

ANIMAL MODEL ASSESSMENT OF THE BIOCHEMICAL EFFECT OF ADMINISTRATION OF AQUEOUS EXTRACTS OF *A. INDICA* STEM BARK

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ABSTRACT

This study investigated the biochemical implication of administration of aqueous extracts of *Azadirachta indica* stem bark in animal model. Eighteen (18) Albino rats of first filial generation were used randomly divided into six groups of three rats each based on their body weight. Group A served as the control while group B which served as the safe dose group received 547.23mg/kg body weight in 1mL of extract. In the same manner, group C, D, E and F respectively received 1mL of extract that were 10% below safe dose, 10% above safe dose, 20% above safe dose and 30% above safe dose respectively. Administration was for fourteen (14) days. On the fifteenth (15th) day, all animals were humanely sacrificed and appropriate tissues for analysis were used to assess Lactate dehydrogenase (LDH), Glutathione peroxidase (GPx) or reductase (GSH), Malondialdehyde (MDA), Gamma glutamyltransferase (GGT) among others. Mean values of triplicate analysis were subjected to single analysis of variance and Turkey krammer multiple comparison post hoc test using GraphPad Prism version 7. Results were considered significant at 95% confidence. Group A recorded comparable G-6-PDH activity as group B, 167.21±1.98. Group B recorded the highest GPx activity. GGT observed in group B, 2.32±0.67U/L was significantly (P<0.05) lower than observed in group A, 6.18±1.39U/L. Administration of the extract resulted in increased LDH activity. Evidence from the study suggest5 beneficial effect of usage of adequate dosage of the extract.

KEYWORDS: Biochemical, Tissue, Sacrifice, Gamma Glutamyltransferase, Glucose-6-Phosphate Dehydrogenase.

INTRODUCTION

Azadirachta indica (neem) tree has been admitted by the United States National Assembly of Science as a tree for solving global problems (Biswas *et al.*, 2002). It gives maximum useful non-wood products like leaves, barks, gum, oil, cake, flowers, fruits or seeds than any other tree species known to have antiallergic, antifungal, antibiotic, antidermatic, antibacterial, anti-inflammatory, insecticidal, larvicidal, antimalarial, antiulcer and other biological activities (Biswas *et al.*, 2002). Due to these activities, neem has found several applications making it a green treasure. *In vivo* studies have strongly suggested that extracts of *A. indica* possesses significant anticancer activities (Arivazhaganet *et al.*, 2003; Subapriya *et al.*, 2003). The bark of *A. indica* is known to possess tannins, phenolic compounds like salicylic acid and gallic acid reported to have anti-inflammatory principles (Lewis, 2001; Isaac *et al.*, 2011).

A study has established that the safe dose of administration of aqueous *A. indica* stem bark is

547.723mg/kg body weight in animal model (Chukwu, 2019).

In this study, the effect of administration of different doses of aqueous extracts of stem bark of *A. indica* was investigated in animal model. The focus was on biochemical consequences. For instance, the level of glucose-6-phosphate dehydrogenase (G-6-PDH, E.C.1.1.1.49), a rate limiting enzyme in pentose phosphate pathway (Kornberg and Horecker, 1956). G-6-PDH deficiency has been associated with hemolysis and lipid metabolism (Horton, 2002; Nkhoma *et al.*, 2009). Also investigated in this study is lactate dehydrogenase (LDH) activity. It was used to assess the integrity of the reversible reaction involving the oxidation of lactate to pyruvate (Stryer, 1982). Also it was used to assess the wholesomeness of the liver, heart, erythrocytes, skeletal muscle and kidney (Calbreath, 1992). This is because when these organs are dysfunctional as in conditions of renal infarction, myocardial infarction and hemolysis significant elevation in total blood LDH activity is observed. Other analyte used to assess the integrity of the

usage of the extract include haemoglobin level, blood urea nitrogen (BUN), glutathione peroxidase/reductase and MDA amongst others.

MATERIALS AND METHODS

Apparatus

Centrifuge (centrifuge 80-3 Lab Science, England), Micropipette (Huma Pette Smart Line, Germany), Spectrophotometer (721 visible spectrophotometer, PEC medicinal U.S.A), Weighing Balance (Shimadzu, TX323L, England). Other devices and apparatus are of medical/analytical standard.

Reagent

All reagents used are of high analytical/medical grades and included Draklin's solution, cyanmethemoglobin standard, Dettol antiseptic, Chloroform (British Drug House, Poole, UK), Alkaline Phosphatase kit and sodium carbonate.

Collection of Sample

The stem bark of *Azadirachta indica* was obtained from 3 different trees in Ewu, Esan Central L.G.A, Edo state, Nigeria (6.8058° N, 6.2270° E). The trees were identified and authenticated by a Taxonomist in Botany Department, Ambrose Alli University, Ekpoma, Edo state, Nigeria.

Processing of Samples

The aqueous extracts of *Azadirachta indica* stem bark was prepared according to the method described by

Ashata *et al.* (2012) with slight modification. The stem bark was cut into pieces, oven dried at 40°C to a constant weight. Sample was then grounded or pulverized in a hammer mill sieved through 1mm diameter pores and 40g soaked in 100ml of distilled deionized water for 72 hours. The mixture was thereafter filtered through Whatman filter paper number I. The filtrate was concentrated at 40°C in a rotary evaporator (RES 2X Labscience, England).

The concentrate was then stored in a fridge till required for investigation.

Study Design

Eighteen (18) normotensive male wistar rats used for the investigation were kept in the animal house of Biochemistry Department, Ambrose Alli University, Ekpoma under well ventilated tropical rain forest environment with diurnal variation of light and darkness characteristics of period of June. The animals were allowed free access to feed and water *ad libitum*. The investigation lasted for 14 days after 1 week of acclimatization.

The rats in this study were randomly divided into six (6) groups consisting of three (3) rats each in a group A – F. Group A served as control and was administered distilled water. Group B – F respectively were administered extract of *A. indica* stem bark extracts as shown below.

Group	Treatment	Concentration
A	Control	Distilled water
B	Safe dose (SD)	547.72mg/kg body weight
C	10% below SD	495.95mg/kg body weight
D	10% above SD	602.49mg/kg body weight
E	20% above SD	657.26mg/kg body weight
F	30% above SD	711.89mg/kg body weight

Management of Animals

The animals were managed in accordance with international guidance for the care and use of laboratory animals (NRC, 2011).

Collection of Data

Twenty four (24) hours after the last dose treatment, the animals were anaesthetized with chloroform and humanely sacrificed (Rowell, 1977). Blood sample was collected by cardiac puncture into plain sample bottle/or EDTA bottle. Muscle or bone tissues were excised from left thigh leg of the respective rats. Also portion of liver/heart tissue were excised.

Treatment of Excised Tissues

Respective excised tissues (2g) of muscle or bone were homogenized in mortar containing sand (acid washed and rinsed) and 9ml of 0.1M phosphate buffered saline (pH 6.9).

Processing Blood for Sera/Plasma

Procedure reported by Dave and Lewis (1986) was used. Whole blood obtained by cardiac puncture into sterile test tubes was left for 2 hours then kept at 4°C in a refrigerator overnight for clot retraction and centrifuged (Baird and Tatlock, Mark IV, England).

For the plasma, cardiac puncture in heparin syringe was centrifuged at 3000rpm for 5 minutes to obtain clear slightly yellow supernatant fluid.

Determination of Glutathione Reductase Activity

The method described by Jollow *et al.* (1974) was adopted. An aliquot of each liver homogenate was deproteinized with the addition of an equal volume of sulphosalicylic acid (4%) and centrifuged at 4000 rpm for 5 minutes. Then 50µL of each of the supernatants obtained was added. 1.5mL of 10mM Ellmans reagent. Absorbance of the respective mixtures were read against

blank at 412nm. Results were calculated from regression equation ($Y = 0.2128x - 4E-06$, $R^2 = 1$).

Determination of Total Protein

Procedure described by Gornall *et al.* (1949) was adopted. Briefly, 0.02ml of 0.9 physiological 0.1M phosphate buffered saline pH 6.9 or 0.02ml plasma or 0.02ml standard serum protein (Randox Laboratories, England) were placed into three different test tubes. Subsequently 1ml Biuret reagent was added respectively to each test tube. The mixtures were allowed to stay for 30 minutes at room temperature.

Then absorbance of standard or sample (plasma) were respectively read against blank at 540nm. Total protein was calculated from the relationship.

$$\frac{A_p}{A_s} \times C = g/dl$$

Where A_p = absorbance of plasma

A_s = absorbance of sample

C = concentration of standard

Determination of Gamma Glutamyl Transferase (GGT) Activity

Determination of GGT activity in the serum of respective rats were determined according to colorimetric method described in a manufacturer's guide (Randox Laboratories Ltd, United Kingdom).

The substrate L- γ -glutamyl-3-carboxy-4-nitroamide, in the presence of glycylglycine is converted by GGT in the sample to 5-amino-2-nitrobenzoate which was measured at 405nm in a spectrophotometer. Briefly, 100 μ L of sample was added to 1000 μ L of reagent and mixed. Then the initial absorbance was read at 405nm simultaneously after 30 seconds interval until 2 minutes. GGT activity (U/L) was calculated from the equation.

$$U/L = 1158 \times \Delta Abs \text{ 405nm/min}$$

Where ΔAbs = change in absorbance per 30 seconds

Determination of Glutathione Peroxidase (GPx) Activity

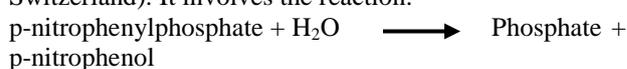
Determination of glutathione peroxidase (GPx) activity was determined with the method of Rotruck *et al.* (1997). The reaction mixture contained 500 μ L 0.1M phosphate buffer pH 6, 100 μ L of 10mM sodium azide, 200 μ L of 4mM GSH, 100 μ L of 25mM H_2O_2 and 500 μ L sample. The mixture was incubated for three minutes at 37°C and reaction terminated with addition of 0.5mL of 10% trichloroacetic acid. Then the mixture was centrifuged and the supernatant used to evaluate residual level of GSH after addition of 2mL of 0.3M dipotassium hydrogen phosphate and 1mL of Ellman's reagent. The absorbance was read at 412nm in a spectrophotometer against a blank and the activity of the GPx was expressed as μ g residual GSH.

Determination of Malondialdehyde

The concentration of malondialdehyde (MDA), the end product of lipid peroxidation (LPO) was examined using the method of Varshney and Kale (1990). It involves the reaction between thiobarbituric acid and MDA to form a pink coloured complex. Briefly 0.2mL each from liver homogenate was mixed with Tris-KCl buffer (1.6mL) containing 0.5mL of Trichloroacetic acid (30%). Subsequently, 0.5mL of 0.75% thiobarbituric acid was added to each of the respective test tubes. The mixtures in their respective test tubes were then incubated at 80°C for 45 minutes in a water bath. Thereafter, the mixture were centrifuged for 10 minutes and absorbance of each supernatant was read against blank in a spectrophotometer at 532nm. Lipid peroxidation expressed as μ mol MDA formed/g protein was calculated with a molar extinction coefficient of 0.156 μ M⁻¹cm⁻¹ (Adam-Vizi and Seregi, 1982).

Determination of Alkaline Phosphatase (ALP)

Quantitative *in vitro* determine of alkaline phosphatase in tissue homogenate was carried out using principle described by Reitman and Frankel (1957) contained in the kit of a manufacturers guide (Agappe Diagnostic, Switzerland). It involves the reaction.



Briefly, 10 μ L of sample or standard or blank were placed into different test tubes. Then 1000 μ L of reagent mixture of R1 and R2 mixed equally were added to each tube. The mixtures were incubated for 5 minutes at room temperature before reading the absorbance against blank at 405nm. The ALP in each sample was calculated from the relationship

$$\frac{A_{\text{sample}}}{A_{\text{standard}}} \times 10$$

Determination of Haemoglobin in Blood or Muscle Homogenate

The determination of haemoglobin level was carried out by cyanmethaemoglobin method using Drabkin's solution. Briefly, into test tubes containing 4mg of Drabkin's solution, 0.02mL of blood or muscle tissue homogenate or blank containing 0.1M phosphate buffer saline pH 6.9 were added. The mixture was allowed to stay for 10 minutes at room temperature before reading the absorbance against blank at 540nm.

The haemoglobin concentration of each sample was calculated from the relationship

$$Cb \text{ (g/100mL)} = \frac{(V+0.02)}{0.02} \left(\frac{Ab \times Cx}{As} \right) \frac{1}{10000}$$

In which

C_b = concentration of the haemoglobin in a sample (blood or homogenate)

A_b = Absorbance of the corresponding sample cuvette

C_s = Cyanmethaemoglobin concentration of the standard

V = volume of Drabkin's solution used

As = Absorbance of the standard cyanmethaemoglobin solution

Determination of Blood Urea Nitrogen (BUN)

Modified procedure of Chaney and Marbach (1962) as reported by Kaplan and Szabo (1979) was used. The principle involved three reactions but basically urea is hydrolyzed to ammonium carbonate by urease. The ammonia released from the carbonate by alkali reacts with phenol and sodium hypochlorite to form a blue indophenol that is measured spectrophotometrically at 560nm.

The procedure briefly involved transferring 1.0mL of working urease solution into different test tubes for standard, blank, control or test tubes. Then with ultramicropipette, 10 μ L water and 10 μ L working standard were added to the blank and standard test tubes respectively. Subsequently, 10 μ L appropriate sera was added to control and respective test tubes for test subjects. Then the mixtures were properly mixed, incubated for 5 minutes at 55°C. Thereafter, 1.0mL phenol-nitroferricyanide was added to each test tube followed by addition of 1.0mL alkaline hypochlorite solution and the mixtures were incubated in a water bath at 55°C for 5 minutes before 7mL of water was added to each tube. The absorbance of the resulting mixture was thereafter read at 560nm in a spectrophotometer against blank. BUN was calculated

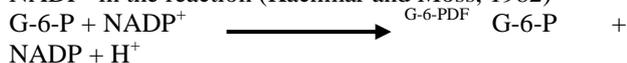
$$\frac{A(\text{test})}{A(\text{standard})} \times C = \text{mg urea N/dL}$$

C = concentration of standard

A = absorbance

Determination of Glucose-6-Phosphate Dehydrogenase (G-6-PDH) Activity

The activity was determined by measurement of the rate of absorbance change at 340nm due to the reduction of NADP⁺ in the reaction (Kachmar and Moss, 1982)



Briefly 0.1mL of plasma or that of bone or muscle homogenate were respectively pipetted into different test tubes. Then, following procedure described in a kit found in a manufacturers guide for quantitative determination of G-6-PDH addition were made to each test tubes of different reagents R1, R2 or R3 at different times. After the addition of 0.05mL of R3, the mixture was poured into a cuvette and their absorbance read against blank at 340nm after 1, 2, 3 and 4 minutes. The activity of G-6-PDH in U/10¹² cell was calculated from the relationship

$$= \frac{(Rt - Rp)}{N \times \frac{1}{0.001}} \times \frac{3.01}{6.22} \times \frac{1}{0.01} \times 10^6$$

Where

Rt = $\Delta A/\text{min}$ in cuvette containing combined GPD + PGD reactions.

Rp = $\Delta A/\text{min}$ in cuvette containing PGD reaction only

0.01 = mL suspension used in assay

N = red cell count of the suspension expressed in 10⁶ cell/ μ L

0.001mL = 1 μ L = volume of suspension containing N x 10⁶ cells

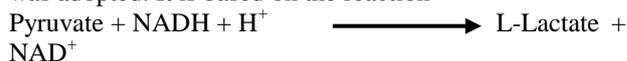
10⁶ = factor to give activity in 10¹² cells

3.01 = total reaction volume in cuvette in mL

6.22 = mmole absorptivity of NADP at 340nm

Determination of Lactate Dehydrogenase (LDH) Activity

The procedure described by Kaplan and Szabo (1979) was adopted. It is based on the reaction



The rate of decrease in absorbance of NADH at 340nm is proportional to LDH activity, Briefly 0.04mL of sample or blank were pipetted into test tubes respectively. Subsequently 1.0mL of reagent as supplied by manufacturer was added. After 5 minutes another reagent was added before leaving the mixture for 30 minutes at room temperature. Within help of a stop watch with the interval of 1 minute absorbance of each were read against blank at 340nm consecutively thrice.

The LDH activity, U/L was calculated from the relationship

$$= \Delta A/\text{min} \times \frac{1}{6.22} \times \frac{0.200\text{mL}}{0.010\text{mL}} \times 1000$$

$$= \Delta A/\text{min} \times 3215$$

Where $\Delta A/\text{min}$ = absorbance change per minute

6.22 = millimolar absorption of NADH at 340nm

0.200mL = total volume in the cuvette

0.010mL = volume of serum sample

1000 = factor to convert millimolar absorption to micromolar

RESULTS

Table 1: Effect of administration of aqueous extracts of *A. indica* stem bark on serum protein, lipid peroxidation and some enzyme activities.

Group	Treatment	GPx xg residual GSH	Serum Protein U/L	Serum GGT U/L	Glutathione reductase x/g/g	MDA nmol/mg	Muscle ALP
A	Control	0.7±0.01 ^b	93.8±0.13 ^a	6.18±1.39 ^{ab}	3.33±0.02 ^b	5.08±0.04 ^c	556.6±18.67 ^a
B	Safe dose (SD)	0.93±0.01 ^a	59.85±0.29 ^b	2.32±0.67 ^a	2.11±0.01 ^a	3.43±0.01 ^a	529.0±13.55 ^a
C	10% below SD	0.78±0.01 ^c	138.2±0.23 ^c	18.53±4.68 ^b	3.68±0.03 ^c	1.11±0.5 ^b	605.36±75.93 ^a
D	10% above SD	0.64±0.01 ^d	38.32±0.23 ^d	2.32±0.67 ^a	3.03±0.03 ^d	3.83±0.01 ^a	222.64±39.11 ^{bc}
E	20% above SD	0.48±0.01 ^e	81.07±0.17 ^e	38.21±1.77 ^c	2.24±0.04 ^a	1.74±0.01 ^b	129.72±29.77 ^c
F	30% above SD	0.93±0.01 ^a	118.2±0.12 ^f	18.53±5.05 ^b	4.39±0.04 ^e	3.51±0.01 ^a	388.24±15.32 ^{ab}

Data is presented as Mean ± Standard error of mean (SEM) of triplicate determinations. Values in the same column with different alphabetic superscripts are considered significantly different (P < 0.05).

Table 2: Effect of administration of aqueous extracts of *A. indica* stem bark on blood parameters, G-6-PDH activity and LDH activity.

Group	Treatment	Haemoglobin blood U/L	Haemoglobin muscle U/L	Blood Urea Nitrogen mg/dL	G-6-PDH plasma	Muscle	Bone	Lactate dehydrogenase U/L
A	Control	1600±0.00	13±0.00	8.13±0.04 ^c	157.78±1.95 ^{ad}	601.37±26.7 ^a	10.7±6.18 ^a	327.4±7.28 ^a
B	Safe dose (SD)	1900±0.00	9±0.00	5.11±0.02 ^a	167.21±1.98 ^d	1109.6±36.68 ^b	644.04±485.5 ^a	124.5±1.38 ^b
C	10% below SD	900±0.00	2±0.00	3.74±0.14 ^b	188.64±7.24 ^b	984.34±23.67 ^c	82.28±5.27 ^a	841.91±2.38 ^c
D	10% above SD	1300±0.00	20±0.00	4.09±0.14 ^b	141.71±0.35 ^{ce}	590.2±20.39 ^a	153.39±7.93 ^a	484.23±44.6 ^d
E	20% above SD	1400±0.00	15±0.00	6.34±0.34 ^d	130.17±1.82 ^c	944.5±30.34 ^c	83.32±2.66 ^a	671.33±7.66 ^e
F	30% above SD	1500±0.00	14±0.00	5.19±0.14 ^a	149.23±1.29 ^{ae}	639.98±12.68 ^a	193.01±5.37 ^a	1013.9±3.64 ^f

Data is presented as Mean ± Standard error of mean (SEM) of triplicate determinations. Values in the same column with different alphabetic superscripts are considered significantly different (P < 0.05).

RESULTS

The result of assessment of effect of administration of extract of *A. indica* stem bark extract in animal model are presented in Table 1 and Table 2.

As shown in Table 1, group B or group F recorded significantly (P < 0.05) higher GPx values compared to as observed in group A. Administration of other doses respectively recorded lower values compared to as seen in control. The serum protein levels observed in groups B, group D or group E respectively were significantly lower than observed in groups administered other doses respectively. Serum GGT level observed in group B was the least and compared to those observed in groups D. Significantly (P < 0.05) higher values were observed in rats in group F or group C which are lesser than observed in group E. Glutathione reductase activity observed in rats in group B or group E were respectively lower when compared to as observed in control subjects i.e. group A, except for group F that recorded higher value compared to as observed in control subjects. The highest MDA content was observed in control subjects. Statistical significantly lower (P < 0.05) values were observed in other studied groups.

Haemoglobin level in blood of rats in group B was the highest. As shown in Table 2, muscle ALP observed in group A was comparable with observed value in group B, group C and group F. Result of other groups recorded significantly (P < 0.05) lower values. Those of the

control group i.e. group A and the other studied subjects were significantly (P < 0.05) less than as observed in group B. However in muscle of group B, haemoglobin content was significantly (P < 0.05) lower than observed in control subjects (group A). Values observed in subjects of group D, group E or group F were respectively higher than observed in control (group A). The highest blood urea nitrogen was observed in group A. Values observed respectively in the other subjects were respectively lower than observed in group A. Plasma G-6-PDH was highest in group C. Value observed in group B was statistically higher than observed in group A. Other studied subject groups respectively recorded statistically (P < 0.05) lower values compared to as observed in group A or group B. All studied subjects except group C recorded higher muscle G-6-PDH compared to as observed in muscle of rats in group A and Muscle of rats in group B recorded the highest G-6-PDH. This group, i.e. group B recorded the highest G-6-PDH level in bone tissue. The bone of group A recorded least G-6-PDH. The LDH in plasma of rats administered any dose of the extract was significantly (P < 0.05) higher than observed in control group i.e. group A except in group B that recorded the least value. The highest value was observed in rats in group F.

DISCUSSION

The use of medicinal plants for treating certain diseases and ailments have been on the increase from time immemorial. They are believed to be nature's pharmacy to us. Their potency depends on their chemical

constituent's ability to elicit beneficial biochemical effect or activity.

Glutathione peroxidase (GPx) (EC·1·11·1·9) is biologically relevant in protection of organisms tissues from oxidative damage (Nachiappan *et al.*, 2010). It reduces lipid hydroperoxide to their corresponding alcohols or free hydrogen peroxides to water (Muthukumar *et al.*, 2011). This activity was highest in group B than observed in control subjects (group A). In subjects in group D or E, lower levels of activity was observed compared to as seen in group A. this indicates reduced enzyme activity associated with accumulation of free radicals and a contributing factor to vitiligo and other diseases (Zedan *et al.*, 2015). High values observed in other groups indicate that the extract when appropriately administered can enhance antioxidant properties of the enzyme GPx to scavenge free radicals. This will help to maintain intracellular homeostasis as well as redox balance.

The serum total protein levels across all experimental groups significantly ($P < 0.05$) differed. This is not in agreement with the findings of Ashata *et al.* (2012) that recorded non-significant ($P > 0.05$) effect on administration of 50, 100, 200 and 300 mg/kg body respectively of ethanolic fraction of *Azadirachta indica* stem bark on serum protein level. The level of the respective extracts are below the least level in this study and might be too low to exert adequate physiological effect.

The evaluation of serum protein is a criterion for assessing the secretory ability and functional capacity of the liver (Naganna, 1989). In this study, group B recorded significantly lower and least functional capacity as revealed by lower level of total serum protein observed compared as seen in group A. This reduction in secretory and functional capacity of the liver was still observed in group D or group E. The liver is an important site for biosynthesis of many proteins (Wudil and Sarki, 2015). The levels of proteins are decreased in hepatic diseases and liver injury (Sturgil and Lambert, 1997; Wudil and Sarki, 2015). Hence there will be need to further study group B for integrity of their liver/kidney function. High serum plasma level seen in group F compared to group A is as in conditions of chronic infection/inflammation as well as in autoimmune disorders and paraproteinemia-myeloma (Marshall, 2012).

Enzymes such as the aminotransferase are membrane bound and leakage from the tissue into the serum occur due to tissue damage or disruption of cell membrane (Ashata *et al.*, 2010). In this study, group C, group E or group F caused significant ($P < 0.05$) increase in GGT levels into their serum when compared with group A. But in group B or group D, reduction was observed. Ashata *et al.* (2010) noticed significant reduction in serum GGT activity in rats when administered 50 or 100

or 200mg/kg body weight of ethanolic extracts of *A. indica* stem bark. Biochemically, serum enzyme measurement offers valuable tool in clinical diagnosis. They provide information on the effect and nature of pathological damage to tissues (Ashata *et al.*, 2010). GGT elevation occurs in liver damage and has been shown to be sensitized in detecting obstructive jaundice, cholangitis, cholecystitis and secondary liver cancer (Tietz, 1987). The low GGT activity noticed in group B or group D when compared to group A could likely present potential protective effect to their liver.

In cellular reaction GSH effectively scavenges free radicals and other reactive oxygen species-hydroxylradical, lipid peroxyradical, peroxylnitrite and H_2O_2 directly and indirectly through enzymatic reaction (Fang *et al.*, 2002; Wu *et al.*, 2004). In such reactions, GSH is oxidized to form GSSG which is then reduced to GSH. The reaction is made possible by the activity of NADPH dependent glutathione reductase (GR). A defect in this activity is reflected in assessment of the activity of this enzyme and point to a threat to the body's antioxidant system.

Evidence from this study points to the fact that the extracts of *A. indica* stem bark has an effect on the activity of glutathione reductase. The result in table 1 showed a significant ($P < 0.05$) reduction in GR activity in the group administered with different doses of the extracts except group F. In a similar studies, there have been various reports of decreases in GR activity in rats administered various doses of extracts of different parts of neem plants- leaves, seed, e.t.c. Dkhil *et al.* (2013) determined the role of *A. indica* treatment on cisplatin-induced hepatotoxicity and oxidative stress in female rats and reported a decline in GR activity along with other antioxidant enzymes – glutathione peroxidase, catalase and superoxide dismutase. Kumbar *et al.* (2012) studied *In vitro* effect of nimbolide an isoprenoid of neem leaf on antioxidant system of rats *Canda epididymis* spermatozoa and also reported a decline in GR activity. The main source of MDA in biological sample is the peroxidation of poly-unsaturated fatty acids (PUFA) with two or more methylene interrupted double bonds (Del-Rio *et al.*, 2005). This is widely used as a reliable biomarker for estimating tissue damage from reactive oxygen species (ROS) and lipid peroxidation. The level of MDA observed in control subjects (group A) is lower than reported in a biochemical study of oxidative stress marker in obese rats (Noeman *et al.*, 2011). However the pattern of change after administration of the extract in this study compared with that observed in the obese rat study that recorded decreased value compared to control (group A). The values observed in group C or group E compared to the values reported for control subjects used in a study of methotrexate oxidative stress in rat kidney tissue (Devrim *et al.*, 2005). The observed decrease in MDA after administration of the different doses of extract suggest protective low lipid peroxidation occasioned by administration of the extract.

Haemoglobin (Hb) is the iron-containing oxygen transport metalloprotein in the red blood cell i.e. erythrocytes of all vertebrates as well as some invertebrates (Maton *et al.*, 1993). Transport of oxygen from the environment to cells and metabolically produced carbon IV oxide and H⁺ in the opposite direction are essential for vertebrate life (Frank *et al.*, 1998). Decrease of haemoglobin with or without an absolute decrease of red blood cells leads to symptoms of anaemia (Fischbach, 2000). Elevated level of haemoglobin are associated with increased numbers or size of red blood cells called polycytherma. The elevation may be caused by congenital heart disease, pulmonary fibrosis, polycythernia vera, smoking, dehydration, advanced lung disease and certain tumors (Barrel, 2017). From the results of this study, administration of extract of *A. indica* stem bark increased the level of haemoglobin in the blood of rats in group B. whereas in other groups, decrease was observed respectively when compared to group A. This suggest that when the extract is administered at an appropriate dose, the constituents that can enhance haemoglobin synthesis exhibited their potential.

In muscle tissues, administration of *A. indica* stem bark extract leads to a decrease in haemoglobin in group C and increase in group D or group E or group F. These implies that the extract when administered could lead to a disruption in the transport of oxygen to muscle tissue. In a similar study, Nwosu *et al.* (2015) after administration of extract of *A. indica* orally at different doses (100mg/kg, 200mg/kg and 300mg/kg) and reported non-significant ($P > 0.05$) increase in haemoglobin content in experimental groups compared to the control subjects. The level of extract used in this study is higher and could be responsible for elicited physiological effect observed.

Urea in urelitic animals provide means for elimination of amino nitrogen (Bishop *et al.*, 2005). It do occur in the mitochondria of liver cells when ammonia is converted to urea via urea cycle (Jain *et al.*, 2016). It is a cyclic process in which ornithine plays a role resembling that of oxaloacetate in Citric Acid Cycle (CAC). A mole of ornithine combines with one mole of ammonia and one mole of carbon IV oxide to form citrulline. In a second reaction, an amino group is added to citrulline to form arginine that is hydrolyzed to urea for elimination (Jain *et al.*, 2014). In this study, administration of the different extract resulted in significantly ($P < 0.05$) lower values in respective groups compared to as observed in control group i.e. group A. The value observed in group A is lower than as reported for control subjects in a similar toxicological study (Asomugha *et al.*, 2013). The pattern of change observed on administration of the respective doses of extracts simulate that reported for a study of the effect of ginger extract on blood urea nitrogen (Modaresi *et al.*, 2007). It have been opined that reduction of BUN in animals suggest a mechanism of reabsorption inhibition of urea in the nephron (Kawamura and Kokko,

1976; Green *et al.*, 1981; Seel and Levy, 1981). The relation of urea to water reabsorption can cause cellular contraction and high concentration of substances like creatinine in plasma (Modaresi *et al.*, 2007). The significant decrease in BUN observed with administration of the different doses can be seen as having protective effect on renal tissue as elevated values have been considered to be toxic to renal tissue (Asomugha *et al.*, 2013).

Glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1.1.1.149) is critical to maintenance of nicotinamide adenine dinucleotide pool and redox homeostasis in the health of an individual (Luzzatto *et al.*, 2016). The enzyme act as a classical oxidoreductase (Stanton, 2012). It is acetylated by elongator complex protein 3(EL P3) at lysine 430, the acetylation inhibit its homodimerization and enzyme activity. However, if it is deacetylated by sirtuin 2 (SIRT 2) at lysine 403, the deacetylation stimulate its enzyme activity. G-6-PDH is highly susceptible to phosphorylation and is believed to be a major reason for the loss of enzyme activity leading to haemolytic anemia (Kirkman and Gaetani, 1986). The balance level of G-6-PDH is required for normal function of cells, whereas increase or decrease causes cell damages (Lee *et al.*, 2011). G-6-PDH is a major enzyme that produce nicotinamide adenine dinucleotide phosphate (NADPH) that plays crucial role in many essential metabolic pathways like lipid metabolism, fatty acid or cholesterol biosynthesis and in control of generation of reactive oxygen species and inflammation (Diaz-flores *et al.*, 2006).

This study investigated the effect of aqueous extracts of *A. indica* on G-6-PDH activity in the blood, right hinge, leg muscle and bones. As shown in Table 2, the activity of G-6-PDH in blood of rats administered the extract were statistically ($P < 0.05$) significantly different. Study subjects in group B and or group C recorded high G-6-PDH activities compared to group A. High G-6-PDH activity helps to protect red blood cells from harmful chemicals or toxins that build up in the body during illness when taking some medications and or food such as Tava bean (Boros *et al.*, 1998). Results for group D or group E or group F that were low compared to those of group A or group B is as in decrease or low level associated with disease conditions such as diabetes mellitus, hemolytic anemia and new born jaundice (Niaza, 1991).

In the muscle, G-6-PDH activity observed in group B, group C, group E or group F recorded high G-6-PDH activity when compared with control. A study has suggested that increased G-6-PDH gives rise to increase in cell growth as occur in mouse embryonic fibroblast (NIH3T3) that stimulate growth of cell (Kuo *et al.*, 2000). Increase in G-6-PDH is also associated with increase in glucose uptake by skeletal muscle (Stanton, 2012). Group D recorded lower G-6-PDH activity compared to group A or group B as in amyotrophic lateral

sclerosis (ALS) (Babu *et al.*, 2018). ALS is a rare group of neurodegenerative disease that occurs due to death of neuron controlling voluntary muscles. It leads to progressively weakening of muscles, muscle atrophy and twitching. The activity of G-6-PDH in bone of rats administered the extract were high when compared with control as occur in growth/normal functioning in cells of bone or as occur in association with provision of ribose-5-phosphate for nucleic acid synthesis (Cappai *et al.*, 2011).

Many different types of cells in the body contain lactate dehydrogenase (LDH) and organs relatively rich in the enzymes are the heart, kidney, liver and muscle (Butt *et al.*, 2002). The enzyme plays an important role in energy production in the body as it helps to convert lactate to pyruvate or pyruvate to lactate (McKee and McKee, 1999). This in turn, leads to more conversion between NAD^+ and NADH which store energy. Generally, administration of the extract resulted in increase in LDH activity. The highest was observed in group B. There are a number of conditions that are known to elevate lactate dehydrogenase activity in blood including bone growth, cancer, meningitis, heart dysfunction, HIV, anemia, liver failure, aortic disorder, brain disorders and muscle damage (Butt *et al.*, 2002). However, lactate dehydrogenase itself typically does not cause health problems rather exposes an underlying disease state.

CONCLUSION

Administration of high doses as in group D to F is likely implicated to cause polycytherma associated with congenital heart diseases, pulmonary fibrosis, lung diseases and tumor (Barrel, 2017). These group also recorded reduction in BUN values compared to control, a condition usually associated with mechanism of reabsorption inhibition of urea in nephron that lead to concentration of substances like creatinine in plasma and cellular contraction. Hence, this study has suggested that adequate usage of appropriate dose of the extract has beneficial effect. Some biochemical implications of sub-acute and acute dosages were also highlighted.

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