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Different Extraction Methods of Colchicine from Flowers of Daylily

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Abstract—The news media and network information often reported that " fresh daylily are toxic". The aim of this study was to extract colchicine from fresh daylily by traditional organic solvent extraction (OSE) and Supercritical fluid extraction (SFE) respectively. The *colchicine* was analyzed by High performance liquid chromatography (HPLC) and Liquid chromatography-tandem mass spectrometry (LC/MS/MS).The daylily flowers were extracted with different solvents such as 95% methanol, 95% ethanol, petroleum ether and methylene chloride, and supercritical fluid extraction. The extracts were analyzed by HPLC-PDA and *colchicine* could not be detected. However, the analysis of trace amounts of petroleum ether and methylene chloride extracts of the daylily flowers by LC/MS/MS can detect trace *colchicines.* It was shown that the *colchicin*e was extracted with petroleum ether/dichloromethane, and then analyzed by LC/MS/MS to determine the *colchicine* content effectively. The recovery rate of this analytical method is 58% and the minimum detectable limit is 0.1 ppb.

***Keywords*—Daylily flowers, *Colchicine*, Extract.**

I. INTRODUCTION

Colchicine is a tricyclic, lipid-soluble alkaloid with a long terminal half-life (20 to 40 hours) and molecular weight of 399.437. *Colchicine* has the effect of inhibiting cell mitosis, primarily for gout treatment [1]-[3].

The early and reversible stage of *colchicine* toxicity (nausea and diarrhea) is well known to clinicians [4], [5], The first oral dose of *colchicine* <0.5 mg / kg causes diarrhea or vomiting in up to 15% of patients. [3], [6].

Daylily flower (the flower of *Hemerocallis fulva L.*) is traditionally used for soothing in Chinese dietary therapy. that has antibacterial [7], antioxidant [8], nitrite-eliminating activities [9], and antidepression [10].

The aim of this study was to extract *colchicine* from fresh daylily by traditional organic solvent extraction (OSE) and Supercritical fluid extraction (SFE) respectively. The colchicine were analyzed by High performance liquid chromatography (HPLC) and Liquid chromatography-tandem mass spectrometry (LC/MS/MS).

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II. MATERIALS AND METHODS

A. Plants materials

The daylily flowers bud (Taitung 6 ; TD6) were plucked from Taitung District Agricultural Research and Extension Station, Taiwan, at the day before flowering. The mixture was lyophilized at -60 ° C for 48 hours and the dried sample was pulverized and passed through a sieve to provide a homogeneous powder for analysis. The powdery material was maintained at -20 ° C until analysis.

B. Organic solvent extraction of colchicine

1) Methanol extraction (ME) and ethanol extraction (EE) of *colchicine*

2 g of the sample powder were immersed in 40 ml of 95% methanol [11] or 95% ethanol [9] at 25 ± 2 ° C. Ultrasonic shaker for 30 minutes, filtered by suction, filtered by Whatman # 1. filter paper. The filtrate was concentrated under reduced pressure and set to 70% ethanol to 1 ml. The extracts were sealed in brown glass bottles and stored at -20 °C for further use.

2) Petroleum ether extraction (PEE) and dichloromethane extraction (DE) of *colchicine*

Dried and powdered (2.5g) were defatted by shaking with 125ml of petroleum ether in closed Cone bottle (250mL) for 60min [12]. Followed by suction filtration, Filter by Whatman # 1. filter paper, repeat this operation once, mix the filtrate twice. The filtrate was concentrated under reduced pressure (BUCHI Rotavaper R-200/205, Switzerland) and then packed in 70% ethanol to 1 ml, stored in a brown bottle, frozen and kept; the remaining solid added after filtration was added to 50 ml of Chloromethane extraction, shake at room temperature for 30 minutes, add 10% ammonia 2.5 ml, shake at room temperature for 10 minutes, put it aside for 30 minutes, filtered by suction, Whatman # 1. filter paper filter, once again using 50 ml of dichlor Methane is cleaned twice, filtered by suction, filtered by Whatman # 1. filter paper. The sample solution was concentrated under reduced pressure (BUCHI Rotavaper R-200/205, Switzerland) and fixed in 70% ethanol to 1 ml, stored in a brown bottle and frozen. An aliquot of the extract was filtered through an 0.22μm before the HPLC analysis.

C. *Supercritical-fluid extraction (SFE) of colchicine*

In this study, all SFE-CO2 extractions were performed using an Applied Separations supercritical fluid extractor (Speed SFE, Allentown, PA). Ethanol was chosen as the extraction solvent because it is safer and more environmentally friendly than other organic solvents. Approximately 6 g of powder from the lyophilised daylily flowers was mixed with 25 ml 95% ethonal, and was placed in the extractor vessel (capacity: 50 ml). fitted with a plug of polypropylene wool at the top and bottom of the vessel to provide a continuous flow of CO_2. The SFE- CO_2 based extraction process consisted of static extraction for 25 min followed by dynamic extraction for 25 min. Supercritical-fluid extractor with CO_2 at 35ºC and pressures of 3700 psi. The CO_2 flow rate was maintained at a constant value of 4 mL/min. All samples were collected in brown bottles to prevent UV-activated degradation. Each sample was extracted three times at the specified temperature and pressure. Ten milligrams of the extract was dissolved in 1 mL of ethanol for HPLC analysis; the remainder was stored in the dark under -20°C [13], [14].

D. HPLC analysis

1) HPLC determination of *colchicine*

The HPLC analysis conditions were: Li Chrospher C18 (250 mm x 4 mm, Merk) [13], The TD6 Extract was filtered through a 0.22 μm membrane, then run through HPLC for analysis. The mobile phase consisted of acetonitrile and water using the linear gradient programed described in TABLE I. the absorbance was measured at a detection wavelength of 350 nm and The flow-rate was 1ml/min and the injected volume was 20 μl. The detection limit (DL) for *colchicine* was calculated based on a signal-to noise ratio (S/N) of 0.6 ppm.

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TABLE I
THE LINEAR GRADIENT PROGRAMME FOR HPLC ANALYSIS

Time (min)	Acetonitrile (%)	D.I. water (%)
1.0	10	90
12.0	30	70
18.0	70	30
19.0	70	30
20.0	90	10
25.0	90	10
25.1	10	90
30.0	10	90

2) HPLC determination of *Gloriosia superb* of the bulb

In *Gloriosia superb* other *colchicine* derivatives such as lumicolchicine, 3-demethyl-N-deformyl-N-deacetylcholine, 3-demethylcolchicine, N-formyl deacetylcholine and others have been isolated [15].

In order to confirm the feasibility of *colchicine* extraction method, *colchicine* containing *Gloriosia superb* of the bulb with petroleum ether and methylene chloride extraction, extraction of the sample and then by HPLC analysis to determine this extraction method can be *colchicine* was detected, and then extracted by the same method of extraction of daylily samples, and HPLC analysis, and then determine whether the daylily contains *colchicine.*

3) HPLC determination of Thiocolchicoside

Thiocolchicine is a semisynthetic chalcone derivative containing sulfur derived from *Gloriosia superb* flower seeds _[16], [17]. Colchicoside is the precursor of thiocolchicoside, a semisynthetic derivative with a relaxant effect on skeletal muscle[18].

E. LC/MS/MS determination of colchicine

The mass spectrometer is a positive ion mode electrospray ionization using a three-stage tandem quadrupole mass spectrometer with multiple Reaction Monitoring. Parameters for the three-stage tandem mass spectrometer were set as follows: Curtain Gas: 15.00, Collision Gas: 4.00, IonSpray Voltage: 5.5 kV, Temperature: 550.00 ° C, Ion Source Gas1 55.00 psi, Ion Source Gas2 55.00 psi. Detection of ion pairs 358 and 282 were quantitative and qualitative. The detection limit (DL) for *colchicine* was calculated based on a signal-to noise ratio (S/N) of 0.1 ppb.

F. Preparation of standard curve

Five amounts (1、2、3、4、5 ppm) of *colchicine* were injected into HPLC; Five amounts (0.5、1、5、10、25 ppb) of *colchicine* were injected into LC/MS/MS; the linear regression equation for each standard curve was obtained by plotting the amount of standard compound injected against the peak area. The regression equation and correlation coefficient (r2) were calculated using a Waters 2996 PDA.

For the purpose of analyzing the recovery rate of *colchic*um in the flowers, refer to the method of Alali (2004) for extraction [12], using the Native species flower as a substrate and adding 1 ml of 0.1 μg/mL and 1 ml during the extraction process. The 1 mg/L *colchicine* standard was used to determine the recovery of the *colchicine* standard.

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G. Statistical analysis

The standard calibration equations for *colchicine*, recoveries and quantitative analyses were performed in triplicate and the mean values were calculated.

III. RESULTS AND DISCUSSION

1) Total *Gloriosia superb* of the bulb content by HPLC

The *Gloriosia superb* of the bulb was extracted with petroleum ether (GB-PEE) and dichloromethane(GB-DE), and the extracted sample was analyzed by HPLC. In Fig. 1, a peak appeared at 16 minutes, and the retention time and the UV scan pattern were compared with the standard (Fig. 2). Therefore, it was found that the extraction method and the analysis conditions were detectable for *colchicine*.

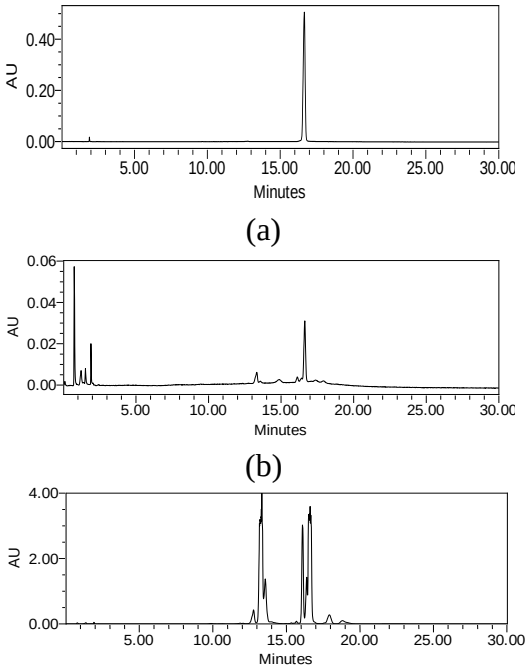
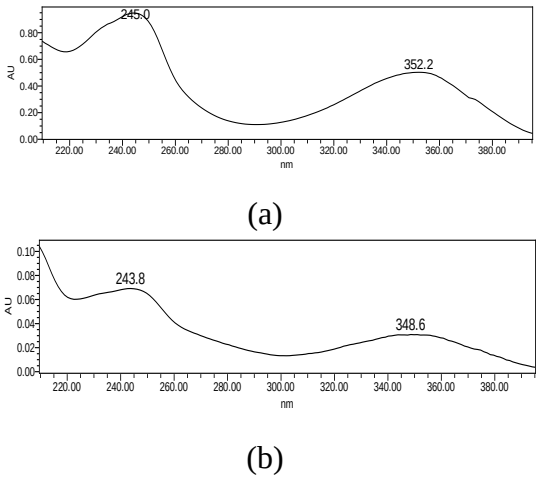


Fig. 1 HPLC chromatograms . (a) *colchicine* standard. (b)GB-PEE (c)GB-DE.



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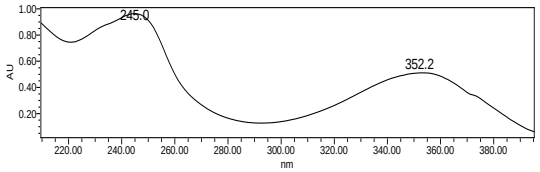
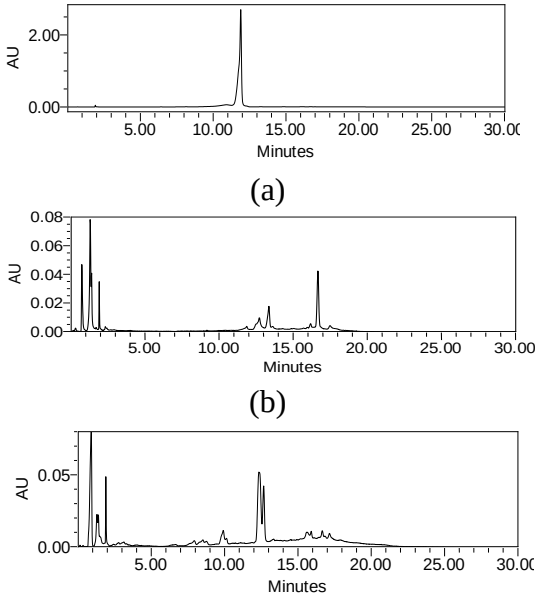


(c)

Fig. 2 UV scan of HPLC chromatograms. (a) *colchicine* standard. (b)GB-PEE (c)GB-DE.

2) Total thiocolchicoside content of daylily flowers bud extract by HPLC

It can be seen that the thiocolchicoside standard exhibits a peak at about 12 minutes, and the absorption wavelengths are 258 nm and 378 nm (Fig. 3a). In the chromatogram of a petroleum ether extract of TD6 (Fig. 3b), peaks appear at the same time, but it is understood from Fig. 4 that the absorption wavelength is not the same. The TD6 methylene chloride extract shows a peak at 12 minutes (Fig. 4b), but it is known from Fig. 4c that TD6 dichloromethane extract and thiocolchicoside standard. The comparison of UV scans of the product shows that the absorption wavelength is not the same. Therefore, it is presumed that the material analyzed by HPLC may have the same retention peak as the thiocolchicine standard, but it is not thiocolchicoside.

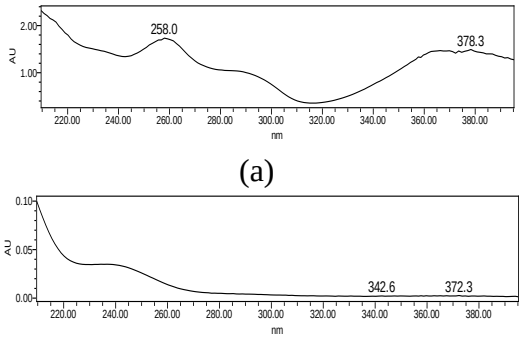


(a)

(b)

(C)

Fig. 3 HPLC chromatograms . (a) thiocolchicoside standard. (b)TD6-PEE (c)TD6-DE.



(a)

(b)

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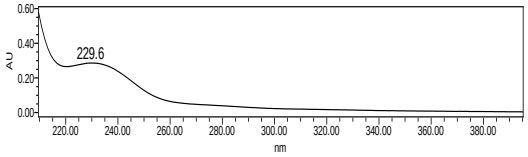
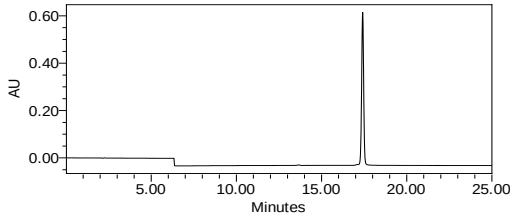


(c)

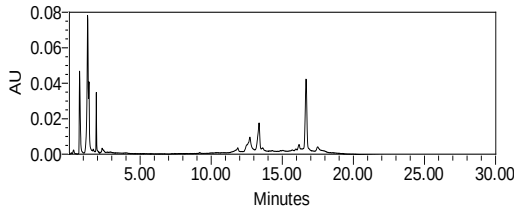
Fig. 4 UV scan of HPLC chromatograms. (a) thiocolchicoside standard. (b)TD6-PEE(c)TD6-DE

3) Total *colchicin*e content of daylily flowers bud extract by HPLC

In this study, extraction of daylily takes four types of extraction solvents including 95% Methanol (MeOH), 95% Ethanol (EtOH), Petroleum ether / dichloromethane (PE/CH_2Cl_2) and supercritical-fluid extractor (SFE) were prepared. Afterwards the *colchicine* compounds in the extracts



(a)



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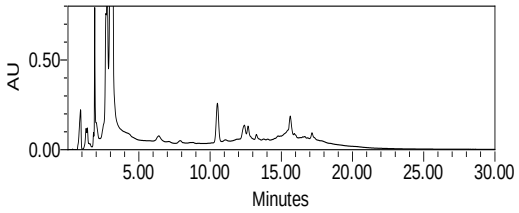

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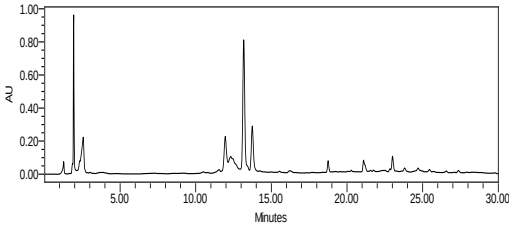
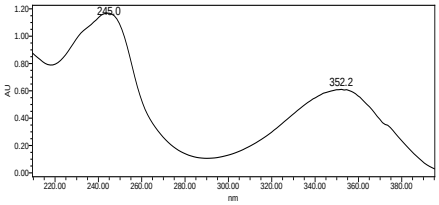


(d)

Fig. 5 HPLC chromatograms . (a) *colchicine* standard. (b) TD6-PEE. (c) TD6-DE. (d) TD6-SFE.



(a)

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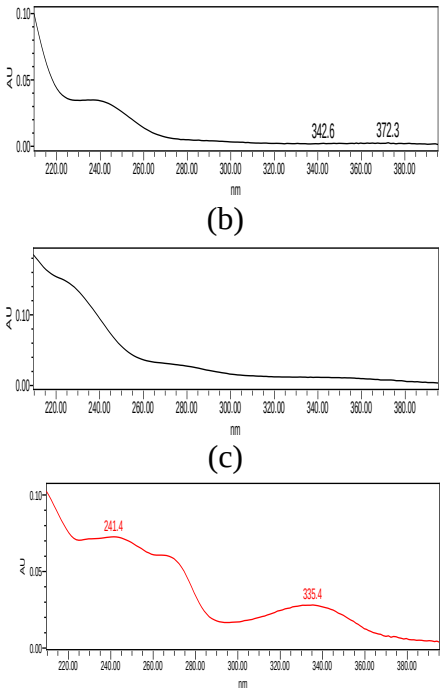

Fig. 6 UV scan of HPLC chromatograms. (a) *colchicine* standard. (b). TD6-PEE. (c) TD6-DE. (d) TD6-SFE.

were identified by HPLC. Colchicine compounds in extracts from daylily flowers HPLC identification of *colchicine* compounds was done by comparing the retention time and UV spectral matching with authentic standards. The colchicine standard was successfully appeared at 17-18 min after the injection of the sample (Fig. 5a), Absorption wavelength of 245nm and 352nm (Fig. 6a). MeOH (Data not shown) and SFE in 17-18 minutes there is no significant retention between the peak (Fig. 5d), EtOH (Data not shown) and PE+CH2Cl2 are 17-18 minutes between the peak (Fig. 5b and 5c), but the UV spectral and standard comparison, the peak is not colchicine (Fig. 6b and 6c). So the use of HPLC-UV or PDA analysis can't be completely detected daylily flowers contained in *colchicine*.

The extract of TD6 daylily three day flowers was extracted with methanol, ethanol, acetone, chloroform and dichloromethane, and the extracts were analyzed by HPLC. The test results show that the above extract did not appear with the standard of *colchicine* retention peak speculated that the lack of *colchicine*, and the results of this test is not much different. [23]. The use of water and different concentrations (30%, 70%, 100%) ethanol extract daylily, HPLC analysis of *colchicine*, the results show that the best use of 100% ethanol extraction, measured daylily *colchicine* content. It is speculated contains about 5ppm colchicine .The results only show the HPLC chromatogram of the ethanol extract and do not confirm the UV scan of the ethanol extract further [9].

SFE studies involving alkaloids such as thebaine, codeine and morphine from poppy [19], vindoline from Catharanthus roseus [20], and the purine alkaloids from herbal mate tea, Ilex paraguariensis [21]. *colchicine* was extracted from the bulbs of lily by using Supercritical fluid extraction (SFE) and traditional organic solvent extractlon (OSE) respectively, the total *colchicine* in the SFE extract (0.0485%) was a little more than that in the OSE extract (0.047%). It could be attributes to the higher extraction efficiency of SFE [22].

(4) Total *colchicine* content of daylily flowers bud extract by LC/MS/MS

Because the HPLC - UV method could not be used to detect the *colchicine* contained in the flower, it was extracted with different solvents (petroleum ether / dichloromethane, 95% methanol and 95% ethanol). The sample was analyzed by LC / MS / MS. It can be seen that the retention peak of *colchicine* standard appeared

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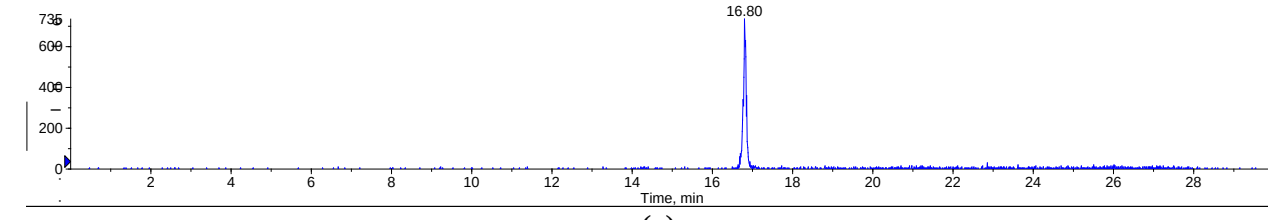
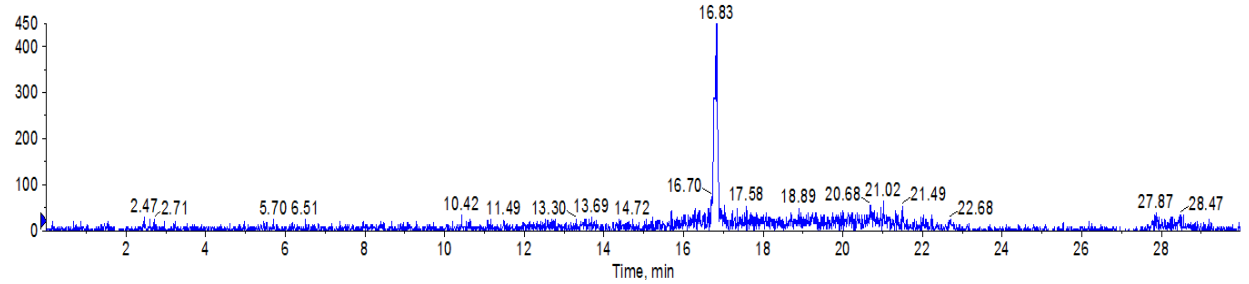
at about 16.8 minutes (Fig. 7a). The mass spectrum chromatogram of TD6 was extracted with petroleum ether / dichloromethane in 16.8 minutes also appeared retention peak (Fig. 7b and 7c), Make sure the substance is *colchicine*. In TD6 methanol and ethanol extract there is no retention peak (Data not shown), suggesting that the use of 95% methanol and 90% ethanol can't extract the *colchicine*

Extracted from petroleum ether / dichloromethane in TD6, an be extracted in day flower of *colchicine*, the content of up to 2.23ppb, but the use of MeOH and EtOH can not be extracted, to further understand whether the daylily harvest in different buds of different changes in the content of *colchicines*, so the petroleum ether / dichloromethane, MeOH and EtOH extract TD6, two day flower and three day flower, TABLE II. the results are similar to those of the extract of TD6 day flower.



(a)



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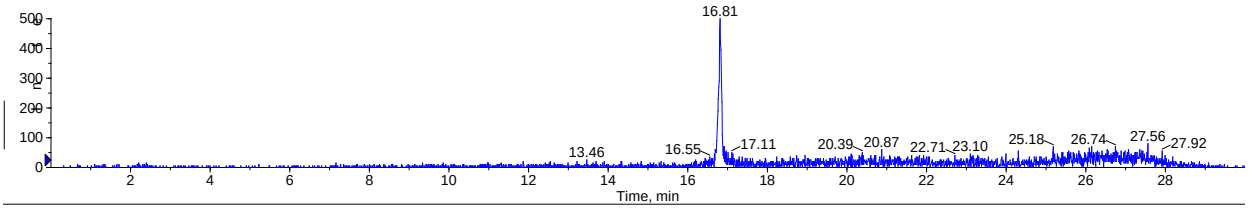

(c)

Fig.7. LC/MS/MS chromatograms . (a) *colchicine* standard. (b) TD6-PEE. (c) TD6-DE.

TABLE II

DETERMINATION OF *COLCHICINE* CONTENT IN TD6 FLOWER BY DIFFERENT EXTRACTION METHODS (ppb)

Extraction method	**day flower**	**two day flower**	**three day flower**
PE+CH_2Cl_2	2.23±0.18	0.56±0.10	0.55±0.01
MeOH	N.D. [a]	-	-
EtOH	N.D. [a]	-	-

a. N.D, not determined

The results showed that the content of *colchicine* could be determined by LC / MS / MS. The extraction method was the best with petroleum ether / dichloromethane. From this, it is speculated that the content of *colchicine* may be too low, less than HPLC-UV detection limit of 0.6ppm, leading to not be able to use

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HPLC-UV or HPLC-PDA method for the analysis of *colchicine* contained *colchicine*. Xiao (2004) using LC / MS / MS analysis of daylily acetone extract, the results show that the retention of the peak *colchicine* standard peak is about 13 minutes, but the daylily acetone extract did not appear peak at 13 minutes, and the standard extract of daylily the MS^2 ion fragment is not the same, which concludes that daylily flower does not contain *colchicine* [23]. Annika (2010) extracted daylily from methanol extraction and analyzed from the daylily using LC / MS / MS. However, *colchicine* from daylily was not detected. The results of the two tests differ greatly from the results of this study, suggesting the differences caused by the different extraction methods [24].

In different buds of the harvest, the *colchicine* content of day flowers compared with the two day flowers and three day flowers, *colchicine* day flower highest content of 2.23ppb (TABLE **II)**.

TABLE III
ADDITION OF *COLCHICINE* STANDARD 1 ml 0.1μg/mL AND 1 ml 1 mg/L

Recovery (%), n=3					
	PE	**CH_2Cl_2-1st**	**CH_2Cl_2-2nd**	**CH_2Cl_2-3rd**	**Total**
0.1μg/mL	4.27	52.64	1.27	0.68	58.86
1 mg/L	4.07	51.08	1.01	0.36	56.53

IV. CONCLUSION

Because of the very low content of the daylily flowers *colchicine*, it can not be used for quantitative analysis by HPLC-UV method. In this study, the determination of the content of *colchicine* in the daylily flowers by LC / MS / MS was established by extraction with petroleum ether / dichloromethane, Petroleum ether and methylene chloride extracts were collected and analyzed by LC / MS / MS. The recovery was 58% and the detection limit was 0.1 ppb (TABLE **III)**.

According to the European Medicines Agency *colchicine* assessment report, the body intake of 0.1mg / kg will have gastrointestinal discomfort. The results of this study showed that 60 kg of adult, for example, about 3kg TD6 fresh daylily buds may cause gastrointestinal discomfort, suggesting that consumption of daylily buds caused by factors other than *colchicine*, speculated that the flower bud may contain other alkaloids cause diarrhea, it is recommended that future toxicity testing against plants and buds of daylily, to confirm the cause of diarrhea ingredients.

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