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Anti-diabetic effect of the ethyl acetate fraction of *Clerodendrum volubile*: protocatechuic acid suppresses phagocytic oxidative burst and modulates inflammatory cytokines

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ABSTRACT

The antidiabetic effects of the ethyl acetate (EtOAc) fraction of *Clerodendrum volubile* leaves was investigated in this study. EtOAc extract was also fractionated to isolate the active compounds. The structure of the isolated compound (Protocatechuic acid) was established using ^{1}H and ^{13}C NMR spectroscopies and mass spectrometry. Protocatechuic acid was investigated for its anti-oxidative burst in polymorphonuclear neutrophils (PMNs) and macrophages. It was also docked with α–glucosidase and TNF-α. Acute treatment with EtOAc fraction of *Clerodendrum volubile* leaves significantly ($p < 0.05$) decreased blood glucose level and hepatic biomarkers, and significantly ($p < 0.05$) increased serum insulin level and β–cell function. It had little or no effect on serum lipid profile and atherogenic indices. Protocatechuic acid significantly ($p < 0.05$) suppressed phagocytic oxidative burst and docked well with α–glucosidase and TNF-α. These results indicate the therapeutic effect of EtOAc fraction of *C. volubile* on type 2 diabetes and its complications, which can be attributed to the main bioactive compound, protocatechuic acid.

1. Introduction

Diabetes is rapidly becoming a major scourge in African communities with change in life style and increasing urbanization being the primary factor. In 2015, 14.2 million cases of diabetes were reported in Africa [1], which has been predicted to rise to 34.2 million in 2040, indicating a more than 100% increment in next 25 years [1]. This increase is a major drain on the health resources of sub-Saharan Africa with low income coupled with other infectious diseases [2]. Diabetes is a complex metabolic disease characterized by inability of the pancreatic β − cells to secret insulin as seen in type 1 diabetes or the insulin secreted is not utilized as seen in type 2 diabetes leading to progressive impairment of glucose tolerance and hyperglycemia [3].

Type 2 diabetes has been recognized as a common diabetes since more than 90 or almost 95% people are suffering from this type of diabetes [1]. It is mostly characterized by insulin resistance with concomitantly reduced pancreatic β–cell function [4]. Subclinical chronic inflammation has been implicated as one of the pathogenic factors underlying insulin resistance [5]. Increased metabolic activities of islet cells on elevation of blood glucose level causes an elevated production of Reactive Oxygen Species (ROS) [4,5]. This has been attributed to increase respiratory oxidative burst upon stimulation of neutrophils and macrophages [6]. This in turn leads to the release of inflammatory cytokines and

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chemokines such TNF–α, IL-1β, IL-8 and MCP1 which promotes insulin resistance [7,8].

Recently there has been a growing interest in the use of medicinal plants for the treatment and management of diabetes and its complications [9–11]. This can be attributed to their phytochemical constituents notably phenolics with reported antioxidant and hypoglycemic activities [12]. *Clerodendrum volubile* is one of the major candidates in such plants. It is locally known as *obenetete* amongst the Urhobos and Itsekiris of the Niger Delta, who use it as a common food ingredient [13]. Due to its numerous folkloric uses, it is often regarded as magic leaf [14]. These folkloric uses include the treatment of diabetes, ulcer, arthritis, rheumatism, and dropsy [14]. Erukainure et al. [13] reported the anti-oxidative potential of an isolated iridoid glycosides from the leaves in brain and hepatic tissues. Fatty acids from the leaves have been shown to possess an anticancer activity in human breast cancer cells [6]. Adefegha and Oboh [12] reported the ability of the leaves to inhibit key enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (Angiotensin-I converting enzyme). However, there are a dearth of information still needs to be elucidated on the compounds responsible for the acclaimed antidiabetic properties as well as molecular modelling of the mechanism involved.

To the best of our knowledge, we hereby reported for the first time the isolation and characterization of Protocatechuic acid (3,4-dihydroxybenzoic acid) from the leaves of *C. volubile* with its effects on complications linked to type 2 diabetes vis-à-vis antioxidative burst activity on polymorphonuclear neutrophils (PMNs) and macrophages; and anti-proliferative activity on T − cells. Consequently, the current study also reported the effect of acute treatment with ethyl acetate fraction of *C. volubile* leaves in type 2 diabetic rats followed by isolation, structure elucidation of the active compound and its immunomodulatory effect *in vitro*; as well as molecular modelling of its interaction with key enzymes and cytokine linked to type 2 diabetes.

2. Materials and methods

2.1. Instrumentation

^{1}H and ^{13}C NMR spectra (400 MHz and 100 MHz respectively) and two dimensional correlation spectroscopy (COSY), NOSEY, HMQC, and HMBC, were recorded on a Bruker AV-400 spectrometer in MeOD. Chemical shifts were reported in δ (ppm) values. The UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. Thin layer chromatography (TLC) were carried out on precoated silica gel 60 F_{254} plates (E. Merck, 0.25 mm), and detected under UV light (254 nm) and by spraying with ceric sulphate reagent. The EI-MS was recorded on a JEOL JMS-HX-110 mass spectrometer.

2.2. Plant materials

Fresh leaves of *Clerodendrum volubile* were purchased from local farmers at Ifon, Ondo State, Nigeria. They were identified, authenticated and assigned the voucher number UBH_{C284} at the Herbarium, Department of Botany, University of Benin, Benin City, Nigeria. The leaves were air-dried, pulverized to fine powder and stored in air-tight containers for further analysis.

2.2.1. Extraction and isolation

The pulverized sample was extracted with methanol (MeOH) at room temperature. The combined extract was evaporated to yield 450 g crude extract. Then 100 g of the crude extract was dissolved in distilled water and fractionated using gradient polarity solvents in the order: [*n*-hexane (Hex), *n*-dichloromethane (DCM) and ethyl acetate (EtOAc)]. The EtOAc fraction was concentrated *in vacuo* and further fractionated using a gravity column chromatography loaded with silica gel. The column was eluted with mixtures of Hex and DCM (50:50) in increasing order of polarity up to DCM (100). Then continued with DCM and MeOH (97:03) up to DCM: MeOH (93:07). The last eluate was subjected to thin-layer chromatography on pre-coated silica gel 60 F_{254} sheets to confirm its purity. The compound of interest was detected by UV light (254 nm) and also by spraying with ceric sulfate reagent. 3,4-dihydroxybenzoic acid (Protocatechuic acid), a phenolic acid was isolated and characterised for the first time from *Clerodendrum volubile*. The spectral data obtained were identical with literatures [15,16].

Protocatechuic acid (3,4-dihydroxybenzoic acid): (white), EI-MS *m/z* (rel. int.%): 154 (84), 137 (100), 109 (18). ^{1}H NMR (CD_3OD, 500 MHz): δ 7.40 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6), 7.42 (1H, *d*, H-2), 6.76 (1H, *d*, $J_{5,6}$ = 8.0 Hz, H-5). ^{13}C NMR (CD_3OD, 150 MHz): δ 123.7 (C-1), 117.7 (C-2), 145.8 (C-3), 150.8 (C-4), 115.5 (C-5), 123.6 (C-6), 170.5 (CO).

Other pure isolates obtained where of very minute quantities (<1 mg), thus we focused on Protocatechuic acid.

2.3. Animals

Twenty-four male albino rats of Wistar strain weighing about 150–200 g were used for the study. They were fed on standard rat pellet diet and allowed to acclimatize for one week. They were provided water *ad libitum* and maintained under standard laboratory conditions of natural photo period of 12-h light-dark cycle. The animals used in the present study were maintained in accordance with the ethical guidelines of International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan.

2.3.1. Induction of type 2 diabetes using nicotinamide and streptozotocin

Type 2 diabetes was induced using the streptozotocin-nicotinamide model. The rats were first injected intraperitoneally with nicotinamide (120 mg/kg bw) and 15 min later streptozotocin (60 mg/kg bw) were injected intravenously. Streptozotocin was dissolved in citrate buffer (0·1 M, pH 4·5) immediately before injection. This is due to the short half-life of streptozotocin (35–40 min), thus ensuring its effectiveness. The animals in the control groups were injected with a required volume of citrate buffer and normal saline only.

After 2 weeks, animals with blood glucose level > 190–200 mg/dl were considered as diabetic and used for the study.

2.3.2. Experimental design

The rats were divided into four groups, each consisting of six animals.

Group 1–Normal rats + Pelletized mouse chows
Group 2–Diabetic (Untreated)
Group 3–Diabetic + *C. volubile* crude extract (400 mg/kg bw)
Group 4–Diabetic + *C. volubile* EtOAc fraction (400 mg/kg bw)

The rats were monitored daily for food and water intake, and body weight. Blood glucose levels of the rats were monitored on weekly basis with a glucometer (Accu-Chek). Treatment lasted for one week. At the end of the experiment, the rats were fasted overnight and anaesthetized with sodium thiopental (60 mg/kg), then sacrificed.

2.3.3. Serum preparation

Blood was collected via cardiac puncture. The blood samples were centrifuged within 30 min at 3000 rpm for 10 min, and serum was separated, aliquoted and stored at −80 °C for further