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Effect of diets supplemented with Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] on some biochemical parameters in normal rats

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ABSTRACT

Objective: To investigate and compare the *in vitro* and *in vivo* antioxidant capacity of Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and seeds of Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)]. **Methods:** Both spices were each extracted with methanol and 1M HCl (1:1, w/v) mixture to give the phenolic-rich extracts, which were used for the *in vitro* analyses [total phenol, total flavonoid and antioxidant properties (reducing properties, 1,1 diphenyl-2- picrylhydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzo-thiazoline-6-sulfonate (ABTS*) radical scavenging abilities]. Thereafter, thirty male albino rats were divided into five groups of six. Group I represented control group; Group II & III were fed diet containing 2% Ethiopian pepper & 4% XA while Group IV & V ate diet supplemented with 2% and 4% Ashanti pepper. **Results:** The results suggest that diet supplemented with 2% and 4% Ethiopian pepper and Ashanti pepper could enhance some *in vivo* antioxidant status, maintain membrane integrity and protect the liver against oxidative stress. **Conclusions:** This could be attributed to the phenolic contents and the *in vitro* antioxidant properties of the Ethiopian pepper and Ashanti pepper. However, dietary supplementation with 4% Ethiopian pepper showed the most promising protective potentials.

1. Introduction

Both epidemiological and clinical studies have shown that one of the practical strategies to fight against degenerative diseases such as diabetes, cardiovascular diseases, cancer and neurodegenerative diseases, is to improve body antioxidant status, which could be achieved by consumption of food rich in antioxidant phytochemicals[1]. Although, the human body is well equipped with antioxidant defense system that deactivates and detoxify highly reactive oxygen species[2-3]. Moreover, the body still requires some exogenous sources of antioxidant (mainly from food) to maintain oxidative stability in the body[3]. Foods from plant origin such as fruits, vegetables, spices and legumes are rich in natural antioxidant phytochemicals that can scavenge free radicals and prevent oxidative stress[3]. These foods are now considered "functional food" because of their additional benefits of health promotion and disease prevention, apart from their conventional nutrient provision[4]. The health promoting and disease preventing roles of food from plant sources have been attributed to their polyphenolic content[5].

Spices are food adjuncts usually added to food to impart characteristic flavour, aroma and colour in order to increase palatability and sensory quality of foods[6]. Apart from the vital role of spices in nutrition, their global use in folklore medicine for the treatment of several ailments are well known[6].The use of spice supplements in food has received global attention due to their potential use as antimicrobial, antihelminthic, antioxidant, antidiabetic, neuroprotective, hypocholesterolaemic, antihypertension, antinflammatory, cancer preventive and antimutagenic agents[7-8]. In Nigeria, Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] are used as value-added ingredients in the preparation of several delicacies and in

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traditional medicine for the treatment of several diseases[8]. They are usually ground into powder and used in cooked foods or in the spicing of beverages. As components of traditional medicines, they are used as a carminative, insecticides, stimulant, and additive to other remedies for the treatment of skin infections, as digestive, appetizer, and for the management of cough and fever, cough, bronchitis, dysentery, and female sterility[9-10].The property of many herbs and spices, including Ashanti and Ethiopian pepper to conserve food and protect against oxidative damage have been attributed to their phenolic content[1,3,7-9]. Adaramoye *et al*.[11] reported the protective effect of the fruit of Ethiopian pepper on the whole body upon exposure to radiation. Recently, Ashanti pepper was also reported to protect the liver from ethanol induced hepatic injury [12]. Although, several reports abound on the antioxidant properties of different spices commonly consumed globally, there are dearths of information on the antioxidative properties of these pepper seeds *in vitro* and the safety of spice supplemented diets on some biochemical parameter on normal rats. Therefore, this study was designed to investigate the antioxidative properties of polyphenol-rich extracts of Ashanti and Ethiopian pepper and evaluate the effects of diets supplemented with these spices on the serum enzymes activities, lipid profile, bilirubin concentrations and antioxidant status in normal rats.

2. Materials and methods

2.1. Materials

Fruits of Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and seeds of Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] were purchased from the Akure main market, Akure Nigeria and authenticated at the Department of Botany and Microbiology, University of Ibadan, Oyo State, Nigeria. The fruits and seeds were air dried and ground into fine powder using a laboratory mill until they could pass through a 1.0 mm screen.

2.2. Chemicals and reagents

The following chemicals and reagents: DPPH (1,1-diphenyl-2-picrylhydrazyl), thiobarbituric acid (TBA), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), 5,5- dithio-bis(2-nitrobenzoic) acid (DTNB), gallic acid, Folin-Ciocalteau′s reagent, Tris HCl, sodium dodecyl sulphate, potassium ferricyanide, ferric chloride, iron (II) sulphate (FeSO4), Sodium bicarbonate (NaHCO3), disodium hydrogen phosphate (Na2HPO4), sodium dihydrogen phosphate (NaH2PO4) and Potassium Persulfate (K2S2O8), sodium carbonate and sodium dodecyl sulphate hydrochloric acid, reduced glutathione (GSH), trichloroacetic acid (TCA), bovine serum albumin (BSA) and thiobarbituric acid (TBA), were purchased from Sigma -Aldrich Chemical Co. (St. Louis, MO), Chemie GmbH (Steinheim, Germany) and BDH Chemicals Ltd., (Poole, England). Alanine aminotransferase (ALT) kit, aspartate aminotransferase kit, triglyceride kit and alkaline phosphatase kit were obtained from Randox laboratories Ltd. (Admore, Crumlin, Co-Antrim, UK). All chemicals and reagents used were of analytical grade while the water was glass distilled.

2.3. Preparation of phenolic-rich extract

Twenty grams of the powdered seeds were extracted with methanol and 1N HCl (1:1 w/v) and filtered (filter paper Whatman No. 2) under vacuum. The filtrate was then evaporated to dryness using a rotary evaporator at 40℃ at the pressure of about 150-300 mbar. The flask containing the powdered dried extract was thoroughly washed with distilled water and the solution was kept in the refrigerator at -20℃ . The frozen extract solution was recovered as dried extract with the aid of a freeze drier. The dried extracts were later reconstituted in water, stored at 4℃ and used for subsequent analysis[13].

2.4. Animal ethics

All of the animals received humane care according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health (USA). The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animals′ welfare during experiments[14]. The experiment was carried out at the Functional foods and Nutraceuticals Laboratory, Biochemistry Department, Federal University of Technology, Akure, Nigeria. The experimental animals were kept at the experimental room.

2.5. Bioassay

The bioassay was carried out according by the modified method by Oboh and Ogunruku[13]. Male Wistar rats weighing 176 - 188g were purchased from the Department of Veterinary Medicine, University of Ibadan, Nigeria. The rats were acclimatized for four weeks. Commercial diet and water were provided for the animals ad libitum. After the four weeks of acclimatization, the rats were weighed (210.5 - 238.7g) and subsequently divided into five (5) treatment groups with 6 rats per group. Rats in each group were placed in wired net cages (a single cage per rat) for easy monitoring of both the daily feed intake and faecal output of each rat. Group I was placed on Basal diet (without spices) and the animals in this group were regarded as the control group, rats in groups II & III were placed on diet containing 2 & 4% Ethiopian pepper (XA) while groups IV & V animals were fed with diet supplemented with 2 & 4% Ashanti pepper (PG). Rats in each cage (based on their group diet) were fed with 20g of their respective diets daily. This allowed for easy monitoring of body weight, daily feed intake and faecal output at 3-day interval in the course of the experiment. The experiment lasted for 14 days[13].

2.6. Preparation of serum and liver homogenates

At the end of the 14-day feeding period, the rats were decapitated by cervical dislocation; blood was collected by heart puncture into non-heparinized tubes and allowed to clot. Serum was separated by centrifugation of the clotted blood at 3000 revolutions for 10 minutes and the serum was subsequently prepared and stored at -4℃ until analysis. Then, the liver tissue was rapidly dissected, excised and placed on ice and weighed. The liver was subsequently homogenized in cold saline (1/10 w/v) with about 10-up- and -down strokes at approximately 1500 rpm in a Teflon glass homogenizer. The homogenate was centrifuge for 10 min at 3000 g to yield a pellet that was discarded, and a low-speed supernatant (S1) containing mainly water, proteins and lipids (cholesterol, galactolipid, individual phospholipids, gangliosides) that was kept for subsequent analysis[15].

2.7. Determination of total phenol content

The total phenol content was determined on the extracts using the method reported by Singleton *et al.*[16]. Appropriate dilutions of the extracts were oxidized with 2.5 mL of 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45℃ and the absorbance was measured at 765 nm in the UV-Visible spectrophotometer (Model 6305, Barloworld Scientific, Dunmow, Essex, United Kingdom). The total phenol content was subsequently calculated using gallic acid as standard.

2.8. Determination of total flavonoid content

The total flavonoid content of both extracts was determined using a slightly modified method reported by Meda *et al.*[17]. Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 mL methanol, 50 μL of 10% AlCl3, 50 μL of 1 mol/L potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm in the Jenway UV-Visible spectrophotometer. The total flavonoid was calculated using quercetin as standard.

2.9. Analytical HPLC chromatography of the phenolic-rich extracts

The analytical HPLC chromatography of the phenolic extracts was carried out. The HPLC system was Smartline, composed by Manager 5000, Degasser, Solvent organizer K1500, HPLC Pump K-1001 with 10mL pump head, mixing chamber, 2 x UV Detector 2600 (K-2501), and column oven (Knauer). The column was a C18-Eurosphere 100, 250 mm x 4.6 mm, 5μm and Autosampler (Basic Marathon). The column temperature was set at 30 ℃. The phenolic-rich extracts were filtered using 0.45μm HPLC membrane filter. A total of 20 μL of the filtered sample was injected. Elution was achieved by a gradient of a 0.1% acetic acid aqueous solution (solvent A) and 0.1% acetic acid and 1% acetonitrile, 10% Acetic acid in 90% methanol (solvent B) at a flow rate of 0.7 mL/min. The gradient was as follows: 20 - 90% solvent B for 40 mins, maintained 90% solvent B for 10 mins, 20% of solvent B for 1 min and equilibration for 9 min at 20% solvent B. The phenolic content was detected at two wavelengths; 280nm and 320nm. The evaluation software was Euro Chrom for Windows, Version 3.05.

2.10. DPPH free radical-scavenging ability

The free radical-scavenging ability of the spice extracts against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated as described by Gyamfi *et al.*[18]. Briefly, an appropriate dilution of the spice extracts (1 mL) was mixed with 1 mL of 0.4 mmol/L methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm in the spectrophotometer. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference, which contained all the reagents without the test sample.

$$\text{DPPH radical scavenging ability (\%)} = (Abs_{ref} - Abs_{sam}) / Abs_{ref} \times 100$$

Where, Abs_{ref} = Absorbance of Reference

Abs_{sam} = Absorbance of Sample

2.11. 2,2- azinobis (3-ethylbenzo-thiazoline- 6-sulfonate (ABTS) radical scavenging ability)

The ABTS* scavenging ability of both extracts was determined according to the method described by Re *et al.*[19]. ABTS* was generated by reacting an ABTS aqueous solution (7 mmol L-1) with $K_2S_2O_8$ (2.45 mmol L-1, final concentration) in the dark for 16 h and adjusting the Abs734nm to 0.700 with ethanol. 0.2mL of appropriate dilution of the extract was added to 2.0mL ABTS* solution and the absorbance were measured at 734nm after 15 min. The trolox equivalent antioxidant capacity was subsequently calculated.

2.12. Determination of reducing property

The reducing property of the extract was determined by assessing the ability of the extract to reduce FeCl3 solution as described by Oyaizu [20]. 2.5 mL aliquot was mixed with 2.5 mL 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated at 50 ℃ for 20 min. and then 2.5 mL 10 % trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. Five milliliter of the supernatant was mixed with an equal volume of water and 1 mL 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated as Ascorbic acid equivalent.

2.13. Lipid peroxidation and thiobarbibutric acid reactions

The extent of lipid peroxidation was determined as the concentration of thiobarbituric acid reactive substances (TBARS), according to the method of Ohkawa *et al.*[21], briefly 100 μL of S1 fraction or serum was mixed with a reaction mixture containing 160 μL of 0.1 M pH 7.4 Tris-

HCl buffer. The volume was made up to 300 μL by water before incubation at 37 ℃ for 1 hr. The colour reaction was developed by adding 300 μL 8.1% SDS (Sodium duodecyl sulphate) to the reaction mixture containing S1 or serum, this was subsequently followed by the addition of 600 μL of acetic acid/HCl (pH 3.4) mixture and 600 μL 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100 ℃ for 1 hr. TBARS (Thiobarbituric acid reactive species) produced were measured at 532nm. The clear supernatant was collected and absorbance measured on spectrophotometer against a reference blank of distilled water at 532nm. Lipid peroxidation in units/mg protein was computed with a molar extinction coefficient of 1.56 x 10^5 M-1Cm^{-1}.

2.14. Estimation of reduced glutathione (GSH)

GSH was determined at 412nm using the method described by Jollow *et al*.[22]. 0.2ml of serum was added to 1.8ml of distilled water and 3ml of the precipitating solution was mixed with sample. The mixture was then allowed to stand for approximately 5 minutes and then filtered. At the end of the fifth minute, 1ml of filtrate was added to 4ml of 0.1M phosphate buffer (pH 7.4). Finally 0.5ml of the Ellman's reagent was added. A blank was prepared with 4ml of the 0.1M phosphate buffer, 1ml of diluted precipitating solution (3 parts to 2 parts of distilled water) and 0.5ml of the Ellman's reagent. The optical density was measured by spectrophotometer at 412nm. GSH was proportional to the absorbance at that wavelength and the estimate was obtained from the GSH standard curve.

2.15. Protein determination

Protein concentration was determined by the method of Lowry *et al*. [23]. 1ml of S1 fraction or serum was dissolved in 39ml of 0.9% saline to give a 1 in 40 dilution. 3ml of Biuret reagent was added to 2ml of diluted sample. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read by spectrophotometer at 540nm. And the protein content of sample was thereafter calculated from the standard using Bovine Serum Albumin (BSA).

2.16. Enzyme assays

The following biochemical assays were determined using commercial kits according to manufacturer's instructions. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to Reitman and Frankel [24]; alkaline phosphatase (ALP) was measured according to the standard method [25]. Serum bilirubin concentration [26]; was also determined using the Randox diagnostic Kits (Random Laboratories Limited, UK).

2.17. Assessment of the lipid profiles

Cholesterol concentration was determined by the method of Searcy and Bergquist [27]. Triglyceride concentration was determined according to the principle described by Trinder [28].

2.18. Statistical analysis

The results of the three replicates were pooled and expressed as mean ± standard deviation (S.D.). Student t-test, one-way analysis of variance (ANOVA) and the least significance difference (LSD) between the control and treatment groups were carried out. $P<0.05$ was considered statistically significant. Microsoft excel 2003 and Origin 6.1 version software were used.

3. Results

The results of the phenolic content of Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] extracts were shown in Table 1 and reported as gallic acid equivalent (GAE) for total phenol content and quercetin equivalent antioxidant capacity (QEAC) for total flavonoid content. The result revealed that there is no significant ($P>0.05$) difference in total phenol contents of Ashanti pepper (4.6 mg/g) and Ethiopian pepper (4.2 mg/g). However, the total flavonoid contents of Ethiopian pepper extract (3.5 mg/g) was significantly higher ($P<0.05$) than Ashanti pepper (2.5 mg/g). Furthermore, the analytical HPLC of the phenolic-rich extracts was carried out using RP-HPLC-UV at 280nm and 320 nm. The chromatograms are shown in Figure 1 and 2.

The *in vitro* antioxidant indices (free radical scavenging ability and reducing property) of both spice extracts were presented in Figure 3-5 respectively. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2- azinobis (3-ethylbenzo-thiazoline- 6-sulfonate (ABTS) radicals are established models for assessing the free radical scavenging ability of plant foods. The DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical scavenging ability of both spice extracts were determined and presented in Figure 3. Judging by the EC_{50} (extract concentration causing 50% radical scavenging) values as shown in Table 1, Ethiopian pepper (0.3 mg/mL) had a significantly higher ($P<0.05$) DPPH free radical scavenging ability than the Ashanti pepper (0.5 mg/mL), in a concentration dependent manner (0.08- 0.53mg/mL) as shown in Figure 3. Furthermore, ABTS* scavenging ability was carried out and reported as Trolox equivalent antioxidant capacity (TEAC). The result also showed that Ethiopian pepper extract has a significantly higher ($P<0.05$) ABTS radical scavenging ability (2.07x10^{-3}mmol.TEAC/g) than the Ashanti pepper (1.42x10^{-3} mmol. TEAC/g). However, the trend in the result of total phenol and total flavonoid content agrees with the free radical scavenging ability of both spices; in that Ethiopian pepper showed significantly higher ($P<0.05$) radical scavenging abilities when compared to Ashanti pepper . This difference could be attributed to their flavonoid contents.

Also, the ferric reducing antioxidant property of the extracts was assessed for their ability to reduce Fe (III) to Fe (II) and expressed as ascorbic acid equivalent (AAE). The result revealed that both extracts possess good reducing potentials. There was no significant difference between the reducing powers of Ethiopian pepper (3.5 x10^{-2} mmol AAE/g) than Ashanti Pepper (3.8 x10-2 mmol AAE/g).

The *in vivo* antioxidant indices were evaluated in normal rats fed with diets supplemented with Ethiopian pepper and Ashanti pepper. The feed formulations for each group are presented in Table 2. The supplementation of Ashanti and

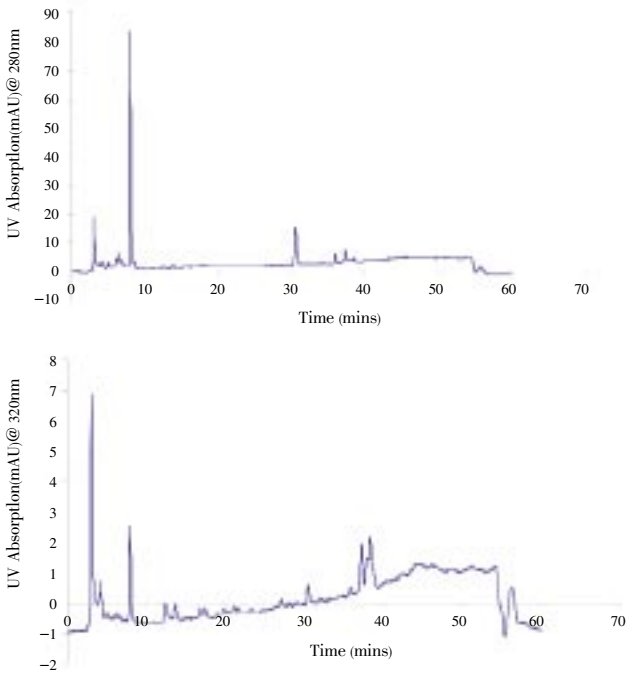

Figure 1. Analytical HPLC chromatograms showing the UV absorption of phenolic-rich extracts of Ashanti pepper at 280 nm and 320 nm.

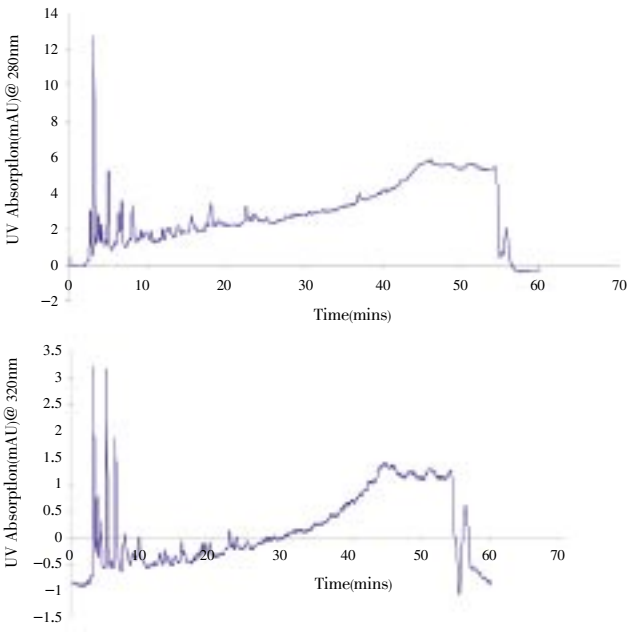

Figure 2. Analytical HPLC chromatograms showing the UV absorption of phenolic-rich extracts of Ethiopian pepper at 280 nm and 320 nm.

Ethiopian pepper in rat's diet at 2% and 4% equal weight replacement in basal diet did not cause any significant change (P>.05) in the average daily feed intake (9.8 −12.4 g), weight gain (2.4 −3.5 g) and faecal output (4.7− 5.9 g) in rats (Table 3). The *in vivo* antioxidant status (reduced glutathione and lipid peroxidation), triglyceride, total cholesterol and serum activities of ALT, AST & ALP and total bilirubin levels of rats were subsequently assessed.

The effect of diets supplemented with Ashanti and Ethiopian pepper (2% and 4 %) were assessed on the serum reduced glutathione (GSH) levels of normal rats. There was no significant change in the serum GSH levels of rats fed with diet supplemented with 2% Ethiopian pepper and 2% Ashanti pepper when compared with the control (rats fed with the basal diet) group. Moreover, rats fed with diet containing 4% Ethiopian pepper and 4% Ashanti pepper showed a significant (P<0.05) higher GSH levels when compared with the control group (Table 4).

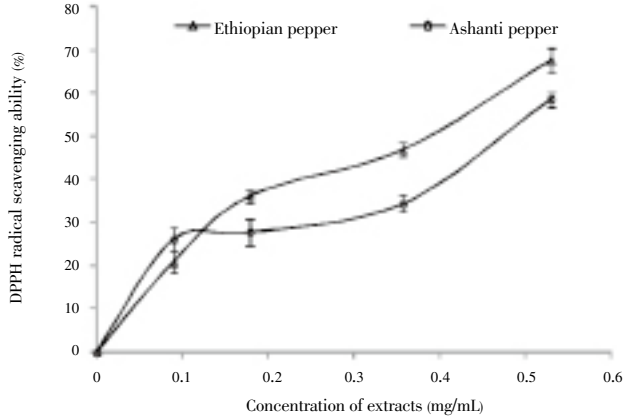

Figure 3. DPPH radical scavenging ability of phenolic-rich extracts of Ethiopian pepper and Ashanti pepper.
Values represent mean ± standard deviation of triplicate readings, n = 3

Table 1
Phenolic contents and EC_{50} values for DPPH radical scavenging ability of Ethiopian and Ashanti pepper extracts.

Pepper	Total phenol (mg/g)	Total flavonoid (mg/g)	EC_{50} values for DPPH radical scavenging ability (mg/mL)
Ethiopian pepper	4.2±0.0	3.5±0.6	0.3
Ashanti pepper	4.6±0.4	2.5±0.2	0.5

Values represent mean ± standard deviation of triplicate readings, n = 3, *P<0.05 comparing with Ethiopian pepper

Table 2
The feed formulation for each group.

Feed	Basal diet (%) (Group I)	Spice supplemented diet	
		(2 %) (Group II)	(4 %) (Group III)
Skimmed milk	28	28	28
Corn starch	58	56	54
Premix	4	4	4
Groundnut oil	10	10	10
Spices	−	2	4

Furthermore, diets containing 2% − 4% Ashanti pepper and Ethiopian pepper were evaluated on serum and liver malondialdehyde (MDA) of rats, as presented in Table 4. Diet supplemented with 2% Ashanti peppers did not alter serum MDA significantly when compared with the control group (rats fed with the basal diets). However, supplementation with 4% Ashanti pepper, 2% and 4% Ethiopian pepper showed significant (P<0.05) reductions in blood concentrations of MDA when compared with the control group (Table 4).

Subsequently, the effects of spice supplemented diets were assessed on serum and tissue (liver) protein in normal rats. The spice supplemented diets did not cause any significant change in both the serum and tissue (liver) proteins in rats fed with different percentages of both spices when compared with the control group (Table 5).

Table 3
The average daily feed intake, weight gain and faecal output within the 14- day feeding trial.

	Average daily feed intake (g)	Average weight (g)			Faecal output (g)
		Initial (g)	Final (g)	Gain (g)	
Control group	12.4±3.7	225.3±2.3	227.7±2.1	2.4	5.5±2.2
2%-XA	10.9±2.5	222.1±3.0	225.4±2.6	3.3	4.7±1.7
4%-XA	11.3±4.3	229.4±2.5	233.2±3.3	2.8	4.8±3.0
2%-PG	9.8±3.4	217.7±1.9	221.4±1.8	3.5	3.7±1.5
4%-PG	12.1±2.8	224.2±3.2	227.2±2.7	3.0	5.9±2.5

Values represent mean ± standard deviation of four or five readings, n = 4 or 5.
Control group (Group I rats) were fed with the basal diet (without spices); 2%-XA (Group II rats): were placed on diet supplemented with 2% Ethiopian pepper; 4%-XA (Group III rats): were placed on diet supplemented with 4% Ethiopian pepper; 2%-PG (Group IV rats) were placed on diet supplemented with 2% Ashanti pepper; 4%-PG (Group IV rats) were placed on diet supplemented with 4% Ashanti pepper.

Table 4
The effect of diet supplemented with Ethiopian pepper and Ashanti pepper on serum glutathione and markers of oxidative stress.

	Glutathione (μg GSH/mg protein)	Serum MDA (μg/mg protein)	Liver MDA(μg/mg protein)
Control	25.4 ±3.5	1.2±0.2	1.5±1.1
2%-XA	26.9±3.1	0.7±0.3*	1.3±0.5
4%-XA	36.0±2.2*	0.5±0.2*	1.2±0.9
2%-PG	24.6±4.0	1.0±0.6	1.5±0.5
4%-PG	33.2±2.5*	0.7±0.5*	1.2±0.7

*P<0.05 comparing with the control group.
Control group (Group I rats) were fed with the basal diet (without spices); 2%-XA (Group II rats): were placed on diet supplemented with 2% Ethiopian pepper; 4%-XA (Group III rats): were placed on diet supplemented with 4% Ethiopian pepper; 2%-PG (Group IV rats) were placed on diet supplemented with 2% Ashanti pepper; 4%-PG (Group IV rats) were placed on diet supplemented with 4% Ashanti pepper.

Table 5.
The effect of diet supplemented with Ethiopian pepper and Ashanti pepper on total protein (serum and liver) and lipid profile in rats (mg/mL).

	Serum total protein	Liver total protein	Total cholesterol	Triglycerides
Control	3.0±1.6	3.2±2.0	32.9±4.4	115.4±6.0
2%-XA	3.1±1.3	3.4±2.3	31.2±1.9	122.2±4.2
4%-XA	3.1±1.1	3.4±1.2	23.9±2.6*	118.5±5.0
2%-PG	3.2±1.5	3.5±1.8	28.6±3.4	109.0±5.9
4%-PG	3.2±1.2	3.3±0.7	20.7±1.1*	108.2±3.7

*P<0.05 comparing with the control group.
Control group (Group I rats) were fed with the basal diet (without spices); 2%-XA (Group II rats): were placed on diet supplemented with 2% Ethiopian pepper; 4%-XA (Group III rats): were placed on diet supplemented with 4% Ethiopian pepper; 2%-PG (Group IV rats) were placed on diet supplemented with 2% Ashanti pepper; 4%-PG (Group IV rats) were placed on diet supplemented with 4% Ashanti pepper.
Values expressed as mean±SD where (n=6). Superscripts indicate significant (P<0.05).
a = significantly difference when compared with control (group I).

Table 6.
The effect of diet supplemented with Ethiopian pepper and Ashanti pepper on serum enzyme activities and total bilirubin levels in rats

	ALP (U/L)	ALT (U/L)	AST (mg/mL)	Total Bilirubin
Control	29.0 ±4.7*	10.9±2.5	37.4±5.8	2.1±0.4
2%-XA	27.6±3.0	10.2±1.3	41.5±4.0	2.3±0.4
4%-XA	24.3±1.8	9.9±3.1	42.3±3.7	2.2±0.8
2%-PG	28.8±2.7	11.1±1.3	43.8±2.0	2.3±0.5
4%-PG	24.5±3.5	8.6±1.9	37.5±2.6	2.0±0.7

*P<0.05comparing with the control group.
Control group (Group I rats) were fed with the basal diet (without spices); 2%-XA (Group II rats): were placed on diet supplemented with 2% Ethiopian pepper; 4%-XA (Group III rats): were placed on diet supplemented with 4% Ethiopian pepper; 2%-PG (Group IV rats) were placed on diet supplemented with 2% Ashanti pepper; 4%-PG (Group IV rats) were placed on diet supplemented with 4% Ashanti pepper.

The effects of spice supplemented diets on lipid profile in rats revealed a significant (P<0.05) reduction in the total cholesterol levels of rats fed with 4% Ashanti pepper and 4% Ethiopian pepper when compared with the control group, whereas diet containing 2% Ashanti pepper and 2% Ethiopian pepper did not alter the total cholesterol levels in rats (Table 5). The serum triglyceride levels of the rats fed with the spices and control rats were also assessed. The result revealed that there was no significant change in serum triglyceride levels between the rats fed with 2% and 4% Ashanti and Ethiopian pepper and the control groups, as shown in Table 5.

The serum enzymes (ALT, AST and ALP) and serum bilirubin concentrations were estimated in this study. There were no significant changes in ALT, AST, ALP levels and serum bilirubin concentrations of diet fed with 2% and 4% spices when compared with the control groups (Table 6).

4. Discussion

The level of antioxidant activity in functional foods and nutraceuticals should be quantified by both the *in vitro* and *in vivo* studies. In this study, the phenolic (total phenol and flavonoid) contents and *in vitro* antioxidant properties of phenolic rich extract of Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] were assessed. Then, the effects of diets supplemented with Ethiopian and Ashanti pepper were evaluated on some biochemical parameters in normal rats.

Phenolics are secondary metabolites found in plants and their beneficial effects have recently been linked to the antioxidant capacities of plant foods [3,5, 9,10,13]. Flavonoids are known to be the most abundant group of phenolics in plants and have been reported to possess antioxidant activity [3,29]. In this study, the phenolic content of Ethiopian pepper [*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae)] and Ashanti pepper [*Piper guineense* Schumach. et Thonn (Piperaceae)] extracts were determined. No significant difference was observed between the total phenol contents of Ashanti pepper and Ethiopian pepper. However, the total flavonoid contents of Ethiopian pepper extract was significantly higher ($P<0.05$) than Ashanti pepper (Table 1). Therefore, we propose that the difference in their phenolic contents could be due to their total flavonoid contents. This was further validated by their signal intensity on the RP-HPLC-UV when both extracts were tested at wavelengths of 280 nm and 320 nm (Figure 1& 2). Although, the peaks were not yet identified, the peak area of the chromatograms showed that Ethiopian pepper may contain higher phenolic contents than Ashanti pepper.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) ability is an important model for assessing *in vitro* antioxidant activities in plants[30] because it is a stable free radical with an unpaired valence electron at one atom of nitrogen bridge and the effect of antioxidants on DPPH is due to their hydrogen donating ability[30-31]. The DPPH free radical scavenging ability of both spice extracts were determined and presented in Fig. 3. In this study, Ethiopian pepper (EC_{50} = 0.3 mg/mL) had a significantly higher ($P<0.05$) DPPH free radical scavenging ability than the Ashanti pepper (EC_{50} = 0.5 mg/mL), in a concentration dependent manner (0.08-0.53mg/mL) as shown in Figure 3. DPPH is frequently used in the determination of free radical scavenging ability; however, it has the limitation of colour interference and sample solubility. Therefore, the free radical scavenging ability of both spice extracts was further studied using a moderately stable nitrogen-centered radical species: ABTS* (2,2- azinobis (3-ethylbenzo-thiazoline- 6-sulfonate). The ABTS radical based model of free radical scavenging ability has the advantage of being more versatile as both non-polar and polar samples can be assessed and spectral interference is minimized as the absorption maximum used is 760 nm, a wavelength not normally encountered with natural products[19]. Therefore, we investigated the ABTS* scavenging ability of both spice extracts and the study revealed that Ethiopian pepper extract has a significantly higher ($P<0.05$) ABTS radical scavenging ability ($2.07x10^{-3}$mmol.TEAC/g) than the Ashanti pepper ($1.42x10^{-3}$ mmol. TEAC/g). However, the trend in the result of total phenol and total flavonoid content agrees with the free radical scavenging ability of both species; in that Ethiopian pepper showed significantly higher ($P<0.05$) radical scavenging abilities when compared to Ashanti pepper. Hence, this difference could be attributed to their flavonoid contents.

Furthermore, the reducing power of antioxidant-rich food could be linked to their hydrogen or electron-donating potentials[32]. Therefore, the ferric reducing antioxidant property of the extracts was assessed and the study revealed that both extracts possessed good reduction potentials, although there was no significant difference between the reducing power of Ethiopian pepper ($3.5 x10^{-2}$ mmol AAE/g) than Ashanti Pepper (3.8 x10-2 mmol AAE/g).

The effects of diets supplemented with Ethiopian pepper and Ashanti pepper were evaluated in normal rats. The feed formulations for each group are presented in Table 2. The supplementation of Ashanti and Ethiopian pepper in rat's diet at 2% and 4% equal weight replacement in basal diet did not cause any significant change in the average daily feed intake (9.8 -12.4g), weight gain (2.4 -3.5 g) and faecal output (4.7- 5.9g) in rats (Table 3). The *in vivo* antioxidant status (reduced glutathione and lipid peroxidation), triglyceride, total cholesterol and serum activities of ALT, AST & ALP and total bilirubin levels of rats were subsequently assessed. Antioxidants may exert their action *in vivo* by inhibiting the generation of reactive oxygen species (ROS)/free radicals, by directly scavenging free radicals, a process known as mopping up; or by retarding the process of lipid peroxidation; or by raising the levels of endogenous antioxidant defenses by up-regulating the expression of the genes[33]. Serum antioxidant status may be considered as an index for the body's global antioxidant status. Glutathione (GSH) is a water-soluble tripeptide containing amino acids glutamine, cysteine, and glycine. It possesses thiol groups which makes it a potent reducing agent. GSH is the most abundant intracellular small molecule thiol, reaching millimolar concentrations in some tissues. As an important antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds. GSH is the most abundant redox system and the GSH/GSSG ratio represents the cellular ability to prevent oxidative damage[34]. Therefore, we investigated the effect of diets supplemented with Ashanti and Ethiopian pepper (2 and 4 %) on the serum reduced glutathione (GSH) levels. There was no significant ($p<0.05$) change in the serum GSH levels of rats fed with diet supplemented with 2% Ethiopian pepper and 2% Ashanti pepper when compared with the control (rats fed with the basal diet) group. Moreover, rats fed with diet containing 4% Ethiopian pepper and 4% Ashanti pepper showed a

significant ($P<0.05$) higher GSH levels when compared with the control group (Table 4) . This may be due to the fact that dietary supplementation of 4% Ethiopian pepper and 4% Ashanti pepper could have initiated the expression of genes which are necessary for the synthesis of glutathione, thereby enhancing the antioxidant status in rats. Furthermore, lipid peroxidation is a known index of increased oxidative stress and subsequent oxidative damage[35]. It could impair cell membrane fluidity and alters the activity of membrane bound enzymes and receptors resulting in membrane malfunction[35]. Assessment of lipid peroxidation and thiobarbituric acid (TBARS) is often used to measure serum and tissue concentrations of malondialdehyde (MDA); a decomposition product of oxidized lipids, and an index of serum and tissue lipid peroxidation. MDA measurement may provide further indication of oxidative injury. The present study revealed that diet supplemented with 2% Ashanti peppers did not alter serum MDA significantly when compared with the control group (rats fed with the basal diets). However, supplementation with 4% Ashanti pepper, 2% and 4% Ethiopian pepper showed significant ($P<0.05$) reductions in blood concentrations of MDA when compared with the control group (Table 4). This is an indication that dietary supplementation of the two spices could provide additional antioxidant effect and protect the body against oxidative stress. Furthermore, supplementation of rat's diet with 2% and 4% Ethiopian and Ashanti peppers did not cause a significant change in liver MDA when compared with the control group (Table 4). This could be attributed to the antioxidant and hepatoprotective potentials of the two spices as reported by previous authors[10-12].

Protein and lipid are the major components of the biological membrane. The membrane integrity is a crucial redox balance that should be maintained in cells[36]. Disruption of membrane integrity arising from the formation of protein adducts or lipid peroxidation could lead to several pathologies[36]. In this study, the effects of spice supplemented diets were evaluated on serum and tissue (liver) protein in normal rats. The spice supplemented diets did not cause any significant change in both the serum and tissue (liver) proteins in rats fed with different percentages of both spices when compared with the control group (Table 5). Moreover, data on lipid profile revealed a significant ($P<0.05$) reduction in the total cholesterol levels of rats fed with 4% Ashanti pepper and 4% Ethiopian pepper when compared with the control group, whereas diet containing 2% Ashanti pepper and 2% Ethiopian pepper did not alter the total cholesterol levels in rats (Table 5). The reduction in total cholesterol levels in rats fed with diet supplemented with 4% Ethiopian pepper and Ashanti pepper could support the assertion in folklore medicine that spices possess hypocholesterolaemic potentials. Furthermore, the serum triglyceride levels of the rats fed with the spices and control rats were assessed. The result revealed that there was no significant change in serum triglyceride levels between the rats fed with 2% and 4% Ashanti and Ethiopian pepper and the control groups, as shown in Table 5. This may suggest that both spices could play crucial role in stabilizing membrane fluidity and maintenance of membrane integrity. Hepatoprotective potentials of plant foods or extracts may be assessed by estimating the concentration or levels of some hepatic enzymes released into the blood [37-38]. The liver is prone to oxidative insult because of its roles in xenobiotic metabolism[39-40]. The serum enzymes (ALT, AST and ALP) and serum bilirubin concentrations were estimated in this study. There were no significant changes in ALT, AST, ALP levels and serum bilirubin concentrations of diet fed with 2% and 4% spices when compared with the control groups (Table 6). This could be attributed to possible protection of the liver against oxidative insults by phenolic phytochemicals from both Ethiopian and Ashanti pepper.

This study revealed the protective effects of diets supplemented with 2% and 4% Ethiopian pepper and Ashanti pepper, as typified by enhancement of *in vivo* antioxidant status, maintenance of membrane integrity and protection of the liver from oxidative stress. This could be attributed to the phenolic contents and the *in vitro* antioxidant properties of the Ethiopian pepper and Ashanti pepper. Moreover, dietary supplementation with 4% Ethiopian pepper showed the most promising protective potentials.

Conflict of interest statement

We declare that we have no conflict of interest.

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