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Research Article

Liver histology of female rat (*Rattus norvegicus*) fed diet containing Calliandra leaf and Pineapple peel during gestation

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ABSTRACT

Calliandra calothyrsus leaf contains 17-28% protein and very potential as protein source on animal diet. However, Calliandra also contain a quite high antinutrition of condensed tannins. The addition of protease enzymes (bromelain) in the diet containing tannins was expected to overcome the negative effects of tannins. Bromelain can be obtained from the pineapple plant including the peel. This study used a completely randomized design of 4x4 factorial design. The main factor was Calliandra leaf meal substitution levels of 0, 10, 17.5 and 25% in the diet. The sub factor was pineapple peel additive levels of 0, 4.35, 8.70 and 13.05 g/rat/day (body weight 200 g). Pregnant rats were divided into 16 treatment groups and given the diet during gestation. After the birth, the mothers were sacrificed and their liver were taken to make histological preparation (paraffin method, Hematoxylin and Eosin staining). Data were statistically analyzed using SPSS. Results showed there was a significant interaction ($P<0.05$) between Calliandra leaves and pineapple peels on sinusoids congestion and nucleus pyknosis of hepatocyte cells. The pyknotic nuclei of hepatocyte cells had a linear increase with increase in the level of dietary calliandra leaf meal. Sinusoids congestion increased significantly only at the highest level (25%) of Calliandra in the diet. Although there was addition of pineapple peel containing bromelain protease, Calliandra leaf up to 25% in the diet still affected rat liver histology adversely when given during gestation.

Keywords: Calliandra leaf, pineapple peel, rat, gestation, liver histology.

INTRODUCTION

Calliandra calothyrsus (17-28% of protein content), was a legume plant that adaptable, pest-resistant and easy to develop in a variety of soil and environmental conditions[1]. Calliandra was relatively abundant in the highlands of Tabanan and Singaraja[2]. Although in the region maintained a lot of non-ruminant i.e. rabbits, pigs and chickens, farmers do not use Calliandra as feed source for those animals. Calliandra also contain a quite high antinutrition of condensed tannins.

Tannins are polyphenolic polymers (relatively high molecular weight), which are widely distributed in trees, shrubs, legumes, cereals and grains. Tannins were considered as antinutritional compounds due to their adverse effects on intake and animal performance[3]. Tannin-rich feed can decrease protein digestibility because tannins can inhibit the activity of trypsin and other digestive enzymes in the intestine of mice and chickens[4]. Dietary sorghum tannins

markedly reduce the digestibility of crude protein and starch in caged White Leghorn layers. The absorption of minerals i.e. calcium, magnesium, iron, sodium and potassium is also adversely affected[5].

Besides, tannins naturally contain polyphenols, which have been considered to play a vital antioxidant role as dietary antioxidants in the prevention of oxidative damage[6]. Due to its antioxidant activities, polyphenols could effectively repair the injury and improve the pathological changes, may be attributed at least in part to the inhibition of lipid peroxidation, the suppression of inflammatory reactions, the induction of apoptosis in hepatocytes[7].

Anti-nutritional effects of tannins can be reduced with many treatments, latest study used herbivores host tannin-degrading bacteria (TDB) to overcome the toxic challenges posed by plant tannins. Lab rats given TDB showed higher maintenance of body mass and lower indicators of liver damage compared with control animals. Thus, TDB significantly increase host performance on tannin-rich diets[8].

Our study tried to reduce anti-nutritional effect of Calliandra tannin by added a protease (enzyme bromelain), obtained from the waste of pineapple peel. These wastes are produced in the market or traders of fruit in Bali, and during this time the waste is simply dumped. Bromelain enzymes could be obtained up to 0,05-0,075% from pineapple peel[9]. The use of plants and agricultural wastes was expected to reduce the use of commercial feed, thus reduced production costs.

We also tried to find out if combination of mixed calliandra leaf, pineapple peel wastes and concentrates could reduce adverse effect of Calliandra tannin when given to pregnant rats. The role of nutrition is vital for pregnant mother. Poor nutrition can reduce the rate of ovulation, low conception rates, high loss of the embryo and fetus, reducing the length of postpartum anestrus, lack of milk, the high perinatal mortality and low performance of a newborn child.

MATERIALS AND METHODS

Levels of calliandra leaves and pineapple peel

Leaves of *Calliandra calothyrsus*, diameter <5 mm, were hand-picked from the trees that grow in Baturiti area, Tabanan, Bali. Calliandra leaf were wind-dried up to a constant weight, blended then sifted into powder. Pineapple (*Ananas comosus*) peel waste were fresh collected from Badung market, Denpasar, Bali, then blended and stored in refrigerator to keep the content of bromelain (protease) enzyme. This study used pig complete feed (18-20% of protein), which was processed into powder.

Calliandra leaf meal in this study contained 7.43 g/ 100 g of condensed tannins and 23.24 g/ 100 g of protein. Fresh pineapple peel contained 0.058 g/ 100 g of bromelain enzyme, and the obtained LD_{50} was 29 g. This study used a completely randomized design of 4x4 factorial design. The main factor was Calliandra leaf meal substitution levels of 0, 10, 17.5 and 25% in the diet. The sub factor was pineapple peel additive levels of 0, 4.35, 8.70 and 13.05 g/rat/day (Table 1). Pregnant rats were divided into 16 treatments of different diet which were given during gestation period (Table 2).

Feed processing

Percentage of Calliandra leaf meal in the feed, was calculated as substitution of concentrates. CMC (carboxymetyl cellulose, feed binder) was added 2% of the total concentrate and Calliandra leaf meal. Fresh pineapple peel was mashed without added water. The feed was homogenized with a mixer and processed in a pelleting machine. Pellets were dried with freeze dryer (8 hours) then stored in a refrigerator to keep condensed tannins and enzyme bromelain levels.

Experimental procedure

Female albino rats (*Rattus norvegicus*), 3 months old, 180-200 g weight, were acclimatized in the laboratory. Animals were housed in plastic cages and placed on a reverse light cycle of 12D:12L, at 26^0C temperature and 40% relative humidity. Standart diets and water were given ad libitum. Estrous cycle was determined by vaginal smear and Giemsa staining method. Estrous female rats were put in the same cage with a male rat (3:1) in the evening. If plug or sperm was found in vagina in the next morning, the next day will be determined as day 1 of gestation[10].

Forty eight pregnant rats were randomly divided into 16 groups of different diet, treatments were given during the gestation period. At the end of the treatment, animals were sacrificed by intramuscular ketamine injection and dissected to collect organs. Liver preparation used paraffin method and Hematoxylin-Eosin staining. Histological observation used an electric microscope (Olympus, magnification of 400x). All procedures were approved by the Animal Research Center Committee, Faculty of Veterinary, Udayana University, Denpasar, Bali.

Data analysis

Data were analyzed using SPSS for Windows version 20. If the data are normally distributed with homogeneous variance, the data were analyzed using One Way Anova (p=0.05), and if there is a significant

difference then continued with Duncan Multiple Range Test. If the data is not normally distributed, it will be analyzed with non parametric Kruskal Wallis test, and if there is a significant difference will be followed by Mann-Whitney U test.

RESULTS AND DISCUSSION

Results showed there was no interaction between tannin (T) of Calliandra leaves and bromelain (B) or protease enzyme of pineapple peels on hepatocyte fatty degeneration and necrosis, and also on inflammatory cells infiltration. Diets containing Calliandra leaves or pineapple peels or combination of both, did not significantly cause any damage in the form of fatty degeneration and necrosis, and inflammatory cells infiltration (Tabel 3).

On the other hand, there was a significant interaction ($P<0.05$) between Calliandra leaves and pineapple peels on sinusoids congestion and pyknotic nuclei of hepatocyte cells. Calliandra level in the diet increased the pyknotic nuclei of hepatocyte cells. However, sinusoids congestion increased significantly only at the highest level (25%) of Calliandra in the diet. Pineapple peel levels did not significantly affect liver damage in this study (Table 4).

The hepatic tissues in control group (diet B0T0) showed a normal structure or few cellular structural changes (Figure 1.A). Normal hepatocytes cells were polygonal, oval-shaped nuclei, and cytoplasm with organelles[11]. Normal hepatic sinusoids in control group usually had persisted their regular organization[12]. In contrast, the hepatocytes of liver from group treated with fed containing different levels of calliandra leaf meal and pineapple peel showed histological changes i.e. small darker nuclei (pyknotic nuclei) with irregular nuclear membrane as the chromatin condensed (Figures 1.B, 1.C and 1.E). There were also accumulation of inflammatory cells around the blood vessels i.e. central vein and hepatic sinusoids (Figures 1.C, 1.D and 1.F). The hepatic tissue showed numerous spots of densely collected inflammatory cells composed mainly of macrophages and lymphocytes at the center and a number of fibrocytes at the periphery[11]. This indicating the presence of inflammation in the area of hepatic tissue.

Tannins have been associated with fatal liver damage from extensive use on burns or in enemas in human. Previous research showed that calliandra leaf extract up to dose 6 mg/kg bw significantly affected the damage of male mice liver i.e. nucleus pyknotic, fatty degeneration, inflammatory cells infiltration, and sinusoid congestion of the liver[13]. On the other hand, diets containing Calliandra leaf in this research, did not significantly cause any damage in the form of fatty degeneration and necrosis, and inflammatory cells infiltration.

Calliandra leaf meal in this study contained 23,24% of crude protein, 1.50% of total tannin and 7,43% of condensed tannin. The content of condensed tannin in each level of 10, 17.5 and 25% of Calliandra leaf meal in the diets were 0.74, 1.30 and 1.86%, respectively[14]. Calliandra leaf extract may contain higher level of bioactive compounds i.e. condensed tannin, which could affected the liver. Therefore, the effect on liver histology showed more pathological changes or damage.

In the liver, there is a variety of important processes such as energy storage process, the formation of proteins, regulation of cholesterol metabolism and neutralization of toxins or drugs into the body. When materials containing toxins or poisons entering the liver, the liver will work very hard to neutralize it. This causes the liver is exposed to toxins so that it becomes easily damaged. Liver damage can occur in the form of structural damage or impaired liver function[12].

Toxic substances that affect the liver in a long time would cause necrosis, which begins with changes in the morphology of the cell nucleus (pyknotic nuclei). The next phase, the nucleus rupture (karyorrhexis) then disappears (karyolysis). Pyknotic nuclei can occur because of damage in the cell, such as membrane damage followed by damage to the mitochondria and Golgi apparatus so that the cells are not able to eliminate the water and triglycerides that accumulate in the cytoplasm of cells[15].

Molecular weights and their chemical structures are the primary factors determining the influence of condensed tannins on animal nutrition. The impact was more pronounced for the condensed tannin fraction with a higher molecular weights[16]. In this study, diet containing calliandra leaf and pineapple peel changed liver histology of female rat if given during gestation. The expected antioxidant properties of Calliandra and bromelain protease of pineapple peel still could not overcome the anti-nutritional effect of condensed tannin on animal nutrition.

CONCLUSION

In this study, diet containing calliandra leaf and pineapple peel given during gestation changed rat liver histology by the increase of pyknotic nuclei of hepatocyte cells and sinusoid congestion.

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Table 1
Level of calliandra leaf and pineapple peel

Level	Calliandra leaf meal (%)	Condensed tannin (g/100 g)	Level	Pineapple peel juice (g/rat/day)	Protease bromelain (mg)
T_0	0 (control)	0	B_0	0 (control)	0
T_1	10	0.743	B_1	4.35	2.5
T_2	17.5	1.30	B_2	8.70	5
T_3	25	1.858	B_3	13.05	7.5

T= tannin calliandra, B= bromelain enzyme of pineapple

Table 2
Diet composition

Diet 1 PK, T_0, B_0	**Diet 2** PK, T_1, B_0	**Diet 3** PK, T_2, B_0	**Diet 4** PK, T_3, B_0
Diet 5 PK, T_0, B_1	**Diet 6** PK, T_0, B_2	**Diet 7** PK, T_0, B_3	**Diet 8** PK, T_1, B_1
Diet 9 PK, T_2, B_1	**Diet 10** PK, T_3, B_1	**Diet 11** PK, T_1, B_2	**Diet 12** PK, T_2, B_2
Diet 13 PK, T_3, B_2	**Diet 14** PK, T_1, B_3	**Diet 15** PK, T_2, B_3	**Diet 16** PK, T_3, B_3

PK= standard commercial feed, T = calliandra leaf meal, B = fresh pineapple peel.

Table 3
Rat Hepatic Histopathology

		B0	B1	B2	B3	Average
Sinusoids congestion	T0	5.0 ± 0 a	10.3 ± 9.79 ab	21.5 ± 13.55 c	5.5 ± 3.37 a	10.58 ± 1.45 A
	T1	6.6 ± 4.42 a	20.0 ± 7.82 c	20.0 ± 8.82 c	6.5 ± 5.15 a	13.28 ± 1.45 AB
	T2	4.5 ± 2.17 a	18.3 ± 14.36 bc	9.8 ± 10.81 ab	8.5 ± 4.74 a	10.28 ± 1.45 A
	T3	21.5 ± 8.83 c	5.7 ± 2.45 a	20.8 ± 18.52 c	20.5 ± 9.84 c	17.13 ± 1.45 B
Average		9.40 ± 1.45 A	13.58 ± 1.45 A	18.03 ± 1.45 B	10.25 ± 1.45 A	
Pyknotic nuclei	T0	2.4 ± 0.89 ab	1.6 ± 0.89 ab	7.2 ± 0.89 d	4.2 ± 0.89 bc	3.85 ± 0.44 A
	T1	2.1 ± 0.89 ab	4.1 ± 0.89 bc	0.4 ± 0.89 a	2.2 ± 0.89 ab	2.20 ± 0.44 B
	T2	0.8 ± 0.89 a	6.0 ± 0.89 cd	0.5 ± 0.89 a	2.8 ± 0.89 ab	2.53 ± 0.44 B
	T3	1.5 ± 0.89 ab	1.1 ± 0.89 a	1.8 ± 0.89 ab	2.3 ± 0.89 ab	1.68 ± 0.44 B
Average		1.70 ± 0.44 A	3.20 ± 0.44 B	2.48 ± 0.44 AB	2.88 ± 0.44 AB	

Values with the same lowercase letters in the same row are not significantly different. Values with the same capital letter in same row or column are not significantly different (P>0.05). Interaction of Calliandra leaf (T) and pineapple bromelain (B) was significant at P<0.05.

Table 4
Rat Hepatic Histopathology

		Fatty degeneration	Inflammatory cells infiltration	Necrosis
Tannin	T0	3.00 ± 0.31 ab	7.15 ± 1.22 a	2.58 ± 0.13 a
	T1	2.38 ± 0.31 a	7.03 ± 1.22 a	2.58 ± 0.13 a
	T2	3.38 ± 0.31 b	7.35 ± 1.22 a	2.85 ± 0.13 a
	T3	2.50 ± 0.31 ab	8.73 ± 1.22 a	2.63 ± 0.13 a
Bromelain	B0	3.15 ± 0.31 a	8.75 ± 1.22 a	2.58 ± 0.13 a
	B1	2.30 ± 0.31 a	5.68 ± 1.22 a	2.78 ± 0.13 a
	B2	2.83 ± 0.31 a	8.23 ± 1.22 a	2.63 ± 0.13 a
	B3	2.98 ± 0.31 a	7.60 ± 1.22 a	2.65 ± 0.13 a

Values with the same letter in the same column indicate no significant difference (P>0.05). Interaction of Calliandra leaf (T) and pineapple bromelain (B) are not significant (P>0.05).

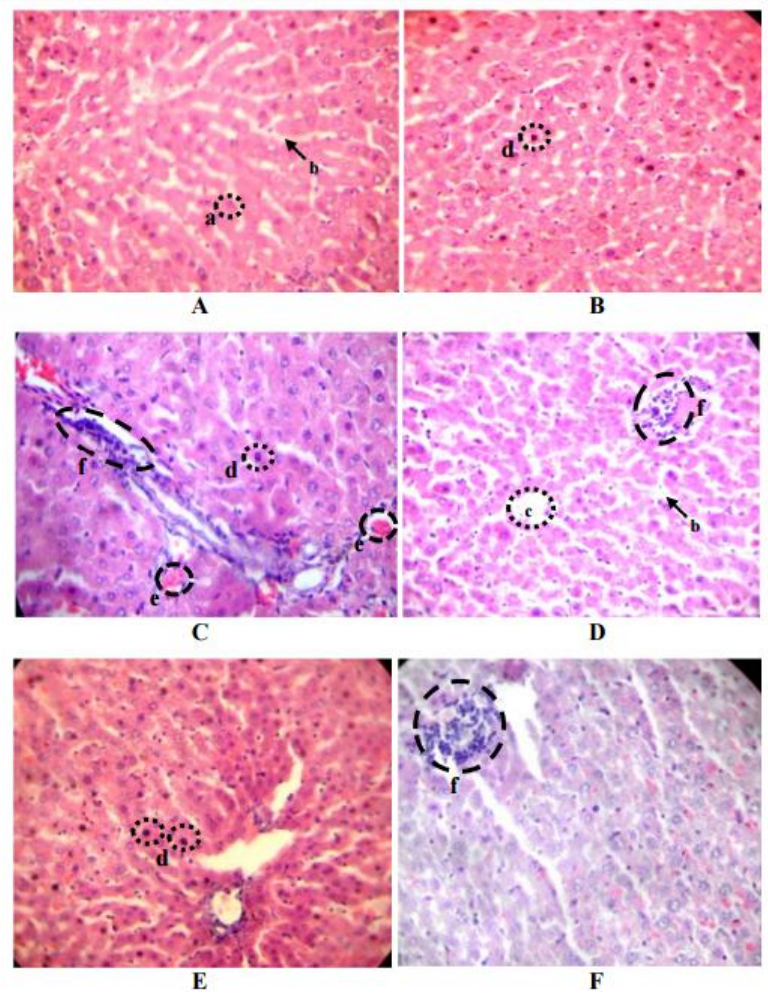

Figure 1.
Liver histology of rat fed diet containing calliandra leaf and pineapple peel (Paraffin method and Hematoxylin-Eosin staining, magnification 400x)

A. Control group (diet B0T0), B. Diet B0T2, C. Diet B0T3, D. Diet B3T0, E. Diet B1T3, F. Diet B3T2 (T= calliandra leaf meal, B= pineapple peel)

a. Hepatocyte cells, b. Sinusoids, c. Central vein, d. Pyknotic nuclei of hepatocytes cells, e. Congestion of blood vessels and sinusoids, f. Inflammatory cells infiltration.

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