Plant Biotechnol Rep (2011) 5:127–134
DOI 10.1007/s11816-011-0165-4

ORIGINAL ARTICLE

# Biotransformation of withanolides by cell suspension cultures of *Withania somnifera* (Dunal)

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Received: 27 April 2010 / Accepted: 20 December 2010 / Published online: 25 January 2011
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**Abstract** The biotransformation potential of cell suspension cultures generated from *Withania somnifera* leaf was investigated, using withanolides, i.e. withanolide A, withaferin A, and withanone as precursor substrates. Interestingly, the cell suspension cultures showed inter-conversion of withanolides, as well converted to some unknown compounds, released to the culture media. The bio-catalyzed withanolide was detected and quantified by TLC and HPLC, respectively. There is noticeable conversion of withanolide A to withanone, and vice versa though at a lower level. The type of reaction of this biotransformation appears to be substitution of 20-OH group to 17-OH in withanolide A. In this paper, we present for the first time the possibility of biotransformation by inter-conversion of withanolides of pharmacological importance through cell suspension culture of *W. somnifera*. The possible role of putative cytochrome $P_{450}$ hydroxylases is implicated in the conversion.

**Keywords** Ashwagandha · Biotransformation · Cell suspension cultures · Withanolide · *Withania somnifera*

## Introduction

Withanolides are triterpenoids, steroidal lactone synthesized in the plant *Withania somnifera*, popularly known as ashwagandha. Withanolides, chemically nomenclatured as 22-hydroxy ergostane-26-oic acid 26, 22-$\delta$-lactones, are C28-steroidal lactones based on an intact or rearranged ergostane frame through appropriate oxidations at C-22 and C-26 to form a $\delta$-lactone ring (Fig. 1). Production of withanolides in plants appears to be restricted to only a few genera within Solanaceae with their most prodigal amounts and structurally diversified forms found in *W. somnifera* (Misra et al. 2008; Sangwan et al. 2005). Major withanolides, like withaferin A and withanolide A, have been demonstrated to possess significant and specific therapeutic action in carcinogenesis, Parkinson's disease and Alzheimer's disease (Choudhary et al. 2005; Jayaprakasam et al. 2003; Kaileh et al. 2007; Kuboyama et al. 2005; Matsuda et al. 2001; Su et al. 2004). Withanolides are credited with a range of bio-activities including adaptogenic, antimicrobial, anti-inflammatory, anti-tumour, and immunomodulatory activity (Singh et al. 2003). Various types of withanolides have been isolated from *W. somnifera* and are reported in the literature (Chaurasiya et al. 2008). Mainly withanolide A, withaferin A, withanone, withanolide D, are present in the plants with their proportions varying according to their developmental age and tissue, chemotype, and geographical locations (Sangwan et al. 2004; Chaurasiya et al. 2008). These withanolides are attributed to different biological activities, such as withaferin A is recognized for inhibition of growth of tumourous cells, while withanolide A has recently been credited as nootropic agent for recovery from nervous degeneration, dendrites formation and its branching, and hence can be a promising compound for the treatment of neural degeneration type of diseases like Parkinson's and Alzimer's diseases (Kuboyama et al. 2005). Though there is considerable information regarding the chemistry of withanolides, however, only a few reports are available on biosynthesis and biogenetic relationship of withanolides. Our laboratory

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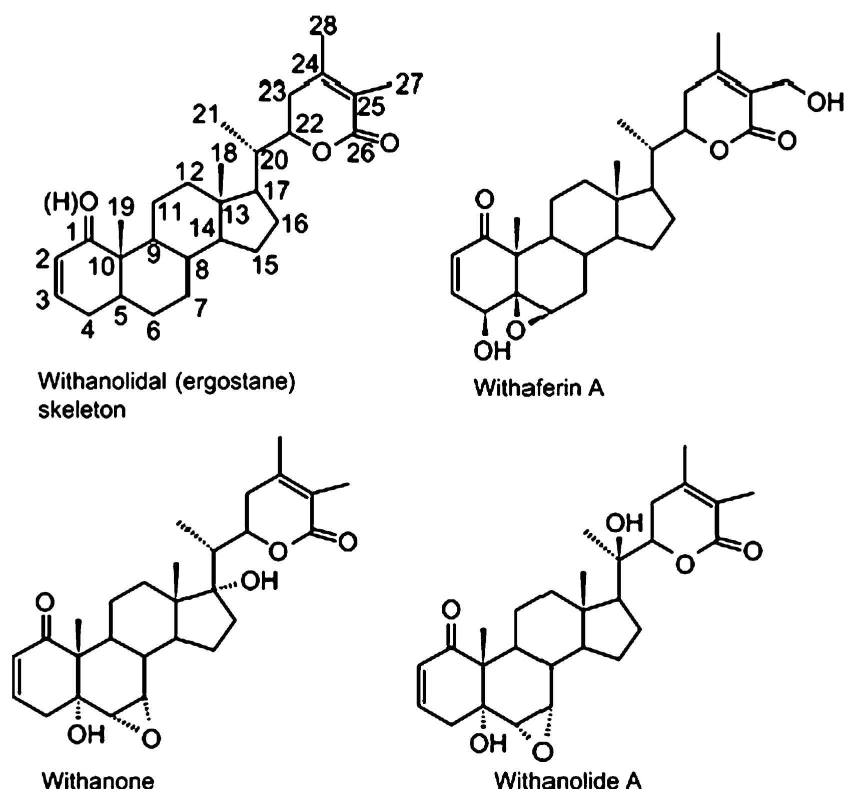



**Fig. 1** Structures of withanolides from *Withania somnifera*

has published work related with the biosynthesis of withanolides in cultures (Sabir et al. 2008; Sangwan et al. 2008). Biotechnologically, there is also hardly any information on the cell suspension cultures of *W. somnifera* except from Sabir et al. (2008) concerning their catalytic capabilities. Inter-conversion of these important phytomolecules through biotransformation can be a promising way for obtaining the type of molecule of interest. Many new and novel derivative compounds are likely to get generated and increase the spectrum of molecules to be tested for their novel drug action. In this paper, we report our attempt on cell suspension culture of *W. somnifera* to be used for the biotransformation of different withanolides to some new compounds, as well as to different types of known withanolides. This is the first report on the study of bio-enzymatic conversion of withanolides through biotransformation by cell suspension culture of *W. somnifera.*

## Materials and methods

### Plant material

*W. somnifera* plants, experimental line RSS 118, were raised at the experimental farm of the Central Institute of Medicinal and Aromatic Plants (Lucknow, India), and these plants were used for raising sterile cultures.

### Cell culture

Stably maintained, friable callus of *W. somnifera* with repeated subculturing for 2 years was used for the generation of cell suspension culture as reported earlier (Sabir et al. 2008). These calli were obtained from the sterile leaves of the plant. The cell suspension cultures were initialized and maintained on the Murashig and Skoog (MS) medium with 3% sucrose and supplemented with 2, 4-D and kinetin in 3 and 0.5 mg $l^{-1}$ concentration, respectively. The cultures were subcultured after every 2 weeks in the same media, having 20 ml of cells in 100 ml fresh medium, on a rotator shaker (100*g*) at 25°C for uniform synchronized growth (Fig. 2a, b).

### Incorporation of substrates

Withanolides such as withanolide A, withaferin A, and withanone at 1 mg/10 ml of culture were added to the synchronously growing cell suspension cultures of *W. somnifera*. The withanolide substrates were dissolved in absolute ethanol at concentration of 1 mg in 0.5 ml. These cells were incubated for different number of days in the above-mentioned conditions. The cultures were kept in a culture room maintained at 25 ± 1°C under 14-h photoperiod (30 µmol $m^{-2}$ $s^{-2}$) supplied from a cool white fluorescent tube.

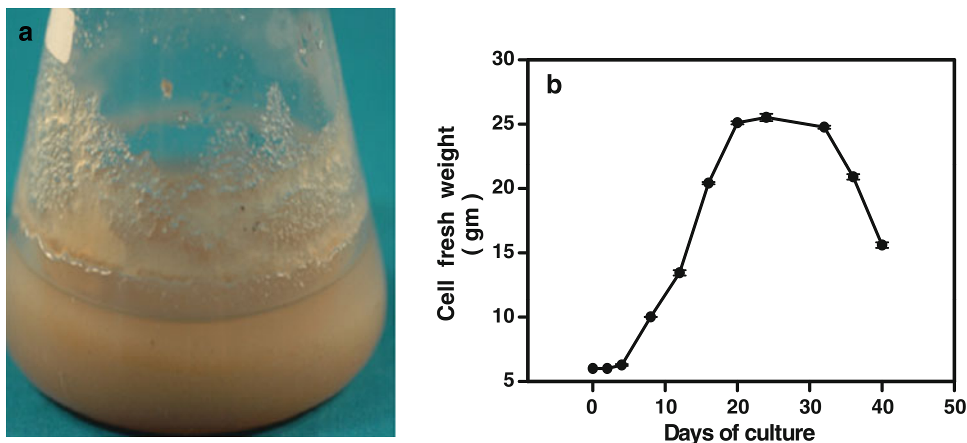



**Fig. 2** **a** Cell suspension cultures of *W. somnifera*, **b** growth kinetics of cell suspension cultures

Identification of withanolides

Isolation, purification, and spectral characterization of withanolide A, withaferin A, and withanone was done as reported earlier (Misra et al. 2008; Sangwan et al. 2008). Withanolides were isolated and purified as white crystals in ethyl acetate according to previously reported methods (Sangwan et al. 2007) and subjected to structure determination through melting point characterization (282–283°C), IR spectroscopy, NMR spectroscopy and mass spectrometry. IR spectrum was recorded on a Shimadzu IR-408 spectrometer. 1H-NMR spectra were recorded on a Bruker AV-300 FT-NMR system at 300 MHz, and chemical shifts were recorded in $\delta$-units using tetramethylsilane as an internal standard. $^{13}$C-NMR spectra were recorded on the same instrument at 75 MHz. The fast atom bombardment (FAB) mass spectrum was recorded on a JEOL SX 102/DA-6000 Mass Spectrometer coupled to Data System using argon/xenon (6 kV, 100 mA) as the gas for FAB. The crystal molecular identity of withanolides was ascertained by matching its spectral information with previous reports (Anjaneyulu and Rao 1997).

Withanolide extraction and estimation

Cells were filtered out from the media, ground in methanol–water (25:75, v/v), incubated for 3 h in a shaker then filtered. This extraction was repeated thrice and the pooled filtrate of methanolic fraction was further extracted three times with chloroform in a separatory funnel. The air-dried pooled chloroform fraction was dissolved in HPLC-grade methanol, filtered through a Millipore clarification kit and subjected to TLC and HPLC analysis as reported earlier (Sangwan et al. 2008). In the case of the media, the withanolides were extracted directly by adding equal volumes of chloroform and repeating the extraction thrice. The chloroform fractions were collected, pooled, air-dried and dissolved in methanol. Further, three volumes of water was added to the dissolved methanol fraction and again re-extracted with chloroform. The air-dried pooled chloroform fractions of both cells and media were dissolved in HPLC-grade methanol, filtered through a Millipore clarification kit and subjected to TLC and HPLC analysis.

HPLC analysis was performed on Waters PAD (Model 996; Milford, MA, USA) and separations were achieved using reversed phase column (150 × 3.9 mm i.d.; 4 µm) subjected to gradient elution. The solvent system (0.6 ml per min) comprised of methanol and water (each containing 0.1% acetic acid) in the gradient mode 45:55–65:35 (45 min). Detection was done at 227 nm using an online UV detector (SPD-10A) and the chromatogram reports were generated through integrated software (Empower). Amounts of 10 µl of the withanolide sample (methanolic solution) or 5 µl of marker withanolide was injected for each run.

## Results and discussion

Different lines of synchronized cell suspension culture of *W. somnifera* were generated by subculturing the homogenously growing cells. Cells were synchronised by transferring the visibly similar size of upper layer of cells to fresh media and routinely subculturing it. Some of these lines expressed the withanolide content, especially withanolide A, whilst some cell lines could not produce the valuable secondary metabolite. Selected synchronized cell suspension culture was tested for the biotransformation of withanolides and also for the growth kinetics (Fig. 2a). The selected cells grew homogenously and achieved around fivefold increase in cell fresh weight in about 20 days of culture (Fig. 2b). Both types of producing and non-producing cells were utilized for biotransformation of withanolides, which gave the same pattern of bioconversion, but quantitatively their amount of bio-converted products and utilization of substrates are different. The non-

**Table 1** Biotransformation of withanolides using cell suspension cultures of *W. somnifera*

| Biotransformation system | Withaferin A detected (mg/g FW) | | | Withanolide A detected (mg/g FW) | | | Withanone detected (mg/g FW) | | | Total withanolides content (mg/g FW) | % Conversion |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cell (mean ± SD) | Media (mean ± SD) | Mean ± SD (cell + media) | Cell (mean ± SD) | Media (mean ± SD) | Mean ± SD (cell + media) | Cell *M* ± SD) | Media (mean ± SD) | Mean ± SD (cell + media) | | |
| Control cell suspension culture | Nil | – | – | 0.0277 | – | – | 0.0047 | – | – | 0.0325 | Nil |
| Withaferin A added in the suspension culture | 0.046 ± 0.01 | 0.036 ± 0.005 | 0.116 ± 0.047 | 0.007 ± 0.005 | 0.018 ± 0.006 | 0.025 ± 0.015 | – | – | – | 0.141 | Nil |
| Withanolide A Added in the suspension culture | – | – | – | 0.450 ± 0.18 | 0.567 ± 0.27 | 1.02 ± 0.36 | 0.04 ± 0.011 | 0.122 ± 0.013 | 0.161 ± 0.02 | 1.181 | 16.1 |
| Withanone added in the suspension culture | – | – | – | 0.013 ± 0.014 | – | 0.013 ± 0.014 | 0.60 ± 0.19 | 0.444 ± 0.052 | 1.04 ± 0.13 | 1.052 | 1.3 |

producing cells were preferred for using in biotransformation studies as there is either very low amount or an absence of endogenous withanolides. Three types of withanolides, withaferin A, withanolide A, and withanone (Fig. 1), were added to the cell suspension cultures of *W. somnifera.* The most noticeable inter-conversion of withanolide A to withanone by the cells could be detected by TLC (Table 1; Fig. 3) and also monitored by HPLC (Fig. 4c, d). Few new peaks also appeared when withanone was fed to the suspension cells (Fig. 4g, h). The basic skeletons of withaferin A and withanolide A are slightly different, in withanolide A, the 5,6 epoxy group and 27-hydroxyl group of withaferin A is replaced by the 6,7 epoxy and 20 hydroxyl groups. There is marked bioconversion of withanolide A to withanone both in cells and media in both types of cells (Table 1). The fundamental frame of both withanolide A and withanone are similar except that the 20-OH group of withanolide A is shifted to the 17-OH group in withanone. Structurally, withanone and withanolide A are quite similar except that there is $\beta$-hydroxylation at C-20 in the latter whilst there is $\alpha$-hydroxylation at C-17 in the former.

Nevertheless, synthesis of withanolide A from withanone seems less favorable biochemically as it would involve complexity of elimination of the $\alpha$-hydroxyl group

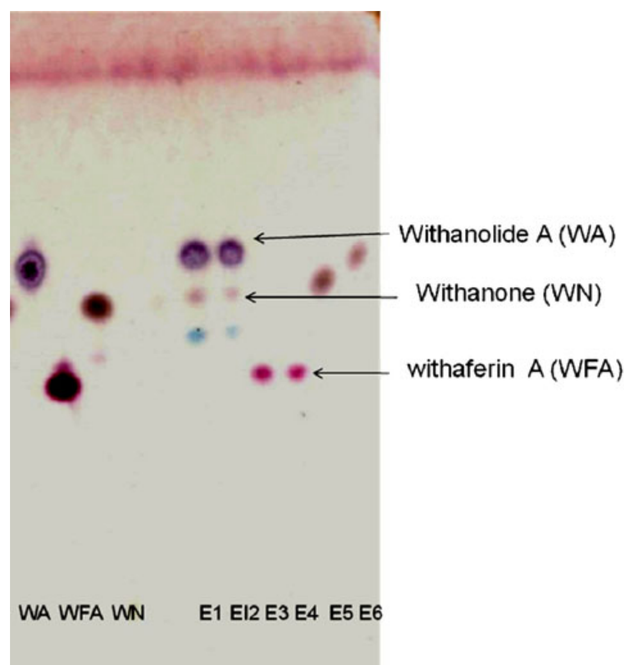



**Fig. 3** TLC-based detection of biotransformation of withanolides by cell suspension cultures. *WA* withanolide A, *WFA* withaferin A, *WN* withanone, *E1*, *E2* withanolide A added to the suspension cell culture (two lanes), *E3*, *E4* withaferin A added to the suspension cell culture (two lanes), *E4*, *E5* withanone added to the suspension cell culture (two lanes)

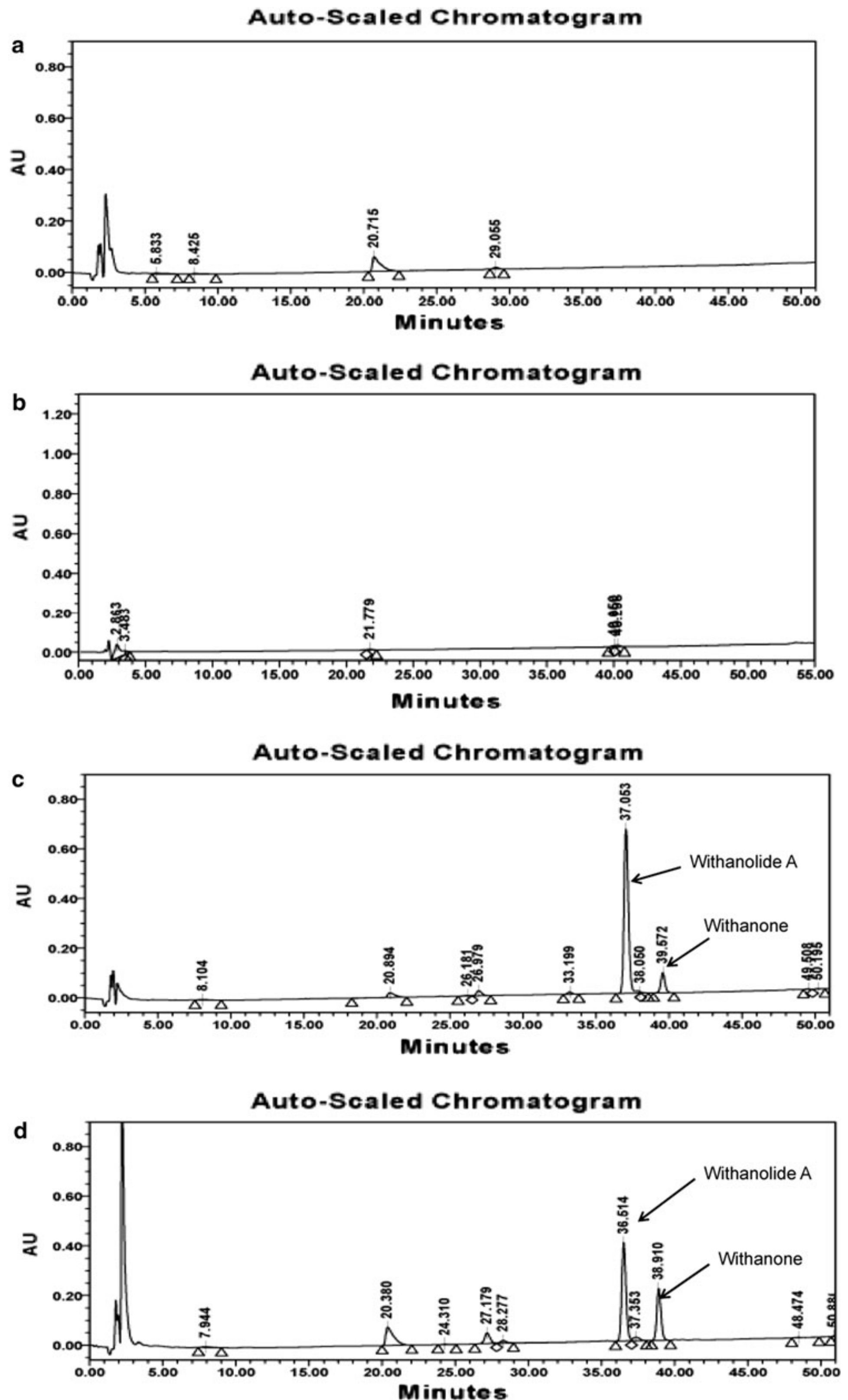
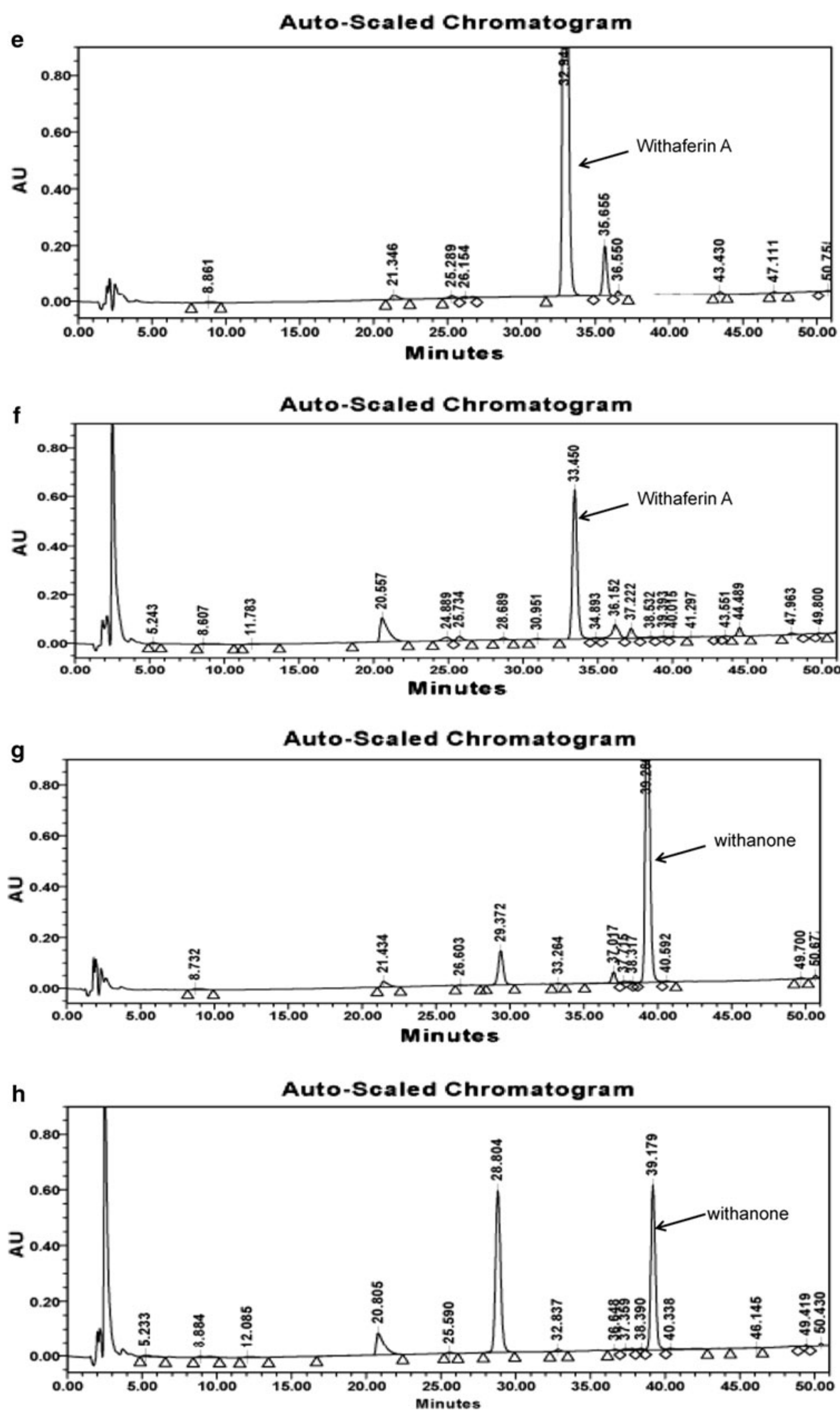



**Fig. 4** HPLC profile of biotransformations through cell suspension cultures of *W. somnifera* under control conditions. **a** Withanolides extracted from the cells of the biotransformation system of the control cell suspension culture; **b** withanolides extracted from the medium of the biotransformation system of the control cell suspension culture (no exogenous substrate is added); **c** withanolides extracted from the cells of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withanolide A; **d** withanolides extracted from the medium of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withanolide A; **e** withanolides extracted from the cells of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withaferin A; **f** withanolides extracted from the medium of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withaferin A; **g** withanolides extracted from the cells of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withanone; **h** withanolides extracted from the medium of the biotransformation system of the experimental cell suspension culture incubated with exogenously provided withanone

from C-17 and incorporation of $\beta$-hydroxylation at C-20. A common progenitor metabolic intermediate for the two may be more likely. In media, there is a greater amount of conversion which is almost equal in both producing and non-producing cells. Withanone added to the suspension cells very minutely bio-transformed to withanolide A

**Fig. 4** continued




(Fig. 4g, h). In media, more conversion was observed than in cells alone. Producing cells had more capacity to biotransform the substrates.

The results suggested that plant cells can be exploited for biotransformation of one important compound to another important molecule. There is no report in the

literature regarding the biotransformation of withanolides except that *Arthrobacter simplex* bio-transformed withaferin A under submerged conditions to 7 new derivatives and 4,27-di-*O*-acetylwithaferin A was transformed to another 9 derivatives. Derivatives also differed in their biological effects on P388 cells. A marked inhibitory effect on the synthesis of nucleic acids and proteins in the leukemia cells P388 was found in 12 out of 16 new derivatives (Funska et al. 1985).

Our results suggest a biogenetic probability of the withanolide terminal transformations: (1) withaferin A is not involved in the withanolide A to withanone conversion; or (2) in the vice versa route, there is a hydroxylation and dehydroxylation possibility between withanone and withanolide A. As there is conversion detected both ways, the study provides a potential to convert easily available quantities of withanone into withanolide A. Withanolide A is one of the most promising phyto-pharmaceuticals because of its recently reported impressive pharmacological properties: (1) induction of neurite regeneration and synaptic reconstruction (Kuboyama et al. 2005; Zhao et al. 2002) that is important in dealing with neurological disorders particularly Alzheimer's and Parkinson's diseases, and (2) strong inhibition of carcinogenesis (Kinghorn et al. 2004). Our ongoing work on identifying the enzymes and genes involved in the putative position-specific hydroxylation (cytochrome P450) in a metabolic model drawn from withania-root chemo-informatics, wherein withanolide B occupies an anaplerotic position for diverse metabolic transformations into withanolide A, withanolide R, withanone and 27 hydroxywithanolide B, is expected to provide more insights/support to the hypothesis on the transformation products of this study. The low recovery of withaferin A from cells and media after incubation may account for the facilitated degradation of the molecule for unknown reasons in the culture conditions. It has been shown earlier during enzymatic studies that withaferin A has two facile C for oxidation as well as for dimerization (Misra et al. 2008). Such reasons may be accountable for the relative disappearance of withaferin A and its newly synthesized compound being formed and or degraded while being under incubation. 2-Mercaptoethanol has been shown to react selectively with the 5$\beta$,6$\beta$-epoxy steroids isolated from *W. somnifera* substituting the epoxide by a six-membered oxyethylene-2′-thio ring, whereas it failed to show such reactivity on 6$\alpha$,7$\alpha$-epoxy withasteroids (Misra et al. 2008). The structure of the product has been elucidated by spectroscopic methods, especially applying extensive 2D NMR methods. Thus, the results reported in the paper are interesting from two angles: (1) they provide a clue for the biogenetic relationship in the withanolide biosynthesis pathway; and (2) provide a possibility of biotransforming withanone, an abundant withanolide, into the more valuable withanolide A and vice versa. Cell-free extracts and cell cultures are important tools for biotransformation studies as reported earlier (Sangwan et al. 1993) and provide important clues for the biogenetic route for the synthesis of important natural products where information is limited.

**Acknowledgments** The authors at CIMAP are thankful to the Director, CIMAP, for providing the necessary facilities and encouragement. The financial grant NWP-09 Program of CSIR, Government of India, New Delhi is gratefully acknowledged. F.S. and J.S. are grateful to CSIR, New Delhi for the award of senior and junior research fellowships, respectively.

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