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Research Article

Isolation and Characterisation of Andrographolide from *Andrographis paniculata* (Burm. F) Wall. Ex Nees and Its Total Flavonoid Effects from Kemaman, Malaysia

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ABSTRACT

Background: Many medicinal plants have wide range of applications from food and aesthetic materials to pharmacological effect on various disease conditions. Traditionally, these plants have been used to treat many diseases including diabetes, inflammation, upper respiratory tract infections and diarrhoea either in single or combined form with other plant parts or materials. Objective: This study investigated the isolation and characterisation of andrographolide from *Andrographis paniculata* (Burm. f) Wall. ex Nees. Methodology: The isolation of the constituents was done using column total flavonoid conducted while the analysis was done using ultraviolet, infrared spectrum analysis (UV, IR), melting point and elemental analysis. Also, proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) of the isolated compound was performed using different equipment. Results: Although, the total flavonoid content was low, ethyl acetate extract indicated the highest total flavonoids content and it's statistically different from methanol, ethanol: water (1:1 v/v) and aqueous extracts. The compound obtained is colorless solid with melting point range of 228^{o}C-238^{o}C. This was further supported by ^{13}C NMR spectral analysis, which displayed 20 signals for all carbon atoms and is like standard compound Andrographolide. Also, the ultraviolet analysis suggesting α, β-unsaturated lactone ring similar andrographolide while the elemental analysis shows the different elements present. Conclusion: Different extracts of *A. paniculata* exhibit multiple pharmacological effects. However, due to the much similarities with the isolated compound and Andrographolide the compound is suggested to be Andrographolide.

Keyword: Andrographolide, ^{1}H NMR, ^{13}C NMR, IR and UV.

INTRODUCTION

A long history of natural products in ancient times and in folk medicine around the world is the basis for the use of many therapeutic agents in modern day medicine[1]. The quest to find new therapeutic candidate compounds from natural biodiversity, particularly plants, has been the prime interest among researchers[2]. Furthermore, a growing worldwide interest in the use of phytopharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, has been noted in recent years. It is believed that about 80% of world's population use plants as their primary source of medicinal agents[3-5]. The current research on plant-based medications focuses on the isolation of biologically active compounds from potent plants, their characterisation and commercialisation. However, studies in this aspect has been greatly influenced by modern physicochemical techniques of isolation and structural elucidation[6]. Proton and carbon NMR (^{1}H & ^{13}C NMR) provides a non-destructive means of determining the contents of individual analyses in a complex matrix, thus requiring no external reference and short analysis time[7], making it a simple technique for the identification of chemical structure of a compound.

Natural products have been used as a potential source of anti-inflammatory drugs. Endotoxins such as lipopolysaccharide-induced pro-inflammatory mediators and cytokines production contribute to the sequence of events in activated macrophage cells, which lead to the kill of microorganisms and causing tissue damage during the inflammatory process[8]. Overproduction of nitric oxide (NO) has been implicated in the pathogenesis of inflammation via inducible nitric oxide synthase (iNOS), which reflects the degree of inflammation process[9]. The liver is the largest internal organ in vertebrates, including humans. It is vital to survival, as it is the principal organ of metabolism and excretion, and support every other organ[10]. Due to continuous exposure to xenobiotics,

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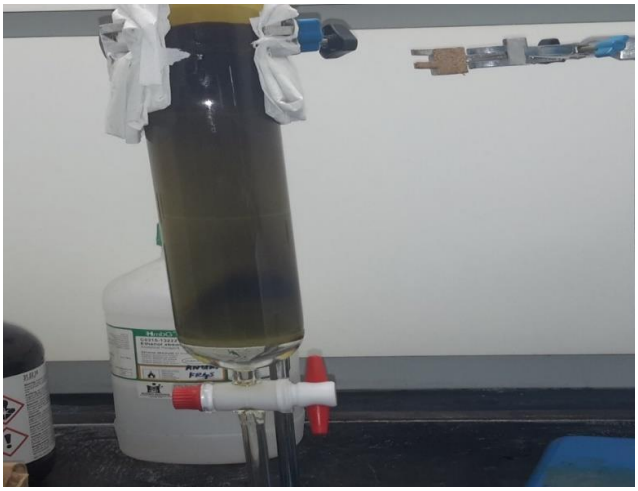
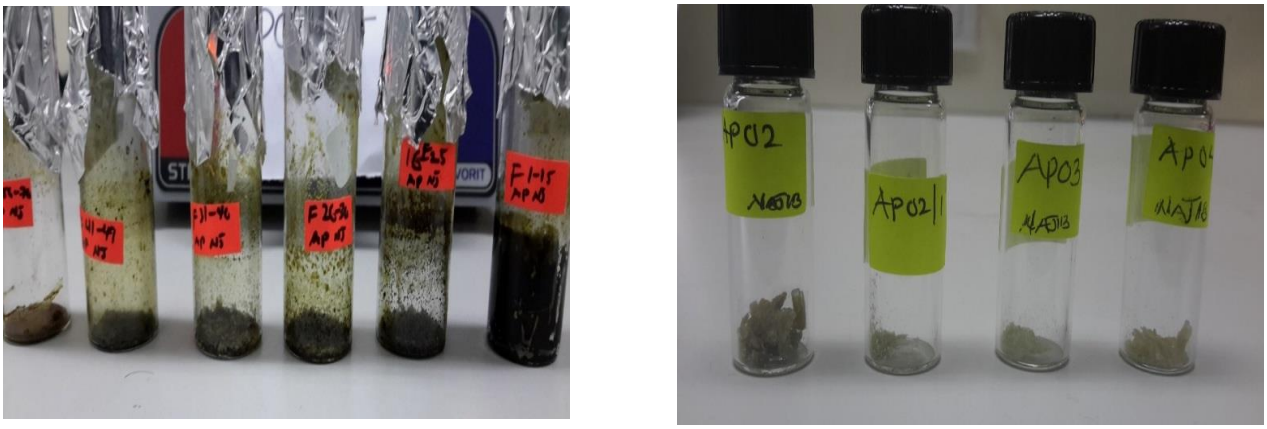


Figure 1: Column chromatography of *A. paniculata.*



a b

Figure 2: Fractions of *A. paniculata* obtained from column chromatography before (A) and after (B) washing to obtain crystals.

Table 1: Total flavonoid contents of *A. paniculata* extracts.

Extracts	Total Flavonoid Contents (mg of QE/g)
Methanol	0.43 ± 0.00[a]
Ethyl acetate	0.44 ± 0.01[b]
Ethanol: water (1:1 v/v)	0.43 ± 0.00[a]
Water	0.43 ± 0.00[a]

chemicals, it is prone to many diseases including cancer[10]. Cancer is one of the most common devastating diseases affecting millions of people per year. Cancer has been estimated as the second leading cause of death in humans. There has been an intense search of anti-inflammatory and anti-cancer drugs from various biological sources[11].

On the other hand, Insulin is a hormone produced in the pancreas that allows glucose from the food to enter the body's cells and convert into energy needed by the muscles and tissues for proper functioning. In a diabetic person, there is improper absorption of glucose that remains circulating in the blood a condition called hyperglycaemia, which causes damage to the body tissues and muscles over time. This damage can lead to disability and life-threatening health complications for the patients[12]. Many natural products or their extracts have claimed to have multiple therapeutic benefits against life-threatening diseases *Andrographis paniculata* (Burm. F) wall. Ex Nees belonging to the family Acanthaceae or Kalmegh, commonly known as "king of bitters" and in Malaysia called "Hempedu bumi". It is widely distributed throughout tropical Asian countries. Is extensively used in Ayurveda, Unani and Siddha medicines as a home remedy for various diseases in India[1]. In Malaysia and other countries, it is reported to have multiple clinical applications. It's an important cold property herb, used in fevers to remove toxins from the body[13]. The plant extracts are known to contain diterpene, and flavonoids that have been reported to exhibit many pharmacological benefits. The present study employed simple isolation technique (column chromatography) to isolate chemical constituents from *A. paniculata* and elucidate the chemical structure of andrographolide using nuclear magnetic resonance spectroscopy (^{1}H, and ^{13}C NMR) and investigating the multiple therapeutic benefit of *A. paniculata* extracts and isolated compound via *in vitro* antidiabetic effect and anti-inflammatory effect against

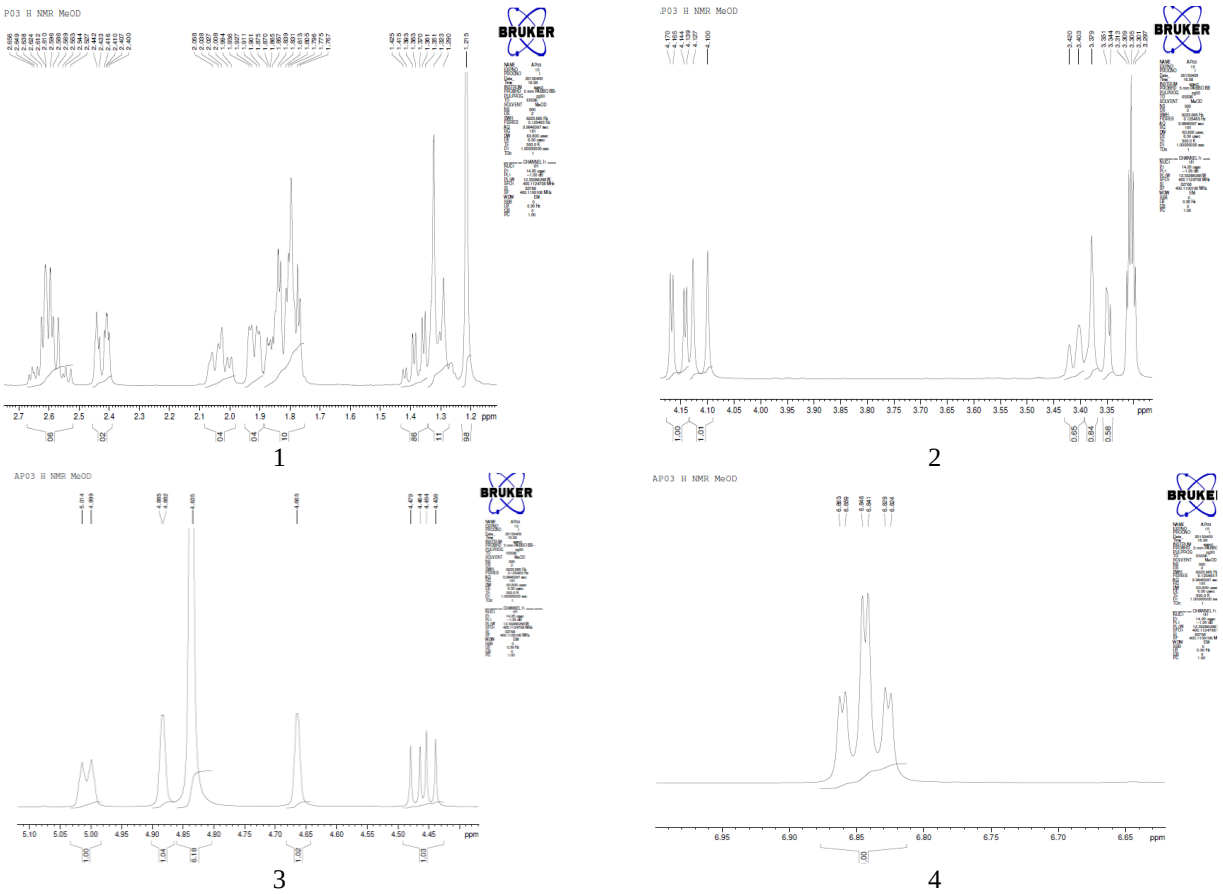


Figure 3: ^{1}H NMR of crystal obtained from *A. paniculata.*

alpha-glucosidase enzyme and nitric oxide scavenging activity respectively.

MATERIALS AND METHODS

Silica gel (purchased from Merck, Germany), column (35 x 5 mm) from Merck, all other solvents were of analytical grade.

Collection of plant material

A. paniculata plant was obtained in September from Kemaman in Terengganu, Malaysia. The plant was identified, authenticated by Norhaslinda Haron from Faculty of Agriculture and Animal Sciences, Universiti Sultan Zainal Abidin, and deposited at the herbarium unit with specimen voucher number 00266.

Preparation of plant extracts

The plant was washed with distilled water, dried at 40^{o}C and then grinded into powdered form. The powdered plant was weighed and soaked in different solvents (methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous) in a ratio of 1:10 respectively. The extracts were then decanted and filtered through filter paper. The filtrate obtained was concentrated using rotary evaporator at temperature 40^{o}C. Extracts were then dried at 50^{o}C and kept in a freezer at 4^{o}C untill use.

Total flavonoid content (TFC)

Total flavonoid content was determined with little modification[14]. A 0.25 mL of the diluted extracts of 250 µg/mL concentration were mixed with solution containing 50 µL of 1 M aqueous potassiun acetate, 50 µL of 10 (%w/v) aluminium chloride and 2.15 mL of 95 % ethanol. The mixtures were mixed and incubated at room temperature in a dark for 40 min, and the absorbance (*A*) was recorded spectrophotometrically at 415 nm. The samples were assayed in triplicate. TFC was calculated using quercetin as standard and expressed as mg per gram from the calibration curve.

Thin layer Column chromatography (TLC)

Isolation of andrographolide was determined as prescribed[15]. Ten-gram slurry of methanol extract was chromatographed on a column of silica gel 60 particle size 0.040-0.063 mm (70-230 mesh) and eluted with ethyl acetate-methanol in an increasing order of ratio (10:0 and 0:10). Fractions 16-25, 26-30 and 31-40 afford 1.61g, 1.63g and 1.58g of colourless needles crystals and labelled APO2, APO3 and APO4 respectively. The compounds visualisation was done under UV light and by spraying with anisaldehyde, it gives a positive test for terpenoids.

Proton and carbon NMR (^{1}H and ^{13}C NMR)

The structure elucidation of the compound (APO2) was further conducted by adopting and modifying method[16]. ^{1}H NMR and ^{13}C NMR spectra were run on Bruker Avanve iii, 400 MHz (Bruker Faellanden, Switzerland) in deuterated methanol. Chemical shifts are reported as values in ppm relative to d_4-methanol with TMS as an internal standard.

Ultraviolet, infrared spectrum analysis (UV, IR), melting point and elemental analysis

The compound was subjected to further analysis to

Table 2: Comparing the ^{13}C-NMR chemical shift of the unknown compound (Isolated) and andrographolide.

S. No.	Isolated compound		Andrographolide		
	^{13}C	SHIFT	^{13}C	SHIFT	Reference
1.	CH_2	38.19	CH_2	37.4	(24)
2.	CH_3	29.09	CH_2	29.1	(24)
3.	CH	80.97	CH	80.0	(24)
4.	R-C_4	43.73	Q	43.9	(24)
5.	CH	56.38	CH	55.4	(24)
6.	CH_2	25.25	CH_2	24.5	(24)
7.	CH_2	38.19	CH_2	38.3	(24)
8.	=C	149.37	Q	148.0	(24)
9.	CH	56.38	CH	56.5	(24)
10.	R-C_4	39.02	Q	39.3	(24)
11.	CH_2	25.76	CH_2	25.1	(24)
12.	=C	148.81	CH	147.0	(24)
13.	=C	129.85	Q	130.3	(24)
14.	CH	66.70	CH	66.1	(24)
15.	CH_2	76.17	CH_2	75.4	(24)
16.	C=O	170.67	Q	170.7	(24)
17.	CH_2	109.26	CH_2	108.8	(24)
18.	CH_3	23.42	CH_3	23.8	(24)
19.	CH_2	65.02	CH_2	64.2	(24)
20.	CH_3	15.58	CH_3	15.3	(24)

Table 3: Elemental analysis of AP03 crystal.

	Elements (%)			
	N	C	H	S
APO 3	0.0433	62.412	8.047555	0

determine the functional groups and their relative transition. The Method[17] was adopted for determining the UV, melting point and elemental analysis while modified method[18] was adopted for determination of IR for the isolated compound. The IR and UV spectra were recorded using Fourier transform infra-red (FTIR) Tracer-100 (Shimadzu) spectrophotometer while using KBr pellets for IR spectroscopy. All spectra were recorded in a range of 400-4000cm-1. However, melting point was recorded using melting point analyser (Stuart Scientific, UK), while the percentage of nitrogen, carbon, hydrogen and oxygen (elemental analysis) was determined using elemental analyser FLASHEA 1112 series. All data were analysed and then recorded.

RESULTS AND DISCUSSION

Although, high total flavonoids content in *A. paniculata* had been reported by earlier researchers[19,20], contrasting results with low flavonoid contents have been reported by many researchers using plants. It is for instance,[13] expressed total flavonoid content of fruits and vegetables as mg catechin equivalent (CE/ 100 g) and analyzed in duplicate, a lower total flavonoid content was observed. Also,[21] reported a lower flavonoid content of *A. paniculata* extracts especially aqueous extracts with TFC value of 0.16 mg/g. Similarly,[22] reported a protective effect of aqueous extracts of *A. paniculata* against various oxidants with lower concentration of flavonoid content. The highest flavonoid content in this study is like that research reported[23] who found that ethanol extracts of *A. paniculata* has total flavonoid of 0.86 mg/g. Therefore, plants with low TFC as reported in this research can exhibit its pharmacological effects.

Note: SPSS software was employed to analyze the mean difference of the TFC between the extracts using One-Way ANOVA and alphabets were assigned to differentiate them from least to highest TFC, where a = extracts with least TFC, b = extract with highest TFC. The mean flavonoid content of *A. paniculata* extracts were significantly different ($p<0.005$). The data expressed as mean ± SD (n=3 for each extract); means were compared by Bonferroni test ($p<0.05$). The mean flavonoid content of *A. paniculata* extracts indicated by the same lower case identical alphabets showed no significant difference between them ($p>0.05$), and the mean flavonoid content of ethyl acetate extract is significantly different from the other three extracts ($p<0.05$). The column chromatography for methanol extract of *A. paniculata* was carried out as shown in Figure 1 and it revealed the presence of bands or layers of compounds which were then eluted and collected.

Collection of similar fractions

Fractions from column chromatography were pooled together based on the spot of TLC and grouped into 6 fractions (1-15, 16-25, 26-30, 31-40, 41-49 and 50-70 respectively). The solvents from these six different fractions were removed using rotary evaporator and the dried fractions were transferred to vials as shown in Figure 2 for further processing. However, only fractions 16-25, 26-30 and 31-40 yielded some crystals which were further washed to obtain cleaned crystals (Figure 2B).

The compound obtained is colourless solid with melting point range of 228°C-238°C. This was further supported by ^{13}C NMR spectral analysis, which displayed 20 signals for all carbon atoms in the molecule, including one carboxyl, four methyl's, eight non-protonated carbons, two methylene's, three methane's, one cyclic alkene, and one benzylic carbon atom (Table 1). This corresponds to similar report by[18], suggesting the isomer to be similar to andrographolide ($C_{20}H_{30}O_5$). However, the ^1H NMR spectrum had several signals comparable to that of ^{13}C NMR of the isolated compound Viz are methylene signalling at (δ 6.84, t), cyclic alkene (δ 4.99, s) and carboxyl (δ 4.45, s). Thus, the strong similarities to andrographolide as described[24].

Moreover, ultraviolet spectrum of the compound at λmax 223 nm (Figure 4) suggesting α, β-unsaturated lactone ring similar andrographolide as reported by (16, 24).

The infra-red spectrum (Figure 5) indicates the presence of hydrogen group, alcoholic group, carboxylic group, and alkene groups at 3401, 1012, 1733 and 1674 cm^{-1} respectively. Thus, implying a close similarity between the compound and andrographolide as reported[24]. Moreover, these are the main functional groups present in andrographolide and therefore, the compound isolated could be andrographolide, a major constituent in *A. paniculata.*

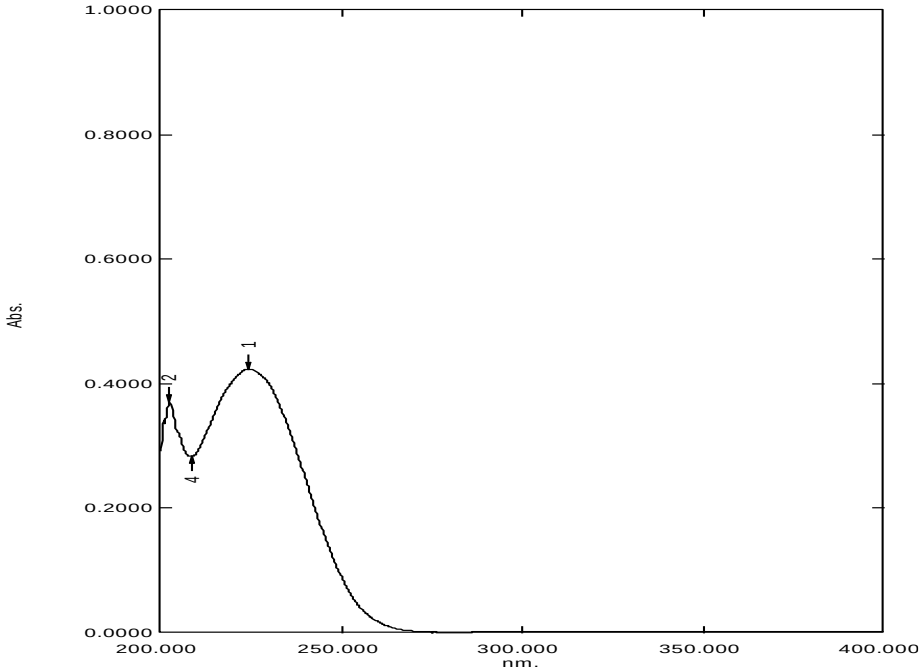


Figure 4**:** Ultra-violet (UV) spectrum of the isolated compound from *A. paniculata.*

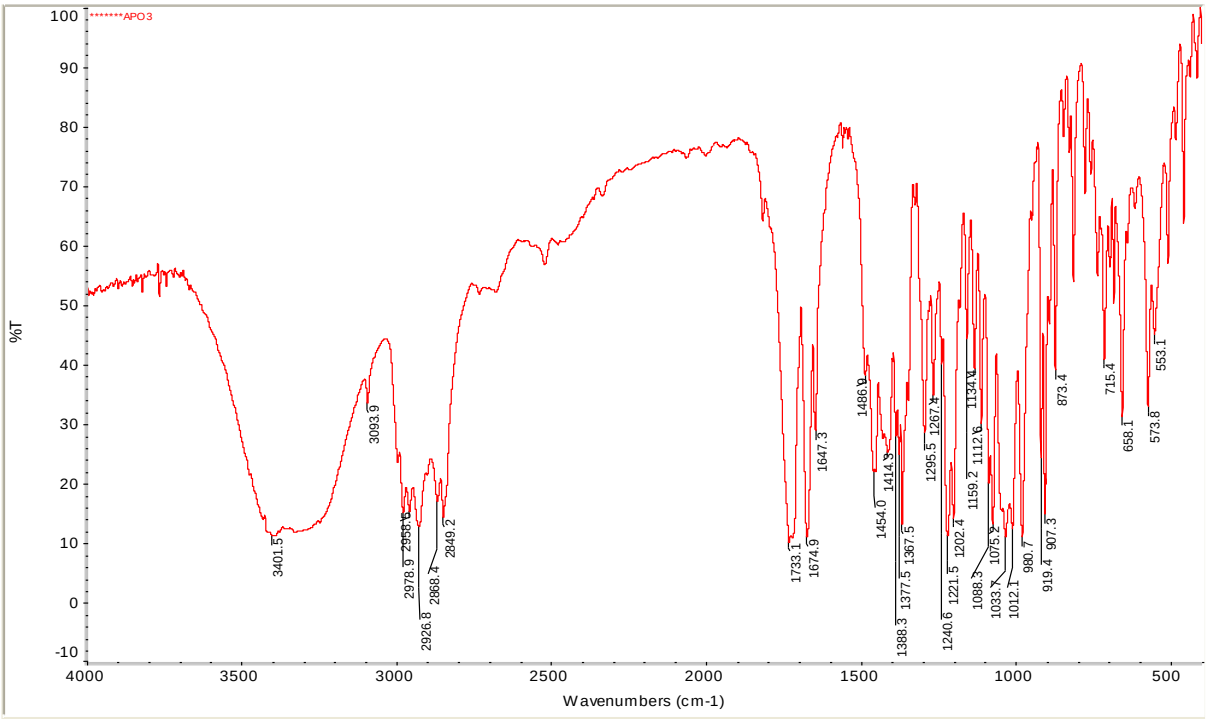


Figure 5: Infra-red spectrum of APO3 crystals obtained from *A. paniculata.*

Key: APO3 = crystal isolated from *A. paniculata*

Hydrogen, nitrogen, sulphur and oxygen analysis (HNSO), showed the percentage of carbon, hydrogen and oxygen in andrographolide obtained as 62.41, 8.04, and 0 %. However, this corresponds to the theoretical value of carbon and hydrogen (68.57, 8.57 %) respectively. Therefore, indicating some strong similarities between the isolated compound and andrographolide from *A. paniculata.*

CONCLUSION

This study demonstrates that column chromatography coupled with ^{1}H and ^{13}C NMR is efficient technique to isolate and confirm andrographolide from crude extracts of the whole of *A. paniculata* that is used as a medicinal plant. Therefore, the method could be used as means of quality control test of the active ingredient.

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CONFLICT OF INTEREST
The research declared no conflict of interest.

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