Phytoestrogen Content and Estrogenic Effect of Legume Fodder (43825)

HANNU SALONIEMI,*[,1] KRISTIINA WÄHÄLÄ,† PÄIVI NYKÄNEN-KURKI,‡ KAARLO KALLELA,* AND ILKKA SAASTAMOINEN*

Department of Basic Veterinary Medicine, College of Veterinary Medicine, Section of Animal Hygiene, FIN-00581 Helsinki; Department of Chemistry,† University of Helsinki, FIN-00014 Helsinki; and Agricultural Research Center,‡ Southern Savo Research Station, FIN-50600 Mikkeli, Finland*

Abstract. **This study is a summary of Finnish investigations of the phytoestrogen content of legume plants, red clover, white clover, alfalfa, and goat's rue. In addition to the chemical analyses, biological studies were performed. Uterine weight of immature rats was used as an indicator of the estrogenic effect of the fodder used. All red clover varieties studied contained estrogenic isoflavones, especially formononetin and biochanin-A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter. The biological study of white clover showed a clear estrogenic effect not visible through chemical analysis. Alfalfa contains small quantities of formononetin and biochanin-A, but 25–65 ppm coumestrol in dry matter. The estrogenic effect of alfalfa was obvious in the biological study. Goat's rue did not contain any known phytoestrogens, and the biological study was completely negative.** [P.S.E.B.M. 1995, Vol 208]

Studies of the effects of phytoestrogens have been conducted since the early 1940s as a result of widely spread fertility problems observed in Australian sheep (1). In Finland, studies started in the 1960s were aimed primarily at determining whether phytoestrogens were involved in cow fertility disorders which frequently occurred in the spring at the beginning of the grazing season (2). Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth rate of animals and the milk yield of cows (3, 4). According to recent studies, they may also have a prophylactic effect against some hormone-related human malignancies (5, 6). The phytoestrogenic effects of legume fodder need to be understood because of increasing consumer interest in so-called organic farming products. This type of farming, which uses no artificial fertilizers, relies heavily on legume fodder which because of the plant's ability to bind nitrogen from air for its use.

The known phytoestrogens are either isoflavonoids or coumarins. Of the isoflavonoids, biochanin-A and genistein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants into the inactive para-ethylphenol (3). Two other phytoestrogens, daidzein and formononetin, are converted by ruminal microbia into the active equol. Coumestrol, which is related to the coumarins, is absorbed and is active as such (3).

Many Finnish fodder and pasture plants contain small amounts of phytoestrogens. The highest concentrations of phytoestrogens occur in red clover, where all varieties contain phytoestrogens. Abundant feeding of a diet based on red clover silage has been shown to cause fertility problems in cattle (7).

Attempts have been made to experiment with new legume varieties suitable for the Finnish climate which might have a positive effect on the fodder quality and palatability. These plants include white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), and goat's rue (*Galega orientalis*). At present, their adaptability to Finnish conditions is being studied at agricultural research stations.

In this paper we summarize the Finnish investiga-

[1] To whom requests for reprints should be addressed at College of Veterinary Medicine, Department of Basic Veterinary Medicine, Section of Animal Hygiene, Box 6, FIN-00581 Helsinki, Finland.

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tions of the phytoestrogen content of some legume plants and their estrogenic effects on rats.

Materials and Methods

Legume species studied and the date and place of sample collection are summarized in Table I. Details for cultivation of sampled fodder have been described in earlier reports (8, 9).

The samples consisted of the entire aboveground part of the plant. The samples were ground in a meat chopper immediately after cutting. Thereafter, they were allowed to stand for 30 min at 37°C for the conjugated phytoestrogens to hydrolyse (10) before mixing the samples in absolute ethanol. The samples were then stored in a refrigerator for closer chemical analyses and biological studies.

Studies conducted on subterranean clover have revealed that crushing the leaf tissue release free isoflavones (aglucones) from glucosides through enzymatic hydrolysis (11, 12). Accordingly, when different parts of red clover are milled, enough enzyme (β-glucosidase) is released from every part of the plant (stems not included) to allow complete hydrolysis (13). The adequacy of the hydrolysis method (maceration and incubation for 30 min at 37°C) was established in tests with red and white clover, in which the results of this method and acid hydrolysis were compared. It was found that maceration and incubation for 30 min at 37°C gave the same results as incubation of 4.6 g dry matter for 2 hr at 80°C in a solution of 1 ml of 4 *N* HCl in 30 ml of absolute ethanol or incubation of 4.6 g dry matter for 2 hr at 80°C in a solution of 10 ml of 4 *N* HCl in 30 ml alcohol or incubation of 4.6 g dry matter for 1 hr at 75°C in 10 ml of 25% HCl 10 ml in 80 ml alcohol (9).

Chemical Analyses. The method described earlier (14) was adapted for these studies. The plant samples warmed up at room temperature (50 g in 50 ml absolute ethanol) were mixed intensely for 5 min. The procedure was repeated the next day, after which the samples were filtered through a Büchner funnel. The filtrate was evaporated using a vacuum evaporator (40°C) to reach 100 ml. An aliquot was diluted and filtered through an Acrodisc CR filter (Gelman) before high-performance liquid chromatography (HPLC). The conditions for analysis are described in Table II.

Daidzein, genistein, formononetin, and biochanin-A were determined using a UV detector, and coumestrol was determined by fluorometry.

The following commercial preparations were used as standards: daidzein and formononetin (K & K Laboratories ICN, Cleveland, OH), genistein (Sigma Chemical Co., St. Louis, MO), and biochanin-A (Aldrich-Chemie, Germany). Standards were diluted in absolute alcohol to concentrations of 1–20 μg/ml. The

Table I. Legume Species Studied and the Date and Place of Sample Collection (9, 16)

Legume (variety)	Research station	Growth stage	Date of sample collection
Red clover (Tepa, Venla, Bjursele)	Southern Savo 61°, 40′	Pasture stage	June 18, 1987
		Silage stage	July 2, 1987
		First aftermath	August 14, 1987
		Second aftermath	August 28, 1987
	Northern Ostrobothnia 64°, 40′	Pasture stage	July 2, 1987
		Silage stage	July 15, 1987
		First aftermath	August 20, 1987
		Second aftermath	September 5, 1987
White clover (Jögeva, Sandra, Tammisto, Undrom)	Southern Savo	Pasture stage	June 17, 1991
		Silage stage	July 4, 1991
		First aftermath	August 6, 1991
		Second aftermath	September 2, 1991
White clover (Undrom)	Karelia, 62°, 20′	Pasture stage	June 19, 1991
	Kainuu, 65°, 50′	Silage stage	July 4, 1991
	N. Ostrobothnia	First aftermath	August 20, 1991
Alfalfa (Jokioinen)	Sata-Häme 61°, 30′	Bud stage	June 17, 1991
		Early blossom	July 27, 1991
		First aftermath	August 1, 1991
		Second aftermath	August 12, 1991
Goat's rue	Karelia, Sata-Häme	Bud stage	June 17, 1991
		Early blossom	June 27, 1991
		First aftermath	August 1, 1991
		Second aftermath	August 12, 1991

Table II. Analytical Conditions in Phytoestrogen Analysis of Red Clover (16) and That of Some Other Legume Fodder (9)

	Red clover analysis	Other legume analysis
Equipment	Perkin-Elmer 1220 liquid chromatograph UV detector Perkin-Elmer LC-55 at 254 nm Sigma 10 lab data system	Hewlett Packard 1050 liquid chromatograph, automatic sampler and UV detector at 254 nm Fluorometer Perkin-Elmer LS-4, Ex. 304, Em. 454 nm HP Chem Station data system
Pre column	3 μm 3 cm C-18 258-0160	Lichrospher 100 RP-18, 5 μm, 4 × 4
Column	5 μm 10 cm HS-5 HCODS 258-0152 P-E	Lichrospher 100 RP-18, 5 μm, 250 × 4 mm Hewlett Packard
Eluent	40% acetonitrile in water	acetonitrile/water: 40%, after 5 min: 70%, after 8.5 min: 80%, after 9 min: 100% run time 15 min, post time 7 min
Flow rate	1 ml/min	1 ml/min
Temperature	55°C	ambient
Sample size	2 μl	10–20 μl

injection volume used was 20 μl, while on the fluorometer it was 10 μl or less.

Biological Studies. Uterine weight of immature rates was used as an indicator of the estrogenic effect of the fodder used. The method has been described in details by Kallela (15) and Saloniemi *et al.* (9). For rat experiments, ether was chosen for extraction of fodder samples (due to its high volatility), whereas alcohol extraction was used for ordinary chemical analysis. The concentrations of isoflavones and coumestrol in prepared rat fodder paralled those observed in chemical studies (9).

Results

Phytoestrogen Content and Estrogenic Effects of Red Clover (*Trifolium pratense*). The most important Finnish legume used for fodder today is red clover. Its estrogenic effect and factors affecting it have been studied in Finland and in other countries. According to several studies (2, 3, 8, 15, 16), all the red clover varieties studied in the Nordic countries contain estrogenic isoflavones, especially formononetin. There are, however, clear differences between varieties. It has also been observed that the growth stage and the temperature affect the quantity of phytoestrogens. They are formed most abundantly in the spring during the rapid growth period or in the autumn in the abundant aftermath (8, 16) (Table III). The uterine weight of immature rats increased from 20 mg to 50–60 mg when rats received 2 g red clover dry matter per day for 5 days (7).

Cool weather during the growing season increases the amounts of phytoestrogens (3, 13). A clear difference was observed in the phytoestrogen concentrations of red clover varieties between North and South Finland (16). The amount of nutrients also have an effect. The formononetin concentration of red clover is higher in a phosphorus-poor soil than in a soil fertilized with phosphorus (13). It has also been observed that with increasing nitrogen doses the phytoestrogen concentration and raw protein concentrations in red clover decrease (8). The possible effect of a number of factors such as soil or rain has not yet been studied as far as we know.

Phytoestrogen Content and Estrogenic Effects of White Clover (*Trifolium repens*). The quantities of estrogenic isoflavones discovered in the white clover varieties were small, only 0.02%–0.06% in dry matter (Table IV). Formononetin was the main component (90%–95% of the total) and a very small amount of genistein (5%–10%) was detected. In most of the material, the amount of daidzein and biochanin-A was below the detection limit. Also small quantities of coumestrol were found, but it was not recovered in all samples. The effect of white clover samples on the

Table III. Phytoestrogen Content of Red Clover as Percentage of Dry Matter at Different Growth Rates (Range of Means, Three Varieties and Two Research Stations) (16)

Growth state	Daidzein	Genistein	Formononetin	Biochanin-A
Pasture stage	n.a.[a]	0.04–0.09	0.66–1.05	0.39–1.46
Silage stage	0.02–0.03	0.04–0.08	0.48–0.77	0.32–1.40
First aftermath	n.a.	0.04–0.14	0.70–1.04	0.55–1.45
Second aftermath	n.a.	0.03–0.11	0.60–0.93	0.40–1.17

Note. Variety Tepa had higher values than Venla and Bjursele, but the differences are not statistically significant. Values in Northern Ostrobothnia research station were higher than in Southern Savo ($p < 0.01$).
[a] n.a. = not analyzed.

Table IV. Phytoestrogen Content of White Clover at Different Growth Rates and the Effects on the Weight of Immature Rat Uterus (Range of Means, Four Varieties and Four Research Stations) (9)

Growth state	Isoflavones (% in DM[a])	Coumestrol (ppm in DM)	Increase in uterine weight[b] (mg)
Pasture stage	0.01–0.06	<1–5.8	15.5 (8.6–18.9)
Silage stage	0.02–0.03	<1–4.2	17.7 (15.0–21.0)
First aftermath	0.02–0.04	<1–8.7	15.6 (8.6–20.6)
Second aftermath	0.02–0.03	<1–8.9	36.0 (31.1–45.0)

Note. Biological study. Duration of experiment, 5 days; experimental extract, 3 g dry matter/day; rats/test group, 5 rats; rats/control group, 8 rats, uterine weight 21.0 ± 2.0 mg. No statistically significant differences were found between varieties and research stations.
[a] DM = dry matter.
[b] Mean (and range) of samples of different varieties.

weight of the rat uterus was, however, clearly positive (Table IV).

There are only a few observations on the estrogenic disorders caused by white clover in domestic animals (17). As in alfalfa, the most active phytoestrogen in white clover is coumestrol, which may increase considerably as a result of, or be solely attributable to, fungal diseases (18, 19).

According to the present HPLC study, the phytoestrogen concentrations of all white clover varieties were small at all Finnish research stations. The samples contained only 0.02%–0.06%estrogenic isoflavones, whereas red clover may contain more than 2%. Also coumestrol was found in small quantities and inconsistently.

A biological study of the efficacy of the estrogenic effect of white clover varieties was clearly positive, but inconsistent with the results of the chemical study. It is obvious that a small quantity of isoflavones does not explain the increased weight of the uterus, since formononetin is almost inactive in rats. The slightly elevated coumestrol content in the late autumn samples of varieties, Tammisto and Undrom, could have been due to the colder weather in the autumn and may to some extent have affected the results of the biological studies. The cold autumn weather and especially the night frosts may increase coumestrol concentrations considerably, as they do phytoestrogen concentrations in general (3). Small and inconsistent coumestrol concentrations do not, however, explain the obvious discrepancy between the results of the biological and chemical studies. In fact, there were apparently other substances increasing the estrogenic potency which were shown in the biological studies but not in the chemical studies. These might be substances of the coumarin group, which might explain the inconsistent results of alfalfa and especially of white clover. Their contribution should be studied further in the future.

Phytoestrogen Content and Estrogenic Effects of Alfalfa (*Medicago sativa*). The estrogenic efficacy of alfalfa was studied in the early days of phytoestrogen research in Finland. The criteria then were the changes in the weight of the uterus and vaginal smear of ovariectomized rats (2). Of the varieties then studied, Rhizoma had an obvious effect, Normad a smaller effect.

According to the present HPLC study, the estrogenic effect of alfalfa is most obviously attributable to coumestrol. The samples contained very small quantities of estrogenic isoflavones (formononetin and biochanin-A), whereas the coumestrol content was remarkably high in the spring and especially in the autumn (Table V). According to the present biological study, the estrogenic effect of alfalfa was also quite obvious. However, no seasonal changes in phytoestrogen content or biological activity were seen in alfalfa, unlike in red clover where there are high estrogen concentrations in early spring, a clear decrease by midsummer, and an increase again in aftermath (8).

Coumestrol is known to be about 30 times more effective than genistein in mice (11), and to cause estrogen-related disorders in animals (20). In addition, coumestrol seems to have a cumulative effect. Consequently, even quite small amounts (25 ppm) in the fodder may have an adverse effect and reduce the fertility of sheep (21). Whitten *et al.* (22) have found that coumestrol induces uterine growth in rats over a 90-hr period at a dietary concentration of 0.01%–0.1%. Lower doses not active over this period were active when provided over a longer period. A coumestrol concentration as low as 0.005% induced uterine enlargement if the diet was provided for over 180 hr.

Table V. Phytoestrogen Content of Alfalfa (variety Jokioinen) and the Effects on the Weight of Immature Rat Uterus (Sata-Häme Research Station) (9)

Growth stage	Coumestrol (ppm in DM[a])	Increase in uterine weight (mg)
Bud stage[b]	33.7	21.2
Early blossom	64.8	57.9
First aftermath	25.3	19.3
Second aftermath	63.0[c]	46.2

Note. Biological study. Duration of experiment, 5 days; experimental extract, 3 g dry matter/day; rats/test group, 5 rats; rats/control group, 8 rats, uterine weight 21.0 ± 2.0 mg.
[a] DM = dry matter.
[b] Small amount of formononetin and signs of biochanin-A in samples of bud stage.
[c] HPLC result of ether extract.

Coumestrol has been observed to accumulate in alfalfa following insect or fungal attack (23). According to some studies, the coumestrol concentration of alfalfa is a consequence of fungal diseases (21). Healthy plants contain only very small quantities of coumestrol (19). There are, however, differences between varieties (19, 24). In the samples investigated here, there were no indications of fungal or other diseases.

Phytoestrogen Content and Estrogenic Effects of Goat's Rue (*Galega orientalis*). Of the plants investigated, goat's rue did not contain any known phytoestrogens. Only traces of isoflavones (Karelia Research Station) and coumestrol (Sata-Häme Research Station) were observed in the plant samples of early spring and late autumn. The biological study of the effect of phytoestrogens on the weight of the rat uterus was completely negative. As far as we known, there are no studies or information in the literature concerning the estrogenic properties of goat's rue (9).

Discussion

Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. Formononetin and biochanin-A concentrations are highest, 0.3%–1.5% of dry matter. The amount of genistein is at the level 0.03%–0.15%, but the daidzein concentration is less than 0.03% of dry matter. The biological effect on the uterus of immature rats is large.

All white clover varieties contain very small quantities of estrogenic isoflavonoids (0.01%–0.06% of dry matter) and coumestrol (less than 10 ppm). However, in biological studies white clover still caused an increase in uterine weight which was about half of that of red clover. Until now, we have not found the chemical substance causing this biological effect.

Alfalfa contains 25–65 ppm coumestrol and has as large an effect as red clover on uterine weight of immature rats.

Goat's rue does not contain any known phytoestrogens. Even in biological studies, it has no estrogenic effect.

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