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Chemical composition and cytotoxic and antimicrobial activity of *Calycotome villosa* (Poiret) Link leaves☆

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Abstract

The chemical composition of the essential oil and methanol extract of *Calycotome villosa* (Poiret) Link leaves collected in Sardinia (Italy) has been studied by analytical and spectroscopic methods. Falcarinol and some alcohols, terpenes, furan derivatives, and paraffins have been isolated from the essential oil. Thirteen alkaloids and falcarinol have been identified in the chloroform fraction of the basic methanol extract. Six flavonoids and four anthraquinones have been isolated in the chloroform fraction after acidification of the basic methanol extract. The cytotoxic and antimicrobial activities have also been evaluated. The essential oil, the methanol extract in toto, and the fraction of the basic extract showed strong cytotoxicity, whereas the fraction of the acid extract showed lower cytotoxicity. Furthermore, this fraction showed good antibacterial activity against *Staphylococcus aureus*, *Bacillus lentus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Providencia rettgeri*, and *Morganella morganii*. It can therefore be stated that this plant's cytotoxicity is prevalently due to falcarinol. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: *Calycotome villosa*; Essential oil; Methanol extract; Falcarinol; Alkaloids and flavonoids

1. Introduction

Calycotome villosa (Poiret) Link [1] is an erect shrub that can grow up to 2 m tall. It has intricate, angular, pubescent branches, which are green when young and become greyish when mature. It shows sharp terminations and alternate leaves; the lower leaves are elongate, oval and trifoliate, and covered by sericeous down. The flowers are yellow and grouped, and have a bell-shaped calyx, thickly downy legume with a protuberant upper rib, and round dark olivaceous seeds. This shrub grows mostly in cool places in central Sardinia, on asperities from 500 to 900 m above sea level [2]. Very little research has been carried out on *C. villosa* [3] and the essential oil of the aerial part has not yet been reported.

As part of a systematic research study on the constituents and biological activity of Sardinian natural plants [4–7], we here examine the chemical composition and cytotoxic and antimicrobial activity of the essential oil and methanol extract of *C. villosa* leaves.

2. Experimental

2.1. Plant material

C. villosa leaves were collected in the countryside around Quartu S. Elena (Cagliari), Sardinia, Italy, during full blossom (April–May 1999). The plant was identified by Prof. M. Ballero of the Institute of Botany and Botanical Gardens, University of Cagliari, and a voucher specimen was deposited in the Herbarium of the Dipartimento Farmaco Chimico Tecnologico, Faculty of Pharmacy, University of Cagliari, Italy.

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2.2. Essential oil

The fresh aerial parts of the plant (1000 g) were distilled in a Clevenger-type apparatus [8] for 4 h. The essential oil, which was pale yellow, was obtained with a 0.05% yield, and was dried over molecular sieves, and stored at 4°C.

2.3. GC/FTIR of essential oil

The analysis was performed with a Perkin Elmer System 2000 GC/FTIR, with interfaced FID (300°C) and FTIR (200°C) detectors, using a DB-5 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness), at an oven temperature programmed to increase from 60 to 240°C at × 3°C/min, and an injector temperature of 300°C. The carrier gas was helium at a flow rate of 1 ml/min, the split ratio was 1:20, and the sample 0.15 μl.

The main components were identified by comparing the retention times with those of pure samples [9]. GC quantitative analyses were based on computer calculated peak area normalisations with the response factor method. GC/FTIR was performed by comparing the absorption of each peak of the Gram–Schmidt interferogram with that of pure samples inserted in the computer integrator [10] as suggested by Herres [11].

2.4. GC/MS of essential oil

Mass spectra were carried out with a QMD 1000 instrument (Fisons Instrument) at 70 eV using a gas chromatograph equipped with the same column and under the same conditions as described in GC/FTIR. The compounds were identified by comparison of the retention times with those of the authentic samples and of the mass spectra with those of the NBS/NIST Library [12] and other published mass spectra [13]. The assignments have also been confirmed by calculating the relative retention indices (RR_t) by a Van den Dool and Kratz operation [14] (data not shown).

2.5. Methanol extract

A soxhlet apparatus was employed to extract 200 g of the air-dried leaves with 1000 ml of methanol for 48 h. The methanol extract was concentrated in vacuo and stored at 4°C. The crude oil, which was yellow-green, was obtained with a 1.5% yield. It was treated with a 5% NaOH solution, and repeatedly extracted with chloroform. The chloroform extract was dried over molecular sieves and concentrated under reduced pressure (fraction I). The residue was chromatographed on a silica gel column (petroleum ether–ethyl acetate 5:1 as eluents, the eluent ratio was varied by increasing the ethyl acetate up to a 1:1 petroleum ether–ethyl acetate ratio). Several compounds were separated and identified by comparison of their physical properties with the values reported in the literature [15–18] and with the pure samples in our laboratory. The analyses showed that the main constituents were falcarinol and the following known alkaloids: lupinine, lupinine-4-ene, calycotomine, *N*-methyl-2-acetyloxycalycotomine, sparteine, anagyrine, lupanine, 13-hydroxylupanine, 3,13-dihydroxylupanine, 13-methoxylupanine, 3-hydroxy-13-tigloyloxylupanine, 3-methoxy-13-tigloyloxylupanine and 3-hydroxy-2-*O*-glucopyranosyl-13-tigloyloxylupanine.

The basic aqueous phase was acidified with diluted HCl and repeatedly extracted with chloroform. The chloroform extract was dried over molecular sieves and concentrated under reduced pressure to yield a very thick oil (fraction II), which shows maximum absorption at 220 and 270 nm. The obtained oil was analysed by a Milton Roy HPLC (column C18, 5μ, 250 mm, mobile phase acetonitrile–water 60:40, flow 0.5 ml/min, detector at 220 and 270 nm). With the obtained chromatogram it was possible to identify by comparison with commercial standards the following flavonoids: chrysin, genistein, luteolin, and quercetin-4′-methyl ether [9]. The remaining oil had to be chromatographed on a silica gel column (ethyl acetate and methanol as eluents). Several compounds were separated and identified by comparison of the physical properties with the values reported in the literature [19,20] and with the pure samples in our laboratory [21]. The analyses revealed that these compounds were the following known flavonoids and the anthraquinones: 6,8-dimethoxyluteolin, 2-(3′,4′-diacetyloxy)-6,8-dimethoxyluteolin, 1,8-diacetyloxy-3-methyl-9,10-anthracenedione, 10[H]-*O*-glucopyranosyl-1,8-dihydroxy-3-methyl-9[10H]anthracenone, 10[H]-*O*-glucopyranosyl-(6′-methyl)-9[10H]-anthracenone ether, 1,8-dihydroxy-3-methylen-(*O*-glucopyranosyl)-9,10-anthracenedione.

2.6. Cytotoxicity assay

Cytotoxic activity was evaluated on VERO cells (a line of green monkey kidney cells from ICN-Flow), grown on Dulbecco's modified MEM with 2% foetal calf serum (Gibco Laboratories Inc.). The assays were performed in duplicate in 24-well plates with about 5×10^4 cells per well. Cytotoxicity was read after 48 h of incubation in an atmosphere of 5% CO_2 at 37°C, and assessed as inhibition of cell multiplication in the presence of decreasing amounts of the compounds under study using a light microscope. The maximum non-toxic dose ($MNTD_{50}$) (μl/ml) was considered to be the dose of each compound that reduces cell multiplication by no more than 50%, as compared to the controls.

2.7. Antimicrobial activity

Antimicrobial activity was tested on *Staphylococcus aureus* ATCC 25923, *S. aureus* C, *Streptococcus faecalis* 69, *S. faecalis* 3208, *S. faecalis* 119, *S. faecalis* 3141, *S. faecium* VIC 5, *S. faecium* VIC 2, *S. faecium* VIC 1, *Bacillus lentus* B 60, *Escherichia coli* ATCC 25922, *E. coli* 143, *E. coli* 150, *Pseudomonas aeruginosa*, *P. fluorescens*, *Klebsiella pneumoniae* 52, *Providencia rettgeri*, *Morganella morganii*, *Candida albicans* CDC, *C. tropicalis* CBS, *Cryptococcus neoformans*, *Botrytis cineraria* 644, *Rhizoctonia solanii* 433, *Drechslera graminia* DG 2, and *Phomopsis* sp. 717. Antimicrobial activity was detected with an agar dilution method, using Mueller–Hinton agar for bacteria and Casitone agar for the yeasts, at the dose of 40 μl/well for crude samples. The inoculum was standardised at 10^5 CFU/ml for all micro-organisms. Inhibition of micro-organism growth was assessed after 48 h of incubation at 37°C and was reported in Table 3 as an inhibition zone (mm).

Table 1
Retention time (RT), molecular ion [M^+], and percentage (%) of the components identified in the essential oil of *Calycotome villosa* using GC/FTIR and GC/MS

Comp.	RT exp. (s)	RT lit. (s) [13]	[M^+]	Yield (%)
1. Methyl valerate	188	190	116	0.6
2. Furfural	196	194	96	1.1
3. 2-Methylfuran	240		92	0.2
4. Furfuryl alcohol	288		98	0.6
5. Falcarinol	360		260	1.4
6. 1,8-cineole	486	485	154	1.3
7. Acetophenone	551	551	120	0.2
8. Fenchone	601	605	152	1.8
9. Camphor	733	734	152	1.2
10. Methyl salicilate	857	856	152	1.2
11. Eugenol	1278	1279	164	1.8
12. Isoeugenol <(Z)–>	1407	1405	164	1.8
13. β-eudesmol	1990	1993	222	1.3
14. Farnesol <E,Z)–>	2202	2201	222	0.7
15. *n*-Eicosane	2740	2740	282	1.5
16. *n*-Heneicosane	2929	2931	296	2.8
17. *n*-Tricosane	3290	3292	310	1.9
18. *n*-Tetracosane	3462	3462	338	2.6
19. *n*-Pentacosane	3628	3627	352	2.6

Table 2
Cytotoxic activity of the analysed fractions from *Calycotome villosa*

Cytotoxic activity	Comp.			
	Essential oil	Crude extract	Fraction I	Fraction II
$MNTD_{50}$ (μl/ml)	0.04	0.04	0.04	10

3. Results and discussion

Thanks to GC/FTIR and GC/MS analyses of the essential oil, it has been possible to identify various alchohols, terpenes, furan derivatives, and paraffins (Table 1) from the retention times and mass spectra: in particular, methyl salicilate and camphor of known pharmacological activity, and falcarinol, a highly toxic diacetylenic derivative [22]. This essential oil showed strong cytotoxicity, which accounts for the fact that *C. villosa* is not widely used in popular medicine.

Because the yields of the essential oil of *C. villosa* were very low, we continued our research on its methanol extract. However, since the crude extract was as toxic as the essential oil itself (0.04 μl/ml), we tried fractionating it. First, we basified it with sodium hydroxide and then extracted it with chloroform. The residue (fraction I) was column chromatographed and falcarinol and the following 13 alkaloids were separated: lupinine, lupinine-4-ene, calycotomine, *N*-methyl-2-acetyloxycalycotomine, sparteine, anagyrine,

Table 3
Antimicrobial and antimycotic activity of fractions of *C. villosa* as an inhibition zone (mm)

	Crude extract	Fraction II
Staphylococcus aureus ATCC 25923	10	20
Staphylococcus aureus C		
Streptococcus faecalis 69		
Streptococcus faecalis 3208		
Streptococcus faecalis 119		
Streptococcus faecalis 3141		
Streptococcus faecium VIC 5		
Streptococcus faecium VIC 2		
Streptococcus faecium VIC 1		
Bacillus lentus B 60	11	10
Escherichia coli ATCC 25922	10	15
Escherichia coli 143	8	12
Escherichia coli 150	8	8
Pseudomonas aeruginosa		14
Pseudomonas fluorescens		
Klebsiella pneumoniae 52	10	12
Providencia rettgeri	8	8
Morganella morganii	10	16
Candida albicans CDC		
Candida tropicalis CBS		
Cryptococcus neoformans		
Botrytis cineraria 644		
Rhizoctonia solanii 433		
Drechslera graminia DG 2		
Phomopsis sp. 717		

lupanine, 13-hydroxylupanine, 3,13-dihydroxylupanine, 13-methoxylupanine, 3-hydroxy-13-tigloyloxylupanine, 3-methoxy-13-tigloyloxylupanine, and 3-hydroxy-2-*O*-glucopyranosyl-13-tigloyloxylupanine.

The basic aqueous phase was then acidified and repeatedly extracted with chloroform, to yield a very thick oil (fraction II), which shows its maximum absorption at 220 and 270 nm. Part of this oil was chromatographed by HPLC and chrysin, genistein, luteolin and quercetin-4′-methyl ether flavonoids were identified. The remaining part of the oil was chromatographed by silica gel column and the following flavonoids and anthraquinones were separated in very small quantities: 6,8-dimethoxyluteolin, 2-(3′,4′-diacetyloxy)-6,8-dimethoxyluteolin, 1,8-diacetyloxy-3-methyl-9,10-anthracenedione, 10[H]-*O*-glucopyranosyl-1,8-dihydroxy-3-methyl-9[10H]anthracenone, 10[H]-*O*-glucopyranosyl(-6′-methyl)-9[10H]anthracenone ether, 1,8-dihydroxy-3-methylen-(*O*-glucopyranosyl)-9,10-anthracenedione.

Cytotoxic analysis of fraction II expressed as maximum non-toxic dose ($MNTD_{50}$) was 10 μl/ml (Table 2), which was found to be very active on *Staphylococcus aureus*, *Bacillus lentus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Providencia rettgeri*, and *Morganella morganii*, and inactive against fungi (Table 3). Antibacterial tests proved positive also on the crude extract, but this datum was hardly reliable, considering the toxicity of the extract itself.

Although the essential oil and methanol extract of *C. villosa* leaves are potentially very toxic, by appropriately treating the extract so as to separate fraction I, we obtained fraction II, which is 250 times less toxic than fraction I, and very active against several gram (±) bacteria, especially *Staphylococcus aureus* (inhibition zone: 20 mm). Furthermore, considering the cytotoxic analyses carried out on the samples, it can, clearly, be stated that the toxicity of *C. villosa* mainly depends on falcarinol, whose presence or absence is crucial to its use in popular medicine.

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