Ife J. Agric 1 (2) 1979 209 - 234

Preliminary studies on the oestrogenic activity of guinea grass [panicum maximum] var. S112 and stylo [Stylosanthes gracilis]

AMOS ADEPOJU, A.A. ADEGBOLA AND S.I. AYININUOLA
Department of Animal Science, University of Ife, Ile-Ife, Nigeria.

Abstract

Purified oestrogenic extracts were obtained from 25 g samples of gound air dried leaf and stem portions of guinea grass (*Panicum maximum* var. S112) and stylo (*Stylosanthes gracilis*) at four stages of growth. 0.05 ml of each of these extracts was injected into immature female mice weighing 8 to 10 g in order to determine the oestrogenic activities of the forages at different stages of growth. The mean values obtained for the oestrogenic potency of guinea grass were 0.30, 0.23, 1.41 and 0.86 mcg of diethyl stilbestrol per kg of the leaf portion and 0.28, 0.15, 1.01 and 0.49 mcg of diethyl stilbestrol per kg of the stem portion at early vegetative, full vegetative, early bloom and full bloom growth stages respectively. Mean values for stylo leaf portion were 0.62, 0.85, 1.50 and 0.75 mcg of diethyl stilbestrol per kg, and those for the stem portion were 0.43, 0.62, 0.80 and 0.55 mcg of diethyl stilbestrol per kg at the early vegetative, full vegetative, early bloom and full bloom growth stages, respectively. The results indicated that the oestrogenic content of *Stylosanthes gracilis* was significantly higher (P .05) than that of guinea grass. For both forages, oestrogen activity was higher (P .05) in the leaf than in the stem, and the differences observed during the various stages of growth were significant.

Introduction

Since the observation of the occurrence of oestrogens in the inflorescence and peduncle of female willow, (Loewe and Sphor, 1926) there has been considerable interest in the determination of oestrogenic potencies of various plants. In recent years, more interest has been shown in the oestrogenic potencies of grasses and legumes not only because forages form the basis of ruminant feeding all over the world but also because of the effect of oestrogen on fertility and gain efficiency in livestock. Forage plants have been shown to contain oestrogens (Bartlett *et al.*,

1948; Legg *et al.*, 1950 and Bickoff *et al.*, 1957), and also saponins in addition to other unidentified factors (Lindahl *et al.*, 1957). The oestrogenic material present in subterranean clover was shown to be largely genistein (Curnow and Benette, 1952) while coumestrol was isolated as the dominant oestrogen in alfalfa and ladino clover (Bickoff *et al.*, 1959).

The question of oestrogen dosage required to produce adverse effects on reproductive performance was investigated by Underwood *et al.* (1959). They found that 10 mcg stilbestrol daily for six months produced barely detectable effects on fertility. In a trial with ewes treated with 8 or 15 mcg stilbestrol daily for 21 to 33 days, Morley *et al.* (1966) obtained slight decrease in fertility and fecundity. Little and Lambourne (1976) indicated that a potency of less than 10 mcg stilbestrol is most unlikely to produce adverse effects on reproduction in cattle, whereas the implantation with 30 mg diethyl stilbestrol in steer calves in a feeding trial resulted in marked weight gain and feed efficiency stimulation (Preston *et al.*,1978). Newsome and Kitts (1977) concluded from their investigation on the effects of alfalfa phyto-oestrogens on circulating levels of endogenous oestrogens in ewes, that the consumption of alfalfa could be high enough to compete with endogenous oestrogen for sites on the oestrogen binding protein in the hypothalamus of the ewes and thus influence the production of gonadotrophins, and so, depress the functioning of the ovary.

This preliminary study was undertaken to determine the oestrogenic activity of the leaf and stem portions of guinea grass *Panicum maximum* var.S112) and of stylo (*Stylosanthes gracilis*) at four different stages of growth so as to provide a guide to the safe level of their inclusion in feeding programmes

MATERIALS AND METHODS

Guinea grass and stylo were established on a forage collection plot of the University farm. These were harvested at four different stages of growth, with the harvest from each stage divided into the leaves and stems portions. The stages of growth at which the forages were harvested were (a) the early vegetative growth stage, when the plants were just becoming established with a high leaf to stem ratio

(b) the full vegetative growth stage, when the plants were fully established and the growth of the vegetative parts was at a maximum (c) the early bloom stage, when the inflorescence heads of the plants were beginning to form and (d) the full bloom stage, when the flower heads of the plants were fully formed and mature.

The leaf and stem portions were wilted in the shade for 24 hours before being transferred to an oven set at 60°C for further drying for 24 hours. They were then ground and the oestrogen extracted from 25 g of each sample using the extraction procedure of Beck and Braden (1951), slightly modified by adding 100 ml of 95% ethanol to 25 g of the dried ground sample, and refluxing for one hour. Sufficient alcoholic NaOH was then added to make the solution 0.5 M NaOH, followed by a further refluxing for 10 minutes. After cooling, the alcoholic extract was removed by filtration and saved. The residue was extracted a second time with 50 ml of 95% ethanol by refluxing for 15 minutes and then filtered. The two extracts were combined and neutralized with alcoholic H_2SO_4 to pH 6.6 and allowed to stand overnight in a refrigerator at 10°C. The solution was then filtered and evaporated to dryness under vaccum. The crude extract was purified by the procedure adopted by Evans *et al.*, (1941), slightly modified by adding 50 ml of saturated $NaHCO_3$ and 100 ml of ether to the crude extract, which was left standing for 15 minutes and then transferred to a separating funnel. Further extraction was done by adding another 100 ml of ether. The ether portion from the separating funnel was concentrated to 50 ml and the active fraction removed by three consecutive extractions with an equal volume of 0.1M NaOH. The alkaline solution thus obtained was acidified with HCL and extracted twice with a small volume of ether. After washing with water, the other phase was evaporated to dryness. The resulting purified extract was weighed and then placed in olive oil.

Twenty one immature female mice weighing between 8 and 10 g were divided into seven groups for administration of standardized doses of diethyl stilbestrol (DES) for the purpose of obtaining a standard curve of the response of uterine weights to different levels of DES. Of the two groups of control mice receiving no DES, each mouse in one group was injected with 0.02 ml and the other with 0.08 ml of sterile distilled water. Each mouse in the remaining five treatment

groups was injected with DES concentrations of 0.01, 0.02, 0.025, 0.03 and 0.04 mcg in each group respectively for four consecutive days, after which they were sacrificed. Their uteri were removed, fixed in Souin's fluid for 24 hours, dried by pressing against filter paper, trimmed of any adhering tissues and then weighed. 0.05 ml of each purified extract in olive oil of leaf and stem samples at the stages of growth being considered was injected subcutaneously into each of three mice in a treatment for four consecutive days after which they were sacrificed and their uteri removed. The potencies of the purified extracts injected were determined by plotting the uterine weights obtained on the standard curve of mice receiving known doses of diethyl stilbestrol.

The data obtained were subjected to analysis of variance, and significance of differences were assessed by applying Duncan's Multiple Range Test at 5% level of probability (Steel and Torrie, 1960).

RESULTS

Table 1 shows the effect of injecting diethyl stilbestrol (DES) at concentration of 0.01 to 0.04 mcg on uterine weights. Uterine weights increased with increasing concentrations of diethyl stilbestrol with a mean of 7.6 mg at 0.0 mcg DES and 49.0

TABLE 1: UTERINE WEIGHT RESPONSES TO VARYING CONCENTRATION OF DIETHYL STILBESTROL (DES)

Mice Groups	DES Concentration (mcg)	Uterine weight (mg) Mice No.			Mean uterine wt. (mg)
		I	II	III	
A	0	7.6	7.8	7.4	7.6
B	0	7.8	7.5	7.3	7.5
C	.01	18.0	17.2	17.8	17.5
D	.02	28.2	28.5	28.2	28.3
E	.025	33.9	33.8	34.3	34.0
F	.03	38.0	37.8	38.5	38.1
G	.04	49.7	48.3	49.0	49.0

mg at 0.04 mcg DES. The injection of 0.02 and 0.08 ml of sterile distilled water to mice in groups A and B respectively did not produce any increase in uterine weight.

Table 2 shows the weight of purified oestrogen extract obtained from 25 g of dried ground sample from the leaf and stem portions of guinea grass at the four stages of growth studied. It also shows the calculated oestrogenic activity per kilogram of dry sample at each of the four stages of growth. The leaf portion extract weights were significantly higher (P .05) than the corresponding stem portion extract weights at the early vegetative, full vegetative, early bloom and full bloom growth stages respectively. The mean oestrogenic activity of the guinea grass leaf portion at the early vegetative growth stage, though higher, was not significantly different (P .05) from that of the full vegetative growth stage. These values were, however, significantly lower (P .05) than the value obtained for the early bloom stage, which in turn was significantly higher (P .05) than the value for the full bloom state. A similar pattern of oestrogenic activity was obtained for the stem portions, although the activity of the leaf portion was higher (P .05) at each of the four stages of growth.

TABLE 2: OESTROGENIC ACTIVITY IN GUINEA GRASS (PANICUM MAXIMUM VAR. S112)

Part of plant	Stage of growth	Weight of purified extract (gm)	Wt. of extract ejected (gm)	Calculated wt. of forage from which extract injected was obtained (gm)	Mean uterine wt. (mg)	Mean calculated potency/kg of forages (mcg DES)
Leaves	Early vegetative	0.21	0.09	9.0	10.5	.31
Stem	"	0.06	0.02	8.3	10.0	.28
Leaves	Full vegetative	0.14	0.06	10.1	11.0	.23
Stem	"	0.05	0.02	10.0	9.6	.15
Leaves	Early blood	0.14	0.04	7.1	18.1	1.41
Stem	"	0.06	0.02	8.3	15.6	1·01
Leaves	Full bloom	0.08	0.03	9.4	14.0	0.86
Stem		0.04	0.02	12.5	12.0	0.49

TABLE 3: OESTROGENIC ACTIVITY IN *STYLOSANTHES GRACILIS*

Parts of plant	State of growth	Weight of purified extract (gm)	Wt. of extract injected (gm)	Calculated weight of forage from which extract injected was obtained (gm)	Mean Uterine wt. (gm)	Mean calculated potency/kg of forage (mcg DES)
Leaves	Early vegetative	0.20	0.05	7.0	12.3	0.62
Stem		0.11	0.03	7.0	10.6	0.43
Leaves	Full vegetative	0.27	0.09	5.3	14.7	0.83
Stem	"	0.09	0.05	14.0	0.67	0.63
Leaves	Early bloom	0.08	0.02	6.3	17.7	1.50
Stem	"	0.03	0.01	8.3	14.6	0.80
Leaves	Full bloom	0.06	0.03	12.5	17.3	0.75
Stem	"	0.04	0.02	12.5	14.2	0.55

Table 3 shows that, as observed for guinea grass, significantly higher amounts of extracts were obtained from the leaf portions of stylo as compared with the stem portion. Also, the estimated oestrogenic activities of the stem and leaf portions of stylo followed a similar pattern as described for guinea grass in Table 2.

Discussion

The consistently higher oestrogenic activity in the leaf portion as compared with the stem portion in both forages at the four stages of growth studied may be due to the higher content of chloroplasts in the leaves than stem, as chloroplasts have been known to be the site of formation of oestrogens in plants (Bartlett *et al.*, 1948; Legg *et al.*, 1950; Pieterse and Andrews, 1956). The same factor could explain why high values were obtained at the early vegetative growth stage in the two forages. As the plants mature, however, there would be fewer chloroplasts per unit area, hence the observed slight decrease in activity in the full vegetative growth stage.

The higher oestrogenic activity obtained in the subsequent early bloom stage was probably due to the fact that the plants were in their active reproduc-

tive form at this stage. Legg *et al.*, (1950) observed that high oestrogen concentration normally proceeds flowering. Alexander and Watson (1951), Pieterse and Andrews (1956), indicated that oestrogenic activity will generally decline after initiation of flowering. This finding is consistent with the data reported herein, in which the activity declined in both forages at the full bloom stage.

That there were differences in oestrogenic activities of the leaf and stem portions of the two forages and at different growth stages was in conformity with the results obtained by Legg *et al.* (1950) who found oestrogenic activity in the leaf, petiole, stem and inflorescence of plant species with considerable seasonal variations and with the different plant parts not showing maximal concentrations simultaneously.

The oestrogenic potencies obtained for guinea grass and stylo in this study are lower than the value of 1.60 - 5.40 mcg stilbestrol/kg for alfalfa reported by Cheng *et al.* (1953). Different forage species grown on different soils and climatic environment may contain varying levels of oestrogen.

Cox and Braden (1974) reported that the effect of oestrogen is restricted in ruminants because the metabolism of such substances in the rumen is known to alter the oestrogenic potency substantially, but Bindon and Lamond (1966)observed that ewes fed on *Leucaena* during pregnancy and lactation produced small lambs exhibiting low viability and growth rate. The problem of ovine infertility due to phyto-oestrogens in subterranean clover has also been reported by Moule *et al.*, (1963). Underwood *et al.* (1959) found that 10 mcg stilbestrol daily for six months produced barely detectable effects on fertility and Little and Lambourne, (1976) observed that potency of less than 10 mcg stilbestrol is most unlikely to produce adverse effects in cattle. However, Preston *et al.* (1978) concluded that implantation with 30 mg diethyl stilbestrol in steer calves produced marked weight gain and stimulated efficiency. The figures reported here in for guinea grass and stylo should, therefore, be of interest to the livestock feeder as to the safe level of inclusion of these forages at the different growth stages in feeding programmes.

References

Alexander, G. and R.H. Walson, 1951. The assay of oestrogenic activity of *Trifolium subterranea* by increase in uterine weight in sprayed guinea pig. Austr. J. Agric. Res. *2*: 480

Barlett, S.S., J. Folley, S.S., Rowland, D.N., Curnow and A. Simpson, 1948. Oestrogens in grass and their possible effects on milk secretion. Nature *162*: 845

Beck, A.B. and A.W. Braden, 1951. Studies on the oestrogenic substances in subterreanean clover. Austr. Jour. Exp. Med. Sci. *29*: 273

Bickoff, E.M., R.L. Lyman, C.R. Thompson and F. Deeds, 1957. Coumestrol, a new oestrogen isolated from forage crops. Science *126*: 969

Bindon, B.M. and D.R. Lamond, 1966. Harmful effects of *Indigofera Spicata, Leucaena Leucocephala* and *Trifolium repens* on reproduction in mice and sheep. Proc. Austr. Soc. Anim. Prod. *6*: 109

Cheng, E., C.D. Story, L.C. Payre, L. Yoder and W. Burroughs, 1953. Detection of oestrogenic substances in alfalfa and clover hays fed to fattening lambs. J. Anim. Sci., *12*: 507

Cox, R.I. and A.W.H. Braden, 1974. The metabolism and physiological effects of phyto-oestrogens in livestock. Proc. Austr. Soc. Anim. Prod. *10* 122

Curnow, D.H. and H.W. Bennett, 1952. Oestrogenic hormones in plants in relation to animal physiology. Abstract of papers from 6th International Grassland Congress.

Evans, J.S., R.F. Vanney and F.C. Koch. 1941. The mouse uterine weight method for the assay of oestrogens. J. Endoc. *28*: 747

Legg, S.P.; D.H. Curnow and S.A. Simpson, 1950. The seasonal and species distribution of oestrogen in British pasture plants. Biochem. J. *46*: 19

Lindahl, I.L., R.T. Tertelli, G.E. Whitmore, W.T. Shalkop, R.W. Dougherty, C.R. Thompson, G.R. Van Atta, E.M. Bickoff, E.D. Walter, A.L. Livingstone, J. Guggols, R.H. Wilson, M.B. Sideman and F. Deeds, 1957. Alfalfa soponins on the chemical, pharmocological and physiological properties in relation to ruminant bloat. U.S.D.A. Bull. 1161.

Little, D.A. and L.J. Lambourne, 1976. The detection of oestrogenic activity by measurement of nucleic acids in serial samples of ovine uterine tissue obtained by biopsy. Austr. J. Agric. Res. *27*: 675

Loewe, S. and E. Spohr, 1926. Nachweis and Gehalts bestimung des weiblichen Brunsthormons in weiblichen organes des pflanzenreiches Anz. Akad. Wiss. Wein. *63*: 167

Lyman, R.L., E.M. Bickoff, A.N. Booth and A.L. Livingstone, 1959. Detection of coumestrol in leguminous plants. Arch. Biochem. Biophysics *80*: 61

Morley, F.H.W., A. Axelsen and D. Bennett, 1966. Recovery of normal fertility after grazing on oestrogenic red clover. Austr. Vet. J. *42*: 204

Moule, G.R., A.W.H. Draden and D.R. Lamond, 1963. The significance of oestrogen in pasture plants in relation to animal production. Anim. Breed. Abstr. *31*: 139

Newsome, F.E. and W.B. Kitts, 1977. Effects of alfalfa consumption on oestrogen levels in ewes. Can. J. Anim. Sci. *57*: 531

Pieterse, P.J.S., and F.N. Andrews, 1956. The oestrogenic activity of alfalfa and other feedstuffs. J. Anim. Sci. *16*: 850

Preston, R.L., F. Byers and K.R. Stevens, 1978. Oestrogenic activity and growth stimulation in steers fed varying protein levels. J. An. Sci. *46*: 541

Steel, R.G.D. and J.H. Torrie, 1960. Principles and procedures of statistics. McGraw-Hill Book Company Inc., New York, Toronto, London.

Underwood, E.J., F.L. Shier, N. Davenport and H.W. Bennetts, 1959. Further studies of the effects of prolonged injections of stilbestrol on the ewe. Austr. Vet. J. *35*: 84