International Journal of Phytomedicine 8 (2016) 29-36
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Synergistic effect of spices in a decoction regulates the energy metabolism in liver cancer cells

Sheetal Ramachandran[1] and Darsan B Menon[1,*]

*Corresponding author:

Darsan B Menon

[1]Centre for Evolutionary and Integrative Biology (CEIB),
University of Kerala, Thiruvananthapuram, Kerala, 695581 India

Abstract

Lactic acid fermentation and not oxidative phosphorylation is reported to be one of the major bioenergetics mechanisms by which cancer cells thrive and proliferate with such rapidity in non-ambient hypoxic conditions. The objective of the study was to determine the synergistic effect of spices in a decoction; Turmeric (*Curcuma longa*), Pepper (*Piper nigrum*) and Garlic (*Allium sativum*) in combination, on the metabolism of Hep G2 liver cancer cells. The biochemical studies of 2 major enzymes involved in cellular metabolism of cells namely; Pyruvate dehydrogenase (PDH) and Lactate dehydrogenase A (LDHA) showed that the decoction down regulated the activity of LDHA and increased the activity of PDH in cancer cells, thereby shifting the metabolic mechanism towards normal functioning in the cancer cells. The expression studies of major molecules involved in regulating metabolic machinery in cells *viz*., tumour suppressor p53 and oncogene MYC mRNAs; and western blot analysis of Hypoxia inducible factor-1-alpha (HIF-1α) and Vascular endothelial growth factor (VEGF) showed that the decoction regulated the expression of these factors helping to revert the metabolism in cancer cells, which could be attributed to the effect of turmeric and also garlic, rich in pantothenic acid, that aids in turning on oxidative phosphorylation in cancer cells. With the above results, it could be concluded that the decoction alters the metabolic mechanism in a cancer cell from lactic acid fermentation to oxidative phosphorylation, like in any other normal cell, thus inhibiting further growth of the cancer.

Keywords: Decoction, synergistic activity, lactate dehydrogenase, hypoxia, metabolism.

Introduction

One of the major differences between a normal cell and a cancer cell is the ability of the cancer cell to survive and flourish in severely hostile and harsh conditions. This adroitness of the cancer cell can be attributed to many factors, one of the most prominent being, the capability to shift its basic bioenergetics mechanism from oxidative phosphorylation to aerobic glycolysis followed by lactic acid fermentation. This shift is mainly triggered by critical changes in the microenvironment of the cell, resulting from inadequate supply of oxygen and hypoxia [1]. The hypoxic environment results in aggressive and metastatic cancer phenotypes which are associated with resistance to radiation therapy, chemotherapy and a poor prognosis [2]. The responses to hypoxia are highly dependent on the activation of hypoxia inducible factor-1-alpha (HIF-1α) [3]. HIF-1α trans-activates a large number of genes that are involved in cellular processes, such as glucose uptake and metabolism, angiogenesis, cell proliferation, differentiation and apoptosis [4, 5]. Over expression of HIF-1α also plays a major role in initiating lactic acid fermentation in cancer cells by modulating the activity of tumor suppressor p53 and oncogene MYC and through the overexpression of VEGF, which stimulates angiogenesis in tumor microenvironment.

Natural products from plants have played a crucial role in lives of humans especially as source of food and medicines throughout history. The anti-tumour compounds act by several mechanisms such as inducing apoptosis (programmed cell death) through DNA cleavage mediated by topoisomerase I or II inhibition, mitochondrial permeabilization, inhibition or regulation of key enzymes involved in signal transduction (e.g. proteases), or cellular metabolism, and by inhibiting tumour-induced angiogenesis (recruitment of new blood vessels) [6]. Curcumin, a major active component of turmeric has been shown to decrease HIF-1α in hepatocellular carcinoma cells [7]. Black pepper is rich in piperine, which is found to increase the bioavailability of curcumin along with possessing anticancer activity against many cancer types. Garlic has been found to be rich in Vitamin B5 (Pantothenic acid) which is a precursor for the formation

of Acetyl Co-A and has also been reported to possess anti-tumor activity.

The decoction used for our study comprises of Curcuma longa, Piper nigrum and Allium sativum added in equal parts, which is part of daily culinary use all over the world. The objective of this study is to determine the synergistic effect of the spices in the decoction on regulation of the bioenergetics mechanism in liver cancer cell line, Hep G2 by focussing on, the activity of enzymes namely pyruvate dehydrogenase and lactate dehydrogenase A and molecular factors viz., p53, MYC, HIF-1α and VEGF.

Materials and Methods

Preparation of the decoction

Dried powder of, rhizome of Curcuma longa , bulb of Allium sativum and fruits of Piper nigrum were taken in equal parts (10 mg each) together (30 mg) in 30 mL water and mixed well using mortar and pestle. The decoction was then heated at 100ºC for 10 minutes, diluted to different concentrations with distilled water and filtered through Whatmann filter paper (No: 4) and was used for the assays.

Cell culture and treatment

The Human liver cancer (Hep G2) and normal (Chang Liver cells) cells were obtained from NCCS, Pune. The cells were maintained in DMEM supplemented with 10 % FBS 75 and 100 mg/L streptomycin and 100 U/L penicillin. Cells were maintained at 37ºC in a humidified atmosphere of 5 % CO2 and 95 % air. All cell culture reagents were purchased from Hi-media (Mumbai, India).

Enzyme assays

Pyruvate dehydrogenase (PDH) activity assay

Pyruvate dehydrogenase activity was measured using a modified protocol of Prabhu et al., (2014) [8]. Rapidly homogenize the Hep G2 cells (1 x 106) untreated control and decoction treated (7.5, 15 and 30 µg/mL), with 100 µL ice cold PDH assay buffer and keep on ice for 10 min. Centrifuge this at 10,000 x g for 5 min. Transfer the supernatant to a fresh tube. Take 50 µL samples for the assay along with 46 µL of PDH assay buffer, 2 µL PDH developer and 2 µL PDH substrate. Absorbance was measured at 450 nm for 10-60 min at 37oC. The PDH activity was calculated according to the formula:

Pyruvate dehydrogenase activity (mU/mL) = B/(ΔT x V) X D

where: B = NADH amount; ΔT = reaction time; V = sample volume added and D = dilution factor.

Lactate dehydrogenase A (LDHA) activity assay

LDH activity was measured according to a modified protocol of Wang et al., (2013) [9]. The assay was based on the detection of absorption value of β-NADH at 340 nm, in vitro LDHA enzymatic system was established. In a 3 ml reaction mix, the final concentrations were 100 mM sodium phosphate, 0.12 mM β-NADH, 2.3 mM pyruvate, 0.033% (W/V) bovine serum albumin and 20 mg of total protein extracts in untreated control or decoction-treated (7.5, 15 and 30 µg/mL) Hep G2 cancer cells. The value of absorbance at 340 nm was continuously recorded for 5 mins at 37oC. The LDHA activity was calculated according to the formula:

LDH activity (U/mg) = (Test A340 nm/ min – Blank A340 nm/min) x 3 x df/6.22 x 0.1 x PC

where: 3 is the total volume of assay, df is the dilution factor, 6.22 is the mM extinction coefficient of β-NADH at 340 nm, 0.1 is the volume of added protein extracts and PC is the protein concentration.

Cell Viability Assay

The cells were grown in 12-well culture plates. MTT assay was used to assess cell viability according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). The assay was quantitated by measuring the absorbance at 570 nm [10]. Chang Liver cells were used as control (noncancerous cells) to check the activity of the decoction on normal cells. After measuring the absorbance at 570 nm at the end of the MTT assay, the % growth inhibition was determined using the formula:

% growth Inhibition = 100 – (Abs (sample) / Abs (control) X 100)

Reverse Transcriptase-PCR

The mRNA expression levels of p53 and MYC were studied using reverse-transcriptase PCR [11, 12]. The Hep G2 cells were grown in 60 mm culture plates. The first set of Hep G2 cells was untreated and taken as control. The second set of cells was treated with the decoction at 7.5 µg/mL concentration. The third set of cells was treated with 15 µg/mL decoction and the last fourth set of cells was treated with 30 µg/mL decoction. After incubation, total RNA was isolated from the treated and untreated cells using manufacturer's protocol (Chromous Biotech Ltd, Bangalore, India). cDNA was synthesized from 1 µg of total isolated RNA by incubation for 1 h at 37oC with M-MLV reverse transcriptase (Promega, Madison, WI). After the 1 h incubation, the mixture was again incubated at 700C for 15 minutes to deactivate the reverse transcriptase. The cDNA synthesized (2 µL) was added with 5 µL reaction buffer (1X), 2 µL dNTPs (0.2 mM each), 1 µL each of Forward and Reverse primer (0.4 µM each), 3 µL MgCl2, 0.2 µL Taq polymerase (Promega, Madison, WI, USA) and made up to 20 µL with nuclease free water for PCR reaction. Human specific primers were used for p53, MYC and beta-actin. The forward and reverse primers used for p53, MYC and beta-actin amplification are given in table. 1. The synthesized

PCR products were separated on 1.5 % agarose gels and analyzed using ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA).

Table. 1. Forward and reverse primers for the genes

Genes	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
p53	AACGGTACTCCGCCACC	CGTGTCACCGTCGTGGA
MYC	AATGAAAAGGCCCCCAAGGTAGTTATCC	GTCGTTTCCGCAACAAGTCCTCTTC
β-actin	GTTTGAGACCTTCAACACCCC	GTGGCCATCTCCTGCTCGAAGTC

Western blot analysis

The protein expression levels of Hypoxia inducible factor-1α (HIF-1α) and Vascular endothelial growth factor (VEGF) were studied using western blotting assay. The Hep G2 cells were grown in 60 mm culture plates. The first set of Hep G2 cells was untreated and was taken as control. The second set of cells was treated with decoction at 7.5 µg/mL concentration. The third set of cells was treated with decoction at 15 µg/mL and the last fourth set of cells was treated with decoction at 30 µg/mL concentrations. The protein was separated by SDS-PAGE on a 10 % separating gel and transferred to nitrocellulose membrane [13, 14]. Addition of primary mouse monoclonal anti-HIF-1α and rabbit polyclonal anti-VEGF antibodies were done after the transfer of protein on to the membrane. The nonspecific protein binding sites on the blotted nitrocellulose membrane were blocked with 5% non-fat dry milk in 0.1 % Tween-20 in PBS (pH 7.4) at 4°C overnight. The membrane was incubated with monoclonal antibodies for HIF-1α, VEGF and β-actin at the dilution of 1:500 (in 3 % BSA), 1:2000 (in 3 % BSA) and 1:2000 (in 3 % BSA) respectively at 40C overnight with gentle shaking. The membrane after incubation was washed thrice with PBS-Tween-20 (5 minutes each) and incubated with goat anti-mouse monoclonal antibody (1:3,000) for HIF-1α and goat anti-rabbit HRP tagged antibody (1:10,000) for VEGF (in 5 % non-fat milk solution) (Santa Cruz Biotechnology, Inc., CA) at RT for 1 hour. After incubation, the membrane was washed twice with PBS-Tween-20 (5 minutes each) and for 10 minutes with the same. The protein bands were quantified by autoluminographs. The amount of protein in each well was confirmed by stripping the membrane with stripping buffer (at 70°C for 1 hour) and reprobing with monoclonal antibody to β-actin by following the manufacturer's instruction (Santa Cruz Biotechnology, Inc., CA). Immunodetection of the protein expressions under study was performed using ECL prime western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) and visualized in a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA).

Statistical Analysis

Results refer to mean ± standard deviation and are average of three values per experiment; each experiment was repeated at least three times. Statistical evaluations were assessed using the Student's *t* test, and $p < 0.05$ was considered significant.

Results

Effect of decoction on enzymes

The decoction treated Hep G2 cells showed an increase in the activity of Pyruvate dehydrogenase (PDH) enzyme than the untreated cells (Figure 1). In untreated liver cancer cells, PDH activity was found to be 0.76 ± 0.07 mU/mL. This PDH activity significantly increased to 1.29 ± 0.09, 3.67 ± 0.15 and 4.65 ± 0.11 mU/mL when treated with 7.5, 15 and 30 µg/mL concentrations of the decoction respectively. The activity of the enzyme was found to increase nearly 5 folds when treated with 15 µg/mL concentration of the decoction.

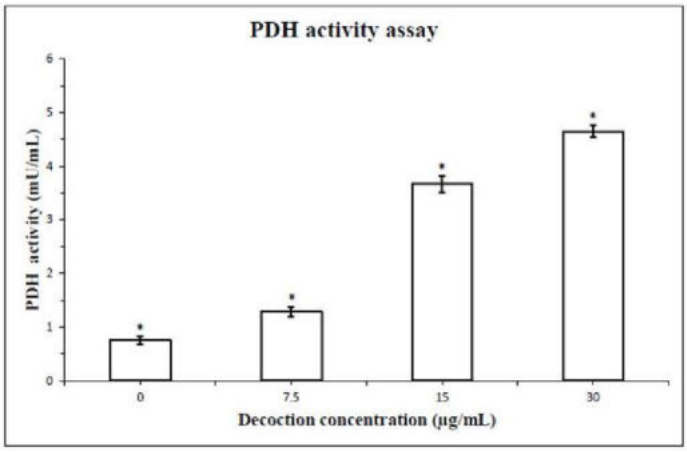

Figure 1. PDH enzyme activity

Figure 2 represents the status of Lactate dehydrogenase A (LDHA) enzyme activity in both untreated and decoction treated liver cancer (Hep G2) cells. In untreated Hep G2 cells, LDHA activity was found to be 1.64 ± 0.04 U/mg protein which decreased to 1.44 ± 0.05, 0.64 ± 0.03 and 0.38 ± 0.04 U/mg protein when treated with 7.5, 15 and 30 µg/mL concentrations of the decoction respectively. The results showed that there was 2.5 times reduction in the activity of the LDHA activity when treated with 15 µg/mL concentration of the decoction.

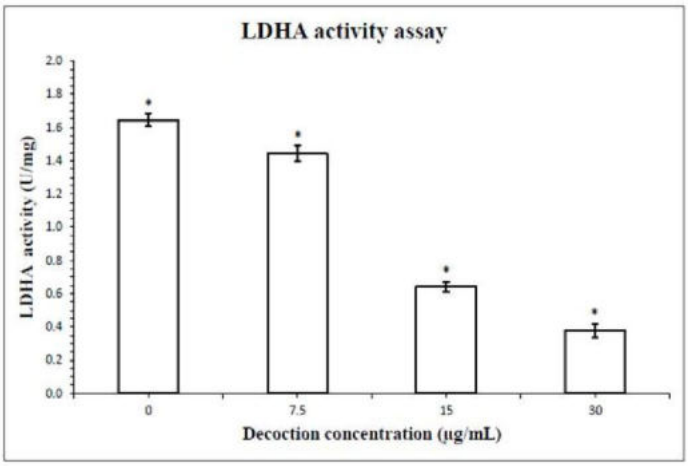

Figure 2. LDHA enzyme activity

Effect of decoction on cell viability

Concentration-dependent study performed using MTT assay to check the effect of the decoction on the cell viability of normal Chang liver cells and Hep G2 liver cancer cells showed that an increase in concentration of the decoction (0, 25, 50, 75 and 100 µg/mL) selectively reduced the cell viability in the cancer cells at higher concentration than normal cells (Fig. 3). Total (100%) growth inhibition was observed at a concentration near 100 µg/mL of the decoction on the Hep G2 cancer cells whereas this concentration produced only 50 % cell death in the normal Chang liver cells which showed that the decoction has reduced cytotoxic effect on non-cancerous normal cells.

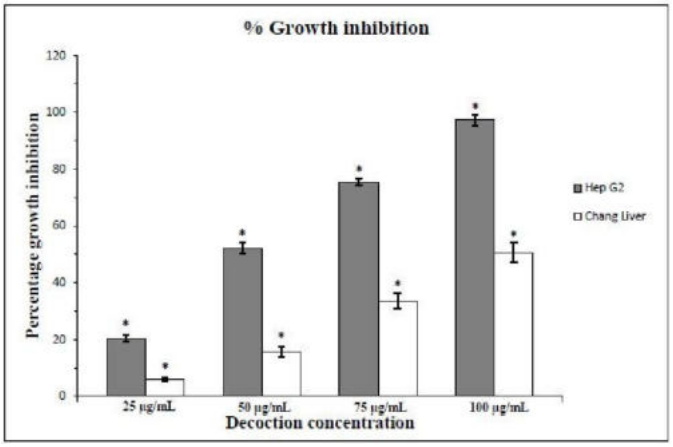

Figure 3. Percentage growth inhibition after treatment with decoction on Hep G2 and Chang liver cells. x-axis shows concentration in µg/mL and y-axis shows percentage of inhibition.

Molecular analysis of the factors in Hep G2 cells

RT-PCR

The mRNA expression level of tumor suppressor p53 from the untreated and decoction treated liver cancer cells were found to increase whereas the expression of oncogene MYC mRNA was found to decrease in the decoction treated Hep G2 cells (Fig. 4); which can be compared to the expression of house-keeping protein beta-actin providing a relative expression pattern (Fig. 5a and 5b). So, it was evident that as the concentration of the decoction increased, the expression of tumor suppressor p53 increased and the expression of oncogene MYC decreased, which confirmed the ability of the decoction to return the overall regulatory mechanism in the cancer cell like that in those of normal cells.

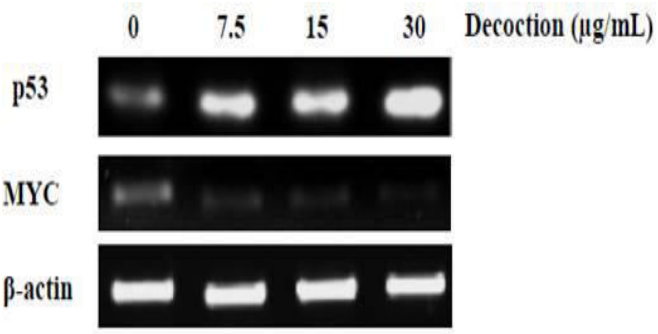

Figure 4. RT-PCR gel showing expression of p53 and MYC genes

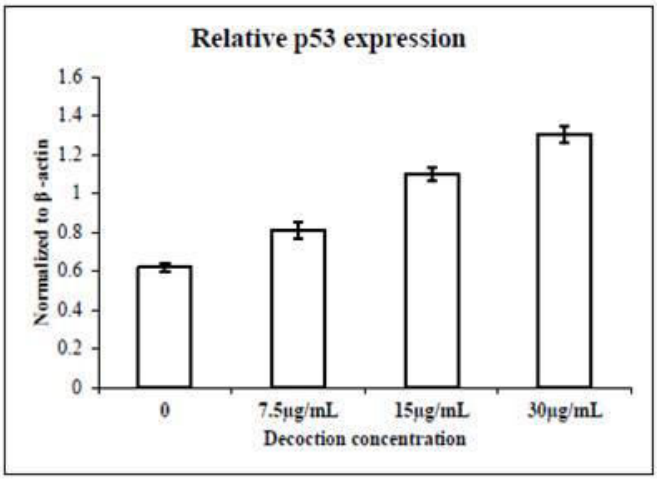

Figure 5a. Relative p53 expression normalized to β-actin

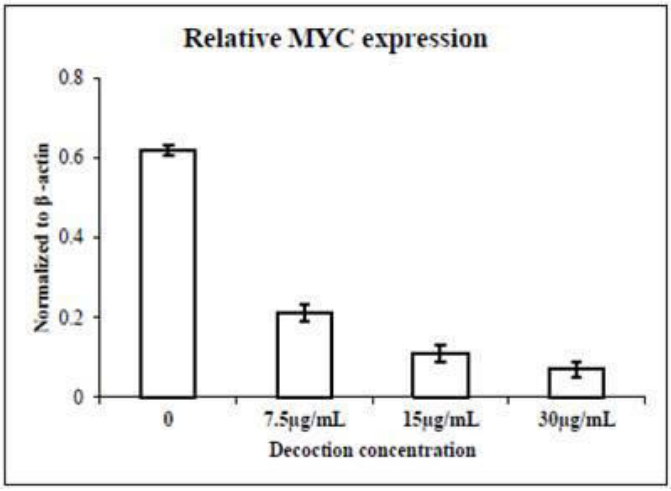

Figure 5b. Relative MYC expression normalized to β-actin

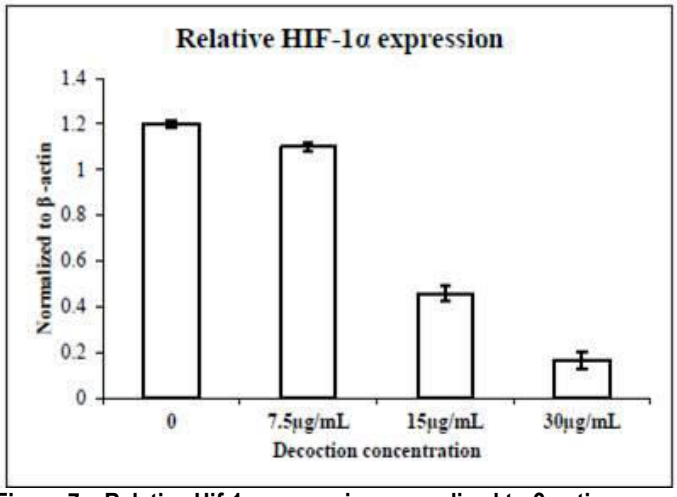

Figure 7a. Relative Hif-1α expression normalized to β-actin

Western blotting

The protein expression studies of Hypoxia inducible factor-1-alpha (HIF-1α) and Vascular endothelial growth factor (VEGF), which would lead to angiogenesis and further helping the cancer cells in survival and metastasis; showed that the decoction induced decreased expression of both the factors (Fig. 6); which can be compared to the expression of house-keeping protein beta-actin providing a relative expression pattern (Fig. 7a and 7b). As these factors play a very significant role in the cancer cell growth and metastasis, the decrease in the expression of HIF-1α and VEGF could be vital in triggering the cancer cell to revert back to oxidative phosphorylation from anaerobic glycolysis and lactic acid fermentation.

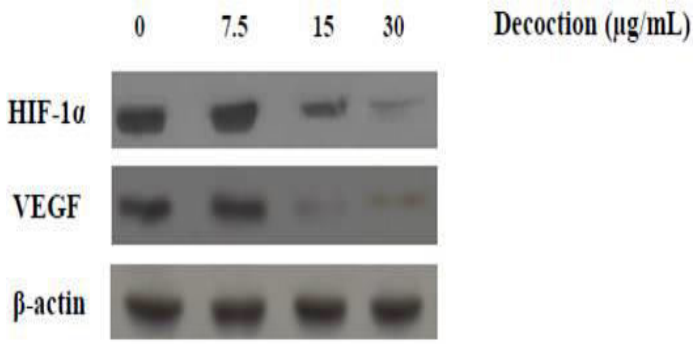

Figure 6. Western blot of Hif-1α and VEGF proteins

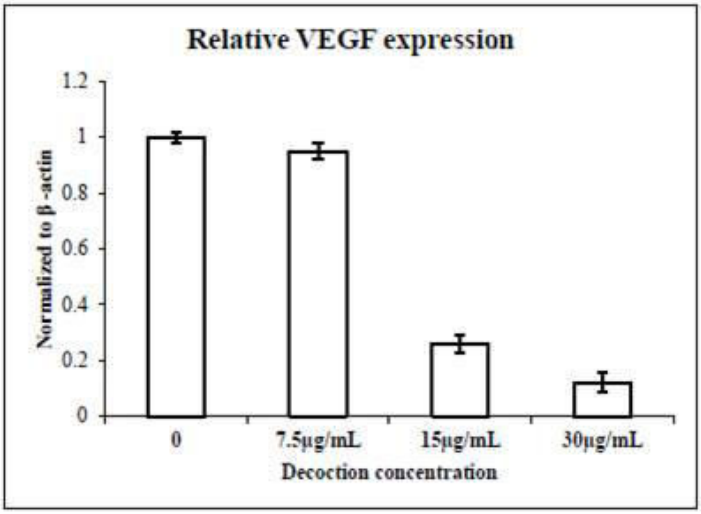

Figure 7b. Relative expression of VEGF normalized to β-actin

Discussion

Cancer cell metabolism is a direct result of the modulation of intracellular signaling pathways that are disrupted by mutated oncogenes and tumor-suppressor genes. The persistent activation of aerobic glycolysis in cancer cells can be linked to activation of oncogenes or loss of tumor suppressors, thereby fundamentally advancing cancer progression. In this respect, inhibition of glycolytic capacity may contribute to an anticancer effect on malignant cells and thus, interrupting lactate fermentation and/or other cancer-promoting metabolic sites may provide a promising strategy to halt tumor development. Unlike normal cells that produce energy mostly through the oxidation of pyruvate in the mitochondria through oxidative phosphorylation, cancer cells predominantly produce energy via enhanced glycolysis in the cytosol followed by lactic acid fermentation which is regulated mainly by factors like HIF-1α [15].

Natural products have played a very vital role in the prevention, treatment and management of diseases in humans from time immemorial. Throughout human evolution, the importance of natural products has been enormous from being a source of energy as food, to being miracle drugs for a plethora of diseases. Turmeric is one such spice that has been used worldwide, especially in Indian cuisines. It is known to be one of the oldest spices that have been used in India for thousands of years and is a major part of Ayurvedic medicine. Curcumin, which is isolated from turmeric, has been found to possess anticancer activities via its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis. Curcumin is reported to possess anti-bacterial, antioxidant, anti-inflammatory, anti-hepatotoxic and anti-tumor properties [16]. Curcumin as a natural phytochemicals could communicate with many novel targets and show synergism to chemotherapy. Additionally, curcumin is well tolerated in humans [17]. Although turmeric has been used in cuisines world-wide, one of the problems associated with it, is its low bioavailability after it is taken orally. But piperine, a component of Black pepper is reported to increase the bioavailability of curcumin by 2000 % [18]. Black pepper (Piper nigrum) can be used for different purposes such as human dietaries, as medicine, as preservatives, as biocontrol agent and as an agent to increase bioavailability of other bioactive compounds. This plant and its active component piperine can stimulate the digestive enzymes of pancreas and intestines and also increases biliary bile acid secretion when orally administered. Other activities include anti-inflammatory activity, antioxidant activity and chemopreventive activity. Medicinally black pepper can be used for digestive disorders like large intestine toxins, different gastric problems, diarrhea and indigestion [19, 20]. Garlic is yet another spice which is used extensively in most cuisines worldwide. Ajoene, a compound isolated from garlic has been found to possess antiproliferative activity and also preferentially suppresses the growth of neoplastic cells [21].

Food provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention. Previous epidemiologic studies have consistently shown that diet plays a crucial role in the prevention of chronic diseases. One of the key issues in this field of research is, however, that purified phytochemicals do not necessarily exert the same beneficial health effect as when the compound source is in a food or even a complete specific diet. Although relatively high doses of single bioactive agents may show potent anticarcinogenic effects, the chemopreventive properties of interactions among various dietary ingredients that potentiate the activities of any single constituent may better explain the observed preventive effect of whole foods and diets in many epidemiological studies [22].

Hot water as the extraction medium was used because water is the main medium used to prepare food generally and for diets in case of patients suffering from medical conditions. Most foods of plant origin contain components which are active regulators of various pathways and processes in the human cells. These are mainly compounds of natural origin, which are formed by regulated biosynthesis in plants [23].

Curcumin (diferuoyl methane) is a phenolic compound and a major component of Curcuma longa L. Curcumin has been reported to regulate various different pathways in a cancer cell. Curcumin is found to inhibit the expression of oncogenes including MYC [24]. In a study by Bae et al., (2006) it was shown curcumin suppressed the transcriptional activity of HIF-1 under hypoxia, leading to a decrease in the expression of vascular endothelial growth factor (VEGF), a major HIF-1 target angiogenic factor. Curcumin also blocked hypoxia-stimulated angiogenesis in vitro and down-regulated HIF-1α and VEGF expression in vascular endothelial cells [25, 26]. Piperine, a principal bioactive compound present in black pepper, is reported to inhibit the proliferation and G1/S transition in endothelial cells without causing cell death. It also inhibited the expression of matrix metalloproteinase MMP-9, thereby interfering with tumor cell migration and invasion [27]. Piperine inhibited the PI3K/Akt signaling cascade that promotes angiogenesis [28]. Garlic and garlic derived sulphur compounds have shown to alter activation of carcinogens and to cause growth inhibition of tumor cells [21]. Several mechanisms have been presented to explain cancer chemopreventive effects of garlic-derived products. These include modulation in activity of several metabolizing enzymes that activate and detoxify carcinogens and inhibit DNA adduct formation, antioxidative and free radicals scavenging properties and regulation of cell proliferation, apoptosis and immune responses. Recent data show that garlic-derived products modulate cell-signaling pathways in a fashion that controls the unwanted proliferation of cells thereby imparting strong cancer chemopreventive as well as cancer therapeutic effects [29]. Garlic is reported to possess 12 % Vitamin-B5 (Pantothenic acid) [30]. Pantothenic acid, which is a precursor for the synthesis of coenzyme A, acts as an acyl group carrier to form acetyl-coA which is important for the conversion of pyruvate formed by glycolysis to enter the TCA cycle and further into the oxidative phosphorylation [31]. The garlic in the decoction could thus acts as a source of pantothenic acid, which would combine with the pyruvate formed in the cancer cell to revert back to TCA cycle and further into oxidative phosphorylation rather than entering lactic acid fermentation.

Even though all the spices are reported to possess anti-proliferative activity, no study has been conducted till date on the synergistic activity of all the three spices in combination, which is commonly used in culinary recipes on daily basis world over. Our study shows that the spices when used in combination triggered decrease in the level of LDHA enzyme and an increase in the level of PDH enzyme and also down regulated the expression of HIF-1α and VEGF, factors which help cancer cells in their survival, promoting angiogenesis and thus resulting in invasion and metastasis (Fig 10). The decoction induced considerable lower percentage of cell death in normal Chang liver cells thus proving its ability to differentiate between normal cells and rapidly dividing cancerous cells.

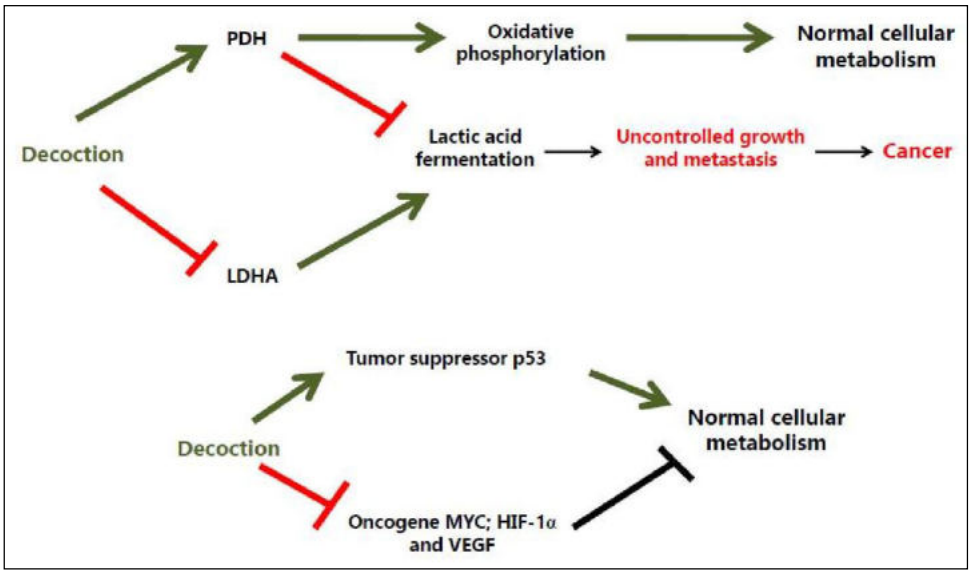

Figure 10. Proposed mechanism of action of the decoction on the cells based on the study

Conclusion

The growing interest in biological actions of plant extracts is a consequence of, among others, an increasingly high incidence of various cancers and a need to find safe and effective method for prophylaxis and therapy. In this study, the decoction of the spices was found to decrease the expression of angiogenesis promoting factors and also increase PDH enzyme activity than LDHA activity which modifies the cancer cell bioenergetics mechanism to that of the normal cells. The decoction induced lower apoptotic activity on the normal Chang liver cells showing that the decoction by certain means manages to differentiate between normal and cancerous cells. The combined activity of aqueous extract of the spices in the decoction is found to have a synergistic growth regulatory activity in the liver cancer cells. This is the first report on the combined synergistic activity of extract of two or more spices in a decoction on shifting the bioenergetics balance in a cancer cell and these findings would provide yet another method of targeting cancer cells using combination of extracts exploiting their synergistic activity.

Acknowledgements

We are thankful to the Director, CEIB, for providing all facilities and encouragement.

Author's Contributions

Both authors have contributed equally to the manuscript and work

References

[1]. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 2000; 60: 4010-4015.

[2]. Greco O, Marples B, Joiner MC, Scott SD. How to overcome (and exploit) tumor hypoxia for targeted gene therapy. J Cell Physiol 2003; 197: 312-325.

[3]. Zhou Y, Lin L, Wang Y, Jin X, Zhao X, Liu D, Hu T, Jiang L, Dan H, Zeng X, Li j, Wang J, Chen Q. The association between hypoxia-inducible factor-1 alpha gene G1790A polymorphism and cancer risk: a meta-analysis of 28 casecontrol studies. Cancer Cell Int 2014; 14: 37-47.

[4]. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995; 270: 1230–1237.

[5]. Dery MA, Michaud MD, Richard DE. Hypoxia-inducible factor 1: regulation by

hypoxic and non-hypoxic activators. Int J Biochem Cell Biol 2005; 37: 535–540.

[6]. Demain AL, Vaishnav P. Natural products for cancer chemotherapy. Microbial biotechnology 2010; 4: 687-699.

[7]. Bae MK, Kim SH, Jeong JW, Lee YM, Kim HS, Kim SR, Yun I, Bae SK, Kim KW. Curcumin inhibits hypoxia-induced angiogenesis via downregulation of HIF-1. Oncol Rep 2006; 15: 1557–1562.

[8]. Prabhu A, Sarcar B, Kahali S, Yuan Z, Johnson JJ, Adam KP, Kensicki E, Chinnaiyan P. Cysteine catabolism: a novel metabolic pathway contributing to glioblastoma growth. *Cancer Res 2014;* 74: 787-796.

[9]. Wang Z, Wang D, Han S, Wang N, Mo F, Loo TY, Shen J, Huang H, Chen J. Bioactivity-Guided Identification and Cell Signaling Technology to Delineate the Lactate Dehydrogenase A Inhibition Effects of *Spatholobus suberectus* on Breast Cancer. PLoS ONE 2013; 8: e56631-e56642.

[10]. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63.

[11]. Hans-Joachim D, Annette D, Gr€unewald-Janho ED, Joe SK. PCR Applications Manual (3rd ed.). Roche Diagnostics, Mannheim, Germany. (2006).

[12]. Xi L, Nicastri DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE. Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers. Clin Chem 2007; 53: 1206–1215.

[13]. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a laboratory manual (2nd ed.) Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory Press, USA. (1989).

[14]. Eslami A, Lujan J. Western blotting: sample preparation to detection. J Vis Exp 2010; 44: 2359-2360.

[15]. Jang M, Kim SS, Lee J. Cancer cell metabolism: implications for therapeutic targets. Exp Mol Med 2013; 45: e45-e52.

[16]. Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. Clin Exp Pharmacol Physiol 2012; 39: 283-299.

[17]. Wilken R, Veena MS, Wang MB, Srivatsan ES. Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer 2011; 10: 12-30.

[18]. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 1998; 64: 353-356.

[19]. Mona AM, Abo-Zeid, Ayman A, Farghaly. The Anti-mutagenic Activity of Piperine against Mitomycine C induced Sister Chromatid Exchanges and Chromosomal Aberrations in Mice. JGEB 2009; 7: 45-50.

[20]. Selvendiran K, Sakthisekaran D. Chemopreventive effect of piperine on modulating lipid peroxidation and membrane bound enzymes in benzo (α) pyrene induced lung carcinogenesis. Biomed Pharmacother 2004; 58: 264-267.

[21]. Li M, Ciu JR, Ye Y, Min JM, Zhang LH, Wang K, Gares M, Cros J, Wright M, Leung-Tack J. Antitumor activity of Z-ajoene, a natural compound purified from garlic: antimitotic and micro tubule-interaction properties. Carcinogenesis 2002; 23: 573-579.

[22]. de Kok TM, van Breda SG, Manson MM. Mechanisms of combined action of different chemopreventive dietary compounds: a review. Eur J Nutr 2008; 47: 51-59.

[23]. Chrpová D, Kouřimská L, Gordon MH, Veronika Heřmanová, Roubíčková I, Pánek J. Antioxidant Activity of Selected Phenols and Herbs Used in Diets for Medical Conditions. Czech J Food Sci 2010; 28: 317-325.

[24]. Sa G, Das T. Anti cancer effects of curcumin: cycle of life and death. Cell division 2008; 3: 14.

[25]. Kim JW, Gao P, Liu YC, Semenza GL, Dang CV. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. Mol cell biol 2007; 27: 7381–7393.

[26]. Doucette CD, Hilchie AL, Liwski R, Hoskin DW. *Piperine, a dietary phytochemical*, *inhibits angiogenesis*, J. Nutr Biochem 2013; 24: 231–239.

[27]. Hwang YP, Yun HJ, Kim HG, Han EH, Choi JH, Chung YC, Jeong HG. Suppression of phorbol-12-myristate-13-acetate-induced tumor cell invasion by piperine via the inhibition of PKCα/ERK1/2-dependent matrix metalloproteinase-9 expression. Toxicol Lett 2011; 203: 9–19.

[28]. Somanath PR, Razorenova OV, Chen J, Byzova TV. Akt1 in endothelial cell and angiogenesis. Cell Cycle 2006; 5: 512–518.

[29]. Shukla Y, Kalra N. Cancer chemoprevention with garlic and its constituents. Cancer Lett 2007; 247: 167-181.

[30]. Olusanmi, MJ, Amadi, JE. Studies on the Antimicrobial Properties and Phytochemical Screening of Gallic (*Allium sativum*) Extract. Ethnobotanical Leaflets 2010; 1: 537 – 545.

[31]. Daugherty M, Polanuyer B, Farrell M, Scholle M, Lykidis A, de Crécy-Lagard V, Osterman A. Complete Reconstitution of the Human Coenzyme A Biosynthetic Pathway via Comparative Genomics. J Biol Sci 2002; 277: 21431–21439.