Proteomics Profiling and Cytotoxic Effect of *Curcuma longa* on Prostate Cancer

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Abstract: Turmeric (*Curcuma longa*) has been shown to possess anti-inflammatory, antioxidant and antitumor properties. Extraction, partition and column chromatography of the dry powder of *C. longa* rhizomes showed presence of biological activity only in ethyl acetate eluted fraction in clonogenic assays using highly metastatic PC-3M prostate cancer cell line. HPLC, UV-Vis and Mass spectra studies showed presence of three curcuminoids in this fraction. Accordingly, we have made an attempt to identify the proteins modulated by purified turmeric fraction in PC-3M human prostate cancer cell line using high-resolution two-dimensional gel electrophoresis (2-DE). Isoelectric focusing and 2-DE analysis showed a total of 29 proteins altered by treatment with ethyl acetate fraction (EAF) of *C. longa*. Out of 29 differentially expressed proteins, 15 were identified by peptide mass fingerprinting through LC/MS/MS sequencing. From these, a total of 7 were down-regulated and 8 were up-regulated spots. The down-regulated proteins identified by Peptide Mass Fingerprinting are Elongation Factor 2 (eEF2), Stress-induced phosphoprotein 1, Glutathione S-transferase (GST) Omega-1, Parvalbumin alpha, Succinyl-CoA: ketoacid, Lamin-A/C and Annexin A2. The up-regulated proteins identified by Peptide Mass Fingerprinting include 78 kDa Glucose-regulated protein precursor (GRP78), Protein disulfide isomerase (PDI) precursor, Actin cytoplasmic 2, protein SET, Calreticulin precursor, Nucleophosmin, Vimentin, and Aortic alpha-actin (ACTA2). The identified proteins have diverse cellular functions such as ER stress, Unfolded Protein Response (UPR), cytoskeletal, structural, regulatory, and apoptotic proteins that will require further in-depth studies to understand the biological significance.

Keywords: *Curcuma longa*, ethyl acetate fraction, curcuminoids, proteomics, 2-DE, LC/MS/MS sequencing.

INTRODUCTION

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 [1]. It has been shown to suppress multiple signaling pathways and inhibit cell proliferation, invasion, metastasis, and angiogenesis [2-4]. Its safety combined with its low cost, and multiple targeting potential makes turmeric an ideal agent to be explored for prevention and treatment of various cancers and fits very well as a candidate for chemoprevention by edible phytochemicals [5, 6]. However, despite the progress, still there is a big lacuna in the information on the active principles and the molecular targets of turmeric.

Curcuma longa (C. longa) is known to have several components that may contribute to the observed beneficial effects besides the major chemical component curcumin (diferuloylmethane) either alone or in combination, 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 [7] 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 *C. longa* has higher efficacy than individual components, there is also a possibility of some components present in *C. longa* may not contribute for chemopreventive activity. In our earlier studies, in order to increase the efficacy, an active fraction free from components non-contributory to chemopreventive activity was isolated by successive fractionation using different solvents and column chromatography. The fraction eluted with ethyl acetate (EAF) containing mainly curcuminoids (curcumin, demethoxycurcumin, and bisdesmethoxycurcumin) possessed the biological activity, up-regulated the cell cycle associated genes p57kip2 and Rad9 and reduced invasive ability of highly metastatic PC-3M prostate cell line (unpublished data).

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

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United States will die of prostate cancer in 2008 and accounts for about 9% of cancer related deaths in men [8]. Although numerous proteome studies have been performed recently to identify cancer-related changes in protein expression, it is still not clear how these proteins are modulated during chemoprevention. Accordingly, we have made an attempt to identify the proteins modulated by purified *C. longa* fraction in PC-3M human prostate cancer cell line using high-resolution two-dimensional gel electrophoresis (2-DE). Proteomic technologies have been used extensively hitherto to identify proteins that are useful for cancer diagnosis, progression, and therapeutic targets and how their use in chemoprevention will contribute to the deeper understanding of underlying mechanisms during human cancer prevention.

MATERIALS AND METHODS

Cell Culture

The effect of purified C. longa fraction was studied on highly metastatic human prostate cancer cell line PC-3M obtained from American Type Culture Collection (ATCC). The 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 cells were maintained in a humidified, 95% air and 5% CO_2 atmosphere incubator at 37°C.

Clonogenic Assays

Clonogenic assays using logarithmically growing cells were performed according to the published methods [9-13]. In brief, approximately 400 cells obtained from subconfluent culture flasks were seeded per 60 mm tissue culture dishes in 5 ml of medium (five dishes per point). Cells were allowed to settle after 24h and test compounds were added at two different concentrations. Control cultures received only solvent in the place of test chemical. Dimethyl sulphoxide (DMSO) served as the vehicle to solubilize 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 number of colonies per dish was counted using computer based Gene tools software (Syngene, MD, USA). The assays were repeated three independent experiments.

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 (40-45°C) temperature in rotary vacuum evaporator. The dried extract was stored at -20°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 (EAF) 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 EAF

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.

Treatment of Cells with EAF

Logarithmically growing cells obtained from subconfluent culture flasks were seeded at 3 x 10^5 for each 25 cm2 flask in 5 ml of medium (five flasks per point). Twenty-four hours after seeding the cells ethyl acetate purified fraction was added at a selected concentration of 5 μg / ml to the medium. Control cultures received only solvent in the place of test chemical. Dimethyl sulphoxide (DMSO) served as the vehicle to solubilize compounds at a final concentration of 0.4% in the culture medium, volume per volume. Flasks were further incubated for 24 h and after that washed with PBS solution and cells were harvested using Trypsin/EDTA solution (ScienceCell Laboratories) and utilized for 2D and MS analysis. The analysis was repeated three times and depicted for one assay. The results were the same in all three analyses.

High-Resolution Two-Dimensional Gel Electrophoresis

One hundred and fifty micrograms of protein from cell lysates were solubilized for 30 min with 2D rehydration/ sample buffer (7 M urea, 2 M thiourea, 1% ASB-14, 40 mM

Tris) and 2% Bio-Rad IPG buffer, pH 3–10. Bio-Rad 11 cm ReadyStrip pH 3–10NL IPG strips were used to separate proteins according to charge. Solubilized proteins were adsorbed into the gel strip overnight and were then focused according to their isoelectric point with the Bio-Rad Protean IEF System. The program used was the following: 250 V rapid voltage ramping for 30 min, 10,000 V slow voltage ramping for 60 min, and 10,000 V rapid voltage ramping for 50 kV hours. The strips were incubated first in equilibration buffer I with 6 M urea, 20% glycerol, 2% SDS, 2% DTT, and 0.375 M Tris, pH 8.8, for 10 min at room temperature, then in equilibration buffer II with 6 M urea, 20% glycerol, 2% SDS, 2% iodoacetamide, and 0.375 M Tris, pH 8.8. The strips were then loaded onto 6-18% SDS-PAGE gels on 13.3 X 8.7 cm and run at 50 V overnight to complete the second dimension of protein separation. ReadyPrep Overlay Agarose was added on top of the strip to secure it and included bromophenol blue tracking dye. A molecular standard was used to estimate relative mass (Mr). Gels were pre-rinsed with water, then stained overnight with Bio-Safe Coomassie Stain as stated in manufacturers protocol. Gels were destained in water, and scanned with the Versadoc Model 1000 system (Bio-Rad, CA). Gel image analyses were performed with PD Quest software (Bio-Rad) version 7.4.0. Individual spot volumes for each gel were normalized relative to the total spot volume of that gel. Normalized spot volume data from each experimental set were analyzed the Student's t-test ($P < 0.05$ was regarded as significant). Spots ≥2-fold higher/lower were considered to be differentially regulated. All experiments were performed in biological and technical triplicates starting with the collection of cells.

Protein identification by Peptide Mass Fingerprinting (PMF)

Protein spots were excised and tryptic digests were analyzed at the University of Texas Medical Branch Proteomics Core Facility by LC-MS/MS. Briefly, protein spots from 2D gel were destained with 50% acetonitrile in 50 mM ammonium carbonate. In-gel tryptic digest was performed using reductively methylated trypsin (Promega, Madison, WI). Before digestion, samples were reduced with DTT (10 mM in 50 mM ammonium carbonate for 60 min at 56°C) and subsequently alkylated with iodoacetamide (55 mM in 50 mM ammonium carbonate for 45 min in the dark at room temperature). The digestion reaction was performed overnight at 37°C. Digestion products were extracted from the gel with a 5% formic acid/50% acetonitrile solution (2x) and one acetonitrile extraction followed by evaporation using an APD SpeedVac (ThermoSavant). The dried tryptic digest samples were cleaned with ZipTip (CB18B; Millipore) before analysis by tandem mass spectrometry for protein identification. The digested sample was resuspended in 10 µl of 60% acetic acid, injected *via* autosample (Surveyor; ThermoFinnigan, San Jose, CA) and subjected to reverse phase liquid chromatography using ThermoFinnigan Surveyor MS-Pump in conjunction with a BioBasic-18 100 x 0.18 mm reverse-phase capillary column (ThermoFinnigan). Mass analysis was done using a ThermoFinnigan LCQ Deca XP Plusion trap mass spectrometer equipped with a nanospray ion source using a 4.5 cm long metal needle (Hamilton; 950–00954) in a data-dependent acquisition mode. Electrical contact and voltage application to the probe tip took place *via* the nanoprobe assembly. Spray voltage of the mass spectrometer was set to 2.9 kV and heated capillary temperature at 190°C. The column was equilibrated for 5 min at 1.5 µl/min with 95% solution A and 5% solution B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) before sample injection. A linear gradient was initiated 5 min after sample injection ramping to 35% A and 65% B after 50 min and 20% A and 80% B after 60 min. Mass spectra were acquired in the mass-to-charge ratio 400–1800 range. Protein identification was performed with the MS/MS search software Mascot 1.9 (Matrix Science, Boston, MA) with confirmatory or complementary analyses with TurboSequest as implemented in the Bioworks Browser 3.2, build 41 (ThermoFinnigan).

Western Blot Analysis

To validate the differentially expressed proteins, two down-regulated proteins namely elongation factor 2 (eEF2), GST-Omega-1 and two up-regulated protein disulfide isomerase (PDI) and GRP78 were selected for Western blotting analysis in PC-3M cells treated with EAF at 5 µg/ml. Cell pellets were lysed by lysis buffer (Pierce, IL) with 1x complete cocktail of protease inhibitors. Total cellular protein in equal amounts were resolved by 4-15% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and were incubated with primary rabbit polyclonal eEF2, PDI and GRP78 antibodies, and mouse monoclonal glutathione S-transferase Omega 1 antibody, β-actin antibody followed by corresponding secondary goat anti-rabbit and goat anti-mouse IgG antibodies respectively. Immunoreactive bands were visualized using a chemiluminescence's Western blotting system according to the manufacturers' instructions (Amersham).

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. 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 dichloro-

methane fraction was further 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 showed that only the ethyl acetate eluted fraction showed significant inhibitory effect on colony formation in clonogenic assays (Fig. **1**). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. Fig. (**2**) shows the HPLC profile of the ethyl acetate fraction showing that the main components present are curcuminoids (curcumin, demethoxycurcumin, and bisdesmethoxycurcumin). Fig. (**3**) shows the UV-Vis and mass spectra of peaks 1 and 2 from Fig. (**1**) confirming that peak 1 consists of a mixture of curcumin and demethoxycurcumin and that peak 2 contains bisdemethoxycurcumin. Besides main components of all curcuminoids, presence of some minor components was also observed (Fig. **4**) in the ethyl acetate fraction. Further studies are required to identify these components and their biological significance.

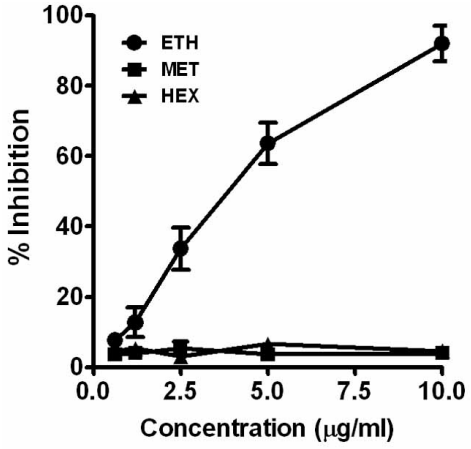


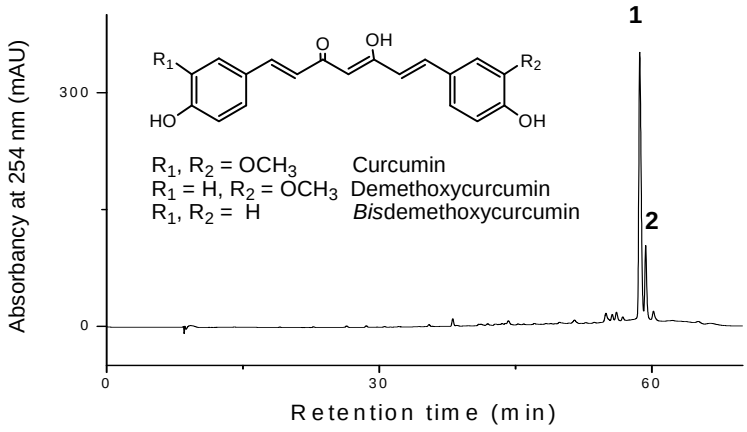
Fig. (1). Effect of the purified fractions from C. longa on highly metastatic PC-3M prostate cancer cell line in clonogenic assays. EA- Ethyl acetate fraction; Hex- Hexane fraction; Met- Methanol fraction; Eth- Ethanol fraction.

Proteomics Profiling of PC-3M Cells Treated with EAF

Solubilized cell lysates from solvent treated control cells and cells treated with ethyl acetate fraction were adsorbed into the gel strip overnight and were then focused according to their isoelectric point with the Bio-Rad Protean IEF System and separated by agarose 2-DE, and proteins were visualized by Bio-Safe Coomassie Stain. Both the samples were examined in triplicate biological and technical experiments, and a total of 29 spots altered by treatment with *C. longa*. Out of 29 differentially expressed proteins 15 were identified by PMF through LC/MS/MS sequencing. From these, a total of 7 were down-regulated and 8 were up-regulated spots. (Fig. **5** and Table **1**).

Identification of EAF-modulated Protein Fingerprints Markers from PC-3M cells

A total of 15 differentially expressed spots from *C. longa* treated cells were identified by mass spectrometry through peptide mass fingerprinting. The significantly differentially expressed proteins were selected and marked in the master gel image displayed in Fig. (**5**). These spots were manually excised from Coomassie blue-stained gels, subjected to in-gel trypsin digestion, and further processed for LC/MS/MS sequencing. A list of sequence–identified proteins is presented in Table **1**. The identified proteins have diverse cellular functions such as ER stress, cytoskeleton, structural, regulation, and apoptotic and proteins. All experiments were performed in biological and technical triplicates starting with the collection of cells. 29 protein spots met the criterion for a 2-fold or greater change in expression relative to control. Of these, 16 proteins were increased in expression in prostate cancer cells, as measured *via* Bio-Safe Coomassie Stain and imaging analyses. Conversely, 13 spots in the prostate cancer cell group were identified as having decreased expression relative to control. For identification by LC-MS/MS peptide sequencing, 15 differentially expressed spots that were significant at $p < 0.05$ were selected from 2 categories: (i) in prostate cancer EAF-induced up-regulation (8 spots) and (ii) prostate cancer EAF-induced down-regulation (7 spots),



Peak	Retention Time (min)	UV max (nm)	Mass $[M+H]^+$ (Da)	Possible compound [M]
1	58.7	426	369, 339	Curcumin [368] + Demethoxycurcumin [338]
2	59.3	418	309	*Bis*demethoxycurcumin [308]

Fig. (2). HPLC profile of the ethyl acetate fraction from *C. longa*, showing that the main components present in the extract are curcuminoids (peaks 1 and 2). UV-Visible and mass spectral data are given in the Table.

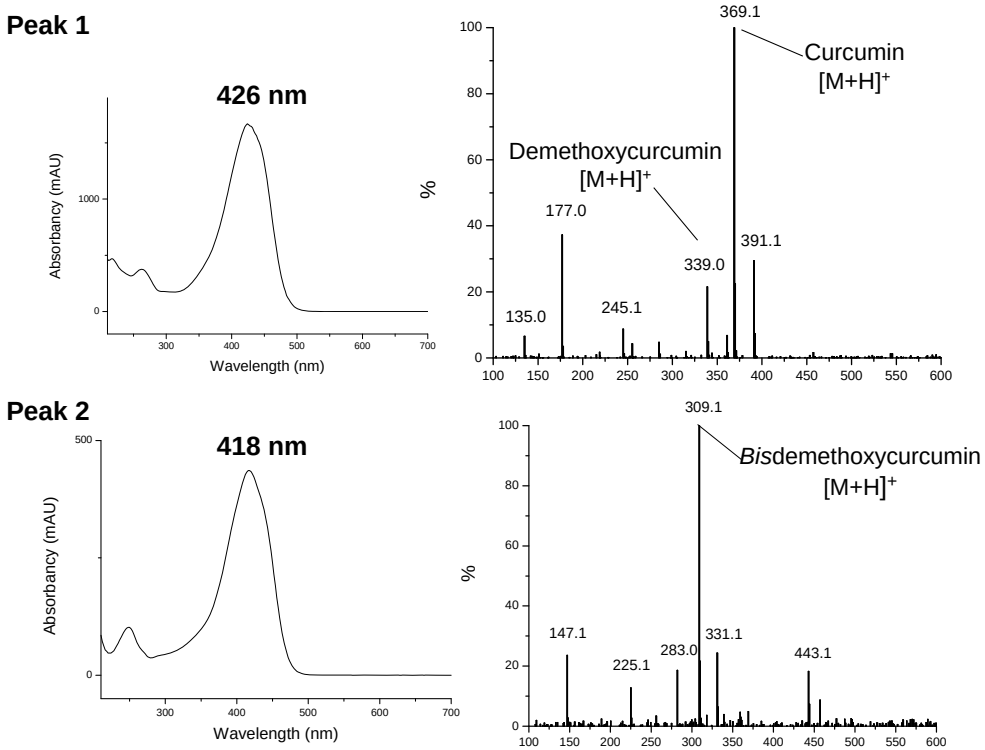
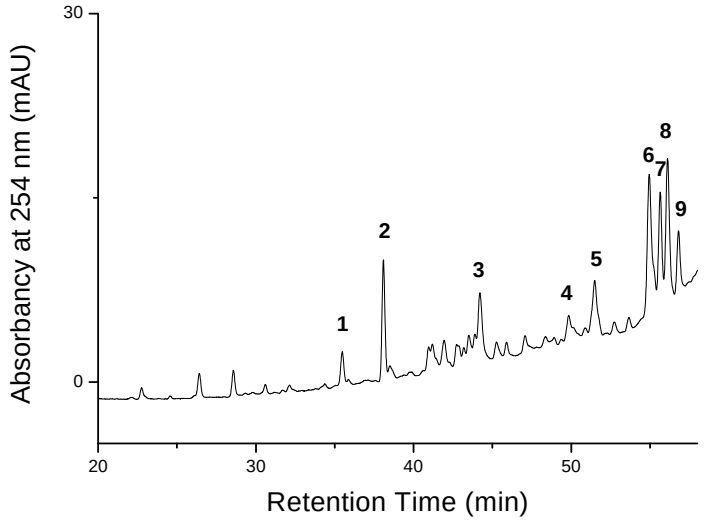


Fig. (3). UV - Vis and mass spectra of peaks 1 and 2 from Fig. (**1**). These data confirm that peak 1 consists of a mixture of curcumin and demethoxycurcumin and that peak 2 contains bisdemethoxycurcumin.



Peak	R.T. (min)	UV max (nm)	Mass $[M+H]^+$ (Da)
1	35.5	338	275
2	38.1	242	275
3	44.2	348	391
4	49.8	390	393
5	51.5	236	257
6	54.9	366	257
7	55.6	372	371
8	56.1	370	273
9	56.8	238	255

Fig. (4). Zoomed HPLC profile of the minor peaks in Fig. (**2**), along with UV - Visible and mass spectral data. The structures of these components cannot be ascertained from these data alone and further studies are required.

(Fig. **5** and Table **1**). The up-regulated proteins identified by peptide mass finger printing (PMF) include 78 kDa glucose-regulated protein precursor (GRP78), protein disulfide isomerase (PDI) precursor, Actin cytoplasmic 2, protein SET, Calreticulin precursor, Nucleophosmin, Vimentin, and Aortic alpha-actin (ACTA2). The down-regulated proteins identified by PMF include Elongation Factor 2, Stress-induced phosphoprotein 1, Glutathione S-transferase Omega-1, Parvalbumin alpha, Succinyl-CoA: ketoacid, Lamin-A/C and Annexin A2 (Fig. **6A** and **B**).

Validation of the Differentially Expressed eEF2, GST-Omega-1 and PDI, Proteins by Western Blot Analysis

The quantitative estimation of altered expression levels of EAF-modulated proteins in PC-3M cells was evaluated by PDQuest Software and validated by Western blot analysis.

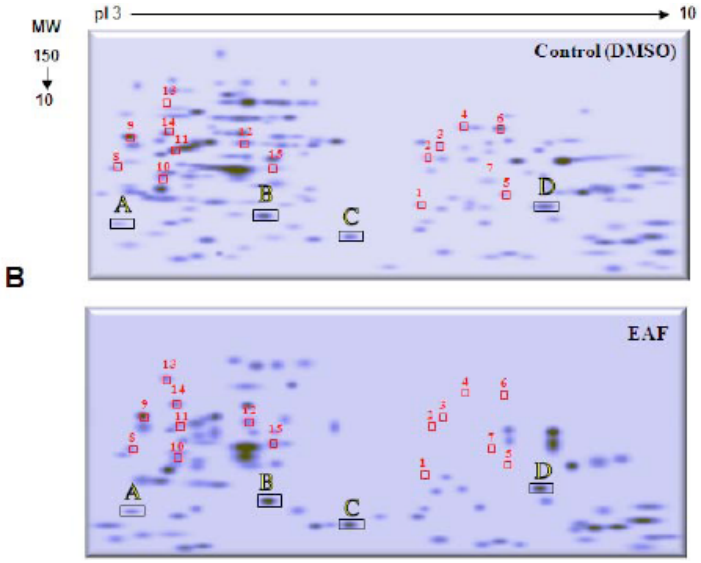


Fig. (5). Differentially expressed human prostate cancer cell line PC-3M proteins identified by peptide mass fingerprinting are indicated with solid red squares in the PC-3M cells control 2D gel image and in the PC-3M cells treated with Curcuma longa (EAF) gel image. The standard common proteins are indicated with black squares and letters.

Table 1. Proteins Differentially Expressed in Human Prostate Cancer Cell Line PC-3M Treated with *Curcuma longa* (EAF) and Identified by Peptide Mass Fingerprinting

Spot #	Protein Name	Accession Number	[1]MW [2]Exp./ [3]Theor.	Calc. [4]pI Exp./Theor.	[5]Score	[6]P	[7]Fc	[8]E
1	Glutathione transferase omega-1	P78417	22.85/27.83	6.91/6.23	282	9	--	↓
2	Parvalbumin alpha	P20472	36.05/12.05	7.02/4.98	77	8	--	↓
3	Succinyl-CoA: 3-ketoacid	P55809	41.57/56.57	7.16/7.14	98	12	--	↓
4	Lamin-A/C	P02545	52.26/74.37/	7.45/6.57	217	16	--	↓
5	Annexin A2	P07355	25.07/38.80	7.96/7.57	1,080	18	--	↓
6	Elongation factor 2	P13639	64.01/96.24	7.76/6.41	435	19	--	↓
7	Stress-induced-phosphoprotein 1	P31948	51.54/63.22	7.91/6.4	269	22	--	↓
8	Protein SET	Q01105	31.38/33.46	3.72/4.23	268	6	3.1	↑
9	Calreticulin precursor	NP004334	42.07/48.28	3.84/4.29	793	17	2.4	↑
10	Nucleophosmin	P06748	29.12/32.72	4.18/4.64	340	8	7.6	↑
11	Vimentin	P08670	38.65/53.67	5.06/4.19	632	24	3.0	↑
12	Actin cytoplasmic 2	P63261	19.35/42.10	5.09/5.31	684	16	4.3	↑
13	78 kDa glucose-regulated protein precursor	NP005338	59.83/72.40	4.07/5.07	1130	32	3.2	↑
14	Protein disulfide isomerase precursor	P07237	46.97/57.47	4.18/4.76	581	26	2.1	↑
15	Aortic, actin smooth muscle	NP001135417	39.85/42.38	5.14/5.23	343	11	2.0	↑

1MW: Molecular Weight (kDa), 2Exp: Experimental, 3Theor: Theoretical, 4pI: Isoelectric Point, Number, 5MS/MS Score: Tandem Mass Spectromety, 6P: Peptide, 7Fc: Fold Change 8E: Expression: ↓: Down-regulated or ↑: Up-regulated, --: No present in Treatment.

We observed the over expression of GST-Omega-1, the ER stress markers: PDI and GRP78, which have been reported in breast cancer [14] and eEF2, which is a key protein involved in the initiation of the unfolded protein response (UPR) [15]. We speculate that increased expression of PDI could be a biomarker of anti-cancer. To confirm the altered expression of two selected proteins namely eEF2 and PDI, we used Western blotting in PC-3M cells treated with EAF at 5 µg/ml. As observed in Fig. (**5**), there was a general concordance between the quantitative estimation of (2D-GE) images and Western blot analysis of the four selected proteins. Data presented in Fig. (**7**) shows that eEF2 and GST-Omega-1 are down-regulated and PDI and GRP78 are up-regulated in PC-3M cells treated with EAF at 5µg/ml compared to solvent treated controls corroborating the observations with 2D-GE images.

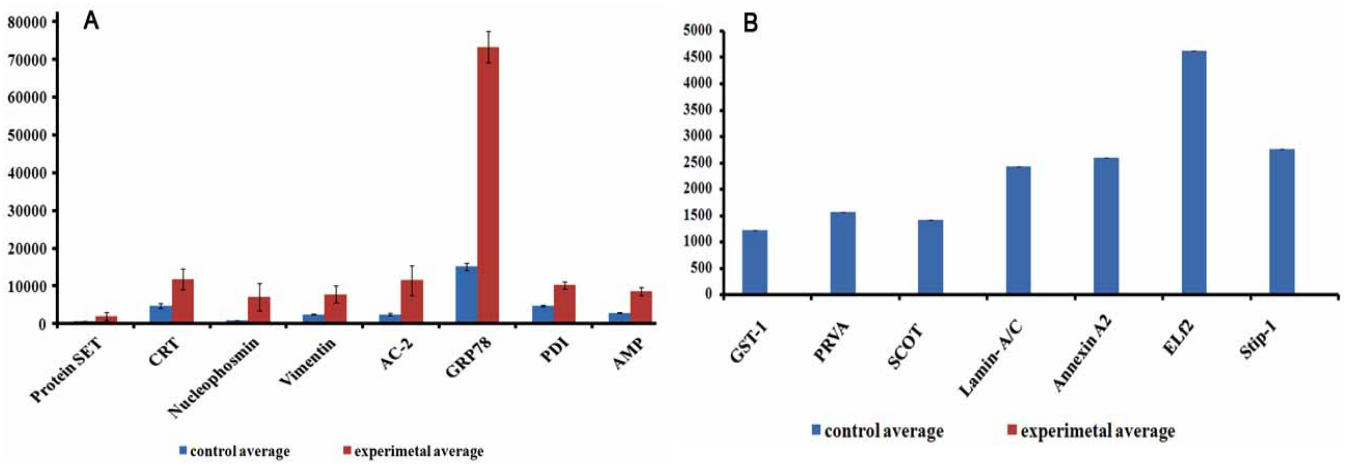


Fig. (6). The accuracy of their quantitative estimation of altered expression levels of up-regulated (**A**) and down-regulate EAF-modulated proteins (**B**) in PC-3M cells by PD Quest Software. These values are expressed by the representative for three separate experiments.

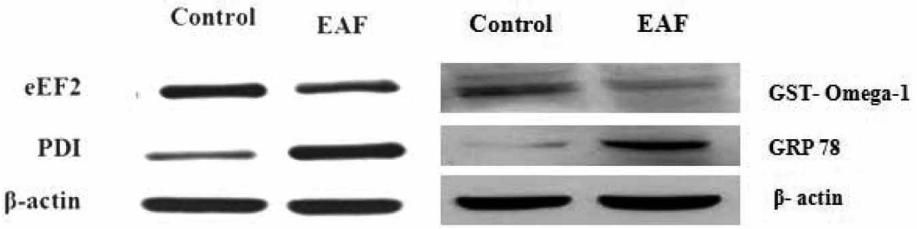


Fig. (7). Validation of the differentially expressed two selected proteins by Western blotting analysis in EAF treated PC-3M cells at 5 µg/ml compared to solvent treated controls. Cell lysates were separated in 4% to 15% linear gradient SDS-PAGE gels and were probed against the respective antibodies. β- Actin was used as the loading control. The gel shown is a representative for three separate experiments.

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 [2]. *C. longa* is traditionally known as a blood purifier and is reported to be useful for the common cold, intermittent fevers, and 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 [3, 4, 16-19]. Recently Aggarwal and co-workers using a commercially available curcumin mix reported that different analogs of curcumin present in *C. longa* showed variable anti-inflammatory and anti-proliferative activities [7]. Curcumin or diferuloylmethane, a major component present in turmeric is a powerful antioxidant and is linked with the suppression of mutagenesis, inhibited nuclear factor- kB (NF-kB) 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) [20-24]. Curcumin is currently in clinical trials for treatment of various cancers [25-26], and for Alzheimer's disease [27].

In our earlier studies *C. longa* powder extract showed significant inhibitory effect on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line [28]. Further, partition and fractionation studies showed that only the ethyl acetate eluted fraction possessed significant inhibitory effect on colony formation in clonogenic assays (Fig. **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 (Fig. **2**, **3** and **4**). Despite the progress, the identification of the molecular targets of *C. longa*, as well as its purified derivatives and delineation of the molecular mechanisms underlying their chemopreventive effects are not explored fully as yet.

The use of proteomics technology enabled us to identify an array of hitherto unknown proteins that may be critical for chemoprevention by *C. longa* and its purified derivatives. Elongation factor 2 is a single polypeptide 857 amino acids (96kDa) which catalyzes the last step of the elongation cycle, translocation, during protein synthesis. Studies have shown that phosphorylated eEF2 has been found in many tumor cells and tissues [29, 30] and that the elongation factor 2 (eEF2) kinase is up-regulated in several cancer cells and tissues. These observations raise the possibility that blocking the activation of eEF2 kinase may represent a potential therapeutic strategy to promote cell death induced by metabolic stress [31]. EAF extract inhibits cell growth and causes death of tumor cells mediated by eEF2 significantly. Also decreased eEF2 was observed in E10, an immortalized but

nontumorigenic cell line derived from alveolar type II pneumocytes with functional GJIC compared to E9, a spontaneous transformant of E10, deficient in GJIC and is tumorigenic upon injection into a syngeneic mouse [32].

Further, over-expression of eEF2 in gastrointestinal cancer cell lines promoted G2/M progression and enhanced their cell growth *in vitro* and *in vivo* compared to eEF2 knockdown which induced G2/M arrest and inactivation of Akt and cdc2 [33]. It is quite interesting that in our study also EAF-treated cells showed down-regulation of eEF2 compared to prostate cancer cells PC-3M. Stress-induced phosphoprotein 1, also known as Hop, acts as an adaptor or co-chaperone and is involved in the formation of protein complexes of heat-shock protein 90 (Hsp90) and heat-shock protein 70 (Hsp70) resulting in an assembly thus enhancing the chaperone activities of these heat-shock proteins [34]. Hop is one of the most extensively studied co-chaperones that is able to directly associate with both Hsp70 and Hsp90. Hsp90 assembles with other chaperones and co-chaperone Hop to form a 'super-chaperone machine' in tumour cells which is 100 times more sensitive to Hsp90 inhibitor, 17-AAG and is toxic to tumour cells compared to normal cells where most of the Hsp90 exists in a free form. Accordingly, inhibitors of Hsp90 could mount a multi-pronged assault on cancer cells that, if not lethal itself, might leave them sufficiently debilitated to allow control by chemotherapy or radiotherapy [35]. It is possible that down regulation of Hop in EAF-treated cells may be a factor involved in chemoprevention.

GRP78 an endoplasmic reticulum (ER) stress marker, is involved in sensing and responding to the accumulation of unfolded or misfolded proteins in the ER. It is a highly conserved protein that resides within the ER of virtually all cells and has a high degree of amino acid sequence homology with the Hsp70 and is, therefore, considered a member of the hsp70 family [36]. GRP78 associates transiently with nascent proteins, facilitating their translocation into the ER and is important for both normal cell growth and stress protection [36]. Consistently observed with our results EAF treatment with PC-3M cells significantly up-regulate the GRP78 protein.

Glutathione S-transferases (GSTs) of the new class, named Omega (27kDa) were down-regulated in our study. This protein exists in several mammalian species and Caenorhabditis elegans. In humans, GSTO 1-1 is expressed in most tissues and exhibits glutathione-dependent thiol transferase and dehydroascorbate reductase activities characteristic of the glutaredoxins. The Omega class GSTs exhibits an unusual N-terminal extension that abuts the C terminus to form a novel structural unit. Unlike other mammalian GSTs, GSTO 1-1 appears to have an active site cysteine that can form a disulfide bond with glutathione [37].

Parvalbumin alpha is a 12 kDa member of the parvalbumin family of Ca2+-binding proteins was down-regulated in our study. In human, it is expressed in intrafusal muscle fibers, plus GABAergic interneurons and cerebellar Purkinje and basket cells. It presumably acts as a Ca2+ buffer that shortens the duration of fiber contraction [38, 39]. Succinyl Co-A: ketoacid-3 (OXCT) a 56 kDa was down-regulated as compared to EAF treated cells. It is a key enzyme for ketone body catabolism, responsible for the transfer of CoA moiety from succinate to acetoacetate. The formation of the enzyme-CoA intermediate proceeds *via* an unstable anhydride species formed between the carboxylate groups of the enzyme and substrate. Defects in OXCT1 are a cause of ketoacidosis [40, 41].

Lamin –A/C a 70 kDa protein was down-regulated by EAF. Lamins are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane, which is thought to provide a framework for the nuclear envelope and may also interact with chromatin. Lamin A and C play an important role in nuclear assembly, chromatin organization, nuclear membrane and telomere dynamics [42, 43].

Annexin A2 (ANXA2) which shows two isoforms (38 and 40 kDa) was down-regulated as compared to EAF treated cells. Annexin A2 is a profibrinolytic co-receptor for plasminogen and tissue plasminogen activator that stimulates activation of the major fibrinolysin, plasmin, at cell surfaces. In human subjects, overexpression of annexin A2 in acute promyelocytic leukemia leads to a bleeding diathesis reflective of excessive cell surface annexin A2-dependent generation of plasmin [44]. In addition, mice completely deficient in annexin A2 display fibrin accumulation within blood vessels and impaired clearance of injury-induced thrombi [45]. Endothelial cell annexin A2, a protein that lacks a typical signal peptide, translocates from the cytoplasm to the extra cytoplasmic plasma membrane in response to brief temperature stress both *in vitro* and *in vivo* in the absence of cell death or cell lysis. This regulated response is independent of new protein or mRNA synthesis and does not require the classical endoplasmic reticulum-Golgi pathway. Temperature stress-induced annexin A2 translocation is dependent on both expression of protein p11 (S100A10) and tyrosine phosphorylation of annexin A2 because annexin A2 release is completely eliminated on depletion of p11, inactivation of tyrosine kinase, or mutation of tyrosine 23. Translocation of annexin 2 to the cell surface dramatically increases tissue plasminogen activator-dependent plasminogen activation potential and may represent a novel stress-induced protein secretion pathway [46].

Calreticulin (CALR) a 46 kDa protein was up-regulated by 2.4 fold. This molecular calcium-binding chaperone promoting folding, oligomeric assembly and quality control in the ER *via* the calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglucosylated glycoproteins that are synthesized in the ER. Interacts with the DNA-binding domain of NR3C1 and mediates its nuclear export [47, 48].

Nucleophosmin (NPM1) a 32 kDa protein was up-regulated by 7.6 fold as compared to control cells. Nucleosphosmine is involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors TP53/p53 and ARF. Binds ribosome presumably to drive ribosome nuclear export and associated with nucleolar ribonucleoprotein structures and bind

single-stranded nucleic acids [49, 50]. Vimentins (VIM) a 53kDa class-III intermediate filament found in various non-epithelial cells was up-regulated by 3.0 fold. Its molecular function is protein C-terminus binding, structural constituent of cytoskeleton. The protein is highly expressed in fibroblasts, some expression in T- and B-lymphocytes, and little or no expression in Burkitt's lymphoma cell lines [51, 52]. Multitasking protein SET of 33 kDa was up-regulated by 3.1 fold. Protein SET is involved in apoptosis, transcription, nucleosome assembly and histone binding [53, 54].

The enzyme PDI was up-regulated by 2.1 fold and is another ER stress marker, multi domain, multi-functional member of the thioredoxin superfamily. PDI can catalyse thiol-disulfide oxidation, reduction and isomerization, the last of which occurs directly through intramolecular disulphide rearrangement or through cycles of reduction and oxidation and catalyses all the reactions that are involved in native disulphide [55].

Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. In vertebrates 3 main groups of actin isoforms, alpha, beta and gamma have been identified. Actin cytoplasmic 2, also known as Gamma-actin, was up-regulated by 4.3 fold and is involved in the internal cell motility [56]. Aortic smooth muscle (ACTA 2) a 42 kDa protein was up-regulated by 2.0 fold as compared to control cells. Up-regulation of these four proteins namely GRP78, PDI, actin cytoplasmic 2 and aortic alpha-actin (ACTA2) was observed in our study.

CONCLUSION

In this study, we have presented the proteomic profiling and cytotoxic effect of *Curcuma longa* extract ethyl acetate fraction (EAF) on prostate cancer cells. The use of 2D-GE separation and a MS-based proteomic approach revealed a total of 29 proteins differentially expressed proteins, among which 13 were down-regulated and 16 up-regulated. Overall these differentially expressed proteins could provide potential drug targets for the development of small molecule therapeutics. Accordingly, detailed analysis of the functional role of novel candidate molecular targets identified in this study would extend our understanding of the chemopreventive effects of *C. longa* derived products and, in the future, enable more specific and concurrent targeting of multiple key molecular pathways leading to more effective chemoprevention.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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