Cancare—a herbal formulation inhibits chemically induced tumours in experimental animals

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Cancer chemopreventive potential of Cancare, a multi-herbal formulation on chemically induced tumours was studied by N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats and 20-methylcholanthrene (20-MC) induced sarcoma development in mice. Oral administration of Cancare was found to inhibit the liver tumour development induced by N-nitrosodiethylamine. Animals administered with NDEA had visible liver tumours by the end of 30th weeks and the liver weight was raised to 6.1 ± 1.4 g/100 g body wt. None of the animals treated with Cancare (150 mg/kg) developed any visible liver tumours by this period and the liver weight was 3.0 ± 0.6 g/100 g body wt. γ-Glutamyl transpeptidase, a marker of hepatocellular carcinoma, which was raised to 83.7 ± 8.9 U/l in serum of NDEA treated group was reduced to 55.2 ± 6.1 U/l by simultaneous administration of Cancare. Elevated levels of serum alkaline phosphatase, glutamate pyruvate transaminase, bilirubin, liver glutathione S-transferase, glutathione and γ-Glutamyl transpeptidase in the NDEA administered group was significantly reduced by Cancare administration. Cancare administration inhibited the sarcoma development and increased the life span of mice administered with 20-MC dose dependently. All animals in the control group developed sarcomas by 150th day and dead by 174th day after 20-MC administration. Cancare administration (30 mg and 150 mg/kg) inhibited the sarcoma development (46.7 and 60%) as well as increased the life span (53.3 and 66.7%) as estimated on 240th day after 20-MC administration. The results are indicative of the chemopreventive potential of Cancare against chemically induced neoplasmas.

Chemoprevention is being tested as a major means of inhibition of cancer, suppression of neoplasia during recurrence and at times reversal of carcinogenic processes. Plants are loaded with chemicals with chemopreventive activity and some of them are under-going clinical trials. Most of the studies on chemoprevention is based on individual chemicals with defined mechanism of action and have reported to inhibit carcinogenesis in animal models. As the carcinogenic process is multifactorial, individual drugs may not always be effective to inhibit the cancer of various types and etiology seen in human beings. We formulated a combination drug for chemoprevention of cancer, which is based on the activity of individual extracts and biological property of the isolated active ingredients.

Cancare is a multi-herbal preparation consisting of 75% methanol extracts of Curcuma longa, Phyllanthus amarus, Allium sativum, Emblica officinalis, Picrorhiza kurroa and Spirulina platensis. These plants are being used in indigenous medical practice or as food additives and hence their non-toxic nature is well established. The individual extracts as well as the active ingredients have been reported to possess anti-oxidant, anti mutagenic, anti genotoxic and immunomodulatory activities. Moreover the extracts and its active ingredients has been reported to inhibit the activity of several protein kinases, carcinogen metabolising enzymes, cell cycle regulators and regulates gene expression. The active ingredients reported are given in Table 1. In the present study we report the anticarcinogenic potential of Cancare on N-nitrosodiyethylamine induced liver tumours in rats and 20-methylcholanthrene induced sarcoma development in mice.

Materials and Methods

Preparation of Cancare—Authenticated rhizomes of Curcuma longa and Picrorhiza kurroa were purchased from Amala Ayurveda Hospital, Thrissur. Fresh fruits of Emblica officinalis and Allium sativum bulbs were purchased from the local market. Spirulina platensis was kindly supplied by Recon Laboratories, Bangalore. Aqueous extract of authenticated aerial
parts of Phyllanthus amarus was supplied by Dr. S.S. Gandhi, Lyka Research Laboratories, Mumbai. Rhizomes of Curcuma longa, Picrorhiza kurroa and fruit pulps of Emblica officinalis were air dried and finally powdered. Allium sativum bulbs were crushed gently. The plant materials were stirred separately with 75% methanol for 24 hours, filtered and evaporated to dryness under a water bath. 1 g of each individual extract was mixed thoroughly and suspended in distilled water so as to get the desired concentrations; i.e., 30, 150, 750 mg/kg body weight in 1 ml of water.

Chemicals—N-nitrosodiethylamine and 20-methylcholanthrene were purchased from Sigma Chemical Co. St. Louis, USA. 1-chloro 2-4-dinitrobenzoic acid (CDNB), 5′5′-dithio-bis 2-nitrobenzoic acid (DTNB) and glutathione (GSH) were purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical reagent grade.

Induction of hepatocarcinogenesis—The study was performed on 8-10-week-old female Wistar rats weighing 160-180 g. Protocols for the animal testing was approved by the Animal Ethics Committee of our Centre. Rats were randomly divided into four groups (n=10 each). Animals in the group I were kept as normal animals receiving vehicle alone. Animals in the group II to IV were administered with 0.02% NDEA, 2.5 ml/rat, 5 days weekly for 20 weeks. Rats in the group III to IV were administered with Cancare 30 and 150 mg/kg respectively immediately after NDEA administration and continued for 20 weeks. After that animals were kept without any drug treatment and sacrificed at the end of 30th week by diethyl ether anaesthesia. Blood was drawn by cardiac puncture and serum was separated. Liver was surgically excised, weighed and homogenate prepared was used for biochemical estimations. A small piece of the liver was fixed in 10% formalin. The formalin fixed specimens were embedded in paraffin and sectioned (3-5 μm), sections from each group were stained with haematoxylin and eosin and histological sections were evaluated by light microscopy.

Protein in liver homogenate was assayed by the method of Lowry et al. Serum γ-Glutamyl transpeptidase activity was assayed by using glutamyl para-nitroanilide as the substrate. Liver γ-Glutamyl transpeptidase activity was assayed by the method described by Tate and Meister. Cytosolic glutathione S-transferase activity was determined by the method described by Haibig et al. Alkaline phosphatase (ALP) was assayed by the method described by King and Armstrong. Glutamate pyruvate transaminase (GPT) was assayed by the method of Lowry et al. Serum bilirubin was assayed by the method described by Jendrassik and Jor.

Induction of sarcomas—Female Balb/c mice (6-8 week old, 20-25 g) were used for the study. Hair was shaved from the dorsal side of mice. All animals were administered with a single dose of 20-MC (200 μg/0.1 ml DMSO/mouse) subcutaneously on the dorsal side. This dosage has been shown to produce sarcoma development in mice by 8-12 weeks. Thereafter animals were randomly divided into four groups (n=15 each), group I was kept without any drug treatment. Animals in the group II to IV were orally treated with Cancare 30, 150 and 750 mg/kg respectively, thrice weekly for 8 weeks. The animals were observed for the onset of sarcoma as well as their survival up to 240 days.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Active ingredients</th>
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<tbody>
<tr>
<td>Emblica officinalis</td>
<td>Emblicanin A, Emblicanin B, Phyllembolin</td>
</tr>
<tr>
<td>Phyllanthus amarus</td>
<td>Phyllanthin, Pedundagin, Ascorbic acid</td>
</tr>
<tr>
<td>Allium sativum</td>
<td>Allin, Diallyl sulphide, Allyl methyl sulphide</td>
</tr>
<tr>
<td>Curcuma longa</td>
<td>Cereumolin, Turmerin, Ferulic acid, Santonin, Cimoneole</td>
</tr>
<tr>
<td>Picrorhiza kurroa</td>
<td>Picroliv (Picroside I &amp; Kutkoside), Picroside II, Apecynin</td>
</tr>
<tr>
<td>Spirulina platensis</td>
<td>β-Carotene, α-Tocopherol, Phycocyanin, Chlorophyll</td>
</tr>
</tbody>
</table>
Results

Effect of Cancare administration on NDEA induced hepatocarcinogenesis—All animals in the carcinogen-administered group developed liver tumours by the end of 30th week. Cancare administration was found to inhibit the tumour development in liver induced by NDEA, at 30 mg/kg dose, 50% of animals developed tumours while at 150 mg/kg no tumours were seen (Table 2). Liver weight of NDEA treated animals were raised as compared to normal rats. Cancare administration significantly lowered the liver weight (Table 2). Increased γ-glutamyl transpeptidase, a marker of hepatocellular carcinoma¹⁹, in the serum as well as in liver was found to be effectively lowered by the administration of Cancare, indicating that Cancare could reduce the proliferation of tumour cells (Table 2). Similarly GSH and GST values which were increased after NDEA treatment was found to be lowered by the administration of Cancare (Table 3). ALP and GPT activity in the serum of NDEA administered group were raised as compared to that of normal value. Cancare administration significantly inhibited the rise of ALP (Table 3) and GPT (Table 4). Similarly elevated levels of bilirubin in the serum of NDEA treated group was found to be lowered by the administration of Cancare (Table 4). Cancare administration was found ineffective in lowering the elevated lipid peroxidation levels significantly.

Histopathological analysis of rat liver after NDEA treatment revealed that of well differentiated hepatocellular carcinoma. The normal architecture of the liver was distorted with the formation of nodules, hepatokaryomegaly, increased nuclear cytoplasmic ratio, prominent nucleoli and chromatin dots were detected in most parts of the liver section. Hepatocytes remained normal in Cancare treated group (150 mg/kg) except for the presence of a few alterations such as hepatocytomegaly and karyomegaly in certain areas. The hepatocytes are separated by uniform sinusoids.

Effect of Cancare administration on 20-MC induced sarcoma development—Animals in the 20-MC administered group (group I) started developing...
sarcomas by 57th day after 20-MC administration and all animals developed sarcomas by 150th day. Cancare administration inhibited the sarcoma development in a dose dependent manner. On 240th day after 20-MC administration 13.3, 46.7 and 60% reduction in the sarcoma development was observed by the administration of Cancare 30, 150 and 750 mg/kg respectively (Fig. 1). Cancare administration was also found to increase the life span of mice administered with 20-MC. All animals in the group I died by 174th day after 20-MC administration, while there was 13.3, 53.3 and 66.6% increase in the life span of animals treated with Cancare (group II to IV) on 240th day (Fig. 2).

Discussion

Nitrosodiethylamine and 20-MC are ubiquitous environmental carcinogens and their carcinogenicity has already been demonstrated in several animal species. The presence of NDEA has been detected in edible vegetable oils, in alcoholic drinks, steamed and fried fish and is formed endogenously in the body20, 21. N-nitroso compounds has been associated with an increased risk of cancer development in various organs22, 23. The limited treatment options as well as poor treatment success makes HCC a leading cause of death in developing countries. The survival time among patients with clinically detectable HCC is extremely short; therefore HCC incidence and mortality rates are roughly equivalent24, 25. Hence a major effort should be directed towards its prevention.

The results presented in this study demonstrate that hepatocarcinogenesis induced by NDEA is effectively inhibited by simultaneous administration of Cancare (150 mg/kg). Liver morphology and histopathological analysis are in good agreement that the Cancare...
administration protects the liver against the hepatocarcinogenesis induced by NDEA. The tumour markers and liver injury markers, which were elevated in the NDEA treatment were significantly reduced by Cancare administration. The extract also prevented the sarcoma formation induced by 20-MC and increased the life span of animals treated with 20-MC.

The extracts used in this study have been reported to have a broad spectrum of biological activities. The extracts as well as the isolated ingredients were reported to have potential antioxidant activity and could scavenge hydroxyl, superoxide radicals and inhibited lipid peroxidation in vitro\textsuperscript{12}. \textit{Embliva officinalis} extract was found to inhibit P-450 enzyme aniline hydroxylase and aminopyrene demethylase in vitro\textsuperscript{12}. \textit{Embliva officinalis} extract significantly reduced the elastogenic effect of 3,4-benzpyrene in mice\textsuperscript{26} and inhibited the hepatocarcinogenesis induced by NDEA in rats\textsuperscript{27}. \textit{Picrorhriza kurroa} extract contains a well-known hepatoprotective agent Picroliv, which was found to reduce the toxicity of a number of ingested xenobiotics\textsuperscript{5}. \textit{Picrorhriza kurroa} extract was found to inhibit the 20-MC induced sarcoma development in mice and reduced the volume of transplanted solid tumours\textsuperscript{6}. \textit{Phyllanthus amarus} extract was found to inhibit DNA topoisomerases of \textit{Saccharomyces cerevisiae} mutant cell cultures and inhibited cdc25 tyrosine phosphatase, a key enzyme in cell cycle regulation and reduced the hepatocarcinogenesis in rats\textsuperscript{28}. Curcumin, isolated from the rhizomes of \textit{Curcuma longa}, inhibited TPA induced tumour promotion and inhibited the carcinogenic potential of several carcinogens in animal models\textsuperscript{9,30}. In mice, \textit{Curcuma longa} extract has been shown to increase the activity of glutathione-S-transferase and to suppress chemically induced aberrations in bone marrow cells, and in rats, to decrease the levels of chemically induced DNA adducts in the liver. Curcumin was reported to inhibit the activity of nuclear oncogene c-Jun and inhibited the activity of ornithine decarboxylase, cyclooxygenase and protein kinase C\textsuperscript{31}. Sulfur containing compounds purified from \textit{Allium sativum} has been shown to inhibit experimentally induced cancer of various sites in animal studies and to increase the activity of detoxification enzymes\textsuperscript{12,33}. Diallyl sulfide isolated from \textit{Allium sativum} extract was found to inhibit the carcinogenesis induced by tobacco specific carcinogen 4-methylnitrosamo-1-(3) pyridyl-1-

butanone (NNK), 1,2-dimethyl hydrazine, benza(a) pyrene and N-nitrosomethylbenzylamine\textsuperscript{34}. \textit{Spirulina platensis} contain rich amounts of β-carotene and α-tocopherol, which are well-known antioxidants and anticarcinogens\textsuperscript{35}.

The protection offered by Cancare against chemical carcinogenesis is attributed to the combined effects of various plant constituents rather than any single component. The combined action such as scavenging of oxygen free radicals, activation of detoxifying enzymes, inhibition of carcinogen activation, inhibition of DNA topoisomerases, cell cycle regulators and oncogenes may be responsible for the anticarcinogenic activity of Cancare reported in this study.

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