Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2371-2377
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.20

Original Research Article

Hepatoprotective and antioxidant properties of the methanol leaf extract of *Diaphananthe bidens* in acetaminophen-induced hepatotoxicity in rats

Patrick E Aba[1], Kenneth O Ugwueze[1], Samuel O Onoja[2]*, Christian O Okorie-Kanu[3], Aruh O Anaga[1]

[1]Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, [2]Department of Veterinary Physiology and Pharmacology, 3Department of Veterinary Pathology, Michael Okpara University of Agriculture, Umudike, Nigeria

For correspondence: **Email: samonreal@yahoo.com, onoja.samuel@mouau.edu.ng; samuelonoja19@yahoo.com*

Sent for review: 3 April 2019 *Revised accepted: 17 October 2019*

Abstract

Purpose: *To investigate the hepatoprotective and antioxidant properties of the methanol extract of Diaphananthe bidens leaf using acetaminophen-induced hepatotoxicity rat model.*
Methods: *Thirty albino Wistar rats, randomly assigned into 6 groups (A - F, n = 5), were used for the study. Groups A and B received distilled water (10 ml/kg), group C received silymarin (0.10 g/kg) while groups D - F received D. bidens extract 0.15, 0.30 and 0.60 g/kg, respectively, for 7 days. On day 8, groups B - F rats received acetaminophen (2 g/kg) orally. About 48 h later, pentobarbitone sodium (0.035 g/kg) was injected intraperitoneally for sleeping time studies. The time of sleep, time of awake and the duration of sleep were recorded. On awakening, blood samples were collected for evaluation of serum biochemical parameters and antioxidant profile. Thereafter, the rats were humanely euthanized and the liver excised for histopathological evaluation. In vitro antioxidant activity of the extract was evaluated using ferric reducing antioxidant power and 2,2-diphenyl-1-picrylhydrazine (DPPH) scavenging assay.*
Results: *Treatment of the rats with D. bidens decreased ($p < 0.05$) malondialdehyde values and activities of ALT, AST and ALP, but increased glutathione levels and catalase activities when compared to negative control group. The extract significantly ($p < 0.05$) decreased sodium pentobarbitone-induced sleeping time relative to the negative control group and produced concentration-dependent increase in antioxidant activities in vitro. The extract protected the hepatocyte against acetaminophen-induced damage.*
Conclusion: *D. bidens extract elicits antioxidant activities in vitro and in vivo and thus, protects rat liver against acetaminophen-induced damage. Therefore, the extract can potentially be developed as a hepatoprotective agent for the clinical management of liver damage.*

Keywords: *Diaphananthe bidens, Hepatoprotection, Antioxidant; Histopathology, Biochemical parameters*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The liver is a very important organ involved in detoxification and biotransformation of xenobiotics [1]. Detoxification render xenobiotic less toxic and sometimes more toxic through lethal synthesis. This is accomplished through conjugation, hydration, sulfation, oxidation and reduction and the resulting metabolite are excreted from the system. The liver also plays a role in synthesis of plasma proteins such as albumin and fibrinogen [2]. The metabolic role of the liver exposes it to insults capable of causing hepatotoxicity [3]. Alcohol, carbon tetrachloride, thioacetamide and drugs like acetaminophen are the most common causes of hepatotoxicity [4]. The toxicity of acetaminophen is occasioned by its toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI) [5]. The NAPQI is normally metabolized by the liver into cysteine and mercaptate complexes which are excreted in the urine, but at high dose and when the liver function is compromised it will accumulate leading to hepatic damage [6].

Acetaminophen-induced liver damage is the major cause of acute liver failure worldwide. This is attributed to overdose and indiscriminate use of acetaminophen [7]. In traditional medicine, hepatotoxicity is managed with herbal preparations. Herbs such as *Azadirachta indica*, *Allium cepa*, *Curcumin longa* and *Aegle marmelos* have been reported to be hepatoprotective [1].

Diaphananthe bidens is an epiphyte or climber belonging to the family Orchidaceae. The plant is used in folklore medicine in the treatment of inflammatory conditions, diabetes mellitus and asthma [8]. The antihyperglycemic activities of *D. bidens* in streptozotocin-induced hyperglycemic rats has been documented [8]. There is dearth of information in scientific literature on the hepatoprotective effects of *D. bidens*, despite the folkloric use of the leaf decoction in the treatment of hepatic conditions in South Eastern, Nigeria.

This study evaluated the possible hepatoprotective and antioxidant properties of methanol extract of *D. bidens* leaf in acetaminophen-induced hepatic damage in rats.

EXPERIMENTAL

Plant collection and identification

The plant material was collected from Eha-Alumona in Nsukka LGA, Enugu State, Nigeria in June 2017 and authenticated by a taxonomist at the Bioresources Development and Conservation Programme (BDCP), Aku Road, Nsukka, Enugu State, Nigeria. The voucher specimen no. was BDCP/H.8910.

Animals

The albino Wistar rats of either sexes, weighing between 140 - 150 g were used in this study. They were obtained from Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka. They were acclimatized for two weeks before the commencement of the experiment. They were fed with pelleted grower feed (Top feed®) and clean drinking water *ad libitum.*

Ethical approval was obtained from Ethical Committee, University of Nigeria (no. ECUN/185442). The rats were managed according to the Guide for the Use and Care of Laboratory Animals of National Research Council [9].

Plant extraction

The leaves of *D. bidens* were air-dried on a laboratory bench at ambient temperature (25 ^{o}C). They were pulverized into a coarse powder using hammer mill (JS- 390, Japan) and 125 g of the powder was soaked in 400 ml of 80% methanol for 48 hours and shaken at 2 h interval. The extract was filtered using Whatmann No 1 filter paper. The filtrate was concentrated *in vaccuo* using rotary evaporator. The extract labeled as methanol extract of *D. bidens* leaf (MEDBL) was kept in a refrigerator at 4 ^{o}C till time of use. The percentage yield was calculated.

Experimental design

Thirty (30) Wistar rats of either sex, weighing 240 - 250 g were randomly assigned into 6 groups (A-F, n = 5). Rats in groups A and B received distilled water (10 ml/kg), group C received silymarin (0.10 g/kg) while groups D-F received MEDBL 0.15, 0.30 and 0.60 g/kg, respectively for 7 days. On the 8th day, 2 g/kg of acetaminophen was administered to the rats in groups B-F. All treatments were *per os*.

Induction of narcosis

Forty-eight hours (48 h) post acetaminophen-induced hepatotoxicity, 0.035 g/kg sodium pentobarbitone was administered intraperito-neally to induce narcosis. The time of injection, time of sleep (when the rat lost reflex), time of awakening (when the rat regains reflex) and the duration of sleep (time between sleep and awakening) were recorded.

Sample collection

On awakening following narcosis, blood samples were collected via the retrobulbar plexus into plain bottles. The blood samples were centrifuged at 4,000 g for 5 min and the sera harvested for serum biochemistry. Thereafter, the rats were humanely sacrificed under mild ether anaesthesia and the liver excised for histopathology studies.

Determination of liver function parameters

The activities of ALT, AST and ALP in serum and serum levels of total protein, albumin, total bilirubin and direct bilirubin were determined spectrometrically with commercially available Randox reagents kit (Randox Laboratories, UK). The serum globulin and conjugated bilirubin levels were calculated using standard formular [10].

In vivo determination of antioxidants

Catalase activity was determined by measuring the formation of chromic acetate from dichromate in the presence of hydrogen peroxide according to the method of Goth [11]. Reduced glutathione in the serum was estimated by measuring its ability to reduce 5,5'-dithiobis (2- nitrobenzoic acid) (DTNB) as described by Moron *et al* [12]. The serum malondialdehyde level was determined by estimating the level of thiobarbituric and reactive substance as described by the method of Stocks and Dormandy [13].

In vitro determination of antioxidants

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of MEDBL was evaluated as described by Mensor *et al* [14]. DPPH, a stable violet coloured free radical, is decolourized by antioxidants to form a stable yellow colour in methanol within 30 minutes.

The ferric reducing antioxidant power (FRAP) of MEDBL was carried out as described by Benzie and Strain [15]. The FRAP assay measures the ability of the extract to reduce iron from the ferric state to the ferrous state.

Histopathological examination

This was done according to the method of Drury *et al* [16]. The tissue was embedded in wax, sectioned, stained with hematoxylin and eosin dye and visualized with electronic microscope at x400 magnification.

Statistical analysis

Data collected were analyzed using one-way-analysis of variance (ANOVA) with the aid of SPSS version 20 and presented as Mean ± SD. The variant Means were separated using Duncan Multiple Range Test (DMRT) Post hoc test $p < 0.05$ was considered significant.

RESULTS

Extract yield

The yield of the extract was 16.9 % w/w and it was pasty brown in consistency.

Effect of MEDBL on Na-pentobarbitone-induced sleeping time

There was significant ($p < 0.05$) increase in sleeping time in the acetaminophen-treated group (B-F) when compared with the untreated group (A). The sleeping time of groups C, E & F were lower when compared with the sleeping time of group B (Fig. 1). There was no significant difference ($p > 0.05$) in the sleeping time of group A and groups C, E & F (Figure 1).

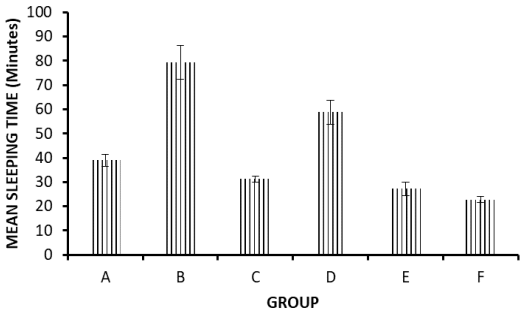

Figure 1: Effect of MEDBL on Na-pentobarbitone-induced sleeping time in acetaminophen –induced liver damage in rats. ***Key:*** A = distilled water 10 ml/kg only; B = distilled water 10 ml/kg + acetaminophen 2 g/kg; C = silymarin 0.10 g/kg + acetaminophen 2 g/kg; D = MEDBL 0.15 g/kg + acetaminophen 2 g/kg, E = MEDBL 0.30 g/kg + acetaminophen 2 g/kg; F = MEDBL 0.60 g/kg + acetaminophen 2 g/kg

Effect of MEDBL on liver function

The serum activities of AST, ALT and ALP of the acetaminophen-treated groups (B - F) were higher ($p < 0.05$) when compared with group A (Table 1). The serum activities of AST, ALT and ALP of groups C, D, E and F were reduced ($p < 0.05$) when compared with group B. There were no significant ($p > 0.05$) differences in total protein levels among the groups. The albumin level of group F was reduced ($p < 0.05$) when compared with other groups while the serum

globulin level of group F was significantly (p < 0.05) increased when compared with other groups (Table 1). There was no significant (p < 0.05) difference in the serum levels of total and indirect bilirubin among the treatment groups when compared with group A. However, the serum direct bilirubin level of group B was significantly (p < 0.05) decreased when compared to other groups (Table 1).

Effect of MEDBL on *in vivo* antioxidant profile

The malondialdehyde levels of groups A, C, D, E and F rats were lower (p < 0.05) when compared with rats in group B (Table 2). However, serum catalase activities and reduced glutathione levels of groups A, C, D, E and F rats were elevated (p < 0.05) when compared with rats in group B (Table 2).

Effect of MEDBL on FRAP and DPPH-based *in vitro* antioxidant profile

MEDBL elicited concentration-dependent increase in FRAP values, though very low when compared with the FRAP values of ascorbic acid (Figure 2). The MEDBL produced concentration-dependent increase in DPPH radicals scavenging activities that was relatively low when compared to DPPH radicals scavenging activities of ascorbic acid (Figure 3).

Effect of MEDBL on histopathology of liver of acetaminophen-poisoned rats

Figure 4 A show the liver from normal rats (group A) showing normal hepatocytes and portal triads. Group B rats showed area of diffused hepatocellular degeneration and necrosis (Figure 4 B). Group C showed fewer area of hepatocyte degeneration relative to group B rats (Figure 4 C). The MEDBL treated groups (groups D-F) showed normal hepatocytes (Figure 4 D - F).

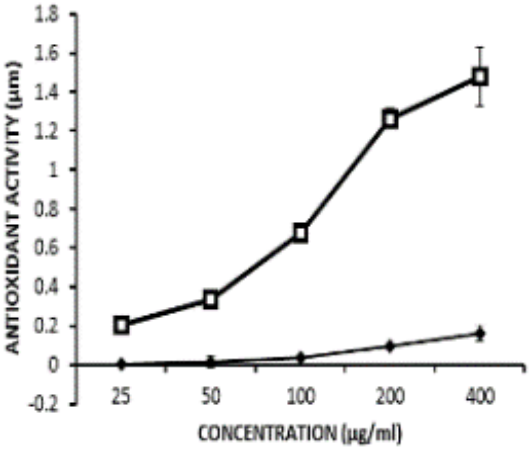

Figure 2: The ferric reducing antioxidant power of MEDBL and ascorbic acid. ***Key:*** -♦-, MEDBL; -□-, ascorbic acid

Table 1: Effect of MEDBL on liver function parameters of acetaminophen-induced hepatotoxicity in rats

Parameter	A	B	C	D	E	F
ALP (IU/L)	53.8± 29.85^{a}	341.7 ± 15.46^{b}	263.50 ±21.26^{a}	264.0 ± 19.59^{a}	256.00 ± 26.36^{ab}	167.00 ±3.17^{a}
ALT (IU/L)	46.35 ± 1.68^{a}	90.79 ± 11.8^{b}	57.05 ± 3.17^{a}	61.89 ± 5.3^{a}	58.30 ± 4.86^{a}	43.02 ± 4.86^{a}
AST (IU/L)	172.5 ± 7.63^{a}	237.0 ± 21.85^{b}	171.70 ± 7.39^{a}	171.4 ± 8.93^{a}	173.9 ± 15.66^{a}	154.7 ± 6.29^{a}
Total protein (g/dL)	6.24±0.35	5.72 ± 0.19	6.62±0.48	6.07±0.18	6.38±0.21	6.30±0.14
Albumin (g/dL)	3.76±0.20^{b}	4.11 ± 0.11^{b}	4.05±0.32^{b}	4.06±0.19^{b}	4.23±0.19^{b}	2.42±0.36^{a}
Globulin (g/dL)	2.48±0.39^{a}	1.61 ± 0.29a	2.56±0.24^{a}	2.01±0.13^{a}	2.14±0.34^{a}	3.97±0.37^{b}
Total bilirubin (mg/dL)	0.35±0.01	0.34 ± 0.06	0.36±0.07	0.34±0.03	0.37±0.02	0.43±0.02
Direct bilirubin (mg/dL)	0.11±0.02^{b}	0.03 ± 0.00^{a}	0.12±0.03^{b}	0.09±0.01^{b}	0.07±0.01^{b}	0.10±0.10^{b}
Indirect bilirubin (mg/dL)	0.24±0.03	0.31±0.06	0.24±0.06	0.25±0.02	0.28±0.03	0.33±0.03

Different letter superscripts along the same row indicate significant difference at p < 0.05. ***Key:*** A = distilled water 10 ml/kg; B = distilled water 10 ml/kg + acetaminophen 2 g/kg; C = silymarin 0.10 g/kg + acetaminophen 2 g/kg; D = MEDBL 0.15 g/kg + acetaminophen 2 g/kg, E = MEDBL 0.30 g/kg + acetaminophen 2 g/kg; F = MEDBL 0.60 g/kg + acetaminophen 2 g/kg

Table 2: Effect of MEDBL on *in vivo* antioxidant profile

Group	A	B	C	D	E	F
Malondialdehyde (AU/ml)	0.02±0.00^{a}	0.06±0.00^{b}	0.03±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}
Catalase (KU/L)	47.14±2.55^{b}	19.08±1.62^{a}	63.96±5.21^{b}	51.07±7.29^{b}	59.62±9.78^{b}	62.36±2.75^{b}
Reduced glutathione (nMoles/ml)	27.12±1.96^{b}	8.73±2.21^{a}	18.61±1.10^{b}	14.75±3.47^{ab}	16.95±1.31^{b}	24.87±10.58^{b}

Different letter superscripts along the same row indicate significant difference *at* p < 0.05. ***Key:*** A = distilled water 10 ml/kg; B = distilled water 10 ml/kg + acetaminophen 2 g/kg; C = silymarin 0.10 g/kg + acetaminophen 2 g/kg; D = MEDBL 0.15 g/kg + acetaminophen 2 g/kg, E = MEDBL 0.30 g/kg + acetaminophen 2 g/kg; F = MEDBL 0.60 g/kg + acetaminophen 2 g/kg

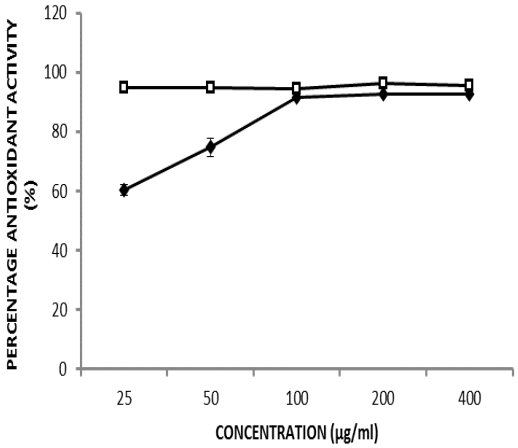

Figure 3: DPPH scavenging activity of MEDBL and ascorbic acid. ***Key:*** -♦-, MEDBL; -□-, ascorbic acid

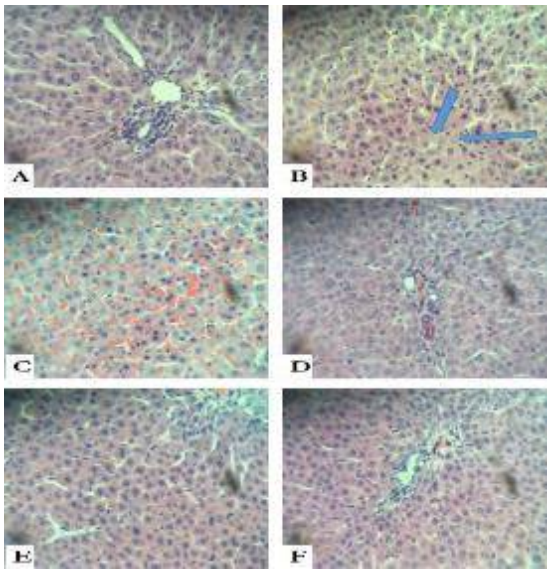

Figure 4: Photomicrographs showing the effect of MEDBL on liver of acetaminophen-poisoned rats (H & E, x400). The arrows show areas of hepatocyte degenerations. ***Key:*** A = distilled water 10 ml/kg; B = distilled water 10 ml/kg + acetaminophen 2 g/kg; C = silymarin 0.10 g/kg + acetaminophen 2 g/kg; D = MEDBL 0.15 g/kg + acetaminophen 2 g/kg, E = MEDBL 0.30 g/kg + acetaminophen 2 g/kg; F = MEDBL 0.60 g/kg + acetaminophen 2 g/kg

DISCUSSION

Hepatoprotective effects and antioxidant properties of *Diaphananthe bidens* were investigated in this study. *Diaphananthe bidens* reduced pentobarbitone-induced sleeping time, elicited antioxidant property and protected the liver against acetaminophen-induced hepatotoxicity in rats.

Treatment of the rats with acetaminophen caused significantly increased serum activities of ALP, AST and ALT in the group B rats when compared with group A rats. This indicates hepatobiliary and hepatocellular damages. Acetaminophen is known to induce liver damage by production of N-acetyl-p-benzoquinoneimine which depletes reduced glutathione level and predisposes to cell membrane fragility and damage [6]. The pretreatment of the rats with MEDBL reduced the serum activities of ALP, AST and ALT in the treated rats relative to the group B rats. This indicates that the extract at the doses used in this study had protective effect on the hepatocytes.

The serum total protein levels in all the groups did not differ statistically. This suggests that the damage induced by acetaminophen may not have affected the synthetic ability of the liver probably because the effect was not prolonged (as the damage lasted for 48 h). Albumin has long half-life of about 19 days [17].

The decreased level of conjugated bilirubin in group B is attributable to hepatic damage that might have impaired bilirubin conjugation [18]. The conjugated bilirubin level of MEDBL- and silymarin-pretreated groups were not statistically different relative to normal control group. This suggests that MEDBL and silymarin protected the liver against the deleterious effects of the acetaminophen and preserved the bilirubin conjugation potentials of the liver.

The MEDBL and silymarin reduced the MDA levels in the treated rats. This suggest that MEDBL enhanced acetaminophen metabolism and reduced the accumulation of N-acetyl-p-benzoquinoneimine that attack cellular organelles and polyunsaturated membrane lipids with resultant elaboration of lipid peroxidation product, MDA [19].

The MEDBL increased the catalase activities and reduced glutathione levels in the treated groups, which indicates that MEDBL stimulated catalase and reduced glutathione release. The antioxidant enzyme, catalase and reduced glutathione, scavenge free radicals and might be responsible for depleted MDA and hepatoprotection in the MEDBL-treated groups. Acetaminophen-induced hepatotoxicity is linked to deleterious effects of reactive metabolite, NAPQI, that causes hepatocyte damage [19]. Pentobarbitone is metabolized mainly in the liver. In the presence of liver damage, pentobarbitone metabolism is compromised due to reduced cytochrome P_{450} level, thus leading to prolonged sleeping time [20]. In this study, the sleeping time of MEDBL treated groups were shorter than the sleeping time of the negative control, group B. The reduced sleeping time could be attributed to enhanced synthesis of enzymes (cytochrome

P_{450}) involved in the metabolism of pentobarbitone [21]. This suggest that MEDBL might have induced enzymes that metabolize acetaminophen, thus inhibited hepatic damage that might have occurred due to accumulation of NAPQI [6].

In the *in vitro* antioxidant studies, the antioxidant property of the extract was more pronounced in DPPH assay than FRAP assay. The FRAP assay measures the ability of the extract to reduce Fe^{3+} to Fe^{2+} while DPPH assay measures the free radical scavenging capacity of the extract [14,15]. DPPH assay is more sensitive and robust than FRAP assay [14,16].

The liver sections of MEDBL-pretreated rats showed normal hepatic architecture. This suggests that the extract protected the hepatocytes against acetaminophen-induced damage. The mechanism of MEDBL hepatoprotection is not known but could be linked to the antioxidant activities. Elevated reduced glutathione levels and catalase activities are capable of protecting cell membranes against oxidative damage [6]. Acetaminophen-induced hepatotoxicity is associated with the generation of N-acetyl-p-benzoquinone imine which produces oxidative damage of hepatocytes [5].

CONCLUSION

The methanol extract of *Diaphananthe bidens* elicited *in vitro* and *in vivo* antioxidant activities and protected the liver against acetaminophen-induced damage in rats. The hepatoprotective effects could be attributed to the antioxidant potentials of the extract. Further studies aimed at isolation and characterization of the active principle of *D. bidens* are recommended.

DECLARATIONS

Acknowledgement

The authors are grateful to Mr AO Ozioko for the collection and identification of the plant material.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

Open Access

REFERENCES

1. *Rane J, Kadhai R, Bakal RL. Liver diseases and herbal drugs: A review. J Innov Pharm Biol Sci 2016; 3: 24-36*
2. *Pradhan SC, Girish C. Hepatoprotective herbal drug-silymarin. From experimental pharmacology to clinical medicine. Indian Med J Resour 2006; 124: 491–504.*
3. *Aba PE, Ozioko IE, Udem ND, Udem SC. Some biochemical and haematological changes in rats pretreated with aqueous stem bark extract of Lophira lanceolata and intoxicated with paracetamol (acetaminophen). J Compl Integr Med 2014; 11(4): 273-277.*
4. *Francesca C. Liver diseases in developing countries. World J Hepatol 2012; 4: 66–67.*
5. *McGill MR, Jaeschke H. Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis. Pharm Res 2013; 30: 2174-2187.*
6. *Yoon E, Barbar A, Choudhary M, Kutner M, Pyrsopoulos A. Acetaminophen–induced hepatotoxicity: a comprehensive update. J Clin Transl Hetatol 2016; 28: 131-142.*
7. *Lancaster EM, Hiatt JR, Zarrinpar A. Acetaminophen hepatotoxicity: an updated review. Arch Toxicol 2015; 89(2): 193-199.*
8. *Anaga AO, Onuoha A, Ogboeli OO, Ezeja M. Antihyperglycaemic effects of methanol leaf extract of Diaphananthe bidens in normoglycemic and streptozotocin-induced hyperglycaemic rats. Asian Pac J Trop Med 2012; 5: 192-196.*
9. *National Research Council. Guide for the care and use of laboratory animals. National Academies Press; 2010.*
10. *Sunheimer RL, Graves L. Clinical Laboratory Chemistry, Pearson Education, New York, 2011: pp 172, 425.*
11. *Goth AK. Colorimetric assay of catalase. Analy Biochem 1972; 47(2): 389-394.*
12. *Moron JH, Tietz NW, Silverman LM. Fundamentals of clinical chemistry. W.B Saunders Co., Philadelphia. 1999: 617-721.*
13. *Stocks J, Dormandy TL. The autoxidation of human red cell lipids induced by hydrogen peroxide. British J Haematol 1971; 20: 95-111.*
14. *Mensor F, Luciana L, Fabia S, Menezes G, Leitao AS, Reis TC, Dos-santos S, Coube S, Leitao G. Screening*

of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 2001; 15: 127-130.

15. *Benzie FF, Strain JJ. Ferric reducing/Antioxidant power Assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 1999; 299: 15-23.*
16. *Drury RA, Wallington A, Cameroun SR. In: Carlleton's Histological Techniques. Oxford University press, New York, 1967; pp 1-420.*
17. *Chaudhury C, Mehnaz S, Robinson JM, Hayton WL, Pearl DK, Roopenian DC, Anderson CL. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J Exp Med 2003; 197: 315-322.*
18. *Thapa BR, Wali A. Liver function test and their interpretation. Indian J Pediatr 2007; 74: 663–671.*
19. *Sies H. Glutathione and its role in cellular functions. Free Radical Biol Med 1999; 27(9-10): 916–921.*
20. *Mujumdar AM, Dhuley JN, Deshmukh VK, Raman PH, Thorat SL, Naik SR. Effect of piperine on pentobarbitone induced hypnosis in rats. Indian J Exp Biol 1990; 28(5): 486-487.*
21. *Singh S, Rehan HM, Majumdar DK. Effect of Ocimum sanctum fixed oil on blood pressure, blood clotting time and pentobarbitone-induced sleeping time. J Ethnopharmacol 2001; 78(2-3): 139-143.*