In vitro Evaluation of Andrographolide Stability in Rumen Fluid to explore oral Administration of *Andrographis paniculata* in Ruminants

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ABSTRACT

Andrographis paniculata (Burm. f.) Wall. Ex Nees is an important medicinal plant used in both human and veterinary healthcare, and andrographolide is the major active constituent of *A. paniculata.* This study communicates the stability of andrographolide in the rumen fluid when administered in different forms. The stability of andrographolide was assessed using an *in vitro* method, in which coarsely powdered aerial parts of *A. Paniculata* (10 mg/mL), a standardized extract of *A. paniculata* (200 µg/mL), and pure andrographolide (5 µg/mL) were separately incubated with rumen fluid, and the level of andrographolide in the rumen fluid was estimated at 1, 2, 4, 8, and 24 h after incubation. This study showed that approximately 90.9% of andrographolide vanished rapidly from the rumen fluid when it was added to the rumen fluid in its pure form, whereas the concentration of andrographolide progressively increased by 167.91 and 200% when the hydroalcoholic extract of A. paniculata or dried aerial part of the plant was incubated with rumen fluid, respectively. This study provides confidence in administering the *A. paniculata* extract or the dried aerial parts orally in ruminants; however, it warrants further detailed investigation on the pharmacokinetics of andrographolide in ruminants upon oral administration of the *A. paniculata* plant or extract.

Keywords: Ruminants, Andrographolide, *Andrographispaniculata*

INTRODUCTION

The genus *Andrographis* belongs to the family Acanthaceae, and it is represented by 25 species worldwide, most of which are endemic to India. *A. paniculata* (Burm. f.) Wall. Ex Nees is a well-known species of this genus; it is native to the Indian subcontinent, but is introduced and cultivated from Myanmar to Indonesia and some parts of Mexico. It is abundant along the plains of the Indian Subcontinent. It is a sub-shrub with quadrangular stems and linear-lanceolate leaves, zigzag panicle branches, white flowers with a pink tinge, and capsules (Manickam *et al*., 2022).

A. paniculata has been known as *Kalmegh* and *Mahathiktha* (king of bitters) in Sanskrit, as *Nilavembu* in Tamil and as green chiretta in English. It has been widely used in Indian traditional medicinal systems as a bitter tonic and as a febrifuge; it is official in the Indian Pharmacopoeia (Nadkarni, 1976) and standardized. *A. Paniculata* extract has been used as an over-the-counter drug for treating the common cold in many countries. In this species, 344 phytochemicals have been reported to date; terpenoids and flavonoids are the major reported compounds. Terpenoids are responsible for their bitterness and therapeutic efficacy; among them, andrographolide is the major terpenoid of this species (Kumar *et al*., 2021) and has been reported to exhibit various biological activities (Zeng *et al*., 2022; Chidambaram, 2024).

In veterinary medicine, *A. paniculata* has been used either as a whole plant (Melchior, 2000) or as an extract or stem for poultry and farm animals (Anonymus, 2002). Supplementation with *A. Paniculata* has been shown to lower the relative abundance of harmful bacteria in the intestines, while increasing the body mass of chickens (Jahja *et al*., 2023; Mathivanan & Kalaiarasi, 2007) and

Muscovy ducks (Liu *et al*., 2023). It's supplementation, in addition to improving gut health, also ameliorates heat shock-induced oxidative stress and immune suppression (Fayed *et al*., 2024).

Supplementation with *A. paniculata*was also shown to improve meat quality and lower fat content (Yusuf *et al*., 2014), as well as improve rumen concentration of n-6 and n-3 fatty acids without affecting the microbiota profile in the rumen (Yusuf *et al*., 2017). Previous studies have attempted to evaluate the health benefits of *A. Paniculata* on ruminants, which communicates the stability of andrographolide, the active constituent of *A. paniculata*, in the rumen fluid when administered in different forms.

METHODOLOGY

Materials

The aerial parts of *A. paniculata*were coarsely powdered and used in the present study. A standardized hydroalcoholic extract of *A. paniculata,* 'KalmCold™', which contains ~30% andrographolide, was procured from Natural Remedies, Bangalore.The aerial parts of *A. Paniculata* were collected from Xavier Solai, the medicinal plant garden of St Xavier's College at Vickiramasingapuram (8.67°N, 77.33°E) in Tirunelveli District. The botanical identity of the specimen was confirmed by Protein Profiling and I. Kumar, Botanist, and one of the authors of the manuscript, and a voucher specimen of the material (XCH 41110) was deposited in the herbarium of the Xavier Research Foundation, St Xavier's College, Palayamkottai.

Instruments and Reagents

Solvents and reagents were of analytical grade, unless otherwise mentioned. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded with CD_3OD using a Bruker Avance Onebay NMR spectrometer at the School of Chemistry, Madurai Kamaraj University, Madurai 625021.

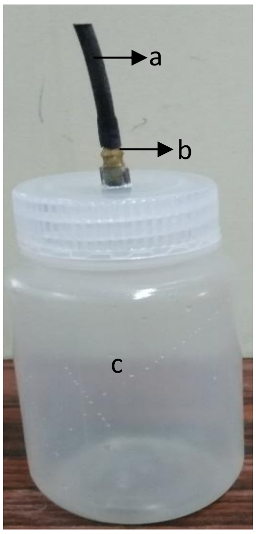
Isolation of Andrographolide

The plants were shade-dried, coarsely powdered (500 g), and extracted three times with five volumes of methanol using the cold percolation method. The contents were filtered and concentrated to 1/4th of their original volume; with this 2% of activated charcoal (w/v), the contents were refluxed for 15 min and filtered through Whatman #1 filter paper. The filtrate was concentrated to 100 mL and allowed to crystallize at 4°C to obtain crude andrographolide. Repeated crystallization with methanol yielded pure andrographolide (yield: 400 mg, purity: 98.5%) (Toppo *et al*., 2017).

Set-up of Mini-Rumen Incubation Kit

Andrographolide stability was assessed using a newly designed *in vitro method*, which was a modified version of the method of Tilley and Terry (1963). This kit consisted of a100 mL polypropylene bottle with a screw-cap and a tube attached to a valve (Figure 1). This method allows the release of excess gas produced during incubation while maintaining anaerobic conditions of the rumen fluid.

The rumen cud was collected from a freshly slaughtered bull at the slaughterhouse in an ice box, immediately taken to the laboratory, and filtered using a muslin cloth folded four times. The collected rumen liquid was immediately transferred to each Mini-Rumen kit at a volume of 10 mL along with 40 mL of McDougall's artificial saliva (Table I). The salts were dissolved in MilliQ water, except for $CaCl_2$, which was added, and the total volume was adjusted to 1 L.



a. Bi-cycle valve tube
b. Bi-cycle valve invertedly fixed to allow the release of gas generated during incubation
c. Container for incubating rumen fluid with extract, dried aerial part, or API

Figure 1: Mini-rumen Incubation Kit

Table I: Composition of McDougall's Artificial Saliva

Ingredient	g/L
Sodium bicarbonate ($NaHCO_3$)	9.8
Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4.12H_2O$)	9.3
Sodium chloride (NaCl)	0.47
Potassium chloride (KCl)	0.57
Calcium Chloride anhydrate	0.04
Magnesium Chloride anhydrate	0.06

Evaluating the stability of andrographolide

The stability of andrographolide in rumen fluid was assessed in three different formulations: coarsely powdered aerial parts of *A. paniculata* (*AP* Powder) at a concentration of 10 mg/mL, KalmCold™, a standardized extract of *A. paniculata* (*AP* extract) at a concentration of 200 µg/mL, and andrographolide (*AP*compound) at a concentration of 5 µg/mL. Rumen cud and McDougall artificial saliva were incubated with either of the test materials. The kit was airtight and flushed with CO_2 through a vent fitted with a valve. The contents were incubated at 29°C; following incubation with the rumen content, samples were collected at 1, 2, 4, 8 and 24 h after incubation. Samples were centrifuged at 3000 rpm for 10 min, and the supernatant (0.5 mL) was mixed with cold methanol to precipitate and remove proteins. The mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered through 0.22 µ filter before HPLC analyses.

HPLC Quantification of Andrographolide

The andrographolide content in the test samples was quantified using a reverse-phase HPLC method, which was performed in isocratic mode with a mobile phase of methanol and water (65:35%) at a flow rate of 1 mL/min in a C_{18} column (Thermo Scientific, Part No.97105-254630, Syncronis C_{18}, 250 × 4.6 mm, 5 µm particle size, LOT-14720 with Serial No. 10511392). The absorbance was set at 223 nm, in accordance with the literature. Standard curve was plotted using different concentrations of and rographolide (0.5 to 50 µg/mL) (Seema *et al*., 2018).

Statistical Analysis

Results are presented as Mean ± SD for triplicates; andrographolide content was estimated using the linear regression method in Microsoft Excel.

RESULTS

NMR Spectra of Andrographolide

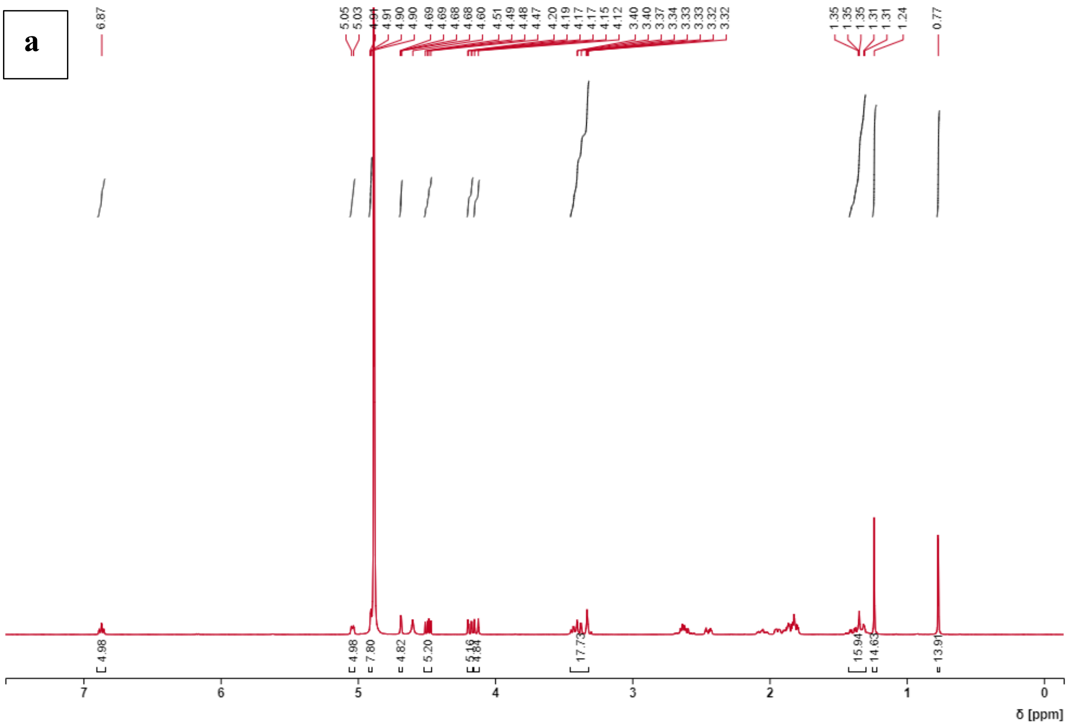
Andrographolide was isolated as colourless crystals, analysed for $C_{20}H_{30}O_5$, and the NMR spectra are shown in Figure 2. The ^1H NMR assignments of andrographolide (CD_3OD, 400 MHz) were δ 6.86-6.88 (t, 1H), 5.04 (d,*J* = 8 Hz, 1H), 4.91 (d, *J* = 4 Hz, 1H), 4.69 (d, *J* = 4 Hz, 1H), 4.49 (dd, *J* = 10.2 & 1.8 Hz, 1H), 4.14 (d, *J* = 12 Hz, 1H), 3.32-3.40 (m, 2H), 1.35-1.31 (m, 1H), 1.24 (s, 3H) and 0.77 (s, 3H). ^{13}C NMR assignments of andrographolide (CD_3OD, 100 MHz) were δ 14.15(C-20), 21.99 (C-19), 23.81 (C-11), 24.32(C-6), 27.62(C-2), 36.73(C-1), 37.57(C-7), 38.57(C-10), 42.28(C-4), 54.92(C-5), 55.99(C-9), 63.57(C-14), 65.23(C-18), 74.79(C-15), 79.51(C-3), 107.84(C-17), 128.38(C-13), 147.38(C-12), 148.02 (C-8) and 171.3(C-16).These values are in accordance with those of a previous report (Su *et al*., 2020).

HPLC Quantification of Andrographolide

The retention time of andrographolide was identified as 5.6 min; chromatogram images of various concentrations of andrographolide are shown in Figure3. Linear regression analysis was done by plotting the area of the peak at 5.6 min on *y* axis and the concentration of andrographolide on the *x-axis*. The slope of the equation was calculated as $y = 57380x + 69036$, with an R^2 value of 0.999.

Estimation of Andrographolide Content in the Rumen Fluid

The test materials (AP powder, AP extract, or AP compound) were incubated with rumen fluid, and the andrographolide content in each sample was assessed at various time intervals; the results are given in Table 2 and Figure 4. In *AP* compound-incubated vials, the andrographolide content decreased progressively compared to the initial quantity. In contrast, *the AP* extract and *AP* powder-treated groups showed a progressive increase in and rographolide content. In the rumen fluid, 90.9% of andrographolide vanished when it was applied in the pure form, whereas the concentration of andrographolide progressively increased by 167.91 and 200% when it was used either as an extract or raw, respectively.



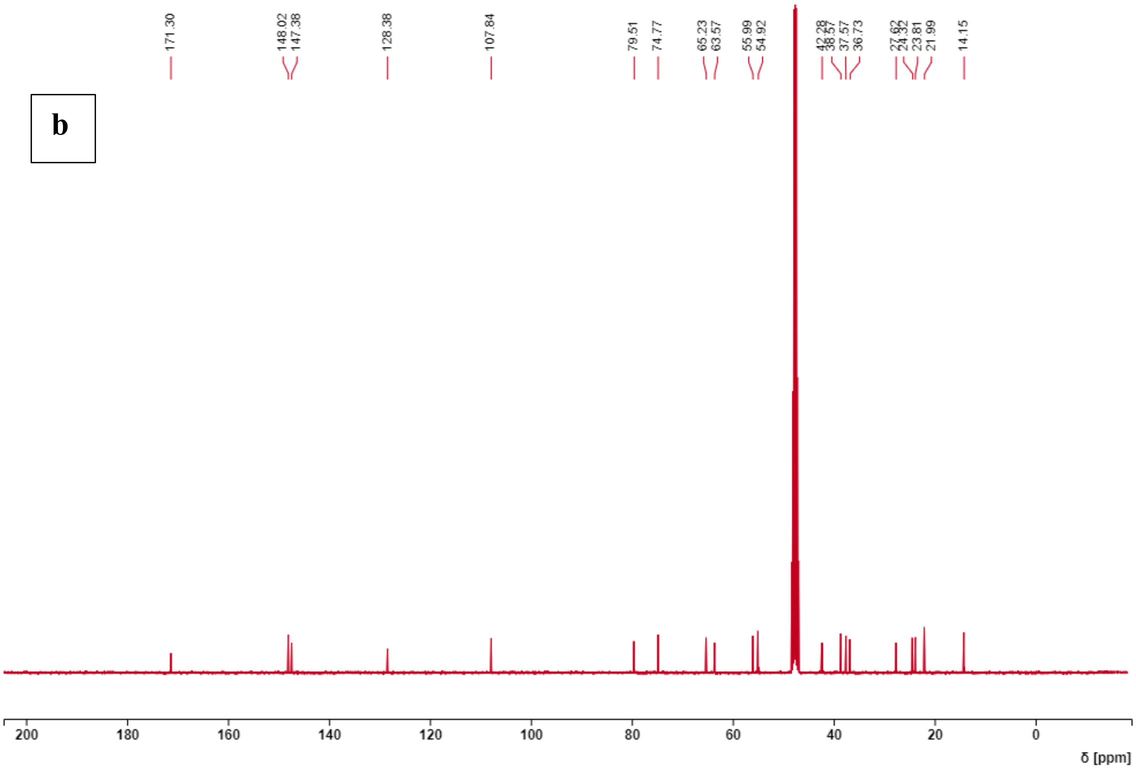


Figure 2: a) ^{1}H and b) ^{13}C spectra of andrographolide

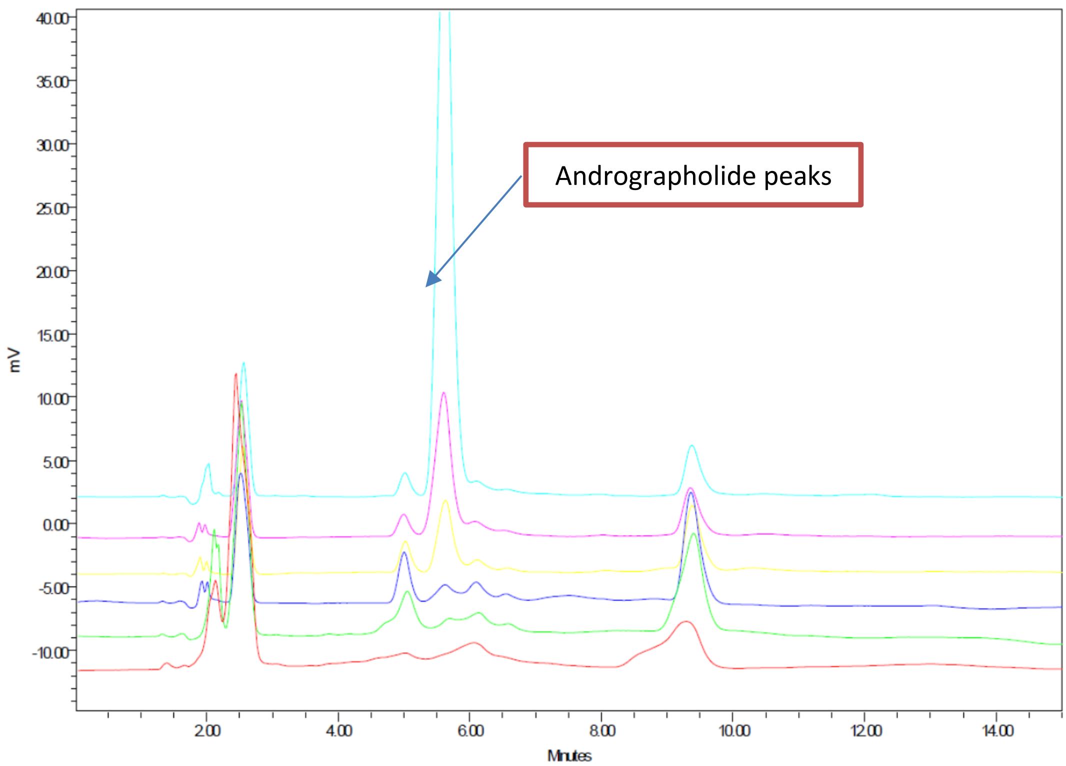


Figure 3: HPLC chromatograms of pure andrographolide at various concentrations

Chromatogram with red line indicates the blank; the others, such as green (0.5 µg/mL), purple (1 µg/mL), yellow (5 µg/mL), pink (10 µg/mL) and blue (20 µg/mL) lines indicate andrographolide at various concentrations

Table II: Concentration of andrographolide after incubation of different forms of *A. paniculata*in rumen liquid at various time points

Formulation	1 h	2 h	4 h	8 h	24 h
AP Compound (andrographolide) (5 µg/mL)	2.64 ± 0.82	1.82 ± 0.48	0.59 ± 0.54	0.93 ± 0.54	0.24 ± 0.21
AP extract (0.2 mg/mL)	3.73 ± 1.69	4.10 ± 1.22	5.05 ± 2.14	6.57 ± 0.97	6.27 ± 1.27
AP powder (10 mg/mL)	26.65 ± 6.35	36.88 ± 6.14	48.01 ± 12.12	75.04 ± 28.62	53.51 ± 15.18

Values (in µg/mL) are means ± SD of three replicates

DISCUSSION

Andrographolide estimation in rumen fluid after incubation with three different *A. paniculata* formulations were performed using an *in vitro method*. The results showed that andrographolide was stable in the rumen fluid under experimental conditions for 24 h. The concentration of andrographolide drastically decreased in the *AP* compound group at 24 h, which could be due to the metabolism of andrographolide by rumen microflora. Previous studies have documented the biotransformation of andrographolide and related terpenoids by various microbes into bioactive compounds with novel structures (Kolat and Patil, 2022).

In contrast to the *AP* compound, the level of andrographolide increased progressively in the other two groups. This could be due to the release of andrographolide by microbial digestion of the extract or plant material. Yusuf *et al.* (2017) showed that there was no significant effect on the populations of protozoa and methanogens in the rumen of *A. paniculata*-fed *goats*. These studies indicated that *A. paniculata* did not have any adverse effects on the rumen microflora or the digestibility of nutrients. Furthermore, it indicated the stability of andrographolide in rumen fluid and supported the oral administration *of A. paniculata* to ruminants.

The results of our preliminary study suggest that supplying raw *A. paniculata* plant material (in dried aerial parts) might help maintain a constant systemic level of andrographolide, thereby exerting maximum health benefits. Further detailed, comparative, and *in vivo* investigations are required to determine the bioavailability of andrographolide on oral administration of raw *A. paniculata* aerial parts or extracts to ruminants and overall health outcomes in ruminants.

CONCLUSION

The stability of andrographolide in the rumen fluid was assessed using a newly designed in vitro method. This study showed that the concentration of andrographolide was reduced rapidly in the rumen fluid when it was applied in the pure form, whereas the concentration of andrographolide progressively increased when it was applied either as an extract or raw material. This study warrants further detailed investigation on the advantages of raw *A. paniculata* on oral administration in ruminants.

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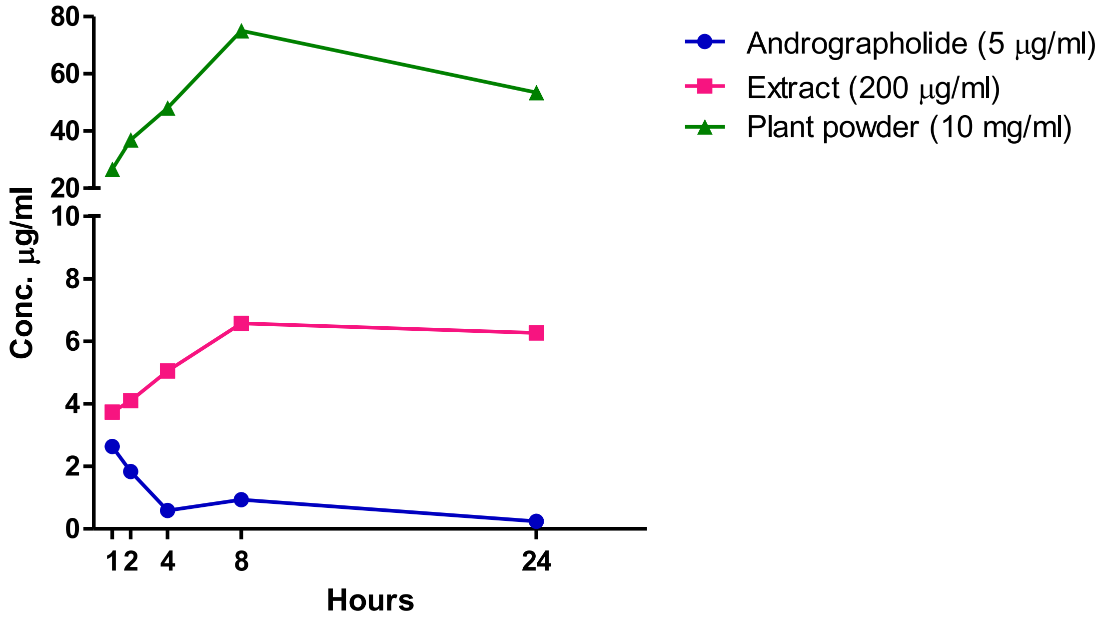

Figure 4: Concentration of Andrographolide in μg/ml of rumen fluid after incubation of different forms of *A. paniculata* in rumen fluid.