FLAVONOIDS OF *ENHYDRA FLUCTUANS* EXHIBITS ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY IN DIFFERENT ANIMAL MODELS

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ABSTRACT

Enhydra fluctuans (Compositae), an edible semi aquatic herbaceous vegetable plant, widely used in traditional system of Indian medicine. Total flavonoids of *E. fluctuans* (TFEF) were screened for analgesic and anti-inflammatory activity. Analgesic activity was studied in acetic acid induced writhing response and by hot plate method in Swiss albino mice. Anti-inflammatory activity was estimated by carrageenan and histamine induced acute inflammation and Freund's complete adjuvant (FCA) induced chronic inflammation in rats. Two flavonoids, baicalein 7-*O*-glucoside and baicalein 7-*O*-diglucoside, were isolated from the ethyl acetate fraction. Oral administration of TFEF at the doses of 200 and 400 mg/kg provide 27.05 and 55.49% protection respectively in acetic acid induced writhing method. It also increased the pain threshold in mice evidenced by hot plate method. TFEF showed more potent anti-inflammatory activity. The results of this study may be attributed to high free radical scavenging and antioxidant potential of the flavonoids present in ethyl acetate fraction of *Enhydra fluctuans.*

Keywords: *Enhydra fluctuans*, adjuvant arthritis, flavonoids, analgesic and anti-inflammatory potential.

INTRODUCTION

Enhydra fluctuans Lour. (Compositae), an edible semi aquatic herbaceous vegetable plant with serrate leaves, grows all over India. The leaves, which are slightly bitter, are used to treat inflammation, skin diseases, and small pox. The leaves are also antibilious and are used in nervous diseases, and in torpidity of liver (Chopra *et al.,* 2000). The plant possesses nutritional value and its methanol extract has been reported to have antidiarrheal activity (Uddin *et al.,* 2005). Recently the free radical scavenging potential of crude extract and different fractions has been reported (Sannigrahi *et al.,* 2010a). Ethyl acetate fraction of the plant exhibits anticancer activity against EAC cell in mice (Sannigrahi *et al.,* 2010b). The leaves of *E. fluctuans* have been reported to have hypotensive activity (Joshi and Kamat, 1972). Sesquiterpene lactones (Ali *et al.,* 1972), gibberelins (Ganguly *et al.,* 1972) and cholesterol derivatives (Krishnaswamy and Prasanna, 1975) have been reported to be present in this plant.

Rheumatic arthritis is a destructive inflammatory and systemic auto-immune disease of unknown cause (Hong-MeiXu *et al.,* 2007). Large numbers of plants are screened for potent analgesic and anti-inflammatory agent from natural origin (Gautam and Jachak, 2009). It is found that the plants having antioxidant activity showed promising anti-inflammatory activity (Nguemfo *et al.,* 2009; Rathee *et al.,* 2009). So, research on plants having free radical scavenging and antioxidant activity is therefore viewed as a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory agents.

The present study evaluated the analgesic and anti-inflammatory activities of flavonoid rich ethyl acetate fraction of *Enhydra fluctuans* Lour. and isolation of some bioactive flavonoids from it.

MATERIALS AND METHODS

Plant material

Fresh aerial part of the plant was collected locally in the rural belt of Bankura, West Bengal, India in the month of December, 2006 and identified by taxonomist of Botanical Survey of India, Howrah, West Bengal, India. The voucher specimen has been deposited in our laboratory for further reference. The plant material was shade dried and milled in mechanical grinder for further studies.

Extraction and fractionation

Air-dried and powdered aerial part (1.8 kg) of the plant was extracted successively with petroleum ether (60-80°C) and methanol using Soxhlet apparatus. The solvents were then removed under reduced pressure and sticky residues were obtained. The crude methanol extract (105 g), after removal of the solvent, was dissolved in 10% sulfuric acid solution and subsequently partitioned with chloroform, ethyl acetate, and *n*-buatnol. The ethyl

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acetate fraction was evaporated under reduced pressure and concentrated *in vacuo* to obtain the total flavonoids of *Enhydra fluctuans* (TFEF). Weighed amount of TFEF was suspended in Tween 80 prior to administration.

Isolation of flavonoids

Ethyl acetate fraction of *E. fluctuans* (7.5 g) was subjected to column chromatography for isolation of flavonoids on silica gel (300 g) and eluted with gradient solvent system (petroleum ether-ethyl acetate). Fractions were collected and monitored by TLC analysis. Based on the R_f value, fractions were mixed and 16 fractions were obtained. Fraction 12 and 13 gave good resolution with petroleum ether and ethyl acetate (7.5:2.5). Both the fractions were subjected to preparative TLC in the above solvent system to afford compound 1 and 2.

Compound 1. UV λ_{max} MeOH nm: 254, 332; MeOH+NaOAc: 362, 278; MeOH+$AlCl_3$: 266, 307, 344; MeOH+$AlCl_3$+HCl: 266, 310, 347; Positive ESI-MS *m/z:* 433 $(M+H)^+$; 271 $[(M+H)-162]^+$. H^1 NMR data was found to be similar with baicalein 7-*O* glucoside (Chen *et al.,* 2005).

Compound 2. UV λ_{max} MeOH nm: 258, 334; MeOH+NaOAc: 261, 331; MeOH+$AlCl_3$: 264, 305, 348; MeOH+$AlCl_3$+HCl: 266, 310, 348; Positive ESI-MS *m/z:* 595.2 $(M+H)^+$; 475 $[(M+H)-120]^+$; 433 $[(M+H)-162)]^+$; 271$[(M+H)-2(162)]^+$. H^1 NMR data was found to match with baicalein 7-*O* diglucoside (Chen *et al.,* 2005).

Quantification of flavonoids in ethyl acetate fraction

The amount of total flavonoid of *Enhydra fluctuans* in the ethyl acetate fraction was determined according to the method described by Moreno *et al* (2000). The sample solution (1 ml) containing 1 mg of the fraction, in methanol was added to test tube containing 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M potassium acetate and 3.8 ml of methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The total amount of flavonoids was calculated according to the equation obtained from the standard quercetin graph:

Absorbance = 0.0342 µg quercetin +0.01338 (R^2 = 0.9965)

Animals

Adult male Wistar albino rats weighing 150-200 g and male Swiss albino mice 20-25 g were used for the present investigation. They were housed in clean polypropylene cages and were fed with standard pellet diet and water *ad libitum* with light-dark cycle. All the studies were approved by Animal Ethical Committee, Jadavpur University, Kolkata, India.

Treatment

Animals were divided into four groups of six animals in each group. Group I served as control treated with normal saline (5 ml/kg), group II and group III served as test treated with TFEF 200 mg/kg and 400 mg/kg respectively and group IV served as standard group and treated with reference drug.

Analgesic activity

Analgesic activity was performed in Swiss albino mice by acetic acid induced writhing method (Pal *et al.,* 2003) and hotplate method (Eddy and Leimbach, 1953).

Anti-inflammatory activity

Male Wister albino rats were selected for the study. Carrageenan-induced paw edema was performed according to the process described by Winter *et al* (1962). Anti-inflammatory activity was also carried out against histamine (1%) induced inflammation. Paw volume was measured prior to and 1, 2, 3, 4 and 5 hours after carrageenan administration using a mercury plethysmograph (Gupta *et al.,* 2005). Chronic anti-inflammatory activity was performed against Freund's complete adjuvant (FCA) induced arthritis (Suleyman *et al.,* 1991).

Histological analysis

All the rats were sacrificed on day 21, limbs were removed above the stifle joints, and fixed in 10 % buffered formalin. They were decalcified and then embedded in paraffin, sectioned, stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope to perform the histological analysis.

STATISTICAL ANALYSIS

Results were expressed as mean ± S.D. (n=6). The statistical significance of differences between groups was determined by one way analysis of variance (ANOVA), followed by Tukey's test for comparisons among groups by using GraphPad Prism4, La Jolla, CA 92037, USA. Differences of $p<0.05$ were considered statistically significant.

RESULTS

Ethyl acetate fraction of *E. fluctuans* was subjected to adsorption chromatographic separation analysis (column chromatography, TLC and preparative TLC) to isolate flavonoids. Compound 1 and compound 2 isolated from the flavonoids rich ethyl acetate fraction was characterized by physical and spectroscopic analysis. Structure of compound 1 and compound 2 were identified by spectral data analysis and confirmed by comparing the data with the published literature as baicalein-7-O glucoside and baicalein-7-O diglucosides.

Total flavonoids of *E. fluctuans* in the ethyl acetate fraction was expressed as µg of quercetin equivalent per mg of the sample. The flavonoid content in the ethyl acetate fraction was found as 92.32 ± 12.25 µg-mg^{-1}.

Oral administration of TFEF significantly decreased the number of acetic acid induced writhes in mice (table 1) and increased the reaction time in hot plate method (table 2).

TFFF produced significant anti-inflammatory activity in carrageenan and histamine induced paw edema in rats (fig. 1).

Total flavonoids of *E. fluctuans* showed significant decrease in paw swelling in chronic treatment against adjuvant induced inflammation (fig. 2).

Fig. 3 shows the effect of TFEF on histology of FCA induced rats. The inflammation induced by FCA was associated with cellular infiltration, bone destruction and edema on day 21 (fig. 3a). Treatment with TFEF at the doses of 200 and 400 mg/kg reduced arthritic changes compared to FCA control rats.

DISCUSSION

Two flavonoids isolated from the aerial parts of *Enhydra fluctuans* was baicalein 7-O diglucosides and baicalein 7-O glucosides. The UV spectral data of the compounds in methanol and with different shift reagents gives valuable information about the nature of the flavonoids and the position of the hydroxyl groups (Mabry *et al.,* 1970). The methanol spectra of the compound 1 and 2 showed maximum absorption at 254, 332 and 258, 334 nm respectively, similar to that reported for flavones. Bathochromic shift of band II of both the compounds were observed with $AlCl_3$ suggesting presence of 5-hydroxyl group in A ring. No change in spectra on addition HCl with $AlCl_3$ confirms the acid stable nature of the complex. It also showed the absence of 3',4' dihydroxy group in the B ring. No change in the spectra was observed on addition of NaOAc confirms the absence of free hydroxyl group at 7 position in the A ring. Mass spectra of the compounds confirm the nature of glycoside as O-glycosides. O-glycosides generate abundant aglycone ions by loss of sugar molecules but C-glycosides do not generate abundant aglycone ions but characteristic ions of the fragmentation of the C-glycoside unit itself (Renee *et al.,* 1998). The $[M+H]^+$ at m/z 433 of the compound 1 suggested the molecular mass of the compound as 432 and $[(M+H)-162]^+$ at m/z 271 indicate the removal of sugar fragment from the compound. The $[M + H]^+$ at m/z 595 of the compound 2 suggested the molecular mass of the compound as 594. The $[(M+H)-162]^+$ at m/z 433 and $[(M+H)-324]^+$ at m/z 271 mean consecutive cleavages of two sugar units from the compound.

Acetic acid induced writhing response and hot plate methods are the two most widely used methods for screening of peripheral and central analgesic activity (Sood *et al.,* 2009). Different mechanism was proposed to explain acetic acid induced abdominal constrictions. This effect may be due to sensitization of nociceptors (Berkenkopf and Weichmann, 1988), or interference with cholinergic nervous system (Ghelardini *et al.,* 1997), or angiotensin II receptor (Georgieva and Georgiev, 1999). The analgesic effect of TFEF may directly or indirectly

Table 1: Effect of total flavonoids of *E. fluctuans* (TFEF) on acetic acid induced writhing in mice[#].

Group	Treatment	Number of writhes	% protection
Group I	Control (Normal saline)	75.82 ± 6.6	-
Group II	TFEF (200 mg/kg)	55.31 ± 8.6**	27.05
Group III	TFEF (400 mg/kg)	33.74 ± 3.3***	55.49
Group IV	Aspirin (100 mg/kg)	12.09 ± 2.6***	84.05

Values are mean ±s.d. (n=6), **p<0.01, ***p<0.001, Results were compared with control group.

Table 2: Effect of total flavonoids of *E. fluctuans* (TFEF) on the pain threshold of mice in the hot plate method[#]

Treatment	Dose	Reaction time (Sec) after drug administration					
		0 min	15 min	30 min	60min	90min	120min
Control (5% tween 80)	5 ml/kg	5.13 ± 0.56	4.76 ± 0.41	5.16 ± 0.38	5.18 ± 0.60	5.13 ± 0.92	4.48 ± 0.71
TFEF	200 mg/kg	5.05 ± 0.73	6.83 ± 0.79***	8.48 ± 0.33***	9.86 ± 0.72***	10.63 ± 1.34***	10.68 ± 0.84***
TFEF	400 mg/kg	5.20 ± 0.21	7.86 ± 0.52***	10.28 ± 1.03***	12.40 ± 0.81***	13.48 ± 1.07***	>15
Pentazocine	10 mg/kg	5.10 ± 0.61	8.66 ± 0.68***	13.30 ± 0.98***	14.37 ± 0.66***	>15	>15

Values are mean ±s.d. (n=6) *** p<0.001, Results were compared with control group.

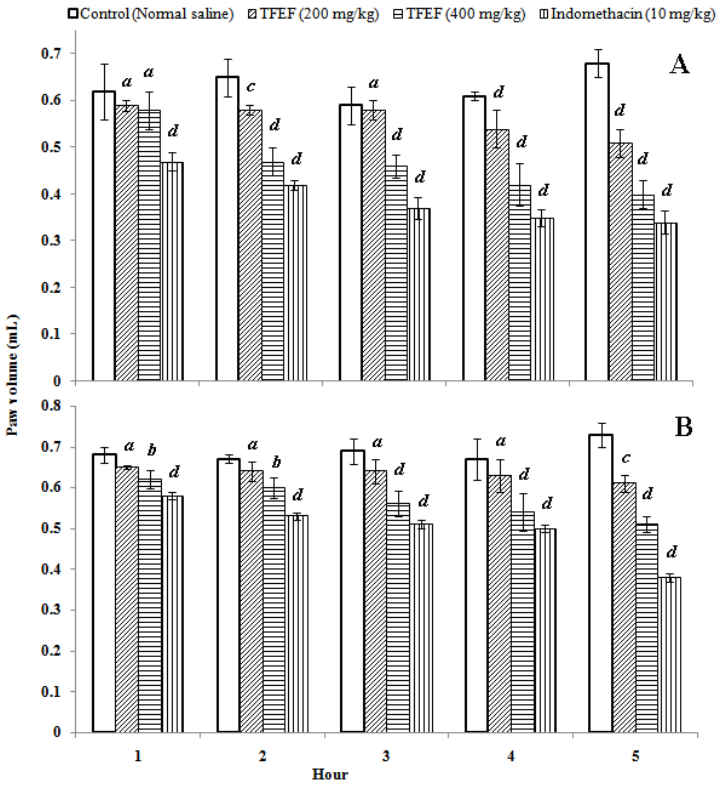

Fig. 1: Effect of TFEF on carrageenan (A) and histamine (B) induced paw edema in rats. Values are given in mean ±s.d. (n=6). Drug treated groups were compared with the control group. *b* - p<0.05, *c*- p<0.01, *d* - p<0.001, *a* - not significant.

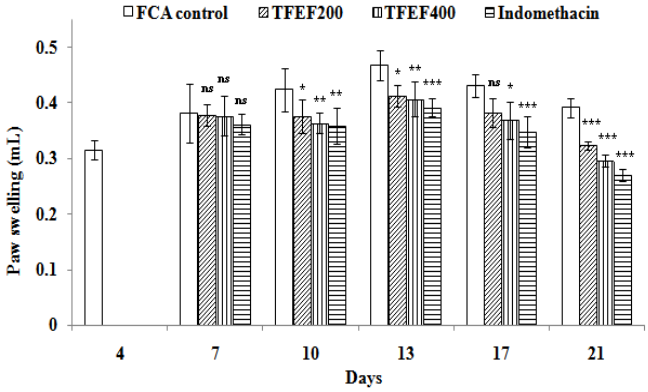

Fig. 2: Effect of TFEF on paw swelling on adjuvant induced arthritis in rats. Values are given in mean ±s.d. (n=6). Drug treated groups were compared with the control group. *p<0.05, **p<0.01, ***p<0.001, ns- not significant.

interfere with these mechanisms. Central analgesic effect, studied by hot plate method showed moderate increase in pain threshold on treatment with TFEF which may be due to interference with opioid receptor (Gray *et al.,* 1998), or with central cholinergic system (Yin and Zhu, 2005).

Inflammation induced by carrageenan has been commonly used as experimental model for acute inflammation study and is believed to be biphasic. Early phase is mediated by histamine, serotonine and increased synthesis of prostaglandin whereas, late phase is sustained

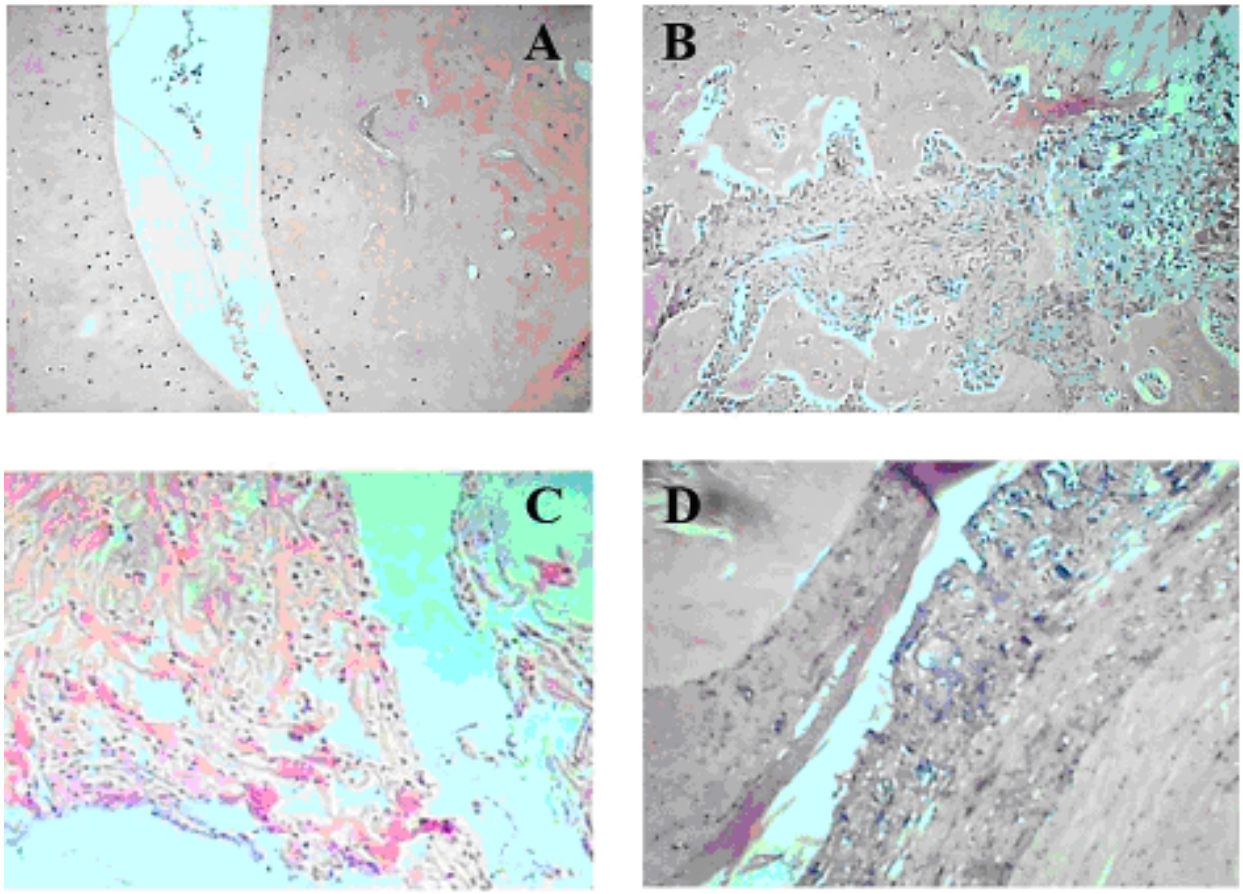

Fig. 3: Histological evidence of TFEF on adjuvant arthritis. (A) Histology of normal rat (B) Histology of adjuvant induced arthritic rat (C) Histology of adjuvant induced arthritic rat with TFEF200 (D) Histology of adjuvant induced arthritic rat with TFEF400.

by prostaglandin release and mediated by different arachidonic acid metabolites (Vinegar *et al.,* 1969; Sugishita *et al.,* 1981). In our study, administration of histamine caused greater inflammation than carrageenan. TFEF significantly decreased the inflammation in dose dependent manner in both carrageenan and histamine induced inflammation. TFEF produced more potent effect on carrageenan induced edema compared to histamine induced inflammation model. This effect may be due to their interference with the first phase of inflammation.

FCA induced arthritis model in rats is suggested as most suitable model of chronic and sub-chronic inflammation. Inflammation in arthritis involves complex response of chemical mediators, chemotactic factors, leukocytes and phagocytes causing injury to cartilage and other tissues. A beneficial effect of total flavonoids of different plants in adjuvant induced arthritis was proved by some authors (Wang *et al.,* 2008; Zhang *et al.,* 2008). The anti-arthritic effect of total flavonoids of *E. fluctuans* may be due to interference with any of the chemical mediators involved in inflammation.

Crude methanol extract of *E. fluctuans* showed less analgesic activity compared to TFEF. Fractionation of crude methanol extract with ethyl acetate pulled up the flavonoids present in the extract to give a flavonoid rich fraction. Flavonoids are polyphenolic compounds and their antioxidant and anti-inflammatory activity was reviewed by some authors (Terao, 2009; Garcia-Lafuente *et al.,* 2009). Flavonoids have been found to have anti-inflammatory activity in both proliferative and exudative phases of inflammation (Rathee *et al.,* 2009). Dietary bioflavonoids supplementation was shown to significantly reduce molar crestal alveolar bone-cement enamel junction distances in rats (Nelson, 2004). The antioxidant defense modulation is also considered as a major factor for their anti-inflammatory activity (Shanmugarajan *et al.,* 2009). A relation was found between plant extracts having anti-inflammatory activity and their antioxidant potential (Amral *et al.,* 2009). Ethyl acetate flavonoids rich fraction of *E. fluctuans* showed high free radical scavenging potential (Sannigrahi *et al.,* 2010a), and in vivo antioxidant and hepatoprotective activity (Sannigrahi *et al.,* 2009).

Results of this study indicate that flavonoids of *E. fluctuans* have significant analgesic and anti-inflammatory activities. The result of this study can be attributed to free radical scavenging and antioxidant potential of polyphenolics and flavonoids present in ethyl acetate fraction. However, the exact structure of the flavonoid(s) and mechanism(s) involved in the analgesic and anti-inflammatory activities of the TFEF are yet to be elucidated.

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