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In-vivo anticancer activity of combined ethanolic extract of *Annona muricata* and *Rauvolfia vomitoria* (AR) in N-Nitroso-N-methylurea (NMU) induced Swiss albino mice

Oloyede Michael Adeola.[1*], Khalifa Muyideen[1], Bolarinwa Kehinde[2]., Olakanmi,Akinde[3].

[1]Department of Cell Biology and Genetics, University of Lagos, Akoka, Nigeria
[2]Distance Learning Institute, University of Lagos, Akoka, Nigeria
[3]College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria
*Corresponding Author: moloyede@unilag.edu.ng,

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Abstract

Annona muricata and *Rauvolfia vomitoria* have been combined traditionally to manage and cure many malady in southwestern Nigeria. This study sought to investigate chemotherapeutic anticancer potential of diherbal preparation *Annona muricata* and *Rauvolfia vomitoria* (AR) against N-nitroso-N-methylurea (NMU) induced mammary carcinogenesis in mice. Twenty-five female mice (17 -20g) were divided into five groups of five animals. Group A received distilled water. Groups B, C and D received cisplatin, 50 mg kg^{-1} and 100 mg kg^{-1} of the herbal preparation 24 hours after NMU administration respectively, while group E served as tumour placebo. Mice were treated for 12 weeks, and sacrificed via jugular puncture to harvest blood, breast, liver, heart, lungs and kidneys for analysis. Haematology revealed dose-dependently increased haemoglobin, PCV, RBC and MCV in treated mice. Antioxidant expression was observed in treated mice. Histology of breast of treated mice were conserved. Alterations like, mononuclear inflammatory cells admixed with tumour-like single cells, hyperplastic basophilic and neoplastic nuclei were observed in tumour placebo. Internal organs of treated mice did not show alteration, however, tumour placebo revealed perivascular mononuclear cuffing, blood vessel congestion, loss of alveoli sac, hyperplastic basophilic nuclei, glomerular infiltrate and renal haemorrhage. Biochemical analytes did not show any significant difference, also breast of treated mice possessed high concentration of salicylic acid. Increased WBC differentials suggest potential of AR in mitigating NMU. Enhanced status of antioxidant in treated mice evident in decreased MDA, SOD and CAT and increased level of GSH level, ensured decline in lipid peroxidation, thereby conferring AR its anticancer potency via apoptotic induction or neovascularization inhibition. ALP and ALB decrease, in treatment suggest that AR is hepatoprotective. Insignificant level of AST, ALT, protein and LDH connotes that AR is not hepatotoxic. Absence of alterations in treated mice portends AR as antitumorigenic, possibly due to synergy exhibited by bullatacin, annonacin, annomuricin, salicylic acid, sapentine and alstonine in AR.

Keywords: Anticancer; Antioxidants; Extract ; Histology; Biochemical analytes

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Introduction

Medicinal plants have been prominent as therapeutic agents from ancient time in Africa, Asia and other continents to cure diseases (Petrovska 2012; Mahomoodally 2013). It has been vital in health care and served as major raw materials for both traditional and conventional medicine preparations. In modern society, the use of traditional medical systems where two or more plants or plant extracts are combined and implored in health remedies is on tremendous

increase (Abera 2014). These combinations of cocktail of natural extracts may exert additive or synergistic effects in the management of diseases. *Annona muricata* is a popular plant in southwestern Nigeria, and very common in the tropics. It belongs to the family Annonaceae (Ferreira *et al*., 2013). It is known as soursop or sharp sharp in southwestern Nigeria (Minari and Okeke, 2014) also found in the rainforest of Asia, America and Africa also widely known as Graviola and Guanabana (Rady et al., 2018). *A. muricata* grows to about 5 – 6m height as a small erect fruit tree (Minari and Okeke, 2014). Ethnobotanical investigations revealed its traditional use in treating cancer (Minari and Okeke, 2014; Gavamukulya *et al*., 2017; Adaramoye *et al*., 2019), malaria and hypoglycemia (Gavamukulya *et al*., 2017). It has been used as anticonvulsant, antipyretic and antispasmodic agent (Ferreira *et al*., 2013). The juice from its fruits and leaves infusion have been used against fever (Boyom 2011) and also topically applied on pain site (WHO, 2009). Leaves decoction serves as analgesic (Ross, 2010). Annona muricata leaves have been described to be externally and internally antiparasitic (Badrie and Schauss, 2009) and also effective against hypertension (Motoosamy and Fawzi, 2014). It has been therapeutically useful in treatment of diabetes (Ezuruike and Prieto, 2014). The leaves, roots and unripe fruits are used as repellants against insects, thus serving as bioinsecticides and biopesticides (Cosia-Tellez *et al*., 2018) Several Annonaceous acetogenins (AAs) have been isolated from the Annona genus and utilized as alternative chemotherapy against several health malaise (Kim *et al.,* 2018). Two hundred and twelve (212) bioactive phytochemicals have been reportedly present in Annona muricata, of which acetoginins are abundantly predominant, followed by alkaloids, phenols and other phytochemicals (Cosia-Tellez *et al*., 2018). Seventy four (74) of these bioactive compounds elicits a variety of anticancer activities in preclinical cell culture and animal model systems (Rady *et al*., 2018).

Rauvolvfia vomitoria, commonly called serpent wood or swizzler stick (Ekong and Nwakanma, 2017) is a shrub abundant in the tropics (Yu *et al*., 2013) and traditionally known as Asofeyeje in Yoruba, Ira in Igbo and Wadda in Hausa (Igimi *et al*., 2018). It belongs to the family Apocynaceae (Ekong *et al* 2015). In Nigeria it has been implored in traditional folk medicine to manage and cure myriads of health malaise. It is used traditionally for the therapy of psychosis and diabetes (Bisong *et al*., 2011) It has been reported to be anti-inflammatory, antipyretic and analgesic (Bisong *et al*., 2011; Igimi *et al*., 2018), antipsychotic, anticardiovascular (Ekong and Nwakanma, 2017), and anti parasitic (Kutalek and Prinz, 2007). Other pharmacologic studies reported its anticancer properties due to the presence of β-carboline alkaloidsalstonine (Bisong *et al*., 2011), hypoglycemic and antihyperglycemic activities (N'doua *et al*., 2016). Rauvolvia vomitoria is traditionally used against fever, malaria, snake bite, nervous disorder (Amole *et al.,* 2009) jaundice, cerebral cramps, gastrointestinal disorders (Ezeigbo *et al*., 2012) and haemarrhoids (Sharma *et al*., 2004). Several studies have revealed the presence of about 420 alkaloids in the plant (Yu *et al*., 2013). Some of the biologically active compounds include ajmalicine, raucafficine, rescinnamine, ajmaline, yohimbine and reserpine (Azeem *et al*., 2005). The aim of the study was to investigate the anticancer therapy of combined ethanolic extract of *Annona muricata* and *Rauvolfia vomitoria* in NMU induced Swiss albino mice so as to validate the folkloric antitumour claims.

Materials and Methods

Plant Materials

The plants *Rauwolfia vomitoria* LUT 6090 and *Annona muricata* LUT 7386 were first obtained from Oyingbo market, Lagos, Nigeria in March 2016. They were identified and authenticated taxonomically by Mr. Oyebanji, in the Department of Botany, University of Lagos, Nigeria, where voucher specimens were deposited.

Carcinogen

Chemicals and Reagent: Methyl-Nitrosomethylurea (MNU), was obtained from Sigma Aldrich in Germany.

Experimental Animals

Healthy 25 young female Swiss albino mice, weighing between 17 and 20g were obtained from BioVaccine Centre, NAFDAC, Yaba, Lagos. Experimental procedure was approved by the University of Lagos Ethical Committee on Animal Use. Animals were accommodated in aerated plastic cages in Animal House of University of Lagos and allowed to acclimatize for one week. Mice were fed with normal mice pellet manufactured by Ladokun feeds limited, Ibadan, Nigeria and water also provided.

Extract preparation

The plants were air dried and granulated separately to powder form. 300g (ratio 1: 1) of each plant was measured, the and mixed together. 2500ml of 95% cold Ethanol was added to the mixed plants and left for 5 days. The beakers containing the extract were heated on a hot plate at 40 ±1°C till the ethanol evaporated. Two concentrations (50 and 100 mg kg^{-1}) of the fresh extract were prepared for bioactivity.

Experimental design

The mice were divided into five groups of five animals each. A, B, C, D and E where A represented negative control (distilled water treatment only), B represented the positive control (cisplatin treatment), C and D were orally administered 50 mg kg^{-1} 100 mg kg^{-1} doses respectively, E was administered NMU only as tumour placebo. Each mouse in all the groups was intraperitoneally induced with 0.2ml carcinogenic agent of NMU solution. After 24hours of induction, experimental groups C and D were administered 50 and 100 mg kg^{-1} doses per Os (orally) respectively. The positive control mice were treated with 0.2ml of cisplatin. Animals were treated for twelve weeks after which the mice were sacrificed by jugular puncture.

Haematology

Blood was drained and vital organs which included liver, kidney, lungs and heart were harvested and their weights were recorded. The breast tissue was also harvested. Haematology test was carried out to determine parameters such as the haemoglobin, mean cell haemoglobin, mean cell haemoglobin concentration, mean cell volume and platelets. Salicylic acid test was carried out on the breast tissue.

Estimation of Salicylic Acid

Breast tissues were separately weighed and recorded, they were homogenized using porcelain mortar and pestle using 2.5mL phosphate buffer saline by 0.5mL stepwise. 1mL of the homogenate was reacted with 1mL of chilled acetonitrile and vortex mixed for about 1 minute. The resulting suspension was centrifuged for 10 minutes at 4000rpm. The supernatant was aliquoted using Pasteur pipette and filtered with 0.5µm acrodysc syringe filter followed by injecting into HPLC Agilent make, 1200 series.

Homogenization of Breast and Liver tissues

Breast and Liver samples were harvested separately, washed in ice-cold saline to ensure it is blood-free. The tissues were separately homogenated the different homogenates (5%w/v) were prepared in cold 50 mmol/L potassium phosphate buffer at pH 7.4 in ice homogenizer. It was then centrifuged at 5000 r/min for 15min at 4 °C so as to remove the cell debris. The supernatant of the breast tissue was used to estimate catalase (CAT), glutathione peroxidase (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA). Supernatant of the liver tissue was used to estimate aspartate transaminase (AST), alanine transaminase (ALT), alanine phosphatase (ALP), lactate dehydrogenase (LDH) and protein concentration.

Determination of liver enzymes

The liver enzymes, serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were determined with Randox diagnostic test following manufacturer's instruction.

Lipid peroxidation level

Estimation of malondialdehyde (MDA) is often used to measure lipid peroxidation. Tissue MDA was measured using the assay method of measuring thiobarbituric reacting substances (TBARs) by Nagababu et al., (2010). Twenty-fold dilution of

supernatant (100 µL) in 0.15M Tris-KCl buffer was mixed with 30% trichloroacetic acid (500 µL) and 0.75% TBA (500 µL). The mixture was heated at 80 °C for 1 h then extracted with 1 mL butanol. The organic phase was separated by centrifugation for 5 min at 3000 g and measured at 532 nm. The molar extinction coefficient 1.56×10^5/M/cm was used in calculating the amount of MDA formed. The concentration of TBARS in the breast tissues were expressed as nmol MDA/mg protein.

CAT Assay

This was estimated in triplicate according to Aebi (1984). The disappearance of H_2O_2 was monitored at 240nm. 30 µL breast homogenate was suspended in 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0) (Aebi 1984). CAT assay commenced by adding 0.5 mL of 0.1 mol/L hydrogen peroxide solution and absorbance at 240 nm was recorded every 10 seconds during 2 min and used to calculate CAT activity. Phosphate buffer was used to substitute H_2O_2 solution, serving as negative control. Molar extinction coefficient 39.4 M^{-1} cm^{-1} for H_2O_2 was used to determine activity of CAT, and expressed as nmol of hydrogen peroxide converted per min per mg total protein where 1 IU activity=1 µmoL H_2O_2 converted to H_2O per min.

SOD Assay

SOD activity was estimated using the method of Marklund and Marklund (1974) with slight modifications by Thanh *et al.,* (2015). This method is predicated upon the ability of SOD to inhibit the autoxidation of pyrogallol. In 970 µL of buffer (1 mmol/L EDTA, 100 mmol/L Tris-HCl, pH 8.2), 20 µL pyrogallol 13 mmol/L and 10 µL of breast homogenates were mixed. Assay was carried out in thermostated cuvettes at 25 °C and changes of absorption were recorded by a spectrophotometer) in triplicate at 420 nm. One unit of SOD activity was defined as the amount of enzyme that can inhibit the auto-oxidation of 50% of the total pyrogallol in the reaction.

GSH Assay

GSH was measured according to (Thanh *et al.,* 2015). 1 mL of the assay mixture contained 100 µL of 10 mmol/L GSH, 770 µL of 50 mmol/L sodium phosphate (pH 7.0), 10 µL of 1.125 mol/L sodium azide,100 µL of 2 mmol/ L nicotinamide adenine dinucleotide phosphate (NADPH), 10 µL 100 IU/mL glutathione reductase and 10 µL deproteinized breast supernatant. The mixture was equilibrated for 10 min. The assay began by adding 50 µL of 5 mmol/ L H_2O_2 to the mixture, NADPH oxidation was measured during 5 min at 340 nm. One unit of glutathione peroxidase was defined as the amount of enzyme able to produce 1 µmol NADP+ from NADPH per min. GSH activity was determined using the molar extinction coefficient 6 220 M^{-1} cm^{-1} for NADPH at 340 nm and reported as IU per mg total protein.

Statistics

The statistical analyses were performed using Microsoft Excel. The data was expressed as the Mean ± SEM. The data was subjected to sample T-test, evaluating the statistical significance of the difference between two means of various parameters between the control and experimental group. The p value was found by means of Microsoft Excel.

Results

Week-dependent weight reduction was observed in group administered distilled water +NMU (tumour placebo). By the 11th week drastic reduction in weight was observed in this group. However the 50 and 100 mg kg^{-1} showed considerable increase in body weight dose-dependently, whereas the cisplatin group showed slight weekly increase but significantly different from control.

Table 1: Average weight of the mice from week 1 to week 12

Weeks	Distilled water +NMU	Cisplatin	50 mg kg^{-1}	100 mg kg^{-1}	Distilled water
1	18.70±1.44	19.80±0.41	17.90±0.59	19.60±1.5	19.70±0.37
2	19.40±1.57	19.80±0.37	18.00±0.55	19.00±1.78	19.50±0.5
3	21.60±0.93	24.00±0.55	23.60±0.93	23.30±2.40	22.00±1.08
4	21.60±0.93	23.00±0.41	22.75±0.63	24.60±2.19	22.25±1.31
5	21.60±0.87	22.50±0.50	23.00±0.71	24.70±2.19	23.25±1.70
6	20.25±0.85	21.00±0.41	22.25±0.85	25.00±2.00	23.25±1.25
7	20.25±0.85	20.75±0.48	21.50±0.87	25.60±2.19	24.25±1.25
8	19.00±0.70	22.50±0.58	22.00±0.58	25.00±2.52	24.30±1.20
9	18.00±1.00	22.00±0.58*	24.00±1.52	25.00±2.52	24.30±1.20
10	17.00±1.00	22.00±0.58*	24.00±1.52	25.00±2.52	25.50±1.50
11	15.00±1.00	23.00±0.58*	24.00±1.52	25.10±2.50	25.70±1.40
12	15.00±1.00	23.00±0.58*	24.00±1.52	25.00±2.51	26.50±1.00

All data are expressed as mean ± SEM. (*) significant $P \leq 0.05$ different from control (distilled water)

There is reduction in the weight of the liver in the negative control group and the group treated with 100mg/kg of the di-herbal formulation. The weight of liver and kidney organs of all the groups are constant. The lung of the positive control group weighed more when compared to the weight of the normal group.

Table 2: Weight of mice organs after sacrificing

Treatments	Livers	Kidneys	Hearts	Lungs
Distilled water	1.30±0.03	0.10±0.02	0.10±0.03	0.10±0.02
Negative contrl	1.10±0.03	0.10±0.02	0.10±0.03	0.10±0.02
Cisplatin	1.10±0.08	0.10±0.02	0.10±0.02	0.20±0.02
50 mg kg^{-1}	1.30±0.11	0.10±0.01	0.10±0.03	0.10±0.02
100 mg kg^{-1}	1.30±0.12	0.10±0.02	0.10±0.02	0.10±0.01

All data are expressed as mean ± SEM.

There is an increased level of haemoglobin, pcv, wbc, rbc and mcv in the group treated with di-herbal formulation when compared to the normal control. The mean cell volume of the positive control group is elevated compared to the normal control.

TABLE 3: Effect of cisplatin and di-herbal formulation on hematological parameters in MNU induced breast cancer in mice.

SAMPLE	Hb g/dl	pcv %	wbc mm3	Rbc	Mcv	Mch	mchc	Neut	lym	Mon	eos	Bas
Dist. water	12.58±0.01	35±0.4	3800±0.41	4.80±0.06	73.76±0.65	26.20±0.40	35.93±0.40	26.25±0.63	70.75±0.85	1.50±0.30	0	0
NEGATIVE CONTROL	12.80±0.01**	25±0.4	5000±0.40*	2.05±0.03	72.05±0.63*	25.40±0.43	35.52±0.41	20±0.40*	68±0.41*	2.5±0.30*	0	0
Cisplatin	11.48±0.02**	35±0.4	4150±0.41**	3.92±0.01*	88.74±0.60*	28.80±0.64*	32.14±0.44	29±0.40*	70±0.40	0.75±0.00	0	0
50 mg/kg-1	14.58±0.01**	41±0.40**	4300±0.40**	5.45±0.06*	75.07±0.74	26.13±0.51	35.54±0.41	35±0.41*	65±0.41**	00±00	0	0
100 mg/kg-1	15.58±0.02**	44±0.4**	4000±0.40**	5.61±0.00*	78.45±0.43*	28.24±0.64*	38.90±0.41	21±0.41	76.5±0.65**	2.00±00	0	0

All data are expressed as mean ± SEM. (*) significant $P \leq 0.05$ different from control Haemoglobin(Hb), packed cell volume (pcv), white blood cell(wbc), red blood cell (rbc), mean cell haemoglobin (mch), mean cell haemoglobin concentration (mchc), neutrophil (neut), lymphocyte (lymp), monocyte (mon), eosinophil (eos), basophils (bas).

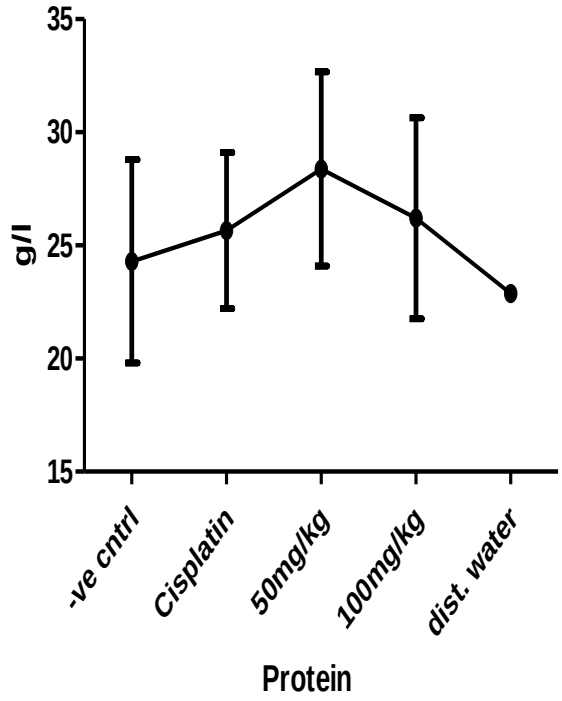


FIG 1A. Activities of treatments on Protein (MDA)

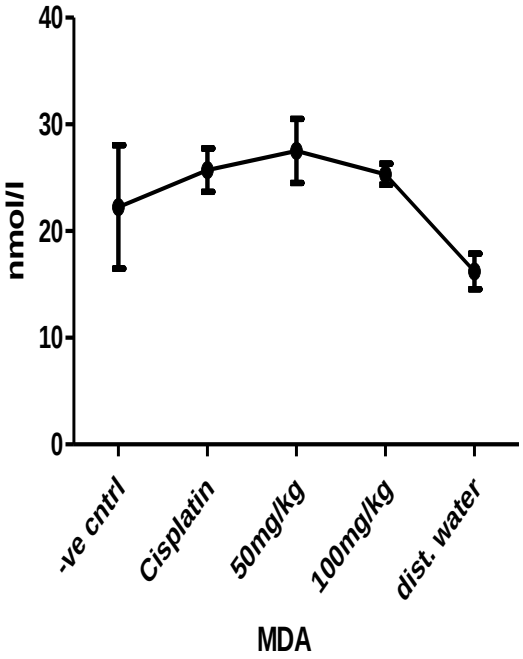


FIG. 1B. Activities of treatments on Malondialdehyde

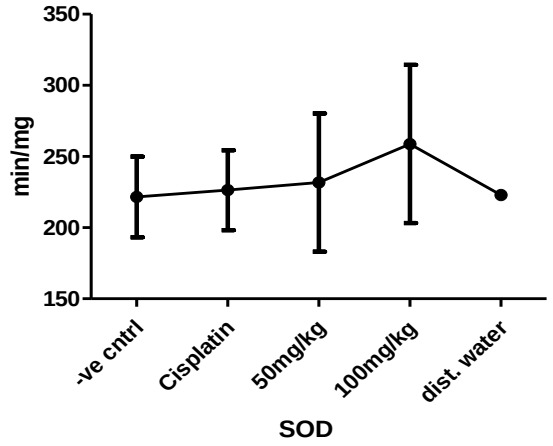


FIG 1C Activities of treatments on Superoxide dismutase (SOD)

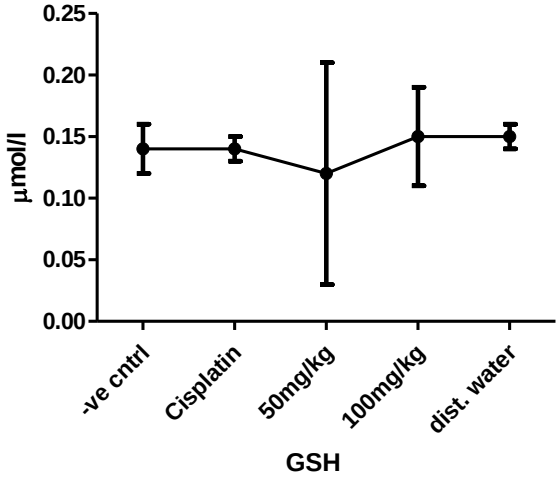


FIG 1D Activities of treatments on Glutathione peroxidase (GSH)

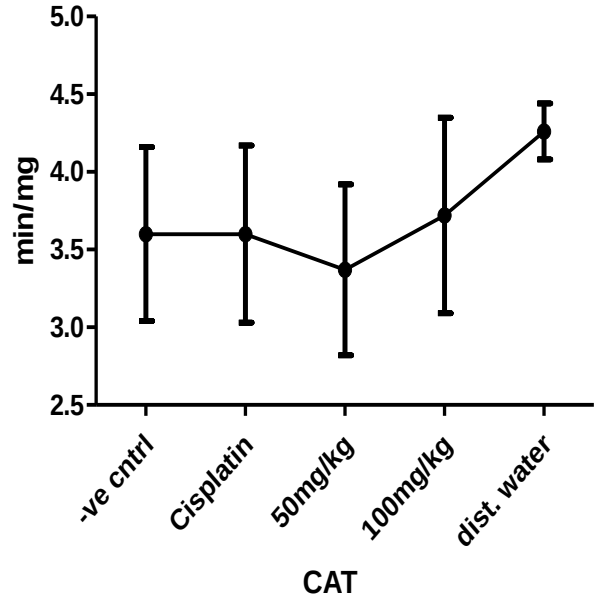


FIG 1F Activities of treatments on Catalase (CAT)

In the figures above there was gradual elevation of protein (1A) with a sharp fall in 100mg/kg, MDA and SOD (1B & C) showed significant increase compared to normal control (distilled water only). Catalase (1F) dropped when compared to normal control.

Figure 1A-E: showing levels of breast protein, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) in NMU induced breast cancer in mice.

Table 4: Effect of the diherbal formulation on enzymes and biochemical analytes in **NMU induced breast cancer in mice**

Treatment	Pro	AST µ/l	ALT µ/L	ALP µ/L	ALB mg/dl	LDH
-VE CNTRL	28.38±0.7	18.37±1.8	25.11±0.4**	104.88±3.2**	4.53±0.0**	180.21±3.6**
Cisplatin	28.11±1.4	17.54±2.2	24.84±2.3*	96.60±1.6**	4.49±0.0**	144.44±12.6
50 mg/kg^{-1}	27.83±0.4	16.89±2.4	24.61±3.0*	86.48±2.4**	4.45±0.0**	134.80±6.0
100 mg/kg^{-1}	27.11±1.2	12.22±0.6	12.53±1.2	79.12±2.4*	2.96±0.0*	123.81±4.1
Distilled water	24.04±1.3	12.43±1.5	10.83±0.7	70.84±1.84	4.07±0.0	125.19±1.4

Data represents mean ± SEM of biochemical analytes from liver of mice (N=5).
* P<0.05; **P<0.001 significantly different from control (Student's t-test).

There is a significant increase in the level of Albumin, lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and alanine transaminase (ALT) in the negative group while all diherbal formulation groups compared favourably with normal control group. The positive control group (Cisplatin) showed significant increase in AST and ALP activities when compared to the normal control group.

Table 5:The mean peak area of Salicylic acid content and the concentration per gram in mice breast after NMU induced breast cancer

Treatment	Mean Peak Area	xdf(2)	Concentration (µg/g)
			2.250
-VE cntrl	1.127	2.254	
			3.027
Cisplatin	5.057	10.114	
			5.566
50mg/kg^{-1}	3.599	7.198	
			8.618
100mg/kg^{-1}	4.76	7.52	
			3.363
Distilled water	0.794	1.588	

All treatment elicited dose-dependent increase in salicylic acid when compared to the normal control. The 100 mg/ml of the di-herbal formulation contained the highest concentration of salicylic acid when compared with group normal control of distilled water.

Histology of Breast and internal organs of mice: Showing various degree from mild to severe histo-achitectural distortions

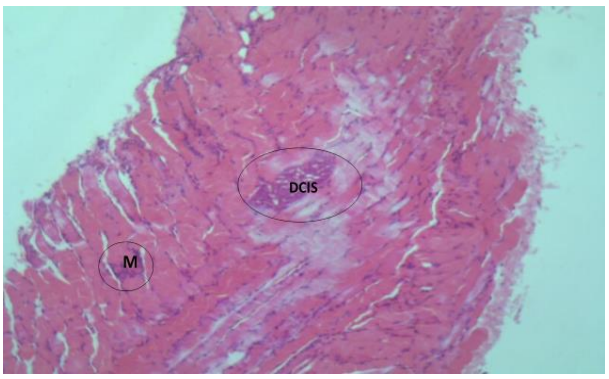


Plate 1. X40 (50mg/kg^{-1})

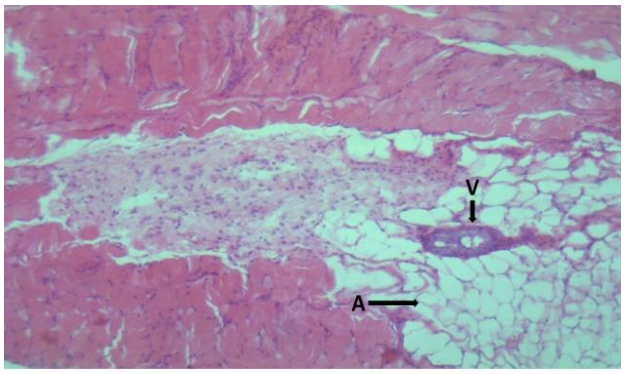


Plate 2. X40 (100mg /kg^{-1})

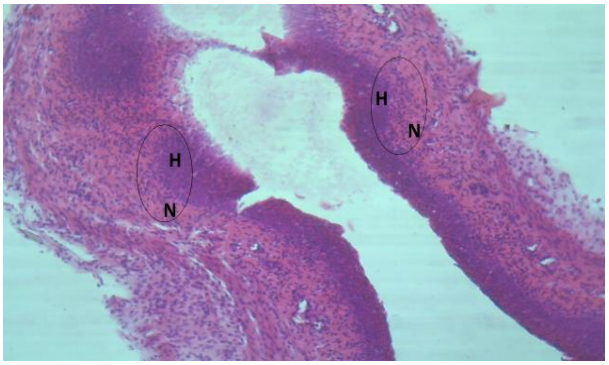


Plate 3. **X40** Control (distilled water +MNU)

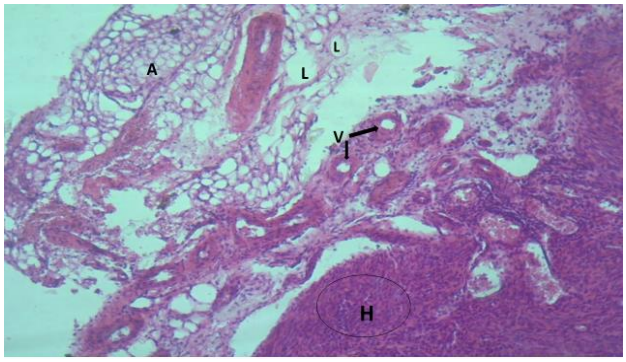


Plate 4. **X40** Cisplatin

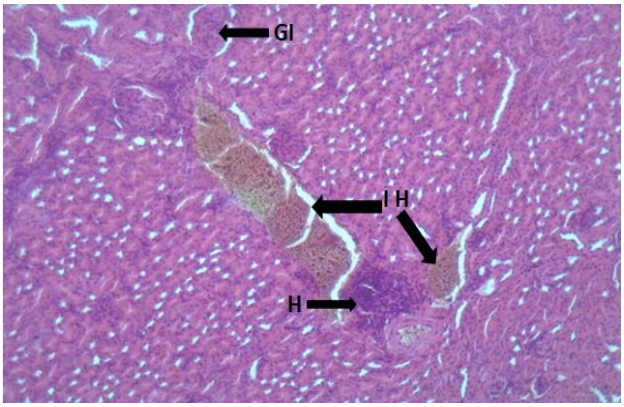


Plate 5. X40 (50mg/kg^{-1})

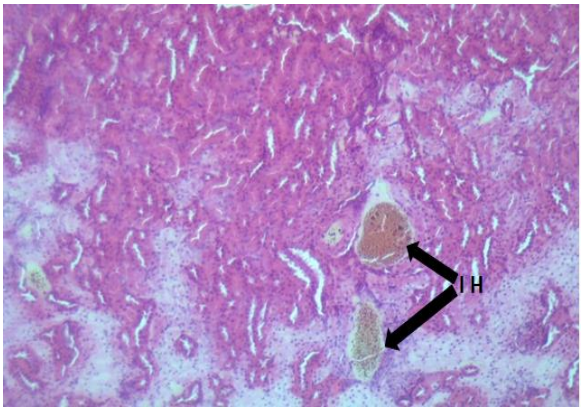


Plate 6. X40 (100mg /kg^{-1})

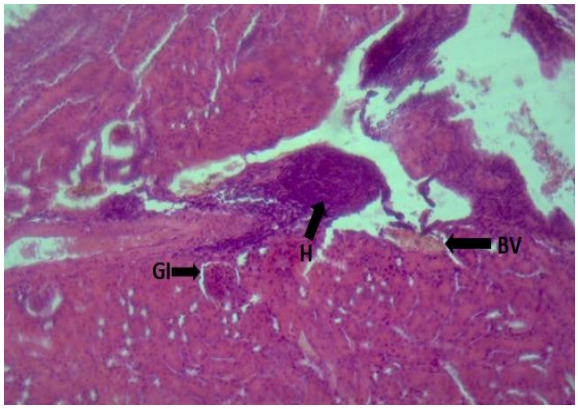


Plate 7. **X40** Control (distilled water +MNU)

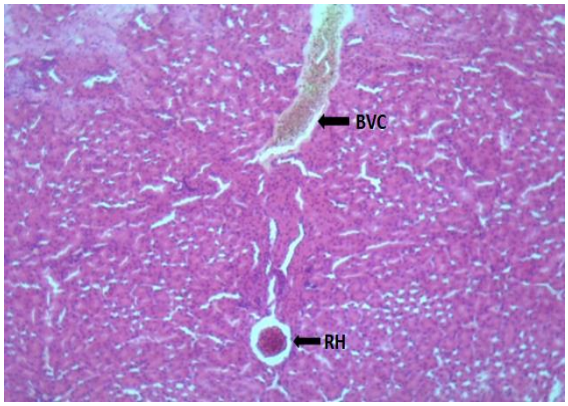


Plate 8. **X40** Cisplatin

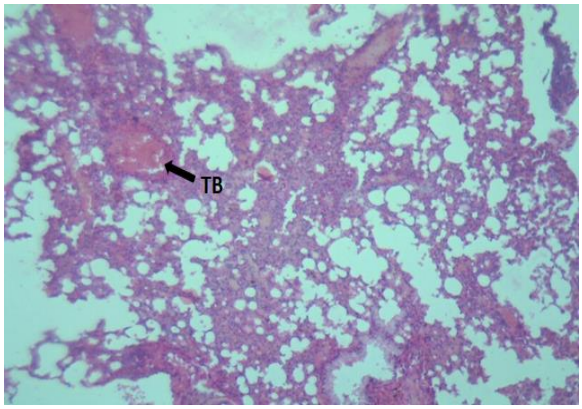


Plate 9. X40 (50mg/kg-1)

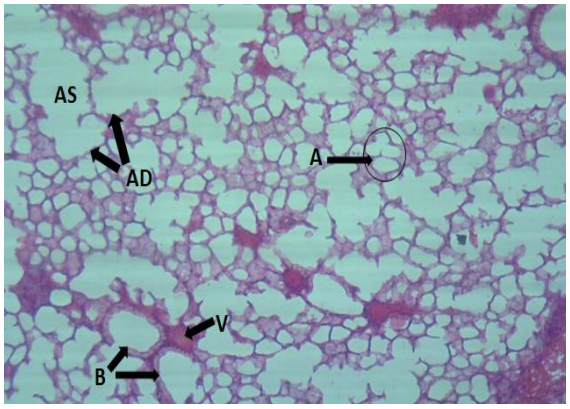


Plate 10. X40 (100mg /kg-1)

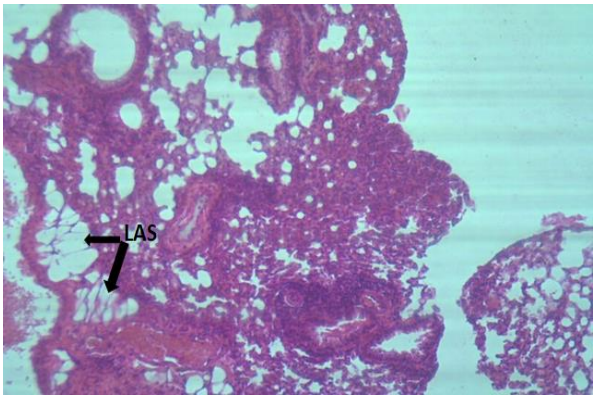


Plate 11. **X40** Control (distilled water +MNU)

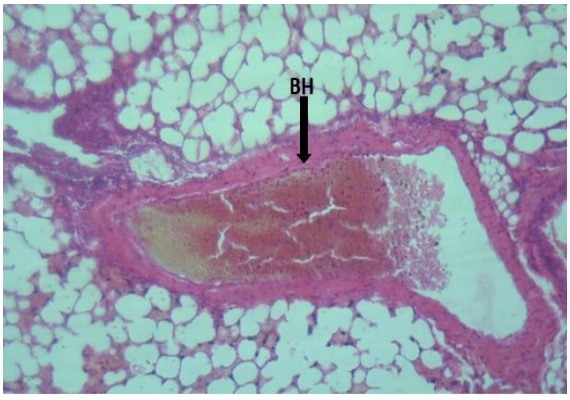
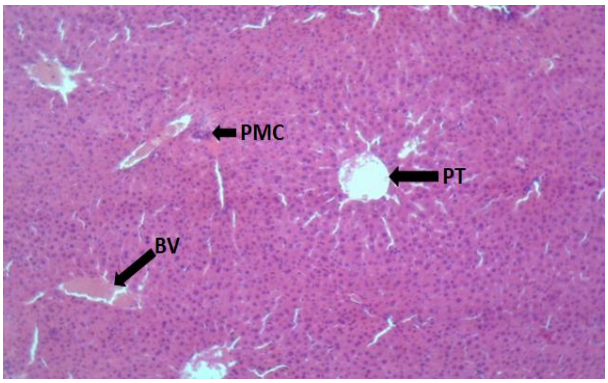


Plate 12. **X40** Cisplatin



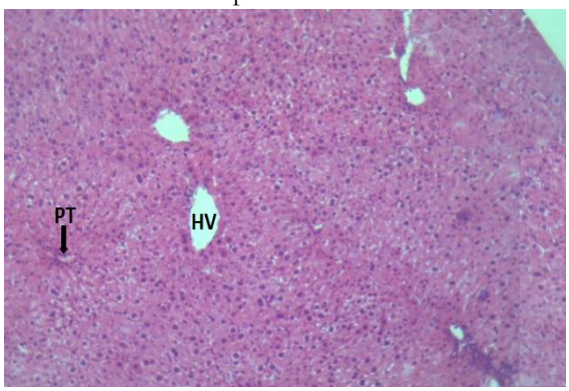


Plate 13. X40 (50mg/kg-1)

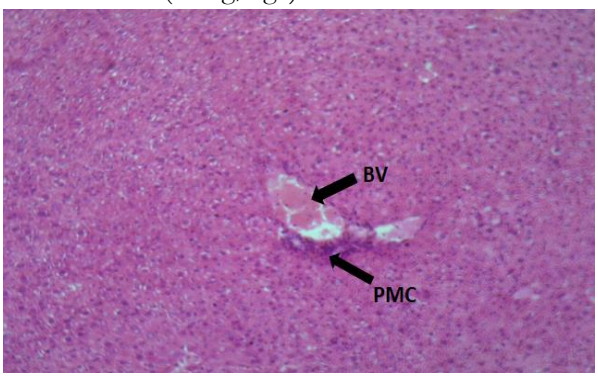


Plate 14. X40 (100mg /kg-1)

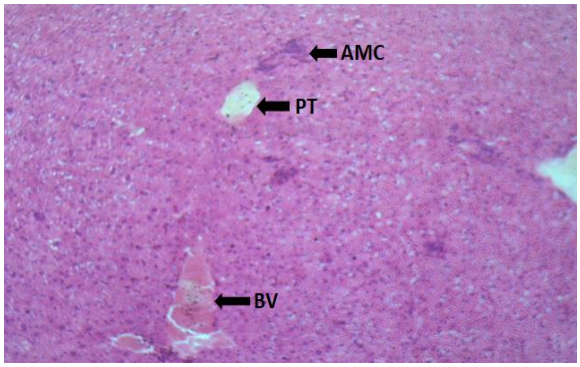


Plate 15. **X40** Control (distilled water +MNU)

Plate 16. **X40** Cisplatin

Plate 1; **M** - mononuclear inflammatory cells infiltrates admixed with tumour-like single cells deposit, also showing Ductal carcinoma insitu (DCIS). Plate 2; Conserved breast with **A**-adipose tissues and **V**-vessel. Plate 3; showing **H**-hyperplastic basophilic nuclei, and **N**- neoplastic nuclei. Plate 5-8 (kidney). Plate 5- histoachitectural changes were minimal. Glomerulus with mononuclear cell infiltrates (GI), interstitial haemorrhage (IH) and hyperplastic basophilic nuclei (H) observed. Plate 6- most features were conserved, but interstitial haemorrhage observed. Plate 7-gross malformation such as glomerulus with mononuclear infiltrates (GI), hyperplastic basophilic nuclei (H), increased renal tubules and glomeruli granulation reaction seen. Plate 8- shows blood vessel congestion (BVC) and renal haemorrhage (RH). Plate 9-12 (Lungs). In Plate 9, mild malformation such as terminal bronchiole (TB) congested with blood observed. Most of the features observed in plate 10 such as alveoli spaces (AS), alveoli ducts (AD), alveoli (A), pulmonary veins (V) and bronchi (B) appeared normal. Gross malformation such as loss of alveoli sac architecture (LAS), alveoli haemorrhage, granulation reaction changes and hyperplastic basophilic nuclei were observed. Bronchus haemorrhage was observed in plate 12. Plate 13-16 (Liver). Minor changes such as perivascular mononuclear cell cuffing (PMC), blood vessel (BV) congestion were seen in plate 13. Features of plate 14 including portal tract (PT) and hepatocentrilobular venule (HV) were conserved. In plate 15, perivascular mononuclear cell cuffing (PMC), blood vessel (BV) dilation and congestion were noticed. Plate 16 showed blood vessel (BV) congestion, aggregates of mononuclear cells (AMC) and congestion of blood vessel.

Discussion

Numerous extracts from plants have been reported to play important role in disease management and eradication, some possessing significant antioxidant properties which play vital roles in alleviating the effects of cancers in the body (Abd Eldaim *et al.*, 2019). Several others are therapeutic due to their pharmacological, biological and antioxidant properties (Abd Eldaim *et al.,* 2019). It has been estimated that 80% of Africa and Asia population utilize traditional medicinal plants, while 70 – 80% of developed countries entertain some form of alternative or complimentary medicine (Araujo *et al.*, 2017). This study investigated some antitumour and toxicologic activities of combined extract of *Annona murucata* and *Rauvolvia vomitoria* (AR).

Body weight is an important criteria in evaluating effect of toxicity of drugs or toxic substances (Fereira *et al.*, 2014). Changes in weights can be one of the first critical evidence of toxicity (Ajayi *et al*, 2019). Evaluation of growth in animals utilizing body weight index is a routine in toxicological studies as it assist in interpreting compound-related effects (Murbach *et al.,* 2017) Moreso, decreased body weight is an indicator of treatment-induced changes and sensitive index of toxicity (Fereira *et al.*, 2014). In

this investigation, weight of mice assessed weekly for 12 weeks showed considerable dose-dependent increase in groups administered 50 and 100 mg kg^{-1} of AR respectively. There was weekly increase in those administered cisplatin (reference drug), though lesser than AR and significantly ($P < 0.05$) lesser than control. The group administered distilled water + NMU showed a significant decrease compared to control and grossly emaciated body. NMU may be responsible for emaciation and decreased body weight observed in group not administered with AR. The toxic agents in the NMU may elicit abnormal metabolic reaction that may affect the body weight. AR administered to other groups may have mitigated the effect of NMU. Abnormality in internal organ weight may suggest toxicity either related to administered extract or inducing agent (NMU). The decreased weight of liver in the negative control may be attributed to the effect of NMU. Alteration in organ weight such as liver and kidney may result in hepatocellular hypertrophy (Greaves, 2011) and nephropathy (Hard and Seely, 2005; Sellers *et al.,* 2007) respectively. Ethanolic extract of AR did not cause alteration in major organs, hence no toxic effect was conferred. Myelosuppression and anaemia are predominantly common in cancer chemotherapy (Rajeshwar *et al.*, 2005). The anaemia observed in the group not administered with AR may be attributed the presence of tumour cells, either caused by deficiency in iron or due to myelopathic and haemolytic conditions.

Assessing haematologic parameters provides vital signs of local and systemic toxicity manifestations elicited by drugs (Ferreira *et al.,* 2014; Raza *et al.*, 2002). It also serves as index of physiological and pathological status in humans and other animals providing vital information pertaining to abnormalities in body metabolism and consequent response of the body to lesions, injuries, stress and deprivation (Dal Bosco *et al.,* 2014). Also haematologic profile provide relevant information on haematopoietic and immunological systems (Balogun *et al.*, 2014)

The hematological parameters commonly evaluated include RBCs, WBCs, neutrophils, eosinophils, lymphocytes, HGB, HCT, MCV, MCH, MCHC, PLT, MPV, RDW-SD, RDW-CV, PDW, P-LCR, PCT and monocytes (Ali *et al.*, 2019). Our study showed dose-dependent increase in Hb, PCV, RBC and MCH. This elevation may be due to the protective activities of the diherbal preparation on NMU induced mice so as to eliminate or induce apoptosis on the carcinomas in the tissues, as they are important indicators in cases of cell carcinoma, hypoxia and pulmonary fibrosis (Ali et al., 2019). In toxicity investigation, excessive WBC may indicate the effect of chemicals in inducing the animals, which elicits immune response of treated animals. In this study NMU was used to induce tumour and AR extract introduced as treatment. Therefore, WBCs and its differentials such as lymphocytes, neutrophils, basophils, eosinophils, and monocytes play vital role in immunity against infections, whereas excessive production of leukocytes can serve as a veritable biomarker for inflammatory and stress related disorders (Ross, 1999). However, significant decrease in WBCs indicates leucopenia which implies the body inability to fight against infections (Tousson *et al.*, 2011).

Our results revealed a dose-dependent decline in MDA from lesser dose to higher dose of AR. It is an indication that the AR reduces free radicals production, consequently minimizing distortion to the cell membrane and reduction in MDA synthesis (Rajeshwar *et al*, 2005). The cell possess effective antioxidant mechanism comprising of SOD, GSH and CAT which prevents damage due to free radicals. Imbalance between production of reactive oxygen species and antioxidant defense results in oxidative stress, thereby causing cellular functions deregulation. SOD, CAT and GSH are metalloenzymes that constitutes the main endogenous enzymatic defense machinery against ROS (Susan *et al.*, 2019). Glutathione (GSH) is highly protective to the cells, in the endogenous antioxidant system it plays a very vital role as it protect the function and structure of the cell, partake in detoxifying the tissues by maintaining redox homeostasis and quenching free radicals(Demir *et al.,* 2011) . It is a potent inhibitor of neoplastic process.

Previous studies observed that tumor presence in any part of the body interferes with functions of the vital organs (Ajayi *et al*., 2019). In this study GSH increased in the highest dose, indicating the AR extract possess the ability of eradicating free radicals, thereby preventing oxidative stress that may culminate in damage to macromolecules (Rajeshwar *et al*., 2005)

Medicinal Plant extracts have been described to be rich in antioxidant that somehow exert certain chemopreventive effect, through scavenging for reactive oxygen species and improving on the antioxidant defense system (Dongre, *et al.,* 2008). AR recorded increased SOD and CAT level at the highest dose, implying that the extract possess some antioxidant properties and displayed antitumor activities in the treated mice. *Annona muricata* which constitute 50% of AR extract had been reported to restore activities of CAT, GSH, MDA and SOD and also possess the ability to reduce cellular ROS (Coria-Tellez *et al*., 2018). Decreased SOD and CAT activities in the non-treated groups may serve as a marker of malignant transformation (Dongre, *et al.,* 2008). Anti-cancer activity in most antioxidants have been reported to be either by induction of apoptosis or through inhibition of neovascularization (Rajeshwar *et al*., 2005). Numerous reports have been made on implication of free radicals in tumours (Ravid and Korean 2003; Feng *et al*., 2001). It is therefore affirmed that antioxidants inhibits tumour effectively.

In recent studies, anticancer activities of salicylic acid have been described (Wu 2000; Liu *et al*., 2017; Xu *et al*., 2018). The presence of high concentration of salicylic acid in AR may have contributed to its dose-dependent antitumour activities.

Histologic examination of tissues can reveal the relationship between the concentrations of toxins and their effect on the target organs **(**Ali *et al*., 2019). Neoplastic activities and necrosis can be distinguished in liver, breast, kidney, stomach, heart, lung, pancreas, spleen, and brain which are the common examined organs **(**Ali *et al*., 2019). This study, exposed remarkable morphological and pathological changes in the major organs of mice not subjected to AR treatment. However mice administered AR extract for 12 weeks showed conserved histoachitecture of major organs.

The liver enzymes AST, ALT, ALP, TP, GLB, and ALB serve as the main sensitive biomarkers of liver toxicity or damage (El-Demerdash, 2004; Ali *et al*., 2019), such that their elevation suggests injury to the hepatocytes(Thapa, and Walia, 2007)**.** The liver is also reported to be susceptible to effects relating to anti-tumour drugs. Elevated AST and ALT are observed when permanent injury is inflicted on hepatocellular cytoplasm and mitochondria (Giboney, 2005; Zou *et al*., 2019). However, increased AST and LDH activity in serum may also imply myocardial damage, since both enzymes occur in heart and liver (Ali *et al*., 2019) Decreased level of serum TP and albumin somewhat provide information on severity of necrosis in the liver (Tanvir *et al*., 2015). Increased serum ALP indicates cholestasis (Do 2010; P'ng *et al*., 2013). In our study, AR extract did not exert adverse effect on the analytes evaluated for liver function markers. It exhibited considerable decrease dose-dependently, the highest dose (100mg/kg) exerting a significant decrease in ALP and ALB. This implies that the hepatocyte integrity is not compromised and may be attributed to high presence of phenolic compounds that possibly elicits some protective effects and sustaining hepatocytes integrity (Ali *et al*., 2019). The levels of AST, ALT, Protein and LDH in the treated mice were similar to the control, indicating AR is not toxic on hepatocytes. Arthur *et al.* (2012a, 2012b) reported the hepatoprotective potential of *A. muricata*.

The antitumour activities observed may be due to the synergy between the two combined herbs (*A. muricata and R. vomitoria*). *A. muricata* had been reported to elicit several antitumorigenic activities (Hamizah *et al*., 2012; Dai *et al*., 2011; Ko *et al.,* 2011; Wang *et al*., 2002; Yang *et al*., 2015 ; Abdul Wahab *et al*., 2018). Acetogenin have been implicated in the inhibition of mitochondrial complex 1 (Lannuzel *et al.,* 2003) and ubiquinone-linked oxidase in the plasma membrane of tumour cells, thereby causing apoptosis (Ali *et al*., 2019). Some acetogenins such as

bullatacin and annonacin found in *A. muricata* have been implicated as necessary active agents that may contribute in eliciting antitumorigenic activities (Ko *et al*., 2011). The mechanism of action may be that AR possibly downregulated proliferating cell nuclear antigen and proteins of Bcl-2, it may also have upregulated proteins of Bax and also embarked on restoring antioxidant enzymes. AR may also have elicited inhibition of several signaling pathways that regulates necrosis, metastasis, metabolism and stimulate arrest of cell cycle (Coria-Tellez *et al*., 2018). Another agent found in *A.muricata* that possibly contributed to antitumour activities is Annomuricin E (Coria-Tellez *et al*., 2018). It has been implicated in depleting mitochondrial membrane potential(MMP), causing mitochondrial permeability transition pores to open and exude pro-apoptotic proteins to cytosol, resulting in apoptosome formation and activation of caspase cascade adduced to mitochondrial death pathway (Coria-Tellez *et al*., 2018). R. vomitoria have been reported to contain alkaloids such as sapentine and alstonine that exhibited antitumorigenic activities (Yu *et al*., 2013).

Conclusion

The likely synergy of bullatacin, annonacin and annomuricin from *A. muricata* on one hand and sapentine and alstonine in *R. vomitoria* which constitute the AR extract may have mediated the effect of NMU induced tumour by reducing lipid peroxidation thereby enhancing antioxidant enzymes. It also significantly improved the biochemical analytes and abrogated rapid proliferation as neoplasm. The high concentration of salicylic acid extracted from breast tissue of treated mice derived from the diherbal mixture "AR" probably contributed to the antitumourigenicity of "AR". These results therefore provides veritable data in the improvement of a promising, high therapeutic potential of antitumorigenic and hepatoprotective natural agent for the management of tumour and liver toxicity. Further investigation on precise mechanism of action of "AR" should be explored.

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Conflict of interest statement

I declare that there was no conflict of interest whatsoever.

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