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Immunological Investigations, 2012; 41(8): 799–819

ISSN: 0882-0139 print / 1532-4311 online
DOI: 10.3109/08820139.2012.689406

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Supplementation with aqueous leaf extract of *Morinda lucida* enhances immunorestoration and upregulates the expression of cytokines and immunostimulatory markers

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Morinda lucida Benth (Rubiaceae) is a versatile plant used in traditional medicine of many countries for the treatment of a variety of ailments and the claims of efficacy are particularly remarkable in the treatment of infections and immuno-inflammatory disorders. In this study, we investigated the immunostimulatory and immunorestorative properties of the aqueous leaf extract of *Morinda lucida* (AML) in cultures of murine splenic lymphocytes and in cyclophosphamide-induced immunosupression models, respectively. Administration of AML (100 and 250 mg/kg; *per os*) in alternate days significantly ($P < 0.05$) increased specific total IgG, IgG1, and IgG2a responses to ovalbumin by as much as 2-10 fold when compared to untreated controls. In cyclophophamide treated mice, the rate of wound healing, leukopoiesis , and body weight recovery were all enhanced by oral supplementation with AML (100 and 250 mg/kg) in a dose-dependent manner. *In vitro* cultures of BALB/C splenocytes treated with AML (12.5 and 50 μg/ml) for 24 h resulted in 5-10 fold increase in IFNγ and IL-4 measured by cytokine capture ELISA. Surface expression of immunostimulatory markers, CD69 and CD25, measured

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flow cytometrically by FACS analyis, were also significantly ($P < 0.05$) upregulated on splenic T and B cells by as much as 8-20 fold. Taken together, the results of these studies show the potent immunostimulatory and immunorestorative properties of the aqueous leaf extract of *Morinda lucida,* which may explain some of the beneficial effects of the plant in the treatment of infections and immuno-inflammatory disorders.

Keywords Immunomodulation, Immunorestoration, immunosuppression, *Morinda lucida*, Noni, ovalbumin.

INTRODUCTION

The immune system is a remarkably sophisticated defence system found in vertebrates which protect them from invading agents. It is made of varieties of cells and molecules capable of recognizing and eliminating limitless varieties of foreign and undesirable agents. Modulation of the immune system connotes any change in the immune response that may involve induction, expression, amplification, or inhibition of any part or phase of the immune response. Advances and the growing body of knowledge on immune cells and how they act in a complex and intricately interwoven manner to ensure the protection of the body against infections and tumour have kindled interest in the development of immunomodulatory therapies.

Unlike conventional anti-infective agents that are designed to act against pathogens, immunomodulatory therapies exert their protective effects by acting on the host immune cells. The potential uses of immunodulators in clinical medicine include the reconstitution of immune deficiency (e.g., the treatment of AIDS) and the suppression of normal or excessive immune function (e.g., the treatment of graft rejection or autoimmune disease, and inflammatory conditions). Herbal and complementary therapies have shown remarkable potentials in modulating the cells involved in the immune responses (Alamgir and Uddin, 2010).

Morinda lucida Benth (Rubiaceae) is a versatile plant used in traditional medicine of many countries for the treatment of a variety of ailments and the claims of efficacy are particularly remarkable in the treatment of infections and immuno-inflammatory disorders. One major concern, that has limited acceptability of herbal medicine, is the 'cure-all' claims often made by herbalist on many of these medicinal plants. This is a situation where one herb is claimed, either rightly or wrongly, to be beneficial in so many conditions. A plausible explanation that has been advanced for the versatile effects on many disease conditions and systems is the modulatory effect these herbal therapies could have on the immune system (Spelman et al., 2006; Nworu et al, 2010a, b, c).

For instance, the stimulation of the body immune system can, in general, strengthen other systems either directly or through mediators such as cytokines. The overall effect is to strengthen the body's resistance against diseases, in the restoration of altered body tissues and functions, and in the fight against

infections or cancers which invariably lead to the quickening of healing and recovery processes (Waldmann, 2003; Tan and Vanitha, 2004).

Many plant metabolites have been shown to activate or supress the functions of the immune cells such as the cytotoxic effector cells, lymphocytes, macrophages, cytotoxic T-lymphocytes, dendritic cells, natural killer (NK), and neutrophils (Dixon, 2001). Several medicinal herbs have been shown to promote immunity in different ways; they have shown to augment specific cellular and humoral immune response (Duke, 1985; Tan and Vanitha, 2004)

The plant, *Morinda lucida,* is a medium-sized tropical West Africa rainforest tree also called Brimstone tree and is widely used as a medicinal plant in West Africa, especially in Nigeria. The leaves are used as an ingredient of "fever teas," which are usually taken for the traditional treatment of malaria. Different parts of the plant are attributed with diverse therapeutic benefits. For example, in Southern Cameroon, cold decoction of the plant leaves is used for the treatment of fever (Dalziel, 1937). However, in most parts of West Africa, the bitter water decoction of the plant bark, root and leaf are used as bitter tonic and as astringent for dysentery, abdominal colic and intestinal worm infestation (Dalziel, 1937).

The Europeans sometimes use the decoction of the plant root or stem to make "bitters" (Dalziel, 1937). *M. lucida* has been reported to be effective against malaria (Makinde and Obih, 1985; Obih et al., 1985; Tona et al., 1999), sleeping sickness (Asuzu and Chineme, 1990), diabetes (Adeneye and Agbaje, 2008), infections (Ogundare and Onifade, 2009), inflammations (Awe et al., 1998), among many other therapeutic properties.

Although extracts and polysaccharide-rich precipitate of the fruit pulp of a related plant, *Morinda citrifolia* (popularly consumed as 'Noni'), is reputed, and widely used as very potent immunostimulatory agent, there are no data on the immunomodulatory potentials of *Morinda lucida*.

Noni is a very popular immune-boosting remedy reported to be beneficial in immunosuppression, tumour, and in other immuno-inflammatory disorders (Hirazumi et al., 1996; Hirazumi and Furusawa, 1999; Furusawa et al., 2003; Zhang et al., 2009, Brooks et al., 2009). This motivated the present study in which the immunostimulatory and immunorestorative effects of the aqueous leaf extract of *M. lucida* were investigated in cultures of murine lymphocytes and in immunocompetent and immunocompromised rodent models.

MATERIALS AND METHODS

Preparation of Plant Material

The fresh leaves of *Morinda lucida* were collected in Nsukka in the month of August and authenticated by a plant taxonomist, Mr Alfred O. Ozioko, of the

Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka, Nigeria. The leaves were washed, air-dried, and pulverized. A portion of the leaf powder (500 g) was extracted by maceration in warm distilled water for 3 h with intermittent agitation. The extract solution was filtered through Whatman No. 1 filter paper and then concentrated at 40°C and a yield of about 7.5 % (w/w) was estimated. The portion of aqueous *Morinda lucida* (AML) extract used for the *in vitro* cell culture assays was extracted similarly, but lyophilised and stored in aliquots in −20°C refrigerator and used subsequently for the experiments. The level of endotoxin in AML was tested by Limulus Amoebocytes Lysate (LAL) kit (Endosafe®, Charles River, Sulzfeld, Germany).

Phytochemistry and HPLC Identification and Analysis of Major Constituents

Preliminary phytochemical tests were carried out on AML using standard procedures (Harborne, 1998). The various constituents present in AML and their relative abundance was determined by comparing their peaks and peak areas with those of known standard compounds in an analytical HPLC 'finger-printing' procedure.

A solution of 1 mg AML/mL was prepared in HPLC grade methanol. The solution was centrifuged and HPLC analysis was carried out on the supernatant with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S). Detection was at 235 nm. The separation column (125 X 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent.

Animals

Adult Swiss albino rats (150–250 g) and mice (15–25 g) of both sexes were obtained from the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka (UNN). BALB/C mice (20–25 g) obtained from Janvier (Le Genest-ST-Isle, France) were used for the study. The mice were housed under standard conditions (25 ± 2°C and 12 h light/dark cycle) and were maintained on standard livestock pellets with unrestricted access to drinking water. The use and care of laboratory animals in the study were in accordance with ethical guidelines as contained in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC) of 1986.

Preparation of Mouse Spleen Cells for Experiments

Mice (BALB/C) were euthanized and the spleens removed aseptically into ice-cold Hanks' Balance Salt Solution (HBSS, Gibco). Single cells suspension

was prepared by gentle dispersion of the cells and straining through BD Falcon™ nylon cell strainer (70 μM). Red blood cells were lysed by the addition of about 3 ml of ACK® lysing buffer (Lonza, Walkersville) per mouse spleen for 5 min. The cells were washed with cold phosphate buffered saline (PBS) and suspended in R-10, consisting of RPMI 1640 medium (Gibco, Germany) supplemented with 10% heat-inactivated foetal calf serum (FBS), 50 μM 2-mercaptoethanol (Gibco, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Estimation of Cytotoxic and Lymphotoxic Effects of Aqueous Morinda lucida Extract (AML)

The cytotoxicity of the AML was evaluated on 293 T cells (human embryonic kidney cells expressing SV40 large T-antigen). The cells were cultured in D-10 medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Germany) with glucose (4.5 g/L), L-glutamine (2 mM), and supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. A modification of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay procedure, as originally described by Mosmann (1983) was employed. The cells were seeded in triplicate into 96-well culture plates at a density of 2×10^4cells/well in 150 μl volume. After 24 h incubation at 37°C under 5% CO_2, the cells were then treated with graded concentrations (5–1000 μg/ml) of the AML. The D-10 medium was used as the 'no-drug' control.

After incubation for 48 h at 37°C under 5% CO_2, a solution of MTT (5 mg/ml, 20 μl per well) was added and further incubated at 37°C in 5% CO_2 for 2 h to allow for formazan formation in viable cells. Thereafter, the medium was removed and 150 μl DMSO was added to dissolve the blue formazan crystals formed in viable cells. The plate was shaken for 30 min. on a plate shaker and the optical density (OD) determined at 550 nm using a multi-well microtiter plate reader (Tecan, Austria). Each single value of the triplicate determination was expressed as a percentage of the mean of triplicates of the 'no-drug' control cultures and the mean calculated for each measurement. The concentration of AML producing 50% cellular toxicity (TC_{50}) was calculated graphically by regression.

Similarly, the lymphotoxic effect of AML was evaluated on naïve murine splenocytes using a modification of the trypan blue exclusion and viability protocol (Freshney, 1987). Spleenocytes of BALB/C mice were prepared, seeded at a density of 1×10^6 cells/well into 96-well cell culture plates and then treated with increasing concentrations of AML (0, 50, 100, 250, 500, 750, and 1000 μg/ml) in triplicates. The treated plates were incubated at 37°C under 5% CO_2 and cultured for 24 h. Samples of the spleen cells preparation were drawn and diluted 1: 4 with trypan blue to assess the viability of the cells before and after

incubation. Dead, necrotic or apoptotic cells, which have lost viability were stained by the trypan blue while viable cells appeared bright under a light microscope. The viable cells and non-viable cells were enumerated and the viable cells expressed as a fraction of the total cells.

For each treatment well, four different samples were enumerated and the mean values recorded and expressed as a percentage of the mean viability for the 'no drug' control. A plot of mean viability (%) versus Log. concentration values was made and the TC_{50}, which is the concentration of AML producing 50% reduction in cell viability, was estimated by regression.

Acute Toxicity (LD_{50}) Test on AML

The acute toxicity (LD50) of the aqueous extract of *M lucida* (AML) was estimated in BALB/C mice by the oral route using the method described by Lorke (1983). Briefly, the tests involved two phases. The first phase involved the determination of the toxic range. The mice were placed in 3 groups (n = 3) and AML (10, 100, and 1,000 mg/kg) was administered orally. The treated mice were observed for 24 h for any deaths. The death pattern in the first phase determined the doses used for the second phase according to the Lorke (1983) estimation.

In the second phase, 4 different doses of AML were administered (*per os*) as predetermined in the earlier phase of the study. The animals were observed for lethality and signs of acute intoxication for the next 24 h. The LD_{50} was calculated as the geometric mean of the highest nonlethal dose and the least toxic dose.

Determination of the Effect of AML on the Expression of CD69 and CD25 Activation Markers on Splenic Lymphocytes

The expression of surface molecular markers, CD69 and CD25, was determined, flow cytometrically, in naïve murine spleen cells to demonstrate the ability of AML to induce activation of these cells. Spleenocytes were prepared from BALB/C mice as earlier described and seeded at a density of 1×10^5cells/well into a 96-well culture plate and treated with AML (0, 12.5, and 50 μg/ml) in triplicate wells. Concanavalin A, Con A (2 μg/ml) (Sigma, Germany) was used as standard agent in the assay. The plates were incubated at 37°C in 5% CO_2.

After culturing overnight for 18 h, the cells were washed with PBS/BSA/Azide and then preincubated with anti-CD16/CD32 (FcR-blockers) for 25 min at 4°C. Thereafter, the cells were stained with 1:300 dilutions of FITC conjugated anti-CD69 or co-stained with anti-CD69-FITC, anti-B220-APC, and anti-CD4/CD8-PerCP monoclonal antibodies (BD Bioscience) for 25 min at 4°C before FACS acquisition on FACScalibur™ flow cytometer (Becton Dickinson).

In the determination of CD25 molecules, after the blocking of non-specific binding by pre-incubation with anti-CD16/CD32, the cells were co-stained with APC conjugated anti-CD25 and PerCP conjugated anti-CD4/CD8 monoclonal antibodies for 25 min at 4°C before FACS acquisition. The FACS data was analysed with FCS Express™ V3.

Determination of the Effects of AML on the Secretion of Interferon-Gamma (IFN-γ) and Interleukine-4 (IL-4) by Splenocytes

Single cell suspension of total spleen cells of BALB/C mice were prepared in R-10 medium containing only 2% FCS and seeded at a density of 5×10^5cells/ml into a 96-well culture plate. The splenocytes culture in the wells were treated with AML (0, 12.5, and 50 μg/ml) and ConA (2 μg/ml) in triplicate wells. The plates were incubated at 37°C in 5% CO_2. After culturing for 48 h, the plates were centrifuged and the supernatants were aspirated and stored at −80°C until cytokines were measured. Commercial cytokine Elisa kits (PeproTech GmbH, Hamburg Germany) were used to assess the concentrations of IL-4 and IFN-γ in the cell-free culture supernatants.

The Effect of Oral AML Supplementation on Antibody Responses to Ovalbumin in Mice

Twenty-five mice were randomised into five groups (n = 5) after acclimatisation and used for the immunogenicity studies. Group I served as the naïve animal and received neither the extract nor immunized with any antigen. Mice in Group II served as the negative control and were immunized on days 2 and 14 with 100 μg/mouse of ovalbumin (OVA) on the hind foot pads (50 μg in 50 μl per footpad), but without the extract.

Groups III and IV are the treatment groups and were, in addition to similar OVA immunisation on days 2 and 14, given AML (100 and 250 mg/kg; p.o) in alternate days. Group V mice were, in addition to similar OVA immunisation on days 2 and 14, given Noni (100 μg/mouse) and served as the standard for comparison. All mice were bled by retro-orbital puncture on day 21 (7 days after boost immunisation) and the sera recovered for antibodies titer measurement.

Determination of Antibody Response by Enzyme Linked Immunosorbent Assay (ELISA)

The titer of antibodies raised against ovalbumin (OVA) in the sera of treated and control groups were estimated by enzyme-linked immunosorbent assay. Microtiter plates with 96 wells were coated with OVA (1 μg/ml) in carbonate buffer pH 9.6 and incubated overnight at 4°C with a proper cover. After the incubation the unbound OVA was washed off thrice with 0.01M PBS buffer containing 0.05% Tween-20 (PBS-T). The nonspecific binding sites were blocked

with 1% solution of bovine serum albumin (BSA) in PBS and incubated for 1 h at room temperature.

The wells were washed again with PBS-T and incubated with 100 μl of 1 in 20 diluted sera samples in duplicates for 1 h at 37°C. The unbound serum proteins and other constituents were washed off. To assess the antibody levels, 100 μl of HRP-conjugated goat anti-mouse total IgG, IgG1, and IgG2a secondary antibodies were added at a dilution of 1:1000 and incubated for another 1h at room temperature. Finally the unbound conjugates were washed off with PBS-T and 100 μl/well of freshly prepared ABTS substrate and stopped with 100 μl of peroxidase stop solution. The colour developed was read at 405 nm using an automatic ELISA reader (Thermomax® ELISA Plate Reader). The data represent the mean absorbance values of the sera samples assayed in duplicates. Naïve mice sera or preimmune sera were included in the assay as control.

The Effect of AML on Weight Recovery and Weight Gain in Immunocompromised Mice

Four groups of mice (n = 5) were randomised and used in this model to assess the effect of AML oral administration on weight gain and weight recovery in cyclophosphamide-induced immunosuppression. Mice in group I were left untreated and served as the negative control, while mice in groups II, III, and IV were treated in alternate days with ALM (100 mg/kg, p.o.), AML (250 mg/kg; p.o.), and Noni (100 mg/kg; p.o.), respectively. On day 3 after commencement of treatment, cyclophosphamide (30 mg/kg; i.p.) was administered to each mouse in the groups. The mice were weighed in alternate days for 12 days and expressed as a percentage of the initial body weight. The mean percentage body weight for the groups were calculated and plotted for each time point.

The Effects of AML on Leukopoiesis in Immunocompromised Rats

The baseline total leukocyte counts of the rats placed in four groups (n = 5) were determined on the first day of the experiment. Thereafter, the immune system was compromised by a single bolus intraperitoneal injection of cyclophosphamide (30 mg/kg) to each of the rats. Rats in the 1st group were not treated and served as the negative control, rats in the 2nd and 3rd groups received daily oral administration of AML (100 and 250 mg/kg; p.o.), respectively. Animals in 4th group were given Noni (100 mg/kg) and served as the standard control group. The total leukocyte counts were determined again on the 7th and on the 14th day post-treatment.

The Effects of AML on Excision Wound Healing Rate in Immunocompromised Rats

Rats were divided into five groups (n = 5) and the hairs on the central dorsal thoracic region of the rats were shaved and the region sterilized with alcoholic

solution. An excision wound of 20 mm diameter (314 mm^2 area) was surgically created on the depilated region under ether anaesthesia. After achieving complete haemostasis by blotting the wound with cotton swab soaked in warm saline, animals were placed in their individual cages. The animals were treated as follows.

Group I received no treatment; group II was given cyclophosphamide (30 mg/kg, i.p.) alone on day 3; group III received cyclophosphamide (30 mg/kg; i.p.) on day 3 and AML (100 mg/kg; p.o.) on alternate days; group IV received cyclophosphamide (30 mg/kg; i.p.) on day 3 and AML (250 mg/kg; p.o.) on alternate days; group V received cyclophosphamide (30 mg/kg; i.p.) on day 3 and Noni (100 mg/kg; p.o.) on alternate days. The wound area was measured in alternate days by tracing with a translucent paper and estimating on 1-mm^2 graph sheet. Wound contraction was calculated as a percentage of the original wound size for each animal.

Determination of the Effect of Oral Supplementation of AML on Relative Spleen Weight of Mice

Mice were grouped and treated with either AML (100 and 250 mg/kg; p.o.) or Noni (100 mg/kg; p.o.) daily for 10 days and at the end the body weight of each mouse was determined. The animals were sacrificed and the weight of spleen was determined and expressed as relative organ weight in mg/100 g body weight of the animal (Davis and Kuttan, 2000).

RESULTS

Phytochemical Studies

Preliminary phytochemical studies on AML showed positive reaction for the presence of glycosides, alkaloids, saponins, flavonoids, terpenoids, carbohydrates, and resins. The HPLC analysis identified fourteen major peaks (compounds) in the extract and three major classes of compounds were found to be abundant- alkaloids (51.35%); flavonoids-Quercetin glycosides (30.7%) and phenolic acids -caffeic acid derivatives (13.96%) (Table 1).

Acute Toxicity Tests (LD_{50})

In the acute toxicity tests in mice, AML administered orally at doses up to 5000 mg/kg body weight did not cause lethality or any sign of acute intoxication in the mice after a 24-h observation period. The LD_{50} is therefore greater than 5 g/kg.

Table 1: Major phytochemical constituents of the aqueous extract of *Morinda lucida* extract (AML) determined by HPLC analysis.

Fraction number	Retention time in (minutes)	Relative area (%)	Type of compound
1	2.1	2.73	Phytallic acid
2	10.41	4.79	Alkaloid
3	12.14	35.6	Alkaloid
4	14.97	2.32	Alkaloid
5	15.50	6.1	Alkaloid
6	16.02	2.3	Quercetin glycoside
7	16.32	2.56	Alkaloid
8	17.78	4.38	Caffeic acid derivative
9	18.45	3.32	Quercetin glycoside
10	19.04	7.3	Quercetin glycoside
11	20.12	1.22	Kaempferol glycoside
12	20.38	17.78	Quercetin glycoside
13	22.94	4.18	Caffeic acid derivative
14	23.93	5.4	Caffeic acid derivative

Cytotoxicity and Lymphotoxicity

In the *in vitro* toxicity studies, the viability of cultured 293T cells and total spleen cells were not significantly affected at the maximum concentration of 1000 mg/ml tested in the assays.

The effect of AML on the expression of CD 69 and CD 25 activation markers on splenic lymphocytes

The expression of early activation molecule, CD69, on the surface of total spleen cells as well as on splenic T and B cells were significantly ($P < 0.05$) up-regulated after an overnight (about 18 h) stimulation of murine splenocytes culture with the aqueous *Morinda lucida extract* (AML). The expression measured flow cytometrically by FACS analysis showed that stimulation of spleen cells with AML (12.5 and 50 μg/ml) increased the mean fluorescence intensities of CD69-FITC on total spleen cells (Figure 1A), T cells (Figure 1B), and B cells (Figure 1B) by as much as 8–20-fold and in a concentration-related manner. Similarly, the expression of another transmembrane activation marker, CD25 molecules, an α-chain of IL-2 receptor, were also significantly ($P < 0.05$) up-regulated on spleenic T by as much as 5-10 fold compared to the untreated control wells (Figure 2).

The effects of AML on the secretion of interferon-gamma (IFN-γ) and interleukine-4 (IL-4) in the culture supernatant of mice spleen cells

Treatment of naïve BALB/C mice splenocytes culture with *AML* (12.5 and 50 μg/ml) induced an increased secretion of *IFN-γ* and IL-4 in cell-free culture supernatant after 48 h of incubation (Figure 3). The concentration of *IFN-γ* and

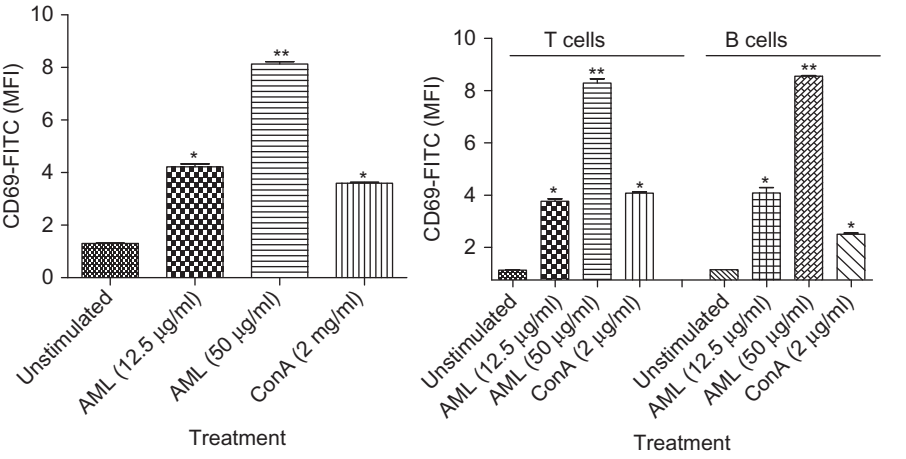

Figure 1: The effect of AML on the expression of CD69 molecules on total spleen cells, T cells, and B cells. BALB/C mice splenocytes were prepared and seeded at a density of 1×10^5cells/well into a 96-well culture plate and treated with AML (12.5 and 50 µg/ml) or conA (2 µg/ml) in triplicates. Negative control consisted of un-stimulated wells containing R-10 medium alone. The plates were incubated at 37°C in 5% CO_2 for 18 h. The cells were pre-incubated with anti-CD16/CD32 and thereafter stained with FITC conjugated anti-CD69 or co-stained with anti-CD69-FITC, anti-B220-APC, and anti-CD4/CD8-PerCP monoclonal antibodies before FACS acquisition. The figures show mean±SEM of mean fluorescence intensity (MFI) of CD69 expression on total spleen cells (Fig. 1a), or gated on B220+ or CD4/CD8+ lymphocytes (Fig. 1b). *P < 0.05 and **P < 0.01 vs. un-stimulated control.

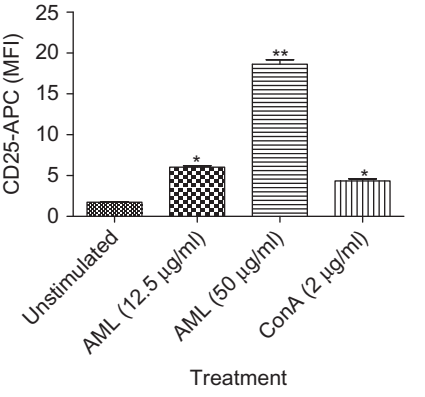

Figure 2: The effect of AML on the expression of CD25 molecules on T cells. BALB/C mice splenocytes were prepared and seeded at a density of 1×10^5cells/well into a 96-well culture plate and treated with AML (12.5 and 50 µg/ml) or conA (2 µg/ml) in triplicates. Negative control consisted of un-stimulated wells containing R-10 medium alone. The plates were incubated at 37°C in 5% CO_2 for 18 h. The cells were pre-incubated with anti-CD16/CD32 and thereafter co-stained with APC conjugated anti-CD25 and PerCP conjugated anti-CD4/CD8 monoclonal antibodies before FACS acquisition. The figure show mean ± SEM of mean fluorescence intensity (MFI) of CD25 expression gated on CD4/CD8+ lymphocytes. *P < 0.05 and **P < 0.01 vs. un-stimulated control.

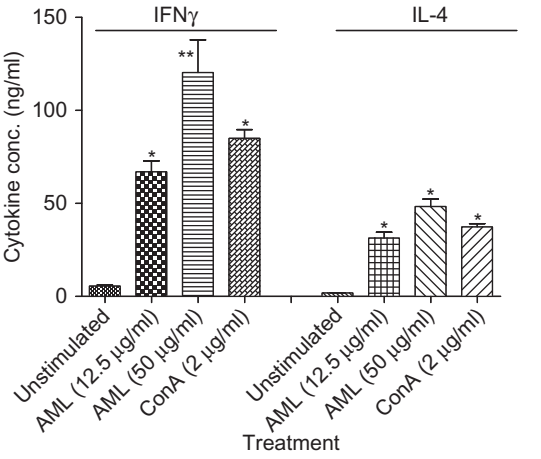

Figure 3: The effect of AML on the expression of IL-4 and IFN-γ bynaïve murine spleenocytes. Single cell suspension of total spleen cells of BALB/C mice were prepared and seeded at a density of 5×10^5cells/well into a 96-well plate. Cells were treated with AML (12.5 and 50 µg/ml) and ConA (2 µg/ml) in triplicate wells. The plates were incubated at 37°C in 5% CO_2 for 48 h. Concentrations of IFN-γ and IL-4 were measured in cell-free supernatant using cytokine Elisa kits. $^*P < 0.05$ and $^{**}P < 0.01$ vs. un-stimulated control.

IL-4 measured by ELISA showed that stimulation with AML caused an increase in the levels of both cytokines in a concentration-dependent manner. The levels of the cytokines in the AML-treated wells were 5-10 times higher than their respective levels in the unstimulated control wells.

The effects of oral supplementation of AML on antibody responses in mice

Daily oral administration of AML (100 and 250 mg/kg body weight) followed by a homologous prime-boost immunisation with ovalbumin (OVA) caused a significantly (P<0.05) higher titers of OVA-specific total IgG (Figure 4A), IgG1 (Figure 4B), and IgG2a (Figure 4C) in the sera of immunized mice compared to the negative control animals. The antibody levels were between 2–10-fold higher in immunized animals that received daily oral supplementation of AML.

The increases in antibody responses were similar to the increase produced by the group that received daily oral administration of the standard drug, 'Noni', a commercial extract of *Morida citrifolia*, reported to possess potent immunostimulant properties, including boosting antibody responses (Hirazumi et al., 1996; Hirazumi and Furusawa, 1999; Zhang et al., 2009).

The effects of AML on weight gain and recovery in immunocompromised mice

Groups of mice treated daily with AML (100 and 250 mg/kg body weight; *per os*) as well the group that received 'Noni' as a standard treatment showed

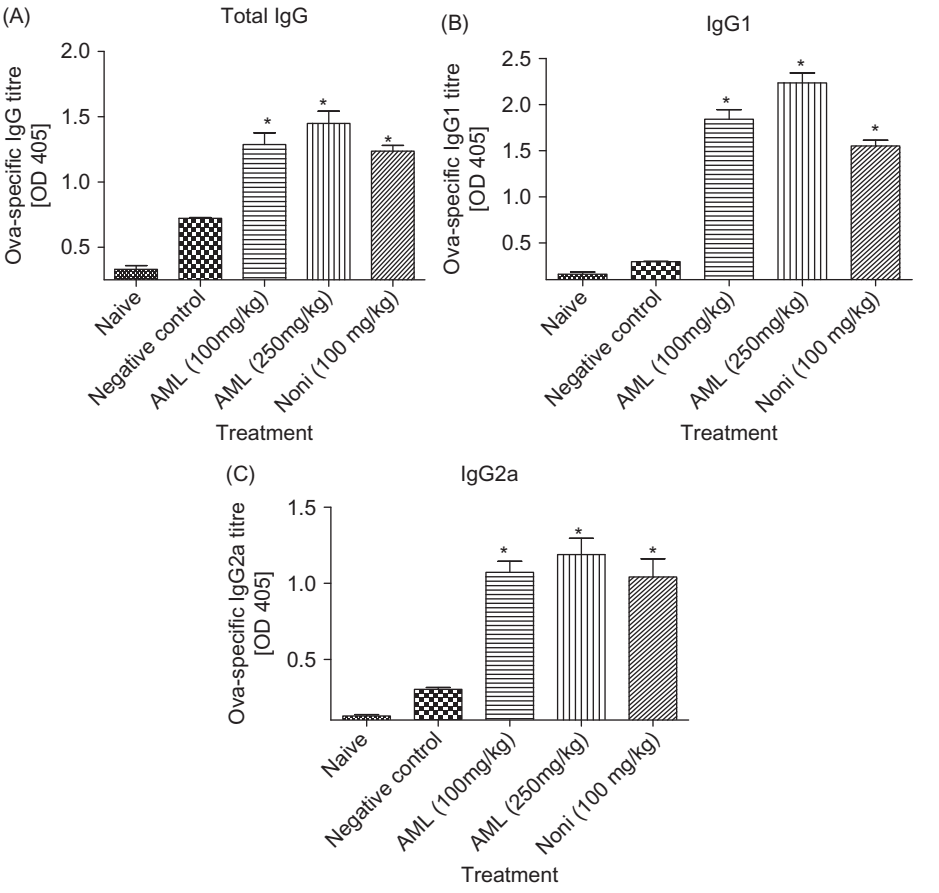

Figure 4: The effect of AML on ovalbumin specific total IgG, IgG1, and IgG2a. Groups of mice (n=5) were treated orally in alternate days with or without AML (100 and 250 mg/kg) or Noni (100 mg/kg) and immunized twice (prime-boost) with ovalbumin 100 μg/mouse. Mice were bled after 7 days after boost immunisation and the titers of ovalbumin-specific total IgG (Fig 4a), IgG1 (Fig 4b), and IgG2a (Fig 4c) were determined in the sera. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. negative control.

significantly ($P<0.05$) higher rate of weight gain and recovery after treatment with cyclophosphamide (30 mg/kg; i.p) (Figure 5). The weight of all the mice receiving cyclophosphamide decreased remarkably between 3–6 days after cyclophosphamide-treatment and their weights recovered gradually thereafter.

The effects of AML on leucopoeisis in immunocompromised rats

Oral administration of AML (100 and 250 mg/kg) ameliorated the cyclophosphamide-induced leukopenia in rats. On the 7th day after cyclophosphamide, the total leukocyte count reduction was 53.50% and 56.87%, respectively, for 100 and 250 mg/kg of AML treatment. This is significantly lower than

the 71.62% reduction recorded for the group that received cyclophosphamide alone (Figure 6). On the 14th day after cyclophosphamide administration, the total leukocytes count was reduced by 71.55% in the cylophosphamide treatment group, whereas a reduction of 43.12% and 27.63% was recorded, respectively, for 100 and 250 mg/kg of AML treatment (Figure 6).

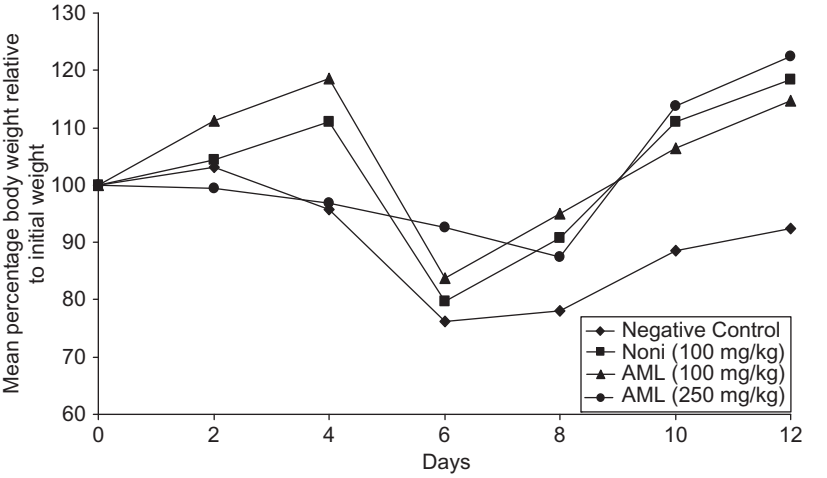

Figure 5: The effect of AML on weight recovery and weight gain in immunocompromised mice. Groups of mice (n = 5) were treated in alternate days with or without AML (100 and 250 mg/kg) or Noni (100 mg/kg) and on the 3rd day they received a bolus injection of cyclophosphamide (30 mg/kg; i.p.). The weight of the mice were monitored every 2 days and expressed as a percentage of the initial body weight. Mean body weight for each of the group is plotted against days.

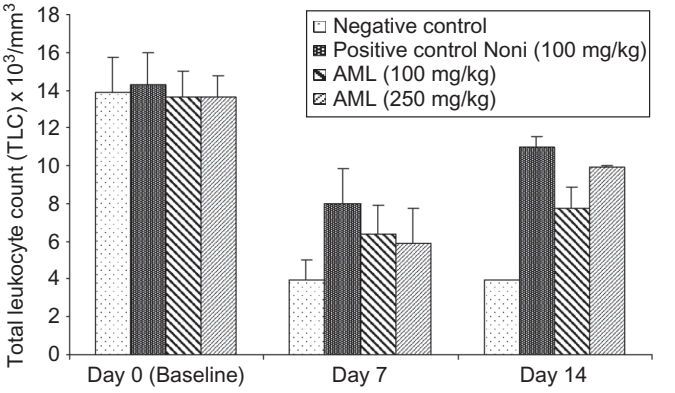

Figure 6: The effect of aqueous leaf extract of *Morinda lucida* on leucopoeisis in immunocompromised mice. Groups of mice (n = 5) were treated in daily with or without AML (100 and 250 mg/kg) or Noni (100 mg/kg) and they received a bolus injection of cyclophosphamide (30 mg/kg; i.p.) thereafter. The animals were bled and the baseline total leucocytes count (TLC) was determined for each mouse on day 0 and then on days 7 and 14 following cyclophosphamide treatment. Mean total leucocytes counts are plotted against post-treatment days.

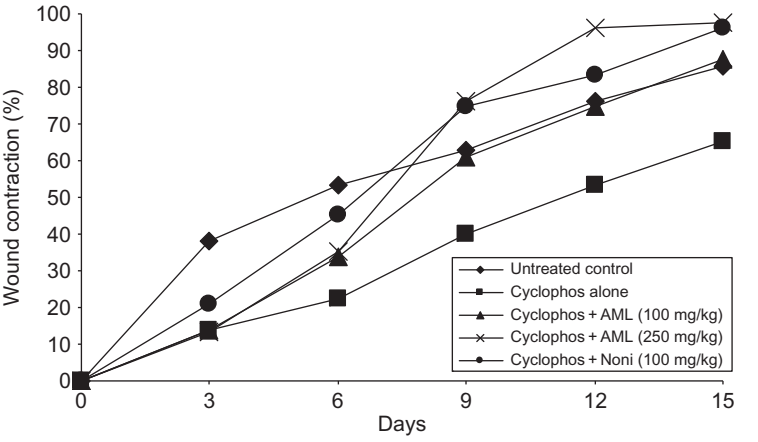

Figure 7: The effects of AML on excision wound healing rate in immuno-compromised rats. An excision wound of 20-mm diameter was surgically created on groups (n = 5) of rats and treated with or without AML (100 and 250 mg/kg) or Noni (100 mg/kg) and on the 3rd day received a bolus injection of cyclophosphamide (30 mg/kg; i.p.). The wound size was measured for each mouse in alternate days and the mean wound contraction rate was determined for each group as a percentage of the original wound size and plotted against days.

The effects of AML on the rate of excision wound healing in immunocompromised rats

The rate of wound closure in rats was impaired in the group administered with cyclophosphamide alone when compared to the wounded, but untreated control group (Figure 7). The administration of AML (100 and 250 mg/kg, *per os*) with cyclophosphamide increased the rate of excision wound healing when compared to groups treated with cyclophosphamide alone.

The effects of oral supplementation of AML on mean relative weight of spleen

The mean relative weights of mice spleens were determined after 10 days of treatment. The groups treated with alternate days of AML (100 and 250 mg/kg body weight; *per os*) showed a remarkable increase in the mean relative weights of the spleen from 1150±250 mg/100 g recorded for the negative control to 1200±200 and 1650±300 mg/100 g body weight, respectively (Figure 8).

DISCUSSION

This study investigated the immunostimulatory and immunorestorative effects of the aqueous leaf extract of *Morinda lucida* in cultures of murine lymphocytes and in immunocompetent and experimentally induced immunocompromised rodent models. *Morinda lucida* Benth belongs to the Rubiaceae family of

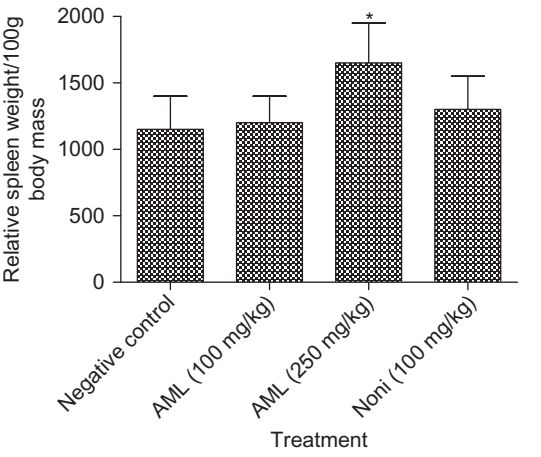

Figure 8: The effect of AML on relative spleen weight of mice. Mice were grouped (n = 5) and treated with either AML (100 and 250 mg/kg; p.o.) or Noni (100 mg/kg; p.o.) daily for 10 days and at the end the body weight and the weight of spleen was determined and expressed as relative organ weight of spleen (mg/100 g body weight). *$P < 0.05$ vs. negative control.

medicinal plants and has versatile uses in traditional medicine of many countries for the treatment of a variety of ailments.

Acute toxicity studies in mice, as well as *in vitro* cytotoxicity studies on 293T cells and total spleen cells show that the aqueous extract of *Morinda lucida* (AML) is reasonably safe for all practical purposes. At doses up to 5,000 mg/kg body weight, AML did not cause lethality nor induce signs of acute intoxications when administered orally to mice (Lorke, 1983). Similarly, in the *in vitro* toxicity studies, the viability of cultured 293T cells and spleenocytes were not significantly affected at the maximum concentration of 1000 mg/ml tested in the assay. However, the amount or quantity consumed or taken by the local population may not be certainly determined because of lack of standardization which is one of the major drawbacks of the practice. One of the major concerns in research in medicinal plants is to provide standardization, and create room for reproducibility by using definite dose/concentration of the extracts/fractions. This aim was achieved in this study by using doses that are safe and with minimal adverse effects.

The ability of AML to activate immune cells was ascertained by measuring the surface expression of molecular activation markers, CD69 and CD25, on splenocytes and splenic lymphocytes. The CD69 molecule is one of the earliest inducible cell surface glycoproteins acquired by immune cells during lymphoid activation and is involved in lymphocyte proliferation and functions as a signal-transmitting receptor (Cambiaggi et al., 1992), while the CD25 molecule is the alpha chain of the IL-2 receptor and a type I transmembrane protein present on activated T cells and B cells. The measurement of the expression of these molecules on treatment with substances is, therefore, a reliable method of

assessing the ability of such treatments to produce activation of these important immune cells. In the study, treatment of spleen cells with AML induced significant and concentration-related increases in the expression of both CD69 and CD25 molecules on total spleenoctyes, and on B and T lymphocytes.

The concentration of interferon-gamma (IFNγ), a Th1 cytokine, and interleukine-4 (IL-4), a Th2 cytokine, were increased many fold in cell-free supernatant of spleen cells treated with AML. IFN-γ is critical for both the innate and adaptive immunity against tumour, viral and intracellular bacterial infections. The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly and most importantly from its immunostimulatory and immunomodulatory effects.

IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007). IL-4 is a key regulator in humoral and adaptive immunity and has many biological roles, including the stimulation of activated B-cell and T-cell proliferation and the differentiation of CD4+ T-cells into Th2 cells (Apte et al., 2008).

In the *in vivo* studies, oral supplementation with the aqueous extract of *Morinda lucida* in alternate days produced a significant and dose related increases in specific total IG, IgG1, and IgG2a antibodies against ovalbumin in mice. Antibodies (or immunoglobulin) are produced by plasma cells and are used by the immune system to identify and neutralize foreign objects such as bacteria and viruses by recognising unique part of the foreign target (the antigenic epitope) for specific binding (Janeway, 2001).

Using this binding mechanism, an antibody can tag a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly (for example, by blocking a part of the microbe that is essential for its invasion and survival). The production of antibodies is the main function of the humoral immune system. The IgG antibody isotype provides the majority of antibody-based immunity against invading pathogens (Pier et al., 2004). It is therefore possible that some of the therapeutic benefits attributed to *M. lucida* leaf extracts could be due to its effect in strengthening the antibody-producing humoral immune system. The specific mechanism and cell type(s) that are activated to produce this effect are not yet elucidated.

Restoration of immune status in immunocompromised condition is one of the clinical applications of immunostimulatory therapies. In this study, immunosuppressed status was induced in rodents by administration of cyclophosphamide (30 mg/kg; i.p). Cyclophosphamide itself is not toxic to lymphocytes or tumor cells *in vitro;* it exerts its alkylating effects only after metabolic conversion to 4-hydroxycyclophosphamide (Sladek, 1972). In animals, a single high dose leads to thymus involution, loss of B cells in lymph nodes and spleen, inhibition of NK activity, and decrease in circulating monocytes.

Peritoneal macrophages show impaired degradation of antigen-antibody complexes. Later, lymphopenia involving both T and B cells also can be observed (Zhang et al., 2006). Depending on the dose used, cyclophosphamide can cause immunosuppression or augmentation of B cell function to T-dependent and T-independent antigens. Delayed-type hypersensitivity also may be impaired or augmented, and tolerance can be induced or terminated (Zhang et al., 2006).

There was a sharp and rapid decrease in the body weight of mice following a single bolus injection of cyclophosphamide, followed by a recovery period 5–7 days after the treatment. The mean rate of weight recovery and weight gain in the groups of mice treated with AML was higher than the untreated control. Cyclophosphamide treatment was marked by gross leucopoenia after 7 days of treatment followed by a recovery phase. Total white blood cells count in groups treated with AML was consistently higher than in the untreated control group showing higher rate of leukopoiesis.

In the study, treatment with cyclophosphamide caused a reduced rate of excision wound healing in rats. This was partially reversed by treatment with AML, which showed a dose-dependent higher rate of wound healing in the immunocompromised rats. Taken together, the studies on immunocompromised rodent models show that extracts of *Morinda lucida* when administered orally could enhance the rate of recovery from immunosuppression as shown by a more rapid rate of weight gain, increased leukopoiesis, and increased rate of excision wound healing.

In immunocompetent mice, the relative weight of spleen was also increased by oral supplementation with AML in a dose-related manner. In the absence of infection and obvious pathology, the relative weight of the spleen reflects the overall immune function of an organism and immunopotentiating therapy are known to increase the relative weights of lymphoid organ such as the spleen (Zhang et al., 2011).

Phytochemical and HPLC analyses identified 14 major constituents in the extract of which three classes of compounds- alkaloids, caffeic acid derivatives (phenolic acid compounds), and quercetin glycosides (flavonoids) were found to be present in abundance. Previous studies demonstrated that these compounds possess useful activities in immunoinflammatory disorders (Natarajan et al., 1996; Prasad et al., 2010).

The results of these studies, therefore, show the potent immunostimulatory and immunorestorative properties of the aqueous leaf extract of *Morinda lucida,* which may explain some of the beneficial effects of the plant in the treatment of infections and immuno-inflammatory disorders.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Humboldt Postdoctoral Research Fellowship award and sponsorship to C. S. Nworu by the Alexander von Humboldt Foundation.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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