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Antioxidant Potential of an Extract of *Calendula officinalis* Flowers *in Vitro* and *in Vivo*

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Abstract

An extract of *Calendula officinalis* Linn. (Compositae) was evaluated for its antioxidant potential *in vitro* and *in vivo*. *Calendula officinalis* extract was found to scavenge superoxide radicals generated by photoreduction of riboflavin and hydroxyl radicals generated by Fenton reaction and inhibited *in vitro* lipid peroxidation. Concentrations needed for 50% inhibition (IC_{50}) were 500, 480, and 2000 μg/mL, respectively. Extract scavenged ABTS radicals and DPPH radicals and IC_{50} were 6.5 and 100 μg/mL, respectively. IC_{50} values were compared with that of ginger extract, which is a standard antioxidant extract. The drug also scavenged nitric oxide, and the IC_{50} was found to be 575 μg/mL. Extract also produced dose-dependent scavenging of nitric oxide in culture. The oral administration of *Calendula* extract inhibited superoxide generation in macrophages *in vivo* by 12.6% and 38.7% at doses of 100 and 250 mg/kg b.wt. Oral administration of *Calendula officinalis* to mice for 1 month significantly increased catalase activity. The extract produced significant increase in glutathione levels in blood and liver. Glutathione reductase was found to be increased, whereas glutathione peroxidase was found to be decreased after administration of *Calendula* extract. These results indicated *Calendula officinalis* has significant antioxidant activity *in vitro* and *in vivo*.

Keywords: ABTS, antioxidant, *Calendula officinalis*, DPPH, free radicals, glutathione.

Introduction

Free radicals generated either exogenously or endogenously inside the body have been implicated in causation of several diseases such as liver cirrhosis (Slater, 1987), inflammation, atherosclerosis (Halliwell & Gutterridge, 1985), diabetes, cancer (Dreher & Junod, 1996), neurodegenerative disease (Knight, 1997), and so forth. The link between free radicals and disease processes has led to considerable research into nontoxic drugs that can scavenge the free radicals. Several plant extracts and plant products have been shown to possess significant antioxidant potential (Soudhamini & Kuttan, 1989; Jose & Kuttan, 1995; Sabu & Kuttan, 2003). *Calendula officinalis* Linn. (Compositae), an herb employed in traditional medicine, has been reported to have several pharmacological activities. Topical application of *Calendula officinalis* extract was found to possess significant anti-inflammatory activity (Della Logia et al., 1994). It also possessed wound-healing activity (Rao et al., 1991). In clinical trials, *Calendula officinalis* extract was found to possess preventive activity against acute dermatitis during irradiation (Pommier et al., 2004) *Calendula officinalis* flower extract was reported to possess an antigenotoxic effect (Perez-Carreon et al., 2002). These reported pharmacological activities might be related to the antioxidant activity of *Calendula* extract, although this was not fully substantiated. In fact, there are only very few reports on the antioxidant activity of *Calendula* extract (Cordova et al., 2002; Herold et al., 2003), and in this report we have done a systematic investigation on the antioxidant potential of this extract *in vitro* as well as *in vivo*.

Materials and Methods

Nitro blue tetrazolium (NBT), glutathione (GSH), glutathione oxidized (GSSG), nicotinamide adenine

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dinucleotide phosphate reduced (NADPH), and 5-5′-dithio*bis* (2-nitro benzoic acid) (DTNB) were purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). 2,2-Diphenyl-1-picryl hydrazyl (DPPH) and 2,2-*azo bis*-3-ethylbenzthiazoline-6-sulfonicacid (ABTS) were purchased from Sigma Aldrich (St. Louis, MO, USA). RPMI 1640 was purchased from Himedia Lab (Mumbai, India). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Phorbol-12-myristate-13-acetate (PMA) was a gift from Dr. Allen Conney. All other chemicals and reagents used were of analytical grade.

Preparation of the extract

Fresh *Calendula* flower tops were used for extraction of the active components. They were collected from Government Botanical Gardens, Ooty, Nilgiris, during January 2005 and were authenticated with the voucher specimen. Extraction was done as per standard pharmacopoeia (Committee on Pharmacopoeia, 1954). *Calendula* flowers (700 g) were extracted with 450 mL ethyl alcohol. 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 every day for 2 weeks. Thereupon the clear liquid was decanted and the residue was pressed out through clean linen, which was added to the decanted liquid. Volume was made up to 1 L with alcohol. One hundred milliliters of this tincture of *Calendula officinalis* was evaporated to dryness in a shaker water bath at 42°C. The yield was found to be 1.1 g. One gram of the dried extract was redissolved in known amount of distilled water and used for all experiments.

Experimental animals

Female Swiss albino mice (20–25 g) were obtained from the animal house of Amala Cancer Research Centre. They were housed in well-ventilated cages and fed with normal mouse chow (Sai Durga Feeds and Food, Bangalore, India) and water *ad libitum*. All the animal experiments were done after approval from the institutional animal ethical committee.

In vitro antioxidant activity

Determination of superoxide radical scavenging activity

Superoxide radical scavenging activity was determined by the NBT reduction method (Mc Cord & Fridovich, 1969). The reaction mixture contained EDTA (6 μM), NaCN (3 μg), riboflavin (2 μM), NBT (50 μM), various concentrations of the extract (0.22 to 2.2 mg), and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 mL. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and the optical density was measured at 560 nm before and after illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes.

Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compound for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose, which results in thiobarbituric acid reacting substance (TBARS) formation (Elizabeth & Rao, 1990). The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), and various concentrations of the extract (0.22 to 2.2 mg) in a final volume of 1 mL. The reaction mixture was incubated for 1 h at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated (Ohkawa et al., 1979).

Determination of inhibition of lipid peroxidation

Reaction mixture (0.5 mL) containing rat liver homogenate (0.1 mL, 25% w/v) in Tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous ion (0.16 mM), and ascorbic acid (0.06 mM) were incubated for 1 h at 37°C in the presence (0.22 to 2.2 mg) and absence of the extracts. The lipid peroxide formed was measured by TBARS formation (Ohkawa et al., 1979). Incubation mixtures (0.4 mL) were treated with sodium dodecyl sulfate (SDS; 8.1%, 0.2 mL), thiobarbituric acid (TBA; 0.8%, 1.5 mL), and acetic acid (20%, 1.5 mL, pH 3.5). The total volume was then made up to 4 mL with distilled water and kept in a water bath at 100°C for 1 h. After cooling, 1 mL of distilled water and 5 mL of a mixture of *n*-butanol and pyridine (15:1 v/v) were added and vortexed. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of the control not treated with the extract.

Determination of DPPH radical scavenging activity

In this method, a commercially available and stable free radical DPPH (2,2-diphenyl-1-picryl hydrazyl) soluble in methanol was used. DPPH in its radical form has an absorption peak at 515 nm, which disappeared on reduction by an antioxidant compound (Aquino et al., 2001). Different concentrations of the extract (22 to 550 μg) were added to 1.5 mL of freshly prepared DPPH solution (0.25 g/L in methanol). Absorbance was measured at

515 nm, 20 min after the reaction was started. The percentage inhibition of $DPPH^+$ in the reaction medium was calculated by comparing with the control.

Determination of ABTS radical scavenging activity

ABTS radical scavenging activity of the extract was determined using ferryl myoglobin/ABTS protocol (Alzoreky & Nakahara, 2001). The stock solutions of 500 μM ABTS diammonium salt, 400 μM myoglobin (MbIII), 740 μM potassium ferricyanide, and 450 μM H_2O_2 were prepared in phosphate-buffered saline (PBS; pH 7.4). Metmyoglobin was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (2 mL) contained ABTS (150 μM), MbIII (2.25 μM), varying concentrations of *Calendula* extract (11 to 110 μg), and PBS. The reaction was initiated by adding 75 μM H_2O_2, and lag time in seconds was recorded before absorbance of $ABTS^+$ at 734 nm began to increase.

Determination of nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent (Green et al., 1982). Stock solution (10 mM) of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of the extract (0.25 to 2 mg) and sodium nitroprusside (1 mM) in PBS in a final volume of 3 mL were incubated at 25°C for 150 min. After incubation, 0.5 mL of the solution was removed and diluted with 0.5 mL of the Griess reagent (1% sulfanilamide, 2% orthophosphoric acid, and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm. The percentage inhibition of nitric oxide was calculated by comparing with the control.

Determination of inhibition of nitric oxide produced by macrophages

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in Swiss albino mice. Peritoneal macrophages were isolated from peritoneum with 5 mL of sterile PBS. Macrophages were washed with PBS and resuspended in RPMI 1640 medium with 10% FCS. The macrophages (1×10^6 cells/well) were plated in 96-well culture plate and incubated for 2 h at 37°C in 5% CO_2. After incubation, nonadherent cells were removed and adherent macrophages were incubated in complete culture medium, in the presence and absence of different concentrations of the extract (10 to 150 μg) for 24 h at 37°C with 5% CO_2. After 24 h, the plate was centrifuged, and the supernatant was used for the estimation of nitric oxide by the Griess reaction (Green et al., 1982). The percentage inhibition of nitric oxide formation is calculated by comparing with that of control.

Determination of the effect of Calendula officinalis *on PMA-induced superoxide radical generation in peritoneal macrophages*

Female Swiss albino mice (4–6 weeks) weighing 20–25 g were used. Animals were divided into three groups (three animals/group). All the animals were injected (i.p.) with sodium caseinate (5%) to elicit macrophages. Group I was kept as control. Groups II and III were treated with single dose of *Calendula* extract (100 and 250 mg/kg b.wt., respectively). On the fifth day after 1 h of drug administration, peritoneal macrophages elicited by sodium caseinate were activated *in vivo* by injecting PMA (100 ng/animal). After 3 h, peritoneal macrophages were harvested. The effect of *Calendula* extract on the inhibition of superoxide generation in the macrophages was measured by inhibition in the reduction of NBT to formazan by the method of Dwivedi et al. (1992). The percentage inhibition was determined by comparing the absorbance values of untreated and treated animals.

Determination of in vivo *antioxidant activity*

Thirty-two Swiss albino female mice were divided into four groups of eight animals and they were treated orally with *Calendula officinalis* extract at different doses for 30 days.

Group I: Normal
Group II: *Calendula officinalis* extract 50 mg/kg b.wt.
Group III: *Calendula officinalis* extract 100 mg/kg b.wt.
Group IV: *Calendula officinalis* extract 250 mg/kg b.wt.

At the end of the experiment, animals were sacrificed, and blood was collected by heart puncture and liver was excised and washed in ice-cold Tris-HCl buffer (0.1 M, pH 7.4), and cytosolic samples of liver homogenate were prepared by centrifuging at 10,000 rpm for 30 min at 4°C.

The total protein was estimated by the method of Lowry et al. (1951). Hemoglobin was estimated by the cyanmethemoglobin solution using Drabkin's method (Drabkin et al., 1932). The following parameters were assayed in both blood and liver to assess the oxidative stress. Superoxide dismutase activity was measured by the NBT reduction method of McCord and Fridovich (1969). Catalase activity was estimated by the method of Aebi (1974) by measuring the rate of decomposition of hydrogen peroxide at 240 nm. Glutathione activity was assayed by the method of Moron et al. (1979) based on the reaction with DTNB. An assay of glutathione peroxidase followed the method of Hafeman et al. (1974)

based on the degradation of H_2O_2 in the presence of GSH. The method of Racker et al. (1955) was followed to assay the activity of glutathione reductase, where the amount of reduced form of NADP consumed during the conversion of GSSG to GSH was measured. Lipid peroxides were measured in liver by using the TBA method of Ohkawa et al. (1979).

Statistical analysis

Data was expressed as mean ± standard deviation (SD). Significance levels for comparison of differences were determined using Student's *t*-test.

Results

Calendula officinalis extract was found to scavenge superoxide and hydroxyl radicals and inhibited tissue lipid peroxidation *in vitro* in a concentration-dependent manner (Fig. 1). The concentration of the extract needed for 50% scavenging of superoxide generated by photoreduction of riboflavin was 500 µg/mL, whereas for the inhibition of hydroxyl radicals generated by Fe^{3+}/ascorbate/EDTA/H_2O_2 system it was 480 µg/mL. IC_{50} for the inhibition of lipid peroxidation of *Calendula officinalis* extract was 2000 µg/mL.

Stable free radicals DPPH and ABTS were effectively scavenged by *Calendula officinalis* extract, and the IC_{50} was found to be 100 and 6.5 µg/mL, respectively (Fig. 1). A standard antioxidant ginger was used to compare the antioxidant potential, and it was found that the IC_{50} of *Calendula officinalis* extract was higher than that of ginger extract (Table 1).

Table 1. Free radical scavenging activity of *Calendula officinalis* and comparison with ginger extract.

Concentration needed for 50% inhibition (IC_{50})		
	Calendula officinalis extract	Ginger extract
Superoxide radical scavenging	500 µg/mL	22 µg/mL
Hydroxyl radical scavenging	480 µg/mL	150 µg/mL
Inhibition of lipid peroxidation	2000 µg/mL	30 µg/mL
DPPH radical scavenging	100 µg/mL	7 µg/mL
ABTS radical scavenging	6.5 µg/mL	2.75 µg/mL
Nitric oxide scavenging	575 µg/mL	ND

ND, not determined.

Nitric oxide, yet another free radical in biological system, which is produced during oxidative stress and has a major role in disease causation, was also found to be scavenged by *Calendula officinalis in vitro* system. The IC_{50} for nitric oxide scavenging was 575 µg/mL (Fig. 1).

Nitric oxide produced by macrophages in culture was also scavenged by incubation with *Calendula officinalis*.

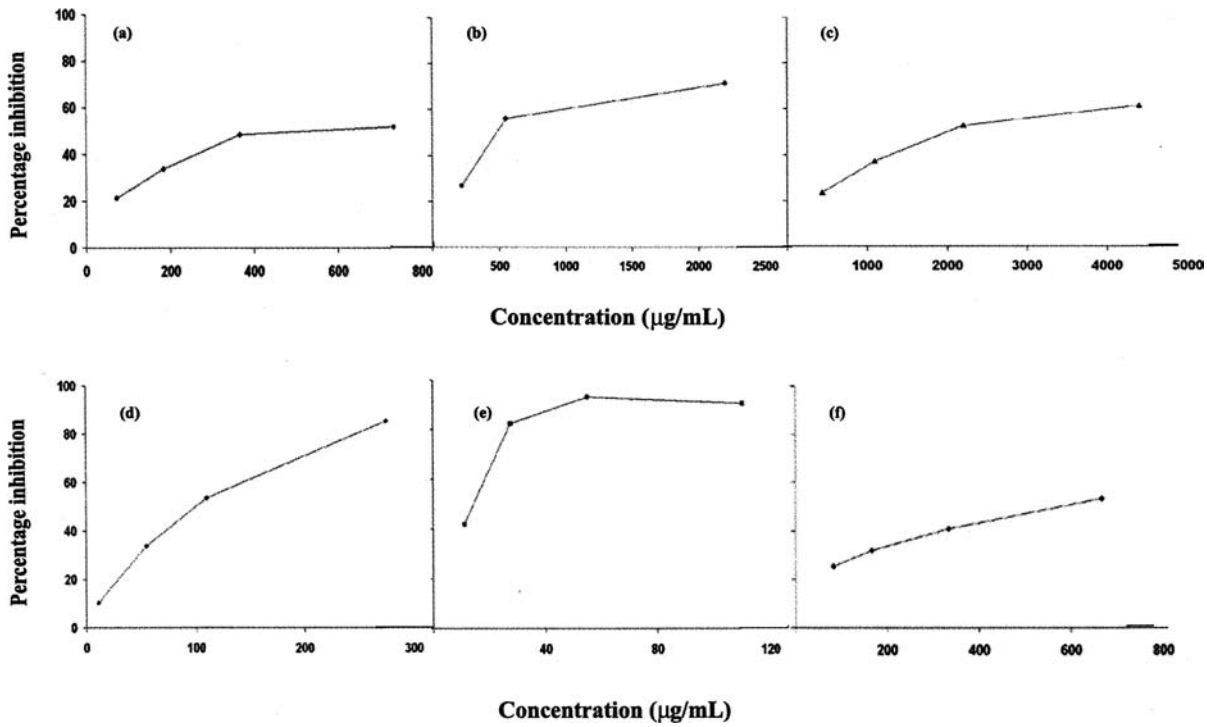

Figure 1. In vitro antioxidant activity of *Calendula officinalis*. (a) Superoxide radical scavenging activity. (b) Hydroxyl radical scavenging activity. (c) Inhibition of lipid peroxidation. (d) DPPH radical scavenging activity. (e) ABTS radical scavenging activity. (f) Nitric oxide radical scavenging activity.

Table 2. Effect of *Calendula officinalis* administration on antioxidant system in blood.

Treatment	Catalase (K/g Hb)	Superoxide dismutase (U/g Hb)	Glutathione peroxidase (U/mL of hemolysate)	Glutathione (nmol/mL)	Glutathione reductase (U/mg protein)
Normal	112.5 ± 26.5	555.6 ± 72.3	2.1 ± 0.24	19.1 ± 0.64	2.9 ± 1.5
50 mg/kg b.wt.	$172.6 \pm 45.5^{**}$	$393.8 \pm 52.3^{***}$	2.13 ± 0.25	$23.8 \pm 1.4^{***}$	2.0 ± 0.5
100 mg/kg b.wt.	$209.9 \pm 83.9^{**}$	469.3 ± 72.2	2.24 ± 0.25	$22.9 \pm 3.4^{**}$	3.1 ± 1.4
250 mg/kg b.wt.	160.7 ± 46.2	609.6 ± 97.3	$1.54 \pm 0.14^{***}$	$31.8 \pm 2.3^{***}$	4.5 ± 2.7

$^{**}p < 0.005$, $^{***}p < 0.001$.
K = the measure of catalase activity (the difference in extinction at 240 nm per 15 seconds).

At concentration of 10 to 150 μg, inhibition of nitric oxide formation was between 3.2% and 32.6%.

The effect of *Calendula officinalis* on *in vivo* superoxide scavenging was determined by PMA-induced superoxide production method. Superoxide radical generated during the activation with PMA in sodium caseinate–induced macrophages was found to be scavenged after oral administration of *Calendula officinalis*. The percentage inhibition was 12.6% and 38.7% for 100 and 250 mg/kg b.wt., respectively.

The effect of *Calendula officinalis* on the antioxidant system in the blood and serum of mice after given for a period of 30 days is shown in Table 2. Catalase was found to be significantly increased in animals treated with *Calendula* extract ($p < 0.005$). Superoxide dismutase was found to be significantly decreased in 50 mg/kg b.wt. group ($p < 0.001$), whereas it was unaltered at higher concentration. Glutathione peroxidase enzyme was found to be decreased in 250 mg/kg b.wt. group ($p < 0.001$). Glutathione was significantly enhanced in all treated groups ($p < 0.005$, $p < 0.001$). Even though glutathione reductase was found to be increased in the 250 mg group, the increase was not significant.

Table 3 shows the effect of *Calendula officinalis* extract on the antioxidant system in mice liver after treatment for 30 days. Catalase was found to be increased significantly in 50 mg/kg b.wt. ($p < 0.01$) and 100 mg/kg b.wt. ($p < 0.005$) groups. Superoxide dismutase was unaltered. Glutathione peroxidase did not change significantly in any of the treated groups. Glutathione was found to be significantly increased in the 250 mg/kg b.wt. group ($p < 0.001$). Glutathione reductase was found to be elevated significantly in both 100 and 250 mg/kg b.wt. groups ($p < 0.001$). Lipid peroxidation was found to be decreased, but the decrease was not significant. Results of the antioxidant levels indicate that increase in catalase and intracellular glutathione are the major changes in antioxidant defense mechanism induced by *Calendula* extract.

Discussion

The body's innate mechanism has many enzymes and nonprotein compounds that protect it from the free radicals and reactive oxygen species produced inside the body during normal metabolism and also due to external stimuli. These include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione, which also play a major role in detoxification and coordinate the body's antioxidant defense processes. Superoxide dismutase is a metalloprotein that scavenges superoxide anions. Catalase is a heme protein, localized in the peroxisome or the microperoxisome, which catalyzes the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage produced by H_2O_2. Glutathione peroxidase catalyzes the reaction of hydroperoxides, which reduces

Table 3. Effect of *Calendula officinalis* administration on antioxidant system in liver.

Treatment	Catalase (K/mg protein)	Superoxide dismutase (U/mg protein)	Glutathione peroxidase (U/mg protein)	Glutathione (nmol/mL)	Glutathione reductase (nmol of NADPH consumed/min per mg protein)	Lipid peroxidation (nmol of MDA formed/mg protein)
Normal	7.5 ± 2.0	1.23 ± 0.1	6.5 ± 1.4	16.4 ± 0.5	100.4 ± 23.9	0.92 ± 0.59
50 mg/kg b.wt.	$13.7 \pm 5.9^{*}$	1.08 ± 0.4	7.8 ± 2.1	16.6 ± 1.5	110.8 ± 43.1	1.5 ± 0.8
100 mg/kg b.wt.	$12.7 \pm 4.0^{**}$	$1.01 \pm 0.1^{***}$	7.5 ± 1.4	17.4 ± 1.7	$137.3 \pm 12.7^{***}$	0.88 ± 0.3
250 mg/kg b.wt.	10.0 ± 3.3	1.07 ± 0.2	8.0 ± 1.8	$19.5 \pm 1.6^{***}$	$135.0 \pm 13.6^{***}$	0.87 ± 0.5

$^{*}p < 0.01$, $^{**}p < 0.005$, $^{***}p < 0.001$.
K = the measure of catalase activity (the difference in extinction at 240 nm per 15 seconds).

glutathione to form glutathione disulfide (GSSG) and the reduction product of the hydroperoxide. Glutathione reductase is involved in the regeneration of glutathione that has been converted to GSSG by oxidation and thiol transfer reactions. Glutathione, a major nonprotein thiol, is mainly involved in detoxification (Halliwell & Gutterridge, 1985).

The current study indicates that *Calendula officinalis* extract effectively scavenged superoxide, hydroxyl, and nitric oxide radicals *in vitro*. These radicals are generated inside the body during the normal metabolism or in presence of xenobiotics. The stable free radicals DPPH and ABTS were also scavenged by *Calendula officinalis* extract. *Calendula officinalis* also scavenged the superoxide generated *in vivo* after the administration of phorbol esters in the mice. Moreover, administration of *Calendula officinalis* significantly increased the catalase and glutathione levels in blood and liver. Glutathione reductase was increased in liver of treated groups. However, lipid peroxidation remained unchanged. These results show that *Calendula officinalis* has a profound effect on the antioxidant defense system both *in vitro* and *in vivo*.

Calendula officinalis has been reported to contain flavonoids (including lutein, quercetin, protocatechuic acid, etc.), triterpenoids (including faradiol, oleanolic acid, beta-amyrin, calenduladiol, etc.), and the alkaloid narcissin (Matysik et al., 2005). Flowers also are rich in carotenoids of which flavoxanthin has been reported to be present at 28.5% of total carotenoids followed by luteoxanthin (Kishimoto et al., 2005). Flowers are also found to contain lycopene and β-carotene. Coumarins are also an active ingredient in *Calendula officinalis*. These ingredients may contribute to the antioxidant potential of this extract.

Calendula officinalis flower extract has been reported to possess several pharmacological activities. The homeopathic preparation of *Calendula officinalis* is reported to possess antiviral (Barbour et al., 2004) and antibacterial (Dumenil et al., 1980) activity. The extract also possesses cytotoxic and antitumoral activity (Boucaud-Maitre et al., 1988). Several pharmacological activities have been associated with the isolated active ingredients of *Calendula officinalis* including antimutagenic activity by saponins (Elias et al., 1990) and hypoglycemic, gastric emptying inhibitory, and gastroprotective activity by triterpene oligoglycosides, calendasaponins A, B, C, D (Yoshikawa et al., 2001). Many of the active principles have profound wound-healing (Rao et al., 1991) and anti-inflammatory activity (Della Logia et al., 1994).

Effect of free radicals on DNA can be minimized by the use of combination therapies that act at sequential steps in the DNA destruction process. Inhibition of DNA strand-breaks can reduce the mutagenicity and further carcinogenicity. Antioxidants have direct effects on transcription through antioxidant response elements present in the promoters of many genes (Palmer & Paulson, 1997). *Calendula* has several ingredients with reported antioxidant and anticlastogenic activities. *Calendula officinalis*, with profound antioxidant potential and having the ability to trigger cellular antioxidants, can be exploited for its use against a number of disorders including cardiovascular diseases, inflammation, and cancer.

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