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In vitro biological assessment of *Homalium zeylanicum* and isolation of lucidenic acid A triterpenoid

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gallic acid (PubChem CID: 370)
Quercetin (PubChem CID: 5280343)
EDTA (PubChem CID: 6049)
acarbose (PubChem CID: 41774)
Diclofenac sodium (PubChem CID: 5018304)

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ABSTRACT

Homalium zeylanicum (Gardner) Benth. (Flacourtiaceae) is a medicinal plant useful in controlling rheumatism, inflammation and diabetes. The objective of this work evaluates *in vitro* antioxidant, antidiabetic, and antiinflammatory properties of hydroalcohol extract of bark of *H. zeylanicum* (HAHZ). It also describes isolation and structure determination of lucidenic acid A, which is the first report in this plant. In order to explain the role of antioxidant principles, DPPH, nitric oxide, hydroxyl, superoxide and metal chelating assays were performed. Antidiabetic and anti-inflammatory activities were investigated by quantifying α-amylase, α-glucosidase and protein denaturation inhibitory activities of HAHZ. Biochemical estimations were performed. The chemical structure of the triterpenoid was elucidated using ^{1}H, ^{13}C NMR and high resolution-MS. IC_{50} of DPPH, nitric oxide, hydroxyl, superoxide and metal chelating activities were of 36.23 ± 0.27, 40.11 ± 0.32, 35.23 ± 0.57, 43.34 ± 0.22 and 11.54 ± 0.08 µg/mL, respectively. IC_{50} of α-amylase and α-glucosidase activities were 29.12 ± 0.54, and 18.55 ± 0.15 µg/mL. Total phenolic and total flavonoid contents were recorded at 233.65 mg/g GAE and 172.7 mg/g QE. Regarding kinetic behaviour, HAHZ showed competitive inhibition on α-glucosidase and mixed competitive inhibition on α-amylase. Lucidenic acid A was confirmed by spectroscopic studies. Anti-inflammatory activity of lucidenic acid A was determined by using protein denaturation assay with IC_{50} 13 µg/mL but HAHZ showed 30.34 ± 0.13 µg/mL. Phenols and flavonoids could be attributed to inhibition of intestinal carbohydrases for anti-diabetic activities whereas triterpenoids could be responsible for anti-inflammatory activity of *H. zeylanicum*.

1. Introduction

Oxidative stress is the major cause of a number of chronic diseases such as diabetes, rheumatic arthritis, cancer, atherosclerosis, hematological and neurodegenerative disorders. Generation of free radicals due to oxidative stress factors associated with inflammation and other diseases became major health issues in recent years. The oxi-flammation effect is induced by oxidative stress factors which may affect various organ systems, and progression of insulin-resistance in the body. Increased inflammation, oxidative stress, dyslipidemia, and glucotoxicity are interlinked with each other which may cause an extra demand on β-cells to stimulate insulin. In this process β-cells are no longer able to meet the over increasing demand of insulin, resulting in the development of frank diabetes and may contributes to several diabetes-associated complications like cardiovascular diseases, nephropathy, neuropathy, retinopathy, urological diseases, and cancer [1,2]. India stands first in the whole world, having the highest number of diabetes patients and this disorder is increasing very fast across the globe. The global incidence stress induced diabetes for all age groups would reach to 4.4% in 2030 CE [3]. Within few decades; it will become one of the world's commonest forms of disease. As per ethnobotanical reports, more than 800 plant species having antidiabetic and antioxidant activities are found in literature. This protective roles can be mainly attributed to the presence of secondary metabolites, which are defined as bioactive phenols, flavoniods and alkaloids in fruits, vegetables, grains, and other parts of plants [4,5]. Various pharmacological approaches

Abbreviations: HAHZ, hydro alcoholic extract of *Homalium zeylanicum*; TLC, thin layer chromatography; R_f, Retardation factor; TPC, total phenolic contents; TFC, total flavonoid contents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; NO, Nitric oxide; OH, hydroxyl; SOD, superoxide anion; GAeqv/g, gallic acid equivalents *per* gram; Queqv/g, Quercetin equivalents *per* gram; NBT, nitroblue tetrazolium; PMS, phenazine methosulphate; IC_{50}, half maximal inhibitory concentration; pNPG, p-nitrophenyl-α-D-glucopyranoside; DNS, dinitrosalicylic; ROS, reactive oxygen species; PBS, phosphate buffer saline; NSAIDs, nonsteroidal anti-inflammatory drugs

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have been introduced in diabetes treatment. These treatments include different modes of action of herbal drugs including stimulation of insulin release, inhibition of gluconeogenesis, increasing the number of glucose transporters and reduction of glucose absorption from the intestine [6]. One of the beneficial therapies is to impair the glucose absorption by the inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase in the digestive organs.

Homalium zeylanicum (Gardner) Benth. (Flacourtiaceae), commonly known as 'Kalladamba', is distributed in Western Ghats, Andhra Pradesh, Tamil Nadu, and Kerala of India. Ethnobotanically, *H. zeylanicum* is used in many ailments such as diabetes, rheumatism, and wound healing activities [7,8]. Our earlier report on antioxidant activities of Indian species of *Homalium* established that the ethyl acetate extract of leaves and bark of *H. nepalense*, *H. tomentosum* and *H. zeylanicum* were found to be better active than other successive extracts [9]. This work is presented here to establish further the antioxidant, anti-diabetic, and anti-inflammatory activity of hydroalcohol extract of *H. zeylanicum*. The kinetic behaviour of *H. zeylanicum* on α-glucosidase and α-amylase were performed while evaluating the antidiabetic properties of this plant. The current investigation reported first the presence of a triterpenoid lucidenic acid A in the bark of *H. zeylanicum*.

2. Materials and methods

2.1. General

^{1}H spectra was measured on high resolution 700 MHz NMR Spectroscopy (Agilent DD2700 MHz NMR), ^{13}C NMR was measured on Bruker AvIII HD–300 MHz FT NMR with low and high temperature facility −90 °C to 80 °C in $CDCl_3$, with tetramethylsilane (TMS) as an internal standard. Mass spectra data was recorded on a JMS-T100L; AccuTOF Mass spectrometer. FT-IR was recorded on Agilent Cary 630. All the spectroscopic analysis of compound **1** had done at Central Drug Research Institute (CDRI), Lucknow, India. Column chromatography was performed by using a glass column (Borosil, 500 × 18 mm) and it was filled with silica gel (100–200 mesh, Himedia, India). TLC was performed using silica gel 60 F_{254} (Merck, India) precoated plates and detection was visualized at 254 nm and 365 nm UV. Other chemicals and reagents used in the study were of analytical grades and procured from Himedia, India, Sigma-Aldrich, India and SRL, India.

2.2. Plant collection and identification

Barks of *Homalium zeylanicum* were collected from Tirumala Hills, Chittoor District, Andhra Pradesh, India. The plant was botanically identified by Dr. P.C. Panda, Principal Scientist, Regional Plant Resource Centre, Bhubaneswar, Odisha. Voucher specimen was deposited in the herbarium of RPRC for future references (Voucher No. 7545/T).

2.3. Extraction and isolation

The dried powdered bark materials of *H. zeylanicum* (1 kg) were extracted with 70% hydro-alcoholic (HAHZ) (3 L × 4) by cold maceration. HAHZ was concentrated (9.6%; w/w) and preliminary phytochemical investigation was carried out for HAHZ in order to assess the presence of different phytochemicals [10]. HAHZ (20 g) was chromatographed on a column eluted successively with stepwise gradients of hexane (100%), followed by hexane:chloroform in the proportion of 99:1, 98:2 and continued upto 0:100 with chloroform. Then the elution was followed by chloroform:methanol in the proportion of 99:1, 98:2, 97:3 and continued to 90:10. Around 415 fractions were collected with each fraction collection capacity was of 15 mL and accordingly similar fractions were re-pooled into a single fraction by TLC profiling with same R_f values. Each fraction was tested by following *in vitro* anti-inflammatory protein denaturation activity study by using multimode microplate reader (Synergy H1 M, BioTEK, USA). It was found that the fraction no. 106–113 with the eluent of hexane:chloroform (66:34%), were shown better activities than other fractions. Further purification of the fraction no. 106–113 was done by washing number times with methanol and a powdered isolated pure compound **1** was obtained (6.7 mg; with respect to HAHZ of 0.0022%). Purification was further cross checked by performing TLC with hexane:chloroform (1:1, v/v; R_f 0.20).

2.4. Estimation of total phenolic contents (TPC)

TPC of HAHZ was determined by using the Folin-Ciocalteu reagent [11]. About 10 µL of 1 mg/mL HAHZ, 450 µL of distilled water and 2.5 mL of 0.2 N Folin-Ciocalteu reagents was added. After 5 min, 2 mL of 10% sodium carbonate was added. The absorbance of the resulting blue-colored solution was measured at 765 nm after incubation at 37 °C for 30 min by using a multimode micro plate reader (SynergyH1MF, BioTek, USA). Gallic acid was used as a reference drug and phenolic content was expressed as mg/g gallic acid equivalents (GAE) *per* gram of dried extract (mg GAeqv/g).

2.5. Estimation of total flavonoid contents (TFC)

TFC of HAHZ was assayed according to standard protocol with a slight modification to it [11]. About 500 µL of 1 mg/mL HAHZ was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm by using a multimode micro plate reader (SynergyH1MF, BioTek, USA). Quercetin was used as reference drug and the results were expressed as mg/g Quercetin equivalents (mg Queqv/g).

2.6. DPPH free radical scavenging assay of H. zeylanicum

The scavenging activity of HAHZ on the stable free radical DPPH was assayed using the modified protocol in which the bleaching rate of DPPH was monitored at a characteristic wavelength in the presence of the sample [12]. Stock solution of 1 mg/mL was prepared in methanol. Various concentrations (10–100 µg/mL) of HAHZ were mixed with 0.1 mL of a 0.15% DPPH solution in methanol. The mixture was kept for 30 min in the darkness, and then the absorbance was read at 517 nm (SynergyH1MF, BioTek, USA).% of decrease in DPPH absorbance was calculated by measuring the absorbance of the sample by applying the following equation:

$$\text{Inhibition (\%)} = (A_{Control}\text{-}A_{Sample}/A_{Control}) \text{ X } 100$$

Different concentrations of ascorbic acid as a reference drug were used as positive controls.

2.7. Nitric oxide (NO) free radical scavenging activity of H. zeylanicum

The reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in phosphate buffer saline (PBS) and different concentrations of HAHZ (10–100 µg/mL) were incubated at 25 °C for 150 min. Then 1 mL of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylene diaminedihydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm (SynergyH1MF, BioTek, USA).% of inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test compounds, whereas quercetin was taken as reference drug. The procedure followed here was the modified [13].

$$\text{Inhibition (\%)} = (A_{Control}\text{-}A_{Sample}/A_{Control}) \text{ X } 100$$