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Synergistic Hepatoprotective Effects of Zingiber Officinale Rosc (Ginger) and Capsicum Annuum Linn (Hot Pepper) on Hematological, Biochemical and Histopathological Alterations in Hepatocellular Carcinoma-induced Albino rats

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ABSTRACT: Several treatment options have been sought to improve the blood indices against hepatocellular carcinoma cases. Despite this treatments, patient continue to suffer from several side effects. The alternative therapeutic agent in the management of hepatocellular carcinoma is imperative. The plant-derived anti-carcinoma are widely accepted and generally perceived as safe. The ethanol extract of Zingber officinale Rosce and Capsicum annuum Linn at doses 200 and 400mg/kg were investigated for anti -hepatocellular carcinoma activities on blood and histopathological indices of rats induced with 300mg/kg thioacetamide. Forty albino rats were divided into 4 groups of 10 rats each and the following treatments were given; Group 1) received feed and water only. 2) received thioacetamide (300mg/kg) + 200mg/kg of extracts. 3) received thioacetamide (300mg/kg) + 400mg/kg extracts. 4) received thioacetamide (300mg/kg) only. Treatment was stopped after 28days. Blood samples were taken for both hematology and biochemical examinations and liver tissue for histopathology. Results: The result showed a significant decrease ($p<0.05$) in HGB, RBC, MCV, MCH and MCHC levels following administration of thioacetamide to the TC group and a relative significant increase ($p<0.05$) in 200mg/kg and 400mg/kg group. There was also a significant increase ($p<0.05$) in ALP, ALT, urea and creatinine levels following administration of thioacetamide (TC) when compared to NC group. . The synergistic use of the extracts

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and essential oil of these plants on hepatocellular induced rats showed an impressive recovery rate both in blood parameters and liver histopathology.

1. INTRODUCTION

Hepatocellular carcinoma, the primary cancer of the liver, is derived from hepatocytes and occurs in more than approximately 80% of cases of liver cancer. The development of hepatocellular carcinoma results from the interaction between environmental and genetic factors. Control of cancer may be accomplished by a variety of treatments including: suppressing, blocking, and transforming agents. The use of suppression agents prevent the formation of new cancers from pro-carcinogenesis, while blocking agents prevent carcinogenic compounds from reaching critical initiation sites but transformation agents act to facilitate the metabolism of carcinogenic components into less toxic materials or to prevent the biological actions of the carcinogen. Other methods for controlling cancer involve blocking metastatic cascades through inhibiting cancer cell invasion into surrounding tissues or by inhibiting cancer cell mobility in circulatory systems **[1].** The Expert Committee of the World Health Organization (WHO) has recommended that traditional herbs and alternative medicines should be screened to identify safe natural products with hepatoprotective potential for patients in developing countries **[2]**. This recommendation is in keeping with the use of *Zingiber officinale* and *Capsicum annuum* as folk medicine to treat hepatitis and liver cirrhosis **[3].** Medicinal plants are a rich source of bioactive compounds with desirable health benefits and are traditionally used to prevent or manage chronic liver disease **[4]**. There are several active compounds which have been discovered from plants on the basis of ethnobotanical information and used directly as potential or bases in the production of synthetic drugs. This makes the use of plants reliable and effective. Fortunately, studies done across the world demonstrated and reported the awareness and the use of herbal medicine among the general population. According to Hosseinzadeh et al. the majority of the poor and the less advantageous people living in the rural areas and urban centres are dependent on medicinal plants for common diseases **[5].** Several factors are responsible for the dependence on the use of herbal medicines **[5].** These are; drug resistance, cost effectiveness and availability. Herbal drugs are safe and can be consumed over a period of time with minimal or without side effects of any scientific proof **[5].** Among some of the herbs with proven pharmaceutical potential include *Capsicum annuum* Linn and *Zingiber officinale Rosce* (common ginger).
Capsicum annuum Linn belonging to the family of *Solanaceae* is an extremely valuable medicinal herb, **[6].** Many research studies have been conducted to prove the plant's potential as being analgesic, anti-angiogenic, anti-parasitic, antiplatelet, anti-arthritic, antioxidant, anti-viral, anti-fungal, hypoglycemic, gastro protective and tumoral effects. The pungency is the most outstanding property of the so-called "hot" pepper, resulting from the accumulation of capsaicin and other related compounds commonly known as capsaicinoids. These are organic compounds which are only found in the capsicum genus and bioactive molecules currently relevant in medical and food science. Its chemical structure is composed of a vanillylamide moiety and an acyl chain of 8-13 carbon atoms **[7].** Two major capsaicinoids present in most varieties of hot pepper are capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnona-namide). In addition to the two major components, other minor capsaicinoids also existed in hot pepper, including nordihydrocapsaicin, norcapsaicin, homocapsaicin, homodihydrocapsaicin, nornorcapsaicin, nornornorcapsaicin, and nonivamide **[8]**. Some minor capsaicinoids have also been identified but are present at very low levels and are not thought to make any contribution greatly to the overall pungency of the capsicum fruits. In particular, capsaicinoids are ant-mutagenic, antitumor and antioxidants compounds **[8].** Recently, the capsaicinoids have attracted the interest of many researchers because they show promise of being powerful antioxidants, protecting the human body from free radicals. In addition, one benefit of hot pepper is that they contain bioactive components such as polyphenol that can reduce oxidative stress and modulate harmful biological pathways, thereby reducing some chronic diseases.

Zingiber. officinale (ginger) has been used as a spice and as natural additives for more than 2000 years **[9]**. Studies have shown that, the long term dietary intake of ginger has hypoglycemic and hypolipidaemic effect **[10].** Ginger has been identified as herbal medicinal product with pharmacological effect. It suppresses prostaglandin synthesis through inhibition of cyclooxygenase- 1 and cyclooxygenase- 2. The anti-cancer effects of ginger are linked to its ability to induce apoptosis (programmed cell death) in cancer cells, inhibit cell proliferation, and prevent angiogenesis (the formation of new blood vessels that supply nutrients to tumors). Ginger compounds have been found to modulate various signaling pathways involved in the development and progression of cancer, such as the mitogen-activated protein kinase (MAPK) pathway, the extracellular signal-regulated kinase (ERK) pathway, and the phosphoinositide 3-kinase (PI3K)/Akt pathway. By targeting these pathways, ginger

can exert a therapeutic effect against cancer cells. The volatile oil of ginger consists mainly of the mono- and sesquiterpenes; camphene, *β*-phellandrene, curcumene, cineole, geranyl acetate, terphineol, terpenes, borneol, geraniol, limonene, *β*- elemene, zingiberol, linalool, *a*-zingiberene, *β*- sesquiphellandrene, *β*-bisabolene, zingiberenol and *a*- farmesene **[11].**

Zingiberol is the principal aroma contributing component of ginger rhizome. The species contains biologically active constituents including the non-volatile pungent principles, such as the gingerols, shogaols, paradols and zingerone that produce a "hot" sensation in the mouth **[12].** The gingerols, a series of chemical homologs differentiated by the length of their unbranched alkyl chains, which were identified as the major active components in the fresh rhizome. The pungency of dry ginger mainly results from shogaols, which are dehydrated forms of gingerols. Gingerols are thermally labile because of the presence of a *β*- hydroxy keto group and readily undergo dehydration to form the corresponding shogaols **[13].** Paradol is similar to gingerol and is formed on hydrogenation of shogoal. Oleoresin, which is isolated by acetone and ethanol extraction, contains 4-7.5% of dried powder, pungent substances namely gingerol, shogaol, zingerone and paradol **[14].**

In traditional Chinese and Indian medicine, ginger has been used to treat a wide range of ailments including stomach aches, diarrhoea, nausea, asthma and respiratory disorders. Ginger has a long history of being used as a medicine and has been found to counteract oxidative stress and restore antioxidant enzyme levels in the liver, contributing to its protective role against cancer development and other diseases **[15,16].**
Ginger extracts have been found to decrease white blood cell (WBC) counts in mice, suggesting an immunomodulatory effect and increase red blood cell (RBC) counts, suggesting a potential erythropoietic effect. Hemoglobin can be affected by liver cancer due to factors like reduced liver function impacting blood cell production or treatment side effects **[17, 18]**. Ginger aqueous extract have been identified to induces change on the levels of liver enzymes, biochemical parameters, and histological changes in male Wistar rats following treatment with streptozotocin, a chemical that induces diabetes and liver damage **[19].** These changes suggested that ginger extracts may have a protective effect on liver function by reducing the levels of these enzymes, which are indicative of liver damage **[20, 21].**

In addition to streptozotocin, TAA represents a second widely used model for the induction of experimental liver fibrosis, but can also be employed for the development of acute liver failure and liver tumours. While thioacetamide itself is not hepatotoxic, its reactive metabolites covalently bind to proteins and lipids thereby causing oxidative stress and centrilobular necrosis. Compared with CCl_4, thioacetamide leads to more per portal infiltrates and more pronounced ductal proliferation. TAA could cause hepatoxicity due to cellular damage by increasing the serum levels of aminotransferase (ALT) **[22, 23].**

This research work employed the use of thioacetamide on experimental animals to induce hepatocellular carcinoma in order to determine the effect of combination of ginger and hot pepper on blood parameters, biochemical and histological changes in liver damage.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh ***Zingiber officinale*** **Rosc (**ginger) rhizome and *Capsicum annuum* Linn (hot pepper) with the herbarium code **(common ginger) ZINOF used in the EPPO Global database and The letter "C" is the code for Capsicum annuum (hot pepper) though no universal code CAPSI_ANN from UPOV,** used in this study were obtained from Federal University of Technology Owerri farm and authenticated as common *Gingiber officinale* and *Capsicum annum* respectively by Dr M.C Nwoko (a botanist) in the Biology department of the University. The albino rats, aged 2-4 months and weighing 119-161g were supplied by the Animal Care Services in Michael Okpara Federal University of Agriculture Umudike, Abia State, Nigeria. Thioacetamide used as induction agent was obtained from Biochemistry Unit, department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria and identified by Dr E U Ejiofor (a chemist and researcher) in the University.

2.2 Extraction Procedure

Fifty grams (50 g) of the dried grounded pepper and ginger were weighed and each extracted with 400 ml of ethanol using Enegide et al. extraction method **[24].** The processes were run for 2 hours each, after which the samples were evaporated to dryness using water bath. The dried extracts were weighed and kept in a well labeled sterile specimen bottles and stored in a refrigerator at 4degree celsius until is required.

2.3 Analytical Methods

2.3.1 Acute toxicity/lethal dose (LD_{50}) test

The acute toxicity/medium lethal dose of the crude extracts of ginger and hot pepper were determined by Jonsson et al. method using the oral routes with the assistance of Mr Dave of Animal house, Federal University of Technology Owerri, Imo State, Nigeria **[25]**. The acute oral toxicity study was conducted in compliance with OECD guideline 425, which stipulate the use of only three animals. The test was divided into two stages. Twelve (12) male albino rat of body weights (*b.w.*) mean of 119-161g were purchased from the Animal Breeding Unit, Federal University of Agriculture, Umudike in Abia state, Nigeria. The animals were kept in stainless–steel cages in a well–ventilated room of temperature 25 ± 2°C and relative humidity of 55–65% with a diurnal 12 hours light cycle. The rats had access to water and pelletized standard finishers. A period of 2 weeks was allowed for acclimatization of the rats to environmental conditions.

Experimental design for toxicity:

Stage One: Determination of the toxic range of the extracts. Rats were divided into 3 groups of 3 animals in each group. Each group received a dose (10, 100, 1000mg/kg) of the ethanol extracts of *Z. officinale* and *C. annuum*. The doses were administered orally and the treated animals observed for 72hrs for number of death.

Stage Two: Determination of lethality of extracts; Doses used in this stage were determined from the number of deaths per dose recorded in the stage one test. As no death occurred in the stage one test, three different higher doses; 1600mg/kg, 2900mg/kg and 5000mg/kg were administered to another group of animals at one dose per animal. The treated animals were monitored for number of deaths for 24hrs and continued to 72hrs. The LD_{50} in this test is determined by calculating the geometric mean of the test and most toxic doses.

LD_{50} Determination

The index of acute toxicity is the LD_{50}. LD_{50} is the dose of a substance capable of producing death in 50% of the population of animal exposed to the substance. Enegide et al. method was used for this determination **[24].**

$$LD_{50} = \sqrt{\text{minimum toxic dose x maximum tolerated dose}}$$

2.4 Phytochemical and proximate analysis

Both quantitative and qualitative analysis were carried out to determine the active phytochemicals and nutritional composition of ginger and hot pepper extract. Phytochemical analysis helps identify the presence of bioactive compounds like alkaloids, flavonoids, phenolic acids, saponins etc, which are responsible for the medicinal and biological properties of plants. The proximate analysis of the samples for moisture content, crude protein, ash, fat, crude fibre, carbohydrates was determined using the methods of the **[25].**

Determination of rats' weights: This was done using an appropriate weighing balance. The procedure was repeated on weekly basis to ascertain the difference in weights.

Milligram equivalent (mg-eq) of rats' weights: the milligram equivalent of each rat's weight in a group was calculated using the standard dose.

Mineral Composition determination

The samples were wet digested with concentrated nitric and perchloric acids. The minerals calcium (Ca) and magnesium (Mg) were determined by atomic absorption spectrophotometer (Model 3030 Perkin Elmer, Nortwalk, USA). Potassium (K) and Sodium (Na) were determined with the aid of corning 400 flame photometer according to the method of **[26]**. Phosphorus was determined using spectrophotometer (JASCO V-630). The concentration of phosphorus was determined through the measurement of the yellow phosphor vanado - molybdate complex using Cecil Digital Spectrophotometer series. The mineral Fe, Zn, Cu and Mn contents of raw Capsicum and Zingiber flour were determined using Buck Scientific Atomic Absorption Spectrophotometer (AAS, Buck scientific, East Norwalk, USA), model 201 VGP at 248.3, 213.9, 324.7 and 279.6 nm, respectively.

Amino acid profile determination

The amino acid profile of the plant extracts were determined by the method described by Olubunmi et al using an amino acid analyzer (Technicon Sequential Multisample Amino Acid Analyzer, Tech-nicon Instruments Corporation, New York) **[27].** Briefly, the extracts were defatted using chloroform methanol. About 40 mg of the each defatted sample was weighed into a glass ampoule, and 7 mL of 6 M HCL was added, and oxygen was expelled by passing nitrogen gas into the ampoule. The glass ampoule was then sealed with a Bunsen flame and put into an oven at 105 °C for 22 hrs. The sample was allowed to cool and then break open at the tip and the content was filtered through filter paper to remove the residue. The filtrate was evaporated to dryness at 40 °C under vacuum with a rotary evaporator. The residue was dissolved with 5 mL acetate buffer (pH_2). About 5-10 mL was injected into the cartridge of the analyzer for amino acid determination.

Gas Chromatography-Mass Spectrometry GC-MS

The experimental GC-MS was carried out on a (Agilent technologies GC- MS. CA, USA) which are obtained in the form of mass spectra, relative intensity (RI) against mass to charge ratio (m/z) for all the compounds studied using electronionization (EI). Cinnamic acid, cinnamyl alcohol and methyl cinnamate are used as standard for identification. Chemical composition of ethanol extracts of ginger was tested by GC–MS) with an Agilent 7890B Series auto-injector, coupled to an Agilent 5977A Mass Selective Detector. The carrier gas used was He with the flow rate of 40 ml/min with DB 1 as the columns (pressure 8.8085 psi) and Electron Impact (EI) as the ionizer. The sample was heated from 70°C up to 250°C with a heating rate of 10°C/min. The detector and injector temperature was 250°C with the initial time of 1 minute. Helium was used as the carrier gas with a constant flow of 1.2 mL/min. The initial temperature of oven temperature program was set at 40°C and continued for 4 min, rising by 5°C/min to 250°C, which continued for 10 min. The volume of injected sample was 1µL. Electron ionization (EI) was used in the MS and standard mass spectra with 70uv ionization energy were recorded m/z from 0-500 at one hour.

High Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) method is considered the most reliable and accurate method for determining capsaicinoids. Preliminary purification of the extract has been applied before HPLC analysis of capsaicinoids. Thin Layer Chromatography (TLC) and Column Chromatography (CC) methods are also used. Extracted capsaicinoids using column chromatography on silica gel were then quantitatively evaluated with a reverse phase-high performance liquid chromatography/photodiode array detection (RP-HPLC/PAD).

Induction with Thioacetamide

Thioacetamide was dissolved in 0.9% of normal saline to make up total volume of 1-1.5ml per rat approximately one hour before injection; treatment groups received 300mg/kg of TAA twice per week for a total of four weeks. After four weeks of treatment with TAA, virtually all the treated animals have developed the histological features of cirrhosis when examined such as bulging eyes, running nose and yellowish eyes.

Preparation of Extract for Treatment

One gram each of the extract of ginger and hot pepper (capsaicinoid) were weighed and dissolved in 10 ml distilled water. The volume used was calculated according to the body weight of the animals using Tedong et al equation. A low (200mg/kg) and high (400mg/kg) were used.

$$\text{Vml} = \frac{DxP}{C}$$

D = dose used

P = Body weight

C = concentration

V= volume

Experimental Design for the treatments

Forty (40) adult male and female albino rats (119 - 161 g) were used in this study. The study was carried out and the animals were handled according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals. The 40 albino rats were divided into 4 groups of 10 rats each and the following treatments were carried out;

Groups	Treatment
Group 1 (NC)	Feed and water only (normal control).
Group 2	Thioacetamide (300mg/kg)+200mg/kg of the combination extract.
Group 3	Thioacetamide (300mg/kg)+400mg/kg of the combination extract.
Group 4 (TC)	300mg/kg of Thioacetamide only

The different treatments were given using a gavage to ensure safe ingestion. On the 28th day, the treatment was stopped, and the animals were observed for another 28 days.

Determination of Hematological parameters

An automated hematology analyzer (Beckman Coulter, Inc, USA) was used to determine blood parameters; while a chemistry analyzer (Abaxis Inc, Union City, CA, USA.) was used for the biochemical indices. Hematological parameters evaluated included hemoglobin, red blood cell (RBC), white blood cell (WBC) and platelet (PLT) counts. Other parameters include hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), etc.

Blood samples were taken for both hematology and biochemical examinations. Blood was drawn through the eyes using a 2ml tube and transferred to two tubes, one with EDTA for immediate hematological analysis and the second one with lithium heparin tube for centrifugation at 4000 rpm for 10 minutes to separate plasma for biochemical estimation.

Histology determination

Liver samples were also collected for histopathology by opening the abdomen. The liver tissues collected were immersed in 10% formalin and further prepared for histology using method described by **[28].**

Sub-acute toxicity

Sub-acute toxicity was conducted to ascertain the safety of the extract on the internal organs. The test animals that recieved doses (200, and 400 mg/kg per body weight) of the extract by oral gavage and the group (control) received that received feed and distilled water were used for this experiment. Blood samples were collected through the 2 common carotid arteries of the animals under anesthesia (3 % pentobarbital sodium; dose: 50mg/kg).

2.5 Statistical Analysis

One-way analysis of variance was used, followed by Data are expressed as mean ± standard deviation (SD, n = 10). A single-factor parametric Duncan's Multiple Range Test was used to determine significant differences among treatment means. Data were considered significant at $p < 0.05$. All statistical analyses were performed using the Minitab Student Version 12 for Windows Software (Minitab, Inc. State College, Pennsylvania) Minitab version 12).

3. RESULTS AND DISCUSSION

3.1 Preliminary Analysis

3.1.1 Acute toxicity or lethality test study

No clinical signs of toxicity were observed in any of the treated rats in stage one at the dose levels of ginger and hot pepper extract. No mortality was observed in the rats. In stage two when the extract was increased. No mortality was observed,

clearly showing that the extract was non-toxic; body weights were recorded prior to oral administration (day 1) and after 72 hours following oral administration.

LD_{50} > 5000mg/kg.

3.2 Instrumental Analysis

3.2.1 Phytochemical screening result

Extracts obtained from the previously mentioned extraction methods were subjected separately to phytochemical screening tests. Alkaloid, Flavonoid, Carbohydrate, Protein, Phenol, Tannin, Saponins, Steroid were observed to be present in different concentrations**.**

3.2.2 Mineral composition

There were presence of macro minerals such as Fe, Zn, Cu, K and Ca in the extracts of *Z. officinale* and *C. annum* in different concentrations, which might contribute to the result.

3.2.3 Amino acid profile

The nutritive value of a protein depends on the ability to provide the needs for nitrogen and the essential amino acids. The presence of the essential amino acids such as threonine, methionine, isoluecine, phenylalanine, lysine, histidine, valine, argunine and luecine in *Z. officinale* and *C. annuum* in different concentrations, suggested that these extracts could be used as food supplement and food additive for the purpose of flavor or as preservative that kills harmful bacteria or prevent their growth. These amino acids have been reported to alleviate or reverse hepatic damage abnormalities in animals via declined oxidative stress.

GC-MS and HPLC results of the characterization and components of *Z. officinale* and *C. annuum* ethanolic extract.

The GC-MS and HPLC results of the characterization of ethanol extract of *Z. officinale* and *C. annuum* are shown in tables 1 and 2.

Table 1: GC-MS chemical components of *Z. officinale* extract

Peak	Compound	M/z	Tt(min)	Amount(mg/l
1	Alpha- curcumene	202	17.804	35.46
2	Zingiberene	202	17.870	22.16
3	Alpha-Ylangene	204	22.401	2.10
4	Zingerone	194	25.234	3.62
5	Iso- Jasmone	166	54.023	0.20
6	Cyclohexane	202	38.535	1.30
7	Beta-Bisabolene	204	18.290	12.34
8	Beta-sesquiphellandrene	206	17.942	21.07
9	Cyclohexanemethanol 4- ethenyl, alpha,alpha 4-trimethyl-3-(1-methylethenyl)-	208	19.206	1.33
10	Nephthalene	204	39.568	9.09

11	2-oxabicyclo-octane 1,3,3 trimethyl	196	17.930	7.17
12	Cyclohexene	202	39.796	6.21
13	Baros camphor	197	24.822	2.96
14	- (-)Alconfor	186	23.559	2.75
15	Camphene	202	14.047	2.60
16	2-methyl-3-phenyl propanal	204	28.032	1.85
17	Acintene A	202	13.264	1.80
18	∞-Terpinyl propionate	186	25.815	1.32
19	β-Eudesmol	204	45.660	1.28
20	Elemol	204	41.667	0.99
21	1,7-di-epi-a-cedrene	197	38.345	1.14
22	Nerolidol	197	41.688	0.99
23	Delta-cadinene	202	40.088	0.95
24	alpha cubebene	204	13.586	0.67
25	Beta- elemene	204	14.165	0.51
26	Thymene	216	26.134	3.51
27	beta-Eudesmol	222	22.486	2.17
28	Germacrene B	222	21.760	0.36
29	Geranyl acetal	167	12.279	6.00
30	Alloaromadendrene	204	14.655	0.42

Table 2: HPLC analysis of components of the capsaicinoids found in *C. annuum* sample expressed as dihydrocapsaicin equivalent (µg/g DW) and their relative amount ratio (%)*

Peak	Type of Compound	M/z	Rtime(min)	Relative amount **(µg/g DW)**
1	Nornordihydrocapsaicin	278	7.40	9.7±0.2 (0.1%)

2	Nordihydro-capsaicin	288	8.07	448.7±4.4 (5.9%)
3	Capsaicin	305	9.01	3462.9±6.9(45.1%)
4	Dihydrocapsaicin	307	9.20	3208.7±6.5(43.1%)
5	N-vanillyl-nonanamide	305	7.90	73.1±2.4 (0.8%)
6	Homocapsaicin	305	8.50	176.3±2.4 (2.4%)
7	N-vanillyl-decanamide	320	8.62	22.7±0.2 (0.3%)
8	Homodihydrocapsaicin Isomer 1	321	9.99	141.2±4.4 (0.4%)
9	Homo dihydrocapsaicin Isomer 11	321	9.96	30.7±4.7 (0.4%)
	Total capsaicinoid analyzed	-		7574 (100.0%)

Based on the normalized value(100.0%) of total capsaicinoid. mean±%SD.

Table 3: Characterization, hematological and biochemical parameters measured after treatment

GROUPS	NC (G1)	200mg/kg extract (G2)	400mg/kg extract (G3)	TC (G4)
Initial weight	161.07±15.78^{a}	119.38±6.83^{a}	155.81±15.50^{a}	45.25±2.35^{a}
Final weight	145.81±15.50^{b}	144.53 ± 7.20^{b}	173.12± 6.89^{b}	130.18±10.08^{a}
WBC /10^{9}/L	4.6±1.2^{a}	5.6±1.3^{a}	5.2 ±1.9^{a}	20.1±2.8^{b}
HB /10g/L	10.6±3.1^{b}	7.5±1.9^{ab}	8.5±2.1^{b}	4.8±2.1^{a}
RBC/10^{12}/L	5.7±1.1^{c}	3.7±0.7^{b}	5.1±0.8^{bc}	1.8±0.2^{a}1
HCT/10^{9}/L	21.1±6.1^{b}	14.3±4.1^{a}	20.8±3.2^{b}	10.3±2.3^{a}
MCH/10^{9}/L	3.4±0.2^{b}	2.3±0.2^{ab}	3.8±0.1^{b}	1.7±0.1^{a}
MCHC/10^{9}/L	36.0±3.1^{b}	43.6±4.2^{bc}	50.3±2.2^{c}	20.5±2.8^{a}

MCV/10^9/L	56.8±4.2^b	47.7±5.8^b	51.2±2.4^b	26.4±3.9^a
PLT/10^9/L	134±14^c	113±8^b	129±4^c	120.5±6^b
MPV/10^9/L	9.6±4.0^a	10.1±1.2^a	12.6±1.0a	9.2±1.8^a
PCT (%)	0.128±0.02	0.18±0.1^a	0.21±0.1^a	0.12±0.0^a
ALP	30.04±2.34^a	33.52±2.81^a	30.56±83.12^a	42.05±4.71^b
ALT	5.60 ± 1.34^a	9.18±0.67^b	5.12± 1 .45^a	11.31±1.96^b
AST	11.23±2.10^a	13.79±2.11^a	10.93 ±1.90^a	13.55±1.88^a
Urea	14.31±1.60^a	18.2±2.12^{ab}	15.22±3.05^a	21.31±3.78^b
Creatinine	2.34 ± 1.90^a	3.01±0.58^a	2.56 ± 1.22^a	5.90± 0.13^b

The results are expressed as mean ± SEM; P≤0.05. $^{a-c}$Different superscripted letters for a given value within a row are significantly different from each other

Table 3 shows the observable features and the hematological changes in the animals. The animals that received the extract at doses 200mg/kg and 400mg/kg (Group 2 and Group 3) showed significant weight gain which may likely be as a result of the nutritional content of the extract thus indicating suppression of hepatocellular disorder. Those animals that received only 300mg/kg TAA (TC) (Group 4) showed significant weight loss indicating distress or ill health. The results also showed a significant increase ($p<0.05$) in WBC following administration of thioacetamide in the TC group (group 4) when compared to the normal control group (NC) (group 1), however this value was significantly reduced following administration of ginger and hot pepper extract combination at doses of 200 and 400 mg/kg (group 2 and 3). The result also showed a significant decrease in HGB, RBC, MCV, MCH and MCHC levels following administration of thioacetamide to the TC group (group 4) and relatively increased in the 200mg/kg and 400mg/kg groups (groups 2 and 3). There was also a significant increase ($p<0.05$) in ALP, ALT, urea and creatinine levels following administration of thioacetamide to experimental rats in group 4 (TC) when compared to the normal control (NC) group 1. However, the result also showed that administration of combined ginger and hot pepper extract at the study level of 200 and 400 mg/kg (groups 2 and 3) significantly reduced the levels of these parameters when compared to the TC group 1 ($p<0.05$) but showed no significant difference ($p>0.05$) when compared to the normal control group. There was no significant difference in AST in all treatment groups ($p>0.05$).

3.3 Biological Activity

Histopathological examination was carried out by an independent investigator blinded to treatment groups Histopathological scores were determined using a modified semi-quantitative, histopathology scoring method as descried by Veterlainen *et al.,*{29}.

Table 4: Histopathology Score method (Liver)

Histopathological Parameter	Severity	Description	Score
Vacuolar Change	Absent	<10%	0
	Mild	10 – 30 %	1

	Moderate	31 – 60%	2
	Severe	>60%	3
Inflammation	None	None	0
	Mild	Focal	1
	Moderate	Multifocal	2
	Severe	Diffuse	3
Necrosis	Absent	0%	0
	Mild	<10%	1
	Moderate	10 – 50%	2
	Severe	>50%	3

Citation: Veteläinen, R. I et al . (2006).

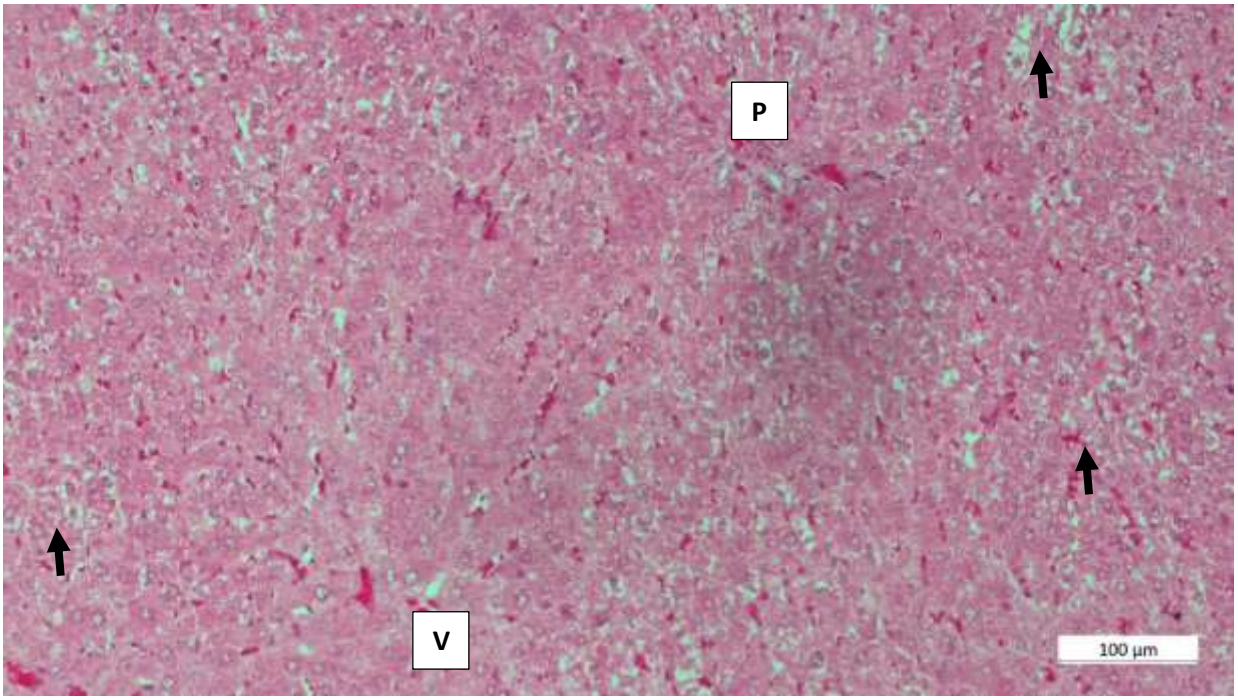

Fig 1 : Liver section of rats given feed and water only (control group 1)

Sections of the liver presented in this group showed mild random microvesicular vacuolation of the hepatocytes (arrow). Central vein (V); Portal triad (P).HE x200.

When TAA was given to the experimental animals, there was hepatic lesions and injury resulting in the enlargement of the liver.

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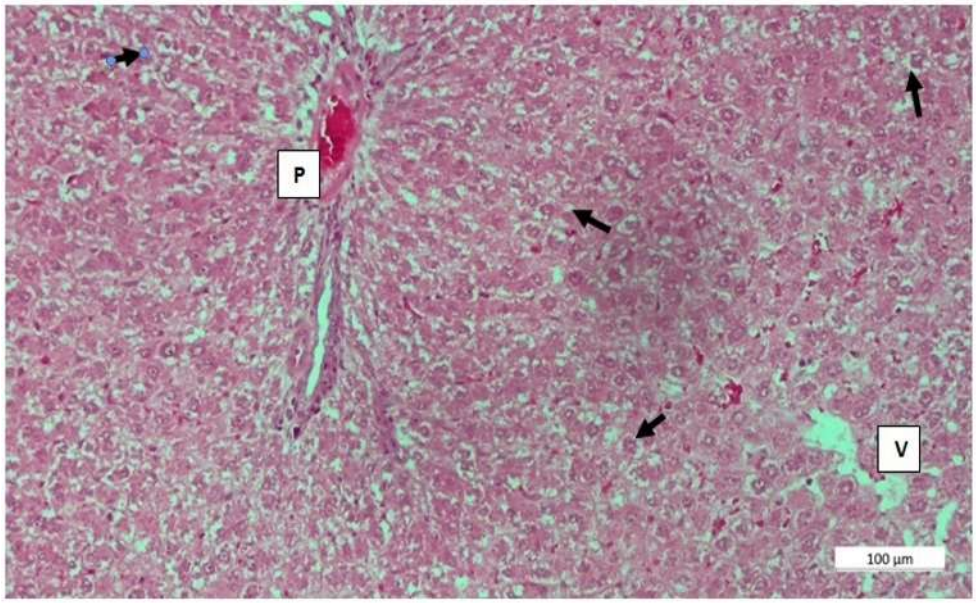

Fig 2: Histopathological changes in the liver of Thioacetamide- induced untreated rats (group 4) ..

Liver sections showed microvesicular vacuolation of the hepatocytes predominantly in the periportal areas of the hepatic lobules (arrow). The affected hepatocytes appeared swollen, causing partial occlusion of adjacent hepatic sinusoids and contained numerous small clear cytoplasm vacuoles.. Hepatocytes surrounding the central veins (centrilobular hepatocytes) exhibited varying degrees of cellular alteration compared to normal hepatic architecture. Central .vein (V); Portal triad (P).HE x200.

The oral administration of 200 and 400mg/kg of the extract significantly reduced the relative liver weight.

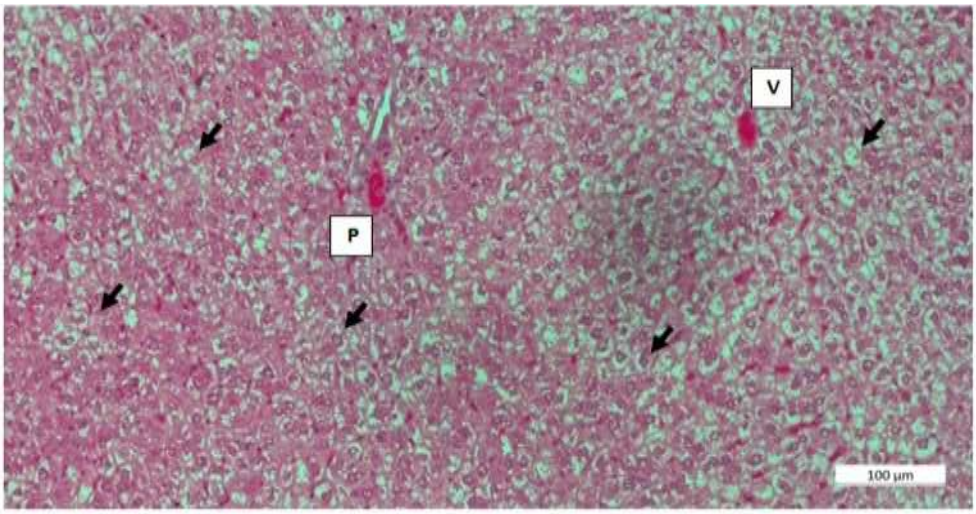

Fig 3: Histopathological appearance of liver in the Thioacetamide- induced treated rats with 200 and 400mg/kg (groups 2 and 3) extracts .

Sections of the liver showed diffuse, mild-to-moderate microvesicular vacuolation of the hepatocytes (arrow) characterised by presence of numerous small cytoplasmic vacuoles. The affected hepatocytes were not markedly swollen and did not significantly compress adjacent hepatic sinusoids. Compared with the untreated thioacetamide- induced group, there was a noticeable reduction in hepatocellular degeneration necrosis , inflammatory cell infiltration and vascular congestion, with preservation of the normal hepatic architecture. Central vein (P) and portal triad (P) were clearly discernible indicating

amelioration of thioacetamide induced hepatic injury following extract treatment not swollen. Central vein (V); Portal triad (P). HE x 200.

Table 5: Histopathological Score of the Rats Liver

PARAMETER	GROUPS			
	G1	G2	G3	G4
vacuolarchange	0	1	1	2
Inflammation	0	0	0	3
Necrosis	0	1	1	3
Total score	0	2	2	8

Sub-acute Toxicity Results

The sub- acute toxicity showed that the extract had little or no effect on the blood indices of the animals when taken, indicating that consumption of both 200mg/kg and 400mg/kg (groups 2 and 3) would not significantly affect the internal organs of the animal. When 200 and 400mg/kg (groups 2 and 3) of the extract were given, the values for AST and ALT were slightly different but not significant from the normal values indicating that the extract would not cause a deleterious effect on the internal organs.

3.4 Discussion

This study presents the results obtained from the evaluation of the effects of the combination of *Z. officinale* and *C. annuum* on blood indices and histology of rats induced for hepatocellular carcinoma using thioacetamide. The screening of the toxicity of the plant was crucial to assure the safety and effectiveness of the plant extract. Signs of toxicity such as pain, distress, allergic reactions, and physical changes in the tested animals were not detected in the *in vivo* assays. The LD_{50} of the test extracts was determined to be above 5000mg/kg and according to Enegide et al. any substance with an LD_{50} of above 2000 mg/kg should be regarded as safe [24].

The phytochemical and nutritional benefits of plants are increasingly tremendous in the world today due to their unrestrained roles in human health and constituent active ingredients. The elucidations of these properties by plants are held greatly to the biological active substances possessed by them such as secondary metabolites, high vitamin and mineral contents. They equally help in the amelioration and prevention of diseases [15]. Ginger (*Z. officinale)* contains several bioactive compounds, which have been shown to have anticancer effects in in vitro and in vivo models.

Antitumor Effects

In this study, hot pepper exhibited a high content of saponins, which have been reported to exhibit cytotoxic effects against various cancer cell lines. Hot pepper and ginger had high content of alkaloids. Alkaloids, such as those present in ginger, have been shown to inhibit the growth and proliferation of cancer cells and induce apoptosis [3]. Ginger and pepper in this study possessed high level of flavonoids and alkaloids which are antioxidant capable of neutralizing or mopping up free radicals that might have been generated by TAA administration.

GC-MS / HPLC analysis OF *Z. officinale* and capsiacinoid from *C. annuum*

The extracts of *Z. officinale* and *C. annuum* were analyzed for chemical composition using GC-MS and HPLC respectively, different organic compounds were determined as shown in Tables 1& 2 respectively. The main organic products of *Z. officinale* was alpha-curcumene present in high quantity, beside others (Zingiberene, beta Sesquiphellandrene, Zingerone) are found in small quantities. The chemical compounds act individually or in synergy with one another and with chemical component from *C. annuum* to counteract oxidative stress and restore antioxidant enzyme levels in the liver, contributing to its protective role against disease development. In this study nine components of major and minor capsaicinoids were analysed by HPLC from capsaicinoid extracted from *C. annuum* which included nornordihydrocapsaicin, nordihydro-

capsaicin, capsaicin, (4) dihydrocapsaicin, N-vanillyl-nonanamide, homocapsaicin, N-vanillyl-decanamide, homo-dihydrocapsaicin isomer I and homo dihydrocapsaicin isomer II. The contents of the capsaicinoid were determined based on the standard curve of dehydrocapsaicin equivalent. The amount of total capsaicinoid in the sample was found to be 7574 (100.0%) as shown in table 2. Capsaicin was the highest capsaicinoid found in *C. annum* and according to literature was considered responsible for pungency of *C. annum*. From the results, it could be reasonably suggested that the combination of the extracts of ginger and hot chilli seemed to exhibit a wide range of normalcy in the hematological and biochemical parameter altered by TAA administration and so could be a good remedy against several blood disorders **[17,18,19].**

Hematological and Biochemical evaluation (Hepatoprotective effects)

The evaluation of hematology and biochemical characteristics has become an important health indicators and means of understanding normal and pathological processes and toxicological impacts. This study results indicated that thioacetamide administration showed significant alteration in the concentration of HB, RBC, PLT, MCV, MCH, MCHC, etc. Altered peripheral blood composition is a reflection of disrupted hematopoietic process and interfering with different stages of red cell synthesis and mature red blood cells. From this study, the significant changes observed (table 3), showed that the administered thioacetamide had potential toxic alteration to hematopoietic process at the study level. Kim et al. reported that toxicants induce hematological alterations leading to microcytic hypochromic anemia in albino rats on the same blood parameters **[16, 20, 22].**

The primary function of erythrocytes is to carry oxygen bound to HB from the lungs to tissues. Furthermore, HB in erythrocytes is an excellent acid-base buffer (for the majority of proteins), thus erythrocytes are mostly responsible for the buffer capacity of whole blood cell. Decrease in HB levels was seen in albino rats used for this study after exposure (TC). Decreased HB% as well as PCV was reported in wild Libyan rats treated with mixture of toxicant **[17].** Hematocrit (HCT) or PVC is used to measure the blood carrying capacity and it is directly associated with the HB% and RBC ability to conduct tissue oxygenation activity. Therefore, PCV shares the reasons for cause of fall in its level with HB% declination. Decrease in PCV may be due to suppression of bone marrow hematopoietic system, causing iron deficiency in synthesis of hem protein of Hemoglobin **[16, 20].** The significant decrease in PCV in group 4 (TC) could indicate anemia or oligohaemia. The changes in the blood PVC value has often been shown to be a good indicator of toxicity.

The white blood cells are the regulators of the immune system and the increase in WBCs count observed in this study (TC group 4) could be due to generalized immune responses. Stimulation of lymphopoiesis and/or enhanced release of lymphocytes from lymph myeloid tissue under toxic stress may lead to an increase in WBCs number **[18].**

The MCV gives an indication of the status or size of the red blood cells and reflects an abnormal or normal cell division during erythropoiesis. An alteration in MCV may show the extent of the shrinking or expanding of cell size due to toxicant **[17]**. But it was interesting fact in this result that a significant decrease (TC group 4) was seen in MCV which is in line with the work of **[18].** The result further showed that administration *Z. officinale* and *C. annuum* extract significantly ($P<0.05$) normalized most of the hematological parameters that were altered by TAA administration with the high dose group showing an impressive recovery rate when compared to the control group (table 3). The therapeutic ability of the extract in this study could be as a result of some bioactive constituents of these herbs.

Alanine aminotransferase (ALT) enzymes are sensitive biomarkers of hepatic status **[17].** Although ALT and AST are synthesized in the liver, they are also present in serum and in various tissues. In particular, ALT serum levels become elevated during liver diseases, and therefore, it is considered a more specific marker for liver injury than AST **[19]**. The significant change observed in ALT activity in the TC group was an indication of liver dysfunction **[23]**. Aspartate aminotransferase (AST) is primarily found in the liver mitochondrial and cytoplasm, it is also found in heart, muscle, kidney and brain. Its serum level increases in hepatic necrosis, myocardial infarction and muscle injury. The result of this study is in agreement with the report of other investigators following administration of toxicants **[19].**

Alkaline phosphatase (ALP) is a biomarker enzyme for assessing the integrity of plasma membrane. Increase in the activities of alkaline phosphatase is an indication that there could be damage due to cytotoxic effect of TAA, thereby resulting to leakage of this enzyme from the liver into the serum. Such increase in alkaline phosphatase activities can constitute threat to the life of cells that are dependent on a variety of phosphate esters for their vital processes since there may be indiscriminate hydrolysis of phosphate esters in the tissue. Increased activity of ALP which occurs due to novo synthesis

by liver cells is a reliable marker of hepatobilliary dysfunction due to damage. .The oral administration of extracts at doses 200 and 400mg/kg (groups 2 and 3) significantly reduced the relative liver weight compared with the untreated thioacetamide induced group ($p<0.05$). Histopathology score showed reduced inflammation and necrosis (table 5).

4. CONCLUSION

The result of this study had shown that administration of *Z. officinale* and *C. annuum* extract could significantly normalize the biochemical, hematological and histopathological alterations caused by hepatocellular carcinoma induced with activated TAA. Therefore synergistic use of ginger and hot pepper might be considered in modulating hematological and hepatic biomarkers in hepatocellular carcinoma.

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AUTHOR CONTRIBUTIONS

***Nmezi Stella N, Designed the study and wrote the first manuscript**

Ubah Venatius C.S, Protocol and logistics.

Nwoko Magnus C, Identification of the plants materials.

Ezea Celestine O, Literature searches

Ajuluchukwu Charles C, Instrumentation.

Nwaneri Chioma B, Animal experiment/study

Nwogwugwu Ngozi U, Literature searches

Igbojionu Vincent O, Methodology, Laboratory analysis.

Iwuala Mirian C, Data collection

Iwu Gina I, Investigation, Statistical analysis

Okechukwu Ugonna P, Interpretation of results

Ugwuegbulam Ozioma P, Proof reading

Okoro Jane C, Plant extraction

Okwueze Ogechukwu E, Histopathology preparation

Korie Maximus C. Writing and review

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

Ethical clearance was given by Ethical committee of Michael Okpara Federal University of Agriculture Umudike, Umuahia Abia State Nigeria on 8th day of March 2024 with ref code: Ref. COLHAS/REC/0754B/25.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this article.

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