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Short communication

HPLC method for the determination of ecdysterone in extractive solution from *Pfaffia glomerata*

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Abstract

A RP-LC method was developed and validated to quantify ecdysterone in extractive solution from subterraneous parts of *Pfaffia glomerata*. The analysis was performed using a RP-18 column with acetonitrile:water isocratic elution and the detection was carried out by UV at 242 nm. The standard curve for ecdysterone was linear over the range of 5.2–41.6 μg/ml (R^2 = 0.9995). The extractive solution showed linear response in the range of 25.05–175.35 μg/ml (R^2 = 0.9977). This method showed excellent repeatability (relative standard deviation, R.S.D. < 2.0%), intermediary precision (R.S.D. = 2.13%) and accuracy (101.04; R.S.D. = 1.51%). The limit of detection (LOD) was 0.036 μg/ml and the limit of quantification (LOQ) was 0.110 μg/ml, demonstrating the sensitivity of the method. This assay can be readily utilized as quality controlled method for *P. glomerata* preparations.

Keywords: *Pfaffia glomerata*; Ecdysterone; Liquid chromatography; Validation; Quality control

1. Introduction

Species of the genus *Pfaffia* (Amaranthaceae) are commercialized in Brazil as substitutes for *Panax* spp. (ginseng, Araliaceae). Due to the similar morphology of its roots to those of ginseng, they are popularly known as "Brazilian ginseng", being used as a tonic, and to treat gastric disturbances and rheumatism [1,2].

Around 90 species of *Pfaffia* are known in Central and South America. In Brazil, 27 species have been described [3]. Although *Pfaffia paniculata* is the most employed in commercial preparations in Brazil, *Pfaffia glomerata* is commonly used as its substituent mainly in South of Brazil [4]. Gosmann et al. [5] investigated some botanical and chemical parameters to the unequivocal differentiation between *P. paniculata* and *P. glomerata* raw materials and extracts. Ecdysterone (Fig. 1) was only found in *P. glomerata*, as already described [6], so it seems that this compound could be a good marker for the differentiation of both species.

The main constituents of *Pfaffia* roots isolated so far are stigmasterol, sitosterol, allantoin, ecdysteroids, triterpenoids and nortriterpenoids [1,7].

Few pharmacological studies have been reported to *P. glomerata*. De-Paris et al. [4] reported a study using six different behavioral animal models for the central activity, suggesting that the ethanol extract of *P. glomerata* roots can act as a central nervous system depressant. Freitas et al. [8] showed that aqueous extract of *P. glomerata* possessed gastroprotective activity. Neto et al. [9] demonstrated that the hydroalcoholic extract of *P. glomerata* roots showed both anti-inflammatory and analgesic effects, similar to those observed for non-steroidal drugs, such as indomethacin.

Several Brazilian pharmaceutical industries produce phytopharmaceuticals containing *P. glomerata*. Brazilian health agency requires validated methodologies to the quality control of phytomedicines, in order to obtain their registration [10]. As far as we know, a validated methodology to quantification of ecdysterone in *P. glomerata* preparations is not

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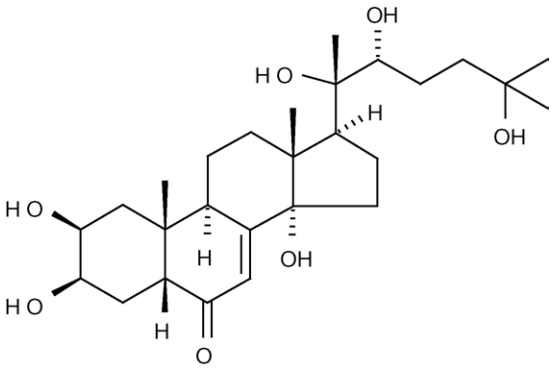

Fig. 1. Ecdysterone.

reported. Thus, in this paper, a simple and fast method by high-performance liquid chromatography (HPLC) was developed and validated to quantify ecdysterone in *P. glomerata* extract.

2. Experimental

2.1. Plant material and preparation of the extractive solution from P. glomerata

P. glomerata (Spreng.) Pedersen subterraneous parts were obtained from the cultivated area of the "Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas" (CPQBA), UNICAMP, Campinas, SP, Brazil, and a voucher specimen is kept in the herbarium UNICAMP (CPQBA 0238). Roots and rhizomes (subterraneous parts) from *P. glomerata* were reduced to small pieces, dried in a circulating air stove (40 °C) and triturated.

The subterraneous parts were extracted with ethyl acetate using soxhlet during 10, 15, 20, 25, 30 and 40 h in order to establish the time necessary to obtain an exhaustive extraction. The ethyl acetate was removed under "vacuum". The dried extract obtained for each time of extraction was determined in relation to 100 g of triturated herbal raw material (%, m/m). The results represented the average of three determinations. The data were compared by ANOVA followed Tukey's test ($\alpha = 0.05\%$).

2.2. Chemicals and solvents

Ecdysterone was purchased from Sigma Co. (St. Louis, MO). Acetonitrile and methanol, HPLC grade were obtained from Merck (Darmstat, Germany) and water purified by Millipore Milli-Q system (Bedford, MA).

2.3. Chromatographic conditions

The analysis was performed on a Shimadzu LC-10A equipped with a pump LC-10AD, an UV–vis-detector SPD-10A, an autosampler SIL-10A and CLASS-LC10 software. A Nova-Pak RP-18 column (150 mm × 3.9 mm i.d., 4 μm, 60 Å) protected by a pre-column Nova-Pak RP-18 was used throughout this study. The mobile phase composed of acetonitrile:water (20:80, v/v) was filtrated (0.45 μm, Millipore) and degassing with helium. It was employed a flow rate of 1 ml/min, an injection volume of 20 μl and the peak was detected at 242 nm.

2.4. Calibration curve

Solutions of ecdysterone Sigma® were prepared in acetonitrile containing 5.2, 10.4, 15.6, 20.8, 26.0, 31.2 and 41.6 μg/ml. The extractive solution from subterraneous parts of *P. glomerata* was diluted with acetonitrile to obtain 25.05, 50.10, 75.15, 100.20, 125.25, 150.30 and 175.35 μg/ml of extract.

The solutions were filtered through a 0.45 μm membrane (Millipore). The calibration curve was made by linear regression and the results represented the average of three injections of each concentration. The specificity, linearity, accuracy, precision (repeatability and intermediary precision), limits of detection and quantification were evaluated according to the International Conference on the Harmonization (ICH) guidelines [11] and to Brazilian resolution [12].

3. Results and discussion

In this report, a method based on reversed phase liquid chromatography combined with UV spectrometric detection was developed to ecdysterone assay in *P. glomerata* extract (Fig. 1).

3.1. Extractive solution

Initially, aiming to determine the time necessary to obtain an exhaustive extractive solution from *P. glomerata*, extractions were tested during 10–40 h using soxhlet. Table 1 presents the yields of the dried extracts obtained to each time. ANOVA analysis showed significant differences among the yields of dried extracts. Tukey's test showed that there are not significant differences between 30 and 40 h of extraction

Table 1
Influence of the time of extraction on the yield of the dried extract from *Pfaffia glomerata*

Time of extraction (h)	Dried extract (%, m/m)[a] ($n = 3$)
10	0.55 d
15	0.84 c,d
20	1.09 b,c
25	1.22 b
30	1.71 a
40	1.79 a

Different letters (a–d) indicate statistical difference in agreement with ANOVA and Tukey's test ($\alpha = 0.05\%$).

[a] Grams from dried extract per 100 g of triturated herbal raw material (%, m/m).

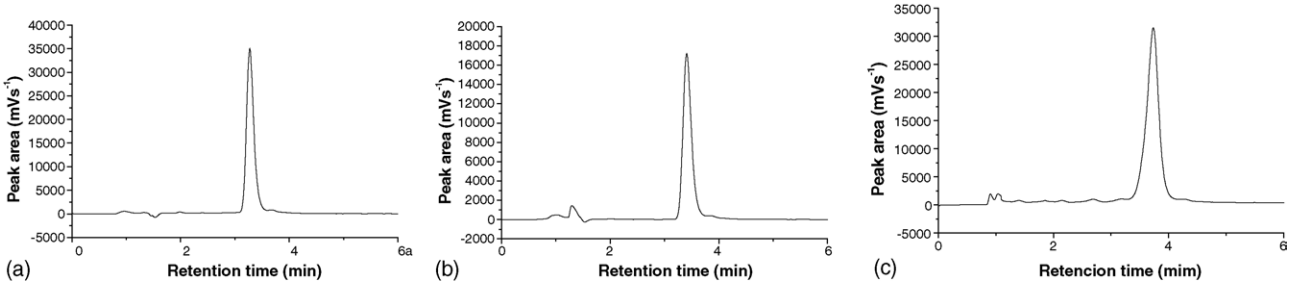

Fig. 2. HPLC chromatograms: (a) ecdysterone Sigma®, R_t = 3.40 min; (b) extractive solution from *Pfaffia glomerata*, R_t = 3.51 min; (c) extractive solution from *Pfaffia glomerata* spiked with ecdysterone Sigma®, R_t = 3.47 min. Chromatographic conditions: Nova-Pak RP-18 (150 mm × 3.9 mm i.d., 4 μm, 60 Å); acetonitrile:water (20:80, v/v); flow rate, 1 ml/min; detection at 242 nm.

in relation to the yield of the dried extract. So, 30 h of extraction was chosen to prepare the extractive solution from *P. glomerata*.

The optimized HPLC conditions were achieved after preliminary assays, where different combination of acetonitrile, methanol and water were tested. Fig. 2 showed the chromatograms of ecdysterone reference substance, extractive solution from *P. glomerata*, extractive solution from *P. glomerata* spiked with ecdysterone. Comparing these chromatograms a coincidence of retention times could be observed (R_t = 3.40, 3.51, 3.47 min, respectively), indicating that other components of the matrix have few interference to ecdysterone peak profile.

The ecdysterone content of the extractive preparation from *P. glomerata* was 20.50% (w/w) in relation to the dried extract or 0.35% (w/w) in relation to subterraneous part.

3.2. Validation

The linearity of the method was determined through the ecdysterone calibration curve and the calibration curve of the corresponding peak of the extract from *P. glomerata*. The slope and the other statistical parameters of the calibration curves were calculated by regression. The calibration curves presented a linear response within the ranges of 5.2–41.6 μg/ml to ecdysterone and 25.05–175.35 μg/ml to the extractive solution. The regression equations were $y = 19248x - 3150.7$ ($R^2 = 0.9995$; $n = 7$; p-value = 0.2049) for ecdysterone and $y = 3658.6x + 4103.8$ ($R^2 = 0.9977$; $n = 7$; p-value = 0.3795) for the extractive solution. The relative standard deviation (R.S.D.) for all data was less than 3%.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation (S.D.) and the slope (S) of the calibration curve [11]. The limit of detection was 0.036 μg/ml and the quantification limit was 0.110 μg/ml, demonstrating the sensitivity of the method.

The accuracy was evaluated through recovery studies by adding known amounts of ecdysterone to the extractive solutions of *P. glomerata*. Controls from all samples were prepared and analyzed. The recovery experiment was performed by preparing samples of the extractive solution of *P. glomerata* spiked with 50, 100 and 150% of ecdysterone. Each solution was prepared in triplicate. The recovery was determined by comparing the peak area of the control matrix preparation with those samples spiked with ecdysterone. The expected and observed quantities are shown in Table 2. The results of the accuracy test demonstrated low interference of the matrix onto the recovery of ecdysterone. The method showed a medium recovery of 101% with R.S.D. below 2% in all analyzed concentrations.

Precision of the HPLC method was evaluated through the determination of the repeatability and intermediary precision as presented in Table 3. According to the R.S.D. values obtained for nine samples evaluated on the same day, it could be observed that the chromatographic system showed a satisfactory response with R.S.D. < 2.0%. The assay of intermediary precision was performed during four consecutive days in order to determine the accumulation of the random errors between different extracts and days. The intermediary precision showed a R.S.D. of 2.13%, demonstrating that the method showed high reproducibility and thus, has low interference of the sample preparation.

Table 2
Recovery studies of ecdysterone in *Pfaffia glomerata* extractive solution

Theoretical quantity (μg)	Experimental quantity (μg)	Recovery (%) (X; R.S.D.%)	Total recovery (%) (X; R.S.D.%)
5.075	5.16	101.80; 1.65	101.04; 1.51
10.15	10.27	101.19; 1.54	
15.225	15.24	100.11; 1.36	

Table 3
Repeatability and Intermediary precision tests of ecdysterone in *Pfaffia glomerata* extractive solution

Days	Ecdysterone concentration ($X \pm$ S.D., $n = 9$) (μg%, w/w)	R.S.D. (%)
1	20.41 ± 0.36	1.76
2	20.05 ± 0.19	0.95
3	20.56 ± 0.30	1.46
4	20.96 ± 0.30	1.43
$X \pm$ S.D. (inter-day)	20.50 ± 0.44	–
R.S.D.% (inter-day)	–	2.13

4. Conclusion

A simple, specific, precise, rapid and reproducible RP-LC method has been developed to identify and quantify ecdysterone, a relevant marker compound in the extractive solution from roots of *P. glomerata*. The validation procedure confirms that this is an appropriate method to the quality control of extractive solutions from *P. glomerata* that can be used by the pharmaceutical industry.

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