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High resolution UPLC-MS/MS profiling of polyphenolics in the methanol extract of *Syzygium samarangense* leaves and its hepatoprotective activity in rats with CCl_4-induced hepatic damage

Mansour Sobeh[a,*], Fadia S. Youssef[b], Ahmed Esmat[c,d], Ganna Petruk[e], Ahmed H. El-Khatib[f,g], Daria Maria Monti[e], Mohamed L. Ashour[b], Michael Wink[a,**]

[a] Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, 69120 Heidelberg, Germany
[b] Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University Abbassia, 11566 Cairo, Egypt
[c] Department of Pharmacology, Faculty of Medicine, King Abdulaziz University, 21589 Jeddah, Saudi Arabia
[d] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, 11566 Cairo, Egypt
[e] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, Via Cinthia 4, 80126 Naples, Italy
[f] Department of Chemistry, Humboldt-Universität zu Berlin, Berlin, Germany
[g] Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt

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ABSTRACT

Oxidative stress plays a crucial role in the development of several liver diseases. Many natural polyphenols can attenuate oxidative stress and liver injury. In this study, a phytochemical profiling of a methanol extract from leaves of *Syzygium samarangense* revealed 92 compounds belonging to flavonoids, phenolic acids, condensed tannins, and ellagitannins. The *S. samarangense* extract exhibited a noticeable antioxidant activity with an EC_{50} of 5.80 µg/mL measured by DPPH scavenging capacity assay, 2632 Trolox equivalents, 10 mM Fe^{2+} equivalents/mg of samples by TEAC and FRAP assays, respectively. The total phenolic content was 419 mg gallic acid equivalent GAE/g extract. In a cell-based model (HaCaT cells), the extract completely inhibited ROS production induced by UVA, and prevented GSH-depletion and p38 phosphorylation. In addition, the extract exhibited a substantial antioxidant and hepatoprotective activities in CCl_4-treated rats, with an increase in GSH (reduced glutathione) and SOD (superoxide dismutase) activities by 84.75 and 26.27%, respectively, and a decrease of 19.08, 63.05, 52.21, 37.00, 13.26, and 15.15% in MDA, ALT, AST, TB (total bilirubin), TC (total cholesterol), and TG (total glycerides), respectively. These results were confirmed by histopathological analyses. We believe that *Syzygium samarangense* is a good candidate for further evaluation as an antioxidant and liver protecting drug.

1. Introduction

The liver constitutes the largest and most critical detoxifying organ within the human body. In spite of the necessity of oxygen to maintain life, sometimes it can become toxic through the generation of reactive oxygen species (ROS), which play an important role in the liver pathology because of lipid peroxidation and DNA damage (Panovska et al., 2007).

Infections, autoimmune disorders, multiple hazardous substances such as acetaminophen, isoniazid, carbon tetrachloride, non-steroidal anti-inflammatory drugs, and alcohol can cause liver inflammation, serious damage and liver cirrhosis. Although the liver is known to possess a great regeneration power after damage, the aforementioned factors can eventually lead to serious hepatic ailments (Pandit et al., 2012).

In modern medicine, evidence-based synthetic liver-protective

Abbreviations: $ABTS^{\cdot+}$, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; ALT, alanine transaminase; ANOVA, one way analysis of variance; AST, aspartate transaminase; CCl_4, carbon tetrachloride; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; ESI, electrospray ionization; FRAP, Ferric reducing antioxidant power; GAE, gallic acid equivalent; GSH, reduced glutathione; HaCaT cells, normal human keratinocytes; HR-UPLC-MS/MS, high resolution ultra-high performance liquid chromatography mass-mass spectrometry; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TB, total bilirubin; TC, total cholesterol; TEAC, Trolox equivalent antioxidant capacity; TG, total glycerides; UVA, near ultra violet radiation (315–400 nm)

* Corresponding author.
** Corresponding author.
E-mail addresses: sobeh@uni-heidelberg.de (M. Sobeh), wink@uni-heidelberg.de (M. Wink).

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agents are nearly absent. However, several medicinal plants with phenolic secondary metabolites are known for their hepatoprotective properties. A lignan mixture from *Silybum marianum*, termed silymarin, is well-known and clinically applied (van Wyk and Wink, 2015, 2017).

The Myrtaceae comprises about 131 genera and 5500 species that are characterized by their abundant antioxidants, mainly flavonoids, flavonols, anthocyanins, ellagitannins as well as phenolic acids (Nair et al., 1999). Among its genera, *Syzygium* is a large genus that includes about 1100 species, many of which had been taxonomically confused with the genus *Eugenia* (Wrigley and Fagg, 2013).

Syzygium samarangense (Blume), Merr. & Perry (synonym *Eugenia javanica* L.) is commonly known as wax apple and characterized by its bell-shaped edible fruit which shows different colors. Extracts from this plant possess antibacterial, anti-inflammatory, analgesic, and spasmolytic properties. Additionally, immune-stimulant, antipyretic, and diuretic activities were reported from *S. samarangense*, as well as anti-hyperglycemic activity in diabetes mellitus type II (Shen et al., 2013). These pharmacological properties are probably attributed to the existence of various secondary metabolites, such as flavonoids, chalcones exemplified by 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone and its isomer 5-*O*-methyl-4′-desmethoxy matteucinol. Gallic and ellagic acids, in addition to many tannins and ellagitannins as vescalagin are present in *S. samarangense* (Nair et al., 1999).

Although *S. samarangense* has been investigated for a plethora of biological activities, its antioxidant activity and its potential hepatoprotective activity, related to the ability to counteract liver deterioration caused by xenobiotics, has not been reported in literature. Thus, in this study, the chemical profiling of a leaf extract was carried out using HR-UPLC-MS/MS. In addition, we investigated the antioxidant potential of the methanol leaf extract both *in vitro*, using DPPH, TEAC, and FRAP assays and in a cell-based model, using normal human keratinocytes (HaCaT cells) in which oxidative stress was induced by UVA irradiation. Finally, the antioxidant and hepatoprotective activities were analyzed *in vivo* using rats with CCl_4-induced hepatic toxicity.

2. Materials and methods

2.1. Plant material

Leaves of *S. samarangense* were collected from fully mature trees that were cultivated in a private botanical garden during the spring season in the period between April to May 2014. The plant was authenticated morphologically by Mrs. Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt (Sobeh et al., 2016). A voucher specimen of the plant material is being kept at Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University with voucher number PHG-P- SS-182.

2.2. Preparation of the plant extract

Air dried leaves (100 g) of *S. samarangense* were ground into a coarse powder and exhaustively percolated in methanol (3 × 0.5 L). After filtering the extract, it was consequently evaporated under reduced pressure at 40 °C until dryness. Upon lyophilization, 15 g dried extract was obtained.

2.3. Chemicals and kits

Diphenylpicrylhydrazyl (DPPH) and carbon tetrachloride (CCl_4) were purchased from Sigma® (Sigma-Aldrich, St. Louis, USA). Quercetin and gallic acid were purchased from Gibco® (Invitrogen; Karlsruhe, Germany). All kits for the assessment of the biochemical parameters as alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TB), total cholesterol (TC), total glycerides (TG), superoxide dismutase (SOD) activity, concentration of reduced glutathione (GSH), and lipid peroxidation marker malondialdhyde (MDA) were obtained from Biodiagnostics (Cairo, Egypt). LC-MS analysis was performed using UPLC solvents grade. All other chemicals and kits were of the highest grade commercially available.

2.4. Chemical profiling

The phytochemical profiling of the plant polyphenolics was done using LC consisting of Agilent 1200 series. The LC column was Gemini® 3 μm C18 110 A°, 100 mm × 1 mm i. d (Phenomenex, USA). The column was protected with a guard column (RP-C18, 5 μm 100 A°, 5 mm × 300 μm i. d). Two solvents (A) water, and (B) 90% MeOH (2% acetic acid each) were used as a mobile phase at a flow rate of 50 μL/min 10 μL sample was injected *via* autosampler. Gradient from 5% B at 0 min to 50% B in 60 min and then increased to 90% in 10 min and kept for 5 min was adopted. A Fourier transform ion cyclotron resonance mass analyzer coupled with electrospray ionization (ESI) system was used (Thermo Fisher Scientific, Bremen, Germany). The instrument was set to the following conditions: capillary voltage of 36 V, a temperature of 275 °C. Voltage of 5 kV, and desolvation temperature of 275 °C were used for the API source. As a nebulizing gas, nitrogen with a flow rate of 15 L/min was used. The ions were collected in a high resolution up to 100,000 and full mass scan mass range of 150–2000 *m/z*. Xcalibur® software (Thermo Fisher Scientific, USA) was used for recording and integrating the chromatograms.

2.5. In vitro antioxidant evaluation

The Folin-Ciocalteu method was applied to determine the total phenolic content as previously described by Zhang et al. (2006), DPPH$^•$ (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extract was assessed using the standard technique described by Blois (1958), trolox equivalent antioxidant capacity (TEAC) persulfate decolorizing kinetic assay was carried out as previously reported by Re et al. (1999), ferric reducing antioxidant power (FRAP) assay was used to measure reduction ability of the extract to convert the ferric complex (2, 4, 6-tripyridyl-*s*-triazine - Fe^{3+}-TPTZ) to its ferrous form (Fe^{2+}-TPTZ) at low pH following the protocol described by Benzie and Strain (1996) and they were adapted to 96-well plate as previously described (Ghareeb et al., 2017).

2.6. Cell culture and MTT assay

Human epidermal keratinocytes (HaCaT), provided by Innoprot (Biscay, Spain), were cultured in Dulbecco's Modified Eagle's Medium (EuroClone), supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics (EuroClone) in a 5% CO_2 humidified atmosphere at 37 °C. Every 48 h, HaCaT cells were sub-cultured in a ratio of 1:3. Briefly, the culture medium was removed and cells were rinsed with PBS (EuroClone), detached with trypsin-EDTA (EuroClone) and diluted in fresh complete growth medium.

For dose and time dependent cytotoxicity experiments, cells were seeded in 96-well plates at a density of 2×10^3/well. 24 h after seeding, increasing concentrations of the methanol extract (from 25 to 200 μg/mL) were added to the cells. After 24 and 48 h incubation, cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described in Monti et al. (2015). Cell survival was expressed as the percentage of viable cells in the presence of the extract compared to controls. Two groups of cells were used as control, i.e. cells untreated with the extract and cells supplemented with identical volumes of buffer. Each sample was tested in three independent analyses, each carried out in triplicates.

2.7. Oxidative stress

UVA light (100 J/cm^2) was used as source of oxidative stress. To investigate oxidative stress, cells were plated at a density of