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Sambulin A and B, non-glycosidic iridoids from *Sambucus ebulus*, exert significant *in vitro* anti-inflammatory activity in LPS-induced RAW 264.7 macrophages *via* inhibition of MAPKs's phosphorylation

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ABSTRACT

Ethnopharmacological relevance: The leaves of *Sambucus ebulus* L. (Adoxaceae) are widely used in Turkish folk medicine particularly against inflammatory disorders. The fresh leaves after wilted over fire or the poultices prepared are directly applied externally to heal burns, edema, eczema, urticarial and abscess. Two iridoids were recently isolated (sambulin A, sambulin B) from the leaves of *S. ebulus*.

Aim of the study: This study aims to investigate the *in vitro* anti-inflammatory activities of these iridoids on LPS-induced RAW 264.7 macrophages.

Materials and methods: Raw 264.7 macrophages were treated with 12.5, 25 and 50 μg/ml Sambulin A and 6.25, 12.5 and 25 μg/ml Sambulin B and induced with 1 μg/ml lipopolysaccharides (LPS). Effect of the compounds on nitric oxide (NO) production and cytokines (TNFα, IL-6) were determined by Griess and ELISA assays respectively. iNOS and the phosphorylation levels of MAPKs (ERK, JNK) were examined by Western Blot.

Results: Sambulin A and sambulin B inhibited 52.82% and 72.88% of NO production at 50 and 25 μg/ml concentrations respectively. The levels of iNOS were significantly decreased by both molecules, sambulin B at 25 μg/ml almost completely decreased iNOS levels (97.53%). Both molecules significantly inhibited TNFα productions. However, only sambulin B inhibited IL-6 production. Consequently, it was shown that sambulin B exerted its effect through the inhibition of ERK and JNK phosphorylations.

Conclusion: The prominent bioactivities exerted by two iridoids will contribute to explanation of the usage of *S. ebulus* in traditional medicine against rheumatoid diseases.

1. Introduction

Sambucus ebulus L. (Adoxaceae) or dwarf elder is a widespread annual herbaceous plant which is known as 'cüce mürver' in Anatolia. Particularly the leaves of the plant are used against various types of inflammatory disorders in Turkish folk medicine (Sezik et al., 1992; Yesilada et al., 1999b). The fresh leaves are either externally applied on the affected body part after wilted over open fire or the poultice prepared by boiling in water to treat a wide range of dermatological problems including burns, infectious wounds, snake bites, edema, eczema, and urticaria, while its decoction is used to bath the affected extremities to relieve rheumatic pain (Sezik et al., 1992; Yesilada et al., 1999b).

Previous experimental studies have indicated that *S. ebulus* leaves exert cytotoxic (Shokrazadeh et al., 2009), antiulcer (Yesilada et al., 2014), anti-*Helicobacter pylori* (Yesilada et al., 1999a), anti-microbial (Smee et al., 2011), anti-oxidative (Ebrahimzadeh et al., 2009) and wound-healing activities (Süntar et al., 2010). Anti-inflammatory and anti-nociceptive activities of the leaves have also been investigated in detail by using several *in vivo* experimental models such as: hot plate test, writhing test, tail flick test and carrageenan- or formalin-induced edema, adjuvant-induced chronic arthritis models (Yesilada, 1997;

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Ahmadiani et al., 1998; Ebrahimzadeh et al., 2006). A wide range of phytochemicals have been isolated from the leaves of *Sambucus* species including lignans, iridoids, flavonoids, anthocyanins, and cyanogenins (Gross and Sticher, 1986; D'abrosca et al., 2001; Jordheim et al., 2007; Süntar et al., 2010; Atay et al., 2015).

Inflammation is an elaborated process that involves various pathways. Signaling proteins which are synthesized during these pathways such as prostaglandins, leukotrienes, platelet-activating factors and cytokines such as IL-1α and TNFα are known to contribute to the progression of inflammation (Yesilada et al., 1997; Yesilada, 1997). Eventually antagonizing these inflammatory pathways would inhibit the productions of inflammatory mediators. Therefore these pathways are mainly targeted for the discovery of anti-inflammatory drug candidate. Key players in these pathways including enzymes such as inducible nitric oxide synthase (iNOS), cytokines such as interleukin-1 beta (IL-1*β*), tumor necrosis factor-alpha (TNF*α*) (Tincani et al., 2007) or mitogen activated protein kinases (MAPKs) are frequently employed as the main targets in anti-inflammatory research (Schmitz and Bacher, 2005; Yoon et al., 2010).

Macrophages regulate inflammation by using a wide range of pro-inflammatory mediators (Yoon et al., 2010; Choi et al., 2011; Lu et al., 2012). Lipopolysaccharides (LPS) which is the principal component of the outer membrane of gram-negative bacteria, is one of the most potent activators of macrophages (Schumann et al., 1990). Once macrophages treated with LPS are known to produce inflammatory mediators, such as nitric oxide (NO) and cytokines such as TNFα, IL-1 and IL-6 (Yoon et al., 2010; Choi et al., 2011; Lu et al., 2012). LPS activates MAPKs including extracellular signal-regulated kinases (ERK)-1 and -2, c-Jun N-terminal kinase (JNK), and p38. The activated MAPKs increase inflammatory disease states by regulating the biosynthesis of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and TNFα (Geppert et al., 1994; Swantek et al., 1997; Chan and Riches, 2001). Raw 264.7 is a murine macrophage cell line and *in vitro* LPS-induced Raw 264.7 macrophage model is frequently used as an experimental model to determine the effect on inflammatory response (Yoon et al., 2010; Choi et al., 2011; Lu et al., 2012).

In our previous study, we reported the isolation and structure elucidation of two non-glycosidic iridoids from the methanol (MeOH) extract of *S. ebulus* leaves namely; sambulin A and sambulin B, the latter being a new compound (Atay et al., 2015). The chemical structures of these compounds were shown in Fig. 1. In the present study, the anti-inflammatory activity of sambulin A and sambulin B have been investigated further on several *in vitro* parameters using LPS induced Raw 264.7 macrophages. For this purpose, effects of the compounds on NO, TNF*α*, IL-6 levels were studied by Griess and ELISA methods. The variations in the iNOS protein expressions and phosphorylation levels of MAPKs (ERK1/2, JNK) in response to Sambulin A and Sambulin B treatment were investigated by Western Blotting.

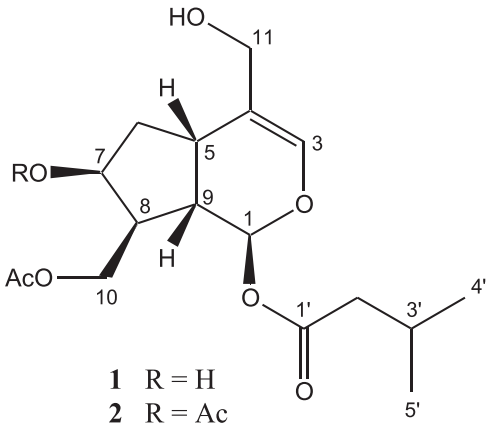

Fig. 1. The chemical structures of Sambulin A (1) and B (2).

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysachharides (LPS), dimethylsulfoxide (DMSO), HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), penicillin-streptomycine was obtained from Invitrogen/Gibco (Grans Island, NY, USA). Antibodies for phospho-ERK1/2, ERK, phospho-JNK, JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). ECL reagent was obtained from Amersham (Buckhinghamshire, UK). Enzyme linked immunosorbent assay (ELISA) kits for TNFα and IL-6 were from R & D Systems (MN, USA). iNOS and β-actin primary antibodies were purchased from Santa Cruz (CA, USA). Griess Reagent System was from Promega (CA, USA). L-NIL was obtained from Calbiochem, Sigma and Cayman respectively. WST-1 reagent was from Roche Applied Science (Mannheim, Germany).

2.2. Plant material

S. ebulus leaves were collected from Uludağ-Bursa (Turkey) in June 2009. The plant was identified by one of the authors (E. Yesilada). A voucher specimen is deposited at the Herbarium of Yeditepe University (YEF 09017). The plant material was dried under shade and powdered prior to extraction.

2.3. Extraction, isolation and structure elucidations of sambulin A and sambulin B

Sambulin A and sambulin B (Fig. 1) were isolated as described previously from the methanolic extract of *S. ebulus* leaves and their structures were elucidated by using 1-D and 2-D nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) techniques (Atay et al., 2015).

2.4. Cells and cell culture

The Raw 264.7 macrophages (ATCC TIB-71) were grown in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. WST-1 assay for cell viability

The non-toxic concentrations of the compounds were determined by using a WST-1 assay kit (Roche Applied Sciences) according to the manufacturer's instructions. WST-1, a tetrazolium-based salt dye, is reduced to a purple formazan salt by metabolically active cells, and it is directly quantified by spectrophotometric measurements. Raw 264.7 cells (22.500 cells/well) in 10% FBS-DMEM were seeded into 96-well plates and various concentrations of compounds were added to the wells with or without 1 μg/ml LPS, and incubated at 37 °C for 24 h. After the supernatant was removed, WST-1 was added directly to the cultures to a final concentration of 5% (v/v), and cells were incubated at 37 °C for an additional 60 min. Absorbance was then read between 420 and 480 nm (λ_{max} 450 nm) using a plate reader. All test compounds were dissolved in DMSO and diluted with DMEM to the appropriate concentrations. The final concentration of DMSO in the culture medium was not more than 0.1% (v/v).