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Phytochemical and ameliorative effects of Peanut skin ethanol extract against Lead toxicity in *Drosophila Melanogaster*

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Abstract

The processing byproduct, peanut skin is a hub of antioxidant compounds such as phenol and flavonoids and vital metal chelators including Cu^{2+} and Zn^{2+}. Adverse exposures to heavy metals like Pb^{2+} could cause oxidative stress, inflammation and neurological and reproductive disorders. This research aimed to study the phytochemical profile and the rescue effects of peanut skin ethanol extract against lead acetate-induced oxidative stress in Drosophila melanogaster. Phenols, flavonoids, alkaloids, saponins, steroids, terpenoids, anthraquinones, cardiac glycosides, Methyl palmitate, Ethyl palmitate, Methyl linoleate, Ethyl linoleate, Methyl oleate, Methyl stearates, 156.15mg/g total phenol and 124.83mg/g total flavonoids were detected by phytochemical screening and GC-MS analysis. Peanut skin extract from 31.25µg/ml -500µg/ml exhibited a dose dependent scavenging activity against DPPH and H_2O_2 radicals at nearly equal strength compared to the standard vitamin C. Lead acetate treated flies had reduced activities of AChE, SOD, and GPx with concomitant increase in NO and MDA concentrations. This was ameliorated by co-treatment with peanut skin extract or resveratrol through elevation of AChE and SOD, normalization of GPx activities and a corresponding decrease in NO and MDA productions. GSH activity was not significantly affected by lead or the co-treatment with peanut skin extract or resveratrol. Thus, this study revealed that peanut skin ethanol extract could be a source of intervention for prevention and treatment of oxidative stress and inflammation related to lead toxicity.

Keywords: Peanut skin, phytochemical profile, GCMS, Oxidative stress, Pb^{2+} toxicity, *Drosophila melanogaster*

1. Introduction

Peanut (*Arachis hypogaea*) is an important nutritional supplement that is so affordable and widely cultivated. Although, its skin is mostly considered as a processing byproduct yet it possesses some strong antioxidant phytochemicals such as linoleic acids, polyphenols, and flavonoids [1]. In a study, ethanol extract of peanut skin has yielded greater antioxidant activity and total phenol content compared to other solvents used for extraction [2].

Oxidative stress and inflammation play a vicious cycle in the pathogenesis of neurodegenerative diseases. Cellular damage resulting from alterations of macromolecules (nucleus, mitochondria, DNA, protein, lipids) by free radicals such as NO, H_2O_2, N_2O_2, -OH can led to the activation of brain immune cells [3]. Sustained oxidative stress can lead to continuous generation of neurotropic or inflammatory factors such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α) by the glia cells, inducing an inflammatory cascade that results to neurotoxicity and cognitive dysfunction [4]. Also sustained inflammation of the nervous system could lead to excessive accumulation of inflammatory factors such as NO that will contribute to oxidative stress [5].

Lead is one of the toxic heavy metals with heavy presence in the environment. Interaction with lead is high in localities of mining activities and at a less extent in industries [6]. Lead enters into the body by inhalation as dust particles and oral ingestion via drinking contaminated waters of streams, rivers and well [7]. Exposures via respiratory and digestive tract could lead to lead assimilation into the cells and ultimately disrupts the cellular activities, including production of free radicals that could damage nucleic acids and affect cellular or organelle membrane integrity and function. This cellular perturbation can accelerate the onset of degenerative diseases in adults and children such as neurodegeneration, reproductive decline [8].

Drosophila melanogaster, the Cinderella of genetics is commonly known as the fruit fly or vinegar fly. It is a holometabolous insect with short life cycle and high reproductive rates, inexpensive maintenance and short regeneration time. A female drosophila would lay about 40eggs in 24hours. The fly has high genetic homology with humans with over 70% genetic similarity. The fly has been used as a model for the study of several human diseases both at protein and molecular level. It is also being used in the study of toxicity of heavy metals, behavioral and social interactions including aggression

and courtship, reproduction, oogenesis and neuronal diseases including Parkinson's, Alzheimer's disease etc. [9].

The objective of this study was to determine the protective effects of peanut skin ethanol extract against lead-induced oxidative stress in *Drosophila melanogaster*. This was achieved through the assessments of phytoconstituents and the DPPH and H_2O_2 radical scavenging activities of peanut skin extract ethanol (PSE), as well as SOD, GSH, GPX, MDA, NO and AChE activities in whole Drosophila tissue homogenate.

2. Materials and methods

Resveratrol analytical grade (Candlewood Stars Incorporated, Danbury, USA). Lead acetate (PbAc) analytical grade (Fisher Scientific Company, USA). ELISA Kits: Nitric oxide Cat No: CK-bio 15075 and Acetylcholinesterase Cat No: CK-bio-14126 (Shanghai Coon Koon Biotech Company Ltd, China).

2.1 Preparation of peanut skin extract (PSE)

Peanut skin was obtained as a processing by-product of groundnut used for making fried peanut cakes from a microscale local industry in Keffi Town, Nasarawa State, Nigeria. An optimized method for the extraction of phenols, flavonoids, resveratrol and strong antioxidant activity from peanut skin was adopted with some modifications [2]. 70% ethanol was used to soak non-crushed peanut skin in a 20ml/g solvent/solid ratio for 4days. Mixture was filtered using Waltman filter paper. Filtrate was condensed and dried by evaporating the solvent in a water bath and air-dried under a biosafety cabinet.

2.2 Drosophila culture

Wild Harwich strains of *Drosophila melanogaster* were maintained on standard cornmeal medium (containing 50g cornmeal, 10g yeast, 8g of agar–agar, 1g of Nipagin and 850ml distilled water) 10, under 12hour dark/light cycle, at 25℃ in the Drosophila and Neurogenetics laboratory, Department of Zoology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria [11]. Drugs concentrations were made per 10ml diet, as described by Kruger and Denton [12]. Drugs concentrations for induction of toxicity, PbAc 4.8mg, and ameliorations, PSE 7.2mg, 14.4mg and 28.8mg or resveratrol (RSV) 37.2mg were selected from a preliminary acute toxicity assay (Figure 3.2 A, B and C) [11].

2.3 Experimental Design

Table 2.1: Experimental data

S/N	Group	Remark
1	Group N	Normal
2	Group L	PbAc 4.8 mg
3	Group LP1	PbAc 4.8mg + PSE 7.2mg
4	Group LP2	PbAc 4.8mg + PSE 14.4mg
5	Group LP3	PbAc 4.8mg + PSE 28.8mg
6	Group LR	PbAc 4.8mg + RSV 37.2mg

2.4 Phytochemical screening

Ethanol extract of the peanut skin was subjected to different chemical tests for the qualitative detection of phytoconstituents as described by Khan [13]. Total phenols and flavonoids contents was analyzed as described by Nayak et al. and Ainsworth et al. [14-15].

GC-MS analysis of the extract was conducted using Agilent Technologies Auto-system GCMS-QP2010 PLUS Shimadzu, Japan at the Multi-User Research Laboratory, Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria [16].

2.5 DPPH and H_2O_2 radical scavenging assay for Peanut Skin Extract

DPPH and H_2O_2 radical scavenging activities of the PSE were analyzed by spectrophotometry as described by Oyedemi *et al.* [17].

2.6 Preparation of the Fly Tissue Lysate and Biochemical Assays

The protocol from Experiments with Drosophila for Biology Courses [18] was adopted with slight modification. 25 Drosophila flies were collected in a microcentrifuge tube, weighed then homogenized in 1.5mL Phosphate buffered saline with the help of a hand homogenizer. The homogenates were centrifuged at 10,000 rpm for 15min; the supernatants were taken for biochemical analysis.

2.6.1 Superoxide dimutase (SOD) activity

Superoxide dismutase (SOD) was determined by the method described by Fridovich [19]. The principle is based on the ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2 form the basis of this assay.

2.6.2 Assay of glutathione concentration

Reduced glutathione (GSH) concentration measurement was done following the method described by Rajagopalan *et al.* [20]. The principle is based on the reaction of 5, 5- dithiobis nitro benzoic acid (DNTB) and reduced Glutathione (GSH).

2.6.3 Lipid peroxidation (MDA)

Lipid peroxidation is evidenced by formation of TBARS and was measured by spectrophotometry as described by Akanji *et al.* [21].

2.6.4 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined as demonstrated by Rotruck et al. 22. The whole reaction mixture was incubated at 37℃ for 3minutes after which 0.5ml of TCA was added and thereafter centrifuged at

3000rpm for 5minutes. To 1ml of each of the supernatants, 2ml of K_2HPO_4 and 1ml of DTNB were added and the absorbance was read at 412 nm against a blank.

2.6.5 Enzyme linked immunosorbent assay (ELISA) for determination of Nitric oxide and Acetylcholinesterase Activities.

Nitric oxide and Acetylcholine esterase were assayed using Rat Nitric oxide and Acetyl choline esterase ELISA Kits respectively. The principle was based on double antibody sandwich technology enzyme linked immunosorbent assay (ELISA). Procedure: 50µL standard and 50µL sample (homogenate 10µL + diluent 40µL) were added into respective wells of pre-coated objective antibody. 100µL HRP conjugate reagent was added to the wells, covered with seal plate membrane, mixed by shaking gently and incubated for 60mins at 37℃ to form an immune complex. The wells were washed (5 times) using 20X washing solution to remove unbound enzymes. For color development, 50µL of chromogen A solution, then 50µL chromogen B solution was added to each well, the reaction mixtures were shaken gently and incubated for 15min at 37℃ in dark. The reaction was stopped by the addition of 50µL stop solution (an acid); this is indicated by immediate change of color from blue to yellow. Blank well was taken as zero and absorbance (optical density) of each well was taken at 450nm (within 15mins after addition of the stop solution). The color depth or light is a positive correlation of the Nitric oxide or acetylcholine concentrations. Standard curves were calculated by linear regression equation using the standard's concentration and the corresponding optical density. The samples optical densities were used to calculate the concentrations of the corresponding samples.

2.7 Statistical analysis

One-way ANOVA test was used to calculate the differences between groups with the mean and standard error of mean ($M \pm S.E.M$). P value at ≤ 0.05 was considered as statistically significant. IBM SPSS Statistics 20 software was used for the analysis.

3. Results and Discussion

3.1 Results

The phytochemical screening of PSE conducted revealed the presence of phenols, flavonoids, alkaloids, saponins, steroids, terpenoids, anthraquinones and cardiac glycosides. The total flavonoids and phenolics contents were 124.83mg/g and 156.15mg/g respectively. GC-MS analysis detected the presence of ethyl and methyl esters of 8 long chain fatty acids, Methyl palmitate, Ethyl palmitate, Methyl linoleate, Ethyl linoleate, Methyl oleate, Methyl heptadecanoate, 10-Octadecenoic acid methyl ester and Octadecanoic acid 17-methyl-, methyl ester (Table 3.1).

Table 3.1: Preliminary qualitative phytochemistry and GCMS analysis

Phytoconstituents	Inference	GC-MS Detected compounds
Alkaloids	+	Hexadecenoic acid, methyl ester (Methyl palmitate)
Cardiac Glycosides	+	Hexadecenoic acid, ethyl ester (Ethyl palmitate)
Sapo+nins	+	9,12-Octadecadienoic acid (Z, Z)-, methyl ester (methyl linoleate)
Phenolic compounds	+	9-Octadecenoic acid (Z)-, methyl ester (methyl oleate)
Tannins	+	Heptadecanoic acid, 16-methyl-, methyl ester (Methyl margarate)
Steroids	+	Linoleic acid ethyl ester (Ethyl linoleate)
Carbohydrates	+	10-Octadecenoic acid, methyl ester (Methyl stearate)
Flavonoids	+	Octadecanoic acid, 17-methyl-, methyl ester (Methyl stearate)
Total Flavonoid Content	124.83 mg/g	
Total Phenol Content	156.16mg/g	

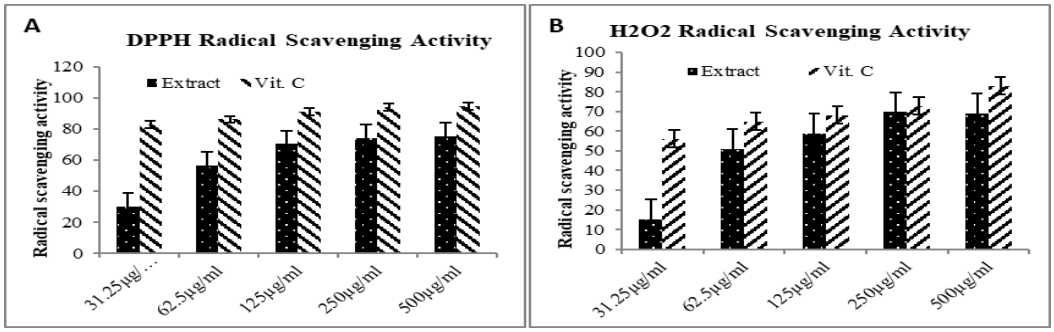

Figure 3.1: A and B: DPPH and H_2O_2 radical scavenging activities

The extract showed a free radical scavenging activity in concentration dependent manner (from 3.125µg/ml to 500µg/ml). The activity is nearly equal to vitamin C. Result presented in mean and SEM.

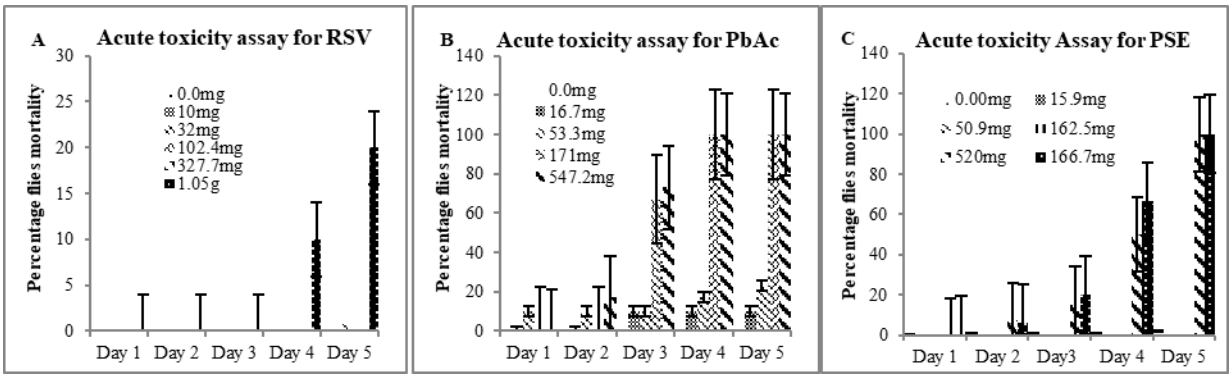

Figure 3.2 A, B and C: Acute toxicity Assay

Resveratrol concentrations from 10mg to 327.7mg were found to be practically nonlethal to adult flies after 5 days exposure as no incidence of mortality was observed. However, at 1.05g RSV 20% mortality was observed, indicating its relative non-lethality to the adult Drosophila (Figure 3.2-A). Lead acetate was lethal to adult flies in a concentration dependent manner, lead acetate from 16.7mg to 53.3mg killed 10-20% of flies and 100% lethality was observed at 171mg to 547.2mg (Figure 3.2-B). Peanut skin extract at lower doses from 15.9mg-162.5mg did not kill any adult fly but the doses of 520mg and above practically killed 100% of the flies (Figure 3.2-C). Results are presented as Mean ± SEM.

The *in vivo* antioxidant and acetylcholinesterase activity in Drosophila whole tissue homogenate is shown in Table 3.2 below, compared to the normal control (N), SOD activity was significantly decreased by PbAc treatment (L), however, co-treatment with PSE 7.2mg (LP1), 14.4mg (LP2), 28.8mg (LP3) or RSV (LR) abolished PbAc toxicity thereby increasing the SOD activity to near normal level. GPx activity was increased significantly by PbAc exposure of flies, upon co-treatment with RSV 37.2mg (LR) or PSE 14.4mg and 28.8mg (LP2 and LP3) this toxicity by PbAc was abrogated and GPx was restored to near normal level. Conversely, co-treatment with PSE 7.2mg (LP1) further reduced the activity of GPx even far below the normal control (N). The treatment of flies with PbAc or combined with RSV or PSE across all concentrations did not significantly affect the level of GSH activity. Although the treatment of flies with PbAc just slightly increased MDA concentration, the co-treatment with PSE 7.2mg (LP1) significantly reduced the MDA concentration to a below normal level. Co-treatment with RSV or PSE 14.4mg or 28.8mg did not have any effect on the MDA concentrations. Compared to the normal control (N), PbAc elevated the concentration of NO, co-treatment with PSE 7.2mg or RSV 37.2mg (LP1 or LP2) further increased NO concentration. PbAc with PSE 14.4mg (LP4) or 28.8mg (LP3) reduced the NO concentration. AChE activity was significantly reduced by PbAc, it was however observed that co-treatment with PSE at 14.4mg (LP2) and 28.8mg (LP3) significantly elevated the AChE activity while PSE 7.2mg and RSV did not affect the PbAc toxicity.

Table 3.2: Antioxidant enzymes and acetylcholinesterase activities assay

Group	N	L	LP1	LP2	LP3	LR
AChE (U/L)	346±4	296±5*	296±2	458±8*	431±10*	274±26*
NO (µmol/L)	188±2	192±2	228±3*	164±3*	161±5*	209±9*
SOD (U/ml)	85±14	53±1*	65±3*	62±3*	58±1*	78±4
GPx (µmol/mg protein)	46±8	60±5*	18±13	54±1	42±8	56±4
GSH (µg/ml)	22±1	22±1	24±0	23±1	22±2	25±4*
MDA (µmol/ml)	25±3	26±2	20±3*	25±1	26±4	26±1

Values with * denotes significant difference from the control at $p<0.05$

3.2 Discussion

Peanut (*Arachis hypogaea*) is an important nutritional supplement; the skin is mostly considered as a processing byproduct. However, its ethno-pharmacological applications have been reported in traditional Chinese medicine for the treatment of hyperglycemia and hemorrhage [23] as well as improving memory via modulation of anti-oxidative stress and regulation of brain-derived neurotrophic factor pathway in animal models [24].

In Table 3.1, this study detected the presence of fatty acid esters of palmitic acid, linoleic acid, oleic acid, margaric

acid and stearic acid as well as other important bioactive compounds including cardiac glycosides, flavonoids, phenols, and alkaloids which have been extensively studied for their cardio-protective, antioxidant, anti-inflammatory, coronary disease preventive and antibacterial activities [25-27], other phytochemicals detected are saponins, steroids and tannins.

Peanut skin ethanol extract has shown a doses dependent strong radical scavenging activity against DPPH and H_2O_2 radicals at nearly equal capacity compared with ascorbic acid. The extract contains 124.83mg/g of flavonoids and 156.15mg/g of phenolics, this result has agreed with the findings by Nepote et al. 2 that peanut skin extracted in 70% ethanol had highest antioxidant activity and total phenolic contentment compared to aqueous, absolute methanol and absolute ethanol (Figure 3.1 A and B).

In the acute toxicity study (Figures 3.2 A, B and C), no flies mortality was recorded following the administration of resveratrol from the minimal to the highest doses (1mM-105mM) (Figure 3.2 A) justifying its relative nontoxicity, nutritional activity and therapeutic applications in mitigating oxidative stress, inflammation and neurotoxicity as similarly reported by Abolaji *et al.* [28]. Exposure to lead acetate was toxic and lethal to the flies from the lowest to highest concentrations (Figure 3.2 B), lead (Pb^{2+}) has no known biological role and exposure even at a very low concentration can cause reproductive, developmental and neuropsychiatric disorders 7. From the study, peanut skin extract is safe but toxic and lethal to flies at doses greater than 162.5mg per 10ml diet. The peanut skin extract is dense in fat and excessive fatty acid intake has been reported in several studies to induce coronary diseases, heart failure and ultimately death [29-30], this would explain the mortality observed in flies administered higher doses of peanut skin extract (Figure 3.2 C).

As shown in Table 3.2, groups treated with lead only exhibited lower SOD and AChE activities with an increased MDA and NO concentrations, while GPx activity was upregulated and GSH activity unaffected compared to the untreated group. The extract protected the flies against lead acetate-induced oxidative stress by increasing the activities of SOD and GSH, normalizing the cellular activity of GPx and inhibiting the cellular generation of MDA and NO. Although lead (Pb^{2+}) exposure is known to induce a reduction of antioxidant enzymes activity such as GPx, other reports such as this, and in Shilpa *et al.* [31] have shown that exposure to organic lead concentrations can induce an increase in GPx activity beyond normal level. Similarly, increased GPx activity alongside MDA has been reported in polyethylene microplastics induced oxidative stress in coral *Goniopora columna* by Chen *et al.* [32]. Furthermore, chronic oxidative stresses in diabetes, glucose intolerance and aging have been reported to cause an increased GPx activity as a result of upregulation of the enzyme [33-34]. Also, the extract at 14.4mg and 28.8mg exhibited a strong neuroprotective activity by increasing acetylcholinesterase activity. Exposure to Pb^{2+} and other heavy metals such as mercury have been reported to cause a significant decrease in acetylcholinesterase activity in several studies [35-36]. Although the extract exhibited a better neuroprotective activity than resveratrol, in *vivo* antioxidant activity is similar to resveratrol. These protective effects could be attributed to its high phenolics and flavonoids contents as evidenced by its strong DPPH and H_2O_2 radical scavenging activities (Table 3.1, and Figures 3.1 A and B) [37].

4. Conclusions

From this research work, it is evident that the PSE possess a strong DPPH and H_2O_2 radical scavenging activities. Across all tested concentrations the extract protected the flies against oxidative stress by significantly increasing SOD concentrations and reducing MDA productions at 14.4mg and 28.8mg extract concentration. The extract inhibited lead induced increase of GPx activity across all doses. GSH activities were not significantly affected by lead acetate or its coadministrations with peanut skin extract. The extract at 14.4mg and 28.8mg exhibited a strong neuroprotective and anti-inflammatory activity by increasing Acetylcholinesterase activity and reducing NO concentration.

Conflict of Interest: The authors declare no conflict of interest.

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