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THE INTERACTION OF ISOLATED TANNIN FROM *Calliandra Calothyrsus* WITH CU^{2+} AND FE^{2+} AND ITS EFFECT ON THE *In Vitro* DIGESTIBILITY

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ABSTRACT

Tannin is found in many forages that are utilized for animal feed. Tannin has the ability to bind not only protein but also mineral and carbohydrate, however, the property of tannin binds mineral or carbohydrate is not well studied by animal nutritionists. The present work studied the interaction of isolated tannin from *C. calothyrsus* or tannic acid with Cu^{2+} (copper) and Fe^{2+} (iron) and the effect of this complex on the *in vitro* digestibility of protein and carbohydrate. Iron ion (Fe^{2+}) is more reactive than Copper (Cu^{2+}) to bind tannin or tannic acid. The optimum level of iron (0.25mM) to bind tannic acids was lower than that bound isolated tannin (require 2 mM), while the optimum concentration of Cu^{2+} to bind tannic acid and isolated tannin was 0.25 mM and 20mM, respectively. Fe-tannin complex only dissolved in acid solution when the solution was heated, but did not dissolve in basic solution. The presence of Fe and tannin in the *in vitro* fermentation tended to cause a synergistic negative effect on carbohydrate but not protein digestibility. In conclusion, the presence of Fe and tannin may cause a more negative effect on fiber digestibility than the presence of tannin in the feed.

Keywords: *tannin, tannic acid, iron, copper, digestibility*

INTRODUCTION

Tannin is found in many plants include legumes that are utilized for animal feed. The most concern of many studies is the negative effect of tannin, which binds protein and reduces protein degradation in the rumen. Therefore, several efforts have been conducted to deactivate tannin by addition of polyethylene glycol (Yildiz *et al.*, 2005), soaking in acidic or basic solution (Wina *et al.*, 2005) or addition of wood ash (Ben Salem *et al.*, 2005). Tannin has the ability to bind not only protein but also mineral and carbohydrate but these properties of tannin are not well studied in animal nutrition. The interaction of tannin with mineral has several applications in industries, such as a complex of Copper (II)-tannin as wood preservative, a complex of Chromium (III)-tannin as leather dye, or the complex tannin-heavy metals as water purifier.

Despite the useful application of mineral-tannin complex, there are several reports on the negative effect of this complex. Drinking a lot of tea causes less absorption of iron (Fe) in the body, hence, causes anemia since tannin in tea binds iron (Kim & Miller, 2005). So far, there is no report of the beneficial or negative effect of mineral-tannin complex in the animal. The present work is studying the interaction of

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tannin with Cu^{2+} and Fe^{2+} and the effect of this complex on the *in vitro* digestibility of protein and carbohydrate.

MATERIALS AND METHODS

Tannin-mineral interaction studies

- Tannin was isolated from freeze dried milled *Calliandra calothyrsus* according to the method of Hagerman & Butler (1980). Tannin was extracted in methanol and later put into a Sephadex LH-20 column. Tannin was eluted from the column using 50% acetone. Tannin fraction was freeze dried after the acetone was evaporated.
- Mineral solution was prepared by dissolving $FeSO_4$ and $CuSO_4$ at different concentration in water (1, 1.5 and 2 mM for Fe^{2+} and 2.5, 5, 10, 20, 25 and 50 mM for Cu^{2+}).

Effect of concentrations

One milliliter tannic acid or tannin solution in distilled water (1 mg/ml) was reacted with 1 ml $CuSO_4$ or $FeSO_4$ solution at different concentrations in a glass test tube. After mixing, the reaction was left for 30 minutes at room temperature. The solution was then centrifuged at 3000 rpm for 15 minutes. The supernatant was separated and one ml of the supernatant was pipetted into a new glass test tube and mixed with 1ml of Bovine Serum Albumin in acetate buffer pH 5.0 (2 mg/ml). The reaction tube was left in a cool room at 5°C for 20 minutes and then was centrifuged for 15 min at 3000 rpm. The supernatant was separated and the precipitate was dissolved in 4 ml of mixture 1% SDS-5% TEA (1:1). After dissolved, 1 ml of 0.01 M $FeCl_3$ solution was added, mixed and kept for 20 min at room temperature. A purple color solution was formed and its absorbance was measured by spectrophotometer at 510 nm wavelength.

Effect of acid or basic solution to the tannin-cation complex

One milliliter tannin solution in distilled water (1 mg/ml) was reacted with 1 ml $CuSO_4$ or $FeSO_4$ solution (20mM for Cu^{2+} and 2 mM for Fe^{2+}) in a glass test tube. After mixing, the reaction was left for 30 minutes at room temperature. The solution was then centrifuged at 3000 rpm for 15 minutes. The supernatant was separated and the precipitate was dissolved in 1ml HCl or NaOH solution (0.01M) and mixed with 1ml of Bovine Serum Albumin in acetate buffer pH 5.0 (2mg/ml). The reaction tube was left in a cool room at 5°C for 20 minutes and then was centrifuged for 15 min at 3000 rpm. The supernatant was separated and the precipitate was dissolved in 4 ml of mixture 1% SDS-5% TEA (1:1). After dissolved, 1 ml of 0.01 M $FeCl_3$ solution was added, mixed and kept for 20 min at room temperature. A purple color solution was formed and its absorbance was measured by spectrophotometer at 510 nm wavelength.

Effect of tannin-mineral complex on digestion (in vitro studies)

- Tannin was extracted from fresh *Calliandra calothyrsus* by grinding the leaves with 70% acetone and dry ice. Acetone was evaporated and the residue was then extracted by diethyl ether 3 times. Diethyl ether was discarded and the aqueous fraction was freeze dried and called as crude tannin fraction. Twenty four mg of tannin was added to each tube.

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- Filter paper Whatman no 41 cut into 0.5 x 0.5 cm pieces is used as cellulose substrate and casein is used as protein substrate and each tube contained 0.3 g of substrate.
- Rumen liquor was taken from a fistulated local goat fed with elephant grass
- $CuSO_4.5H_2O$ solution was added at the level of 50.8 and 101.6 ppm (10 and 20 mM)
- $FeSO_4.7H_2O$ solution was added at the level of 5.6 and 11.2 ppm (1 & 2 mM)

Different levels of tannin and minerals solution reported above were placed in the *in vitro* system to study the effect of tannin-mineral interaction to digestion. A substrate of casein or filter paper (0.3 g) was added to a 50 ml plastic propylene tube. Twenty four ml of buffer (pH 6.9) was added together with 6 ml of rumen liquor at 39°C. The tube was incubated at 39°C for 48 hours under anaerobic condition. Then, it was filtered through a filter paper using vacuum pump. The residue was dried at 105°C for 24 hours and weighed. Dry matter digestibility can be calculated. Supernatant after casein fermentation was taken for ammonia determination.

RESULTS AND DISCUSSION

Different concentrations of Copper (II) and Iron (II) solution reacted with tannic acid or isolated tannin solution (1 mg/ml) formed a precipitate; a bluish purple precipitate for tannin-Fe complex and a yellowish white precipitate for tannin-Cu complex. The residual tannin (unreacted tannin) which present in the supernatant, was analyzed by Protein precipitation method and expressed as Absorbance (Figure 1).

The reaction of tannic acid (1 mg/ml) with either Cu or Fe formed a precipitate at the lowest concentration of Cu or Fe (0.25mM). It was a complete reaction between tannic acid and Cu or Fe as the residual tannic acid was undetected. The reactions of the isolated tannin from Calliandra with Fe and Cu are presented in Figure 1A and 1B, respectively. Figure 1A shows that the unreacted tannin was very low (almost undetectable) when the concentration of Fe solution reached 2mM, while Figure 1B shows that the unreacted tannin remained stable after the concentration of Cu solution was above 20mM. It means that Iron is more reactive than Copper when reacted with the isolated tannin and tannic acid is more reactive than the isolated tannin from *C. calothyrsus*. McDonald *et al.* (1996) reported that more condensed tannin (such as legume tannin) was required as compared to hydrolysable tannin (such as tannic acid) to precipitate similar amounts of copper. The chemical structure of tannins and in particular the type of hydroxyl substitution explain the difference in their ability to precipitate Fe or Cu since one copper atom requires o-dihydroxylphenyl (two hydroxyl groups in the position of ortho to each other) in the tannin molecule to form a complex or chelate.

The reaction of tannin and Fe has a strong bonding as 71.63% of tannin still bound with Fe and only 28.37% of Calliandra tannin released after dissolved in hot acid and did not dissolve at all in basic solution (Table 1). It has been reported that the pH condition influenced not only the precipitation but also the stability of cation-tannin complexes. (McDonald *et al.*, 1996).

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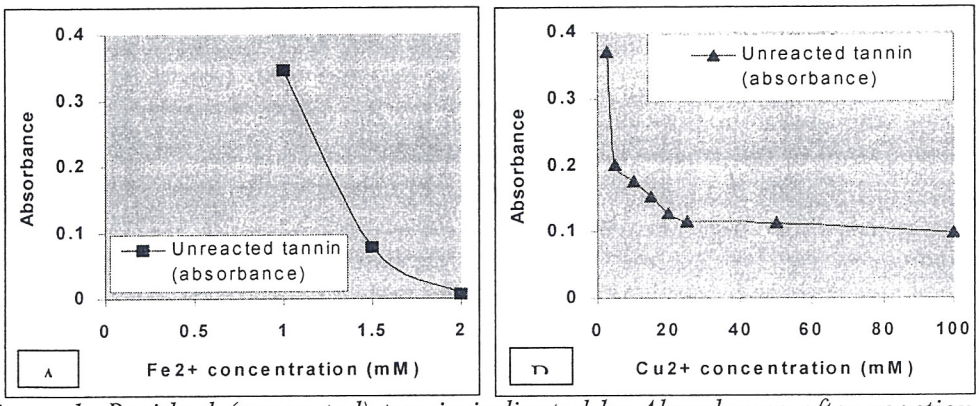

Figure 1: Residual (unreacted) tannin indicated by Absorbance after reaction between tannin and FeSO4 solution (Left) or between tannin and CuSO4 solution (Right) at different concentration

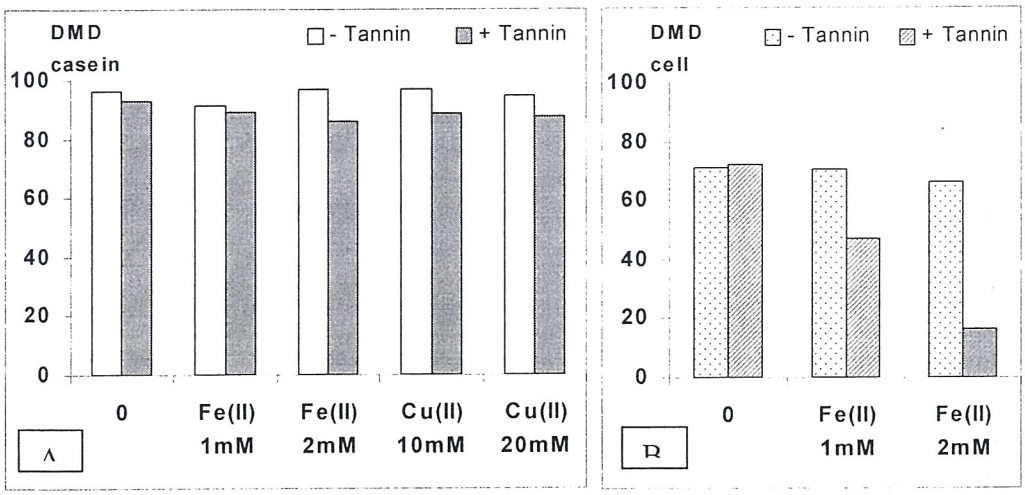

Figure 2. In vitro DM digestibility (DMD) of casein (Fig 2A) and DMD of cellulose (Fig 2B) in the presence of Fe or Cu with and without tannin.

Table 1: Solubility of Complex tannin-cation in acid or basic solution

Cation	Concentration (mM)	Complex T-C* +HCl	% tannin released by HCl	Complex T-C + NaOH
Fe	2	Dissolved when heated	28.37	Not dissolved
Cu	20	Readily dissolved	71.63	Not dissolved

*T-C= Tannin-Cation

Tannin from *Pinus radiata* bark at pH 3 could bind 53% Fe from waste water (Palma *et al.*, 2003). From this work, Palma *et al.* (2003) suggested to use tannin to clean the waste water. Makkar (2003) used the principle reaction of tannin with Fe to analysis tannin in plant materials. The strong bonding between tannin and Fe, however, becomes a disadvantage for the animal since this tannin-Fe complex could exist in the undissociated form in the abomasum suggesting the availability and absorption of Iron could decrease in the presence of tannin (Afsana *et al.*, 2004). Therefore, it is possible that the animals especially those require high amount of Iron will suffer from anemia if

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the animals consume high tannin containing forages. Iron requirement for beef cattle is about 50ppm and for dairy calves and dairy cow is between 30-100 mg/day (Harris *et al.*, 1994)

The *in vitro* fermentation by adding tannin together with Iron or Copper was conducted and the result is presented in Figure 2. As casein is very soluble, the digestibility of casein in the *in vitro* fermentation was very high (Fig 2A). However, the presence of tannin slightly reduced the digestibility of casein (from 96.28% to 92.68%). When Fe or Cu was added together with tannin to the fermentation, the DM digestibility of casein tended to reduce. Tannin will bind protein (casein) by hydrogen bonding which is between hydroxyl group of tannin to the hydrogen atom of protein, hence, cause less degradation of protein in the rumen. Ammonia production, which is the product of casein degradation was depressed in the presence of tannin (from 93.09 mM to 87.27mM) and more depressed in the presence of Cu (from 93.09 mM to 20.62mM). Cu did not affect casein solubility but it was clearly shown it negatively affected the degradation of casein to ammonia. Cu might be toxic to the proteolytic rumen microbes and depressed their growth. Cations such as Zn or copper are required for the growth of rumen bacteria. Copper is also required for metabolism in the animal body such as activity of enzymes associated with iron metabolism, elastin and collagen formation, melanin production, and the integrity of the central nervous system etc. Copper requirement of beef cattle is 4 to 10 ppm in the total diet (http://www.saltinstitute.org/47o.html, 2001).

Figure 2B. shows that Fe did not affect cellulose digestibility at low concentration but it slightly decreased cellulose digestibility at higher concentration. This was in agreement with the result of Harrison *et al.* (1992) which showed the reduction of DM digestibility with increasing level of iron sulfate in the in vitro fermentation. The negative effect of Fe was much stronger in the presence of tannin. The complex of tannin and Fe may be depressed the activity of fibrolytic rumen microbes in degrading cellulose or was toxic to the fibrolytic rumen microbes so that the growth of these microbes was depressed. It was not possible to study the effect of Cu to the *in vitro* fermentation in the presence of tannin since Cu, at the concentration of 10 and 20 mM in the *in vitro* fermentation (equals to 100 to 200 ppm) has killed the fibrolytic microbes totally so that there was no cellulose degradation at all. Engle & Spears (2000) reported that *In vitro* DM digestibility was not affected if the addition of Cu to the in *vitro* fermentation was only up 20 ppm.

The complex of tannin with Cu or Fe did not show any positive effect to the *in vitro* fermentation. In contrast, the presence of tannin with Cu or Fe seemed to be synergistically depressed the digestibility of cellulose in the fermentation and more over this complex may not be easily degraded in the duodenum and caused deficiency of Iron for the animals, which are in the certain physiology state require high intake of Iron.

More work should be conducted in the *in vitro* and *in vivo* trials especially with different mixtures of metal ions since the presence of several metal ions can be synergism or antagonism to their availability.

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