NPC Natural Product Communications

2007
Vol. 2
No. 0
1 - 4

# A Cytotoxic and Hepatoprotective Agent from *Withania somnifera* and Biological evaluation of its Ester Derivatives

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**Received: December 12th, 2006; Accepted: March 24th, 2007**

Detailed chemical investigation of *Withania somnifera* roots resulted in the isolation and identification of a cytotoxic and hepatoprotective agent, palmitic acid (**1**), which was converted to eight semi-synthetic ester derivatives **2**-**9**. *t*-Butyl palmitate (**8**) and amyl palmitate (**9**) were 4-6 times more active than **1** against adherent and suspension colon cancer cell lines. Interestingly, palmitic acid (**1**) and its ester derivatives **2–9** also showed hepatoprotective activity which is being reported for the first time.

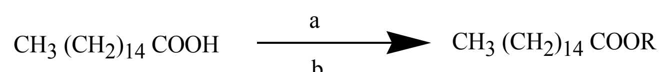
**Keywords:** *Withania somnifera*, palmitic acid, chemical derivatization, cytotoxicity, hepatoprotection.

Medicinal plants have been known and highly esteemed all over the world as a key source for the discovery of new drugs and are an important part of the Asian cultural heritage. Asia is a continent endowed with an enormous wealth of plant resources, which produces a diverse range of bioactive molecules, making them a rich source of different types of medicines. In Asia, a large part of primary care is still derived from plants and large volumes of plant material or their extracts are sold in the informal and commercial sectors of the economy [1].

*Withania somnifera* (L.) Dunal (Solanaceae), also known as Ashwagandha, Indian ginseng, and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years [2]. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia [2]. Clinical trials and animal research support the use of ashwaganda for anxiety, cognitive and neurological disorders [2], inflammation [3], and Parkinson's disease [2]. Ashwaganda's chemopreventive properties make it a potentially useful adjunct for patients undergoing radiation and chemotherapy. Ashwaganda is also used therapeutically as an adaptogen for patients with nervous exhaustion, insomnia, and debility due to stress [2], and as an immune stimulant [4] in patients with low white blood cell counts. Recently, the plant has also been found to possess strong antibacterial activity and can be effectively used in the treatment of murine salmonellosis [5]. A recent report demonstrated the anti-tumor [6] effect of Ashwagandha, which prompted a detailed chemical investigation of *W. somnifera* roots to isolate and characterize the anticancer principle and carry out its chemical transformation into compounds having potential anticancer activity.

For this, purpose, roots of *W. somnifera* were extracted and processed as discussed in the experimental section, which resulted in the isolation of palmitic acid (**1**) which was characterized on the basis of its GC-MS, $^{1}$H, and $^{13}$C NMR spectroscopic data. It is reported that palmitic acid possesses antibacterial and cholesterolaemic effects [7-11]. Recently it has shown selective cytotoxicity [12] to human leukemic cells but no cytotoxicity to normal

human HDF cells, at a concentration ranging from 12.5 to 50µg/mL. It also showed *in vivo* anti tumor activity in mice. One molecular target of palmitic acid in tumor cells is DNA topoisomerase I [12]. However, it does not affect DNA topoisomerase II; this suggests that palmitic acid may be a lead compound for anticancer drug discovery. This encouraged us to prepare derivatives and evaluate their antiproliferative and hepatoprotective activities.




**Scheme 1**: Reagent and conditions: (a) $(COCl)_2$, stirring at RT 3-4 hr under $N_2$ atm. (b) ROH (1.2eq), triethyl amine kept overnight, 80 -82.5 % [ R = $CH_3$ methyl palmitate (**2**), R = $CH_2CH_3$ ethyl palmitate (**3**), R = $CH(CH_3)_2$ isopropyl palmitate (**4**), R = $(CH_2)_3CH_3$ butyl palmitate (**5**), R = $CH_2CH(CH_3)_2$ isobutyl palmitate (**6**), R = $CH(CH_3)CH_2CH_3$ *sec*-butyl palmitate (**7**), R = $C(CH_3)_3$ *tert*-butyl palmitate (**8**), R = $(CH_2)_4CH_3$ amyl palmitate (**9**).

**Table 1**: Antiproliferative activities [a] ($IC_{50}$ µg/mL) of palmitic acid (**1**) and its ester derivatives **2-9** against the human breast (MCF-7), liver (WRL-68), adherent colon (Caco-2), and suspension colon (Colo-320-DM) cancer cell lines

| Compound | MCF-7 | WRL-68 | Caco-2 | Colo-320-DM |
|---|---|---|---|---|
| | $IC_{50}$ | $IC_{50}$ | $IC_{50}$ | $IC_{50}$ |
| **1** | 0.55 | 1.0 | 0.75 | 0.35 |
| **2** | 0.52 | 0.85 | 0.62 | 0.25 |
| **3** | 0.65 | 1.25 | 1.20 | 0.82 |
| **4** | 1.25 | 4.86 | 2.65 | 1.86 |
| **5** | 0.75 | 1.42 | 1.25 | 1.0 |
| **6** | 1.25 | 4.65 | 1.86 | 0.85 |
| **7** | 0.8 | 2.62 | 0.95 | 1.20 |
| **8** | 0.25 | 0.64 | 0.15 | 0.08 |
| **9** | 0.35 | 1.25 | 0.12 | 0.07 |
| **Vinblastine** | 0.02 | 1.45 | 0.46 | 0.52 |

[a]Reference 16

The antiproliferative activities of **1** and its semi-synthetic ester derivatives **2-9** against the hormone-dependent human breast (MCF-7), liver (WRL-68), adherent colon (Caco-2), and suspension colon (Colo-320-DM) cancer cell lines are presented in Table 1 using vinblastine as the control. All the compounds had antiproliferative activity against the various cancer cell lines. Palmitic acid (**1**) itself was more active than vinblastine with respect to human liver (WRL-68), and suspension colon (Colo 320-DM) cancer cell lines. From Table 1 it is evident that the activity of palmitic acid ester derivatives from 1-3 carbon atoms (**2-4**) decreased. This decrease in activity was also observed in the 4 carbon atom ester derivatives **5-7**, but dramatic enhancement in activity was observed in the 4 and 5 carbon ester derivatives **8** and **9** of palmitic acid. Compound **8** was 4 and 5 times more active with respect to palmitic acid and 6 and 3 times more active with respect to vinblastine against suspension colon (Colo 320 DM) and adherent colon (CaCo2) cancer cell lines, respectively. Similarly, compound **9** was 5 and 6 times more active with respect to palmitic acid and 7 and 4 times more active with respect to vinblastine against suspension colon (Colo 320 DM) and adherent colon (CaCo2) cancer cell lines, respectively.

**Table 2**: Hepatoprotective activities [a] ($IC_{50}$ µg/mL) of palmitic acid (**1**) and its ester derivatives **2-9** against galactosamine.

| No. | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10**[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| $IC_{50}$ | 0.65 | 0.85 | 0.20 | 1.0 | 0.40 | 1.2 | 0.10 | 0.10 | 0.25 | 2.6 |

[a]References 17-20, [b]Silymarin

The hepatoprotective activity of palmitic acid (**1**) and its semi-synthetic ester derivatives **2-9** were also tested against galactosamine with reference to the known hepatoprotective agent silymarin [13]. The results are presented in Table 2, which showed that all the compounds protected hepatocytes from being damaged by galactosamine and reduced the toxicity by 2 to 12 times with respect to 26 times by silymarin. The results show that palmitic acid, methyl palmitate, *iso*-propyl palmitate *and iso*-butyl palmitate, exhibited significant hepatoprotective activity.

From the above it may be concluded that the palmitic acid ester derivatives, **8** and **9** possess potential anticancer activity and moderate hepatoprotective activity. These results may be of great help in anticancer drug development from a very common, inexpensive, and non-toxic natural product.

## Experimental

***General:*** $^1H/^{13}C$ NMR spectra were measured at 300/75 MHz in $CDCl_3$ solution at 25°C. The signal of the deuterated solvent ($CDCl_3$) were taken as the reference (the singlet at δ7.25 for $^1H$ NMR and triplet centered at 77.00 ppm for $^{13}C$ NMR data). Carbon atom types (C, CH, $CH_2$, $CH_3$) were determined with DEPT pulse sequence. Silica gel G or H (Merck) was used for TLC and Flash chromatography, respectively. All the spots on TLC plates were visualized by spraying with 10% $H_2SO_4$ solution followed by heating at 100°C for a few minutes. Reactions which required inert atmosphere were carried out under $N_2$ with oven dried glass ware.

***Plant material*:** The plant material was identified and collected by Dr. S. C. Singh, Scientist, Botany Division, CIMAP in December, 2004 from the CIMAP Research Farm, Lucknow. A voucher specimen has been deposited in the Herbarium section of the Botany Department, CIMAP, Lucknow.

***Extraction:*** The dried and powdered roots (125g) of *Withania somnifera* were extracted with ethanol:water (1:1). The dried aqueous ethanolic extract (72.3 g) was then extracted with 3% HCl solution. The acid insoluble portion so obtained was dissolved in distilled water, neutralized to pH-7 and successively extracted with hexane, $CH_2Cl_2$, EtOAc, and *n*-BuOH.

***Isolation of palmitic acid:*** TLC [$CHCl_3$: MeOH (90: 10)] profiles of hexane (0.5g) and dichloromethane (DCM) extracts (2.35g) were more or less similar and found to be a mixture of 3-4 compounds; therefore both extracts were pooled and purified on a flash chromatographic column (460 mm X 36 mm), using TLC grade silica gel H (150 g, without binder, Qualigen) and fractions of 50 mL each were collected. Gradient elution of the column was carried out with mixtures of solvents, hexane, hexane: $CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH in various proportions. Fractions were monitored and pooled on the basis of their TLC profile. A total of 799 fractions were collected. Biological activity analysis of various pooled fraction was carried out, which revealed that palmitic acid (**1,** 0.07%) isolated from fractions 19-23 eluted with (hexane:$CHCl_3$, 75:25) possessed significant cytotoxicity against the MCF-7, WRL-68, CaCo2, Colo-320 DM cancer cell lines and hepatoprotection against galactosamine.

***Chemical derivatization:*** Palmitic acid (200 mg, 0.74 mmoles) was dissolved in dry DCM (3 mL). To this reaction solution, oxalyl chloride (1 eq, 0.065 mL) was added under $N_2$ atmosphere, and the reaction mixture was stirred for 3-4 hr at room temperature [14]. After 4hr, dry alcohols (1.2 eq) were added to this solution in the presence of triethylamine (0.1 mL). The air tight reaction mixture was kept overnight at room temperature and the progress of the reaction was monitored by TLC (98.5:1.5, hexane:acetone). After completion of the reaction, the mixture was worked up in usual way and the product was purified using preparative thin layer chromatography (PTLC) silica gel 60 $F_{254}$TLC plates (20x20 cm) to afford the desired compound in 80-82.5 % yield. The $^1$H and $^{13}$C NMR data were in agreement with the proposed structures [15]

***Cytotoxicity assay:*** The following four human cancer cell lines were procured from the cell repository of the National Center for Cell Sciences (NCCS) Pune, India. Cytotoxicity testing was done by the method of Woerdenbag *et al.*[16] in which 2 X $10^3$ cells/well were incubated in the 5% $CO_2$ incubator for 24 hr to enable them to adhere properly to the 96-well polystyrene micro plates (Grainer, Germany). At least five doses of the test compounds dissolved in 100% DMSO and were added and left for 6 hrs after which the compound plus media was replaced with fresh media and the cells were incubated for another 48 hr in the $CO_2$ incubator at 37°C. The concentration of DMSO used in the experiments never exceeded 1.25%, which was found to be nontoxic to cells. Then, 10 µL MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliun bromide, Sigma M 2128] was added, and the plates were incubated at 37°C for 4 hr. 100 µL Dimethyl sulfoxide were added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Spectra Max 190 Micro plate Elisa reader (Molecular Devices Inc., USA), at 570 nm. Plates were normally read within 1 h of adding the DMSO. The experiment was done in triplicate and the inhibitory concentration (IC) values were calculated as: % inhibition = [1-OD (570 nm) of sample well/ OD (570 nm) of control well] X 100. The results are presented in Table 1

***Hepatoprotective assay:*** Isolated hepatocytes were plated in 96-well polystyrene plates having galactosamine as a hepatotoxic control in different dilutions. A calorimetric assay measuring succinate dehydrogenase release was performed on the treated and untreated cells as described by Carmichael *et al* [17,18]**.** Galactosamine as a hepatotoxic agent was added in different wells in different dilutions (GalN in 1% PBS). The tetrazolium assay [19,20] was performed to identify 50% inhibition (IC-50) of the hepatotoxic agents. Further it was found that 0.1 µg/mL of GalN was required to kill 50% of the hepatocytes. Silymarin, a known plant compound with hepatoprotective activity from *Silybum marianum,* was taken as a positive control which prevented the damage of hepatocytes by galactosamine. The $IC_{50}$ values of the tested compounds were compared with the $IC_{50}$ value of the

positive control to establish the hepatoprotective activity.

**Supplementary data:** Physical and spectral data for palmitic acid (**1**), methyl palmitate (**2**), ethyl palmitate (**3**), isopropyl palmitate (**4**), *n*-butyl palmitate (**5**), isobutyl palmitate (**6**), *sec*-butyl palmitate (**7**), *tert*-butyl palmitate (**8**) and amyl palmitate (**9**).

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