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Evaluation of unconventional oil cakes on *in vitro* Methanogenesis and fermentation of feed in rumen liquor of Buffalo

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Abstract
In the present experiment four unconventional oil cakes (UOC) *viz*. karanj (*Pongamia glabra*), mahua (*Madhuca indica*), neem (*Azadirachta indica*) and castor bean (*Ricinus communis*) were tested for their effect on methane production, total gas production, IVTD, and ammonia nitrogen concentration. In Experiment I all the UOCs (Karanj, Mahua, Neem and castor bean) were evaluated for their feed fermentation in *in vitro* where 70% of UOCs and 30% maize was used as a substrate (50:50 Concentrate : Wheat straw). The methane production (ml/g DM) was significantly reduced ($p<0.01$) by 48.48, 45.02, 37.06 and 33.19 percent in karanj cake, mahua cake, neem cake and castor bean meal, respectively as compared to control. The IVTD (%) in karanj and mahua cake inclusion was found comparable with control unlike in neem and castor bean meal inclusion where the IVTD was significantly lower in comparison to control. Based on the first experiment, karanj and mahua cake was selected for further *in vitro* experiment for selecting the ratios of the UOC. The karanj and mahua cake were selected and tested in *in vitro* at different combinations *viz*. 25:75, 50:50 and 75:25 ratios. The total gas production in karanj and mahua cake (75:25) inclusion was comparable as compared to control. The methane production (ml/g DM) was significantly lower ($p<0.01$) in karanj and mahua cake mixture (75:25) inclusion as compared to control group. The IVTD (%) in karanj and mahua cake mixture at 75:25 inclusion was comparable with control, unlike other combinations.

Keywords: Unconventional oil cake, Total gas, *in vitro* True digestibility, Methane, Ammonia N

Introduction
Ruminants are mainly fed on lignocellulosic agricultural by-products like cereal straws, stover, sugarcane bagasse etc. The rumen microbes convert these un-utilizable plant tissues into valuable animal products like meat and milk. The rumen microbial ecosystem is highly diversified which comprises bacteria, protozoa, fungi, and bacteriophages. Unfortunately, fermentation in the rumen is associated with various losses because of the fermentation of different nutrients like carbohydrate (structural and non- structural) and protein. CO_2, methane and ammonia are the metabolic end products which are wasteful as far as the nutrition of animal is concerned but they are unavoidable products of rumen fermentation. During rumen fermentation, the methane production leads to 2-15% of loss in the GE [1, 2, 3]. Domestic livestock since long has been blamed for substantial contribution to global anthropogenic GHG emissions. The world population of domesticated ruminants is thought to be responsible for 29% of total CH_4 emissions due to anaerobic enteric fermentation of feeds. The contribution of India's livestock to global methane emissions is 10.63% (NIANP, 2017). Also, methane is a potent greenhouse gas having 23 times more global warming potential than CO_2, enteric methane production contributes significantly to global warming.
There is a need for effective manipulation of rumen fermentation to inhibit the various losses due to gas production for increasing feed efficiency. Manipulation of rumen fermentation also produces environmental, social and economic benefits to the global population. All these aspects of enteric nutrients losses had directed the scientific community to find alternative ways to manipulate the rumen fermentation pattern in an efficient manner. Numerous chemical additives and antibiotics have been tested and used for this purpose [1].
There are many unconventional feed resources available in India which can be included in the ration of ruminant animals [4]. Among all, the most promising are castor bean, mahua, neem and karanj oil cakes which contain quality nutrients like that of conventional cakes.

These unconventional oil cakes (UOC) are rich sources of phytochemicals such as glycoside, saponins, tannins, essential oils and others [5]. Also, it has been reported that these cakes having various functions and beneficial effects for livestock production other than feed value.

Materials and Methods

Preparation of substrate and UOC

Individual raw materials of concentrate mixture and UOC were dried in hot air oven at 100±2 ^{0}C overnight, ground to pass through to 1 mm sieve separately and stored in airtight container.

Experimental Design

Experiment I: *In vitro* screening of four most promising unconventional oil cakes on the basis of rumen fermentation

The promising unconventional oil cakes (Karanj cake, Neem seed cake, Mahua seed cake and Castor bean meal) were taken as a substrate for *in vitro* experiment.

Table 1: Amount of substrate (200 mg) to be used for the *in vitro* study

Treatments	Concentrate mixture	Roughage
G_0	Concentrate mixture (100 mg)	Finely ground wheat straw (100 mg)
G_1	Karanj cake (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)
G_2	Mahua cake (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)
G_3	Neem cake (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)
G_4	Castor bean cake (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)

Experiment II: *In vitro* screening of unconventional oil cakes combination on the basis of rumen fermentation

On the basis of rumen fermentation in experiment-I two most promising unconventional oil cakes were selected for *in vitro* evaluation. Selected cakes i.e., A and B combination will be taken as substrate at the level of 25:75, 50:50 and 75:25 for *in vitro* experiment.

Table: 2 Amount of substrate (200 mg) to be used for the *in vitro* study

Experimental study	Concentrate mixture	Roughage
D_0	Concentrate mixture (100 mg)	Finely ground wheat straw (100 mg)
D_1	UOC combination 25:75 (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)
D_2	UOC combination 50:50 (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)
D_3	UOC combination 75:25 (70 mg) + concentrate mixture (30 mg)	Finely ground wheat straw (100 mg)

Preparation of inoculum

Rumen liquor was collected from two fistulated buffaloes fed on a diet of wheat straw and concentrate mixture in 1:1 ratio. The wheat straw had organic matter (OM), 909 g; crude protein (CP), 31 g; ether extract (EE), 15 g; neutral detergent fibre (NDF), 835 g and acid detergent fibre (ADF), 535 g/kg and the concentrate mixture (consisting of maize, 32; solvent extracted soybean meal, 20; wheat bran, 45; mineral mixture, 2 and salt, 1 kg/100 kg) had OM, 895 g; CP, 195 g; EE, 40 g; NDF, 385 g and ADF, 164 g/kg on DM basis. The animals were fed at the rate of 2.5 kg dry matter/100 kg of body weight. The rumen liquor was sampled just before feeding (0 h) from two animals and transported in insulated flasks under anaerobic conditions to the laboratory, pooled in equal proportions and used as a source of inoculum.

In vitro gas production test

The substrate (wheat straw and concentrate mixture in 1:1 ratio) was milled to pass through 1mm sieve and 200±10 mg was weighed in glass syringes of 100 ml capacity. The incubation medium was prepared as described by Menke and Steingass (1988) [6] and 30 ml was dispensed anaerobically in each syringe. Syringes were incubated at 39 ◦C for 24 h.

Estimation of gas and methane production

After 24 h incubation gas production was estimated by the displacement of piston during incubation. The gas produced due to fermentation of substratewas 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. The gas produced in standard syringe (containing standard maize hay) was used to check day to day variation in the quality of inoculum. For methane estimation 100l gaswas sampled from the headspace of syringe in an airtight syringe and injected into Nucon-5765 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q. The gas flow rates for nitrogen, hydrogen and air were 30, 30 and 300 ml/min, respectively. Temperature of injector oven, column oven and detector were 40, 50 and 50 ◦C, respectively. A50/50 mixture of methane and carbon dioxide (Spancan; Spantech Products Ltd., England) was used as a standard.

In vitro true digestibility (IVTD)

The IVTD of feed was determined after termination of incubation. The contents of syringes were transferred quantitatively, into spoutless beakers by repeated washings with 100 ml neutral detergent solution. The flask contents were refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade G1). The dry matter content of the residue was weighed and *in vitro* true digestibility of feed was calculated as follows by Van Soest *et al.* (1991) [7].

$$\textit{In vitro} \text{ true DM digestibility} = \frac{\text{(Initial dry matter of feed} - \text{NDF residue in crucible)}}{\text{Initial DM of feed taken}} \times 100$$

Ammonia nitrogen
Ammonia nitrogen in the fermented medium was estimated by the method of Weatherburn, (1967) [8] as follows:

Table 3: Tubes were prepared in duplicate for standard curve as follows

Tube	1	2	3	4	5	6	7
Distilled water (ml)	1.0	0.90	0.80	0.60	0.40	0.20	0.00
Standard solution (ml)	0.10	0.20	0.40	0.60	0.80	1.00	
Ammonia nitrogen (µg)	0.00	1.00	2.00	4.00	6.00	8.00	10.0

Procedure
To the rumen liquor (0.1 ml), 0.9 ml of distilled water was added, followed by addition of 5.0 ml of solution A and then immediately 5.0 ml of solution B and mixed thoroughly. The tubes were incubated at 39 °C for 15 min for color development. Samples were then read spectrophotometrically at 625 nm against a reagent blank. In a similar way, standard samples (ammonia nitrogen concentration ranging from 0.5 to 10.0 µg) were processed and a calibration curve was plotted. The concentration of the unknown sample was calculated by the standard curve.

Proximate analyses
The DM (ID number 930.15), OM and ash (942.05) and CP (N×6.25, ID number 954.01) and EE (ID number 920.39) of substrates were determined by AOAC (1995) procedures. NDF (estimated without amylase and expressed inclusive of ash), ADF (also expressed as inclusive of ash) and ADL were analyzed according to the methods described by Van Soest *et al*. (1991) [7].

Statistical Analysis
The experimental data generated were analyzed using SPSS computer package (SPSS version 20.0, SPSS Inc., Chicago, USA) [9] adopting standard statistical procedures. The periodic alterations in blood parameters were analyzed using repeated measures design (General linear model; GLM, Multivariate). Other parameters were analyzed using one way ANOVA with Dunken's post hoc testing to compare experimental groups. For all statistical analyses, probability values less than 0.05 were considered as significant.

Results and Discussion
Experiment I: Effect of unconventional oil cakes on the production of total gas, methane and *in vitro* feed digestibility (IVTD)
Gas production (ml/g DM) was significantly (P<0.001) differed with higher production in control 123.63, karanj 118.10 and mahua 117.07 compared to neem 90.39 and castor bean cake 88.69. IVTD (%) was significantly (P<0.001) differed with higher in control 54.75, karanj 52.95, and mahua 52.76 compared to neem 44.28 and castor bean cake 43.29. The Methane production (ml/gm DM) was 12.68, 13.53, 15.49, 16.44 and 24.61 in karanj, mahua, neem, castor bean and control, respectively. The methane production significantly (P<0.001) lowers in karanj and mahua oil cake as compared to others Fig 4.1. The ammonia nitrogen concentration (mg/dl) was 21.72, 13.96, 15.07, 16.94 and 18.68 in control, karanj, mahua, neem and castor bean cake, respectively. The ammonia nitrogen concentration in karanj and mahua was found lower than the other treatment group.

Table 4: Effect of unconventional oil cakes on *in vitro* feed fermentation

Attributes	Gas (ml/g DM)	Methane (ml/g DM)	IVTD (%)	NH_3-N (mg/dl)
Control	$123.63^{a}\pm4.65$	$24.61^{a}\pm0.76$	$54.75^{a}\pm0.11$	$21.72^{a}\pm0.36$
Karanja cake	$118.10^{a}\pm1.25$	$12.68^{c}\pm0.13$	$52.95^{a}\pm0.03$	$13.96^{d}\pm0.48$
Mahua cake	$117.07^{a}\pm2.00$	$13.53^{c}\pm0.16$	$52.76^{a}\pm0.07$	$15.07^{cd}\pm0.44$
Neem seed cake	$90.39^{b}\pm3.88$	$15.49^{b}\pm0.20$	$44.28^{b}\pm0.53$	$16.94^{bc}\pm0.53$
Castor bean meal	$88.69^{b}\pm3.70$	$16.44^{b}\pm0.16$	$43.29^{b}\pm0.86$	$18.68^{b}\pm0.32$
SEM	4.18	1.14	1.25	0.75
P-value	<0.001	<0.001	<0.001	<0.001

abcd Superscript bearing means values within row differ significantly

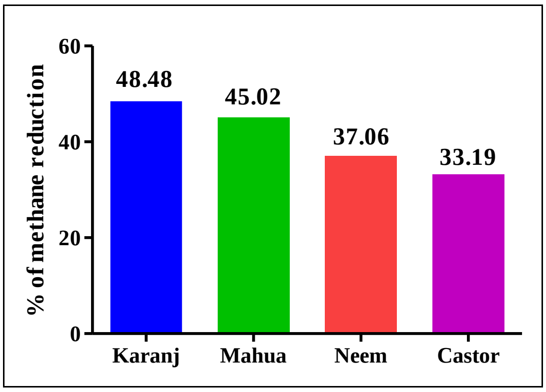

Fig 4.1: Percent inhibition in *in vitro* methane production by unconventional oil cakes

Experiment II: Effect of combination unconventional oil cakes on the production of total gas, methane and *in vitro* feed digestibility (IVTD)
The results of *in vitro* feed fermentation as affected by inclusion in a combination of karanj + mahua were presented in Table 4. It was observed that the total gas production were 117.08, 100.57, 93.34 and 113.10 ml/g DM in control, karanj + mahua (25:75), karanj + mahua (50:50) and karanj + mahua (75:25), respectively. The methane production was 22.82, 12.44, 13.69 and 10.70 ml/g DM in control, control, karanj + mahua (25:75), karanj + mahua (50:50) and karanj + mahua (75:25), respectively. The methane production significantly lowers in karanj + mahua (75:25) as compared to others. Similarly, the IVTD per cent was found lower in the combination of karanj + mahua (25:75) and karanj + mahua (50:50) inclusion as compared to control. However, the inclusion of karanj + mahua (75:25) level was found comparable with control. The ammonia nitrogen concentration mg/dl was 20.60, 13.70, 14.79 and 11.79 in control, karanj + mahua (25:75), karanj + mahua (50:50) and karanj + mahua (75:25), respectively.

Table 4: Effect of combination of karanj and mahua oil cakes on *in vitro* feed fermentation

Attributes	Gas (ml/g DM)	Methane (ml/g DM)	IVTD (%)	NH_3-N (mg/dl)
Control	117.08^a±0.87	22.82^a±0.19	52.57^a±0.25	20.60^a±0.31
Karanj + Mahua cake (25:75)	100.57^b±0.24	12.44^c±0.07	49.40^b±0.14	13.70^c±0.15
Karanj + Mahua cake (50:50)	93.34^c±1.17	13.69^b±0.15	45.23^c±0.47	14.79^b±0.12
Karanj + Mahua cake (75:25)	113.10^a±3.37	10.70^d±0.12	50.95^{ab}±0.31	11.79^d±0.22
SEM	2.98	1.41	0.83	1.00
P-value	<0.001	<0.001	<0.001	<0.001

^{abcd}Superscript bearing means values within row differ significantly

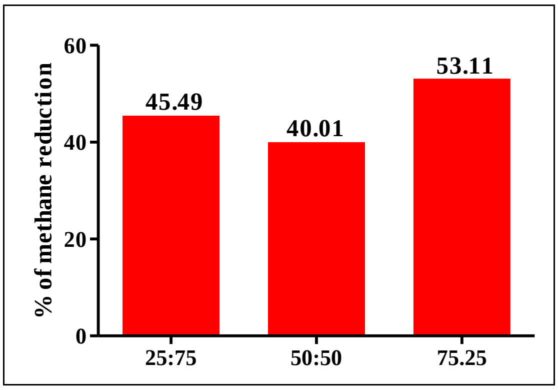

Fig 4.2: Percent inhibition in *in vitro* methane production by Karanj and mahua combinations.

In vitro evaluation of unconventional oil cake on the basis of rumen fermentation

The total gas production was found comparable in karanj and mahua cake inclusion as compared to castor and neem cake. Similarly mixture of karanj and mahua cake at 75:25 level gas production was similar as compared to control. These findings are in concurrence with Yatoo *et al.* (2014) [10] reported significant inhibition (P<0.001) in total gas and methane production by inclusion of eucalyptus oil or combination of three essential oils *i.e*, ajwain oil, garlic oil and cinnamon leaf oil in equal proportions using graded levels (0, 0.66, 1.33, 1.66 and 2.0µl/ml of incubation medium). Out of seven oils tested, there was a linear decrease *in vitro* gas production by the inclusion of six essential oils at graded levels. Kumar *et al.* (2011) [11] screened the extracts of different leaves and found either no change or decreased gas production by the inclusion of different extracts. Pawar *et al.* (2014) [12] conducted *in vitro* trial to study the effect of several essential oils at low to moderate dose levels and observed a significant reduction in gas and methane production. On the contrary to above results Arif *et al.* (2015) [13] who tested various plant parts and few oil cakes rich in different secondary metabolites like tannins, saponins, essential oils, flavonoids etc. There was no effect on *in vitro* gas production by the inclusion of any of the plant parts at the rate of 10% of the substrate. In his discussion he reported that *in vitro* gas production may decrease, increase or remained unaffected depending upon the type and quantity of plant secondary metabolites included in the system.

The methane production was found lower in the experimental group as compared to control. Similar results were obtained by Kamra *et al*. (2008) [14] who observed 93 plant extracts for their potential to inhibit *in vitro* methanogenesis and ciliate protozoa using buffalo rumen liquor and reported that 20 extracts abated CH_4 production by more than 25%, accompanied by a sharp decline in methanogen numbers. Some plant species have a more pronounced effect on fermentation in which are rich in saponins (*Sapindus mukorossi*), tannins (*Terminalia chebula*, *Populus deltoids*, *Mangifera indica* and *Psidium guajava*) or essential oils (*Syzygium aromaticum*, *A. sativum*). The unconventional oil cake contains a mixture of plant parts rich in tannins, saponins and essential oils which have anti-methanogenic activity by Patra *et al.* (2006) [15]. Agarwal *et al.* (2007) [16] observed a lower methane production on *in vitro* with water, ethanol and methanol extract of soapnut (*Sapindus mukorossi*) however the highest efficiency was reported in ethanol extract. Malaiyappan *et al.* (2012) [17] found that methane production was reduced by 40% after 24 hours incubation when wheat straw based diet was incubated with neem (*Azadiracta indica*) leaf extract (2ml/ 30 ml medium). Inamdar *et al.* (2015) [18] reported that de-oiled mahua seed cake (*Madhuka longifolia*) either alone or in combination with harad seed pulp (*Terminalia chebula*) was evaluated for its anti-methanogenic activity under *in vitro* as well as *in vivo* conditions. Chaturvedi *et al.* (2016) [19] reported that methane production (mg/g of substrate DM) was significantly (P<0.05) reduced by the inclusion of combinations of amla and neem.

The finding of the present investigation the IVTD (%) was found comparable in karanj and mahua cake inclusion as compared to castor and neem cake. Similarly karanj + mahua at 25:75 level the IVTD was similar as compared to control. It was in agreement with finding of Kumar *et al.* (2007) [20], who reported that true digestibility was highest with Soyabean cake (SBC) followed by Groundnut cake (GNC), Cotton seed cake (CSC), Mustard seed cake (MSC), Karanja seed cake expeller extracted (KCEE), Karanja seed cake solvent extracted (KCSE), Castor bean cake solvent extracted (CBCSE) and Castor bean cake expeller extracted (CECEE). Patra *et al.* (2005) [21, 22] observed *A. concinna* and *A. indica* have defaunating properties and these extracts also suppressed *in vitro* DM and *in vitro* OM digestibility. Pawar *et al.* (2014) [12] observed a significant linear reduction in IVOMD (%) of the substrate by the inclusion of six essential oils by gradually increasing the inclusion levels of essential oils but, garlic oil did not affect IVOMD of feed at the lowest level. Agarwal *et al.* (2009) [16] reported that the inclusion of peppermint oil at higher levels decreased IVTD was observed.

The ammonia nitrogen concentration was found lower in the experimental group as compared to control. These findings are in concurrence with Kumar *et al.* (2007) [20] who reported ammonia nitrogen level was positively correlated with the amount of protein present in the cake. Dey *et al.* (2006) [23] studied the effect of tropical tree leaves on the *in vitro* nitrogen degradability of Groundnut cake (GNC) and observed significant reduction in degradation with condensed tannin at the level of 1-2% irrespective of the source of tree leaves. Cardozo *et al.* (2004) [24] reported that cinnamon oil modified the N metabolism of rumen microorganisms by inhibiting peptidolysis. However, the higher doses of cinnamon oil and cinnamaldehyde decreased ammonia N concentrations and cinnamaldehyde was more effective as to cinnamon oil (Busquet *et al.* 2006) [25]. Gradual decrease in ammonia nitrogen level on the inclusion of increasing level of garlic oil. They also reported a decrease in ammonia nitrogen level inclusion of cinnamon oil at lower doses but by inclusion of clove oil at higher doses the level increased by Pawa *et al.* (2014) [12].

Conclusion

All four promising unconventional oil cakes (karanj, mahua, neem and castor bean) inclusion was found effective to reduce the *in vitro* methane production. Out of four unconventional oil cakes tested, karanj and mahua were found most effective in lowering the methane production without any effect on IVTD. Combination of karanj and mahua cake in the ratio of 75:25 was found most effective on lowering methane production as compared to other combinations.

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