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In-vitro sucrase enzyme activity of alcoholic extracts of certain plants belonging to families *Meliaceae, Podocarpaceae* and *Fabaceae* resulted in isolation of novel polyphenolic compound

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ABSTRACT

80% ethanolic extracts of *Azadirachta indica, Podocarpus gracilior, Acacia glauca, Acacia Senegal and Acacia nilotica* were biologically tested using Sucrase enzyme inhibitory activity test to evaluate their anti-diabetic activity. The alcoholic extracts exhibited significant sucrase inhibitory activity. Thus these plants have great potential as a source for natural health products. The *Podocarpus gracilior* exhibited the highest sucrase enzyme inhibitor which subjected for chromatographic separation of polyphenolic compounds. The isolated compounds from *Podocarpus gracilior* resulted in identification of four known compounds, gallic acid, ellagic acid, cinnamic acid and apigenin. In addition of isolation of new polyphenolic compound. This compound was identified as 4“, 4“‘ -dimethoxy-32 ,82 2 -Biapigenin.

KEYWORDS

Meliaceae;
Podocarpaceae;
Fabaceae,
Anti-diabetic activity.

INTRODUCTION

Herbal medicines have been used since centuries by different cultures worldwide for treatment of diabetes. Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin action, insulin secretion, or both[1]. Control of postprandial hyperglycemia is critical in the early treatment of diabetes mellitus[2] as it could induce non enzymatic glycosylation of various proteins, resulting in the development of chronic complications such as, micro- and macrovascular diseases[3], and it has also been proposed as an independent risk factor for cardiovascular diseases[4&5]. Postprandial hyperglycemia can be controlled by decreasing the absorption of glucose through the inhibition of enzymes responsible for hydrolysis carbohydrate such as sucrase enzyme, in

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the digestive tract[6]. Phytochemicals exhibit their hypoglycemic effect by several mechanisms, such as, inhibition of carbohydrate metabolizing enzymes, manipulation of glucose transporters, α-cell regeneration, and enhancing the insulin releasing activity[7]. The present study deals with the investigation of the in-vitro anti-diabetic activity of several 5 plants belong to families *Meliaceae* (such as *Azadirachta indica*), Podocarbaceae (such as *Podocarpus gracilior*) and *Fabaceae* (such as *Acacia glauca, Acacia Senegal and Acacia nilotica*) through the performance of Sucrase inhibitory activity test.

MATERIALS AND METHODS

Preparation of the extracts

Powdered, air-dried leaves of *Azadirachta indica, Podocarpus gracilior, Acacia glauca, Acacia Senegal and Acacia nilotica* (250 g) were exhaustively extracted with hot 80% Ethanol (4x1 L), under reflux. After filtration the extracts were concentrated under vacuum at 50 C. The dry residues obtained were kept in the refrigerator.

Apparatus

JOEL GX-500 (500 and 125 MHz for ^{1}H and ^{13}C NMR), NMR department, National Research Center (NRC), and NMR in Strathclyde Institute of Pharmacy and Biomedical Science, Glasgow, United Kingdom were used. The δ-values were reported as ppm relative to TMS in DMSO-d_6 and *J*-values were in Hz. ESI-MS spectra were measured on Walters ACQUITY/TQD triple quadrupole, Center for Applied Research and Advanced Studies, Faculty of Pharmacy Cairo University. The UV analyses for pure samples were recorded on a Shimadzu UV 240 spectrophotometer, separately as solutions in methanol and with different diagnostic UV shift reagents[8&9]. Rotary evaporator (Bűchi, G, Switzerland). Fractionation of the extracts was done by columns chromatography using polyamide 6S (Riedel-De Hän Ag, Seelze Hannover, Germany), isolation and purification of compounds were done on either cellulose (Pharmacia, Uppsala, Sweden) or Sephadex LH-20 (Fluka, Switzerland) columns of different dimensions and eluted with different solvent systems (Figures 1 and 2). Separation processes were followed up by 2D-PC and CoPC using Whatmann No. 1 paper with (S_1) and (S_2) as in TABLE 1. Ultraviolet lamp (VL-215 LC, Marne La Vallee, France): It was used for visualization of spots on paper and thin layer chromatograms and follow up the columns fractionation on columns at 254 and/or 365 nm and also with sprayed Naturstoff reagent[10]. Gas liquid chromatography TRACE GC ULTRA was used for analysis of both total fatty acids (TFA) and unsaponifiable matter (USM) using GC/MS HP 6890 series (Agilent) MSD, Faculty of Agriculture, Cairo University according to the following conditions: Capillary column HP6890 series (30 m x 0.25 mm i.d. and 0.25 μm film thickness); detector: MSD; carrier gas: Helium, with flow rate: 1 ml/min; injector temperature: 270ºC; detector temperature: 280ºC; initial column temperature: 70ºC, programmed by 8ºC/min up to final temperature 270ºC within 20 min. GLC conditions for total fatty acids analysis: Capillary column: Thermo TR-FAME (70% cyanopropyl polysilphenylene siloxane) (30 m x 0.25 mm i.d. and 0.25 μm film thickness); detector: flame ionization; carrier gas: N_2, with flow rate 30 ml/min; injector temperature: 200°C; detector temperature: 220°C; initial column temperature: 140°C, programmed by 5°C/min up to final temperature 200°C within 12 min. Fluostar Optima microplate ELISA reader and 96 well cell culture microplates were used for pharmacological studies.

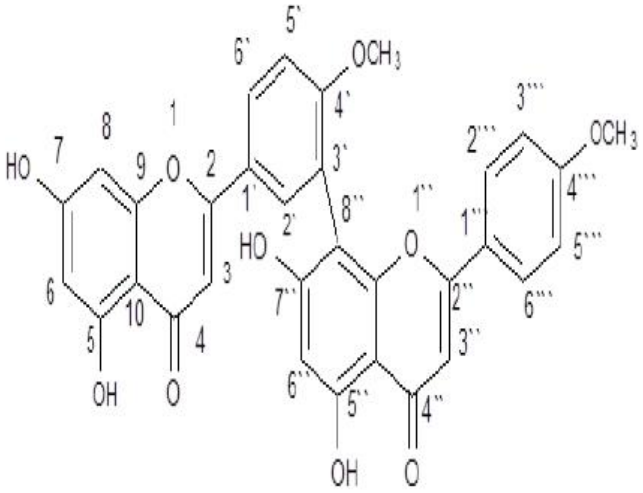

Figure 1: structure of 4“, 4“‘ -dimethoxy-32 ,82 2 -Biapigenin

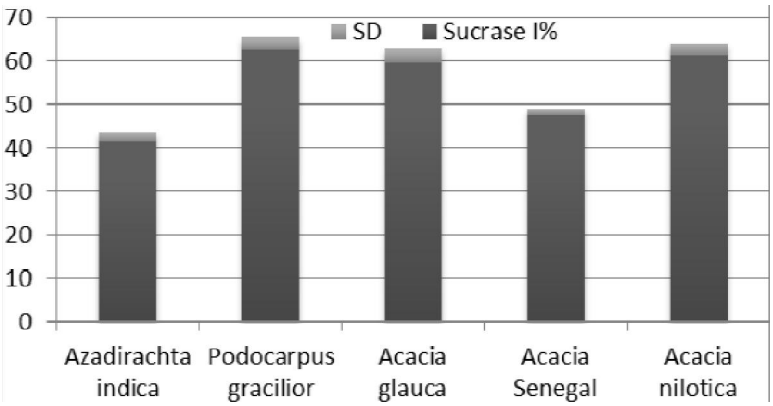

Figure 2: Sucrase enzyme activity

TABLE 1: Sucrase enzyme activity

Tested extract	Sucrase I%±SD
Azadirachta indica	41.34 ± 2.37
Podocarpus gracilior	62.54 ± 2.76
Acacia glauca	59.43 ± 3.42
Acacia Senegal	47.54 ± 1.33
Acacia nilotica	61.38 ± 2.46

Assay of sucrase inhibitory activity

A crude enzyme solution of rat intestinal sucrase enzyme was prepared according to the method of Dahlqvist[11]. The effect of samples on sucrase enzyme activity was assayed according to the method of Honda and Hara[12]. Enzyme solutions (10 µL) were incubated together for 10 min at 37°C, and the volume was made up to 200 µL with maleate buffer (pH 6.0) in case of control or up to 200 µL with buffer solubilized sample (100 µg/mL in maleate buffer with pH 6.0). The enzyme reaction was initiated by adding 100 µL of sucrose solution (60 mM). After 30 min, the reaction was terminated by adding 200 µL of 3,5-dinitrosalysilic acid reagent and placing the mixture in a boiling water bath for five min. The absorbance of solution was read at 540 nm. The percent inhibitory activities were calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and the Abs sample is the absorbance of the test sample; an untreated enzyme solution was used as the control. All experiments were carried out in triplicate.

RESULTS

Results of the anti-diabetic activity (Sucrase inhibitory activity)

Podocarpus gracilior decreases the activity of sucrase enzyme by (62.54 ± 2.76) followed by *Acacia nilotica* (61.38 ± 2.46), *Acacia glauca* (59.43 ± 3.42), *Acacia Senegal* (47.54 ± 1.33) and *Azadirachta indica* (41.34 ± 2.37), as shown in TABLE 1.

The isolated compounds from *Podocarpus gracilior* resulted in identification of four known compounds, gallic acid (1), ellagic acid (2), cinnamic acid (3) and apigenin (4). In addition of isolation of new polyphenolic compound this identified according to the following:

Compound (5): Yellow amorphous powder (30 mg). Chromatographic properties: R_f values; 0.72 (S_1), 0.03 (S_2); dark purple spot under UV-light turned to yellow fluorescence on exposure to ammonia vapors. It gave green color and yellowish green fluorescence with $FeCl_3$ and Naturstoff spray reagents respectively. UV-spectral data λ_{max} (nm) (MeOH): 270, 294,331; (+NaOMe): 278, 384; (+NaOAC): 277, 304, 354; (+$AlCl_3$): 280, 302(sh), 344, 392; (+$AlCl_3$/ HCl): 281, 301(sh), 344, 390. ^{1}H-NMR

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(500 MHz, DMSO-d_6): δ ppm 13.02 (1H, s, H-bounded OH-5), 12.88 (1H, s, H-bonded OH-5“), 8.14 (1H, dd, *J*=10.0, 2.0 Hz, H-6‘), 8.01 (1H, d, *J*=2.0 Hz, H-2‘), 7.56 (2H, d, *J*=8.9 Hz, H-2“‘/6“‘), 7.46 (1H, d, *J*=10.0 Hz, H-5‘), 7.31 (2H, d, *J*=8.9 Hz, H-3“‘/5“‘), 6.85 (1H, s, H-3“), 6.76 (1H, s, H-3), 6.67 (1H, d, *J*=1.8 Hz, H-8), 6.33 (1H, d, *J*=1.8 Hz, H-6), 6.16 (1H, s, H-6“), 3.75 (3H, s, O-CH_3), 3.71 (3H, s, O-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): δ ppm 182.67 (C-4“), 182.35 (C-4), 164.79 (C-2/2“), 163.93 (C-7), 162.79 (C-7“), 162.37 (C-5/5“), 162.01 (C-4“‘), 161.19 (C-4‘), 157.99 (C-9), 154.88 (C-9“), 131.47 (C-6‘), 128.58 (C-2“‘/6“‘), 128.37 (C-2‘), 123.41 (C-1‘), 123.12 (C-1“‘), 122.16 (C-3‘), 116.37 (C-5‘), 115.10 (C-3“‘/5“‘), 104.33 (C-8“), 103.80 (C-10/10“), 103.13 (C-3/3“), 99.46 (C-6“), 99.14 (C-6), 94.68 (C-8), 56.48 (C-OCH_3), 56.07 (C-OCH_3). Negative ESI-MS: *m/z* 565.17 $[M-H]^-$.

Compound 5 was expected to be a free flavone aglycone based on its chromatographic properties. The UV- spectrum in MeOH exhibited the two characteristic absorption bands at λmax (nm) 270 nm (band II) and 331nm (band I) of apigenin nucleus, together with the remaining diagnostic shift reagents confirming an apigenin structure[8]. Negative ESI-MS showed a molecular ion peak at *m/z* 565.17 ascribable to $[M-H]^-$ and corresponding to the molecular formula $C_{32}H_{22}O_{10}$ and M.wt. of 566. This information led us to suggest a biflavone structure of compound. The absence of any proton resonance signals in the aliphatic region in ^{1}H-NMR spectrum together with the splitting pattern of aromatic protons and the presence of two singlet signals at δ ppm 13.02 and 12.88 assigned for the protons of 5-OH and 5“-OH respectively gave the evidence of having aglycone biflavone structure. The AX coupling system of two ortho doublets, each integrated for two protons at δ ppm 7.56 and 7.31 assign to H-2“‘/6“‘ and H-3“‘/5“‘ respectively of 1“‘,4“‘-disubstituted ring-B, together with the two singlet signal resonance at δ ppm 6.85, 6.16 assignable to H-3“of ring-C and to

H-6“of ring-A respectively characteristic for apigenin flavone structure. The absence of H-8“ resonance signal gave the expectation of C-linkage on C-8“ of flavone to flavone. Also the ^{1}H-NMR spectrum showed the AM coupling system of the two meta-coupled douplets at δ ppm 6.67 and 6.33 assignable for H-8 and H-6 respectively characteristic for ring-A of flavones (unit I), in addition to singlet signal resonance at δ ppm 6.76 assignable for H-3 of ring-C and the signals at δ ppm 8.14 (dd), 8.01 (d), 7.46 (d) assignable to H-6‘, H-2‘and H-5‘ respectively of ring-B with the absence of H-3‘ resonance signal suggesting the C-linkage of the two flavone units between C-3‘ and C-8“. The two resonance singlet signals each integrated for three protons at δ ppm 3.75 and 3.71 was indicative for presence of two methoxy groups, the location of one methoxy group on C-7 of flavone unit I was deduced from the downfield shift of H-6 and H-8 at δ ppm 6.33 and 6.67 (≈+0.2), while the location of other methoxy group on C-4“‘ of flavones unit II was detected through the downfield shift of H-3“‘/5“‘ at δ ppm 7.31 (≈+0.5) and upfield shift of H2“‘/6“‘ at 7.56 (≈-0.4) in comparison with previously published data[13&14]. Further structure confirmation was obtained from ^{13}C-NMR spectrum that displayed typical carbon resonance signals for biflavone structure among which the characteristic two C-signals at δ ppm 182.64 and at δ ppm 182.35 that are describable for the two carbonyl carbons C-4“ and C-4 respectively. Moreover the C-linkage between the two flavone units was confirmed to be between C-3‘ and C-8“ from the downfield shift of C-3‘ at δ ppm 122.16 and of C-8“ at δ ppm 104.33 (+≈10). The presence of two methoxy groups in the biflavone structure was concluded from the two carbon resonance signals at δ ppm 56.48 and 56.07.

All 1D NMR signals, assigned in NMR spectra were finally proved from hetero-nuclear long range coupling 2D HMBC spectra which showed $^{2,3}J_{CH}$ coupling between O-Me protons at δ ppm 3.75 and 3.71 with C-7 at δ ppm 163.93 and C-4“‘ at δ ppm 162.01 respectively that proved the attachment of methoxy gps on these carbons. Also showed $^{2,3}J_{CH}$ coupling between (OH-5) at δ ppm 13.02 with C-10 at δ ppm 103.80, while in the spectrum showed the most important $^{2,3}J_{CH}$ coupling between H-2‘ at δ ppm 8.01 with C-8“ at ä ppm 104.33 confirming the position of C-linkage in biflavone structure between C-3‘ and C-8“. Other $^{2,3}J_{CH}$ coupling were between H-6‘ (8.14) with C-2‘(128.37), H-3“‘/5“‘ (7.31) with C-1“‘ (123.12), H-3“ (6.85) with C-1“‘

(123.12), H-8 (6.67) with C-10 (103.80), H-6 (6.33) with C-10 (103.80), and H-6" (6.16) with C-8" (104.33). The $^{2,3}J_{CH}$ coupling between H-6' (8.14) with C-4' (161.19), H-2' (8.01) with C-4' (161.19), H-5' (7.46) with C-4' (161.19), H-2"'/6"' (7.56) with C-4"' (162.01), H-3" (6.85) with C-2" (164.79), H-3 (6.76) with C-2 (164.79), H-8 (6.67) with C-7 (163.93), H-6 (6.33) with C-5 (162.37).

Therefore, the structure of compound was finally identified after the comparison of corresponding peaks in 1D and 2D NMR spectra with reported literature of similar compounds[14-17]. Thus compound was confirmed as 4", 4"' -dimethoxy-32 ,82 2 - Biapigenin (Figure 1) which is isolated for the first time from *Podocarpus gracilior.*

DISCUSSION

It is a general opinion that medicinal plants inhibit sucrase activity due to the presence of several possible factors and mechanisms, such as polyphenolic concentration[18]. In the our present investigation, the effect of various extracts of tested plants on carbohydrate hydrolyzing enzyme, namely, rat intestinal sucrase, have been studied using *in vitro* model systems. The extracts of tested plants significantly inhibited sucrase enzyme activity (Figure 1). The Sucrase inhibitory activity of *tested plants* supposed to be due to the presence of flavonoid glycosides and/or hydrolysable tannins[19]. With a constant rise in the incidence of type II diabetes around the world it appears that more anti-diabetic drugs with complementary mechanisms of action should be developed, in order to achieve durable glycemic control by inhibiting, in a reversible way, the hydrolysis of disaccharides and the ultimate steps of the digestion of dietary polysaccharides, to reduce the rise of postprandial blood glucose in diabetics[20]. Rat intestinal sucrase occurs as a complex of sucrase and isomaltase, which converts sucrose into glucose[21]. So the tested plants (i.e. *Azadirachta indica, Acacia nilotica, Acacia glauca* and *Acacia Senegal)* may offer a support in treatment of diabetic disease.

CONCLUSION

The ethanol extract of the leaves of *Podocarpus gracilior* (family Podocarbaceae), *Azadirachta indica* (family *Meliaceae), Acacia nilotica, Acacia glauca* and *Acacia Senegal* (family *Fabaceae*) leaves exhibited significant in-vitro anti-diabetic activity using Sucrase inhibitory activity test. But previous studies observed that those plants which belonging to the family *Fabaceae* (Mimosaceae) contain the toxic alkaloid mimosine[18&22], so we recommend in vitro and in-vivo toxic test to be done to evaluate their safety to be used as complementary drugs to help main medicines in treatments of diabetic patients.

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