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IN VITRO INHIBITORY ACTIVITIES OF SHORT PEPTIDES PURIFIED FROM *COMBRETUM MICRANTHUM* AGAINST *α*-AMYLASE AND α-GLUCOSIDASE

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ABSTRACT

This work is aimed at evaluating the inhibitory activities of peptides partially purified from *Combretum micranthum* (CM)methanol leaf extract against α-glucosidase and α-amylase which are two key enzymes involve in starch degradation and considered as model pathway for new drug discovery for the treatment and prevention of diabetes. Liquid chromatography- Mass Spectroscopy was used to identify the peptides present in the most potent fraction of CM methanol extract and Column chromatography-TLC was used to further fractionate it into sub fractions. The *invitro* inhibitory activities of the subfractions were carried out using spectrometric method. The results showed a significant ($p<0.05$) Inhibitory activity of sub fractions A, B and C against α-amylase compared to acarbose as control. Significance ($P<0.05$) difference was found between acarbose and fractions; A, B and C at all substrate concentration (10, 20, 30, 40, and 50 µg/ml respectively) of CM sub fractions against α-glucosidase. In conclusion, short peptides isolated from *C. micranthum* have antidiabetic potentials.

Keywords: Diabetes, Chromatography, Peptides, α-glucosidase, and α-amylase Spectroscopy.

Introduction

Combretum micranthum commonly known as kinkeliba belongs to the family of *Combretacea*. It is known as Farargeza in Hausa, Nzaotego in Igbo, and Okan in Yoruba. It's an undomesticated shrub species found in the tiger bush region of western Africa (Welch *et al*., 2018). It was reported to have antidiabetic potentials (Ibrahim *et al*., 2018). Methanol leaf extract of *Combretum micranthum* has been reported to reduce oedema in rats (Olajide *et al*., 2003).Given the special concern over the recent increase in the prevalence of diabetes worldwide and the side effect of antidiabetic drugs, the role of short peptides on glucose metabolism and insulin resistance in the human body is of paramount importance.

Globally, diabetes is one of the most frequent non-communicable, lifestyle-related diseases with a permanent growth in the incidence. Every 5 seconds someone somewhere in the world is diagnosed with diabetes, and every 10 seconds someone dies of it (Colagiuri, 2010). The latest prevalence figure published by International Diabetes Federation (IDF) is 425 million persons living with diabetes worldwide with nearly 50% of these figures undiagnosed and its projecting to over 500 million by the year 2030 (Sabir *et al*., 2018). Peptides structurally related to the insulin receptor (IR)-binding protein mcIRBP-19 have been identified in various plants that have IR kinase-activating abilities similar to mcIRBP-19 that has a proven blood glucose-lowering activity that exhibits IR-binding potentials (Lo *et al.*, 2016; Sanchez and Vazquez, 2017). The control of some key enzymes such as α-glucosidase and α-amylase which are involved in starch degradation is considered a model pathway for new drug discovery for the treatment and prevention of diabetes. These enzymes can degrade carbohydrate into glucose, which can be further absorbed into body. Alpha-glucosidase and dipeptidyl peptidase IV (DPP-IV) are enzymes that play a significant role in the development of type 2 diabetes (T2D). The beneficial effects of these inhibitory peptides can be explained through several mechanisms, such as the satiety response, regulation of incretin hormones, insulinemia levels, and reducing the activity of carbohydrate degrading digestive enzymes (Patil *et al.*, 2015; Sanchez and Vazquez, 2017). This work is aimed at evaluating the *invitroα*-amylase and α-glucosidase activities of short peptides isolated from *Combretum micranthum* methanol leaf extract.

Materials and Methods

Leaves of *Combretum micranthum* (CM) were collected fresh from Filing Shagari, Bauchi State and identified at the herbarium section of the Department of Biological Sciences, Bayero University Kano. A voucher number BUKHAN 0349 was deposited (Ibrahim *et al*., 2017). The most potent fraction used in this work was partially purified by Ibrahim *et al*., 2017 in a bioassay guided fractionation using column chromatography – TLC and analytical preparative thin layer chromatography.

Identification of the Purified Fraction

The purified fraction was identified and further Separated at ZULIANA BINTI ZAINAL ABIDIN INFRA Analytical Laboratory, Level 3 Research Management

and Innovation Complex, University of Malaya, Kaula Lumpur, using Ultra High-Performance Liquid Chromatography (UHPLC). The UHPLC system (Agilent infinity 1290, USA) was incorporated with a binary pump (G4220A), an auto sampler (HiP - ALS, G4226A), degasser (G1322A), Thermostatted Column Compartment (G1316C) and a DAD detector (G4212A) with a light-pipe flow cell (recording at 280 and 320 nm). This was coupled to Ultra-High-resolution-Quadrupole-Time-of-Flight Mass Spectrometer (G6550A) (USA) equipped with an ESI source (DUAL AJS ESI) operating on Auto-MS/MS mode. The analysis was achieved in the negative ion mode in a mass range from m/z 100-2000.

Further Separation of the Potent Fraction Using Column Chromatography-TLC

The potent fraction of *Combretum micranthum* (CM) methanol leaf extract as reported by Ibrahim *et al*., 2017 to have the highest hypoglycemic effect was further separated at the Biochemistry Laboratory, Bayero University, Kano using Column chromatography thin layer chromatography as described by Ode *et. al* (2011). Twenty-five 25 g of the potent fraction of CM was further separated into its component fractions. Sixty grams (60g) of Silica gel (60 – 120G) was used as the stationary phase while ethylacetate: methanol in varying proportion (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100) of increasing polarity were used as the mobile phase. In the setting up of column chromatography, the lower part of the glass column (5.5 x 80cm) was stocked with glass wool with the aid of glass rod. The slurry was prepared by mixing 90g of silica gel and 100 ml of ethyl acetate.A measured volume (100 ml) of each solvent combination was collected gradually with a 10 ml syringe. The eluted fractions were collected in aliquots of 50ml in conical flasks, thus resulting in collecting 54 fractions. Which were subjected to analytical TLC.

The content of each conical flask was spotted on pre-coated silica gel (XtraSIL G/UV_{254}) on aluminum plates in a small chromatographic tank to separate the different fractions based on their relative mobilities in solvent systems ethyl acetate: methanol (8:2). A spot of the sample was applied on the plate about 1.0cm from the edge allowed to dry at room temperature (27^0C). The strip was introduced into a small chromatographic jar containing the solvent system ethyl acetate: methanol (8:2). The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried at room temperature (27^0C). The plates were sprayed with a solution of freshly prepared 0.5ml p-anisaldehyde in 50ml glacial acetic acid and 10 ml 97% sulphuric acid and heated to 105°C until maximum visualization of spots were identified (mostly violet, purple, and red as observed).The fractions with same Rf values were pooled together and kept at 4^0C in the refrigerator for further study

The visible spot was measured with a ruler and the relative Retention factor (Rf) value calculated based on the formula:

Rf = Distance travelled by the streak from the starting point / Distance travelled by the

solvent from the starting point to the solvent front

α -Amylase Inhibition Assay

The α-amylase inhibitory assay was performed using Oboh *et al*., (2010) method. Three (3) tubes were set up. Aqueous dilution of the fractions (50µl) and 500µl of 20mM sodium phosphate buffer (pH, 6.9, with 6mM NaCl) containing porcine pancreatic α-amylase (SigmaAldrich) (0.5mg/ml) was incubated at 25^{o}C for 10 minutes (one unit of the enzyme liberated 1.0mg of maltose from starch in 3 minutes). Then, 500µl of 1% starch solution in 20mM sodium phosphate buffer (pH, 6.9, with 6mM NaCl) was added to each tube. The reaction mixtures were incubated at 25^{o}C for 10 minutes and stopped with 1.0ml of 3,5-dinitrosalicylic acid (DNSA) colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 minutes and cooled to room temperature (25^{o}C). The reaction mixture was then diluted by adding 10ml of distilled water, and absorbance was measured at 540nm. The control experiment was performed without the test sample, and the α-amylase inhibitory activity was expressed as percentage inhibition using the following expression.

Percentage of inhibition (% I)= [($Abs_{control}$ − Abs_{sample})] ×100/Absorbance of control

Where $Abs_{control}$ is the absorbance of the mixtures without the sub-fraction, and Abs_{sample} is the absorbance of the mixture with the sub-fraction.

α-Glucosidase Inhibition Assay

The assay was performed as described by Apostolidis *et al.,* (2007). Five (5) dilutions of the vegetable oils (50µl) and 100µl of α-glucosidase (SigmaAldrich) solution (1.0U/ml) in 0.1M phosphate buffer (pH, 6.9) was incubated at 25^{0}C for 10 minutes. (One unit of the enzyme liberates 1.0 µmol of D-glucose from p-nitrophenyl-α-D-glucoside per minute) Then, 50µl of 5mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1M phosphate buffer (pH, 6.9) was added. The mixtures were then incubated at 25^{0}C for 5 minutes, and the absorbance was measured at 405nm. The control experiment was performed without the test sample, and the α-glucosidase inhibitory activity was expressed as percentage inhibition using the following expression:

Percentage of inhibition (I) = [($Abs_{control}$ − Abs_{sample})] ×100 / Absorbance of control

Where $Abs_{control}$ is the absorbance of the mixture without the potent fraction, and Abs_{sample} is the absorbance of the mixture with the potent fraction.

Phosphorylated Insulin Receptor Tyrosine Kinase Activity Assay:

The assay was performed according to the protocol described by Macrini *et al.* (2009), with some modifications. Aliquots of 10 µL of a solution composed of 125 UmL-1 of mushroom tyrosinase (Sigma-Aldrich, USA) were added to 96-well microplates, and then 70 µL of pH 6.8 phosphate buffer solution and 60µL of the Amazonian native flora oils (350µg mL-1, in 25% dimethyl sulphoxide, DMSO) were also added. For the positive control, 60µL of kojic acid (17.5µg mL-1 in 25% DMSO) was used instead of the Amazonian oil, and for the negative control, 60µL of 2.5% DMSO was added. To the

resultant mixture, 70µL of tyrosine kinase (Sigma-Aldrich, USA) were added at a concentration of 0.3mgmL-1 in distilled water (the final volume in the wells was 210µL). The absorbance of the microplate wells was read using a microplate spectrophotometer (SpectraCount, Packard, USA) at 510nm.

Statistical Analysis

All analysis were carried out in triplicate and data obtained were subjected to analysis of variance (ANOVA) using SPSS version 16.0 and values with $p<0.05$were considered to be significantly different.

RESULTS AND DISCUSSION

Table 1 showed the compounds identified in *Combretum micranthum* sub fractions A, B and C through liquid chromatography-mass spectroscopy (LC-MS) technique. The compounds include Desacetylcolchicine, C16 Sphinganine, C17 Sphinganine, Convallasaponin A, Ile-Arg-Ile, Thioridazine, Trp-Lys-Lys, Tyr-Ser-Arg, Phloionolic acid, 24-Nor-5β-cholane-3α,7α,12α,22,23-pentol, 2-deoxy-20-hydroxy-5alpha-ecdysone 3-acetate, Nonoxynol-9, 2-amino-hexadecanoic acid and N-cyclohexanecarbonylpentadecyl-amine. Table 2 showed the retention factors of the pooled sub fractions A, B and C respectively and they weighed 0.63g, 1.71g and 0.82g respectively. Inhibitory activity of sub fractions A, B and C against α-amylase (Table 3), revealed a significant ($p<0.05$) between acarbose as control and fraction; A, B, and C respectively at 10µg/ml, 30µg/ml, 40µg/ml and 50µg/ml concentration, respectively. But there was no significant($P>0.05$) difference at substrate concentration of 10µg/ml and 30µg/ml. Significance ($P<0.05$) difference was found between acarbose and fraction; A, B and C at all substrate concentration (10, 20, 30, 40, and 50 µg/ml respectively) of CM sub fractions against α-glucosidase. Also fraction A was statistically different ($p<0.05$) from fraction B and C, respectively at 10µg/ml, 20µg/ml,40µg/ml, and 50µg/ml. This implies synergistic activities of the different fractions. Similar observation was made by Kaur and Singh (2015) who reported dual inhibitory activity of peptides isolated from *Aspergillus awamori*. *Combretum micranthum* have also demonstrated the ability to control diabetes through inactivation of α-glucosidase and α-amylase, the key enzymes that hydrolyze polysaccharide into glucose. These enzymes (α-amylase and α-glucosidase) are found to be effective in retarding glucose absorption to suppress hyperglycemia (Patil *et al.*, 2015; Sanchez and Vazquez, 2017). Inhibition of these enzymes reportedly decreases blood glucose levels in diabetic patients (Ibrahim *et al*., 2018). The research on the structure-activity correlation for peptides as potential alpha-glucosidase has not been reported, because the chemical structures of alpha-glucosidase inhibitory peptides were limited (Yu *et al*., 2012). However, some researchers have established the quantitative structure activity relationship (QSRA) model for xanthones and phthalimide derivatives as potent alpha-glucosidase inhibitor and suggested that inhibiting of alpha-glucosidase was a result of multiple interactions with the enzyme (Yu *et al*., 2012).

Table 1:Identified Compounds in the most Potent Fraction of *Combretum micranthum* Methanol Extract

Name	**Formula**	**Mass**	**RT**	**MFG Diff (ppm)**	**DB Diff (ppm)**	**Hits (DB)**
Desacetylcolchicine	C20 H23 N O5	357.1568	2.645	2.29	2.29	1
C16 Sphinganine	C16 H35 N O2	273.2666	18.139	0.57	0.57	1
C17 Sphinganine	C16 H22 O4	287.2821	20.605	1.28	1.27	1
Convallasaponin A	C17 H37 N O2	278.1516	21.086	4.86		2
Ile Arg Ile	C20 H23 N O5	287.2822	22.377	-1.63	-1.63	10
Thioridazine	C32 H52 O9	580.3583	22.941	-3.98	-3.98	2
Trp Lys Lys	C18 H36 N6 O4	400.2805	22.986	-0.17	-0.17	4
Tyr Ser Arg	C21 H26 N2 S2	370.1552	23.115	-1.25	-1.25	10
Phloionolic acid	C23 H36 N6 O4	460.2799	23.128		0.46	4
24-Nor-5β-cholane-3α,7α,12α, 22,23-pentol	C18 H28 N6 O6	424.2076	24.287		0.58	4
2-deoxy-20-hydroxy-5alpha-ecdysone 3-acetate	C18 H36 O5	332.2561	24.762	5.02	5.02	4
Nonoxynol-9	C23 H40 O5	396.2873	25.514		0.57	1
2-amino-hexadecanoic acid	C29 H46 O7	506.3218	25.81	1.16	1.16	8
N-Cyclohexanecarbonylpentadecyl-amine	C33 H60 O10	616.4183	25.837	1.14	1.14	2

Table 2: Retention factor (R_f) Values of Pooled Subfractions A, B and C

Number of Spots	Rf of A (cm)	Rf of B (cm)	Rf of C (cm)
1	0.044	0.174	0.044
2	0.721	0.147	0.088
3	0.838	0.221	0.131
4	0.882	0.294	
5	0.971	0.441	

Table 3: Alpha-amylase inhibitory activity of the Subfractions A, B and C of the Potent Fraction of C. *Micranthum* methanol leaf extract in IU (µmol/ml)

	10µg/ml (% I)	**20µg/ml (% I)**	**30µg/ml (% I)**	**40µg/ml (% I)**	**50µg/ml (% I)**
Fraction A	91.63±0.383*[a]	91.49±0.046*[a]	91.56±0.038*[a]	92.49±0.038*[a]	92.76±0.268*[a]
Fraction B	75.64±0.116*[ab]	75.75±0.116*[a]	75.73±0.156*[a]	76.19±0.155[a]	76..03±0.193[a]
Fraction C	92.96±0.000*[b]	93.09±0.072[a]	93.31±0.097*[a]	93.55±0.038*[a]	93.81±0.043*[a]
Acarbose	98.70±0.489*	101.3±0.097*	110.8±0.077*	118.9±0.100*	121.5±0.141*

Values are expressed as mean ± standard error of mean with those bearing same (* or [a,b,c]) superscripts in the same column are being Significantly ($P< 0.05$) different.

Table 4: Alpha-glucosidase inhibitory activity of the Subfractions A, B and C of the Potent Fraction of C. *Micranthum* methanol leaf extract in IU (µmol/ml)

	10µg/ml (% I)	20µg/ml (% I)	30µg/ml (% I)	40µg/ml (% I)	50µg/ml (% I)
Fraction A	21.03±0.133*[a]	27.10±0.109*[b]	39.76±0.105*[a]	75.62±0.109*	74.53±0.109*
Fraction B	21.75±0.044*[a]	29.05±0.040*[b]	71.81±0.024*[b]	83.31±0.125*	82.76±0.072*
Fraction C	22.73±0.141*[a]	29.29±0.109*[b]	43.96±0.142*[a]	83.15±0.142*	89.85±0.117*
Acarbose	8.438±0.109*[b]	52.15±0.142*[c]	92.07±0.189*[c]	165.8±0.109*[b]	246.7±0.114*[c]

Values are expressed as mean ± standard error of mean with those bearing different superscripts in the same column are Significantly (P< 0.05) different.

Conclusion

Peptides Ile-Arg-Ile, Trp-Lys-Lys and Tyr-Ser-Arg isolated from *C. micranthum* methanol leaf extract had inhibitory activities against α-amylase and α-glucosidase as shown by the *invitro* analysis.

Conflict of Interest

There was no conflict of interest declared

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