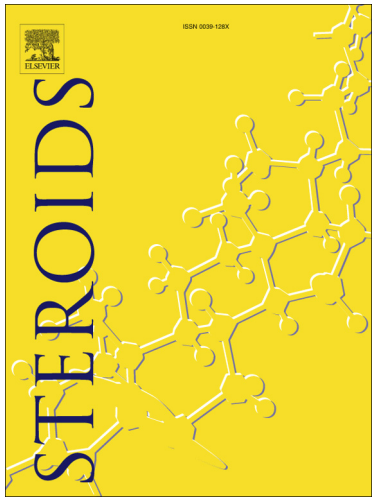

Ichnocarpus frutescens root derived phyto-steroids defends inflammation and algesia by pulling down the pro-inflammatory and nociceptive pain mediators: an *in-vitro* and *in-vivo* appraisal

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Ichnocarpus frutescens root derived phyto-steroids defends inflammation and algesia by pulling down the pro-inflammatory and nociceptive pain mediators: an *in-vitro* and *in-vivo* appraisal.

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Running title: *Ichnocarpus frutescens* derived phytosterols: promising anti-inflammatory agent.

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ABSTRACT

Ichnocarpus frutescens, a climber plant, is distributed all over India. As its different parts are used as anti-inflammatory agent, so we re-investigated the roots to isolate compounds and evaluate its biological efficacy. Also, *in-silico* molecular docking was carried out to elucidate the structure activity relationship (SAR) of isolated compounds toward identifies the drug target enzyme with validation, which was further supported by anti-inflammatory *in-vitro* and *in-vivo* experimental models. The compounds have been undertaken mainly to investigate the anti-inflammatory and analgesic efficacy along with molecular docking investigation followed by anti-proteinase, anti-denaturation and cyclo-oxygenase (COX) inhibition studies. Inflammatory cytokines like TNF-α and IL-6 were assayed from lipopolysaccharides (LPS) and Concavallin (CON A) stimulated human PBMC derived macrophages by Enyme linked immune sorbent assay (ELISA) method. The purity index of the lead compound was determined by HPLC. The compounds were illustrated as 2-hydroxy tricosanoic acid (**1**), stigmasterol glucoside (**2**), stigmasterol (**3**), *β*-sitosterol (**4**) and *β*-sitosterol glucoside (**5**).The test molecules showed significant anti-denaturation, anti-proteinase and analgesic effect validated with docking study. Compounds exhibited anti-inflammatory and pain killing action due to dexamethasone like phytosterol property. Promising anti-denaturation and anti-proteinase activity offered by the compound **5**, may hold its promise to fight against arthritis by rejuvenating the osteoblast cells and destroying the bone-resorpting complex of hydrated protein, bone minerals by secreting the acid and an enzyme collagenase along with pain management. The lead bioactive compound i.e. *β*-sitosterol glucoside (compound 5) demonstrated considerable anti-inflammatory activity showing more than 90% protection against the inflammatory cytokines at 50μM dose. The anti-denaturation and COX-2 inhibition shown by the compound 5 was also noteworthy with the significant IC_{50} (ranging from 0.25 to 2.56 μM) that also supporting its future promise for developing as anti-inflammatory agent. Since the most bio-active compound (5) elicit promising acute anti-inflammatory action and peripheral anti-nociceptive pain killing action with a

significant ED_{50} dose of 3.95 & 2.84 mg/kg i.p. respectively in the *in-vivo* animal model. It could suggest its potentiality as a good *in-vivo* bio available agent to be an emerging anti-inflammatory drug regimen scaffold in the future. It also establishes significant *in-vitro* and *in-vivo* result co-relation.

Therefore, the compound 5 could be believed as a potent lead for designing anti-inflammatory, anti-arthritic drug or pain killer without showing any untoward effect.

Keywords: *Ichnocarpus frutescens, Plant Sterol, Cyclooxygenase-2, proteinase, cytokine, anti-inflammatory.*

1. Introduction

The species of genus, *Ichnocarpus* of family Apocynaceae are climbing shrubs with slender branches. About 15 species are distributed in China, India, Bangladesh, Malaysia, Ceylon, and Australia. Only 2 species are distributed in India [1]. The former species is dispersed through Indian subcontinents and in the adjacent countries, whilst the latter species is scattered in temperate and subtropical Himalaya from Kumaon to Arunachal Pradesh and in Assam, Meghalaya up to 2100 m [2]. *I. frutescens* has been mostly exploited in the traditional remedy for the several diseases. The roots are said to hold diaphoretic, diuretic, demulcent, and alterative tonic properties. It is used in dyspepsia, fevers, and skin troubles. The root powder is provided with milk for the remedy of diabetes, elimination of stone in the bladder and as blood cleanser. Leaves of this plant are boiled with oil and apply in the complication like headaches, fevers and wounds between fingers [3, 4]. The root decoction of the plant along with the Colocynth, Anantamul and Hedyotis root biflora are administered with powdered form of long pepper in persistent skin ailments, loss of sensation, syphilis, and hemiplegia [5]. The roots from *I. frutescens* is used by local human being for healing various types of diseases, such as reduce fever, tonic, snake bite, rheumatism, liver problems and diabetes. We have also reported earlier, the roots of the plant showed significant antioxidant activity comparable to that of standard anti-oxidant di-*tert*-butylhydroxytoluene [6]. In the existing exploration on the roots of *I. frutescens*, we document the isolation and identification of five known compounds in aid of widespread spectroscopic analysis.

Phytochemical study on the whole plant in addition to different parts of the plant has provided the way in identification of 12-pentacyclic triterpenoids, 4-steroids, 7-flavonoids, 3-aliphatics, 4-phenolic acids and 2-carbohydrates [7-15]. The crude extracts of the diverse components of the plant exposed the array of pharmacological outcomes involving antitumor, anti-inflammatory, antioxidant,

ant diabetic, antipyretic, and hepatoprotective and analgesic activities. The plant exhibited antitumor activity against Ehrlich Ascites Carcinoma (EAC) in albino mice [16]. Methanol extract derived from the roots showed substantial anti-inflammatory endeavour in carrageenan-stimulated paw oedema and cotton pellet induced granuloma models in rats. The methanol extract of the plant root showed potential anti-pyretic effect in yeast-induced rat pyrexia model [17]. 70% Alcoholic extracts of leaves, stems and roots showed analgesic activity in hot plate and tail immersion models in rats along with anti-inflammatory action[18].Moreover, this undertaken plant is employed as traditional folk medication to deal with inflammatory ailment akin to piles and blazing sensation [19]. It is well documented from earlier reports that phytosterols like stigmasterol and β-sitosterol have proven promising immunomodulatory effect via regulating B, T-lymphocytes [20]. Therefore, present study is conducted to separate the phyto-steroid enriched fraction and subsequent isolation of the phytosterol. We further extended our undertaken work to inspect the anti-inflammatory, anti-arthritic and pain killing action of the isolated molecules aided with molecular docking study.

2. Materials and Methods

2.1. Plant material

The plant, *Ichnocarpus frutescens* was collected from Kalsi (Jolaibari), Tripura in March 2014. Taxonomist, Prof. B.K.Datta, Tripura University, Department of Botany identified the plant with a voucher specimen (#BD/06/08).

2.2 Investigational Animals

Either sex, Swiss Albino mice, weighing 20–25 g and healthy male albino adult rats weighing 120–180 g were undertaken for *in vivo* work. Animals were endorsed by Institute animal ethical committee (**Ref.No.41/IAEC-IPT/2018**) and set apart under preferred laboratory prerequisites for pharmacological evaluation. Carrageenan induced acute-inflammation in Wistar rat model was implemented to assay the anti-inflammatory property of the test compounds. Acetic acid intervened peritoneal constrictions develop peripheral nociceptive ache in albino mouse that appraise the analgesic activity of test compound as per recognised model.

2.3. Extraction and isolation

Fresh air-dried roots of *I. frutescens* were dried in shaded ground and mashed into coarse powder. Dehydrated coarse powder (1.5 kg) was extracted with MeOH (4L × 3) at room temperature for seventy two hours. The MeOH extract used to be concentrated underneath decreased pressure in vacuum to a gummy mass (90 g). The residue (90 g) was once suspended in H_2O (100 mL) and fractionated into hexane (5 g), petroleum ether (8 g), chloroform (15 g), ethyl acetate (18 g) and *n*-butanol (25 g) soluble fractions by partition between water and hexane (1:1), water and petroleum ether (2:3), water and chloroform (2:3), water and ethyl acetate (2:3), water and *n*-butanol (1:1), successively.

Petroleum-ether fraction was further passed to Silica gel CC. The elution of the column with petroleum-ether & ethyl acetate (9:1) gave a solid, which on crystallization from petroleum ether-$CHCl_3$ mixture rendered a white amorphous powder, mp 76–77°C, which was homogeneous in TLC and was once recognised as 2-hydroxy tricosanoic acid (**1**) as per NMR and MS spectral study.

The $CHCl_3$ fraction was passed to Silica gel CC. Extraction of the column materials with PE-$CHCl_3$ (6:1) afforded two needle shaped white compounds on TLC and on repeated CC afforded two compounds in colourless needles, mp 136°C and mp 156°C. These compounds on spectral (MS and 1H-NMR) studies were identified as *β*-sitosterol (**4**) and stigmasterol (**3**).

The EtOAc fraction was passed through CC over Silica gel. The elution of the column with EtOAc-MeOH (9:1) gave a crystalline solid, mp 270°C, which was homogeneous in TLC and was identified as stigmasterol glucoside (**2**) on the basis of ^{1}H- and ^{13}C-NMR and MS spectral study. Elution of the column with EtOAc-MeOH (7:1) gave another crystalline solid, mp 283–285°C, homogeneous in TLC and was identified as *β*-sitosterol glucoside (**5**) based on spectral (^{1}H- and ^{13}C-NMR and MS) analysis and consultation of literature.

2.4. General Experimental Procedures

Melting points were determined by using of Kofler type electrical melting point apparatus. All the analytical samples were examined for homogeneity on TLC plates in various solvent systems. TLC plates were organized in glass plates using slurry of Silica gel G (Merck, India) in EtOAc/ethanol and the spots of plates were visualized by either exposing the plates in iodine chamber or spraying with 10% H2SO4 in ethanol observed by heating at 1100C. Silica gel (mesh 60-120, Merck, India) was utilized for column chromatography (CC). CC and TLC were performed at room temperature

(20–30^{0}C). UV-VIS spectra were recorded Perkin Elmer Lambda 25 spectrophotometer and were expressed in λ_{max} solvent nm (log ε). IR spectra in KBr disc were documented on a Shimadzu 8100 FT-IR spectrophotometer and were expressed in ν_{max} cm^{-1}. ^{1}H, ^{13}C and 2D-NMR spectra were recorded by using Varian XL-300, 400 and 600MHz NMR/Bruker Avance II 600 MHz NMR spectrometer. Chemical shifts were articulated in δ (ppm) with tetramethylsilane (TMS) as an internal standard, and the coupling constants were in hertz (Hz). EI-MS, HR-EI-MS were taken using a JEOL JMS 700 mass spectrometer and HR-FAB-MS were evidenced on a Jeol JMS-HX 110 mass spectrometer. In MS the mass ion peaks were given in *m/z* values with their relative abundances in % with respects to the base peak in a spectrum. NMR-DEPT experiments were carried out with flip angle θ of 45°, 90° and 135°. Spectral data of all isolated compounds has been given in the supplementary file. .

2.5. Evaluation of in-vitro anti-inflammatory assessment

2.5.1. Anti-protein denaturation evaluation

5% aqueous 2.40 mL bovine serum albumin and 0.10 mL of 0.2 % DMSO solution consisting of compound 2,3,4, 5 (1, 5, 10 and 50 µM concentrations) were added in the reaction tube. Then it was adjusted at 6.3 using a small amount of 1 N HCl. The incubation was carried out of all the reaction tubes at 37^o C for 20 min. Then the samples were heated at 57^{0}C for 30 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube to adjust the final volume of 5 ml. Turbidity was determined in spectrophotometer [21] at 660 nm wavelength compared to untreated controls. Test 0.10 mL DMSO was used alternatively of fraction while bovine serum albumin was absent in the product control test as per approach adopted. The percentage inhibition of protein denaturation was computed as follows.

$$\% \text{ of inhibition} = 100 - \frac{(\text{O.D. of test} - \text{O.D. of product control}) \times 100}{\text{O.D. of Control}} \quad [21]$$

2.5.2. Proteinase inhibitory action assay

The reaction mixtures (2.0 mL) contained 0.06 mg trypsin, 1.0 mL. 25 mM tris-HCl buffers (pH 7.4) and 1.0 mL solution[22] containing 1, 5, 10 and 50 µM of compound 2, 3, 4, and 5 dissolved in 0.2% DMSO. The mixtures were incubated at 37^{o}C for 5 minutes, and then 1.0 mL of 0.8% (w/v) casein

was added. The mixtures were further incubated for 20 minutes. 2.0 mL of 70% (v/v) perchloric acid was added to the mixtures to stop the reaction proceeding. The cloudy suspension was once centrifuged. The absorbance of the supernatant solution was determined at 280 nm wavelength against buffer as blank [22]. The percentage of inhibition was calculated as per the following the formula and compared with positive controls of dexamethasone [22].

% of Inhibition = 100 - [(O.D of test solution- O.D. of only STF-HAENS fraction without casein-trypsin enzyme reaction) / (O.D of control) × 100][22]

2.5.3. Membrane stabilization studies with the isolated phytosterol compounds.

The assay mixtures (4.5 mL) contained 1 mL 0.15 M phosphate buffer saline (pH 7.4), 2 mL hypotonic saline (0.25% NaCl), and 1.0 mL aqueous solution containing 0.2% DMSO of compound 2,3,4, 5 (1, 5, 10 and 50 µM of final volume). 0.5 mL HRBC suspension [10% v/v] in normal saline was added. For controls, 1 mL of isotonic saline was used instead of test solution while red blood cells was absent in the product control [23]. The mixture was incubated at 56^{o} C for 30 minutes. The tubes were chilled under running tap water for 20 minutes. Then centrifugation was carried out for the cooled mixture tube and absorbance of the supernatants was determined at 560 nm [23]. Percent membrane stabilizing activity was calculated as follows:-

$$\% \text{ of stabilization} = 100 - \frac{(\text{O.D. of test} - \text{O.D. of product control}) \times 100}{\text{O.D. of Control}} \quad [23,24]$$

The control represents 100% lysis. The result was compared with dexamethasone treated samples.

2.6. In-vitro COX-1 and COX-2 inhibition assay with recombinant enzyme

Assay of COX-1 and COX-2 inhibition was carried out with commercially accessible colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, MI; lot 184104) containing the recombinant enzyme. Compound 2, 3, 5 was added to the reaction system at graded concentrations (ranging from 1 to 50 µM), dissolved in 0.2% DMSO, and prepared just before use. In this assay, the COX activity was evaluated by using *N, N, N', N'*-tetraethyl-*p*-phenylenediamine (TMPD) as a co-substrate with AA (reduction of PGG2 to PGH2) [24]. TMPD oxidation was once determined with ELISA reader at 590 nm. No colorimetric variation was observed in control where incubations were carried out through leaving out enzymes or heat-denatured enzymes and without fraction in combination with TMPD [24].

2.7. HPLC assessment of lead compound: β-sitosterol glucoside

In order to characterize the lead compound(β-sitosterol glucoside) HPLC study was done in the C-18 reversed-phase column (Waters; 2.1 ×50 mm; 1.7 μM) at a flow rate of 120 μL/min using an isocratic mobile phase (methanol:acetonitrile:water:formic acid = 45:40:14.8:0.2 (v/v/v/v)). After injection of the test compound 5, the sample run time was set for 30 minutes. UV detector was set at 380 nm for developing chromatograms. The standardisation of the test compound within the chromatogram was inferred based on their individual specified retention-time and area under the curve (AUC).

2.8. In vitro pro-inflammatory cytokine assay

PBMCs (Peripheral blood mononuclear cells) were extracted from the healthy volunteers, taken approval before withdrawing blood (without known significant health disorders who participated in this study approved by the Institute ethical committee). PBMCs were purified using Ficoll-Paque gradient centrifugation method[24]. The tubes were centrifuged for 20 min at 1,020 × *g*. The cell interface layer was washed twice in PBS carefully (for 10 min at 640 × *g* followed by 10 min at 470 × *g*) after harvesting. It was then reconstituted in RPMI 1640 medium supplemented with penicillin (50 U/mL)-streptomycin (50 μg/mL) ,10 mM HEPES and Glutamax before counting. PBMCs (2 $\times 10^6$) were added in 1 mL of above medium with colony stimulating factor-1(CSF-1), incubated for 5 days. Macrophages were developed for further study in 6^{th} day. Then the cultures were incubated for 24 h with 20 ng/mL of lipopolysaccharide (derived from *Escherichia coli* O55:B5,Sigma) and Concanavalin A(2.5 μg/mL) to stimulate the proinflammatory cytokines TNF-α and IL-6. Compound 5 was added at graded concentrations (5-50μM) for 24 h incubation after lipopolysaccharide (*Wild E. coli 055: B5*, Sigma) and CON A treatment. After incubation, the cells were separated by centrifugation for 10 min at 1500 rpm. TNF-α and IL-6 were estimated from the culture media cell supernatant with ELISA kit as per manufacturers' protocol (BD pharmingen) and earlier methods accordingly [24].

2.9. In vivo acute anti-inflammatory evaluation

A carrageenan induced paw oedema model was performed in rat as our preceding report [25]. Wistar rats of either sex was weighed, randomized and segregated into 5 groups (n = 6). Firstly, the right paw volume of each animal was estimated using a plethysmometer. Group I was served as control (V_c) receiving vehicle only (10% Tween 80 in distilled water). Group II was received Indomethacin (10 mg/kg) as positive

control(standard), and Group III, IV & V were treated with the test compounds of β-sitosterol glucoside (5 and 10 and 20 mg/kg), selected based on the earlier published literature. Plantar side of the left hind paw of each experimental rat of all groups received 0.05 ml of 1% w/v freshly prepared carrageen an after 30 minutes The paw volume (Vt) was determined every 30 min up to 2 h. % inhibition of inflammation in-terms of peripheral edema in each group was calculated using the following formulae:

% inhibition=(Vc- Vt)/Vc x 100; [25]

where V_c = mean variation of oedema for the control group;

Vt = mean variation of oedema.

2.10. Peripheral nociceptive activity study (acetic acid induced writhing response model)

Twenty-four hours prior to actual testing a large number of mice (20–25 g) were taken. Writhing movements were checked in all animals to investigate analgesic effect following the method of [26, 24]. All the positive respondent mice [26] were divided into 5 groups consisting of 6 mice in each group. The first group was marked as control and treated with 10% Tween 80 in distilled water (vehicle). The second group was received with standard drug Diclofenac **1**0 mg/kg i.p. as positive control. The third, fourth and fifth groups were received test drug (β-sitosterol glucoside) intra-peritoneally at a dose of 1, 3, and 6 mg/kg i.p. (chosen based on the earlier reported literature and LD_{50} dose of β-sitosterol glucoside) half an hour prior to glacial acetic acid challenge respectively. The test phytomolecule was injected intraperitoneally to the experimental mice as stated above. Then 0.6% glacial acetic acid was once administered intraperitoneally to each experimental mouse@10 ml/kg body wt dose. Finally, each mouse was monitored for the total number of writhing performed for 15 min following glacial acetic acid injection.

2.11. Statistical analysis

All the data and results obtained from the experimental studies were interrelated statistically using Graph Pad Prism Software (version 5). Every experiments, three replication was executed and the results have been presented as mean ± standard error mean. Statistical correlation between compounds and biological activities was also additionally investigated among untreated controls, and various treated groups by unpaired student *t-test*; *P* values *<0.05 or <0.001 and p < 0.0001*

were considered statistically significant. The IC_{50} values were determined by sigmoidal fitting of the data in the Graph Pad Prism version-V program by linear regression analysis (probit calculation).

3. Results and Discussion

3.1. Effect of compound 2, 3 and 5 on protein denaturation, proteinase inhibitory action, membrane stabilization

In vitro protein denaturation in BSA model was remarkably inhibited by compound **5** showing 80.45% to 99.67% protection at the doses ranging from 1 to 50 µM respectively. These findings showed 41.6 times better activity compared to results obtained by standard drug dexamethasone (positive control) at equivalent dose. All the tested compounds **5**,**4**,**3**,**2** demonstrated anti protein denaturation effect in dose dependent manner with the $\mathbf{IC_{50}}$ values of 0.25, 35.4,1.25 and 25.4 µM respectively. Furthermore compound **5** protected 90% RBC lysis triggered by the hypotonic saline solution at 7.75 µM. As per result, the compound **5** was found to be 11.9 times more potent than standard drug dexamethasone at equivalent dose as depicted in Table 2. The rest of the compounds **4**, **3** and **2** also showed stabilisation in human RBC with the ED_{50} dose of 4.56, 5.89 and 21.4 µM correspondingly. Compound **5** at 4 different graded doses (1, 5, 10 and 50 µM displayed significant protection against trypsin (proteinase) mediated casein degradation activity by 20.58, 80.35, 94.79 and 99.1 % inhibition respectively. Anti-proteinase activity of Compound **5** was 2.94 fold higher than the standard drug (dexamethasone) at IC_{50} dose.

Denaturation of proteins is a well recognized cause of inflammation and rheumatoid arthritis. Several anti-inflammatory drugs have demonstrated dose dependently inhibition on thermally induced protein denaturation. Amendment in hydrogen, electrostatic, disulphide & hydrophobic bonding in the protein structure is taken place during the denaturation of protein. Hence we assessed the defensive role of test compounds defends the protein denaturation elicited by heat and tissue protein damages triggered by the proteinase enzymes (MMP) during the onset of arthritis and inflammatory disease progression. This phenomenon may guide to understand the *in vitro* anti-arthritic mechanism. [27, 21]. Our results may be suggested that the compound **5**, **4** and **3** may possess the antiproteinase activity that may lead to attenuate the arthritic syndrome by repairing the cartilage injury in the bone collagen [28].

The result obtained from the study reveals that compound **5** demonstrates very significant activity against the anti-inflammatory and anti-arthritic drug screening model. As compound **5** is phytosterol

glucoside called as β-sitosterol glucoside**.** This compound is structurally *very* much similar to standard drug dexamethasone which is already been found as potential anti-arthritic and disease modifying anti-inflammatory agent [29]. In earlier publication, we accounted that the bioactive fraction enriched with phytosterol could be used as potential anti-inflammatory agent by as well [25].The anti-inflammatory activity of β-sitosterol in experimental IBD and in atherosclerosis has been reported [30, 31].In atherosclerosis, the phytosterols exhibited their anti-inflammatory activity by up-regulating the anti-inflammatory cytokine, IL-10 and down-regulating the pro-inflammatory cytokine, IL-6. Here, the 4 hydroxyl groups in the aglycone part of compound **5** have given enough binding capacity to inhibit the proteinase activity. The saturation at 22nd position of the tested compound has given enough flexibility to bind with the active site of proteinase for propagating the anti-arthritic effect. Moreover, the compound **5** exhibited best activity in all anti-inflammatory *in vitro* models among all tested compounds. The best result had been shown by the compound **5** may due to its higher binding capacity with the active sites of the test enzyme (proteinase). Glycosylated 4 hydroxyl functional group in the glucose moiety of compound **5** may be the contributing factor for strong binding with proteinase activity to demonstrate the strong and better inhibition in comparison to standard drug dexamethasone. The significant anti-proteinase and anti-denaturation effect of compounds **5** and **3** may lead to explore these compounds as promising anti-arthritic and protective agent against inflammatory and nociceptive pain as validated by earlier methods [25, 32].

3.2. Effect of compounds 2, 3 and 5 on COX Activity

The inhibitory effects of compounds 2, 3 and 5 on COX-mediated TMPD oxidation activity were evaluated using purified COX as enzyme sources. Although COX-1 activity was not significantly turned down on exposure of compounds **5**,**3**,**2** up to 50 µM. Whereas COX-2 activity was strongly inhibited by compounds **5**,**3**,**2** treatment, demonstrating IC_{50} of 0.75, 5.6 and 1.2 µM. The inhibitory magnitude of COX-2 by the compounds **5**, **3**, and **2** was markedly higher than that of COX-1. Compound 5(*β*-sitosterol glucoside) and other test compounds experimentally validated the binding score of docking studies against COX-2 in a significant manner. The increased binding affinity of compound 5 towards Ser 531 due to presence of larger group of hydrophobic region in the molecule has correlated with the strong inhibition against COX-2 in the wet experiment. These findings may deduce that the compound **5** could be recommended as a selective COX-2 inhibitor with a significant selectivity index of 45. Because, its selectivity index related to COX-2 selectivity was found to be

best among all tested compounds. This result was quit comparable to the selective COX-2 inhibitor, celecoxib (Table 3). The data indicated that the compounds **5** and **2** could be featured to the down regulation of prostaglandins synthesis by selectively blocking COX-2, which was expressed in the subsequent site of inflammatory disorders and nociceptive pain. The selectivity of COX-2 over COX-1 suggested that the compound **5** might be applied for acute pain management devoid of some unpleasant side effect which was associated with specific COX-1 inhibitor drugs. These findings implied that the compound **5** could be emerged as a potential therapeutic alternative for several inflammatory pathologies. [33].

Our lead compound may block the all possible ecosanoids mediators including prostaglandin related to nociceptive pain by selective inhibition of COX-2 like other phytosterol and Celastrol with new therapeutic frontiers [34, 35]. This outcome may recommend that test compound could be implicated in the management of additional cartilage degeneration along with inflammation and pain [36].

3.3. Characterization of compound 5 by HPLC investigation

As depicted in Figure 3, preliminary HPLC investigation recommended 1 major peak in the chromatogram. The chromatogram represented the lead test compound *β*-sitosterol glucoside, showing its peak in the retention time at 13.6 minutes. However, one single small peak in the retention time at 7.8 minutes with an AUC 209 indicating the very little concentration of impurity is there. As per the AUC level of both the sample peak including active major lead compound **5**, 93.56% purity was found.

*3.4. Compound 5(β-*sitosterol glucoside) *elicit significant anti-inflammatory action by diminishing pro-inflammatory cytokines, TNF-α and IL-6.*

LPS and CON A treated Human PBMC derived macrophage cells demonstrated a substantial up regulation in the levels TNF-α and IL-6 in the culture supernatants (Figure 4). Compound 5 reduced the levels of both the inflammatory cytokines significantly in a dose dependent manner. Compound 5 demonstrated 9.29 (89.25%) fold reduction ($P < 0.0001$) on LPS+CON-A induced IL-6 production at 50 µM compared to untreated controls. Other doses like 5 and 10 µM executed 1.88 ($P < 0.001$) and 6.58 ($P< 0.0001$) fold suppression of IL-6 production significantly in comparison to untreated controls, respectively (Figure 4b). Whereas, attenuation of the potent pro-inflammatory cytokine TNF-α by 74 %($P< 0.0001$), 82.67% ($P < 0.0001$) and 94.67 % ($P < 0.0001$) was executed by the compound 5 on exposure to 5,10 and 50 µM dose, in comparison to untreated controls respectively

(Figure 4a). The results of the anti-inflammatory cytokine activity elicited by Compound 5 (β-sitosterol glucoside) illustrated that it could be the promising immunoregulator to ameliorate the strong inflammation triggered by any kind of infection, wound, oedema and allergy etc, as it showed powerful anti-inflammatory effect in the human cytokine model. These findings were also supported by the earlier reports [37] of same kind of phytosterol in the murine macrophages model of inflammatory cytokines. Compound 5 has undertaken for the anti-inflammatory cytokine assay, as it demonstrated best results among the all tested compounds (2, 3, 4 and 5) against COX-2, MMP-13, protein denaturing and anti-proteinase activity assessment. This compound could be explored as promising anti-inflammatory agent against the numerous chronic immune-mediated abnormalities, including chronic viral infections, tuberculosis, rheumatoid arthritis, allergies, cancer, and auto-immune diseases via targeting the T-helper lymphocytes and Natural killer [38].

3.5. Compound 5 i.e. β-sitosterol glucoside exhibited in-vivo acute anti-inflammatory action in rat model

Current study, we have validated our *in-vitro* anti-inflammatory findings elicited by compound 5 by performing the *in-vivo* acute inflammation induced by carrageenan in the rat paw oedema model, as well. Earlier study [39] suggested that β-sitosterol glucoside isolated from *Mentha cordifolia Opiz* protected 16.67% peripheral oedema in carrageenan induced hind paw oedema model of mouse at single dose of 100 mg/kg. But it is very difficult to calculate the ED_{50} dose (very necessary to investigate the therapeutic window/index related to future drug development) of any molecule with one single dose study. Hence, in order to understand the drug mediated better protection level against acute inflammation through *in-vivo* system, we have carried out the 0.1 ml of 1% w/v freshly prepared carageenan induced paw oedema in established well-known wistar rat model with the graded doses of *β-sitosterol glucoside*. It shows superior level of % inhibition against rat paw hind oedema intruded acute inflammation in wistar rat very specifically in comparison to β-sitosterol [40] related to structural activity relationships owing to presence of β-D glucosyl moiety (aglycone part) in compound 5 glycosides. The test compound 5 has exhibited significantly 85.56%, 95.56% and 98.89% reduction (Table 5) of rat hind paw oedema at 5, 10 and 20 mg/kg doses respectively by concentration dependent manner after 2 hrs of carrageenan injection. These results could be more rational than the earlier reports [39]. Carrageenan induced paw oedema model is a biphasic pharmacological response, in which initial phase histamine, bradykinin and serotonin like

inflammatory mediators [41] are released within 2 hrs of paw hind carrageenan injection . Therefore, the β-sitosterol glucoside mediated significant anti-inflammatory effect has been obtained at 2 hours. These have been shown as maximum protection against the acute inflammation. These noteworthy anti-inflammatory actions may occur perhaps due to presence of sterol moiety [42] in the glycoside molecule (compound 5). The ED_{50} dose of lead compound as acute anti-inflammatory agent has been found to be 3.95 mg/kg i.p. (determined from the probit calculation), which is quit significantly comparable to the reference anti-inflammatory drug indomethacin. Carrageenan is a complex polysaccharide. It can attach Toll-like receptor 4, activating B cell lymphoma/leukemia 10(Bcl10), the NF-kβ and IκBα pathway to induce inflammation [43]. Probable stimulation of inflammatory bio-markers [44], like COX, IL-6, MMP-13 etc could be triggered by the above mentioned cascade. Furthermore, during carrageenan induced inflammatory paw-oedema, vasodilatation of blood vessels/capillaries occurs may due to upregulation of proteinase and protein denaturation [45] that may mediate the acute-inflammation. Thus the evaluating agents (compound 5) might be showing acute anti-inflammatory properties by blocking the above pathway. All the above cited cascade of carrageenan mediated *in-vivo* pathway has been revalidated and co-related with the execution of anti-TNF-α, anti-IL-6, anti-proteinase and anti-denaturation *in-vitro* results of β-sitosterol glucoside. Thus we may deduce that the *in-vitro* and *in-vivo* observation have been nicely established for better understanding of anti-inflammatory activity.

3.6. Compound 5 mediates peripheral analgesic effect in mouse model.

The *in-vitro* COX inhibition assay has also been corroborated *in-vivo* system through the acetic acid mediated peripheral nociceptive pain remedy. Nirmal *et al*., 2012 [40] reported the β-sitosterol phytosteroid, derived from medicinal plant, *Nyctanthes arbortristis* leaves and it demonstrated 52.25% , 49.13% and 43.53% protection against the peripheral nociceptive pain at 20, 10 and 5 mg/kg dose respectively. Whereas present study, our lead compound β-sitosterol glucoside has illustrated 58.73%, 50.79% and 46.03% protection(Table 4) against nociceptive peripheral pain at 1, 3 and 6 mg/kg dose with much improved level of pain killing effect compared to parent compound (β-sitosterol) probably may due to better absorption and distribution of the molecule towards the affected pain site owing to linkage of glycosidic bonded glucose moiety. Since, the compound 5 has shown significantly better results even in 3.34 fold lesser dose in comparison to parent compound β-sitosterol (20 mg/kg) [40]. Furthermore, β-sitosterol glucoside isolated from *Mentha cordifolia Opiz* [39], had been reported for 73% nociceptive pain inhibition at 100 mg/kg dose in 0.7% acetic acid

induced writhing model with single dose. However, their test dose was 16.67 times higher than our highest analgesic dose (6 mg/kg). Furthermore, it was very difficult to assess the ED_{50} dose with one single dose study. Herein, we have evaluated the analgesic ED_{50} dose (2.84 mg/kg i.p.; determined based on the linear regression analysis of probit calculation from the dose vs. peripheral analgesic response curve) of β-sitosterol glucoside in 0.6% acetic acid induced writhing mouse model by taking 3 graded concentrations (1, 3 and 6 mg/kg) to standardise the pharmacological activity against nociceptive pain as peripheral analgesic. Standard drug diclofenac sodium has demonstrated 68.26% nociceptive pain protection at 3 mg/kg body weight i.p., which is quite comparable with the therapeutic dose of compound 5. Our test molecule may elicit the analgesic activity due to prevention of liberation of bradykinin and prostaglandin which produces pain through the activation of chemosensitive nociceptors or irritation of visceral surface triggered by the intraperitonal booster of acetic acid [46]. Hence the note-worthy *in-vitro* COX inhibition results elicited by the β-sitosterol glucoside have been authenticated with the *in-vivo* peripheral analgesic effect, remarkably.

Conclusions

I. frutescens root has rendered five known phytochemicals through phytochemical isolation followed by the chemical characterization. First time, the isolation of compounds **1-5** derived from this plant has been reported. This plant derived ethyl acetate and chloroform fraction separated from crude methanol extract gave the few phytosterols. However these fractions also gave flavonoids, but steroids enriched bioactive fractions demonstrated better anti-inflammatory activity compared to flavonoids riched fractions (data not shown). Hence present study we have interested to isolate those phytosteroids for focusing them as anti-inflammatory agents. The roots of this plant is used by the local traditional people for the treatment of various kinds of diseases, such as reduce fever, tonic, snake bite, **rheumatism**, liver disorders, pain, **inflammation** and diabetic as folk medicine. The isolated compounds specially compound 5 (β-sitosterol glucoside) could be focused as potential nociceptive pain killer and anti-inflammatory agent for the treatment of rheumatic arthritis and other cartilage degenerative disorders. 4 hydroxyl groups of the aglucone part and saturation in the 22 **position** of the side-chain of phytosterol may play crucial role for showing better activity against inflammatory arthritis and pain. They help to form a stronger binding with the target enzyme's active site residue.

From the earlier literature and the present results, it could be inferred that carrageenan & acetic acid as external chemical stimulators may generate ROS in the *in-vivo* models. This ROS further may activate the T-cells to liberate the pro-inflammatory cytokine TNF-α by auto-immune response. Thus it may cause inflammation. This pro-inflammatory cytokine may again triggers the NF-kβ (nuclear factor k-beta) pathway via IKK-β/α (inhibitor of nuclear factor k-beta/alfa kinase), which may further expresses the cyclooxygenase enzymes to produce prostaglandins. Hence it creates nociceptive pain in the body. This activated over-expressed COX pathway further stimulates iNOS & IL-6 (pro-inflammatory cytokine). Finally this cascade may cause activation of MMP-13 like proteinase at downstream, which may induce chronic inflammatory disorder of arthritis at end-stage due to joint cartilage protein denaturation. Our test compound 5 may attenuate the inflammation, pain and chronic arthritis syndromes by down-regulating the above cited cell-signalling pathway mediators like TNF-α, COX-2, IL-6 , MMP-13 and protein denaturation as supported by the *in-vitro* and *in-vivo* assay.

Thus, we have clarified how peripheral nociceptive pain (COX) and pro-inflammatory mediators (TNF-α, IL-6, proteinse and MMP-13) were knocked down by the lead compounds to alleviate the cascade of pain and inflammation. We have hypothesized the future prospect of β-sitosterol glucoside as anti-rheumatoid arthritis agent in connection to its anti-denaturation and anti-proteinse inhibitory action. This explanations and findings have properly been depicted by us in comparison to the earlier reports [39, 47, 40].

It concludes that compound **5**(β-sitosterol glucoside) may confirm to be a helpful therapeutic approach to the treatment of RA, inflammatory and nociceptive pain. These phytosterol could also be highlighted as promising anti-inflammatory as well as immunity booster agent as validated by previous report [47]. Moreover our isolated phyto-steroidal the lead molecule (β-sitosterol glucoside) had shown better anti-inflammatory activity compared to earlier reports of withanolides [48] and steroidal saponin [49]respect to inflammatory mediators. Simultaneously, the lead compound might be exhibited very amazing *in-vivo* anti-inflammatory results possibly due to existence of novel phytosterol scaffold linked with glucose moeity in the molecule. Since, this phytosterodal glycoside moiety may possess optimum pharmako-kinetic parameters like good absorption and distribution. Its hydrogen bond donor and acceptors are 4 and 6 respectively. The pKa and logP values are within the desirable range to acquire the drug like properties. Moreover, the test compound does not exhibit as

such apparent untoward effect and adverse reaction on the experimental mice and rats with the therapeutic doses (data not shown). The maximum anti-inflammatory effect has been found in rat model as much more impressive and significantly better than the maximum *in-vivo* peripheral analgesic effect within their therapeutic window level of dosing range. However, this differentiation has not been reflected in case of the both the *in-vitro* results of analgesic and anti-inflammatory activity. These findings may suggest that the compound's pharmacokinetic parameters (ADME) related to absorption in systemic circulation and distributions towards the locus of action could be superior in case of Wistar rat species rather than swiss albino mice. Therefore the *in-vivo* anti-inflammatory activity shows enhanced results compared to *in-vivo* analgesic action. Consequently, these results may provide valuable informations for designing emerging potential lead with further modification of existing scaffold through Q-SAR studies against the inflammatory disorders like arthritis and nociceptive pain with safe drug target with impressive biological action.

However, whether this lead or its derivative are active specifically against rheumatoid arthritis and osteo-arthritis disease model or not, needs to be assessed with prime interest coupled with their mechanism of alleviating action in the path physiological signalling pathway, in the future studies.

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Conflicts of interest

The authors have declared that there is no conflict of interest.

Figure legends

Figure 1: Isolated phyto-compounds 1-5 have been represented with their structure. 2-Hydroxy tricosanoic acid (1), Stigmasterol glucoside (2), Stigmasterol (3), β-Sitosterol (4), β-Sitosterol glucoside (5)

Figure (2X): Figure (**A**) and (**B**) shows docking pose of test compounds 3 and 5 with MMP-13(**PDB ID: 5BOT**). Compound 3 and compound 5 have involved in the common hydrophobic interactions with Ala 186, His 232 and Tyr 244. In case of compound 5, the hydroxyl groups of the sugar moiety provided strong H-bond (----) interactions with Gly 183 and Tyr 244. These interactions are playing a crucial role for activity. (**C**) Illustrate the binding interactions crystal structure of reference compound. Figure (**D**) and (**E**) confirmed almost similar binding mode of compound 3 and 5 with dexamethasone (yellow) and prednisolone (white) respectively. Again (**F**) superimposition of dexamethasone and prednisolone shows their almost similar binding mode towards the binding pocket of reference compound.

Figure (2Y): (**A**), (**B**) and (**C**) indicate the docked conformation of compound 2, 3 and **5** with cyclooxygenase-2 enzyme (**PDB ID: 4PH9**)**.** These are shows better activity towards the cyclooxygenase-2 enzyme binding domain. Among them, compound 5 shows h-bond (----) interaction with Ser 531. With respect to Ibuprofen, best docked Compounds **5** shows ideal binding affinity with Tyr 116, Val 117, Arg121, Tyr 356 etc. Superimposition of compound 2 (**D**), 3 (**E**) and 5 (**F**) with Ibuprofen (cyan) clearly indicate their affinity towards common binding pocket of cyclooxygenase-2 enzyme.

Figure 3 : HPLC characterization of lead compound 5(β-Sitosterol glucoside), showing single major peak with an C-18 reversed-phase column (Waters; 2.1 ×50 mm; 1.7 µm) @ 120 µl/min flow rate using an isocratic mobile phase (formic acid: water:acetonitrile: methanol = 0.2:14.8:40:45 (v/v/v/v)) with run time for 30 minutes at 380nm for undertaking chromatograms. The retention time was found to be 13.5 minutes with a maximum area under the curve with 93.56% purity.

Figure 4: Effect of compound 5 on liberation of pro-inflammatory cytokines TNF-α (a) and IL-6 (b) stimulated by the LPS (20ng/mL) + CON-A (2.5 µg/mL) at dose dependent manner. The cytokines

were determined by ELISA techniques. The results are expressed as mean ± S.E.M. (n= 3 per group), representative of two similar independent experiments. ***P < 0.001 & ***P < 0.0001 were* regarded as significant compared to LPS+CON-A treated controls without exposure to compound 5 Assessed by unpaired student *t test* of two tailed.

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Table 1a: Binding energy of all test compounds against PDB-5BOT(Matrix metallopeptidase 13)
Software: Auto Dock 4.0

Compound	Gibbs free energy (ΔG)	No. of hydrogen bonds	H-bond interaction sites
Compound 3	-7.12	0	No h-bond interactions
Compound 5	-8.48	2	ILE 243, TYR 244
Reference	-9.24	3	PHE 241, TYR 246, THR 247
Prednisolon	-9.02	4	LEU 185, ALA 186, ILE 243, TYR 244
Dexamethasone	-9.11	**4**	LEU 185, AlA 186, ALA 188, TYR 244

Table 1b: Binding energy of all test compounds against PDB-4PH9 (human cyclooxygenase-2)

Compound	Gibbs free energy (ΔG)	No. of hydrogen bonds	H-bond interaction sites
Compound 2	-8.14	0	No h-bond interactions
Compound 3	-8.01	0	No h-bond interactions

Compound 5	-8.66	1	SER 531
Ibuprofen	-8.35	**2**	ARG 121, TYR 356

Table 2. Effect of compound 2,3,4,5 derived from *Ichnocarpus frutescens* on the inhibition of proteinase inhibitory action, membrane stabilization & protein denaturation,

Experiment	**IC_{50} µM**				
	5	**4**	**3**	**2**	**dexamethasone**
Inhibition of protein denaturation	0.25±0.09	35.4±2.34	1.25±0.08	25.4±2.12	10.4±1.58
proteinase inhibition	0.85±0.18	10.5±1.01	7.56±0.94	20.4±1.56	2.5±0.45
Membrane stabilization	2.56±0.45	4.56±1.02	5.89±0.89	21.4±3.12	30.4±3.12

Table 3: Comparison of IC_{50} values (µM) of various test compounds along with the reference compound as COX inhibitors.

Compound	COX-1	COX-2	COX-1/COX-2 Ratio[a]	Reference
	IC_{50}(µM)			
5	33.75±4.12	0.75±0.28	45*(P < 0.001*)	this work
3	58.02±3.45	5.6±1.31	10.36	this work
2	23.4±1.40	1.2±0.49	19.5	this work
Celecoxib	26.64	0.45	59.2*(P < 0.001*)	[50]

[a]Proportion of the IC_{50} values for COX-1 and COX-2 can be used as an indication of the COX-2 selectivity of inhibitors. A COX-1/COX-2 ratio of more than 1 signifies preferential COX-2 selectivity. $P < 0.001$ is significant difference between COX-1 and COX- 2, assessed by un-paired *student t test.*

Table 4: pharmacological effect of isolated phytosterol β-sitosterol glucoside of *Ichnocarpus frutescens* root on 0.06% acetic acid mediated writhing on mouse

Treatment	no. of abdominal writhing
Untreated vehicle control	63±2.3
Diclofenac 3mg/kg i.p.	20±0.42***
β-sitosterol glucoside 1 mg/kg i.p.	34±1.02**
β-sitosterol glucoside 3 mg/kg i.p.	31±3.45 **
β-sitosterol glucoside 6 mg/kg i.p.	26±2.08***

All the datas are represented as mean ± S.E.M.; n=6; **p < 0.01; **p < 0.001; ***p < 0.0001* have been considered as significant as compared to vehicle control.

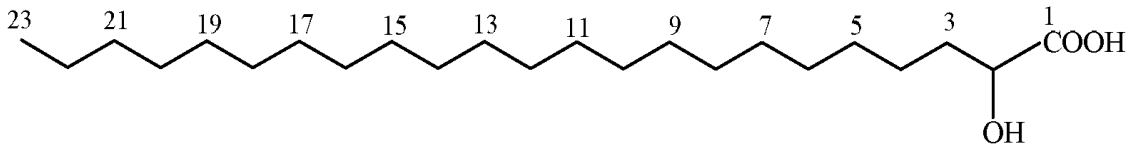
Table 5: pharmacological effect of isolated β-sitosterol glucoside on paw oedema of rat triggered by the carrageenan.

Treatment	peripheral paw oedema by mean increase in paw volume (ml) ± SEM			
	Time in minutes			
	0	60	90	120
Carrageenan control	0.18	0.80	0.91	1.08
(0.05 ml of 1% w/v)	±0.01	±0.05	±0.07	±0.10
Indomethacin	0.17	0.21	0.19	0.18
(10 mg/kg b.w. i.p.)	±0.04	±0.01*	±0.03*	±0.01*
β-sitosterol glucoside	0.18	0.27	0.26	0.31
(5 mg/kg i.p.)	±0.01	±0.02*	±0.07*	±0.09*
β-sitosterol glucoside	0.18	0.25	0.24	0.22
(10 mg/kg i.p.)	±0.03	±0.02*	±0.06*	±0.08*
β-sitosterol glucoside	0.18	0.24	0.22	0.19

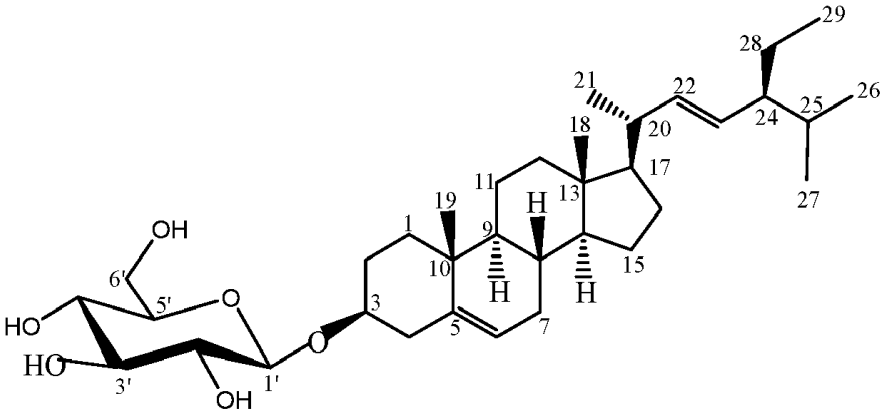
(20 mg/kg i.p.)	±0.03	±0.05*	±0.03*	±0.01*

All values are expressed as mean ± SEM; n = 6, * $P < 0.05$ significant compared to carrageenan control

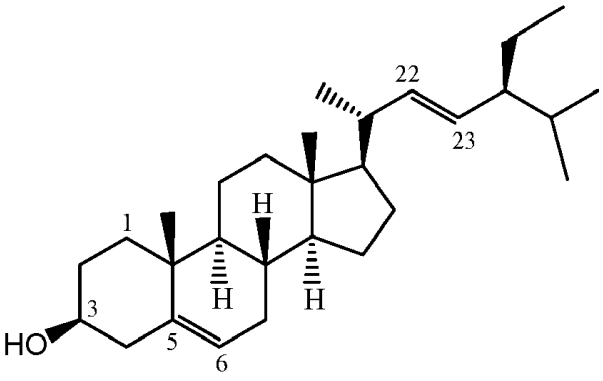
- The bioactive phytosterols have been isolated from the ***Ichnocarpus frutescens*** **roots** for the first time and characterized their structure.
- Those compounds showed their potential biological activity against the various *in vitro* inflammatory and pain models.
- The compound 5 i.e. *β*-sitosterol glucoside demonstrated note worthy anti-inflammatory activity and pain killing activity in a dose dependent manner both *in-vitro* and in-vivo studies.
- The docking studies against the MMP-13 and COX-2 of the tested compounds along with compound 5 also supported and validated with the experimental results.
- *In-vitro* and *in-vivo* results were significantly co-related and supported by each other's findings.
- Therefore the compound 5 could be explored as potential anti-inflammatory agents and anti-arthritis agent for probable management of nociceptive pain.


23
21
19
17
15
13
11
9
7
5
3
1
COOH
OH

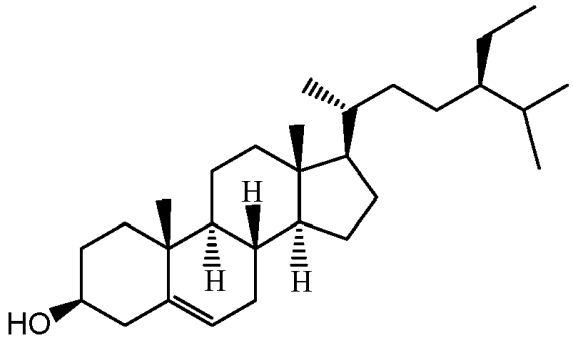
1


29
28
21
22
26
25
24
20
18
17
27
11
19
13
H
1
9
10
15
H
H
OH
6'
5'
O
HO
3
5
7
HO
3'
1'
O
OH

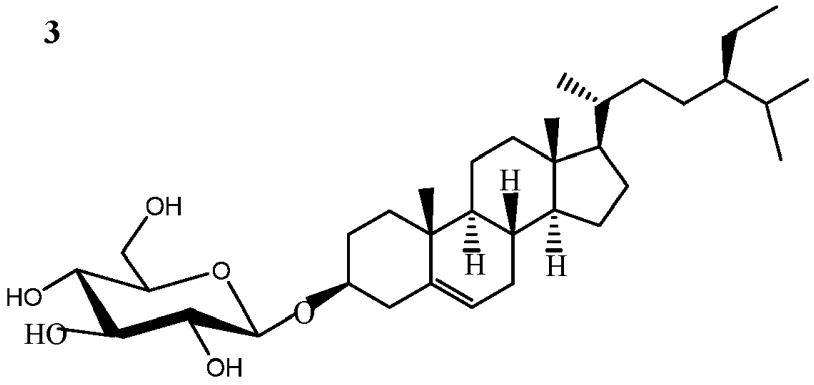
2


22
23
1
H
H
H
3
HO
5
6

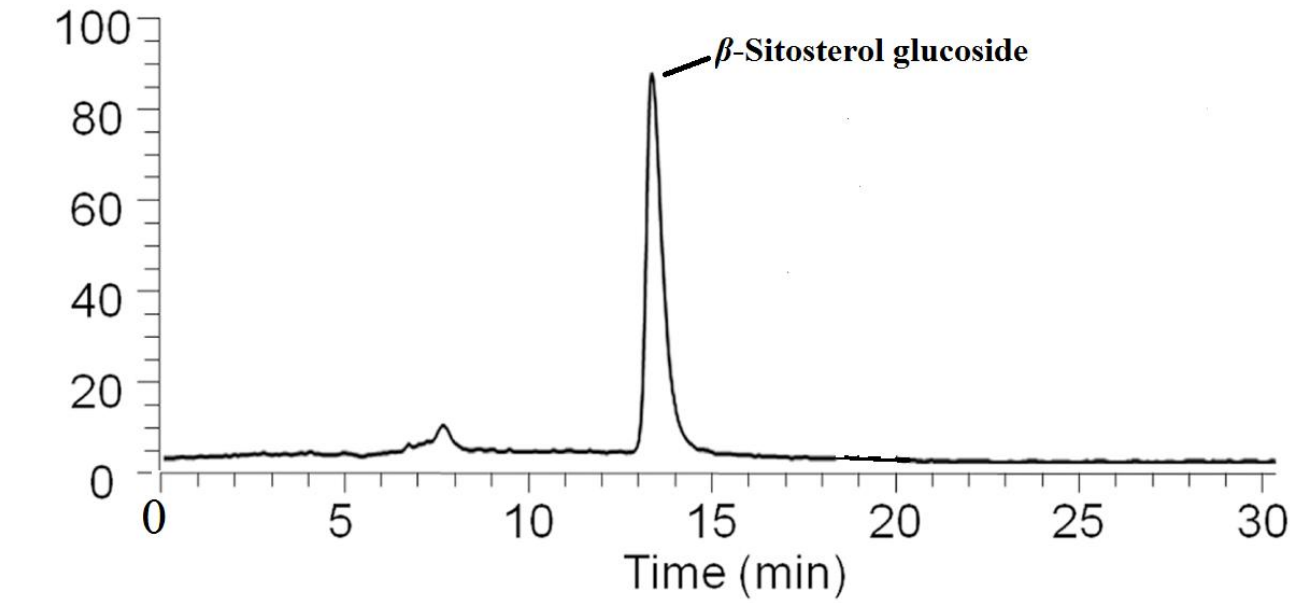
3

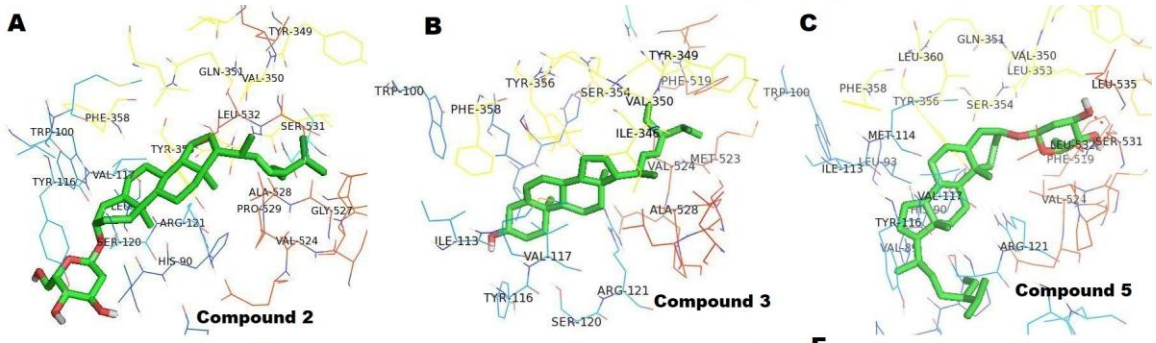

H
H
H
HO

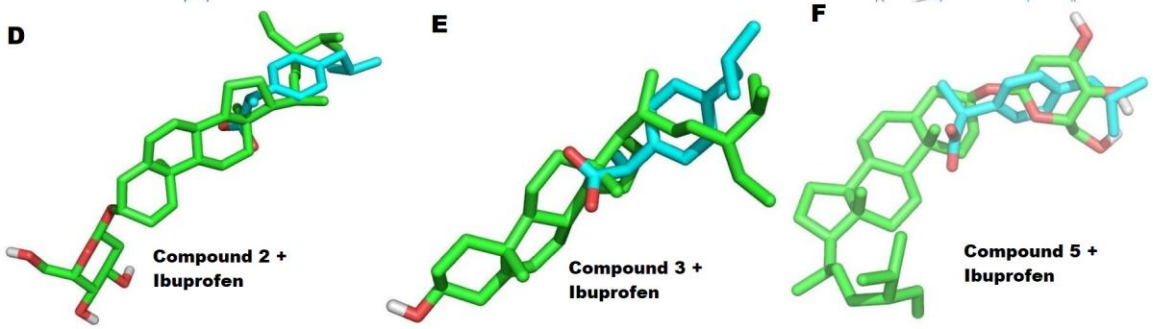
4

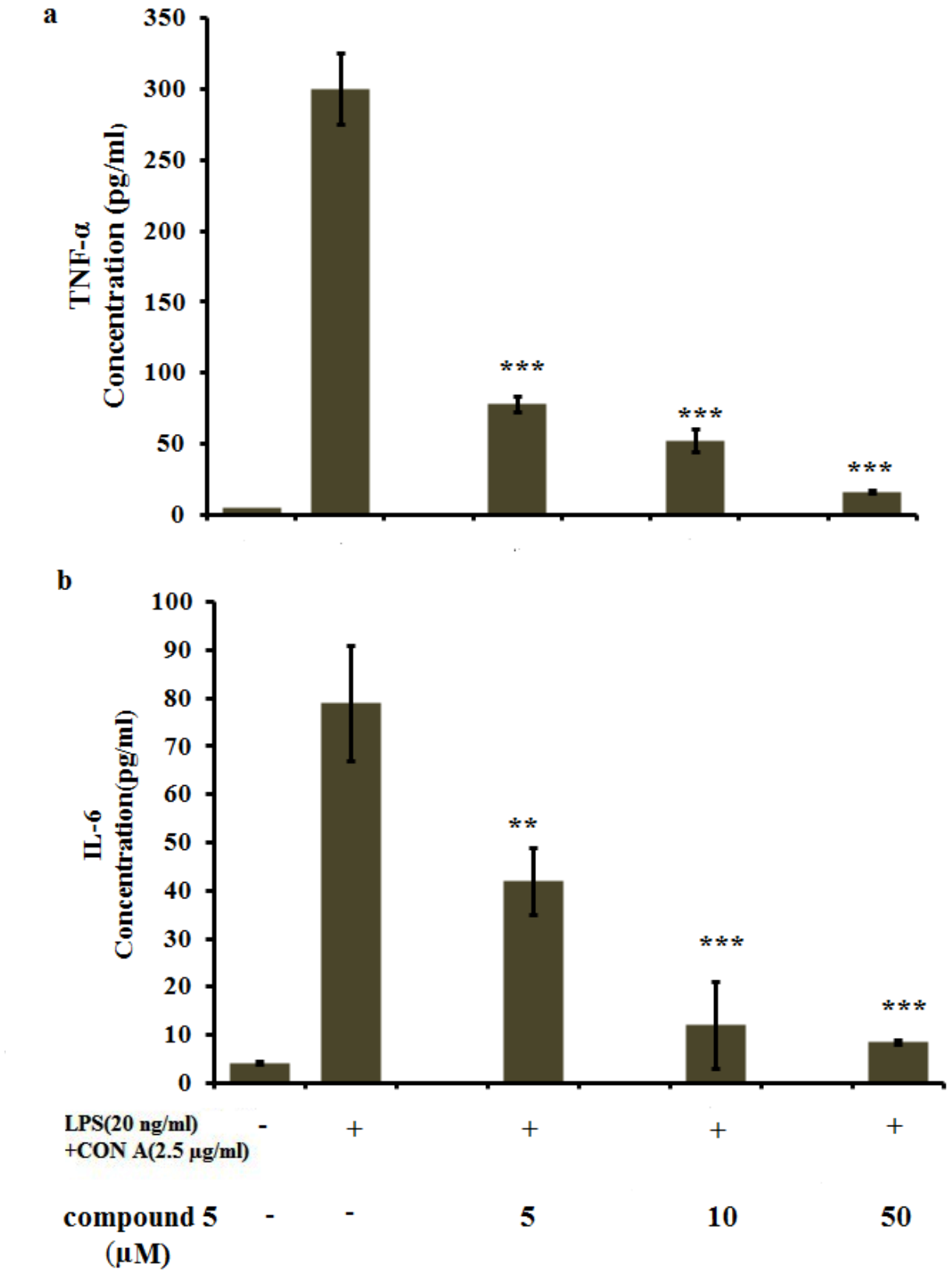

H
H
H
OH
O
HO
HO
OH

5

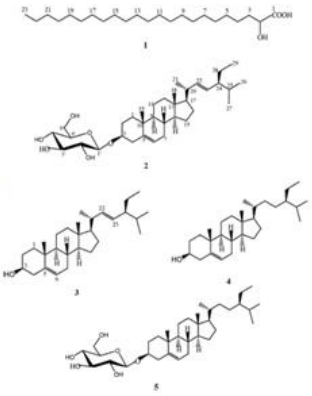

β-Sitosterol glucoside
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (min)


A
TYR-349
GLN-351
VAL-350
PHE-358
LEU-532
SER-531
TRP-100
TYR-35
VAL-117
TYR-116
ALA-528
PRO-529
GLY-527
ARG-121
SER-120
VAL-524
HIS-90
Compound 2
B
TYR-349
TYR-356
PHE-519
TRP-100
SER-354
VAL-350
PHE-358
ILE-346
MET-523
VAL-524
ALA-528
ILE-113
VAL-117
ARG-121
TYR-116
SER-120
Compound 3
C
GLN-351
LEU-360
VAL-350
LEU-353
LEU-535
PHE-358
TYR-356
SER-354
TRP-100
MET-114
SER-531
ILE-113
PHE-519
VAL-117
VAL-524
TYR-116
ARG-121
Compound 5

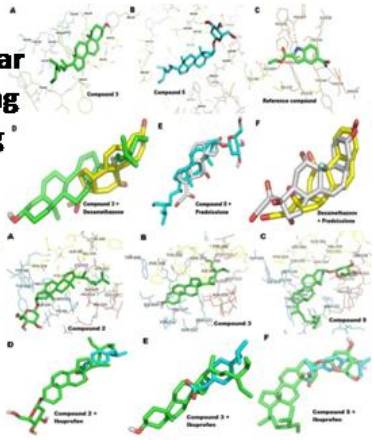

D
Compound 2 +
Ibuprofen
E
Compound 3 +
Ibuprofen
F
Compound 5 +
Ibuprofen


a
TNF-α Concentration (pg/ml)
350
300
250
200
150
100
50
0
***
***
***
b
IL-6 Concentration(pg/ml)
100
90
80
70
60
50
40
30
20
10
0
**
***
***
LPS(20 ng/ml) - + + + +
+CON A(2.5 μg/ml)
compound 5 (μM) - - 5 10 50

Phytochemical Extraction and isolation Characterization



In-silico molecular screening for drug target



Anti-inflammatory and anti-arthritic assay through *in vitro* protein denaturation, proteinase inhibition model for compound 2, 3 and 5

COX inhibition assay for analgesic effect with the test compound 2,3 and 5

Compound 5 optimised as lead analgesic and anti-inflammatory agent

Compound 5 :purity assessed and characterized by HPLC

β-sitosterol glucoside(5) protected acute-inflammation and peripheral nociceptive pain in rat and mice model in pharmacological dose dependent manner

Human PBMC derived pro-inflammatory cytokine TNF-α and IL-6 down regulated by β-sitosterol glucoside(5)