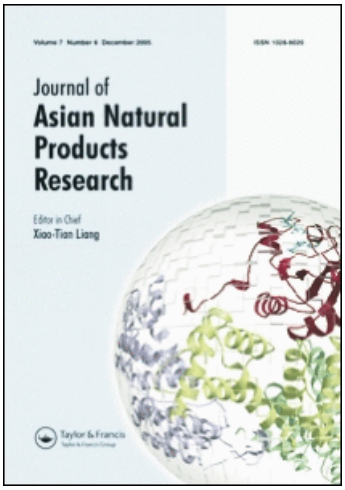

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ORIGINAL ARTICLE

Role of 7,8-dimethoxycoumarin in anti-secretary and anti-inflammatory action on pyloric ligation-induced gastritis in rats

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The present study was designed to investigate the effect of 7,8-dimethoxycoumarin (DMC) isolated from ethyl acetate extract of *Citrus decumana* peels on gastritis in rats. Isolation of 7,8-DMC from ethyl acetate extract of *C. decumana* peels was done by column and preparative thin layer chromatography using different solvents on polarity basis. Furthermore, effect of 7,8-DMC (50, 75, and 100 mg/kg, *i.p.*) in pyloric ligation-induced gastritis was studied in rats. The highest dose of 7,8-DMC showed significant decrease in the gastric volume, total acidity, ulcerative index, thiobarbituric acid reactive species levels, and myeloperoxidase activity, whereas there was an increase in the glutathione level. However, the lowest and medium doses did not produce significant results as compared to omeprazole and *N*-acetyl cysteine-treated groups. Compound 7,8-DMC (100 mg/kg) showed ameliorative effect on gastric inflammation and may be used as a therapeutic agent in the treatment of gastritis.

Keywords: 7,8-dimethoxycoumarin; *Citrus decumana*; gastritis; *N*-acetyl cysteine

1. Introduction

Phytogenic agents have been traditionally used by herbalists and indigenous healers for the prevention and treatment of gastritis. There are various plant-originated 'gastroprotectors' with different compositions which have been used in clinical and folk medicine in many countries due to their beneficial effects on the gastro-intestinal (GI) tract mucosa. *Glycyrrhiza glabra* which belongs to family Papillionaceae possesses antiulcer potential due to the presence of coumarins, flavonoids, chalcones and triterpenoids [1]. Gastritis, an inflammation or irritation of the lining of the stomach, is a condition that has many causes. It can be caused by drinking too much alcohol, prolonged use of non-steroidal anti-inflammatory drugs such as aspirin or ibuprofen, or infection with bacteria such as *Helicobacter pylori*. Gastritis also develops after major surgery, traumatic injury, burns, stress, or severe infections. Many cases are asymptomatic, but dyspepsia and GI bleeding are the common symptoms. Treatment of gastritis depends on the specific cause but often includes drugs causing a decrease in acid secretion (such as H^+K^+-ATPase pump inhibitors and histamine H_2-receptor blockers) and with antibiotics to treat *H. pylori*-related gastritis [2]. But, these synthetic drugs have various side effects such as diarrhea, headache, drowsiness,

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fatigue, and muscular pain [3]. However, it has also been suggested that free radicals are closely related to gastritis [4]. Hence, these days natural compounds having antioxidant potential are being explored so that they could replace these synthetic drugs. Clinical research has confirmed the efficacy of several plants for the treatment of gastrointestinal diseases such as *Momordica charantia, Garcinia indica, and Carpolobia lutea* [5].

In recent years, there is a growing interest in citrus fruits (family Rutaceae) because their consumption decreases the risk of cancer, inflammation, heart disease, ulcers, etc. Citrus juices are considered to be a rich source of antioxidants including vitamin C, phenolic compounds, and carotenoids, which are responsible for their health benefits [6]. Citrus fruit peels from *Citri reticulatae pericarpium*, *Citri reticulatae viride pericarpium*, *Aurantii immaturus fructus,* and *Aurantii fructus* are an important group in Chinese crude drugs and are usually listed in various prescriptions. Citrus herbal products, prepared from mature or immature peels of citrus fruits, have been traditionally used to promote the flow of liver energy. Citrus herbal products have also been used to reduce energy accumulations, such as food stagnation, with pain and distention symptoms. Furthermore, it has also been described in traditional Chinese medical literature that they are utilized to dry dampness and transform phlegm [7]. However, most people throw away the peels after enjoying the citrus fruit. Even during the processing of citrus fruit or juice in food industries, peels are the primary byproducts. Some citrus peel extract has also been used traditionally in Maranda, Nagrota, and Panchrukhi region of Himachal Pradesh, India, for its beneficial effect in relieving rheumatic pain, headache, and as an astringent in cosmetic preparations. Our previous research also reported that *Citrus decumana* peel extracts possess potent anti-inflammatory and analgesic potential [8].

Recently, the peels of many citrus species have been evaluated for antioxidant activity due to the presence of flavonoids, coumarins, and other phenolic compounds [9]. It also shows that coumarins possess strong antioxidant and other pharmacological activities including anti-ulcer effects [10–12]. So, these coumarins can be used to treat gastrointestinal disorders, including gastritis and inflammatory bowel disease. Therefore, the present study was undertaken to evaluate the effect of 7,8-dimethoxycoumarin (DMC) on gastritis in rats.

2. Results and discussion

2.1 Spectral analysis

Isolation of 7,8-DMC (Figure 1) was carried out from ethyl acetate extract of peels of *C. decumana* using column and preparative thin layer chromatography (PTLC) techniques and the proposed compound structure was characterized using IR and NMR spectral techniques.

IR (KBr): the IR spectrum of 7,8-DMC showed absorption bands at 1725 (C═O), 1638 (C═C), 1560 and 1413 (aromatic ring) cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.66 (s, 3H, 8-OCH_3), 3.94 (s, 3H, 7-OCH_3), 6.27 (d, 1H, $J = 9.7$ Hz), 6.89 (d, 1H, $J = 9.6$ Hz), 7.37 (d, 1H, $J = 9.6$ Hz), 7.65 (d, 1H, $J = 9.7$ Hz).

2.2 Phamacological evaluation

Effect of 7,8-DMC on gastric volume, total acidity, and ulcerative index in pyloric ligation (PL)-induced gastritis model is depicted in Table 1. Significant

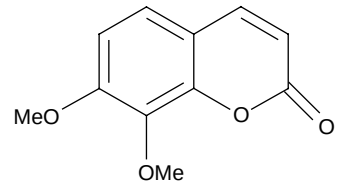

Figure 1. Structure of 7,8-DMC.

Table 1. Effect of 7,8-DMC on gastric volume, total acidity, and ulcerative index changes in rat.

Groups	Dose (mg/kg)	Gastric volume (ml/100 g)	Total acidity (mEq/l)	UI
Sham	–	–	–	0.02 ± 0.24
PL control	–	2.81 ± 0.48	109.43 ± 2.09	2.17 ± 0.73
OP	40	1.14 ± 0.28* (59.4%)*	61.69 ± 1.36* (43.6%)*	0.36 ± 0.08* (83.3%)*
NAC	20	1.25 ± 0.21* (55.5%)*	59.83 ± 1.29* (45.3%)*	0.35 ± 0.13* (83.7%)*
DMC	50	2.41 ± 0.89†‡ (14.2%)†‡	95.58 ± 2.83†‡ (12.7%)†‡	1.79 ± 0.41†‡ (16.7%)†‡
DMC	75	1.89 ± 0.39†‡ (32.7%)†‡	83.71 ± 1.97†‡ (23.5%)†‡	1.54 ± 0.17†‡ (28.4%)†‡
DMC	100	1.24 ± 0.19* (55.9%)*	64.17 ± 2.78* (41.4%)*	0.51 ± 0.11* (76.3%)*

Notes: Values are mean ± S.E.M. of six animals. *$p < 0.05$, as compared to PL (PL model group); †$p < 0.05$, as compared to OP-treated group and ‡$p < 0.05$, as compared to NAC-treated group. Data in the parentheses represent inhibitory rate (%).

difference in the above parameters was observed in the rats treated with lower and medium doses of 7,8-DMC (50 and 75 mg/kg) as compared to that of omeprazole (OP) (40 mg/kg) and *N*-acetyl cysteine (NAC) (20 mg/kg)-treated groups. However, higher dose (100 mg/kg) showed significant reduction in gastric volume, total acidity, and ulcerative index similar to that of OP and NAC-treated groups.

Table 2 shows the biomarker changes in PL-induced gastritis in rat. There was an increase in the thiobarbituric acid reactive species (TBARS) level and myeloperoxidase (MPO) activity and a decrease in the level of glutathione (GSH) in the PL model group. Furthermore, pretreatment with 7,8-DMC (50, 75, and 100 mg/kg), OP, and NAC showed reversible changes in the above parameters. However, low and medium dose-treated groups showed significant difference in the above parameters as compared to OP and NAC-treated groups, but only higher dose (100 mg/kg) showed similar results to that of OP and NAC-treated groups. Moreover, oral

Table 2. Effect of 7,8-DMC on tissue biomarker changes in rat.

Groups	Dose (mg/kg)	TBARS (nmol/g of protein)	GSH (μmol/g of protein)	MPO (U/min/g of protein)
Sham	–	3.52 ± 0.34	1.58 ± 0.26	0.34 ± 0.01
PL control	–	5.05 ± 0.21	0.72 ± 0.05	1.11 ± 0.06
OP	40	3.56 ± 0.31* (97.4%)*	1.49 ± 0.04* (89.5%)*	0.36 ± 0.09* (97.4%)*
NAC	20	3.62 ± 0.29* (93.5%)*	1.52 ± 0.03* (93.0%)*	0.35 ± 0.05* (98.7%)*
DMC	50	4.36 ± 0.46†‡ (45.1%)†‡	0.83 ± 0.02†‡ (12.8%)†‡	1.06 ± 0.04†‡ (6.5%)†‡
DMC	75	4.31 ± 0.21†‡ (48.4%)†‡	0.97 ± 0.04†‡ (29.1%)†‡	0.87 ± 0.06†‡ (31.2%)†‡
DMC	100	3.60 ± 0.65* (94.8%)*	1.46 ± 0.06* (86.1%)*	0.41 ± 0.19* (90.9%)*

Notes: Values are mean ± S.E.M. of six animals. *$p < 0.05$, as compared to PL (PL model group); †$p < 0.05$, as compared to OP-treated group; ‡$p < 0.05$, as compared to NAC-treated group. Data in the parentheses represent inhibitory rate (%) for TBARS and MPO levels, whereas, it represents an increase (%) rate for GSH level.

administration of 7,8-DMC (50, 75, and 100 mg/kg) has not shown any mortality and behavioral alterations.

2.3 Discussion

In the present study, 7,8-DMC was evaluated for its *in vivo* antiulcer activity in PL-induced peptic ulcer models. The development of peptic ulcer due to generation of free radicals and excess of acid secretion causes are mucosal damage and change in antioxidant enzymes. The accumulation of acid due to PL in the stomach causes an increase in the gastric volume and total acidity which in turn lead to the gastric mucosal damage. The excess free radical generation causes enhanced lipid peroxidation which was indicated by an increase in the levels of TBARS. Due to increased TBARS level, antioxidant defense mechanisms fail to prevent the formation of excess free radicals which leads to tissue injury [13]. GSH acts as an important endogenous antioxidant against the reactive oxygen species. This GSH level is reduced in rats subjected to PL process [14]. Moreover, the gastric mucosal damage is also caused by increased recruitment of neutrophils via excessive free radical accumulation which is evidenced by elevated MPO (specific inflammatory marker) activity in the PL model [15].

Phytochemical screening revealed the presence of coumarin in the ethyl acetate extract of peels of *C. decumana*. Coumarin and OP have been well documented to possess H^+K^+ ATPase-inhibitory action [16]. Moreover, coumarins and NAC also possess antioxidant potential [17]. Hence, this action of 7,8-DMC may be through anti-secretary and free radical scavenging mechanisms. The decrease in gastric volume and total acidity is an indication of its potent anti-secretary action, whereas a decrease in lipid peroxidation, i.e. TBARS level and MPO activity, and increase in the levels of reduced GSH in 7,8-DMC pre-treated group compared to those in gastritis group suggest its ability to therein protect the gastric mucosal inflammation against free radical-mediated gastric tissue injury.

PL leads to potential alteration of physiological oxidant and antioxidant biomarkers. Hence, in the present study, 7,8-DMC showed potential amelioration of gastric inflammation and oxidative stress marker changes in gastric tissue.

3. Materials and methods

3.1 Animals

Wistar rats of either sex were obtained from Sanjay Biologicals, Amritsar, India. They were kept at standard laboratory diet, environmental temperature, and humidity. A 12h light–dark cycle was maintained throughout the experimental protocol. The experimental protocol was duly approved by Institutional Animal Ethics Committee and care of the animals was as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg No. 874/ac/05/CPCSEA).

3.2 Plant material

For the present investigation, the fruits of *C. decumana* were collected from the northern region of India. The plant material was authenticated and a voucher specimen, No. 0353, has been deposited in the Botanical and Environmental Science Department, Guru Nanak Dev University, Amritsar, India. The fruits were washed and dried properly before removing peels. The peels were then dried under shade at room temperature. The dried peels were ground into a coarse powder in a mixer. The powder was sieved through a 1 mm metal sieve to obtain a uniform particle size.

3.3 Extraction and isolation

The dried peel powder (100 g) of the fruit peel was extracted by a maceration process

using solvents of increasing polarity; hexane, chloroform, ethyl acetate, and methanol. The powdered material was extracted with each solvent three times at room temperature over a period of 24 h. The material was kept for 24 h between each successive solvent for proper drying. The extracts were filtered and concentrated under vacuum on a rotary evaporator at 40°C and stored in a refrigerator for further analysis.

3.4 Phytochemical screening

The crude extracts were analyzed for alkaloids, tannins, saponins, flavonoids, coumarins, steroids, terpenoids, and phenolic acids using standard procedures of analysis [18]. The ethyl acetate extract showed the presence of coumarins, flavonoids, and phenolic acids. Ammonia-UV, shinoda, and ferric chloride tests were carried out for the confirmation of coumarins, flavonoids, and phenolic acids, respectively.

3.5 Isolation and characterization

The ethyl acetate extract of *C. decumana* peels (12.45 g) was fractionated by silica gel column chromatography. Column chromatography was carried out using toluene–acetone as the eluting solvent with an increasing amount of acetone. Fractions of 50 ml were collected and monitored by thin layer chromatography (TLC) (8 cm × 3 cm) using toluene–acetone (9:1) as mobile phase. The fractions showing the same R_f value on TLC plates were pooled and three pools were obtained. The solvent was removed *in vacuo* and the residues thus obtained were further purified by PTLC (20 × 20 cm). Pool 2 was subjected to PTLC using toluene–acetone (9:1) as the solvent system. Three bands were observed under UV light. Sufficient amounts of material of these bands was collected by scrapping off the TLC plates. The material was dissolved in methanol, filtered, and then concentrated (98% purity). The spectral analysis of the isolated compound was carried out using IR and NMR techniques. The isolated compound was further used for gastritis studies.

3.6 Induction of gastritis by PL in rats

Gastritis study was performed by PL process in rats as described in the literature [19]. Animals were fasted for 24 h before pylorus ligation with water *ad libitum*. Normal saline (1 ml/rat, *i.p.*) was administered twice daily to all the animals during the fasting period. Under light ether anesthesia, the abdomen was opened by midline incision below the xiphoid process. The pyloric portion of the stomach was slightly lifted out and ligated, avoiding damage to its blood supply. The stomach was placed back carefully and the abdominal muscular and skin layers were closed with sutures. Animals were sacrificed 6 h after pylorus ligation by euthanasia (thiopental sodium 50 mg/kg *i.p.*). The rat stomach was removed and opened along the greater curvature and then washed with serum physiological solution (0.9% w/v NaCl). Gastric content was subjected to measurement of gastric volume and total acidity. Moreover, isolated tissue was employed for the measurement of ulcerative index. Furthermore, biochemical estimation was also performed in the gastric tissue.

3.7 Experimental design

In the present gastric inflammation study, seven groups, each comprising six rats, were used:

group I: Sham control group;
group II: PL model group (PL);
group III: PL + OP (40 mg/kg, *i.p.*)-treated group;
group IV: PL + *N*-NAC (20 mg/kg, *i.p.*)-treated group;
group V: PL + 7,8-DMC (50 mg/kg, *i.p.*)-treated group;

group VI: PL + 7,8-DMC (75 mg/kg, *i.p.*)-treated group, and
group VII: PL + 7,8-DMC (100 mg/kg, *i.p.*)-treated group.

All standard and test compounds were administered 1 h before the PL process in groups III–VII.

3.8 Pharmacological study

3.8.1 Estimation of gastric volume and total acidity

The gastric juice was collected and its volume was measured. Furthermore, the gastric juice was centrifuged and the clear supernatant was analyzed for total acidity as described by the method previously [20]. Briefly, 1 ml of supernatant liquid was pipetted out and diluted to 10 ml with distilled water. The solution was titrated against 0.01 N sodium hydroxide using phenolphthalein as an indicator. The end point was indicated by the appearance of a pink color in solution. The total volume of NaOH was noted, which corresponds to the total acidity.

3.8.2 Measurement of ulcerative index

Ulcerative index was measured by the previously described method [21]. The stomach was opened briefly and washed with running tap water. Then, it was placed on a flat glass plate to count the ulcerative area. Standardization was made with a 10 cm × 10 cm square glass plate. The opened stomach was laid on the glass plate and the mucous was exposed, allowing the counting of injuries per square mm. The ulcer index was determined using the formula, Ulcer index = $10/X$, where X = total mucosal area/total ulcerated area.

3.8.3 Biochemical estimation

Tissue homogenate was prepared with 10 volumes of 0.1 mol/l of Tris–HCl buffer (pH 7.4), and a supernatant of homogenate was employed to estimate TBARS, reduced GSH, and total protein content as well as MPO activity. Lipid peroxide content was determined in terms of TBARS [22]. The concentration of TBARS in tissue homogenate was expressed in terms of nmol/g of protein and 1,1,3,3-Tetramethoxypropane (1–10 nmol) was used as a standard. Reduced GSH levels were estimated according to the method of Ellman [23]. An equal quantity of tissue homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate protein. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5′-dithio,bis(2-nitrobenzoic acid), and 0.4 ml of double-distilled water were added. The mixture was vortexed and the absorbance was taken at 412 nm within 15 min. The concentration of reduced GSH was expressed as μmol/g of protein.

Moreover, MPO enzyme activity measurement is an indication of neutrophil infiltration. MPO activity was measured using a procedure described by Hillegass *et al.* [24]. Gastric tissue samples were homogenized briefly in 50 mmol/l potassium phosphate buffer (pH 6.0) and centrifuged at 2500 rpm (10 min); pellets were suspended in 50 mmol/l phosphate buffer containing 0.5% hexadecyltrimethylammoniumbromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 2500 rpm for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of the reaction mixture containing 50 mmol/l phosphate buffer, *O*-dianisidine, and 20 mmol/l H_2O_2 solution. The presence of MPO was measured at 460 nm for 3 min and MPO activity was expressed as U/min/g tissue. One unit of this activity was defined as that degrading 1 μmol/l peroxide/min at 25°C. Furthermore, protein concentration was determined using bovine serum albumin as a standard [25]. The results are expressed as mg/g of tissue.

3.9 Statistical analysis

All the results are expressed as mean ± standard error of means (S.E.M). The data were statistically analyzed by one-way analysis of variance followed by Tukey's multiple range tests using Sigmastat Version-2.0 Software. The p-value < 0.05 was considered to be statistically significant.

4. Conclusion

The above studies showed that 7,8-DMC possesses a gastroprotective effect at a dose level of 100 mg/kg in PL-induced gastritis in rat. Thus, 7,8-DMC may be a potent therapeutic agent for the treatment of gastric inflammatory disorder.

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