

RESEARCH COMMUNICATION

Chemopreventive Potential of an Ethyl Acetate Fraction from *Curcuma Longa* is Associated with Upregulation of p57^{kip2} and Rad9 in the PC-3M Prostate Cancer Cell Line

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Abstract

Background: Turmeric (*Curcuma longa*) has been shown to possess anti-inflammatory, antioxidant and antitumor properties. However, despite the progress in research with *C. longa*, there is still a big lacuna in the information on the active principles and their molecular targets. More particularly very little is known about the role of cell cycle genes p57^{kip2} and Rad9 during chemoprevention by turmeric and its derivatives especially in prostate cancer cell lines. **Methods:** Accordingly, in this study, we have examined the antitumor effect of several extracts of *C. longa* rhizomes by successive fractionation in clonogenic assays using highly metastatic PC-3M prostate cancer cell line. **Results:** A mixture of isopropyl alcohol: acetone: water: chloroform: and methanol extract of *C. longa* showed significant bioactivity. Further partition of this extract showed that bioactivity resides in the dichloromethane soluble fraction. Column chromatography of this fraction showed presence of biological activity only in ethyl acetate eluted fraction. HPLC, UV-Vis and Mass spectra studies showed presence three curcuminoids in this fraction besides few unidentified components. **Conclusions:** From these observations it was concluded that the ethyl acetate fraction showed not only inhibition of colony forming ability of PC-3M cells but also up-regulated cell cycle genes p57^{kip2} and Rad9 and further reduced the migration and invasive ability of prostate cancer cells.

Keywords: *Curcuma longa* - ethyl acetate fraction - up-regulation - p57^{kip2} and Rad9 genes - invasive ability

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Introduction

Chemoprevention has been acknowledged as an important and practical strategy for the management of cancer. Many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents (Wattenberg, 1997; ; Krishnan et al., 1998; Rock, 1998; Vecchia & Tavani, 1998; Mukhtar & Ahmad, 1999; Aggarwal, 2008). The rhizome of the plant turmeric/ *Curcuma longa* has been used for centuries in traditional medicine and is known to have cancer preventive or therapeutic capabilities (Anand et al., 2008). Accordingly, turmeric fits very well and is an ideal candidate for chemoprevention by edible phytochemicals, which is now recognized as a plausible and cost-effective approach to reduce cancer morbidity and mortality by inhibiting precancerous events before the occurrence of clinical disease as well as for treatment of clinical disease (Thangapazham et al., 2006). However, despite the progress, still there is a big lacuna in the information on the active principles and their molecular targets of turmeric.

Prostate cancer is the second leading cause of cancer death in American men, behind only lung cancer. The American Cancer Society estimates that 28,660 men in the United States will die of Prostate Cancer in 2008 and accounts for about 9% of cancer related deaths in men (American Cancer Society, 2008). The epidemiological studies have shown significant correlations between prostate cancer incidence and dietary habits and the potential of dietary substances to act as chemopreventive agents against prostate cancer is increasingly appreciated (Syed et al., 2007; 2008). In this regard long-term well-designed and optimized intervention trials are required to delineate the potential clinical usefulness of *C. longa* both in normal populations as well as in high- risk groups and warrant further studies in the treatment and prevention of human neoplasm.

The p57^{kip2}, one of CDK inhibitors of the Cip/Kip family, shares sequence homology with p27^{kip1} and p21^{CIP1/WAF1} in the NH₂ terminal domain, which is involved in the binding to cyclin-CDK complexes (Lee et al., 1995; Matsuo et al., 1995). Human p57^{kip2} is located

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on chromosome 11p15.5, a region implicated in sporadic cancers and Beckwith-Wiedemann syndrome, a familial cancer syndrome (Matsuoka et al., 1995). The p57^{kip2} has been implicated in the modulation of many cellular events, including cell cycle control, differentiation, apoptosis, tumorigenesis, and development. Because of its chromosomal location, biochemical activities, and imprinting status, p57^{kip2} has been considered a candidate tumor suppressor gene (Zhang et al., 1997). HRad9 is an evolutionarily conserved human gene important for promoting resistance to DNA damage and regulating cell cycle checkpoints (Lieberman et al., 1996). The encoded protein can induce apoptosis (Komatsu et al., 2000), and regulate genomic stability (Hopkins et al., 2004). It has 3' to 5' exonuclease activity (Bessho et al., 2000), can bind p53 consensus DNA-binding sequences and up-regulate transcription of p21, as well as other downstream genes (Aiping et al., 2008). It can bind and stimulate activity of several DNA repair proteins involved primarily in base excision repair (Lieberman, 2006). AR can bind human Rad9 and represses androgen-induced AR transcription activity in prostate cancer cells thus regulating prostate function (Wang et al., 2004; Hsu et al., 2005). However, very little is known about the role of p57^{kip2} and Rad9 during chemoprevention by turmeric and its derivatives especially in prostate cancer cell lines.

Curcuma longa (turmeric) is known to have several components besides the major chemical component curcumin (diferuloylmethane) which may contribute to the observed beneficial effects, either alone or in combination with curcumin, although the precise identity and the role of the active fractions are yet to be elucidated. Further, it has been reported that curcumin alone is less effective than the combined in suppressing NF-KB activation (Sandu et al., 2007) suggesting that the unidentified other constituents in *C. longa* are critical for the total biological activity. However, despite the fact that the combination of compounds present in turmeric has higher efficacy than individual components, there is also a possibility of some components present in turmeric may not contribute for chemopreventive activity. Accordingly, in the present study, in order to increase the efficacy, we have made an attempt to identify the active fraction free from components non-contributory to chemoprevention by successive fractionation using different solvents, testing biological activity, identifying the components and see the effect of this active fraction on cell cycle associated genes p57^{kip2} and Rad9 as well as on invasive ability of highly metastatic PC-3M prostate cell line. Our results indicate that the fraction separated using ethyl acetate possess the biological activity, up-regulated the cell cycle associated genes and reduced invasiveness.

Materials and Methods

Cell Culture

The effect of *C. longa* fractions were studied on highly metastatic human prostate cancer cell line PC-3M and normal prostate epithelial cells (RWPE-1), both obtained from American Type Culture Collection (ATCC). The PC-

3M cells were grown in 25 cm² flasks / 60x15mm tissue culture dishes containing RPMI 1640 (GIBCO/Invitrogen) with 10% fetal bovine serum (GIBCO/Invitrogen) and antibiotics. The prostate epithelial cells were grown in 25 cm² flasks / 60x15mm tissue culture dishes containing keratinocyte Serum Free Medium (K-SFM)(GIBCO/Invitrogen) supplemented with bovine pituitary extract (BPE)(0.05 mg/ml) and epidermal growth factor (EGF) (5 ng/ml). The cells were maintained in a humidified, 95% air and 5% CO₂ atmosphere incubator at 37 °C.

Extraction, partition and column chromatography of *C. longa*

The ground powder (100g) of dried rhizomes of *C. longa* was extracted using a combination of isopropyl alcohol: acetone: water: chloroform: and methanol in the ratio of 4:4:6:3:3 for 48 h at room temperature in dark. The extract was filtered and the residue left was re-extracted with the same solvents. The filtrates were combined, concentrated under reduced pressure at low temperature (40-45 °C) in rotary vacuum evaporator. The dried extract was stored at 40 °C for further analysis. A small quantity of this extract was dissolved in dimethylsulfoxide (DMSO) and tested for biological activity and another portion of this extract was partitioned with water: dichloromethane (1:1). The aqueous layer was concentrated under vacuum and dried. The organic (dichloromethane) layer was concentrated under reduced pressure at 60 °C in rotary vacuum evaporator. Also a third interface fraction was obtained between the aqueous and organic layer. All the three fractions were dissolved in DMSO and tested for biological activity. Only the dichloromethane fraction showed significant inhibitory effect on colony formation in clonogenic assays suggesting that potential chemopreventive activity resides in dichloromethane soluble fraction. This dichloromethane fraction was subjected to column chromatography (silica gel, 200-400 mesh, column size 20 cm) and eluted successively with hexane, ethyl acetate, methanol and ethanol. All the four elutes were evaporated to dryness and dissolved in DMSO and tested for biological activity. Results indicated that only the ethyl acetate fraction showed significant inhibitory effect on colony formation in clonogenic assays and accordingly utilized for identification of the components present and for mechanism studies.

Analysis and identification of ethyl acetate fraction

A solution of the extract in dimethylsulfoxide (10 mg/ml) was diluted about 1:100 in methanol and water (v/v, 1:1). HPLC-DAD-MS analysis was performed with an Agilent 1100 liquid chromatography system consisting of an automatic injector, a gradient pump, a Hewlett-Packard series 1100 diode array detector, and an Agilent series 1100 VL on-line atmospheric pressure ionization electrospray ionization mass spectrometer. Separations were done on a C18 reversed phase column (Vydac 218TP52, 2.1 mm diameter x 250 mm; 5 µm particle size). The column was eluted at a flow rate of 0.35 ml/min with a gradient of water with 1% (v/v) formic acid (A) and acetonitrile with 1% (v/v) formic acid (B) using the following elution program: 0 min, 95% A, 5% B, 0-40 min, a linear gradient

to 30% A, 70% B, 40-45 min, a linear gradient elution to 15% A, 85% B, 45-50 min, isocratic elution at 15% A, 85% B; 50-55 min gradient elution to 95% A, 5% B, and re-equilibration with the latter solvent for 15 min. The mass spectrometer was run in the positive ion mode.

Clonogenic Assays

Clonogenic assays using logarithmically growing cells were performed as described earlier (Panandiker et al., 1992; 1994; Mahudawala et al., 1999; Nair et al., 2004; Rao et al., 2004). In brief, approximately 1000 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dishes in 5 ml of medium (five dishes per point). Twenty four hours after seeding the cells, test compounds were added at selected concentrations to the medium. Control cultures received only solvent in the place of test chemical. dimethylsulfoxide (DMSO) served as the vehicle to dissolve compounds at a final concentration of 0.4% in the culture medium, volume per volume. Preliminary experiments have shown that 0.4% volume per volume DMSO has no effect on cell survival. Dishes were returned to the incubator for up to 7 to 8 days and surviving cells were allowed to form colonies. When the colonies were discrete and well defined, the dishes were washed with PBS solution, fixed with methanol, stained with Giemsa and allowed to dry. The colonies per dish were counted using Computer based Quantity One BIO-RAD version 4.6.3.Windows and Macintosh Software. The assays were repeated two to three times and depicted for one assay. The results were the same in other assays.

RNA extraction and quantitative real-time PCR (QRT-PCR)

The total RNA from approximately 1×10^6 PC-3M cells both control and curcumin treated was extracted using RNA purification Qiagen kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was eluted, quantified by absorbance at 260 nm and stored at -80°C . Total RNA (5 μg) from all the samples were reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA). The cDNA was stored at -20°C until further analysis. Expression of mRNAs for both Rad9 (Hs00270240_m1) and p57^{kip2} (Hs00175938_m1) were measured by one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan Gene Expression Assays (Applied Biosystems) (For pre-designed assays, the manufacturer does not provide the primer and probe sequences). The probe anneals to a complementary sequence between the forward and reverse primer sites on the target. When the DNA polymerase extends the upstream primers and encounters the downstream probe, the 5' to 3' nuclease activity of the polymerase cleaves the probe and the reporter fluorophore is released into the solution and the amount of fluorescence is measured at each amplification cycle (Cunningham, 2001) using Mx3000P instrument. The PCR cycle at which fluorescence measured by the instrument reaches instrument defined threshold value (Ct or threshold cycle) was measured

to quantify the target gene. β -actin was used as an internal control and the primers used were: Forward (5' 5'-TGACGGGGTACCCACACTGTGCCCATCTA-3') Reverse (3'5- AGTCATAGTCCGCCTAGAAGCATTTG CCGT-3'). All the results were expressed as the ratio of the expression of the target gene normalized to β -actin gene in treated cells compared to the normalized expression of the target genes in untreated cells (Shively et al., 2003).

Cell Invasion Assay

The invasive activity of PC-3M prostate cancer cells and normal prostate epithelial cells in vitro was tested using a QCMTM 24-Well Collagen-Based Cell Invasion Assay kit (Chemicon International, Inc.). The invasion assay was performed using a 24-well tissue culture plate with 12 cell culture inserts. The insert contains an 8 μm pore size polycarbonate membrane coated with a thin layer of polymerized collagen. Prior to initiating assay, the plates and reagents were brought to room temperature. The collagen layer was rehydrated for 15-30 minutes at room temperature by adding 300 μl of pre-warmed serum free media to the interior of the inserts. After rehydration, 250 μl of media was carefully removed from the inserts without disturbing the collagen-coated membrane. A cell suspension containing 1.0×10^6 cells/ml from serum starved media was prepared in chemo-attractant-free media and 250 μl of prepared cell suspension was added to each insert. To the lower chamber 500 μl of media in the presence of chemo-attractant was added. Ethyl acetate extract at 5 $\mu\text{g}/\text{ml}$ was added to the experimental inserts. Controls received only solvent in the place of test chemical. Dimethylsulfoxide (DMSO) served as the vehicle to dissolve the compounds at a final concentration of 0.4% in the medium, volume per volume. The plates were covered and incubated for 72 hours at 37°C in a CO_2 incubator (5% CO_2). After the incubation period, the media was discarded and the cells were stained and inserts were dipped into a beaker of water several times to rinse. While the inserts were still moist, the non-invading cells and collagen layer were gently removed from the interior of the inserts using a cotton-tipped swab, air dried and cells that migrated through the porous membrane and adhered to the lower surface were counted. The experiments were done in quadruplicate wells.

Statistics

The results were expressed as mean \pm SD and significance was evaluated using Student's t-test (GraphPad Software). *P* values of ≤ 0.05 and less were considered significant. The final values were expressed as either per cent relative plating efficiency or per cent decrease in colony formation compared to solvent controls.

Results

Extraction, partition, column chromatography and identification of *C. longa* derivatives

Chemoprevention has been acknowledged as an important and practical strategy for the management of

cancer. Recent research provides evidence that many daily consumed dietary compounds possess cancer preventive properties. From this point of view, we have selected 13 plant materials, which are in common use either as dietary supplements or traditional medicine and extracted with a mixture of isopropyl alcohol, acetone, water, chloroform and methanol for testing the growth inhibitory effects on prostate cancer cell lines (unpublished observations). Out of 13 lyophilized plant extracts evaluated for growth inhibitory effects, *Curcuma longa*/turmeric powder extract showed significant inhibitory effect on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at 20 and 40 $\mu\text{g/ml}$ concentrations. In order to see whether whole turmeric extract is effective or only a fraction, the turmeric extract was partitioned with dichloromethane: water (1:1) as described in the Materials and Methods section. The three fractions obtained were tested for biological activity using clonogenic assays. Only the dichloromethane fraction showed major inhibitory effect on colony formation in clonogenic assays suggesting that potential chemopreventive activity resides in dichloromethane soluble fraction. The water and Interface fractions showed relatively marginal effects. To increase the efficacy, the dichloromethane fraction was further subjected to column chromatography (silica gel, 200-400 mesh column size 20 cm) and eluted

Table 1. Effect of Hexane, Ethyl Acetate, Methanol, and Ethanol Eluted Fractions from Dichloromethane Extract of *C. longa* on Highly Metastatic PC-3M Cell Line in Clonogenic Assays.

Inhibition of Colony Formation (APE \pm SD % Inhibition)				
Treatment	Hexane	Ethyl Acetate	Methanol	Ethanol
($\mu\text{g/ml}$)				
DMSO	354 \pm 13.8	432 \pm 23.6	432 \pm 23.6	432 \pm 23.6
0.625	393 \pm 4.8	397 \pm 15.1 ^a	8	502 \pm 18.2
1.25	391 \pm 27.6	371 \pm 42.5 ^a	14	492 \pm 25.5
2.5	381 \pm 29.2	319 \pm 37.2 ^b	26	474 \pm 24.3
5	360 \pm 21.8	164 \pm 22.7 ^c	62	472 \pm 35.9
10	349 \pm 21.4	14 \pm 4.7 ^c	97	440 \pm 22.5
				517 \pm 20.6

* ^aAPE-Absolute Plating Efficiency. ^ap < 0.022; ^bp < 0.0004; ^cp < 0.0001

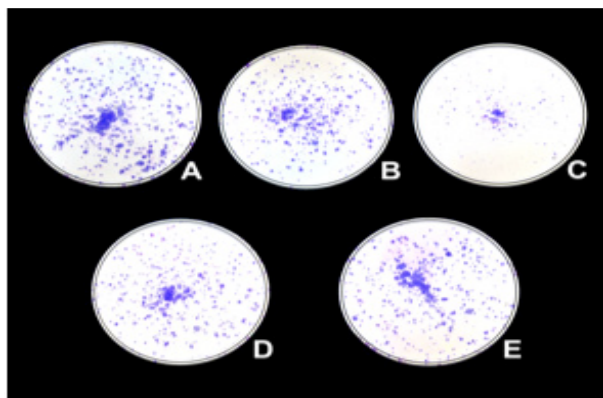


Figure 1. Representative Petri Dishes Showing the Effect of the Purified Fractions from *C. longa* on Highly Metastatic PC-3M Prostate Cancer Cell Line in Clonogenic Assays. A. DMSO control; B. Hexane fraction; C. Ethyl acetate fraction; D. Methanol fraction; E. Ethanol fraction.

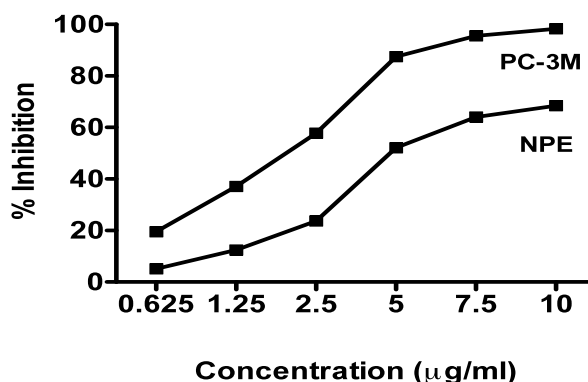


Figure 2. Effect of Ethyl Acetate Fraction from *C. longa* on Highly Metastatic PC-3M Cell Line and Normal Prostate Epithelial Cells in Clonogenic Assays.

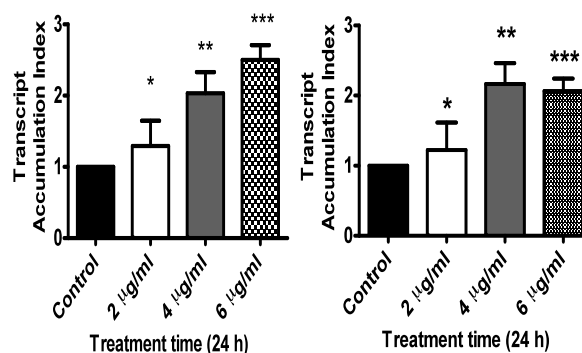


Figure 3. Effect of Ethyl Acetate Fraction from *C. longa* on p57^{kip2} and Rad9 Expression in Highly Metastatic PC-3M cell line. * Not significant; **p<0.02; ***p<0.009

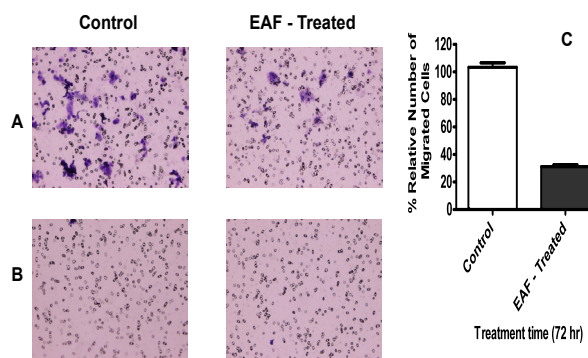


Figure 4. Effect of Ethyl Acetate Fraction from *C. longa* on the Invasive Ability of Highly Metastatic PC-3M Cell Line and Normal Prostate Epithelial Cells (x100). A. PC-3M cells; B. Normal prostate epithelial cells; C. % Relative number of migrated cells in PC-3M cell line. p<0.0002

successively with hexane, ethyl acetate, methanol and ethanol. All the four elutes were evaporated to dryness and dissolved in DMSO and tested for biological activity. Results showed that only the ethyl acetate eluted fraction showed significant inhibitory effect on colony formation in clonogenic assays (Table 1 and Figure 1). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. The HPLC profile of the ethyl acetate fraction showed that the main components present are curcuminoids (curcumin,

demethoxycurcumin, and bisdesmethoxycurcumin) (Rao et al., 2011). The UV-Vis and mass spectra of peaks 1 and 2 further confirmed that peak 1 consists of a mixture of curcumin and demethoxycurcumin and that peak 2 contains bisdesmethoxycurcumin (Rao et al., 2011). Besides main components of all curcuminoids, presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction. Further studies are required to identify these components and their biological significance.

Effect of Ethyl acetate Fraction on PC-3M and Normal Prostate Epithelial Cells

The ethyl acetate fraction showed significant inhibitory effects on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at different concentrations tested (Figure 2). On the normal prostate epithelial cells ethyl acetate fraction showed similar effects but magnitude was less.

Up-regulation of p57^{kip2} and Rad9 genes

Data presented in Figures 3 show the dose dependent effect of ethyl acetate fraction on p57^{kip2} and Rad9 gene expression in PC-3M cell line. PC-3M cells were treated with ethyl acetate fraction at different concentrations for 24 h. RNA was extracted and reverse transcribed and both p57^{kip2} and Rad9 gene expression was quantified by real time PCR using β -actin as a house keeping gene. PC-3M cells treated with ethyl acetate fraction both at 4 and 6 μ g/ml for 24 h showed significant up-regulation of about two fold higher the amount of both p57^{kip2} and Rad9 genes when compared to corresponding controls. No significant difference in gene expression was observed at 2 μ g/ml concentrations for both the genes (Figure 3).

Up-regulation of p57^{kip2} and Rad9 is associated with decreased the invasive ability

To understand functional significance of up-regulation of both p57^{kip2} and Rad9 during chemoprevention by ethyl acetate fraction, Collagen-Based Cell Invasion Assay was performed. The ability of cells to invade collagen and migrate to the underside of the inserts was determined by a 72-hour response to medium containing chemoattractant i.e. 10% FBS in the lower chamber. In the presence of ethyl acetate fraction, significantly fewer cells migrated to the underside compared with the absence of ethyl acetate fraction (Figure 4) suggesting that both p57^{kip2} and Rad9 expression is associated with the reduced migration and invasive ability of prostate cancer cells. No invasive ability was observed either in the presence or absence of ethyl acetate fraction in normal prostate epithelial cells.

Discussion

Curcuma longa or turmeric, a widely cultivated tropical plant has been used since ancient times as a spice, as a beauty care agent and therapeutically for a wide range of ailments and as well as in traditional medicine (Kunnumakkara et al., 2008). Turmeric is traditionally known as a blood purifier and is reported to be useful for the common cold, intermittent fevers, afflictions of the

liver, indolent ulcer and wound healing. It has been found to possess anti-inflammatory and antioxidant activities and chemopreventive activity for a wide variety of cancers like colon, breast, prostate, esophagus, lung, oral and has potential as an antiviral and antibacterial agent (Mazumder et al., 1995; Anto et al., 1996; Aggarwal et al., 2003; Dorai & Aggarwal, 2004; Duvoix et al., 2005; Olszanecki et al., 2005; Park et al., 2005; Alpers, 2008). Recently Aggarwal and co-workers using a commercially available curcumin mix reported that different analogs of curcumin present in turmeric showed variable anti-inflammatory and anti-proliferative activities (Sandur et al., 2007). Curcumin or diferuloylmethane, a major component present in turmeric is a powerful antioxidant and is linked with the suppression of mutagenesis, inhibited nuclear factor- κ B (NF- κ B) activation, suppressed cyclin D1 and anti-apoptotic gene products, induced cytochrome C release, activated caspases and have anti-angiogenic effects through down-regulation of vascular endothelial growth factor (VEGF) (Choudhuri et al., 2005; Aggarwal et al., 2006; Aggarwal et al., 2007; Liu et al., 2007; Shankar & Srivastava, 2007). Curcumin is currently in clinical trials for treatment of various cancers (Sharma et al., 2004; Garcea et al., 2005; Dhillon et al., 2006; Rafailov et al., 2007) and for Alzheimer's disease (Yang et al., 2005).

In the present study *Curcuma longa*/turmeric powder extract showed significant inhibitory effect on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line. Further, partition and fractionation studies showed that only the ethyl acetate eluted fraction possessed significant inhibitory effect on colony formation in clonogenic assays (Table 1 and Figure 1). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. HPLC, UV-Vis and mass spectra analysis showed the presence of three main curcuminoids namely curcumin, demethoxycurcumin, and bisdesmethoxycurcumin in ethyl acetate fraction (Rao et al., 2011). Besides main components of all curcuminoids, the presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction. Further studies are required to identify these components and their biological significance. In order to see a possible differential effect of the ethyl acetate fraction on PC-3M and normal prostate epithelial Cells, the effects were studied on both the cells. The ethyl acetate fraction showed significant inhibitory effects on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at different concentrations tested (Figure 2). The ethyl acetate fraction showed similar effects on normal prostate epithelial cells but magnitude was less (Figure 2).

Over-expression of p57^{kip2} has been shown to cause cell growth arrest and senescent phenotype in many cell types (Jin et al., 2004; Williams et al., 2005). P57^{kip2} has been considered a candidate tumor suppressor gene due to its location in the genome, biochemical activities, and imprinting status (Jin et al., 2008). Previously, many studies have focused on p21^{CIP1/WAF1} or p27^{kip1} whereas little is known about the biological function of p57^{kip2} during tumorigenesis and cancer progression (Jin et al., 2008). Recently, it has been shown that the expression of p57^{kip2}

is significantly decreased in human prostate cancer (Jin et al., 2008) and the over-expression of p57^{kip2} in prostate cancer cells significantly suppressed cell proliferation and reduced invasive ability (Jin et al., 2008). Further, in LNCaP cells, over-expression of p57^{kip2} inhibited tumor formation in nude mice and the prostate of p57^{kip2} knockout mice developed prostatic intraepithelial neoplasia and adenocarcinoma suggesting that p57^{kip2} is an important gene in prostate cancer tumorigenesis, and the p57^{kip2} pathway may be a potential target for prostate cancer prevention and therapy, more specifically, during turmeric chemoprevention (Jin et al., 2004; 2008; Williams et al., 2005). The Rad9 gene has many functions that could bear on carcinogenesis, including a role in maintaining genome integrity and regulating cell cycle checkpoints (Lieberman et al., 1996; Bessho & Sancar, 2000; Komatsu et al., 2000; Hopkins et al., 2004; Aiping et al., 2008). Rad9 protein can bind AR and this protein-protein interaction represses the ability of testosterone to induce a conformational change in the receptor, to activate a receptor transcription regulatory function, and subsequently to express downstream target genes critical for proper prostate function (Wang et al., 2004; Hsu et al., 2005; Lieberman, 2006). In our studies, PC-3M cells treated with ethyl acetate fraction both at 4 and 6 µg/ml for 24 h showed significant up-regulation of about two fold higher the amount of both p57^{kip2} and Rad9 genes when compared to corresponding controls (Figures 3). No significant difference in gene expression was observed at 2 µg/ml concentration for both the genes (Figures 3). Therefore, these results suggest that up-regulation of both p57^{kip2} and Rad9 genes are associated with inhibition of cell proliferation and accordingly reduced colony formation in cells treated with ethyl acetate fraction. P57^{kip2} has important functions, such as binding to proliferating cell nuclear antigen to prevent DNA replication and inhibit cell transformation (Tsugu et al., 2000). It is also involved in glucocorticoid-induced anti-proliferation (Samuelsson et al., 1999). Recently, it has been shown that over-expression of p57^{kip2} in prostate cancer cells significantly suppressed the cell proliferation and arrested the cell cycle at G0-G1 stage (Jin et al., 2008). Studies are in progress to see whether similar mechanisms are operative during PC-3M cell exposure to ethyl acetate fraction.

Invasion through the extracellular matrix is an important step in tumor metastasis. By releasing proteolytic enzymes such as MMPs and collagenases, cancer cells are able to breach the membrane and penetrate the blood vessel wall and invade (Albini, 1998). Collagen, the primary structural element of the basement membrane and tissue scaffolding protein, represents the main deterrent in the migration of tumor cells. The ability to study cell invasion through a collagen barrier, is of vital importance for developing possible metastatic inhibitors and therapeutics. The objective of our study is to see whether up-regulation of both p57^{kip2} and Rad9 genes in PC-3M cells treated with ethyl acetate is associated with a decrease in the invasive potential of the tumor cells. Consistent with the known metastatic potential, the untreated PC-3M cells were relatively more invasive compared to the cells treated with ethyl acetate fraction.

In the presence of ethyl acetate fraction, very few cells migrated to the underside of the insert compared to the cells in the absence of ethyl acetate fraction (Figure 4). These results indicate that inhibition of colony forming ability of PC-3M cells by ethyl acetate fraction is not only associated with up-regulation of p57^{kip2} and Rad9 but also reduced the migration and invasive ability of prostate cancer cells. Normal prostate epithelial cells were not invasive at all either in the presence or absence of ethyl acetate fraction (Figure 4). It is known that invasiveness of cells through a gel of basement membrane proteins correlates well with metastatic potential *in vivo* (Dedhar et al., 1993; Albini, 1998).

Acknowledgments

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