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ANTI CANCEROUS EFFICACY OF AYURVEDIC MILK EXTRACT OF *SEMECARPUS ANACARDIUM* NUTS ON HEPATOCELLULAR CARCINOMA IN WISTAR RATS

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Abstract

The objective of the study was to determine the anticancerous efficacy of Ayurvedic preparation made of *Semecarpus anacardium* (SA) nuts. Five groups of rats were used for the study. Group I served as water control. Hepatocellular carcinoma (HCC) was induced in groups II, III and IV animals using N-nitrosodiethylamine as inducing agent followed by phenobarbitone as promoter for 13 weeks. Group-II animals were kept untreated as hepatocellular carcinoma control. Group-III animals were treated with Ayurvedic milk extract of *Semecarpus anacardium* nuts at dose mentioned in Ashtangahridaya, an authentic book of Ayurveda for 49 days and group-IV animals were treated with doxorubicin as reference drug at dose of 1mg/kg twice a week for 7 weeks. Group V animals were kept as drug (SA nut milk extract) control for studying the effect of nut milk extract on normal rats. After 154 days of experiment, all animals were subjected to screening for HCC by estimation of liver enzymes, HCC marker (alpha-2 macroglobulin) and histopathology. Both liver enzymes and HCC marker were increased in hepatocellular carcinoma control along with neoplastic changes in liver and were decreased in *Semecarpus anacardium* nut milk extract treated group. The Ayurvedic drug showed positive correlation with the action of doxorubicin. This study demonstrated the efficacy of *Semecarpus anacardium* nut milk extract for the treatment of hepatocellular carcinoma either alone or along with chemotherapy.

Key words: Hepatocellular carcinoma, N-nitrosodiethylamine, *Semecarpus anacardium*, Wistar rats

Abbreviations: SA - *Semecarpus anacardium*, HCC - Hepatocellular carcinoma , NDEA - N-nitrosodiethylamine, IAEC - Institutional Animal Ethical Committee, SEM - Standard error of mean, A2M - Alpha-2 macroglobulin, ALT - Alanine aminotransferase, AST - Aspartate aminotransferase, AKP - Alkaline phosphatase, GGT - Gamma-glutamyl transferase

Introduction

Liver cancer is one of the most common malignancies worldwide, especially in Asia and Africa. Hepatocellular carcinoma accounts for about 80 – 90 % of all liver cancers and is the most common cause of cancer mortality. It has been reported in different species of animals also. Synthetic drugs used for cancer therapy can lead to many side effects and undesirable hazards. There are a lot of medicinal plants with anticancerous potential. *Semecarpus anacardium* (Varnish tree) is one among them. It is a deciduous tree distributed in the Sub Himalayan tract and tropical parts of India (Kirtikar and Basu, 1975). This medicinal plant has long been used in Siddha and Ayurveda for treatment of various ailments. Siddha preparation of *Semecarpus anacardium* has already proved its anticancerous efficacy on hepatocellular carcinoma (Premalatha et al., 1999). Similar preparations are in Ayurveda also. The purpose of this study is to determine the anticancerous efficacy of nut milk extract of *Semecarpus anacardium* on hepatocellular carcinoma.

N-nitrosodiethylamine (NDEA) used for HCC induction in this study is known to induce damage of many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models. This chemical is also a constituent in tobacco smoke, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics, pharmaceutical products, etc.

Materials and Methods

Preparation of *Semecarpus anacardium* nut milk extract

Nut milk extract was made as per standard technique described in system of Ayurvedic medicine by boiling nuts (g), milk (ml) and water (ml) in the ratio 1:15:15 (Rao, 2008). *Semecarpus anacardium* nuts were boiled with water and then mixed with Ksira (milk). This preparation was boiled till the volume of extract reduced to the level of 2ml (for oral administration to rats). Nut extract was filtered using a muslin cloth and filtrate was removed. Freshly prepared drug was used for the experiment.

Calculation of rat dose of *Semecarpus anacardium* nut milk extract

Dose of the test formulation was calculated by extrapolating the human dose according to Ashtangahridaya

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(Warrier, 1942) to rat dose based on the body surface area ratio by referring to the standard table of Paget and Barnes (1969). Human dose × conversion factor for rat (0.018) = "x"/200 g body weight of rat. Dose for rats = Human dose x 0.018 for rat weighing 200g (Table 1).

Animals

The experiment was carried out in conformity with the Institutional Animal Ethical Committee (IAEC No. 2010/Med/85), Veterinary College, Anand, Gujarat, India. Female adult rats of Wistar strain weighing 150 – 200 g were procured from Zydus Research Centre, Ahmedabad, Gujarat. Animals were acclimatised for 7 days in the animal house. They were housed in well-ventilated polypropylene cages with an air conditioning system and regulated temperatures at 24^0C and humidity at 50-60% on a 12 hrs day and night cycles using artificial lights. Animals were fed with commercial feed for rats and distilled water *ad libitum*. The experiment was carried out in conformity with the Institutional Animal Ethical Committee (IAEC No. 2010/Med/85), Veterinary College, Anand, Gujarat, India.

Experimental Design

Rats were selected randomly and divided into 5 groups (Group-I, II, III, IV and V). Group I and II were comprised of six animals, while the remaining groups consisted of eight animals each. Group I served as water control made up of healthy animals. Hepatocellular carcinoma was induced in group II, III and IV animals using N-nitrosodiethylamine (Sigma Chemical Company, St. Louis, MO, USA) as inducing agent (single administration of NDEA at the rate of 200 mg/kg body weight mixed in saline) followed by phenobarbitone (0.05% in drinking water up to 13 weeks) as promoter. Group II animals were kept untreated. After diagnosis of hepatocellular carcinoma in rats by histopathology (Joice, 2011) and A2M estimation, Group-III animals were treated with nut milk extract of *Semecarpus anacardium* according to dose mentioned in Ashtangahridaya (Warrier, 1942). Group-IV animals were treated with reference drug (doxorubicin at the dose of 1mg/kg body weight twice a week for 7 weeks) and Group V animals were kept as drug (nut milk extract) control for studying the effect of nut milk extract on normal rats. At the end of experimental period, activity of liver function enzymes and concentration of A2M in serum were compared among the groups.

Biochemical analysis

Blood samples were collected and allowed to clot at room temperature. The blood samples were centrifuged after clotting at 3000 rpm for 20 minutes at room temperature. Separated serum samples were stored at -20^0C. Stored samples were analysed for estimation of alanine aminotransferase (Mod. IFCC method), aspartate aminotransferase (Mod. IFCC method), alkaline phosphatase (PNPP Kinetic method), gamma-glutamyl transferase (Carboxy substrate method) and lactate dehydrogenase (Mod. IFCC method) using commercial diagnostic kits procured from Crest Biosystem (Division of Coral Clinical System, Goa).

Table 1: Calculation of rat dose of *Semecarpus anacardium* nuts

Day	Human dose (g)	Rat dose (g)	Day	Human dose (g)	Rat dose (g)
1	12	0.216	26	55.5	0.999
2	13.5	0.243	27	51	0.918
3	15	0.27	28	46.5	0.837
4	16.5	0.297	29	42	0.756
5	18	0.324	30	40.5	0.729
6	19.5	0.351	31	39	0.702
7	21	0.378	32	37.5	0.675
8	22.5	0.405	33	36	0.648
9	24	0.432	34	34.5	0.621
10	25.5	0.459	35	33	0.594
11	27	0.486	36	31.5	0.567
12	28.5	0.513	37	30	0.54
13	30	0.54	38	28.5	0.513
14	31.5	0.567	39	27	0.486
15	33	0.594	40	25.5	0.459
16	34.5	0.621	41	24	0.432
17	36	0.648	42	22.5	0.405
18	37.5	0.675	43	21	0.378
19	39	0.702	44	19.5	0.351
20	40.5	0.729	45	18	0.324
21	42	0.756	46	16.5	0.297
22	46.5	0.837	47	15	0.27
23	51	0.918	48	13.5	0.243
24	55.5	0.999	49	12	0.216
25	60	1.08			

Approximate weight of 1 SA nut ≈ 1.5 g

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Estimation of alpha-2 macroglobulin in serum samples

Concentration of alpha-2 Macroglobulin in serum of animals was estimated using enzyme linked immunosorbent assay (ELISA) kit (Immunology Consultants Laboratory, Newberg, USA).

Statistical analysis

Data obtained were analysed using standard statistical procedure described by Snedecor and Cochran (1992) and were expressed as mean ± SEM (Standard error of mean).

Results

Effect of different treatments on liver enzymes in different groups of Wistar rats are given in Table 2. The activity of enzymes specific for liver function and the concentration of alpha-2 macroglobulin in serum were in normal range in group I and group V animals.

ALT (Alanine aminotransferase)

After the experimental period, ALT activity was significantly ($P < 0.05$) high in Group II animals (563.54 ± 42.49 U/L) compared to animals in group I (49.86 ± 4.59 U/L). In groups treated with SA nut milk extract and doxorubicin, a significant ($P < 0.05$) reduction (225.24 ± 25.26 and 156.35 ± 8.98 U/L, respectively) was observed in this enzyme activity compared to group II.

AST (Aspartate aminotransferase)

A significant ($P < 0.05$) elevation of activity of AST was noted in group II (389.00 ± 52.66 U/L) compared to group I (185.51 ± 43.46 U/L). Group III animals showed significant reduction ($P < 0.05$) of this enzyme activity (179.30 ± 44.29 U/L) compared to group II, while non significant reduction of this enzyme activity was noted in group IV animals (282.75 ± 71.34 U/L) compared to group II.

AKP (Alkaline phosphatase)

Serum AKP activity was significantly ($P < 0.05$) elevated in group II animals (587.06 ± 122.72 U/L) compared to group I animals (120.46 ± 23.08 U/L). In group III animals a non-significant reduction of AKP activity was noted (444.31 ± 127.15 U/L) compared to group II. But group IV animals showed significant ($P < 0.05$) reduction of this enzyme activity (350.68 ± 30.60 U/L).

GGT (Gamma-glutamyl transferase)

Serum GGT activity was found significantly increased ($P < 0.05$) in group II animals (70.64 ± 4.56 U/L) compared to normal animals (43.04 ± 6.05 U/L) in group I. Both SA nut milk extract and doxorubicin reduced its activity non-significantly (53.10 ± 8.72 and 50.95 ± 11.04 U/L).

Table 2: Effect of different treatments on liver enzymes in different groups of Wistar rats

Group	ALT (U/L)	AST (U/L)	AKP (U/L)	GGT (U/L)	LDH (U/L)
I(Water control)	49.86±.59	185.51±43.46	120.46±23.08	43.04±6.05	148.87±6.41
II(HCC control)	563.54*± 42.49	389.00*±52.66	587.06*±122.72	70.64*±4.56	250.32*±14.96
III(NDEA+PB+SA)	225.24*±25.26	179.30*±44.29	444.31± 127.15	53.10 ± 8.72	212.99±19.01
IV(NDEA+PB+Doxo)	156.35* ± 8.98	282.75 ± 71.34	350.68* ± 30.60	50.95±11.04	200.09±17.41
V(SA control)	68.78 ± 57.35	189.41 ± 28.66	108.00 ± 14.07	36.48 ± 7.81	161.46 ± 6.22

* Significant at $P < 0.05$, HCC= Hepatocellular carcinoma, NDEA= N-nitrosodiethylamine, PB=Phenobarbitone, Doxo = Doxorubicin, SA= *Semecarpus anacardium*

LDH (Lactate dehydrogenase)

Serum LDH activity was significantly ($P < 0.05$) elevated in group II (250.32 ± 14.96 U/L) compared to group I (148.87 ± 6.41 U/L), and it was non-significantly reduced in group III and IV (200.09 ± 17.41 U/L and 212.99 ± 19.01 U/L, respectively).

Table 3: Alpha-2 macroglobulin concentrations at the end of experimental period indifferent groups

Group	Description of group	Mean ± SE (ng/ml)
I	WC	1.07 ± 0.25

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II	HCC Control (NDEA + PB)	40.10* ± 12.82
III	NDEA + PB + SA	1.38* ± 0.38
IV	NDEA + PB + Doxorubicin	2.38* ± 0.92
V	Drug control (SA nut milk extract)	0.95 ± 0.37

* Significant at P < 0.05, HCC= Hepatocellular carcinoma, NDEA= N-nitrosodiethylamine, PB=Phenobarbitone, SA= *Semecarpus anacardium*

Alpha-2 macroglobulin

After 13 weeks of promotion period, alpha-2 macroglobulin concentration was significantly (P < 0.05) increased in serum (9.40 ± 1.79 ng/ml) of HCC induced animals compared to normal animals (1.07 ± 0.25 ng/ml). Concentration of alpha-2 macroglobulin was compared before and after treatment with nut milk extract in group III animals. It was elevated before treatment (12.7 ± 2.63 ng/ml) and came down to normal level (1.38 ± 0.38 ng/ml) after treatment with SA nut milk extract in group III. After completion of experimental period, concentration of alpha-2 macroglobulin was compared among the groups (Table 3). It was significantly (P < 0.05) elevated in group II animals (40.10 ± 12.82 ng/dl) compared to normal animals (1.07 ± 0.25 ng/dl). Concentration of alpha-2 macroglobulin was significantly (P < 0.05) lesser in group III (1.38 ± 0.38 ng/dl) and group IV animals (2.38 ± 0.92 ng/dl) than in group II animals.

Histopathological examination of HCC induced animals showed neoplastic changes like loss of lobular architecture, necrosis, fatty changes, cytomegaly with karyomegaly as well as vesicular active nuclei and presence of more than one nucleolus. Nuclei of many hepatocytes appeared malignant or showed features of degenerating and dividing process. At the end of experimental period, those lesions were reduced in animals treated with SA nut extract and doxorubicin (group III and IV). Both group I and group V animals revealed normal architecture of liver sections with polyhedral shaped hepatocytes and cytoplasm granulated with small uniform nuclei (Joice, 2011).

Discussion

Neoplasm or cancer is an abnormal mass of tissue, which exceeds and is uncoordinated with that of normal tissues and persists in the same excessive manner even after cessation of the stimulus that evoked the change (Willis, 1952). N-nitrosodiethylamine (NDEA) used in this study is a powerful heaptocarcinogen (Pereira et al., 1984) and generates free radicals to exert its carcinogenic effects (Verna et al., 1996; Sundaresan and Subramanian, 2003). Phenobarbitone acts as a tumour promoter (Hudig et al., 1979) when administered subsequent to an initiating carcinogen like N-nitrosodiethylamine.

In vitro studies on SA (Chitnis et al., 1980) have already revealed its anticancerous property. Nut milk extract prepared according to Siddha system of medicine has proved its anticancerous efficacy against hepatocellular carcinoma in wistar rats (Premalatha et al., 1999). Results of present investigation also points towards the anticancerous nature of Ayurvedic nut milk extract. Elevation of serum aminotransferases in group II indicates the damage to liver during hepatocellular carcinoma induction. It could be reversed significantly (p < 0.05) by this Ayurvedic preparation and was comparable to the effects of doxorubicin in HCC induced animals. Development of tumour results in tissue damage that leads to the release of AKP into circulation and this enzyme level will be elevated in serum of the tumour-bearing animals (Ramakrishnan et al., 2007). Elevated activity of AKP in serum indicates the presence of HCC in group II and SA nut milk extract and doxorubicin treatment reduced the activity of this enzyme in group III and group IV indicating anticancerous nature of these drugs. Elevation of GGT activity reflects the progress of carcinogenesis, since its activity correlates with tumour growth rate. Increased activity of this enzyme noted in group II animals indicates the basic tumour burden, and SA and doxorubicin treatment decreased the activity of this enzyme indicating their curing action. An increased activity of LDH has been reported in malignant cells spreading through the organs of tumour bearing rats. An increase in LDH in malignant liver disorder depends upon the extent of metastasis (Jahan et al., 2011). The cancer cells tend to synthesise ATP mainly through glycolysis, a metabolic state that is linked to high glucose uptake and local acidification owing to lactate production. Glycolytic enzymes are induced by oncogenesis. The reduction in LDH activity in the treatment with SA nut milk extract owes to controlled glycolysis and renders the protection to membrane integrity. Agreeing with these results, Premalatha et al. (1999) observed reduction in elevated activity of enzymes for hepatic injury in HCC induced rats by Siddha preparation of SA nut milk extract. Similar results were also obtained in HCC induced rats treated with different preparations having anticancerous nature (Jeena et al., 1999; Shaarawy et al., 2009; Jahan et al., 2011).

Tumour markers comprise a wide spectrum of biomacromolecules synthesised in excess concentration by a wide variety of neoplastic cells. Alpha-2 macroglobulin (A2M) is a homotetrameric major acute-phase glycoprotein and novel cytochemical marker characterising preneoplastic and neoplastic rat liver lesions negative for hitherto established cyto-chemical markers (Sukata et al., 2004). It is tightly linked to the rat hepatocarcinogenesis from the initial stage to tumour progression even in conditions, which are undetectable, by established cytochemical markers such as placental glutathione-S-transferase (GST-P) and gamma -glutamyl transferase (gamma - GT) positive lesions. A2M functions as a carrier protein and regulator for various growth factors and cytokines such as transforming growth factor-α (known to be involved in the onset of hepatocyte apoptosis) (James, 1990). Furthermore, A2M partially counteracts the inhibitory effects of transforming growth

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factor-α on proliferation of neoplastic hepatocytes, suggesting that under some conditions, A2M can promote hepatocarcinogenesis by perturbing transforming growth factor-α-induced apoptosis (Wollenberg et al., 1991). Increased concentration of this tumour marker in serum indicates the induction or progression of hepatocellular carcinoma. Both doxorubicin and SA nut milk extract reduced the concentration of A2M indicating curing action of both drugs. Reductions in elevated concentration of A2M were also noticed with HCC induced rats treated with blueberries (Sadik et al., 2008).

Semecarpus anacardium consists of many principles having anticancer properties, including both flavanoids and bhilawanols. Flavonoids have many biological effects that play a role in cancer prevention and treatment including free radical scavenging, antimutagenic and antiproliferative properties, regulation of cell signalling and cell cycle, and inhibition of angiogenesis (Moon et al., 2006). In vitro and in vivo experimental studies suggested that flavonoids influence signal transduction pathways (Frigo et al., 2002). The antitumour effects of plant flavonoids have been reported to induce cell growth inhibition and apoptosis in a variety of cancer cells (Di Carlo et al., 1999). Bhilawanols, which is localised maximally in the cell membrane of *Semecarpus anacardium*, exert its effect through changing the permeability of the membrane, affecting cellular growth (Patwardan et al., 1988) and this may also contribute to its anticancer property.

Conclusions

Semecarpus anacardium known as 'Ardha Vaidhya' have been used in Ayurveda and Siddha for the treatment of many diseases. Nut milk extract of this plant has already proven its anticancerous efficacy on HCC in the form of a Siddha preparation. This study was conducted for assessing the anticancerous property of nut milk extract of *Semecarpus anacardium* made according to Ayurveda system of medicine. The analysis of data indicates that Ayurvedic preparation of *Semecarpus anacardium* nut milk extract is equally effective to the Siddha preparation for the treatment of hepatocellular carcinoma. Anticancerous effect of this drug can be compared with that of doxorubicin. Even though this study was specific to rats, clinical trials can be conducted to determine curative effect of this Ayurvedic drug on human hepatocellular carcinoma.

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