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QSAR, docking and *in vivo* studies for immunomodulatory activity of isolated triterpenoids from *Eucalyptus tereticornis* and *Gentiana kurroo*

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ABSTRACT

Two triterpenoids ursolic acid (**1**) and lupeol (**2**) isolated and characterized from *Eucalyptus tereticornis* and *Gentiana kurroo* were subjected to *in silico* QSAR modeling and docking studies and later the predicted results were confirmed through *in vivo* experiments. QSAR modeling results showed that both the triterpenoids possess immunomodulatory and anti-inflammatory activity comparable to boswellic and cichoric acids, but were less active than levamisol. Docking results suggested that both the triterpenoids (**1** and **2**) showed immune modulatory and anti-inflammatory activity due to high binding affinity to human receptors *viz*., NF-kappaB p52 (−50.549 kcal/mol), tumor necrosis factor (TNF-alpha) (−47.632 kcal/mol), nuclear factor NF-Kappa-B P50 (−16.798 kcal/mol) and cyclooxygenase-2 (−55.244 kcal/mol). Further both the triterpenoids (**1** and **2**) were subjected to *in vivo* immunomodulatory activity in female Swiss albino mice. The experimental mice were divided into nine groups, each comprised of six mice. These received oral treatment for a period of 28 days. The triterpenoids (**1** and **2**) showed significant increased in humoral immune function, but no significant changes were observed in cell mediated immune response and hematological parameters. The *in silico* and *in vivo* experimental data suggested that both the triterpenoids **1** and **2** may be considered as potential immunomodulatory drug-like molecules.

1. Introduction

A substance which modulates, alters or helps in regulating the immune system of an organism is called immunomodulator. There are two types of immunomodulators; the one which enhance the immune response are called immunostimulants and those which suppress the immune response are called the immunosuppressants. Apart from the above there are certain agents which normalize the overactive or underactive immune system. Immunomodulators are most often used in autoimmune diseases such as rheumatoid arthritis and in organ transplantation to prevent rejection of the new organ. A number of plants which are used in traditional systems of medicine for vitality and against long enduring diseases have shown to alter the immune system (Fedson, 2009; Chandrashekar et al., 2011; Ragupathi et al., 2008; Ross et al., 2001; Zhao et al., 2001). Since people have started realizing the importance of a healthy immune system, immunomodulators are becoming famous in natural health care worldwide and modulation of immune system by therapeutic agents is emerging as a major area in pharmacology (Geetha et al., 2005). Hence, there is growing interest among the scientific community to isolate and characterize novel natural immunomodulators for the use in human health care.

Triterpenoids, a large class of diverse and ubiquitous group of C_{30} pentacyclic compounds (Connolly and Hill, 2002), biosynthetically derived from squalene cyclization (Prestwich et al., 1999) are mostly present in higher plants. They are stored as glycosides in various parts of the plants and are regarded as an important and promising source of medicinal compounds (Matsuo et al., 2009). There are a large number of plants and their isolated constituents which potentiate immune response (Chiang et al., 2003). Recently two triterpenoids isolated from *Luffa cylindrica* have shown dose related immunostimulatory effect on *in vivo* immune functions in mice (Khajuria et al., 2007). This prompted us to investigate Indian medicinal plants for immunomodulatory agents using *in silico* and *in vivo* studies. Traditionally the process of drug development has revolved around a screening approach and trial-and-error method.

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This discovery process was very time consuming and laborious and discovery of a new drug used to take around 8–14 years and costs about US $1.8 billion. In order to minimize the time and cost in this drug discovery process, scientists around the world contributed tremendously and come up with a modern drug-designing program. The beauty of this modern drug designing is that now we can tailor the drug with desired combinations computationally before going for experimental laboratory work (Khan et al., 2011).

Eucalyptus hybrid (Myrtaceae, Mysore Gum) extensively grown in various states of India is pure *Eucalyptus tereticornis* Smith (up to say 99%) (Qureshi, 1966). It is a valuable quick growing species used in large scale for afforestation programmes throughout the country. It is mainly used as raw material for pulp in wood industry while the leaves are a good source of essential oil. Cineol is the major constituent (Rao et al., 1970) of its essential oil and used as an ingredient in traditional medicines.

Gentiana calycosa Griseb. (Gentianaceae) is true gentian (http://www.ubcbotanicalgarden.org/potd/2010/02/gentiana_calycosa.php) and is commonly known as mountain bog gentian, while Indian gentian, *Gentiana kurroo* Royle is commonly known as kuru or "Kurtki" and occurs as a perennial herb in the Himalayan region of India at an altitude of 1500–3300 m. The dried rhizomes and roots of this plant species are used as a substitute for the true gentian. In India, it has been used medicinally as a bitter to stimulate gastric secretion, to cure debility and in case of fever and urinary complaints. It is also useful in syphilis and leucoderma (Sharma et al., 1993). Fresh roots and rhizomes are the source of the glycosides-gentiopicrine, gentiain and the alkaloid gentianin (Sharma et al., 1993). The compounds from the gentians were found to be anthelmintic, anti-inflammatory, antiseptic and febrifuge. It is taken internally in the treatment of liver complaints, indigestion, gastric infection, etc. This species is one among the several being used as a source of medicinal gentian roots (Uphof, 1959; Usher, 1974).

In the present communication, immunomodulatory and anti-inflammatory drug-like potential of the two triterpenoids **1** and **2** have been discussed on the basis of their *in silico* and *in vivo* activity studies.

2. Materials and methods

2.1. Chemicals and instruments

All the solvent, chemicals and silica gel used for the isolation of triterpenoids were purchased fem E. Merck India, The Vacuum Liquid Chromatography (VLC, G1,) was purchased from Vensil, India. ^{1}H, ^{13}C and DEPT-135 NMR performed on a Bruker 300 MHz instrument (^{1}H 300 MHz; ^{13}C 75.5 MHz). Chemical shifts (δ) are reported in ppm relative to the residual solvent (internal standard) signals (C_5D_5N δH 7.19, 7.55, 8.71 ppm and δC 123.5, 135.5, 149.9 ppm). ESI-MS experiments were performed on a LCMS-2010V (Shimadzu, Kyoto, Japan) simultaneously in positive (detector voltage 1.6 KV) ionization under scan mode.

2.2. Plant material

The leaves of *E. tereticornis* were collected from the medicinal farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh, India during the month of January, 2008 and a voucher specimen No. 12510 has been deposited at the Botany Division of the institute. The roots of *G. kurroo* Royle were supplied by the Department of Forest Products, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan Himanchal Pradesh, India in the month of January 2006 where a voucher specimen no. 2023 is deposited in the Botany Division of the Y.S. Parmar University.

2.3. Extraction and isolation of triterpenoid 1

The leaves of *E. tereticornis* were air dried under shade, powdered (1.5 kg) and defatted with hexane (4 × 6 l, 24 h each) at room temperature, which yielded hexane extract (4 g). The defatted plant material was later extracted with methanol (4 × 5 l). The combined methanol extract was subjected for complete solvent removal at 40 °C under vacuum. The methanolic extract so obtained was dissolved in distilled water (2 l) and successively extracted with hexane (4 × 400 ml) and ethyl acetate (4 × 400 ml). The combined hexane and ethyl acetate extracts were separately washed with water, dried over anhydrous Na_2SO_4 and subjected under vacuum distillation at 40 °C to yield hexane (2 g) and ethyl acetate extracts (34 g) respectively. Further ethyl acetate extract (34 g) was resolved over Vacuum Liquid Chromatography (VLC, G1, Silica gel-H, 260 g). Gradient elution of VLC column was carried out with solvents of increasing polarity viz. hexane, chloroform, methanol in various proportions. A total of 110 fractions were collected and pooled on the basis of their TLC profiles visualized with vanillin-sulphuric acid. The fractions 3–42 (7.1 g) eluted with Hexane: $CHCl_3$ (1:1) to $CHCl_3$: MeOH (99:1) were further subjected to VLC (G1, Silica gel-H, 15 g). A total of 285 fractions were collected. The fractions 175–182 (1.5 g) eluted with chloroform (100%), afforded homogeneous triterpenoid **1** (300 mg).

Triterpenoid 1 (Ursolic acid 8) 300 mg, mp. 291–292 °C, ^{1}H NMR (300 MHz, C_5D_5N) δ 0.77, 0.78, 0.98, 1.09, 1.14 (3H each, all s, 5 × Me) 0.92 and 0.96 (3H each, each d, J = 6.4 and 7.3 Hz, 25 × Me), 2.82 (1H, d, J = 9.9 Hz, H-18 β), 3.20 (1H, dd, J = 6.8 and 8.7 Hz, H-3α) 5.28 (1H, m, H-12); ^{13}CNMR (75 MHz, C_5D_5N) δ C-1(39.1_t), C-2 (28.5_t), C-3 (78.8_d), C-4 (39.8_s), C-5 (56.4_d), C-6 (19.3_t), C-7 (34.1_t), C-8 (39.7_s), C-9 (48.5_d), C-10 (39.7_s), C-11 (24.4_t), 12 (126.1_d), C-13 (139.7_s), C-14 (43.0_s), C-15 (29.1_t), C-16 (25.4_t), C-17 (48.6_s), C-18 (54.1_d), C-19 (40.0_d) C-20 (39.0_d), C-21 (31.6_t), C-22 (37.1_t), C-23 (29.2_q), C-24 (16.1_q), C-25 (16.8_q), C-26 (17.9_q), C-27 (24.1_q), C-28 (180.0_s), C-29 (17.9_q), C-30 (21.8_q), ESI-MS: m/z 457 $[M + H]^+$.

2.4. Extraction and isolation of triterpenoid 2

The air dried roots of *G. kurroo* (400 g) were crushed, powdered and extracted six times over night with methanol (65 × 2 l, 24 h each) at room temperature. The combined methanol extract was completely dried under vacuum to yield MeOH extract (95 g), which was dissolved in distilled water (2 l) and successively extracted with petroleum ether, benzene, and chloroform. The TLC profile of petroleum ether (29 g), benzene (3 g) and chloroform (2 g) extracts was more or less same, hence they were pooled together. Purification of this pooled extract (34 g) was carried out on silica gel (SiO_2 60–120 mesh) column. Gradient elution of the column was carried out with solvents of increasing polarity viz. petroleum ether, chloroform, methanol in various proportions. A total of 1110 fractions were collected and pooled on the basis of their TLC profile. The fractions 239–296 (2 g) eluted with $CHCl_3$: MeOH (98:2) were further subjected to flash chromatography (SiO_2, 43 g, 230–400 mesh). The flash chromatographic fractions 40–58 eluted with hexane: chloroform (80:20) were further purified by preparative TLC [hexane: dichloromethane: acetone (50:49:1)], which afforded a homogeneous triterpenoid **2** [127 mg; Hexane: DCM: Me_2CO (50:50:2)], visualized with spraying reagent vanillin-sulfuric acid.

Triterpenoid 2 (Lupeol 7) 127 mg, mp. 213–215 °C, ^{1}H NMR (300 MHz, C_5D_5N) δ 0.25, 0.82, 0.78 (each 3H, s, H-25, H-28,

H-24), 0.97 (3H, s, H-23), 0.98 (3H, s, H-27), 1.67 (3H, s, H-30), 3.16 (1H, dd, J = 4.7 Hz, H-3), 4.69 (1H, s, H-29b), 4.57 (1H, s, H-29a); ^{13}CNMR (75 MHz, C_5D_5N) δ C-1 (39.2_t), C-2 (27.6_t), C-3 (78.6_d), C-4 (38.8_s), C-5 (55.8_d), C-6 (18.2_t), C-7 (34.4_t), C-8 (41.2_s), C-9 (50.5_d), C-10 (37.9_s), C-11 (21.6_t), C-12 (25.7_d), C-13 (38.7_d), C-14 (42.9_s), C-15 (27.3_t), C-16 (36.1_t), C-17 (43.5_s), C-18 (49.2_d), C-19 (48.4_d) C-20 (151.3_s), C-21 (30.0_t), C-22 (40.2_t), C-23 (27.8_q), C-24 (15.8_q), C-25 (16.8_q), C-26 (16.2_q), C-27 (15.1_q), C-28 (18.3_s), C-29 (110_t), C-30 (19.3_q), ESI-MS: m/z 427 $[M+H]^+$.

2.5. In silico molecular docking studies

The 3D chemical structures of drugs/compounds *viz*., aristolochic acid, azimexon, boswellic acid, ciamexon, cichoric acid, emetin, imemixon, isopteropodin, levamisol, lupeol, ursolic acid and oleanolic acid were retrieved through the PubChem-compound and PubChem-substance database at NCBI webserver, USA (www.pubchem.ncbi.nlm.nih.gov). The chemical structures of standard drugs and lupeol, ursolic and oleanolic acid were cleaned, energy optimized and docked to receptors through Scigress Explorer v7.7.0.47 software (Fujitsu Ltd., Japan). The optimization of the cleaned molecules was done through MO-G computational application that computes and minimizes an energy related to the heat of formation. The MO-G computational application solves the Schrodinger equation for the best molecular orbital and geometry of the ligand molecules. The augmented Molecular Mechanics (MM2/MM3) parameter was used for optimizing the molecules up to its lowest stable energy state. This energy minimization was done until the energy change was less than 0.001 kcal/mol or else the molecules get updated almost 300 times. Crystallographic 3D structures of human target proteins were retrieved through Brookhaven protein databank (www.pdb.org). The valency and hydrogen bonding of the ligands as well as target proteins were subsequently satisfied through the Workspace module of Scigress Explorer (Meena et al., 2011; Yadav et al., 2010). Hydrogen atoms were added to protein targets for correct ionization and tautomeric states of amino acid residues such as His, Asp, Ser and Glu. Molecular docking of the drugs and the isolated compounds with immuno-modulatory receptors was done through Fast-Dock-Manager and Fast-Dock-Compute engines available with the Project-leader module of Scigress Explorer. For the automated blind docking of ligands into the active sites a Genetic algorithm with a fast and simplified Potential of Mean Force (PMF) scoring scheme was used (Muegge, 2000; Muegge and Martin, 1999). PMF uses atom types which are similar to the empirical force-field's used in Mechanics and Dynamics. The blind docking method was used which allowed the terpenoids to bind anywhere in the surface unbiased or the interior of the target protein under standard docking parameters. A minimization was performed by the FastDock engine, which uses a Lamarkian Genetic algorithm so that individuals adapt to the surrounding environment. The best fits were retained by analyzing the PMF scores of all chromosomes and were given more reproductive opportunities to those with lower scores. This process was repeated for 3000 generations with 500 individuals and 100,000 energy evaluations. The Genetic algorithm parameters used during docking studies were- population size 50, crossover rate 0.8, elitism 5, maximum generation 3000, mutation rate 0.2, convergence (kcal) 1.0, while the active site bounding box parameters were X coordinate 15.0 Å, Y coordinate 15.0 Å and Z coordinate 15.0 Å. Under united atom parameter the grid spacing was set to 0.3 Å and local search parameters include 300 maximum iterations and 0.06 rate. Other parameters were left as their default values. Structure based screening involves docking of candidate ligands into protein targets, followed by applying a PMF scoring function to estimate the likelihood that the ligand will bind to the protein with high affinity or not (Dilber et al., 2008).

2.5.1. Selection of chemical descriptors for QSAR modeling

QSAR analysis is a mathematical procedure by which chemical structures of molecules are quantitatively correlated with a well defined parameter, such as biological activity or chemical reactivity. A QSAR model attempts to find consistent relationships between the variations in the values of molecular properties and the biological activity for a series of compounds which can be then used to evaluate properties of new chemical entities (Lipinski et al., 2001; Yoshida and Topliss, 2000). Before the novel compound is used as potential drugs, the prediction of toxicity/activity ensures the calculation of risk factor associated with the administration of that particular drug. To identify the immunomodulatory activity of triterpenoids **1** and **2**, QSAR study was performed. A total of 52 chemical descriptors were used for model development. Some of the important chemical descriptors used were: Atom Count (all atoms), Atom Count (carbon), Atom Count (hydrogen), Atom Count (oxygen), Bond Count (all bonds), Conformation Minimum Energy (kcal/mole), Connectivity Index (order 0, standard), Connectivity Index (order 1, standard), Connectivity Index (order 2, standard), Dipole Moment (debye), Dipole Vector X (debye), Dipole Vector Y (debye), Dipole Vector Z (debye), Electron Affinity (eV), Dielectric Energy (kcal/mole), Steric Energy (kcal/mole), Total Energy (Hartree), Group Count (amine), Group Count (carboxyl), Group Count (ether), Group Count (hydroxyl), Group Count (methyl), Heat of Formation (kcal/mole), HOMO Energy (eV), Ionization Potential (eV), Lambda Max UV–Visible (nm), Lambda Max far-UV–Visible (nm), Log$P$, LUMO Energy (eV), Molar Refractivity, Molecular Weight, Polarizability, Ring Count (all rings), Size of Smallest Ring, Size of Largest Ring, Solvent Accessibility Surface Area (Å^2). Effective dose (ED_{50}) was considered as the biological activity parameter of compounds. Multiple linear regression (MLR) mathematical expression was then used to predict the biological response of lupeol (**7**, triterpenoid **2**), ursolic acid (**8**, triterpenoid **1**) and oleanolic acid (9).

2.5.2. Parameters for QSAR model development

Initially, a total of 51 immunomodulatory and anti-inflammatory compounds/drugs with reported activities were used as training data set (see suppl. file 3), while developing the QSAR model. The antimalarial activity was in inhibitory concentration (IC_{50} in nM) form. Total 52 chemical descriptors (physico-chemical properties) were calculated for each compound (see supp file 4). Various descriptors like electronic, steric, and thermodynamic were calculated by the Scigress Explorer software (Fujitsu, Poland). Compounds selection was made on the basis of structural similarity, to ensure diverse training data set rather than same family. Similarly, when selecting the best subset of chemical descriptors, highly correlated descriptors were excluded. Finally, model was developed based on forward stepwise multiple linear regression method.

2.5.3. Statistical calculations used in QSAR modeling

2.5.3.1. Selecting a statistical method: stepwise multiple linear regression. The stepwise multiple linear regression method calculates QSAR equations by adding one variable at a time and testing each addition for significance. Only variables that are found to be significant are used in the QSAR equation. This regression method is especially useful when the number of variables is large and when the key descriptors are not known. In the forward mode, the calculation begins with no variables and builds a model by entering one variable at a time into the equation. In backward mode, the calculation begins with all variables included and drops variables one at a time until the calculation is complete; however, backward regression calculations can lead to over fitting.

2.5.3.2. Multiple correlation coefficient (r). Variation in the data is quantified by the correlation coefficient (r), which measures how closely the observed data tracks the fitted regression line. This is a measure of how well the equation fits the data (i.e., it measures how good the correlation is). A perfect relation has $r = +1$ (positively correlated) or -1 (negatively correlated); no correlation has $r = 0$. The regression coefficient (r^2) is sometimes quoted, and this gives the fraction of the variance (in %) that is explained by the regression line. The more scattered the data points, the lower the value of r. A satisfactory explanation of the data is usually indicated by an r^2 of at least 0.9; compare $r = 0.9$ ($r^2 = 0.81$; 81% of the variance is explained) with $r = 0.7$ ($r^2 = 0.49$; 49% of the variance is explained; 51% is unexplained). Errors in either the model or in the data will lead to a bad fit. This indicator of fit to the regression line is calculated as:

$$r^2 = (\text{sum of the squares of the deviations from the regression line})/(\text{sum of the squares of the deviations from the mean}) \quad (1)$$

$$r^2 = (\text{regression variance})/(\text{original variance}) \quad (2)$$

where the regression variance is defined as the original variance minus the variance around the regression line. The original variance is the sum of the squares of the distances of the original data from the mean.

2.5.4. Validation of QSAR model

QSAR model validated to test the internal stability and predictive ability by the internal, external validation and randomization test procedure:

2.5.4.1. Internal validation. Internal validation was carried out using leave-one-out (LOO) method. For calculating cross validation regression coefficient (r^2_{cv}), each molecule in the training set was eliminated once and the activity of the eliminated molecule was predicted by using the model developed by the remaining molecules. The cross validation regression coefficient (r^2_{cv}) was calculated using the equation which describes the internal stability of a model (Khan et al., 2011).

$$r^2 = 1 - \frac{\sum(Ypred - Y)^2}{\sum(Y - \ddot{Y})^2}$$

where r^2_{cv} refers cross validation regression coefficient, $Y_{\text{experimental}}$ and Y_{pred} activity of the molecule in the training set, respectively, and $\ddot{Y}$ is the average activity of all molecules in the training set.

2.5.4.2. External validation. For external validation, the activity of each molecule in the test set was predicted using the model developed by the training set. The regression coefficient (r^2) value is calculated as follows.

$$r^2_{cv} = 1 - \frac{\sum(Y_{\text{pred(test)}} - Y_{\text{(test)}})^2}{\sum(Y_{\text{(test)}} - \ddot{Y}_{\text{(training)}})^2}$$

where r^2 refers regression coefficient, $Y_{\text{experimental}}$ and Y_{pred} are activity of the molecule in the training set, respectively, and $\ddot{Y}_{\text{training}}$ is the average activity of all molecules in the training set. Both summations are over all molecules in the test set. Thus, the regression coefficient (r^2) value is indicative of the predictive power of the current model for external test set. For this we have used only eight compounds for test. Generally, a QSAR model was considered to have a high predictive power only if the r^2_{cv} was >0.6 for the test set (Zhang et al., 2007; Zheng and Tropsha, 2000) (Eq. (1)).

2.5.4.3. Randomization test. To evaluate the statistical significance of the QSAR model for an actual dataset, one tail hypothesis testing was used (Yadav et al., 2010; Yoshida and Topliss, 2000). The robustness of the models for training sets was examined by comparing these models to those derived for random datasets. Random sets were generated by rearranging the activities of the molecules in the training set. The statistical model was derived using various randomly rearranged activities (random sets) with the selected descriptors and the corresponding q^2 were calculated in Eq. (2). The significance of the models hence obtained was derived based on a calculated Z_{score} (Zheng and Tropsha, 2000).

A Z_{score} value is calculated by the following formula:

$$Z_{score} = \frac{(h - \mu)}{\sigma}$$

where h is the q^2 value calculated for the experimental dataset, μ the average q^2, and σ is its standard deviation calculated for various iterations using models build by different random datasets. The probability (α) of significance of randomization test is derived by comparing Z_{score} value with Z_{score} critical value as reported in (Zheng and Tropsha, 2000) if Z_{score} value is less than 4.0; otherwise it is calculated by the formula as given in the literature. For example, a Z_{score} value greater than 3.10 indicates that there is a probability (α) of less than 0.001 that the QSAR model constructed for the real dataset is random. The randomization test suggests that all the developed models have a probability of less than 1% that the model is generated by chance. The resulting QSAR model exhibited a high regression coefficient.

2.5.5. In silico druggability test

For analyzing druggability, Lipinski's rule of five pharmacokinetics filter was used as drug-likeness test (Lipinski et al., 2001). Briefly, this rule is based on the observation that most orally administered drugs have a molecular weight (MW) of 500 or less, a Log P no higher than 5, five or fewer hydrogen bond donor sites and 10 or fewer hydrogen bond acceptor sites (N and O atoms). In addition, the bioavailability of all drugs or test compounds was assessed through topological polar surface area analysis. The polar surface area (PSA) was calculated by using method, termed topological PSA (TPSA), based on the summation of tabulated surface contributions of polar fragments (ChemAxon-Marvin-View 5.2.6 software by PSA plugin (Norinder et al., 1999). Polar surface area (PSA) is formed by polar atoms of a molecule. This descriptor was shown to correlate well with passive molecular transport through membranes and therefore, allows prediction of transport properties of drugs and has been linked to drug bioavailability. Generally, it has been seen that passively absorbed molecules with a PSA > 140 Å^2 are thought to have low oral bioavailability (Ertl et al., 2000).

2.6. In vivo immunomodulatory study

Animal study was undertaken in accordance with the "Committee for the Purpose of Control and Supervision of Experimentation on Animals" (CPCSEA) guidelines and approved experimental protocol by Institutional Animal Ethics Committee. Female Swiss albino mice of 18–23 g body weight were obtained from the laboratory animal house of Central Institute of Medicinal and Aromatic Plants, Lucknow India. These animals were maintained at a room temperature of 22 ± 1 °C with 50–70% relative humidity and cycles of 12:12 h of light and dark with *ad libitum* food and water. Animals were divided into nine groups each comprising of six animals. The first groups served as normal vehicle control, fed with distilled water, second to fourth groups were fed with the triterpenoid **1**; fifth to seventh group were fed with the triterpenoid **2** at doses of 1, 3 and 10 mg/kg body weight. Eighth group was served as a positive control, which was given levamisole hydrochloride (Sigma–Aldrich, USA) as a immunostimulatory agent at a dose of 0.68 mg/kg body weight. These mice were fed orally with test compounds using intragastric cannulae for the period of 28 days. Ninth group served as a negative

control, which was given cyclophosphamide monohydrate (Sigma–Aldrich, USA) as an immunosuppressive agent at 200 mg/kg body weight. Cyclophosphamide monohydrate was administered 3 days prior to antigen stimulation as a single dose.

The parameters related to the humoral, cell mediated immune response along with the hematological parameters and body weight variation was performed as follows:

2.6.1. Humoral immune response

Rabbit red blood corpuscles (rRBCs) isolated from New Zealand white rabbits were used as antigen. Blood was collected from the central artery of the ear and mixed with heparin as an anticoagulant. It was immediately centrifuged at 2000 rpm at 4 °C for 10 min and the supernatant containing the plasma was discarded. The pellets containing the rRBCs were resuspended in an equal volume of Alsever's solution of the following composition: dextrose (2.05 g), sodium citrate (0.80 g), and sodium chloride (0.42 g) in 100 ml distilled water, and centrifuged again after the discarding the supernatant. The process of washing was repeated three times before suspending the rRBCs in sterile normal saline to make it a 10% suspension. Two schedules of immunization were used. In the first, mice were intra-peritoneally injected with 200 μL (25×10^8 cell/ml) of rRBCs on the seventh day from the start of the experiment. A booster immunization was given 1 week later (day 14) and the animals were bled on 28th day to detect the presence of antibodies. About 0.5 ml blood was collected from the retro orbital plexus of the mice using hematocrit capillaries (HiMedia, India). The blood was allowed to clot at room temperature for 1 h and then kept at 4 °C for 60 min, followed by centrifugation at 2500 rpm for 10 min. The serum was collected and stored at −20 °C till further use. To quantify the antibodies, hemagglutination was performed. Briefly, this involved serial two-fold dilutions of serum samples in Alsever's solution, to which 100 μl of 10% rRBCs were added to 100 μL of the diluted test samples in U-bottom microtiter plates (Greiner, Germany). The plates were incubated for 1–2 h at 25 °C before rRBC setting patterns were read. The HA titer was expressed as the reciprocal of the highest dilution of the serum showing definite positive pattern (flat sediment or shield formation) as compared to the negative pattern (smooth dot in the center of the well). The respective antibody titer was expressed as the serial dilution of the serum per well, as per the method described (Zhao et al., 2001).

2.6.2. Delayed type hypersensitivity test (DTH)/footpad thickness test in mice

The mice were immunized in the same way as described for humoral immune response, but in addition, on day 28th all the mice were challenged with rRBCs (50 μL; 2×10^8 cells/ml) in the intraplantar region of the hind right paw. The differences in the footpad thickness of the two paws were measured 24 h later by using standard vernier calipers.

2.6.3. Body weight and hematological parameters

To observe the effect of triterpenoids **1** and **2** on the body weight variation and hematological parameters such as total RBCs, WBCs counts using hemocytometer (Rohem, India) and hemoglobin using Drabkin method were performed.

2.6.4. Statistical analysis

Data were statistically evaluated using one-way ANOVA, followed by Dunnet test using statistical software (Graph Pad prism-4). The values were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Isolation and characterization of triterpenoids

The dried leaves of *E. tereticornis* were powdered and defatted with hexane followed by extraction with methanol. Further, methanol extract was dissolved in distilled water and successively extracted with hexane and ethyl acetate. The usual work-up yielded 34 g of ethyl acetate extracts, which on chromatographic separation afforded a homogeneous triterpenoids (**1**). The triterpenoids (**1**) was characterized as ursolic acid on the basis of its ^{1}H, ^{13}CNMR (Seebacher et al., 2003), ESI-MS spectroscopic data and Co-TLC with an authentic sample. Similarly the air dried and powdered roots of *G. kurroo* were extracted with methanol. The methanol extract so obtained was dissolved in distilled water and successively extracted with petroleum ether, benzene, and chloroform. Due to similar TLC profile benzene and chloroform extracts were pooled together, which on chromatographic separation afforded a homogeneous triterpenoids (**2**). The triterpenoids (**2**) was characterized as lupeol on the basis of its ^{1}H, ^{13}CNMR (Imam et al., 2007), ESI-MS spectroscopic data and Co-TLC with an authentic sample.

3.2. Druglikeness test

Druglikeness is a qualitative concept used in drug design for how "druglike" a substance is with respect to factors like bioavailability. It is estimated from the molecular structure before the substance is even synthesized and tested. It can be estimated for any molecule, and does not evaluate the actual specific effect that the drug achieves (biological activity). A traditional method to evaluate druglikeness is to check compliance of Lipinski's Rule of Five (Lipinski et al., 2001) Lipinski's Rule of Five is a rule of thumb to evaluate druglikeness, or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion (ADME). However, the rule does not predict if a compound is pharmacologically active. The rule is

Table 1
Compounds following parameters of Lipinski's rule for drug likeness.

Compound	MW	Log*P*	Amine group count	Sec-amine group count	Hydroxyl group count	Nitrogen atom count	Oxygen atom count	Rule of five violations	MW > 500	Log*P* > 5	H-bond donors > 5	H-bond acceptors > 10	TPSA (Å^2)
Cyclophosphamide	261.088	0.782	0	1	0	2	2	0	0	0	0	0	41.6
Levamisole	204.289	3.259	0	0	0	2	0	0	0	0	0	0	15.6
Glycyrrhetinic acid	470.691	6.101	0	0	1	0	4	1	0	1	0	0	74.6
Ibuprofen	206.284	3.83	0	0	0	0	2	0	0	0	0	0	37.3
Indomethacin	357.793	3.969	0	0	0	1	4	0	0	0	0	0	68.5
Naproxen	230.263	2.989	0	0	0	0	3	0	0	0	0	0	46.5
Lupeol	426.724	8.028	0	0	1	0	1	1	0	1	0	0	20.2
Ursolic acid	456.707	7.214	0	0	1	0	3	1	0	1	0	0	57.5
Oleanolic acid	456.707	7.317	0	0	1	0	3	1	0	1	0	0	57.5

Note: MW: molecular weight, Log*P*: octanol-water partition coefficient. TPSA: topological polar surface area.

Table 2
Comparison of structure properties of compounds with immunomodulatory (1, 2) and anti-inflammatory (3–6) drugs.

Compound	Name/MF	AC	BC	CME (kcal/mole)	DM (debye)	EA (eV)	DE (kcal/mole)	SE (kcal/mole)	TE (Hartree)	HF (kcal/mole)	HOMO energy (eV)	IP (eV)	LUMO energy (eV)	MR	P	RC	SI1	SASA (A^2)
1	Cyclophosphamide $C_7H_{15}N_2O_2PCl_2$	29	29	35.223	5.93	0.329	−0.629	35.646	−123.949	−149.621	−9.645	9.647	−0.33	58.478	21.587	1	12.071	253.846
2	Levamisole $C_{11}H_{12}N_2S$	26	28	4.964	3.782	0.194	−0.432	4.951	−96.549	56.186	−9.107	9.106	−0.191	60.744	22.892	3	9.242	227.871
3	Glycyrrhetinic acid $C_{30}H_{46}O_4$	80	84	62.47	3.693	0.179	−0.642	62.444	−252.29	−226.394	−10.182	10.183	−0.179	134.361	54.753	5	25.641	429.75
4	Ibuprofen $C_{13}H_{18}O_2$	33	33	−17.473	2.15	−0.042	−0.326	−17.541	−110.323	−100.672	−9.564	9.565	0.039	60.732	24.365	1	13.067	255.281
5	Indomethacin $C_{19}H_{16}NO_4Cl$	41	43	−23.131	0.927	0.606	−0.57	−23.127	−187.003	−112.564	−8.697	8.697	−0.594	93.55	39.451	3	19.753	350.842
6	Naproxen $C_{14}H_{14}O_3$	31	32	−27.913	3.367	0.565	−0.44	−28.07	−124.703	−96.479	−8.698	8.698	−0.563	64.854	27.03	2	13.432	260.371
7	Lupeol $C_{30}H_{50}O$	81	85	73.62	1.266	−1.097	−0.363	73.62	−241.931	−183.706	−9.322	9.322	1.097	133.698	54.288	5	24.684	409.78
8	Ursolic acid $C_{30}H_{48}O_3$	81	85	101.526	1.745	−1.089	−0.147	101.526	−219.337	−90.574	−9.832	9.832	1.089	130.908	53.481	5	22.776	401.209
9	Oleanolic acid $C_{30}H_{48}O_3$	81	85	76.077	2.25	−1.068	−0.402	76.06	−241.926	−181.585	−9.367	9.367	1.064	133.624	54.267	5	24.684	417.806

Note: Molecular Formula (MF): the molecular formula of the molecule. Atom Count (AC): the number of atoms. Bond Count (BC): the number of bonds. Weak and ionic bonds are ignored. Conformation Minimum Energy (CME): energy calculated for an optimized conformation of the compound. Dipole moment (DM): the magnitude of the molecule's dipole. Electron Affinity (EA): the change in the total. energy of a molecule when an electron is added. Dielectric Energy (DE): the dielectric energy is a portion of the total energy of a molecule embedded in a dielectric. It is the stabilizing portion that results from screening the charges in the molecule by a dielectric. Steric Energy (SE): the steric energy of a molecule is the sum of the molecular mechanics potential energies calculated for the bonds, bond angles, dihedral angles, nonbonded atoms and so forth. It is specific to Mechanics and depends upon the force-field used. Total Energy (TE): the total energy is the work required to separate the electrons and nuclei infinitely far apart. Heat of Formation (HF): the energy released or used when a molecule is formed from elements in their standard states. HOMO energy: the energy required to remove an electron from the highest occupied molecular orbital. Ionization Potential (IP): the energy required to remove an electron from a molecule in its ground state. LUMO energy: the energy gained when an electron is added to the Lowest Unoccupied Molecular Orbital (LUMO). Molar Refractivity (MR): the molar refractivity of the compound. It should be in the range of 40–130 for drug likeness. Polarizability (P): the molecule's average alpha polarizability. Ring Count (RC): the number of rings present in the compound. Rings with more than 12 atoms are ignored. The number of rings with 12 or fewer atoms (All = all aromatic, small, 5-membered aromatic, 6-membered, 6-membered aromatic, large, large aromatic). Shape index order 1 (SI1): a topological index quantifying the shape of a chemical sample. The shape index of order 1 (Kappa 1) quantifies the number of cycles in the chemical sample. Solvent Accessible Surface Area (SASA): the molecular surface area accessible to a solvent molecule.

important for drug development where a pharmacologically active lead structure is optimized step-wise for increased activity and selectivity, as well as drug-like properties as described by Lipinski's rule. The modification of the molecular structure often leads to drugs with higher molecular weight, more rings, more rotatable bonds, and a higher lipophilicity. Over the past decade Lipinski's profiling tool for druglikeness has led to further investigations by scientists to extend profiling tools to lead-like properties of compounds in the hope that a better starting point in early discovery can save time and cost.

Results revealed that studied compounds follow drug-likeness except Log *P*, which correspond to compound solubility and hydrophobicity. In the context of pharmacokinetics, the partition coefficient has a strong influence on ADME properties (Absorption, Distribution, Metabolism, and Excretion) of the drug. Hence the hydrophobicity of a compound (as measured by its partition coefficient) is a major determinant of how drug-like it is. More specifically, in order for a drug to be orally absorbed, it normally must first pass through lipid bilayers in the intestinal epithelium (a process known as transcellular transport). For efficient transport, the drug must be hydrophobic enough to partition into the lipid bilayer, but not so hydrophobic, that once it is in the bilayer, it will not partition out again. Hydrophobicity plays a major role in determining where drugs are distributed within the body after adsorption and as a consequence how rapidly they are metabolized and excreted. Compound **7**, **8** and **9** showed a Log *P* value larger than five thus violating the Lipinski's rule of five, but since the number of violations is no more than one, thus acceptable. This feature of compounds **7**, **8** and **9** is similar to that of known anti-inflammatory drug glycyrrhetinic acid, therefore studied compounds with *in vivo* immunomodulatory activity considered as potential drug-like molecules (Tables 1 and 2, Fig. 1).

3.2.1. Development of QSAR model for immunomodulatory activity

Structure activity relationship has been represented by QSAR model showing significant relationship between immunomodulatory activity and chemical descriptors with 99% accuracy (r^2 = 0.99) and prediction accuracy of 96% ($r^2_{cv} = 0.96$). A total of 51 drugs were used for QSAR modeling against 52 chemical descriptors (Table 3). A forward feed MLR method was used for finding correlation between dependent and independent variables by using leave-one-out approach. For validation of QSAR model, test set compounds i.e., berberine, indomethacin, diethyl dithiocarbamate, methyl enosine monophasphate, gelsemin and plumbagin were tested and found predicted activity similar to experimental activity. Only three chemical descriptors found significant correlation and seems responsible for *in vivo* immunomodulatory activity (Table 3).

Anti-inflammatory and immunomodulatory drugs fit well into this correlation, which intuitively seems very reasonable. QSAR studies indicated that connectivity index (order 2), sec. amine group count and ether group count correlates well with activity (Tables 2 and 3). The QSAR mathematical model equation derived through MLR is given below, showing relationship between *in vivo* experimental activity (ED_{50}) and highly correlated chemical descriptors:

$$\begin{aligned}\text{Predicted Log } ED_{50}\ (\text{mg/kg}) = {} & +0.11890 \\ & \times (\text{Connectivity Index, order 2}) \\ & - 0.517676 \\ & \times (\text{sec. amine group count}) \\ & - 0.334085 \times (\text{Ether group count}) \\ & + 1.60503 \quad [r^2_{cv} \\ = {} & 0.845319 \quad \text{and} \quad r^2 = 0.97158]\end{aligned}$$

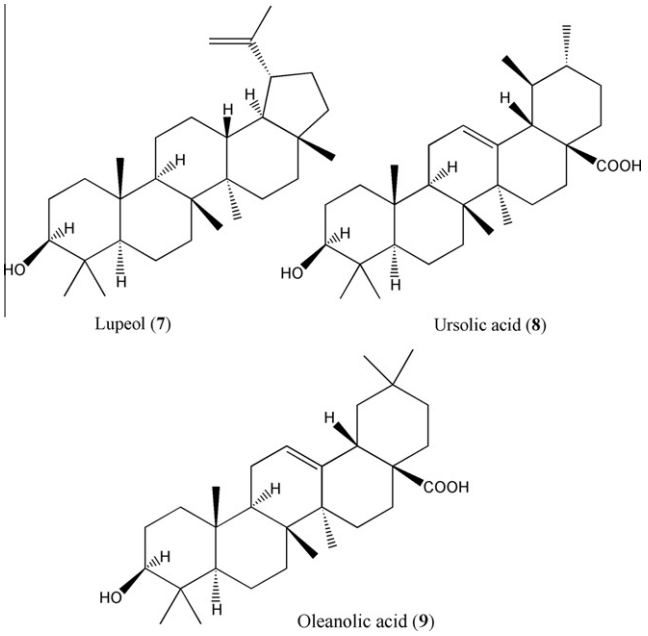

Fig. 1. Chemical structures of lupeol, ursolic acid and oleanolic acid.

Since experimental *in vivo* activity (ED_{50}) was reported for immunomodulatory drugs (Table 3), thus we aimed to predict the activity of test data set compounds i.e., lupeol (tritepenoid-2), oleanolic acid and ursolic acid (tritepenoid-1) through QSAR model. We successfully developed the QSAR model for immunomodulatory activity and no significant changes were found in the predicted activities of lupeol (tritepenoid-2) and ursolic acid (tritepenoid-1). More than 51 drugs with reported immunomodulatory activity were included in the training data set for comparison and evaluation of prediction accuracy of QSAR model. Results showed that predicted activity of lupeol and ursolic acid molecules were comparable with experimental immunomodulatory activity. Results indicate that lupeol and ursolic acid molecules possess immunomodulatory activity comparable to boswellic acid and cichoric acid but were less active than levamisol. Moreover, on molecular docking lupeol and ursolic acid showed high binding affinity with immunomodulatory receptors such as, Cyclooxygenase-2 (PDB: 1CX2), Human NF-kappaB p52 (PDB: 1A3Q), tumor necrosis factor (TNF-alpha) (PDB: 2AZ5), Viral Interleukin-10 (PDB: 1VLK), and Phosphatidyl inositol 3-Kinase Regulatory alpha subunit (PDB: 2IUG), thus were considered as most active compounds (Table 4).

Further, isolated compounds were also checked for compliance of Lipinski's Rule-of-five for drug-likeness. Results indicated that all the studied compounds violates the Lipinski's rule-of-five e.g., Log $P > 5$, but under acceptable limit i.e., not showing more than one violation (Table 1). This helped in establishing the pharmacological activity of these isolated compounds for their use as potential drugs. Moreover, on calculation the topological polar surface area (TPSA) as chemical descriptor for passive molecular transport through membranes, results showed lower TPSA for lupeol than standard drugs (Table 1). TPSA allows prediction of transport properties of drugs and has been linked to drug bioavailability. Generally, it has been seen that passively absorbed molecules with a TPSA > 140 $Å^2$ are thought to have low oral bioavailability (Lipinski et al., 2001). On the basis of bioavailability scores, we concluded that isolated compounds have marked immunomodulatory activity and higher bioavailability as compared to standard drugs. The isolated compound ursolic acid (tritepenoid-1) showed comparatively higher TPSA than lupeol (tritepenoid-2).

3.2.2. In silico molecular interaction study

Results of molecular docking showed that except aldehyde dehydrogenase (PDB:1AD3), alkaline phosphatase (PDB:1ALK & 1KH9), collagenase type IV (PDB:1GKD), aldehyde oxidoreductase (PDB:1SIJ), dihydroorotate dehydrogenase (PDB:1UUO), lupeol (**7**), ursolic (**8**) and oleanolic acid (**9**) showed strong binding affinity to human immunomodulatory receptors such as NF-kappaB p52 (PDB:1A3Q), nuclear factor NF-Kappa-B P50 (PDB:1BFS), viral interleukin-10 (PDB:1VLK), tumor necrosis factor (PDB:TNF-alpha) (PDB:2AZ5), phosphatidylinositol 3-kinase regulatory alpha subunit (PDB:2IUG) and anti-inflammatory receptor such as cyclooxygenase-2 (PDB:1CX2). Molecular docking results of studied compounds are comparable with standard immunomodulatory drugs e.g., levamisole (CID 26879). Anti-inflammatory target COX2 also showed good binding affinity for the studied compounds and docking results are comparable with standard anti-inflammatory drugs e.g., glycyrrhetinic acid, ibuprofen, indomethacin and naproxen (Table 4, Figs. 2–4).

3.2.3. Binding affinity revealed by molecular docking studies

The aim of the molecular docking study was to elucidate whether compounds lupeol (**7**), ursolic (**8**) and oleanolic acid (**9**) modulate the immunomodulatory targets, and to study their possible mechanisms of action. The results of the molecular docking suggest that derived compounds inhibit the activity of nuclear factor NF-kappa-B, tumor necrosis factor (TNF-alpha), interleukin, phosphatidylinositol 3-kinase and anti-inflammatory receptor cyclooxygenase-2. In the work presented here, we explored the orientations and binding affinities (in terms of the docking energy in

Table 3
Comparison of experimental and predicted *in vivo* activity data calculated through QSAR model based on best correlated chemical descriptors.

Drug/compound	Exp. ED50 (mg/kg)	Exp. Log ED50	Pred. Log ED50	Pred. ED50	Connectivity index (order 2)	Group count (secamine)	Group count (ether)
Aristolochic acid	81	1.908	1.937	86.50	11.224	0	3
Azimexon	170	2.23	2.388	244.34	6.582	0	0
Boswellic acid	5000	3.699	3.529	3380.65	16.184	0	0
Ciamexon	130	2.114	2.014	103.28	6.246	0	1
Cichoric acid	1750	3.243	3.379	2393.32	14.919	0	0
Emetin	32	1.505	1.51	32.36	14.792	1	4
Imemixon	150	2.176	2.051	112.46	3.754	0	0
Isopteropodin	162	2.21	2.205	160.32	12.209	1	1
Levamisol (2)	180	2.255	2.328	212.81	6.081	0	0
Lupeol (triterpenoid-2) (7)			**3.455**	**2851.02**	15.556	0	0
Ursolic acid (triterpenoid-1) (8)			**3.533**	**3411.93**	16.218	0	0
Oleanolic acid (9)			**3.602**	**3999.45**	16.792	0	0

Table 4
Comparison of docking scores of isolated compounds *viz.*, lupeol, ursolic acid and oleanolic acid with standard drugs.

Name	Compound	Docking score (kcal/mol)										
		Human NF-kappaB p52 (1A3Q)	Viral interleukin-10 (1VLK)	Tumor necrosis factor (TNF-alpha) (2AZ5)	Phosphatidyl inositol 3-kinase regulatory alpha subunit (2IUG)	Nuclear factor NF-kappa-B P50 (1BFS)	Cyclo-oxygenase-2 (1CX2)	Aldehyde dehydrogenase (1AD3)	Alkaline phosphatase (1ALK)	Aldehyde oxidoreductase (1SJ)	Dihydroorotate dehydrogenase (1UUO)	Type IV collagenase (1GKD)
Cyclophosphamide (immuno suppresent)	1	−65.067	−57.131	−46.11	−40.036	−56.344	−70.748	−84.927	21.498	−48.271	−2.46	−60.14
Levamisole (immuno stimulant)	2	−60.665	−49.107	−70.019	−63.094	−63.915	−73.686	−70.867	146.42	−36.971	−51.692	−64.638
Glycyrrhetinic acid	3	−64.387	−52.853	−43.719	−43.125	−33.054	−43.029	9.263	33990.87	501.222	522.47	−33.905
Ibuprofen	4	−65.089	−44.65	−68.681	−54.818	−59.429	−67.601	−40.094	56.779	−21.483	−48.8	−59.799
Indomethacin	5	−94.317	−60.391	−78.731	−74.554	−41.315	−99.944	−13.411	1694.662	−7.227	−49.363	−81.558
Naproxen	6	−68.965	−47.359	−83.086	−70.739	−41.671	−88.196	−80.616	145.335	−30.885	−69.545	−68.495
Lupeol (triterpenoid-2)	7	−48.463	−35.517	−46.254	−29.242	−16.132	**−49.433**	0.246	12549.77	980.864	440.76	55.137
Ursolic acid (triterpenoid-1)	8	−49.478	−42.487	−47.95	−26.94	−25.135	**−64.043**	−2.563	16517.3	1039.637	395.77	28.669
Oleanolic acid	9	−51.366	−41.15	−13.526	−34.64	−21.107	**−63.351**	19.378	24892.89	485.595	399.886	−11.666

Note: Docking energy in bold face indicate high affinity while positive values indicate low binding affinity to corresponding receptors.

Fig. 2. Ursolic acid (triterpenoid-1) (**8**) docked with high affinity of cyclooxygenase (1CX2) anti-inflammatory receptor with docking score −64.043 kcal/mol.

Fig. 3. Lupeol (triterpenoid-2) (**7**) docked with high affinity on cyclooxygenase (1CX2) anti-inflammatory receptor with docking score −49.433 kcal/mol.

kcal mol^{-1}) of studied compounds towards immunomodulatory and anti-inflammatory targets.

The binding affinity obtained in the docking study allowed the activity of the lupeol (**7**), ursolic (**8**) and oleanolic acid (**9**) to be compared to that of the standard drugs/compounds. The compounds 7, 8 and 9 showed high binding affinity (low docking energy) to immunomodulatory and anti-inflammatory targets. When we compared how the binding pocket residues of target interacted with the compounds, we found that compound ursolic acid (8) showed interaction with conserved amino acid residues thus lead to more stability and potency in these cases (Table 4). The docking results showed that compound 8 docked onto immunomodulatory and anti-inflammatory target cyclooxygenase with low interaction energy (−64.043 kcal mol^{-1}) (Fig. 2). In this complex, the conserved binding pocket amino acid residues within a selection radius of 4 Å from bound ligand were belongs to hydrophilic class e.g., Thr-212 (Threonine; neutral/polar), His-214, 386, 388 (Histidine; polar basic), Gln-303 (Glutamine; polar amide) and rest residues belongs to hydrophobic category e.g., Val-291, 295, 447 (Valine; non-polar aliphatic), Asn-382 (Asparagine; polar amide), Leu-391, 408 (Leucine; non-polar aliphatic). Aliphatic amino acids do not contain N, O, S in side chain e.g., Valine, Leucine. Similarly, neutral amino acids e.g., Threonine, Asparagine, and Glutamine contain hydroxyl or amide groups in side chain. The hydrophobic residues are responsible for hydrophobic bond formation, thus lead to more stability and potency.

The docking results showed that compound 7 docked onto immunomodulatory and anti-inflammatory receptor cyclooxygen-

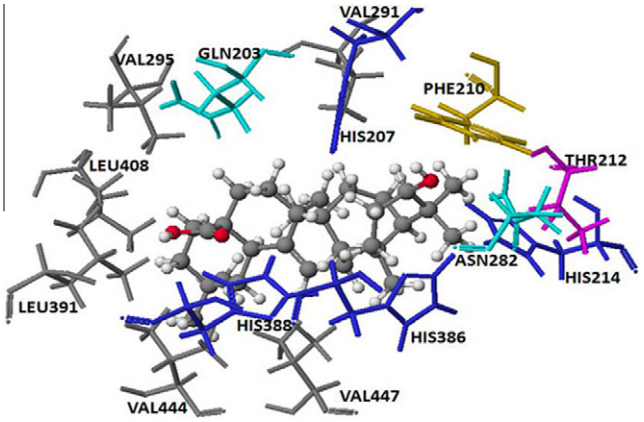

Fig. 4. Oleanolic acid (**9**) docked with high affinity on cyclooxygenase (1CX2) anti-inflammatory receptor with docking score −63.351 kcal/mol.

Table 5
Effect of orally administered triterpenoid-1 and triterpenoid-2 on humoral and cell mediated immune response in mice.

Treatment	Hummoral immunity	Cell-mediated immunity
	Heamagglutination titer (well) mean ± SE	Foot paw thickness (cm) mean ± SE
Vehicle control	4.83 ± 0.76	0.57 ± 0.11
Triterpenoid-1 (1 mg/kg)	8.50 ± 0.77[a]	0.51 ± 0.10
Triterpenoid-1 (3 mg/kg)	7.67 ± 0.48[a]	0.54 ± 0.11
Triterpenoid-1 (10 mg/kg)	6.67 ± 0.66[a]	0.54 ± 0.09
Triterpenoid-2 (1 mg/kg)	6.50 ± 0.43[a]	0.56 ± 0.10
Triterpenoid-2 (3 mg/kg)	6.83 ± 0.61[a]	0. 5 ± 0.08
Triterpenoid-2 (10 mg/kg)	8.17 ± 0.52[a]	0.43 ± 0.09
Levamisole HCl (0.68 mg/kg)	9.17 ± 0.61[a]	0. 57 ± 0.08
Cyclophosphamide (200 mg/kg)	1.40 ± 0.19[b]	0.37 ± 0.11

Note: $n = 06$, $P < 0.05$ using student t test (vehicle control verse treatment).
[a] Significantly increased.
[b] significantly decreased.

ase with low interaction energy -49.433 kcal mol^{-1}) (Fig. 3). In this complex, the conserved binding site pocket amino acid residues within a radius of 4 Å from bound ligand were belongs to two classes *viz*., hydrophilic and hydrophobic. The residues belongs to hydrophilic class were His-207, 214, 388 (Histidine; polar basic), Gln-203 (Glutamine; polar amide), Lys-211 (Lysine; polar basic). The residues belongs to hydrophobic class were Val-291, 295, 444, 447 (Valine; non-polar aliphatic), Asn-382 (Asparagine; polar amide), Leu-408, 391 (Leucine; non-polar aliphatic), Phe-404 (Phenylalanine; non-polar aromatic; benzene ring in side chain). Due to hydrophobic residues compound 7 able to form strong hydrophobic molecular interaction, thus lead to more stability and potency.

Similarly, the docking results showed that compound 9 docked onto immunomodulatory and anti-inflammatory receptor cyclooxygenase with low interaction energy −63.351 kcal mol^{-1}) (Fig. 4). In this complex, the conserved binding site pocket amino acid residues within a radius of 4 Å from bound ligand were belongs to two major physical classes *viz*., hydrophilic and hydrophobic. The residues belongs to hydrophilic class were Thr-212 (Threonine; neutral/polar), His-207, 214, 388, 386 (Histidine; polar basic), Gln-203 (Glutamine; polar amide). The residues belongs to hydrophobic class were Val-291, 295, 444, 447 (Valine; non-polar aliphatic), Asn-282 (Asparagine; polar amide), Leu-391, 408 (Leucine; non-polar aliphatic), Phe-210 (Phenylalanine; non-polar aromatic; benzene ring in side chain). Due to these hydrophobic residues compound 9 able to form strong hydrophobic interaction, thus lead to more stability and potency. These results showed compliance with docking results of standard drug levamisole (Immuno-stimulant) with cyclooxygenase, which showed a comparable docking energy of −73.686 kcal mol^{-1} (Table 4).

3.3. In vivo immunomodulatory activity

In the present study the *in vivo* immunomodulatory effect of two purified triterpenoids **1** and **2** isolated from *E. tereticornis* and *G. kurroo* was undertaken. In a 28 days oral administration study, triterpenoids **1** and **2** exhibited significant increase in antibody titer in mice immunized with rabbit red blood cells (rRBC) in a dose dependent manner when compared with normal vehicle control ($P < 0.05$). The representative data are depicted in Table 5.

When compared with the vehicle control versus treatment, significant changes were not observed in cell mediated immune response/delayed type hypersensitivity test (DTH), gain in body weight, total red blood cell counts, total white blood cell counts and hemoglobin parameters (Table 6). Previous reports suggested that agents induced increase in hemagglutinin antibody titers serve as immunostimulatory agents for the immune system and several Indian medicinal plants possess immunostimulatory activity (Wagner, 1983; Atal et al., 1986; Godhwani et al., 1988; Dua et al., 1989). The result of this study concludes that triterpenoid-1 (ursolic acid) exhibited higher antibody titer at the lower dose whereas the triterpenoid-2 (lupeol) exhibited higher antibody titer at higher dose. Since triterpenoid-1 exhibited higher antibody titer at lower dose, so it can be consider as potential immunomodulatory agents and can be further explored for detail target based study using molecular biology approach.

4. Conclusion

The isolated triterpenoids (**1** and **2**) and test set triterpenoid oleanolic acid (**9**) were evaluated for druglikeness using the traditional Lipinski's rule of five, which revealed that all the three triterpenoids followed druglikeness parameters, similar to known

Table 6
Effect of triterpenoid-1 and triterpenoid-2 on rate of body weight gain and hematological parameters after 28 days oral administration.

Treatment	Body weight gain (gm)	RBC's (millions/mm^3)	WBC's (thousands/mm^3)	Hemoglobin (gm/dl)
Vehicle control	3.30 ± 0.81	6.85 ± 1.50	12.55 ± 3.44	12.007 ± 0.45
Triterpenoid-1 (1 mg/kg)	4.06 ± 0.68	8.05 ± 2.14	12.97 ± 0.30	11.636 ± 0.81
Triterpenoid-1 (3 mg/kg)	3.69 ± 1.07	7.42 ± 1.15	11.96 ± 1.75	11.493 ± 1.20
Triterpenoid-1 (10 mg/kg)	3.39 ± 4.48	7.05 ± 4.46	11.68 ± 3.41	10.952 ± 1.02
Triterpenoid-2 (1 mg/kg)	3.65 ± 0.91	6.69 ± 0.15	11.59 ± 1.08	11.634 ± 0.90
Triterpenoid-2 (3 mg/kg)	3.88 ± 0.32	7.10 ± 0.24	12.86 ± 4.08	13.868 ± 0.94
Triterpenoid-2 (10 mg/kg)	4.06 ± 0.95	8.12 ± 0.54	12.08 ± 4.44	10.952 ± 1.02
Levamisole (0.68 mg/kg)	5.16 ± 7.35	8.00 ± 1.71	12.08 ± 4.44	11.634 ± 0.90
Cyclophosphamide (200 mg/kg)	1.61 ± 0.83	3.42 ± 2.23	10.59 ± 1.08	11.12 ± 1.56

Note: $n = 06$, $P < 0.05$ using student t test (vehicle control verse treatment).

anti-inflammatory and immunomodulatory drug glycyrrhetinic acid, therefore all the three triterpenoids were considered as potential drug-like molecules. Later, these results were found comparable to experimental *in vivo* activity of triterpenoid **1** (ursolic acid) and **2** (lupeol). Since, oleanolic acid possesses much structural similarity with ursolic acid hence may possess similar *in vivo* activity as predicted *in silico* results.

Further a forward feed multiple linear regressions QSAR model was developed using leave-one out approach for the prediction of anti-inflammatory and immunomodulatory activity and no significant differences were observed in the predicted and observed activities of triterpenoids **1** and **2**.

The results of QSAR study suggest that both ursolic acid (**8**, triterpenoids-1) and lupeol (**7**, triterpenoids-2) possess immunomodulatory activity comparable to boswellic acid and cichoric acid but are less active than levamisol, while results of *in silico* molecular docking experiments suggest that both the triterpenoids **1** and **2** possess immunomodulatory and anti-inflammatory activity due to high binding affinity to human receptors *viz*., NF-kappa-B P52, tumor necrosis factor (TNF-alpha), nuclear factor NF-Kappa-B P50 and cyclooxygenase-2. Further, theoretical results were validated experimentally and found comparable to *in vivo* data for immunomodulatory activity in female Swiss albino mice.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2012.05.009.

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