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Anti-inflammatory effect of new kopetdaghins A, C and E from *Dorema kopetdaghense*

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Nitric oxide (NO) is synthesised by activated inflammatory macrophages and is involved in the pathogenesis of inflammatory conditions. *Dorema kopetdaghense* Pimenov (Apiaceae) has been used in traditional medicine from ancient times. In order to investigate anti-inflammatory potential, new kopetdaghins A, C and E were isolated and their non-toxic concentrations, as defined using MTT assay, were evaluated for anti-inflammatory activity. J774A.1 macrophages were stimulated with lipopolysaccharide (1 μg/ml) and kopetdaghins (10–100 μg/ml) for 24 h. The amount of NO production was assessed using Griess reagent. Western blot analysis was employed for evaluating the inducible nitric oxide synthase (iNOS) expression. Kopetdaghins at tested concentrations (10–100 μg/ml) did not reduce cell viability, while they significantly inhibited NO release. The expression of induced iNOS was decreased. Present experiment for the first time revealed the remarkable anti-inflammatory activity of new isolated kopetdaghins from *D. kopetdaghense* which might be potential candidates for further therapeutic investigations.

Keywords: kopetdaghin; inflammation; J774A.1 macrophages; nitric oxide; inducible nitric oxide synthase

Introduction

During the last decades, discovery of endogenous formation of nitric oxide (NO) has led to an explosion in research on NO-induced cellular injury (Abramson, Attur, Amin, & Clancy, 2001; Brown, 1995). NO that is synthesised from L-arginine in several mammalian cells and tissues is a well-established marker of inflammation. NO plays various physiological roles and may also contribute towards pathological processes. When NO is synthesised in large amounts by activated inflammatory macrophages, it shows cytotoxic properties that may be involved in the pathogenesis of acute and chronic inflammations (Moilanen, Whittle, & Moncada, 2003). Supraphysiological amount of NO is produced by inducible nitric oxide synthase (iNOS), which is induced by several stimuli such as bacterial lipopolysaccharide (LPS). During the inflammation, NO production increases remarkably and may show cytotoxic activity. Moreover, the free radical

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nature of NO makes it as a potent pro-oxidant molecule, which is able to provoke oxidative damage and to be potentially harmful for cellular targets (Epe, Ballmaier, Roussyn, Brivida, & Sies, 1996; Kharitonov et al., 1994). Therefore, inhibition of NO production in response to inflammatory stimuli might be a valuable therapeutic approach in inflammatory diseases (Hobbs, Higgs, & Moncada, 1999; Sautebin, 2000).

The genus *Dorema* (Apiaceae) is represented by seven species in Iranian flora (Mozaffarian, 2003, 2007). Among them, *Dorema kopetdaghense* Pimenov and *D. ammoniacum* D. Don are endemic to Iran and have been collected for their resins in Persia since 4000 years ago (Duthie, 1956; Hooper, 1937; Jafari, Chahouki, Tavili, Azarnivand, & Amiri, 2004;). Moreover, members of the *Dorema* species possess several biological activities (Asnaashari, Dadizadeh, Talebpour, Eskandani, & Nazemiyeh, 2011; Bahraminejad, 2012; Nabavi, Nabavi, & Ebrahimzadeh, 2012; Prashanth Kumar, Chauhan, Padh, & Rajani, 2006; Rajani, Saxena, Ravishankara, Desai, & Padh, 2002; Ram, Balachandar, Vijayananth, & Singh, 2011; Yousefzadi et al., 2011).

D. ammoniacum has been used in folk medicine for the treatment of dermatitis, inflammation, spasm, asthma and bronchitis (Ram et al., 2011). It exhibits remarkable antibacterial and antifungal properties that support its folkloric application in the treatment of a number of infectious diseases (Prashanth Kumar et al., 2006; Rajani et al., 2002). Moreover, the essential oil from its ripe fruits shows *in vitro* toxicity on human cancer cell lines (Yousefzadi et al., 2011). Extracts and derivatives of *D. aitchisonii* Korovin ex Pimenov show excellent antihaemolytic and antifungal but weak antioxidant activities (Bahraminejad, 2012; Nabavi et al., 2012). Sesquiterpenes of *D. glabrum* Fisch. & C.A. Mey. exhibited free radical scavenging activity. Also, based on common folk beliefs, it can treat some disorders especially different cancers (Asnaashari et al., 2011; Ibadullayeva, Movsumova, Gasymov, & Mamedli, 2001).

There are only a few reports concerning the chemical constituents of plants within the genus *Dorema*. However, we previously reported the isolation of three new compounds including two prenylated coumarins (kopetdaghins A, C) and a sesquiterpene derivative (kopetdaghin E) from *D. kopetdaghense* (Iranshahi, Shaki, Mashlab, Porzel, & Wessjohann, 2007). In the present study, three kopetdaghins were isolated from the root of *D. kopetdaghense* and their structures were elucidated using various nuclear magnetic resonance (NMR) and high-resolution positive ion ESIMS techniques. Then, their anti-inflammatory effects were evaluated on LPS-induced NO release by J774A.1 macrophages. Finally, we examined if these compounds diminish the expression of iNOS enzyme.

Materials and methods

Plant material

The plant material (*D. kopetdaghense* M. Pimen.) was collected in 2007 from Khor valley, Khorasan Razavi province, Iran. It was identified by Mohammad Reza Joharchi, Ferdowsi University of Mashhad Herbarium (FUMH). A voucher specimen (No. 1001) has been deposited at the herbarium of Faculty of Pharmacy, Mashhad University of Medical Sciences (MUMS).

Isolation of kopetdaghins

Isolation of kopetdaghins (A, C and E) from *D. kopetdaghense* and elucidation of their structures have been done as described in previous paper (Iranshahi et al., 2007).

Cell culture

The J774A.1 murine macrophages were obtained from National Cell Bank of Iran (NCBI, Tehran, Iran) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Laboratories, Detroit, MI) in 95% air and 5% CO_2 humidified atmosphere at 37°C.

Determination of cell growth

Cell growth was assessed by the mitochondrial respiration-dependent reduction method of MTT 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide to formazan (Emami, Zamani Taghizadeh Rabe, Ahi, Mahmoudi, & Tabasi, 2009; Ghazanfari, Zamani Taghizadeh Rabe, Tabasi, & Mahmoudi, 2011; Mahmoudi, Zamani Taghizadeh Rabe, Ahi, & Emami, 2009; Mosmann, 1983; Zamani Taghizadeh Rabe, Mahmoudi, Ahi, & Emami, 2011). J774A.1 macrophages were mechanically scraped and seeded 125×10^3 cells/well in a 96-well plate. Then, cells were incubated with increasing concentrations of kopetdaghins (10–100 µg/ml) in 5% CO_2 at 37°C. After 24 h of incubation, 25 µl of MTT solution (Sigma Chemical Co, St Louis, MO) was added in each well. After that, the medium was removed and cells were lysed with DMSO to dissolve the formazan crystals produced in viable cells. The optical density of the formazan product in each well was measured with a microplate reader at 545 nm. The cell growth percent was calculated by the following formula: Cell growth (%) = $[(OD_{control} - OD_{treated})/OD_{control}] \times 100$.

Measurement of NO production

J774A.1 macrophages were seeded in 24-well plates at 8×10^5 cells/well. After 3 h, cells were treated with various concentrations of kopetdaghins (10–100 µg/ml) and stimulated for 24 h with or without 1 µg/ml of LPS (Serotype 0111:B4, Sigma Chemical Co, St Louis, MO). Nitrite accumulation, as an indicator of NO synthesis, was measured in the culture medium using Griess method (Emami, Zamani Taghizadeh Rabe, Iranshahi, Ahi, & Mahmoudi, 2010; Green et al., 1982). Briefly, equal amounts of cell culture supernatants were mixed with Griess reagent [equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylenediamine-HCl] and incubated at room temperature for 20 min. Then the absorbance at 545 nm was measured in a microplate reader. Nitrite concentration (in µM) was calculated from a sodium nitrite standard curve.

Western blot analysis

Treated and untreated cells were lysed in freshly prepared lysis buffer [20 mM HEPES, pH 7.9, 400 mM NaCl, 0.1% Nonidet (N) P-40, 10% glycerol, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) along with protease inhibitor cocktail] for 45 min on ice. The protein concentrations of cytosolic extracts were determined by Bradford assay. Equal amounts of proteins (30–50 µg/ml) of each cell lysate were dissolved in Laemmli's sample buffer and subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed by transferring proteins from a slab to a sheet of polyvinylidene difluoride (PVDF) membrane at 240 mA for 40 min. The filter was then blocked with 5% skim milk in phosphate buffered saline (PBS) overnight at

4°C. After extensive washing with PBS, the membrane was incubated with anti-mouse iNOS polyclonal antibody (Panomics, Inc, Redwood city, CA) as primary antibody at room temperature for 2 h. Membranes were then incubated with a goat anti-rabbit-horseradish peroxide conjugated antibody (KOMA biotech, Seoul, Korea) as secondary antibody for 1 h at room temperature. β-actin protein was used as housekeeping control. Subsequently, blots were extensively washed with PBS and developed using ECL-detection reagents (Amersham, Cardiff, UK).

Statistical analyses

Data are reported as mean ± SEM values of three independent determinations. All experiments were performed at least three times. Statistical analysis was performed using analysis of variance (ANOVA) test and multiple comparisons were made using Bonferroni's test. *P*-values less than 0.05 were considered statistically significant.

Results

Elucidation the structure of kopetdaghins

Three new compounds were isolated from *D. kopetdaghense*. Elucidation of their structures using various techniques revealed that kopetdaghins A and C were prenylated coumarins, but kopetdaghin E was a sesquiterpene derivative (Figure 1).

Effect of kopetdaghins on cell growth

Growth of macrophages treated with kopetdaghins A, C and E (10–100 μg/ml) was measured using MTT colorimetric assay. Exposure to 10–100 μg/ml of kopetdaghins A,

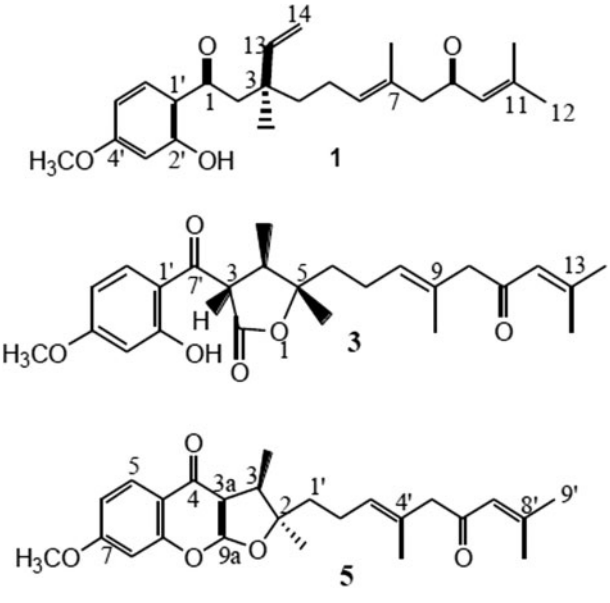

Figure 1. Chemical structures of kopetdaghin A (1), kopetdaghin C (3) and kopetdaghin E (5) isolated from *D. kopetdaghense*.

C and E for 24 h did not change the growth of J774A.1 macrophages (Figure 2). These results suggest that isolated kopetdaghins were not toxic for J774A.1 macrophages up to the concentration 100 μg/ml. Therefore, macrophages were treated with kopetdaghins A, C and E in the concentration range of 10–100 μg/ml during follow-up experiments. The concentrations producing 50% growth inhibition (IC_{50}) values of kopetdaghins A, C and E on J774A.1 macrophages were 474.1 ± 0.9, 496.4 ± 0.7 and 514.3 ± 0.4 μg/ml, respectively.

Inhibitory effects of kopetdaghins on LPS-induced NO production

To assess the effects of kopetdaghins A, C and E on LPS-induced NO production by macrophages, cell culture supernatants were assessed for quantitation of their nitrite contents using Griess reagent. A released basal level of NO in unstimulated J774.A1 macrophages was 0.7 ± 0.01 μM, while LPS-stimulation increased NO production (24.2 ± 0.17 μM). However, pre-treatment with kopetdaghins A, C and E (Figure 3A, B and C, respectively) significantly decreased LPS-induced NO production in a concentration-dependent manner. The inhibitory percent of produced NO after pre-treatment with 10, 20, 50 and 100 μg/ml concentrations of kopetdaghins A, C and E were 57.30 ± 0.66, 77.81 ± 0.16, 94.20 ± 0.38, 100; 14.97 ± 0.32, 43.68 ± 0.28, 75.75 ± 0.12, 100 and 43.59 ± 0.24, 63.09 ± 0.10, 75.37 ± 0.11, 100%, respectively.

Effects of kopetdaghins on LPS-induced iNOs expression

To determine the anti-inflammatory mechanism of kopetdaghins on NO production as an inflammatory mediator, we evaluated the expression of iNOS using Western blot analysis. As demonstrated in Figure 4, the experiment showed a concentration-dependent inhibitory activity of kopetdaghins A, C and E (20 and 50 μg/ml) on LPS-stimulated iNOS expression. The inhibitory profile of kopetdaghins examined on iNOS expression overlapped with their inhibitory activity on NO production.

Discussion

Activated macrophages produce a large amount of NO through the action of iNOs. This inflammatory mediator stimulates other immune cells and can cause inflammatory diseases such as rheumatoid arthritis and endotoxemia-induced multiple organ injury (Hattori, Kasai, & Gross, 2004; Katsuyama, Shichiri, Marumo, & Hirata, 1998; Stuehr, 1997). Some anti-inflammatory drugs prevent the development of human acute and chronic inflammatory diseases by suppressing the production of pro-inflammatory mediators including NO. Moreover, inhibition of iNOS with neutralising antibodies or gene targeting considerably alleviates the development and progression of inflammatory diseases (Hattori et al., 2004; Katsuyama et al., 1998; Stuehr, 1997).

Natural products have played a significant role in drug discovery and development for the treatment of several diseases that have been existed from ancient times to the present. Plants contain many phytochemical agents with various bioactivities (Mollazadeh et al., 2011; Neshati et al., 2009). Numerous experiments have been reported on the effectiveness of isolated natural compounds as anti-inflammatory agents (Maia et al., 2009).

The present study was undertaken to elucidate the anti-inflammatory activity of isolated kopetdaghins on the production of inflammatory mediators *in vitro*. Accordingly,

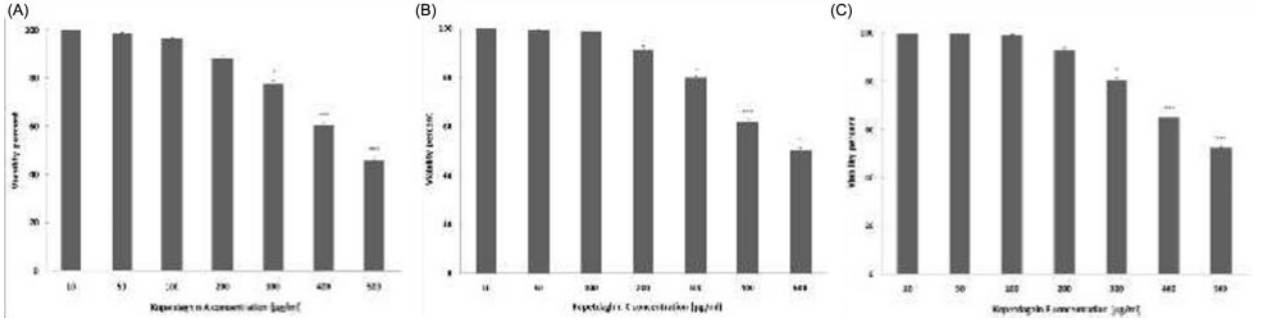

Figure 2. Effect of 10–100 μg/ml of kopetdaghin A (A), kopetdaghin C (B) and kopetdaghin E (C) on the viability of J774A.1 macrophages. Results are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to untreated macrophages.

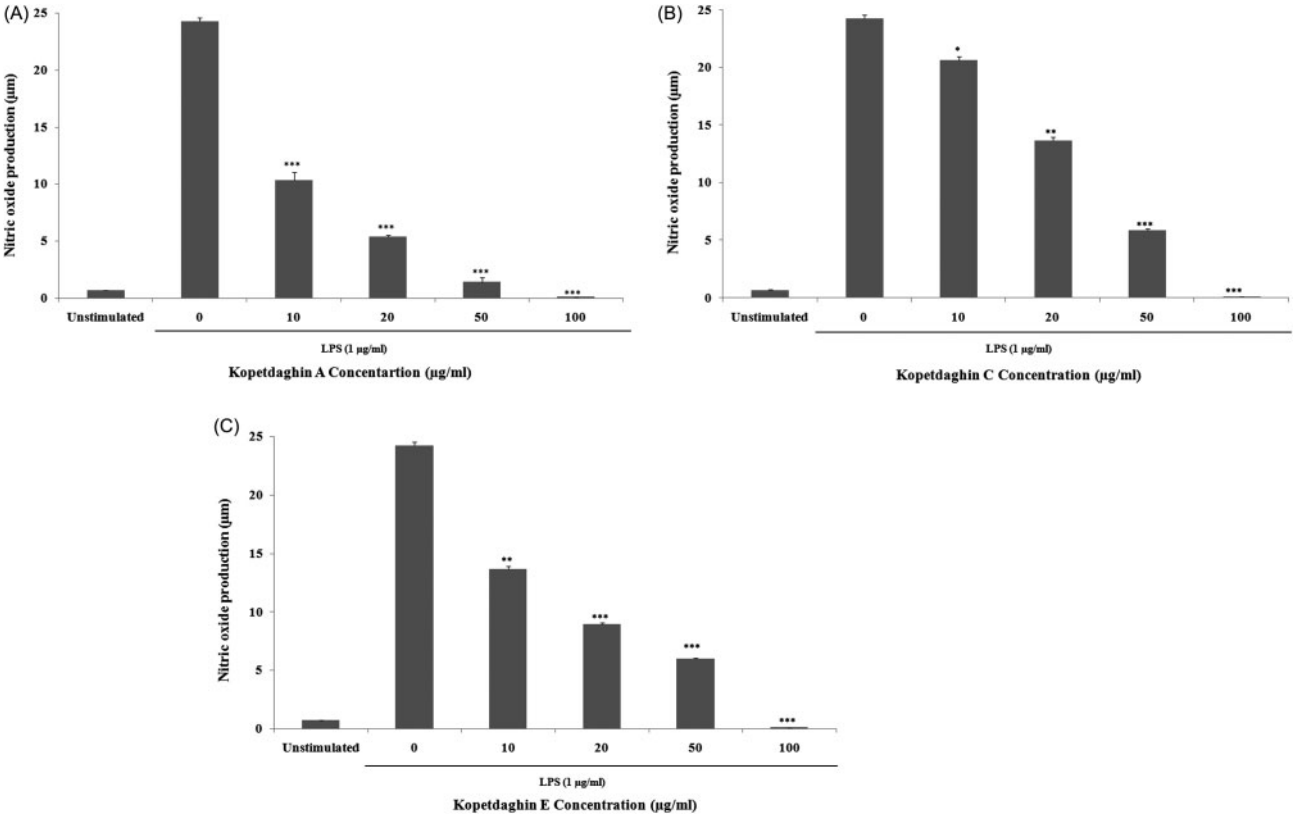

Figure 3. Evaluation of NO production by J774A.1 macrophages stimulated for 24 h with LPS alone or in combination with 10–100 µg/ml of kopetdaghin A (A), kopetdaghin C (B) and kopetdaghin E (C). Results are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to LPS-stimulated macrophages.

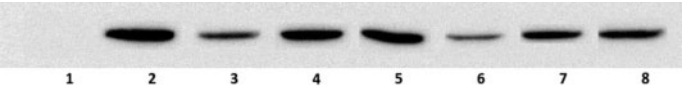

Figure 4. Effect of kopetdaghins A, C and E on LPS-induced iNOS expression. Lysates were prepared from control or LPS (1 μg/ml) stimulated macrophages alone or in combination with kopetdaghins for 24 h. Different lanes were represented as follow: (1) unstimulated macrophages, (2) LPS-stimulated macrophages, (3, 4) stimulated macrophages with 20 and 50 μg/ml of kopetdaghin A, (5, 6) stimulated macrophages with 20 and 50 μg/ml of kopetdaghin C, and (7, 8) stimulated macrophages with 20 and 50 μg/ml of kopetdaghin E.

three kopetdaghins (A, C and E) were isolated from *D. kopetdaghense*. Initially, we determined their toxicity in J774A.1 macrophages and utilised their non-toxic concentrations for further examinations. Subsequently, their anti-inflammatory effects were examined on LPS-induced inflammation in J774A.1 macrophages.

We showed that isolated kopetdaghins (A, C and E) inhibited the production of NO by LPS-stimulated J774A.1 macrophages. Moreover, the expression of iNOS was suppressed in kopetdaghins-treated macrophages. These findings may account for their anti-inflammatory properties.

Conclusions

In summary, we established that isolated kopetdaghins (A, C and E) from *D. kopetdaghense* possessed anti-inflammatory activity through the inhibition of NO production and iNOS expression in LPS-stimulated macrophages. As a result, this inhibitory effect has important implications for the development of anti-inflammatory drugs and strategies to limit pathological inflammation.

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