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Inhibition of α-amylase, α-glucosidase and oxidative stress by some common apple varieties

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Abstract

In recent times, the consumption of apples has been encouraged for the management of chronic diseases such as diabetes, but biochemical evidence to support this practice is lacking. Therefore, this study investigated α-amylase and α-glucosidase inhibitory activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability, Fe^{2+}-induced lipid peroxidation potential as well as the total phenol and flavonoid contents of aqueous extracts of the apple varieties *Malus sylvestris* (green apple), Malus pumila (red apple) and *Syzygium samarangense* (wax apple).

The results showed that all apple varieties inhibited α-amylase (IC_{50}=12.66–16.98 μg/ml) and α-glucosidase (13.55–16.23 μg/ml) in a dose-dependent manner, with green apple showing the highest inhibitory activity while wax apple had the least. Similarly, all apple varieties showed dose-dependent DPPH radical scavenging activity (EC_{50}=222.92–278.71 μg/ml) with green apple also showing the highest scavenging activity while wax apple showed the least. Furthermore, the aqueous extracts of the apple varieties dose-dependently inhibited Fe^{2+}-induced lipid peroxidation in rat pancreas (38.60–53.57 μg/ml), with wax apple exhibiting the highest inhibitory potential. Also, the total phenol content of the apple varieties ranged from 16.14 to 17.45 mg GAE/100 g, while the flavonoid content ranged from 4.17 to 5.56 mg QUE/100 g, with green apple having the highest total phenolic and flavonoid contents. The biological activities exhibited by the apple varieties could be attributed to the presence of biologically active photochemicals. Furthermore, the apple variety (green apple) with the highest phenolic content showed the best overall activity, indicating the potent role of phenolic compounds in the management of diabetes, thereby providing biochemical support for the use of apples as a functional food in diabetes management.

Introduction

Diabetes mellitus (DM) is a chronic disease that affects millions of people worldwide. The prevalence of this disease is increasing annually, with the number of people with diabetes projected to exceed 300 million before 2025 [1]. In Nigeria, 15% of all DM deaths are attributed to cardiovascular disease [2]. There are two types of diabetes: type 1 and type 2, with type 2 being the most com-

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mon and accounting for 90% of all cases. Type 2 diabetes is a metabolic disorder resulting from the body's inability to produce enough or properly utilize insulin and is characterized by hyperglycaemia [3]. Previous research indicated that hyperglycaemia-induced vascular dysfunction may be triggered by reactive oxygen species (ROS) produced in the mitochondrial electron transport chain [4]. ROS such as the superoxide anion radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), are physiological metabolites formed as a result of respiration in aerobic organisms. ROS are very unstable and react rapidly with other substances including DNA, membrane lipids and proteins. ROS are believed to be associated with DM in humans [5].

Recent reports have suggested that inhibition of some carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase could help control postprandial hyperglycaemia [6]. Complex polysaccharides are hydrolyzed by α-amylase to oligosaccharides which are further hydrolyzed by α-glucosidase to release glucose before its absorption into the intestinal epithelium and blood circulation. Thus, α-amylase and α-glucosidase inhibitors may help reduce postprandial hyperglycaemia through inhibition of the enzymatic hydrolysis of carbohydrates.

National and international agencies recommended the consumption of fruit because of its health benefits. Studies have shown that fruit is a rich source of antioxidants such as flavonoids, carotenoids and hydroxycinnamic acids [7]. These antioxidants may help to protect the human body against functional damage caused by ROS as well as retard the absorption of glucose through inhibition of the carbohydrate-hydrolyzing enzymes α-glucosidase and α-amylase in the digestive tract [8, 9]. Apple is the common name for some trees and fruit belonging to the rose family. It is a widely cultivated tree fruit, and the best known of the genus *Malus*. Different cultivars are bred for various tastes and uses, including cooking, fresh consumption and cider production [10]. The green apple variety Granny Smith is a tip-bearing apple cultivar, which originated in Australia in 1868. It is thought to be a hybrid of *Malus sylvestris* (the European wild apple) with the domestic apple *Malus domestica* as the polleniser. Granny Smiths are green when picked but become yellow when over-ripe [11]. The red Fuji apple is an apple hybrid developed in Japan in the late 1930s. Fuji apples contain 9–11% sugars by weight and have a dense flesh that is sweeter and crisper than many other apple cultivars, making them popular with consumers around the world. Fuji apples also have a very long shelf life compared to other apples and can remain fresh for up to a year [12]. *Syzygium samarangense* (known as the wax apple, love apple, java apple, royal apple and bell fruit) is a tropical, heavy-cropping apple tree bearing up to 700 apples annually [13]. Apples contain a variety of phytochemicals, including quercetin, catechin, phloridzin and chlorogenic acid, all of which are strong antioxidants, although it has been reported that the phytoconstituents of apples vary greatly between different varieties [14]. Phytochemicals in plant foods have been demonstrated to exhibit antidiabetic potential as a result of their inhibitory effect on the activities of plant-hydrolizing enzymes [15]. Previous research has also demonstrated that women who ate apples had a 13–22% decrease in cardiovascular disease risk [16], while De Oliviera *et al*. reported that apple and pear intake was associated with weight loss in middle-aged overweight women in Brazil [17].

In recent times, research on the consumption of apples has attracted the interest of scientists following suggestions that it could lower the risk metabolic syndrome diseases like type 2 diabetes. However, there is little information on the inhibitory effect of the different apple varieties on key enzymes involved in the digestion of carbohydrates. This study therefore investigated the antioxidant properties and inhibitory potential of some selected apple varieties on key enzymes associated with type 2 diabetes.

Materials and Methods

Materials

Collection of samples

Wax apples (*Syzygium samarangense*), red apples (*Malus pumila*) and green apples (*Malus sylvestris*) were purchased from Oja-Oba market in Akure, south-west Nigeria. The samples were authenticat-

ed at the Department of Crop, Soil and Pest Management (CSP), Federal University of Technology, Akure, Nigeria.

Sample preparation

The apples were washed with distilled water and sliced, the seeds were removed, and the juices were extracted using a juice extractor (Panasonic MJ-66PR). The juices were then freeze dried using a laboratory freeze dryer. The freeze-dried juice extract was later reconstituted by dissolving 0.1 g of the freeze-dried juice in 10 ml of distilled water and used for further analysis.

Animal ethics

All animals used received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals under EU Directive 2010/63/EU for animal experiments. The ethics regulations have been followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments. The experiments were carried out at the Functional Food, Nutraceuticals and Phytomedicine Research Laboratory of the Department of Biochemistry, Federal University of Technology, Akure, Ondo State, Nigeria.

Chemicals and reagents

Chemicals and reagents including α-amylase, α-glucosidase, dinitrosalicyclic acid colour reagent, *p*-nitrophenyl-α-D-glucopyranoside and quercetin, were procured from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, acetic acid, sodium carbonate, potassium acetate, phenol and sodium hydroxide were sourced from BDH Chemicals (Poole, Dorset, UK). Gallic acid was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and the water used was glass distilled.

Methods

α-Amylase inhibition assay

Diluted extracts of apple juice (0–200 µl) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase (EC 3.2.1.1; 0.5 mg/ml) were incubated at 25°C for 10 min. Then, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures were incubated at 25°C for 10 min and stopped with 1.0 ml of dinitrosalicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance was measured at 540 nm using a spectrophotometer. The α-amylase inhibitory activity was expressed as percentage inhibition [18].

α-Glucosidase inhibition assay

Briefly, appropriately diluted apple juice extracts (0–200 µl) and 100 µl of α-glucosidase (EC 3.2.1.20) solution in 0.1 M phosphate buffer (pH 6.9) were incubated at 25°C for 10 min. Then, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25°C for 5 min and absorbance was read at 405 nm using a spectrophotometer. The α-glucosidase inhibitory activity was expressed as percentage inhibition [19].

1,1-Diphenyl-2-picrylhydrazyl free radical scavenging ability

The free radical scavenging ability of the apple juice extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was evaluated as described by Gyamfi *et al*. [20]. Briefly, appropriately diluted fruit juice extracts (0–400 µl) were mixed with 1 ml of 0.4 mmol/l methanol solution containing DPPH radicals. The mixture was left in the dark for 30 min and absorbance was read at 516 nm in a UV-visible spectrophotometer (Model 6305; Barloworld Scientific, Dunmow, Essex, UK). DPPH free radical scavenging ability was subsequently calculated with respect to the reference, which contained all reagents without the test sample.

Lipid peroxidation and thiobarbituric acid reactions

Rats were euthanized by cervical dislocation after which the pancreas was rapidly isolated, placed in ice and weighed. The tissue was subsequently homogenized in cold 0.9% (w/v) saline with about 10 up-and-down strokes at approximately 1,200 rev/

min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3,000×g to yield a pellet, that was discarded, and a low-speed supernatant (Sl) that was kept for lipid peroxidation assay [21]. The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.* [22]. Briefly, 100 µl of Sl fraction was mixed with a reaction mixture containing 30 µl of 0.1 M pH 7.4 Tris–HCl buffer, extracts (0–100 µl) and 30 µl of 250 µM freshly prepared $FeSO_4$ (the procedure was also carried out using 7 µM sodium nitroprusside). The volume was made up to 300 µl with water before incubation at 37°C for 1 h. The reaction was developed by adding 300 µl 8.1% sodium dodecyl sulphate (SDS) to the reaction mixture and this was followed by the addition of 600 µl of acetic acid/HCl (pH 3.4) and 600 µl 0.8% thiobarbituric acid (TBA). This mixture was incubated at 100°C for 1 h and the thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm. Subsequently, lipid peroxidation was calculated as malondialdehyde (MDA) produced (percentage of control) using MDA as the standard.

Determination of total phenol content

Total phenol content in the apple juice extracts was determined using the method reported by Singleton *et al.* [23]. Appropriate dilutions of the extracts were oxidized with 2.5 ml of 10% Folin-Ciocalteu reagent (v/v) and neutralized with 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance measured at 765 nm in the UV-visible spectrophotometer (Model 6305; Barloworld Scientific). The total phenol content was subsequently calculated using gallic acid as the standard.

Determination of total flavonoid content

The total flavonoid content of the apple juice extracts was determined using a slightly modified method as reported by Meda *et al.* [24]. Briefly, 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 µl of 10% $AlCl_3$, 50 µl of 1 mol/l potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was measured at 415 nm in the UV-visible spectrophotometer. The total flavonoid content was calculated using quercetin as the standard.

Data analysis

The results of triplicate experiments were pooled and expressed as mean±standard deviation. A one-way analysis of variance (ANOVA), followed by Duncan's multiple range test and least significant differences was performed with $p \leq 0.05$ considered significant [25].

Results and discussion

The inhibitory effects of the different apple juice extracts on the enzyme α-amylase are presented in Fig. 1. The result revealed that all juice extracts inhibited α-amylase activity in a dose-dependent manner (0–16.22 µg/ml). However, as revealed by the IC_{50} values (Table 1), the green apple extract had the highest inhibitory activity on α-amylase (IC_{50}=13.55 µg/ml), while the wax apple extract showed the least (IC_{50}=16.22 µg/ml). Similarly, the α–glucosidase inhibitory activity of the apple juice extracts was assessed. As shown in Fig. 2, all juice extracts inhibited α-glucosidase activity in a concentration-dependent manner (0–16.98 µg/ml). However, the difference between green (IC_{50}=12.66 µg/ml) and red (IC_{50}=13.11 µg/ml) apple α-glucosidase inhibitory activity was not significant (p>0.05), while wax apple juice showed the least α-glucosidase inhibitory activity (IC_{50}=16.98 µg/ml) following the same trend as seen with α-amylase inhibitory activity.

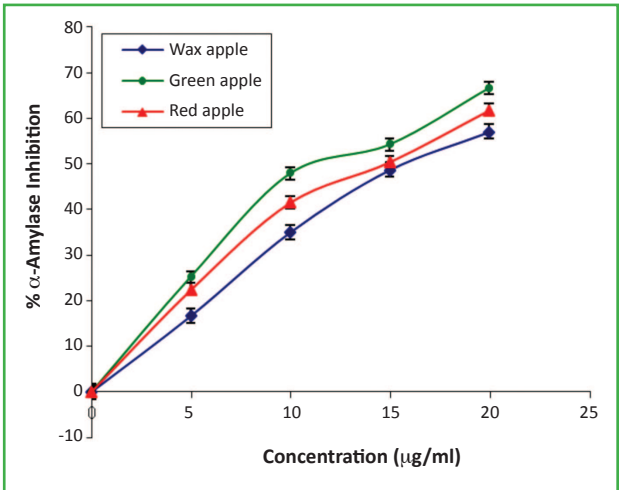

Figure 1 - α-Amylase inhibitory activities of juice extracts of three apple varieties

	Green apple	Red apple	Wax apple
Total flavonoid (mg QUE/100 g)	5.56±0.30[a]	4.17±0.75[b]	4.9±0.25[b]
DPPH radical scavenging ability (μg/ml)	222.92±4.20[a]	234.96±6.00[b]	278.71±8.50[c]
Fe^{2+}-induced lipid peroxidation (μg/ml)	44.79±3.86[c]	53.57±4.34[b]	38.60±5.20[a]
α-Amylase inhibition (μg/ml)	13.55±0.35[a]	14.85±0.28[b]	16.22±0.47[c]
α-Glucosidase inhibition (μg/ml)	12.66±0.51[a]	13.11±0.62[a]	15.98±0.78[b]
Values represent mean±standard deviation (n=3)			
Values with the same superscript number on the same row are not significantly different (p<0.05)			

Table 1 - Results of analysis of the juice extracts of green, red and wax apples

Control of postprandial hyperglycaemia is recognized as an effective therapeutic approach in the management of type 2 diabetes and in reducing chronic vascular complications [26]. It is believed that inhibition of the enzymes involved in the digestion and uptake of carbohydrates can significantly decrease the postprandial increase in blood glucose level after a mixed carbohydrate intake and therefore could be an important strategy in the management of hyperglycaemia linked to type 2 diabetes [27, 28]. Hence, inhibition of enzymes involved in the digestion of polysaccharides by dietary phytochemicals has shown promising potential [27–29]. Inhibition of the enzyme α-glucosidase slows the breakdown of disaccharides to simple glucose, thus reducing the amount of glucose absorbed into the blood. This forms the basis for the hypothesized mechanism of action of α-amylase and α-glucosidase inhibitors in reducing the glycaemic index of foods [30].

Our results indicate that the three apple varieties studied demonstrate ability to inhibit α-amylase and α-glucosidase activity in vitro (Figs. 1 and 2). It is worth noting that all apple varieties showed higher α-glucosidase inhibitory activity (IC_{50}=12.66–15.98 μg/ml) than their respective α-amylase (13.55–16.22 μg/ml) inhibitory activity. This agrees with previous reports that phytochemicals found in fruit are mild inhibitors of α-amylase and strong inhibitors of α-glucosidase activity [6, 31]. This ability may confer an advantage over or an additive effect to synthetic drugs currently used in the management of postprandial blood glucose, such as acarbose, which strongly inhibit α-amylase. There are indications that excessive pancreatic amylase inhibition could result in abnormal bacterial fermentation of undigested saccharides in the colon [32], which may account for the side effects that accompany the constant use of synthetic drugs. Hence, the stronger inhibition of α-glucosidase activity and mild inhibition of α-amylase exerted by apple juice extracts could minimize the side effects of α-glucosidase and α-amylase inhibitors such as abdominal distention, flatulence, meteorism and possibly diarrhoea [32]. Therefore, this study supports the assertion that natural inhibitors from fruits have a lower inhibitory effect against α-amylase activity and stronger α-glucosidase inhibitory activity, and could be effective in the management of post-prandial hyperglycaemia with minimal side effects [6, 29].

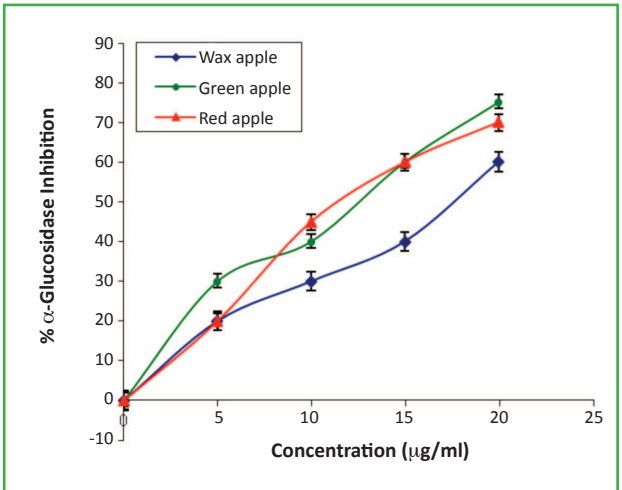

Figure 2 - α-Glucosidase inhibitory activities of juice extracts of three apple varieties

The DPPH free radical scavenging ability of the apple juice extracts was assessed and is shown in Fig. 3. The results revealed that all apple juice extracts scavenged DPPH radicals in a dose-dependent manner (0–278.71 μg/ml), with green apple (222.92 μg/ml) showing the highest DPPH free radical scavenging ability while wax apple (278.71 μg/ml) showed the least, as revealed by the IC_{50} values given in Table 1. Furthermore, incubation of rat pancreas in the presence of 250 μM Fe^{2+} caused a significant increase (p<0.05) in the MDA content (Fig. 4). However, the juice extracts of all apple varieties inhibited MDA production in the pancreas in a dose-dependent (0–53.57 μg/ml) manner; IC_{50} values are presented in Table 1. Wax

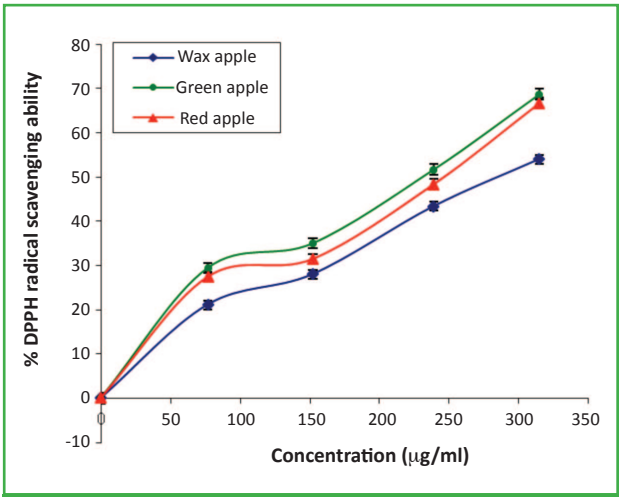

Figure 3 - 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability of juice extracts of three apple varieties

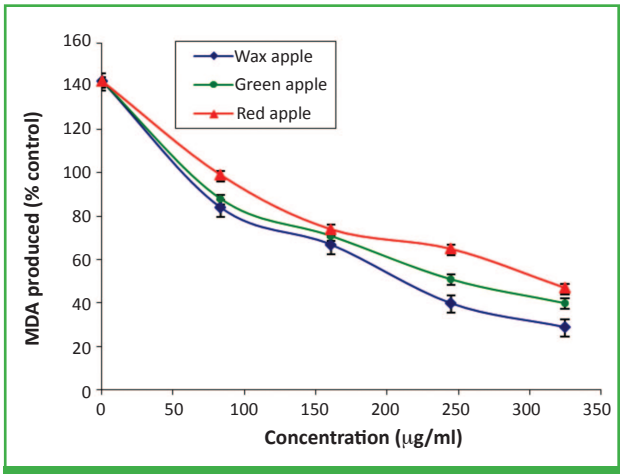

Figure 4 - Inhibition of Fe^{2+}-induced lipid peroxidation in rat pancreas by juice extracts of three apple varieties. *MDA* malondialdehyde

apple showed the highest inhibition of MDA production, while red apple showed the least.

The juice extracts of all apple varieties also demonstrated strong free radical scavenging activity as exemplified by their scavenging activity of moderately stable DPPH in vitro. The free radical DPPH shows a characteristic absorption at 517 nm (purple in colour), which decreases significantly on exposure to radical scavengers (apple varieties) by providing hydrogen atoms or by electron donation [20]. The potent antioxidant activity of these apple varieties may be due to the redox properties of their hydroxyl groups attached to the chemical structure of the phenolic compounds, most especially the flavonoids [15, 33, 34]. Hence, a steady supply of dietary antioxidants found in apples may help augment or boost endogenous antioxidant defence mechanisms and thereby reduce free radical-mediated oxidative stress in type 2 diabetes. Also, increased oxidative stress is a widely accepted to contribute to the development and progression of diabetes [35]. The mechanisms whereby increased oxidative stress induces diabetic complications include glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins [35]. Abnormally high levels of free radicals and the simultaneous deterioration of the antioxidant defence system could cause damage to cellular organelles and enzymes, increased lipid peroxidation and the development of insulin resistance, which is a major contributor to the progression of type 2 diabetes and its complications [36]. Incidentally, inhibition of lipid peroxidation induced by Fe^{2+} in pancreas homogenate by the various apple varieties is an indication of potent antioxidant capacity as inhibition of lipid peroxidation has been identified as an antioxidant mechanism of action. The peroxidation of biomolecules and biological membranes has been linked to the aetiology and progression of a number of diseases including DM. Furthermore, free cytosolic Fe has been implicated in the initiation of lipid peroxidation in biological systems [37]. This is suggested to be due to the participation of Fe in the generation of ROS such as OH radical with potency to attack the polyunsaturated fatty acids of the cell membrane, thereby triggering a chain reaction of peroxidized molecules and eventually leading to cell disruption and death.

The total phenol and flavonoid contents of aqueous extracts of the apple varieties are shown in Table 1. Green apple (17.45 mg GAE/100 g) had the highest total phenol content, while there was no significant (p>0.05) difference between the wax apple (16.58 mg GAE/100 g) and the red apple (16.14 mg GAE/100 g) total phenol content. Furthermore, the total flavonoid content of the apple varieties showed the same trend as with total phenol content with green apple (5.56 mg QUE/100 g) having the highest total flavonoid content, while there was no significant (p>0.05) difference between the wax apple (4.9 mg QUE/100 g) and red apple (4.17 mg QUE/100 g) total flavonoid content.

Apples are a widely consumed, rich source of phytochemicals, and epidemiological studies have

linked the consumption of apples to a reduced risk of diabetes. Previous research has reported that apples have very strong antioxidant activity, inhibiting key enzymes involved in digestion [14]. Apples contain a variety of phytochemicals, including quercetin, catechin, phloridzin and chlorogenic acid, all of which are strong antioxidants. The phytochemical composition of apples varies greatly between different varieties, while small changes in phytochemicals are observed during maturation and ripening of the fruit [38]. Phenolics are pharmacologically active components of plants which can scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce tocopherol radicals and inhibit oxidizing enzymes in biological systems [39, 40]. Findings from this study revealed that the apple varieties studied are rich in phenolics. Recent studies have described the beneficial role of plant-based phenolic compounds in the management of various cardiovascular diseases such as diabetes and hypertension [29, 34]. This is consistent with previous research that found that the biological activities of plant used as food are directly proportional to their phenolic contents [34, 41].

Conclusion

Apples contain a wide variety of phytochemicals, many of which have strong antioxidant activity as demonstrated in this study by their radical scavenging ability. The inhibition of α-amylase and α-glucosidase activity exhibited by the apple varieties in this study supports the biochemical justification for recommending the consumption of apples by patients with diabetes. In view of these findings, we encourage patients with diabetes to consume, preferably green (*Malus sylvestris*), apples.

Conflict of Interest

The authors declare they have no conflicts of interest.

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