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Multiple pharmacological targets, cytotoxicity, and phytochemical profile of *Aphloia theiformis* (Vahl.) Benn.

Marie Carene Nancy Picot[a], Onur Bender[b], Arzu Atalay[b], Gokhan Zengin[c], Loïc Loffredo[d], Francis Hadji-Minaglou[d], Mohamad Fawzi Mahomoodally[a,*]

[a] Department of Health Sciences, Faculty of Science, University of Mauritius, 230 Réduit, Mauritius
[b] Biotechnology Institute, Ankara University, 06100, Ankara, Turkey
[c] Department of Biology, Science Faculty, Selcuk University, Campus, 42250 Konya, Turkey
[d] BotaniCert, Botanical and Phytochemical Authentication, 4, trav. Dupont, 06130 Grasse, France

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ABSTRACT

Aphloia theiformis (Vahl.) Benn. (AT) is traditionally used in Sub-Saharan African countries including Mauritius as a biomedicine for the management of several diseases. However, there is a dearth of experimental studies to validate these claims. We endeavoured to evaluate the inhibitory effects of crude aqueous extract as traditionally used together with the crude methanol extracts of AT leaves on urease, angiotensin (I) converting enzyme (ACE), acetylcholinesterase (AChE), cholesterol esterase (CEase), glycogen phosphorylase a (GPa), and glycation *in vitro*. The crude extract showing potent activity against the studied enzymes was further partitioned using different solvents of increasing polarity. The enzyme inhibitory and antiglycation activities of each fraction was assessed. Kinetic of inhibition of the active crude extract/fractions on the aforementioned enzymes was consequently determined using Lineweaver-Burk plots. An ultra-high performance liquid chromatography (UHPLC-UV/MS) system was used to establish the phytochemical profile of AT. The real time cell analysis system (iCELLigence™) was used to monitor any cellular cytotoxicity of AT. Crude methanolextract (CME) was a potent inhibitor of the studied enzymes, with IC_{50} ranging from 696.22 to 19.73 μg/mL. CME (82.5%) significantly ($p < 0.05$) inhibited glycation and was comparable to aminoguanidine (81.5%). Ethyl acetate and *n*-butanol fractions of CME showed non-competitive, competitive, and uncompetitive mode of inhibition against ACE, CEase, and AChE respectively. Mangiferin, a xanthone glucoside was present in CME, ethyl acetate, and *n*-butanol fractions. Active extract/fractions were found to be non-cytotoxic ($IC_{50} > 20$ μg/mL) according to the U.S National Cancer Institute plant screening program. This study has established baseline data that tend to justify the traditional use of AT and open new avenues for future biomedicine development.

1. Introduction

Therapeutic virtues of plant metabolites have been substantially used for the management of several human ailments. Indeed, traditional herbal medicinal knowledge and utilisation bequeathed from generation to generation represent a fundamental source of study for the discovery of novel drugs [1]. Interestingly, medicinal plants are known to possess an array of pharmacological activities and are thus considered as emerging therapeutic tools to achieve a multi-target strategy for the management of complex chronic diseases. For instance, *Azadirachta indica*, commonly known as neem was reported to exhibit immunomodulatory, antifungal, anti-inflammatory, antioxidant, antidiabetic, antibacterial, antimalarial, anticarcinogenic, antiviral,

Abbreviations: DT2, diabetes type 2; AT, *Aphloia theiformis*; ACE, angiotensin (I) converting enzyme; AChE, acetylcholinesterase; CEase, cholesterol esterase; GPa, Glycogen phosphorylase a; CME, crude methanol extract; CAE, crude aqueous extract; U.S, United States; UHPLC, UV/MS Ultra High Performance Liquid Chromatography-Ultra Violet/Mass Spectrometry; MSIRI, Mauritius Sugarcane Industry and Research Institute; HHL, hippuryl-glycyl-glycine; DTNB, 5 5-dithio-bis (2-nitrobenzoic) acid; PNPB, *p*-nitrophenylbutyrate; AGEs, advanced glycation end products; BSA, bovine serum albumin; HEK, human embryonic kidney cells; DMEM, Dulbecco's Modified Eagle's Medium; CO, $_{22}$carbon dioxide; RTCA, real time cell analysis system; DMSO, dimethyl sulfoxide; PAD, photodiode array detection; ELSD, evaporative light scattering detector; ANOVA, one way analysis of variance; DCM, dichloromethane; IC_{50}, concentration of inhibitor required to reduce response by half.

* Corresponding author.
E-mail address: f.mahomoodally@uom.ac.mu (M.F. Mahomoodally).

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antimutagenic, and antiulcer properties [2]. *Morinda citrifolia* or noni is another plant which has been found to possess several therapeutic properties namely, analgesic, antibacterial, antifungal, immune enhancing, anthelminthic, antiviral, hypotensive, anti-inflammatory, and antitumor properties [3]. Medicinal plants have become cynosure for the development of new drugs in modern medicine.

Aphloia theiformis (Vahl.) Benn. (AT), a traditionally used medicinal plant in Sub-Saharan African countries including Mauritius and has attracted much interest. This plant belonging to the botanical family Flacourtiaceae, is used for the management of leucorrhoea and dizziness in Comoros [4]. In Mauritius, therapeutic use of AT include the treatment of cataract, DT2, and cholesterol [5]. AT is also used as an abortive, emetic, anti-malarial, anti-tuberculosis, and antacid in Madagascar [6]. Previous scientific studies have reported the immunostimulatory effects [4], anti-microbial activities [7], and photo protective properties [8] of AT leaves.

Despite the availability of ethnopharmacological data on AT, there is still a dearth of scientific information on the potential of AT as inhibitor of key enzymes linked to diabetes type 2 (DT2), obesity, urolithiasis, hypertension, and Alzheimer's disease. Additionally, to our knowledge, there have been no attempt to evaluate the antiglycation potential and cytotoxicity activity of this plant. The main aim of this *in vitro* study was to investigate the inhibitory activity of AT on glycogen phosphorylase a, cholesterol esterase, urease, angiotensin (I) converting enzyme, and acetylcholinesterase. The kinetics of inhibition of AT on these enzymes were also elucidated. Finally, phytochemicals present in AT were identified using the UHPLC-UV/MS and cytotoxicity of the active extracts was evaluated using real time cell analysis iCELLigence system.

2. Materials and methods

2.1. Collection of plant material

The leaves of AT were collected from MontVert Nature Reserve situated on the upper humid region of Mauritius. Authentication was carried out by Mr. K. Pynee, Senior Technical Assistant/Botanist of the Mauritius Herbarium at the Mauritius Sugarcane Industry and Research Institute (MSIRI), Réduit, Mauritius. A voucher specimen bearing the reference number MAU26544 was deposited at the MSIRI herbarium.

2.2. Chemicals and reagents

4-Nitrophenyl butyrate (PNPB), hydroxyurea, urease from *Canavalia ensiformis* (EC number 232.656.0), sodium hypochlorite, pancreatic cholesterol esterase (EC number 232-808-6), simvastatin, angiotensin (I) converting enzyme (EC number 3.4.15.1), N-Hippuryl-His-Leu (HHL), captopril, acetyl cholinesterase (EC number 232-559-3), galantamine, 5, 5-dithio-bis (2-nitrobenzoic) acid (DTNB), acetyl thiocholine iodide, glycogen phosphorylase a (EC number 232-878-8), glycogen, α-D-glucose-1-phosphate and albumin from bovine serum were purchased from Sigma-Aldrich, Germany. All other chemicals used were of analytical grade.

2.3. Extraction

The leaves were washed under running tap water and shade dried. The dried leaves were then grounded using a Pacific mixer-grinder. Crude methanol extract (CME) was prepared by exhaustively extracting 250 g of ground plant material with methanol at 70% (v/v). Crude aqueous extract (CAE) was prepared following decoction method which involves boiling 50 g of ground plant material into 200 mL distilled water for 30 min. The filtrates were concentrated under vacuum using a rotary evaporator (Bibby Scientific Ltd, UK).

2.4. Solvent partitioning

CME was suspended in distilled water and sequentially partitioned using four solvents of increasing polarity, namely hexane, dichloromethane, ethyl acetate, and *n*-butanol. The resulting solvent fractions were concentrated under vacuum using a rotary evaporator [9].

2.5. Urease inhibition assay and kinetic studies

Urease inhibitory activity was assessed following the modified Berthelot reaction method [10]. Briefly, 15 μL of urease solution (1 mg/mL) prepared in phosphate buffer saline pH 7.4 was added to 135 μL sample solution and pre-incubated at 37 °C for 15 min. The reaction was initiated by adding 850 μL urea (0.06 mM) and the mixture was allowed to stand at 37 °C for 60 min. The reaction was terminated by adding 500 μL of solution A (0.5 g phenol and 2.5 mg sodium nitroprusside in 50 mL distilled water) and 500 μL of solution B (250 mg sodium hydroxide and 820 μL sodium hypochlorite at 5% in 50 mL in distilled water). The mixture was incubated at 37 °C for 30 min and the absorbance was measured at 625 nm. Percentage inhibition was calculated as follows: $[(Abs_{control}-Abs_{sample})/Abs_{control}]$ x100. The concentration of sample required to inhibit urease activity by 50% (IC_{50}) under standard assay conditions was calculated from the percentage inhibition values.

The mode of inhibition of active samples on urease was determined by varying urea concentration based on the modified method as described by Biglar, Sufi, Bagherzadeh and Mojab [10]. Sample solution (135 μL) was pre-incubated with 15 μL urease (1 mg/mL in PBS pH 7.4) at 37 °C for 15 min. Graded concentration of urea (850 μL) was then added to the mixture to initiate the reaction. To ensure steady reaction time, solutions A and B were added to each mixture after exactly 30 min to terminate the reaction. The absorbance of ammonia formed was read at 625 nm using a spectrophotometer after 30 min. Microsoft Office Excel 2007 was used to generate appropriate lines derived from appropriate Lineweaver-Burk plots. The kinetic parameters namely, the Michaelis-Menten constant affinity (K_m) and maximum velocity (V_{max}) were then calculated from the linear equations.

2.6. Angiotensin (I) converting enzyme (ACE) inhibition assay and kinetic studies

ACE inhibitory activity was determined following the method described by Cushman and Cheung [11]. A volume of 50 μL of ACE solution (0.25 mU/mL) prepared in phosphate buffer pH 8.3 was mixed to 50 μL of sample solution and incubated at 37 °C for 15 min. To start the reaction, 150 μL hippuryl-glycyl-glycine (HHL) (8.3 mM) was added to the enzyme-sample mixture and the reaction was carried out at 37 °C for 30 min. The reaction was stopped by the addition of 250 μL hydrochloric acid (1 M). A volume of 500 μL of ethyl acetate was added to the reaction mixture, vortexed and centrifuged at 3000 rpm for 15 min to extract hippuric acid formed. After centrifugation, 200 μL of the supernatant was removed and ethyl acetate was evaporated under reduced pressure. Distilled water was added to dissolve the residual hippuric acid and the absorbance was measured at 228 nm. The% inhibition and IC_{50} were calculated as previously described.

The mode of inhibition of active samples on ACE was determined by varying HHL concentration [11]. Briefly, 50 μL sample solution was pre-incubated with 50 μL ACE (0.25 mU/mL in phosphate buffer pH 8.3) at 37 °C for 15 min. Graded concentration