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Medemia argun seed extract ameliorates the lipopolysaccharide-induced acute inflammation

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ABSTRACT

Medemia argun (MA) is a relatively unknown species of fan palm that has been reported to possess antioxidant properties. This study explored the effect of *Medemia argun* seed extract on mice with lipopolysaccharide (LPS)-induced sepsis, using albino mice as the experimental model. The animals were divided into three groups of five. One group received alpha-lipoic acid (ALA) as a standard anti-septic drug, another group was treated with MA seed extract in five consecutive doses administered every other day, and the final group served as the positive control. As expected, LPS exposure significantly elevated serum inflammatory markers. However, treatment with MA seed extract or ALA resulted in a marked reduction in serum pro-inflammatory cytokines (TNF-alpha, IL1B, and IL6) and improved liver function parameters (AST, ALP, and TB) compared to the LPS-positive control group. Although ALT levels increased noticeably in the LPS-only group, they showed a modest reduction in the treated groups. Additionally, MA seed extract protected the mice against elevated serum nitric oxide levels and liver DNA damage, as indicated by the comet assay. Histological analysis also revealed significant improvement in liver tissue inflammation in the MA-treated animals compared to the LPS-only group. Collectively, these findings demonstrate the hepatic protective and anti-inflammatory effects of MA extract in this experimental model.

Keywords: Sepsis, Alpha lipolic acid, Antioxidants, Comet

1. INTRODUCTION

Sepsis is a life-threatening organ dysfunction. It can be caused by a dysregulated host response to lipopolysaccharide (LPS) infection [1,2]. Sepsis is considered to be amajor cause of death worldwide [3].

Anyone can be affected by sepsis but some people such as elderly people, neonates, pregnant women, hospitalized patients, and people with autoimmune diseases, cancer are at higher risk [4]. It was demonstrated that LPS is one of the most important causes of sepsis [5]**.** LPS is a normal component of most of Gram-negative bacteria that can enhance inflammatory process. Toll-like receptors (TLRs) are the most important pattern-recognition receptors; TLR4 is the major receptor for LPS [6] that is activated after binding of LPS leading to downstream signaling cascades and expression of inflammatory cytokines. Overwhelming LPS infection can induce sepsis due to the resulted over-expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF-alpha), interleukin-1b (IL-1B) and interleukine-6 (IL-6). LPS also can induce over-expression of reactive nitrogen species (RNS), such as nitric oxide (NO) [7,8**]**. NO exerts anti-microbial and inflammatory effects [9], but over-expression of this radical causes damage to various tissues and DNA damage [10**,**11]**.** Antioxidants have been suggested to reduce mortality and prevent the damage of organs of the animal models in the LPS-induced endotoxinomia **[**8,12]. Treatment with antioxidants as a support in the standard management of patients with sepsis was suggested [13,14]. Antioxidants are molecules which reduce oxidation that may lead to the production of highly reactive species and free radicals that can cause damage to cell structures [15]. Oxidative stress resulted during sepsis can trigger DNA damage through strand breaks as well as base damage and also cellular signaling disruption can be occurred by some ROS that may act as cellular messenger. Protection of the cell against oxidative stress may be through chelation of trace metals related to free radical generation or through the actions of antioxidants [16]. Alpha-lipoic acid (ALA) is a naturally occurring derivative of octanoic acid. It serves as a potent antioxidant and has been reported to have anti-inflammatory effects [17]. ALA has a preventive and a therapeutic effect on severe endotoxemia in rats [18]. It inhibits LPS-induced inflammatory gene expression in mouse tissues [19]. ALA also attenuates hypothermia induced by LPS through decreasing excessive NO production during sepsis [20]. *Medemia argun* (MA) is a little known species of fan palm from the Nubian Desert Oases of Northern Sudan and Southern Egypt [21]**.** It was exhibited that the proanthocyanidins (PAC) fraction from MA nut can be considered as a protecting factor against oxidative/nitrative stress that can cause different diseases such as cancer, neurodegenerative and cardiovascular diseases). This importance MA nut is due to its content of proanthocyanidins, phenolic acids and flavonoids. PACs of MA nuts may be promising antioxidants [22].

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

LPS (from Escherichia coli o111:4) was purchased from Sigma Aldrich, USA. It was dissolved in PBS as 5mg/ml for stock solution. ALA was purchased from EVA pharma TNFα (tumor necrosis factor alpha) ELISA kit, catalog NO: E-EL-M0049, Interleukin 6 ELISA kit, catalog NO: E-EL-M0037, and Interleukin1B ELISA kit, catalog NO: E-EL-M0037, were purchased from Elabscience, USA. Nitric oxide assay (Colorimetric Determination of Nitrate), catalog NO: 25-33, was purchases from BIODIAGNOSTIC. Total bilirubin, SPINREACT, Alkaline phosphatase ALP (IFCC), AST, ALT/GPT-kinetic Bio Med-GPT were purchased from BIOMED DIAGNOSTICS

2.2. Preparation of *M. argun* seed extract

Extract was prepared using the method of [23]. Medemia ripe dried fruits were collected from palms grown in Aswan University desert garden. The fruits were crushed to take out the embryos and the brown colored endocarp layer surrounding the embryo was scratched and used for the extraction process. The endocarp materials were powdered and exhaustively extracted with ethanol at room temperature until

clearness. The extract was concentrated in vacuo giving a crude residue left at air for dryness. Two grams crude extract were chromatographed on C300 silica gel column chromatography (3 cm × 60 cm). The column was performed using a gradient solvent system starting with CH2Cl2, CH2Cl2:MeOH (9:1~1:9), MeOH, and MeOH:H2O (9.5:0.5–9:1) as eluents to give 71 sub-fractions. *M*.A´s seeds extract was dissolved in ethanol as 0.1g/ml for stock solution.

2.3. Animals and experimental design

Twenty male albino mice (*Mus musculus*), weighing 25±5g were purchased from **Misr University for Science & Technology** and were acclimated to the lab for a week before the experiment. Animals were categorized into four groups (n=5) including normal control, positive control (LPS) (4mg/kg) [20, 24], MA extract (70mg/kg) [25] and ALA (40mg/kg) [20]; injection was intraperitoneally. In MA group, animals received five separate doses which are considered as small therapeutic doses of the extract (three doses per week) and then injected with a single dose of LPS after the last dose of the extract. In the ALA group, animals received only a single dose, 30 min before, LPS injection. Animals in all groups, excluding the negative control group, received the single dose of LPS then sacrificed after eight hours from LPS administration.

2.4. Biochemical analysis

Blood was let to clot at room temperature for about 15 minutes followed by centrifugation at 4000 rpm for 10 minutes in the cooling centrifuge (SIGMA 1-14K, item no. 10020). Serum was collected and kept at -20°C for measuring some of sepsis-specific physiological parameters. TNFα interleukin 1B were determined by ELISA kit (see materials and methods) according to manufacturer recommendation. However, nitric oxide, bilirubin and alkaline phosphatase, ALP, AST and ALT were measured colorimetrically (see materials and methods) according to manufacturer recommendation.

2.5. Histological section preparation

After scarification, liver tissue samples were collected and immediately washed by cold PBS, then fixed by 10% formaldehyde and preparation of paraffin-embedded blocks was according to the standard protocol of [26]. Tissues were sliced (5um) and stained with hematoxylin and eosin according to [27]. All images were obtained using calibrated standard digital microscope camera (Tucsen ISH1000 digital microscope camera) using Olympus® CX21 microscope, with resolution of 10MP (megapixels) (3656 x 2740 pixel each image). Images were processed using "IS Capture" software for capture and image enhancements. All slides captured at original magnification 400x (objective 40x), UIS optical system (Universal Infinity System, Olympus®, Japan).

2.6. Comet assay

The method of Comet test was according to [28], homogenization of liver tissue in 1 ml cooled phosphate-buffered saline (PBS) was performed then filtration occurred. A mixture of cell suspension (100 μL) and low-melting agarose (600 μL) was prepared and spread onto pre-coated slides. 2.5% sodium dodecyl sulfate (SDS) in 0.045 M Tris-borate-EDTA, pH 8.4 was used as lysis buffer in which slides

were immersed for 15 minutes followed by electrophoresis in TBE buffer at a constant voltage of 2 V/cm for 2 minutes, then a constant current of 100 mA. Ethidium bromide (20μg/ml at 4°C) was used to stain slides. For each dose level, the migration patterns of DNA fragments of 100 cells were assessed by the fluorescence microscope with excitation filter of 420-490nm [issue 510nm]). The lengths of comets tails were measured from the center of the nucleus to the tail end. Observation and imaging were performed by the fluorescent microscope (40X objective). A comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) connected to a CCD camera was used for assessment of DNA damage in the cells. DNA damage is assessed by measuring the length of DNA migration and the DNA migration percentage. Finally, tail moment was calculated by the program. Generally, 50 to 100 randomly selected cells are analyzed for each sample.

2.7. Statistical analysis

Results were expressed as means ± standard errors (SE). Statistical Package for Social Science (SPSS) version 16 was used for data analysis to assess any significant differences among the groups. The effects of *M. argun* seed extract (MA) and alpha lipolic acid (ALA) were analyzed using the test of one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare means with control. Differences between groups were considered to be statistically significant when $P < 0.05$. Data appeared as mean ±SE in all experiments.

Ethical approval

This study was confirmed by the Scientific Research Ethics Committee No. 18 (18 November 2024) at the faculty of Science, Port Said University (ERN: PSU. Sci. 80).

3. RESULTS

3.1. Levels of pro-inflammatory cytokines

To evaluate the anti-inflammatory effects of MA seed extract, we utilized an LPS-induced sepsis model in experimental mice (refer to Methods). As anticipated, LPS administration with the tested dose resulted in markedly elevated serum levels of pro-inflammatory markers, including TNF-alpha, IL-2B, and IL-6, compared to the untreated control group. Notably, pretreatment with either MA extract or ALA significantly reduced these elevated pro-inflammatory markers. For instance, serum TNF-alpha levels in LPS-treated septic animals were significantly elevated (69.26 ± 2.24 pg/ml) compared to the untreated control group (26.38 ± 2.11 pg/ml). However, pretreatment with MA extract (33.87 ± 3.56 pg/ml) or ALA (33.55 ± 2.59 pg/ml) led to a substantial reduction in TNF-alpha levels ($p < 0.001$), as illustrated in Figure 1A.

Similarly, other inflammatory markers, such as IL-1B and IL-6, were significantly elevated in the sera of the LPS-treated group (127.47 ± 5.63 pg/ml and 48.93 ± 3.02 pg/ml, respectively) compared to the untreated control group (30.17 ± 3.0 pg/ml and 19.20 ± 0.60 pg/ml, $p < 0.001$). Notably, pretreatment with MA extract resulted in a marked reduction in the levels of both markers (36.17 ± 1.10 pg/ml and

27.95 ± 1.25 pg/ml, respectively). A comparable effect was observed with ALA pretreatment, which also significantly reduced the levels of IL-1B and IL-6 (34.88 ± 2.99 pg/ml and 28.84 ± 2.48 pg/ml, respectively) when compared to the LPS-treated group (127.47 ± 5.63 pg/ml and 48.93 ± 3.02 pg/ml, $p < 0.001$). (Fig. 1A, C).

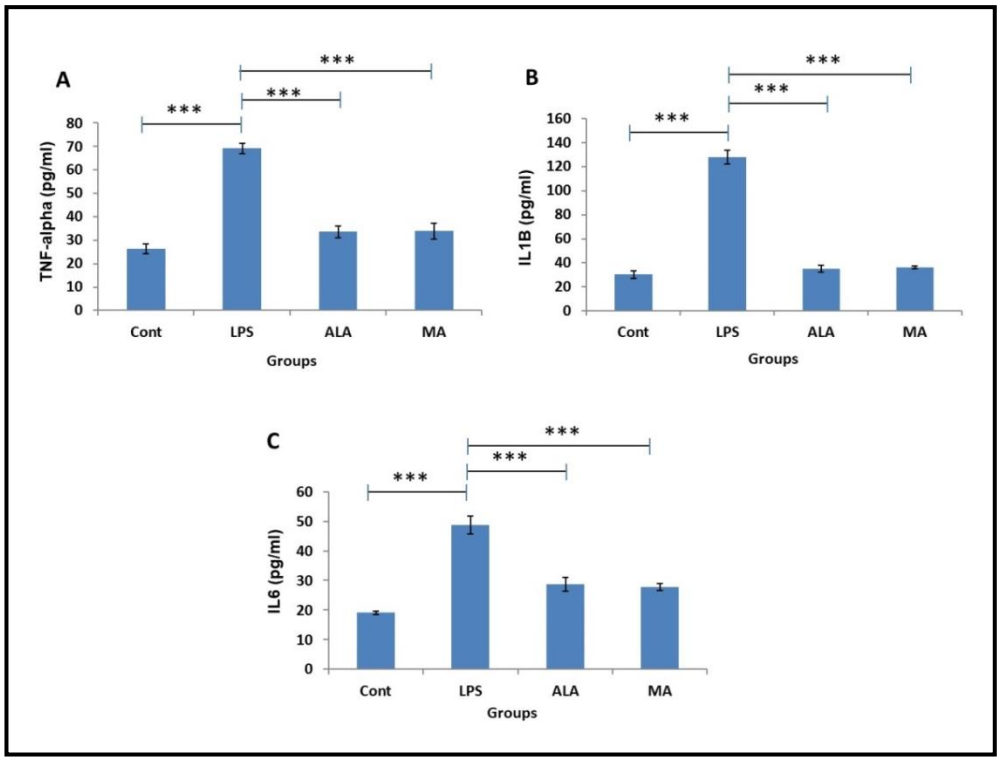

Figure 1. Effect of MA and ALA on the level of pro-inflammatory cytokines in serum of LPS-induced septic mice; tumor necrosis factor alpha (TNF-alpha) **(A)**, interleukin 1B (IL1B) **(B)** and interleukin 6 (IL6) **(C);** in LPS-induced septic mice. Three asterisk showed a highly significant difference (***p<0.001) in normal control group, MA-protected group and ALA-protected group compared to septic group using one way ANOVA statistical analysis test.

3.2. Levels of liver parameters

To study the impact of MA extract on the liver function, liver parameters (ALT, ALP, AST and TB), were measured in the animal sera from different groups. Although LPS with the tested dose enhances ALT levels in the septic groups (46.70±0.45 U/l), which was significantly observable compared with non-treated animals (37.0±1.17 U/l), neither MA nor ALA showed appreciated changes when pre-administered to animals **(Fig.2A).** However, the enhanced levels of serum AST, ALP and TB in the septic groups (27.73±0.91, 85.66±3.31 U/l and 2.60±0.10 mg/dl, respectively) when each is compared with the corresponding control. These values are significantly improved in MA-pretreated group (18.06±0.92 U/l, 53.33±3.31 U/l and 2.18±0.09 mg/dl, respectively) p<0.001**,** which showed values very close to non-treated control **(Fig.2B, C, D).**

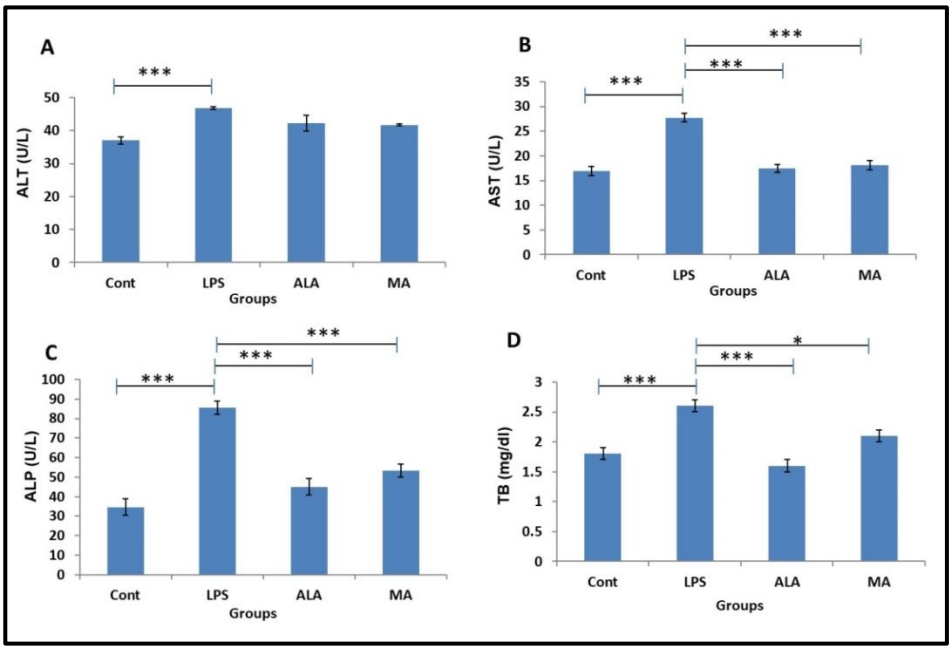

Figure 2. Effect of MA and ALA on the liver function in serum of LPS-induced septic mice. (A) indicates levels of ALT enzyme (Alanine aminotransferase), **(B)** indicates levels of AST enzyme (Aspartate transaminase) and **(C)** indicates level of total bilirubin (TB). Serum ALT level showed highly significant differences in normal control group compared to septic group (***$p<0.001$) while there was non-significant decrease in serum ALP of MA-protected group and ALA-protected group compared to septic group ($p>0.05$). Levels of ALP and AST in serum of normal control, MA-protected group and ALA-protected group were significantly decreased compared to septic group (***$p<0.001$). TB level showed a highly significant difference between both LPS and normal control group and LPS and ALA group; TB level also showed a significant decrease between LPS and MA-protected group (*$p<0.05$). One way ANOVA statistical analysis test was used.

3.3. Levels of nitric oxide

As an indicator for oxidative stress, nitric oxide was measured in the different groups' sera. Average level of serum nitric oxide was very high under LPS treatment (septic group; 109.33±1.17 μM/ml) when compared with non-treated (62.66±3.45 μM/ml; $p<0.001$). These NO values were significantly decreased in MA-protected group (65.00±3.84 μM/ml) as well as ALA-protected group (74.66±2.59 μM/ml) compared to septic group (109.33±1.17 μM/ml), $p<0.001$ **(Fig.3).**

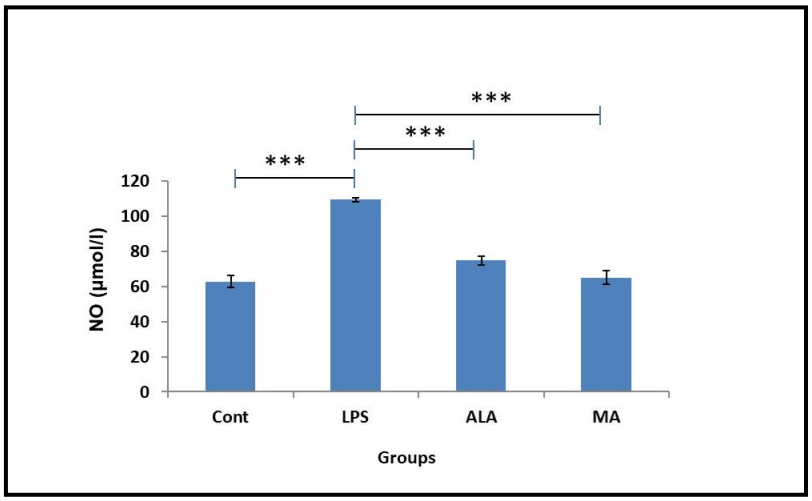

Figure 3. Effect of MA and ALA on NO (Nitric Oxide) level in serum of LPS-induced septic mice. A highly significant differences was indicated in normal control group, MA-protected group and ALA-protected group compared to septic group (***p<0.001).

3.4. Comet assay

Additionally, the comet assay was employed to evaluate the ability of MA extract to mitigate LPS-induced genotoxic stress in liver tissues. The assay revealed a significant increase in DNA damage in the LPS-only treated group (5.38 ± 0.26) compared to the untreated control group (91.50 ± 0.11, $p < 0.001$). Notably, pretreatment with MA extract (2.32 ± 0.45) or ALA (2.66 ± 0.02) significantly ($p < 0.001$) reduced the comet tail moment, bringing it closer to normal levels. This demonstrates a substantial reduction in LPS-induced DNA damage (Fig. 4).

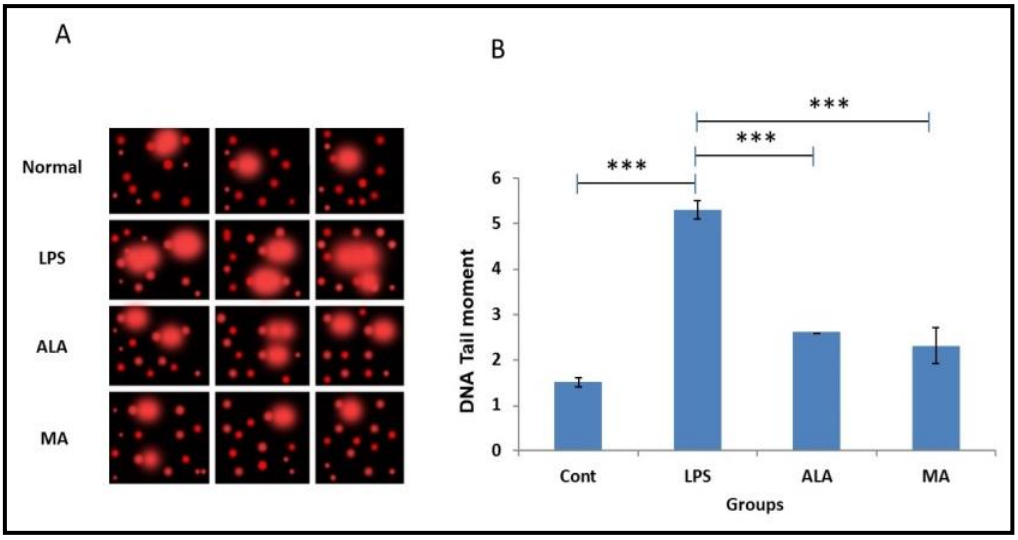

Figure 4. (A) DNA in the liver cells of the study groups using comet assay. MA and ALA inhibited the DNA damage caused by LPS administration. **(B)** Highly significant differences in DNA tail moment of normal control group, MA-protected group and ALA-protected group compared to septic group (***p<0.001).

Table1. Effect of MA and ALA administration on the studied parameters in LPS induced septic mice.

	TNF-alpha	IL1B	IL6	ALT	AST	ALP	TB	NO	DNA Tail moment
control	26.38±2.11	30.17±3.0	19.20±0.60	37.0±1.17	16.90±0.99	34.66±4.35	1.85±0.09	62.66±3.45	1.50±0.11
LPS	69.26±2.24*	127.47±5.63*	48.93±3.02*	46.70±0.45*	27.73±0.91*	85.66±3.31*	2.60±0.10*	109.33±1.17*	5.38±0.26*
ALA	33.55±2.59	34.88±2.99	28.84±2.48	42.10±2.43	17.53±0.81	45.0±4.11	1.96±0.10	74.66±2.59*	2.66±0.02
MA	33.87±3.56	36.17±1.10	27.95±1.25*	41.66±0.37	18.06±0.92	53.33±3.31*	2.18±0.09	65.00±3.84	2.32±0.45

Values are expressed as mean ± SE (n = 5/group)

*Significant differences between normal control and LPS, ALA and MA administrated groups using one way ANOVA statistical analysis test.

3.5. Histopathological examination

Images were illustrated in **fig.5** and showed that liver tissue of the normal control group (5A) showed preserved hepatic architecture. Hepatocytes (black arrow) are arranged in thin plate separated by thin blood sinusoids (arrowhead), each cell showed eosinophilic homogenous cytoplasm and central rounded vesicular nucleus. Central veins patent and thin walled. (5B) Liver tissue of LPS group showed focally disrupted architecture. Hepatocytes showed moderate ballooning degeneration (vacuolar degeneration of the cytoplasm; black arrow). There is inflammatory infiltrate (dashed arrow) formed mainly of lymphocytes, located within hepatic lobules or in the portal areas with perivascular condensation. There is a focal marked vascular congestion (arrowhead). (6C) MA-pretreated group showed residual moderate degenerative changes in the form of hydropic degeneration of hepatocytes (arrow) and moderate congestion and dilatation of vessels. There is marked improvement of inflammatory infiltrate. There is only residual minimal focal infiltration by small lymphocytes (dashed arrow). (5D) ALA group showed marked improvement of pathological changes with residual focal mild degenerative changes in the form of hydropic degeneration of hepatocytes (arrow) and mild congestion and dilatation of vessels (arrow head). There is no inflammatory infiltrate; dashed arrow showed periportal areas with no infiltration by inflammatory cells).

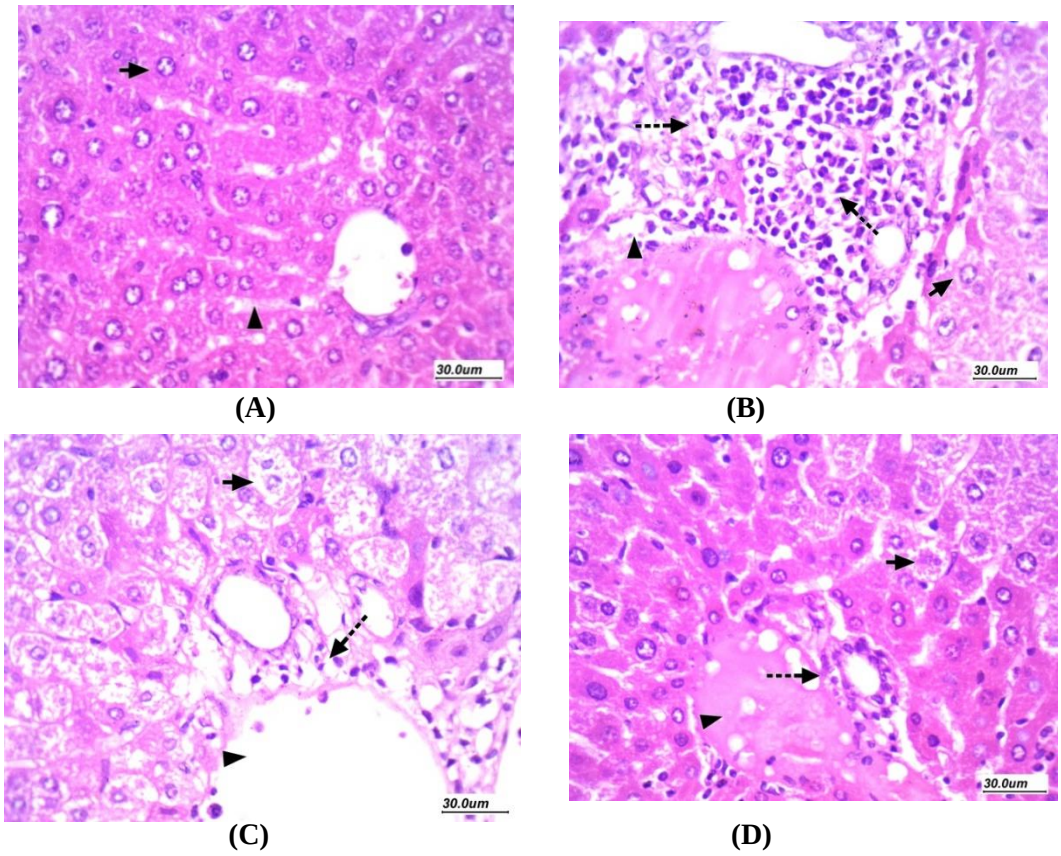

Figure 5. T.S sections of experimental animal's liver tissue (A) liver tissue of the normal control group. (B) Liver tissue of LPS group. (C) MA group. (D) ALA group.

4. DISCUSSION

LPS is a TLR-4 ligand. It firstly binds Lipid Binding Protein (LBP) in the serum which in turn binds to CD14 expressed by macrophages, dendritic cells and other cell types, and finally the complex interacts with TLR4/MD-2 [29]. Subsequent intracellular signals transduction then occurred causing release of pro-inflammatory cytokines such as the cytokines TNF-α, IL-1B and IL-6 **[**30**].** The elevation of pro-inflammatory cytokines causes liver injury and can disrupt liver function [31,32]. LPS also induces excessive production of nitric oxide [33]**.** Management of the overwhelming inflammatory response is an important issue for sepsis treatment**.** Previous studies have indicated that, treatment with anti-inflammatory and antioxidant agents is beneficial in protection against LPS-induced hepatic injury [34,35].

In this study, LPS induced a very good model of sepsis in experimental animals as expected. This model showed detectable elevation in serum inflammatory markers including TNF-alpha, IL1B and IL6. Our results indicate that *M. argun* seed extract exhibit protective effect of similar to alpha lipolic acid against LPS-induced liver injury by decreasing the levels of pro-inflammatory cytokines (TNF-alpha, IL1B and IL6) in serum significantly compared to their levels in LPS positive control group. The liver function results revealed that the serum ALP, AST and TB were also significantly decreased compared to

LPS control group. The improvement of the Pro-inflammatory cytokines, ALP, AST and TB values were close to those of the non-treated controls which showed non-significant difference.

Comet assay is used to evaluate double and single strand DNA damage. Here, our data revealed that the DNA damage by LPS was inhibited by pretreatment by MA extract as indicated by the comet tail measurements. These data may indicate that MA not only inhibit the pro-inflammatory marker but also inhibits the subsequent genotoxic effect expected in the liver. Interestingly, these observations were in parallel with those from histopathological evaluation of the hepatic tissues from different groups in this study as the liver tissues clearly showed improved architecture almost like ALA. This protective effect may be due to their content of proanthocyanidins, phenolic acids and flavonoids as anti-oxidants [22].

The fact that natural antioxidant can show brolyphractic effect against toxins was reported **[**36**]**. One of such markers to assess the oxidative stress release is NO [37]. Our results show that there was a significant decrease in serum NO of MA-pretreated animals as well as ALA-pretreated ones. This also may indicate that MA may induce its effect through antioxidant compounds. It was reported that MA seeds may have Proanthocyanidin which proved to induce antioxidant against different oxidative stress inducers [38].

Taken together, our data show that MA seed extract fraction may contain antioxidant enough to protect from LPS. This protection includes inhibition of the pro-inflammatory cytokines and liver DNA damage and subsequently improves liver function. To our knowledge, the present study could be the first study revealing the protective effect of *M. argun* seed extract against LPS-induced septic mice.

5. CONCLUSION

M. argun seed extract has a similar protective effect of alpha lipolic acid in the LPS-induced septic features in mice including liver DNA degradation, biochemical inflamatory markers and histological changes and finally hepatic function. This may be through suppression of the systemic inflammation caused by LPS due to the plant antioxidant capacity. Further studies are still needed to explain the molecular mechanism behin such protective effect of *Medemia argun* seed's extract on sepsis.

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