Synthesis and characterization of amino acid conjugates of oleanolic acid and their *in vitro* cytotoxic effect on HCC cell lines

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Abstract: Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid; OA-01), a pentacyclic triterpene, exhibit a wide range of pharmacological ad biological activities. We have isolated oleanolic acid from methanolic extract of *Periploca aphylla*, collected from surroundings of Karachi in the month of February. Furthermore, four known and two new C-28 amino acid conjugates of oleanolic acid were prepared to explore potential of these compounds on HCCs and one breast cancer cell line. Cytotoxic effects revealed that as compare to parent compound (OA-01), two derivatives OA-04 ($p<0.0001$) and OA-06 ($p<0.01$) showed significantly increased /higher inhibition rates.

Keywords: Oleanolic acid, amino acid, HCC cell lines, amides, *Periploca aphylla*.

INTRODUCTION

Pentacyclic triterpenoids of lupane, ursane and oleanane series are reported to posses high pharmacological potential for different cancer cell lines (Silva *et al*., 2012). Prominent of these are betulinic acid, ursolic acid and oleanolic acid (Cichewicz *et al*., 2004, He X *et al*., 2007, Jie Li *et al*., 2002, Jie Liu., 2005, Laszczyk., 2009, Liu *et al*., 2012, Meng *et al*., 2009, Patlolla *et al*., 2012, Petronelli *et al*., 2009, Silva *et al*., 2012, Tae *et al*., 2011, Wang *et al*., 2009). C-28 amino acid conjugates of betulinic acid (Jeong *et al*., 1999) and ursolic acid have exhibited promising activity against human melanoma. Hence, despite striking structural similarity of oleanolic acid with betulinic acid and ursolic acid its C-28 amino acid conjugates have been not studied against a number of cancer cell lines with an exception of N-[(3*β*)-3-(acetyloxy)-28-oxoolean-12-en-28-yl]-glycine methyl ester (Lu *et al*., 2007). Although, oleanolic acid derivatives obtained through modification at C-28 carboxylic moiety displayed promising inhibition against human Hepatocellular carcinoma cell line (HepG2) and colon cancer cell line (Col-02) (Tae *et al*., 2011, Jie Li *et al*., 2002).

Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid; OA) is a naturally occurring pentacyclic triterpenoid found in more than 1600 species (Yeung *et al*., 2010). Literature demonstrates that oleanolic acid and its derivatives are reported to exhibit a wide range of biological and pharmacological properties including anti-inflammatory (Lee *et al*., 2013), antitumor (Lu *et al*., 2007), Anti HIV (Zhu *et al*., 2001), antioxidant (Gao *et al*., 2009), cardiotonic (Somova *et al*., 2004), diuretic (Jadhav *et al*., 2010) and hepatoprotective properties (Jeong., 1999).

Further structural conversion of C-3 hydroxyl group in to acetoxy, oxo or hydroxy imino group showed strong cytotoxic activity as compare to oleanolic acid (Bednarczyk-Cwynar *et al*., 2012). Anticancer activities of oleanolic acid and its derivatives are reported against four human liver cancer cell lines HepG2, Hep3B, HUH7 and HA22T (Yan *et al*., 2010), Non-Small Cell lung cancer cell lines A459 and H460 (Lu cio *et al*., 2011), human colon carcinoma cell line HCT15 (Jie Li *et al*., 2002), ovarian carcinomas IGROV1 and breast cancer cell line MDA-MB-231 (Linwei *et al*., 2010).

RESULTS

Oleanolic acid was isolated from *Periploca aphylla* applying classical separation techniques in four steps shown in Scheme-1(*vide experimental*). *Periploca aphylla* is shrub common in southern region of Pakistan (Zhu *et al*., 2001). The aerial parts (leaves and stem bark) of *Periploca aphylla* were collected from surroundings of Karachi region during January and February 2009.

Structural modifications of organic compounds often enhance biological activities of secondary metabolites. For this reason six amino acid conjugates of oleanolic acid, have been synthesized including N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]-glycine methyl ester (OA-2), N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]-*L*-cysteine

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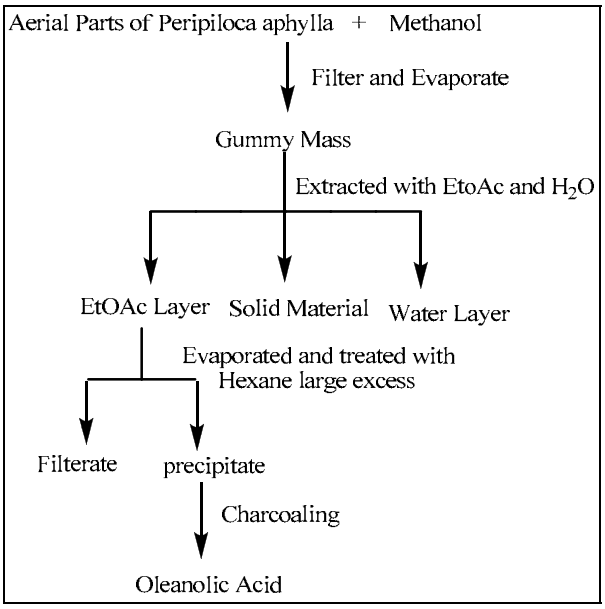
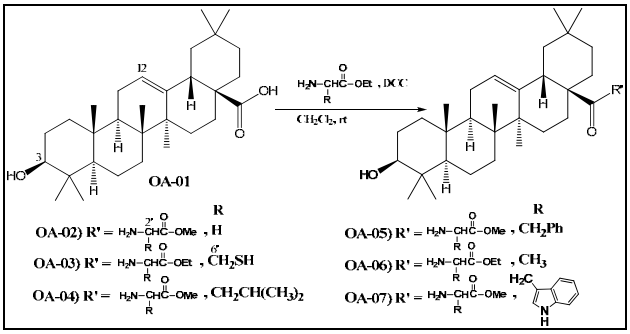
methyl ester (OA-3), N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]- *L*-leucine methyl ester (OA-4), N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]-*L*-phenyl alanine methyl ester (OA-5), N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]]-*L*-alanine ethyl ester (OA-6), N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]]-*L*-tryptophane methyl ester (OA-7) were prepared out of which two (OA-3 and OA-7) are hitherto unreported.



Scheme 1: Schematic diagaram of isolation

The amide bond between OA-1 and amino acid esters was achieved by applying most common strategy of amide formation i.e. carbodiimide protocol (Gao *et al*., 2009). In a general procedure 1 equivalent of OA-1 in dichloromethane (CH_2Cl_2), 2 equivalent of respective amino acid ester, 1 equivalent of dicyclocarbidiimide (DCC) and 0.5 equivalent of dimethylaminopyridine (DMAP) were stirred at room temperature for 2 hours. After usual workup respective amino acid derivatives (OA-2, OA-7) were obtained, scheme-2 (*vide experimental*).



Scheme 2: Synthesis of amino acid conjugates of Oleanolic acid (OA-01)

The structures of synthesized compounds were confirmed through ESI, IR and ^{1}H-NMR spectroscopic data and comparison with reported literature (Somova *et al*., 2004, Jadhav *et al*., 2010). Cytotoxic evaluation of these compounds against eight hepatocellular carcinoma (HCC) cell lines including HUH7, Hep3B, HepG2, SNU449, Mahlavue, PLC, SNU387 and SNU475 and one breast cancer line MCF7 was carried out using Sulforhdamine B (SRB) method. Results displayed superior activity of all synthesized compounds over parent compound against aforementioned cell lines. This behavior suggests further investigation of structurally modified oleanolic acid derivatives for anti-tumor/ anticancer research as potential drug candidates.

DISCUSSION

In recent study, a total of six OA derivatives were screened for their cytotoxic effect on one breast cancer and eight HCC cells by SRB technique as described in experimental part. Trial drugs OA-02, OA-03, OA-04, OA-05, OA-06, OA-07 and parent compound OA-01 were introduced in two different (50uM and 10uM) concentrations in triplicate for each sample.

Out of six OA derivatives, OA-04 (100%) and OA-06 (100%) showed higher inhibitory effects on both HUH7 and MCF7 cell than parent compound OA-01 (41% and 73%) at 50uM concentration. Similarly, as presented in table 1; comparatively significant increased of inhibitory effects was recorded at 10uM of both OA-04 and OA-06.

Based on primary screening results (fig. 1), OA-04 containing valine as amino acid conjugate was found to be the most potent with 100% growth attenuation at 50uM concentration on HUH7 and MCF7 cells; and significantly ($p<0.0001$) high inhibitory effect at 10uM on MCF7 cells. Therefore, most potent trial drug OA-04 was further subjected for systematic primary screening with eight HCC and one-breast cancer cell lines to examine the % inhibition at 10uM concentration. (fig. 2).

As per above-mentioned findings, MCF7 and HUH7Cells were used. 2000-2500 cells were cultured in each well of 96 well-plate and incubated for 24 hours. After 24hours, cells were treated with OA-04 drug at different concentrations in triplicates ranging from 10uM to 0.313uM; for controls up to 0.1% DMSO final concentration was maintained with respective media for each cell line; camptothecin as a positive drug indicator was also used in triplicate at 5uM concentration. The IC_{50} values against on both MCF7 and HUH7 cell lines were determined against each drug concentration. Camptothecin showed 95%-100% growth attenuation at 5uM concentration on both cell lines.

As presented in inhibition curve in fig. 3, IC_{50} value of OA-04 was calculated as 18.9uM and 43.8uM on MCF7 and HUH7 cells respectively;, indicating that this drug might have comparatively higher therapeutic index on MCF7 cells than other trial drugs of the study.

Table 1: Comparison of inhibition rates (%)

Compound Code	Inhibition rates (%)			
	HUH7		MCF7	
	50µM (±SD)	10µM (±SD)	50µM (±SD)	10µM (±SD)
OA-01	40,98 (0,215)	29,11 (0,261)	72,91 (0,004)	16,4 (0,039)
OA-02	0	0	40,81 (0,012)	0
OA-03	12,6 (0,046)	0	27,63 (0,028)	0
OA-04	100 (0,001)	33,42 (0,187)	100 (0,001)	66,54* (0,009)
OA-05	89,81 (0,024)	11,49 (0,192)	97,44 (0,002)	2,04 (0,008)
OA-06	99,8 (0,004)	44,58* (0,115)	100 (0,001)	30,01* (0,017)
OA-07	93,43 (0,011)	25,99 (0,049)	99,21 (0,001)	30,27* (0,029)

*Statistically significant ($p<0.01$) increased inhibition rates than parent compound (OA-01) Values are means of three experiments after exposure of 72 hours of trial drugs. Negative values approximated to zero growth attenuation.

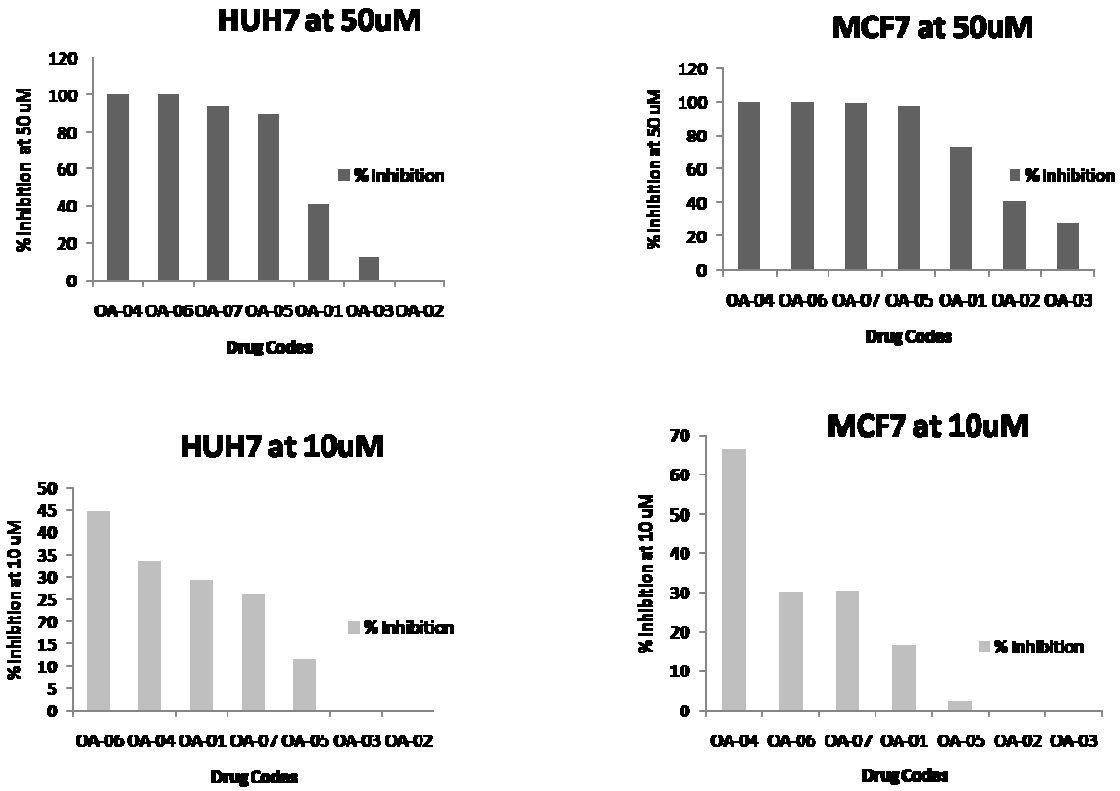


Fig. 1: Primary screening results

Each inhibition bar represents mean of three experiments. Inhibition rates were Calculated using following formulae; % inhibition=100- (Average OD of samples) X100/Average OD of Control)

In short, the study elaborates isolation of oleanolic acid in large amounts from *Periploca aphylla* using very simple, time saving and comparatively inexpensive strategy.

Finally, we devised comparatively inexpensive, rapid and simple isolation protocol for synthesis of bulk amount of oleanolic acid from aerial parts of *Periploca aphylla.* Secondly, six C-28 amino acid conjugates of oleanolic acid were prepared to explore the role of structural manipulations and potential effects of these compounds on HCCs and one breast cancer cell line. Cytotoxic effects revealed that as compare to parent compound (OA-1), three amino acid conjugate derivatives OA-04, OA-06 and OA-07 showed significantly high inhibition rates at 10uM; indicating enhanced cytotoxic efficiency of structurally manipulated derivate.

MATERIALS AND METHODS

General: All reagents were purchased from Sigma–Aldrich and Merck and were used without further purification. Technical grade solvents were used for

chromatography and distilled prior to use. Thin layer chromatography was performed on M-N ALUGRAM (registered Germany) Silica gel/ UV 254 sheets, and detection of spots was made by UV light and/or iodine vapors. Column chromatography was performed using silica gel 70-230-mesh.

Extraction and Isolation of Oleanolic acid

The fresh, un-dried and uncrushed aerial parts including leaves and stem (100 kg) were repeatedly extracted with methanol five times at room temperature. The solvent from the combined extract was evaporated in vacuum and the concentrated into gummy extract. The gummy plant extract (10 Kg) was stirred with water and Ethylacetate (EtOAc) resulting in three layers, aquous layer, EtOAc soluble layer and solid particles sandwiched between two solvents.

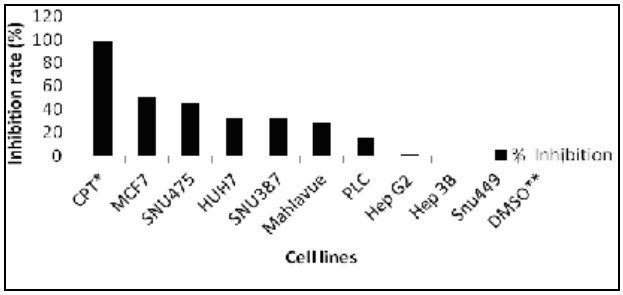


Fig. 2: OA-04 Response at 10uM concentration on cell lines (n=9)

*Camptothecin (CPT) used as positive control with 5uM concentration on each cell line. **DMSO upto 0.1% of final concentration in respective media used as negative Control. Each bar represents average of three values with the exposure of 72 hours of trial drug.

Mr. Jan Alam of Botany department identified it and a G. H. # 85609 was given. A voucher specimen has been deposited in the herbarium of the same department. The fresh, un-dried and uncrushed leaves (100kg) were repeatedly extracted with methanol (five times) at room temperature. The solvent from the combined extract was evaporated in vacuum and the concentrated into gummy extract. The gummy plant extract (*vide experimental*) from aerial parts of the plant was stirred with water and Ethylacetate (EtOAc) resulting in three layers, aquous layer, EtOAc soluble layer and solid particles sandwiched between two solvents. The EtOAc part was evaporated to obtain gummy mass was obtained, which after dissolving in minimum amount of EtOAc treated with excessive amount of hexane resulting in precipitation. The hexane insoluble part (precipitates, 250g) was subjected for charcoaling in $CHCl_3$-MeOH (9.5:0.5) yielded <95% pure crystalline oleanolic acid (OA-1) identified through comparison TLC with authentic sample and comparison with spectroscopic data reported literature.

General procedure for the preparation of Amides from amino acids

To a solution of oleanolic acid (1 mole) in dichloromethane, dicyclohexyl carbodiimide (DCC, 1 mole), 4-dimethyl amino pyridine (DMAP, 0.5 mole) and amino acid esters (1 mole) were added, the resulting mixture was stirred at room temperature for two hour. After completion of reaction 50 ml of ethyl acetate was added and filter, to the filterate add 20 ml of water and separate the organic layer. Again add 20 ml of water and separate the organic layer. Dry with anhydrous sodium sulfate and evaporate.

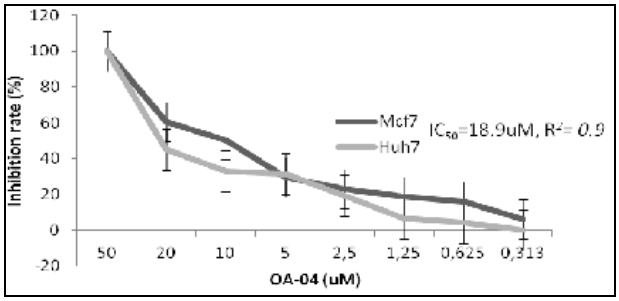


Fig. 3: Inhibition Curve with IC_{50} values of MCF7 and HUH7

In order to determine IC_{50} values, Modelling laboratory (MLAB), and interactive mathematical modelling software is used (Civilized Software, Inc.; Gary Knott; Revision Date: Oct. 8, 1996). Each point represents average of three values with the exposure of 72 hours of trial drug and compared with negative control. The bar over each concentration represents standard of error mean.

N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]-L-cysteine methyl ester (OA-3)

Yield: 90%; Amorphous solid, IR (KBr): υ_{max}=3401, 2931, 2858, 2550, 1740, 1646, 1507, 1451, 1388, 1207 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$) δ: 2.93 (1H, dd, *J*=13.1, 3.6 Hz, H-18), 3.23 (1H, dd, *J*=12.3, 5.0 Hz, H-3), 5.40 (1H, s, H-12), 5.08 (1H, t, *J*=6.8 Hz, H-2'), 2.83 (1H, d, *J* =6.8 Hz, H-2'); HRMS-ESI calcd for $C_{35}H_{57}O_4NS$ $(M+H)^+$ 588.4087, found 588.3954.

N-[(3β)-hydroxy-12-en-28-oxoolean-28-yl]]-L-tryptophane methyl ester (OA-7)

Yield: 91%; Amorphous solid; IR (KBr): υ_{max} =3401, 2931, 2858, 1740, 1646, 1507, 1451, 1388, 1207 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$) δ: 2.93 (1H, dd, *J*=13.1, 3.6 Hz, H-18), 3.23 (1H, dd, *J*=12.3, 5.0 Hz, H-3), 3.750 (2H, s, H-2'), 5.40 (1H, s, H-12), 7.071 (1H, s, H-10'), 7.107 (1H, t, *J*=7.2 Hz, H-7'), 7.174 (1H, t, *J*=7.2 Hz, H-6'), 7.311 (1H, d, *J*=8.0 Hz, H-8'), 7.591 (1H, d, *J*=7.6 Hz, H-5'); HRMS-ESI calcd for $C_{42}H_{60}O_4N_2$ $(M+H)^+$ 657.4631, found 657.4569.

Stock solution preparation

All stock solutions were prepared in 100% DMSO with a concentration of 20mM. Further dilutions were made up to 0.1% of DMSO with the help of respective media used for each cell line.

Cell lines

Eight hepatocellular carcinoma (HCC) cell lines including HUH7, Hep3B, HepG2, Snu449, Mahlavue, PLC, Snu387 and Snu475. While, one breast cancer line MCF7 was also used for primary screening. The cell lines were provided by MÖ (Mehmet Özturk) group, Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey.

Cytotoxicity screening protocol

Sulforhdamine B (SRB) is an aminoxanthene dye with two phenolic groups, responsible to attach with the cell proteins in slightly acidic environment, therefore 10% TCA is used to fix the attached cells by maintaining acidic condition; cells become decolorized and detached in basic phase by using 10mM trisma base. Absorbance in terms of optical Density (OD) for each sample is measured using microplate reader in between SRB dye reference range. The SRB assay is non-destructive, very stable and colorimetric screening protocol, was first carried out by Skehan *et al* [21-22]. In current study, SRB method was used for screening. One hepatocellular carcinoma (HCC) cell line HUH7 and one breast cancer line MCF7 were used for initial screening of trial drugs. For both HUH7 and MCF7 cell lines, 2000 cells/ well were cultured in 96 well plate in incubator at 37^{o}C with 5 % CO_2 in complete medium (DMEM, 10% FBS, 1% NEA, 1% L-Glutamine and 1% P/S) and wait for 24 hours. All stock solutions were prepared in 100% DMSO with a concentration of 20mM. Further dilutions were made with the help of respective media used for each cell line. After 24hours, all trial drugs were introduced in two different (50uM and 10uM) concentrations in triplicate for each sample and plates were further incubated for next 72 hours. After 72 hours, media were discarded and cells were washed once by using 1XPBS. For fixation, 50ul of ice cold 10% TCA was added into each well and kept in dark at 4^{o}C for one hour. After fixation, the TCA was removed by tapping and plates were washed 4-5 X with ~ 200 µl dH_2O. Plates were left over night for drying under hood. Finally, 50ul of 0.4% sulforhodamine B (SRB) in 1% acetic acid solution was added to each well and left at room temperature for 10 min. SRB was removed and the plates washed 4-5 times with 1% acetic acid before air drying. Bound SRB was solubilized with 100ul of 10 mM un-buffered chilled Tris-base solution and plates were left on a plate shaker for at least 1-2 min. Absorbance was recorded using µ-Quant microplate reader with a wave length range of 405-515nm. The test OD values were defined as the absorbance of each sample. Mean values were determined and standerd deviation was found satisfactory ranging in between 0.001 to 0.25 in all respective samples from triplicates wells which was calculated automatically using Excel 7.0 software for Windows 2007.

Abbreviations List

CPT: Camptothecin, DCC: Dicyclohexylcarbodiimide, DMAP:N,N-Dimethyl amino pyridine**,** DMEM: Dulbecco's modified eagle medium, , FBS: fetal Bovine serum, IC_{50}: The half maximal inhibitory concentration, NMR: Nuclear magnetic resonance, OA: Oleanolic acid, OD: Optical density, P/S: Penicillin/Streptomycin, PBS: Phosphate buffer saline, RPMI: Roswell Park Memorial Institute medium, SD: Standard deviation, SRB: sulforhodamine B, TBS: Tris-buffered saline, TCA: Trichloroacetic acid, UV: Ultra violet.

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