Research Article

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Anti-inflammatory effect of D-pinitol isolated from the leaves of *Colutea cilicica* Boiss et Bal. on K562 cells

Colutea cilicica Boiss et Bal. yapraklarından izole edilen D-pinitol'ün K562 hücreleri üzerindeki anti-inflamatuar etkisi

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Abstract

Aim: D-pinitol, a natural compound has shown various biological and pharmacological effects. Last studies are focused on the determination of its further pharmacological activities including mainly biological activity. Therefore, isolation of D-pinitol from the leaves of *Colutea cilicica* Boiss et Bal. and investigation of its apoptotic and anti-inflammatory activity on K562 cell lines were aimed in the concept of the study.

Materials and methods: Isolation of D-pinitol was performed by column chromatography. Chemical structure of the compound was confirmed by spectroscopic methods including ^{1}H NMR, ^{13}C NMR, 2D NMR, HPLC-TOF/MS, and IR. Cell viability was evaluated by dose and time dependent in K562 cell lines. D-pinitol was isolated from *C. cilicica* leaves for the first time.

Results: Stimulation of cells with D-pinitol (0–80 μM) was observed for 24, 48 and 72 h. It is determined that D-pinitol inhibited protein expression of Cox-2 in K562 cells. We observed that Poly (ADP-ribose) polymerase (PARP) protein expression did not change, but Cox-2 protein expression reduced with non-cytotoxic concentrations of D-pinitol.

Conclusion: It is concluded that D-pinitol did not affect cell proliferation and apoptosis in K562 cells however reduced the inflammation, significantly. These results show that D-pinitol may be anti-inflammatory agent for the treatment of K562 cells.

Keywords: Anti-inflammatory activity; K562; Colutea cilicica Boiss et Bal.; Isolation; D-pinitol.

Özet

Amaç: Doğal bir bileşik olan D-pinitol, çeşitli biyolojik ve famakolojik etkilere sahiptir. Son çalışmalar, başlıca biyolojik aktiviteyi de kapsayan farmakolojik aktivitelerinin belirlenmesi üzerine odaklanmıştır. Bu nedenle, çalışma kapsamında *Colutea cilicica* Boiss et Bal. bitkisinin yapraklarından D-pinitol'ün izolasyonu, K562 hücre hattına karşı apoptotik ve antiinflamatuvar aktivitesinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: D-pinitol'ün izolasyonu kolon kromatografisi ile gerçekleştirildi. Saf bileşiğin kimyasal yapısı ^{1}H NMR, ^{13}C NMR, 2D NMR, HPLC-TOF/MS ve IR gibi spektroskopik yöntemler ile kesinleştirildi. Hücre canlılığı K562 hücrelerinde zamana ve doza bağlı olarak değerlendirildi. D-pinitol, *C. cilicica* yapraklarından ilk defa izole edildi.

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Bulgular: D- pinitol (0–80 μM) ile hücrelerin uyarılması 24, 48 ve 72 saat boyunca gözlendi. D-pinitol'ün, K562 hücrelerinde Cox-2 protein ekspresyonunu inhibe ettiği belirlendi. D-pinitol'ün sitotoksik olmayan konsantrasyonlarında PARP protein ekspresyonu değişmezken, Cox-2 protein ekspresyonunun azaldığı gözlemlendi.
Sonuç: D-pinitol'ün K562 hücrelerinde hücre proliferasyonu ve apoptozu etkilemediği ancak belirgin biçimde inflamasyonu azalttığı sonucuna varıldı. Bu sonuçlar bize D- pinitol'ün K562 hücrelerinin tedavisi için anti-inflamatuar bir ajan olabileceğini göstermektedir.

Anahtar kelimeler: Anti-inflamatuar aktivite; K562; Colutea cilicica Boiss et Bal.; izolasyon; D-pinitol.

Introduction

Plant derived phytochemicals have a great importance owing to their curative properties on various diseases. Due to the side effects of several allopathic drugs and the increase of resistance to currently used drugs canalized people to use plant materials in the treatment of several diseases. It is reported that more than 80,000 plants have exhibited medicinal property among 250,000 plant species, all over the World [1]. Investigations are focused on the plant derived natural products not only their minimal side effect, but also their medicinal value. The studies reveal that plants have been natural source of anticancer compounds [2–4].

The genus *Colutea* comprises about 28 species (Leguminosae), is growing from 2 to 5 m tall, the leaves are pinnate and light green to glaucous grey-green. The flowers are yellow to orange, pea-shaped and produced in racemes throughout the summer. *Colutea cilicica* Boiss. & Bal., generally known as "bladder senna", is native to the Mediterranean, and it is mostly grown for its attractive yellow flowers and fruits [5]. Previous studies showed that ethanol extract of *C. cilicica* exhibited antibacterial and inhibitory activities [6]. Branches and ash of the plant are used for the treatment of wounds and making ointment, respectively [7]. Secondary metabolites, such as flavonoids, triterpenes and alkaloids are responsible from the wound healing activity of the plant [8].

Previous studies displayed that root extract of *C. cilicica* were rich in isoflavonoids [9]. Phytochemical studies revealed the presence of coluteol (3′,5′,-dihydroxy-7,2′,4′-trimethoxyisoflavan) and colutequinone B (7,4′,6′-trimethoxyisoflavan-2′,5′-quinone) in the root bark of *C. cilicica* [10].

Previous phytochemical studies revealed that the major compounds of the fruits of the aqueous extract of *C. cilicica* were flavonoids. In addition, tannins were also observed in the extract [6].

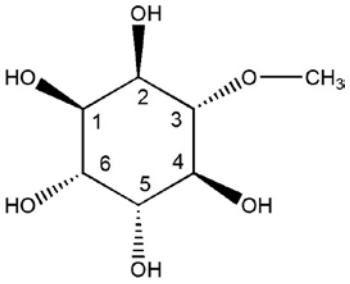

Figure 1: Chemical structure of D-pinitol.

D-pinitol (Figure 1), a 3-methoxy analogue of D-chiro-inositol, has been reported to reduce metastasis of human lung cancers [11]. Chaubal et al. [12] isolated D-pinitol, from the EtOH extract of *Acacia nilotica*, which showed larvicidal activity. In addition, it has biological activities such as anti-inflammatory, anti-hyperlipidemic, anti-oxidant, and cardioprotective [13, 14]. Last studies reveal that D-pinitol is a potent chemotheraphy agent against cancers of the lung, bladder and breast [15, 16]. It was reported that, D-pinitol is effective in prostate cancer via inhibition the migration and invasion of prostate cancer cells [17]. The cyclooxygenases (COXs) are a family of enzymes, which catalyze the rate-limiting step of prostaglandin biosynthesis. Cox-2 is a member of COXs family. Cox-2 was described to modulate cell proliferation and apoptosis mainly in solid tumors, that is colorectal, breast and prostate cancers, and more recently, in hematological malignancies [18]. Several diseases are associated to chronic inflammation, such as cancer (chronic myeloid leukemia). The biological effects of D-pinitol on K562 cells are largely unknown. In this study, we reported the isolation of D-pinitol from *C. cilicica* leaves and investigated apoptotic and anti-inflammatory effects of isolated D-pinitol on K562 cells.

Materials and methods

General experimental procedures

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1D and 2D NMR spectra were recorded on a 400 MHz Bruker Avance III spectrometer, in DMSO-d_6 or D_2O, with TMS as an internal standard. IR spectra were measured by using Jasco FT/IR-430 Fourier Transform Infrared Spectrometer. HPLC-TOF/MS analysis of the compound was

performed using an Agent Technologies 6210 Time-of-Flight LC-MS. Melting point was determined by Barnstead Electrothermal 9100 model apparatus. Column chromatography was carried out using silica gel (70–230 mesh, Merck). TLC was performed with precoated silica gel 60 F_{254} (0.25 mm, Merck) plates. Spots were visualized under UV light (254 nm) and spraying with 10% H_2SO_4 followed by heating. Chemical structure of D-pinitol was confirmed with the spectral data that reported previously in the literature [19, 20].

Procurement of plant material

Colutea cilicica Boiss et Bal. was collected from central region of Tokat, in May 2010. The plant was identified by Dr. Bedrettin Selvi. A voucher speciman was deposited the sample (no. GOPU 2562) in the herbarium of Gaziosmanpasa University, Tokat, Turkey.

Extraction and isolation

Dried and ground leaves of *C. cilicica* (740 g) were extracted with $CHCl_3$: MeOH (1:1) (3×6 L) at room temperature by maceration. After the end of the period, it was filtered and concentrated under reduced pressure to obtain the residue (65 g). The crude extract was subjected to silica gel column chromatography (70–230 μm×5 cm) with elution using a gradient of increasing amounts of $CHCl_3$ concentration in hexane (25%–100%), ethyl acetate (EtOAc) in hexane (50%–100%) and methanol (MeOH) in EtOAc (25%–100%) (5 L for each solvent/solvent mixture) yield 13 main fractions. All fractions were concentrated using evaporator until to obtain 50 mL solution. D-pinitol (1803 mg) was precipitated from the fraction of EtOAc-MeOH (3:1). The compound was not subjected to further purification. Structure elucidation of D-pinitol was performed using spectroscopic data (IR, HPLC-TOF/MS, ^{1}H NMR, ^{13}C NMR and 2D NMR).

Cell culture

Human Chronic Myelogenous Leukemia cell line (K562) was purchased from the American Type Culture Collection. K562 cells were maintained at 37°C, 5% CO_2, in RPMI-1640 medium supplemented with 20 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were incubated with various concentrations (0–80 μM) of D-pinitol for 24, 48, and 72 h.

WST-1 assay

Cell viability was determined by WST-1 assay. After treating with D-pinitol for 24, 48 and 72 h, 10 μL WST-1 (cell proliferation reagent) was added to each well, and the mixture was incubated at 37°C for 2 h. The mixture was shaken and then incubated for 5 min, at room temperature. Absorbance of the each well was determined at 450 nm using a microplate reader (Molecular Device, USA).

Western blot analysis

For western blot analysis of total cell lysates, control and treatment cells lysed in 200 μL cold lysis buffer (50 mM Tris–HCl, pH 6.8, 15 mM EDTA, 15 mM $MgCl_2$, 50 mM β-glycerol, 150 μg/mL digitonin containing 1 mM dithiothreitol and 100 mM phenylmethylsulfonyl fluoride). Samples were incubated on ice for 15 min and the supernatant was collected after centrifugation at 18,000×*g* for 10 min. Protein concentration in lysates was measured using BCA™ Protein Assay Kit (Thermo Scientific, USA) according to manufacturer instructions. Cellular lysates were prepared and approximately 30 μg of total proteins were loaded to each well. Protein was resolved by SDS-PAGE. Proteins were transferred to nitrocellulase membranes. The blots were blocked with 5% bovine serum albumin for 1 h at room temperature and probed with rabbit anti-human antibodies against PARP (1:1000), Cox-2 (1:100) and GAPDH (1:1000) for 12 h at 4°C (Thermo Scientific, USA). After three washes, the blots were incubated with HRP-linked goat anti-rabbit secondary antibody (1:1000) for 1 h at room temperature (Thermo Scientific, USA). The blots were visualized with ChemiDoc MP System (Bio-Rad Laboratories, USA). Band intensities were analyzed by Image Lab Software (Bio-Rad).

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc.) program was used for statistical analyses. Statistical Analysis Data are expressed as the mean SD and were statistically compared by Tukey's multiple comparison tests. Values with $p < 0.05$ were considered as statistically significant.

Results

D-pinitol was obtained as white powder. The negative HPLC-TOF/MS analysis showed a molecular ion at m/z

193.0724 $[M-H]^-$ which confirmed the corresponding formula of $C_7H_{14}O_6$. Melting point of the compound was determined as 185°C–186°C. IR absorptions of D-pinitol indicated the presence of hydroxyl groups (3393 and 3305 cm^{-1}), C-H stretch (2918 cm^{-1}) and C-O stretch (1124 cm^{-1}). The ^1H NMR spectrum of the compound exhibited signals at δ 3.44 (1H, m), δ 3.35 (1H, m), δ 3.01 (1H, t, $J = 9.04$ Hz), δ 3.50 (1H, m), δ 3.63 (1H, m), δ 3.62 (1H, m).

The signal at δ 3.43 (3H, s) showed the presence of methoxy group. ^1H-NMR spectra indicated the presence of five -OH groups between 4.3 and 4.8 ppm which were exchangeable with D_2O. TOCSY correlations display the connectivity of the H atoms which confirm the structure is a ring. The DEPT spectrum of the compound showed six methine carbons and a methyl carbon that belongs to the methoxy group. The COSY couplings were observed between δ_H 3.01 and δ_H 3.35 (H-3/H-2), δ_H 3.44 and δ_H 3.62 (H-1/H-6), respectively. In the HMBC spectrum methoxy carbon was correlated with H-3. Correlations of C-3 and C-6 were observed with H-1 and H-5, respectively. The following ^1H–^{13}C couplings were assigned in the HETCOR spectrum; C-1 (δ_C71.38) δ_H 3.44 (H-1), C-2 (δ_C73.04) δ_H 3.35 (H-2), C-3 (δ_C84.21) δ_H 3.01(H-3), C-4 (δ_C70.53) δ_H 3.50 (H-4), C-5 (δ_C72.40) δ_H 3.63 (H-5), C-6 (δ_C72.85) δ_H 3.62 (H-6), and methoxy carbon (δ_C60.07) δ_H 3.43 (Table 1). The absolute configuration was determined by comparison of ^1H and ^{13}C-NMR data with those obtained from the literature [19, 20].

The proliferative effect of D-pinitol in K562 cell lines was examined by the WST-1 assay, which is reliable to detect proliferation of cells. The results of the WST-1 assay are presented in Figure 2.

Table 1: Carbon and proton NMR data of D-pinitol.

C/H	δ_c, ppm	δ_H, ppm (Hz)
1	71.38	3.44, m
2	73.04	3.35, m
3	84.21	3.01, (t, $J = 9.04$ Hz)
4	70.53	3.50, m
5	72.40	3.63, m
6	72.85	3.62, m
OCH_3	60.07	3.43, s

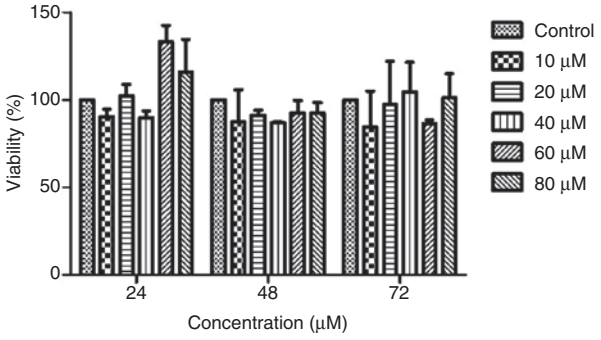

Figure 2: K562 cells were incubated with various concentrations (0–80 μM) of D-pinitol for 24, 48 and 72 h.
The cell viability was examined by WST-1 assay (n = 3). Dose dependent and time dependent inhibition of the growth human chronic myelogenous leukemia (K562) cells by D-pinitol. Results are expressed as a percentage of growth inhibition obtained from three separate experiments. Results are expressed as the mean ± SD.

Discussion

Proliferative and anti-inflammatory effect of D-pinitol in K562 cells

It was observed that K562 cell proliferation was not changed with D-pinitol treatment at different time and dosages so we selected non-cytotoxic concentrations. Several studies were conducted for D-pinitol in terms of biological activity. The inhibition of MCF-7 cell population was above 50% at the concentration of 60 μM for 24 h, indicated the antiproliferative and cytotoxic nature of D-pinitol [21]. Another study was carried out by Lin et al. They observed that cell migration decreased in the presence of D-pinitol at the concentrations of 0–30 μM while cell viability was not affected in human prostate cancer cells [17].

Apoptosis is a complex cell death program which plays a part in physiological and pathological processes via activation of the caspase enzyme. In the current study, we aimed to determine the apoptosis and examine its relationship with PARP cleavage. We showed that Full-PARP protein expression was not changed. Based on this D-pinitol does not cause cell apoptosis at noncytotoxic dosages in K562 cells (Figure 3).

Secondary metabolites that present in natural products are responsible biological activity of the plant such as anticancer, antioxidant, etc. D-pinitol was isolated simply from the leaves of the *C. cilicica* plant. There are few studies regarding with the isolation of secondary metabolites from *C. cilicica* and D-pinitol presence in *C. cilicica* plant was reported for the first time. Nonsteroidal anti-inflammatory drugs (NSAIDs) and their anti-inflammation activities were determined anti-cancer agents [22]. The objectives of customary NSAIDs are cyclooxygenases 1 and 2 (COX-1 and COX-2), enzymes complicated in the production of prostaglandins from arachidonic acid [23]. From this point of view, NSAIDs are known as inhibition agents for tumor cells, to inhibit tumor growth by utilizing

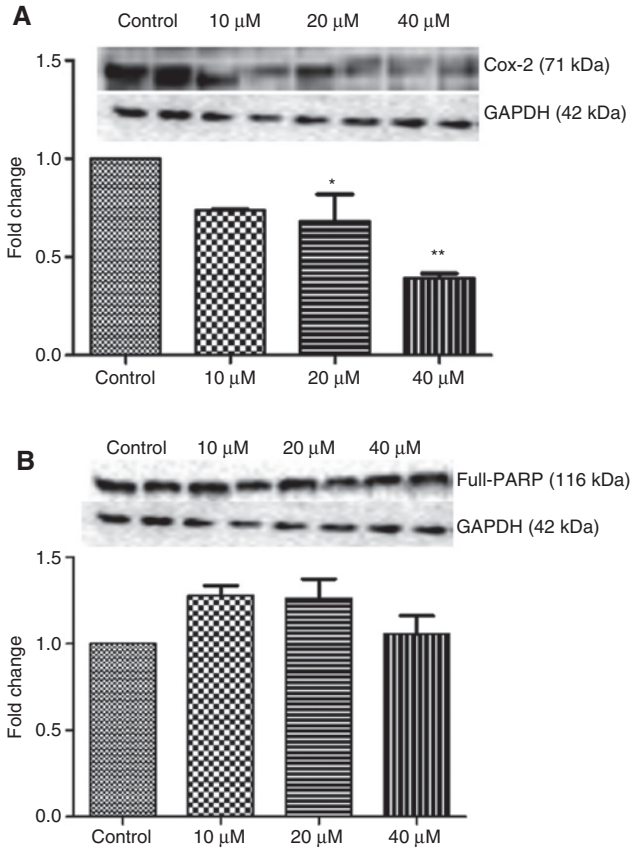

Figure 3: (A) The effect of Cox-2 protein expression in K562 cells. (B) The effect of PARP protein expression in K562 cells. (A) Cells were incubated with 0–40 μM noncytotoxic-concentrations of D-pinitol for 24 h; the protein expression was examined by Western blotting. Values are expressed as mean ± SD. $p^* < 0.05$, $p^{**} < 0.01$ compared with control. (B) Cells were incubated with 0–40 μM noncytotoxic-concentrations of D-pinitol for 24 h; the protein expression was examined by Western blotting. Band intensities were analyzed by Image Lab Software (Bio-Rad). GAPDH was used as an internal control. Changes in protein expression are presented as a fold change.

antimetastatic and antiangiogenic effects over inhibition of COX activity [24]. However, additional experimental studies must be carried out in order to determine the effect of D-pinitol on the other types of cells. Present study gives opportunity for better understanding of anti-inflammatory properties of D-pinitol. The previous studies showed that COX-2 inhibitors used in the cancer treatment. But, anti-inflammatory agents, especially COX-2 inhibitors, may induce serious damages to stomach, kidney and heart, which have restricted the clinical uses of these drugs. Some anticancer agents are not effective in the killing of the cells due to the activation of NF-κB [25]. So, prevention of cancer could be possible with the anticancer agents which inhibit NF-κB activity. NF-κB linked proinflammatory diseases may treat with D-pinitol through the blocking of NF-κB pathway. Previous studies revealed that D-pinitol exhibits anti-inflammatory activity via suppression of the NF-κB pathway [11, 26]. In the current study, we found that D-pinitol is effective in the inhibition of inflammation at non-cytotoxic concentrations (0–40 μM) in K562 cells (Figure 3). All of these functions are related to the ability of pinitol suppress inflammatory process. Therefore, the natural substances have used for anti-inflammation effects [27]. In the light of the obtained results, it is concluded that;

- D-pinitol can be isolated simply from the leaves of *C. cilicica* plant.
- Treatment with non-cytotoxic concentrations of D-pinitol did not affect cell viability for 24, 48 and 72 h.
- Change of protein expression showed (Cox-2 and PARP) in K562 cells. PARP expression did not change but Cox-2 expression reduced with non-cytotoxic concentrations of D-pinitol. Unfortunately, we found that D-pinitol did not affect cell proliferation and apoptosis in K562 cells however reduced the inflammation, significantly at non-cytotoxic concentrations.
- Importantly, the current study was the first demonstration showing the anti-inflammatory activity of D-pinitol on K562 cells. Our results show a better understanding of the effects of D-pinitol.
- Application of natural anti-inflammatory agents (such as D-pinitol) may be important for the prevention of cancer.
- The present study supports the ethnopharmacological value of *Colutea cilicica* in terms of phytochemical applications.

Supplementary material

Supplementary material may be found in the online version of this article

Conflict of interest statement: Authors have no conflict of interest

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Supplemental Material: The online version of this article (DOI: 10.1515/tjb-2016-0120) offers supplementary material, available to authorized users.