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Department of Pharmacognosy with Medicinal Plant Laboratory, Medical University of Lublin

TOMASZ BAJ, DOROTA KOŁTUNOWSKA, KAZIMIERZ GŁOWNIAK, TADEUSZ WOLSKI

Determination of aucubin in aerial parts of Buddleja davidii Franch. using different TLC-detection methods

Oznaczanie zawartości aukubiny w nadziemnych częściach *Buddleja davidii* Franch. przy użyciu różnych metod detekcji TLC

Aucubin is a naturally ocurring glycoside of cyclopentano [c] pyran monoterpenoid aglycone. It is commonly included among the iridoids group that at present contains almost one thousand compounds [6]. Aucubin is widespread in plant's kingdom, especially in *Scrophulariaceae, Plantaginaceae, Rubiaceae,* where it plays a role as a useful chemotaxonomic marker and also as a plant defensive chemical against generalist herbivores [6, 18, 29].

Aucubin, as well as its aglycone, has numerous well known biological activities such as antimicrobial [5], antifungal [1], antiinflammatory [22], hepatoprotective [3] and antispasmodial [20]. Recent studies on aucubin have demonstrated its possible mechanism of action due to its direct binding reactions with proteins and nucleic acids [12, 14, 17]. It have been also reported that its anti-inflammatory action originates from an inhibition of κB nucleic factor that results in a decline in pro-inflammatory cytokines TNF-α and IL-6 in mast cells [12, 21]. Photoprotective and antioxidative activities of aucubin have been also reported to be more potent than ascorbic acid [11]. Aucubin is also topoisomerase-I inhibitor [9].

The content of aucubin has been marked by different methods, mostly UV/Visible spectrophotometry [7, 8, 26] or chromatographic methods: HPLC [10, 23], capillary chromatography (MECC) [24, 25] and TLC or HPTLC [2, 10, 16, 19]. Except for HPLC and MECC, above-mentioned methods need derivatization. There are many different spray reagents for visualization, i.e. sulfuric acid anisaldehyde reagent [28], sulfuric acid vanillin reagent [2], EP or Ehrlich's reagent, Trim and Hill's reagent. Both EP and Ehrlich's reagents contain 4-dimethylaminobenzaldehyde in acidic solutions and form a stable blue product when heated with aucubin [15, 27].

Densitometry is a fast detection method in both qualitative and quantitative analysis of natural substances performed on thin-layer chromatographic plates. It is also applied to identify and distinguish different plant species [4].

Despite numerous publications on the presence of different compounds by TLC or HPTLC methods combined with classical i.e. slit-scanning densitometry, little information is available on the videodensitometrical method of detection. This study aims to compare both mentioned methods of quantitative determination of aucubin in *Buddleja davidii* extracts. It is also the first approach to quantitatively determine the content of aucubin in this plant.

MATERIAL AND METHODS

Plant material. Buddleja (*Buddleja davidii* Franch.) was grown in the herb garden at Faculty of Pharmacy, Medical University of Lublin, Poland (N 51°16' E 22°34'). Aerial parts of Buddleja were harvested during flowering stage in August 2005. The taxonomic identification was confirmed by plant taxonomist, Stanisław Kwiatkowski, in the Dept. of Pharmacognosy with Medicinal Plant Laboratory, Faculty of Pharmacy, Medical University of Lublin, Poland. After identification, the plant material was dried at 35°C and ground. The voucher specimen was deposited at the Herbarium of the Department of Pharmacy, Medical University of Lublin, Poland (No. TBB0505).

Preparation of Buddleja davidii extracts. The powdered plant material (500 mg) was weighed to accuracy within 0.001 g. It was put into round-bottom flasks. To the flask with plant material 300 mg of calcium carbonate and 100 mL of methanol were added. This was followed by the reflux extraction (RE). After cooling, crude extract (BD) was filtered through 1.2 g aluminium oxide neutral throwing out the first 5 mL. The extract was concentrated to dryness under vacuum at 60–80°C. The dry residue was dissolved with 25 ml of methanol.

Instrumentation and chemicals. Chromatography was performed on 10x20 glass HPTLC plates coated with 0.20 mm layer of silica gel Si 60F254 (Merck, Darmstad, Germany). Samples were spotted by the TLC III Sampler (Camag, Switzerland, Version 2.12) and developed in DS-horizontal chamber (Chromdes, Lublin, Poland). Documentation of the results was achieved by the use of video scanning equipment UV Reprostar 3 (Camag, Muttenz, Switzerland). The documentation was processed with Video Store 2 software. Video-densitograms were obtained by the use of Camag VideoScan TLC/HPTLC Evaluation Software version 1.01.00. Classical densitograms were obtained by the use of a TLC Scanner 3, with WinCats software (Camag, Muttenz, Switzerland). All chemicals and solvents were of analytical grade. A HPLC standard of aucubin was purchased from Carl Roth (Karlsruhe, Germany).

HPTLC-Chromatography. Before use, HPTLC plates were washed with methanol and after drying at room temperature activated at 100°C for 1 h. Samples of standard aucubin solution (5, 10, 15, 20, 25 µL) and extract BD (15 µL) were applied bandwise by spraying. Sample BD were spotted 3 times each 15 µL per spot and the final results are arithmetic means. The plates were conditioned for 10 min and then developed upside down in DS-horizontal chamber with ethyl acetate:methanol:water (77:15:8, v/v) as a mobile phase on 80 mm distance. The procedure was repeated three times at room temperature (25°C ± 2°C). HPTLC chromatograms were visualized by spraying with a mixture of a 1% solution of 4-dimethylaminobenzaldehyde in ethanol and 36% hydrochloric acid, then heating at 100°C for 5 min. Aucubin reference substance was dissolved in methanol to prepare the solution 0.15 mg mL^{-1}.

Densitometry methods. Video densitograms were obtained in absorption mode under VIS light by 3CCD colour camera. Color filtration options were red: 50%; green : 50%; blue : 0%. Classical slit-scanning densitometry was performed at 590 nm in absorption mode with 20 mm/s scanning speed. The slit dimensions were 8 x 0.9 mm. Both calibration methods were linear regression.

Limit of Detection and Limit of Quantification. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times following the same method as explained above and the signal-to-noise ratio was determined. The limit of detection was considered as 3:1 and LOQ as 10:1.

RESULTS AND DISCUSSION

A rapid colour reaction with 4-dimethylaminobenzaldehyde directly on the plate allows fast quantification of aucubin with not only classical densitometry but also with videodensitometry. Classical densitometer is generally used in both qualitative and quantitative analysis. Its advantages are inter alia wide scan spectrum and a possibility of obtaining an absorption maximum of analyzed compounds. Reprostar 3 is usually used to perform a video documentation of TLC/HPTLC plates. However, its modern software also allows obtaining quantitative and qualitative analysis after getting a photo of a plate.

There are two methods of construction of the calibration curve in the densitometric determination of natural substances. Densitometers are equipped with modern software that allows drafting that curve according to the height or area of analyzed spot peak. In present investigation higher values were achieved when analysed the amount of aucubin by peak area although the correlation for peak height was satisfying (see Tab. 1).

Table 1. Correlation ratio for aucubin standard calibration curves obtained by different detection methods

Detection methods	Equation of the linear graph and correlation ratio			
	Height		Area	
Video densitometry	0.9576	y = 552.69x + 551.67	0.9884	y = 5797.4x + 3568.2
Slit densitometry	0.9718	y = 85.129x + 118.98	0.9915	y = 2719.3x + 1996.5

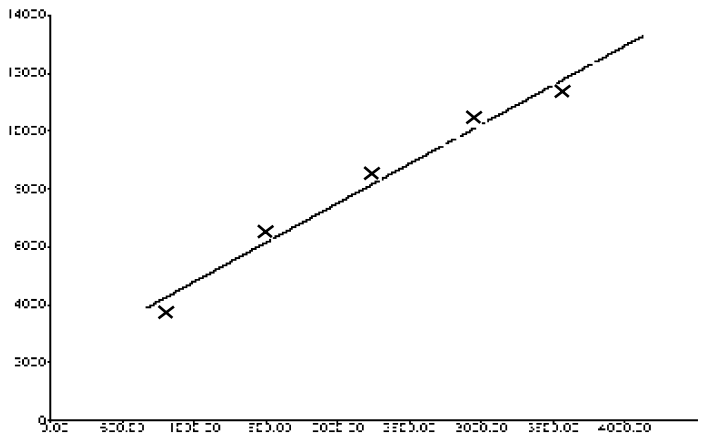

Fig. 1. Linear relationship between the peak area and aucubin standard amount. Correlation ratio r=0.9915

The developed TLC method for estimation of aucubine content showed a good correlation coefficient r = 0.9576 (height calculation), 0.9884 (area calculation) for video-densitometry detection and 0.9718 (height), 0.9915 (area) for slit densitometry detection in a concentration range from 5 to 25 µl solution aucubine standards. Presented results show good linearity for both detections methods.

The densitometric quantification of aucubin was possible due to high separation of peaks on the plate. Although other iridoids were present, they did not interfere with aucubin. R_F values of aucubin in ethyl acetate:methanol:water (77:15:8, v/v) as mobile phase was 0.44. Iridoids were usually isolated

from the sample matrix by maceration solvents, mainly methanol, ethanol or hot water [13]. Quantitative determination in both TLC-detection methods demonstrated that the methanol used in extraction was an appropriate solvent for isolation of aucubin. It is also remarkable than the amount of aucubin is relatively high (ca. 1.2% of dry mass) in *Buddleja davidii,* which can suggest this plant as a good source of this compound. Slit densitometry and video densitometry gave comparable results (see Tab. 2). A comparison of video and slit densitograms of the same track is shown (see Fig. 2). Absorption spectrum of aucubin in colour complex with 4-dimethylaminobenzaldehyde obtained from the plate show Fig. 3.

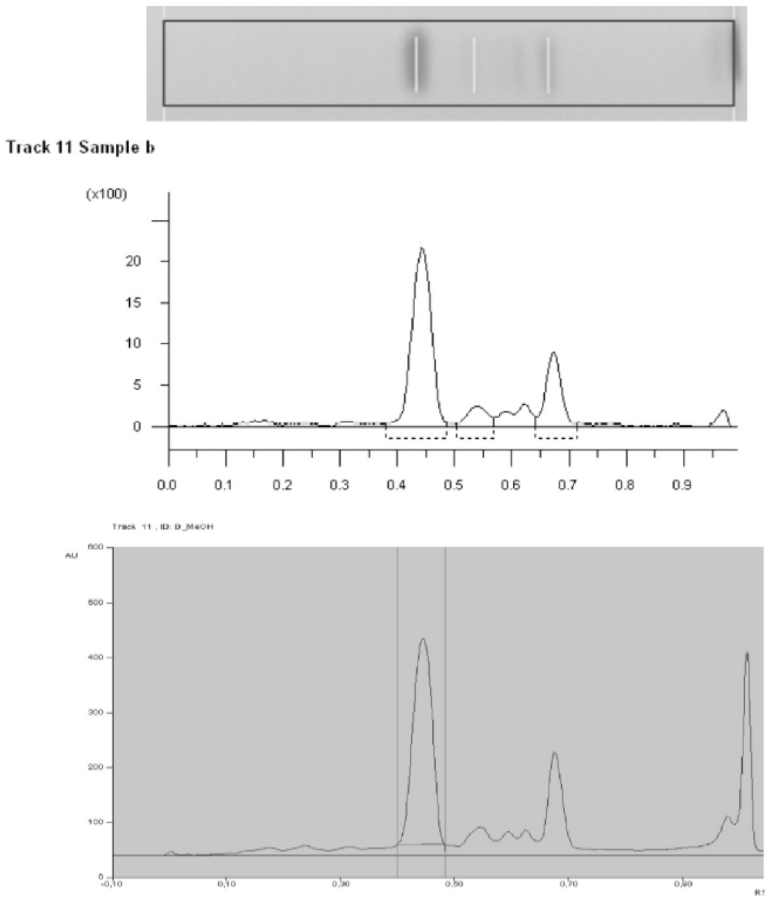

Fig. 2. Comparison of video (1) and slit (2) densitograms of the same track

Tab. 2. shows that the content of aucubin of examined raw material was in the range from 0.89 to 1.21% of dry mass depending on the methods of detection and calculation. The received results of the contents of the substance were lower than the appointed limit of detection LOD (0.20–0.25 μg per spot) and limit of quantification LOQ (0.67–0.83 μg per spot), which confirms reliability of the method. However, both methods of TLC detection are commonly used but the slit-densitometry method is more sensitive. This method resulted in 2.97 μg per spot (measured from peak height) and 3.43 μg per spot (measured from peak area) of acubin. The differences of the content of determined compound between detection methods were 0.46 μg per spot (measured from peak height) and 0.32 μg per spot (measured from peak area).

Table 2. Data of aucubin content determined by different methods of calculation, limit of detection (LOD) and limit of quantification

Detection methods	Aucubin content n=3					
	Height		Area		LOD (µg per spot)	LOQ (µg per spot)
	µg per spot	% of dry mass	µg per spot	% of dry mass		
Video densitometry	2.51±0.13	0.89	3.11±0.28	1.10	0.25	0.83
Slit densitometry	2.97±0.31	1.05	3.43±0.40	1.21	0.20	0.67

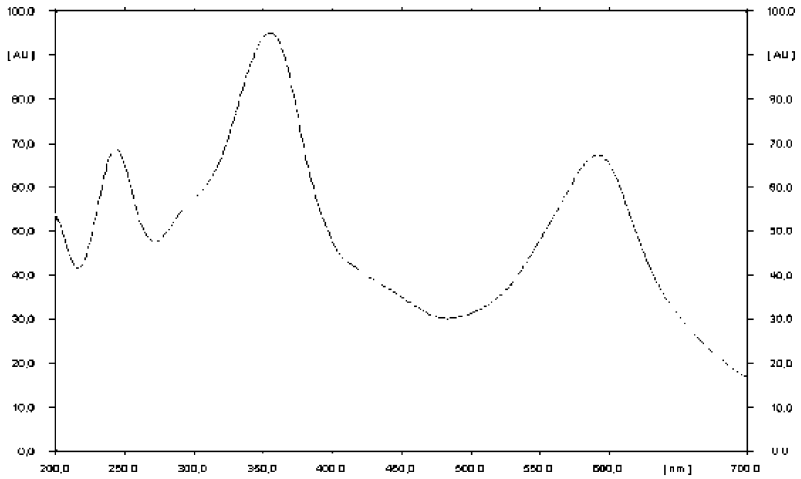

Fig. 3. Absorption spectrum of aucubin in colour complex with 4-dimethylaminobenzaldehyde obtained from the plate

CONCLUSIONS

Modern methods of HPTLC analysis allow preliminary quick evaluation of bioactive substances in plant material. Video densitometry, as well as slit densitometry, permit a qualitative and quantitative analysis of plant extracts and the results of these two methods are only slightly different. That implies that there is a possibility to use a video densitometer not only to perform a video documentation but also to obtain qualitative and quantitative full value analysis.

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SUMMARY

In the present study a quantitative analysis of the content of aucubin in *Buddleja davidii* Franch. was performed. The experiment was based on the colour reaction between iridoids and 1% solution of 4-dimethylaminobenzaldehyde in ethanol and 36% hydrochloric acid. Iridoids were visualized on the HPTLC plate and their content was measured by means of classical TLC densitometer and videodensitometer. The quantitative determination was carried out comparing peak area or peak height with standard aucubin solution. The classical densitometry resulted in 1.05% (measured from peak height) and 1.21 (measured from peak area) of aucubin in dry mass of aerial parts of *Buddleja davidii* and was about 10% higher than video densitometry concentrations.

STRESZCZENIE

W pracy przedstawiono analizę ilościową zawartości aukubiny w zielu *Buddleja davidii* Franch. W prowadzonych oznaczeniach wykorzystano zdolność tworzenia barwnych kompleksów irydoidów z 1% roztworem 4-dimetylaminobenzaldehydu w etanolu i 36% kwasem solnym. W pracy zastosowano derywatyzację irydoidów na płytce HPTLC, a następnie oznaczono ich zawartość densytometrycznie, używając klasycznego densytometru TLC oraz viedeodensytometru. Ilościowe oznaczenia prowadzono dwiema metodami detekcji, porównując pola powierzchni pod pikiem oraz wysokość pików z wzorcowymi roztworami aukubiny. W przypadku klasycznej densytometrii oznaczona zawartość aukubiny w nadziemnych częściach *Buddleja davidii* była wyższa o ok. 10% i wynosiła 1,05% sm (obliczone z wysokości piku) i 1,21% sm (obliczone z pola powierzchni piku).