

Chemical characterization and anti-inflammatory activity of phytoconstituents from *Swertia alata*

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Abstract

Background: *Swertia alata* C.B Clarke (*Gentianaceae*) is well reported in Indian Traditional system of medicine and plant was known for its febrifuge, tonic, laxative and antimalarial properties.

Objective: To isolate the phytoconstituents from the plant species *S alata* (*Gentianaceae*) and to study *in vitro* COX-1/COX-2, *in vivo* anti-inflammatory and ulcerogenic activity.

Material and methods: With intent to explore newer phytoconstituents, the ethanolic extract of aerial parts of *S. alata* was partitioned into petroleum ether and chloroform soluble fractions. The isolation of phytoconstituents was performed using silica gel base column chromatography, afforded two phytoisolates (one new and one known) characterized as oleanolic acid (**SA-1**) and 3-hydroxylup-12-(13)-ene-17-carboxylic acid (**SA-4**). The structures of the isolated compounds were established based on melting point (MP), Ultraviolet (UV), Attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR), 1D (¹H NMR & ¹³C NMR) 2D Heteronuclear Multiple Bond Correlation (HMBC) Nuclear magnetic resonance (NMR) and Mass spectrometry. Pharmacological screening was performed to evaluate *in vitro* Cyclooxygenase (COX-1 /COX-2) inhibitory activity, *in vivo* anti-inflammatory and ulcerogenic activity.

Results: Among the compounds, **SA-4** (COX-1: COX-2 :: 104 : 61.68 μM, % inhibition = 61.36) found to be more effective than **SA-1**(COX-1:COX-2:: 128.4:87.25 μM, % inhibition = 47.72) Ulcerogenic study was also performed on the isolated compounds (**SA-1 and SA-4**) and found to possess significant gastric tolerance than indomethacin.

Conclusion: Ayurvedic knowledge supported by modern science is necessary to isolate, characterize, and standardize the active constituents from herbal sources for anti-inflammatory and antiulcer activity.

Background

Swertia alata C.B. Clarke (*Gentianaceae*) is a perennial herb and is a widely distributed plant in the west and north-west Himalayas particularly in Kashmir to Kumaon, Mussorie, Dehradun, and Nainital region of India. (1). Numerous species of *Swertia* are being used as substituents or adulterants of *S. chirata*. *S. alata* is used and unknowingly collected as *S. chirata* by traders for the preparation of several ayurvedic drugs (2). *S. alata* is widely used indigenous medicine possessing various properties. The bitterness, anthelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin, swerchirin, swertiamarin and other active principles of the herb (3). The most potential specie of *Swertia* genus i.e. *S. chirata* is now nearly extinct from India. *S. alata* is known to possess oleanolic acid (4), swertisin (4), swertiamarin (5), swertianin (6), methyl swertianin (6), methylbellidifolin (6), bellidifollin (7) swertiaperennine and decussatin (8), but still very limited data available over phytochemistry *S. alata*.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most broadly utilized medications in the world due to their showed efficacy in reducing pain and inflammation (9). Inflammation is a typical

defensive response to tissue injury and it includes a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (10).

Inflammatory injuries actuate the release of a variety of systemic mediators, cytokines and chemokines, which increases the rate of synthesis of prostaglandin (11). Its production relies upon the activity of prostaglandin G/H synthases, colloquially known as COXs, bifunctional enzymes that contain both cyclooxygenase and peroxidase activity, which exist as distinct isoforms COX-1 and COX-2 (12).

The essential method of activity is inhibition of the pro-inflammatory enzyme cyclooxygenase (COX). NSAIDs as a class, involves both of traditional nonselective NSAIDs (tNSAIDs) that nonspecifically repress both COX-1 and COX-2, and selectively inhibits COX-2. Albeit effective at relieving pain and inflammation, tNSAIDs are related with a significant risk of serious gastrointestinal adverse effects (13).

To overcome this adverse effects, specific inhibitors of the COX-2 isoenzyme were created, thus opening the possibility to provide anti-inflammatory and analgesic advantages, while hypothetically leaving the gastro protective activity of the COX-1 isoenzyme in place. However, vital concerns have recently been raised with respect to the potential cardiovascular toxicity of COX-2 inhibitors (14).

Looking for selective COX-2 inhibitors without affecting the normal physiological functions of COX-1 has remained a noteworthy thrust area of anti-inflammatory pharmaceutical research. Nevertheless, the anti-inflammatory agents having greater activity towards COX-2, yet less receptive towards COX-1 are acknowledged as novel anti-inflammatory agents in the mainstream of anti-inflammatory research (15). Due to the inherent problems associated with the current non-steroidal as well as steroidal anti-inflammatory agents, there is continuous search of phytoconstituents having anti-inflammatory activity with reduced gastrointestinal side effects.

Based on the aforementioned findings, an attempt has been made to isolate and characterize the phytoconstituents from *S. alata* and to evaluate against in vitro COX-2, in vivo anti-inflammatory and ulcerogenic activity.

Methods

General

The chemical and solvents used were purchased from commercial vendors and used without purification. The melting points of all isolates were determined in open capillary tube and were uncorrected. Ultraviolet spectra were recorded in methanol (MeOH) on a Shimadzu UV-160A UV visible recording spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). IR spectra were recorded on Bruker ATR-FTIR spectrophotometer. Mass spectra were recorded using electron impact ionization at 70 eV on an ESIMS analyst QS TOF (Canada) mass spectrometer. The ¹H-NMR, ¹³C-NMR, and HMBC spectra were taken on a Bruker-Advance III-010601AM-500 spectrometer (Bruker Corporation, Karlsruhe, Germany) in CDCl₃ and D₂O using TMS as an internal standard expressing coupling constants (J values) in Hertz

(Hz). Silica gel G (Qualigen, 60–120 mesh) was used for column chromatography. TLC was performed on plates coated with silica gel G (E. Merck, Germany).

Plant material and extract preparation

The plant material was supplied by Almas Pharmaceutical Ltd, Uttar Pradesh, India and identified by Dr. H.B Singh, Scientist F and Head, Raw Material Herbarium and Museum, NISCAIR (National Institute of Science Communication and Information Resources) Pusa Gate, New Delhi. The voucher specimen (NISCAIR/RHMD/2013/2185/190) of the test drug has been deposited in the herbarium of NISCAIR, India for future reference. The aerial parts were carefully collected and air dried under shade. The air dried materials were reduced to coarse powder. The coarse powdered material (1.2 kg) was subjected to exhaustive extraction with 95% ethanol in a SOXHLET apparatus for 50 hours. The extract was concentrated in rotary evaporator to yield greenish brown color 52.63 gms (4.385%) residue.

Isolation and Purification

The residue was dissolved in the minimum amount of ethanol and absorbed on column grade silica gel (60–120 mesh, stationary phase) to obtain slurry, dried in air to free flow and subjected to silica gel based column loaded in petroleum ether. The column was successively eluted using solvent system of petroleum ether and chloroform offered two phyto-isolates SA-1 and SA-4. The structures of the compound were established by spectroscopic method and by comparison with the previous reported works. All spectroscopic data are available as Supplementary Figures S1.

Animals

The animals (Wistar albino rats) used in the study were procured from Animal House Center and were divided and housed in different cages at 25–28 °C, under well maintained hygienic and environmental conditions with relative humidity of 50–65%, under 12 hr light and dark cycles. All animals were acclimatized for a week before use. All experimental work was conducted after receiving the approval from Institutional Animal Ethics Committee (IAEC) via protocol no. IAEC/2015-I/Prot no. 09, 10 and IAEC/2016-I/Prot no. 10, Delhi Institute of Pharmaceutical Sciences and Research, New Delhi.

In-vitro activity

In-vitro COX-1 and COX-2 inhibitory assay

The isolated compound was screened for their in vitro COX-1 and COX-2 enzymatic activity using enzyme immunoassay kit (catalog No. 560131, Cayman Chemicals Inc., Ann Arbor, MI, USA). Enzymatic assay was performed as per the manufacturer's assay instructions and literature procedure (16). The efficiencies of the test compounds that causes 50% inhibition of COX-2 was calculated as IC₅₀ from the concentration response curve. The compounds were further tested for their in vivo ant-inflammatory activity.

In-vivo activity

Anti-inflammatory activity

The anti-inflammatory activity of isolated compound was evaluated on Wistar albino rat by carrageenan induced rat paw edema method as described by earlier (17). The animals were divided into groups, consisting of five rats in each group. Prepared compounds were administered orally at dose of 2, 4, 8 mg/kg body weight (b.wt) and the volume of paw was determined plethysmographically (Ugo-Basyl, Italy). Control group received equivalent volume of normal saline and indomethacin (8 mg/kg b.wt.) was administered orally to the reference group. Carrageenan (0.1 ml, 1.0% w/v in 0.9% of normal saline) was injected after half an hour into the sub-plantar tissue of the rat's hind paw. The paw volume was measured at hourly interval for 3 h (0, 1, 2 and 3 h) and the percent inhibition of edema was calculated using formula:

$$\% \text{ inhibition} = (1 - V_s/V_c \times 100)$$

Where,

V_s = paw volume in sample treated group

V_c = paw volume in control group.

Acute ulcerogenic activity

The ulcerogenic activity of the isolated compound was performed according to previous reported method (18). Each study group consisted of five Wistar albino rats. The animals were fasted for 18 h before the administration of the test compound, while water was given continuously. The dose quantity was made three times (6, 12, 24 mg/kg) of the administered dose for anti-inflammatory studies (2, 4, 8 mg/kg). The control group received only normal saline. After 6 h of the drug administration the rats were sacrificed, stomach was removed and opened around the greater curvature. Inner lining was washed properly with distilled water followed by normal saline. The mucosal damage was examined, and number of ulcers and severity index was calculated on a scale of 0–3, where: 0 = no lesions; 0.5 = redness; 1.0 = spot ulcers; 1.5 = hemorrhagic streaks; 2.0 = ulcers > 3 but ≤ 5; 3.0 = ulcers > 5.

Statistical analysis

Experimental data are expressed as mean. Statistical difference between the treated and control group was evaluated by one way analysis of variance (ANOVA) followed by Turkey's test as a post ANOVA (Graph Pad Prism 5, San Diego, CA, USA) to determine the statistical significance. The significance level was #p > 0.05, ##p > 0.01 and ###p > 0.001, when compared to control and *p > 0.05, **p > 0.01 and ***p > 0.001.

Results

Structural elucidations of isolated compounds were achieved by melting point, UV, ATR-FTIR, 1D (^1H NMR & ^{13}C NMR) and 2D (HMBC) NMR and Mass spectrometry.

Structure Elucidation Of Compounds

Oleanolic acid (SA-1)

SA-1 (Fig. 1a) was obtained as white amorphous powder from elution of the column with chloroform: petroleum ether (2:8), 50 mg, Melting Point 71 °C. ATR-FTIR (cm^{-1}): 2917, 2849, 1730, 726; ^1H NMR and ^{13}C NMR (data given in Table 1); ESI-MS m/z : 457 $[\text{M} + \text{H}]^+$ and 456 $[\text{M}]^+$ (Fragmentation pattern given in Supplementary scheme S2).

Table 1
¹HNMR and ¹³CNMR data for compound SA-1

Position	¹ H NMR	¹³ C NMR
1	1.18, m, 2H	29.26
2	1.79, m, 2H	29.61
3	3.56, m, 1H	29.38
4	–	77.02
5	1.18, m, 1H	29.26
6	1.42, m, 2H	29.38
7	1.18, t, 2H (J = 7.22)	29.26
8	–	33.75
9	2.13, t, 1H (J = 7.19)	35.24
10	–	29.61
11	2.30, d, 2H (J = 7.19)	35.32
12	5.21, t, 1H (J = 15.12)	125.77
13	–	127.28
14	–	35.46
15	1.89, t, 2H (J = 7.26)	29.61
16	2.5, t, 2H (J = 7.26)	24.71
17	–	35.52
18	2.27, t, 1H (J = 7.12)	35.62
19	1.18, t, 2H (J = 7.12)	29.26
20	–	31.94
21	1.18, m, 2H	29.26
22	2.13, t, 2H (J = 7.25)	23.76
23	0.4, s, 3H	14.1
24	0.45, s, 3H	14.1
25	0.52, s, 3H	22.71

Coupling constants in Hertz are provided in Parenthesis

Position	¹ H NMR	¹³ C NMR
26	0.78, s, 3H	29.06
27	0.81, s, 3H	29.06
28	–	178.33
29	0.47, s, 3H	24.71
30	0.47, s, 3H	24.71
Coupling constants in Hertz are provided in Parenthesis		

3.1.2 3-hydroxylup-12-(13)-ene-17-carboxylic acid (SA-4)

SA-4 (Fig. 1b) was obtained as yellow amorphous powder from elution of the column with chloroform: petroleum ether (3:2), recrystallized from methanol, 86 mg (0.071%) and having MP 110 °C. ATR-FTIR (cm⁻¹): 2917, 2849, 1730, 724; ¹H NMR and ¹³C NMR (data given in Table 2); ESI-MS m/z: 456 [M]⁺ and 455 [M-H]⁺ (Fragmentation pattern given in supplementary scheme S3).

Table 2
¹HNMR and ¹³CNMR data of compound SA-4

Position	¹ H NMR	¹³ CNMR
1	1.18, m, 2H	27.2
2	1.70, m, 2H	27.96
3	3.72, m, 1H	72.53
4	–	27.82
5	1.18, m, 1H	27.2
6	1.45, m, 2H	27.82
7	1.18, m, 2H	27.2
8	–	33.25
9	1.18, m, 1H	35.28
10	–	27.96
11	2.13, t, 2H (J = 7.6)	35.32
12	5.20, t, 1H (J = 15.1)	125.42
13	–	127.35
14	–	35.46
15	1.18, m, 2H	27.96
16	1.70, m, 2H	24.97
17	–	35.52
18	2.23, d, 1H (J = 7.2)	35.62
19	1.56, m, 1H	51.63
20	1.90, d 2H, (J = 12.2)	35.62
21	1.96, d, 2H, (J = 12.2)	35.92
22	0.81, s, 3H	17.97
23	0.85, s, 3H	17.97
24	0.95, m, 3H	24.97
25	0.95, m, 3H	18.01

Coupling constants in Hertz are provided in Parenthesis

Position	¹ H NMR	¹³ CNMR
26	0.95, m, 3H	27.2
27	–	27.2
28	1.56, m, 1H	
29	0.65, d, 3H (J = 7.22)	17.97
30	0.65, d, 3H (J = 7.22)	17.97
Coupling constants in Hertz are provided in Parenthesis		

Biological Activity

In-vitro COX-1 and COX-2 inhibitory assay

The inhibitory activity of isolated compounds (SA-1 and SA-4) was evaluated against ovine COX-1 and human recombinant COX-2 using enzyme immunoassay kit (Supplementary Figure S4) and IC₅₀ (μM) values were determined (Table 3).

Table 3

IC₅₀ of the synthesized compounds by in vitro COX-1 and COX-2 enzymatic assay and COX-2.

Compound	IC ₅₀ (μM) ^a	
	COX-1	COX-2
SA-1	128.4	87.25
SA-4	104	61.68
Indomethacin	53	36.56

^a IC₅₀ value is the concentration of the compound required to produce 50% of inhibition of COX-1 and COX-2 respectively using enzyme immunoassay kit (Catalog No. 560131, Cayman Chemicals, Inc., Ann Arbor, MI, USA).

In-vivo activity

Anti-inflammatory Activity

The degree of swelling of the carrageenan injected paws was maximal at the 3rd hour after injection. Statistical analysis revealed that SA-4 (8 mg/kg) significantly inhibited the development of edema at the third hour after treatment ($P > 0.05$). The biological data for SA-1 and SA-4 is shown in Table 4, which clearly implies that the isolated compounds exhibited varying degree of anti-inflammatory activity (Figure 2).

Table 4: *In-vivo* anti-inflammatory activity of compounds using carrageenan-induced rat paw edema method

Compound		Increase in paw edema (mL) ^{a, b} (Mean ± SEM)
SA-1	2mg/kg	0.29±0.00*
	4mg/kg	0.27 ±0.22*
	8mg/kg	0.23 ±0.01*
SA-4	2mg/kg	0.28 ±0.00*
	4mg/kg	0.26 ± 0.22*
	8mg/kg	0.17 ±0.01*
Control		0.44 ±0.04
Indomethacin		0.15 ±0.02***

p values were compared with control group (3 h after inducing edema) (Turkey's test). Number of animals (rats) in each group = 5.

^a Values are determined after 3 h and are expressed as Mean ± SEM.

^b $p > 0.05$ (significant difference).

Ulcerogenic activity

The result (Table 5) (Supplementary Figure. S5) showed that the tested compounds **SA-1** and **SA-4** showed better G.I. safety profile with the ulcer score of 2.5 ± 0.61 and 1.8 ± 0.44 , respectively in comparison to standard drug Indomethacin 2.7 ± 0.273 . Further, SA-4 possesses significantly less ulcerogenic potential as compared with SA- 1 in a dose of 12 mg/kg.

Table 5: *In-vivo* ulcerogenic activity of the compounds in rat model.

Compound	Ulcerogenic activity ^(a) (severity index) ^{(b), (c)} (Mean ± SD)
SA-1 (6mg/kg)	0.0 ±0.00
(12mg/kg)	0.0 ±0.00
(16mg/kg)	2.5 ± 0.61
SA-4 (6mg/kg)	0.0 ±0.00
(12mg/kg)	1.8 ± 0.44
(16mg/kg)	1. ±0.44
Control	0.0 ±0.00
Indomethacin (21mg/kg)	2.7 ± 0.27

^a Number of animals in each group is 5

^b Severity Index=Mean score of treated group-Mean score of control group

^c $p < 0.5$ (Significant difference)

Discussions

Characterization of Phyto-isolates

The compound SA-1 identified as oleanolic acid was obtained as white amorphous powder. It was acid hydrolysed and responded positively to Liebermann-Burchard test for steroids. The presence of sterol nucleus was confirmed by acid hydrolysis using 2M H₂SO₄. The IR spectrum showed characteristic absorption bands at (2917, 2849) for C-H stretching, and (1730) for C=O stretching thereby confirmed the presence of carbonyl group. The generation of the prominent ion peaks at m/z 457 [C₃₀H₄₈O₃]⁺ was attributed to molecular ion of oleanolic acid. Other fragment ions peaks at m/z 439 [C₃₀H₄₇O₂]⁺, 411 [C₂₉H₄₇O]⁺, 395 [C₂₉H₄₇]⁺, 248 [C₁₆H₂₄O₂]⁺, 235 [C₁₅H₂₄O₂]⁺ supported presence of steroidal structure (Supplementary Scheme S2).

The ¹H NMR spectrum of compound SA-1, methyl protons displayed one singlet at δ 0.47 for 6H assigned to H-29 & H-30 protons, two singlet at δ 0.4 & 0.45 assigned to H-23 & H-24 protons, and three singlet at δ 0.52, 0.78, 0.81 were assigned to H-25, H-26, & H-27 protons respectively. There was a characteristic triplet signal at δ 5.21 with *J* value of 15.12 assigned to H-12. The protons of OH and COOH group displayed characteristic broad signal at δ 12.42 & 12.6 respectively. Remaining methylene and methine unit protons

displayed broad multiplet signals between δ 0.47 to 2.13. The ^{13}C NMR spectrum data of SA-1, exhibited important signals at δ 14.21 for C-23 and C-24, signal at δ 24.71 for C-29 & C-30. The signal at δ 29.06 was assigned to C-26 & C-27. A signal at δ 22.71 was assigned to C-25. The signal at δ 32.02 for C-31, signal at 178 assigned to carbonyl carbon. The two characteristic signals at δ 125.77 and 127.28 were assigned to C12 and C13 respectively. The carbonyl group signal appeared at δ 178.45. The remaining 19 carbons displayed their signals from δ 14.1 to 77.02 (Table 1).

The HMBC correlations of H-C(3) with C(1), C(2), C(4), C(23), and C(24), of H-C(9) with C(5), C(8), and C(10), of H-C(12) with C(11), C(13), C(14), and C(18), of H-C(18) with C(13), C(14), C(16), and C(28), and of H-C(22) with C(28), indicated the relative positions of these groups in the molecule SA-1 (Figure. 3a).

The IR data, Mass fragmentation pattern, ^1H NMR and ^{13}C NMR chemical shifts of the isolated compound SA-1 were comparable with related compound viz, oleanolic acid. On the basis of spectral data analysis and acid hydrolysis based chemical identification, the structure of isolated compound SA-1, was finally elucidated as oleanolic acid.

The compound SA-4 identified as 3-hydroxylup-12-(13)-ene-17-carboxylic acid, was obtained as yellow amorphous powder. It is obtained chemically by acid hydrolysis. It responded positively to Liebermann-Burchard test for steroids and formed effervescence with sodium bicarbonate solution indicating the presence of carboxylic acid group in the molecule. The chemical identification was done by acid hydrolysis method using 2M H_2SO_4 which confirmed the presence of sterol nucleus. The IR spectrum showed characteristic absorption bands for (2917, 2848) for C-H stretching, and (1735) for C=O stretch to confirm the presence of carbonyl group. The +ve ion mass spectrum showed molecular ion peak at m/z 456 $[\text{C}_{30}\text{H}_{48}\text{O}_3]^+$ corresponding to M^+ ion, and 455 $[\text{C}_{30}\text{H}_{47}\text{O}_3]^+$ corresponds to $\text{M}-1$ peak, which indicated saturated nature of molecule. The characteristic signals at m/z 439 $[\text{C}_{30}\text{H}_{47}\text{O}_2]^+$, 411 $[\text{C}_{29}\text{H}_{47}\text{O}]^+$, 395 $[\text{C}_{29}\text{H}_{47}]^+$, and 235 $[\text{C}_{15}\text{H}_{24}\text{O}_2]^+$ suggested presence of steroidal structure (Supplementary scheme S3).

The ^1H -NMR spectrum of compound SA-4, methyl protons displayed one singlet at δ 0.65 for 6H assigned to H-29 & H-30, two singlet at δ 0.81 & 0.85 assigned to H-22 & H-23 proton respectively, and one singlet at δ 0.95 was assigned to H24, H-25, & H-26 protons. There was a characteristic triplet signal at δ 5.20 with J value of 15.1 Hz assigned to H-12. The protons of hydroxy and COOH group displayed characteristic broad signal at δ 12.49 & 12.56 respectively. Remaining methylene and methine unit protons displayed broad multiplet signals at δ 0.95-3.72. The ^{13}C NMR spectrum data of SA-4, exhibited important signals for two methyl carbons at δ 17.97 C-22, C-23, C-29 & C-30. The signal at δ 27.2 was assigned to C-26 & C-27. A signal at 18.01 was assigned to C-25. The signal δ 178 assigned to carbonyl carbon. The two characteristic signals at δ 125.42 and 127.35 were assigned to C12 & C13 respectively. The remaining 19 carbons displayed their signals from δ 14.1 to 72.53 (Table 2).

The HMBC correlations of H-C(3) with C(1), C(2), C(4), C(23), and C(24), of H-C(9) with C(5), C(8), and C(10), of H-C(12) with C(11), C(13), C(14), and C(18), of H-C(18) with C(13), C(14), C(16), C(19) and C(27),

and of H-C(21) with C(27), indicated the relative positions of these groups in the molecule SA-4 (Figure 3b).

The IR, data, Mass fragmentation pattern, ^1H and ^{13}C NMR chemical shifts of the isolated compound SA-4 were comparable with related compound viz, Hydroxylupic acid. On the basis of spectral data analysis and acid hydrolysis based chemical identification, the structure of isolated compound SA-4, was finally elucidated as 3-hydroxylup-12-(13)-ene-17-carboxylic acid. This is a new compound reported for the first time in *S. alata* species. The spectral characterization of molecular structure of isolated compounds SA-1 and SA-4 was confirmed and supported using standard literature (19–22).

***In-vitro* COX-1 and COX-2 inhibitory assay**

COX-1 and COX-2 catalyze the biosynthesis of prostaglandin H₂ from the arachidonic acid substrate. The inhibition of COX-1 results in some undesirable side-effects, whereas COX-2 inhibition provides therapeutic effects in pain, inflammation, cancer, glaucoma, Alzheimer's and Parkinson disease (23). COX-2, which is an inducible enzyme, COX-1 is constitutive, that is, present even in the absence of inflammatory conditions. In addition to the pro-inflammatory prostaglandins, COX-1 is responsible for the synthesis of those prostaglandins that are necessary for maintaining the integrity of gastro-intestinal mucosa. A higher inhibition of COX-1 increases the tendency of a drug to induce gastric ulcers and related complications (24).

The IC₅₀ values of indomethacin for COX-1 and COX-2 were observed as 53.00 μM and 36.56 μM respectively. The results of the *in vitro* COX-1 and COX-2 inhibitory studies revealed that the isolated compounds (SA-1 and SA-4 potentially inhibit COX-2 (IC₅₀ = 61.68-87.25 μM range) over COX-1 104-128 μM range). Whereas, isolated compound SA-4 (COX-1/COX-2 = 104/61.68) were found to be potent inhibitor of COX-2 than SA-1 (COX-1/COX-2= 128.4 / 87.25) (Figure 4). To understand the inhibitory activity of these isolated compounds, they are further evaluated for their *in vivo* anti-inflammatory activity.

Anti-inflammatory activity

Anti-inflammatory activity of the phytocompounds (**SA-1 and SA-4**) was evaluated as per the method of Winter et al with minor modifications. Carrageenan was used to induced paw Edema in Wistar albino rat. Carrageenan induced paw edema is a standard assay for acute inflammation that is effectively employed to evaluate the phytoisolate against anti-inflammatory activity (25). Edema is produced by a sequential release of inflammatory mediators such as histamine, serotonin, kinins, prostaglandins and bradykinins which leads to fluid accumulation (26). Edema is characteristic of acute inflammatory response (27). The release of histamine or serotonin occurs in the first phase (Upto 1 hr) and the second phase (over 1 hr) is associated with the production of bradykinins (28). It is well known that the third phase of the edema

induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction (29,30).

Indeed, previous studies have shown that this compound play a pharmacological role in inflammation (31). The finding of the present study revealed that the phytoisolates **SA-1** and **SA-4** shows dose dependent anti-inflammatory activity by suppressing the rat paw edema.

Ulcerogenic activity

It has been reported for that non-steroidal anti-inflammatory agents are inadequately dissolvable in gastric acid and stay in contact with the stomach wall for a more extended period, thus producing a highly dangerous local concentration. This leads to local irritation of stomach wall followed by ulceration. This prompts to local irritation of the stomach wall took after by ulceration (32). Both the compounds **SA-1** and **SA-4** possessing *in vivo* anti-inflammatory activity were further screened for their ulcerogenic activity according to Cioli method. **SA-4** showed significant ulcerogenic potential compared to the control treated group at a dose of 12 and 24 mg/kg. A strong correlation between the potency of NSAID's as inhibitor of prostaglandin synthesis and ulcerogenic activity has been observed (33).

Most potent compound SA-4 showed severity index lower than the standard drug Indomethacin. Hence, this compound may ascertain to have better safety margin on gastric mucosa than indomethacin (34).

Conclusion

The present work reports the chromatographic isolation and spectroscopic identification of newer compounds i.e SA-1 and SA-4. The isolated compounds were studied for their anti-inflammatory and ulcerogenic properties. The compound SA-4 showed comparable anti-inflammatory activities with indomethacin but were less irritant to the gastric mucosa and No significant ulcerogenicity was observed on comparing with indomethacin and control.

Abbreviations

Melting point : MP, Ultra violet : UV, Attenuated total reflection-Fourier-transform infrared spectroscopy : AT-FTIR, Heteronuclear Multiple Bond Correlation : HMBC, Nuclear magnetic resonance : NMR, Electrospray ionization Mass spectrometry: ESI-MS, Cyclooxygenase : COX, Traditional nonselective anti-inflammatory drugs : tNSAIDs, Methanol : MeOH, Deuterated chloroform : CDCl₃, Deuterium oxide: D₂O, Tetramethylsilane : TMS, Hertz : Hz, Thin layer chromatography : TLC, Grams : gms, Half maximal Inhibitory concentration : IC₅₀, Milligrams : mg, Kilogram : Kg, Hours: h, one way analysis of variance : ANOVA, Standard error mean: SEM, Millilitre : mL, Positive :+ve.

Declarations

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Authors' contributions

Sakshi Bajaj and Sharad Wakode contributed to the conception and design of the study; Sakshi bajaj, Avneet kaur and Sachin kumar performed the experiments; Himangini Bansal, Satish Manchanda and Neeraj Fuloria organized the database and performed the statistical analysis; Sakshi Bajaj and Shivkanya Fuloria wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Please contact corresponding authors for data requests.

Ethics approval and consent to participate

All experiments in this study were supervised and approved from Institutional Animal Ethics Committee (IAEC), Delhi Institute of Pharmaceutical Sciences and Research, New Delhi and strictly followed the norms of animal experiment ethic to reduced number as well as suffering of the animals.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

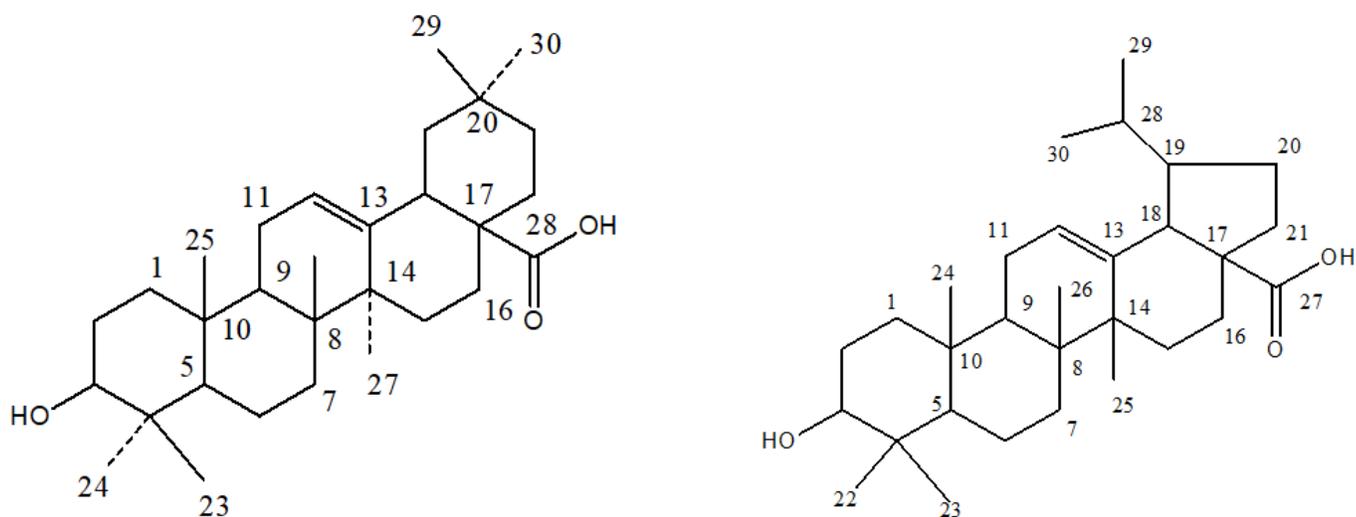


Figure 1

a: Oleanolic acid (SA-1) b: 3-Hydroxylup-12-(13)-ene-17-carboxylic acid (SA-4)

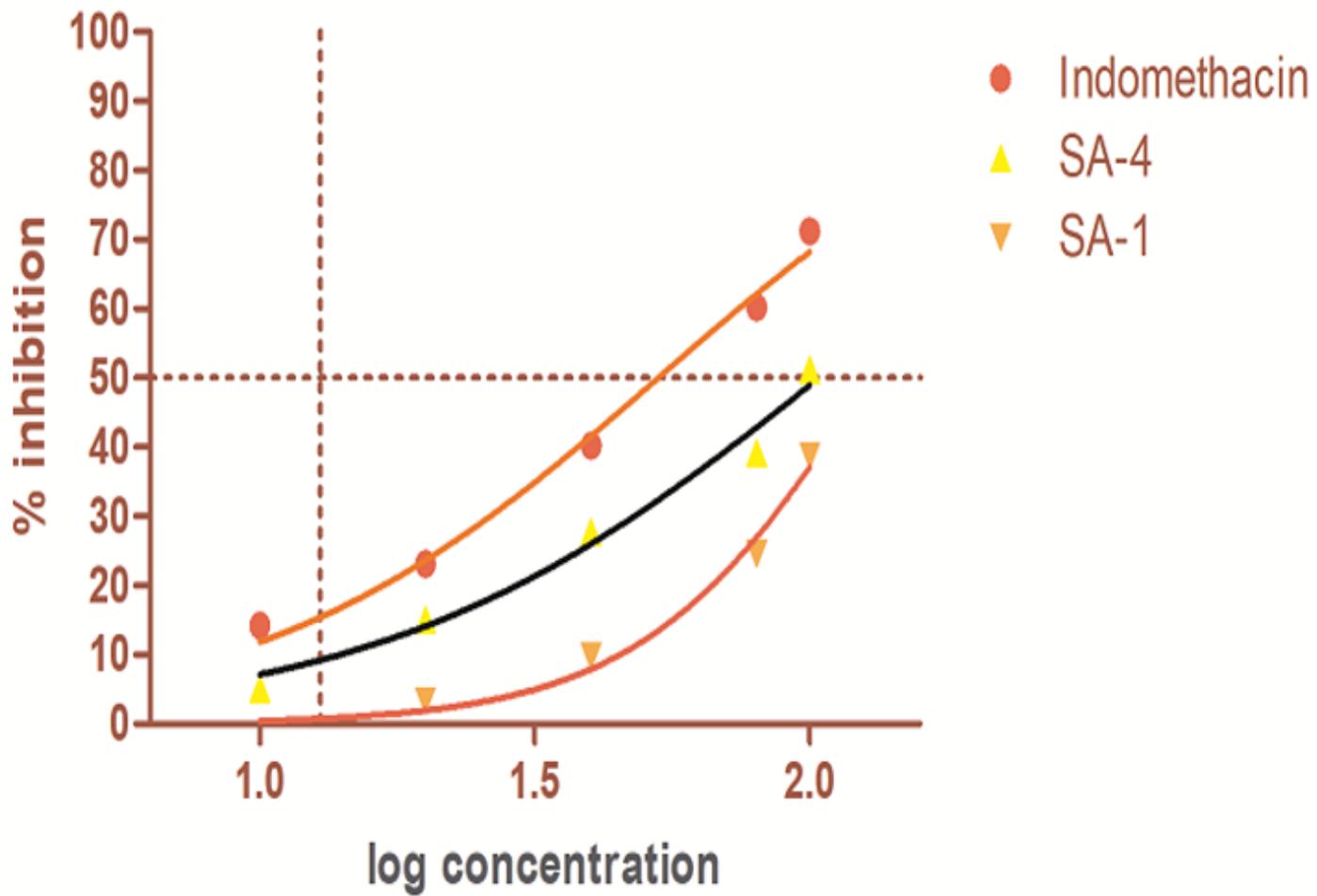


Figure 2

In vitro percentage inhibition of COX-2 versus log concentration curve of SA-1, SA-4 and indomethacin.

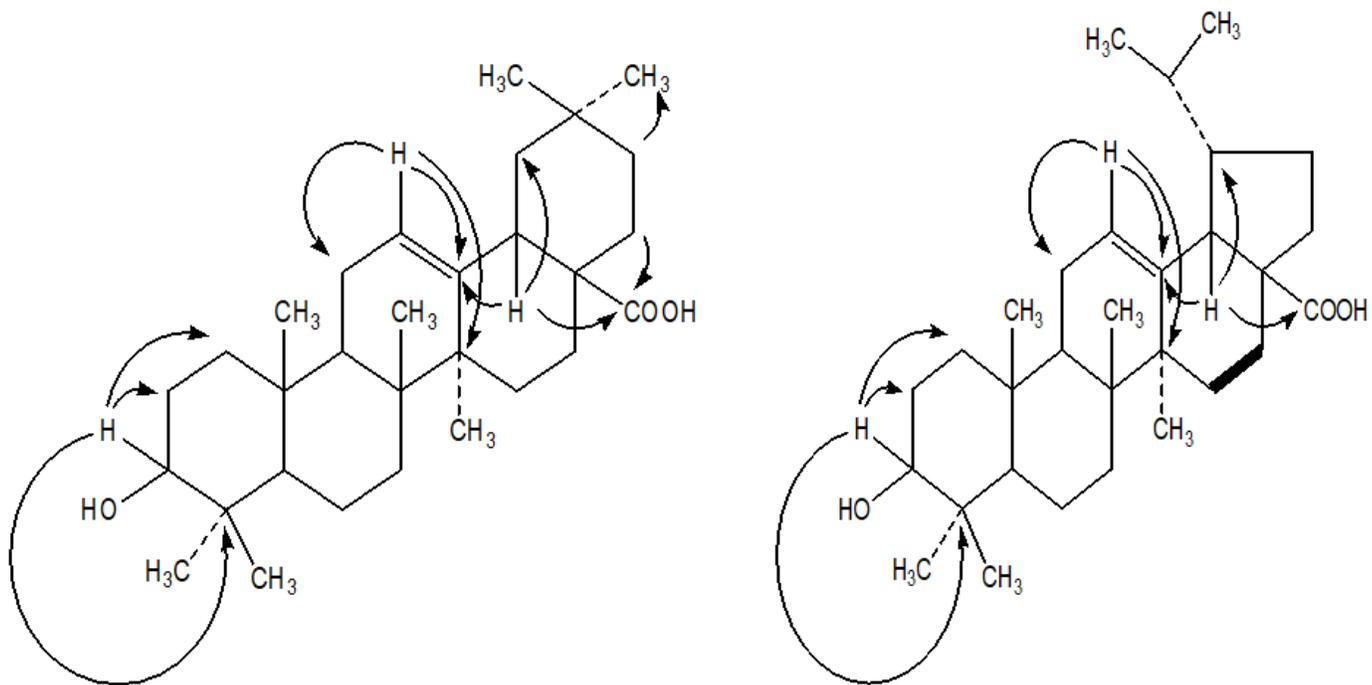


Figure 3

a: HMBC interaction in SA-1 b: HMBC interaction in SA-4

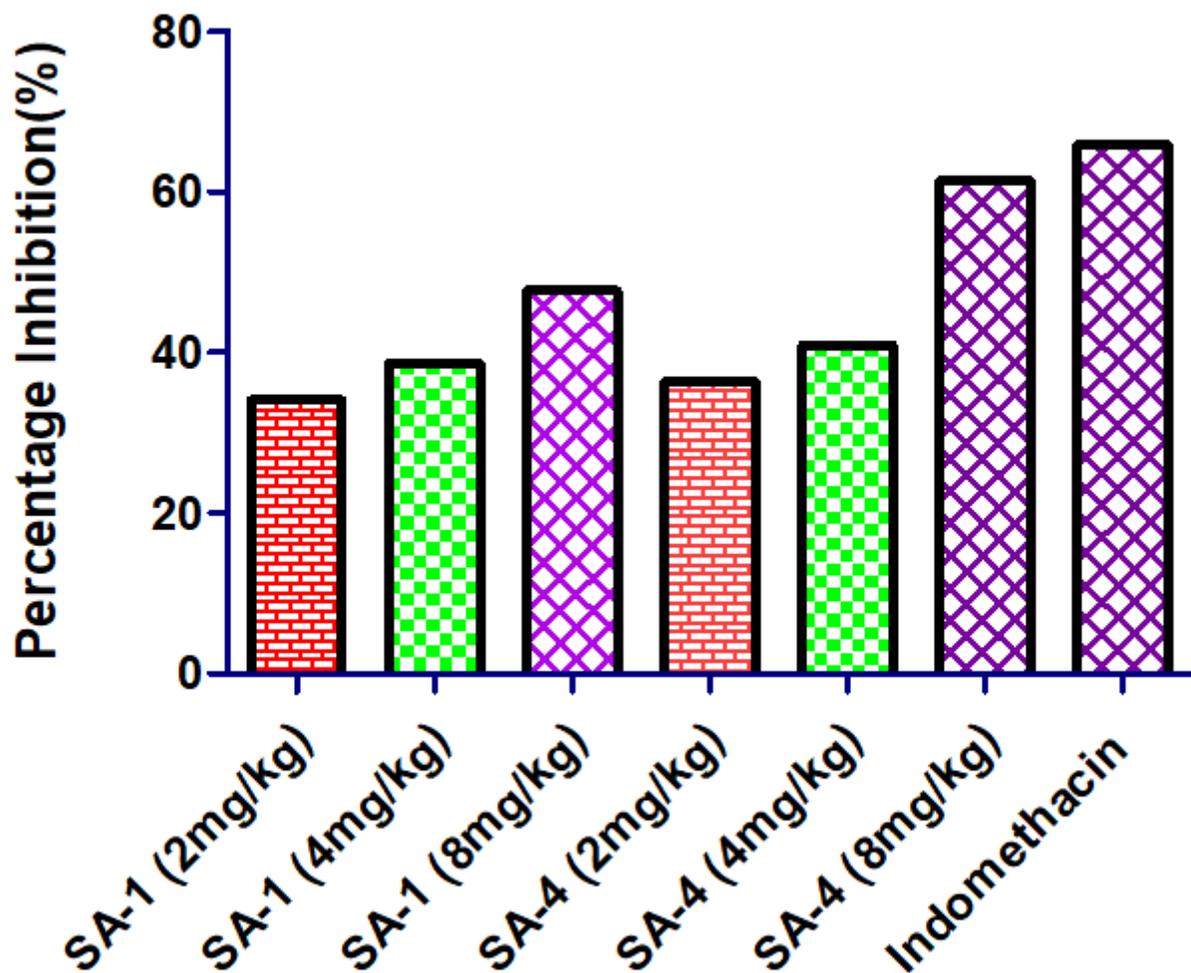


Figure 4

In vivo percentage inhibition of SA-1, SA-4 and indomethacin

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