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ORIGINAL PAPER

CONTENT OF ISOFLAVONES AND MACROELEMENTS IN FIVE CLOVER SPECIES (*TRIFOLIUM* SPP.) GROWN ON PASTURE: THE INFLUENCE OF A SLOPE ASPECT*

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Abstract

This study aimed to determine the content of isoflavones (biochanin A, daidzein, formononetin and genistein) and macroelements in 5 clover species: 1) hare's-foot clover (*Trifolium arvense* L.), 2) field clover (*T. campestre* Schreb.), 3) suckling clover (*T. dubium* Sibth.), 4) alsike clover (*T. hybridum* L.), 5) zigzag clover (*T. medium* L.) collected from different slope aspects (north- and south-facing slopes) of pasture in Northeastern Bulgaria. The content of biochanin A (0.317 mg g^{-1}), daidzein (0.201 mg g^{-1}) and genistein (0.205 mg g^{-1}) was found to increase significantly on the south-facing slope of pasture. Zigzag clover (0.117 mg g^{-1}) and hare's-foot clover (0.119 mg g^{-1}) samples from the north-facing slope had the lowest content of daidzein, which was higher in the other clover samples. Alsike clover on the south-facing slope of pasture had significantly higher genistein content (0.219 mg g^{-1}) than the other clovers. The highest concentration of formononetin was detected in alsike clover (0.167 mg g^{-1}) and field clover (0.175 mg g^{-1}) on the south-facing slope of pasture. The south-facing slope resulted in significantly higher potassium (27.00 g kg^{-1}), calcium (24.50 g kg^{-1}), magnesium (2.30 g kg^{-1}) and phosphorus (3.24 g kg^{-1}) content in the plants than the north-facing slope of pasture. The content of these isoflavones and macroelements depended on the slope aspect and clover species. The clover grown on the southern slope of pasture had high content of isoflavones and macroelements. The content of isoflavones and macroelements revealed in the present study could provide useful information for development of clover cultivation management strategies, such as the selection of species, to ensure healthy animal feeding.

Keywords: clover species, isoflavones, mineral content, pasture, slope aspect.

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INTRODUCTION

Legume-based (*Fabaceae* L.) systems are known to contribute to sustainable, environmentally friendly and energy-efficient agriculture, and are likely to increase in importance, also in view of the reduced unit costs of production (Porqueddu et al. 2003). On the other hand, the results of several trials show that less intensive production schemes based on pasture or forage legumes and forage legume-grass (*Poaceae* L.) mixtures in animal feeding have markedly positive direct effects (e.g. high carotene content improves milk, butter and cheese color) and indirect effects (e.g. the concentration of proteolytic enzyme affects cheese maturation and texture) on several characteristics on animal products (Coulon, Priolo 2002). Among the main aspect, we might consider the possibility of connecting the botanical composition of an animal diet with the quality of end final products, thereby being able to identify and evaluate feeds. Among the compounds that can be directly transferred from pasture to animal tissue are the terpenes present in herb-rich pasture swards. Some of these molecules can still be used as feed tracers. Also, flavonoids and phenolic compounds are known for their influence on the taste and preservation of many human foods, and the improved health of the consumer (Porqueddu et al. 2003). However, the positive effect of flavonoids and phenolic compounds on the productivity and health of animals as well as rumen fermentation and control of nutritional stress such as bloat and acidosis have been demonstrated in several researches (Patra et al. 2006, Rochfort et al. 2008, Kalantar 2018, Yildirim et al. 2021), but their concentration in most feedstuff, forage crops and forage crops mixtures are not known.

Isoflavones are a subclass of flavonoid compounds produced almost exclusively by legumes. Their structures are composed of a 3-phenylchromen skeleton, which is chemically derived from the 2-phenylchromen skeleton by an aryl-migration mechanism (Das et al. 2019). Common isoflavones include biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), daidzein (*4',7-dihydroxyisoflavone*), formononetin (*7-dydroxy-4′-methoxyisoflavone*), genistein (*4',5,7-trihydroxyisoflavone), and prunetin (4′,5-dihydroxy-7-methoxyisoflavone)*. They are classified as phytoestrogens because they are plant-derived and have estrogenic activity (Fukuda et al. 2017). These isoflavones have been identified in various forage legumes, including *Trifolium* spp., *Medicago* spp., *Lotus* spp., *Melilotus* spp., sainfoin (*Onobrychis sativa* L.), sulla (*Hedysarum coronarium* L.), pitch trefoil (*Psoralea bituminosa* L.) and fenugreek (*Trigonella foenum-graecum* L.). Nevertheless, the environmental conditions and agricultural practices affect yields and chemical compositions (proteins, minerals, carbohydrates, secondary metabolites, etc.) of these forage crops on pasture or field.

The slope and altitude as well as the faunistic and floristic compositions are among the most important environmental factors that affect pasture traits. The botanical composition of a pasture may influence animal production by affecting the feeding value of herbage, seasonality of herbage accumulation, and rate of herbage accumulation. At the same time, the feeding value and palatability of herbage varies with the slope aspect, which is different in each plant community on a pasture. Understanding this aspect is important for forage crop management and planning because of its influence on the growth and morphological characteristics, nutritive value and productivity of forage crops. The differences between southern and northern slopes arise mainly from differences in solar radiation, which affect the soil surface temperature, evaporation, and water retention capacity of the soil (Ates 2017, 2021). In general, south-facing slopes receive more sunlight and become more xeric and warmer, supporting drought resistant vegetation and less conducive for plant growth. In contrast, north-facing slopes retain moisture and are cold and humid, supporting moisture-loving plants. Most studies on the slope aspect and altitude have focused on effects on morphological characteristics of plant species, plant communities and grassland productivity. Besides, previous studies addressing the isoflavone distribution in some clover species (*Trifolium* spp.) have reported that the year, environment, growth stage, fertilization, and number of cuts could be sources of variation in the isoflavone content (Amezaga et al. 2004, Du et al. 2013, Lemežienė et al. 2015, Tava et al. 2019). However, few studies have focused on effects of the slope aspect on vitamin, mineral and other nutritive values of forage legumes and grasses (Ates 2011, 2017, 2021, Ates, Tekeli 2011, Tenikecier, Ates 2019). There are no reports on the isoflavone content of clover species under a different slope aspect of pasture. Thus, this study aimed to determine the content of isoflavones (biochanin A, daidzein, formononetin and genistein) and macroelements in 5 clover species: hare's-foot clover (*T. arvense* L.), field clover (*T. campestre* Schreb.), suckling clover (*T. dubium* Sibth.), alsike clover (*T. hybridum* L.) and zigzag clover (*T. medium* L.) collected from different slope aspects (north- and south-facing slopes) of pasture in Northeastern Bulgaria.

MATERIAL AND METHODS

Pasture conditions and experimental design

The studied area is located on a pasture of the village Topchii, in the province of Razgrad, in Northeastern Bulgaria (43°40′N, 26°30′E) – Figure 1, in the Danube plain, at 287 meters above sea level, and it has all the characteristics of the continental climate, with hot summers and cold winters (Köppen-Geiger climate classification: Dfa) – Sadovski, Ivanova (2020). The pasture has a total area of 78.5 ha. The pasture replaced the original oak (*Quercus* spp.) and linden (*Tilia* spp.) forests and has been maintained for one hundred and sixty-five years by the grazing activity of wild (deer, *Cervus* sp.) and domestic animals: sheep (*Ovis aries*), cattle (*Bos taurus*),

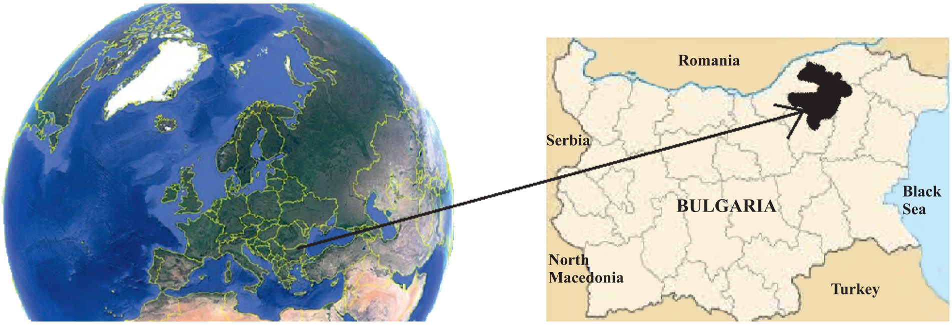

Fig. 1. Geographical location of the experimental site

goat (*Capra hircus*), donkey (*Equus africanus asinus*) and horse (*Equus ferus caballus*). Generally, the first grazing started each year in the middle of April and lasted until the beginning of November. The climatic data were obtained from the National Institute of Meteorology and Hydrology (NIMH 2011) in Sofia, Bulgaria. The average annual temperature of the region is 10.8°C. The hottest month of the year is July, with an average high of 27.78°C and low of 15.56°C. January is the coldest month, with an average low of -4.44°C and high of 2.22°C. The mean annual rainfall is 615 mm. Precipitations appear throughout the year in this site. The total precipitation ranges from 12.7 mm in the driest months (January) to 48.3 mm in the wettest (June). The soils of the pasture on the north- and south-facing slopes are described as PHl-Luvic Phaeozems (USDA soil taxonomy: Mollisol) – Hristov, Filcheva (2017). The pasture soil where the research was conducted was good in organic matter (north-facing slope: 4.78%, south-facing slope 4.45%), rich in phosphorus (P) content (north-facing slope: 12.7 mg kg^{-1}, south-facing slope 12.4 mg kg^{-1}), but moderate in potassium (K) content (north-facing slope: 672 mg kg^{-1}, south-facing slope 655 mg kg^{-1}), and with pH 6.6-6.8. The north- (28%) and south-facing slope (30%) of the pasture are steep. A two-factor experiment was set up in a randomized complete block design with three replications. The north- (20.1 ha) and south-facing (19.6 ha) slopes of the pasture were divided for three blocks. Five plots (50 x 50 m) in randomly selected areas of each block were defined (Ates, Tekeli 2011).

Collection and preparation of clover samples

For analyses of the content of isoflavones and minerals, six plants from each clover species (hare's-foot, field, suckling, alsike and zigzag clovers) per plot were collected each year at the full-bloom stage (Ates, Culpan 2018). Plants were collected once a month from June to August. All plants were cut at the ground level on the same day. Plant samples were collected during 2008-2009, and plants damaged by biotic and abiotic factors were discarded. Dead biomass and litter were removed from each sample and then the green parts were washed thoroughly with tap water, rinsed with distilled water and blotted on filter paper (Lemežienė et al. 2015). The plant material was air dried between two sheets of filter paper, which was done in shadow, at a temp. of 18-25°C and air humidity of 55-65%. The drying process was regularly controlled for damaged or discolored samples. The process lasted for seven days and, after its completion, the difference in mass before and after the drying process was used to determine the air-DM content. Then, dried clover samples were ground to small (≤1 mm) pieces and stored at -25°C in sterile, airtight bags until extraction and analyses (Mustonen et al. 2018, Ates et al. 2020, Ates 2021, Petrović et al. 2021, Tenikicier, Ates 2022). Analyses were made in 2018.

Chemical analyses

The milled clover samples (0.50±0.01 g) were placed each in a porcelain crucible of approximately 15 ml in volume. Then, the samples were ashed in a muffle furnace at 550°C for ~8 h (until obtaining a whitish ash, indicating the complete elimination of organic material). The cold ash was dissolved in 3 ml concentrated HCl, and the solution was diluted with 2% HCl (v/v) to a final concentration of 0.1 N. The solution of acidified ash was filtered through filter paper, and the consecutive rinses, with 2% HCl (v/v), from crucibles were collected in a 50 ml volumetric flask. The samples were diluted to a final concentration of 8%. Interference by phosphate in the measurement of calcium (Ca) was prevented by the addition of La_2O_3 and Al-Cs respectively, dissolved in 2% HCl to a final concentration of 10% (v/v) – Fernandez-Hernandez et al. (2010). Then, the Ca, K, and magnesium (Mg) content (g kg^{-1}) was found using a flame atomic absorption spectrophotometer – FAAS (Varian - Spectra AA-220 model, California, USA). The P content (g kg^{-1}) was determined colorimetrically by the vanadomolybdate method (AOAC, 2019). All samples were analyzed in duplicate.

The extraction of isoflavones and HPLC (quantitative high performance liquid chromatography) analyses were performed according to slightly modified procedures described by Saviranta et al. (2008), Du et al. (2013), Lemežienė et al. (2015), Mustonen et al. (2018), and Petrović et al. (2021). In brief, for isoflavone extraction, the 300 mg of a representative sample was weighed into a 25 ml glass flask with a screw cap, and 12 ml of methanol (CH_3OH)/water (8:2, v/v) containing 2 mol l^{-1} hydrogen chloride (HCl) was added. This mixture was sonicated for 45 min at 25°C and then incubated in a water bath at 80±2°C for 90 min with magnetic stirring. The samples were filtered through Acrodisc® syringe filter GHP (0.45 µm pore size, 25 mm diameter) before HPLC injection, and solutions were prepared in 10 ml volumetric flasks. The concentration of isoflavone standards: biochanin A (purity≥98%, Sigma-Aldrich Inc.), daidzein (purity≥98%, Sigma-Aldrich Inc.), formononetin (purity≥99%, Sigma-Aldrich Inc.), and genistein (purity≥98%, ChromaDex) standards in 80% ethanol were 0.4-12.5 µg ml^{-1}, and standard dilutions were made and analyzed daily. Each calibration curve used for quantification was characterized by a coefficient of determination (R^2) better than 0.9989. The concentration of stock solution was 25 µg ml^{-1}. For the separation of isoflavones, a 250 x 4.6 mm Agilent Zorbax® SB-C$_{18}$ 5 µm HPLC column with a mobile phase consisting of acetonitrile and water was used. The gradient profile was acetonitrile increased from 20% to 70% in 20 min, and the flow rate was 1 mL min^{-1} at room temperature. The isoflavone was detected at 254 nm wavelength. The injection volume was 10 µL. Each sample was analyzed by duplicate injections. Isoflavone contents (mg g^{-1}) were expressed on the air DM basis (Lemežienė et al. 2015).

Data analyses

The results were analyzed using the SPSS statistical program (Winer et al. 1991). There were no significant differences between years (P>0.05). The means of two years were compared by the Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

The slope aspect affected the content of isoflavones and macroelements (Tables 1 and 2). Differences between clover species were observed in their two-year average content of isoflavones and macroelements. In addition, the effects of the slope aspect x clover species interaction on isoflavone and macroelement concentrations were significant.

Isoflavone concentrations

As shown in Table 1, the slope aspect affected all the biochanin A, daidzein, formononetin and genistein concentrations in the five clover species and their interactions (P<0.01). The biochanin A (0.317 mg g^{-1}), daidzein (0.201 mg g^{-1}) and genistein (0.205 mg g^{-1}) contents were found to increase significantly on the south-facing slope of pasture. The highest biochanin A (0.503 mg g^{-1}) content was detected from alsike clover on the south-facing slope. Our findings that biochanin A is the dominant isoflavone in alsike, hare's-foot, zigzag, suckling and field clovers are in agreement with numerous studies on the quantification of isoflavones in clover species (Ramos et al. 2008, Saviranta et al. 2008, Lemežienė et al. 2015, Butkutė et al. 2018). As for the daidzein concentration, zigzag clover (0.117 mg g^{-1}) and hare's-foot clover (0.119 mg g^{-1}), samples on the north-facing slope had the lowest content, while the other clover samples had the highest levels of this isoflavone. Alsike clover on the south-facing slope of pasture had a significantly higher genistein content (0.219 mg g^{-1}) than the other clovers. The highest concentration of formononetin was detected in alsike clover (0.167 mg g^{-1}) and field clover (0.175 mg g^{-1}) on the south-facing slope of pasture. Specialized meta-

Table 1

Effect of slope aspect on isoflavone concentrations (mg g^{-1} of air-dry matter) of 5 clover species at the full bloom stage on pasture (mean of two years)

Species (S)	Biochanin A			Daidzein			Formononetin			**Genistein**		
	slope aspect (Sa)			slope aspect			slope aspect			slope aspect		
	north	south	mean	north	south	mean	north	south	mean	north	south	mean
Hare's-foot clover	0.174*g*	0.253*e*	0.213*d*	0.119*d*	0.185*b*	0.152*c*	0.088*d*	0.114*b*	0.101*b*	0.168*e*	0.211*b*	0.189*b*
Field clover	0.158*g*	0.220*f*	0.189*e*	0.123*c*	0.192*b*	0.157*c*	0.110*b*	0.175*a*	0.142*a*	0.145*g*	0.197*c*	0.171*d*
Suckling clover	0.254*e*	0.321*c*	0.287*b*	0.144*c*	0.224*a*	0.184*b*	0.091*d*	0.120*b*	0.105*b*	0.152*f*	0.206*b*	0.179*c*
Alsike clover	0.441*b*	0.503*a*	0.472*a*	0.186*b*	0.227*a*	0.206*a*	0.114*b*	0.167*a*	0.140*a*	0.177*d*	0.219*a*	0.198*a*
Zigzag clover	0.215*f*	0.289*d*	0.252*c*	0.117*d*	0.178*b*	0.147*c*	0.080*d*	0.106*c*	0.093*b*	0.133*h*	0.191*c*	0.162*e*
Mean	0.248*b*	0.317*a*	0.283	0.138*b*	0.201*a*	0.170	0.097*b*	0.136*a*	0.116	0.155*b*	0.205*a*	0.180
LSD	S: 0.023** Sa: 0.054** S x Sa: 0.031**			S: 0.017** Sa: 0.051** S x Sa: 0.023**			S: 0.013** Sa: 0.029** S x Sa: 0.011**			S: 0.008** Sa: 0.033** S x Sa: 0.0067**		

** significant at 0.01 level. Slope aspect means and species x slope aspect interactions with a different letter in the same column are significantly different. Clover species means with a different letter in the same row are significantly different.

Table 2

Influence of slope aspect on macroelement content (g kg^{-1} of air-dry matter) of 5 clover species at the full bloom stage on pasture (two-year means)

Species (S)	K			Ca			P			Mg		
	slope aspect (Sa)			slope aspect			slope aspect			slope aspect		
	north	south	mean	north	south	mean	north	south	mean	north	south	mean
Hare's-foot clover	22.63*c*	25.98*b*	24.31*c*	27.75*a*	29.60*a*	28.68*a*	2.48*b*	2.82*b*	2.65*b*	2.40*a*	2.48*a*	2.44*a*
Field clover	21.87*c*	25.41*b*	23.64*c*	22.13*d*	24.77*c*	23.45*b*	2.97*b*	3.84*a*	3.41*a*	2.37*b*	2.41*a*	2.39*a*
Suckling clover	22.00*c*	25.12*b*	23.56*c*	19.05*e*	27.03*b*	23.04*b*	2.99*b*	3.85*a*	3.42*a*	2.07*d*	2.20*c*	2.14*b*
Alsike clover	29.75*a*	31.17*a*	30.46*a*	18.70*e*	22.06*d*	20.38*c*	2.31*c*	2.76*b*	2.54*b*	2.00*e*	2.24*c*	2.12*c*
Zigzag clover	26.78*b*	27.33*b*	27.06*b*	17.97*e*	19.04*e*	18.50*c*	2.41*c*	2.92*b*	2.67*b*	2.11*d*	2.19*c*	2.15*b*
Mean	24.61*b*	27.00*a*	25.81	21.12*b*	24.50*a*	22.81	2.63*b*	3.24*a*	2.94	2.19*b*	2.30*a*	2.25
LSD	S: 2.006* Sa: 1.887* S x Sa: 2.223*			S: 2.645* Sa: 1.008* S x Sa: 1.851*			S: 0.621** Sa: 0.470** S x Sa: 0.540**			S: 0.266** Sa: 0.104** S x Sa: 0.102**		

*, ** significant at 0.05 and 0.01 level, respectively. Slope aspect means and species x slope aspect interactions with a different letter in the same column are significantly different. Clover species means with a different letter in the same row are significantly different.

bolites generally are low-molecular-weight compounds normally present in plants, where they are involved in various metabolic processes or stored as inactive precursors being activated by specific mechanism when a need arises. For instance, the synthesis of these metabolites can be induced by biotic and abiotic stresses (Bennet, Wallsgrove 1994, Tava et al. 2021). Clover species are the richest in specialized metabolites, with compounds such as isoflavones, flavones, cyanogenic glycosides, coumarins, etc. that have been researched (Butkutė et al. 2014, Zhang et al. 2018, Tava et al. 2016, Upton et al. 2017, Tava et al. 2019). Comparable amounts of isoflavones can be detected in clover's roots, stems, and leaves but smaller quantities appear in flowers. The isoflavones genistein and daidzein are among the most active phytoestrogens. In red clover (*T. pratense* L.), four isoflavones, such as genistein, daidzein, biochanin A, and formononetin are primarily found. Some sources claim that formononetin in ruminants is estrogenically more active than genistein degraded to daidzein and then metabolized to equol, which is a weak estrogenic compound. Although formononetin itself shows no estrogenic activity, its metabolite causes so-called clover disease, occurring mainly in small ruminants, especially sheep (Chen et al. 2010, Kašparová 2013, De Lucas et al. 2015, Hloucalová et al. 2016). De Rijke et al. (2001) also determined isoflavone concentrations in red clover. According to their findings, the content of individual substances was as follows: biochanin A 0.33 mg g^{-1}, formononetin 0.6 mg g^{-1} and daidzin 0.042 mg g^{-1}. In their experiment, daidzein and genistein were not detected in red clover leaves. Visnevschi-Necrasov et al. (2013) obtained 0.03 mg g^{-1} daidzein, 0.02 mg g^{-1} genistein, 0.04 mg g^{-1} formononetin and 0.02 mg g^{-1} biochanin A in DM from white clover (*T. repens* L.) leaves. Their values for daidzein, genistein, formononetin and biochanin A are much lower than the values measured in our experiment. Lemežienė et al. (2015) found that the formononetin, biochanin A, daidzein and genistein concentrations in red clover stems at the flowering stage ranged between 1.49-2.25, 0.44-0.86, 0.06-0.22 and 0.37-0.56 mg g^{-1} DM, respectively. Hloucalová et al. (2016) investigated the phytoestrogen content in leaves of freshly cut red clover, and obtained higher values for formononetin (4.31 mg g^{-1}) and biochanin A (3.70 mg g^{-1}). They reported 0.16 mg g^{-1} daidzein, 0.03 mg g^{-1} genistein, 0.11 mg g^{-1} formononetin and 0.31 mg g^{-1} biochanin A in leaves of berseem clover (*T. alexandrinum* L. Cent.). Ates (2017) emphasized that the feeding value and palatability of herbage varied depending on a slope aspect and are different in each plant community of natural flora. Butkutė et al. (2018) detected higher concentrations of formononetin in leaves of some clover species (3.98 mg g^{-1} DM in red clover cv. Sadūnai to 15.1 mg g^{-1} DM in zigzag clover) than in their stems (1.65 mg g^{-1} DM in red clover cv. Vyčiai to 10.8 mg g^{-1} DM in zigzag clover) or flowers (0.517 mg g^{-1} DM in red clover cv. Sadūnai to 5.28 mg g^{-1} DM in zigzag clover). Mustonen et al. (2018) reported that the respective concentrations of genistein, 0.49-0.55 mg g^{-1} DM and daidzein, 0.23-0.30 mg g^{-1} DM in red clover were considerably smaller than the concentrations of the main isoflavones. Tenikecier, Ates (2019) and Ates (2021) stated that the chemical traits in clovers were changed depending on growth stage, leaf/stem ratio; climatic and edaphic factors, such as the geographic location and topographic traits, seasonal and yearly variation, illuminance-associated diurnal variation, soil traits, biotic damage, conservation methods of herbage or hay (dehydration, ensiling and drying, etc.), and storage conditions of forage. Petrović et al. (2021) obtained the values of 0.062 to 0.160 mg g^{-1} for daidzein, 0.206 to 0.210 mg g^{-1} for genistein, 0.018 to 0.318 mg g^{-1} for formononetin and 0.197 to 0.646 mg g^{-1} for biochanin A in air-DM of different clover species. The current findings are similar to those earlier results.

Macroelement contents

The results presented in Table 2 showed that the south-facing slope gave significantly higher K (27.00 g kg^{-1}), Ca (24.50 g kg^{-1}), Mg (2.30 g kg^{-1}) and P (3.24 g kg^{-1}) contents than the north-facing slope of pasture. The effects of species were significant for the macroelement content. The highest K content was determined in alsike clover (29.75-31.17 g kg^{-1}) on the north- and south-facing slopes of pasture. The highest Ca (27.75-29.60 g kg^{-1}) content was determined in hare's-foot clover on the north- and south-facing slopes of pasture. The lowest P (2.31-2.41 g kg^{-1}) content was found in alsike and zigzag clovers on the north-facing slope while the minimum Mg (2.00 g kg^{-1}) content was found in alsike clover on the same slope aspect. The general functions of macro- and microelements for ruminant and non-ruminant animals are as follow: (a) give rigidity and strength to the skeletal structure, (b) engage in mineral-vitamin relationships, (c) activate enzyme systems, (d) serve as constituents of the organic compounds, such as proteins and lipids, which make up the muscles, organs, blood cells, and other soft tissue of the body, (e) exert characteristics effects on the irritability of muscles and nerves, (f) control fluid balance-osmatic pressure and excretion, (g) regulate acid-base balance. There is a direct and most important relationship between the content and availability of mineral elements in the soil and the mineral composition of forage crops. Sometimes the concentration of an essential mineral in the soil is so low that forage crops growing on it will not contain enough of minerals to meet the dietary requirements of ruminant and non-ruminant animals. At other times, forage crops may contain such high concentrations of a certain mineral(s) that they are toxic to the animals that eat them. Such soil/plant/animal relationships are particularly important in macro- and microelements. Acute and chronic dietary deficiencies of macro- and microelements have a significant impact on productivity of rangelands throughout the world (Pinchak et al. 1989, Ensminger et al. 1990, Tenikecier, Ates 2021). Amezaga et al. (2004) emphasized that the negative effect of a slope aspect on the P concentration as well as the same aspect on pH could be due to nutrient loss through surface runoff. Northern aspects usually have higher levels of precipitation, thus facilitating loss of nutrients through surface runoff, a parameter also greater on steep slope sites. The K, Ca and Mg levels in plants are usually in the range 1.39-2.50%, 0.77-3.00% and 0.20-1.20%, respectively, which is adequate for plant growth (Tekeli et al. 2003). The results were similar to those reported by these researchers. Suttle (2010) reported that mineral deficiencies cause species--specific symptoms, such as reduced feed intake, growth retardation, impaired development, and even death. For example, a low concentration of Mg^{2+} in the brain or cerebrospinal fluid (<0.25 mM) causes the NMDA receptors to be activated by glutamate and aspartate, and reduces the threshold at which neurons repetitively fire nerve impulses (action potentials), resulting in nervous disorders and consequently in tetany. However, the potassium deficiency syndrome includes muscle weakness, spasms, tetany, paralysis, numbness (particularly in legs and hands), excessive loss of body water (due to the inability to concentrate urine), low blood pressure, frequent urination, and thirst in animals. Finally, hypokalemia can also cause cardiac rhythm abnormalities, cardiac arrest, and even death in animals (Wu 2018, Tenikecier 2021). Ates (2011) and Ates, Tekeli (2011) reported that the Mg and P contents of plant species did not change depending on a slope aspect; whereas Ca and K ratios of plants changed depending on this parameter. The results of Mg and P contents by Ates (2011) and Ates, Tekeli (2011) were different from our results. Kohler et al. (2013) found that the Ca content was greater and the P content was lower in alpine compared to lowland forage. Ates (2021) investigated α-tocopherol, β-carotene, ergocalciferol contents and some nutritive values of six clover species on north- and south-facing slopes of natural flora, and reported that the south-facing slope gave significantly higher K (2.71% DM), Ca (2.90% DM), Mg (0.31% DM) and P (0.35% DM) contents than the north-facing slope, which coincides with our findings. NASEM (2021) reported that the demand for major mineral nutrients for gestating beef cows or lactating beef cows is 0.6-0.8% (w/w) for K, 0.1-0.4% for Ca, 0.1-0.4% for P and 0.04-0.1% for Mg, which resembles the present findings.

CONCLUSION

Our investigation provides new insights into the biochanin A, daidzein, formononetin and genistein concentrations and macroelement content in five clover species on different slope aspects of a pasture. The contents of these isoflavones and macroelements in alsike clover, field clover, zigzag clover, hare's-foot clover and suckling clover are dependent on a slope aspect and species. In clover species grown on the south aspect of a pasture, high contents of isoflavones and macroelements were determined. The isoflavone and potassium contents of alsike clover were found to be higher than in the other clovers, whereas the highest calcium and magnesium contents were determined in hare's-foot clover. Essentially, the mineral contents in the dry matter of species grown on both slope aspects were adequate for ruminant and non-ruminant animals. Besides, our results show that high and possibly harmful concentrations of these isoflavones for ruminant and non-ruminant animals can be formed on the south-facing slope of a pasture in certain clover species growing under the mentioned ecological conditions. On the other hand, these isoflavones can be used as feed additives in ruminant and non-ruminant production. The contents of isoflavones and macroelements revealed in the present study could be useful for developing management strategies for cultivation of these clovers, such as selection of species, to ensure healthy animal feeding.

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