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Kaempferol ameliorates aflatoxin B1 (AFB_1) induced hepatocellular carcinoma through modifying metabolizing enzymes, membrane bound ATPases and mitochondrial TCA cycle enzymes

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ABSTRACT

Objective: The present study was aimed to scrutinize the anticancer consequence of kaempferol against aflatoxin B1 induced hepatocarcinogenesis. Epidemiological studies of the incidence of liver cancer in the population, where dietary aflatoxin exposure is high, have provided much circumstantial evidence for the development of aflatoxin B1 induced primary liver cancer in humans. **Methods:** In the present investigation, aflatoxin B1 (2 mg/kg body weight i.p) was used as a hepatocarcinogen to induce hepatocellular carcinoma in experimental animals. **Results:** In the present analysis, on treatment with bioflavonoid kaempferol (100 mg/kg body weight p.o) the nucleic acids levels were brought back to normal and also the altered levels of biological enzymes such as membrane bound ATPase, carbohydrate metabolizing enzymes and mitochondrial TCA cycle enzymes levels ($P<0.01$). **Conclusions:** Membrane bound ATPase, carbohydrate metabolizing enzymes and mitochondrial TCA cycle enzymes were modulated by kaempferol evaluated on aflatoxin B1 induced primary liver carcinogenesis.

1. Introduction

Aspergillus flavus and *A. parasiticus*, are widespread fungi and members of the Aspergillus group, are aflatoxin-producing strains [1]. Aflatoxins (AF) are naturally notorious toxic secondary metabolites found on foods which contaminate cereal grains, nuts and are known for their impacts on human and animal healthiness mainly found in tropical regions of the world [2,3]. Out of the four major aflatoxins (B1, B2, G1, G2), B1 is usually found in the greatest concentrations. Aflatoxin B1 (AFB1), chemically classified as a furocoumarin, is known to be the most potent hepatocarcinogen [4,5]. AFB1 is exclusively produce by *Aspergillus flavus* which is most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma [6,7]. It is the most prevalent carcinogenic of the aflatoxins and has been classified as a class I human carcinogen by the International Agency for Research on Cancer [8]. The carcinogenic activity of AFB1 derives from metabolically activated reactive intermediates that covalent binds to hepatocellular DNA, which leads to mutations in the host genome. Hence, liver is responsible for many vital and complex functions in an organism and it is the target organ for the toxic effects of different compounds [9].

A variety of lines of indication suggest that nutrition and specific nutritional components, as well as dietary habits, in various parts of the world may play an necessary role in the causation and development of a number of important cancer types [10]. In fact, several cancer epidemiologists opine that minor changes in nutrition and lifestyle could tilt the balance in favour of anticarcinogenesis

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and dramatically reduce the incidence of cancer [11]. Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and a leading in cancer related deaths [12]. Epidemiology of HCC has distinct demographic and geographic variations, occurring mainly in Africa and Asia and incidence is also increasing in America and Europe in recent years [13]. Among several etiological factors HBV and HCV infections and AFB1 exposure are responsible for approximately 80% of all HCCs [14]. The development of primary HCC is a complex, multistep process [15] and experimental hepatocarcinogenesis induced by aflatoxin B1 (AFB1) is a favourite model as it facilitates the study of the mechanism of chemical carcinogenesis and response of HCC to anticancer drug therapy [16].

Plant extracts and plant derived biological products have been identified as excellent defender against the free radicals by triggering antioxidants [17]. Hence, natural antioxidants from plant sources have been viewed as promising therapeutic drugs [18]. Flavonoids are clusters of natural occurring polyphenolic biological products that are everywhere in vegetables and fruits, and are components in human diet and potent antioxidants [19]. Natural bioflavonoid like kaempferol has been isolated from tea, broccoli, grapefruit, and other plant sources know to possess several interesting biological functions such as anticancer, antiviral, anti-inflammatory and anticoagulant activities [20]. Consequently, the present investigation was undertaken to elucidate the therapeutic outcome of kaempferol with reference to the status of nucleic acids, membrane bound ATPases and mitochondrial TCA enzymes in aflatoxin B1 induced heptocellular carcinoma in experimental rats.

2. Materials and Methods

2.1. Chemicals

Aflatoxin B1 and Kaempferol were purchased from Sigma St Louis, MO, U.S.A. All other chemicals including solvents used were of high purity and of analytical grade.

2.2. Experimental Animals

Healthy adult Wistar albino rats of male sex weighing between (150±20) g were used for the study. They were obtained from the Central Animal House Facility, Dr.ALMPGIBMS, University of Madras, Chennai (IAEC No: 06/02/11). The animals were kept in polypropylene cages and received standardized rat pellet and water *ad libitum*. All the procedures were done in compliance with the guidelines issued by the Institutional Animal Ethics Committee.

2.3. Cancer induction

Aflatoxin B1 was used as a carcinogen for the present investigation. Hepatocellular carcinoma was induced by a single dose of 2 mg kg bw; i.p of AFB1 diluted in DMSO (1 mL) given using a gavage [21]. After 45 days of experimental period, all the animals were sacrificed for further analysis.

2.4. Experimental design

The animals were divided into four groups of six animals each. Group I – Control animals were administered orally with 1ml of Dimethyl sulfoxide (DMSO). Group II – Animals were administrated of Aflatoxin B1 (2 mg kg bw; i.p) in a single dose by dissolving in DMSO (1ml) to induce Hepatocellular carcinoma. Group III – After 45days, hepatocellular carcinoma bearing animals were treated with Kaempferol (100 mg kg bw p.o) dissolved in 1ml of DMSO for a period of 28 successive days. Group IV – Animals received kaempferol (100 mg/ kg bw p.o) dissolved in 1ml of DMSO for a period of 28 successive days.

2.5. Collection of Blood and tissue samples

At the closing stage of the investigational period, all the animals were sacrificed by cervical dislocation. Animals were fasted overnight before sacrifice. Blood was collected in tubes containing EDTA and centrifuged at 3000 rpm for 15 min. The buffy coat was removed and the packed cells were washed thrice with physiological saline. The washed cells were lysed by suspending in hypotonic buffer and centrifuged at 15000 *g* for 30 min. The resulting pellet is the erythrocyte membrane and the supernatant represent the hemolysate. Liver were perfused *in situ* with cold 0.15 M NaCl at 37 ℃.

2.6. Mitochondrial Isolation

Tissues were rinsed with ice-cold saline immediately after removal from the animal. A 20% homogenate was prepared using 0.02M Tris-HCl buffer containing 0.25 M sucrose. It was centrifuged at 5000 rpm for 10 min to remove nuclear fraction and the broken cell debris and then centrifuged at 12000 rpm for 10 min to sediment mitochondrial fraction. The resulting mitochondrial pellet was washed twice or thrice with 1.15% potassium chloride solution and finally suspended in 0.25 M sucrose solution. All operations were performed at 4 ℃. The mitochondrial fractions were used for the analysis of TCA cycle enzymes.

2.7. Biochemical analysis

Deoxyribonucleic acid (DNA) was estimated by the method of Burton [22]. Ribonucleic acid (RNA) was estimated by the method of Rawal *et al*., [23]. Na^+K^+-ATPase was estimated by the method of Bonting [24], The activity of Ca^{2+}-ATPase was assayed according to the method of Hjerten and Pan [25], Mg^{2+}-ATPase activity was assayed by the method of

Ohinishi *et al*., [26]. Hexokinase was assayed by the method of Brandstrup *et al*., [27], Phosphoglucoisomerase was assayed by the method of Horrrocks *et al*., [28], Aldolase and Glucose-6-phosphatease was estimated by the method of King [29], Fructose-1,6-diphosphatase was assayed by the method of Gancedo and Gancedo [30]. Estimation of Isocitrate dehydrogenase by the method of King [31], Succinate dehydrogenase was estimated by the method of Slater and Borner [32]. Malate dehydrogenase assayed by the method of Mehler *et al*., [33], α-ketoglutarate dehydrogenase by the method of Reed and Mukherjee [34].

2.8. Statistical Analysis

Data are presented as the mean±standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used to compare the means of different groups of by using SPSS 12.5 student's versions. Comparisons were made between group II and IV with group I and III for animal studies. $P<0.01$ was considerable statistically significant in all cases.

3. Results

The nucleic acids (DNA and RNA) in liver of control and experimental animals were presented in Table 1. In group II primary liver cancer bearing animals, the levels of nucleic acids were significantly elevated ($P<0.01$). On treatment with kaempferol these levels were significantly decreased in group III animals ($P<0.01$). On the contrary, no statistical dissimilarity was observed in group IV drug control animals when compared to the control animals.

The activites of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase in erythrocyte membrane and liver respectively are shown in Figures 1 and 2 respectively. In aflatoxin B1 induced group II hepatoma bearing animals, significant reduction in the levels of Na^+/K^+ ATPase, Mg^{2+} ATPase ($P<0.01$) and Ca^{2+} ATPase ($P<0.01$), were observed in erythrocyte membrane and liver when compared to control animals. These ATPases levels were increased in the kaempferol treated group III animals when compared with group II carcinoma induced animals. No significant alterations of all these enzymes level were observed in group IV animals when compared with group I control animals.

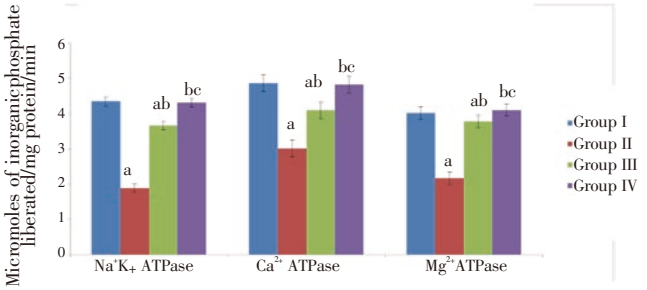

Figure 1. Effect of Kaempferol on ATPases in erythrocyte membrane of control and experimental animals.

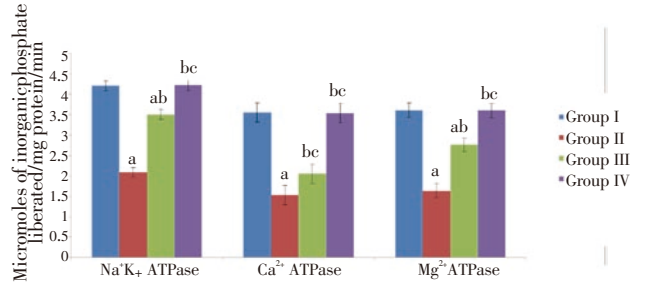

Figure 2. Effect of Kyaempferol on ATPases in liver of control and experimental animals.

Table 2 illustrate the activities of the carbohydrate metabolizing enzymes in liver of control and experimental group animals. In group II, aflatoxin B1 induced animals,

Table 1
Effect of kaempferol on the levels of nucleic acids in liver of control and experimental animals.

Tissue	Parameters (mg/g wet tissue)	Group I Control	Group II AFB1	Group IIIAFB1 + kaempferol	Group IV kaempferol
Liver	DNA	8.81±0.61	11.35±0.96[a]	9.52±0.21[ab]	8.14±0.14[bc]
	RNA	5.16±0.34	7.65±0.57[a]	6.32±0.31[ab]	5.43±0.18[bc]

Values are expressed as mean±SD for six animals in each group.
[a] – Group I Vs Group II, III and IV, [b] – Group II Vs Group III and IV, [c] – Group III Vs Group IV. The significance at the level of $P<0.01$.

Table 2
Effect of kaempferol on the levels of carbohydrate metabolizing enzymes in liver of control and experimental animals.

Parameters	Group I Control	Group II AFB1	Group III AFB1 + kaempferol	Group IV kaempferol
Hexokinase n moles of glucose-6- phosphate liberated/mg protein/min	16.60±1.20	31.25±2.00[a]	24.30±1.31[ab]	16.15±0.30[bc]
Phosphoglucoisomerase n moles of fructose liberated/mg protein/min	28.15±3.12	42.56±1.11[a]	35.34±2.16[ab]	27.99±2.14[bc]
Aldolase n moles of glyceraldehydes liberated/ mg protein/min	26.45±0.01	39.70±1.01[a]	32.61±2.11[ab]	25.33±1.21[bc]
Glucose-6-Phosphatase n moles of inorganic phosphate liberated/mg protein/min	25.39±1.42	14.58±2.13[a]	20.17±0.01[ab]	24.89±2.10[bc]
Fructose-6-Phosphatase n moles of inorganic phosphate liberated/mg protein/min	56.14±2.04	39.26±1.00[a]	45.14±0.16[ab]	55.97±3.01[bc]

Each bar expressed as mean±SD for six animals in each group.
a – Group I Vs Group II, III and IV, b – Group II Vs Group III and IV, c – Group III Vs Group IV. The significance at the level of $P<0.01$.

Table 3
Effect of kaempferol on the levels of TCA Cycle enzymes in liver of control and experimental animals.

Parameters	Group I Control	Group II AFB1	Group III AFB1+ kaempferol	Group IV kaempferol
Isocitrate dehydrogenase (n mol of α – ketoglutarate formed / mg protein / min)	6.83±0.24	3.88±0.56[a]	5.14±0.92[ab]	6.29±0.96[bc]
Succinate dehydrogenase (μ mol of succinate oxidized / mg protein / min)	5.91±0.28	3.85±0.94[a]	4.16±0.14[ab]	5.67±0.53[bc]
Malate dehydrogenase (μ mol of NADH oxidized/ mg protein / min)	8.93±0.39	6.22±0.87[a]	7.74±0.86[ab]	8.36±0.59[bc]
α – ketoglutarate dehydrogenase (μ mol of potassium ferrocynade liberated / mg protein/min)	1.45±0.61	0.84±0.93[a]	1.20±0.021[ab]	1.40±0.02[bc]

Values are expressed as mean±SD for six animals in each group.
[a] – Group I Vs Group II, III and IV, [b] – Group II Vs Group III and IV, [c] – Group III Vs Group IV. The significance at the level of $P<0.01$.

the activities of carbohydrate metabolizing enzymes such as hexokinase, phosphogluco-isomerase and aldolase were significantly increased and the gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1,6-diphosphatase enzymes were significantly decreased ($P<0.01$) when compared to control. On kaempferol treatment in group III animals reveal these enzyme were significantly ($P<0.01$) brought back to near normal levels when compared with cancer bearing animals. No considerable changes in the activities of glycolytic and gluconeogenic enzymes were observed in kaempferol alone treated group IV animals when compared with control groups.

Table 3 depict the levels of mitochondrial TCA cycle enzymes in liver of control and experimental animals. There was significantly decrease ($P<0.01$) in the levels of TCA cycle enzymes in Group II animals when compared to the control group I animals. Upon administration of kaempferol in Group III animals, these levels were brought back to near normal comparable to that of group II aflatoxin B1 induced liver cancer bearing animals ($P<0.01$). Kaempferol alone treated Group IV animals did not illustrate much dissimilarity when compared to control Group I animals.

4. Discussion

Neoplasms are associated with abnormalities in their DNA content, which increase with the degree of malignancy. The determination of DNA content was more important with regard to biological and functional aspects of the tumor, because it is an indicative of the proliferating activity in tumor conditions. DNA content is an independent indicator and often correlates with DNA content of tumor [35]. It has been proposed that protein, lipids and DNA are the major targets of oxidative injury [36]. In neoplasms, the size of the tumors often correlates with its DNA content and therefore it is found to be an independent indicator of prognosis [35]. The increased DNA content in cancer bearing liver may be due to the increased expression of enzymes which are necessary for DNA synthesis in tumour cells with repression of many enzymes related to differentiated cell function [37]. The RNA level in liver and kidney of cancer-bearing animals was also increased but not as significantly as DNA. The increased DNA content may lead to an increased transcription, which might have resulted in the moderately elevated RNA content in tumour cells. Decreased levels of DNA and RNA content to near normal levels were observed in kaempferol treated animals. Since, kaempferol are potent inhibitors of tumour cell proliferation and anticancer agents [38-40]. It imparts a significant inhibition on the rate of development of tumour.

The lipid dependent membrane bound enzymes are Adenosine Tri Phosphatases (ATPase). ATPases are vital enzymes play an imperative role in the upholding of ionic gradients between the intracellular and extracellular compartments of the cell and providing metabolic energy to the living processes [41]. Na^+K^+ ATPase play a momentous role in active transport of Na^+ and K^+ ions across the plasma membrane. Similarly, Ca^{2+}ATPase clearly linked with Ca^{2+} pump and transport of Ca^{2+} ions. Active transport of Na^+, K^+ directly depends on the active calcium transport and resultant low calcium concentration. Magnesium is one of the intracellular ions and has a key role in the intermediately metabolism. It is a component of the high energy for the organism. The Mg^{2+} ion forms Mg^{2+}ATP complex, which is the substrate for the enzyme. Mg^{2+}ATPase is to control the intracellular Mg^{2+} concentration, changes which can modulate the activity of Mg^{2+} dependent enzymes and regulate the rates of protein synthesis and cell growth [42].

Any alteration in membrane lipid leads to change in membrane fluidity, which in turn alters the ATPase activities and cellular functions. A certain degree of membrane fluidity seems to be essential for Na^+-K^+ATPase [43]. In the present investigation there was a noteworthy decrease in the activities of membrane bound ATPases in erythrocyte membrane and liver tissues of primary liver carcinoma induced experimental animals which may be due to oxidative stress, lipid peroxidation and formation of free radicals leads to cellular damage. It was reported that inhibition of lipid dependent membrane bound ATPase enzymes in various types of cancer [44]. Since free radicals are deeply associated with pathogenesis of various diseases including cancer [45]. Free radicals exert their cytotoxic effects by causing peroxidation of membrane phospholipids. Kaempferol treatment showed a notable increase in the

membrane bound ATPase enzyme activities which recovered the distorted cell membrane fluidity. Since kaempferol is a natural bioflavonoid which is known to influence the permeability of biomembranes and intermingle with Na^+, K^+-ATPases pumps. Because of this property of bioactive kaempferol, the activities of ATPases might have regained normal efficiency and cell whispered normal properties [46].

It has long been acknowledged that cancer cells exhibit an increased rate of glucose metabolism compared with healthy cells from the same tissue of origin. The development of different types of tumours is accompanied by distinguishing alterations in the activities of enzymes, particularly those involved in carbohydrate metabolism [47]. The degree of elevation of carbohydrate metabolism enzymes is directly related to the degree of morphologically undifferentiating and growth rate of cancer [48]. The early changes of the carbohydrate metabolism are of particular interest, since anomalies of glycolytic and gluconeogenic pathways are well known from biochemical examination of cancer conditions [49]. Hexokinase levels occupy an important place in determining the glycolytic capacity of cancer cells [50]. It is a rate-limiting enzyme which catalyses the conversion of glucose to glucose-6-phosphate in the first step of the glycolytic pathway. The proliferating cells undergo a shift from oxidative to glycolytic metabolism, where the energy requirements of the rapidly dividing cells are provided by ATP from glycolysis. The increased hexokinase activity may not only be the consequence of altered metabolic requirements of cancer cells but also be a modification to increased mitotic activity [51]. In the present investigation, increase in the activity of hexokinase in AFB1 intoxicated animals might be due to the increased metabolic need of proliferating cancer cells for its energy requirement.

Phosphoglucoisomerase serves as an excellent manifestation of cancer condition and which act as a catalyst in the conversion of glucose-6-phosphate to fructose-6-phosphate and it is a marker of metastatic growth with elevated levels in patients with neoplasms, especially after metastasis [52]. In the present investigation, increased levels of phosphoglucoisomerase were observed in hepatoma bearing experimental animals which may be due to the higher glycolytic rate in hepatic tissues and further leakage from destruction of neoplastic tissues. Alterations in the activity of phosphoglucoisomerase might be expected to influence the proportion of glucose-6-phospatase metabolized via the glycolytic pathway [53]. Aldolase is another key enzyme in the glycolytic pathway is also increased in metastatic condition [54]. Glucose-6-phosphatase is a marker enzyme for liver microsomal activity and it is greatly inhibited in cancer bearing animals. The decreased activities of glucose-6-phosphatase and fructose-1-6-diphosphatase during cancer phase and the decreased activities of glucose-6-phosphatase reveal the progressive failure of gluconeogenesis in cancerous conditions [55,56]. Hence, the observed reduction in activities of carbohydrate metabolizing enzymes in cancer bearing animals in the present investigation may be due to the higher lactate production in neoplastic tissues. On treatment with kaempferol, notably altered the levels of these enzymes and were reverted to back near normal, which may be due to inhibition of glycolytic pathway and activation of gluconeogenesis. This illustrate that kaempferol may interrupt the energy requirement of neoplastic tissues leading to the suppression of cancer development.

Mitochondria are dynamic intracellular organelles that play a central role in oxidative metabolism and apoptosis responsible for generating energy as adenosine triphosphate (ATP), since mitochondria are the powerhouses of our cells. Damage to mitochondria is now understood to play a role in the pathogenesis of a wide range of seemingly unrelated disorders [57]. Mitochondrial oxidative process plays a vital role in the maintenance of cellular energy supply and it is the major intracellular source during oxidative phosphorylation and is the primary target of reactive oxygen species (ROS). It has been established that imperfection in the respiratory chain leads to enhanced production of ROS and free radicals in mitochondria resulting in mitochondrial DNA mutations which indirectly impairs glucose sensing by reducing intracellular concentrations of ATP [58,59] and any damage inflicted in the mitochondria would ultimately result in the reduction of energy production and leads to cell death [60]. The citric acid cycle enzymes were decreased under oxidative stressed circumstance because the stressed animals are meeting its energy requirement through anaerobic oxidation [61]. Declined actions of these enzymes may be due to the alteration in cell morphology, ultra structure and ability of mitochondria to undergo metabolic changes also due to severe reduction in the number of mitochondria in cancerous cells when compared with normal cells and this could be due to noticeable deficiency in one or more electron transport chain components [62]. In the present study, the activities of citric acid cycle enzymes such as isocitrate dehydrogenase, succinate dehydrogenase, α-KGDH and malate dehydrogenase were found to be decreased in AFB1 induced hepatocellular carcinoma bearing animals. This may be due to the mitochondrial damage caused by AFB1-induced oxidative damage. Increased free radical production thus compromises the ability to meet the energy demands of the cell by reducing the levels of mitochondrial TCA cycle enzymes. In the present investigation the levels of ICDH, SDH, MDH and α-KGDH were increased significantly due to kaempferol administration which may be due to its efficient in preserving the mitochondrial membrane integrity.

In conclusion, biochemical alterations observed in HCC condition seem to be mainly due to oxidative stress and formation of oxy radicals. In recent times more research has been looking on the role of bioflavonoids in cancer prevention because epidemiological investigations suggest that increased intake of fruits and vegetables are associated

with the reduced risks of certain cancers. Consequently, it has been concluded that, kaempferol is a tremendous bioactive flavonoid with potent anticarcinogenic property. It proves its antineoplastic nature through modulating the levels of nucleic acids, membrane bound enzymes, carbohydrate metabolizing enzymes and mitochondrial enzymes.

Conflict of interest statement

We declare that we have no conflict of interest.

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