PRODUCTO NATURAL

Harvest time influences on coumarin and umbelliferone contents in extracts of *Justicia pectoralis* Jacq. (tilo)

Influencia del tiempo de cosecha sobre el contenido de cumarina y de umbeliferon hallado en los extractos de *Justicia pectoralis* Jacq. (tilo)

Dr.C. Jorge Enrique Rodríguez Chanfrau, Téc. Carlos Rodríguez Ferrada

Centro de Investigación y Desarrollo de Medicamentos (CIDEM). La Habana, Cuba.

ABSTRACT

Introduction: *Justicia pectoralis* Jacq. (Acanthaceae) is medicinal plant species commonly used in Cuba for the treatment of nervous disorders because of its sedative effect. Coumarin is one of its main active phytochemicals present in the extracts obtained from this plant and used as analytic marker in quality control. On the other hand, this compound contributes to the sedative effect attributed to this plant.
Objective: to evaluate the influence of harvest time on the coumarin and umbelliferone (7-hydroxycoumarin) in J*usticia pectoralis* extracts.
Methods: the experiment lasted two years. The harvest was performed at 4, 6 and 8 months after planting. Aqueous and hydroalcohol extracts were produced and the coumarin and umbelliferone contents were determined by high resolution liquid chromatography.
Results: the achieved results showed the presence of coumarin and umbelliferone in both extracts. Both methods can be used for the extraction of these components from the plant, although in the case of umbelliferone, the best extraction results were achieved by using aqueous extract. In both cases, the recovery percentages were more than 98 %. This study confirmed that the harvest time significantly influences on the coumarin and umbelliferone contents.
Conclusions: the best results are observed in the first two harvests (4 and 6 months at summer time), which indicates that the industry should process the vegetal material in these two periods of the year.

Keywords: coumarin, HPLC, influence of harvest time, *Justicia pectoralis* Jacq., umbelliferone.

RESUMEN

Introducción: *Justicia pectoralis* Jacq. (Acanthaceae) es una planta medicinal comúnmente usada en Cuba para el tratamiento de enfermedades nerviosas por su efecto sedante. La cumarina es uno de los fitocomponentes mayoritarios en los extractos obtenidos con esta planta y empleado como marcador analítico en los controles de calidad. Por otro lado, este componente contribuye con el efecto sedante atribuido a esta planta.
Objetivo: evaluar la influencia del tiempo de cosecha sobre el contenido de cumarina y umbelliferona (7 hidroxicumarina) en extractos de *Justicia pectoralis*.
Métodos: se desarrolló el experimento durante 2 años. Se realizaron las cosechas a los 4, 6 y 8 meses de plantada. Se elaboraron extractos acuosos e hidroalcohólicos y se determinó el contenido de cumarina y umbelliferone por cromatografía líquida de alta resolución.
Resultados: se mostró la presencia de cumarina y umbelliferona en ambos extractos. Además, en el caso de la umbelliferona, los mejores resultados se alcanzaron al aplicar extracción acuosa. En ambos casos, los por cientos de recobrados fueron superiores al 98 % Se confirmó que el tiempo de cosecha influyó significativamente sobre el contenido de cumarina y umbelliferona.
Conclusiones: los mejores resultados se obtienen en la primeras dos cosechas (4 y 6 meses que coincide con el verano), lo que sugiere que el material de la planta debe procesarse por la industria en esos periodos del año.

Palabras clave: cumarina, CLAR, influencia del tiempo de cosecha, *Justicia pectoralis* Jacq., umbelliferona.

INTRODUCTION

The medicinal plants have been used during centuries for the treatment of diverse illnesses due to phytochemical active contents. Among these components, the coumarins have been identified to have anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, sedative and anticarcinogenic activities.[1-5]

Coumarin (1, 2-benzopyrone), the parent molecule of coumarin derivatives, is the simplest compound of a large class of naturally occurring phenolic substances formed by the fusion of benzene and α-pyrone rings. On the other hand, some studies have been published on the umbelliferone (7-hydroxycoumarin) as main active metabolite of coumarin. Both compounds are present in the *Justicia pectoralis* Jacq., commonly known in Cuba as Tilo and used in the treatment of nervous affections by its sedative effect.[2,3,5-9] The cultivation of this plant in Cuba can be done any time of year, but the best results are obtained during the months of December to March.[10]

Technological processes to obtain dry extract from hydroalcoholic and aqueous extracts of *J. pectoralis* Jacq. by spray dried were developed.[11,12] Coumarin is the main component in theses extracts and was used as analytic marker in quality control. The objective of this work was to evaluate the influence of harvest time on the coumarin and umbelliferone contents in *J. pectoralis* Jacq. extract.

METHODS

PLANT MATERIAL

The aerial parts of *J. pectoralis* (var. pectoralis) were collected in the Experimental Station of Medicinal Plants "Dr. Juan Tomás Roig", in Artemisa, Cuba. Herbarium specimens was marked 4636 and deposited in herbaria at the Experimental Station. The experiments were developed during two years. The harvests were carried out at 4, 6 and 8 months after planting the *J. pectoralis* Jacq. The vegetal drugs were washed with H_2O and 2 % sodium hypochlorite solution (SHS), dried at 45 ºC, and stored in nylon bags until its use. In parallel, the quality control was carried out according to method described.[10,13]

PREPARATION OF PLANT EXTRACTS

A sample of dry material was used to elaborate hydroalcoholic and aqueous extracts. Thirty percent of hydroalcoholic extract was obtained by repercolation method (4 extractions, drug/solvent ration 1:20) according to *Sánchez et al*.[13] The aqueous extract was obtained by the decoction method (drug/solvent ration 1:20 and an extraction time of 15 min) according to *Rodríguez et al*.[11]

PREPARATION OF SAMPLE SOLUTIONS

In all experiments we determined the amount of coumarin and umbelliferone extracted. For coumarin, 2 mL of the extract were dissolved in 100 mL of methanol. Then 1 mL of this solution was dissolved in 25 mL of mobile phase and conserved for analytical determination. In the case of umbelliferone, 2 mL of the extract were dissolved in 100 mL of mobile phase and conserved for analytic determination.

The total content was expressed as g/100 g dry matter content in the extract and mg/100 g of dry matter content in the extract of coumarin and umbelliferone, respectively.

HPLC ANALYSIS

The coumarin content was determined in the extracts by HPLC with UV detection according to a previously reported method with some modifications.[14] The detector was used at 274 nm which corresponds to the maximum absorption value experimentally founded for the coumarin standard RS (Aldrich Chemical Co.) An aliquot of the sample solution were filtered through a 0.45 µm syringe filter prior to HPLC-UV analysis. Coumarin was separated using a Lichrospher® 100, RP 18 column (5 µm, 250 mm × 4 mm, Merck, Germany). The solvent flow rate was 1 mL/min and the mobile phase was composed of methanol**:** water (40:60). A calibration curve (6 points between 5 and 40 µg**/**mL) to quantify coumarin content was used. The analysis was carried out in triplicate. The injection volume was 20 µL each time.

The umbelliferone was determined in the extracts by HPLC with UV detection according to a previously reported method with some modifications.[15] The detector was used at 330 nm which corresponds to the maximum absorption value experimentally founded for the umbelliferone standard RS (Aldrich Chemical Co.)**.**

An aliquot of the sample solution were filtered through a 0.45 µm syringe filter prior to HPLC-UV analysis. Umbelliferone was separated using a Lichrospher® 100, RP 18 column (5 µm, 250 mm × 4 mm, Merck, Germany). The solvent flow rate was 1 mL/min. and the mobile phase was composed of methanol:KH_2PO_4 buffer at pH 2.5:Tetrahidrofurane:Acetonitrile (20:150:17:10 v/v). A calibration curve (6 points between 0.5 and 10 µg/mL) to quantify umbelliferone content was used. The analysis was carried out in triplicate. The injection volume was 20 µL each time.

Dry matter content and pH were determined according to Farmacopeia Brasileira.[16]

INTERFERENCE TEST

Previously, an interference test was conducted to know if HPLC analytical procedures would be selective. For this, an amount equivalent to 10 µg/mL and 5 µg/mL of coumarin and umbelliferone, respectively were mixed before injection. The chromatograms applying both analysis methods were carried out.[17]

RECOVERY TEST

From each extract, a sample solution was divided into four parts. One of these was used as a control and the rest were added known quantities of coumarin and umbelliferone reference standards at following concentrations: 10, 20, and 30 µg/mL. The recovery percentage was determined.[17]

STATISTICAL ANALYSIS

All experimental results were expressed as mean/standard deviation (SD) and assessed by an analysis of variance (ANOVA), afterwards, a Ducan test was carried out. The results were considered significant when $p < 0.05$.

RESULTS

The vegetal drugs compliment the quality specifications established. Table 1 shows the analytical results of the extracts obtained from the vegetal matter harvested at three different times. A transparent brownish gray liquid with fragrant characteristic was obtained to the aqueous extract. The dry matter content and pH of the extract was 4.2/0.7 % and 6.3/1.0, respectively.

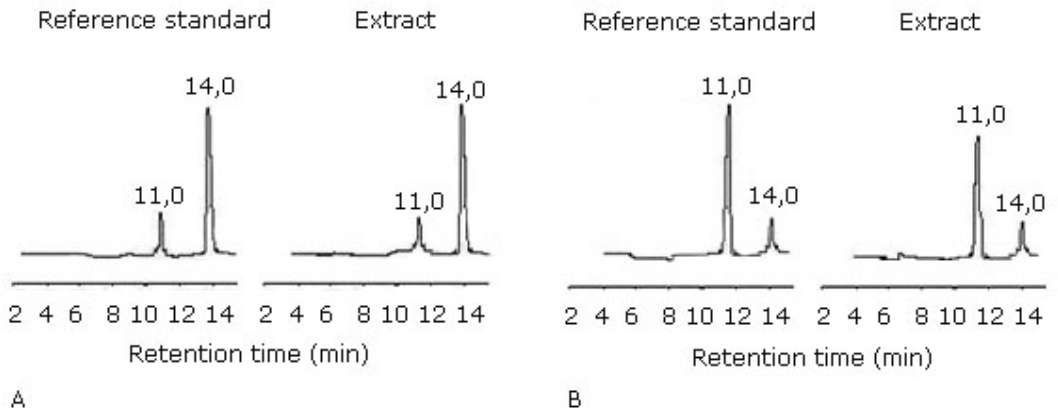
In the case of the hydroalcoholic extract, a transparent amber liquid and fragrant was obtained. The dry matter content and pH of the extract was 16.0/4.9 % and 6.9/0.7 respectively. The hydroalcoholic content in this extract was 24.7/1.1 %.

Chromatograms from interference test from both extracts showed two peaks at retention times of 14 and 11 min to coumarin and umbelliferone, respectively (Fig. 1). In coumarin determination method (wavelength to 274 nm), a small intensity peak at 11 min and a big intensity peak at 14 min were obtained. In the case of umbelliferone determination method (wavelength to 330 nm) an inverse behaviour was observed. Both methods provided sufficient resolution being specific for the determination of these components.

Table 1. Analytical results of the extracts harvested at three different times during two years

Year	Harvest time (months)	pH	Dry matter (%)	Alcoholic Content (%)	Coumarin Content (g/100 g dry matter)	7 OH coumarin Content (mg/100 g dry matter)
Aqueous extract						
1	4	6,6	4,86/0,65	-	4,97/1,65	97/1,53
	6	4,9	3,90/0,43	-	5,26/2,41	94/1,05
	8	7,9	3,60/1,08	-	2,46/0,98	95/0,96
2	4	6,1	4,85/0,99	-	4,44/1,70	95/0,65
	6	5,7	4,10/1,65	-	5,40/2,12	93/1,15
	8	6,9	3,70/0,54	-	3,05/1,47	94/1,05
Hydroalcoholic extract						
1	4	6,1	21,6/4,65	24,0 / 0,65	4,33 / 2,01	49/3,65
	6	6,0	17,1/5,01	24,0 / 0,61	4,95 / 2,96	43/2,74
	8	7,8	11,6/4,14	25,5 / 0,48	2,44 / 1,52	47/2,82
2	4	6,5	23,6/5,30	25,0 / 0,78	4,22 / 2,13	48/2,01
	6	7,2	15,6/4,32	23,0 / 0,24	4,04 / 1,86	41/3,14
	8	6,9	12,1/4,25	26,0 / 0,71	3,01 / 1,02	45/2,27

The results correspond to mean/DS the average of three determinations (n= 3).



A: coumarin method; B: umbelliferone method.

Fig. 1. Interference study chromatograms to aqueous extract.

Coumarin recovery rates were 99.1 %; 99.8 % and 99.9 % in aqueous extract portions and 99.3 %; 99.5 % and 99.7 % in hydroalcoholic extract portions. While umbelliferone recovery rates were 98.7 %; 99.1 % and 99.5 % in aqueous extract portions and 98.3 %; 99.2 % and 99.5 % in hydroalcoholic extract portions. In both case, the recovery percentages obtained were more than 98 % according to the acceptable limit for chromatographic methods.

Table 2 shows that the harvest time significant influences on coumarin concentration in both extracts (p= 0.0089 and p= 0.0398, from aqueous and hydroalcoholic extracts, respectively). Harvest time difference evaluations by Duncan test showed no significant differences among the first two harvests, but there were significant differences between the first two and the last one harvest times for both extraction types (Fig. 2). However, the harvest time does not have significant influence on the umbelliferone concentration in the studied extracts (p= 0.1822 and p= 0.2677, from aqueous and hydroalcoholic extract, respectively), showing a bigger quantity of this component in the aqueous extract (approximately 50 %). There was not difference among the coumarin extraction methods (p= 0.1654), but significant difference was obtained among the umbelliferone extraction methods (p= 0.0000).

Table 2. Statistical parameters obtained from ANOVA

Assays evaluated	Source	Sums of squares	Means squares	t ration	P value
Coumarin analysis					
Aqueous extract	Between groups	7,2158	3,6079	33,3	0,0089
	Inter groups	0,32	0,11		
Hydroalcoholic extract	Between groups	3,7225	1,8612	9,59	0,0398
	Inter groups	0,58	0,19		
Umbelliferone analysis					
Aqueous extract	Between groups	6,3333	3,1666	3,17	0,1822
	Inter groups	3,0	1,0		
Hydroalcoholic extract	Between groups	6,3333	3,1666	2,11	0,2677
	Inter groups	4,5	1,5		

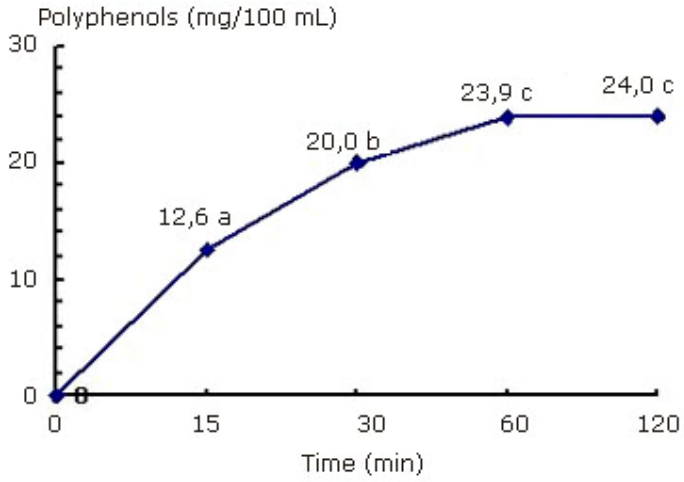


Fig. 2. Extraction process in the time (solid liquid ratio: 1:20 and 30 % alcoholic concentration). Similar letter no significant for p<0,05.

A significant influence of harvest time on dry matter content in the extracts (p= 0.0020 and p= 0.0045, to aqueous and hydroalcoholic extracts, respectively), was observed (Fig. 3).

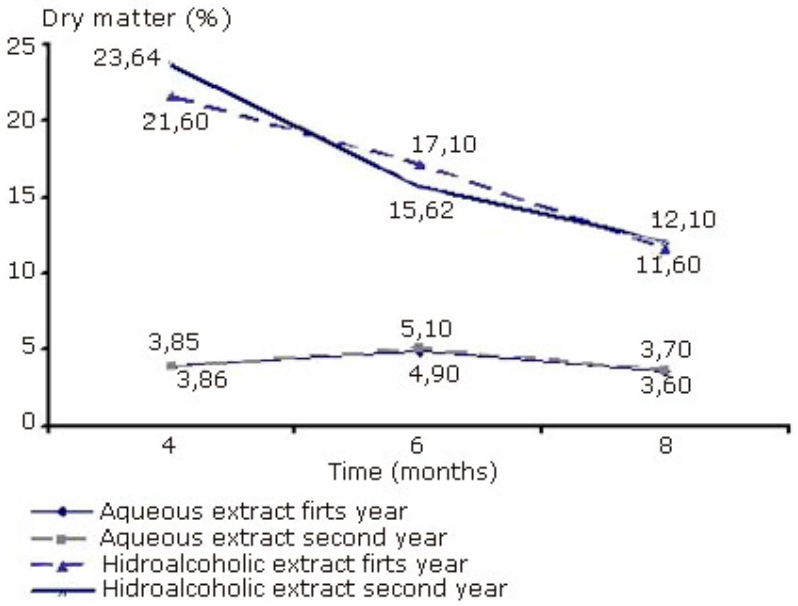


Fig. 3. Harvest time influences on dry matter content in the studied extracts.

DISCUSSION

Analytic results showed the presence of coumarin and umbelliferone in both extracts. Both extraction methods can be used for the extraction of these components from the plant, although, in the case of umbelliferone extraction method best results were obtained with aqueous extract. The quantities of coumarins detected in the extracts were similar to those detected by *Rodríguez, et al.* in works previously.[11,12]

The results of this study confirmed that the harvest time significantly influences on the coumarin and umbelliferone contents. The best results were obtained in the first two harvests (4 and 6 months, coinciding with the summer), which suggests that the plant material must be processed by the industry at that time of year.

This period coincides with the interval of highest yield of fresh foliage agree with *Silva et al.*,[10] whose yields were 20 ton/ha of fresh foliage, approximately equivalent to 4 ton/ha of dry material. On the other hand, the dry matter content in the extract decreases with time of harvest, yielding the highest percentage in the first crop.

These results are important because this plant is used for obtaining of pharmaceutical raw material according to technological process performed by *Rodríguez et al.*[11] It requires a high content of coumarin because this is the main active phytochemical in the raw material used for the manufacture of sedative pharmaceutical preparations. Furthermore, a high content of dry matter in the

extract ensures adequate drying process during the step of obtaining of the raw material by spray dried.

In summary, the harvest time significantly influences on the extracted coumarin content, being the 4 and 6 months of harvest the best in achieving the highest amounts of this active component as well as dry matter content in the extracts. In the case of the umbelliferone the results showed that the harvesting time does not significantly affect the concentration of this component.

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Jorge Enrique Rodríguez Chanfrau. Centro de Investigación y Desarrollo de Medicamentos (CIDEM). BioCubaFarma. Ave. 26, No. 1605, e/ Ave. Boyeros y Calzada de Puentes Grande, Nuevo Vedado, CP 10600, La Habana, Cuba. Correo electrónico: jorge.rodriguez@infomed.sld.cu