RESEARCH ARTICLE

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Clausenidin from *Clausena excavata* Burm. causes Apoptosis of Liver Cancer in Mice

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Abstract

Background: Clausenidin is a pyranocoumarin that was isolated from the roots of *Clausena excavata* Burm. Previously, the pure clausenidin crystals were successfully used to treat HepG2 cells (liver cancer) in vitro; however, no in vivo study had been conducted to support these findings. Therefore, the current study was designed to investigate the in vivo anti-liver cancer effect of pure clausenidin in hepatocellular carcinoma-induced BALB/c mice. **Method:** DNA fragmentation, caspases, and histological assays were conducted to evaluate the cytotoxic effect of the pure clausenidin, while real-time qPCR was performed to monitor the expression of apoptosis-inducing genes in the clausenidin-treated mice. **Result:** Clausenidin significantly ($p<0.05$) decreased the liver damage-induced levels of alanine and aspartate aminotransferases and also caused fragmentation of the genomic DNA of the tumors. This was followed by a significant increase ($p<0.05$) in the expression of *caspases 3, 8* and 9 proteins in the clausenidin-treated mice. In addition, clausenidin upregulated the expression of pro-apoptotic genes associated with the extrinsic and intrinsic pathways. However, it was observed that clausenidin may have inhibited angiogenesis by downregulating the expression of the *VEGF* gene in the treated mice. **Conclusion:** Therefore, clausenidin can be potentially used as an anti-liver cancer agent.

Keywords: Clausenidin- apoptosis- hepatocellular carcinoma- P-53- MDM2- VEGF

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Introduction

Cancer is a multistage disease characterized by uncontrolled and spontaneous cell division that affects millions of people worldwide. It is a debilitating disease that has caused over 9.6 million deaths, with lung, breast, and liver cancer being among the main causes of cancer [1]. Sub-saharan Africa is the worst hit by the cancer crisis because the region is unprepared and lacks quality healthcare facilities. For instance, in Nigeria, cancer kills 240 Nigerians daily [2]. Liver cancer is a leading cause of death in Nigeria and other sub-Saharan countries in Africa. The most common and devastating form of liver cancer is hepatocellular carcinoma (HCC), which accounts for more than 80% of all liver cancer cases and causes mortality. Cancer care is expensive and out of reach for many Nigerians. To contain the surge in cancer, middle and low-income countries have resorted to the use of herbs with purported therapeutic benefits. The consumption certain therapeutic herbs remains the only way to manage of certain cancer for many poor people in Nigeria and Africa.

Plants constitute a common alternative for cancer treatment in many countries, and more than 3000 plants globally have been reported to exert anticancer properties [3, 4]. Presently, some herbs are accepted in certain developed countries as adjuvants or complementary agents in cancer therapy. Plants contain many secondary metabolites that contribute to their therapeutic potential. For instance, pyranocoumarin such as clausarin, nordentatin, and clausenidin are anticancer compounds that were isolated from *Clausena excavata* Burm., a local herb used in many parts of Africa and Asia for traditional cancer treatment. Pyranocoumarins are a subclass of the phenolic compound family, and their major advantage is that they are cytotoxic to malignant tumors [5, 6]. Secondary metabolites in plants boost immunity because pharmaceuticals are primarily based on plant agents [7].

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Some plant secondary metabolites are used clinically as anticancer agents, while others are undergoing clinical trials [8, 9].

Previously, we isolated the novel pyranocoumarin clausenidin (Figure 1) that was used to treat HepG2 cells (liver cancer) in vitro. The isolated clausenidin crystal is promising but lacks in vivo data to support its anticancer properties. Therefore, the present study aimed to investigate the in vivo anticancer effects of pure clausenidin crystals in hepatocellular carcinoma-induced mice. Based on our literature search, the current study is the first to investigate the in vivo anti-liver cancer effect of pure clausenidin crystals.

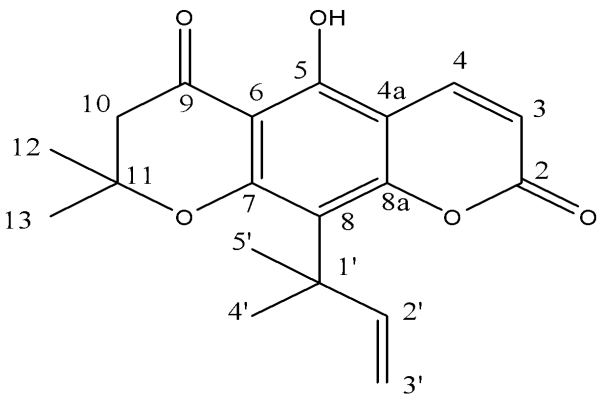

Figure 1. Clausenidin

Materials and Methods

Extraction of clausenidin

Pure crystals of clausenidin were extracted in our previous study from the roots of *Clausena excavata*, as described by Waziri et al. [10].

Animals

Male and female balb/c mice weighing 20 to 25 g were purchased and used in this study. Mice were kept in a metal cage inside a well-ventilated room with a 12-h/12-h light/dark cycle. The animals were handled based on National and International guidelines and protocols. Ethical approval was granted before the start of the study (AEC/2023/001).

Experimental Design

The animals were randomly divided into 5 groups of 4 mice each (Table 1). Hepatocellular carcinoma was induced using diethylnitrosamine (DEN), as described by Waziri et al. [11]. Following tumor development, the mice were treated with either clausenidin, PBS (1% v/v DMSO), or doxorubicin (positive control) for about one (1) week (Table 1). Thereafter, the animals were sacrificed and blood samples and liver tissue were collected for bioassays.

Liver enzyme analysis

Blood samples from treated and untreated mice with hepatocellular carcinoma were centrifuged to obtain serum that was used for alanine amino transferase (ALT) and aspartate amino transferase (AST). The analysis of serum liver enzymes was performed using a BK-200 Chemistry analyzer (Biobase, China) following the manufacturer's instructions.

Table 1. Experimental Design

Group	Treatment	Treatment Dose
A (Negative Control)	DEN + 1% (v/v) DMSO	-
B	DEN + CLS	50 mg/kg b.w.
C	DEN + CLS	100 mg/kg b.w.
D	DEN + CLS	150 mg/kg b.w.
E (Positive Control)	DEN + Doxorubicin	50 mg/kg b.w.

DEN, diethylnitrosamine; CLS, clausenidin; mg, milligram; kg, kilogram, b.w, body weight

Caspases assays

caspases 3, 8, and 9 assays were performed on liver samples using separate Caspase colorimetric assay kits (Biovision, USA). Briefly, 50 µl of the chilled lysis buffer was added to 20 mg of homogenized liver tissue in 1.5 ml Eppendorf tube and incubated at room temperature for 2 hours at 37°C. The lysed liver sample was centrifuged at 1000 rpm for 1 min to collect 50 µl of the supernatant, which was mixed with 50 and 5 µl of 2x reaction buffer and 4 mM LEHDpNA substrate, respectively, and incubated at 37°C for 2 h. Thereafter, the absorbance of the samples was recorded at 470 nm using a microtiter plate reader (Biorad, USA).

DNA fragmentation analysis

DNA fragmentation analysis of the liver tissues was performed using an Apoptotic DNA assay kit (Roche, Germany) following the manufacturer's instructions. Briefly, DNA was extracted from the liver tissues of mice and quantified using a UV/Vis Biophotometer (Eppendorf, Germany). The extracted DNA sample was mixed with loading buffer at a ratio of 5:1 and resolved by 1% agarose electrophoresis, which was run at 80 V for 30 min. The Gel images were captured using an EGel imager (Life Technologies, UK).

Histology

The harvested liver samples were fixed in 10% formalin for 48 h after the completion of animal treatment. A microtome (Reichert-Jung, UK) was used to cut the parafilm-embedded liver tissue into thin sections of about 3.5 µm. The cut sections were successively treated with xylene followed by 100, 95, 85, and 75% (v/v) ethanol before staining with hematoxylin and eosin (H & E) dye at room temperature. The stained sections of the liver tissue were viewed and captured under a microscope (Olympus, Japan).

Gene expression studies

Primer Design

Primer sequences were designed using primer blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the quality of each primer was evaluated using

OLIGOCAL software (http://biotools.nubic.northwestern.edu/OligoCalc.html). The primers were synthesized by Integrated DNA Technologies (IDT, Singapore), as presented in Table 2.

RNA extraction

RNA extraction from mouse livers was performed using a TRIpure Total RNA Extraction kit (ELK Biotechnology, USA) according to Manufacturer's instruction. Pure RNA was quantified using a nanospectrophotometer (Eppendorf, UK).

Reverse transcription quantitative PCR (RT-qPCR)

Reverse transcription quantitative PCR was performed using real-time PCR (Biorad, USA) and β-actin gene was used for normalization. Reverse transcription was performed using RT Master mix with gDNA remover (Toyobo, Japan) under the following conditions: genomic DNA removal at 37°C for 5 min, cDNA synthesis at 37°C for 15 min, and the reaction was terminated by incubating at 98°C to inactivate the reverse transcription enzyme. The PCR analysis of apoptotic genes was performed using qPCR Master Mix (Bridgen, China) under the following conditions: initial denaturation for 1 min at 95°C, 40 cycles of 15 s of denaturation, 30 s of annealing 60°C and final extension for 1 min at 74°C. Cycle threshold (ct) values were obtained and used to analyze fold changes in gene expression using Livak's ($2^{-\Delta\Delta Ct}$) method.

Statistical Analysis

Values obtained were reported as mean ± standard error of mean (SEM), and analysis of variance (ANOVA) was performed at 95% confidence level to determine the level of significance between treatment groups. The analysis was performed using SPSS software (IBM Inc, USA) version 27.

Results

Liver enzyme assay

The induction of hepatocellular carcinoma in mice caused liver damage and increased production of the liver enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST) above the normal range. However, treatment of mice with pure clausenidin

Table 2. Primer Sequences of Amplified Genes

S/no	Gene	Primer sequence
1	*P-53*	Forward–ACATCGTCGCTTTGGTGGAG
		Reverse: AAAACAAGCATCTGGGGTCCA
2	*P-27*	Forward–TTAGCCGAATCCAGCCTGTCA
		Reverse: CGGGGAACCGTCTGAAACAT
3	*P-21*	Forward–TTAGCCGAATCCAGCCTGTCA
		Reverse–TCCTGGATGTCTACGCCGTT
4	*MDM2*	Forward–TAGCAGCCAAGAAAGCGTGA
		Reverse: GGTTCGATGGCATTCAGGGA
5	*Bax*	Forward–TCCGGCGAATTGGAGATGAA
		Reverse: CCAGTTGAAGTTGCCATCAGC
6	*Bcl-2*	Forward–CTTTGAGTTCGGTGGGGTCA
		Reverse: GCCGGTTCAGGTACTCAGTC,
7	*Caspase 3*	Forward–ATGGGAGCAAGTCAGTGGAC
		Reverse: GTCCACATCCGTACCAGAGC
8	*Caspase 8*	Forward–AGGACCATGCTGGCAGAAAA
		Reverse: AGTCACACAGTTCCGCCATT
9	*VEGF*	Forward–ACGAAAGCGCAAGAAATCCC
		Reverse: GCAACGCGAGTCTGTGTTTT
10	*Cytochrome C*	Forward–TCTGTCTTCGAGTCCGAACG
		Reverse: ACTGGGCACACTTCTGAACA
11	*Catalase*	Forward–CACTGACGAGATGGCACACT
		Reverse: TGTGGAGAATCGAACGGCAA
12	*Cyclin A*	Forward: ACAGTTTCCCCAATGCTGGT
		Reverse: CGGCTGCTTCCTCATGTAGT
13	*Apaf 1*	Forward: CTGCTCTTCCCAGCACAACT
		Reverse: TGACGAGCAACAGGATGTGT
14	*Bid*	Forward–TCACGCACCATCTTTGCTCC
		Reverse: TCTTTCCATGGTCGCGGTTC

MDM2, murine double minute 2; VEGF, vascular endothelial growth factor; bcl-2, B cell lymphoma 2; bax, bcl-2 associated x apoptosis regulator; bid, BH3 interacting domain against death; apaf 1, apoptotic protease activating factor 1.

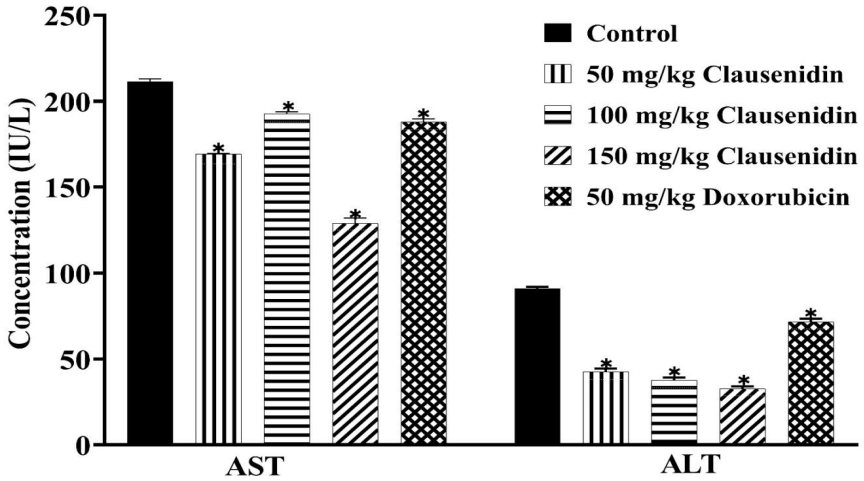

Figure 2. Serum AST and ALT Levels in Treated and Untreated Mice (IU – International Unit, L – liter, mg = milligram, kg - kilogram). *Means are significantly ($p<0.05$) different from control, n = 4.

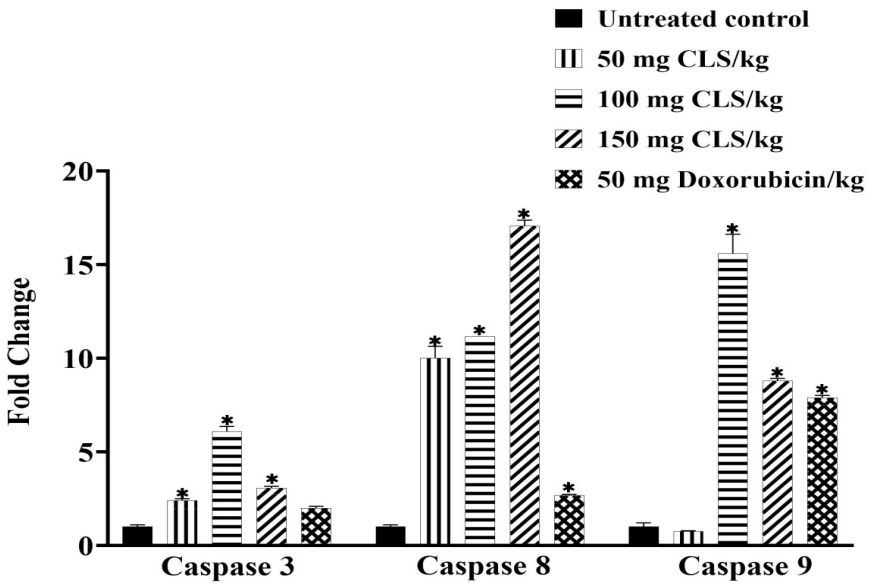

Figure 3. *Caspases 3, 8* and 9 Protein Level of Treated and Untreated Mice. *Means are significantly ($p<0.05$) different from control, n = 4.

improved the levels of liver enzymes ALT and AST (Figure 2). In fact, the ALT and AST levels of clausenidin-treated mice were significantly lower ($p<0.05$) than those of untreated mice (control group).

caspases 3, 8, and 9

Clausenidin (CLS) treatment significantly increased the concentrations of caspase 3, 8, and 9 proteins in mice (Figure 3). The concentration of caspase 8 proteins increased in a dose-dependent manner, whereas those of caspase 3 and 9 proteins were highest at a treatment dose of 100 mg/kg body weight.

DNA fragmentation analysis

Clausenidin treatment caused fragmentation of the genomic DNA of mice (Figure 4). The untreated control had intact genomic DNA (lane B) whereas the treated mice livers (lanes C to E). The positive control sample treated with actinomycin D also produced fragments of different sizes (Lane F).

Histology

Histological micrographs of mouse livers treated with diethylnitrosamine and clausenidin are shown in Figure 5. DEN treatment caused hepatocellular damage, including high nuclear polymorphism and disarrayed hepatic plate arrangement. The damage was more prominent in the untreated control, which showed hepatocellular fibrosis (Figure 5B). However, clausenidin helped slow the carcinogenesis as the treatment dose increased, and some of the cells showed nuclear condensation, especially at the highest treatment dose (Figure 5E). Liver damage was less severe in clausenidin- and doxorubicin-treated mice.

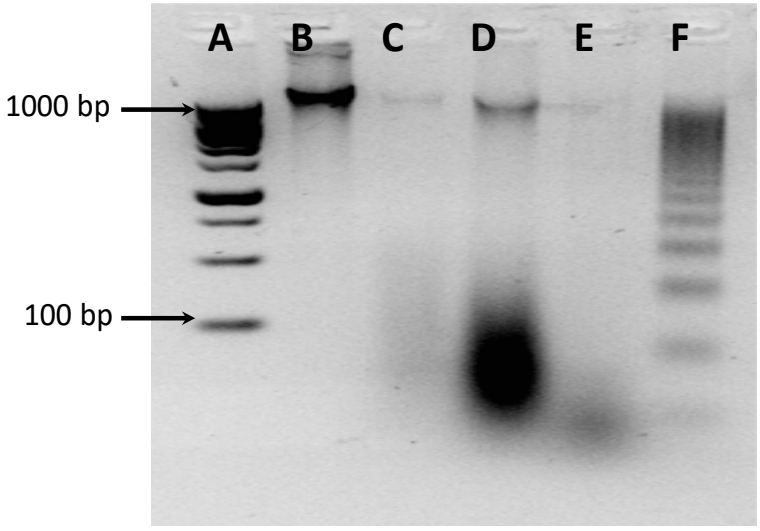

Figure 4. Agarose Gel Image of Treated and Untreated Mice DNA (Lane A – DNA ladder, B – untreated control, C – 50, D – 100 and E – 150 mg of CLS/kg bw of mice; F – positive control); CLS, clausenidin; bp, base pair; mg, milligram; kg, kilogram; bw, body weight.

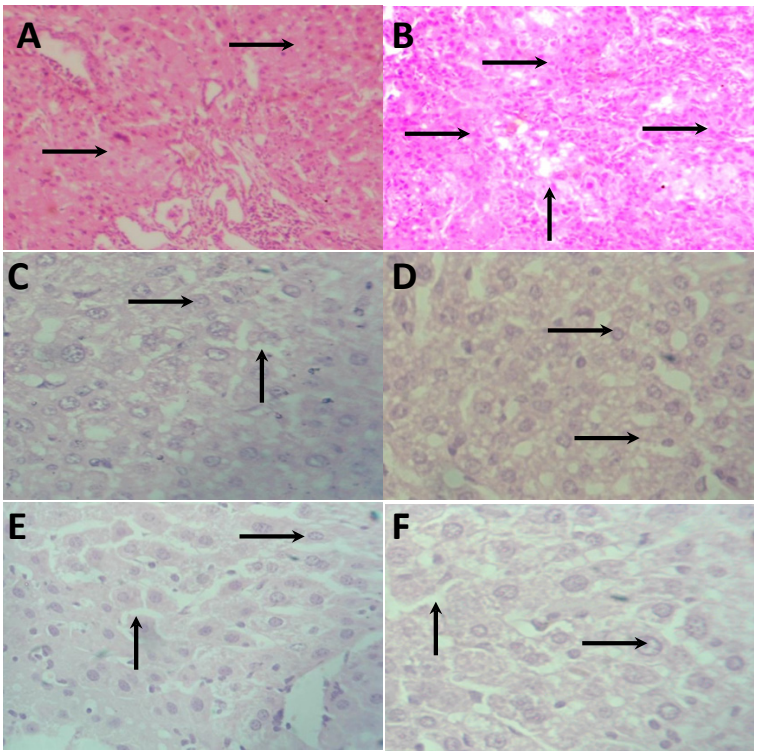

Figure 5. Hematoxylin and Eosin Stained Micrographs of the Mice Liver Tissues (A – normal mice liver, B – DEN treated liver, C – DEN + 50 mg CLS/kg, D – DEN + 100 mg CLS/kg, E – 150 mg CLS/kg and F – DEN + 10 mg DOX/kg bw of mice) (DEN – diethylnitrosamine, CLS – clausenidin, DOX – doxorubicin and bw – body weight). The arrows show regular hepatic portal tract (A); high nuclear polymorphism, non-linear arrangement of tumors in (B) (untreated control); moderate nuclear polymorphism, disarrayed arrangement of hepatic plates and mild fibrosis in C, D and E (clausenidin treated) as well as F (positive control); Magnification = x400).

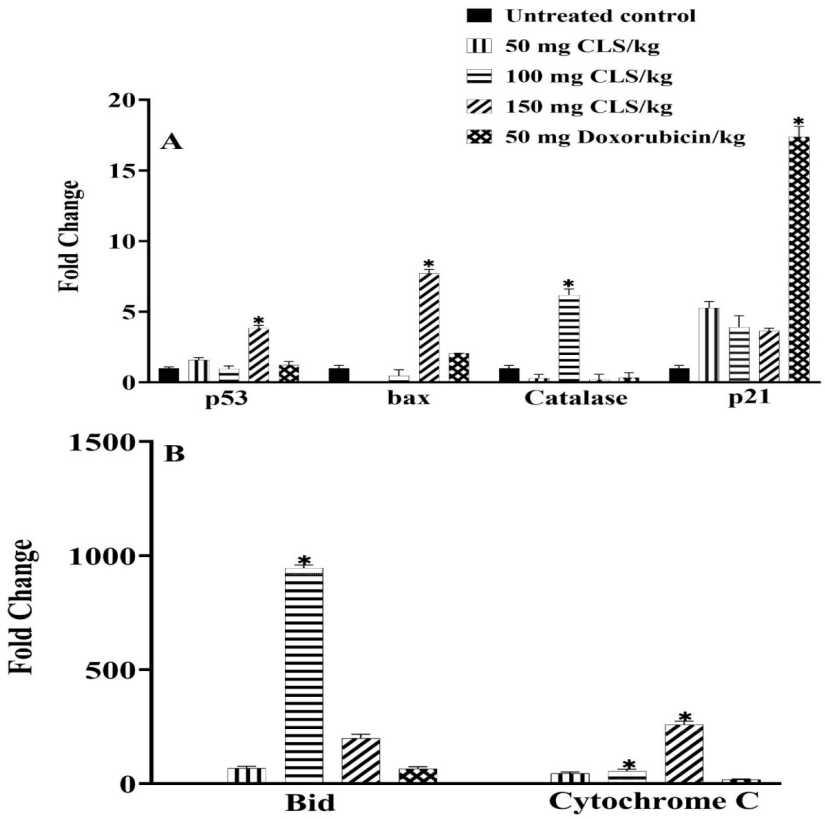

Figure 6. Effect of Clausenidin on the mRNA Expression of the *P-53, bax, catalase,* and *P-21* Genes (A); bid and cytochrome c (B). CLS, Clausenidin; mg, milligram; kg, kilogram. *Means are significantly ($p<0.05$) different from the control, n = 4

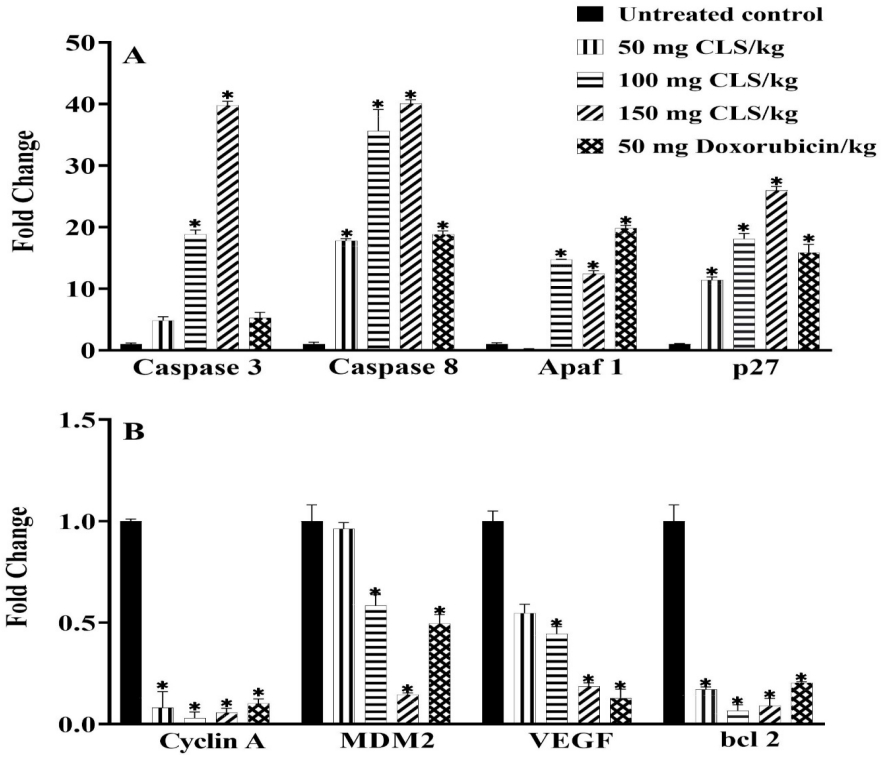

Figure 7. Effect of Clausenidin on the mRNA Expression of *caspases 3 &* 8, *apaf-1*, and *P-27* (A); *cyclin A, MDM2, VEGF*, and *bcl-2* (B). CLS, Clausenidin; mg, milligram; kg, kilogram. *Means are significantly ($p<0.05$) different from the control, n = 4.

RT-qPCR analysis of liver gene expression

Clausenidin significantly increased the levels of *P-53*, *P-21*, *bax, bid,* and *cytochrome* c mRNAs (Figure 6). The mRNA levels of bid and cytochrome c mRNAs were the highest among all genes investigated in the current study (Figure 6B). In addition, treatment caused a significant increase in the mRNA expression of *catalase, apaf-1, P-27, caspases 3 & 8* mRNAs (Figures 6A and 7A).

Conversely, the mRNA expression of *cyclin A, MDM2, VEGF*, and *bcl-2* decreased significantly following treatment with clausenidin (Figure 7B).

Discussion

Previously, we reported that clausenidin is a potent and promising anti-liver cancer compound based on in vitro studies on HepG2 cancer cell lines [10, 12]. Since then, numerous studies have been conducted to investigate the anticancer effect of pure clausenidin on different cancer cell lines. However, to date, no study has demonstrated the anti-liver cancer effect of pure clausenidin crystals in an animal model. Therefore, the current study aimed to investigate the in vivo anticancer effect of clausenidin in hepatocellular carcinoma-induced Balb/c mice.

Clausenidin was isolated from the roots of *Clausena excavata* as described by Waziri et al. [10] and was used to treat hepatocellular carcinoma-induced mice for in a dose-dependent manner. The induction of hepatocellular carcinoma with diethylnitrosamine triggered the production of ALT and AST, which are key markers of hepatocyte damage. Moreover, increased production and expression of ALT and AST in the blood are a feature of deranged protein metabolism and liver cancer [13, 14]. It was observed that treatment of mice with pure clausenidin crystals halted and decreased the liver damage-induced production of ALT and AST, which is typically observed with anticancer drugs.

Cancer is the result of dysfunctional cell growth, and apoptosis is the preferred route of cancer cell death [15]. The fragmentation of nuclear DNA, cell membrane blebbing, and nuclear condensation are features of apoptosis. Histological analysis of the mouse liver showed that some cell nuclei were condensed. We observed that clausenidin treatment caused fragmentation of the nuclear DNA of mice, which is often caused by caspase 3-activated DNases (CAD). CAD is usually activated in tumors undergoing apoptosis [16, 17]. It is suspected that clausenidin treatment activated the DNases that cleaved genomic DNA in mice with hepatocellular carcinoma.

Caspase protein assays were conducted to confirm the occurrence of apoptosis in clausenidin-treated mice. The assays revealed that clausenidin significantly increased the expression of *caspases 3*, 8, and 9 in mouse liver tissue. Caspases act as tumor suppressors [18]. Caspase is a major target in cancer cell therapy because its activation primes tumors for programed cell death. Based on the modes of activation and substrate preference, caspases are divided into initiators (*caspases 8 & 9*) and executioners (*caspases 3* and 7). *Caspase 8* initiates the extrinsic pathway of apoptosis because signals are generated from outside the cell, whereas *caspase 9* initiates the intrinsic pathway of apoptosis because signals are generated from within

the cell. Our observations showed that both external and internal signals were generated for clausenidin-induced apoptosis in mice tumors.

Dysfunctional gene expression is a hallmark of cancer. In this study, we observed that clausenidin treatment affected the expression of some cancer-related genes. Specifically, the mRNA expression of *P-53*, *P-21*, *p-27*, *caspases 3* and 8, *catalase, apaf 1, bax, bid,* and *cytochrome c* increased, whereas those of *bcl-2*, *cyclin A, VEGF,* and *MDM2* decreased in the mice after treatment. Of the genes analyzed, bid and cytochrome c were the most upregulated following treatment with clausenidin. These genes are tightly involved in the intrinsic pathway of apoptosis, suggesting that apoptosis occurs more through the intrinsic pathway in clausenidin-treated mice. Upon activation of the intrinsic apoptotic pathway by caspase 9, bax and bid genes are activated, leading to the production of their corresponding proteins and other downstream proteins in mitochondria. The mitochondrial permeability increases and allows the passage of downstream proapoptotic proteins like cytochrome c, into the mitochondria to combine with dATP and apaf 1. The trio of cytochrome c, dATP, and apaf 1 completes the formation of the apoptosome complex, leading to the activation of *caspase 3* that helps to execute apoptosis [19-21]. We also observed increased expression of the catalase gene, which we suspect was triggered in response to the oxidative stress generated in the intrinsic pathway.

The findings of this study also suggest the involvement of the extrinsic pathway of apoptosis. The increased expression of caspase 8 protein and mRNA indicates that clausenidin treatment activates external signals of apoptosis in the mice. The external and intrinsic pathways of apoptosis are closely linked together [22].

In addition, clausenidin treatment led to downregulation of *bcl-2*, *MDM2*, and *VEGF* expression. Overexpression of the *bcl-2* gene and its protein is responsible for many cancers [23]. Mutation of the *bcl-2* gene usually leads to a gain of function and consequent overexpression of the gene and increased proliferation of cancer cells. The *bcl-2* gene is an oncogene that prevents apoptosis. Some drugs have been designed to target the *bcl-2* gene and prevent its overexpression in tumors [24]. The decreased expression of the *bcl-2* gene following treatment in mice in the present study could have enhanced the therapeutic effect of pure clausenidin crystals.

Murine double minute 2 (*MDM2*) is also an oncogene that regulates the expression and activity of the tumor suppressor *P-53*. *P-53* is the guardian and protector of the genome for many reasons. It maintains the integrity of DNA, provides genome stability, surveillance, and repair of DNA damage. *P-53* generates numerous signals that lead to the apoptosis of cancer cells. However, *MDM2* is a negative regulator of *P-53* and other proteins involved in DNA repair and apoptosis [25]. This leads to the sequestration and subsequent destruction of *P-53*, which allows cells to evade apoptosis and become cancerous. For this reason, many anticancer agents target the *MDM2*-P53 interaction [26, 27]. The lower the expression of *MDM2* in cells, the higher the activity of *P-53* and the rate of tumor cell death. In the present study, we observed decreased expression of *MDM2*. By this we postulate that one of the possible mechanisms of action of clausenidin in mice is through downregulation of the *MDM2* gene, which allowed *P-53* gene activity.

Furthermore, clausenidin induced anti-cancer effects in treated mice by inhibiting angiogenesis, which involves a multi-step process in which tumors grow and spread to other tissues [28]. Angiogenesis is the major driver of cancer metastasis and is accomplished via the transcription of angiogenic genes [29, 30]. Specifically, the vascular endothelial growth factor *(VEGF)* gene is under strict transcriptional control because it drives the sprouting and formation of new blood vessels that provide the requisite nutrients needed to sustain and maintain tumors [30]. In fact, *VEGF* gene damage enables the transformation of benign to malignant tumors. Hence, the *VEGF* gene and its product are target molecules for many anti-angiogenic drugs used in cancer chemotherapy [31]. Previously, clausenidin was reported to inhibit *VEGF* expression in HepG2 liver cancer cell lines in vitro [12]. In the present study, clausenidin downregulated *VEGF* gene expression in mice in a dose-dependent manner. Thus, it is evident that the anticancer effect of pure clausenidin crystals occurs through the inhibition of angiogenesis.

In conclusion, bioactive compounds from herbs continue to play significant roles in human health. The investigation of the biological activity of compounds is sine qua non for drug discovery. The present study provides evidence of the in vivo anticancer effect of pure clausenidin crystals. Clausenidin treatment significantly upregulated the expression of pro-apoptotic extrinsic and intrinsic genes while downregulating the expression of anti-apoptotic genes. This suggests that the major mechanism of clausenidin-induced cancer cell death is apoptosis. Thus, clausenidin has the potential to serve as an anti-hepatocellular carcinoma drug.

Author Contribution Statement

Peter M Waziri: Manuscript preparation/ Research Design. Richard Auta: In vivo assay. Mustapha Umar Imam: Gene expression studies. Ben Chindo: In vivo assay. Zakari Ladan: Research Design. Zainab Mohammed: In vivo assay. Samson Wayah: Statistical analysis. Jaafar M Sani : Gene expression studies. Godwin I Ayuba: Histology assay. Oluwafemi T Ige: Gene expression studies. Daniel Tyoapine: In vivo assay. Abel Agbaji: Research design/protein assays.

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Ethical Approval

The ethical approval for this study was granted by

the Research Ethics Committee (KASU/AEC/2023/001), Kaduna State University.

Data availability

All data are available on request

Declaration of conflict of interest

The authors declare that there is no conflict of interest.

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