

Anti-Hepatocarcinogenic activity of a Polyherbal Formulation (Chathurmuka Chooranam) against Aflatoxin-B1 induced Male Wistar Rats

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Abstract: Hepatocellular carcinoma is one of the primary liver malignancies and is prevalent in developing countries. The present study was designed on evaluating the hydro-ethanolic extract of Chathurmuka Chooranam (CMC) herbal formulation against Aflatoxin-B1 (AFB1) induced hepatic carcinoma in Wistar strain. Hepatic carcinoma was induced in male Wistar rats by AFB1 (250 µg/kg/i.p) for 7 days. The administration of the polyherbal extract at a dose of 250 mg/kg and 500mg/kg were given orally for a period of 14 days. Estimation of enzymic antioxidants and non-enzymic antioxidants, Total proteins, DNA, RNA and LPO were measured. Elevated levels of DNA and RNA were observed in AFB1 induced rats when compared to the control rats. The administration of the polyherbal extract to the AFB1 treated group restored the normal levels of DNA, RNA and protein content. Lipid peroxidation was found to be decreased whereas increased levels of antioxidant enzymes were seen in polyherbal extract treated group when compared to the AFB1 group. From the present study, it might be concluded that the antioxidant potential of the polyherbal extract was responsible for its anti-hepatocarcinogenic potential.

Keywords: Chathurmuka chooranam, Aflatoxin-B1, Enzymic antioxidants, Nucleic acids, Proteins.

Introduction:

Liver is the vital organ for various metabolic pathways in the body. The induction by toxic agents or by infection causes abnormalities in liver which pave the way for the hepatic cancer. Liver cancer is one of the 5th most cancers. Aflatoxins are the primary consequence for Hepatocellular carcinoma (HCC) which results in deleterious effects to human beings with Hepatitis B, Hepatitis C infection and Cirrhosis. Aflatoxins are difuranocoumarins which are fungal secondary metabolites derived from three major genera of mycotoxins *Aspergillus sp.*, *Fusarium sp.*, and *Penicillium sp.*^[1].

A study by Van rensberg *et al.*, (1985) reported that the degree of food contamination by aflatoxin is directly proportional to the incidence of HCC^[2]. There are several types of aflatoxins out of which aflatoxin-B1 (AFB1) is the most potent. AFB1 itself is not mutagenic but it requires a bioactivation pathway to bind with DNA. Aflatoxin-B1 is metabolized by cytochrome p450 to form exo 8,9 epoxide. This covalent adduct formation by metabolically activate reactive intermediates with hepatocyte DNA causes mutations in the p53 gene on chromosome 17^{[3][4]}. The expression of p53 gene on Hep G 2 cell line by CMC formulation was already demonstrated by Revathi *et al.*, (2014)^[5]

Many anticancer drugs are available in the market and one of the main drug is Methotrexate (MTX). It is a folic acid antagonist used against several solid tumors (Breast, Liver), Leukemia and inflammation diseases. Widespread use of MTX causes a major side effect mainly hepatotoxicity^[6]. In the recent years, several plant derived chemopreventive agents have received considerable attention due to their pharmacological properties. These agents are composed of several antioxidant enzymes, secondary metabolites and phytochemicals which will repair the DNA mutation and rejuvenate the necrotic cells in hepatic tissues.

In traditional medicine, CMC formulation was used for several disorders, relieve toxicity from blood, antianemic property and so on. The herbal formulation contains several active secondary metabolites like Plumbagin, 3-chloroplumbagin, Hexadeconic acid, Curculigosides, Stearic acid and Asparagine. From these observations, the present study was sought to investigate the advantages of the formulation and its possible hepatoprotective role against aflatoxin-B1 induced hepatic carcinoma.

Materials and Methods:

Chemicals:

Aflatoxin-B1 was purchased from Sigma-Aldrich chemicals Co., USA. All other chemicals used were of analytical grade.

Preparation of the plant extract:

Poly herbal formulation contains *Semecarpus anarcadium* (seed), *Curculigo orchoides* (rhizome), *Asparagus racemosus* (root), *Plumbago zeylanica* (root) and *Tinospora cordifolia* (whole plant). The plants were authenticated in Botanical survey of India, Coimbatore and ABS botanical garden, Salem.

These plants are equally weighed, shade dried and coarsely powdered. 100 g of dried powder was cold macerated with 50 % hydro ethanol with occasional stirring for 3 days. After 3 days, the suspension was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature (<40⁰ C) under reduced pressure in a rotary vacuum evaporator. The yield of plant extract was found to be 9.64%. The sample was stored in an air-tight desiccator and used for further analyses.

Experimental protocol:

Male Wistar rats (100±120 g) were divided into 5 groups (n= 6). During the study, the animals received normal laboratory diet and water ad libitum. The rats were acclimatized to laboratory conditions for 10 days before commencement of the experiment. The clearance of the ethical committee for experimentation on animals was obtained from PSG institute of Medical Science and Research before the start of the experiment (Proposal No: 202/2013/IAEC).

Grouping of animals

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

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

Group III: Test received a total of 7 doses of AFB1 (250 µg/kg/ *i.p*) for 7 days and 250 mg/kg/*p.o* of hydro-ethanolic extract of CMC formulation from 7th day to 14th day

Group IV: Test received a total of 7 doses of AFB1 (250 µg/kg/ *i.p*) for 7 days and 500 mg/kg/*p.o* of hydro-ethanolic extract of CMC formulation from 7th day to 14th day

Group V: Received Methotrexate 0.5 mg/kg/dose/*i.p* for 7 days after AFB1 pre treatment.

Biochemical parameters:

The amount of DNA^[7], RNA^[8] and Protein^[9] in different groups were estimated. The activities of enzymic antioxidants like Superoxide dismutase (SOD)^[10], Catalase (CAT)^[11], Glutathione peroxidase (GPx)^[12], Glutathione reductase (GR)^[13] and Glutathione S transferase (GST)^[14] were carried out. The non-enzymic

antioxidants like Vitamin-C^[15], Vitamin-E^[16] and Glutathione^[17] were estimated. LPO was analyzed by the method of Nichans and Samuelson,(1968)^[18].

Statistical analysis:

The results were articulated as mean \pm standard deviation. Statistical analysis was carried between the experimental groups using one way analysis of variance (ANOVA) employing Statistical Package for Social Science (SPSS Version 1.6).Post hoc testing was performed for inter-group comparison using Duncan 's multiple range test. The level of significance was set as ($P < 0.05$)

Results:

Table: 1 depicts the variations in the DNA, RNA and protein content between the groups of animals. An increased level of Nucleic acids with a decreased level of protein was observed in the hepatocellular carcinoma induced group. In group-III and group-IV the levels of DNA and RNA were significantly ($p < 0.05$) decreased to near normal and also the protein content increased with the polyherbal extract treatment (250 mg and 500mg).But with the methotrexate treatment the levels were similar to hepatocellular control.

The activities of enzymic antioxidants were shown in Table-2.The activities of SOD, CAT, GPx, GST and GR were found to be dropped in the group-II animals. But in the polyherbal extract treated groups (III & IV) the activities were retrieved nearer to control group. The levels of these antioxidants in methotrexate group were significantly decreased when compared to normal group.

Table-3 comprises the non-enzymatic antioxidants (Vitamin-E, Vitamin-C and GSH) and Lipid peroxidation levels. Similar to enzymatic antioxidants, the levels of non-enzymatic antioxidants were found to be depleted in the AFB1 group which was revised in the groups III and IV. Lipid peroxidation level was found to be greater in the methotrexate treated group followed by AFB1 treated group. The LPO level returns to near normal after the treatment with polyherbal extract.

Table 1: Effect of CMC formulation on Nucleic acids and total protein content

Groups	DNA(mg/g)	RNA(mg/g)	Total protein(mg/g)
GROUP I	7.27 \pm 0.95	10.04 \pm 0.94	500.00 \pm 1.67
GROUP II	9.19 \pm 0.39 ^a	14.20 \pm 1.52 ^a	250.00 \pm 1.87 ^a
GROUP III	7.02 \pm 0.47 ^b	13.87 \pm 0.51 ^a	312.00 \pm 1.41 ^{ab}
GROUP IV	7.05 \pm 0.14 ^b	12.41 \pm 2.00 ^{ab}	364.33 \pm 1.21 ^{ab}
GROUP V	8.90 \pm 0.48 ^{ab}	14.57 \pm 1.22 ^{ab}	270.67 \pm 0.82 ^{ab}

Values are mean \pm SD (n=6)

a – Group I vs Group II, III, IV, V ; b – Group II vs Group III, IV, V. a, b are significant at 5% ($p < 0.05$)

Table 2: Effect of CMC formulation on enzymic antioxidants activities.

Groups	SOD(U/mg protein)	CAT(μ moles of H2O2 utilized per min/mg protein)	GPX(μ moles of GSH utilized per min/mg protein)	GR(n moles of NADPH oxidized per min/mg protein)	GST(n moles of CDNB utilized per min/mg protein)
GROUP I	2.30 \pm 0.98	1.28 \pm 0.85	5.19 \pm 1.28	0.17 \pm 1.89	0.79 \pm 1.62
GROUP II	1.41 \pm 0.54 ^a	0.14 \pm 1.20 ^a	2.86 \pm 1.85 ^a	0.07 \pm 1.63 ^a	0.36 \pm 1.95 ^a
GROUP III	1.80 \pm 1.52 ^{ab}	1.28 \pm 1.25 ^{ab}	3.85 \pm 1.15 ^{ab}	0.15 \pm 1.04	0.51 \pm 1.54 ^{ab}
GROUP IV	1.76 \pm 1.25 ^{ab}	1.30 \pm 0.91 ^{ab}	3.93 \pm 1.06 ^{ab}	0.18 \pm 1.48	0.52 \pm 1.68 ^{ab}
GROUP V	1.46 \pm 1.41 ^{ab}	1.16 \pm 1.42 ^{ab}	2.90 \pm 1.27 ^a	0.09 \pm 1.08 ^a	0.40 \pm 1.42 ^a

Values are mean \pm SD (n=6)

a – Group I vs Group II, III, IV, V ; b – Group II vs Group III, IV, V. a, b are significant at 5% ($p < 0.05$)

Table 3: Effect of CMC formulation on non-enzymic antioxidants and LPO

Groups	Vit-C(mg/g)	Vit-E(mg/g)	GSH (mg/g)	LPO(moles of MDA/g tissue)
GROUP I	1.54 ± 1.07	1.45 ± 1.12	1.46 ± 0.52	25.68 ± 0.45
GROUP II	0.83 ± 1.20^a	0.69 ± 1.52^a	0.64 ± 0.67^a	42.74 ± 0.70^a
GROUP III	1.09 ± 1.58^a	1.00 ± 1.02^{ab}	1.25 ± 0.54^{ab}	28.98 ± 0.64^{ab}
GROUP IV	1.15 ± 1.65	1.13 ± 1.32^{ab}	1.37 ± 0.84^{ab}	31.14 ± 0.96^{ab}
GROUP V	0.66 ± 1.56^a	0.71 ± 1.05^a	0.70 ± 0.91^a	44.38 ± 0.92^{ab}

Values are mean ± SD (n=6)

a – Group I vs Group II, III, IV, V ; b – Group II vs Group III, IV, V.a, b are significant at 5% (p<0.05)

Discussion:

Oxidative stress injury mainly targets the lipids, DNA and protein for their conjugative mechanism leading to cell damage. In tumorigenesis, the DNA content directly resembles the size of the tumor and it is an independent pointer of prognosis. The determination of DNA content was more important with regard to biological and functional aspects in proliferating tumor cells^[19]. A significant increase in the DNA content of AFB1 group might be due to the expression of enzymes present in the DNA of the tumor cells essential for the synthesis of AFB1-DNA adduct formation and the repression of many enzymes related to differentiated cell function^[20]. Moderate elevation in RNA was observed with response to DNA content which may involve in the transcription process. The present study was in accordance with the findings of Sharma *et al.*, (2011)^[21]. The polyherbal extract treated animals showed a significantly decreased levels of DNA and RNA which proves that the CMC formulation is a potent inhibitor of cancer cell proliferation.

The decrease in the protein content of AFB1 control may be due to the interlude in transcription process of hepatic protein metabolism in tumor cells. The reason behind is that AFB1-DNA adduct interrupt the protein synthesis^[22]. Moreover, it was observed that the combined effect of protein degradation with the formation of AFB1-DNA adduct resulted in a decreased amount of amino acid uptake by the experimental animals. Several studies have shown that AFB1 causes the depression of protein synthesis in the tested tissues^[23]^[24]. There is a marked rise in protein level observed in the plant treated and the MTX group indicating rejuvenation of hepatocytes stimulating the protein synthesis in membrane attached ribosomes.

A significant reduction in the activities of enzymatic antioxidants and non- enzymatic antioxidants could be due to an increase in lipid peroxidation in aflatoxin-B1 control rats. Cells are equipped with enzymatic antioxidant mechanisms that play an important role in the elimination of free radicals. These include free radical scavengers and the chain reaction terminator enzymes such as SOD, CAT and GPx system^[25]. Their function is to maintain a balance between reactive oxygen species and antioxidant defense mechanism. SOD protects the cells from damage by breaking the free radical superoxide anion into hydrogen peroxide and oxygen in which Catalase and GPx plays a potential role in the decomposition of hydrogen peroxide into water and respective alcohol^[26]

A reduction in the hepatic SOD level in the rats treated with AFB1 might be due to two possible mechanisms, one is by higher production of superoxide radical in mitochondria present in the cancerous hepatic cells or another by loss of mitochondrial-superoxide dismutase complex in the damaged hepatic cells^[27]. The reduction in the catalase activity in the AFB1 treated group might due to the free radical intermediates affects the production of enzymic antioxidants.

Catalase was more concentrated in peroxisomes and under high free radical input increases the autocatalytic process of ROS. Protection of mammalian cells from oxidative damage by GPx is by the reduction of hydrogen peroxide with the expense of the reduced GSH^[28]. When there is reduction in the availability of substrate GSH it directly affects the rate of GPx production in the AFB1 control animals. Moreover, it was noted that the activities of SOD, CAT and GPx increases nearly to normal after treatment with the formulation proves the presence of natural antioxidants in the formulation.

Glutathione transferase plays an important role in the protective mechanism against cell injury. It catalyzes the conjugation of reactive electrophilic agent to glutathione (GSH). GR is the key enzyme in maintaining the glutathione in reduced form rather than oxidized form. Liver GR was reduced in AFB1 treated

group confirmed the higher production of GSSG due to the higher rate of detoxification of GPx. The increased activities of GST and GR in the formulation treated rats might be due to the protective effect of the formulation against any singlet oxidation mediated injury, including tissue damage, owing to intracellular and extracellular response with hydrogen peroxide.

GSH detoxify the toxic chemicals and xenobiotic compounds like AFB1^[29]. AFB1 conjugates readily with GSH resulting in marked alterations in Ca⁺⁺ homeostasis. The excessive accumulation of Calcium ions cause the dysfunction of mitochondria and denaturation of proteins in AFB1 induced rats^{[30] [31]}. The presence of flavonoids and phenolic compounds in the CMC formulation have been reported by Revathi *et al.*, (2013)^[32]. The secondary metabolites have the capacity to increase GSH levels, modifying its redox rate and actively participating in the elimination of AFB1 metabolite^[33].

Vitamin-E is the lipid soluble peroxy radical scavenger of lipid peroxidation by terminating the chain reaction. In the present study, AFB1 control and MTX treated animals were found to have increased amount of lipid peroxides when compared to plant treated group due to the limited amount of Vitamin-E in the rats. The decreased amount of vitamins might be due to the excess utilization of the antioxidants for quenching of enormous amount of free radicals produced by ROS species. Vitamin-C is a water soluble biological antioxidant, and in the present study it was found to be significantly reduced in AFB1 control when compared to the formulation treated group because of its utilization to scavenge of free radicals produced by the cancerous cells or its synthesis may be affected by AFB1 metabolite^[34]. For free radical scavenging process, ascorbate is required which is obtained by the conversion of vitamin-C → dehydroascorbate → ascorbate^[35]. For this conversion reduced glutathione is required which was found to be reduced in AFB1 induced group which further significantly lowered the level of ascorbic acid.

LPO is an autocatalytic free radical process formed by the oxidative damage in the cells. The oxidative damage of plasma membrane and initiation of carcinogenesis starts with the oxidation of lipid peroxides in the cell membrane. Reactive Oxygen species formed in malignant tissues results in lipid peroxidation and subsequently it enhances the level of malondialdehyde (MDA) which is the major end product and index of lipid peroxidation^[36]. These levels arises to the formation of the metabolite, AFB1-8-9-epoxide which covalently bind to DNA to form AFB1-DNA adduct continuous to several mechanism to form tumorigenesis^[37]. This was evident in the present study where there is an increase of MDA level in the AFB1 induced rats and MTX group identifies the toxicity of the synthetic drugs. There was a significant decrease in the level of MDA in the poly herbal extract treated group. This may be due to the free radical scavenging action of flavonoids in the plant extract. Flavonoids possess free radical quenching activity and protect against lipid peroxidation by enhancing host detoxification system^[38].

From the present study, it was observed that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver tissue and decreasing the levels of enzymic and non-enzymic antioxidants. These results were consistent with other studies^{[39] [40]}. The increased MDA level observed in the present study can be attributed to the lipid peroxidation which can be induced by MTX itself or an increase in the ROS level by MTX induction.

Even at the minimal dose MTX have the ability to cause hepatotoxicity. The mechanism by which MTX causes hepatotoxicity due to its binding to the enzyme dihydrofolic reductase, thus preventing conversion of folic acid to its active form, folinic acid. This in turn blocks the synthesis of nucleic acids, certain amino acids and indirectly proteins which lead to damage of organelles and plasma membranes of hepatic parenchymal cells interfering with their function^[41].

Conclusion:

From the above results, it can be concluded that the biochemical changes studied in AFB1 induced hepatotoxicity is mainly due to the formation of oxidative stress. The herbal formulation (CMC) has a definite beneficial role against AFB1 induced hepatic carcinoma.

Conflict Of Interest:

The author declared no conflict of interest

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