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6-Gingerol Inhibition of NF-κB Expression in Diethylnitrosamine-Initiated, 2-Acetylaminofluorene-Promoted Liver Dysfunction and Inflammation Involves Induction of Antioxidant Enzymes and Suppression of Pro-Inflammatory Cytokines

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Abstract

This study investigates the hepatoprotective effects of [6]-gingerol against liver dysfunction and inflammation induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) in mice. BALB/c mice were divided into four groups and treated with DEN, 2-AAF, [6]-gingerol, or a combination thereof. Biochemical, histological, and immunohistochemical analyses were conducted to evaluate liver function, oxidative stress, and inflammation. Mice exposed to DEN and 2-AAF exhibited significant hepatic injury, characterized by elevated serum liver enzymes (ALT, AST, ALP, γ-GT), reduced antioxidant enzyme activities (SOD, CAT, GPx, GST), increased lipid peroxidation, nitric oxide production, and expression of pro-inflammatory markers (NF-κB, iNOS, COX-2). Histopathological examination confirmed substantial liver damage. Pre-treatment with [6]-gingerol significantly mitigated these effects, restoring antioxidant enzyme activities, reducing oxidative and nitrosative stress, and suppressing the expression of inflammatory mediators. Liver histoarchitecture also improved markedly in [6]-gingerol-pretreated mice. These findings suggest that [6]-gingerol confers protective effects via antioxidant and anti-inflammatory mechanisms, primarily through modulation of the NF-κB signaling pathway. The results highlight the therapeutic potential of [6]-gingerol in preventing liver diseases associated with xenobiotic-induced oxidative stress and inflammation.

Keywords: Diethylnitrosamine, 2-Acetylaminofluorene, Liver Toxicity, NF-kB, Oxidative Stress

Abbreviations

2-AAF	:	2-Acetylaminofluorene	**DEN**	:	Diethylnitrosamine
ALP	:	Alkaline Phosphatase	**GSH**	:	Reduced glutathione
ALT	:	Alanine Transaminase	**GST**	:	Glutathione S-transferase
AST	:	Aspartate Aminotransferase	**H2O2**	:	Hydrogen peroxide
CAT	:	Catalase	**iNOS**	:	Inducible nitric oxide synthase
COX-2	:	Cyclooxygenase-2	**LPO**	:	Lipid peroxidation
			MPO	:	Myeloperoxidase

NF-kB	**:**	Nuclear Factor kappa-B
NO	**:**	Nitric oxide
SOD	**:**	Superoxide dismutase
XO	**:**	Xanthine oxidase
y-GT	**:**	Gamma-Glutamyl Transferase

Introduction

Persistent liver inflammation arises from a combination of chronic effects triggered by various harmful substances [1-3]. This condition can ultimately progress to cirrhosis, characterized by the formation of fibrous tissue and hepatocyte nodules. However, the intricate processes leading to liver dysfunction and inflammation are not fully understood. The characteristics of this condition include alterations in biochemical pathways, programmed cell death, activation of hepatic stellate cells (HSCs), free radical production, necrosis, and oxidative stress, all of which are essential factors in these mechanisms [4-6]. Exposure to environmental and occupational contaminants, such as diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), specific dietary factors, viruses, and agrochemicals, is associated with a heightened risk of developing liver preneoplasia [7-10]. DEN, a potent carcinogen and hepatotoxic agent, is commonly employed in animal studies to investigate the onset and progression of hepatic cancer. It induces liver lesions ranging from degenerative to neoplastic changes, offering insights into the development of hepatic carcinogenesis [11-13].

When combined with other toxins like 2-AAF, phenobarbital, or carbon tetrachloride, DEN's ability to induce liver dysfunction has garnered significant scientific interest [14]. These substances often stimulate the overproduction of chemical mediators, including cytokines, chemokines, NF-kB associated proteins. While these molecules play roles in pathogen elimination and tissue repair, their dysregulated expression can lead to chronic inflammatory disorders such as endotoxemia and fulminant hepatitis [15-17].

Efforts to mitigate these harmful effects have turned to natural nutritional phytochemicals, which are gaining recognition as a foundation for chemoprevention. Among these, the ginger rhizome (Zingiber officinale), used for its antioxidant and anti-inflammatory properties, has been a staple in traditional medicine and culinary practices worldwide [18, 19]. Its chemical constituents, notably [6]-gingerol and [6]-shogaol, have shown to exhibit various biological effects, including the inhibition of tumor growth and cell proliferation [20, 21]. Numerous studies indicate that [6]-gingerol suppresses NF-κB-regulated proteins linked to cell proliferation and angiogenesis, while also inhibiting inflammatory mediators like TNF-α and IL-1β [22]. Preclinical and experimental studies further highlight its potential to reduce nitric oxide synthesis and counteract inflammation [23-26].

Despite these findings, the specific mechanisms by which [6]-gingerol exerts its hepatoprotective effects are still uncertain. This study is designed to uncover the mechanism by which [6]-gingerol prevents sub-chronic liver inflammation and oxidative damage in a DEN+2-AAF-induced mouse model.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) were bought from Sigma-Aldrich Co., St. Louis, MO, USA. Polyclonal antibodies targeting nuclear factor (NF-kB), cyclo-oxygenase-2, and inducible nitric oxide synthase (iNOS) procured from Elabscience, China. Other analytical reagents were purchased from British Drug Houses, located in Poole, Dorset, UK.

Authentication and Extraction of [6]-Gingerol

Fresh ginger roots and foliage were purchased at Bodija Market in Ibadan, Nigeria, from a local vendor. The collected plant samples were authenticated at the herbarium (voucher number: UIH-22390) at the Department of Botany, University of Ibadan. The extraction of [6]-gingerol, with a purity exceeding 91%, was conducted using a method described by Ajayi et al [27].

Animals

Forty BALB/c mice (16-18g) were sourced from the University of Ibadan's Faculty of Veterinary Medicine. Prior to the experiment, the animals underwent a one-week acclimatization period in suspended plastic cages. The mice were given free access to standard laboratory feed and water and kept on a 12-hour light-dark cycle throughout the study. Animal care and handling adhered to internationally recognized guidelines, ensuring humane treatment and compliance with institutional regulations. The research proposal involving animal subjects was granted ethical approval by the Animal Care and Use Research Ethics Committee at the University of Ibadan, under reference number UI-ACUREC/21/122.

Treatment Schedule

The experimental animals were divided into four groups (I–IV), each comprising ten mice (n=10).

Group I: received an oral treatment of corn oil at a dose of 2 mL/kg.

Group II: was administered a single intraperitoneal (i.p.) dose of DEN (75 mg/kg) in normal saline, followed by a 7-day recovery period, and subsequently fed a diet formulated with 2-AAF (200 mg/kg).

Group III: was treated daily with [6]-gingerol (100 mg/kg) in corn oil.

Group IV: underwent a 7-day pre-treatment with [6]-gingerol in corn oil, after which they were subjected to the same regimen as Group II.

The selection of doses for [6]-gingerol (100 mg/kg), 2-AAF (200 mg/kg in feed), and DEN (75 mg/kg) was guided by findings from a pilot study conducted in our laboratory, as well as data from previous study [28-30]. All treatments were administered via intraperitoneal injection and oral delivery for a period of four weeks.

Preparation of Homogenates

After the final treatment, all animals were weighed and euthanized. Following a 24-hour interval, they were sacrificed, and their livers were promptly excised. Part of the liver tissue was fixed in 4% buffered formalin for histological analysis while the other remaining liver tissue rinsed in cold phosphate-buffered saline, carefully blotted dry using filter paper, and weighed to facilitate immunohistochemical procedures and analysis of biochemical parameters.

Determination of Liver Function Indices

The serum activities of AST, ALT, ALP, and GGT were analyzed using Randox's commercially available diagnostic kits.

Determination of Hepatic Oxidative Damage

Liver tissues were homogenized in ten volumes of 0.1 M phosphate buffer (pH 7.4) using a Teflon homogenizer from Thomas Scientific, Swedesboro, NJ, USA. The homogenates were subsequently centrifuged at 15,000 g for 10 minutes at a temperature of 4 °C using Thermo Scientific Sorvall TM WX Floor Ultra Centrifuges. The resulting supernatant was collected and stored for biochemical analysis. Protein concentration was determined using Bradford, a spectroscopic method that measures protein concentration based on the binding of Coomassie Brilliant Blue dye to protein molecules at 595 nm [31].

The activity of superoxide dismutase (SOD) was evaluated by monitoring the inhibition of adrenaline autoxidation under alkaline conditions at pH 10.2, as described in the method by Misra and Fridovich, this method measures the enzyme's ability to convert superoxide radicals into hydrogen peroxide and oxygen, while catalase (CAT) activity was determined using hydrogen peroxide as a substrate, following the protocol by Clairborne, this assay quantifies the enzyme's efficiency in breaking down hydrogen peroxide into water and oxygen. The hepatic activities of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, following the methods by Rotruck et al. and Habig et al. respectively. Also, reduced glutathione (GSH) levels were measured by quantifying the chromophoric complex formed between 5',5'-dithiobis-2-nitrobenzoic acid (DTNB) and endogenous liver glutathione, as described by Jollow et al [32-36].

Lipid peroxidation (LPO) was assessed using a modified protocol, as described by Farombi et al [29]. This method involves measuring the formation of malondialdehyde (MDA), a pink-colored end product generated when 2-thiobarbituric acid (TBA) reacts with lipid peroxides. Hepatic hydrogen peroxide (H_2O_2) levels were evaluated following the method outlined by Wolff which is based on the oxidation of ferrous ions (Fe^{2+}) coupled with sorbitol, leading to color development. Xanthine oxidase (XO) activity was assessed following Stripe and Della Corte method [37, 38]. This assay is based on the catalytic conversion of xanthine to uric acid, with the reaction being measured spectrophotometrically at 290 nm. Nitric oxide (NO) levels were quantified by determining hepatic nitrite end-products using the Griess reagent, as described by Green et al. Myeloperoxidase (MPO) activity was evaluated by monitoring the oxidation of o-Dianisidine in the presence of hydrogen peroxide, following the method of Granell et al. with slight modifications [39, 40].

Histological Examination

Liver samples were freshly excised and fixed in 10% formalin diluted with phosphate-buffered saline. Following 48 hours of fixation, the tissues were dehydrated, washed in xylene, and processed for infiltration and embedding. Subsequently, they were sectioned into slices of 5 µm thickness using a rotary microtome (Leica RM2125 RTS, Germany). The sections were stained with hematoxylin and eosin, and a pathologist, who remained unaware of the study groups, performed a detailed light microscopic examination of the liver tissue.

Immunohistochemistry

Liver tissue samples embedded in paraffin wax on poly-L-lysine-coated slides were rehydrated by immersion in xylene and a graded series of ethanol concentrations (100% to 50%). Heat-induced epitope recovery was achieved by treating the sections with citrate buffer (pH 6) for 20 minutes, followed by immersion in cold water for 10 minutes. To block the activity of endogenous peroxidase, the tissue sections marked with a PAP pen were treated with 5% hydrogen peroxide in a dark environment. The slides were then incubated overnight at 4 °C with primary polyclonal antibodies targeting anti-iNOS, anti-NF-kB, and anti-COX-2.

After thorough washing with tris-buffered saline, the sections were incubated with horseradish peroxidase-labeled anti-mouse polyclonal secondary antibodies (Dako, Agilent Technologies, USA). The immune complexes formed were visualized by treating the sections with 0.05% 3,3-diaminobenzidine (DAB) for 5 minutes, followed by counterstaining with hematoxylin. Subsequent dehydration of the slides was performed using increasing concentrations of ethanol, followed by a brief immersion in xylene before mounting. Liver images were captured using a Leica DM 500 digital light microscope (Germany) equipped with a detachable ICC50 digital camera (Germany). The images were processed and analyzed using ImageJ-Fiji software (National Institutes of Health, USA).

Statistical Analysis

Data analysis utilized single-factor ANOVA via SPSS (version 17), with Bonferroni post hoc tests at a significance level of $p < 0.05$.

Results

Effects of DEN and 2-AAF on Body Weight Gain, Liver Weight, and Relative Liver Weight

Administration of 75 mg/kg DEN followed by exposure to a diet containing 2-AAF (200 mg/kg) markedly reduced the final body weight in the treated mice in comparison to the control group, as illustrated in Table 1. Additionally, treatment with DEN and 2-AAF caused a statistically significant ($p < 0.05$) increase in liver weight and/or relative liver weight in comparison to the control. Pre-treatment with [6]-gingerol significantly ($p < 0.05$) mitigated these effects, reversing the impact of DEN and 2-AAF on both the final body weight and organ weight of the mice. Notably, the administration of [6]-gingerol alone did not alter the organ weight of the mice in comparison to the control group, as shown in Table 1. These findings align with observations reported by Sultana et al. [41].

Table 1: Chemopreventive effect of 6-gingerol on body and organ weight (g) and relative organ weight (g/100g bw) of mice treated with DEN+2-AAF for 4 weeks. Data expressed as mean ± SD of 10 mice/group. a Values are significantly different when compared to control ($p<0.05$) and bValues are significantly different from DEN+2-AAF ($p<0.05$).

	CTRL	DEN+2AAF	6G	6G+DEN+2AAF
Final body weight (g)	25.69±2.01	20.05±1.63[a]	26.11±1.24	22.45±1.11[b]

Initial body weight (g)	16.02±1.02	16.13±1.08	17.05±1.27	16.10±1.07
Liver weight (g)	1.00±0.11	1.27±0.12[a]	1.01±0.46	1.10±0.20[b]
Relative liver weight (g/100g bw)	3.15±0.36	3.80±0.14[a]	3.19±0.69	3.34±0.66[b]

Effects of [6]-Gingerol Pretreatment on the Activities of Serum Hepatic Enzymes Following DEN and 2-AAF Exposure

Figure 1 demonstrates that administering DEN (75 mg/kg) and 2-AAF (200 mg/kg in the diet) significantly ($p < 0.05$) increased serum levels of ALT, AST, ALP, and γ-GT in comparison to the control group. However, pre-treatment with [6]-gingerol (100 mg/kg) led to a notable decrease ($p < 0.05$) in the levels of these hepatic enzymes.

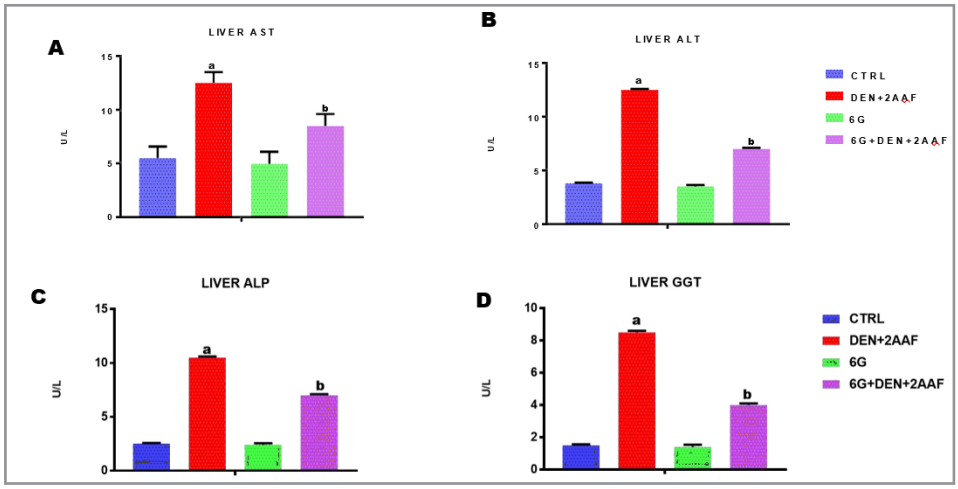

Figure 1: Effect of 6-Gingerol on serum enzymes markers AST, ALT, GGT and ALP in DEN+2-AAF exposed mice for 4 weeks. Each bar represents mean± SD of 10 animals. a Values differ significantly from control ($p<0.05$). b Values differ significantly from DEN+2-AAF ($p<0.05$)

Effects of [6]-Gingerol Pre-Treatment on Liver Redox Status Biomarkers

Figure 2 reveals that mice exposed to 75 mg/kg DEN and a formulated diet of 2-AAF (200 mg/kg) exhibited a marked decline within the functionality of antioxidant enzymes, such as SOD, CAT, and GST relative to the control group. However, [6]-gingerol (100 mg/kg) administration effectively prevented the reduction in these enzyme activities in DEN and 2-AAF-treated mice ($p<0.05$). Furthermore, [6]-gingerol restored hepatic glutathione (GSH) concentrations, reversing the depletion caused by DEN and 2-AAF exposure. Mice subjected to DEN and dietary 2-AAF treatment showed a marked decline in glutathione peroxidase (GPx) activity, alongside a rise in lipid peroxidation, indicated by heightened levels of malondialdehyde. Conversely, the administration of [6]-gingerol significantly mitigated oxidative stress ($p < 0.05$) in these mice. Additionally, DEN and 2-AAF exposure substantially increased hepatic myeloperoxidase (MPO) activity and nitric oxide (NO) levels compared to the control group. Pre-treatment with [6]-gingerol proved to be significantly effective ($p < 0.05$) in reducing MPO activity and NO levels, restoring them to near-normal values in DEN and 2-AAF-exposed mice, as shown in Figure 3.

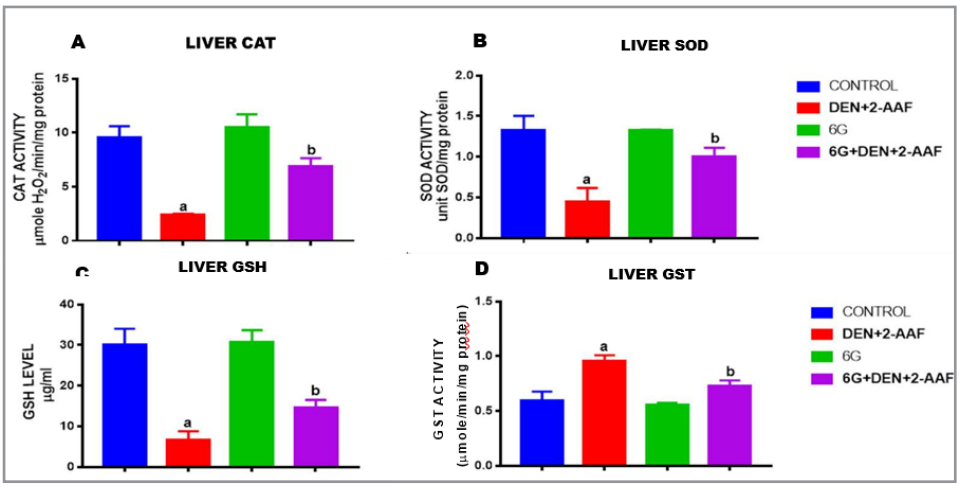

Figure 2: Effect of 6-Gingerol on the activities of SOD, CAT, GSH and GST in liver of DEN+2AAF-treated mice respectively for 4 weeks. Each bar represents mean ± SD of 10 mice. a: Values differ significantly from control ($p <0.05$). (b: Value differ significantly from DEN+2AAF group ($p < 0.05$)

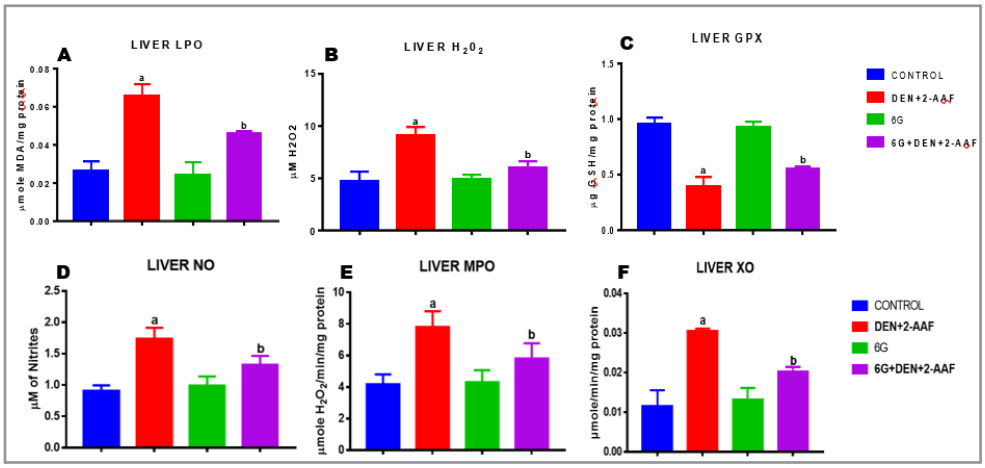

Figure 3: Effect of 6-Gingerol on MDA, H2O2, GPX, NO, MPO and XO levels in liver of DEN+2AAF-treated mice for 4 weeks. Each bar represents mean ± SD of 10 mice. a: Values differ significantly from control ($p < 0.05$). (b: Values differ significantly from DEN+2AAF group ($p < 0.05$)

Effects of [6]-Gingerol Pre-Treatment on Mice Liver Architecture

Hematoxylin and eosin staining of liver sections from both the control and the [6]-gingerol-treated mice revealed normal histological features, including intact central veins, organized liver cords, healthy hepatocytes, and thin sinusoidal spaces. In contrast, mice exposed to DEN (75 mg/kg) and a diet containing 2-AAF (200 mg/kg) exhibited significant histopathological abnormalities. These included disrupted central veins, distorted portal spaces infiltrated with inflammatory cells such as lymphocytes and macrophages, lipid-laden swollen hepatocytes, and dilated sinusoidal spaces. Pre-treatment with [6]-gingerol (100 mg/kg) followed by DEN (75 mg/kg) and dietary 2-AAF exposure led to a notable improvement in liver histology. Treated mice displayed mild sinusoidal distortions, moderate hepatocyte hypertrophy, slight neutrophil infiltration, and a moderately intact central vein (Figure 4).

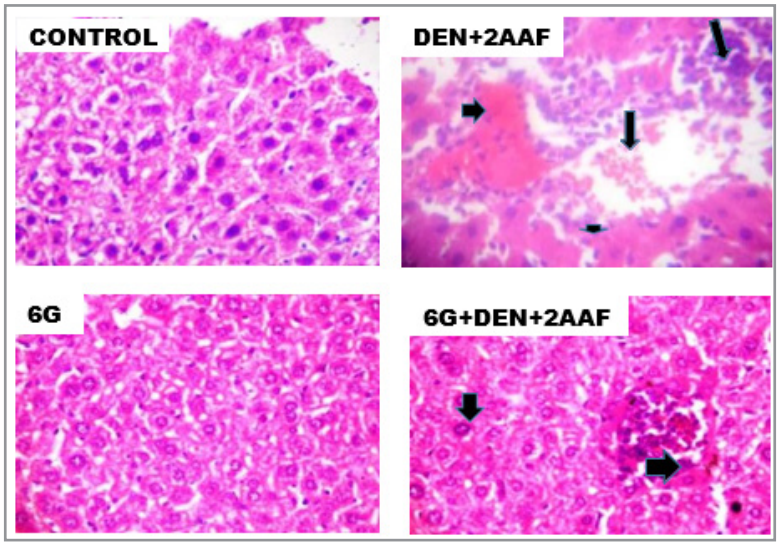

Figure 4: Representative photomicrographs of histopathological examination of experimental mice liver after 4 weeks treatment. H&E (X400). (A) Control orally administered corn oil (2mL/kg): showed normal liver architecture. (B) DEN+2- AAF: showed fibrosis and inflammatory cells, mainly lymphocytes and macrophages in the distorted portal space. (C) 6[G]: showed normal liver architecture. (D) 6[G]+DEN+2-AAF: showed restored integrity of the liver with mild hepatic congestion.

Effects of [6]-Gingerol on the Expression of Pro-Inflammatory Enzymes

The pro-inflammatory biomarkers iNOS, COX-2, and NF-kB have been linked to stimulation caused by DEN+2-AAF exposure. The anti-inflammatory effects of [6]-gingerol were assessed by analyzing its impact on DEN+2-AAF-induced iNOS and COX-2 expression (Figures 5A and 5B) through immunohistochemical examination of liver tissue in the treated mice. Mice challenged with DEN (75 mg/kg) and subsequently exposed to 200 mg/kg 2-AAF in their diet exhibited a significant ($p < 0.05$) expression of pro-inflammatory cytokines within the inflammatory foci of the liver relative to control mice. Nonetheless, pretreatment with [6]-gingerol successfully suppressed the immune expression of these inflammatory biomarkers in the DEN+2-AAF-treated mice.

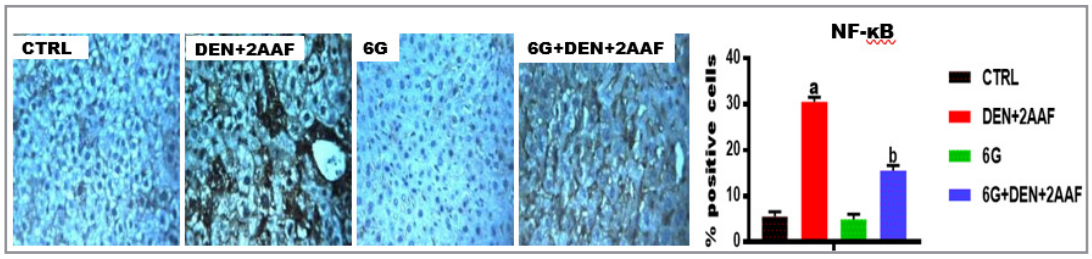

Figure 5A: Influence of 6G on hepatic NF-κB protein expression in mice exposed to DEN+2AAF for 4 weeks. Brown colour indicates positive staining for NF-κB antibody on 5 µm section of paraffin fixed mice liver. (x40). Each bar represents mean ± SD of 10 mice. a: Values differ significantly from control ($p < 0.05$).b: Values differ significantly from DEN+2AAF group ($p < 0.05$).

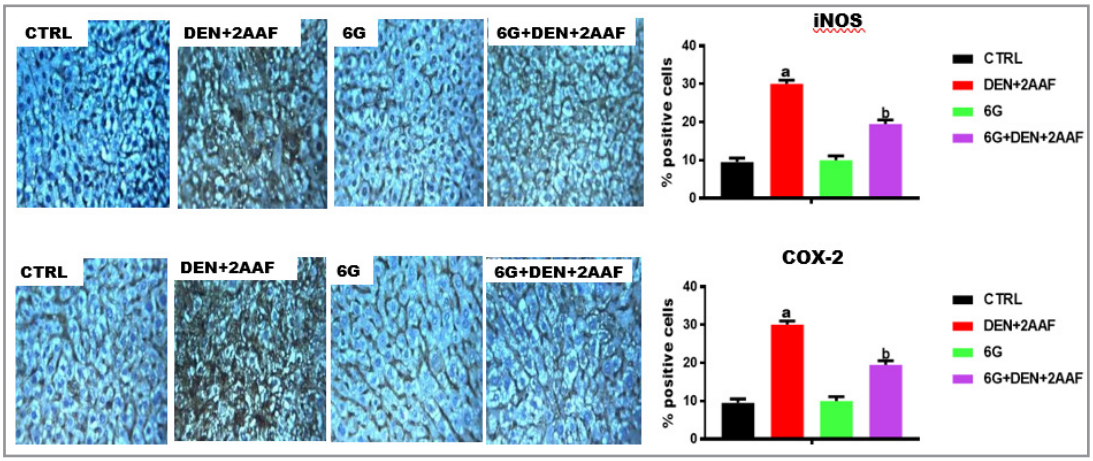

Figure 5B: Influence of 6G on hepatic iNOS (A) and COX-2 (B) protein expression in mice exposed to DEN+2AAF for 4 weeks. Brown colour indicates positive staining for iNOS and COX-2 antibody on 5 µm section of paraffin fixed mice liver. (x40). Each bar represents mean ± SD of 10 mice. a: Values differ significantly from control ($p < 0.05$).b: Values differ significantly from DEN+2AAF group ($p < 0.05$).

Discussion

The toxicological impact of DEN combined with 2-AAF in the diet has been associated with its production of highly reactive intermediates and reactive oxygen species (ROS) during the process of bioactivation. Recent studies have highlighted its potential to induce hepatotoxicity, causing liver damage, inflammation, oxidative stress, and abnormal cell proliferation [42]. In this experimental model, a notable decrease in the final body weight, accompanied by an increase in liver weight, was observed in mice treated with an initial single dose of DEN and fed a 2-AAF-supplemented diet, highlighting the harmful effects of DEN+2-AAF metabolites on growth and liver function (Table 1). Interestingly, the hepatoprotective properties of [6]-gingerol were evident without any associated changes in body weight, suggesting that [6]-gingerol administration exhibits minimal toxicity. A previous study has demonstrated [6]-gingerol tolerance with minimal or no toxicity [43].

A key indicator of hepatic injury is the release of intracellular enzymes into the plasma, resulting from chemical disruptions in hepatocyte transport functions [44]. Assessing serum enzymes, including AST, ALT, ALP, and γ-GT, provides a dependable indicator of liver health. Elevated levels of these enzymes in the blood are often indicative of impaired hepatic function or damage [45]. Based on our findings (Fig. 1), exposure to DEN and 2-AAF had detrimental effects on liver function markers in mice. This was demonstrated by a marked rise in serum activities of AST and ALT, along with increased levels of ALP and γ-GT in DEN+2-AAF-treated mice relative to the control. Numerous studies attribute hepatoprotective properties of both pharmaceutical drugs and herbal agents to their ability to combat oxidative stress through antioxidant activity and free radical scavenging mechanisms [46-48]. However, the administration of [6]-gingerol effectively mitigated these effects, promoting liver health by significantly suppressing the elevated liver function enzymes.

To further validate our serum findings, Figure 4 highlights the histopathological evaluation of liver tissue. Exposure to DEN+2-AAF in mice resulted in notable structural damage, including hyperchromatic nuclei, infiltration of inflammatory cells, and severe congestion, indicative of compromised hepatic integrity. However, the administration of [6]-gingerol significantly alleviated these hepatic lesions in DEN+2-AAF-treated mice, restoring the liver tissue architecture to near-normal conditions comparable to that of the sham-treated control group. The observed restoration of serum transaminase levels, accompanied by hepatic parenchymal repair and hepatocyte regeneration, aligns with the findings of Salihu et al [26]. The hepatoprotective effects of [6]-gingerol can be ascribed to its antioxidant characteristics and its capacity to stabilize cellular membranes, thereby preventing the release of intracellular enzymes.

Superoxide dismutase is a crucial enzymatic antioxidant responsible for the rapid conversion of superoxide ions into hydrogen peroxide, while catalase detoxifies reactive peroxide radicals, converting them into harmless water and oxygen [49]. Multi-

ple studies recognize these enzymes as a robust protective duo, functioning as a defense system against oxidative damage [50-52].

This study demonstrated a decline in superoxide dismutase and catalase activities in mice exposed to DEN and 2-AAF, indicating the suppression of these enzymes. As a result, this leads to a buildup of deleterious superoxide radicals and hydrogen peroxide within the liver tissue of the treated mice. However, the administration of [6]-gingerol significantly restored SOD and CAT activities in the liver of DEN+2-AAF-challenged mice. This finding indicates the potential antioxidant properties of [6]-gingerol, as evidenced in Figures 2A and 2B. Many hepatoprotective agents, including Sylimarin, Commiphora berryi, and Phyllanthus niruri, effectiveness in inhibiting lipid peroxidation induced by ROS has been well-established, owing to their strong free radical neutralizing capabilities [53-55].

Glutathione (GSH) plays a crucial role in protecting against oxidative stress by directly neutralizing hydroxyl radicals and singlet oxygen, facilitating drug detoxification, and restoring antioxidant vitamins C and E to their functional states. In contrast, glutathione peroxidase (GPx), a family of selenium-dependent enzymes, interacts with GSH to break down hydrogen peroxide and lipid hydroperoxides [56, 57]. Both GSH and GPx play crucial roles in maintaining the body's antioxidant defense mechanisms. Meanwhile, glutathione-S-transferase is instrumental in catalyzing the conjugation of toxic electrophilic compounds with GSH, functioning as a key enzyme in phase II drug detoxification processes [58]. Non-enzymatic antioxidants like glutathione and ascorbic acid safeguard against xenobiotic toxicity by neutralizing free radicals, a process that results in their oxidation [59]. Consequently, the hepatotoxic effects of DEN and 2-AAF are largely attributed to the free radicals generated during their metabolic activation. Our study revealed a substantial decrease in GSH levels, along with diminished activities of GPx and GST, in mice treated with DEN+2-AAF (Figures 2C, 2D, and 3C). However, the administration of [6]-gingerol effectively counteracted these reductions, revitalizing antioxidant enzyme functions and replenishing the levels of GSH in treated mice.

The observed elevation in hepatic GSH levels in mice exposed to DEN+2-AAF-exposed mice suggests that [6]-gingerol plays a role in facilitating the neutralization of reactive oxygen species generated during the P450 bioactivation of DEN+2-AAF. The DEN+2-AAF+ [6]-gingerol-treated groups exhibited nearly normal levels in enzymic and non-enzymic antioxidants, underscoring the significance of polyphenols in preserving the integrity of the endogenous cellular antioxidant defense system. Moreso, the antioxidant properties exhibited by [6]-gingerol in this study align with previous research, which has consistently highlighted its efficacy against reactive oxygen-mediated damage [60, 61].

Lipid peroxidation serves as a key indicator of oxidative damage triggered by ROS, leading to compromised membrane functionality, reduced fluidity, and the inactivation of membrane-bound receptors and enzymes [62]. It also leads to the production of toxic by-products, such as malondialdehyde (MDA) and 4-hydroxynonenal, which can target cellular components like DNA, causing mutagenesis and cancer [63, 64]. In this study, an elevated level of liver MDA was observed in mice treated with DEN+2-AAF, indicating heightened ROS production and significant lipid peroxidation (Fig. 3A). However, the administration of [6]-gingerol effectively reduced liver MDA levels in DEN+2-AAF-challenged mice, demonstrating its antioxidant and free radical scavenging potential.

These findings align with prior reports [27, 28, 65]. which further emphasized [6]-gingerol liver protecting capacity on lipid peroxidation. The oxidation reaction driven by xanthine oxidase mainly yields hydrogen peroxide, making it a crucial reactive oxygen species of interest when investigating xanthine oxidase involvement in inflammation and cellular communication [66]. In this study, treatment with DEN+2-AAF in mice led to a substantial increase in H_2O_2 production, accompanied by elevated XO activity (Figures 3B and 3F). However, the administration of [6]-gingerol effectively suppressed the heightened XO activity and H_2O_2 generation in the liver of DEN+2-AAF-treated mice, attributed to its anti-inflammatory properties. These findings are consistent with the observations, who reported increased XO activity along with enhanced H_2O_2 production in the liver of rats [67].

To understand the role of nitrosative stress, we measured iNOS and NO levels in DEN+2-AAF-induced liver inflammation. Evidence suggests that NO contributes to nitrosative stress and subsequent cellular damage by directly or indirectly interacting with biomolecules, especially in cases where the antioxidant defense system has been depleted [68]. Furthermore, inflammatory markers such as NO and MPO are upregulated via the expression of iNOS and COX-2 [24, 25, 69].

Infiltrated neutrophils and macrophages exhibit elevated MPO activity, a key enzymatic marker of inflammation. Activated neutrophils also release additional pro-inflammatory cytokines, amplifying the inflammatory response [70]. Mounting scientific evidence strongly supports the intrinsic link between inflammation and liver dysfunction [71, 72]. A key mechanism linking inflammation to hepatic disease is the transcription factor NF-κB, which is rapidly activated by cytokines, viruses, or chemotherapeutic agents and plays a regulatory role in COX-2 expression [73, 74].

During prostaglandin biosynthesis, COX-2 has demonstrated significant potential as a molecular target for various anti-inflammatory and therapeutic agents [75]. Elevated NO and MPO levels, coupled with increased iNOS, COX-2, and NF-κB expression (Figures 3D, 3E, and 5A and B), confirm liver inflammation in DEN+2-AAF-treated mice. Our result indicated that [6]-gingerol's anti-inflammatory actions might involve downregulating iNOS and COX-2 by inhibiting NF-κB. Collectively, our data revealed that [6]-gingerol confers protection by downregulating iNOS and COX-2 via NF-κB pathway suppression. This emphasizes that its liver protection is tightly associated with its potent anti-inflammatory abilities.

Conclusion

In conclusion, [6]-gingerol has been identified in several studies as an effective hepatoprotective agent, with its beneficial properties largely attributed to antioxidant and anti-inflammatory effects [49, 76, 77]. Furthermore, the findings from our study highlight the significance of [6]-gingerol in combating liver in-

flammatory conditions. Evidence from this study demonstrated that [6]-gingerol successfully mitigated DEN+2-AAF-induced hepatic inflammation associated with preneoplasia in mice by inhibiting the excessive release of inflammatory cytokines, enhancing non-enzymatic and enzymatic antioxidant activity, and effectively suppressing oxidative stress and tissue damage. Consequently, [6]-gingerol shows promise as a therapeutic agent for the prevention and management of liver diseases induced by toxic xenobiotics.

Contributing Authors

Ikenna Maduako: Investigation and analysis, drafting original manuscript, editing, and review. Olatunde Farombi: Concept idea, supervision, review, and editing

Ethical Approval/Consent

The research proposal involving animal subjects was granted ethical approval by the Animal Care and Use Research Ethics Committee at the University of Ibadan, under reference number UI-ACUREC/21/022.

Conflict Declaration

We declared that there were no conflicting interests between the authors with regard to this publication.

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Declaration of Generative AI and AI-assisted Technologies

During the preparation of this work, the author(s) used Grammarly to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and took full responsibility for the publication's content.

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