AJEHS
African Journal of Environmental
Health Sciences
Volume 4, November, 2017
pp 61-68

Anti-anaemia Potential and Safety of *Morinda lucida* Leaf Extracts in Balb/c Mice Induced with Rhabdomyosarcoma Cells

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Keywords:
Anaemia, Cancer, Safety, *Morinda lucida,* Haematological parameters

Mots-clés:
Anémie, Cancer, Sécurité, *Morinda lucida*, Paramètre hématologique

Abstract
Anaemia, a major health challenge, results from factors including dietary deficiencies and malaria. Although reports from previous studies show that *Morinda lucida* is effective in the treatment of anaemia resulting from these conditions, little information exist to suggest its beneficial effects in treating anaemia resulting from cancer. This study therefore examined the anti-anaemia potential of *M. lucida* leaf extracts in Balb/c mice inoculated with rhabdomyosarcoma (RD) cells. Forty mice inoculated intra-peritoneally with RD cells were randomly divided into eight groups: A (untreated); Treatment groups —B1, B2 and B3 (100, 200 and 300 mg/kg aqueous *M. lucida* extracts respectively); C1, C2 and C3 (100, 200 and 300 mg/kg *M. lucida* ethanolic extracts respectively) and D (Cisplatin). The mice were treated for 2 weeks and thereafter, blood samples were collected through the orbital sinus for haematological and serum biochemical analyses. Compared with group A (3.25 ± 0.03), ethanolic leaf extracts of *M. lucida* increased white blood cell count (x10^3ul) significantly (p<0.05) in groups C1, C2, and C3 (5.45 ±0.03, 6.00 ±0.05, and 5.60 ±0.10 respectively) but there was no significant increase of the packed cell volume in groups C and D. Compared with group A, aspartate aminotransferase (AST) levels were significantly lowerin all treatment groups, and alkaline phosphatase levels (ALP) significantly lower in group B. The treatments therefore did not improve all haematological parameters in RD cell-bearing mice but elicited increased immune responses, were safe at the doses administered and consequently may be beneficial in cancer therapy.

Potentiel anti-anémique et innocuité des extraits de feuilles de Morinda lucida chez des souris Balb/c induites par des cellules de rhabdomyosarcomess

Résumé
L'anémie, un problème de santé majeur, résulte de facteurs tels que les carences alimentaires et le paludisme. *Morinda lucida* est efficace dans le traitement de l'anémie résultant de ces conditions. Peu d'informations existent pour suggérer ses effets bénéfiques dans le traitement de l'anémie résultant du cancer. Cette étude a donc examiné le potentiel anti-anémique des extraits de feuilles de M. lucida chez des souris Balb/c

inoculées avec des cellules de rhabdomyosarcome (RD). Quarante souris inoculées par voie intra-péritonéale avec des cellules RD ont été divisées au hasard en huit groupes : A (non traité); Groupes de traitement -B1, B2 et B3 (100, 200 et 300 mg / kg extraits aqueux de *M. lucida* respectivement); C1, C2 et C3 (respectivement 100, 200 et 300 mg / kg d'extraits éthanoliques de *M. lucida*) et D (cisplatine). Les souris ont été traitées pendant 2 semaines et par la suite, des échantillons de sang ont été prélevés à travers le sinus orbital pour des analyses biochimiques hématologiques et sériques. Par rapport au groupe A (3,25 ± 0,03), feuille d'extraits éthanoliques de comptage *M. lucida* accrue de globules blancs (x103ul) significative (P0.05) dans les groupes C1, C2, C3 et (5,45 ± 0,03, 6,00 ± 0,05, et 5,60 (AST) étaient significativement plus faibles dans tous les groupes de traitement, et les niveaux de phosphatase alcaline (ALP) étaient significativement plus faibles dans les groupes C et D. Par rapport au groupe A, les taux d'aspartate aminotransférase (AST) étaient significativement plus faibles dans tous les groupes de traitement et les niveaux de phosphatases alcalines (ALP) significativement plus faibles dans le groupe B. Les traitements n'ont donc pas amélioré tous les paramètres hématologiques chez les souris porteuses de RD. Des réponses immunitaires, étaient sans danger aux doses administrées et par conséquent peuvent être bénéfiques dans le traitement du cancer.

Introduction

Anaemia is a condition characterised by a decrease in number of red blood cells (RBCs) or their oxygen-carrying capacity and it is defined as a haemoglobin concentration of less than 12 g/dl in males and below 11 g/dl in females (Deyan, 2012). As the most common haematological disorder, anaemia constitutes a major health problem in both developed and developing countries of the world with devastating consequences on human health as well as social and economic development (Christopher, 2012). The 2011 global estimates suggest anaemia affects around 800 million children and women (WHO, 2015). About 1 million deaths a year can be attributed to anaemia, with a very large proportion occurring in Africa and South-East Asia; the cause is multifactorial, including nutritional deficiencies, pathological states and haemoglobin disorders (Kayode and Adeolu,2012). Although it commonly occurs in children and pregnant women, it affects all stages of life (Okafor *et al.,* 2013).

Over the years, various forms of cancer, including lung cancer and rhabdomyosarcoma, have been associated with anaemia. Its prevalence among cancer patients varies widely, ranging from 30% to 90%, depending on the type of cancer (Wanitchar and Mongkol, 2012). Several case studies have frequently found rhabdomyosarcoma (the most common malignant soft tissue tumor in children) patients, to be anaemic (Ocheke *et al.,* 2011). The presence of anaemia reduces the quality of life and shortens survival periods for several cancers, thus increasing mortality in cancer patients since it is also responsible for low immunity to infections. (Ocheke *et al.,* 2011; Dia and Ismael, 2012). The mechanisms underlying the incidence of anaemia in cancer patients have been noted to include bleeding from tumor sites, nutritional deficiencies (including iron metabolism disorder), bone marrow damage, tumor infiltration of bone marrow, and myelosuppressive effect of anticancer therapy (Wanitchar and Mongkol, 2012). Also, cancers are often associated with the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF- α), interferon-gamma (INF-ᵞ) and interleukin-1 (IL-1) which may cause decrease in hemoglobin levels by haemolysis, suppression of erythropoiesis, and impairment of erythropoietin response to erythroid medullary precursors (Wanitchar and Mongkol, 2012). The result, anaemia.

Morinda lucida (Rubiaceae) is a tropical West Africa rainforest tree called Brimstone

tree. It is known as 'Oruwo' or 'Oowo' in Yoruba dialects of Nigeria. In Nigeria, *M. lucida* is one of the commonly used medicinal plants for the management/treatment of various types of ailments. Various extracts of its leaf have been recommended for the treatment of trypanosomiasis (Alli *et al.,* 2011), coccidiosis (Ola-Fadunsin and Ademola, 2014), diabetes (Adeneye and Agbaje, 2008; Adejuwon, 2013) and microbial infections such as pneumonia and yellow fever (Bright *et al.,* 2013), while Adejo *et al*. (2015) postulated the efficacy of *M. lucida* in managing diarrhoea that may result from *Ebola*. More so, the effectiveness of the raw liquid extract of the leaf of *M. lucida* in the treatment of human malaria has been reported by Mathew and Elizabeth (2014).

Alli *et al.* (2011) and Saganwan *et al.* (2014) demonstrated that extracts of *M. lucida* leaf improved packed cell volume (PCV) and haemoglobin (Hb) concentration in animal models. There is however little information on the anti-anaemic activity of this plant in cancer or in tumor-bearing animals. Therefore, the present study was carried out to determine the potentialof extracts of *M. lucida* leaf to correct or alleviate anaemia associated with cancer and the safety of the extracts in therapy.

Materials and Methods

Plant materials

The fresh leaves of *Morinda lucida* were collected from Amina way, University of Ibadan, Ibadan, Oyo State and authenticated at the Forestry Research Institute, Ibadan, Nigeria (FRIN), where specimen number (FHI.110162) was deposited. The collected leaves were cleaned, air dried and macerated. The macerated samples were then used for aqueous and ethanol extractions.

Extraction of plant extracts

The method described by Gyamfi *et al*. (1999) was used with little modification. 120g of the macerated leaf was dissolved in 1000ml of distilled water for 24hrs; another 120g of the macerated leaf was dissolved in 1000ml of ethanol for 72hrs. Each of the mixtures was then filtered first with muslin cloth and with a Whatman filter paper into a beaker. The filtrate was evaporated to dryness on a water bath and the dried concentrated extract was stored in a refrigerator until required for use.

Experimental animals

Forty Balb/C mice of both sexes aged 8-10weeks old weighing 20-25g were purchased from the International Institute of Tropical Agriculture (IITA) and were housed five per cage at the Animal house, Department of Zoology, University of Ibadan, Oyo state, Nigeria receiving standard feed pellets and water *ad libitu.* They were allowed to acclimatise to the new environment for one week. All experiments were conducted in accordance with the NIH Guidelines on the care and use of laboratory animals.

Rhabdomyosarcoma (RD) cells

Rhabdomyosarcoma (RD) cells were obtained from the Virology unit of University of Ibadan College Hospital (UCH), Ibadan. 0.4ml of RD cell suspension at $1x10^6$/ml was given to all the groups of the mice by intraperitoneal (i.p) injections (taken as day 0).A second dose was given 10 days after the first dose. Treatment with plant extracts was commenced three days after the second dose.

Experimental design

After acclimatisation, the mice were randomly assigned to one of the following experimental groups: Group A was implanted intraperitoneally with rhabdomyosarcoma cells, no treatment was given; Group B1 received 100 mg/kg body weight (b.w) aqueous *M. lucida* leaf extract; Group B2 received 200 mg/kg b.w aqueous *M. lucida* leaf extract; Group B3 received 300 mg/kg b.w aqueous *M. lucida* leaf extract; Group C1 received 100 mg/kg b.w ethanolic *M. lucida* leaf extract; Group C2 received 200 mg/kg b.w ethanolic *M. lucida* leaf extract; Group C3: received 300 mg/kg b.w ethanolic *M. lucida* leaf extract. Group D received 5mg/kg b.w cisplatin (standard anticancer drug). Distilled water was

administered to group A while the extracts were given to groups B and C by oral gavage daily for two weeks. Cisplatin was given by intraperitoneal injections twice a week. 24hrs after the administration of last dose, blood samples for hematological and biochemical assays were collected from each mouse through the orbital sinus with heparinised capillary tubes into EDTA-treated bottles and plain bottles respectively. Thereafter, mice were sacrificed. Haematological parameters were determined using standard procedures (Mukherjee *et al.*, 2007).The procedure described by Reitman and Frankel (1957) was employed for the assay of Alanine Aminotransferase (ALT), Alkaline phosphatase (ALP) and Aspartate Aminotransferase (AST).

Statistical Analysis

The results were expressed as Mean ± Standard Error (Mean ± S.E). The statistical analyses were performed by One-way Analysis of variance (ANOVA), followed by Tukey's Multiple Comparison Test, using Graphpad Prism (5.02 version). Differences were considered significant at $p<0.05$.

Results

Extracts of *M. lucida* did not significantly improve all the haematological parameters in rhabdomyosarcoma implanted mice (Tables 1 and 2). PCV, Hb, and RBC values in the extract treated mice were not significantly higher ($p> 0.05$) than those of untreated mice. However, WBC values were significantly increased ($p< 0.05$) by ethanolic extracts and cisplastin when compared with the untreated control, (Table 2). Lymphocyte count was significantly lower ($p< 0.05$) in aqueous extract treated mice when compared with the untreated control. Neutrophil levels were significantly higher ($p< 0.05$) in all treatment groups (27-36%) when compared with the untreated control group (21%). Platelet count in group A was significantly lower ($p<0.05$) ($74,0000x10^3/\mu l$) when compared with all treatment groups ($92,000\ x10^3/\mu l$ -$166,000\ x10^3/\mu l$) except group B2 ($64,000\ x10^3/\mu l$).

AST level was significantly higher in the untreated control (140.05 U/L) when compared with the treatment groups (98.33-123.03U/L) (Table 3). ALP was significantly higher ($p< 0.05$) in untreated mice (Group A) (69.04) when compared with mice treated with aqueous extract in groups B1, B2, B3 and group D, cisplastin (45.19-61.54).

Table 1: Effect of *Morinda lucida* extracts on PCV, Hb and RBC counts.

GROUPS	**PCV (%)**	**H.b (g/dl)**	**RBC ($x10^6$ul)**
A (untreated, RD only)	45.00 ± 1.00^a	15.00 ± 0.15^a	8.67 ± 0.00^a
Aqueous extract			
B1 (100mg/kg)	45.00 ± 0.50^a	15.00 ± 0.25^a	8.76 ± 0.01^a
B2 (200mg/kg)	46.00 ± 0.50^a	15.60 ± 0.05^a	7.40 ± 0.05^{ab}
B3(300mg/kg)	40.00 ± 0.50^b	13.30 ± 0.15^b	8.59 ± 0.01^a
Ethanol extract			
C1(100mg/kg)	45.00 ± 1.00^a	15.00 ± 0.10^a	7.11 ± 0.01^b
C2(200mg/kg)	45.00 ± 1.00^a	15.00 ± 0.25^a	7.22 ± 0.05^b
C3(300mg/kg)	46.00 ± 1.00^a	15.30 ± 0.05^a	8.83 ± 0.02^a
D (cisplatin)	31.00 ± 0.50^c	10.30 ± 0.05^c	6.44 ± 0.00^c

*Means with the same superscripts along columns are not significantly different ($p<0.05$)

Table 2: Effect of *Morinda lucida* extracts on WBC, lymphocyte, neutrophil and platelet counts

GROUPS	WBC (x10^3ul)	Lymphocyte (%)	Neutrophil (%)	Platelet (10^3/µl)
A (untreated, RD only)	3.25 ± 0.03^c	74.00 ± 0.02^a	21.00 ± 0.05^b	74,000 ± 5.00^c
Aqueous extract				
B1 (100mg/kg)	5.00 ±0.31^b	66.00 ± 0.50^b	31.00 ± 0.50^a	92,000 ± 5.50^b
B2 (200mg/kg)	3.50 ± 0.05^c	67.00 ± 0.50^b	28.00 ± 0.25^a	64,000 ± 50.00^c
B3(300mg/kg)	7.00±0.10^a	63.00 ± 0.50^b	31.00 ± 0.50^a	166,000 ± 489.00^a
Ethanol extract				
C1(100mg/kg)	5.45 ±0.03^b	64.00 ± 0.50^b	32.00 ± 0.50^a	92,000 ± 450.00^b
C2(200mg/kg)	6.00 ±0.05^b	55.00 ± 0.50^c	36.00 ± 0.50^a	117,000 ± 5.00^b
C3(300mg/kg)	5.60 ±0.10^b	61.00 ± 0.50^{ab}	33.00 ± 0.50^a	95,000 ± 25.00^b
D (cisplatin)	6.00 ±0.03^b	70.00 ± 0.02^b	27.00 ± 0.50^a	133,000 ± 250.00^a

*Means with the same superscripts along columns are not significantly different ($p<0.05$)

Table 3: Effect of *Morinda lucida* extracts on biochemical parameters

GROUPS	AST (U/L)	ALT (U/L)	ALP (U/L)
A (untreated, RD only)			
Aqueous extract	140.05 ±7.04^a	25.51 ± 1.84^b	69.04 ± 0.58^a
B1 (100mg/kg)	98.33 ± 21.33^c	26.35 ± 13.35^b	61.54 ± 3.75^b
B2 (200mg/kg)	112.55 ±12.04^c	33.64 ± 16.64^a	47.31 ± 4.23^c
B3(300mg/kg)	104.96 ± 14.40^c	21.82 ± 2.67^b	45.19 ± 1.73^c
Ethanol extract			
C1(100mg/kg)	104.93 ± 6.59^c	27.50 ± 12.20^b	57.31 ± 3.46^b
C2(200mg/kg)	123.03 ± 23.03^b	18.36 ± 9.36^c	68.85 ± 34.43^a
C3(300mg/kg)	99.99 ± 9.62^c	30.34 ± 3.50^a	56.35 ± 5.58^b
D(cisplatin)	102.61 ± 13.62^c	25.91 ± 7.26^b	55.20 ± 2.89^b

*Means with the same superscripts along columns are not significantly different ($p<0.05$)

Discussion

Anaemia rarely occurs alone, it often co-exists with underlying infection or health disorder (Kayode and Adeolu,2012). Low PCV, RBC and Hb have been previously reported in cancer induced mice (Subramanian *et al.,* 2011; Baskar *et al*., 2012). Anaemic conditions in tumour bearing mice is mainly due to reduction in RBC or haemoglobin percentage, which occurs either as a result of iron deficiency or haemolytic myelopathic conditions (Subramanian *et al.,* 2011).In this study, the PCV, Hb and RBC levels in the untreated mice were not significantly lower than those of extract treatment groups, and were still within normal ranges which may be because tumours were not fully established in the RD cell bearing mice. However, the lowest PCV value (31%) obtained in group D- cisplatin treated mice was significantly lower than in the untreated mice (group A) which indicates the contribution of chemotherapy to the incidence of anaemia in

cancer (Demetri, 2001).

The significantly reduced WBC count observed in the untreated control (Group A) shows the immunosuppressive ability of rhabdomyosarcoma cells. This concurs with the report of Khanna *et al.* (2012) that rhabdomyosarcoma cancer damages the bone marrow which could then lead to reduction in white blood cell count. The WBC counts were significantly elevated by the administration of extracts of *M. lucida,* suggesting the ability of *M. lucida* to improve immune system function.

Platelets play major role in mediating blood clottingand in modulating inflammatory and immune responses. The significant increase in the platelet counts observed in both cisplatin and all extract treated groups except B2 (200 mg/kg aqueous extract) may be an indication that cisplatin and extracts of *M. lucida* have stimulatory effects on megakaryocytes that regulate platelet production and release (Arise *et al*., 2013). Our results agree with the study of Basakar *et al*. (2012), who reported lower platelet count in untreated tumour bearing mice when compared with mice treated with ethanol extract of *Impatiens balsamina*.

The measurement of the activities of certain enzymes in tissues and body fluids serves as a useful tool in diagnosis, disease investigation and in the assessment of safety or toxicity risk of drug or plant extract (Bamisaye *et al*., 2013). Enzymes AST and ALT are majorly present in hepatocytes, serving as useful biomarkers in assessing the degree of inflammation (Ghouri *et al.,* 2010).In pathological conditions of the liver which include cirrhosis and many times, side effects of some drugs, there is a leak of these enzymes into the serum, thus raising their activity (Bamisaye *et al*.,2013). AST values at all administered doses of the extracts showed significantlylower serum levels when compared with the untreated control, indicating that extracts of *M. lucida* administered at these doses are safe. ALP is a marker enzyme for the plasma membrane and endoplasmic reticulum and often employed in assessing the integrity of the plasma membrane such that any alteration in the activity of the enzyme in the serum would indicate likely damage to the plasma membrane (Adeyemi *et al.,* 2015). RD cell inoculation raised the level of liver ALP in the serum, implying that the integrity of hepatocyte plasma membrane has been compromised, thereby causing increase in the release of the enzyme to the serum. The loss of the enzyme from liver in to the serum may probably be attributed to disruption of ordered lipid bi-layer of the membrane structure. Groups B1-B3 which were givenaqueous extracts of *M. lucida* had significantly lower levels compared with group A. This suggests the hepatoprotective activity of *M. lucida*, inhibiting the leakage of the enzyme.

Conclusion

The present study demonstrated that although *M. lucida* leaf extracts did not stimulate erythropoiesis, the extracts improved immune system function and can safely be used in therapy at the administered doses. The use of this plant will therefore be beneficial in normalizing compromised immune systems particularly associated with cancer.

Acknowledgement

We wish to thank Prof Georgina N. Odaibo of the Department of Virology, College of Medicine, University of Ibadan, Nigeria for kindly providing the rhabdomyosarcoma cells.

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Volume 4, November, 2017
ISSN: 2476-8030
pp 61-68