

Effect of *Ipomea carnea* Jacq. flowers on hematological changes in toluene diisocyanate-induced inflammation in Wistar rats

Jain Smita¹, Patil U.K.^{2*}

¹ Department of Pharmacognosy, VNS Institute of Pharmacy, Bhopal (M.P.) 462044, India;

² Department of Pharmaceutical Sciences, Dr. H.S. Gour Central University, Sagar (M.P.) 470003, India

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[ABSTRACT]

AIM: To investigate the active chloroform fraction of the ethanol extract of *Ipomoea carnea* flowers on hematological changes in toluene diisocyanate-induced inflammation in Wistar rats.

METHOD: Except for the control group, all of the rats were sensitized with intranasal application of 5 μ L of 10% toluene diisocyanate (TDI) for 7 days. One week after second sensitization, all of the rats were provoked with 5 μ L of 5% TDI to induce airway hypersensitivity. After the last challenge, blood and bronchoalveolar lavage (BAL) fluid were collected and subjected to total and differential leucocytes count. Flash chromatography was performed on the most active chloroform fraction to isolate an individual component.

RESULTS: Treatment with the ethanolic extract and its chloroform fraction at an oral dose of 200 mg·kg⁻¹ showed a significant decrease in circulating neutrophil and eosinophil in blood and BAL as compared with standard dexamethasone (DEXA). The structure of the compound obtained from chloroform fraction of *Ipomea carnea* was elucidated as stigmast-5, 22-dien-3 β -ol on the basis of spectral data analysis.

CONCLUSION: The chloroform fraction was found to be more effective to suppress airway hyper reactivity symptoms, and decreased count of both total and differential inflammatory cells.

[KEY WORDS] *Ipomea carnea*; Toluene diisocyanate; Bronchoalveolar lavage; Neutrophils; Stigmastrol

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Introduction

According to the National Institutes of Health, asthma is defined as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, as mast cells, eosinophils, T-lymphocytes, neutrophils, and epithelial cells [1]. Asthma is caused by a very complex interaction between inflammatory cells and mediators. Occupational asthma is the most prevalent lung disease reported in many industrialized countries. Toluene diisocyanate (TDI), a low molecular weight compound, widely used as an industrial chemical, is the most commonly identified cause of occupational asthma, as demonstrated by Bernstein [2]. It is estimated that 5%–15% of chronically exposed workers develop

symptoms of asthma in response to very low levels of exposure [3–4]. Plant-based approaches for the treatment of asthma have regained their popularity, with their safety and efficacy aspects being supported by controlled clinical studies [5].

The plant *Ipomoea carnea* Jacq. (Besharam, Behaya) (Convolvulaceae) is a large, diffuse, or struggling shrub with a milky juice, leaf ovate cordate, entire, acuminate, flower large campanulate, pale rose, pink or light violet in lax, dichotomously branched axillary and terminal, pedunculate cymes; fruits glabrous capsule; seed silky [6–7]. It is well-distributed in India, and is found particularly in Chhattisgarh and Madhya Pradesh [8–9]. It is also called Chokob Kat, and morning glory has been traditionally used in the Ayurvedic and Chinese systems of medicine for stimulating allelopathic effects on the body and mind. Traditional healers have used its milky juice for treating skin disorders like leucoderma. It is traditionally used for the treatment of Reuma IK, which is a cold-natured illness characterized with generalized discomfort and pain in the bones and joints in rheuma-

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[*Corresponding author] Patil U.K.: jainsmita09@gmail.com

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tism^[10]. It is rich in iron and *S*-methyl methionine (vitamin U), and is used traditionally to treat gastric and intestinal disorder and diabetes mellitus, probably because of its anti-hyperglycemic activity^[11]. It is used to decrease the teratogenic effects resulting from cyclophosphamide^[12]. The plant also shows anti-allergic, anti-inflammatory, anti-microbial, and anticancer activity^[13] and is also reported to have wound healing activity^[14]. Preliminary qualitative phytochemical screening of *I. carnea* revealed the presence of phenolic compounds, terpenoids, flavonoids, and steroids. Some of these constituents have antioxidant and antimicrobial activities^[15]. An aqueous extract of *I. carnea* shows neuromuscular blocking activity. It is also used as aphrodisiac, purgative and cathartic^[16]. The leaves of *I. carnea* contain flavonol glycosides and ergine (D-lysergic acid amide)^[17]. Polyhydroxylated alkaloids were isolated from the leaves, flowers, and seeds^[18]. Chromatographic separation of the leaf extract resulted in the isolation of swainsonine, 2-epilantiginosine, calystegines B (1), B (2), B (3), and C (1) and *N*-methyl-*trans*-4-hydroxy-*L*-proline and β -sitosterol^[19-21]. The flavonoid content of the flowers was quite high compared to that of the leaves and the stem^[22].

The present study was planned to experimentally assess the effect of a flash chromatographic fraction of the chloroform soluble portion of a 90% hydroalcoholic extract of *I. carnea* flowers on hematological changes in toluene diisocyanate-induced inflammation in Wistar rats.

Materials and Methods

Plant material

The flowers of *I. carnea* were collected in June, 2011 from Bhopal (M.P.), India, and were identified and authenticated by Dr. Zia ul Hassan, Department of Botany, Saifia College of Science & Education, Bhopal. A voucher specimen no.181/Bot/Safia/ 2011 is deposited in the herbarium of the botany department.

Extraction and fractionation

The dried flowers were coarsely powdered and then exhaustively extracted with 90% ethanol in a Soxhlet apparatus. The ethanolic extract obtained was evaporated under vacuum to afford a dark brown mass (48 g, 6.89% yield). The solvent-free ethanolic extract was further dissolved and extracted with chloroform. The ethanolic extract and its chloroform-soluble fraction were thus obtained.

Phytochemical profiling

Preliminary phytochemical studies were performed to detect the presence of various phytoconstituents: namely, alkaloids, glycosides, tannins, coumarins, flavanoids, terpenoids, and sterols^[23].

Drugs and chemicals

All the solvents used for extraction, qualitative tests, and chromatographic studies were of analytical grade, and obtained from Hamidia Laboratories and Loba Chemie, Mumbai, India. 2, 4-Toluene diisocyanate, Field's stain, and dex-

amethasone (DEXA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of test materials

The ethanolic extract and chloroform fraction were suspended in distilled water and polyoxy-ethylene sorbiton mono-oleate (Tween 80).

Experimental animals

The Wistar albino rats were selected and housed in polypropylene cages maintained under controlled conditions. The animals were fed with pellet food and water *ad libitum*. The animals were fasted for 12–14 h before experimentation, and were allowed free access to water. Rats of either sex, 6–8 weeks old, and weighing 100–120 g, were used for the experiments. The Institutional Animal Ethical Committee of the Research Center, following CPCSEA Reg. No. –778/03/c/CPCSEA, approved the study.

Sensitization and provocation of animals

2, -Toluene diisocyanate (TDI) was used as antigen for the sensitization. TDI was prepared in ethyl acetate to obtain a concentration of 10% and 5% for provocation.

Albino rats were divided into eight groups of six rats each. Group I served as normal control, while Group II served as TDI-sensitized control, and both received distilled water (vehicle). Group III was kept as reference standard and administered with dexamethasone 2.5 mg·kg⁻¹. Groups IV and V were treated orally with the ethanol extract at doses of 100 and 200 mg·kg⁻¹, respectively. Groups VI and VII were administered the chloroform fraction at the oral dose of 100 and 200 mg·kg⁻¹, respectively. Group VIII were treated orally with the standard drug and the isolated compound stigmasterol at 2.5 mg·kg⁻¹^[24].

Except for control group I, all of the rats were sensitized with an intranasal application of 5 μ L of 10% TDI for seven days. All the rats were re-sensitized again for seven consecutive days, after being given a week of rest. One week after the second sensitization, all of the rats were provoked with 5 μ L of 5% TDI to induce airway hypersensitivity. All the animals of group II to group VII were administered the respective drug treatment from one week prior to the first course of sensitization and continued daily up to the final challenge with 5% TDI. The treatment was also continued during the rest period^[25].

Blood collection and cell count

After the last challenge, blood was collected from cervical vein, and was diluted at 1 : 10 with 1% acetic acid to lyse the red blood cells for total leucocyte counts. Blood smearing following staining with Field's stain was used to observe leucocyte subtype counts.

Bronchoalveolar lavage (BAL) fluid collection

Bronchoalveolar lavage was performed with 0.9% normal saline (37 °C) through a tracheal cannula introduced into the trachea. The BAL fluid was centrifuged at 500 g for 10 min and the supernatant was separated. The cell pellet which settled was washed with 250 μ L of 0.9% saline containing

bovine serum albumin. This aliquot of cells was used for total and differential cell count using an automated cell counter to investigate the inflammatory status in the airways [26].

Statistical analysis

Statistical evaluation of the data was done by Student's *t* test. (Graph PAD Instat software, Kyplot). $P < 0.05$ was considered to be significant.

Chromatographic studies and isolation of compound

TLC of the ethanol extract and its chloroform fraction was carried out using various solvent systems. A mixture of *n*-butanol–ethanol–acetic acid–water (30 : 10 : 10 : 10) gave the best resolution for the ethanol extract. The chloroform fraction was best resolved in a mixture of CHCl_3 –*n*-butanol–ethanol (5 : 2 : 1). Flash chromatography (Büchicontroller C-610) was performed on the most active chloroform fraction found on the basis of pharmacological studies with a view to isolate an individual component. The column was eluted with petroleum ether, chloroform, and methanol successively. Elution of the column was with CHCl_3 –methanol (70 : 30) [Fractions 41–57] yielded a colorless amorphous powder which was recrystallised from methanol afford compound **1** (42 mg, 0.034% yield). TLC of the powdered sample was carried out using various solvent systems. The most appropriate was found to be chloroform–ethanol–acetic–acid (18 : 6 : 1)

Characterization of the isolate

The UV spectra were recorded on a Shimadzu UV 1700 model spectrometer EtOH. The IR spectra were measured on a JASCO FT/IR-5300 spectrometer. The ^1H spectra and ^{13}C spectra were recorded on a Bruker AV300/2 FT-NMR spectrometer, using DMSO and CDCl_3 as solvents. Mass spectra were recorded on a Scan AP spectrometer/JEOL JMS AX-500 spectrometer.

Isolate compound has R_f : 0.45; mp 169–170 °C; λ_{max} in EtOH: 257 nm; IR (KBr) ν_{max} : 3420, 2942, 2852, 1640, 1463, 1378, 1164, 965 cm^{-1} ; Positive ion FAB-MS m/z 412 $[\text{M}]^+$ ($\text{C}_{29}\text{H}_{48}\text{O}$), 397, 394, 382, 253, 229, 213; ^1H NMR (CDCl_3) and ^{13}C NMR (CDCl_3) data are shown in Table 3. Compound was characterized as stigmast-5, 22-dien-3 β -ol (stigmasterol) by comparing the physical and spectral data with the results reported by Khatun [27].

Results

Preliminary phytochemical screening revealed the presence of tannins, coumarins, alkaloids, and glycosides in the ethanolic extract of *I. carnea*, while the chloroform fraction revealed the presence of sterols and flavonoids.

After provocation with 5% TDI, the TDI-sensitized rats exhibited symptoms like irritability, sneezing, and hyperrhinorrhea with a little elevation of body temperature as ± 2 °C. The control, DEXA, and *I. carnea* flower extract-treated rats did not show such symptoms, but a significant reduction was found in body temperature. TDI exposure resulted in an increase of circulating neutrophils and eosinophils in the blood of TDI-sensitized rats. The numbers of leucocytes and basophils, lymphocytes, and monocytes were not significantly different among the treated groups (Table 1). Treatment with the ethanolic extract and its chloroform fraction at a dose of 200 $\text{mg}\cdot\text{kg}^{-1}$ showed a significant decrease in circulating neutrophils and eosinophils in blood as compared with standard DEXA.

The total cells and each leucocyte subtype were significantly increased in the BAL fluid of TDI-sensitized rats (Table 2). A significant reduction was found in these counts in DEXA, the ethanolic extract, and its chloroform fraction in the 200 $\text{mg}\cdot\text{kg}^{-1}$ treated groups of animals. The combined effect with standard drug, and the isolated compound from the chloroform fraction was found to be almost identical to standard DEXA to decrease the total cells and each leucocyte subtype in BAL fluid of TDI-sensitized rats.

Discussion

Zheng *et al.* developed an asthmatic model in Wistar rats by intranasal application of TDI, which involves determination of the total and differential cell count and estimation of cytokines in blood and BAL fluid [25]. Toluene diisocyanate (TDI) is known as a potent sensitizer that causes occupational asthma in a significant proportion of individuals exposed. In the present study, the inflammatory status in airways was investigated with evidence from of blood and airway cellular events. The differential count, particularly circulating

Table 1 Total cells and leukocytes subtypes in blood ($\times 10^3 \text{ cells}\cdot\text{mL}^{-1}$) (mean $\pm$ SEM, $n = 6$)

G.N.	Groups	Body Temp./°C	Total cells	Eosinophils	Lymphocytes	Monocytes	Neutrophils
I	Normal control	37.3 $\pm$ 1.21	14.78 $\pm$ 0.74	0.08 $\pm$ 0.05	13.69 $\pm$ 0.60	0.05 $\pm$ 0.03	1.53 $\pm$ 0.05
II	TDI-control	39.8 $\pm$ 0.89	15.48 $\pm$ 0.08	0.39 $\pm$ 0.13*	12.19 $\pm$ 0.82	0.034 $\pm$ 0.17	2.98 $\pm$ 0.34*
III	TDI + DEXA (2.5 $\text{mg}\cdot\text{kg}^{-1}$)	36.9 $\pm$ 1.12*	14.82 $\pm$ 0.38	0.06 $\pm$ 0.91**	12.25 $\pm$ 0.37	0.023 $\pm$ 0.87	1.74 $\pm$ 0.54**
IV	TDI + Ethanolic Extract (100 $\text{mg}\cdot\text{kg}^{-1}$)	37.4 $\pm$ 0.67	15.41 $\pm$ 0.16	0.28 $\pm$ 0.16	12.68 $\pm$ 0.29	0.032 $\pm$ 0.05	2.16 $\pm$ 0.13*
V	TDI + Ethanolic Extract (200 $\text{mg}\cdot\text{kg}^{-1}$)	36.1 $\pm$ 1.23	14.96 $\pm$ 1.38	0.19 $\pm$ 0.07*	12.46 $\pm$ 1.32	0.029 $\pm$ 1.07	1.98 $\pm$ 0.79*
VI	TDI + Chloroform Fraction (100 $\text{mg}\cdot\text{kg}^{-1}$)	35.9 $\pm$ 1.47*	15.12 $\pm$ 0.42	0.12 $\pm$ 0.79*	12.46 $\pm$ 2.01	0.031 $\pm$ 1.23	1.96 $\pm$ 0.43**
VII	TDI + Chloroform Fraction (200 $\text{mg}\cdot\text{kg}^{-1}$)	37.1 $\pm$ 0.34*	14.89 $\pm$ 1.31	0.09 $\pm$ 1.06**	12.38 $\pm$ 1.28	0.028 $\pm$ 0.09	1.83 $\pm$ 0.89**

* $P < 0.05$, ** $P < 0.01$, Group I vs Group II, Group II vs Group III-VII

Table 2 Total cells and leukocytes subtypes in BAL ($\times 10^5$ cells·mL⁻¹) (mean $\pm$ SEM, $n = 6$)

G.N.	Groups	Total cells	Eosinophils	Lymphocytes	Macrophages	Neutrophils
I	Normal control	14.68 $\pm$ 0.63	0.04 $\pm$ 0.08	1.18 $\pm$ 1.23	10.39 $\pm$ 0.36	0.08 $\pm$ 0.06
II	TDI-control	46.36 $\pm$ 1.23	1.38 $\pm$ 0.15* (97%)	3.45 $\pm$ 1.34 (93%)	39.43 $\pm$ 0.58 (15%)	0.89 $\pm$ 1.14* (98%)
III	TDI + DEXA (2.5 mg·kg ⁻¹)	20.23 $\pm$ 1.36* (56%)	0.46 $\pm$ 1.63** (67%)	1.36 $\pm$ 0.38 (61%)	15.58 $\pm$ 1.02** (60%)	0.43 $\pm$ 0.56** (52%)
IV	TDI + Ethanolic extract (100 mg·kg ⁻¹)	32.23 $\pm$ 1.46 (30%)	0.93 $\pm$ 1.21 (33%)	3.12 $\pm$ 0.89* (10%)	21.83 $\pm$ 1.56 (45%)	0.69 $\pm$ 1.14 (22%)
V	TDI + Ethanolic extract (200 mg·kg ⁻¹)	29.46 $\pm$ 1.38 (36%)	0.74 $\pm$ 1.02* (46%)	2.89 $\pm$ 0.76 (16%)	19.46 $\pm$ 1.31** (51%)	0.58 $\pm$ 1.06* (35%)
VI	TDI + Chloroform fraction (100 mg·kg ⁻¹)	26.12 $\pm$ 0.32* (44%)	0.71 $\pm$ 0.26 (49%)	3.02 $\pm$ 1.31 (12%)	18.87 $\pm$ 0.94 (52%)	0.52 $\pm$ 0.65 (42%)
VII	TDI + Chloroform fraction (200 mg·kg ⁻¹)	24.94 $\pm$ 0.75* (46%)	0.67 $\pm$ 0.38** (51%)	2.72 $\pm$ 1.13* (21%)	17.12 $\pm$ 0.63** (57%)	0.49 $\pm$ 0.14** (45%)
VIII	TDI + DEXA + Stigmasterol (2.5 mg·kg ⁻¹)	16.38 $\pm$ 1.21* (65%)	0.43 $\pm$ 0.12* (69%)	1.31 $\pm$ 0.76* (62%)	14.32 $\pm$ 0.84* (64%)	0.38 $\pm$ 0.32** (57%)

* $P < 0.05$, ** $P < 0.01$, Group I vs Group II, and Group II vs Group III-VIII

eosinophils and neutrophils in blood and total cells and differential leucocyte subtypes, were found to be significantly elevated in TDI-sensitized rats as already discussed by Mali *et al.* An increased number of neutrophil and eosinophil counts were investigated in the BAL fluid of TDI-sensitized rats [28]. TDI-sensitized rats exhibited a small elevation of body temperature as ± 2 °C. The control, DEXA, and *I. carnea* flower extract treated rats did not show such symptoms. Packman

et al. [29] reported the body temperature lowering effect of antihistamines on rats, and a parallelism exists between the anti-inflammatory and anti-histaminic activity and the temperature lowering effect.

Treatment with the ethanol extract and its chloroform fraction at an oral dose of 200 mg·kg⁻¹ and DEXA (2.5 mg·kg⁻¹) significantly decreased the count of these inflammatory cells. Infiltrated eosinophils of the bronchial mucosa are activated in asthma, and are thought to induce disease through release of pro-inflammatory molecules and granular proteins like eosinophils cationic protein (ECP) and major basic protein (MBP) [30-31]. (These eosinophils released biochemicals causing airway injury, including epithelial damage, mucus hypersecretion, and increased reactivity of smooth muscles [32-33]. The combination of the standard drug and the isolated compound from the chloroform fraction to treat the TDI-sensitized rats was found to almost identical to standard DEXA effective to suppress airway hyper-reactivity symptoms, and decreased count of both total and differential inflammatory cells, particularly neutrophils and eosinophils, with no or minimal side effects. This may be due to the synergistic effects of the standard drug and stigmasterol from the active chloroform fraction. Stigmast-5, 22-dien-3 β -ol, also known as Wulzen anti-stiffness factor, is a phytosterol. Stigmasterol, unlike dexamethasone, possesses the hydroxyl group in β -configuration at C-3 of ring A. Both compounds showed significant anti-inflammatory activity, consistent with the indications revealed in the second phase (inflammatory) [34]. It also possesses expectorant, anti-inflammatory, antioxidant, thyroid inhibiting properties and combats cholesterol build up and is antifungal [35]. It was demonstrated that it inhibits several pro-inflammatory and matrix degradation mediators typically involved in osteoarthritis induced cartilage degradation [36]. Stigmasterol does seem to be playing a role in reducing inflammation [37] and exerts a significant topical anti-inflammatory activity [38]. It is a Precursor of compounds which can limit inflammatory processes.

Table 3 NMR data of isolate compound

Position	δ_H	δ_C
1		36.33
2		31.09
3	3.52 (1H, br s)	69.74
4		40.92
5		141.08
6	5.16 (1H, br s)	119.83
7		32.23
8		34.97
9		50.18
10		36.68
11		21.09
12		38.78
13		41.92
14		54.98
15		24.34
16		27.29
17		55.33
18		12.48
19		20.31
20		35.09
21		18.65
22	5.12 (1H, m)	137.38
23	5.06 (1H, m)	128.69
24		45.08
25		29.52
26		25.73
27		18.69
28		23.50
29		18.71

Conclusion

The present work characterized an isolated component stigmast-5, 22-dien-3 β -ol from the active chloroform fraction of the ethanol extract of *I. carnea* flowers. These results allow the conclusion that the flower extract of *I. carnea* is having a protective effect on hematological changes occurring on TDI-exposure in Wistar rats, possibly due, in part, to the presence of stigmastrol. However, future studies are necessary to evaluate the effect of *I. carnea* on the release of chemical mediators, like tumor necrosis factor (TNF- α) and various cytokines, which are also known to enhance the symptoms of asthma.

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