Original Research Article

In-vivo Antioxidant's Modulatory properties of *Justicia carnea* Aqueous Leave Extract on Sheep Red Blood Cells Immunized Mice

ABSTRACT

Aims: Oxidative stress emanating from inflammatory cytokines, Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), exogenously introduced antigens among others are keenly involved in the generation of oxidative stress related diseases, which are regulated by endogenous antioxidants. This study aimed at evaluating the In-vivo Antioxidant's Modulatory properties of Justicia carnea Aqueous Leave Extract on Sheep Red Blood Cells Immunized Mice.

Study design: The animals were grouped into six with five animals per group (Vehicle (VEH); distilled water 10 mL/Kg , Sheep Red Blood Cells (SRBC) 0.2 mL/Kg, Justicia carnea (JC) 125 mg/Kg , JC 250 mg/Kg, JC 500 mg/Kg, and Levamisole (LEV) 7.5 mg/Kg)

Place and Duration of Study: The animals used for this study were breed, feed and housed at ambient temperature and well-ventilated animal house of the Basic Medical Sciences in Delta State University, Abraka and Emma-maria laboratory Abraka. The study lasted for two (2) weeks.

Methodology: All groups were immunized at day zero and day 5 with SRBC inducing immune response except for the VEH group that was not immunized. Treatment of the various groups with respective treatment agents commenced on day 3 to day 9. On day 10 the antioxidant enzymes {Determination of Superoxide Dismutase (SOD); Catalase (CAT); Myeloperoxidase (MPO); Malondialdehyde (MDA); Glutathione Peroxidase (GPx) ; Nitric oxide (NO) and Xanthine Oxidase (XO)} of the brain, liver, kidney and Spleen were carried out.

Results: The findings in this study reveal the antioxidant enhancement by the plant *Justicia carnea* being significant at $P < 0.05$ when compared to the VEH and SRBC. LEV is a standard drug used in this research as immunostimulants.

Conclusion: The study was able to showcase the antioxidants enhancing activities of the plant *Justicia carnea* as evaluated. Pharmacological antioxidants may be developed that might be helpful in immune related diseases.

Key words; *Immunized, Immunostimulant, antigenic, Antioxidants, Free-radicals, Justicia carnea*

1. INTRODUCTION

Oxidative stress is an immunological expression that is characterized by the level of stress expressed by the orga within its systemic components, which result from the stressors such as Reactive Nitrogen Species (RNS), R Oxygen Species (ROS), Free-radicals and other inducers that might be exogenously introduced. ROS is lir hyperactivation in inflammation that can cause tissue damage and pathology [1].
Nevertheless, in attempt to meet with the demand of ROS for the activation of T cells, NADPH oxidase in conjuncti mitochondria activates ROS generation which have direct effect on the activation of B cells production and prolif upon interaction with the receptor of B cell (BCR) [2][3]. In other words, TCR and BCR transduction pathways need generation to illicit well-established inflammatory response.
These findings have been revalidated following in-vivo studies. The engine room for the reactive oxygen species generation has highly been attributed to the mitochondria [4] [5]. Strategically maneuvering the mitochondria using pharmacological and genetic modification approach can reduce the activation of T cells invitro and in-vivo.
Adaptively, upon sensing the presence of pathogens the Cells of T and B pathogen's-specific are proliferated to meet the demand of precipitating, presenting and deactivating the pathogens. In vitro experimental investigation reveals use of pharmacologic antioxidant as being suppressive to the production and proliferation of IL-2 as a result of activated T cell

Comment [A1]: Very extensive. It can be written in no more than 15 words and state the essentials of the study, which are the antioxidant modulating properties of the extract of Justicia Carnea.

Comment [A2]: It is not recommended to define acronyms in the abstract.

Comment [A3]: If you talk about the factors that are related to oxidative stress, it would be more correct to say: the processes triggered by inflammatory cytokines...

Comment [A4]: This is not a result of this study.

Comment [A5]: It was already raised in the results, it is not necessary to repeat it.

Comment [A6]: The acronyms must be defined from the introduction and before using them. Once an acronym is defined, it should be used whenever it refers to that defined word or words.

Comment [A7]: There are more recent concepts that better define what oxidative stress is. This is more than an immunological expression…

Comment [A8]: What pathology?

Comment [A9]: Write in another way so that it maintains agreement with the previous paragraph. May the end of one give way to the beginning of the other.

receptor (TCR) [2]. IL-2 is a player in inflammation processes, whose regulation is required in antiflammatory therapeutic goal.

In this study the antioxidants of interest are Superoxide dismutase (SOD) [6][7], Catalase (CAT) [8][9][10], Myeloperoxidase (MPO), Malondialdehyde (MDA) [11][12], Glutathione peroxidase (GPx) [13], Nitric oxide (NO) [14] and Xanthine Oxidase (XO) [15]. These enzymes are responsible for the clearance of oxidants such as ROS, Reactive Nitrogen Species (RNS) and possible measure of lipid peroxidation. Also, these oxidants are required to initiate certain cellular physiological process like inflammation, apoptosis, and mutagenesis [9][10]. In other words, Sheep Red Blood Cells (SRBCs) being antigenic might elicit the generation of oxidants capable of inducing oxidative stress, a process suspected to occur [16]. Hence the rationale of the study, in which selective organs (Brain, Liver, Kidney and Spleen) of animal such as mice is of focus.

The plant *Justicia carnea* popularly called “hospital too far” phytochemical analysis supplied by previous studies have indication of antioxidant secondary metabolites such as flavonoid, phenol, vitamin C and E and other medicinal enrich phytochemicals [17][18][19]. Going by the phytochemical constituents, the plant might have cytoprotective properties against possible cytotoxic damage by SRBC-induces oxidative stress [16]. Levamisole (LEV) is a standard drug used in most research as immunostimulant, primarily used pharmacological in the past as antihelminth. The idea is to study the influence of immunomodulators such as the standard drug (LEV) and *Justicia carnea* on possible SRBC instigated oxidative stress in an immunological response paradigm.

2. MATERIAL AND METHODS

Determination of Aqueous *J. carnea* Extract Yield

Cool Maceration Extraction Technique; in a suitable loosely covered containers 10 g weight of 600 μm of particle sizes of the pulverized leaves was submerged in distilled water such that the volume of water was noted. The filtrate after 24 hours of soaking was concentrated at 60^0C until a paste-like semi-solid extract was obtained. It was then stored in an air thigh container and was stored in the refrigerator at 4^0C unstill further used [20].

The plant *Justicia carnea* was authenticated by Dr. Akinnibosun Henry Adewale of the Department of Plant Biology and Biotechnology, Herbarium Unit, with Specimen Voucher Number: UBH-J386.

The concentrated extract was weighed and noted as the Yield, and the Percentage (%) Yield was calculated as follows;

Equation 1; % Yield of Aqueous *J. carnea* Extract

$$\text{Percentage Yield } = \frac{\text{Yield (g)}}{\text{Weight of Pulverized plant leaves}} X\ 100$$

Phytochemical analysis

Phytochemical analysis of the plant was carried out according to the methods described by Rahman, [21] to determine the presence of anthraquinones, tannin, saponins, flavonoids, glycosides, terpenoids, steroids and phlobatannins was presence determined by Ejikeme, [22]. Reducing sugar was determine by the method describe by Pedreschi, [23]

Toxicity; Acute LD_{50} Determination

Lorke’s method was used for the LD_{50} determination of the plant extracts [24][25][26].

Preparation of Drugs and Plant Extract.

Preparation of Levamisole (Lev 4 mg/mL)

The drug levamisole hydrochloride is in tablet dosage formulation (40 mg) that was obtained from Pharmacy Unit of the Health Centre of Delta State University. Levamisole hydrochloride tablet is formulated in 40 mg. A stock concentration of 4 mg/mL was prepared by dissolving 1 tablet it in 10 mL of Distilled water using 50 mL beaker stirring continuously until the dissolution is ascertained.

Preparation of Aqueous *J. carnea* Extract Stock Concentration

The quantity of aqueous *J. carnea* extract stock concentration was prepared in accordance to the demand at moment of it use. The doses are related to body weight of the mice. A stock solution of 20 mg/mL was prepared by weighing 200 mg of paste-like aqueous *J. carnea* extract and dissolving it in 10 mL of Distilled water using 50 mL beaker stirring continuously until the dissolution is ascertained. Individual volume of extract administration was calculated following the formula;

$$\text{Volume of Extract to be administered } = \frac{\text{Dose(mg/Kg) X Body Weight of mice (G)}}{\text{Extract Conc. (mg/mL) } X\ 1000}$$

Preparation of Antigens

Sheep Red Blood Cells (SRBCs) and Candida albicans are the antigens used in this study. SRBCs is obtain aseptically with a 5 mL syringe from the left ear lobe of a male sheep into an anticoagulant sample container. The SRBCs were washed with sterilized normal saline in 1:3 ratio (SRBCs: Normal Saline) by centrifugation at 3000 rmp for 10 minutes. This process was repeated for three times. The washed cells were suspended in normal saline adjusted to 1 X 10^9 Cells/mL. This suspended solution of SRBCs was used subsequently for the immunization exercise according to the experimental design [25].

Comment [A10]: They should not clarify what is in this study specifically, in case they wish to do so it will be in the Methods.

Don't hypercite. This text does not need to have so many citations.

Comment [A11]: Do not use this connector here because it is not clarifying what was said above.

Comment [A12]: The foregoing does not justify that it be in these organs.

Comment [A13]: Why mice and not another animal?

Comment [A14]: I recommend separating it from the previous paragraph because it deals with a new idea.

Comment [A15]: The objective...

Comment [A16]: They have unnecessarily cited articles. The description of the steps followed do not carry citations.

UNDER PEER REVIEW

Preparation of Animals

The animals used for this study were breed, feed and housed at ambient temperature and well-ventilated animal house of the Basic Medical Sciences in Delta State University, Abraka. They were allowed to acclimatize in their respective groupings in accordance with the experimental design for two (2) weeks. The animals were handled in compliance with the animal use Act as approved by Research Ethics Committee (UPH/CEREMAD/REC/MM74/003) of the University of Port Harcourt. The total of five (5) Wistar Rats weighing between 140 – 160 g and 73 mice weighing (17 – 34g) were used [25].

Administration and Samples Collection

All groups were immunized on day zero and day 5 with SRBC in other to induce immune response except for the VEH group that was not immunized. Treatment of the various groups with respective treatment agents commenced on day 3 to day 9 and on day 10 the animals were sacrificed. The animals were humanely sacrificed via cervical dislocation and surgical peritoneum was done with sterile dissecting kits, the organs (brain, liver, kidney and Spleen) were carefully collected, weighed and placed into a well labelled sample container with cooled phosphate buffer (pH 6.8) solution. The antioxidant enzymes {Determination of Superoxide Dismutase (SOD); Catalase (CAT); Myeloperoxidase (MPO); Malondialdehyde (MDA); Glutathione Peroxidase (GPx)**;** Nitric oxide (NO) and Xanthine Oxidase (XO)} were carried out.

Tissue Homogenization Technique

The test tube was halve-way submerged in an ice-Berge parked beaker, the tissue in the test tube was homogenized with an electrical hand drilling device with a smooth tightly fitted plastic cork. [27][13][28]. The homogenate is centrifuge at 3000rpm for 10 minutes and supernatant is filtered with whatman (90 mm) filter paper into a well labelled container and kept in refrigerator for subsequent analysis.

Determination of the Average Body Weight of Animals

The animals' average body weight was calculated for each group (n=5) for Day 0, 2, 4, 6, 8, and 10 follows the formula;

$$\text{Average body weight of the individual animal} = \frac{\text{(Sum of individual body weight of animal taken over the duration of study)}}{\text{(the number of time interval the weights were taken)}}$$

Note; this process was done for all groups in the study, data were further analyzed using statistical tools such as ANOVA also multiple comparison using TurkeyHSD post hoc.

Determination of the Percentage (%) Change in Body Weight

From the animal weight taken the **%** change in body weight of the experimental animals was calculated as:

$$\text{The \% change in body weight of the experimental animals} = \frac{\text{Final body weight} - \text{initial body weight}}{\text{final body weight}}\, X\, 100$$

Note that, weight of subsequent day interval weight of the animal (day 2, 4, 6, 8, 10) was regarded as the final body weight peculiar to that day – day 0 of the same animal was regarded as initial body weight / weight of subsequent day interval of the animals (day 2, 4, 6, 8, 10).

Determination of the Percentage (%) Relative Organ Weight

The Experimental animals (mice) in their respective grouping under study were humanely sacrificed via Cervical Dislocation as stated earlier, and organs (Brain, Liver, Kidney and Spleen) were carefully harvested, weighed (in grams) and transported into Phosphate Buffer of pH 6.8 at temperature of 4ºC in the sample container, immediately were homogenized as explained earlier and then undergo biochemical analysis [29][30]. The formula to determine Percentage Relative Organ Weight is as follows;

$$\text{\% Relative Organ Weight } = \frac{Organ\ weight}{Live\ Body\ Weight\ of\ the\ Animal}\, X\, 100$$

In-vivo Antioxidant Biochemical Analysis

Determination of Superoxide Dismutase (SOD) Activities

SOD activities on Homogenate of Tissue are spectrophotometric based, principled on indirect inhibition of the substrate epinephrine oxidation to adrenochrome by 50 % for every of its 1 unit. This reaction occurs over 60 seconds. As describe by Sun and Zigma, [31].

Determination of Catalase (CAT) Activities

The catalase activity was determined by spectrophotometric at 620 nm and was expressed as µmoles of H_2O_2 consumed/min/mg protein at 25ºC [32].

Determination of Myeloperoxidase (MPO) Activities

Myeloperoxidase (MPO) activity of supernatant of tissue homogenates were determine following Bradley methods [33].

Determination of Malondialdehyde (MDA) Activities

MDA determination was described by Gutteridge and Wilkins as pinpointed by lipid peroxidation product a Thiobarbituric Acid Reactive Substance (TBARS) that can be measured spectrophotometrically [34]. The principle of this test is premise on the fact that lipid peroxidation generates the generation of Malondialdehyde (MDA) from polyunsaturated fatty acid (PUFA) which is reactive with barbituric acid forming red coloration that is measured at wavelength 532 nm. The formed MDA concentration is proportional to absorbance.

Determination of Glutathione Peroxidase (GPx) Activity

UNDER PEER REVIEW

Glutathione Peroxidase (GPx) Activity of supernatant of tissue homogenates were determine as described Lawrence and Burk [35]

Scavenging of Nitric oxide Assay

The chelating of nitric oxide ability of the supernatant of tissue homogenate was adopted from the method explained by Green, and colleagues [36]. The principle behind this method is the synthesis of nitric oxide from Sodium nitroprusside in an aqueous physiological pH environment. The nitric oxide reaction with oxygen produces nitrite ions that forms coloration with Griess reagents measured spectrometrically.

Determination of Xanthine Oxidase Activities

Xanthine Oxidase (XO) Activity of supernatant of tissue homogenates were determined following the description of Hideharu (2013) which is continuous spectrophotometric rate determination [37].

3. RESULTS AND DISCUSSION

The Yield of *J. carnea*

A yield of 15% of the aqueous leave extract of *J. carnea* was obtained.

Phytochemicals analysis result of *J. carnea*

The Phytochemicals of the aqueous leave extract of *J. carnea* results is presented in **table 1** below.

Table 1. Phytochemical's analysis of *J. carnea*

S/N	Phytochemical Analyzed	Status
1	Reducing Sugar	+
2	Glycosides	+
3	Flavonoids	+
4	Saponins	+
5	Alkaloids	+
6	Terpenoids	+
7	Tannins	+
8	Phlobatannins	+
9	Steroids	+

Key; + means present

Acute toxicity

Lock's method results shown in **Table 2** that no death occurred at all doses of the aqueous leave extract of *J. carnea* as well as the vehicle.

Table 2. Result of Acute toxicity Lock's Method

PHASE I			
Dose (mg/Kg)	10	100	1000
Number of Mice Used	3	3	3
Number of Death Recorded	0	0	0
PHASE II			

UNDER PEER REVIEW

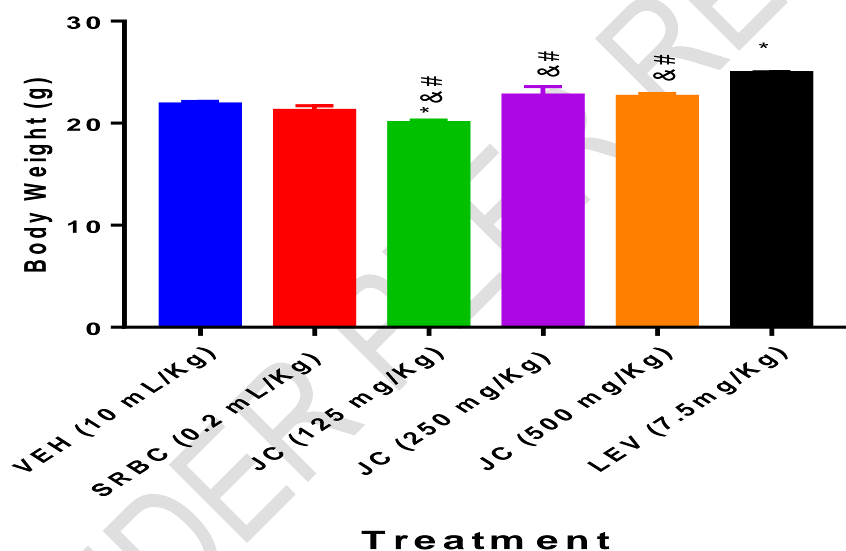
Dose (mg/Kg)	1600	2900	5000
Number of Mice Used	1	1	1
Number of Death Recorded	0	0	0
CONTROL (Vehicle - Water)			
Dose (mL/Kg)			10
Number of Mice Used			1
Number of Death Recorded			0

Key; 0 means no death

Comment [A17]: The result of these tables can be expressed in text form. It is not necessary to make these tables.

The Results Presentation of Body Weight of Animals and Percentage Relative Organs Weight of respective Groups

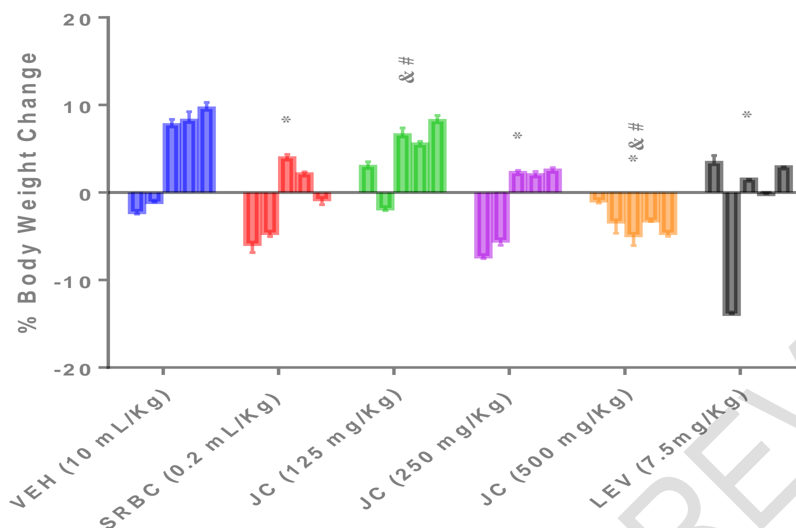
The Body Weight of Animals of respective Groups



VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 1. The Average Body Weight of Animals of Various Groups

UNDER PEER REVIEW



VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 2. % Body Weight Change of Various Groups

The Percentage Relative Organ Weight

The percentage relative organ weight of Brain, Liver, Kidney and Spleen were considered, the results are presented table 3 below.

Table 3. Percentage Relative Organs Weight of Mice.

Treatment / Dose	**Brain (%)** **Mean ± SEM**	**Liver (%)** **Mean ± SEM**	**Kidney (%)** **Mean ± SEM**	**Spleen (%)** **Mean ± SEM**
VEH (10 mL/Kg)	1.5 ± 0.08	4.7 ± 0.04	1.5 ± 0.06	0.79 ± 0.03
SRBCS (0.2 mL/Kg)	$1.3 \pm 0.06^{*}$	$3.9 \pm 0.04^{*}$	1.5 ± 0.05	$0.43 \pm 0.02^{*}$
JC (125mg/Kg)	$1.6 \pm 0.07^{*\&\#}$	$4.1 \pm 0.09^{*}$	$1.0 \pm 0.04^{*\&\#}$	$0.32 \pm 0.01^{*\#}$
JC (250 mg/Kg)	$1.5 \pm 0.02^{\&\#}$	$4.9 \pm 0.04^{\&\#}$	1.7 ± 0.22	$0.62 \pm 0.01^{*\&\#}$
JC (500 mg/Kg)	$1.6 \pm 0.09^{*\&\#}$	$4.9 \pm 0.12^{\&\#}$	1.4 ± 0.05	$1.5 \pm 0.03^{*\&\#}$
LEV (7.5 mg/Kg)	$1.3 \pm 0.05^{*}$	$4.2 \pm 0.18^{*}$	1.5 ± 0.08	$1.0 \pm 0.05^{*}$

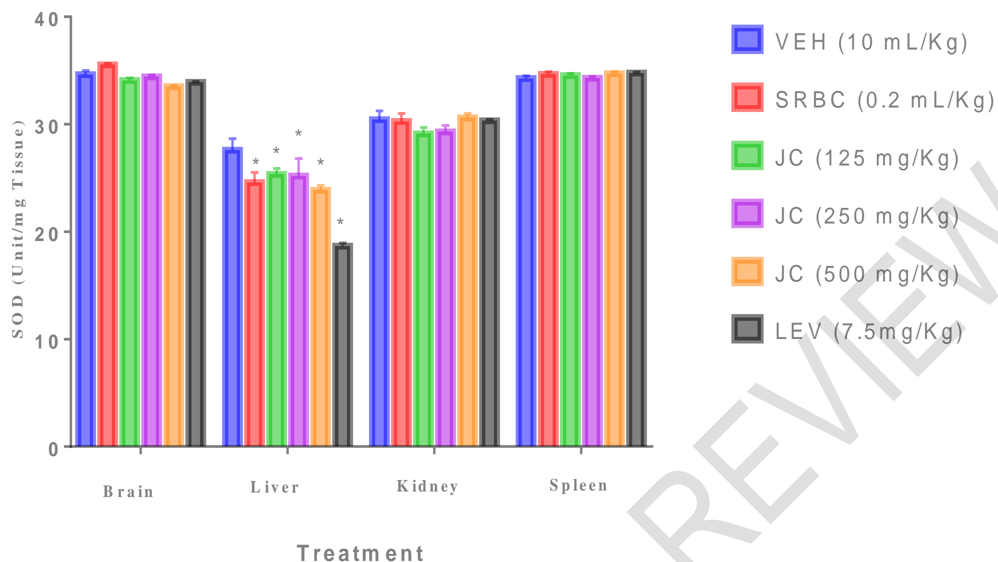
VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

3.4 Biochemical (In-vivo Antioxidants) Analysis

The biochemical assay results presented are as follows; Superoxide dismutase (SOD), Catalase (CAT), Myeloperoxidase (MPO), Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Nitric Oxide (NO), and Xanthine Oxidase (XO).

3.4.1 Superoxide dismutase (SOD)

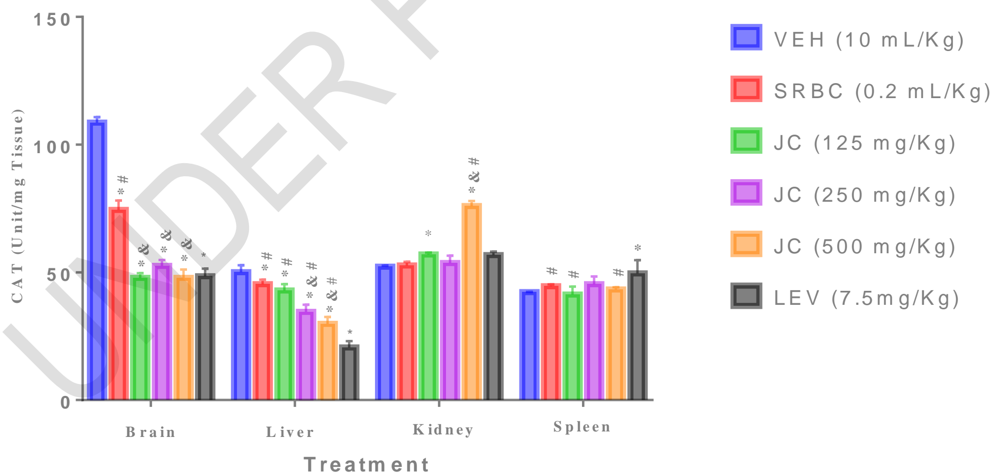
UNDER PEER REVIEW



VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), * indicate significant to VEH.

Fig 3. SOD of Brain, Liver, Kidney and Spleen homogenate

3.4.2 Catalase (CAT)

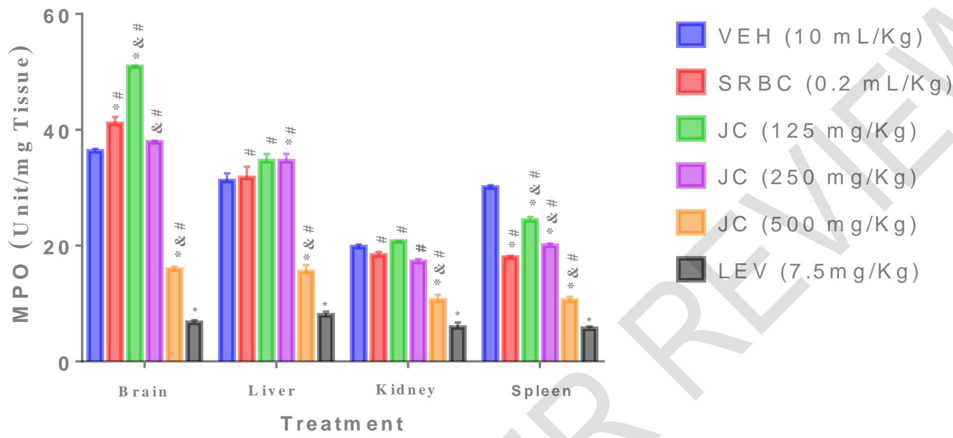


VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 4. CAT of Brain, Liver, Kidney and Spleen homogenate

UNDER PEER REVIEW

3.4.3 Myeloperoxidase (MPO)

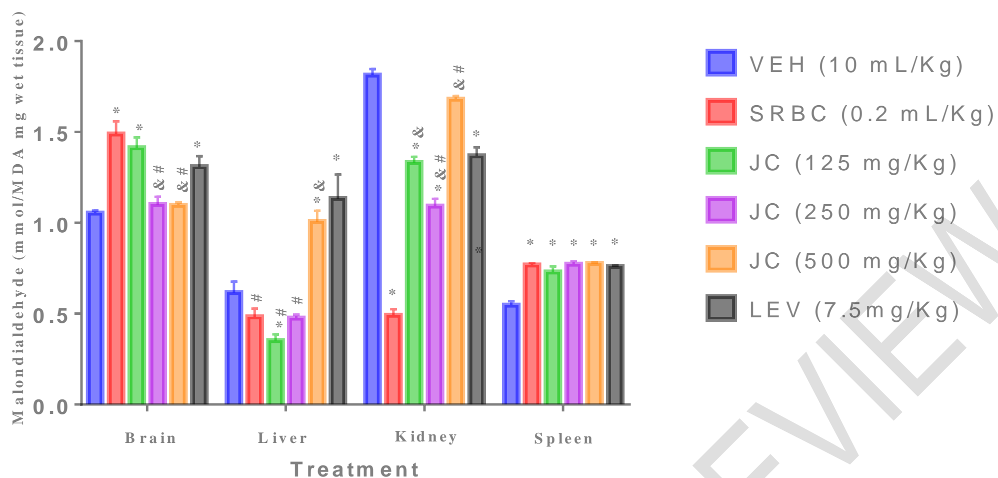


VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 5. MOP of Brain, Liver, Kidney and Spleen homogenate

UNDER PEER REVIEW

3.4.4 Malondialdehyde (MDA)

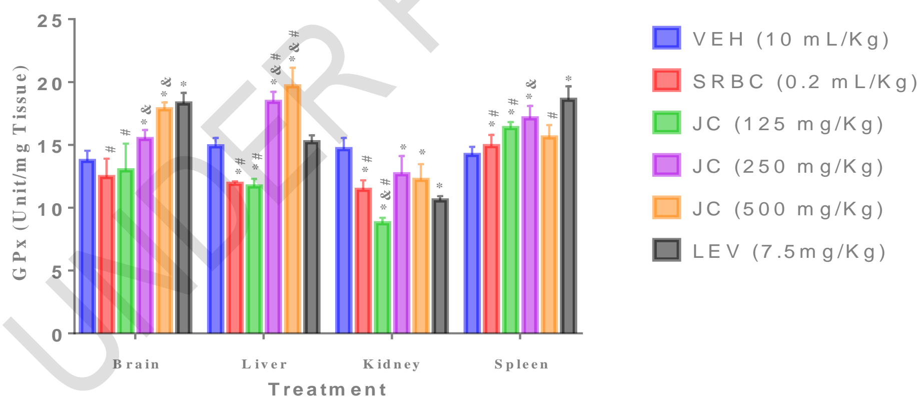


VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 6. MDA of Brain, Liver, Kidney and Spleen homogenate

UNDER PEER REVIEW

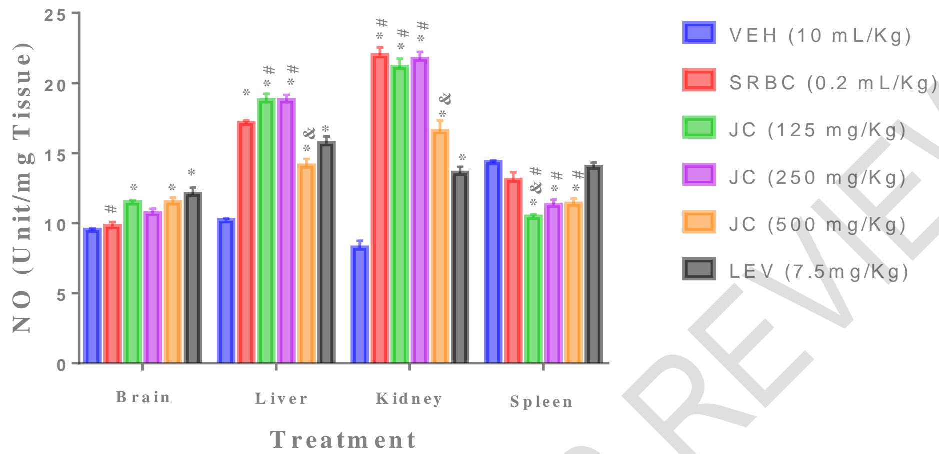
3.4.5 Glutathanione Peroxidase (GPx)



VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 7. GPx of Brain, Liver, Kidney and Spleen homogenate

3.4.6 Nitric Oxide (NO)

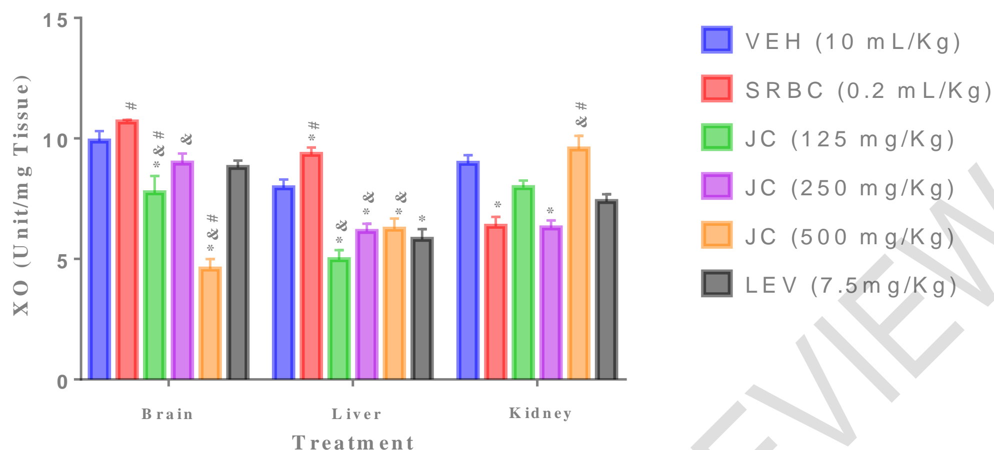


VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 8. NO of Brain, Liver, Kidney and Spleen homogenate

3.4.7 Xanthine Oxidase (XO)

UNDER PEER REVIEW



VEH; Vehicle, JC; *Justicia carnea*, LEV; Levamisole, SRBCs; Sheep Red Blood Cell. Mean ± SEM (n = 5), '*, &, and #' indicate significant to VEH, SRBCs, and LEV respectively.

Fig 9. XO of Brain, Liver and Kidney homogenate

The Body Weight of Animals and Percentage Relative Organs Weight of Respective Groups

The Average Body Weight of Animals of respective Groups

In **Fig 1** the body weight of the treatment group shows that there is no significant difference between the vehicle versus SRBCS, JC 250 mg/mL, and JC 500 mg/mL. However, there was significant difference between the vehicle versus JC 125 mg/mL and Lev 7.5 mg/mL. In addition, there is also significant difference between SRBCS versus JC 250 mg/mL, JC 250 mg/mL, JC 500 mg/mL and Lev 7.5 mg/mL. The latter is significant to JC 250 mg/mL, JC 250 mg/mL, JC 500 mg/mL. The statistical analysis was done at P < 0.05.

Fig 2 All groups showed significantly reduced % Body Weight Change when compared to the VEH group except for JC 125 mg/mL group that is not significant at P < 0.05. However, there are significant difference between JC 125 mg/mL group versus SRBC and LEV groups as well as JC 500 mg/mL group versus SRBC and LEV groups at P < 0.05.

The Percentage Relative Brain Weight

The Percentage Relative Brain Weight was computed with respect to life animal weight in all groups at P < 0.05. The comparative studies among the various treatment groups are to compare the effect of the treatments on the weight of the brain as shown in (**Table 3**). All groups are showed to be significant to the VEH group except for JC 250 mg/Kg that is statistically non-significant. However, the SRBC and LEV groups are significantly lower in their Percentage Relative Brain Weight compared to the VEH group. Meanwhile the three (3) doses of the plant extract have relatively higher Percentage Relative Brain Weights compared to the SRBC and LEV groups.

The Percentage Relative Liver Weight

The Percentage Relative Liver Weight comparative studies among the various groups was done at P < 0.05 as presented in (**Table 3.**). SRBC, JC 125 mg/mL and LEV groups Percentage Relative Liver Weight are significantly lower compared to the VEH group, while JC 250 mg/mL and JC 500 mg/mL groups are statistically non-significant to the VEH group. However, JC 250 mg/mL and JC 500 mg/mL groups are increasingly significant to both SRBC and LEV groups.

The Percentage Relative Kidney Weight

All groups tend to show that there is non-significant effect of Percentage Relative Kidney Weight among the various groups except for JC 125 mg/mL that is significantly reduced compared to the VEH, SRBC and LEV groups at P < 0.05 as expressed in (**Table 3.**).

The Percentage Relative Spleen Weight

UNDER PEER REVIEW

The Percentage Relative Spleen Weight comparative studies among the various groups was done at P < 0.05 see (**Table 3.**). All groups are significant to the VEH group. JC 125 mg/mL, JC 250 mg/mL and JC 500 mg/mL are significant to LEV and while JC 250 mg/mL and JC 500 mg/mL are both significant to SRBC group.

Comment [A18]: What relationship do these weights have with the central theme of the work (the antioxidant activity of...)?

Biochemical (In-vivo Antioxidants) Analysis

Superoxide dismutase (SOD)

Statistically at P < 0.05 as presented in (**Fig 3**). SOD activities were relatively nonsignificant among the various treatment group when compared with VEH and SRBC in the Brain, Kidney and Spleen. However, the various treatment groups were significantly lower when compared to the VEH.

Catalase (CAT)

Statistically at P < 0.05 as presented in (**Fig 4.**). The CAT activities of the SRBC group were significantly reduced in the Brain and Liver and nonsignificant in Kidney and Spleen when compared with VEH. However, the CAT activities of plant extract were significantly lower in the Brain and Liver, significantly higher in the Kidney and nonsignificant in the Spleen when compared to the SRBC and VEH groups.

Myeloperoxidase (MPO)

Statistically at P < 0.05 as presented in (**Fig 5.**) MPO activities of the SRBC group was significantly high in Brain, nonsignificant difference in Liver and Kidney and significantly lower in Spleen when compared to VEH. In all organs the plant extract was able to significantly reduce MPO activities when compared with SRBC and VEH.

Malondialdehyde (MDA)

Statistically at P < 0.05 as presented in (**Fig 6.**) MDA activities of the SRBC group was significantly increased in the Brain and Spleen when compared to the VEH, whereas there was decrease in the Liver and Kidney that was significant only in the latter when compared to the VEH. Extract of *Justicia carnea* was able to significantly reduced MDA activities in the Brain. However, MDA activities of the extract and standard drug LEV was significantly high in the Liver when compared to VEH and SRBC. The plant extract was significantly higher than the SRBC but nonsignificant when compared to VEH. The plant extract was nonsignificant when compared to SRBC in the Spleen but significantly higher than the VEH.

Glutathanione Peroxidase (GPx)

Statistically at P < 0.05 as presented in (**Fig 7.**) GPx activities were significantly reduced by SRBC in the Brain, Liver and Kidney except for Spleen where there was significant increase when compared to the VEH. The plant extract was shown to significantly increase the GPx activities in the Brain, Liver, Kidney and Spleen with respect to VEH and SRBC.

Nitric Oxide (NO)

Statistically at P < 0.05 as presented in (**Fig 8.**) NO in the SRBC group indicates no significant difference in the Brain and Spleen when compared to the VEH. However, there was significant increment of NO in the SRBC group of the Liver and Kidney. Furthermore, the plant extract (*Justicia carnea*) was able to significantly reduce NO in later two organs mentioned.

Xanthine Oxidase (XO)

Statistically at P < 0.05 as presented in (**Fig 9.**) Xanthine Oxidase activity of SRBC was significantly increased when compared to the VEH. However, the plant extract displays significant reduction of XO of the Brain, Liver and Kidney

Discussion

Phytochemicals

The phytochemicals screening indicates the presence of ample of phytochemicals which are in accord with previous research findings of leaves extract of *Justicia carnea* [30][19].

Flavonoids are phytochemical present in Justicia carnea its claim of having antioxidant property was recently reported [19][38]. The anti-inflammatory property of flavonoids was premised on the fact that they can exterminate free radicals such as superoxide anions, hydroxyl radicals and lipid peroxy which might have result from metabolism and oxidative stress. Its helps in the immunity of the human body as the case maybe by modifying, enhancing and increasing response to allergens and other disease-causing mediators like viruses and carcinogenic agents [39][40][19][38].

Phenolic compounds are highly implicated in antioxidant activities, while the flavonoids are quite effective in anti-inflammatory actions. Both phytochemicals are present in the aqueous extract of the plant as showed in the result which was in accordance with the findings of Udedi and colleagues [38].

Discussion of Acute Toxicity Findings

Acute toxicity testing by Lock's method results revealed no death at all doses including the highest dose (5000 mg/Kg) of the aqueous leaves extract of *Justicia carnea* which was in line with the works done by Alozie and colleagues [41]. In accordance with the United State Environmental Protection Agency (U.S. EPA) Toxicity of Chemicals Classification,

UNDER PEER REVIEW

Justicia carnea is considered as Not Acutely Toxic. Similar research done with rat reveal same, even though different laboratory animal (Rat) was used.

Discussion of the Average Body Weight of Animals of respective Groups

From the research the average body weight of the animals at termination day (day 10) pattern shows a stepwise increase which might not necessarily indicate the true change in body weight of the groups. The relative change in weight indicated weight lost with the plant extract at JC 500 mg/Kg. Similarly, weight lost was also recorded in the SRBC group. However, the vehicle and JC 125 mg/Kg groups were able to gain weight. With increase in dose of the plant extract the relative change in weight moves to the negative scale, an indication of weight lost. These results trend suggest that the plant might have antiobesity property [42].

Discussion of the Percentage Relative Organs Weight

The gain in Percentage Relative Brain Weight with respect to the various doses of plant extract groups indicates its effect(s) on the organ which might be a possibility as reported in folk central nervous system protection so claimed [43]. The need to carryout investigation on the behavioral performances on SRBC immunized mice treated with the plant extract might reveal the effects of the mentioned agents on brain functionality. Since SRBC might be possibly considered as clinical toxicosis based on the explanation by Nayakwadi and colleagues as well as other bioanalysis that can be used to establish the degree of functionality of the brain [42]. It also can be hypothesized that the plant extract might have the tendency of either accessing the brain or aid the passage of substances that promote neuronal cells growth. Considering the SRBC and LEV groups where brain atrophy occurred might be attributed to the immunizing agent (SRBC) the resultant effect recognized as an attribute of clinical toxicosis, upon a close comparison with VEH group [42]. Taking a cue from the relative weight lost that occurred also with the SRBC group at the terminal day (day 10). In otherwards one can say that relative weight lost was experienced in both body mass and the brain tissue in the SRBC group.

The reduced weight of the liver observed in groups treated with SRBC, JC 125 mg/mL and LEV groups deducing that there might be lost of hepatic cells as a result of the immune responses of a sort regarded as one of the possible clinical toxicosis as explained by Nayakwadi and colleagues [42]. And the group treated with JC 250 mg/mL and JC 500 mg/mL groups was able to show some kind of hepatoprotective properties by maintaining baseline value of Percentage Relative Liver Weight in spite of the fact that SRBC was used to stimulate the immune reaction noticed in the group administered SRBC alone, the hepatoprotective properties claim can be support by the liver function test result as well the work done by Udedi, and colleagues [38]. Be mindful of the fact that all the groups except the VEH were immunized at Day 0 and Day 5. While the plant extract and standard drug were given at Day 4 through to Day 9.

The reduced Percentage Relative Kidney Weight cannot be clearly explained for the fact that the SRBC group which serves as the antigen for the Negative Control is statistically increasingly significant to the JC 125 mg/Kg, meaning the activities of the Kidney in this group might be alter in some kind sort which was the exceptional case to the works done by Onyeabo, and colleagues [30] in which there was no significant difference in relative percentage weight of the organs.

There is spleen significant weight gain in JC 500 mg/mL group probably due to the trapping of antigens as well as increase in size to meet with the challenge of getting rid of the antigen in circulation. This was also the case observed in the standard drug. The spleen happens to be an organ in the immune response process. The findings which was similar to the works done by Onyeabo, and colleagues [30].

Biochemical (In-vivo Antioxidants) Analysis

In summary, the SOD activities were not significantly altered in all the organs except for the liver. Research have pointed out that SOD expression in the Brain is lower as reported by Crapo [6] which was in line with the findings of this research, also reported was the work done by Ragıp and colleagues [44]. The liver is a major player in the immunological responses in the human body and as well as in animal models. The enzyme NAPDH is activated in the presence of antigen that leads to the production of superoxide in attempt to attack the antigen or any other process that produces superoxide into the physiological environment of the organs [14]. This will then necessitate the activities of the enzymes superoxide dismutase (SOD) to dismutate the reactive oxygen species (ROS) in other to produce hydrogen peroxide H_2O_2 [7].

Catalase enzymes can covert H_2O_2 to H_2O and O_2. The degree of this enzymatic action determines its activities [38]. However, enzymes other than catalase such as glutathione peroxide (GPx) can as well breakdown H_2O_2 [8][10]. In addition, it is expected to have H_2O_2 turnover from SOD activities for CAT and GPx to be optimally functional as will be indicated by the level of activities of these two enzymes [10]. As earlier explained, the brain catalase enzymes might be competing for the substrate with other enzymes sharing the functional role with that of the CAT [10]. CAT activities were more in the JC 500 mg/kg in the kidney, indicating selectivity in expression of activities probably due to decrease activities of other enzymes that can initiate same actions in the kidney [10]. In addition, the dose of the extract appears to contribute to the activities of the enzymes as also witnessed in the study done by Udedi and colleagues [38]. The spleen as an immune organ shows no significant H_2O_2 production increased. It seems catalase activities was relatively within the physiological baseline despite SRBC immunization.

Catalase activities tends to be higher in term of the brain organ compared to other organs. This increased activities as noticed in the SRBC group in the brain compared to that of other organs and also the VEH in the brain compared to other

Comment [A19]: What is the relationship of these results with the antioxidant antioxidant modulating activity of the plant extract?

Comment [A20]: NADPH is not an enzyme. It is a reduced cofactor. They may refer to NADPH oxidase.

Comment [A21]: Do not redefine already defined acronyms.

Comment [A22]: In brackets.

VEH of other organs. The SRBC groups in all organs respectively did not indicate higher CAT activities compared to their respective VEH which indicated that less H_2O_2 was available for it to breakdown.
In the Brain at lesser concentration such as JC 125 mg/kg MPO expression was high which means that there might be increase in the production of HOCl while as the concentration of dose of the plant extract increases there was lesser MPO activities associated with the plant extract groups in a dose-response related fashion where as the standard drug (LEV) group which appears to have the lowest MPO activities [7]. The increase in HOCl might create an unconducive environment for the brain cells most especially the structural integrity of the vascular walls [45].
Interestingly, the Liver function test for JC 500 mg/kg and LEV groups show that the liver is functional and was protected from possible dysfunctional tendency as indicated in SRBC group compared alongside with the VEH group. MPO activities were lowered by the plant extract at JC 500mg/kg and LEV groups.
Meanwhile, in the Kidney MPO activities was at baseline with the VEH group in which no intervention was made. The generation of H_2O_2 and in the presence of halides to generate toxic chemical such as HOCl is highly decreased when the various doses of the plant extract was used which happens to be protective to the kidney, also acknowledged by the works done by Ragıp and colleagues using antioxidant such as Vitamin C [44].
The spleen being an immune organ expressing high activities of MPO as we have in JC 125mg/kg group can help in destroying trapped antigen and again it can also be detrimental to the spleen by the possible cytotoxic generated hypochloride. However, in JC 500mg/kg and Lev groups have a lowered MPO activities which might possibly be devoid of hypothesized cytotoxic attack. Going by this hypothesis the spleen becomes protected in either high or low dose of the plant extract against the SRBC stimulated immune-derived cytotoxic generated compounds as carbon tetrachloride induces increased generation of MPO in reports of Ragıp and colleagues [44]. Meaning SRBC stimulated immune-derived cytotoxicity might have similar consequence to that induced by CCl_4 as recorded by Ukpoabi-Ugo, and colleagues [46] and also clinical toxicosis based on the explanation by Nayakwadi and colleagues [42].
The activities of MPO decreases as the concentration of the plant extract increases across all organs which might be protective. Significant increase of MPO was found in the brain tissue meaning SRBC was able to alter the MPO baseline activity as observed in SRBC group alone as well as that of the JC 125mg/kg group.
The innate immunity might be enhanced by the increased MPO activities which might lead to the generation highly reactive compounds capable of attenuating foreign substance (antigen). This phenomenon was expressed by the plant extract at JC 125 mg/Kg in all tissue examined. However, the higher doses of the plant extract in a dose-dependent fashion implying the concept explained by Odobasic and colleagues [47] might be able to improve the adaptive immunity since the activities of MPO was reduced or inhibited. These abilities can be associated with the plant extract as the result have shown.
Lipid Peroxidation estimation using MDA as the end product has been a yardstick for measurement. In this study the SRBC instigate lipid peroxidation in the brain which was reduced by the plant extract at both JC 250 mg/kg and JC 500 mg/kg significantly to baseline. There was also significant reduction of lipid peroxidation of the plant extract at the later doses when compared to the standard drug. The plant extract reversed the lipid peroxidation that occurred when SRBC was administered which was in line with the works done by Udedi, and colleagues [38] using alloxan to induced diabetic in rat.
Even though result showed increased lipid peroxidation in the liver at JC 500 mg/kg and the standard drug groups, the phenotypical expression in terms of liver functionality appears to be normal, data not supplied. This goes to say that it might be that the trapped antigen underwent peroxidation responses which might be the expression measured.
There was significantly increased lipid peroxidation in the kidney observed in the plant extract groups as well as the in the standard drug when compared to the SRBC group. However, the increase in lipid peroxidation in these groups did not exceed the baseline peroxidation observed in the VEH group. The kidney function test appears normal for the plant extract group, data not supplied.
This result showed that there are peroxidation activities at the spleen level. It should be however be noted that the spleen is an immune organ responsible for the breakdown of antigen tripped.
In summary, MDA is an indication of peroxidation in lipid which was observed in liver and spleen at JC 500mg/kg and Lev groups. The increased activities of MDA of the various tissues might be a sense of concern for consideration most especially in the case of hepatic cells functions, the question will be if the integrity of the organ is intact or it was a mere peroxidation of antigen [48].
GPx an antioxidant that can break down H_2O_2 which is an oxidant capable of causing oxidative stress. The plant extract at JC 250mg/kg, JC 500mg/kg and as well as Lev were able to show case their antioxidant properties in a dose-response relationship fashion in the brain.
There was more expression of GPx in the liver in the plant extract at JC 250mg/kg and JC 500mg/kg groups as well as Lev group. Indicating the protection of the liver by the antioxidant, which may be further validated by the function of the liver assay.
In the kidney the GPx activities are significantly reduced beyond baseline in the group immunized with SRBC and also treated with the plant extract and the standard drug. However, the plant extract at doses JC 250mg/kg and JC 500mg/kg are both at the same level with the SRBC expression of GPx.

UNDER PEER REVIEW

The spleen been an immune organ, expression of GPx actions is significant in JC 250mg/kg and Lev indicating spleen antioxidant properties enhanced by the plant extract and as well as the standard drug 'Lev'.
GPx was well expressed in the all organs in dose dependent fashion for the groups treated with the plant extract compared to the SRBC group. This implies that the organ might be protected by the antioxidant (GPx) activities enhanced by the plant. GPx increased activities can be said to be protective to the various organs which was reduced by the SRBC used in immunized animals. The plant extract was able to reverse oxidant activities posed by the SRBC. This protection is witnessed in a dose-response related fashion. This finding corroborates with the work done by Jaqueline and colleagues [13].
The slight increase in NO in the plant extract related groups as well as the standard drug Lev is an indication that the brain is challenged with possible stressors. Stressors such as NO might be of merit or demerit, depends on the duration and target on the antigen or pathogen in focus with strict restriction against bystanders [49].
The result reveals that NO production is significantly increased in the liver by the SRBC which was reduced by the plant extract at JC 500mg/kg as well as the standard drugs. The standard drugs are statistically non-significant to JC 500mg/kg. The NO production was significantly increased by the immunizing agent (SRBC) compared to the Vehicle group which was significantly reduced by the JC 500mg/kg and the standard drugs Lev groups. However, the reduction was not entirely to baseline as we will have in the VEH group.
The result reveals the suppressive property of the plant extract on NO production in the spleen.
Statistically there was no significant difference in the production of NO by SRBC compared to the VEH in the brain and spleen respectively. However, in the liver and kidney the NO production was high in the SRBC group which might be attributed to the body response to the presence of antigen this response might be of benefit and the same time cytotoxic if not regulated. The plant extract tends to reduce the NO expression which is a function of nitric-oxide-synthase, as noticed in the plant extract groups and also the standard drug group. Following the claim that Isonicotinic acid N-oxide present in ethanolic extract of *Justicia carnea,* having nitric-oxide-synthase inhibitory effect serves as an antidote for anaphylactic reaction, buttresses the findings of this research [43].
The plant extract is distinct in its ability to suppress the activities of XO instituted by the SRBC. However, there was no significant difference in the level of XO activities in the VEH group compared to the SRBC alone. This means that SRBC did not alter the synthesis of NO in the brain.
The expression of XO in the liver by SRBC is significantly increased compared to the VEH group. There was significant reduction of XO activities by the plant extract as well as the standard drug.
The activities of XO were non-significantly altered by JC 500mg/kg of the plant extract when compared to the VEH. In this case the kidney shows relatively lower activities of XO instituted by SRBC. The fact that XO activities helps in the generation of superoxide that requires catalase or glutathione peroxidase to breakdown, which in this case was not beyond the baseline.
Xanthine oxide is implicated in the production of superoxide O_2^- which in this result have revealed that xanthine oxidase activities are reduced in the brain, liver significant but remain the same in the kidney with that of the VEH (i.e., unaltered). This suppressed xanthine oxidase activities might be attributed to the presence of anti-nutrition factors such as phytate in the plant *J. carnea* as reported by Orjikor and colleagues [19]. Xanthine Oxidase (XO) enzyme can facilitate the generation of superoxide from oxygen in the process, hypoxanthine is converted to xanthine, and is further converted to uric acid [14]. Whereas SOD needs the generated superoxide to be converted to hydrogen peroxide. In other words, SOD activity is partly a function of xanthine oxidase. The dose-dependent response observed is in line with the research done by Udedi, and colleagues [38] using the ethanol extract of plant's leave was able to show significant reduction of MDA in alloxan induced diabetic rat and also was able to increase in activities of SOD and CAT. This implies partly that if the activities of xanthine oxidase increase the activities of SOD will also increase provided the enzyme is efficiently and sufficiently available. The relevance of the suppression of xanthine oxidase are emphasized in the oxidative stress patients such as cardiovascular related diseases; stable angina, coronary spasm and inflammatory gout as a result of increase uric acid production.

4. CONCLUSION

This study however was able to reveal that at higher concentration of the plant extract, MPO and MDA activities were decreased in the respective organs, which are key components in both innate and adaptive immune responses. There was dose-dependent increase in GPx activities connoting the enhanced antioxidant activities of the plant extract. The plant extract in a dose-response related fashion was able to reduce the activities of xanthine oxidase. Increase in NO was established by the SRBC which was reversed by the plant extract and as well as the standard drug in their respective groups' organs such as liver, kidney and spleen. The study was able to showcase the antioxidants enhancing activities of the plant Justicia carnea as evaluated. Pharmacological antioxidants may be developed from the plant that may be helpful in immune related diseases. With further focus on the plant, as substantial source for vaccine adjuvants development.

Comment [A23]: ... it's not necessary

UNDER PEER REVIEW

ETHICAL APPROVAL

The animals were handled in compliance with the animal use Act as approved by Research Ethics Committee (UPH/CEREMAD/REC/MM74/003) of the University of Port Harcourt.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

UNDER PEER REVIEW

REFERENCES

References

1. Michael S, Navdeep SC. ROS Function and in Redox Signaling and Oxidative Stress. Curr. Biol, 2014; 24(10): 453-462. https://pubmed.ncbi.nlm.nih.gov/24845678

2. Kaminski M, Kiessling M, Suss D, Krammer PH, Gulow K. Novel role for mitochondria: protein kinase Ctheta-dependent oxidative signaling organelles in activation-induced T-cell death. Mol Cell Biol. 2007; 27:3625–3639. https://doi.org/10.1128/MCB.02295-06

3. Wheeler ML, Defranco AL. Prolonged production of reactive oxygen species in response to B cell receptor stimulation promotes B cell activation and proliferation. J Immunol. 2012; 189:4405–4416. http//doi.org/10.4049/jimmunol.1201433

4. Kaminski MM, Sauer SW, Klemke CD, Suss D, Okun JG, Krammer PH et al. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and IL-4 expression: mechanism of ciprofloxacin mediated immunosuppression. J Immunol. 2010; 184:4827–4841. http//: doi:10.4049/jimmunol.0901662

5. Sena, LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity, 2013; 38:225–236. http//: doi: 10.1016/j.immuni.2012.10.020

6. Crapo JD. Oxidative stress as an initiator of cytokine release and cell damage. Eur Respir J, 2003; 22(44):4-6. http//: doi: 10.1183/09031936.03.00000203a

7. Winterbourn CC, Kettle AJ, Hampton MB. Reactive oxygen species and neutrophil function. Annu. Rev. Biochem., 2016; 85, 765–792. doi: 10.1146/annurev-biochem-060815-014442

8. Halliwell B, Gutteridge JM. “Oxidative stress: adaptation, damage, repair and death,” in Free Radicals in Biology and Medicine, Oxford Science Publications, New York, NY, USA, 3rd edition, 1999; Chapter 2, pp.36–104.

9. Veal E, Day A. “Hydrogen peroxide as a signaling molecule,” Antioxidants & Redox Signaling, 2011; 15(1): 147–151. doi: 10.1089/ars.2011.3968

10. Ankita N, Liang-Jun Y, Chandan KJ, Nilanjana D. Role of Catalase in Oxidative Stress- and Age-Associated Degenerative Diseases. Oxidative Medicine and Cellular Longevity, 2019; 10: 1-19. https://dx.doi: 10.1155/2019/9613090

UNDER PEER REVIEW

11. Całyniuk B, Grochowska-Niedworok E, Walkiewicz KW, Kawecka S, Popiolek E, Fatyga E. Malondialdehyde (MDA) – product of lipid peroxidation as marker of homeostasis disorders and aging. Ann Acad Med Siles, 2016; 70:224–8. https://doi.org/10.18794/aams/65697

12. Ahsani DN, Fidianingsih I. Age-related changes of malondialdehyde, body weight and organ weight in male mice. Univ Med, 2018; 37:115-26. https://doi:10.18051/UNIVMED.2018.V37.115-126

13. Jaqueline DB, Leonardo ST, Fabio EM, Elisabeth CU. The immune system is limited by oxidative stress: Dietary selenium promotes optimal antioxidative status and greatest immune defense in pacu Piaractus mesopotamicus. Fish & Shellfish Immunology, 2015; 47: 360-367. https://doi.org/10.1016/j.fsi.2015.09.022

14. Valko M, Dieter L, Jan M, Mark TD, Cronin MM, Joshua T. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology, 2007; 39: 44–84. https://doi.org/10.1016/j.biocel.2006.07.001

15. Lorne E, Zmijewski JW, Zhao X, Liu G, Tsuruta Y, Park YJ et al. Role of extracellular superoxide in neutrophil activation: Interactions between xanthine oxidase and TLR4 induce proinflammatory cytokine production. Am. J. Physiol. Cell Physiol., 2008; 294: 985–993. PubMed ID 18287332

16. Gregory SL. Primary Immune Response to Sheep Red Blood Cells (SRBC) as the Conventional T-Cell Dependent Antibody Response (TDAR) Test. Journal of Immunotoxicology, 2007; 4:149–152. https://doi.org/10.1080/15476910701337357

17. Fasuyi AO. Nutritional potentials of some tropical vegetable leaf meals. Chemical characterization and functional properties. Afr. J. Biotechnol., 2006; 5: 49–53. http://www.academicjournals.org/AJB

18. Habtamu FG, Negussie R. Antinutritional factors in plant foods: Potential health benefits and adverse effects. International Journal of Nutrition and Food Sciences, 2014; 3(4): 284-289.

19. Orjikor CA, Uroko RI, Njoku OU, Ezeanyika US. Nutritive properties of aqueous extract Justicia carnea leaves and its effects on haematological and some biochemical indices of anaemia induced male wistar albino rats. Biomedical Research, 2019; 30 (4): 645-654. https://dx.doi.org/10.35841/biomedicalresearch.30-18-666

20. Trease GE, Evans WC. Test Book of Pharmacognosy. 11th Edition. London: Brailliare, Tindall and Macmillian Publishers, 1989; 176-180.

21. Rahman G, Syed UJ, Syed F, Samiullah S, & Nusrat J. Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from Ephedra intermedia Indigenous to Balochistan. The Scientific World Journal, 2017; Article ID 5873648. https://doi.org/10.1155/2017/5873648

UNDER PEER REVIEW

22. Ejikeme CM, Ezeonu CS, Eboatu AN. “Determination of physical and phytochemical constituents of some tropical timbers indigenous to Niger Delta Area of Nigeria,” European Scientific Journal, 2014; 10: (18), p. 247–270. https://doi.org/10.19044/esj.2014.v10n18p%25p

23. Pedreschi FM, María S, Granby K. "Current issues in dietary acrylamide: formation, mitigation and risk assessment". Journal of the Science of Food and Agriculture. Society of Chemical Industry, 2013; 94 (1): 9–20. https://doi.org/10.1002/jsfa.6349

24. Lorke DA. New Approach to Practical Acute-Toxicity Testing. Arch. Toxicol., 1983; 53: 275 – 289. https://doi.org/10.1007/BF01234480

25. Okoye CT, Peter AA, Adaobi CE, Philip FU, Uchenna EO, Sebastian OI, et al. Immunomodulatory effects of Stachytarpheta cayennensis leaf extract and its synergistic effect with artesunate. BMC Complementary and Alternative Medicine, 2014; 14; 376. https://doi.org/10.1186/1472-6882-14-376

26. Adumanya O, Obiloma AA, Iwuanyanwu C. Assessment of oral acute toxicity (LD50) of “Green Health Herbal Mixture” in wistar albino rats. International Journal of Advances in Pharmaceutics, 2015; 4(4): 29 – 33.

27. Belle NAV, Dalmolin GD, Fonini G, Rubim MA, Rocha JBT. Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. Brain Res, 2004; 1008:245–51.

28. Abdel-Hakeemi SS, Abdel-Zaher M, Abed GH. Haematological Parameters and Cellular Immune Response Associated with Administration of Polyvalent Schistosoma mansoni Vaccine in Mice. International Journal of Science and Research (IJSR), 2016; 5(12); 1548-1555.

29. Bailey SA, Zidell RH, Perry RW. Relationships Between Organ Weight and Body/Brain Weight in the Rat: What Is the Best Analytical Endpoint? Toxicologic Pathology, 2004; 32:448–466.

30. Onyeabo C, Achi NK, Chima AE, Ebere CU, Okoro CK. Haematological and biochemical studies on Justicia carnea leaves extract on phenylhydrazin induced anemia in albino rats. Acta Sci Pol Technol Aliment, 2017; 16:217-230.

31. Sun M, Zigma S. An Improved Spectrophotometer Assay of Superoxide Antioxidation. Analytical, 1978; 90:81-89.

32. Sinha AK. Colorimetric Assay of Catalase. Analytical Biochemistry, 1972; 47(2):389- 394.

UNDER PEER REVIEW

33. Bradley PD, Friebal DA, Christensen RD. Measurements of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol, 1982; 78: 206–209.

34. Gutteridge JMC, Wilkins S. Copper-Dependent Hydroxyl Radical Damage to Ascorbic Acid: Formation of a Thiobarbituric Acid Reactive Product. FEBS Letters, 1982; 137: 327-330.

35. Lawrence RA, Burk RF. “Glutathione peroxidase activity in selenium-deficient rat liver,” Biochemical and Biophysical Research Communications, 1976; 71(4): 952–958.

36. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem, 1982; 126: 131-138.

37. Hideharu, S. Determination of Xanthine Oxidase. Pharm Anal Acta, 2013; S7: 004

38. Udedi SC, Ani ON, Asogwa KK, Maduji FC, Okafor CN. In-vitro and In-vivo Antioxidant Activity of Ethanol leaf Extract of Justicia carnea. International Journal of Biochemistry Research & Review, 2020; 29(4): 48 – 60.

39. Okwu DE, Josiah C. Evaluation of the chemical composition of two Nigerian medicinal plants. African Journal of Biotechnology, 2006; 4:357-361.

40. Reşat A, Kubilay G, Birsen D, Mustafa Ö, Saliha E, Çelik B et al. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules, 2007; 12:1496-1547.

41. Alozie SC, Wegwu OM, Amadi AB, Amadi UP, Njoku CU. The Modulatory Activity of Justicia carnea in Plasmodium Infected Mice. Trends Journal of Sciences Research, 2018; 3(4):151-160.

42. Nayakwadi S, Ramu R, Kumar SA, Kumar GV, Rajukumar K, Kumar V et al. Toxicopathological studies on the effects of T-2 mycotoxin and their interaction in juvenile goats. PLoS ONE, 2020; 15(3): e0229463.

43. Otuokere IE, Amaku AJ, Igwe KK, Chinedum GC. Medicinal Studies on the Phytochemical Constituents of Justicia carnea by GC-MS Analysis. American Journal of Food Science and Health, 2016; 2(4):71-77.

44. Ragıp B, Haluk D, Hanefi Ö, İrfan B, Mehmet RŞ. (2008). Protective Effects of Antioxidants on the Experimental Liver and Kidney Toxicity in Mice. Eur J Gen Med, 5(3),157-164.

UNDER PEER REVIEW

45. Alexander L, Sascha L, Gianina N, Alva R, Catherine MS, Jun O et al. Myeloperoxidase deficiency ameliorates progression of chronic kidney disease in mice. Am J Physiol Renal Physiol, 2014; 307, 407–417.

46. Ukpabi-Ugo JC, Ndukwe PAC, Iwuoha AG. Hepatoprotective Effect of Methanol Extract of Justicia carnea Leaves on Carbon Tetrachloride-Intoxicated Albino Rats. Biochemistry & Analytical Biochemistry, 2019; 8(2); 381.

47. Odobasic D, Kitching AR, Yang Y, O'Sullivan KM, Muljadi RCM, Edgtton KL et al. Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. Blood, 2013; 121, 4195–4204.

48. Kapun AP, Salobir J, Levart A, Kotnik T, Svete AN. Oxidative stress markers in canine atopic dermatitis. Res. Vet. Sci., 2012; 92: 469–470.

49. Medzhitov R. Origin and physiological roles of inflammation. Nature, 2008; 454: 428–435.

UNDER PEER REVIEW