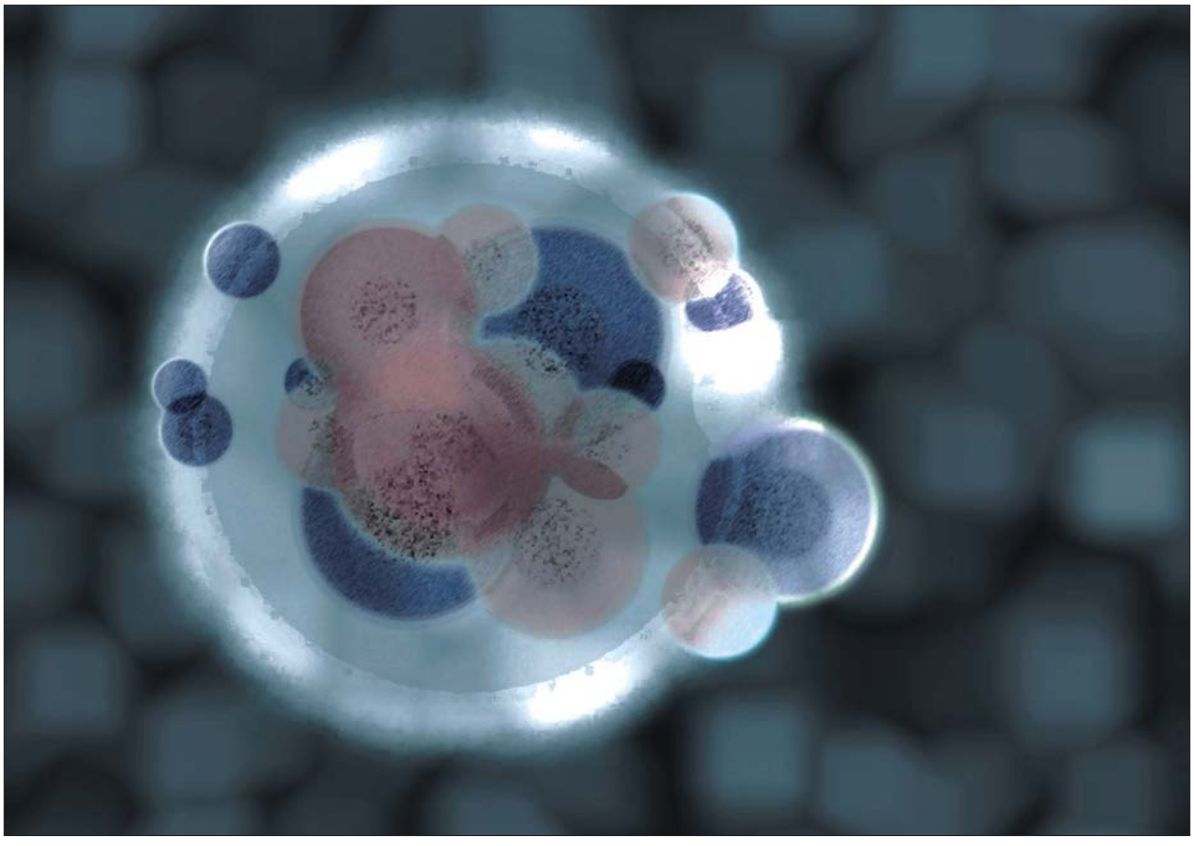

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 10 (3): 231-236, 2010
ISSN 1727-3048

Effect of *Citrus karna* Peel Extract on Stress Induced Peptic Ulcer in Rat

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Abstract: The present study was designed to evaluate the antioxidant and anti-ulcerogenic potential of *Citrus karna* peel extract. Extraction of *Citrus karna* peels was carried out using different solvents. Phytochemical screening and evaluation of antioxidant activity of all the extracts were carried out. Further, anti-ulcerogenic activity of ethyl acetate extract of *Citrus karna* peels (EtCK) was assessed in water immersion (WIS) and hypothermic restraint (HRS) stress models at different doses (200, 300 and 400 mg kg^{-1}). EtCK showed ulcer protective effect in both models in a dose dependent manner, which was indicated by decrease in the ulcerative index and thiobarbituric acid reactive species (TBARS) level in the blood and gastric tissue samples as compared to the disease control group. Moreover, antioxidant markers i.e., reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in the blood and tissue samples were found to be significantly increased in medium and high doses of EtCK (300 and 400 mg kg^{-1}) treated groups similar to that of ranitidine treated group. The present study concluded that EtCK has a significant anti-ulcer effect at a dose level of 300 and 400 mg kg^{-1}. Hence, it may be considered as a useful herbal therapeutic agent for the treatment of peptic ulcer disease.

Key words: *Citrus karna*, water immersion stress, hypothermic restraint stress, peptic ulcer

INTRODUCTION

Peptic ulcer is a very common global problem today. It is one of the life style disorders. The etiology of peptic ulcer includes age, inheritance, cigarette smoking and diet habits (Malyshenko *et al*., 2005). Other common causes are physical or psychological stress (Caso *et al*., 2008), use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) (Kim, 2008) and bacterial infection (Ernst and Gold, 2000; Goodwin *et al*., 1986). Gastric lesions occur due to the loss of delicate balance between gastro-protective (bicarbonate, mucin and prostaglandins) and aggressive factors (*H. pylori*, acid and pepsin) (Desai *et al*., 1997). Therefore, treatment with antioxidants and synthetic drugs such as H^+ K^+- ATPase pump inhibitors and H_2-receptor blockers can decrease gastric mucosal damage (Salim, 1994; Waldum *et al*., 2005). But these synthetic drugs have various side effects such as diarrhoea, headache, drowsiness, fatigue and muscular pain (Zimmerman, 1984). Therefore, natural products and medicinal plant extracts are now considered as an alternative approach for the control of this disease. These are considered to be safer because of natural ingredients with no side effects (Borrelli and Izzo, 2000; Garg *et al*., 1993; Reyes-Chilpa *et al*., 2006).

In recent years, there is a growing interest in citrus fruits (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 (Gattuso *et al*., 2007; Proteggente *et al*., 2003). Peels of various citrus fruits such as *Citri reticulatae pericarpium*, *Citrus aurantium* and *Citrus sinensis* are an important group in Chinese crude drugs and 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. It has also been described in traditional Chinese medical literature that they are utilized to dry dampness and transform phlegm (Dan and Andrew, 1986). Our previous studies on *Citrus medica* peels revealed their use in inflammatory pain due to anti-oxidative potential (Sood *et al*., 2009). However, most people throw away the peels after enjoying citrus fruit. Even during the processing of citrus fruit or juice in food industries, peels

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are the primary byproducts. Many studies show that bioactive flavonoids present in the citrus peel possess strong antioxidant, anti-atherogenic, anti-viral, anti-aggregatory, anti-mutagenic, antiulcer and antitumor effects (Del-Rio *et al*., 1992; Parmar and Kar, 2008; Zia, 2006). It is also observed that oxidative stress plays a role in ulcer formation (Das *et al*., 1997).

Further, water immersion and hypothermic restraint conditions produced stress in rats. This stress leads to the mucosal erosion and peptic ulcer formation by generation of free radicals, membrane lipid peroxidation and alteration in the prostaglandin and histamine levels. Moreover, ramitidine (H_2- receptor antagonist) is well reported to possess antioxidant action, which may also be responsible for its antiulcerogenic activity (Ardestani *et al*., 2004; Lapenna *et al*., 1994). So, the present research work was undertaken to study the antiulcer potential of *Citrus karna* peel extract in stress induced peptic ulcer in rats by using ranitidine as a standard drug.

MATERIALS AND METHODS

Plant material: The fruits of *Citrus karna* were collected from Northern region of India in the month of January, 2009. The plant material was identified and authenticated in the P.G. Department of Horticulture, Khalsa College, Amritsar (Voucher No. HD -1108). 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 1mm metal sieve to achieve a standard size of particles. Further extraction process and anti-ulcer studies were carried out in the month of August, 2009.

Animals: Wistar rats of either sex (180-200 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 dried peel powder of plant material was subjected to maceration 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.

Phytochemical screening: The crude extracts were analyzed for alkaloids, tannins, saponins, flavonoids, steroids, terpenoids and phenolic acids using standard procedures of analysis (Evans, 2002; Harborne, 2007). The ethyl acetate and methanol extracts showed the presence of flavonoids and phenolic acids. Shinoda test and ferric chloride test were carried out for the confirmation of flavonoids and phenolic acids.

Induction of peptic ulcer in animal models

Water immersion- induced stress (WIS): The rats were fasted 24 h prior to experiment and test samples were administered 1 h before stress induction. Rats were immobilized in a stress cage and then immersed to the level of the xiphoid in a water bath at 23±0.2°C for 4 h (Hayase and Takeuchi, 1986). The blood samples were collected by the retro-orbital sinus puncture for the estimation of biomarker components. Chemical euthanasia (thiopental sodium 50 mg kg^{-1}, i.p.) was used before sacrificing the rats under study and stomach of each animal was removed and cut open along the greater curvature and pinned on wooden board after washing it with running tap water. Further, measurement of ulcerative index and biochemical parameters was carried out on the isolated gastric tissues.

Hypothermic Restraint Stress (HRS): The rats were fasted 24 h prior to experiment and test samples were administered 1 h before stress induction. Rats were immobilized in a restraint cage at 4°C for 3 h (Senay and Levine, 1967). The blood samples were collected by the retro-orbital sinus puncture for the estimation of biomarker components. Chemical euthanasia (thiopental sodium 50 mg kg^{-1}, i.p.) was used before sacrificing the rats under study and stomach was removed. Tissue samples were collected for the measurement of ulcerative index and biochemical parameters.

Experimental design: Eleven groups, each comprising of six rats, were included in the antiulcer studies.

- **Group I:** Normal control group
- **Group II:** WIS control group
- **Group III:** WIS + Ranitidine 50 mg kg^{-1}, p.o. treated group
- **Group IV:** WIS + EtCK 200 mg kg^{-1}, p.o. treated group

- **Group V:** WIS + EtCK 300 mg kg^{-1}, p.o. treated group
- **Group VI:** WIS + EtCK 400 mg kg^{-1}, p.o. treated group
- **Group VII:** HRS control group
- **Group VIII:** HRS + Ranitidine 50 mg kg^{-1}, p.o. treated group
- **Group IX:** HRS + EtCK 200 mg kg^{-1}, p.o. treated group
- **Group X:** HRS + EtCK 300 mg kg^{-1}, p.o. treated group
- **Group XI:** HRS + EtCK 400 mg kg^{-1}, p.o. treated group

All the doses of ethyl acetate extract of *Citrus karna* were adininistered for 10 consecutive days and 1 h before ulcer induction on the day of experiment.

Pharmacological evaluation

Measurement of Ulcerative Index (UI): Ulcerative index was measured according to the method of Takagi *et al.* (1969). Briefly, the stomach was opened and washed with running tab water. Then it was placed on a flat glass plate to count the ulcerative area. Standardization was made with a 10×10 cm squared 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 by using the formula:

$$\text{Ulcer index} = 10/X$$

Where:

$$X = \frac{\text{Total mucosal area}}{\text{Total ulcerated area}}$$

Preparation of tissue samples: Gastric tissue samples were homogenized by using 10% w/v of trichloroacetic acid after the measurement of ulcerative index. Centrifugation (5000 g) of the homogenized tissues and the collection of supernatants were carried out. These supernatants were stored under refrigeration for the further estimation of various biochemical markers.

Estimation of tissue and plasma TBARS: Estimation of lipid peroxide content as Thiobarbituric Acid Reactive Species (TBARS) was done according to the method of Niehaus and Samuelson (1968) in tissue and according to Yagi (1998) in plasma. 1, 1, 3, 3-tetramethoxy propane was used as a primary standard. The results were expressed as nM g^{-1} of protein in tissue and nM mL^{-1} in plasma.

Estimation of tissue and plasma GSH: Reduced glutathione level in different samples were determined by the enzymatic method of Tietze (1969). The results were expressed as μmol g^{-1} of protein in tissue and μM mL^{-1} in plasma.

Estimation of superoxide dismutase (SOD) and Catalase (CAT) activity in tissue and plasma: Activities of SOD and CAT in tissue and plasma were determined using commercially available kits (Span Diagnostics, Gujarat, India). SOD and CAT activities were expressed as U mg^{-1} of protein in tissue and U mL^{-1} in plasma.

Estimation of tissue and plasma protein content: Protein concentration was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard and the results were expressed as mg g^{-1} of tissue and mg mL^{-1} in plasma.

Statistical analysis: All the results were expressed as mean±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-value<0.05 was considered to be statistically significant (Sood and Muthuraman, 2009).

RESULTS

Effect of ethyl acetate extract of *Citrus karna* peels in both WIS and HRS model had shown a decrease in ulcerative index (Fig. 1). There was an increase in the

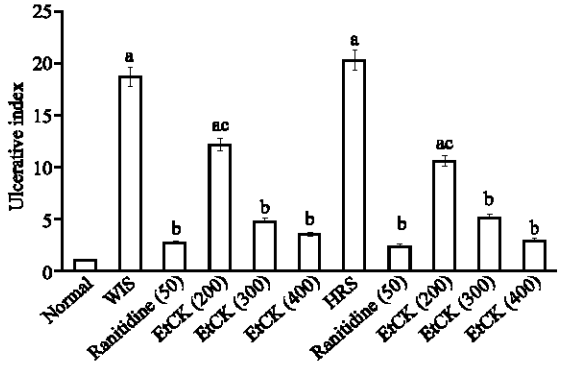

Fig. 1: Effect of EtCK on ulcerative index in WIS and HRS induced peptic ulcer. Values are Mean±SEM of 6 animals. [a]p<0.05, as compared to normal control group; [b]p<0.05, as compared to WIS and HRS control group; [c]p<0.05, as compared to ranitidine treated group, in parenthesis indicated the dose in mg kg^{-1}

Table 1: Effect of EtCK on biomarker changes in WIS model

	TBARS		GSH		SOD		CAT	
Groups (mg kg^{-1})	Tissue (nM g^{-1})	Plasma (nM mL^{-1})	Tissue (μM g^{-1})	Plasma (μM mL^{-1})	Tissue (U mg^{-1})	Plasma (U mL^{-1})	Tissue (U mg^{-1})	Plasma (U mL^{-1})
Normal	3.29±0.06	3.55±0.21	1.38±0.06	15.96±1.43	4.86±0.16	2.02±0.02	5.92±0.93	0.41±0.06
WIS	4.92±0.04^{a}	5.08±0.13^{a}	0.70±0.05^{a}	8.72±1.21^{a}	2.67±0.67^{a}	1.36±0.05^{a}	2.41±0.13^{a}	0.25±0.01^{a}
Ranitidin (50)	3.31±0.03^{b}	3.59±0.11^{b}	1.36±0.03^{b}	16.52±1.95^{b}	4.83±0.24^{b}	1.98±0.04^{b}	5.86±0.56^{b}	0.40±0.02^{b}
EtCK (200)	4.74±0.07^{ac}	4.81±0.42^{ac}	0.67±0.01^{ac}	9.24±1.26^{ac}	3.09±0.71^{ac}	1.39±0.06^{ac}	2.65±0.59^{ac}	0.27±0.01^{ac}
EtCK (300)	3.52±0.02^{b}	3.72±0.43^{b}	1.18±0.03^{b}	13.77±1.58^{b}	4.35±0.27^{b}	1.78±0.07^{b}	4.93±0.26^{b}	0.36±0.02^{b}
EtCK (400)	3.34±0.05^{b}	3.64±0.35^{b}	1.27±0.04^{b}	14.63±1.42^{b}	4.54±0.17^{b}	1.87±0.65^{b}	5.52±0.62^{b}	0.39±0.03^{b}

Values are Mean±SEM of 6 animals. $^{a}p<0.05$, as compared to normal control group; $^{b}p<0.05$, as compared to WIS control group; $^{c}p<0.05$, as compared to ranitidine treated group

Table 2: Effect of EtCK on biomarker changes in HRS model

	TBARS		GSH		SOD		CAT	
Groups (mg kg^{-1})	Tissue (nM g^{-1})	Plasma (nM mL^{-1})	Tissue (μM g^{-1})	Plasma (μM mL^{-1})	Tissue (U mg^{-1})	Plasma (U mL^{-1})	Tissue (U mg^{-1})	Plasma (U mL^{-1})
Normal	4.52±0.05	4.89±0.04	1.44±0.07	15.92±1.17	4.71±0.04	1.99±0.09	6.65±1.31	0.39±0.04
WIS	5.82±0.07^{a}	6.08±0.06^{a}	0.64±0.09^{a}	7.42±0.96^{a}	2.32±0.09^{a}	1.32±0.01^{a}	2.12±1.01^{a}	0.23±0.02^{a}
Ranitidin (50)	4.48±0.0^{b}	4.82±0.03^{b}	1.41±0.06^{b}	15.86±1.05^{b}	4.67±0.03^{b}	1.83±0.09^{b}	6.61±1.22^{b}	0.37±0.04^{b}
EtCK (200)	5.45±0.03^{ac}	5.92±0.09^{ac}	0.79±0.13^{ac}	9.17±0.81^{ac}	2.69±0.05^{ac}	1.38±0.05^{ac}	334±1.06^{ac}	0.25±0.02^{ac}
EtCK (300)	4.82±0.09^{b}	5.24±0.02^{b}	1.33±0.04^{b}	13.56±0.76^{b}	4.15±0.02^{b}	1.64±0.02^{b}	5.57±0.94^{b}	0.32±0.02^{b}
EtCK (400)	4.65±0.08^{b}	4.96±0.07^{b}	1.38±0.11^{b}	14.79±1.07^{b}	4.51±0.03^{b}	1.79±0.03^{b}	6.24±1.01^{b}	0.36±0.01^{b}

Values are Mean±SEM of 6 animals. $^{a}p<0.05$, as compared to normal control group; $^{b}p<0.05$, as compared to HRS control group; $^{c}p<0.05$, as compared to ranitidine treated group

ulcerative index in disease control groups whereas, EtCK pretreated groups showed reduction in the ulcerative index. However, only medium and high doses of EtCK produced significant reduction in the ulcerative index similar to that of ranitidine (50 mg kg^{-1}) treated group.

Effects of EtCK on tissue and plasma biomarker changes in WIS and HRS models were evaluated by the estimation of oxidative stress marker (TBARS and GSH) along with enzymatic activity (i.e., SOD and CAT). These results have been presented in Table 1 and 2. There was an increase in the TBARS level and a decrease in the levels of GSH, SOD and CAT in the disease control groups as compared to normal control group. Further, pretreatment with EtCK (200, 300 and 400 mg kg^{-1}) for 10 consecutive days and ranitidine (50 mg kg^{-1}) showed reversible changes in the above parameters. However, only medium and higher doses of EtCK showed significant ameliorative effect on various biomarkers which was similar to that of ranitidine treated group.

DISCUSSION

In the present study, the peel extracts of *Citrus karna* in different solvents were evaluated for their antioxidant and antiulcer activity. Results showed the presence of flavonoid and phenolic acid in ethyl acetate and methanolic extracts. EtCK was further found to possess highest *in vitro* antioxidant potential so, it was analysed for its *in vivo* antiulcerogenic activity. EtCK showed dose dependent antiulcer effect in both water immersion and hypothermic restraint stress models. The induction of stress generate free radicals, which cause mucosal damage and change in antioxidant enzymes (Das and Banerjee, 1993). Excessive free radical generation causes enhanced lipid peroxidation which was indicated by an increase in the level of TBARS and hence, it leads to gastric tissue injury (Vendemiale *et al*., 1999). GSH acts as an important endogenous defense substance against the Reactive Oxygen Species (ROS). This GSH level was found to be reduced in rats subjected to increased stress level (Sahin and Gumuslu, 2007). Moreover, various antioxidant enzymes like SOD and CAT prevent the accumulation of ROS but stress results in an imbalance in the activity of these enzymes which lead to faulty disposal of free radicals and their accumulation (Halliwell, 1981).

Results indicated that low dose (200 mg kg^{-1}) was not effective in treating ulcer as compared to ranitidine treated group but medium and higher doses (300 and 400 mg kg^{-1}) showed significant effect. In the disease control group GSH, SOD and CAT level decreased due to increased free radical generation caused by stress condition. However, EtCK medium and high doses pretreated groups has resulted in the attenuation of the above stress induced biomarker changes. Moreover, the same doses caused significant decrease in the TBARS level and the ulcerative index.

Many *Citrus* species such as *Citrus medica*, *Citrus reticulate*, *Citrus limon* etc. have been reported to possess antioxidant potential (Sood *et al*., 2009). Antioxidant activity and phenolic composition of *Citrus bergamia*, *Citrus limon* and *Citrus aurantium* peel and seed extracts have also been studied. Methanolic extract

of these species were found to contain free phenolic compounds (Bocco *et al*., 1998) but very less reports are available on the therapeutic potential of *Citrus karna* peels. In our studies, phytochemical screening revealed the presence of flavonoids and phenolic acids in the EtCK extract, which are known to possess antioxidant and gastroprotective activities (Kandaswami and Middleton, 1994; Zayachkivska *et al*., 2005). Thus, the action of EtCK extract may be through free radical scavenging mechanism. The WIS and HRS stress models cause potential alteration of physiological antioxidant status and imbalance in the free radical defense enzymatic system. Hence, in both models EtCK showed potential amelioration of ulcerative and oxidative stress marker changes in tissue and plasma.

CONCLUSION

Therefore, the above studies showed that EtCK possess gastroprotective effect at a dose level of 300 and 400 mg kg^{-1} in both water immersion and hypothermic restraint stress models. Thus, EtCK may be a potent herbal therapeutic agent for the treatment of peptic ulcer disorders.

ACKNOWLEDGMENTS

Thanks to Professor A.C. Rana and all faculty members of Rayat Institute of Pharmacy for their encouragement and support. We are also grateful to Rayat and Bahra Educational and Research Trust for their unconditional helps to carry out this project.

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