UNIVERSITI PUTRA MALAYSIA

PROTECTIVE EFFECT OF PHENOBARBITONE AND GRISEOFULVIN AGAINST SIGNAL GRASS (BRACHIARIA DECUMBENS) TOXICITY IN SHEEP

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By

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Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Medicine and Health Sciences Universiti Putra Malaysia

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"To my beloved family and all those individuals behind the scenes who make me possible to finish my study."

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

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Chairman : Professor Dr. Abdul Salam Abdullah

Faculty : Medicine and Health Sciences

The protective effect of phenobarbitone and griseofulvin against signal grass (*B. decumbens*) toxicity were studied in fifty-three Wiltshire x Malin (Indigenous Malaysian) male sheep. Twenty-six animals were used in phenobarbitone experiment and twenty-seven animals were used in griseofulvin experiment. Grazing on signal grass significantly decreased the concentration of cytochrome P-450 and the activity of drug metabolizing enzyme, *viz.* aminopyrine-N-demethylase, aniline-4-hydroxylase, UDP-glucuronyltransferase and glutathione-S-transferase in liver and kidney of sheep. The concentration of cytochrome P-450 was determined in microsomal fraction according to the method of Omura and Sato (1964a) as described by Mazel (1971). The activities of aminopyrine-N-demethylase and aniline-4-hydroxylase were determined using Mazel, 1971 method by estimating the

concentration of formaldehyde and ρ-aminophenol, respectively. The method of Dutton and Storey (1962) was used to determine UDP-glucuronyltransferase activity by estimation of o-aminophenyl-glucuronide concentration by using o-aminophenol as a substrate. The activity of glutathione-S-transferase was determined in cytosolic fraction by estimation of 2,4-dinitrophenylglutathione according to the method described by Habig *et al* (1974). Oral administration of phenobarbitone (30 mg/kg body weight) for five consecutive days every two weeks resulted in significant increases in hepatic and renal activities of drug metabolizing enzymes. The induction in sheep grazing on Signal grass group was found to be lower than in animals given phenobarbitone alone. Induction of drug metabolizing enzyme activity by phenobarbitone provided a degree of protection for sheep against the toxic effect of *B. decumbens* as indicated by the delay in the appearance of toxicity signs. Furthermore, the toxicity signs were much milder compared to those in the intoxicated sheep not treated with phenobarbitone. Griseofulvin administration (5 mg/kg body weight) only induced phase II drug metabolizing enzymes (UDP glucuronyltransferase and glutathione-S-transferase) in liver and kidney of sheep. This drug provided less protection when five out of seven animals in group treated with griseofulvin and grazed *B. decumbens* affected by this plant toxicity. This present study suggests that phenobarbitone-type cytochrome P-450 isoenzyme induction may increase resistance against signal grass and the lack of potency may preclude griseofulvin as a drug inducer to protect sheep from *B. decumbens* toxicity.

KESAN KETAHANAN PHENOBARBITONE DAN GRISEOFULVIN TERHADAP KERACUNAN RUMPUT SIGNAL (BRACHIARIA DECUMBENS) DI DALAM BIRI-BIRI

Oleh

HASIAH BT AB HAMID

Februari 2001

Pengerusi : Profesor Dr. Abdul Salam Abdullah

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Kesan penggunaan ubat phenobarbitone dan griseofulvin terhadap keracunan rumput signal (*B. decumbens*) telah dikaji di dalam lima puluh tiga ekor biri-biri jantan kacukan Wiltshire dan Indigenous Malaysia. Dua puluh enam ekor biri-biri digunakan didalam kajian phenobarbitone dan dua puluh tujuh ekor biri-biri digunakan di dalam kajian griseofulvin. Rumput signal telah menurunkan paras sitokrom P-450 dan merencatkan aktiviti enzim metabolik melalui enzim. aminopyrine-N-demethylase, aniline-4-hydroxylase, UDP glucuronyltransferase and glutathione-S-transferase secara signifikan di dalam hati dan buah pinggang biri-biri. Kepekatan sitokrom P-450 ditentukan mengikut kaedah Omura dan Sato (1964a) seperti yang dinyatakan oleh Mazel (1971). Aktiviti enzim aminopyrine-N-demethylase dan aniline-4-hydroxylase ditentukan menggunakan kaedah Mazel, 1971 dengan menganggarkan kepekatan formaldehyde dan ρ-aminophenol. Kaedah Dutton and Storey (1962) digunakan untuk menentukan aktiviti enzim UDP-

glucuronyltransferase dengan menganggarkan kepekatan o-aminophenylglucuronide dimana o-aminophenol digunakan sebagai substrat Aktiviti enzim glutathione-S-transferase ditentukan di dalam fraksi sitosolik dengan menganggarkan kepekatan 2,4-dinitrophenylglutathione mengikut kaedah Habig *et al* (1974). Pendedahan secara oral phenobarbitone (30 mg/kg berat badan) selama lima hari setiap dua minggu telah meningkatkan aktiviti enzim metabolisma di dalam hati dan ginjal biri-biri. Walaupun peningkatan aktiviti enzim yang berlaku di dalam biri-biri yang memakan *B. decumbens* lebih rendah berbanding dengan biri-biri yang diberi phenobarbitone sahaja, tetapi peningkatan aktiviti enzim ini dapat menyediakan ketahanan kepada biri-biri terhadap keracunan rumput signal (*B. decumbens*). Ini dibuktikan dengan kelewatan munculnya tanda-tanda keracunan berbanding dengan biri-biri yang hanya memakan *B. decumbens* sahaja. Penggunaan griseofulvin (5 mg/kg berat badan) hanya mampu meningkatkan aktiviti enzim metabolisma fasa II sahaja (UDP-glucuronyltransferase and glutathione-S-transferase) di dalam hati dan ginjal biri-biri. Griseofulvin menyediakan ketahanan yang kurang berkesan berbanding dengan phenobarbitone apabila terbukti dimana lima daripada tujuh ekor biri-biri telah menunjukkan tanda-tanda keracunan rumput signal. Dengan itu kajian ini telah menunjukkan bahawa peningkatan isoenzim sitokrom jenis phenobarbitone boleh meningkatkan ketahanan biri-biri terhadap keracunan *B. decumbens* dan kekurangan potensi menyebabkan griseofulvin tidak digalakkan penggunaannya sebagai agen perangsang enzim untuk mengurangkan kesan keracunan rumput signal di dalam biri-biri.

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I certify that an Examination Committee met on 26th February 2001 to conduct the final examination of Hasiah bt Ab Hamid on her Master thesis entitled "Effect of Phenobarbitone and Griseofulvin Against Signal Grass (Brachiaria decumbens) Toxicity in Sheep" accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Member of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master Science.

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Date: 14 JUN 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been fully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

HASIAH BT AB HAMID

Date: 18th April 2001

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LIST OF ABBREVIATION/GLOSSARY OF TERMS

mM	millimolar
M	molar
µl	microlitre
µ mole	micromole
µg	microgram
N	normal
nm	nanometre
w/v	weight per volume
APS	adenosine-5'-phosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
DHA	dehydrogenase acid
MARDI	Malaysian Agriculture Research Development Institute
MFO	mixed function oxidases
NADPH	nicotinamide adenine dinucleotide phosphate
PAP- 3',5'	phosphoadenosine
PAPS	phosphoadenosine-5'-phosposulphate
P-P_i	pyrophosphate
UDP	uridine diphosphate
UDPGA	uridine diphosphoglucuronide acid

CHAPTER 1

INTRODUCTION

The rearing of sheep especially on small farm holdings in tropical and subtropical countries play an important role in the production of meat (Abas Mazni and Sharif, 1987). There is a considerable increase in the production of this animal in Malaysia. Availability of suitable pasture is a prerequisite towards achieving this goal. Signal grass (*B. decumbens*) is an important source of fodder for livestock in Malaysia, because of its high nutritive value and high resistance towards pest and drought (Loch, 1977). Unfortunately, this grass is highly toxic to sheep as it causes liver and kidney damage (Salam Abdullah *et al.*, 1988; Salam Abdullah and Rajion, 1990). At terminal stages sheep develop anorexia and they die a few weeks after continuous grazing on this grass. The grass is not toxic *per se* but certain compounds, which as a result of ruminal activities of the sheep were converted to their derivatives (episarsasapogenin and epismilagenin) responsible for causing toxicity.

Attempts to prevent the toxicity to this grass by adding zinc sulphate to the drinking water were not very successful (Salam Abdullah *et al.*, 1994). The mechanism of protection by zinc from the toxic effect of *B. decumbens* may be due to the binding of toxic compounds, episarsasapogenin and epismilagenin,

with zinc in the rumen of sheep, perhaps in a way similar to the binding of zinc to saponin (Price *et al.*, 1987).

Chemoprotection or chemoprevention is an emerging strategy for disease prevention and treatment (Eaton and Groopman, 1994). Chemoprotection is now known as an effective approach for cancer prevention in human, particularly against aflatoxin induced liver cancer (Hong *et al.*, 1990; Eaton and Groopman, 1994). For instance, administration of oltipraz, a schistosomicidal drug, was found to induce the activity of glutathione-S-transferase and several other drug-metabolizing enzymes, which are involved, either directly or indirectly, in the detoxification of carcinogens (Roebuck *et al.*, 1991). The possibility that increases in these enzymes may lead to enhanced detoxification of carcinogens and other toxic substances provided the evidence to predict excellent chemoprotection following the use of drug-metabolizing enzymes inducers. Many studies showed that phenobarbitone (Cook and Wilson, 1970; Shetty *et al.*, 1972; Ford *et al.*, 1976 and Swick *et al.*, 1983) and griseofulvin (Olantunde *et al.*, 1999) have a potential to induce drug-metabolizing enzymes and can increase the effectiveness of xenobiotic detoxification in human and animal.

The objectives of this study were to investigate the following:-

1. To determine the protective effect of phenobarbitone and griseofulvin in *B. decumbens* toxicity by inducing drug metabolizing enzyme activities in sheep.

2. To compare the effectiveness of phenobarbitone and griseofulvin in inducing drug metabolizing enzymes activities for the protection of *B. decumbens* toxicity in sheep.

CHAPTER 2

LITERATURE REVIEW

2.1 Signal Grass (*Brachiaria decumbens*)

Brachiaria decumbens (Signal grass) (plate 1 – see APPENDIX C-1) is an important source of fodder for livestock production in Malaysia. This grass is widely grown in ruminant livestock farms not only in Malaysia but also in countries such as South America, Australia, Indonesia, Papua New Guinea and Africa (Loch, 1977). This high yielding stoloniferous grass can adapt to a wide range of well-drained soils. Grows well even with substandard management and during drought. Research conducted locally has shown that *Brachiaria decumbens* is well adapted the local conditions and give impressive yields of both green and dry matter (Table 2.1).

Table 2.1: Average dry matter yield of various fodder cut at six weeks.

Fodder	Average dry matter yield (ton/hectare/year)
Signal grass	25.5
Para grass	12.1
Guinea grass	18.6
Napier grass	23.2
Splendid grass	17.6
Kazungula grass	18.2

(Adapted from A-Z on Pasture and Fodder. Department of Veterinary Service Malaysia, 1991).

High productivity, tolerance towards low fertility conditions, drought resistance and relative freedom from pest and diseases account for the current interest. Apparently, *B. decumbens* could provide all the forage requirements of ruminant in the tropics and this has helped to promote the growth of this livestock sector. However, this interest has been short lived because numerous reports have shown that *B. decumbens* is hepatotoxic to sheep and goats (Abas Mazni *et al.*, 1983; Salam Abdullah *et al.,* 1987). Research should be conducted to determine methods to prevent and minimize the adverse effect.

2.1.1 *Brachiaria decumbens* Toxicity

Toxic signs developed in sheep as early as two weeks after grazing on *B. decumbens* pasture (Salam Abdullah, 1990) and usually died four weeks after grazing this grass. The affected sheep exhibited signs of jaundice, emaciation, photosensitization (plate 2 – see APPENDIX C-1) and oedema of the ears, submandibular area and eyelids in intoxicated sheep. In severe cases, exudation and necrosis occurred in the affected parts. All carcasses showed ulcerations on the skin particularly around the eyelids, lips, muzzle, ears (plate 3 – see APPENDIX C-2) and vulva indicating photosensitization.

Necropsy revealed varying degrees of jaundice of the subcutaneous, omental and mesentric fat including serous and mucous membranes (plate 4 – see APPENDIX

C-2). The livers were enlarged and firm, mottled and icteric with distended gall bladders. Liver pathology was further confirmed by presence of necropsy where evidence of liver changes was shown. The livers were consistently slightly enlarged and containing marked thickened bile. The animals with haemolytic anaemia, and the kidneys were darker than normal. Most of which showed acute hepatonecrosis. Cholangitis and mild bile retention were also observed. In animals with haemoglobinuria, a few tubules in the kidney were seen with haemoglobin casts which probably due to haemolysis (Salam Abdullah, *et al.*, 1990)

The neurological dysfunction corresponds with the pathological changes observed in the brain, particularly in the white matter (Salam Abdullah *et al.*, 1989). *B. decumbens* toxicity also affects reticulo-rumen motility and microbial activity of sheep (Salam Abdullah *et al.*, 1990). Ruminal stasis occurred within 3 weeks of grazing on this grass and the toxicity also produced changes in the rumen microbial population and the levels of volatile fatty acids of affected sheep.

2.1.2 Clinical Biochemistry

Salam Abdullah *et al.*, (1994) found that *B. decumbens* toxicity increased plasma icterus index almost 40 folds during the 8 weeks period of the study in sheep. This increase coincided with appearance of jaundice. Other significant changes in the serum biochemistry of the affected sheep included increased total bilirubin levels,

blood urea nitrogen (BUN) creatinine, activities of the enzymes such as aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH) and gamma glutamyltransferase (GGT).

2.1.3 Toxic Compound

It has been reported that the ethanolic extract of rumen liquor from *B. decumbens* intoxicated sheep contain a hepatotoxic substance (s) causing marked enlargement of the liver and severe necrosis of hepatocytes of rats (Salam Abdullah, 1987). The infusion of rumen liquor from *B. decumbens* intoxicated sheep into the rumen of cattle caused hepatic and renal dysfunction where as the grass itself when fed directly to cattle did not produce toxic symptoms (Nordin *et al.*, 1989). These observations suggested strongly that the grass is not toxic *per se* but certain compounds, which as a result of ruminal activities of the sheep were converted to their derivatives responsible for causing the toxicity.

Spectroscopic examination of purified extracts of the above rumen liquors revealed the presence of a mixture of sapogenin, 3- spirostanols (Salam Abdullah *et al.*, 1992) and later were identified as episarsasapogenin and epismilagenin (Figure 1-APPENDIX A-1) by Nordin Lajis *et al.*, (1993). The same compounds were also isolated by a group of scientist in New Zealand (Miles *et al.*, 1993) from the bile of