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ORIENTAL JOURNAL OF CHEMISTRY
An International Open Free Access, Peer Reviewed Research Journal

www.orientjchem.org

ISSN: 0970-020 X
CODEN: OJCHEG
2015, Vol. 31, (Spl Edn):
Month : Oct.
Pg. 53-58

Antimicrobial and Antioxidant Effects of Some Turkish Fodder Plants Belongs to Fabaceae Family (*Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima*)

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(Received: March 20, 2015; Accepted: May 05, 2015)

http://dx.doi.org/10.13005/ojc/31.Special-Issue1.06

ABSTRACT

In this study, the antimicrobial and antioxidant activities of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* samples were investigated. For this purpose, plant extracts were tested against 3 fungus, 4 Gr(-) and 3 Gr(+) bacteria; additionally the glutathione (GSH) level and total phenolic content were measured spectrophotometrically and total antioxidant capacity (TAC) was done by ELISA method. According to results methanolic extracts of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* exhibited no antimicrobial activities against test microorganisms. *Trifolium ochroleucum* extract has the highest levels for GSH and TAC (462.06±25.89 mmol/L, 30.35±0.003 mg GAE/g dw respectively). *Vicia villosa* extract is showed the highest antioxidant capacity (4.416±0.107 mM).

Key words: *Vicia villosa*, *Trifolium ochroleucum, Onobrychis altissima*, Antioxidant, Antimicrobial, Glutathione, Total phenolic content.

INTRODUCTION

The flowering plant family Fabaceae (Leguminosae) contains over 18000 species distributed throughout the world in many ecological settings, from deserts of high latitudes to seasonally dry or wet tropical forests of equatorial regions[1]. *Trifolium*, the clover genus, is one of the largest genera of the legume family. Clover (*Trifolium*), or trefoil, is a genus of about 300 species of plants in Fabaceae. The genus *Trifolium* is distributed in temperate and subtropical regions of both hemispheres[2]. This genus widely grow in the Mediterranean region especially in Turkey where it is widely spread and represented by 103 species. They are small annual, biennial, or short-lived plants and have small white, yellow, purple or red flowers[3]. *Trifolium* is one of the two most important legume genera in live stock agriculture[4].

Vicia sp. is one of the most important annual forage legumes in the world because it has multiple uses such as hay, grain, straw, silage and green manure; its high nutritional value, and its ability to grow over a wide range of climatic and soil conditions. It's known as vetch and Turkey is one of the centre of domestication of it. Several species of the *Vicia* genus have been cultivated from ancient times and their use as feed crops dates back to antiquity[5]. 150 species of *Vicia* grow as natural populations in various parts of the world, and 59 of them grow in Turkey. It's recorded that wild, weedy forms of vetch exist in almost all part of Turkey at altitudes from sea level to 2200 m[6].

The genus *Onobrychis* comprises about 130 species. Its distribution ranges from the Mediterranean region to Caucasia, the Zagros Mountains and central Asia. Most species are concentrated in north western Asia, especially in Iran and Anatolia in Turkey[7, 8]. *Onobrychis altissima* have significant agricultural use as a perennial forage and fodder legume, for increasing the nutritive value of drought-resistant pastures through nitrogen fixation, and for soil conservation[9, 10].

In the last decade, studies have been concerned with antimicrobial and antioxidant properties of different nutritional products. Antioxidant ability has usually been attributed to the activity of antioxidant enzymes (mainly superoxide dismutase, glutathione peroxidase, catalase) as well as to the content of low-molecular antioxidants such as glutathione (GSH), carotenoids, tocopherols, ascorbicacid, phenolic substances[11]. It is widely known that significant antioxidant activity of food is related to high total phenolic content. Plants contain a large variety of phenolic derivatives, including simple phenols, phenylpropanoids, benzoicacid derivatives, flavonoids, stilbenes, tannins, lignans, and lignins. Flavonoids are the predominant components in some reported legumes[12]. Glutathione (GSH), the tripeptide g-glutamylcysteinylglycine, is the major source of non-protein thiols in most plant cells. GSH plays a central role in protecting plants from environmental stresses, including oxidative stress due to the generation of active oxygen species, xenobiotics, and some heavy metals[13].

The purpose of the present study was to determine glutathione levels (GSH), total phenolic contents, antimicrobial and antioxidative effects of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* widely consumed by animals as feed.

MATERIALS AND METHODS

Plants materials

Vicia villosa, *Trifolium ochroleucum* and *Onobrychis altissima* were collected from Ardahan region (Northeast of Turkey) and were identified with the help of Flora of Turkey and The East Aegean Island[14] and were kept in the herbarium of Scientific Research Center of Ardahan University. These samples were cleaned from debris, dried in the shade at room temperature, and finally powdered.

Preparation of Extracts

Aerial parts of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* used in this study. Powdered plant materials (10 g) were loaded to Soxhlet apparatus and the extraction was carried out using methanol (pi: 5.1) (300 ml) for 6 h. The resulting mixture was then filtered and concentrated under vacuum at 40 °C (Buchi, Rotavapor R-210, Labortechnik, AG, Flavil, Switzerland). Filter-sterilized and concentrated extracts were refrigerated (-18 °C) until use.

Determination of Antimicrobial Activity

Agar well diffusion method used for determination of the antimicrobial activity of the methanol extracts obtained from *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima.* For this aim, 4 Gram negative bacteria (*Enterobacter aerogenes* ATCC 27859, *Pseudomanas aeroginosa* 9027, *Escherichia coli, Klebsiella pneumoniae*), 3 Gram positive bacteria (*Bacillus subtilis, Bacillus megaterium* DSM 32, *Staphylococcus aureus* 6538) and 3 fungi species (*Yarrovia lipolytica, Candida albicans, Sacharomyces cerevisiae*) were used as test microorganisms. The various concentrations of extracts (10, 20, 40, 60, 80 and 200 µl) were tested against test microorganisms and ampicillin/ sulbactam 10 mcg bioanalyse disk, amikasin 30 mg oxoid disk and rifampisin 5 µg oxoid disk were also used as control for comparing the antimicrobial effects.

The petri dishes were incubated at 35 °C for 18-24 h, except for *Candida albicans, Saccharomyces cerevisiae* and *Yarrovia lipolytica* which were incubated at 27 °C. Inhibition zones were measured by vernier caliper and recorded as the mean diameter of 3 replications in mm. All tests were performed in triplicate and mean were centred.

Biochemical Analyzes

Reduce Glutathione levels were measured by the spectrophotometric method of Sedlak and Lindsay[15]. GSH is reacted with 5,5-dithiobis-2-nitrobenzoic acid resulting in the formation of a product with has a maximal absorbance at 410 nm. The results were expressed as µmol/L.

Total antioxidant capacity was determined by commercial ELISA kit. The assay relies ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{®+}$. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration. The phenolic content of samples was estimated by Folin–Ciocalteu reagent using the method of Lister and Wilson[16]. Plant extracts (100 µl) were mixed with 0.2 ml of Folin–Ciocalteu reagent and 2 ml of H_2O, and incubated at room temperature for 3 min. Following the addition of 1 ml of 20% sodium carbonate to the mixture, total polyphenols were determined after 1 h of incubation at room temperature. The absorbance of the resulting blue colour was measured at 765 nm with spectrophotometer. Quantification was done with respect to the standard curve of gallic acid. All determinations were performed in triplicate (n = 3).

Statistical Analyzes

Statistical analyses were accomplished with the use of the SPSS (Statistical Package for Social Sciences) computer program (version 16.0).

RESULTS AND DISCUSSION

Interdependence between the level of different phenolics and antioxidant ability of fruits, vegetables and medical plants has been studied for many years. The high ability of phenolic constituents to neutralize the active oxygen species is strongly associated with their structure, such as the conjugated double bonds and the number of hydroxyl groups in the aromatic ring, mostly attributed to flavonoids and cinnamic acid derivatives[11].

Table 1: The levels of antioxidant parameters and total phenolic content of plant extracts

	Trifolium ochroleucum	*Onobyrchis altissima*	*Vicia villosa*
GSH (mmol/L)	462.06±25.89	304.83±10.33	366.87±9.06
TAC (mM)	2.546±0.191	3.314±0.110	4.416±0.107
Total Phenolic Content (mg GAE/g dw)	30.35±0.003	25.06±0.010	23.58±0.009

The results of glutathion levels (GSH), total antioxidant capacities and total phenolic contents were presented in Table 1. According to these results methanolic extacts of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* are of glutathion levels (GSH), total antioxidant capacities and total phenolic contents at different rates. The highest total phenolic levels as well as *GSH* levels have been detected in *Trifolium ochroleucum*. Total antioxidant capacity have been observed as 2.546±0.191 mM in *Trifolium ochroleucum*, 3.314±0.110 mM in *Onobyrchis altissima* and 4.416±0.107 mM in *Vicia villosa*, respectively.

The results obtained from the present study show that the species used for the studies which is best known as forage, have significant levels of antioxidants and total phenolic content. Djeridane et al.[17] found in their studies with various species including *Trifolium* that the amount of total phenolic compounds in all tested plants was higher than some Asian vegetables some herbs and medicinal plants such as *Armoracia rusticana*, *Fallopia convolvulus, Matricaria matricarioides.* Leja

Table 2: Antimicrobial activities of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima*

Microorganisms		***Vicia villosa***	***Trifolium ochroleucum***	***Onobrychis altissima* 10 mcg Bioanalyse Disk (mm)**	**Ampicillin/ Sulbactam 30 mgOxoid Disk (mm)**	**Amikasin 5 µg Oxoid Disk (mm)**	**Rifampisin**
Gram Positive Bacteria	*B. subtilis*	-	-	-	14	11	21
	S. aureus 6538	-	-	-	10	9	18
	*B. megaterium*DSM 32	-	-	-	-	10	16
Gram Negative Bacteria	*E. aerogenes* ATCC 27859	-	-	-	10	9	16
	E. coli	-	-	-	13	13	18
	P. aeroginosa 9027	-	-	-	-	14	8
	K. pneumonia	-	-	-	16	10	19
Fungi	*Y. lipolytica*	-	-	-	-	-	-
	C. albicans	-	-	-	-	-	-
	S. cereviciae	-	-	-	-	-	-

et al.[11] demonstrated that *Trifolium sp.* has medium levels of antioxidant activity. In the one of the study about *Onobrychis viciifolia* reported that the extracts from the *O. viciifolia* possess hydrogen donating capabilities to act as antioxidant. The extract also caused significant elevation of reducing power potential, highest in methanolic extracts, in accordance with increasing doses[18]. *Vicia sp.* is valuable part of animal feeds because of it has a high protein content[19]. Chaieb *et al.*[20] indicated that *Vicia faba L.* is a good source of natural antioxidants because of their high levels of total phenolic content and antioxidant capacity.

Results of this study showed that glutatahione levels (GSH) of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* determined as 366.87±9.06, 462.06±25.89 and 304.83±10.33 mmol/L respectively and *Trifolium ochroleucum* had the higest GSH level

It is determined in this study that plant extracts obtained from *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* have significant GSH levels at different rates. Knowledge of the functions of glutathione (GSH) in plants has expanded rapidly in recent years. It now appears potential that glutathione could act both as a direct link between environmental stress and a number of the key adaptive responses plants have evolved[21]. The studies demonstrated that legume nodules have highest levels of GSH, which is the effective scavenger of free radicals[22, 23]. Legumes are an interesting plant material with which to study thiol metabolism for various reasons. First, there is an active ascorbate-GSH cycle in the root nodules, which requires a continuous supply of GSH to protect nitrogen fixation against toxic oxygen species[23].

Results showed that methanolic extracts of *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* did not have antimicrobial properties against test microorganisms used in this study (Table 2). However, this case did not show that *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* possess no antimicrobial activity. Several scientific reports have described the inhibitory effect of plants on a variety of microorganisms, although considerable variation for resistance of different microorganisms to a given plant and of the same microorganisms to different plants[24]. Differences in the activity of many species may be explained due

to variations in the nature and combinations of phytocompounds present in the solvent extract, strain sensitivity, antimicrobial procedure adopted in tests, or may be largely depending on the plant species and/or geographical sites[25, 26, 27]. The extraction product also varied in terms of quality, quantity and composition according to climate, soil composition, plant organ, age *etc*.[28].

Some of previous studies showed that different members of Fabaceae family (*Syzygium aromaticum* and *Glycyrrhiza glabra*) exhibited antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv at different rate[29]. Moreover, the trypsin inhibitör purified from seeds of *Mucuna pruriens* (Fabaceae) showed inhibitory activity against *Aspergillus niger* and *Trichoderma viridae*[30].

Consequently, *Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima* used as animal feeds have glutathione (GSH), total antioxidant capacity (TAC) and total phenolic content levels at different rate. These properties are very important for livestock.

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