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Semisynthetic modification and Immunomodulatory activity studies of 19α-H lupeol esters

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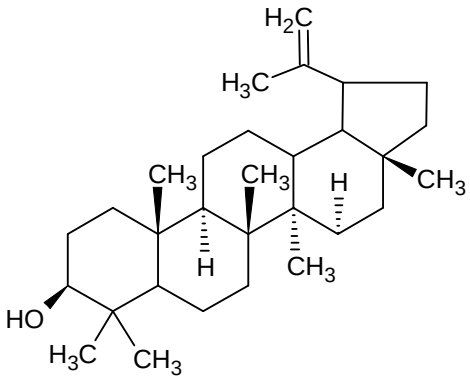
ABSTRACT

Isolation, semisynthetic modification of 19α-H lupeol to its various ester derivatives and their immunomodulatory activity studies has been envisage in this research work. 19α-H lupeol is a triterpenic alcohol which reacts with different acid chlorides or anhydride to give esters. Firstly, the acids are converted to their respective acid chloride derivative in the presence of thionyl chloride. 19α-H lupeol is then react with various acid chloride derivatives in the presence of dry pyridine to give different ester derivatives. The immunomodulatory activity of various ester derivatives was evaluated using Delayed type hypersensitivity (DTH) model. Cyclophosphamide was used as immunosuppressant. The different derivatives in the doses 15mg/kg and 30mg/kg produced minimum foot pad thickness as compared to control and CP. The derivatives also significantly reduced CP induced myelosuppression and thrombocytopenia.

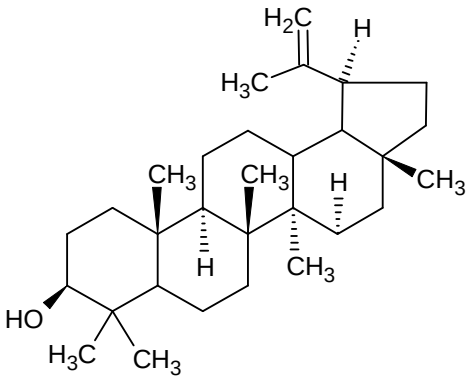
Key words: Strobilanthus ixiocephala, 19α-H lupeol, derivatization, immunomodulator.

INTRODUCTION

19α-H lupeol is a rare triterpenoid reported in *Strobilanthus heyneanus* and *Strobilanthus ixiocephala*[1](Family: *Acanthaceae*). It is a pentacyclic triterpenic alcohol which is a stereoisomer of lupeol. Lupeol is found abundantly in *Strobilanthus Callosus* roots. The main distinguishing feature was seen in the melting points of lupeol (M.P. 213-215) and 19α-H lupeol (M.P. 204-206).



Lupeol



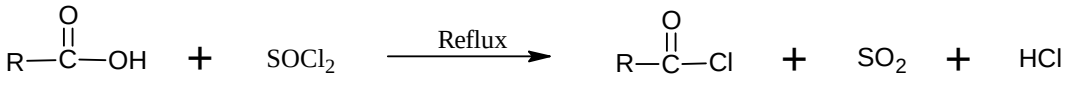
19α-H Lupeol

Lupeol has been reported as a competitive inhibitor of both trypsin and chymotrypsin whereas its palmitic and linoleic esters are non competitive inhibitor of trypsin [2]. Triterpenoid as such are reported to be devoid of any ulcerogenic action [3].

Presently, many steroidal, non steroidal and immunosuppressant drugs are used which help in controlling inflammatory symptoms but are associated with certain undesirable side effects [4]. Hence, the search for novel anti-inflammatory agents has attracted the attention of many researchers. Semisynthetic modifications of triterpenoids to ester derivatives have been reported by Kweifio-Okai et. Al. (1995) [5] which enhances the activity of parent compounds. Literature review also revealed that esterified lupeol with linoleate [6] and ecosopentanoic acid (EPA)[7] shows better results. Literature review indicates that 19α-H lupeol exhibits similar action as that of lupeol and therefore it's mechanism of action is similar to that of lupeol. The ester derivatives of lupeol such as linoleate and palmitate have been reported to have better anti-inflammatory and anti-arthritic activity. Hence, semisynthetic modification of 19α-H lupeol to it's various ester derivatives have been envisage in this research project.

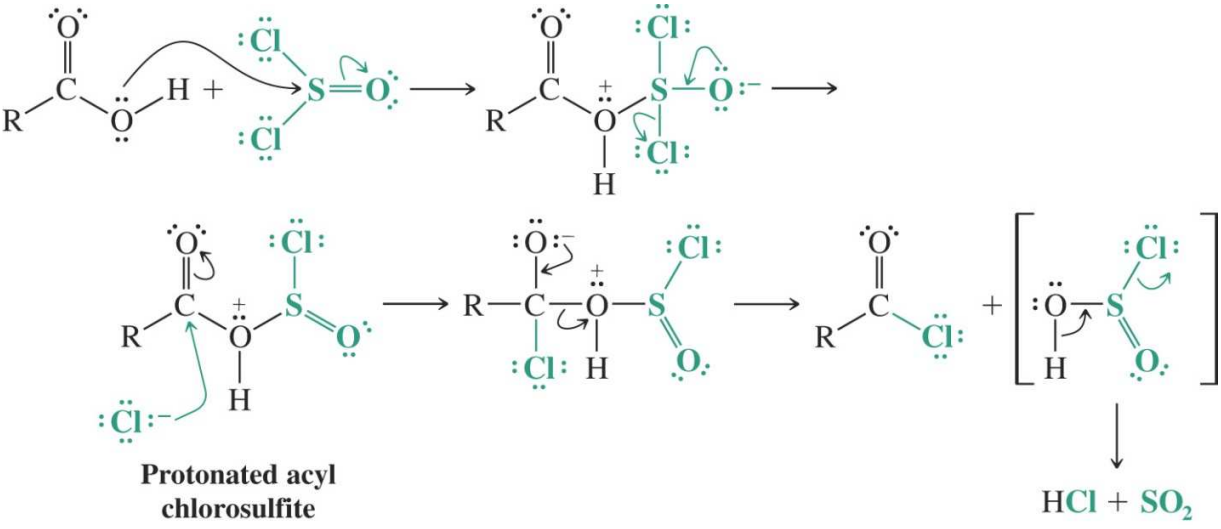
19α-H lupeol was isolated from the roots of *S. ixiocephala.* 19α-H lupeol is coded as AL. The title compounds were synthesized through the reaction sequence shown in scheme II. The key intermediate acid chlorides (or anhydride in case of succinic acid) were prepared by the reaction between various aromatic and fatty acids with thionyl chloride (acetic anhydride) Scheme I. This acid chlorides and anhydride on reaction with 19α-H lupeol which is a triterpenic alcohol in the presence of dry pyridine converted to various ester derivatives coded as ABD (19α-H lupeol benzoate), ASD (19α-H lupeol salicylate), ACD (19α-H lupeol cinnamate), ASC (19α-H lupeol succinate), AOD (19α-H lupeol oleate) and APD (19α-H lupeol palmitate).

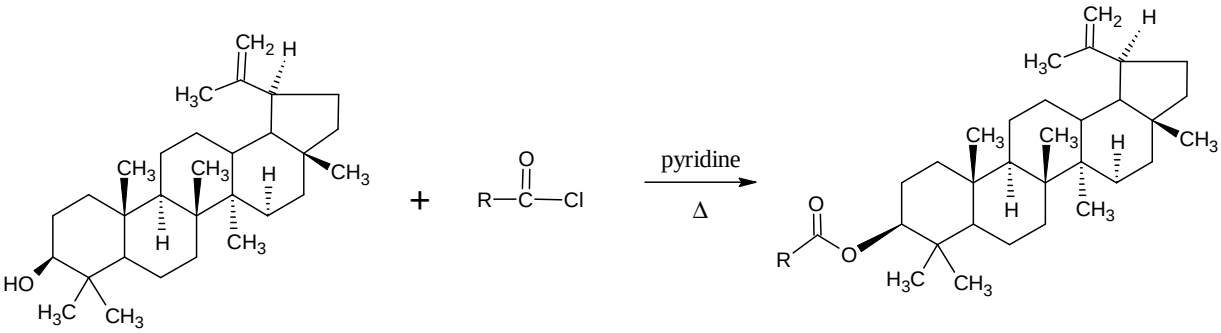
The structural assignments of the isolated compound were based on their elemental analyses and spectral data (IR, ^{1}H-NMR, MS). The isolated compound (19α-H lupeol) showed characteristic peak at 3350 for –OH group whereas the title compounds showed characteristic peak at 1715-1750 for ester group.



Scheme I

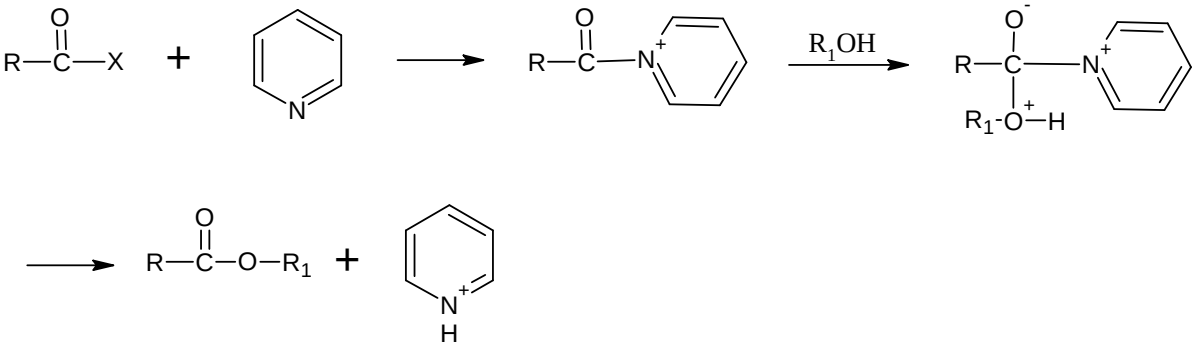
Mechanism





Scheme II

Mechanism:



MATERIALS AND METHODS

19α-H lupeol was isolated from the root of *Strobilanthus ixiocephala* (family-Acanthaceae) using petroleum ether (60^0-80^0 C). Plant *S. ixiocephala* was authenticated by the Botanical survey of India, Pune with voucher specimen no. 153115 .

Melting points of the isolated compound and the ester derivatives were recorded in open capillaries in an Fourtech melting point apparatus. IR spectra of various derivatives were recorded on a Perkin-Elmer 1800 FT-IR spectrometer.

General procedure for the preparation of different acid chlorides from acids [8,9]
Fit a reflux condenser into the short neck of a 50 ml claisen flask, a dropping funnel into the long neck and plug the side arm with a small cork. Place (0.1 mole) of commercial acid (aromatic or fatty acid) in the flask, add 17.84 g (10.94 ml, 0.15 mole) of redistilled thionyl chloride through the separatory funnel, shake the flask from time to time to ensure thorough mixing. Reflux the mixture for 30 min. Arrange the apparatus for distillation from an air bath; the excess of thionyl chloride passes over first, followed by acid chloride.

General procedure for the preparation of succinic anhydride from succinic acid
In a100 ml bolt head flask, provided with a 4^{ll} or 5^{ll} reflux condenser protected by a loose plug of cotton wool inserted into the upper end, place 11.8 g of succinic acid and 20.4 g (19.0 ml) of redistilled acetic anhydride. Warm the mixture gently over a small luminous flame with continuous swirling until a clear solution is obtained (2-3 min.) and then reflux gently (Small luminous flame) for 15 min. Remove the flame, allow to cool and finally cool in ice. Collect the crystalline succinic anhydride on small buckner or sintered glass funnel. Wash it with two 10 ml portions of ether, spread the solid on a clock glass and dry in a dessicator. Yield is 8.9 g. M. P. 119-120^0C.

General procedure for the preparation of different ester derivatives
In a 100 ml round bottom flask place a mixture of 0.426 g (0.001 mole) 19 α-H lupeol,0.0015 mol of acid chloride (or acid anhydride) and 10 ml dry pyridine. Add small chips of porous porcelain, attach reflux condenser and boil the mixture for few hours at a temperature of 60^0-70^0C. Check the completion of the reaction using thin layer chromatography until a single spot is obtained. After completion of the reaction, shake the reaction mixture with cold petroleum ether (60-80^0). The excess of acid chloride remains undissolved in cold petroleum ether, collect the cold petroleum ether part and allow it to evaporated. The residue is washed with several times with cold petroleum ether to give the ester derivative.

Immunomodulatory activity:
All synthesized compound were screened for immunomodulator activity by delayed type hypersensitivity model.

Materials
1. Cyclophosphamide (Dabur Health Care Ltd.) was used as standard immunosuppressant.

2. Antigenic material - The sheep RBCs (SRBCs) were used as antigenic material. The sheep blood was withdrawn from external jugular vein of sheep (Government Vetneiry college, Nagpur). It was mixed in 1:1 proportion in Alsever's solution & stored at 2^0 to 8^0C in refrigerator.

Preparation of SRBC suspension

Sheep blood was collected from Veterinary College in Alsevar's solution (1:1) and centrifuged at 2500-3000 rpm for 10 minutes. Supernatant was removed with Pasture pipette, and packed SRBCs were washed thrice with sterile Alsevar's solution. The resulting SRBCs were suspended in sterile Alsevar's solution to obtain a cell density of 10^6 SRBCs/mm^3, using improved Neubaur chamber.

Table I: Characterization data for different ester derivatives

Compounds	Melting point (°C)	R_f value*	Wave number (cm^{-1})	Function
19α-H lupeol	204-206	0.38	3350	-OH Stretching
ABD	117	0.97	1715	C=O Stretching (ester)
ASD	262	0.97	1750	C=O Stretching (ester)
ACD	98	0.97	1715	C=O Stretching (ester)
ASC	190	0.97	1750	C=O Stretching (ester)
AOD	-	0.97	1740	C=O Stretching (ester)
APD	75	0.97	1735	C=O Stretching (ester)

**Solvent system used is Benzene: ethyl acetate (97.5:2.5).*

Table II: Various ester derivatives of 19α-H lupeol and their yields

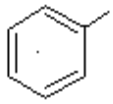
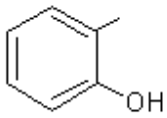
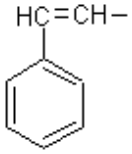
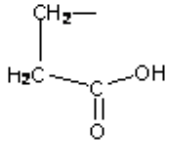
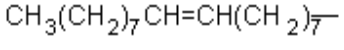
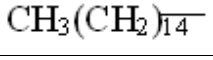
Compounds	R	%Yield
ABD		76.21
ASD		57.37
ACD		51.68
ASC		48.02
AOD		68.29
APD		72.80

Table III: Effect of various Semisynthetic derivatives of 19α-H lupeol on Mean Foot Pad Oedema in DTH model

Group No.	Group Specification		Mean foot pad thickness (mm)			
			24 hrs	48 hrs	72 hrs	96 hrs
I	Control Tween-80 (2%)		2.717* ± 0.076	2.353±0.084	1.913±0.110	1.837±0.067
II	AL	L	2.198± 0.110	1.710±0.321	1.401±0.025	1.392±0.059
		H	1.786*±0.237	0.971±0.355	0.804±0.098	0.798±0.091
III	ABD	L	1.260± 0.313	1.172±0.431	1.167±0.254	1.156±0.101
		H	1.104* ± 0.237	0.698±0.118	0.671±0.033	0.659±0.076
IV	ASD	L	1.762± 0.237	1.324±0.110	1.297±0.025	1.270±0.160
		H	1.276± 0.397	0.764±0.364	0.673±0.287	0.673±0.228
V	ACD	L	1.472± 0.237	1.286±0.169	1.210±0.016	1.164±0.245
		H	1.427* ± 0.262	0.798±0.118	0.692±0.135	0.691±0.050
VI	ASC	L	1.976 ± 0.194	1.424±0.177	1.302±0.050	1.298±0.033
		H	1.619 ± 0.101	0.826±0.063	0.724±0.127	0.719±0.063
VII	AOD	L	1.987± 0.262	1.526±0.220	1.370±0.110	1.367±0.033
		H	1.630 ± 0.152	0.897±0.203	0.762±0.008	0.762±0.135
VIII	APD	L	2.052± 0.237	1.677±0.169	1.387±0.186	1.375±0.220
		H	1.756± 0050	0.972±0.110	0.801±0.084	0.796±0.059
IX	Cyclophosphamide (20mg/kg)		3.149* ±0.237	2.556±0.114	2.142±0.063	1.964±0.076
X	AL + CP	L	2.683± 0.237	2.286± 0208	1.879±0.093	1.803±0.321
		H	2.641* ± 0.330	2.192±0.341	1.780±0.220	1.695±0.287
XI	ABD+CP	L	2.297± 0.254	1.765±0.270	1.447±0.050	1.440±0.026
		H	1.984* ± 0.136	0.974±0.254	0.872±0.025	0.869±0.169
XII	ASD+CP	L	2.328± 0.262	1.913±0.220	1.625±0.050	1.598±0.067
		H	2.276± 0.524	1.012±0.592	0.976±0.186	0.975±0.262
XIII	ACD+CP	L	2.303± 0.237	1.899±0.235	1.566 0.110	1.540±0.050
		H	2.147* ± 0.431	0.986±0.347	0.930±0.254	0.930±0.143
XIV	ASC+CP	L	2.387± 0.270	1.989±0.169	1.642±0.186	1.608±0.093
		H	2.310± 0.042	1.137±0.160	1.137±0.110	1.136±0.135
XV	AOD+CP	L	2.480 ± 0.237	2.041±0.245	1.761±0.220	1.617±0.110
		H	2.396 ± 0.389	1.196±0.254	1.656±0.152	1.656±0.313
XVI	APD+CP	L	2.599± 0.270	2.235±0.321	1.837±0.016	1.693±0.135
		H	2.512 ± 0.330	2.176±0.270	1.768±0.033	1.697±0.159

Statistical method: One way ANOVA followed by Bonferronis multipal comparison test
N = 6; values represent mean ±S.D.;
**P <0.001 (I when compared with IX)*
**P<0.05 [I when compared with II (H), III (H), V (H)];*
**P<0.05 [IX when compared with X (H), XI (H), XIII (H)]; ABD, ASD, ACD, ASC, AOD and APD: Different semisynthetic derivatives of 19α-H lupeol,*
CP: Cyclophosphamide

Preparation of suspension of dose of tested drugs:

5 ml of 3mg/ml (Lower dose) and 5 ml of 6mg/ml (Higher dose) suspension of different derivatives were prepared in distilled water using 2% w/v Tween-80 as a suspending agent.

Preparation of solution of Cyclophosphamide

10 ml of 2mg/ml solution of cyclophosphamide was prepared in sterile normal saline.

Statistical analysis:

Data were analyzed using one way analysis of variance followed by Bonferronis multiple comparison test. The values are expressed as mean ± SD.

Table IV: Effect of Semisynthetic derivatives on Leukocyte and Platelet Count in DTH model.

Group No.	Group Specification		Haematological Parameters	
			WBC Count (thousand/mm^3)	Platelet Count (thousand/mm^3)
I	Control		8.7 ± 1.022	1140± 124.675
II	AL	L	7.5 ± 1.143	1110± 267.087
		H	6.8 ± 0.694	1119± 243.764
III	ABD	L	6.6 ± 0.843	975± 187.480
		H	6.3 ± 0.896	915± 197.548
IV	ASD	L	4.8 ± 0.542	831± 208.764
		H	4.0 ± 0.584	869 ± 234.671
V	ACD	L	5.7 ± 0.846	1134 ± 167.872
		H	4.2 ± 0.984	1161 ± 142.407
VI	ASC	L	6.6 ± 1.584	1167 ± 196.584
		H	5.7 ± 1.475	963 ± 152.600
VII	AOD	L	7.8 ± 1.717	777 ± 247.370
		H	7.2 ± 1.099	783 ± 251.728
VIII	APD	L	9.1* ± 1.728	804* ± 312.473
		H	8.4 ± 1.576	762 ± 338.622
IX	Cyclophosphamide (20 mg/kg)		5.6* ± 1.152	675* ± 156.360
X	AL+CP	L	8.4 ± 1.752	1227± 137.441
		H	9.3* ± 1.603	1245* ±182.607
XI	ABD+CP	L	7.1 ± 1.566	849 ± 176.882
		H	8.6 ± 1.798	892 ±162.601
XII	ASD+CP	L	7.1 ± 0.769	941 ± 179.564
		H	7.3 ± 0.856	976 ± 267.967
XIII	ACD+CP	L	8.1 ±1.736	1098 ±382.706
		H	8.2 ± 1.845	1089 ± 341.384
XIV	ASC+CP	L	8.4 ± 1.664	1170 ± 278.743
		H	8.7 ± 1.420	1203 ± 269.081
XV	AOD+CP	L	8.7 ± 1.642	1113 ± 192.768
		H	9.6* ± 1.708	1248* ± 164.873
XVI	APD+CP	L	7.8 ± 1.077	1143 ± 285.946
		H	9.4* ± 1.604	1230* ± 291.772

Statistical method: One way ANOVA followed by Bonferronis multipal comparison test
*N = 6; values represent mean ± S.D.; *P<0.01 (comparison of I with IX)*
**P<0.05 [VII (L), X (H), XV(H), XVI(H) when compared with I]*
CP: Cyclophosphamide

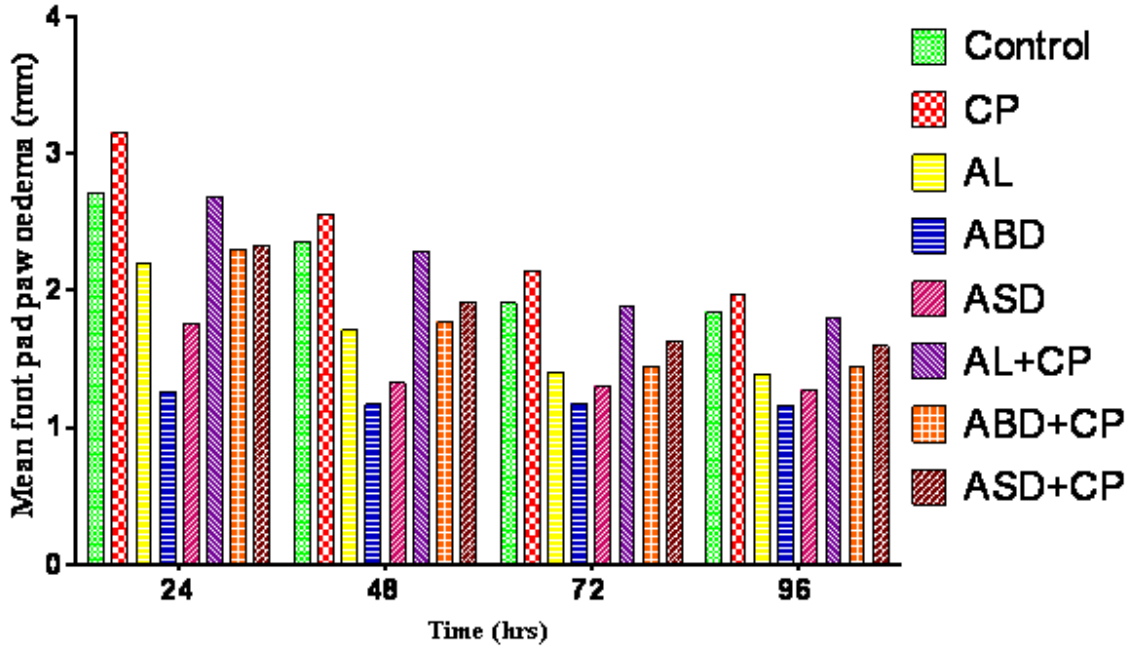


Fig. No. 1: Effect of ABD and ASD (15 mg/Kg) on Mean foot pad oedema in DTH model.

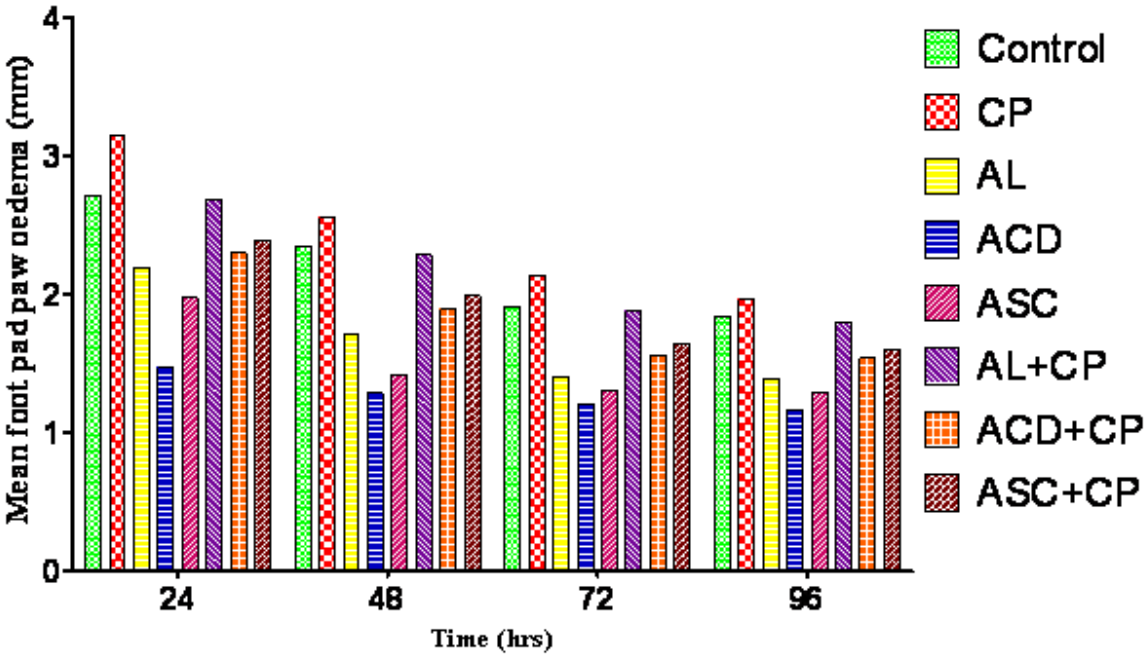


Fig. No. 2: Effect of ACD and ASC (15mg/kg) on Mean foot pad oedema in DTH model

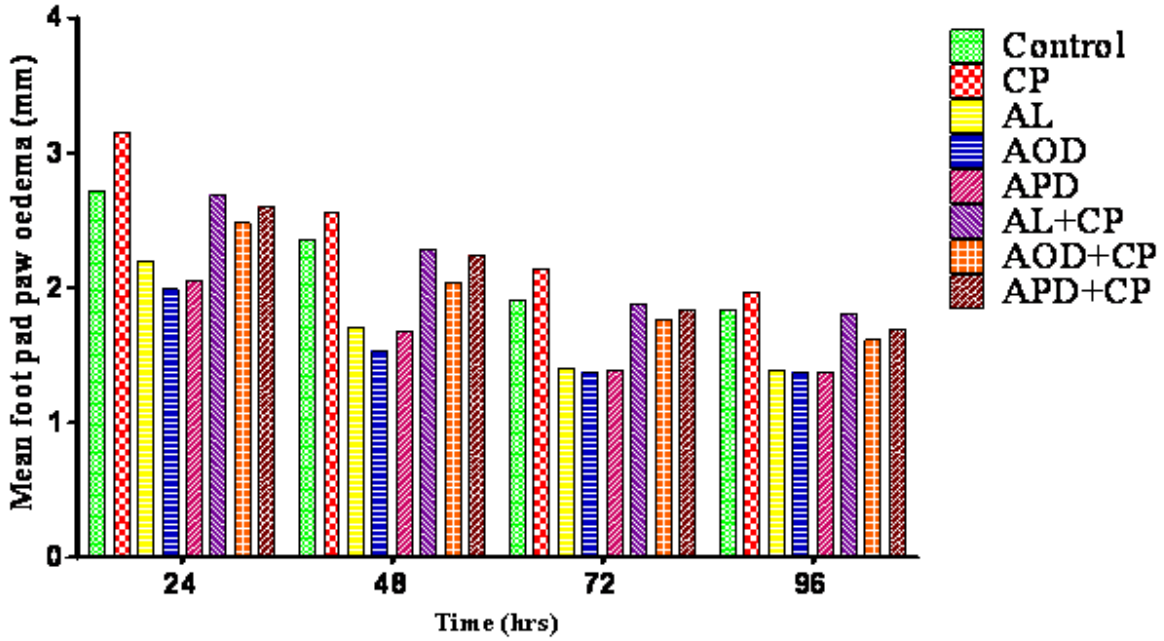


Fig. No. 3: Effect of AOD and APD (15 mg/kg) on Mean foot Pad Oedema in DTH model.

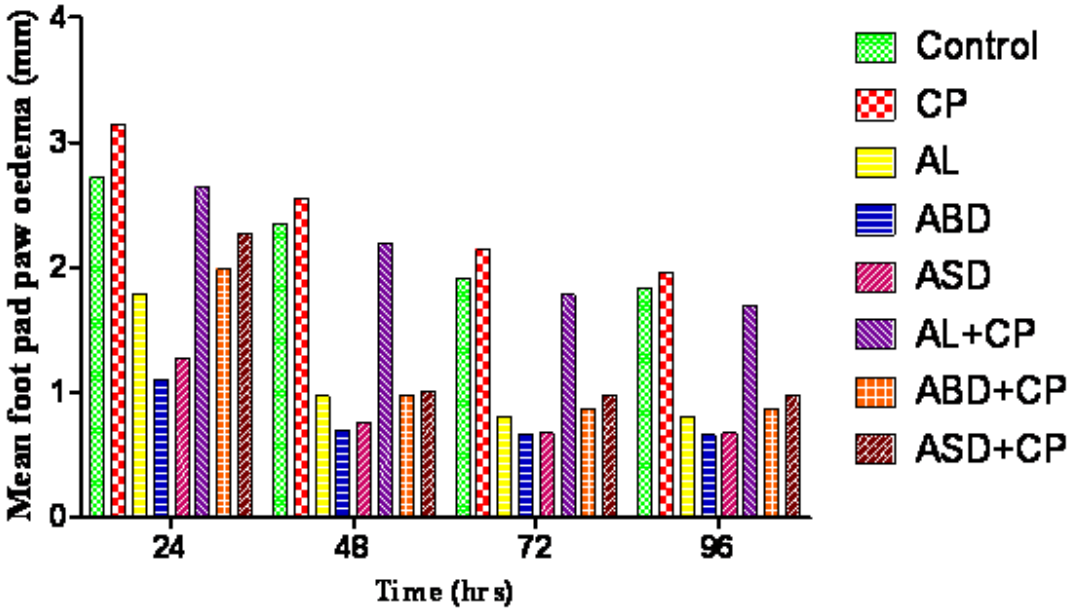


Fig. No. 4: Effect of ABD and ASD (30 mg/kg) on Mean foot pad oedema in DTH model.

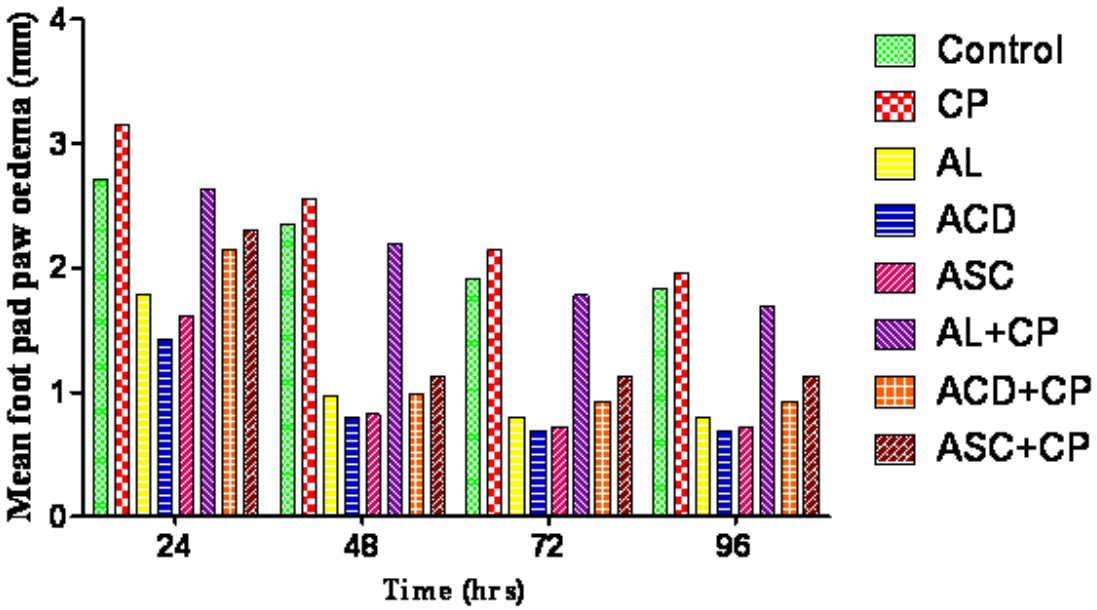


Fig. No. 5: Effect of ACD and ASC (30 mg/kg) on Mean foot pad oedema in DTH model.

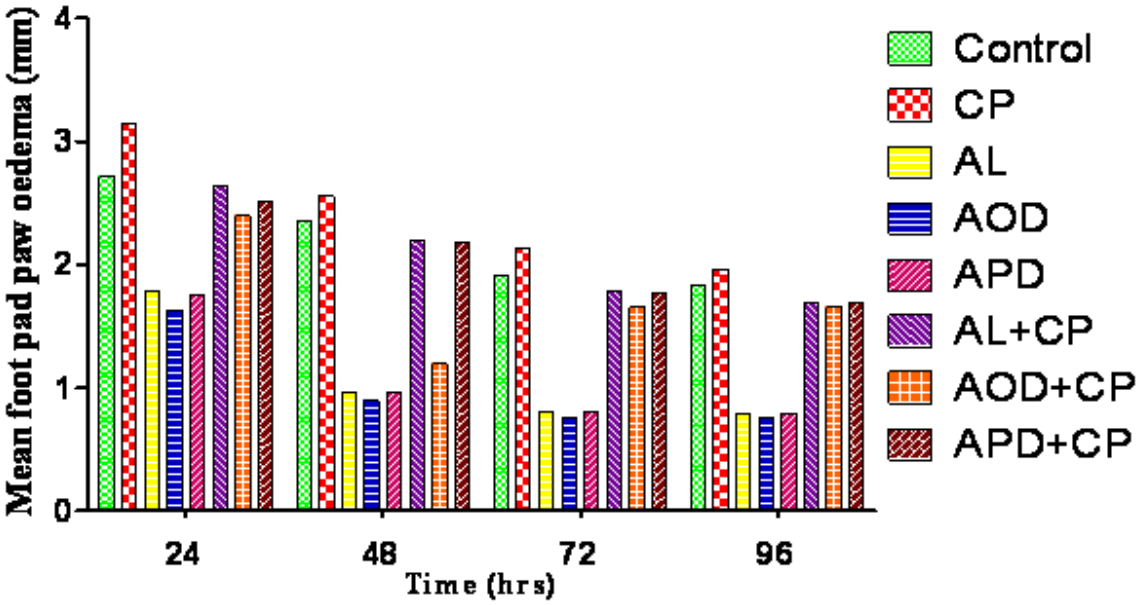


Fig. No. 6: Effect of AOD and APD (30 mg/kg) on Mean foot pad oedema in DTH model

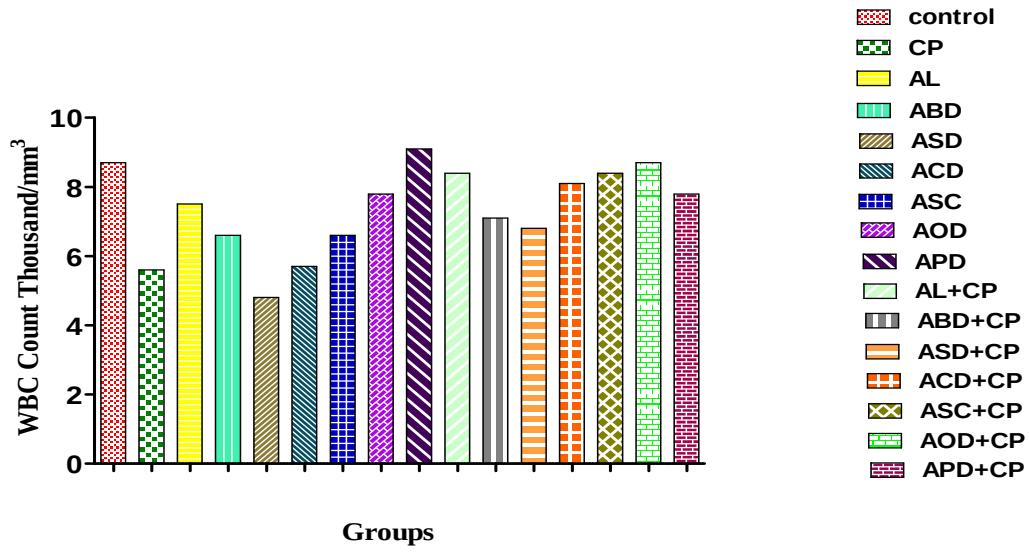


Fig. No. 7: Effect 0f ABD, ASD, ACD, ASC, AOD and APD (15 mg/kg) on Leukocyte Count in DTH model

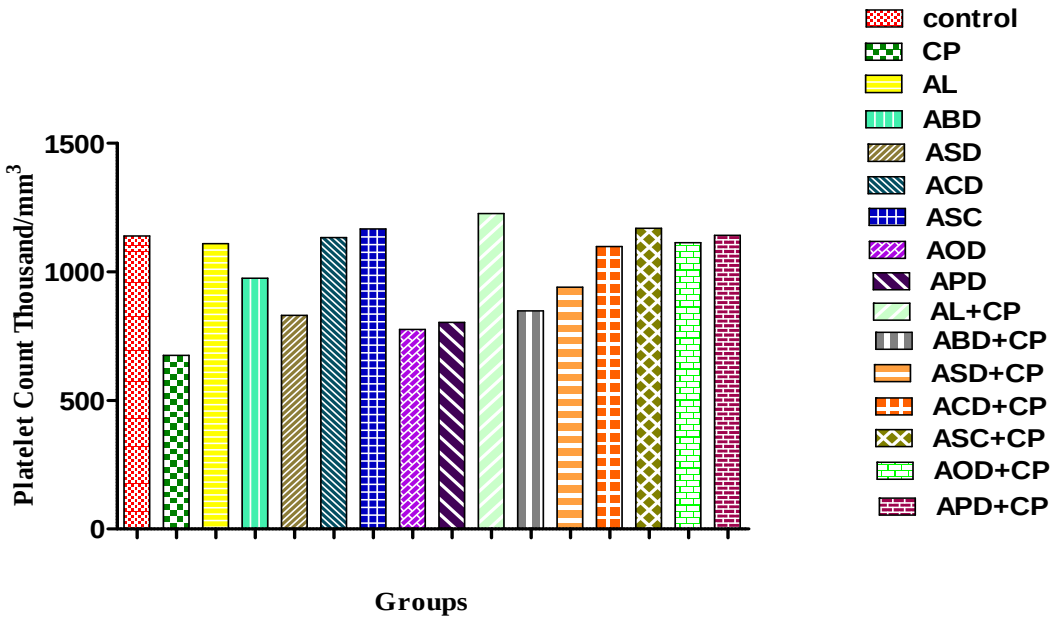


Fig. No. 8: Effect 0f ABD, ASD, ACD, ASC, AOD and APD (30 mg/kg) on Leukocyte Count in DTH model.

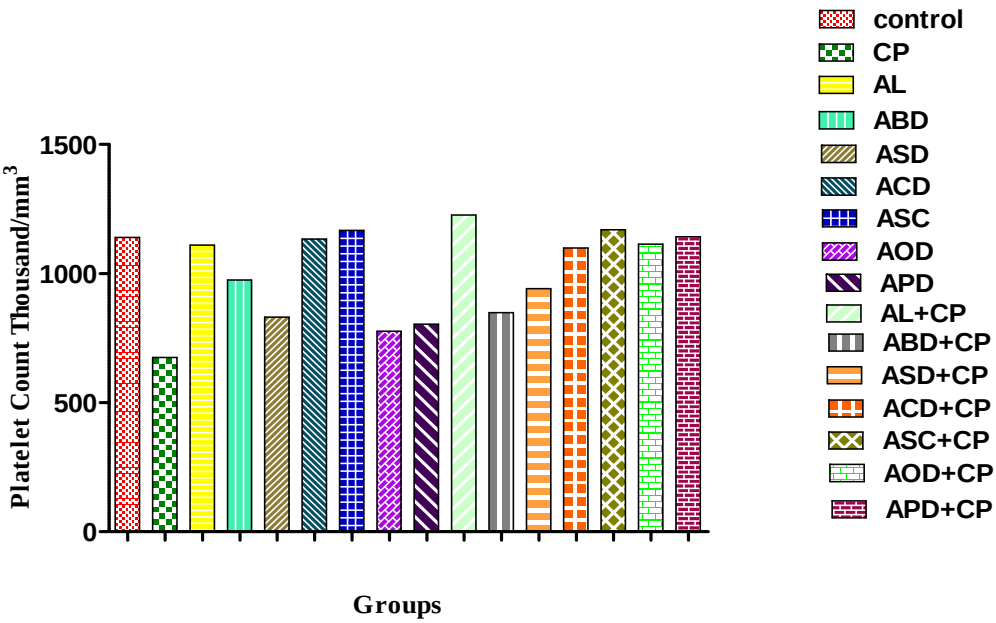


Fig. No. 9: Effect 0f ABD, ASD, ACD, ASC, AOD and APD (15 mg/kg) on Platelet Count in DTH model.

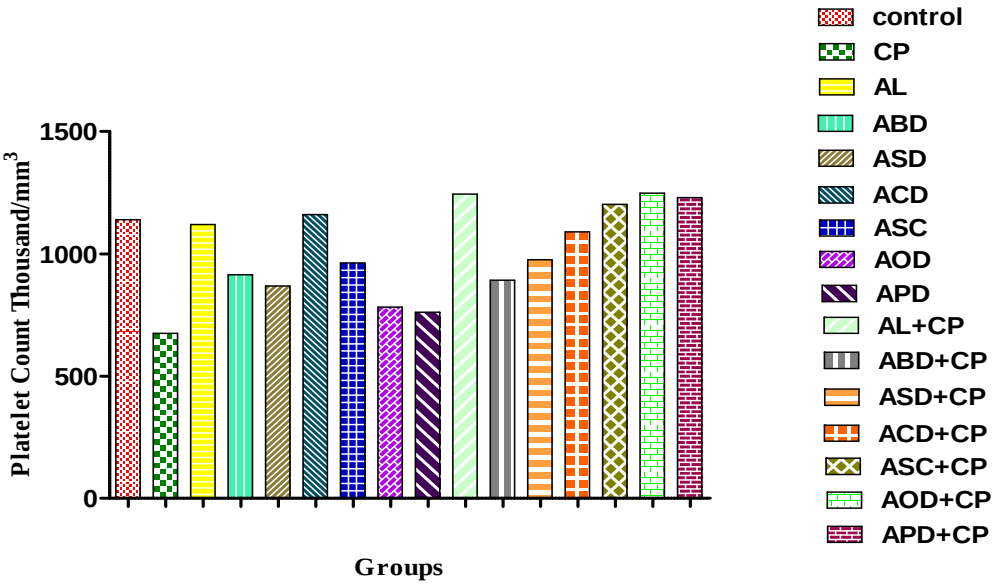


Fig. No. 10: Effect 0f ABD, ASD, ACD, ASC, AOD and APD (30 mg/kg) on Platelet Count in DTH model.

RESULTS AND DISCUSSION

The bands at 3350 cm for hydroxyl stretch, 2947 cm for CH stretch, 1641 cm for C=C stretch, 1039 cm for C-O stretching are the important vibrations observed in IR spectrum[10] of the isolated compound (19α-H lupeol).The various ester derivatives of 19α-H lupeol showing a ester functional group in the region 1715-1750. This indicates that the hydroxyl group is converted to ester group.

^{1}H-NMR spectrum of 19α-H lupeol in $CDCl_3$ (Spectrum No. 2, Table No. 2) indicates all methyl protons appeared as singlets in the area of 0.97 to 1.68 ppm. C_3 proton appeared at 3.17 ppm as a double doublet and two unsaturated protons of C_{29} appeared at 4.57 and 4.69 ppm.

Mass spectrum of 19α-H lupeol (Spectrum No. 3, Table No. 3) depicted a strong molecular ion peak at m/e 426 (35.5%). It has indicated two major fragments[11] at m/e 218 and m/e 207 due to Ring C breakdown. A base peak with 100% relative abundance was observed at m/e 69.

All the ester derivatives synthesized in the present study were tested for immunomodulatory activity using delayed type hypersensitivity (DTH) model. The results of the effect of various semisynthetic derivatives of AL on mean foot pad thickness in DTH model indicates that animals treated with cyclophosphamide (CP) 20 mg/kg exhibited maximum potentiation of DTH as observed from an increase in mean foot pad thickness, 24 hour after challenge. The derivatives when administered orally at the dose of 15mg/kg and 30 mg/kg produces minimum foot pad oedema compared to control (Table 3, Fig. No. 1-6), in this case ABD, ASD and ACD showed significant results ($P<0.05$). Animals treated with CP and receiving the derivatives significantly suppressed the potentiation induced by CP as compared with animals treated with CP alone. It indicates the derivatives alone and in combination with CP ($P<0.05$) suppresses or favorably modulate the CP potentiated DTH reaction.

The results of the effect of Various derivatives of AL on total WBC and platelet count indicates that the WBC Count was significantly decreased in animals treated with CP and different derivatives except APD at lower (15 mg/kg) and ACD, ASC at higher (30 mg/kg) dose levels. The CP induced myelosuppression was significantly counteracted by the different derivatives at both dose levels. The Platelet Count was also significantly decreased in animals treated with CP. In lower dose i.e 15 mg/kg AL, ACD and ASC did not have any significant effect on Platelet Count but ABD, ASD, AOD and APD decreases the platelet count whereas in higher dose i.e 30 mg/kg AL and ACD did not have any significant effect on Platelet Count but ABD, ASD, ASC AOD and APD decreases the platelet count when administered alone. The CP induced thrombocytopenia was prominently counteracted by all the derivatives in both lower and higher doses (Table No. 4, Fig. No. 7 - 10).

CONCLUSION

Semisynthetic modification of 19α-H lupeol to it's various ester derivatives have been envisage in this present research work. 19α-H lupeol was isolated from the roots of *S. ixiocephala*. The different ester derivatives of 19α-H lupeol like benzoate, salicylate, cinnamate, succinate, oleate and palmitate have been synthesized which are coded as ABD, ASD, ACD, ASC, AOD and APD. The derivatives were confirmed by TLC studies. All the derivatives are giving R_f value at 0.97. The derivatives were further confirmed by IR spectral studies which gives ester functional group (-0-C=0) at region 1715-1750. The derivatives were subjected to immunomodulator activity studies, the derivatives alone cause potentiation of DTH but in combination of CP it suppresses or favorably modulates the CP potentiated DTH reaction. The CP induced myelosuppression was significantly counteracted by the different derivatives at both dose levels

i.e at 15 mg/kg and 30 mg/kg. The CP induced thrombocytopenia was prominently counteracted by all the derivatives in both lower and higher doses.

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