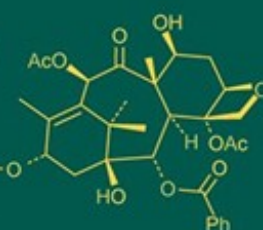
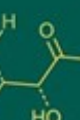
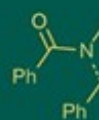


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Evaluation of the safe level of Karanja oil cake using in vitro gas production kinetics

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ABSTRACT

This study was conducted to determine a safe inclusion level of Karanja (*Pongamia pinnata*) oil cake, a non-edible protein source rich in plant secondary metabolites, in ruminant feed using the in vitro gas production technique. A mixed substrate of roughage and concentrate (70:30) was fortified with Karanja oil cake at 1, 3 and 5 percent levels and compared with an unfortified control. Gas production was recorded at intervals up to 72 hours of incubation, and fermentation kinetics parameters (lag time, half time of gas production, rate constants, maximum gas volume and rate of fermentation) were derived along with in vitro dry matter digestibility. Total gas production and cumulative gas production were higher in the control than in the treated groups. Fermentation kinetics differed significantly ($P < 0.01$) among the control and the 1, 3 and 5 percent Karanja cake groups. Half time of gas production was below 12.75 hours at the 3 and 5 percent levels compared with 13.53 hours in the control and the 1 percent group, while rate constants were highest in the treated groups. In vitro dry matter digestibility was 74.08, 64.55, 64.53 and 60.84 percent in the control, 1, 3 and 5 percent groups, respectively, and differed significantly among groups ($P < 0.01$). The study concluded that fermentation kinetics declined as the proportion of Karanja oil cake in the substrate increased, owing to its content of plant secondary metabolites, and that 1 percent inclusion was the safer level, having a negligible effect on gas production, fermentation kinetics and dry matter digestibility relative to the control.

Keywords: Karanja oil cake, *Pongamia pinnata*, In vitro gas production, Fermentation kinetics, Plant secondary metabolites, Dry matter digestibility

1. Introduction

India possesses a wide range of unconventional feed resources that can be incorporated into the diets of ruminant animals (Gowda *et al.*, 2000) [1]. Among these, karanja, mahua, neem and simarouba oil cakes are considered promising alternatives, as they contain nutrient levels comparable to those found in conventional oil cakes. Rising costs of conventional protein sources reduce dairy farm profitability, and unconventional oil cakes have therefore been evaluated as alternative protein sources in dairy cattle diets. Karanja (*Pongamia pinnata*) is a forest-grown tree found throughout India, particularly in Andhra Pradesh, Karnataka and Tamil Nadu, owing to its ecological benefits (Vinay *et al.*, 2008) [2]. Karanja seed cake is an unconventional, protein-rich feed resource obtained in large quantities as a by-product of biodiesel extraction from Karanja seeds. After oil extraction it contains 24-34% crude protein and, after detoxification, can replace groundnut cake at up to 50% inclusion in ruminant diets without adverse effects on health or nutrient digestibility (Nagalakshmi *et al.*, 2021; Thakur *et al.*, 2015) [3, 4]. However, karanja seed cake is less palatable and less digestible owing to *anti*-nutritional factors such as karanjin (0.132%), tannin (6.55%), and glabrin and trypsin inhibitors (3.17%) (Panda *et al.*, 2006; Dinesh *et al.*, 2013) [6, 5]. Non-edible oil cakes are also reported to have beneficial effects on livestock beyond their feed value. Parasitic infections are a major constraint to livestock production in tropical regions, and no single control strategy is fully effective against parasitic load, which in turn affects the growth performance of growing animals. Feeding non-edible oil cake containing plant secondary metabolites is therefore a simple and inexpensive approach to controlling helminth infestation in ruminants (Garg *et al.*, 2012; Molan *et al.*, 2000) [7, 8]. Ethnoveterinary use of plant secondary metabolites has similarly been reported to act against helminth and liver-fluke infestation in ruminants (Nguyen *et al.*, 2005) [9]. The present study was therefore carried out to evaluate the safe level of Karanja oil cake fortification on the fermentation kinetics of a mixed feed substrate using the in vitro gas production technique.

2. Materials and Methods

2.1 Experimental design

In vitro experiments were conducted to evaluate safe levels of plant secondary metabolites from Karanja (non-edible) oil cake on the in vitro fermentation kinetics of a mixed substrate. The change in substrate fermentation after 1%, 3% and 5% fortification with Karanja cake (*Pongamia pinnata*) was assessed using the in vitro gas production (IVGP) technique (Menke *et al.*, 1988) [10].

The substrate consisted of mixed green fodder, dry fodder (straw) and a concentrate supplement in the ratio 40:30:30, giving a roughage-to-concentrate ratio of 70:30. This total mixed substrate (TMS) served as the basal substrate and was fortified with Karanja oil cake at 1%, 3% and 5% levels. In vitro fermentation of TMS fortified with Karanja oil cake was arranged in a completely randomized design with four treatment levels (0%, 1%, 3%, and 5% Karanja oil cake inclusion), using 10 replicates per treatment (Table 1).

2.2 In vitro gas production test

The substrate (mixed substrate and concentrate mixture, 70:30) was milled to pass through a 1 mm sieve, and 200 mg was weighed into 100 ml glass syringes. Incubation medium was prepared as described by Menke *et al.* (1988) [10], and 30 ml was dispensed anaerobically into each syringe. Syringes were incubated at 39 °C, and gas production was recorded at 0.5, 1, 2, 4, 6, 12, 18, 24, 36, 48, 60 and 72 h of incubation.

2.3 In vitro digestibility

At the end of 72 h of incubation, unfermented substrate residue was recovered using double-layered muslin cloth and dried in a hot-air oven at 100 ± 5 °C for 12 h prior to further analysis.

2.4 Estimation of plant secondary metabolites in Karanja oil cake

Sample preparation: A 0.2 g sample of Karanja oil cake, passed through a 1 mm sieve, was extracted with 10 ml of 70% acetone by shaking in a water bath at 30 °C (130 cycles/min) for 2 h. The extract was filtered through Whatman filter paper No. 1 and used for further analysis (Makkar, 1993) [11].

Total tannin: Tannin content was determined by the Folin-Ciocalteu method. Extract (0.1 ml) was mixed with 0.5 ml distilled water, 0.25 ml of 1N Folin-Ciocalteu reagent and 20% sodium carbonate solution, and held at room temperature for 40 min. Standard solutions of tannic acid (20-100 µg/ml) were prepared similarly, and absorbance of test and standard solutions was read against a blank at 725 nm.

Total alkaloid: A 1 mg sample was dissolved in dimethyl sulphoxide and 1 ml of 2N HCl, transferred to a separating funnel with 5 ml bromocresol green solution and 5 ml phosphate buffer, and shaken with 4 ml chloroform; the extract was made up to 10 ml with chloroform. Standard solutions of atropine (20-100 µg/ml) were prepared similarly, and absorbance was read against a reagent blank at 470 nm.

Total flavonoid: Flavonoid content was determined by the aluminium chloride colorimetric method. Extract (1 ml) and 4 ml distilled water were treated with 0.30 ml of 5% sodium nitrite, followed after 5 min by 0.3 ml of 10% aluminium chloride and, after a further 5 min, 2 ml of 1M sodium hydroxide, and made up to 10 ml with distilled water. Standard solutions of quercetin (20-100 µg/ml) were prepared similarly, and absorbance was read against a reagent blank at 510 nm.

2.5 Chemical analysis

Karanja oil cake and the mixed substrate were analysed for proximate composition as per AOAC (2012) [12].

2.6 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) to test the significance of differences among treatments. Gas production at successive fermentation intervals was analysed using a repeated-measures ANOVA model, with treatment as a between-group factor and time interval as a within-group factor. In vitro gas production, cumu-

lative gas production, and fermentation kinetics parameters were analysed using the model $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} is the observation, μ is the overall mean, T_i is the effect of the i th treatment, and e_{ij} is the random error.

3. Results and Discussion

3.1 Chemical composition of substrate and Karanja oil cake

The proximate and cell-wall composition of the feed ingredients and Karanja oil cake used in the study is presented in Table 2. Mixed green fodder contained 25.01% dry matter (DM), 91.33% organic matter (OM), 5.17% crude protein (CP), 1.49% ether extract (EE), 8.56% total ash (TA), 84.76% total carbohydrate (TCHO), 66.04% neutral detergent fibre (NDF) and 43.43% acid detergent fibre (ADF) on a DM basis. Straw contained 94.14% DM, 9.70% OM, 4.81% CP, 2.82% EE, 8.29% TA, 84.07% TCHO, 73.14% NDF and 47.17% ADF, and the concentrate mixture contained 89.41% DM, 87.16% OM, 17.44% CP, 1.91% EE, 12.94% TA, 67.66% TCHO, 45.03% NDF and 11.76% ADF. These values were comparable to those reported previously for similar feed ingredients (ICAR, 1998; ICAR, 2013) [13, 14].

Karanja oil cake contained 90.13% DM, 96.34% OM, 24.69% CP, 13.44% EE, 9.84% TA, 58.09% TCHO, 48.82% NDF and 27.36% ADF (Table 2). Its plant secondary metabolite content, also given in Table 2, was 2.72 mg/ml tannin, 0.18 mg/ml alkaloid and 0.19 mg/ml flavonoid (extract basis), and these differed significantly among metabolite classes ($P < 0.01$).

3.2 Effect of Karanja oil cake fortification on gas production kinetics

Karanja cake was evaluated for its optimum level of fortification of the substrate at 1%, 3% and 5%, compared with an unfortified control (CG), using the in vitro gas production technique over 72 h of incubation; fermentation kinetics were subsequently derived using a first-order kinetic model.

Gas production at successive fermentation intervals differed significantly between the control and treated groups with increasing proportion of Karanja cake fortification (Table 3, Fig 1). In vitro gas production is considered a simple, reliable and safe tool for assessing the fermentative value and potential rate of fermentation of a feedstuff (Krishnamurthy *et al.*, 2005) [15]. Gas production was highest in the control at most fermentation intervals, rose to a peak at 12 h of incubation and declined thereafter, with smaller secondary peaks at 36 and 48 h (Fig 1).

Cumulative gas production over the incubation period is presented in Fig 2. Cumulative gas production was statistically comparable between the control and the 1% group, and was higher in both than in the 3% and 5% groups. The linear rate of cumulative gas production was 10.09 ml/h in the control and 1% groups, compared with 8.83 and 7.22 ml/h in the 3% and 5% groups respectively, indicating that the rate of cumulative gas production decreased with increasing proportion of Karanja cake fortification. A significant increase in cumulative gas production was observed from 4 h of incubation onward in the fortified groups. Although cumulative gas production expressed as a percentage of total gas production increased between 6 and 36 h in the 5% group (Fig 3), the absolute cumulative gas volume in this group remained lower than in the control or other treated groups throughout incubation.

Fermentation kinetics parameters differed significantly ($P < 0.01$) among the control, 1%, 3% and 5% Karanja cake groups (Table 3). Total gas production was significantly higher in the control than in the treated groups, reflecting the variable composition of the substrate as well as the plant secondary metabolite content of the oil cake, which can alter fermentation rate. This variation may be attributed to the phenolic compound, alkaloid and flavonoid content of Karanja cake (2.72, 0.18 and 0.19 mg/ml, respectively), since microbial colonisation of feed particles is influenced by chemotactic attraction to released soluble carbohydrates. Although total gas production was low

est in the 5% group, its lag time (about 13 min) was shorter than in the control, whereas lag time was longer at the lowest (1%) proportion of Karanja cake; this suggests that rumen microbes require time to adapt to the more concentrated Karanja cake-fortified substrate relative to the control.

Half-time of gas production ($T_{1/2}$) was below 12.75 h in the 3% and 5% groups, compared with 13.53 h in the control and 1% groups; this contrast in $T_{1/2}$ may be useful in regulating the fermentation kinetics of Karanja cake-fortified substrate at different inclusion levels. Rate constants (K_1 and K_2) were highest in the treated groups compared with the control. Rate of fermentation is known to vary with non-fibre carbohydrate (Singh *et al.*, 2018) [16], cell-wall carbohydrate or feed protein content (Rathod *et al.*, 2018) [17], but the present findings indicate the importance of Karanja oil cake proportion in determining fermentation rate. Lag time and $T_{1/2}$ were directly related to, while K_1 , K_2 and fermentation rate were inversely related to, particle size, indicating that K_1 , K_2 and $T_{1/2}$ reflect the complexity of feed nutrient composition (Swain *et al.*, 2009) [18], whereas the kinetic rate of gas production and lag time reflect microbial characteristics governing fermentative degradation of feed particles (Krishnamurthy *et al.*, 2005) [15].

3.3 In vitro dry matter digestibility

In vitro dry matter digestibility (IVDMD) differed significantly among the control and treated groups (Table 3), with an overall difference of about 10 percentage points between the control and the treated groups. The lowest fermentation and IVDMD were observed in the 5% group, attributable to its content of total phenols, alkaloids and flavonoids (2.72, 0.18 and 0.19 mg/ml, respectively). The decline in IVDMD with increasing Karanja cake inclusion may be attributed to the higher phenolic concentration, which is known to inhibit trypsin digestion. Although these secondary metabolites occur naturally in Karanja cake, their concentration depends on the processing method; tannin content, for example, has been reported to be slightly

higher in solvent-extracted karanj cake than in expeller-pressed cake (Panda *et al.*, 2006) [6], the latter containing 0.94% tannin along with 0.65% phytate and 31 units/mg trypsin inhibitor (Vinay *et al.*, 2008) [2]. A negative relationship between in vitro fermentation parameters, fibre fraction and tannin concentration has similarly been reported (Salem, 2005) [19], and a negative correlation between secondary compound content and both in vitro gas production and dry matter degradability has been reported for some tree fodders (Jung *et al.*, 2006) [20]. In contrast, supplementation with an oil-press cake at 400 g/kg has been reported not to affect gas production or dry matter digestion, an effect commonly associated with high dietary lipid content, which can reduce bacterial growth.

4. Conclusion

Non-edible Karanja oil cake, fortified into a mixed feed substrate at 1%, 3% and 5%, was evaluated using in vitro gas production and fermentation kinetics parameters. Based on quantitative comparison of gas production, fermentation kinetics and dry matter digestibility, 1% inclusion of Karanja oil cake was identified as the safer level compared with 3% and 5%, as it had a negligible effect on in vitro gas production, fermentation kinetics and dry matter digestibility relative to the control. Increasing the proportion of Karanja oil cake delayed the onset of fermentation and reduced the rate of gas production, owing to higher residues of tannin, alkaloid and flavonoid content. These findings establish a threshold level of Karanja oil cake supplementation that can guide future in vivo feeding studies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Table 1. Control and treatments under the in vitro completely randomized design

Energy (Main factor)	Supplementation (Sub factor)	Replicates
Blank*	No substrate	5
Total mixed substrate (TMS)	Without	10
TMS + 1% Karanja cake	With	10
TMS + 3% Karanja cake	With	10
TMS + 5% Karanja cake	With	10

*Blank contains rumen inoculum and buffer only, without substrate.

Table 2. Chemical composition (% DM basis) of feed ingredients and Karanja oil cake, and its plant secondary metabolite content

Parameter	Mixed green fodder	Straw	Concentrate mixture	Karanja oil cake
DM	25.01	94.14	89.41	90.13
OM	91.33	9.70	87.16	96.34
CP	5.17	4.81	17.44	24.69
EE	1.49	2.82	1.91	13.44
TA	8.56	8.29	12.94	9.84
TCHO	84.76	84.07	67.66	58.09
NDF	66.04	73.14	45.03	48.82
ADF	43.43	47.17	11.76	27.36
Plant secondary metabolite (mg/ml extract)	Karanja oil cake			
Tannin	2.72			
Alkaloid	0.18			
Flavonoid	0.19			

DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; TA = total ash; TCHO = total carbohydrate; NDF = neutral detergent fibre; ADF = acid detergent fibre.

(continued on next page)

Values bearing different superscript letters within a column differ significantly ($P < 0.01$). CG = control group; K_1 , K_2 = rate constants; Y_{max} = maximum gas volume; IVDMD = in vitro dry matter digestibility.

Table 3. In vitro fermentation kinetics and dry matter digestibility of substrate fortified with Karanja oil cake

Treatment	Total gas (ml)	Lag time (h)	T½ (h)	K1 (%/h)	K2 (%/h)	Ymax (ml)	Ferm. rate (%/h)	IVDMD (%)
CG	49.63 b	0.29 b	13.53 b	5.13 a	9.62 a	49.02 b	5.49 a	74.08 c
1%	49.72 b	0.29 b	13.58 b	5.13 a	9.71 a	49.07 b	5.50 a	64.55 b
3%	46.05 b	0.22 b	12.75 b	5.47 a	10.68 b	45.62 b	5.89 a	64.53 b
5%	41.55 a	0.22 a	11.39 a	6.11 b	12.51 c	41.33 a	6.67 b	60.84 a
SEM	1.43	0.01	0.30	0.11	0.32	1.40	0.14	0.02
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

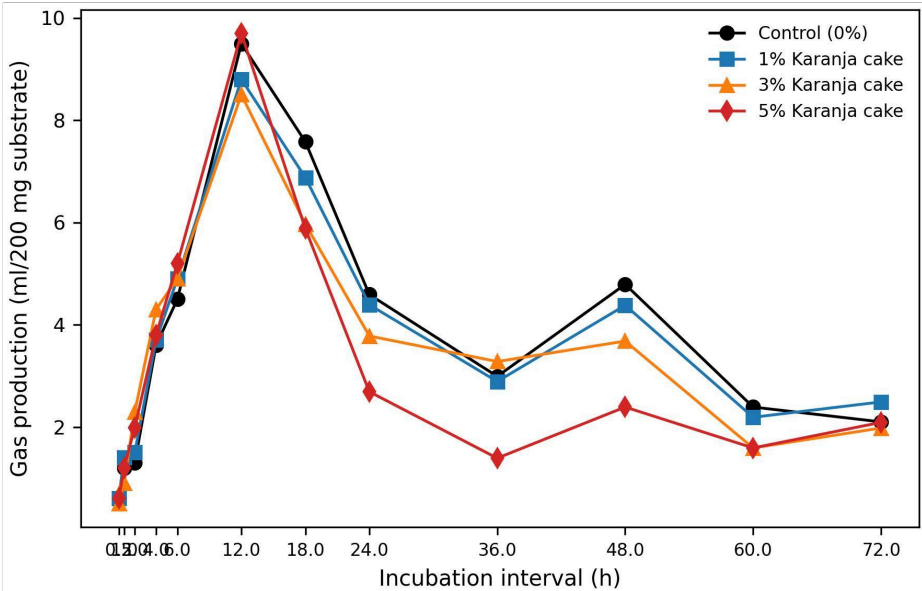


Figure 1. In vitro gas production (ml/200 mg substrate) at successive incubation intervals in substrate fortified with 0 (control), 1, 3 and 5% Karanja oil cake

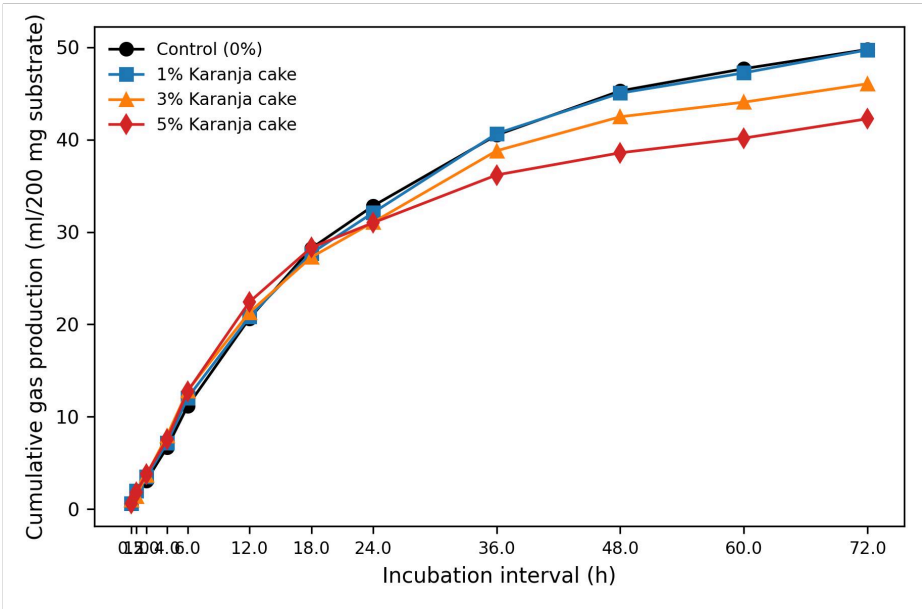


Figure 2. Cumulative in vitro gas production (ml/200 mg substrate) over 72 h of incubation in substrate fortified with 0 (control), 1, 3 and 5% Karanja oil cake

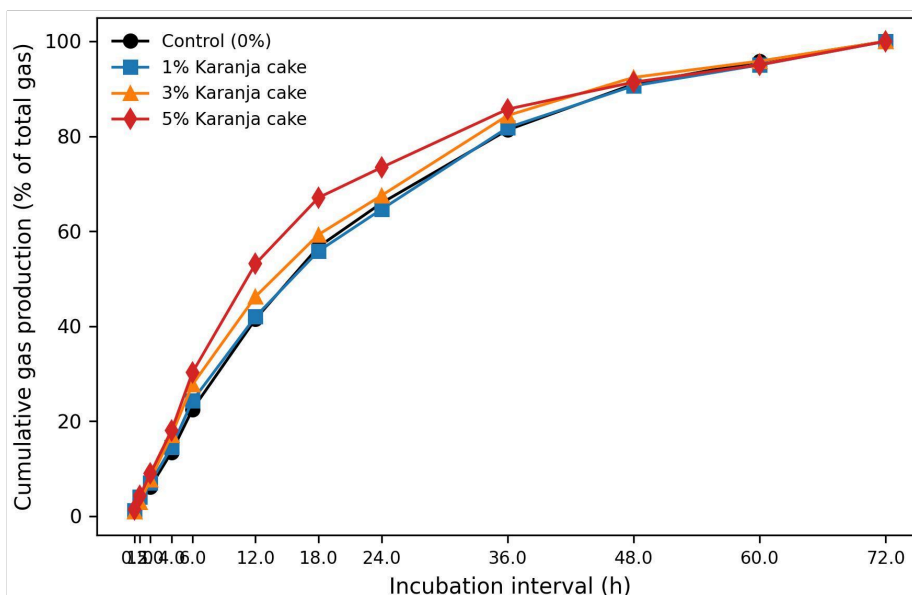


Figure 3. Cumulative in vitro gas production expressed as a percentage of total gas production over 72 h of incubation in substrate fortified with 0 (control), 1, 3 and 5% Karanja oil cake

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