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Antiglycation, antioxidant and toxicological potential of polyphenol extracts of alligator pepper, ginger and nutmeg from Nigeria

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ABSTRACT

Objective: To evaluate the antioxidant and antiglycation potential of polyphenols from three spices; alligator pepper, ginger and nutmeg. **Methods:** Polyphenol extracts of these spices were subjected to brine-shrimp lethality assay, phytotoxicity test, DPPH and superoxide anion radical scavenging as well as BSA-glucose antiglycation assay. **Results:** Results obtained showed that polyphenol extract of ginger has the highest antioxidant potential with IC_{50} 0.075 and 0.070 mg/mL for DPPH and superoxide anion radical scavenging assay while alligator pepper displayed highest antiglycation activity with IC_{50} 0.125 mg/mL. However, nutmeg extract exhibited weakest cytotoxic and phytotoxic potential with LD_{50} 4359.70 and 1490 μg/mL respectively. **Conclusions:** It can be concluded that the polyphenol extracts of alligator pepper, ginger and nutmeg displayed good antioxidant as well as antiglycation potential and are safe for consumption.

1. Introduction

Glycation is a non enzymatic condensation reaction between reducing sugars and amino groups of proteins that undergo rearrangements to stable ketoamines, leading to the formation of advanced glycation end products (AGEs)[1]. It is a spontaneous reaction which is dependent on the degree and duration of hyperglycaemia, half-life of the protein and permeability of the tissue to free glucose[2]. Increased glycation and build-up of tissue AGEs have been implicated in diabetic complications because they can alter enzymic activity, decrease ligand binding, modify protein half-life and alter immunogenicity[3]. Hyperglycemia is considered as a clinical hallmark of diabetes, the phenomenon that results into the formation of AGEs. Therefore glycation is also important therapeutic target for the treatment of diabetic complication.

Polyphenolic compound on the other hand, is a diverse class of plant secondary metabolites[4,5] characterised by a polyphenol structure, which means that they have several hydroxyl groups on two or more benzene rings[6]. Several plant species such as fruits, spices and vegetables are reported to possess polyphenols which confer on them the ability to remove free radicals formed from glycation and its endproducts from the system. In this regard, several spices are among the top dietary sources of polyphenolics[7]. Not only do culinary spices provide high concentrations of bioactive compounds, they also tend to provide few calories which is an advantage in type 2 diabetes, which is often associated with abdominal obesity[8]. For the purpose of this study, alligator pepper, ginger and nutmeg are the spices of interest.

Alligator pepper (*Aframomum melegueta* [Roscoe] *K. Schum*), also known as Guinea pepper or grains of paradise, is a member of the Zingiberaceae family native to West Africa. The seeds are used as a spice for flavouring food and have a wide range of ethnobotanical uses. They are used as a remedy for treating stomachache, diarrhoea, and snakebite[9,10]. *Zingiber officinale* Roscoe [family: Zingiberaceae], commonly known as 'ginger', is one of the frequently used spices in many parts of the world. It

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has been cultivated for thousands of years for medicinal purposes and as a spice. It is used extensively in traditional medicine to treat headaches, nausea, febrile conditions, colds, arthritis, rheumatic disorders and muscular discomfort[11,12]. *Myristica fragrans* Houtt (Myristicaceae) with a common name, nutmeg, is an aromatic tree cultivated in many tropical countries. Its dried kernel has been claimed to possess medicinal properties (digestive, carminative and expectorant) in Indian medicine[13]. It also possesses hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, and anti-inflammatory activities[14].

Due to the global widespread incidence of diabetes and its complications in the recent time, the aim of this study is to evaluate the antiglycation and antioxidant potential of the polyphenol-rich extract of these spices. Their ability or inability to constitute danger to their consumer is also assessed through the cytotoxicity and phytotoxicity assay.

2. Materials and methods

2.1. Plant materials

Ginger, nutmeg and alligator pepper were purchased from the Central Spices Market in Mile 12 area, Ketu, Lagos. They were dried under room temperature, grounded into powder and kept in plastic containers in the refridgerator before the commencement of the study.

2.2. Chemicals

BSA (Bovine serum albumin) was obtained from the Research Organics Cleveland USA. 1, 1-diphenyl, 2-picryl hydrazyl (DPPH), ferric chloride and trichloroacetic acid were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). All the chemicals were of analytical grade and the water was glass distilled.

2.2. Preparation of crude polyphenol extracts

The powdered samples were extracted with 80% acetone (1:2 w/v) thrice each for 72 hours each time at room temperature. Pooled extracts were filtered with Whatman filter paper (type 2) under vacuum and the filtrate was concentrated under reduced pressure on rotatory evaporator (BÜCHI, R-3000, Switzerland) at 40 ℃ temperature. The concentrated extract was further lyophilized. The lyophilized extract was then used for the experiments[15].

2.3. Brine shrimp cytotoxicity assay

The test was conducted by taking half filled hatching tray (22 x 32 cm) with brine solution (sea salt 38 g/L), 500 mg eggs of brine shrimp (*Artemia salina*) were sprinkled and after covering with a lid, it was incubated at 27 ℃ for 2 days for hatching. The brine shrimp larvae were collected through a light source and Pasteur pipette. The extracts were tested by using initial concentrations of 10, 100 and 1000 μg/mL in vials containing 5 mL of brine and 10 shrimps in each of the three replicates. Survivors were counted after 24 h. The data was analyzed to determine LD_{50} values at 95% confidence intervals[16].

2.4. Phytotoxicity assay

This test was performed according to the modified protocol of McLaughlin *et al* [16]. The test fractions were incorporated with sterilized medium at different concentrations i.e. 10, 100, 1000 μg/mL in methanol. Sterilized conical flasks were inoculated with fractions of desired concentrations prepared from the stock solution and allowed to evaporate overnight. Each flask was inoculated with 20 mL of sterilized E-medium and then ten Lemna minor each containing a rosette of three fronds were placed on media. Other flasks were supplemented with methanol serving as negative control and reference inhibitor i.e. Paraquat served as positive control. Treatment was replicated three times and the flasks incubated at 30 ℃ in Fisons Fi-Totron 600 H growth cabinet for seven days, (56±10)% relative humidity and 12 h day length. Growth of *Lemna minor* (*L. minor*) in fraction containing flask was determined by counting the number of fronds per dose and growth inhibition was calculated with reference to negative control.

2.5. Diphenylpicrylhydrazine (DPPH) radical-scavenging activity

The radical scavenging property of the extract was evaluated by assessing the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity[17]. The reaction mixture was prepared by mixing 5 μL extract (dissolved in DMSO) with 95 μL of DPPH (dissolved in ethanol). Different concentrations of extract were prepared, while the concentration of DPPH was kept as 300 μM in all reaction mixture. These reaction mixtures were dispensed in 96-well plate and incubated at 37 ℃ for 30 min and the absorbance was measured at 515 nm. The radical scavenging activity was calculated as a percentage of DPPH decolorization compared to control. Propyl gallate was used as a positive control.

$$\% \text{ Inhibition} = [1 - (\text{Absorbance extract} / \text{Absorbance control})] \times 100$$

2.6. Superoxide anion scavenging (SAS) assay

The method of Ferda[18] was used in the determination of superoxide scavenging activity of the samples. Superoxide scavenging was assayed in phosphate buffer (0.1 M, pH 7.5). Xanthine oxidase (0.003 unit /well) and test samples in DMSO were mixed in 96 - well microliter plate and pre-incubated for 10 min at room temperature, WST-1 (15 μM) was added. The reaction was initiated by adding 0.1 mM of xanthine and uric acid formation was measured spectrophotometrically at

295 nm and the reduction of WST-1 was read at 450 nm by using molecular devices, spectrama x 340. The percentage inhibitory activity by the samples was determined against DMSO blank and calculated using the formula:

% Inhibition =[1 − (Absorbanceextract/ Absorbancecontrol)] x 100.

2.7. Antiglycation assay

In vitro antiglycation activity of the spices was examined by testing the ability of the extracts to inhibit the methyl glyoxal mediated development of fluorescence of bovine serum albumin (BSA)[19]. In 96-well plate assays, each well contained 60 μL reaction mixture of 20 μL BSA (10 mg/mL), 20 μL of glucose anhydrous (50 mg/mL), magnesium oxide (14 mM) and 20 μL test sample (extract). Glycated control contained 20 μL BSA, 20 μL glucose and 20 μL sodium phosphate buffer (0.1 M, pH 7.4) containing NaN3 (30 mM), while blank control contained 20 μL BSA and 40 μL sodium phosphate buffer. Reaction mixture was incubated at 37 ℃ for 9 days in the presence or absence of various concentrations of the extract. After 9 days of incubation, 60 μL TCA (100 %) was added in each well and centrifuged (15,000 rpm) for 4 minutes at 4 ℃. After centrifugation, the pellet was washed with 60 μL 5 % TCA. The supernatant containing glucose, inhibitor and interfering substance, was removed and pellet containing advanced glycation endproducts (AGEs)-BSA was dissolved in 60 μL PBS. AGEs formation was measured by the fluorescence's intensity excitation (370 nm) to emission (440 nm) by using the spectrofluorometer RF-1500 (Shimadzu, Japan). Rutin was used as a positive control. The results are reported as follows:

% Inhibition = [1 − (Absorbance extract/Absorban cecontrol)] x 100.

3. Results

The result of phytotoxic activity of these extracts is displayed in Figure 1. All the extracts did not inhibit the growth of the plant at 10 μg/mL and showed low activities at 100 μg/mL. At a high concentration of 1 000 μg/mL, alligator pepper and ginger showed very high activity. The activities of the different extracts were significantly different ($P<0.05$) from one another at 100 and 1 000 μg/mL. Nutmeg displayed the highest LD_{50} (1490 μg/ml) as against alligator pepper (350) and ginger (600) as shown in Table 1.

The effect of the extracts on the survival of brine shrimps (*Artemia salina*) is shown in Figure 2. At low concentrations (10 and 100 μg/mL), the extracts showed low activity (less than 50%) against the organism but were active at high concentration (1 000 μg/mL) except nutmeg which still possesses less than 50% mortality of the organism. However, ginger showed the highest activity against the brine shrimps (46.67 and 73.33 at 100 and 1 000 μg/ml respectively). So the result of ginger was significantly different ($P<0.05$) from the other spices at these concentrations. Their LD_{50} (μg/mL) is in this order; ginger (187.85), alligator pepper (749.67), nutmeg (4359.70) (Table 1).

Table 1
LD_{50} values of polyphenolc extracts of alligator pepper, ginger and nutmeg in cytotoxity and phytotoxicity assays (μg/mL).

Spices	LD_{50}	
	Cytotoxicity	Phytotoxicity
Alligator pepper	749.67±9.56	350.00±3.85
Ginger	187.85±3.25	600.00±5.29
Nutmeg	4359.70±12.33	1490.00±6.12

Table 2
IC_{50} values of the extracts of alligator pepper, ginger and nutmeg in antiglycation and antioxidant assays (mg/mL).

Spices	IC_{50}		
	Antiglycation	[1]DPPH Assay	[2]SAS Assay
Alligator pepper	0.125±0.020	0.110±0.010	0.105±0.020
Ginger	0.285±0.010	0.075±0.000	0.070±0.010
Nutmeg	0.200±0.030	0.100±0.010	0.135±0.010

1DPPH; Diphenylpicrylhydrazine, 2SAS; Superoxide anion scavenging.

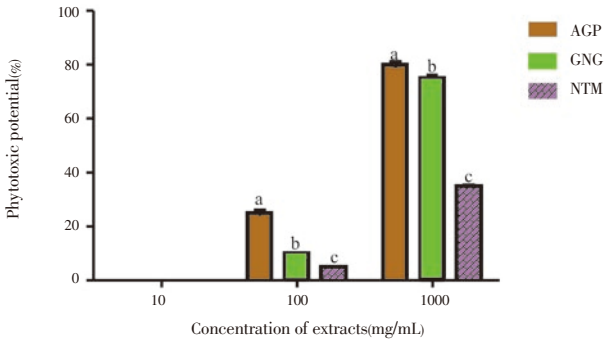

Figure 1. Phytotoxic effect of the polyphenolic extracts of spices against *L. minor*.
The values are expressed as means±SEM of triplicate tests. Means not sharing a common letter at the same concentration were significantly different ($P < 0.05$) when analysed by ANOVA and Bonferroni post hoc test. AGP: Alligator pepper, GNG: Ginger, NTM: Nutmeg.

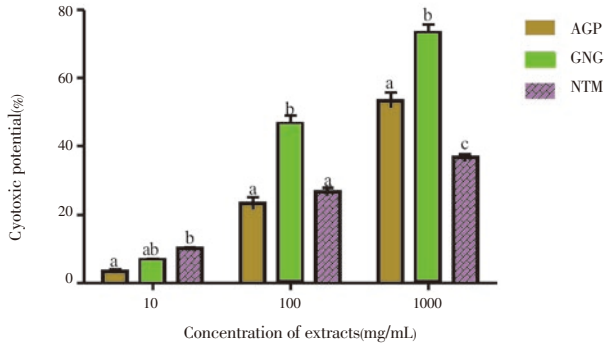

Figure 2. Cytotoxic effect of polyphenolic extracts of selected spices on *Artemia salina*.
The values are expressed as means±SEM of triplicate tests. Means not sharing a common letter at the same concentration were significantly different ($P< 0.05$) when analysed by ANOVA and Bonferroni post hoc test. AGP: Alligator pepper, GNG: Ginger, NTM: Nutmeg.

The DPPH radical scavenging activity of the spices is shown in Figure 3. All the extracts showed dose-dependent inhibition. At all the concentrations tested, the extracts

exhibited more than 50% inhibition of the radical. Though, the extracts had different inhibitions at lower concentrations, their percentage inhibition at 0.5 mg/mL were not significantly different ($P<0.05$) from one another. However, the IC_{50} shown in table 2 depicted that ginger had the lowest IC_{50} value (0.075) when compared to nutmeg (0.1) and alligator pepper (0.11).

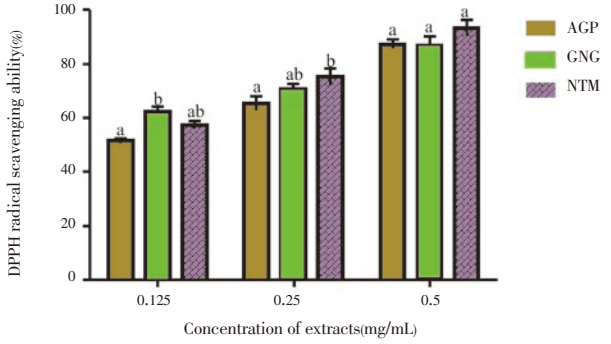

Figure 3. Inhibitory capacities of the polyphenolic extracts of alligator peppper on the formation of DPPH radicals.
The values are expressed as means±SEM of triplicate tests. Means not sharing a common letter at the same concentration were significantly different ($P< 0.05$) when analysed by ANOVA and Bonferroni post hoc test. AGP: Alligator pepper, GNG: Ginger, NTM: Nutmeg.

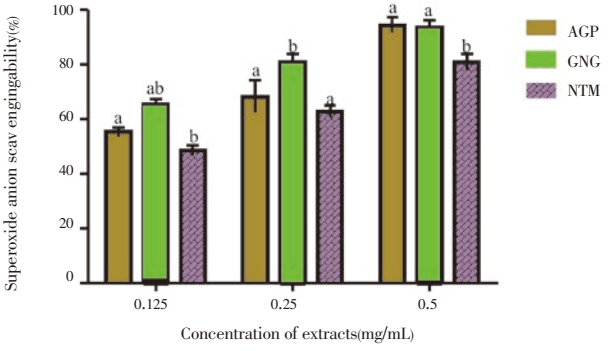

Figure 4. Inhibitory capacities of the polyphenolic extracts of selected spices on the formation of superoxide anion radicals.
The values are expressed as means±SEM of triplicate tests. Means not sharing a common letter at the same concentration were significantly different ($P < 0.05$) when analysed by ANOVA and Bonferroni post hoc test. AGP: Alligator pepper, GNG: Ginger, NTM: Nutmeg.

Figure 4 showed that ginger offered the highest inhibition to the superoxide anion radicals; 65.52%, 80.66% and 93.18% at 0.125, 0.25 and 0.5mg/ml concentration respectively. However, both alligator pepper and ginger were not significantly different ($P<0.05$) at the highest concentration tested. This trend is corroborated by the lowest IC_{50} displayed by ginger (0.07), followed by alligator pepper (0.105) and nutmeg (0.135)

Figure 5 presents the antiglycation capacities of the polyphenolic extracts, as evaluated by their inhibition of the formation of AGEs in the BSA/glucose system. All the extracts inhibited the glucose-mediated formation of fluorescent AGEs in a dose-dependent manner. At 1.0 mg/mL, all the extracts exhibited high inhibition towards the formation of AGEs which was retained by alligator pepper at all concentrations. Its inhibitory capacity was significantly different ($P<0.05$) compared to ginger and nutmeg at all concentrations tested. At 0.25mg/ml, the antiglycation capacity of the extracts followed this order; alligator pepper (73.4%), nutmeg (55.5%), ginger (46.4%). Table 2 also showed that alligator pepper had the lowest IC_{50} (0.125) compared to that of ginger (0.285) and curry (0.200).

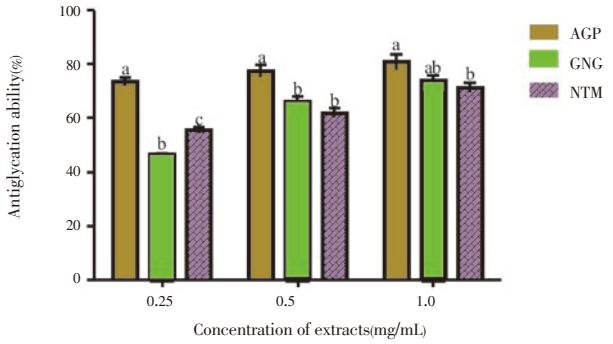

Figure 5. Inhibitory capacities of the polyphenolic extracts of alligator pepper, ginger and nutmeg on the formation of advanced glycation endproducts (AGEs)
The values are expressed as means±SEM of triplicate tests. Means not sharing a common letter at the same concentration were significantly different ($P < 0.05$) when analysed by ANOVA and Bonferroni post hoc test. AGP: Alligator pepper, GNG: Ginger, NTM: Nutmeg.

4. Discussion

Brine shrimp lethality is a general bioassay, which is indicative of cytotoxicity of an extract or compound[16]. The result indicates that crude polyphenolic extract of ginger is the most cytotoxic of the three. This is attested to by the large difference in their LD_{50} values (187, 749 and 4359 μg/mL for ginger, alligator pepper and nutmeg respectively). However, all the extracts can still be considered safe due to their relatively high LD_{50}. Anderson *et al* [20]. stated that extracts which showed $LD5_0$ higher than 100 μg/ml in the brine shrimp lethality test can be considered inactive and so safe for consumption.

To assess the possible phytotoxic potential of these extracts, their effect on the growth of plant Lemna minor was determined. From the LD_{50} values generated from their phytotoxic study, it showed that all the extracts had high LD_{50}, the implication of which is that they are non-toxic to the plant except at very high concentrations. According to Khan *et al*[21], a number of plants, their extracts or their purified active constituents can act as allelochemicals to other plants. These spices are not suitable for such purpose due to their inactivity.

The DPPH free radical scavenging ability of the extracts showed that all the spices inhibited the free radicals in concentration-dependent manner. These high inhibition possessed by the spices at all concentrations may not be unconnected with the large quantities of phenolics[15] in

them. This is also shown by their low IC_{50} signifying that ginger seems to be the most active against DPPH radicals due to its least IC_{50} of 0.075 mg/mL.

In order to further confirm the antioxidant activity of these extracts, their ability to scavenge superoxide anion radical was evaluated. Extract of ginger also displayed the highest inhibition at all concentrations. This is testified to by its IC_{50} 0.07 mg/mL as against 0.105 mg/mL and 0.135 mg/mL for alligator pepper and nutmeg. The antiradical activity of polyphenols is principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure[22,23]. In accordance, several reports had established a correlation between the total phenol content of plant food and their antioxidant properties[15,24]

Free radicals especially reactive oxygen species (ROS) had been implicated in a lot of degenerative diseases such as diabetes and cardiovascular diseases. Overproduction of ROS can directly attack the polyunsaturated fatty acids of the cell membranes and induce lipid peroxidation[25]. Antioxidants carry out their protective properties on cells either by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body[26]. The free radical scavenging ability of all these extracts is an indication that these spices promise to be excellent dietary source of antioxidants.

Hyperglycemia is regarded as a key factor in the development of diabetic complications. Chronic exposure of tissues to high levels of blood glucose can lead to adverse intracellular effects, leading to what is known as glucose toxicity. Several major mechanisms have been proposed for hyperglycemia induced-tissue damages, including increased polyol pathway flux, increased de-novo diacylglycerol synthesis with resultant activation of protein kinase C isoforms, hexoamine pathway and AGEs formation [27-29]. A large body of evidences point to glycation as a key molecular basis of diabetic complications. The fact that glycoxidative reactions cause protein dysfunction strongly suggests a role for such reactions as pathogenic factors in diabetes[30-39]. AGEs contribute to diabetic complications through a series of pathological changes such as increased atherogenicity of LDL, increased basement membrane permeability and decreased insulin binding to its receptors. AGEs play, as well, an important role in diabetic micro- and macroangiopathy where it deposits under endothelial cells[40,41].

The extracts of all the spices tested displayed good antiglycation ability which increases with the increase in concentration, though alligator pepper extract showed highest potential at all concentrations tested. Glycation and AGE-induced toxicity are known to be associated with increased free radical production. Therefore, agents that possess good antioxidant activity by mopping up free radicals can simultaneously inhibit the formation of advanced glycation endproducts[42,43]. As such, polyphenols from these spices can effectively serve as an antioxidant and antiglycation agent in the diets of diabetics.

Conflict of interest statement

We declare that we have no conflict of interest.

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