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Phytochemical and anti-inflammatory property of *Solanum nigrum*

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Abstract

Two new steroidal saponins, solanigrosides and on known saponins, degalactotigonin , were isolated from the whole plant of solanum nigrum. Their chemical structures were elucidated using spectroscopic analysis, chemical degradation, and derivatization. All two the alcoholic extract was obtained through Soxhlet heat extraction method, while the antioxidantive properties were assessed via 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, ferric reducing antioxidant power (FRAP) and xanthine oxidase inhibition (XOI) assays. Further, anti-inflammatory property of the extract was evaluated on rat's model of carrageenan induced paw model of edema viaphysical measurements and histology. The extract exhibited antioxidant activity with an EC_{50} value of 1482 µg/ml in the DPPH radical scavenging assay, an EC_1 value of 2191 µg/ml in the FRAP assay and 10.15 ± 6.20% in the XOI assay. Rats pretreated with the extract have shown dose dependent decrease in paw edema when compared to non-treated group of rats. The highest dose (50 mg/kg) of extract exhibited similar effects to aspirin in terms of reducing paw thickness, leucocytes infiltration sand disruption of collagen. In conclusion, the E. cuneatum alkaloid leaf extract possesses both antioxidative and anti- inflammatory properties suggesting its potentials for future development of antioxidant and anti-inflammatory drugs.

Introduction

The plant *Solanum nigrum* L., a popular medicinal herb in China, is believed to have various therapeutic properties, especially against certain types of cancer.[1] Its therapeutic mechanism remains unknown. In a previous study, we have identified three steroidal glycosides β_2 –solamargine, solamargine, and degalactotigonin) from this plant, all of which exhibited cytotoxicity in six cultured human solid tumor cell lines: HT-29, HCT-15, LNCaP, PC-3, T47D, and MDA-MB-231.[2] The current study was conducted to extend our search for cytotoxic saponins from this plant.[3] This paper describes the structure elucidation of six new saponins, solanigrosides isolated from the extract of the whole plant and their anti-inflammatory properties study on rat paws inflammation.

Inflammation is the body response towards foreign substances such as pathogens, allergens, chemical irritants as well as injury which involves infiltration of leukocytes and generation of pro-inflammation factors to the injured site.[4] Inflammation helps in mitigating the effects of harmful microorganisms and remove dead cells which may prevent further developmentof irritation and allows the injured tissue to recover to normal condition.

Reactive oxygen species (ROS) is the product of cellular aerobic metabolism giving rise to both harmful and beneficial effects to the body. Inequality between antioxidants and oxidants in the body is detrimental to the integrity of macromolecules and body cells.[6] Free radicals generation is one of the causes of inflammation, while excessive and persistent inflammation leads to undesirable pathologic conditions such as rheumatoid arthritis, neurogenerative diseases, cancer, asthma and inflammatory bowel disease.[7]

Drugs that are commonly used for the treatments of inflammatory conditions are known as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids.[8] Non-selective NSAIDs are associated with gastric ulcerogenesis which occurs in patients after long term exposure, while selective NSAIDs have displayed better gastrointestinal tolerability and safety. However, toxicities and kidney problems are not uncommon to both the non-selective and selective NSAIDs. Further, selective NSAIDs are also identified for increasing the risk of developing cardiovascular diseases such as myocardial infarction andstroke which led to worldwide withdrawal of rofecoxib . Corticosteroids, like the NSAIDs are also associated with numerous side effects such as gastrointestinal ulcers, hyperglycaemia and steroids withdrawal symptoms.[9]

Experimental

General Experimental Procedures. Melting points were determined with an X- 5 hot stage microscope melting point apparatus (uncorrected). Optical rotations were obtained on a P-1020 digital polarimeter (JASCO corporation). IR spectra were measured on a JASCO FT/IR-480 plus instrument. 1D and 2D NMR spectra were recorded on a Bruker AV400 spectrometer in C_5D_5N solution.

ESIMS spectra were acquired using a Bruker Esquire 2000 mass spectrometer. HRESIMS spectra were recorded using a Micromass Q-TOF mass spectrometer. Column chromatography was done on Diaion D-101 (Mitsubishi Kasei), silica gel (200-300 mesh, Qingdao Factory of Marine Chemical Industry, Qingdao, China), and ODS (40-63 μm, Merck). TLC was performed using Merck TLC plates precoated silica gel 60 F_{254}, and the spots were detectedby spraying with 10% H_2SO_4-EtOH and heating. Preparative HPLC was performed using an ODS column (19 mm × 300 mm, 10 μm, XTerra Prep. Rp18, detector: RID). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). RPMI-1640 medium, fetal bovine serum (FBS), and trypsin-EDTA solution (1×) were obtained from GIBCOBRL (Grand Island, NY).

Plant Material. The herb *S. nigrum* L. was collected from habitat of Ayodhya u.p (India) in June 2013 and identified by Prof. R.P. Singh (Division of Pharmacognosy, Banaras Hindu University Varanasi). A voucher specimen (No. 250) is available at the Department of Natural Products Chemistry, Department of Pharmacognosy, B.H.U. Varanasi (U.p).

Extraction and Isolation. The dried, whole plant of *S. nigrum* (0.8 kg) was extracted with 60% EtOH (200 L × 2). The solvent was removed under vacuum to yield the crude extract (380 g). A suspension of the extract in H_2O was centrifuged and then applied to a D-101 macroresin column (120 mm × 1500 mm) and eluted with H_2O (40 L), 30% EtOH (40 L), 60% EtOH (40 L), and 95% EtOH (40 L) successively. The 60% EtOH elute (130 g) was dried and then extracted with MeOH. The MeOH extract was separated by silica gel (3000g) using $CHCl_3$–MeOH gradient mixtures (10:0–6:4) to give 10 fractions (1– 10). Compound **1** (44.0 mg, 0.0445‰ yield)[10] was separated from fraction 7, eluted with $CHCl_3$–MeOH (7:3). Fraction 9 (57 g), eluted with $CHCl_3$–MeOH (6:4), was further separated by silica gel column chromatography eluting with $CHCl_3$–MeOH-H_2O (90:10:0.5; 80:20: 1; 75:25:2; 70:30:5; 65:35:5) and then purified by ODS column chromatography eluting with MeOH–H_2O (3:7;

5:5; 7:3) and repeated RP-18 HPLC preparation to yield compounds **2** (9.5 mg, 0.0005% yield),[11] column chromatography eluting with $CHCl_3$–MeOH–H_2O (80:20:1; 75:25:2; 70:30:5; 65:35:4; 60:40:5), ODS column chromatography eluting with MeOH–H_2O (6:4; 7:3).

Anti-inflammatory Activity

These ethanolic extracts were tested for anti-inflammatory activity by carrageenan induced rat paw edema. Healthy albino rats of either sex, weighing 100 -160 gm were selected and provided a standard rat food (I have procured animal feed from Central Drug research Institute Lacknow) and water *ad libitum.* Before the experiment, food was withdrawn overnight but adequate water was given to the rats. Doses selected were 50 mg, 100 mg and 200 mg/kg for each extract. Since 50 mg and 100 mg/kg did not show significant activity; the results at these doses are not presented and discussed. The animals were divided into 5 groups of 6 animals each. The first group (control group) receives acacia (5%, 10ml/kg). The second group received Diclofenac sodium (5 mg/kg, positive control). The third, fourth and fifth groups received ethanolic extract (200 mg/kg) of alcoholic extract of *S. nigrum*. All the drugs were given orally half an hour before the administration of carrageenan suspension. Acute inflammation was produced by the sub-planter administration of 0.1 ml of 1% carrageenan in normal saline in the left hind paw of the rats. The paw volume was measured at 0, 1, 3 and 5 hours with the help of plethysmometer. The average paw swelling in the group of extract treated rat was compared with control group and standard group and percent change in edema was calculated.

Results and Discussion

Compound **1** was obtained as a white, amorphous power. It showed a quasi-molecular ion peak of $[M + Na]^+$ at m/z 1057. The ^1H and ^{13}C NMR data of this compounds were identical to hose of degalactotigonin, namely, (3β, 5α,25*R)* – spirostan -3-*O*-*β*-D-glu-copyranosyl-(1→2)-*O*-[*β*-D-xylopyranosly- (1→3)] –*O*-*β*-D-glucopyranosyI-(1→4)-*O*-*β*-D-galactopyranoside.[1]

Spectral data of compound 1

IR (K Br) was 3440, 3360, 3290, 2840, 1650, 840, 690, cm-^{1}

Table:-1 ^{13}C NMR Spectral data of compound 1

Position	^{13}C NMR	Position	^{13}C NMR	Position	^{13}C NMR

1	37.0	11	21.1	21	14.8
2	29.0	12	39.9	22	109.0
3	77.2	13	40.5	23	31.6
4	34.6	14	56.2	24	29.7
5	44.4	15	31.9	25	30.4
6	28.7	16	81.1	26	66.6
7	32.2	17	62.8	27	17.1
8	35.0	18	16.4		
9	54.2	19	12.1		
10	35.6	20	41.8		

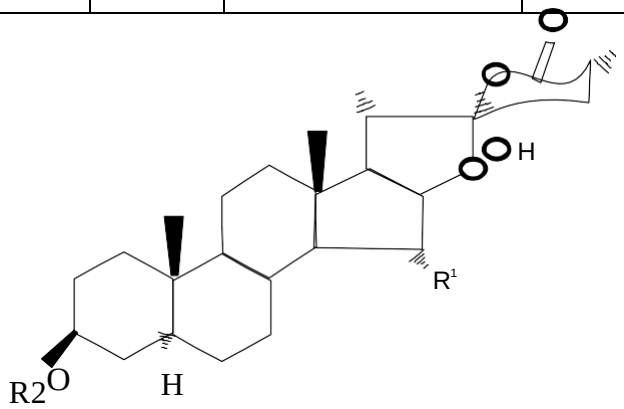

Figure:-1 Structure of compound 1

Spectral study of compound 2

The molecular formula of compound **2**, solanigroside C, was deduced as C_{51} $H_{82}O_{26}$ by HRESIMS (*m/z* 1133.4938 [M + Na]$^{+}$, calcd 1133.4992). its IR spectrum showed an absorption band at 1757 cm^{-1}, suggesting the presence of a δ-lactone carbonyl group . The ^{1}H NMR spectrum displayed signals for two tertiary methyl groups at δ 0.64 (3H, s) and 0.96 (3H, s), two secondary methyl's at δ 1.13(3H, d, *J* = 6.4 Hz) and 1.26 (3H, d, *J* = 6.9 Hz), attributesto a steroidal aglycone moiety, and four anomeric signals appearing at δ 5.56 (d, *J* = 7.8 Hz), 5.28 (d, *J* = 7.8 Hz), 5.13 (d, *J* = 7.9 Hz), and 4.84 (d, *J* = 7.9 Hz). Additionally, the ^{14} C NMR spectrum showed an ester carbonyl resonance at δ

180.4, four oxygenated methines at δ 77.3, 77.5, 78.8, and 91.9, ant a spiroketal carbon at δ 109.7, which was closely analogous to tigogenin glycoside.[3] In the $^{1}H - ^{1}H$ COSY spectrum, there were correlations in a sequence of H_3-21 [δ 1.26(3H,d)], H-20 [δ 2.75(1H,m)], H-17 [δ 2.11 1H, (m)],H-16[δ 4.96 (1H,dd, *J* = 3.9, 9.1 Hz)], and H-15[δ 4.24 (1H,m)] and in a sequence of H_3-27 [δ 1.13 (3H, d, *J* = 6.4 Hz), H-25 [δ2.96 (1H, overlapped)], H-24 [δ 2.97 (1H, overlapped) and 1.96 (1H, m)], and H-23 [4.67 (1H, br d, *J* = 8.3 Hz)]. This indicated that two hydroxyl groups were attached to C-15 (δ 77.5) of tigogenin.[15] The location of the lactone ring was detrmined from the HMBC spectrum. The cross-peaks observed for H_3-27/C-26 (δ 180.4) and H-24,H-25/C-26 were consistent with the presence of the F-ring lactone moiety.

ROESY correlations of H-18/H-15, H-20; H-23/H_3-21, H-20; and H-27/H_2-24 indicated that the orientations of the hydroxyl groups at C-15 and C-23 and the methyl group at C-25 were α. The chemical shift for C-20 (δ 37.1) supports an R-configuration at C-22, since C-20 for a 22 *S*-isomer showed a 6 ppm downfield shift, compared to the corresponding value in a 22 *R*-ismor . On the basis of these data., the structure of the aglycone was identified as (22*R*,25*R*)-3β,15α,23α-thrihydroxy-5α-spirostan-26-one.[26] The monosaccharides obtained after acid hydrolysis of compound 2 were derivatized into aldononitrile peracetataae derivatives and analyzed by GC-MS using authentic samples as references. Glucose and galactose in the relative proportions of 3; 1 were detected.The absolute configurations of the sugar residues were assumed to be S-galactose and D-glucose. These assumptions were based on the usual configuration of naturally occurring monosaccharides. NMR coupling constants ($^{3}J_{1,2}$>7Hz) for anomeric protons indicated that the anomeric carbon configuration was β for the D-galactopyranosyl and D-glucopyranosyl moieties.The attachment points of the sugar chain and interglycosidic linkage were established by an HMBC experiment.[17] Long-range correlations were observed between H-1 (δ 4.84) of galactosyl and C-3 (δ 77.3) of the aglycone, H-1 (δ 5.13) of glucosyl I and C-4 (δ 80.0) of galactosyl, H-1 (δ 5.56) of glucosyl II and C-3 (δ 81.2) of glucosyl I, and H-1 (δ 5.28) of glucosyl III and C-3 (δ 88.3) of glucosyl I.[18] The ^{13}CNMR chemical shifts of the sugar chain were in good agreement with reported data.[5] On the basis of the above data, the structure of **2**

was established as (22*R*,25*R*)–3β,15α, 23α-trithydroxy-5α-spirostan-26-one 3-*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→3)]-*O*-*β*-D- glucopyranosyl-(1→4)-*O*-*β*-D-galactopyranoside.[19]

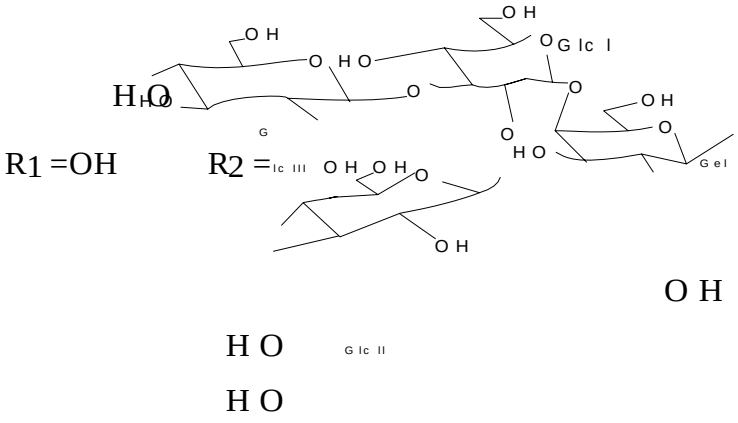

Figure:-2 Structure of compound 2

Anti –Inflammatory study

Ethanolic extract of *solanum nigrum*, showed a significant ($p<0.01$) inhibition of carrageenan induced rat paw edema and the results are presented intable -2. The extract of *S. nigrum* showed 40.81%, 39.43% and 46.47% edema inhibition respectively after third hour at 200 mg/kg dose. Maximum activity was found at 3.0 hr intervals with each dose.[20] Among these plant of *S. nigrum* showed maximum anti-inflammatory activity every hour. The inflammation induced by carrageenan is biphasic in nature.[21] The initial phase of edema has been attributed to the release of histamine and serotonin; the edema maintaining during the plateaue phase, attribute to kinin like substances and the second accelerating phase of swelling is attributed to the release of prostaglandin.[22] Since the extract of *S. nigrum* inhibited the carrageenan induced edema that involves release of histamine and serotonin in the first phase; hence the inhibitory effect of the extracts could be partly due to inhibition of mast cellmediator release.[23]

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Table.2-Percent Protection Comparison of Ethanolic Extract of *S. nigrum*

Treatment	Edema Volume					
	1.00 hr		3.00 hr		5.00 hr	
	(Ml)	%Inhibition	(ml)	%Inhibition	(ml)	Inhibition
Control	0.65 ±0.03	----	0.70 ±0.04	----	0.69 ±0.05	----
S. nigrum (100mg/kg)	0.40±0.05	25.66	0.42±0.03	40.81*	0.35±0.04	33.78*
S. nigrum (200mg/kg)	0.38 ±0.02	28.33	0.38 ±0.06	46.47*	0.32± 0.02	38.13*
S. nigrum (300mg/kg)	0.39 ± 0.03	26.74	0.43 ±0.04	39.43*	0.48± 0.02	30.43*
Standard (Diclofenac Sodium) (5mg/kg)	0.03	±0.06 55.57	0.25	0±.03 65.00*	0.29	0±.03 58.00*

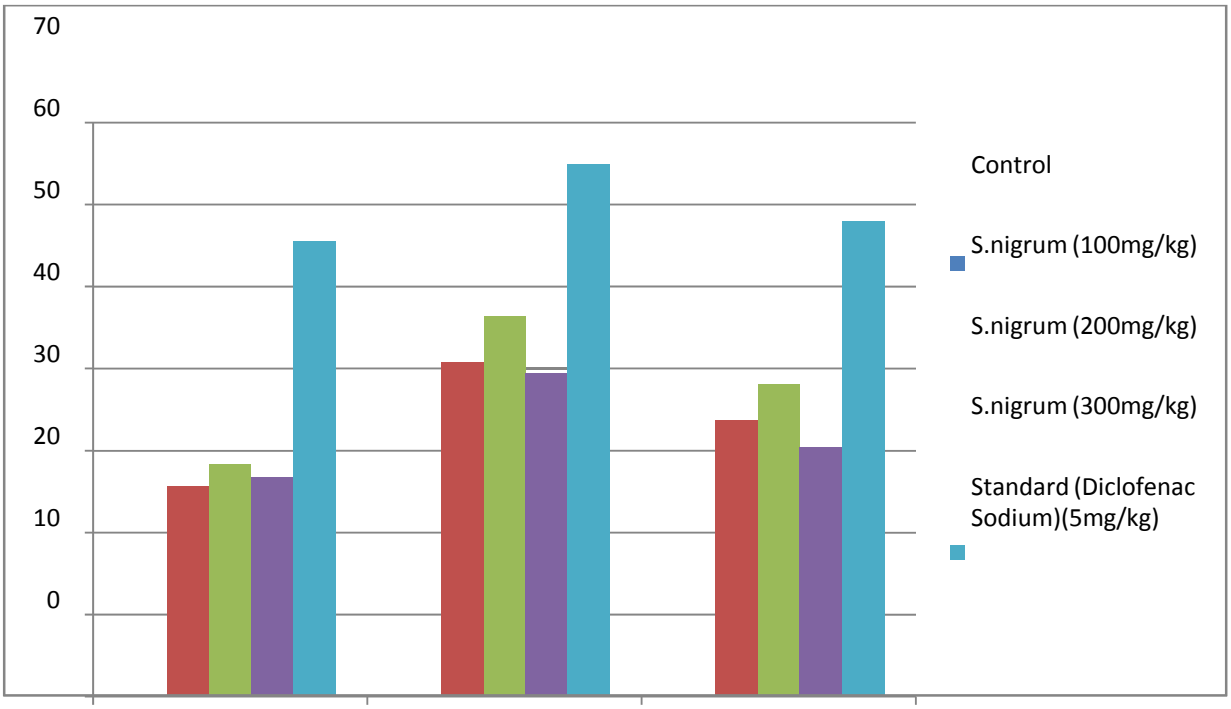

Figure.3-Percent Protection Comparison of Ethanolic Extract of *S. nigrum*

Conclusion

The present study suggest that *E. cuneatum* alkaloid leaf extract is a potential source of antioxidant as it showed an antioxidative properties in DPPHantioxidant assay. The extract also possesses anti-inflammatory properties as it has similar effects to aspirin in carrageenan-induced model of paw edema test. The alkaloids extracts showed a reduction of the edema in a dose dependent manner.[24] This study confirmed the traditional use of this plant for medicinal purposes. However, since the present study do not proceed for further isolation of alkaloids, the exact alkaloids which is exhibiting the major properties of *E. cuneatum* extract need to be further investigated in the future.

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