Anticancer potentials of Morinda lucida and Annona muricata on Ki67 and Multidrug resistance1 genes expressions in Sodium arsenite-induced hepato-toxicity in rats

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ABSTRACT

This study evaluated anticancer potentials of *Morinda lucida* (ML) and *Annona muricata* (AM) on Ki67 and Multi-drug resistance1 (MDR1) concentrations in livers of rats in Sodium arsenite (SA)-induced hepato-toxicity. 60 adult female rats were randomly divided into 12 groups (n= 5). Groups 1 and 2 received physiological saline and 10mg/kg bodyweight of SA respectively. Groups 3-6 received SA followed by treatments with ML and AM doses. Groups 7-10 received extracts only. Groups 11-12 received co-administrations of SA with extracts. Drugs/extracts were administered orally. Experimental procedure was 5 weeks. Consequently, Liver histo-pathology and ELISA concentrations of Ki67 and MDR1 were evaluated. Data were statistically analyzed (P≤0.05). Results showed decreased levels of Ki67 (Groups 11-12) and MDR1 (Groups 3-4 and 11) compared with Group 2, indicating that ML ameliorated SA-induced hyperplasia and drug resistance, while AM ameliorated SA-induced hyperplasia. Therefore, ML possesses anti-proliferation and

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anti-drug resistance potentials, while AM possesses anti-proliferation potentials.

INTRODUCTION

Morinda lucida (ML) is a medium size tree with short crooked branches. Although it is very bitter, different parts of ML have been reported to possess medicinal properties. The leaf extract of the plant was reported to possess trypanocidal, antimalarial activities and aortic vaso-relaxant effect. The use of a weak decoction of the stem bark has been documented to treat severe jaundice. ML leaf extract has also been reported to have a strong oral hypoglycemic property. In Southern Nigeria, numerous people treat malaria by drinking aqueous leaf extract of ML. It is well documented that ML leaf extract has various therapeutic benefits with no known adverse effect among the users[1,2].

Annona muricata (AM) is a member of the Annonaceae family and is a fruit tree with a long history of traditional use. It's also known as soursop, graviola and guyabano. It is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. The fruits of AM are used to prepare syrups, beverages. A wide array of ethno-medicinal activities has been attributed to different parts of AM, and indigenous communities in Africa extensively use this plant in their folk medicine. Numerous researches have substantiated these activities, including anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepato-protective and antidiabetic activities[3,4].

Arsenic is an established human clastogen that exists naturally either in organic or inorganic forms in the environment with attending potential for neurotoxicity, cardiac dysfunction and hepatotoxicity. Arsenic-induced cytotoxicity is via increased generation of free radicals and further confinement of oxidative stress in body organs resulting in damages to DNA, proteins and lipids[5], as well as resulting in increased micronuclei frequency and chromosomal aberrations[6]. Arsenic-induced toxicity can lead to cancers of the skin, lung, bladder, liver and kidney in exposed organisms[5,6].

Furthermore, cancers comprise of cancer stem cells (CSCs), macrophages and vascular endothelial cells, with CSCs having tumourigenic capacity while others do not[7,8]. Cancer treatment regimens kill most cancer cells, but do not eliminate CSCs, which have protective and resistance mechanisms[7,8] via up-regulation of biomarkers of proliferation (Ki67) and drug resistance (multidrug resistance1 (MDR1) gene or P-glycoprotein and Aldehyde dehydrogenase

1)[9]. CSCs are, therefore, able to regenerate other cancer cells well after completion of treatment regimens. Hence, the characteristic survival of CSCs provides explanations for failures of cancer treatments, as well as informed directions for the development of more potent anticancer drugs from plants or other sources.

Ki-67 protein is detected during all the active phases of the cell cycle and it is usually used as a complement to grading systems that include mitotic counting as a sign of proliferation[10,11]. It is one of the five genes (out of 16 cancer-associated genes) of proliferation that is of important weight to the Oncotype score. Ki-67 is not expressed by quiescent or resting cells in the G0phase, hence it is an excellent operational marker for evaluation of the proliferation of a given cell population and the aggressiveness of malignancies[10-12]. The MDR1 gene or P-glycoprotein is localized in the cell-membrane and it functions pharmacologically as an active drug efflux transporter protein of various substances including drugs and toxins[9,13,14]. The MDR1 protein is physiologically expressed at the bile canalicular membrane of the liver functioning in biliary excretion of lipophilic drugs[15]. The MDR1 protein has affinity for hydrophobic compounds and efforts have been made to by-pass its efflux effect using reversal agents such as R-verapamil, Tween-80 and Cremophor EL. These reversal agents have, however, been reported to induce significant toxicity at required doses for MDR1's inhibition[9,13,14].

The characteristic abnormal cellular proliferation (hyperplasia) with accompanied increased expressions of Ki67 and MDR1 by CSCs makes the treatment of cancers a very challenging task. It is, therefore, very relevant to evaluate plants sources towards the isolation of drugs compounds that can specifically target CSCs and reduce or eliminate drug resistance. Arsenic-induced toxicity is of global health concerns; hence it is relevant to search for edible plants' sources which can prevent or counteract the adverse effects of Arsenic-induced toxicity. Therefore, this study evaluated the effects of *Morinda lucida* and *Annona muricata* on immunomodulations of Ki67 and MDR1 protein expressions in the liver tissues of rats in Sodium arsenite-induced hepato-toxicity in-order to further determine which plant fractions possess hepato-protective, anti-proliferation, anti-drug resistance and anticancer potentials.

METHODOLOGY

Collection, Authentication and Deposition of *Morinda Lucida* (ML) and *Annona muricata* (AM) Leaves

Freshly cut leaves of ML and AM were obtained locally from forest reserves in Ilorin and samples identified and authenticated by a Pharmaceutical Botanist of the Department of Botany, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria. ML and AM leaves were deposited at the herbarium of the Department of Botany, Faculty of Life Sciences, University of Ilorin, and assigned Herbarium Identification Numbers UITH/004/1103 and UITH/003/1106 respectively.

Preparations and Ethanolic extractions of *Morinda Lucida* (ML) and *Annona muricata* (AM) Leaves

ML and AM leaves were air-dried at the laboratory unit of the Department of Chemistry, University of Ilorin, Ilorin, Nigeria. The dried leaves of ML and AM were grinded to powder form to enable proper absorption of solvent and weighed using the electronic compact scale. Extraction was carried out using distilled ethanol in-order to remove impurities, and the resultant product was put in a conical flask and heated. Liquid ethanol flowed from the condenser into a container and was continuously recycled to keep the process running. Boiling chips/anti-bumping granules were put in the conical flask to prevent liquid ethanol from 'bumping' into the condenser[16].

The mixture was decanted and then sieved after 24 hours. After decantation, another distilled ethanol was added to the sieved ML and AM and left for another 24 hours. When the colour quality and texture of the dissolved ML and AM in ethanol became evidently low (compared to previous solutions decanted), the procedure was halted. Ethanol was separated from ML and AM and Column chromatography was done to get different fractions of ML and AM[16]. Column Chromatography Fractionation of Ethanol Extract of *Morinda Lucida* (ML)

The ethanol extract of ML was were fractionated in a silica gel open column, using n-hexane, dichloromethane, ethyl acetate and ethanol in an increasing order of polarity (N-hexane: Dichloromethane [3;1,3:2,1:1,1:2,1:3]; Dichloromethane, Dichloromethane: Ethylacetate [3:1,3;2, 1:1, 1:2, 1;3]; Ethylacetate; Ethylacetate: Methanol [3:1, 3;2, 1:1, 1:2, 1:3] and Methanol, to afford thirty-six eluents of 250ml each. The resulting eluents were pooled based on the colour of the solvents that elute them to give a total of nine combined fractions[16]. The fraction MLF1 which had the best preliminary antioxidant potential out of the 9 fractions was used in this study to evaluate the effects of ML on Sodium arsenite-induced hepato-toxicity in rats.

Column Chromatography Fractionation of Ethanol Extract of *Annona muricata* (AM)

The ethanol extract of AM was fractionated in a silica gel open column, using n-hexane, dichloromethane, ethyl acetate and ethanol in an increasing order of polarity (N-hexane: Dichloromethane [3;1,3:2,1:1,1:2,1:3]; Dichloromethane, Dichloromethane: Ethylacetate [3:1,3;2, 1:1, 1:2, 1;3]; Ethylacetate, Ethylacetate: Methanol [3:1, 3:2, 1:1, 1:2, 1:3] and Methanol, to afford thirteen eluents of 250ml each. The resulting eluents were pooled based on the colour of the solvents that elute them to give a total of five combined fractions[16]. The fraction AMF1 which had the best preliminary antioxidant potential out of the 5 fractions was used in this study to evaluate the effects of ML on Sodium arsenite-induced hepato-toxicity in rats.

Animal Care and Feeding

A total number of sixty (60) female Wistar rats with an average weight of 156g and 2 months of age were used in this study. The rats were of different initial bodyweights (Kg) depending on time of birth as available from the same colony bred. Male rats were used in our previous study which evaluated the effects of plants' extracts on Ki67 and MDR1 levels in 7,12-Dimethylbenz[a]anthracene-induced cancer model [16]. Hence, female rats were used in this study in-order to provide comparative gender analyses on the ameliorative effects of plants' extracts on Ki67 and MDR1 levels. The rats were acclimatized for 5 days, received water ad libitum and kept in the animal house located in the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin. The animals were fed daily with pelletized grower feed from Ogo-Oluwa Livestock and Aqua Feed enterprise, Kwara State, Ilorin. The grower feed contains 15% crude protein, 7% fat, 10% crude fibre, 1% calcium, 0.35% phosphorus and 2.55% kcal/kg of metabolized energy as indicated on the pack. The animals were grouped into ten with five animals each in a wire gauzed cage. The animals were kept under a normal room temperature of 37°C and double-crossed ventilation.

Chemicals and Reagents

Sodium arsenite (SA) was a product of Sigma–Aldrich Japan Co. (Tokyo, Japan), and was purchased from Emed Ejeson enterprises in Ilorin, Kwara State, Nigeria. Normal Saline was obtained from MOMROTA pharmaceutical company in Ilorin, Kwara State, Nigeria.

Experimental Procedures and Drugs Administration

The experimental procedures and drugs administration are as detailed in Table 1. The number of rats employed in this study was determined based on the guidelines and approval of the University Ethical Review Committee (UERC) of University of Ilorin, Nigeria. The dose of Sodium Arsenite was determined from a previous study[17], while the doses of ML and AM were determined from previous study on anticancer potentials of ML and AM in Lead acetate-induced toxicity in rats[18]. In addition, bodyweights (g) of all rats were measured on Day 1 of experimental procedure and at the end of each week.

The Experimental treatments and toxicological profiling Groups were in six categories as detailed below.

Anticancer potentials of ML: Groups 3 and 4.

Anticancer potentials of AM: Groups 5 and 6.

Toxicological profiling of ML: Groups 7 and 8.

Toxicological Profiling of AM: Groups 9 and 10.

Chemo-preventive potentials of ML: Group 11 and

Chemo-preventive potentials of AM: Group 12.

Table 1. Doses of Drug/Extract administered and Period of Administration.

Groups of rats	Doses of drug/extract administered
1	Physiological saline (5 weeks)
2	10mg/Kg bodyweight Sodium arsenite (SA) (5 weeks)
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)
7	7.5mg/Kg bodyweight *Morinda lucida* (5 weeks)
8	15mg/Kg bodyweight *Morinda lucida* (5 weeks)
9	7.5mg/Kg bodyweight *Annona muricata* (5 weeks)
10	10mg/Kg bodyweight *Annona muricata* (5 weeks)
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA (5 weeks)
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA (5 weeks)

Animal Sacrifice

At the end of experimental procedures, all rats were sacrificed by cervical dislocation.

Histo-pathological Evaluations of the Liver

The liver tissues of all rats were excised and fixed in 10% formal saline of at least five times of its volume. Tissue preparation and staining of the sections were carried out via Haematoxylin and Eosin method as previously described[18].

Enzyme Linked Immunosorbent Assay (ELISA) of Concentrations of Ki67 and Multidrug Resistance1 (MDR1) Genes in Liver Tissues of Rats

Liver parts were cut from each rat and placed in 10% formalin for histo-pathological examinations, processed for light microscopy using conventional histological procedures and obtained slides were stained with Hematoxyline and Eosin[17]. In addition, separate liver tissues were isolated and then subjected to thorough homogenization using porcelain mortar and pestle in ice-cold 0.25M sucrose, in the proportion of 1g to 4ml of 0.25M sucrose solution. The tissue homogenates were filled up to 5ml with additional sucrose and collected in a 5ml serum bottle. Homogenates were thereafter centrifuged at 3000 revolution per minute for 15 minutes using a centrifuge (Model 90-1). The supernatant was collected with Pasteur pipettes and placed in a freezer at -4°C, and thereafter assayed for concentrations of Ki67 (Sigma-Aldrich AB9260) and MDR1 (Sigma-Aldrich HPA002199-100UL) proteins in the liver tissues of all rats of Control and Experimental Groups using ELISA technique[16].

The ELISA assay technique employs the quantitative sandwich enzyme immunoassay technique. Antibodies specific for Ki67 and MDR1 proteins were pre-coated onto a microplate. Standards and samples were pipetted into the wells, and Ki67 and MDR1 proteins present were bound by the immobilized antibodies. After removing any unbound substances, biotin-conjugated antibodies specific for Ki67 and MDR1 proteins were added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of Ki67 and MDR1 proteins bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

Statistical Analyses

All data obtained were expressed as arithmetic means ± standard error of mean, and were subjected to statistical analyses using T-test to compare Group 2 with

Groups 1 and 3 – 12. Differences were tested and considered statistically significant when p≤0.05 using Graph Pad Prism software package (Graph Pad Software Inc., San Diego, CA, USA; version 7 for Windows) and Microsoft Excel 2016.

Rats of Group 2 received only the clastogen (Sodium arsenite) used for the induction of toxicity in this study, and without further treatment with plants' extracts. Hence, Control Group 1 was compared with Group 2 to establish the adverse effects of Sodium arsenite (SA) on Ki67 and MDR1 levels. Similarly, Groups 3 – 12 were compared with Group 2 to confirm the degree of ameliorative potentials of doses of ML and AM on the effects of SA on Ki67 and MDR1 levels.

RESULTS AND DISCUSSION

Gross Morphological and Behavioural Observations

Morphological observations showed normal gross morphology of liver of rats of Groups 1 - 12. In addition, no behavioural anomalies were observed in rats of Groups 1 - 12. This implied that administrations of doses of SA, ML and AM to rats did not result in adverse effects on the gross morphology of rats, the liver and behavioural functions of rats.

Changes in Bodyweights of Rats

Results showed decreases in bodyweight of rats per week, and decreases in the final bodyweight compared to the initial bodyweight of Group 2, which received only SA. This implied that SA-induced toxicity resulted in decreased bodyweights of rats.

Does AM have ameliorative potentials against SA-induced adverse effects on bodyweight of rats? Results showed increases in bodyweight of rats per week, and increases in the final bodyweight compared to the initial bodyweight of Control Group 1 and Experimental Groups 3 - 4 and 6 - 11 (Table 2). This implied that post-treatments of SA-induced toxicity with 7.5 and 15mg/kg bodyweight of ML ameliorated the adverse effects on bodyweight of rats of Groups 3 and 4.

Table 2. Changes in Bodyweight (g) of rats.

Groups of rats	Doses of drug/extract administered	Initial Bodyweight (g)	Final Bodyweight (g)	% Bodyweight change
1	Physiological saline	119.2±0.49	178.6±3.78	49.79±2.66
2	10mg/Kg bodyweight Sodium arsenite (SA)	192.6±8.02	170.0±12.05	13.61±6.29
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	170.8±7.67	199.0±7.12	17.11±5.23
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	132.4±8.79	183.8±6.98	41.68±12.20
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	201.0±5.26	185.8±12.35	12.34±5.72
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	138.0±4.37	171.2±3.65	24.64±5.32
7	7.5mg/Kg bodyweight *Morinda lucida*	142.0±9.06	185.8±12.35	33.31±10.85
8	15mg/Kg bodyweight *Morinda lucida*	124.4±0.40	169.0±11.78	33.90±9.66
9	7.5mg/Kg bodyweight *Annona muricata*	110.8±0.49	170.4±4.55	53.79±4.03
10	10mg/Kg bodyweight *Annona muricata*	111.2±0.49	182.0±11.70	63.75±10.74
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA (5 weeks)	109.6±0.40	150.6±8.82	37.46±8.26
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA (5 weeks)	171±5.69	165.4±16.03	11.83±4.73

Does ML have preventive potentials against SA-induced adverse effects on bodyweight of rats? Our findings implied that co-administration of 10mg/kg bodyweight of SA with 15mg/kg bodyweight of ML prevented the adverse effects of SA-induced toxicity on bodyweight of rats of Group 11.

Does AM have preventive and/or ameliorative potentials against SA-induced adverse effects on bodyweight of rats? Results showed decreases in the final bodyweight compared to the initial bodyweight of rats of Groups 5 and 12, which received 10mg/kg bodyweight of SA and were treated or co-administered with doses of AM (Table 2). This implied that post-treatments of SA-induced toxicity with 7.5mg/kg bodyweight of AM did not ameliorate the adverse effects on bodyweight of rats of Group 5. In addition, the co-administration of 10mg/kg bodyweight of SA with 10mg/kg bodyweight of AM did not prevent the adverse effects of SA-induced toxicity on bodyweight of rats of Group 12.

Histo-pathological Evaluations of the Liver

Histo-pathological evaluations showed normal histo-architectures of the liver (Figures 1 - 12) in all rats of Groups 1 - 12. There were normal cellular density and staining characteristics of hepatocytes, hepatic sinusoids and central veins. The nuclei of hepatocytes were well characterized with no apparent large vacuolations around them. This implied that administrations of doses of SA,

ML and AM to rats did not result in evident histopathology of the liver after 5 weeks of exposure. This is due to the fact that the cyto-toxicity of adverse chemical agents is exposure-dependent and drug-induced toxicity is usually first elicited on molecular markers, while further exposure will result in evident histo-pathology at tissue level.
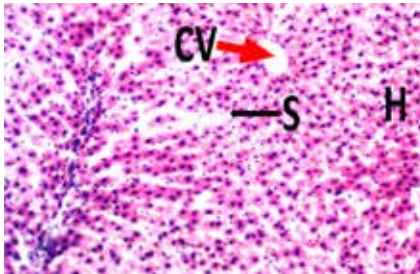

Figure 1. Photomicrograph of liver of rat of Control Group 1, which received Normal Saline. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.
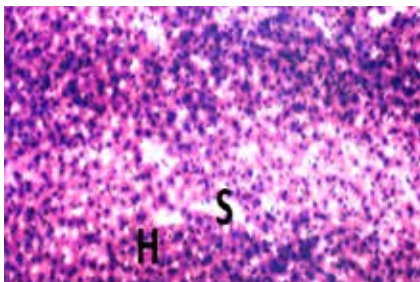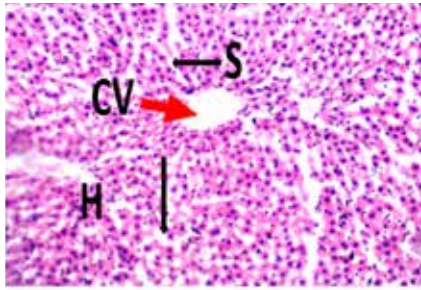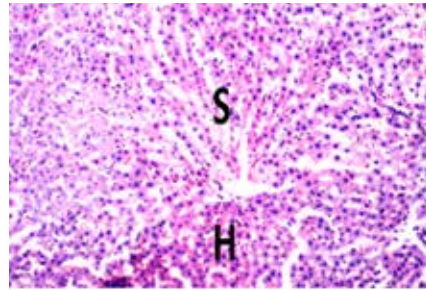

Figure 2. Photomicrograph of liver of rat of Experimental Group 2, which received 10mg/kg bodyweight of Sodium arsenite only. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.
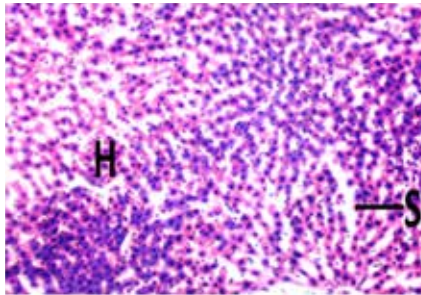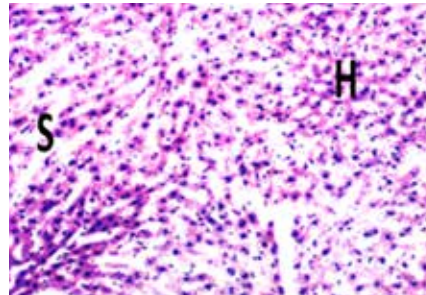
Figures 3 & 4. Photomicrograph of liver of rat of Experimental Groups 3 and 4, which received 10mg/Kg bodyweight Sodium arsenite (2 weeks) + 7.5 and 15mg/Kg bodyweight *Morinda lucida* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

Figures 5 & 6. Photomicrograph of liver of rat of Experimental Groups 5 and 6, which received 10mg/Kg bodyweight Sodium arsenite (2 weeks) + 7.5 and 10mg/Kg bodyweight *Annona muricata* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.
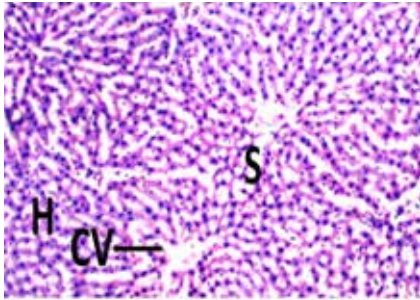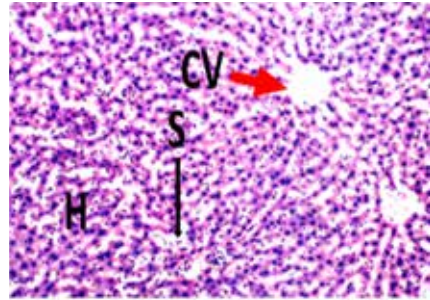
Figures 7 & 8. Photomicrograph of liver of rat of Experimental Groups 7 and 8, which received only 7.5 and 15mg/Kg bodyweight *Morinda lucida* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

Figures 9 & 10. Photomicrograph of liver of rat of Experimental Groups 9 and 10, which received only 7.5 and 10mg/Kg bodyweight *Annona muricata* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.
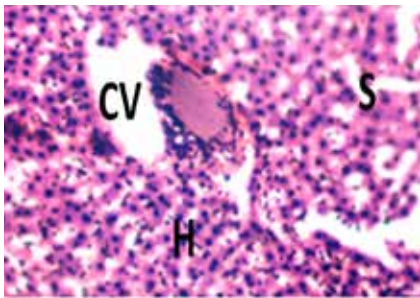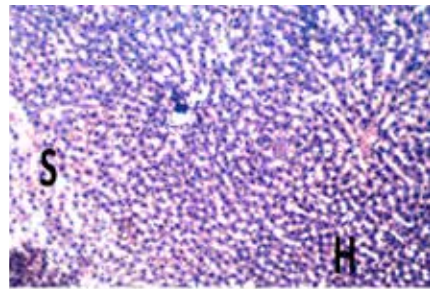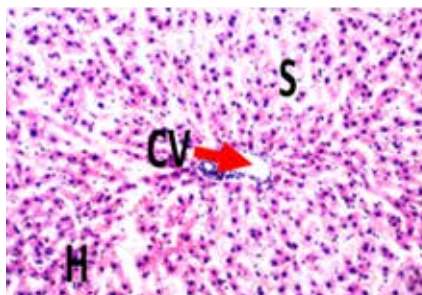
Figure 11. Photomicrograph of liver of rat of Experimental Group 11, which received co-administration of 10mg/Kg bodyweight SA + 15mg/Kg bodyweight *Morinda lucida* (5 weeks). Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

Figure 12. Photomicrograph of liver of rat of Experimental Group 12, which received co-administration of 10mg/Kg bodyweight SA + 10mg/Kg bodyweight *Annona muricata* (5 weeks). Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.
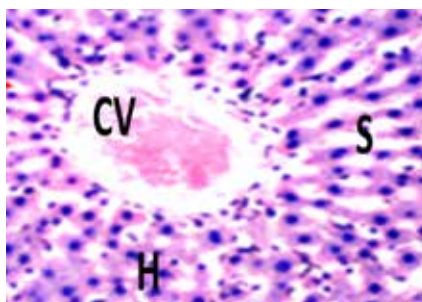
ELISA Concentrations of Ki67 in Liver Tissues of Rats

Ki-67 protein is an established biomarker of cellular proliferation[10-12]. Hence, increased Ki67 concentrations is associated with hyperplasia and aggressiveness of malignancies[10-12].

Does SA have any adverse effects on Ki67 levels? Results showed statistically non-significant higher ($p \geq 0.05$) levels of Ki67 in rats of Group 2 when compared with Group 1 (Table 3). This result implied SA-induction of abnormal proliferation (hyperplasia) and upregulation of Ki67 in rats of Group 2. This observation agrees with the views of[12], which opined that all proliferating cells tested expressed Ki67, and that there is no evidence to the contrary that proliferating cells do not express Ki67.

Can ML and AM ameliorate SA-induced upregulations of Ki67 and hyperplasia? Post-treatments of SA-induced hepato-toxicity with 7.5 and 15mg/kg bodyweight of ML, 7.5 and 10mg/kg bodyweight of AM resulted in statistically non-significant higher (p≥0.05) Ki67 levels in rats of Groups 3 - 6 when compared with Group 2 (Table 3). This implied that the tested doses of ML and AM did not ameliorate SA-induced abnormal proliferations in rats of Groups 3 - 6 respectively.

Table 3. Ki67 concentrations (Mean±SEM) (ng/ml) in Liver tissues of rats.

Groups of rats	Doses of drug/extract administered	Ki67 (Mean±SEM) (ng/ml)	P≤0.05: Group 2 versus Groups 3 - 12
1	Physiological saline	4.37±1.21	0.11
2	10mg/Kg bodyweight Sodium arsenite (SA)	9.99±1.70	
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	11.31±3.81	0.78
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	15.47±4.79	0.39
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	12.46±9.66	0.83
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	14.67±4.34	0.42
7	7.5mg/Kg bodyweight *Morinda lucida*	9.10±3.09	0.65
8	15mg/Kg bodyweight *Morinda lucida*	2.84±0.29	0.05[*]
9	7.5mg/Kg bodyweight *Annona muricata*	3.85±0.89	0.09
10	10mg/Kg bodyweight *Annona muricata*	2.28±0.19	0.05*
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA	6.954±0.39	0.22
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA	2.92±0.84	0.07

*Means with superscript are significantly different (P<0.05).

Do ML and AM have cyto-protective potentials against SA-induced upregula-

tions of Ki67 and abnormal proliferations in rats? Results showed statistically non-significant lower (p≥0.05) Ki67 levels in rats of Groups 11 and 12 when compared with Group 2 (Table 3). Our findings implied that co-administrations of 10mg/kg bodyweight of SA with 15mg/kg bodyweight of ML and 10mg/kg bodyweight of AM resulted in downregulation of Ki67 levels and, therefore, offered cyto-protective potentials against SA-induced abnormal proliferations in rats. These observations are in agreement with previous studies which noted anti-proliferation and tumour inhibitory potentials of ML leaves[19] and AM leaves[20] against HL-60 leukemia cells and breast cancer MCF-7 cells respectively.

Are there any adverse effects on Ki67 levels following exposures of rats to only the evaluated doses of ML and AM? Results showed statistically significant (p≤0.05) decreased Ki67 levels in rats of Groups 8 and 10, which received only 15mg/kg bodyweight of ML and 10mg/kg bodyweight of AM respectively when compared with Group 2 (Table 3). In addition, it must be noted that the Ki67 concentrations in Groups 8 and 10 were non-significant statistically lower (p≥0.05) than that of Control Group 1, which received physiological saline (Table 3). These results implied that the administrations of doses of ML and AM only, resulted in the downregulation of Ki67 levels. Therefore, ML and AM possess anti-proliferation potentials.

Furthermore, Ki67 is a biomarker of Cancer Stem Cells (CSCs), hence our findings indicate that ML and AM possibly possess anti-cancer compounds that can specifically target and eliminate CSCs.

ELISA Concentrations of MDR1 in Liver Tissues of Rats

MDR1 or P-glycoprotein is a cell membrane protein, which by its pharmacological function as an active drug efflux transporter protein enhances drug resistance capacity of CSCs[9,13-15]. Hence, significant upregulation of MDR1 is characteristic of drug resistant tumours and has been associated with cancer cells survival[9,13-15].

Does SA have any adverse effects on MDR1 levels? Results showed statistically significant higher (p≤0.05) levels of MDR1 in rats of Group 2 when compared with Group 1 (Table 4). This result implied SA-induction of drug resistance and significant upregulation of MDR1 and in rats of Group 2.

Table 4. MDR1 concentrations (Mean±SEM) (ng/ml) in Liver tissues of rats.

Groups of rats	Doses of drug/extract administered	MDR1 (Mean±SEM) (ng/ml)	P≤0.05: Group 2 versus Groups 3 - 12
1	Physiological saline	17.44±1.12	0.04*
2	10mg/Kg bodyweight Sodium arsenite (SA)	22.59±0.07	
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	17.92±2.45	0.20
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	16.54±2.93	0.12
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	35.30±5.27	0.13
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	36.89±4.93	0.11
7	7.5mg/Kg bodyweight *Morinda lucida*	18.77±3.32	0.26
8	15mg/Kg bodyweight *Morinda lucida*	18.59±3.23	0.23
9	7.5mg/Kg bodyweight *Annona muricata*	21.54±1.30	0.52
10	10mg/Kg bodyweight *Annona muricata*	15.37±0.58	0.02*
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA	15.33±0.75	0.01*
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA	25.69±1.88	0.27

*Means with superscript are significantly different (P<0.05).

Can ML and AM ameliorate SA-induced upregulations of MDR1 and induction of drug resistance? Post-treatments of SA-induced hepato-toxicity with 7.5 and 15mg/kg bodyweight of ML resulted in statistically non-significant lower (p≥0.05) levels of MDR1 in rats of Groups 3 and 4 when compared with Group 2 (Table 4). These results implied that ML ameliorated SA-induced upregulation of MDR1 and drug resistance in rats of Groups 3 and 4.

In contrast, post-treatments of SA-induced hepato-toxicity with 7.5 and 10mg/kg bodyweight of AM resulted in statistically non-significant higher (p≥0.05) levels of MDR1 in rats of Groups 5 and 6 when compared with Group 2 (Table

4). These results implied that AM did not ameliorate SA-induced upregulation of MDR1 and drug resistance in rats of Groups 5 and 6.

Do ML and AM have cyto-protective potentials against SA-induced drug resistance in rats? Results showed statistically significant ($p \leq 0.05$) lower levels of MDR1 in rats of Group 11 when compared with Group 2 (Table 4). Our findings implied that 15mg/kg bodyweight of ML offered cyto-protective potentials against SA-induced upregulation of MDR1 and drug resistance when co-administered with 10mg/kg bodyweight of SA.

In contrast, results showed statistically non-significant higher ($p \geq 0.05$) levels of MDR1 in rats of Group 12 when compared with Group 2 (Table 4). This result implied that 10mg/kg bodyweight of AM did not offer cyto-protective potentials against SA-induced upregulation of MDR1 and drug resistance when co-administered with 10mg/kg bodyweight of SA.

Furthermore, MDR1 is a biomarker of CSCs, hence our findings indicate that ML possibly possesses anti-cancer compounds that can specifically target and eliminate CSCs.

There is paucity of studies, which evaluated the anticancer potentials of ML and AM on MDR1 levels (drug resistance) for comparative analyses, hence the interpretations and implications of results were limited to the observations of this study.

In conclusion, our findings in this study implied that post- and preventive-treatments of SA-induced hepato-toxicity with doses of ML resulted in decreased Ki67 and MDR1 levels. Therefore, ML possibly contains chemical components that may target cancer stem cells, and it possesses hepato-protective, anti-proliferation, anti-drug resistance and anticancer potentials. Hence, the use of ML as nutritional supplements may be further evaluated. In contrast, post-treatments of SA-induced hepato-toxicity with doses of AM resulted only in decreased Ki67 levels. Hence, AM though possibly possesses hepato-protective and anti-proliferation potentials, it does not possess anti-drug resistance potentials.

STATEMENTS OF ETHICS

Ethical approval for this study was sought and received via UERC/ASN/2018/1161 from the University Ethical Review Committee (UERC) of the institution where the study was primarily conducted. This research study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as provided in the European Community guidelines

(EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

CONFLICT OF INTEREST STATEMENT

The authors wish to confirm that there are no known conflicts of interest associated with this study.

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AUTHORS CONTRIBUTION: All listed authors contributed equally to the conduct of the study based on their academic status; and the manuscript was read and approved by all listed Authors.

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