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In vitro anthelmintic effect of *Vicia pannonica* var. *purpurascens* on trichostrongylosis in sheep

Esma Kozan [a,*], Serap Arabaci Anul [b], I. Irem Tatli [b,*]

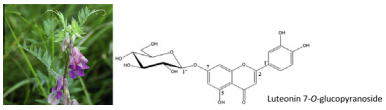
[a] Department of Parasitology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200 Afyonkarahisar, Turkey
[b] Department of Pharmaceutical Botany, Faculty of Pharmacy, Hacettepe University, Sihhiye, 06100 Ankara, Turkey

HIGHLIGHTS

- *Vicia* species are used in Turkish traditional medicine.
- We examined the *in vitro* anthelmintic effects of *Vicia pannonica* extracts.
- This plant possesses a significant *in vitro* anthelmintic activity.

GRAPHICAL ABSTRACT

The acetone extract of *Vicia pannonica* var. *purpurascens* and its flavonoid glucosides possesses a significant *in vitro* anthelmintic activity which support its traditional utilization.



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ABSTRACT

Vicia species are used for the treatment of malaria, diarrhea, hemorrhoids, kidney problems and infertility in Turkish traditional medicine. The present study was carried out to evaluate the *in vitro* anthelmintic effects of *Vicia pannonica* Crantz. var. *purpurascens* (DC). Ser. extracts. Larval motility test was used to determine anthelmintic activity of this plant. Motility of the larvae is measured by observation. The methanol, *n*-hexane, chloroform, acetone, and aqueous extracts of the aerial parts of the plant including the leaves and flowers were applied to developing trichostrongylus larvae at 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/ml doses. Thiabendazole and distilled water with 5% DMSO was used as positive and negative control. All of the extracts were 100% effective. Two flavone and flavonol glycosides; luteolin-7-*β*-*O*-glucopyranoside (**1**) and quercetin-3-*O*-*β*-glucopyranoside (**2**) were isolated from the acetone extract and their structures were elucidated by spectral techniques. The solutions prepared from two flavonoid fractions at several doses were performed *in vitro* to larvae in the same way. Both of them were 100% effective at 1 and 0.8 mg/ml doses. Results of the present study support the utilization of these plant species employed in Turkish folk medicine.

1. Introduction

Gastrointestinal parasites are recognized as major problems to livestock production in tropical and developing countries (Adejinmi and Harrison, 1997; Hounzangbe-Adote et al., 2005). The economic impacts of these parasites, as production diseases in ruminants lie not only in direct losses such as mortality associated with the clinical forms of diseases but also indirect insidious losses as a result of weaknesses (Gibbs, 1986).

In generally modern synthetic anthelmintics are used in control of these parasites (Kaplan, 2004; Coles et al., 2006). However helminth control programs, base on improvement of farm management and regular anthelmintic treatment, are often impracticable in developing countries because of relatively high price of modern anthelmintics for smallholder (Satrija et al., 2001). In addition, the development of anthelmintic resistance in nematodes has been reported throughout the world (Waller, 1994; Jackson and Coop, 2000). Furthermore some drugs may cause food residues and environmental pollution (Hammond et al., 1997). For these reasons, recently, the new alternative methods or nonchemical agents have been needed. A variety of *in vitro* assays have been developed for anthelmintic properties, in which the parasite stages are directly incubated in the chemical compound (Taylor et al., 2002). Larval motility assays measure the ability of anthelmintic to paralyse the infective third larval stage. Therefore, the overall principle of these assays relies on the assessment of larvae motility. The larval

* Corresponding authors. Fax: +90 312 3114777.
E-mail addresses: esmakozan@aku.edu.tr (E. Kozan), itatli@hacettepe.edu.tr (I.I. Tatli).

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motility tests are useful for detecting benzimidazole and macrocyclic lactones resistance. The larvae are incubated in the drugs and then motility of the larvae is measured by observation (Gill et al., 1991). Anthelmintic plants offer a traditional alternative to manufactured anthelmintics that is both sustainable and environmentally acceptable. Such plants could have a more important role in the future control of helminth infections in the tropics (Hammond et al., 1997).

Fabaceae (Leguminosae) family with 69 genera and 1145 taxon is the second largest family in Turkey as well as the genus *Vicia* with 85 taxon is the third largest genera in the family Fabaceae. The genus *Vicia* is represented by 64 species, 9 of which are endemic, in the flora of Turkey (Davis and Plitmann, 1969). *Vicia pannonica* is also widely distributed in the world including France, Germany and Hungary (Leht and Jaaska, 2002).

Vicia species are used for the treatment of malaria, diarrhea, hemorrhoids, kidney problems and infertility in woman in Turkish traditional medicine (Honda et al., 1996; Sezik et al., 2001). It is an important nutrient source for animals and also humans (Artık and Pekşen, 2005). In Italy, *Vicia* species are used in folk veterinary medicine for gastrointestinal complaints and as food supplement (Viegi et al., 2003). Some Fabaceae species are also used against parasitic diseases in the world traditional medicine (Ahua et al., 2007). Phytochemical studies on these species have revealed the presence of lectins, flavonoids, condensed tannins, procyanidins, and saponins (Webb and Harborne, 1991; Torck and Pinkas, 1992; Eisele et al., 1993; Desroches et al., 1995; Kinjo et al., 1998; Merghem et al., 2004). Several flavonoid aglycones (apigenin, luteolin, kaempferol, quercetin, myricetin, diosmetin and vitexin) and glycosides (apigenin glucopyranoside, luteolin glucopyranoside, kaempferol dirhamnopyranosyl glucopyranoside, quercetin glucopyranoside) were identified in *Vicia* species (Waage and Hedin, 1985; Webb and Harborne, 1991; Torck and Pinkas, 1992; Gamal-Eldeen et al., 2004). Phenolic compounds including condensed tannins and flavonoids have been implicated in pharmacological activities such as antioxidant, anti-inflammatory, antinociceptive, chemopreventive, antimicrobial and anthelmintic effects (Waage and Hedin, 1985; Gamal-Eldeen et al., 2004; Spanou et al., 2008; Azando et al., 2011).

There are no studies with this plant about its anthelmintic effects. So the aim of this study is to evaluate the *in vitro* anthelmintic effects of *V. pannonica* var. *purpurascens* extract and flavonoids derived from the this plant.

2. Materials and methods

2.1. Plant materials

V. pannonica Crantz. var. *purpurascens* (DC.) Ser. (Fabaceae) was collected from the vicinity of Kastamonu province in Turkey, Ilgaz Mountain, 20 km from Kastamonu to Safranbolu in September, 2008. Voucher specimens were authenticated by Serap Arabaci Anul and Ceren Arituluk (Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Ankara, Turkey). A specimen of the original collection (coded as HUEF 09890) is preserved at the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

2.1.1. Bioassay-guided fractionation and isolation of active ingredients
2.1.1.1. Preparation of the acetone extract from the aerial parts of V. pannonica var. purpurascens. The air-dried and powdered aerial parts including the leaves and flowers of *V. pannonica* var. *purpurascens* (32.5 g) were extracted with MeOH (3 × 2000 ml) at 40 °C for 8 h using rotary evaporator without vacuum. Extracts were filtered and the combined MeOH extracts were concentrated under reduced pressure at 35–40 °C (MeOH extract: 7.5 g; yield: 23.1%, w/w). Because of the complex composition of the plant extracts, the liquid–liquid fractionation was used as a rapid and basic method. This method was based on relative solubility of the compounds in solvents of different polarity. The resultant residue was dissolved in H_2O (100 ml, each) and the water-soluble portion was partitioned in *n*-hexane, $CHCl_3$ and acetone (2 × 100 ml), respectively, in a separating funnel to remove chlorophyll and other lipophilic constituents (*n*-Hexane extract: 596.5 mg; yield; 7.9%, w/w; $CHCl_3$ extract: 892.9 mg; 11.9%, w/w; Acetone extract: 1.9 g; 25.3%, w/w). The rest of the H_2O phase was obtained as 415.6 mg (yield: 5.5%, w/w). The highest extraction yield was obtained from acetone. Therefore, the most yielded acetone extract containing flavonoid fractions as determined by TLC was administered for activity assessments.

2.1.2. Fractionation of the acetone extract
The acetone phase (1.9 g) was fractionated over a silica gel column [Merck, 230–400 mesh, 130 g, $CHCl_3$/MeOH (95:5 → 65:35)] to afford six main fractions: Fr. A (148 mg), Fr. B (209 mg), Fr. C (176.5 mg), Fr. D (98 mg), Fr. E (253.1 mg) and Fr. F (156.8 mg). Because of high purity of the fractions and intensive yellow colors of the compounds on the TLC plate, fractions C and E were chosen to apply on column chromatography for the isolation of pure compounds.

2.1.3. Chromatographic separation and isolation of the constituents
Fraction C (176.5 mg) was further purified on a Sephadex LH-20 (20 g) column using MeOH to give compound **1** (9.4 mg). Purification of Fraction E (253.1 mg) by Sephadex column chromatography LH-20 (20 g) furnished compound **2** (24.8 mg).

*2.1.4. Structure elucidation of the compounds **1–2***
Structure elucidation of the isolated compounds **1**–**2** from fractions C and E, respectively, was carried out by spectral techniques; UV, IR, 1D- and 2D-NMR, and mass spectroscopy (HR-ESIMS), and detailed data were recently submitted elsewhere (Akdemir et al., 2001; Tatli et al., 2008). The structures of compounds **1**–**2** were as follows (Fig. 1): luteolin-7-*O*-*β*-glucopyranoside (**1**) and quercetin-3-*O*-*β*-glucopyranoside (**2**).

2.2. Preparation and maturation of trichostrongylid larvae

Natural trichostrongylosis was diagnosed by Flotation technique in flocks. One kilogram of faeces was collected from flocks natural infected with trichostrongylosis. This faeces were mixed with medium thick wood sahvings in a plastic container. The mixture was then incubated at 24–26 °C for 7–8 days. This allows the eggs of trichostrongylid nematodes to hatch and develop to infective L3 larvae. At the end of incubation, the mixture was placed into Baermann's apparatus and left overnight to allow these larvae to migrate. The water containg the infective *Trichostrongylus* sp. L3 larvae was tapped off and cleaned 3 times with water. The larvae were then stored at 8 °C for later use (Molan et al., 2003a).

2.3. In vitro assay

The rumen fluid was obtained from sheep slaughtered in the abattoir in the morning and centrifuged twice at 10, 000*g*. Rumen fluid was used on day of collection (Molan et al., 2003a). The dried extracts were dissolved distilled water with 5% dimethyl sulfoxide (DMSO) (Barrau et al., 2005).

Previous studies had decribed several *in vitro* test s that can be conducted to evaluate the anthelmintic efficacy of any potential plants. In this study the preliminary modification was done to the larval motility assay (Kotze et al., 2004) to determine the