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Studies on the effects of methanolic extract of *Cinnamomum zeylanicum* on *in vitro* methane inhibition and rumen fermentation patterns

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ABSTRACT

Cinnamomum zeylanicum (Cinnamon) belongs to the family Lauraceae and is used to cure bronchitis, asthma, diarrhoea, nausea and vomiting, flatulence, fever, headaches, bad breath and toothaches. Its powder is a very good appetizer and improves digestion, while its oil "cinnamon oil" has been shown to exhibit antibacterial, antifungal, antispasmodic and anti-infectious properties. The aim of the current study was to estimate the plant secondary metabolites in aqueous methanol extract of C. zeylanicum and their effect on in vitro methanogenesis and rumen fermentation on adding different level (1, 2 and 3ml) of aqueous methanol extract of C. zeylanicum bark powder on three different sorghum based diets i.e. low, medium and high fiber diets (LFD, MDF and HFD). Aqueous methanol extract contain the 5.04%, 0.03%, 0.11% and 0.05% total sugar, saponins, total protein, and total tannins on dry matter basis respectively. Evaluation of aqueous methanol extract of C. zeylanicum bark powder was carried out using in vitro gas production technique. Results showed the maximum methane reduction (45.37% in term of mM/gDDM) in HFD at 3 ml of extract. In case of HFD diet and 1 ml level of extract TVFA, propionate and butyrate production were increased 59.13, 27.81 and 56% respectively.

Key words: *C. zeylanicum*, plant secondary metabolites, *in vitro* gas production, methane and rumen fermentation.

INTRODUCTION

Methane is one of the major end products of anaerobic fermentation of feeds in the rumen and its emissions to the atmosphere may result in a detrimental impact on the environment because of its greenhouse effect. Ruminal methanogenesis represents a loss of feed energy for ruminants. Energy lost as enteric methane from mature ruminants ranges from 2–12% of gross energy intake [1] depending on diet composition [2, 3, 4].

In ruminants, many ionophores, antibiotics have been used to improve the rumen fermentation [5], improving the some end product (propionate) and decreasing the total amount of methane [6]. Since, January 2006, European Union banned the use of antibiotics as a feed additive due to the risk of its residue in animal products (e.g.: milk and meat) and its subsequent effects on human health [7]. Therefore safe and cost effective new alternatives are needed to maintain efficient animal production systems. Several secondary compounds contained in plants can be used as a safe means of ruminal fermentation modulators. However, only a small number of plant species have been tested to date, and only few studies have dealt specifically with the possibility of decreasing methane production using phytogenic additives. The present experiment was planned to see the effect of *C. zeylanicum* on rumen fermentation and methane reduction under *in vitro* conditions.

C. zeylanicum, popularly known as "Cinnamon" belongs to the family Lauraceae. It is an evergreen tree about 8-18 meter tall and around 50 cm in diameter with reddish brown soft bark. The bark of cinnamon has sugar, saponins, protein, tannins and 0.5 to 1% essential oil *i.e.* cinnamon oil. The main active component of cinnamon oil is cinnamaldehyde, a phenylpropanoid with antimicrobial activity, accounting for up to 75% of its composition [8] and lesser percentages of other phenols and terpenes, including eugenol, trans-cinnamic acid, hydroxycinnamaldehyde, o-methoxycinnamaldehyde, cinnamyl alcohol and its acetate, limonene, alpha-terpineol, tannins, mucilage, oligomeric procyanidins, and trace amounts of coumarin [9,10]. Cinnamon oil shows the inhibition of peptidolysis of rumen micro organisms [11] and its main active component cinnamaldehyde reduced the concentration of *Prevotella* spp. bacteria which are involved in deamination [12]. Cinnamaldehyde also reduced the molar proportion of acetate and increased the proportion of propionate, and the effects of cinnamaldehyde on rumen VFA profiles occurred in a dose-dependent manner [13].

EXPERIMENTAL SECTION

Plant material

The bark of *C. zeylanicum* was purchased from local market of Karnal district, Haryana, India. The bark was crush, oven dried at low temperature (30-50^{0}C) and ground in mills to pass through 1 mm sieve.

Preparation of Plant Extract

The plant extracts were prepared according to prescribed method [14] with some modifications. The plant materials were dried at 50°C and ground in mills to pass a 1mm sieve. Take 25 g powder in 500 ml conical flask and add 250 ml 50% aqueous methanol (1:10 dried bark to solvent). Flask was tightly sealed and kept in a shaker at 25^{0}C and 120 rpm for 24 hour. After

shaking the content of the flask, it is squeezed through four layers of muslin cloth and then filtered through Whatman No. 1 filter paper. The residue was reextracted with 125ml in same condition and be filtered through Whatman 1 filter paper. Extracts was combined and stored at 4^0C for further use.

Estimation of plant secondary metabolites

Aqueous methanol extract of *C. zeylanicum* was subjected to plant secondary metabolites estimation. Total protein, total sugar and total saponins were determined using calorimetric method [15, 16, 17] while total tannins according to the standard prescribed method [18].

Preparation of Treatment Systems and *In Vitro* Fermentation

The method used for *in vitro* fermentation was based on the technique described earlier by Menke *et al.* [19]. Different sorghum based diets (HFD, MFD and LFD) was milled to pass through 1 mm sieve and used as substrate. The different levels (1, 2 and 3 ml) of extract was added to the diet sample in glass syringe (100ml) containing 200 ±10 mg of milled (1mm) three type sorghum based diets. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage and syringes were closed using clamps. A set was also incubated devoid of substrate with and with out extract which served as blanks for particular treatment and values were corrected for different parameters with these blanks. The buffer and rumen liquor were prepared as described by [19]. Rumen samples were obtained after manual mixing of rumen contents from three rumen fistulated mature male buffalo (*Bubalus bubalis*). The buffalo were kept on a standard diet comprising concentrate and roughage in a ratio 50:50.

Estimation of total gas production and estimation of methane

After 24 h incubation, total gas production was estimated by the displacement of piston during incubation. The gas produced due to fermentation of substrate was calculated by subtracting gas produced in blank syringe (containing no substrate, but only the inoculum and buffer) from total gas produced in the syringe containing substrate and inoculum and buffer. Methane content in fermentation gas was determined by gas chromatography (GC) using Nucon-5765 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q (length 6';o.d.1/8" i.d. 2 mm; mesh range 80-100). Temperatures were 40, 50 and 50^0 C, in injector oven, column oven and detector respectively and the flow rates of carrier gas (nitrogen), hydrogen and air were 30, 30 and 300 ml/min, respectively. For methane estimation, each gas sample (250µl) was manually injected using Hamilton airtight syringe. Methane content in sample was calculated by external calibration, using a certified gases mixture with 50% CH_4 and 50% CO_2 (Spantech calibration gas, Surrey, England).

Total volatile fatty acid (TVFA) estimation

TVFA concentration (mM/100 ml) in the supernatant was estimated according to prescribed method [20].

Estimation of individual volatile fatty acids (IVFA)

Individual volatile fatty acid estimated by gas chromatograph according to the prescribed method [21].

Partitioning factor and microbial biomass yield

The PF is calculated as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor [22].

Microbial mass = Substrate truly degraded - (gas volume × stoichiometrical factor)

Where the stoichiometrical factor used was 2.25.

Estimation of ammonia nitrogen

The supernatant of each syringe including that of blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (12 ml) and steam passed on this 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.

***In vitro* true DM degradability**

To estimate true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per the prescribed method [23].

Proximate analyses and Cell wall constituents

The proximate analysis of substrate was carried out as per the methods of AOAC [24]. The cell wall constituents of substrates were determined according to described method [25].

Statistical analysis

Experimental data of different parameters were analyzed in randomized block design with three replicates for analysis of variance [26].

RESULTS AND DISCUSSION

Physical and chemical composition of sorghum based high, medium and low fiber diets used as substrate in *in vitro* incubation is shown in Table 1 and 2.

Result of plant secondary metabolites estimation was shown in Table 3. Aqueous methanol extract of *C. zeylanicum* bark powder was found to contain the 5.04%, 0.03%, 0.11% and 0.05% total sugar, saponins, total protein, and total tannins on percentage dry matter basis respectively. Results of different levels of extract on *in vitro* rumen fermentation and methanogenesis were represented in Table 4. Results indicate that the highest methane reduction (in term of mM/gDDM) 45.37%, 24.44% and 25% was found in HFD, MFD and LFD at 3 ml level of extract in comparison with control diet without addition of extract. Effect of all levels of treatments on dry matter digestibility was non significant, only 8.39% rise was found in LFD at 1 ml level. Partition factor value and microbial biomass (mg) yield were decreased in all cases instead of MFD and LFD at 3 ml levels. Microbial biomass (18.79%) and partition factor value (15.31%) were increased of MFD and LFD at 3 ml levels, respectively.

The methane synthesis in rumen is usually associated with increased propionate production and reduced acetate to propionate ratio [27]. In the present study at 3ml levels on HFD and 3ml levels on MFD, 53.65 and 58.54% acetate production decreased. These levels on HFD and LFD also reduced 45.37 and 24.44% methane production. Ammonia nitrogen concentration was also decreased (49.5%) at 3ml levels on HFD.

Previous work indicates that cinnamon oil and cinnemaldehyde decreased acetate production increased propionate production and decreased NH3-N concentration and both are the main active components of C. zeylanicum bark powder. Thus aqueous methanol extract of *C. zeylanicum* bark powder showed the similar results of previous results.

Table –1: Ingredients of sorghum based diets used as substrate in *in vitro* incubation

Roughage	
Particulars	**g/kg on DM basis**
Wheat straw	700
Sorghum fodder	300
Concentrate	
Particulars	**g/kg on DM basis**
Maize	330
Ground nut cake	210
Mustard cake	120
Wheat bran	200
Deoiled rice bran	110
Mineral mixture	20
Salt	10

Table-2: Chemical composition of Sorghum based diets used as substrate in *in vitro* incubation

Diets	**Chemical constituents (g/kg on DM basis)**						
	OM	**CP**	**EE**	**NDF**	**ADF**	**HC**	**TA**
HDF(80R:20C)	893.4	115.5	18.5	575.2	391.4	183.8	106.6
MFD(50R:50C)	900.0	178.1	22.6	422.6	290.1	132.5	100.0
LFD(20R:80C)	**9**01.9	196.3	35.2	279.0	192.2	86.8	98.1

OM= Organic matter, CP= Crude protein, EE= Ether extract, NDF= Neutral detergent fiber, ADF= Acid detergent fiber, HC= Hemicelluloses

Table –3: Plant secondary metabolites in aqueous methanol extract of *C. zeylanicum* (%DM Basis)

Plant secondary metabolites	**% DM Basis**
Total sugar	5.04
Total Protein	0.11
Total Tannin	0.05
Total saponins	0.03

Table-4: Effect of different levels of *C. zeylanicum* extract on *in vitro* rumen fermentation pattern and methane inhibition

DDM= Digestible dry matter, PF= Partition factor, MBM= Microbial biomass, CH_4=Methane, TVFA=Total volatile fatty acid, NH_3-N= Ammonia nitrogen, SEM=standard error of means.

Diets	Dose	Parameters									
		DDM (mg)	PF	MBM (mg)	CH4 (ml/gm DMD)	CH4 (mM/gm DMD)	TVFA (mM/100ml)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	NH3-N (mg/100ml)
HFD	control	132.00	3.45	45.75	32.97	2.16	5.75	8.76	1.28	0.48	18.01
	1 ml	134.00	3.33	43.44	38.19	2.54	9.15	6.37	2.02	0.75	11.90
	2 ml	85.00	2.99	20.88	27.93	1.18	7.05	5.05	1.47	0.53	8.68
	3 ml	92.50	3.34	30.06	24.52	1.13	5.63	4.06	1.34	0.33	9.10
MFD	control	135.00	3.63	47.67	28.89	1.80	17.45	7.55	2.92	1.71	10.61
	1 ml	109.50	2.21	40.69	45.28	2.47	9.30	6.88	2.43	0.84	11.20
	2 ml	86.00	2.07	34.82	52.21	2.23	7.13	5.08	1.35	0.69	8.26
	3 ml	114.00	2.31	56.63	24.02	1.36	4.55	3.13	1.03	0.40	10.08
LFD	control	137.00	3.33	44.33	36.95	2.52	6.17	4.14	1.48	0.55	19.41
	1 ml	148.50	2.80	29.25	68.79	5.08	6.05	4.17	1.39	0.49	17.78
	2 ml	125.50	2.94	29.31	50.96	3.18	5.95	3.91	1.46	0.57	9.10
	3 ml	119.00	3.84	49.25	31.91	1.89	5.05	3.41	1.16	0.47	9.66
SEM	Diet	1.10	0.05	1.42	0.55	0.04	0.08	0.06	0.04	0.02	0.13
	Dose	1.56	0.08	2.01	0.78	0.06	0.11	0.09	0.06	0.03	0.18
	D*D	2.70	0.17	3.49	1.35	0.11	0.20	0.15	0.10	0.05	0.312

CONCLUSION

The results of this study suggested that aqueous methanol extract of *C. zeylanicum* at 3 ml levels on high HFD modulate the rumen fermentation and reduced the methane production. Considering the above results, further research is required to identify optimal dose, mechanism of its action and used as a powder in *in vitro* and *in vivo* condition to get a significant reduction in methanogenesis without affecting feed digestibility and animal performance.

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