

ORIGINAL CONTRIBUTION

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Mitigative effects of *Moringa oleifera* against liver injury induced by artesunate-amodiaquine antimalarial combination in *wistar* rats

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Abstract

Background: Artesunate-amodiaquine (AS-AQ) is an antimalarial drug. It is associated with improved cure rates, accelerated response to therapy and delayed development of resistance. However, liver damage, neurotoxicity and agranulocytosis have been reported as adverse effects whose origins have been linked to free radicals generated by the drug. According to native *materia medica*, *Moringa oleifera* (MO) has wide utility in ethnomedicine. However, there is paucity of information on the hepatoprotective efficacy of this plant. The present study evaluated the mitigative effects of MO leaf extracts against liver injury induced by AS-AQ combination in female *Wistar* rats.

Methods: Dry leaf powder of MO was extracted with water and a 20:80 v/v mixture of water and methanol to give aqueous (AQ) and aqueous-methanol (AQ-ME) MO leaf extracts respectively. In vitro hydroxyl free radical scavenging activity of serial dilutions (10–100 µg/ml) of each of the extracts was then evaluated using an assay model where butylated hydroxytoluene (BHT) served as a reference standard. The extract with better free radical scavenging activity was then evaluated for hepatoprotective effects against AS-AQ intoxication in female *Wistar* rats based on the Acute Toxic Class method (OECD 2000). Serum aspartate amino transferase (AST), alanine amino transferase (ALT), total bilirubin and histological examination of rat liver sections were used to evaluate the hepatoprotective activity of the selected MO leaf extract. Siliphos[®] (standard hepatoprotectant) was used for comparison.

Results: There was a concentration dependent increase in the hydroxyl free radical scavenging activity of MO leaf extracts and standard (BHT) with values ranging from 46.36–66.36% for the AQ extract, 41.04–60.95% for the AQ-ME extract and 44.93–65.23% for BHT with corresponding IC₅₀ values of 26.84 µg/ml, 51.88 µg/ml and 32.58 µg/ml respectively. A 1000 mg/kg dose of the AQ-ME MO leaf extract significantly ($p < 0.05$) lowered AST values of AS-AQ intoxicated rats to a level comparable to the standard hepatoprotectant; Siliphos[®]. Serum ALT and TB were also lowered but this was not statistically significant ($p > 0.05$). The 1000 mg/kg dose also reduced hepatocyte degeneration in rats treated with four times the clinical dose of AS-AQ. This study suggests that the hepatoprotective activity of the leaves of MO may have some relation to its free radical scavenging properties. These leaves may thus be useful in mitigating free radical initiated disease conditions.

Conclusion: The aqueous-methanol *Moringa oleifera* leaf extract exhibits free radical scavenging and hepatoprotective properties. Further investigations on the structural identity of the phytoconstituents and their mechanisms of action should be performed to facilitate the development of a potent medicinal agent.

Keywords: *Moringa oleifera*, Artesunate-amodiaquine, Hepatoprotective activity, *Wistar* rats

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Background

Intimate proximity of the liver to the small intestines and blood circulation exposes it to many foreign substances or their metabolites which may induce injury [1]. Access to antimalarials in the African region is unabated resulting in unwarranted self-medication practices whose net result may be untoward effects such as toxicity [2, 3]. Artesunate-amodiaquine is a fixed dose combination currently in use for the treatment of uncomplicated malaria [4]. At high doses, the drug causes liver damage, neurotoxicity and agranulocytosis associated to free radicals generated by the drug [5, 6]. Silyphos[®] is an antioxidant substance made up of a complex of silybin (a flavonoid) and phosphatidylcholine (a phospholipid). This complex has been reported to have good hepatoprotective activity [7]. *MO* is a plant associated with antioxidant properties [8]. Positive correlations have been made between the antioxidant and hepatoprotective properties of other plants such as *Acacia catechu*, *Camellia sinensis*, *Magnifera indica*, *Punica granatum* and *Phyllanthus emblica* [9]. However, there is paucity of information on the antioxidant and hepatoprotective effects of *MO*. The present study aims to determine the antioxidant and protective effects of leaves of *MO* on the liver of artesunate-amodiaquine intoxicated female *Wistar* rats.

Methods

Chemicals

Pure artesunate drug powder (SIGMA A3731), pure amodiaquine drug powder (FLUKA A2799) and phosphate buffer saline (PBS) tablets were purchased from Sigma-Aldrich chemical company (St. Louis, MO). Siliphos[®] was purchased from iherb, California USA and diagnostic kits (MIND-RAY) were purchased from SHENZEN MINDRAY Biomedical electronics, Shenzhen, China. All other reagents were analytical grade and of high purity.

Collection and identification of plant material

Fresh aerial plant material of *MO* was collected from Kibwezi, Makueni county in Kenya (2°25'S/37°58'E) (Fig. 1). The leaves were carefully separated from the

plant, duplicate samples prepared and authenticated by a botanist at the National Museums Herbarium. A voucher specimen number (NMK/BOT/CTX/1/2) was assigned to the specimen for future reference.

Preparation of extracts

Leaves of *MO* were air-dried under shade for 1 week and powdered using an electric mill. A slightly modified method of Anwar et al., [10] was used to prepare water and aqueous-methanol extracts. For the aqueous extract, one hundred grams of dry *MO* leaf powder was weighed and poured into a 500 ml volumetric flask wrapped in tin foil. Water was gradually added to this flask and the flask gently shaken until a slurry of uniform consistency was formed. The contents were then stirred by use of a magnetic stirrer for 48 h and subsequently centrifuged at 3000 RPM for 10 min. The supernatant was collected into amber coloured plastic bottles and freeze dried to obtain a dry lyophilized powder which was weighed and kept in air-tight containers awaiting further work.

Similarly, the aqueous-methanolic extract was prepared by weighing one hundred grams of dry *MO* leaf powder into a 500 ml volumetric flask wrapped in tin foil. A mixture of water and methanol (20:80 v/v) was gradually added to this flask and the flask gently shaken until a slurry of uniform consistency was formed. The contents were then stirred using a magnetic stirrer for 48 h and subsequently centrifuged at 3000 RPM for 10 min, supernatant collected and poured into a round bottomed flask. Owing to the different boiling points of water and methanol, the round bottomed flask was then attached to a rotary evaporator whose operating temperature was then set at 40 °C. The setup was allowed to run for 6 h to remove methanol after which the semisolid mass was transferred to a 40 °C sand bath and left overnight. The resulting solid mass was then collected and stored in air-tight containers awaiting further work.

Determination of hydroxyl radical scavenging activity

The method of Klein et al., [11] was used. Briefly, extract concentrations (10–100 µg/ml) were added to a 3 ml mixture containing 1.5 mM Ferrous sulphate, 6 mM

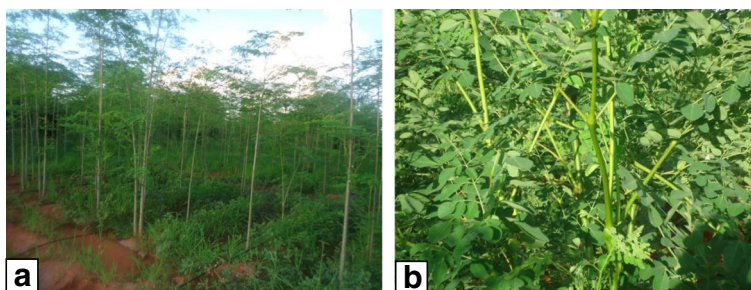


Fig. 1 a *Moringa oleifera* whole plant b aerial parts of *Moringa oleifera*

hydrogen peroxide and 20 mM sodium salicylate. The resulting solution was then incubated at 37 °C for 1 h. The intensity of the colour developed was measured at 532 nm using a Spectronic 21D Milton Roy UV-VIS Spectrophotometer (USA). The capacity of the samples to scavenge hydroxyl free radicals was calculated using the formula as described by Singh et al., [12].

$$\begin{aligned} & \% \text{Scavenging capacity of sample extracts on hydroxyl radicals} \\ &= \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100 \end{aligned}$$

Where;

A_{Control} = Absorbance of the control (Ferrous sulphate, hydrogen peroxide and sodium salicylate).

A_{Sample} = Absorbance of the sample (Ferrous sulphate, hydrogen peroxide, sodium salicylate and varied concentrations of leaf extracts of *MO*).

The concentration of the extracts which inhibited 50% of hydroxyl radicals was calculated using regression analysis in MS Excel 2007.

Ethical considerations

Before commencing in vivo studies in *Wistar* rats, ethical clearance was sought from the biosafety, animal use and ethics committee of the University of Nairobi. A reference number was given for future reference BAUEC/J56/76385/2014.

Occupational health and personal protective equipment (PPE)

Established protocols [13] for handling laboratory animals were followed throughout the study. Latex hand gloves and protective masks were used at all times. In addition, anti-tetanus and anti-rabies vaccines were made available and stored under refrigeration.

Preparation of animals

Thirty-six healthy, 8–10 week old female *Wistar* rats weighing 180–200 g were used for the study. The animals were nulliparous and non-pregnant and were purchased from the animal holding unit of the Department of Public Health, Pharmacology and Toxicology of the University of Nairobi. They were then housed in polypropylene cages measuring 35 (L) × 25 (W) × 18 (H) and lined with wood shavings. Temperature of the animal house was maintained at 25 ± 3 °C and 50–60% relative humidity for a period of 10 days to enable the animals to acclimate to the laboratory conditions. A 12-h light and dark cycle was maintained and the animals were sustained on water ad libitum and standard rat pellets from a commercial feed supplier (Unga feeds, Kenya Limited).

Experimental design

From our previous studies on acute oral toxicity of *MO* [14], a 1000 mg/kg dose of the aqueous-methanol *MO* leaf extract was used in evaluating hepatoprotective activity against AS-AQ intoxication. Thirty six rats were randomly assigned to 12 groups (each 3 animals) based on the Acute Toxic Class method [15]. The animals were labelled to enable identification. All test substances; Siliphos[®], *MO*, AS-AQ were dissolved in physiological buffer saline (PBS) for administration to rats by oral gavage over a 5-day period as follows;

Group I- Orally received 2 ml of PBS once daily.

Group II- Orally received a 1000 mg/kg dose of *MO* leaf extract once daily.

Group III- Orally received 200 mg/kg of Siliphos[®] once daily.

Group IV- Orally received 4, 2, 2, 2, 2 mg/kg of artesunate (AS) and 10.8, 5.4, 5.4, 5.4 and 5.4 mg/kg dose of amodiaquine (AQ). This is equivalent to the clinical dose of AS-AQ for the treatment of uncomplicated malaria [16, 17] and is referred to in this experiment as clinical dose of AS-AQ.

Group V- Orally received 8, 4, 4, 4 and 4 mg/kg dose of artesunate (AS) and 21.6, 10.8, 10.8, 10.8 and 10.8 mg/kg dose of amodiaquine (AQ). This is equivalent to double the clinical dose of AS-AQ (2 × clinical dose of AS-AQ).

Group VI- Orally received 16, 8, 8, 8 and 8 mg/kg of artesunate (AS) and 43.2, 21.6, 21.6, 21.6 and 21.6 mg/kg of amodiaquine (AQ). This is equivalent to four times the clinical dose of AS-AQ (4 × clinical dose of AS-AQ).

Group VII- Rats were first orally pre-treated with a 200 mg/kg dose of the standard hepatoprotectant (Siliphos[®]) followed an hour later by treatment with the clinical dose of AS-AQ as previously described.

Group VIII- Rats were orally pre-treated with a 200 mg/kg dose of the standard hepatoprotectant (Siliphos[®]) followed an hour later by treatment with 2 × clinical dose of AS-AQ as previously described.

Group IX- Rats were first orally pre-treated with a 200 mg/kg dose of the standard hepatoprotectant (Siliphos[®]) followed an hour later by treatment with 4 × clinical dose of AS-AQ as previously described.

Group X- Rats were first orally pre-treated with a 1000 mg/kg dose of *MO* leaf extract followed an hour later by treatment with the clinical dose of AS-AQ as previously described.

Group XI- Rats were first orally pre-treated with a 1000 mg/kg dose of *MO* leaf extract followed an hour later by treatment with 2 × clinical dose of AS-AQ as previously described.

Group XII- Rats were first orally pre-treated with a 1000 mg/kg dose of *MO* leaf extract followed an hour later by treatment with 4 × clinical dose of AS-AQ as previously described.

Blood collection, necropsy and disposal of rat carcasses

Twenty-four hours after administration of the last treatment, individual animals were held with a warm cloth to dilate the blood vessels. The lateral tail vein was then gently punctured using a 21-gauge hypodermic needle attached to a 2 ml syringe. One ml of blood was allowed to collect in the syringe and then transferred to vacutainers lined with a clot activator. The clotted blood was then centrifuged at 3000 RPM for 10 min and serum collected by use of micropipettes into cryo-vials which were capped and stored at 4 °C awaiting biochemical analysis. The levels of serum aspartate aminotransferase (AST), alanine amino transferase (ALT) and total bilirubin (TB) in the different treatment groups were assayed using standard protocols of MIND-RAY commercial kits [18]. Animals were then humanely euthanized by use of a 150 mg/kg dose of intravenous sodium pentobarbital injection. Livers were harvested, weighed and preserved in 10% neutral buffered formalin solution for 24 h awaiting histological examination. Rodent carcasses were then placed in zip locked non-polyvinylchloride (non-PVC) transparent plastic bags and incinerated.

Histological examination

A slightly modified method of Palipoch and Punsawad [19] was used. Formalin preserved liver tissues were washed with 70% ethanol. The tissues were then placed in metallic caskets, stirred by use of a stirrer followed by dehydration in graded ethanol (70–100%) and embedded in paraffin wax by use of an embedding machine. The paraffinized blocks were then sectioned using a rotatory ultra microtome, transferred to glass slides and allowed to dry overnight. The slides were then stained by haematoxylin and eosin (H&E) dye and mounted on a light microscope for observation.

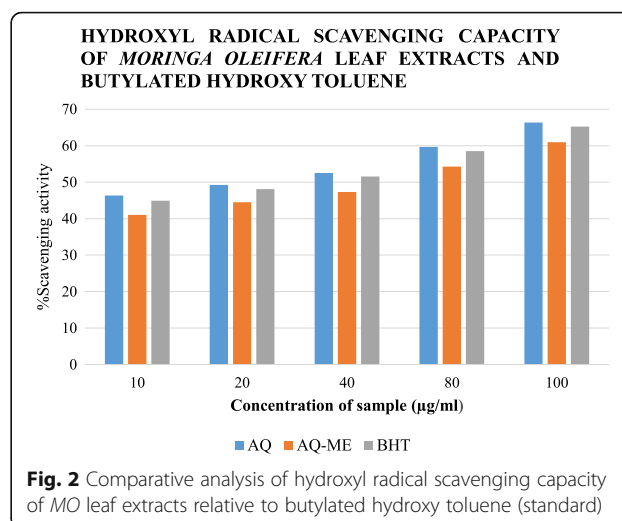
Statistical analysis

Results of the analysis of the hepatospecific markers of liver injury were expressed as mean \pm SEM and analyzed using one-way analysis of variance (ANOVA) followed by least significant difference as post hoc test using Gen Stat Statistical Software 4th edition. ($p \leq 0.05$) was considered significant.

Results

Hydroxyl radical scavenging capacity of *Moringa oleifera* leaf extracts

It was shown on Fig. 2 that there was a concentration dependent increase in the in vitro hydroxyl radical scavenging activity of *MO* extracts and butylated hydroxyl toluene (BHT). The scavenging activity of the AQ extract was in the range 46.36–66.36% compared to 41.04–60.95% of the AQ-ME extract and 44.93–65.23% of BHT. However, the ability of the AQ extract to



scavenge these free radicals (66.36%) was found to be higher than both the AQ-ME extract (60.95%) and BHT (65.23%). Regression analysis in MS excel established the concentration of the extracts and standard necessary to inhibit 50% of the hydroxyl radicals (IC_{50}) Table 1.

Changes in serum liver markers

It was shown on Table 2, that there was a dose-dependent increase in the levels of AST, ALT and TB following treatment with the AS-AQ antimalarial combination. Moreover, pre-treatment with Siliphos® (200 mg/kg body weight per oral) and AQ-ME leaf extract of *MO* at a dose of 1000 mg/kg body weight per oral significantly reduced the levels of serum AST but non-significantly reduced the levels of ALT and TB when compared to the AS-AQ intoxicated groups.

Histological examination of rat liver sections

It was shown on Fig. 3 that the administration of four times the clinical dose of AS-AQ resulted in vacuolation and necrosis of hepatocytes.

It was shown on Fig. 4 that pre-treatment of rats with a 1000 mg/kg dose of *MO* before administration of four times the clinical dose of AS-AQ resulted in mild improvement in the degree of vacuolation and hepatic congestion.

Table 1 IC_{50} values of *MO* leaf extracts and butylated hydroxy toluene (BHT) against hydroxyl free radicals

Sample	OH radical scavenging activity (µg/ml)
Water (AQ) extract	26.84
Aqueous-methanol (AQ-ME) extract	51.88
Butylated hydroxyl toluene (BHT)	32.58

Table 2 Evaluation of the effect of *MO* leaf extract on AS-AQ intoxicated *Wistar* rats by using biomarkers of liver injury

Group	Treatment code (n = 3)	AST (IU/L)	ALT (IU/L)	TB (mg/dl)
1	PBS only	122.37 ± 13.73	65.18 ± 20.08	0.83 ± 0.67
2	SCG	127.67 ± 25.07	78.95 ± 24.11	3.08 ± 1.30
3	MCG	113.93 ± 5.46*	80.73 ± 21.65	1.69 ± 0.38
4	CD-ASAQ	138.97 ± 13.14	76.44 ± 0.85	2.92 ± 2.06
5	2 × CD-ASAQ	158.8 ± 20.08	85.70 ± 17.55	1.19 ± 0.94
6	4 × CD-ASAQ	220.33 ± 53.97*	90.39 ± 13.62	2.70 ± 0.96
7	S + CD-ASAQ	130.97 ± 16.61	58.01 ± 20.16	1.15 ± 0.57
8	S + 2CD-ASAQ	125.74 ± 45.13	64.47 ± 23.65	2.00 ± 2.07
9	S + 4CD-ASAQ	184.00 ± 23.60	68.00 ± 28.83	2.93 ± 2.32
10	M + CD-ASAQ	138.77 ± 24.33	66.37 ± 13.29	0.53 ± 0.35
11	M + 2CD-ASAQ	171.13 ± 52.88	80.38 ± 21.56	1.61 ± 0.80
12	M + 4CD-ASAQ	140.03 ± 24.45*	76.77 ± 14.61	1.17 ± 0.66

Values are expressed as mean ± SEM (n = 3) significantly different at * $p \leq 0.05$

PBS physiological buffer saline, SCG siliphos[®] control group, MCG moringa control group, CD-ASAQ clinical dose of artesunate-amodiaquine, 2 × CD-ASAQ double the clinical dose of artesunate-amodiaquine, 4 × CD-ASAQ four times the clinical dose of artesunate-amodiaquine, S + CD-ASAQ pre-treatment with Siliphos[®] followed an hour later by treatment with a clinical dose of artesunate-amodiaquine, S + 2* CD-ASAQ pre-treatment with Siliphos[®] followed an hour later by treatment with double the clinical dose of ASAQ, S + 4 × CD-ASAQ pre-treatment with Siliphos[®] followed an hour later by treatment with four times the clinical dose of ASAQ, M + CD-ASAQ pre-treatment with the aqueous-methanol *MO* leaf extract followed an hour later by treatment with the clinical dose of AS-AQ, M + 2 × CD-ASAQ pre-treatment with the aqueous-methanol *MO* leaf extract followed an hour later by treatment with double the clinical dose of ASAQ, M + 4 × CD-ASAQ pre-treatment with the aqueous-methanol *MO* leaf extract followed an hour later by treatment with four times the clinical dose of AS-AQ

Discussion

The hydroxyl free radical is one of the most potent reactive oxygen species in biological systems [20, 21]. It undergoes chemical reactions with many components of biological cells such as amino acids, sugars, lipids and nucleotides which may result in cell damage [20, 21]. Within biological systems, hydroxyl free radicals may be generated from the catalytic degradation of hydrogen peroxide [22]. In the in vitro context, the hydroxyl free radical may be generated by the Fenton reaction which involves the interaction of transition metals such as Iron II (Fe^{2+}) and hydrogen peroxide [23]. From the present study, we established a concentration dependent increase

in the in vitro hydroxyl free radical scavenging effect of *MO* leaf extracts. This observation is in agreement with the work of other authors [21, 22, 24]. Moreover, butylated hydroxy toluene (standard antioxidant) showed better scavenging capacity than both the aqueous and the aqueous-methanolic *MO* leaf extracts. Based on the work of other workers [25–27], butylated hydroxytoluene is a synthetic compound made up of polyphenolics with marked antioxidant activity.

The inhibitory concentration value at 50% (IC_{50}) refers to the concentration of the antioxidant substance sufficient to inhibit free radical activity by 50% [22]. The lower this value, the higher the antioxidant capacity of medicinal

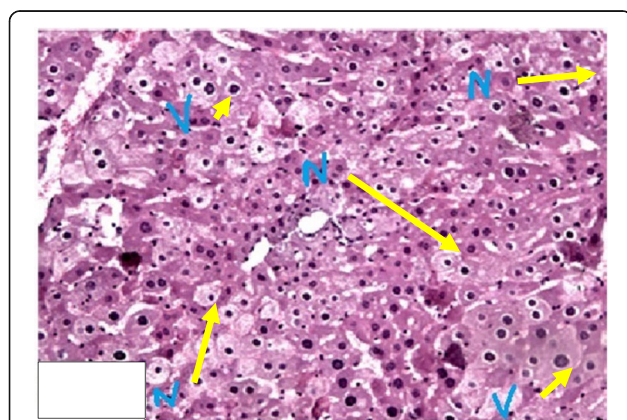


Fig. 3 Photomicrograph of liver section from a rat treated with four times the clinical dose of AS-AQ. (Magnification ×400). V = Hepatic vacuolation, N = Hepatic cell necrosis

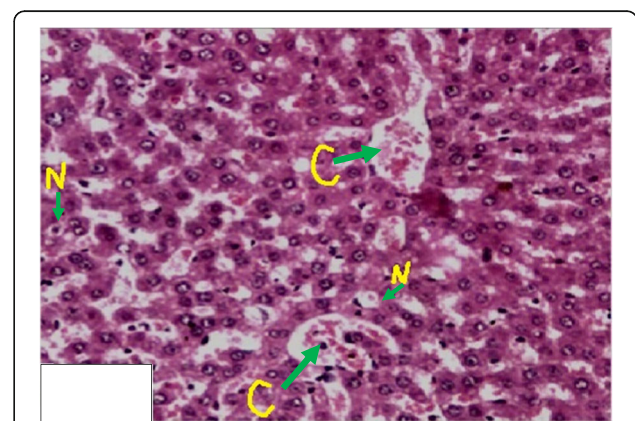


Fig. 4 Photomicrograph of liver section from a rat pre-treated with a 1000 mg/kg dose of aqueous-methanolic *MO* leaf extract and four times the clinical dose of AS-AQ. (Magnification ×400). C = Hepatic vein congestion, N = Hepatic cell necrosis

plants [22, 24]. The aqueous *MO* leaf extract had a lower IC_{50} value than both the aqueous-methanolic *MO* leaf extract and butylated hydroxytoluene. This may imply that the AQ *MO* leaf may have better antioxidant properties than the AQ-ME *MO* leaf extract. However, according to Moon and Shibamoto [28], at least two antioxidant assays should be used to evaluate the antioxidant properties of medicinal plants.

In our previous work [29], we reported the total phenolic, flavonoid and ascorbic acid contents of *MO* leaf extracts. It is on the backdrop of these results that the aqueous-methanolic *MO* leaf extract was selected for evaluation of hepatoprotective activity in *Wistar* rats.

There is a large body of literature available on the hepatoprotective activity of medicinal plants against carbon tetrachloride, ethanol or paracetamol toxicity [30–33]. However, the level of exposure of these chemicals to man may be very low. To the best of our knowledge, this is the first report of the protective effects of the aqueous-methanolic *MO* leaf extract against AS-AQ induced liver injury in *Wistar* rats. This report is based on evidence of biochemical and histologic findings. Similar effects of liver protection were also observed in rats dosed with Siliphos[®], which was used as a positive control.

Siliphos[®] is a complex of silybin and phosphatidylcholine [7]. The former is a flavonoid produced from the fruit of the milk thistle, *Silybum marianum* while the latter is a lipid-soluble derivative of soy. Given that silybin is lipid-incompatible, the combination with phosphatidylcholine facilitates passage of the flavonoid through biological membranes [7]. Some workers have reported on the protective effect of Siliphos[®] against carbon tetrachloride, acetaminophen, alcohol and mushroom poisoning [7, 34–36].

Artesunate-amodiaquine is an antimalarial agent. It comprises of artesunate and amodiaquine. The former is a potent and rapidly acting blood schizonticide while the latter is used in prophylaxis and treatment of malaria [37, 38]. The drug is safe at therapeutic doses but high doses of artesunate causes congestion and dilation of hepatic sinusoids, vacuolation as well as necrosis [37]. A cytotoxic mechanism characterized by protein carbonylation, formation of reactive oxygen species and lipid peroxidation has been associated with the clinical use of amodiaquine [38].

In the present study, liver injury due to AS-AQ intoxication was observed as elevated levels of hepatospecific parameters like AST, ALT and TB. Studies on the histopathology of liver sections from rats treated with high doses of AS-AQ also corroborate findings of biochemical analysis. Hepatocytes carry out a host of metabolic activities under the influence of enzymes [39]. AST and ALT are found in higher concentrations in the cytoplasm. High levels of these enzymes in the serum may be indicative of loss of the functional integrity of

hepatocytes and subsequent leakage of cell contents [40]. Group 6 rats (4 × AS-AQ dose) registered the highest elevation of these enzymes. However, since AST is also present in the kidney and cardiac muscles [40], it may be suggested that AS-AQ may have some toxic effects on these organs as well. Pre-treatment with 1000 mg/kg of the *MO* leaf extract (group 12) significantly lowered the elevated levels of AST. This reduction was comparable to the control (group 3) and the hepatotoxic agent (group 6).

Bilirubin is a principle indicator of cholestatic liver injury [41]. In the present study, AS-AQ intoxicated rats were characterized by high serum bilirubin activity (group 4 and 6). However, the levels were not significantly different from the control group. This observation may suggest that AS-AQ may not be associated with cholestatic liver injury.

Histological examination of the liver sections of AS-AQ intoxicated rats (group 6) showed significant hepatotoxicity characterized by vacuolation, necrosis of hepatocytes and congestion of central veins (Fig. 3). However, animals pre-treated with the aqueous alcoholic extract of *MO* (group 12), the extent of hepatic distortion was decreased relative to the damage observed in the AS-AQ intoxicated group (Fig. 4).

In our previous work on the aqueous methanolic extract of *MO* leaf extract, we identified flavonoids, phenolics and ascorbic acid as phytoconstituents [29]. These secondary plant metabolites have been identified as natural antioxidants [23]. Thus, the observed free radical scavenging and hepatoprotective activities of the aqueous-methanolic *MO* leaf extract may be attributable to the antioxidant phytoconstituents.

Discussions are still ongoing as to what effect is produced when AS-AQ and the *MO* leaf extract are concurrently used in a *Plasmodium falciparum* infected animal model. Some published works [42, 43] have reported on the effectiveness of *MO* seeds on *Plasmodium falciparum* and *Schistosoma cercariae*. Nonetheless, similar activity is yet to be reported on the leaves. Other authors have suggested that antioxidants and supplements of herbal origin (grape fruit juice, orange fruit juice, ascorbic acid) may alter the efficacy of antimalarial drugs in clearing parasitaemia [44–46].

Conclusion

Based on our preliminary investigations it can be concluded that the aqueous-methanol *Moringa oleifera* leaf extract exhibits free radical scavenging and hepatoprotective properties. However, in a bid to develop a potent medicinal agent, further investigations on the structural identity of the phytoconstituents as well as the mechanism behind the observed effects should be performed.

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Authors' contributions

MOO, AWK, POO participated in the conduction of experiments. FOO, JMM, LWK and DWG made substantial contributions to concept design and conduction of research. Data analysis and interpretation was done by MOO and POO. MOO, FOO, JMM and DWG participated in drafting the manuscript and LWK, SGK, POO, DWG revised the manuscript critically for intellectual content. MOO and AWK made the necessary corrections in the write up and JMM, DWG and SGK gave final approval for the submission of revised version. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

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