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Research

Protection against diethylnitrosoamine-induced hepatocarcinogenesis by an indigenous medicine comprised of *Nigella sativa, Hemidesmus indicus and Smilax glabra*: a preliminary study

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Abstract

Background: A decoction comprised of *Nigella sativa* seeds, *Hemidesmus indicus* root and *Smilax glabra* rhizome is used to treat cancer patients in Sri Lanka. However, the anti-carcinogenic properties of this decoction have not been experimentally confirmed. The purpose of this study was to determine whether the above decoction could protect against chemically induce hepatocarcinogenesis.

Methods: The effects of this decoction on diethylnitrosamine (DEN) induced hepatocarcinogenesis were examined in male Wistar rats using the medium term bioassay system of Ito, based on a 2-step model of hepatocarcinogenesis. Rats were randomly divided into 6 groups of 10 each. Groups 1 to 4 were injected with DEN (200 mg/kg) to initiate carcinogenesis. Twenty-four hours later groups 1 and 2 were administered the decoction at 4 g/kg body weight/day (dose 1) and 6 g/kg body weight/day (dose 2), respectively. Group 3 and group 4 were given distilled water instead of the decoction and a suspension of garlic powder (20 g/kg body weight/day) in distilled water (positive control), respectively. Group 5 and 6 were injected with normal saline and twenty-four hours later group 5 was given distilled water (normal control) while group 6 was given decoction dose 2 (decoction control). Oral feeding continued for two weeks after which all rats were subjected to 2/3 partial hepatectomy to promote carcinogenesis. Oral feeding continued for eight more weeks. At the end of the 10th week, rats were sacrificed and samples of livers taken for immunohistochemical studies.

Carcinogenic potential was scored by comparing the number, area and staining intensity of glutathione S-transferase placental form (GST-P) positive foci and the number of cells/cm^2 of the positive foci in the livers of the six groups of rats.

Results: The number and area of DEN-mediated GST-P positive foci, number of cells/cm^2 of foci and staining intensity of the foci were significantly ($P > 0.001$) reduced by the decoction and garlic in the order dose 2 = garlic >dose 1.

Conclusion: Overall results indicate that the decoction comprised of *N. sativa*, *S. glabra* and *H. indicus* has the potential to protect rat liver against DEN induced hepatocarcinogenesis

Background

Cancer has become an important topic in medicine since it is a major cause of death in both the developed and developing countries and it is now only secondary to that of myocardial infarction [1]. A great majority of human cancers (about 80%-90%) are attributable to environmental factors [2]. However, it is not an easy task to eliminate carcinogenic causes from the environment. While modern surgery has significantly reduced the cancer mortality, the use of additional treatment such as radiotherapy and chemotherapy has resulted in no more than 5% reduction in the number of deaths [2]. Therefore, there is a continuing search for better control and preventive methods in order to reduce cancer mortality and related side effects. Many investigations are now being carried out to discover naturally occurring compounds, which can suppress or prevent the process of carcinogenesis [3–6].

A herbal remedy that has been developed from ancient Ola leaf inscription and prescribed to cancer patients (personal communication, Ayurvedic physician, Dr. N. Jayathilake) contains *Nigella sativa* (seeds), *Hemidesmus indicus* (root) and *Smilax glabra* (rhizome). Although, this plant mixture in the form of a decoction has been prescribed to cancer patients for so many years, it has, to date, not been subjected to any form of scientifically controlled investigation to determine if this herbal formulation truly has the potential to be of benefit to these patients. Traditionally, all the three plant types used in the formulation of this decoction are considered useful in the preparation of medications used in the treatment of boils and other skin conditions [7].

Glutathione S-transferase, a detoxifying enzyme in the liver has many isoforms. In adult rats, the GST-P form is strongly expressed during the early stage of chemically induced hepatocarcinogenesis. Previous investigators have demonstrated that garlic could significantly protect against diethylnitrosamine (DEN) induced expression of GST-P in rat livers [5]. No records can be found in published literature regarding the use of a decoction containing *Nigella sativa*, *Hemidesmus indicus* and *Smilax glabra* for the specific treatment of cancer patients. In view of this and the increasing global incidence of cancer [1], an investigation of the anti-tumor potential of this decoction was considered important.

Investigations have therefore been initiated to determine the ability of the decoction comprised of *Nigella sativa* (seeds), *Hemidesmus indicus* (root) and *Smilax glabra* (rhizome) to protect against DEN-induced hepatocarcinogenesis. In the present study, the anti-carcinogenic potential was assessed by its effects on DEN-mediated GST-P expression in rat liver.

Methods

Experimental animals

In all experiments Wistar rats (8 week old littermates, 190 ± 10 g) were used and maintained in a temperature controlled room (25°C ± 2°C) under 12 hours light/dark cycle (dark phase 6 p.m. to 6 a.m.).

Rats were fed with a standard laboratory diet containing 19% crude proteins, 3.8% fiber and 4400 kcal of energy, prepared by the Medical Research Institute, Sri Lanka, based on a formula recommended by the WHO and water *ad libitum* [8].

Plant material

Dried rhizome of *Smilax glabra*, dried seed of *Nigella sativa* and dried root of *Hemidesmus indicus* were purchased locally, and identities were confirmed by Botanist, Bandaranayake Memorial Ayurveda Research Institute, Navinna, Maharagama, Sri Lanka. Garlic bulbs were oven dried at 60°C and powdered (kindly supplied by Aushada Lanka (Pvt) Ltd, Colombo 03, Sri Lanka).

Chemicals

Diethylnitrosamine (DEN) and Diaminobenzidine (DAB) were purchased from Sigma Diagnostics Inc, USA. Normal Swine serum, Rabbit polyclonal anti GST-P antibody, Biotin labeled anti Rabbit IgG and. Avidin Biotin-peroxidase Complex (ABC) were purchased from DAKO, Denmark.

Preparation of the decoction

The plant decoction was prepared according to the method recommended by traditional Medical practitioners for the administration to cancer patients (Personal communication, Dr. N. Jayathilake, Bandaranayake Memorial Ayurvedic Research Institute, Navinna, Maharagama, Sri Lanka).

20 g each of *Nigella sativa* (seeds), *Hemidesmus indicus* (root) and *Smilax glabra* (rhizome) were mixed and boiled in 1.6 l of distilled water and final volume was reduced to 200 ml by boiling over 3 hours.

Dosage and administration of decoction

The decoction was administered to rats using a Sondi needle by gastric gavage method. The effect of two doses of the decoction was studied. Dose 1 was 4 g/kg-body weight/day. This dose corresponded to normal therapeutic dose administered to adult humans as calculated based on relative surface areas of human and rat. Dose 2 provides a higher dose of 6 g decoction/-kg body weight/day.

Experimental procedure

Sixty male Wistar rats were randomly divided into 6 groups of 10 animals in each group (groups 1–6). Groups 1 and 2 served as the test groups to which the two doses of the decoction under investigation were administered. Animals in these two groups were injected with a single dose of DEN dissolved in normal saline (200 mg/kg-body weight) to initiate hepatocarcinogenesis [9]. Twenty-four hours later, the animals were orally administered the decoction at doses of 4 g/kg/day (group 1) and 6 g/kg/day (group 2) respectively.

The decoction treatment was continued for two weeks after which the animals were subjected to two-third partial-hepatectomy (PH) by the technique recommended by Higgins and Anderson [10] for the promotion of hepatocarcinogenesis. Treatment with the respective doses of the decoction was then continued for six more weeks. Groups 3 (DEN control) and 4 (positive control) were treated with DEN and subjected to partial hepatectomy in the same manner as groups 1 and 2. However, instead of the decoction, animals in-group 3 were orally administered distilled water, while those in group 4 received a dose of garlic (20 mg/kg/day) that had previously been shown to protect against DEN induced hepatocarcinogenesis in rats [5].

Groups 5 and 6 (negative control and decoction control) animals received saline (i.p) instead of DEN and subjected to PH after 2 weeks. In place of the decoction, animals in-group 5 received only distilled water, for the same time period as all the other animals. Rats in each group were sacrificed for examination after 8 weeks. Weights of rats in each group were recorded at the beginning of the experiment and at the end of every week.

Tissue processing

At autopsy, livers were excised and slices of 2–3 mm thick (six slices of liver, two each from the right posterior, right anterior and caudate lobes) were cut with a surgical blade, fixed in 10% phosphate buffered formalin and embedded in paraffin. They were used for immunohistochemical examination of GST-P positive foci.

GST-P immunohistochemistry

The Avidin Biotin peroxidase Complex (ABC) method described by Hsu *et al* [11] was used to demonstrate GST-P liver foci. Deparaffinized sections were treated with normal swine serum (1:10), rabbit polyclonal anti GST-P antibody (1:150), biotin labeled anti rabbit IgG (1:300) and ABC. The sites of peroxidase binding were visualized using Diaminobenzidine (DAB) method [11]. Sections were counter stained with Carazzis Haematoxylin for microscopic examination. As positive control for the specifity of anti-GST-P antibody binding human thyroid sections were used. The number of foci, number of cells in each focus, total area of the liver sections, area of GST-P positive foci and staining intensity of each focus were measured using an OLYMPUS research microscope (X400).

Statistical analysis

The results were expressed as Mean ± Standard Error of Mean (S.E.M). The significance of difference in the number of foci, area of foci, number of cells and staining intensity between the control 1 and test groups were analyzed by Student's t-test.

Results

In this study, "the modified method of the medium term bioassay system" of Ito based on the two-step model of hepatocarcinogenesis was used as an assay system [10]. This system was initially introduced in order to screen environmental and naturally-occurring carcinogens. However, it was later used successfully for identifying different anti carcinogens [12,13]. DEN was used as a carcinogen to initiate hepatocarcinogenesis because it is a proven and specific carcinogen for hepatocarcinogenesis [14].

The protective actions of the decoction treatment on hepatocarcinogenesis induced by DEN are summarized in Tables 1 and 2. The effects of the decoction have also been compared with those produced by a dose of garlic which had in a previous study [5] been shown to significantly protect against DEN-induced hepatocarcinogenesis of Wistar rats. From the results in Table 1, it is evident that the animals treated with the decoction dose 2, shows a significant reduction in GST-P positive foci number, foci area and number of cells/cm^2 of foci when compared to those treated with DEN and distilled water (group 3). The effects produced by the decoction dose 2 were very similar to those produced by garlic. In the test groups (groups 1 and 2), the number of positive cells /cm^2 of foci reduced by 71.4%in test 1 and 83.8% in test 2 when compared with those of group 3. The reduction in the garlic treated group

Table I: Numbers and areas of DEN-initiated GST-P positive foci in the livers of rats treated with decoction or garlic.

Group	Treatment	Foci/cm^2	Area mm^2 / cm^2	Cells/cm^2
Test I	DEN+ Decoction dose I	16.0 ± 9.2*	0.09 ± 0.01*	121.2 ± 31.5*
Test 2	DEN+Decoction Dose 2	5.3 ± 1.8**	0.07 ± 0.01**	68.5 ± 24.7**
Control I	DEN+Distilled water	24.4 ± 3.9	0.37 ± 0.03	424.2 ± 77.4
Control 2 (Positive control)	DEN+Garlic	5.3 ± 2.6**	0.16 ± 0.04**	70.8 ± 29.1**
Control 3 (Negative control)	Saline+Distilled water	3.2 ± 2.1	0.02 ± 0.01	33.1 ± 25.2
Control 4 (Decoction control)	Saline+ Decoction dose 2	ND	ND	ND

Data shown are the Mean ± SEM of 10 determinations * Significantly different from control I at P < 0.01 (student's *t* test) ** Significantly different from control 1at P < 0.001 (student's *t* test) ND – Not Detected

Table 2: Staining intensity of DEN-induced liver GST-P positive foci in Wistar rats treated with decoction or garlic.

Group	Treatment	++++%	+++%	++%	+%	Positive foci not detected%
Test I	DEN+Decoction Dose I	ND	4.3*	21.7	21.7	52.2*
Test 2	DEN+Decoction Dose 2	ND	ND	16.7	16.7	58.3*
Control I	DEN+Distilled water	21.4	42.9	14.3	21.4	ND
Control 2	DEN+garlic	ND	ND	25.0	25.0	50.0*
Control 3	Saline+Distilled Water	ND	ND	ND	36.4	63.6
Control 4	Saline+Decoction Dose 2	ND	ND	ND	ND	ND

Data shown are the percentage values of 10 determinations ++++: very strongly stained foci +++ :strongly stained foci ++ :moderately stained foci + :weakly stained foci ND : Not Detected

was 83.3%. Results in Table 2 demonstrate that in animals treated with decoction dose 1 and 2, there was a marked reduction in staining intensity of cells when compared to group 3 animals treated with DEN and distilled water. There was no significant difference between the final body weights of the control rats and the rats treated with the decoction or garlic.

Discussion

Rat GST-P, which is related to human GST-π in enzymatic and immunological properties, is used by many researchers as a reliable marker for preneoplastic lesions, since it is strongly and specifically expressed in the very early phase of chemically induced hepatocarcinogenesis, but not in normal hepatocytes [14]. The degree of induction of GST-P positive foci and nodules in this bioassay system for hepatocarcinogenesis has been proven to correspond with incidence of hepatocellular carcinomas observed in long-term in vivo assays [15,16].

In the present investigation it was observed that that decoction comprised of *Nigella sativa* (seeds), *Hemidesmus indicus* (root) and *Smilax glabra* (rhizome) can significantly inhibit DEN-mediated GST-P expression in rat livers. Previous investigation by Samaranayake *et al.*, (2000) has shown that garlic at a dose of 20 g/kg body weight / day can significantly inhibit DEN-mediated GST-P expression in rat livers. It is interesting to note that the alterations in DEN-induced changes in the hepatocytes mediated by dose 2 of the decoction were very similar to those produced by garlic at a dose of 20 mg/kg body weight/day.

The overall results obtained in the present study indicate that the decoction under investigation, which was prepared from *N. sativa* seed, *S. glabra* rhizome and *H. indicus* root has the potential to inhibit the early DEN initiated phase of hepatocarcinogenesis. Pre cancerous inhibition by the decoction may be taking place after initiation with DEN, after promotion by partial hepatectomy, or during both stages of carcinogenesis. Further studies are required to determine exactly at what stage the inhibition takes place.

The mechanism by which the decoction mediates its anti-carcinogenic effects is not clear. Anti-tumor effect of the decoction may be mediated by one or more of the following mechanisms:

(a) detoxification of the carcinogen by inducing detoxification enzymes such as GSTs.

(b) anti-oxidant activity.

(c) immuno modulatory action.

(d) cytotoxicity.

Recent in vitro studies have demonstrated that *N. sativa* can be cytotoxic to several cancer cell lines [17]. Thymoquinone and dithymoquinone, two isolated active components of *N. sativa,* have also been shown to be cytotoxic to several parental and multi drug resistant (MDR) human cell lines [18]. Further *Nigella sativa* and *Hemidesmus indicus* have been shown to possess anti-oxidant activities [19–22].

Investigations on the mechanisms of action of the decoction used in the present study is in progress. Anti-carcinogenic effect of the extracts of individual plants in the decoction was not studied because only the decoction comprised of *N. sativa* seed, *S. glabra* rhizome and *H. indicus* root is traditionally used in cancer therapy. Recent *in vivo* studies have shown that tumour development in mice skin could be inhibited by the active principles of *Nigella sativa* [17,23]. Whether *Nigella sativa* is mainly responsible or all the three plants are equally responsible for the anti-tumor potential demonstrated by the decoction in the present study is not clear and further studies are required before definite conclusions can be reached.

Conclusion

It can be concluded from the study that the decoction under investigation can protect the liver against chemically mediated pre-cancerous lesions in a similar manner to garlic.

List of abbreviations

DEN: Diethylnitrosamine

GST-P: Glutathione S-transferase

WHO: World Health Organization

DAB: Diaminobenzidine

ABC: Avidin Biotin-peroxidase Complex

PH: Partial-Hepatectomy

S.E.M: Standard Error of Mean

Author's contribution

SSI did feed preparation, animal handling and feeding under the supervision of MGT. SSI and MGT participated in the surgical procedure for partial hepatectomy. SSI under the supervision of NR performed the immunohistochemical process and the interpretation of the stained sections. NW and IT conceived, designed and coordinated the study. IT, SSI and NW participated in writing the manuscript.

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