Anti-inflammatory and NF-κB inhibitory activity of aerial parts of *Cestrum diurnum*

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Research

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Abstract

Background

Cestrum diurnum L. (Solanaceae) is used in various traditional medicine for pain and related disorders. The Malayali tribe of Tamil Nadu in India use the leaf in joint pain. In the Chinese traditional medicine it is used for the treatment of burns and swellings. Present study was designed to evaluate its traditional use in pain and inflammation.

Methods

Methanol extract of the aerial parts of *C. diurnum* was tested by carrageenan induced paw oedema and formalin induced paw licking test in mice at the oral doses of 150 and 300 mg/kg body weight. NF-κB inhibitory activity was evaluated by TNF-α induced NF-κB activation assay in RAW 264.7 macrophage cells at the concentration of 100 μg/ml.

Results

The extract, at the doses of 150 and 300 mg/kg, showed significant inhibition ($p < 0.05$) of carrageenan induced paw oedema and the effect persisted throughout the entire experimental period of 3 h with the highest activity (50% inhibition) at 3rd h. In formalin induced paw licking test, the extract exhibited significant ($p < 0.05$) inhibition of paw licking, both in the early and late phase of the experiment at the aforementioned dose levels. At the concentration of 100 μg/ml, the extract did not inhibit the nuclear translocation of NF-κB. Rather, the extract was found to downregulate NF-κB p65 protein expression.

Conclusions

The present work supports the folkloric use of the plant for its analgesic and anti-inflammatory action which might involve downregulation of NF-κB p65 protein expression and/or inhibition of autacoid (histamine, serotonin, prostaglandin) synthesis.

Background

The day-blooming Jasmine (*Cestrum diurnum* L., Family: Solanaceae) is an erect evergreen woody shrub with numerous leafy branches and simple leaves. The sweet smelling white flowers appear in short clusters, and the black fruits are nearly globular shaped berries. The younger parts are covered with a very sparse glandular scruff. Although native to West Indies, this plant is cultivated in gardens as well as grows in the wild throughout India and Bangladesh.

Many *Cestrum* species are used in the Chinese traditional medicine for the treatment of burns and swellings [1]. The plant is traditionally used by the Malayali tribe of Tamil Nadu to treat weeping illness and joint pain [2]. The leaf paste is used in joint pain while the flower fragrance is used to reduce chemical pollutions in atmosphere [3]. The plant is reported to possess cytotoxic, thrombolytic [4], and antimicrobial [2] properties. Previous phytochemical studies resulted in the isolation of alkaloids [5, 6], norlignans, glycosides [1], saponin glycosides (diurnoside having antifungal activity) [7, 8], triterpene (ursolic acid, β-amyrin) and steroids which include β-sitosterol, vitamin D_3 and its derivatives including 1,25-$(OH)_2D_3$ [8–10]. Leaves of *C. diurnum* was found to be rich in β-carotene, lutein, xanthine, calcium, and vitamin D_3 [11]. *C. diurnum* also contains different fatty acids including myristic, palmitic, stearic, oleic and linoleic acid [10]. The plant was reported to enhance calcium and phosphate uptake and can be effective in the prevention and treatment of bone metabolic disorders [12, 13]. The plant is a component of cosmetic preparation used in the prevention or treatment of skin disorders with wrinkled, flaky, aged, photo-damaged skin caused by the lack of vitamin D [14].

Although reported to be used in pain and inflammatory disorders, *C. diurnum* has not been studied before for such activity. This study was designed to investigate the aerial part of *C. diurnum* for anti-inflammatory activity and its role on the inflammatory mediator NF-κB.

Methods

Collection and identification of plant material

The aerial parts of the plant *Cestrum diurnum* L. (Family: Solanaceae) was collected from Dumuria area of Khulna district, Bangladesh (*Coordinate* 22.8083° N, 89.4250° E). The species was identified by the experts at Bangladesh National Herbarium (voucher specimen no. DACB 38792).

Test animals

Young Swiss albino mice of either sex, 3-4 weeks old, weighing 20-25 g were purchased from the Animal Resources Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B) and used for *in vivo* anti-inflammatory activity screening. The animals were kept in the animal house of Manarat International University under standard laboratory conditions (relative humidity 55-65%, room temperature 25.0 ± 2.0 °C and 12 h light-dark cycle) for adaptation. They were fed with ICDDR,B formulated pelleted standard diet and water *ad libitum*.

Extract preparation

The collected aerial part of the plant was separated from undesirable materials and shade-dried. The dried plant materials were ground into a coarse powder with the help of a grinder. The powdered sample was stored in an airtight container and kept in a cool, dark and dry place until extraction commenced.

Powered plant material (135 g) was taken in a clean, dry, flat-bottomed glass container and soaked with 400 ml of methanol (Merck Germany). The container was sealed and kept for 3 days with occasional shaking or stirring. It was filtered through a cotton plug and evaporated to dryness using a rotary vacuum evaporator to get the crude extract (yield 7.4% of the dry powder).

Carrageenan-induced paw oedema test

Anti-inflammatory activity was tested by carrageenan induced paw oedema test in mice [15, 16]. In brief, 0.1 ml of 1% w/v carrageenan suspended in 1% CMC was injected into the sub-plantar tissue of the left hind paw of each mouse. Mice were divided into four groups containing six animals in each. Test groups received plant extract at the doses of 150 and 300 mg/kg body weight, while control and positive control group received vehicle and diclofenac sodium (10 mg/kg body weight), respectively. The paw thickness was measured using a calliper at 0, 60, 120 and 180 min after the carrageenan injection. The anti-inflammatory activity was calculated as percentage inhibition of oedema in the animals treated with extract/standard as compared to the control. Percentage (%) inhibition of oedema was calculated using the following formula where T_t and T_0 are the thickness of paw oedema in test/positive control and control group, respectively.

[Please see the supplementary files section to view the equation.]

Formalin induced paw licking test

Mice were grouped in control, test and positive control receiving vehicle, extract (150 and 300 mg/kg) and diclofenac sodium (10 mg/kg), respectively. Formalin solution (0.2 ml of freshly prepared 5% v/v formalin in distilled water) was injected into the dorsal surface of the right hind paw 30 min after the treatments. The mice were observed for the time spent licking the injected hind paw during the early phase (0-5 min) and late phase (15-30 min) of post formalin injection with the help of a stop watch and recorded [17, 18].

TNF-α induced NF-κB activation assay

RAW 264.7 cells (Sigma-Aldrich) were cultured in RPMI supplemented with 10% foetal bovine serum, penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. NF-κB activation kit (Thermo Scientific) was used as previously described [19]. RAW 264.7 cells were seeded in 96-well plate overnight and the cells were stimulated with tumour necrosis factor α (TNF-α) for 1 h. The extract was added into the medium and further incubated for 4 h. Cells were fixed, permeabilised and incubated with NF-κB p65 antibody for 1 h. Staining solution (containing DyLight 488 Goat Anti-Rabbit and Hoechst dye) was then added and further incubated for 1 h. The plate with stained cells was evaluated using a Cellomics ArrayScan HCS Reader (Cellomics, PA, USA). Data

were captured, extracted and analysed with ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics).

LC-MS analysis

The HR-ESIMS spectra were recorded on 6530 Accurate-Mass Q-TOF LC/MS spectrometer (Agilent Technologies) equipped with ZORBAX Eclipse XDB-C18 Rapid Resolution column (HT 4.6 mm i.d. × 50 mm × 1.8 µm). The extract was injected at a volume of 10 µl (1 mg/ml) with a flow rate of 1 ml/min. A gradient elution with solvent A (1% trifluroacetic acid in water) and B (MeOH) starting at 95%A5%B to reach 100%B in 40 min was adopted. Other parameters include capillary voltage of 3500 V, nebuliser pressure 35 psi, drying gas flow 8 L/min, drying gas temperature 200ºC. The mass recorded in the range of m/z 100-1000 and expressed as total ion chromatogram (TIC).

Data analysis

Values are expressed as mean ± SEM. Results were analysed using one-way ANOVA followed by Dunnet's test. Differences were considered as statistically significant at $p<0.05$.

Results

Carrageenan-induced paw oedema

C. diurnum extract, at the doses of 150 and 300 mg/kg, prevented carrageenan-induced paw oedema for the entire experimental period of 3 h. The results were comparable to that of positive control and significantly different from that of control (Table 1).

Table 1 Effect of *C. diurnum* extract on carrageenan induced paw oedema in mice

Treatment	Dose (mg)	Paw oedema in mm (% inhibition)		
		1st h	2nd h	3rd h
Control	-	4.67±0.33	3.67±0.33	2.33±0.21
C. diurnum extract	150	3.83±0.17*	2.67±0.21*	1.67±0.21*
		(18%)	(27%)	(29%)
	300	3.50±0.34*	2.50±0.18*	1.17±0.11*
		(25%)	(32%)	(50%)
Diclofenac sodium	10	4.00±0.13	2.3±0.21*	1.75±0.25
		(14%)	(36%)	(25%)

Values expressed as mean ± SEM; *n*=6; *p<0.05.

Formalin induced paw licking in mice

C. diurnum extract, at the doses of 150 and 300 mg/kg extract significantly (p<0.05) suppressed the licking activity both in the early and late phase of formalin-induced pain in test mice as compared to the control (Table 2).

Table 2 Effect of *C. diurnum* extract on formalin induced paw licking test in mice

Treatment	Dose (mg)	Duration of licking (% Inhibition)	
		Early phase (0-5 min)	Late phase (15-30 min)
Control	-	8.50±0.76	23.00 ± 1.32
C. diurnum extract	150	7.67 ± 1.21* (10%)	15.00 ± 1.00* (35%)
	300	7.50 ± 0.35* (12%)	11.33 ± 1.21* (51%)
Diclofenac sodium	10	5.33 ± 0.49* (37%)	8.00 ± 1.67* (65%)

Values expressed as mean ± SEM; *n*=6; *p<0.05.

Effect on TNF-α induced NF-κB activation

In the absence of TNF-α, NF-κB p65 remained in the cytoplasm of RAW 264.7 cells. In response to TNFα stimulation, NF-κB p65 translocates from cytoplasm into the nucleus implying NF-κB activation. When tested at the concentration of at 100 µg/ml, *C. diurnum* extract did not inhibit TNF-α -mediated nuclear translocation of NF-κB p65 very strongly although the result was significantly different from that of control (**Fig. 1**). Interestingly, we found that total NF-κB p65 protein expression was down-regulated, indicating the extract exerts its anti-inflammatory effect by inhibiting NF-κB p65 protein expression (**Fig. 2**).

Results of LC-MS analysis

LC-MS analysis of *C. diurnum* revealed the presence of some secondary metabolites already reported from this species that include vitamin D_3 and its derivatives (Table 3). Total ion chromatogram and MS spectrum for the major peaks is given in supplementary file.

Table 3. Detection of compounds previously reported from *C. diurnum*.

Name	Molecular formula	Class	M+H	M+Na	Exact mass	t_r	Reference
Medusaside B	$C_{26}H_{34}O_{12}$	Lignan	539.2319	-	538.2050	17.54	[1]
Liriodendrin	$C_{36}H_{50}O_{18}$	Lignan	-	793.3956	742.2684	24.72	[1]
Cholecalciferol	$C_{27}H_{44}O$	Steroid	385.2925	-	384.3392	32.26	[6]
Calcifediol	$C_{27}H_{44}O_2$	Steroid	401.2182	-	400.3341	24.97	[6]
Calcitriol	$C_{27}H_{44}O_3$	Steroid	417.1596	439.3579	416.3290	21.58	[6]
Nicotine	$C_{10}H_{14}N_2$	Alkaloid	-	185.1173	162.1157	23.28	[6]
Nornicotine	$C_9H_{12}N_2$	Alkaloid	149.0224	171.1018	148.1000	29.44	[6]
Tigogenin	$C_{27}H_{44}O_3$	Saponin	417.1569	439.3579	416.3290	21.58	[9]
Ursolic acid	$C_{30}H_{48}O_3$	Triterpene	457.2570	479.3513	456.3603	32.69	[9]

Discussion

Carrageenan-induced acute inflammation is one of the most acceptable test procedures to screen anti-inflammatory activity of crude extracts or pure compounds. The total duration of carrageenan-induced paw oedema in mice model is generally represented by a biphasic curve. The first phase of inflammation occurs immediately after the carrageenan injection mediated by histamine and serotonin release and lasts for about two and half hour [20]. The second phase of inflammation is due to the prostaglandin synthesis which starts after the first phase and can last up to six hours [15, 21]. Both the extract and diclofenac sodium produced mild inhibition of paw oedema in the early phase of inflammation but the inhibition was more noticeable during the late phase indicating their key inhibitory role on cyclooxygenase mediated prostaglandin synthesis.

The biphasic model of formalin induced nociception is represented by neurogenic (0–5 minute) and inflammatory pain (15–30 minute), respectively. The early phase of neurogenic pain is due to the chemical stimulation of the nociceptors predominantly C-fibres, while the late phase of tonic pain is a combination of peripheral inflammatory responses and functional changes in spinal cord level [22]. Drugs that act primarily on the central nervous system such as narcotic analgesics inhibit both phases while steroids and NSAIDs suppress mainly the late phase [23]. Suppression of both phases by the extract implies that it contains constituents that act both centrally and peripherally. Formalin-induced paw oedema is also one of the most suitable test procedures to evaluate chronic inflammation, as it

closely resembles human arthritis [24]. Thus, the present investigation supports the use of this plant in ethnobotanical practice for alleviating arthritic pain.

To investigate the underlying mechanism of anti-inflammatory effect, RAW 264.7 cells were treated with the extract before stimulating them with TNF-α, a potent inducer and activator of nuclear factor-κB (NF-κB). The NF-κB protein family is a central mediator of inflammatory responses and plays a crucial role in the development of acute or chronic inflammation [19]. It consists of five subfamilies that share same DNA binding domain and dimerisation domain. The NF-κB proteins are associated with each other to form homo- or heterodimeric complexes, which remain inactive in the cytoplasm by sequestering with inhibitory proteins (IκB). Upon stimulation by various factors including LPS or TNF-α, IκB is degraded and dissociated from NF-κB. As a result, NF-κB migrates from the cytoplasm into the nucleus and triggers the transcription of numerous genes involved in pro-inflammatory responses [25, 26]. Various classes of natural products have been identified with the capability to downregulate NF-κB; prevent nuclear translocation of NF-κB and/or inhibit their DNA binding [27, 28]. In the present study, *C. diurnum* extract was found to downregulate the expression of NF-κB p65 protein. Reviews on NF-κB inhibitory natural products reveal that most of the identified compounds prevent the nuclear translocation of NF-κB while very few of them including catechin, epigallocatechin gallate act through the downregulation of NF-κB expression [29, 30]. Development of a suitable anti-inflammatory agent working through the inhibition of NF-κB is still underway [28, 31].

LC-MS analysis of the crude extract revealed the presence of a number of bioactive compounds including ursolic acid, nicotine and nornicotine. In a previous study ursolic acid, a pentacyclic triterpene, ubiquitously present in many plants inhibited prostaglandin biosynthesis through the inhibition of COX-2 enzyme [32]. However, another study showed that the anti-inflammatory effect of ursolic acid involves the inhibition of NF-κB activation and its DNA binding [33]. Thus, the downregulation of NF-κB by *C. diurnum* extract might involve compound(s) other than ursolic acid. Nicotine and nornicotine cannot be ruled out for the observed anti-inflammatory activity since anti-inflammatory activity has been reported for nicotine [34] and analgesic activity for nornicotine [35].

Inflammation is a physiological response to injury, microbial attack or malignancy [36]. Pain is just one of the manifestations of the inflammatory responses and prolonged inflammation can aggravate an already existing disease as well as new disease progression including cancer [37]. Present study suggests that *C. diurnum* extract possesses anti-inflammatory and anti-nociceptive activity; and the plant can be useful in the discovery of new leads for the treatment of diseases linked to NF-κB mediated inflammatory responses.

Conclusions

Present investigation supported the ethnobotanical uses of *C. diurnum* in inflammatory pain. Further, we found an interesting mechanism of the anti-inflammatory action of *C. diurnum* through the

downregulation of NF-κB p65 protein. The plant also proves itself interesting enough for bioassay guided study to find new natural anti-inflammatory agent.

Abbreviations

NF-κB: Nuclear factor kappa B; ICDDR,B: International Centre for Diarrhoeal Diseases and Research, Bangladesh; CMC: Carboxymethyl cellulose; TNF-α: Tumor necrosis factor alpha; RAW 264.7 cells: Murine macrophage cell line; RPMI: Roswell Park Memorial Institute Medium; LC-MS: Liquid chromatography mass spectrometry; HR-ESI: High resolution electrospray ionisation; QTOF: Quadropole time-of-flight; SEM: Standard error of mean; ANOVA: Analysis of variance; NSAID: Nonsteroidal anti-inflammatory drug; IκB: inhibitor of κB proteins; COX-2: Clycooxygenase enzyme-2

Declarations

Acknowledgement

Not applicable.

Author contributions

AK and MR designed the study, MLN, WFW and HH did the *in vivo*, *in vitro* assay, and LC-MS analysis, respectively. KA, AK, MR, CYL, MAM, SJU, JAS assisted in the preparation of the manuscript.

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No.

Availability of data materials

Datasets used in the study are available from the corresponding author on request.

Ethics approval and consent to participate

The research was carried out according to the standard guidelines for research with animals and the experimental protocol approved by the Animal Ethics Committee, Manarat International University (Reference no: MIU/AHEC/01/2013).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

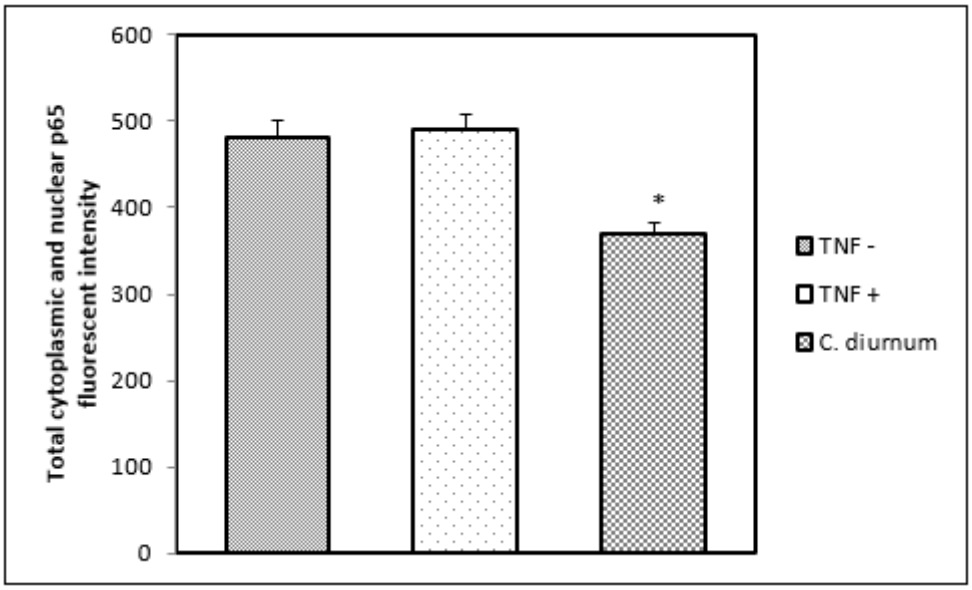

Figure 1

C. diurnum extract induced inhibition of TNFα-induced nuclear translocation of NF-κB in RAW 264.7 cells (*p<0.05)

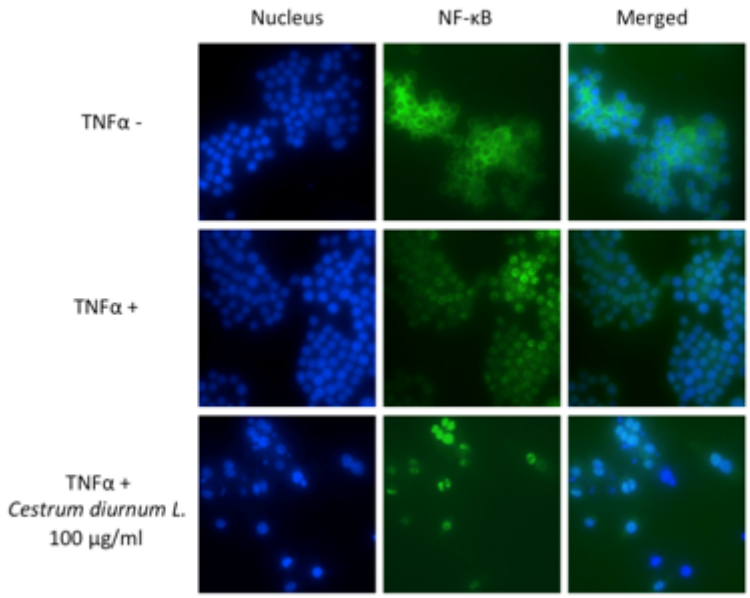

Figure 2

TNFα induced nuclear translocation of NF-κB in RAW 264.7 cells. Cells were pre-treated with 100 µg/ml of C. diurnum extract prior to stimulation with 1 µg/ml of TNFα for 30 min. Cells were fixed, stained for NF-κB and visualized using HSC

Supplementary Files

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