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Research Article

INHIBITORY ACTIVITIES OF ALKALOID FROM *COCCINIA GRANDIS* AGAINST ALDOSE REDUCTASE AND GENERATION OF ADVANCED GLYCATION ENDPRODUCTS

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Abstract:

*The aim of the present study was to evaluate the anti-diabetic complications of *Coccinia grandis* leaf extract. Anti-diabetic complications activities were evaluated via rat lens aldose reductase (RLAR), human recombinant aldose reductase (HRAR), and advanced glycation endproducts (AGE) assays. Among the different solvent-soluble fractions obtained from the methanolic extract, the dichloromethane (CH₂Cl₂) fraction was found to exhibited potent inhibitory activities against RLAR, HRAR and AGE with IC₅₀ values of 8.26 ± 0.10, 10.20 ± 0.13 and 28.18 ± 0.06 µg/mL, respectively. Silica gel column chromatography of the CH₂Cl₂ fraction yielded a alkaloid, 1-tert-butyl-5,6,7-trimethoxyisoquinolene, based on the comparison with reported ¹H- and ¹³C-NMR spectroscopic data. 1-tert-butyl-5,6,7-trimethoxyisoquinolene showed potential inhibitory activity against RLAR, HRAR and AGE with the respective IC₅₀ values of 3.26 ± 0.11, 5.40 ± 0.25, and 18.54 ± 0.55 µM, respectively. Our results clearly indicate the potential RLAR, HRAR and AGE formation inhibitory activities of *C. grandis* as well as its isolated constituents, which could be further explored to develop therapeutic modalities for the treatment of diabetes and related complications.*

Keywords: *C. grandis; Diabetic complications; Aldose reductase; Advanced glycation end products.*

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INTRODUCTION:

Diabetes mellitus is an intensifying metabolic disorder associated with carbohydrate, fat and protein metabolism when the body does not produce adequate insulin or does not respond to the actual produced insulin, contributing to an increase of regarding blood glucose levels (hyperglycemia) and also triggering considerable and irreparable harm to body systems, for example blood vessels and nerves [1]. It is envisioned that there are about 366 million people will tend to be diabetic throughout the world by the year 2030 [2]. In Asia, the prevalence of diabetes is raising as an alarming rate and anticipated to increase 2-3 folds by 2030 [3]. Consistent hyperglycemia is responsible for long-term diabetes complications for example retinopathy, cataracts, neuropathy, atherosclerosis, nephropathy, and delayed of healing of wounds [4]. Despite the fact that mechanisms ultimately causing diabetic complications usually are not entirely recognized, several biochemical path ways regarding hyperglycemia are already implicated. Hyperglycemia-induced formation of increased polyol pathway flux, activation of protein kinase C isoforms, increased hexosamine pathway flux, and advanced glycation end products have been thought to be major factors from the pathogenesis of long-term complications associated diabetes [5]. Among these, aldose reductase (AR)-associated polyol pathway has been extensively studied. Several evidences suggest a strong correlation between diabetes-related complications and downstream effects of osmotic and oxidative stress resulting from polyol pathway hyperactivation in hyperglycemic conditions [5, 6]. On the other hand, prolonged exposure to hyperglycemia and carbonyl stress also induces increased production and accumulation of AGE in body tissues. Increased formation of AGE molecules can lead to protein cross-linking and contribute to the development and progression of several diabetic complications such as peripheral neuropathy, cataracts, impaired wound healing, vascular damage, arterial wall stiffening, and decreased myocardial compliance [7-11]. Thus, AR and AGE inhibitors are great potential therapeutic strategies for the prevention of diabetic and other pathogenic complications.

The search for alternative, effective, and safe anti-diabetic agents is of paramount importance in mainstream pharmaceutical research as synthetic anti-diabetic drugs possess numerous side effects including liver disorders, flatulence, abdominal pain, renal tumors, hepatic injury, acute hepatitis, abdominal fullness, and diarrhea [12-14]. Medicinal plants play a vital role in the development of new drugs. A vast body of literature has accumulated recently outlining potential roles for compounds from medicinal plants in the management of diabetes and the efficacy of these compounds in the amelioration of

secondary complications of diabetes such as cataracts [15]. *C. grandis* L. Voigt. commonly known as "Ivy gourd" is a tropical plant belonging to the family Cucurbitaceae. It has been found in many countries in Asia and Africa. The plant parts of *C. grandis* such as roots, leaves and fruits are used for numerous medicinal purposes like wound healing, ulcers, jaundice, diabetes and antipyretic. The leaf possesses hypoglycemic, antihyperglycemic, antibacterial, antioxidant properties and is also used to treat infective hepatitis [16-19]. However, there have been no studies on *C. grandis* that show which active components are responsible for RLAR, HRAR as well as AGE inhibitory activities. The objective of the present study was to investigate the anti-diabetic complications activity of the different fractions of the methanolic extract of the leaves of *C. grandis* using *in vitro* models. Our data suggest that *C. grandis* may perhaps represent a source of new way for the management and treatment of diabetes-associated complications.

MATERIALS AND METHODS:

General Experimental Procedures

Column chromatography was conducted using silica (Si) gel 60 (70–230 mesh, Merck, Darmstadt, Germany), Si gel 60 (230–400 mesh, Merck, Darmstadt, Germany), Sephadex LH20 (20–100 µm, Sigma, St. Louis, MO, USA), Lichroprep RP-18 (40–63 µm, Merck, Darmstadt, Germany). All thin layer chromatography (TLC) was conducted on pre-coated Merck Kiesel gel 60 F₂₅₄ plates (20 × 20 cm, 0.25 mm, Merck) and RP-18 F_{254S} plates (5 × 10 cm, Merck), using 10% H₂SO₄ as the spray reagent.

Chemicals and Reagents

Ethylenediaminetetraacetic acid (EDTA), b-nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), DL-glyceraldehyde dimer, D-(–)-fructose, D-(+)-glucose, aminoguanidine hydrochloride, and quercetin were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium azide was purchased from Junsei Chemical Co. (Tokyo, Japan). All chemicals and solvents used in the assays were of reagent grade, and were purchased from commercial sources.

Plant Material

The plant sample of *C. grandis* leaves were collected in September, 2016 from local area of Bangladesh. The plant was identified by Bangladesh National Herbarium, Dhaka, where a voucher specimen (20161012) has been deposited. At first, Plants were washed properly to remove dirty materials and air-dried for several days. These were then ground with a hammer grinder for better grinding. The dried leaves were ground into a

coarse powder. Then, the dried powder was preserved in an airtight container against the re-absorption of moisture, oxidation, excessive heat or humidity, growth of moulds and bacteria and infestation by insects and rodents.

Extraction, Fractionation and Isolation of *C. grandis*

Dried powder of *C. Grandis* was refluxed with MeOH (3×3 L) for 3 h, and each filtrate was concentrated until dry in vacuo at 40°C , resulting in MeOH extract (175.0 g). This extract was suspended in distilled H₂O and then successively partitioned with CH₂Cl₂, EtOAc, and *n*-BuOH, to yield the CH₂Cl₂ (29.6 g), EtOAc (51.5 g), and *n*-BuOH (39.6 g) fractions, respectively, as well as an H₂O residue (52.5 g). The active CH₂Cl₂ fraction (29.6 g) obtained from *C. grandis* was subjected to chromatography on a silica gel column, with CH₂Cl₂-MeOH (50:1 to 5:1) as the eluent, yielding twelve subfractions (CF01-CF12). Repeated column chromatography of CF06 (5.78 g) was conducted with a solvent mixture of CH₂Cl₂ and MeOH, yielding eight subfractions (CF0601-CF0608). CF0605 (0.17 g) was purified on an RP-18 column and eluted with aqueous MeOH (20% MeOH-100 % MeOH, gradient elution) to yield 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene. The chemical structure of this compound was identified by spectroscopic methods, including ¹H- and ¹³C-NMR as well as published data [20]. The structure is shown in Fig. 1.

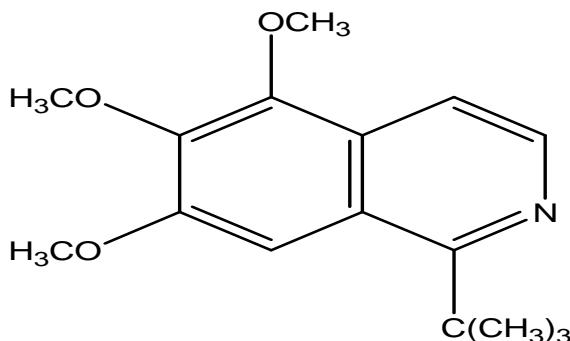


Fig. 1: Structure of 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene

Alkaloid 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene

In the ¹H NMR spectral analysis signal near 8.491 (singlet) is for ⁷CH proton, peaks at 7.996, 7.963, 7.931 (multiplet) is for ¹¹CH₃, ¹²CH₃, ¹³CH₃ protons, peaks at 3.486, 3.194, 3.161 (multiplet) is for ¹⁵CH₃, ¹⁶CH₃, ¹⁷CH₃ protons. Peak at 3.152 (singlet) is for ⁸CH and peak at 2.500 (singlet) is for ⁴CH proton. In the ¹³C NMR spectra, the peak around 78.754 is due to ¹¹C, ¹²C, ¹³C, the peak

around 78.624 is due to ¹C, ²C, ³C. The peak around 78.426 is due to ⁵C and the peak around 78.098 is due to ⁷C. The peak around 39.919 is due to ⁹C and ¹⁰C, the peak around 39.714 is due to ⁸C, at 39.500 is due to ⁴C. The peak at 39.294 is due to ¹⁵C, ¹⁶C, ¹⁷C, the peak at 39.081 is due to ¹⁴C.

Assay for RLAR inhibitory activity

We followed the Guidelines for Care and Use of Laboratory Animals as approved by Inje University (Republic of Korea). Rat lens homogenate was prepared according to the modified method of Hayman and Kinoshita [21]. Briefly, the lenses were removed from the eyes of Sprague-Dawley rats weighing 250-280g. The lenses were homogenized in sodium phosphate buffer (pH 6.2), which was prepared from sodium phosphate dibasic (Na₂HPO₄H₂O, 0.66 g) and sodium phosphate monobasic (NaH₂PO₄H₂O, 1.27 g) in 100 mL of double-distilled water. The supernatant was obtained by centrifugation of the homogenate at 10,000 rpm at 4°C for 20 min and was frozen until use. A crude AR with a specific activity of 6.5 U/mg was used in the evaluations of enzyme inhibition. The partially purified material was separated into 1.0 mL aliquots and stored at -40°C . Each 1.0 mL cuvette contained equal units of enzyme, 100 mM sodium phosphate buffer (pH 6.2), and 1.6 mM NADPH, either with or without 50 mM of the substrate, DL-glyceraldehyde, and an inhibitor. AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 4-min period using an Ultrospec 2100 pro UV/visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, Piscataway, NJ, USA). Quercetin, a well-known AR inhibitor, was used as a positive control. Inhibition percentage (%) was calculated as $[1-(\Delta A_{\text{sample}}/\text{min} - \Delta A_{\text{blank}}/\text{min}) / (\Delta A_{\text{control}}/\text{min} - \Delta A_{\text{blank}}/\text{min})] \times 100$, where ΔA sample/min represents the reduction in absorbance over a period of 4 min for the test sample and substrate, respectively, and ΔA control/min represents the same but with 100 % DMSO instead of sample. The RLAR inhibitory activity of each sample was expressed in terms of the IC₅₀ value ($\mu\text{g/mL}$ for extracts and μM for compounds) as calculated from the log (dose inhibition) curve.

Assay for HRAR inhibitory activity

The HRAR inhibitory activities were examined as described by Nishimura et al [22]. The reaction mixture was prepared as follows: 100 μL of 0.15 mM NADPH, 100 μL of 10 mM DL-glyceraldehyde as a substrate, 5 μL of the HRAR, and the sample (dissolved in 100% DMSO) in a total volume of 1.0 mL of 100 mM sodium phosphate buffer (pH 6.2). The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a period of 1 min on an

Ultraspec®2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Quercetin, a well-known ARI, was used as a positive control. The inhibition percentage (%) was calculated as described in the RLAR assay, except that the ΔA sample/min represents the reduction of absorbance for 1 min with the test samples and substrate. The HRAR inhibitory activity of each sample was expressed in terms of the IC_{50} value, which was calculated from the log-dose inhibition curve.

Assay for AGE formation inhibitory activity

The inhibitory activity of AGE formation was examined according to the modified method of Vinson and Howard [23]. To prepare the AGE reaction solution, 10 mg/mL of BSA in 50 mM sodium phosphate buffer (pH 7.4) with 0.02 % sodium azide to prevent bacterial growth was added to 0.2 M fructose and 0.2 M glucose. The reaction mixture (950 μ L) was then mixed with various concentrations of sample (50 μ L, final concentration 200 μ g/mL for extract and 200 μ M for the compounds) dissolved in 10 % DMSO. After incubating at 37° C for 7 days, the fluorescence intensity of the reaction products was determined using a spectrofluorometric detector (FLx800 microplate fluorescence reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) with excitation and emission wavelengths set at 350 and 450 nm, respectively. The activity of each sample to inhibit AGE formation was expressed graphically in terms of the IC_{50} value (μ g/mL for extracts and μ M for compounds) in triplicate. The

nucleophilic hydrazine compound aminoguanidine hydrochloride was used as a reference in the AGE formation inhibitory assay.

Statistical analysis

One-way ANOVA and Student's t test were used to determine the statistical significance of differences between values for various experimental and control groups (Systat; Evaston, IL, USA). Each result is expressed as the mean \pm SEM of triplicates.

RESULTS:

RLAR and HRAR inhibitory activities of the MeOH extract and its solvent soluble fractions from *C. grandis*

To find out potential aldose reductase activities, we successively partitioned the crude extract with several solvents using a bioassay-guided fractionation strategy. The MeOH extract of *C. grandis* was dissolved in H₂O and successively partitioned with CH₂Cl₂, EtOAc, and *n*-BuOH to obtain the respective solvent-soluble fractions and water residue. We then evaluated the RLAR as well as HRAR inhibitory activities of the different solvent-soluble fractions. As shown in Table 1, CH₂Cl₂ fraction exhibited significant RLAR inhibitory activities with IC_{50} values of 8.26 \pm 0.10 μ g/mL. In accordance with RLAR inhibitory activities, the highest HRAR inhibitory activity was also observed in the CH₂Cl₂ fraction with an IC_{50} value of 10.20 \pm 0.13 μ g/mL compared to Quercetin (IC_{50} = 0.49 \pm 0.10 μ g/mL).

Table 1: RLAR and HRAR inhibitory activities of the methanolic extract and its solvent-soluble fractions from *C. grandis*

Extract/Fractions	IC_{50} values (μ g/mL) ^a \pm SEM	
	RLAR	HRAR
MeOH extract	60.21 \pm 2.10	61.81 \pm 1.93
CH ₂ Cl ₂ fraction	8.26 \pm 0.10	10.20 \pm 0.13
EtOAc fraction	28.31 \pm 0.23	36.97 \pm 0.64
<i>n</i> -BuOH fraction	32.78 \pm 0.82	48.61 \pm 1.43
H ₂ O fraction	>100	>100
Quercetin ^b		0.49 \pm 0.10

^a The concentration that caused 50% inhibition (IC_{50}) is given as the mean \pm SEM of triplicate experiments

^b Used as positive control in RLAR and HRAR inhibitory assay

Table 2: AGE formation inhibitory activities of the methanolic extract and its solvent-soluble fractions from *C. grandis*

Extract/Fractions	IC_{50} values (μ g/mL) ^a \pm SEM	
	AGE	
MeOH extract	193.28 \pm 0.56	
CH ₂ Cl ₂ fraction	28.18 \pm 0.05	
EtOAc fraction	96.46 \pm 0.36	
<i>n</i> -BuOH fraction	145.32 \pm 0.40	
H ₂ O fraction	>100	
Aminoguanidine ^b	53.82 \pm 0.10	

^a The concentration that caused 50% inhibition (IC_{50}) is given as the mean \pm SEM of triplicate experiments

^b Used as positive control in AGE formation inhibitory assay

Table 3: RLAR, HRAR and AGE formation inhibitory activities of compound isolated from *C. grandis*

Compounds	IC ₅₀ (µM) ± SEM ^a		
	RLAR	HRAR	AGE
1- <i>tert</i> -butyl-5,6,7-trimethoxyisoquinolene	3.26 ± 0.11	5.40 ± 0.25	18.54 ± 0.55
Quercetin ^b	6.80 ± 0.87	7.63 ± 0.45	
Aminoguanidine ^c			67.68 ± 1.22

^a The concentration that caused 50% inhibition (IC₅₀) is given as the mean ± SEM of triplicate experiments

^b Used as positive control in RLAR and HRAR inhibitory assay

^c Used as positive control in AGE formation inhibitory assay

AGE formation inhibitory activity of the MeOH extract as well as different fractions from *C. grandis*

AGE formation inhibitory activities of the MeOH extract and its different solvent-soluble fractions of *C. grandis* are presented in Table 2. The MeOH extract exhibited weak AGE formation inhibitory activity at the concentration tested. In contrast, its different solvent-soluble fractions exhibited potential inhibitory activity, with the CH₂Cl₂ fraction displaying the highest AGE formation inhibitory activity with an IC₅₀ value of 28.18 ± 0.05 µg/mL compared to the positive control aminoguanidine with an IC₅₀ value of 53.82 ± 0.10 µg/mL. In addition, the EtOAc and *n*-BuOH fractions also showed significance inhibitory activity with corresponding IC₅₀ values of 96.46 ± 0.36 and 145.32 ± 0.40 µg/mL, respectively.

RLAR, HRAR and AGE formation inhibitory activities of compound isolated from *C. grandis*
In order to determine the active compounds responsible for potential inhibition of aldose reductase as well as advanced glycation endproducts, compounds isolated from *C. grandis* were further evaluated for RLAR, HRAR, and AGE inhibiting assay. The isolated alkaloid compound, 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene, exhibited potential inhibitory activity against RLAR as well as HRAR, with IC₅₀ values of 3.26 ± 0.11, and 5.40 ± 0.25 µM, respectively. The IC₅₀ values of the positive control, quercetin, against RLAR and HRAR was 6.80 ± 0.87 and 7.63 ± 0.45 µM, respectively. In addition, 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene also exhibited significant inhibitory activity against AGE, with an IC₅₀ value of 18.54 ± 0.55 µM compared to the positive control aminoguanidine with IC₅₀ value of 67.68 ± 1.22 µM results were shown in Table 3.

DISCUSSION:

Although the mechanisms remain unclear, diabetes and hyperglycemia cause increased oxidative stress, which presumably has an important role in the onset of diabetic complications, including cataracts, neuropathy, nephropathy, and retinopathy [24,25]. Several mechanisms for the pathogenesis of diabetic complications were proposed, including

the AR-related polyol pathway, AGE formation, AGE receptors, protein kinase C isoforms, and the hexosamine pathway. Among them, the AR-related polyol pathway and AGE formation are deemed as the major contributors to oxidative stress in some tissues; particularly, eye lenses and nerves [25-27]. Accumulated evidence suggests that the development and progression of various complications in type 1 and type 2 diabetes are clearly linked to elevated blood glucose levels. Although glucose is preferentially metabolized through the glycolytic pathway, under hyperglycemic conditions as are present in DM, elevated blood glucose levels saturate the normal pathways of glucose metabolism, resulting in a dramatic increase in flux through the polyol pathway. The polyol pathway involves two enzymatic reactions. In the first reaction, glucose is reduced to sorbitol by the action of AR with concomitant conversion of NADPH into NADP. In the second reaction, sorbitol is oxidized to fructose by the action of sorbitol dehydrogenase [28]. Clinical evidence has established that sugar-induced cataractogenesis is associated with AR-mediated generation of sugar alcohols such as sorbitol [29]. Regardless the numerous efforts made over recent decades, to date, epalrestat is the only AR inhibitor commercially available and in Japan alone, while fidarestat has already undergone phase III clinical trial for diabetic neuropathy and was found to be safe. In many cases, the failure of new candidates can be ascribed to poor pharmacokinetic properties, undesirable side effects, and low efficacy due to reduced binding affinity to AR. For these reasons and for the newly described therapeutic potentials of AR inhibitors, there is still a great interest in the identification of novel AR inhibitors [30-33]. In addition to the increased polyol pathway flux, prolonged hyperglycemia also accelerates the formation of AGE in body tissues. AGE are a chemically heterogeneous group of compounds formed by the Maillard reaction, when reducing sugars react nonenzymatically with amine residues, predominantly lysine and arginine, on proteins, lipids, and nucleic acids ultimately leading to their chemical modification [34]. Increased formation of AGE relates to the development of cataracts, diabetic complications, uremia, Alzheimer's

disease, and other disorders [35]. The harmful effects of AGE (both endogenous and exogenous) result from structural and functional alterations in plasma and extracellular matrix proteins, in particular, from the cross-linking of proteins and the interaction of AGE with their receptors and/or binding proteins. This leads to enhanced formation of reactive oxygen species with subsequent activation of nuclear factor- κ B and release of pro-inflammatory cytokines, growth factors, and adhesion molecules [36].

In the present study, we found that methanol extract of *C. grandis* showed potent inhibitory activities against RLAR, HRAR and AGE. Solvent partitioning of the MeOH extract yielded four different solvent-soluble fractions. Among them, the CH₂Cl₂ fraction was found as the most active fraction by RLAR and HRAR assays. The CH₂Cl₂ fraction was also shown to possess strong inhibitory activities against AGE formation. Considering the inhibitory potential, CH₂Cl₂ fraction was selected for chromatographic separation in order to determine the active compounds from *C. grandis*. Repeated chromatography of the CH₂Cl₂ fraction yielded alkaloid compound, 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene, was found to be the most active compound in the RLAR and HLAR inhibitory assays as well as AGE formation inhibitory assay. In summary, the MeOH extract of *C. grandis* as well as its different solvent-soluble fractions showed potential RLAR, HRAR and AGE formation inhibitory activity. One alkaloid compound, 1-*tert*-butyl-5,6,7-trimethoxyisoquinolene was isolated by repeated chromatography of the CH₂Cl₂ fraction showed strong RLAR and HRAR inhibitory activity along with potential AGE formation inhibitory activity at the concentrations tested. In this study, we also revealed for the first time the AR and AGE formation inhibitory activity of the isolated alkaloid. Our results clearly demonstrate the potential health benefits of *C. grandis* in preventing diabetic complications as well as in developing remedies for the treatment of diabetic complications. However, further extensive biological experiments should be carried out to fully evaluate its therapeutic potential as natural anti-diabetic agent as well as to elucidate the mechanism of action of the active compounds.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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