



REGULATION OF ANTIOXIDANT ENZYMES AND APOPTOSIS INDUCTION VIA THE MITOCHONDRIAL PATHWAY IN LIVER CANCER CELLS BY THE SYNERGISTIC EFFECT OF SPICES IN A DECOCTION

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ABSTRACT

Natural products from plants have played a key role in lives of humans especially as source of food and medicines throughout history. The objective of the study was to determine the synergistic effect of the regularly used spices; Turmeric (*Curcuma longa*), Pepper (*Piper nigrum*) and Ginger (*Zingiber officinale*) in combination, on the antioxidant enzymes, Super oxide dismutase, Glutathione peroxidase and Catalase and to determine the activity of the decoction on oxidative stress marker; Malondialdehyde levels in liver cancer cell line, Hep G2 and also to check the ability of the decoction to induce apoptosis in the liver cancer cells. In the cell viability assay, the decoction induced cell death in cancer cells and showed distinctively very less effect on the normal cells showing the capacity to differentiate between normal and cancerous cells. The transcriptional expression studies of the pro and anti-apoptotic molecules namely Bcl-2, Bax and Cox-2; and the western blot analysis of Cytochrome-c and Caspase activated DNase (CAD) showed that the decoction induces apoptosis in decoction treated liver cancer cells. With the above results, it could be concluded that the decoction induced apoptosis in the liver cancer cells via the intrinsic or the mitochondrial pathway.

Key words: Decoction, synergistic activity, Antioxidant enzymes, Apoptosis.

INTRODUCTION

One inevitable end for all living cells is death characterized by aging, a process which cannot be reversed or prevented. Research has established the fact that the probability of a human developing cancer increases with age and the oxidative stress is a major cause for aging and development of cancers [1]. Oxygen free radicals are created through aerobic metabolism and are mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [2]. Exogenous antioxidants such as vitamin C, E and β -carotene, prevent the cascade of oxidative reactions by combining with free radicals [3-5].

Tumor cells represent an example whereby oxygen toxicity is accomplished mainly due to deficiency of scavenger enzymes [SOD, GPx, CAT] [6]. Bize *et al.*, showed that both total SOD and manganese containing superoxide dismutase (MnSOD) activities were lower in the tumor cell homogenates compared to normal liver. A deficiency of hepatic antioxidant enzymes and its partners or an increase in toxic free radical species, may contribute to the progression of liver disease such as alcoholic liver disease, viral hepatitis and liver cancer [7]. Natural products from plants have played a key role in lives of humans especially as source of food and medicines throughout history [8].

The decoction used for our study comprises of *Curcuma longa*, *Piper nigrum* and *Zingiber officinale* 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 the

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antioxidant enzymes SOD, GPx and CAT and also on MDA, which is an oxidative stress marker, in liver cancer cell line, Hep G2 and its ability to induce apoptosis in the liver cancer cells.

MATERIALS AND METHODS

Preparation of the decoction

Dried powders of rhizomes of *Zingiber officinale* and *Curcuma longa* 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 100°C for 10 minutes, diluted to a concentration of 10 µg/mL 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 37°C in a humidified atmosphere of 5 % CO₂ and 95 % air. All cell culture reagents were purchased from Hi-media (Mumbai, India).

Enzyme assays

The 70 – 80 % confluent cells were washed twice with Phosphate Buffered Saline (PBS), pH 7.2 and treated with 0.25 % trypsin (Sigma-Aldrich, St. Louis, MO, USA), 2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 min. The cell suspension was centrifuged for 10 minutes. Cell pellets were then lysed in 50 mM phosphate buffer pH 7.0, followed by sonication (Sonicator ultrasonic processor, XL Heat Systems, USA) for 2 min on ice. The mixture was then centrifuged for 10 minutes at 10,000 x g and the supernatant was assayed for enzyme activities and protein concentration. SOD, GPx and CAT activities were measured by Beyer and Fridovich's [9], Paglia and Valentine's [10] and Aebi's [11] methods which were modified respectively. Measurement of MDA formation was carried out through its reaction with thiobarbituric acid, TBA (Sigma-Aldrich, St. Louis, MO, USA) using the method of Ohkawa [12].

Superoxide dismutase (SOD)

Supernatant (20 µL) was added to a reaction mixture consisting of 0.033 mM EDTA, 3.3 mM methionine, 0.33 µg/ml riboflavin and 0.01 mM KCN (Sigma-Aldrich, St. Louis, MO, USA). The cuvette containing the reaction mixture was placed in a box illuminated with 20 W neon lamp for 10 min. Results were calculated by subtracting the changes in absorbance of the blank from the sample and dividing it by 0.012 to obtain the McCord - Fridovich units of activity.

Glutathione peroxidase (GPx)

Both the blank and the sample cuvettes contained 0.1 M KPO₄ buffer (pH 7.0), 2 µM EDTA, 10 U/mL glutathione reductase, 4 mM sodium azide, 200 mM NADPH (Sigma-Aldrich, St. Louis, MO, USA) and supernatant of cell lysate. In addition, the sample cuvette contained 1.0 mM glutathione, GSH (Sigma-Aldrich, St. Louis, MO, USA). After 10 minutes of pre incubation at 37° C, the reaction was started by the addition of 1.0 mM H₂O₂ (Sigma-Aldrich, St. Louis, MO, USA) to the blank and sample cuvettes. An additional blank assay in which the buffer was substituted for the supernatant was performed in order to correct the non-enzymatic oxidation of GSH and NADPH by H₂O₂.

Catalase (CAT)

CAT activity was determined by the method described by Aebi [11] using an ultraviolet spectrophotometer (UV-160A, Shimadzu, Japan). The decomposition rate of the substrate H₂O₂ was monitored at 240 nm. A molar absorptivity of 43.6 L mol⁻¹ cm⁻¹ was used to calculate the activity. One unit is equal to 1 µM of H₂O₂ decomposition/min.

Malondialdehyde (MDA) level studies

Supernatant samples (200 µL) were mixed with 200 µL of 8.1 % SDS, 1.5 mL of 0.8 % 2-thiobarbituric acid, TBA and 1.5 mL of 20 % acetic acid (Sigma-Aldrich, St. Louis, MO, USA). After heating at 95°C for 60 minutes, the reaction mixture was cooled under tap water and a mixture consisting of 1 mL of distilled water and 5 mL of n-butanol: pyridine (15 : 1) was added, mixed and incubated for 10 min at 4°C. The mixture was then centrifuged at 200 g for 15 min at 4°C. The absorbance of the resulting organic layer was measured spectrophotometrically (UV-160A, Shimadzu, Japan) at 532 nm and compared to with an external standard of MDA.

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 [13]. 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

$$\% \text{ growth Inhibition} = 100 - (\text{Abs (sample)} / \text{Abs (control)} \times 100)$$

Reverse Transcriptase-PCR

The mRNA expression levels of Bcl-2, BAX and COX-2 were studied using reverse-transcriptase PCR [14, 15]. The Hep G2 cells were grown in 60 mm culture

plates. The first set of Hep G2 cells was untreated. The second set of cells was treated with PMA at a concentration of 50 ng/mL. The third set of cells was treated with the decoction alone at 10 µg/mL concentration. The last fourth set of cells was treated with both decoction and PMA at 10 µg/mL and 50 ng/mL concentrations respectively. 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 37°C with M-MLV reverse transcriptase (Promega, Madison, WI). After the 1 h incubation, the mixture was again incubated at 70°C 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 Bcl-2, BAX, COX-2 and beta-actin. The forward and reverse primers used for Bcl-2, BAX, COX-2 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).

Western blot analysis

The protein expression levels of Cytochrome c and Caspase activated DNase (CAD) 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. The second set of cells was treated with decoction at 10 µg/mL. The last and third set of cells was treated with decoction at 20 µg/mL. The protein was separated by SDS-PAGE on a 10 % separating gel and transferred to nitrocellulose membrane [16, 17]. Addition of primary mouse monoclonal anti-Cytochrome c and rabbit polyclonal anti-CAD 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 Cytochrome c, CAD, and β-actin at the dilution of 1:1000 (in 3 % BSA), 1:1000 (in 3 % BSA) and 1:2000 (in 3 % BSA) respectively at 4°C overnight with gentle shaking. The membrane after incubation was washed thrice with PBS-Tween-20 (5 minutes each) and incubated with anti-mouse monoclonal antibody for Cytochrome c and goat anti-rabbit HRP antibody for CAD (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

The decoction showed altered activities on the normal and the cancerous cells. Figure 1 represents the status of SOD activity in normal (Chang liver cells) and cancerous cell line (Hep G2 cells) with and without treatment of the decoction. In untreated normal and liver cancer cell lines, SOD activities were 78.62 ± 4.76 unit/mg protein and 165.89 ± 9.23 unit/mg protein respectively. This SOD activity significantly increased to 88.89 ± 5.83 unit/mg protein and 184.71 ± 7.65 unit/mg protein in normal and Hep G2 cell lines respectively when treated with 10 µg/mL decoction.

Figure 2 represents the status of GPx activity in normal (Chang liver) and liver cancer (Hep G2) cell lines with and without treatment of decoction. In untreated Chang liver cells and Hep G2 cell lines, GPx activities were 0.66 ± 0.48 unit/mg protein and 0.59 ± 0.34 unit/mg protein respectively. GPx activity significantly increased to 0.83 ± 0.53 unit/mg protein and 0.96 ± 0.47 unit/mg protein in normal and Hep G2 cell line respectively when treated with decoction at 10 µg/mL.

The catalase enzyme activity is depicted in the Figure 3. In untreated normal and Hep G2 cell lines, CAT activities were 0.86 ± 0.04 unit/mg protein and 1.06 ± 0.05 unit/mg protein respectively. After treatment with 10 µg/mL decoction, CAT activity increased to 0.89 ± 0.03 unit/mg protein and 1.28 ± 0.02 unit/mg protein in both normal and Hep G2 cells respectively.

Figure 4 depicts the concentration level of MDA (µM) in normal and cancer cell lines with and without treatment of the decoction. In untreated Chang liver cells and Hep G2 cell lines, MDA levels were 78.62 ± 0.55 µM and 85.89 ± 0.36 µM respectively. The MDA levels decreased to 71.89 ± 0.47 µM and 75.03 ± 0.29 µM in the normal and cancer cells after treatment with 10 µg/mL decoction.

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, 10 and 20 $\mu\text{g}/\text{mL}$) selectively reduced the cell viability significantly in the cancer cells than normal cells (Fig. 5). The IC_{50} value of the decoction was found to be $9.89 \pm 0.83 \mu\text{g}/\text{mL}$ on Hep G2 cancer cells. Total (100 %) growth inhibition was observed at a concentration of 20 $\mu\text{g}/\text{mL}$ of the decoction on the Hep G2 cancer cells whereas this concentration produced only 20 % cell death in the normal Chang liver cells which showed that the decoction has reduced cytotoxic effect on non-cancerous normal cells.

The mRNA expression level of pro-apoptotic BAX from the normal and decoction treated liver cancer cells were found to increase whereas the expression of anti-apoptotic Bcl-2 and COX-2 mRNAs were found to decrease in the decoction treated Hep G2 cells (Fig. 6). So, it was evident that as the concentration of the decoction increased, the expression of pro-apoptotic BAX increased and the expression of anti-apoptotic Bcl-2 and COX-2 decreased, which confirmed the ability of the decoction to disturb the integrity of mitochondrial membrane thereby leading to release of cytochrome c into the cytoplasm which would form the apoptosome

complex by binding with pro-caspase-9 and Apaf-1 triggering the downstream steps of Caspase cascade in the intrinsic pathway of apoptosis.

The protein expression studies of enzymes Cytochrome c and Caspase activated DNase (CAD), which would lead to cell apoptosis when found in cytoplasm; showed that the decoction induced increased expression of both the enzymes (Fig. 7); which can be compared to the expression of house-keeping protein beta-actin providing a relative expression pattern (Fig. 8). As these enzymes play a very significant role in the mitochondria mediated apoptosis pathway, the increase in the expression of Cytochrome c and CAD could be vital in the induction of intrinsic pathway of apoptosis.

Finally, a significant increase in the caspase-3 activity was observed in decoction treated cells compared to control, which indicated the involvement of caspase-3 in decoction induced apoptosis in the Hep G2 liver cancer cells. The activity of the decoction at 10 $\mu\text{g}/\text{mL}$ ($109.74 \pm 3.8 \mu\text{mol}$ of pNA released/min/mL) was comparable to the activity of 5-FU ($105.99 \pm 7.37 \mu\text{mol}$ of pNA released/min/mL) at IC_{50} 5 $\mu\text{M}/\text{mL}$ (Fig. 9).

Fig 1. Activity of Superoxide dismutase (SOD) in normal Chang liver cell line and in hepatoma cell line Hep G2 with and without decoction treatment.

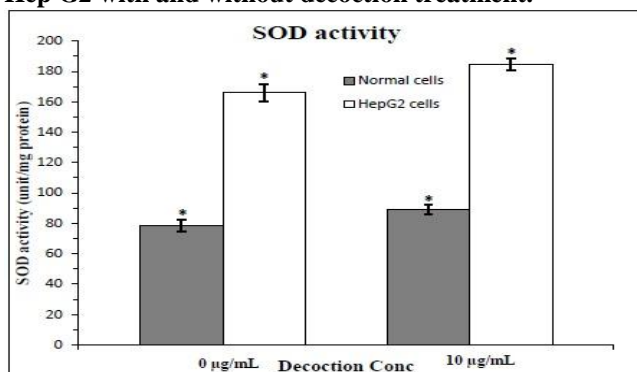


Fig 2. Activity of GPx in normal Chang liver cell line and in hepatoma cell line Hep G2 with and without decoction treatment.

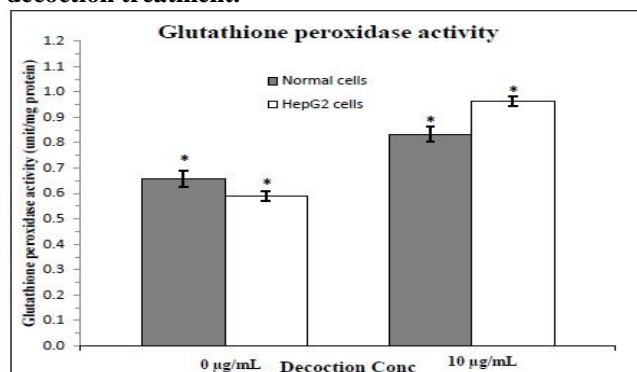


Fig 3. Activity of catalase in normal Chang liver cell line and in hepatoma cell line Hep G2 with and without decoction treatment.

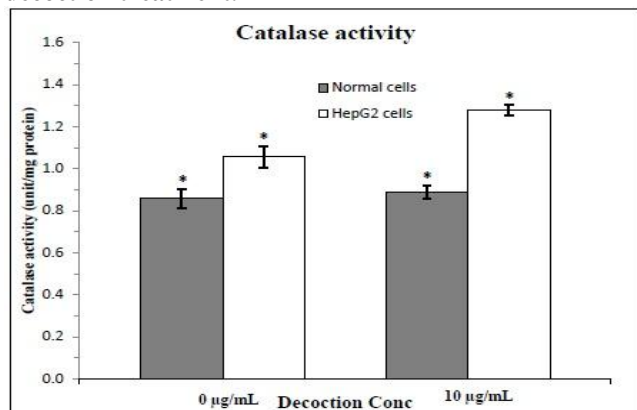


Fig 4. Status of malonaldehyde (MDA) in Hep G2 and in normal Chang liver cell lines with and without treatment of decoction

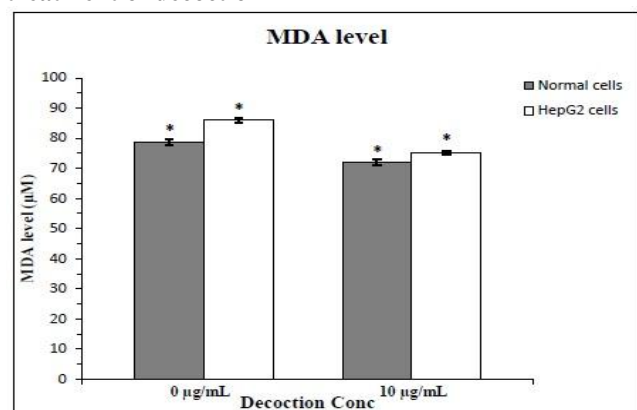


Fig 5. Dose dependent response of decoction on the cell viability of Hep G2 and normal Chang liver cells

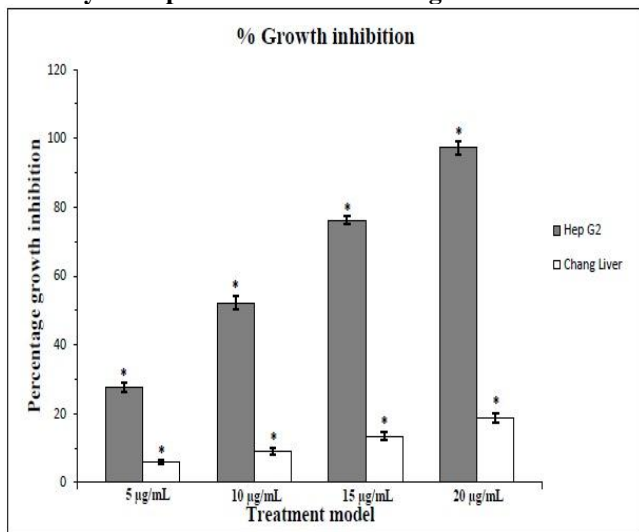


Fig 6. RT-PCR gel showing mRNA expression of pro-apoptotic BAX and anti-apoptotic Bcl-2 and COX-2

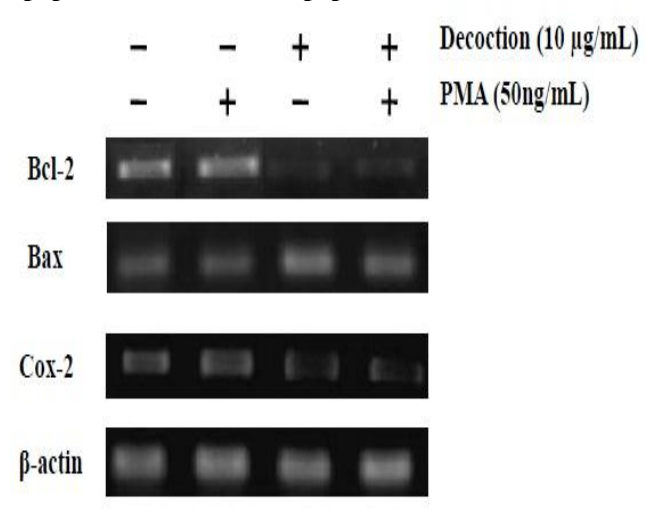


Fig 7. Western blot of pro-apoptotic proteins Cytochrome c and Caspase activated DNase (CAD)

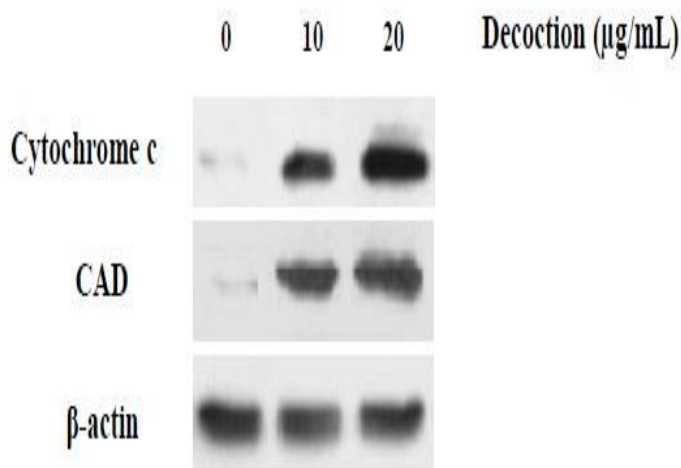


Fig 8. Relative protein expression of Cytochrome c and CAD compared to β-actin

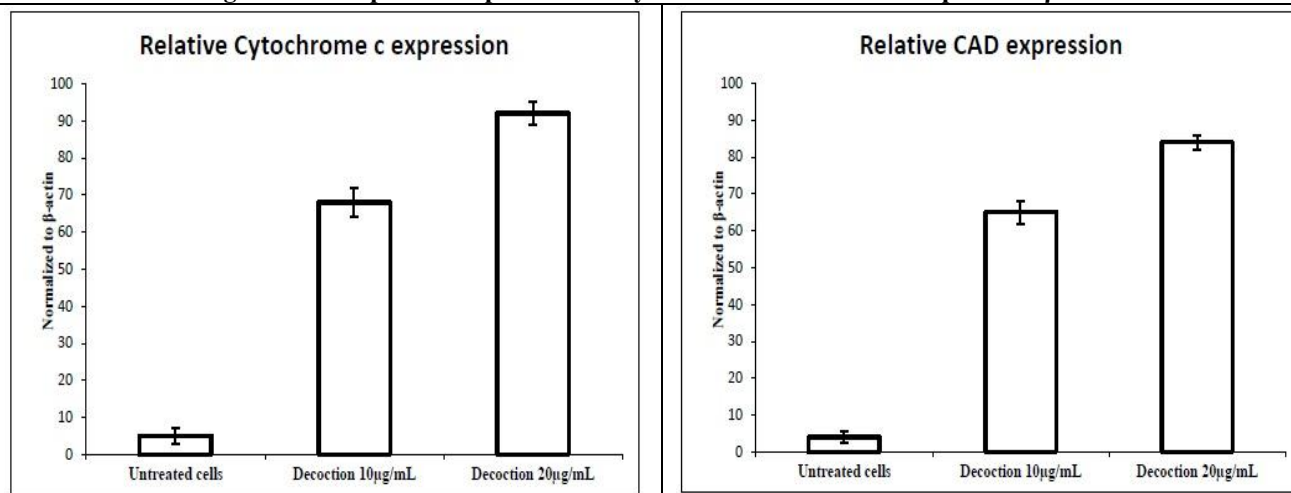


Fig 9. Effect of decoction on the Caspase-3 release in Hep G2 cells when treated with decoction at different concentrations.

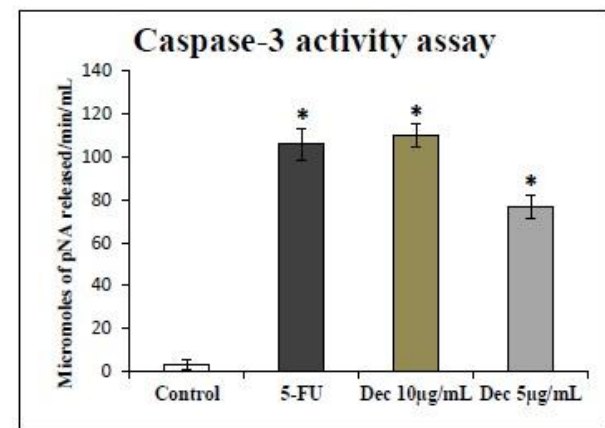


Fig 10. Multiple mechanisms of action of the decoction on the hepatic normal and cancerous cell lines.

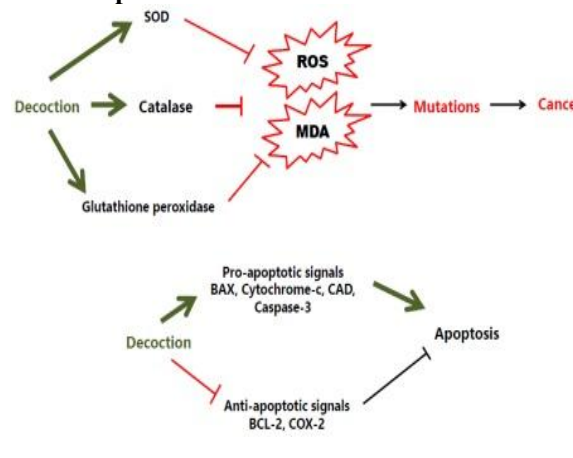


Table 1. Forward and reverse primers for the genes

Genes	Forward primer (5' - 3')	Reverse primer (5' - 3')
Bcl-2	ATGGACGGGTCCGGGGAG	TCAGCCCATCTTCTTCCA
BAX	CAGCTGCACCTGACG	ATGCACCTACCCAGC
Cox-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT
β-actin	GTTTGAGACCTTCAACACCCC	GTGGCCATCTCCTGCTCGAAGTC

DISCUSSION

In India, the earliest mention of the use of medicinal plants is to be found in Rigveda which was written between 4500-1600 BC [18]. Turmeric (*Haridra*) is one such medicinal plant explained extensively in Indian material medica (*Dravyaguna Sastra*). It is known to be one of the oldest spices that have been used in Western and Southern parts of India for thousands of years and is a major part of Ayurvedic medicine. *Curcuma longa* Linn is used as medicinal plant in day to day practice in Indian homes for various ailments. 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. Additionally, curcumin is well tolerated in humans [19]. Black pepper (*Piper nigrum*) and its active component piperine can stimulate the digestive enzymes of pancreas and intestines and also increases biliary bile acid secretion when orally administered. Medicinally black pepper can be used for digestive disorders like large intestine toxins, different gastric problems, diarrhea and indigestion [20, 21]. Ginger (*Zingiber officinale*) is widely used around the world in foods as a spice. Ginger rhizome has a long history of use in Chinese and Ayurvedic medicine as an antiemetic, antipyretic, and anti-inflammatory agent. The anticancer effects of ginger are thought to be attributed to various constituents including vallinoids, viz. gingerol and paradol, shogaols, zingerone, and Galanals A and B.

Ginger has a long history of use as an anti-inflammatory and many of its constituents have been identified as having anti-inflammatory properties [22].

Food provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention. 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 [23]. Hot water as the extraction medium was used because water is the main medium used to prepare foods for diets for patients suffering from medical conditions and also because of similar procedures in the literature. Most foods of plant origin contain components which are active as inhibitors of undesirable oxidative processes not only in food but very often in the human body as well [24].

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress, which plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process. Antioxidants are substances that when present in low concentrations, compared to those of an oxidisable substrate significantly delay or prevent oxidation of that substance. At present most of the

antioxidants used for this are manufactured synthetically. The main disadvantage with the synthetic antioxidants is their side effects when taken *in vivo*. Plants are a potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are primary or secondary metabolites of plants [25].

Curcumin (diferuoyl methane) is a phenolic compound and a major component of *Curcuma longa* L. Curcumin inhibited 97.3% lipid peroxidation of linoleic acid emulsion at 15 µg/mL concentration (20 mM). On the other hand, butylated hydroxyanisole (BHA, 123 mM), butylated hydroxytoluene (BHT, 102 mM), α -tocopherol (51 mM) and trolox (90 mM) as standard antioxidants indicated inhibition of 95.4, 99.7, 84.6 and 95.6% on peroxidation of linoleic acid emulsion at 45 µg/mL concentration, respectively. In addition, curcumin had an effective DPPH scavenging, ABTS scavenging, DMPD scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, ferric ions (Fe^{3+}) reducing power and ferrous ions (Fe^{2+}) chelating activities [26]. In a study by Gulcin I, it was found that the aqueous extract of *Piper nigrum* at 75 µg/mL concentration of showed 95.5% inhibition on peroxidation of linoleic acid emulsion. On the other hand, at the same concentration, standard antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and alpha-tocopherol exhibited 92.1%, 95.0%, and 70.4% inhibition on peroxidation of linoleic acid emulsion, respectively [27]. *Zingiber officinale* contains a number of antioxidants such as beta-carotene, ascorbic acid, terpenoids, alkaloids, and polyphenols such as flavonoids, flavones glycosides, rutin, etc [28]. Easily cultivable, *Zingiber officinale* with its wide range of antioxidants can be a major source of natural or phytochemical antioxidants [29]. In a study by Stoilova *et al.*, [30] it was found that the aqueous extract of ginger controlled the quantity of free radicals generated during various antioxidant assays and the extract also reduced the peroxidation of lipids. Each spice used in the decoction had good antioxidant activity individually and when combined to form a decoction, it showed considerably higher activity compared to the untreated control.

Curcumin has been found to possess anti-cancer activity by acting on various biological pathways including oncogene expression, mutagenesis, apoptosis and metastasis. Curcumin is found to be well tolerated in humans, which makes it a potential candidate as both a prophylactic and therapeutic agent. The only concern with regard to curcumin is its limited systemic bioavailability [19]. But studies have shown that piperine present in the black pepper increases the bioavailability of curcumin by 2000% and that piperine enhances the serum concentration, extent of absorption and bioavailability of

curcumin in both rats and humans with no adverse effects [31]. Moreover, studies have shown that extracts of *P. nigrum* showed anti-mutagenic and anti-tumor activity by modulating lipid peroxidation and through the activation of antioxidant enzymes [32]. A study by Choudhury *et al.*, [33] showed that the aqueous extract of ginger possessed anti-proliferative activity by interacting with the microtubule machinery and thus inducing apoptosis in the cancer cells.

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 increase in the levels of antioxidant enzymes and also elicited the same apoptosis inducing ability in the liver cancer cells by down regulating the anti-apoptotic factors and up regulating the pro-apoptotic factors (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.

CONCLUSION

The growing interest in biological activities 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 activate the antioxidant enzymes thereby blocking the damaging activity of the free radicals. The decoction induced apoptosis in the Hep G2 liver cancer cells via the mitochondria-dependent pathway. The decoction induced lower apoptotic activity on the normal Chang liver cells showing that the decoction could differentiate between normal and cancerous cells. The combined activity of aqueous extract of the spices in the decoction is found to have a synergistic antioxidant and growth inhibitory apoptotic inducing activity in the liver cancer cells. This is the first report on the mechanism of apoptosis induction by the combined synergistic activity of extract of two or more spices in a decoction and these findings would provide yet another method of targeting cancer cells using combination of extracts exploiting their synergistic activity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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