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Original Research Article

Antimammary Tumour Effects of *Calliandra portoricensis* Fraction Via Pro-Apoptotic and Anti-Inflammatory Actions in Female *Wistar* Rats

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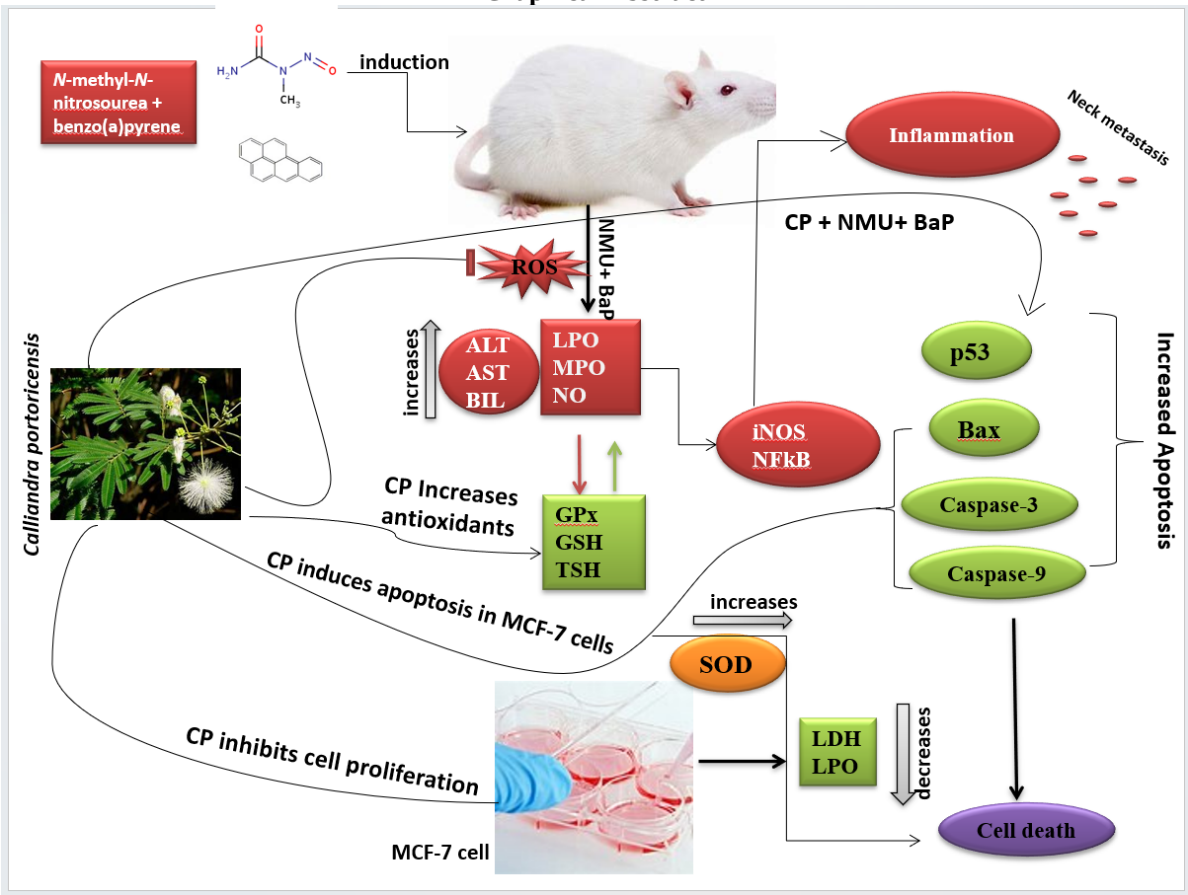
Abstract

Graphical Abstract



Calliandra portoricensis (CP) is used in ethnomedicine to manage breast inflammation. We investigated the anti-mammary tumour effects of fraction from CP in rat model of mammary tumorigenesis induced with *N*-methyl-*N*-nitrosourea (NMU)

and benzo (a)pyrene (BaP) and MCF-7 cells. *In vivo*, thirty-two female *Wistar* rats were assigned into four equal groups: Group 1 (control), group 2 received [NMU (50 mg/kg) +BaP (50 mg/kg)], group 3 received [NMU (50 mg/kg) +BaP (50 mg/kg) + CP (100 mg/kg)] and group 4 received [NMU (50 mg/kg) +BaP (50 mg/kg) + vincristine (VIN) (500 µg/kg)]. The NMU and BaP was injected intraperitoneally to rats at age 7, 10 and 13 weeks for twelve weeks. Thereafter, CP (orally) and vincristine (i.p) was administered for two weeks. *In vitro*, CP and VIN concentration-dependently inhibited the growth of MCF-7 cells by over 80% at 100 µg/mL. The CP and VIN elevated Bax by 4.2 and 1.5 folds, and decreased myeloperoxidase by 75% and 82%, respectively, while CP alone decreased interleukin-1β by 34% *in vitro*. *In vivo*, [NMU+BaP] increased weight and organo somatic weight of mammary gland by 3 and 2.9 folds; total bilirubin, nitric oxide and malondialdehyde by 23%, 51% and 52%, respectively. In [NMU+BaP] rats, weak expression of caspase-3, Bax, and strong expression of iNOS and NF-kB activities were observed, with histological alteration. The GC-MS fingerprint of CP fraction revealed the presence of hexadecanoic acid methyl ester as the most abundant constituent. Treatment with CP ameliorates mammary tumour through mechanisms that involve anti-inflammatory and pro-apoptotic reactions.

Keywords: Apoptosis, *Calliandra Portoricensis*, Inflammation, Oxidative Stress, Cancer, Mammary Gland.

INTRODUCTION

Breast cancer is currently among the most frequent cancers afflicting women all over the world [1]. Although extensive studies have been conducted, it remains a leading cause of death both in developed and developing countries, making it the biggest public health challenge in the world when it comes to women's health [2]. Over the decades, many chemotherapeutic drugs developed and used for treatment of breast cancer (such as vincristine) are yet to produce the desire results in clinical settings [3]. However drug side effects such as unconciousness, weight loss, weakness, neuropathy, headache, hyponatremia, gastrointestinal distress have limited these treatments [4, 5]. In view of this, natural agents with good anticancer properties may be suitable for breast cancer treatment if they can be evaluated for their efficacy and low toxicity [6,7]. For many years now, traditional medicine has gained popularity in prevention and treatment of different diseases world wide. A growing body of research indicates that natural products often outperform commercial drugs and contain bioactive components that may be used to treat or prevent cancer. This is partially due to the fact that natural products contain bioactive components that have a variety of target locations and can inhibit various signaling pathways [8]. Natural products offer important resources for the creation of new cancer treatments through chemoprevention drugs [9, 10].

Calliandra portoricensis (CP) belongs to Leguminosae family and its common name is corpse awakener. Traditional medicine frequently uses the root and leaves to treat breast engorgement and sickle cell disease. Additionally, a variety of conventional oral and dental medications investigated the use of CP for the treatment of acute ulcero-membranous gintivitis, coated tongue, swollen tonsils, and toothaches [11]. CP extracts have been studied for array of biological activities, including antioxidants, cytotoxic effects, antihelmintic properties, bactericidal properties, antiparasitic properties, and antiviral effects [12-14]. *Calliandra portoricensis* (CP) extracts were reported to inhibit cell proliferation by inducing apoptosis in human prostate cancer cells [13], and also exerted similar effects during mammary gland tumorigenesis in female rats [15-17]. Due to the promising therapeutic potential of CP, we investigated the anti-proliferative activity of chloroform fraction from CP in MCF-7 cells as well as its anti-tumour effects in rats administered NMU and BaP.

MATERIALS AND METHODS

Plant Material Processing and Extraction

CP were obtained fresh from Odofin Agbegi village, Ikire, Osun State. The CP was authenticated at Forest Herbarium Ibadan with FHI number 111949. The roots were cleaned, stripped and dried in the laboratory for two weeks before being pulverized and weighed. Cold extraction was used to extract the powdered roots using *n*-hexane and methanol. The methanol extracts was partitioned to get the chloroform fraction of CP. The chloroform fraction was evaporated to dryness (40^0C). The yield from this extraction was 5.9%.

Gas-Chromatography-Mass Spectrometry Analysis

A Agilent technologies 7890 GC system was used for this analysis, and an Agilent technologies 5975 MSD (Mass Spectrometry Detector) was used as a detector [18]. An auto injector sample compartment was used to store the vial bottle containing the chloroform fraction of CP. The automatic injector injects the CP into the liner. Separation of CP took place in the column, where different components were retained for different lengths of time based on the mobile phase. By analyzing the spectrum mass to charge ratio, the molar mass and structure of the compounds was determined by the mass spectrometry.

Cell Proliferation and Viability Tests

To assess the effects of chloroform fraction of CP on the growth of MCF-7 cells, MTT assay was conducted according to the procedure of Zhou [19]. MCF-7 cells were grown in Dulbecco's modified Eagle medium (DMEM, Hyclone, UT, USA) containing 10% fetal bovine serum. In 96-well plates, cells were seeded at a density of 5×10^3 cells per well. Following incubation for 24 hours, the cells were treated for 72 hours with various concentrations of CP (5, 25, 50 and 100 µg/mL) for 72h. The MTT (thiazolyl blue tetrazolium bromide, Sigma, St Louis, MO, USA), 7% FCS and penicillin/streptomycin in PBS was added for 4 hours. After incubation, the dye was solubilized with

DMSO. The concentration of MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 550 nm in a micro-plate reader.

Biochemical Parameters on MCF-7 Cell Lysates

MCF-7 cells were seeded in 96-well plates. After 24 h of incubation, the cells were treated with 49.3 µg/ml (IC_{50}) of CP for 24 h. The cells were washed with cell lysate buffer 3 times and collected into a centrifuge tube. The collected cell lysates were centrifuged at 10,000xg for 10 mins to separate the supernantant from cell debris. SOD, CAT, MPO, LDH were assayed using the supernatant.

Determination of Lactate Dehydrogenase (LDH) Activity on MCF-7 cell

LDH activity was determined according to the method of Weisshaar [20].

Assessment of Inflammatory and Pro-Apoptotic Proteins on MCF-7 cell

The activities of IL-β, caspases-3, -9, and BAX were assessed in cells using commercial available ELISA kits, following manufacturers' instructions (Elab science ELISA kits).

Chemicals

Benzo(a)pyrene (BaP) and N-methyl-N-nitrosourea (NMU) were purchased from Sigma in St. Louis, Missouri, USA and stored under dark conditions at 4°C. All other reagents were of analytical grade and obtained from the British Drug Houses (Poole, Dorset, UK). Prior to use, NMU was dissolved in normal saline, while BaP was dissolved in corn oil.

Animals Protocol

Six-weeks-old female *Wistar* rats was obtained from the Central Animal House, Department of Veterinary Medicine and weighed between 60-70 grams. Animals were housed in plastic cages in four groups at the following conditions: 25 ± 3°C, 60±10% humidity, 12 hours of daylight. During the experiment, animals were fed laboratory feeds and water every day. Rats were used for this experiment after the University of Ibadan's Animal Ethics Committee reviewed and approved the protocol, handling, and treatment methods on November 19, 2015 (UI-ACUREC/App/2015/061). The experimental etiquettes were followed in accordance with the guidelines set by the University of Ibadan Ethical Committee. The rats were randomly assigned into four groups of eight animals per group and were treated as follows: Group 1 served as control, group II received [NMU+BaP-only], group III received [NMU+BaP] and treated with CP, while group IV received [NMU+BaP] and treated with vincristine (VIN). At age 7, 10 and 13 weeks, a dose of NMU (50 mg/kg) and BaP (50 mg/kg) was injected intraperitoneally to the rats and animals were allowed twelve weeks to develop mammary tumour with proof of metastasis to a distant organ (neck). Thereafter, CP (100 mg/kg) was administered by oral gavage and vincristine (100 µg/kg) administered intraperitoneally for two consecutive weeks. The doses of CP and vincristine used in the present study were selected from pilot studies and previously published data [15].

Serum Preparation

The blood was collected using ocular puncture, which was then centrifuged after an hour to obtain a clear supernatant by using a bench centrifuge at 3000 g for 10 minutes. Biochemical analyses were carried out on the clear supernatant.

Protein Determination Level

Lowry *et al*., [21], method was used to determine protein levels in serum and mammary tissue. An alkaline solution of 0.7 mL (NaOH and Na_2CO_3) was mixed with 0.5 mL of diluted sample or standard and 20 minutes incubation at room temperature was conducted. Additionally, 6 mL of distilled water and 2N Folin and Ciocalteu's phenol reagents (0.1 mL) were added, and allowed to incubate for 30 minutes at room temperature after mixing. By extrapolating from the BSA calibration curve, the protein values were estimated based on the measured absorbance at 750 nm after incubation.

Assessment of Biomarkers of Mammary Oxidative Stress

The mammary glands was removed, washed with ice-cold 1.15% KCl solution to remove blood stain, dried and weighed. The post-mitochondrial fraction (PMF) was obtained by homogenizing the gland with 50mM phosphate buffer solution at pH 7.4 in 4 volumes for 15 minutes and centrifuging it at 10,000g for 15 minutes. The entire procedure took place at 4°C. Superoxide dismutase (SOD) activity was determined according to the method of McCord and Fridovich [22]. Reduced glutathione (GSH) was determined at 412 nm according to the method of Moron *et al*., [23]. Catalase (CAT) activity was determined using hydrogen peroxide as a substrate according to the method of Aebi [24]. Glutathione-s-transferase (GST) activity was assessed by the methods described by Habig *et al*., [25], using CDNB as a substrate. Glutathione peroxidase (GPx) activity was determined according to the method of Rotruck *et al*., [25]. Lipid peroxidation was quantifed as MDA according to the method described by Buege and Aust [26]. Mammary nitrites content was obtained using a sodium nitrite curve as standard and expressed as µM of nitrites/mg protein as described by Palmer *et al*., [27]. Myeloperoxidase (MPO) activity was determined in the mammary tissues according to the method of Trush *et al*., [28]. The amounts of total sulphydryl (TSH) in mammary tissue was measured according to Ellman [29].

Histology

Mammary tissues were fixed in 10% phosphate-buffered formalin for 48 h. Fixed sections of dehydrated mammary glands in formalin were cleared in xylene before embedding in paraffin. Hematoxylin-eosin

staining was done on micro sections (3 µm) of the tissue. Microanatomy of mammary tissue was examined under a light microscope and images were captured with a digital camera.

Immunohistochemical Analysis

DAKO Chemical Inc. provided the kits required for immunohistochemical staining of mammary glands to detect the expression of p53, NF-KB, Caspase 3, iNOS, and BaX activities. A modified version of the Chakravarthi *et al*., [30], immunohistochemistry method was used. Using a 1:100 dilution of a primary antibody (or as instructed by the manufacturer) to bind to specific antigens is the principle behind the procedure. After the antibody-antigen complex is formed, a secondary, enzyme-conjugated antibody is added and incubated. A binocular microscope revealed coloured deposits at the sites of antibody-antigen binding when the enzyme is in contact with substrate and chromogen. In contrast to external controls, cells with specific distinct colours in their cytoplasm, cell membranes, or nuclei were considered positive. Antigen was retrieved from the sections by heating them at 100°C for 15 minutes in citric acid buffer. Image Jsoftware was used to quantify band intensities.

Statistical Analysis

As a result, five-eight rats from each group were summed up to obtain mean and standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the data followed by the post-hoc Duncan's multiple range test for analysis of biochemical data using SPSS (22.0). Statistical significance was determined at $p<0.05$.

RESULTS

Identification of Compounds Present in Chloroform Fraction of CP by Gas Chromatography Mass Spectrometry

The GC-MS spectral results and database searches identified ten (10) major compounds in chloroform fraction of CP (Figures 1a and b). Compounds identified includes: Hexadecyl pentyl ether (1), Hexadecanoic acid methyl ester (2), Tretradecanoic acid (3), 1,8,11-Heptadecatriene (4), 6-octadecenoic acid methyl ester (5), 1,19-Eicosadiene (6), Z-7-tetradecenoic acid (7), 13-tetradecenal (8), 1-undecene,11-nitro- (9), Squalene (10). The GC-MS analysis showed compound 2 (Hexadecanoic acid methyl ester) to be the most abundant, whereas compound 9 (1-undecene,11-nitro-) was the least abundant of all the compounds identified (Table 1a).

Table 1a: Putatively identified compounds from chloroform fraction of *Calliandra portoricensis*

GC Peak No	Compounds	Retention Time	Amount %
1	Hexadecyl pentyl ether	13.941	3.10
2	Hexadecanoic acid methyl ester	21.946	32.17
3	Tretradecanoic acid	22.662	6.25
4	1,8,11-Heptadecatriene	25.500	4.63
5	6-octadecenoic acid methyl ester	25.654	25.60
6	1,19-Eicosadiene	25.786	9.05
7	Z-7-tetradecenoic acid	26.215	11.51
8	13-tetradecenal	26.433	2.77
9	1-undecene,11-nitro-	26.450	1.10
10	Squalene	27.720	2.29

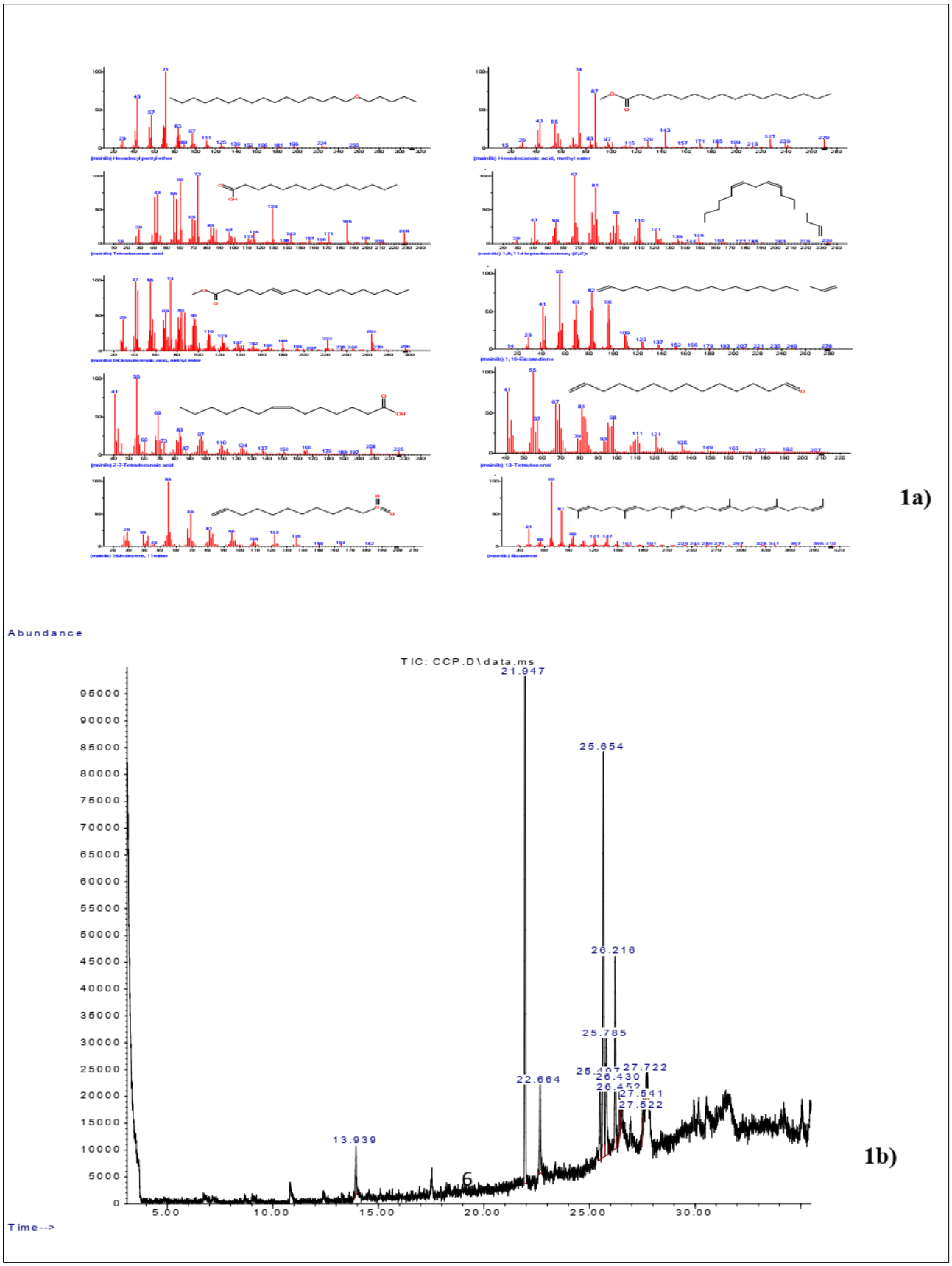


Figure 1: Chromatogram and finger printing of chlroform fraction *Calliandra portoricensis* by Gas Chromatography and Mass Spectrometry

Effect of CP on Cell Viability and Possible Mechanism of Action in MCF-7 Cell Lysates

The effect of CP on cell viability in MCF-cells was investigated (Table1b). Growth inhibition increased in a concentration-dependent manner from 5 to 100 µg/mL of CP with values ranging from 1.7 to 87.5% at 72h. At 100 µg/mL, the growth inhibition of CP (87.5%) is statistical similar to VIN (reference drug; 87.6%). Furthermore, the LDH activities and MDA level (From cell lysates) of cells treated with CP and VIN were reduced compared to cancer cells control (Figures 2a and b), while the activities of SOD increased in cells treated with CP and VIN respectively (Figure 2c). In the same manner, the activities of caspases-3, -9 and BAX in cells treated with CP and VIN increased significantly ($P<0.05$) relative to controls (Figures 2d-2f). In contrast, inflammatory markers; myeloperoxidase and interleukin-1beta were reduced significantly ($P<0.05$) in cells treated with CP and VIN (Figures 2g and 2h).

Table 1b: Effect of chloroform fraction of CP and VIN on cell viability in MCF-7 cells

Conc. (µg/mL) (%)	CP Growth Inhibition (%)	VIN Growth Inhibition
5	1.7±0.08	49.0±4.71
25	28.6±3.50*	62.3±5.40*
50	49.2±6.11*	87.6±7.03*
100	87.5±5.20*	87.6±4.00*

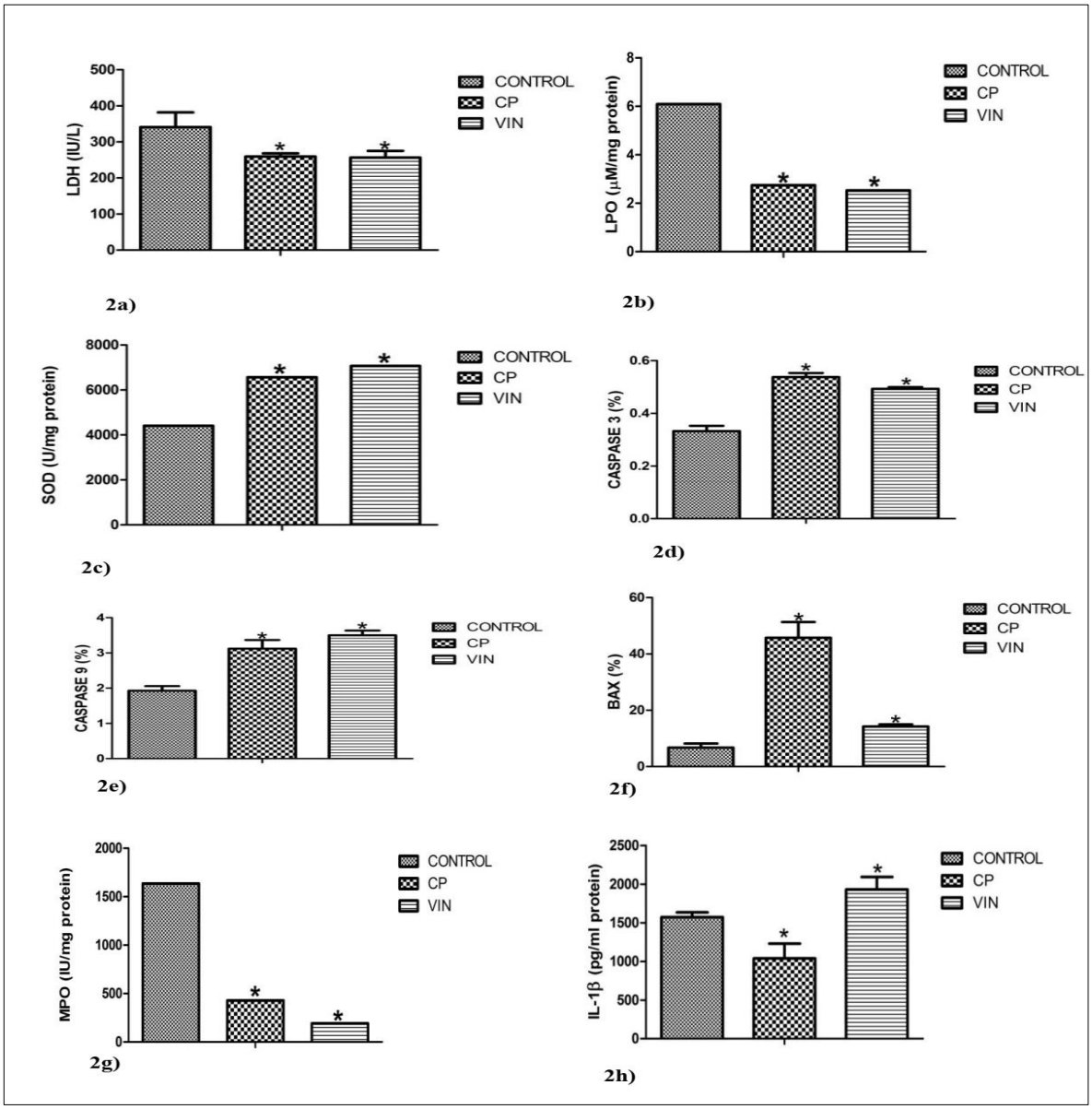


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Figure 2: Effects of CP and VIN on lactate dehydrogenase, superoxide dismutase, malondialdehyde, caspases-3, -9, BAX, myeloperoxidase and Interleukin-1β activities in MCF-7 cells.

*Significantly different from control ($p< 0.05$); CP= *Calliandra portoricensis*; VIN= Vincristine

Effects of CP on Body Weight, Mammary Glands' Weight and Biochemical Indices in Rats Administered NMU and Bap

As shown in table 3, there were decreased in body weight-gain in [NMU+BaP]-administered rats relative to control. In contrast, the weight of mammary gland increased in [NMU+BaP]-administered rats, while treament with CP and VIN reduced the weight of the mammary gland. Furthermore, the organo somatic weight of mammary gland which increased in [NMU+BaP] rats, was attenuated upon post-treatment with CP and VIN (Table 2a). In table 4, there were notable increase in the activities of serum ALT, AST, and total bilirubin level in [NMU+BaP]-administered rats when compared to controls (Table 2b). However, oral exposure of CP and VIN to [NMU+BaP]-administered rats decreased the activities of AST, ALT and total bilirubin level.

Table 2a: Effect of fraction of CP and VIN on body weights and mammary tissue's weight of rats given NMU and BaP

TREATMENTS	BODY WEIGHTS		MAMMARY TISSUE	ORGANO-SOMATIC	
	Initial (g)	Final (g)	Weight Gained (g)	Weight (g)	Weight (as % body wt)
CONTROL	83.55±6.02	175.83±5.25	92.28±5.70	0.36±0.07	0.23±0.05
NMU+BaP	83.43±6.66	164.08±8.28	80.65±8.14[a]	1.46±0.40[a]	0.89±0.04[a]
NMU+BaP+CP	81.51±5.89	177.67±22.22	96.16±6.81	1.00±0.21[b]	0.63±0.07[b]
NMU+BaP+VIN	85.48±6.94	147.78±25.38	62.30±32.21[b]	1.12±0.37[b]	0.76±0.03[b]

Values are expressed as Mean ± Standard deviation of 5-8 animals per group; [a] Significantly different from control ($p < 0.05$); [b] Significantly different from [NMU+BaP] ($p < 0.05$); NMU= *N*-methyl-*N*-nitrosourea, BaP= Benzo(a)pyrene, CP= *Calliandra portoricensis* (100 mg/kg)

Table 2b: Effect of chloroform fraction of CP and VIN on the activities of ALT, AST and T-Bil levels in rats treated

TREATMENTS	ALT (U/L)	AST (U/L)	T-BIL (µmol/L)
CONTROL	153.0±2.99	369.38±4.77	81.03±19.29
NMU+BaP	178.97±7.54	393.90±3.21[a]	99.35±8.13
NMU+BaP+CP	148.01±4.22[b]	364.00±4.51[b]	82.02±6.58
NMU+BaP+VIN	145.73±2.99[b]	368.75±8.76	86.06±0.74

Values are expressed as Mean ± Standard deviation of 5-8 animals per group; [a] Significantly different from control ($p < 0.05$); [b] Significantly different from [NMU+BaP] ($p < 0.05$); NMU= *N*-methyl-*N*-nitrosourea; BaP= Benzo(a)pyrene; CP= *Calliandra portoricensis* (100 mg/kg); ALT= Alanine aminotransferases; AST= Aspartate aminotransferases; T-BIL= Total bilirubin

Effects of CP on Oxidative Stress, Apoptosis and Inflammation in [NMU+BaP] Rats

Mammary SOD, GST, and catalase activities were significantly ($P<0.05$) reduced following administration of NMU and BaP when compared to controls while oral exposure to CP treatment restored the enzyme activities across the treated rats (Figures 3a-3c). Similarly, administration of CP and VIN to [NMU+BaP]-rats increased the levels of mammary GSH, TSH and GPx activity (Table 2c). In addition, [NMU+BaP]-administration elevated mammary MPO activity, NO and MDA levels relative to controls (Table 2c, Figures 3d and 3e). The CP and VIN treated groups had decreased in MPO activity as well as NO and MDA levels when compared to [NMU+BaP] rats (Table 2c, Figures 3d and 3e). As shown in Figures 4a - 4c, we observed a very low expression in the activities of bax, p53 and caspase-3 proteins (apoptotic indices) in [NMU+BaP]-rats while post-treatment with CP and VIN significantly increased the apoptotic indices when compared to [NMU+BaP] rats (Figures 4a - 4c). In Figure 4d and Figure 4e, NMU and BaP administration elevated the mammary activities of iNOS and NF-kB (Inflammation markers) significantly ($P<0.05$) while treatment with CP and VIN attenuated the changes in these proteins.

Table 2c: Effect of chloroform fraction of CP and VIN on NO, GPx, GSH and TSH levels in rats treated with NMU and BaP

TREATMENTS	NO (µmol/L)	GPx (nmol/mg protein/min)	GSH (µg/ml/ mg protein)	TSH (µmol/L)
CONTROL	25.47±1.27	12.14±0.72	11.52±0.77	11.82±0.64
NMU+BaP	38.45±1.52[a]	9.37±0.93[a]	7.89±0.48[a]	8.90±0.81
NMU+BaP+CP	22.2±0.90[b]	13.66±1.38	12.69±1.00[b]	14.28±1.24[b]
NMU+BaP+VIN	23.65±3.43[b]	12.47±1.23[b]	12.06±0.74[b]	13.25±1.21[b]

Values are expressed as Mean ± Standard deviation of 5-8 animals per group; [a] Significantly different from control ($p < 0.05$); [b] Significantly different from [NMU+BaP] ($p < 0.05$); NMU= *N*-methyl-*N*-nitrosourea, BaP= Benzo(a)pyrene, CP= *Calliandra portoricensis* (100 mg/kg); NO= Nitric oxide; GPx= Glutathione peroxidase; GSH= Reduced glutathione; TSH= Total thiol

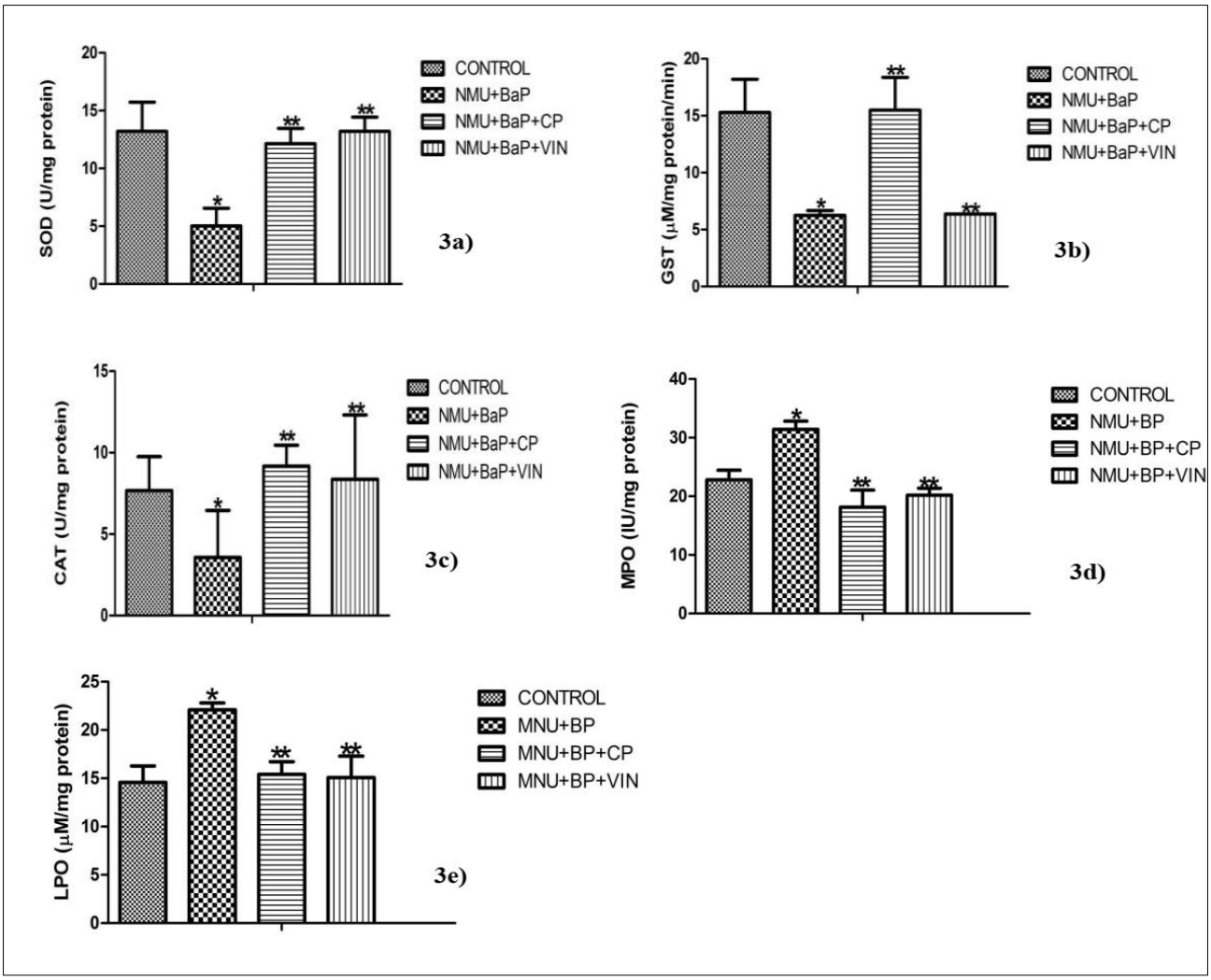
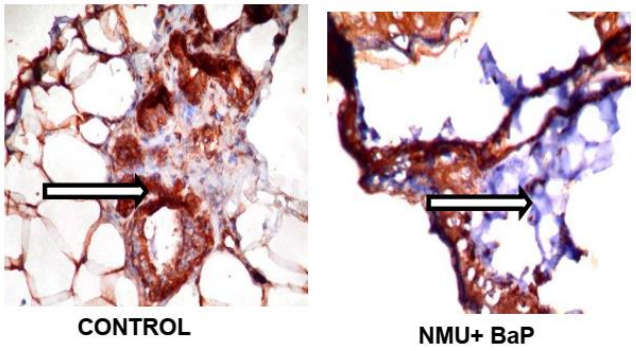


Figure 3: Effects of CP and VIN on mammary activities of superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), myeloperoxidase (MPO) and malondialdehyde (MDA) level in rats treated with NMU and BaP. *Significantly different from control ($p < 0.05$);

**Significantly different from [NMU+BaP] ($p < 0.05$); NMU= N methyl N nitrosourea; BaP= Benzo(a)pyrene; CP= *Calliandra portoricensis* (100mg/kg), VIN= Vincristine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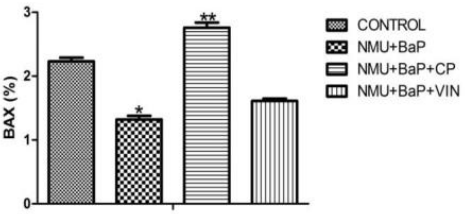
NMU+ BaP


BAX (%)
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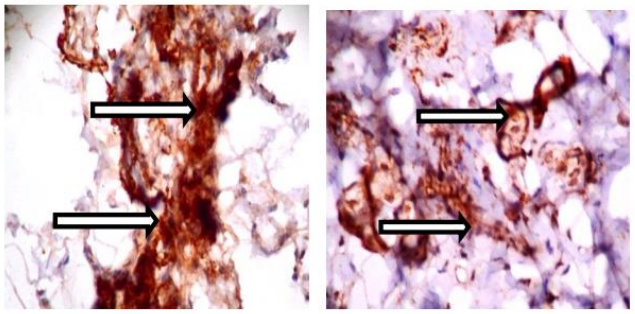
CONTROL
NMU+BaP
NMU+BaP+CP
NMU+BaP+VIN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NMU+ BaP+VIN

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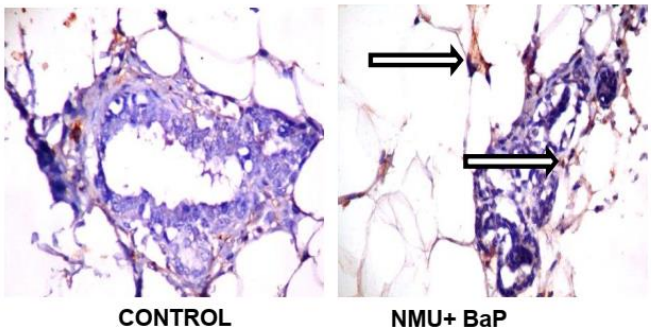
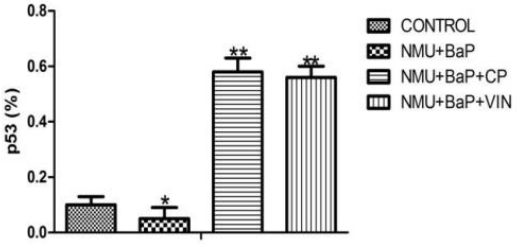

CONTROL
NMU+ BaP


p53 (%)
0.8
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CONTROL
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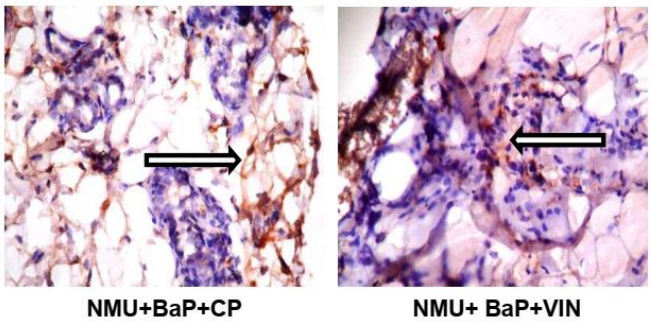
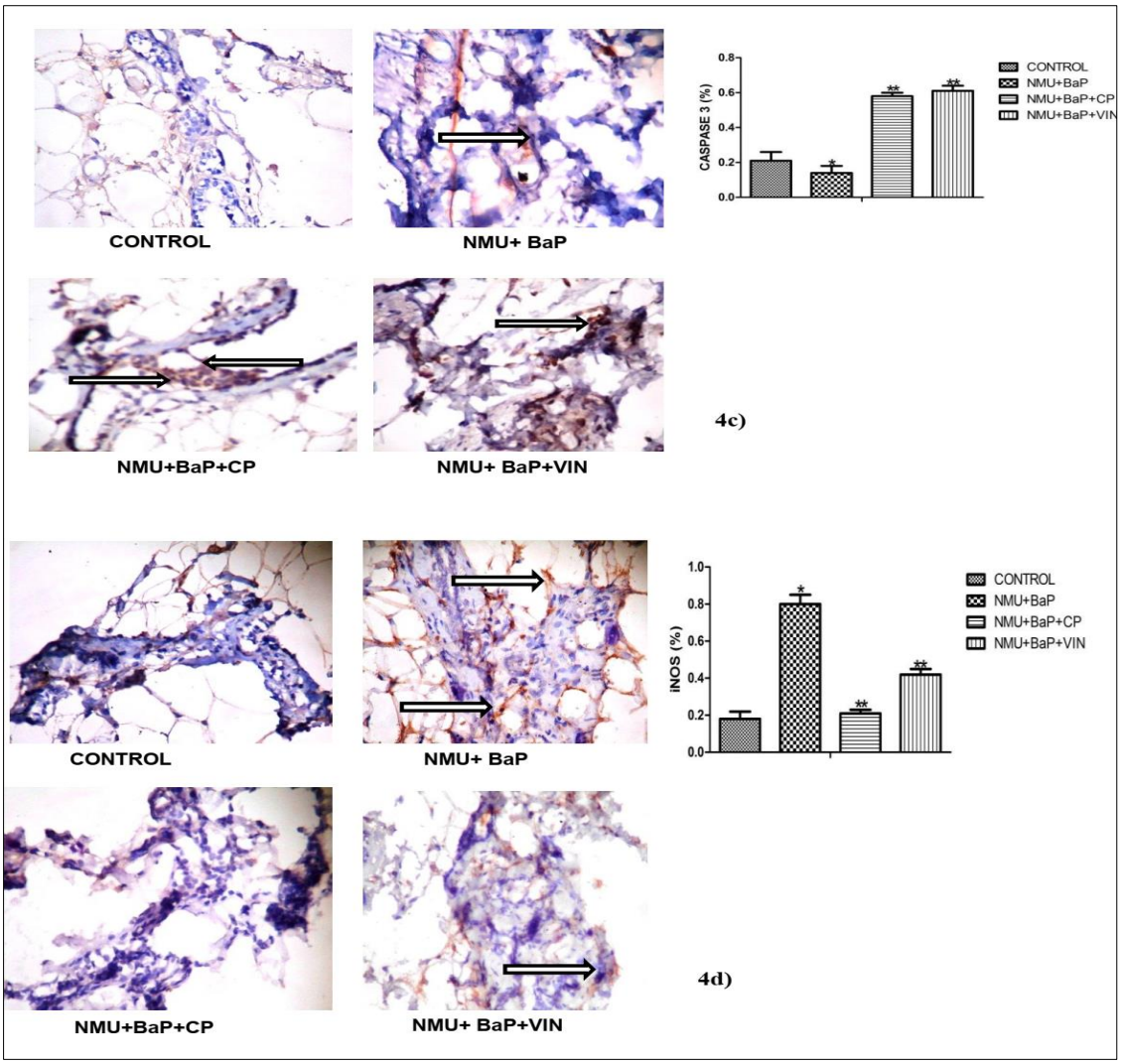
NMU+BaP+CP
NMU+BaP+VIN


NMU+BaP+CP
NMU+ BaP+VIN

4b)



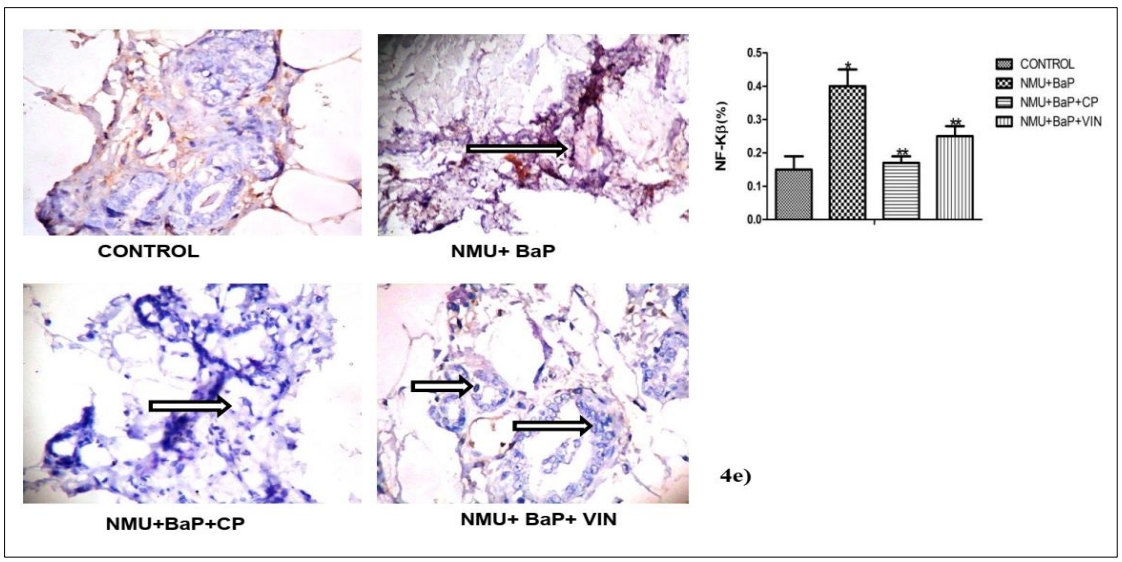


Figure 4: Effect of CP and VIN on Bax, p53, caspase-3, inducible nitric oxide synthase (iNOS) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activities in rats administered BaP and NMU.

*Significantly different from control ($p < 0.05$); ** Significantly different from [NMU+BaP] ($p < 0.05$); NMU= N methyl N nitrosourea; BaP= Benzo(a)pyrene; CP= *Calliandra portoricensis* (100mg/kg), VIN= Vincristine. The white arrows showing the expression of NF-kB.

Effect of CP on Cyto-Architecture of Mammary Glands in Rats Administered NMU and BaP

The presence of maglinant cells with piknotic nuclei and a high nucleocytoplasmic ratio was confirmed by the histology of the mammary gland from [NMU+BaP] rats (Figure 5a), while glands from [NMU+BaP] rats treated with CP and VIN lacked maglinant cells and appear like control. The NMU and BaP-induced mammary tumourigenesis was characteristically associated with massive neck tumor (metastasis) as confirmed by histology (Figure 5b).

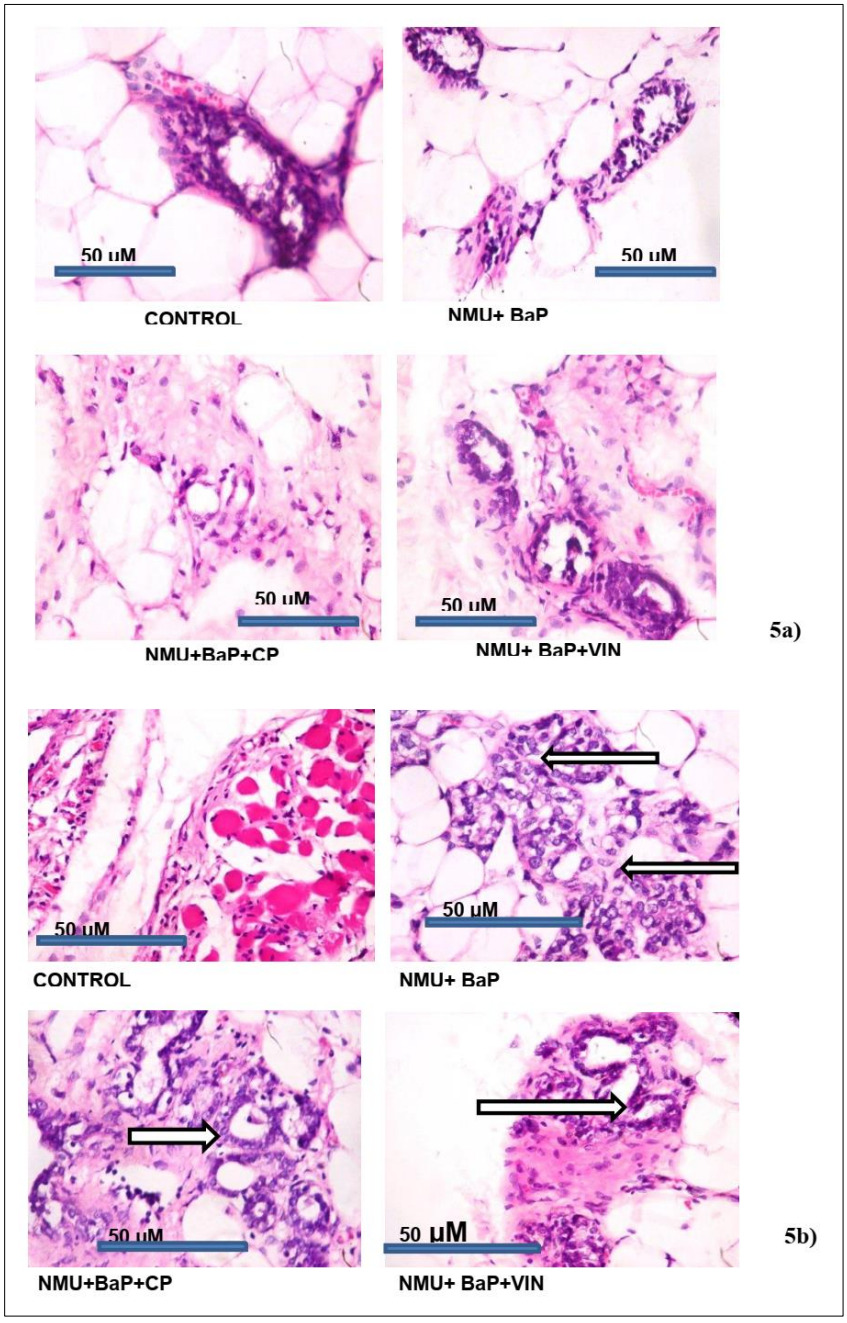


Figure 5: Photomicrographs of mammary gland and neck tissue in [NMU+BaP]-administered rat and then treated with CP and VIN (M×400). The white arrows show severe metastasis of mammary glandular tissues in the neck tissues

DISCUSSION

Safer anticancer agents are ceaselessly been identified and evolved from natural plants which have been reported to be effective and less toxic. Plant extracts and phytoconstituents have been found to exhibit powerful anti-proliferative effects *in vitro* and in animal studies [31]. In this study, the cytotoxic effect of CP on MCF-7 cells was investigated. Our findings revealed that exposing MCF-7 cells to CP inhibited their proliferation in a dose-dependent way. The apoptosis of MCF-7 cells was found to be considerably boosted after treatment with CP. This suggests that CP inhibits the cancer cells growth by trigerring apoptosis. Tumor proliferation, growth, invasion, and metastasis have been reported to

be associated with elevated levels of lactate dehydrogenase in many types of cancer [32]. It has been demonstrated that inhibiting lactate dehydrogenase activity can reduce metastasis and invasion of cancer cells by reducing their energy supply [33]. Our results demonstrated inhibition of lactate dehydrogense activities by CP and vincristine treatment which further corroborates previous reports and supports the anti-proliferarive activity of CP.

In animal studies, the potential proliferation associated with NMU-induced mammary gland tumors using BaP as a promoter in regular mammary gland tissue was also investigated. Findings from this study showed that animals receiving NMU and BaP exhibited reductions in body weight gain suggesting that mammary gland tumors may interfere with the coordinated metabolic network in the animal, resulting in rapid weight loss and tissue wasting. However, animals orally exposed to CP showed progressive weight gain, which implies that CP improves and supports positive energy metabolism. This results are consistent with [34], and [31], who found that neem oil decreased the proliferation of breast cancer cells and dimethylbenz(a)anthracene-induced breast cancer in high-fat and sugar-fed rats. On the contrary, VIN-treated rats couldnt restored the weight loss and tissue wasting in NMU and BAP-administered rats which is one of the strong side effects of VIN therapy in breast cancer patients [17].

When analyzing the mechanisms by which CP ameliorates NMU and BaP-induced mammary tumors, it is also necessary to consider changes in organ systems such as liver. In the current investigation, we found a notable increase in the liver toxicity indicators (ALT, AST, and T-BIL levels) in NMU and BaP-administered rats, and these values were subsequently nearly restored to control after CP and VIN treatment. This observation further corroborates previous reports by Adefisan *et al*., [16], which indicates that fraction of CP is non-toxic and may protect the integrity of the liver. In addition, further analysis showed that scavenging of oxygen radicals is one of the possible mechanisms of anti-tumorigenic and anti-proliferative action of fraction of CP both in animal studies and *in vitro*. Research has shown that chemical carcinogens such as NMU and DMBA generate reactive oxygen species (ROS) after exposure and it is essential that free radicals and antioxidants are in balance for proper physiological function of the body [35]. A condition known as oxidative stress occurs when these free radicals overwhelm the body's ability to regulate them. This causes free radicals to damage lipids, proteins, and DNA, which in turn leads to various human diseases, including cancer [36]. Thus, two sets of analyses were used to determine the chloroform fraction of CP's antioxidant capacity: (1) antioxidant enzyme activities of SOD, GPx, GSH, TSH, GST, CAT and (2) the levels of biochemical indices; MDA, MPO and NO. Our data demonstrate significant depletion in the enzyme activities of SOD, GST and CAT, accompanied by notable decrease in GPx activity, GSH and TSH levels in the mammary gland of NMU and BaP-rats. Post-treatment with chloroform fraction of CP and VIN attenuated the alteration in enzymatic activities and restored the values close to the control group. In this regard, chloroform fraction of CP improved the antioxidant defense system of NMU and BaP-administered rats due to the protective antioxidant mechanisms employed by both enzymatic and non enzymatic substances such as phenolic and flavones. Also, NMU and BaP significantly increased MPO activity, MDA and NO levels in the serum and mammary tissues but these increases were reduced by oral treament of CP. These results indicate a strong anti-oxidative capacity of CP in combating chemically-induced oxidative stress, which further support our previous findings [13].

In tumorigenesis, carcinogenic factors disrupt the homeostatic molecular signaling networks, which result in cell physiological alterations leading to tumor growth. The NMU and BaP models result in high levels of oxidative stress in the mammary gland [37], which promotes the expression of genes related to inflammation, cell proliferation and invasion [38]. The NF-κB is one of the transcription factors that are activated by numerous stimuli and its activation increases the expression of genes related to tumor progression, including genes that promote cell proliferation and survival, angiogenesis, metastasis, and inflammation [37]. Numerous malignancies have been discovered to have increased NF-κB activity [39], and this is usually prevalent in breast cancer that is ER-negative [40]. In this investigation, the findings showed that CP treatment significantly decreased the levels of NF-kB in the mammary tissues of NMU and BaP-administered rats. This outcome is consistent with reports by Biwas *et al*., [41], who found that Neem had anti-proliferative and pro-apoptotic effects on human breast cancer tissues when they were cultured *in vitro*. Additionally, NMU and BaP drastically reduced Bax, p53, and caspase-3 activities in the animals while inducing mammary gland tumors, whereas CP therapy was observed to enhance the amount of these proteins.

Increased iNOS levels have been observed in many cancers as a result of studies demonstrating that iNOS increases tumor aggression and is related with poor survival in patients with breast cancer [38-42]. It's significant to note that aberrant iNOS and NF-kB cascade pathway activation is frequently associated with carcinogenesis, medication resistance, and carcinoma progression [19-43]. Our results demonstrated that NMU and BaP-administered rat's iNOS levels were decreased upon post-treatment with CP. According to Adaramoye *et al*., [13] this decrease may be attributed to CP's high quantities of phenolic components including anthocyanin and polyphenols, indicating its anti-inflammatory activities in this breast cancer model. The mammary tumor specimen's histological findings

revealed severely malignant epithelial cells with piknotic nuclei and high nucleocytoplasm. However, the lesions brought on by NMU and BaP in the rats were lessened and alleviated by post-treatment with CP. The NMU and BaP-induced mammary tumourigenesis was characteristically associated with massive neck tumor (metastasis) as confirmed by histology findings. The presence of malignant cells from mammary gland in the neck tumor of NMU and BaP rats is a major indication of tumourigenesis caused by these chemicals. More so, the GC-MS fingerprint of CP fraction identified ten major compounds. Out of the compounds identified, the GC-MS result and data based search revealed the presence of hexadecanoic acid methyl ester as the most abundant constituent while 1-undecene,11-nitro- as the least of the identified compounds. The presence of hexadecanoic acid methyl ester may be responsible for the effective activity CP in MCF-7 cells and in this breast cancer model.

Overall, we have demonstrated that CP was effective in inhibiting cell proliferation of MCF-7 cells by inducing apoptosis. In addition, CP and vincristine attenuated NMU and BaP-induced inflammation and oxidative stress through suppression of pro-inflammatory cytokines, inhibition of NF-kβ, IL-1β and iNOS activities, elevated antioxidant enzymes, and reduces NO accumulation in the mammary tissue of NMU and BaP-administered rats. Also, CP activated apoptotic-related proteins; caspases-3, -9, Bax, p53 *in vitro* and *in vivo*. This study could serve as a basis for new experimental and clinical studies that examine the therapeutic role of CP in breast cancer.

STATEMENTS AND DECLARATIONS

Ethical Approval

All animal procedures followed the general regulations and guidelines of the Experimental Animal Ethics Committee of the Faculty of Basic Medical Sciences,Rats handling was approved by the University of Ibadan Animals' Ethics Committee (UI-ACUREC/App/2015/061).

Competing Interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors Contributions: Oluwatosin Adaramoye conceived and designed the research; Material preparation, data collection and analysis were performed by Adedoyin Adefisan and Toluwanimi Emmanuel Akinleye; The first draft of the manuscript was wriiten by Adedoyin Adefisan; All authors read and review the manuscript; Oluwatosin Adaramoye, Olubusuyi Adewumi supervised the work.

Availability of data and Materials: Enquiries about data availability should be directed to the authors.

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