

African Journal of Pharmacy and Pharmacology

Volume 10 Number 34, 15 September, 2016
ISSN 1996-0816



*Academic
Journals*

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ARTICLES

- Ameliorative effect of grape seed extract on metabolic disorders caused by high fat diet induced obesity in rats by reversing the increase in hepatic miR-33a and miR-122** 699
Hoda E. Mohamed, Dina M. Abo-ELmatty, Same M Saleh and Amr T. Sakr
- Toxicological assessment of the aqueous dried leaf extracts of *Senna alata* L. in wistar rats** 709
Eziuche Amadike Ugbogu, Emmanuel Okezie, Iheanyichukwu Elekwa, Friday Uhegbu, Emmanuel Akubugwo, Chinyere Godwin Chinyere, Faith Ewuzie and Chizoba Jennifer Ugorji
- In vitro* and *in vivo* assessment of genotoxic activity of *Petiveria alliacea*** 718
João Paulo Bastos Silva, Fábio Rodrigues de Oliveira, Thiago Portal da Paixão, Nívea Silva Malcher, Patrícia Carara dos Santos, Ana Cristina Baetas, Maria do Carmo Pimentel Batitucci, Marta Chagas Monteiro and Marcieni Ataíde de Andrade

Full Length Research Paper

Ameliorative effect of grape seed extract on metabolic disorders caused by high fat diet induced obesity in rats by reversing the increase in hepatic miR-33a and miR-122

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Received 16 August, 2016; Accepted 5 September, 2016

MiR-33a and miR-122 are major regulators of lipid metabolism in the liver and their deregulation has been linked to the development of metabolic diseases such as obesity and metabolic syndrome. The aim of this study was to evaluate whether the level of miR-33a and miR-122 in rat liver correlate with obesity and potential anti-obesity effect of grape seed extract (GSE) and calorie restricted diet with special emphasis on dyslipidemia, oxidative stress and inflammation. Rats received high fat diet (HFD) for four months to induce obesity. Animals which had $\geq 30\%$ increase in body weight were selected in this study. Obese rats were divided into 4 groups (n=12/each) and treated for 8 weeks with caloric restriction, GSE (30 mg/kg daily orally) alone or in combined form. Obese rats developed increased body weight and up-regulation of miR-33a and miR-122 in the liver. Also obesity provoked dyslipidemia, oxidative stress and inflammatory status. Importantly, GSE alleviated all deleterious effects of HFD especially when administered with calorie restricted diet. They counteracted the increase of these two miRNAs with improvement in dyslipidemia, oxidative and inflammatory processes. The results reported suggested that beneficial metabolic effects of GSE in combination with dietary treatment could be useful to treat obesity and metabolic disorders.

Key words: Obesity, GSE, dyslipidemia, oxidative stress, hepatic miRNAs

INTRODUCTION

Obesity is a public health concern characterized by excessive fat deposition into adipocytes and non-adipose tissues, which is accompanied by a cluster of chronic metabolic disorders, including cardiovascular diseases,

type 2 diabetes, steatohepatitis and dyslipidemia. Obesity and metabolic disorders are also linked to an overt oxidative stress and chronic inflammatory status. Oxidative stress along with a decline in antioxidant

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defenses cause an irreversible damage to macromolecules (Levine and Stadtman, 2000) and a disruption in redox signaling mechanisms (Kamata and Hirata, 1999). High-fat feeding has commonly been used to induce visceral obesity in rodents because of the similar pathogenesis with abdominal obesity found in human (Katagiri et al., 2007).

Dyslipidemia is a known complication of obesity (Sowers, 2003). Obese children and adults particularly those with a central or abdominal distribution of fat, have elevated concentration of serum triacylglycerol (TAG) surrogate measure of very low density lipoprotein cholesterol (VLDLc) and low concentration of high density lipoprotein cholesterol (HDLc) (Freedman et al., 2002). Obesity is also associated with higher levels of total cholesterol (Tc) and low density lipoprotein cholesterol (LDLc) (Knopp et al., 2008). Obesity enhances oxidative stress in young and old populations as shown by elevations in lipid peroxidation or protein oxidation. Lipid peroxidation is associated with several indices of adiposity and low systemic antioxidant defense that is, antioxidant enzymes, glutathione (GSH) (Vincent et al., 2007). It plays a role in the initiation of inflammation and development of insulin resistance (Tilg and Moschen, 2008). Insulin resistance (IR), hyperinsulinemia and hyperglycemia are linked with obesity (Sowers, 2003). Obesity-induced inflammation results in increased infiltration of macrophages and release of cytokines like tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6) and interleukin-1-beta (IL-1 β) and contributes significantly to insulin resistance (Larsen et al., 2007).

MiRNAs are known to modulate more than 60% of human transcripts and thus play important regulatory roles in a variety of biological processes and are implicated in almost all metabolic pathways (Friedman et al., 2009). Moreover, there is much evidence that the deregulation of miRNAs is related to the development of chronic diseases (Rottiers and Naar, 2012). Specifically, miR-33 and miR-122 are known as major regulators of lipid metabolism in the liver, and their deregulation may contribute to the development of metabolic diseases such as obesity and metabolic syndrome (Rottiers and Naar, 2012; Ramirez et al., 2011). MiR-122 plays a critical role in liver homeostasis by regulating genes with key roles in the synthesis of triglycerides (TGs) and fatty acids (FAs), such as FA synthase (FAS) and sterol regulatory element-binding protein 1C (SREBP1C), as well as genes that regulate FA β -oxidation (Tsai et al., 2012; Hus et al., 2012).

Additionally, miR-33a plays an important role in the regulation of cholesterol homeostasis in the liver, regulating the ATP-binding cassette transporters (ABC transporters) ABCA1 and ABCG1 in addition to its role in FA β -oxidation by targeting the carnitine palmitoyltransferase 1a (CPT1a) (Moor et al., 2011). Since current medical treatments fails to stop the progress of metabolic disorders, polyphenol-rich grape products are being widely investigated as an additional strategy to

combat obesity (Chuang and McIntosh, 2011). The grape seed skin extract (GSSE) exerts numerous biological activities and health-promoting properties such as antioxidant (Belvir'nli et al., 2012), lipid lowering (Quesada et al., 2012), anti-tumor (Nandakumar et al., 2008) and anti-obesity effects (Ohyama et al., 2011) by inhibiting lipid absorption from the intestine which has been shown to occur partly via inhibition of lipase (Moreno et al., 2003).

In the present study, we evaluate whether the level of miR-33a and miR-122 in rat liver correlate with obesity and potential anti-obesity effect of grape seed extract with or without calorie restricted diet with special emphasis on dyslipidemia, oxidative stress and inflammation.

MATERIALS AND METHODS

Chemicals and drugs

Gervital (grape seed extract: GSE) was purchased from Arab Co. for Pharmaceuticals and Medicinal Plants (MEPACO-MEDIFOOD), Sharkeya-Egypt. All other chemicals used in this study were of analytical grade obtained from Sigma Aldrich, USA, unless otherwise noted.

Animals and experimental design

Male Wistar rats (n= 100, 160 \pm 15 g) were purchased from Egyptian Organization for Biologic Products and Vaccines (Cairo, Egypt). All experimental protocols were approved by the Animal Experimental Ethics Committee of Faculty of Pharmacy, Zagazig University. Every effort was made to minimize the number of animal used and their suffering. Rats were housed in stainless steel rodent cages at room temperature (25 \pm 2°C) and with 12 h dark/light cycle. Animals were fed rodent chow and allowed free access to drinking water. One week after acclimatization, eighty eight were switched from rodent chow to high fat diet (25% total fat (including 11% unsaturated fat, 44% carbohydrate, 18% protein and 13% fiber and other ingredients) for four months to induce obesity (Alzoubi et al., 2009). The animals which had \geq 30% increase in body weight were selected in this study. The obese rats were trained to lick suspension of gum acacia in distilled water (1 ml) which was used as the vehicle and randomly divided into four groups: Obese control (OC; n=12, kept on normal chow diet), calorie restricted group (CR; n=12, 25% food restriction of commercial chow) (Luvizotto et al., 2010), gervital group (Ger; n=12 receiving GSE 30 mg/kg daily orally dissolved in distilled water using gum acacia as suspending agent kept in normal chow diet) and calorie restricted + gervital group (CR + Ger; n=12) for 8 weeks. In addition to normal control (NC, n=12).

Biochemical studies

At the end of experimental period, body weight was determined for all groups, then rats were anesthetized with urethane (1.3 g/kg) and blood samples were collected from orbital sinus of rats according to (Sorg and Buckner, 1964) then centrifuged at 3000 rpm for 15 min. Serum was collected, divided into aliquots and stored at -20°C for the determination of total cholesterol (Tc), triacylglycerol (TAG), high density lipoprotein (HDLc) using commercially available kits spinreact sant Esteve de Bas Spain. Low density lipoprotein (LDLc)

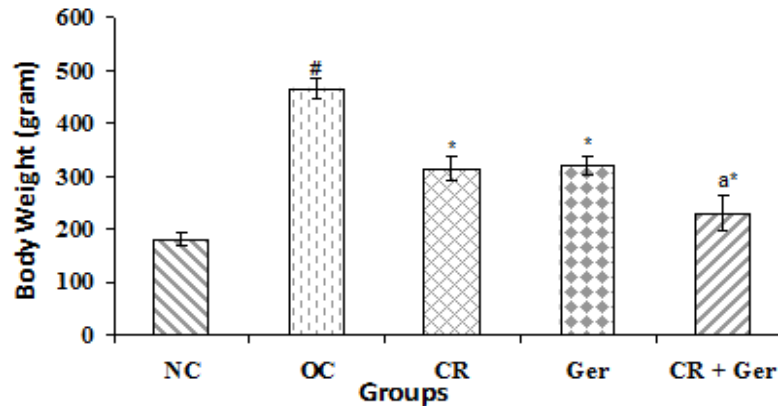


Figure 1. Effect of CR, Ger and their combination on body weight. Values are expressed as mean \pm SD (n=12) ($P < 0.05$). #Significantly different from NC group. *Significantly different from OC. ^aSignificantly different from CR. NC: Normal control, OC: Obese control, CR : Calorie restricted, Ger: Gervital

was calculated according to Friedewald formula (Friedewald et al., 1972):

LDLc (mg/dl): Tc-(HDLc + TAG/5).

Serum glucose level was done using kit according to the method of Trinder (Trinder 1969), serum insulin was estimated by radioimmunoassay technique using insulin kit purchased from Siemens Medical Solutions Diagnostics, Los Angeles, USA according to method of Bennett (Bennett, 1983). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using serum glucose and insulin level according to Matthews et al. (1985) $HOMA-IR = (\text{glucose} \times \text{insulin})/405$. Serum TNF α was assayed according to Chen et al. (1998) by ELISA using rat TNF α kits (Ray Biotech, USA). Serum andiponectin was measured using enzyme assay (ELISA) kits (Invitrogen) according to the manufacturer's instruction.

After blood collection, rats were euthanized by CO₂ asphyxiation, livers were removed, washed with 0.9% NaCl. Part of harvested organs was quickly frozen in liquid nitrogen (-170°C) for analysis of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), total antioxidant capacity (TAC) using spin react kits Spain. Malondialdehyde (MDA) was measured spectrophotometrically by modified method of Buege and Aust (Buege and Aust, 1978). Reverse transcription-polymerase chain reaction (PCR) was used to analyze the miR-33a and miR-122 gene expression. To analyze the expression of each miRNA, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster city, USA). miRNA-Specific reverse-transcription primers were provided by TaqMan MicroRNA Assay (Applied Biosystems, Foster City, USA). For the reverse transcription, a Biometra thermal cycler was used. The reaction was performed at 42°C for 30 min and 95°C for 5 min. A total of 1.33 ml of this diluted cDNA was used in a subsequent quantitative reverse transcriptase polymerase chain reaction (RT-PCR) amplification using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, USA) and the specific probe provided in the TaqMan MicroRNA Assay (Applied Biosystems). Specific Taqman probes were as follows: microRNA-122 (miR-122: hsa-mir-122), 5'-UGGAGUGUGACAAUGGUGUUUG-3', and microRNA-33 (miR-33: hsa-mir-33), 5'-GUGCAUUGUAGUUGCAUUG-3'. The results were normalized with U6 small nuclear RNA (U6 snRNA), 5'-TGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAA GAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTC

GTGAAGCGTTCATATTTT-3', that was used as an endogenous control. Amplification was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, USA) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale of the equation $2Ct$, where $Ct = Ct \text{ miRNA} - Ct \text{ U6}$ and $Ct = Ct$ treated samples Ct untreated controls.

Statistical analysis

All data were measured as mean \pm standard deviation. Results were analyzed by analysis of variance ((ANOVA); 1-way) followed by Tukey Post Hoc test, $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS (version 16 SPSS Inc. Chicago, USA). However the correlations between the studied parameters were assessed using the person's correlation coefficient (r).

RESULTS

Body weight

Body weight increased significantly in obese group by 158.2% after four months high fat diet compared to NC group while significant decreases were observed after 8 weeks in the CR, Ger either individually or in combination by 32, 31 and 50.6%, respectively compared to OC animals and CR + Ger group showed a significant reduction by 27% than CR only ($P < 0.05$) (Figure 1).

Biochemical evaluation

Serum parameters

Serum lipid profile: Obese rats demonstrated significant increase in Tc, TAG, LDLc and atherogenic index by 61.9, 44.6, 161 and 27.6%, respectively associated with

Table 1. Effect of Cr, Ger either individually or in combination in lipid profile in obese rats.

Concentration (mg/dl)	Normal	Obese	CR	Ger	CR + Ger
Tc	84 ± 4.5	136.5±8.1 [#]	109±11.1 [*]	107±10.5 [*]	87.9±6.9 ^{*a}
TAG	62.2 ± 4.5	112±10.2 [#]	88.1±8 [*]	70.9±3.1 [*]	64.1±9.2 ^{*a}
LDLc	34.5 ± 2	88.9 ±1.8 [#]	55.4±4 [*]	57±9.5 [*]	37.2±2 ^{*a}
Atherogenic index	0.93 ± 0.1	3.5±0.4 [#]	1.5±0.17 [*]	1.6±0.28 [*]	1.0±0.07 ^{*a}
HDLc	37.1 ± 4.3	25.2±3.9 [#]	36±6.1 [*]	35.9±6.1 [*]	37.5±3.9 [*]

Values are expressed as mean ± SD (n=12) (P<0.01). [#]Significantly different from NC group. ^{*}Significantly different from OC group. ^aSignificantly different from Cr group

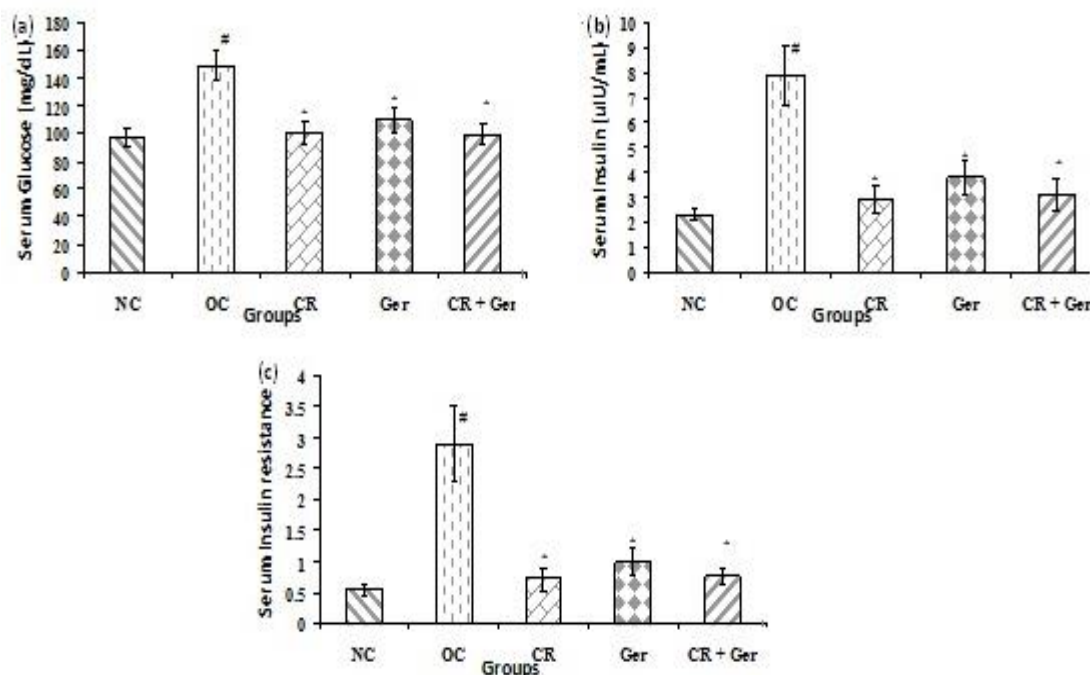


Figure 2. Effect of CR, Ger and their combination on serum glucose, insulin and insulin resistance (HOMA) (a,b,c). Values are expressed as mean ± SD (n=12) (P<0.05). [#]Significantly different from NC group. ^{*}Significantly different from OC. ^aSignificantly different from CR.

significant decrease in HDL by 32.4% in comparison with NC group. These alterations in lipid profile were significantly ameliorated in all treated groups as compared to OC Rats. Co-administration of gervital with caloric restriction significantly lowered Tc, TAG, LDLc and atherogenic index by 20.1, 27.2, 32.7 and 33%, respectively compared to calorie restricted group (P<0.05). The HDLc showed non-significant change (Table 1).

Serum glucose, insulin level: Obese rats had a significant increase in serum glucose level by 52.2% and as compared to NC group. This elevation in glucose level was accompanied by a significant increase in insulin level and insulin resistance by 243.5 and 427%, respectively.

Obese rats received CR or Ger either individually and in combined form showed a remarkable improvement in these parameters in comparison with OC group. Co-administration of gervital with calorie restricted diet demonstrated non significant change in comparison to CR group (P<0.05) (Figure 2).

Serum adiponectin: Obese rats demonstrated significant (P<0.05) decrease in serum adiponectin by 45.5% in comparison with normal group. Treatment with calorie restricted diet or gervital either individually and in combined form significantly increased adiponectin level by 27.7, 44.4 and 42.3%, respectively as compared to obese rats. Combination treatment showed remarkable increase in its level by 34.8% in comparison with CR group

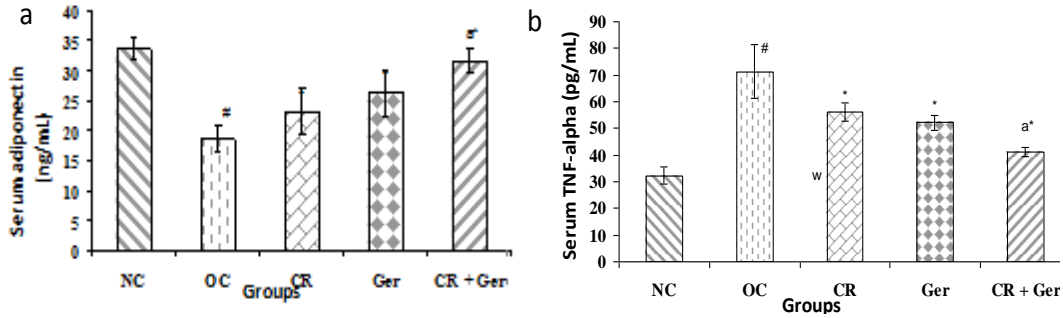


Figure 3. Effect of CR, Ger and their combination on serum adiponectin and TNF α (a,b). Values are expressed as mean \pm SD (n=12) (P<0.05). #Significantly different from NC group. *Significantly different from OC. ^aSignificantly different from CR

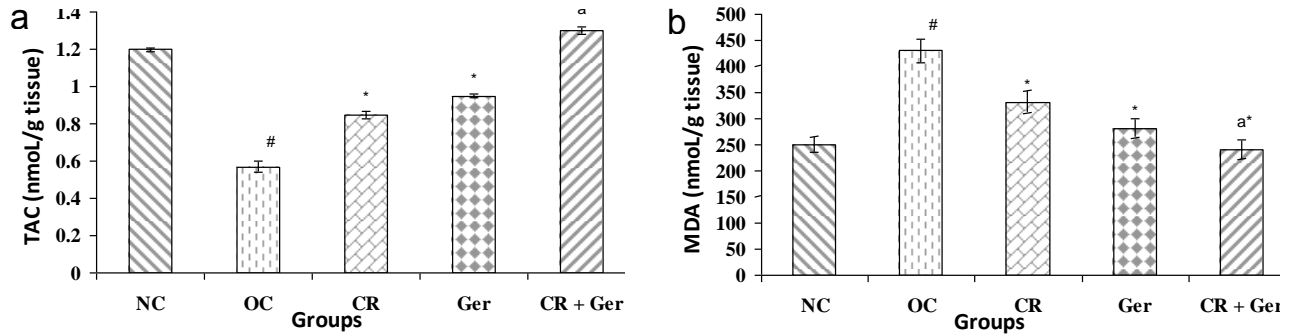


Figure 4. Effect of CR, Ger and their combination on hepatic TAC and MDA (a,b). Values are expressed as means \pm SD (n=12) (P<0.005). #Significantly different from NC group. *Significantly different from OC. ^aSignificantly different from CR.

(P<0.05) (Figure 3).

Serum TNF α : The serum TNF α level was significantly higher in obese rats by 121% than normal group (P<0.05). After treatment with calorie restricted diet and gervital, this cytokine was lowered especially in CR plus gervital group (Figure 3).

Tissue parameters

Hepatic TAC and MDA: The result of present study have demonstrated that obese rats showed a significant decrease in hepatic TAC capacity by 52.5% compared to normal group. This decrease in TAC is accompanied by increase in hepatic MDA by 72%. Caloric restriction or gervital administration either individually and in combined form notably inhibited the elevation in of hepatic MDA by 23.2, 34.9 and 44.2%, respectively and exert significant enhancement on hepatic TAC by 49.1, 66.7 and 128% in comparison with obese rats. Dietary regimens and drug therapy significantly increase TAC by 52.9% and decrease MDA by 27.3% in comparison with CR group

(P<0.05) (Figure 4).

Hepatic antioxidant enzyme activity: Key antioxidant enzymes including SOD and CAT were measured. Obese rats showed significant decrease in all antioxidant enzyme activities by 53.9 and 30%, respectively when compared to the normal control. However, calorie restricted diet or gervital either individually and in combined form significantly (P<0.05) increased the antioxidant enzyme activities compared to that of obese control group. Co-administration of gervital with calorie restricted diet significantly elevates antioxidant enzyme activities by 36.4 and 25.3% compared to CR group (Figure 5).

Hepatic GSH: Liver GSH level were decreased significantly in obese rats by 44.4% in comparison to normal control (P<0.05). Calorie restricted diet or gervital therapy either individually or in combination significantly restored GSH levels near normal values compared to obese rats. However calorie restricted diet in combination with gervital therapy induced a significant elevation in GSH level by 21.9% in comparison with CR group (Figure 6).

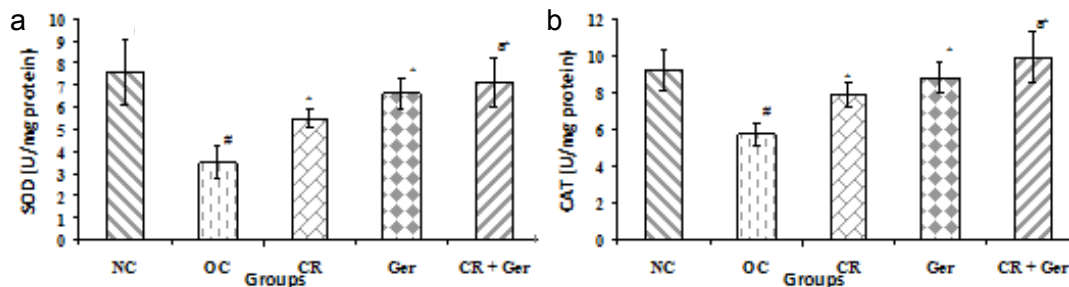


Figure 5. Effect of CR, Ger and their combination on hepatic antioxidant enzyme activity (a,b). Values are expressed as means \pm SD (n=12) (P<0.05). [#]Significantly different from NC group. ^{*}Significantly different from OC. ^aSignificantly different from CR.

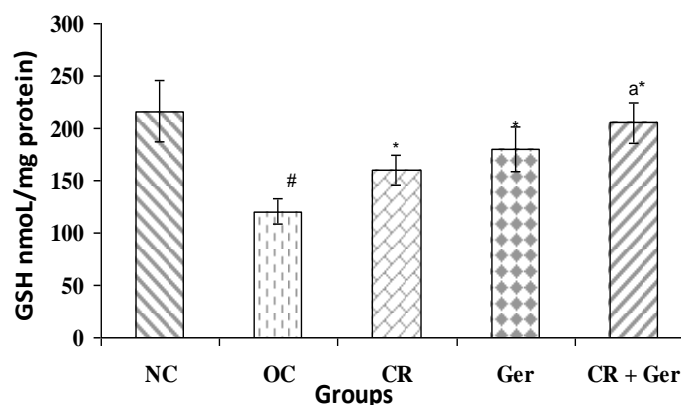


Figure 6. Effect of CR, Ger and their combination on hepatic GSH. Values are expressed as means \pm SD (n=12) (P<0.05). [#]Significantly different from NC group. ^{*}Significantly different from OC. ^aSignificantly different from CR.

Hepatic miR-33a and miR-122: High fat diet significantly increase miR-33 and miR-122 gene expression in liver tissue by 358 and 267%, respectively in comparison to normal rats (P<0.05). Treatment with caloric restriction or gervital either individually or in combination significantly reversed the increase in miR-33a and miR-122 leading to reduction by (36.4, 40.9, and 75%) and (40, 44 and 70%), respectively in their level compared to obese rats. However when gervital and caloric restriction were administered in combination, the reduction was greater by 60.7 and 51.3%, respectively than caloric restriction alone (P<0.05) (Figure 7 to 9).

Correlation study

Using the combined results from all groups, we illustrated that both hepatic miR-33a and miR-122 were positively correlated with all lipid fractions (except HDLc), tumor necrosis factor alpha and adiponectin. On the other hand, they were negatively correlated with hepatic antioxidant parameters but positively correlated with MDA.

DISCUSSION

The current work handled the effect of high fat diet in the induction of obesity. The latter was manifested by a marked increase in body weight. Obese rats developed dyslipidemia as manifested by a significant increase in Tc, TAG, LDLc, atherogenic index and reduction on HDLc accompanied by significant elevation in blood glucose level and remarkable increase in insulin intolerance. With regard to the liver, HFD also provoked a clear oxidative stress status as evidenced by increased MDA, decreased reducing power (GSH and TAC) and inhibition of antioxidant enzyme activities as CAT and SOD and inflammation as shown by elevation in hepatic TNF α . Interestingly, in association with dyslipidemia, inflammation and oxidative stress, the level of miR-33a and miR-122 were upregulated in obese rats.

Previous study, Kappes and Loffler (2000) suggested that there is increasing evidence that obesity impairs adipocyte function and secretion of adipocytokines (adiponectin and TNF- α). The reduction in adiponectin level is due to increasing TNF- α which inhibits adiponectin expression in adipose tissue (Li et al., 2009). Moreover circulating adiponectin is inversely correlate with plasma TNF- α (Bruun et al., 2003). Reduced levels of adiponectin contribute to development of insulin resistance. Adiponectin is negatively correlate with insulin resistance (Hotta et al 2000). Havel (2002) reported that adiponectin can reduce glucose level so this hypoglycemic effect is associated with increase insulin sensitivity. MiRNAs have been described as regulators of gene expression and the deregulation of several miRNAs that are related to chronic diseases has been reported (Rottiers and Naar 2012).

Specifically, miR-33a and miR-122 play key roles in lipid metabolism. It is well known that these two miRNAs are involved in cholesterol and TAG metabolism (Krutzfeldt et al 2005 and Esau et al 2006). So deregulation of miR-33a and miR-122 in obese rats has been related to the development of dyslipidemia. Inhibition of miRNA-122 in mice results in a significant

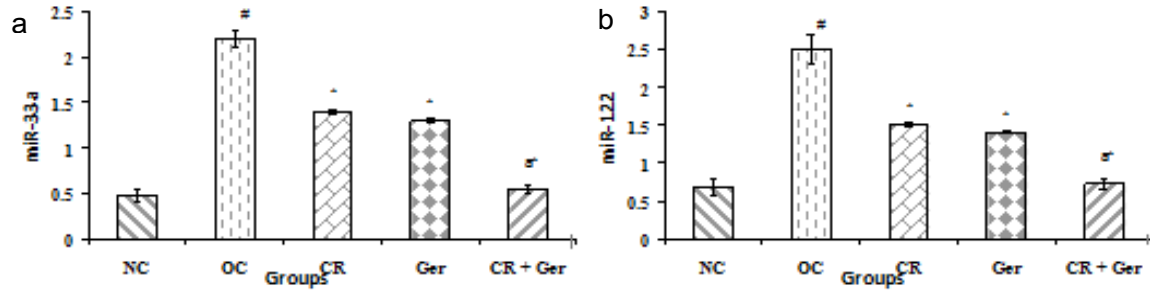


Figure 7. Effect of CR, Ger and their combination on hepatic miR-33a and miR-122 (a,b). Values are expressed as means ± SD (n=12) (P<0.05). [#]Significantly different from NC group. ^aSignificantly different from OC. ^bSignificantly different from CR.

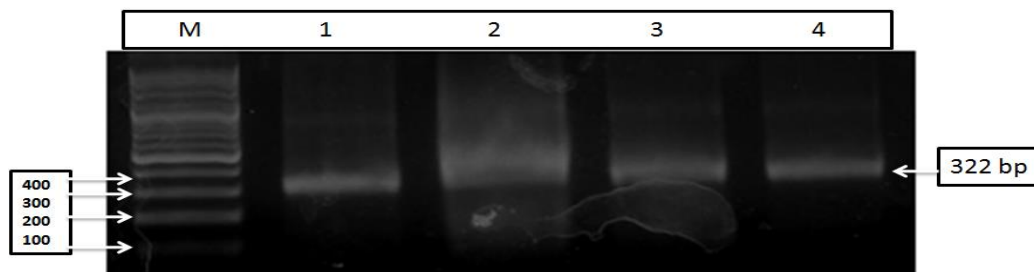


Figure 8. Agarose gel electrophoresis showed PCR product of mir RNA 33a gene with 322 bp size in RNA extract. Lane M: DNA ladder (100, 200, 300, 400 bp.....etc) .Lane 1-4: PCR products of positive samples in all studied groups

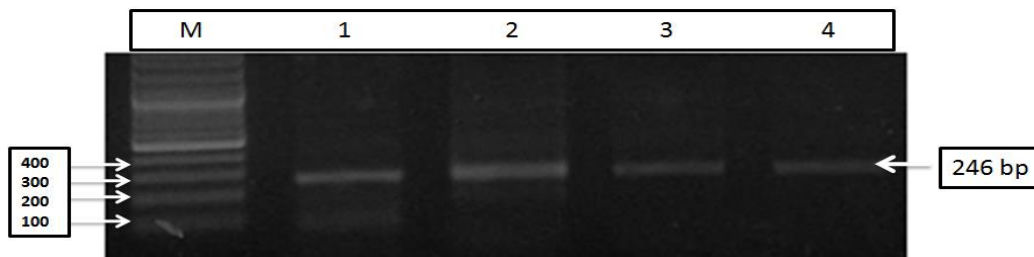


Figure 9. Agarose gel electrophoresis showed PCR product of mir RNA 122 gene with 246 bp size in RNA extract. Lane M: DNA ladder (100, 200, 300, 400 bp.....etc) .Lane 1-4: PCR products of positive samples in all studied group.

Table 2. Correlation between hepatic miR-33a and miR-122 with serum lipid fractions, tumor necrosis factor alpha (TNF) adiponectin.

Serum parameter	MiR-33 a	miR- 122 a
TC	r=0.82	r=0.72
TAG	r=0.72	r=0.81
LDL	r=0.85	r=0.69
HDL	r=-0.59	r= -0.79
LDL / HDL	r=0.69	r=0.62
TNF	r=0.81	r=0.88
adipotectin	r=0.71	r=0.79

Significant at P<0.0001

reduction in plasma cholesterol and TAG levels, Mechanistically, Tsai and colleagues (2012) found that the absence of miR-122 results in a significant reduction of microsomal transfer protein (MTTP) expression, thereby decreasing very low density lipoprotein (VLDL) secretion from liver Furthermore, the silencing of miR-33a by knockout or antisense techniques in mice results in an improvement in plasma lipid profile with increasing (HDLc) level (Rayner et al., 2011). These results were confirmed by correlation between miR-33a miR122 with Tc, TAG, LDLc and negative correlation with HDLc (Table 2).

Generally dyslipidemia observed in obesity is mainly

Table 3. Correlation between hepatic miR-33a and miR-122 with hepatic oxidant and antioxidant parameters.

hepatic oxidative and antioxidant parameters	MiR-33 a	miR- 122 a
MDA	r= 0.81	r= 0.62
GSH	r= -0.75	r= -0.77
TAC	r=-0.85	r= -0.81
CAT	r= -0.72	r= -0.59
SOD	r= -0.66	r= -0.69

Significant at P<0.0001

attributed to decreased activity of lipoprotein lipase. The LPL is an insulin sensitive enzyme which demonstrates significant alteration in many diabetics (Adiels et al., 2006). The reduction of LPL activity is sufficient to reduce the clearance of chylomicrons and VLDL in insulin resistance syndrome (Markel et al., 2002). Hosogai et al. (2007) demonstrated that hypoxia in adipose tissue associated with obesity results in endoplasmic reticulum (ER) stress due to accumulation of unfolded proteins in the ER. The ER stress causes down regulation of adiponectin through activation of C/EBP homologous protein (CHOP) that heterodimerize with C/EBP and form inactive complex. It is stated that C/EBP is critical for regulation of adiponectin transcription.

Obesity is linked to an overt oxidative stress along a decline in antioxidant defenses (Kamel et al., 2013) HFD cause reactive oxygen species (ROS) accumulation, an increase in lipoperoxidation and protein carbonylation and decrease in thiols radicals and glutathione. Consequently our data further confirmed that HFD also inhibit SOD, CAT and GPx activity leading to depletion of glutathione and reduction in TAC (Lee et al 2008). Inhibition of miR-33a also decrease the expression of proinflammatory and prooxidant genes including inducible nitric oxide synthase and tumor necrosis factor alpha (Ho et al., 2011). Also inhibition of miR122 decreases the basic leucine zipper transcription factor-1 (BACH-1) and increases heme oxygenase -1 (OH-1) a key cytoprotective enzyme with antioxidant properties repressed by BACH-1 (Shan et al., 2007). These results were confirmed by correlation studies (Table 3)

The current study demonstrated that obese rats treated with CR or GSE exhibited an improvement in lipid profile where Tc, TAG, LDLc and atherogenic index were decrease while HDL was elevated, significant decrease in blood glucose, insulin and IR, an elevation in TAC, CAT, SOD and glutathione and reduction in MDA. Adiponectin is significantly increased while TNF- α is decreased in addition to miR-33a and miR-122 are also elevated compared to obese rats.

Lipid lowering property of CR is possibly due to increase level of adiponectin (Ding et al., 2012) who have proposed that CR have a role in protecting against insulin resistance through lowering adiposity and up-regulation of adiponectin expression. Adiponectin plays major role in

the regulation of glucose, insulin and fatty acids and has anti-obesity effect. It can increase β -oxidation in tissues and causes weight loss and is inversely related with fat mass (Hu et al., 2012). Also adiponectin over expression confers dramatic metabolic improvement that includes decreased TAG, Tc, Fat cell size and increased fat cell number and improved glucose tolerance (Trujillo and Scherer, 2006). The marked decreased in TNF- α level is greatly joined with elevated adiponectin concentration (Cawthorn and Sethi, 2008). The ability of CR to restore antioxidant defense mechanism and elevation of miR-33a and miR-122 may be due to its weight lowering effect.

The current study demonstrated that obese rats treated with gervital exhibited the powerful ability of grape seed extract (GSE) to counteract most of the HFD induced disturbance such as weight gain, dyslipidemia, IR oxidative stress, low grade inflammation and upregulation of miR-33a and miR-122 the major regulators of lipid metabolism in the liver. In line with our results, previous studies have suggested that GSE could be an effective therapeutic agent for obesity. Pajuelo et al. (2012) showed that GSE administration protected against weight gain in wistar rats with obesity induced by high fat diet. Improvement of lipid fraction to be close to normal level after GSE administration indicating its effect in intestinal absorption (Sugiyama et al., 2007).

Our data are fully in line with those of Caimari et al. (2013) who showed the beneficial anti-obesity effect of grape seed procyanidins which increased lipase activity in white adipose tissue in hamsters. Literature survey revealed that GSE exhibit insulinomimetic properties (Adisakwattana et al., 2010) and antihyperglycemic effects (Suwannaphet et al., 2010). These effects may be due to antidiabetic activity of the natural plant phenolic compounds (You et al., 2012). Consequently GSE increases the level of liver enzyme activities of SOD, CAT as well as GSH. Such treatment caused augmentation in serum insulin, hepatic TAC levels accompanied by decrease in serum glucose and liver MDA. The antioxidant activity of GSE may be due to the inhibition of oxidation of plasma lipids. Moreover it is able to scavenge hydroxyl radicals, peroxy radicals superoxide anion radicals (Yilmaz and Toledo, 2004).

When weight gain was reduced with GSE, the capabilities of GSE as an anti-inflammatory was identified

in our study. Therapeutic inhibition of C-reactive protein (CRP) by GSE is considered as a promising new approach to cardioprotective and myocardial infarction or to other inflammatory, infective and tissue damaging conditions characterized by increased CRP level (Pepys et al., 2006). Altogether, our data highlighted the antioxidant and anti-inflammatory role of GSE (Bagchi et al., 2000).

Interestingly, GSE treatment counteracted the overexpression of miR-133 and miR-122 induced by HFD. Our data is in agreement with previous studies (Laura et al., 2013) who stated that the repression of rat liver miR-33a and miR-122 induced by GSE was clearly associated with the improvement in plasma lipid profile, it could be suggested that the modulation of miR-33a could be one of the molecular mechanism used by GSE to improve the plasmatic atherogenic profile that was induced by HFD.

Our study revealed that using a combination between CR and GSE might synergically improve lipid profile except HDLc which showed non significant change in comparison to CR group. We evaluated effect of this combination in blood glucose, insulin and IR. In this case the effect was showed to a similar degree to that found when administering separately and the effect was not additive or synergistic.

In the present study, the tested combination of both CR diet and GSE on oxidative stress markers may reflect the sum of efficiency of either administration alone. Thus acting with more than one mechanism of action including, the reduction of ROS and scavenging of free radicals as well as improvement of the tissue enzymatic and non enzymatic antioxidant activities. When the two treatments were orally administered together the increment of hepatic adiponectin content showed an additive effect in CR-Ger group accompanied with synergic decrease in hepatic TNF- α . Additionally, co-administration repressed the liver expression of miR-33a and miR-122 greater than when the treatments were administered separately. Previously, we have shown that GSE repress miR-33a and miR-122 liver expression in rats treated with an acute dose which also induced postprandial hypolipidemia in normal rats (Baselga et al., 2012).

Conclusion

The level of miR-33a and miR-122 in the liver correlate with metabolic disorders induced by high fat diet. Grape seed extract in combination with caloric restriction is considered a promising therapy for treatment of metabolic disorders associated obesity by down regulation of hepatic miR 33a and miR 122. More studies are necessary to elucidate the exact mechanism by which CR and GSE repress miR-33a and miR-122.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Toxicological assessment of the aqueous dried leaf extracts of *Senna alata* L. in wistar rats

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Received 20 June, 2016; Accepted 21 July, 2016

Senna alata is a medicinal plant that belongs to Leguminosae family. Different parts of the plant are used in folklore medicine for curing skin infections, abdominal pain, and gonorrhoea. Despite the use of *S. alata* in the treatment of various ailments, there is limited or no comprehensive scientific information on the toxicological evaluation of the dried leaf extract in the folklore medicine in Africa. This study therefore evaluated the extensive toxicological effects of the aqueous dried leaf extract of *S. alata* in wistar rats. In acute toxicity test, aqueous dried leaf extract of *S. alata* were administered orally up to 10 g/kg body weight to male wistar rats. In sub-acute study, the wistar rats were daily administered orally with aqueous dried leaf extract of *S. alata* at doses of 250, 500 and 1000 mg/kg for 14 days and haematological and biochemical parameters were determined and a histopathology of the liver and kidney were analysed. The results revealed that in acute toxicity study, no death was recorded within 24 h after oral administration. In the sub-acute study, the extract did not exhibit any significant difference ($p > 0.05$) on haemoglobin, red blood cells and mean corpuscular haemoglobin concentration in all the tested doses. Significant differences ($p > 0.05$) were observed on white blood cell, platelet, urea, aspartate aminotransferase, alkaline phosphatase, total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol. The histopathology of the liver and kidney did not reveal any pathological changes. Our findings revealed that aqueous dried leaf extract of *S. alata* is not toxic at the tested doses, indicating that it is safe for therapeutic uses at the tested doses.

Key words: Haematology, hepatic and renal function, sub-acute toxicity, histopathology, *Senna alata*.

INTRODUCTION

The practice of herbal medicine is as old as the origin of mankind (Petrovska, 2012). Today, the use of plant-based herbal remedies is spreading worldwide and is gradually gaining general acceptance, because it is used in both the developing countries as the major primary

health care of the poor and also in developed countries where modern medicine is dominant in the national health care system (Tahvilian et al., 2014). For example, the use of quinine and quinidine extracted from *Cinchona* tree and also artemisinin obtained from

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Artemisia annuain for treating malaria is an indication that medicinal plants are essential sources of novel drugs (Igoli et al., 2005).

Herbal medicine plays vital role as hypolipidemic (Yadav et al., 2008), abortifacient and contraceptive (Yakubu et al., 2010), antihypertensive (Nworgu et al., 2008), effective for treating skin diseases (Ajibesin et al., 2008), wound healers, and hypoglycemic effects and antimicrobial activities (Lee et al., 2009). Herbal medicine has numerous advantages such as low-cost, affordability, availability, accessibility, acceptability, and low toxicity; however, there are various disadvantages of consuming herbal products which include inappropriate formulations, lack of adequate scientific proof of the plant, imprecise diagnosis dosage and unstandardized usage which can lead to serious health risk to the patients (Elujoba et al., 2005).

Senna alata (L.) Roxb. is a medicinal plant that belongs to Leguminosae family. It is an important ornamental flowering plant commonly known as candle tree, ringworm plant and candle bush (Singh et al., 2012; Otto et al., 2014) and exist in diverse environments like ditches, rivers, margins of ponds, drainage channels and alongside water ways in Africa. In traditional medicine, the leaves, roots and stems are widely used for treatment of skin infections (Lifongo et al., 2014), abdominal pain (Hennebelle et al., 2009), gonorrhoea and heart failure (Otto et al., 2014). It is used as anti-bacterial, laxative, diuretic, treatment of flu, malaria and other infectious diseases (Hennebelle et al., 2009). It has been reported that *S. alata* leaves possess antimutagenic (Villaseñor et al., 2002), anti-inflammatory, and antimicrobial properties (Khan et al., 2001; Somchit et al., 2003).

Previous studies on *S. alata* have shown that it possesses antifungal (Palanichamy and Nagarajan, 1990), anti-bacterial activities (Somchit et al., 2003), antioxidant properties (Sagnia et al., 2014) and can be used as abortifacient (Yakubu et al., 2010). Hennebelle et al. (2009) also reported the laxative and purgative potentials of *S. alata*, but to the best of our knowledge there is limited or no comprehensive scientific documentation on the toxicological evaluation of the leaf extract of *S. alata* in the folklore medicine. Therefore, this present study is aimed at evaluating extensively the toxicological effects of the leaf extract of *S. alata* in wistar rats.

MATERIALS AND METHODS

Plant collection and authentication

Fresh leaves of *S. alata* were collected from Amaku Nvosi in Isiala Ngwa South Local Government Area of Abia State, Nigeria in July, 2015 (Figure 1). The plant was identified and authenticated by Mr. Onyeukwu Chijioko of the Department of Plant Science and Biotechnology (Botany), University of Nigeria Nsukka. A voucher specimen (UNH/118b) was deposited in the herbarium of the department.

Preparation of plant extract

Fresh leaves of *S. alata* were destalked and washed with deionized water before sun drying for 7 days. The dried leaves were milled into fine powder and then macerated in sterile distilled water (300 g in 2 L) for 24 h. The extract was decanted and filtered with Whatman filter paper No. 1 and the filtrate was concentrated to dryness in a water bath for 3 days at 50°C giving a greenish brown colour. The dried extract was dispensed into airtight sterile container and stored at 4°C in the refrigerator until usage. Extracts were later reconstituted in distilled water to give the required doses of 250, 500, 1000, 2000, 5000, and 10000 mg/kg body weight used in this study.

Phytochemical and proximate analysis of leaves of *S. alata*

Phytochemical analysis of the *S. alata* leaf was determined using standard analytical methods. Alkaloids, phenolics, saponins, and flavonoids were quantitatively determined by the method of Harborne (1973). Tannin was determined using the Folin-Denis spectrophotometric method (Shabbir et al., 2013) and total oxalate was estimated according to the methods of Day and Underwood (1986).

The proximate compositions of *S. alata* leaf, namely, moisture, ash crude lipid, nitrogen content, crude fibre, and carbohydrate were determined according to the recommended methods of the Association of Official Analytical chemists (AOAC, 2005).

Animals

Forty-five (45) healthy male albino rats (80 to 100 g) used in this study were purchased from animal house of University of Nigeria, Nsukka, Enugu State, Nigeria. The rats were transported to Department of Biochemistry, Abia State University, Uturu, Abia State, Nigeria. The albino rats were kept under normal standard environmental conditions of humidity (35 to 60%), temperature (25 to 28°C) and a 12 h/12 h light/darkness cycle and were fed *ad libitum* with standard feed and allowed free access to water. The albino rats were allowed to acclimatize to laboratory conditions for two weeks before the commencement of the study. The albino rats were handled in accordance with the World Health Organization (WHO) good laboratory practice regulations of 1998 and United State guidelines for experimental animal (NIH publication #85-23, revised 1996). Ethical principles in animal care and handling were strictly adhered throughout the study (Neuwing, 2000).

Acute toxicity test

The rats were randomly divided into 7 groups of 3 animals per group. Graded oral doses of plant extract (250, 500, 1000, 2000, 5000, and 10000 mg/kg) were separately administered orally to the rats in each group. The control group was orally given 0.25 ml of distilled water. All the rats were allowed free access to food and water and were observed for a period of 24 h post-treatment for behavioural changes, signs of toxicity, and mortality.

Sub-chronic toxicity study

The albino rats were randomly divided into 4 groups of 6 rats per group. The rats were orally administered *ad libitum* an aqueous dried leaf extract of *S. alata* at doses of 250, 500, and 1000 mg/kg daily for 14 days. The control group was orally administered 0.25 ml of distilled water daily. The rats were weighed daily throughout the course of the experiment.



Figure 1. The leaves of *Senna alata*.

Collection of blood and organ samples

Fourteen days after feeding the rats with the aqueous dried leaf extracts of *S. alata*, they were fasted overnight, anaesthetized with chloroform and sacrificed. Blood samples were collected through cardiac puncture with the help of syringe and needle and dispensed into ethylenediaminetetraacetic acid (EDTA) containers for haematological analysis and heparinized containers for blood chemistry test.

The organs, namely, liver, kidneys, heart, lungs, spleen, and testes were dissected and removed carefully and absolute weights of each organ were determined. The relative organ weight of individual wistar rats was calculated as follows:

Relative organ weight = (Absolute organ weight (g) / Body weight of rat on sacrificed day (g)) × 100

Procedures used for haematological and serum chemistry analysis

Packed cell volume (PCV), haemoglobin level (Hb), white blood

cells count (WBC), platelets and red blood cell indices (mean corpuscular volume [MCV]; mean corpuscular haemoglobin [MCH]; mean corpuscular haemoglobin concentration [MCHC]) were analyzed using the methods outlined by Dacie and Lewis (1991).

The renal function tests; urea, creatinine, sodium, potassium, chloride and bicarbonate and liver enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were spectrophotometrically determined by using standard ready to use kits from Randox Laboratory Ltd, Co. Antrim, United Kingdom. The assay kits used for total cholesterol (TC), triacylglycerol (TAG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were also products of Randox Laboratory Ltd, Co. Antrim, United Kingdom. The manufacturer's instructions for the entire biochemical test were strictly adhered to.

Histopathological studies

The liver and kidney were removed carefully and fixed in 10% formalin saline in labelled sample bottles after sacrificing the rats. The tissues were processed routinely and embedded in paraffin

Table 1. Phytochemical constituents of aqueous dried leaf extract of *S. alata*.

Parameter	Leaves
Alkaloids	3.17 ± 0.29
Flavonoids	1.53 ± 0.76
Saponins	2.83 ± 0.29
Oxalate	1.37 ± 0.03
Phenol	0.87 ± 0.01
Tannin	0.82 ± 0.15

Values represent the mean ± SD for N=3.

Table 2. Proximate composition of dried leaf of *S. alata*.

Parameter	Leaves (% w/w)
Moisture content	7.33 ± 1.53
Ash content	7.33 ± 1.53
Lipid content	3.27 ± 0.23
Crude protein	11.20 ± 0.31
Crude fibre	53.87 ± 0.98
Carbohydrate	17.00 ± 3.87

Values represent the mean ± SD for N=3.

Table 3. Acute (oral) toxicity study of albino rats after 24 h of administration of aqueous dried leaf extract of *S. alata*.

Group	Dose (mg/kg)	D/T	Signs of toxicity
A	0.25 ml (H ₂ O)	0/3	No toxic effects observed
B	250	0/3	No toxic effects observed
C	500	0/3	Scratching of body and became restless within 2 min
D	1,000	0/3	Dullness was observed with 5 min
E	2,000	0/3	Scratching of body, dullness and calmness
F	5,000	0/3	Scratching of mouth and weakness
G	10,000	0/3	Very weak and felt sleepy within 2 h

D/T: Number of albino rat deaths/Total number of albino rats used.

wax. Sections of 5 µm thickness were cut and stained with haematoxylin and eosin. The processed sections were viewed using the light microscope by an experienced pathologist.

Statistical analysis

One-way analysis of variance (ANOVA) with the RTM Statistic software package, version 3.0.3 and excel package were used for statistical analysis. The normal distribution of the data and the homogeneity of variance were tested by Bartlett homogeneity test. One-way ANOVA with a Tukey test post-hoc was used to identify statistical differences among groups. A p-value of ≤0.05 was considered statistically significant.

RESULTS

The quantitative phytochemical estimation revealed that

aqueous dried leaf extract of *S. alata* contains alkaloid (3.17%), followed by saponin (2.83%), flavonoids (1.53%), oxalate (1.37%), phenol (0.87%), and tannin (0.82%) are shown in Table 1. The result of the proximate analysis shows the presence of crude fibre (53.87%), carbohydrate (17.00%), crude protein (11.20%), moisture content (7.33%), ash content (7.33%), and lipid content (3.27%) are shown in Table 2.

In acute toxicity study, no deaths were recorded in wistar rats after *S. alata* extracts were orally administrated at various doses ranging from 250 to 10 g/kg body weight, but scratching of body, calmness, dullness, and weakness of body within 2 h were noticed among rats treated with higher doses ranging from 500 to 10 g/kg of the post-treatment (Table 3).

In the sub-acute toxicity study, the percentage weight

Table 4. Effects of aqueous dried leaf extract of *S. alata* on the body weight of rats after 14 days administration (n=6).

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg
Weight at day 0	100.01 ± 9.98 ^b	83.87 ± 6.26 ^a	94.00±2.76 ^b	87.50±9.87 ^a
Weight at day 14	132.63 ± 12.41 ^c	109.73±2.21 ^a	117.97±3.09 ^b	110.70±24.42 ^a
Weight gain (g)	32.62	25.86	23.97	23.20
Weight gain (%)	24.59	23.57	20.32	20.96

Values represent the mean ± SD for N=6. Values in the same row bearing the same alphabets are not significantly different from each other (P > 0.05).

Table 5. Effect of aqueous dried leaf extract of *S. alata* on the relative organ weight of wistar rats.

Organ	Relative organ weight (g) of albino rats			
	Control	Group I (250 mg/kg)	Group II (500 mg/kg)	Group III (1000 mg/kg)
Liver	5.53 ± 0.08 ^a	4.67 ± 0.43 ^a	4.51 ± 0.43 ^a	4.48 ± 1.07 ^a
Spleen	0.52 ± 0.02 ^a	0.31 ± 0.09 ^a	0.36 ± 0.04 ^a	0.34 ± 0.03 ^a
Kidneys	2.44 ± 0.08 ^a	1.86 ± 0.05 ^a	1.80 ± 0.01 ^a	1.78 ± 0.11 ^a
Lungs	0.84 ± 0.07 ^a	0.86 ± 0.09 ^a	0.87 ± 0.09 ^a	0.89 ± 0.32 ^a
Testes	3.08 ± 0.09 ^a	2.00 ± 0.11 ^a	1.96 ± 0.30 ^a	2.58 ± 0.30 ^a
Heart	0.47 ± 0.04 ^a	0.34 ± 0.03 ^a	0.38 ± 0.04 ^a	0.34 ± 0.05 ^a

Values represent the mean ± SD for N=6. Values in the same row bearing the same alphabets are not significantly different from each other (P > 0.05).

Table 6. Effects of aqueous dried leaf extract of *S. alata* on haematological parameters of wistar rats.

Parameter	Control	Group I (250 mg/kg)	Group II (500 mg/kg)	Group III (1000 mg/kg)
PCV (%)	41.33±1.53 ^c	38.00± 2.00 ^{bc}	33.33±1.53 ^a	35.33±3.51 ^{ab}
Hb (g/dl)	13.76±0.51 ^a	12.66±0.65 ^a	11.12±0.50 ^a	11.88±1.32 ^a
RBC (×10 ¹² /L)	6.22±0.02 ^a	5.74±0.02 ^a	4.77± 0.15 ^a	5.84±0.02 ^a
MCV(fl)	66.49±2.45 ^b	66.21±3.72 ^b	69.28±2.24 ^c	60.52±6.22 ^a
MCH (pg)	22.13±0.81 ^a	22.05±1.21 ^a	23.38±1.72 ^a	20.18±2.07 ^a
MCHC (g/dl)	33.28±0.05 ^a	33.31±0.05 ^a	33.37±0.06 ^a	32.33±1.75 ^a
WBC (× 10 ⁹ /L)	5.27±0.31 ^{bc}	6.97±0.25 ^c	4.73±0.97 ^{ab}	4.13±0.32 ^a
Neutrophil (%)	46.33±2.08 ^a	45.67±0.58 ^a	46.67±1.15 ^a	46.00±1.00 ^a
Lymphocyte (%)	49.00±1.00 ^a	49.00±2.00 ^a	49.33±1.15 ^a	50.00±2.65 ^a
Eosinophil (%)	1.33±0.58 ^a	1.33±0.58 ^a	1.33±0.58 ^a	1.67±1.15 ^a
Basophil (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Monocytes (%)	3.67±0.58 ^b	3.00±1.00 ^{ab}	2.67±1.15 ^{ab}	2.33±1.58 ^a
Platelet (×10 ⁹ /L)	176.67±15.28 ^c	153.33±20.82 ^a	153.33±7.64 ^a	163.33±15.28 ^b

Values represent the mean ± SD for N=6. Values in the same row bearing the same alphabets are not significantly different from each other (P > 0.05). PCV: Packed cell volume; HB: haemoglobin; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell.

gain and relative weight of organs were not altered in all the groups (250, 500, and 1000 mg/kg body weight) treated with *S. alata* when compared with the control rats (Tables 4 and 5).

The results of the haematological studies as shown in Table 6, showed that aqueous dried leaf extract of *S. alata* did not show any significant difference (p > 0.05) on

haemoglobin, red blood cells, mean corpuscular haemoglobin, lymphocytes, neutrophil, eosinophil, and mean corpuscular haemoglobin concentration in all the tested doses. However, significant difference (p > 0.05) was observed in the white blood cell, mean corpuscular volume, packed cell volume, monocytes and platelet when compared with the control rats.

Table 7. Effects of aqueous dried leaf extract of *S. alata* on hepatic enzymes and renal function of wistar rats.

Parameter	Control	Group I (250mg/kg)	Group II (500mg/kg)	Group III (1000 mg/kg)
Urea (mg/dl)	40.02 ± 0.19 ^b	37.38 ± 0.71 ^{ab}	34.74 ± 1.28 ^a	34.58 ± 1.17 ^a
Creatinine (mg/dl)	1.67 ± 0.09 ^b	0.81 ± 0.08 ^a	1.23 ± 0.07 ^b	1.30 ± 0.10 ^b
Na ⁺ (mEq/L)	140.59 ± 0.70 ^a	136.13 ± 4.47 ^a	138.55 ± 3.75 ^a	137.04 ± 2.76 ^a
Cl ⁻ (mEq/L)	104.61 ± 1.26 ^c	101.92 ± 0.34 ^b	98.71 ± 0.50 ^a	98.11 ± 0.82 ^a
K ⁺ (mEq/L)	5.06 ± 0.07 ^b	5.11 ± 0.07 ^b	4.20 ± 0.09 ^a	4.26 ± 0.59 ^a
HCO ₃ ⁻ (mMol/L)	29.18 ± 0.75 ^c	27.73 ± 0.50 ^b	27.83 ± 0.63 ^b	22.33 ± 1.96 ^a
ALT (U/L)	8.43 ± 0.56 ^a	12.74 ± 2.34 ^a	7.68 ± 0.42 ^a	9.99 ± 0.64 ^a
AST (U/L)	18.38 ± 0.82 ^b	9.33 ± 2.08 ^a	15.49 ± 3.75 ^b	18.16 ± 1.18 ^b
ALP (U/L)	44.47 ± 1.85 ^b	57.20 ± 1.66 ^c	38.15 ± 1.38 ^a	61.28 ± 4.67 ^c

Values represent the mean ± SD for N=6. Values in the same row bearing the same alphabets are not significantly different from each other (P > 0.05). Na⁺: Sodium ion; Cl⁻: chloride ion; K⁺: potassium ion; HCO₃⁻: bicarbonate; ALT: alanine transaminase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

Table 8. Effects of aqueous dried leaf extract of *S. alata* on lipid profile of wistar rats.

Parameter	Control	Group I (250 mg/kg)	Group II (500 mg/kg)	Group III (1000 mg/kg)
Total cholesterol (mg/dl)	120.91 ± 2.68 ^b	122.34 ± 2.05 ^b	105.26 ± 10.29 ^a	115.75 ± 7.84 ^b
Triglycerides (mg/dl)	126.47 ± 4.99 ^a	145.50 ± 5.07 ^b	121.10 ± 10.67 ^a	127.87 ± 5.00 ^a
HDL-C (mg/dl)	42.89 ± 0.57 ^a	43.67 ± 0.11 ^a	43.18 ± 0.87 ^a	43.78 ± 1.04 ^a
LDL-C (mg/dl)	45.02 ± 10.92 ^c	46.62 ± 5.91 ^c	37.57 ± 8.43 ^a	43.19 ± 8.70 ^b
VLDL-C (mg/dl)	25.30 ± 1.00 ^a	29.10 ± 1.00 ^b	24.22 ± 2.14 ^a	25.57 ± 1.00 ^a

Values represent the mean ± SD for N=6. Values in the same row bearing the same alphabets are not significantly different from each other (P > 0.05). HDL-C: High-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol.

In the renal function test, a slight decrease in the results of urea, creatinine, sodium ion, potassium ion, and bicarbonate was observed across the tested groups when compared with the control rats. On the liver enzymes parameters, there was no significant difference (P > 0.05) on ALT. AST decreases when administered the dried leaf aqueous extract of *S. alata* at 250 and 500 mg/kg to 9.33 and 15.49 U/L, respectively, and increased to 18.16 U/L at 1000 mg/kg body weight, while the control had 18.38 U/L. ALP showed statistical significance (P > 0.05) with values of 44.44, 57.20, 38.15 and 61.28 U/L for the control, 250, 500 and 1000 mg/kg doses of the extract, respectively (Table 7).

The results of the lipid profile (Table 8) showed slightly higher values at 250 mg/kg dose for total cholesterol (122.34 mg/dl), triglycerides (145.5 mg/dl), low-density lipoprotein cholesterol (46.62 mg/dl), and very low-density lipoprotein cholesterol (29.10 mg/dl) when compared with the other doses as well as the control. The histopathological examination of the liver (Figure 2) and kidney (Figure 3) of the wistar rats in all the treated doses of the aqueous dried leaf extract of *S. alata* did not reveal any damage or pathological changes when compared with the control rats.

DISCUSSION

Despite the tremendous use of medicinal plants in treatment of various ailments in Africa and other parts of the world, there are still few documented scientific studies on toxicological evaluation of medicinal plants to ascertain the efficacy and safety usage of herbal remedies for human consumption. This present study, therefore evaluated the selected phytochemicals, proximate compositions, and toxicological effects of aqueous leaf extract of *S. alata* using wistar rats.

The quantitative phytochemical estimation revealed that aqueous dried leaf extract of *S. alata* contains alkaloid, which is one of the most efficient therapeutic bioactive compounds in plants because of its analgesic and bactericidal effects (Ahmad et al., 2013). Saponin was 2.83% and can be considered as safe and non-toxic (Asuk et al., 2015) as high levels of saponin >10% have been associated with gastroenteritis, manifested by diarrhoea and dysentery, flavonoids (1.53%), indicating that *S. alata* can help fight against microbes and hepatic toxicity (Georgiev et al., 2014). The results of the proximate composition showed the presence of high crude fibre (53.87%), which is a good indication that the

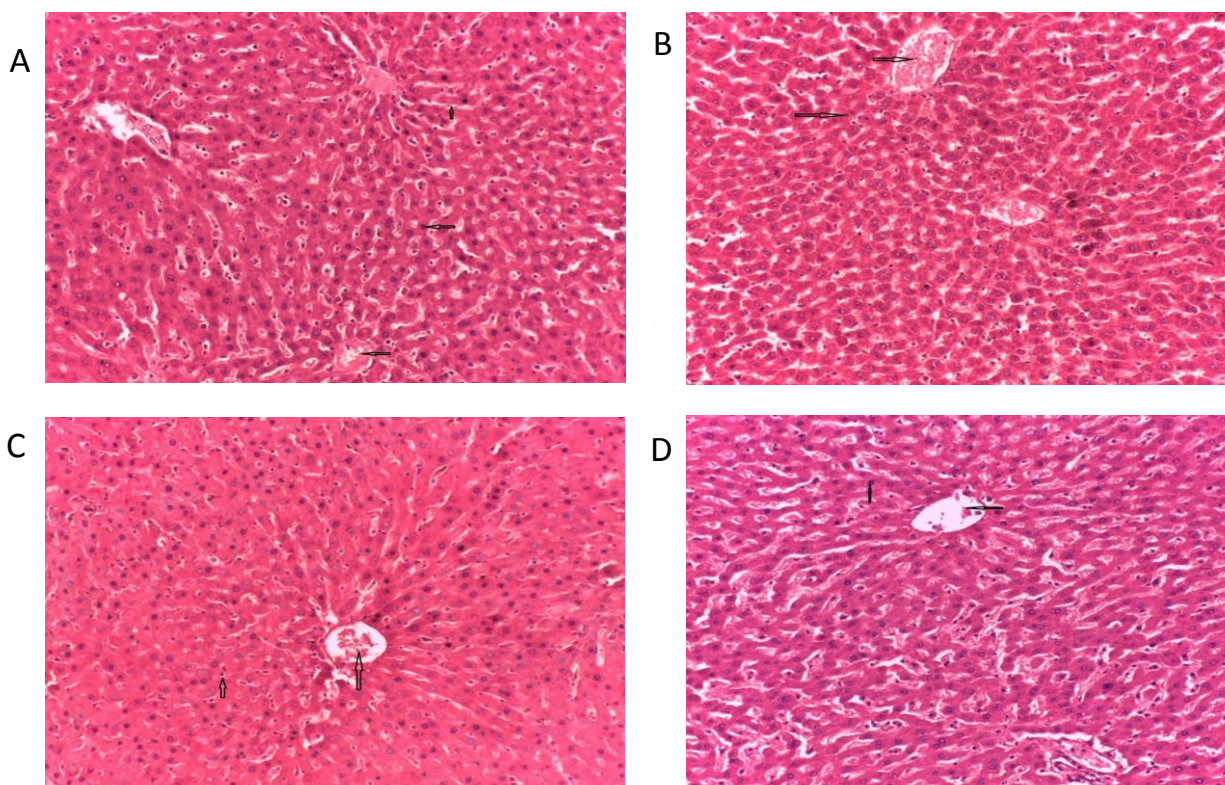


Figure 2. Micrographs of the liver sections obtained from untreated (control) and treated wistar rats with various doses of aqueous dried leaf extract of *Senna alata*. Haematoxylin and eosin staining (H&E), Magnification (40x). (A) control, (B) wistar rats treated with 250 mg/kg leaf extract of *Senna alata*., (C) wistar rats treated with 500 mg/kg leaf extract of *Senna alata*. (D), wistar rats treated with 1000 mg/kg leaf extract of *Senna alata*.

plant can prevent diverticulosis and also aid in the absorption of trace elements in the guts (Chiba et al., 2015) and carbohydrate (17.00%), which can serve as energy source while moisture content and ash content fell within the range of acceptable limits (6 to 15%) for most vegetable drugs (Asuk et al., 2015).

The acute toxicity evaluation of the aqueous dried leaf extract of *S. alata* showed no mortality, although scratching of body, calmness, dullness, and weakness of body within 2 h were noticed among rats treated with higher dose of up to 10 g/kg body weight, suggesting LD₅₀ of *S. alata* to be above 10.0 g/kg. This result shows that *S. alata* is safe for oral usage at tested doses and can be categorized as non-toxic plant based on report that LD₅₀ above 5000 mg/kg body weight is considered as non-toxic (Zbinden and Flury-Roversi, 1981). In the sub-acute toxicity study, there were no significant differences ($P > 0.05$) in percentage of weight gain of the rats when compared with the control groups throughout the study, but there was observable general improvement in body weight of all the rats; this weight gain could be attributed to the nutritive constituents present in feed or aqueous leaf extract used (Ashafa et al., 2012). Also, increase or decrease in relative organ weight has been implicated to be a sensitive indicator of organ toxicity (Aly

and El-Gendy, 2015). The relative weight of organs (liver, spleen, kidneys, lungs, testes, and heart) were not altered in all the groups treated with *S. alata* when compared with the control rats, thus serving as good indicator that the extract may not be toxic. Similar result was obtained from the findings of Silva et al. (2011) on acute and sub-acute toxicity of *Cassia occidentalis* L. stem and leaf in wistar rats.

The results of all haematological parameters were within the internationally accepted reference range for each tested parameter. Therefore, it could be suggested that the aqueous dried leaf extract of *S. alata* did not produce adverse effect on the bone marrow, which is the chief organ for haematopoietic processes and susceptible targets of toxic compound (Kifayatullah et al., 2015). This implies that *S. alata* may not have negative effects to the immune system since the white blood cell and differential counts are not impaired.

It has been previously reported that some herbal remedies have hepatotoxic (Movahedian et al., 2014) and nephrotoxic effects (Asif, 2012). The maintenance levels within the internationally accepted reference range of creatinine, urea and electrolytes (Na^+ , Cl^- , HCO_3^- and K^+) of the wistar rats treated with aqueous dried leaf extract of *S. alata* suggests that the short term treatment of

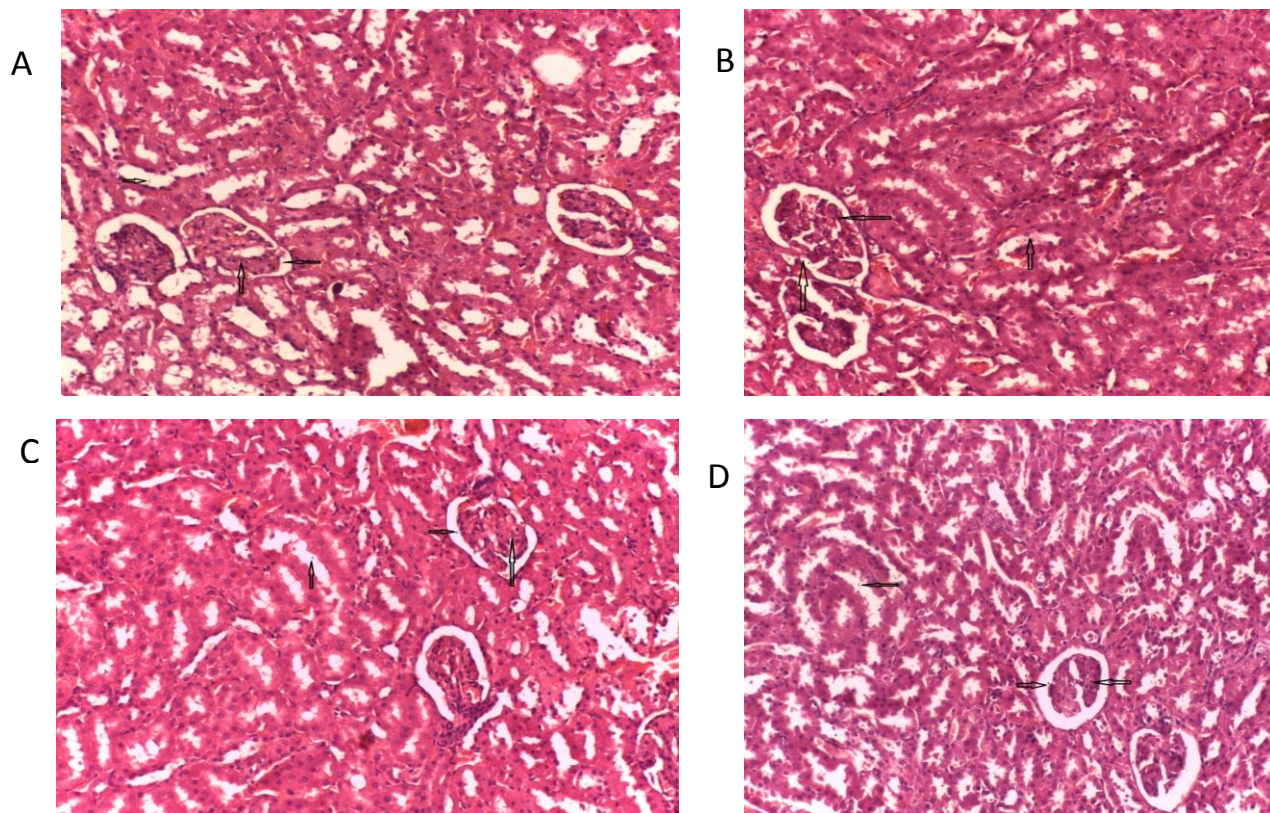


Figure 3. Micrographs of the kidney sections obtained from untreated (control) and treated wistar rats with various doses of aqueous dried leaf extract of *Senna alata*. Haematoxylin and eosin staining (H&E), Magnification (40×). (A), control (B), wistar rats treated with 250 mg/kg leaf extract of *Senna alata*. (C), wistar rats treated with 500 mg/kg leaf extract of *Senna alata*. (D), wistar rats treated with 1000 mg/kg leaf extract of *Senna alata*.

extract has no detrimental effect on the kidney function of the wistar rats. Also, lack of alteration in the liver biomarkers (AST, ALT and ALP) from internationally accepted reference range to the treated wistar rats showed that the administration of the aqueous dried leaf extracts of *S. alata* cannot cause an impairment of liver function of the wistar rats. However, Yagi et al. (1998) reported that ethanol extract and compounds isolated from *S. alata* caused subtle hepatorenal toxicity on rats.

Lipid profile showed slightly higher values at 250 mg/kg dose for total cholesterol, triglycerides, low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol when compared with the other doses and the control. Interestingly, the results of all the tested lipid profile parameters were within the internationally accepted reference range for each tested parameters. The histopathological examination of liver and kidney harvested from the control rats and rats treated with various doses of *S. alata* showed that aqueous dried leaf extract of *S. alata* did not damage or produce any pathological changes in the organs.

In conclusion, this study has presented strong evidence that *S. alata* is non-toxic and safe for consumption and for therapeutic uses in folk medicine at the tested doses.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank the dynamic Vice Chancellor of Abia State University, Professor E.U. Ikonke for providing favourable environment to carry out this research. The authors also wish to thank Mr Uche Arukwe for his technical assistance.

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Full Length Research Paper

In vitro* and *in vivo* assessment of genotoxic activity of *Petiveria alliacea

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Received 24 April, 2016; Accepted 25 July, 2016

***Petiveria alliacea* L. (Phytolaccaceae) is an Amazonian shrub used in traditional medicine for many purposes. This study investigated the genotoxicity of the hydroalcoholic extract of *P. alliacea* (EHPa). In addition, we conducted pharmacognostic characterization and phytochemical investigation. Phytochemical screening and thin layer chromatography (TLC) were used to determine the chemical composition of the extract. Genotoxicity was evaluated using an *in vitro* comet assay in human lymphocytes (50, 100, and 250 mg/ml) and an *in vivo* micronucleus assay in mice orally treated with the extract (50, 100, and 250 mg/kg b.w., p.o.). The phytochemical screening identified the metabolites reducing sugars, alkaloids and saponins. TLC analyses identified organosulfur compounds in EHPa. Comet assay data showed that EHPa induced exacerbated DNA damage at 100 mg/ml treatments (EHPa 100 mg/ml: TL 219.13 ± 48.38 mm, % DNA-t 59.33 ± 18.98%, TM 134.99 ± 61.34, OM 87.48 ± 32.98) as compared with negative control (Roswell Park Memorial Institute (RPMI): TL 37.79 ± 24.79 mm, % DNA-t 9.73 ± 6.56%, TM 4.86 ± 5.79, OM 6.90 ± 5.80; p<0.01). These effects were similar to positive control (hydrogen peroxide). The micronucleus assay, however, showed that EHPa induce slight genotoxicity *in vivo*. However, EHPa induced an exacerbated DNA damage *in vitro*, but this effect was slight in the animal model. These *in vitro* effects may be related to the chemical composition of the extract.**

Key words: *Petiveria alliacea*, genetic toxicity, DNA damage, comet assay, micronucleus assay.

INTRODUCTION

Petiveria alliacea L. (Phytolaccaceae) is an Amazonian indigenous perennial shrub, slender and erect, which reaches about 1 m in height (Rocha et al., 2006).

Popularly, the plant is known as herb to “tame the master”, mucuracaá, guiné, pipi, tipi, anamu, apacin, embayayendo, ouoembo, among others (Camargo, 2007).

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This variety of names is directly related to its large geographical distribution that includes countries in tropical America, Africa, Europe, and the Caribbean, West Indies, and India (Alonso, 2007).

P. alliacea has been used for many purposes in traditional medicine. A decoction of the roots is used in the Amazonian region as an abortifacient, antispasmodic, analgesic, sudorific, and antirheumatic (Corrêa, 1984; Elisabetsky and Castilhos, 1990). An ethnobotanical survey pointed out that the plant has been widely used for the treatment of respiratory diseases, including asthma, bronchitis, chest congestion, and common cold (Vandebroek et al., 2010). Several pharmacological studies have demonstrated the therapeutic potential of *P. alliacea* as antimicrobial, anticancer and immunostimulant agent (Guedes et al., 2009; Mata-Greenwood et al., 2001; Santander et al., 2012). Regarding phytochemistry, *P. alliacea* possesses a broad spectrum of compounds in its constitution, including flavonoids and terpenoids (Delle Monache and Suarez, 1992; Delle Monache et al., 1996; Neves et al., 2011), as well as higher amounts of organosulfur compounds that justifies its pungent smell of garlic (Kubec and Musah, 2001; Kubec et al., 2002, 2010). Among the sulfur-containing compounds present in *P. alliacea*, previous investigations have highlighted the polysulfides and thiosulfonates as responsible for several biological activities shown in the extracts (Benevides et al., 2001; Cifuentes et al., 2009; Kubec and Musah, 2001; Kubec et al., 2002, 2010; Rosado-Aguilar et al., 2010; Uruëña et al., 2008).

The DNA of all living organisms is constantly exposed to injury. Genomic and cellular integrity is maintained by effective maintenance and repair mechanisms. Despite the existence of such pathways, some substances found in medicinal plants and herbal drugs can damage the genetic material and provide genotoxic stress triggering cell aging and carcinogenic processes (Abdelmigid, 2013). International organizations, such as the International Committee on Harmonization (ICH), have recommended the investigation of the potential DNA damaging effects of medicinal plants, through *in vitro* and *in vivo* experimental models (ICH, 2008). In this regard, these *in vitro* and *in vivo* models in the field of genetic toxicology can identify and measure the DNA damage, including frequency of DNA adducts, DNA strand break (single or double), DNA cross-linking, mutations or chromosomal aberrations (Abdelmigid, 2013).

Although *P. alliacea* possesses great pharmacological potential, some studies have pointed out its genotoxic hazards. In this sense, Hoyos et al. (1992) have demonstrated that ethanol extract of *P. alliacea* induces DNA damage evaluated by sister-chromatid exchange assay in *in vitro* and *in vivo* models, and also Soares et al. (2014) reported that different aqueous extracts of the plant caused single- and double-strand breaks in plasmid DNA from an *Escherichia coli* strain. However, both investigations evaluated the genotoxicity of low

concentrations of *P. alliacea* extracts in nonhuman models.

In this spite, this study was conducted to predict the potential genotoxic hazards of high concentrations of the hydroalcoholic extract of *P. alliacea* (EHPa) through *in vitro* and *in vivo* experimental models. In addition, the pharmacognostic parameters of the dry powder of the aerial parts of *P. alliacea* and the phytochemical composition of EHPa was examined.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol, methanol, dimethyl sulfoxide (DMSO), RPMI-1640, Triton X-100, TRIS buffer, sodium hydroxide, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), trypan blue dye, hydrogen peroxide (H₂O₂), ethidium bromide, low melting-point agarose, and normal melting point agarose (Sigma Chemical Com., St. Louis, MO, USA), fetal bovine serum (Gibco Life Technologies, Lofer, Austria), phytohemagglutinin (Life Technologies, Carlsbad, CA, USA), and cyclophosphamide (Asta Medica, Frankfurt, Germany) were used for the purpose of this study.

Plant

The aerial parts of *P. alliacea* (stem, leaves, and flowers) were collected in April, 2011 at Acará, Pará, Brazil (S 01°29'09.0/W 48°17'94.8). A voucher specimen was identified by the Botanist consultant Mário Augusto G. Jardim and was deposited in the herbarium of the Museu Paraense Emílio Goeldi (MPEG) under registry MG 94354. After collection, the plant material was first dried at room temperature (25°C/24 h) and subsequently, was dried in a forced-air oven to 40°C for 15 h. *P. alliacea* dry aerial parts were then powdered in a knife mill (Willey, Marconi MA 680 model).

Pharmacognostic characterization

The pharmacognostic characterization of *P. alliacea* was conducted from its aerial parts and encompassed the following determinations: granulometric analysis and mean particle diameter, loss on drying (oven/infrared moisture balance), total ash content, foaming index, and content of extractable matter (Allen et al., 2000; Brasil, 2010; World Health Organization 2011).

Extract preparation

The hydroalcoholic extract was chosen because previous studies of our group have demonstrated its potential effects on central nervous system when comparing with traditionally preparations (Andrade et al., 2012; Silva et al., 2015). Crude EHPa was prepared through the maceration method. Thus, plant drug (930 g) was extracted with 70% ethanol (v/v) for 7 days. The hydroalcoholic extract was filtered and evaporated under vacuum (40°C, 167 mbar, and 120 rpm) to provide 92.07 g of EHPa (1 g of extract equivalent to 10.1 g of dried aerial parts).

Preliminary phytochemical screening

The phytochemical screening of EHPa was conducted according to

Table 1. Reactions, reagents, and positive controls used in preliminary phytochemical screening of EHPa according to Barbosa et al. (2001).

Metabolites	Reagent / Reaction	Positive control
Reducing sugars	Formation of a red precipitate with Fehling's solutions	<i>Eupatorium ayapana</i>
Alkaloids	Precipitate formation after addition of Bouchardat's, Dragendorff's, Mayer's or Bertrand's reagents	<i>Psychotria ipecacuanha</i>
Deposides and depsidones	Chromogenic reaction after addition of ferric chloride (1%) to ether extract residue resuspended in methanol	<i>Portulaca pilosa</i>
Sesquiterpene lactones	Chromogenic reaction after addition of ferric chloride (1%) to an acidified solution of extract diluted in methanol plus hydroxylamine hydrochloride (10%) and KOH (10%)	<i>Achryrocline satureioides</i>
Coumarins	Blue fluorescence after addition of NaOH (1 N) drops to ether extract spotted in filter paper	<i>Eupatorium ayapana</i>
Flavonoids	Shinoda reaction	<i>Citrus aurantium</i>
Polysaccharides	Lugol's reagent	<i>Zea mays</i>
Tannins	Precipitate formation after addition of 1% ferric chloride to aqueous extract	<i>Stryphnodendron adstringens</i>

Barbosa et al. (2001) for the identification of the following metabolites: reducing sugars, alkaloids, deposides and depsidones, sesquiterpene lactones, coumarins, flavonoids, polysaccharides, and tannins. The respective reactions and reagents for identification of the metabolites mentioned, as well as vegetable drugs used as positive controls, are shown in Table 1

Thin-layer chromatography of EHPa

Thin-layer chromatography (TLC) was performed on a precoated 20 x 20 cm silica gel 60 F₂₅₄ plate (Merck, Germany) for the identification of sulfur compounds in EHPa as described by Wagner and Bladt (2001). Briefly, aliquots of EHPa dissolved in dichloromethane (10 mg/ml) were spotted on the plate. TLC analysis was developed using toluene-ethyl acetate (100:30, v/v) as mobile phase. As positive controls, the organic phase of aqueous extracts of *Allium sativum* and *Allium cepa* fresh bulbs extracted with dichloromethane were used. After chromatography, the plate was sprayed with 10 ml of vanillin-glacial acid reagent (VGA) and heated at 110°C for 3 min. Then the spots were observed under visible light. The retention factor values (R_f) of each spot were calculated using the equation: R_f = distance the spot moved above the origin/distance the solvent front moved above the origin.

In vitro comet assay

Cell culture and sample preparation for comet assay

Peripheral human blood samples were obtained from healthy volunteers (non-smoking and non-drinking males and females between 20 and 45 years old) after approval by the Research Ethics Committee of UFPA (CAEE 0154.0.073.000-11, Registry 165/11 CEP-ICS/UFPA). The blood samples were obtained as previously described by Albuquerque et al. (2015) for lymphocyte culture. In brief, 0.3 ml of venous blood were added to 4 ml of RPMI-1640 medium containing 20% of Fetal bovine solution (FBS)

and 50 µg/ml phytohemagglutinin (PHA) to stimulate lymphocyte proliferation. The mixture was then incubated under humidified environment at 37°C and 5% CO₂ for 24 h. The lymphocytes cultures (4.5 ml) were then treated with different concentrations of EHPa (50, 100, and 250 mg/ml; 0.4 ml) for 3 h at 37°C. As a negative and positive control, the lymphocytes were treated with RPMI-1640 medium and H₂O₂ at 35%, respectively, for 30 min at 37°C. Afterwards, cell viability was assessed with trypan blue dye exclusion test. Only cell samples whose viability was over 90% were measured by the comet assay (single cell gel electrophoresis).

DNA damage using comet assay

The comet assay was performed as described by Singh et al. (1988) and modified by Anderson et al. (1994). Treated lymphocytes, as already described were mixed with 0.5% low melting-point agarose, and the mixture (100 µl) was pipetted onto pre-coated slides with 1.5% normal melting point agarose. The drop containing the cells was placed under a glass cover slip (24 mm x 24 mm) and left at 4°C for 5 min. The cover slips were gently removed and the slides were treated with a lysis solution (2.5 M NaCl, 100 mM EDTA, 100 mM TRIS, 1% Triton X-100 and 10% DMSO, pH 10.2) for 24 h at 4°C. The slides were immersed in freshly prepared electrophoresis alkaline buffer (2.5 M NaOH, 100 mM EDTA, pH>13) and electrophoresis was performed using an electric field of 23 V/cm for 20 min. After electrophoresis, the slides were neutralized with distilled water for 5 min, fixed with ethanol for 3 min and air-dried. The microscope slides were then stained with ethidium bromide (20 µg/ml), and the cells were visualized and photographed under a fluorescence microscope (ZEISS AxioCam HRC) using a 510 to 560 nm emission/barrier filter at 400x magnification. A total number of 100 cell images (50 cells/slide) were collected randomly for each treatment and analyzed using Comet Score 1.6 (Tritek) software. The parameters of DNA damage analyzed included tail length (TL), percentage of DNA in the tail (% tail DNA), tail moment (TM), and Olive moment (OM). To avoid interference of additional DNA damage this experiment was conducted in a dark room and performed in duplicate.

Table 2. Pharmacognostic characterization of the dry powder of aerial parts of *P. alliacea*.

Parameter	Mean \pm SD
D_{50} (mm)	0.276 \pm 0.0012
Foaming index	133.95 \pm 12.6219
Loss on drying (%)	
Oven	9.67 \pm 0.2887
Infratest	8.93 \pm 0.1155
Total ash content (%)	9.43 \pm 0.2309
Content of extractable matter (%)	0.75 \pm 2.8868

In vivo micronucleus assays

Experimental animals

All experimental protocols were conducted in accordance with the National Institute of Health (Institute of Laboratory Animal Resources) for animal research and were approved by the Committee for Animal Care and Use of the Universidade Federal do Espírito Santo (UFES, Approval number: 074/2011). Eight-week-old adult male Swiss mice (*Mus musculus*) weighing between 25 and 35 g were obtained from the animal colony at UFES. The animals were housed in polypropylene cages, maintained under *ad libitum* food and water diet, and controlled laboratory conditions (22 to 25°C) with an artificial 12 h light/dark cycle.

Micronucleus assay

The micronucleus assay protocol was modified from Schmid (1975). Briefly, 30 mice were separated into five groups of six mice each. Thus, three groups were administered by oral gavage with EHPa at 50, 100, and 250 mg/kg b.w., and two groups received physiological saline (0.9% NaCl) and cyclophosphamide (50 mg/kg b.w., i.p.). After 24 h the animals were euthanized by cervical dislocation, and their femurs were dissected immediately for bone marrow extraction. The bone marrow samples were homogenized twice with 1 ml of fetal bovine serum (FBS) and centrifuged for 10 min at 1000 rpm. The supernatant was discarded, and the pellet was re-suspended in 0.5 ml of FBS. The cells were dropped on microscope slides and air-dried. Afterward, the slides were fixed with methanol and stained in two different concentrations of Leishman stain to allow visual differentiation between immature polychromatic erythrocytes (PCE) and mature normochromatic erythrocytes (NCE): (a) Leishman at 100% for 3 min., and (b) Leishman-distilled water (1:6, v/v) for 15 min (Krishna and Hayashi, 2000). The microscope slides were air-dried and then analyzed under light microscopy (Olympus, CX41) at a magnification of 1000x. To evaluate the genotoxicity of EHPa, the ratio of micronucleated polychromatic erythrocytes (MNPCE) per 2000 PCE was considered for each treatment and animal. To evaluate the cytotoxic effects of EHPa, 200 erythrocytes (PCE and NCE) were counted for the PCE/(PCE + NCE) ratio following Hayashi et al. (2000).

Data analysis

All results are expressed as mean \pm SD values. For the *in vitro* comet assay, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test for

multiple comparisons. The data was analyzed by Graph-Pad Prism software version 5.0 (San Diego, USA). For the *in vivo* micronucleus assay, normality test followed by Kruskal-Wallis test for nonparametric data were performed using Assisat software version 7.6 beta (UFPB, Brazil). In all experiments, differences were considered statistically significant if $p < 0.01$.

RESULTS

Pharmacognostic characterization

Table 2 summarizes the results obtained from pharmacognostic characterization of the dry powder of the aerial parts of *P. alliacea*. As shown, the powder possessed a mean particle diameter (D_{50}) of 0.275 mm. The powder was considered as moderately coarse according to the classification of pharmacopeia. All values for moisture content and total ash content, using the Brazilian Pharmacopoeia (Brazil, 2010) techniques, were within the limits established for plant drugs. The foaming index and the content of extractable matter corresponded to the values for qualitative assays of the plant drug.

Phytochemical screening of EHPa

Preliminary phytochemical screening of the EHPa was regarded as positive for the presence of metabolites, reducing sugars, alkaloids, and saponins.

Thin-layer chromatography of EHPa

TLC of the organic phase of *A. sativum* and *A. cepa* extracts showed the development of organosulfur compounds spots as previously reported by Wagner and Bladt (2001). TLC chromatogram of EHPa revealed the presence of three blue or blue-gray colored majority spots having R_f values of 0.55, 0.87, and 0.95, after spraying with VGA reagent. These spots were similar to the positive controls used and indicate the presence of

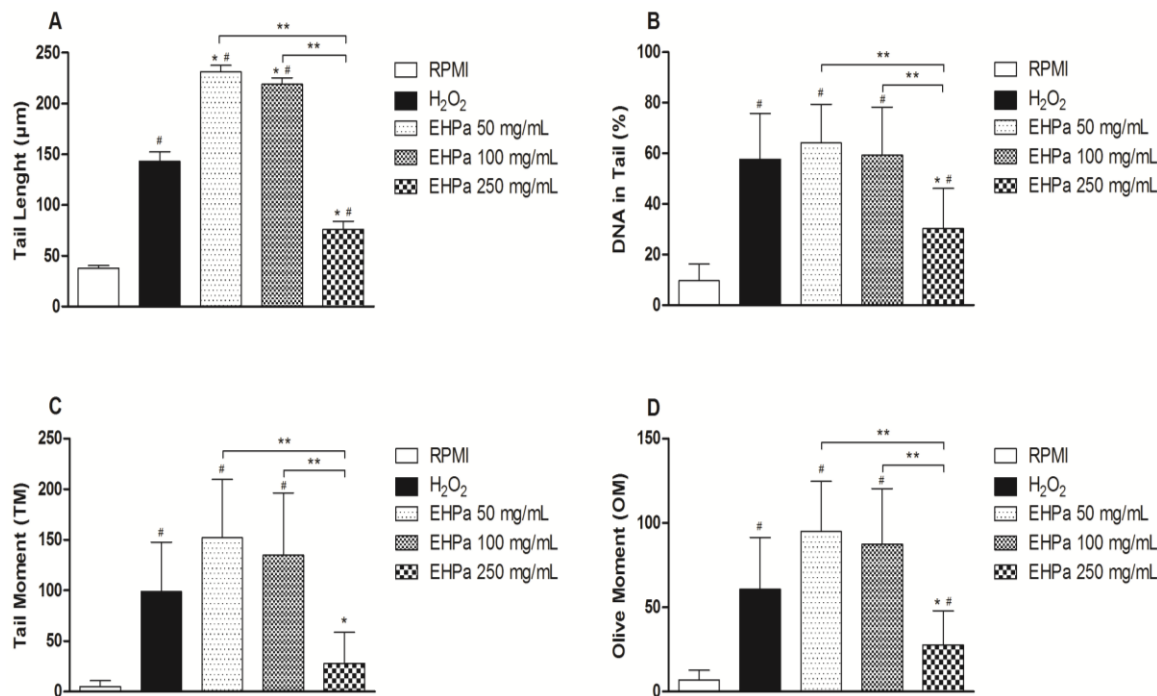


Figure 1. *In vitro* genotoxic effects of *P. alliacea*. The parameters tail length (A), percentage of DNA in the tail (B), tail moment (C) and Olive moment (D) were used as markers for DNA damage in human lymphocytes through comet assay. All values are represented as mean \pm SD. # $p < 0.01$ compared to the negative control (RPMI); * $p < 0.01$ compared to the positive control group (H₂O₂); ** $p < 0.01$ compared to greater EHPa concentration.

sulfur-compounds in EHPa (for example, thiosulfinates).

In vitro comet assay

The comet assay was performed for *in vitro* evaluation of some potential DNA damage of EHPa on human lymphocytes. According to the parameters for DNA damage analyzed (Figure 1), EHPa exhibited a dual effect on DNA. Treatment with EHPa at the concentrations of 50 and 100 mg/ml induced significant DNA damage when compared to the negative control (EHPa 50 mg/ml: TL 231.25 ± 48.56 mm, % tail DNA 64.12 ± 15.26 , TM 152.09 ± 57.63 , OM 94.93 ± 29.72 ; EHPa 100 mg/ml: TL 219.13 ± 48.38 mm, % tail DNA 59.33 ± 18.98 , TM 134.99 ± 61.34 , OM 87.48 ± 32.98 ; negative control: TL 37.79 ± 24.79 mm, % tail DNA 9.73 ± 6.56 , TM 4.86 ± 5.79 , OM 6.90 ± 5.80 ; $p < 0.0001$). These values were statistically higher than DNA damage caused by H₂O₂ for TL, TM, and OM parameters (H₂O₂: TL 143.23 ± 89.51 mm, TM 96.54 ± 51.35 , OM 60.67 ± 30.67 ; $p < 0.0001$). On the other hand, the highest concentration evaluated of EHPa (250 mg/mL) exhibited minor effects on DNA for TL, % tail DNA, and OM parameters when comparing to negative control (EHPa 250 mg/mL: TL 76.24 ± 56.77 mm, $p = 0.0017$; % tail DNA 24.01 ± 19.26 , $p = 0.0001$; OM 23.03 ± 20.67 , $p = 0.0021$).

In vivo micronucleus assay

The micronucleus assay was carried out in rodents for assessment of genotoxic and cytotoxic activities of EHPa. Table 3 summarizes the micronucleated polychromatic erythrocytes (MNPCE) frequencies and PCE/(PCE + NCE) ratio - ratio between immature polychromatic erythrocytes (PCE) and immature polychromatic erythrocytes (PCE) plus mature normochromatic erythrocytes (NCE); ratio in bone marrow of mice treated with different doses of EHPa. As for the genotoxic parameter, our results demonstrated that EHPa at doses of 50 and 250 mg/ml induced changes in MNPCE frequency compared to negative control ($p < 0.01$); however, these values were at least threefold lower than the positive control ($p < 0.01$). With regards to the cytotoxicity parameter, EHPa did not decrease significantly the PCE/(PCE + NCE) ratio when comparing mice treated with the negative control for all tested doses ($p > 0.01$). As observed with cyclophosphamide treatment, the PCE/(PCE + NCE) ratio was statistically lower than negative control and EHPa 50 mg/ml ($p < 0.01$), but not significant, compared to the EHPa 100 mg/ml and EHPa 250 mg/ml treatment groups.

DISCUSSION

Petiveria alliacea is an important Amazonian herb with

Table 3. Micronucleated polychromatic erythrocytes (MNPCE) frequencies and polychromatic/normochromatic erythrocytes ratio (PCE/[PCE + NCE]) in mouse bone marrow cells treated with oral doses of EHPa and controls.

Treatment (mg/kg b.w.)	MN/2000 PCE	PCE/(PCE + NCE)
EHPa 50	10.00 ± 2.04 ^b	0.49 ± 0.04 ^b
EHPa 100	8.75 ± 2.60 ^{ab}	0.45 ± 0.04 ^{ab}
EHPa 250	9.58 ± 2.10 ^b	0.43 ± 0.14 ^{ab}
Positive control	32.98 ± 4.13 ^c	0.41 ± 0.05 ^a
Negative control	5.50 ± 1.97 ^a	0.48 ± 0.03 ^b

Values are expressed as mean ± SD. Same letters in the same column – $p > 0.01$; different letters in the same column – $p < 0.01$. Negative control: saline; positive control: cyclophosphamide.

several pharmacological properties already reported. Recent investigations of our group have demonstrated that ethanolic preparations of *P. alliacea* possess anxiolytic, antidepressant and mnemonic effects upon central nervous system (Andrade et al., 2012; Silva et al., 2015). Although previous studies have demonstrated that *P. alliacea* induces genotoxic hazards in low concentrations, there is a lack of information about the effects of high concentrations of ethanolic preparations in both *in vitro* and *in vivo* models.

As alluded to earlier, we performed some pharmacognostic tests aiming to determine the quality control parameters of the powder obtained from *P. alliacea*. With regards to pharmacognostic characterization, powdered dry aerial parts of *P. alliacea* showed quality control parameters which were within the limits set forth in Brazilian Pharmacopoeia. These results are also similar to those observed by Audi et al. (2001) for loss on drying and content of extractable matter tests.

Phytochemical screening encompasses a set of qualitative tests for the identification of metabolites present in medicinal plants preparations. In this study EHPa showed positive results for the presence of reducing sugars, alkaloids, and saponins. Carillo et al. (1997) also identified the presence of alkaloids and saponins in ethanol extract of *P. alliacea*, differently however, we did not identify tannins, flavonoids, and sesquiterpene lactones. On the other hand, Villar et al. (1997) did not identify saponins and alkaloids in *P. alliacea* extracts.

Thin layer chromatography was also performed in order to identify the presence of organosulfur compounds in EHPa. TLC chromatograms demonstrated the presence of organosulfur compounds similar to Kubec and Musah (2001) with a spot of $R_f = 0.55$. Other sulfur compound spots identified in the chromatogram may belong to the class of thiosulfinates and other polysulfides, when compared to positive controls *A. cepa* and *A. sativum* spots (Wagner and Bladt, 2001). In this regard, several studies have reported the identification of organosulfur compounds in EHPa, including polysulfides (for example, dibenzyl trisulfide) and thiosulfinates (Benevides et al.,

2001; Hernández et al., 2014; Kubec et al., 2002, 2010; Rosado-Aguilar et al., 2010; Urueña et al., 2008; Cifuentes et al., 2009).

Other phytochemical studies have revealed the presence of different compounds in *P. alliacea*, such as the flavonoids engeletin, astilbin, myricetin, leridal, leridol, and petiveral (Delle Monache and Suarez, 1992; Delle Monache et al., 1996; Hernández et al., 2014); terpenoids; and benzenoids (Neves et al., 2011). The non-identification of some metabolites previously reported in *P. alliacea* does not characterize their absence in EHPa because several factors, such as environmental temperature, time of collection, height, UV radiation, storage, drying, and extraction, influence the constancy of metabolite content in plants (Gobbo-Neto and Lopes, 2007; Sahoo et al., 2010).

In addition to pharmacognostic and phytochemical investigations, the evaluation of toxicity of medicinal plants and their derivatives is an important step in predicting potential hazards to human health (Abdelmigid, 2013; Netto et al., 2006). In the acute toxicity assay (data not shown), we did not observe any death in animals treated with EHPa even at the maximum dose administered (1000 mg/kg b.w.). Hernández et al. (2014) evaluating the acute toxicity of a *P. alliacea* fraction (doses ranged between 5 to 2000 mg/kg b.w., i.p.), calculated an LD₅₀ of 1545 mg/kg b.w.

The possible genotoxic effects of *P. alliacea* was evaluated through the *in vitro* comet assay in the present study; through the *in vitro* comet assay it was observed that EHPa significantly induced DNA damage of human lymphocytes. However, the EHPa induced high DNA damage in low concentrations (50 to 100 mg/ml), while the greatest concentration (250 mg/ml) induced a small DNA damage. This different effect could be due to the synergistic and antagonistic relationship between some substances in the extract, like thiosulfinates (Xiao and Parkin, 2002), that can lead to an oxidative stress imbalance and can also activate DNA repair pathways, reducing the genotoxic effects (Cooke et al., 2003). In this context, Hageman et al. (1997) reported that the water extract of raw garlic and two organosulfur

compounds, diallyl sulfide and S-allylcysteine, were able to inhibit the DNA adduct formation induced by benzo[a]pyrene by antioxidant mechanisms, such as increase of the glutathione S-transferase activity and reduction of ROS. In addition, other studies reported that another antioxidant compound, such as phenolic compounds, mainly caffeoylquinic acid derivatives, from root extracts of *Rhaponticum carthamoides* may stimulate in CHO cells to repair oxidative DNA damage and protect DNA by increased antioxidant gene and protein expression levels (SOD2 and CAT) regulating intracellular antioxidant capacity (Skala et al., 2016).

In this regards, reactive oxygen species (ROS) and free radicals generation may play a pivotal role in DNA damage (Cadet et al., 1999; Del-Rio et al., 2005; Subhashinee et al., 2005). Previous studies have demonstrated that *P. alliacea* exerted both pro-oxidant and antioxidant effects on *in vitro* and *in vivo* models (Desmarchelier et al., 1997; Andrade et al., 2012). Desmarchelier et al. (1997) observed that preparations obtained from aerial parts of *P. alliacea* (1 mg/ml) presented slight anti-oxidant activity with lowest TRAP values (aqueous and dichloromethane extracts: <100 μ M Trolox; methanol extract: 275 μ M Trolox). On the other hand, our group also reported that animals orally treated with 900 mg/kg of *P. alliacea* L. hydroalcoholic extract exhibited pro-oxidant status (Andrade et al., 2012). In this study, it was shown that the extract was able to increase Trolox equivalent antioxidant capacity (TEAC), nitric oxide (NO) and malondialdehyde (MDA) levels, as well as increase methemoglobin formation in the human plasma. These data suggest that *P. alliacea* extract has pro-oxidant action and can lead to cell death (cytotoxicity) *in vivo*, because the increase of MDA is used as a marker of lipid peroxidation and cell damage caused by the action of reactive species in the body, as well as oxidative processes in the membrane (Janero, 1990; Del-Rio et al., 2005). In addition, *in vitro* and *in vivo* studies have reported that the MDA is able to interact with nucleic acid bases, as deoxyguanosine and deoxyadenosine, to form several different adducts, which may be mutagenic and these were quantified in several human tissues (Marnett, 1999a; Marnett, 1999b; Lykkesfeldt, 2007). Thus, high levels of reactive species and MDA formation induced by *P. alliacea* may be one of the mechanisms that lead to the high EHPa-induced DNA damage *in vitro* observed in this study.

In order to confirm the results from *in vitro* comet assay the *in vivo* micronucleus assay was performed using mouse bone marrow. In this study, only the treatment with EHPa at the concentrations of 50 and 250 mg/kg b.w. induced small changes in the frequency of MNPCE compared to negative control. In this regard, the micronucleus assay has been employed for genotoxicity and mutagenicity detection of substances that can cause cytogenetic damage, thus the increased MNPCE frequency can be related to cancer, though the MN can

be a target of carcinogenesis (Bonassi et al., 2006). On the other hand, the comet assay was used to detect genotoxic effect of compound by measuring its DNA damage at single cell (Hartmann et al., 2003). Therefore, the present data showed that *P. alliacea* induces a slight mutagenic effect *in vivo*, but a high genotoxic effect that was detected by Comet assay. Thus, the data suggest that *P. alliacea* is associated with high genotoxicity, but a low risk for carcinogenesis, as described by Lee et al., (2015). These different effects detected by *in vitro* (comet assay) and *in vivo* (micronucleus assay) assays, also was reported by other studies, as Schauss et al. (2015) that showed that the Dahurian larch tree derived lavitol led to DNA damage in marrow cells, liver and blood, but did not increase the number of binucleate cells containing micronuclei. These effects can be explained by the concentrations/doses used in both assays. As well established, herbal derivatives, including plant extracts, possess poor lipid solubility or improper molecular size, which results in poor absorption and consequently poor bioavailability (Kesarwani and Gupta, 2013). In this regard, the activity of some enzymes on *P. alliacea* compounds could decrease their bioavailability on tissues, as well as the time of administration of the plant extract, and as mentioned before, the possible synergistic and antagonistic interactions between the compounds presents in the EHPa and their effects on oxidative balance. These findings may suggest that the oral administration route can allow some substances present in the plant extract to suffer from drug biotransforming reactions involving hepatic enzymes, which can facilitate their excretion and consequently the decrease of plasmatic concentrations. In spite of this, our results suggest that at least a concentration of 50 mg in plasma level could trigger DNA damage, as observed through comet assay.

Furthermore, other factors that justify the differences obtained in the *in vivo* versus *in vitro* experiments cannot be excluded. These factors include the DNA repair mechanisms present in biological environment, namely base excision repair, nucleotide excision repair, mismatch repair, direct repair, and recombination repair, which could repair some DNA damage induced by EHPa in animal model (Ramos et al., 2011). Furthermore, it was hypothesized that the DNA damage induced by EHPa can also lead to cell death because it is already well-reported that *P. alliacea* extracts exhibited cytotoxic activity against normal fibroblasts and peripheral blood mononuclear cells (Urueña et al., 2008), and several tumor cell lines, such as melanoma, leukemia, and lymphoma (Pérez-Leal et al., 2006; Rossi, 1990). Regarding cellular toxicity, our data showed that none of the doses led to cytotoxic effects. However, there was a tendency to increase the cytotoxicity with increasing concentration of extract, as seen in Table 3. Urueña et al. (2008) observed that an aerial parts fraction of *P. alliacea* exhibited low toxicity to normal fibroblasts and peripheral

blood mononuclear cells in the presence or absence of phytohemagglutinin stimulation (IC₅₀ of 440, 151, and 121 µg/ml, respectively). Differing from our results, previous studies using the crustaceans *Artemia salina* and *Thamnocephalus platyurus* have demonstrated cytotoxic activity in *P. alliacea* extracts (Desmarchelier et al., 1996; Berger et al., 1998; Mayorga et al., 2010). These differences could be due to the different variables involved in the preparation of *P. alliacea* extracts (that is, plant part, period of collection, nature of solvent, method of extraction, and time of extraction).

In this context, Hernández et al. (2014) also reported other mechanisms by which the *P. alliacea* induced 4T1 cells apoptosis, such as caspase-3 activation and DNA fragmentation without mitochondrial membrane depolarization, this was also shown by Uruña et al. (2008) using a leukemia cell line. Accordingly, Hartmann et al. (2003) and Azqueta and Collins (2013) also reported that the cytotoxicity is associated with increased levels of DNA damage, which leads to the generation of false positive results. In addition, a previous study showed that a *P. alliacea* ethanol extract was able to induce sister-chromatid exchanges in human lymphocytes at the final concentration of 100 µg/ml, as compared with the positive (N-ethyl-N-nitrosourea) and saline control groups (Hoyos et al., 1992).

Therefore, the variation in these factors may yield extracts with different chemical composition which could explain the different pharmacological profiles found in the cytotoxic evaluation. The susceptibility of each cell model employed in these studies may also explain these differences.

Conclusion

Our data suggest that *P. alliacea* induces DNA damage *in vitro*, but this effect was slight in an *in vivo* model. The different effect of the EHPa in *in vitro* model may be related to the chemical composition of the extract, which includes thiosulfonates compounds. Further investigations on the mechanism of the DNA damage induced by *P. alliacea* are required to understand if it could trigger deleterious effects, such as an induction of mutations and carcinogenic processes.

Conflict of interests

The authors have not declared any conflict of interest

ACKNOWLEDGEMENTS

The authors are grateful to the technical assistance of Parasitology Division of Instituto Evandro Chagas and Toxicology Laboratory (Faculty of Pharmacy-UFGA). We gratefully acknowledge the financial support of Pró-

Reitoria de Pesquisa e Pós-Graduação from UFGA (PROPESP/UFGA).

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