

Corosolic Acid Inhibits Secretory Phospholipase A₂Ila as an Anti-Inflammatory Function and Exhibits Anti-Tumor Activity in Ehrlich Ascites Carcinoma Bearing Mice

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Background: Inflammation is generally connected to tumour progression and development. The secretory phospholipase A₂Ila (sPLA₂Ila) is an important inflammatory enzyme that catalyse the hydrolysis of membrane phospholipids into arachidonic and lysophosphatidic acid, which are the precursors for production of a lot of pro-inflammatory mediators like prostaglandins, prostacyclins, thromboxanes, leukotrienes and platelet activating factors, which involved in the proliferation, migration, invasion, and metastasis. Therefore, investigating safe and effective sPLA₂Ila inhibitors as a therapeutic agent to treat cancer is indeed in need.

Methods: Anti-inflammatory function of corosolic acid was evaluated by docking it with sPLA₂Ila enzyme, sPLA₂Ila inhibition, calcium and substrate concentration-dependent assays; intrinsic fluorescence and UV-CD analysis; neutralisation of sPLA₂Ila induced indirect hemolytic and edema. Evaluated the anticancer activity of corosolic acid by MTT assays and caspase-3 expression; the anti-tumour activity by EAC-induced cell line and interleukin 6 expression.

Results: The corosolic acid inhibits sPLA₂Ila activity to 82.21±2.82%. The inhibition was evaluated by increasing calcium from 2.5 to 15 µM and substrate from 20 to 120 nM, it did not affect the level of inhibition. Corosolic acid altered the intrinsic fluorescence and UV-CD spectra of sPLA₂Ila enzyme, indicating the direct interaction. It neutralised sPLA₂Ila induced hemolytic activity from 97 ±1.23% to 15.75±1.44% and edema from 171.51±2.39% to 119.3±2.6%. Further, as antiproliferative activity, corosolic acid reduced the PC3 cell viability from 99.66±0.57% to 23±2.64% and suppressed LPS-induced IL-6 level from 94.35±2.2% to 34.36±2.4%. It increased mean survivability time from 30 to 38 days and displayed the drug-like qualities.

Conclusion: All the experimental results have proven the corosolic acid as an anti-inflammatory and anticancer molecule that may further be used to develop it as a drug.

Keywords: corosolic acid, antioxidant, secretory phospholipase A₂ Ila inhibition, caspase 3, IL-6, prostate cancer, EAC, ADME-toxicity, Anti-inflammatory

Introduction

Inflammation generally promotes or suppresses the tumour progression.¹ Neoplasia initiates the inflammation-related pathway (intrinsic pathway) that leads to the development of the microenvironment and inflammatory reactions (extrinsic pathway) that

facilitate cancer development.² The immune response against cancer development is closely related to the inflammatory reaction.^{3,4} Also, currently available cancer therapy includes anti-inflammatory drugs like non-steroidal anti-inflammatory drugs (NSAIDs), which have been postulated for anti-cancerous properties.⁵⁻⁷ Hence, modulating inflammation is the principal strategy for cancer prevention and therapy.⁸

Inflammation is mainly mediated by secretory phospholipase A2IIa (sPLA2IIa) enzymes. It catalyse the membrane phospholipids into arachidonic acid and lysophosphatide, which further converted into pro-inflammatory mediators like prostaglandins, thromboxanes, leukotrienes, prostacyclins and platelet activation factors⁹ (Figure 1). The products of the sPLA2IIa enzyme contribute to pathophysiological conditions of inflammatory diseases like sepsis, trauma, asthma,¹⁰ atherosclerosis¹¹ and cancers.^{12,13} The prostaglandins and leukotrienes play a significant role in prostate cancer cell proliferation, migration, invasion, and metastasis.¹⁴ They are overexpressed in almost all human prostate cancer specimens.¹⁵

Since long ago the NSAIDs have been used to cure inflammatory diseases by targeting lipoxygenases (LOX) or cyclooxygenase (COX-1/2) enzymes. Despite their benefits, these drugs cause serious side effects like gastrointestinal toxicities, renal injuries, and cardiovascular risks.¹⁶⁻¹⁸ Furthermore, COX-1/2 or LOX inhibitors are unable to control the production of platelet activating factor (PAF), which intensifies the inflammation.¹⁹ Though it looks like a simple approach,

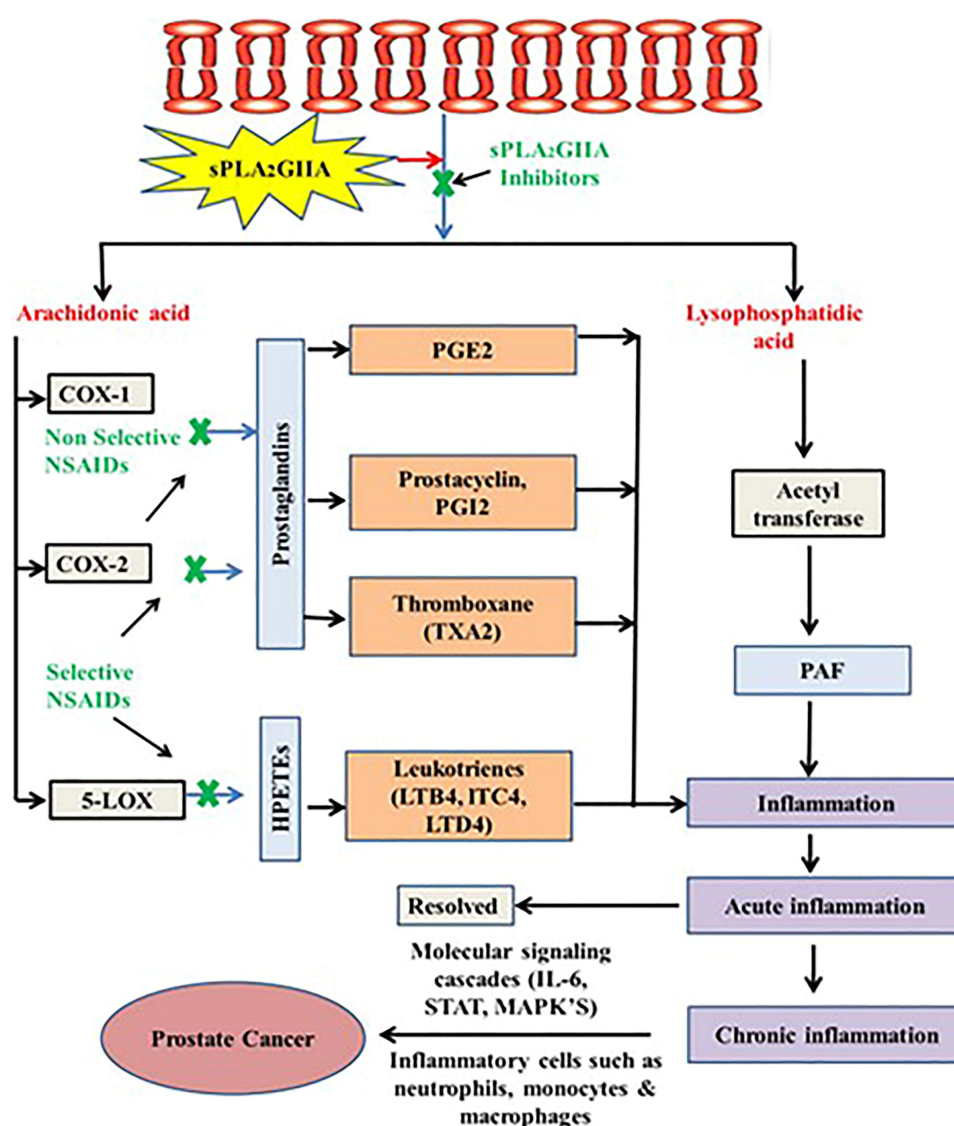


Figure 1 The schematic representation shows sPLA2IIa cascade in the arachidonic acid and lysophosphatidic metabolic pathway and action of non-selective and selective NSAIDs on prostaglandins and HPETE and Inflammation itself a double-edged sword, has two types acute and chronic inflammation, acute gets resolved and chronic inflammation through molecular.

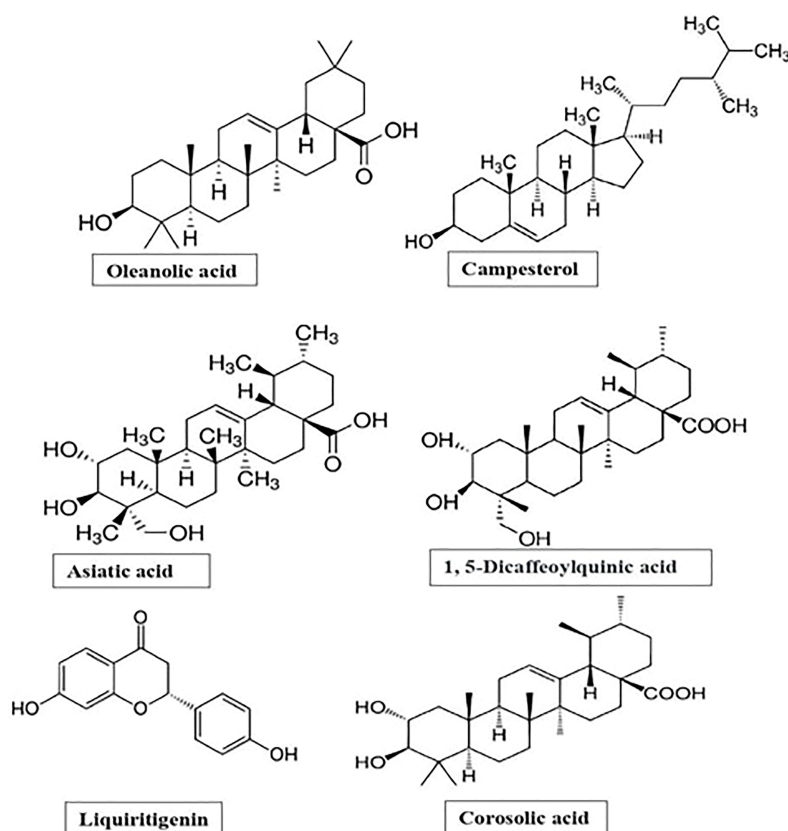


Figure 2 Structures of pharmacologically important molecules from *Centella asiatica*.

a potent and safe inhibitor of sPLA2IIa reduces pro-inflammatory mediators.^{20,21} For half a century, hundreds of sPLA2IIa inhibitors were invented and developed, few of them trialled at clinical level. But none of them reach the market even though they inhibit sPLA2IIa enzymes in nanograms that might be due to problems related with formulation or cytotoxicity^{22–24} or it could be bad pharmacodynamics and pharmacokinetic features. Hence, there is a great demand for effective sPLA2IIa inhibitors derived from natural resources to treat inflammatory diseases as well as cancers.

In the current study, an important medicinal plant, *Centella asiatica* widely used to treat several inflammatory diseases like leprosy, ulcers, psoriasis, and female genitourinary tract diseases²⁵ etc., was considered for the evaluating its anti-inflammatory function. Initially, a few pharmacologically important bioactive molecules from *C. asiatica* like Liquiritigenin, Asiatic acid, 1, 5 Dicaffeoylquinic acid, Campesterol and Corosolic acid (Figure 2) (Table 1) were docked with sPLA2IIa enzyme, among them, corosolic acid showed the greater binding affinity. It also showed a variety of pharmacological activities like anti-diabetic,²⁶ anti-inflammatory,²⁷ anti-obesity activity^{28,29} and apoptosis in prostate cancer cells.³⁰ But, till date, the corosolic acid has not been evaluated for the anti-inflammatory function by inhibiting sPLA2IIa, an important inflammatory enzyme. Therefore, this study intended to evaluate the potency of corosolic acid for sPLA2IIa inhibition as an anti-inflammatory and anticancer effect by in vitro, in situ and in vivo methods. The oleanolic acid is a triterpenoid and isomer of corosolic acid has proven to be a very good sPLA2IIa inhibitor³¹ and anticancer molecule³² used as reference control.

Materials and Methods

Chemicals and Reagents

Sephadex G-25, -50 and -75, CM sephadex C25 Corosolic acid, Oleanolic acid and Lipopolysaccharide (#L2018), Ultima Gold Scintillation Cocktail, Bovine serum albumin (BSA), were procured from Sigma-Aldrich, USA. ¹⁴C-oleic acid obtained from Perkin Elmer Life Sciences Inc. Boston. Mouse Anti-Human IL-6 (Cat No: 340527) was from B.D Biosciences. Six-well cell culture plates from BioLite - Thermo. The PC3 Cell line was obtained from the national centre for cell science (NCCS), Pune, the cell culture medium (RPMI 1640, #AL028A), Fetal Bovine Serum (#RM10432) and

Table I Molecular Docking of Selective Bioactive Compounds and Their Energy Values Against sPLA₂ (IPOE)

Compound Name	MW(D)	TPSA (Å)	Hydrogen Bond Interaction	van der Waals Interaction	Docking Energy Value (KCal/ Mole)
Liquiritigenin	256.2534	66.76	Asp48, Leu2	Cys44, Cys28, Gly29, His47, Phe98, Ala17, Phe5, Ala21, Ile9, His6	-7.63
Asiatic acid	488.6991	97.99	–	His6, Ala17, Leu2, Gly22, Gly29, Ala18, Phe5, Val30, Cys28, Asp48, His47	-9.14
1,5-Dicaffeoylquinic acid	516.4509	211.28	Gly29, Asp48, Gly31	Lys62, Leu2, Val30, Tyr5, Gly29, Ala18, Ala17, His47, Cys44, Phe98, Phe5	-7.14
Campesterol	400.6801	20.23		Phe5, Ala18, Leu2, His6, Ile9, Ala17, Gly22, Tyr21, Val30, Lys2, Asp48, His47, Gly29, His27, Tyr51	-7.29
Corosolic acid	472.6997	77.76	His47, Asp48	Gly44, Phe5, Gly22, Gly28, Leu2, Ala18, Val30	-9.71
Oleanolic acid	456.709	90.9	His47, Asp48	Val30, Cys44, Tyr21, Gly29, Gly22, Gly28, Leu2, Phe5	-9.55

Note: Binding energy-KCal/Mole.

Abbreviations: MW, molecular weight in dalton; TPSA, topological polar surface area in Å.

dulbecco's phosphate buffered saline (D-PBS) (#TL1006) were procured from HiMedia Laboratories, Mumbai, India. Ethylenediaminetetraacetic acid (EDTA) and dimethyl sulphoxide (DMSO) were acquired from SRL (Sisco Research Laboratories), India. The solvents and chemicals utilised in the entire investigation were standard laboratory grade.

Human Sample

Institutional Human Ethical Committee, Mangalore University approved the usage of blood samples (MU/IHEC/2018/1). The blood was collected from healthy volunteers after taking an informed consent letter.

Animals

Swiss albino mice (20–25g) were used for anti-tumour activity and neutralization of edema study. The animals were handled and supervised according to India's Regulations passed by the laboratory animal welfare act. The approval number of the Institutional Animal Ethical Committee is NGSMIPS/IAEC/NOV-2019/166 (NGSM Institute of Pharmaceutical Science, Mangalore).

sPLA₂ IIA Enzyme

The sPLA₂Ia was purified according to the method of Kasturi and Gowda 1989³³ and homogeneity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as per the method of Laemmli, 1970.³⁴

In Silico Docking Study

The sPLA₂Ia structure was obtained from Protein Data Bank (PDB) (1POE) (<https://www.rcsb.org/structure/1POE>). Structures of phytoconstituents were obtained from the PubChem database. Details of the active site were obtained from PDB and literature.^{35,36} The graphical user interface (AutoDock Tools 4.2.6) was used to carry out intermediary steps like energy minimization, grid box formation; protein-inhibitor preparation. The prepared file was saved in PDBQT format by AutoDock using iterated local search global optimizer.^{37,38} The hydrophobic and H bond interactions between the ligands and receptors of the docked complex were analyzed using the LigPlot tool. After the successful completion of the docking runs, different conformations of the ligands known as binding modes were obtained with their respective binding affinity and the stable one which happens to be the one with the lowest binding affinity was picked and aligned with receptor structure for further analysis.^{39,40}

ADME Toxicity Prediction

Corosolic acid and oleanolic acid MOL files were obtained from the ChemSpider database (<http://chemspider.com>) and used as a source of data for in silico analysis. The in-silico absorption, distribution, metabolism, excretion, toxicity (ADME-T) properties were estimated using the PreADME-T web-based tool (<https://preadmet.bmdrc.kr/adme> and <https://preadmet.bmdrc.kr/toxicity/>).⁴¹

Secretory Phospholipase A₂ (sPLA₂Ia) Assay and Inhibition

The sPLA₂Ia activity was determined using ¹⁴C-oleic acid labelled *E. coli* cells (autoclaved) as per the method of Patriarca et al⁴² For sPLA₂Ia inhibition assay, dissolved 10 mg of corosolic acid in 1 mL DMSO and made upto required concentration using Tris-HCl buffer. The sPLA₂Ia activity was estimated by treating corosolic acid from 2 to 16 µM concentration. Oleanolic acid is a well-known sPLA₂Ia inhibitor and isomer of corosolic acid was used as standard.⁴³ The highest DMSO concentration used in this assay was 0.022%.

Effect of Calcium and Substrate Concentration

The nature of sPLA₂Ia inhibition was tested by increasing the concentration of calcium from 2.5 to 15 µM and substrate from 30 nM to 120 nM in presence and absence of IC₅₀ concentration of corosolic acid (The separate assays were carried out for calcium and substrates).

Intrinsic Fluorescence Analysis

Intrinsic fluorescence of sPLA₂Ia enzyme with and without corosolic acid was recorded in the Fluorolog-3 spectrofluorometer. The 2.0 mL reaction mixture consists of sPLA₂Ia (20µg/mL) and corosolic acid range from 0.02 to 0.1µM was subjected for analysis. Fluorescence spectra were recorded between 300 nm and 380 nm and were empirically corrected using the tryptophan standard.⁴⁴

Circular Dichroism Analysis

The UltraViolet Circular Dichroism (UV-CD) spectra of sPLA₂Ia (30µg/mL) was recorded with and without IC₅₀ concentration of corosolic acid using Jasco J-810 spectropolarimeter between 200 and 240 nm. The response time was 2s and the bandwidth was 1 nm. Total of 10 scans were made into the final spectrum. The spectrum of standard reaction mixture was subtracted to correct the spectra.

Neutralisation of Indirect Hemolytic Activity

The experiment was carried out according to the method of Boman and Kaletta 1957.⁴⁵ The 1mL of red blood cell (RBC) and 1mL of egg yolk in 8mL of phosphate buffered saline (PBS) was used as substrate. The corosolic acid was pre-incubated with sPLA₂Ia at 37°C for 30 minutes, then added 1mL of the substrate and incubated at 37°C for 45 minutes. Added 9 mL of ice-cold PBS to halt the reaction and centrifuged at 1500×g for 20 minutes. The hemolytic activity of sPLA₂Ia enzyme in terms of released hemoglobin was measured at 530 nm.

Neutralization of Edema Inducing Activity

The method of Yamakawa et al 1976⁴⁶ altered by Vishwanath et al 1987⁴⁷ was followed. The sPLA₂ IIA (5µg) alone or with corosolic acid (range from 0 to 21 µM) in total volume of 20 µL injected into right hind footpad of mice. A 20 µL of saline was injected into the respective left-hind footpad to serve as control. The animals were euthanized after 45 minutes by giving anaesthesia (30 mg/kg of pentobarbital i.p.). The limbs were amputated at the ankle joint and weighed individually.

$$\text{Edema ratio} = \frac{\text{Weight of the edematous leg}}{\text{Weight of the normal leg (saline - injected)}} \times 100$$

MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide) Assay

The protocol of Alley et al⁴⁸ and the MTT cell proliferation instruction guidelines were used to determine prostate cancer (PC3) cell viability. The cells were grown in T-25 flasks, harvested and plated in a 96-well plate in DMEM at cell density of 10,000 cells per well. After 24h incubation, the cells were treated with different concentrations of corosolic acid. After 24h incubation, MTT reagent was added to get 0.5mg/mL concentration and incubated in the CO₂ incubator for 3h. The media containing excess MTT was removed, and formazan crystals were dissolved using 100 µL DMSO. Reading was taken at 570 nm for the purple colour formazan solution and at 630 nm to subtract the background reading. The percentage viability of PC3 cells was calculated.

Determination of Caspase 3 Level by Flow Cytometry

The 3×10^5 cells/2 mL density were cultured and treated with corosolic acid. The cells were harvested, washed with PBS, fixed with 0.5mL of 2% paraformaldehyde and incubated for 20 minutes. The cells were washed with 0.5% BSA in PBS and permeabilized with 0.1% Triton-X 100 of 0.5% BSA solution. Staining was done with 20µL anti-caspase-3 antibody and incubated for 30 minutes in the dark at room temperature. Unbound antibodies were removed by washing with PBS twice, then 0.5 mL of PBS was added, mixed thoroughly and recorded the fluorescence intensity. Caspase-3 FITC (green) fluorescence was detected in the FL1 detector using a 525 nm bandpass filter.^{49–51}

Anti-Tumor Activity

The anti-tumor activity of the corosolic acid was evaluated by injecting Ehrlich Ascites Carcinoma (EAC) cells into Swiss albino mice.⁵² In brief, 24 mice of either sex were grouped into 4 sets containing 6 animals in each group. Group 1 received only buffers and served as vehicle control and Group 2 injected 1×10^6 EAC cells (i. p.) served as disease control; Group 3 animals received corosolic acid (10 mg/kg, p.o). Group 4 was treated with a standard molecule (5 fluorouracil). The inhibitor was prepared by dissolving in Tris HCL buffer (10mM) to the final concentration of 1 µg/µL. Animals were treated after 24 h of tumour induction and continued for the 10 days. Animals were observed for clinical signs and mortality. The weight of the animals was recorded and percentage increase in mean survival time (% MST) was calculated.

$$\% \text{ MST} = (1 - \text{TC}) \times 100$$

Where C= Mean survival time of the control group; T= Mean survival time of the treated group.

Interleukin 6 Expression Studies by Flow Cytometry

IL-6 expression was measured following the manufacturer's instructions (BD Biosciences PE Mouse Anti-Human IL-6; Cat No: 340527)⁵³ and analysed by FACS – Cell quest pro software.

Statistical Analysis

The obtained test results were given as the mean standard deviation (n=3). IC₅₀ values were calculated by Graph Pad Prism Version 5.0 (La Jolla, USA).

Results and Discussion

The AutoDock4 version (v4.2.6)³⁷ module was used to study the binding pattern of corosolic acid and oleanolic acid with human sPLA2IIa (PDB ID: 1POE organism: *Homo sapiens*). The His47/Asp48 residues and calcium-binding loop of the sPLA2IIa (1POE) enzyme are necessary for activity.⁵⁴ The majority of sPLA2IIa inhibitors interact with His47/Asp48 and weaken the Ca²⁺ association that lowers the enzyme activity.^{55,56} Some of the sPLA2IIa inhibitors bind to substrate binding pouches that interfere with substrate binding to the active site. The amino acids like Phe-23, Phe-5, Leu-31, and Leu-2⁵⁷ of substrate binding pouch of sPLA2IIa enzyme establish van der Waals interactions with inhibitors. The corosolic acid (PubChem ID 6918774) interacts with active site His47 and Asp48 via hydrogen bonding and van der Waals interaction with Gly-44, Phe-5, Gly-22, Gly-28, Leu-2, Ala-18, Val-30. Similarly, the standard reference oleanolic acid (PubChem ID 10494) interacts with His-47, Asp-48 through hydrogen bonding and van der Waals interaction with Val-30, Cys-44, Tyr-21, Gly-29, Gly-22, Gly-28, Leu-2 and Phe-5 (Figure 3).

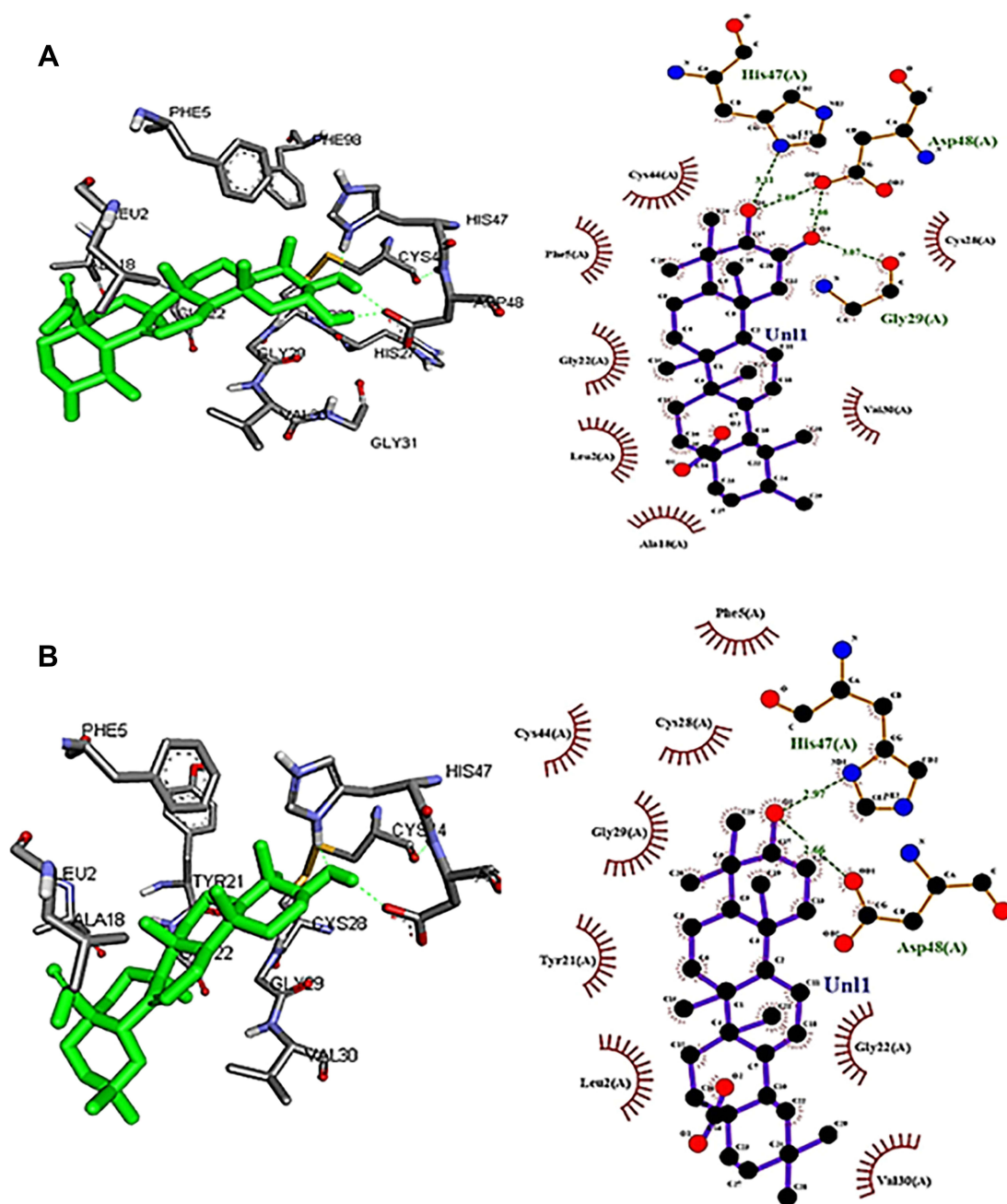


Figure 3 Docked images of corosolic acid and oleanolic acid with sPLA2IIa enzyme: Stereo view of corosolic acid (**A**) and oleanolic acid (**B**) docked with active site of sPLA2IIa.

The pharmacokinetic profiling at the initial stages of drug discovery using in silico methods is necessary because it is cheap and timesaving compared to the experimental approach.⁵⁸ Hence, corosolic acid was subjected to ADME-toxicity profiling to qualify it as a drug candidate. The ligand physical descriptors and pharmaceutically essential properties were analysed using the Pre ADME-T program. The parameters used in ADME-Toxicity prediction are based on Lipinski's rule of 5; the corosolic acid showed 94.278% intestinal absorption, 99.224% of plasma protein binding and 2.788% of blood-brain barrier (BBB); thus, corosolic acid displayed drug-like qualities (Table 2).

As corosolic acid exhibits better binding energy (negative E value) and displayed drug-like qualities, further subjected to sPLA2IIa enzyme inhibition. The corosolic acid inhibited sPLA2IIa enzyme to $82.21 \pm 2.82\%$ at $16 \mu\text{M}$ concentration in a concentration dependent manner (Figure 4). The IC_{50} value of corosolic acid was $9.44 \pm 0.59 \mu\text{M}$, whereas standard reference oleanolic acid showed the IC_{50} value of $6.74 \pm 0.71 \mu\text{M}$.

Table 2 Predicted ADME-Toxicology of Corosolic Acid Compared with Standard Oleanolic Acid

ADME							Toxicity		
Compound	HIA (%)	PPB (%)	BBB (%)	Caco- 2(nm/sec)	Skin Permeability	MDCK(nm/se)	Mutagenicity	Carcino Mouse	Carcino Rat
Corosolic acid	94.278	99.224	2.788	21.264	-2.585	0.043	Non-mutagen	Positive	Positive
Oleanolic acid	95.996	100	7.879	21.887	-2.354	0.043	Non-mutagen	Positive	Positive

Abbreviations: HIA, human Intestinal Absorption; PPB, plasma protein binding; BBB, blood-brain barrier; Caco-2 cells, human colorectal carcinoma; MDCK, Madin-Darby canine kidney.

Some of the inhibitors limit sPLA2IIa activity either by binding to substrate or chelating metal ion, calcium. Hence, the nature of sPLA2IIa inhibition was examined by increasing the calcium concentration from 2.5 to 15 μM in presence and absence of corosolic acid (IC_{50} concentration), it showed the constant inhibition of $47.90 \pm 1.37\%$ over all the ranges of calcium (Figure 5A). Also, estimated sPLA2IIa inhibition by increasing substrate concentrations from 30 to 120 nM in presence and absence of corosolic acid (IC_{50} concentration), which showed the constant inhibition of $49.63 \pm 0.94\%$ over

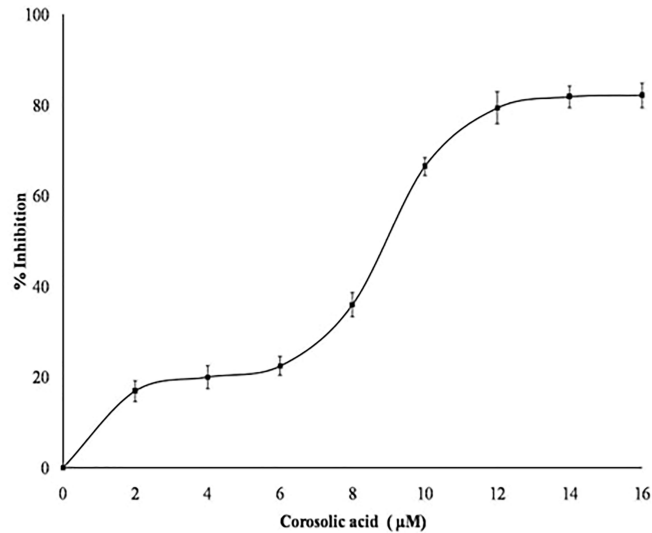


Figure 4 Inhibition of sPLA2IIa enzyme: The reaction mixture consists of labelled *E. coli* cells, Tris-HCl buffer, calcium, sPLA2IIa and corosolic acid ranging from 0 to 16 μM . The data showed mean $\pm$ SD ($n = 3$).

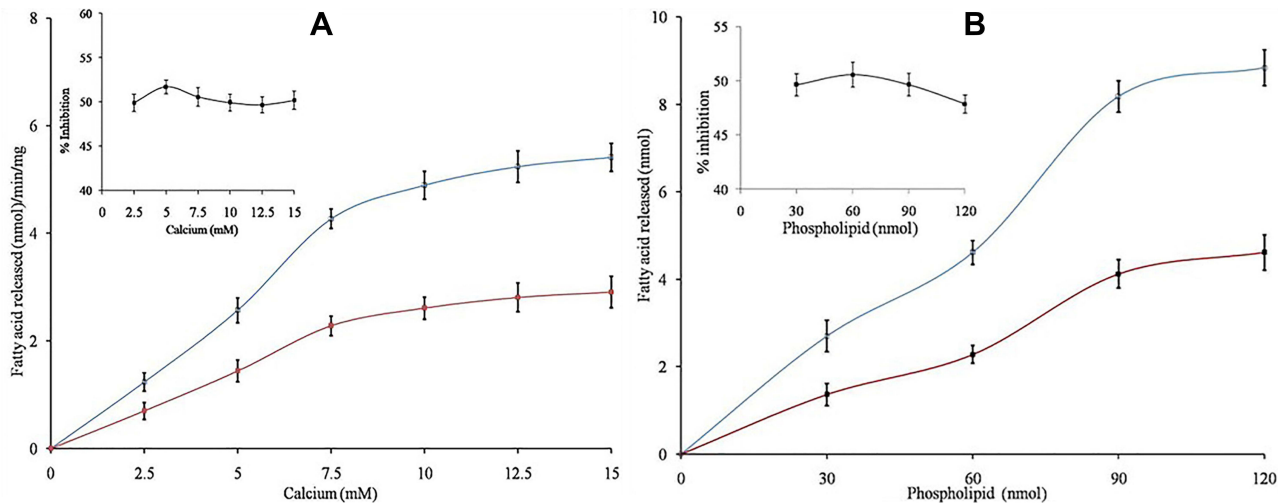


Figure 5 (A) Effect of calcium concentration on sPLA2IIa inhibition: Estimated sPLA2IIa activity alone (blue line) and with IC_{50} concentration of corosolic acid (red line) with indicated concentrations of calcium. The data are given in mean $\pm$ standard deviation ($n=3$). **(B)** Effect of substrate concentration on inhibition: Estimated sPLA2IIa activity alone (blue line) and with IC_{50} concentration of corosolic acid (red line) via increasing the substrate concentration from 30 to 120 nM. The data expressed as mean $\pm$ standard deviation ($n=3$).

all the ranges of substrates (Figure 5B). The results suggested that sPLA2IIa inhibition does not change due to either increased calcium or substrate concentrations.

Generally, the interaction of inhibitors with the sPLA2IIa enzyme exposes amino acids like phenylalanine, tryptophan and tyrosine to the surrounding media that leads to quenching of intrinsic fluorescence.⁵⁹ Altering the intrinsic fluorescence indicates structural change in proteins due to substrate or ligand interaction. Increased fluorescence intensity of sPLA2IIa upon binding to corosolic acid indicating the formation of enzyme-inhibitor complex (Figure 6A). Increasing relative intrinsic fluorescence was observed with increasing corosolic acid concentration from 0.02 μ M to 0.1 μ M (Figure 6B).

Far UV-CD studies provide the information regarding the changes in sPLA2IIa structure upon binding to an inhibitor, which validates the findings of fluorescence study. The far UV-CD spectrum of sPLA2IIa without corosolic acid gave two characteristic negative bands (peaks) at 210 and 222nm. The addition of corosolic acid (IC_{50} concentration) reduces peak height and shifts peaks to 217 and 220 nm (Figure 7), which indicates the changes

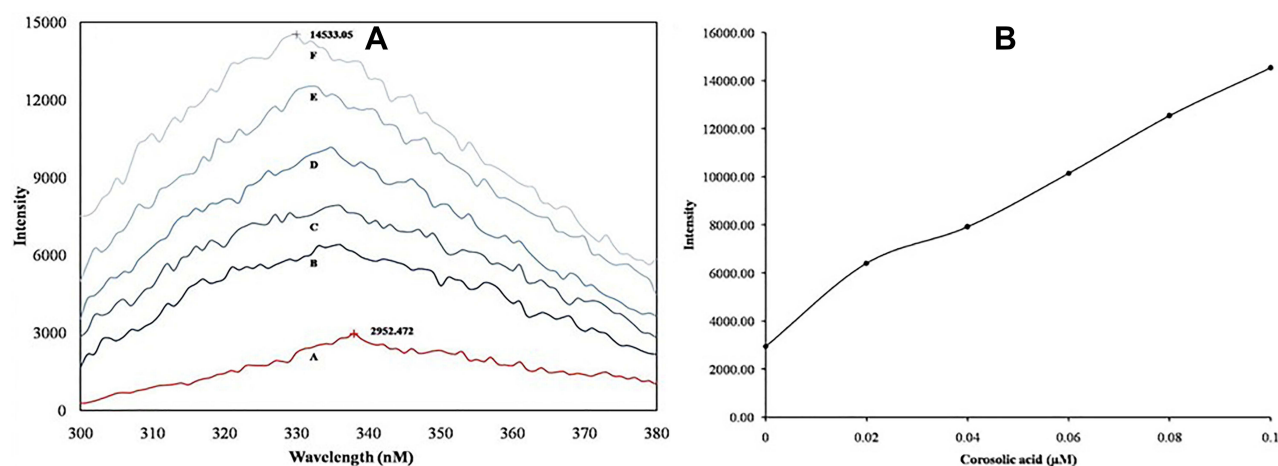


Figure 6 Intrinsic fluorescence study: (A) Spectra of sPLA2IIa enzyme alone (a); and with corosolic acid of 0.02 μ M (b), 0.04 μ M (c), 0.06 μ M (d), 0.08 μ M (e) and 0.1 μ M (f) concentrations. (B) Maximum fluorescence emission of the sPLA2IIa enzyme upon addition of each concentration of corosolic acid.

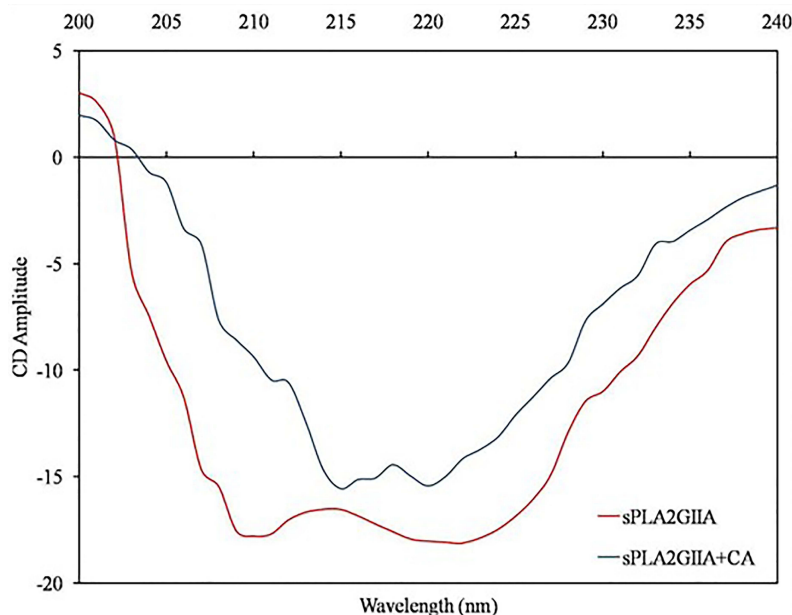


Figure 7 UV CD spectra of sPLA2IIa enzyme: Recorded UV-CD spectra of sPLA2IIa enzyme alone (red line) and with IC_{50} concentration of corosolic acid (blue line).

Table 3 Secondary Structures of sPLA₂Ila Enzyme Before and After Corosolic Acid Treatment

Secondary Structures	sPLA ₂ Ila	sPLA ₂ Ila+Corosolic Acid (IC ₅₀)
α-helix	47.94%	26.75%
β-turn	13.67%	28.81%
Random coil	38.39%	44.44%

in percentage of secondary structure of sPLA₂Ila enzyme. The changes in percentage of the secondary structures of sPLA₂Ila in presence of corosolic acid were calculated (Table 3).

Further, evaluated the efficacy of corosolic acid for neutralising indirect haemolytic activity of sPLA₂Ila. The sPLA₂Ila enzyme exhibits 97±1.23% hemolysis. Addition of corosolic acid reduced the sPLA₂Ila mediated indirect hemolytic activity to 15.75 ± 1.44% in a dose-dependent manner (Figure 8). Distilled water was used as positive control, which showed 100% hemolysis.

In general, the in vitro studies show positive results but do not show efficiency by in-vivo models that is due to heterogeneity of the environment. Administration of the purified sPLA₂Ila enzymes into joints induce inflammatory response with edema (171.51 ± 2.39%), swelling of synovial cells and hyperplasia.^{60–62} Injection of corosolic acid with sPLA₂Ila enzyme (pre-incubated) reduced the inflammatory response (edema) from 171.51±2.39% to 119.3±2.6% with an apparent IC₅₀ value of 7.19 ± 0.34 μM (Figure 9). Conclusively, the in vivo experimental result was in accordance with results of in vitro and in situ experiments.

Many sPLA₂ inhibitors have been widely explored for cancer prevention and treatment strategies^{63–65} by targeting different pathways. The studies showed that corosolic acid can modulate a diverse range of cancer signalling pathways like NF-κB, PI3K/Akt, and Wnt/-catenin and apoptosis.^{66–68} Hence, we hypothesised the current investigation to evaluate the anticancer potency of corosolic acid. Initially treated corosolic acid to PC3-cell line which reduced the viability to 23 ± 2.64% at 30 μM concentration and IC₅₀ value was 9.362 ± 0.56 μM/mL, which was compared to reference molecule oleanolic acid (Figure 10A and B). Oleanolic acid has great structural and functional similarity with corosolic acid and also a known anticancer molecule.⁶⁹

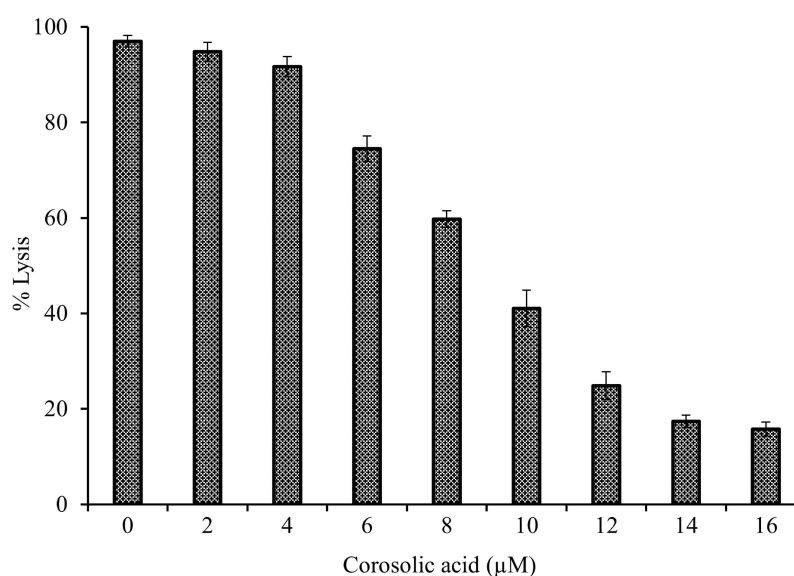


Figure 8 Neutralization of sPLA₂II mediated indirect hemolytic activity by corosolic acid: The 1mL substrate was incubated with sPLA₂Ila enzyme for 30 min at 37°C. The haemolysis effect of sPLA₂Ila enzyme was measured at 540 nm. The reaction mixture without enzyme served as positive control. The data represents mean ± SD (n=3).

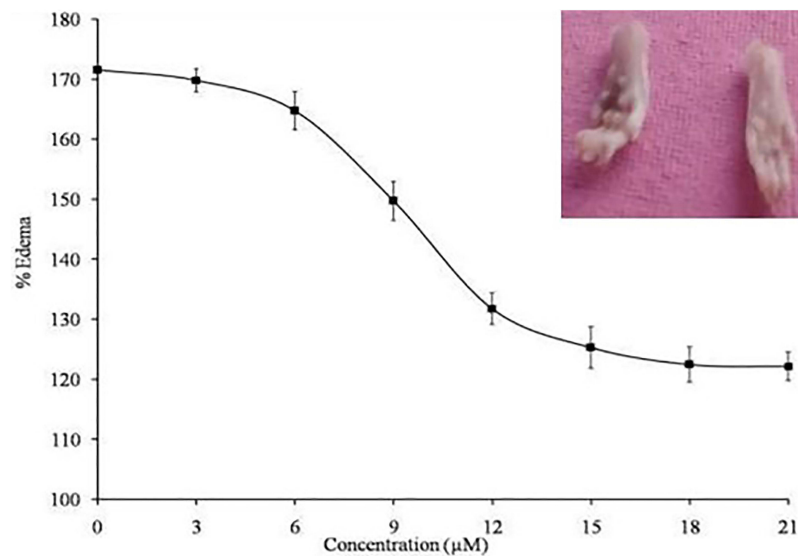


Figure 9 Neutralization of edema-inducing activity of sPLA2IIa: The corosolic acid from 0 to 21 μM concentration incubated with sPLA2IIa and administered to right foot pad of mice. Corosolic acid neutralized edema in dose dependent manner. The data are expressed in mean ± standard deviation (n=3).

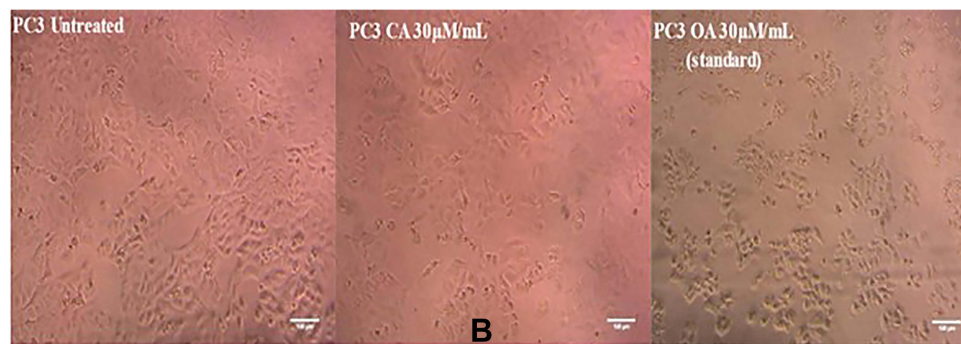
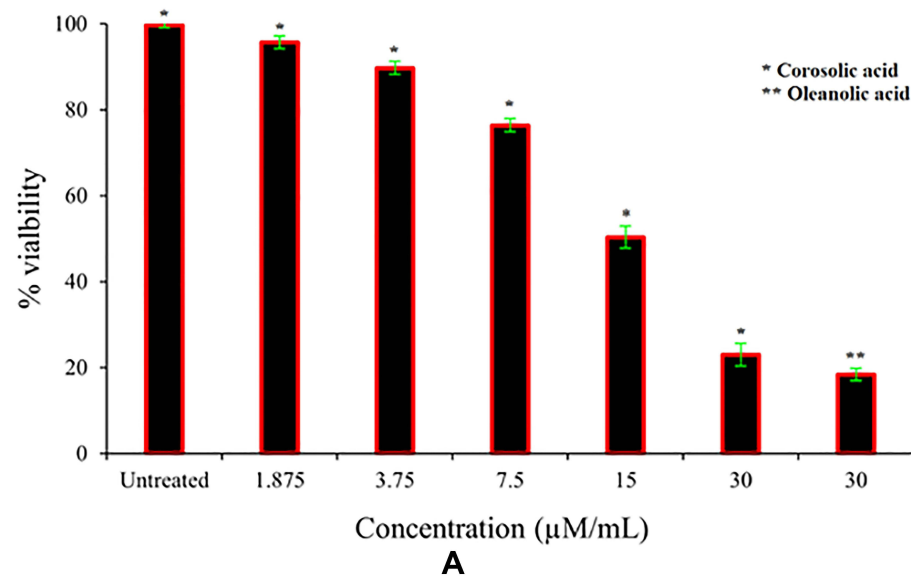


Figure 10 Anti-cancer activity of corosolic acid. (A) Anti-cancer activity of corosolic acid against PC3 cell line. (B) The cell population was recorded after 24h of treatment of corosolic acid and compared to control oleanolic acid (OA) and untreated cells.

Caspases play a crucial role in cell apoptosis or programmed cell death.⁷⁰ Effector caspase 3 is responsible for the actual cleavage of cellular components during apoptosis. Hence, in the present study, caspase-3 level was estimated using FITC rabbit anti-active caspase-3 IgG antibody assay kit (BD Biosciences, catalogue no. 559341). The increase in caspase-3 expression was measured by cell positive FITC (green) fluorescence in the FL1 detector using a 525 nm bandpass filter. The present study showed that cells treated with corosolic acid showed a moderate increase in caspase-3 level (584 MFI) in FITC fluorescence and compared to untreated cells (Figure 11).

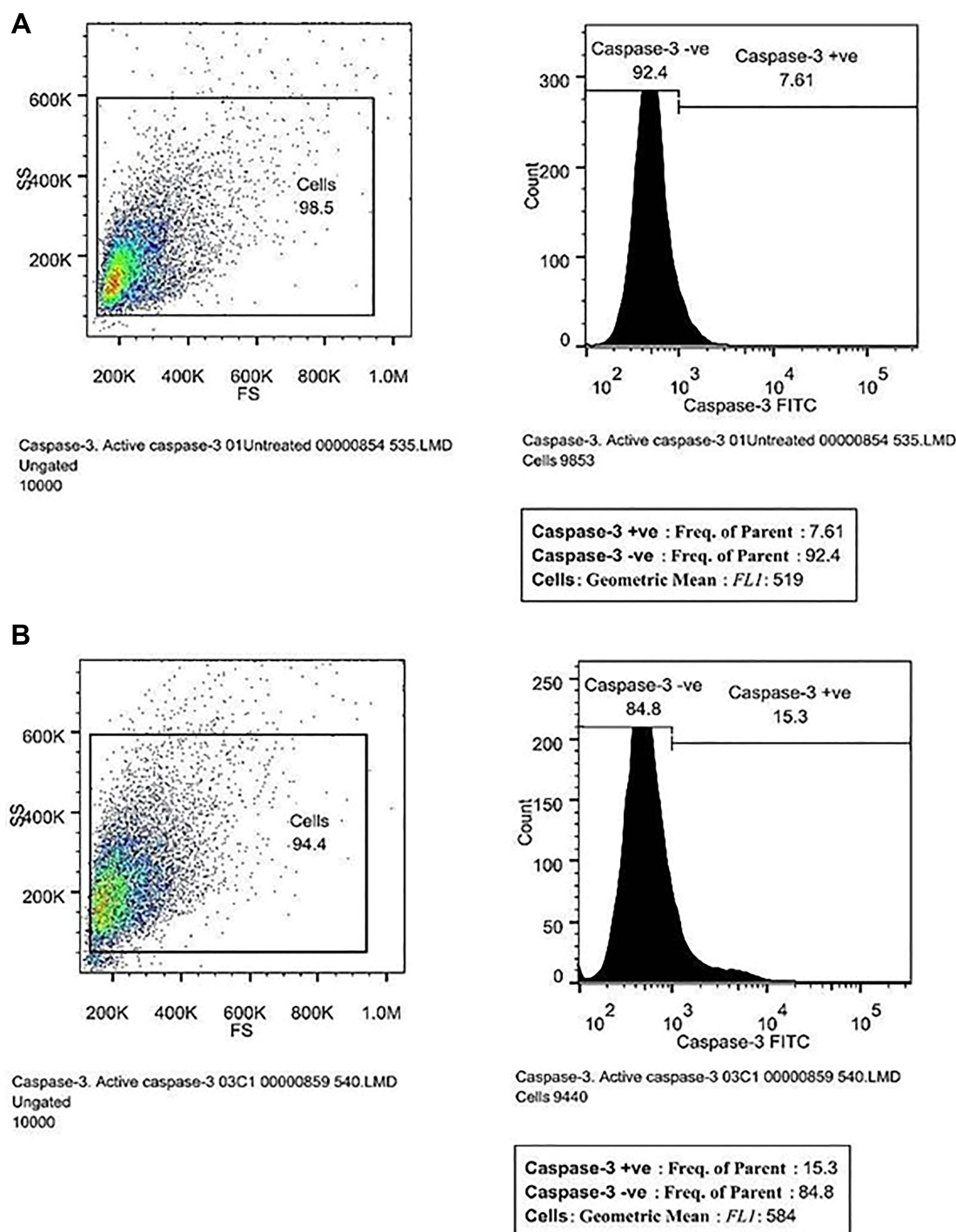


Figure 11 Caspase-3 level was estimated in (A) untreated and (B) corosolic acid-treated PC3 cells.

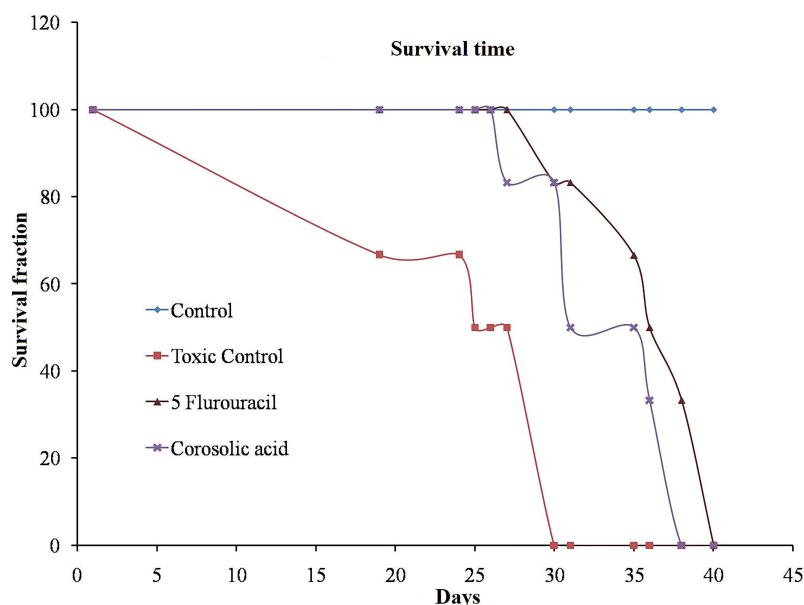


Figure 12 Effect of corosolic acid in mice bearing Ehrlich Ascites Carcinoma tumour.

Animal studies have been of great importance in understanding treatments. Therefore, we have used this EAC-induced mice model to estimate the effect of corosolic acid. Corosolic acid and 5-Fluorouracil (standard molecule) showed a protective effect against EAC-induced mortality. Animals treated with corosolic acid (G3) showed increased MST to 38 days compared to toxic control (G2), which was 30 days, while 5 fluorouracil treatments showed MST upto 40 days (G4) (Figure 12). The toxic control group (G2) injected with EAC showed a significant rise in body weight up to 46.6 ± 4.0 g compared to normal control (G1) body weight of 33.3 ± 2.8 g, in contrast, corosolic acid-treated groups (G3) showed a reduced body weight of 39.1 ± 3.7 g. In addition, toxic control (G2) showed a survival fraction of EAC cells of 56.4 ± 5.9 , whereas the corosolic acid-treated group (G3) decreased the survival fraction to $49.1 \pm 20.9\%$, whereas the standard (G4) showed by 50.9 ± 10.48 (Table 4). Thus, corosolic acid showed significant anti-cancer activity against EAC-induced tumour in mice.

There are reports showing that IL-6 mediates the expression of inflammatory phospholipase A₂ enzymes in most chronic inflammatory diseases.⁷¹ IL-6 increases the intracellular phospholipid (PL), phosphatidylethanolamine (PE) and sphingomyelin (SM); they act as signalling compounds for either inflammation or apoptosis.⁷² The elevated level of IL-6 correlates with prostate cancer burden.^{73,74} Hence, targeting IL-6 would be a novel treatment strategy for various immune-mediated diseases and cancers. Treating LPS ($2\mu\text{g/mL}$) to the PC3 cells results in the pro-inflammatory marker IL-6 expression upto 99.35%. Treatment of different concentrations of corosolic acid to LPS treated PC3 cells reduced the IL-6 expression from 99.35% to $34.36 \pm 2.4\%$ (Figure 13).

Table 4 Antitumor Activity of Corosolic Acid

Parameters	Group 1, Normal Control	Group 2, Toxic/ Disease Control	Group 3, (Corosolic acid)	Group 4, Standard (5-Fluorouracil)
% Viability	NA	56.4 ± 05.9	49.1 ± 20.9	50.9 ± 10.48
% Survival Fraction	33.3 ± 2.8	46.6 ± 4.0	39.1 ± 3.7	34.8 ± 2.8
MST	NA	33	38	40

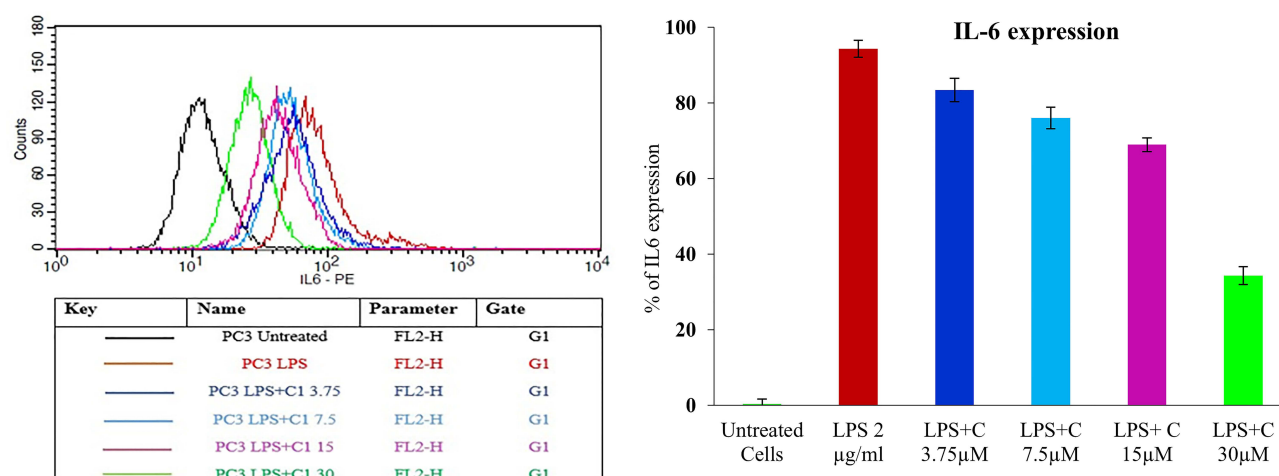


Figure 13 Effect of corosolic acid of 3.75 µM (*), 7.5 µM (*), 15µM (*) and 30µM (*) concentration on LPS Induced IL6 expression PC3 cell line (*).

Conclusion

The sPLA2IIa is an important inflammatory enzyme which plays a very significant role in inflammatory diseases and tumour progression. Investigating effective and safe sPLA2IIa inhibitors is indeed necessary to treat inflammatory diseases and cancers. The current study reports that the corosolic acid has proven as a potential sPLA2IIa inhibitor which neutralised the sPLA2IIa induced indirect haemolytic activity and mouse paw edema in concentration dependent manner, thus proven as an anti-inflammatory molecule. Further, corosolic acid tested for anti-proliferative properties, which reduced the PC3 cell viability, increased expression of caspase 3 level and decreased IL-6 expression. Finally, corosolic acid displayed drug-like qualities by ADME-toxicity analysis. Hence, this molecule may further develop as an anti-inflammatory and anticancer agent for treating the inflammatory diseases and cancers.

Institutional Review Board Statement

The studies on edema and antitumor assays by using Swiss albino mice were conducted according to the guidelines of the Declaration of Indian National Regulations for Animal Research, India and approved by the Institutional Ethical Committee of NGSM Institute of Pharmaceutical Science Mangalore, India (NGSMIPS/IAEC/NOV-2019/166).

Data Sharing Statement

All the data are available within this manuscript. We declare that “The datasets used and/or analysed during the current study available from the corresponding author on reasonable request”.

Informed Consent Statement

All experiments were performed in accordance with AVMA guidelines Animal experiments were performed after obtaining animal ethical approval NGSMIPS/IAEC/NOV-2019/166 (NGSM Institute of Pharmaceutical Science Mangalore) and the human blood sample was collected from healthy volunteers after obtaining ethical approval and an informed consent letter (IHEC-No. MU/IHEC/2018/1).

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Disclosure

The authors report no conflicts of interest in this work.

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