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ISOLATION OF THREE POLYMETHOXYLATED FLAVONES FROM *Ageratum conyzoides* L. GROWING IN CAN THO CITY

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ABSTRACT

This paper introduces the extraction and purification of some polymethoxylated flavones from Ageratum conyzoides L., (Family-Asteraceae), growing in Can Tho city. Different extraction methods were used to study on the aerial part of the herb. From 1% HCl in water extracts, three polymethoxylated flavones have been isolated and identified. They were O-methyl apigenin, sinensetin, and scutellarein tetramethyl ether. Structures of isolated compounds were elucidated according to their ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC, MS spectra as well as referring to published article.

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1 INTRODUCTION

Ageratum conyzoides L., commonly called Cỏ Hôi (Ho, 2000; Loi, 2004), was popular annual weed in many regions in the world including Vietnam (Ming, 1999). Because of vitality and significant adaptation, it can be found easily in the harvested farms, pastures, and wild lands with huge reserves.

In traditional medicine, *Ageratum conyzoides* was widely utilized systems wherever it grows, although applications vary widely by region (Nyemb *et al.*, 2009). A decoction of *A. conyzoides* is used for the treatment of pneumonia, and to cure wounds and burns. In India, it is also used as a bactericide, antidysenteric and antilithic. Whole plants have been used to treat colic, colds, and fevers, diarrhoea, rheumatism, spasms, orasa tonic (Xuan *et al.*, 2004). What is more, modern medicine proves that there are numerous pharmacological effects reported highlighted antiulcerogenic, analgesic, anti-inflammatory, anticataleptic, antidiabetic, antitumor, cytotoxic, hepatoprotective, anticonvulsant, radioprotective, antidotal, antioxidant, antiprotozoal, antimicrobial, anthelmintic, allelopathic, insecticidal, haematopoietic, wound healing, gastroprotective, uterine and bronchodilating potential of *A. conyzoides* (Dogra, 2015).

According to recent researches, a large number of bioactive chemical compounds have been found in *A. conyzoides* including sterols, flavonoids, terpenoids, lignans, pyrrolones, chromenes and pyrrolizidine alkaloids (Dogra, 2015). Within the group of flavonoids, there were reported polymethoxylated flavones (Okunade, 2002).

From the modern medical point of view, polymethoxylated flavones, a group of natural products, play vital role in the prevention of cancer, obesity and cardiovascular due to their antioxidant property (Atindehou *et al.*, 2013). In addition, they appear to correlate well with several pharmacological activities such as: anti-inflammatory (Huang *et al.*, 2010), cell growth inhibition in human neuroblastoma SH-SY5Y cells

(Akao *et al.*, 2008), prevent lipopolysaccharide-induced inflammatory bone loss (Tominari *et al.*, 2012), enhanced inhibition basophilic leukemia RBL-2H3 combination (Itoh *et al.*, 2008), induction of apoptosis in human cervical carcinoma HeLa cells (Kim *et al.*, 2010), and etc.

Evidences show that *A. conyzoides* is a potential herb that growing along the length of our country with great reserves; however, it has not been taken full advantages and studied extensively in Vietnam. Hence, research towards the extraction and isolation of bioactive products - polymethoxylated flavones - from *A. conyzoides* L., is necessary.

2 MATERIALS AND METHODS

2.1 Materials

Sample collection and preparation: 7 kg of *A. conyzoides* in flowering stage was harvested on the roadside of Nguyen Van Cu Street. After removing of chlorosis leaves, 2 kg of aerial parts was washed and cut into small pieces; the rest was dried at room temperature without exposure to direct sunlight for several days. Thereafter, dried grass was milled into fine powder and this powder was used for further steps of this research.

Petroleum ether (PE), chloroform, ethyl acetate (EA), and methanol were purchased from Chemsol, Vietnam. Hydrochloric acid was from China (Xilong). Silica gel 60 for column chromatography and silica gel F_{254} (0.2 mm thickness) for thin layer chromatography (TLC) were purchased from Merck (Germany). ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC spectra were measured on Bruker Avance 500 MHz.

2.2 Experimental procedure

Survey of the best extraction process: the structures of target molecules in this research could be converted into flavylium cations under acidic condition, so that polymethoxylated flavones are able to dissolve in acidified solution. This property directs us to choose the appropriate solvent. Besides acidified solutions (1% HCl in methanol, 1% HCl in H_2O), methanol, 1% NaOH in H_2O solution, and EA were used for comparison.

Fresh and dried samples were used and each of them was soaked in 4 different solvents (MeOH, 1% HCl in water, 1% HCl in MeOH, and EA) for 48 hours; however, sample in EA was basified by 1% NaOH solution before the extraction. The extraction processes are described and denoted in Table 1.

Table 1: Extraction process summary

Solvent	Symbol		
	General	Fresh sample	Dried sample
Methanol	A	A_1	A_2
1% HCl in H_2O	B	B_1	B_2
1% HCl in methanol	C	C_1	C_2
Soak basified sample in ethyl acetate	D	D_1	D_2

Extraction for column chromatography: 400 g of dried herb were soaked in 4 L 1% HCl in H_2O at room temperature for 48 hours. Filtered extraction was poured into the separatory funnel and shaked thoroughly with 8 L EA, combined extraction was evaporated under reduced pressure until semi-dried. Ethyl acetate solution was washed with 1% NaOH in H_2O (4×200 mL). Treated extraction was dried under vacuum with rotary evaporator, then kept in container for further analysis.

Column chromatography: 3.063 g of crude extract of flavones was subjected to column chromatography to separate the extract into its components fractions. Silica gel 60 was used as the stationary phase while PE:EA with gradual increase in polarity (7:4, 6:4, 5:5, 4:6, 3:7, 0:1) was the mobile phase. As the result, seven fractions were collected, numbered from no.1 to no.7.

Alk1 (12 mg), alk2 (7 mg) and alk3 (5 mg) were isolated from fraction no.7, no.6 and no.4, respectively after appropriate fraction was purified by column chromatography. For fraction no.7 and fraction no.6, a mixture of PE:EA 1:1 (v/v) was used as the mobile phase while PE:EA 7:4 (v/v) was used for fraction no.4. All steps are presented succinctly in the diagram below.

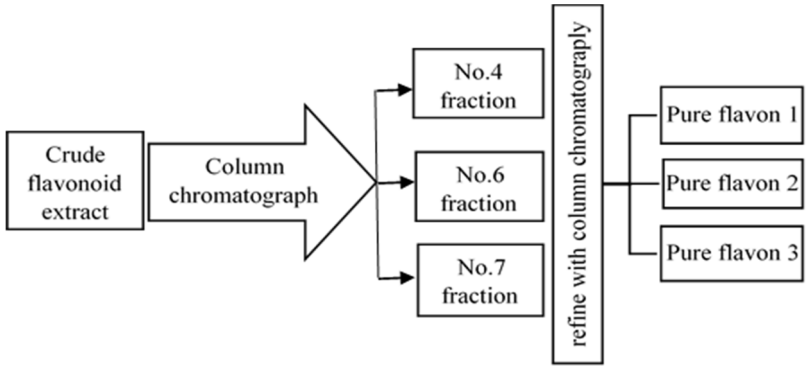


Fig. 1: Illustration of the experimental procedure

3 RESULTS AND DISCUSSION

3.1 Extraction process

Table 2 shows that all control processes (A, C, D) contain more impurities (more spots) than process B. Besides, process using dried sample extract more substances than the fresh ones in both A and C. In contrast, B and D are similar results in 2 types of sample.

Process B analysis: There were two big dark black spots at starting line, detected at 254 nm in both B_1 and B_2. That could be sugar, organic salt or hydrophilic molecule (called impurities); however, polymethoxylated flavone, which was in flavylium cation form, was able to dissolve in 1% HCl as well. Hence, these compounds were transferred from 1% HCl solution to ethyl acetate, then semi-dried under vacuum with rotary evaporator (notate as 1). Next, ethyl acetate solution was washed by 1% NaOH solution in order to eliminate impurities in 1% NaOH layer and collect polymethoxylated flavones (neutralised form) in ethyl acetate layer (notate as 2). Components of both (1) and (2) were examined by running TLC in PE:EA 4:7 (v/v) system. The result was shown in the Figure 3.

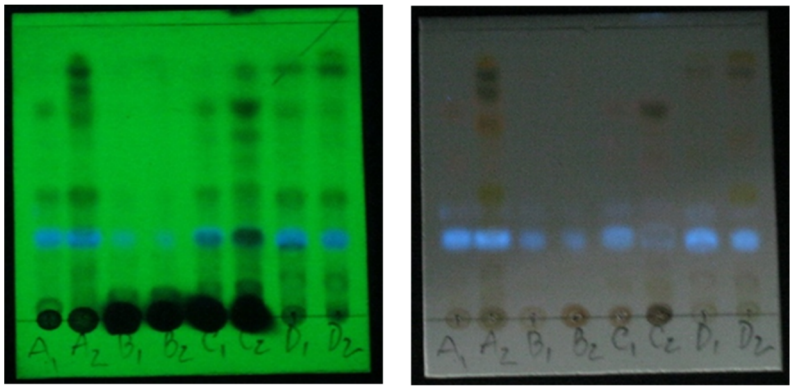
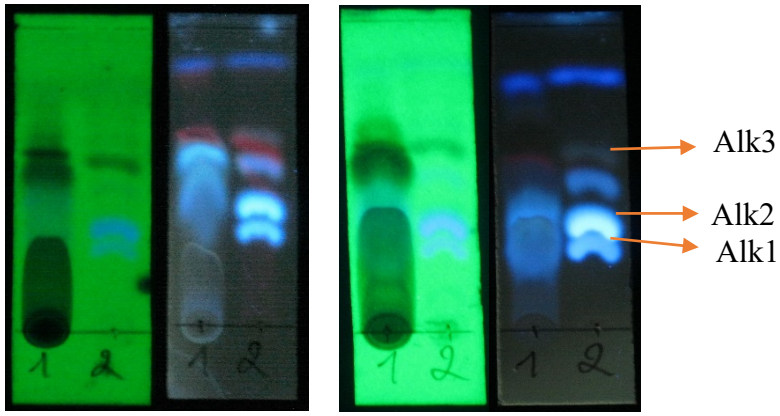


Fig. 2: TLC of all extractions is at 254 nm, 365 nm in succession

Table 2: Overview of TLC's results

Ordinal	Name of process	Observation	
		254 nm	365 nm
1	A_1	5 spots	Multiple spots, 2 distinct spots
2	A_2	Multiple spots, 6 distinct spots	Multiple spots, 5 distinct spots
3	B_1	1 large distinct spot	2 distinct spots
4	B_2	1 large distinct spot	2 distinct spots
5	C_1	Multiple spots, 4 distinct spots	Multiple spots, 2 distinct spots
6	C_2	Multiple spots, 6 distinct spots	Multiple spots, 3 distinct spots
7	D_1	Multiple spots, 6 distinct spots	Multiple spots, 4 distinct spots
8	D_2	Multiple spots, 6 distinct spots	Multiple spots, 5 distinct spots



Dried sample **Fresh sample**

Fig. 3: TLC of ethyl acetate extraction at 254 and 365 nm

It is clear that after washing by 1% NaOH, there are 4 spots (three of them are polymethoxylated flavone) instead of big dark black spot initially. It means impurities have been eliminated and flavylium cations have been neutralized, so HCl 1% in H_2O is the best solution for polymethoxylated flavone extraction.

Apply this process for column chromatography in order to extract polymethoxylated flavones as much as possible.

3.2 Structural identification

3.2.1 Alk1

Light-brown amorphous solid, fluoresced blue spot under 365 nm light (R_f = 0.45; EA:PE 9:1). It was well soluble in chloroform.

^{1}H-NMR spectrum of Alk1 (Table 3) revealed 5 signals of 7 aromatic protons in which two couple of them were equal chemical shift, it indicated the existence of at least one symmetric benzene ring.

^{13}C-NMR combine with DEPT, HMBC showed typical signals of a tri-substituted flavone backbone. Actually, excepting three methoxyl groups (δ_C 55.4, 55.7 and 56.3, confirmed by HMBC spectrum), there were 15 carbons of a flavone unit with symmetric B-ring. The carbon δ_C 177.6 was carbonyl (C-4), two signals δ_C 114.3 and 127.5 were 2 couples of magnetically equivalent methine carbons of B-ring. The signals δ_C 160.7, 160.8, 163.9, 159.8 and 162.0 were 5 oxygenated quaternary carbons, signals δ_C 109.1 and 123.8 were two aromatic quaternary carbons, the others were aromatic methine carbons of A and C-ring (Table 3).

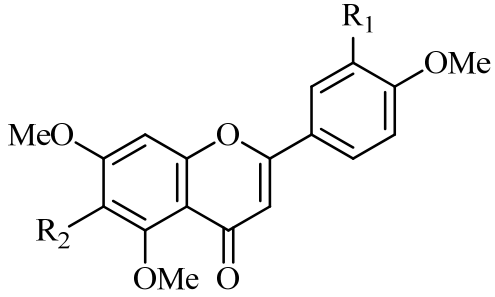
Table 3: NMR spectral data of Alk1 (δ) and reference compound ($\delta^{\#}$)

C/H	δ_H	$\delta^{\#}_H$	δ_C	$\delta^{\#}_C$	DEPT	HMBC
2			160.7	160.6	C	
3	6.58 (1H, *s*)	6.56 (1H, *s*)	107.6	107.6	CH	2, 4, 10, 1′
4			177.6	177.6	C	
5			160.8	160.7	C	
6	6.36 (1H, *d,* 2.0)	6.32 (1H, *d,* 2.0)	96.0	96.0	CH	5, 7, 8, 10
7			163.9	163.8	C	
8	6.54 (1H, *d,* 2.0)	6.51 (1H, *d,* 4.0)	92.8	92.8	CH	6, 7, 9, 10
9			159.8	159.7	C	
10			109.1	109.1	C	
1′			123.8	123.7	C	
2′, 6′	7.81 (2H, *d,* 9.0)	7.78 (2H, *d,* 9.0)	127.5	127.5	CH	2, 4′, 6′
3′, 5′	6.99 (2H, *d,* 9.0)	6.96 (2H, *d,* 9.0)	114.3	114.3	CH	1′, 4′, 5′
4′			162.0	161.9	C	
5-OMe	3.95 (3H, *s*)	3.92 (3H, *s*)	56.3	56.4	CH_3	5
7-OMe	3.90 (3H, *s*)	3.88 (3H, *s*)	55.7	55.7	CH_3	7
4′-OMe	3.87 (3H, *s*)	3.85 (3H, *s*)	55.4	55.4	CH_3	4′

Note: δ recorded in $CDCl_3$, 500/125 MHz; $\delta^{\#}$ recorded in $CDCl_3$, 400/100 MHz

The molecular formula of Alk1 was speculated to be $C_{18}H_{16}O_5$ (calcd. for 312 amu) on the basis of the ESI-MS (*m/z* 313 $[M+H]^+$) and above NMR spectral data.

As interpreting and comparing spectral data to those in reference (Gupta, 2010), Alk1 was identified as 5,7-dimethoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (*O*-methyl apigenin) (Figure 4). It was quoted as having antibacterial, antiplasmodial, radical scavenging, chemopreventive, and inhibiting 17β-hydroxysteroid dehydrogenase type 1 activities (Lee *et al.*, 2015).



Alk1: R_1=R_2=H
Alk2: R_1=R_2=OMe
Alk3: R_1=H; R_2=OMe

Fig. 4: Structure of three polmethoxylated flavones

3.2.2 Alk2

Light yellow amorphous powder, detection at 365 nm (blue spot; R_f = 0.25; chloroform). It was able to dissolve easily in chloroform.

1D-NMR spectra of Alk2 (Table 4) were basically similar to those of Alk1. However, Alk2 had two fewer protons and two more methoxyl groups than Alk1, instead. Alk2 also had two more oxygenated quaternary carbons and two less aromatic methine carbons than Alk1. It indicated that Alk2 was a penta-substituted flavone. Alk2 had no equivalent protons and carbons which proved that it was non-symmetric flavone.

Table 4: 1D-NMR spectral data of Alk2 (δ) and sinensetin ($\delta^{\#}$)

C/H	δ_H	$\delta^{\#}_H$	δ_C	$\delta^{\#}_C$	DEPT
2			161.2	162.1	C
3	6.61 (1H, *s*)	6.61 (1H, *s*)	108.7	108.4	CH
4			177.2	178.1	C
5			152.6	152.9	C
6			140.4	141.4	C
7			157.7	157.8	C
8	6.80 (1H, *s*)	6.81 (1H, *s*)	96.3	97.3	CH
9			154.5	154.8	C
10			107.4	108.1	C
1′			124.1	121.2	C
2′	7.33 (1H, *d*, 1.5)	7.35 (1H, *s*)	111.2	109.8	CH
3′			149.3	149.9	C
4′			151.8	150.3	C
5′	6.97 (1H, *d*, 8.5)	6.99 (1H, *d*, 8.5)	112.9	112.2	CH
6′	7.51 (1H, *dd*, 8.5, 2.0)	7.52 (1H, *d*, 8.5)	119.6	120.6	CH
$^{(*)}C_5$–OCH_3	3.92 (3H, *s*)	3.89 (3H, *s*)	62.2	63.2	CH_3
$^{(*)}C_6$–OCH_3	3.96 (3H, *s*)	3.94 (3H, *s*)	61.5	62.5	CH_3
$^{(*)}C_7$–OCH_3	4.00 (3H, *s*)	4.01 (3H, *s*)	56.3	57.3	CH_3
$^{(*)}C_{3'}$–OCH_3	4.00 (3H, *s*)	4.00 (3H, *s*)	56.2	57.2	CH_3
$^{(*)}C_{4'}$–OCH_3	3.98 (3H, *s*)	3.98 (3H, *s*)	56.1	57.1	CH_3

Note: δ recorded in $CDCl_3$, 500/125 MHz; $\delta^{\#}$ recorded in $CDCl_3$, 400/100 MHz

(*) These assignments may be interchanged

Chemical structure of Alk2 has been identified as 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one (sinensetin) (Figure 4) by using NMR results in comparing with published data (Yam *et al.*, 2010). Chemical formular: $C_{20}H_{20}O_7$. It was quoted as having anticancer, antioxidant properties and in preventing obesity (Atindehou *et al.*, 2013).

3.2.3 Alk3

Green amorphous solid, detection at 365 nm (dark spot, R_f = 0.27, chloroform), It was well soluble in chloroform.

1D-NMR spectra of Alk3 (Table 5) were similar to Alk1. It was also a tetra-substituted and symmetric flavone. The chemical structure was confirmed by the existence of couples of magnetically equivalent protons and carbons, 4 methoxy groups, 1 carbonyl group, 6 oxygenated quaternary carbons and comparing to reference data (Sunhee *et al.*, 2013).

Table 5: 1D-NMR spectral data of Alk3 (δ) and scutellarein tetramethyl ether ($\delta^{\#}$)

C/H	δ_H	$\delta^{\#}_H$	δ_C	$\delta^{\#}_C$	DEPT
2			161.1	160.3	C
3	6.80 (1H, *s*)	6.70 (1H, *s*)	107.0	106.1	CH
4			177.2	175.6	C
5			152.5	151.6	C
6			140.3	139.7	C
7			157.6	157.4	C
8	6.58 (1H, *s*)	7.20 (1H, *s*)	96.2	97.3	CH
9			154.5	153.9	C
10			112.8	112.0	C
1′			123.8	123.0	C
2′, 6′	7.82 (2H, *d*, 9)	8.01 (2H, *d*, 9)	127.6	127.8	CH
3′, 5′	7.00 (2H, *d*, 9)	7.10 (2H, *d*, 9)	114.4	114.5	CH
4′			162.1	161.9	C
C_5–OCH_3	3.92 (3H, *s*)	3.80 (3H, *s*)	62.2	61.8	CH_3
C_6–OCH_3	3.88 (3H, *s*)	3.77 (3H, *s*)	61.5	61.0	CH_3
C_7–OCH_3	3.99 (3H, *s*)	3.95 (3H, *s*)	56.2	56.4	CH_3
$C_{4'}$–OCH_3	3.98 (3H, *s*)	3.90 (3H, *s*)	55.4	55.5	CH_3

Note: δ recorded in $CDCl_3$, 500/125 MHz; $\delta^{\#}$ recorded in $CDCl_3$, 400/100 MHz

Alk3 was finally identified as scutellarein tetramethyl ether [5,6,7-trimethoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one] (Figure 4). Chemical formula: $C_{19}H_{18}O_6$. It was anti-inflammatory (Pandith *et al.*, 2013), and quoted as having anticancer, antioxidant properties and in preventing obesity (Atindehou *et al.*, 2013).

4 CONCLUSIONS

In this research, three pharmalogical natural products have been identified from *Ageratum conyzoides* collected in Can Tho city, they are *O*-methyl apigenin, sinensetin, and scutellarein tetramethyl ether. Using inorganic solution (1% HCl in water) as primary solvent, it is not only cheap, safe to people and the environment, but also efficient for the extraction of polymethoxylated flavones.

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