Research Article

FLAVONOIDS FROM *RHYNCHOSIA PSEUDO-CAJAN* AS SUITABLE α -GLUCOSIDASE INHIBITORS AND FREE RADICAL SCAVENGERSMuhammad Athar Abbasi^{1*}, Ghulam Hussain¹, Aziz-ur-Rehman¹ and Viqar Uddin Ahmad²¹Department of Chemistry, Government College University, Lahore, Pakistan²HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

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ABSTRACT

A series of nine flavonoids, 1-9, isolated from *Rhynchosia pseudo-cajan* Cambess., has been investigated in present work for their biological potential against α -glucosidase, lipoxigenase (LOX), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes; and antioxidant activity against diphenylpicrylhydrazyl (DPPH) radical respectively. The results revealed that these flavonoids are very suitable inhibitors of α -glucosidase and moderate ones of AChE enzyme. Against BChE enzyme, only myricetin (7) while against LOX, quercetin (6) and myricetin (7) remained active as shown by their IC₅₀ values. Moderate to excellent scavenging activity was also depicted by these molecules against DPPH. The modes of interaction of some active compounds against α -glucosidase and BChE were computationally observed and correlated with the experimental results.

Keywords: α -Glucosidase, AChE, BChE, DPPH, flavonoids, LOX.

INTRODUCTION

Flavonoids are the part of many plants including fruits and vegetables as well as beverages^{1,2}. Out of known 4000 flavonoids, many are part of our regular diet³. Flavonoids have gained much importance because of their multiple activities including antimicrobial⁴, cytotoxicity⁵, anti-inflammatory⁶, anti-tumor⁷ and antioxidant⁸⁻¹¹. α -Glucosidase (α -D-glucosideglucohydrolase, EC 3.2.1.20), a family of hydrolase enzymes¹², hydrolyze the 1,4-glycosidic linkage to produce α -D-glucose and other monosaccharide that are utilized as carbon and energy source¹³. The inhibitors of this enzyme hinder the liberation of D-glucose and hence reduced postprandial hyperglycemia and type-2 diabetes¹⁴. Lipoxigenase (LOX, EC 1.13.11.12) are implied in arachidonic acid metabolism and generation of various biologically active lipids owning a primary role in inflammation. The inhibitors of this enzyme are employed for the treatment of a variety of disorders such as bronchial asthma, inflammation, cancer and autoimmune diseases^{15,16}. Cholinesterase enzymes (Acetyl cholinesterase, AChE, EC 3.1.1.7 and butyryl cholinesterase, BChE, EC 3.1.1.8) include serine hydrolases. This enzyme system is responsible for the termination of acetylcholine at cholinergic synapses. The major function of AChE and BChE is to catalyze the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses¹⁷. BChE is significantly present in Alzheimer's plaques than in the normal age related non dementia of brains¹⁸. In the present work nine known flavonoid molecules (Figure 1), namely flavone¹⁹ (1), apigenin²⁰ (2), kaempferide²¹ (3), kaempferol²² (4), catechin²³ (5), quercetin²⁴ (6), myricetin²⁵ (7), hesperidin²⁶ (8) and rutin²⁷ (9) were isolated from different fractions of *Rhynchosia pseudo-cajan* and their spectral data was coherent with reported literature as referenced. These molecules were then assessed for their anti-enzymatic and antioxidant activities.

MATERIALS AND METHODS

General

The structures of isolated molecules, 1-9, were corroborated through ¹H- and ¹³C-NMR spectral data. All chemicals used in the experiments were of analytical grade and were used after purification by distillation. For column chromatography (CC), silica gel (70-230 mesh) was used. TLC was performed on pre-coated silica gel G-25-UV254 plates. Detection was carried out at 254 nm and by ceric sulphate reagent/aniline phthalate reagent. Purity was checked on TLC with different solvent systems by using methanol, acetone and chloroform giving single spot. ¹H and ¹³C-NMR spectra were recorded in CD₃OD on Bruker spectrometers operating at 400, 500 or 600 MHz. For further assistance, literature on spectral data has been referenced for each molecule.

Plant Material

The plant *Rhynchosia pseudo-cajan* Cambess. was collected from Kotli, Azad Kashmir in July 2012 and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen (GC. Herb. Bot. 623) has been deposited in the herbarium of the same university.

Extraction, Fractionation and Isolation

The shade-dried ground whole plant (11 kg) was exhaustively extracted with methanol (16 L $\times$ 4) at room temperature. The extract was evaporated on rotavapour to a greenish-black residue (861 g), which was dissolved in distilled water (1.5 L) and partitioned with *n*-hexane (1.5 L $\times$ 4), chloroform (1.5 L $\times$ 4), ethyl acetate (1.5 L $\times$ 4) and *n*-butanol (1.5 L $\times$ 4), respectively. The chloroform soluble fraction (113 g) was subjected to the CC over the silica gel using solvent system of *n*-hexane with the gradient of CHCl₃ (0 to 100 %) to get ten fractions (Fr. 1-10). Fraction 9 (1.2 g) was subjected to CC and eluted with *n*-hexane:CHCl₃ (3:7) to get compound 1 (9.7 mg). Fraction 10 (1.9 g) was loaded on silica gel and

eluted with *n*-hexane: CHCl₃ (0.5:9.5) to afford compound 2 (14 mg). The ethyl acetate soluble fraction (150 g) was subjected to the CC over the silica gel by using CHCl₃ with gradient of acetone and then CH₃OH to get thirteen fractions (Fr. 1-13). The fraction 6 (1.7 g) was purified on CC using CHCl₃:acetone (6.5:3.5) as eluent to afford compound 3 (15.2 mg). Fraction 7 (2.4 g) showed two major spots, subjected to the preparative TLC to isolate compound 4 (16 mg) and 5 (18.4 mg). Fraction 9 and 10 were further purified by using gradient elution of CHCl₃:acetone (6:4 and 5:5 respectively) to get compound 6 (23.4 mg) and 7 (19.8 mg) respectively. Similarly compounds 8 (22.9 mg) and 9 (12.6 g) were obtained by using chloroform:acetone:methanol (7.5:1.5:1 and 6.5:2.5:1 respectively) as eluent in column chromatography.

Lipoxygenase Assay

Lipoxygenase activity was assayed according to the reported method²⁸ with minor modifications. 200 µL lipoxygenase assay mixture containing 150 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL test compound and 15 µL purified lipoxygenase enzyme was prepared. The contents were mixed, pre-read at 234 nm and pre-incubated for 10 minutes at 25°C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance noticed after 6 minutes at 234 nm was used as index for the inhibition. All reactions were performed in triplicates. The positive and negative controls were incorporated in the assay. Baicalein (0.5 mM well⁻¹) was used as a positive control. The percentage inhibition (%) was calculated as,

$$\text{Inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Where Control = Total enzyme activity without inhibitor;
Test = Activity in the presence of test compound

IC₅₀ values (concentration at which there is 50 % enzyme inhibition) of compounds was calculated using EZ-Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA). IC₅₀ values were determined by serial dilution of the compounds from 0.5 mM to 0.25, 0.125, 0.0625, 0.03125, 0.015625 mM and from the graph. Values are mean of 3 independent experiments.

α-Glucosidase Assay

The α-glucosidase inhibition activity was performed in relation to the slightly modified method²⁹. Volume of the reaction mixture was made 100 µL by adding 70 µL of 50 mM phosphate buffer saline with pH of 6.8, 10 µL (0.5 mM) test compound and 10 µL (0.057 units) enzymes. The contents were mixed, pre-incubated for 10 minutes at 37°C and pre-read at 400 nm. The reaction was initiated by the addition of 10 µL of 0.5 mM substrate (*p*-nitrophenylglucopyranoside). Acarbose was used as positive control. After 30 minutes of incubation at 37°C, absorbance was measured at 400 nm using Synergy HT micro plate reader. All experiments were carried out in duplicates. The percent inhibition was calculated by the same procedure as mentioned for the lipoxygenase assay.

Cholinesterase Assays

The AChE and BChE inhibition study was performed according to the established method³⁰ with slight modifications. 100 µL reaction mixture contained 60 µL Na₂HPO₄ buffer having conc. of 50 mM with pH 7.7 was prepared. Test compound of volume ten µL and conc. of 0.5 mM well⁻¹ was poured, accompanied by the accession of ten µL enzyme of conc. 0.005 unit well⁻¹. All contents were immixed and pre-read at a wavelength of 405 nm. After that pre-incubation of the contents for 10 minutes at 37°C was performed and the initiation of the reaction was done through 10 µL of conc. 0.5 mM well⁻¹ substrate i.e acetylthiocholine iodide (for AChE) or butyrylthiocholine chloride (for BChE). Then the ten µL of DTNB with conc. 0.5 mM well⁻¹ were added. After incubation of 15 minutes at 37°C, absorbance at 405 nm was measured by 96-well plate reader Synergy HT, Biotek, USA. All the observations were carried out in triplicate with their respective controls. Eserine of conc. 0.5 mM well⁻¹ was applied as a positive control. The results were calculated as per formula mentioned for the lipoxygenase assay.

Scavenging Activity by DPPH Method

The stable 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) was used for the determination of antioxidant activity³¹. Different concentrations of compounds in relevant solvents (10 µL) were added to 90 µL of 100 µM methanolic DPPH in a total volume of 100 µL in 96-well plates. The contents were mixed and incubated at 37°C for 30 minutes. The absorbance was measured at 517 nm (using Synergy HT BioTek® USA microplate reader). The synthetic quercetin was used as standard antioxidants. The experiments were carried out in triplicates. The decrease in absorbance indicates increased radical scavenging activity which was determined by same procedure as mentioned for the lipoxygenase assay.

Statistical Analysis

All the measurements were measured in triplicate and statistical analysis was performed by Microsoft Excel 2007. Results are presented as mean ± sem.

Protein Preparation

The protein molecules included in our study, α-glucosidase and BChE were retrieved from Protein Data Bank. Water molecules were removed and the 3D protonation of the protein molecule was performed using MOE applications. The energy of the protein molecules were minimized *via* energy minimization algorithm of MOE tool. The following parameters were used for energy minimization; gradient: 0.05, Force Field: MMFF94X+Solvation, Chiral Constraint: Current Geometry. Energy minimization was terminated when the root mean square gradient falls below the 0.05. The minimized structure was used as the template for Docking.

Molecular Docking

The binding mode of the ligands into the binding pocket of protein molecule was predicted by MOE-Dock implemented in MOE. After the completion of docking we analyze the best poses for Hydrogen Bonding/π-π interactions by using MOE applications³².

Figure 1: Structures of compounds 1-9

Table 1: Physical characteristics of flavonoids 1-9

Compound	Name	Physical state	Molecular formula	Molecular mass	Melting point
1	Flavone	White amorphous solid	C ₁₅ H ₁₀ O ₂	222	95°C
2	Apigenin	Light green amorphous solid	C ₁₅ H ₁₀ O ₅	272	345-350°C
3	Kaempferide	Yellowish amorphous powder	C ₁₆ H ₁₂ O ₆	300	229-232°C
4	Kaempferol	Yellowish amorphous powder	C ₁₅ H ₁₀ O ₆	286	276-278°C
5	Catechin	Green crystalline solid	C ₁₅ H ₁₄ O ₆	290	178-180°C
6	Quercetin	Yellowish amorphous powder	C ₁₅ H ₁₀ O ₇	302	316°C
7	Myricetin	Yellowish green amorphous powder	C ₁₅ H ₁₀ O ₈	318	360°C
8	Hesperidin	Yellowish green amorphous powder	C ₂₈ H ₃₄ O ₁₅	610	250-255°C
9	Rutin	Greenish yellow amorphous solid	C ₂₇ H ₃₀ O ₁₆	610	195°C

Table 2: Enzyme inhibition activities of flavonoids 1-9

Compound	α -Glucosidase enzyme		LOX enzyme		AChE		BChE	
	%age Inhibition	IC ₅₀ (μ M)	%age Inhibition	IC ₅₀ (μ M)	%age Inhibition	IC ₅₀ (μ M)	%age Inhibition	IC ₅₀ (μ M)
1	2.41 $\pm$ 0.88	-	37.33 $\pm$ 1.17	-	36.36 $\pm$ 0.68	> 500	32.12 $\pm$ 0.91	> 500
2	90.54 $\pm$ 1.24	54.12 $\pm$ 0.75	49.56 $\pm$ 1.21	-	55.11 $\pm$ 0.91	242.23 $\pm$ 0.35	23.84 $\pm$ 0.63	> 500
3	93.13 $\pm$ 1.45	48.23 $\pm$ 0.98	31.83 $\pm$ 1.09	-	25.92 $\pm$ 0.67	> 500	11.45 $\pm$ 0.98	-
4	83.41 $\pm$ 0.98	69.75 $\pm$ 0.85	44.56 $\pm$ 1.12	-	64.61 $\pm$ 0.95	166.71 $\pm$ 0.27	48.79 $\pm$ 0.88	> 500
5	65.18 $\pm$ 0.95	375.21 $\pm$ 0.88	43.67 $\pm$ 1.06	-	34.31 $\pm$ 0.82	> 500	44.24 $\pm$ 0.36	> 500
6	84.35 $\pm$ 0.55	18.21 $\pm$ 0.44	92.94 $\pm$ 1.22	160.71 $\pm$ 1.01	95.42 $\pm$ 0.85	155.22 $\pm$ 0.23	41.34 $\pm$ 1.11	> 500
7	99.21 $\pm$ 0.75	14.72 $\pm$ 0.25	93.44 $\pm$ 1.23	177.92 $\pm$ 1.26	114.61 $\pm$ 1.16	37.12 $\pm$ 0.24	89.37 $\pm$ 0.79	95.9 $\pm$ 0.25
8	8.45 $\pm$ 0.88	-	26.72 $\pm$ 1.05	-	24.86 $\pm$ 0.76	> 500	10.25 $\pm$ 0.52	-
9	14.64 $\pm$ 0.86	-	85.22 $\pm$ 1.29	-	25.94 $\pm$ 0.76	> 500	51.44 $\pm$ 0.49	487.21 $\pm$ 0.25
Control	92.23 $\pm$ 0.14 ^a	38.25 $\pm$ 0.12 ^a	93.79 $\pm$ 1.27 ^b	22.4 $\pm$ 1.3 ^b	91.27 $\pm$ 1.17 ^c	0.04 $\pm$ 0.0001 ^c	82.82 $\pm$ 1.09 ^c	0.85 $\pm$ 0.0001 ^c

All compounds were dissolved in methanol and experiments were performed in triplicate (mean $\pm$ sem, n = 3). a = Acarbose, b = Baicalein, c = Eserine, AChE = acetylcholinesterase, BChE = butyrylcholinesterase LOX = lipoxigenase

Table 3: Antioxidant activity of flavonoids 1-9

Compound	DPPH	
	%age Inhibition	IC ₅₀ (μM)
1	1.98 ± 0.87	-
2	5.33 ± 0.54	-
3	85.83 ± 0.91	270.31 ± 0.13
4	88.92 ± 0.98	163.32 ± 0.27
5	88.17 ± 0.76	65.32 ± 0.11
6	93.21 ± 0.97	16.96 ± 0.14
7	83.92 ± 0.93	33.53 ± 0.62
8	21.76 ± 0.55	-
9	86.42 ± 0.93	43.74 ± 0.15
Control	93.21 ± 0.97 ^a	16.96 ± 0.14 ^a

All compounds were dissolved in methanol and experiments were performed in triplicate (mean ± sem, n = 3). a = Quercetin (same as 6), DPPH = 1, 1-diphenyl-2-picrylhydrazyl radical

Figure 2: The 2D interaction analysis of myricetin (7) against α -glucosidase

Figure 3: The 2D interaction analysis of myricetin (7) against BChE

RESULTS AND DISCUSSION

The physical data, enzyme inhibition and antioxidant activity results have been tabulated in the Table 1, Table 2 and Table 3, respectively. The results revealed that the isolated molecules exhibit a suitable inhibitory potential against α -glucosidase enzyme along with optimum scavenging activity. A computational approach was also adopted to find out the binding modes of these inhibitors against α -glucosidase and BChE enzymes. Molecular Operating Environment (MOE) was used for docking studies³² and the results exposed that the isolated inhibitors have decent affinity with the binding cavity of target enzymes.

α -Glucosidase Inhibition

Almost all the molecules depicted inhibition activity except flavone (1), hesperidin (8) and rutin (9). The potent inhibitory potential (almost three times of reference standard) was executed by myricetin (7) and almost two times better by quercetin (6) with IC_{50} values of 14.72 ± 0.25 and 18.21 ± 0.44 $\mu\text{mol/L}$ respectively, relative to acarbose, the reference standard having IC_{50} value of 38.25 ± 0.12 $\mu\text{mol/L}$. The promising activity of both 7 and 6 was credibly because of presence of 3, 4, 5-trihydroxy and 3, 4-dihydroxy substituted phenyl rings, respectively. The docking study of 7 revealed that it interacted with six amino acids residues from the binding cavity of target enzyme. The hydroxyl groups were involved in most of the observed interactions. Gly109 and Asp267 interacted with hydroxyl group of substituted resorcinol moiety, whereas the other four interactions were observed with hydroxyl group of benzenetriol moiety (Figure 2). Likewise all the compounds showed good binding pattern. Other compounds with relatively better inhibitory action were 3, 2 and 4 with IC_{50} values of 48.23 ± 0.98 $\mu\text{mol/L}$, 54.12 ± 0.75 , 69.75 ± 0.85 , respectively, relative to the reference standard. The decreasing order of the active compounds was as under: 7 > 6 > 3 > 2 > 4 > 5.

Lipoxygenase (LOX) Inhibition

Only two flavonoids, myricetin (7) and quercetin (6) exhibited moderate inhibitory potential against lipoxygenase enzyme having IC_{50} value of 177.92 ± 1.26 and 160.71 ± 1.01 $\mu\text{mol/L}$ respectively, relative to baicalein, a reference standard with IC_{50} value of 22.4 ± 1.3 $\mu\text{mol/L}$ (Table 2). The series overall showed the least activity against this enzyme.

Cholinesterases Inhibition

It is clearly evident from the results (Table 2) that the only myricetin (7) was found to be promising inhibitor against acetyl cholinesterase enzyme having IC_{50} value of 37.12 ± 0.24 $\mu\text{mol/L}$ relative to eserine, a reference standard with IC_{50} value of 0.04 ± 0.0001 $\mu\text{mol/L}$. The remaining compounds were moderately less active with the order of 6 > 4 > 2. The butyryl cholinesterase enzyme was inhibited by myricetin (7) and rutin (9) with IC_{50} values of 95.9 ± 0.25 and 487.21 ± 0.25 $\mu\text{mol/L}$ respectively, relative to eserine, a reference standard with IC_{50} value of 0.85 ± 0.0001 $\mu\text{mol/L}$. The active compound 7 showed good binding pattern with the active site of BChE. Five interactions were observed between binding pocket and docked compound. Asp285 makes hydrogen bond linkage with benzenetriol moiety, Ser186 interacts with hydroxyl group of hydroxypyran moiety. Trp279 makes arene-arene bond with substituted resorcinol

ring while Tyr121 interacts with hydroxyl group of the same moiety (Figure 3).

Antioxidant Activity

The results have shown that all the compounds expressed promising activity but 1, 2 and 8 remained inactive (Table 3). The better scavenging activity was depicted by compounds 6 (same molecule as standard), 7 and 9 with IC_{50} values of 16.96 ± 0.14 , 33.53 ± 0.62 and 43.74 ± 0.15 , respectively, relative to quercetin, the reference standard with IC_{50} value of 16.96 ± 0.14 $\mu\text{mol/L}$. The decreasing order of all the active natural compounds was 6 > 7 > 9 > 5 > 4 > 3.

CONCLUSION

It may be concluded from the present investigation that the isolated flavonoids have moderate to talented activity against α -glucosidase enzyme and DPPH radical; moderate against acetyl cholinesterase; and low activity against lipoxygenase and butyryl cholinesterase enzymes. So, such molecules might serve as possible therapeutic agents for diabetes and suitable antioxidants to rescue the oxidative stress.

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