCINOMA OF METHANOLIC EXTRACTS OF *HIBISCUS CANNABINUS LINN* ON AFLATOXCIN B1 INDUCED RATS

*¹Sundarrajan T, ¹Edwin Singh S R, ¹Balakrishnan M, ²Vijey Aanandhi M
¹Seshachala College of Pharmacy, Puttur, A.P, India.

²Department of Pharmaceutical Chemistry, School of Pharmaceutical Science,
 Vels university, Chennai, T.N, India.

Abstract

The methanolic extraction of *Hibiscus cannabinus Linn* (MEHC) was pharmacological evaluation of Aflatoxin B1 (AFB1) induced hepatocellular carcinoma, AFB1 causes oxidative stress and cellular the enhanced formation of reactive oxygen species (ROS) and is associated with increases in biochemical parameters like serum glutamyl pyruvate transaminase (SGPT), serum glutamyl oxalacetic acid (SGOT), serum alkaline phosphatase (SALP), α -glutamyl transpeptidase (GGT), bilirubin, lipid levels as well as decrease in the levels of total protein and uric acid. MEHC was administered orally (100 & 200 mg/kg) for 14 days to hepatocarcinoma bearing rats. The levels of lipid peroxides and activity of enzymic antioxidants level were determined in liver homogenates. Marked increase in lipid peroxide levels and concomitant decrease in enzymic antioxidants levels were observed in carcinoma induced rats, while MEHC treatment reversed the conditions to near normal levels. Liver histopathology showed that MEHC reduced the Incidence of liver lesions, lymphocytic infiltrations and hepatic necrosis induced by AFB1 in rats. These results suggest that MEHC could protect liver against the AFB1-induced oxidative damage in rats, which may be due to its capability to induce the *in vivo* antioxidant system.

Keywords: Hepatocellular carcinoma, *Hibiscus cannabinus Linn*, Reactive oxygen species.

Introduction

Primary hepatocellular carcinoma (HCC) is a tumour of considerable epidemiologic, clinical and pathological interest. It is the sixth most common cancer worldwide and the third most common cause of death from cancer¹. Exposure to aflatoxins is probably also an important contributor to the high incidence of liver cancer. Aflatoxins are a group of structurally related secondary fungal metabolites that are carcinogenic, hepatotoxic, teratogenic and immunosuppressive.² Antioxidants

may protect against reactive oxygen species toxicity by the prevention of ROS formation, by the interruption of ROS attack, by scavenging the reactive metabolites and converting them to less reactive molecules. Flavonoids have been known to possess antioxidant property, antimutagenic and anticarcinogenic activity. Flavonoids also possess specific inhibitory action on AFB1-DNA adduct formation and they reflect their ability to afford

Author for Correspondence:

T Sundarrajan,
 Seshachala College of Pharmacy.
 Puttur, Chittoor District, A.P, India.
 E-mail: chemistrysundar@gmail.com

protection against development of AFB1-induced neoplasia in susceptible species.³

Hibiscus cannabinus (Malvaceae) is an annual or perennial herbaceous bush and has several forms with varying colors of flowers. It is native to China and grown widely as an ornamental plant throughout India. The flowers are considered emollient, and an infusion of the petals is used as a demulcent. Its decoction is given in bronchial catarrh in India. Previous studies show that the plant possesses anti-complimentary, antidiarrhetic and antiphlogistic activities⁵. The leaves and flowers have been found to be effective in the treatment of heart disorders. No reports are available on the antidiabetic activity of *Hibiscus cannabinus* leaves. Hence, the present study focuses on the scientific investigation of antidiabetic activity of *Hibiscus cannabinus* leaves.⁴

Materials and Methods

Chemicals

AFB1 (Sigma, St. Louis, Mo) was dissolved in dimethylsulphoxide (DMSO) immediately before administration. Reduced Glutathione (GSH), 1-Chloro-2,4-dinitrobenzene (CDNB), Glutathione Oxidised (GSSG), Epinephrine, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) was obtained from Sigma, Aldrich Ltd.

Animals

Adult Wistar albino male rats. Weighing 100-120g were used in the pharmacological studies. Animals were obtained from Nantha College of Pharmacy, Erode, Tamil Nadu. The animals were maintained in well-ventilated room temperature with natural 12h \pm 1h day. Night cycle in the polypropylene cages. India and tap water ad libitum throughout the experimental period. The animals were housed for one week, prior to the experiments to acclimatize to laboratory temperature. All the animal experiments were performed according to the rules and regulations of Animal Ethics Committee, Government of India.

Plant collection and Authentication

The plant material of *Hibiscus cannabinus* Linn leaf used for the investigation was collected from ABS botanical garden, Yercaud. The plant was identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC),

Chennai and a voucher specimen is kept in the Herbarium

Extraction

The leaves were dried under shade and powdered with help of mechanical grinder and made coarse powder. The powdered material was defatted with petroleum ether (40-60°C) and extracted with methanol by continuous Hot Percolation extraction. It was fractionated by n-hexane, chloroform, ethyl acetate and methanol. The methanol extract was concentrated using rotary evaporator and dried under vacuum.⁵ As the methanolic extract contains different types of phytochemicals, the methanolic fraction was found to be positive for the presence of flavonoids. So the methanolic fraction was used for further investigation.

Experimental groups

The rats were divided into five groups with six animals in each group and were given dose schedule as given below:

Group .I: Animals were given a single administration (p.o) of 0.5 ml vehicle (2% v/v aqueous Tween 80) and after 6 weeks, 0.5 ml of vehicle/day was administered (p.o) for next 14 days. This group served as normal control.

Group .II: Animals were given a single dose (i.p) of 0.5ml dimethyl sulphoxide (DMSO) and after 6 weeks, 0.5ml of vehicle /day was administered (p.o) for 14 days. This group served as DMSO control.

Group .III: Hepatocellular carcinoma was induced in these animals by a single dose of AFB1 in DMSO (2mg/kg body weight i.p). After 6 weeks, the animals were sacrificed by cervical decapitation and the hepatocellular carcinoma this group served as AFB1 intoxicated hepatocarcinoma control.

Group .IV: Hepatocellular carcinoma was induced in these animals by a single dose of AFB1 in DMSO (2mg/kg body weight i.p). Six weeks after the induction of AFB1, the animals were treated with 100 mg/kg bodyweight (p.o) of methanolic fraction of *Hibiscus cannabinus* Linn, For 14 days. On the 15th day, the animals were sacrificed by cervical decapitation and various biochemical parameters were analyzed.

Group .V: Hepatocellular carcinoma was induced in these animals by a single dose of AFB1 in DMSO (2mg/kg body weight i.p). Six weeks after the induction of AFB1, the animals were treated with 200 mg/kg bodyweight (p.o) of methanolic

fraction of *Hibiscus cannabinus* Linn for 14 days. On the 15th day, the animals were sacrificed by cervical decapitation and various biochemical parameters were analyzed.⁶

Biochemical analysis

At the end of the experimental period, animals were sacrificed by cervical decapitation under light ether anesthesia and blood was collected, serum was separated by centrifuging at 3,000rpm for 10 min.⁷ The above collected serum was used for the assay of marker enzymes, such as glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), serum total bilirubin, serum uric acid, serum total cholesterol, HDL cholesterol and total protein. Serum α -glutamyl transpeptidase (α -GT) was determined by the methods of Szasz 1969.⁸ The liver a 10% homogenate was prepared in Tris-HCl buffer (0.1 M, pH.7.4). The homogenate was centrifuged and the supernatant was used for the assay of total protein and cytoprotective enzymes, namely glutathione peroxidase (GPx) glutathione-Transferees (GST), glutathione reductase (GR), superoxide dismutase (SOD) catalase (CAT) and lipid peroxidation (LPO).^[50] All the enzymatic assays were taken at particular nm using Shimadzu spectrophotometer, UV-1601 model.^{9, 10}

Statistical analysis

Values are expressed as Mean \pm SD. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnet.s.t. Test. Comparisons were made between Group I and Group II, Group I and Group III, Group III and Group IV, Group III and Group

V. p values <0.05 were considered as significant

Results and discussion

Effect of MEHC on serum enzymes Level, bilirubin, uric acid α -GT and protein.

The effect of methanol fraction of *Hibiscus cannabinus* Linn on serum transaminases, alkaline phosphatase, bilirubin, uric acid, α -GT and total protein levels in AFB1 intoxicated rats are summarized in Table No. 01. Hepatic damage induced by AFB1 caused significant ($p < 0.001$) rise in marker enzymes SGPT, SGOT, ALP, bilirubin, and α -GT in Group III hepatocarcinoma bearing animals when compared with that of normal control group animals. Whereas treatment with MEHC at the doses of 100 and 200 mg/kg, p.o, significantly

($p < 0.001$) decreased the elevated serum marker enzymes, bilirubin and α -GT levels as compared with that of respective hepatocarcinoma control group. The uric acid and total protein levels were significantly decreased in AFB1 intoxicated rats. Administration of MEHC at tested dose levels reversed the altered uric acid and total protein to near normal levels as compared with that of hepatocarcinoma bearing control group. Comparison between Group I and Group II shows no significant Variation in marker enzyme levels, bilirubin, uric acid, α -GT and protein levels indicating no appreciable adverse side effect due to the administration of DMSO in Group II animals

Effect of MEHC on serum cholesterol and HDL cholesterol

It is clear that the total cholesterol level were increased significantly ($p < 0.001$) and HDL cholesterol level were decreased significantly ($p < 0.001$) in Group III hepatocarcinoma bearing animals, when compared with that of Group I normal control animals. Treatment with them ethanolic fraction of *Hibiscus cannabinus* Linn Linnat 100 and 200 mg/kg, p.o dose level significantly ($p < 0.001$) decreased the total cholesterol level, when compared with that of Group III hepatocarcinoma bearing animals. MEHC at 100 and 200 mg/kg dose level showed significant ($p < 0.001$) increase in serum HDL-cholesterol levels in AFB1 challenged rats. The DMSO treated group II did not show any significant difference in the level of total cholesterol and HDL-cholesterol, when compared to normal control group I.

Effect of MEHC on liver enzymic antioxidants

The effect of MEHC on the activities of enzymic antioxidants viz., superoxide dismutase, catalase, Glutathione peroxidase, glutathione-S-transferase and glutathione reductase in liver homogenates is shown in Table No. 02. The levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase were significantly decreased ($p < 0.001$) in Group III hepatocarcinoma bearing animals, when compared with that of group I normal control rats. Treatment with MEHC at doses 100 and 200 mg/kg recovered these decreased enzyme activities produced by AFB1 towards normalization in a dose dependent manner. Comparison between Group I and Group II shows

no significant variation in enzymic antioxidant levels indicating no appreciable adverse side effect due to the administration of DMSO in Group II animals.

Effect of MEHC on *invivo* lipid peroxidation

The localization of radical formation resulting in lipid peroxidation, measured as MDA in rat liver homogenate, content in the liver homogenate was significantly ($p < 0.001$) increased in AFB1

intoxicated group compared to normal control group. Treatment with MEHC resulted in significant decrease in the level of lipid peroxidation products (MDA) in AFB1 challenged rats. This observation leads to the inference that the MEHC treatment counteracts the abnormal increase in lipid peroxidation induced by AFB1. The DMSO treated group II did not show any significant difference in the level of lipid peroxides, when compared to normal control group I.

Table No. 01: Effect of methanolic fraction of *Hibiscus cannabinus* Linn on serum transaminases, alkaline phosphatase, bilirubin, uric acid, α -GT and total protein levels in AFB1 intoxicated rats

| Parameters | Group I (Normal Control) | Group II (DMSO Control) | Group III (AFB1 intoxicated) | Group IV (AFB1 + 100mg/kg MEHC) | Group V (AFB1 + 200mg/kg MEHC) |
|-----------------------|--------------------------------|-------------------------------|------------------------------------|--|---|
| SGOT (U/ml) | 42.71±4.81 | 42.33±4.12NS a | 176.81±7.83b* | 120.59±4.28c* | 87.46±5.21d* |
| SGPT (U/ml) | 48.16±3.24 | 51.24±4.14a | 135.92±7.48b* | 115.8±4.52c* | 76.32±3.42d* |
| ALP (KAunits) | 67.43±5.34 | 66.44±3.24a | 164.44±7.22b* | 122.45±5.60c* | 89.45±5.12d* |
| Bilirubin (mg/dl) | 0.63±0.18 | 0.66±0.16a | 2.50±0.35b* | 5.46±0.33c* | 0.98±0.22d* |
| Total protein (mg/dl) | 7.14±0.12 | 6.99±0.28a | 4.89±0.32b* | 5.74±0.43c* | 6.51±0.54d* |
| Uri acid(mg/dl) | 2.98±0.32 | 2.72±0.28a | 1.33±0.18b* | 2.11±0.23c* | 2.46±0.22d* |
| α -GT (mU/ml) | 28.62±3.26 | 29.48±4.12 | 192.51±7.21b* | 121.35±6.24c* | 85.32±5.14d* |
| HDL | 23.22±2.31 | 17.70 ± 5.10a | 24.10±4.11b | 24.30 ± 3.20c* | 25.21±34.12d* |

Values are given as Mean \pm SD of six animals in each group.

Comparisons were made between (a) group I and group II, (b) group I and group III, (c) group III and group IV, (d) group III and group V.

Statistical differences are expressed as * $p < 0.001$, NS-non significant.

Table No. 02: Effect of methanolic fraction of *Hibiscus cannabinus* Linn on activities of on antioxidant enzyme levels in liver

| Parameters | Group I Normal | Group II DMSO Control | Group III AFB 1 Intoxification | Group IV AFB 1+100mg MEHC | Group V AFB 1+200mg MEHC |
|------------|-------------------|--------------------------|--------------------------------------|------------------------------|-----------------------------|
| SOD | 7.21±0.49 | 6.97±0.11a NS | 2.16±0.12b* | 4.12±0.22c* | 5.42±0.33d* |
| CAT | 98.32±0.49 | 98.83±5.62aNS | 49.27±4.16b* | 64.15±4.48c* | 89.33±5.14d* |
| GPx | 5.17±0.38 | 4.96±0.29aNS | 1.33±0.12b* | 3.63±0.14c* | 4.38±0.21d* |
| GST | 19.84±1.63 | 20.22±1.46aNS | 12.13±1.12b* | 15.23±1.21c* | 17.16±1.13d* |
| GR | 0.812±0.06 | 0.83±0.04aNS | 0.23±0.01b* | 0.38±0.02c* | 0.61±0.03d* |
| LPO | 4.47±0.45 | 33.47±3.25aNS | 40.31±3.32b* | 8.19±0.22c* | 6.21±0.22d* |

Values are given as Mean \pm SD of six animals in each group.

Comparisons were made between (a) group I and group II, (b) group I and group III, (c) group III and group IV, (d) group III and group V.

Statistical differences are expressed as * $p < 0.001$, NS-non significant.

Conclusion

In conclusion, biochemical alterations observed in HCC status seem to be mainly due to an oxy radical mediated mechanism, involving LPO, under conditions of reduced antioxidant defenses. Recently more research has been focused on the role of flavonoids in cancer prevention because epidemiological investigations suggest that

increased intake of fruits and vegetables are associated with the reduced risks of certain cancers. In preliminary phytochemical studies the methanolic fraction of *Hibiscus cannabinus* Linn used in this study showed positive results for the presence of flavonoids, which correlates with earlier reports. In this study, the hepatoprotective effects of MEHC may be due to its ability to

scavenge free radicals and thus can prevent the AFB1-induced hepatotoxicity in rats by its antioxidant function which may be in part due to the presence of these flavonoids. Further studies on isolation of active constituent(s) present in the methanolic fraction of *Hibiscus cannabinus* Linn is under progress.

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