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Antioxidant activities of developed natural smoothies and their effects on blood glucose levels and expression of pro-inflammatory genes in streptozotocin-induced diabetic rats

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Abstract

Fruits and vegetables are laden with antioxidants and have been used to quench reactive oxygen species. The combination of two or more fruits and vegetables to produce smoothies is expected to yield a synergistic effect that could further improve functionality. This study investigated the antioxidative and blood glucose-lowering potentials of smoothies made from selected fruits and vegetables as well as their effect on the expression of pro-inflammatory genes in streptozotocin-induced diabetic Wistar rats. Standard procedures were used for the *in vitro* antioxidant determination while pancreas tissue was excised for molecular study. The inclusion of beetroot increased the total phenolic contents by 30-50% while the flavonoid content was increased by more than 3-fold. Similarly, smoothies with beetroot and bitter gourd exhibited stronger free radical scavenging abilities as shown by DPPH (1, 1- diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid), Nitric oxide (NO) scavenging ability, metal chelation (Fe^{2+}) and ferric reducing antioxidant properties (FRAP) compared to the sample devoid of the duo. The blood glucose levels of the rats fed the smoothie were significantly lowered with a similar trend in the animals that were administered metformin. The smoothies significantly ($p<0.05$) reduced the malondialdehyde (MDA) content thus reducing lipid peroxidation in the animals. The endogenous antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the pancreas were all significantly ($p<0.05$) increased when the animals were fed smoothies. The expression of pro-inflammatory genes, IL-1β, IL-6 and TNF-α, was down-regulated in the animals fed with smoothies. The findings showed that the smoothies made from the combination have the potential for managing type-2 diabetes mellitus.

Keywords Smoothies, Type-2 diabetes mellitus, Genetic expression, Antioxidant properties, Endogenous antioxidant enzymes

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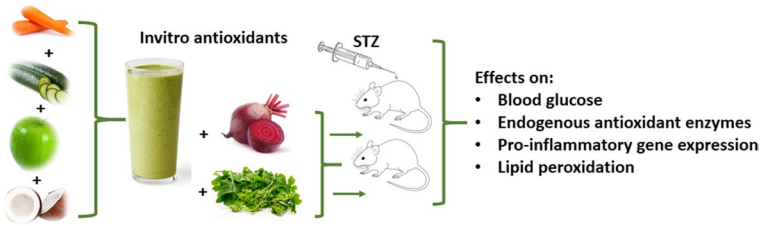
Graphical Abstract



Introduction

In recent decades, there has been increased interest in nutrition, fitness, and beauty, leading to hightened concerns regarding human diet and health. Consequently, there has been a growing emphasis on the positive aspects of diet. Epidemiological surveys have consistently reported that fruits are naturally rich sources of antioxidants, which can help to reduce the incidence of degenerative diseases such as ageing, arteriosclerosis, arthritis, cancer, heart disease, and inflammation (Harasym and Oledzki 2014). Additionally, each daily serving of fruit can reduce mortality risks and improve health and well-being (Gehlich et al. 2020). Whether consumed in whole form, as part of a salad, or in mixes (smoothies), fruit and vegetable intake is critical for a well-balanced diet.

Smoothies are a type of semi-liquid product primarily based on fruit and/or vegetable and possesses a smooth consistency and nutritional qualities essential for the human body. Smoothies are low in energy but rich sources of bioactive compounds (Castillejo et al. 2016; Formica-Oliveira et al. 2017). Consumption may serve as an effective means of increasing the number of fruits and vegetables in one's diet. Additionally, smoothies offer a way to extend the storage stability of highly perishable fruits and vegetables while providing improved aesthetic qualities as an added benefit (Castillejo et al. 2016). The storage stability of fruit smoothies remains a significant challenge due to their susceptibility to spoilage. However, several natural antimicrobials like nisin, natamycin and green tea extract have been used to enhance the storage stability (Nieva et al. 2022).

Bitter gourd (*Momordica charantia* L.) is native to India, Malaysia and tropical Africa. The different parts of the plant are commonly consumed as food, and vegetables for medicinal purposes. The leaf and fruits are used as a preventive measure or in the management of diabetes, hypertension and liver-related diseases (Saeed et al. 2018). It has been used in combination with other plant materials to produce beverages with enhanced antioxidant properties (Badejo et al. 2020). The leaf and powder have been reported to lower blood sugar in diabetic rats (Virdi et al. 2003).

Beetroot (*Beta vulgaris L.*) is a root vegetable from the Chenopodiaceae family that is laden with betalains, nitrates, phenolic acids, and flavonoids. It is used as a food additive and for medicinal purposes because of its analgesic, hepatoprotective, antioxidant, antimicrobial, and anti-inflammatory properties (Chen et al. 2021). The betalain it contains is effective in scavenging radicals and preventing DNA damage while the nitrate is the main component for lowering blood lipids, glucose, and pressure. Aliahmadi et al. (2021) reported a positive impact on cognitive function, glucose metabolism and other metabolic markers when diabetic patients were fed raw red beetroot for eight weeks.

The study of the relationship between antioxidants and the prevention of non-communicable diseases, such as cardiovascular disease, cancer and diabetes has continued to increase with free radicals suggested to be playing key roles (Kubola & Siriamornpun 2008). Fruits and vegetables that are laden with antioxidants can protect against many of these diseases, including diabetes and cardiovascular diseases by reducing oxidative stress and inhibiting macromolecular oxidation (Senevirathne et al. 2006).

It has been estimated that by 2045 over 700 million people will be living the diabetes (IDF 2019). This non-communicable disease can contribute to the increase of other diseases such as blindness, cardiovascular disease, and cancer, thus greatly impacting public health globally. The cost of medication is exorbitant and the attendant side effect of synthetic drugs is a drawback to consumers and people suffering from type 2 diabetes. Several studies have shown that fruits and vegetables have an impact on type-2 diabetes (Bazzano et al. 2008; Halvorsen et al. 2021). However, many published reports on fruit and vegetable intake and type 2 diabetes risk have not been consistent because most have only analysed a few specific fruit and vegetable subtypes (Bazzano et al. 2008;

Bondonno et al. 2021). Vegetables alone have been found to pose a lower risk of post-transplantation diabetes in patients with renal transplants (Gomes-Neto et al. 2019).

This study is aimed at producing smoothies from apple (*Malus* spp), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), beetroot (*Beta vulgaris*) and coconut (*Cocos nucifera*) milk with the inclusion of bitter gourd (*Momordica charantia*) leaf powder to be used as a food-based intervention in the management of non-communicable diseases such as type-2 diabetes.

Materials and methods

Source of materials used

Mature apple (*Malus domestica*), cucumber (*Cucumis sativus*), carrot (*Daucus carota*), beetroot and local coconut *(Cocos nucifera)* were purchased from the local stores around the Federal University of Akure, Nigeria. At the same time, the bitter gourd (*Momordica charantia*) was harvested from the Teaching and Research Farm of the University.

Source of chemicals used

6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), phosphate buffer, 2,4,6-tri(2-pyridyl)-striazine (TPTZ), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), 2,2′-azinobis(3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Sodium dodecyl sulphate (SDS) potassium ferrocyanide, streptozotocin, and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (USA) and were of analytical grade.

Preparation of bitter gourd powder

Freshly harvested *Momordica charantia* leaves were sorted; thoroughly washed under running potable water and drained. The leaves were then dried in a Genlab drying cabinet (Model DC 125) at 40°C for 48 h. Kenwood Blender (Model BL335) was used to mill the dried leaves into fine powder.

Coconut extracts production

The coconut shells were carefully broken to prevent coconut water spillage and the water was collected in a clean container and kept safely for further use. The endosperm was collected, and washed and a total of 100 g was weighed, grated and blended with the addition of 100 ml of coconut water. A white, clean muslin cloth was used for the extraction.

Beetroot juice production

The beetroots were sorted and washed under running potable water. They were peeled and 75 g weighed and grated. A very clean muslin cloth was used for the juice extraction.

Production of smoothies

The smoothies were produced using the method of Nieva et al. (2022) with slight modifications. The carrot, cucumber and apple were washed with potable water and scraped (where applicable).

Two hundred grams of each were weighed and blended using Kenwood BL335 with the addition of 100 ml of coconut extract (coconut milk) until a homogeneous smooth, semi-viscous slurry was achieved. This was used as the base mixture. The beetroot extract and bitter gourd leaves powder were combined in different proportions as shown in Table 1. The smoothies were prepared freshly for all the analyses. They were diluted appropriately where necessary to reduce the interference of the colour from the extracts for antioxidant determination.

Determination of total phenolic contents

The determination of the total phenol content of the extract was conducted according to the method of Singleton et al. (1999). Briefly, 0.2 ml of the smoothie was combined with 2.5 ml of 10% Folin Ciocalteau's reagent and 2 ml of 7.5% Sodium carbonate and incubated at 45°C for 40 min. The absorbance was measured at 700 nm in a spectrophotometer with Gallic acid used as a standard. The results are expressed as mg gallic acid equivalent (GAE) /ml of sample.

Determination of total flavonoid contents

The smoothie's entire flavonoid composition was quantified using the colorimetric assay (Bao et al. 2005). Briefly, 0.2 ml of the extract was introduced into 0.3 ml of 5% $NaNO_3$, after 5 min, 0.6 ml of 10% $AlCl_3$ was included, followed by 2 ml of 1M NaOH and 2.1 ml of distilled

Table 1 The mixing ratio of the smoothies prepared in this study

Sample	Bas (%)	Beetroot extract (%)	Bitter gourd powder (%)
Bas	100	Nil	Nil
Bas_BR	98	2	Nil
Bas_BG	98	Nil	2
Bas_BR_BG	98	1	1

Where

Bas: Smoothie mixture of carrot, cucumber, apple, and coconut extract (coconut milk)

Bas_BR: Bas plus Beetroot

Bas_BG: Bas plus Bitter gourd powder

Bas_BR_BG: Bas plus Beetroot plus Bitter gourd powder

Nil: Nothing added

water. The absorbance was measured at 510 nm, and the flavonoid content was measured as milligrams of rutin equivalent per ml.

Determination of ferric-reducing property

The determination of the reducing property of the smoothie was carried out as described by Pulido et al. (2000). Briefly, 0.25 ml of the smoothie was added to equal volumes of 200mM Sodium phosphate buffer pH 6.6 and 1% Potassium ferrocyanide. The resultant mixture was then subjected to incubation at a temperature of 50°C for 20 min. Following this, 0.25 ml of 10% TCA was added, and the mixture was centrifuged. Subsequently, 1 ml of the supernatant was mixed with 1 ml of distilled water and 0.1% of $FeCl_3$. The absorbance of the resultant mixture was then measured at 700 nm.

Determination of DPPH scavenging ability

The method of Gyamfi et al. (1999) was used to determine the free radical scavenging ability of the smoothie against DPPH (1, 1-diphenyl-2-picrylhydrazyl). Briefly, a 1 ml aliquot of the smoothie was combined with a 1 ml portion of 0.4 mM methanolic solution of DPPH, and the resulting mixture was left in the dark for 30 min before measuring the absorbance at 516 nm.

$$\text{DPPH inhibition} = \frac{(Absorbance\ of\ Standard - Absorbance\ of\ Sample)}{Absorbance\ of\ Standard} \times 100$$

Determination of NO radical scavenging ability

A solution containing 5 mM sodium nitroprusside in phosphate-saline was mixed with the smoothie and incubated at 25°C for 150 min. Following this, the reaction mixture was added to Greiss reagent (0.2% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2% sulfanilamide in 5% phosphoric acid), and the absorbance at 546 nm was measured in a spectrophotometer relative to the absorbance of a standard solution of potassium nitrate treated in the same way with Greiss reagent (Jagetia and Baliga 2004).

$$\text{OH Radical Scavenging}(\%) = \frac{(Absorbance\ of\ Standard - Absorbance\ of\ Sample)}{Absorbance\ of\ Standard} \times 100$$

$$\text{NO Radical Scavenging}(\%) = \frac{(Absorbance\ of\ Standard - Absorbance\ of\ Sample)}{Absorbance\ of\ Standard} \times 100$$

Determination Fe^{2+} chelation

The smoothie's capacity to chelate Fe^{2+} was evaluated using a modified method of Puntel et al (2005). A reaction mixture composed of 168 ml of 0.1M Tris-HCl pH 7.4, 218 ml of saline, and the smoothie was supplemented with 150 mM $FeSO_4$. Following a 5 min incubation period, 13 ml of 1, 10-phenanthroline was introduced to the mixture, and absorbance was measured at 510 nm.

Determination of ABTS scavenging ability

The method of Re et al., (1999) was used to determine the ABTS scavenging ability. The ABTS was produced through the reaction of 7mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM/l, final concentration) in the dark for 16 h while adjusting the absorbance at 734nm to 0.700 with ethanol. Subsequently, 0.2 of the appropriate dilution of the extract was added to 2.0 ml of ABTS solution, and the absorbance was read at 732nm after 15 min. Trolox was used as standard and the Trolox equivalent antioxidant capacity was then calculated.

OH radical scavenging ability

The OH radical scavenging ability was determined using the method of Gutteridge et al. (1981). Freshly prepared smoothie (100 µl) was added to a reaction mixture comprising 120 µl, 20 mM deoxyribose, 400 µl, 0.1 M phosphate buffer (pH 7.4), 40 µl, 20 mM hydrogen peroxide, and 40 µl, 500 µM $FeSO_4$. The volume was then made up to 800 µl with distilled water and incubated at 37°C for 30 min. It was then terminated by adding 0.5 ml of 2.8% TCA and 0.4 ml of 0.6% TBA solution. The tube was subsequently incubated in a water bath at 95°C for 20 min and the absorbance was measured in a spectrophotometer at 532 nm.

Animal care and handling

Forty male Wistar rats with a weight range of 180-200g were acclimatized for 2 weeks and treated as described by Nwanna et al. (2019). The animals were kept in wire-mesh cages under a controlled light cycle (12 h light/12 h dark) at a temperature of 22 ± 2°C and placed on commercially available feed and water administered *ad libitum* during the period of acclimatization.

Inducing type-2 diabetes and measurement of blood glucose

Ethical clearance was obtained for the study. The rats were fed with a formulated basal diet and water *ad libitum* before the dietary manipulation. Following two weeks of acclimatization, the animals were allocated to two dietary regimens. Six rats were fed only the basal diet (44.4% skimmed milk, 41.6% corn flour, 10% oil and 4% mineral/primary premix), while the remaining rats were fed a high-fat diet (44.4% skimmed milk, 21.6% corn flour, 30% lard and 4% mineral/primary premix) for an initial period of 2 weeks. After 2 weeks, the rats fed with a high-fat diet were injected intraperitoneally (IP) with a low dose of streptozotocin (STZ) (35 mg/kg) for the induction of diabetes. The diabetic state was checked after 72 hours. Blood samples were collected by tail vein puncture, and glucose level was monitored using an auto-analyzer (Accucheck glucometer ROCHE Germany). The animals with baseline blood glucose levels ≥ 200 mg/dl after 72 hours were considered diabetic and were used for the study. Non-diabetic animals received 1 ml of 0.1M citrate buffer intraperitoneally. The diabetic rats were divided into six groups randomly and further kept on a diet of smoothie supplementation (as a percentage of total weight), for 21 days. During the experiment, fasting blood glucose was monitored at three- to four-day intervals. At the end of the experiment, the animals were sacrificed after an overnight fast. The rats were decapitated under a light diethyl ether anaesthetic, and the organs were swiftly separated, chilled, and weighed.

Animal groupings

The animals were randomly divided into six (6) groups of six (6) animals each.

Group 1 - Control + Basal: Normal rats (control) received citrate buffer (pH 4.5) (1 ml/kg, IP) and were fed with basal diet;
Group 2 - Diabetic + Basal: diabetic rats fed with basal diet.
Group 3 - Diabetic + Met: diabetic rats that received 100mg/kg IP metformin orally and fed with basal diet;
Group 4 - Diabetic + Bas: diabetic rats fed with a diet supplemented with 100% Smoothie base;
Group 5 - Diabetic + Bas_BR: diabetic rats fed with a diet supplemented with 98% smoothie base with 2% beetroots.
Group 6 - Diabetic + Bas_BR_BG: diabetic rats fed with a diet supplemented with 98%, 1% beetroots and 1% bitter gourd).

All animals remained on the assigned diet throughout the twenty-one days of the study.

Determination of protein content

Protein content in the tissues used was determined using the method of Lowry et al. (1951). Briefly, to 1 ml of the test solution, 5mL of solution of Copper reagent (0.5 volume 1% (w/v) copper sulphate, 0.5 volume 2% (w/v) sodium potassium tartrate and 50 volume 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide) was added and mixed thoroughly by vortexing and stand at room temperature for 10 min. To the mixture, 0.5 ml of solution of Folin-Ciocalteau reagent diluted to 1 M acid, was added mixed rapidly, and incubated for 30 min at room temperature. The absorbance was measured in a spectrophotometer at 600 nm against a reagent blank not containing protein. The concentration was estimated against a standard curve obtained using a known concentration of bovine serum albumin (BSA).

Determination of Lipid Peroxidation

The lipid peroxidation assay was determined in the tissue according to the modified method of Ohkawa et al. (1979). Briefly, a mixture containing 300 µl of tissue homogenate, 300 µl of 8.1% SDS (Sodium dodecyl sulphate), 500 µl of acetic acid/HCl (pH, 3.4) and TBA (thiobarbituric acid) was incubated at 100°C for 1 h. After that, the thiobarbituric acid reactive species (TBARS) produced was measured at 532 nm and subsequently calculated as malondialdehyde (MDA) equivalent and expressed as mmol MDA per mg protein.

Determination of Glutathione Peroxidase (GPx) activity

The activity of GPx was quantified as described by Godin et al. (1988). Specifically, a solution consisting of 0.2 ml of 0.4M phosphate buffer (pH7.0), 0.1 ml of 10mM sodium azide, 2 ml of plasma homogenate, 0.2 ml of 10 mM glutathione, and 0.1 ml of 0.2 mM hydrogen peroxide was prepared. Following a 10 min incubation period, 0.4 ml

of 10% TCA was introduced to terminate the reaction. The resulting mixture was subjected to centrifugation at 3,200×g for 2 min, and the supernatant was carefully analyzed for glutathione content utilizing ELLMAN'S reagent (consisting of 19.8 mg of DTNB in 100 ml of 0.1% sodium nitrate). Ultimately, the activity of GPx was expressed in terms of mg of GSH consumed per minute per gram of protein.

Determination of Catalase (CAT) activity

The reaction mixture was comprised of 50 mM potassium phosphate buffer with a pH of 7, 10 mM H_2O_2, and 20 ml of the supernatant. The evolution rate of H_2O_2 was observed at 240 nm over a period of 2 min at room temperature. Enzymatic activity was quantified in units per mg of protein, whereby one unit of the enzyme is defined as the quantity of catalase that decomposes one millimole of H_2O_2 per minute at 25°C and a pH of 7 (Fridovich 1989).

Determination of Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was performed following the method of Fridovich (1989). Briefly, epinephrine was subjected to auto-oxidation under conditions of pH 10.2, resulting in the formation of adrenochrome, a pigmented species that was subsequently quantified at a wavelength of 480 nm. The introduction of samples (10, 20, 30 µl) into a cuvette containing 1 ml of 50 mM glycine buffer at pH 10.5 was followed by the addition of 17 µl of 60 mM adrenaline. The degree of inhibition was then monitored over 180 s at 480 nm.

Expression of diabetes-related genes

The RNA was extracted using Bio-Rad RNA extraction kit according to the manufacturer's instructions. Total RNA isolated from the pancreas was treated with RNAase-free DNase to remove any contaminating DNA in the sample completely. The concentration of the RNA yield was determined using a Nanodrop and the sample was stored at -80°C. The RNA obtained was then used for cDNA synthesis using BioRad cDNA synthesis kit according to the manufacturer's instructions. The polymerase chain reaction (PCR) of the genes related to diabetes was carried out in a total volume of 20 µl containing 2 µM of each primer, 50 ng of cDNA and 10 µl of SYBR Premix ExTag. The mixture was subjected to cycling conditions of initial denaturation for 5min at 95°C, 35 cycles of 95°C for 30 s, 55-58°C for 30 s and 72°C for 30 s. GAPDH was used as the housekeeping gene and the relative transcript was calculated. Table 2 shows the primer design used in the study.

Table 2 The primer design for the pro-inflammatory genes expression used in this study

Gene	Forward	Reverse
IL-1β	5′-GCAATGGTCGGGACATAGTT-3′	5′-AGACCTGACTTGGCAGAGGA-3′
IL-6	5′-TCTCTCCGCAAGAGACTTCCA-3′	5′-ATACTGGTCTGTTGTGGGTGG-3′
TNF-α	5′-ACCACGCTCTTCTGTCTACTG-3′	5′-CTTGGTGGTTTGCTACGAC-3′
GAPDH	5′-AGACAGCCGCATCTTCTTGT-3′	5′-CTTGGTGGTTTGCTACGAC-3′

IL-1β Interleukin-1β, *IL-6* Interleukin-6, *TNF-α* Tumor Necrosis Factor- α, *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase

Data analysis

All parameters were analyzed in triplicates unless otherwise stated. The data sets of the *in-vivo* parameters were subjected to the Anderson-Darling test using Minitab Software 19.0 (Minitab Inc., USA). After checking the normality (p>0.05) of the data distribution, the biochemical parameters of the studied groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range post hoc test, with the Statistical Package for the Social Sciences (SPSS) version 17.0 software (SPSS Inc., Chicago, IL, USA). P-value<0.05 was considered statistically significant. Pearson correlation was performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and was used to determine the relationship between phenolic content, flavonoid content and antioxidant capacities.

Results and discussion

Total phenolic and flavonoid contents of the smoothies

The total phenolic content of the base smoothie was 10.1 mg GAE/ml. With the addition of beetroot and /or bitter gourd leaf powder, the total phenolic content increased to a maximum of 15 mg GAE/ml (Fig. 1A). The significant (p<0.05) increase may be a result of the addition of beetroot juice. Beetroot is very high in phenolic contents with values ranging from 50-60 µmol/g fresh weight and has hydroxybenzoic and hydroxycinnamic acid derivatives as the major phenolic acids (Chhikara et al. 2019). Although only 2% of the juice was added to the Base smoothie, it was able to achieve over 50% increase in the total phenolic contents (Fig 1A). With the addition of 2% bitter gourd leaf powder, the total phenolic content also increased significantly. The total phenolic content of the bitter gourd plant has been reported to range from 224 to 474 mg GAE/100g dry sample with the leaf having the highest value and gallic acid and caffeic acid being predominant (Kubola and Siriamornpun 2008).

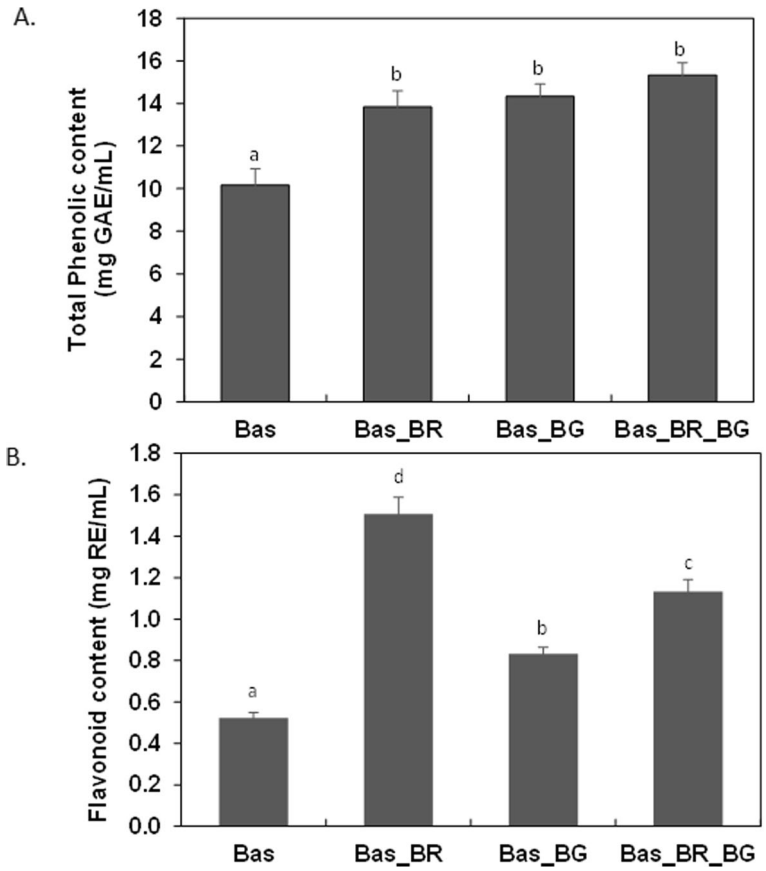


Fig. 1 The total phenolic (**A**) and flavonoid (**B**) contents of the smoothies. Values are Mean ± SD (n≥3). Bars with different alphabets are significantly different ($p<0.05$). Bas is the combination of all the fruits and vegetables mix. Bas_BR: Bas plus Beetroot. Bas_BG: Bas plus Bitter gourd powder. Bas_BR_BG: Bas plus Beetroot plus Bitter gourd powder

Phenolic compounds are secondary metabolites found in plant-based foods with diverse biological properties including antioxidant, anti-inflammatory, anticancer, and cardioprotective effects (Sadef et al. 2022), thus the total phenolic content is key to evaluating the health benefits inherent in the smoothies.

Phenolics, in particular flavonoids, are often directly linked to antioxidant activity. The flavonoids in plants are biologically active compounds that possess good antioxidants and are laden with numerous health benefits. The flavonoid contents of the smoothies ranged from 0.52 mg RE/ml to 1.1 mg RE/ml (Fig 1B). Like the phenolic content, the flavonoid contents of the smoothies also increased significantly ($p<0.05$) with the addition of beetroot and bitter gourd. The presence of low-molecular antioxidants has been reported to protect cells and their structures against oxidative damage (Harasym and Oledzki 2014). Pearson correlation showed the correlation between phenolic, flavonoids and antioxidant activities.

Antioxidant properties of the smoothies

Antioxidants in foods and beverages are powerful supplements that can reduce oxidative stress, by quenching reactive oxygen species (ROS) (Badejo et al. 2020). The scavenging activities of DPPH· and ·$ABTS^{+}$ were determined by measuring the decrease in the absorbance caused by the addition of the smoothie samples which serves as the antioxidant. The DPPH scavenging ability of the smoothies was highest in the sample with base and beetroot only with a value of 15% while the lowest was in the sample containing only the base (5%) (Fig 2A). The 3-fold increase in the DPPH showed that the beetroot contributed significantly to the radical scavenging ability of the smoothies.

The ABTS scavenging activities followed a similar trend to the DPPH. The lowest value (0.5 mMol TE/ml) (Fig 2B) was recorded in the base sample while the sample with the beetroot had over 100% increase in the ABTS scavenging ability. The presence of bitter gourd

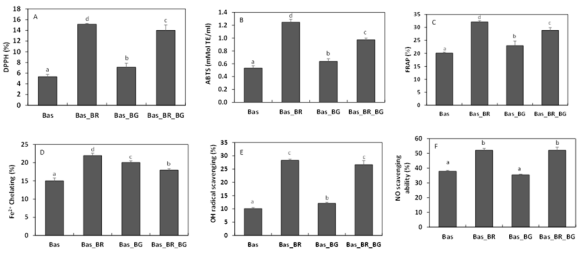


Fig. 2 The *in vitro* antioxidant capacities of the smoothies as expressed by DPPH (**A**), ABTS (B), FRAP (**C**), Metal Chelation (**D**), OH radical scavenging (**E**) and NO radical scavenging (**F**). Values are Mean ± SD (n≥3). Bars with different alphabets are significantly different ($p<0.05$). Bas is the combination of all the fruits and vegetables mix. Bas_BR: Bas plus Beetroot. Bas_BG: Bas plus Bitter gourd powder. Bas_BR_BG: Bas plus Beetroot plus Bitter gourd powder

also contributed to increasing the ABTS scavenging ability. A similar finding was reported when bitter gourd was included in tigernut beverage and antioxidant scavenging properties increased significantly (Badejo et al. 2020).

The ferric-reducing antioxidant power ranged from 20.1 to 32.1 % in all the smoothie blends. The Base sample had the lowest FRAP value (Fig 2C) while the inclusion of beetroot and bitter gourd raised the FRAP value significantly by up to 70%. In this assay, the smoothie samples were able to reduce the ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex to a ferrous form with an intense blue colour.

ROS are generated as by-products of metabolic reactions and an outcome during mitochondrial electron transport within the system. Transition metal (Fe^{2+}) ion possesses the ability to perpetuate the formation of free radicals by gain or loss of electrons. To reduce the formation of ROS within the system, there is a need for chelation of the metal ions with chelating agents. The smoothies produced showed that they have metal chelation ability with Fe^{2+} chelation ability of 15 to 22 % (Fig 2D) with the sample having beetroot showing the highest chelation ability (Fig. 2D).

Hydroxyl radicals (.OH) are the major active oxygen species causing oxidation of polyunsaturated fatty acid in food and oxidative damage to cells because they unspecifically attack biomolecules resulting in cellular disorders such as neurodegeneration and cardiovascular diseases (Lipinski and Pretorius 2012). Hydroxyl radical scavenging activity assay is a widely used method to determine antioxidant activity in fruits and vegetables. The smoothies had OH^- radical scavenging activity ranging from 20 to 35% with the Base sample having the lowest value while the sample with 2% beetroot inclusion had the highest value of 35% (Fig 2E).

The NO scavenging ability of the smoothies increased by 40% when beetroot was added to the base sample (Fig 2F). There was no significant ($p>0.05$) difference between the base sample and the one with bitter gourd leaf powder. Also, there was no significant ($p>0.05$) difference between the sample with only beetroot and the one with both beetroot and bitter gourd added (Fig 2F).

Pearson correlation (Supplementary Table 1) showed that all the antioxidant properties were positively correlated with the flavonoid content with R^2 values 0.80, 0.83, 0.92, 0.94, 0.96 and 0.98 for Fe^{2+}, NO, OH, DPPH, FRAP and ABTS, respectively.

The effect of the smoothies on blood glucose levels in diabetic rats

The blood glucose of the animals induced with streptozotocin was significantly higher than the control animals that were not induced. The sudden spike may be attributed to the possible decrease in the beta cell mass of the pancreas occasioned by the impact of the streptozotocin. The cytotoxic effect of streptozotocin on beta cells has been reported by Elekofehinti et al. (2023). It induces severe and irreversible hyperglycemia in experimental animals. The administration of metformin and the developed smoothies significantly ($p<0.05$) reduced the blood glucose level at the end of 21 days of treatment (Fig 3).

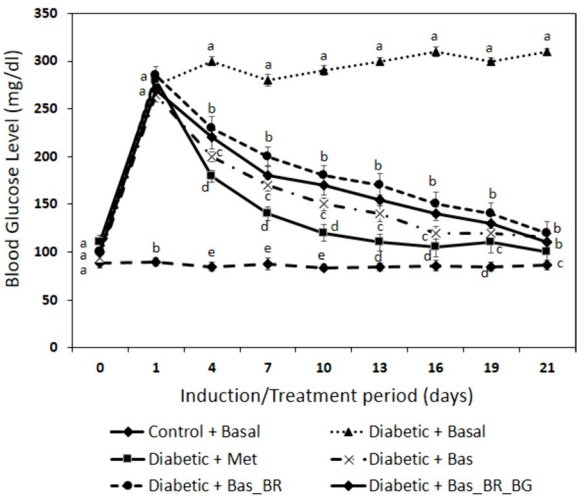


Fig. 3 Blood glucose lowering potential of the smoothies. Values are Mean ± SD ($n=6$). Values in each day across the treatment having different alphabets are significantly different ($p<0.05$). Control + Basal: Control animal fed basal diet. Diabetic + Basal: Diabetic animal fed Basal diet. Diabetic + Met: Diabetic animal treated with Metformin drug. Diabetic + Bas: Diabetic animal fed Bas (combination of all the fruits and vegetables mix).Diabetic + Bas_BR: Diabetic animal fed Bas plus Beetroot mix. Diabetic + Bas_BR_BG: Diabetic animal fed Bas plus Beetroot plus Bitter gourd powder mix

Some plant extracts have been reported to exert blood glucose-lowering effects by preventing the death of β cells and/or permitting the recovery of partially destroyed β cells (Prasad et al. 2009). The blood glucose-lowering potential observed in the current study may be due to the antioxidative properties of the smoothies.

The effect of the smoothies on lipid peroxidation

Lipid peroxidation is the reaction between oxidants such as free radicals and lipids containing carbon-carbon double bond(s), mostly polyunsaturated fatty acids (PUFAs) leading to the formation of many different aldehydes as secondary products such as malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE). MDA is an indicator of lipid peroxidation, and its level is related to the extent of damage in lipid membranes as a result of free radical accumulation (Gilani et al. 2021). Excessive MDA production has been associated with different pathological states (Garcia et al. 2013). Rat induced with STZ showed a spike in the MDA level, which indicates that diabetes damages the lipid membranes. The administration of Metformin drug significantly (p<0.05) lowered the MDA level. Similarly, when the animals were fed with the smoothies, the MDA level decreased significantly (p<0.05) (Fig 4). This can be due to decreased adiposity and this decrease in adipose tissue mass can lower lipid peroxidation by reducing ROS production (Vuolo et al. 2020). Stress conditions can induce excessive production of ROS which mostly correlates with the production of MDA. The antioxidant capacities of the smoothies are very potent in scavenging ROS and may have been critical to the observed decline in MDA. The smoothie in the current study was able to significantly decrease the MDA level and thus can serve as a functional beverage.

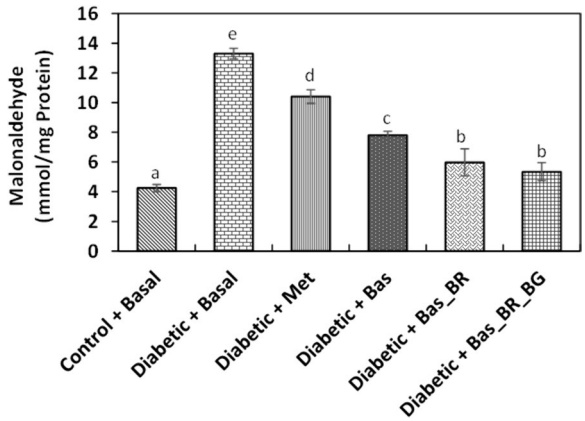


Fig. 4 Effect of the smoothies on oxidative stress depicted by malondialdehyde in STZ-induced rats. Values are Mean ± SD (n=6). Bars with different alphabets are significantly different (p<0.05). Control + Basal: Control animal fed basal diet. Diabetic + Basal: Diabetic animal fed Basal diet. Diabetic + Met: Diabetic animal treated with Metformin drug. Diabetic + Bas: Diabetic animal fed Bas (combination of all the fruits and vegetables mix). Diabetic + Bas_BR: Diabetic animal fed Bas plus Beetroot mix. Diabetic + Bas_BR_BG: Diabetic animal fed Bas plus Beetroot plus Bitter gourd powder mix

Effect of the smoothies on endogenous antioxidants

The mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes are the primary sources of endogenous ROS production within the cell. However, there are several other exogenous stimuli, such as ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicides/insecticides (Moldovan and Moldovan 2004). Overproduction of ROS has been implicated in the development of various chronic and degenerative diseases. Fruits and vegetables laden with antioxidants have been used to regulate the concentration of ROS in order to ensure redox balance (Liu et al. 2018).

The endogenous SOD activity in the STZ-induced animals fed with the smoothies was significantly (p<0.05) higher (5.5-8.4 mmol/min/mg protein) than that in the diabetic animals feeding on basal diet (3.7 mmol/min/mg protein) (Fig 5A). There was no significant (p>0.05) difference between the SOD activity of the control animal feeding on a normal diet and diabetic animals fed with the base sample of the smoothies. An increase in the SOD activity resulting from feeding on the smoothies is an indication of a decrease in intracellular ROS levels in an attempt to restore redox balance (Gupta and Chari 2006). Superoxide radicals can be dismutated into H_2O_2 by SOD in the mitochondria and the cytoplasm. H_2O_2 can diffuse across the cell membrane and it can be very toxic. There is therefore the need to get rid of it as fast as possible and the developed smoothies is showing the potential to do it effectively.

Catalase plays a vital role in defending aerobic cells against the toxic effects of superoxide radicals and acts as an antioxidant enzyme, decomposing hydroxyl radicals. It is the main enzyme that eliminates H_2O_2 in the system by reducing it to H_2O as part of the defense system against oxidative injury. The CAT activity in the STZ-induced animal was the lowest (0.012 Units/mg protein) (Fig 5B). The increased production of ROS by STZ could be the source of the decrease in CAT activity. When the animals were administered Metformin, there was a significant (p<0.05) increase of 3-fold in the CAT activity. Similarly, STZ-induced animals fed with the smoothies also showed a significantly high CAT activity (Fig 5B). CAT activity has been reported to follow a similar trend in STZ-induced diabetic rats administered

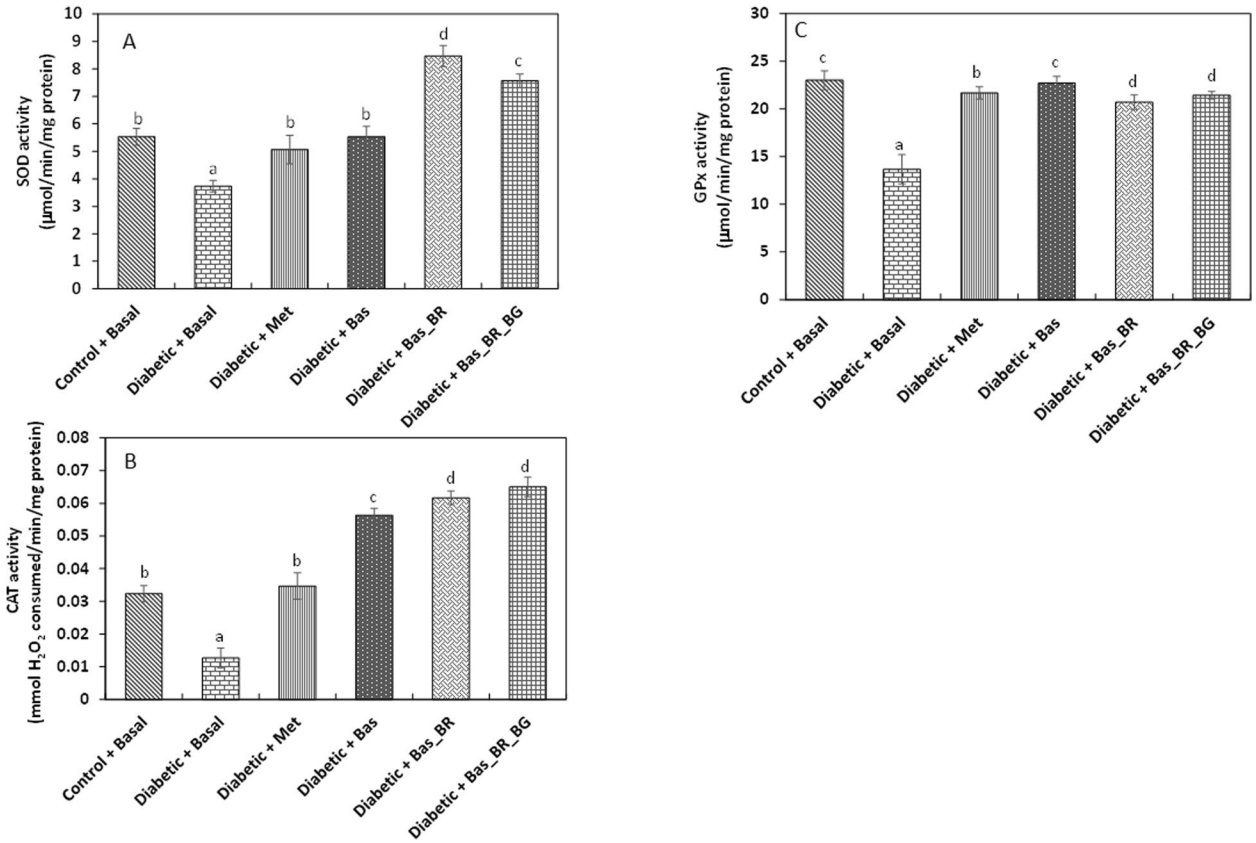


Fig. 5 Effect of the smoothies on endogenous antioxidant enzymes; SOD (**A**), CAT (**B**) and GPx (b). Values are Mean ± SD (n=6). Bars with different alphabets are significantly different (p<0.05). Control + Basal: Control animal fed basal diet. Diabetic +Basal: Diabetic animal fed Basal diet. Diabetic + Met: Diabetic animal treated with Metformin drug. Diabetic + Bas: Diabetic animal fed Bas (combination of all the fruits and vegetables mix). Diabetic + Bas_BR: Diabetic animal fed Bas plus Beetroot mix. Diabetic + Bas_BR_BG: Diabetic animal fed Bas plus Beetroot plus Bitter gourd powder mix

fustin a compound derived from traditional herbal plant *Rhus verniciflua* (Gilani et al. 2021). As the smoothies are laden with antioxidative potentials, they may be very effective in scavenging reactive species or by enhancing endogenous antioxidant activity.

GPX is widely distributed in mammalian tissues and can be found in the cytosol, nuclei and mitochondria. They are very vital in catalysing the reduction of H_2O_2 to water using glutathione (GSH) as a reductant. The GPX activity was significantly (p<0.05) low in STZ-induced animals (13.67 mmol/min/mg protein) as a result of the stress created by diabetes (Fig 5C). Upon the administration of metformin, the GPX activity increased significantly (p<0.05) to 21.67 mmol/min/mg protein (Fig. 5C). A similar trend was observed with the animals fed the smoothie samples. The increase in the GPX activity in diabetic animals administered smoothies shows the ability to scavenge free radicals as GPX will act against the accumulation H_2O_2 within the cells and protect them from damage and dysfunction (Gupta and Chari 2006). A low level of GPX could lead to dysfunction and failure of β-cells (Benáková et al. 2021). Antioxidant systems including CAT and GPX have been very effective in scavenging free radicals.

Effect of the smoothies on pro-inflammatory genes

Chronic hyperglycemia can directly promote an inflammatory state in which cytokines can lead to the destruction of the pancreatic beta cells resulting in diabetes. Proinflammatory cytokines including interleukin (IL-)1β, IL-6, and tumor necrosis factor (TNF-)α can significantly relate to the pathogenesis of diabetes mellitus (Navarro et al. 2007).

Interleukin 1β (IL-1β), a regulator of the body's inflammatory response plays a role in various diseases, associated with metabolic syndrome such as atherosclerosis, chronic heart failure and type 2 diabetes (Maedler et al. 2009). A relatively high expression of IL-1β in the pancreas of diabetic rats (Fig 6A) could indicate an inflammatory response which could affect beta cell function and survival, and contribute to the development of diabetic retinopathy. A low expression

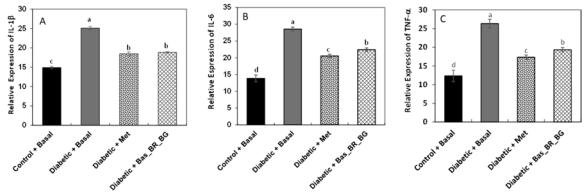


Fig. 6 The relative gene expression of IL-1β (**A**), IL-6 (**B**) and TNF-*α* (**C**). Values are Mean ± SD (*n*=6). Bars with different alphabets are significantly different ($p<0.05$). Control + Basal: Control animal fed basal diet. Diabetic + Basal: Diabetic animal fed Basal diet. Diabetic + Met: Diabetic animal treated with Metformin drug. Diabetic + Bas_BR_BG: Diabetic animal fed Bas plus Beetroot plus Bitter gourd powder mix

of IL-1β in diabetic rats (as seen in the control group) could indicate improved beta cell function and glucose metabolism, improved glycemic control (as seen with the lower blood glucose level in Fig 3), and reduced inflammation.

Interleukin 6 (IL-6) is a multifunctional cytokine that has been implicated in the pathophysiology of type 2 diabetes. At high expression levels, IL-6 is an independent predictor of type 2 diabetes and is involved in the development of inflammation, insulin resistance and β-cell dysfunction (Akbari and Hassan-Zadeh 2018). A low expression could indicate a favourable social environment, reduced acute phase response and inflammation, potential effects on glucose and lipid metabolism, and reduced production of acute phase proteins and neutrophils.

Tumor necrosis factor-alpha (TNF-*α*) is a potent pro-inflammatory cytokine and immunomodulator produced primarily by adipocytes in a variety of metabolic disorders such as diabetes mellitus. In humans, serum concentration of TNF- *α* is elevated in type 2 diabetes mellitus and has been used to predict glycemic control in obese diabetic patients (Alzamil 2020). The expression of TNF- *α* was significantly higher in diabetic rats compared to the control. The administration of the smoothie to the diabetic rat significantly reduced the expression of TNF- *α* (Fig 6C). Chronic hyperglycemia induces inflammation which can result in the production of cytokines that may eventually lead to pancreatic dysfunction causing the destruction of beta-cells.

The antioxidant capacities of the smoothies may have contributed significantly to the observed changes in the expression of pro-inflammatory genes in this study. Beetroot has a protective effect in diabetic rats due to its antioxidant properties. It also has the potential to reduced fasting blood glucose (Fig 3) and increase insulin levels (Al-Harbi et al. 2021). Similarly the *M. charantia* in the smoothie may be exerting its hypoglycemic effect through preservation of islet β cells and their functions. Joseph and Jini (2013) reported that *M. charantia* can stimulate insulin secretion from the endocrine pancreas and elicit glucose uptake in the liver. This may be part of the several pathways by which the smoothie exerts its hypoglycemic effects.

Conclusion

The study showed that smoothies from the selected fruits and vegetables are effective in the management of diabetes-induced oxidative stress. The smoothies made with carrots, cucumbers, apples, coconut milk were very effective in lowering blood glucose levels in diabetic rats. With the inclusion of beetroot extract and bitter gourd leaf powder, the smoothies showed promising antioxidant potentials and a positive impact on stress biomarkers by reducing pancreatic inflammation caused by diabetes mellitus and down-regulating the expression of IL-1 β, IL-6 and TNF- *α* thus protecting the pancreas from oxidative stress. The formulated smoothies might be suitable for type-2 diabetics.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
JAM, VTO, GMO and AOA developed the product and performed the experiments; AIF: Product formulation, development and testing; AAB: Conceptualization and Draft Manuscript; EEN: Performed animal care and handling, OOE: Performed the molecular studies

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Declarations

Ethics approval and consent to participate
Ethical approval was obtained from the Ethical Committee of the School of Agriculture and
Agricultural Technology with the number FUTA/SAAT/ETH/2021/016.

Consent for publication
The consent of all the authors was sought before submitting for publication

Competing interests
The authors declare that they have no competing interests

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