IN VITRO GAS PRODUCTION CHARACTERISTICS OF THE EXTRACTS AND RESIDUES OF FOUR MULTIPURPOSE TREE SEEDS

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ABSTRACT

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The extracts from the seeds of four tropical multipurpose trees Leucaena leucocephala (LL), Pterocarpus santalinoides (PS), Treculia africana (TA) and Enteolobium cyclocarpum (EC) in two solvents (ethanol and methanol) and two levels (0.5ml and 1.0ml) were evaluated for their effect on rumen fermentation and in vitro gas production. In vitro gas production of the residues of the seeds after extraction of the secondary metabolites was carried out in a 24 –hour incubation. Gas production per 200 ml DM of substrate (guinea grass and concentrate mixture in ratio 3:2) was significantly higher ($p<0.05$) with ethanolic extracts of all the seeds at both levels except EC at 0.5 ml, than the control (i.e. substrate without plant extracts). Methanolic extracts of all the seeds had significantly ($p<0.05$) lower gas production due to the secondary metabolites (saponin, tannin and steroid) extracted from the seeds which enhance low gas production during fermentation than the control except EC and PS at 0.5ml. Gas production from the methanol residue of the seeds was significantly ($p<0.05$) higher than that of the ethanol residue. Low gas production prevents the accumulation of greenhouse gases in the atmosphere and also confirms absence of bloat ln the animals and efficient utilization of the feeds for production purposes thereby meeting farmers expected goals.

Keywords: *In vitro gas production, multipurpose trees extracts, rumen fermentation, solvents.*

INTRODUCTION

Tree and shrub leaves have the potential to alleviate some of the feed shortages and nutritional deficiencies being experienced during dry season by small holder farmers. However, the use of tree and shrub leaves for ruminant feeding should be done with caution because of the presence of deterring mechanisms related to their high tannin content (Provenza, 1995). Seed supplementation to low quality pastures and crop residue used as ruminant feed in the tropics is of particular interest as the seeds might be good sources of protein, minerals, vitamins and energy.

Tree legume seeds possessed an enhanced protein content and lower fat than the oilseeds (Ismartoyo, 2000), indicative of its suitability for ruminants. On the other hand, seeds might possess secondary metabolite such as saponin, phenols, steroids and alkaloids, which are substances the plant does not need for its growth, development and production although they are used as defense for the plant against insect invasion and predation. These compounds may induce beneficial as well as detrimental effects on ruminants. D'Mello (2000) reported the sensitivity of cattle and sheep to condensed tannins, which are freely present in leguminous forage seeds and sorghum. However, at moderate level, it resulted to nutritional gain in respect of increased bypass protein availability and bloat suppression in cattle. Recently, Hess *et a*l., (2003) established the depressive effect of a saponin rich tropical fruit (*Sapindus saponaria*) on methanogenesis in faunated and defaunated rumen fluid, being in favour of both animal (energy gain) and the environment (reduced greenhouse emission). This study was carried out to identify the ethanolic / methanolic exract characteristics of four multipurpose tree seeds.

MATERIALS AND METHODS

Pods of *Enterolobium cyclocarpum, Pterocarpus santalinoides* and *Leucaena leucocephala* were handpicked among those that fell down from their respective tree stands, as well as the fruit of *Treculia africana.* They were processed and thoroughly sundried, bagged and kept in the pasture and range management laboratory.

Qualitative determination of secondary metabolites

Saponin, phenols and steroids were determined as reported by Babayemi *et al*. (2004a). Two grams of ground seed was weighed into extraction bottle. Added into the different extraction bottles were 30ml petroleum ether (PE) and 25ml methanol water (MW, 9/1, v/v) and 30ml petroleum ether (PE) and 25 ml ethanol water (EW, 9/1, v/v). The mixtures were shaken at 180 revolutions per minute for 1.5hr, filtered and separated by funnels. The residues were rinsed several times with a mixture of petroleum ether and methanol/ethanol water as the case may be. Two layers were distinctly formed: the lower layer, being methanol/ethanol-water (polar fraction) and the upper layer being petroleum ether (non-polar fraction), were emptied into 50ml volumetric flasks. Each of the fractions in the flasks was indicated as the methanol water, ethanol water and petroleum ether from methanol/ethanol. The secondary metabolites of the seeds were determined quantitatively as described (Larrahondo, 1985) as modified (Babayemi *et al*., 2004a).

Saponin determination

From the MW/EW fraction, 1.67ml was dispensed into 9ml distilled water, from this, 1ml was taken into a test tube. The test tube was shaken for 30 seconds and left to stand for 15 minutes. Saponin content was evaluated from the height of the foam layer as negative (< 5mm), low (5-9mm), medium (10-14mm) and high (>15mm).

Phenol determination

1ml from the MW/EW fraction was dispensed into test tubes and 1% iron chloride ($FeCl_2$) was added into it at 0.2ml. Phenols form complexes with ferric iron, resulting in a blue solution and hence, their presence was scored as: no phenols (no colour change), hydrolysable (dark blue) and condensed tannins (dark green).

Steroids determination

10ml from the PE fraction was evaporated in a water bath at 45 °C and 0.5 ml chloroform, 0.25ml acetic anhydride and 0.125 ml conc. H_2SO_4 were added. The mixture was agitated briefly and the colour reaction was accessed as being steroids (blue or green), triterpenoids (red, pink or purple) or saturated steroids (light yellow).

***In vitro* gas production**

The rumen fluid used as inoculum in this trial was obtained from three west african dwarf does on the Teaching and Research farm University of Ibadan through suction tube before the morning feed. The animals were on a diet containing 40% concentrate feed (40% corn, 10% wheat offal, 10% palm kernel cake, 20% groundnut cake, 5% soybean meal, 10% dried brewers grain, 1% common salt, 3.75% oyster shell and 0.25% fish meal) and 60% guinea grass. Incubation was as reported (Menke and Steingass, 1988) using 120 ml calibrated syringes in three batches at 39 °C. To 200mg sample in the syringes was added 30ml inoculum containing cheese cloth strained rumen liquor and buffer (9.8g $NaHCO_3$ +2.77g Na_2HPO_4 + 0.57g KCl +0.47g NaCl + 0.12g $MgSO_4$. $7H_2O$ + 0.16 g per litre $CaCl_2$ O) (1:4) under continuous flushing with CO_2. The gas production was measured at 3, 6, 9, 12, 15, 18, 21 and 24 hour. After incubation the volume of the gas produced was plotted against the incubation time, rate and extent of gas production was determined for each samples by fitting gas production data to the non- linear equation $Y=b(1-e^{-ct})$ described by Qrskov and McDonald (1979), where Y = volume of gas produced at time 't', b = potential gas production (ml g^{-1} DM) and c = fractional rate of gas production. There were total number of nine samples subjected to in vitro fermentation which include: (a) the extracts of the four multipurpose tree seeds (LL, EC, PS and TA) (b) the residue of the four multipurpose tree seeds after extraction of the secondary metabolites and (c) the pure substrate which served as control.

Statistical analysis

Data obtained were subjected to one way analysis of variance (ANOVA) in a Completely Randomized Design (CRD) and the means were separated by Duncan multiple range test using Statistical Analysis System Package (SAS,1999). Probability was measured at 5% level of significance.

RESULTS

The qualitative contents of the secondary metabolites in the four multipurpose tree seeds were shown in Table 1. For saponin content, only *E.cyclocarpum* and *P. santalinoides* show negligible quantity while it was totally absent in *L. leucocephala* and *T. africana.* None of the four multipurpose tree seeds has phenols except *P. santalinoides* which has condensed tannins. Apart from *T. africana* which has triterpenoid, all the other three multipurpose tree seeds have steroid. Table 2 shows the *in vitro* gas production characteristics of the methanol and ethanol extraction residue. There was a significant ($p<0.05$) variation in the potential gas productioeachn (b $mlg^{-1}DM$) from the residue of the four multipurpose tree seeds. The values of b was highest ($p<0.05$) in *T. africana* (51.57 $mlg^{-1}DM$) for methanol residue and lowest in both *L. leucocephala* (42.57 $mlg^{-1}DM$) and *P. santalinoides* (41.54 $mlg^{-1}DM$). In ethanol residue *T. africana* and *P. santalinoides* had the highest ($p<0.05$) b values while the lowest value was recorded in *L. leucocephala*. The values of the fractional rate of gas production (c) also vary significantly ($p<0.05$) in the methanol and ethanol residues of the four multipurpose tree seeds. *E. cyclocarpum* (0.12) and *P. santalinoides* (0.14) had the highest c values from methanol residue while *L. leucocephala* (0.02) had the least value. In the ethanol residue *P. santalinoides* (0.12) had the highest c value while *L. leucocephala* (0.04) recorded the least value.

Table 3 shows the effect of solvents on the *in vitro* gas produced by the residue where comparison was made between the two solvents used to know which of them will be better in terms of extracting secondary metabolites as they can affect gas production. The table also compares the gas produced by the residues with that of the normal (unextracted) seeds. The result showed that the unextracted seeds of *L. leucocephala* and *P. santalinoides* have higher gas production (13.17 and 26.50 respectively) when compared with the gas produced from their methanol and ethanol residues (10.75 and 23.92) and (8.33 and 16.58) respectively which do not have secondary metabolites. The methanol residue of *E. cyclocarpum* and *T.africana* produced higher gas (25.79 and 25.50 respectively) upon incubation than their unextracted seeds (19.67 and 18.54) and ethanol residue (17.29 and 21.71) respectively.

The *in vitro* gas produced by incubating a supplement feed with different levels of plant extracts of each of the solvents was as presented in table 4. The volume of gas produced from the incubation of the extracts of the four multipurpose tree seeds vary significantly ($p<0.05$) from the control (i.e feed without plant extract) as shown in Figures 1-4.The gas produced by 1.0ml ethanolic extracts of the four multipurpose tree seeds were higher than the

control. The same result was obtained in the 0.5ml ethanolic extract of *T. africana, P. santalinoides* and *L. leucocephala*. Gas production by the control was significantly ($p<0.05$) lower than the gas produced by the 0.5ml methanolic extract of *E. cyclocarpum* and *P. santalinoides* but higher than the 0.5ml ethanolic extract of *E. cyclocarpum* and the methanolic extracts of *T. africana* and *L. leucocephala*. However, the gas produced by the methanolic extracts of the four multipurpose tree seeds were lower than the control. Hence, the result showed that for all the seeds, the 1.0ml ethanolic extracts had significantly higher ($p<0.05$) values of gas production than the 1.0ml of methanolic extracts while means were separated by Duncan multiple range test along the column

Table 1: Qualitative contents of secondary metabolites in four multipurpose tree seeds

Tree species	Saponin	Phenols	Steroids
Leucaena leucocephala	Absent	condensed tannin	steroid
Enterolobium cyclocarpum	Negligible	Absent	steroid
Pterocarpus santalinoides	Negligible	Absent	steroid
Treculia africana	Absent	Absent	Triterpenoid

Table 2: Gas production characteristics of the residues of four multipurpose tree seeds by methanol and ethanol

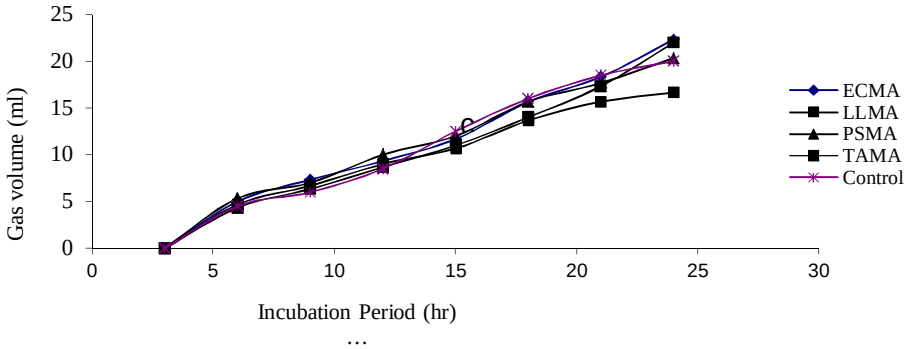
Tree species	B	C	Lag time	RSD
Methanol residue				
E. cyclocarpum	46.78b	0.12a	4.4	1.21
L. leucocephala	42.57c	0.02c	0	0.52
P. santalinoides	41.54c	0.14a	4.9	0.53
T. africana	51.57a	0.09b	4.3	1.03
Ethanol residue				
E. cyclocarpum	41.68b	0.07b	4.7	0.61
L. leucocephala	12.66c	0.04c	0	0.85
P. santalinoides	47.19a	0.12a	4.8	1.05
T.africana	50.50a	0.07b	4.6	0.88

a,b,c= means along the same column having different superscript are significantly different ($p<0.05$). B = potential gas production (ml 200mg^{-1} DM); C = fractional rate of gas production (mlh^{-1}). RSD = Residual Standard Deviation

Table 3: Effect of solvents on *in vitro* gas production of the residues

Tree species	Unextracted seeds	Methanol residue	Ethanol residue
L. leucocephala	13.17d	10.75c	8.33d
E. cyclocarpum	19.67b	25.79a	17.29b
P. santalinoides	26.50a	23.92ab	16.58bc
T.africana	18.54bc	25.50a	21.71a
SEM	1.79	2.13	2.05
Level of significance	*	*	*

a,b,c=Means with different superscript(s) along the same column are significantly different ($p<0.05$).



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DISCUSSION

There was reduction in gas production from the plant extracts when compared with the control, which did not have plant extracts. This could be due to the secondary metabolites contained in the extracts. Methanol extracts at higher dose had a reduction in gas production when compared with ethanol extracts. Methanol extracts agree with the findings of Patra *et al*. (2006) that there was 95% reduction in methane emission with the lower dose of the extract and the inhibition was almost complete at the double level of extract. The reduction in gas production from

the extracts of *L. leucocephala* might be due to the presence of condensed tannin in it. Phenolic acid has been found to decrease methane, acetate and propionate production (Ushida *et al.*, 1989 and Asiegbu *et al*., 1995).

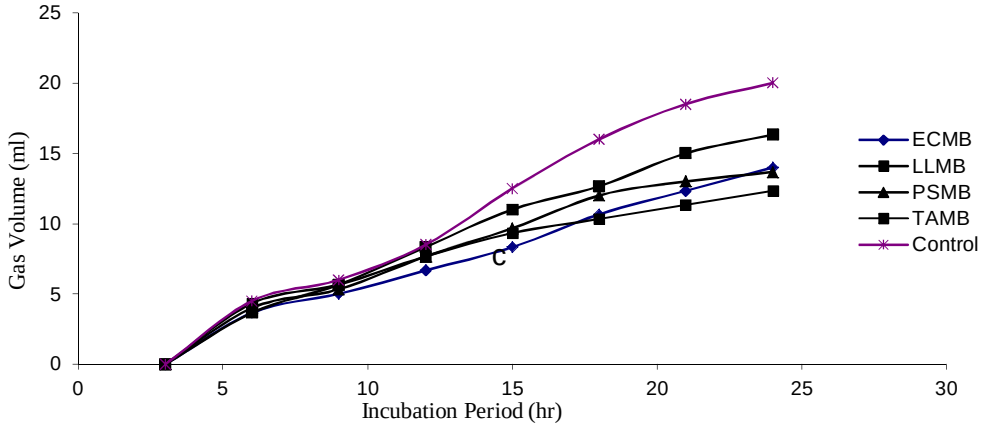

Fig 2. Effect of extract of four MPTS on the fermentation of a pure substrate…

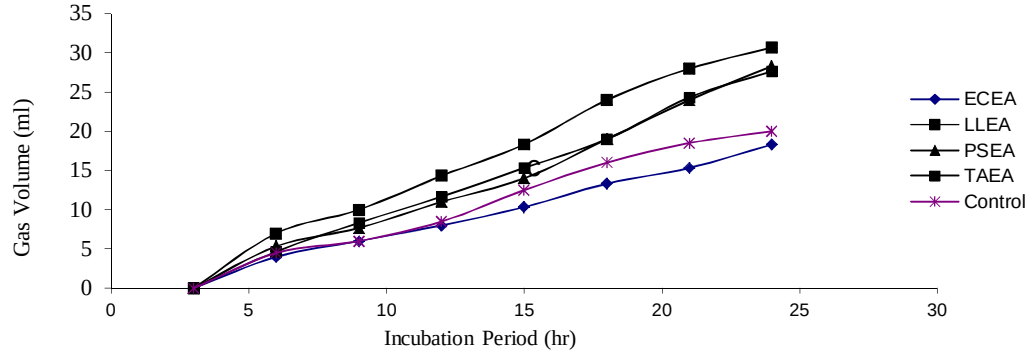

Fig.3. Effect of ethanol (0.5ml) extract of four MPTS on the fermentation of a pure substrate

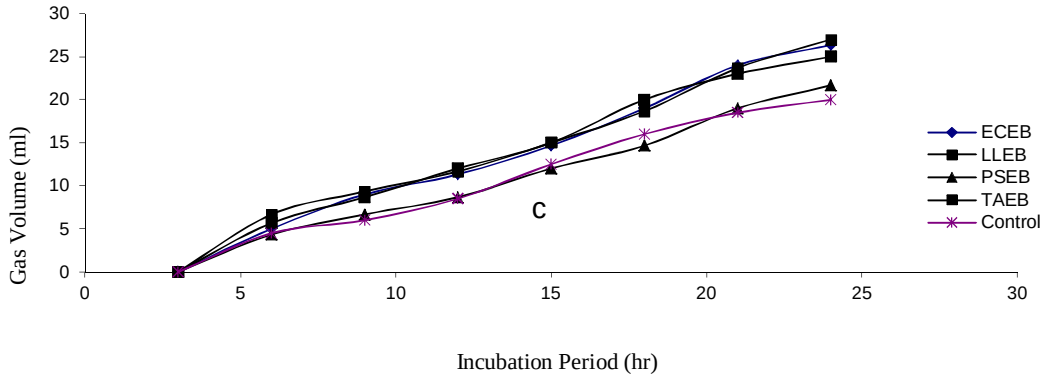

Fig 4. Effect of ethanol (1.0ml) extract of four MPTS on the fermentation of a pure substrate

The same reason could be traced to the reduced gas production from the extracts of Enterolobium and Pterocarpus at 1.0ml inclusion as they contain certain amount of saponin which could possibly have had inhibitory effect on the rumen protozoa. *Treculia africana* on the other hand contained triterpenoid which do not have any effect on protozoa. Chevallier (1996) carried out similar experiment on pods of *Acacia concinna* which was found to contain triterpenoids and steroid saponin. The inhibitory effect of the extracts of Acacia could be due to its saponin content. Decreased protozoan counts with supplementation of saponins rich extract (Hristov *et al.*, 1999 and Kamra *et al.*, 2000) or saponins rich forages (Newbold *et al.*, 1997 and Teferedegne *et al*., 2000 or fruits (Thalib *et al.*, 1998 and Hess *et al.*, 2003) have been reported The residue from methanol extracts had a higher gas

production than the residue from ethanol extracts. Higher gas production implies that there will be high level of acid production in the rumen and inefficient feed digestion. Higher gas production by the residue could be because methanol has higher potential to extract the secondary metabolites which could have helped in reducing gas production than ethanol. Low gas production ensures that much of the nutrients in the feed had been utilized by the animal for production purposes and also reduction in the emission of greenhouse gases into the atmosphere (Patra et al., 2006). This is confirmed in the lower gas produced from the extracts of methanol when added to a substrate (Patra *et al.*, 2006)

Table 4: Cumulative effect of levels of plant extracts on *in vitro* gas production

Tree species	Ethanol extract at 0.5ml	Ethanol extract at 1.0ml	Methanol extract at 0.5ml	Methanol extract at 1.0ml
L. leucocephala	13.88b	14.00a	9.63c	7.63c
E. cyclocarpum	9.42c	13.67a	11.21a	7.58c
P. santalinoides	13.67b	10.88b	11.13a	8.17ab
T. africana	16.54a	13.67a	10.21ab	9.08a
SEM	1.98	1.70	1.33	0.98
Control	9.71	9.71	9.71	9.71

a,b,c= means with different superscripts along the column are significantly ($p<0.05$) different. Note: SEM means Standard Error of Mean obtained from the four MPTs aside the control.

CONCLUSION AND RECCOMENDATION

Methanol and ethanol actually extracted the secondary metabolites in the multipurpose seeds (EC- saponin (<5mm) and steroid; PS-saponin (<5mm) and steroid; TA-triterpenoid and LL-tannin and saturated steroid), studied no matter how minute they are and this was confirmed in the lower gas production in the substrates that contained them when incubated. When low gases are produced from the fermentation of feedstuff, it implies that there will be low emission of greenhouse gases (CO_2 and CH_4) hence, the damage(s) caused by such gases will be minimized. It also shows that much of the nutrients in the feedstuff had been utilized by the animals for synthesis and muscle growth. Low gas production also indicated that there was low acid production and high rate of feed digestion and efficient utilization for production purposes to generate the desired or expected products by the farmer. However, it is recommended that further studies should be carried out to validate the result obtained in this research.

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