RESEARCH COMMUNICATION

Inhibition of Metastasis of B16F-10 Melanoma Cells in C57BL/6 Mice by an Extract of *Calendula Officinalis* L Flowers

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Abstract

Aim: To determine the effect of a *Calendula officinalis* flower extract on lung metastasis by B16F-10 melanoma cells in C57BL/6 mice. Materials and Methods: Male mice were injected with B16F-10 melanoma cells through the tail vein and simultaneously treated with *C.officinalis* flower extract. Parameters studied were lung tumor nodule count, life span of animals, gamma glutamyl transpeptidase activity, sialic acid, TNF-α, IL-1β, IL-6, IL-2, GM-CSF, VEGF and TIMP-1 levels in serum, and lung hydroxyproline, uronic acid and hexosamine levels, as well as histopathological features. Effects of *C.officinalis* on the expression of various genes involved in metastasis like matrix metalloproteases (MMPs), tissue inhibitor of metalloproteases (TIMPs), prolyl hydoxylase, lysyl oxidase, nm23, and proinflammatory cytokines were also investigated. Results: Simultaneous administration of *C.officinalis* extract to tumor bearing C57BL/6 mice reduced the lung tumor nodules by 74% with 43.3% increase in life span. Elevated levels of hydroxyproline, uronic acid, hexosamine, serum sialic acid and γ-glutamyl transpeptidase in the metastatic controls were found to be significantly lowered in the *C.officinalis* treated animals. The extract also inhibited expression of MMP-2, MMP-9, prolyl hydroxylase and lysyl oxidase and activated TIMP-1 and TIMP-2 and downregulated proinflammatory cytokines. Conclusions: The present investigation indicated antimetastatic effects of *Calendula officinalis* flowers through the inhibition of key enzymes involved in processes of metastasis.

Keywords: *Clendula officinalis* flowers - lung metastasis - B16F-10 melanoma - metalloproteases - cytokines

Asian Pacific J Cancer Prev, **11**, 1773-1779

Introduction

Cancer, which is the second leading cause of death among human population is multifactorial in its origin, heterogeneous nature and characterized is by uncontrolled proliferation. The major cause of cancer death is due to the spread of malignant cells from primary site to distant target organs by a process called metastasis (Fidler, 1978).

The flowers of *Calendula officinalis* (family Astraceae) is traditionally used as antiinflammatory agent in alternative and complementary system of medicine (Basch et al., 2006). Folk medicine healers in Europe used Calendula to induce menstruation, produce sweat during fevers, and cure jaundice. Preparations of *Calendula officinalis* were also used to treat stomach ulcers, liver complaints, conjunctivitis and wounds (Chevallier, 1996). Calendula flowers are used in the form of tincture and ointments to promote the granulation and facilitate healing of skin inflammations, wounds, burns, bruises, and cuts, as well as prevent the spread of infection. The flowers are rich sources of carotenoids including lutein, lycopene and β-carotene. *Calendula officinalis* has been reported to be cytotoxic to tumor cell lines (Jiménez-Medina et al., 2006) and shown to have antitumor activity (Boucaud-Maitre et al., 1988). Other pharmacological activities reports with *Calendula officinalis* extract includes antioxidant activity (Preethi et al., 2006), wound healing (Preethi and Kuttan, 2009a), hepato and reno protective activity (Preethi and Kuttan, 2009b), antimutagenic activity (Elias et al., 1990), hypoglycemic, gastric emptying inhibitory and gastro protective activity (Yoshikawa et al., 2001). Our previous study revealed the antiinflammatory activity of *Calendula officinalis* flower extract in mouse model. It was also found that the extract administration could inhibit the cyclooxygenase-2 enzyme, which plays regulatory role in inflammation (Preethi et al., 2009). Dietary lutein present in *Calendula officinalis* extract has been found to suppress mammary tumor growth, increase tumor latency, and enhanced lymphocyte proliferation in mouse models (Chew et al., 1996).

Calendula officinalis flowers are reported to contain pharmacologically active components like coumarins, quercetin, protocatechuic acid, faradiol, oleanolic acid, betaamyrin, calenduladiol and narcissin (Matysik et

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al., 2005). Flowers are also rich in carotenoids like flavoxanthin, luteoxanthin, lycopene, auroxanthin, lutein, β-carotene etc (Kishimoto et al., 2005). The carotenoids present in Calendula flowers like lycopene has been reported to consistently reduce transcript levels of proinflammatory cytokines (Herzog et al., 2005). β-carotene, lycopene and lutein were reported to inhibit colonic aberrant crypt foci formation in rats (Narisawa et al., 1996). β-carotene has been reported to have antimetastatic potential against B16F-10 melanoma cells (Pradeep and Kuttan, 2003).

Many herbal drugs such as alcoholic extract of *Thuja occidentalis* (Sunila and Kuttan, 2006), aqueous-methanol (3:7) extract of *Boerhaavia diffusa* (Leyon et al., 2005), methanolic extract of *Withania somnifera* roots (Leyon and Kuttan, 2004), naturally occurring allyl and phenyl isothiocyanates (Manesh and Kuttan, 2003), curcumin (Menon et al., 1999), sulphorafane (Thejass and Kuttan, 2006), etc. have been reported to inhibit metastasis.

Metastases is a multi-step event which involves detachment of metastatic cells from the primary tumor, migration through blood and lymphatics, adhesion to the extracellular matrix, invasion after the proteolytic cleavage of the basement membrane and adhesion and formation of new metastatic colonies. The appearance of metastases in latter stages of neoplastic disease results in poor prognosis as the metastatic cells become resistant to the majority of drugs and treatment strategies (Condeelis et al., 2000; Engers and Gabbert, 2000). Hence there is an urge to find out newer effective non-toxic drugs to inhibit metastasis.

In the present study we have evaluated the antimetastatic activity of *Calendula officinalis* flower extract in B16F-10 melanoma cell induced metastasis in C57BL/6 mice. We have also studied the molecular mechanism of inhibition in the metastasis by regulating expression of various genes involved in metastasis.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Himedia (Mumbai, India). Fetal bovine serum (FBS) was procured from Life Technologies (Grand Island, NY). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma Chemicals (St Louis, MO). Oligonucleotide primer sequences of genes for reverse transcription polymerase chain reaction were purchased from Maxim Biotech (San Francisco, CA). Highly specific quantitative sandwich enzyme-linked immunosorbent assay kits for mouse TNF-α, IL-1β, IL-6, IL-2 and GM-CSF were purchased from Pierce Biotechnology (Rockford, IL). ELISA kits for VEGF and TIMP-1 was purchased from R & D system, USA. All other reagents and chemicals used were of analytical reagent grade.

Cell line

B16F-10 melanoma cell line was originally obtained from the National Centre for Cell Science (Pune, India). Human Umbelical Vein Endothelial Cells (HUVEC) was isolated human umbilical cord according to the protocol described by Baudin et al., (2007). The cells were maintained in DMEM and Medium 199 respectively, supplemented with 10% FCS and antibiotics at 37°C in a humidified incubator with 5% CO_2.

Animals

C57BL/6 mice (20-25g body wt, 6-8 weeks old males) were purchased from National Institute of Nutrition, Hyderabad, India. The animals were fed with mouse chow (Sai Feeds, India) and water *ad libitum*. Animal experiments were conducted after getting prior permission from Institutional Animal Ethics Committee (IAEC) and as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Preparation of Calendula flower extract

Fresh *Calendula officinalis* flower tops were used for extraction of the active components. They were collected from Government Botanical Gardens, Ooty, Nilgiris and were authenticated by Dr. S. Rajan, Field Botanist, Central Council for Research in Homeopathy, Ooty, India. The voucher specimens (no# Co05) after authentication were deposited at the herbarium of Amala Cancer Research Centre, Amala Nagar, Kerala.

Calendula flowers (700gm) were extracted with 450ml ethyl alcohol by masturation. 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. Clear liquid was decanted and the residue was pressed out through clean linen, volume was made upto 1 L with alcohol. 100mL of this tincture was evaporated to dryness in a shaker water bath at 42°C. The yield was found to be 1.1gm/100mL. Dried extract (1g) was redissolved in a known amount of distilled water and used for all experiments. The extract was administered through oral gavage at a dose of 250mg/kg body wt for 10 consecutive days. The selection of dosage for in vivo experiments was based on our previous studies (Preethi et al., 2009).

Cell viability

B16F-10 melanoma and HUVEC cells were seeded (5000 cells/well) in 96-well flat bottomed titre plate and incubated for 24 hr at 37°C in 5% CO2 atmosphere. Different concentrations of *Calendula officinalis* extract (0.5-500μg/ml water) were added and incubated further for 48 hr. Before 4 hr of completion of incubation, 20μL MTT (5 mg/ml) was added (Cole, 1986; Campling et al., 1991). Percentage of dead cells was determined using an ELISA plate reader at 570 nm.

Determination of the antimetastatic activity of Calendula officinalis flower extract in vivo

C57BL/6 mice were divided into 3 groups (12 animals/ group). Group I was kept as normal without any treatment. The animals in group II and group III were injected with B16F-10 melanoma cells ($1x10^6$cells/ animal) through lateral tail vein. Animals in group III were administered with *Calendula officinalis* flower extract through oral

gavage at a dose of 250mg/kg body wt for 10 consecutive days.

Six animals from each group were sacrificed on the 21st day of tumor inoculation. Blood was collected by heart puncture and lungs were excised out. Serum was separated from the blood and used for determining the sialic acid level (Skoza and Mohos, 1976) and γ-glutamyl transpeptidase (GGT) activity (Szasz, 1976). The level of inflammatory cytokines like TNF-α, IL-1β, IL-6, IL-2 and GM-CSF and TIMP-1 and VEGF levels were determined by ELISA method, as per manufacturer's protocol.

Morphological examinations for metastatic tumor nodules were done and a portion of the lung was used for histopathology and gene expression analysis. Estimation of collagen hydroxyproline (Bergman and Loxley, 1940), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) in lung tissue were done accordingly. Lung tissue was fixed in 10% formalin, dehydrated in different concentrations of alcohol and embedded in paraffin wax. Sections (4 μm) were stained with eosin and hematoxylin for histopathological analysis.

Total RNA was isolated from the lungs, and cDNA was synthesized using RT-PCR. Amplification was performed using specific primers of MMP-2, MMP-9, TIMP-1, TIMP-2, prolyl hydroxylase, lysyl oxidase, nm23 and proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and GM-CSF (Table 1). The amplified products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

The rest of the six animals in each group were observed for their survival. The mortality of the animals was observed everyday and the percentage increase in life span (%ILS) was calculated using the formula %ILS=T–C/C×100, where T represents the number of survival days of treated animals and C represents the number of survival days of control animals.

Statistical analysis

All data were expressed as mean ± S.D. The statistical analysis was done by one way ANOVA using Graphpad InStat version 3.00 for Windows 98, GraphPad Software, San Diego, California, USA. P value less than 0.05 was considered significant.

Results

Cytotoxic activity of Calendula officinalis towards B16F-10 melanoma and HUVEC cells by MTT assay

Cytotoxicity of *Calendula officinalis* towards B16F-10 melanoma and HUVEC cells in culture is shown in Table 2. *Calendula officinalis* was found to have a dose dependent toxicity to B16F-10 melanoma and HUVEC cells. The extract showed similar cytotoxic activity towards B16F-10 melanoma and HUVEC cells.

Effects of Calendula officinalis extract on survival of tumor bearing animals

Administration of *Calendula officinalis* extract significantly increased the life span of tumor bearing animals by 43.3% (Table 3).

Table 1. Primer Sequences of the Genes Studied

Mouse Product	Forward and Reverse Sequences	Size bp
MMP-2	F 5'-GAGTTGGCAGTGCAATACCT-3'	354
	R 5'-GCCGTCCTTCTCAAAGTTGT-3'	
MMP-9	F 5'-AGTTTGGTGTCGCGGAGCAC-3'	327
	R 5'-TACATGAGCGCTTCCGGCAC-3'	
TIMP-1	F 5'- CTGGCATCCTCTTGTTGCTA - 3'	414
	R 5'- AGGGATCTCCAGGTGCACAA - 3'	
TIMP-2	F 5'- AGACGTAGTGATCAGGGCCA - 3'	525
	R 5'- GTACCACGCGCAAGAACCAT - 3'	
nm23	F 5'-CTCAGCCTTAATTTTTTCCCCC-3'	310
	R 5'-TTAACTTCCGACACTGGGTGT-3'	
GM-CSF	F 5'-TGTGGTCTACAGCCTCTCAGCAC-3'	368
	R 5'-CAAAGGGGATATCAGTCAGAAAGGT-3'	
IL-1β	F 5'-ATGGCAACTGTTCCTGAACTCAACT-3'	563
	R 5'-CAGGACAGGTATAGATTCTTTCCTTT-3'	
IL-6	F 5'-ATGAAGTTCCTCTCTGCAAGAGAC-3'	638
	R 5'-CACTAGGTTTGCCGAGTAGATCTC-3'	
TNF-α	F 5'-ACTCCCAGAAAAGCAAGCAA-3'	688
	R 5'-TGGAAGACTCCTCCCAGGTA-3'	
LO	F 5'-CTACATCCAGGCTTCCACG-3'	283
	R 5'-TCTCCTCTGTGTGTTGGCAT-3'	
PH	F 5'-CGGGATCCTAGACCGGCTAACAAGTA-3'	317
	R 5'-GGAATTCCAAGCAGTCCTCAGCTG-3'	
GAPDH	F 5'-CGTCCCGTAGACAAAATGGT-3'	527
	R 5'-CCTTCCACAATGCCAAAGTT-3'	

LO, lysyl oxidase; PH, prolyl hydroxylase

Table 2. Cytotoxicity of *Calendula officinalis* Extract Towards B16F-10 Melanoma and HUVEC Cells

Concentration of Calendula officinalis extract (μg/ml)	Percentage cytotoxicity B16F-10 cells	Percentage cytotoxicity HUVEC cells
0.5	1.3	0
1	2.0	0.66
5	29.9	5.53
10	42.2	18.05
50	48.3	57.84
100	85.4	71.41
250	93.2	92.23
500	97.6	100

B16F-10 melanoma and HUVEC cells were incubated with different concentrations (0.5-500 μg/ml) of *Calendula officinalis* extract. % cytotoxicity was determined by MTT assay

Table 3. Effect of *Calendula officinalis* on Lung Colonization of B16F-10 Melanoma Cells and Survival

Treatment	Number of lung tumor nodules	%inhibition of nodule formation	Percentage increase in lifespan
Control	250[a]	-	-
Calendula officinalis extract (250 mg/Kg b.wt)	65.4 ± 7.57*	74%	43.29%

The lungs were dissected out and observed for metastases on the 21st day after injection of B16F-10 melanoma cells through the lateral tail vein. Treated animals simultaneously received 10 doses of *Calendula officinalis* extract. Values are mean±S.D; * p<0.001

Effects of Calendula officinalis extract on lung tumor nodule formation

Metastatic tumor bearing animals treated with *Calendula officinalis* showed significant reduction in

Table 4. Effect of *Calendula officinalis* Extract on Serum Sialic Acid Level and GGT Activity in B16F-10 Melanoma Bearing Animals

Treatment	Sialic acid (μg/ml)	GGT activity (nmol P-nitroaniline/ml)
Normal	22.68 ± 0.61	30.36 ± 5.41
Control	117.66 ± 0.80*	116.82 ± 4.85*
Calendula officinalis extract (250 mg/Kg b.wt)	31.50 ± 0.39*	54.12 ± 3.23*

The serum was collected on 21st day of tumor challenge by B16F-10 melanoma cells through lateral tail vein and assayed for serum biochemical parameters. Values are mean±S.D.* p<0.001

This elevated level was reduced to 0.98 ± 0.14mg/100 mg tissue dry wt. when *Calendula officinalis* was administered simultaneously with the tumor cells. In control metastatic tumor bearing animals, the lung uronic acid level were drastically elevated (279.8 ± 3.09μg/100 mg wet wt tissue), as compared to normal level (30.1 ± 0.89μg/100 mg wet wt tissue) which was significantly reduced with the simultaneous administration of *Calendula officinalis* (69.6 ± 3.4μg/100 mg wet wt tissue).

Effect of Calendula officinalis extract on proinflammatory cytokine, VEGF and TIMP-1 levels in metastatic tumor bearing animals

The effect of *Calendula officinalis* extract on cytokines level is given in Table 5. There was a drastic increase in the level of proinflammatory cytokines like IL-1β, IL-6, TNF-α, GM-CSF and growth factor-VEGF level in the sera of B16F10 melanoma induced animals on 7th as well as 21st day of tumor inoculation when compared to normal. This increase was found to be significantly inhibited by the administration of the Calendula extract. TIMP-1 expression level which was significantly reduced in control animals was almost normalized on 21st day in treated animals.

Effect of Calendula officinalis *extract on the expression of genes involved in metastasis*

Expression of MMP-2 and MMP-9 were markedly elevated in the lungs of control animals. This was found to be inhibited in *Calendula officinalis* treated metastatic lungs, indicating a preferential downregulation of MMP-2 and MMP-9 by the extract in B16F-10 cells (Figure 1).

The tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2 were both found to be expressed in the Calendula extract treated group whereas there was no expression of these genes in normal or control animals, which indicates the extract treatment increased the synthesis of TIMP 1 & 2. Lysyl oxidase and prolyl hydroxylase, extracellular matrix-modulating enzymes, were found to be expressed in metastatic tumor bearing animals. Treatment with *Calendula officinalis* reduced expression in lungs. The antimetastatic gene - nm23 expression was neither seen in control nor in the treated group.

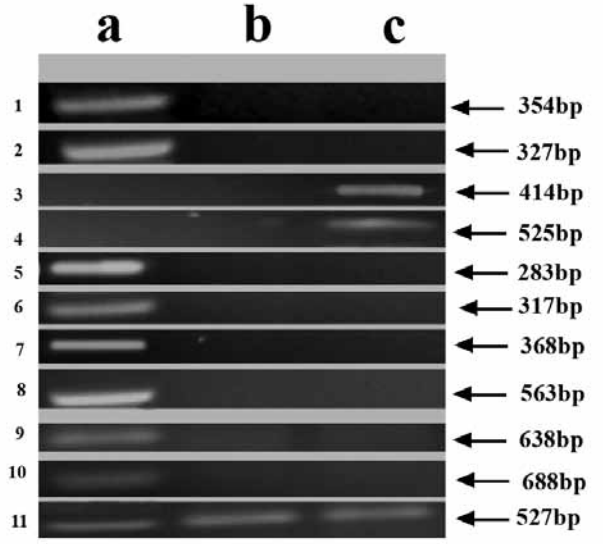

Figure 1. Gene Expression in Mouse Lungs. Lane (a) Untreated control; Lane (b) Normal; Lane (c) Treated with 250mg/kg b.wt *Calendula officinalis* extract; (1) MMP-2, (2) MMP-9, (3) TIMP-1, (4) TIMP-2, (5) Lysyl oxidase, (6) Prolyl hydroxylase, (7) GM-CSF, (8) IL-1β, (9) IL-6, (10) TNF-α, (11) GAPDH

We also evaluated the effect of *Calendula officinalis* on the expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and GM-CSF. Our result indicates that *Calendula officinalis* could inhibit the expression of these proinflammatory cytokines in metastatic lungs (Figure 1).

Histopathology

Histopathology of lung sections of control animals showed prominent tumor nodules and clear area of necrosis. Alveolar passage could not be distinguished due to the massive infiltration of neoplastic cells. Metastatic

Table 5. Effect of *Calendula officinalis* on Cytokines, VEGF and TIMP-1 Level in Metastatic Tumor Bearing Animals

	Normal	Control		Treated (*C.officinalis* 250 mg/Kg b.wt)	
		7th day	21st day	7th day	21st day
IL-1β	16.22 ± 0.35	43.50 ± 0.58*	57.72 ±1.18*	34.51 ± 0.87*	29.44 ± 1.18*
IL-6	35.64 ± 2.01	326.58 ± 4.68*	459.28 ± 4.88*	255.57 ± 4.54*	313.81 ± 3.02*
TNF-α	20.57 ± 0.65	263.62 ± 4.05*	327.56 ± 4.05*	176.12 ± 2.24*	189.21 ± 5.65*
GM-CSF	18.13 ± 0.15	38.69 ± 0.37	40.41 ± 0.15*	27.30 ± 1.59*	26.63 ± 2.35*
IL-2	23.73 ± 0.29	20.20 ± 0.18	20.47 ± 0.22	22.98 ± 0.16	23.28 ± 0.41
VEGF	15.99 ± 0.77	65.45 ± 1.39*	138.04 ± 0.45*	53.81 ± 0.65*	83.08 ± 1.64*
TIMP-1	597.24 ± 5.05	349.12 ± 2.42*	342.48 ± 2.26*	493.61 ± 3.21*	576.94 ±4.36*

C.officinalis inhibits the production of proinflammatory cytokines, VEGF and enhances TIMP-1 production. On 7th and 21st days of tumor induction serum was analysed to determine the concentrations of IL-1β, IL-6, TNF-α, GMCSF, IL-2, VEGF and TIMP-1 by quantitative enzyme-linked immunosorbent assay. Values are expressed as the mean ± SD. *p<0.001

Table 6. Effect of *Calendula officinalis* Extract on Biochemical Parameters in Lungs of B16F-10 Melanoma Bearing Animals

Treatment	Uronic acid (μg/100 mg wet wt. tissue)	Hexosamine (mg/100 mg dry wt. tissue)	Hydroxyproline (μg /mg protein)
Normal	30.10±0.89	0.44±0.09	2.45±0.18
Control	279.80±3.09*	2.05±0.13*	22.55±0.53*
Calendula officinalis	69.60 ± 3.40*	0.98±0.14*	8.89±1.44*

The lungs were dissected out and assayed different biochemical parameters on the 21st day of induction of B16F-10 melanoma cells through the lateral tail vein. Values are mean±S.D.,* p<0.001

tumor bearing animals treated with *Calendula officinalis* showed prominent reduction in the tumor mass. Alveoli and bronchioles were relatively tumor free, alveolar passage and other areas were similar to that of normal lung.

Discussion

The present study evaluated the anti-metastatic activity of *Calendula officinalis* and its molecular mechanism of action in the molecular level. B16F-10 melanoma cells are highly metastatic and form tumor cell colonies in the lungs when administered through tail vein. Initial studies indicated that Calendula extract was cytotoxic to B16F-10 cells.

In vivo analysis through various biochemical, molecular and histopathological analysis confirmed the antimetastatic potential of *Calendula officinalis*. There was significant inhibition of lung tumor colonies in the mice when treated with *Calendula officinalis* extract with significant increase in life span of tumor bearing mice. This is also reflected in the levels of sialic acid, an acetylated derivative of neuraminic acid which is often found increased in neoplasm and are shed or secreted by tumor cells and thus found to be increased in blood (Khadapkar et al., 1975; Kloppel et al., 1977). In our study, the increased sialic acid level in the control metastatic tumor bearing animals was significantly reduced in the animals treated with *Calendula officinalis*. Gamma glutamyl transpeptidase (GGT) is a cellular proliferation marker and high level of GGT was found in the serum of control metastatic tumor bearing animals. Administration of *Calendula officinalis* significantly reduced the serum GGT level.

Extracellular matrix, especially collagen is deposited massively in the alveoli of lungs during metastasis. As ten percent of collagen is hydroxyproline, lung collagen hydroxyproline content is a direct marker of lung fibrosis. Hexosamine, an integral part of many structural polysaccharides and glycosaminoglycans found in the ECM, which serves as ground substratum for collagen synthesis was also found to be significantly reduced in extract treated animals. The enhanced level of hexosamine in the control metastatic tumor bearing animals indicates the active growth and proliferation of tumor cells (West et al., 1985). Administration of *Calendula officinalis* flower extract resulted in significant reduction of hydroxyproline, uronic acid and hexosamine content in the tumor bearing animals, which indicates a reduction in lung fibrosis. This was well in correlation with histopathological analysis and significant reduction of tumor nodules in *Calendula officinalis* treated animals. This is also supported by a reduction in the expression of genes related to collagen synthesis.

Calendula officinalis could inhibit the mRNA expression of prolyl hydroxylase and lysyl oxidase, a copper- containing amine oxidase in B16F-10 melanoma bearing animals. Lysyl oxidase deaminate the side chains of lysine residues in collagen and elastin, thereby catalyzing their cross-linking in the extracellular matrix (Akiri et al., 2003). The increased expression of lysyl oxidase suffices to induce collagen accumulation and fibrosis *in vivo*. Another major enzyme that plays an important role in the biosynthesis of various types of collagen is prolyl hydoxylase (Fahling et al., 2004). It was found that *Calendula officinalis* could inhibit the expression of both these enzymes, which lightens to one of the mechanism involved in the inhibition of metastasis by the extract.

Matrix metalloproteinases are a family of zinc dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix and thereby up regulates invasion and metastasis (Stetler-stevenson et al., 1996; Chambers and Matrisian, 1997). Among the MMPs reported earlier, MMP-2 and MMP-9 are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Zucker et al., 1993; Bernhard et al., 1994). Several experiments also proved that MMPs not only break down the physical barrier of extracellular matrix but also modulates the growth factors and cytokines stored in the extracellular matrix, which may promote neoplastic progression (Voet and Voet, 1995). Our results indicate that *Calendula officinalis* could inhibit the expression of MMP-2 and MMP-9 in B16F-10 melanoma bearing animals. We also found that *Calendula officinalis* could activate the expression of tissue inhibitor of metalloproteinase TIMP-1 and TIMP-2, the inhibitors of MMP-2 and MMP-9.

In conclusion, in the present study, oral administration of *Calendula officinalis* flower extract simultaneously with tumor inoculation showed significant reduction in the number of lung colonization. The extract treatment also produced significant increase in the life span of metastatic lung tumor bearing C57BL/6 mice. This data correlated with the levels of markers such as lung hydroxyproline, major building block for collagen, and levels of the structural monosaccharides such as uronic acid and hexosamine, which are known promoter of metastasis. Also the serum gamma glutamyl transpeptidase activity, a marker of cellular proliferation, and sialic acid levels were brought back to near normal levels in the calendula extract treated animals.

Numerous studies have indicated that tumor cells exhibit an elevation in the constitutive production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and GM-CSF (Chen et al., 1999; Dong et al., 1999). Our results indicate that *Calendula officinalis* treatment could inhibit the expression of these cytokines in metastatic

lungs. More over there was a drastic reduction in the level of these cytokines in serum after treatment with the extract, which reveals the potent inhibitory effect of *C.officinalis* flower extract. The level of serum VEGF was reduced with the extract treatment. Also the production of TIMP was increased with treatment of mice with calendula extract.

The above experimental evidences clearly underline the anti-metastatic property of *Calendula officinalis* flower extract on experimentally induced metastasis in C57BL/6 mice.

Acknowledgement

The authors report no conflicts of interest.

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