Original Article

Sub-Acute Toxicological Evaluation of An Artemesia-Based Moringa Supplement (AMS)

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Abstract

Background: In developing nations, several natural medicine practitioners explore the use of plant-based supplements and other forms of phytotherapies to combat viral infection via immune-boosting mechanisms. One such supplement is a locally compounded and consumed Moringa supplement containing *artemisia annua* and named, artemisia-based Moringa supplement (AMS). The aim of this study was to evaluate the effect of a sub-acute (30 days) administration of AMS on the toxicological indices in albino Wistar rats of both sexes.
Materials and Methods: Thirty-two albino Wistar rats (170-220 g) were randomly assigned into four experimental groups: Group 1, served as control, while Groups 2-4 were administered 125, 250 and 500 mg/kg/day of AMS consecutively for 30 days. The animals were anesthetized, and cardiac puncture was used to obtain whole blood for hematology and serum biochemistry. The *in vivo* liver antioxidant assays were also carried out on the liver homogenates.
Results: There were no adverse hematological effects because of AMS treatment. Serum electrolytes levels were not affected across the doses of AMS administered for 30 days. Creatinine levels were not significantly altered, however a significant ($p = 0.037$) reduction in urea level was observed in the highest dose of 500 mg/kg. Liver function assays showed no significant alterations in the liver function enzymes, although, a significant decrease in the liver malonaldehyde (MDA, product of lipid peroxidation) level was observed. There was no adverse effect on kidney function parameters and serum lipid profile.
Conclusion/ Recommendations: The artemisia-based polyherbal supplement seems to be relatively safe at the doses considered for this experiment. However, further safety studies will be required to ascertain more detailed organ safety.

Keywords: *Artemisia annua*, Nutraceuticals, Hepatotoxicity, Serum biochemistry, Hematology

Introduction

The use of herbal medicines and other forms of phytotherapies, provides ample potentials for the development of new agents either as supplements, drugs, extract, pure compounds and their respective derivatives.[1,2] Over the years, there has been a folkloric misconception that natural originating supplements are safe for medicinal purposes. This however lacks scientific validation.[3,4] Several herbal therapies used in folk medicine have compelling proof regarding their bio-medicinal activities.[5,6] However, the major limitation to the use of these remedies is the paucity of scientific, mechanistic and clinical data in support of the efficacy and safety of these drugs.[7] Toxicological evaluations of plant-derived medicinal in the form of supplements and therapeutic agent must be carried out to ascertain their preclinical safety profile, as this will increase the assurance of their safety in humans, particularly for use in the development of pharmaceuticals.

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Herbal formulations, medicinal plants or dietary supplements constitute a major part of our existence.[8,9] Investigation of plants or their constituents as medicinal foods dates to the very history of man. Research findings suggest that a great percentage of Africans and Asians depend on natural product medicines as a key element of therapy and not just a

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complementary aspect.[10] The increased subscription for herbal medicines is linked to their accessibility, availability and affordability. Many constructs for the use of nutraceuticals/phytomedicines as sources of therapeutic agents are being explored, profiled and scientifically validated to herbal-based pharmacopoeia, like the West African Herbal Pharmacopoeia.[11] This affords a compendium of several plants, their derivatives, and vital information about their safety and possible efficacy. Plants produce several secondary metabolites, forming complex compounds that may be harmful or beneficial to humans.

Artemisia annua usually referred to as "annual absinthe" is an annual herbaceous plant, where it derived its name, "annua". The plant is grown in Asia, Africa, Central Europe and Australia tropical regions.[12,13] It is commonly used as a dietary condiment, herbal tea and medicinal plant in the mild climates of Asia, such as China and Korea; and some African countries, such as Nigeria.[14,15] For several decades, Artemisia annua plant extract has been a main therapy in Asia and Africa for the treatment of malaria and fever, in the form of tea or herbal concoction.[16,17]

We previously reported a 30-day toxicological assessment of a Nigerian polyherbal Moringa supplement,[18] this present study evaluates a 30-day preliminary safety profile of a new Artemisia-based Moringa supplement (AMS). This will help understand the safety role of artemisia or otherwise in the previously reported herbal formula.

Materials and Methods

Experimental animals and Sub-acute Toxicological Evaluation: Rats (32) of both sexes were randomly assigned to four groups, one as control and the others for doses of 125, 250 and 500 mg/kg of AMS respectively. AMS extract and vehicle (distilled water, 600 µL for the control), were administered daily for 30 days using an orogastric tube. Wistar albino rats of both sexes weighing between 180 to 220g were purchased from *McTenny Farms*, a commercial private colony near Ladoke Akintola University of Technology (LAUTECH), Ogbomosho, Oyo State, Nigeria, and housed under natural light and temperature conditions within the Laboratory Animal House facility of the College of Health Sciences, University of Ilorin, Nigeria. Rat pellets (Growers' Mash, Ogo Oluwa, Ilorin, Kwara State, Nigeria) and water were made available *ad libitum*. The animals were acclimatized for one week prior to the experiment. Procedures involving animal handling followed the guidelines published by the National Institute of Health (NIH, 1996) and the University of Ilorin Ethical Review Committee (UIERC) with approval number: UERC/ASN/2020/2034.

Reagents and Solvents: All reagents used were of analytical grade. Anesthetic agent (pentobarbital) and absolute ethanol, were obtained from Sigma Aldrich, USA.

Preparation of Artemisia based Moringa supplement (AMS): AMS, a natural product supplement containing, medicinal plants: leaves of *Moringa oleifera* (75%w/w), *Artemisia annua* (5%w/w) *Ocimum gratissimum* (5%w/w), *Garcinia kola* (bitter kola) (5%w/w), and *Zinginber officianale* (ginger) (5%w/w) were compounded by Biofuel® and Natural Product Herbal Supplement; NAPHERBS). AMS 500g was constituted in absolute ethanol (98.7 %) for 72hr, filtered using Whatman filter paper 1. The filtrate was concentrated using a rotary evaporator at 40°C (BUCHI Rotavapor® Model R-215, Switzerland) with the vacuum Model V-801 EasyVac® Switzerland. The concentrate was weighed and tagged AMS extract.

$$\text{Percentage yield} = \frac{\text{Weight of Extract (g)}}{\text{Weight of PHS powder (g)}} \times 100$$

Hematological Assays: After 30 days of AMS administration, the rats were sacrificed by an intraperitoneal administration of 30 mg/kg pentobarbital and blood was drawn by cardiac puncture. A portion of the blood sample was put in a heparinized bottle for hematological assays, while the other portion was centrifuged for 20 min at 3000 *g*, and the serum was harvested and frozen for serum biochemical assay.

A wide-range of investigative hematological parameters was explored to access the percentage lymphocyte (% LYM), white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), hemoglobin concentration (HBG), etc., using an automated hematology analyzer from *HORIBA Pentra™ XL 80,* USA.

Serum Biochemical Assays: As previously described by Afolabi et al., serum electrolyte levels were assessed using instruments from TECO Diagnostics (CA, USA). Renal function determinants such as serum creatinine and urea levels were evaluated using kits supplied by *Spinreact™ autoanalyzer* (Girona, Spain). Liver function enzymes such as alanine transaminase (ALT), aspartate transferase (AST), and bilirubin levels were evaluated using the *Beckman Coulter LH 780 analyzer*. Lipid profile [total cholesterol (TC), triglycerides (Trig), high-density lipoprotein (HDL-c), and low-density lipoprotein (LDL-c] were analyzed using commercial kits obtained from Randox Laboratories Ltd (Crumlin, UK), with strict adherence to the

manufacturer's protocol.[18]

Liver Antioxidant assays and MDA levels: Catalase, Superoxide dismutase (SOD) and reduced glutathione (GSH) activities were carried out on the liver homogenate post 30 days administration with AMS. Analytical kits obtained from *MyBiosource Inc*. (San Diego, USA) were used for the assay. Glutathione peroxidase and malonaldehyde (a marker of lipid peroxidation) levels were estimated using analytical kits obtained from *MyBiosource Inc.* (San Diego, USA) and *Elab Sciences* (Texas, USA) respectively, and following the manufacturer's protocol as previously reported.[18] To assay for the product of lipid peroxidation (MDA levels), three major reagents containing clarificant, acid reagent (thiobarbituric acid) and a chromogenic agent were added successively to each experimental group in triplicate (including standard and experimental controls) according to the manufacturer's protocol, and absorbance was read at 532 nm with a microplate reader.

Statistical Analysis: The statistical analysis used was the one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test (*GraphPad prism IX software*, Boston, USA). Significance was considered at P< 0.05. All results are expressed as the mean ± standard error of the mean

Results

General Observation: A percentage extract yield of 15.25 % w/w was extrapolated for AMS extract. On an observatory note, the control and treatment groups did not show any physical sign of pain by writhing, stretching, or paw licking during the 30-day administration. There was no daily observance of fatigue or anxiety during the course of the experiment. Food and water intake was not different from the control group (data not presented).

Hematological Assays: After administering doses of AMS daily for 30 days, there were no statistical differences in the hematological indices such as WBC, LYM (%), PCV, RBC, MCV, HGB, etc. (Table 1).

Serum Electrolyte: At the dose of 250 mg/kg/day, the sodium level was significantly (*P*= 0.032) decreased (Table 2). Potassium (*P*= 0.12), calcium (*P*= 0.09) and bicarbonate levels (*P*= 0.11) were not significantly affected by the 30-day administration of AMS.

Liver and Kidney Function Parameters: Across the groups, ALT, AST, and bilirubin levels were not altered by the 30-day AMS treatment. In the same trend, for the kidney function indices, creatinine and urea concentrations were not significantly (*P*<0.05) altered

Table 1. Effect of AMS on hematological indices

Parameters	Control (*n* = 4)	AMS (125 mg/kg) (*n* = 4)	AMS (250 mg/kg) (*n* = 4)	AMS (500 mg/kg) (*n* = 4)	p values
PCV	51.50 ± 1.60	50.67 ± 1.74	46.17 ± 2.34	52.33 ± 1.94	0.9318
HBG (g/dL)	16.65 ± 0.47	16.57 ± 0.48	15.41 ± 0.77	17.30 ± 0.49	0.7990
RBC (10^6/µL)	8.46 ± 0.21	8.34 ± 0.19	7.70 ± 0.40	8.59 ± 0.30	0.6021
WBC (10^3/ µL)	5.72 ± 0.20	5.38 ± 0.16	5.27 ± 0.61	4.60 ± 1.01	0.1171
Platelet (10^3/ µL)	67.83 ± 3.56	67.33 ± 12.42	66.00 ± 10.54	71.00 ± 10.72	0.3249
Lymphocytes (%)	77.50 ± 1.36	76.33 ± 2.02	74.00 ± 0.57	76.83 ± 1.19	0.0732
Neutrophils (%)	18.67 ± 1.36	19.67 ± 2.70	23.67 ± 0.61	20.17 ± 1.37	0.2320
Monocytes (%)	1.83 ± 0.30	2.17 ± 0.48	1.83 ± 0.40	1.67 ± 0.33	0.0928
Eosinophils (%)	2.00 ± 0.58	1.83 ± 0.79	1.17 ± 0.17	1.33 ± 0.42	0.0768
MCV (fL)	60.87 ± 0.57	60.71 ± 1.17	59.93 ± 0.38	60.90 ± 0.44	0.2196
MCH (pg)	19.69 ± 0.16	19.86 ± 0.31	20.01 ± 0.14	20.15 ± 0.18	0.5386
MCHC (g/dL)	3.23 ± 0.01	3.27 ± 0.03	3.34 ± 0.03	3.31 ± 0.05	0.1570

n = 4, **P*<0.05; statistical analysis: One-way Analysis of Variance (ANOVA)

Table 2. Effect of AMS on serum electrolyte indices.

n = 4, **P*<0.05; statistical analysis: One-way Analysis of Variance (ANOVA)

Parameters	Control (*n* = 4)	AMS (125 mg/kg) (*n* = 4)	AMS (250 mg/kg) (*n* = 4)	AMS (500 mg/kg) (*n* = 4)	p values
Na (mg/L)	149.50 ± 1.70	147.83 ± 1.85	145.16 ± 1.10	148.83 ± 1.77	0.5147
K (mg/L)	7.95 ± 0.14	7.70 ± 0.12	7.60 ± 0.04	7.80 ± 0.11	0.3277
Ca (mg/L)	11.28 ± 0.36	11.00 ± 0.29	11.00 ± 0.24	11.41 ± 0.37	0.8935
HCO3 (mg/L)	24.50 ± 0.62	23.50 ± 0.76	22.17 ± 0.54	22.82 ± 0.31	0.5863

Table 3. Effect of AMS administration on liver and kidney function indices
n = 4, *$P<0.05$; statistical analysis: One-way Analysis of Variance (ANOVA)

Parameters	Control (*n* = 4)	AMS (125 mg/kg) (*n* = 4)	AMS (250 mg/kg) (*n* = 4)	AMS (500 mg/kg) (*n* = 4)	p values
AST (U/L)	109.87 ± 3.62	101.82 ± 9.82	96.93 ± 7.17	93.10 ± 2.80	0.1857
ALT (U/L)	67.98 ± 2.95	61.89 ± 8.31	65.07 ± 5.71	55.22 ± 2.69	0.2235
Creatinine (mg/dL)	46.83 ± 3.27	56.83 ± 4.72	43.50 ± 9.38	51.00 ± 4.28	0.3161
Total Bilirubin (mg/dL)	3.16 ± 0.26	3.09 ± 0.33	3.24 ± 0.38	3.10 ± 0.24	0.8708
Urea (mg/dL)	52.68 ± 1.90	53.66 ± 1.10	55.77 ± 1.39	44.95 ± 5.35*	**0.0365**

Table 4. Effect of AMS on lipid profile indices

Parameters	Control (*n* = 4)	AMS (125 mg/kg) (*n* = 4)	AMS (250 mg/kg) (*n* = 4)	AMS (500 mg/kg) (*n* = 4)	p values
Triglyceride (mg/dL)	73.79 ± 4.89	79.51 ± 9.21	79.52 ± 6.48	70.23 ± 4.32	0.6028
Total cholesterol (mg/dL)	99.07 ± 9.48	92.18 ± 9.07	91.87 ± 2.61	86.62 ± 4.12	0.1701
HDL (mg/dL)	24.50 ± 1.53	26.01 ± 2.98	28.58 ± 2.06	24.31 ± 1.71	0.9992
LDL (mg/dL)	59.36 ± 8.94	50.27 ± 8.35	47.40 ± 2.72	48.27 ± 4.52	0.2593
VLDL (mg/dL)	14.76 ± 0.98	15.90 ± 1.84	15.90 ± 1.30	14.05 ± 0.87	0.6094
AI	2.00 ± 0.23	2.20 ± 0.45	1.86 ± 0.33	1.93 ± 0.19	0.5147
HDL/LDL	0.51 ± 0.14	0.60 ± 0.13	0.61 ± 0.06	0.55 ± 0.09	0.5608
CRI	4.01 ± 0.35	3.62 ± 0.30	3.27 ± 0.18	3.68 ± 0.37	0.7070

n = 4, *$P<0.05$; statistical analysis: One-way Analysis of Variance (ANOVA)

Table 5. Effect of AMS treatment on liver antioxidant enzymes and MDA levels.
n = 4, *$P<0.05$; statistical analysis: One-way Analysis of Variance (ANOVA)

Parameters	Control (*n* = 4)	AMS (125 mg/kg) (*n* = 4)	AMS (250 mg/kg) (*n* = 4)	AMS (500 mg/kg) (*n* = 4)	p values
GSH (μmol/ mg protein)	4.91 ± 0.17	6.64 ± 0.29	7.20 ± 0.19	7.08 ± 0.19	0.8060
MDA (ηmol/ mg protein)	1.56 ± 0.24	1.09 ± 0.13	0.57 ± 0.10*	0.40 ± 0.10*	**0.0027**
Nitrite (μmol/ mg protein)	1.78 ± 0.23	1.74 ± 0.17	1.78 ± 0.41	1.79 ± 0.18	0.4112
Catalase (U/ mg protein)	2.84 ± 0.19	5.44 ± 0.62	5.97 ± 0.56	5.52 ± 0.32	0.2796
SOD (U/ mg protein)	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.5232

when compared with the experimental control.

Serum Lipid Profile: Across the treatment groups, there was no statistically significant ($P<0.05$) difference in the concentration of triglycerides, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL), Atherogenic index (AI) and coronary risk index (CRI) when compared with the control.

Liver Antioxidant Parameters and MDA Levels: There was a significant ($P<0.05$) increase in the reduced glutathione (GSH) levels across the treatment groups, when compared with the control (Table 5). The product of lipid peroxidation, malonaldehyde levels were also significantly ($P<0.05$) decreased in both 250 and 500 mg/kg/day treatment groups. The concentration of catalase enzyme was also significantly ($P<0.05$) increased across the treatment

groups when compared to the control. Sodium dismutase (SOD) and nitrite concentrations were not significantly altered as a result of AMS treatment, when compared with the control.

Discussion

This study evaluated the sub-acute toxicity profile of an artemisia based moringa supplement (AMS) consumed for it's immune-boosting folkloric claims. In the sub-acute toxicological assessment, AMS did not affect the PCV, RBC, WBC counts and its differentials such as the lymphocytes, monocytes and granulocytes across the groups. Studies have shown that a reduction in neutrophils could denote an immunocompromised status in the animals, leading to susceptibility to several opportunistic infections.[19-20] An increase in WBC and lymphocytes suggests an active immunologic response to infections or stress, while a decrease in these differentials may connote a chronic state of infection.[21] AMS does not portend an adverse effect on the animal's hematological indices which is crucial for optimal wellness.

Serum lipid profile is a vital investigatory tool to ascertain optimal cardiac functions, cardiovascular well-being and more pertinently, the risk of developing atherosclerosis and other cardiac-related diseases.[22] Coronary risk index (CRI) (TC/HDL-C) and Atherogenic index (AI) (LDL-C/HDL-C) are vital indices for checking the risk of coronary heart diseases and atherosclerosis.[23] Our findings show that AMS did not significantly alter all the lipid parameters assayed for and their corresponding index, after the 30 days administration. Marked increase in serum LDL-c and total cholesterol levels have been linked to increased cardiovascular morbidity and mortality, whereas a high HDL-c level is cardioprotective.[24] This suggests that AMS does not increase the risk of atherosclerosis and other cardiovascular diseases.

At the doses considered in this study, AMS dis not affect the serum electrolytes: Na^+, K^+, Ca^{2+} and HCO_3^- levels. assayed for. For example, Sodium ion is an osmotically active cation and a main electrolyte in the extracellular fluid. It is responsible for the maintenance of the extracellular fluid volume and regulating the membrane potential of cells.[25] A significant decrease in the serum concentration of Na+ could be the result of increased Na+ excretion alongside water, similar to a natriuretic or diuretic effect, or an underlying kidney or heart failure. [26] However, AMS didn't cause any electrolyte imbalance.

Creatinine levels as indicators of kidney functions was not significantly altered by AMS administration. Creatinine is a known an indicator of chronic kidney disease (CKD). [27] Creatinine is a metabolic byproduct in the serum, produced by the catabolism of muscle cells. Physiologically, optimal-functioning kidneys clear creatinine out of the blood and excrete it from the system via the urine.[28] In the case of an impaired kidney function, creatinine level increases because of its build-up in serum. AMS at the highest dose of 500 mg/kg caused a significant reduction in the level of urea.[29] A reduction in serum urea levels alone cannot be used as standalone diagnostic parameter. The assessment of liver and kidney functions is crucial, as these metabolic organs have several detoxifying mechanisms.[30] While the liver and kidney are pivotal to the process of detoxifying, the organs are also susceptible to damage during the process of detoxification. Therefore, several biochemical parameters are used in the assessment of hepatic dysfunction and liver cell damage.[31] Aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) are accessors of liver functions. An increase in ALT, AST, ALP, and bilirubin denotes hepatocellular disease. An elevation in ALP and bilirubin suggests cholestatic disease.[32] AMS did not significantly alter these liver enzymes.

Free radicals are produced in the body as a result of several metabolic processes. The body mops up these radicals via the machinery of antioxidants. The impaired ability of these antioxidants to clean up these radicals lead to oxidative stress, which underlies several cardiovascular and metabolic disorders.[33] Our findings showed no significant alterations in the liver antioxidant enzymes evaluated. Catalase is a vital antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to oxygen and water; thus, neutralizing the cytotoxicity of peroxyl radicals. Catalase deficiency in mice has been shown to increase the possibility of developing type 2 diabetes, obesity and fatty liver.[34] Malonaldehyde (MDA) concentration was dose-dependently reduced in the 250 and 500 mg/kg groups. MDA, a product of lipid peroxidation gives a scientific idea of a functional antioxidant system. A reduction in the MDA levels suggest a robust antioxidant defense system.[36] AMS could prevent lipid peroxidation in the liver, which is a major hepatoprotective mechanism.

Conclusion

Our findings suggest that AMS could be hepatoprotective via antioxidant mechanisms, although there is the possibility of hyponatremia at doses considered for this study. Further research into more specific toxicities such as neurotoxicity, genotoxicity reproductive toxicity and histological examination of vital organs will establish a more robust safety profile.

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Conflicts of Interest

The authors declare no conflict of interest.

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