Asian Journal of Chemistry Vol. 20, No. 2 (2008), 1491-1496

Antiinflammatory and Antioxidant Activities of *Trifolium resupinatum* var. *microcephalum* Extracts

T. SABUDAK*, D. DOKMECI†, F. OZYIGIT†, E. ISIK‡ and N. AYDOGDU§
Department of Environmental Engineering, Faculty of Corlu Engineering
Namik Kemal University, 59860, Corlu-Tekirdag, Turkey
Fax: (90)(282)6529372; Tel: (90)(282)6529476; E-mail: tsabudak@yahoo.com

The antiinflammatory and antioxidant activities of the ethanol extract of *Trifolium resupinatum* L. var. *microcephalum* (Leguminosae) have been assessed on arthritic rats. The extract (1.35 and 13.5 mg/kg, i.p.) significantly reduced the paw edema induced by the complete Freund's adjuvant. Furthermore, it also inhibited lipid peroxidation. These effects of *T. resupinatum* extract may be directly linked to the content in triterpene saponins and flavonoid compounds and consequently to their free radical scavenging activities.

Key Words: Antiinflammatory, Antioxidant activity, Persian clover, *Trifolium resupinatum* var. *microcephalum*.

INTRODUCTION

Herbal medicines are increasingly used by the general population for several diseases such as rheumatoid arthritis[1,2]. *Trifolium* with 67 taxa is one of the most important genera of the Leguminosae family both in its agricultural value and the number of species about 300. The Mediterranean region is rich for *Trifolium* species[3], especially in Turkey where it is wide spread and represented with 103 species[4]. In Turkish folk medicine, some *Trifolium* species such as *Trifolium repens*, *T. arvense* and *T. pratense* are used as expectorant, antiseptic, analgesic, sedative and tonic[5]. It is reported that in Egyptian folk medicine, the seeds of *T. alexandrinum* are used as an antidiabetic agent[6,7]. Previously, oleanene-type triterpenoidal saponins[8], megastigmane glycosides[6] and chalcanol glucosides[7] have been isolated from the seeds of this plant.

†Department of Pharmacology, Faculty of Medical, Trakya University, 22030, Edirne, Turkey.
‡Department of Chemistry, Faculty of Science and Arts, Trakya University, 22030, Edirne, Turkey.
§Department of Physiology, Faculty of Medical, Trakya University, 22030, Edirne, Turkey.

Trifolium resupinatum (Persian clover) is an annual pasture crop originating from the Mediterranean region, where it has been cultivated for centuries. Recently, triterpene saponins (aglycones) such as soyasaponin, soyasapogenol and flavonoid compounds such as quercetin[9,10] were isolated and characterized from the seeds of *T. resupinatum*.

To our knowledge the role of *T. resupinatum* L. var. *microcephalum* in antiinflammatory and antioxidant effects are not defined in literature. In the current study we examined whether there is a protective effect of *T. resupinatum* L. var. *microcephalum* on inflammation and oxidant status in experimental animals. This is the first report on the biology of *T. resupinatum* L. var. *microcephalum*.

EXPERIMENTAL

Trifolium resupinatum L. var. *microcephalum* was collected from Edirne, Turkey in May 2002 and identified Dr. N. Basak (Trakya University). A voucher specimen is deposited in the Herbarium of the Biology Department, Trakya University (EDTU 8328).

Previously isolated classes of constituents: During recent years, triterpene saponins (aglycones) such as soyasaponin **I**, 3-O-[α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl(1→2-β-D-glucuronopyranosyl]-22-O-[β-D-glucuronopyranosyl(1→2)-β-D-glucopyranosyl]soyasapogenol B, soyasaponin **II**, 3-O-β-D-glucopyranosyl sitosterol, 3-O-β-D-glucopyranosylstigmasterol and 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl]oct-1-ene-3-ol, 3-O-α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-22-O-β-D-glucopyranosyl soyasapogenol B and flavonoid compounds such as quercetin[9,10] were isolated and characterized from the seeds of *T. resupinatum*.

Extraction and isolation: Dried aerial parts of plant material were cut into small pieces and macerated with CH_2Cl_2 (three times) and then EtOH (three times) successively. The combined EtOH extracts were concentrated under reduced pressure. The extracts (1350 mg) were solved EtOH:H_2O (2:8) and these crude extracts were used for the experiments.

Animals: Male Sprague-Dawley rats weighing 180-200 g were used in the experiments. They were housed at 22 ± 1 °C under a 12 h light/12 h dark cycle and had free access to standard pellet diet for rats and tap water. The experimental protocol was approved by the Ethical Committee of Trakya University Medical Faculty Animal Breeding and Research (29 April 2004, No TUTFEK-2004/048). Complete Freund's Adjuvant (CFA) was purchased from Sigma Chemical (St. Louis, Mo. USA).

Toxicity study: Two groups of six rats were administered *T. resupinatum* L. var. *microcephalum* (from 1.35 to 135 mg/kg, i.p.) and observed continuously for 2 h to detect changes in autonomical and

behavioural responses[11]. Both the extracts were administred i.p. to different groups of mice in doses ranging from 1.35-135 mg/kg for the LD_{50} study using the method of Miller and Tainter[12]. Mortality, if any, during the experiment and the following 7 d was also recorded. A group of animals treated with the vehicle served as control. There was no lethality in any of the groups after 7 d of treatment.

Antiinflammatory activity: In this study adjuvant arthritis was induced according to previously described methods for the evaluation of rheumatoid arthritis[13]. Under light ether anesthesia, rats were inoculated intradermally into the plantar surface of right hand paw with 0.1 mL of CFA containing 10 mg/mL of heat-killed *Mycobacterium tuberculosis* suspended in paraffin oil. The antiinflammatory activity was assessed by measuring the paw thickness of the experimental rats.

The rats were divided into four groups each consisting of six animals. The first group served as control which received 0.1 mL of CFA. The second and third groups animals, after being injected with CFA, were treated with *T. resupinatum* L. var. *microcephalum* (1.35-13.5 mg/kg, i.p., respectively). The fourth group was administered the standard drug (diclofenac, 1 mg/kg). The day of inoculation was regarded as day 0. Thereafter, the paws were measured on days 0, 11, 13, 15 and 18. *T. resupinatum* L. var. *microcephalum* (1.35 and 13.5 mg/kg, i.p.), vehicle (10 mg/kg, i.p.) and diclofenac (1 mg/kg, i.p.) were administered everyday from the 11th to 18th day post-adjuvantly to arthritic rats.

Antioxidant activity: At the end of the study, the animals were sacrificed by cervical dislocation and the blood was collected by cardiac puncture prior to the sacrifice. Blood samples were collected into heparin-treated (50 IU/mL) tubes by cardiac puncture. Plasma samples were obtained by centrifugation at 3000 rpm for 10 min at 4 ºC to determine the plasma malondialdehyde (MDA) levels. The MDA in plasma, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS)[14].

Statistical analysis: The data were analyzed using a one-way analysis of variance (Anova) followed by Student's Newman-Keuls test. The values are expressed as mean ± the standard error of the mean (SEM) of six independent values. p values less than 0.05 were considered as significance.

RESULTS AND DISCUSSION

Antiinflammatory activity: The results obtained from adjuvant arthritis-induced rats paw edema are shown in Fig. 1. Injection of CFA induced in intense inflammatory reaction in the injected paw. A significant reduction in paw-volume was observed in both *T. resupinatum* L. var. *microcephalum* and standard drug-treated rats compared to the control group (Table-1).

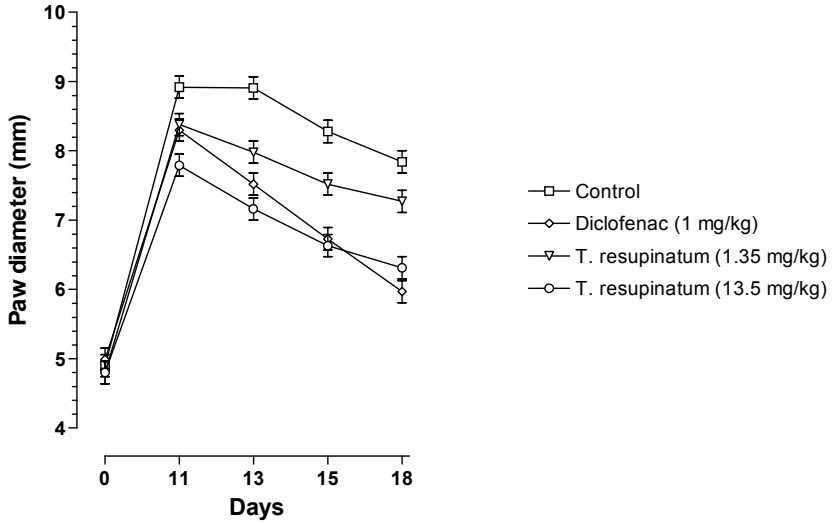

Fig. 1. Changes in paw thickness (mm) in rats

TABLE-1
EFFECT OF *T. resupinatum* AND DICLOFENAC ON FOOT THICKNES (mm) IN ADJUVANT ARTHRITIS

Groups	Day 0	Day 11	Day 13	Day 15	Day 18
Control (CFA)	4.90 ± 0.15	8.92 ± 0.25	8.91 ± 0.12	8.27 ± 0.75	7.84 ± 0.25
T. resupinatum					
1.35 mg/kg	4.79 ± 0.05	8.37 ± 0.05	7.98 ± 0.25	7.52 ± 0.25	7.26 ± 0.75†
13.5 mg/kg	4.79 ± 0.75	7.79 ± 0.90	7.15 ± 0.75	6.62 ± 0.75	6.31 ± 0.15†‡
Diclofenac	4.99 ± 0.15	8.30 ± 0.52	7.52 ± 0.38	6.72 ± 0.75	5.96 ± 0.75‡

†$p < 0.05$ *vs.* diclofenac group on day 18.
‡$p < 0.05$ *vs.* control (CFA) group on day 18.

Antioxidant activity: The lipid peroxidation activity significantly increased in the plasma of the arthritis-induced animals when compared to control. *T. resupinatum* L. var. *microcephalum*-treated animals showed remarkably reduced lipid peroxidation, which was comparable to that of control group (Table-2).

Adjuvant-induced arthritis in rats has been employed widely as a model for rheumatoid arthritis and chronic systemic inflammation and possesses many features in common with human rheumatoid arthritis[13]. The model is useful for investigations examining the therapeutic effects of various anti-inflammatory drugs[15]. It involves most of the joints and associated tissues. Oxygen free radicals have long been implicated in damage of connective tissues in inflammation and rheumatoid arthritis[16]. In arthritic condition, the granulocytes and macrophages accumulate in the affected area and

TABLE-2
EFFECT OF *T. resupinatum* ON LIPID PEROXIDATION IN PLASMA OF CONTROL AND EXPERIMENTAL RATS

Groups	Lipid peroxidation (nmol/mL) mean ± SEM
Control	5.54 ± 0.34
T. resupinatum	
1.35 mg/kg	4.66 ± 0.37*
13.5 mg/kg	2.76 ± 0.50*

Each value represents mean ± SEM (n = 6); *p < 0.05 *vs.* control group.

produce large amounts of superoxide anion, hydrogen peroxide and hydroxyl radicals[17,18] and the estimation of these active species in the disease-induced and the drug-treated animals help in assessing the free radical scavenging property and indirectly the antiarthritic potential of the plant-drug. In the present study, the alcohol extract of the plant *T. resupinatum* L. var. *microcephalum* was considered for the study based on the plant's antiarthritic potential. Administration of various doses of *T. resupinatum* L. var. *microcephalum* to the animals did not show any toxicity thereby confirming the safety of the extract. Recently, triterpene saponins (aglycones) and flavonoid compounds such as quercetin[9,10] were isolated and characterized from the seeds of *T. resupinatum*.

Flavonoids and isoflavinoids play an important role in human nutrition as health promoting natural chemicals[19]. Flavonoids are found throughout the plant kingdom, whereas isoflavonoids are more restricted. Isoflavonoids are particularly prevalent in the Papilonoideae subfamily of the Leguminosae[20]. Isoflavones exhibit estrogenic, antiangiogenic, antioxidant and anticancer activities[21,22] and are now popular as dietary supplements.

All known classes of terpenoids have been reported within the Leguminosae. Particularly interesting are the triterpene saponins, whose biological activities can positively and negatively impact plant traits[20]. Some saponins display allelopathic, antimicrobial and antiinsect activity and saponins also have useful pharmacological activities. Health beneficial activities include antiinflammation, antiulcer, antiallergic and anticarcinogenic and this triterpene saponins may account for many of these properties. Furthermore, health promoting activities are shown also by some saponins, including soyasapogenol glycosides[23]. Concentration of these glycosides in seeds of *Trifolium* species is similar to the concentration in other leguminous plants. High concentration of quercetin and presence of soyasapogenol B glycosides make seeds of some *Trifolium* species, a promising plant material to be used in human nutrition as nutraceuticals or food additives. This high concentration of quercetin can be advantageous for seeds due to its high antioxidant and radical scavenging properties. As shown in several studies quercetin is one of the most potent antioxidant and radical scavenging compound from the group of flavonoids[24].

In conclusion, the data obtained in this study demonstrated that *T. resupinatum* L. var. *microcephalum* might have antiinflammatory and antioxidant activities. Further studies are necessary to elucidate the mechanisms behind its effects and the results suggest that the inhibition of lipid peroxidation is likely to be a component of its antiinflammatory activity.

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(*Received*: 23 March 2007; *Accepted*: 10 October 2007) AJC-5997