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Anti-hepatocarcinogenic Ayurvedic herbal remedy reduces the extent of diethylnitrosamine-induced oxidative stress in rats

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ABSTRACT
Previous studies have shown that a decoction prepared from a mixture of *Nigella sativa* seeds, *Hemidesmus indicus* roots and *Smilax glabra* rhizome, has the potential to protect against diethylnitrosamine (DEN) – induced hepatocarcinogenic changes in rats. The present in-vivo investigation with Wistar rats was conducted to determine whether the treatment with above decoction (6 g / kg / day) for a period of 10 weeks can provide protection against DEN (200 mg / kg by a single i.p. injection) mediated changes in (a) lipid peroxidation, (b) glutathione (GSH) concentration, (c) activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), and (d) activity of the phase II detoxification enzyme glutathione S-transferase (GST). DEN administration resulted in a significant ($p<0.05$) enhancement (+ 46.2%) of lipid peroxidation (as assessed by formation of thiobarbituric acid reactive substances, TBARS), accompanied by a decreased GSH concentration (- 21.7% in liver; - 5.9% in blood), and activities of SOD (- 34.3%), GPx (- 49.1%) and GST (- 19.38%). Administration of the decoction to DEN treated animals resulted in a significant ($p<0.05$) reduction in TBARS production, along with a restoration towards the normal levels, of the other biochemical parameters evaluated. The overall results obtained suggests that, protection against DEN – mediated changes in oxidative stress and enhancement of the activities of enzymes participating in carcinogen detoxification are possible mechanisms utilized by the decoction to mediate its anti-hepatocarcinogenic action.
KEY WORDS: Antioxidants, Phase II enzymes, Diethylnitrosamine, *Nigella sativa, Hemidesmus indicus, Smilax glabra*.

INTRODUCTION

A decoction prepared from a mixture of *Nigella sativa* (family Ranunculacae) seeds, *Hemidesmus indicus* (family Asclepiadaceae) roots, and *Smilax glabra* (family Smilacaceae) rhizome, has been used for many years by a particular family of Ayurvedic physicians in Sri Lanka to treat cancer patients (personal communication). Recent investigations carried out by Iddamaldeniya et al., [1,2] have demonstrated that the decoction comprised of *N. sativa* seeds, *H. indicus* roots and *S. glabra* rhizome can significantly protect against chemically (diethylnitrosamine) - induced hepatocarcinogenic changes in rats, thus providing a rational basis for the ethnopharmacological use of this decoction for cancer treatment.

Many studies have highlighted the role of reactive oxygen intermediates (ROS) in the multistage events of carcinogenesis [3, 4, 8]. It has been postulated that chemopreventive agents that can exert significant antioxidant activity may be able to protect against

carcinogenesis by preventing ROS-induced oxidative tissue damage and improving the host antioxidant defence mechanisms [4, 6, 8].

Similarly, substances that can enhance the activities of phase II drug metabolizing enzymes such as glutathione S-transferase (GST), possibly accompanied by a decrease in the activities of phase I enzymes, are considered to assist in promoting the metabolic detoxification of carcinogens, thereby inhibiting the initiation step in chemical carcinogenesis [7, 8]. The present investigation was therefore carried out with the aim of examining whether antioxidant activity and enhanced activity of liver detoxification enzymes are possible mechanisms by which the decoction comprised of *N. sativa* seeds, *H. indicus* roots and *S. glabra* rhizomes mediates its anti-hepatocarcinogenic actions.

MATERIALS AND METHODS

Plant material

Seeds of *N. sativa* (family Ranunculacae), roots of *H. indicus* (family Asclepiadaceae) and rhizome of *S. glabra* (family Smilacaceae) were purchased from an Ayurvedic medicine shop at Borella, Sri Lanka. Plants were authenticated by the botanist at the Bandaranayake Memorial Ayurvedic Research Institute (BMARI), Nawinna, Maharagama, Sri Lanka. Voucher specimens (UKFM / B / 2006 / 01, UKFM / B / 2006 / 02, UKFM / B / 2006 / 03) have been deposited at the Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, Sri Lanka.

Preparation of decoction

The plant decoction was prepared according to the method recommended traditionally for administration to cancer patients (personal communication, Dr. N. Jayathilake, BMARI, Nawinna, Maharagama, Sri Lanka). Equal portions (20 g each) of *N. sativa* (seeds), *H. indicus* (roots), and *S. glabra* (rhizome) were mixed and boiled in 1.6 L of distilled water and the final volume reduced to 200 ml by boiling for approximately 3 h. The extract was then filtered, freeze dried, and stored in a vacuum desiccator at -4 ^{0}C. The percentage yield of the freeze dried plant material was 13.6 %. For experimental purposes, the required weight of the freeze dried powder was reconstituted in distilled water.

Experimental animals

In all experiments, male Wistar rats were used. Healthy male Wistar rats aged 6 weeks (average weight 150-200 g) were purchased from the Medical Research Institute, Colombo, Sri Lanka. Animals were housed in polypropylene cages (four per cage) and maintained at 25±2 ^{0}C with 12 h dark cycle. They were fed with normal rat chow prepared by Medical Research Institute, based on a formula recommended by the W.H.O [9]. The experiment was performed in accordance with the ethical guidelines for investigations in laboratory animals and was approved by Ethical Committee of the Medical Research Institute, Colombo, Sri Lanka.

Chemicals

Commercial reagent kits for the estimation of glutathione peroxidase and superoxide dismutase activities were purchased from Randox Laboratories Ltd., Antrim, UK. All other chemicals were purchased from Sigma Chemicals Co. Ltd (Poole, Dorset, UK).

Dosage and administration of decoction

The decoction at a dose of 6g / kg / day was administered to rats by gastric gavage. Dose was chosen because it has been reported by Iddamaldeniya et al., [1] to mediate the maximum anti-hepatocarcinogenic effects in Wistar rats. This dose also corresponds to the normal human therapeutic dosage as calculated on the basis of relative surface area to weight [40].

Evaluation of anti-oxidant activity

Both *in vivo* and *in vitro* methodologies were utilized for assessment of antioxidant activity of the decoction..In the *in vivo* experiments, effects of the decoction on lipid peroxidation, liver glutathione (GSH) concentration and activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), were evaluated.

Forty (n = 40) male Wistar rats were randomly divided into four groups of ten each. Group 1 and 2 served as normal and decoction controls respectively. Both groups were injected with normal saline (NS) intra-peritoneally (i.p.) on day one. Twenty four hours after NS injection, the animals in group 2 were orally administered the decoction at the dose of 6 g/kg/ day. This treatment continued for two weeks. Animals in group 1 received distilled water (5 ml/kg/day) for the same period.

Each rat in Group 3 (DEN control group) was administered a single dose (200 mg / kg, i.p.) of diethylnitrosamine (DEN) dissolved in NS on day one, to initiate hepatocarcinogenesis. Distilled water (5 ml / kg/day) was then orally administered to these animals for two weeks, commencing twenty four hours after the i.p. administration of DEN.

On day one, animals in group 4 (DEN + decoction treated group) were injected with DEN (200 mg / kg, i.p.), and 24 h later, the decoction treatment was commenced. The decoction (6 g/kg/day) was

administered for a period of two weeks.

At the end of two weeks, all animals in the four groups were subjected to partial hepatectomy under general anaesthesia to promote carcinogenesis [1]. Stimulation of hepatocytes proliferation and tissue regeneration due to partial excision of the liver significantly promotes the carcinogenesis induced by DEN [10]. Subsequently the same treatment schedule started on day 2 was continued for a further eight weeks. At the end of this period, all animals were anaesthetized with diethyl ether and blood was collected by cardiac puncture for the estimation of SOD (E.C.1.15.1.1) and GPX (E.C. 1.11.1.9) activities. Liver sections were excised for the estimation of reduced glutathione, and glutathione S-transferase (E.C.2.5.1.18) activities.

Assessment of GPx activity

GPx activity in blood was estimated by the method of Paglia and Valentine [5] using a commercially available reagent kit purchased from Randox Laboratories Ltd. Enzyme activity was expressed as U/g haemoglobin.

Assessment of SOD activity

Erythrocyte SOD activity was assayed according to the method described in the assay kit purchased from Randox Laboratories. In this method, xanthine and xanthine oxidase are used to generate superoxide anion radicals which react with 2-(4-iodophenyl)-3-(4-nitophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. Enzyme activity was expressed as U / g haemoglobin.

Estimation of reduced glutathione (GSH)

i) Liver GSH

Reduced glutathione in liver was determined by the method of Jollow et al [11]. An aliquot of 1.0 ml liver homogenate (10 % in 0.1M phosphate buffer) was precipitated with 1.0 ml sulfosalicylic acid (4 %). The samples were kept at 4 ^{0}C for 1h and then subjected to centrifugation at 1200 g for 15 min at 4 ^{0}C. The assay mixture contained 0.1 ml aliquot from the supernatant, 2.7 ml 0.1M phosphate buffer (pH 7.4) and 0.2 ml dithionitrobenzene (DTNB) (40 mg in 10 ml 0.1M phosphate buffer, pH 7.4) in a total volume of 3.0 ml. The optical density of the yellow colour developed was read immediately at 412 nm in a spectrophotometer. GSH was expressed as µg / mg protein using a pre-plot GSH standard curve.

ii) Blood GSH

GSH in the haemolysate was estimated according to the method of Patterson and Lazarow [12]. Whole blood collected (0.2 ml) was haemolysed by adding 1.8 ml of distilled water. Proteins in haemolysate were precipitated by addition of 3.0 ml precipitating solution containing 1.6 g metaphosphoric acid, 0.2 g disodium EDTA and 30 g of NaCl in 100 ml of distilled water. The mixture was then allowed to stand for 5 min at 4 °C and filtered using Watmann No.1 filter papers.

The reaction mixture contained 2.0 ml of filtrate, 8.0 ml of 0.1M phosphate buffer, (pH 7.4) and 1.0 ml DTNB (40 mg in 10 ml 0.1M phosphate buffer, pH 7.4) in total volume of 11.0 ml. The optical density of the yellow colour developed was read immediately in a spectrophotometer at 412 nm against a reagent blank containing 8.0 ml of 0.1M phosphate buffer (pH 7.4), 2.0 ml of diluted precipitation solution and 1.0 ml of DTNB (40 mg in 10 ml of 0.1 M phosphate buffer, pH 7.4). GSH in the haemolysate was expressed as µg / g Hb. using a pre-plot standard curve.

Assessment of Lipid peroxidation in liver

The assay for lipid peroxidation followed the method of Wright et al [13]. The reaction mixture in a total volume of 1.0 ml contained 0.58 ml 0.1M phosphate buffer (pH 7.4), 0.2 ml liver homogenate, 0.2 ml ascorbic acid (100mM) and 0.02 ml ferric chloride (100mM). The reaction mixture was incubated at 37 °C in a shaking water bath for 1 h. The reaction was stopped by addition of 1ml 10 % trichloroacetic acid (TCA). Reaction mixture was then mixed with 1 ml of 0.67 % thiobarbituric acid (TBA) and placed in a boiling water bath for a period of 20 min. After this time, the tubes were shifted to a crushed ice bath and then centrifuged at 2500 g for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed in each sample was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank, using a spectrophotometer. The results were expressed as nmol TBARS formed / h / g tissue at 37°C using a molar extinction coefficient of 1.56X105 / M / cm.

Free radical (DPPH and nitric oxide radicals) scavenging assays were used for the *in-vitro* assessment of antioxidant activity

1, 1 - Diphenyl - 2 - picrylhydrazyl (DPPH) radical scavenging assay

DPPH scavenging activity determination was based on the reduction of a methanolic solution of the violet coloured free radical [14]. Aliquots (190 µl) of a solution of DPPH dissolved in absolute methanol (2 mg / dl) were pipetted into the wells of a 96 well ELISA titre plate containing the decoction. Each well triplet contained 10 µl of the decoction at different concentrations (15, 30, 62, 125, 250, and 500 µg / ml).

A triplet of wells containing 190 µl of the DPPH solution and 10 µl of distilled water served as the control. After 5 min incubation at room temperature (28 – 31 ^{0}C) the absorbance of the solutions in each well was measured spectrophotometrically at 517 nm against a methanol blank (190 µl of methanol with 10 µl of distilled water) in a MULTISCAN ELISA reader (Thermolab Systems). The percentage scavenging activity (decolorisation) with respect to the control was calculated. L-ascorbic acid was used as the positive control.

NO radical scavenging assay

NO generated from sodium nitroprusside (SNP) was measured according to the method of Sreejayan and Rao [15]. Briefly, SNP (5 mM) in phosphate buffered saline (pH 7.4) was mixed with test samples of the decoction at different concentrations (25 - 1000 µg / ml) and incubated at 25^0 C for 150 min. Cucumin was used as a reference compound. The amount of nitric oxide radical produced was assayed by measuring nitrite accumulation using a microplate assay method based on the Griess reaction [16].

Assessment of liver glutathione S-transferase (GST) activity

A portion of the liver homogenate prepared for estimation of liver GSH was used for evaluation of liver GST activity. Estimation of the liver GST activity was carried out by the method of Habig et al. [17]. The reaction mixture consisted of 1.425 ml 0.1M phosphate buffer (pH 6.5), 0.2ml of 1mM reduced glutathione (40 mg GSH in 100 ml of 0.1M phosphate buffer, pH 7.4), 0.025 ml 1mM 1-chloro-2,4-dinitrobenzene (CDNB) and 0.3 ml 10% liver homogenate in a total volume of 2.0 ml. The changes in absorbance were recorded at 340 nm and enzymic activity was calculated as nmol CDNB conjugate formed / min / mg protein using a molar extinction coefficient of 9.6X10^3 / M / cm. Protein content of the liver homogenate was measured by the method of Lowry et al. [18].

Statistical analysis

Data are expressed as Means ± S.E.M. Statistical analysis of difference between groups was evaluated by one-way ANOVA followed by student's t-test. Statistical significance was set as $p<0.05$.

RESULTS

Effects of the decoction on lipid peroxidation

Effect of the decoction containing *N. sativa, H. indicus,* and *S. glabra* on lipid peroxidation, as assessed by the formation of TBARS, in livers of control and DEN-treated animals are summarized in Fig.1. TBARS formation was found to be significantly increased ($p<0.05$) in DEN treated animals (Group 3) compared to animals in the distilled water treated group (Group 1). Conversely, oral administration of the decoction resulted in a significant reduction (by 31.9 %) in lipid peroxidation ($p<0.05$) in DEN treated rats (Group 4). The decoction mediated reduction of TBARS formation in normal rats untreated with DEN (Group 2) as compared to distilled water treated controls (Group 1), provides further confirmatory evidence of its ability to inhibit lipid peroxidation.

Effects of the decoction on enzymic and non-enzymic antioxidants in liver and blood of rats

The effects of the decoction on reduced glutathione (GSH) concentrations in livers and blood of control rats and DEN treated rats are shown in Tables 1 and 2 respectively. DEN treatment resulted in a significant ($p<0.05$) decrease in the concentration of GSH in livers and blood of rats (Group 3) when compared to animals of the distilled water treated group (Group 1). However, in the DEN plus decoction treated group (Group 4), the GSH concentrations in both liver and blood was significantly ($p<0.05$) higher (by 36.1 % and 9.9 % respectively) than that observed in Group 3.

Similarly, DEN treatment resulted in a significant ($p<0.05$) reduction in activities of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutatse (SOD) in blood (Table 2). By administration of the decoction, these DEN-mediated changes in the activities of the antioxidant enzymes could also be significantly ($p<0.05$) reversed (Group 4). It was noted that the stimulation of SOD activity was more prominent (by 59.9 %) when compared with the activity of GPx (by 14.9 %) with the treatment of the decoction.

DPPH scavenging activity

The decoction demonstrated a significant dose-dependent (r^2 = 0.98, $p < 0.01$) DPPH radical scavenging activity, as indicated by the % decolourisation of DPPH in methanol (Fig. 2) although the activity was not as powerful as that demonstrated by the positive control L-ascorbic acid. The EC_{50} values of the decoction and ascorbic acid were 69.59 ± 2.9 µg / ml and 6 ± 0.3 µg / ml, respectively.

Nitric oxide radical scavenging activity

As evident from Fig. 3, the decoction demonstrated a significant, dose dependent (r^2 = 0.9425, $p<0.001$) NO radical scavenging activity, with an EC_{50} value of 685.3 µg / ml. The EC_{50} value of the positive control, cucumin was 20.4 µg / ml.

Table 1: Effects of the decoction on the concentration of reduced glutathione and activity of glutathione S-transferase in livers of control and DEN - treated rats.

Parameters	Distilled water control (Group 1)	Decoction control (Group 2)	DEN control (Group 3)	DEN + Decoction (Group 4)
Glutathione (mmol/g tissue)	3.95±0.12	4.87±0.23 [a*]	3.08±0.18 [a*]	4.19±0.2 [b*]
Glutathione-S-transferase (nmol CDNB formed/min/mg protein)	93.78±2.44	103.11±4.93 [a*]	75.61±2.77 [a*]	97.33±3.93 [b*]

Note: Each value represents the mean ± SE ; [a] Groups 2, and 3 compared with Group 1 ; [b] Group 4 compared with Group 3 ; [*] p < 0.05

Table 2: Effects of the decoction on blood levels of reduced glutathione, glutathione peroxidase (GPx) and superoxide dismutase (SOD), in rats treated or untreated with DEN.

Parameters	Distilled water control (Group 1)	Decoction control (Group 2)	DEN control (Group 3)	DEN + Decoction (Group 4)
Glutathione (mmol/g Hb.)	1.51±0.057	1.62±0.082 [a*]	1.42±0.042 [a*]	1.56±0.056 [b*]
Glutathione peroxidase (U/g Hb)	84.97±3.83	96.1±3.41 [a*]	42.49±2.4 [a*]	48.83±2.1 [b*]
Super oxide dismutase (U/g Hb)	28.17±0.98	37.56±1.3 [a*]	18.5±0.87 [a*]	29.59±0.93 [b*]

Note: Each value represents the mean ± SE ; [a] Groups 2, and 3 compared with Group 1 ; [b] Group 4 compared with Group 3 ; [*] p < 0.05

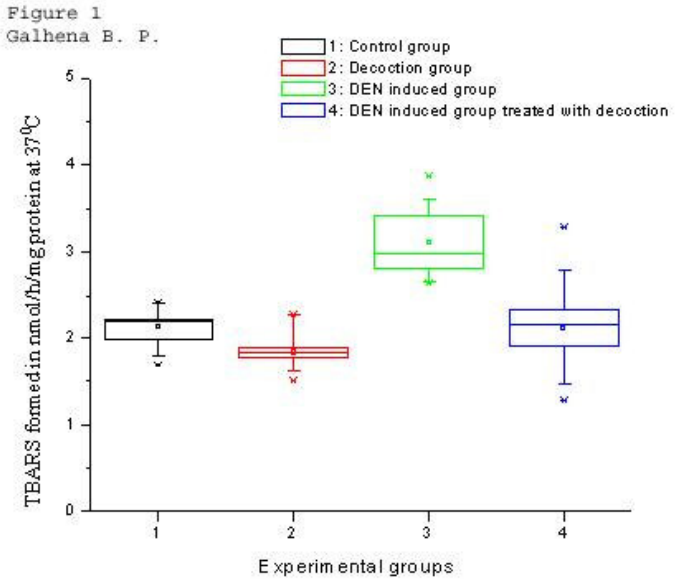

Fig.1 Levels of thiobarbituric acid reactive substances (TBARS) in livers of control and experimental rats. Data are expressed as Means ± S.E.M.

*[a] – Group 2 compared with Group 1.; [b] – Group 4 compared with Group 3. ; * - p<0.05*

Fig. 2 - B. P. Galhena

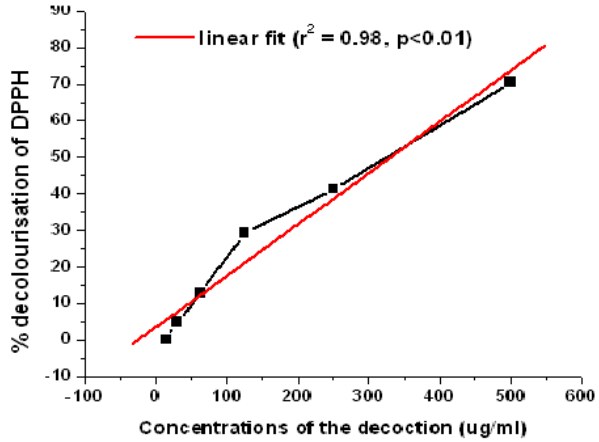

Fig. 2 DPPH scavenging activity of the decoction comprised of Nigella sativa seeds, Hemidesmus indicus roots and Smilax glabra rhizome. Data are presented as Means ± S.D.

Fig. 3 - B. P. Galhena

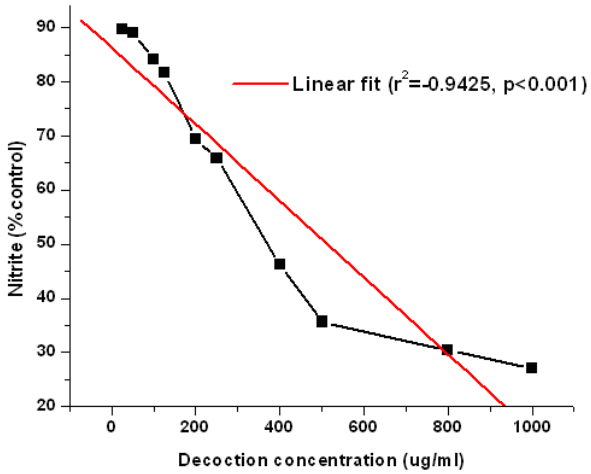

Fig. 3 Nitric oxide radical scavenging activity of the decoction comprised of Nigella sativa seeds, Hemidesmus indicus roots and Smilax glabra rhizome. Data are presented as Means ± S.D.

DISCUSSION

Reactive oxidants produced in biological systems, either by normal metabolic pathways or as a consequence of exposure to chemical carcinogens have been reported to contribute to the multi-stage process of carcinogenesis [6, 19, 20]. Lipid peroxidation is regarded as one of the basic mechanisms by which free radicals cause cellular damage [20]. Thiobarbituric acid reactive substances (of which malondialdehde is a major component) that are generated during lipid peroxidation cause cross-linking of proteins as well as nucleotides in the same and opposite strands of DNA [21]. Protection against the adverse effects of oxidant or reactive metabolites of pre-carcinogens is afforded by the collective action of both antioxidants (enzymic and non-enzymic) and phase II drug metabolizing enzymes such as glutathione S-transferase [6, 19, 20]. Depletion of antioxidant enzymes and increased lipid peroxidation in test animals following exposure to carcinogens and / or tumour promoters is well documented [19, 20, 22-24]. Conversely, a decreased lipid peroxidation along with an elevation in the above enzymes in the test animals, following administration

of chemopreventive agents has also been reported [22-26]

Diethylnitrosamine (DEN) is a well known hepatocarcinogen that has been reported to generate free radicals to exert its carcinogenic effects [10, 27]. Administration of DEN to test animals has been reported to generate free radical products that mediate lipid peroxidation and assists in initiation of carcinogenesis [10, 27]. Subsequent action of tumour promoting agents is thought to enhance the hepatic damage caused by these free radicals [28].

In the present investigation also, in accordance with the observations of previous researchers [22-24, 29], DEN treatment resulted in a significant increase in the generation of thiobarbituric acid reactive substances (TBARS), thus demonstrating the occurrence of enhanced lipid peroxidation in the carcinogen treated rats. The observed increase in lipid peroxidation is probably due to the DEN-mediated generation of free radicals overwhelming the antioxidant status, thus leading to oxidative stress. Support for this view is provided by the fact that DEN administration also resulted in a significant reduction in (a) activities of antioxidant enzymes SOD and GPx, and (b) GSH levels, in the test animals.

Antioxidant activity has been reported to be a major mechanism by which many plants and plant compounds mediate anti-cancer actions [8, 23, 24, 30-33]. The significant reduction in TBARS generation as well as the enhancement of the activities of SOD and GPx and GSH levels mediated by the decoction in both DEN treated and untreated rats suggests that the decoction comprised of *N. sativa* seeds, *H. indicus* roots and *S. glabra* rhizome also contains components that can exert significant antioxidant activity. The percentage improvement in liver GSH mediated by the decoction after 10 weeks in DEN treated rats, is comparable with those produced by other plants such as *Solabnum trilobatum* [23] and *Terminalia arjuna* [24] in the same experimental model. The percentage changes mediated by the decoction on TBARS and activities of the antioxidant enzymes SOD and GPx in DEN treated rats, are also similar to those reported for *S. trilobatum* [23], although the effects of the decoction in SOD and GPx are reversed when compared to *T. arjuna* [24]. According to Sivalokanathan et al., [24], *T. arjuna* treatment produces a greater improvement in GPx (33.3%) than in SOD (17%). Reasons for such differences are not clear, but may be related to their different modes of action. The ability of this decoction to demonstrate significant radical scavenging activity (both DPPH and NO radicals) *in vitro*, also provides further support for the view that the decoction under investigation has strong antioxidant potential, although on consideration of the overall antioxidant effects of the decoction, radical scavenging does not seem to be as important as protection against free radical mediated oxidative damages. DPPH and NO radical scavenging assays have been used by many researchers to reliably evaluate *in vitro*, antioxidant properties in plant materials [14, 33-35].

Houghton et al., [36] have demonstrated that *N. sativa* fixed oil and thymoquinone (a compound isolated from *N.* sativa seed oils) can inhibit lipid peroxidation *in vitro*. Similarly, by use of *in vitro* methods, a methanolic extract of *H. indicus* has been reported to inhibit lipid peroxidation and exhibit radical scavenging activity [37]. Therefore, both these plant materials may be contributing to the strong antioxidant activity demonstrated in *vitro* and *in vivo*, by the decoction under investigation.

Promotion of carcinogen detoxification by enhancing the activities of detoxification enzymes (especially the phase II enzymes) is also considered to help reduce genetic instability that can lead to carcinogenesis [7, 8]. In the literature there are conflicting reports of the effects of DEN administration on liver GST activity in rats. According to Marinho et al., [38], DEN administration results in an enhanced liver GST activity while Bishayee [39] reports a decreased activity. Results of the present investigation are in accordance with the findings reported by Bishayee [39]. The fact that the decoction under investigation in the present study could assist at least in partial restoration of GST activity towards normal levels in DEN treated rats suggests that it has the potential to modulate carcinogen detoxification.

Based on the ability of the decoction comprised of *N. sativa* seeds, *H. indicus* roots and *S. glabra* rhizome to enhance the GSH concentration and activities of SOD and GPx as well as the phase II enzyme GST in both DEN treated and untreated rats, it may be concluded that antioxidant activity and carcinogen detoxification may be two mechanisms by which this herbal preparation mediates its reported [1, 2] anti-hepatocarcinogenic effects.

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