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Bio-modulation of specific biochemical markers linked with oxidative stability in frozen beef

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Abstract

Cold storage has been implicated in the alteration of several biochemical parameters and other cellular components in meat, despite its beneficial attributes. Thus, this research seeks to mitigate the breakdown process in meat, during cold storage (-18 °C) using plant extracts for 28 days. Fresh beef samples were aseptically collected, evaluated, and treated using 5, 10, and 15% *Xylopia aethiopica, Rhaphiostylis beninensis, Piper guineense,* and butylated hydroxytoluene, respectively, alongside the control setup for this experiment. The endogenous enzyme activities, haem iron content and lipolytic parameters were estimated at the beginning and end of the experiment. The results showed that the beef samples immersed in the spice formulations had increased activities in their endogenous antioxidant enzymes i.e., Peroxidase (11.21×10^{-4} units/mg tissue), Catalase (8.89×10^{-4} units/mg tissue), Superoxide Dismutase (13.60×10^{-4} units/mg tissue) at 5, 5, and 10% *X. aethiopica*, respectively, compared to the control (Peroxidase = 2.57×10^{-4} units/mg tissue, Catalase = 2.86×10^{-4} units/mg tissue, and Superoxide Dismutase = 4.04×10^{-4} units/mg tissue). It also improved the level of haem iron content (3.02 mg/kg at 5% combined spice formulation), while decreasing the rate of lipolysis (free fatty acid = 0.06 g Oleic acid/100 g fat at 5% *P. guineense* and acid value = 0.08 at 10% *P. guineense*). Therefore, the outcome of this investigation further affirms that the spices are promising replacements for synthetic chemical antioxidants for sustaining the oxidative quality of frozen beef.

Keywords Antioxidant spices, Endogenous enzymes, Haem iron, Cold storage, Lipolysis, Preservation

Introduction

Meat contains vital food components that render a significant portion of a human's daily nutritional requirement. Thus, the chemical quality of meat plays a crucial role in sustaining, as well as enhancing consumer health. Notwithstanding, the chemical constitution of meats (physicochemical, proximate, vitamin and mineral contents) makes them prone to oxidative processes that could negatively impact their freshness, nutritive value, oxidative quality, shelf life, and other vital assessors of meat quality [1]. To forestall the detrimental impacts of the said processes in meat, an array of preservative techniques have been employed, the most prominent is freezing. Meat preserved through freezing lasts the longest

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with the least degree of quality impairment. However, the meat's structure can be torn apart by big ice crystals, liberating several cellular components, including enzymes, free fatty acids, amino acid residues, and haem iron during frozen storage [2]. This causes the degradation of the sensory qualities of the meat and the depletion of meat nutrients. Moreover, phospholipids present in cell membranes are susceptible to oxidation owing to the double bonds found in their structures [3].

Given the foregoing, the necessity for an additional technique of meat preservation has become more apparent. One supplementary approach is the incorporation of antioxidants. The synthetically made antioxidant, butylated hydroxytoluene (BHT) has been widely employed in the delay or suppression of oxidation and elongation of the shelf life of meat products consequent upon its free radical scavenging potency. However, there is rising market demand for natural antioxidants because of some reported toxicological impacts of synthetic antioxidants on the biological system [4]. Accordingly, the counter-intuitive effects of synthetic antioxidants, have made plant-based natural antioxidants such as spices more prominent than ever. In this regard, numerous researchers around the globe have investigated spices. For example, Hes et al. [5] reported that the incorporation of extracts of thyme and rosemary in ground fried meatballs subjected to frozen storage, inhibited lipid oxidation and preserved the meat's nutritional value. Similarly, the reports of Nikousaleh and Prakas [6] on the preservative properties of clove, cinnamon and sumac spices on meat sausages stored under refrigeration for 30 days revealed that the addition of the spices to the meat sausages resulted in a lower free fatty acid level in the sausages after the storage period. Moreover, Draszanowska et al. [7] have shown ginger rhizome is a promising substitute for synthetic antioxidants in preserving pasteurised canned meat products. Additionally, Lopez et al. [8] demonstrated that essential oils from Hop (*Humulus lupulus)* and Oregano (*Origanum vulgare)* are viable alternatives for synthetic antioxidants as peanut oils rich in oleic acid and fortified with the essential oils, showed less susceptibility to oxidation compared with sunflower oils and other high oleic acid peanut oils enriched with BHT. In another study, Lopez et al. [9] reported that the essential oils also protected the high oleic peanut oils from oxidation occasioned by deep frying of the oils. Moreover, various investigations on *Xylopia aethiopica, Rhaphiostylis beninensis, and Piper guineense* spices have revealed their food preservative potentials, renowned antioxidant and other biological attributes [10–13].

Xylopia aethiopica, sometimes known as "African pepper" (Family: Annonaceae), is said to thrive in forest areas, frequently beside rivers and areas lacking sufficient rainfall. The fruits of *Xylopia aethiopica* are tiny, swirled pods containing beans and are cylindrical, dark brown, about 2.5–5 cm long, and 4–6 mm thick. Five to eight kidney-like seeds, each measuring around five millimetres in length, are contained in each pod. After drying, the ripe fruit's colour (green) normally turns brownish-black [14, 15]. The plant's bioactive components, vital nutrients and antimicrobial attributes [16–18] made it increasingly common among several indigenous Africans for the management of various disease conditions and meal preparation.

In the Western and Southern parts of Africa, the woody climbing *Rhaphiotylis beninensis* (Icacinaceae), thrives. Locally, the plant's root is utilized as a soup relishing agent. There have been reports of the plant's antibacterial, growth inhibition, anti-inflammatory, cytotoxic, analgesic, aphrodisiac, hepatoprotective, anti-sickling, and anti-oxidant effects [19–21]. *Piper guineense*, a member of the *Piperaceae* family commonly referred to as *Ashanti pepper, West African Black pepper, Guinea pepper, Benin pepper, and false cubeb* in the English language; is a perennial climber that uses its adventitious rootlets to scale trees up to 12 m high. It is indigenous to Africa's tropical rain forests, mostly in the wild. *Piper guineense* has exhibited a range of pharmacological effects that have been confirmed [22, 23].

Previous investigations on *R. beninensis, X. aethiopica* and *P. guineene* spices [10–14, 18–26] have established their antioxidant properties, their plethoric bioactive chemical components, proximate composition, and mineral constituents, their ability to attenuate the formation of toxic lipid-protein peroxidation products and maintain an optimum pH and peroxide values in beef samples during frozen storage. Findings from these studies invariably show that the spices are more than just ingredients for relishing foods and components in therapeutic formulations for a variety of illnesses; they also hold great promise for the food Industry.

Therefore, the current study aims to provide fresh perspectives and insights on using these biologically active spices as promising strategies to minimise the detrimental impacts of lipid oxidation in foods, building on and corroborating the findings of previous research. This will be accomplished by assessing their modulatory efficacies on a few biochemical indices in frozen beef, including the free fatty acid content, haem iron content, acid value, and the activities of endogenous antioxidant enzymes.

Methodology

Plant samples

The roots of *Raphiostylis beninensis*, fruits of *Xylopia aethiopica*, and seeds of *Piper guineense*, spices, were obtained from *Koko* market located at Oghara, Delta

state, Nigeria. Afterwards, a plant taxonomist from the Department of Plant Biology and Biotechnology, University of Benin, Nigeria, verified them. To acquire dried, pulverised plant materials, a waring mechanical blender (Fisher Inc. USA) was used to homogenise a considerable quantity of fresh spices that had been subjected to drying at 27.0 ± 2.0 °C (room temperature) for two weeks. Equivalent amounts of the three ground spices (1:1:1) were weighed into a big bowl for the combined extract formulation. Every ground component was then subjected to an extraction process using a maceration method at a ratio of 500 g ground spice to 2.5 L of methanol. The mixture was then agitated, allowed to stand for 72 h, and then filtered through muslin cloth. Additionally, using a RotoVap RE-501, USA rotary evaporator set at 40 °C, the different extracts were concentrated in a vacuum into a slurry. Subsequently, the concentrated extracts were kept at 4 °C in airtight containers until they were needed.

Collection and evaluation of fresh beef samples

A portion of beef tenderloins from a freshly slaughtered White Fulani cattle was bought at an abattoir in Oghara, Delta State (Latitude: 5.5908°N, Longitude: 6.1001°E) and delivered right away in an ice pack to the laboratory section of the Department of Biochemistry at Western Delta University Oghara, Nigeria. Subsequently, all fatty parts of the beef samples were meticulously excised with the aid of a sterilized knife before being submerged in various spice extracts.

Experimental design

The experimental design included a control group (beef samples immersed in distilled water only) and experimental groups comprising beef samples (1.5 g) immersed in 5, 10, and 15% formulations of pulverized *X. aethiopica, R. beninensis,* and *P. guineense* spices, their combined (1:1:1) extract formulations and butylated hydroxyl toluene (standard antioxidant) respectively. Following that, beef samples were subjected to 28 days of freezing at −18 ^{0}C. At the beginning of storage and on days 4, 8, 12, 16, 20, 24, and 28, the beef samples were routinely examined for free fatty acid content, acid value content, haem iron content, catalase, superoxide dismutase, and peroxidase activities after being defrosted in a biosafety compartment.

Assessment of the indices of lipolysis in frozen beef samples

Determination of free fatty acid

Percentage Free Fatty Acids (FFA) level was evaluated according to the methods described by Rukunudin et al. [27] and Cunniff [28] with slight modifications. In a dried mortal crucible, 1.5 g of each of the samples was homogenized using 15 mL of neutralized hexane/ethanol mixture (1:1). The filtration procedure employed a Whatman filter paper to exclude meat fragments from the filtrate. Following that, the filtrate was mixed after receiving 2–3 drops of 1% ethanolic phenolphthalein indicator. Subsequently, the sample was titrated with NaOH solution (0.01N) until an unchanging pale pink colour was achieved.

$$\%\text{of FFA as equivalent to oleic acid} = \frac{T \times N \times 28.24}{W} \times \frac{100}{1} \quad (1)$$

Where: T = the titre value
N = the normality of NaOH
W = Weight of sample
28.24 is the molecular weight of oleic acid divided by a conversion factor of 10

Determination of acid value

The acid values of frozen beef samples were determined by the technique of the American Oil Chemists' Society [29]. 1.5 g of each sample was homogenized with 15 mL of chloroform. To separate meat particles from the filtrate, the homogenized sample was run through the Whatman #1 filter paper. To the filtrate, 1.5 mL of neutralized ethanol and 0.5 mL of a 1% phenolphthalein solution were then added. Subsequently, the mixture was thoroughly stirred before being titrated with a 0.01N NaOH solution to an endpoint of a stable faint pink colour. Using the following formula, the acid value was determined:

$$\text{Acid value} = \frac{\text{S} \times \text{N} \times 56.1}{\text{W}} \quad (2)$$

Where: S = Titer value
N = Number of ml of 0.01 sodium hydroxide required
W = Weight of sample

Determination of Haem iron content

Utilizing acidified acetone extraction and spectrophotometry, the method of Hornsey [30] was used to measure the amount of haem iron in the meat samples. In summary, A 50 mL centrifuge tube containing two grammes (2 g) of each sample was filled with 9 mL of an acid acetone mixture made up of 90% acetone, 8% deionized water, and 2% HCl. A glass rod was used to thoroughly stir the mixture, which was then allowed to stand at room temperature for an hour. After that, the extract underwent a 10-min centrifugation at 2200 g (1186 rpm). The supernatant was additionally filtered using Whatman

filter paper, and the absorbance was measured at 640 nm in comparison to an acid-acetone blank. The following formula was used to compute the total pigments as haematin [31]:

Total pigment (mg/kg) = $A_{640} \times 680$ and haem iron will be calculated thus:

$$\text{Haem iron (mg/kg)} = \text{total pigment (mg/kg)} \times 8.82/100 \quad (3)$$

Determination of Enzymatic Antioxidant Activities

The beef samples were processed into 10% tissue homogenates in 0.86% physiological saline and centrifuged at 2000 rpm (444 g) for 10 min. Following that, the activity of the catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) enzymes in the supernatant were all measured [32].

Catalase activity

Catalase activity was assessed using the Aebi [33] and Luck [34] protocols. The reaction mixture (3 mL) contained 100 µL of the tissue homogenate, 400 µL of 60 mM H_2O_2, and 2500 µL of 50 mM potassium phosphate buffer (pH 7) to begin the reaction. For three minutes, the reaction was monitored at 240 nm. Using a molar extinction value of 43.6 mM^{-1} cm-^{1}, the CAT activity was calculated.

Superoxide Dismutase Activity

The method described by Misra and Fridovich [35] was used to evaluate SOD activity spectrophotometrically. In brief, a test tube with a total reaction volume of 3000 mL received 200 µl (µL) of the tissue homogenate, 2500 µL of a 50 mM carbonate buffer (pH 10.4), and 300 µL of a 0.03 mM adrenaline solution. After the test tube had equilibrated at room temperature, the absorbance was measured at 480 nm every 30 s for three minutes against a reagent blank. Then, SOD activity was calculated using a molar extinction coefficient of 4.02 mM-1 cm-1 to yield units/mg protein.

Peroxidase Activity

With modest modifications, the pyrogallol oxidation method [36] was used to investigate POD activity. Briefly, a 100 µL of 0.05 M pyrogallol, 100 µL of the tissue homogenate, 2500 µL of 50 mM potassium phosphate buffer (pH 7), and 300 µL of 1% or 40 mM H_2O_2 were all included in the 3 mL reaction mixture. The 300 µL of H_2O_2 served as the reaction's initiator. For a total of three (3) minutes, the variations in absorbance were measured at 470 nm every 30 s, and POD activity was calculated using 12 mM^{-1} cm^{-1}. The activities of CAT, SOD, and POD in frozen beef tissues incorporated with extracts of the spices were computed following the mathematical model below:

$$\text{Enzyme activity (units/mL)} = \frac{\Delta A/\text{min} \times V_T \times D}{M_a \times V_S} \quad (4)$$

Where; ΔA/min = Change in absorbance at a given wavelength per unit time

V_T = Total volume of assay mixture (mL)

D = Dilution factor

Vs = Volume of the test sample in the assay mixture (mL)

Ma = Molar absorption coefficient at a given wavelength for a specific enzyme (M^{-1} cm^{-1})

$$\text{Specific enzyme activity (units/mg protein)} = \frac{E_a}{P_c} \quad (5)$$

Where; E_a = Enzyme activity expressed in units/mL

P_c = Protein concentration expressed in mg/mL

Data Analysis

Data collected from the analyses carried out were presented as Mean ± SEM (standard error of mean) for three distinct observations. Randomized complete block design (RCBD), two-way analysis of variance (ANOVA), was employed in the evaluation of raw data while the homogeneity of means was determined using Dunnett's multiple comparison test (DMCT) at $P < 0.05$. The software used for the statistical analysis was Minitab 16.0, CoStat 6.451, SPSS v.20 and GraphPad Prism (v.8.0.2).

Results

Effects of spice extracts on some indices of lipolysis of frozen beef samples

Free fatty acid levels

It was observed that 10% spice extract of *Piper guineense* gave the best reduction in the amount of free fatty acid in stored beef sample at day 28 i.e., 0.06 g Oleic acid per 100 g fat (Table 1), compared to that of the standard antioxidant "butylated hydroxytoulene" (BHT = 0.08 g Oleic acid/100 g fat) and control setup for the experiment (2.14 g Oleic acid/100 g fat), as shown in Fig. 1. Inferably, 5 and 15% spice formulation of *Xylopia aethiopica* (0.10 and 0.15 g Oleic acid/100 g fat, respectively) also effectively reduced the level of free fatty acid in the pretreated beef.

Table 1 Biochemical markers associated with oxidation in pretreated frozen beef samples

Parameter	Day	Treatment	Concentration		
			5%	10%	15%
Peroxidase (x10^{-4}) Units/mg tissue	0	Fresh beef	6.43	6.43	6.43
	0	Control	2.57	2.57	2.57
	0	BHT	13.10	12.50	12.17
	28	Spice A	11.21	11.30[a]*	9.19
	28	Spice B	9.52	9.81	8.26
	28	Spice C	9.21	9.62	8.60
	28	Combination	10.50	10.48	10.00
Catalase (x10^{-4}) Units/mg tissue	0	Fresh beef	4.44	4.44	4.44
	0	Control	2.86	2.86	2.86
	0	BHT	10.28	9.32	8.77
	28	Spice A	8.85	8.90[a]*	7.81
	28	Spice B	5.96	6.30	6.33
	28	Spice C	6.12	6.08	5.32
	28	Combination	8.89	7.60	7.25
Superoxide Dismutase (x10^{-4}) Units/mg tissue	0	Fresh beef	9.39	9.39	9.39
	0	Control	4.04	4.04	4.04
	0	BHT	15.17	16.17	12.06
	28	Spice A	13.60	14.90[a]*	12.06
	28	Spice B	13.00	11.38	13.10
	28	Spice C	11.00	11.50	13.00
	28	Combination	13.31	14.05	12.94
Free Fatty Acid g Oleic acid/100g fat	0	Fresh beef	0.90	0.90	0.90
	0	Control	2.14[a]	2.14	2.14
	0	BHT	0.08	0.08	0.11
	28	Spice A	0.10	0.09*	0.15
	28	Spice B	0.16	0.24	0.30
	28	Spice C	0.19	0.06	0.34
	28	Combination	0.11	0.11	0.17
Acid Value	0	Fresh beef	1.28	1.28	1.28
	0	Control	3.04[a]	3.04	3.04
	0	BHT	0.11	0.11	0.16
	28	Spice A	0.15*	0.13	0.21
	28	Spice B	0.23	0.35	0.43
	28	Spice C	0.27	0.08	0.48
	28	Combination	0.16	0.16	0.24
Haem iron content (mg/Kg)	0	Fresh beef	1.38	1.38	1.38
	0	Control	0.46	0.46	0.46
	0	BHT	3.10	3.20	3.12
	28	Spice A	2.98	3.04	2.70
	28	Spice B	1.78	2.40	1.98
	28	Spice C	1.96	2.60	2.40
	28	Combination	3.02	3.06[a]*	3.02

The superscript "a" indicates the highest value while * shows the best spice extract

Spice A = *Xylopia aethiopica;* Spice B = *Rhaphiostylis beninensis;* Spice C = *Piper guineense;* BHT = Butylated hydroxytoluene (Standard Antioxidant)

Acid value content

The experiment carried out on the pretreated stored beef samples showed that 10% spice formulation of *Piper guineense* had the highest reduction in the acid value content of the frozen meat samples at day 28 i.e., 0.08, compared to that of the standard treatment used

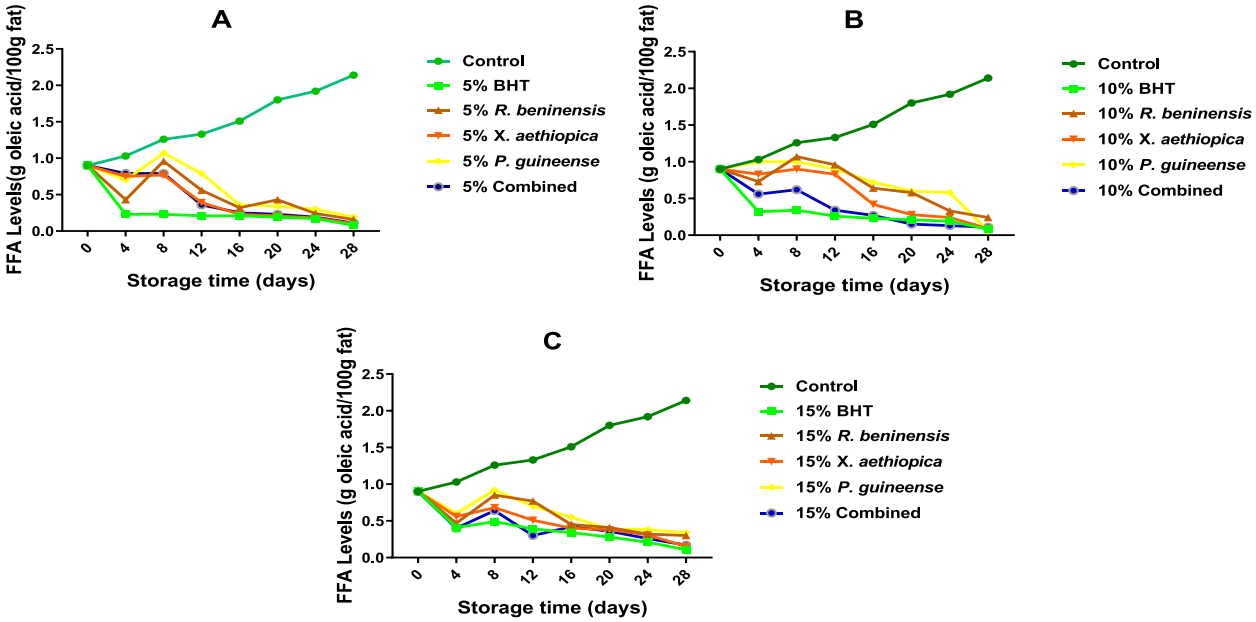

Fig. 1 Effects of graded concentrations of spices on the free fatty acid (FFA) levels of frozen beef samples

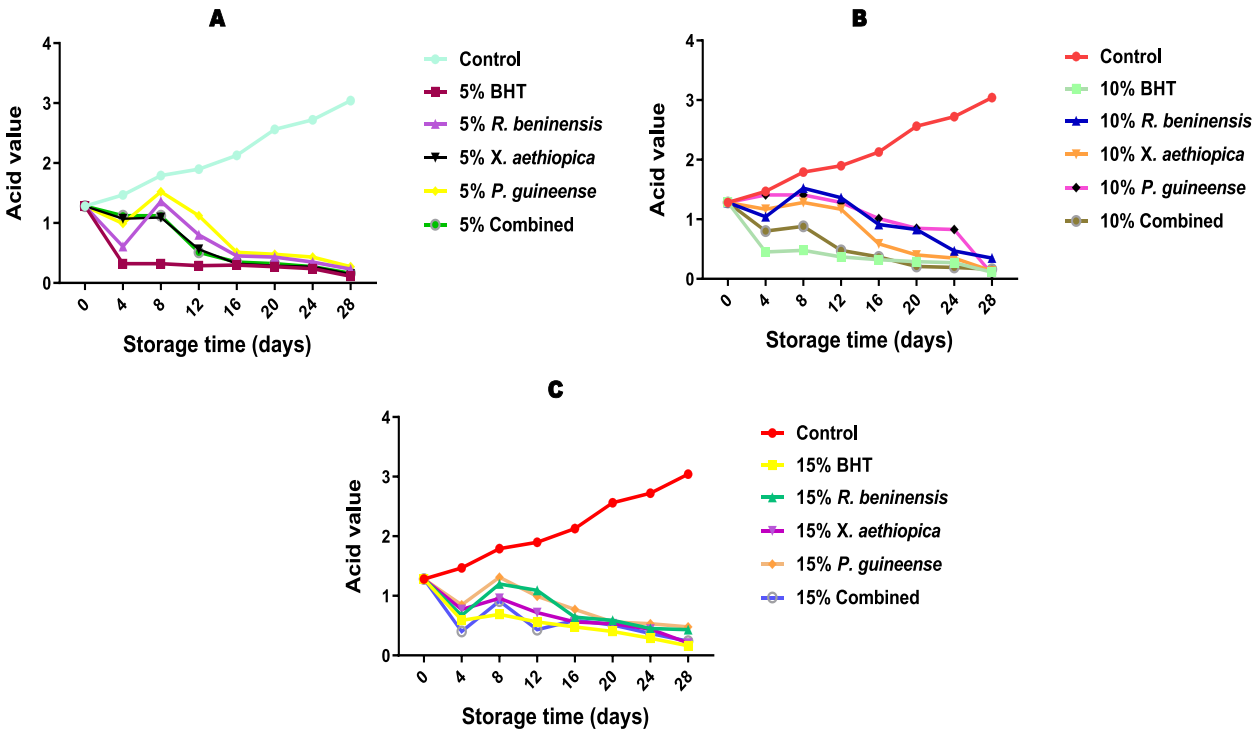

Fig. 2 Effects of graded concentrations of spices on the acid values of frozen beef samples

commercially (BHT = 0.11) and the control setup for the experiment (3.04), as illustrated in Fig. 2. Other spice formulations were also observed to have positive effects in decreasing the acid value of the stored beef i.e., 5 and 15% *X. aethiopica* (0.15 and 0.21, respectively), as shown in Table 1.

Effect of Spice extracts on Haem iron contents of frozen beef

At the end of the storage period (day 28), it was noted that 10% combined formulation of the spices effectively increased the haem iron content of the pretreated beef samples i.e., 3.06 mg/Kg, compared to that of the standard antioxidant "butylated hydroxytoulene" (BHT = 3.20 mg/Kg) and the control setup for the experiment (0.46 mg/Kg), as shown in Fig. 3. Furthermore, 5 and 15% combined formulations of the spices respectively increased the haem iron content of the stored beef samples (3.02 and 3.02 mg/Kg), as shown in Table 1.

Effects of spice extracts on endogenous enzyme activities of frozen beef

Peroxidase (POD) activity

The peroxidase activity of frozen beef samples incorporated with 10% *X. aethiopica* spice extracts had the highest increase at day 28 i.e., 11.30×10^{-4} units/mg tissue (Table 1), compared to that of the standard, BHT (12.50×10^{-4} units/mg tissue) and control setup for the experiment (2.57×10^{-4} units/mg tissue), as shown in Fig. 4. Beef samples pretreated with 5% *X. aethiopica* and 15% combined spice formulation (11.21×10^{-4} and 10.00×10^{-4} units/mg tissue) respectively, also recorded increased peroxidase activities.

Superoxide dismutase (SOD) activity

The outcome of the experiment on day 28 (Fig. 5), showed that 10% *Xylopia aethiopica* spice-treated samples gave the best superoxide dismutase activity (14.90×10^{-4} units/mg tissue), compared to that of the standard antioxidant "butylated hydroxytoluene" (BHT = 16.17×10^{-4} units/mg tissue), the control setup for the experiment (4.04×10^{-4} units/mg tissue), 5% *Xylopia aethiopica* (13.60×10^{-4} units/mg tissue), and 15% *Rhaphiostylis beninensis* spice-treated beef samples (13.10×10^{-4} units/mg tissue), as shown in Table 1.

Catalase (CAT) activity

The 10% spice extract of *Xylopia aethiopica* gave an outstanding increment in the catalase activity of stored beef samples among other spice formulations at day 28 i.e., 8.90×10^{-4} units/mg tissue (Table 1), compared to that of the standard antioxidant "butylated hydroxytoulene" (BHT = 9.32×10^{-4} units/mg tissue) and control setup for the experiment (2.86×10^{-4} units/mg tissue). Notwithstanding, the 5 and 15% spice formulations of the combined extract and *Xylopia aethiopica* (8.89×10^{-4} units/mg tissue and 7.81×10^{-4} units/mg tissue, respectively) also increased the catalase activity in the pretreated beef samples (Fig. 6).

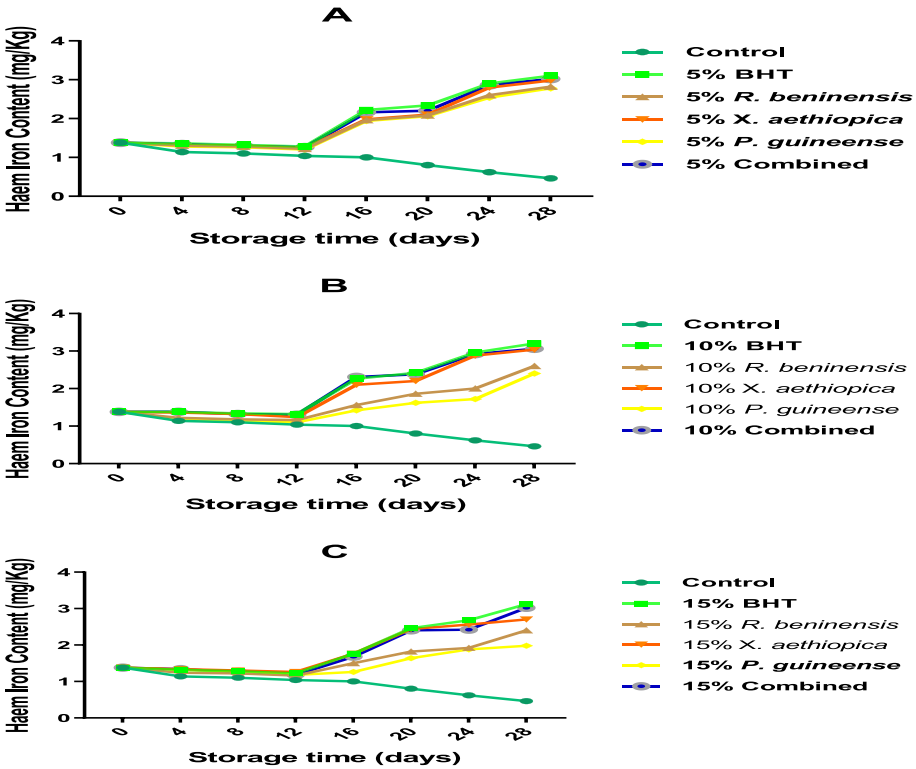

Fig. 3 Effects of graded concentrations of spices on the haem iron contents of frozen beef samples

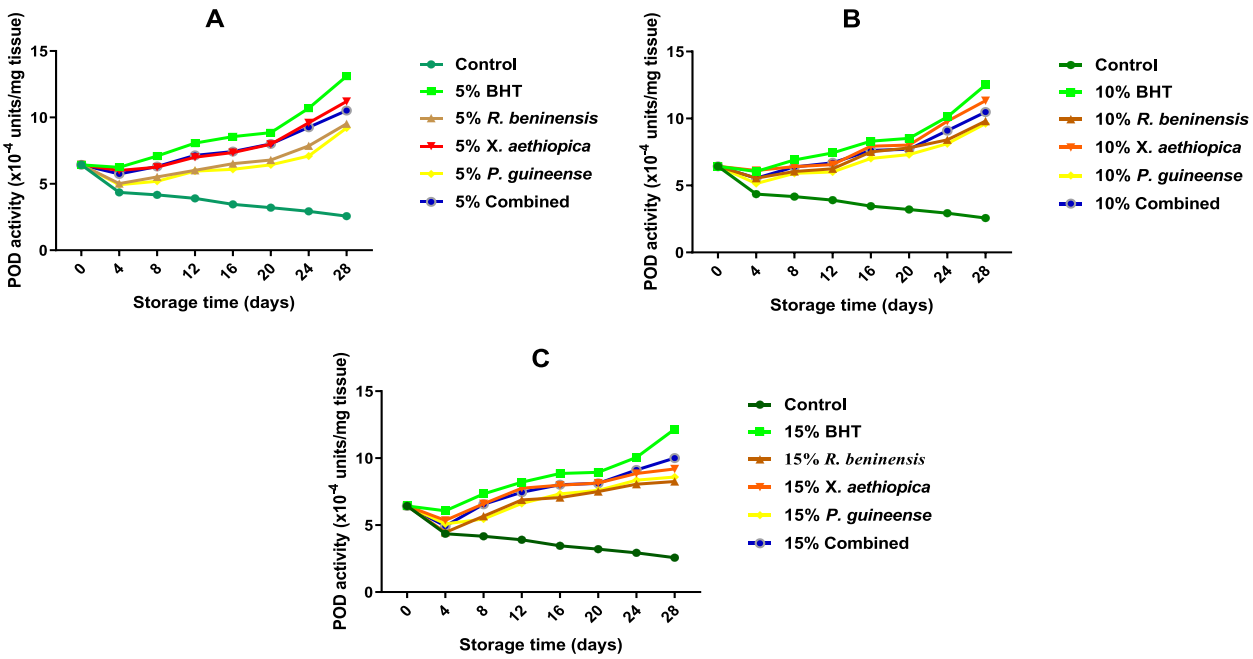

Fig. 4 Effects of graded concentrations of spices on the peroxidase activities in frozen beef samples

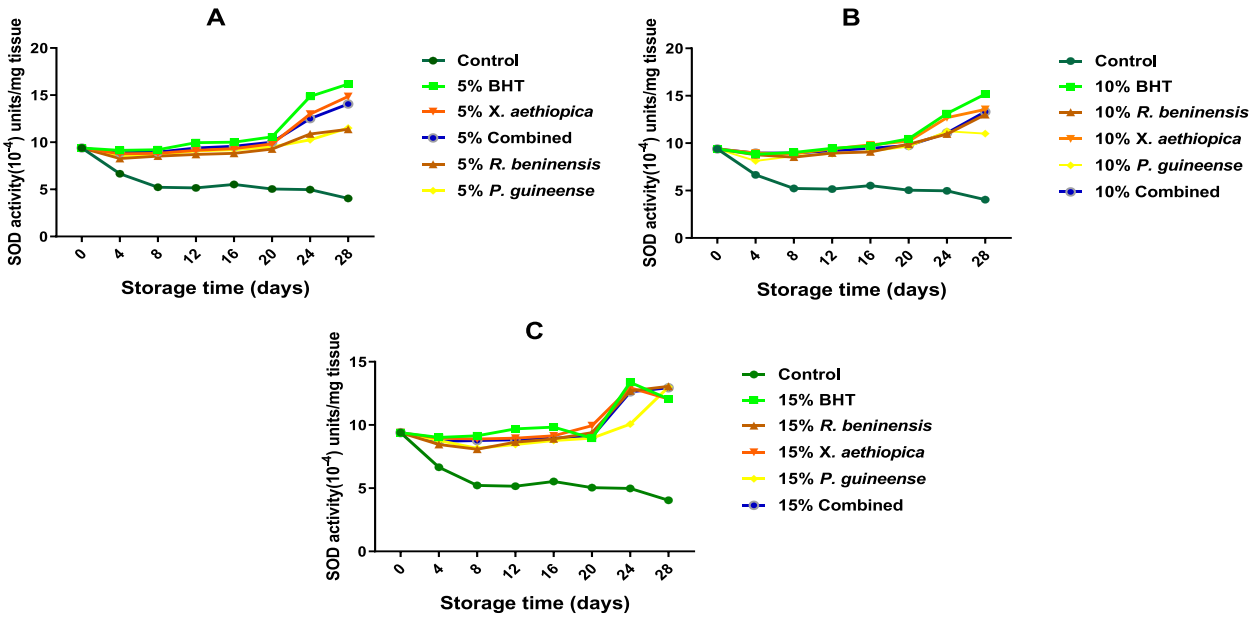

Fig. 5 Effects of graded concentrations of spices on the superoxide dismutase activities in frozen beef

Discussion

Effect of the spices on indices of lipolysis of frozen beef

The significance of free fatty acids (FFA) is to provide insight into the stability of lipids during storage [37]. As a further indicator of hydrolytic rancidity, the acid value (AV) test examines free fatty acids [38]. Thus, the direct correlation between FFA and AV indicates that both values are indicators of the level of lipolysis occurring during the storage of lipids. The outstanding reductions in the FFA and AV values in the beef samples brought about by 10% *Piper guineense* spice at the end of the storage period, may be associated with the growth

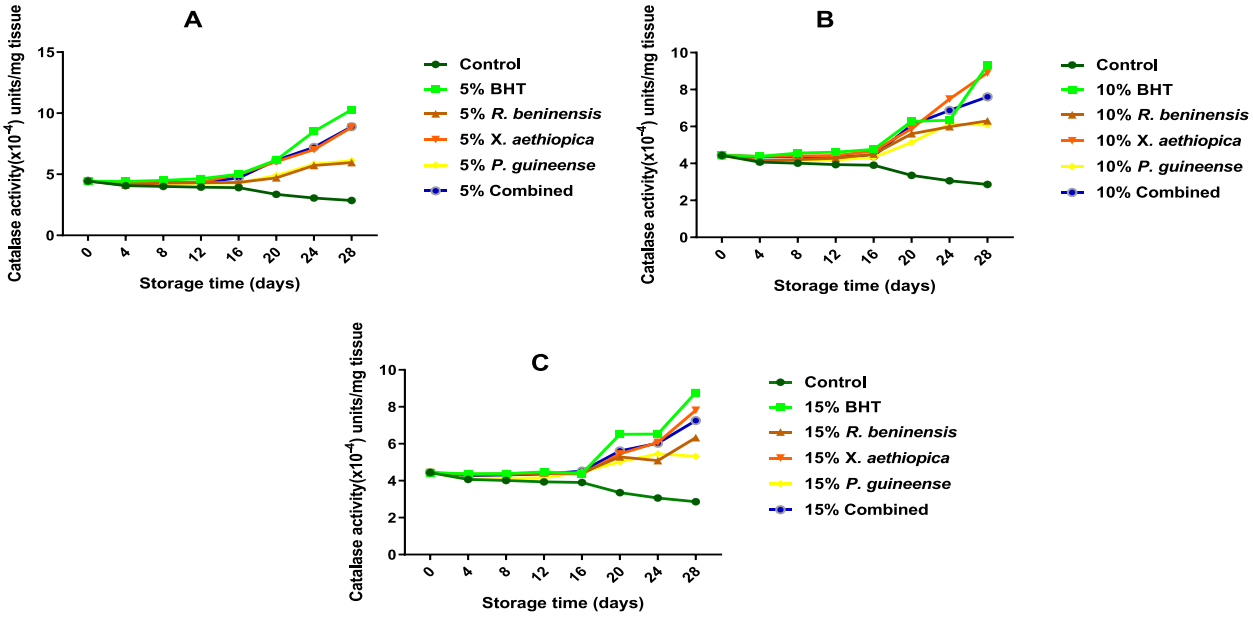

Fig. 6 Effects of graded spice concentrations on the catalase activities in frozen beef samples

inhibition of lipolytic microbes, total myofibrillar protein solubility, and inhibition of lipolytic enzymes respectively. This observation was corroborated by the reports of Cilla [39] who stated that, microbial lipases, phospholipases, acid lipases, neutral lipases, phospholipase A, and lysophospholipase are among the potential lipolytic enzymes found in beef. Thus, the presence of bioactive substances with antioxidant capabilities in the spice may have impeded the action of lipolytic enzymes and lipid auto-oxidation in the beef during frozen storage, leading to lower FFA and AV values. This result is consistent with the studies of Jummai et al. [40] who reported that application of local spices on fish chubs during frozen storage(-18^0C) yielded lower free fatty acid content in the fish chubs compared to that of the control (without spices). Thus, the inability of the control sample to inhibit the activities of lipolytic enzymes and auto-oxidation of lipids culminating in increased FFA and AV values with increasing storage time, could be attributed to the absence of potent bioactive compounds; indicating that meat's natural lipolytic activities could not be prevented/impeded by freezing alone [2]. Moreover, a stunning outcome was noted in this research as the spice formulation of *Piper guineense* performed better than the standard synthetic antioxidant, BHT conventionally employed in the preservation of meat and meat products. This observation is similar with the findings of Amera et al. [41], who reported that Rosemary spice extract-treated ground beef samples during frozen storage exhibited a higher reductive ability on the FFA content of the beef when compared with those treated with an equivalent mixture of butylated hydroxytoluene and butylated hydroxyanisole antioxidants. This outcome is a mark of promise that *P. guineense* could be explored as a natural preservative to prevent oxidative rancidity in beef during cold storage, in place of the synthetic antioxidant.

Effects of graded concentrations of the spices on the haem iron content of frozen beef

The colour of meats is partially attributed to haem iron, which is an essential component of myoglobin in meat. Hence, the dissociation of haem from myoglobin may result in loss of meat colour [42, 43]. The reductions in values of haem iron for all beef samples treated with various concentrations (5 −15%) of *X. aethiopica, R. beninensis, P. guineense* spices, and BHT extracts respectively from day 4 to day 12 may be due to the inability of the beef samples to absorb the extracts sufficiently during the aforementioned days, culminating in their inability to prevent the disruption of haem and release of free iron from haem [44]. Thus, the visual appearance of the beef samples during the early days of storage may have been compromised. However, increasing frozen storage times and the absence of antioxidants to prevent the eventual oxidation of myoglobin during frozen storage of the beef could have led to the resultant reduction in haem iron content observed in the control sample. Moreover, the destruction of certain porphyrin rings via oxidative cleavage and the subsequent dissolution of the haem iron complex may have contributed to their low haem iron

content [45]. Additionally, the noticeable increments observed in post-mortem haem iron content of the frozen beef for all samples containing various formulations of the extracts and BHT relative to the control on day 28, suggests that the spices which at this time may have permeated the beef tissues, could have prevented the release of more free iron from haem iron and plausibly, the elevated level of soluble haem pigments in the spice treated samples is attendant to a greater extractability of haem pigments [44]. Furthermore, the greatest inclination to forestall the liberation of free iron from haem iron exhibited by the 10% combined extract formulation may be due to the synergistic actions among the various antioxidant spices [46]. This finding concur with the report of Nishad, et al. [46], who revealed that the synergistic effects of a combined formulation of extracts from *Citrus paradisi* peels and *Myristica fragrans* (0.5 and 1%) gave better protection of frozen meatballs against oxidation compared to similar concentrations of the individual extracts.

Time-dependent effect of graded concentrations of the spices on the activities of endogenous enzymes of frozen beef

Endogenous antioxidant enzymes such as Peroxidase (POD), Superoxide dismutase (SOD), and Catalase (CAT) have been reported to act as inhibitors of oxidative processes by facilitating the conversion of hydrogen peroxides, superoxide anions, and hydroperoxides into non-toxic molecules. As a result, the build-up of secondary peroxidation products is forestalled [47]. The outstanding increment in the POD, SOD and CAT activities that were detected on day 28 for beef samples treated with 10% *X. aethiopica*, suggests an improvement in the functional role of the endogenous antioxidant enzyme system in the beef to decompose hydrogen peroxide and superoxide radicals formed during lipid peroxidation. Furthermore, the trend in CAT and SOD values is consistent with previous research by Kim and Lee [48]. According to the authors, proven antioxidant compounds, catechin and pcynogenol enhanced the CAT and SOD activities of ground beef samples subjected to refrigerated storage for 10 days. Interestingly, catechin has been reported to be the most prevalent antioxidant compound in *X. aethiopica* [25] and *X. aethiopica* possessed higher concentrations of phenolic compounds than *P. guineense* and *R. beninensis* spices [24]. Plausibly, the reported phenolic components present in the spice is a contributor to the sustained CAT and SOD activities observed in the *X. aethiopica*-treated beef samples. However, the gradual reductions in the POD, SOD and CAT activities seen for beef samples without antioxidant treatment (control) is in line with previous studies [32, 49] where muscle tissues devoid of antioxidants and kept under frozen storage, showed reducing SOD and CAT activities in the tissues as storage progresses. Zheng et al. [32] reported that the activities of SOD in muscle tissues of three Duck breeds subjected to freezing for 5 days recorded a downward trend in values. Correspondingly, El-Zeftawy et al. [50] reported that the activities of the said antioxidant enzymes in the muscle tissues of farmed fish at the end of a two-day frozen storage was considerably reduced. The findings of these studies further confirms the deleterious outcomes of freezing meat and meat products in the absence of antioxidants. Moreover, this study reveals that among the antioxidant enzymes, CAT recorded the lowest activity in beef at the end of the storage period. Thus, it is reasonable to state that; Catalase being a heme-containing enzyme, may have undergone oxidative degradation into carbonyls consequent upon its utilization during the protection of cells from post-slaughter autooxidation [51, 52].

Conclusion

The present study investigated the practicability of employing *R. beninensis, X. aethiopica and P. guineense* spices as viable replacements for synthetic antioxidants in the protection of meat against toxicity occasioned by frozen storage. Furthermore, it also affirms that frozen storage alone does not prevent the oxidative deterioration of beef. The spice-treated beef samples exhibited robust antioxidant features that were comparable to those of the reference antioxidant. Therefore, the spices are sustainable alternatives to the synthetic antioxidant, butylated hydroxytoluene. These findings have validated the pharmaceutical and preservative efficacies of these spices presented in our previous studies and those of other scientific reports. It would also aid meat industries in the provision of supplementary preservative strategies that are capable of averting the detrimental impacts of cold storage on meat and meat products devoid of antioxidants while improving consumer safety. Further investigation is necessary, nevertheless, to better understand the influence of the spices on the water-holding capacity of meats in the course of frozen storage and to determine the relationship between various chemical compositions that may represent distinct locations of plants employed in food systems.

Abbreviations

BHT	Butylated Hydroxytoluene
FFA	Free Fatty Acids
SOD	Superoxide Dismutase
POD	Peroxidase
CAT	Catalase
AV	Acid Value

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
All authors contributed to the study's conception and design. UFE: Conceptualization, Investigation, Resources, Writing – original draft. NPO: Conceptualization, Methodology, Supervision. AA: Investigation, Formal analysis, Validation, Writing – review & editing. PME: Formal analysis & Validation. EDK: Validation, Writing – review & editing.

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Data availability
The authors are willing to supply the datasets utilized and/or analyzed in the current work upon reasonable request.

Declarations

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The authors declare no competing interests.

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