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Influence of the Flavonoic Contents of *Taraxacum officinale* and *Anacyclus clavatus* on Ruminal Methane Production in Batch System

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Abstract: Food degradation in the rumen is an anaerobic biological process in which organic matter is converted to organic acids (volatile fatty acids) and biogas (mainly composed of methane and carbon dioxide). The ruminal methane production represents loss of the animal energy and is a factor of global warming. Different processes have been deployed to minimize its production; these processes are chemical (antibiotics) or biological (defaunation). However, they are expensive and not allowed by the new European legalisation. Over the last decade more than 200, 000 structures of plant secondary metabolites (essential oils, flavonoids, saponins, etc.) have been exploited as feed additives to inhibit ruminal methanogenesis via inhibition of protozoal growth. The main objective of this study is to evaluate the ability of flavonoic contents, obtained from two plants belonging to the *Asteraceae* namely : *Taraxacum officinale and Anacyclus clavatus* in the reduction of ruminal methanogenesis and the impact of these molecules on the quantitative composition of protozoa to which the *archae* (methanobacteria) are mainly fixed. The inoculum consists of the total flora implanted in the rumen. The results indicated that the extracts of the two plants did not influence the gas production *in vivo* ($P > 0.05$). On the other hand, they lead to a significant reduction in the *in vitro* methane production ($P < 0.05$). For *Taraxacum officinale*, 250 and 500 µl extract reduced for the production of dichloromethane (40.9 and 63.3%) and n-butanol (27.2 and 50%), respectively. This reduction in methane production could be attributed to a change in the metabolic pathways. Both 250 and 500 µl of *Anacyclus clavatus* extract not only reduced the production of n-butanol extract by 60 and 68% but also decreased the number of protozoa.

Key words: Taraxacum officinale · Anacyclus clavatus · Flavonoids · Methane · Rumen · Protozoa

INTRODUCTION

Polyphenols are molecules synthesized by plants; they belong to their secondary metabolism. They participate in the plants defense against environmental aggressions. These are phytomicronutrients and are, generally, pigments responsible for the autumn colors of the leaves and the colors of the flowers and fruits. They contain about 8000 compounds which are divided into several groups: phenolic acids, flavonoids, tannins obtained by flavonoid polymerization and lignins. They are found everywhere in the roots, stems, flowers and leaves of all plants [1]. Their nature and concentration are highly variable [2]. They are renowned for their powerful antioxidant power. In fact, they have the ability to trap free radicals such as the superoxide (O_2^-), the perhydroxy group (H_2O^-), hydrogen peroxide (H_2O_2), the hydroxyl group (OH) -) and the peroxide group (ROO) [3].

Ruminants are polygastric animals, the only ones able to use the energy stored in plants (cellulose). They depend, for this, on a microbiota implanted in the rumen. The latter is an open biotope where the environmental

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conditions promote the development of an extremely large and diversified anaerobic microflora. The microbial communities that populate this biotope belong to the three living domains: *Archaea*, *Bacteria* and *Eucarya*. The representatives of the viral world are also present [4]. Anaerobic digestion of substrates is a biological process that takes place in the absence of oxygen and during which the organic matter is converted into organic acids (volatile fatty acids) and biogas (mainly composed of methane and carbon dioxide). The production of methane represents an energetic loss and an environmental pollution factor, given its enormous radiative power [5].

Different processes have been deployed to minimize its ruminal production; these methodes are in general chemical (antibiotics) or biological (defaunation and acetogenesis). However, these techniques are expensive and not allowed by the new European legislation [6]. Thus, the use of other substitutes is essential. In this context, our study aimed to investigate the ability of phenolic compounds, in particular the flavonoids of *Asteraceae* family, to reduce ruminal methanogenesis and the impact of these molecules on the quantitative composition of rumen fauna to which the *archae* (methanogenic bacteria) are mainly fixed.

MATERIALS AND METHODS

Vegetable Material: The study involved two plants belonging to the family *Asteraceae*. They were collected from Tlidjene province, situated in the chief place of the state of Tebessa. They were collected during the spring season. The botanical identification of the plants was carried out in the laboratory of plant biology, University of Tebessa, according to the recommendations of Quezel and Santa [7]. The selected plants included *Taraxacum officinale* and *Anacyclus clavatus* which are commonly called "Telma" and "Boumlala" by the local population.

Phytochemical Analysis

Flavonoids Extraction: After drying, the plants have been crushed into calibrated pieces of 1 mm. Then, the crushed material (200 g) has been macerated in ethanol / water (30/70: v / v) for 72 hours with solvent renewal every 24 hours and alternated agitation. The macerates have been pooled and filtered on Wattman paper No. 1. The filtrates have been evaporated to near dryness using a rotary evaporator. The dry residue has been suspended in 100 ml of boiled distilled water. After decantation (one night), the solution has been filtered three times on filter paper (Wattman paper No. 1). The solution thus obtained has been putted in three solvent solutions: dichloromethane, ethyl acetate and n-butanol, into decanting bulbs. The aqueous phase and the solvent are mixed and vigorously stirred, then left to rest for 30 min. The aqueous phase which is at the bottom of the bulb and the phase charged of specific molecules have been separately recovered. The various recovered phases (dichloromethane, ethyl acetate and n-butanol) were allowed to evaporate to dryness, then suspended in 10 ml of methanol which concentrations of methyl alcohol???, the extracts obtained are stored in dark flasks, at room temperature.

Analytical Thin-Layer Chromatography: The qualitative analysis of the extracts was carried out by adsorption chromatography (T.L.C.). It had been performed on aluminum plaques with silica gel. The mobile phase is constituted by a mixture of (ethyl acetate / methanol / water) in the proportions 100: 13.5: 10, v / v / v. The deposit (10 µl) of the extracts has performed using micropipettes. The spots were revealed using aluminum chloride (Al_2Cl_3, 1%). After drying, the chromatogram could be directly observed. The flavonoic samples were colored in yellow. The the frontal ratio (FR) was used to identifiy the flavonoids [8]: polyhydroxyflavones have low values of FR (0.00-0.25 cm), oligohydroxy and oligomethoxyflavones have values of FR ranging between (0.3-0.5 cm) and flavanones, flavonols, methoxyflavones have the highest FR values ??(0.5-0.75 cm)

Spectrophotometric Dosage

Determination of Phenolic Compounds: The content of the phenolic compounds of our extracts was estimated by the Folin-Ciocalteu method, using the following reaction medium: 10 µl of extract + 990 µl of distilled water, 500 µl of Folin-Ciocalteu reagent (1 N), 2.5 ml of Na_2CO_3 (2%). The mixture was vigorously stirred and then incubated for 2 h in the dark at room temperature. The absorbance has been checked-in at 765 nm. The concentration of the total phenols in the extracts was expressed in terms of tannic acid equivalent (y = 0.01482 x [tannic acid], R2 = 0.8985).

Dosage of Flavonoids: 0.5 ml of an ethanolic solution of Al_2Cl_3 (2%) was added to 0.5 ml of the plant extract. After 30 minutes of incubation at room temperature, the absorbance of the mixture is checked-in at 420 nm. The concentration of flavonoids was expressed in quercetin equivalent (y = 0.0939 x [quercetin], R2 = 0.895).

Evaluation of the Biological Activity of the Extracts

***In vitro* Fermentation:** The fermentation was carried out into 60 ml polypropylene syringes. Two hundred milligrams (200mg) of alfalfa hay was introduced into each syringe and fermented with 30 ml of the mixture (10 ml of rumen juice + 20 ml of artificial saliva) [9]. Two syringes had beenincubated with 250 µl and 500 µl of each extract. Under the same conditions, two syringes contained neither the substrate nor the additives (rumen + artificial saliva juice; white) and another two control syringes contained only the substrate had been incubated.

Rumen juice was taken from sheep sacrificed in slaughterhouse situated in the state of "Tebessa". It has been recovered immediately after slaughter and evisceration. It has been collected in thermos previously heated to 39°C and saturated with CO_2. In the laboratory, the contents of the rumen were filtered through 4 layers of surgical gauze. The inoculum was obtained by mixing rumen juice and artificial saliva in 1: 2 (v / v) proportions.

The inoculated syringes were horizontally incubated in a rotating incubator with 9 rotation/min at 39°C. for 24 hours. The monitoring of the fermentation kinetics was carried out by measuring the total volume of the gases produced at 2, 4, 6, 8 and 24 hours.

The production of methane was determined according to the protocol described by Jouany [10]; a volume of 4 ml of alkaline solution of sodium hydroxide (NaOH, 10N) was injected in a sealed manner into each syringe. Sodium hydroxide reacts with CO_2 to produce sodium carbonate (blackish color) pushing the piston back. The production of methane gas was determined by subtracting the volume of CO_2 from the volume of total gas produced.

Protozoa enumeration: after 24 hours of fermentation, 100 µl of the contents of each syringe was mixed with 100 µl of the MFS solution. This mixture was homogenized and kept in the dark for 30 minutes. The enumeration was carried out on a cell of Malassez. The counting was then carried out under a microscope using the (x40) objective. The number of protozoa was expressed according to the following formula: $N= n_1 x\ v\ x\ n_2 x\ f$ with: N: number of cells per ml, n_1: number of cells counted, v: volume of a rectangle in ml, n_2: number of counting rectangles And F: dilution factor.

Statistical Analysis: *In vitro* gas production data were subjected to an analysis of variance (ANOVA) with four (04) factors (plant, extract, dose and incubation time) using the "Statitcf" computer software. The classification of the averages is done according to the Newman-Keuls test ($P < 0.05$).

RESULTS AND DISCUSSION

Chemical Characterization: From table (1), dry matter (DM) and mineral content of *Taraxacum officinale* and *Anacyclus clavatus* are statistically comparable ($P > 0.05$). The low dry matter content (65 g / kg) and the high water content refer to the deep root system which allows them to draw water in the depth. In addition, these plants are classified among Mediterranean plants growing in water-rich areas. Similarly, this result can be explained by the fact that these plants were collected during their flowering period.

Both plants have high concentrations of mineral matter, with 387.5 g / kg and 238.0 g / kg for *Anacyclus clavatus* and *Taraxacum officinale*, respectively. Several factors such as soil type, climate, stage of maturity and season contribute to a notable variation in the concentration of mineral elements in forages [11, 12]. Likewise, contamination by silica could also explain this high percentage of mineral matter [13].

The total phenol content of the plants has been determined in different solvent systems. All the solvents used in this study allowed to solubilize a portion of the phenolic compounds (Table 2). *Taraxacum officinale* had high phenolic compounds (6.31 mg / ml) compared to *Anacyclus clavatus* (3.29 mg / ml). Regarding the solvent systems used, ethyl acetat seems to be the best for the extraction of total phenols (Table 2). This result is in perfect agreement with other studies that have tested the capacity of various extraction solvents [14]. The classification of the solvent systems according to the total solubilized phenol content is: ethyl acetate> dichloromethane = n-butanol. The concentration of flavonoids (Table 2), tested with colorimetric method, using the aluminum chloride of *Taraxacum officinale* was significantly ($P < 0.05$) high (2.63 mg / ml) compared those of *Anacyclus clavatus* (1.14 mg / ml). However, it is surprising to find that dichloromethane allows the solubilization of a higher proportion of flavonoids, compared to ethyl acetate (the solvent commonly used for this type of extraction).

Chromatographic Analysis: For the detection and characterization of the flavonoic contents of the extracts obtained in the various phases, thin layer chromatography has been applied. The stripping solvent consists of three moderately polar solvents. The spots were revealed by spraying with aluminum chloride (Al_2Cl_3). The qualitative analysis was carried out by calculating of the frontal ratio (FR).

Table 1: Dry (DM), organic (OM) and mineral (MM) content (g/ kg) of *Taraxacum officinale* and *Anacyclus clavatus*

	DM	OM	MM
Taraxacum officinale	65	61, 9	23, 8
Anacyclus clavatus	65	612, 5	387, 5
S.E.M.	0	74, 4	74, 4
Pr.	> 0.05	< 0.05	< 0.05

Table 2: Total Phenols and flavonoids concentrations (mg/ml) of *Taraxacum officinale* and *Anacyclus clavatus* extracts in various solvent systems

	Total Phenols			Total Flavonoids		
	Dichloromethane	Diethyl acetate	N-butanol	Dichloromethane	Diethyl acetate	N-butanol
Taraxacum officinale	4.10	10.1	4.74	3.82	1.21	2.88
Anacyclus clavatus	2.70	4.54	2.63	1.60	1.27	0.55
S.E.M.	0.98	3.93	1.49	1.56	0.04	1.64
Pr.	> 0.05	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05

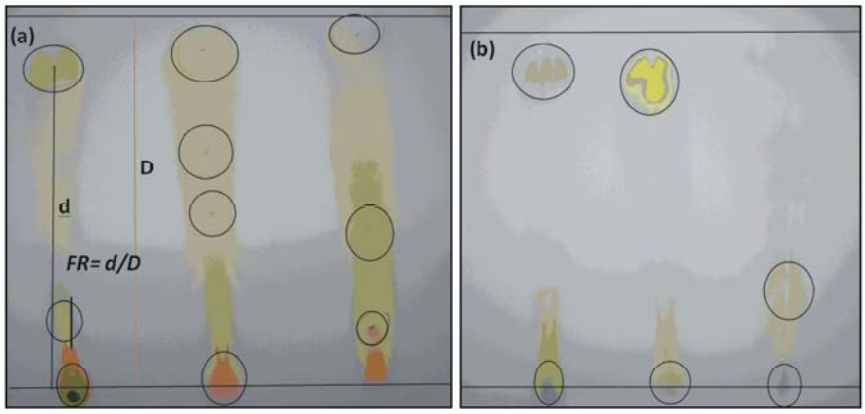

Fig. 1: Chromatographic profile of *Taraxacum officinale* (a) and *Anacyclus clavatus* (b) extracts in the mobile phase of ethyl acetate / methanol / H_2O (100: 13.5: 10; v / v / v)

The results of the chromatographic migration of the extracts (Figure 1) and the qualitative identification according to the FR of the components of each extract is illustrated in Tables 3 and 4. . On this basis, the flavonoid contents of each extract and each plant are represented in Tables 3 and 4.

The revelation by aluminum chloride, which is a specific colorimetric reaction of flavonoids, allowed us to confirm our assay by spectrophotometry. Indeed, the different solvent systems solubilize the flavonoids (yellow spots, Figure 1). For the identification of the spots structure (Figure 1), the calculated frontal ratios (FR) were compared with those of Lahouel [8] under the same conditions (Tables 3, 4).

Gas Production: From Figure (2), The production of gas from the alfalfa hay with the extracts of *Taraxacum officinale* and *Anacyclus clavatus* has no influence on the gas production, except the ethyl acetate extract, where the gas production of increased significantly (P <0.05). This result can be explained by the fact that the ethyl acetate extract contains factors that stimulate the microbial activity. Indeed, ethyl acetate is a polar solvent which mainly allows the solubilization of the glycosylated flavonoids. Furthermore, some flavonoic structures have been shown to activate the membrane transport system.

The results of gas production resulting from the *in vitro* fermentation of *Taraxacum officinale* and *Anacyclus clavatus* extracts, administered at different doses were presented in figure 2. They indicate that for *Taraxacum officinale* the administration of 250 µl of the Dichloromethane, n-butanol and ethyl acetate extracts induces a slight increase in gas production.

This difference is, still, statistically comparable (P> 0.05). On the other hand, no improvement in gas production has been noted for the various extracts of *Anacyclus clavatus* added at the same dose (250 µl). Moreover, the batch system of 500 µl, did not influence the production of gas (P> 0.05). However, it should be noted that the ethyl acetate extract generated high gas production, whereas that of n-butanol reduced the gas production, compared to the control (Figure 3).

Table 3: Relationship between the colored spots of *Taraxacum officinale* and the structure of the flavonoids

Phases	Colore	FR	Probable types of flavonoids
Dichloromethane	*deposit= brown	0	/
	* green- yellow	0, 15	* Free 5 OH or substituted 5 OH
	*green	0, 75	*(chlorophyll)
Ethyle acetate	* deposit= clear brown	0	/
	* clear violet	0, 41	*Isoflavone, flavanones, flavone 5 OH and 4 OH
	*tan	0, 55	* absent 3 OH ou substituted 3 OH
	* green- yellow	0, 79	* Free 5 OH or substituted 5 OH
n-butanol	* deposit= clearer brown	0	/
	*orange	0, 13	*Free Flavonol 3-OH with or without free 5 OH.
	*tan	0, 37	* absent 3 OH ou substituted 3 OH
	* bright green-yellow	0, 83	* Free 5 OH or substituted 5 OH

Table 4: Relationship between the colored spots of *Anacyclus clavatus* and the structure of the flavonoids

Phases	Colores	FR	Probable types of flavonoids
Dichloromethane	*deposit= brown	0	/
	*green	0, 75	*(chlorophyll)
Ethyl acetate	* deposit= clear brown	0	/
	*yellow	0, 74	* Free Flavonol 3-OH with or without free 5 OH.
n-butanol	* deposit= pale brown	0	/
	*orange	0, 25	* Free Flavonol 3-OH with or without free 5 OH.

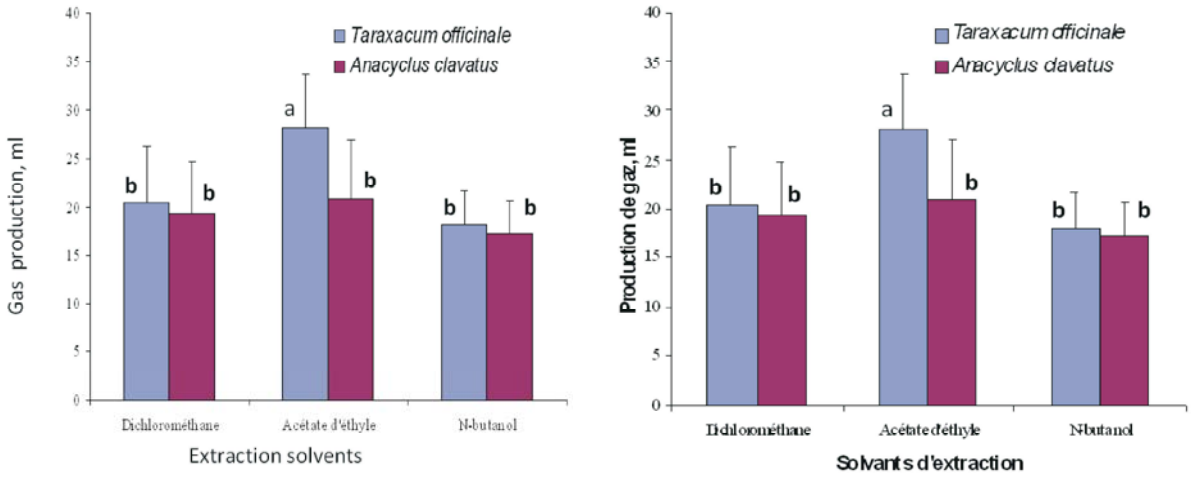

Fig. 2: Mean Gas production resulting from the *in vitro* fermentation of *Taraxacum officinale* and *Anacyclus clavatus* extracts, indicating a significant difference at P <0.05

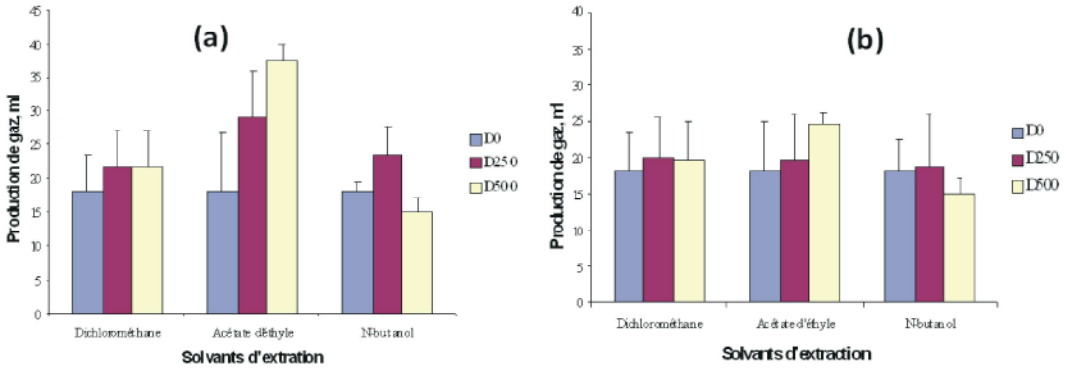

Fig. 3: Gas production resulting from *in vitro* fermentation of *Taraxacum officinale* (a) and *Anacyclus clavatus* (b) extracts, administered at different doses in batch systems with error bars

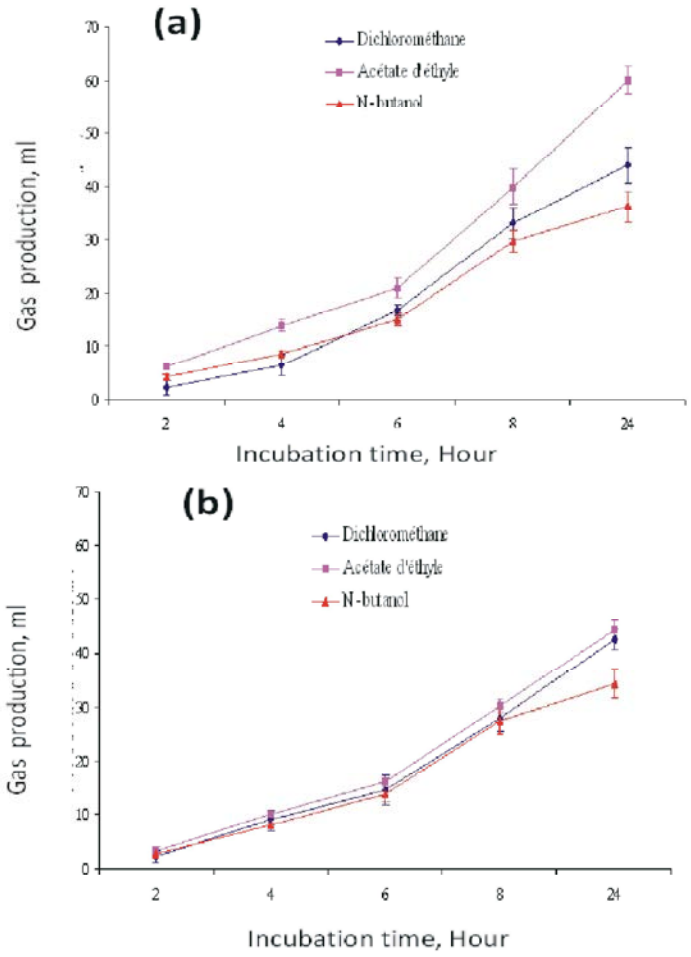

Fig. 4: Kinetics of *in vitro* gas production of *Taraxacum officinale* (a) and *Anacyclus clavatus* (b) extracts of, obtained in various extraction systems

Kinetics of Gas Production: The extracts resulting from the maceration of *Anacyclus clavatus* of dichloromethane, ethyl acetate and n-butanol (Figure 4) had the same gas production profile (P> 0.05). The same trend is noted for *Taraxacum officinale*, except for the ethyl acetate extract where the gas production is high at different incubation times. This increase could be due to the richness of these extracts in soluble sugars that act as a source of carbon by the microorganisms in the rumen.

Regarding *Taraxacum* officinale gas production from alfalfa hay supplemented with dichloromethane, ethyl acetate and n-butanol extracts introduced in two doses 250 µl and 500 µl in batch systems (Figure 5) was greater than that of the control beyond 6 hours of incubation. This difference is statistically comparable (P> 0.05). However, in the case of *Anacyclus clavatus* and for both doses, the stimulatory effect of the dichloromethane extract is more pronounced beyond 8 h of incubation. It should also be noted that this difference is statistically identical (P> 0.05).

The ethyl acetate extract for the two doses introduced into the syringes generated an increase in gas production at different incubation times for both *Taraxacum officinale* and *Anacyclus clavatus*; for the latter, the effect due to the 250 µl dose was more remarkable after 8 hours of fermentation. Here again, it should be noted that the differences are significantly comparable (P> 0.05). A different trend was observed for the n-butanol extract where the 250 µl dose induced an increase in gas production, compared to the control. Whilst 500 µl of the two plants decreased the production of gas for different incubation times. The fact that the majority of the extracts increased the gas production suggests that either they allow solubilization of energy

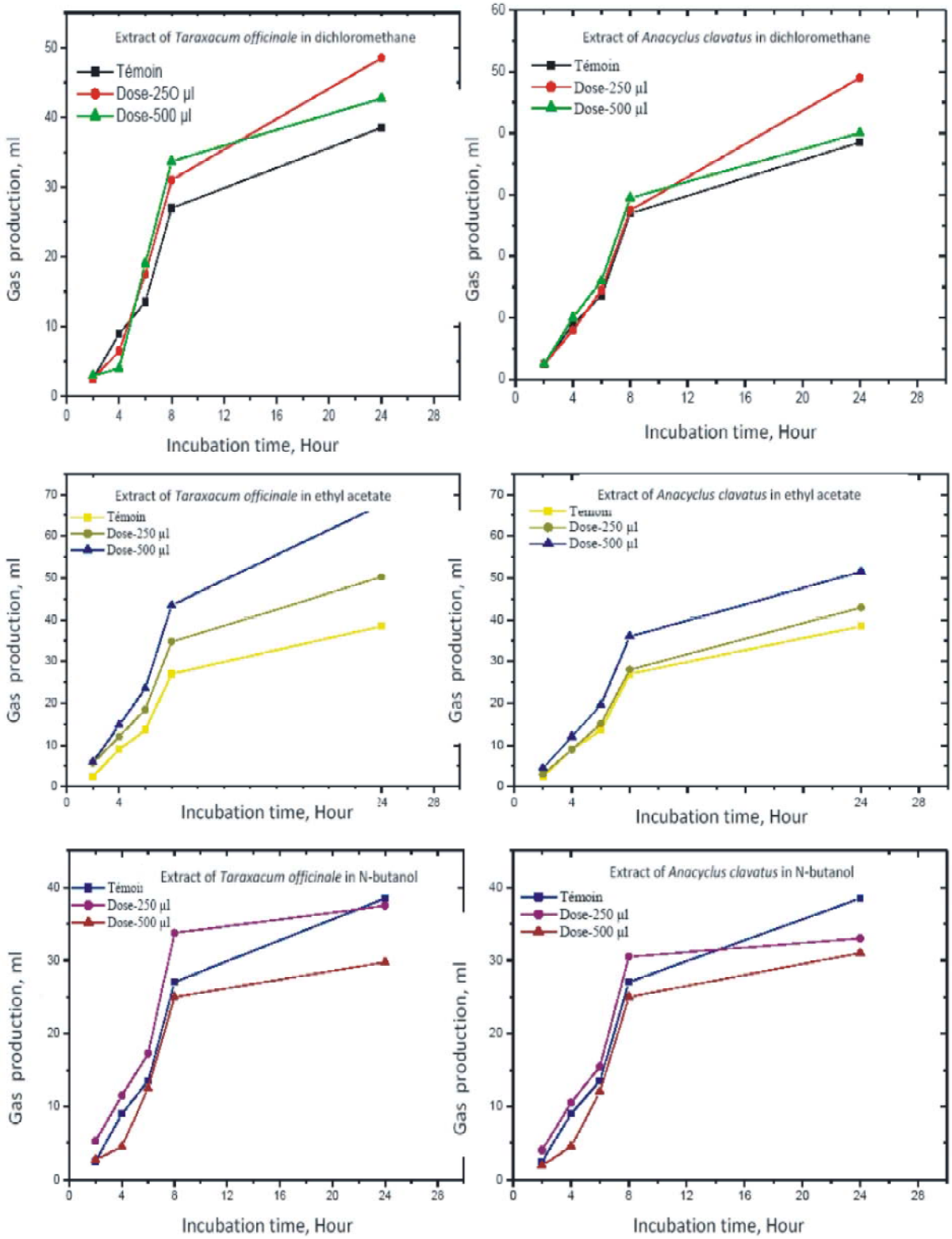

Fig. 5: Kinetics of *in vitro* gas production of *Taraxacum officinale* and *Anacyclus clavatus* extracts, administered at doses of 250µl and 500µl in batch systems

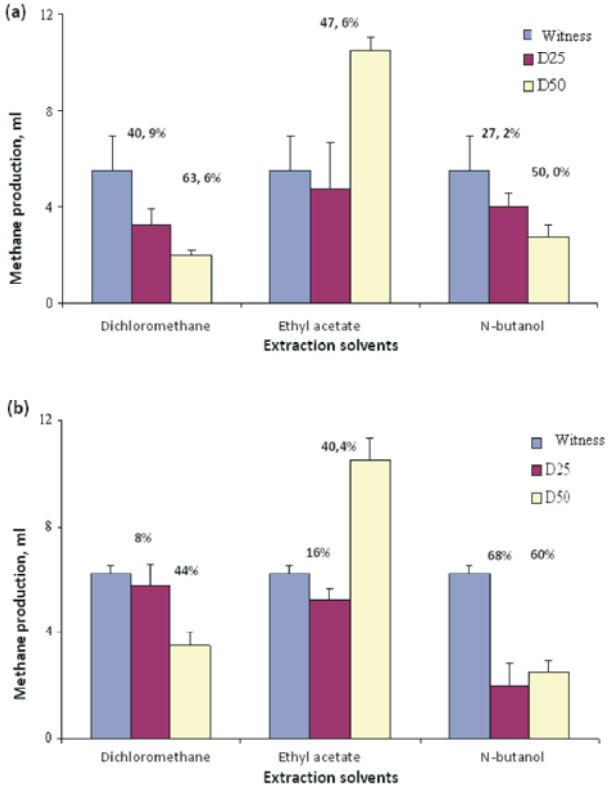

Fig. 6: Influence of *Taraxacum officinale* and *Anacyclus clavatus* (b) extracts, administered at doses of 250µl and 500µl on *in vitro* methane production

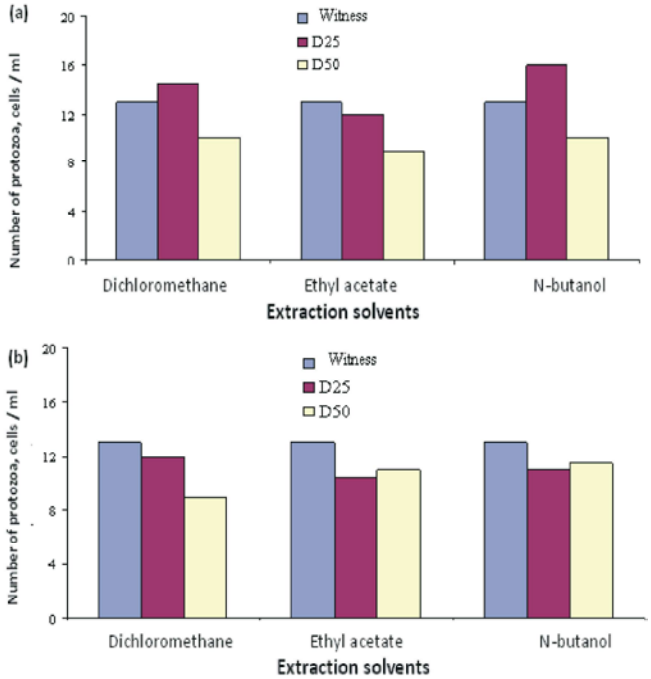

Fig. 7: Influence of *Taraxacum officinale* and *Anacyclus clavatus* (b) extracts, administered at different doses, on ruminal fauna

sources from the studied plants (the case of dichloromethane which precipitates lipids) or that these solvents are metabolized by the Ruminal microbiota as a source of carbon. Similar results have been obtained by Patra *et al*. [15], who indicated that extraction of various condiments and *hirsuta* using methanol, ethanol and n-butanol increases the gas production.

Influence of Extracts on Ruminal Methanogenesis: It appeard that at the doses of 250 µl and 500 µl of Taraxacum officinale and *Anacyclus clavatus* extracts lead to a significant reduction in the methane production (Figure 6), except for the ethyl acetate extract and the 500 µl dose. For *Taraxacum officinale*, the decrease varies between 13.6 and 63.6%. Indeed, the highest reductions have been noted for the dichloromethane (40.9 and 63.6%) and n-butanol (27.2 and 50%) extracts, for the two doses 250 and 500 µl, respectively. The same profile was recorded for *Anacyclus clavatus*, with only one difference compared to *Taraxacum officinale*; The n-butanol extract greatly reduced methane production (60 and 68%) for the 250 and 500 µl doses, respectively.

4-6- Enumeration of Protozoa: The count of the protozoa after 24 h of incubation of the various treatments performed is illustrated in figure 7. It indicated that dichloromethane and n-butanol extracts from *Taraxacum officinale* generated a proliferation of the ruminal fauna at a dose of 250 µl. However, for the same dose, the ethyl acetate extract reduced the number of protozoa. The different extracts caused a considerable reduction of the fauna at the dose 500 µl. For *Anacyclus clavatus*, the different extracts also induced a reduction in the protozoan population.

CONCLUSION

The study revealed that the different solvents used are able to solubilize the phenolic compounds. This result is demonstrated by chromatographic (yellow spot) and spectral analysis (colorimetric assay). However, one result is noteworthy, for the dichloromethane where the capacity to extract the flavonoids is greater than that of ethyl acetate. This is likely due to the nature of the flavonoic compounds present in *Taraxacum officinale* and *Anacyclus clavatus*.

Regarding the influence of these flavonoic extracts on gas production, this study showed that dichloromethane and n-butanol macerates reduced the gas production. On the other hand, this production increased, compared to the control (syringe without additive). This result could be explained by the fact that this solvent causes the solubilisation of energetic substances stimulating the growth of the ruminal population. Similarly, various extracts induced a significant reduction in methane production, which is probably due to the inhibition of the activity of methanobacteria, essentially associated with protozoa. This result is corroborated by the decrease in ruminal fauna for *Anacyclus clavatus*. On the other hand, for *Taxaracum officinale*, the reduction in methane production could be attributed to a change in the metabolic pathways.

Overall, this study allowed us to demonstrate the ability of crude extracts of flavonoids (polyphenols) to reduce methane. It is now necessary to define the composition of each extract by CG-MS and to evaluate the isolated molecular effect on the ruminal methanogenesis.

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