Original Research Article

THE ANTI-OXIDATIVE POTENTIALS OF ETHANOLIC EXTRACT OF AVOCADO PULP *(Persea americana mill)*ON CAFFEINE INDUCED OXIDATIVE STRESS ON WISTARRATS

ABSTACT

This study evaluated the effect of ethanolic extract of *Persea americana* pulp on caffeine induced oxidative stress on male wistar rats. Fifty-four (54) mature male rats were used and divided into six (6) groups of nine (9) rats per group. Group two (2) to group six (6) were induced with 200mg/kg caffeine for two weeks. Three rats were sacrificed from each group after two weeks of induction, and blood collected to check for caffeine toxicity. The rats were treated as follows for another four weeks, Group 1: Normal control(feed and water only)., Group 2:positive control (caffeine only), Group 3: 100mg/kg extract, Group 4: 300mg/kg extract, Group 5: 500mg/kg extract, Group 6: 0.107mg/kg proviron. The rats were sacrificed twice after treatment: two weeks after treatment and four weeks after treatment and blood, was collected for oxidative stress markers. Oxidative stress (superoxide dismutase, glutathione peroxidase, catalase, glutathione reduced and malondi- aldehyde) were comparable to the negative control and positive control group. From the findings of this study, it can be concluded that oral administration of ethanolic pulp extract of *Persea americana* has a dose ameliorating effect on caffeine induced oxidative stress damage on male rats.

KEY WORDS: Proviron,*Persea americana*, Caffeine, Peroxidase, Glutathione, Catalase, and Superoxide dismutase.

INTRODUCTION

The use of plants for therapy has been in existence before modern medicine. There is a great relationship between food and health. The application of food in medicine was advocated by Hippocrattess (10). Phytochemicals in plants are known to reduce the risk of some severe disorders such as autoimmune and cardiovascular diseases as well as neurodegenerative diseases. Some known polyphenols such as curcumin, ferulic acid, pro-anthocyanidin, quercetin, and resveratrol exhibits curative effects non inflammation and oxidation. These phytochemicals have also shown their neuro-protective effects. The carvacrol is a monoterpenic phenol which exhibits anti-inflammatory effect, analgesic effect, anti-arthritic effect, anti-allergic effect, anti-diabetic effect, cardio-protective effect, hepatoprotective effect, and neuroprotective effect (20). It is responsible for the regulation of human ion channel regulator transient receptor potential causing sensation of warmth. Another important plant phytochemical with health benefit is ferulic acid. It exhibits strong oxidative effect (35). It is derived from phenylalanine which is converted ton 4-hygroxycinnamic acid and then caffeic acid. It has anti-inflammatory effects, anti–tumor, neuroprotective and anti-diabetic properties . L-theanine which amino acid component of some herds has been demonstrated to have some health benefits such as improving concentration and learning ability, functioning as an anti-tumor, lowering of blood pressure, improving the immune system, and displaying neuroprotective effect. L-theanine shows some effects in the central nervous system including the potentiation of gamma–amino-butyric acid, dopamine and serotonin and inhibition of glutamate uptake (25). Pro-anthocyanidins are oligomeric flavan-3-ols which are found in plants such as apple, cocoa, bean, grape and tea. It has been proven from research that these compounds exhibit pharmacological properties such as cardio-protective effect, and anti-oxidant effect (29). Proanthocyanidin administration to mice and 50mg/kg body weight for seven days reduces immobility time in both forced swimming and tail suspension test in mice. Another polyphenolic phytochemical with pharmacological benefit is quercetin. It is present in many fruits, vegetables and medicinal plants. It functions as a strong anti-oxidant by scavenging of free radicals.

Majority of the products generated from plants have not been tested to for their safety and efficacy. There was a policy formulated by the World Health Organisation on the use of traditional medicine in 1991. Researches have been carried out on plants used in traditional medicine with a multidisciplinary approach with more than 10000 plants having being studied in the past five years which results to enough scientific evidence on the pharmacological effects of these plants (15). This knowledge has relevant application in the pharmacological industry as this has led to the synthesis of many active substances isolated from the plants. The Food and Agricultural Organisation estimated in 2002 that more than fifty plants which have therapeutic effects are used in the world. The medicinal plants have great applications in modern medicine as about one third of the drugs prescription for patients originates from medicinal plants. According to World Health Organisation, eight percent of the persons in the world depend majorly on traditional medicine . Many successful drugs in the market contain asprin and texomefen (37). Drugs derived from plants such as opiates, cocaine, and cannabis have both medical and recreational uses. Many plants that have their application in traditional medicine have exhibited effects such as anti-mutagenic and anti-oxidant properties(24) anti-diabetic activity , neuro-protective effect, anti-depressant effect, anti-ulceral effect, anti-microbial effect and anti- bacterial effect (22), hepatoprotective effect, and neuroprotective effect(20).

In traditional medicine, it has been pointed to the theurapeutic effects of *Persea americana* (avocado) pulp. Avocado is an energetic fruit which is rich in its nutritional content and it is an important tropical fruit because it is rich in protein. Avocado also contains fat soluble vitamins which are absent in other fruits such as vitamins A, B, D, and E. The high oil content of the pulp is employed in the pharmaceutical and cosmetic industries for obtaining commercial oil similar to olive oils because of similar fatty acid composition. Also the fruit has lots of health benefits because of compounds contained in its lipid fraction such as omega fatty acids, tocopherols, phytosterols and squalene (33).

Caffeine is a psychoactive compound which intake is on the high side (13). The food substances that contain caffeine include: beverages, like coffee, tea, energy drinks, carbonated beverage products containing cocoa or chocolate. When caffeine is taken in low concentration, it can lead to alertness and positive effects in the heart. But when caffeine is taken in high concentration, it can cause a wide range of undesirable mental and physical conditions such as nervousness, restlessness, irritability, insomnia, headache and heart palpitation after caffeine intake (23). This study set out to ascertain the therapeutic effect of *Persea americana* pulp on caffeine induced oxidative stress on male wistar rats.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

Fresh fruits of *Persea americana* were bought from oil Mill market in Port Harcourt Rivers State of Nigeria. The avocado fruits were washed and peeled to remove the epidermal layer and the pulp was used for the crude ethanolic extraction using (14).

DETERMINATION OF CHEMICAL COMPOSITION

ESTIMATION OF MOISTURE CONTENT

The method that was adopted for moisture content determination was the method described by (7).

PRINCIPLE: The exposure of tissues to high temperature makes them to lose water gradually leading to loss in weight. The weight loss continues until there is no longer weight loss which is evidenced by constant weight. The weight loss indicates the moisture content while the constant weight indicates the dry matter.

PROCEDURE: A crucible was subjected to heat in an oven at 110°C to make it dry and then cooled in a desicator until a constant weight W1 is obtained. A crucible was weighed and then 3g of sample was introduced into the crucible and the sample reweighed W2. The crucible with the sample was dried in an oven to a constant temperature, W3

$$\%\text{moisture content} = \frac{W2-W3X100}{W2-W1}$$

DETERMINATION OF THE CRUDE LIPID CONTENT

The method of (7) was adopted to determine the lipid content

PRINCIPLE: Solvent such as petroleum ether was used to extract the lipid which is then measured by the method. The extraction of the lipids by the solvent is based on the inability of the lipids to dissolve readily in water but readily soluble in non- polar solvents. The evaporation of the solvent leaves behind the residue which is the lipid component.

PROCEDURE: A round bottom flask of capacity 500ml was cleaned and dried. Anti-bumping granules were introduced into the flask and the flask and the anti-bumping granules were weighed together and the weight taken as W1. Petroleum ether of 300ml was introduced into the flask with soxhlex extraction unit for the purpose of extraction. The round bottom flask was connected to the soxhlet extractor and then a cold water circulation and condenser was introduced into it. The mantle which introduces heat was switched on and there was adjustment on the heating rate until there was a steady reflux of the solvent. The solvent was recovered and the oven was used to dry the oil at 70°C for one hour. The oil and the round bottom flask was then allowed to cool and then reweighed(W2)

CALCULATION $\%\text{LIPID CONTENT} = \frac{W2-W1}{\text{Weight of sample}} \times 100$

DETERMINATION OF ASH CONTENT

The method used for ash estimation was according to (7).

PRINCIPLE: When substances are subjected to high temperatures of between 540°C and 600°C, their organic components are changed to volatile components leaving behind a grayish-white residue

which constitutes the inorganic components. The weight of the resulting residue gives the ash content.

PROCEDURE: The oven was used to dry the crucible at 100° C for 10mins and then cooled in a desicator and weighed (w1). 2g of the sample was weighed into the crucible which had been previously weighed and then reweighed (W2). It was then ignited and then taken into a furnace, and then read at 560° C . The sample was allowed to stay in the furnace for 8hours to make sure it was properly ashed. The crucible containing the ash was then removed, cooled in a desicator and weighed(W3).

CALCULATION

$$\%\text{ASH CONTENT} = \frac{W3-W2X100}{W2-W1}$$

ESTIMATION OF FIBRE CONTENT

The method used for the determination of fiber content was according (7) .

PRINCIPLE: The addition of boiling sulphuric acid to a fat free sample then boiling sodium hydroxide removes the ash leaving behind a residue which constitute the fiber content of the sample.

PROCEDURE: Two grams of the sample was introduced into a round bottom flask followed by the addition of 100ml of 0.25M sulphuric acid solution and then boiled under reflux for 30mins. The filtration of the hot solution was done under suction. Hot water was used to wash the non- soluble matter many times to make that all acid was eliminated. It was then transferred into the flask followed by the introduction of 100ml 0f hot 0.30M NAOH under reflux for 30mins and then subjected to suction filtration. To ensure total elimination of base, the soluble residue was washed with boiling hot water until all base were eliminated. Drying to constant weight was done with an oven at 100° C, cooled in a desicator and weighed (W1). The weighed sample was then incinerated in a furnace at 550° C for 2 hours, cooled in a desicator and reweighed(W2).

CALCULATION

$$\%\text{CRUDE FIBRE} = \frac{\text{W1-W2X100}}{\text{WEIGHT OF ORIGINAL SAMPLE}}$$

ESTIMATION OF CRUDE PROTEIN

The crude protein content was determined using kjeldahl method described by (27).

PRINCIPLE: The principle involves the digestion of the sample with concentrated H_2SO_4 which results to the conversion of nitrogen to ammonium ions. The catalyst employed in the digestion is $CUSO_4$. Alkali is added and the ammonia given out is distilled in an excess boric acid solution . Hydrochloric acid is used to filter the distillate to determine the ammonia absorbed in the boric acid

PROTEIN DIGESTION: Exactly 1.5 g of defatted sample in a filter paper which was free from ash was dropped into 300ml kjeldahl flask, then followed by the addition of 25ml of concentrated sulphuric acid and three grams(3g) of digestion mixed with catalyst. Then, the flask was taken to the kjeldahl digestion apparatus. The digestion of the sample went on until a clear green colour was obtained .This was followed by the cooling of the digest and subsequently dilution with 100ml of distilled water

DISTILLATION OF DIGEST

To 500ml of kjeldahl flask which contained anti-bumping chips and 40% sodium was added 200ml of diluted digest . This was done slowly by the side of the flask. The ammonia that was evolved was trapped in a 250ml conical flask which contained a mixture of 50ml 2% boric acid and four drops of mixed indicator. The kjedahl flask and the conical flask were placed on the distillation apparatus with the tubes inserted into the conical flask and the kjeldahl flask. The heating of the flask led to the distillation of the ammonia and the distillate was collected in the boric acid solution. When colour change from purple to pale green occurred in the receiving flask, the distillation was stopped. O.1M HCL was used to titrate against distillate until the colour changed to pink.

CALCULATION

$$\%\text{NITROGEN} = \frac{\text{14XMXV1XTVX100}}{}$$

WEIGHT OF SAMPLE (mg)XV2

%CRUDE PROTEIN =%NITROGENX6.25

WHERE M=actual molarity of acid

TV=Titre volume of HCl used;V1=Total volume of diluted digest; V2=Aliquot volume distilled

ESTIMATION OF CARBOBYDRATE CONTENT

The method adopted by (27) was employed with some modifications.

PRINCIPLE: The principle involves the use of perchloric acid for the digestion of the food sample to bring about the hydrolysis of starches and other soluble sugars which are determined with the use of a spectrophotometer at 630nm. The result is expressed as percentage glucose

PROCEDURE:

EXTRACTION OF CARBOHYDRATE: Two grams of sample was weighed and introduced into 100ml graduated measuring cylinder then 100mls of water was added. The sample was thoroughly stirred with a glass rod to bring about dispersion of sample. 10mls of 52% perchloric acid was added and the solution was thoroughly stirred . for 30mins and then diluted to 100mls with distilled water. The solution thoroughly mixed and filtered into a 250ml graduated flask. The volume was made up with distilled water and thoroughly mixed to obtain the sample extract.

Carbohydrate determination: Forty-five mills (45ml) of the extract was diluted with 450ml of distilled water.

1ml of the filtrate was pipetted into each of three test tubes. One mill of water (blank) was pipetted into two of the tree test tubes and one mill of glucose was pipette into two of the tubes (standard). 5ml of freshly prepared 0.10% Anthrones reagent was added into each of the three test tubes and the tubes were stoppered and the contents thoroughly mixed . The contents of the tubes were placed in a water bath (37 ° C) for 12munites and then allowed to cool in a room temperature. The absorbance of the sample and the standards were read against the reagent blank using a

spectrophotometer at 630nm. The total available carbohydrate was calculated as percentage glucose as follows:

Weight of sample (g)= X; Absorbance of diluted sample=A1

Absorbance of diluted standard=A2

Therefore % glucose $= \frac{25A1\ X1}{XA2}$

DETERMINATION OF PHYTOCHEMICAL CONTENT

QUALITATIVE PHYTOCHEMICAL ANALYSIS

Preliminary phytochemical analysis was done for the extract using the methods adopted by (6) with some modification..

QUANTITATIVE PHYTOCHEMICAL DETERMINATION

DETERMINATION OF FLAVONOID CONTENT

The method described by (7) was used to determine the flavonoid content

One hundred mills of 80% aqeous methanol was used for the extraction of the sample at room temperature then followed by filtration. The filtrate was introduced into a crucible and evaporated o dryness . The residue was weighed . The flavonoid content was calculated thus:

% Flavonoid $= \frac{\text{weight of flavonoid (residue)x100}}{\text{Weight of sample}}$

ESTIMATION OF SAPONIN CONTENT

The quantitative saponin content was determined using the method described by (6). The extraction of saponin was achieved using two different solvents. The extraction of crude lipid from the sample was done with acetone while the extraction of saponin proper was done with methanol.

Two grams of the sample was introduced in a soxhlet extractor and a reflux condenser fitted on top. The process of extraction was achieved with acetone in a 250ml round bottom flask for four hours after which the apparatus was removed and 100ml of methanol was introduced into another round bottom flask and the flask was fitted to the extractor and extraction lasted for another three hours. The flask was weighed before and after the second extraction. When the second extraction was completed, the methanol was recovered by distillation and the flask was dried in the oven to ensure all solvents inside the flask were eliminated. The flask was kept in the room temperature for it to cool and then its weight taken. The saponin content of the sample was calculated thus:

$$\%\text{SAPONIN} = \frac{\text{WEIGHT OF SAPONIN X100}}{\text{WEIGHT OF SAMPLE}}$$

DETERMINATION OF ALKALOID CONTENT

Total alkaloid content was estimated using method described by (21).

Five grams of sample was added to 50ml of acetic acid solution in ethanol. The mixture was thoroughly shaken and allowed to stand for four hours before filtering. It was evaporated to one quartyer of its original volume followed by addition in drops of NH_4OH to precipitate the alkaloid. Filtration of the precipitate was done using a pre-weighed filter paper and washed with 1% NH_4OH solution. The oven was used to dry the precipitate at 60°C for thirty minutes and reweighed. The alkaloid content of the sample was determined as follows:

$$\%\text{ALKALOID} = \frac{\text{WEIGHT OF ALKALOID X 100}}{\text{WEIGHT OF SAMPLE}}$$

DETREMINATION OF PHYTIC ACID CONTENT

The phytic acid content was determined by the method described by (6). Two grams of sample were weighed into a 250ml conical flask. The sample was soaked with 100ml of 2% concentrated hydrochroric acid for three hours then followed by filtration. Fifty mills of filtrate was added to 10mls of distilled water to reduce the PH to acidic level, then followed by the addition of 10mls of 10% ammonium thiocyanate solution and then titrated against standard iron (11) chloride solution. The presence of a yellowish coloration which persisted for five minutes signifies end point. The percentage phytic acid content is calculated thus:

%PHYTIC ACID= Y X1.9 X 10 X100

Where Y = Titre value x 0.00195g

DETERMINATION OF TANIN CONTENT

Tannin estimation was done using the method described by (7). Fifthy grams (50g) of sodium tungstate was dissolved in 30cm^3 of distilled water then folled by preparation of Folin-Denis reagent. To the Folin-Denis reagent prepared, 10g of phosphomolybdic acid and 25cm^3 of orthophosphoric acid were added. The mixture was allowed to reflux for two hours , cooled and then diluted to 500cm^3 with distilled water. One gram of the sample was introduced into a conical flask folled by addition of 100cm^3 of distilled water. The mixture was boiled in a water bath for 30mins and then filtered with Whatman filter paper .Then 5cm^3 of Folin-Denis reagent and 10cm^3 of sodium carbonate solution was added into 50cm^3 of distilled water and an aliquot volume of the diluted extract (10cm^3)was added to the solution above and allowed to stand for twenty munites for colour development. The solution was boiled in a water bath for thirty minutes at 25°C after agitation. The optical density was read spectrophometrically at 700nm and compared to a standard tannic acid curve.

CALCULATION

$$\text{TANNIC ACID (mg/100g)} = \frac{C \times \text{EXTRACT VOLUME} \times 100}{\text{ALIQUOT VOLUME} \times \text{WEIGHT OF SAMPLE}}$$

Where C = Concentration of tannic acid read from the graph.

DETERMINATION OF OXALATE

AOAC method (1990) was used in the determination of oxalate. An aliquot weight of the sample (0.1g) was weighed into 250ml beaker followed by the addition of 30mls of 1M HCl. The mixture was thoroughly shaken and the boiled in a water bath at 100°C for thirty minutes. Half gram (0.5 g) of 5% calcium chloride was added to the mixture and the mixture was shaken thoroughly to ensure adequate mixing thereby precipitating calcium oxalate. Centrifugation of the suspension was carried out for fifteen minutes and the supernatant discarded. Two mills (2mls) of 0.35M NH4OH was used to wash the pellets, and then the pellet was dissolved in 0.5M H2SO4.The solution was titrated with standard solution of 0.1M KMNO4 while still maintaining the temperature at 60°C until a faint violet color that lasted for at least twenty seconds was observed. The same procedure was adopted for soluble oxalate. The only difference was that in the case of soluble oxalate, the extraction was done with 30mls of distilled water instead of extracting with 30mls of 1MHCl.

DETERMINATION OF STEROID

Aliquots of sample was transferred to similar test tube. The sample was evaporated to dryness by warming it slightly under a stream of nitrogen. Half mill (0.5ml) of methanol was used for the dissolution of the residue.Half mill (0.5ml) DNPH reagent was added to each test tube and then thoroughly mixed. The tubes were boiled in a water bath at 59°C. Direct light was prevented from entering the tubes and reaction was allowed to proceed for 90mins, then the tubes were cooled .This was followed by the addition of 0.5ml of 4N NaOH to the tubes and then shaken, then followed by dilution with 5mls of methanol. The solutions were thoroughly shaken for proper mixing and then allowed to stand foe thirty minutes at room temperature. The optical density of the content of each tube was read against the reagent blank at 475nm. The quantity of steroid present was obtained in terms of cortisone by reference to a calibration curve which had been prepared from a series containing 0.5ml of methanol 0 to 20y of cortisone.

DETERMINATION OF CYANOGENIC GLYCOSIDE

The method used for the estimation of cyanogenic glycoside was the method described by (7). Five grams of the sample was dissolved in 50mls of distilled water. The set up was kept to stay overnight and after which, it was filtered. Variable concentrations of potassium cyanide solutions which contained 0.1mg/ml-1.omg/ml cyanide was prepared. Four mills of alkaline picrate solution was introduced into a test tube containing one mill of sample filtrate and standard cyanide solution and then kept in the water bath for fifteen minutes for incubation. Blank solution was prepared by adding one mill of distilled water to alkaline picrate solution. The absorbance of the test solution was read at 490nm against the blank after colour development. The cyanide content was extrapolated from the cyanide standard curve.

$$\text{CYANOGENIC GLYCOSIDE} = \frac{C(\text{mg}) \times 10}{\text{Weight of sample}}$$

Where C = Concentration of cyanide content read off the graph.

(54) healthy and sexually mature male wistar rats were used for this study.

PROCUREMENT OF ANIMALS

Fifthy-four (54) healthy and sexually mature male wistar rats were used for this study. The rats werwe housed in a conventional wire mesh cages. The rats were given free access to water and pellet feed throughout the period of the experiment.

PROCUREMENT OF CAFFIENE AND STANDARD DRUG (PROVIRON)

Foreign nescafe which contains 80% caffeine was purchased from Eddys supermarket, Rumuokoro, Port Harcourt, Rivers State. A satchet of standard drug(proviron) was purchased from Chimes pharmaceutical shop, Rumuokoro, Port Harcourt, Rivers State.

PREPARATION OF ETHANOLIC PULP EXTRACT

The crude extraction was carried out using the method described by (14).

Procedure: An empty reagent bottle was dried in an oven and weighed. Four hundred grams of blended sample was weighed and placed in the bottle. Five hundred mills of ethanol was then added and vortexed. The extraction was allowed to occur for 24hrs. when twenty- four hours elapsed, the sample was filtered and the residue was re-extracted and then subjected to refiltration. The concentration of the filtrate was done at low temperature using water bath. The crude extractplus the reagent bottle was weighed. The difference in weight of the reagent bottle plus the crude extract and the weight of the reagent bottle only was taken as the weight of the crude extract. The crude extract was then stored in the refrigerator.

EXPERIMENTAL DESIGN

The fifthy-four (54) male rats were divided into six (6) groups of nine rats each based on their body weights. The rats were subjected to one week acclimatization before the commencement of the study. The treatment lasted for forty-two (42) days and the protocol is shown in the table below:

Table 1. Protocol for daily treatment of experimental animals

GROUPS	TREATMENTS	DESCRIPTION OF TREATMENT
1	Normal control	No extract and no caffeine
2	Positive control (caffeine only)	200mg/kg body weight orally only
3	Caffeine+ extract	Caffeine 200mg/kg body weight for two weeks, extract 100mg/kg body weight body for four weeks; both orally
4	Caffeine+ extract	Caffeine 200mg/kg body weight for

		two weeks, extract 300mg/kg body weight body for four weeks; both orally.
5	Caffeine+ extract	Caffeine 200mg/kg body weight for two weeks, extract 500mg/kg body weight body for four weeks; both orally.
6	Caffeine+ proviron	Caffeine 200mg/kg body weight for two weeks, proviron 0.107mg/kg body weight for four weeks; both orally.

The rats were sacrificed under the chloroform anesthesia 24hours after the last treatment. Blood was collected using a sterile stringe for oxidative stress markers assay.

ASSAY OF OXIDATIVE STRESS MARKERS

ASSAY OF CATALASE ACTIVITY

The method used for catalase estimation was described by (12).

PRINCIPLE: The principle is based on the ability of catalase to bring about the catalysis hydrogen peroxide to water and oxygen.

$2H_2O \rightarrow O_2 + H_2O$

The amount of hydrogen peroxide can be obtained by reading the absorbance at 420nm. After the incubation of hydrogen peroxide with the enzyme and reading absorbance with a spectrophotometer, the concentration of hydrogen peroxide dissociated can be obtained and hence activity of the enzyme can be determined.

PROCEDURE: Three tubes were used and labeled A1(blank), A2(test sample), and A3(standard). A1 contained 0.1ml of distilled water, A2 contained 0.1ml of sample and A3 contained 1.1ml of

0.05M phosphate buffer. One mill (1.0ml) of hydrogen peroxide was introduced into A1 and A2. The mixing of the mixture was done by inversion and allowed to stand for 30mins. This was followed by the addition of 0.2ml of 6M H_2SO_4 to the three test tubes and then the mixtures were thoroughly mixed. After one minute, the absorbance of the content of the tubes was read at 450nm against distilled water using a spectrophotometer. The serum catalase activity in the sample was calculated as follows:

CALCULATION:

Absorbance of blank=Ab ; Absorbance of standard=As ; Absorbance of test=At

So =As-Ab; S3=As-At

KC=Inverse of log SoX203

S3

ASSAY OF SUPEROXIDE DISMUTASE (S.O.D) ACTIVITY.

Superoxide dismutase was estimated using the method described by (26)

PRINCIPLE: The principle involves auto-oxidation of adrenaline which occurred in aqeous solution to give adrenochrome whose concentration is estimated at 520nm. The decomposition of superoxide ion is catalyzed by superoxide dismutase and hence inhibiting the auto-oxidation of adrenaline. The rate at which superoxide dismutase inhibits the above reaction is a function of its activity and it is determined at 520nm.

PROCEDURE: Two test tubes labeled A1(blank), and A2(test sample) were set up. A1 contains 0.2ml of distilled water, A2 contained 0.2ml of serum. This was followed by the introduction of 2.5mls of 0.05M carbonate buffer into each of the two test tubes. There was thorough mixing of the content and then equilibrated at room temperature. Then adrenaline was added to each of the tubes. The blank contained 3mls of distilled water. There was proper mixing of the content of each tube and the absorbance read at 520nm one minute after addition of homogenate.

CALCULATION:

Percentage inhibition = (absorbance of reference- absorbance of test)

absorbance of reference

Therefore, Superoxide Dismutase activity in units/mg protein=%inhibition

50 XT

Where T= milligram protein in the volume of supernatant used

ESTIMATION OF PLASMA GLUTATHIONE PEROXIDASE (GPX) LEVEL

Serum glutathione peroxidase estimation is carried out using the method according to (31)

PRINCIPLE: The principle is based on the measurement of the residual glutathione left after the catalytic action of glutathione peroxidase. The absorbance was read at 412nm.

PROCEDURE: 0.5ml of sodium phosphate buffer was mixed with 0.1ml of sodium azide, 0.1ml, 0.2ml of reduced glutathione, 0.1ml of hydrogen peroxide and 0.5ml of serum and the volume was made up to 2mls with distilled water. This was followed by the incubation of the mixture at 37°C for 3mins .The reaction was stopped by the addition of 0.5ml 10% TCA. The mixture was centrifuged and the supernatant removed. To estimate the residual glutathione content, 4.0mls of disodium hydrogenphosphate solution and 1ml of DTNB reagent were added to the supernatant. It was allowed to stand for 3mins for color development. The absorbance was read at 412nm against the reagent blank containing only phosphate solution and DTNB reagent.

CALCULATIONS

GPx activity (unit/mg) =change in absorbance x V1 X50

$0.OO373um^{-1}$ x V2

Where V1= Volume of sample; V2 = Total volume of reaction mixture; 50= sample dilution factor

$0.00373um^{-1}$=extinction coefficient of NADPH

ASSAY OF GLUTATHIONE REDUCED (GSH)LEVEL

PRINCIPLE: Glutathione reduced (GSH) was determined by the method (7)
Which is based on the observation that the addition of Elimans reagent t sulfhydryl compounds produced a colored complex which absorbs at 412nm. The formation of a colored complex is due to the 2-nitro-5-thio-benzoic acid which results from the reaction of DTNB with GSH.
PROCEDURE: An aliquot of the sample (0.2ml) was mixed with 1.8ml of distilled water and 3ml of sulphosalicyclic acid (a precipitating solution) .The centrifugation of the mixture was done after allowing it to stand for five minutes. Then 1ml of the filtrate was mixed with 0.1M Phosphate buffer then followed by the addition of DTNB (0.5ml). The solution was thoroughly mixed.. For the preparation of the blank, 4mls of 0.1M phosphate buffer was added to 0.5ml of DTBD. The solution was allowed to stand for 10munites at room temperature and absorbance read at 412nm using a spectrophotometer. GSH concentration was proportional to the absorbance at the wavelength and it was estimated from GSH standard curve.

ESTIMATION OF LIPID PEROXIDATION

Lipid peroxidation was quantified as malondi-aldehyde(MDA) according to the method described by (7) and expressed as micro-mol MDA/g tissue.

RESULTS AND DISCUSSION

RESULTS

Table 2. Proximate content of *Persea americana* pulp

PARAMETERS	MEAN/S.D.
MOISTURE (%)	75.33±2.33
ASH (%)	1.93±0.30
FIBRE (%)	0.90±0.10
LIPID (%)	11.46±1.25
PROTEIN (%)	4.64±0.86

CARBOHYDRATE (%)	5.72±1.40	

*Values are shown in means of triplicate analysis ± Standard Deviation. Values bearing different alphabetical superscript are significantly different ($p<0.05$) from each other down the same column

Table 3: . Qualitative Phytochemical content of *Persea americana* pulp

PARAMETERS (mg/kg)	**PRESENCE(+) OR ABSENCE (-)**	
TRITERPENOIDS	+	
ALKALOIDS	+	
FLAVONOIDS	+	
PHYTATE	+	
STEROIDS	+	
SAPONIN	+	
GLYCOSIDES	+	
OXALATE	+	
TANIN	+	

Table 4: Quantitative . Phytochemical content of *Persea americana* pulp

PARAMETERS (mg/kg)	**MEAN±S.D**	
TRITERPENOIDS	1.00±0.08	
ALKALOIDS	4.48±0.46	
FLAVONOIDS	2.25±0.14	

PHYTATE	0.06±0.09
STEROIDS	1.64±0.15
SAPONIN	4.53±0.89
GLYCOSIDES	0.05±0.01
OXALATE	8.14±0.30
TANIN	9.14±1.37

*Values are shown in means of triplicate analysis ± Standard Deviation. Values bearing different alphabetical superscript are significantly different ($p<0.05$) from each other down the same column

Table 5: (14days):Effect of caffeine on some oxidative stress markers, levels in different groups of rats. Superoxide Dismutase, Glutathione Peroxidase , and catalase and reduced glutathione levels of the caffeine induced group significantly($p<0.05$) decreased when compared to the control group. There was a significant($p<0.05$) increase in the Malondi-Aldehyde level of the caffeine group when compared with the control group .

Groups	**Group description**	**SOD (mmol/ g Hb)**	**GPX mmol/ g Hb**	**CAT mmol/gtissue**	**GSH mmol / tissue**	**MDA mmol/gtissue**
1	control	73.67±3.17[a]	55.33± 0.11[a]	56.65 ± 1.07[a]	67.03±0.58[a]	3.86 ± 0.21[a]
2	200 mg / kg BW caffeine	33.10±9.12[b]	20.003±0.89[b]	36.80 ± 1.19[b]	29.43±1.20[b]	16.44 ± 2.94[b]
3	200 mg /	32.67±1.01	23.63±1.13[b]	35.14±0.49[b]	31.57±0.57	15.95 ±0.41[b]

	kg BW caffeine	[b]			[b]	
4	200 mg / kg BW Caffeine	34.00±2.59 [b]	21.07±0.57[b]	38.07± 1.68[b]	32.70±0.86 [b]	15.45± 0.44[b]
5	200 mg / kg BW caffeine	28.07±0.37 [b]	21.67±1.79[b]	31.50± 1.91[b]	36.43±0.78 [b]	14.93 ± 1.73[b]
6	200 mg / kg BW caffeine	30.04±0.38 [b]	24.47± 2.52[b]	30.10± 1.77[b]	38.07±0.73 [b]	15.52 ± 0.06[b]

*Values are shown in means of triplicate analysis ± Standard Deviation. Values bearing different alphabetical superscript are significantly different($p<0.05$) from each other down the same column.
LEGEND: SOD = Superoxide Dismutase; GPX = Glutathione Peroxidase; CAT= Catalase ; GSH= Reduced Glutathione; MDA= Malondi-aldehyde; BW= Body Weight

Table 6 (28days): Effect of different concentrations of avocado fruit pulp extract and proviron on selected biochemical and oxidative stress marker enzyme levels of rats induced with caffeine. The superoxide Dismutase, glutathione peroxidase ,catalase and reduced glutathione levels of groups treated with extract increased while Malondi-Aldehyde level decreased significantly($p<0.05$) when compared with the group induced with caffeine without treatment(positive control).

Groups	**Group description**	**SOD mmol/g Hb**	**GPX mmol/g Hb**	**CAT mmol/gtissue**	**GSH mmol/gtissue**	**MDA mmol/gtissue**

1	Control	75.67±0.16[a]	58.63±0.21[a]	54.21± 0.01[a]	70.03± 0.28[a]	3.91 ± 0.36[a]
2	200 mg / kg BW caffeine	31.10±1.10[b]	22.05±1.05[b]	22.08± 0.04[b]	30.43 ± 1.00[b]	14.08 ± 1.87[b]
3	200 mg / kg BW caffeine + 100 mg / kg BW extract	34.60±0.01[b]	26.03±1.10[b]	28.60 ± 1.02[b]	40.57± 0.52[c]	11.17 ± 2.05[b]
4	200 mg / kg BW Caffeine + 300 mg / kg BW extract	34.00±2.59[b]	28.47±0.27[b]	28..07± 0.27[b]	43.70± 0.36[c]	7.99 ± 1.31[c]
5	200 mg / kg BW caffeine + 500mg / kg BW extract	39.17±1.02[c]	26.68±1.09[b]	31.67±1.09[c]	40.53±0.08[c]	5.02± 1.21[c]

6	200mg/kg BW Caffeine + 0.107 mg / kg BW proviron	42.20±0.24^{c}	36.20±0.04^{c}	45.89± 0.02^{d}	48.87± 0.33^{d}	6.60 ± 0.86^{c}

Values are shown in means of triplicate analysis ± Standard Deviation. Values bearing different alphabetical superscript are significantly different ($p<0.05$) from each other down the same column.

LEGEND: SOD = Superoxide Dismutase ; GPX = Glutathione Peroxidase; CAT= Catalase ; GSH= Reduced Glutathione; MDA= Malondi-Aldehyde; BW= Body Weight

Table 7 (42days): Effect of different concentration of avocado fruit pulp extract and proviron on oxidative stress marker enzyme levels Glutathione and MDA levels in different groups of rats induced with caffeine. There was a significant increase ($P<0.05$) in the Superoxide Dismutase, Glutathione peroxidase, catalase and GSH and a significant ($p<0.05$) decrease in MDA level of groups treated with extract compared with the group induced with caffeine without treatment(positive control). The effects of the 500mg/kg BW extract is comparable to that of the standard drug

Groups	**Group description**	**SOD mmol/ g Hb**	**GPX mmol/ g Hb**	**CAT mmol/gtissue**	**GSH mmol/gtissue**	**MDA mmol/gtissue**
1	Control	77.47±0.36^{a}	60.13±0.06^{a}	56.28± 0.31^{a}	74.24± 1.18^{a}	3.23 ± 0.26^{a}

2	200 mg / kg BW caffeine	33.15±1.50^{b}	26.15±1.13^{b}	26.34± 0.54^{b}	34.43 ± 1.20^{b}	12.08 ± 1.68^{b}
3	200 mg / kg BW caffeine + 100 mg / kg BW extract	46.60±0.31^{c}	30.23±0.13^{b}	38.68 ± 1.08^{c}	49.34± 0.12^{c}	6.12 ± 2.24^{c}
4	200 mg / kg BW Caffeine + 300 mg / kg BW extract	54.00±0.23^{d}	38.17±0.22^{b}	46..08± 0.28^{d}	50.30± 0.36^{c}	5.80± 1.06^{c}
5	200 mg / kg BW caffeine + 500mg / kg BW extract	58.16±0.07^{d}	52.62±1.12^{c}	48.37±1.03^{d}	65.83± 0.13^{d}	4.22 ± 1.01^{d}
6	200mg/kg BW Caffeine + 0.107 mg / kg BW	65.04±1.04^{e}	58.25±0.14^{c}	52.34± 2.12^{d}	64.82± 0.34^{d}	4.66± 1.26^{d}

proviron

Values are shown in means of triplicate analysis ± Standard Deviation. Values bearing different alphabetical superscript are significantly different ($p<0.05$) from each other down the same column.
LEGEND: SOD = Superoxide Dismutase; GPX = Glutathione Peroxidase; CAT= Catalase; GSH= Reduced Glutathione; MDA= Malondi-Aldehyde; BW= Body Weight

DISCUSSION

The study carried out on *Persea americana* pulp revealed the presence of active phytochemical constituents . The highly present phytochemicals in ethanolic pulp extract of *Persea americana* pulp are tannin, oxalate, and terpenoids. Tanins are non-toxic but can become toxic to filamentous fungi such as yeast, and bacteria and organisms that take them may experience some physiological responses (34). The availability of tannin shows that *Persea americana* pulp can function as anti-fungal, anti-diarrheal, anti-oxidant, anti-haemorrhagic and anti-hemorrhoidal agents (8). The result of phytochemical analysis of
the pulp also showed the presence of saponin. Saponins exhibit antimicrobial effects but its harmful effect to animals that consume them is low. Saponins play important role in intracellular histochemistry staining so as to enable antibodies to have free access to intracellular proteins. The saponins are used as antioxidant, weight reducing agent, anti-inflammatory and anti-cancer agents. The phytochemical result also showed the presence of flavonoid. Flavonoids are used to combat bacteria. Viruses, and it equally exhibits anti- neoplastic effect (2). Some health challenges such as heart disease, cancer, diabetes can be addressed with the use of flavonoids and the mechanism of action is by its ability to neutralize free radicals (36).The result also shows the presence of alkaloids. Alkaloids is used in the management of diabetes, bacterial and fungal infection (9) also it stimulates anaesthetic action (16).

Proximate analysis of *Persea americana* pulp showed the content of carbohydrate, protein, dietary fat, fibre, moisture and ash. Carbohydrate content of *Persea americana* pulp was lower than that of *Amaranthus incurvatus* (39.05%) (19) . The protein content was found to be lower than those of *Amaranthus viridus*(28), 31% for *Talinum triangulare* (1). The proximate analysis of the pulp showed the high content of dietary fat. Dietary fats increases palatability by flavor absorption and retention but consumption of dietary fats in large quantity can lead to cardiovascular disorders (4). The result showed high moisture content. Moisture content is one of the most important parameters evaluated in foods because it gives the economic value of the food. The crude fiber value of *Persea americana* pulp obtained from the proximate result was lower than that for leaves of O. gratissimum,(11.3%). Adequate intake of dietary fiber can decrease the plasma cholesterol level , heart disease, hypertension and breast cancer(22).The ash content of persea Americana pulp was lower than those reported for the leaves of A. viridus (22.84%) (28). The ash content represents the mineral contents of the food sample.

In this research, the antioxidant defense system both enzymatic (superoxide dismutase, glutathione peroxidase and catalase) andnon- enzymatic defense system (glutathione reduced and malondi-aldehyde) had been studied. From the result, there is a decrease in the anti-oxidant defense mechanism which is determined by low level of plasma superoxide dismutase, glutathione peroxidase, catalase, reduced glutathione and increased level of plasma malondi-aldehyde(MDA) in caffeine induced rats when compared with the control group in response to oxidative stress .

Reduced glutathione is a major protein thio which play important roles in different cellular metabolism and regulation. Reduced glutathione plays a major role in cellular anti-oxidant defense mechanism by scavenging free radicals and other reactive oxygen species (37). Reduced glutathione exhibits anti-oxidative effect in the cells and its reduced level was reported in oxidative stress (11). A significant reduction of plasma reduced glutathione (GSH) level was observed in testicular damage. The reduced level in reduced glutathione level is as a result of lipid utilization as a result of

oxidative stress (5).The reduction of the reduced glutathione level may reduce the anti-oxidant activity as reduced glutathione is required as a substrate for anti-oxidant activity (30). The damage was reversed with *Persea americana* pulp extract to a level comparable to that recorded in control rats and standard drug (proviron).

Superoxide dismutase and catalase are two major enzymes that play vital roles in the scavenging of free radical. Superoxide dismutase alternates the catalysis of dis-mutation of superoxide radicals into either molecular oxygen or hydrogen peroxide. Superoxide is generated during the metabolism of oxygen and if not removed, may be detrimental to the cell. CAT and peroxidase protect SOD against inactivation by hydrogen peroxide also SOD protects CAT and GPX against inhibition by superoxide anion and peroxide generated by sub-cellular compartment (3). The decreased activity of SOD observed in caffeine induced rats may indicate an inhibition by hydrogen peroxide as a result of corresponding decrease in testicular CAT activity.

An increase in the level of MDA is an indication of high lipid peroxidation which may indicate an increased oxygen free radicals and has been associated with abnormalities and decreased spermatozoa counts (18). However the high level of MDA in the caffeine induced rats may be that hydrogen peroxide from SODactivity becomes elevated to the point that it overwhelms CAT thereby allowing its deleterious effects. hydrogen peroxide is the primary ROS responsible for the loss of spermatozoa function since cat, which selectively degrades this ROS, is the only scavenger to confer complete protection to the spermatozoa. (17)Hydrogen peroxide is equally dangerous to the cell but is being degraded by other enzymes such as catalase glutathione peroxidase (GPX) plays an important anti-oxidative role by reducing lipid peroxidation to their corresponding alcohols and reduces the free hydrogen peroxide to water. However improvement in the catalase, superoxide dismutase and glutathione peroxidase activities of treatment groups could be due to the restoration of glutathione of reduced glutathione (GSH).

On the administration of different doses of *Persea americana* pulp extract after two weeksof treatment prior to induction with caffeine, there is a slight increase in the level of plasma superoxide dismutase, glutathione peroxidase, catalase , and reduced glutathione when compared with the untreated caffeine induced group. There is a slight decrease in the level of MDA of the treated groups when compared with the caffeine induced untreated group. The elevated level of plasma reduced glutathione of rats treated with *Persea americana* pulp extract may be one factor responsible for inhibition of lipid peroxidation.

The damage was reversed with *Persea americana* pulp extract to a level comparable to that recorded in control rats and standard drug (proviron) after four (4) weeks of treatment with the extract as evidenced in the increased level of catalase , superoxide dismutase, glutathione peroxidase and reduced glutathione as well as decreased level of MDA .

CONCLUSION,

This study reveals that *Persea americana* pulp is a source of carbohydrate, lipid , moisture and ash and hence can serve as alternative source of energy and other nutrients needed to fight malnutrition in developing countries . The presence of biologically active compounds such as flavonoid, steroids , alkaloids are evidence that it could be used in the management of different ailments and can be used as a potential source of useful drug . It also demonstrated that *Persea americana* pulp extract exhibited anti-oxidant properties on caffeine induced rats on dose and duration dependent manner. Therefore *Persea americana* pulp represents potentially useful pulps for the treatment of oxidative stress.

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