EFFECT OF PLANT EXTRACTS ON *IN VITRO* RUMEN MICROBIAL FERMENTATION

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE
IN
DAIRYING
(ANIMAL NUTRITION)

BY

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KARNAL - 132001 (HARYANA), INDIA

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Dedicated To
My Late Brother,

Dear DADA

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Approved by

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This is to certify that the thesis entitled, **"EFFECT OF PLANT EXTRACTS ON *IN VITRO* RUMEN MICROBIAL FERMENTATION"** submitted by **SHETE SHANKAR MOHAN** towards the partial fulfilment of the award of the degree of **MASTER OF VETERINARY SCIENCE in DAIRYING (ANIMAL NUTRITION)** of the NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: June 17 , 2008

(BHUPINDER SINGH)
MAJOR ADVISOR

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ABSTRACT

Ten plant extracts were screened for their potential to reduce methane production and/or to improve microbial protein synthesis without adversely affecting digestibility and out of these Rutin and *Terminalia belerica* aqueous methanol extract were selected for use in the *in vitro* studies to evaluate their effects on rumen fermentation parameters. Rutin was used at 15 and 30 mg levels while *T. belerica* extract was used at 2 and 3 ml levels. Berseem, oat and wheat straw (DM and isolated NDF) were used as substrates. The parameters studied were total gas and methane production, true DM and OM degradability, partitioning factor, microbial biomass, ammonia nitrogen, individual volatile fatty acids, IVCPD. In addition, degradation kinetic parameters viz. rate and extent of gas production for DM and isolated NDF were also studied. The kinetic parameters for cell solubles were estimated by difference.

Rutin at both the levels did not affect total gas and methane production while *T. belerica* aq. methanol extract significantly ($P< 0.001$) decreased total gas as well as methane production. Gas production from NDF was not affected by treatment and the pattern of gas production was similar in DM and cell solubles. This suggests that gas production form DM is mostly contributed by cell solubles. Degradation kinetic parameters were not affected by the treatments. Rutin at 15 and 30 mg level slightly increased DM and OM degradability whereas *T. belerica* at both the levels significantly ($P< 0.001$) decreased true degradability of DM as well as OM. Microbial biomass was not affected by treatment of Rutin but, decreased due to addition of *T. belerica* aq. methanol extract. Rutin significantly ($P< 0.001$) decreased ammonia nitrogen concentration and A:P ratio while *T. belerica* increased ammonia nitrogen concentration in all substrates. Rutin at 15 and 30 mg levels increased the rate of NH_3-N uptake for microbial protein synthesis but *T. belerica* was not having significant effect on IVCPD.

Rutin was effective in modulating rumen fermentation and may have potential as feed additives but, *Terminalia belerica* aqueous methanol extract needs further evaluation.

सारांश

दस पादपीय सत्वों का अन्तः पात्र मिथेन उत्पादन तथा पाचकता पर प्रभाव आकलन हेतु प्राथमिक परीक्षण किया गया जिनमे यूकेलिप्टस पत्तियों का सत्व रूटीन और टरमिनालिया बेलेरिका (बेहड़ा) का जलीय मिथेनाल सत्व अन्तः पात्र शोध हेतु चुने गये। रूटीन (15 और 30 मि. ग्रां.) तथा बेहड़ा सत्व (2 और 3 मि.ली.) पर प्रयोगित किये गयें। कार्यद्रव्य रूप में बरसीम, जई और भूसा लिये गये तथा अन्तः पात्र शुष्क पदार्थ (अशुपा), कार्बनिक पदार्थ पाचकता (अकापा), सकल गैस तथा मिथेन उत्पादन, विभाजक घटक, जीवाणविय जैवभार, अमोनिया नाईट्रोजन तथा प्रत्येक वाष्पशील वसीय अम्लों (वावअ) सान्द्रता का अध्ययन करने के साथ साथ उपरोक्त कार्यद्रव्यों में शुष्कपदार्थ व पृथककृत एन. डी. एफ. से गैस उत्पादन गतिज कारकों का अध्ययन भी किया गया।

रूटीन के दोनों स्तरों पर सकल गैस तथा मिथेन उत्पादन पर सार्थक प्रभाव नही पाया गया जबकि बेहड़ा सत्व के दोनो स्तरों पर सकल गैस तथा मिथेन उत्पादन सार्थक रूप से निम्न हुए । दोनों पादपीय सत्वों ने गैस उत्पादन गतिज कारकों पर सार्थक प्रभाव नही दर्शाया। रूटीन के दोनो स्तरों पर अशुपा तथा अकापा में बढ़ोतरी हुई किन्तु सार्थक नही थी। जब कि बेहडा सत्व के दोनों स्तरो ने अशुपा व अकापा में सार्थक रूप से कमी दर्शायी। दोनों स्तरों पर रूटीन ने जीवाणवीय जैवभार पर कोई प्रभाव नही दर्शाया। जबकि यह बहेडा सत्व के दोनों स्तरों पर दुष्प्रभावित हुआ। सभी कार्यद्रव्यों में रूटीन सम्पूरण से अमोनिया नाइट्रोजन और एसिटेटः प्रोपिओनेट अनुपात में कमी आयी। रूटीन के दोनों स्तरों पर जीवाणवीय प्रोटीन संश्लेषण हेतु अमोनिया नाइट्रोजन ग्राहयता मे बढ़ोतरी हुई। बेहड़ा सत्व के दोनो स्तरो पर सम्पूरण द्वारा सभी कार्यद्रव्यों में अन्तः पात्र प्रोटीन पाचकता अप्रभावित रही।

रोमन्थी किण्वन में लाभकारी बदलाव हेतु रूटीन प्रभावशील सिद्ध हुआ और इसे पशु आहार सम्पूरक रूप में प्रयोगित किया जा सकता है। पशु आहार हेतु बेहडा सत्व के प्रभावशील उपयोग के लिए अधिक शोध की आवश्यकता हैं।

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LIST OF ABBREVIATIONS

A:P	:	acetate to propionate ratio
'b'	:	Potential gas production (ml)
BW	:	body weight
'c'	:	Specific degradation rate
CT	:	condensed tannins
DM	:	dry matter
DMI	:	dry matter intake
DOMI	:	digestible organic matter intake
EMNS	:	efficiency of microbial nitrogen synthesis
HT	:	hydrolyzable tannins
IVDN	:	in vitro degradable nitrogen
IVDMD	:	in vitro dry matter digestibility
IVGP	:	in vitro gas production
IVTOMD	:	in vitro true organic matter degradability
ME	:	metabolizable energy
MN	:	microbial nitrogen
mM	:	millimole
MPS	:	microbial protein synthesis
NDFD	:	neutral detergent fiber digestibility
NH_3-N	:	ammonia nitrogen
OM	:	organic matter
PF	:	partitioning factor
ppm	:	parts per million
PSM	:	plant secondary metabolites
R^2	:	coefficient of determination
RUSITEC	:	rumen simulation technique
TD	:	true degradability

1. INTRODUCTION

The value of ruminant animals lies in their ability to utilize low quality feeds, upgrading low quality inputs to high quality outputs. This ability is due to the symbiotic relationship between ruminants and the large microbial population within their rumen. The rumen microbial population is able to digest and ferment plant cell wall polymers. Associated with this process is the production of volatile fatty acids (VFA), the major energy source in ruminants, accounting for approximately 80% of the energy disappearing from the rumen (France and Siddons, 1993). In addition, the outflow of microbes from the rumen to the lower digestive tract accounts for a large percentage (50-75) of the protein digested in and absorbed from the small intestine (AFRC, 1992). The ruminant has evolved a unique digestion system, where the slowly degradable fibre originating from newly ingested large feed particles is selectively retained in the rumen at the expense of older, smaller, and more digested particles. Compared to random passage, this approach increases fiber digestibility.

The VFA, microbial cells and gases produced per unit of substrate degraded are not constant. Microbial yield relative to VFA produced (i.e., microbial growth efficiency) in the rumen is variable (Leng, 1993). Therefore the efficiency of rumen metabolism has a profound effect on the production efficiency of the animal. The efficiency of rumen metabolism is also an important factor influencing the output of environment-polluting waste products. Methane gas produced during rumen fermentation causes loss of 8-10 % of gross energy intake of animal and it is also a major gas contributing in global warming. Efficient rumen fermentation results in reduced methane production and emission. Inefficient N retention by rumen microorganisms leads to the excretion of N-rich wastes. As a consequence there is considerable interest in the manipulation of rumen metabolism.

Various rumen manipulation techniques have been tried to increase the efficiency of rumen fermentation which aim toward increasing fiber digestion, propionate production, yield and efficiency of microbial protein synthesis and decreasing methane

production. Feed additives used for rumen manipulation include enzymes, antibiotics, ionophores, probiotics, herbal plants (Martin *et al*., 1999). Use of ionophore antibiotics improved performance and health in beef and dairy cattle. However, the use of antibiotics as feed additive in beef and dairy cows has been banned by European Union (EU regulation No. 1831/2003) due to the risk of antibiotics residue in animal products (milk and meat) and subsequent effects on the human health (Casewell *et al*., 2003). For this reason, attention has recently shifted to natural antimicrobials as a safe means of beneficially modifying ruminal fermentation. Further, there is an increasing consumer demand for organically produced meat and milk. Thus, there is increasing interest in exploiting natural products as feed additives that have no public health hazard. The natural products include probiotics, prebiotics, enzymes, organic acids and plant secondary metabolites or their nature-identical chemicals (Wallace, 2004).

It is estimated that there are 250,000 to 500,000 species of plants on earth. A relatively small percentage (1 to 10%) of these is used as foods by both humans and other animal species. Plants in the context of animal production are thought of first and foremost as the principal source of nutrients for animals, and sometimes as an unwelcome source of anti-nutritional factors that interfere with an animal's ability to maximize utilization of ingested nutrients. Scientists from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro* (Harborne, 1993; Wink, 1999a,b). The increased interest in phytochemicals in animal diets has been prompted by the disapproval and decline in the use of 'in feed' antibiotics, the removal of animal proteins from the diet and thus the increased variety and inclusion levels of vegetable protein sources. Thus, there are increasing numbers of novel plant species and by-products that are being identified and studied for their potential use in the agricultural industries.

Plant secondary metabolites (PSM) have been extensively studied because of the adverse effects that they have when ingested by animals (D'Mello, 1997; Cheeke, 1998; Acamovic *et al.*, 2004). However, more recently, the beneficial effects of PSM in animals have also been investigated (Douglas *et al.*, 1995; Kamra *et al*., 2000;

Pfannhauser *et al.*, 2001; Cross *et al.*, 2004; James *et al.*, 2004; Nash, 2004; Bento *et al.*, 2005; Alexander, 2005; Patra *et al*., 2006; Anamika, 2007; Alexander *et al*., 2008). Plants and their extracts with 'specific' properties could be used to manipulate rumen metabolism.

Keeping the above facts in view, the present study was carried out with the following **objectives:-**

1. **To investigate the effect of plant bioactives on *in vitro* ruminal fermentation parameters and microbial protein synthesis**

2. **To investigate the effect of plant bioactives on dry matter and fiber degradation kinetics**

2. REVIEW OF LITERATURE

The increased concentration of greenhouse gases (CO_2, CH_4 and N_2O) in the troposphere has been implicated in the consistent increase in atmospheric temperature and global warming over the last few decades (IPCC, 2001). The rising concentration of CH_4 is correlated with increasing world populations and currently about 70% of CH_4 production arises from anthropogenic sources and the remainder from natural sources (Moss *et al*., 2000).

In the rumen, hydrogen (H_2) is produced during the anaerobic fermentation of glucose. This H_2 can be used during the synthesis of VFA and microbial organic matter. The excess of H_2 from NADH is eliminated primarily by the formation of CH_4 by methanogens found in the rumen ecosystem (Baker, 1999). The stoichiometric balance of VFA, CO_2 and CH_4 indicates that acetate and butyrate promote CH_4 production whereas propionate formation conserves H_2, thereby reducing CH_4 production (Wolin, 1960).

Use of ionophore antibiotics improved performance and health in beef and dairy cattle. However, the use of antibiotics as feed additives in beef cattle and dairy cows has been banned in the EU due to the risk of antibiotic residues in animal products (e.g., milk and meat) and its subsequent effects on human health (Russell and Houlihan, 2003). For this reason, attention has recently shifted to natural antimicrobials as a safe means of beneficially modifying ruminal fermentation.

2.1 Plant secondary metabolites

Plant secondary metabolites (PSM) is a generic term used for more than 30,000 different substances which are exclusively produced by plants. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. The beneficial effects of most of the medicinal plants typically result from the combination of secondary metabolites present in the plants. Although PSM have historically been defined as chemicals that do not appear to have

vital biochemical role in the process of building and maintaining plant cells, recent research have shown their pivotal role in the eco-physiology of plants. They protect plants from pathogens, herbivores, inter-plant competition and abiotic stress and attract beneficial organisms such as pollinators or symbionts (Wink, 1999a). NAPRALERT, a database of world literature of natural products, lists over 135000 isolated and characterized natural products (Cordell, 2000), which is probably just a small percentage if it is considered that only an estimated 5-15% of the terrestrial plants have been investigated for the presence of secondary metabolites (Cragg *et al*., 1997). Complexity in terms of number and the variability of secondary metabolites in plants, and the interrelationship between them, is a major limitation to the rate of progress that is made in this area of research, where raw plant materials and plant extract preparations are being studied (Cordell, 2000).

2.2 Plant extracts as rumen fermentation modulators

Under roughage based feeding systems, rumen manipulation techniques aim toward increasing fiber digestion, propionate production, yield and efficiency of microbial protein synthesis and decreasing methane production. However practical methods to achieve these goals are yet to be developed. Various physical, chemical, biological and biotechnological methods have been applied to optimize rumen fermentation with limited success. Recent studies have shown that PSM at lower concentrations could be used to manipulate rumen fermentation and to improve animal productivity. Saponins and tannins are the group of secondary metabolites that have been widely investigated for their pro nutritive effects in ruminants. Therefore, studies involving saponins and tannins in relation to manipulation of rumen fermentation are reviewed while rutin is discussed separately.

2.3 Saponins

Saponins are natural detergents found in many plants. Saponins have detergent or surfactant properties because they contain both water-soluble and fat-soluble components. They are found in various parts of the plant: leaves, stems, roots, bulbs, blossom, and fruit. Saponins dissolve in water to form a stable soapy froth; which is thought to be due

to their amphiphilic nature. The word sapon means 'soap', referring to the permanent froth saponins make on being mixed with water. They are also characterized by their bitter taste, and their ability to hemolyze red blood cells. They consist of a fat-soluble nucleus, having either a steroid or triterpenoid structure, with one or more side chains of water-soluble carbohydrates

Saponins are the glycosides of 27 carbon atom steroids, or 30 carbon atom triterpenes. Removal of the sugar moiety (hexoses, pentoses, and saccharic acids) from a saponin by complete hydrolysis yields the aglycone, sapogenin. Diosgenin from the Mexican wild yam when subjected to the Marker degradation yields the synthetic hormone progesterone, the basis for combined oral contraceptive pill or simply "the pill." It was also the starting material for a cheap and plentiful supply of cortisone. In cultivated crops, including many legumes such as soy, bean, pea and lucerne, triterpenoid saponins are generally predominant. Saponins are highly toxic to cold-blooded animals, due to their ability to lower surface tension. Saponin as the sapogenin aglycone have also been identified in the animal kingdom in snake venom, starfish, and sea cucumber.

2.3.1 Effect of saponins on rumen microbial ecology

Rumen protozoa

A major source of ruminal ammonia is proteolysis of bacterial protein, occurring as a result of ingestion of ruminal bacteria by protozoa. Saponins have pronounced antiprotozoal activity. The mechanism of the anti-protozoal effects is that saponins form irreversible complexes with cholesterol. Cholesterol and other sterols are components of the cell membranes of all organisms except prokaryotes (bacteria). Thus, reductions in ruminal protozoa numbers observed when saponins are fed (Lu and Jorgensen, 1987; Wallace *et al.*, 1994; Klita *et al.*, 1996) and within *in vitro* ruminal fermentation systems (Makkar *et al.*, 1998; Wang *et al.*, 1998) are caused by reaction of saponins with cholesterol in the protozoal cell membrane, causing breakdown of the membrane, cell lysis, and death. The antiprotozoal activity requires the intact saponin structure with both the nucleus and side chain(s) present. Saponins may have potential as natural ruminal defaunating agents. However, a complicating factor is that saponins are

hydrolyzed by ruminal bacteria that remove the carbohydrate side chains (Makkar and Becker, 1997; Wang *et al.*, 1998). Because there may be an adaptation of ruminal bacteria for metabolism of saponins, one approach for retaining antiprotozoal activity would be to feed saponins intermittently. Such a regimen might suppress protozoa, but without the continuous presence of saponins bacterial adaptation might also be suppressed. Thalib *et al.* (1996) found that administering saponins to sheep every 3 d was effective in suppressing protozoa and reducing ruminal ammonia concentrations. Primarily as a result of suppression of ruminal protozoa, dietary saponins increase the outflow of bacterial protein from the rumen (Wallace *et al.*, 1994; Makkar and Becker, 1996).

Makkar and Becker (1997) observed that Quillaja saponins were stable in the rumen for up to 6 h after administration. It is possible that this time period may be adequate for the saponins to have antiprotozoal activity. Thus, the fact that saponins are rapidly degraded in the rumen may not necessarily eliminate their capacity to suppress ruminal protozoa. Ruminal protozoa are unable to adapt to or detoxify saponins (Newbold *et al.*, 1997). The practicality of using yucca extract to suppress rumen protozoa have been questioned (Killeen *et al.*, 1998), because effective concentrations (1,000 to 10,000 mg/L) are much higher than those commonly applied to livestock feeds (60 to 250 mg/kg).

Rumen bacteria and fungi

The mode of action of antibacterial effects of saponins seems to involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium (Killeen *et al.*, 1998). Thus, their inhibitory activity is associated with adsorption to microbes and is, therefore, influenced by microbial population density. Sen *et al.* (1998) observed a concentration-dependent growth response of *E. coli* K-12 to Quillaja and yucca saponins, with growth-promoting activity at low concentrations and inhibition at higher levels. Thus, the impact on a mixed bacterial population such as in the rumen is difficult to predict.

Pure cultures also indicate possible antibacterial effects of saponins. *Y. schidigera* extracts abolished growth of the fiber digester, *Butyrivibrio fibrisolvens*, and prolonged the lag phase of *Streptococcus bovis* (Wallace *et al.*, 1994). Similar sensitivity of *S. bovis*

to *Y. schidigera* extracts was found by Wang *et al.* (2000a), who additionally found that cellulose digestion by *Ruminococcus* spp. and *Fibrobacter succinogens* was inhibited.

The anaerobic ruminal fungi, *Neocallimastix frontalis* and *Piromonas rhizinflata* were highly sensitive to *Y. schidigera* saponins (Wang *et al.*, 2000a).

Valdez *et al.* (1986) observed that sarsaponin, from *Y. schidigera*, decreased protozoal numbers but not bacterial number in a 22 d semi-continuous system, and Lu *et al.* (1987) found that the bacterial population was changed in the presence of alfalfa saponins from a morphologically diverse one in controls to one in which fewer morphotypes were present in vessels receiving saponins.

2.3.2 Effect of saponins on end products of rumen fermentation

Rumen fluid characteristics

The effect of saponins on total ruminal VFA production and molar proportions are variable. Some studies have observed increased propionate production at the expense of acetate and butyrate production, whilst other have seen no effect (Hart *et al.*, 2007). Part of this variability is explained by differences in saponin type and concentration, but interestingly, the effects seems to be more pronounced (increase in propionate) when a diet is rich in grain or starch (Hristov *et al.*, 1999; Lila *et al.*, 2003) in contrast to diets rich in fiber or mixed forages (Hess *et al*., 2003b). This suggests that composition of the diet may influence the response to saponin supplementation.

Many saponins cause decrease in rumen ammonia concentrations (Lu *et al.*, 1987; Wallace *et al.*, 1994; Makkar *et al.*, 1998). However this effect is not consistent across studies. Muetzel *et al*. (2003) reported that saponin from *Sapindus rarak* did not inhibit feed protein degradation *in vitro*, whilst Wang *et al*. (1998) found that extracts from *Y. schidigera* enhanced casein breakdown in the continuous culture fermenters. Decreased NH_3-N in the rumen may be due to higher uptake of NH_3 for the synthesis of bacterial cells or decreased dietary protein degradation or increase rate of passage or bacterial predation by protozoa. Increased propionate is desired since it acts as alternate hydrogen

sink in the rumen thereby reducing methane formation. Decrease in branched chain fatty acids can occur due to inhibition of deamination of amino acids.

Makkar *et al*. (1998) compared the fractions with saponins from *Y. schidigera*, *Quillaja saponaria* and *Acacia auriculoformis* on NH_3 production in an *in vitro* ruminal system. The NH_3-N concentration in the rumen fermentation medium was 29.7, 14.9 and 14.7% lower after 24 incubation in the presence of yucca, quillaja and acacia saponins, respectively. A similar trend was observed when hay was replaced by hay plus concentrate (in the ratio of 70:30) conforming superior nature of yucca saponins to reduce NH_3 concentrations over other saponins.

Effect of saponin containing yucca extract (De-Odorase) on TVFA concentration was investigated by Ryan *et al*. (2003) using hay, straw and concentrate as substrates. During *in vitro* studies, De-Odorase (100 mg/l) caused initial increased in TVFA levels in hay (6.4%), or straw (3.1%) as substrates. Dietary incorporation of some saponin containing extracts enhances propionate production in the rumen (Hristov *et al.*, 1999; Lila *et al.*, 2003; Wina *et al*., 2005; Anamika, 2007).

Methane production

Only few reports are available showing the effects of saponins on CH_4 production. Wang *et al.*, (1998) reported that yucca extract addition at 0.5 mg/ml did not affect total gas or CH_4 production *in vitro*. In contrast, Takahashi *et al*. (2000) found significant decrease in total gas and CH_4 production due to addition of yucca extract (0.01ml/ml) to an *in vitro* ruminal system. The decrease in the CH_4 production may be due to decrease in protozoal number to which some of the methanogens are attached and partially due to increase in the propionate production which provides alternative sink for hydrogen which otherwise is used for CH_4 production.

Hess *et al*. (2003a) observed *S. saponaria* fruit supplementation decreased daily CH_4 production by 20% in RUSITEC. The effect of *S. saponaria* was pronounced in defaunated versus faunated rumen fluid. When related to OM apparently fermented, differences relative to control diet persisted, but CH_4 production per unit of fiber degraded did not differ between *S. saponaria* diet and control diet. The ratio of CH_4 to

CO_2 was also lower with *S. saponaria* supplementation, indicating variation in gas release pattern also. Hess *et al*. (2003b) further reported that *S. saponaria* reduced methanogenesis by 11% on average in grass alone or in legume supplemented diets.

Partitioning of nutrients and microbial protein synthesis

Different saponins influence partitioning of nutrients in different ways. The microbial protein synthesis was higher for yucca and acacia saponins for which gas production was lower than the control (Makkar *et al*., 1998). For Quillaja saponins, the gas production was similar to that of control and higher microbial mass was observed. In the presence of acacia saponins, there was a shift in the partitioning of fermented substrate. Acacia saponins decreased gas production and increased microbial mass production while Quillaja saponins increased the extent of truly degraded substrate from 300 to 323 mg without change in gas production. These results suggested that there could be (1) the same amount of truly degraded substrate but different microbial mass and gas production as for acacia saponins, (2) the same volume of gas production but different microbial mass production depending upon the extent of truly degraded substrate as for Quillaja saponins, and (3) occurrence of phenomena 1 and 2 together as for the yucca saponins. They concluded that saponins partition the nutrients in such a manner that a higher proportion of the digested substrate goes into the formation of microbial mass and a lower proportion to SCFA and gas.

Wang *et al*. (2000b) reported an increase in the microbial protein synthesis when steroidal saponin isolated from yucca extract was added @ 15 µg/ml to buffered suspension of mixed ruminal microbes. Microbial efficiency (g of microbial N/kg OM apparently fermented in the rumen) significantly increased due to supplementation of *S. saponaria* fruit in sheep fed grass hay as sole diet (Abreu *et al*., 2004).

2.3.3 Effect of saponins on microbial protein supply

According to Dewhurst *et al*. (2000), synchrony between the availability of fermentable energy and degradable nitrogen in the rumen, level of feeding and subsequent dilution rate and nitrogen recycling by protozoa are the main factors affecting the microbial protein synthesis in the rumen and microbial protein supply at intestinal

level. Lu and Jorgenson (1987) observed decrease in bacterial nitrogen flow to duodenum due to intra ruminal administration of alfalfa saponins (2 or 4% of DMI) in sheep fed roughage or concentrate diet. The effect was more pronounced in roughage than in concentrate diet. In contrast, Klita *et al*. (1996) reported that duodenal flow of microbial nitrogen was increased by intra ruminal administration of same source and dose of saponin in sheep. In both the experiments, diaminopimelic acid (DAPA) was used as marker to measure duodenal flow of bacterial nitrogen. However, Lu and Jorgenson (1987) prepared saponins by solvent extraction followed by acid hydrolysis. This method has been shown to yield sapogenins and saponin artifacts. Abreu *et al*. (2004) used total purines as marker to quantify microbial nitrogen and reported that duodenal microbial N flow was 34% higher due to intra ruminal administration of *S. saponaria* at 10% level. Hristov *et al*. (1999) found no significant results with yucca extracts by measuring urinary purine derivatives.

2.3.4 Saponins and ruminant production

Saponins have selective effects on ruminal microorganisms might be useful in livestock production. A safe, persistent suppression of ciliate protozoa may have widest application. Ciliate protozoa are primarily responsible for the substantial turnover of bacterial protein which occurs during fermentation (Ushida *et al.*, 1991; Wallace and McPherson 1987; Williams and Coleman, 1991). As a consequence, nitrogen retention is improved by defaunation, which has amply demonstrated in many studies where the protozoa were removed by chemical or physical means, or where the animals had been isolated from birth and thus had not been colonised by protozoa (Williams and Coleman 1991). The argument in favour of defaunation depends on other factors as well, however. As some species of protozoa are cellulolytic, there are implications for fibre breakdown of removing protozoa (Demeyer & Van Nevel 1986; Kayouli *et al.*, 1984). Also, the protozoa are proteolytic, so there would be consequences there too (Ushida *et al.*, 1991). However, it is generally agreed that removing or suppressing protozoa would make the best use of nitrogenous resources, particularly on low-protein diets.

Effects of saponins on the bacterial population merit further examination. Wang *et al.* (2000a) suggested that *Y. schidigera* extract would be best used with high grain

diets, because of its suppressive effect on *S. bovis S. bovis* is a starch-digesting, lactate-producing Gram-positive species which is a major cause of rumen fermentation lapsing into lactic acidosis (Stewart *et al.*, 1997). The suppression of bacteria involved in fibre digestion could have serious consequences to overall digestion.

In animal feeding trials, there have been mixed observations concerning fermentation and productivity. Lu and Jorgensen (1987) found that alfalfa saponins caused a decrease in the efficiency of microbial protein synthesis in sheep, because the growth of bacteria as well as protozoa was depressed. A 36% fall in the efficiency of protein synthesis occurred in cattle receiving *Y. schidigera* extract (Goetsch and Owens, 1985). In contrast, inclusion of *Enterolobium cyclocarpum* increased the rate of body weight gain in sheep by 24% (Leng *et al.*, 1992) and 44% (Navas-Camacho *et al.*, 1993) and wool growth by 27% (Leng *et al.*, 1992), which was attributed to a decrease in protozoal numbers. These differences therefore imply that the effects of saponins on ruminant nutrition are complex, and depend on diet, and the saponins involved. General observations with saponins, where changes in ruminal fermentation characteristics occur, that saponins administration decreases NH_3 concentration (Lu and Jorgensen 1987; Lu *et al.*, 1987; Makkar *et al.*, 1998) and, where VFA are affected, increases propionate concentration (Lu *et al.*, 1987; Hristov *et al.*, 1999) are typical effects of decreased protozoal numbers (Williams and Coleman 1992). Saponin-containing *Y. shidigera* extract appeared to have ammonia-binding properties (Headon *et al.*, 1991). However, the reduction in rumen ammonia concentrations when *Y. shidigera* extract was fed is most likely due to suppression of ciliate protozoa (Wallace *et al.*, 1994; Wang *et al.*, 1998).

2.3.5 Metabolism of saponins in ruminants

Rumen metabolism

Makkar and Becker (1997) showed that quillaja saponin could be degraded by rumen microbes. The degradation rate was slow initially but much more rapid after 6 h of *in vitro* incubation. The degradation products of saponins have not been investigated in detail, but it is suggested that quillaic acid is one of the degradation products of quillaja

saponin (Makkar and Becker, 1997) and medicagenic acid that of alfalfa saponin. *Butyrivibrio* strains were presumed to degrade alfalfa saponins; however, isolated strains failed to degrade any saponin. Saponins from *Y. schidigera* and *Narthecium ossifragum*, which have the same aglycone (sarsapogenin), were degraded in the rumen to sarsapogenin as the major degradation product and five other derivative products of sarsapogenin: smilagenin, episarsapogenin, epismilagenin, sarsapogenone and smilagenone (Flaoyen and Wilkins, 1997; Flaoyen *et al*., 2002). However, Meagher *et al*. (2001) showed that saponins from an extract of *Costus speciosus* rhizome were hydrolyzed not to its aglycone (diosgenin) as the major degradation product but to epismilogenin, the epimerized product of diosgenin. The formation of sapogenin and its several derivative products in the rumen indicates that several processes such as hydrolysis of saponin, epimerization and hydrogenation of sapogenin occur in the rumen (Meagher *et al*., 2001; Flaoyen and Wilkins, 1997).

Intestinal metabolism

All the sapogenins are transported further along the digestive tract and are excreted in the feces. They may be absorbed in the duodenum and transported to the liver via the portal vein (Flaoyen and Wilkins, 1997; Flaoyen *et al*., 2002). In the liver, free sapogenins may be conjugated with glucuronide and excreted into the bile. The appearance of salts of the sapogenin glucuronide in the liver and in the bile has been reported in sheep suffering photosensitization. These salts are insoluble and precipitate in the bile duct as crystals. The disruption of bile excretion results in retention of phylloerythrin and induction of photosensitization (Miles *et al*., 1994).

2.4 Tannins

Tannins are oligomeric compounds with multiple structure units that have free phenolic groups. They range anywhere from 500 to sometimes greater than 20,000 in molecular weight. Tannins are usually soluble in water (Haslam, 1982) except for some with high molecular weight structures. They are also capable of binding proteins and forming soluble and insoluble tannin-protein complexes. Tannins are usually divided into

two groups, hydrolyzable tannins (HT) and CT (proanthocyanidins), based on their chemical structure and properties (Athanasiadou *et al.*, 2001).

2.4.1 Hydrolyzable Tannins

Hydrolyzable tannins are molecules with a carbohydrate, generally D-glucose as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid (ellagitannins) (Waghorn and McNabb, 2003). Hydrolyzable tannins are usually present in low amounts in plants (Mueller-Harvey, 2001). These tannins are found in oak (*Quercus* spp.), Acacia, Eucalypts and a variety of browse and tree leaves (Waghorn and McNabb, 2003). The browse that contain these leaves and apices can contain anywhere from 200g per kg of dry matter (DM) and in some species they can contain phenolic compounds that can exceed 500g per kg of dry matter (Reed, 1995; Lowry *et al*., 1996). Hydrolyzable tannins are potentially toxic to animals, but most ruminants can adjust to a diet of these tannins (Waghorn and McNabb, 2003). Ruminants are able to adjust to these toxic tannins by reducing their urinary excretion of degradation products, thus allowing them to consume these diets (Lowry *et al*., 1996). Although ruminants have this ability, an excessive amount of this tannin diet can lead to liver and kidney lesions, as well as death (Waghorn and McNabb, 2003). Death usually occurs five to ten days after the first excessive consumption; the toxic compound responsible is not known.

2.4.2 Condensed Tannins

Of the tannins, condensed tannins are the most widely distributed. Condensed tannins are oligomers or polymers of flavonoid units linked by carbon-carbon bonds (Waghorn and McNabb, 2003) not susceptible to cleavage by hydrolysis (Reed, 1995). They are called condensed tannins because of their condensed chemical structure. CT, are also termed proanthocyanidins (PA), which is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins through heating of PA in acidic alcohol solutions (Haslam, 1982). Cyanidin (procyanidin) and delphinidin (prodelphinidin) are the most common anthocyanidins produced (Reed, 1995).

Condensed tannins can contain as little as two or greater than fifty flavonoid units. Due to the variability of flavonoid units to some substituents and because of the variable sites for

interflaven bonds, condensed tannin polymers have complex structures. Condensed tannins may or may not be soluble in aqueous organic solvents, depending on their chemical structure and degree of polymerization. Condensed tannins have a complex chemistry. The heterocyclic C-rings can be formed via 2,3-cis or 2,3-trans, which determine "how monomeric units are attached relative to one another" (Barry *et al*., 1999). The number of monomeric units are variable (Foo *et al*., 1996, 1997) making an "infinite variety of chemical structures, which in turn affect the biological properties of the CT" (Barry *et al*., 1989). For example, *Lotus corniculatus* and *L. pedunculatus* are considerably different concerning their chemical structure (Foo *et al*., 1996, 1997) It is speculated that plants containing CT evolved over time to implore them as a defense mechanism, which protected them against pathogenic microorganisms and against being consumed by insects or grazing animals (Swain, 1979). Condensed tannins found in tropical forages are thought to promote plant growth by reducing the release of leaf litter into the soil (Palm and Sanches, 1991) and reducing the release of animal feces (Waghorn and McNabb, 2003). Because of the substantial benefits of CT for ruminant health and productivity, much of research has been focused on hese tannins (Waghorn and McNabb, 2003). There are many different types of foliage that contain condensed tannins: lotus, sainfoin (*Onobrychus viciifolia*), sulla (*Hedysarum coronarium*), dock (*Rumex obtusifolia*), *Sericea lespedizia* (*L. cuneata*) and *Dorycnium rectum*, all of which can do well even in average or poor soil as well as acidic soil (Waghorn and McNabb, 2003). Condensed tannin ontaining forages have different benefits for ruminants, depending on the species of plant. For example, lotus has been proven beneficial in the prevention of bloat (Beddows, 1956). Other CT has been efficient at improving live-weight gain (Waghorn *et al*., 1994). In sheep, they have been shown to increase milk protein concentration (Wang *et al*., 1994), improve lambing percentages (Min *et al*., 2002), and reduce, gastrointestinal nematode infection (Niezen *et al*., 1998), incidence of fly strike (Leathwick *et al.,* 1995), and methanogenesis in sheep (Waghorn *et al*., 1994).

2.4.3 Effect of tannins on nutrient digestion and absorption

By forming hydrogen bond between the phenolics sub units of the polymer and the carbonyl groups of peptides of the protein, tannins bind with protein at near neutral pH

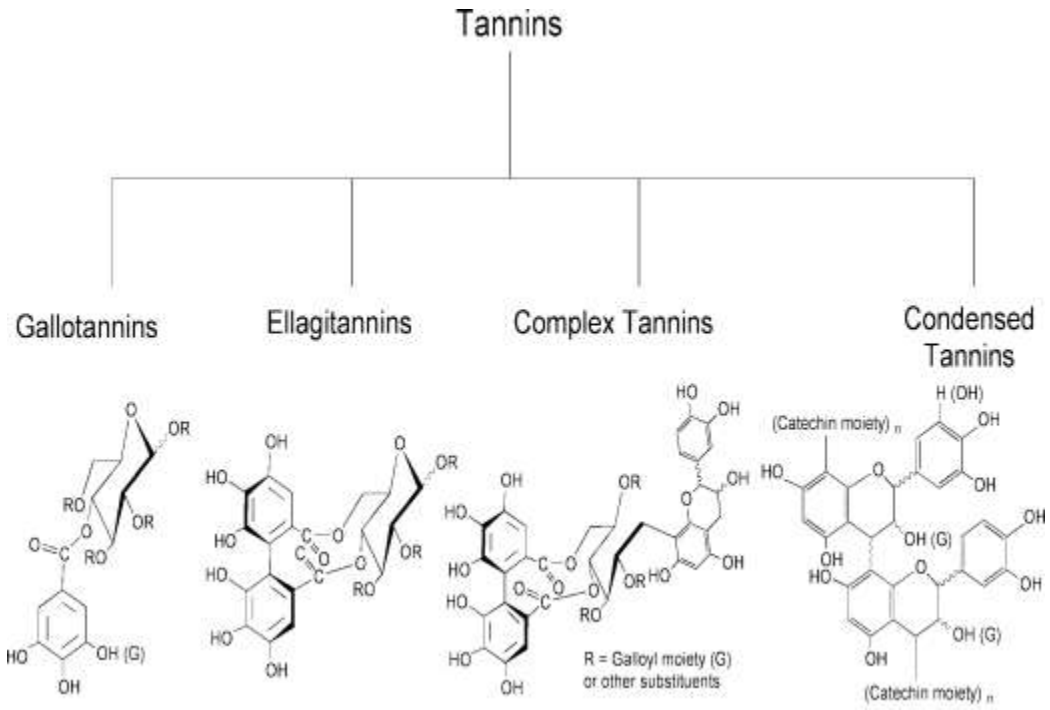


Fig. 1: Classification of tannins

(4.0 to 7.0) to form tannin protein complexes, but dissociates and release protein at acidic pH (<3.5) (Jones and Magnum, 1977). Specificity of tannin for protein is dependent on the conformational structure of proteins including size and charge of the protein molecule (Hagermann and Butler, 1981). Martin *et al*. (1985) demonstrated that protein tannin complex is stable in pH range of 5.6 to 7.0 but above or below this range the complex dissociates. Once the complex passes through the rumen, it becomes subjected to gastric (pH 2.5 to 3.5) and pancreatic secretions and dissociates, making the protein available for absorption from the small intestine.

Waghorn *et al*. (1994) found a difference due to CT level on the apparent absorption of essential amino acids (EAA) from the small intestine of sheep fed on fresh *Lotus corniculatus* (2.2% CT) and *L. pedunculatus* (5.5% CT). The CT of *L. corniculatus* increased both abomasal flow and the net absorption of EAA from the small intestine (53

and 59% respectively), with no effect on apparent digestibility in the small intestine, while CT of *L. pedunculatus* increased abomasal flow by 30% in sheep fed on this forage. McNabb *et al.* (1998) demonstrated that the differences in CT structure between *L. corniculatus* and *L. pedunculatus* did not cause appreciable difference in the *in vitro* precipitation of leaf protein (Rubisco) from white clover. However, *L. pedunculatus* CT extract was more effective in reducing Rubisco degradation by ruminal microbes than the CT extract from *L. corniculatus* (Aerts *et al*., 1999). These findings suggest that protein precipitation by CT may be more responsive to relative molecular mass of CT, but the effect of CT on the degradation of protein by microorganisms may be more responsive to difference in flavon-3-ol composition of CT.

Devarajan (1999) observed that addition of CT or HT decreased *in vitro* degradable nitrogen (IVDN) values in *Artocarpus lakoocha* and *Grewia optiva* while it increased in *Acacia catechu, Albizia Stipulata, Bauhinia variegate, Dendrocalamus hamiltonii, Ficus roxburghii, Leucaenea leucocephala, Morus alba* and *Quercus incana.* Santos *et al*. (2000) reported that tannic acid treatment was effective in decreasing rapidly soluble fraction of alfalfa and Bermuda grass silages. However, tannic acid reduced the effective crude protein degradability of alfalfa grass, but not the Bermuda grass.

2.4.4 Effect of tannins on rumen microbial ecology

Although the mode of action of tannins is not fully known, the reduction in proteolysis may be attributed to direct effects of tannins on microbial enzyme activity or to indirect effects on rumen metabolite concentrations which can regulate proteolytic activity in some bacteria (Waghorn *et al*., 1994). Jones *et al*. (1994) found that sainfoin CT had profound effects on 5 strains of proteolytic rumen bacteria. Polya and Foo (1994) reported that increasing degree of catechin polymerization isolated from the cladodes of *Phyllocadus trichomanoides*, the bark of *Psedotsuga menziesii* and heartwood of *Acacia melanoxylon* correlated with increased inhibitory activity of phospholipid dependent protein kinase and Ca^{2+} dependent protein kinase. Molan *et al*. (2001) studied the effect of CT from *L. corniculatus* and *L. pedunculatus* on the growth of four strains of proteolytic rumen bacteria. Both the sources CT inhibited the growth of all the four strains of bacteria. However, growth of *Eubacterium* sp., *Prevotella bryantii* and

Butyrivibrio fibrisolvens was inhibited more by CT of *L. pedunculatus* than by *L. corniculatus*. McSweeny *et al*. (2001b) observed that total number of cellulolytic bacteria including *Fibrobacter succinogens* and *Ruminococcus flavifaciens* was lower in sheep supplemented with calliandra forage rich in CT while fungal population was less affected.

Studies related to influence of tannins on rumen protozoa are of relatively recent origin. Terrill *et al*. (1992) found an increase in protozoal numbers in sheep grazing sulla (CT). Higher protozoal population in sheep fed with *L. corniculatus* plus polyethylene glycol than those fed *L. corniculatus* alone was observed suggesting possible antiprotozoal effect due to CT (Wang *et al*., 1994),. Makkar *et al*. (1995b) found decrease in protozoal population in the presence of quebracho tannins *in vitro*. However, McSweeny *et al*. (2001b) reported that protozoal population was less affected by calliandra tannins. In contrast, quebracho powder fed to cattle at 0.6 % of dietary DM reduced protozoal populations (Baah *et al*., 2002). Ruiz *et al*. (2004) observed an absence of holotrichs and low numbers of endodiniomorph protozoa in the rumen of goats fed olive leaves (CT, 11.1mg/g DM) alone. However, when animals received a diet based on olive leaves, barley and faba beans, holotrichs appeared and endodiniomorph populations increased.

Hydrolysable tannins from *Castanea sativa* wood extract did not influence protozoal count both *in vitro* (Sliwinski *et al*., 2002a) and *in vivo* (Sliwinski *et al*., 2002b) Recent study by Hristov *et al*. (2003) showed that HT (tannic acid) also exhibit antiprotozoal activity.

2.4.5 Effect of tannins on end products of rumen fermentation

Rumen fluid characteristics

Tannins generally exhibit inhibitory effect both on carbohydrate and nitrogen metabolisms in the rumen. Makkar *et al*. (1995a) during *in vitro* studies found decrease in TVFA and molar proportion of acetate and increase in propionate in the presence of tannins. Singh *et al*. (2001) observed lower NH_3-N concentrations in the presence of tannic acid in an *in vitro* ruminal system. Tannin might have complexed with deaminase

and urease (Sliwinskii *et al*,, 2002a). Hristov *et al*. (2003) showed that tannic acid not only decreased NH_3-N, it also reduced acetate, propionate, isobutyrate, iso valerate and TVFA concentrations and A:P ratio. However, daily intra-ruminal administration of quebracho extract (75 % CT) to sheep did not affect pH, NH_3-N and VFA concentrations.

Methane production

Roth *et al*. (2001) compared the effect of tannin rich extracts from chestnut and mimosa (HT) and quebracho (CT) on methane production *in vitro* at very high concentrations. Chestnut tannins were very effective in reducing methanogenesis. However, methane production was not reduced by chestnut extract during both *in vitro* (Sliwinskii, *et al*., 2002a) and *in vivo* studies (Sliwinskii, *et al*., 2002b) at lower concentrations. Puchala *et al*. (2005) compared the effects of feeding tannin rich *Lespedeza cineata* (17.7 % CT) and tannin free mixed crab grass (*Digitaria ischaemum* and *Festuca arundinacea*) (0.5 % CT). Methane emission expressed as both quantities per day or relative to dry matter intake was lower for *Lespedeza cineata* than for crab grass (7.4 vs. 10.6 g/d and 6.9 Vs. 16.2 g/kg DMI). Substantial differences between the forages in CT concentration and methane emission by goats suggested that CT decrease methane emission.

Partitioning of nutrients and microbial protein synthesis

Microbial protein synthesis *in vitro*, expressed as ^{15}N incorporation into microbes per unit of short chain fatty acid production was more efficient in the presence of tannins (Makkar *et al*., 1995a). The incorporation of ^{15}N into microbial cells and efficiency of microbial protein synthesis were higher with CT (quebracho tannins) compared with HT (tannic acid). Recent study by Hristov *et al*. (2003) also showed that presence of tannic acid at lower concentrations (0.1 %) in *in vitro* incubation medium increased ^{15}N incorporation into bacteria while decreased protozoa. Beever and Siddons (1986) indicated an increase in microbial protein supply after feeding moderate concentration of CT from sainfoin in sheep. Calliandra tannins (2 to 3 %) in the diet failed to alter microbial protein flow from the rumen in sheep (Mc Sweeny *et al*., 1998). McNeill *et al*. (1998) also found similar results in response to feeding of tannin rich *Leucaena*

leucocephala (7.3 % CT). McNeill *et al*. (2000) further demonstrated that there was no change in microbial protein flow in sheep fed 100 % diet of dried Leucaena hybrid (11.6 % CT). Min *et al*. (2002) also observed no change in total microbial protein in the rumen or microbial protein flow to abomasum in sheep fed *L. corniculatus*.

2.4.6 Effect of tannins on microbial protein supply

Egan and Ulyatt (1980) observed an increase in nitrogen retention in sheep fed sainfoin (CT 10 to 11 %) when compared to those fed white clover and rye grass. The CT of *L. corniculatus* (2.2 % CT) increased both abomasal flow and net absorption of essential amino acids from small intestine (53 and 59 %, respectively) (Waghorn *et al*., 1987). However, feeding of CT rich *L. pedunculatus* (5.5 % CT) increased abomasal flow of amino acids by 30 %, but increase in intestinal apparent absorption of essential amino acids was only 10 %. (Waghorn *et al*., 1994). It was postulated that the mechanism through which CT may reduce amino acid absorption could be due to free CT inhibiting endogenous enzyme activity or by associating with intestinal mucosa. In an attempt to define the interrelationship between tannin-protein binding ability and nitrogen digestion post ruminally, tannins in several species of Leucaena were examined for their effect on protein digestion (McNeill *et al*., 1998). Purified CT from the Leucaena was assessed for their ability to bind protein as well as to release complexed protein, between the abomasums and the end of small intestine in sheep. These studies demonstrated that Leucaena species differed markedly in apparent digestion of nitrogen and this was correlated with the ability of the purified tannins to bind protein. However, protein complexed with tannin had a digestibility greater than 78 % in small intestine. It has been suggested that gains in delivery of digestible dietary protein to small intestine is offset by enhanced losses of endogenous protein due to interactions between the dissociated CT and secreted and structural protein from the intestinal tissue.

2.4.7 Ruminal degradation of tannins and tannin-protein complex

There is sufficient evidence suggesting degradation of HT by rumen microbes (Makkar *et al*., 1995a,b). *Selenomonas ruminantium* and *Streptococcus sp*. probably cleave the ester bonds with the help of enzymes esterase and tannin acylhydrolase to

produce gallic acid and ellagic acid (Nelson *et al*., 1998). Gallic acid is then decarboxylated to pyrogallol and converted to resorcinol and phlorglucanic ring to acetate and butyrate (Murdiaty *et al*., 1992). The rumen bacteria involved in this degradative pathway includes *Eubacterium oxidoreducens*, *Streptococcus bovis*, *Syntrophococcus sucromutans* and *Coprococcus sp.* Singh *et al.* (2001) showed degradation of tannic acid to gallic acid, pyrogallol and resorcinol using rumen fluid of cattle which had no prior exposure to tannin rich diets.

However, little evidence exists for degradation of CT in the rumen. Robbins *et al*. (1991) reported that ingested quebracho tannin was completely recovered from the faeces of mule and deer, but only 75% was recovered from faeces of sheep. It suggested that CT might have been metabolized in the digestive tract and absorbed. Similarly, studies on sheep and goats fed tannin rich *Acacia saligna* showed absence of CT in faeces (Degen *et al*., 1995). Makkar *et al*., (1995a) showed that rumen microbes are not capable of degrading oligomeric CT during *in vitro* incubations. Rumen microbes were exposed to small amount of quebracho tannins using rumen simulation technique for eight days and there was no degradation of CT. (Makkar *et al.,* 1995b). In another study, CT (Sephadex LH-20 purified quebracho and *Acacia nilotica* tannins) were degraded within seven days under aerobic conditions in artificial fermenters (Makkar, 2003).

Anaerobic bacteria have been isolated from non-ruminant animals which can dissociate HT protein complexes, but not complexed with CT (Nemoto *et al*., 1995; McSweeny *et al*., 1999). The enzymes (tannin acylhydolases; esterases) depolymerise the tannin polymer by cleaving ester linkages between glucose and the phenolic subunits (Skene and Brooker, 1995). A similar mechanism for dissociation of the CT-protein complex would require depolymerisation of CT by cleavage of carbon-carbon bonds, which has only been demonstrated under aerobic conditions and is probably unlikely in the rumen (McSweeny *et al*., 2001a).

2.5 Rutin

Rutin is a bioflavonoid, a flavonol glycoside comprised of the quercetin and the disaccharide rutinose (rhamnose and glucose). Pure rutin is yellow or yellow-green colored needle-shaped crystal. Rutin is found in many plants, fruits and vegetables. The richest source is buckwheat. Rutin is also found in citrus fruits, noni, black tea, apple peel. During digestion much of the rutin is metabolized to its aglycone, quercetin. Rutin has strong antioxidant properties. Rutin has also the property to chelate metal ions, such as iron, thereby reducing the Fenton reaction (production damaging oxygen radicals). Rutin has anti-inflammatory effects. Animal studies have shown that rutin has preventive and healing effects. There are indications that rutin can inhibit some cancerous and pre-cancerous conditions. Rutin may help to prevent atherogenesis and reduce the cytotoxicity of oxidized LDL-cholesterol.

Quercetin and rutin are used in many countries for blood vessel protection and are ingredients of numerous multivitamin preparations and herbal remedies.

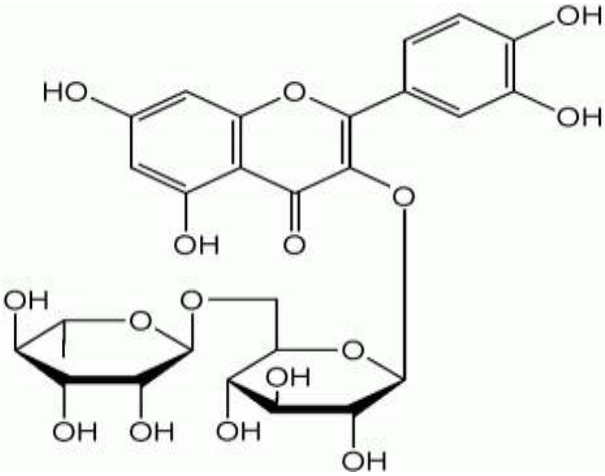


Fig. 2: Chemical structure of Rutin

2.5.1 Extraction of Rutin

Rutin is highly soluble in a number of organic solvents like methanol, ethanol, pyridine, etc. these solvents dissolve other substances present in the plant besides rutin. Therefore, the choice of these solvents is highly unattractive (Thappa *et al.*, 1982).

Though rutin is poorly soluble in cold water, but is fairly soluble in boiling water. Also water does not dissolve most of the other unwanted substances and it involves practically no expense at all.

Rutin is commercially extracted from leaves of *Eucalyptus macrorhyncha* and *Eucalyptus youmanii* with hot water at high temperature and starts settling down at about 900C (Sood and Kalia, 1996). Fine powdered dry leaves 30-40 mesh are boiled with water (1:20) for about 25-30 min. in closed vessel at elevated pressure of 15-20 lbs. Excessive heating for long period causes hydrolyses of rutin into Quercetin. The hot extract is filtered immediately and fine yellow coloured rutin is separated out on cooling. The crystallization of pure golden yellow coloured rutin is done with methanol (Sood and Kalia, 1996).

2.6 *In vitro* gas production

The *in vitro* and *in situ* techniques now in use (Tilley and Terry, 1963; Goering and Van Soest, 1970; Ørskov *et al.*, 1980) have the disadvantage that the contribution of the soluble components of the original feed cannot be assessed, since they rely mainly on the measurement of particle loss. Fermentation, on the other hand, is the anaerobic decomposition of substrates by microorganisms and can be assessed according to the production of end-products, of which VFA and gas are the most important (Lin *et al.* 1985).

The *in vitro* gas production technique is used widely in animal nutrition for feed evaluation and to study the kinetics of microbial fermentation processes in the digestive tract. At present, cumulative gas production is increasingly being used to measure the fermentation kinetics of ruminant feeds (Beuvink and Spoelstra, 1992; Pell and Schofield, 1993; Theodorou *et al.* 1994). The technique relies on mathematical modelling to estimate the rate and extent of feed digestion from cumulative gas production profiles. The amount of gas which is released when feedstuffs are incubated *in vitro* with rumen liquor is closely related to digestibility and therefore to the energetic value of feedstuffs for ruminants (Menke *et al.* 1979).

Hohenheim *in vitro* gas production test

A working group in Germany (Menke *et al.*, 1979; Raab, 1980; Menke and Steingass, 1988) developed a feed evaluation test mainly based on the measurement of *in vitro* gas production. The system they developed is basically a Tilley and Terry (1963) system but gas production rather than dry matter loss is measured. Huang (1986) reported that gas production of hay, grain and cotton fibre measured by Hohenheim *in vitro* gas production test followed first order reaction kinetics. Blümmel (1994) concluded that gas volume exclusively described substrate fermentation to SCFA and that there was inverse relationship between the production of short chain fatty acids and microbial biomass yield when both were related to a given unit of substrate truly fermented.

Production of CO_2 and CH_4 can be regarded as a measure of energy available for protein synthesis (Raab *et al.,* 1983). The principles of techniques using gas production measurements have been reviewed by Theodorou *et al.* (1998) and Getachew *et al.* (1998a).

There are a number of factors that affect fermentation of feeds *in vitro*. These are mainly associated with the nature of rumen fluid inoculum, breed of animal, its physiological condition, diet, time of feeding, time of collection of rumen fluid relative to feeding time (Craig *et al.,* 1987), method of rumen fluid collection (i.e. liquid or solid phase) (Craig *et al.,* 1987; Cecava *et al.,* 1990), and time elapsed between rumen fluid sampling and inoculation (Robinson *et al.,* 1999).

Fermentations are conducted in large (100 ml) calibrated glass syringes containing the feedstuff and a buffered rumen fluid. In this system, gas production in 24 h observed on incubation of 200 mg feed DM correlated well with OMD determined *in vivo* with sheep. The volume of gas in 24 h from 200 mg feed DM was used together with the concentration of other chemical constituents to predict ME (Menke and Steingass, 1988).

2.7 Kinetics of gas production

Digestion rate determines the amount of nutrients supplied to the animal. Rate estimation from gas production depends on a predictable relationship between forage digestion and microbial metabolism. Kinetics of feed stuffs fermentation can be determined from fermentative gas and the gas released from buffering of the short chain fatty acids (SCFA). Kinetics of gas production is dependent on the relative proportions of soluble, insoluble but degradable, and undegradable particles of the feed. Mathematical description of gas production profiles allows analysis of data, evaluation of substrate and media related differences, and fermentability of soluble and slowly fermentable components of feeds. Various models have been used to describe gas production profiles. The exponential model by Ørskov and McDonald (1979) is widely used in ruminant feed evaluation to describe degradation kinetics as measured with the nylon bag technique, but the model has also been used to describe kinetics of gas production data (Blümmel *et al.*, 1990; Siaw *et al.*, 1993; Khazaal *et al.*, 1993). This model is based on first-order kinetics, assuming a constant fractional rate of fermentation (Groot *et al.*, 1996). Since some feed particles ferment at different rates, the assumption in exponential model is not universally valid. Beuvink and Kogut (1993) evaluated various curve fitting models and reported that the exponential model resulted in larger residual mean squares as compared to sigmoidal models.

Groot *et al.* (1996) introduced three-phasic model which differentiates soluble, insoluble but fermentable, and microbial turnover. Conceptually, this model should provide useful data; however, it requires sophisticated equipment to record gas production at different time of incubation. Furthermore, the model performed poorly when used in the prediction of voluntary feed intake of 24 roughages from Ethiopia (Blümmel *et al.*, 1998).

Dry matter and NDF digestion rates may be obtained using standard *in vitro* techniques, but these methods are labor-intensive (Pell and Schofield, 1993; Cone *et al.*, 1996). Gas production is a promising technique to measure the digestion rates of the soluble and insoluble fractions of forages (Menke and Steingass, 1988; Pell and Schofield, 1993; Cone *et al.*, 1996).

Wolin (1960) presented calculations on the relationship among VFA, gas production, and the fermentation of glucose. These calculations are often used to predict changes in gas production caused by metabolic shifts during fermentation (Beuvink and Spoelstra, 1992). Gas arises directly from microbial metabolism and indirectly from the reaction of acid end products with bicarbonate, an important component of the buffering system (Beuvink and Spoelstra, 1992). The use of gas measurements to estimate the rate of forage digestion depends on a close relationship between gas yield and VFA production (Blümmel and Ørskov, 1993). Microbial population size and metabolism may vary over the course of fermentation (El-Shazly and Hungate, 1965; Naga and Harmeyer, 1975; Krishnamoorthy *et al.,* 1991). Changes in microbial metabolism or yield may alter the relationship between substrate digestion and gas production and could affect the estimation of digestion rate from gas measurements.

Since, feed fermentation and microbial biomass synthesis in the rumen are the major components of digestion in ruminants; it would be desirable to have feed descriptive indices capable of describing feedstuffs for these characteristics. As the fermentation characteristics of feed protein and carbohydrates influence efficiency of microbial biomass synthesis (EMBS; Clark *et al.,* 1992; Sinclair *et al.,* 1995), the feed or diet specific differences in microbial efficiency is considered in diet formulation for ruminants (NRC, 2001). Blümmel *et al.*. (1997) reported that the ratio of *in vitro* substrate true digested to gas volume, defined as the "partitioning factor" (PF), could serve as an index to assess the differences in EMBS of feedstuffs on the assumption: (i) carbon of TDOM is quantitatively distributed between microbial biomass and the sum of fermentative gas and SCFA; and (ii) gas production is stoichiometrically related to SCFAs. Further, it was observed that the PF of the mixed diets had a significant relationship with the microbial efficiency *in vivo*, indicating the possibility of using PF of the diet to influence EMBS *in vivo* (Blümmel and Lebzien, 2001; Blümmel *et al.*, 2003). However, PF for concentrate feed stuffs reported in the literature is meager (Blümmel *et al.,* 2003). The PF, for the respective group of feedstuffs varied from 3.86 to 6.48, 3.28 to 4.53, 2.73 to 3.72 and 2.55 to 3.23. Kiran and Krishnamoorthy (2007) concluded that the range in the PF of the tested feed ingredients was wide enough to facilitate ingredient selection for higher PF.

2.8 Gas production from NDF and cell solubles

NDF is a mixture of polymers with variable sugar composition and digestibility. Thus, the mixture of hexoses and pentoses changes constantly over the course of the fermentation (Van Soest, 1994). These factors may partially explain the low gas volumes early in the NDF fermentations. Because the changing ratios seem to affect only the first 1 to 3 ml of gas produced for any of the samples analyzed, rate calculations are unlikely to be seriously affected. The gas and VFA production were correlated with NDF disappearance using the isolated NDF samples. When gas production from the isolated NDF was regressed against NDF disappearance for each forage, the resulting slopes ranged from 0.31 ml/ mg for mature bromegrass to 0.37 ml/mg for corn stover. The mean slope (gas yield from NDF digestion) from the NDF data was 0.35 ml of gas/mg of NDF disappearance (r^2 = 0.92, n= 171) (Doane *et al.*, 1997). *In situ* studies on ADF degradation in alfalfa (Nocek and English, 1986) suggested two distinct pools. Several dual pool models, including a dual-pool logistic model different in format from the one presented here, were tested by Robinson *et al.*. (1986) and gave a relatively unbiased fit to in situ NDF digestion data from various feedstuffs. Gas yield was 0.35 ml/mg (r^2 = 0.92) of NDF digested for the isolated NDF.

Little is known about the digestion kinetics of the NDS fraction, because most *in vitro* kinetic methods have studied the disappearance of insoluble cell wall components and, in addition, gravimetric techniques do not usually measure the soluble faction. However, knowledge of the degradation characteristics of both fractions is very important to formulate balanced diets for high-yielding animals. For this purpose the curve subtraction method is useful, as reported by many authors (Schofield and Pell, 1995; Stefanon *et al.*, 1996; Calabrò *et al.*, 2001). The contribution of the NDF fraction to gas production increases while that of the NDS decreases with time.

Previous kinetic studies of fiber digestion have considered the process as a first order chemical reaction in which microbial growth does not play a major role (Waldo *et al.*, 1971; Mertens and Loften, 1980). Models in common use include the exponential, logistic, and Gompertz (France and Thornley, 1987). These models differ both in their

mathematical forms and in the underlying assumptions about the effects of microbial numbers and substrate levels on growth. Their application to gas production curves from fiber digestion can potentially yield information useful for predicting the digestion of fiber in animal feeds and for analyzing other gas-yielding fermentations.

The gas production profiles are not necessarily linearly related to degradation or fermentation of substrate. The higher predicted compared with measured gas production for CS indicated that the stoichiometric relations (Van Soest, 1994) are probably not valid for rapidly fermenting substrates. For CSC, the approximately 25 % higher gas production predicted by stoichiometric calculations than measured was similar to overestimations of gas production reported for cellulose (by 20%) and glucose (by 10%)by Beuvink and Spoelstra (1992). Typically, these overestimations occur for substrates producing high proportions of propionic acid. The use of pure substrates by Beuvink and Spoelstra (1992) seems to rule out the possible mechanism of reduction of gas production by bonding of $C0_2$ to NH_3. A second process which could reduce the gas produced per mol VFA, would be the conversion of CO_2 and H_2 to acetate instead of CH_4 (Miller, 1995), which would result in the net consumption of one mol $C0_2$. This process mainly occurs when low roughage diets containing high proportions of sugars and protein are fed (Leedle and Greening, 1988). A third possibility could relate to the large crude protein fraction of CC. Little work has been done in the area of the stoichiometric relations for the fermentation of protein, and it is likely that these differ from those for hexose equivalents.

Relatively large amounts of propionic and butyric acid from CS are more frequently reported when easily digestible substrates are supplied to rumen microbes (Van Houtert, 1993). When the rate of gas production was high, the proportions of propionic and butyric acid increased and consequently the calculated gas yield was low. After 24 h of incubation, the gas production rate was lower, and mainly acetic acid was produced which led to an increase in calculated gas yield**.** This corresponds with the more frequently observed two-phasic gas production (Pell and Schofield, 1993; Groot *et al.,* 1996). However, multiphasic analysis of the gas production profiles was not possible, due to the limited number of observations per curve. For CW, the value of calculated gas

yield increased throughout the incubation, due to the higher production of acetic acid compared with other VFA. This resulted in a higher half time for gas production (265h) than for fermentation of OM (23.7 h).

The proportion of gas associated with the rapidly digesting portion of the unfractionated forage exhibited the same basic trends that were reported by Schofield and Pell (1995). As the fiber content of the unfractionated forage increased, the amount of gas in the rapid pool decreased. The improved fit of the single pool logistic model for the NDS fraction of the mature alfalfa and bromegrass samples is inconsistent with earlier research. Stefanon *et al.*. (1996) presented parameter estimates from the two-pool logistic model from fermentations of the water-soluble fractions of alfalfa and bromegrass samples of varying maturity. The extraction methods (water vs. neutral detergent solution) may account for the differences. Schofield and Pell (1995) also reported parameter estimates from the two-pool logistic model for alfalfa NDS. Two reasons why the one-pool model better fits the data from this study may be 1) the greater maturity and NDF content of the mature alfalfa sample and 2) the use of washed and resuspended bacteria rather than whole ruminal fluid.

The fast pool of the unfractionated forage is identified from a mathematical analysis of the gas profile rather than from a chemical or physical entity. The values for the specific rates of the rapidly digested pool in the isolated NDF samples (0.107 to 0.194 h^{-1}) were similar to the fast rate of the unfractionated forage (0.095 to 0.170 h^{-1}) and comparable to rates previously reported (Schofield and Pell, 1995; Stefanon *et al.,* 1996). The gas volume associated with the fast specific rate in the unfractionated forage may be a composite pool originating from both the NDF and NDS fractions and must be interpreted cautiously in relation to chemical composition. Similarly, the slower rates of the NDS fraction presented here for the immature alfalfa (0.04 h^{-1}) and by Stefanon *et al* (1996) for water-soluble components (0.03 h^{-1} to 0.07 h^{-1}) approach the values of the slower rates within the NDF fraction (0.03 h^{-1}). This supports the contention that the gas associated with the slower rate in unfractionated forage may arise from both the NDF and NDS fractions.

Doane *et al.* (1997) studied the effect of ensiling on the NDS fraction of forages at different maturity stage. Calabro *et al.*. (2007) studied the fermentation of NDS using a 2x3x2 factorial arrangement of two intercropped forages (barley/broad bean and vetch/oats) collected at three growth stages and preserved by haymaking. Each feed sample and its isolated NDF were fermented *in vitro* and gas production was monitored utilizing the IVGPT. Haymaking decreased gas yield and rate of gas production from the unfractioned forage. Increasing maturity did not change the final gas volume, but reduced the rate of gas production from the NDS fraction. The rate of gas production from the NDS fraction of hay decreased on average by 2.9 ml/h compared with fresh forage. On average, haymaking reduced the gas yield from the NDS fraction by 18.70%. The fermentation of the fibre within the unfractioned forage and the isolated NDF were similar, as also reported elsewhere (Schofield and Pell, 1995; Stefanon *et al.*, 1996; Doane *et al.*, 1997; Calabrò *et al.*, 2001). Krishnamoorthy *et al.*. (1991) documented a curvilinear relationship between total microbial synthesis and the gas production over 2 h. This indicated that smaller amounts of gas were produced per unit of microbial yield at higher rates of fermentation. Thus, the digestion rate and microbial synthesis may affect the balance of metabolic end products between gas and VFA and partially account for the poor correlation between the A: P ratio and gas produced per mM of VFA across forages. The variability in the VFA measurements from multiple *in vitro* fermentations for this experiment is similar to that reported by other authors (Peters *et al.*, 1989).

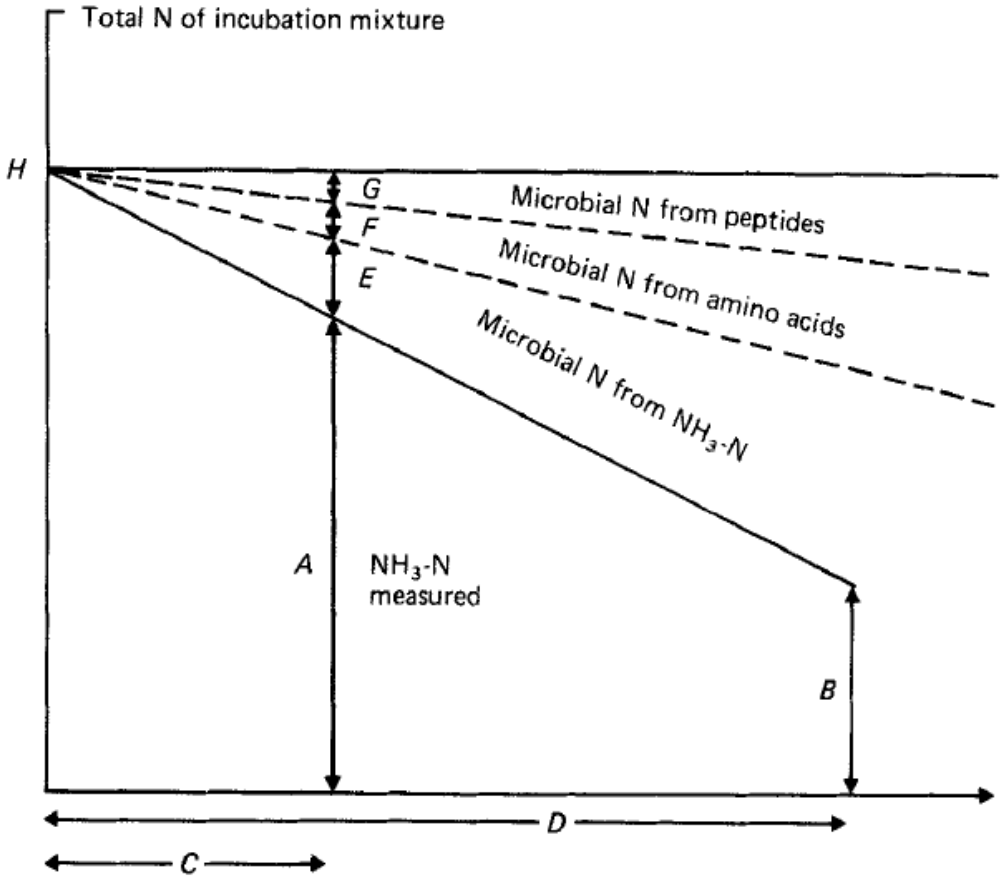
2.9 *In vitro* degradable nitrogen

Degradability of protein in the rumen is an important value to be used for the prediction of protein passing undegraded to the small intestine and for the calculation of protein utilization and protein requirements of ruminants. Attempts have been made to estimate protein degradation from N solubility (Crooker *et al*., 1978), commercial proteases (Krishnamoorthy *et al*., 1983) and by the use of polyester bags to be incubated in the rumen of a fistulated animal (Orskov & McDonald, 1979). Difficulties in measuring protein degradation from the release of ammonia arise from the fact that

protein degradation and bacterial protein synthesis are processes which occur simultaneously (Alexander, 2005).

Raab *et. al.* (1983) proposed a method to estimate ruminal protein degradation based on measurements of ammonia concentration and gas production (Menke *et al.* 1979) when a feedingstuff was incubated with rumen fluid *in vitro*. The method described here uses the known relationship between fermentation of carbohydrates and microbial protein synthesis for the determination of NH_3-N incorporated into microbial proteins. The total amount of NH_3-N liberated is found by extrapolation of the linear regression to zero protein synthesis. NH_3 liberated during incubation is in part used for microbial protein synthesis. Production of carbon dioxide and methane can be regarded as a measure of energy available for protein synthesis. The ratio, gas production: incorporation of NH_3-N was estimated by addition of starch to the substrate. The noresponse in gas production was linear when starch was added to the substrate (Fig. 3). Linear regression between NH_3-N concentration (y, mg) and gas production (x**,** ml) yielded an intercept (b_0) representing that amount of NH_3-N which would be released when no fermentable carbohydrates were available and consequently no bacterial protein synthesis took place. The difference between this intercept b_0**,** and NH_3-N content in the blank (rumen fluid without substrate added) indicated the amount of NH_3 liberated from protein and other N-containing compounds of the feedingstuff incubated. *In vitro*-degradable N (IVDN) was calculated as a proportion of total N by the equation:

$$IVDN = \frac{\text{NH}_3\text{ - N at zero gas production} - \text{NH}_3\text{ - N of blank}}{\text{Total nitrogen of feedstuff incubated}}$$



A= ammonia-nitrogen content after incubation of feedingstuff alone;
B= ammonia-nitrogen content after incubation of feedingstuff + starch;
C= gas production from feedingstuff alone;
D= gas productions from feedingstuff + starch;
E, F, G= microbial N expected to be derived from NH_3-N, amino acids and peptides, when the feedingstuff is incubated alone;
H= NH_3-N expected to be present at zero gas production

Fig. 3: Schematic representation of the relationship between protein degradation and protein synthesis in rumen fluid *in vitro***.**

3. MATERIALS AND METHODS

A brief description of experimental techniques followed during the course of study is presented in this chapter. The study was conducted in two phases.

PHASE I

3.1 Selection of plants

Based on their traditional medicinal use, ten medicinal plants and one purified compound, Rutin, isolated from leaves of *Eucalyptus macrorhyncha* were screened to select two best plants for evaluation of their effects on in *vitro* rumen fermentation. The plant parts were collected from the 'Herbal Garden' of Central Soil Salinity Research Institute, Karnal, Haryana and are listed in Table 3.1.

Table 3.1 List of medicinal plants selected for screening their potential as rumen fermentation modulators

Botanical name	**Common Name**	**Part selected**	**Known active Principle**
Acacia concinna	Shikakai	Pods	Saponins
Albizzia lebbeck	Siris	Seeds	Saponins
Aloe barbadanis	Aloe vera	Laves	Steroids
Alstonia scholaris	Devil tree	Leaves	Alkaloids
Azadirachta indica	Neem	Leaves	Azadirachtin
Eucalyptus globulus	Eucalyptus	Leaves	Essential oils
Terminalia arjuna	Arjuna	Leaves	Tannins
Terminalia belerica	Behara	Leaves	Tannins
Tribulus terrestris	Gokhru	Seeds	Saponins
Withania somnifera	Ashwagandha	Leaves	Alkaloids, Steroids

3.2 Preparation of plant extracts

Water and 50% aqueous methanol were used as solvents to prepare plant extracts. The protocol followed was as per Alexander, (2005) and is described below.

1. The plant part was cut into small pieces and oven dried at < 50^{o}C.
2. The dried plant material was ground to pass through a 1 mm screen.
3. A known quantity of finely ground sample was weighed into 250 ml conical flask. The respective solvent was added in ratio of dry weight of the sample to solvent as 1:10.
4. The flask was tightly sealed and kept on a mechanical shaker (20^{o}C, 120 rpm) for 12 h.
5. The contents of the flask were squeezed through four layers of muslin cloth.
6. The filtrate obtained from aqueous extract was centrifuged at 5000 rpm for 20 min. and filtered through Whatman filter paper No. 41 while aqueous methanol extract was filtered using Whatman filter paper No. 1.
7. The filtrate was collected and stored in a deep freeze at -15 to -20^{o}C until use.

3.3 Screening of plant extracts for their effect on i*n vitro* rumen fermentation

The plant extracts were screened for their effect on *in vitro* rumen fermentation parameters in an *in vitro* gas production test as per Menke and Steingass (1988). Berseem, Oat and Wheat straw were used as substrates. The parameters studied during screening were total gas production, methane production, TD and IVTOMD. *Morus alba* was used as the internal standard for *in vitro* gas production method.

Extracts from selected plants were ranked based on their effects on *in vitro* methane production and digestibility in an initial screening and five potential extracts (*Aloe barbadanis, Azadirachta indica, Terminalia arjuna, Terminalia belerica* and rutin) were selected for further screening to select two best plant extracts. Two plant extracts causing significant decrease in gas and methane production with little or no effect on the digestibility were selected for further detailed study (in Phase II).

3.4 Chemical composition of substrates

Berseem, Oat and Wheat straw were dried at 60^0C and ground to pass 1mm sieve. The ground samples were stored in air tight plastic bottles for further analysis.

3.4.1 Proximate Composition

Standard methods as described in AOAC (1995) were followed for the determination of proximate composition.

Dry matter

Representative samples of feed ingredients were weighed in moisture cups and kept overnight in a hot air oven at 100 ± 2^0C. Dried samples were weighed and DM calculated as follows:

$$DM(\%) = {}^{a}\!/\!_{b} \times 100$$

where, a = weight of the sample after oven drying

b = fresh weight of the sample

Nitrogen

Nitrogen content of the samples was determined by the standard Kjeldahl method using a digestion unit (Pelican) and digested samples were distilled in a distillation unit (Pelican). The distillate was collected in boric acid (2%) with mixed indicator (methyl red and bromocresol green in 0.7:1). The titration was done using standard N/100 sulphuric acid.

Ether extract

A known quantity of ground sample was taken in the thimble (Whatman) and extracted for 7-8 h with petroleum ether (B.P. 40-60^0C) using Soxhlet apparatus. The extracted oil along with oil flask was dried at 100 ± 2^0C to a constant weight. The ether extract was

estimated as the difference in the weight of oil flask with and without oil and also as the loss of weight in sample in the thimble due to extraction.

Total ash

A known quantity of ground sample was taken in a preweighed silica crucible and charred over the heater to make it smoke free. The crucible along with the sample was ignited at 550^0C for 4 h. The residue on ashing was taken as total ash and was expressed on DM basis.

3.4.2 Fiber composition

Fiber composition was determined as per the methods of Robertson and Van Soest (1981).

Neutral detergent fiber

A known quantity of ground sample (0.5 or 1.0 g) was taken in spoutless beaker and a known quantity of neutral detergent solution (50 or 100 ml) was added to it. The beaker along with the contents was heated to boil and refluxed for 60 min. The contents were filtered through a preweighed Gooch crucible (G-1, 50 ml) under vacuum with 3-4 washings of hot distilled water and a final washing of acetone. The crucibles were dried to a constant weight at 100 ± 2^0C and weighed. Cell wall contents or NDF was calculated as follows:

$$\text{NDF}(\%) = \frac{(\text{wt.of crucible} + \text{cell wall contents}) - \text{wt.of crucible}}{\text{wt.of the sample}} \times 100$$

Acid detergent fiber

A known quantity of ground sample (0.5 or 1.0 g) was taken in spoutless beaker and a known quantity of acid detergent solution (50 or 100 ml) was added to it. The beaker along with the contents was heated to boil and refluxed for 60 min. The contents were filtered through a preweighed Gooch crucible (G-1, 50 ml) under vacuum with 3-4 washings of hot distilled water and a final washing of acetone. The crucibles were dried

to a constant weight at 100 ± 5^0 C and weighed. Acid detergent fiber was calculated as follows:

$$\text{ADF}(\%) = \frac{(X - Y)}{S} \times 100$$

Where, X = weight of oven dried crucible including ADF

Y = weight of empty oven dried crucible

S = sample weight on DM basis

The NDF and ADF determinations were in triplicate.

3.5 Preparation of isolated neutral detergent fiber

Neutral detergent fiber was prepared as per the method of Schofield and Pell (1995). About 5 g of feed sample was weighed in to 1L spoutless beaker, to which 500 ml of neutral detergent solution was added. The contents were heated to boil and refluxed for 1 h and filtered through nylon screen under vacuum and thoroughly washed with hot tap water (10-12 washings) and a final washing with acetone. The residue was transferred to another 1L flask containing 500 ml of 1M ammonium sulphate. The flask with its contents was incubated over night at 39 ± 1^0C. The contents, after incubation, were filtered under vacuum with repeated washings with hot distilled water and dried in a hot air oven maintained at 50 ± 1^0 C. The NDF obtained was free of sodium lauryl sulphate.

PHASE II

3.6 *In vitro* studies

The amount of gas produced, extent of degradation of DM and NDF of selected feeds were determined by *in vitro* gas production technique.

3.6.1 *In vitro* gas production (IVGP) technique

The amount of gas which is released when feedstuffs are incubated *in vitro* with rumen liquor is closely related to digestibility and therefore to the energetic value of feedstuffs for ruminants (Menke *et al.* 1979). The DM, fiber and protein degradation was studied by *in vitro* gas production method (Menke *et al.* 1979; Raab *et al* 1983; Alexander, 2005).

The incubations were carried out in 100 ml calibrated glass syringes (Fortuna,Germany) as described by Menke *et al*., (1979) and Menke and Steingass (1988). The substrate was weighed on a plastic boat with removable stem and was placed into the bottom of the glass syringe ensuring that it did not stick to the sides of the syringe. The piston was lubricated with petroleum jelly and pushed inside the glass syringe. Plant extracts were injected prior to incubation. Incubation was done in triplicate for each substrate (three each for blank, respective plant extract, control and two for standard) at 0, 2 and 3 ml plant extract/30ml of inoculum. The syringes were incubated in a oven at $39^0 \pm 0.5^0$C for 24 h. The following table gives details of solutions and the order in which they were added prior to the filling in syringes.

Item	30 syringes	45 syringes	60 syringes
Solution			
Distilled water	365 ml	550 ml	730 ml
Micromineral solutiona	0.1 ml	0.15 ml	0.185 ml
Rumen buffer solutionb	183 ml	275 ml	365 ml
Macromineral solutionc	183 ml	275 ml	365 ml
Resazurin solution	0.95 ml	1.45 ml	1.90 ml
Rumen liquor	330 ml	500 ml	660 ml
Reducing solution			
1N NaOH	1.6 ml	2.4 ml	3.1 ml
$Na_2S.7H_2O$	220 mg	330 mg	440 mg
Distilled water	37 ml	55 ml	73 ml

Micromineral solution

$CaCl_2.2H_2O$	13.2 g	
$MnCl_2.4H_2O$	10.0 g	
$CoCl_2.6H_2O$	1.0 g	
$FeCl_3.6H_2O$	8.0 g	Dissolved in 100 ml of water.

Rumen buffer solution

NH_4HCO_3	4.0 g	
$NaHCO_3$	35.0	Dissolved in 1000 ml of water.

Macromineral solution

Na_2HPO_4 anhydrous	5.70 g	
KH_2PO_4 anhydrous	6.20 g	
$MgSO_4.7H_2O$	0.60 g	Dissolved in 1000 ml of water

Resazurin solution

Dissolved 100 mg in 100 ml distilled water

The incubation medium without rumen liquor was prepared by mixing appropriate amount of different solutions depending upon no. of syringes to be incubated. The medium mixture solution was prewarmed to 39ºC and bubbled with CO_2 just before the collection of rumen liquor. Rumen liquor was collected from two fistulated male cattle maintained on a roughage based diet (1.0 kg concentrate mixture in equal proportions at 10.00 am and 4.00 pm and wheat straw *ad lib*.) before morning feeding into a pre-warmed thermo-flask and brought to the laboratory. The rumen liquor was bubbled with CO_2 for about 2 minutes and filtered through 4 layers of muslin cloth. Then reducing solution was added to medium mixture and CO_2 was bubbled. The colour of the medium changed from blue over pink to colourless, indicating complete reduction of resazurin. Once the medium became colourless, the required amount of filtered rumen liquor was added to it. The ratio of medium mixture solution to rumen liquor was 2:1. 30 ml of buffered rumen liquor was pumped with an automatic pipette into each syringe, prewarmed at 39^{o}C. The syringes were shaken gently and residual air or air bubbles if any was removed and the outlet was closed. The level of piston was recorded and the syringes were placed in a oven (39º ± 0.5ºC). The syringes were shaken every 30 minutes for first 2 h from the start of the incubation and thereafter every 2 h up to 10 h of

incubation. The blank values for gas production (with or without treatments) at each hour of incubation was subtracted from the volume of gas produced from the sample at that particular hour to get the net volume of gas produced. For estimating the rate and extent of gas production of DM and isolated NDF, the volume of gas produced was recorded at 0, 1, 2, 3, 6, 9, 12, 24, 30, 36, 48, 60, 72 and 96 h.

3.7 Fermentation end products

The IVGP test was used to study the influence of plant extracts on DM and OM degradability, total gas and methane production, NH_3-N and individual VFA concentrations and microbial biomass. The amount of substrate used was 200 mg while volume of incubation medium was 30 ml. The substrate was incubated with or without plant extract in triplicate. A similar set without substrate was also incubated which served as blanks. After 24 h incubation, the volume of gas production was recorded and suitable aliquot of gas was withdrawn from tip of the incubation syringes using gas tight syringe and analysed for methane by gas chromatograph. The syringe contents were transferred to centrifuge tubes and centrifuged at 3000 g for 20 minutes. The NH_3-N in the supernatant was determined immediately.

3.7.1 Methane

The chromatograph system and the analytical conditions followed for methane estimation were same as those for VFA except that the column was made up of stainless steel and packed with porapak-q (length 6'; o.d. 1.8''; i.d. 2 mm; mesh range 80-100). The temperature of injection port was, 150ºC; column 60ºC and detector 130ºC. Injection volume was 5 ml. The injection was performed by means of 10 ml glass syringe. The standard gas used for methane estimation comprised of 50% methane and 50% carbon dioxide (SPANCAN Calibration gas, Spantech, Surrey, England). The peak was identified by comparision of above standard and the response factor obtained was used to calculate methane concentration in the gas sample. The volume of methane produced (ml) was calculated by multiplying the gas produced (ml) by the concentration of methane in the sample. The methane produced from the substrate after 24 h incubation was calculated by correcting for the corresponding blank values.

3.7.2 Ammonia nitrogen

5 ml Supernatant from centrifuge tube was mixed with 1 N NaOH (2 ml) and steam distilled using KEL PLUS - N analyzer (Pelican, India) and the NH_3 evolved was collected in boric acid solution having mixed indicator and titrated against N/ 100 H_2SO_4. Ammonia nitrogen concentration was calculated as follows:

$$\text{ammonia nitrogen (mg/100ml)} = \text{Vol. of acid used} \times \frac{1}{100} \times \frac{1}{5} \times 14 \times 100$$

3.7.3 Volatile fatty acids

4 ml of supernatant from centrifuge tube was transferred to a tube containing 1 ml of 25 % metaphosphoric acid and centrifuged at 3000 rpm for 15 min. The supernatant was transferred to another tube and stored in a freezer (-20ºC) for estimation of VFA. Individual VFA in the rumen fluid samples were determined using Gas chromatograph (Nucon 5700, Nucon Engineers, New Delhi) equipped with flame ionization detector and stainless steel column packed with chromosorb –101 (length 4'; o.d ¼"; i.d. 3 mm; mesh range 80-100). Analytical conditions for fractionation of VFA were as follows: Injection port temperature, 250ºC; column temperature, 190ºC and detector temperature, 260ºC. The flow rate of carrier gas (nitrogen) was 40 ml/min; hydrogen 30ml/min; air 300 ml/min. Injection volume was 3 µl. The injection was performed by means of 10 µl Hamilton syringe (Hamilton, Nevada, USA). The concentration of various VFA (mM/l) in the standard mixture were, acetic acid, 65.07; propionic acid, 20.01 and butyric acid, 8.06. The peaks were identified by comparision of above standards and the response factor obtained using standard VFA mixture for each fraction was used to quantify VFA fractions (mM/l) in the sample. The analysis and calculations were performed using Aimil chromatography data system (WINACDS). The amount of VFA produced (mM) was calculated after deducting the corresponding blank values.

3.7.4 True digestibility

True digestibility was estimated as per the method outlined by Goering and Van Soest (1970) with little modifications. The incubations were carried out in 100 ml

graduated glass syringes in triplicates for each sample as described earlier . After 24 h incubation, the contents of the syringe were directly transferred in a 500 ml spoutless beaker. The syringe was washed with 50 ml of double strength NDS and washings added to the beaker. The contents in the beaker were refluxed for 1 h, filtered under vacuum through preweighed sintered (G1) crucibles. NDF content of the residue was determined. True digestibility was calculated as follows.

$$TD = \frac{\text{wt.of sample - wt.of residual NDF}}{\text{wt.of sample}} \times 100$$

Truly degradable organic matter (TDOM) was calculated as the amount of substrate OM incubated minus the amount of substrate OM recovered as residue after ND solution treatment, and the partitioning factor (PF) was calculated as the ratio of TDOMR (mg) to gas volume (ml) produced from it during 24 h of incubation. Microbial biomass production (MBP) was calculated from TDOMR using equation:

MBP (mg) = TDOMR (mg) – (2.25 x net gas volume)

where constant 2.25 is the stoichiometric factor (Blümmel and Fernandez-Rivera, 2002).

3.8 *In vitro* ruminal nitrogen degradation (IVDN)

Protein degradation in rumen fluid *in vitro* was determined by the method described by Raab *et al.* (1983). The method is based on measurement of ammonia nitrogen concentration and gas production (Menke *et al.* 1979) when a feedingstuff is incubated with rumen fluid *in vitro*. The method uses the known relationship between fermentation of carbohydrates and microbial protein synthesis for determination of NH_3-N incorporated in to the microbial proteins. The total amount of NH_3-N liberated is found by extrapolation of linear regression to zero protein synthesis.

Incubations were carried out in 100 ml calibrated syringes (Fortuna, Germany) according to the method described by Menke *et al.* (1979). Berseem, oat (DM and NDF) and a diet containing roughage to concentrate ratio (R:C) as 50:50 were taken as substrates. Sample weight was chosen so as to correspond 26 mg crude protein. 100 mg

starch (an energy supplement) was incubated alongwith as an aid to microbial protein synthesis. Incubation was done in triplicate (3 each for blank, blank + treatments, sample, sample + starch, sample + treatments, sample + starch + treatments and standard). Rest of the procedure was essentially the same as described earlier for IVGPT. The incubation was terminated at 24 h. When gas production exceeded 80 ml, the piston was pushed back to the initial position. At the end of incubation, the amount of gas produced was measured by reading the position of piston (accuracy 0.5 ml). The contents of the syringes were transferred in to a 100 ml beaker on ice. A 5 ml of aliquot was transferred to the Kjeldahl distillation tube for determination of NH_3-N by distillation with 2 ml of 1N NaOH (found to be sufficient for evolution of all the ammonia present; ammonia was not evolved from amino acids under these conditions) (Makkar and Becker 1996). The liberated NH_3 was trapped in 25-30 ml of 2 % boric acid and titrated with N/100 H_2SO_4

Nitrogen degradability was calculated from the linear regressions of NH_3-N concentration (y; mg) vs. gas production (x; ml) observed on incubation of feed with and without endogenous energy source (starch), as described in Raab *et al* (1983). The intercept at Y axis represents the amount of NH_3-N which is released when no fermentable carbohydrate is added and hence no synthesis of bacterial protein takes place. The difference between the Y intercept and NH_3-N content in the blank indicated the amount of NH_3-N liberated from protein and other nitrogen containing compounds of the feedstuff incubated. The IVDN at 24 h was calculated as a proportion of total nitrogen incubated by the equation:

$$IVDN = \frac{\mathrm{NH3-N\ at\ zero\ gas\ production} - \mathrm{NH3-N\ of\ blank}}{4.16} \times 100$$

3.9 Kinetics of gas production: Model Fitting

The rate and extent of gas production were obtained by fitting the data to appropriate models. The models fitted were Modified Ørskov model with lag, Logistic and Gompertz.

I) **Modified Ørskov model with lag**

The first order substrate limited model (modified Mitscherlich equation) of Ørskov and McDonald (1979) was described by the following equation:

$$y = b*(1-e^{-c*(t-L)})$$

Where, y = gas production (ml) after time t

b = potential gas production (ml)

c = rate constant

L =discrete lag time

II) **Logistic model**

A single pool logistic model is derived on the assumption that the rate of gas production is proportional to both the accumulated microbial mass and to the amount of digestible substrate remaining (Schofield *et al.*, 1994). The logistic model is described by the following equation:

$$y = \frac{b}{\left\{1+e^{\left(2+4c\left(L-t\right)\right)}\right\}}$$

Where, y = gas production (ml) after time t

b = potential gas production (ml)

c = rate constant

L = lag time

III) **Gompertz model**

The data for gas production for DM and NDF for the various times of incubation for each sample were also fitted with the Gompertz model (Bidlack and Buxton, 1992),

without considering the washing losses. The Gompertz model assumes that gas production rate is proportional to the microbial activity, but the proportionality parameter decreases with time, according to the first order kinetics, which can be ascribed to loss of efficiency in fermentation with time (Bidlack and Buxton, 1992).

$$y = B \times e^{-C \times e^{-A \times t}}$$

where, y = fraction degraded at time t

B = asymptotic value (total potentially degradable fraction)

C = relative degradation rate

A = constant factor of microbial efficiency.

3.10 Statistical Analysis

All the data was subjected to the statistical analysis by unpaired t-test using a statistical software SAS® (version 8.12; SAS Institute, Cary, NC)

4. RESULTS AND DISCUSSION

4.1 Chemical composition of the substrates

The chemical composition of berseem, oat and wheat straw is presented in Table 4.1. The DM content varied from 11.90 % for berseem to 92.58 % for wheat straw. The OM, CP, EE and total ash ranged from 83.62 to 90. 54, 3.18 to 18.84, 0.49 to 3.47 and 9.45 to 16.37 % respectively. These values are close to the values reported by Ranjhan (1998). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) ranged from 40.27 to 79.53 and 29.31 to 51.79 % respectively. The NDF and ADF content was similar to the values reported by Ayyappan (2004).

Table 4.1: Chemical composition of substrates used in the *in vitro* studies (% in DM)

Substrate	**DM**	**OM**	**CP**	**EE**	**Ash**	**NDF**	**ADF**
Berseem	11.90	83.62	18.84	3.47	16.37	40.27	31.30
Oat	14.60	88.93	15.56	1.46	11.06	48.35	29.31
Wheat straw	92.58	90.54	3.18	0.49	9.45	79.53	51.79

n=3

4.2 Screening of plant extracts

Results of screening of four aqueous plant extracts and Rutin are presented in Table 4.2. Total gas production ranged from 20.50 to 40.00 ml for berseem and 36.50 to 47.50 ml for oat. In both the substrates, Rutin caused maximum reduction in the total gas production. In berseem, the decrease was from 38.25 to 20.50 ml while in oat from 40.00to 36.50 ml. Other plant did not affect in reducing total gas. Methane production varied from 4.47 to 13.64 ml in berseem and from 9.96 to 21.93 ml in oat. Though Neem extract resulted in maximum reduction in methane from berseem but, it was not effective

Table 4.2: Effect of aqueous plant extracts on total gas, methane production and degradability

Parameter	Control	Aloe vera	Neem	Rutin	*T. arjuna*	*T. belerica*
Berseem						
Total gas (ml/24h)	38.25	39.00	40.00	20.50	38.00	33.50
Methane (ml/24 h)	8.47	13.64	4.47	9.14	11.04	11.53
TD (%)	82.37	72.77	75.20	77.30	78.02	73.05
IVTOMD (%)	80.77	72.85	74.14	75.18	77.54	74.34
NDFD (%)	56.23	32.39	38.42	43.63	45.43	33.08
Oat						
Total gas (ml/24h)	40.00	42.25	44.25	36.50	45.75	47.50
Methane (ml/24 h)	12.85	16.47	15.52	9.96	21.93	17.05
TD (%)	77.35	81.17	78.02	81.02	79.25	78.97
IVTOMD (%)	77.96	77.34	76.47	79.00	78.63	78.91
NDFD (%)	53.15	61.06	54.55	60.75	57.08	56.51

TD- true DM degradability, IVTOMD- *in vitro* true OM degradability, NDFD- NDF degradability

Table 4.3: Effect of aqueous methanol plant extracts on total gas, methane production and degradability

Parameter	Control	Aloe vera	Neem	*T. arjuna*	*T. belerica*
Berseem					
Total gas (ml/24 h)	38.00	32.5	37.00	34.75	29.50
Methane (ml/24 h)	10.91	14.33	12.33	14.92	11.20
TD (%)	73.20	72.62	75.82	67.45	72.95
IVTOMD (%)	68.93	72.49	73.03	68.07	73.21
NDFD (%)	33.45	32.02	39.97	19.17	32.83
Oat					
Total gas (ml/24 h)	46.00	37.50	36.50	35.00	28.25
Methane (ml/24 h)	21.98	17.16	19.38	23.89	14.23
TD (%)	78.12	81.45	72.7	73.22	58.95
IVTOMD (%)	77.93	80.32	70.39	70.17	56.51
NDFD (%)	54.75	61.63	43.53	44.62	45.09

TD- true DM degradability, IVTOMD- *in vitro* true OM degradability, NDFD- NDF degradability

in oat whereas in both the substrates, methane production by Rutin was similar. In oat, Rutin caused about 22 % reduction in methane production. In berseem, Rutin caused reduction in true DM degradability from 82.77 to 75.18 % whereas in oat it was increased from 77.35 to 81.02 %. Other plant extracts caused more reduction in TD of berseem and did not affect TD of oat. Similar trend was observed for IVTOMD and NDFD.

Results of screening of four aqueous methanol plant extracts are presented in Table 4.3. Total gas production varied from 29. 50 to 38.00 ml in berseem and from 28.25 to 46.00 ml in oat. In both the substrates, *T. belerica* caused maximum reduction in total gas. Methane production in berseem varied from 11.20 to 14.92 ml and in oat from 14.23 to 23.89 ml. *T. belerica* was most effective in reducing methane production in both the substrates. In oat, it caused about 12 % reduction in methane production. There was not much difference in the true DM, OM and NDF degradability of control and treatments in both the substrates.

From the results of screening study, Rutin and *T. belerica* aqueous methanol extract were identified as most potent plant extracts and used for further studies.

4.3 *In vitro* gas production

Gas arises directly from microbial metabolism and indirectly from the reaction of acid end products with bicarbonates, an important component of the buffering system (Beuvink and Spoelstra, 1992). The degradation of feedstuff in the rumen requires sequential processes of solubilization and fermentation. Both processes depend on the chemical composition and structure of the substrate (Salvador *et al.* 1993; Merchen and Bourquin, 1994).

The values for total gas production from DM and isolated NDF at 24 h of incubation are presented through Table 4.4 to 4.15. In berseem total gas production ranged from 24.66 to 35.75 ml (DM) and 0.51 to 2.5 ml (NDF). Rutin at 15 and 30 mg levels was not having any significant effect on the total gas production from berseem DM and NDF but, *T. belerica* at 2 and 3 ml level significantly decreased total gas production ($P< 0.001$) in berseem DM. In oat total gas production ranged from 25.00 to 42.00 ml (DM) and 3.16 to 9.50 ml (NDF). Rutin at both levels was not having any significant

effect on the total gas production from oat DM and significantly decreased total gas production from oat NDF. *T. belerica* at 2 and 3 ml level significantly decreased total gas production (P< 0.001) in berseem DM and NDF. In wheat straw, total gas production ranged from 8.25 to 20.83 ml (DM) and 3.35 to 5.68 ml (NDF). Rutin at both levels did not show any significant effect on the total gas production from wheat straw DM and NDF but, *T. belerica* at 2 and 3 ml level significantly decreased total gas production (P< 0.001) in wheat straw DM. Total gas production from the fermentation of NDF of all the three substrates was lower than expected. Possible reasons for this maybe that traces of detergent remained at the time of isolation of NDF may have decreased the microbial activity or the lag time of fermentation maybe high in these substrates.

Although gas production is a nutritionally wasteful product but provides a useful basis from which metabolizable energy (ME), organic matter digestibility (OMD) and short chain fatty acids (SCFA) may be predicted.

4.4 *In vitro* true DM degradability, *in vitro* true OM degradability and Partitioning factor

In vitro true dry matter degradability (TD) of all the substrates with or without plant extracts is presented in Table 4.4 through 4.15. The TD for berseem DM and NDF ranged from 59.20 to 84.16 % and 14.33 to 30.60 % respectively. Rutin at 15 and 30 mg levels not significantly affected TD though there was slight increase in the TD in berseem DM as well as NDF. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased TD in berseem DM and NDF. The TD of oat DM and NDF ranged from 55.35 to 90.38 % and 15.11 to 53.03 % respectively. Rutin at both the levels increased the TD of berseem DM and NDF though it was not significant. This increase was more pronounced at 30 mg level. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased TD in oat DM and NDF. Decrease was more at 3 ml level than 2 ml. The TD for wheat straw DM and NDF ranged from 28.31 to 59.41 % and 16.25 to

Table 4.4: Effect of Rutin on *in vitro* rumen fermentation parameters of berseem

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	34.83±0.16	32.50 ±2.00	33.00±0.50
Methane (ml/200 mg, 24 h)	7.59± 0.17	7.08 ±0.47	7.21±0.13
TD (%)	78.25±0.45	79.52 ±0.57	84.16±1.04
IVTOMD (%)	76.11±0.09	75.87 ±1.58	80.01±0.97
PF	4.46± 0.001	5.38 ±0.41	4.79±1.09
Microbial mass (mg)	47.54±0.70	47.698±1.75	51.31±0.78
NH_3-N (mg/100 ml)	34.30±0.42	30.24±1.40	30.98±0.83
Acetate (mM)	12.30a±1.10	13.95a ±0.25	16.52b ±0.76
Propionate (mM)	4.95a ±0.16	5.57a±1.96	9.03b±0.80
Butyrate (mM)	3.87±0.01	4.027 ±0.02	4.12±0.48
A:P ratio	2.48	2.50	1.82

n = 3, Means with different superscripts in a row differ significantly (P< 0.001).

Table 4.5: Effect of Rutin on *in vitro* rumen fermentation parameters of berseem NDF

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	2.50±0.57	2.00±0.28	1.83±0.44
Methane (ml/200 mg, 24 h)	0.12±0.09	0.22±0.06	0.25±0.03
TD (%)	25.60±2.46	30.60±6.25	22.73±1.32
IVTOMD (%)	14.59a±2.31	18.01b±6.88	19.09a±1.22
Microbial mass (mg)	15.61±4.91	18.01±1.68	21.28±2.51
NH_3-N (mg/100 ml)	26.78±0.97	27.53±0.49	27.34±0.33
Acetate (mM)	7.34a±0.02	9.85b±0.23	9.06b±1.77
Propionate (mM)	2.61±1.48	3.89±0.76	3.13±1.10
Butyrate (mM)	2.22±0.06	3.33±0.28	2.57±0.09
A:P ratio	2.81	2.52	2.89

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.6: Effect of Rutin on *in vitro* rumen fermentation parameters of oat

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	40.16±1.45	37.66±1.59	39.33±0.92
Methane (ml/200 mg, 24 h)	8.45±0.71	7.81±0.29	8.16±0.23
TD (%)	85.28±0.53	88.30±1.00	90.38± 0.13
IVTOMD (%)	83.37±0.28	85.01±1.55	88.17±0.09
PF	4.20±0.14	4.60±0.21	3.86±0.62
Microbial mass (mg)	56.63±2.92	60.63±1.59	60.40±1.98
NH_3-N (mg/100 ml)	32.29±1.80	32.10±0.56	29.02±0.81
Acetate (mM)	20.31±1.80	17.09±0.99	17.38±1.40
Propionate (mM)	11.20±3.01	11.60±1.09	11.39±2.49
Butyrate (mM)	7.71±0.20	6.24±0.09	7.24±0.43
A:P ratio	1.81	1.47	1.52

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.7: Effect of Rutin on *in vitro* rumen fermentation parameters of isolated oat NDF

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	8.50a±2.93	4.00b±0.86	6.66a,b±1.64
Methane (ml/200 mg, 24 h)	1.88± 0.74	0.88±0.22	1.59±0.37
TD (%)	46.40±7.25	46.31± 0.65	53.03±1.29
IVTOMD (%)	42.78±7.57	40.25±0.40	46.72±1.59
Microbial mass (mg)	54.10±9.39	55.10±2.62	58.53±2.66
NH_3-N (mg/100 ml)	20.25±1.41	21.93±0.18	20.06±0.56
Acetate (mM)	12.29 ±1.71	10.05±0.73	13.81±1.21
Propionate (mM)	6.71±3.07	5.54±1.14	6.29±2.86
Butyrate (mM)	4.92±1.479	3.70±0.28	4.92±0.22
A:P ratio	1.83	1.81	2.19

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.8: Effect of Rutin on *in vitro* rumen fermentation parameters of wheat straw

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	20.83±0.88	20.50±1.00	20.33±2.92
Methane (ml/200 mg, 24 h)	4.49±0.22	4.54±0.26	4.24±0.51
TD (%)	53.81±0.76	59.41±0.59	58.13±0.87
IVTOMD (%)	52.15±0.65	55.19±0.89	52.67±1.29
PF	5.26±0.18	5.47±0.18	5.01±1.43
Microbial mass (mg)	45.31±2.13	47.15±2.27	40.75±4.76
NH_3-N (mg/100 ml)	23.70a±0.56	21.09a±0.56	11.01b±3.32
Acetate (mM)	11.12±2.75	11.47±0.26	12.32±0.36
Propionate (mM)	5.79±2.87	5.99±0.18	6.19±0.71
Butyrate (mM)	4.58±1.09	5.19±0.07	4.71±0.20
A:P ratio	1.92	1.91	1.98

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.9: Effect of Rutin on *in vitro* rumen fermentation parameters of isolated wheat straw NDF

Dose (mg)	**0**	**15**	**30**
Total gas (ml/200 mg, 24 h)	12.16±0.66	14.00±1.04	12.66±1.64
Methane (ml/200 mg, 24 h)	2.71±0.17	3.23±0.15	2.92±0.35
TD (%)	34.81a±1.18	41.20b±1.82	44.50b±2.61
IVTOMD (%)	32.90±1.36	36.21±1.91	38.04±2.86
Microbial mass (mg)	29.51±1.09	27.08±5.62	31.11±8.02
NH_3-N (mg/100 ml)	16.52a±1.83	13.06a±2.32	21.00b±0.85
Acetate (mM)	10.16±1.53	12.01±0.35	9.21±2.73
Propionate (mM)	5.99±0.51	6.04±0.29	5.94±0.001
Butyrate (mM)	4.01±0.24	5.16±0.22	5.14±0.89
A:P ratio	1.69	1.98	1.55

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

45.46% respectively. Increase in the TD with addition of Rutin at 15 and 30 mg levels was not significant in wheat straw DM but in wheat straw NDF, it significantly increased TD at both the levels. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased TD in wheat straw DM and NDF. The decrease was more at 2 ml than 3 ml level.

In vitro true organic matter degradability (IVTOMD) of all the substrates with or without plant extracts is presented in Table 4.4 through 4.15. The IVTOMD of berseem DM and NDF ranged from 57.26 to 80.01 % and 14.59 to 27.01 % respectively. Rutin at 15 and 30 mg levels was not having any significant effect on IVTOMD of berseem DM but, it significantly increased IVTOMD of berseem NDF at both the levels. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased IVTOMD of wheat straw DM but it has not affected IVTOMD of berseem NDF though there was slight decrease in IVTOMD. The IVTOMD of oat DM and NDF ranged from 50.13 to 88.17 % and 13.11 to 47.77 % respectively. Rutin at 15 and 30 mg levels non-significantly increased IVTOMD of berseem DM and NDF. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased IVTOMD of oat DM as well as NDF. IVTOMD of wheat straw DM and NDF ranged from 21.23 to 55.19 % and 12.86 to 40.26 % respectively. Rutin at both the levels did not significantly affect IVTOMD of wheat straw DM and NDF. *T. belerica* aq. methanol extract at both the levels significantly decreased IVTOMD of wheat straw DM and NDF.

McSweeny *et al*. (2001b) observed that total number of cellulolytic bacteria including *Fibrobacter succinogens* and *Ruminococcus flavifaciens* was lower in sheep supplemented with calliandra forage rich in CT while fungal population was less affected. Due to possible decrease in the cellulolytic bacterial population, degradability of the substrate might have decreased.

The ratio of milligram true degradable organic matter to ml of gas produced gives the partitioning factor (PF). PF of DM of all the substrates with or without plant extracts is presented in Table 4.4 through 4.15. The PF for berseem, oat and wheat straw was found between 4.41 to 6.00, 3.55 to 10.21 and 5.01 to 9.52 respectively. There was not significant change in PF of all the substrates due to addition of Rutin at 15 and 30 mg

level. The PF was higher on addition of *T. belerica* aq. methanol extract at 2 and 3 ml levels with all the three substrates. This increase is a result of significant reduction in the gas production from the fermentation of these substrates with addition of *T. belerica* extract.

For conventional feeds (roughages), the ratio of substrate truly degraded to gas volume termed as partition factor, range from 2.74 - 4.65 mg ml^{-1} (Blümmel *et al.* 1997b). The partitioning factor as high as 16.1 has been obtained for *A. barteri* which is well beyond the theoretical (2.75 - 4.41) or observed (2.74 - 4.65) ranges of partitioning factors (Blümmel *et al.* 1997b). This result also reinforce the observation of Makkar *et al.* (1998) that due to various artifacts the partitioning factors cannot be determined for tannin-rich forages by the approach of residue determination using NDS. This high partitioning factor could be due to: i) leaching of tannins from the feed during fermentation contributing to the dry matter loss but without contributing to the gas production, and ii) inhibition of cell solubles by tannins, or combination of i) and ii) (Makkar *et al.* 1998; Getachew *et al.* 1998). All these results suggest again that the detergent system of fibre analysis should be used with caution for tannin-containing feeds.

The partitioning factor is regarded as an index of efficiency of microbial biomass synthesis (EMBS) on assumption that carbon of true degradable OM is quantitatively distributed between microbial biomass and the sum of fermentative gas and short chain fatty acids (SCFA) and gas production is stoichiometrically related to short chain fatty acids.(Kiran and Krishnamoorthy, 2007). Partitioning factor calculated by concomitant true DM degradability and gas volume measurements after 24 h incubation have been used to assess the partitioning of nutrients between gases (TVFA) and microbial cells (Blümmel *et al.*, 1997b) and the high PF value (low gas production per unit of substrate degraded) is indicative of proportionally higher microbial yield.

The main active principle in the *T. belerica* is tannins. The high values for tannin-rich feeds appear to be due to the solubilization of tannins from the feeds during fermentation, contributing to the dry matter loss but without contributing to gas, and inhibition of gas production from cell solubles by tannins through inhibition of rumen

fermentation. Getachew *et. al.* (2000) has reported the range of PF for tannin-rich feeds from 3.1 to 16.1.

4.5 Methane production

The methane production in the present study is presented in the Table 4.4 through 4.15. It ranged from 3.35 to 10.68 ml for substrate DM and from 0.12 to 5.67 ml for NDF. In control, it was highest from oat DM and lowest from berseem NDF. Rutin at 15 and 30 mg level was not having any significant effect on the methane production from DM as well as NDF of the substrates used in the experiment. *T. belerica* aq. methanol extract at 3 ml level significantly decreased methane production in berseem and oat DM while at 2 ml level also, there was not significant decrease in the methane production. In oat NDF, it significantly decreased methane production at both the levels while it was not effective with NDF of other substrates.

Feeding condensed tannin (CT) containing forages to ruminants reduces methane emissions (Woodward *et al*., 2001; Puchala *et al*., 2005). Two mechanisms are proposed whereby condensed tannins reduce methane emissions from ruminants: 1) indirectly through a reduction in fiber digestion, which decreases H_2 production, and 2) directly through an inhibition of the growth of methanogens (Tavendale *et al.,* 2005).

The tannin-containing forages used to reduce methanogenesis include sulla (*Hedysarum coronarium,* 2.7 to 6.8% CT), red clover (*Trifolium pretense,* 0.3% CT), big trefoil (*Lotus pedunculatus,* 5.3% CT), and *Sericea lespedeza* (*Lespedeza cuneata,* 17.7% CT). In most of these studies, the reduction in methane due to feeding forages containing CT was confounded with changes in forage quality, such as lower NDF content. Woodward *et al*. (2001) reported that in sheep, methane emission relative to digestible DMI was decreased by 24 to 29% when *L. pedunculatus* (8% CT) was fed compared with ryegrass or lucerne. A similar decrease (23%) in methane emission relative to DMI was observed by the same authors when cows were fed *L. corniculatus* (2.6% CT) silage compared with ryegrass silage. Feeding *Acacia mearnsii* (black wattle tree) extract (2.5% of DMI) to sheep decreased methane per kilogram of intake by approximately 12%. Whether CT from other sources also

reduce methane emissions, and the dietary concentration of condensed tannins necessary for such a reduction, is not clear. Thus there is still considerable uncertainty about the effectiveness of CT extracts and CT containing forages to reduce enteric methane emissions from cattle.

Patra *et. al.*, (2006) reported 95 % decrease in methane production by adding methanol extract of *Terminalia chebula*, a tannin containing plant extracts to an *in vitro* system. Phenolic acids such as *p*-coumaric acids, cinnamic acids, ferulic acids and phloretic acids and some monomeric phenolics have been found to decrease methane production (Asiegbu *et al*., 1995). Decrease in methane production maybe caused due to reduction in protozoal population. Hristov *et al.,* (2003) reported antiprotozoal effect of tannins. Sliwinski *et al*. (2002a) also showed that with addition of *Castanea sativa* wood extract (0.5-2.5 g tannins/kg) to RUSITEC, caused significant reduction in methane emission. Carulla *et al*. (2005) reported about 13% reduction in methane production with supplementation of *Acacia mearnsii* tannins to sheep at a level of approximately 0.025% of diet DM. In the present study, *T. belerica* aq. methanol extract increased propionate production in DM of all the substrates. Increase in propionate production is associated with decrease in the methane production due to availability of alternative sink for H_2. Observations in the present study are similar to those reported already.

The inhibition of methanogenesis by tannins was probably the result of a suppression of fiber degradation. However, a direct effect of CT on rumen methanogens cannot totally be excluded (Field *et al*., 1989). Extracted tannins as well as tannin-rich legumes can be useful in limiting methane emissions but, when employing tannins in the form of very tannin-rich legumes, a simultaneous depression of the feeding value of the diet may question the viability of this feeding measure (Hess *et al*., 2006).

Only few reports are available to date, almost all from *in vitro* studies, relating the effects of saponin containing plants or extracts on CH_4 production. Wang *et al.* (1998) reported that yucca extract addition at 0.5 mg/ml did not affect total gas or CH_4 production *in vitro*. In contrast, Takahashi *et al.* (2000) found significant decrease in total gas and CH_4 production due to addition yucca extract (0.01 ml/ml) to an *in vitro* ruminal system. The CH_4 or H_2 production or H_2 balance was not affected due to incorporation of

yucca saponins up to 100 mg/kg DM in RUSITEC (Sliwinskii *et al.* 2002a). As the concentration of sarsaponin increased from 1.2 to 3.2 g/l, fermentation of soluble potato starch, corn starch, or hay plus concentrate decreased CH_4 production from 20 to 60 % (6 h) and 17 to 50 % (24 h), 21 to 58 % (6 h) and 18 to 52 % (24 h), and 23 to53 % (6 h) and 15 to 44 % (24 h), respectively (Lila *et al.*, 2003). However, total gas production increased in a dose dependent manner. Hess *et al.* (2003a) observed *S. saponaria* fruit supplementation decreased daily CH_4 production by 20 % in RUSITEC. The effect of *S. saponaria* was more pronounced in defaunated versus faunated rumen fluid. When related to OM apparently fermented, differences relative to control diet persisted, but CH_4 production per unit of fiber degraded did not differ between *S. saponaria* diet and control diet. The ratio of CH_4 to CO_2 was also lower with *S. saponaria* supplementation, indicating variation in gas release pattern also occurred. Hess *et al.* (2003b) further reported that *S. saponaria* reduced methanogenesis by 11 % on average in grass alone or in legume supplemented diets.

Klita *et al.* (1996) studied the effect of intra ruminal administration of saponins @ 0, 4 % or 8 % of DMI on CH_4 production in sheep. There were no significant differences in O_2 consumption, CO_2 or CH_4 production. Similarly, CH_4 production was not influenced when lambs were fed with yucca saponins @ 2 or 30 ppm of DMI (Sliwinskii *et al.*, 2002b). As some methanogens live in association with protozoa, it was expected that reducing protozoa would also reduce methanogens thus decreasing methane production. The supplements of *S. saponaria* influenced daily methane release in a similar way to that in which they affected ciliate protozoa counts (Hess *et al.*, 2004) .

Roth *et al.* (2001) compared the effect of tannin rich extracts from chestnut and mimosa (HT) and quebracho (CT) on methane production *in vitro* at very high concentrations. Chestnut tannins were very effective in reducing methanogenesis. However, methane production was not reduced by chestnut extract during both *in vitro* (Sliwinskii, *et al.*, 2002a) and *in vivo* studies (Sliwinskii, *et al.*, 2002b) at lower concentrations. Puchala *et al.* (2005) compared the effects of feeding tannin rich *Lespedeza cineata* (17.7 % CT) and tannin free mixed crab grass (*Digitaria ischaemum* and *Festuca arundinacea*) (0.5 % CT). Methane emission expressed as both quantities

per day or relative to dry matter intake was lower for *Lespedeza cineata* than for crab grass (7.4 vs. 10.6 g/d and 6.9 Vs. 16.2 g/kg DMI). Substantial differences between the forages in CT concentration and methane emission by goats suggested that CT decrease methane emission.

In recent years, there is growing interest in the use of PSM (tannins, saponins, essential oils) as a CH_4 mitigation strategy because of their natural origin in opposition to chemicals additives. Most trials with plant extracts have been done *in vitro* and the response of these molecules on methanogenesis is highly variable. For tannin containing plants, the antimethanogenic activity has been attributed mainly to CT. Two modes of action of tannins on methanogenesis have been proposed by Tavendale *et al*. (2005): a direct effect on ruminal methanogens and an indirect effect on hydrogen production due to lower feed degradation. The mode of action of saponins seems to be clearly related to their anti-protozoal effect (Newbold *et al*., 1997). However, the effect of saponins on protozoa may be transient.

4.6 *In vitro* ammonia nitrogen

In vitro ammonia nitrogen (NH_3-N) content in present study is presented in the Table 4.4 through 4.15 for different substrates. It ranged from 11.01 to 34.30 mg/100 ml for DM and from 12.42 to 27.34 mg/100 ml for NDF of the experimental substrates. Table 4.10: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of berseem Rutin at 15 and 30 mg levels was not having any significant effect on NH_3-N for berseem and oat DM as well as NDF but, it significantly decreased NH_3-N content for wheat straw DM at both the levels whereas it decreased NH_3-N content at 15 mg level and increased NH_3-N content at 30 mg level for wheat straw NDF. *T. belerica* aq. methanol extract at 2 and 3 ml levels increased NH_3-N concentration significantly in all substrates DM while it significantly increased NH_3-N content at both the levels in oat and wheat straw NDF.

Table 4.10: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of berseem

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	35.75a±5.25	25.83b±6.17	24.66b±2.89
Methane (ml/200 mg, 24 h)	9.64a±1.02	8.07a±1.22	5.89b±0.48
TD (%)	77.30a±0.15	65.41b±0.85	59.20b±0.92
IVTOMD (%)	77.54a±0.149	65.36b±0.95	57.26b±8.91
PF	4.41±0.64	6.00±1.96	4.97± 0.73
Microbial mass (mg)	51.04±11.30	52.48±14.83	33.44±8.79
NH_3-N (mg/100 ml)	18.76a±2.24	25.76b±1.48	28.65b±1.13
Acetate (mM)	10.10a±0.20	10.62b±0.21	8.82c±0.06
Propionate (mM)	3.58a±0.26	4.04b±0.02	4.43c±0.06
Butyrate (mM)	1.12a±0.06	0.64b±0.02	0.51c±0.05
A:P ratio	2.82	2.62	1.99

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.11: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of isolated berseem NDF

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	1.00 ±1.04	0.66 ±0.44	0.51±0.44
Methane (ml/200 mg, 24 h)	0.73±0.20	0.33±0.26	0.25±0.04
TD (%)	24.86a±3.74	17.31b,c±0.64	14.33c±2.41
IVTOMD (%)	27.01±3.56	25.37±0.86	22.35±1.55
Microbial mass (mg)	21.84±5.96	26.78±2.86	35.18±2.71
NH_3-N (mg/100 ml)	19.98±2.42	21.93±0.65	22.77±0.97
Acetate (mM)	7.44a±0.08	3.71b±0.25	7.06a±0.02
Propionate (mM)	2.52a±0.01	2.17b±0.01	2.39c±0.08
Butyrate (mM)	0.62a±0.013	0.06b±0.001	0.311c±0.06
A:P ratio	2.95	1.71	2.95

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.12: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of oat

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	42.00a±0.86	26.50b±0.28	25.00b± 0.50
Methane (ml/200 mg, 24 h)	10.68a±0.29	8.37a±0.08	6.75b±0.09
TD (%)	83.80a±1.27	58.80b±3.49	55.35b± 0.87
IVTOMD (%)	81.18a±0.38	55.77b ±2.54	50.13b± 1.49
PF	3.55± 0.04	10.21±4.61	5.46±0.75
Microbial mass (mg)	41.38a±2.55	30.28b ±4.66	23.54b± 3.71
NH_3-N (mg/100 ml)	14.00a±4.93	25.48b±0.64	22.77b± 1.48
Acetate (mM)	14.80a±0.15	13.21b±0.15	17.05c± 0.06
Propionate (mM)	7.66a±0.03	7.51a±0.06	9.82b±0.06
Butyrate (mM)	0.40±0.001	0.216±0.06	0.311± 0.04
A:P ratio	1.93	1.75	1.73

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.13: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of isolated oat NDF

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	9.50a±0.57	3.66b±0.88	3.16b±2.61
Methane (ml/200 mg, 24 h)	5.67a± 0.10	3.03b± 0.19	2.28b ±0.59
TD (%)	52.46a±0.82	15.11b± 3.15	18.18b±5.92
IVTOMD (%)	47.77a± 0.59	13.11b± 1.41	14.69b±2.39
Microbial mass (mg)	9.44±1.53	4.64± 1.62	8.54±1.73
NH_3-N (mg/100 ml)	12.42a± 0.09	21.46b± 0.09	24.64b±0.64
Acetate (mM)	12.74a± 0.16	8.82b± 0.06	14.20c±0.05
Propionate (mM)	5.50a ±0.02	5.10a± 0.06	6.33b±0.03
Butyrate (mM)	2.64a± 0.02	2.66a±0.03	3.50b±0.06
A:P ratio	2.31	1.72	2.24

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table 4.14: Effect of *T. belerica* aq. methanol extract on *in vitro* rumen fermentation parameters of wheat straw

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	19.83a±0.88	6.50b±1.26	8.25b±2.25
Methane (ml/200 mg, 24 h)	5.68±0.09	3.89±0.36	3.35±0.96
TD (%)	53.06a±0.27	28.31b±2.39	34.72b±6.02
IVTOMD (%)	48.84a±0.37	21.23b±2.52	28.54b±6.68
PF	5.37±0.26	9.46±2.14	9.52±4.06
Microbial mass (mg)	30.98a±2.54	10.31b±6.53	19.69b±1.70
NH_3-N (mg/100 ml)	14.48a±0.90	20.25b±2.51	22.26b±0.42
Acetate (mM)	13.23a±0.14	10.43b±0.07	10.40b±0.10
Propionate (mM)	3.73a±0.02	4.31b±0.06	4.37b±0.03
Butyrate (mM)	0.44a±0.02	0.88b±0.02	0.89b±0.002
A:P ratio	3.54	2.41	2.37

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

Table4.15:Effect of *T.belerica* aq.methanol extract on *in vitro* rumen fermentation parameters of isolated wheat straw NDF

Dose (ml)	**0**	**2**	**3**
Total gas (ml/200 mg, 24 h)	16.50±2.25	11.00±4.44	11.75±2.25
Methane (ml/200 mg, 24 h)	5.52±0.13	2.90±1.02	5.256±3.83
TD (%)	45.46a±0.31	16.25b±7.00	26.37c±2.12
IVTOMD (%)	40.26a±0.24	12.86b±4.52	23.32c±0.30
Microbial mass (mg)	22.78±0.43	17.24 ±1.90	14.19±0.55
NH_3-N (mg/100 ml)	15.97a±1.37	20.72b±1.13	24.22b± 2.38
Acetate (mM)	10.19a±0.21	11.25b±0.22	10.06a±0.06
Propionate (mM)	3.37± 0.004	3.49 ±0.06	3.06±0.01
Butyrate (mM)	3.83a±0.02	3.72b±0.06	3.69c±0.01
A:P ratio	3.02	3.22	3.29

n=3, Means with different superscripts in a row differ significantly (P< 0.001)

The NH_3-N concentration at any given time in the rumen is considered to be a balance between two entry sources, i.e., degradability of feed nitrogen and nitrogen recycling and three outputs, incorporation of NH_3-N into microorganisms, NH_3-N absorption and outflow through omaso-reticular orifice (Doreau and Ferlay, 1995). The decrease in rumen NH_3-N concentration could be attributed to lower degradability of feed nitrogen, lower bacterial nitrogen recycling and higher incorporation of NH_3-N into microbial cells (Alexander, 2005). Tannins by forming complexes with dietary proteins, decrease ruminal degradation of proteins at near neutral pH of rumen (Waghorn *et al*., 1994). Since bacterial mass was low in the treatment group, uptake of NH_3-N by microbes was less and that might have increased NH_3-N concentration. Salem (2002) also reported increased NH_3-N concentration in sheep fed quebracho extract with no variation in VFA concentration.

Alexander (2005) observed a sharp decrease in ammonia concentration, and production of isoacids in the presence of *P. kurroa* extract which could be due to decreased degradation of substrate protein. However, lower gas production could be attributed to increased propionate production (Getachew *et al.,* 1998). It appears that positive effects of *P. kurroa* extract on rumen fermentation might be due to the presence of higher concentration of saponins or related compounds. *P. zeylanica* aqueous extract seems to enhance substrate degradability leading to increased VFA production. The decrease in ammonia concentration with a simultaneous decrease in isobutyrate and isovalerate in the presence of *P. zeylanica* extract could be due to low levels of HT, CT and saponins (Alexander, 2005). Very high concentration of HT (32 g/kg DM) in *T. bellerica* extract might have decreased ammonia concentration, isobutyrate and isovalerate levels. Decrease in ammonia due to addition of hydrolysable tannins of *Castanea sativa* wood extract or tannic acid has been reported (Śliwinskii *et al.*, 2002; Hristov *et al.,* 2003). Failure of *Z. officinale* aqueous extract to elicit a positive response on rumen fermentation might be due to the inability of water to extract the bioactive compounds, or the plant may not have bioactive compounds that modify rumen fermentation.

Most of the reported research found inconsistent effects of saponins on ammonia N concentration. Studies in sheep (Klita *et al.*, 1996), dairy cows (Wu *et al.*, 1994; Wang *et al.*, 1998), and steers (Hussain and Cheeke, 1995) found no effect of saponins on ammonia N concentration. In contrast, other *in vitro* studies indicated that saponins reduce ammonia N concentration (Makkar *et al.*, 1998: level of inclusion 1,200 mg/L of pure saponins). Hristov *et al.* (1999) also observed a lower ammonia N concentration 2 h after supplying 60 g/d of *Yucca schidigera* (containing 4.4% of saponins) to heifers.

4.7 Individual volatile fatty acid production

Individual volatile acid production in the present study is presented in the Table 4.4 through 4.15 in different substrates with or without plant extracts. The acetate production recorded was from 3.21 to 17.38 mM in DM and 3.71 to 14.20 mM in NDF. The propionate production varied from 2.04 to 11.60 mM in DM and 2.17 to 6.71 mM in NDF. Rutin at 15 and 30 mg levels slightly decreased acetate to propionate ration by increasing propionate production in DM whereas there was not much change in the ratio in NDF of experimental substrates. *T. belerica* aq. methanol extract at 2 and 3 ml level significantly increased propionate production in DM while in NDF, results were variable.

Increase in the propionate production is associated with corresponding decrease in the methane production because propionate provides alternative sink for hydrogen used for methane production. In the present study also when there is increase in the propionate production, decrease in the methane production was observed. Patra *et. al.*, (2006) reported decrease in the acetate to propionate ratio on addition of *T. belerica* extract to *in vitro* system. The VFA concentration was not affected by the addition of quebracho, A:P ratio were 41.0 and 1.93 and 39.5 and 3.15 in cows fed on barley or maize based diets, respectively. Lower acetate to propionate ratio was attributed to faster and extensive starch degradation in the rumen. Sinclair *et al.*(1995) recorded higher VFA concentration (120 mM) on feeding synchronous diet than on asynchronous diet (105 mM) 3-4 h post feeding, thereafter concentration was reduced to the minimum level i.e. 90 and 80 mM recorded o h of feeding on synchronous and synchronous diet, respectively.

Rymer and Givens (2002) reported from an *in vitro* study that the dietary inclusion of maize grain at 20, 50 and 80% levels resulted in molar proportion of acetate and propionate as 61.4 and 23.3; 60.4 and 27.1; and 58.8 and 31.1, respectively, and TVFA concentration on corresponding diets was 51.8, 66.4 and 58.3 mM. They recorded higher molar proportion of propionate at the expense of acetate on higher proportion of readily fermentable carbohydrate (such as starch) in the diet.

Total VFA concentration is the result of diet fermentation, and lower acetate:propionate reflects a shift in ruminal fermentation that is more efficient for beef production systems (Wolin and Miller, 1988). Previous research reported limited effects of saponins on total VFA concentration or profile in steers (Hussain and Cheeke, 1995; Hristov *et al.*, 1999: level of inclusion 3.0 and 2.6 g/d of pure yucca saponins, respectively), sheep (Klita *et al.*, 1996: level of inclusion 520 mg/d of pure alfalfa saponins), or dairy cows (Wu *et al.*, 1994; Wang *et al.*, 1998: level of inclusion 1.2 g/ d and 22 mg/L of pure yucca saponins, respectively). High doses of all extracts resulted in lower VFA concentration, which confirms their antimicrobial effect (Cowan, 1999). Evans and Martin (2000) also reported that high doses of thymol (400 mg/L) inhibited VFA production in mixed ruminal microbial fermentation.

Kaswari *et al.* (2007) reported that the TVFA concentration from the diets having SI of 0.52, 0.76 and 0.82 was 106.5, 96.5 and 109.0 mM/l, respectively. In another experiment, the diets having SI of 0.72, 0.85 and 0.90 resulted in TVFA concentration of 105.5, 98.4 and 100.3 mM/l, respectively. However, the variation among the diets was not significant. Mean TVFA concentration in both experiments (104.0 and 101.4 mM/ l) was within the normal range (70-130 mM/l) as suggested by France and Siddons (1993). The diets having SI of 0.90 and 0.72 had lower molar proportions of acetic acid and higher molar proportion of propionic acid than that having SI (0.85). Wheat grain based diet produced lower molar proportion of acetic acid and higher proportion of propionic acid than the corn based diet and hence the lower acetate to propionate ratio.

Lu and Jorgenson (1987) reported that the NH_3-N and TVFA concentration linearly decreased due to intra ruminal administration of alfalfa saponins (2 or 4 % DM) in sheep fed roughage or concentrate diet. In contrast, Diaz *et al.* (1993) found non

significant effect on feeding different levels of (25 or 50 g) saponin rich *S. saponaria* seed pericarp to sheep on rumen pH, NH_3 concentrations and VFA proportions.

Klita *et al.* (1996) observed linear increase in total, acetic, propionic, butyric and valeric acid concentrations and a linear decrease in the branched chain acids with increasing level of alfalfa root saponins in sheep. Total, acetic and propionic acid concentrations did not differ on day 14, but butyric and valeric acid continued to show a linear increase in concentration with saponin dose. A decrease in NH_3 concentration and increase in propionate concentration was observed (2h post feeding) in heifers fed yucca extract @ 20 or 60 g/d (Hristov *et al.*, 1999). The rumen pH and TVFA were not altered while NH_3-N was 21 % lower when yucca extract was supplied at 100 mg/kg diet in RUSITEC (Sliwinskii *et al.*, 2002a). However, rumen NH_3-N as well as pH, TVFA or proportion of individual VFA was not influenced in lambs fed low level of yucca saponins (2 and 30 ppm) (Sliwinski *et al.*, 2002b). Lila *et al.* (2003) studied the influence of yucca extract (sarsaponin) at different concentrations (0, 1.2, 1.8, 2.4, and 3.2 g/l) on *in vitro* rumen fermentation of soluble potato starch, corn starch or hay plus concentrate. The pH of the medium and NH_3-N concentration significantly decreased with all levels added sarsaponin while TVFA concentration increased as concentration of sarsaponin increased from 0 to 3.2 g/l. Molar proportion of propionate and butyrate significantly increased and acetate decreased in a dose dependent manner with corresponding decrease in A:P ratio. Hess *et al.* (2003a,b) compared the effects of supplementation of three saponin containing fruits on rumen fermentation and found that molar proportion of butyrate and valerate increased with *E. cyclocarpum* and *P. saman*. Rumen fluid NH_3-N, proportion of isobutyrate and isovalerate decreased with *P. saman*. Supplementation of *S. saponaria* fruit decreased NH_3 concentrations and A: P ratio but ruminal pH and TVFA were not influenced. Abreu *et al.* (2004) and Hess *et al.* (2004) also observed similar results in sheep fed with *S. saponaria* fruit.

Tannins generally exhibit inhibitory effect both on carbohydrate and nitrogen metabolisms in the rumen. Makkar *et al.* (1995a) during *in vitro* studies found decrease in TVFA and molar proportion of acetate and increase in propionate in the presence of tannins. Singh *et al.* (2001) observed lower NH_3-N concentrations in the presence of

tannic acid in an *in vitro* ruminal system. Tannin might have complexed with deaminase and urease (Sliwinskii *et al*,, 2002a). Hristov *et al.* (2003) showed that tannic acid not reduced acetate, propionate, isobutyrate, isovalerate and TVFA concentrations and A:P ratio. However, daily intra-ruminal administration of quebracho extract (75 % CT) to sheep did not affect pH, NH_3-N and VFA concentrations.

4.8 Microbial biomass

There is inverse relationship between gas volume (or short chain fatty acids production) and microbial mass production particularly when both are expressed per unit of substrate truly degraded. In the present study, microbial biomass varied from 10.31 to 60.63 mg for DM and 4.64 to 58.53 mg for NDF of all the three substrate. This wide variation in the microbial biomass production can be explained by the distinct fermentation pattern of each substrate than other. Rutin at 15 and 30 mg levels was not having any significant effect on the microbial biomass production but, *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased microbial biomass from fermentation of oat and wheat straw DM. Decrease was more pronounced at 2 ml level than 3 ml. Due to decrease in the DM and OM degradability with addition of *T. belerica* extract, the energy supply for microbial protein synthesis is reduced and that may have resulted in the decreased microbial biomass production. The decrease in gas production is generally associated with increased microbial yield. However, in case of *T. belerica* aq. methanol extract, the decrease in gas production failed to improve microbial yield indicating that the extract might be toxic to microbes leading to under estimation of microbial yield.

The ATP produced during fermentation of OM to VFA is used as main energy source for microbial growth. There is inverse relationship between VFA production and microbial biomass yield due to utilization of ATP with variable efficiency under different feeding conditions. Microbial efficiency (g of microbial N/ kg of OM apparently fermented in the rumen) significantly increased due to supplementation of *Sapindus saponaria* fruit (12% crude saponins) in sheep fed grass hay as sole diet (Abreu *et al.* 2004). Makkar *et al* (1998) observed a higher microbial mass for Yucca and Acacia saponins. There was a shift in the partitioning of fermented substrate in presence of

Acacia saponins. On the other hand, quillaja saponins increased the extent of truly degraded substrate from 300 to 323mg without change in gas production. Microbial efficiency (g of microbial nitrogen/kg of OM apparently fermented in the rumen) significantly increased due to supplementation of *S. saponaria* fruit in sheep fed grass hay as sole diet (Abreu *et al.* 2004).

Microbial protein synthesis *in vitro*, expressed as ^{15}N incorporation into microbes per unit of short chain fatty acid production was more efficient in the presence of tannins (Makkar *et al.*, 1995a). The incorporation of ^{15}N into microbial cells and efficiency of microbial protein synthesis were higher with CT (quebracho tannins) compared with HT (tannic acid). Recent study by Hristov *et al.* (2003) also showed that presence of tannic acid at lower concentrations (0.1 %) in *in vitro* incubation medium increased ^{15}N incorporation into bacteria while decreased protozoa. Beever and Siddons (1986) indicated an increase in microbial protein supply after feeding moderate concentration of CT from sainfoin in sheep. Calliandra tannins (2 to 3 %) in the diet failed to alter microbial protein flow from the rumen in sheep (Mc Sweeny *et al.*, 1998). McNeill *et al.* (1998) also found similar results in response to feeding of tannin rich *Leucaena leucocephala* (7.3 % CT). McNeill *et al.* (2000) further demonstrated that there was no change in microbial protein flow in sheep fed 100 % diet of dried Leucaena hybrid (11.6 % CT). Min *et al.* (2002) also observed no change in total microbial protein in the rumen or microbial protein flow to abomasum in sheep fed *L. corniculatus*.

4.9 *In vitro* crude protein degradability

The influence of plant extracts on *in vitro* crude protein degradability (IVCPD) is shown in the Table 4.16 and 4.17. Regression equations revealed that Rutin at 15 and 30 mg levels increased the rate of NH_3-N uptake for microbial protein synthesis. Compared to control, NH_3-N concentration at both the levels was either decreased or not changed (Table 4.9 through 4.12). IVCPD was increased at 15 mg level but decreased at 30 mg level in berseem DM and NDF and oat while it decrease at both the levels in oat NDF and R:C 50:50 diet. *T. belerica* aq. methanol at 2 and 3 ml levels not showed any pattern in uptake of NH_3-N and IVCPD. In most of the substrates, IVCPD was decreased more at 3

ml level than 2 ml level. This suggests that at 3 ml level, there is more binding of dietary proteins by tannins or there is suppression of rumen microbial activity.

Difficulties in measuring protein degradation from the release of NH_3-N arise from the fact that protein degradation and microbial protein synthesis occur simultaneously. Moreover, modification of microbial population like suppression of protozoa can also decrease NH_3-N due to reduced bacterial nitrogen recycling in the rumen. Devarajan (1999) observed that addition of CT or HT decreased *in vitro* degradable nitrogen (IVDN) values in *Artocarpus lakoocha* and *Grewia optiva.*

The decrease in the NH_3-N in presence of rutin might be through the three different mechanisms viz. decreased dietary protein degradation, increased microbial uptake and decreased bacterial protein breakdown. This suggestion is based on the observation that rutin at 30 mg level decreased IVDN (Table 4.16) and increased regression coefficient (an indirect measure of NH_3-N uptake).

The IVDN method is based on the principle that linear regression between NH_3-N concentration (y mg) and gas production (x ml) yield an intercept (b_0) representing that amount of NH_3-N which would be released when no fermentable carbohydrates (or energy) are available. The difference between this intercept (b_0) and NH_3-N content of blank indicates the amount of NH_3-N that is liberated from the breakdown of protein and other N containing compounds of feedstuff incubated (Raab *et al*., 1983). The regression coefficient is related to efficiency of energy utilization for microbial protein synthesis and is expressed as microbial nitrogen synthesized per g of truly degraded OM (MN/TDOM) (Raab *et al.*, 1983).

Degradability of protein in the rumen is an important value to be used for the prediction of protein passing undegraded to the small intestine and for the calculation of protein utilization and protein requirements of ruminants. Attempts have been made to estimate protein degradation from solubility tests (Crooker *et al.* 1978; Chamberlain and Thomas, 1979) or by the use of polyester bags to be incubated in the rumen of a fistulated animal (Orskov and Mehrez, 1977). Difficulties in measuring protein degradation from the release of ammonia (Chamberlain and Thomas, 1979) arise from the fact that protein

degradation and bacterial protein synthesis are processes which occur simultaneously. For separate determination of bacterial protein synthesis either certain characteristic constituents of micro-organisms such as diaminopimelic acid, RNA or DNA (Ling and Buttery, 1978; Siddons *et al.* 1979), or isotopes such as ^{35}S, ^{32}P or ^{15}N can be used (Pilgrim *et al.* 1970; Walker and Nader, 1975; Van Nevel and Demeyer, 1977). Rumen degradability of protein, for example, is not only related to the kind of feedingstuff and nature of protein, but it is also a function of the residence time of the feedingstuff in the rumen.

Bartley *et al.* (1986) estimated feed protein degradation and microbial protein synthesis using modification of the Hohenheim *in vitro* gas production test. Simultaneously, a procedure based on 6 h gas production and residual NH_3 in a rumen liquor-buffer solution was developed at Bejing Agricultural University (Xiong *et al.* 1990). Xiong *et al.* (1990) proposed a technique based on 6 h gas production and residual NH_3 in the liquid to estimate both ruminal starch availability and ruminal protein degradation for processed sorghum grain. Getachew *et al.* (1998) modified this method for quantification of *in vitro* degradability of protein in low quality roughages.

It is well recognized that ammonia concentration alone cannot be used to assess CP degradation and/or microbial yield since both CP degradation and microbial protein synthesis occur simultaneously. Further, a common observation in the rumen of ciliate-free ruminants was lower ammonia concentration and a higher molar proportion of propionate (Eugène *et al.*, 2004). IVDCP was not affected by the *Moringa oleifera* extract, the slope of the regression equations (0.0568 *versus* 0.0334) suggested that the decrease in ammonia might be due to higher uptake of ammonia for microbial protein synthesis (Alexander *et al*., 2008). The decrease in CP degradability (87.0 to 62.5) appears to be the main reason for decreased ammonia concentration with *P. kurroa* extrac*t* suggesting inhibition of one of the degradation step from protein to ammonia.

Getachew *et al.* (1998) studied the effect of 30 ml (as in original method) and 40 ml buffered rumen fluid (containing double the amount of hydrogen carbonate buffer as in the original method) on rumen degradation of N from low quality roughages. Nitrogen degradability of several cereal straws (barley, millet, oat, rice, sorghum, triticale and

Table 4.16 Effect of Rutin on IVCPD and uptake of NH_3-N for microbial protein synthesis

Substrate	**Dose** (mg)	**Regression equations**	R^2	**IVCPD** (%)
Berseem	0	y = 8.027-0.0436 x	0.92	35.98
	15	y = 8.520-0.0559 x	0.92	47.83
	30	y = 7.758-0.0831 x	0.91	29.51
Berseem (NDF)	0	y = 6.724-0.0768 x	0.99	4.992
	15	y = 7.176-0.0865 x	0.98	15.52
	30	y = 6.593-0.0865 x	0.93	1.51
Oat	0	y = 7.299-0.0367 x	0.86	18.48
	15	y = 8.026-0.0572 x	0.87	35.96
	30	y = 6.971-0.0699 x	0.92	10.60
Oat (NDF)	0	y = 7.649-0.0562 x	0.87	26.89
	15	y = 7.074-0.0439 x	0.99	13.07
	30	y = 6.955-0.0352 x	0.94	10.21
R:C 50:50	0	y = 7.548-0.0639 x	0.91	24.47
	15	y = 7.523-0.0755 x	0.99	23.87
	30	y = 7.422-0.0712 x	0.99	21.17

Table 4.17 Effect of *T. belerica* aqueous methanol extract on IVCPD and uptake of NH_3-N for microbial protein synthesis

Substrate	**Dose** (ml)	**Regression equations**	R^2	**IVCPD** (%)
Berseem	0	y = 7.598-0.0462 x	0.95	75.28
	2	y = 7.477-0.0558 x	0.92	72.37
	3	y = 7.734-0.0470 x	0.91	78.55
Berseem (NDF)	0	y = 4.802-0.0738 x	0.95	8.07
	2	y = 5.697-0.0589 x	0.96	29.59
	3	y = 5.165-0.0534 x	0.79	16.80
Oat	0	y = 8.429-0.0706 x	0.94	95.26
	2	y = 7.658-0.0848 x	0.90	76.73
	3	y = 6.934-0.0622 x	0.68	59.32
Oat (NDF)	0	y = 5.968-0.0256 x	0.41	36.10
	2	y = 6.042-0.0946 x	0.78	37.88
	3	y = 5.371-0.743 x	0.59	21.75
R:C 50:50	0	y = 7.929-0.0672 x	0.86	78.43
	2	y = 6.425-0.0681 x	0.94	47.09
	3	y = 5.511-0.0398 x	0.63	25.12

wheat) and one grass hay was calculated from the linear regressions of NH_3-N concentrations vs. *in vitro* gas production. Using both systems, the IVDN for triticale straw was virtually nil and for others the values obtained using 40 ml system were either similar (oat straw, rice straw, sorghum stover and wheat straw) or higher (barley straw, grass hay and millet stover) than those obtained using 30 ml system. IVDN was 0.48% for *F. roxburghii*, 11.66% for *L. leucocephala* and -9.14% for *Q. incana* (Devarajan, 1999).

Raab *et al.* (1983) reported close relationship between *in vivo* and *in vitro* values when incubation was terminated after 17 h. When conventional proteins feed was used about 80 % of the 24 h value was degraded in the first 8 h incubation whereas in protected protein feed only 60% of the value measured after 24 h incubation were degraded (Raab *et al.* 1983). The appropriate incubation time for *in vitro* degradability studies may depend on the rate of degradability of protein. The presence of phenolic compounds reduce the rate of degradation of feeds (Khazaal *et al.* 1993; Broderick & Albrecht, 1997) making the choice of incubation time more complicated. Raab *et al.* (1983) reported that *in vitro* degradable nitrogen (proportion of total N) after 24 h of incubation in oil cakes, hay and by products ranged from 0.84 to 0.94, 0.36 to 0.38, and 0.64 to 0.69, respectively.

Kaitho *et al.* (1998) used a range of browse species with different level of phenolic compounds and reported degradation rate of –0.1 to 19.0 % h-1. Broderick & Albrecht (1997) using the *in vitro* inhibitor system for tannin-containing feeds reported low rates of degradability of protein. The rate of protein degradability can be affected not only by the amount of phenolic compounds but also by their biological activity (Broderick & Albrecht, 1997). Condensed tannins from different plant species have been reported to show different physical and chemical properties (Mangan, 1988).

4.10 Kinetics of gas production

The development of new gas production methods and the automation of existing ones have led to the urgent need for suitable mathematical models to describe and interpret cumulative gas production profiles. The slope of the profile tends to be zero in the early stages of fermentation, as there is little or no gas production, giving rise to a lag phase.

Thereafter, a steady increase is observed until substrate depletion causes the profile to approach an upper asymptote. Ideally, a function is required which is capable of modeling both a range of shapes with no inflexion point and a range of sigmoidal shapes in which the inflexion point is variable (France *et al.*, 2000). In the analysis of gas production data many mathematical functions have been adopted (Beuvink and Kogut, 1993; Blümmel and Orskov, 1993; France *et al.*, 1993; Groot *et al.*, 1996).

France *et al.* (1993) proposed a new function which is capable of modeling sigmoidal and non-sigmoidal shapes encountered in gas production studies Following the discussion of France and Thornley (1987), the Gompertz model assumes that substrate limitations have no effect on growth, that the rate of growth is proportional to cell mass, and that the growth rate decays exponentially with time due to inactivation of the bacteria. Feed is not a uniform substrate, and the use of a more complex mathematical model to describe the gas production curves may provide insight into changes in the digestion kinetics of the feed fractions. The logistic and Gompertz models differ primarily in the substrate-dependence term; the logistic model assumed a linear and the Gompertz a logarithmic dependence. These models fit the data equally well, but the assumptions behind the logistic model are simpler.

Digestion rate determines the amount of nutrients supplied to the animal. Conventional techniques for estimating digestion rates are time-consuming and often unsuited to analyzing many samples. Rate estimation from gas production depends on a predictable relationship between forage digestion and microbial metabolism. Kinetics of gas production is dependent on the relative proportions of soluble, insoluble but degradable, and undegradable particles of the feed. Mathematical description of gas production profiles allows analysis of data, evaluation of substrate and media related differences, and fermentability of soluble and slowly fermentable components of feeds. Gas production is a promising technique to measure the digestion rates of the soluble and insoluble fractions of forages (Menke and Steingass, 1988; Pell and Schofield, 1993; Cone *et al*., 1996).

Gas production profiles are not necessarily linearly related to degradation or fermentation of substrate (Van Soest, 1994). Previous kinetic studies of fiber digestion have considered the process as a first-order chemical reaction in which microbial growth does not play a major role (Waldo *et al.*, 1971; Mertens and Loften, 1980). Models in common use include the exponential, logistic, and Gompertz (France and Thornley, 1987). These models differ both in their mathematical forms and in the underlying assumptions about the effects of microbial numbers and substrate levels on growth. Their application to gas production curves from fiber digestion can potentially yield information useful for predicting the digestion of fiber in animal feeds and for analyzing other gas-yielding fermentations.

The proportion of gas associated with the rapidly digesting portion of the unfractionated forage exhibited the same basic trends that were reported by Schofield and Pell (1995). As the fiber content of the unfractionated forage increased, the amount of gas in the rapid pool decreased. The improved fit of the single pool logistic model for the NDS fraction of the mature alfalfa and bromegrass samples is inconsistent with earlier research. Stefanon *et al.* (1996) presented parameter estimates from the two-pool logistic model from fermentations of the water-soluble fractions of alfalfa and bromegrass samples of varying maturity. The extraction methods (water vs. neutral detergent solution) may account for the differences. Schofield and Pell (1995) also reported parameter estimates from the two-pool logistic model for alfalfa NDS. Two reasons why the one-pool model better fits the data from this study may be 1) the greater maturity and NDF content of the mature alfalfa sample and 2) the use of washed and resuspended bacteria rather than whole ruminal fluid.

The fast pool of the unfractionated forage is identified from a mathematical analysis of the gas profile rather than from a chemical or physical entity. The values for the specific rates of the rapidly digested pool in the isolated NDF samples (0.107 to 0.194 h^{-1}) were similar to the fast rate of the unfractionated forage (0.095 to 0.170 h^{-1}) and comparable to rates previously reported (Schofield and Pell, 1995; Stefanon *et al.*, 1996). The gas volume associated with the fast specific rate in the unfractionated forage may be a composite pool originating from both the NDF and NDS fractions and must be

interpreted cautiously in relation to chemical composition. Similarly, the slower rates of the NDS fraction presented here for the immature alfalfa (0.04 h^{-1}) and by Stefanon *et al* (1996) for water-soluble components (0.03 h^{-1} to0 .07 h^{-1}) approach the values of the slower rates within the NDF fraction (0.03 h^{-1}). This supports the contention that the gas associated with the slower rate in a unfractionated forage may arise from both the NDF and NDS fractions.

Little is known about the digestion kinetics of the NDS fraction. In this study, curve subtraction proved a reliable method to also describe changes in the soluble fraction of feed samples, confirming the findings of Doane *et al.* (1997) and Calabrò *et al.* (2005),the contribution of the NDF fraction to gas production increases while that of the NDS decreases.

The curves in Figures (4 through 9) represent the gas produced by fermentation of substrates from DM, NDF and NDS. These curves are nearly parallel in DM and NDS. This indicates that the gas production in the unfractionated substrate is mostly contributed by the cell solubles. The pattern of gas production was also confirmed by various models (Table 4.7 and 4.8). NDF is a mixture of polymers with variable sugar composition and digestibility. Thus, the mixture of hexoses and pentoses changes constantly over the course of the fermentation (Van Soest, 1994). These factors may partially explain the low gas volume early in the NDF fermentation. Because the changing ratios seem to affect only the first 1 to 3 ml of gas produced for any of the samples analysed, rate calculation are unlikely to be seriously affected.

Blümmel and Ørskov (1993) adapted gas production technique to describe the kinetics of fermentation based on the exponential model P= a+b (1^{-e-ct}) and to predict feed intake in cattle. The results showed that total gas production (a+b) value as described by the equation were correlated with intake (r^2 =0.88), digestible DM intake (r^2 =0.93) and growth rate (r^2 =0.95) in a multiple regression model. In general, there is positive correlation between gas production and VFA production, and a negative correlation between and microbial biomass yield (Blümmel *et al.*, 1994).

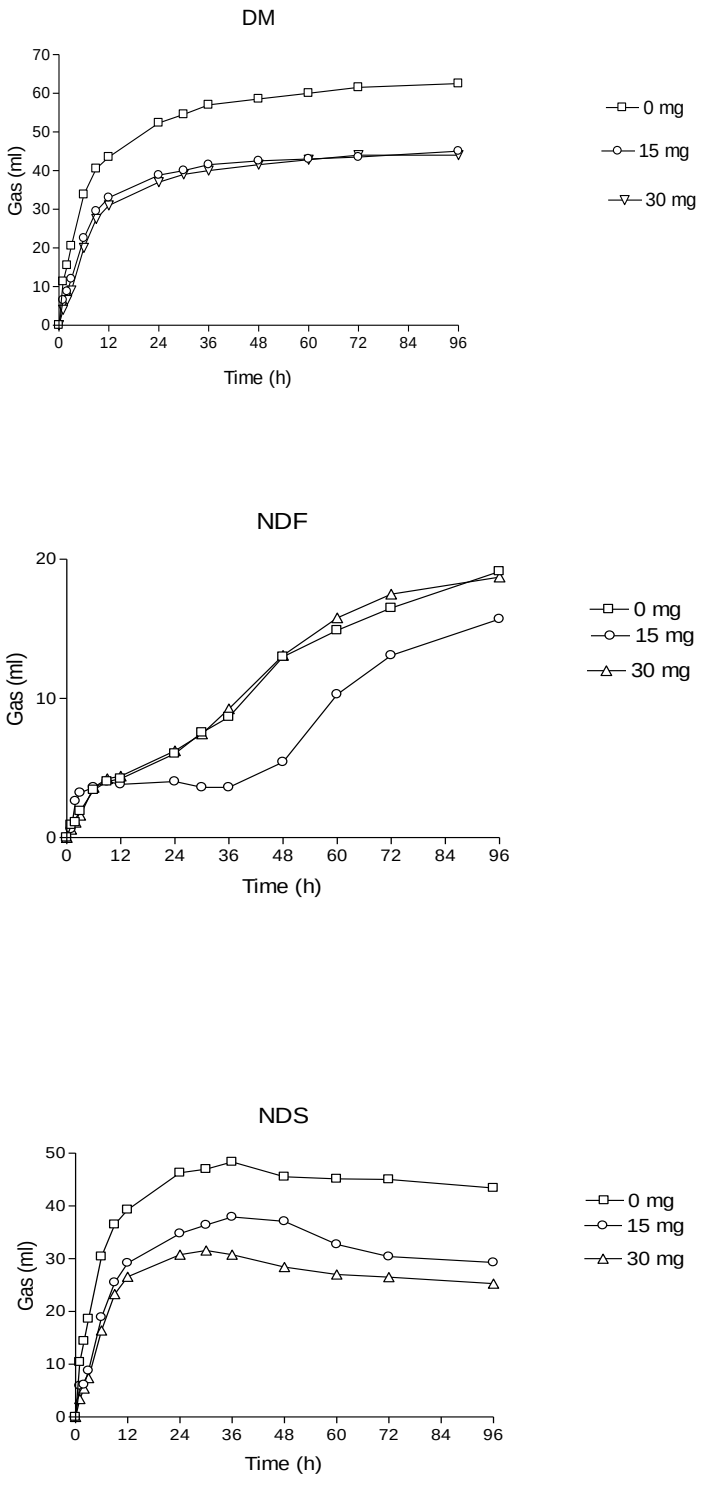


Fig. 4. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of berseem with (15 and 30 mg) or without Rutin

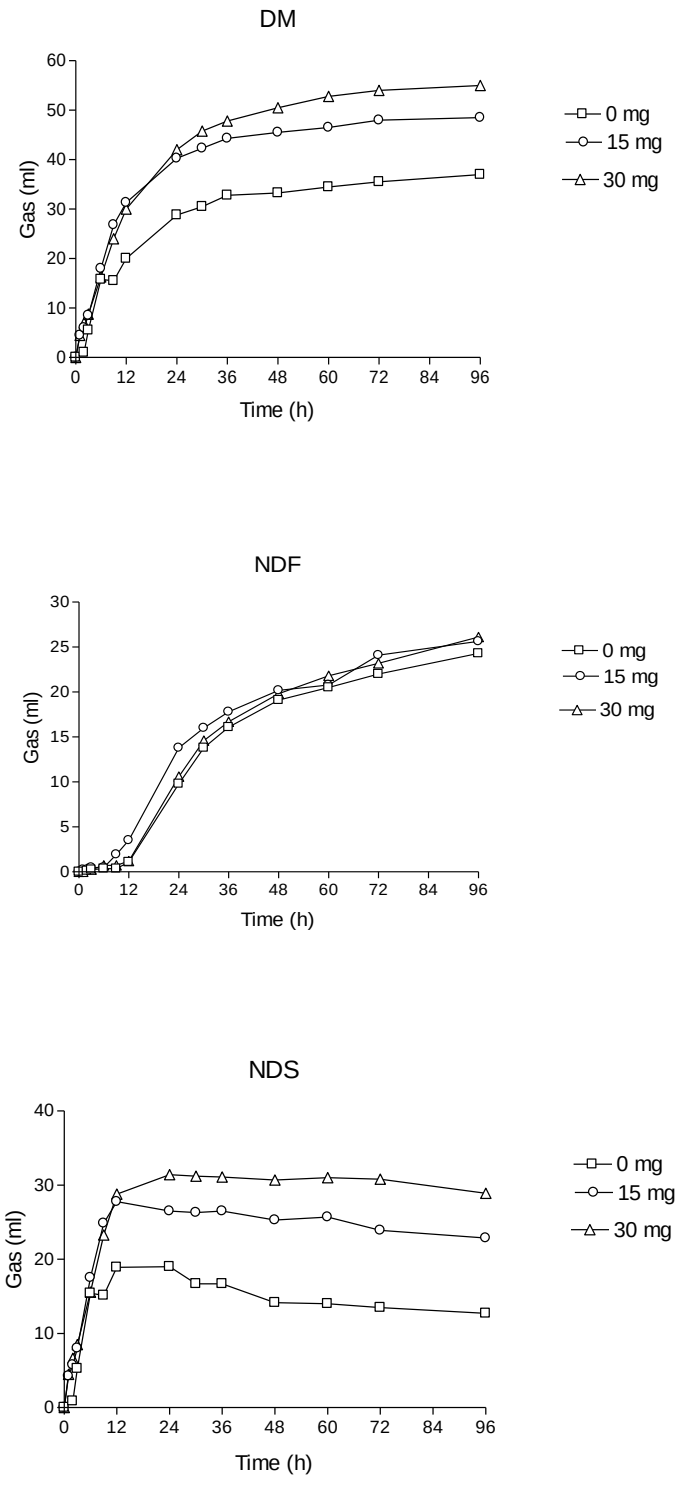


Fig. 5. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of oat with (15 and 30 mg) or without Rutin.

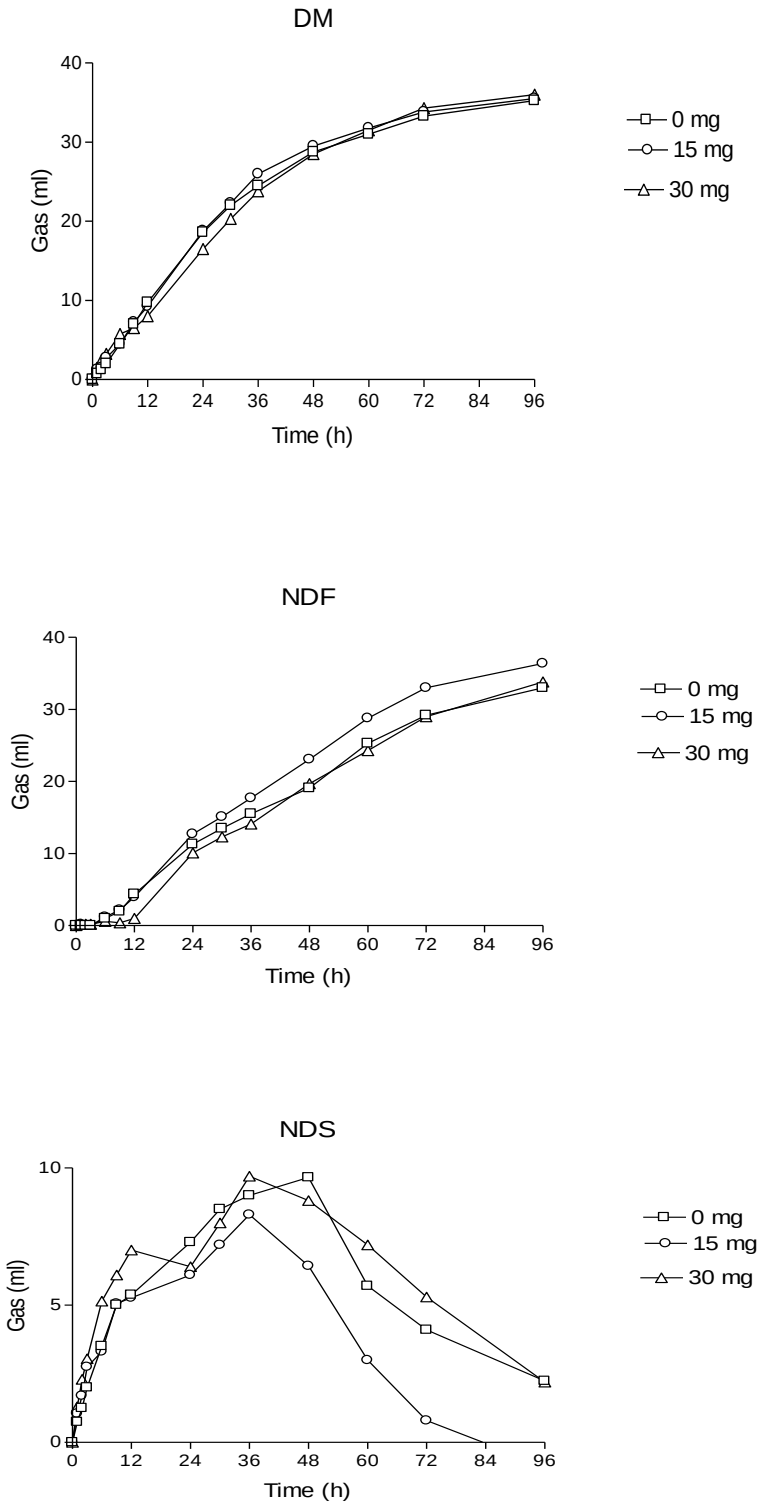


Fig. 6. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of wheat straw with (15 and 30 mg) or without Rutin.

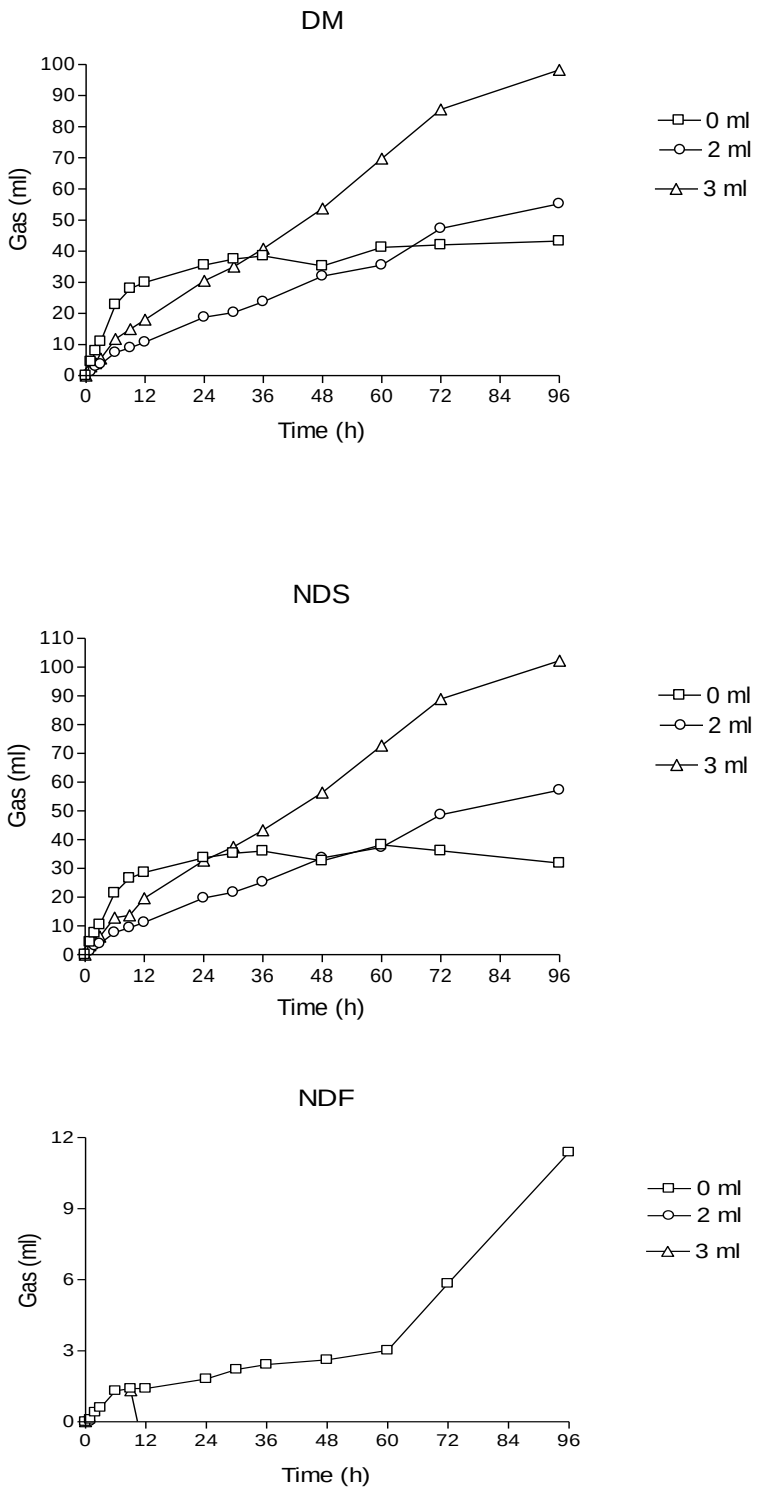


Fig. 7. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of berseem with (2 and 3 ml) or without *T. belerica* aq. methanol extract.

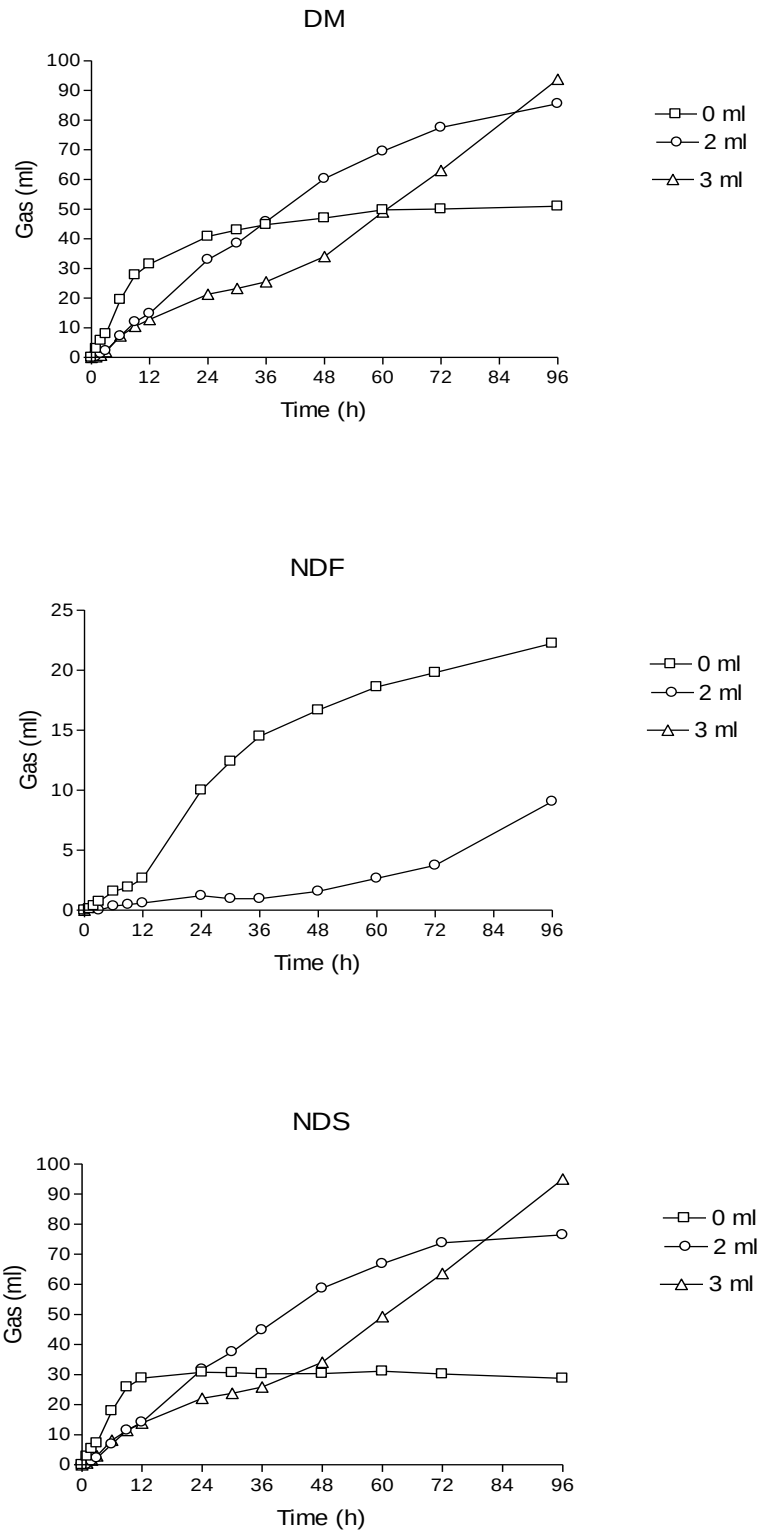


Fig. 8. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of oat with (2 and 3 ml) or without *T. belerica* aq. methanol extract.

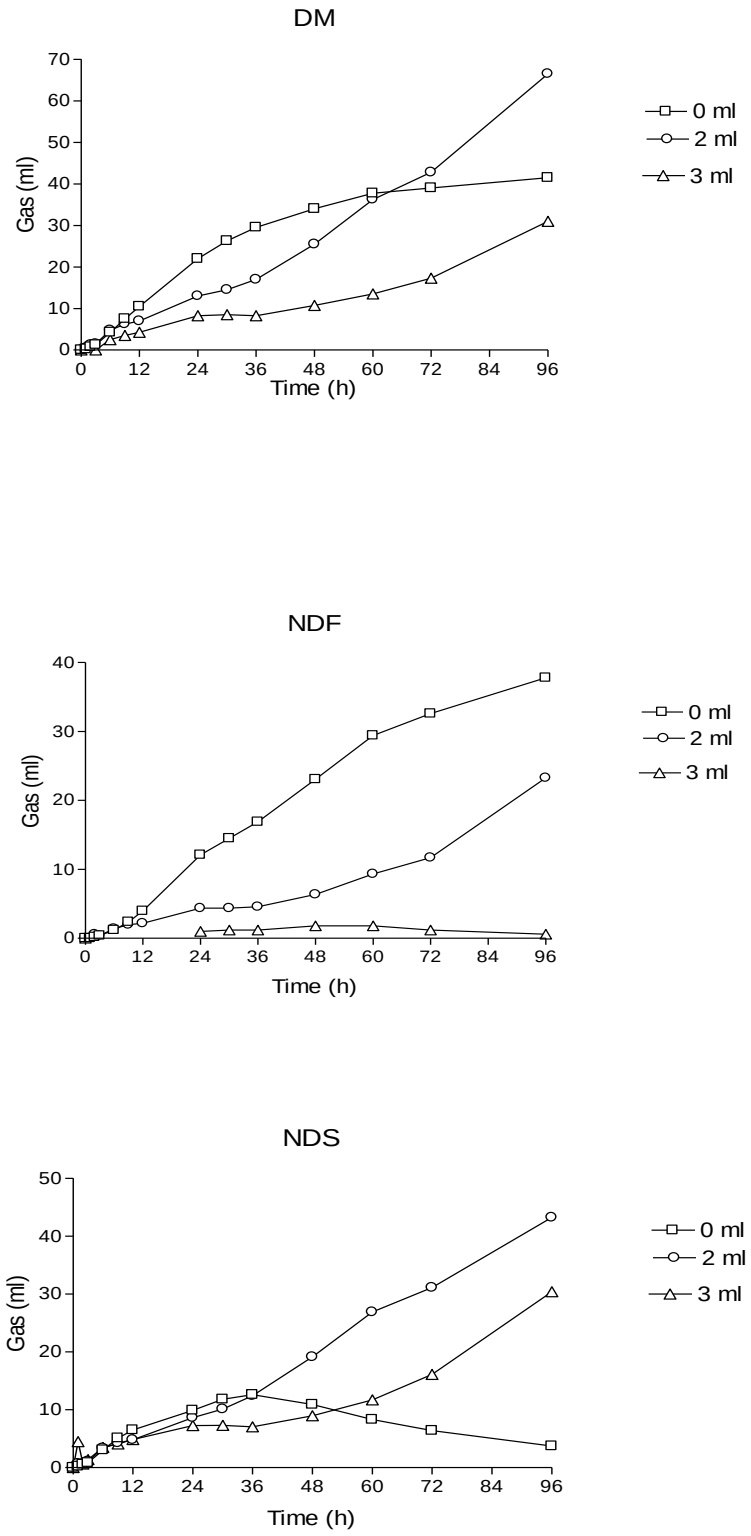


Fig. 9. Cumulative gas production (ml) from the fermentation of DM, NDF and NDS of wheat straw with (2 and 3 ml) or without *T. belerica* aq. methanol extract.

Table 4.18: Potential extent and rate of gas production for berseem, oat and wheat straw with (15 and 30 mg) or without Rutin

Substrate	**Rutin** (mg)	**b** (ml)	**c** (h^{-1})	R^2
Modified Ørskov model with lag				
Berseem	0	59.09	0.117	0.99
	15	42.84	0.119	0.99
	30	35.39	0.073	0.99
Oat	0	35.39	0.074	0.99
	15	47.15	0.085	0.99
	30	54.24	0.062	0.99
Wheat straw	0	38.29	0.028	0.84
	15	38.81	0.028	0.99
	30	42.46	0.021	0.99
Logistic model				
Berseem	0	57.75	0.070	0.96
	15	41.83	0.079	0.98
	30	41.01	0.08	0.98
Oat	0	33.33	0.063	0.96
	15	45.07	0.071	0.98
	30	50.51	0.051	0.98
Wheat straw	0	32.80	0.026	0.98
	15	33.37	0.027	0.99
	30	34.39	0.022	0.99
Gompertz model				
Berseem	0	58.13	2.072	0.97
	15	42.10	2.466	0.99
	30	41.33	2.816	0.99
Oat	0	33.93	2.951	0.97
	15	45.60	2.789	0.99
	30	51.96	2.500	0.99
Wheat straw	0	34.01	3.072	0.99
	15	34.56	3.075	0.99
	30	36.13	2.889	0.99

b- potential gas production (ml/200 mg DM), c- rate of gas production (h^{-1}), R^2- coefficient of determination

Table 4.19: Potential extent and rate of gas production for berseem, oat and wheat straw with (2 and 3 ml) or without *T. belerica* extract

Substrate	***T. belerica*** (ml)	**b** (ml)	**c** (h^{-1})	$\mathbf{R^2}$
Modified Ørskov model with lag				
Berseem	0	39.69	0.124	0.98
	2	Did not fit		
	3	Did not fit		
Oat	0	49.15	0.081	0.99
	2	117.12	0.014	0.99
	3	Did not fit		
Wheat straw	0	45.27	0.029	0.99
	2	Did not fit		
	3	Did not fit		
Logistic model				
Berseem	0	38.68	0.085	0.96
	2	56.96	0.013	0.97
	3	101.87	0.014	0.98
Oat	0	46.6	0.072	0.97
	2	80.88	0.020	0.98
	3	119.34	0.011	0.98
Wheat straw	0	38.66	0.029	0.99
	2	81.54	0.011	0.99
	3	54.15	0.008	0.94
Gompertz model				
Berseem	0	38.94	2.579	0.97
	2	64.78	2.833	0.98
	3	116.43	3.014	0.99
Oat	0	47.22	2.856	0.98
	2	86.64	3.370	0.99
	3	Did not fit		
Wheat straw	0	40.01	3.425	0.99
	2	127.31	3.868	0.99
	3	121.34	4.230	0.95

b- potential gas production (ml/200 mg DM), c- rate of gas production (h^{-1}), R^2- coefficient of determination

Fardin (2005) reported that the total gas production and rate of gas production (c/h) from wheat straw was 43.38 (ml/0.5 g substrate) and 0.021 at 24 h incubation, however, corresponding value for TMR (wheat straw: concentrate 60:40) was 67.78 and 0.041 at 24 h incubation. Srinivas and Gupta (1994) concluded that gas production was influenced by the CP, OM as well as cell wall constituents of the ingredients. Total gas production (ml/ g DM) of mustard, groundnut, cottonseed cake, maize, barley grain and wheat bran was 208.0, 208.6, 179.6, 219.8, 265.8 and 201.8, respectively. The relative proportion of concentrate and forage in the diet will have a considerable influence on *in vitro* gas production. The difference in donor animal's diet and interaction with type of feed incubated reflected variation in total gas volume produced *in vitro* (Getachew *et al.*, 2002; Nagadi *et al.,* 2000). The diet of donor animal exerted considerable influence on bacterial concentration and so influenced *in vitro* gas production. Since different feeds can affect the relative proportion of microbes in the rumen, this may influence the extent of fermentation of feeds (Nagadi *et al.,* 2000). Getachew *et al.* (2002) reported that incubation of barley grain, mustard cake and maize grain produced total gas (ml/ 200 mg) for 24 h ranged from 55.0 to 73.8, 37.8 to 43.5 and 60.8 to 75.6, respectively. Blümmel *et al.* (2003) reported that total gas volume (ml/ 500 mg), TDOM (mg) and partitioning factor (PF, mg/ ml) at 16 h incubation of maize grain was 234.0, 445.3 and 4.92, respectively. Kiran and Krishnamoorthy (2007) reported total gas production (ml/ g DM) at 24 h incubation of cottonseed, groundnut, mustard cake, maize grain, wheat bran and paddy straws was 190.5, 211.5, 204.5, 377.0, 286.5, and 145.2, respectively. The potential gas production (D, ml/ g DM), rate of gas production (c/ h) and PF (mg TDOM/ ml gas at t½) of above sources were 241.0, 0.087 and 3.86, 256.0, 0.154 and 5.97, 241.0, 0.130 and 6.31, 476.5, 0.054 and 3.38, 327.0, 0.101 and 4.07 and 254.8, 0.033 and 2.93, respectively. Ayyappan and Tomar (2006) reported that the total gas production (ml/ g DM) from wheat straw, paddy straw, maize grain, mustard cake and wheat bran at 48 h incubation was 6.3, 6.3, 55, 35 and 38, respectively. During the microbial fermentation in the rumen, carbohydrates, protein and glycerol are fermented to acetate, propionate, butyrate, carbon dioxide, ammonia and methane. The methane, formed due to reduction of carbon dioxide by hydrogen via methanogenesis cannot be used as an energy substrate and is eructed in the air as green house gas, contributing in environmental pollution.

Energy value of methane is 13.15 kcal/g, therefore, 3-12 % of dietary gross energy is lost as methane depending on the nature of diet. Efforts have been made to reduce the energy loss as methane to increase the efficiency of animal production (Lee *et al.*, 2003). Blümmel and Orskov (1993) recorded the average proportion of CH_4 in wheat and barley straws were 17.9 % of total gas production at 24 h incubation. Srinivas and Gupta (1994) reported that the CH_4 (% of total gas) in mustard, ground nut, cottonseed cake (CSC), maize, barley grain, and wheat bran was 23.6, 24.6, 39.0, 24.0, 20.7 and 23.5, respectively. Lee *et al.* (2003) reported that CH_4 (ml/0.2g DM) level at 24 h incubation in wheat, maize, oat grain wheat bran, MC, CSC and paddy straw was 11.39,10.33, 6.87, 8.26, 5.69, 4.48 and 2.42, respectively. In the same study, methane production was highest from grains followed by bran and hulls, oil cakes, roughages and lowest from animal by products. Singh and Mohini (2004) reported that the methane proportion in total gas was highest (43%) when the diet contained straw only and reduced gradually with increasing level of concentrate and lowest value (17.4%) was recorded when diet contained only concentrate mixture only. Straws are having high amount of structural carbohydrates such as cellulose and hemi-cellulose with varying level of lignin, which is responsible for their low digestibility. Fardin (2005) reported that the IVDMD and IVOMD of wheat straw was 41.81 and 44.77 at 48 h incubation. TMR at 60:40 roughage and concentrate ratio produced IVDMD and IVOMD of 60.03% and 60.98% at 48 h incubation.

True digestible OM (mg/ kg DM) and partitioning factor (mg TDOM/ ml gas) at t½ of cotton seed, groundnut, mustard cake, maize grain, wheat bran, wheat bran and paddy straw were 494.6 and 3.86; 606.0 and 5.97; 648.2 and 6.31; 825.6 and 3.38 and 658.0 and 4.07, respectively (Kiran and Krishnamoorthy, 2007).

5. SUMMARY AND CONCLUSIONS

The present study was carried out to evaluate the effect of plant extracts on *in vitro* rumen microbial fermentation. Ten plant extracts were used for screening and out of these; Rutin and *Terminalia belerica* aqueous methanol extract were selected for detailed study. Rutin was used at 15 and 30 mg (per 30 ml of incubation medium) levels whereas *T. belerica* was used at 2 and 3 ml levels to find out their effect on rumen fermentation parameters *in vitro*. Berseem, oat and wheat straw (DM and NDF) were used as substrates. The parameters studied were total gas production, methane production, TD, IVTOMD, PF, microbial biomass, NH_3-N, individual VFA, IVCPD and kinetics of gas production.

The dry matter (DM) content varied from 11.90 % for berseem to 92.58 % for wheat straw. The organic matter (OM), crude protein (CP), ether extracts (EE) and total ash ranged from 83.62 to 90.54, 3.18 to 18.84, 0.49 to 3.47 and 9.45 to 16.37 % respectively. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) ranged from 40.27 to 79.53 and 29.31 to 51.79 % respectively.

Rutin at 15 and 30 mg levels did not show any significant effect on the total gas production but, *T. belerica* at 2 and 3 ml level significantly decreased total gas production in all the three substrates used with corresponding reduction in the methane production. *T. belerica* aq. methanol extract at 3 ml level was very effective in reducing methane production. It reduced methane production by about 40% from the DM of substrates used in the experiment.

The TD of substrates ranged from 28.31 to 84.16 % in DM and from 14.33 to 59.41 % in NDF. Rutin at 15 and 30 mg levels slightly increased TD of all the substrates. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased TD of the substrates. The decrease in the TD was more at 2 ml than 3 ml level in wheat straw. The IVTOMD ranged from 21.23 to 88.17 % in DM and from 12.86 to 27.01 % in NDF of various substrates. Rutin at both the levels slightly increased IVTOMD of substrates but, it was not significant. *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased IVTOMD of all the substrates. The PF indicated the efficiency of microbial

synthesis and ranged from 3.55 to 10.21 in DM of substrates. There was not significant change in PF of all the substrates due to addition of Rutin at 15 and 30 mg level. The PF was higher on addition of *T. belerica* aq. methanol extract at 2 and 3 ml levels with all the three substrates. The kinetics of gas production of DM and isolated NDF were determined by IVGPT. The contribution of cell solubles to gas production at various hours was estimated by difference. The potential extent of gas production and rate of gas production was not affected by any of the treatment.

There was inverse relationship between gas volume (or short chain fatty acids production) and microbial mass production. In the present study, microbial biomass (200 mg substrate) was varied from 10.31 to 60.63 mg for DM and 4.64 to 58.53 mg for NDF of all the three substrate. Rutin at 15 and 30 mg levels resulted in non significant effect on the microbial biomass production but, *T. belerica* aq. methanol extract at 2 and 3 ml levels significantly decreased microbial biomass from oat and wheat straw DM. The decrease was more pronounced at 2 ml level than 3 ml. The NH_3-N concentration varied from 11.01 to 34.30 mg/100 ml for DM and from 12.42 to 27.34 mg/100 ml for NDF of the experimental substrates. Rutin at 15 and 30 mg levels was not having any significant effect on NH_3-N for berseem and oat DM and NDF but, significantly decreased NH_3-N content for wheat straw DM at both the levels. *T. belerica* aq. methanol extract at 2 and 3 ml levels increased NH_3-N content significantly in all substrates DM while it significantly increased NH_3-N content at both the levels in oat and wheat straw NDF.

Rutin at 15 and 30 mg levels slightly decreased A:P ration by increasing propionate production in DM whereas there was not much change in the ratio in NDF of experimental substrates. *T. belerica* aq. methanol extract at 2 and 3 ml dose significantly increased propionate production in DM while in NDF, results were not significant. Regression equations revealed that Rutin at 15 and 30 mg levels increased the rate of NH_3-N uptake for microbial protein synthesis. IVCPD was increased at 15 mg level but decreased at 30 mg level in berseem DM and NDF and oat DM while it decrease at both the levels in oat NDF and R:C 50:50 diet. *T. belerica* aq. methanol at 2 and 3 ml levels did not affect uptake of NH_3-N and IVCPD. In most of the substrates, IVCPD decreased more at 3 ml level than at 2 ml level.

Conclusions

- Rutin at 15 and 30 mg levels did not affect total gas production but *T. belerica* aqueous methanol extract at 2 and 3 ml level significantly decreased total gas production from berseem (legume), oats (non-legume) and wheat straw diet.
- Methane production was not affected by Rutin at both the levels tested while *T. belerica* aqueous methanol extract at 2 and 3 ml level significantly reduced methane production in 24 h.
- Rutin at 15 and 30 mg level slightly increased DM and OM degradability whereas *T. belerica* at both the levels significantly decreased true degradability of DM as well as OM.
- Microbial biomass was not affected by Rutin but, decreased due to addition of *T. belerica* aq. methanol extract.
- Rutin significantly decreased ammonia nitrogen concentration while *T. belerica* increased it in all the substrates.
- Rutin at both the levels decreased A:P ratio by increasing propionate production but *T. belerica* aq. methanol extract was not effective in changing A:P ratio.
- Rutin at 15 and 30 mg levels increased the rate of NH_3-N uptake for microbial protein synthesis but *T. belerica* was not having significant effect on IVCPD.
- Rutin and *T. belerica* were not having any effect on gas production kinetic parameters and the effects were substrate specific.

Rutin was effective in modulating rumen fermentation and may have potential as feed additives but, *Terminalia belerica* aqueous methanol extract needs further investigation.

Until recently, there has been very limited research on the effect of plant extracts on ruminal microbial fermentation. Further research is needed to specify the bioactive compound(s) of the plant extracts associated with modulation of rumen fermentation.

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