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INVESTIGATION OF HOW 6-GINGEROL ELICITS ANTICANCER EFFECTS IN A MOUSE MODEL OF EARLY HCC EVENTS VIA DEGRADATION OF PRO-INFLAMMATORY CYTOKINE

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Abstract
Hepatocellular carcinoma (HCC) is among the most fatal cancers globally, ranking third in cancer-related deaths, with particularly high prevalence in Asia and Sub-Saharan Africa. Natural dietary compounds have demonstrated significant potential for both preventing and managing cancer. [6]-Gingerol, a phenolic constituent of ginger (Zingiber officinale), has shown chemoprotective effects against liver cancer development in animal models. The present study examines the molecular mechanisms of anticancer effects of [6]-gingerol in mouse model of early hepatocellular carcinoma events induced by diethylnitrosamine and promoted by 2-acetylaminofluorene. Thirty-two male BALB/c mice were distributed into four groups of ten animals each. Group 1 received corn oil. Group 2: received a single i.p dose of DEN (75 mg/kg) in normal saline after 7 days, followed by 2-acetylaminofluorene (200 mg/kg) 2-AAF in the diet. Group 3 was treated only with 6-GR (100 mg/kg). Group 4: Mice were pre-treated with [6]-gingerol in corn oil for 7 days, then treated with the same regimen as in Group 2, with treatment continuing for 4 weeks. Inflammation was evaluated by measuring IL-1β, IL-6, and TNF-α levels, as well as NF-κB and COX-2 expression. With strong evidence, 6-GR exhibited a potent anticancer effect against DEN and 2AAF-induced early events in hepatocellular carcinoma by inhibiting the proinflammatory cytokines.

Keywords: *Hepatocellular carcinoma, 6-gingerol, Inflammation, Mouse model, Cytokine, Early events*

Introduction

Hepatocellular carcinoma (HCC) recent global spread both clinically and epidemiologically cannot be negated (Heimbach *et al*., 2018). HCC manifestation takes through the process of some crucial molecular events involving commonplace hepatic cells genetical remodeling or expressions of nuclear factors and some immuno-regulatory proteins resulting in the significant number of deaths associated with late detection and lack of adequate treatment regimen (Stefaniuk *et al*., 2010; Tseng *et al*., 2005). Asia and sub-Saharan Africa are the worst hit by HCC, which arises from severe liver damage with cirrhosis or hepatitis origin (Sergio *et al*., 2008). Other predisposing factors include infections from viruses, food preservatives, alcoholism, and aflatoxicosis, toxic chemicals generated from industrial (Tsukuma *et al*., 1993; Farazi and DePinho, 2006). Hence, chemoprevention of HCC is now a call to duty.

The etiology of HCC involves genetic and environmental factors, leading to disruption of epithelial barrier and immune dysregulation. Furthermore, it increases the gut-liver axis permeability, which now allows bacterial translocation, resulting in aberrant immune response (Chen *et al*., 2023). In HCC, oxidative stress from ROS damages hepatocytes while neutrophil infiltration exacerbates inflammation, which in turn promotes tumor progression and immune dysfunction (Yang *et al*., 2022; Liu *et al*., 2023). In the liver, tumor necrosis factor-alpha (TNF-α) can boost inflammation by triggering immune responses, production of pro-inflammatory cytokines and proteases, thus promoting tissue remodeling, angiogenesis, and tumor growth (Refolo *et al*., 2020; Rico Montanari *et al*., 2021). In addition, neutrophils infiltration and extreme release of pro-inflammatory cytokines have been associated with the disruption of hepatocyte tight junctions and liver injury (Zhu *et al*., 2023). Infiltrated neutrophils as evidenced by elevated myeloperoxidase (MPO) activity can generate reactive oxygen species (ROS). These ROS are drivers of chronic liver inflammation due to their damaging effects on cellular macromolecules (Yang *et al*., 2022).

Gingerol is the principal pungent component in fresh ginger and is considered a significant active constituent with many pharmacological and physiological effects (Nakatani *et al*., 2001). Research indicates that [6]-gingerol [6G] has antioxidant activity and anti-inflammatory activity (Kuo *et al*., 1999; Ippoushi *et al*., 2003; Reddy and Lokesh, 1992; Krishnakantha and Lokesh, 1993; Young *et al*., 2005), [6]-gingerol exhibited anti-tumor and anti-proliferative activities by inhibiting cytotoxicity and angiogenesis *in vitro* and *in vivo* (Lee and Surh, 1998; Keum *et al*., 2002; Kim *et al*., 2005). Many experimental pieces of evidence using animal model have revealed that combined treatment with DEN and 2-acetylaminofluorene (2-AAF) can induce HCC two-stage hepatic carcinogens is in mice (Sultana *et al*., 2013). To this end, we explored the expression of interleukins (IL-1β, IL-6) and tumor necrosis factor (TNF-α), which might be targets of the anticancer action of [6]-gingerol.

Materials and Methods

Extraction of [6]-gingerol

Locally purchased ginger plant (rhizome and foliage) from Bodija Market, Ibadan, Nigeria, was later sent for authentication to the Botany Department, University of Ibadan, where it was assigned voucher number UIH-22390 and lodged in the herbarium. Furthermore, the modified procedure of Ajayi *et al*. (2015) was used to isolate and characterized our [6]-gingerol with (> 91%) purity.

Chemicals

Diethylnitrosamine (DEN) and 2-Acetylaminofluorene (2-AAF) were purchased from AK Scientific, Inc. Union City, California, USA. Additionally, ELISA kits for NF-κB, TNF-α, IL-1β, and IL-6 were also obtained from Elabscience, China. All other reagents were of the highest standard from BDH (UK).

Animal and Treatment Regimen

Male BALB/c mice (n=32; 16 – 18 g) were acquired from the Department of Anatomy, University of Benin. The animals had unrestricted access to food and water during their one-week acclimatization period, which was a 12-hour light/dark cycle. All procedures complied with NAS guidelines (NRC, 2011) and were endorsed by the University of Benin Ethics Committee (File No.: 22/018).

Control Group

Mice orally received corn oil (2 mL/kg).

HCC Group

Mice received a single i.p. dose of DEN (75 mg/kg) in normal saline, allowing a 7-day recovery period, followed by exposure to a diet containing 2-AAF (200 mg/kg) for 4 weeks.

[6]-gingerol Group

Mice received [6]-gingerol in corn oil alone (100 mg/kg) daily.

HCC Treated with [6]-gingerol

Mice received the same treatment regimen as the HCC group after pre-treatment with [6]-gingerol in corn oil for 7 days. The therapy lasted for four weeks. The [6]-gingerol (100 mg/kg), 2-acetylaminofluorene (200 mg/kg) 2-AAF/feed, and diethylnitrosamine (75 mg/kg) employed in the investigation were selected based on previously published results (Ajayi *et al*., 2015 and Maduako and Farombi, 2025). All treatments were administered intraperitoneally (i.p.) and orally, respectively.

Sample Collection

Twenty-four hours post-final treatment, animals were anesthetized with ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg), weighed, and euthanized. Blood samples were obtained via cardiac puncture into EDTA tubes, and liver tissues were harvested. A portion was preserved in 4% buffered formalin for histology and immunostaining analyses, while the remainder was rinsed, dried, weighed and stored for biochemical determination. Blood samples for liver function test were separated by centrifugation at 3000 x g for 5 min. Liver homogenates were prepared in 0.1 M phosphate buffer and spun at 10,000 x g for 15 min at 4 ^{o}C.

Determination of Liver Function Test and Damage

The hepatoprotective activity of [6]-gingerol was determined by evaluating the levels of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the plasma using the method (Reitman and Frankel, 1957), also the gamma-glutamyl transferase (γ-GT) activity was analyzed based on the release of p- nitroaniline from γ-glutamyl-p-nitroanilide in the presence of glycyl glycine as glutamyl acceptor at 405 nm (Szasz, 1969), furthermore bilirubin (Tietz, 1995).

Enzyme-linked Immunosorbent Assay

Commercially available ELISA kits with the manufacturer's procedure were used for NF-κB, tumor necrosis factor (TNF-α), and Interleukins (1β and 6) (Elabscience Biotechnology Company, Wuhan, China).

Histological observation of the liver architecture

Briefly, the liver tissue representative was fixed in buffered formalin for 24h. Liver tissues were systematically dehydrated accordingly and immediately fixed in paraffin using tissue embedder (Leica EG 1150H). Furthermore, embedded 5µm liver tissue was sectioned and stained using hematoxylin and eosin (H&E). Examination of liver tissue under light microscope was performed by a pathologist naive to the study.

Data Analysis

One-way ANOVA was used to compare experimental groups, followed by student's t-test, with GraphPad Prism (version 7). At $p < 0.05$, values were considered statistically significant.

Results

[6]-gingerol Ameliorated HCC-induced Increase in Plasma Enzyme Levels, Histological and Gross Morphological Lesions in the Liver of Mice,

Mice challenged with DEN and dietary 2-AAF displayed a notable increase in plasma enzymes in the HCC mice compared to control mice. Dietary exposure to DEN+2-AAF raised ALT, AST, Bilirubin, and γ-GT levels significantly ($p < 0.05$), respectively. However, [6]-gingerol administration ameliorated significantly ($p < 0.05$) the observed increases in plasma enzyme levels in the HCC group (Table 1), and [6]-gingerol was able to restore liver architecture and integrity to near normal, both in the histological and gross morphological assessment (Fig. 1)

Table 1: Effect of 6-Gingerol on Liver Enzyme Markers in DEN/2-AAF-Exposed Mice

Parameters	Control	DEN+2AAF	6G	DEN+2AAF+6G
AST (U/L)	10.12 ± 0.20	42.90 ± 1.55[a]	11.21± 0.01	25.5 ± 1.11[b]
ALT (U/L)	08.01 ± 0.81	26.40 ± 1.23[a]	07.24 ± 0.78	18.2 ± 1.02[b]
γGT (U/L)	12.10 ± 0.16	44.09 ± 0.26[a]	11.63 ± 0.21	18.09 ± 0.19[b]
Bilirubin (mg/dL)	0.08 ± 0.01	0.56 ± 0.05[a]	0.09 ± 0.02	0.21± 0.04[b]

Each bar represents the mean ± SD of 8 animals. [a] Values differ significantly from control (p<0.05). [b] Values differ significantly from DEN+2-AAF (p<0.05).

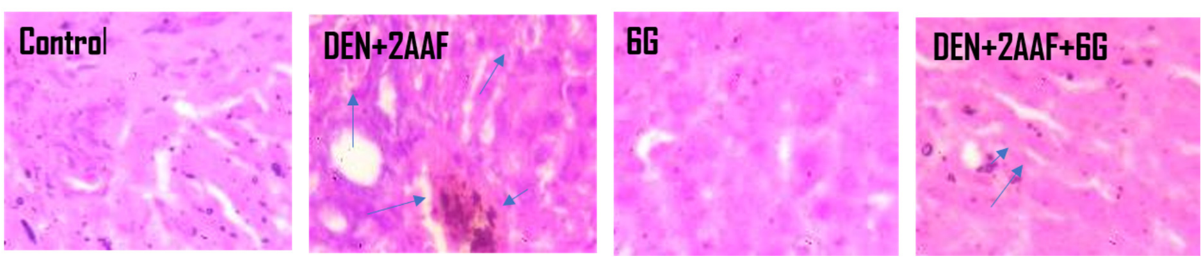

Figure 1 Representative photomicrographs of histopathology of experimental mice liver after four weeks of treatment. H&E (X400). (A) Control orally administered with corn oil; showed normal liver architecture. (B) DEN + 2AFF; showed fibrosis and inflammatory cells infiltration, mainly lymphocytes and macrophages in the distorted portal space. (C) 6G; showed normal liver architecture. (D) 6G + DEN + 2AAF; showed restored integrity of the liver with mild hepatic congestion.

[6]-gingerol Diminishes HCC-induced Level of Pro-inflammatory Cytokines

HCC typically develops against a backdrop of persistent fibrosis and inflammation. Therefore, administration of [6]-gingerol to HCC group mice significantly ($p < 0.05$) suppressed the levels of NF-κB, TNF-α, IL-1β, and IL-6 as compared with the control (Table 2).

Table 2: Effect of 6-Gingerol on inflammatory markers NF-κB, TNF-α, IL-1β, and IL-6 in DEN+2-AAF-exposed mice

Parameters	Control	DEN+2AAF	6G	DEN+2AAF+6G
NF-κB (pg/mL)	3.16 ± 0.47	20.20 ± 0.55[a]	3.00 ± 0.10	10.12 ± 0.11[b]
TNF-α (pg/mL)	1.30 ± 0.10	16.12 ± 0.22[a]	1.04 ± 0.18	9.11 ± 0.02[b]
IL-1β (pg/mL)	1.03 ± 0.17	8.13 ± 0.60[a]	1.50 ± 0.01	4.17 ± 0.12[b]
IL-6 (pg/mL)	0.16 ± 0.01	4.69 ± 0.02[a]	0.19 ± 0.01	3.00± 0.16[b]

Each bar represents the mean ± SD of 8 animals. [a] Values differ significantly from control ($p<0.05$). [b] Values differ significantly from DEN+2-AAF ($p<0.05$).

Discussion

Different experimental animal models have over the years been adopted for studying multistage carcinogenesis in the laboratory. Hepatocellular carcinoma (HCC) which arises upon the background of advanced state of inflammation, fibrosis, and cirrhosis, has gained global attention in the Western clime due to poor lifestyle while poorly developed countries, battle with food additives, preservation, and fungal toxins (Farazi and DePinho, 2006). However, our latest findings demonstrated that [6]-gingerol possesses anticancer potential against DEN and 2-AAF-induced early HCC events in mice after four weeks of exposure. The effects can be explained by the degradation of the inflammatory pathway. Thus, [6]-gingerol, a biologically active molecule from ginger rhizome, has demonstrated a potent candidate in cancer prevention and treatment, which can improve patients' wellness (Ajayi *et al*.,2015; Maduako and Farombi, 2025).

The current study investigated the anticancer effects of [6]-gingerol in HCC mice. Firstly, we assess the early indications of liver inflammation, damage, and function, such as AST, ALT, γ-GT, and bilirubin, which studies have shown leak out into circulation from cells lining the bile ducts and liver cell inflammation during liver injury. [6]-gingerol exhibited potent chemopreventive effects by lowering significantly the levels of these plasma markers when compared with the HCC group. Many scientific and clinical studies have demonstrated that hepatoprotective effects of many conventional drugs and natural agents are due to their abundant antioxidant and free radical scavenging abilities (Jaffar *et al*., 2024; Mittal *et al*., 2025; Chandimali *et al.,* 2025). In order to corroborate the hepatoprotective effect of [6]-gingerol against DEN + 2AAF-induced HCC, we examined the histological architecture of HCC-challenged mice. The restored liver architectural structure and function, as observed with [6]-gingerol administration, were potent against HCC by inhibiting infiltrated inflammatory cells and macrophages, proliferating fibroblasts, and hepatocellular foci with oval cell proliferation. Gross morphological examination of DEN + 2AAF-induced HCC-challenged mice revealed alterations and appearances of karyomegaly of hepatocytic nuclei in the liver. However, treatment with [6]-gingerol resulted in a near-normal morphology with a vascularized appearance. Our data are consistent with previously reported studies (Sayed-Ahmed *et al*., 2010; Ali *et al*., 2022).

Only recently, the correlation between chronic inflammation and cancer has received attention, hence making inflammation a star player as the tumor progresses, since several cancers occur from sites of infection and chronic inflammation (Coussens and Werb, 2002). NF-κB is a heterodimer with p50 and p65 that is strongly linked to cancer, including HCC (Garg and Aggarwal, 2002; Oeckinghaus and Ghosh, 2009). Under normal physiological form, NF-κB is resident in the cytosol and bound to its inhibitor, upon activation, NF-κB migrates into the nucleus initiating the transcription of pro-inflammatory cytokines such as TNF-α, COX-2, and IL-1β and IL-6 (Oeckinghaus and Ghosh, 2009). The increased expression of NF-κB in response to DEN and 2-AAF exposure is consistent with previously documented reports (Sharma *et al*., 2019). Also, COX-2, an enzyme that converts arachidonic acid, is transiently induced in response to proinflammatory stimuli, while IL-6 is released in the course of trauma or tissue damage (Williams *et al* 1999; Xie, 2001). Abounding evidence has shown that cytokines that promote inflammation, for example, TNF-α, COX-2, IL-1β, and IL-6, are all found upregulated in chronic liver inflammation and cancer (Oeckinghaus and Ghosh, 2009); therefore, natural agents that can suppress their production have demonstrated strong therapeutic potential (Rutledge and Adeli, 2007). In the present study, [6]-gingerol pretreatment attenuated the DEN and 2-AAF-mediated HCC increase in the protein expression observed in NF-κB, COX-2, and in the levels of TNF-α, IL-1β, and IL-6, similar to control, demonstrating that [6]-gingerol protected the liver via direct or indirect regulation of the signaling mechanisms associated with the inflammation and cancer.

Conclusion

Overall, our study demonstrated for the first time that the anticancer effects of [6]-gingerol against early HCC events in mice at the molecular level were mediated by the degradation of pro-inflammatory cytokines.

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