Comparative evaluation of *in vitro* cytotoxic effects among parent abietyl alcohol and novel fatty acid ester derivatives against MCF7 and hepatocellular carcinoma cell lines

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Abstract: Synthesis of twelve hitherto unreported esters of abietyl alcohol and screening of these esters against four cancer cell lines including one breast cancer line MCF7 and four hepatocellular carcinoma cell lines (HCC) Huh7, Hep3B, Snu449 and Plc has been determined using SRB assay. The Cell cycle progression showed changes in cellular behaviour after 48 and 72 hours in MCF7 and Huh7 cell lines. Abietyl alcohol was obtained from the reduction of abietic acid, a tricyclic diterpene, isolated from oleoresin of *Pinus longifolia* Roxberghii.

Keywords: Abietic acid, Diterpene, Steglich esterification, Cancer cell lines, SRB assay.

INTRODUCTION

Abietic acid, a resin acid, i.e. a tricyclic monobasic diterpene, with molecular formula $C_{19}H_{29}$ COOH mainly found in rosin of pinus species. Significant pharmacological properties of resin acids and their derivatives have been reported in literature such as antibacterial (James 2006), anti-tuberculosis (James 2005) antitumour (Perry and Foster, 1994); Kwang-Hee *et. al*. 2005; Rao *et. al.* 2008) antiulcer (Wada *et al.* 1985) and antiviral (Miguel *et. al.* 2009) etc. The anticancer activity of abietic acid and derivatives specially against breast cancer and liver cancer cell lines is well documented in literature (Abd El Hady, *et al.* 2002). In view of the above facts, present work undertaken to isolate abietic acid from *Pinus longifolia* Roxb. followed by its reduction into alcohol and esterification of alcohol to furnish new derivatives of abietane series for initial screening against liver and breasr cancer cell lines using SRB assay.

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) 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. NMR spectra were recorded at room temperature on Bruker AM instrument operating at 400 MHz. Infrared spectra were recorded on a Schimadzu system and reported in cm^{-1}. Samples were prepared in thin film technique.

Extraction and isolation

The rosin of turpentine oil from *Pinus longifolia* Roxb, was purchased from local market. Rosin (250g) was dissolved in methanol (2L) and concentrated solution of sodium hydroxide (40%, 1 L) was added with stirring, a gummy suspension floated on surface. The suspension after separation was evaporated. On comparison with authentic sample of abietic acid (AB) it was found that gummy suspension was pure abietic acid (AB). The procedure was attempted to isolate abietic acid.

Synthesis of abietyl alcohol (ABA)

A round bottom flask charged with Lithium Aluminium Hydride ($LiAlH_4$) and THF, covered with drying tube kept in ice bath. After 10 minutes abietic acid is added with stirring. After completion of reaction, monitored through TLC, drop wise addition of water resulted in white ppt. Solution was filtered followed by addition of sodium bicarbonate and extraction with ethylacetate. Ethyacetate part is dried over anhydrous sodium sulfate, evaporated on vacuum and purified through coloumn chromatography (Hexane-Ethyl acetate in order of increasing polarity).

General procedure for the preparation of esters (1-12)

To a solution of Abiet-7, 13-dien-18-ol (1 mole) dissolved in dichlormethane, add 1 mole of acid and 4-dimethyl amino pyridine (DMAP, 0.5moles) followed by stirring in an ice bath at 0°C. Dicyclohexylcarbodiimide (DCC, 1 moles), is added over a five minutes period and reaction mixture is stirred at 0°C for further five minutes and then stirred for two hours at room temperature. The reaction

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mixture is filtered to remove dicyclohexylurea, washed with two 50mL portions of 0.5N hydrochloric acid and two 50mL portions of saturated sodium bicarbonate solution. The organic solution is dried over anhydrous sodium sulfate and concentrated under reduced pressure. Purification of the compounds was done by column chromatography Hexane-ethyl acetate (in order of increasing polarity).

Stock solution preparation

100 % DMSO was used to prepare stock solutions with a concentration of 20mM. With the help of respective media used for each cell line dilutions of solutions were made up to 0.1% of DMSO.

Cell lines

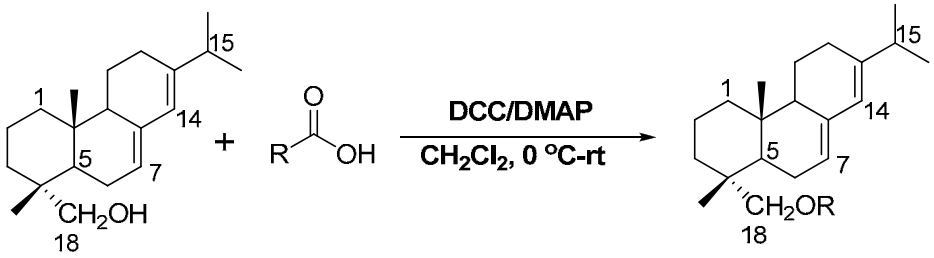
Four hepatocellular carcinoma HCC) cell lines including Huh7, Hep3B, Snu449 and Plc; While, one breast cancer line MCF7 was also used for cytotoxic screening.

Cytotoxicity screening protocol

Sulforhdamine B (SRB) was adopted as described in our previously published work (Mustafa *et. al.* 2014).

Cell cycle progression

Cells were plated in $10cm^2$ Petri plates at 2-3 x 10^5 per plate. After drug treatment, cells were harvested in different intervels (24, 48 and 72 hrs.) by trypsinization and washed with PBS. Cells were fixed in ice-cold 70% ethanol, washed, and resuspended in 3 ml of 70% ethanol for storage at 4^oC; fixed cells treated with RNase A; and stained with propidium iodide for 45 minutes at room temperature. The stained cells were analyzed after washing by flow cytometry using BD FACScalibur.



Scheme 1: Synthesis of abietyl alcohol ester derivatives

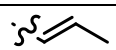
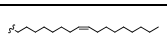
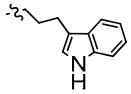
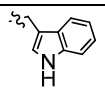
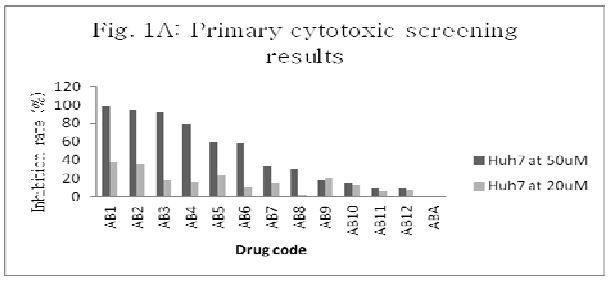
Comp. #	R	Yield (%)	Rf Hexane/EtOAc 9:1	$[\alpha]_D^{26}$ (Con.) $CHCl_3$	HRMS-ESI $(M+H)^+$	IR ν (cm^{-1})
1	-n-$C_{12}H_{25}$	98.04	0.8627	-5.33 (0.0818)	471.4306	2924, 2854, 1736, 1463, 1383, 720
2	-n-$C_{14}H_{29}$	98.32	0.8431	-9.16 (0.0413)	499.4535	2926, 2854, 1736, 1463, 1382, 722
3	-n-$C_{16}H_{31}$	98.57	0.8725	-7.25 (0.0291)	527.4792	2924, 2853, 1736, 1463, 1383, 720
4	-n-$C_{18}H_{35}$	72.9	0.8332	-1.81 (0.0048)	555.5231	2924, 2853, 1736, 1463, 1382, 720
5		97.08	0.7037	-10.51 0.0458)	357.2815	2932, 2854, 1719, 1446, 1382,
6		93.916	0.8529	-5.76 (0.0274)	553.4932	2926, 2853, 1730, 1463, 1382, 721
7		94.78	0.8823	-3.56 (0.007)	609.5702	2927, 2854, 1735, 1463, 1382, 723
8		98.44	0.8666	-3.58 (0.0148)	551.2816	2931, 2859, 1730, 1462, 1382, 720
9		94.04	0.7962	-24.94 (0.0705)	393.2821	2925, 2854, 1719, 1458, 1380
10		96.47	0.6851	-21.35 (0.0088)	407.2942	2930, 2854, 1733, 1459, 1382
11		98.32	0.2962	-29.46 (0.0539)	474.2856	3415, 2927, 2856, 1728, 1458, 1380
12		96.44	0.2314	-27.01 (0.0077)	446.2599	3399, 2929, 2870, 1725, 1458, 1380

Table 1: [1]H-NMR spectral data of compounds 1-12 (400 MHz, $CDCl_3$, δ ppm,)

H #	1	2	3	4	5	6	7	8	9	10	11	12
5[a]	1.98	1.97	1.87	2.07	1.55	1.55	1.58	1.67	1.55	1.56	1.54,	1.35
7[a]	5.37	5.37	5.37	5.37	5.37	5.31	5.32	5.32	5.38	537	5.40	5.28
14[b]	5.74	5.74	5.76	5.76	5.67	5.66	5.67	5.66	5.76	5.76	5.79	5.76
15[c]	2.28	2.81	2.85	2.22	2.24	2.84	2.30	2.84	2.36	2.35	2.24	2.25
16[d]	1.21	-	1.23	0.98	0.99	1.23	1.24	-	0.98	1.26	1.02	1.04
17[d]	1.21	-	1.23	0.99	0.99	1.23	1.24		1.00	1.26	1.02	1.04
18a[e]	3.62	3.92	3.95	3.66	3.76	3.65	3.65	3.65	3.94	3.66	3.63	3.60
18b[e]	3.92	4.05	4.06	3.80	3.84	3.72	3.79	3.78	4.05	4.08	3.84	3.75
19[b]	0.91	0.91	0.91	0.92	0.92	0.90	0.91	0.90	0.86	0.86	0.91	0.85
20[b]	0.87	0.91	0.91	0.80	0.81	0.87	0.87	0.85	0.84	0.76	0.81	0.73
2’	2.28[a]	2.12[g]	2.30[f]	2.25[f]	5.79[h]	2.29[a]	-	2.30[a]	-	3,62[a]	2.37[f]	-
3’	1.21[b]	1.58[a]	1.23[b]	1,24[a]	6.94[h]	1.56[a]	-	2.73[a]	8.03[i]		2,81[a]	
4’	1.21[b]	1.23[b]	1.23[b]	1,24[a]	0.87[j]	1.23[b]	1.24[b]	-	7.44[k]	7.12[i]	-	-
5’	1.21[a]	1.23[a]	1.23[a]	1,24[a]	-	1.23[a]	1.24[a]	-	7.55[l]	7.15[a]	-	7.59[g]
6’	1.21[a]	1.23[a]	1.23[a]	1,24[a]	-	1.23[a]	1.24[a]		7.44[k]	7.14[i]		7.17[m]
7’	1.21[a]	1.23[a]	1.23[a]	1,24[a]		1.23[a]	1.24[a]	2.73[a]	8.03[i]	7.15[a]		7.10[m]
8’	1.21[a]	1.23[a]	1.23[a]	1,24[a]		1.98[a]	1.24[a]	2.00 [a]		7.12[i]	7.18[n]	7.31[i]
9’	1.21[a]	1.23[a]	1.23[a]	1,24[a]		5.31[a]	1.24[a]	5.32[o]			7.11[n]	
10’	1.21[a]	1.23[a]	1.23[a]	1,24[a]		5.31[a]	1.24[a]	5.32[o]			7.33[i]	7.07[b]
11’	1.21[a]	1.23[a]	1.23[a]	1,24[a]		1.98[a]	1.24[a]				7.59[i]	
12’	.87 [p]	1.23[a]	1.23[a]	1,24[a]		1.23[a]		5.32[o]			6.94[b]	
13’			1.23[a]	1,24[a]		1.23[a]	5.38[o]	5.32[o]				
14’		.87[p]	1.23[a]	1,24[a]		1.23[a]	5.38[o]	2.00[a]				
16’		0.87[f]		1,24[a]		1.23[a]	1.24[a]					
18’				0.84[f]		.86[p]	1.24[a]	0.85[p]				
22’							.87[p]					

Multiplicity (*J* in Hz): a=multiplet or broad singlet; b=singlet; c=septet (6.8); d=doublet (6.8); e=doublet (11.2); f=triplet (7.2); g=doublet (7.2); h=doublet (15.6); i=doublet (8.0); j=doublet (6.8); k=double doublet (14.8, 7.2); l=double doublet (13.2, 8.4); m=triplet (7.2); n=triplet (7.6); o=triplet of doublet (10.8, 4.8); p=triplet (6.8).



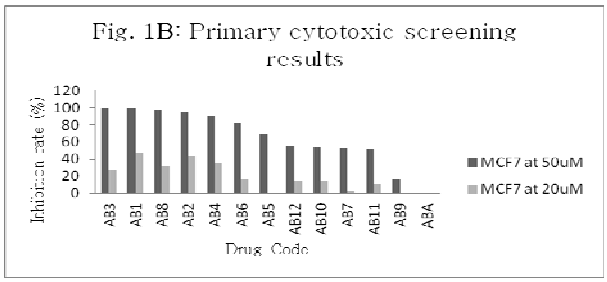


Fig. 1A & 1B: Huh7 and MCF7 cell lines primarily screened against 50µM and 20µM of trial drugs

RESULTS

Abietic acid (AB) was isolated from rosin of *Pinus longi folia* Roxb by Kraft Pulping Process (Jun *et. al.* 2012). The abietic acid (AB) so obtained then subjected to reduction to obtain abietyl alcohol ABA using lithium aluminiumhydride ($LiAlH_4$) and confirmed through EIMS and NMR spectral data found in accordance with reported data (Yadav *et al.* 2007).

Esterification is one of the most common methods used in the synthetic organic chemistry. The most common and classical one is known as Fischer esterification that involves use of sulfuric acid as catalyst and heating and excessive amounts of reagents, and therefore, in case of expensive and heat sensitive substrates (Either acid or alcohol) this reaction fails. Therefore, there is always a need for alternative milder and selective esterification methods.

Table 2: ^{13}C-NMR spectra data of compounds 1-12 (100 MHz, $CDCl_3$)

C #	1	2	3	4	5	6	7	8	9	10	11	12
1	35.8	36.2	37.7	37.9	36.0	37.9	37.4	38.3	36.2	36.20	36.29	36.24
2	18.9	18.9	18.5	18.5	18.1	18.9	18.5	18.9	18.1	18.88	18.07	18.02
3	38.3	38.5	38.5	38.7	38.8	38.3	38.6	43.8	38.8	37.96	38.76	38.55
4	36.0	35.6	35.7	35.6	35.6	33.4	35.7	33.0	35.6	34.91	35.60	35.60
5	40.9	44.3	44.3	44.4	44.4	43.4	44.3	43.4	44.9	43.67	44.16	43.60
6	25.1	24.9	25.0	25.0	23.9	24.9	24.9	24.4	24.1	24.11	23.97	23.79
7	121	121	121	121	121	121	121	121	121	120.7	121.0	120.8
8	132	132	132	135	135	135	135	134	135	135.3	135.4	135.3
9	44.2	51.6	45.5	50.7	50.7	44.2	58.9	44.2	50.7	43.94	50.83	50.42
10	36.6	36.5	37.7	36.5	36.5	35.6	36.6	35.2	36.9	36.69	36.42	36.51
11	22.6	22.6	22.6	21.8	22.6	24.9	22.6	23.9	22.7	23.91	22.65	22.61
12	28.5	27.5	27.6	28.9	27.5	27.2	27.2	27.1	27.4	27.53	27.54	27.51
13	145	146	146	145	145	145	145	145	145	147.1	145.4	145.0
14	123	123	123	123	122	122	122	122	122	122.5	122.5	123.0
15	34.4	33.7	37.7	33.5	34.9	35.6	35.7	35.3	34.9	38.06	34.92	34.94
16	24.5	23.8	23.9	23.8	20.8	23.9	23.8	21.9	20.8	20.90	20.87	20.93
17	23.8	23.9	22.6	23.9	21.4	23.8	23.9	22.2	21.4	21.46	21.45	21.52
18	72.2	72.3	72.3	71.6	72.4	72.3	72.3	71.7	73.4	72.80	72.37	72.53
19	17.5	17.5	17.5	17.5	17.9	17.5	17.5	17.5	17.8	17.46	17.82	17.75
20	16.2	17.1	17.5	17.5	14.1	14.1	14.1	14.5	14.5	14.11	14.18	14.08
1’	173	174	174	174	167	174	174	174	167	173.8	173.8	172.1
2’	34.3	37.9	34.4	34.4	122	34.4	34.4	34.3	137	46.98	33.82	31.49
3’	29.7	25.0	29.7	25.0	124	24.9	24.9	25.6	129	134.2	24.41	108.8
4’	29.7	29.6		29.7	17.8	29.1	29.8	29.0	128	128.5	25.18	127.3
5’	29.2					29.1		29.1	132	129.6	115.5	118.9
6’	29.2					29.1		29.1	128	127.0	128.8	122.1
7’	29.2					31.9		31.5	129	129.6	118.9	119.7
8’	29.2					27.2		27.1		128.6	121.6	111.1
9’	29.2					130		132			119.2	136
10’	31.9					130		128			111.0	122
11’	22.6	29.1				27.1	29.3	25.6			136.4	
12’	14.1	31.8				31.9	27.2	128			121.9	
13’		22.6	25.0			29.0	129	132				
14’		14.1	31.9			29.0	130	27.1				
15’			22.7	29.2		29.1	27.2	29.0				
16’			14.1	31.9		31.9	29.7	32.5				
17’				22.6		22.6		22.5				
18’				14.1		14.1		13.9				
19’							29.3		17.8			
20’							31.9		14.1			
21							21.6					
22’							14.1					

In the present work, we have prepared esters of abietyl alcohol (ABA) using fatty acids, aromatic acids and acids with heterocyclic systems. Fatty acids being larger molecules do not undergo esterifcation by traditional methods. We, therefore, planned to initiate this work employing mild conditions by using Steglich esterification (Ramalinga *et. al.* 2002).

Steglich esterification involves use of DCC and DMAP. DCC activates carboxylic acid and DMAP works as acyl transfer catalyst. The esterification proceeds without the need of a preformed, activated carboxylic acid derivative, at room temperature, under non-acidic, mildly basic conditions. The synthesis of compounds 1-12 was accomplished according to scheme-1 the reaction mixture is stirred for 2 hr at room temperature. Different spectroscopic techniques, ESI-MS, HRMS (ESI), IR, ^{1}H and ^{13}C-NMR were used to characterize esters (table 1 and table 2).

DISCUSSION

The present paper deals with the screening of 12 hitherto unreported derivatives of abietyl alcohol derivatives (1-12) and parent abietyl alcohol (ABA) for their cytotoxic effect on one breast cancer and four HCC cells using SRB technique (Vanicha *et al.* 2006). Trial drugs ABA with 12 derivatives 1-12 were introduced in two different concentrations i.e. 50uM and 20uM; in triplicate for both concentrations for each sample (*vide experimental*). As mentioned in fig. 1A and 1B, comparatively higher inhibitory effects were observed in derivatives 1-12 than parent compound. Although derivatives 1 and 2 were found to be the most potent exhibiting above 95% growth attenuation at 50uM concentration on two cell lines including Huh7 and MCF7 on the other hand no cytotoxic effect was revealed in parent compound ABA on both cell lines.

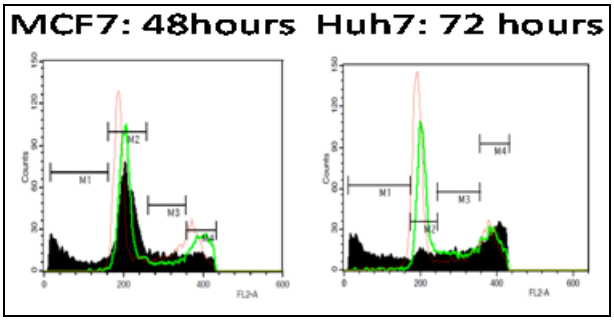


Fig. 2: MCF7 (48hrs.) Huh7 (72hrs) cell Progression after exposure of compound 1

Key: Control: Red: Untreated cells; Sample: Green: compound 1 treated cells; Positive Control: Black: Camptothecin treated cells

On the basis of above results, compound 1 and 2 further subjected for systematic primary screening on one breast cancer cell line and four HCC and to observe the percentage inhibition at 20uM.

Each inhibition bar represents mean of three experiments. Inhibition rates were calculated as mentioned in our previous published work (Mustafa *et. al*. 2014)

IC_{50} Determination

MCF7 and Huh7 Cells were used. 2000-2500 cells were cultured in each well of 96 well plates and incubated for 24 hours. Cells were treated with compound 1 after 24 hours at different concentrations in triplicates ranging from 50uM to 1,25uM; for controls up to 0.1% DMSO final concentration was maintained with respective media for each cell line. At 5uM concentration as a positive drug indicator was used (in triplicate) consists of camptothecin. For compounds 1 and 2, the IC_{50} values were determined against three HCC (Huh7, Hep3B and Plc) and one breast cancer cell line MCF7. For each cell line 2500 cells/well in 96 well plates were seeded before 24 hours of drug introduction. On all treated cell lines, 95-100% growth attenuation observed at 5uM by Camptothecin.

As presented in table 3, compound 2 displayed significant inhibitory effects on two cell lines i.e. MCF7 and Huh7 with IC_{50} values of 16,7uM and 21,5uM respectively. While for Hep3B and Plc cells, less than 10% growth attenuation at 20uM was detected that is why we did no determine IC_{50} values. Compound 1 was found to be more potent derivative with lowest comparative IC_{50} value on MCF7 (15,8uM), Huh7 (20uM), Hep3B (21,2uM) and Plc (31,6uM); suggesting that compound 1 has comparatively significant cytotoxic effects as compare to other derivatives of the study.

Table 3: IC_{50} [µM] for **1** and **2**

Cell Line	AB1		AB2		Control OD
	IC_{50} (µM)	R^2	IC_{50}(µM)	R^2	
MCF7	15,8	0,9	16,7	0,8	1,028
Huh7	20	0,7	21,5	0,8	1,357
Hep3B	21,2	0,8	-	-	0,605
Plc	31,6	0,8	-	-	0,508

Cell Cycle analysis of compound 1 treated MCF7 and Huh7 Cells

Cell cycle is the series of repeated events, responsible for ongoing cell division and duplication process. Mainly, it consists of synthesis (G_1 and S phase), inter-phase (G_2 phase) and mitosis (M phase). Based on IC_{50} values, MCF7 and Huh7 the most potent cell lines were treated with AB1 for 24, 48 and 72 hours. Camptothecin (5µM) was used to validate our experiments as positive control. While, 0.1% DMSO containing media was used for negative control MCf7 Cell line showed around 50% decrease in G_1 phase after 48 hours of AB1 treatment. Same findings were observed in Huh7 cell line after 72 hours in comparison with DMSO control (fig. 2). Deviations in cellular response reiterate our hypothesis about structural changes in chemical structure of parent compound can lead to synergic and conformational response on uncontrolled progression of cancer cells.

REFERENCES

Abd El Hady FK and Hegazi AG (2002). Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. *Z. Naturforsch*, **57c**: 386-394.

James RH (2005). Diterpenoids. *Natural Product Report*, **23**: 594-602.

James RH (2006). Diterpenoids. *Natural Product Report*. **23**: 875-885.

Jun Ai, Ulrike WT and Zachary T (2012). Hemicellulose extraction from aspen chips prior to kraft pulping utilizing kraft white liquor. *Biomass and Bioenergy*, **37**: 229-236.

Kwang-Hee S, Hyun-Mi O, Sung-Kyu C, Dong CH and Byoung-Mog K (2005). Anti-tumor abietane diterpenes from the cones of *Sequoia sempervirens*. *Bioorg & Med. Chem. Lett*., **15**(18): 2019-2021.

Miguel AG, Julieth CR, Lee A, Ana M and Liliana BG (2009). Synthesis and biological evaluation of abietic acid derivatives. *Europ. J. of Med. Chem.*, **44**: 2468-2472.

Mustafa MA, Hashmi IA, Manzoor S, Ahmed A, Ahmad VU, Aslam A, Ozen C, Naqvi NH, Oztruk M and Ali FI (2014). Synthesis and characterization of amino acid conjugates of oleanolic acid and their *in vitro* cytotoxic effect on HCC cell lines. *Pak. J. of Pharma. Sci.,* **27**(5 Special): 1491-1496.

Perry NB and Foster LM (1994). Antitumour lignans and cytotoxic resin acids from a New Zealand gymnosperm, Libocedrus plumose. *Phytomedicine*, **1**(3): 233-237.

Ramalinga K, Vijayalakshmi P and Kaimal TNB (2002), A mild and efficient method for esterification and transesterification catalyzed by iodine. *Tetrahedron Lett*., **43**: 879-882.

Rao X, Song Z and He L (2008). Synthesis and antitumor activity of novel α-aminophosphonates from diterpenic dehydroabietylamine. *Heteroatom. Chem.,* **19**(5): 512-516.

Vanicha V and Kanyawim K (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*, **1**(3): 1112-1116.

Wada H, Kodato S and Kawamori M (1985). Antiulcer Activity of Dehydroabietic acid derivatives. *Chem. Pharm. Bull.,* **33**(4): 1472-1487.

Yadav JS, Gakul B and Uttam D (2007), Synthesis of (+)-amberketal and its analog from L-abietic acid. *Tetrahedron,* **63**(39): 9896-9902.