EFFECTS OF DIFFERENT MOLECULAR WEIGHTS OF CONDENSED TANNINS ON RUMINAL FERMENTATION AND MILK FATTY ACID PROFILE IN DAIRY GOATS

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ผลของน้ำหนักโมเลกุลของคอนเดนซ์แทนนินส์ที่แตกต่างกันต่อกระบวนการหมักในรูเมนและองค์ประกอบกรดไขมันของน้ำนมในแพะนม

นายอนันต์ เพชรล้ำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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อนันต์ เพชรล้ำ : ผลของน้ำหนักโมเลกุลของคอนเดนซ์แทนนินส์ที่แตกต่างกันต่อกระบวนการหมักในรูเมนและองค์ประกอบกรดไขมันของน้ำนมในแพะนม (EFFECTS OF DIFFERENT MOLECULAR WEIGHTS OF CONDENSED TANNINS ON RUMINAL FERMENTATION AND MILK FATTY ACID PROFILE IN DAIRY GOATS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. ปราโมทย์ แพงคำ, 107 หน้า.

การศึกษาวิจัยนี้มีวัตถุประสงค์ เพื่อศึกษาผลของน้ำหนักโมเลกุลของคอนเดนซ์แทนนินส์ที่แตกต่างกันต่อกระบวนการหมักในรูเมนและการปรับเปลี่ยนองค์ประกอบของกรดไขมันในน้ำนมของแพะนม

การทดลองที่ 1 เป็นการศึกษาองค์ประกอบของแทนนินส์ น้ำหนักโมเลกุล และความสามารถในการจับโปรตีนของคอนเดนซ์แทนนินส์ในพืชเขตร้อนที่มีศักยภาพในการนำมาใช้เป็นแหล่งของสารแทนนินส์สำหรับปรับเปลี่ยนกระบวนการหมักในกระเพาะรูเมนได้ โดยได้ศึกษาในใบมันสำปะหลัง *Manihot esculenta*, Cranzt) ใบกระถิน (*Leucaena leucocephala*) ใบสะเดา Siamese neem (*Azadirachta indica* A. Juss. var. Siamensis Valeton) เปลือกมังคุด (*Garcinia mangostana*) และสารคอนเดนซ์แทนนินส์สกัดจากคิวบราโช (quebracho) พบว่า ตัวอย่างจากใบพืช ใบกระถิน มีความเข้มข้นของคอนเดนซ์แทนนินส์ต่ำที่สุด (1.2% สิ่งแห้ง) ในขณะที่ใบสะเดามีสารคอนเดซ์แทนนินส์สูงที่สุด (5.0% สิ่งแห้ง) ส่วนเปลือกมังคุดและสารสกัดคิวบราโช มีระดับแทนนินส์ที่สูงกว่าในใบพืชที่ศึกษา สารคอนเดนซ์แทนนินส์ในพืชตัวอย่างมีน้ำหนักโมเลกุล 2,964 3,222 3,409 3,539 และ 3,612 ดาลตัน (Da) ในเปลือกมังคุด ใบกระถิน ใบมันสำปะหลัง สารสกัดคิวบราโช และใบสะเดา ตามลำดับ นอกจากนี้ ความสามารถของคอนเดนซ์แทนนินส์ในการจับกับโปรตีนสูงขึ้นสัมพันธ์กับน้ำหนักโมเลกุลที่สูงขึ้นด้วย

การทดลองที่ 2 เป็นการศึกษาถึงผลของน้ำหนักโมเลกุลของคอนเดนซ์แทนนินส์ที่แตกต่างกัน ต่อการผลิตแก๊ส และกระบวนการหมัก (ในหลอดทดลอง) โดยใช้สารสกัดคอนเดนซ์แทนนินส์จากใบพืช 2 ชนิดคือ 1) กระถิน สำหรับใช้เป็นแหล่งของสารคอนเดนซ์แทนนินส์ที่มีน้ำหนักโมเลกุลต่ำ และ 2) สะเดา สำหรับใช้เป็นแหล่งของสารสกัดคอนเดนซ์แทนนินส์ที่มีน้ำหนักโมเลกุลสูง โดยแต่ละชนิดใช้เสริมในระดับ 2, 4 และ 6 กรัม ทำการวัดผลผลิตแก๊ส มีเทน และวัดความเข้มข้นของกรดไขมันระเหยได้ พบว่า การเสริมสารสกัดคอนเดนซ์แทนนินส์ที่มีน้ำหนักโมเลกุลสูงจากใบสะเดาทั้ง 3 ระดับมีผลยับยั้งการผลิตแก๊สและการสร้างมีเทน ในขณะที่การเสริมสารสกัดคอนเดนซ์แทนนินส์ที่มีน้ำหนักโมเลกุลต่ำจากใบกระถินมีผลยับยั้งการผลิตแก๊สเฉพาะเมื่อเสริมในระดับสูงสุดเท่านั้น

การทดลองที่ 3 เป็นการศึกษาถึงผลของการเสริมแหล่งของสารคอนเดนซ์แทนนินส์ที่มีน้ำหนักโมเลกุลแตกต่างกันต่อปริมาณการกินได้ การย่อยได้ของโภชนะ กระบวนการหมักในรูเมน และการปรับเปลี่ยนองค์ประกอบของกรดไขมันในน้ำนม โดยศึกษาในแพะรีดนมพันธุ์ลูกผสมซาเนน และวางแผนการทดลองแบบสุ่มบล็อคสมบูรณ์ ทรีตเมนต์ที่ศึกษาประกอบไปด้วย 1) กลุ่มควบคุม 2) เสริมเปลือกมังคุด เป็นแหล่งแทนนินส์ 3) เสริมเปลือกมังคุด เป็นแหล่งแทนนินส์ และมีเสริมสาร PEG ซึ่งมีคุณสมบัติยับยั้งการทำงานของแทนนินส์ (กลุ่มควบคุมของ T2), 4) เสริมสารสกัดคิวบราโช เป็นแหล่งแทนนินส์ 5) เสริมสารสกัดคิวบราโช เป็นแหล่งแทนนินส์ และเสริมสาร PEG ซึ่งมีคุณสมบัติยับยั้งการทำงานของแทนนินส์ (กลุ่มควบคุมของ 4) พบว่า น้ำหนักโมเลกุลของแทนนินส์ไม่มีผลต่อปริมาณการกินได้ การย่อยได้ของโภชนะ กระบวนการหมักในรูเมน ปริมาณน้ำนม ตลอดจนองค์ประกอบของกรดไขมันในน้ำนมแพะ

สาขาวิชาเทคโนโลยีการผลิตสัตว์
ปีการศึกษา 2560

ลายมือชื่อนักศึกษา _____
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ANAN PETLUM : EFFECTS OF DIFFERENT MOLECULAR WEIGHTS OF CONDENSED TANNINS ON RUMINAL FERMENTATION AND MILK FATTY ACID PROFILE IN DAIRY GOATS. THESIS ADVISOR : ASSOC. PROF. PRAMOTE PAENGKOUM, Ph.D., 107 PP.

TANNINS/MOLECULAR WEIGHT/RUMINAL FERMENTATION/FATTY ACID

This research aimed to investigate the effect of different molecular weight of condensed tannins (CT) on ruminal fermentation and milk fatty acid compositions of lactating dairy goats.

Experiment I was conducted to study the tannin contents, molecular weight and protein-binding ability of condensed tannins in tropical feed resources. Leaves of three plant species, namely, leucaena (*Leucaena leucocephala*) (LN), cassava (*Manihot esculenta*, Cranzt) (CV), and Siamese neem (*Azadirachta indica* A. Juss. var. Siamensis Valeton) (SN), and mangosteen (*Garcinia mangostana*) peel (MS) and quebracho tannins (QB) extract were used in this study. Condensed tannin contents in tropical plant leaves ranged from 1.2% DM in LN to 5.0% in SN ($P<0.001$), whereas the MS and the QB contained higher concentrations of condensed tannins (14.38 and 67.34% DM, respectively) ($P<0.001$). The weight-average molecular weight of the purified condensed tannins were 2,964, 3,222, 3,409, 3,539 and 3,612 Da for MS, LN, CV, QB and SN, respectively. Moreover, the protein-binding ability of condensed tannins were increased relative to higher molecular weight.

Experiment II was designed to evaluate the effect of condensed tannins with different molecular weight on ruminal fermentation and gas production *in vitro*. Condensed tannins extracted from two plant species leaves, LN representing condensed

tannins of lower molecular weight (LMW-CT) and SN representing condensed tannins of higher molecular weight (HMW-CT) were selected. Seven treatments including the control (with no CT supplementation) and treatments supplemented with LMW-CT and HMW-CT extracts at 2, 4, and 6 mg/100 mg DM of substrate were assigned. Results showed that, supplementation of HMW-CT from SN (at 2-6 mg/100 mg DM) significantly inhibited *in vitro* total gas and methane productions (24 h of incubation) while supplementation of LMW-CT from LN had no effect, except for total gas production at the highest (6 mg/100 mg DM) level of supplementation. Similarly, HMW-CT had a stronger effect ($P< 0.001$) on *in vitro* volatile fatty acids production.

Experiment III was conducted to investigate the effect of the inclusion of condensed tannins with different molecular weights on voluntary feed intake, nutrient digestibility, ruminal fermentation and modification of the fatty acid profile in milk in lactating dairy goats. Dietary treatments were designed as following : T1) control (with no CT supplementation), T2) supplemented with MS in a concentrate as a source of LMW-CT at 3.0% DM of CT equivalent, T3) supplemented with the same diet with T2 but added with polyethylene glycol (PEG, as tannin inactivator) as the control of T2, T4) supplemented with QB in a concentrate as a source of HMW-CT at 3.0% DM of CT equivalent, and T5) supplemented with the same diet with T4 but added with PEG. No significant change was detected for feed intake, nutrient digestibility, ruminal pH and VFA, and milk yield, milk compositions and the milk fatty acid profile.

School of Animal Production Technology Student's Signature _____

Academic Year 2017 Advisor's Signature _____

Co-advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	degree Celsius
A:P	acetic to propionic ratio
ADF	acid-detergent fiber
AIA	acid insoluble ash
BH	biohydrogenation
BSA	bovine serum albumin
BW	body weigh
C_2	acetic acid
C_3	propionic acid
C_4	butyric acid
CH_4	methane
CLA	conjugated linoleic acid
CP	crude protein
CT	condensed tannin
CV	cassava (*Manihot esculenta*, Cranzt)
Da	Dalton
DM	dry matter
DP	degree of polymerization
EE	ether extract
FA	fatty acid

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

FI	feed intake
HMW	high molecular weight
HT	hydrolysable tannin
IVDMD	*in vitro* dry matter digestibility
LA	linoleic acid
LL	*Leucaena leucocephala*
LMW	low molecular weight
LNA	α-linolenic acid
MSP	mangosteen (*Garcinia mangostana*) peel
MW	molecular weight
M_w	weight-average molecular weight
n.d.	not determined
NDF	neutral-detergent fiber
nm	nanometer
OM	organic matter
P	probability
PEG	polyethylene glycol
PSM	plant secondary metabolites
PUFA	polyunsaturated fatty acids
QB	quebracho tannins extract
RA	rumenic acid
SA	stearic acid

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

SAS	Statistical Analysis System
SEM	standard error of the mean
SFA	saturated fatty acids
SN	Siam neem (*Azadirachta indica* A. Juss. var. siamensis Valeton)
SNF	solid-not-fat
TP	total phenols
TS	total solids
TT	total tannins
UFA	unsaturated fatty acids
VA	vaccenic acid
VFA	volatile fatty acid

CHAPTER I

INTRODUCTION

Currently, the production of healthy ruminant-derived foods is an important topic in ruminant nutrition research. Although tissue lipids of ruminants are recognised to be highly saturated in nature relative to non-ruminants (Banks and Hilditch, 1931 referred by Jenkins et al., 2008), however, ruminant products also contain potentially health-promoting conjugated linoleic acid (CLA), mainly *cis*-9, *trans*-11-CLA isomer (rumenic acid, RA) which shown in many animal studies to contribute to cancer prevention, decreased atherosclerosis, improved immune response, and altered protein or energy metabolism (Pariza, 2004; Palmquist et al., 2005; Gebauer et al., 2011).

The composition of diet fed to ruminant animal is the main factor that influence to the composition of fatty acid of ruminant's products both meat and milk. The composition of fatty acid in meat and milk from ruminant are results of dietary lipids metabolism by rumen microbial (Buccioni et al., 2012). The process namely biohydrogenation (BH) which occur in the rumen is the important process to convers dietary polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA) by rumen microbes. By this process, the transformation of PUFA to SFA results in accumulation of several intermediates in meat and milk of ruminant animals. The CLA isomers are also formed as the intermediates of biohydrogenation process of dietary linoleic acid (*cis*-9, *cis*-12-18:2, LA) in the rumen (Jenkins et al., 2008). Ruminal

biohydrogenation presents a major human health issue (Maia et al., 2010) due to its effect on the fatty acids composition of ruminant derived products. The CLA content of meat and milk is strongly linked to the ruminal BH of dietary linoleic acid and *α*-linolenic acid (*cis*-9, *cis*-12, *cis*-15-18:3, LNA) (Vasta et al., 2009a). During BH process, LA and LNA are gradually isomerized and saturated to form several 18 carbon diene and monoene isomer intermediates; stearic acid (18:0, SA) is the final product of this pathway (Bessa et al., 2007). Moreover, RA can also be formed endogenously in the muscle or in the mammary gland from vaccenic acid (*trans*-11 C18:1, VA) (Griinari et al., 2000) through the action of Δ^9-desaturase enzyme (Corl et al., 2001), and recent studies by Corl et al. (2001) and Piperova et al. (2002) indicated that a high proportion of CLA in milk (78 to 93 %) is derived from this route and CLA contained in meat may also be produced from absorbed VA. Therefore, factors influencing the production of VA in the rumen are of interest in order to be a feasible strategy for increasing the CLA content in milk or meat of ruminant (Fukuda et al., 2006; Kim et al., 2009).

The CLA concentrations in ruminant-derived products can be increased by nutritional and management strategies that facilitate higher forestomach output of CLAs and VA for absorption and incorporated into animal tissues (Bauman et al., 2009; Kouba and Mourot, 2011). Because of the ruminal BH, and the formation of CLA, is catalysed by the ruminal microorganisms, therefore, many researches has focused on developing nutritional strategies which influence BH process and identification of the major microorganisms involved with a view to improving the healthiness of ruminant products (Kim et al., 2009). Kemp and Lander (1984) grouped the bacteria involved in the BH pathways into Group A and B; Group A bacteria were

classified based on their ability to hydrogenate PUFA to VA, whereas Group B bacteria were categorised based on the ability to hydrogenate PUFA through to SA. Many ruminal bacteria species of the genera *Butyrivibrio*, *Ruminococcus*, *Treponema-Borrelia*, *Micrococcus*, *Megasphaera*, *Eubacterium*, *Fusocillus* and *Clostridium* are known to be involved in ruminal BH (Harfoot and Hazlewood, 1997; Maia et al., 2007; Durmic et al., 2008 referred by Patra and Saxena, 2011). However, *Butyrivibrio* spp. are the most active species among the group A bacteria, which form CLA from linoleic acid, while few species of bacteria such as *Fusocillus* spp. and *Clostridium proteoclasticum* which renamed from its 16S rRNA gene sequence by Moon et al. (2008) as *Butyrivibrio proteoclasticus* convert VA acid to SA (group B) (Maia et al., 2007; Paillard et al., 2007; Durmic et al., 2008). Therefore, it has been suggested that selective inhibition of group B bacteria may provide more ruminal concentration of VA and CLAs for absorption and incorporated into animal tissues (Patra and Saxena, 2011).

Whereas the inclusion of fish oil in ruminant diets has been very successful at inhibiting the final BH step from VA to SA by its toxic effect on certain bacterial species (Kim et al., 2008), however, this strategy seem to be made higher cost of production in particularly for smallholder faming scale. Considerable attention has also been given to the action of plant secondary compounds including essential oils, saponins (Wallace, 2004) and catecholamines which possess anti-microbial properties. The ability of plant extracts in particularly tannins to modify the fatty acid composition of ruminant-derived food products (i.e. milk or meat) has recently received greater attention (Patra and Saxena, 2011).

There is very limited information pertaining to the effects of tannins on

ruminal BH process and fatty acids composition of ruminant derived products. Tannins are bioactive compounds that able to interfere with the metabolism of protein and also lipid in the rumen. Moreover, in general, tannins are considered to have both beneficial and detrimental effects on ruminant, depending on their concentration and chemical structure. However, Jayanegara et al. (2011) suggested that the effect of tannins are also depending on other factors, such as species and physiological state of animal and composition of the diet. Condensed tannins have been shown to inhibit the growth of many ruminal bacteria (Jones et al., 1994; Min and Hart, 2003; Patra and Saxena, 2009) including bacteria associated with ruminal BH. Durmic et al. (2008) were screened several plants in Australia for their ability to modify ruminal BH process, it was found that a wide range of the plant extracts had selective inhibitory effect towards *B. proteoclasticus* (which forms SA from LA) without affecting *B. fibrisolvens* (which forms CLA and VA acid, but not SA) and indicated that *B. proteoclasticus* was more sensitive to a tannin-rich plant extract than *B. fibrisolvens*. However, only a few plants, including *Acacia iteaphylla* and *Kennedia eximia*, inhibited LA metabolism or SA formation in the mixed bacterial community *in vitro*. Similarly to a study by Vasta et al. (2010) which demonstrated that inclusion of quebracho tannin (9.57 % of DM intake) to a concentrate and hay (lucerne) diet for sheep increased the population of *B. fibrisolvens*, while the growth of *C. proteoclasticum* reduced.

In addition, both *in vitro* (Khiaosa-Ard et al., 2009; Vasta et al., 2009a) and *in vivo* (Vasta et al., 2009b; 2010; Rana et al., 2012) studies have been shown that addition of condensed tannins inhibited the last step of LNA biohydrogenation, this inhibition led to the accumulation of VA in ruminal fluid, while concentration of SA

were decreased. The effects of tannins on ruminal bacteria are reported to be dependent upon the species of microorganism and type or source of tannin (Jones et al., 1994; Sivakumaran et al., 2004). Moreover, Sivakumaran et al. (2004) demonstrated that condensed tannins from *Dorycnium rectum* forage have varied inhibiting effects on growth of *B. fibrisolvens* and *C. proteoclasticum* depended upon to molecular weighs (i.e. low, medium and high) and concentrations of tannins of *in vitro* medium. However, although supplemented with tannins shown an affected to fatty acid composition (higher RA and total CLA, and lower SA) of ruminant meat (*Longissimus dorsi* muscle) (Rana et al., 2012), however, studies by Toral et al. (2011; 2013) demonstrated that inclusion of tannins in diet not affected on milk fatty acid composition, and they were suggested that structural and chemical dissimilarities between hydrolysable tannin (HT) and condensed tannin (CT) may offer an explanation for differences in their biological effects and, therefore, results obtained using a particular type of tannins cannot be applied to others.

Although effects of tannins are varying in each study, however, many researches have been shown beneficial effects on manipulation of ruminal BH and modification of fatty acids composition of ruminant products. Protein-binding ability found to be the major biological property of condensed tannins (CT), and its molecular weight found to be main factor effect on this property (Osborne and McNeill, 2001). Many studies have demonstrated that larger-size (higher molecular weight) CT has stronger protein-binding ability than smaller-size (lower molecular weight) CT (Porter and Woodruffe, 1984; Osborne and McNeill, 2001; Huang et al., 2010; 2011). Therefore, further research emphasis on effect of condensed tannin with differing structure (particularly molecular weight) on selectively effects on specific

bacteria involved in ruminal BH and alteration of fatty acids composition of ruminant-derived food products would be needed.

1.1 Research hypothesis

Inclusion of higher molecular weight of condensed tannins source increased proportion of unsaturated fatty acids in goat milk.

1.2 Research objectives

1.2.1 To determine the molecular weight of condensed tannins of plant materials and their effect on some ruminal fermentation parameters.

1.2.2 To investigate the effect of molecular weight of condensed tannins on fatty acid profiles of dairy goats milk.

1.3 Scope of the study

This study aimed to examine the molecular weight (MW) of some tropical plant materials and commercial extract of condensed tannins (CTs) and their effect on protein-binding ability and *in vitro* ruminal fermentation parameters included gas production, methane (CH_4) production and volatile fatty acid (VFA) concentration. The response of crossbred-Saanen dairy goats on dietary inclusion of differing molecular weight condensed tannins were measured for milk yield, milk composition, milk fatty acid (FA) profile, feed intake (FI), nutrient digestibility, and ruminal pH and volatile fatty acid concentration.

1.4 Expected benefits

1.4.1 To provide a new information base for MW and protein-binding ability of CTs of some tropical plant materials which potentially used as ruminant feeds.

1.4.2 To provide a knowledge base for effects of differing MW of CT inclusion on modification of milk fatty acid profile and ruminal fermentation of dairy goat.

1.4.3 This knowledge can be used as a new feeding strategy using CT-containing tropical plant material for improving of milk quality.

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CHAPTER II

LITERATURE REVIEW

2.1 Importance/advantages of CLAs

Tissue lipids of ruminants are recognised for a long time to be highly saturated in nature relative to non-ruminants (Banks and Hilditch, 1931 referred by Jenkins et al., 2008) and consumption of ruminant fats has been linked to increased incidence of coronary heart disease (Williams, 2000; Givens, 2005 referred by Kim et al., 2009). However, ruminant products also contain potentially health-promoting conjugated linoleic acid (CLA), mainly *cis*-9, *trans*-11-CLA which shown in many animal studies to contribute to cancer prevention, decreased atherosclerosis, improved immune response, and altered protein or energy metabolism (Pariza, 2004; Palmquist et al., 2005).

2.2 Ruminal biohydrogenation and CLAs synthesis

Lipids entering the rumen are first transformed by microbial lipases in a process called lipolysis. After lipolysis, unsaturated fatty acids undergo BH by ruminal microbes. The pathways of biohydrogenation of the major dietary PUFA, linoleic acid (18:2*n*-6) and linolenic (18:3*n*-3) acids are shown in Figure 1. This process converts the unsaturated fatty acids to SFA via isomerization to *trans* fatty acid intermediates, followed by hydrogenation of the double bonds (Harfoot and Hazlewood, 1988) (Figure 2.1).

The initial step was the isomerization of the *cis*-12 bond to either the C11 or C13 position. Next, one of the bonds was hydrogenated to leave an 18:2, followed by hydrogenation of another bond, producing an 18:1. Hydrogenation of the 18:1 formed stearic acid as the final product (Jenkins et al., 2008). This process may accumulate a wide range of intermediates (Palmquist et al., 2005), including conjugated linoleic acid (CLA) isomers, *cis*-9, *trans*-11 18:2 (rumenic acid), which is mostly reduced to *trans*-11 18:1 (vaccenic acid, VA) and finally to 18:0 (stearic acid, SA) (Belenguer et al., 2010).

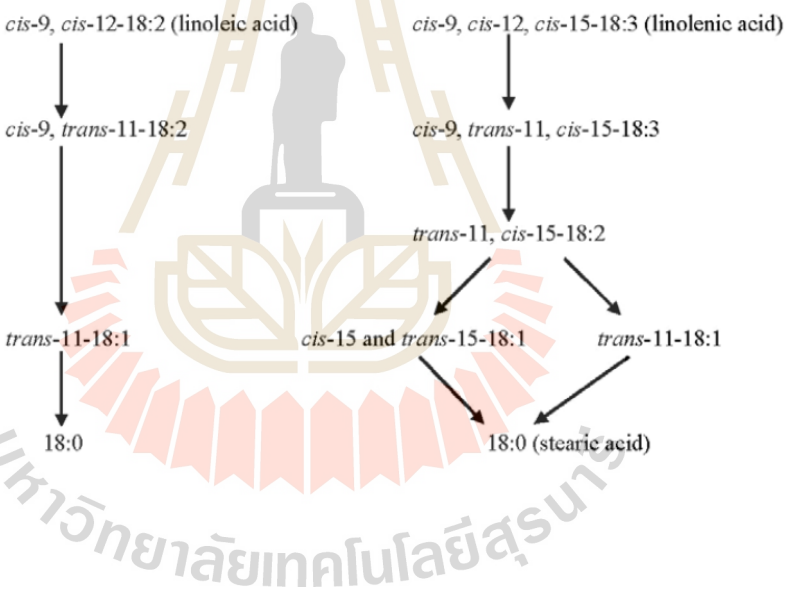


Figure 2.1 Biohydrogenation pathways of linoleic acid and linolenic acid**.**

Source: Adapted from Harfoot and Hazlewood (1997).

2.3 Role of microbes in ruminal biohydrogenation

Ruminal microorganisms play a very important role in transform lipids entering the rumen via 2 major processes, lipolysis and biohydrogenation (Jenkins et al., 2008). Manipulation of rumen microbial community can be altered

biohydrogenation and fatty acid composition of ruminant products, and different nutritional strategies have been used, such as forage feeding, antimicrobial feed additives such as monensin and supplementation of dietary fat particularly vegetable oils or oilseeds, marine products or protected fat sources (Lourenco et al., 2010). However, different fatty acids have different effect on rumen microbes, Harfoot and Hazlewood (1997) reported that unsaturated fatty acids (UFA) have a stronger antimicrobial effect than saturated ones, while different PUFA have differential toxicity toward rumen microorganisms (Maia et al., 2007). Lipid supplementation can lead to a shift in the rumen microbial population (Lourenco et al., 2010) which also can be altered biohydrogenation and fatty acid composition of ruminant products.

Harfoot and Hazlewood (1997) noted that little had changed in review of the role of the different microbial species in the BH of unsaturated fatty acids. Significantly, virtually all the information predated the discovery of the importance of CLA. Modern microbial genetics and molecular phylogenetic techniques for identifying and classifying microorganisms by their small-subunit rRNA gene sequences have provided new information about BH since research interest was rekindled by increasing concerns about fatty acids and human health (Jenkins et al., 2008).

Even though known for many years that protozoal lipids contain proportionally more unsaturated fatty acids than the bacterial fraction (Harfoot and Hazlewood, 1997), whereas in terms of FA flow, protozoa accounted for between 30% and 43% of the CLA and 40% of the VA reaching the duodenum, and thus protozoa could represent a very important source of PUFA, CLA, and *trans*-11-18:1 for incorporation into meat and milk. Recent *in vitro* experiments carried out at the Rowett Research

Institute (Devillard et al., 2006) showed that the protozoa have much higher CLA concentrations than bacteria, but did not possess delta-9 desaturase activity suggesting that protozoa preferentially incorporate CLA and VA formed by bacteria. *In vivo* data also showed that protozoa were proportionally high in PUFA and CLA compared with bacteria (Or-Rashid et al., 2007). However, the extensive ingestion of bacteria by protozoa was considered. Thus, even if protozoa do not themselves produce CLA and VA by their own metabolism, nevertheless they might be expected to have a significant influence on CLA and VA available to the host animal.

In additionally, ruminal fungi have been known for some time to contain a relatively high 18:1 composition and linoleic acid and linolenic acid incubated with *Piromyces communis* resulted in the formation of conjugated products (Kemp et al., 1984). However, although ruminal fungi produce *cis*-9, *trans*-11-18:2 from LA (Nam and Garnsworthy, 2007), their activity is very low in comparison with ruminal bacteria, *Butyrivibrio fibrisolvens* (Maia et al., 2007).

Bacteria play the main role in FA biohydrogenation (Jenkins et al., 2008). In early studies, *B. fibrisolvens* was identified to undertake biohydrogenation of FA and to form CLA and VA as intermediates during the biohydrogenation of LA (Polan et al., 1964 referred by Jenkins et al., 2008), whereas stearic acid was not formed from LA by *B. fibrisolvens*. Later studies, Kemp et al. (1975) identified other bacteria that were capable of biohydrogenation, and bacteria carrying out stearate formation were identified as *Fusocillus spp.* Van de Vossenberg and Joblin (2003) isolated from a grazing cow a bacterium, which could also form stearate from linoleate. It was phenotypically similar to '*Fusocillus*' which phylogenetically close to *Butyrivibrio hungatei*. Subsequently, a species named *Clostridium proteoclasticum* was identified

as a stearate producer with morphological and metabolic properties that were indistinguishable from those reported for *Fusocillus* (Wallace et al., 2006). Moon et al. (2008) renamed *C. proteoclasticum* as *Butyrivibrio proteoclasticus*. Recently, Boeckaert et al. (2008) suggested that other, as-yet-uncultivated bacteria that cluster closely to the *Butyrivibrio* and *Pseudobutyrivibrio* genera might be more important for SA production than *B. Proteoclasticus*. Whereas, other less abundant rumen bacteria, such as strains of *Megasphaera elsdenii,* may aslso have a role in BH (Jenkins et al., 2008).

2.4 Biohydrogenating ruminal bacteria

The bacteria that are involved in the different steps of the biohydrogenation process were classified as group A and B (Harfoot and Hazlewood, 1997). Group A bacteria hydrogenated LA and LNA to VA, whereas group B bacteria convert the same FA to stearic acid. However, Lourenco et al., (2010) was proposed that it is now much more appropriate to describe the bacteria based on their correct taxonomy (Figure 2.2). The main Group A bacterium is believed to be *B. fibrisolvens* (Harfoot and Hazlewood, 1997), whereas the main Group B organism identified to date is '*Fusocillus*' sp. (Kemp et al., 1975). The latter is a genus that no longer exists in modern taxonomy, and stored isolates of this organism are no longer viable (Wallace et al., 2006). Nonetheless, recent attempts using culture-dependent techniques have been made to re-isolate this '*Fusocillus*' or any bacteria capable of biohydrogenating PUFA to 18:0 with some success. Modern phylogenetic analysis, using 16S rDNA sequencing, of recent isolates has now shown that re-isolated 18:0-forming bacteria, like the most active Group A bacteria, are part of the *B. fibrisolvens* group, an ill-

defined taxon that includes the genera *Butyrivibrio* and *Pseudobutyrivibrio* and the species *Clostridium proteoclasticum* (Paillard et al., 2007). Group B bacteria (18:0 producers) form a tight grouping in which strains cluster together close to *C. proteoclasticum* (Wallace et al., 2006). Furthermore, following concentrate feeding it has also been demonstrated that a concomitant rise in *Megasphaera elsdenii* occurs within the rumen. *Megasphaera elsdenii* causes the biohydrogenation of 18:2*n*-6 to the *trans*-10, *cis*-12 CLA (Kim et al., 2002), which explains the increase in this isomer following concentrate feeding (Kim et al., 2009).

Metabolism of LA by *Butyrivibrio* results in the formation of *cis*-9,*trans*-11 CLA and VA, but no *trans*-10,*cis*-12 CLA or *trans*-10-18:1 is formed (McKain et al., 2010). The bacteria responsible for milk fat depression must therefore be different to the *Butyrivibrio* spp (Lourenco et al., 2010). The formation of *trans*-10,*cis*-12 CLA also occurs by a different enzymic mechanism to that of *cis*-9,*trans*-11 CLA (Wallace et al., 2007). Enrichment cultures with starch carried out by Kim et al. (2002) contained abundant large cocci identified as *Megasphaera elsdenii* that formed *trans*-10,*cis*-12 CLA. Devillard et al. (2006) indicate that *Propionibacterium acnes* may be responsible for the formation of *trans*-10,*cis*-12-18:2. Furthermore, when digesta samples from cows producing high amounts of *trans*-10,*cis*-12-18:2 were analysed for *M. elsdenii* by qPCR of 16S rRNA genes, numbers were, 103/g, while much larger numbers of *P. acnes* were detectable (Lourenco et al., 2010). *P. acnes* does not, however, convert *trans*-10,*cis*-12 CLA to *trans*-10-18:1, and this can be accomplished by the *Butyrivibrio* spp. (McKain et al., 2010). A future challenge will be to associate with certainty the role of individual microbial species with cows suffering milk fat depression (Lourenco et al., 2010).

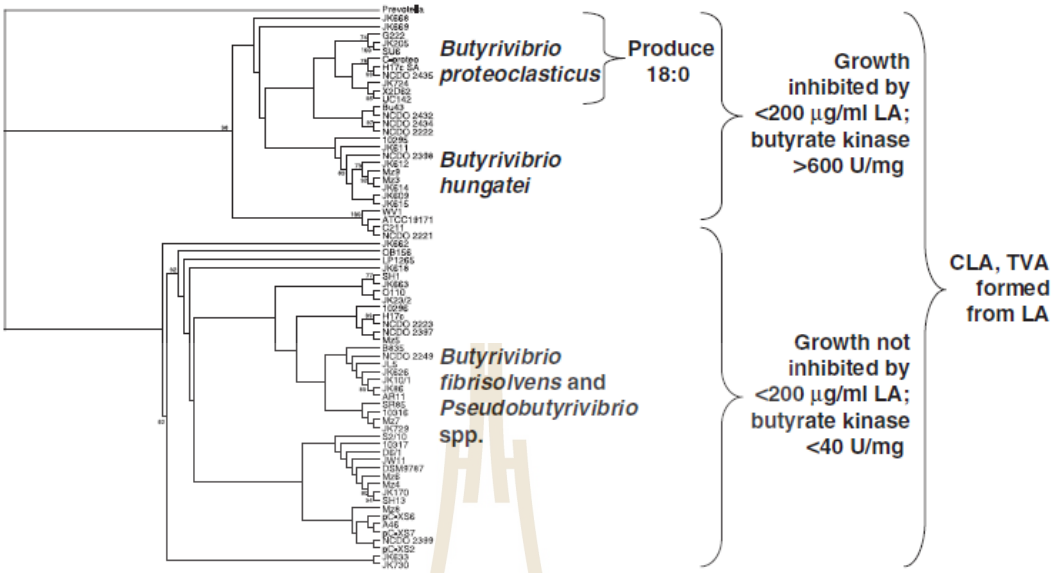


Figure 2.2 Relation between phylogenetic position, formation of biohydrogenation products from LA, sensitivity to growth inhibition and mechanism of butyrate formation in the *Butyrivibrio* phylogenetic tree.

CLA=conjugated linoleic acid; VA=vaccenic acid; LA=linoleic acid.

Source: Lourenco et al. (2010).

2.5 Factors affecting ruminal biohydrogenation

The major factors which influenced biohydrogenation included: ruminal pH, forage to concentrate ratio, level of intake and fish oil supplementation. Glasser et al. (2008) found a significant protective effect of low ruminal pH on *cis*-9, *cis*-12 18:2*n*-6 and 18:3*n*-3. Likewise a high proportion of forage in the diet had a negative effect on the flows of 18:2*n*-6 and 18:3*n*-3 to duodenum, suggesting increases in biohydrogenation. High levels of intake increased 18:3*n*-3 biohydrogenation and fish

oil appeared to act on 18:3*n*-3 and *trans*-18:1, decreasing the proportion of 18:0 in total C18 duodenal flows (Kim et al., 2009). Another dietary strategy to alter biohydrogenation has included the use of copper supplementation (Engle et al., 2001) where copper decreased the concentrations of the 18:1 *trans* isomer and the C18-conjugated dienes in milk. The rates of BH are dependent on the type and amount of fat delivered to the rumen and ruminal pH (Van Nevel and Demeyer, 1996), and also, lipolysis is considered to be rate limiting for biohydrogenation (Harfoot and Hazlewood, 1997).

In addition, considerable attention has also been given to the action of plant secondary compounds including polyphenol oxidase but also essential oils, saponins (Wallace, 2004) and catecholamines which possess anti-microbial properties. The ability of plant extracts in particularly tannins to modify the fatty acid composition of ruminant-derived food products (i.e. milk or meat) has received great attention recently (Patra and Saxena, 2011)

2.6 Potentials of condensed tannins in ruminal BH manipulation

2.6.1 Roles of tannins in ruminant nutrition

Tannins are water-soluble polyphenolic polymers of relatively high molecular weight and have capacity to form complexes mainly with proteins, to a lesser extent with carbohydrates due to the presence of a large number of phenolic hydroxyl groups. Tannins are usually divided into two groups: hydrolysable tannins (HTs) and condensed tannins (CTs) (Figure 2.3).

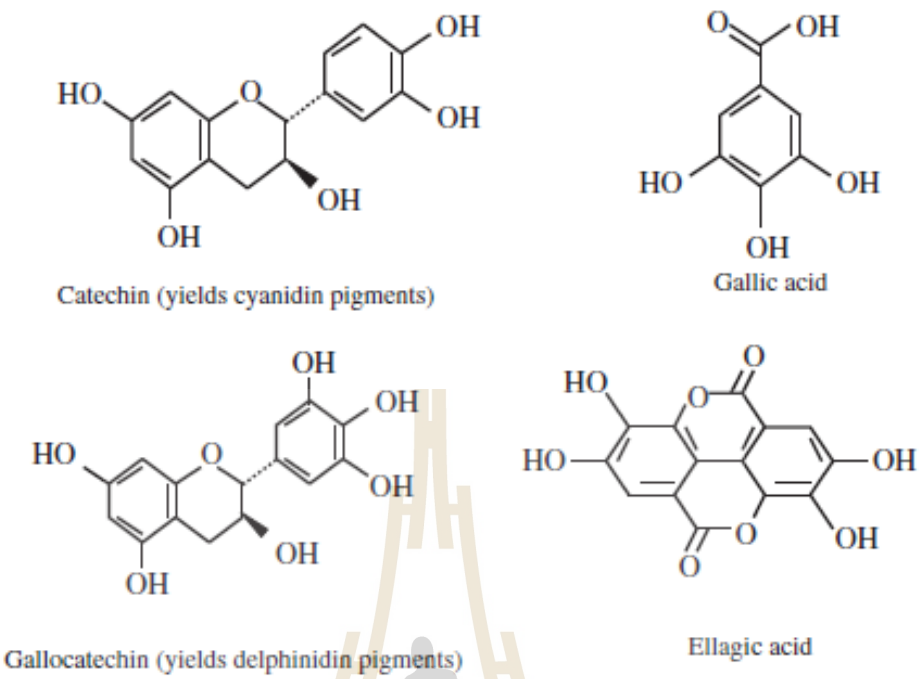


Figure 2.3 Monomeric units of condensed (catechin and gallocatechin) and hydrolysable tannins (gallic and ellagic acid).

Source: Patra and Saxena (2010; 2011).

The HTs are complex molecules with a polyol as a central core, such as glucose, glucitol, quinic acids, quercitol and shikimic acid, which is partially or totally esterified with a phenolic group, i.e. gallic acid (3,4,5-trihydroxy benzoic acid; gallotannins) or gallic acid dimer hexahydroxydiphenic acid (ellagitannins). The resultant phenolic groups may further be esterified or oxidatively cross-linked to yield more complex HTs. Hydrolysable tannins are susceptible to hydrolysis by acids, bases or esterases yielding polyol and the constituent phenolic acids (Haslam, 1989). The CTs, or proanthocyanidins, are mainly polymers of the flavan-3-ol (epi)catechin and (epi)gallocatechin units (Hemingway, 1989 referred by Patra and Saxena, 2011).

Many other monomers of CTs, e.g. profisetinidins, probinetidins and proguibortinidins, are also present (Haslam, 1989). Quebracho tannins are largely profisetinidins. The CTs are degraded to form monomeric anthocyanidins (e.g. cyanidins and delphinidins) pigments upon treatment with acid butanol reaction. The CTs can react by hydrogen bonding with plant protein to form stable and insoluble CT–protein complexes at pH 3.5–7.0, which dissociate and release protein at pH <3.5 (Jones and Mangan, 1977).

Tannins are widely distributed in nutritionally important forage trees, shrubs and legumes, cereals and grains that often limit their utilisation as feedstuffs. Generally, tannin concentrations are greater in vulnerable parts of the plants, i.e. new leaves and flowers, and various factors such as temperature, light intensities, water and nutrient stress, soil quality and topography influences the concentrations of tannins in plants (Frutos et al., 2004). Both HTs and CTs may occur in the same plant. A particular type of CT may be predominant in a particular plant, which may explain different physiological effects and animal performance (Waghorn, 2008). It has been noted that low molecular weight CT oligomers are more reactive and have higher protein-precipitating capacities than high molecular weight polymeric tannins (Butler and Rogler, 1992 referred by Patra and Saxena, 2011). The presence of prodelphinidins and procyanidins in different proportions in forages can also determine different biological efficacy. For example, prodelphinidins were more effective against abomasal nematodes than procyanidins (Brunet et al., 2008).

Tannins were primarily considered as anti-nutritional biochemical due to their adverse effects on feed intake and nutrient utilization (Kumar and Vaithiyanathan, 1990). Nevertheless, in recent years, they have been recognized as useful

phytochemicals for modulating rumen microbial fermentation beneficially (Patra and Saxena, 2011). A number of reviews have been published which focused mainly on adverse effects of tannins on animal systems with some discussion on the positive effects on protein metabolism and bloat prevention (Muller-Harvey, 2006; Waghorn, 2008; Kumar and Vaithiyanathan, 1990; McSweeney et al., 2001; Min and Hart, 2003). Moreover, recent reviews by Mueller-Harvey (2006) and Waghorn (2008) discussed little on methane inhibition by tannin-containing forages. Since the latest reviews, a great number of studies have been published on the effects of tannins on inhibition of methanogenesis and enrichment of CLAs in meat and milk from ruminants, which justify a fresh appraisal of the present scenario on the influences of tannins on rumen metabolism and animal performance (Patra and Saxena, 2011).

It is generally agreed that tannins decrease the rate of protein degradation in the rumen, which may occur due to the formation of tannin–protein complexes in the rumen pH and inhibition of the growth and activities of proteolytic bacterial populations (Muller-Harvey, 2006). Condensed tannins in varies forages slowed down the rates of both solubilisation and degradation of forage protein to ammonia (McNabb et al., 1996; Min et al., 2005). Therefore, a reduction of protein degradation in the rumen will increase the quantity of protein digested in the small intestine (Patra and Saxena, 2011). Nevertheless, shifting excretion pattern of nitrogen from urine to faeces and formation of CT–protein complex are beneficial environmentally. First, faecal nitrogen is mainly in the organic form, which is less volatile, whereas urinary nitrogen is largely in the form of urea, which is more rapidly hydrolysed to ammonia and nitrified to nitrate (Misselbrook et al., 2005; Eckard et al., 2010). Most of studies have shown that the apparent digestion of soluble carbohydrates appears to be

unaffected by tannins (Barry et al., 1986; Waghorn et al., 1987), however, McAllister et al. (2005) showed that CTs from different legume forages had a varied extent of inhibition of rumen fibrolytic bacteria, *Fibrobacter succinogenes*. Tannins with low molecular weight had greater inhibitory effects on rumen microbes. The reduced rate of carbohydrate digestion, especially fibre, may decrease total volatile fatty acid concentrations in the rumen (Beauchemin et al., 2007; Patra et al., 2006)

Free-gas or feed-lot bloat occurs sporadically in feed-lot cattle, but frothy bloat is more common in ruminants grazing legume forages (Cheng et al., 1998) or immature wheat pasture (Min et al., 2005). Tannins have the ability to precipitate soluble protein fractions which have been implicated in the reduction of bloat grazing CT-containing legume forages (McMahon et al., 2000). A reduction in severity of bloat due to feeding CTs has been attributed to reduction of microbial activities, biofilm production, and ruminal gas production (Min et al., 2006). Besides feeding tannin-containing forages, tannin extract can be fed to animals to prevent bloat problems (Min et al., 2006).

The effects of tannins on rumen microbial populations have been recently discussed in detail (Patra and Saxena, 2009). The effects of tannins on ruminal bacteria are reported to be dependent upon the species of microorganism and type or source of tannin (Jones et al., 1994; Sivakumaran et al., 2004). The antimicrobial activities of tannins are ascribed to the interactions of tannins with the extracellular enzymes secreted and the cell wall of bacteria causing morphological changes of the cell wall, tannin-induced membrane disruption, direct action on microbial metabolism, deprivation of substrates for microbial growth and chelation of cations by tannins reducing its availability to microbes (Kumar and Vaithiyanathan, 1990; Jones et al.,

1994; Smith et al., 2005). Vary forms of tannins have varies inhibiting effects of the cellulolysis and zoospore attachment to cellulose by fungus *Neocallimastix frontalis* strain RE1, however, ability of fiber degradation of rumen fungi may be less sensitive to the inhibitory effects of CT compared to cellulolytic bacteria (McSweeney et al., 1998). Nevertheless, the effects of tannins on rumen protozoa are variable and the mechanisms of inhibition of rumen protozoa are not known (Patra and Saxena, 2011).

Tannin-containing forages and tannin extracts have been demonstrated to decrease methane production both *in vivo* and *in vitro* (Patra and Saxena, 2011), and because of tannins have been shown to lower protozoal numbers, therefore, it may also decrease protozoal-associated methanogenesis. Overall, the inhibitory effects of tannins on rumen methanogenesis have been ascribed to direct effects on methanogenic archaea, protozoal-associated methane production and indirectly through a depression of fiber digestion in the rumen (Patra and Saxena, 2011).

2.6.2 Biological properties of CTs as affected by their differing molecular weight (MW)

Factors found to affect the major biological property of CTs, the ability to precipitate protein, include the type of condensed tannins, the type of protein and the environment in which the reaction occurs (Osborne and McNeill, 2001). The report suggests that the larger CTs have a greater binding ability (Porter and Woodruffe, 1984). In addition, because of hydrogen bonding is implicit in the interaction of phenolic groups with protein, therefore, the pH of the reaction environment will have a major effect on the degree of binding (Makkar and Becker, 1996).

According to biological properties of condensed tannins as affected by their

molecular weight, many studies have suggested that larger-sized (higher molecular weight) condensed tannin could precipitate more protein than smaller-sized condensed tannin. Osborne and McNeill (2001) studied the binding affinities of total extractable CTs from *Leucaena* species as compared to reference CTs from *Acacia aneura* and *Lotus pedunculatus* and tannic acid, they have been demonstrated that the larger-sized CT of the accessions *L. pallida* and *L. trichandra* shown higher protein binding affinity than the smaller-sized CT (Table 2.1). In addition, differing molecular weights of hybrid-*Leucaena* and local *Leucaena* (Huang et al., 2010) and fractions of CTs from *Leucaena*-hybrid (Huang et al., 2011) have been demonstrated that protein-binding affinity of CTs increased with increasing of molecular weight.

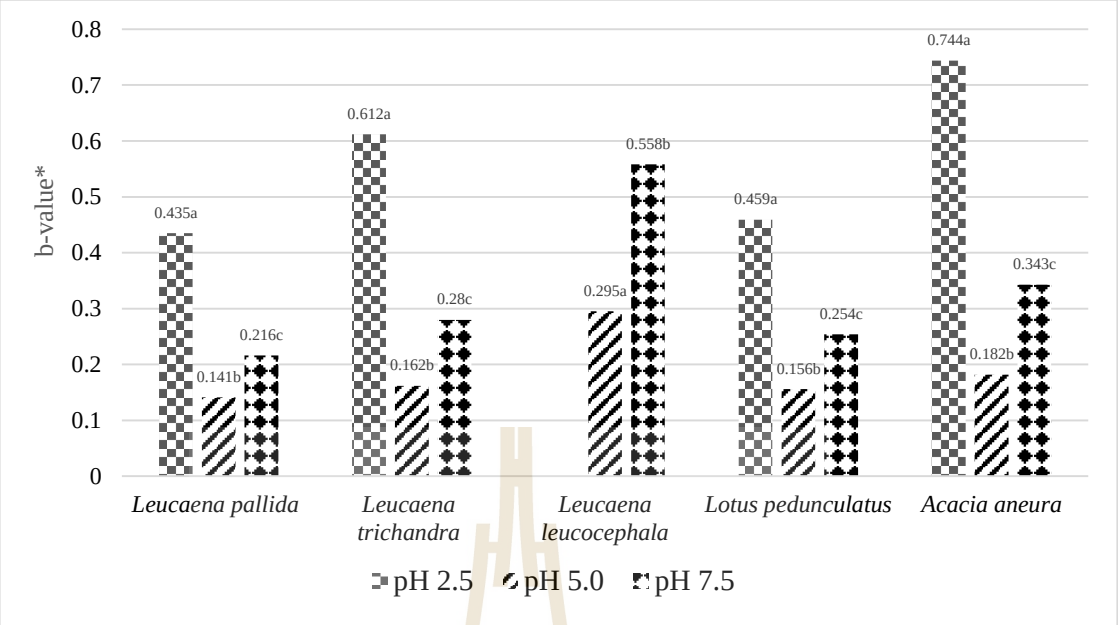
Additionally, ability of tannin to precipitate to protein, however, affected by the reaction environment particularly pH. Osborne and McNeill (2001) suggested that increasing or decreasing the pH of the reaction solution away from pH 5.0 (approximately the isoelectric point of the protein) reduced the ability of condensed tannins to precipitate protein, the decrease being higher at pH 2.5 than at pH 7.5 (Figure 2.4).

Table 2.1 Protein-binding affinities as affected by differing molecular weight of CTs

Sources of CT, molecular weights	**Mw[/1] (range)**	**b-value[/2]**	**References**
Leucaena pallida	larger-sized	0.141[a]	Osborne and McNeill (2001)
Leucaena trichandra	larger-sized	0.162[a]	
Leucaena leucocephala		0.295[c]	
Lotus pedunculatus		0.156[a]	
Acacia aneura		0.182[ab]	
Tannic acid		0.217[b]	
Leucaena-hybrid Bahru (LLB)	2737 (251-31,623)	0.305	Huang et al. (2010)
Local *Leucaena leucocephala* (LLL)	2871 (282-31,623)	0.420	
LLB-fraction-1	1349 (1203–1566)	0.381[a]	Huang et al. (2011)
LLB-fraction-2	857 (848–1203)	0.510[b]	
LLB-fraction-3	730 (726–740)	0.580[bc]	
LLB-fraction-4	726 (726)	0.636[c]	
LLB-fraction-5	495 (442–832)	0.780[d]	

Notes: [/1]Mw=weight-average molecular weight (Da), [/2]The b-value, the CT quantity that is needed to bind half of the maximum precipitable BSA, is used to denote the protein-binding affinity of CTs in this study. That is, when the b value is smaller, the protein-binding affinity of the CTs is stronger

[a,b,c,d] Means with different letters in the same column are significantly different ($P < 0.05$) (within each study)



*the *b*-value, the CT quantity that is needed to bind half of the maximum precipitable BSA, is used to denote the protein-binding affinity of CTs in this study. That is, when the b value is smaller, the protein-binding affinity of the CTs is stronger

[a,b,c] Means with different letters in the bars within the same extracts species are significantly different ($P < 0.05$)

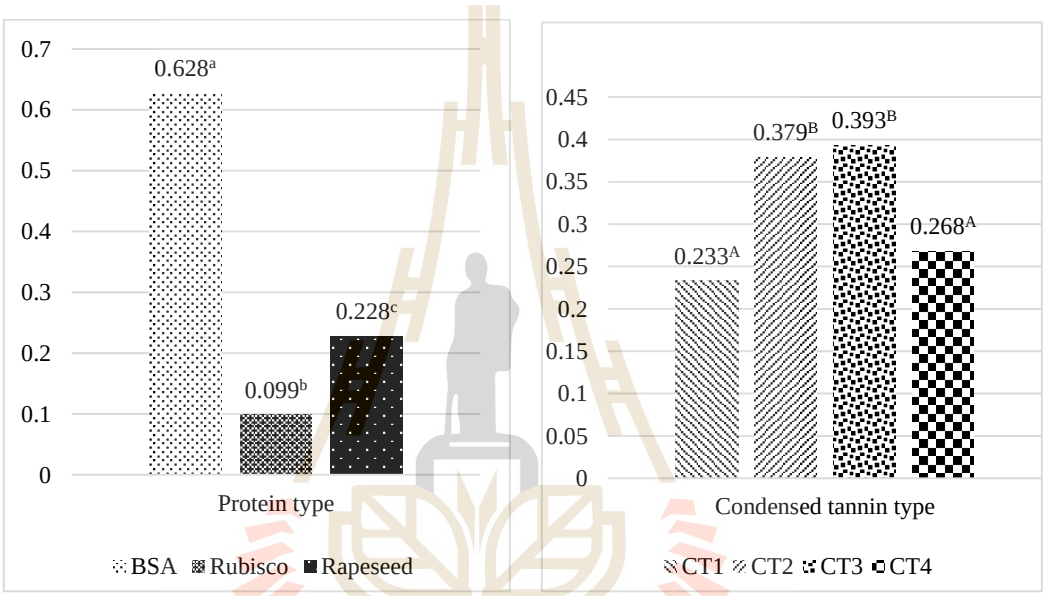
No *b*-value of *L. leucocephala* CT at pH2.5 calculated as maximal precipitation of protein not achieved

Figure 2.4 Protein-binding affinities (b-value) of total extractable CT at pH 2.5, 5.0 and 7.5 from *Leucaena* species as compared to non-*Leucaena* forage legumes.

Source: Adapted from Osborne and McNeill (2001).

Moreover, recently study by Lorenz et al. (2013) has been compared effects of four contrasting CT types isolated from sainfoin (*Onobrychis viciifolia*) (varied in mean of Degree of Polymerization [mDP], proportion of prodelphinidins and trans-flavanol subunits) on the precipitation of proteins extracted from two plants with

bovine serum albumin (BSA) included as a control and indicated that not only structure of condensed tannins affected on protein-binding ability, protein type also be importance factor in tannin-protein interactions (Figure 2.5), however, the authors also suggested that the tree-dimensional structures of proteins may be more important factors in tannin-protein interactions.



[a,b,c] Values followed by lower-case superscript letters are differ ($P < 0.05$)

[A,B] Values followed by upper-case superscript letters are differ ($P < 0.05$)

CT1; mDP = 18.9±0.71, PDs:PCs = 0.683:0.317, *cis*-:*trans*- = 0.841:0.159

CT2; mDP = 16.0±0.80, PDs:PCs = 0.883:0.117, *cis*-:*trans*- = 0.773:0.227

CT3; mDP = 32.3±1.41, PDs:PCs = 0.717:0.283, *cis*-:*trans*- = 0.756:0.244

CT4; mDP = 76.5±0.38, PDs:PCs = 0.865:0.135, *cis*-:*trans*- = 0.725:0.275

When mDP=mean degree of polymerization, PDs=prodelphinidins, PCs=procyanidins

Figure 2.5 Proportion of bovine serum albumin (BSA), Rubisco and rapeseed protein remaining in solution after incubation with different condensed tannin types

Source: Lorenz et al. (2013)

2.6.3 Effects of MW of CTs on ruminal fermentation

Many researches have been suggested that different sources (different structure) and levels of tannins effected on ruminal microorganisms and fermentation parameters. In particularly, inhibitory effect of tannins on ruminal methanogens and methane production have recently attention. Recently researches have been indicated that different sources (and molecular weights) of tannins has different effect on ruminal methane production (Animut et al., 2008a; Huang et al., 2011) and methane production also decreased with increasing of level of condensed tannins (Animut et al., 2008b; Huang et al., 2010; Tan et al., 2011) (Table 2.2).

Moreover, the effects of tannins on *B. fibrisolvens* have also been investigated by Jones et al. (1994), who found *in vitro* that tannins from sainfoin leaves inhibit the growth and activity of *B. fibrisolvens* A38 by causing changes in the bacteria's morphology. This concept was confirmed by study of Huang et al. (2011) who fractionated CTs from *Leuceana leucocephala* hybrid into five fractions by a size exclusion chromatography procedure, and determined the molecular weights and protein-binding affinities of the CT fractions, and indicated that, in general, CTs of higher molecular weight fractions have stronger protein-binding affinity than those of lower molecular weights, and in additionally, CT fractions with the highest MW had in the highest inhibition of CH_4 production, being 62% lower than the control (Huang et al., 2011). In addition, Tan et al. (2011) also reported linear reductions in total methanogens and total protozoa with increasing levels of CT (Table 2.3).

Table 2.2 Effect of sources (and molecular weights) and levels of condensed tannins on rumen fermentation parameters.

Sources/level of CTs	Type of study	Rumen fermentation parameters			
		Gas production (ml/g DM)	CH_4 (ml/g DM)	IVDMD[/1]	c[/2] ml/h
Animut et al. (2008a)	*in vivo (goat)*				
Sericea lespedeza (S)		-	14.6	0.698	
Kobe lespedeza (K)		-	14.1	0.648	
K+ quebracho tannin (K+Q)		-	10.9	-	
KS		-	12.1	-	
Huang et al. (2011)	*in vitro*				
Leucaena leucocephala CT[/3]					
Control (without CT)		65.8[a]	12.1[a]	0.277[bc]	0.045[a]
495 (442–832) of MW		57.5[b]	9.5[b]	0.303[a]	0.021[c]
726 (726) of MW		55.5[b]	9.7[b]	0.283[abc]	0.025[b]
730 (726–740) of MW		56.0[b]	7.8[c]	0.287[ab]	0.021[c]
857 (848–1203) of MW		51.8[c]	5.6[d]	0.277[bc]	0.020[c]
1349 (1203–1566) of MW		49.8[c]	4.6[e]	0.263[c]	0.012[d]

[1/]IVDMD=*in vitro* dry matter digestibility, [2/] c=the rate of gas production, [3/]CT=condensed tannins fractions supplemented at 15 mg/500 mg DM.

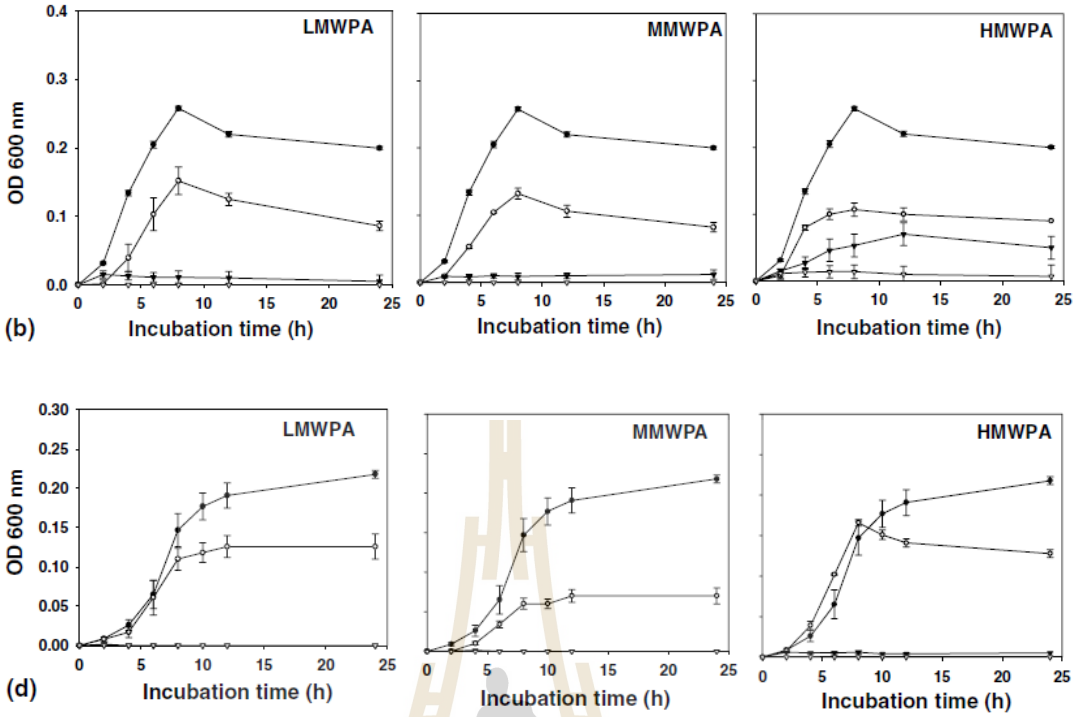
K=Kobe lespedeza (*Lespedeza striata*); KQ=Kobe lespedeza plus quebracho tannin substituted for 50 g/kg DM intake; S=Sericea lespedeza (*Lespedeza cuneata*); KS=1:1 mixture of Kobe and Sericea lespedeza.

Table 2.3 Effect of concentrations of CTs from *Leucaena leucocephala* on ruminal methanogens and protozoa

Items	**CT level (mg/500 mg DM)**						***P*-value**
	0	**10**	**15**	**20**	**25**	**30**	**Linear**
Microbiological methods							
Total methanogens (×10^7 cells/ml)	1.62	1.22	1.90	0.02	0.09	0.08	*<0.01*
Total ciliate protozoa (×10^6 cells/ml)	9.80	1.37	1.70	3.75	4.37	4.38	*<0.01*
16S rRNA gene copies/ml							
Total methanogens (×10^7)	3.25	3.24	2.89	2.30	2.58	1.65	*<0.01*
18S rRNA gene copies/ml							
Total protozoa (×10^7)	5.83	4.73	4.33	4.98	4.41	4.11	*<0.01*

Source: Adapted from Tan et al. (2011).

In addition, Sivakumaran et al. (2004) demonstrated that all three fractions (i.e. low, medium and high molecular weight) of proanthocyanidins from *Dorycnium rectum* forage inhibited the growth of *C. proteoclasticum* at concentrations of 100, 200 and 300 mg L^{-1} of *in vitro* medium, whereas although low and medium molecular weight fractions inhibited the growth of *B. fibrisolvens* at these concentrations, the high molecular weight fraction stimulated the growth of this bacterium at the concentration of 100 mg L^{-1} (Figure 2.6).



(b) *Clostridium proteoclasticum*, and (d) *Butyrivibrio fibriosolvens* CF3 in the absence (●) and presence of 100 (○), 200 (▼), and 300 (▽) μg/ml.

Values are the means of duplicate cultures and the vertical bars represent standard errors of the mean.

Figure 2.6. *In vitro* activity of three proanthocyanidin fractions, low molecular weight proanthocyanidin (LMWPA) fraction, medium molecular weight proanthocyanidin (MMWPA) fraction and high molecular weight proanthocyanidin (HMWPA) fraction, from *Dorycnium rectum* leaves on the growth of the rumen biohydrogenating involved bacteria

Source: Adapted from Sivakumaran et al. (2004)

Tannins are generally regarded as inhibitory to the growth of rumen micro-organisms (Patra and Saxena, 2011). Hence, inhibitory activity of tannins against bacteria has been implicated due to the ability of tannins to form complexes with the

cell wall and membrane of bacteria causing morphological changes of the cell wall and the extracellular enzymes secreted (Jones et al. 1994). This selective activity of tannins to rumen bacteria could be beneficial nutritionally by altering the ruminal biohydogenation process and hence enhancing CLA content of ruminant-derived products (Patra and Saxena, 2011).

2.6.4 Selectively inhibitory effects of tannins on bacteria involved in ruminal biohydrogenation

As described previously, the ability of plant extracts including tannins to modify the fatty acid composition of ruminant-derived food products (i.e. milk or meat) has received great attention recently. The CTs have been shown to inhibit the growth of many ruminal bacteria (Jones et al., 1994; Patra and Saxena, 2009) including bacteria associated with ruminal biohydrogenation.

Recently, Vasta et al. (2010) demonstrated that inclusion of quebracho tannin (9.57 % of DM intake) to a concentrate and hay (lucerne) diet for sheep increased the population of *Butyrivibrio fibrisolvens* (8.76 vs. 4.22% of total bacteria), while the growth of *Clostridium proteoclasticum* (2.77 vs. 3.99% of total bacteria) reduced, and they have suggested that while some bacterial strains were reduced by tannins, others could benefit, which would explain the increase in the population of *B. fibrisolvens* in the rumen of the tannin-fed lambs. However, the total number of bacteria was not affected in their study, while the number of protozoa was higher in the rumen of the lambs supplemented with tannins than in the animals not supplemented (Table 2.4). Similarly to Durmic et al. (2008), who found that *B. proteoclasticus* strain P18 was more sensitive to a tannin-rich plant (*Acacia mearnsii*) extract than *B. fibrisolvens*

JW11, they were screened several plants in Australia for their ability to modify ruminal biohydrogenation process; it was found that a wide range of the plant extracts had selective inhibitory effect towards *C. proteoclasticum* (which forms SA from LA) without affecting *B. fibrisolvens* (which forms CLA and VA acid, but not SA). However, only a few plants, including *Acacia iteaphylla* and *Kennedia eximia*, inhibited LA metabolism or SA formation in the mixed bacterial community *in vitro*, and the active components of these plants have not been elucidated.

Table 2.4 Effect of tannins on ruminal biohydrogenating bacteria

	Microbial population			
Items	No. of bacteria[1/]	B. *fibrisolvens*[2/]	*C. proteoclasticum*[2/]	No. of protozoa[1/]
Vasta et al. (2010)[3]				
Concentrate	9.55	4.22[a]	3.99	5.01[a]
Conc.+ tannins	9.64	8.76[b]	2.77	7.82[b]

[a,b] Means with different letters in the same column are significantly different ($P < 0.05$).

[1/] $\log_{10}$ copies/g fresh matter, [2/] % total bacteria, [3/] Using quebracho (*Schinopsis lorentzii*) powder as source of tannins (at 9.57 % of DM intake) in sheep

2.6.5 Fatty acid compositions of ruminant-derived food products as affected by tannins

Khiaosa-Ard et al. (2009) reported that addition of CT (7.9 % of DM) inhibited the last step of linolenic acid (LNA) biohydrogenation in RUSITEC. This inhibition led to the accumulation of vaccenic acid (VA) in the feed residues but had no effect on the losses of LNA compared with the control treatment. Vasta et al. (2009a) also reported that the concentration of VA increased while concentration of total

conjugated linoleic acid (CLA) did not increase in *in vitro* ruminal fluid (Table 2.5). Moreover, in *in vivo* studies, indicated that addition of quebracho tannins in the diet of sheep resulted in an increased concentrations of VA in the rumen (Vasta et al., 2009b; 2010; Rana et al., 2012) and rumenic acid (RA) and poly unsaturated fatty acids (PUFA) in lamb (Vasta et al., 2009b; Rana et al., 2012) (Table 2.6).

In addition, in vivo study by Rana et al. (2012) found that the concentration of RA (C18:2 *c*9, *t*11) in goat kid groups supplemented with extract tannins of *T. chebula* at 1.06 and 3.18 g/kg BW were higher ($P \leq 0.05$) than in the control group (Table 2.7). The concentration of VA was higher ($P \leq 0.01$) in group supplemented with 3.18 g tannins/kg BW compared to another groups, while the stearic acid (SA) concentration was lower ($P \leq 0.05$) in group supplemented with 3.18 g tannins/kg BW than control and supplemented with 1.06 g tannins/kg BW. However, there was found that supplementation of quebracho tannins to lambs at 95.7 g/kg DM did not affected on fatty acid profile of plasma (Vasta et al., 2009b). Decreased saturated fatty acid (SFA) and increased unsaturated fatty acids (UFA) in *T. chebula* extract supplemented groups indicated an influence on rumen biohydrogenation and resultant higher absorption of the UFA as evident from the fatty acid profile of blood and muscle. It is also suggested from the present investigation that *T. chebula* extract affects rumen biohydrogenation as well as protects the UFA in plasma and tissues from lipid peroxidation (higher enzymatic and nonenzymatic antioxidative activity) which increases the animal product quality (Gladine et al., 2007 referred by Rana et al., 2012).

Table 2.5 Effect of tannins on fatty acid profile of ruminal fluid and biohydrogenation (*in vitro*)

Items	**Fatty acids composition (% of total fatty acids)**					
	RA	**Total-CLA**	**VA**	**SA**	**LA**	**LNA**
Khiaosa-Ard et al. (2009) (RUSITEC)[1/]						
Control (without tannins)	-	1.83	11.7[b]	50.0[a]	4.78	4.32
Tannin-containing forage (sainfoin, 8.12 % condensed tannins in plant DM)	-	2.43	7.6[b]	48.4[a]	5.54	6.81
Tannin extract (from *Acacia mearnsii*, 61.5 % condensed tannins in DM)	-	2.22	30.7[a]	26.4[b]	4.74	4.76
Vasta et al. (2009a) (*in vitro*)[2/]						
0.0 mg of tannins/ml	0.280	0.556	2.692[b]	30.114[a]	2.608	1.524[b]
0.6 mg of tannins/ml	0.245	0.473	3.768[a]	30.708[a]	2.938	2.102[a]
1.0 mg of tannins/ml	0.297	0.453	3.327[ab]	25.350[b]	2.738	1.350[b]

[a,b] Means with different letters in the same column are significantly different ($P < 0.05$).

[1/] Rumen Simulation Technique (RUSITEC), using ruminal fluid obtained from rumen-fistulated lactating Brown Swiss cow fed diet consisting of hay and concentrate in a ratio of 1.5:1; the condensed tannins supplementation level was kept at the same daily amount at 1.12 g/d.

[2/] For each tannins source (carob, *Ceratonia siliqua*; acacia leaves, *Acacia cyanophylla* and quebracho, *Schinopsis lorentzii*) was added at two different levels (mg/ml of buffered ruminal fluid, BRF), and using ruimal fluid from rumen-fistulated non-lactating Friesian–Holstein cow.

RA= *cis*-9, *trans*-11 CLA (rumenic acid), CLA=conjugated linoleic acid, VA=*trans*-11 C18:1 (vaccenic acid), SA=C18:0 (stearic acid), LA=*cis*-9, *cis*-12 C18:2*n*-6 (linoleic acid), LNA= *cis*-9, *cis*-12, *cis*-15 C18:3*n*-3 (linolenic acid).

Table 2.6 Effect of tannins on fatty acid profile of ruminal fluid and biohydrogenation (*in vivo*)

Items	**Fatty acids composition (% of total fatty acids)**					
	RA	**Total-CLA**	**VA**	**SA**	**LA**	**LNA**
Vasta et al. (2009b) (sheep, age: 45-105 d)[1/]						
Herbage (Vetch, *Vicia sativa*; no tannins)	1.09	1.50	3.19[a]	39.5[a]	2.17	1.48
Herbage + tannins (40.6 g/kg DM)	1.41	1.76	3.82[ab]	38.6[a]	2.00	1.68
Concentrate	0.44	1.48	2.80[b]	39.0[a]	1.41	0.42
Concentrate + tannins (40.4 g/kg DM)	1.03	1.91	5.51[a]	19.9[b]	2.72	0.68
Vasta et al. (2010) (Lamb, 45 to 115 d)[2/]						
Concentrate	-	0.23	1.47[a]	50.45	1.17	0.21
Conc.+quebracho tannins (95.7 g/kg DM)	-	0.66	3.06[b]	43.92	1.83	0.28
Rana et al. (2012) (goat, 5-6 to 8-9 months)[3/]						
Control	1.04[a]	1.08[a]	1.12[a]	29.48[b]	-	0.15[a]
1.06 g extract of *T. chebula* /kg BW	1.23[b]	1.27[b]	1.71[b]	23.18[a]	-	0.15[a]
3.18 g extract of *T. chebula* /kg BW	1.23[b]	1.26[b]	2.05[b]	22.07[a]	-	0.32[b]

[a,b] Means with different letters in the same column are significantly different ($P < 0.05$).

[1/] Using quebracho powder as source of tannins (456 g of equivalent tannic acid/kg DM).

[2/] Using quebracho (from *Schinopsis lorentzii*) powder as source of tannins

[3/] Supplemented with aqueous extract of *Terminalia chebula*, and fatty acids composition were expressed in g/100g of FAME

RA= *cis*-9, *trans*-11 CLA (rumenic acid), CLA=conjugated linoleic acid, VA=*trans*-11 C18:1 (vaccenic acid), SA=C18:0 (stearic acid), LA=*cis*-9, *cis*-12 C18:2*n*-6 (linoleic acid), LNA= *cis*-9, *cis*-12, *cis*-15 C18:3*n*-3 (linolenic acid).

Table 2.7 Effect of tannins on plasma fatty acids

Items	Fatty acids composition (% of total fatty acids)					
	RA	**Total-CLA**	**VA**	**SA**	**LA**	**LNA**
Vasta et al. (2009b) (sheep, *in vivo*)[1]						
Herbage (Vetch, *Vicia sativa*; no tannins)	0.52	0.55	0.402	23.3	11.4	4.21
Herbage + tannins (40.6 g/kg DM)	0.33	0.37	0.316	22.7	16.3	6.21
Concentrate	0.29	0.39	0.290	20.3	15.3	1.25
Concentrate + tannins (40.4 g/kg DM)	0.46	0.62	0.241	18.7	18.9	2.18
Rana et al. (2012) (goat, 5-6 to 8-9 months)[2]						
Control	0.56^{a}	0.58^{a}	0.29^{a}	25.96^{b}	-	0.98^{a}
1.06 g extract of *T. chebula* /kg BW	0.69^{b}	0.72^{b}	0.31^{a}	24.88^{b}	-	1.45^{ab}
3.18 g extract of *T. chebula* /kg BW	0.70^{b}	0.75^{b}	0.39^{b}	17.26^{a}	-	1.61^{b}

^{a,b} Means with different letters in the same column are significantly different ($P < 0.05$).

[1/] Using quebracho powder as source of tannins (456 g of equivalent tannic acid/kg DM)

[2/] Supplemented with aqueous extract of *Terminalia chebula*, and fatty acids composition were expressed in g/100g of FAME

RA= *cis*-9, *trans*-11 CLA (rumenic acid), CLA=conjugated linoleic acid, VA=*trans*-11 C18:1 (vaccenic acid), SA=C18:0 (stearic acid), LA=*cis*-9, *cis*-12 C18:2*n*-6 (linoleic acid), LNA= *cis*-9, *cis*-12, *cis*-15 C18:3*n*-3 (linolenic acid)

Rana et al. (2012) reported that the RA concentration of *Longissimus dorsi* muscle was higher ($P \leq 0.01$) in goat fed tannins than in the control group, while total CLA concentration was significantly higher ($P \leq 0.01$) in group fed highest level of tannins. The SA content in muscle of kids of group supplemented with 3.18 g tannins/kg BW was significantly ($P \leq 0.05$) lower than in control group. However, study by Vasta et al. (2009b) was found that supplementation of quebracho tannins to lambs at 95.7 g/kg DM did not affected on fatty acid profile of this muscle (Table 2.8).

Although supplemented with tannins shown an affected to fatty acid composition of ruminant meat, however, studies by Toral et al. (2011; 2013) demonstrated that inclusion of tannins in diet not affected on milk fatty acid composition, and they were suggested that addition of quebracho tannins to a diet rich in linoleic acid did not prove useful to beneficially modify milk FA composition, especially over the long term, and structural and chemical dissimilarities between HT and CT may offer an explanation for differences in their biological effects and, therefore, results obtained using a particular type of tannins cannot be applied to others. A key issue when using plant extracts as feed additives is dosage (Toral et al. (2011). Most doses of tannins evaluated in the studies quoted above ranged from 4.5 g/kg DM (Benchaar et al., 2008) to 44.5 g/kg DM (Wang et al., 1996).

Table 2.8 Effect of tannins on fatty acid compositions of ruminant-derived products

Items	Fatty acids composition (% of total fatty acids)					
	RA	Total-CLA	VA	SA	LA	LNA
Meat (*longissimus dorsi* muscle)						
Vasta et al. (2009b) (sheep)[1]						
Herbage (Vetch, *Vicia sativa*; no tannins)	0.82	-	1.31	14.78[a]	8.2[b]	2.42
Herbage + tannins (40.6 g/kg DM)	0.50	-	1.04	14.77[a]	14.5[a]	3.52
Concentrate	0.46	-	0.69	14.18[a]	9.2[b]	1.00
Concentrate + tannins (40.4 g/kg DM)	0.96	-	1.32	11.51[b]	10.7[ab]	1.38
Rana et al. (2012) (goat, 5-6 to 8-9 months)[2/]						
Control	0.60[a]	0.63[a]	0.33	16.31[a]	-	1.29
1.06 g extract of *T. chebula* /kg BW	0.71[b]	0.75[b]	0.36	14.53[ab]	-	1.01
3.18 g extract of *T. chebula* /kg BW	0.97[c]	1.00[c]	0.38	12.17[b]	-	1.14
Milk						
Toral et al. (2011)[3/]						
Control (TMR+20g sunflower oil/kg DM)	2.15	2.26	0.43	7.62	1.92	0.31
Control diet plus 10 g of tannins/kg DM	2.07	2.16	0.43	7.92	2.00	0.33
Toral et al. (2013)[4/]						
Control	1.936	-	4.670	9.108	3.151	0.624
Quebracho tannin 20 g/kg DM	2.099	-	5.233	9.419	3.218	0.637

[a,b,c] Means with different letters in the same column are significantly different ($P < 0.05$).

[1/] Using quebracho powder as source of tannins (456 g of equivalent tannic acid/kg DM),

[2/] Supplemented with aqueous extract of *Terminalia chebula*, and fatty acids composition were expressed in g/100g of FAME, [3,4/] Studied in dairy ewe.

RA= *cis*-9, *trans*-11 CLA (rumenic acid), CLA=conjugated linoleic acid, VA=*trans*-11 C18:1 (vaccenic acid), SA=C18:0 (stearic acid), LA=*cis*-9, *cis*-12 C18:2*n*-6 (linoleic acid), LNA= *cis*-9, *cis*-12, *cis*-15 C18:3*n*-3 (linolenic acid)

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CHAPTER III

STUDY ON MOLECULAR WEIGHT AND PROTEIN-BINDING ABILITY OF CONDENSED TANNINS OF SOME TROPICAL PLANT MATERIALS

3.1 Abstract

The concentrations of phenolic compounds and molecular weights (MW) of condensed tannins from tropical plant materials which have potential to be used as ruminant feed and their effects on *in vitro* gas, including methane, production were investigated. Leaves of three plant species; namely leucaena (*Leucaena leucocephala*), cassava (*Manihot esculenta*, Cranzt), and Siamese neem (*Azadirachta indica* A. Juss. var. Siamensis Valeton), fruit by-product, 'mangosteen (*Garcinia mangostana*) peel' and commercial condensed tannins extract, quebracho tannins were used in this study. Condensed tannins (CTs) contents in tropical plant leaves ranged from 1.2 % DM in the leucaena to 5.0% in Siamese neem ($P<0.001$), whereas mangosteen peel and quebracho CTs extract contained higher concentration of CTs (14.38 and 67.34 %DM, respectively) ($P<0.001$). The weight-average molecular weight (M_w) of the purified condensed tannins determined using gel permeation chromatography was 2,964, 3,222, 3,409, 3,539 and 3,612 Da for mangosteen peel, leucaena, cassava, quebracho CT extract and Siamese neem, respectively. In addition, the result in this study was found that CTs extracted from Siamese neem which had the highest weigh-average molecular

weight (M_w), had a highest amount of protein precipitated, whereas, CTs extracted from mangosteen peel had the lowest M_w, had a lowest protein-binding capacity. In general, these results indicate that CTs of higher molecular weight have greater protein-binding ability than those of lower molecular weight.

Key words: Forage, Phenolic compounds, Biological property, Bovine serum albumin

3.2 Introduction

In the tropical regions, utilization of tannins-containing plant materials for ruminant feeding and rumen manipulation is received greater interest, due to both its potential in improvement of animal performances and the shortage and rising costs of the conventional feed stuffs. Plant secondary metabolites (PSM) including condensed tannins (CTs) are widely distributed in forages, shrubs, legumes, and also cereals and grains. Although condensed tannins that presents in these plant materials resulting in adverse anti-nutritional effects to the animals which consumed them and have been identified as a major factor in limiting the nutritive value and feeding quality of those plant materials since these secondary metabolites have been reported to cause a variety of detrimental effects, such as decreased feed palatability, voluntary intake and nutrients digestibility (Osborne and McNeill, 2001), however, in ruminant nutrition, condensed tannins have been recognized to be beneficial useful phyto-chemicals for rumen manipulation to increase rumen-undegradable protein supply to the small intestine, bloat prevention (Waghorn, 2008), anthelmintic property (Athanasiadou et al., 2001) and mitigating methane emission (Patra and Saxena, 2011).

Effects of CTs on ruminal fermentation parameters have been varied among studies, and primarily depending on their sources and concentrations used. However,

recent studies seem to suggest that chemical structure and molecular weight of this metabolite have greater influence on their major biological property, protein-binding ability and their efficacy to manipulate rumen fermentation including mitigation of methane production (Huang et al, 2010; Huang et al, 2011). Therefore, this study was purposed to determine tannins conentrations, molecular weight of some tropical plant materials and commercial CTs extract which have potential in rumen fermentation manipulation and as feed supplement for ruminant livestock

3.3 Objective

The objective of this study was to characterize the concentration, molecular weight distribution and protein-binding ability of CTs of some tropical plant materials and commercial CTs extract.

3.4 Materials and methods

3.4.1 Preparation of sample

Tropical available forage and plant materials were selected from their potential use as dietary source of tannins for dairy goat including (i) potentially use as ruminant feeds, and (ii) probably high condensed tannin content. Whereas commercially available condensed tannins extract also has been selected.

Four tropical available forage species and plant material including of 1) leucaena (*Leucaena leucocephala*) (LL), 2) cassava (*Manihot esculenta*, Cranzt) (CV), 3) Siam neem (*Azadirachta indica* A. Juss. var. siamensis Valeton) (SN), and 4) mangosteen (*Garcinia mangostana*) fruit peel (MSP) were selected. All selected tropical forages were harvested from the area of Nakhon Ratchasima Province, Thailand

(between 9:00 a.m. and 10:30 a.m.) by cutting tips of about 30 cm from the youngest fully expanded leaves from several trees. All harvested samples were immediately brought back to the laboratory, and then all leaves of forages were collected. Sample of mangosteen fruit was purchased from the market in the area of Nakhon Ratchasima Province. Similarly to forage samples, peel part of mangosteen fruit was separated and collected. All fresh samples were freeze-dried using a lyophilyzer (GAMMA 2-16 LSC, CHRiST®, Germany) prior to grinding through a 1.0 mm sieve, and stored at 4 °C in an airtight dark container pending further analysis. In addition, commercial available condensed tannin extract, Quebracho (QB) condensed tannin extract (UNITAN ATO, Buenos Aires, Argentina; 75-77 % tannins) which purchased from supplier in Thailand was also evaluated for tannins concentration, molecular weight and protein binding ability.

3.4.2 Preparation of sample extract

Tannins of tropical plant materials and quebracho tannin extract were extracted from dried-ground sample using aqueous acetone as described by FAO/IAEA (2000) with minor modification. In brief, 200 mg of dried-ground samples were dissolved in 10 mL of 70% (v/v) aqueous acetone and kept in a shakable-water bath (MEMMERT, WNB22) for 20 min at room temperature. Samples were then subjected to centrifugation for 10 min at approximately 3000×*g* at 4°C. After that, the supernatant were immediately collected and kept at 4°C for further analysis.

3.4.3 Determination of total phenols, total tannins and condensed tannins of tropical plant materials

Total phenols and total tannins of the samples were determined using the Folin-Ciocalteu method as described by FAO/IAEA (2000). For total phenols determination,

briefly, 0.1 mL of aliquots of the tannin-containing extract of each sample was added into test tubes, distilled water was then added to make up the volume to 0.5 mL, after that, 0.25 mL of the Folin-Ciocalteu reagent and then 1.25 ml of the sodium carbonate solution were added. Vortex the tubes and then absorbance at 725 nm after 40 min was read (Spectronic 200, Thermo Scientific). The amount of total phenols was calculate as tannic acid equivalent and express on a dry matter basis (x%).

In addition, by this method, total tannins were determined using polyvinyl polypyrrolidone (PVPP) as a phenolic binder. In brief, 100 mg of PVPP was weighed and transferred into a 100 x 12 mm test tube. Then, 1.0 mL of distilled water and then 1.0 mL of the tannin-containing extract were added. Test tube contained sample and reagents were mixed and vortex, and kept at 4°C for 15 min, vortex it again (the tannins have been precipitated along with the PVPP), then after, each sample was centrifuged at 3000×*g* for 10 min. Supernatant which has only simple phenolics other than tannins was collected and measured for the phenolic content followed the procedure as mentioned above by took triple volume which used for total phenol estimation. The content of non-tannin phenols were expressed on a dry matter basis (y%), and the percentage of tannins was calculated as equal to x-y and expressed as tannic acid equivalent on a dry matter basis.

Condensed tannins were determined using Vanillin-HCl method as described by Hagerman (2002). By this method, 1 mL of aliquots of each extract were added into test tube and incubated in the water bath. After that, 5.0 mL of the vanillin reagent was added at 1.0 minute intervals to one set of sample, and 5.0 mL of the 4.0 % HCl solution was added at 1.0 minute intervals to another set of sample (as blanks). All samples (including blanks) were incubated in the water bath for exactly 20.0 min, and then read

and recorded the absorbance at 500 nm (Spectronic 200, Thermo Scientific), and the value was compared to the standard curve (catechin equivalents).

3.4.4 Condensed tannins extraction and purification

The condensed tannins (CTs) from each plant materials and commercial CT extract were extracted and purified using the method of Terrill et al. (1992) as described by Huang et al. (2010) with minor modified. Freeze-drying ground samples (200 mg) were extracted in 200 mL of 70 % (v/v) aqueous acetone in shaker at room temperature for 20 min. All samples were centrifuged at 3000×*g* for 10 min, and the supernatant was collected and then filtered to remove any plant residues under vacuum. Chlorophyll, pigments, and low molecular weight phenolic acids were removed by washing with equal volume of diethyl ether in a separation funnel. CTs extracts were further evaporated under vacuum in a rotary evaporator (Rotavapor R-3, Büchi Labortechnic, Switzerland) at <40°C in order to remove traces of diethyl ether and acetone. Crude condensed tannins extracts of each samples were lyophilized (GAMMA 2-16 LSC, CHRiST®, Germany) and stored at 4°C in the dark.

Crude condensed tannin extracts were added with equal volume of 40 % (v/v) methanol prior to purify using Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) which equilibrated in methanol and packed in a 40cm×16mm column as described by Huang et al. (2010; 2011). The CT extract of each plant species were eluted with 40% (v/v) methanol to remove low molecular weight phenolic, and then the CTs were eluted with 80% (v/v) aqueous acetone. The purified CTs were evaporated to remove traces of aqueous acetone by using a rotary evaporator (Rotavapor R-3, Büchi Labortechnic, Switzerland). The purified CTs were freeze-dried and stored in the dark at 4 °C.

3.4.5 Determination of molecular weight of CTs

Molecular weight of the purified CTs from each plant species were determined by Gel Permeation Chromatography (RID-10A, Shimadzu, Columbia, MD, USA) with procedures as described by Huang et al. (2010) using a HSP gel RT 5.0 THF 3µm column (Waters, Milford, MA, USA) and tetrahydrofuran as the mobile phase (at 0.5 mL/min, 25°C). Purified CTs were dissolved in tetrahydrofuran (0.5 mg/mL) and 20 µL was injected. Relative weight-average molecular weight (M_w) were calculated after calibration with polystyrene (molecular weight standards) standards in the range of 162–22,000 Da. Polydispersity index (PDI) were calculated as the M_w divided by the number average molecular weight (M_n). Degree of polymerization (DP) was also estimated on the basis of a constituent proanthocyanidin peracetate M_w of approximately 500.

3.4.6 Determination of protein-binding ability of CTs

The protein-binding ability of the purified CTs from tropical plant materials and commercial CTs extract were determined by using a protein precipitation assay as described by Huang et al. (2010). Bovine serum albumin (BSA) was used to determine the ability of the CTs to bind protein. Different concentrations of BSA, from 0 to 1.2 mg/ml were used to generate the standard curve. The 0.5 ml of CTs from each materials at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.6 mg/ml dissolved in 500 ml/l aqueous methanol were prepared and then mixed with 0.5 ml BSA buffer (1 mg/ml of BSA in 0.2M acetate buffer with 0.17M NaCl at pH 5.0) for sample analysis. After centrifuged at 5000×g for 20 min, the supernatant of each sample was carefully discarded and the unbound protein was removed by washing with 0.2M acetate buffer. All the tubes were oven dried at 100 ◦C overnight. After that, the hydrolysis was carried

out by using 13.5M NaOH in the oven at 120 ◦C for 20 min. The tubes were then cooled after 1.0 ml acetic acid was gently added and an aliquot of 0.1 ml was added to 1.0 ml of ninhydrin solution. After 20 min of incubation in the water bath at 100 ◦C, the tubes were cooled and added with 5 ml of deionized water. The absorbance was recorded at 570nm after vortex using the spectrophotometer (Spectronic 200, Thermo Scientific). The equation for protein-binding data was analyzed using a nonlinear regression procedure. The curve for purified CTs was fitted to a sigmoid curve: $Y = a/(1 + b \times \exp^{(-c \times x)})$, where Y =mg of BSA precipitated and x = mg of extracted CTs incubated. The protein-binding ability of CTs was expressed as the amount of precipitated protein.

3.4.7 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) procedure. Means were separated using Duncan's procedure where differences were $P<0.05$ among treatments. All data were analyzed using SAS software (SAS Institute, 1996).

3.4.8 Location of the study

The study was conducted at the Center for Scientific and Technological Equipment Building 1 and 10, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.4.9 Experimental period

This study was carried out from August 2013 to November 2014.

3.5 Results and discussion

3.5.1 Phenolic compounds of some tropical plant materials

The concentrations of phenolic compounds of leaves of the three plant species, plant material and commercial CT extract are presented in Table 3.1. The concentration

of total phenols (TP), total tannins (TT) and condensed tannins (CT) of the leaves of tested plant species ranged from 3.36 to 6.38, 1.98 to 5.27 and 1.17 to 5.03 % of DM, respectively, and all the above values were lowest for leucaena and highest for Siamese neem (*P<0.001*). However, there was found that mangosteen peel contained significant higher (P<0.001) concentrations of phenolic compounds including TP, TT and CT (16.47, 15.48 and 14.38 % of DM, respectively) than those of leaves of selected plant species, whereas commercial quebracho CT extract contained highest concentration of condensed tannins (nearly 70 % of DM).

As pointed out earlier, there are increasing interests to use unconventional local feed materials to replace the traditional feed ingredients in livestock production in the developing countries due to shortage and high cost of the latter. Because of their high nitrogen content, leaves of leucaena, cassava and Siamese neem are among the local feed resources used for livestock, including ruminant production in the tropical countries (Paengkoum, 2010). In addition to their nutrient content, the presence of secondary phenolic compounds in the above mentioned feed forages has shown to exhibit beneficial capability to increase flow of rumen undegraded protein (Paengkoum, 2011) and mitigating methane production (Beauchemin et al., 2007).

According to concentration of some phenolic compounds of selected plant materials, results of this study showed that, among the leaves of tropical plant species, Siamese neem had the highest concentrations of total phenols, total tannins and condensed tannins followed by cassava with leucaena contained the lowest concentrations of the above compounds. The concentration of condensed tannins in leucaena (1.17 % of DM) recorded in the present study was lower than the 2.76% for leucaena Assession, UHK636 reported by Osbourne and McNeill (2001). However, the

concentrations of condensed tannins of cassava leaves (2.92 % of DM) and Siamese neem leaves (5.03 % of DM) obtained in this study were lower than those reported by Wanapat (2003) (4.3 % of DM for cassava leaves); Paengkoum et al. (2013) (3.9 % of DM for leucaena) and Ngamsaeng et al. (2006) (11.4 % of DM for Siamese neem), respectively. Mangosteen peel is another source for CTs used in ruminant feeding manipulation since it contain high concentration of condensed tannins and saponin. Mangosteen is a common fruit in the South-east Asian countries, particularly in the southern region of Thailand. Concentration of tannins of manosteen peel obtained from this study (15.48 and 14.38 % of DM for total tannins and CTs, respectively) is within the range that have been reported at 14.1 % total tannins (Moosophin et al., 2010), 7.0-15.0 % crude tannin (Ngamsaeng et al., 2006; Kanphakdee and Wanapat, 2008), 15.4 % of CT (Paengkoum et al., 2015), and 17.7 % CT (Norrapoke et al., 2014). Generally, concentration of condensed tannins is affected by various environmental and management factors such as temperature, light intensity, water and nutrient stress, soil quality and topography. Thus differences in the concentrations of condensed tannins among studies are expected.

Table 3.1 Phenolic compounds of some tropical plant materials and commercial CT extract.

Items	Plant materials and commercial extract[/1]						
	CV	LL	SN	MS	QB	SEM	*P*-Values
Phenolic compounds (% of DM)							
Total phenols	4.45[c]	3.36[c]	6.38[b]	16.47[a]	n.d.	0.52	*<0.001*
Total tannins	3.23[c]	1.98[c]	5.27[b]	15.48[a]	n.d.	0.55	*<0.001*
Condensed tannins	2.92[d]	1.17[e]	5.03[c]	14.38[b]	67.34[a]	0.26	*<0.001*

[a,b,c,d,e] Means within the same row with different superscripts are significantly different ($P < 0.001$).

[1/] CV= cassava leaves; LL= leucaena leaves; SN= Siamese neem leaves; MS= mangosteen peel; QB= quebracho CT extract.

3.5.2 Molecular weight of CTs

The molecular weight distribution of CTs of tropical plant materials and commercial CT extract is presented in Table 3.2. The apparent weight-average molecular weight (M_w) of purified CT from cassava, leucaena and Siamese neem leaves, mangosteen peel and quebracho CT, relative to the polystyrene standards was 3,409, 3,222, 3,612, 2,964 and 3,539 Da, respectively, and the degree of polymerization (DP) ranged from 5.93 to 7.22. Similar to those of the concentration of phenolic compounds, the M_w and DP values of CT, compared of those of selected tropical plant leaves, were lowest for leucaena and highest for Siamese neem. In addition, among all the CT sources in this study, M_w and DP values of CT from mangosteen peel was lowest while M_w and DP values of CT from quebracho extract were similar to Siamese neem.

Since molecular weight has been reported to be a major factor affecting the efficacy of condensed tannins to influence rumen fermentation rate and methane gas

production (Huang et al., 2010; 2011; Naumann et al., 2013), this study determined the molecular weight of the above three commonly used feed leaves. The weight-average molecular weights (M_w) of condensed tannins for leaves of cassava (3,409 Da), leucaena (3,222 Da) and Siamese neem (3,612 Da) estimated in this study are within the range reported in the literature. Generally, the molecular weight for temperate plants was reported to range between 3,036 and 7,143 Da (McAllister et al., 2005). In addition, the range of M_w value of CT extracted from selected tropical plant leaves in this study (3,222 to 3,612 Da) was higher than those reported for warm-season perennial legumes which ranged between 552 to 1,483 Da (Naumann et al., 2013). However, the molecular mass of condensed tannins of leucaena estimated in this study were higher than those reported for local *Leucaena leucocephala* (2,737 Da) and Leucaena hybrid Bahru (2,872 Da) (Huang et al. 2010) and for *L. leucocephala* hybrid Rendang (1,266 Da) (Saminathan et al., 2014) grown in Malaysia. We do not know of any published data on the MW of cassava, Siamese neem leaves and quebracho CT extract and thus assume that the present data are the first reported of MW of CTs from cassava, Siamese neem leaves and quebracho CT extract.

Table 3.2 Molecular weight of CTs of some tropical plant materials and commercial CT extract.

Items	Plant materials and commercial extract[/1]				
	CV	**LL**	**SN**	**MS**	**QB**
Relative molecular weights[A]					
M_w	3,409	3,222	3,612	2,964	3,539
M_n	2,856	2,703	3,026	2,384	2,845
PDI	1.194	1.192	1.194	1.194	1.244
DP	6.81	6.44	7.22	5.93	7.08

[A]M_w: weight-average molecular weight (Da), represent relative molecular weights of purified CTs. M_n: number average molecular weight. DPI: polydispersity (M_w/M_n), a measure of the distribution of molecular mass in a given polymer sample in organic chemistry which indicates the distribution of individual molecular mass in a batch of polymers. DP: degree of polymerization, estimated as M_w/500, on the basis of M_w of a proanthocyanidin peracetate unit of approximately 500.

3.5.3 Protein-binding ability of CTs

The protein-binding ability of CTs from tropical plant materials and commercial CT extract is presented in Figure 3.1. In the present study, the result was indicated that the amount of protein (BSA) precipitated with CTs relatively increased with higher molecular weight of CTs. CTs extracted from Siamese neem had the highest weigh-average molecular weight (M_w) of all tropical plant leaves and materials studied, had a highest amount of protein precipitated, whereas, CTs extracted from mangosteen peel had the lowest M_w of all materials studied, had a lowest protein-binding capacity. In general, these results indicate that CTs of higher molecular weight have greater protein-binding ability than those of lower molecular weight.

Protein-binding ability is the major biological property of CTs, since CTs can bind and form insoluble complexes with proteins, which influence to resist both microbial and enzymatic degradation, and subsequent ruminal fermentation (Lorenz et al. 2013), in general, the molecular size of CTs is the major factor influenced on their protein-binding ability (McNabb et al., 1998), however, molecular weight of CTs not a sole factor affected on protein-binding ability, CTs structure (Perez-Maldonado et al., 1995) and environment of reaction such as pH (Makkar and Singh, 1995) were among the factors which could be influenced on protein-binding ability of CTs. According to the literature, it was found that protein-binding ability of CTs differed among plant species and varieties, in addition, protein-binding capacity of CTs also differed among their molecular weight. Osborne and McNeill (2001) studied the ability of CTs with different sizes (by size exclusion chromatography procedure) from the genus *Leucaena*, to bind protein and reported that the larger-sized CTs could precipitated more protein than the smaller-sized CTs. This latter result was agreed by the studies of Huang et al. (2010; 2011) who reported that higher molecular weight fraction of CTs extracted from *Leucaena leucocephala* have stronger protein-binding capacity than those of lower molecular weight.

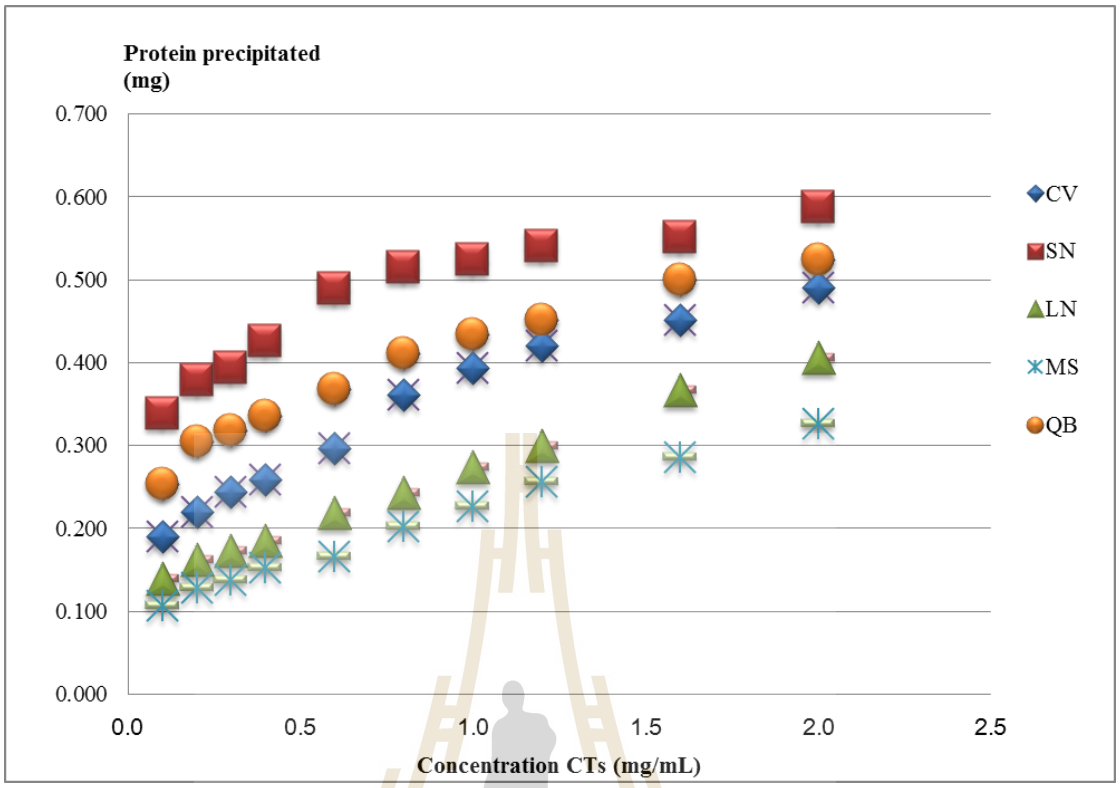

Figure 3.1. Protein-binding ability of CTs with differing molecular weight

3.6 Conclusion

Based on the current results, the content of phenolic compounds, proportions of condensed and hydrolizable tannins were differenced among the tropical plant leaves, material and commercial CT extract. Plant materials, such as leaves of Siamese neem, containing high MW condensed tannins may serve as good methane mitigating agent as compared to leucaena leaves, but supplementing Siamese neem leaves in excessive amount may lead to negative effects on rumen fermentation and thus animal performance. On the other hand, leucaena leaves which is high in protein and contain condensed tannins of relatively lower MW is a suitable natural plant protein supplement and methane mitigation agent for ruminant production including small-scale farming system in the tropic or sub-tropic region where this feed material is available. As the

molecular weight of CTs extracted from all materials examined here were also differenced, this results led us to further speculate that there might be a relationship between molecular weight and protein binding by CT. The result do support this hypothesis. Furthermore, since the protein-binding ability is an importance biological property of CTs, and their molecular weight is among major factors influenced on this property, it will be necessary to elucidate further effect of the different molecular weight of CTs from different plant materials on ruminal fermentation parameters such as pH, volatile fatty acid production, and gas and methane productions.

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CHAPTER IV

STUDY ON INCLUSION OF DIFFERING MOLECULAR WEIGHT OF CONDENSED TANNIN ON RUMINAL GAS PRODUCTION AND SOME FERMENTATION PARAMETERS *IN VITRO*

4.1 Abstract

The objective of this study was aimed to investigate the effects of inclusion of CTs with differing molecular weight on ruminal gas productions and some ruminal fermentation parameters *in vitro*. To evaluate this effect of CTs, the *in vitro* syringe gas production technique was applied. Based on the results of the molecular weight of CTs of some tropical plant species, CTs extracted from leaf samples of two plant species; leucaena (LN), representing CT of lower molecular weight (M_w 3,222 Da) (LMW-CT) and Siamese neem (SN), representing CT of higher molecular weight (M_w 3,612 Da) (HMW-CT) were selected for the *in vitro* gas production study. Seven treatments were set up: control (with no CTs supplementation) and treatments supplemented with LMW-CT and HMW-CT extracts at 2, 4, and 6 mg/100 mg DM of substrate were set up. The total gas produced were recorded at 1, 2, 3, 4, 6, 8, 12 and 24-h of incubation, and at the end of 24-h of incubation, methane (CH_4) gas volume was determined. The contents of incubated ruminal fluid from another three syringes from each treatment were collected at 4-h of incubation for volatile fatty acid concentration measurement. Results

showed that, supplementation of condensed tannins of higher MW from leaves of Siamese neem (at 2-6 mg/100 mg DM) significantly inhibited *in vitro* total gas and methane productions while supplementation of the low MW condensed tannins from leaves of leucaena had no effect, except for total gas production at the highest (6 mg/100 mg DM) level of supplementation. Similarly, condensed tannins from Siamese neem leaves had stronger effect ($P< 0.001$) on *in vitro* volatile fatty acids production. We conclude that the efficacy of condensed tannins from plant materials to reduce methane emission and manipulate ruminal fermentation are affected by particularly their structural characteristics and dose of CTs supplemented, and also plant species and possibly the environment in which they are grown. Leaves of Siamese neem, containing high MW condensed tannins may serve as good methane mitigating agent at excessive amount, it may lead to negative effects on rumen fermentation and thus animal performance. On the other hand, leucaena leaves which is high in protein and contain condensed tannins of relatively lower MW is a suitable natural plant protein supplement and methane mitigation agent for ruminant production including small-scale farming system in the tropic or sub-tropic region where this feed material is available.

Key words: Tannins-containing forages, CTs extract, Methane production, VFAs.

4.2 Introduction

Recently, rumen manipulation in order to improve rumen fermentation efficiency and animal performances using natural compounds has greater interested. Plant secondary compounds (i.e. saponins and condensed tannins) are important feed additives for ruminants (Wanapat et al., 2013). In the tropics, ruminant feeding and rumen manipulation mostly based-on locally available plant materials which generally

contained plant secondary compound particularly condensed tannins (CTs). CTs are widely distributed in tropical plants includings forages, shrubs and legumes. Using tannins-containing plants as feedstuff can be benificial or detrimental for ruminant, depending on source and dose are consumed, and particular the compound's structure and molecular weight (Frutos et al., 2004). In generally, consumption of plant species/diets with high concentration of CT (> 5.0 % of DM) have been reported to cause a variety of detrimental effects, such as decreased feed palatability, voluntary feed intake and nutrients digestibility (Osborne and McNeill, 2001). However, in ruminant nutrition, appropriate concentration of CTs in the diet (generally 2-4 % of DM) have been recognized to be beneficial useful as phyto-chemicals for manipulation of rumen fermentation and particularly mitigating methane emission (Patra and Saxena, 2011).

Recent studies seem to suggest that chemical structure and molecular weight of this metabolite have greater influence on their major biological property, protein-binding ability and their efficacy to manipulate rumen fermentation including mitigation of methane production (Huang et al, 2010; Huang et al, 2011). Therefore, this study was carried out to evaluate the effect of CTs molecular weight on ruminal gas production and ruminal fermentation *in vitro*.

4.3 Objective

The objective of this study was to investigate the effects of inclusion of CTs with differing molecular weight on ruminal gas productions and some ruminal fermentation parameters *in vitro*.

4.4 Materials and methods

4.4.1 Preparation of plant CTs extracts

Based on the results of the molecular weight of CTs of selected tropical plant species from previous study, leaf samples from two plant materials; namely leucaena (LN), representing CT of lower molecular weight (M_w 3,222 Da) (LMW-CT) and Siamese neem (SN), representing CT of higher molecular weight (M_w 3,612 Da) (HMW-CT) were selected for the *in vitro* gas production study. Crude CT extracts of leucaena and Siamese neeem leaves from previous study were brought to use as CT source in this study.

4.4.2 Determination of *in vitro* ruminal fermentation parameters

To determine the effect of inclusion of differing molecular weight of CTs on gas production and ruminal fermentation *in vitro*, the syringe gas technique procedure of Menke and Steingass (1988) was applied in this study. The following seven treatments were set up: control (with no CTs supplementation), supplemented with LMW-CT extracted from LN at 2, 4, and 6 mg/100 mg DM of substrate (LN-2, LN-4 and LN-6, respectively), and supplemented with HMW-CT extracted from SN at 2, 4, and 6 mg/100 mg DM of substrate (SN-2, SN-4 and SN-6, respectively).

Three matured rumen-fistulated Saanen male goats fed 2.5 % dry matter of body weight containing 60% Pangola (*Digitaria eriantha*) hay and 40% commercial concentrate (14% of crude protein) were used as donors of the rumen fluid. Ruminal fluid collected were pooled and strained through 4 layers of cheesecloth and then used immediately. Artificial saliva was prepared under anaerobic conditions in a water bath at 39°C according to Menke and Steingass (1988), the strained rumen fluid was mixed in a 2:1 ratio (artificial saliva: rumen fluid). Thirty mL of buffered rumen fluid solution

was dispensed into 100 mL calibrated glass syringes (Toitu, TOP Surgical Manufacturing Co. Ltd., Japan) containing 200 mg of oven dried (60 °C) Pangola grass as substrate and the assigned levels of CT extract from LN or SN (equivalent to 2, 4 and 6mg CT/100 mg DM of substrate, respectively). Syringes were immediately incubated in water bath at 39◦C for 24 h. For above procedures, a total of three replicates for each treatment per separated run (with total 3 separated runs) were used for total gas and methane production and volatile fatty acid (VFA) measurements.

4.4.3 Data record, sample collection and sample analysis

The total gas produced were recorded at 1, 2, 3, 4, 6, 8, 12 and 24-h of incubation, and net gas production values were corrected by subtracting blank values from the samples (Menke and Steingass, 1988).Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979) described as $Y = a+b(1 - e^{-ct})$; where Y represents the cumulative gas production at time t, a is the gas production from the immediately soluble fraction, b is the gas production from the insoluble fraction, c is the rate of gas production (/h) and $(a+b)$ is the potential gas production. At the end of 24-h of incubation, methane (CH_4) gas volume was determined according to the method as described by Fievez et al. (2005). In brief, after the final gas volume was recorded, 4.0 mL of 10 M NaOH was introduced into the incubated contents of each syringe via the lower end of the glass syringe, which thereby avoiding gas escape. After mixed with NaOH, the CO_2 was absorbed into the content and the remaining gas volume in the syringe was considered to be CH_4.

The contents of three syringes from each treatment were collected after 4-h of incubation. For each sample, 20 mL of content was added into a 50 mL centrifuge tube, then 5 mL of 6N HCl was immediately added and centrifuged at 5,000×g for 20 min at

4 ◦C. The supernatants were used for VFA analysis. The concentrations of VFA were determined by Gas Chromatography (SHIMADZU, Model GC-2014, SHIMADZU CORP., Kyoto, Japan) connected with auto sampler (SHIMADZU, Model AOC-20s) and auto injector (SHIMADZU, Model AOC-20i). Gas chromatography was equipped with a 30 m × 0.25 mm × 0.25 µm column (HP INNOWAX, DB-1, Agilent, USA). Detector and injector temperatures were 200 ◦C. The temperature of column was respectively set as follow: kept 145 ◦C for 3 min, increased at 10 ◦C/min to 200 ◦C and held at 200 ◦C for 5.5 min.

4.4.4 Statistical analysis

Data of *in vitro* gas production and VFA concentrations were analyzed in a Completely Randomized Design (CRD) using the PROC GLM of SAS Institute (1998). Multiple comparisons among treatment means were identified using Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980). Mean differences were considered significant at $P<0.05$.

4.4.5 Location of the study

The study was conducted at the Center for Scientific and Technological Equipment Building 10, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

4.4.6 Experimental period

This study was carried out from January to June 2015.

4.5 Results and discussion

4.5.1 Gas kinetics, total gas production and methane production

Gas production and the kinetics of gas production of each treatment combination and those of the control are presented in Table 4.1. In comparison with control group, the intercept value (*a*) was significantly affected (P <0.01) by CT from both leucaena and Siamese neem leaves. However, only Siamese neem, at all the three supplementation levels significantly decreased (P<0.01) the insoluble fraction (*b*). Similarly, potential gas production (*a*+*b*) and cumulative gas production at 24 h were significantly decreased with supplementation of all levels of CT from Siamese neem but similar effect was observed only at the highest level (6mg/100 mg DM) for CT of leucaena (P <0.01). In addition, methane (CH_4) production for 24 h was decreased with all supplementation levels of CT from Siamese neem (P <0.01).

Currently, emission of methane is consider as the most important gas that impacts on global environmental issue (IPCC, 2001). Furthermore, the enteric methane production is a product of the digestive process by the ruminal microbes which could accounted as a loss of 2-12 % of the energy in the feed consumed. Therefore, mitigating methane production is greater consider for decreasing the emission of the greenhouse gas with improved feed efficiency. Tannins constitute the major class of plant secondary metabolites (PSM) that are greater interesting in using as feed supplement in order to reduce enteric methane emission. In general, there are two modes of action of tannins on ruminal methanogenesis: directly, affecting activity or population of methanogens and resulting in reduction of methane emission, and indirectly, by reduced hydrogen production bylowering feed degradation (Tavendale et al., 2005), furthermore, tannins are known to decrease number of protozoa (Makkar et al., 2005). However, CTs from

different sources resulted in different affected to mitigation of ruminal methane emission. Molecular weight of CTs could be a major factor in the bioactivity of CTs, however, there were varied in the effect of molecular weight of CTs on methane mitigation, such as molecular weight of CTs increases, bioactivity also increases (Peleg et al., 1999) while others have suggested that the bioactivity not relatively increased with an increases of molecular weight of CTs (Huang et al., 2010).

In this current study, condensed tannins from leaves of leucaena (with lowest M_w) and Siamese neem (with highest M_w) among the three leaves tested were used to evaluate their influence on *in vitro* fermentation kinetics and gas production, particularly their role in mitigating methane production. Results of our study, once again demonstrated that condensed tannins of higher MW (Siamese neem) had stronger effect than that of lower MW (leucaena) on methane productions. Supplementation of the higher Mw condensed tannins from leaves of Siamese neem, even at the lowest concentration (2mg/100mg DM) significantly suppressed (P<0.001) methane productions while supplementation of the lower MW condensed tannins from leucaena leaves showed no effect on methane production except at the highest level (6mg/100 mg DM) of supplementation.

Huang et al. (2010) reported that inclusion of condensed tannins from leucaena-hybrid Bahru (LLB) at levels of 2 to 5 mg/100 mg DM significantly decreased *in vitro* total gas and methane production compared to control (P<0.001) even though the MW of LLB (2,737 Da) was lower than that of the leucaena used in this study (3,222 Da). The above authors also demonstrated that although the Mw of LLB was similar to that of the local *Leucaena leucocephala* (LLL, M_w = 2,871 Da), the condensed tannins from hybrid LLB had stronger protein binding and methane mitigation ability than that from

LLL which they demonstrated that the stronger protein binding ability and suppression of methane production of LLB was due to the higher proportion of the larger molecular weight fractions of the condensed tannins of LLB (Huang et al., 2011a; Huang et al., 2011b).

4.5.2 Volatile fatty acid concentrations

Concentrations of VFA including those of acetic acid, propionic acid and butyric acid are presented in Table 4.2. Inclusion of CT from both leucaena and Siamese neem leaves significantly reduced ($P< 0.001$) production of total VFA and acetic acid except for the lowest level (at 2 mg/100 mg DM) of CT from leucaena leaves. On the other hand, production of propionic and butyric acids were significantly increased with all supplementation levels with CT from both, leucaena and Siamese neem leaves ($P< 0.001$) except for the 2 mg/100 mg leucaena group. In general, CT from the Siamese neem leaves had stronger influence on total VFA and the various individual VFA production than CT from leucaena leaves.

Fermentation of carbohydrate produces primarily acetic and butyric acids which are associated with higher methane production and supplementation of legumes contained condensed tannins (Johnson and Johnson, 1995) and quebracho tannin extract (Beauchemin et al, 2008) has been shown to produce a higher ratio of propionic to acetic acids which resulted in lower CH_4 production. Influence of condensed tannins from leaves of leucaena (with lowest M_w) and Siamese neem (with highest M_w) on *in vitro* VFA production was evaluated in this study. Our results showed that condensed tannins of higher MW had stronger inhibitory effect on total VFA and acetic acid production than those of lower MW as well as the control (no condensed tannins). Low MW condensed tannins from leucaena leaves need to be supplemented at the highest

concentrations (6 mg/100 mg DM) to significantly decreased ($P< 0.001$) productions of total VFA and acetic acid as compared to the control group, whereas more pronounced effect can be obtained from supplementation of higher MW condensed tannin from Siamese neem leaves even at the lowest (2 mg/100 mg DM) (Table 4.2).

Table 4.1 Effects of different levels of CTs of differing molecular weights (MW) extracted from leucaena (low MW) and Siamese neem (high MW) on *in vitro* gas and methane productions.

Items	Treatments[1/]							SEM	*P*-value
	Control	LN-2	LN-4	LN-6	SN-2	SN-4	SN-6		
Gas kinetics[2/]									
a	18.1^{a}	10.7^{b}	5.0^{bc}	4.5^{c}	2.0^{c}	2.8^{c}	5.7^{bc}	0.69	0.001
b	130.2^{a}	125.8^{a}	120.4^{a}	111.0^{a}	92.6^{b}	76.5^{bc}	61.1^{c}	2.27	0.001
c	0.050	0.057	0.067	0.070	0.053	0.060	0.073	0.002	0.069
a+*b*	148.3^{a}	136.5^{ab}	125.4^{bc}	115.5^{c}	94.7^{d}	79.2^{de}	66.8^{e}	2.11	0.001
Gas productions[3/]									
Total	107.0^{a}	104.0^{ab}	100.7^{ab}	93.4^{b}	68.7^{c}	60.7^{cd}	55.9^{d}	0.75	0.001
CH_4	10.0^{bc}	12.5^{ab}	16.7^{a}	5.8^{cd}	3.3^{d}	1.7^{d}	0.01^{d}	0.01	0.001

Means within row followed by different letters are significantly different ($P< 0.01$)

[1/]Control: 200 mg DM of pangola hay as substrate. LN-2, LN-4 and LN-6: supplemented with condensed tannins (CTs) extracted from leucaena (LN, representing CTs of lower MW) at 2, 4 and 6 mg/100 mg DM, respectively. SN-2, SN-4 and SN-6: supplemented with CTs extracted from Siamese neem (SN, representing CTs of higher MW) at 2, 4 and 6 mg/100 mg DM, respectively.

[2/]*a*:the gas production from the immediately soluble fraction. *b*: the gas production from the insoluble fraction. *c*: the gas production rate constant. *a*+*b*: the potential extent of gas production.

[3/]Gas production of 24 h of incubation (mL/g DM of substrate); CH_4: methane.

In contrast to the acetic acid, proportion of propionic acid and butyric acid was significantly increased ($P < 0.001$) with supplementation of CT from leucaena and in particularly when CT from Siamese neem leaves was used. The above results are in agreement with that reported by Saminathan et al (2015) which found that addition of CT of higher MW led to lower acetic acid formation and lower methane production. The authors suggested the above result was probably due to enhanced acetogenesis which led to the dispose of metabolic H_2 and thus a decreased in methane production. However, Tan et al. (2011) reported that supplementation of CT extracted from Leucaena-hybrid Rendang (LLR) at levels of 2 to 6 mg/100 mg DM resulted in significant linear decreased *in vitro* total VFA concentration but not acetic acid concentration. Differences in the efficacy to mitigate methane production and volatile fatty acid production between the present study and those of the above mentioned workers, and perhaps also differences between the leucaena and Siamese neem in this study were due to differences in the proportions of the various MW fractions between species.

Table 4.2 Effects of different levels of CTs of differing MW extracted from leucaena (low MW) and Siamese neem (high MW) on *in vitro* ruminal volatile fatty acid (VFA) concentrations.

Items	Treatments[1/]							SEM	*P*-value
	Control	LN-2	LN-4	LN-6	SN-2	SN-4	SN-6		
Total VFA (mM/mL)	14.8^{a}	14.3^{a}	13.2^{b}	12.8^{b}	11.6^{c}	11.0^{cd}	10.5^{d}	0.33	0.001
Acetic (%)	80.7^{a}	79.8^{a}	78.0^{b}	75.3^{c}	72.9^{d}	73.1^{d}	72.6^{d}	0.41	0.001
Propionic (%)	12.4^{d}	13.1^{cd}	14.2^{c}	16.7^{b}	18.2^{a}	17.5^{ab}	17.4^{ab}	0.45	0.001
Butyric (%)	6.9^{d}	7.1^{d}	7.8^{c}	7.9^{c}	8.8^{b}	9.4^{b}	10.0^{a}	0.20	0.001
A:P	6.5^{a}	6.1^{a}	5.5^{b}	4.5^{c}	4.0^{c}	4.2^{c}	4.2^{c}	0.18	0.001

Means within row followed by different letters are significantly different ($P< 0.01$)

[1/]Control: 200 mg DM of pangola hay as substrate. LN-2, LN-4 and LN-6: supplemented with condensed tannins (CT) from leucaena (of lower MW) at 2, 4 and 6 mg/100 mg DM, respectively. SN-2, SN-4 and SN-6: supplemented with CT from Siamese neem (of higher MW) at 2, 4 and 6 mg/100 mg DM, respectively.

4.6 Conclusions

Putting the above together, we conclude that the efficacy of condensed tannins from plant materials to reduce methane emission depends on plant species and possibly the environment in which they are grown. Plant materials, such as leaves of Siamese neem, containing high MW condensed tannins may serve as good methane mitigating agent as compared to leucaena leaves, but supplementing Siamese neem leaves in excessive amount may lead to negative effects on rumen fermentation and thus animal performance. On the other hand, leucaena leaves which is high in protein and contain condensed tannins of relatively lower MW is a suitable natural plant protein supplement

and methane mitigation agent for ruminant production including small-scale farming system in the tropic or sub-tropic region where this feed material is available.

4.7 References

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CHAPTER V

INCLUSION OF DIFFERENT MOLECULAR WEIGHT CONDENSED TANNIN ON RUMINAL FERMENTATION AND MILK FATTY ACID PROFILE OF DAIRY GOATS

5.1 Abstract

The aim of this study was to investigate the effect of CTs with differing molecular weight on their capacity to modify the fatty acid profile in milk. Twenty multiparous crossbred lactating dairy goats were assigned in a RCBD design, and were subjected to receive the dietary treatments as followings; T1: control (with no CTs supplementation), T2: supplemented with mangosteen peel in a concentrate as a source of low molecular weight CTs at level of 3.0 %DM of CTs equivalent, T3: supplemented with the same diet with T2 but added with polyethylene glycol (PEG, as tannin inactivator) as the control of T2, and T4: supplemented with quebracho CT extract (UNITAN ATO, Buenos Aires, Argentina; 75-77 % tannins) in a concentrate as a source of high molecular weight CTs at level of 3.0 %DM of CTs equivalent, and T5) supplemented with the same diet with T4 but added with PEG as the control of T4. No significant change was detected for feed intake and nutrient digestibility indicate that CTs at level of 3.0 %DM of diet did not showed the detrimental effect to feed intake and nutrient digestibility, however, ruminal fermentation parameters and milk yield and

milk compositions did not affected by different source of CT inclusion.

Key words: CTs molecular weight, Ruminal biohydrogenation, Milk compositions

5.2 Introduction

Currently, concerning of healthiness food for consumption have been greater considered. Ruminant food products particularly tissue lipids are recognised to be highly saturated in nature compared to non-ruminants, however, ruminant products also contain potentially health-promoting fatty acid includings conjugated linoleic acid (CLA), mainly *cis*-9, *trans*-11-CLA isomer (rumenic acid, RA), since they in many animal studies to contribute to cancer prevention, decreased atherosclerosis, improved immune response, and altered protein or energy metabolism (Pariza, 2004; Palmquist et al., 2005; Gebauer et al., 2011). The CLA isomers can formed as the intermediates of biohydrogenation of polyunsaturated fatty acids including linolenic and linoleic acids (Jenkins et al., 2008) to the final product of this process, stearic acid (Bessa et al., 2007). In addition, rumenic acid can also be formed endogenously in the muscle or in the mammary gland from vaccenic acid (*trans*-11 C18:1, VA) (Griinari et al., 2000) through the action of Δ^9-desaturase enzyme (Corl et al., 2001) indicating that factors influencing the production of VA in the rumen are of interest in order to manipulate the biohydrogenation for increasing the CLA content in milk or meat of ruminant (Fukuda et al., 2006; Kim et al., 2009). Since many researches have been stated that there are two group of ruminal bacteria involved in ruminal biohydrogenation which have been categorized into Group A (have ability to hydrogenate polyunsaturated fatty acids to VA) and Group B (ability to hydrogenate polyunsaturated fatty acids through to SA) bacteria (Kemp and Lander, 1984). Therefore, strategy which can inhibit the group B

bacteria and resulting in inhibit the final step of biohydrogenation process is greater interest.

Nutritional strategies of supplementation of fish oil in ruminant diets have been very succesful in order to inhibit the final biohydrogenation step from VA to SA by its toxic effect on certain bacterial species (Kim et al., 2008), however, recently, the action of plant secondary compounds including essential oils, saponins and tannins which possess anti-microbial properties is greater attention (Wallace, 2004; Patra and Saxena, 2011). However, information pertaining to the effects of tannins on ruminal BH process and fatty acids composition of ruminant derived products is very limited, in paticularly using of difference source and structure of condensed tannins. Therefore, research emphasis on effect of condensed tannin with differing structure (particularly molecular weight) on ruminal BH and alteration of fatty acids composition of ruminant-derived food products would be further investigated.

5.3 Objective

The objective of this study was to evaluate the effect of inclusion of CTs sources with differing molecular weight in Sunflower oil-riched diet on voluntary feed intake, nutrients digestibility, ruminal fermentation and milk yield and milk fatty acid profile of lactating dairy goats.

5.4 Materials and methods

5.4.1 Experimental animals, design, and diet

This experiments was conducted according to principles and guidelines approved by the Animal Care and Use Committee of Suranaree University of

Technology, Suranaree University of Technology, Nakhonratchasima, Thailand.

Twenty multiparous crossbred-Saanen lactating goats in early lactation period (30 ± 9.5 day in milk) with 0.83 ± 0.32 kg per day of milk yield and 41.2 ± 7.2 kg of body weight were subjected to use in this experiment. All goats were housed in individual pens which able to free access to mineral block and water. All goats were assigned to a randomized complete block design (RCBD) with 4 blocks (replicates) per treatment. The experimental period was divided into a 14-days adaptation period and a 21-days of experimental period. The lactating goats were subjected to hand milking twice a day and fed a pangola hay *ad libitum* as the soul roughage source, and supplemented with the following dietary concentrate treatments twice a day at the milking times (7.00 a.m. and 16.00 p.m.), the dietary treatments composed T1) control (with no CTs supplementation), T2) supplemented with mangosteen peel in a concentrate as a source of low molecular weight CTs at level of 3.0 %DM of CTs equivalent, T3) supplemented with the same diet with T2 but added with polyethylene glycol (PEG, as tannin inactivator) as the control of T2, T4) supplemented with quebracho CT extract (UNITAN ATO, Buenos Aires, Argentina; 75-77 % tannins) in a concentrate as a source of high molecular weight CTs at level of 3.0 %DM of CTs equivalent, and T5) supplemented with the same diet with T4 but added with PEG as the control of T4.

All concentrate treatments were calculated to contain crude protein (CP) at 18.5 % DM by using of the 16 and 21 %CP commercial pelleted concentrates (produced by Suranaree University of Technology Farm, SUT Farm) and molasses as the feed ingredients (Table 5.1). All commercial concentrates used as the feed ingredient were ground in order to make diet mixed well prior to mix in the mixtures. Sunflower oil was

added to the ration at 5 % DM daily before feeding, to avoid the rancidity of the diet.

Table 5.1 Feed ingredient, chemical and major fatty acid composition of dietary treatments

Items	Experimental diets and feed stuff					
	T1	T2,T3[/2]	T4,T5[3/]	Pangola hay	MSP[4/]	QB[5/]
Ingredient composition (% DM)						
SUT concentrate (16%CP)[/1]	26.0	0.0	9.0	-	-	-
SUT concentrate (21%CP)[/1]	68.0	73.0	80.5	-	-	-
Dried-MSP powder	-	21	-	-	-	-
QB extract powder	-	-	4.5	-	-	-
Molasses	1	1	1	-	-	-
Sunflower oil	5	5	5	-	-	-
Chemical composition (% DM)[/6]						
DM (%)	92.49	92.45	92.54	88.82	90.57	91.28
OM	92.47	93.12	92.37	94.40	96.15	91.71
Ash	7.53	6.88	7.63	5.6	3.85	8.29
CP	18.64	18.59	18.53	5.13	14.89	1.36
EE	8.11	7.84	8.05	1.51	n.d.	n.d.
NDF	42.45	48.36	42.21	74.38	n.d.	n.d.
ADF	23.56	31.62	21.65	41.16	n.d.	n.d.
AIA	1.06	1.09	1.10	1.95	n.d.	n.d.
Condensed tannin	-	3.0	3.0	-	14.4	67.8

[1/] Commercial concentrate pellet feed purchased from Suranaree University of Technology (SUT) farm.

[2/] T2 and T3 diets contains CTs form mangosteen peel as the source of low molecular weight CTs.

[3/] T4 and T5 diets contains CTs form quebracho extract as the source of high molecular weight CTs.

[4/] Mangosteen peel powder as the source of low molecular CTs.

[5/]Quebracho condensed tannins extract (UNITAN ATO, Buenos Aires, Argentina).

[6/]DM= dry matter; OM= organic matter; CP= crude protein, EE= ether extract, NDF= neutral-detergent fiber; ADF= acid-detergent fiber; AIA= acid insoluble ash.

n.d.= not determined.

5.4.2 Sample collection, measurements and analysis

All goats were weighed at the beginning and end of the experiment to calculate feed intake. All feed and feed residue samples were collected weekly for chemical analysis. Feeds offered and remained were recorded daily during the last 14 days of experimental period to calculate feed intake and nutrients digestibility measurement. Feces from all goats were sampled about 10 % at last two weeks of the experimental period. All feed samples of each treatments were pooled at the end of experiment and divided into two parts, one for dry mater (DM) measurement by using hot air oven at 100 °C, 48 h, and another part was dried at 60 °C prior to grind pass through a 1.0 mm screen and subjected to proximate analysis. Feed and feces samples were analyzed for DM, crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), ADF, and acid insoluble ash (AIA). All chemical compositions were expressed on DM basis.

In addition, feeds and sunflower oil samples were analysis for fatty acid compositions. Fatty acids in feed samples were extracted using a method of Folch et al. (1957) and Metcalfe et al. (1991) with minor modified. In brief, Fifteen gram of each sample was homogenized with 90 mL of chloroform-methanol (2:1) for 2 min. Then, further homogenized for 2 min with 30 mL of deionized water prior to adding of 5 mL of 0.58% NaCl. The fatty acid methyl esters layer was moved and placed in a screw-cap test tube and stored at -20 °C until further methylation. To prepare the fatty acid sample for determine fatty acid composition, fatty acid methyl esters (FAME) were prepared by the method described by Ostrowska et al. (2000). Briefly, transferred approximately 30 mg of the extracted oil into a screw-cap tube fitted with a Teflon-lined. Adds 1.5 mL of 0.5 M NaOH in methanol into the tube, dried with N_2 about 30 sec and immediately capped the cap. Heat the sample at 100 °C for 2 min with

occasional shaking and then cooled the sample at room temperature. After that, 1.0 mL of C17:0 internal standard (1.0 mg/mL of trimethyl pentane) and 2.0 mL of 14 % boron trifluoride (BF_3) in methanol were added, dry with N2 for 30 sec and immediately capped. Heated sample mixture at 100 °C for 30 min with occasional shaking and cooled at room temperature. Add 5.0 mL of trimethyl pentane, shake and then added with 5.0 mL of saturated NaCl and shake. The upper clear part was collected and transfer to the 1.0 mL vial and capped, FAME samples were kept at -20 °C until further analysis for fatty acid profile using Gas Chromatography (GC).

Milk samples were determined for compositions including fat, protein, lactose, total solids (TS), and solid-not-fat (SNF) by MilkoScan FT2 infrared automatic analyser (FOSS, Hillerod, Denmark)

5.4.3 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) procedure. Means were separated using Duncan's procedure where differences were $P<0.05$ among treatments. All data were analyzed using SAS software (SAS Institute, 1996).

5.4.4 Location of the study

The study was conducted at the Center for Scientific and Technological Equipment Building 1 and 10, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

5.4.5 Experimental period

This study was carried out from January 2015 to November 2016.

5.5 Results and discussion

5.5.1 Chemical composition of experimental diets

The dietary formula and chemical compositions of dietary treatments and some individual feeds used in this experiment is presented in Table 5.1. Concentrate feeds contained 18.5-18.6 % DM of crude protein, EE between 7.8-8.1 % DM and condensed tannins at 3.0 % DM. Concentrate feeds were offered to all goats as the sources of protein, energy and also unsaturated fatty acid. Table 5.2 shows the fatty acid compositions of experimental diets and individual feeds. Sunflower oil contains high proportion of polyunsaturated fatty acid (PUFA) (50 g/100 g FA) particularly C18:2n6c fatty acid (49.49.4 g/100 g FA). Although pangola hay contains high proportion of PUFA (12.34 and 18.11 g/100 g FA for C18:2n6c and C18:3n3 fatty acids, respectively), however, due to low content of fat, it seem to not be a good sources of those fatty acids compared to other species of grass such as *Pennisetum purpureum*.

Table 5.2 Fatty acid compositions of experimental diets and some individual feeds.

Items	Experimental diets and some individual feeds				
	T1	**T2,T3**	**T4,T5**	**SFO**	**Pangola hay**
Fatty acid composition (g/100 g FA)					
C12	13.14	11.86	12.74	0.00	2.15
C14	2.25	2.03	2.18	0.00	2.02
C14:1	0.40	0.36	0.38	0.00	2.27
C16	10.36	9.94	10.23	6.03	11.48
C16:1	0.36	0.32	0.35	0.00	1.88
C18	3.05	3.07	3.05	3.23	2.24
C18:1n9c	30.85	31.70	31.11	39.60	5.17
C18:2n6c	31.73	33.46	32.27	49.44	12.34
C20	0.28	0.28	0.28	0.25	2.23
C18:3n6	0.25	0.23	0.24	0.00	4.00
C20:1	0.37	0.35	0.36	0.21	2.44
C18:3n3	0.49	0.49	0.49	0.54	18.11
C20:2	0.33	0.30	0.32	0.00	3.89
C22:0	0.62	0.63	0.62	0.70	2.27
C20:3 n6	0.32	0.29	0.31	0.00	3.01
C22:1n9	0.24	0.22	0.23	0.00	0.08
C20:3n3	0.18	0.16	0.17	0.00	1.94
C20:4n6	0.19	0.17	0.18	0.00	1.82
SFA[1/]	29.7	27.81	29.1	10.21	22.39
MUFA[2/]	32.22	32.95	32.43	39.81	11.84
PUFA[3/]	33.49	35.1	33.98	49.98	45.11
PUFA : SFA	1.13	1.26	1.17	4.90	2.01

[1/]SFA = sum of saturated fatty acids (C12:0-C14:0, C16:0, C18:0: C20:0, C22:0).
[2/]MUFA = sum of monounsaturated fatty acid (C14:1, C16:1, C18:1n9, C20:1, C22:1n9).
[3/]PUFA = sum of polyunsaturated fatty acid (C18:2n6, C18:3n6, C18:3n3, C18:4n3, C20:2, C20:3n6, C20:3n3, C20:4n6).

5.5.2 Feed intake, nutrients intake and nutrient digestibility

Voluntary feed intake and intake of nutrients are presented in Table 5.3. Level of CTs at 3.0 % DM in concentrate feed seem to be high, however, this experiment were offered concentrate to experimental animal at 60 percent of total feed intake, therefore there were around 1.8 % of total DM feed intake. However, there were very low intake of feed contained CTs source such as mangosteen peel and quebracho extract and some

animal did not accepted the diet at the beginning of the experiment, therefore, we suggests a longer period for adaptation of animal to the CT-containing diet.

Table 5.3 Effects of inclusion of CTs of differing molecular weight on voluntary feed intake in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Feed intake (kg DM/h/d)						
Concentrate	0.6309	0.5963	0.7271	0.6867	0.4732	0.1922
Pangola hay	0.5075	0.6127	0.5884	0.4752	0.6129	0.7317
Total	1.1384	1.2090	1.3156	1.1619	1.0861	0.5659
Feed intake (% BW)						
Concentrate	1.5699	1.4220	1.7159	1.7583	1.2836	0.2190
Pangola hay	1.2913	1.4260	1.2988	1.2150	1.6616	0.2226
Total	2.8612	2.8480	3.0147	2.9732	2.9452	0.4270

This study demonstrated that the diet contains CTs with both high or low molecular weight at 3.0 %DM (1.8 % of total feed) have no effect on voluntary feed intake (*P>0.05*). The amount of feed intakes were in normal range (2.8-3.0 %BW). Moreover, according to the results of this study, molecular weight of CTs have no effected on nutrient intake and nutrient digestibility (Table 5.4).

Table 5.4 Effects of inclusion of CTs of differing molecular weight on nutrient intake and digestibility (calculated using the AIA as eternal marker) in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Nutrients intake (kg DM/d)						
Organic matter	1.0625	1.1336	1.2326	1.0829	1.0157	0.5511
Crude protein	0.14363	0.14228	0.16536	0.15162	0.11913	0.2202
EE	0.059	0.056001	0.065893	0.062453	0.047350	0.2316
NDF	0.64531	0.74407	0.78932	0.64329	0.65560	0.4307
ADF	0.35754	0.44073	0.47212	0.34425	0.35471	0.1024
Nutrients digestibility (% of DM)						
Organic matter	75.854	73.468	71.318	73.477	72.252	0.7517
Crude protein	74.935	74.705	73.736	75.219	75.670	0.9851
NDF	68.979	66.526	66.834	66.333	69.251	0.9067
ADF	60.964	62.178	61.008	58.856	58.748	0.9568

5.5.3 Intake of fatty acids

Intake of dietary fatty acids of lactating dairy goats fed diet contained differing molecular weight of CTs are presented in Table 5.5. There was found that all goats from each treatment received similarly amount of PUFA and intake of total fatty acid (ranged from 17 to 24 g/d and 47 to 65 g/d, respectively).

5.5.4 Ruminal fermentations

Ruminal pH and volatile fatty acids concentration affected by inclusion of CTs of differing molecular weight are presented in Table 5.6. Molecular weight of CTs did not affected on ruminal fermentation. The pH values at 0 h post feeding of ruminal fluid did not significant different ($P>0.05$), however, at 3-h post feeding, ruminal pH of goats fed diet contained CTs from mangoateen peel was significantly lower than others. Moreover, there were found that CTs with differing molecular weight did not affected on ruminal fermentation parameters such as concentration of volatile fatty acids.

Table 5.5 Intake of major fatty acids.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Intake of fatty acids (g/d)						
C12	6.886	5.742	6.950	7.197	5.053	0.2089
C14	1.304	1.134	1.335	1.349	1.017	0.2491
C16	6.182	5.709	6.686	6.480	4.960	0.2613
C18	1.731	1.641	1.947	1.849	1.371	0.2166
C18:1n9c	16.181	15.299	18.532	17.570	12.331	0.1977
C18:2n6c	17.183	16.784	20.171	18.722	13.435	0.1843
C20	0.316	0.337	0.357	0.316	0.314	0.7975
C18:3n6	0.434	0.475	0.484	0.421	0.462	0.8866
C20:1	0.376	0.392	0.419	0.377	0.365	0.8204
C18:3n3	1.638	1.907	1.891	1.571	1.863	0.7832
C20:2	0.469	0.501	0.518	0.458	0.484	0.9134
C22:0	0.491	0.504	0.560	0.507	0.448	0.4471
C20:3 n6	0.394	0.414	0.432	0.387	0.397	0.9262
C22:1n9	0.128	0.108	0.130	0.134	0.096	0.2900
C20:3n3	0.238	0.253	0.263	0.233	0.244	0.9146
C20:4n6	0.235	0.247	0.258	0.231	0.237	0.9324
SFA	16.91	15.07	17.84	17.70	13.16	0.2700
MUFA	16.69	15.80	19.08	18.08	12.79	0.2046
PUFA	20.59	20.58	24.02	22.02	17.12	0.2075
Total FA	54.19	51.45	60.93	57.80	43.08	0.2315

[1/]SFA = sum of saturated fatty acids (C12:0-C14:0, C16:0, C18:0: C20:0, C22:0).
[2/]MUFA = sum of monounsaturated fatty acid (C14:1, C16:1, , C18:1n9, C20:1, C22:1n9).
[3/]PUFA = sum of polyunsaturated fatty acid (C18:2n6, C18:3n6, C18:3n3, C18:4n3, C20:2, C20:3n6, C20:3n3, C20:4n6).

Table 5.6 Effects of inclusion of CTs of differing molecular weight on ruminal pH and volatile fatty acids concentration in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Ruminal pH						
- *0-h post feeding*	7.12	7.07	6.95	7.03	7.05	0.1031
- *3-h post feeding*	6.79a	6.71b	6.76ab	6.83a	6.80a	0.0330
Ruminal VFAs						
- *0-h post feeding*						
Total VFAs (mM)	37.57	40.92	47.46	47.60	43.69	0.0540
Acetic acid (%)	67.39	68.18	67.59	69.07	68.81	0.8195
Propionic acid (%)	21.38	20.96	21.86	19.49	20.49	0.4310
Butyric acid (%)	11.24	10.86	10.55	11.44	10.69	0.5883
Acetic : Propionic	3.31	3.39	3.18	3.59	3.41	0.6158
-*3-h post feeding*						
Total VFAs (mM)	52.153	47.698	49.765	53.715	51.858	0.7026
Acetic acid (%)	65.653	67.805	67.645	69.985	66.313	0.6141
Propionic acid (%)	23.595	21.940	22.575	19.090	23.388	0.4736
Butyric acid (%)	10.7575	10.2525	9.7750	10.9275	10.3025	0.5553
Acetic : Propionic	2.9850	3.1925	3.0875	3.6825	2.9500	0.4921

5.5.5 Milk yield and milk compositions

Milk yield and milk compositions are presented in Table 5.7. Inclusion of CTs of differing molecular weight in the diet contained high level of sunflower oil did not affected on milk yield, milk compositions and milk fatty acids profile (Table 5.8, Table 5.9). Molecular weight of CTs did not affected on the milk fatty acid compositions. Milk from all goats treatments. Stearic acid, the final product of biohydrogenation process in the rumen did not significant different among treatment and ranged between 9 – 14 g/100 g FA, indicated that inclusion of CTs with differing molecular weight may not inhibit the growth of bacteria involved the final step of the biohydrogenation process.

Table 5.7 Effects of inclusion of CTs of differing molecular weight on milk yield and milk composition in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Milk yield (kg/d)	0.96a	0.80b	1.03a	1.00a	0.52	0.0261
Milk yield[1/] (kg/d)	0.94	0.91	0.84	0.75	0.87	0.0261
P-value (covariance analysis)						
T1	*	0.5178	0.0651	0.0025	0.2009	
T2	0.5178	*	0.2070	0.0100	0.4627	
T3	0.0651	0.2070	*	0.0812	0.5982	
T4	0.0025	0.0100	0.0812	*	0.0550	
T5	0.2009	0.4627	0.5982	0.0550	*	
Milk composition (%)						
fat	3.89	3.76	3.88	3.79	3.90	0.1272
protein	3.30a	3.31a	3.22b	3.23b	3.26b	0.0010
Lactose	4.67	4.74	4.65	4.66	4.66	0.1241
Total solids	12.52	12.45	12.45	12.38	12.53	0.7851
Solid-not-fat	8.80	8.76	8.70	8.68	8.75	0.9508

[1/]Yield adjusted (covariance analysis)

Table 5.8 Effects of inclusion of CTs of differing molecular weight on milk fatty acid composition in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Fatty acids (g/100 g FA)						
C4	1.51	1.45	1.24	1.48	0.94	0.0927
C6	2.01	2.16	1.92	2.18	1.67	0.0611
C8	2.24	2.63	2.39	2.64	2.30	0.0709
C10	7.17	8.15	7.71	8.51	8.31	0.2032
C11	0.19	0.17	0.17	0.13	0.14	0.9221
C12	5.35	6.98	6.27	5.60	6.78	0.0706
C13	0.02	0.00	0.03	0.00	0.00	0.6085
C14	10.76	9.73	9.18	10.54	10.81	0.0873
C14:1	0.38	0.26	0.37	0.25	0.34	0.4106
C15	1.11	0.94	1.04	0.76	0.88	0.0574
C15:1	0.16	0.04	0.07	0.14	0.04	0.2238
C16	30.31	24.29	24.93	24.90	25.21	0.1281
C16:1	0.56	0.37	0.44	0.64	0.79	0.8519
C17:1	0.22	0.11	0.11	0.17	0.11	0.5313
C18	9.06	12.86	12.61	13.77	10.58	0.4766
C18:1n9c	2.01	2.61	3.57	2.24	2.42	0.7245
C18:1n9t	19.183	18.27	20.56	20.53	20.915	0.6538
C18:2n6t	0.26b	0.25b	0.34ab	0.33ab	0.443a	0.0340
C18:2n6c	4.13	5.35	4.29	3.4750	5.0050	0.1983
C20	0.32	0.29	0.28	0.19	0.31	0.2006
C18:3n6	4.13	5.35	4.30	3.47	5.01	0.5309
C20:1	0.07	0.04	0.00	0.00	0.05	0.2435
C20:2	0.22	0.49	0.62	0.22	0.59	0.2331
C22:0	0.15	0.13	0.05	0.04	0.00	0.0589
C20:3 n6	0.07	0.00	0.00	0.00	0.00	0.0640
SFA	72.53	72.08	69.58	71.96	69.25	0.5149
MUFA	22.59	21.71	25.12	23.97	24.67	0.6017
PUFA	4.89	6.22	5.30	4.07	6.08	0.2067
PUFA : SFA	0.07	0.09	0.08	0.06	0.09	0.1976

[1/]SFA = sum of saturated fatty acids (C4:0-C14:0, C15:0, C16:0, C18:0: C20:0, C21:0, C22:0).
[2/]MUFA = sum of monounsaturated fatty acid (C14:1, C15:1, C16:1, C17:1, C18:1n9, C20:1, C22:1n9).
[3/]PUFA = sum of polyunsaturated fatty acid (C18:2n6, C18:3n6, C18:3n3, C18:4n3, C20:2, C20:3n6, C20:3n3, C20:4n6).

Table 5.9 Effects of inclusion of CTs of differing molecular weight on milk fatty acid yield in dairy goat.

Items	Treatment					P-value
	T1	T2	T3	T4	T5	
Yield of milk fatty acids (g/d)						
C4	0.53	0.42	0.44	0.52	0.18	0.2196
C6	0.69	0.61	0.74	0.77	0.32	0.3082
C8	0.77	0.73	0.97	0.93	0.43	0.3972
C10	2.47	2.26	3.11	3.02	1.57	0.4682
C11	0.06	0.04	0.06	0.04	0.03	0.6859
C12	1.77	1.93	2.43	1.99	1.30	0.5425
C13	0.00	0.00	0.02	0.00	0.00	0.4895
C14	3.61	2.70	3.32	3.72	2.06	0.3210
C14:1	0.13	0.07	0.16	0.11	0.07	0.3469
C15	0.38	0.26	0.38	0.27	0.17	0.1961
C15:1	0.05	0.02	0.03	0.04	0.01	0.5569
C16	10.02	6.71	8.77	8.74	4.74	0.1590
C16:1	0.22	0.10	0.14	0.22	0.16	0.8499
C17:1	0.08	0.03	0.02	0.05	0.02	0.2204
C18	3.85	3.77	5.01	4.95	2.03	0.4338
C18:1n9c	0.76	0.82	1.59	0.91	0.49	0.5495
C18:1n9t	6.99	5.06	7.24	7.18	4.00	0.2645
C18:2n6t	0.10	0.07	0.12	0.11	0.08	0.4542
C18:2n6c	1.30	1.44	1.81	1.19	0.94	0.6379
C20	0.11	0.08	0.10	0.06	0.06	0.4625
C18:3n6	0.07	0.03	0.03	0.01	0.01	0.3336
C20:1	0.02	0.01	0.00	0.00	0.01	0.4860
C20:2	0.09	0.12	0.25	0.06	0.11	0.3348
C22:0	0.05	0.03	0.01	0.01	0.00	0.0499
C20:3 n6	0.02	0.00	0.00	0.00	0.00	0.1024
SFA	25.01	20.18	25.98	25.43	13.14	0.3473
MUFA	8.25	6.12	9.18	8.51	4.75	0.3596
PUFA	1.57	1.65	2.21	1.37	1.14	0.5829
PUFA : SFA	0.07	0.09	0.08	0.06	0.09	0.1976

[1/]SFA = sum of saturated fatty acids (C4:0-C14:0, C15:0, C16:0, C18:0: C20:0, C21:0, C22:0, C23:0).
[2/]MUFA = sum of monounsaturated fatty acid (C14:1, C15:1, C16:1, C17:1, C18:1n9, C20:1, C22:1n9).
[3/]PUFA = sum of polyunsaturated fatty acid (C18:2n6, C18:3n6, C18:3n3, C18:4n3, C20:2, C20:3n6, C20:3n3, C20:4n6).

5.6 Conclusion

Based on the current results, inclusion of CTs with differing molecular weight in the diet rich-in sunflower oil did not affected on the fatty acid compositions of milk. However, although inclusion of CTs from both mangosteen peel and quebracho extract not altered the fatty acid profile, in the other hand, further study focus on the molecular weight and dose of CTs used would be investigated in particular their effect on bacteria involved in this process.

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CHAPTER VI

OVERALL CONCLUSIONS AND IMPLICATION

6.1 Conclusions

This study aimed to investigate the effect of inclusion of different molecular weight of condensed tannins on ruminal fermentation and milk fatty acid composition in dairy goat, with the hypothesis that, higher molecular weight of CTs had stronger inhibitory effects on ruminal methane production and inhibit the final step of ruminal biohydrogenation process which could results in higher unsaturated fatty acid and lower saturated fatty acid concentration in goat milk. For these objectives of study, the study was carried out comprising 3 experiments. The first experiment was conducted to determine the component of phenolic compounds such as total phenol, total tannins and condensed tannins, and molecular weight and protein-binding ability of condensed tannins in the selected tropical plant materials and commercial condensed tannins extract (Chapter 3). The second experiment was conducted to investigate the effect of different molecular weight (higher and lower) of condensed tannins on *in vitro* gas production kinetics, methane production and fermentation parameters (Chapter 4). The last experiment was carried out to determine the effect of inclusion of condensed tannins sources with differing molecular weight on feed intake and nutrient digestibility, and milk yield and milk fatty acid composition of dairy goats (Chapter 5). The results from this study are summarized as presented below.

Selected tropical plant materials and tannins extract with potential use as the

sources of condensed tannins (CTs) for ruminant feeding, including, leucaena, cassava, Siamese neem leaves, mangosteen peel and quebracho extract. Among of those plant species leaves, Siamese neem contained highest concentration of CTs and highest molecular weight. Furthermore, protein-binding ability, a biological property of CTs which is the major factor influent on CTs activities, had relative high with higher molecular weight of CTs.

Supplementation of CTs of higher MW from leaves of Siamese neem significantly inhibited *in vitro* total gas and methane productions while supplementation of the low MW condensed tannins from leaves of leucaena had no effect, except for total gas production at the highest (6 mg/100 mg DM) level of supplementation. Moreover, Siamese neem leaves had stronger effect ($P< 0.001$) on *in vitro* volatile fatty acids production.

Inclusion of CTs sources with represents the higher and lower molecular weight (quebracho extract and manosteen peel powder, respectively) at 3%DM of CTs equivalent have no detrimental effect to feed intake and nutrient digestibility, similarly, ruminal fermentation parameters and milk yield and milk compositions did not affected by different source of CT inclusion.

6.2 Implication

Currently, concerning of healthiness food for consumption have been greater considered. To produce healthy benificial foods (milk and meat) from ruminats, nutritional strategies in order to manipulate rumen fermentation which results in improving the animal products has greater interested. Utilization of plants containing tannins as the source of natural bioactive agent for ruminant feeding is a part of most

effective strategies. However, there were found that supplementation of tannins-containing plants to ruminant animal had a varied results. Therefore, more understanding of tannins characteristic of these plants could be useful for this strategy application. The novelty of this work is that we explore a molecular weight characteristic of condensed tannins of tropical avialable plant species which has potential use as a source of tannins in ruminant feeding. However, further researchs involving selective inhibitory effect to some baceria involved in ruminal biohydrogenation, dose of using of various sources with differing molecular weight of condensed tannins and molecular-based on experiment in rumen microbes should be investigate.

BIOGRAPHY

Mr. Anan Petlum was born on 18th April 1973 in Khon Kaen, Thailand. In 1992, he graduated high school level from Chumphae Suksa School, Khon Kaen. In 1996, he obtained his Bachelor's degree in Animal Science from the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen. During the year 1996 to 2002, he worked as the research assistant at Research and Improvement of Dairy Cattle Feeding Efficiency in North Eastern Region-Thailand Project (supported by FAO), Khon Kaen University, Thailand. In 2002, he graduated his a Master of Science in Animal Science (Ruminant Nutrition) from the Faculty of Graduate School, Khon Kaen University, Khon Kaen. During the year 2002 to 2005, he worked as the researcher at Livestock-Crop System Research Project (supported by International Livestock Research Institute, ILRI), Khon Kaen University, Thailand. Since 2005, he has been working as a lecturer at the Program in Animal Production Technology, Faculty of Technology, Udon Thani Rajabhat University, Udon Thani. He studied in a field of Animal Production Technology for PhD Program at the School of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima from November 2011 to September 2017 with the thesis entitled "Effects of Different Molecular Weights of Condensed Tannins on Ruminal Fermentation and Milk Fatty Acid Profile in Dairy Goats".