



Antioxidant and anti-inflammatory activities of the major phenolics from *Zygophyllum simplex* L.



Hossam M. Abdallah^{a,b,*}, Ahmed Esmat^c

^a Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia

^b Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt

^c Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt

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ABSTRACT

Ethnopharmacological relevance: *Zygophyllum simplex* L. is a halophyte plant that follows Zygophyllaceae. The plant is growing in arid and semiarid regions. It has been used traditionally in Arabic region to treat gout, asthma and inflammation.

Aim of study: Although anti-inflammatory activity has been reported for this plant, this study aimed to isolate and identify the major constituents of *Zygophyllum simplex* L., as well as assessing their antioxidant and anti-inflammatory activities *in-vitro*. In this study, the mechanism of anti-inflammatory activity of the isolated compounds was assessed.

Materials and methods: Defatted fraction of the total methanol extract of the aerial parts of *Z. simplex* was repeatedly chromatographed on Diaion HP-20, polyamide, and RP₁₈ columns to give five major phenolic compounds. The identity of the purified compounds was established by NMR experiments and comparing with previously known analogs. Moreover, the antioxidant and anti-inflammatory activities of the purified phenolics were investigated *in-vitro* through measuring of NFκB, PGE₂, IL-6, IL-1β and TNF-α levels in human peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA).

Results: Phytochemical investigation of the flowering aerial parts of *Z. simplex* resulted in isolation of five major metabolites identified as isorhamnetin-3-O-β-D-rutinoside (1), myricitrin (2), luteolin-7-O-β-D-glucoside (3), isorhamnetin-3-O-β-D-glucoside (4), and isorhamnetin (5). It is noteworthy to report that compounds 1–3 were isolated from the plant for the first time. It was reported that NFκB represents an important linkage between oxidative stress and inflammation. Compounds 2 and 3 have exhibited the highest antioxidant activity and showed the most efficient in decreasing NFκB p65 at the lowest concentration (1 μM). Moreover, at 1 μM concentrations, only compounds 2 and 3 significantly decreased IL-6, IL-1β and TNF-α levels from PHA treatment. Nevertheless, at 100 μM, all isolated metabolites significantly decreased IL-6 compared to PHA treatment.

Conclusion: Five major phenolic compounds were isolated from *Z. simplex*. Anti-inflammatory activity exhibited by the isolated compounds augment the traditional use of this plant as anti-inflammatory. The effect was mediated *via* inhibition of NFκB through antioxidant mechanism and subsequent inhibition to other inflammatory mediators like TNF-α, IL-1β and IL-6.

1. Introduction

The biological process “Inflammation” could be defined as a series of changes, which arises in living tissue upon injury and/or is the response to damage of living microcirculation [1]. Inflammation includes blood flow alterations, increased permeability of blood vessels, tissue destruction through the activation and migration of leukocytes with synthesis of reactive oxygen species (ROS), and the synthesis of local inflammatory mediators, such as prostaglandins (PGs), leuko-

trienes, and platelet-activating factors induced by phospholipase A₂, cyclooxygenases (COXs), and lipoxygenases (Wiert, 2007).

Therefore; in many cases inflammation is mediated by reactive oxygen species (ROS) which produced from phagocytic leukocytes that invade the tissue. Moreover, ROS promote cytotoxicity and may initiate inflammation through up-regulation of some genes that code for proinflammatory cytokines (Conner and Grisham, 1996). In addition, ROS are included in production of nuclear transcription factor κB (NFκB) which induces production of inflammatory cytokines and COX-

* Corresponding author at: Department of Natural Products, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia.

E-mail address: hmafi2013@gmail.com (H.M. Abdallah).

2 (Schinella et al., 2002). Therefore, using of antioxidants will diminish the effect of free radicals, inhibit formation of NF- κ B and consequently prevent production of inflammatory mediators. For that reason, regular intake of antioxidants either in form of drugs (vitamins C and E) or food (flavonoids in fruits and vegetables) will suppress generation of ROS or protect against lipid peroxidation and inflammation. Flavonoids, a class of naturally occurring metabolite in plant kingdom, possess anti-inflammatory properties (Di Carlo et al., 1999; Gerritsen et al., 1995; Middleton Jr, 1998; Santos, 2004). Luteolin, quercetin and isoflavonoid genistin prevented the release of TNF- α and IL-6 from RAW macrophage induced by LPS (Xagorari et al., 2001). Silymarin from *Silybum marianum* was able to inhibit TNF- α induced NF- κ B activation in human histocytic lymphoma U-937 cells (Manna et al., 1999). Nobiletin from citrus can inhibit PGE₂ production in rabbit synovial fibroblasts (Ishiiwa et al., 2000). Baicalin and wogonin from *Scutellaria baicalensis* are used in treatment of different inflammatory conditions including bronchitis, asthma and dermatitis (Michinori et al., 1984).

Zygophyllum simplex L. is a halophyte plant that follows Zygophyllaceae; and known in Arabic region as (Garmal, Hureim) (Batanouny, 1981; Tackholm and Drar, 1956). It has been used traditionally in Arabic and Indian regions to treat gout, asthma and inflammation (Haroun and Abualghaith, 2015; Kakrani et al., 2011), this effect was referred to ethyl acetate fraction (Kakrani et al., 2011).

Previous reports revealed the isolation of isorhamnetin, isorhamnetin-3-O-glucoside, kaempferol 3-O-rutinoside, 6''-(2-E-butenoyl) isorhamnetin-3-O-glucoside, sitosterol glucoside and quinic acid 3- α -L-rhamnoside (Hassanean and Desoky, 1992).

This work was implemented to evaluate this claim; major phenolic compounds of *Z. simplex* will be isolated and identified. Moreover, their antioxidant and anti-inflammatory activities will be assessed *in-vitro* through measuring of PGE₂, IL-6, IL-1 β , NF- κ B and TNF- α levels in human peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA).

2. Material and methods

2.1. General

UV spectra were recorded on UV IKON 940 spectrophotometer using MeOH. Bruker DRX-400 MHz Ultrashield spectrometer (Bruker BioSpin, Billerica, MA, USA) was used to determine NMR spectra using DMSO-*d*₆ as solvent. Different adsorbents were used in separation process including, polyamide 6 for column chromatography (Merck, Darmstadt, Germany), Sephadex LH-20 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden), Silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany), and Silica gel 100C₁₈- (0.04–0.063 mm, Merck, Darmstadt, Germany). TLC analysis was performed on pre-coated TLC plates with Silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany).

2.2. Plant material

Flowering aerial parts of *Zygophyllum simplex* L. were collected from Jeddah, Saudi Arabia. The collected plant was kept at the Herbarium of the Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University (ZS-4036). The identity of plant material was performed by staff of the Department of Taxonomy, Faculty of Science, King Abdulaziz University.

2.3. Extraction, isolation and identification

Flowering aerial parts of *Z. simplex* (1 kg) were dried, ground and extracted with methanol (4×5 L) at room temperature till exhaustion. Total methanol extract was evaporated under reduced pressure to give 38 g of brown residue. The residue was suspended in the least amount of water and fractionated with chloroform (4×500 ml). The remaining

aqueous mother liquor was evaporated (18 g), dissolved in distilled water and fractionated on polyamide column (5×110 cm, 250 g), starting with water followed by mixtures of MeOH/ H₂O mixtures until pure MeOH. Similar fractions were pooled together to give three collective fractions (A-C) after TLC screening using different spraying reagents including 5% AlCl₃, 1% FeCl₃, or anisaldehyde-H₂SO₄. Fraction A (10–30% MeOH) was chromatographed on a Silica gel 100C₁₈- reversed phase column using MeOH:H₂O, 1:9 as an eluent to afford compounds **1** (40 mg) and **2** (40 mg). Fraction B (40–60 - % MeOH) was subjected to Silica gel 100C₁₈- reversed phase column using MeOH:H₂O, 2:8 as an eluent to afford compounds, **3** (32 mg) and **4** (50 mg). Fraction C (70–90% MeOH) was subjected to silica gel column (25 cm x 2 cm, 50 g) and eluted with a CHCl₃-MeOH mixture (9:1, v/v) to give compounds **5** (150 mg) which was further purified on Sephadex LH-20 using MeOH as an eluent.

2.4. Biological study

2.4.1. Material for biological study

ELISA kits for assessment of TNF- α , IL-1 β and IL-6 were purchased from Orgenium Laboratories' (Vantaa, Finland). NF- κ B p65 was assessed using ELISA kit obtained from Abcam (#: ab 176648; MA, USA) according to the manufacturer instructions. Tissue culture reagents including: fetal bovine serum (FBS), DMEM, penicillin/streptomycin, and phosphate-buffered saline (PBS) were obtained from Lonza (Basel, Switzerland). Ficoll-Paque was obtained from GE Healthcare (Biosciences AB, Uppsala, Sweden) and phytohemagglutinin (PHA) from Gibco (Germany). All other chemicals were of the highest available commercial grade.

2.4.2. Assay for DPPH free-radical scavenging potential

Anti-oxidant characterization was assessed by 2,2'-diphenyl-1-picrylhydrazyl (DPPH). The principle relies on reversible reduction of DPPH resulting in a relatively stable free radical that has a strong absorption at λ 517 nm (Amarowicz et al., 2004). One hundred microliter from each sample in HPLC methanol was added to 3.9 ml, 0.004% (w/v) DPPH in methanol so that three different concentrations (1, 10 and 100 μ M) of each compound were tested. The mixtures were left at room temperature for 30 min and the absorbance was measured at λ 517 nm. The scavenging activity (SCA) was expressed as a percentage of scavenging activity on DPPH:

$$\text{SCA\%} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100\%,$$

where A_{control} is the absorbance of the control (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus scavenger).

2.4.3. Separation of human lymphocytes

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood obtained from healthy volunteers *via* density centrifugation. In brief, diluted blood samples were warily layered on Ficoll-Paque reagent, followed by centrifugation at 400×g for 15 min (20 °C), and then the lymphocyte layer was cautiously transferred out. The cells were washed and pelleted down with three volumes of PBS-BSA-EDTA. Then, they were maintained in supplemented DMEM at 5% CO₂ for 48 h. It is worthy noted that PBMCs should be freshly prepared.

2.4.4. Lymphocyte challenging

Separated PBMCs were seeded at a density of 1.5 × 10⁶ cells/ml; in 6-well plates. Then, three different concentrations of the test compounds (100, 10 and 1 μ M) were used to pretreat the cells. This concentration range is typical for most phenolics having *in-vitro* biological activities in literature. After 24 h, 10 μ g/ml of mitogen "PHA" was utilized to stimulate the cells. A positive control group included cells challenged with PHA but not the extracts. Unchallenged cells were treated with DMSO in a final concentration equal to test

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