Research Article

Inhibition of Alpha-Amylase Activity by *Gymnocarpos decandrus* Forssk. Constituents

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ABSTRACT

The methanolic extract of the aerial parts of *Gymnocarpos decandrus*, the plant growing wildly in the middle east area and north Africa, showed a promising α-amylase inhibitory activity. The ethyl acetate fraction of the plant showed the highest inhibitory activity followed by the methylene chloride fraction. Phytochemical investigation of both fractions led to the isolation of 10 compounds; oleanolic acid (**1**), maslinic acid (**2**), apigenin (**3**), β-sitosterol glucoside (**4**), luteolin (**5**), afzelechin (**6**), epiafzelechin (**7**), catechin (**8**), epicatechin (**9**) and gallocatechin (**10**). The structures of compounds **1-10** were established on the basis of extensive 1D- and 2D-NMR spectroscopic techniques. This is the first report for the isolation of these compounds from *G. decandrus*. The phenolic compounds (**6-10**) isolated from the ethyl acetate fraction showed the highest α-amylase inhibitory activity. The more the hydroxylation pattern of the isolated phenolic compounds (**6**, **8**, **9** and **10**), the higher the ability to inhibit the α-amylase activity. This was confirmed through the molecular docking experiment. The wild plant *G. decandrus* is a promising α-amylase inhibitor that can be used in the management of obesity and diabetes mellitus.

Keywords: *Gymnocarpos decandrus*, α-amylase inhibitor, docking experiment.

INTRODUCTION

Disorders of carbohydrate uptake can cause chronic health problems as diabetes mellitus and obesity. The global prevalence (age-standardized) of diabetes mellitus has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. The death rates caused by diabetes mellitus that occurs prior to age 70 is higher in low- and middle-income countries in Eastern Mediterranean, South-East Asia, and African regions. Its prevalence in 2000 in the Eastern Mediterranean region was estimated to be 15,188,000 and expected to rise to be 42,600,000 by 2030[1].

The International Diabetes Federation (IDF) listed Egypt among the world top 10 countries in the number of patients with diabetes. It is alarming that diabetes mellitus prevalence in Egypt has increased rapidly within a relatively short period from approximately 4.4 million in 2007 to 7.5 million in 2013. It is expected that this number will jump up to 13.1 million by 2035[2]. Long term complications of diabetes mellitus include retinopathy, nephropathy, neuropathy, microangiopathy and increased risk of cardiovascular disease.

Obesity is a complex metabolic disorder, it has become a worldwide public health threat, since it is involved in various serious diseases, including diabetes mellitus (type 2), hypertension, coronary heart diseases and osteoarthritis. One of the greatest public health challenges in the first half of 21st century is preventing the epidemic of obesity.

One therapeutic approach for the management of both diabetes mellitus (type 2) and obesity is to decrease the post-prandial glucose levels. This could be achieved by retarding the absorption of glucose through the inhibition of the carbohydrates-hydrolysing enzymes as α-amylase and α-glucosidase that are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise. Acarbose has been used as potent reversible inhibitor of α-amylase and α-glucosidase in several commercial preparations, however undesirable side effects limit its use[3,4].

Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources. The search for α-amylase inhibitors from natural sources has already become a focus of research[3]. Recent studies indicate that plants rich in flavonoids and polyphenolic compounds inhibit α-amylase and α-glucosidase activity and allow for the control of blood glucose level. Moreover, their consumption is more acceptable due to low cost and relative safety, including a low incidence of serious gastrointestinal side-effect[5,6]. This urged us to search for α-amylase inhibitors from available plants growing wildly or even suitable for cultivation in the area of middle east and north Africa.

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Gymnocarpos decandrus (Caryophyllaceae) is a shrub distributed through the Saharo-Arabian region from Morocco in the west till Egypt in the east and from Saudi Arabia to Jordan, Syria and Pakistan in Asia[7] . In Egypt, *G. decandrus* grows wildly on stony wadis and slopes in the Mediterranean coastal strip, Sinai peninsula[8,9]. The constituents of its essential oil were identified as hexadecanoic acid, dodecanoic acid, vinyl guaiacol, tetradecanoic acid, and linoleic acid[10]. Its phytoconstituents were reported to be belonging to several classes: sterols, triterpenes and flavonoids. *G. decandrus* extract exhibited analgesic, antipyretic, anti-inflammatory, diuretic and local anesthetic activities[11]. The aim of this study is to test *G. decandrus* as a potential *α*-amylase inhibitor, besides the isolation and identification of the pure compounds responsible for this activity.

MATERIALS AND METHODS

General experimental procedures

Silica gel for column chromatography and TLC (G60F$_{254}$) were purchased from E-Merck, Germany. The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6, CD$_3$OD and CDCl$_3$ using TMS as an internal standard, on Bruker AV-400 spectrometer and on Jeol 500 MHz TM spectrometer.

Plant material

The aerial parts of *G. decandrus* were collected from the north Mediterranean coastal region to the west of Alexandria, Egypt in April, 2015. The plant was authenticated by Dr. I. Mashaly, Prof. of Botany, Faculty of Sciences, Mansoura University. A voucher specimen (Gd-4015) is kept in Pharmacognosy Dept. Faculty of Pharmacy, Mansoura University, Egypt.

Extraction and Isolation of compounds

The powdered aerial part of *G. decandrus* (2 kg) was extracted by maceration in methanol at room temperature (5 x 4L). The collected methanolic extract was evaporated under reduced pressure to give 120 g of dry residue. The dried methanolic extract was subjected to fractionation by petroleum ether, methylene chloride and ethyl acetate to produce 45, 15 and 42 g, respectively.

The dried methylene chloride fraction was chromatographed on a silica gel column. Elution was started with petroleum ether - methylene chloride (100:0 to 0:100) then ethyl acetate - methanol (100:0 to 0:100). The eluted sub-fractions were collected, concentrated and screened by TLC, similar sub-fractions were combined together into three groups. Group 1 (sub-fractions 19-26) eluted with methylene chloride (100%) was further purified on silica gel column using petroleum ether - ethyl acetate (85:15) by gradient elution technique yielding compound **1** (41 mg). Group 2 (sub-fractions 51-57) eluted with methylene chloride - methanol (99:1) by gradient elution technique yielded compound **2** (10 mg). Group 3 (sub-fractions 72-73) eluted with methylene chloride - methanol (99:1) was chromatographed on a silica gel column using petroleum ether-ethyl acetate by gradient elution technique to afford compound **3** (14 mg). Group 4 (sub-fractions 96-98) eluted with methylene

chloride - methanol (90:10) afforded compound **4** (34 mg).

The dried ethyl acetate fraction was chromatographed on a silica gel column. Elution was started with ethyl acetate- methanol (100:0 to 50:50). The eluted sub-fractions were collected, concentrated and screened by TLC, similar sub-fractions were combined together into five groups. Group 1 eluted with 2% ethyl acetate-methanol (5 g) was chromatographed on a silica gel column using methylene chloride - methanol (99:1 to 97:3) by gradient elution technique. further purification on silica gel column by isocratic elution using 5% methanol in methylene chloride afforded compound **5** (8 mg) and a mixture of compounds **6** and **7** (26 mg). This mixture was subjected to reversed phase silica Rp-18 by isocratic elution technique using medium pressure column to afford compounds **6** (10 mg) and **7** (9 mg) in pure form. Group 3 eluted with 4% ethyl acetate-methanol (8 g) was chromatographed on a silica gel column using methylene chloride - methanol (98:2 to 96:4) by gradient elution technique. Sub- group 3A eluted with 3% methanol in methylene chloride afforded a mixture of compounds **8** and **9**. This mixture was separated on reversed phase silica Rp-18 by isocratic elution technique using medium pressure column to produce compounds **8** and **9**. Sub-group 3B eluted with 4% methanol in methylene chloride was chromatographed on reversed phase silica Rp-18 by isocratic elution technique using medium pressure column to afford compound **10**.

NMR data of the isolated compounds:

Compound **1** was obtained as white powder. ^{1}H NMR (400 MHz, CDCl$_3$), 3.14 (1H, m, H-3), 5.21 (1H, s, H-12), 2.76 (1H, dd, *J*= 9.8 and 1.5, H-18), 0.70 (6H, s, CH$_3$-24 &26), 0.85 (3H, s, CH$_3$-29), 0.86 (3H, s, CH$_3$-30), 0.92 (3H, s, CH$_3$-25), 1.07 (3H, s, CH$_3$-23), 1.19 (3H, s, CH$_3$-27).

^{13}C NMR (100 MHz, CDCl$_3$), 38.5 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 32.7 (C -7), 39.4 (C-8), 47.7 (C-9), 37.1 (C-10), 23.0 (C-11), 122.7 (C-12), 143.6 (C-13), 41.7 (C-14), 27.7 (C-15), 23.4 (C-16), 46.5 (C-17), 41.1 (C-18), 45.9 (C-19), 30.6 (C-20), 33.6 (C-21), 32.5 (C-22), 28.1 (C-23), 15.5 (C-24), 15.3 (C-25), 17.1 (C-26), 25.9 (C-27), 182.4 (C-28), 33.0 (C-29), 23.6 (C-30).

Compound **2** was obtained as white powder. ^{1}H NMR (400 MHz, DMSO-d_6), 3.44 (1H, m, H-2), 2.77 (1H, d, *J*=12), 5.19 (1H, br s, H-12), 1.19 (3H, s, CH$_3$-23), 1.11 (3H, s, CH$_3$-27), 0.94 (3H, s, CH$_3$-24), 0.93 (3H, s, CH$_3$-30), 0.89 (3H, s, CH$_3$-25), 0.82 (3H, s, CH$_3$-26), 0.80 (3H, s, CH$_3$-29).

^{13}C NMR (100 MHz, DMSO-d_6), 47.2 (C-1), 67.7 (C-2), 82.8 (C-3), 39.5 (C-4), 55.3 (C-5), 18.5 (C-6), 32.9 (C -7), 39.3 (C-8), 47.6 (C-9), 38.2 (C-10), 23.8 (C-11), 121.9 (C-12), 144.4 (C-13), 41.9 (C-14), 27.4 (C-15), 23.1 (C-16), 46.2 (C-17), 41.3 (C-18), 45.9 (C-19), 30.9 (C-20), 33.3 (C-21), 32.6 (C-22), 29.0 (C-23), 17.4 (C-24), 16.8 (C-25), 17.5 (C-26), 26.1 (C-27), 179.0 (C-28), 32.9 (C-29), 23.5 (C-30).
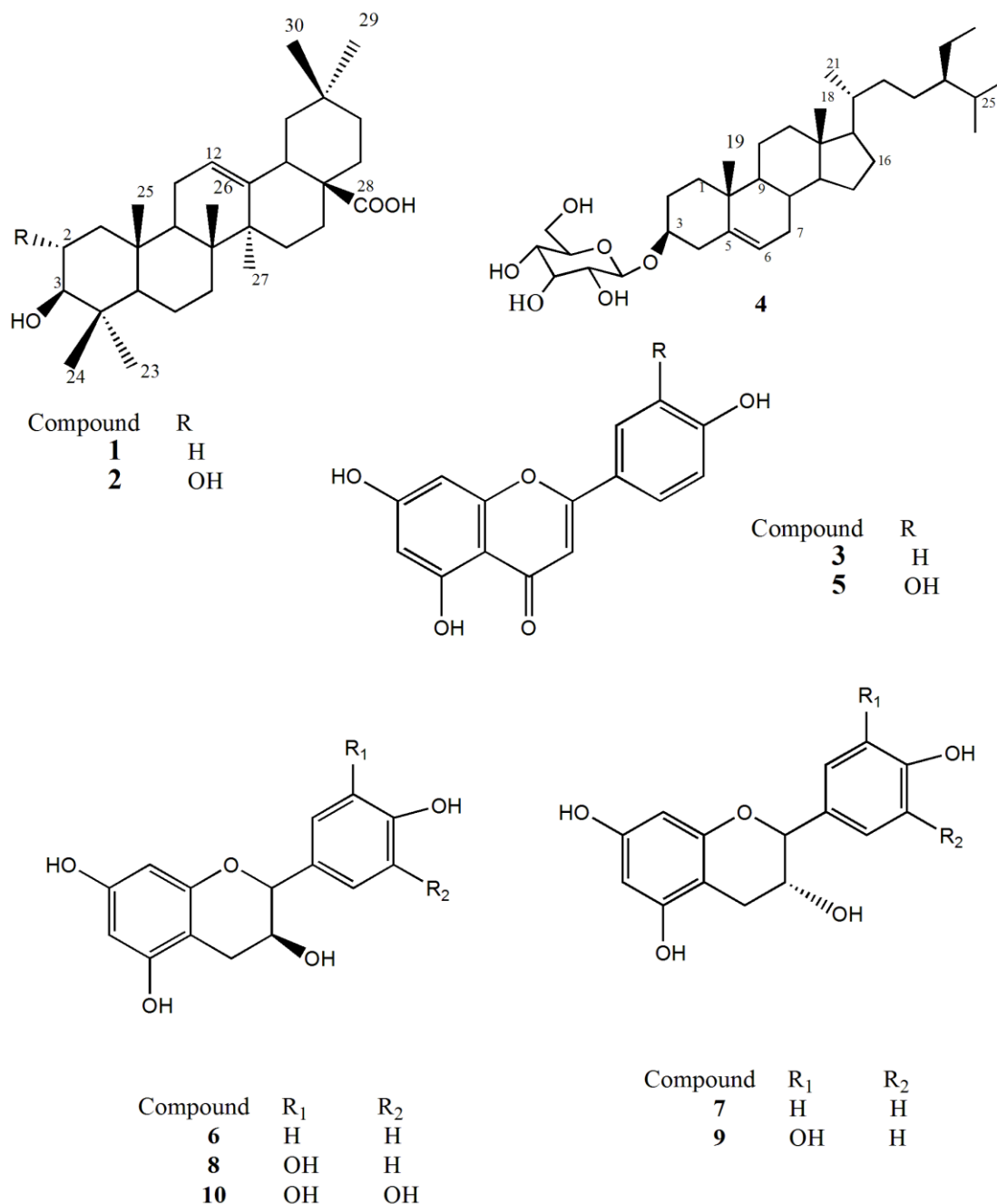
Figure 1: Chemical structures of compounds 1-10.

Table 1: *α*-amylase inhibitory activity of constituents isolated from *Gymnocarpos decandrus*.

Constituent	Inhibition (%)
Methanolic extract	88.0
Pet. ether fraction	58.0
Methylene chloride fraction	75.0
Ethyl acetate fraction	87.6
Compound 6	64.4
Compound 8	67.0
Compound 9	66.7
Compound 10	68.3
Acarbose	57.0
100 % enzyme	0

Compound **3** was obtained as yellow powder. ^{1}H NMR (400 MHz, CD$_3$OD), 6.75 (1H, s, H-3), 6.48 (1H, s, H-6), 6.19 (1H, s, H-8), 7.92 (2H, d, *J*=8 , H-2' and H-6'), 6.64 (2H, d, *J*=8, H-3' and H-5').
^{13}C NMR (100 MHz, CD$_3$OD), 162.0 (C-2), 103.4 (C-3), 182.2 (C-4), 161.8 (C-5), 99.4 (C-6), 164.2 (C-7), 94.5 (C-8), 157.8 (C-9), 103.5 (C-10), 121.7 (C-1'), 128.9 (C-2' and C-6'), 116.5 (C-3' and C-5'), 161.7 (C-4').
Compound **5** was obtained as yellow powder. ^{1}H NMR (400 MHz, DMSO-*d₆*), 6.69 (1H, s, H-3), 6.23 (1H, brs, H-6), 6.48 (1H, brs, H-8), 7.44 (1H, s, H-2'), 6.94 (1H, d, *J*=8, H-5'), 7.46 (1H, d, *J*=8, H-6').
^{13}C NMR (100 MHz, DMSO-*d₆*), 164.9 (C-2), 103.3 (C-3), 182.1 (C-4), 162.0 (C-5), 99.4 (C-6), 164.4 (C-7), 94.3

Figure 2: Interaction diagrams of docked structures of tested compounds with alpha-amylase (PDB:1HNY). Green arrow represents side chain acceptor/donor; blue arrow represents backbone acceptor/donor; blue shadow represents ligand exposure.

(C-8), 157.8 (C-9), 104.1 (C-10), 121.8 (C-1'), 113.8 (C-2'), 146.3 (C-3'), 150.4 (C-4'), 116.5 (C-5'), 119.5 (C-6').

Compound **6** was obtained as white powder. ^{1}H NMR (400 MHz, CD$_3$OD), 4.54 (1H, d, *J*=8, H-2), 3.88 (1H, m, H-3), 2.41(1H, dd, *J*=16 and 8 H-4a), 2.80 (1H, dd, *J*=16 & 5.7 H-4b) 5.85 (1H, brs, H-6), 5.76 (1H, brs, H-8), 7.12 (2H, d, *J*=8, H-2' and H-6'), 6.69 (2H, d, *J*=8, H-3' and H-5').

^{13}C NMR (100 MHz, CD$_3$OD), 81.5 (C-2), 67.5 (C-3), 27.9 (C-4), 156.2 (C-5), 95.0 (C-6), 156.5 (C-7), 94.2 (C-

8), 155.6 (C-9), 99.6 (C-10), 130.1 (C-1'), 128.2 (C-2' and 6'), 114.7 (C-3' and 5'), 157.0 (C-4').

Compound **7** was obtained as white powder. ^{1}H NMR (400 MHz, CD$_3$OD), 4.81 (1H, brs, H-2), 4.11 (1H, m, H-3), 2.64 (1H, dd, *J*=16 & 3.2 H-4a), 2.76 (1H, dd, *J*=16 and 4.5 H-4b), 5.84 (1H, brs, H-6), 5.75 (1H, brs, H-8), 7.21 (2H, d, *J*=8 , H-2' and H-6'), 6.68 (2H, d, *J*=8, H-3' and H-5').

^{13}C NMR (100 MHz, CD$_3$OD), 78.5 (C-2), 66.1 (C-3), 27.5 (C-4), 156.3 (C-5), 95.1 (C-6), 156.5 (C-7), 94.6 (C-8), 156.0 (C-9), 98.7 (C-10), 130.2 (C-1'), 127.8 (C-2' and

Table 2: Free energy of binding (*d*G) in Kcal/mol calculated by MOE for the tested compounds.

Compound	Free energy of binding (*d*G) (Kcal/mol)
10	-5.77
9	-5.51
8	-5.51
7	-5.30
6	-4.58

6'), 114.4 (C-3' and 5'), 156.6 (C-4').

Compound **8** was obtained as white powder. ^{1}H NMR (500 MHz, CD$_3$OD), 4.91 (1H, d, *J* = 8, H-2), 3.95 (1H, m, H-3), 2.83 (1H, dd, *J* = 16 and 5 H-4a), 2.48 (1H, dd, *J* = 16 and 8 H-4b), 5.90 (1H, d, *J* = 2, H-6), 5.83(1H, d, *J* = 2, H-8), 6.81(1H, d, *J* = 2, H-2'), 6.74 (1H, d, *J* = 8, H-5'), 6.69 (1H, dd, *J* = 8 and 2 H-6').
^{13}C NMR (100 MHz, CD$_3$OD), 81.7 (C-2), 67.6 (C-3), 27.3 (C-4), 156.4 (C-5), 95.1 (C-6), 156.6 (C-7), 94.3 (C-8), 155.7 (C-9), 99.6 (C-10), 131.0 (C-1'), 114.1 (C-2'), 145.0 (C-3' and C-4'), 114.9 (C-5'), 118.9 (C-6').

Compound **9** was obtained as buff powder . ^{1}H NMR (400 MHz, CD$_3$OD), 4.57 (1H, d, *J* = 8, H-2), 4.18 (1H, m, H-3), 2.73 (1H, dd, *J* = 16 and 4 H-4a), 2.45 (1H, dd, *J* = 16 and 8 H-4b), 5.94 (1H, s, H-6), 5.86 (1H, s, H-8), 6.97 (1H, s, H-2'), 6.83 (1H, d, *J* = 8, H-5'), 6.73 (1H, d, *J* = 8 H-6').
^{13}C NMR (100 MHz, CD$_3$OD), 78.5 (C-2), 66.1 (C-3), 27.8 (C-4), 156.5 (C-5), 95.2 (C-6), 156.3 (C-7), 94.6 (C-8), 156.0 (C-9), 98.8 (C-10), 130.9 (C-1'), 114.6 (C-2'), 144.8 (C-3') 144.6 (C-4'), 114.8 (C-5'), 118.1(C-6').

Compound **10** was obtained as buff powder. ^{1}H NMR (400 MHz, CD$_3$OD), 4.44 (1H, d, *J* = 4, H-2), 3.87 (1H, m, H-3), 2.71 (1H, dd, *J* = 16 and 4 H-4a), 2.41 (1H, dd, *J* = 16 and 8.2 H-4b), 5.83 (1H, s, H-6), 5.77 (1H, s, H-8), 6.31(2H, s, H-2' and H-6').
^{13}C NMR (100 MHz, CD$_3$OD), 81.5 (C-2), 67.4 (C-3), 26.7 (C-4), 156.2 (C-5), 95.0 (C-6), 158.4 (C-7), 94.2 (C-8), 155.5 (C-9), 99.4 (C-10), 130.3 (C-1'), 105.9 (C-2'), 145.5 (3'), 132.6 (4'), 145.5 (C-5'), 105.9 (C-6').

Reagents for biological assay:
Porcine pancreatic *α*-amylase (EC3.2.1.1, type VI) was purchased from Sigma Chemicals, USA. It was dissolved in ice-cold distilled water to obtain a concentration of 4 unit/mL solution. The substrate solution (potato starch, 0.5% w/v) was dissolved in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride. Dinitrosalicylic acid was prepared by dissolving 1 gm in 50 mL of distilled water then, sodium potassium tartrate - tetrahydrate (30 gm) were added slowly, followed by 20 mL of 2 N NaOH, finally the solution was diluted to 100 mL with distilled water. Test compounds (1mg/mL in DMSO-*d$_6$*) were used as stock solutions. The absorbance was measured at 570 nm by a Bioteck® Elisa plate reader.

Procedure
The *α*-amylase inhibition assay was performed using the chromogenic method adopted from Sigma-Aldrich[12,13].

Docking methodology

A docking experiment was carried out to investigate the favorable binding configurations between the tested compounds and macromolecular target; α-amylase. Docking studies of the isolated compounds were performed using Molecular Operating Environment (MOE), Version 2009.10, Chemical Computing Group, Inc., Montreal, Quebec, Canada, 2009 software. The crystal structure of the enzyme was downloaded from the RCSB Protein Data Bank (PDB, code: 1HNY). The docking experiment was adjusted as described in reference[14]. The pocket atoms were used to define the active site. Alpha spheres within 5 Å were used to guide the placement. We performed 30 docking iterations for each compound. The top scoring configuration of each of the compound-enzyme complexes was selected on energetic ground. The output of docking simulation is the scoring function which reflects the binding free energy *d*G in kcal/mol.

RESULTS AND DISCUSSION

Owing to the high prevalence of diabetes mellitus type 2 and obesity and the limited number of current medicinally used safe natural drugs for their management, there is a growing need to search for safe natural products to help control these diseases.

The methanolic extract of *G. decandrus* showed a promising *α*-amylase inhibitory activity (88.0%). The ethyl acetate fraction exhibited the highest α-amylase inhibitory activity (87.6%) followed by the methylene chloride fraction (75.0%) then the pet. ether fraction (58.0%) (Table 1). This encouraged us to extend our work to isolate pure compounds from the ethyl acetate and methylene chloride fractions using different chromatographic techniques. Identification of these compounds was achieved depending on 1D and 2D NMR data. The compounds were identified as oleanolic acid, maslinic acid[15] (**1, 2**), apigenin[16] (**3**), *β*-sitosterol glucoside[17] (**4**), luteolin[16] (**5**), afzelechin, epiafzelechin, catechin, epicatechin and gallocatechin[18] (**6-10**) (Fig. 1). This is the first report to describe the isolation of these compounds from *G. decandrus*.

α-amylase Inhibitory Assay
Compounds isolated from the ethyl acetate fraction were tested for their *α*-amylase inhibitory activity by chromogenic method using dinitrosalicylic acid as a substrate. Compared to acarbose as positive standard, these compounds showed potent inhibitory activities (Table 1).

The α-amylase inhibitory activity of ethyl acetate extract was higher than that exhibited by the pure isolated compounds (Table 1) indicating the synergistic effect of these compounds. Moreover, the % inhibitory activity showed by the ethyl acetate fraction was nearly equal to that exhibited by the methanolic extract indicating that the activity of the methanolic extract is attributed mainly to the ethyl acetate fraction and its combined constituents. It can also be concluded that the more the hydroxylation pattern of the isolated phenolic compounds, the higher its ability to inhibit the *α*-amylase activity; afzelechin (**6**), catechin (**8**) and gallocatechin (**10**) showed % inhibition

activities; 64.4%, 67.0% and 68.3%, respectively. Compounds with the same hydroxylation pattern but differ only in the configuration of the hydroxyl group at position number 3 exhibited nearly the same % inhibition activity; as in catechin (**8**) and epicatechin (**9**) (Table 1 and Fig. 1).

Molecular docking

A docking experiment was carried out using MOE docking software to explain the mode of interaction between the binding sites of α-amylase enzyme and the isolated phenolic compounds (Table 2). Gallocatechin (**10**) exhibited the highest α-amylase inhibitory activity (68.3 %), this could be explained by the formation of highly stable complex with the active sites of the enzyme. This result was confirmed by running the docking utility, where the binding energy is the lowest among all tested compounds (-5.77 Kcal/mol). The potential binding sites of **10** were found to be Asp 300, Glu 233, Trp 59, Gln 63 through hydrogen bonding (Fig. 2 E). Both catechin (**8**) and epicatechin (**9**) showed the same binding energy (-5.51). The potential binding sites of **8** were Asp 300, Glu 233 and Gln 63 through hydrogen bonding (Fig. 2 C), while the binding sites of **9** were Gln 63, Trp 59, Asp 300, Asp 197 through hydrogen bonding and Trp 59 and Tyr 62 through hydrophobic (π - π) interaction (Fig. 2 D). This supports the nearly similar α-amylase inhibitory activities (67.0 % and 66.7%, respectively). Afzelechin (**6**) and epiafzelechin (**7**) showed binding energy (-4.58 and -5.30, respectively). The binding sites of **6** were Asp 300, Glu 233, Trp 59 through hydrogen bonding and Tyr 62 through hydrophobic (π - π) interaction (Fig. 2 A). The binding sites of **7** were Asp 300, Glu 233 and Gln 63 through hydrogen bonding (Fig. 2 B).

The results of this study open possibilities for the use of *G. decandrus* for controlling diabetes mellitus type 2 and obesity through its α-amylase inhibitory activity. It also suggests that plants belonging to genus *Gymnocarpos* can be evaluated as α-amylase inhibitors, consequently controlling these diseases.

CONCLUSION

This research is significant for the evaluation of the α-amylase inhibitory activity of the wild herb *Gymnocarpos decandrus*. Moreover, the isolation and identification of different fractions and pure compounds responsible for this activity was performed. The results suggest that the ethyl acetate fraction and its isolated phenolic compounds are promising natural sources for further studies on development of new medications which might be useful in management of obesity and diabetes mellitus.

CONFLICT OF INTEREST

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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