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Original Research

Role of fumaric acid in anti-inflammatory and analgesic activities of a *Fumaria indica* extracts

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ABSTRACT

Aim: The aim was to test whether the ethanolic extract of *Fumaria indica* (FI) possesses anti-inflammatory and analgesic activities, and fumaric acid (FA) could be one of its bioactive constituent involved in such activities of the extract. **Materials and Methods:** For anti-inflammatory activity, carrageenan-induced edema and cotton pellet induced granuloma tests in rats and for analgesic activity rat tail flick test and hot plate and acetic acid writhing tests in mice were used. All tests were performed after seven daily oral doses of the FI extract (100, 200, and 400 mg/kg/day) and pure FA (1.25, 2.50, and 5.00 mg/kg/day). **Results:** Anti-inflammatory activities of FI and FA were observed in carrageenan-induced edema and cotton pallet granuloma even after their lowest tested doses. No analgesic activity of lowest tested dose of FA was observed in the acetic acid writhing test, but likewise, all tested dose levels of FI, higher tested dose levels of FA were also possess significant analgesic activity in this test. Further, significant analgesic activities of both FI and FA in hot plate and tale flick tests were observed after all their tested doses. **Conclusions:** These observations are in agreement with our working hypothesis on the connection of FA in mode(s) of action(s) of FI, and reaffirm the conviction that FI could be an herbal alternative against fibromyalgia and other pathologies often associate with, or caused by, inflammatory processes.

KEY WORDS: Analgesic, anti-inflammatory, central sensitivity syndrome, central nervous system function *Fumaria indica*, fumaric acid

INTRODUCTION

Increased sensitivity of the central nervous system to painful stimuli is encountered in numerous chronic inflammatory diseases. A group of psychiatric and pathological symptoms that accompany increased central sensitivity are now often referred to as "central sensitivity syndrome" (CSS) [1]. Comorbidities of depression, anxiety, and other mental health problems are often encountered in almost all patients suffering from CSS. Available, psychoactive, analgesic, anti-inflammatory, and other drugs now commonly used for treatments of chronic diseases with CSS do not appropriately meet the therapeutic demands of patients, and as yet little concentrated efforts have been made to discover and develop drugs potentially useful for prevention or cure of enhanced central sensitivity to pain. Preclinical and therapeutic observations made with diverse types of extracts of several traditionally known medicinal plants have revealed that repeated daily doses of some of them alters central sensitivity to environmental and metabolic stress. Several such herbal preparations, now commonly referred to as herbal adaptogens, are some of the more popular herbal remedies often used not only in traditionally known medical practices, but also are now often recommended by practitioners and scholars of integrative medicine well-trained in modern medical sciences [2]. Despite extensive efforts though, many questions concerning their active principles and modes of action remain open, and their therapeutic potentials for treatments of CSS associated hyperalgesia still remain at the best speculative only.

Fumaria indica (FI) Linn., a wildly growing weed of the fumariaceae family, is one such traditionally known medicinal plant widely used for diverse therapeutic purposes in ayurvedic and other traditionally known medical systems of India and other Asiatic countries. Extensive efforts made by medicinal phytochemists and pharmacologists during several more recent decades have revealed diverse therapeutically interesting bioactivities of different types of extracts of the plant, and numerous structurally and functionally diverse secondary metabolites of the plant are now known also [3]. More recent observations made with a hydro alcoholic extract of FI have revealed that repeated daily oral doses of the extract effectively suppresses central sensitivity to metabolic as well as environmental stress, and that fumaric acid (FA) and its conjugates could as well be its quantitatively major bioactive constituents involved in its anti-stress or adaptogenic activities [4,5].

It had earlier been suggested that monomethyl-fumarate is one of the hepatoprotective constituent of FI extracts [6], and a more recent report has revealed anti-inflammatory and analgesic activities of a hydro alcoholic extract of the plant in animal models [7]. However, in this later-mentioned report only acute oral doses of the tested extract were tested, and the fumarate contents of the extract were not quantified. Since acute dose effects of drugs and other bioactive agents are often not identical and can even be opposite [8,9], it was of interest to test whether anti-inflammatory and analgesic activities of FI can be detected after its repeated daily doses and whether FA is also its bioactive constituent involved in such activities of the extract. Results of the very first set of experiments conducted to verify such possibilities experimentally were described and discussed in this communication.

MATERIALS AND METHODS

Animals

Adult Charles Foster albino rats (150 ± 10 g) and Wistar mice (20 ± 5 g) were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi, and were randomly distributed into different experimental groups of 6 animals each. The animals were housed in polypropylene cages at an ambient temperature of 25 ± 1°C and 45-55% relative humidity, with a 12:12 h light/dark cycle. Unless stated otherwise, the animals were always provided with commercial food pellets and water *ad libitum*. They were acclimatized to laboratory conditions for at least 1 week before using them for the experiments. Principles of laboratory animal care (NIH publication number #85-23, revised in 1985) guidelines were always followed, and prior approval of Central Animal Ethical Committee (Dean/11-12/CAEC/324) of Banaras Hindu University was obtained.

Plant Material and Extraction

A shade-dried and pulverized sample of FI was thoroughly extracted using a soxhlet apparatus and 50% ethanol (v/v) as the extraction solvent. The plant was collected locally in Varanasi, and it was botanically authenticated by Prof. N. K. Dubey, Department of Botany, Faculty of Science, Banaras Hindu University. An herbarium specimen of the plant has been preserved (specimen voucher, January 01, 2009) for reference purposes. Yield of the dried FI extract obtained from the dried plant sample was 10.17% w/w.

Contents of FA and its Conjugates in FI

FA is one of the quantitatively major extractable secondary metabolite of FI, and fairly high oral doses of its esters are now therapeutically used for treatments of inflammatory diseases such as psoriasis and multiple sclerosis [10-14]. Therefore, free and conjugated FA contents of FI were quantified by a well-standardized analytical procedure using high-performance thin layer chromatography and CAMAG TLC Scanner - III and Camag Linomat applicator IV. Commercially available FA and the di-methyl fumarate (Sigma-Aldrich, USA) were used as authentic markers. For quantifying free FA FI and pure FA were dissolved in methanol. For the estimation of FA conjugates, FI and pure di-methyl fumarate were dissolved in 50 mL of 5N HCL and refluxed for 2 h and the hydrolyzed samples were dried on a water bath and re-dissolved in methanol. The test samples and FA solutions of known concentration were applied to pre-coated silica gel plate (Merck $60F_{254}$) and developed up to 90 mm using a solvent system consisting of formic acid:chloroform:butanol:heptane (12:16:32:44). The developed plates were dried and scanned under absorbance mode (scanning wavelength λ 260 nm), and calculations were based on the area of peaks of the sample and corresponding authentic marker FA. Free FA content of FI was 0.45% (w/w), and that of its conjugates (calculated as the di-methyl fumarate) was 0.35% (w/w).

Drugs and Chemicals

Analytically pure FA and dimethyl fumarate were purchased from Sigma-Aldrich, USA. The reference anti-inflammatory agents aspirin (Alkem Laboratories Ltd., India) and indomethacin (Sun Pharmaceutical Industries Ltd., India); and a centrally acting analgesic pentazocine (Ranbaxy, India) were used. Other chemicals and reagents were of the purest grade available from local suppliers.

Treatments

All test agents and the reference drugs were suspended in 0.3% carboxymethyl cellulose for oral administrations. The control groups were always treated with the vehicle. Doses of FI (100, 200, and 400 mg/kg/day) or FA (1.25, 2.5 and 5.0 mg/kg/day) were administered once daily for 7 consecutive days, and their last doses were administered 1 h before the tests. The treatment regimen and doses of FI were based on our earlier observation dealing with psychopharmacological activity profile of the extract. Choice of the dose range of FA used was based on the total fumarate contents of FI (0.8%) and diverse reports on anti-inflammatory or immune function modulating effects of FA esters. Unless stated otherwise, the

reference drugs aspirin (100 mg/kg), indomethacin (5 mg/kg) and pentazocine (30 mg/kg) were orally administered only once (i.e. only 1 h before the tests).

Carrageenan Induced Edema Test

The test procedure described by Winter *et al.* [15] and well standardizes in our laboratories [16] was used. In short, 0.1 mL of a 1% carrageenan suspension in saline was injected in the sub-plantar region of the left hind paw of male rats, and the paw was marked with ink at the level the lateral malleous of the injected paw. The paw volume (mL) was measured before and 3 h after carrageenan injection using a mercury plethysmography. The edema volume was calculated.

Cotton Pellet Granuloma Tests

Sub-acute inflammation was induced in rats by implanting cotton pellets according to the method described by Winter and Potter [17]. In short, sterile cotton (50 ± 1 mg) soaked in 0.2 mL of distilled water containing penicillin (0.1 mg) and streptomycin (0.13 mg) was implanted bilaterally in axilla under the ether anesthesia. The animals were sacrificed on the 7th day. Resulting granulation tissues with a cotton pellet were dried at 60°C overnight and weighed. The weight (mg) of the cotton pellet before implantation was subtracted from the dried weight of dissected granuloma pellet. In this test, not only FI and FA, but also the reference drug, indomethacin, were administered for 7 consecutive days, beginning from the day of cotton pellet implant.

Tail Flick Test

The method described by Davies *et al.* [18] was followed. In short, a rat was placed in a holder, with its tail coming out through a slot in the lid. The tail was kept on the bridge of an analgesiometer (Techno, India) jacket with an electrically heated nichrome wire underneath. The tail received radiant heat from the wire, heated by passing current of 6 mA. Through the water jacket, cold water was continuously passed, so that the bridge did not get heated and tail could be conveniently placed over the bridge. The time taken for the withdrawal of the tail after switching on the current was taken as the latency period, in seconds, of "tail flicking" response. This latency period was considered as an index of nociception. The cut off time for determination of latency period was 30 s [19]. Tail flick latencies were assessed for each rat just before treatments on the test day and 1, 2, and 3 h thereafter, and the means of the tail-flick latencies were used for statistical analysis.

Hot Plate Test

Mice were screened by placing them on a hot plate maintained at 55 ± 1°C and recording the reaction time in seconds for forepaw licking or jumping [19]. Only those mice reacting within 15 sec and did not show large variation when tested on four separate occasions (each 15 min apart) were taken for the test. The preselected animals were used for the test, whereupon FI and FA were administered for 7 consecutive days and the reference analgesic pentazocine only once on the 7th day of the test. The time (s) for forepaw licking or jumping on the heated plate of the analgesiometer was taken as an index of algesic state of the animals.

Acetic Acid Writhing Test

Acetic acid solution (15 mg/mL) at the dose of 300 mg/kg body weight was injected intraperitoneally, and the number of writhings in the following 30 min period were counted [20]. A significant reduction in a number of writhings (N) by treatment was considered as a positive analgesic response.

Statistical Analysis

All data are expressed as means ± standard deviation (SD) for each treatment group. Mean ± SD were calculated for the observed values in each experimental group. Statistical analysis was performed by one-way analysis of variance followed by Student-Newman-Keuls multiple comparison tests. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California, USA) was used for statistical analysis.

RESULTS

Carrageenan Induced Edema

Results summarized in Figure 1, revealed that FI 100 mg/kg/day significantly inhibits carrageenan-induced inflammatory response ($P < 0.05$), and that its maximal efficacy in this test is achieved after its 200 mg/kg daily doses. On the other hand, efficacy of FA in this test increased in dose-dependent manner. It must be noted that with 200 mg/kg, only 1.6 mg/kg FA and its conjugates were administered, and the efficacy of FI after this dose was much higher than those of the highest tested daily dose (5 mg/kg) of pure FA. Quantitatively, the efficacy of a single 100 mg/kg dose of aspirin observed was somewhat higher than that of the maximally observed effect of FI.

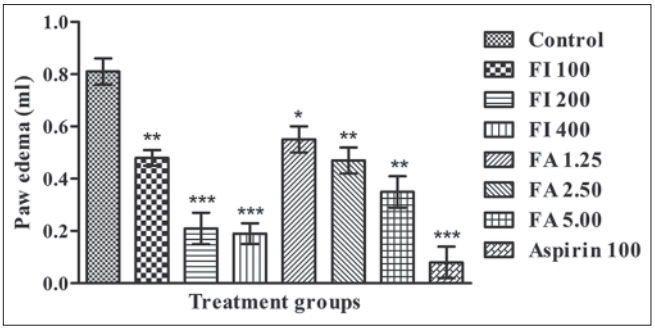

Figure 1: Effects of seven daily oral doses of FI extract and FA on carrageenan-induced paw edema in rats. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, $n = 6$ animals in each group. Values are mean±standard deviation; *,** and ***$P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, in comparison to control

Cotton Pellet Induced Granuloma

Alike indomethacin, significant anti-inflammatory effects of both FI and FA were apparent in this test. Results summarized in Figure 2 reveal again that 200 mg/kg daily dose FI is almost the maximally effective one. The efficacy of this dose of FI was almost equal to that of 5 mg/kg daily doses of FA ($P < 0.01$).

Tail Flick Test

Results summarized in the Table 1 reveal that almost maximal possible analgesic activities of FI, as well as FA, were observed even after their lowest doses tested. Quantitatively, the efficacies of 100 mg/kg/day FI, or of 1.25 mg/kg/day FA were of the same order of magnitude as that of a single 30 mg/kg dose of the centrally acting analgesic pentazocine. Efficacies of all drug treatments were apparent 1 h after the treatments, which remained almost constant up to the last time point of measurements, i.e., 3 h ($P < 0.01$).

Hot Plate Test

It is apparent from Figure 3 that the mean reaction times of all test groups before treatments, or of the control Group 1 h thereafter, were almost equal and not significantly different from one another. Dose-dependent and pentazocine-like analgesic effect of FA was apparent on hour of the treatment, whereas such efficacies of FI after its all tested doses were almost equal.

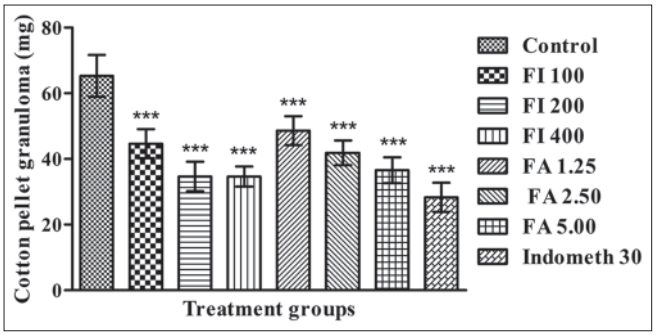

Figure 2: Effects of seven daily oral doses of FI extracts and those of FA in the rat cotton pellet granuloma test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, Indometh: Indomethacin, $n = 6$ animals in each group. Values are mean ± standard deviation, ***$P < 0.001$ in comparison to control

Analgesic efficacies of the highest tested doses of FI (200 and 400 mg/kg/day) and that of FA (5 mg/kg/day) were almost equal but were somewhat lower than that of a single oral dose of pentazocine (30 mg/kg).

Acetic acid writhing test: Statistically significant aspirin-like analgesic activity of FI was observed only after its 200 and 400 mg/kg daily doses, and that of FA after its highest dose (5 mg/kg/day) tested (Figure 4) ($P < 0.05$). Quantitatively, the observed effect of a single oral dose of aspirin was higher than those of the highest doses of FI or FA tested.

DISCUSSION

Observations reported in this communication not only add further experimental evidence in favor of to our conviction that repeated daily treatments with FI suppresses central sensitivity to environmental stimuli, but also strongly suggest that FA is quantitatively the major, but not the only, therapeutically interesting bioactive constituent of the extract. In addition, they reveal that daily treatment with fairly low oral doses of FA protects animal from peripheral inflammatory responses and that 200 mg/kg daily doses of FI is its maximally effective one for its anti-inflammatory and centrally acting analgesics like efficacies. However, quantitatively, the observed efficacies of FI cannot be explained by its analytically estimated FA contents only. Daily FA doses administered with 100 and 200 mg/kg/day doses of FI were 0.45 and 0.9 mg/kg, and the observed effects of 1.25 mg/kg/day doses of pure FA in both the models of inflammation were lower than that of 100 mg/kg/day doses

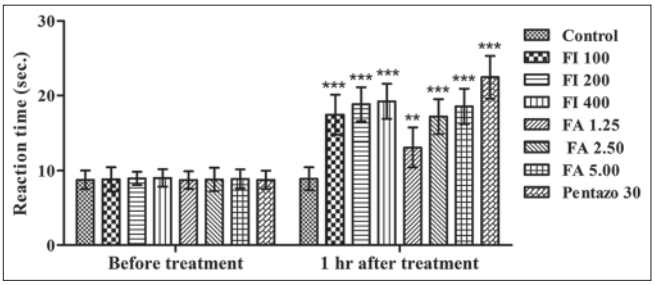

Figure 3: Effect of seven daily oral doses of FI extract and FA in mice hot plate test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, Pentazo: Pentazocine, $n = 6$ animals in each group. Values are mean ± standard deviation, ** and ***$P < 0.01$ and $P < 0.001$, respectively, in comparison to control

Table 1: Effects of FI extract and FA in rat tail flick test

Treatment	Dose (mg/kg)	Reaction time in seconds			
		0 h	1 h	2 h	3 h
Control	-	8.83±0.75	8.33±0.51	6.83±1.47	7.66±1.63
FI	100	9.16±0.75	17.5±1.87**,††	20.83±3.12**,††	19.5±1.87**,††
FI	200	7.83±0.73	16.16±1.60**,††	18.66±2.25**,††	19.16±2.7**,††
FI	400	10.16±1.16	18.66±0.80**,††	20.16±2.31**,††	19.16±2.22**,††
FA	1.25	8.78±0.82	16.24±1.38**,††	18.12±1.98**,††	18.04±1.62**,††
FA	2.50	9.06±0.74	17.58±1.65**,††	19.28±0.86**,††	18.16±1.64**,††
FA	5.00	8.82±0.90	17.85±1.52**,††	19.80±1.76**,††	19.54±2.18**,††
Pentazocine	30	7.66±1.21	18.83±3.60**,††	19.83±3.18**,††	21.16±3.86**,††

FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, n=6 animals in each group. Values are mean±SD, **P<0.01, respectively, in comparison to control, ††P<0.01 in comparison to 0 h. SD: Standard deviation

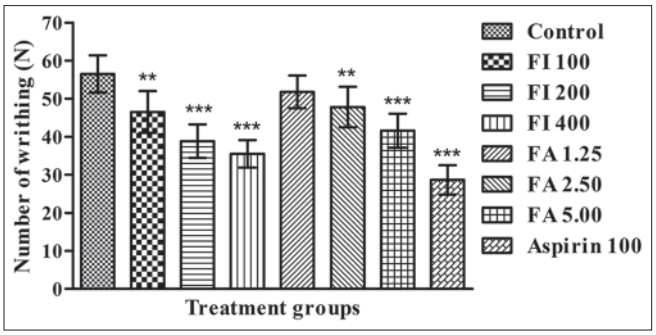

Figure 4: Effect of seven daily oral doses of FI and FA in mice writhing test. FI: Ethanolic extract of *Fumaria indica*, FA: Fumaric acid, $n = 6$ animals in each group. Values are mean ± standard deviation, ** and ***$P < 0.01$ and $P < 0.001$, respectively, in comparison to control

of FI. Analogous were also the observations made in both the rodent models for centrally acting analgesics used in this study.

Although clear dose-dependent and aspirin- or indomethacin-like anti-inflammatory activities of FA in carrageenan and granuloma tests were observed, only after its highest dose tested (5 mg/kg/day) significant aspirin-like analgesic activity of FA were detected in the acetic acid induced writhing test. Thus, it seems reasonable to assume that the biological mechanisms and processes involved in the observed anti-inflammatory activities of FA are not like those of non-steroidal anti-inflammatory drugs. Analogous were also the observations made with FI daily doses up to 200 mg/kg/day. However, some modest, but statistically significant, aspirin-like analgesic activity of FI was observed in the writhing test for peripheral analgesics. These observations could also indicate that apart from FA, FI contains some other bioactive components with mild anti-inflammatory and analgesic activities. Earlier observations in our laboratories have revealed that repeated daily administration of FI induces sedation in laboratory rodents [21,22]. Therefore, the possibility that it's observed analgesics like efficacies in this study could as well be due to behavioral alterations induced by its repeated daily doses. Further, more detailed studies will be necessary to clarify the situation. In any case, our results strongly indicate that FA is an anti-inflammatory constituent of FI.

FA was first isolated from another medicinal plant of the fumariaceae family, and therapeutic potentials of mixtures of FA and its conjugates for the treatment of psoriasis has also been known since long [10,11]. Numerous preclinical studies conducted during more recent decades have revealed that FA esters possess anti-inflammatory properties [12], and several properly controlled clinical trials have consistently demonstrated therapeutic efficacy of fairly high oral doses of methyl fumarates in patients with psoriasis and multiple scleroses [13,14]. However, the possibility that FA could as well be medicinally used as a safe and effective anti-inflammatory agent has never been experimentally verified. This is mainly because in comparison to its esters, oral bioavailability and cellular permeability of FA is almost negligible [23,24], and it is rapidly metabolized or catabolized after its oral intake.

In the light of these reports, the significant inhibitory effects of FA against peripheral inflammation and behavioral responses to thermal stimuli observed in this study after its lowest tested doses (1.25 mg/kg/day) seems to be due to its modulatory actions inside the gastrointestinal tract. Importance of gastrointestinal functions in regulating metabolic and mental processes are well-established and it is now becoming increasingly apparent that gut microbiota ecology and gut-brain axis plays crucial roles in dictating the delicate balance between health and diseases [25]. Therefore, it could as well be that modulating effects of FA on gut functions and its microbial population are also involved in the clinically observed beneficial effects of its esters. Since theoretically the FA conjugates present in FI could also be hydrolyzed to the parent acid, it is not impossible the observed quantitative discrepancies between the efficacy of FA and FI are mainly due to the presence of relatively high concentrations of FA conjugates [26,27]. Observations made during efforts to compare the efficacies of FA and its mono and dimethyl esters strongly suggest that such could indeed the case (unpublished data provided by the authors of this manuscript).

FA or its esters have often been reported to be the bioactive constituents of several medicinal plants [28-34], and it has since long been known that FA possesses antioxidant. Uses of FA enriched forage in veterinary medicine for promoting farm animal growth are also fairly common [35]. These facts taken together with the observations made to date with FI and FA in our laboratories, strongly suggest that efforts to identify the pharmacological targets and mechanisms involved in their therapeutically interesting pharmacological activity profiles could lead to the identification of novel non systemically acting therapeutic leads [4,5,21,22] potentially useful for prevention and cure of CSS in patients suffering from chronic inflammatory diseases. Moreover, since increased central sensitivity to metabolic stress is also often encountered in patients suffering from diabetes and other metabolic disorders and beneficial effects of FI has already been demonstrated in animal models [36]. A pharmacologically and analytically well-standardized FI extract could be more effective and cheaper herbal alternative for prevention and cure of such life-threatening medical conditions still spreading like epidemic in all countries.

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