THE POSSIBILITY OF TOXIC COMPOUNDS PRESENT IN *ACACIA VILLOSA*

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ABSTRACT

Acacia villosa is a leguminous tree in the family of Mimosoideae. It was introduced to Indonesia in 1920 from Curacao (West Indies) and now widely grows in Indonesia especially in the buffering zone of forest area. *Acacia villosa* may be potential as a protein source as its protein content is quite high (22-28%). However, addition of different extracts from *Acacia villosa* to *in vitro* fermentation showed a significant inhibition of these extracts to dry matter digestibility of elephant grass and protein digestibility of casein. Methanol extract gave the highest inhibition compared to 50% methanol or water extracts. The inhibition was greater to fibre than protein digestibility and more significant to rumen microbes than to microbial enzyme. The *in vivo* test on sheep without adaptation period showed 75% mortality in a week. There were several anti nutritive compounds measured in *Acacia villosa* but suspected one or more toxic compounds which are polar compounds need to be clarified and identified.

(Key Words: Toxic Compound, *Acacia Villosa*).

KEMUNGKINAN ADANYA SENYAWA RACUN DALAM *ACACIA VILLOSA*

INTISARI

Acacia villosa atau lamtoro merah termasuk leguminosa pohon/ semak dalam keluarga Mimosoideae. Tanaman ini masuk ke Indonesia tahun 1920 dari Curacao ("West Indies") dan sekarang banyak ditemui pada daerah penyangga hutan. Dengan kadar protein yang cukup tinggi (22-28%), *Acacia villosa* sangat berpotensi menjadi sumber protein bagi ternak ruminansia. Tetapi penambahan ekstrak dari *Acacia villosa* ke dalam fermentasi *in vitro* menunjukkan terjadinya penghambatan oleh ekstrak ini terhadap kecernaan bahan kering rumput gajah dan kecernaan protein casein. Ekstrak metanol memberikan penghambatan terbesar dibanding ekstrak 50% metanol atau air. Serat lebih terhambat dari protein. Penghambatan terhadap mikroba rumen lebih besar dari pada terhadap enzim mikroba. Uji *in vivo Acacia villosa* terhadap domba dilakukan tanpa masa adaptasi dan menunjukkan terjadinya kematian yang tinggi (75%) dalam satu minggu. Ada beberapa senyawa anti nutrisi yang sudah diukur tetapi senyawa yang diduga racun masih harus diidentifikasi lebih lanjut.

(Kata Kunci: Senyawa Racun, *Acacia villosa*).

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Introduction

Alternatives leguminous tree beside Leucaena have always been looked for as a protein source for livestock animals in rural areas especially in dry land area. There are a lot of different types of Acacia in the world and *Acacia villosa* is one of them which grows widely in Indonesia.

Acacia villosa is a leguminous tree belongs to a family of Mimosoideae. It was introduced to Indonesia by Verluijs (forestry Director) from Curacao (West Indies) in 1920 (Backer and van den Brink Jr, 1963). Now, it is widely found in the buffering zones of forest area in Java or Sumatra. It was planted for reforestation and as fire wood for the people in the forest surrounding areas.

Acacia villosa is a small tree (shrub) with highly branches up to 4 m height and has white flower. In Kupang, West Timor, it is called "red leucaena" as the tree resembles to Leucaena except the branches sometimes have red colour. According to Lowry *et al.* (1992), the plant is almost unknown elsewhere. It grows well in dry land area such as Africa or Australia. It produces a lot of seeds and multiplies very quickly, therefore it is very suitable for reforestation. There are hundreds different types of Acacia. There are reports of feeding experiments conducted in Africa on *A.angustissima* leaves (Osuji and Odenyo, 1997), on *A.boliviana* (Maasdorp *et al.*, 1999) and on *A.cyanophylla* (Ben Salem *et al.*, 1999). In Israel, there was an experiment on the utilization of *A.senegal* and *A.saligna* (Degen *et al.*, 1998). There was also report on the utilization of dry fruits *of A. albida*, *A.sieberiana* and *A.tortilis* (Osuji & Odenyo, 1997; Tanner *et al.*, 1990).

Like other legumes, *A.villosa* contains high protein level (Lowry *et al.*, 1992) and several secondary compounds. Some secondary compounds may be toxic to both microbe and/ or host animals (Woodward and Reed, 1989). For example: mimosine, a non-protein amino acid in *Mimosa pudica* and *Leucaena leucocephala* caused hair-loss or wool in Australia but not in Indonesia. In this experiment, the effect of secondary component in *A.villosa* to the rumen microbes and host animal was studied.

Material and Methods

Material

Leaf samples of *Acacia villosa* were collected from the farm station of Research Institute for Animal Production, Ciawi-Bogor which located at 400m above sea level. The leaves were freeze-dried and milled.

Methods

Several analyses were carried out to measure several secondary compounds in *Acacia villosa*. Total phenol analysis using Folin-Denis method (Swain &Hillis, 1959) and tannin analysis using Vanillin-HCl method (Broadhurst & Jones, 1978) were conducted on 3 different extracts (Methanol, 50% methanol and water extracts) of *A.villosa*. Saponin analysis by thin layer chormatography (Gestetner *et al.*, 1966) and non-protein amino acid analysis by high performance liquid chromatography (Wina *et al.*, 1993a) were also done. Qualitative test for cyanide compound was also conducted.

Preparation of plant extracts

A successive extraction was conducted to get different plant extracts. A sample of fresh *A.villosa* leaves (10 g dry matter) was blended in waring blender for 2-3 minutes successively by methanol, 50% methanol and distilled water. After methanol extraction and centrifugation, supernatant was separated and residue was then extracted by shaking it with the next solvent for 30 minutes. All fractions were evaporated by rotary evaporator until dry and redissolved in 20 ml of water. There were 3 extract fractions called methanol (MeOH), 50% methanol (50% MeOH) and water (H_2O) extracts.

Total phenol analysis

One milliliter of a sample aliquot (Methanol and 50% methanol fractions) diluted to 7 ml with distilled water followed by addition of 0.5 ml of Folin-Denis reagent. The mixture was made up to 10 ml with water. After 30 minutes of reation, the mixture was centrifuged at 3000 rpm for 15 minutes. The absorbance of the supernatant was recorded at 725 nm wavelength. Standard solution from 0 to 100 ppm were prepared from para coumaric acid in methanol.

Tannin analysis

Half of millilitre of a sample aliquot (Methanol, 50% methanol and water fractions) was reacted with 3 ml concentrated hydrochloric acid and 1.5 ml of 4% vanillin in methanol solution. The absorbance was recorded at 500nm after 20 minutes reaction. The blank sample was made by reacting 0.5 ml aliquot of sample with 3 ml of concentrated HCl and 1.5 ml of methanol. The standard solution from 0 to 250 ppm were prepared from catechin in 25% methanol.

Saponin analysis

Half a gram of free-fat sample was extracted with 15 ml absolute methanol for 24 hours using a shaker. Then, it was centrifuged for 15 minutes at 3000 rpm. The supernatant was separated and 5 ml of the supernatant was evaporated and redissolved in 1ml of methanol. This solution was put through a Sep-pak (Silica cartridges, Waters Ass.) and washed with hexane, chloroform and then the saponin eluted with 5 ml methanol. All methanol was evaporated and the residue redissolved in 1 ml methanol. Silica Gel 60 tlc plate was divided into half by drawing a line in the middle of the plate. The left side of plate have standard solution (saponin white, 1 mg/ml) spotted in a series of volume from 200 to 1000ul and a spot of sample solution (200ul). The right side of plate have one spot of standard solution and one spot of sample solution. The plate was eluted in a mixture of ethanol : n-butanol : ammonia (2:7:5) for 6 hours. When elution finished, the plate was air dried and cut into two. The right side of plate was used for qualitative analysis by spraying with a mixture of sulfuric acid : glacial acetic acid : 4-methoxy benzaldehyde (1:50:1). The plated was left air-dried and then, it was placed in the oven for 10 minutes at 105°C. The color appeared greenish blue for saponin and recorded the Rf values. The left side of plate was used for quantitative analysis. Exactly the same Rf values, the bands were circled by pencil and scrapped from the plate. The scrappings were extracted twice with methanol in an ultrasonic bath, then centrifuged. The supernatant was separated and evaporated till dry. Two ml of concentrated sulfuric acid was added and left to stand in the cool room for 22 hours. Three ml of glacial acetic acid was added and orange color would appeared and its absorbance was recorded at 530 nm wavelength.

Non-protein amino acid analysis

Fresh leaves of 2.5 g were extracted with 20 ml of 5% tricholoacetic acid in 50% isopropanol in a blender for 2 minutes. For free-fat dried samples, 0.3 g of the sample was extracted with 8 ml of the above solution. Then, it was transferred to a 100ml plastic tube and shaken for 30 minutes. The solution was left at room temperature overnight. The next day, it was centrifuged at 3000rpm for 15 minutes and the supernatant was separated. Two ml of the solution was put into a chromatography column made from a plastic tip containing 2.5 ml of resin (Dowex AG 50 W-X4, 100-200 mesh H+). Then, 8 ml of distilled water was added to wash the column, followed by 8 ml of 2N ammonia solution as an eluent. One milliliter of this solution in a tube was placed in a rotary evaporator to eliminate the amonia which was checked with pH paper (pH8-10). This solution was ready to be spotted on the thin layer plate (20ul) or injected into the HPLC column. The solution before injecting in HPLC was diluted 10 times and filtered through a milipore filter (0.45um). The solution was reacted with OPA solution

(SIGMA product). Ten ul of the solution was injected to C-18 column with a gradient eluent with solution A: buffer with methanol and solution B: 100% methanol. UV detection was used with variable wavelength.

***In vitro* fermentation**

Two substrates, dried-milled elephant grass and casein (0.5 g) were used separately. Strained rumen liquor was taken from sheep fed elephant grass. Different extracts (MeOH, 50% MeOH and H20 extracts) were added into the *in vitro* tube separately (1.0ml). After 48 hours incubation at 39°C, the residue of elephant grass was filtered, dried and weighed. Dry matter digestibility could be calculated from the initial and residue weight of elephant grass. The residue of casein has to be precipitated first by tricholoroacetic acid and then determined its protein content by Kjeldahl. Protein digestibility could be calculated from the protein content of casein and the residue.

***In vivo* experiment**

Feeding trial experiment of *A.villosa* to four sheep was conducted. The sheep has never been exposed to *A. villosa* leaves before. *A.villosa* was given as a total diet without adaptation period and replacing the whole elephant grass without gradual replacement. The offer and residual feed were weighed. The experiment was only lasted for 7 days.

Results and Discussion

Acacia villosa as mentioned before is potential as a protein supplement as the protein content of leaves is high (Table 1). The neutral and acid detergent fibres contents are lower than leaves samples of *C. calothyrsus*, *G. sepium* or *L. leucocephala* analysed at our laboratory and reported by Lowry *et al.* (1992). By this analysis, it could be assumed that *A.villosa* could be easily degraded unless there were some secondary compounds in the leaves that might render the fibre digestion in the rumen.

When different extracts from *A.villosa* were added separately into fermentation tube containing either elephant grass or casein, the most significant reduction was produced when methanol extract was added (Table 2). The reduction of dry matter digestibility of elephant grass was 85.88%, 79.04% and 60.31% caused by MeOH, 50% MeOH and H_2O extracts, respectively. Water extract was added twice volume of methanol extract. This result shows that the compound or compounds that causing reduction in digestibility was mostly found in methanol extract and was still quite polar. MeOH and H_20 extracts gave the same reduction to protein digestibility (only 13.73% reduction). The negative effect of MeOH extract was much less to the protein digestibility than to dry matter digestibility which indicated that majority of casein was still soluble even when the extract was added. The less degradation of casein perhaps was not due to the inhibition of the extract to the rumen microbes but might be due to the complexation of casein to the compounds that existed in MeOH extract and made casein less soluble. The same phenomena has been observed on tannin, an anti nutritive compound, isolated from calliandra bound to casein and made casein less degradable Tannin isolated from calliandra also gave a reduction to the IVDMD of elephant grass but not as drastic as MeOH extract of *A.villosa* (Wina *et al.*, 1993a). Therefore, there must be other compound beside tannin which contributed to the negative effect of MeOH extract in *A.villosa*. Further study showed that increasing volume of methanol extract into the fermentation tube caused more depression to the degradation of elephant grass both by rumen liquor or by pepsin-cellulase (Figure 1). Increasing volume of MeOH extract gave decreasing degradation of elephant grass and addition of 2 ml of MeOH extract to 50 ml of fermentation substrate and rumen liquor caused almost no degradation.

Table 1. Chemical composition of *Acacia villosa* leaves

	%
Crude Protein	26-28
Ether Extract	4-5
Neutral Detergent Fiber	20-28
Acid Detergent Fiber	12-19
Cellulose	7-9
Lignin	5-9
Ash	3-4
P	0.2-0.4
Ca	0.6-0.7

Source: Lowry *et al.* (1992)

Table 2. Effect of different extract of *Acacia villosa* on IVDMD of elephant and protein digestibility of casein

	IVDMD (%) elephant grass	Protein digestibility (%) casein
Blank	48.15	89.91
+ 1 ml MeOH extract	6.80	77.56
+ 1 ml 50% MeOH extract	10.09	82.42
+ 1 ml H_2O extract	19.11*	77.56

* Addition of water extract was 2 ml into *in vitro* fermentation tube

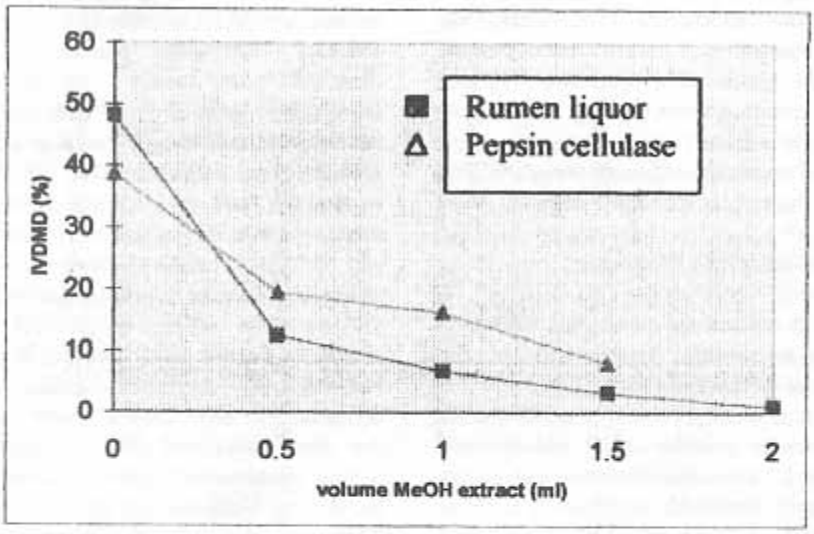

Figure 1. *In vitro* dry matter digestibility of elephant grass affected by volume of methanol extract of *Acacia villosa*.

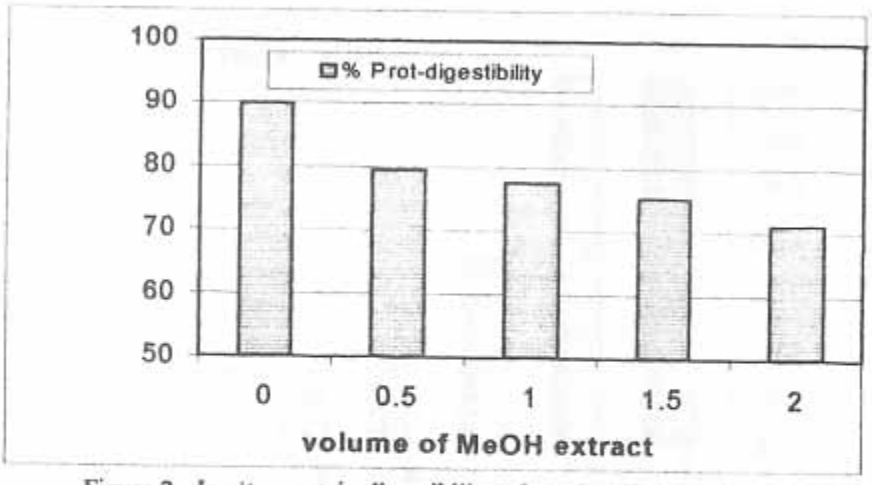

Figure 2. *In vitro* protein digestibility of casein affected by volume of methanol extract of *Acacia villosa.*

The inhibition was bigger to rumen liquor than to pepsin-cellulase fermentation which indicated that compounds in MeOH extract had more negative effect to rumen microbes than to commercial cellulase enzyme preparation. This is strongly supported by the report of Odenyo *et al.* (1997) that 70 % acetone and aqueous extracts from *A.angustisstma* inhibited the growth of most rumen bacteria i.e *R. albus, R. flavefaciens, P. ruminicola, S. ruminantium* and *Streptococcus bovis* which were cellulolytic bacteria. Protein digestibility was also decreasing with the increasing volume of MeOH extract but the reduction was only 20.81%, not as significant as reduction toward the dry matter digestibility (Figure 2).

Feeding fresh *A.villosa* to sheep without adaptation period gave a gradual decrease in the daily consumption (Figure 3). All sheep refused to eat *A.villosa* and at day 5, there was so little consumption of *A.villosa*. This is in contrast to similar experiments done with *Gliricidia sepium* (Wina *et al.* , 1998) or *Calliandra calothyrsus* (Tangendjaja and Wina, 1998). Even with a strong smell, the intake of *Gliricidia sepium* by sheep was increasing gradually and there was no sign of toxicity on sheep fed *G. sepium* (*Wina et al.*, 1998). Feeding fresh Calliandra to Merino sheep without adaptation period gave an increase of Calliandra consumption and no symptoms of toxicity (Tangendjaja and Wina, 1999). But in this experiment, three out of four sheep died. There might be an acute toxicity which caused haemorraghic to all organs, according to the autopsy of one sheep fed *A.villosa*. A similar result was reported when sheep fed *Acacia angustissima* without adaptation period. The sheep was supplemented with *A.angustissima* only 300g/day became ill and died after 9 days. It was suspected that the toxic compound in *A.angustissima* was not tannin but neurotoxin although tannin was found quite high level (16.2% as tannic acid equivalent) in *A.angustissima*. Further study to identify the toxic compund was being carried out (Odenyo *et al.*, 1997). The interesting thing *of A. angustissima* feeding trial was that sheep which was adapted to *A.angustissima* could survive and grew quite well. It was clear that with adaptation period, the rumen microbes that capable to detoxify poisonous compound already develop in the rumen and protect the animal from toxicity.

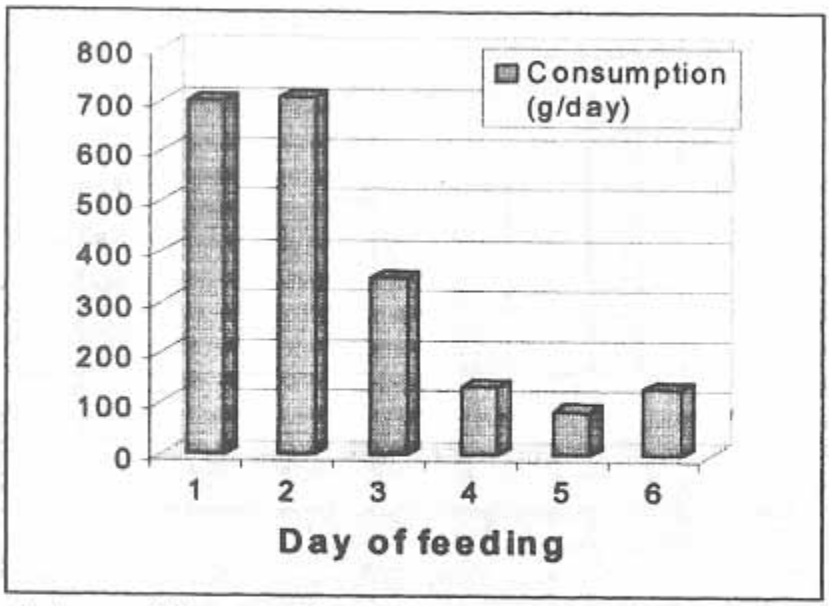

Figure 3. Average daily consumption of *Acacia villosa* (g/day) by Javanese thin tail sheep.

To find out the toxic compounds in *A. villosa*, several compounds which were considered as anti-nutritive compounds were measured qualitatively and quantitatively. In different extracts, total phenol in MeOH extract was quite high (5.91%) than that in other extracts and almost 50% consisted of tannin compounds. High condensed tannin content in forages reduced dry matter and protein digestibilities and the drying process gave more severe effect to those digestibilities (Wina *et al.*, 1993b), but causing no death of animal. Nutritional value of *A.villosa* could be less because of the tannin content in it.

Saponin was detected quite low in *A.villosa*. Saponin was antiprotozoal agent. In low concentration, saponin would be benefial than detrimental because by reducing the protozoa population, it would increase the growth of cellulolytic bacteria. But other compounds in the extract have more stronger negative effect toward the cellolulytic bacteria than saponin in the leaves.

Qualitative analysis for the presence of hydrocyanic acid was conducted and got negative result. Hydrocyanic acid usually indicates the presence of cyanogenic compounds which is also toxic to animal.

Non-protein amino acid that measured in *A.villosa* was 2.88%, the highest level compared to other legumes (Tangendjaja and Wina, 1995). There were other non-protein amino acids that have been detected in legume forages. Mimosine was found in *Leucaena leucocephala* or *Mimosa pudica*. Canavanine has been found in *Sesbania grandiflora*. The mechanism of non-protein amino acid in the body is usually as an essential amino acid analogue. Mimosine acted as a tyrosine analogue and may inhibit tyrosine-utilizng enzyme or may incorporated into protein in place of tyrosine (Tangendjaja, 1983). Odenyo *et al.* (1997) hypothesized that the toxic compound in *A.angustissima* which was suspected as a neurotoxin was a thiamine analogue. It could competed with thiamine and interfered thiamine-utilizing enzyme. Unless there are some rumen microbes degrading this compound or its metabolite, the animal would suffer from toxicity.

Table 3 Some anti-nutritive compounds in *Acacia villosa*

Parameter	%
Total phenol:	
- Methanol extract	5.91
- 50% methanol extract	0.69
Tannin	
- Methanol extract	2.06
- 50% methanol extract	1.30
- Water extract	0.35
Saponin	0.52
Non-protein amino acid	2.88
HCN	negative

This happened to Australian ruminant which had lack of microbes degrading mimosine and its metabolite when consumed *Leucaena leucocephala*. After infusion with rumen liqour containing mimosine degrading microbes, the animals survived well without toxicity (Jones and Lowry, 1984). Adaptating the animals to the new feed which contains anti-nutritive compounds is important to let the rumen microbes develop tolerance to the compound or develop a specific microbe that has the ability to degrade the compound.

Conclusion

There were one or more compounds in Methanol extract of *Acacia villosa* which caused toxicity to rumen microbes and host animal. It was suspected a non-protein amino acid compound but the real chemical structure needs to be clarified by more sophisticated equipments. Work needs to be done on developing technology to overcome the anti-nutritive or toxic compounds in *Acacia villosa*.

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