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Anti-Hepatocarcinogenic activity of hydroethanolic leaf extract of *Ctenolepis garcinii* against Aflotoxin-B1 induced Male Wister Rats.

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Abstract: Hepatocellular carcinoma (HCC) most common liver cancer prevailing among people is due to Aflotoxin B1 (AFB1), a powerful mycotoxin. The current study was undertaken to evaluate the 50% hydroethanolic leaf extract of *Ctenolepis garicnii* Burm. against HCC which was induced in male wistar rats 250µg of AFB1/kg/*i.p*) for 7 days. The administration of the hydroethanolic leaf extract at a dose of 250mg/kg and 500 mg/kg were given orally for a period of 14 days. The levels of antioxidants nucleic acids and LPO were measured. Elevated levels of nucleic acids were observed in cancer induced male wistar rats when compared to the control rats. The administration of the herbal extract to the AFB1 treated group restored the normal quantity of DNA and RNA contents. Lipid peroxidation was found to be decreased near normal whereas of antioxidant enzymes activity were increased in treated group when compared to the AFB1 group. From the present study, it is concluded that the antioxidant potential of the hydroethanolic leaf extract was responsible for its anti-neoplastic potential.
Keywords: *Ctenolepis garcinii*, Aflotoxin B1, Enzymic antioxidants, Nucleic acids.

Introduction

Medically cancer is known as neoplasm causing autonomous growth of tissues[1]. Cancer has been a major cause of death, with increase mortality [2]. Among many types of cancers liver cancer stands in fifth place. The primary liver cancer accounting 70% of all liver cancer is hepatocellular carcinoma(HCC)[3]. People exposed to contaminated food with toxins like aflotoxin B1, T-2 toxins, fuminosin-B1 and chemical carcinogens like diethyl nitrosamine are risking their life to develop HCC. When food stuffs are stored improperly *Aspergillus flavus* and *Aspergillus parasticus* will grow releasing AFB1[4]. Aflotoxin B1 have been considered as potent carcinogen by The International Agency for Research on Cancer (IARC) and it is a genotoxic hepatocarcinogen that causes HCC. AFB1 is converted to AFB1 8,9-epoxide by hepatic microsomal enzymes (*CYP450*) that forms AFB1-N7 guanine adduct leading to DNA strand breakage and oxidative damage that causes HCC[5].

Many anticancer drugs like Mechlroethamine, Melphalan, Uracil mustard, Methotrexate, 6-Mercaptopurine, fluorouracil, Actinomycin D, flutamide etc are available. Methotrexate (MTX) is a folic acid antagonist used for solid tumors like breast cancer, liver cancer, Leukemia and in inflammatory diseases[6]. The

adverse side effects of MTX is mainly hepatotoxicity[7]. Other side effects includes Leukopenia, thrombocytopenia and megaloblastic anemia, diarrhea, intestinal ulceration, Alopecia due to damage of hair follicles. Even though they have many side effects, plants are the supreme sources of effective chemopreventive agents with many pharmacological properties which composed of secondary metabolites, antioxidant enzymes, phytochemicals[8].

There exists great significance in exploiting novel anticancer drugs from medicinal plants. Natural products discovered from medicinal plants are used to treat cancer as they have negligible toxicity. *Ctenolepis garcinii* comes under family cucurbitaceae. It is a climber, distributed in southern part of India and some parts of Ceylon[9]. *Ctenolepis garcinii* have been reported to have anticancer activity, antibacterial activity, antifungal activity and anti-inflammatory activity[10]. All these properties are due to the presence of several secondary metabolites like flavonoids, polyphenols and alkaloids. From these observations, the current study was undertaken to investigate the hepatoprotective role against the mycotoxin, Aflotoxin-B1 induced HCC.

Materials and methods:

Chemicals:

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

Preparation of the plant extract:

Fresh leaves of *Ctenolepis garcinii* was collected randomly in and around Kolli hills near Namakkal, Tamil Nadu, India. The plants were authenticated by Botanical Survey of India,Coimbatore, India and ABS botanical garden, located in Salem, India. The fresh leaves were washed under running tap water; air dried under shed for 3 days and were ground to fine power (each 100g) and stored in polythene containers at room temperature and further used. The sample was analyzed chemically to detect the phytochemicals. About 100g of the each powdered test material was placed onto a Soxhlet apparatus and fractionated sequentially in 200 ml of 50 % hydroethanol over 6-12 hrs and the extraction was continued until the liquid was clear. The extracts obtained was distilled and concentrated under the reduced pressure using rotaevaporator at 40°C to dryness and the residue was used for the biochemical analysis.

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 libtium. 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: 199/2013/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 hydroethanolic extract of CMC formulation from 7^{th} day to 14^{th} 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 hydroethanolic extract of CMC formulation from 7^{th} day to 14^{th} day.
Group V: Received Methotrexate 0.5 mg/kg/dose/*i.p* for 7 days after AFB1 pretreatment.

Biochemical parameters:

The amount of DNA[11], RNA[12] and Protein[13] in different groups were estimated. The activities of enzymic antioxidants like Superoxide dismutase (SOD)[14], Catalase (CAT)[15], Glutathione peroxidase (GPx)[16], Glutathione reductase (GR)[17] and Glutathione S transferase(GST)[18] were carried out. The non- enzymic

antioxidants like Vitamin-C[19], Vitamin-E[20] and Glutathione[21] were estimated. LPO was analyzed by the method of Nichans and Samuelson, (1968)[22].

Statistical analysis:

The results were articulated as mean ± 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:

The effect of hydroethanolic leaf extract on nucleic acids DNA and RNA in liver of experimental animals were presented in Table 1. The animals which are in group II bearing cancer show elevated levels of nucleic acids. On treatment with hydroethanolic extract of *Ctenolepis garcinii* in group III and IV animals these levels were significantly decreased. GR was found to be decreaed in group II animals. But in the hydroethanolic extract treated animals in group III and IV the activities were regained nearer to control group. The levels of these antioxidants in standard control methotrexate group animals were significantly decreased when compared to normal group.

Table 1: Effect of Hydroethanolic leaf Extract on Nucleic acids.

Groups	DNA(mg/g)	RNA(mg/g)
Group I	7.30^{a} ± .15275	10.07^{a} ± .10263
GroupII	9.88^{d} ± .08083	12.88^{e} ± .06658
Group III	7.81^{b} ± .07506	10.87^{b} ± .09713
Group IV	7.71^{b} ± .07550	10.11^{c} ± .12055
Group V	8.87^{c} ± .05292	12.55^{d} ± .11676

Values are mean ± SD (n=6). Each column not sharing a common superscript letter is significantly different at p<0.05 by Duncan's multiple range test.

Table 2: Effect of Hydroethanolic leaf extract on enzymatic antioxidant activity.

Groups	SOD (U/mg protein	CAT (μ moles of H_2O_2 utilised per min/mg protein	GPX (μ moles of GSH utilised per min/mg protein)	GR (nmoles of NADPH oxidized per min/mg protein)	GST (nmoles of CDNB utilized per min/mg protein)
Group I	2.29^{a} ± .09539	$1.26^{b,c}$ ± .10693	4.18^{c} ± .08083	0.17^{c} ± .02000	0.79^{c} ± .07000
GroupII	1.41^{d} ± .07506	0.15^{a} ± .04041	2.85^{a} ± .07024	0.07^{a} ± .01528	0.35^{a} ± .04041
Group III	1.89^{c} ± .10536	$1.22^{b,c}$ ± .06110	3.79^{b} ± .09866	$0.12^{a,b}$ ± .02082	0.57^{b} ± .07024
Group IV	1.97^{b} ± .09074	1.32^{c} ± .07024	3.89^{b} ± .10536	$0.16^{b,c}$ ± .03512	0.65^{b} ± .04509
Group V	1.47^{a} ± .10066	1.16^{b} ± .09504	2.90^{a} ± .09504	0.09^{a} ± .02517	$0.43^{a,b}$ ± .07371

Values are mean ± SD (n=6). Each column not sharing a common superscript letter are significantly different at p<0.05 by Duncan's multiple range test.

Effect of hydroethanolic leaf extract on non enzymatic antioxidants activity is presented in Table 3. Similar to the result of enzymatic antioxidants, the levels of ascorbic acid, tocopherol and glutathione were decreased in group II animals and this was reversed in the group III and IV animals treated with extract. Lipid peroxidation level is greater in Aflotoxin B1 induced group. This LPO level returns to near normal after treatment with hydroethanolic extracts.

Table 3: Effect of Hydroethanolic leaf extract on non enzymatic antioxidants activity.

Groups	Vit C (mg/g)	Vit E (mg/g)	GSH (mg/g)	LPO (moles of MDA/g tissue)
Group I	1.52^{d}± .07767	1.45^{d}± .07000	1.39^{b}± .06083	$26.25^{a,b}$± 2.2053
GroupII	0.84^{b}± .07095	0.68^{a}± .05568	0.65^{a}± .04041	42.9^{c}± 1.7559
Group III	1.30^{c}± .06000	0.91^{b}± .07095	1.41^{b}± .07095	27.85^{b}± 1.2371
Group IV	1.62^{d}± .06658	1.11^{c}± .06110	1.81^{c}± .07095	23.18^{a}± 1.7322
Group V	0.66^{a}± .05508	0.72^{a}± .05132	0.71^{a}± .07550	44.88^{c}± 2.2912

Values are mean ± SD (n=6). Each column not sharing a common superscript letter is significantly different at $p<0.05$ by Duncan's multiple range test.

Discussion:

The degree of malignancy in neoplasms are associated with the cells DNA content. Quantification of DNA in tumor cells infer the proliferating activity of tumor conditions[23]. The cellular components like DNA, lipids and protein content were affected by reactive oxygen species[24]. There is increase in DNA content in tumor cells because there is increase in enzyme expression required for DNA replication[25]. The ribonucleic acid content is increased in group II animals which suffer from cancer but not as significant as DNA. The RNA content is increased due to increased transcription. The results obtained parallels the findings of Sharma et al., [26] and Premalatha & sachdanandam[27]. Decreased levels of nucleic acid contents to near normal levels were observed in hydroethanolic leaf extract treated animals. This significantly proves that the *Ctenolepis garcinii* extract can be a potent inhibitor of cancer cell proliferation.

Cells are gifted with antioxidant mechanisms which play an important role in removing free radicals which includes free radical scavengers and chain reaction terminator enzymes[28].SOD converts superoxide radical to H_2O_2 which inturn is converted to H_2O by catalase. Depletion in the activities of these enzymes are due to enhanced free radical production in Aflotoxin induced tumor bearing rats[29, 30]. This defence enzymatic activity of SOD was increased in rats treated with hydroethanolic leaf extracts and in methotrexate. This may be due to presence of polyphenols supporting antioxidant properly compared to hepatoma control. The peroxisomes are rich in catalase when free radical content is high due to autocatalytic process of singlet oxygen. The level of catalase was quite decreased in cancer animals but activity got increased in treated animals with herbal extraction.

Glutathione peroxidase reduces H_2O_2 giving protection against oxidative damage caused due to autocatalytic process of ROS[31]. In group II animals there is great reduction in the enzyme level but the treated group III and IV animals show increased enzyme due to presence of natural antioxidants. Glutathione reductase (GR) is the key enzyme in maintaining the glutathione in reduced form. In the present study the liver GR got reduced in AFB1 treated group which confirms increased GSSH due to higher rate of detoxification of glutathione peroxidase. The increased activities of GST and GR in the treated rats is due to protective effect of the herbal extract against any singlet oxidation mediated injury, including tissue damage.

Vitamin C is one of the most effective antioxidant. It reduces the risk of diseases associated with oxidative stress like cancer[32]. In the present study vitamin C level got significantly reduced in AFB1 control. But the level become near normal in treated groups. This is because the vitamin C is utilized to scavenge free radicals produced by cancerous cells or the synthesis of ascorbic acid may be affected by AFB1 metabolite[33]. The ascorbate is required for free radical scavenging process[34]. This process requires reduced glutathione which got reduced in AFB1 induced group which is further significantly lowered the level of ascorbic acid.

Vitamin E is lipid soluble peroxyl radical scavenger of lipid peroxidation. Its level got reduced in AFB1 control which might be due to increased utilization of this antioxidant for quenching enormous quantity of free radicals produced in tumor state. But group III and IV animals show increased level than negative control which indicates there is control in cancerous growth. The same result was obtained by Vijaya Ravinayagam et al.,[35]. Thus, vitamin C and E could act synergistically in scavenging a wide variety of ROS. GSH plays an important role in the detoxification of xenobiotic compounds[36]. In tumor induced group II rats AFB1 conjugates with GSH which alters Ca^{2+} homeostasis, this cause accumulation of calcium ions causing dysfunction in mitochondria[37,38]. Hence its level got decreased. But presence of high levels of flavonoids and phenolic

compounds in hydroethanolic leaf extracts of *Ctenolepis garcinii*[39] eliminates AFB1 metabolite in group III and IV animals restoring GSH activity near normal. Also presence of phenolic compounds in the hydroethanolic extracts induces GSH levels which is similar to results obtained by Sharma *et al*., [40].

The free radicals damage tissues by lipid peroxidation[41]. In the group II animals the mycotoxin AFB1 forms AFB1 8,9-epoxide which causes membrane damage through lipid peroxidation and subsequent covalent binding to DNA to form AFB1 –DNA adduct, this leads to hepatocarcinogenesis[42]. Moreover AFB1 treated rats significantly increased lipid peroxidation by measuring the major end product malondialdehyde (MDA). AFB1 induces generation of oxygen free radicals which attacks the cell membrane rich in PUFA, which initiates a chain reaction leading to peroxidation of fatty acids[43]. Also MTX identifies toxicity of synthetic drugs. In the treated animals of group III and IV there is significant decrease in levels of MDA. This may be due to the presence of flavonoids in the plant extract. As mentioned by Chakraborty A et al [44], flavonoids possess free radical quenching activity and protect against lipid peroxidation by augmenting detoxification system. From the results obtained in group V animals, the MTX causes oxidativie tissue damage by increasing lipid peroxidation in liver. This result is same with results of other studies[45]. MTX even at its minimal dose have the ability to cause hepatotoxicity[46].

Conclusion:

The present study concludes that the hydroethanolic extract of Ctenolepis garcinii was evaluated for anticancer activity against AFB1 induced hepatocellular carcinoma in wistar rats. There is significant retrival of altered tissue parameters exposing anticancer activity which is mainly due to the formation of oxidative stress. The herbal extract of Ctenolepis garcinii at both dose levels (250 and 500 mg/kg body weight) has a prominent role in showing anticancer activity. This is due to the presence of phytochemical constituents like alkaloids, flavonoids, tannins and phenolic compounds present in it enabling antioxidant mechanism against oxidative damage.

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