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Title

Antimicrobial and Anti-Inflammatory Activity of Some *Lathyrus* L. (Fabaceae) Species Growing In Turkey

Türkiye'de Yetişen Bazı *Lathyrus* L. (Fabaceae) Türlerinin Antimikrobiyal ve Antienflamatuar Aktivite Değerlendirilmesi

Short title

Antimicrobial and Anti-Inflammatory Activity of Some *Lathyrus* L.

Bazı *Lathyrus* L. Türlerinin Antimikrobiyal ve Antienflamatuar Aktivitesi

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Abstract

Objectives: The present study aimed to evaluate antimicrobial and anti-inflammatory activities of methanol extracts and *n*-hexane, ethyl acetate, chloroform and water fractions of five *Lathyrus* species, *L. armenus, L. aureus*, *L. cicilicus*, *L. laxiflorus* subsp. *laxiflorus* and *L. pratensis* growing in Turkey.

Materials and Methods: The antimicrobial activities were screened against *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10231. Broth dilution method was used to determine the antimicrobial activities of extracts and fractions. In vitro anti-inflammatory activity of these extracts and fractions were determined by using human red blood cell (HRBC) membrane stabilization method.

Results: The results demonstrated that ethyl acetate fractions of the tested species exhibited higher antimicrobial activity than the other extracts. Among all of the tested extracts and fractions, the highest anti-inflammatory activity was detected in water fractions. Furthermore, water fractions of *L. pratensis* showed better anti-inflammatory activity than acetylsalicylic acid and diclofenac sodium which were used as standard drugs in this assay.

Conclusion: The results point out that the membrane stabilizing effect of the various extracts and fractions of the *Lathyrus* species and could be a preliminary study for *in vivo* anti-inflammatory activity experiments.

Keywords: Anti-inflammatory activity, Antimicrobial activity, HRBC membrane, *Lathyrus*

Özet

Amaç: Bu çalışmada Türkiye yetişen beş *Lathyrus* türü, *L. armenus, L. aureus*, *L. cicilicus*, *L. laxiflorus* subsp. *laxiflorus* ve *L. pratensis* türlerinin metanollü ekstreleri ve hekzan, etil asetat, kloroform ve su fraksiyonların antimikrobiyal ve antienflamatuar aktivitesi değerlendirilmiştir.

Gereç ve Yöntemler: Ekstrelerin ve fraksiyonların antimikrobiyal aktivitesi *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 ve *Candida albicans* ATCC 10231 karşı elde edilmiştir. In vitro antienflamatuar ise insan kırmızı kan hücresi kullanarak membran stabilizasyon yöntemi ile değerlendirilmiştir.

Bulgular: Etil asetatlı fraksiyonlar diğer ekstre ve fraksiyonlara göre daha yüksek antimikrobiyal aktivite, sulu fraksiyonlar ise diğer ekstre ve fraksiyonlara göre daha yüksek antienflamatuar aktivite göstermiştir. Ayrıca, *L. pratensis*'in su fraksiyonu, standart olarak kullanılan asetilsalisilik asit ve diklofenak sodyumdan daha yüksek anti-inflamatuar aktivite göstermiştir.

Sonuç: Elde edilen sonuçlara göre *Lathyrus* türlerinin ekstreleri ve fraksiyonlarının membran stabilizasyon aktiviteye sahip olup, ve in vivo anti-inflamatuar aktivite deneyleri için bir ön çalışma olabileceğini belirtmiştir.

Anahtar kelimeler: Antienflamatuar aktivite, antimikrobiyal aktivite, HRBC membran, *Lathyrus*

Antimicrobial and Anti-Inflammatory Activity of Some *Lathyrus* L. (Fabaceae) Species Growing In Turkey

Introduction

Lathyrus L. is one of the largest genus in Fabaceae family, with about 160 species distributed worldwide [1]. Turkey has a rich diversity of *Lathyrus* genus, with 65 species and 75 taxa [2].

Secondary metabolites of Plants, such as tannins, terpenoids, alkaloids and flavonoids, which have been found in plants, have extensively different bioactive properties. Antibiotics are commonly used in fighting against bacterial infections and have been profoundly effective in the health and quality of human life since their invention [3]. However, because of the appearance of the resistance to the antibiotics and some toxic products resulted due to their consumption in last decades antibiotics became less effective against certain illnesses. Therefore antibacterial agents that derived from natural sources have started to play a significant role in the prevention and treatment of infection diseases [4]. Plant extracts have established as a source of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [5].

Inflammation is a protective mechanism of living organisms against abnormal stimulation. It is a complex series of biochemical activities performed by the body in response to the injury or abnormal stimulation caused by a physical, chemical, or biological agent. In general, generation of cytokines is accepted to play a major role in inducing inflammatory process, and free radicals can propagate inflammation by stimulating release of proinflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α [6]. Drugs that are currently used for treatment of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. NSAIDs drugs inhibit prostaglandins and thromboxane inflammatory mediators synthesize by deactivating of cyclooxygenase (COX), COX-1 and COX-2

enzymes. Some of these drugs such as aspirin, diclofenac, ketorolac, naproxen and piroxicam have toxic effects such as risk of gastrointestinal bleeding [7,8].
Moreover, the generation of oxygen free radicals is known to be involved in the development of the inflammatory process. These radicals are highly reactive molecules with an unpaired electron which can initiate radical chain reaction lead to the damaging or destroying the normal function of a living cell and consequently causes many different diseases such as neurodegenerative disorders, cancer, cardiovascular diseases atherosclerosis, diabetes, cataracts and inflammation [9,10]. In addition, inflammation caused by oxidative stress is the origin of many human diseases.

The potential harmful effects of free radicals are usually controlled by endogenous antioxidant mechanisms present in the cells. These mechanisms include cellular enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and other defensive mechanisms, involving antioxidants, such as ascorbic acid, a-tocopherol and glutathione. On biological systems antioxidant agents show their effects by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation [11,12]. Reactive oxygen species such as hydroxyl radical, superoxide anion, and peroxynitrite radicals cause cellular damage by destroying the cellular bio molecules such as nucleic acids, proteins, carbohydrates and lipids that resulted in inflammation. Therefore, the compounds with radical scavenging activities may be expected to have anti-inflammatory properties [13]. Current anti-inflammatory drugs essentially have become ineffective for long term protection since they have unexpected side effects. Hence, new plants and herbal compounds with anti-inflammatory properties are investigated to explore more effective compounds and avoid toxic effects of anti-inflammatory drugs.

Radical scavenging activities of phenolic and polyphenolic compounds which are plants secondary metabolites were shown in previous studies. There are many studies on anti-inflammatory activity of plant extracts and secondary metabolites such as flavonoids [14,15].

The aim of this study was to evaluate the total flavonoid contents, the antimicrobial and anti-inflammatory activities of methanol extracts and *n*-hexane, chloroform, ethyl acetate, water fractions of the aerial parts of *Lathyrus armenus* (Boiss & Huet) Sirj, *L. aureus* (Stev.) Brandza, *L. cicilicus* Hayek & Siehe, *L. laxiflorus* (Desf.) O. Kuntze subsp. *laxiflorus*, *L. pratensis* L. growing in Turkey. In these species, *L. armenus* and *L. cilicicus* are endemic species for Turkey. There are no previous reports dealing with anti-inflammatory activities of the examined five *Lathyrus* species.

The study protocol was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

Material and methods

Chemical Material

The solutions, acetylsalicylic acid, sodium chloride and Mueller Hinton Broth were purchased from Merck (Germany) Sigma-Aldrich (USA), Riedel-de Haën (Germany) and Difco Laboratories (USA) respectively.

Instruments

The absorbances was measured by SpectraMax 190 Microplate Reader (Spectramax molecular devices inc, USA), the centrifugation was carried out by (Sigma 4K15 10740) and vortex by (Labinco L46, Netherlands) were used in this study.

Plant Material

The aerial parts of *Lathyrus armenus, L. aureus, L. cicilicus*, *L. laxiflorus* subsp. *laxiflorus, L*. *pratensis* were collected and identified by Dr. M. Tekin. Voucher specimens were deposited in Ankara University, Faculty of Pharmacy, Kamil Karamanoglu Herbarium (AEF). Data for collected species are given in Table 1.

Preparation of Extracts

The obtained plants dried and powdered. 20 g of the plant materials were extracted separately with methanol using Soxhlet apparatus for 24 hrs. The solvent was evaporated under reduced pressure and dissolved in water and partitioned with *n*-hexane, chloroform and ethyl acetate respectively. All extracts were dried and stored at 4°C.

In vitro Antibacterial and Antifungal activity of *Lathyrus* Species

Methanol extracts and *n*-hexane, chloroform, ethyl acetate, water fractions from the aerial part of five *Lathyrus* species were investigated for their potential *in vitro* antibacterial activities against *S. aureus* ATCC 29213, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and antifungal activity against *C. albicans* ATCC 10231. Stock solution was prepared by dissolving 4 mg of the methanol crude extract and water fraction in 70% (*v/v*) methanol and in water respectively and chloroform, ethyl acetate and *n*- hexane fractions in 20% (*v/v*) DMSO. Broth dilution assay was used for determination of the minimum inhibitory concentrations (MIC). The cultures were obtained in Mueller Hinton Broth; serial two-fold dilutions ranging from 1.000 to 0.0625 mg/ml were prepared in medium. A series of tubes containing only inoculated broth were used as controls. After incubation for 18-24 at 37±1 ºC for bacteria, 48 h for fungi, the last tube with no microbial growth was recorded to represent MIC value (mg/mL) [16, 17].

Total Flavonoid Content

The extracts and fractions (2 mg/mL) were placed in a 3 mL test tube. Then distilled water was added to the test tube to complete 1.5 mL and vortexed. 0.075 mL $NaNO_2$ % 5 (*w/v*) were added and vortexed and waited for 5 minutes. 0.15 ml of $AlCl_3$ % 10 (*w/v*) were added to the tube. After 6 minutes, 1 M NaOH 0.5 mL was added to the mixture. Then the final volume was made to 3 mL with distilled water. This mixture was vortexed and the absorbance was measured against a blank at 510 nm. Quercetin was used as a standard for a calibration curve. The flavonoid content was calculated by using the quercetin calibration equation [18].

$A=0.0245C-0.0417$, $r^2=0.9834$

A: Absorbance

C: Flavonoid content (µg/mg)

Anti-inflammatory Assay

Preparation of Human Red Blood Cells (HRBC) Suspension

Fresh whole human blood was collected from healthy human volunteer who had not taken any anti-inflammatory or steroidal drug for 2 weeks before the experiment and transferred to the centrifuge tubes. The tubes were subjected to the centrifugation at 3000 rpm for 10 min. the supernant part of the tubes were decanted and the participated parts were washed three times with equal volume of isosaline (0.85 %, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Heat Induced Hemolysis

The reaction mixture (2mL) consisted of 1 mL of test sample (methanol extract, water, ethyl acetate, chloroform and *n*-hexane fractions) and 1 mL of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Acetylsalicylic acid and diclofenac sodium were used as standard drugs. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for 30 min. At the end of the incubation the tubes were cooled under streaming tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was measured at 560 nm. The experiment was performed in triplicates for all the test samples [19,20].

The percentage hemolysis and protection was calculated according to the below formula:

Hemolysis% = (Optical density of test sample/ Optical density of control)×100

Protection% = 100 – [(Optical density of test sample/ Optical density of control)×100]

Results

The antimicrobial activity of the methanol extracts and *n*- hexane, chloroform, ethyl acetate and water fractions of *Lathyrus* species are shown in Table 2. The results indicated that the *L. cilicicus* water and *n*-hexane fractions showed no activity against tested microorganism. Methanol extract was effective against *C. albicans* and chloroform fraction was effective against *C. albicans* and *P. aeruginosa*. While *L. armenus* water fraction showed no activity, methanol extract and *n*-hexane, chloroform fractions showed activities against *C. albicans. L. laxiflorus* methanol extract and *n*-hexane, chloroform fractions showed activity against *C. albicans,* and also water fraction of *L. laxiflorus* was found effective against *B. subtilis*. Methanol extract and *n*-hexane, chloroform fractions of *L. aureus* showed activity against *C. albicans,* additionally water fraction of *L. aureus* was found effective against *B. subtilis*. Methanol extract of *L. pratensis* was effective against *C. albicans* and chloroform fraction was effective against *C. albicans* and *P. aeruginosa* additionally water fraction of *L. pratensis* was found effective against *B. subtilis*. Ethyl acetate fractions of all studied *Lathyrus* species were found effective against all tested microorganisms. The antimicrobial effect of plant extracts against the microorganisms may be due to the content secondary metabolites of these extracts like phenolic compounds, saponin and other which are reported to be antimicrobial [3]. There are not so many research carried out about the antibacterial screening of *Lathyrus* species. According to the literature butanolic extracts of *L. aphaca* seeds two triterpenoid saponins were isolated that show antifungal activities against *Colletotrichum dematium* and *Alternaria alternate* [21]. Inhibition growth of *Xanthomonas campestris* pv. citri by *L. odoratus* L. and *L. sativus* L. seeds extracts were studied. While *L. odoratus* showed no antibacterial activity, mean inhibition zone of *L. sativus* seeds extract was showed 1.16 mm [22]. Antifungal activity of ethanolic extract and dicholoromethan and water fractions of *L. pratensis* were expressed as minimum inhibitory concentrations against *Candida albicans, Aspergillus fumigatus* and *Asperigillus niger* [23]. Methanol and ethanol extracts of leaf and body of *L. karsianus* showed antibacterial activity against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Salmonella enteritidis*, *Proteus mirabilis*, *Escherichia coli*, *Enterococcus faecalis* [24]. Butanolic extract of seeds of *L. ratan* and *L. aphaca* were investigated for their antibacterial screening.

The maximum inhibition was shown by *L. ratan* against *Staphylococcus aureus*. As reported *L. ratan* extract was more active than *L. aphaca* [25]. Antimicrobial activity of isolated anthocyanins and the ethanolic extract of *L. odoratus* were tested by using disc diffusion assay against *Staphylococcus aureus, Escherichia coli*, *Bacillus subtilis, Aspergillus niger*, and *Candida albicans* [26].

In the study of Heydari *et al*.,[27] antioxidant activities of these species were investigated by using DPPH radical scavenging method. Based on mentioned previous study, different extracts of *Lathyrus* species exhibited significant free radical scavenging activity. The highest antioxidant activity belongs to *L. laxiforus* subsp. *laxiflorus* . As seen in Table 3, *L. laxiforus* subsp. *laxiflorus* and *L. pratensis* have the highest contents of flavonoids. Recent studies showed that flavonoids possess antioxidant, anti-inflammatory, antinociceptive and cytostatic properties due to their effects on the prostaglandins pathway [28]. Therefore they are effective in reducing oxidative stress and acute inflammation. The human red blood cell membrane is analogous to the lysosomal membrane. Therefore HRBC membrane stabilization has been used as a method to the study of in-vitro anti-inflammatory effects [29]. During the inflammation neutrophils and monocytes are impaired or destroyed resulting in releasing of lysosomal enzymes [30]. The stabilization of the membrane suggests that the extracts might stabilize lysosomal membranes. Most anti-inflammatory drugs show their effects either by stabilizing the lysosomal membranes or inhibiting lysosomal enzymes. Moreover, several studies indicate that herbal products and plants could be effective in stabilizing the red blood cell membrane against hipotonicity, heat or chemicals [31]. Therefore stabilization of HRBC membrane was studied for further establishing the mechanism of anti-inflammatory action of different extracts and fractions of *Lathyrus* species. The anti-inflammatory activity of the methanol extracts and *n*-hexane, chloroform, ethyl acetate and water fractions of *Lathyrus* species investigated by using human red blood cell (HRBC) membrane stabilization method. Most of the extracts and fractions at concentration of 2 mg/ml showed protective effects on human erythrocyte membranes against lysis induced by heat as shown in Table 4. In comparison to the other fractions and extracts, water fractions showed the highest activity. Furthermore, the maximum membrane stabilization effect was observed at water fraction of *L. pratensis* (88%) among all other

extracts and followed by *L. laxiflorus* (86%), methanol extract of *L. laxiflorus* , *L. armenus* (83%) *and L. aureus* (81%), respectively. Methanol extract of *L. laxiflorus* showed the maximum membrane stabilization effect (82%) among the other methonol extracts. Acetylsalicylic acid and diclofenac sodium were used as standard drugs and showed almost 87% protection at concentration of 2 mg/mL.

Discussion

Most of *Lathyrus* species are consumed as a food by animals and human. Despite of this knowledge, there is no enough biological activity research on *Lathyrus* taxa. The aim of this study was to investigate the antimicrobial and anti-inflammatory activities of *L. armenus, L. aureus, L. cicilicus*, *L. laxiflorus* subsp. *laxiflorus* and *L*. *pratensis.* According to the results, ethyl acetate fractions were found as more effective than other extracts and fractions against test microorganisms. Also our results revealed that different extracts and fractions of examined *Lathyrus* species possess anti-inflammatory properties. Methanol extracts and water fractions exhibited membrane stabilization effect by inhibiting heat induced lysis of erythrocyte membrane more than the others. Water fraction of *L. pratensis* showed the maximum activity (almost equal with the standard drugs) among all of the fractions of other examined *Lathyrus* species.

Conclusion

Lathyrus species are consumed as a food by animals and human. Despite of the fact that there is not enough biological activity research about *Lathyrus* the aim of this study was to investigate the antimicrobial and anti-inflammatory activities of *Lathyrus* species, that two of these are endemic for Turkey. According to the results ethyl acetate fractions were more effective than other extracts and fractions against Gram positive, Gram negative and fungal. Also our results revealed that different extracts and fractions of *Lathyrus* species possess anti-inflammatory properties. Methanol extracts and water fractions exhibited membrane stabilization effect by inhibiting heat induced lysis of erythrocyte membrane more than the others. Water fraction of *L. pratensis* showed the maximum activity (almost equal with the standard drugs) among all of the fractions. In conclusion, these experimental results point out that the membrane stabilizing effect of

the various extracts and fractions of the *Lathyrus* species is primarily due to the active phytoconstituents (i.e.flavonoids) in the plant, which seems to support the use of this plant in traditional medicine. In this regard, the isolation from the *Lathyrus* species is preceded simultaneously in our laboratory. To the best of our knowledge, this is the first study evaluating the membrane stabilizing activity of *Lathyrus* species growing in Sivas, Turkey. However, further studies are needed to evaluate exact mechanism and responsible substances of these activities.

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Table 1: Collection data of examined *Lathyrus* species

Species	Collected Location	AEF No.
L. armenus	Sivas (M.Tekin 1278)	26680
L. aureus	Sivas (M.Tekin 1277)	26684
L.cilicicus	Karaman (M.Tekin 1210)	26681
L. laxiflorus subsp. *laxiflorus*	Sivas (M.Tekin 1274)	26682
L. pratensis	Sivas (M.Tekin 1273)	26683

Table 2: MIC values (mg/mL) of examined *Lathyrus* species against tested microorganisms.

Extracts		Microorganisms				
		S. aureus ATCC 29213	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
		MIC (mg/mL)				
L. armenus	Chloroform	-	-	-	-	0.5
	n-Hexane	-	-	-	-	1
	Water	-	-	-	-	-
	Ethyl acetate	0.5	0.5	0.5	0.5	0.5
	Methanol	-	-	-	-	1
L.aureus	Chloroform	-	-	-	-	0.5
	n-Hexane	-	-	-	-	1
	Water	-	1	-	-	-
	Ethyl acetate	1	0.5	0.5	0.5	0.5
	Methanol	-	-	-	-	1
L. cilicicus	Chloroform	-	-	-	1	0.5
	n-Hexane	-	-	-	-	-
	Water	-	-	-	-	-
	Ethyl acetate	1	0.5	0.5	0.5	0.5
	Methanol	-	-	-	-	1
L. laxiflorus	Chloroform	-	-	-	-	0.5
subsp.	*n*-Hexane	-	-	-	-	1
laxiflorus	Water	-	1	-	-	-
	Ethyl acetate	1	0.5	0.5	1	0.25
	Methanol	-	-	-	-	1
L. pratensis	Chloroform	-	-	-	1	0.5
	n-Hexane	-	-	-	-	1
	Water	-	1	-	-	-
	Ethyl acetate	0.5	0.5	0.5	0.5	0.5
	Methanol	-	-	-	-	1

'-'=represents no activity

Table 3: Total flavonoid contents of methanolic extracts of examined *Lathyrus* species

Species	µg $_{Quercetin}$/mg $_{extracts}$ ± SD
L. armenus	55.6 ± 0.75
L. aureus	90.9 ± 0.84
L. cilicicus	36.2 ± 1.32
L. laxiforus subsp. *laxiflorus*	105.4 ± 2.38
L. pratensis	105.3 ± 2.68

Each value represents mean ± standard deviation

Table 4: Protection and hemolysis percentage of examined *Lathyrus* species, on human red blood cell (HRBC) membrane stability method.

Extracts		HRBC	
		Hemolysis %,	Protection %
		Concentration (2 mg/mL)	
Control (distilled water)		100	-
Control (isosaline)		100	-
L. armenus	Chloroform	87.85±0.004*	12.14*
	n-Hexane	99.85±0.005	0.14
	Water	17.20±0.006*	82.79*
	Ethyl acetate	24.65±0.004*	75.34*
	Methanol	43.23±0.001*	56.76*
L. aureus	Chloroform	74.47±0.017*	25.52*
	n-Hexane	86.84±0.002*	13.15*
	Water	19.16±0.002*	80.83*
	Ethyl acetate	23.86±0.009*	76.13*
	Methanol	38.03±0.005*	61.96*
L. cilicicus	Chloroform	62.40±0.003*	37.59*
	n-Hexane	97.90±0.022	2.09
	Water	18.72±0.004*	81.27*
	Ethyl acetate	22.41±0.005*	77.58*
	Methanol	27.62±0.003*	72.37*
L. laxiflorus* subsp. *laxiflorus	Chloroform	75.05±0.003*	24.94*
	n-Hexane	91.03±0.003*	8.96*
	Water	14.09±0.004*	85.90*
	Ethyl acetate	21.25±0.006*	78.74*
	Methanol	17.64±0.003*	82.35*
L. pratensis	Chloroform	97.68±0.014	2.313
	n-Hexane	99.78± 0.001	0.216
	Water	12.07±0.004*	87.92*
	Ethyl acetate	18.72±0.004*	81.27*
	Methanol	30.22±0.003*	69.77*
Acetylsalicylic acid		12.74±0,37*	87.25±0.37*
Diclofenac sodium		12.14±0.02*	87.85±0.02*

Each value represents mean ± standard deviation

* Statistically significant as compared to controls, $p<0.05$ (one-way ANOVA).