


Phytochemical and *in vitro* methane inhibition efficacy of plant extracts as influenced by extractive solvents

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Make today matter

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Outline

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- Objectives
- Material and methods
- Results
  - Effect of plant extracts on gas production
  - Chromatograph representation and tentative identification of metabolites
  - Correlation of methane with metabolites and how extractive solvent affects metabolite concentration
  - How does the metabolites associated with methane reduction and OMD?
- Conclusion and recommendations

Introduction

- Ruminants emit CH4 (a greenhouse gas with a global warming effect), and the bulk comes from enteric fermentation.
- Important to reduce enteric methane emission
- Dietary intervention (use of feed additives) is one among many possible options to reduce enteric methane emission. However, many countries banned the use of antibiotic additives that improve feed efficiency (e.g. Monensin) with co-benefits of reducing methane emission
- Many plant-based compounds tested as alternative solution
    - Many of them reduced enteric methane emission some with a co-benefit of improving digestibility of feed (Akanmu and Hassen, 2018)

Introduction...

- Since the plant extracts are crude, the efficacy is not consistent
- Efficacy varies based on the concentration and interaction of the bioactive metabolites involved and hence difficult to predict the level of response from crude product
- The net effect recorded is as a result of the pull-push effect of many of the bioactive compounds available in that particular product.
- It is important to identify those bioactive compounds associated with methane inhibition
    - We can use them as a marker for selection using breeding of the plants
    - Standardization of the crude plant extract to get consistent effect
- Extraction yield for the bioactive metabolites is also dependent on the solvent extract utilized

Objective

- To evaluate Phytochemical and *in vitro* methane inhibition of plant extracts as influenced by extractive solvents

Moringa oleifera ***Jatropha curcas*** ***Piper betle*** ***Aloe vera***




Material and methods

- Collection of plant materials (Dept of Agric. P0095290)
- Methanolic extraction at 70, 85 and 100%MeOH (Maceration method)
- Ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) analysis of bioactive molecules in plants' extracts- Data was reprocessed using MSDIAL and MSFINDER

Material and methods

- In vitro gas production (Menke et al., 1979; Adejoro & Hassen, 2018)
- In vitro organic matter digestibility (Akanmu and Hassen, 2018)
- Chemical and statistical analysis (correlation, PCAs, etc.)

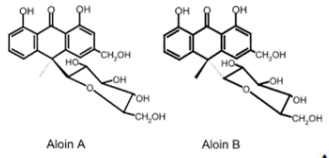
Effect of plant extract and extractive solvent on gas production characteristics

Extract (50mg/kg DM)	CH4 (mL/g DM)	TGP (mL/g DM)	CH4/TGP ($\times 10^{-3}$)	IVOMD (g/kg DM)	TGP/IVOMD (mL/kg DM)	CH4/IVOMD (mL/kg DM)
Aloe vera						
Control	4.24A	166.50B	25.64A	608.41	273.97	6.96A
Aloe vera 70%	3.97B	170.49A	23.39B	609.40	280.91	6.54B
Aloe vera 85%	3.92B	167.73AB	23.49B	608.43	275.44	6.44B
Aloe vera 100%	3.94B	167.72AB	23.68B	604.08	277.10	6.53B
SEM	0.05	1.10	0.41	10.39	3.82	0.12
P-value	0.01	0.04	0.01	0.97	0.35	0.04
Jatropha curcas						
Control	4.24A	166.50	25.64A	608.41	273.97	6.96A
Jatropha curcas 70%	3.79B	169.93	22.52B	616.17	275.20	6.15B
Jatropha curcas 85%	3.90B	172.30	22.78B	604.03	284.89	6.45B
Jatropha curcas 100%	3.75B	164.91	22.95B	608.37	271.01	6.16B
SEM	0.14	2.78	0.45	9.79	8.32	0.13
P-value	0.04	0.13	0.003	0.63	0.41	0.04
Moringa oleifera						
Control	4.24A	166.50	25.64A	608.41	273.97	6.96A
Moringa oleifera 70%	3.71B	165.46	22.69B	608.67	271.95	6.09B
Moringa oleifera 85%	3.71B	165.23	22.73B	613.33	269.55	6.07B
Moringa oleifera 100%	3.69B	165.65	22.55B	613.00	270.02	6.02B
SEM	0.08	2.42	0.38	6.40	2.41	0.12
P-value	0.002	0.95	0.001	0.87	0.35	0.001
Piper betel						
Control	4.24A	166.50	25.64A	608.41	273.97	6.96A
Piper betle 70%	3.97B	168.42	23.65B	614.72	274.19	6.47B
Piper betle 85%	4.00B	171.89	23.41B	609.29	282.45	6.57B
Piper betle 100%	3.91B	166.36	23.66B	613.97	270.92	6.37B
SEM	0.06	2.18	0.43	6.08	3.87	0.09
P-value	0.03	0.15	0.01	0.74	0.09	0.02

Chromatograph representation- *Aloe vera*

CH4-Re

Aa-Aloin A
Ab-Aloin B
Alos-Aloesin
AE-Aloe emodin
HA-Hydroxyaloin A
NA-Nataloin A



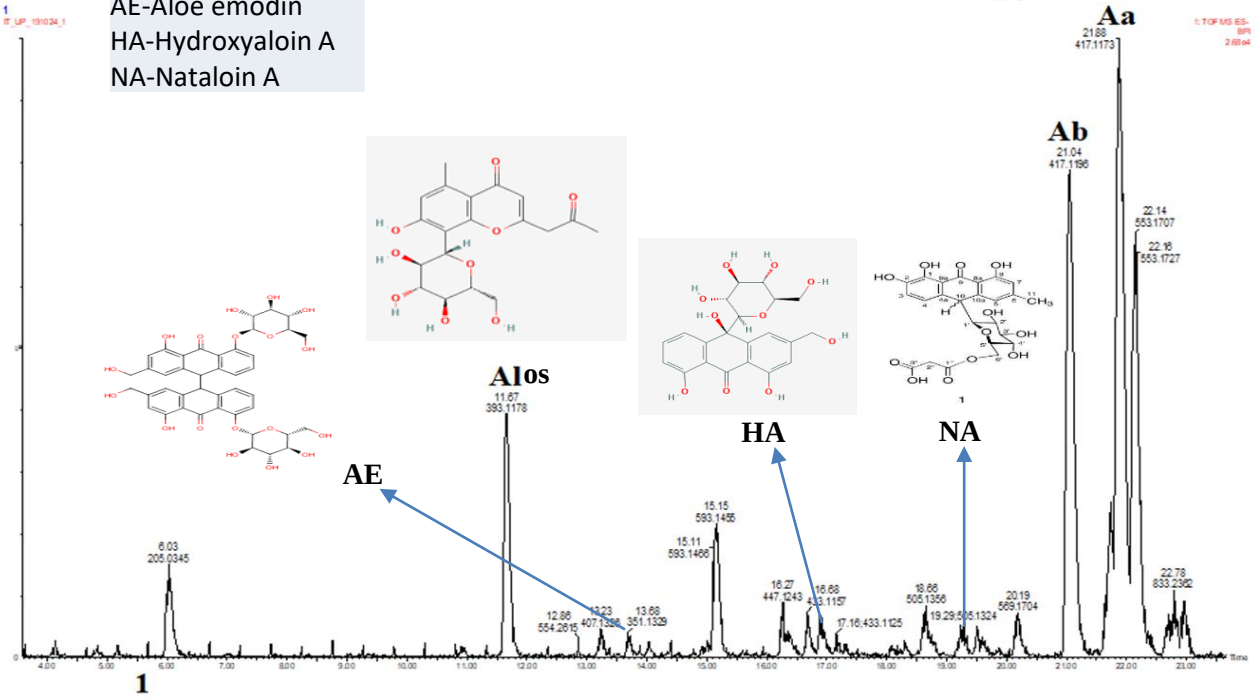


Table 1. Identification of metabolites in *Aloe vera*

RT (min)	Molecular formula	Measured mass(m/z)	UVmax (nm)	Error m/z (ppm)	MS Fragment	Tentative identification	Classification of the compounds
6.03	C7H9O7	205.0345	None	1.5	173,155,111	Homocitric acid	Acid compound
10.94	C16H17O8	337.0928	309	-0.3	273,245,202	Coumaryl quinic acid	Phenolic acid
11.64	C19H21O9	393.1185	296	-0.3	273245202	Aloesin	Chromone (C-glycosylated chromone)
14.02	C27H29O15	593.1526	332	-0.7	473,383,353	Aloe emodin-diglucoside	Anthrone (Anthracene compound)
15.15	C27H29O15	593.1455	269,335	-6.1	431,311,297, 283,282,269	Aloe emodin-diglucoside isomer (Apigenin diglucoside)	Anthrone (Anthracene compound)
16.25	C21H19O11	447.1304				Luteolin-8-C-glucoside (orientin)	Flavonoid (C-glycosylated flavoinoid)
16.66	C21H21O10	433.1138	304	1.6	313,270	10-hydroxyaloin B	Anthrone
16.89	C21H21O10	433.1133	305	0.9	313,270	10-hydroxyaloin A	Anthrone
18.61	C24H25O12	505.1343	264,301	-1.6	343,297,257	6-Malonylnataloin B (nataloin B)	Anthrone
19.24	C24H25O12	505.1355	264,301	-4.4	343,325,297, 257	6-Malonylnataloin A (nataloin A)	Anthrone
20.16	C29H29O12	569.1669	300	0.2	407,243,161	Caffeoyl ester of aloesin	Chromone
21.01	C21H21O9	417.1194	297,354	0.0	297	Aloin B	Anthrone (Anthracene compound)
21.84	C21H21O9	417.1176	297,354	-2.4	297	Aloin A	Anthrone (Anthracene compound)
22.68	C36H37O16	725.2085	297	0.4	297	Pelargonidin 3-(4-p-coumarylrutinoside)	Phenolic acid

Chromatograph representation- *Jatropha curcas*

CH4-Re

Ca-Catechin
ApiA-Apigenin
PDB2-Procyanidin
Vi-Vitexin
Ivi-Isovitexin

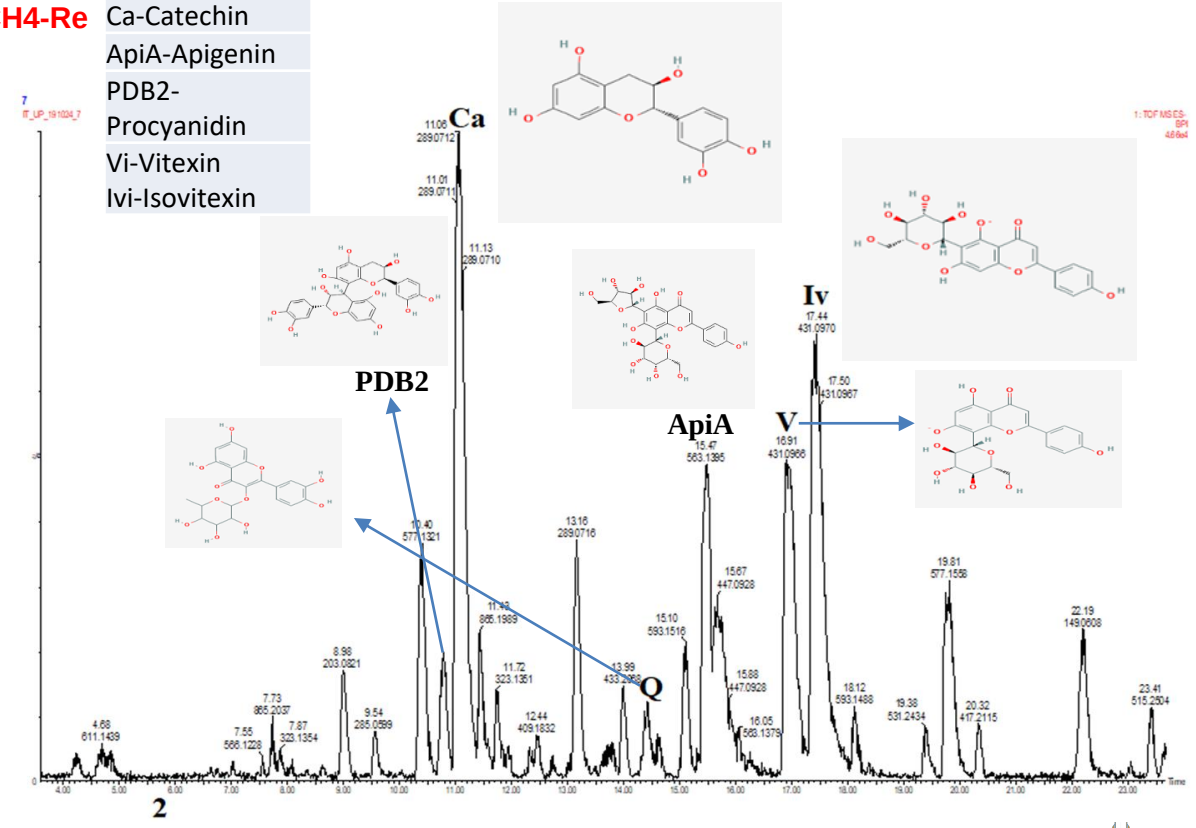


Table 2. Identification of metabolites in *Jatropha curcas*

RT (min)	Molecular formula	Measured mass (m/z)	UVmax (nm)	Error m/z (ppm)	MS Fragment	Tentative identification	Classification of the compounds
7.73	C45H37O18	865.2037	279	3.7	577,407,289,125	Procyanidin trimer C1	Flavonoid
8.98	C11H11N2O2	203.0821	279	-0.3	149	Tryptophan	Amino acid
10.4	C30H25O12	577.1321	279	-4.9	407,289,125	Procyanidin dimer B1	Flavonoid
10.78	C30H25O12	577.1322	279	-4.9	407,289,125	Procyanidin dimer B2	Flavonoid
11.06	C15H13O6	289.0712	279	-1	245,203,151,103	Catechin	Flavonoid
11.44	C45H37O18	865.1969	279	-1.3	577,407,289,125	Procyanidin trimer C2	Flavonoid
13.16	C15H13O6	289.0716	279	-1.4	245,203,151,103	Epicatechin	Flavonoid
15.47	C26H27O14	563.1395	271,335	-2.1	443,383,353	Apigenin-6-C-arabinosyl-8-C-hexoside	Flavonoid (C-glycosylated flavoinoid)
15.69	C21H19O11	447.093	269,349	0.7	357,327,300	Quercetin rhamnoside	Flavonoid
16.91	C21H19O10	431.0966	268,335	-1.9	341,311,283	Vitexin-7-olate	Flavonoid (C-glycosylated flavoinoid)
17.44	C21H19O10	431.097	271,335	-2.1	341,311,283	Isovitexin-7-olate	Flavonoid (C-glycosylated flavoinoid)
19.81	C27H29O14	577.1558	267,335	0.7	269	Apigenin-O-rutinoside	Flavonoid (O-glycosylated flavoinoid)

Chromatograph representation- *Moringa Oleifera*

CH4-Re

Q-Quercetin

K-Kaempferol

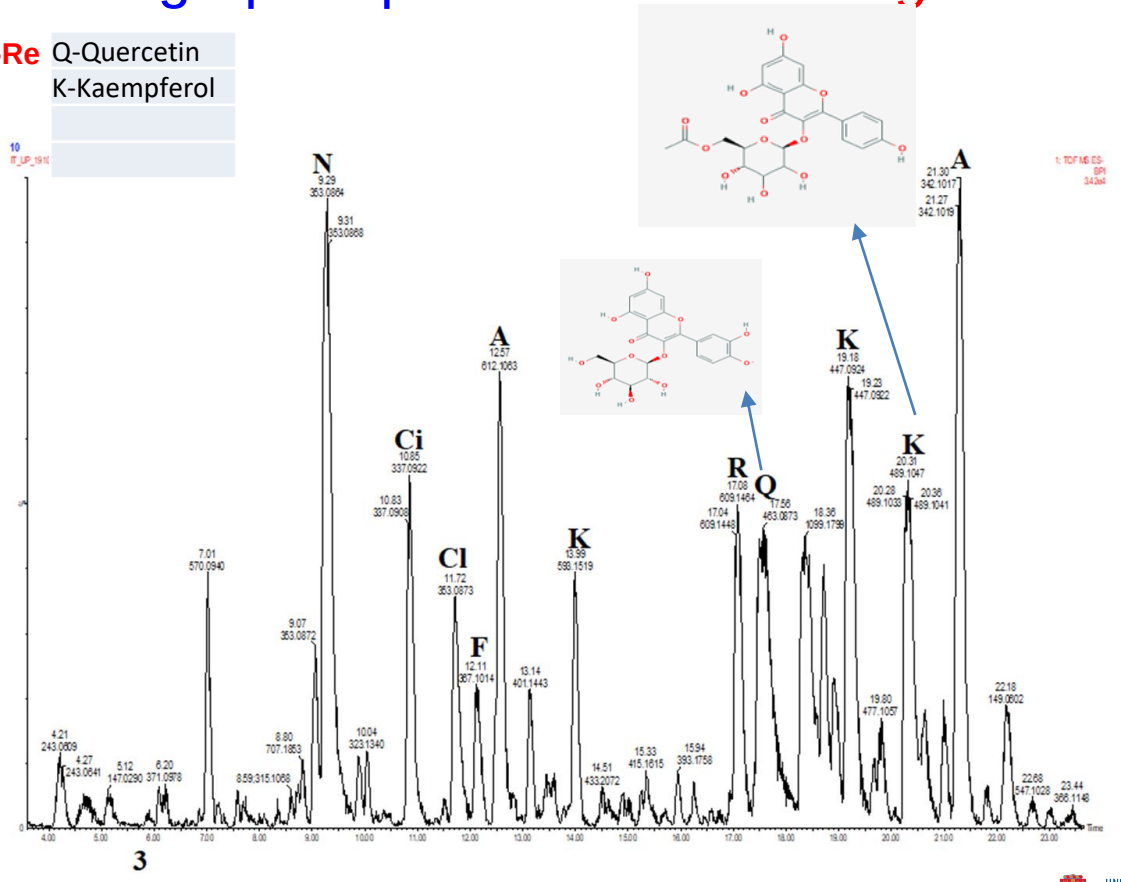


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Table 3. **Identification of metabolites in** Moringa oleifera

RT (min)	Molecular formula	Measured mass (m/z)	UVmax (nm)	Error m/z (ppm)	MS Fragment	Tentative identification	Classification of the compounds
9.29	C15H13O9	353.0864	325	-2.5	191,179,135	neochlorogenic acid	Phenolic acid
10.85	C16H17O8	337.0922	305	-0.3	191,173,163	cinnamoyl quinic acid	Phenolic acid
11.72	C16H19O9	353.088	325	-1.4	191	chlorogenic acid	Phenolic acid
12.11	C17H19O9	367.1014	323	-1.9	193,134	Feruloylquinic acid	Phenolic acid
12.57	$C_{39}H_{18}NO_7$	612.1063	344	-1	97	Alkaloid	Alkaloid
13.99	C27H29O15	593.1519	270,334	2.5	473,383,353,297	Kaempferol 3-O-rutinoside (isomer)	Flavonoid (O-glycosylated flavoinoid)
17.08	C27H29O16	609.1464	256,354	-0.3	300,271,255	Rutin	Flavonoid
17.56	C21H19O12	463.0873	351	-0.2	300,271,255	Quercetin -O-hexoside	Flavonoid (O-glycosylated flavoinoid)
18.36	C23H21O13	505.0983	354	0.2	300,271,255	Quercetin-O-acetyl-glucoside	Flavonoid (O-glycosylated flavoinoid)
18.71	C27H29O15	593.151	265,348	-1.9	285,271,255	Kaempferol 3-O-rutinoside	Flavonoid (O-glycosylated flavoinoid)
19.18	C21H19O11	447.0924	265,348	-0.7	285,255,227	Kaempferol -O-hexoside	Flavonoid (O-glycosylated flavoinoid)
20.31	C23H21O12	489.1047	265,348	3.5	285,255	Kaempferol O-acetyl-glucoside	Flavonoid (O-glycosylated flavoinoid)

Chromatograph representation- *Piper betel*

CH4-Re

Ci-Cinnamoylquinic acid
Cu-Coumaric acid
Di-Dihydrocaffeic acid
R-Rutin

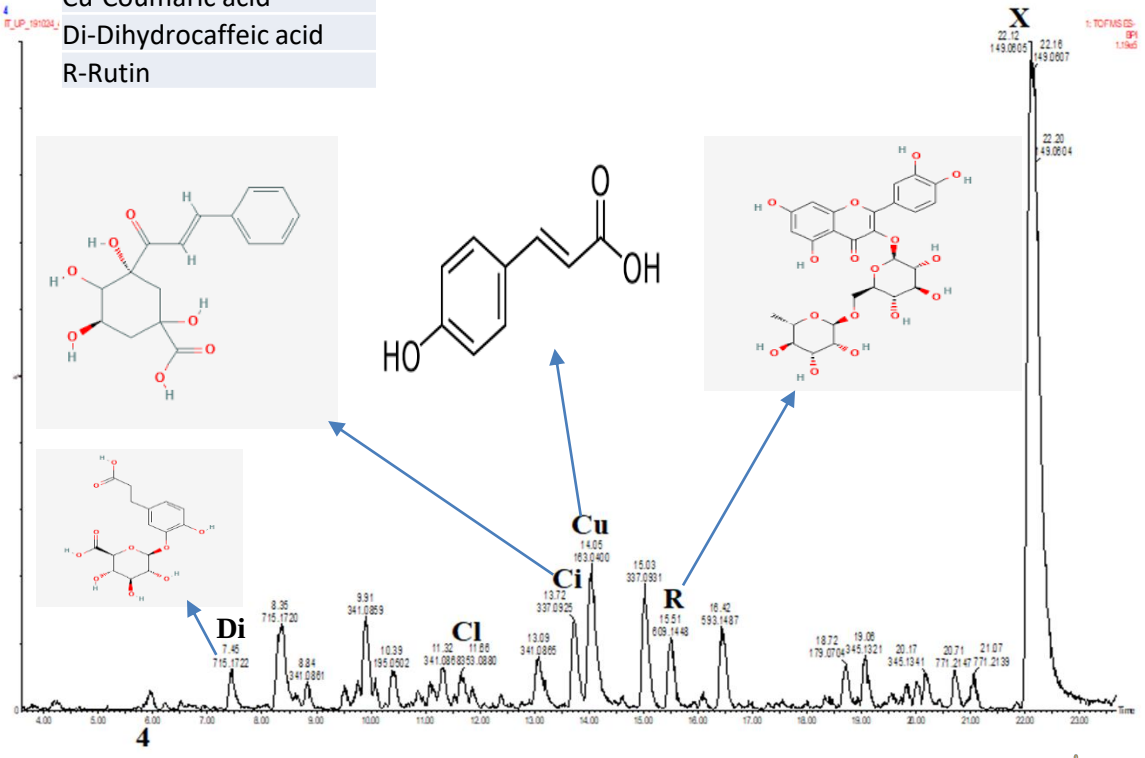


Table 4. Identification of metabolites in *Piper betel*

RT (min)	Molecular formula	Measured mass (m/z)	UVmax (nm)	Error m/z (ppm)	MS Fragment	Tentative identification	Classification of compounds
7.45	C15H17O10	357.0825	326	0.8	345,195	Dihydrocaffeic acid 3'-O-βD-glucuronide	Phenolic acid
8.35	C15H17O10	357.0829	325	2.0	195,129,75	Dihydrocaffeic acid 4'-O-βD-glucuronide	Phenolic acid
9.91	C15H17O9	341.0859	312	0.6	195,163,119	Dihydro-m-coumaric acid 3'-O-β-D-glucuronide	Phenolic acid
10.39	C15H17O9	341.0862	308	-3.2	195.163	Dihydro-p-coumaric acid 4'-O-β-D-glucuronide	Phenolic acid
11.32	C16H19O10	371.0971	313	-1.9	341,195,195	7,8-dihydroxy-6-methoxycoumarin hexoside (fraxetin hexoside)	Phenolic acid
11.66	C16H19O9	353.088	325	-1.4	191	chlorogenic acid	Phenolic acid
13.72	C16H17O8	337.0925	305	-0.3	191,173,163	cinnamoyl quinic acid	Phenolic acid
14.05	C9H7O3	163.04	339	-0.5	163	Coumaric acid	Phenolic acid
15.03	C16H17O8	337.0929	305	1.8	191,173,163	cinnamoyl quinic acid (isomer)	Phenolic acid
15.51	C27H29O16	609.1448	348	1.8	489,429,357, 327,309	Rutin	Flavonoid
16.42	C27H29O15	593.1487	268,334	-1.7	413,293	Apigenin diglucoside	Flavonoid (O-glycosylated flavoinoid)
22.12	$C_9H_{10}O_2$	149.0603				2-Methoxy-4-vinylphenol	Phenolic acid

Correlation of methane yield per unit CH4-Re with metabolites and effect of extractive solvent on metabolite concentration (mg /L)- *Aloe vera*

Metabolites	CH4-Re Correlation	% methanol in water-methanol solvent		
		70%	85%	100%
Aloin A (AA)	0.930*	163.05	310.38	303.57
Aloin B (AB)	0.910*	123.34	275.75	265.68
Aloe emodin (AE)	0.815	28.45	92.67	91.18
Aloesin (Alos)	0.771	59.51	281.46	293.30
Caffeoyl ester of aloesin (Ce)	0.698	3.14	43.32	44.84
cinnamoyl quinic acid (Ci)	0.769	7.65	8.42	2.40
Hydroxyaloin A (HA)	0.693	0.91	58.23	70.58
Hydroxyaloin B (HB)	0.654	1.45	60.38	74.93
Nataloin A (NA)	0.677	1.97	55.97	59.22
Nataloin B (NB)	0.678	3.04	77.87	83.30
Kaempferol (K)	0.901*	8.32	6.50	5.22
Total extract yield (g/10 g leaf)		3,11A	2,42B	2,35B

Correlation of methane yield per unit CH4-Re with metabolites and effect of extractive solvent on metabolite concentration (mg /L)- *Jatripha curcas*

Metabolites	CH4-Re Correlation	% methanol in water-methanol solvent		
		70%	85%	100%
Apigenin (Api)	0.960*	141.07	137.38	137.52
Catechin (Cat)	0.964*	292.48	298.98	312.01
Epicatechin (Epi)	0.935*	101.19	125.93	143.53
Kaempferol (K)	0.955*	7.89	6.31	6.37
Procyanidin dimer B1 (PDB1)	0.957*	96.38	104.34	109.56
Procyanidin dimer B2 (PDB2)	0.809	49.05	95.19	99.05
Procyanidin trimer C1 (PTC1)	0.716	10.54	30.67	34.64
Procyanidin trimer C2 (PTC2)	0.923*	56.84	72.59	74.58
Tryptophan (Try)	0.960*	43.36	46.64	49.83
Vitexin-7-olate (Vi)	0.960*	142.42	143.18	144.79
Isovitexi-7-olate (Ivi)	0.963*	188.89	187.05	190.77
Total extract yield (g/10 g leaf)		2,26A	2,06A	1,77B

Correlation of methane yield per unit CH4-Re with metabolites and effect of extractive solvent on metabolite concentration (mg /L)- *Moringa oleifera*

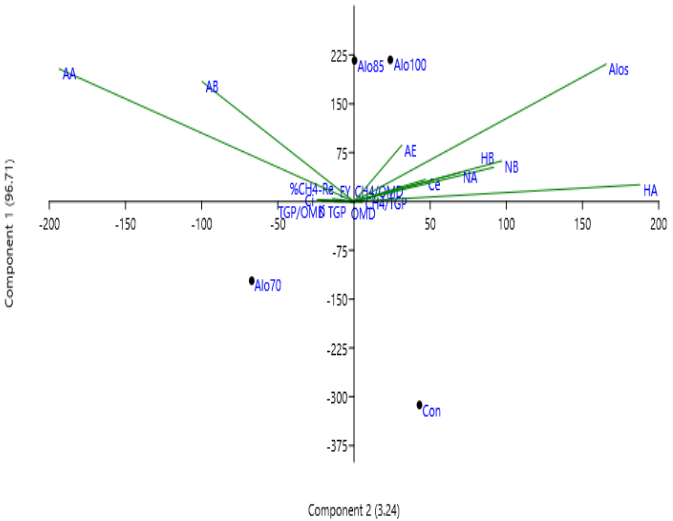
Metabolites	CH4-Re Correlation	% methanol in water-methanol solvent		
		70%	85%	100%
Alkaloids (A)	0.979*	142.73	265.40	272.63
chlorogenic acid (Cl)	0.793	30.44	47.73	19.94
cinnamoyl quinic acid (Ci)	0.998**	108.21	110.85	107.67
Feruloylquinic acid (F)	0.325	39.14	0	0
Kaempferol (K)	0.998**	108.11	115.14	108.24
neochlorogenic acid (N)	0.997**	203.93	205.09	198.07
Quercetin (Q)	0.998**	94.24	94.18	89.58
Rutin (R)	0.997**	102.18	105.07	101.61
Total extract yield (g/10 g leaf)		3,92	3,3.38	3,24

Correlation of methane yield per unit CH4-Re with metabolites and effect of extractive solvent on metabolite concentration (mg /L)- *Piper betel*

		% methanol in water-methanol solvent		
Metabolites	CH4-Re Correlation	70%	85%	100%
cinnamoyl quinic acid (Ci)	0.918*	128.56	129.21	105.34
Coumaric acid (Cu)	0.389	155.02	190.66	12.17
Dihydrocaffeic acid (Dca)	0.893	34.36	54.73	44.62
Dihydrocoumaric acid (Dcu)	0.892	98.09	101.57	88.52
Rutin (R)	0.954*	73.76	98.27	103.70
2-Methoxy-4-vinylphenol	0.972*	675.93	775.91	814.11
Total extract yield (g/10 g leaf)		3,53A	3,07A	2,42B

How does the metabolites associate with methane reduction & OMD?

Aloe vera

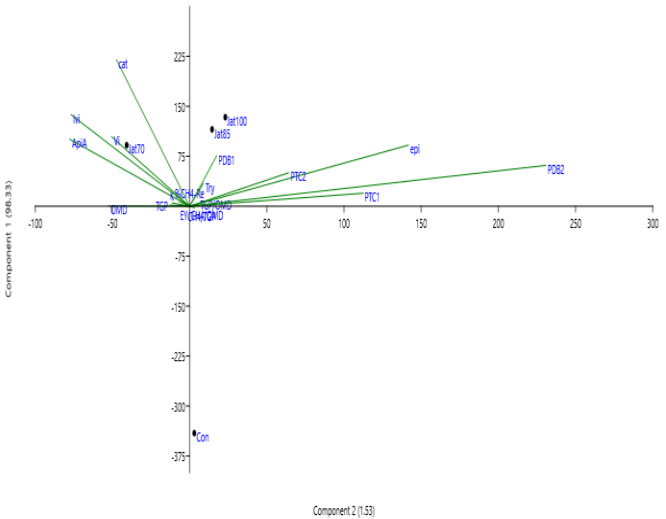


- Higher Aloin A (Aa), Aloin B (Ab)= Higher CH4-Re, Higher OMD
- Higher Aloesin (Aloe), Aloe emodin (AE) = Higher %CH-Re & lower OMD
- Higher Nataloin A (NA), Nataloin B (NB), Hydroxyaloin A (HA), Hydroxyaloin B (HB) = Lower OMD

Figure 1. Principal component analysis plot 1 vs plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with three different aqueous-methanol (70, 85 and 100%) extractions of *Aloe vera* leaf extracts.

How does the metabolites associate with methane reduction & OMD?

Jatropha curcas

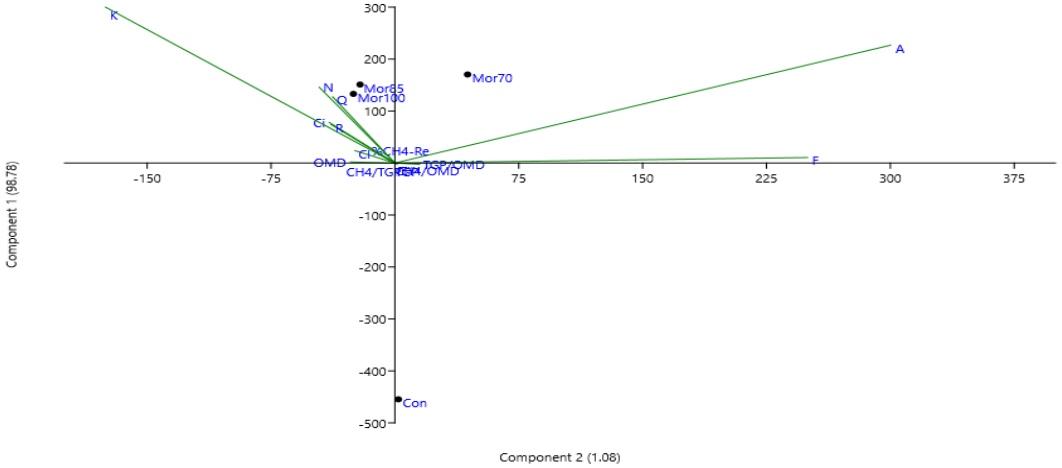


- Higher Catechin (Cat), Isovitexi-7-olate (Ivi), Apigenin (ApiA) and Vitexin-7-olate (Vi)= Higher CH4-Re
- Higher Catechin = Higher OMD
- Higher Procyanidin dimer B2 (PDB2), trimer C1 (PTC1), trimer C2 (PTC2)= Lower OMD

Figure 1. Principal component analysis plot 1 vs plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with three different aqueous-methanol (70, 85 and 100%) extractions of *Jatropha curcas* leaf extracts.

How does the metabolites associate with methane reduction & OMD?

Moringa oleifera

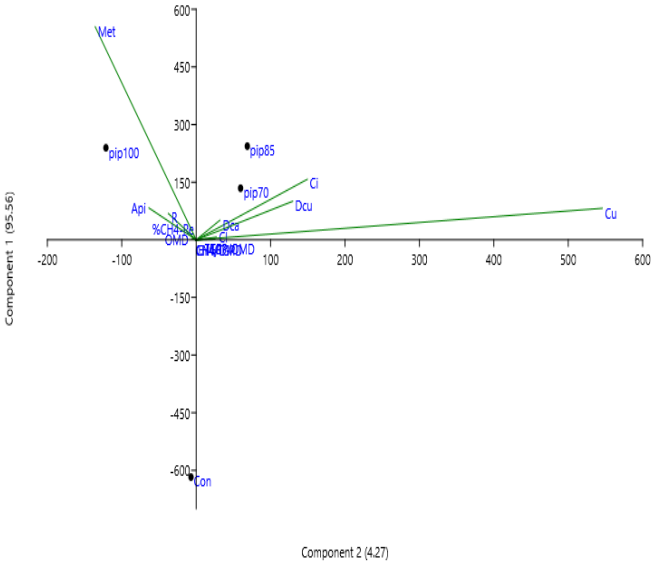


- Higher Alkaloids (A) = Higher %CH4-Re and lower OMD
- Higher Kaempferol (K), Rutin (R), Quercetin (Q), Chlorogenic acid (Cl), Neochlorogenic acid (N), Cinnamoylquinic acid (Ci)= Higher CH4-Re and higher OMD

Figure 1. Principal component analysis plot 1 vs plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with three different aqueous-methanol (70, 85 and 100%) extractions of *Moringa oleifera* leaf extracts.

How does the metabolites associate with methane reduction & OMD?

Piper betle



- Higher Methoxy-4-vinylphenol (Met), Rutin (R), Apigenin (Api)= Higher CH4-Re and higher OMD
- Higher Coumaric acid (Cu), Cinnamoyl quinic acid (Ci), Dihydrocaffeic acid (Dca), Dihydrocoumaric acid (Dcu) = Higher %CH4-Re and Lower OMD

Figure 1. Principal component analysis plot 1 vs plot 2 of all fermentation parameters of *Eragrostis curvula* hay fermented with three different aqueous-methanol (70, 85 and 100%) extractions of *Piper betle* leaf extracts.

Conclusions and recommendations

- The four plant leaves used in this study relatively contain more of water-soluble phytochemicals as their crude extracts' yields increased with an increase in the proportion of distilled water in the extraction solvents.

- Although, the net effect of plants' extracts reduced *in vitro* methane gas indices, the improved extract yield associated with increase in water proportion of the extract didn't translate into higher methane reducing potential or improvement in TGP and IVOMD.

Conclusions and recommendations

- In this study alkaloids, kaempferol, quercetin, aloin A, aloin B, aloesin, catechin, apigenin, vitexin-7-olate and isovitexin-7-olate most likely exhibited higher methane reducing potential than other metabolites and hence, additional studies need to be conducted using pure compounds of the individual metabolites and their cocktails to quantify their effect on:
  - Actual methane reducing effect
  - Digestibility of the feed
  - Rumen microbial population (methanogens, protozoa, etc.)
  - Animal performance, feed effeicency and quality of animal product

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