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ETHNOPHARMACOLOGICAL SCREENING OF STEMS (WOOD WITHOUT BARK) OF *LEUCAENA LEUCOCEPHALA(LAM.)DE WIT*

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Abstract:
Leucaena leucocephala (Lam.) de wit is a plant belonging to family (Leguminosae). It has reported traditionally as a medicinal plant and timber tree in many countries. It is reported for pharmacological and therapeutic action, such as anthelmintic, abortifacient, antibacterial, and antimicrobial. The Leucaena leucocephala (Lam.) de wit stem (wood without bark) has terpenoids, which acts as antioxidant so the present consist of investigation immunomodulatory action. The Leucaena leucocephala (Lam.) de wit Stems (wood without bark) has terpenoids, and terpenoids have antiviral activity, so the present work is the investigation of Anti HIV activity.

Keywords: *Stems, Extract, terpenoids, Anti HIV activity, Immunomodulatory action*

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INTRODUCTION:

Leucaena leucocephala(Lam) de wit (Leguminosea) is tree and shrub known for its long life, high content of nutrition forage and it's medicinally uses. Studies have reported that *Leucaena leucocephala (Lam) de wit* shows the presence of various secondary metabolites such as terpenoids, flavonoids, alkaloid, glycosides, cardiac glycosides, tannins, saponins, and in this species. Immunostimulation can be considered as an alternative to an adjuvant for conventional chemotherapy and prophylaxis of infection. Immunomodulators are becoming very popular in the worldwide today COVID 19 disaster. Hence the present work is undertaken to investigate of immunomodulatory properties of *Leucaena leucocephala (Lam) de wit*.

HIV is an unusual and extraordinary epidemic which needs more attention and care. Plants and natural phytoconstituents reported to have strong antiviral activity. Aqueous and organic extractions have in general proved equally useful for treating HIV Infection. Hence the present work is undertaken to investigate of anti HIV screening *Leucaena leucocephala (Lam) de wit*.

MATERIAL AND METHODS

Collection and Authentication of plant:

The plant *Leucaena leucocephala (Lam.) de wit* was collected in the month of September from the local area, of Sangli region. The plant was identified and authenticated by M. D. Wadmare of Botony Department, Smt. Kasturbai Walchand College, Sangli.

Drugs and Chemicals:

All the drugs and Chemical were of analytical grade.

Animals

The experiment was carried by using male albino rat of Wistar strain. Weight around 150- 200 gm. The experimental protocol was approved by the institutional animal ethics committee and the care of laboratory animal was taken as per the guideline of CPCSEA. Present study was carried out in CPCSEA approved animal house of Appasaheb Birnale College of Pharmacy Sangli, Maharashtra.

Virus and enzyme:

Pepsin has a close resemblance in proteolytic activity with HIV-1 protease inhibitor. Pepsin hence enzyme was used as a substitute of HIV-1 protease to check out anti HIV activity of plants extract in this experiment.

PREPARATION OF EXTRACT:

Soxlet Extraction

Extraction of powder materials was done with a range of solvents, petroleum ether, chloroform, ethyl acetate, ethanol with increasing polarity. The coarse powder of *Leucaena leucocephala (Lam.) de wit* of stems (wood without bark) 50 gm was weighed and placed filter paper bag. This bag was placed in thimble. Solvent was added in the flask in the proportion of 1:3 that is for 50 gm powder, 150 ml solvent. The solvent was boiled by electronic heating mantel. The extraction was continued until the solvent in the thimble became clear.

Maceration:

About 150 gm of powder of *Leucaena leucocephala (Lam.) de wit* was weighed and subjected chloroform water 10 % as per I. P. in 1 liter beaker. The beaker was covered with aluminum foil and kept room at temperature for seven days. Frequent shaking was done daily. On eighth day it was filtered and filtrate was collected. The obtained filtrate was used as aqueous extract.

PHARMACOLOGICAL SCREENING

The immunomodulatory activity is carried out using the following in-vivo immunomodulatory models.

1. **Delayed Type Hypersensitivity Reaction.**
2. **T cell population test**

Delayed hypersensitivity reaction

Delayed hypersensitivity reaction is a reaction is based upon cell mediated immunity and is observed only after 16- 24 hrs. In this test animals were divided into four group comprising 6 animals in eac

Procedure:

1. In this test animals were divided into four group comprising 5 animals in each.
2. Group I was kept as a control and given vehicle only water 10 ml/kg by oral route.
3. Group II was kept as a standard and given standard drug levamisole 50 mg/kg oral route.
4. Group III was kept is given extract 200 mg/kg the aqueous extract.
5. Group IV was given ethanolic extract by oral route. The dosing was continued for 20 days.
6. On day 21st, animals from all groups get challenge with 0.03ml of 1% SRBCs in sub plantar region of right hind paw.
7. Foot pad reaction was observed after 24hrs on 22nd day.
8. Increase in foot pad edema is measured with the help of vernier caliper.

Sr. No	Group	Test Substance	Dose
1.	Group I	Control (water)	10 ml/kg
2.	Group II	Std (Levamisole)	50 mg/kg
3.	Group III	Aqueous extract	200 mg/kg
4.	Group IV	Ethanolic extract	200 mg/kg

Grouping and Treatment Schedule

Preparation of sheep RBCs:

Sheep blood was collected in sterile Alsever's solution in 1:1 proportion, Alsever's solution. Blood was kept in the refrigerator, it was centrifuged at 2000 rpm for 10 min and was washed with physiological saline 4-5 times and then suspending into buffered saline for further use.

Sr. no	Chemicals	Quantity(g/L)
1.	Sodium Chloride	4.2
2.	Sodium Citrate	8.0
3.	Citric acid anhydrous	0.55
4.	Glucose	20.5
5.	Distilled water q.s	1000m

Composition of Alsever's Solution

Statistical analysis:

Result is expressed in mean value ± SEM. The variation in set of data has been estimated by one way analysis of variation (ANOVA) method. Dunnets test is used for the individual comparison of group mean value.

T cell population test

Sr. No	Group	Test Substance	Dose
1.	Group I	Control (water)	10 ml/kg
2.	Group II	Std (Levamisole)	50 mg/kg
3.	Group III	Aqueous extract	200 mg/kg
4.	Group IV	Ethanolic extract	200 mg/kg

Animal and their dosing

Procedure:

1. In this test animals were divided into four group comprising 5 animals in each.
2. Group I was kept as a control and given vehicle only water 10 ml/kg by oral route.
3. Group II was kept as a standard and given standard drug levamisole 50 mg/kg oral route.
4. Group III was given extract 200 mg/kg the aqueous extract.
5. Group IV was given ethanolic extract by oral route for 10 days.
6. On 11th day, blood is collected from the retro-orbital plexus and anticoagulated with Alsever's solution in separate test tubes.

7. This test tube was kept in sloping position 45^0 at 37^0 C for 1 hour. RBCs were allowed to settle at bottom and supernatant was collected from each test tube by using micropipette which contains lymphocytes.
8. 50 µL of lymphocyte suspension & 50 µL SRBC were mixed in test tube and incubated.
9. Resultant suspension is centrifuged at 200 rpm for 5 min and kept in a refrigerator at 40 C for 2 hour. The supernatant fluid was removed and one drop of cell suspension was placed on a glass slide.
10. It is observed under the microscope. Total lymphocytes were counted and a lymphocyte binding with three or more erythrocytes were as considered as rosette and number of rosettes is counted.

Statistical analysis:
Result is expressed in mean value ± SEM. The variation in set of data has been estimated by one way analysis of variation (ANOVA) method. Dunnets test is used for the individual comparison of group mean value.

Procedure of Anti HIV activity

Enzyme inhibition assay

1. For this assay, 50µg pepsin, 800µg hemoglobin and different crude plant extracts were taken in 500µl of reaction mixture.
2. The mixture was allowed to incubate at 37^0C, after 20 min 700µl of 5% TCA is added to stop the reaction.
3. It is then centrifuged at 14000 g for 5 min and the supernatant is collected. Optical Density (OD) is recorded by spectrophotometer at 280 nm.
4. Separate blanks were used or both positive and negative controls as well as for sample.

Sr. No	Parameter	Optimum range
1.	pH	2- 4
2.	Incubation period	30 min.
3.	Reaction volume	1000 µL
4.	Incubation temperature	37 ^{0} C
5.	Centrifugation	14000 rpm.

5. For negative control, enzyme and substrate were taken and followed the above procedure and for positive control protease is taken as a well-known inhibitor of HIV-protease, lopinavir was taken.
6. Each sample was taken in triplicate, so this assay gives reproducible results. Percentage of inhibition is calculated by using a formula.

Inhibition (%) = [(OD of negative control - OD of sample) /OD of negative control] ×100.

IDENTIFICATION OF CHEMICAL CONSTITUENTS

Thin layer chromatography:

Ethyl acetate extract is used for the identification. Silica gel G is used as an absorbent, the slurry is prepared with water in 1:2 proportions. Ethyl acetate extract is spotted by sealed capillary on the TLC plate the diameter of the spot is about 3 mm. These TLC plates were developed chromatography developing chamber, with saturated mobile phase. Mobile phase n-hexane: Acetone (9:1) is selected separation.

Marker Drug:

The marker β-sitosterol was obtained from Central store of Appsaheb Birnale College of Pharmacy, Sangli.

Spectral Analysis:

The isolated compound standard β-sitosterol was analyzed by using UV-visible spectrophotometer. The λ max of standard and isolated compound from the ethyl acetate extract was compared.

FTIR spectra

The isolated compound from the ethyl acetate extract was used to obtain the IR spectra. The fraction is dried in the desiccators. The dried compound is triturated with potassium bromide and the thin palates were prepared. The palate is placed in between IR beam. And IR spectrum is obtained.

Mass spectra

The isolated dried compound from the ethyl acetate extract was dried and subjected to mass analysis.

MOLECULAR DOCKING

Receptor:

A three-dimensional structure of receptor was taken from the Protein Data Bank PDB ID: 1REV.

Structure:

The structure of isolated compound β-sitosterol is used for docking. The structure is drawn by King draw application and converted into Dot mol.

Procedure:

1. The receptor protein was downloaded from the protein data bank in pdb format
2. The downloaded receptor protein was opened in the software by selecting the pdb file from the file menu.
3. From the above missing residue the loop was inserted by missing residue treatment from lower similarity score PRO1A to higher similarity score THR450A.
4. The unwanted chains were deleted by biopredict tools select the unwanted chains and delete.
5. In the Biopredict tools clicked the analyze option and grip docking.
6. Noted the binding energy and minimum docking score.

RESULT AND DISCUSSION:

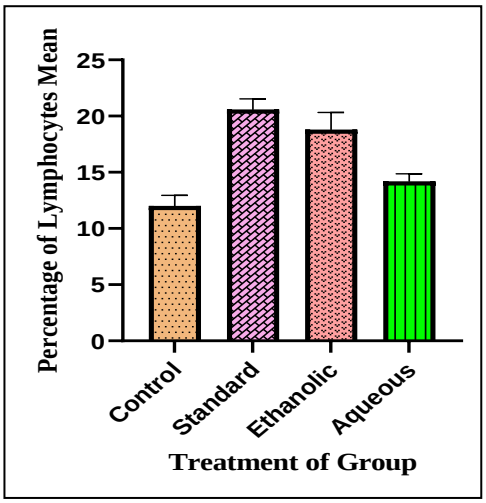
Immunomodulation activity

T cell Population Test

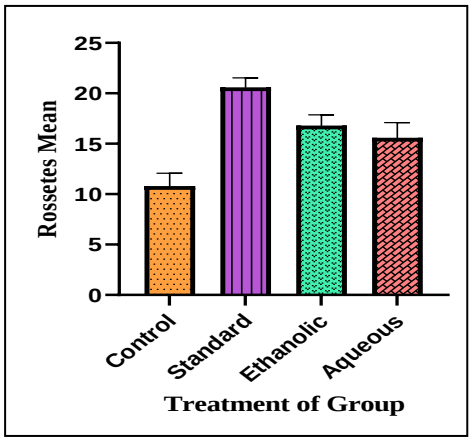
The Ethanolic extract increased Absolute lymphocyte percentage 18.8 ± 0.9487 and No of Rosette count 16.8 ± 1.068. Aqueous extract showed increased Absolute lymphocyte percentage was 16.4 ± 0.9274 andNo of Rosette count was 15.6 ± 1.503. P value obtained is <0.0001.Ethanolic and aqueous extract showed immunostimulant activity. The ethanolic extract was found to be more potent as compared aqueous extract. The, absolute lymphocyte, percentage, and no of rosettes were high in ethanolic extract as compared to control aqueous extract.

Sr. no	Group	Lymphocyte percentage Mean	No of Rosette count Mean
1.	Control	12 ± 0.613	10.8 ± 1.281
2.	Standard	20.6 ± 1.53	20.6 ± 0.9247
3.	Ethanolic	18.8 ± 0.9487	16.8 ± 1.068
4.	Aqueous	16.4 ± 0.9274	15.6 ± 1.503

Result of T cell Population



Graphical representation of Percentage Lymphocyte

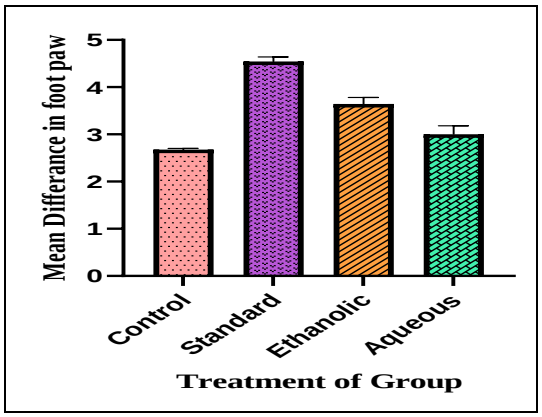


Delayed type of Hypersensivity test:

The Ethanolic extract increased foot paw edema 3.644 ± 0.1367. Aqueous extract showed increased. Aqueous extract increased foot paw edema 2.964 ± 0.1283. P value obtained is <0.0001. Ethanolic and aqueous extract showed immunostimulant activity. The ethanolic extract was found to be more potent as compared aqueous extract. The, foot paw edema was high in ethanolic extract as compared to control aqueous extract.

Sr. no	Group	Mean difference paw edema
1.	Control	2.678 ± 0.1175
2.	Standard	4.544 ± 0.1396
3.	Ethanolic	3.644 ± 0.1367
4.	Aqueous	2.964 ± 0.1283

Result of DTH



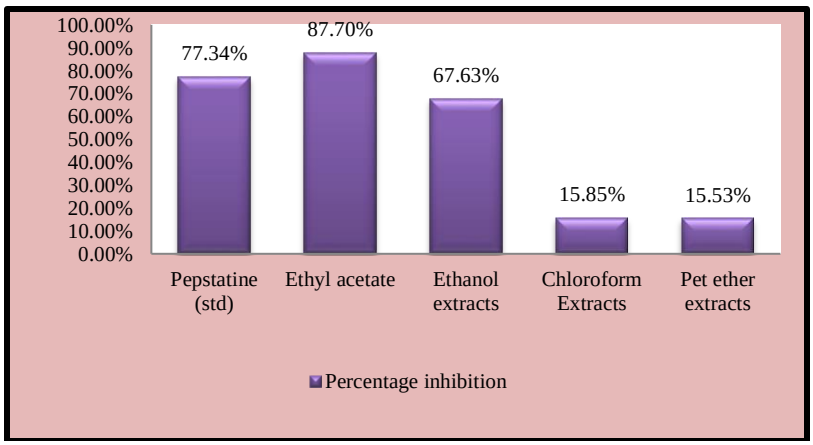
Graphical representation of DTH

Antiviral activity

Anti HIV property of petroleum ether, chloroform, ethyl acetate and ethanol extracts , among all four extract ethyl acetate extract were more potent at conc. 100 µl/ml and have shown inhibitory activity of 87.70 % is higher as compared to standard pepstatine drug.

Sr. no.	Compound	Concentration	Reading	Percentage inhibition
1.	Control	water	0.309	00.00
2.	Pepstatine (std)	100 µg/ml	0.070	77.34 %
3.	Ethyl acetate extracts	100 µg/ml	0.038	**87.70 %**
4.	Ethanol extracts	100 µl/ml	0.100	67.63 %
5.	Chloroform Extracts	100 µl/ml	0.260	15.85 %
6.	Pet ether extracts	100 µg/ml	0.261	15.53 %

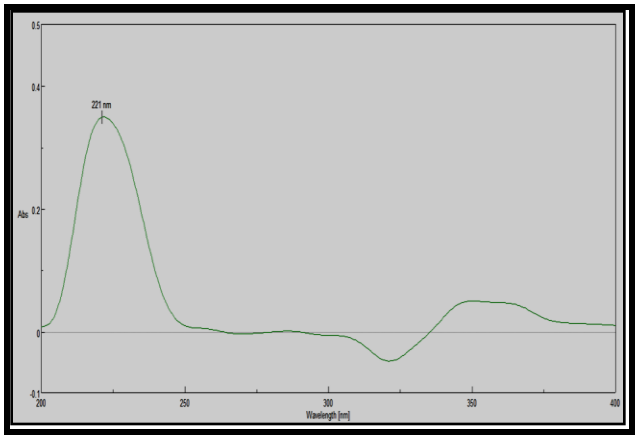
Result of Antiviral Activity



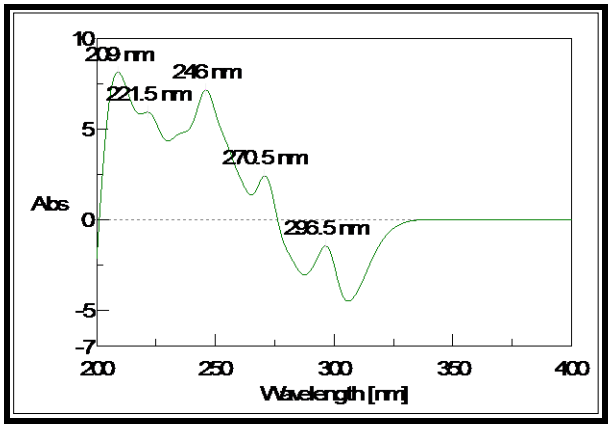
Graphical representation of Antiviral Activity

UV Spectra:

The λ max of standard β sitosterol at 221 nm. There were five peak found in the UV graph. Among them one peak is of 221.5 nm which is perfectly matched with the λ max of standard β sitosterol.



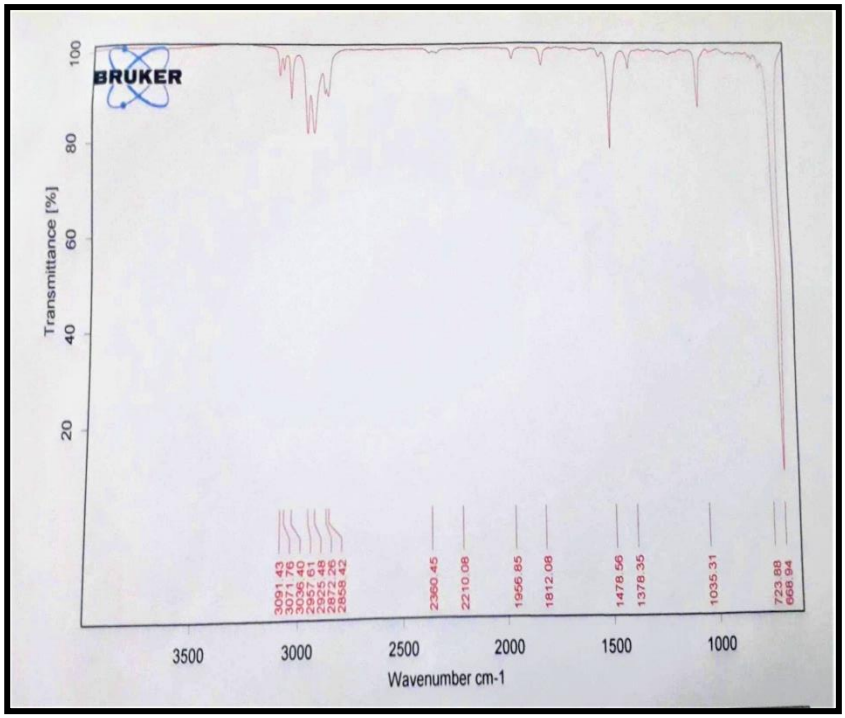
λ max of standard β sitosterol



λ max of sample

IR spectra

Functional group the component isolated from the ethyl acetate extract and β sitosterol are approximately same



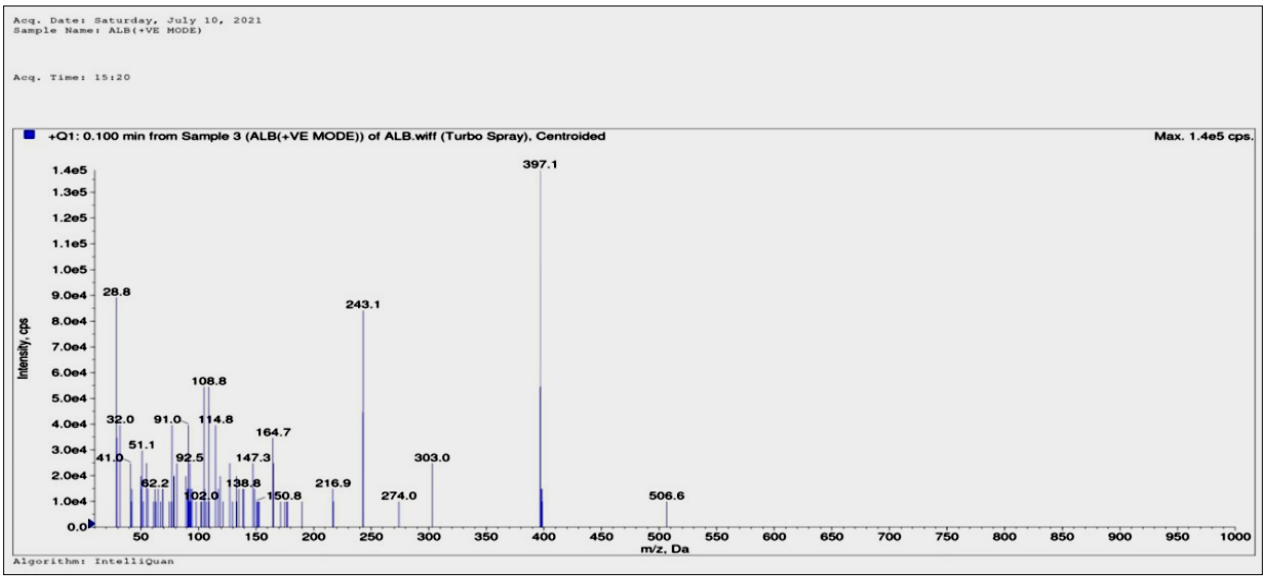
IR spectra of extract

Sr. No	Peak value (cm^{-1})	Functional group	Assignment
1.	3091.43 - 3036.40	Aromatic ring	C-H bend (m)Aromatic ring Confirmed
2.	2957.61 - 2925.48	Alkanes	C-H stretch (m)
3.	2872.26 - 2858.42	Alkanes	C-H stretch (m)
5.	2360.45 - 2210.08	Alkanes	C-H stretch (m)
6.	1956.85 - 1812.08	Alkanes	C-H stretch (m)
7.	1478.56	Aromatic carbon	Aromatic C=C stretch(s)
8.	1378.35	Alkanes	C-H_3 bend (m)
9.	1035.31	Primary alcohol	O-H bend (m)
10.	723.88 - 668.94	Long chain alkane	C-H bend (s)

Result of IR spectra

Mass Spectra

The fragmentation pattern of ethyl extract shows m/z 397 has molecular formula $C_{28}H_{47}O$ Molecular weight of 399 which is nearby to the Molecular weight of β sitosterol 414 ($C_{29}H_{50}O$).Thus β sitosterol may be present in the isolated ethyl acetate extract.



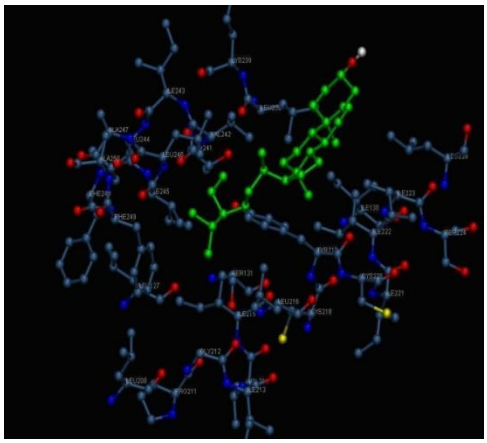
Mass spectra of extract

MOLECULAR DOCKING

β sitosterol structure showed binding tor the 1REV receptor with a dock score of -5.203429.This indicates that by molecular docking ,anti HIV activity is due to β sitosterol.

Hydrophobic interaction:

C28 [3.56]- ILE215 (CG2)
C28 [3.90]- LEU216 (CD2)
C13 [3.15]- TYR219 (CB, CG)
C20 [3.30]- LEU238(CD2)
C29 [3.48]- VAL242 (CB,CG1)
C9 [3.73]- VAL242(CG2)
C24[3.01]- ILE245 (CB,CG2)



The binding of β sitosterol to receptor 1REV

CONCLUSION:

Ethanolic extract showed maximum immunomodulatory activity by both in-vivo and in-vitro method. Ethyl acetate extract showed maximum anti HIV activity (Enzyme inhibition assay) as compared to all other extracts. The terpenoids were isolated from ethyl acetate extract by TLC and β sitosterol was isolated from ethyl acetate extract and its presence was confirmed by performing spectroscopic analysis i. e UV, IR and Mass. It was confirmed by molecular docking that anti HIV activity is due to β sitosterol.

ACKNOWLEDGEMENTS:

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