Indian Journal of Experimental Biology
Vol. 47, March 2009, pp. 163-168

Hepato and reno protective action of *Calendula officinalis* L. flower extract

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Received 3 July 2008; revised 9 January 2009

Flower extract of *C. officinalis* L. was evaluated for its protective effect against CCl_4 induced acute hepatotoxicity and cisplatin induced nephrotoxicity. The activities of serum marker enzymes of liver injury like glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) which were increased by CCl_4 injection was found to be significantly reduced by the pretreatment of the flower extract at 100 and 250 mg/kg body weight. The lipid peroxidation in liver, the marker of membrane damage and the total bilirubin content in serum were also found to be at significantly low level in the extract pretreated group, indicating its protective role. The kidney function markers like urea and creatinine were significantly increased in cisplatin treated animals. However, their levels were found to be lowered in the extract pretreated groups (100 and 250 mg/kg body weight). Moreover, cisplatin induced myelosuppression was ameliorated by the extract pretreatment. Treatment with the extract produced enhancement of antioxidant enzymes — superoxide dismutase and catalase and glutathione. Results suggest a protective role of the flower extract of *C. officinalis* against CCl_4 induced acute hepatotoxicity and cisplatin induced nephrotoxicity. Extract has been found to contain several carotenoids of which lutein, zeaxanthin and lycopene predominates. Possible mechanism of action of the flower extract may be due to its antioxidant activity and reduction of oxygen radicals.

Keywords: Antioxidant, *Calendula officinalis*, CCl_4, Cisplatin, Hepatoprotection, Nephroprotection

Role of free radicals in the causation of diseases has been well established[1]. Several substances have been known to produce excessive free radicals and thereby produce tissue damage[2,3]. Since liver is the major organ involved in the detoxification of xenobiotics, it is the main target of tissue injury produced by these chemicals and their metabolites. Reactive oxygen species produce deleterious effect on membrane lipids of the cellular components thereby producing peroxidation of lipids which leads to cell death[4]. An association of reactive oxygen species with collagen synthesis and fibrosis has also been reported[5].

Many drugs, useful against diseases are known to produce severe side effects. Antitumor drugs have been known to produce myelosuppression, nephrotoxicity and hepatotoxicity which are mainly caused by free radical generation[6]. Scavengers of free radicals can reduce side effects of these drugs. Plant kingdom possesses several non-toxic compounds that can scavenge free radicals and boost the antioxidant defense mechanism in body and have a protective role against tissue damage induced by several chemicals and drugs[7-10].

The flower of *Calendula officinalis* L. (Family Asteraceae) is used to treat various ailments[11]. It has been reported to possess anti-inflammatory[12], antibacterial[13], antimutagenic[14] and antiviral[15] activities. The flower extract possesses potent anti-oxidant activity both *in vitro* and *in vivo*[16].

The present study has been undertaken with aim to determine the hepatoprotective and nephro protective activities of the flower extract of *Calendula officinalis* in animal models.

Materials and Methods

Chemicals—Nitro blue tetrazolium (NBT), glutathione (GSH), 5-5′dithiobis (2-nitro benzoic acid) (DTNB), were purchased from Sisco Research Laboratories Pvt.Ltd, Mumbai, India. Thio barbaturic acid was purchased from HiMedia Laboratories, Mumbai, India. CCl_4 was purchased from E-Merck. Cisplatin was obtained from Dabur Pharma, Himachal Pradesh. The kits for estimating enzyme activities were purchased from Agappe Diagnostics, Ernamkulam. The kits for urea, creatinine, total protein and bilirubin were purchased from Span

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Diagnostics, Surat. All other chemicals and reagents used were of analytical grade and obtained locally.

Preparation of the extract—Fresh *Calendula* flower tops, used for extraction of the active components were collected from Government Botanical Gardens, Ooty, Nilgiris during January and were authenticated by Dr.T.Subbaraju, J.S.S. College, Ooty and the voucher specimen was deposited at Amala Ayurvedic Research Centre (Voucher No: Co05). Extraction was done immediately after shade drying as per standard pharmacopoeia[17]. *Calendula* flowers (700 g) were extracted with 450 ml ethyl alcohol by masturation. For this, the material was placed in a wide mouth bottle and the alcohol was added. The jar was stoppered and sealed to prevent evaporation. It was placed in a dark room at room temperature and shaken everyday for two weeks. Then the clear liquid was decanted and the residue was pressed out through clean linen, and added to the decanted liquid. Volume was made upto 1 litre with alcohol. From this tincture of *Calendula* flowers 100 ml was evaporated to dryness in a shaker water bath at 42°C. The yield was found to be 1.1 g. Dried extract (1 g) was redissolved in a known amount of distilled water and used in all experiments.

HPTLC Analysis—The HPTLC of *C. officinalis* flower extract was performed at Tropical Botanical Garden and Research Institute, Palode. Lutein and Lycopene were used as standards. The HPTLC was performed on precoated silca gel plates 60F 254 (E Merck KGaA) using CAMAG TLC Scanner 3 "Scanner 3_131223" S/N 131223 (1.14.26). The plates were developed in solvent system of ethylacetate: formic acid: glacial acetic acid: water (100:11:11:26). The detection was done at 356 nm and the evaluation was done via peak areas with linear regression.

Animals—Female Wistar rats (150-200 g) and Swiss albino mice (20-25 g) obtained from Small Animal Breeding Station, Mannuthy, Thrissur, Kerala were housed in well-ventilated cages. They were fed with normal mouse chow (Sai Durga Feeds and Food, India) and provided water *ad libitum*. All the animal experiments were done after approval from the Institutional Animal Ethical Committee.

Determination of hepatoprotective activity of C. officinalis—Female Wistar rats were divided into following 4 groups of 6 animals each.

Group I: Normal without any treatment, which served as control, Group II: CCl_4 alone treated control animals, Group III: CCl_4 + 100 mg/kg body weight *Calendula* extract (pre-treated), and Group IV: CCl_4 + 250 mg/kg body weight *Calendula* extract (pre-treated). The doses were determined after toxicity study and ability to induce antioxidant enzymes during a pilot study.

Group II, III and IV animals received single dose of 0.25 ml of CCl_4 in liquid paraffin (1:1)/100 g body weight intraperitoneally (ip) to produce acute hepatotoxicity[18]. Vehicle liquid paraffin by itself does not produce any hepatotoxicity. The extract treatment (orally) was started 3 days prior to CCl_4 administration. After 24 hr of CCl_4 injection all the animals were sacrificed, blood was collected to separate serum and the activity of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), and the content of bilirubin were analyzed using commercially available kits. Liver was excised, washed in ice cold saline and small portion was fixed in 10% formalin for histopathological analysis. A 25% tissue homogenate was prepared and the level of lipid peroxidation was assessed by the method of Ohkawa *et al*[19].

Determination of nephroprotective activity of C.officinalis—Swiss albino mice (32) were divided into 4 groups of 8 animals each and were treated as follows:

Group I: Normal without any treatment which served as control, Group II: cisplatin alone treated animals, Group III: cisplatin + 100 mg/kg body weight *Calendula* extract (pre-treated) and Group IV: cisplatin + 250 mg/kg body weight *Calendula* extract (pre-treated) animals.

Animals in group II- IV received single dose (ip) of cisplatin (16 mg/kg body weight) in saline to produce nephrotoxicity[20]. The extract treatment was started 3 days prior to cisplatin injection and continued for 3 days. After 72 hr of cisplatin injection, animals were sacrificed and as the volume of the blood was very low, it was collected in heparinized vials for total WBC count[21] determination, differential count and hemoglobin content[22] and in non- heparinized vials to separate serum for the analysis of kidney function parameters viz. urea and creatinine using commercially available kits. Bone marrow from both the femurs was flushed out into PBS with 10% goat serum to determine bone marrow cellularity[23]. Kidney was excised out, washed in ice-cold saline and a portion of kidney was fixed in 10% formalin for histopathological analysis. A 10% homogenate was prepared to evaluate the activity of superoxide dismutase[24], catalase[25], glutathione content[26] and level of lipid peroxidation[19].

Statistical analysis—The values were expressed as mean ± SD. The significant levels for comparison of differences compared to that of the control was determined by one way-ANOVA followed by appropriate post-hoc test (Dunnett multiple comparison test) using Graph pad In Stat 3 software.

Results

HPTLC profile—The HPTLC profile of *C. officinalis* is shown in Fig. 1. The flower extract showed nearly 9 peaks absorbing at 356 λ, of which 5 were major ones. By using co-chromatography it was found that lutein (R_f 0.8) and lycopene (R_f 0.87) are present in the extract. Other absorbing peaks were unidentified.

Effect of C. officinalis on CCl_4 induced hepatic damage in rats—There was a significant increase in hepatic marker enzymes SGPT, SGOT and ALP activity of untreated control animals when compared with that of normal control (Table 1). Pretreatment of *Calendula* extract (group III and group IV) could significantly reduce the SGPT, SGOT and ALP activities in serum. The total bilirubin in the group II animals was significantly increased when compared with that of group I animals. Pretreatment with *Calendula* extract decreased the bilirubin level (Table 1).

The lipid peroxidation in liver tissue of untreated control animals was 2.58 ± 0.42 which was significantly higher when compared with that of normal level (0.74 ± 0.22). This increased level was found to be significantly lowered in the extract treated groups (Table 1).

The histopathology of liver of CCl_4 alone administered animals showed severe liver cell necrosis with steatosis, areas of congestion and dysplastic nucleus. In *Calendula* (250 mg/kg body weight) treated animals, the central vein was

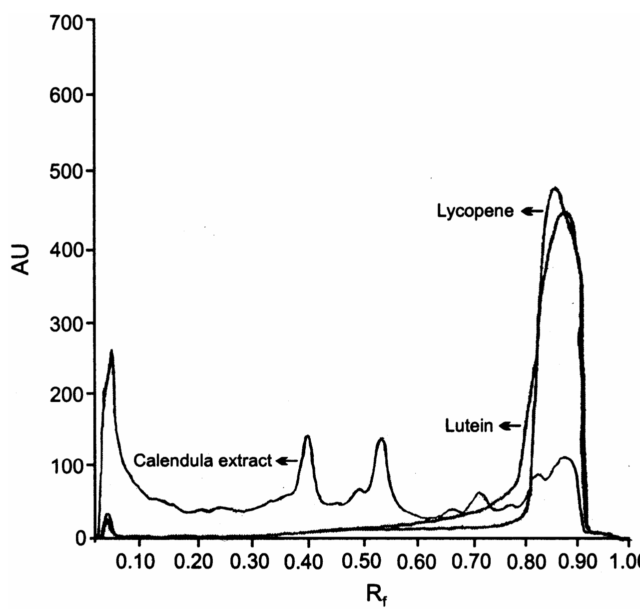

Fig. 1—HPTLC profile of *Calendula officinalis* flower extract

Table 1—Effect of *C. officinalis* flower extract on serum enzymes of CCl_4 administered rats
[Values are mean ± SD from 6 animals in each group]

Group	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	Total bilirubin (mg/dl)	Lipid peroxidation (n mols of MDA formed/mg protein)
Group I (Normal control)	32.22±9.36	142.04±26.36	59.77±8.90	0.56±0.13	0.74±0.22
Group II (Untreated control-CCl_4 alone)	585.71±198.0^c	735.81±146.22^c	133.53±8.55^c	1.20±0.20^b	2.58±0.42^b
Group III (CCl_4 +; flower extract; 100 mg/kg body weight)	206.49±73.10^c	252.15±32.48^c	102.21±21.78^a	0.71±0.26^b	1.83±0.31^b
Group IV (CCl_4 + flower extract; 250 mg/kg body weight)	187.58±77.22^c	169.37±52.33^c	102.45±24.99^a	0.67±0.10^c	0.82±0.24^c

P values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001
Group II compared with Group I and Groups III & IV compared with Group II

prominent with RBCs. Hepatocytes with steatosis were seen only occasionally and there was no necrosis.

Effect of Calendula extract on cisplatin induced nephrotoxic animals—Renal function markers like urea and creatinine were significantly higher in the group II animals when compared to that of group I indicating renal damage (Table 2). This increased level was decreased significantly by extract treatment.

The antioxidant enzyme superoxide dismutase showed no significant change with cisplatin treatment. But SOD activity was increased in the group III and IV animals (Table 2). The catalase activity was reduced in group II animals. The extract treatment significantly enhanced the catalase activity (Table 2). The glutathione content in the *Calendula* extract treated group of animals was significantly enhanced by the treatment which indicates the triggering of antioxidant mechanism by the *Calendula* extract as a defense against the free radicals generated. The lipid peroxidation in all the group of animals was high when compared with that of the normal. Treatment with *Calendula* extract did not decrease the elevated lipid peroxidation (Table 2).

The total WBC count was decreased in group II animals. Treatment with 250 mg/kg body weight extract significantly increased the total count (Table 3). There was no significant change in the hemoglobin content of animals with and without cisplatin treatment. However there was significant increase in the hemoglobin content in group III and IV when compared with that of group II animals. The bone marrow cellularity which was significantly reduced in group II animals was increased after extract treatment (Table 3).

The histopathology of the kidney revealed that there was formation of cast which is usually due to the deposition of proteineceous substances in cisplatin alone treated animals. Such changes were minimum in *Calendula* extract treated animals indicating its protective role.

Discussion

In the present study it was found that the flower extract of *C. officinalis* can modulate the hepatotoxicity induced by CCl_4 and nephrotoxicity induced by cisplatin. The toxicity produced by CCl_4 is mediated through free radical mechanism. The CCl_4 is metabolized by cytochrome P_{450} enzyme and its metabolic products, trichloromethyl free radicals are highly reactive and induces lipid peroxidation of macromolecules leading to tissue injury[27,28]. It was observed that pre-treatment with *Calendula* flower extract significantly reduced the tissue damage produced by CCl_4. This is evident from the decreased level of marker enzymes of tissue injury and total bilirubin level. Similarly lipid peroxidation induced by CCl_4 was significantly lowered by the extract pre-treatment, indicating a protective effect of *Calendula* flower extract against CCl_4 induced hepatotoxicity. Although in the present study involving prior treatment with the extract for three

Table 2—Effect of *C. officinalis* flower extract on kidney damage and antioxidant enzyme status in cisplatin induced mice
[Values are mean ± SD from 6 animals in each group]

Groups	Urea (mg/dL)	Creatinine (mg/dl)	SOD activity (U/mg protein)	Catalase activity (K/mg protein)	Glutathione content (n mols/mg protein)	Lipid peroxidation (n mols MDA/mg protein)
Group I (Normal control)	47.53±8.13	1.19±0.17	1.50±0.42	10.56±2.34	23.44±5.33	1.12±0.25
Group II (Untreated control-Cisplatin alone)	154.29±36.90^c	2.82±1.02^c	1.55±0.36	7.78±1.34	24.19±4.33	2.76±0.66^c
Group III (Cisplatin + flower extract; 100 mg/kg body weight)	83.14±23.88^c	1.42±0.52^c	2.27±0.62	14.26±5.26^b	42.42±10.13^b	2.76±0.38
Group IV (Cisplatin + flower extract; 250 mg/kg body weight)	67.54±18.72^c	1.29±0.49^c	2.51±0.93^a	15.49±3.78^c	41.33±11.64^b	2.56±0.34

P values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001
Group II compared with Group I and Groups III & IV compared with Group II

Table 3—Effect of *C. officinalis* flower extract on hematological parameters and bone marrow cellularity in cisplatin treated mice
[Values are mean ± SD from 6 animals in each group]

Groups	Total WBC count (cells/mm^3)	Haemoglobin content (gm/dL)	Bone marrow cellularity (cells/femur/ml)
Group I (Normal control)	9271±230	13.67±2.14	$16.29 \times 10^6 \pm 9.99 \times 10^5$
Group II (Untreated control- Cisplatin alone)	7157±403	11.59±0.59	$10.76 \times 10^6 \pm 7.28 \times 10^{5c}$
Group III (Cisplatin + flower extract; 100 mg/kg body weight)	7438±432	14.35±1.09^a	$14.51 \times 10^6 \pm 10.92 \times 10^{5c}$
Group IV (Cisplatin + flower extract; 250 mg/kg body weight)	8438±473^b	14.10±1.82^a	$14.90 \times 10^6 \pm 8.54 \times 10^{5c}$

P values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001
Group II compared with Group I and Groups III & IV compared with Group II

days produced only partial reversal of hepatic markers, it is possible that continued administration can produce a complete reversal of the marker levels.

Cisplatin is a potent drug used in the treatment of a wide range of cancers[29]. However, the severe toxic side effects are the major limitation in its usage. Cisplatin induces oxidative stress causing damage to intracellular organelles and alters their functions which lead to inhibition of protein synthesis, glutathione depletion, lipid peroxidation and mitochondrial damage[30]. Controversially there are also reports that the level of glutathione increases in patients undergoing chemotherapy as a mechanism of tumor cells for acquiring drug resistance[31]. In the present study by cisplatin treatment the level of GSH was more or less normal but in *C. officinalis* flower extract pretreatment could significantly increase the GSH level. The catalase activity was also found to be enhanced in *Calendula* flower extract pretreated groups. There was significant lipid peroxidation indicating possible membrane damage. Pretreatment with the extract significantly decreased kidney function markers. The myelosuppression induced by cisplatin was also found to be modulated by the extract pre-treatment. The histopathological section of kidney in cisplatin alone treated showed proteineous casts due to the damage of the basement membrane of glomuruli which causes the proteins to get deposit in the nephrons. These changes were minimum in extract pretreated groups.

Many plants and plant derived products are reported to protect the body from deleterious effects of free radicals[32,33]. *C. officinalis* has been reported to contain flavonoids like quercetin, protocatechuic acid etc., triterpenoids like faradiol, oleanolic acid, beta-amyrin, calenduladiol etc. and the alkaloid, narcissin[34]. Flowers are also rich in carotenoids like lycopene, β-carotene, flavoxanthin, luteoxanthin[35] etc. The phytochemical analysis showed the presence of lutein and lycopene. The active ingredients present in the *Calendula* flowers like β-carotene, lutein, lycopene etc. are reported to possess several pharmacological activities including chemopreventive potential[36]. Lycopene has been reported to ameliorate cisplatin induced renal failure[37].

The present results revealed that the extract could protect the organs from toxicity induced by chemical compounds. This activity can be partially attributed to the free radical scavenging activity and enhancement of the antioxidant system effectively by the extract since many of the active ingredients present in the extract are potent free radical scavengers. However, other mechanism such as its effect on Cytochrome P_{450} enzymes may also be looked into.

Acknowledgement

One of the authors (KCP) thank ICMR, New Delhi for Senior Research Fellowship. Thanks are due to Dr.A.Subramonium for HPTLC analysis.

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