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Original Research Article

In vitro antioxidant and antidiabetic activities of catechin isolated from the stem bark of *Acacia auriculiformis*

Adesegun J Kasimawo[1], Bilqis A Lawal[2], Ini Ekpe[3], Utuekong E Ekong[4], Aniefiok Udobre[4] and Augustine A Ahmadu[5]*

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, [2]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Kwara State, [3]Department of Biochemistry, Faculty of Pure and Applied Sciences, Veritas University, Bwari, Abuja, [4]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom State, [5]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Veritas University, Bwari, Abuja, Nigeria

For correspondence:* ***Email:* *ahmadu2001@yahoo.com;* ***Tel:*** *+2348037033505*

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Abstract

Purpose: *To investigate the antioxidant and in vitro antidiabetic activities of the n-butanol fraction and isolated compounds from the stem bark of Acacia auriculiformis.*

Methods: *Vacuum liquid chromatography (VLC) was performed on the n-butanol fraction using a gradient elution technique. Fractions 6 and 7 eluted with 5% methanol in dichloromethane afforded compound 1, while fractions 12 and 13 eluted with dichloromethane: methanol (80:20) were pooled together and purified over Sephadex LH-20 to afford compound 2. The chemical structures of the purified compounds were established using ^{1}H- and ^{13}C-NMR and mass spectrometry. Antioxidant activity of the n-butanol fraction and isolated compounds was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and nitric oxide (NO) scavenging assays, while in vitro antidiabetic activity was evaluated using α-amylase and α-glucosidase enzymes.*

Results: *Compounds 1 and 2 were identified as ethyl gallate and catechin on the basis of NMR and MS and compared with existing literature in the field. Catechin showed strong antioxidant activity in DPPH and ABTS assays with IC_{50} of 46.74 and 17.15 µg/mL versus ascorbic acid, with IC_{50} of 73.87 and 27.28 µg/mL, respectively, but weak nitric oxide scavenging activity with an IC_{50} of 208.87 µg/mL compared to 99.65 µg/mL for ascorbic acid. The in vitro antidiabetic assay revealed that catechin had weak inhibition against α-amylase (IC_{50} = 545.48 µg/mL) and moderate inhibition against α-glucosidase (IC_{50} = 201.32 µg/mL) compared to acarbose.*

Conclusion: *This study has revealed the potential of catechin as a strong antioxidant against DPPH and ABTS radicals with moderate α-glucosidase inhibitory activity.*

Keywords: *Antidiabetic, Antioxidant, Catechin, Ethyl gallate*

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INTRODUCTION

The pathogenesis of diseases such as diabetes, hypertension, cancer, among others, is known to be mediated by reactive oxygen and nitrogen species [1]. They function as important signaling molecules in physiological processes [2]. These radicals are scavenged by compounds that have the ability to donate a hydrogen atom via the

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activation of antioxidant enzymes [3]. In the case of plant extracts containing high amounts of biologically active compounds such as flavonoids and phenolics, they have the potential to be used in agriculture, nutraceutical and pharmaceutical products [4].

Acacia auriculiformis belongs to the family Fabaceae and is a widely distributed member of the genus Acacia, which is found in tropical and subtropical countries including Nigeria, Ghana and Mali [5]. The Aborigines of Australia use an infusion of the bark against inflammation [6]. The Ibibios of Akwa Ibom State of Nigeria use the infusion from the leaves of this plant as an antimalarial [7]. The antioxidant activity of the ethyl acetate-soluble fraction of the stem bark extract has been reported [8], while Sathya and Siddhuraju [9] reported the antidiabetic and antioxidant activity of the acetone extract of the bark and empty pod. The kinase inhibitory activity of the triterpenoid betulin isolated from the ethyl acetate soluble fraction of *Acacia auriculiformis* has been reported [6]. The present study reports the isolation of two phenolic compounds from the n-butanol fraction of *Acacia auriculiformis* stem bark. The *in vitro* antioxidant and antidiabetic activities of the isolated catechin were also evaluated.

EXPERIMENTAL

Vacuum liquid chromatography was carried out using silica gel G 0.2 mm with 230-400 mesh size. Also, thin-layer chromatography (TLC) was performed on a silica gel TLC chromatographic plate (0.2 mm, Silicycle), aluminum-backed, while column fractionation was performed using Sephadex LH-20 (Sigma). NMR spectroscopy was carried out using a Bruker DRX 500 MHz (^{1}H) and 125 MHz (^{13}C) spectrometer using deuterated methanol as solvent and tetramethylsilane (TMS) as internal standard, while mass spectrometry was carried out using an Orbitrap Thermo Scientific mass spectrometer.

Extraction and Isolation

The plant parts consisting of the leaves and stem bark of *Acacia auriculiformis* were obtained from their natural habitat in Samaru, Zaria, Nigeria, in August, 2024 and authenticated by US Gallah of the Department of Biological Sciences, Kaduna State University, Kaduna. A voucher specimen (Number 4050) was deposited in the herbarium. The air-dried pulverized stem bark, 300 g, was extracted with a total of 5 L of 70% ethanol at room temperature for 7 days. Subsequent removal of the solvent, using a rotary evaporator (ROTA VAP 41996A), afforded 32 g of a dark brown solid of the crude hydroalcoholic stem bark extract. A portion (20 g) of the extract was suspended in 500 mL of water and partitioned with 2.5 L each of ethyl acetate and n-butanol. Removal of the solvent using a rotary evaporator afforded their respective soluble masses of 3.2 and 2.5 g, respectively. The n-butyl alcohol soluble portion (2.0 g) was triturated with a small quantity of silica gel to form a slurry. This was transferred to a Büchner funnel carefully packed with the stationary adsorbent made up of silica gel G and subjected to vacuum liquid chromatography commencing with dichloromethane (100%). This was followed by the stepwise addition of dichloromethane: methanol in the following ratios: 99:1; 98:2; 97:3; 96:4; 95:5; 90:10; 80:20; 70:30; 60:40; 50:50; 30:70; 10:90; methanol (100%) and 5% water in methanol. Solvent systems I: Dichloromethane: ethyl acetate (2:3), II: Dichloromethane: ethyl acetate: methanol: water (15:8:4:1), and III: Dichloromethane: ethyl acetate: methanol: water (6:4:4:1) respectively were used to monitor the separation on TLC. A total of 19 fractions were collected. Fractions 6-7 eluted with 5% methanol in dichloromethane afforded compound 1.

Fractions 12-13 of the VLC fractions eluted with dichloromethane: methanol (80:20) revealed 3 spots on TLC using solvent system I. This was further subjected to column fractionation over Sephadex LH-20 in a column of dimensions (50 × 1.1 cm), eluting with methanol to give 20 sub-fractions. Sub-fractions 10-13, which showed similar Rf values of 0.41 and 0.95 on TLC using solvent systems I and II, were combined together and the evaporation of the pooled fraction afforded compound 2.

Antioxidant study

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was carried out according to the method of Brand-Williams *et al* [10], as previously described. Briefly, 8 mg of 2,2-diphenyl-1-picrylhydrazyl was dissolved in methanol (100 mL) to give a solution of concentration 80 µg/mL. Aliquots of the DPPH reagent (100 µL) were mixed with 100 µL of sample in a 96-well microplate and incubated at room temperature for 30 min. After incubation, the absorbance was measured at 517 nm using a microplate reader and 100 % methanol was used as control. The DPPH scavenging activity was estimated using Eq 1.

DPPH radical scavenging (%) = $((A_{blank} - A_{sample})/A_{blank}) \times 100$ …….. (1)

Where A_{blank} represents the absorbance of the control reaction, and A_{sample} represents the absorbance of the test sample/ascorbic acid, the reference drug.

2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid; ABTS) assay

The ABTS assay was done according to the method of Arnao *et al* [11], with some modifications. Portions of 7 mM ABTS and 2.45 mM potassium persulfate were mixed and dissolved in distilled water. The solution was diluted with distilled water in a 1:9 v/v ratio. A 190 µL volume of reagent was pipetted into a microtiter well, followed by the subsequent addition of 10 µL of sample/standard (ascorbic acid). The absorbance was measured at 735 nm. A reagent blank reading was taken and after the addition of the sample, the absorbance reading was taken after 6 minutes of initial mixing. The antioxidant activity was estimated using Eq 2.

ABTS scavenging (%) = $((A_{blank} - A_{sample})/A_{blank}) \times 100$ …….. (2)

Nitric oxide radical scavenging activity

This assay method involved the generation of nitrogen(II) oxide from sodium nitroprusside and was measured by the Griess reaction as described by Patel *et al* [12]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with varying concentrations of the test sample dissolved in phosphate buffer (0.025 M, pH 7.4) for 5 h. Control experiments without the test compounds, but with equivalent amounts of buffer, were conducted in an identical manner. After 5 h, 0.5 mL of the incubation solution was removed and diluted with 0.5 mL Griess reagent (1% sulfanilamide, 2% o-phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and its subsequent coupling with naphthylethylenediamine was read at 546 nm, with ascorbic acid as the standard reference drug. The percentage inhibition was calculated using Eq 3.

Inhibition (%) = $((A_b - A_s)/A_b) \times 100$ …… (3)

Where A_b is the absorbance of the blank, and A_s is the absorbance of the sample.

***In vitro* α-glucosidase inhibitory activity**

Catechin was tested for in vitro α-glucosidase inhibitory activity and the test involved the colorimetric determination of the rate of release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside by the action of α-glucosidase enzyme [13]. Briefly, 60 µL of the sample solution and 50 µL of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 units/mL) were incubated in 96-well plates at 37 °C for 20 min. After pre-incubation, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 °C for another 20 min. The reaction was stopped after the addition of 160 µL of 0.2 M sodium carbonate to each well. Absorbance readings (A) were recorded at 405 nm using a microplate reader and compared to a control containing 60 µL of buffer solution instead of the compound.

Alpha-amylase inhibitory activity

The assay system comprised the following components as described by Kulkarni *et al* [14] in a total volume of 260 µL: 40 µL of phosphate-buffered saline (PBS; 0.02 M, pH 6.9), 100 µL of glucose oxidase reagent, 40 µL each of soluble starch (2 g/L), inhibitor solution and enzyme solution (2 units/mL). Briefly, the enzyme solution was mixed with the test compound and pre-incubated on a hot plate for 10 min at 37 °C. The reaction was initiated by pipetting the starch solution into the wells and incubating for another 15 min. Finally, 100 µL of glucose oxidase reagent was added and the absorbance was measured at 540 nm after 15 min. For the negative control, 40 µL of phosphate-buffered saline was measured in parallel with the samples. The results are expressed in terms of the IC_{50} values.

Data analysis

All statistical analyses were performed using a two-sample t-test with Microsoft Excel 2021 software. Experiments were carried out in triplicate, and results were expressed as mean ± standard deviation (SD) of triplicate determinations.

RESULTS

Fractions 6 - 7 eluted with 5 % methanol in dichloromethane afforded compound 1 as an off-white solid (6.3 mg). NMR (^{1}H, CD_3OD): δ(ppm): 1.34 (t, CH_3), 4.27 (q, CH_2), 7.04 (2H, s; Ar-H; Supplementary file). NMR (^{13}C, CD_3OD): δ(ppm): 14.1(CH_3), 62.0 (OCH_2), 110.0 (C-2, C-6), 121 (C-1), 140.0 (C-4), 146.0 (C-3, C-5; aromatic carbons), 168(C=O; Supplementary file)

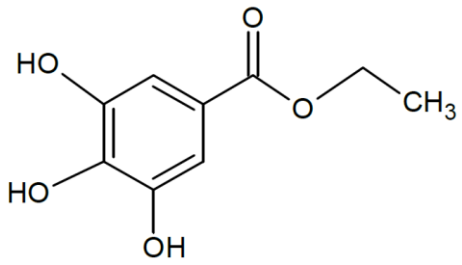


Figure 1: Chemical structure of ethyl gallate

Compound 2 was isolated from 20 % methanol in dichloromethane as a brown solid (10.2 mg)
NMR (^{1}H, CD_3OD): δ(ppm): 2.55 (d, H-4α), 2.85 (d, H-4β), 4.01 (m, H-3), 4.55 (d, H-2), 5.86 (d, H-6), 5.95 (d, H-8), 6.74 (d, H-6′), 6.78 (H-5′), 6.85 (d, H-2′)

NMR (^{13}C, CD_3OD): δ(ppm): 27.0 (C-4α, β), 69.0 (C-3), 83.0 (C-2), 95 (C-8), 96.5 (C-6), 101.0 (C-10), 115.2 (C-2′), 116.0 (C-5′), 120.0 (C-6′), 132.0 (C-1′), 145.5 (C-3′, C-4′), 156.0 (C-9), 157 (C-5), 158.0 (C-7; Supplementary file)

ESI-MS m/z (rel. int.; negative mode): $[M-H]^-$, 289.07 (52), $[M+FA-H]^-$ 335.07 (48), $[2M-H]^-$ 579.14 (100); supplementary file)

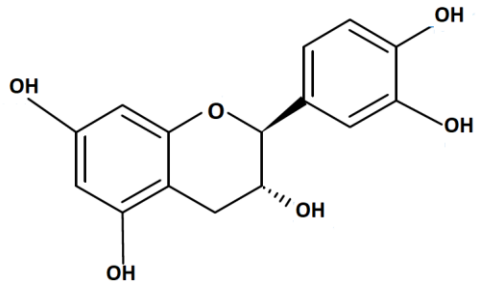


Figure 2: Chemical structure of catechin

Antioxidant activity of n-butanol fraction and catechin

DPPH radical scavenging activity

The DPPH radical scavenging activity of the n-butanol fraction revealed a gradual increase in activity up to a maximum activity at 50 µg/mL. A progressive increase in radical scavenging activity was observed, with a maximum inhibition of 70.45 ± 2.03 % at 50 µg/mL (Table 1). Catechin also showed a gradual increase in scavenging activity with the highest inhibition of 80.53 ± 2.40 % at 50 µg/mL, while ascorbic acid gave the highest inhibition of 84.82 ±1.58% at 150 µg/mL. The IC_{50} of catechin (Table 1) is 46.74 µg/mL, which is lower than that of ascorbic acid, with an IC_{50} of 73.87 µg/mL. This is an indication that catechin showed a higher DPPH scavenging activity than ascorbic acid.

ABTS radical scavenging activity

The ABTS radical scavenging activity of the n-butanol fraction showed a dose-dependent radical scavenging activity, with the highest inhibition of 92.19 ± 2.97% at 150 µg/mL, higher than ascorbic acid with inhibition of 78.77 ±0.22 % at the same concentration (Table 2). Catechin had the highest ABTS inhibition of 83.9 ±3.60 % at 150 µg/mL, which was also higher than the reference antioxidant molecule. The IC_{50} of catechin was found to be 17.15 µg/mL, which showed higher activity than ascorbic acid with an IC_{50} of 27.28 µg/mL (Table 2).

Nitric oxide inhibition activity

Results from the nitric oxide assay indicated a gradual increase in inhibition of nitric oxide by the test samples with increasing concentration, though much lower than ascorbic acid. The n-butanol fraction showed a weak nitric oxide scavenging activity, with the highest inhibition of 16.5% at 150 µg/mL, which was far lower than ascorbic acid, with inhibition of 61% at the same concentration (Table 3). Catechin had the highest inhibition of 36.76 ± 0.76%, which was also far lower than ascorbic acid, with an inhibition of 61.05 ±1.83 % at the same concentration. Comparison of the IC_{50} (Table 3) revealed that ascorbic acid has the highest nitric oxide scavenging activity with an IC_{50} of 99.65 µg/mL.

Table 1: Effect of n-butanol fraction, catechin, and ascorbic acid on DPPH radical

Concentration (µg/mL)	% Inhibition (n-butanol fraction)	% Inhibition (Catechin)	% Inhibition (Ascorbic acid)
10	6.46±4.84	17.48±9.74	30.26±18.02
20	39.17±20.91	34.39±15.67	16.65±1.01
50	79.62±2.03*	80.53±2.10*	42.07±3.45*
100	74.48±3.03*	80.07±0.83*	59.01±5.58*
150	70.45±2.21*	72.18±6.02	84.82±1.58
IC_{50} value (µg/mL)	54.95	46.74	73.87

% Inhibition expressed as mean ± SD. *means % inhibition at the same concentration differs significantly (P value < 0.05), n=3

Table 2: Effect of n-butanol fraction, catechin and ascorbic acid on ABTS radical

Concentration (µg/mL)	% Inhibition (n-butanol fraction)	% Inhibition (Catechin)	% Inhibition (Ascorbic acid)
10	45.81±4.07	43.34±5.67	38.99±4.60
20	47.98±3.49	61.04±12.55	52.98±2.87
50	53.41±16.90	71.40±11.23	60.23±0.19
100	69.63±10.28	82.26±4.60*	62.96±0.52*
150	92.19±2.97*	83.91±3.60	78.77±0.22
IC_{50} value (µg/mL)	29.97	17.15	27.28

Inhibition % expressed as mean ± SD. *means Inhibition % of the same concentration differs significantly (P value < 0.05), n=3

Table 3: Effect of n-butanol fraction, catechin and ascorbic acid on nitric oxide inhibition

Concentration (µg/mL)	n-butanol inhibition (%)	Catechin inhibition (%)	Ascorbic acid inhibition (%)
10	9.02±0.26*	7.40±0.23*	18.27±0.89*
20	10.78±0.45*	15.10±3.11*	28.28±4.77*
50	13.84±2.19*	21.00±0.88*	42.84±2.04*
100	14.89±2.25*	30.70±0.99*	51.94±0.24*
150	16.5±0.95*	36.76±0.76*	61.05±1.83*
IC_{50} value (µg/mL)	824.10	208.87	99.65

% Inhibition expressed as mean ± SD. *means Inhibition % of the same concentration differs significantly (*P* < 0.05), n = 3

In vitro antidiabetic activity of catechin

Alpha-amylase inhibitory activity

The alpha-amylase inhibition result revealed that catechin had weak alpha-amylase inhibitory activity, with an inhibition of 26.50 ±1.14% at 250 µg/mL compared to acarbose with inhibition of 61.37 ± 2.82 % at the same concentration (Table 4). The high IC_{50} value of 545.48 µg/mL for catechin in comparison with that of acarbose at 235.06 µg/mL revealed the weak activity of catechin on alpha-amylase when compared with the reference drug.

Table 4: Alpha-amylase inhibitory activity of catechin

Concentration (µg/mL)	Catechin inhibition (%)	Acarbose inhibition (%)
50	8.39±8.91	8.88±1.42
100	10.54±5.74	10.46±0.84
150	15.45±1.00*	20.65±0.34*
200	17.65±2.12*	36.89±2.48*
250	26.50±1.14*	61.37±2.82*
IC_{50} (µg/mL)	545.48	235.06

% Inhibition expressed as mean ± SD. *means Inhibition % of the same concentration differs significantly (P value < 0.05), n=3

Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibition results present a moderate picture for catechin's antidiabetic potential. Catechin exhibited an IC_{50} of 201.32 µg/mL, which is higher than acarbose with an IC_{50} of 7.83 µg/mL (Table 5).

Table 5: Alpha-glucosidase inhibitory activity of catechin

Concentration (µg/mL)	Catechin inhibition (%)	Acarbose inhibition (%)
50	29.97±2.51*	61.54±13.09*
100	32.07±2.82*	74.66±0.53*
150	48.56±9.52*	80.54±6.45*
200	50.79±4.19*	83.99±4.42*
250	54.13±0.36*	85.86±2.44*
IC_{50} (µg/mL)	201.32	7.83

% Inhibition expressed as mean ± SD. *means Inhibition % of the same concentration differs significantly (*P* < 0.05), n=3

DISCUSSION

Column chromatography of the n-butanol-soluble fraction led to the isolation of compounds 1 and 2. Compound 1 was isolated as an off-white solid. The ^{1}H-NMR spectrum revealed a triplet signal at δ =1.34 ppm ascribable to a terminal methyl proton with a neighboring methylene proton, which was confirmed by an oxymethylene signal at δ= 4.27 ppm. The presence of a singlet aromatic proton at δ = 7.04 ppm integrating for two protons confirmed compound 1 as a trihydroxy-substituted benzene derivative. The 13C-NMR spectrum further confirmed the presence of a methyl carbon, an oxygenated methylene carbon, as well as carbonyl signals at δ=14.1, 62, and 168 ppm, respectively. Compound 1 was found to be ethyl gallate as previously reported [15]. Compound 2 was obtained as a brown solid. The ESI-MS gave an [M-H] ion fragmentation at m/z 289.07, ascribable to a molecular weight of 290.07 and

the molecular formula $C_{15}H_{14}O_6$; the molecular weight was further confirmed from the $[M+FA-H]^-$ and $[2M-H]^-$ peaks at m/z 335.07 and 579.14, respectively, which all point to the molecular weight of 290.

The ^1H NMR spectrum revealed a pair of doublet signals at δ = 2.55 and 2.85 ppm assigned to H-4α and H-4β protons on carbon 4 (C-4); signals at δ = 4.01 and 4.55 ppm are assigned to C-3 and C-2 protons of a flavan nucleus [16]. Other diagnostic signals include aromatic protons at δ=5.86 and 5.95 ppm assigned to H-6 and H-8 of a 5,7-dihydroxy substituted ring A of the flavonoid nucleus, while aromatic proton signals at δ = 6.74, 6.78 and 6.85 ppm are consistent with the ABX protons of the flavonoid nucleus. The C-H correlation spectrum of compound 2 confirmed the attachment of the diagnostic proton signals to their respective carbons. The methylene proton signals at δ = 2.55 and 2.85 ppm correlated with the carbon signal at 27 ppm typical of a methylene carbon of catechin (C-4). The ring A and B aromatic protons all correlated with their respective carbons at δ = 95, 96.5, 115.2, 116, and 120 ppm, respectively, thus confirming compound 2 to be a tetrahydroxy-substituted flavan. The heteronuclear multiple bond correlation (HMBC) experiment also aided in the C- H long-range correlations. The methylene protons at δ = 2.55 ppm (H-4α) and 2.85 ppm (H-4β) showed correlations with carbon signals at δ = 83 ppm (C-2), 69.0 ppm (C-3), and 156 ppm (C-9), while the hydroxymethine proton at δ= 4.01 ppm (H-3) showed correlation with the carbon signal at δ=101 ppm (C-10). Also, correlations between the H-2 proton at δ=4.55 ppm and carbon signals at δ=27 ppm (C-4), 69 ppm (C-3) and 156 ppm (C-9) all aided in establishing the identity of compound 2 as catechin [17].

The antioxidant activity of the n-butanol fraction revealed a dose-dependent radical scavenging activity against DPPH and ABTS radicals, but weak nitric oxide scavenging activity, suggesting that nitric oxide scavenging is not a major mechanism of the antioxidant in this system. Ascorbic acid demonstrated the highest nitric oxide scavenging activity. We have previously reported the antioxidant and *in vitro* antidiabetic activity of compound 1, ethyl gallate isolated from *Acacia nilotica* stem bark [15]. The antioxidant activity of the n-butanol fraction is likely due to the presence of phenolic compounds such as catechin, which is a natural antioxidant that relieves oxidative stress via free radical scavenging and chelates reducing metals, thereby enhancing antioxidant enzyme activity [18]. Catechin is a phenolic compound that belongs to the flavonoid class of phytochemicals, particularly the flavan-3-ol. The antioxidant activity of catechin is due to the presence of the free hydroxyl group at position 3 of the catechin structure, as well as the presence of 5,7-dihydroxy phenolic groups of ring A of the flavan nucleus [19].

Catechin has been reported to significantly ameliorate fasting blood glucose levels by mitigating insulin resistance through the inhibition of digestive enzymes such as α-amylase and α-glucosidase, as well as through the activation of cellular pathways, thereby protecting the pancreatic β-cells and reducing inflammation [20]. The *in vitro* antidiabetic activity of catechin revealed a much lower inhibition of the activity of α-amylase and α-glucosidase in comparison with acarbose, the reference drug. This suggests that catechin might be a selective inhibitor of α-glucosidase, an enzyme involved in complex carbohydrate metabolism, thereby reducing postprandial glucose levels. Catechin alone in this plant might not be responsible for the folkloric use of the plant in the management of diabetes. It probably works in synergy with other phenolic compounds which might be present in the plant.

CONCLUSION

In vitro antioxidant activity of the n-butanol fraction, as well as the chromatographic separation, led to the isolation of ethyl gallate and catechin. Catechin exhibits strong antioxidant activity but moderate alpha-glucosidase inhibition activity; hence, the potential of catechin as a selective alpha-glucosidase inhibitor can be further explored as a lead in the discovery of antidiabetic drugs from folkloric plants used in the management of diabetes.

DECLARATIONS

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Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design,

results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Ethical approval

Not applicable.

Contribution of Authors

We declare that the work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. AAA conceived and designed the study; AJK, BAL, and IE collected and analyzed the data; UEE and AU wrote the manuscript. All authors read and approved the manuscript for publication.

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