Tropical Journal of Pharmaceutical Research February 2012; 11 (1): 51-61

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v11i1.7

Research Article

Effect of Standardized Decoction of *Nigella sativa* Seed, *Hemidesmus indicus* Root and *Smilax glabra* Rhizome on the Expression of *p53* and *p21* Genes in Human Hepatoma Cells (HepG2) and Mouse Liver with Chemically-Induced Hepatocarcinogenesis

Sameera R Samarakoon[1], Ira Thabrew[1], Prasanna B Galhena[2] and Kamani H Tennekoon[1]

[1]Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, 90, Cumarathunga Munidasa Mawatha, Colombo 3, [2]Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, Thalagolla Road, Ragama, Sri Lanka

Abstract

***Purpose*:** To evaluate *in vitro* (using human hepatoma HepG2 cells) and *in vivo* (using mouse liver with diethlynitrosamine (DEN)-induced hepatocarcinogenesis) effect of a standardized decoction on the expression of *p53* (tumour suppressor) and *p21* (cyclin kinase inhibitor) genes with the long-term goal of developing the formulation into a globally acceptable therapy for hepatocellular carcinoma (HCC).
Methods: The effect of the decoction on (a) mRNA and (b) protein expression of *p53* and *p21* genes in HepG2 cells and mouse livers with DEN-induced early hepatocarcinogenesis were evaluated by (a) reverse transcription PCR (RT-PCR) and (b) immunohistochemical and Western blot analysis, respectively.
Results: The results demonstrated that the decoction significantly ($p < 0.001$) enhanced the expression of *p53* and *p21* genes in a time- and dose-dependent manner in HepG2 cells. A dose of 75 µg/ml significantly increased p53 mRNA at 24 and 48 h and p21 mRNA at 12, 24, 48 h of incubation with the decoction ($p < 0.01$). Induction of hepatocarcinogenesis in mice significantly increased hepatic expression of both p53 and p21 compared to distilled water control ($p < 0.001$), while treatment with the decoction further enhanced expression of both genes in DEN-induced hepatocarcinogenesis ($p < 0.01$).
Conclusion: Overall, the findings demonstrate that the decoction may mediate its reported antihepatocarcinogenic effect, at least in part, through the modulating activities of genes involved in tumour suppression and cell cycle arrest.

Keywords: *Hemidesmus indicus, Nigella sativa, Smilax glabra*, HepG2 cells, Diethylnitrosamine, *p53, p21.*

Received: 10 May 2011 *Revised accepted: 11 December 2011*

Corresponding author:* ***Email:* *ranganath148@yahoo.com;* ***Tel:*** *+94 112 552 528;* ***Fax:*** *+94 112 552 529*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the fifth commonest neoplasm in the world. Despite the availability of curative surgical methods, the recurrence rate of HCC is reported to be as high as 50 % at 2 years [1]. Similarly, adjunct therapies such as radiotherapy, chemotherapy and hormonotherapy have not reduced the number of deaths from HCC to the expected level [2]. Further, these treatments produce some very unpleasant side effects such as toxicity to normal cells, bone marrow depletion, adverse effects on the gastro-intestinal tract etc. Therefore, there is an urgent need to search for better control and preventive methods that would not only reduce mortality of HCC patients, but would also have less side effects.

In many Asian countries, some poly-herbal preparations are considered by traditional medical practitioners to be useful for the treatment of cancer, despite lack of evidence from scientifically controlled studies to validate these claims. One such remedy that has been used for many years by a particular family of Ayurveda physicians in Sri Lanka is a decoction comprised of *Nigella sativa* seeds, *Hemidesmus indicus* roots and *Smilax glabra* rhizomes [3,4]. Previous *in vivo* investigations confirmed that this decoction protects against chemically induced carcinogenesis in rat liver, with no significant toxic side effects, thus providing support for its ethnopharmacological use for cancer therapy [3,4]. In the past few years, with the sequencing of the human genome providing us with an enormous number of potential targets associated with cancer, the focus of drug development for cancer therapy has moved from cytotoxic compounds identified by screening to identification of compounds that can act at specific molecular targets associated with carcinogenesis. *p53* is a tumour suppressor gene that plays an important role in maintaining genetic stability and cell cycle regulation after induction of DNA damage. It is the most commonly mutated gene in human cancers [5]. p21, is a cyclin kinase inhibitor (CKI) involved in cell growth arrest [6]. Expression of p21 is controlled at the transcriptional level by both p53 dependent and -independent mechanisms [7]. Since the integrity of p53 has been reported to be critical in cancer predisposition, onset and therapeutic response, interventions to restore wild-type p53 is an attractive approach for cancer therapy [8]. Therefore, identification of natural agents that can restore wild-type p53 (wt p53) may lead to the development of novel drugs for cancer treatment.

The aim of the present investigation was to evaluate in vitro (using human hepatoma HepG2 cells) and *in vivo* (using mouse liver with diethyl nitrosamine induced hepatocarcinogenesis), the effect of a standardized decoction comprised of *N. sativa* seed*, H. indicus* root and *S. glabra* rhizome on the expression of *p53* (tumour suppressor) and *p21* (cyclin kinase inhibitor) genes with the long term goal of developing the above herbal formulation into a form that could be used by itself or as an adjunct to existing therapies for the treatment of HCC.

EXPERIMENTAL

Collection of plant material

Plant materials for the preparation of extracts were purchased from a reputed vendor of herbal material used by traditional medical practitioners in Sri Lanka (D.J. Fernando Pvt Ltd, Gabose Lane, Colombo 13). Their identities were confirmed by a taxonomist (Mr. Gunarathne Silva) of the Bandaranayaka, Memorial Ayurvedic Research Institute (BMARI), Navinna, Maharagam, Sri Lanka. Voucher specimens of *N. sativa* seed*, H. indicus* root*,* and *S. glabra* rhizome (nos. UOC/IBMBB/2009/01, UOC/IBMBB/2009/02 and UOC/IBMBB/2009/03, respectively) were deposited at the herbarium of the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

Preparation of plant decoction

The plant decoction was prepared according to the method recommended traditionally for administration to cancer patients [3,4]. Sixty grams (60 g) of plant material (composed of a mixture of 20 g each of *N. sativa* seeds, *H. indicus* roots and *S. glabra* rhizomes) were ground and boiled gently with 1.6 L distilled water for approximately 3 h to reduce the volume to 200 ml. The extract was then filtered through a layer of muslin, the filtrate centrifuged at 3000 g for 15 min to remove any debris, and the supernatant freeze-dried and stored at -20 ^{0}C until required. Prior to use, the freeze-dried extract was reconstituted with the appropriate volume of distilled water containing 1 % DMSO.

HepG2 cell culture and treatment

HepG2 cells (ECACC, Salisbury, UK) were grown in monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37 ^{0}C in 95 % air / 5 % CO_2 atmosphere, and 95 % humidity. The media were purchased from GIBCO, Invitrogen Corporation, U.S.A, while other chemicals were obtained from Sigma Aldrich (Gillingham, Dorset, UK). The cells were harvested by trypsinization and 2 x 10^5 cells / flask were seeded into 18 T_{25} (25 cm^2) flasks. Cell cultures were initially maintained in DMEM medium (5 ml) for 24 h at 37 ^{0}C in 95 % air/5% CO_2 atmosphere, and 95 % humidity. After 24 h, the medium from each flask was replaced with fresh medium and cell cultures were treated with different concentrations of the decoction. The concentrations used were 75, 150, 300, 600, and 1200 µg/ml, and triplicate cell cultures were exposed to each concentration. Control cell cultures received only medium containing 1 % DMSO. Treated and control cell cultures were incubated for a further 12, 24 or 48 h. At the end of the incubation period, the cells were harvested and (a) total RNA and protein were extracted for the reverse transcription PCR (RT- PCR) quantification of *p53*^{wt} and *p21* genes, and western blot analyses of p53^{wt} and p21 proteins, respectively, or (b) immunohistochemical focusing of the p53^{wt} and p21 proteins.

The viability of cells (2 x 10^5 cells/ml) incubated with the decoction for 12, 24 and 48 h with different doses of the decoction were evaluated by the MTT assay [9] as described previously [10].

Experimental animals and treatments

C3H male mice (5-6 weeks old) were purchased from the Medical Research Institute (M.R.I.), Colombo, Sri Lanka. They were housed in a temperature–controlled room (25 ± 2 ^{0}C) and maintained on a standard laboratory diet, prepared by the M.R.I. according to a formula recommended by the WHO [11], and water *ad libitum*. Ethical clearance for the animal experiments was obtained from the Ethical Review Committee of the M.R.I., Colombo, Sri Lanka. The animals were maintained in accordance with guidelines for ethics review of research proposals involving animals in Sri Lanka [12].

A total of thirty two (n = 32) male C3H mice (5 - 6 weeks old) were randomly divided into four equal groups (8 mice/group). The mice in group 1 (distilled water control group) were orally fed with distilled water for a period of four weeks. Mice in group 2 (decoction control group) were orally fed with the decoction at a dose of 10 g/kg body weight/day for four weeks. This dose was based on the decoction dose which demonstrated maximum anti-hepatocarcinogenic effects against diethyl-nitrosamine (DEN)-induced HCC in rats [3]. Distilled water or decoction was orally administered to mice via a Sondi needle [3]. Mice in group 3 (positive control group) and group 4 (test group) were injected with DEN in 0.9 % NaCl at a dose of 20 µg/g body weight as a single intraperitoneal injection on day 1 to initiate hepatocarcinogenesis [13]. Throughout the experiment, the animals had

free access to standard diet and water. From day 2 onwards, DEN-induced animals in the positive control and test groups were orally administered distilled water and the decoction (at a dose of 10g/kg body weight/day) respectively, for a period of four weeks. Animals in the test and positive control groups were subjected to a second dose of DEN (20 µg/g body weight, i.p.), 30 min. before they were sacrificed [14]. At the end of the fourth week, liver specimens from all animals were excised under diethyl ether anaesthesia, immediately washed with RNase-free ice cold phosphate buffered saline (pH 7.4) and used for (a) RT-PCR quantification of *p53* ^{wt} and *p21* genes, (b) western blot analysis of p53 and p21 proteins, and (c) immunohistochemical focusing of the p53 and p21 proteins.

Isolation of total RNA and RT-PCR analysis

Total RNA was isolated from the HepG2 cells, or liver sections from C3H/H mice, using TRIzol reagent (Invitrogen, Life Technologies; USA) according to the manufacturer's specifications. Total RNA concentration in the final elutes was determined by using a spectrophotometer (UV-1700, pharmasprc, Shimadzu, Japan). Each sample of isolated RNA was reverse-transcribed by M-MLV reverse transcriptase system (A3500, Promega Cooperation, Madisons, U.S.A) to synthesize single stranded cDNA as follows. RNA sample (2µg) was initially mixed with 0.5µg of random primers. Subsequently, RNA-random primer mix was incubated for 60 min at 37^0C in reverse transcription reaction mix containing 5µg M-MLV 5X reaction buffer, 10 mM dNTP mix, 25 units of RNasin and 200 units of M-MLV reverse transcriptase in PCR water adjusted to 25 µl.

GAPDH amplification was performed as an internal control. Each PCR was carried out in a master mix containing 1X Green Go Taq Flexi Buffer, 2 mM $MgCl_2$, 10 mM dNTPs and 1.25 U GoTaq DNA polymerase (Promega Inc. US) with 0.2 mM of respective forward and reverse primers in 50 µl reaction mix. PCR amplification was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) and the PCR conditions for both human and mouse *GAPDH* were 30 cycles at 94 ^{o}C for 30 s, at 54 ^{o}C for 30 s, at 72 ^{o}C for 1 min. PCR conditions for both human and mouse *p53* were 35 cycles at 94 ^{o}C for 1.5 min, at 55 ^{o}C for 1 min, at 72 ^{o}C for 1 min. PCR conditions for mouse *p21* were 30 cycles at 94 ^{o}C for 1.5 min, at 62 ^{o}C for 1 min, at 72 ^{o}C for 1 min and for human *p21* were 35 cycles at 94 ^{o}C for 1.5 min, at 52 ^{o}C for 1 min, at 72 ^{o}C for 1 min. Primers were purchased from Integrated DNA Technologies, USA. These were, human *p53*: forward primer 5`-GTTCCGAGAGCTGAATGAGG-3`, reverse primer 5`-TCTGAGTCAGGCCCTTCTGT-3`; human GAPDH : forward primer 5`-GAAGGTGAAGGTCGGAGTC-3`, reverse primer 5`-GAAGATGGTGATGGGATTTC-3`; human *p21*: forward primer 5-GTCACCCTCCAGTGGTGTCT-3', reverse primer 5'- TGCGTTCACAGGTGTTTCTG-3'; mouse *p53*: forward primer 5'-CCCGAGTATCTGGAAGAC AG – 3', reverse primer 5'- ATAGGTCGGCGGTTCAT – 3'; mouse GAPDH: forward primer 5' - GAGGGGCCATCC ACAGTCTTC – 3', reverse primer 5' - CATCACCATCTTCCAGGAGCG – 3' ;and mouse *p21*: forward primer 5'-AGTGTGCCGTTGTCTCTTCG – 3', reverse primer 5'-ACACCAGAGTGCAAGACAGC–3'.

Amplified PCR products were subjected to electrophoresis at 40 V through 2% agarose gel (Sigma, U.S.A) for 60 min. A 100 bp DNA ladder marker was used as a molecular marker. Gels were stained with 0.5 mg/ml ethidium bromide in TAE buffer. The gel bands were examined by using a Gel Doc imaging system and the intensity of each band was measured by using BIO RAD quantity one soft ware. The size of the amplified PCR products were 159 bp for human *p53*, 150 bp for mouse *p53*, 216 bp for human *p21*, 311 bp for mouse *p21*, and

226 bp for human *GAPDH* and 350 bp for mouse *GAPDH*.

Western blot analysis

For *in vitro* evaluation of *p53* and *p21* proteins in HepG2 cells by western blot analysis, cells were harvested after 12, 24 and 48 h incubation by trypsinization, and centrifuged at 1000 g for 10 min at 4 ^{o}C. The pelleted cells were solubilized for 15 min at 4 ºC in lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 0.25 % Na-deoxycholate, 1 mM PMSF (phenylmethylsulfonyl fluoride) and proteinase inhibitor cocktail]. Lysates were centrifuged at 12,000 *g* for 15 min at 4 ºC to remove insoluble material. The supernatant was collected and protein concentration was determined using the Bradford's reagent. For Western blot analysis, samples (40 µg each) were separated by electrophoresis on 10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membrane.

For evaluation of p53 and p21 protein expression in mouse liver, frozen mouse liver sections (200 mg of each liver specimen) were completely homogenized in 5 ml of ice cold RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1 % NP40, 0.25 % Na-deoxycholate, 1 mM PMSF [phenyl-methylsulfonyl fluoride] supplemented with proteinase inhibitor cocktail containing aprotinin and leupeptin). Homogenate was immediately centrifuged at 12,000 g for 15 min at 4 ^{0}C and the protein content of the supernatant was evaluated by Bradford's reagent using pre-plot standard curve. Equal amount (40µg) of liver protein lysate of each experimental group were separated by 10% SDS-PAGE and transferred overnight on to nitrocellulose paper at 4^{0}C.

Nitrocellulose membranes onto which protein extracted from HepG2 cells and mouse livers were transferred were pre-blocked with 5% non fat dry milk in PBS for 30 min. Then the membranes were incubated for 16 h at 4 ºC before incubating with specific primary antibodies which detect both human and mouse proteins. Bands were normalized using β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After three washes of 10 min each with 1x TBST (50mM Tris-HCl, pH7.4, 150mM NaCl, 1 % Tween 20), each membrane was incubated with the secondary antibody for 30 min at room temperature. The membrane was then washed three times with TBST (10 min/wash) and bands visualized using western blot detection system (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical analysis of p53 expression in HepG2 cells and mouse liver

Sample preparation

HepG2 cells were harvested by trypsinization, and 2 x 10^5 cells/ml were plated on sterile coverslips placed in wells of a 24 well cell culture plate. The cells (on coverslips) were initially maintained in DMEM medium (1 ml/coverslip) for 24 hrs at 37 ^{0}C in 95% air/ 5% CO_2 atmosphere, with 95% humidity. Cultures were then treated with the standardized decoction (600µg/ml or 1200 µg/ml) and incubated for 24 h. The doses of 600 µg/ml and 1200 µg/ml decoction were selected for this experiment based on the doses that produced significant enhancement of *p53* and *p21* gene expression in the experiments described above. Cells were then washed with phosphate buffer saline (PBS), fixed with cold 1:1 acetone: methanol for 15 min and air-dried.

Sections of mouse liver were immediately fixed in 10% formaldehyde, embedded in paraffin. Subsequently, 4 µm sections were used for staining after de-paraffinization in xylene, and rehydration through graded ethanol (AR, 80 – 100 %). p53 and p21 activities were monitored in de-paraffinized tissue sections by exposing each section to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0), using a pressure cooker [15].

Immunohistochemical analysis

Primary and secondary antibodies and the ABC staining kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The coverslips with HepG2 cells and slides containing deparaffinized mouse liver sections were quenched with 1% BSA in 0.05 M Tris/HCl (pH 7.6) for 30 min, and then incubated for 60 min at 37 ^{0}C with anti-p53 (sc- 6343) and anti-p21 (sc-397) rabbit polyclonal antibodies, diluted 1: 200. After washing with PBS (two times), the cells and tissue sections were incubated for 60 min with goat anti-rabbit IgG-HRP (sc-3837 diluted 1: 1000), washed again with PBS (two times) and visualized with ABC staining kit. Cells / tissue sections were counterstained with hematoxylin and examined under a light microscope.

Statistical analysis

The results of RT-PCR were analyzed using Prism 2.01 (Graph Pad Prism, San Diego, USA). One-way analysis of variance (ANOVA) with Dunnett's post test for multiple comparisons was used to find out the concentrations and time points at which the p53 and *p21* expression were significantly increased in comparison to controls. Two-way ANOVA analysis was used to detect the effect of different concentrations of the decoction and duration of the treatment on the mRNA expression of *p53* and *p21* in HepG2 cells. One-way ANOVA with Bonferroni post test was used to compare all pairs of columns and used to analyze the results of *p53* and *p21* mRNA expression in mouse liver.

RESULTS

Preparation of plant decoction

The yield of the freeze-dried decoction was 15.2 ± 0.3.%

Effect of decoction on HepG2 cell viability

Alterations in viability of cells incubated for 12, 24 and 48 h with different doses of the decoction are shown in Fig 1. At all three post-incubation time points, statistically significant differences in viability from zero time were observed only at doses above 1200 µg/ml. Hence, in the experiments to evaluate effects on p53 and p21 expression, doses of decoction at or below 1200 µg/ml were used.

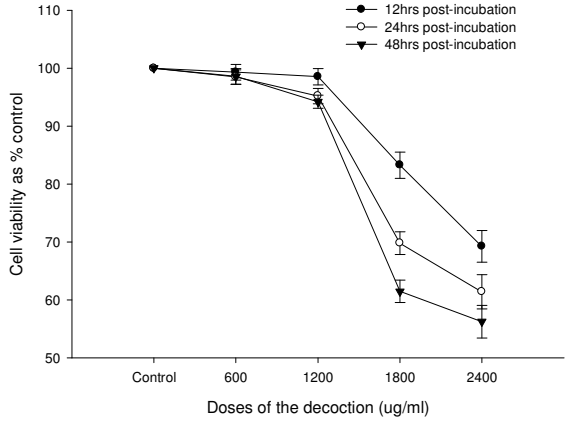

Figure 1: Effect of aqueous extract on overall cell viability-MTT assay (values are mean ±SEM)

Effect of decoction on p53 gene expression in HepG2 cells

In the RT-PCR evaluation, *p53* gene expression was normalized to the house keeping GAPDH gene. Results of the RT-PCR (Fig 2) demonstrated that the standardized decoction enhanced *p53* mRNA expression in human HepG2 cells in a significant ($p < 0.001$) time and dose dependant manner. The lowest doses at which statistically significant ($p < 0.001$) *p53* mRNA up regulation was observed at 12, 24 and 48 h post-incubation were 300, 75 and 75 µg/ml, respectively. The results of RT-PCR analysis were supported by findings of the immunohistochemical evaluation and Western blot analysis of the p53 protein expression in HepG2 cells (Fig 3).

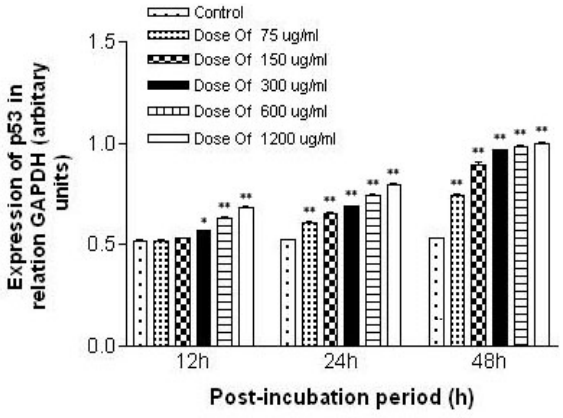

Figure 2: Effect of decoction on mRNA expression of p53 gene in HepG2 cells (*p <0.05, **p < 0.01 when compared to control)

Effect of decoction on p21 gene expression in HepG2 cells

As with *p53, p21* gene expression was also normalized to the house keeping *GAPDH* gene. RT-PCR evaluation (Fig. 4) showed that in HepG2 cells, the standardized decoction induced over expression of *p21* mRNA in a significant (p < 0.001) time and dose dependant manner. The lowest dose at which statistically significant (p < 0.01) *p21* mRNA up regulation was observed at 12, 24 and 48 h post-incubation was 75 µg/ml.

The results of RT-PCR analysis were supported by findings of the western blot analysis and immunohistochemical analysis of the p21 protein expression in HepG2 cells (Figs 3B and 5A).

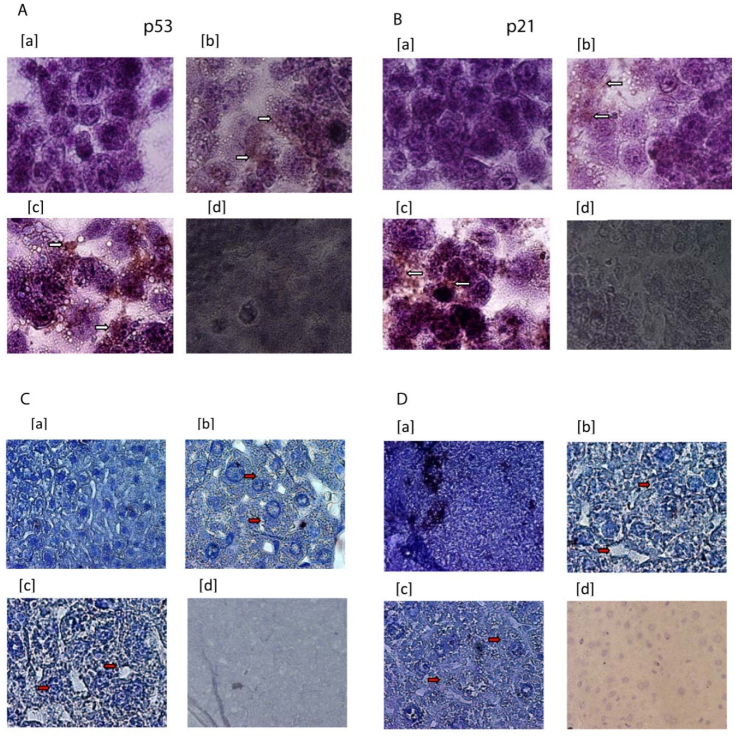

Figure 3: Immunohistochemical analysis of *p53* and *p21* in HepG2 cells and mouse liver. *Expression of p53 (A) and p21 (B) protein in HepG2 cells (shown by arrows) was confirmed by immunohistochemical analysis: [a] control (untreated) [b] treated with the decoction (600 µg/ml), [c] treated with the decoction (1200 µg/ml). Section [d] represents the negative control. Expression of p53 (C) and p21 (D) protein (shown by arrows) in mouse liver [a] neither induced with DEN nor treated with the decoction (normal control), [b] treated with the decoction after induction, [c] induced with DEN, [d] the negative control. Hematoxyline was used for counterstaining*

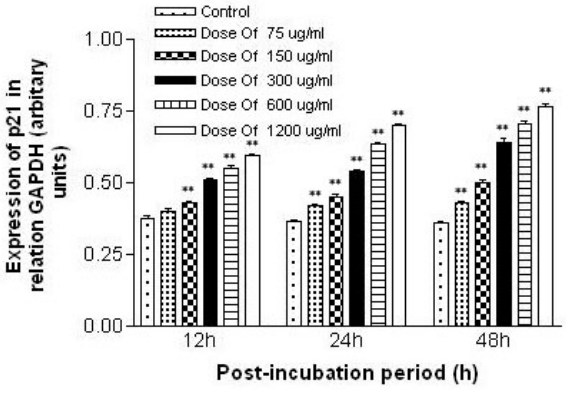

Figure 4: Effect of decoction on mRNA expression of p21 gene in HepG2 cells (*p < 0.05, **p <0.01 when compared to the control)

Effect of decoction on p53 and p21 gene expression in mouse liver

In mice treated only with DEN, an increased *p53* expression was observed (on RT-PCR evaluation of *p53* mRNA) in comparison to livers of animals not exposed to this hepatocarcinogen (Fig 6a). A similar increase was also observed with *p21* (Fig 6b). However, as evident from Fig 6, expression of both *p53* and *p21* genes were significantly (p < 0.01) further up-regulated by administration of the decoction to post-DEN treated mice, although the decoction by itself did not significantly modulate the expression of either gene.

Immunohistochemical and western blot analysis evidence shown in Figs 3 (C,D) and 5 (B) confirm that the decoction can enhance expression of both *p53* and *p21* genes in mouse liver in early stage of hepatocarcinogenesis.

DISCUSSION

A number of studies carried out over the last few decades, especially in China, on prevention and treatment of HCC have led to the identification of several herbal compounds and formulations that can affect the initiation, promotion as well as the progression of HCC [13]. Previous investigations [3,4] have shown that the decoction, comprised of *N. sativa* seed, *H. indicus* root, and *S. glabra* rhizome, has anti-hepatocarcinogenic potential without producing any significant toxic side effects. Although cytotoxicity [10,16], antioxidant activity [17] and anti-inflammatory activity are possible anti-cancer mechanisms, the under-

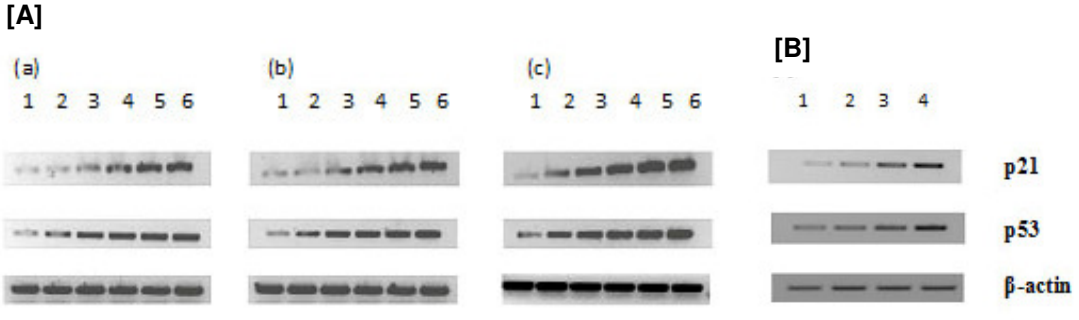

Figure 5: Western blot analysis of effects of the decoction on p53 and p21 expression in HepG2 cells and mouse liver with DEN-induced early hepatocarcinogenesis.
[A]-*Western blot analysis of p53 and p21 expression in HepG2 cells treated with different concentrations (lane 1-control, lane 2-75 µg/ml, lane 3-150 µg/ml, lane 4-300 µg/ml, lane 5-600 µg/ml, lane 6-1200 µg/ml) of the standardized decoction at (a) 12 h post incubation, (b) 24 h post-incubation and (c) 48 h post incubation was performed as described in the Materials and Methods section. Normalization of bands was performed using β-actin protein. [**B**] Western blot analysis of p53 and p21 expression in mouse livers with DEN-induced early hepatocarcinogenesis treated with the decoction was performed as described in the Materials and Methods section. Lane 1 - distilled water control, Lane 2 - decoction only, Lane 3- induced with DEN, Lane 4 - treated with the decoction post DEN- induction.*

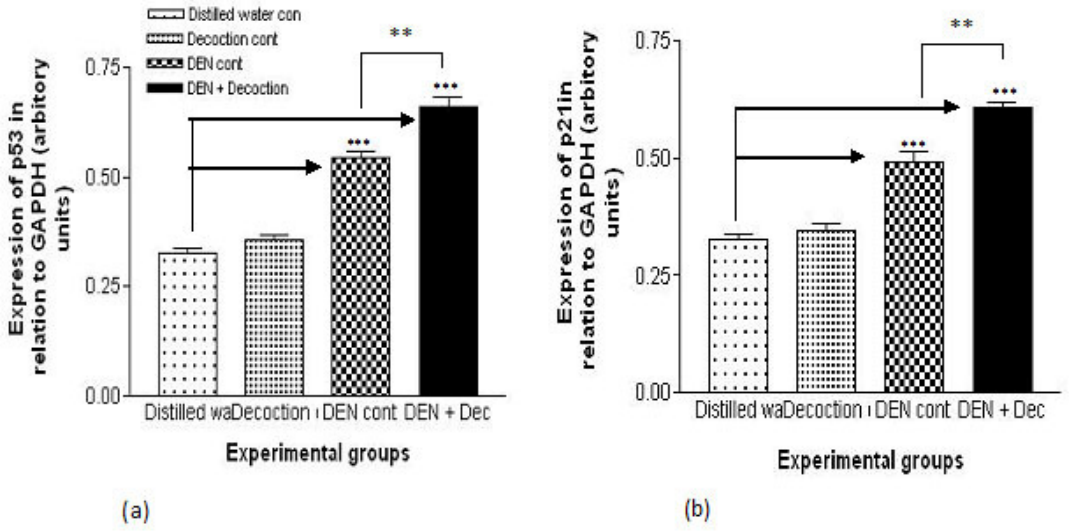

Figure 6: mRNA expression of p53 and p21 in mouse liver (***$p < 0.001$) compared to distilled water control; **$p < 0.01$ compared to DEN control

lying molecular mechanisms by which the decoction mediates its anti-carcinogenic effects have not yet been investigated.

Inappropriate expression of tumor suppressor/pro-apoptotic and cell cycle related proteins, such as p53 and p21, is considered to be one of the major factors contributing to HCC development [18]. Several mechanisms of apoptosis induction by p53 have been identified involving transcriptional and/or nontranscriptional regulation of its downstream effectors. For example, tumor suppressor p53 is known to induce apoptosis by transcriptional up-regulation of pro-apoptotic genes such as *Bax*, *NOXA, PUMA, AIP, Apaf-1*, and by transcriptional repression of *Bcl2* and inhibitors of apoptosis [19].

Supportive evidence for the ability of the decoction under test to enhance the expression of *p53* and *p21* genes is provided by results obtained in the western blot analysis.

The Cdk inhibitor p21 is often responsible for stress-induced p53-dependent and p53-independent cell cycle arrest, and deregulation of the transcriptional protein p53 can induce p21/ WAF1 expression which inhibits cyclin-dependent kinases for the control of both G1 and G2/M checkpoints in the cell cycle or trigger apoptosis [20].

In the present study we have demonstrated that transcription (as shown by RT-PCR) and translation (as shown by immunohisto-chemistry and Western blot analysis) of both *p53* and *p21* genes are significantly enhanced in response to the decoction comprising of *N. sativa* seed, *H. indicus* root, and *S. glabra* rhizome. Up regulation of expression of both *p53* and *p21* genes in a time- and dose-dependent manner was evident in the human hepatocellular carcinoma cell line HepG2 *in-vitro* and in the DEN-induced mouse hepatic cancer model *in-vivo.* Although up regulation of expression of p53 in the mouse liver in response to DEN alone has been previously reported [21,22], we observed further enhancement of p53 expression in response to the decoction over and above DEN-induced p53 expression. It has been suggested that the induction of *p53* by DEN is not related to a cytotoxic effect, but most likely results from DNA strand breaks induced directly by this genotoxic compound or formed as a result of DNA-repair [20,21].

One of the compounds isolated from *N. sativa* seeds is an anticancer compound, thymoquinone. We have previously demonstrated that thymoquinone is not present in the decoction used in the present experiment [10]. Therefore, some other water-soluble compound(s) is(are) likely to be responsible for the up regulation of p53 and p21 mRNA, and protein levels observed in the present study. Other investigators have reported that several plant compounds such as resveratrol, curcumin and eurycomanone [23] mediate their anti-proliferative effects trough apoptosis and cell cycle arrest. Decoction comprising of *N. sativa* seed, *H. indicus* root, and *S. glabra* rhizome investigated in the present study also appears to exert anti-proliferative and anti carcinogenic effects via apoptosis and cell cycle arrest through up regulation of p53 and p21 genes.

CONCLUSION

Up regulation of *p53* and *p21* activity in HepG2 cells and mouse liver with DEN-induced early hepatocarcinogenesis suggests that the decoction comprised of *N. sativa* seed, *H. indicus* root, and *S. glabra* rhizome may mediate its reported antihepatocarcinogenic effects at least in part, through the modulating activities of genes involved in tumour suppression and cell cycle arrest. The results provide a new insight into the possible molecular mechanisms by which the decoction may be of benefit in the therapy of hepatopcellular carcinoma.

ACKNOWLEDGEMENT

The financial support provided by the National Science Foundation (NSF), Sri Lanka, and the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka is gratefully acknowledged.

REFERENCES

1. *Yamamoto J, Kosuge T, Takayama T, Shimada K, Yamasaki S, Ozaki H, Yamaguchi N, Makuuchi M. Recurrence of hepatocellular carcinoma after surgery. Brit J Surg. 1996; 83: 1219-1222.*
2. *Taieb J, Barbare JC, Rougier P. Medical treatments for hepatocellular carcinoma (HCC): What's next? Ann Oncol. 2006; 17 (Suppl. 10): 308–314.*
3. *Iddamaldeniya SS, Thabrew I, Wickramasinhe SMDN, Ranathunga N, Thammitiyagoda MG. Protection against diethylnitrosoamine-indused hepatocarcinogenesis by an indigenous medicine comprised of Nigella sativa, Hemidesmus indicus, and Smilax glabra. J. Carcino. 2003; 2: 6-11.*
4. *Iddamaldeniya SS, Thabrew I, Wickramasinhe SMDN, Ranathunga N, Thammitiyagoda MG, A long term investigation of anti hepatocarcinogenic potential of indigenous medicine comprised of Nigella sativa, Hemidesmus indicus, and Smilax glabra. J. Carcino. 2006; 5: 11-14.*
5. *Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen SB, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. Nuclear Acid Res. 1994; 22: 3551-3555.*
6. *Xiong Y, Hannon GH, Zhang H, Casso D, Kobayashi R, Beach D. P21 is a universal inhibitor of cyclin kinases. Nature. 1993; 366: 701-704.*
7. *Gartel AL, Tyner AL. The Role of the Cyclin-dependent Kinase Inhibitor p21 in Apoptosis. Mol. Can Ther. 2002; 1: 639-649.*
8. *Haupt S, Haupt Y. Importance of p53 for cancer onset and therapy. Anticancer Drug 2006; 17: 725–732.*
9. *Oka M, Maeda S, Koga N, Kato K, Saito T. A modified colorimetric MTT assay adapted for primary cultured hepatocytes: application to proliferation and cytotoxic assay. Bio Biotech Biochem. 1992; 56: 1472-1473.*
10. *Samarakoon SR, Thabrew I, Galhena PB, De Silva D, Tennekoon KH. A comparison of the cytotoxic potential of standardized aqueous and ethanolic extracts of a polyherbal mixture comprised of Nigella sativa (seeds), Hemidesmus indicus (roots) and Smilax glabra (rhizome). Pharm Res. 2010; 2: 335-342.*
11. *Sabourdy MA. Breeding and care of laboratory animals, WHO Health Laboratory Technology Unit, Geneva, Switzerland. 1988; 1: 18–19.*
12. *Guidelines for ethics review of research proposal involving animals in Sri Lanka, Forum of Ethics Review Committees, Sri Lanka, 2009.*
13. *Chuang SE, Kuo ML, Shu CH, Chen CR, Lin JK, Lai GM, Shieh CY, Cheng AL. Curcumin-containing diet inhibits diethylnitrosamine induced murine hepatocarcinogenesis. Carcinogenesis. 2000; 21. 2: 331-335.*

14. Teufelhofer O, Parzefall W, Kainzbauer E, Ferk F, Freiler C, Knasmuller S, Elbling L, Thurman R, Schulte-Herman R. Superoxide generation from Kupffer cells contributes to hepatocarcinogenesis:studies on NADPH oxidase knockout mice. Carcinogenesis. 2005; 26(Part 2)**:** 319-329.
15. Lennartson P, Hogberg J, Stenius U. Wild type p53 expression in liver tissue and enzyme altered foci: an in vivo investigation in diethylnitrosamine treated rats. Carcinogenesis. 1998; 19: 1231–1237.
16. Thabrew MI, Ragai RM, Mohammed A, Morsy Robin D, Hughes. Cytotoxic effects of a decoction of Nigella sativa, Hemidesmus indicus, and Smilax glabra on human hepatoma Hep G2 cells. Life Sci. 2005; 77: 1319-1330.
17. Galhena P, Thabrew I, Tammitiyagodage MG, Hanna, Rachel VA. Anti-hepatocarcinogenic Ayurvedic herbal remedy reduces the extent of diethylnitrosamine-induced oxidative stress in rats. Pharm Magazine. 2009; 5: 19-27.
18. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanab, S, Omata M, Kuriyama S. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 2003; 37: 534-543.
19. Yee KS, Vousden KH. Complicating the complexity of p53. Carcinogenesis 2005; 26: 1317- 1322.
20. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest growth arrest in human fibroblasts. Proc Nat Acad Sci. USA 1995; 92: 8493-8497.
21. Gijssel VHE, Maassen CBM, Mulder GJ, Meerman JHN. p53 protein expression by hepatocarcinogens in the rat liver and its potential role in mitoinhibition of normal hepatocytes as a mechanism of hepatic tumour promotion. Carcinogenesis 1997; 8: 1027–1033.
22. Yin ZZ, Jin HL, Yin YZ, Li TZ, Quan JS, Jin ZN. Effect of Boschniakia rossica on expression of GST-P, p53 and $p21^{ras}$ proteins in early stage of hepatocarcinogenesis and its anti-inflammatory activities in rats. World J Gastroen 2000; 6: 813–818.
23. Srivastava RK, Chen Q, Siddiqui I, Sarva K, Shankar S. Linkage of curcumin induced cell cycle arrest and apoptosis by cyclin-dependent kinase inhibitor p21 (/WAF1/C1P1). Cell Cyc. 2007; 6: 2953–2961.