



Antioxidant Activity of *Annona Squamosa* (L.) and *Annona Muricata* (L.) Fruit Pulp Extracts against *Leishmania major* Induced Oxidative Stress in BALB/c Mice Model

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ABSTRACT

Animals remain the best models for the characterization of any disease and its impact on the host. The BALB/c mice model provides a unique opportunity to study Cutaneous Leishmaniasis (CL) in human in its active form due to its susceptibility to *L. major* infection, developing clinical and pathological features of CL similar to those found in human. During leishmaniasis progression, there is damage of internal organs such as the liver and spleen due to high parasite burden or metabolic processes produced by the host. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated upon macrophages exposure to *Leishmania* leading to the regulation of the inflammatory response controlled by the cellular antioxidant defense system. The ripe fresh fruits of *A. muricata* and *A. squamosa* were collected. The pulp, peel and seeds from the fruits were dried and grinded into fine powder. Aqueous and organic extraction of phytochemicals was undertaken and the extracts used to treat the BALB/c mice. The *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts showed marked antioxidative activities against CL accompanied with DNA protective effects against H₂O₂-induced toxicity both *in vitro* and *in vivo*. Methanol and aqueous extracts of the fruits were evaluated for *in vivo* antioxidant activities using both non-enzymatic and enzymatic antioxidants and organ protection ability using serum. The findings of this study showed that these extracts possess antioxidant potential in BALB/c mice. Extracts

strengthened both enzymatic and non-enzymatic antioxidants to prevent the extent of lipid peroxidation and normalized level of both cardiac and hepatic markers in serum.

Keywords: Antioxidants, Induced Oxidative stress, Leishmaniasis, Reactive Oxygen Species

INTRODUCTION

Animals remain the best model for the characterization of any disease and its impact on the host. Hamster and mouse are the two well-studied and suitable models for studying the infection and chemotherapy while monkey model is used for vaccine trials (Awasthi *et al.*, 2004). The BALB/c mice model provides a unique opportunity to study Cutaneous Leishmaniasis (CL) in human in its active form due to its susceptibility to *L. major* infection, developing clinical and pathological features of CL similar to those found in human (Awasthi *et al.*, 2004). However, different strains of mice show variability in their susceptibility to *Leishmania* parasite (Bradley & Kirkley, 1977). BALB/c mice have been shown to be highly susceptible to *L. major*, showing signs of slow breeding, are small and delicate to breed (Bradley & Kirkley, 1977). The Swiss Albino mice on the other hand are easy to breed. Both have the same appearance and are quite resilient but are known to show resistance to *L. major* (Santos *et al.*, 2003; Santos *et al.*, 2008). Although infection in BALB/c mice is a well-studied susceptible host model, it is not suitable for trial of chemotherapeutic purpose due to much higher effective dose required to cure *Leishmania* infection compared to humans (Croft & Yardley, 2002; Awasthi *et al.*, 2004). However, it can form the basis of selecting higher animal models. The matching physiology with human and availability, BALB/c mice had been chosen as a model in this study.

During leishmaniasis progression, there is damage of internal organs such as the liver and spleen due to high parasite burden (Makwali *et al.*, 2012; Jarallah, 2015; Jarallah, 2016) or increased secondary metabolic processes produced by the host (Oliveira & Cecchini, 2000; Oliveira *et al.*, 2011b). Damage of DNA and nitric oxide production has been reported in mice following infection with *L. chagasi* (Oliveira *et al.*, 2011b; Inacio *et al.*, 2014). Besides, adverse effects of leishmania chemotherapy such as Amphotericin B (Am B) interacting with both parasite and host cell membrane induce lipid peroxidation (LPO) of the plasma membrane (Roy *et al.*, 2012; Fernandes *et al.*, 2013; Alkathiri *et al.*, 2017) complicating further the management of the disease. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated upon macrophages exposure to *Leishmania* leading to the regulation of the inflammatory response controlled by the cellular antioxidant defense system (Inacio *et al.*, 2014; Paiva & Bozza, 2014; Alkathiri *et al.*, 2017).

Internal and external pathological factors such as viral, bacterial, and parasitic infections disrupt the body's oxidant/antioxidant balance initiating oxidative stress mechanisms such as oxidation of lipids, proteins, and nucleic acids (Eissa *et al.*, 2012; Jafari *et al.*, 2014; Aguiar *et al.*, 2010). Accumulation of ROS and RNS in cells, damage membrane lipids if not prevented by an appropriate antioxidant scavenging system (Alkathiri *et al.*, 2017). However, good antioxidant defense system is closely associated with good Protein Energy (PE) or Protein calories (PC) nutrition. Although endogenous antioxidants are available to reduce ROS and RNS accumulation, exogenous antioxidants obtained from appropriate diet can also play a crucial role (Dkhil *et al.*, 2016). The exogenous and endogenous antioxidant defense systems act in

coordination, with their levels being regulated by each other, to avoid oxidative stress (Dkhil *et al.*, 2013; Dkhil *et al.*, 2016). Based on these facts, it is important to carry out studies to investigate the potential antioxidant property of *A. Squamosa* and *A. Muricata* fruit pulp against *L. major* induced oxidative stress in BALB/c mice model to provide insights on the pathogenesis of the disease.

MATERIALS AND METHODS

Collection of Plant Materials

Ripe fresh fruits of *A. muricata* and *A. squamosa* were collected between March and September 2014 from farms in Kilifi and Kwale Counties at the coastal Kenya. The National Museum of Kenya, Nairobi, identified the species where voucher numbers for *A. muricata* and *A. squamosa* were deposited. The harvested fruits were washed with chlorinated water to retard aging and remove fungi and bacteria. The pulp, peel and seeds from the fruits were separated and then dried at 40°C and 95% relative humidity using a constant temperature and humidity chamber (Tokyo Thermo Tech Co. Ltd, Japan). The dry pulp, peel and seeds were then grinded separately into fine powder using a grinding machine (Mitamura Riken, Kogyo Inc. Tokyo, Japan). The ground fruit parts in form of powder were weighed using a top-loading balance, transferred into polythene bags, sealed, and stored at 4°C.

Extraction of Phytochemicals

Aqueous extraction was done by macerating 50g of *A. muricata* and *A. squamosa* fruits powder in 100 mL sterile distilled water in a Warring blender for 10 minutes. The macerate was then filtered through double-layered muslin cloth and centrifuged at 4000g for 30 minutes then the supernatant was filtered through Whatman No.1 filter paper. The extracts were then preserved aseptically in sterile airtight bottle at 4°C for later use (Biba *et al.*, 2013). The organic solvent extraction was carried out using 50g of powdered pulp from *A. muricata* and *A. squamosa* fruits sequentially using 100 mL of solvents of increasing polarity starting with n-hexane followed by ethyl acetate and finally Methanol (MeOH) for 48 hours each with occasional swirling to ensure thorough extraction. The extracts were decanted and filtered through Whatman filter paper and the macerate steeped in solvents (n-hexane, ethyl acetate and MeOH) again for 48 hrs. Extraction process was repeated twice and the filtrates combined and concentrated on a rotary vacuum evaporator (Bibby Sterilin Ltd, RE 100B, UK) under reduced pressure at a temperature of 50°C, packed and stored in an airtight bottle at 4°C for later use (Biba *et al.*, 2013) as described and abbreviated in Table 1.

Table 1: Abbreviations of different crude extracts of *A. muricata* and *A. squamosa* pulp, peel and seeds

Extract Abbreviation	Full name of the extract
ASPUAE	<i>Annona squamosa</i> pulp aqueous extract
ASPUME	<i>Annona squamosa</i> pulp methanol extract
AMPUAE	<i>Annona muricata</i> pulp aqueous extract
AMPUME	<i>Annona muricata</i> pulp methanol extract

Animals

BALB/c mice (3 - 4 weeks old) obtained from Animal House Unit, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya were used. BALB/c mice were maintained with standard rat pellets (Rate pellets®, Unga Feeds Ltd, Kenya) and water ad libitum. They were then kept under

standard temperature 26°C and 60% humidity and a natural light-darkness cycle throughout the experimental period.

Parasites

Metacyclic promastigotes of *L. major* (IDUB/KE/83=NLB-144 strain) maintained by cryopreservation and *in vitro* culture periodically passaged in BALB/c mice at KEMRI were used. Parasites were maintained as described by Titus *et al.* (1994). Briefly, *L. major* parasites were cultured in Schneider's insect medium supplemented with 20% Fetal Bovine Serum (FBS), glutamine (2 mM), Penicillin G (100 U/mL) and streptomycin (100 µg/mL). Stationary phase amastigotes were harvested in 7 days old cultures. Metacyclic promastigotes were isolated from stationary phase cultures by negative selection using peanut agglutination (Tonui & Titus, 2006).

Experimental Design

The BALB/c mice were divided into seven groups each group consisting of six mice as follows; Group I: Negative control non infected (NI); Group II: Positive control infected non treated (INT) (50 mg/Kg bwt DMSO); Group III: 20 mg/Kg bwt Pentostam, Group IV: 50 mg/Kg bwt ASPUAE/AMPUAE, Group V: 50 mg/Kg bwt of ASPUME/AMPUME, Group VII: 50 mg/Kg bwt ASPUAE/AMPUAE + 20 mg/Kg bwt pentostam, Group VII: 50 mg/Kg bwt ASPUME/AMPUME + 20 mg/Kg bwt pentostam. After the infected animals had developed lesions, the respective groups received oral treatments of the extracts, Pentostam and a combination of the extracts and pentostam for a period of 3 to 4 weeks.

After the experimental period, all mice were decapitated and sacrificed, whole blood and serum collected for assays of markers of hepatic damage; Aspartate amino transferase (AST), Alanine amino transferase (ALT), Lactate dehydrogenase (LDH), Creatine Kinase (CK); non enzymatic antioxidants; lipid peroxidation (LPO), Thiobarbituric acid reactive substances (TBARS) and nitric oxide (NO), enzymatic antioxidants Glutathione (GSH), Glutathione-s-transferase (GST), Glutathione peroxidase (GSHPx), Superoxide dismutase (SOD) and Catalase (CAT) using an automated Retroflon® Plus automated analyzer as described by Wheeler *et al.*, (1990). The sizes of Left Footpads (LHFD) swellings were measured using a direct reading using a Vernier caliper whereas the lesion sizes were estimated using the method of Nolan and Farrell, (1987). Liver and spleen impression smears were used to quantitate the parasite loads as described by Bradley and Kirkley, (1977).

Ethical Consideration

All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The KEMRI's Animal Care and Use Committee (ACUC) and Scientific and Ethics Review Unit (SERU) approved all experimental protocol before using them. The ethical issues in animal studies, stress or suffering subjected to the animals through infection with *L. major* and killing of the animals was addressed by treating the animals with drug/extracts and anaesthetizing them using pentobarbitone sodium (Sagatal®) respectively. For invasive procedure, mice were anaesthetized with pentobarbitone sodium (Sagatal®) and those animals that died as results of experimentation were placed in biohazard bags and stored at -20°C until incineration.

Data Analysis

The different data were analyzed using mean separation by GenStat discovery 14th Edition (Pyne *et al.*, 2011) through Fischer least significance difference. Comparisons of multiple means was done by Analysis of variance (ANOVA) followed by Duncan's test, $P < 0.05$ considered statistically significant. The results were expressed as mean $\pm$ standard error of the mean.

RESULTS

In Vitro Antioxidant Activities

Lipid Peroxidation (LPO) Inhibition Activity:

The LPO inhibition activity of aqueous extracts (ASPUAE and AMPUAE) and methanol extracts (ASPUME and AMPUME) is presented in Table 2. The methanol extracts (ASPUME and AMPUME) registered higher LPO percent inhibition at $71.93 \pm 0.22\%$ and $78.20 \pm 0.11\%$ respectively at $500 \mu\text{g/mL}$ compared to the percentage inhibition of aqueous extracts (ASPUAE and AMPUAE) at $55.27 \pm 0.34\%$ and $60.49 \pm 0.43\%$ respectively. In all ranges of concentrations ($15 \mu\text{g/mL}$ to $500 \mu\text{g/mL}$), the LPO percent inhibition of methanol extracts (ASPUME and AMPUME) were significantly higher ($P < 0.05$) compared to that of aqueous extracts (ASPUAE and AMPUAE). The IC_{50} ranged from $433.50 \pm 7.12 \mu\text{g/mL}$ (AMPUAE) to $475.33 \pm 1.50 \mu\text{g/mL}$ (ASPUAE). The slightly lower IC_{50} value of methanol extracts; $433.50 \pm 7.12 \mu\text{g/mL}$ (AMPUME) and $448.39 \pm 11.35 \mu\text{g/mL}$ (ASPUME) in comparison with the aqueous extracts; $465.00 \pm 3.33 \mu\text{g/mL}$ (AMPUAE) and $475.33 \pm 1.50 \mu\text{g/mL}$ (ASPUAE) is an indication that the methanol extracts (AMPUME and ASPUME) have better ability to inhibit LPO as compared to the aqueous extracts (AMPUAE and ASPUAE). However, the IC_{50} values were significantly higher ($P < 0.05$) as compared to standard ascorbic acid of (IC_{50} ; $121.78 \pm 0.93 \mu\text{g/mL}$).

Table 2: Lipid peroxidation inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps

Conc ($\mu\text{g/mL}$)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	$15.53^c \pm 0.34$	$11.52^a \pm 0.22$	$17.37^d \pm 0.16$	$11.01^a \pm 0.17$	$9.76^b \pm 0.15$
30	$30.49^a \pm 0.16$	$16.33^b \pm 0.35$	$32.90^a \pm 0.15$	$19.49^c \pm 0.19$	$15.08^b \pm 0.18$
60	$34.35^b \pm 0.27$	$39.45^a \pm 0.32$	$34.54^b \pm 0.31$	$36.45^c \pm 0.35$	$41.14^d \pm 0.30$
125	$66.92^a \pm 0.36$	$51.78^c \pm 0.12$	$65.44^a \pm 0.62$	$56.06^d \pm 0.16$	$63.55^b \pm 0.30$
250	$74.25^c \pm 0.12$	$50.30^b \pm 0.20$	$76.08^c \pm 0.26$	$58.23^a \pm 0.22$	$82.35^d \pm 0.38$
500	$71.93^a \pm 0.22$	$55.27^b \pm 0.34$	$78.20^c \pm 0.11$	$60.49^d \pm 0.43$	$91.04^e \pm 0.43$
IC_{50} ($\mu\text{g/mL}$)	$448.39^d \pm 11.35$	$475.33^e \pm 1.50$	$433.50^c \pm 7.12$	$465.00^b \pm 3.33$	$121.78^a \pm 0.93$
Mean values ($n=3$) $\pm$ SEM. Values appended by different superscript letters within a row are significantly different ($P < 0.05$)					

Nitric Oxide (NO) Scavenging Activity:

Nitric oxide (NO) scavenging activities were observed for aqueous extracts (ASPUAE and AMPUAE) and methanol extracts (ASPUME and AMPUME) (Table 3).

In all the range of concentrations used ($15 \mu\text{g/mL}$ to $500 \mu\text{g/mL}$), the percent inhibition of the methanol extracts ASPUME ($63.27 \pm 0.38\%$) and AMPUME ($70.78 \pm 0.46\%$) were significantly higher ($P < 0.05$) than those of the aqueous extracts ASPUAE ($42.38 \pm 0.35\%$) and AMPUAE ($46.81 \pm 0.17\%$). The IC_{50} values were $354.39 \pm 4.98 \mu\text{g/mL}$ (ASPUME), $370.00 \pm 1.67 \mu\text{g/mL}$ (ASPUAE), $345.50 \pm 1.94 \mu\text{g/mL}$ (AMPUME) and $354.67 \pm 1.50 \mu\text{g/mL}$ (AMPUAE). Although all

the extracts (ASPUME, ASPUAE, AMPUME and AMPUAE) exhibited certain degree of NO scavenging, the IC₅₀ values were high ranging from 345.67±1.94 µg/mL (AMPUME) to 370.00±1.67 µg/mL (ASPUAE) compared to the standard (Ascorbic acid) value of 124.75±2.93 µg/mL.

Table 3: Nitrous oxide (NO) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps

Conc (µg/mL)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	24.52 ^b ±0.21	10.71 ^a ±0.26	20.89 ^c ±0.48	16.52 ^d ±0.75	29.64 ^e ±0.21
30	42.77 ^a ±0.25	32.57 ^c ±0.29	44.28 ^b ±0.31	36.41 ^d ±0.23	41.83 ^b ±0.27
60	53.87 ^c ±0.68	41.12 ^b ±0.28	56.20 ^d ±0.31	43.10 ^a ±0.32	64.79 ^e ±0.23
125	61.51 ^d ±0.17	42.93 ^a ±0.11	63.94 ^e ±0.22	46.36 ^b ±0.24	81.49 ^c ±0.19
250	59.36 ^a ±0.29	40.16 ^b ±0.29	64.51 ^c ±0.24	45.41 ^d ±0.16	78.75 ^e ±0.19
500	63.27 ^d ±0.38	42.38 ^e ±0.35	70.78 ^c ±0.46	46.81 ^b ±0.17	79.49 ^a ±0.27
IC ₅₀ (µg/mL)	354.39 ^b ±4.98	370.00 ^a ±1.67	345.50 ^c ±1.94	354.67 ^d ±1.50	124.75 ^e ±2.93
Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05)					

Superoxide (O₂⁻) Scavenging Activity:

Superoxide (O₂⁻) scavenging activities were exhibited by the aqueous extracts (ASPUAE and AMPUAE) and methanol extracts (ASPUME and AMPUME) in all concentration ranges (Table 4). The methanol extracts (AMPUME and ASPUME) displayed a greater degree of superoxide scavenging with percentage inhibition of 65.19±0.28% and 56.16±0.45% respectively, than the aqueous extracts (AMPUAE and ASPUAE), which was at 47.45±0.33% and 49.54±0.38% respectively. The IC₅₀ values were lower in the methanol extracts; AMPUME (155.56±2.62 µg/mL) and ASPUME (185.00±3.333 µg/mL) compared to the aqueous extracts; AMPUAE (300.00±16.67 µg/mL) and ASPUAE (310.00±3.333 µg/mL).

However, the IC₅₀ values of all the extracts (ASPUME, ASPUAE, AMPUME and AMPUAE) were significantly higher (P<0.05) than that of the standard ascorbic acid (131.88±3.13 µg/mL).

Table 4: Superoxide inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps

Conc (µg/mL)	Percentage inhibition				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	8.46 ^b ±0.16	4.93 ^a ±0.33	9.60 ^b ±0.12	6.85 ^c ±0.16	12.33 ^d ±0.33
30	17.82 ^b ±0.10	11.24 ^a ±0.27	18.66 ^c ±0.19	10.16 ^a ±0.26	18.48 ^c ±0.18
60	25.77 ^b ±0.37	19.57 ^a ±0.20	27.43 ^c ±0.34	19.61 ^a ±0.21	30.56 ^d ±0.42
125	35.26 ^b ±0.55	31.94 ^c ±0.52	39.80 ^a ±0.17	33.93 ^b ±0.21	49.51 ^d ±0.17
250	50.51 ^a ±0.29	45.46 ^b ±0.26	44.99 ^b ±0.26	44.19 ^b ±0.25	65.19 ^c ±0.29
500	56.06 ^a ±0.45	49.54 ^b ±0.38	65.19 ^c ±0.28	47.45 ^d ±0.33	75.25 ^e ±0.25
IC ₅₀ (µg/mL)	185.00 ^d ±3.33	310.00 ^a ±3.33	155.56 ^c ±2.62	300.00 ^b ±16.67	131.88 ^e ±3.13
Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05)					

Hydroxyl (OH⁻) Scavenging Activity:

The aqueous extracts (ASPUAE, and AMPUAE) and methanol extracts (ASPUME and AMPUME) had the potential of scavenging OH⁻ (Table 5), which increased with increasing concentration of the extracts. In the methanol extracts, OH⁻ inhibition observed was in the range of 13.59±0.13% to 65.53±0.15% (ASPUME) and 13.28±0.14% to 76.65±0.33% (AMPUME) at concentrations ranges of 15 µg/mL to 500 µg/mL. Lower OH⁻ inhibition percentages were obtained in the aqueous extracts ranging from 9.88±0.12% to 42.36±0.34% (ASPUAE) and 8.65±0.34 to 60±0.27% (AMPUAE) at concentrations ranges of 15 µg/mL to 500 µg/mL. The OH⁻ scavenging abilities of the methanol extracts (ASPUME and AMPUME) were found to be higher than those of the aqueous extracts (ASPUAE and AMPUAE). Although the extracts exhibited the potential to scavenge OH⁻, the IC₅₀ values were still significantly higher at 300.00±16.67 µg/mL (AMPUME and ASPUME), 311.33±2.36 µg/mL (ASPUAE) and 316.67±5.09 µg/mL (AMPUAE) than that of the standard (ascorbic acid) 171.77±4.91 µg/mL.

Table 5: Hydroxyl (OH⁻) inhibition activity of methanol and aqueous extracts of *A. muricata* and *A. squamosa* fruits pulps

Conc (µg/mL)	Percentage inhibition of different extracts				
	ASPUME	ASPUAE	AMPUME	AMPUAE	Ascorbic acid
15	13.89 ^a ±0.13	9.88 ^b ±0.12	13.28 ^a ±0.14	8.65 ^b ±0.34	20.39 ^c ±0.35
30	20.19 ^a ±0.25	15.84 ^c ±0.07	28.92 ^b ±0.11	13.08 ^e ±0.21	33.97 ^d ±1.19
60	32.27 ^a ±0.24	21.93 ^c ±0.15	37.39 ^c ±0.17	27.89 ^d ±0.26	45.95 ^b ±0.42
125	30.56 ^b ±0.26	24.92 ^a ±0.22	54.84 ^c ±0.12	37.83 ^d ±0.13	64.03 ^e ±1.58
250	42.79 ^a ±0.30	36.73 ^b ±0.09	63.79 ^c ±0.26	54.72 ^d ±0.24	84.03 ^e ±0.80
500	65.53 ^a ±0.15	42.36 ^b ±0.34	76.65 ^c ±0.33	60.13 ^d ±0.27	94.65 ^e ±0.72
IC ₅₀ (µg/mL)	300.00 ^b ±16.67	311.33 ^a ±2.36	300.00 ^b ±16.67	316.67 ^d ±5.09	171.77 ^d ±4.91
Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05)					

Total Oxidant and Reducing Activities:

The total antioxidant activity and reducing powers of aqueous extracts (AMPUAE and ASPUAE) and methanol extracts (AMPUME and ASPUME) were evaluated as equivalent to µg/mL of α-tocopherol and equivalent to µg/mL of ascorbic acid respectively as presented in Table 6. The reducing powers against the standard ascorbic acid were found to be 57.37±0.71 µg/mL (ASPUME) and 62.92±2.50 µg/mL (AMPUME), 44.28±0.58 µg/mL (ASPUAE) and 46.61±0.32 µg/mL (AMPUAE). Total antioxidant activities were found to be 201.12±1.02 µg/mL (ASPUME), 199.15±1.07 µg/mL (AMPUME), 183.09^a±1.76 µg/mL (ASPUAE) and 181.36±0.87 µg/mL (AMPUAE)

Table 6: Total antioxidant Activity (Equivalent to µg of α-tocopherol) and total Reducing Power (Equivalent to µg of ascorbic acid) of methanolic and aqueous extracts of *A. muricata* and *A. squamosa* fruit pulp

Parameter	Solvents used in extraction and species of the plant			
	ASPUME	ASPUAE	AMPUME	AMPUAE
Total antioxidant activity	201.12 ^b ±1.02	183.09 ^a ±1.76	199.15 ^c ±1.07	181.36 ^a ±0.87
Total Reducing activity	57.37 ^c ±0.71	44.28 ^a ±0.58	62.92 ^b ±2.50	46.61 ^a ±0.32
Mean values (n=3) ± SEM. Values appended by different superscript letters within a row are significantly different (P < 0.05)				

In Vivo Antioxidant Activity

Markers of Liver and Cardiac Damage:

Hepatoprotective role of the extracts were assessed through determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels whereas activities of cardiac markers, lactate dehydrogenase (LDH) and creatinine kinase (CK) depicted cardiac involvement. Infection with *L. major* in BALB/c mice resulted in significant increase ($p<0.05$) in AST, ALT, LDH and CK activities as shown in the INT (positive control) group (Group II). After treatment in IT groups (Group III, Group IV, Group V, Group VI and Group VII), there was a significant decrease ($p<0.05$) in ALT, AST, LDH and CK activities (Table 7 and 8). Further, combined treatment of extracts and Pentostam (Group VI and Group VII) had the highest significant decrease ($p<0.05$) in AST, ALT, LDH and CK activities compared to the extract's groups (Group IV and Group VI) and Pentostam group (Group III) separately.

Enzymatic and Non-Enzymatic Antioxidant Levels:

The *in vivo* antioxidant activity was also analyzed through the level of non-enzymatic antioxidants (GSH, NO, TBARS (MDA), LPO and ROS) and enzymatic antioxidants (GST, GSHPx, CAT and SOD). The infection with *L. major* in BALB/c mice caused a significant ($p<0.05$) elevation of NO, TBARS (MDA) LPO and ROS levels (Table 7 and 8). In all the infected treated (IT) groups of BALB/c mice (Group III, Group IV, Group V Group VI and Group VII), a significant ($p<0.05$) decrease in the NO, TBARS (MDA), LPO and ROS levels was observed after the treatment compared to negative control (Group I) and INT (positive control) group (Group II). The combined treatment of the extracts and Pentostam (Group VI and Group VII) showed the highest significant decrease in NO and TBARS (MDA) levels ($p<0.05$) compared to the IT extracts groups (Group IV and Group V) and Pentostam group (Group III) separately. However, the ROS increased significantly ($P<0.05$) in all the infected treated groups of mice (Group III, Group IV, Group V Group VI and Group VII) compared to the negative control group (Group I) and the positive control group (Group II). The alteration of GSH, GST, GSHPx, SOD and CAT were used to prove the effect of leishmaniasis on the oxidant/antioxidant imbalance. The BALB/c mice infected with *L. major* caused a significant ($p<0.05$) decrease in GSH, GST, GSHPx, SOD and CAT activities (Table 7 and 8).

Table 7: Effects of *A. muricata* and *A. squamosa* aqueous extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice

Antioxidant	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
GSH (IUg ⁻¹ of Hb)	4.18±0.28	1.43±0.15* **	4.02±0.55	4.82±0.75*	4.21±0.47	8.27±1.15**	9.68±1.12* *
GST (IU g ⁻¹ of Hb)	1.62±0.02	1.07±0.04*	2.08±0.09*	3.28±0.09*	3.32±0.05*	7.00±0.44** *	10.90±1.95 ***
GSHPx (IU g ⁻¹ of Hb)	3.55±0.13	2.02±0.17*	4.22±0.74*	3.92±0.14*	4.08±1.08**	8.23±1.06	7.17±2.28* **
CAT (μMmg ⁻¹ Hb)	7.58±0.06	2.15±0.03* **	3.83±0.17*	6.93±0.47*	6.05±0.03*	9.90±0.06*	8.97±0.11*
TBARS/MDA (nMmL ⁻¹)	0.88±0.02	1.20±0.01*	0.76±0.02*	1.03±0.00*	0.86±0.02	0.75±0.02*	0.75±0.02*
ROS (nMmin ⁻¹ mg ⁻¹ protein)	0.17±0.01	0.21±0.01*	0.38±0.03*	0.38±0.03*	0.32±0.03*	0.43±0.03*	0.33±0.03*
SOD (IU mg ⁻¹ protein)	23.70±0.30	18.65±0.44 *	26.52±0.22*	49.62±0.92*	45.00±0.12* *	62.77±0.42* *	69.45±0.67 ***

NO (uMmL ⁻¹)	23.17±0.44	47.17±1.39***	33.33±1.33*	35.43±2.43*	36.03±1.80*	23.83±1.29	26.73±0.63*
AST (UL ⁻¹)	370.58±54.55	783.67±20.64	572.50±19.48**	567.50±31.48**	498.43±12.59**	508.00±11.43**	530.02±5.40**
ALT (UL ⁻¹)	75.17±2.57	96.50±1.55**	75.17±2.57	84.67±3.74*	79.50±2.26*	59.33±1.74*	60.17±2.51*
LDH (UL ⁻¹)	252.17±10.69	541.50±11.33*	255.33±9.48**	328.17±5.21**	247.00±7.30**	242.17±10.69*	249.33±4.96**
CK (UL ⁻¹)	360.67±14.5	390.33±8.58**	255.33±18.19*	265.33±18.19*	252.50±12.60**	249.17±3.07**	256.33±5.70**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs Control (DMSO). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUAE), V: (AMP UAE), Group, VI: (Pentostam + ASP UAE) Group, VII: (Pentostam + AMP UAE). Key: AST; Aspartate amino transferase, ALT; Alanine amino transferase, LDH; Lactate dehydrogenase, CK; Creatine Kinase, TBARS; Thiobarbituric acid reactive substances NO; nitric oxide, GSH; Glutathione, GST; Glutathione-s-transferase, GSHPx; Glutathione peroxidase, SOD; Superoxide dismutase, CAT; Catalase

Compared to the negative control group (Group I) and INT (positive control) group (Group II), the IT groups (Group III, Group IV, Group V Group VI and Group VII), showed a significant increase in GSH, GST, GSHPx, SOD and CAT activities (p<0.05) after or concurrently with the treatment. Further, the combined treatment of the extracts and Pentostam (Group VI and Group VII) showed the highest significant increase in GSH, GST, GSHPx, SOD and CAT activities (p<0.05) compared to the IT extracts groups (Group IV and Group

Table 8: Effects of *A. muricata* and *A. squamosa* methanol extracts on serum non enzymatic and enzymatic antioxidants and markers of hepatic damage in *L. major* infected BALB/c mice

Antioxidant	Group I	Group II	Group III	Group IV	Group V	Group V	Group VII
GSH (IUg ⁻¹ of Hb)	4.18±0.28	1.43±0.15**	4.02±0.55	6.27±0.15**	6.68±0.12*	9.68±0.12*	10.27±0.15*
GST (IU g ⁻¹ of Hb)	1.62±0.02	1.07±0.04*	2.78±0.09*	2.90±0.04*	3.90±0.05*	8.90±0.05*	9.00±0.04**
GSHPx (IU g ⁻¹ of Hb)	3.55±0.13	2.02±0.17*	4.22±0.74*	5.23±0.06	6.17±1.28*	17.17±0.28**	12.23±0.67**
Catalase (CAT) (μMmg ⁻¹ Hb)	7.58±0.06	2.15±0.03**	3.83±0.17*	6.09±0.76**	5.37±0.11*	9.37±0.11*	11.00±0.06*
TBARS/MDA (nMmL ⁻¹)	0.88±0.02	1.20±0.01*	0.76±0.02*	0.86±0.02	1.00±0.11*	1.00±0.01*	0.86±0.02
ROS (nM min ⁻¹ mg ⁻¹ protein)	0.17±0.01	0.21±0.01*	0.38±0.03*	0.43±0.03**	0.33±0.03*	0.63±0.03*	0.53±0.03**
SOD (IU mg ⁻¹ protein)	23.70±0.30	18.65±0.44*	26.52±0.22*	45.67±0.42*	41.50±0.66	74.50±0.77	87.67±0.49*
Nitric oxide NO (uMmL ⁻¹)	23.17±0.44	47.17±1.39***	33.33±1.33**	22.83±0.29	25.83±0.63	18.83±0.63*	20.83±0.29
AST (UL ⁻¹)	375.58±0.5	78.67±1.64**	57.25±0.48**	50.00±1.43**	53.00±1.40**	33.00±1.40*	30.00±1.41*
ALT (UL ⁻¹)	75.17±1.57	96.50±1.55**	75.17±2.57	68.33±0.74*	71.17±1.51*	44.17±0.51*	40.33±0.74*
LDH (UL ⁻¹)	252.17±1.69	541.50±1.33*	255.33±1.48**	205.33±11.48***	194.33±1.96**	149.33±1.46**	155.33±1.48***
CK (UL ⁻¹)	360.67±1.45	390.33±1.58**	355.33±1.19*	195.50±1.260*	176.33±1.30**	156.33±1.70**	152.50±1.60**

Mean values ± SEM, (n=6) *P<0.05; **P<0.01; ***p<0.001 vs Negative Control. Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUME), V: (AMPUME), Group, VI: (Pentostam + ASPUME) Group, VII: (Pentostam + AMPUME). Key: AST; Aspartate amino transferase, ALT; Alanine amino transferase, LDH; Lactate dehydrogenase, CK; Creatine Kinase, TBARS; Thiobarbituric acid reactive substances NO; nitric oxide, GSH; Glutathione, GST; Glutathione-s-transferase, GSHPx; Glutathione peroxidase, SOD; Superoxide dismutase, CAT; Catalase

Lesion Size and Liver and Splenic Length, Size and Parasite Burden:

An increase in lesions sizes with progression of the disease was observed (Figure 1 and 2). A decrease in lesion size was observed in all the IT groups (Group II, Group IV, Group V, Group VI and Group VII) after the initiation of treatment with the extracts and the standard drug (Pentostam). However, the rate of decrease in lesion size was significantly higher ($p<0.05$) in the IT-pentostam group (Group III) and IT-pentostam+extracts groups (Group VI and Group VII) than in the IT-extracts groups (Group IV and Group V). In the INT (positive control) group (Group II), a significant ($p<0.05$) increase in lesion sizes was observed until the end of the experimental period. The parasite load (LDU), relative mean weight and length of liver and spleen increased with increasing time of infection (Table 9 and 10). There was a significant ($p<0.05$) decrease in parasite load (LDU), relative hepatic and splenic length and size in all the IT groups of BALB/c mice (Group III, Group IV, Group V, Group VI and Group VII). In the INT (positive control) group (Group II), a significant ($p<0.05$) increase in parasite load, relative weights of liver and spleen was observed until the end of the experimental period. A combination of the extracts and standard drug (pentosam) (Group VI and Group VII) lead to a significant ($p<0.05$) decrease in parasite load (LDU), hepatic and splenic size and length compared to the IT extracts groups (Group IV and Group V) and standard drug (Pentostam) group (Group III) separately.

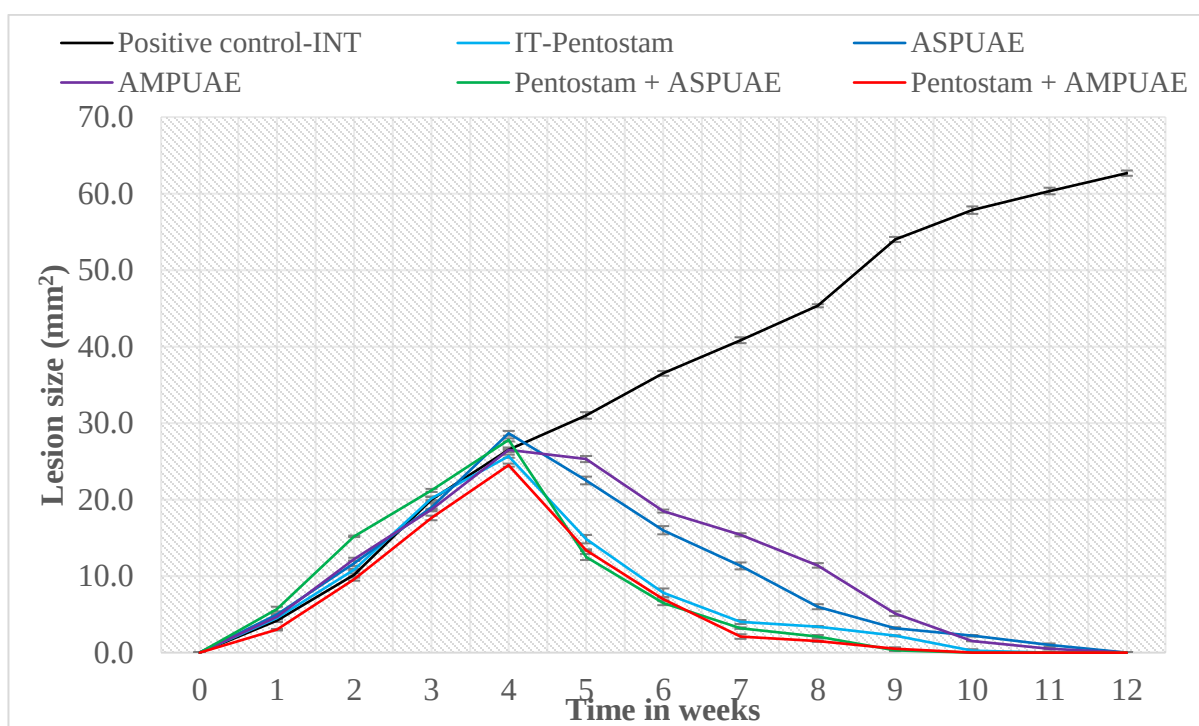


Figure 1: Effects of *A. muricata* and *A. squamosa* pulp aqueous extracts on lesion sizes in *L. major* infected BALB/c mice

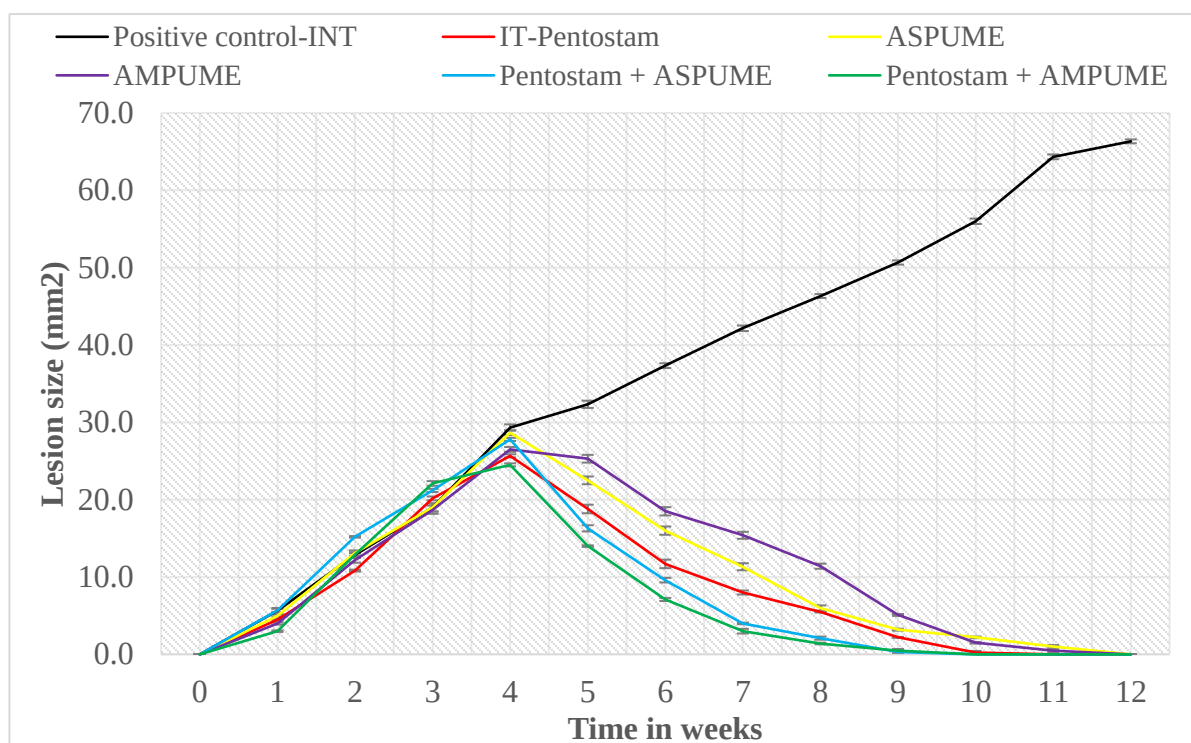


Figure 2: Effects of *A. muricata* and *A. squamosa* pulp methanol extracts on lesion sizes in *L. major* infected BALB/c mice

Correct the units of lesion size on the y-axis {mm²}

Table 9: Effects of *A. muricata* and *A. squamosa* fruits pulp aqueous extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in invivo antioxidant study

Parameter	Organ	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Body Weight	Day 0	19.99 ^a ±0.19	20.76 ^a ±0.07	19.93 ^a ±0.15	19.96 ^a ±0.22	19.93 ^a ±0.13	19.93 ^a ±0.33	19.93 ^a ±0.11
	Day 84	29.74 ^{ab} ±0.12	10.71 ^c ±0.13	25.38 ^e ±0.19	27.38 ^d ±0.17	28.38 ^b ±0.13	33.38 ^e ±1.19	35.38 ^f ±0.29
Relative length (mm)	Liver	22.08 ^c ±0.11	51.10 ^d ±0.38	28.15 ^a ±0.13	25.15 ^e ±0.18	26.15 ^e ±0.27	22.15 ^c ±0.12	23.15 ^c ±0.08
	Spleen	18.83 ^d ±0.32	43.17 ^a ±0.44	21.17 ^c ±0.11	24.17 ^b ±0.14	23.17 ^b ±0.17	19.17 ^d ±0.15	18.17 ^d ±0.19
Relative weight (mg)	Liver	1.44 ^{ab} ±0.06	3.16 ^c ±0.06	1.49 ^b ±0.04	1.51 ^b ±0.01	1.61 ^d ±0.02	1.41 ^a ±0.08	1.45 ^a ±0.05
	Spleen	0.14 ^a ±0.01	2.34 ^c ±0.03	0.21 ^b ±0.01	0.25 ^d ±0.01	0.20 ^b ±0.01	0.17 ^e ±0.01	0.15 ^a ±0.01
LDU (10 ⁶)	Liver	ND	545.44 ^b ±12.58	2.21 ^a ±0.58	7.15 ^c ±12.58	12.93 ^d ±4.13	1.03 ^e ±3.13	0.97 ^e ±0.13
	Spleen	ND	14.02 ^a ±2.24	0.02 ^b ±0.24	0.12 ^c ±0.24	0.98 ^e ±0.19	0.01 ^d ±0.19	0.02 ^b ±0.19

Mean values ± SEM, (n=6). Values appended by different superscript letters within a row are significantly different (P < 0.05). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUME), Group V: (AMPUME), Group VI: (Pentostam + ASPUME) Group VII: (Pentostam + AMPUME), ND: Not done

Table 10: Effects of of *A. muricata* and *A. squamosa* fruits pulp methanol extracts on relative weight (mg) and length (mm) of the liver and spleen and parasitic burden (DU) in *L. major* infected and non-infected BALB/c mice in invivo antioxidant study

Parameter	Organ	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Body Weight	Day 0	19.99 ^a ±0.19	20.76 ^a ±0.07	19.93 ^a ±0.15	20.08 ^a ±0.11	20.10 ^a ±0.11	19.98 ^a ±0.11	20.01 ^a ±0.41
	Day 84	29.74 ^{ab} ±0.12	10.71 ^c ±0.13	25.38 ^e ±0.19	24.35 ^e ±1.19	28.78 ^a ±0.47	32.67 ^d ±1.09	33.68 ^d ±0.79
Relative length (mm)	Liver	22.08 ^c ±0.11	51.10 ^d ±0.38	28.15 ^a ±0.13	29.55 ^b ±1.12	25.55 ^e ±0.01	21.00 ^c ±0.27	22.30 ^c ±0.97
	Spleen	18.83 ^d ±0.32	43.17 ^a ±0.44	21.17 ^c ±0.11	23.11 ^b ±1.01	26.11 ^f ±0.01	19.88 ^e ±0.13	18.78 ^e ±0.66
Relative weight (mg)	Liver	1.44 ^{ab} ±0.06	3.16 ^c ±0.06	1.49 ^b ±0.04	1.55 ^b ±0.03	1.77 ^b ±0.19	1.45 ^b ±0.17	1.56 ^b ±0.33
	Spleen	0.14 ^a ±0.01	2.34 ^c ±0.03	0.21 ^b ±0.01	1.11 ^d ±0.01	1.61 ^g ±0.15	0.61 ^f ±0.14	0.77 ^e ±0.18
LDU (10 ⁶)	Liver	ND	545.44 ^b ±12.58	2.21 ^a ±0.58	15.55 ^c ±2.01	23.55 ^d ±2.01	12.55 ^e ±2.04	9.55 ^f ±1.11
	Spleen	ND	14.02 ^a ±2.24	0.02 ^b ±0.24	1.11 ^c ±0.01	0.51 ^f ±0.01	0.31 ^e ±0.02	0.21 ^d ±0.03

Mean values ± SEM, (n=6). Values appended by different superscript letters within a row are significantly different (P < 0.05). Group I: Normal Control; Group II: (Positive control-INT); Group III: (IT-Pentostam), Group IV: (ASPUME), V: (AMPUME), Group, VI: (Pentostam + ASPUME) Group, VII: (Pentostam + AMPUME), ND: Not done

DISCUSSION

Natural products from plants have an important contribution in the search for new leishmanicidal drugs (Sakthianandeswaren *et al.*, 2005; Tiuman *et al.*, 2011; Al-Olayan *et al.*, 2014; Alkathiri *et al.*, 2017). Thus, conventional therapy combined with natural products has been the trend in management of leishmaniasis (Metwally *et al.*, 2016; Alkathiri *et al.*, 2017). The *L. major* causes inflammation via mast cell stimulation and enhancing secretion of pro-inflammatory mediators (Cragg and Newman, 2013; Alkathiri *et al.*, 2017). Moreover, ROS and RNS produced during an inflammatory response leads to oxidative injury in non-infected cells (Alkathiri *et al.*, 2017). During oxidative damage, free radicals that play a role in collagen damage are released (Sakthianandeswaren *et al.*, 2005; Sheikholeslami *et al.*, 2014).

In this study, *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts revealed marked antioxidative activities accompanied with DNA protective effects against H₂O₂-induced toxicity both *in vitro* and *in vivo*. This indicates that the *A. muricata* and *A. squamosa* fruit pulp can be included in the diet of leishmaniasis patients to remove ROS and RNS produced by the interaction between the parasite and the host macrophages. It has been reported that ROS and RNS production are highly elevated during CL (Faria *et al.*, 2007; Alkathiri *et al.*, 2017), an indication that free radicals and oxidative damage play a vital role in pathogenesis. Natural antioxidants of plant origin have been studied extensively for their effectiveness in the treatment of toxic injury (Abdel-Moneim, 2014a; Othman *et al.*, 2014b). Furthermore, they have been shown to support the skin's underlying structure and lower the synthesis of collagen-degrading enzymes, resulting in younger-looking skin (Aslam *et al.*, 2006; Alkathiri *et al.*, 2017). The evaluated antioxidant activity of *A. muricata* and *A. squamosa* fruits through *in vitro* models are higher compared to earlier conducted studies (Nawwar *et al.*, 2012; Roy *et al.*, 2011; Nandhakumar & Indumathi, 2013; Samuagam *et al.*, 2014). This further expands the therapeutic potential of *A. muricata* and *A. squamosa* fruits. Higher total phenol and flavonoid contents in methanol extracts in this study might be the cause of antioxidant activities as reported in other studies (Nadhakumar & Indumathi, 2013; Gulçin *et al.*, 2010).

Methanol and aqueous extracts the fruits pulp of *A. muricata* (AMPUAE and AMPUME) and *A. squamosa* (ASPUAE and ASPUME) were evaluated for *in vivo* antioxidant activities using both non-enzymatic oxidants (GSH, NO, TBARS, MDA and ROS) and enzymatic antioxidants (GST, GSHPx, CAT and SOD) and organ protection ability using serum AST, ALT, LDH and CK activity. The findings of this study suggested that these extracts possess antioxidant potential in BALB/c mice. This study shows that the extracts strengthened both enzymatic and non-enzymatic antioxidant to prevent the extent of lipid peroxidation and normalized level of both cardiac and hepatic markers in serum. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane in organs producing these enzymes such as the liver (Drotman *et al.*, 1978). Increased levels of ALT and AST assesses the extent of hepatic damage with ALT being more specific than AST (Arthur *et al.*, 2012a; Arthur *et al.*, 2012b, Arthur *et al.*, 2011). The results of this study revealed significance difference seen among negative control groups (Group I) and positive control (Group II) with IT groups (Group III, Group IV, Group V, Group VI and Group VII) where the levels of serum enzymes were lowered. The principle of antioxidant activity is based on the availability of electrons to neutralize any free radicals (Nadhakumar & Indumathi, 2013). During extracts therapy, BALB/c mice showed normalized level of both the cardiac markers LDH and CK suggesting the cardiac potential capacity of phenolic compounds that may be present in the extracts. Further, the present study also showed the antioxidant and radical scavenging mechanism extracts by antioxidant assays and analysis of lipid peroxidation. In free radical elimination, cellular antioxidants play an important role in the existence of equilibrium between enzymes under normal conditions (Binu & Harikumar, 2015). Excess production of free radicals makes biological system to lose this equilibrium resulting in the establishment of an oxidative assault. In this study, the extracts IT groups (Group IV and Group V) showed significant ($p < 0.05$) change in the activity of antioxidant like GSH, GST and GSHPx. Endogenous antioxidants (GSH, GSHPx and GST) play a major role in the maintenance of redox potential and GSH act as a substrate for GST and GSHPx (Yoshida *et al.*, 1997; Ilavenil, 2012). The GSHPx reduces free H_2O_2 to water and lipid hydroperoxides to their corresponding alcohols as described by other studies (Binu & Harikumar, 2015). Several isoenzymes of GSHPx exist and GSHPx1 is the most abundant version found in the cytoplasm of many mammalian tissues (Gulçin *et al.*, 2010).

The GSH level is an important factor in leishmania parasite induced toxicity and elevated GSH level increases GSHPx1 activity. In CL, a decreased GSH levels have been observed (Jafari *et al.*, 2014). The GSH, the main nonenzymatic antioxidant molecules in cells plays a protective role in the metabolism of several toxic agents (Alkathiri *et al.*, 2017) by acting as a free radical-trapping agent to preserve cytochrome P450 via blocking LPO (Moskaug *et al.*, 2005). The results from previous studies on mice infected with *L. major* and treated with plants extracts (Aguiar *et al.*, 2010; Jafari *et al.*, 2014) showed remarkable elevated levels of GSH and GSHPx. In this study, *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts markedly increased and maintained GSH and GSHPx activities in *L. major* infected BALB/c mice. In biological systems, LPO is thought to be a toxicological phenomenon and low concentration of LPO products and byproducts are seen in biological system (Binu & Harikumar, 2015). The byproduct lipid peroxides such as malondialdehyde (MDA) always alter the function of membrane proteins and lead to the production of peroxynitrite ($ONOO^-$) radicals (Binu & Harikumar, 2015). The LPO occurs as a result of oxidative stress resulting from ROS and RNS over-production due to host defense against leishmania parasite (Dkhil *et al.*, 2013) and are responsible for cellular damage (Abdel-Moneim & El-Khadragy, 2013). Moreover, the

production of free oxygen radicals ($O_2^{\bullet-}$) in excess depletes protective enzymatic antioxidants, resulting in cellular injury observed in *Leishmania* infection (Ozbilge *et al.*, 2005). Increased LPO has been described in VL in hamsters (Sen *et al.*, 2001), humans (Neupane *et al.*, 2008) and dogs (Heidarpour *et al.*, 2012). Further LPO in patients with active CL has been reported (Vural *et al.*, 2004; Abdel-Moneim, 2014a). Inhibitory effects of compounds present in extracts on the LPO process may be closely related to the metal reducing activity (Ito *et al.*, 2005; Alkathiri *et al.*, 2017). In human CL, serum antioxidant activities, MDA and NO levels are elevated (Vural *et al.*, 2004; Serarslan *et al.*, 2005). In this study, the extracts IT groups of BALB/c mice (GROUP IV and Group V) showed low level of MDA compared to positive control group of BALB/c mice (Group II). Prevention of LPO after *A. muricata* and *A. squamosa* fruit pulp aqueous and methanol extracts treatment could be attributed to the radical-scavenging effect of the antioxidant constituents of the extracts.

The NO play a role in inducing inflammatory response and its toxicity is propagated only when they react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), which damages biomolecules such as proteins, lipids, and nucleic acids (Abdel-Moneim, 2014b). In the present study, NO generation was restrained by the extracts, suggesting its applicability as a potent and novel therapeutic agent for scavenging NO. Further, the extracts may affect regulation of pathological conditions caused by excessive generation of NO and its oxidation product peroxynitrite ($ONOO^-$). Enhanced levels of NO and peroxynitrite ($ONOO^-$) have been reported in the blood and lesions of mice infected with *L. amazonensis* (Shirbazou & Jafari, 2012). The ROS and RNS in the form of peroxynitrite ($ONOO^-$) produced by the reaction of superoxide radical with NO has been indicated in the production of cytotoxic effects (Ou, *et al.*, 2006; Yukllhami, 2011) thus related in the development of oxidative damage. In this *in vivo* model of the study, the level of NO declined in in the extracts IT groups (Group IV and Group V) compared to the positive control (Group II). Therefore, the extracts could have potential natural compounds that are reactive nitrogen scavenger. A further decrease in the activities of CAT and SOD has been reported in Canine VL (Heidarpour *et al.*, 2012) and *L. major* infected BALB/c mice. (Jafari *et al.*, 2015). In this study, treatment with the extracts (Group IV and Group V) caused a significant increase in CAT and SOD activities. The ability of the extracts treatment to prevent reduction in enzymatic antioxidants may be attributed to host defense for protection against toxic oxygen metabolites (Alkathiri *et al.*, 2017). Results of other studies on other plant species have shown a positive effect of polyphenols and flavonoids on enzymatic antioxidant activities *in vivo* (Abdel-Moneim & El-Khadragy, 2013; Abdel-Moneim, 2014a; Othman *et al.*, 2014b). Further, there was a higher effect that was observed when the extract from *A. muricata* and *A. squamosa* fruits pulp were combined with the standard drug (Pentostam) (Group VI and Group VII). The finding of this study revealed that *A. muricata* and *A. squamosa* fruits aqueous and methanol extracts caused a significant increase in GSH GSHPx, GST, SOD and CAT and decrease in NO, MDA, ALT AST and ALkP in *L. major* infected BALB/c mice. Thus, the extracts may be included in the formulation of the existing leishmania chemotherapy for better management of the disease.

CONCLUSION

The studies on antioxidant activity of medicinal plants extracts against *L. major* induced oxidative stress in BALB/c mice can be used to understand the pathogenesis of leishmaniasis. Overall, the results of this study contribute to understanding of the fruits in family Annonaceae,

especially the genus *Annona* growing in Kenya as a source of antioxidant. Besides, the results affirmed the importance of selection of plant extracts used in folk medicine in screening programs when searching for new antileishmanial agents. The findings from this study highlight the potential usefulness of *A. muricata* and *A. squamosa* fruits paramount in the search for new leishmaniasis fighting strategies such as use of good protein energy (PE) or Protein calorie (PC) as supportive therapy for Leishmaniasis.

RECOMMENDATIONS

The study was to determine antioxidant activity of *A. muricata* (L.) and *A. squamosa* (L.) pulp fruits crude extracts against *L. major* induced oxidative stress in BALB/c mice. Further Investigation be done on the crude extracts from *A. muricata* and *A. squamosa* fruits to focus on isolation and characterization to obtain pure constituents with the aim of understanding mechanisms of action, and continued *in vitro* and *in vivo* investigations. Besides, metabolic studies are also necessary to determine whether digestive processes decrease or increase antioxidant activity of the active compounds is recommended.

AUTHOR'S CONTRIBUTION

This work was carried out in collaboration between all the authors. 'Author CLM' designed the study developed the protocol and performed the experimental analysis of the study and managed the literature searches and statistical analysis. 'Author GOM', 'Author CN' 'Author AMJ' and Author 'GKG' participated in the development of the manuscript. All authors read and approved the final draft of the manuscript.

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