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Research Article

Separation and Identification of Furocoumarin in Fruits of *Heracleum candicans* DC. by HPTLC

A. K. S. Rawat, A. P. Singh, D. P. Singh, M. M. Pandey, R. Govindarajan, and Sharad Srivastava

Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow 226003, India

Correspondence should be addressed to Sharad Srivastava; sharad_ks2003@yahoo.com

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Heracleum candicans Wall. ex DC. (Apiaceae) is widely used in Indian system of medicines as an aphrodisiac, nerve tonic and also in the treatment of skin diseases with reports of being rich in furocoumarins which are extensively used in pharmaceutical industry for their photosensitizing activity on human skin. A simple high performance thin layer chromatography (HPTLC) method has been developed for the simultaneous determination of psoralen (1) and heraclenol (2) in the fruits of *H. candicans*. The analytes were separated on silica gel F_{254} plates with toluene : diethy lether : acetic acid (6 : 4 : 1) and scanned densitometrically at 350 nm. The method was validated in terms of precision, repeatability, and accuracy. The linear range for psoralen and heraclenol was found to be 1–5 μg spot^{-1} and 1–4 μg spot^{-1} with correlation coefficient of 0.973 and 0.964, respectively. The two compounds were quantified in fruits of *H. candicans* and were found to be present in the range of 0.021–0.036% and 0.029–0.043% w/w. The method was found to be very simple, accurate, precise, and economical and can be used for routine quality control.

1. Introduction

Heracleum candicans Wall. ex DC. (syn. *H. lanatum* Michx, *H. nepalense* D. Don.; family Apiaceae), commonly known as "*Gandharajan*" or "Patrala," is a large herb found in temperate forests of Himalayan regions [1]. In Indian system of medicines, it is used as an aphrodisiac, nerve tonic and also used in the treatment of skin diseases [2]. The plant has been shown to possess potent stimulatory effect on melanogenesis with significant enhancement of cell proliferation [3]. Dichloromethane extract of shoots of *H. candicans* has been reported for anti-inflammatory activity [4]. Chemical investigation of the plant has revealed the presence of furocoumarins, namely, bergapten [2], heraclenol, xanthotoxin, phellopterin, angelicin, imperatorin, xanthotoxol [5], heraclenin [6], candibirin [7], and 8-gernoxypsoralen [8]. Activity-guided isolation has also shown heraclenin to be the anti-inflammatory principle present in *H. candicans* [4].

Furocoumarins are polyphenolic compounds, synthesized from L-phenylalanine, which may occur in a linear form with the furan ring attached to the 6, 7 position of the benzo-2-pyrene nucleus. These are biologically active natural compounds found in many plants particularly in members of the Umbelliferae and Rutaceae families [9, 10]. They are typically phototoxic compounds leading to photodermatitis upon exposure to UV light [11]. In humans and experimental animals, chronic furocoumarin treatment, in combination with UV light, is used in the therapy of psoriasis [12]. On the molecular level furocoumarins bind to cellular constituents such as proteins and lipids, can damage lysosomes, lead to the formation of reactive oxygen species, and can contribute to the formation of novel antigens by covalent modification of proteins [13]. Furocoumarins of *H. candicans* are extensively used in pharmaceutical industry for their photosensitizing activity in human skin [14]. There has already been an attempt made at the spectrophotometric estimation of the total furocoumarin bitter principles from roots of *H. candicans* [15], but this seems to be a preliminary report on indiscriminate estimation of total furocoumarins along with a report of the root of *H. candicans* [16].

HPLC as a tool for the quantitative estimation of furocoumarins in fruits of *H. candicans* has been reported [17], but HPTLC is a cheaper and faster technique with similar resolution. HPTLC of the *H. candicans* root for the estimation

of the heraclenin and heraclenol has also been reported [18]. Thus keeping in view the importance of furocoumarins, quantitative estimation of these compounds in *H. candicans* has been carried out using HPTLC. We have made an attempt to qualitatively evaluate *H. candicans* fruit taking two furocoumarins, namely, psoralen and heraclenol as bioactive marker. An attempt has also been made to validate the developed method in terms of precision, repeatability, LOD, LOQ, and accuracy.

2. Experimental

2.1. Plant Material and Reagents. Fruits of *H. candicans* DC. were collected from Nainital (Uttarakhand), India, during April-May 2004. The samples were authenticated and herbarium of the same was deposited in the NBRI, Lucknow, India (voucher specimen no. 217680).

2.2. Chromatographic Conditions. A CAMAG (Muttenz, Switzerland) HPTLC system, comprising of a Linomat 5 automatic applicator, a twin trough plate development chamber, Camag TLC scanner 3, and win-CATs software, was used. Precoated silica gel 60 F_{254} (20 × 10 cm, 0.2 mm thickness, Merck, Darmstadt, Germany) glass plates were used as stationary phase. Conditions for the HPTLC were ambient temperature with 40% relative humidity. The mobile phase consisting of petroleum ether/hexane/toluene : diethyl ether : acetic acid (6 : 4 : 1) and so forth was used for solvent optimization. Solutions of sample and standard compounds 1, 2, and 3 of known concentrations were applied on TLC F_{254} in band form as 6 mm wide and 10 mm from the bottom. The scanner conditions used were slit width 5 × 0.45 mm, wavelength 350 mm, and absorption reflection scan mode. These parameters were kept constant throughout.

2.3. Preparation of Standard Solutions of Psoralen and Heraclenol. Stock solutions of psoralen (**1**) and heraclenol (**2**) (1.0 mg mL^{-1}) were prepared by dissolving 10 mg of accurately weighed 1 and 2 in methanol and making up the volume of the solutions to 10 mL each with methanol. Aliquots (200, 400, 800, 1600, 3200, and 4000 μL) of stock solutions were transferred to 5.0 mL volumetric flask and the volume of each one was adjusted to 5.0 mL with methanol to obtain standard solutions containing 40, 80, 160, 320, 640, and 800 μgmL^{-1} of psoralen and heraclenol, respectively.

2.4. Extraction of Plant Materials. Air dried (40–50°C), powdered fruits (2.0 g each) of *H. candicans* were extracted with methanol (3 × 20 mL) by stirring for 30 min on shaker. The combined extracts were concentrated under vacuum. The extracts were obtained in a yield of 20.22%. Solutions of 10 mg mL^{-1} concentration were prepared by dissolving the extracts in methanol for analysis.

2.5. Calibration. 10 μL of each of the standard solutions (0.4, 0.8, 1.6, 3.2, 6.4, and 8.0 μg $spot^{-1}$) of psorelen and 8 μL of the standard solutions (0.32, 0.64, 1.28, 2.56, 5.12, and 6.4 μg $spot^{-1}$) of heraclenol were applied in triplicate onto silica gel 60 F_{254} HPTLC plates using a Linomat 5 automatic sample applicator. The plates were developed with a solvent system of toluene : diethy lether : acetic acid (6 : 4 : 1 v/v) in a CAMAG glass twin trough chamber, up to a distance of 8.0 cm. After development, the plates were air dried and scanned at 350 nm. The peak areas were recorded. Calibration curves were prepared by plotting peak areas versus concentration.

2.6. Quantification of Psoralen and Heraclenol in Methanolic Extract of H. candicans Fruits. 10 μL of sample solution was applied in triplicate on a silica gel 60 F_{254} HPTLC plate with the CAMAG automatic sample applicator. The plate was developed and scanned as described above. The peak areas were recorded. The amount of 1 and 2 in the sample was calculated using the calibration curves. Peak identification was achieved in comparison of both R_f and UV absorption spectrum with those obtained for standards.

2.7. Method Validation. Precision was checked by repeated scanning of the same spots of 1 (3.2 μg $spot^{-1}$) and 2 (2.56 μg $spot^{-1}$) six times and was expressed in terms of relative standard deviation (% RSD). The repeatability of the method was done by analyzing the spots of standard solutions of 1 and 2 after application on the HPTLC plate ($n = 5$) and expressed in terms of % RSD. Variability of the method was studied by analyzing aliquots of standard solutions of 1 (0.4, 0.8, 1.6, 3.2, 6.4, and 8.0 μg $spot^{-1}$) and 2 (0.32, 0.64, 1.28, 2.56, 5.12, and 6.4 μg $spot^{-1}$) on the same day (intraday precision) and on different days (interday precision) and the results were expressed in terms of % RSD. The accuracy of the method was determined by recovery studies. Plant samples spiked with three different amounts of 1 (1.0, 2.0, and 3.0 μg) and 2 (400, 600, and 800 μg) were analysed and the percent recovery was calculated. Different dilutions of standard solutions of 1 and 2 in methanol were applied. Limit of detection (LOD) and limit of quantification (LOQ) were determined on the basis of-signal to-noise ratios of 3 : 1 and 10 : 1.

3. Results and Discussion

Earlier a simple TLC method has been reported for the simultaneous determination of only two compounds from the roots of *Heracleum candicans* [18]. The quantitative estimation of furocoumarins in fruits was also reported by HPLC only [17]. In the present study different compositions of the mobile phase consisting of petroleum ether/hexane/toluene : diethyl ether : acetic acid (6 : 4 : 1) and so forth were tested for solvent optimization. In systems other than toluene: Diethyl ether: Acetic acid either the resolution of the spots was not very clear or there was interference from the matrix. Best resolution and reproducible peaks were obtained only by using toluene : diethyl ether : acetic acid (6 : 4 : 1) as mobile phase (1: R_f = 0.61, 2: R_f = 0.16). Linearity was observed in the range of 1 to 5 μg per spot for 1 and 1 to 4 μg per spot for 2. The specificity of the method was ascertained by analyzing standards and the

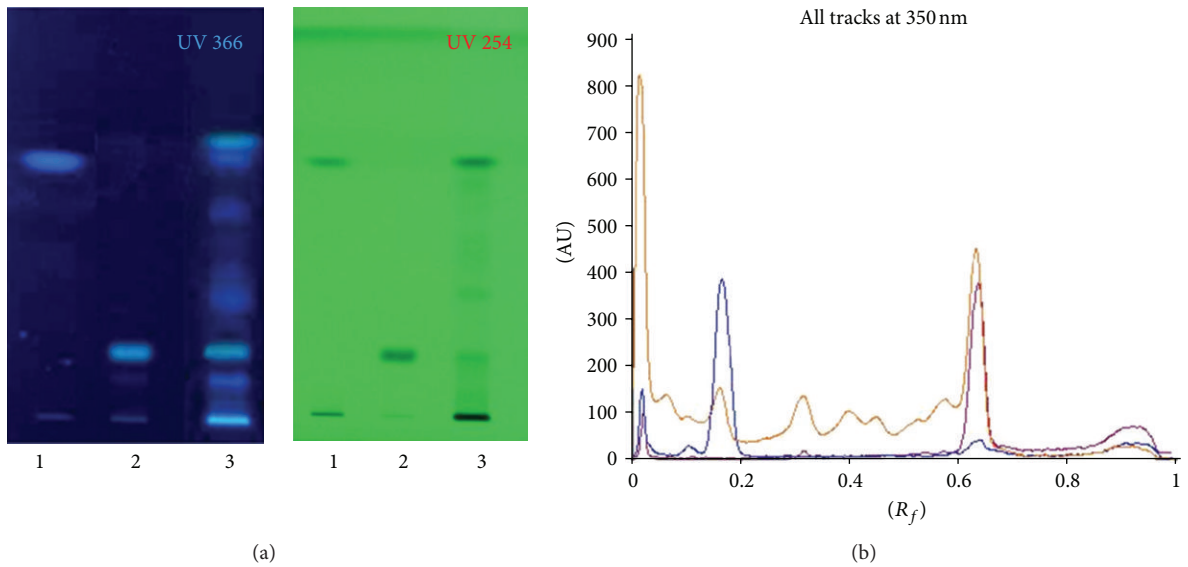

Figure 1: HPTLC fingerprint profile and densitometric scanning profile showing the separation of psoralen (1) and heraclenol (2) in methanolic extract of *H. candicans* fruits (3).

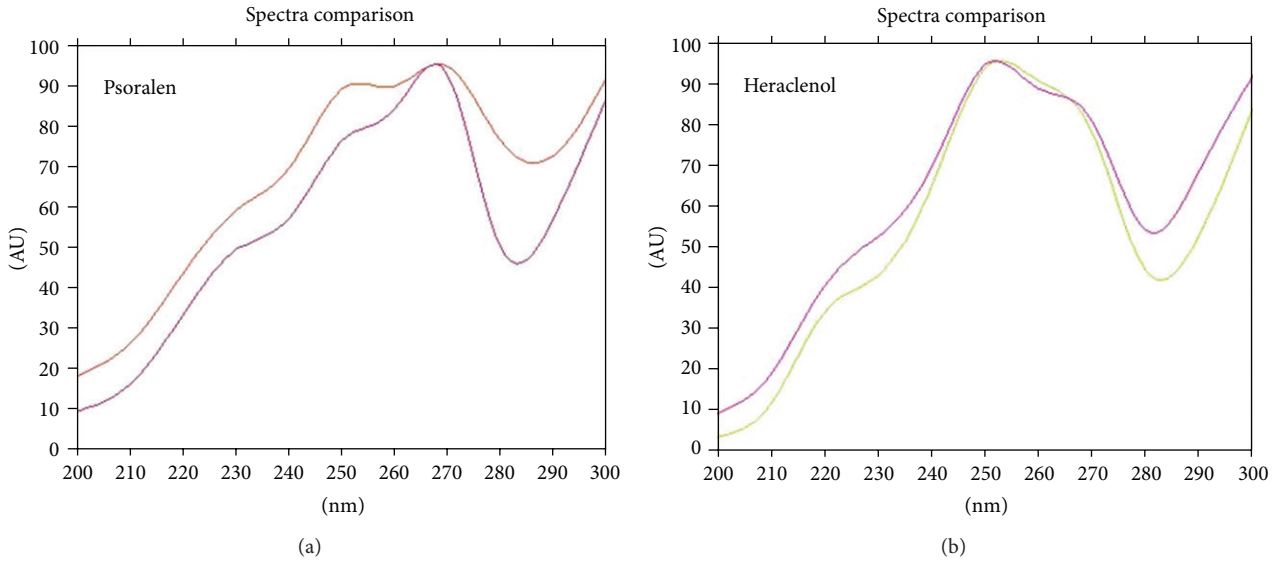

Figure 2: Spectrum of psoralen (1) and heraclenol (2) along with methanolic extract of *H. candicans* fruits.

samples. The spots for psoralen (1) and heraclenol (2) in the sample were confirmed by comparing the R_f values and the spectra of the spots with those of the standards (Figure 1). The overlay spectra of identified peaks in the standards as well as the corresponding peak in the extracted samples showed total superimposition at peak start, peak maximum, and peak end, thus confirming the purity of the peaks in the extracted solutions. The method was validated in terms of precision, repeatability, and accuracy (Table 1). The linear range for psoralen (1) was found to be 1 to 5 μg spot^{-1} with correlation coefficient of 0.973 while for heraclenol (2) it was 1 to 4 μg spot^{-1} with a correlation coefficient of 0.964. These correlation coefficients indicated good linearity between the concentration and peak area in the applied concentration range. The intraday and interday precision values for 1 and 2 indicated that the method was precise and reproducible. Also, the recovery values >95% for 1 and 2 showed that the method was reliable and accurate. Psoralen (1) and heraclenol (2) contents were determined quantitatively in the fruits of *H. candicans* (Figure 2) and were found to be present in yields of 0.025% and 0.039% w/w, respectively, in the samples (Table 2).

Table 1: Method validation parameters for the estimation of psoralen and heraclenol by HPTLC densitometry.

Parameter	Results	
	Psoralen (1)	Heraclenol (2)
Accuracy (%)	85.64	93.51
Precision (% RSD)		
CV (instrument precision)	0.037948	
CV (method precision)	1.31	1.14
Interday precision	0.73	0.80
Intraday precision	0.68	0.75
Limit of detection	0.86 μg	2.79 μg
Limit of quantification	2.62 μg	4.82 μg
Specificity	Specific	Specific
Correlation coefficient	0.973	0.964
Linearity range	1–5 μg spot^{-1}	1–4 μg spot^{-1}
Linear regression coefficient	$y = 4.010x + 8878$	$y = 1.253x + 1790$
Slope	4.010 ± 0.4694	1.254 ± 0.1698
Y-intercept	8878 ± 1285	1791 ± 465.1
P value	0.0134	0.0179

Table 2: Percent of psoralen and heraclenol in *H. candicans* fruits.

Compound	% present in fruit of *H. candicans* (w/w)
Psoralen	0.025
Heraclenol	0.039

4. Conclusion

The method for the quantification of psoralen (1) and heraclenol (2) in the fruits of *H. candicans* was found to be simple, accurate, precise, and suitable for rapid screening of a large number of samples without any special sample pretreatment. The method can be used for the routine quality control of *H. candicans* as well as for the quantification of psoralen and heraclenol in other plant materials as well as in compound herbal formulations containing these furocoumarins.

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Conflict of Interests

This is to inform you hereby that all the authors have no conflict of interests.

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