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Preliminary communication

DEVELOPMENT OF AN OPTIMIZED PROCESSING METHOD FOR *WITHANIA FRUTESCENS*

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Withania somnifera (L.) Dunal originates mainly from Northern and Southern India. Primarily the roots are used in the Ayurvedic medicine as tonic, sedative hypnotic, adstringent, diuretic, emetic, and aphrodisiac. In Europe, it is widely used in food supplements. Due to the many effects and uses of this plant, the analysis of the *Withania somnifera* and optimization of industrial processing is nowadays an important issue. In Europe *W. frutescens* is native, and may be interesting for industrial preparation due to its similar phytochemical profile to *W. somnifera*. The point of our research was to develop an effective extraction and hydrolysis method of the *Withania frutescens* leaves to optimize the industrial processing.

Keywords: *Withania frutescens*, extraction, hydrolysis, withaferin A, optimization

Withania somnifera (L.) Dunal (Solanaceae, Sanskrit name: Ashwaganda) originates mainly from the southern and northern regions of India. This medium sized evergreen bush is widely used in the Ayurvedic medicine for different purposes (Matsuda et al., 2001; Sangwan et al., 2004; Lal et al., 2006). Due to its adaptogenic effects, *Withania somnifera* root is mostly used as general tonic to alleviate fatigue and support the physical and mental activity, which explains its botanically misleading name, Indian ginseng. The main compounds considered to be most important for the bioactivity of *W. somnifera* are the so-called withanolides. The firstly isolated and the most extensively examined withanolide is withaferin A, the main withanolide of *W. somnifera*.

Due to the widespread Ayurvedic use of the plant, the pharmacological properties of *W. somnifera* have been studied in detail. Neuroprotective and acetylcholinesterase inhibitory effects have been observed in animal experiments, whereas human studies confirmed the anxiolytic effect of the *W. somnifera* ethanolic extract (Kulkarni & Dhir, 2008; Bhatnagar et al., 2012). Further preclinical studies with withanolides revealed their antitumor, chemopreventive, and anti-inflammatory effects (Budhiraja et al., 2000). Although the level of evidence is not sufficient to use *Withania* or its active constituents as medicine, the long-standing application of the plant and the available experimental pharmacological data support its safe application. Therefore, *Withania* is available in several food supplements for its health-promoting effects.

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The bioactivities of the plant are mainly related to the withanolides, with withaferin A as the major representative of this group. For standardization of the commercial products, withaferin A is considered as marker compound. Due to the biological activities of this compound, effective industrial processing of *Withania* species and extraction of withaferin A from plant material have become of major interest. Optimization studies usually aim at the extraction of withanolide aglycons (Sangwan et al., 2004; Mirzajani et al., 2010; Sumithradevi et al., 2011). For this purpose, beside the traditionally applied roots, leaves may also be used due to their high withaferin A content (Praveen et al., 2010). Although withanolides are present in the plant material in remarkable quantity, extraction and hydrolysis of their glycosides to obtain free withanolides, such as withanone or withanolide A, has not been studied systematically yet. *W. frutescens*, a species native to Spain, has not been studied chemically extensively so far, however, withanolides were reported as its biologically active components (El Bouzidi et al., 2013).

1. Materials and methods

The present study aimed at the optimization of an extraction and hydrolysis method for the isolation of withaferin A from crude *W. frutescens* extract. *Withania frutescens* (L.) Pauquy plant materials (leaves, twigs, and roots) were collected from 3 years old cultivated plants (Reading, UK) and from wild plants in Spain. For the optimization of extraction method leaves were used. From dried fine ground *W. frutescens* leaves 1–1 g was extracted with different solvents (10 ml) by ultrasonic bath under tempered circumstances (25 °C) for 10 min. The extracts were evaporated to dryness with the help of rotation evaporator (300 mBar, 40 °C) and the dry masses were measured. Immediately after the evaporation, the dry extracts were dissolved in methanol and filtered through 0.45 μm PTFE syringe membrane filters, then analysed with HPLC. For the HPLC analysis, Kinetex 150×4.6 mm, 5μ, RP C-18 column was used on a Waters 600 HPLC system equipped with a DAD detector. Gradient elution was carried out with H_2O–MeOH–EtOH (0 min: 65:17.5:17.5, 25 min: 55:22.5:22.5, 40 min: 0:50:50 ratio; 1 ml min^{-1}, detected at 230 nm). The peak of withaferin A was detected at 10 min (withaferin A standard has been purchased from Sigma-Aldrich, purity of 95.0%). The comparison of HPLC chromatograms of different extracts is presented in Figure 1. The withaferin A content of twigs (6 samples), leaves (4 samples), and roots (8 samples) was measured by the method described above, using 10 ml of MeOH–H_2O 1:1 as extracting solvent. During the hydrolysis experiments, 0.5 g of *Withania* leaf extract (prepared with MeOH–H_2O 1:1) was hydrolysed for 30 min at 100 °C with 20 ml of 10% phosphoric acid, acetic acid, sulphuric acid, and hydrochloric acid. The results of hydrolysis were monitored by TLC (normal phase, CH_2Cl_2–cyclohexane–MeOH 3.7:1:0.3, detection by spraying with vanillin–sulphuric acid followed by heating at 120 °C for 1 min).

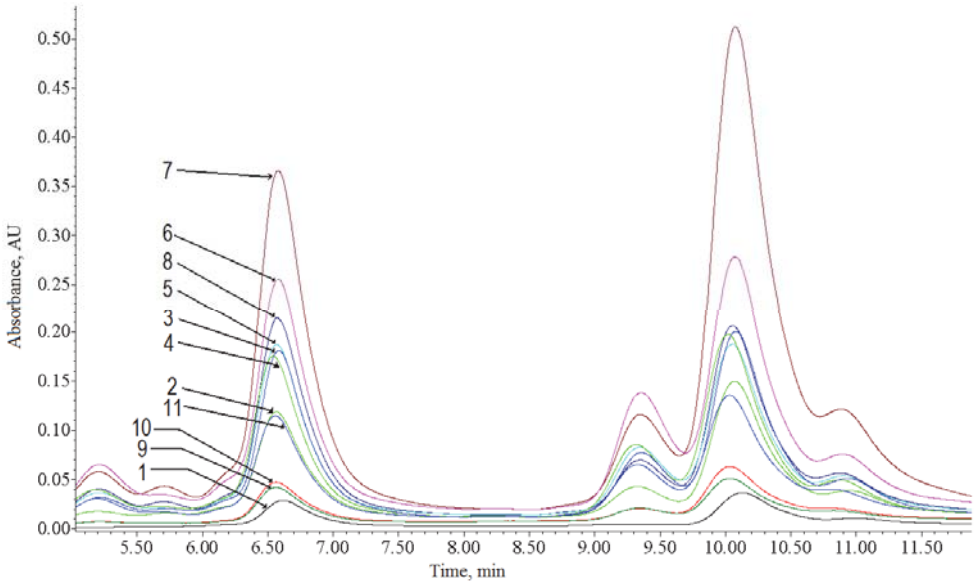

Fig. 1. HPLC comparison of various extracts. Extracting solvents: 1: CH_2Cl_2; 2: CH_2Cl_2–MeOH 75:25; 3: CH_2Cl_2–MeOH 50:50; 4: CH_2Cl_2–MeOH 25:75; 5: MeOH; 6: MeOH–H_2O 75:25; 7: MeOH–H_2O = 50:50; 8: MeOH–H_2O 25:75; 9: EtOH; 10: CH_2CL_2–EtOH 25:75; 11: EtOH–H_2O 75:25

2. Results and discussion

After the extensive extraction trial, the most effective extraction solvent proved to be MeOH–H_2O 1:1 (Table 1). The extract prepared with this solvent resulted in high dry mass and contained the highest amount of withaferin A. Extracts gained with MeOH and H_2O contained also remarkable amounts of withanolide glycosides.

Table 1. Extraction of *Withania frutescens* leaf with different solvents

Extraction solvent		Dry mass (mg)	Withaferin A content (mg)	Percentage of withaferin A in dry mass (%)
CH_2Cl_2	100	9.62	1.72	17.88
CH_2Cl_2:MeOH	75:25	33.41	5.63	16.85
CH_2Cl_2:MeOH	50:50	58.08	7.88	13.57
CH_2Cl_2:MeOH	25:75	85.59	7.93	9.27
MeOH	100	69.54	7.68	11.04
MeOH:H_2O	75:25	129.91	1.83	1.41
MeOH:H_2O	50:50	140.70	20.44	14.52
MeOH:H_2O	25:75	157.18	7.92	5.03
EtOH	100	76.90	2.39	3.11
CH_2Cl_2:EtOH	25:75	42.83	2.77	6.46
EtOH:H_2O	75:25	70.18	5.78	8.24

In the Ayurvedic medicine primarily the roots of *W. somnifera* are used. Taking into account that other plant parts may also contain withanolides, the withaferin A content of different plant parts of *W. frutescens* was also studied (Table 2).

Table 2. Average withaferin A content of plant organs, extracted with MeOH–H_2O 1:1

Plant part	Withaferin A content (mg g^{-1})	Standard deviation (mg g^{-1})
Leaf	4.38	0.1678
Root	1.60	0.0638
Twig	1.53	0.0015

To enhance the efficiency of withaferin A production from the plant material, a hydrolysis study was started. Initial tests (data not shown) revealed that hydrolysis was successful only with sulphuric acid and acetic acid, which were then further used for the optimization of the hydrolysis by modifying acid concentration and reaction time. Withaferin A contents of the hydrolysates were verified by HPLC according to the method presented before. The parameters (hydrolysis time, acid concentration) and resulting withaferin A concentrations are listed in Table 3. The highest yield in free withaferin A was obtained with hydrolysing with sulphuric acid in concentration of 1% for 90 min.

Table 3. Hydrolysis experiments with a *Withania frutescens* leaf extract (MeOH–H_2O 1:1)

Acid name	C (V/V%)	Time of sampling (min)	Free withaferin A (mg)
Sulphuric acid	1.00	30	0.81
	1.00	60	0.83
	1.00	90	0.95
	2.50	30	0.84
	2.50	60	0.86
	2.50	90	0.87
	5.00	30	0.86
	5.00	60	–
	5.00	90	–
	7.50	30	–
	7.50	60	0.86
	7.50	90	–
	10.0	30	0.82
	10.0	60	0.84
	10.0	90	0.82

Table 3 continued

Acid name	C (V/V%)	Time of sampling (min)	Free withaferin A (mg)
Acetic acid	25	4	0.80
	25	5	0.81
	25	6	0.80
	50	4	0.79
	50	5	0.80
	50	6	0.80
	100	4	0.78
	100	5	0.79
	100	6	0.79

3. Conclusions

Our experiments revealed that leaves, twigs, and roots of *W. frutescens* contain remarkable amount of withaferin A. Based on the high withaferin A content (4.38 mg g^{-1}), the high biomass production compared to twigs, and also considering the aspects of sustainable cultivation, leaves seem to be the most promising sources for industrial processing. To further increase the yield of withaferin A, we successfully developed an effective method to gain the withanolide-rich extracts from *Withania frutescens* leaf. Dichloromethane-containing extracting solvents resulted in withaferin A-rich extracts, however the overall yield of this compound could be maximized by using mixtures of methanol and water. Hydrolysis of extracts was also optimized in order to enhance the efficiency of withaferin A production from plant material. This European species may be regarded as a perspective source for withanolide production.

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