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Therapeutic Potential of *Citrus medica* L. Peel Extract in Carrageenan Induced Inflammatory Pain in Rat

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Abstract: In this study, we planned to evaluate the antioxidative, anti-inflammatory and analgesic potential of *Citrus medica* peel extract. Antioxidant activity in different solvent systems was evaluated. Ethyl acetate extract of *Citrus medica* peel (EtCM) showed maximum 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging activity in a dose dependent manner as compared to ascorbic acid. Further, anti-inflammatory and analgesic activities of EtCM (200, 300 and 400 mg kg⁻¹) were studied on carrageenan induced inflammatory pain in rats. Anti-inflammatory activity was assessed by measuring paw volume in rats. Analgesic activity was evaluated for its central and peripheral pharmacological actions by using hot plate, plantar, pin prick and mechanical allodynia tests in rats. EtCM (400 mg kg⁻¹) produced significant decrease in paw volume and pain as compared to diclofenac. Therefore, the *Citrus medica* peel extract may be used as a future antioxidant for the treatment of inflammation and pain.

Key words: Antioxidant, *Citrus medica*, pain, paw edema

INTRODUCTION

Medicinal plants and herbs have been used for many centuries for the treatment or prevention of diseases and for the promotion of good health. Herbal medicine is probably as old as the human species itself. Before the availability of synthetic drugs man was completely dependent on natural medicinal plants for curing diseases. Many of the substances in modern pharmacology are derived from plant. Plant extracts as well as their primary and secondary metabolites have served as antioxidants in phytotherapeutic medicines to protect against various diseases for centuries (Halliwell, 1996, 2006). Natural antioxidants exhibit a wide range of pharmacological activities and have been shown to possess analgesic, antiulcer, anticancer, anti-inflammatory and anti-aging properties because oxidative stress produced by accumulation of free radicals is supposed to play an important role in the pathogenesis of these diseases (Mayne, 2003; Pinnell, 2003). More recently, it has become evident that phenolic compounds obtained from natural products may reduce oxidative stress by antioxidant action (Vinson *et al.*, 1998). For example, various flavonoids, which are found naturally in fruits and vegetables have been demonstrated to exert antioxidant effect through a number of different mechanisms. Numerous vegetables, crops, spices and medicinal herbs have been tested in an effort to identify new and potentially useful antioxidants (Zheng and Wang, 2001; Nijveldt *et al.*, 2001).

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Citrus fruits and juices have long been recognized to contain secondary metabolites including antioxidant such as ascorbic acid, flavanones, phenolics and pectin that are important to human nutrition. Many researchers have found antioxidant in juice and edible parts of oranges of different origin and from different varieties (Miller and Rice-Evans, 1997; Gorinstein *et al.*, 2001). Citrus processing byproducts represent a rich source of naturally occurring flavonoids. The citrus peels represent roughly half of the fruit mass and their extracts were found to have a good antioxidative potential (Zia-ur-Rehman, 2006; Anagnostopoulou *et al.*, 2006). The peel extract was also found to contain the highest concentration of flavonoids as compared to juice or pulp part. Numerous work on citrus peels of different species, like *Citrus sinensis*, *Citrus limon*, *Citrus paradisi*, *Citrus reticulata* and *Citrus junos* particularly dealing with antioxidant effects and pharmacological actions are well documented in previously reported studies (Alicia *et al.*, 2005; Parmar and Kar, 2008; Yi *et al.*, 2008). Our previous studies also reported that *Citrus decumana* peel extract possessed ameliorative potential in stress induced peptic ulcer in rat (Sood *et al.*, 2009). However, little information is available on the potential therapeutic effect of *Citrus medica* peels. Therefore, in the present investigation an attempt has been made to explore the anti-inflammatory and analgesic potential of the peel extract of *Citrus medica*.

MATERIALS AND METHODS

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), hydrogen peroxide, ascorbic acid, carrageenan and diclofenac sodium were obtained from Sigma Chemical Co. All other chemicals and solvents used were of analytical grade.

Plant Material

The fruits of *Citrus medica* were collected in the month of January, 2008 from Northern region of India particularly Himachal Pradesh. The plant material was identified and authenticated in the P.G. Department of Horticulture, Khalsa College, Amritsar (Voucher No. HD-1109). The peels were removed manually and dried under shade at room temperature. The dried peels were grounded into a coarse powder in a mixer. The powder was sieved through a 1 mm metal sieve to achieve a standard size of particles. Further extraction process, analgesic and anti-inflammatory studies were carried out in the month of November, 2008.

Animals

Wistar rats of either sex (180-220 g) were obtained from Sanjay Biologicals, Amritsar. They were kept at standard laboratory diet, environmental temperature and humidity. A 12 h light-dark cycle was maintained throughout the experimental protocol. The experimental protocol was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of 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).

Extraction

The finely grounded and dried peel powder was subjected to maceration using non-polar and polar solvents. First maceration was done using hexane followed by chloroform, ethyl acetate and methanol. The extraction with each solvent was carried out three times for a period of 24 h at room temperature. The material was kept for a period of

24 h between each successive solvent for proper drying. The extracts were dried under vacuum on a rotary evaporator at 40°C and stored in a refrigerator to pursue further analysis.

Phytochemical Screening

The concentrated extracts were used for preliminary screening of phytochemicals such as alkaloids, tannins, saponins, flavonoids, steroids, terpenoids and phenolic acids using standard procedures of analysis (Harborne, 2007; Evans, 2002). Ethyl acetate and methanol extracts showed the presence of flavonoid and phenolic acid. The confirmation of these was carried out using shinoda test and ferric chloride test, respectively (Audu *et al.*, 2007).

In vitro Studies

Radical Scavenging Activity Using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Method

The free radical scavenging activity of the extracts obtained was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Shyu and Hwang, 2002). A solution of 0.5 mmol DPPH in methanol was prepared. The 1.0 mL of this solution was mixed with 0.1 mL of extract solution (250-400 µg mL⁻¹), 2 mL acetate buffer (pH 5.5) and 1.9 mL methanol. The reaction mixture was left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. For each concentration, mixture without DPPH was used as blank and mixture without extract was used as control. Ascorbic acid was used as reference. The scavenging ability was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{(\text{Absorbance}_{\text{control}} - (\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}))}{(\text{Absorbance}_{\text{control}})} \times 100\%$$

Radical Scavenging Activity Using Hydrogen Peroxide Method

Hydrogen peroxide scavenging activity was measured using the method of Hui-Yin *et al.* (2007) with some modifications. An aliquot of 2.4 mL of 0.1 M phosphate buffer (pH 7.4) and 1.0 mL of various concentrations (80, 100, 120, 140 µg mL⁻¹) of extract solution was mixed with 0.6 mL of 43 mmol hydrogen peroxide solution. The absorbance was measured at 230 nm after 10 min. For each concentration, a separate blank sample (without hydrogen peroxide) was used. Ascorbic acid was used as reference. The scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{(\text{Absorbance}_{\text{control}})} \times 100\%$$

Pharmacological Evaluation

Experimental Protocol for Anti-Inflammatory and Analgesic Activity

Six groups, each comprising of six rats, were employed in the present study.

- **Group I (Normal Control Group):** Administration of 1% CMC, p.o. on the day of experiment
- **Group II (Disease Control Group):** 0.1 mL of carrageenan (1%) was injected at the plantar surface of the right hind paw
- **Group III (Reference Standard Group):** Administration of diclofenac sodium (12.5 mg kg⁻¹, p.o.) on the day of experiment
- **Group IV to VI (EtCM Low, Medium and High Dose):** Administration of EtCM (200, 300 and 400 mg kg⁻¹) for 7 consecutive days

In group III to VI pretreatment with diclofenac and extracts was performed 1 h before carrageenan administration on the day of experiment. Further, analgesic activities were assessed at different time interval of 0, 2, 4, 6, 8 and 10 h.

Acute Inflammation Activity

Paw Edema Test

All groups of animal were employed for the paw edema test by using plethysmometer (Winter *et al.*, 1962). To assess anti-inflammatory activity paw volume was measured in all the groups at different time interval of 0, 1, 3, 6 and 12 h.

Analgesic Activity

Hot Plate Test

Thermal nociceptive (conduct heat) threshold, as an index of thermal hyperalgesia was assessed by the hot plate test as described by Andreas and Rainer (2005). Eddy's hot plate was pre-heated and maintained at temperature of $52.5 \pm 0.5^\circ\text{C}$. Rats were placed on the hot plate and pain withdrawal threshold was assessed with respect to hind paw licking. The response of paw licking/jumping was recorded in seconds. Cut-off time of 30 sec was maintained.

Plantar Test

Thermal nociceptive (radiant heat) threshold, as an index of thermal hyperalgesia was assessed in the right hind paw under the radiant heat lamp source as described by Hargreaves *et al.* (1988). The intensity of the radiant heat stimulus was maintained at $25 \pm 0.1^\circ\text{C}$. The response of paw withdrawal latency was noted in seconds. Cut-off time of 30 sec was maintained.

Pin Prick Test

Peripheral nociceptive (pin point stimuli) threshold as an index of mechanical hyperalgesia was assessed as described by Erichsen and Blackburn-Munro (2002). Surface of the injured hind paw was touched with the point of bent 18 gauge needle (at 90° angle) at an intensity sufficient to produce a reflex withdrawal response in normal non-operated animals but at an intensity which was insufficient to penetrate the skin in all other group. The duration of the paw lifting response was recorded in seconds. Cut-off time of 20 sec was maintained.

Mechanical Allodynia Test

Peripheral nociceptive (tactile stimuli) threshold as an index of mechanical hyperalgesia was assessed as described by Field *et al.* (2000). The surface of the hind paw was lightly stroked with a cotton bud. Care was taken to perform this procedure to avoid recording general motor activity. At least three measurements were taken at each time point the mean of which represented the paw withdrawal threshold. The duration of the paw withdrawal response was recorded in seconds. Cut-off time of 15 sec was maintained.

Statistical Analysis

All the results were expressed as Standard Error of Means (SEM). The data was statistically analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple range tests by using Sigmastat Version-2.0 Software. The $p < 0.05$ was considered to be statistically significant.

RESULTS

In vitro* Antioxidant Assay*Scavenging of 1,1-Diphenyl-2-Picryl Hydrazyl**

Antioxidants react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and convert it into 1,1-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate. The various *Citrus medica* peel extracts showed concentration dependent antioxidant activity. Maximum antioxidant activity was found in the ethyl acetate extract followed by methanol, chloroform and hexane extract (Table 1). Ethyl acetate extract showed comparable *in vitro* anti-oxidative potential with that of standard ascorbic acid.

Scavenging of Hydrogen Peroxide

Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells and attack on cellular energy systems. The hydrogen peroxide scavenging activity of various extracts is shown in Table 2. Concentration dependent antioxidant activity was observed for all the extracts. The maximum antioxidant activity was found in the ethyl acetate extract followed by methanol, chloroform and hexane. Ethyl acetate extract showed comparable *in vitro* anti-oxidative potential with that of standard ascorbic acid.

Pharmacological Evaluation**Effect of Ethyl Acetate Extract of *Citrus medica* (EtCM) on Paw Edema Test**

EtCM showed dose dependent anti-inflammatory activity in carrageenan induced paw edema in rats. Low and medium doses i.e., 200 and 300 mg kg⁻¹ of the ethyl acetate extract had shown significant difference in the anti-inflammatory activity as compared to the normal control (*p<0.05) group as well as diclofenac (†p<0.05) treated group whereas, insignificant difference when compared with disease control group. High dose (400 mg kg⁻¹, p.o.) treated group showed insignificant difference in the anti-inflammatory activity when compared to the normal control and standard groups but significant difference (**p<0.05) when compared with disease group. Hence, it was observed that the higher dose of EtCM significantly attenuated carrageenan induced ipsilateral right hind paw edema in rats (Fig. 1).

Table 1: Effect of *Citrus medica* peel extracts on DPPH scavenging activity

Extract concentration (µg mL ⁻¹)	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Ascorbic acid
250	2.140±0.76	2.510±0.51	26.74±1.78	18.52±2.34	65.98±1.74
300	4.590±1.45	6.520±0.46	48.15±3.56	30.69±1.56	78.71±2.60
350	6.740±1.23	8.630±1.19	61.72±3.29	32.80±2.61	85.14±1.48
400	12.24±1.52	17.15±2.25	79.14±3.47	46.08±1.32	93.86±2.45

Effects of *Citrus medica* peel extracts were determined by the 1,1-diphenyl-2-picrylhydrazyl scavenging activity *in vitro*. Data were expressed as standard error of mean for each group (n = 3)

Table 2: Effect of *Citrus medica* peel extracts on hydrogen peroxide scavenging activity

Extract concentration (µg mL ⁻¹)	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Ascorbic acid
80	6.320±1.79	12.87±0.63	31.03±2.64	29.11±1.57	55.91±3.13
100	15.98±0.94	21.92±2.72	55.46±3.13	39.58±0.84	72.82±1.38
120	20.15±2.72	26.62±1.16	64.58±3.35	48.34±2.35	87.34±2.64
140	23.69±2.89	30.44±1.75	81.12±3.56	66.10±2.58	95.78±2.58

Effects of *Citrus medica* peel extracts were determined by the hydrogen peroxide scavenging activity *in vitro*. Data were expressed as standard error of mean for each group (n = 3)

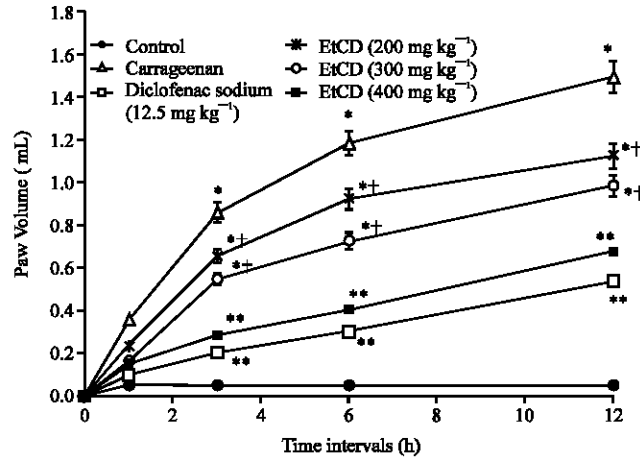


Fig. 1: Time course of paw edema measured by the carrageenan induced increased paw volume in rats. Data were expressed as Mean±SEM, n = 6 rats per group. *p<0.05 vs. normal control group, **p<0.05 vs. carrageenan treated group and †p<0.05 vs. diclofenac sodium control group

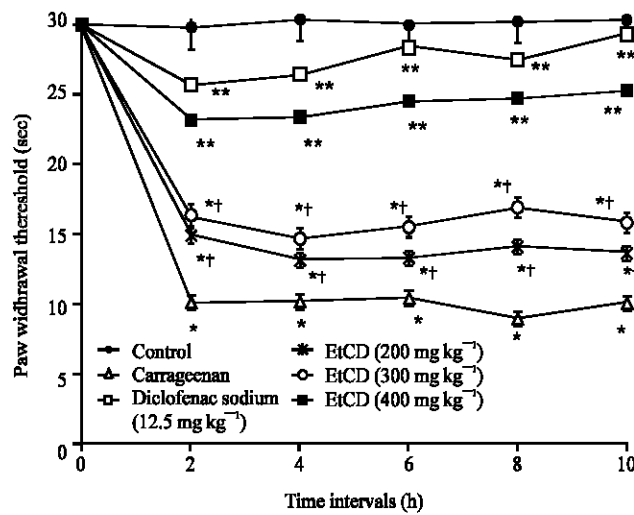


Fig. 2: Time course of paw thermal hyperalgesia measured by the noxious conduct heat evoked paw licking reflex. Data were expressed as Mean±SEM, n = 6 rat per group. *p<0.05 vs. normal control group, **p<0.05 vs. carrageenan treated group and †p<0.05 vs. diclofenac sodium control group

Analgesic Activity in Rat

Effect of Ethyl Acetate Extract of *Citrus medica* (EtCM) on Hot Plate Test

EtCM showed significant analgesic activity against hyperalgesia induced by thermal stimuli as shown in Fig. 2. The oral administration of EtCM showed a dose dependent increase in paw licking threshold. Low and medium doses showed no significant difference as compared to the disease control group but significant difference was observed as

compared to normal control (*p<0.05) and diclofenac ([†]p<0.05) treated group. Whereas, high dose showed significant difference in the analgesic activity when compared with disease (**p<0.05) group but insignificant difference when the comparison was done with that of normal control and standard group. Hence, it was observed that the EtCM significantly reduced the pain sensation in rat at higher dose (400 mg kg⁻¹, p.o.).

Effect of Ethyl Acetate Extract of *Citrus medica* (EtCM) on Plantar Test

EtCM showed significant analgesic activity against hyperalgesia induced by thermal stimuli as shown in Fig. 3. The oral administration of EtCM showed a dose dependent increase in ipsilateral right hind paw withdrawal threshold. Low and medium doses showed no significant difference as compared to the disease control group but significant difference was observed as compared to normal control (*p<0.05) and diclofenac ([†]p<0.05) treated group. Whereas, high dose showed significant difference in the analgesic activity when compared with disease (**p<0.05) group but insignificant difference when the comparison was done with that of normal control and standard group. Hence, it was observed that the EtCM significantly reduced the pain sensation in rat at higher dose (400 mg kg⁻¹, p.o.).

Effect of Ethyl Acetate Extract of *Citrus medica* (EtCM) on Pin Prick Test

EtCM showed significant analgesic activity against hyperalgesia induced by mechanical stimuli as shown in Fig. 4. The oral administration of EtCM showed a dose dependent increase in paw lifting duration. Low and medium doses showed no significant difference as compared to the disease control group but significant difference was observed as compared to normal control (*p<0.05) and diclofenac ([†]p<0.05) treated group. Whereas, high dose showed significant difference in the analgesic activity when compared with disease (**p<0.05) group but insignificant difference when the comparison was done with that of

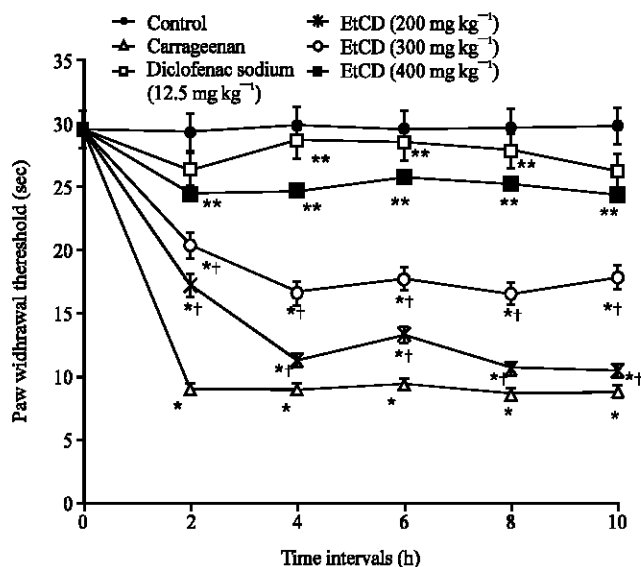


Fig. 3: Time course of paw thermal hyperalgesia measured by the noxious radiant heat evoked ipsilateral right hind paw withdrawal reflex. Data were expressed as Mean±SEM, n = 6 rat per group. *p<0.05 vs. normal control groups, **p<0.05 vs. carrageenan treated group and [†]p<0.05 vs. diclofenac sodium control group

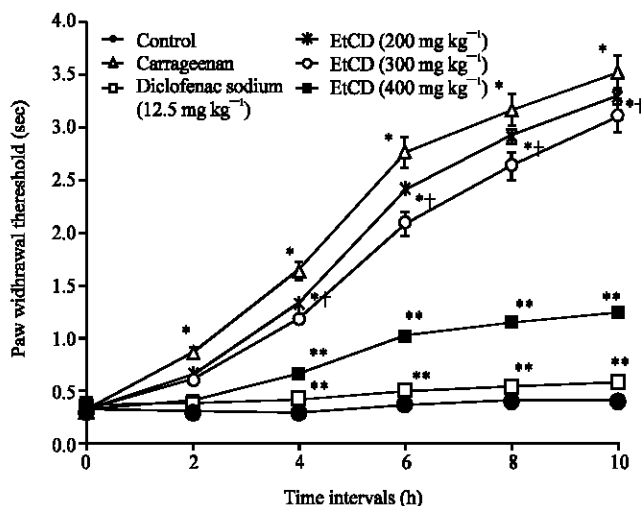


Fig. 4: Time course of paw mechanical hyperalgesia measured by the pin prick evoked rise in paw lifting duration. Data were expressed as Mean±SEM, n = 6 rat per group. *p<0.05 vs. normal control groups, **p<0.05 vs. carrageenan treated group and †p<0.05 vs. diclofenac sodium control group

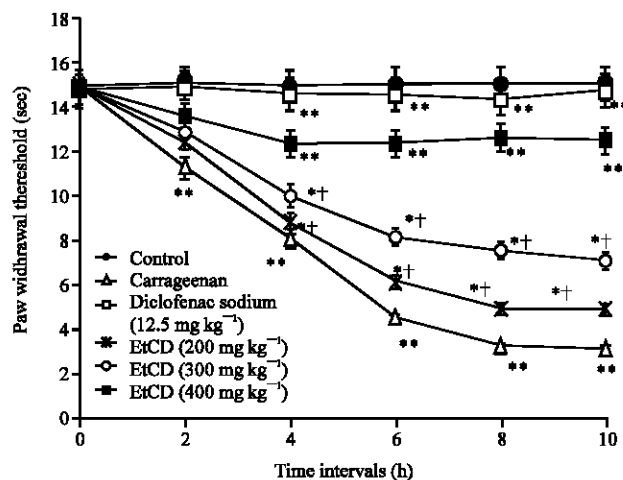


Fig. 5: Time course of paw mechanical allodynia measured by the dynamic cotton bud evoked paw withdrawal reflex. Data were expressed as Mean±SEM, n = 6 rat per group. *p<0.05 vs. normal control groups, **p<0.05 vs. carrageenan treated group and †p<0.05 vs. diclofenac sodium control group

normal control and standard group. Hence, it was observed that the EtCM significantly reduced the pain sensation in rat at higher dose (400 mg kg⁻¹, p.o.).

Effect of Ethyl Acetate Extract of *Citrus medica* (EtcM) on Mechanical Allodynia Test

EtCM showed significant analgesic activity against allodynia induced by mechanical stimuli as shown in Fig. 5. The oral administration of EtCM showed a dose dependent

increase in paw withdrawal latency. Low and medium doses showed no significant difference as compared to the disease control group but significant difference was observed as compared to normal control (* $p < 0.05$) and diclofenac ($p < 0.05$) treated group. Whereas, high dose showed significant difference in the analgesic activity when compared with disease (** $p < 0.05$) group but insignificant difference when the comparison was done with that of normal control and standard group. Hence, it was observed that the EtCM significantly reduced the pain sensation in rat at higher dose (400 mg kg^{-1} , p.o.).

DISCUSSION

In the present study, the peel extracts of *Citrus medica* in different solvents were evaluated for the presence of important phytoconstituents and for their *in vitro* antioxidant activity. Ethyl acetate and methanol extracts showed the presence of flavonoid and phenolic acid. Results also revealed that ethyl acetate extract of *Citrus medica* (EtCM) possessed highest *in vitro* antioxidant activity. Hence, EtCM was further evaluated for its *in vivo* anti-inflammatory and analgesic activity in rats. Carrageenan induced rat paw edema test has frequently been used to assess the anti-edematous effect of natural products. Carrageenan is a strong chemical for the release of inflammation mediators (histamine, kinnin, prostaglandin, leukotriene etc.) and proinflammatory cytokinins (tissue necrosis factor, interleukin etc.) (DiMartino *et al.*, 1987; Di Rosa *et al.*, 1971). Free radicals are known to play an important role in the pathogenesis of inflammation and algisia (Koblyakov, 2001; Winrow *et al.*, 1993; Chung, 2004). Moreover, literature also reveals the antioxidant and anti-inflammatory activity of *Citrus sinensis* peel extract in both acute and chronic inflammatory models due to the presence of free phenolic constituents (Ramachandran *et al.*, 2002). Red orange extract showed interesting antiinflammatory properties in human keratinocyte cells (Cardile *et al.*, 2009). Antioxidant activity and phenolic composition of *Citrus bergamia*, *Citrus limon* and *Citrus aurantium* peel and seed extracts has also been studied. Methanolic extract of these species were found to contain free phenolic compounds (Bocco *et al.*, 1998). Recently, peels of number of commercially available citrus species have also been evaluated for their phenolic and flavonoids content along with their antioxidative potential (Ghasemi *et al.*, 2009). In our studies EtCM had shown statistically significant results in anti-inflammatory and analgesic tests in rats. As EtCM had also shown significant free radical scavenging activity *in vitro*, so this can be the possible reason for the reduction of inflammation and algisia in the carrageenan induced paw edema in rats. Moreover, phytochemical screening revealed the presence of flavonoids and phenolic acids in the EtCM so, these constituents may be responsible for the antioxidant, anti-inflammatory and analgesic activities (Shahidi and Wanasundara, 1992; Hanasaki *et al.*, 1994). However, more elaborative studies are required to identify and characterize the active components and precise mechanism of anti-inflammatory and analgesic actions.

CONCLUSIONS

Ethyl acetate extract of *Citrus medica* peels (EtCM) reduced carrageenan induced paw edema and showed analgesic action in rats. Its free radical scavenging action may be responsible for the observed ameliorative effects. Therefore, this herbal candidate may act as a potent anti-inflammatory and analgesic agent in human subjects.

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