ORIGINAL ARTICLE

Evaluation of the bioavailability of major withanolides of *Withania somnifera* using an *in vitro* absorption model system

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ABSTRACT

Withania somnifera (L.) Dunal, shows several pharmacological properties which are attributed mainly to the withanolides present in the root. The efficacy of medicinally active withanolides constituents depends on the absorption and transportation through the intestinal epithelium. We examined these characteristics by employing the Sino-Veda Madin-Darby canine kidney cells culture system, which under *in vitro* condition shows the absorption characteristics similar to the human intestinal epithelium. Thus, the aim of the present investigation was to assess the bioavailability of individual withanolides. Withanolides were diluted in Hank's buffered saline at a concentration of 2 μg/ml were tested for permeability studies carried out for 1 h duration. Permeability was measured in terms of efflux pump (P_{eff}) in cm/s. P_{eff} values of withanolide A (WN A), withanone (WNN), 1,2-deoxywithastramonolide (1,2 DWM), withanolide B (WN B), withanoside IV-V (WS IV-V), and withaferin A were 4.05×10^{-5}, 2.06×10^{-5}, 1.97×10^{-5}, 1.80×10^{-5}, 3.19×10^{-6}, 3.03×10^{-6} and 3.30×10^{-7} respectively. In conclusion, the nonpolar and low molecular weight compounds (WN A, WNN, 1,2 DWM, and WN B) were highly permeable. As against this, the glycosylated and polar WS IV and WS V showed low permeability. Surprisingly and paradoxically, the highly biologically active withaferin A was completely impermeable, suggesting that further studies possibly using human epithelial colorectal adenocarcinoma (Caco-2) cells may be needed to delineate the absorption characteristics of withanolides, especially withaferin A.

Key words: Absorption model, ashwagandha, bioavailability, Caco-2 cells, Madin-Darby canine kidney cells, withanolides

INTRODUCTION

Withania somnifera, commonly known as Ashwagandha, Winter Cherry, and Indian ginseng, is one of the most important plants used for over 3000 years in Indian Ayurvedic medicinal system. *W. somnifera* shows several pharmacological activities which are mainly attributed to withanolides present in the roots.[1-4] Withanolides are the naturally occurring C_{28} steroidal lactones built on ergostane framework in which C_{22} and C_{26} are oxidized to form a six-member lactone ring [Figure 1]. About 35 withanolides have been isolated from the roots of *W. somnifera* of which withanoside V (WS V), withaferin A, withanolide A (WN A) and withanolide B (WN B) are the major

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components.[5-7] Withaferin A has anti-tumor, apoptotic, anti-angiogenesis, radiosensitizing and anti-inflammatory activities.[8-11] WN A is effective as a neurological, immunological and anti-stress agent.[12-14] WS IV and V play an important neuro-regenerative role. Thus in spinal cord injury WS IV and V improve hindlimb function and increase the myelin layer in the peripheral nervous system.[15] In the light of this, it is important to find out the bioavailability of the individual withanolide compounds to ascertain their therapeutic efficacy.[16]

The intestinal epithelium plays an important role in absorption and transportation of drugs. It has been suggested that *in vitro* cell culture system may be a useful model system to quickly assess the bioavailability of a given drug.[17-19] Sino-Veda Canada has developed and patented a system employing Madin-Darby canine kidney (MDCK) cells which were found to be useful in assessing the bioavailability of various drugs.[20-22] In the present investigation, we report the bioavailability profiles of major withanolides of *W. somnifera* employing the MDCK cell culture system.

MATERIALS AND METHODS

Chemicals

Hanks' buffered saline, 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid (HEPES) and Lucifer Yellow were parched form Sigma-Aldrich, St. Louis, MO, USA. WS IV, WS V, withaferin A (WF A), 1,2 deoxywithastramonolide (1,2 DWM), withanone (WNN), WN A and WN B were obtained from Natural Remedies Pvt. Ltd., Bengaluru, India. The purity of the standards was established by high-performance liquid chromatography (HPLC) analysis [Figure 1].

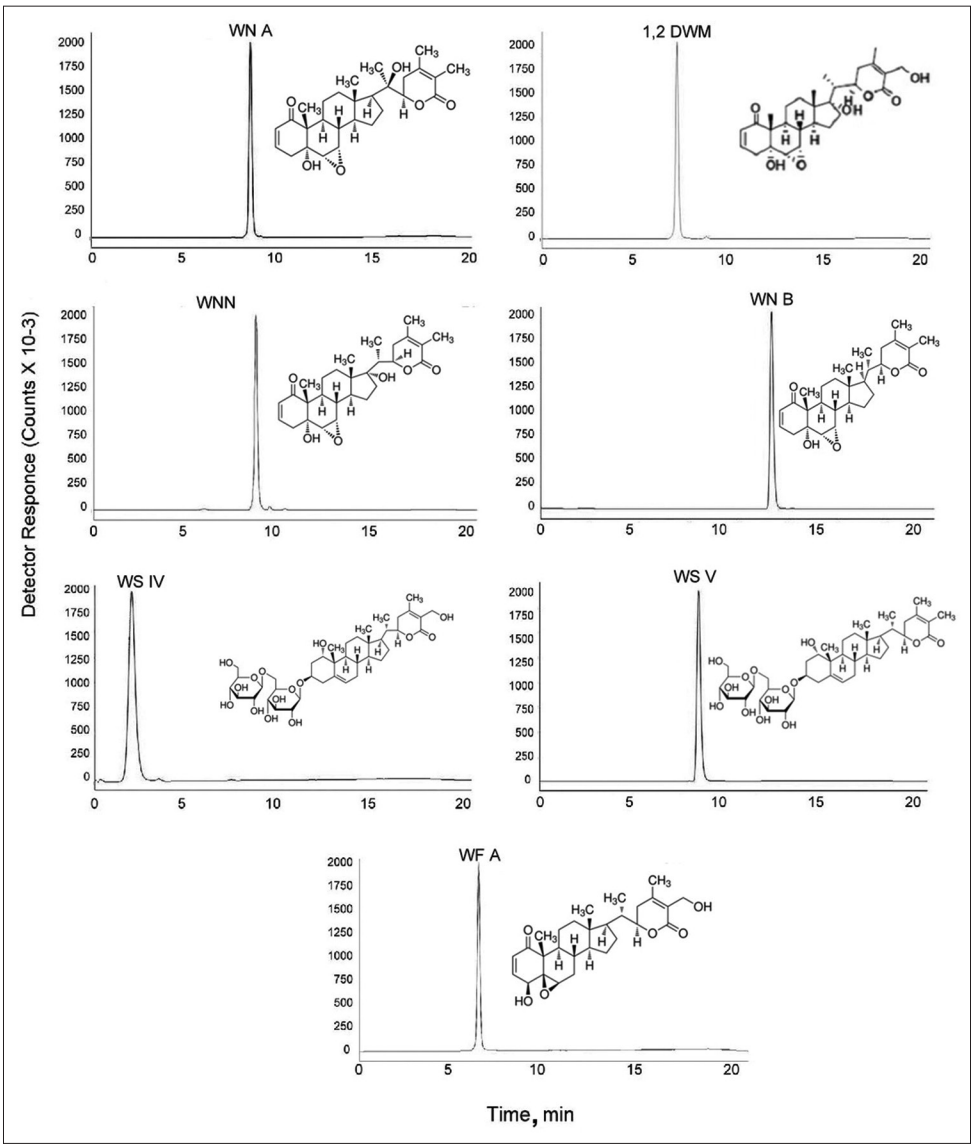

Figure 1: The major withanolides of *Withania somnifera* and their high performance liquid chromatography elution profiles

Bioavailability studies

The individual withanolides were diluted in Hank's buffered saline and tested for permeability by the Sino-Veda's cell culture system [Figure 2]. The concentrations of withanolides in epical and basal chambers were determined by HPLC coupled to diode array absorbance detection (diode array detector [DAD]) and positive mode electrospray ionization mass spectroscopy (MS). A Phenomenex Luna 3 μ C18 (2) 100A 15 cm × 4.60 mm column equipped with a guard column (security guard C18) and column heater set at 40°C was used. For HPLC-MS analysis, mobile phase A was 5 mM ammonium acetate (pH 3.0 adjusted with formic acid) in 18 mega Ω water, and mobile phase B was HPLC grade acetonitrile. Based on the behavior of withanolides in the cell culture system the mobile phase gradient program was modified to obtain better resolution. Flow rate was 0.7 ml/min and solvent gradient program for WF A, WN A, WN B, WNN and 1,2 DWM was 0–15 min A: B (60:40%), 15–20 min A: B (15:85%) and 20–25 min A: B (60:40%). For WS IV 0–15 min A: B (70:30%), 15–20 min A: B (15:85%) and 20–25 min A: B (70:30%). Mobile phase program for WS V was 0–15 min A: B (70:30%), 15–20 min A: B (75:25%) and 20–25 min A: B (70:30%). DAD condition specific signals were collected at 205 nm (bandwidth 16), 210 nm (bandwidth 8), 254 nm (bandwidth 16), 270 nm (bandwidth 16) and 280 nm (bandwidth 16). All spectra were scanned from 190 nm to 400 nm with 2 nm step. Electrospray mass spectrometer conditions were in positive mode, gas temperature 350°C, drying gas 13 L/min, neb pressure 60 psi, vaporizer 350°C and capillary voltage 3000 V. Scan conditions were low mass 150, high mass 600, fragmentor 70, gain 1, threshold 150, stepsize 0.20. Selected ion mode signals were monitored at various ranges. Electrospray mass spectrometer selected ion mode signals were monitored at 471.3, 488.4 (WN A), 455.4, 472.4 477.4 (WN B), 471.2, 453.2, 493.2 (WNN), 471.3, 493.2 (WF A, 1,2 DWM), 411.2, 441.4, 459.4, 805.4 (WS IV) and 407.4, 425.4, 443.4, 767.4, 784.6, 789.4 (WS V) except for WS IV and WS V where the high mass is 850.

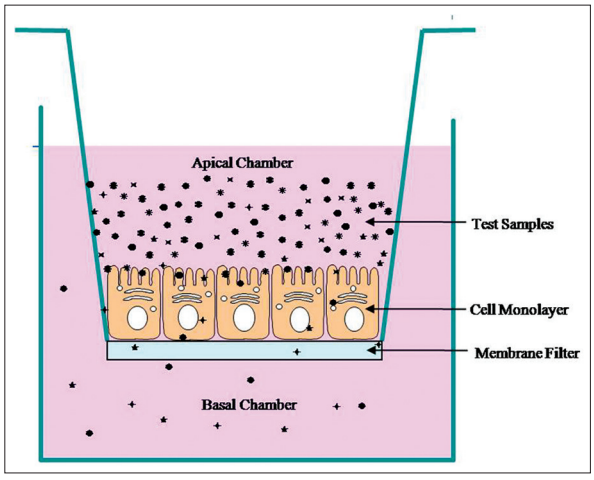

Figure 2: Illustrates the cell culture chamber, the apical medium, to which the apical surface of a cell monolayer is exposed, and basal medium, to which the basal surface of a cell monolayer is exposed are provided to the cell culture chamber via the apical medium flow inlet and the basal media flow inlet. Samples to be tested for permeability are incubated under control conditions and allowed to pass through the cell monolayer. Samples collected from apical and basal chambers are analyzed by high performance liquid chromatography-mass spectroscopy for ascertaining permeability of different withanolides

MDCK cells were cultured for 3 days and monolayers with Trans Epithelial Electric Resistance between 80 and 120 Ωcm^2 were used in the study. Standard stock solutions were prepared in methanol and further diluted with Hanks' buffered saline containing 20 mM HEPES buffer pH 7.4 to desired concentrations. Lucifer yellow was added to the test solution as an indicator for monitoring the integrity of membrane monolayer. Incubation was carried out in a shaker water bath (50–70 rpm) at 37°C for 1 h. Samples were collected from apical donor side before incubation and form basal receiver side after incubation [Figure 2]. All withanolides were tested at the concentration of 2 μg/ml which ascertained that the withanolides did not disrupt membrane integrity and the concentrations at the receiver side were within the permissible range of their quantification limits; it has been reported that the IC_{50} for withanolides in cytotoxicity assay ranged from 0.067–9.3 μM (0.7–9.8 μg/ml). Thus, the concentrations used for the present studies were well within the permissible range.[23] Samples from the apical chamber were diluted 1:20 and the injection volume was 20 μL for LC-MS analysis. For samples from basal chamber, no dilution was necessary, and 40 μL was injected.

RESULTS

HPLC studies revealed that the mean retention time (R_t) for withanolides WS IV, WF A, 1,2 DWM, WN A, WS V, WNN, and WN B, respectively, was 2.25, 6.8, 7.75, 9.01, 9.2, 9.31, and 13.1 min. Representative mass spectrometric selected ion mode chromatograms for the individual withanolides which were collected from apical and basal chambers are shown in Figures 3 and 4. A single peak was observed for all withanolides except for WNN and WNB which showed an extra peak possibly representing a modified form or an adduct formation in the buffer system. WNN and WNB and the unidentified products were highly permeable. By contrast, WS IV and WS V seem to be partially permeable. The amount of individual withanolides in apical and basal chambers was quantified based on the standard curve. Permeability was measured in terms of efflux pump (cm/s) and was calculated as follows:

$$P_{eff} = \frac{\text{Receiver's volume} \times \text{Receiver's concentration}}{\text{Membrane area} \times \text{Donor's concentration} \times \text{Incubation time}}$$

P_{eff} values of WN A, WNN, 1,2 DWM, WN B, WS IV, WS V and WF A were 4.05×10^{-5}, 2.06×10^{-5}, 1.97×10^{-5}, 1.80×10^{-5}, 3.19×10^{-6}, 3.03×10^{-6} and 3.30×10^{-7} respectively [Table 1].

DISCUSSION

The biological activity of some individual withanolides has been evaluated in *in vivo* assay system.[8-15] However, as far as we are aware *in vitro* assay for evaluation of bioavailability of withanolides have not been carried out. Assessment of the bioavailability of a compound is a most important primary step in drug development. The intestinal epithelium plays an important role in the absorption and transportation of a given drug. It has been suggested that the *in vitro* cell culture may be useful systems for rapidly monitoring the absorption behavior of withanolides.[17,18] In view of this we decided to estimate the bioavailability of withanolides by using the Sino-Veda MDCK cell culture system.[20,24]

Table 1: P_{eff} values and permeability of *W. somnifera* standard

Chemicals	P_{eff} (sec/cm) mean	Permeability
WN A	4.05×10^{-5}	High
1, 2 DWM	1.97×10^{-5}	High
WNN	2.06×10^{-5}	High
WN B	1.80×10^{-5}	High
WS IV	3.19×10^{-6}	Low
WS V	3.03×10^{-6}	Low
WF A	3.30×10^{-7}	Impermeable

It has been reported that WNA is the most stable and bioavailable withanolide under *in vivo* condition;[13,14,25-27] results of our previous studies are in conformity of this observation.[27] MDCK cells grown under the specified conditions have a narrow range of paracellular permeability (1×10^{-6} to 1×10^{-5} cm/s). We observed that the P_{eff} value of WN A was 4.05×10^{-5} which indicates high permeability. The permeability observed in these cells correlates directly to the permeability in the human system.[28] Based on our results, we observe that the WNN, 1,2 DWM, and WN B are highly permeable [Figure 3]. Figure 3 also shows that an unidentified product appeared in WNN and WN B. Apparently, only WNN and WNB seem to be susceptible under the experimental conditions. These products of WNN and WNB are unidentified and need further investigations. We observed that P_{eff} values for the WS IV and WS V were 3.19×10^{-6}, 3.03×10^{-6} respectively signifying their low permeability as compared to other above mentioned withanolides. WS IV and WS V have glucose moiety at C_3 position [Figure 1]. Thus they are polar and also have higher molecular weight. It is known that hydrophobic interior of the cell membrane-the lipid bilayer-serves as a barrier to the passage of polar and high molecular weight molecules.[29] The gut has glucosidases which could hydrolyze the glucose moiety, thus facilitating the absorption of such compounds. In view of this it may be suggested that further studies on these lines may be warranted.

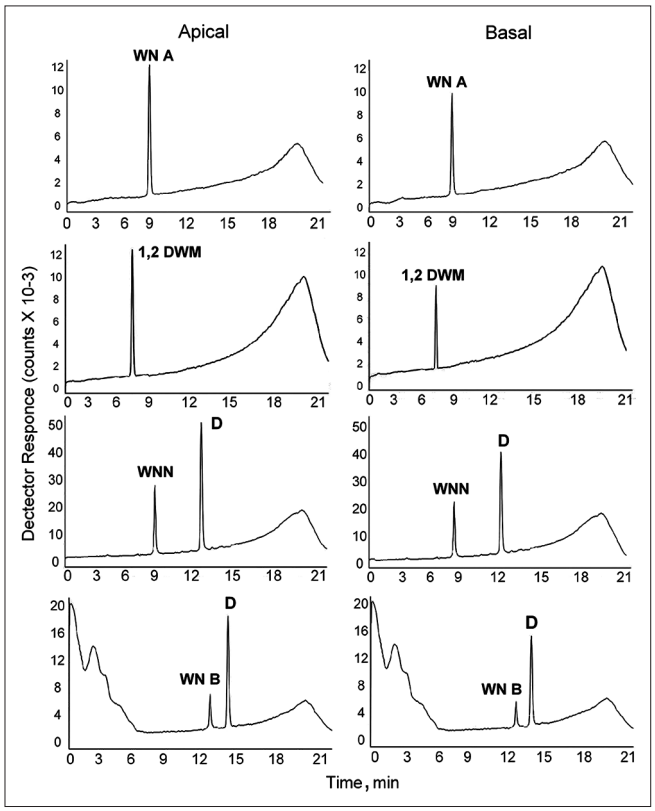

Figure 3: Apical and basal distribution pattern for highly permeable withanolides. Unidentified product is denoted by D

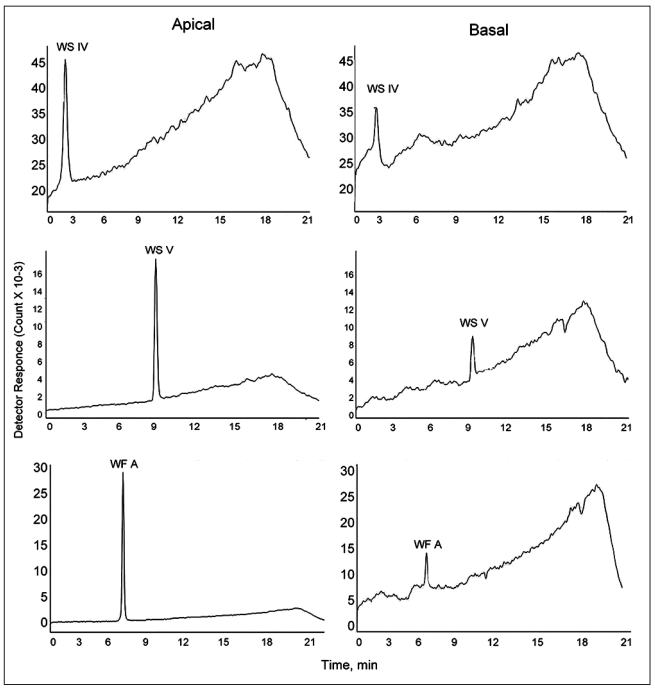

Figure 4: Apical and basal distribution pattern for low and impermeable withanolides

Several researchers have reported that WF A is a biologically highly active compound.[8-11] In our earlier studies, we noted that the anti-oxidant potential of WF A is the highest.[27] After oral administration of aqueous extract of *W. somnifera* bioavailability of WF A was greater compared to WN A.[26] Surprisingly and paradoxically we observed that the P_{eff} value of WF A was the lowest (3.30×10^{-7}) implying that it may be impermeable under *in vitro* system, that is, in the MDCK cells or that it may be metabolized as it passes through the cell layer and we are unable to measure the metabolite. However, as mentioned above, after oral administration of aqueous extract of *W. somnifera* to mice there is significant absorption resulting in a high concentration of WF A in the plasma.[26] It seems that the process of WF A absorption is more complex, and the MDCK *in vitro* model possibly does not provide the exact *in vivo* environment. However, this possibility needs to be verified further by more direct experiments using different cell culture system such as human epithelial colorectal adenocarcinoma (Caco-2) cell monolayer.[21] Although both CaCo2 cells and MDCK cells are predictors for passive properties and lack of active transporters, the discrepancy seen in the present studies could likely be attributed to the lack of active transporters or species difference.

CONCLUSIONS

Based on the present studies on the absorption characteristics of the tested withanolides it may be concluded that WN A, WNN, 1,2 DWM and WN B were highly permeable; whereas WS IV, and WS V showed low permeable. Surprisingly WF A, the highly biologically active withanolide was found to be either impermeable or metabolized on passing through the cell layer. It is likely that absorption of WFA *in vivo* is a complex process and possibly a system employing Caco-2 cells could provide better insight in the absorption characteristics of WF A.

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Conflicts of interest

There are no conflicts of interest.

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