RESEARCH COMMUNICATION

Inhibition of N-Methyl N'-nitro-N-nitrosoguanidine (MNNG) Induced Gastric Carcinogenesis by *Phyllanthus amarus* Extract

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Abstract

Chemopreventive activity of *Phyllanthus amarus* Schum & Thonn (Euphorbiaceae) extract was studied with regard to N-methyl N'-nitro-N-nitrosoguanidine (MNNG) induced stomach cancer in Wistar rats. Administration of the extract with MNNG significantly reduced the incidence of gastric neoplasms in rats (44%) as well as their numbers. Moreover, elevated levels of enzymes in the stomach were found to be reduced by *P. amarus* administration. For example, γ-glutamyl transpeptidase activity was decreased from 20.3 ± 6.7 mmol/min/mg protein to almost normal levels (2.8 ± 0.9) by 750mg/kg body weight of the extract. Similarly glutathione S-transferase activity (1317.6 ± 211 n mol/min/mg protein) and glutathione reductase (368 ± 66) levels in the MNNG treated group were found to be lowered to 494.8 ± 76 and 192 ± 45, respectively, while reduced glutathione (GSH) was increased from 4.6 ± 0.9 to 8.5 ±1.4 n mol/min/mg protein. AgNOR dots and clusters, indicators of cellular proliferation, which were increased by MNNG treatment, became near to normal in *P. amarus* treated animals.

Key Words: Phyllanthus amarus - MNNG - gastric cancer - chemoprevention - AgNORs

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Introduction

In spite of the immense efforts to improve treatment of cancer, overall mortality rates for many forms of cancer have not declined in the past 25 years (Hong and Sporn, 1994). Prevention of carcinogenesis is one of major strategies for cancer control. Several phytochemicals have been isolated and identified and have demonstrated to block or suppress the different stages of carcinogenesis (Sukumaran et al., 1994; Rajeshkumar and Kuttan, 2001; Surh, 2003; Ho et al., 1994; Huang et al., 1994, Tsao et al, 2004). Phyllanthus amarus is a small annual herb with significant medicinal properties. It has been found to reduce viral hepatitis in human and reduce the HbsAg antigen in carrier population and in culture (Yeh et al, 1993; Jayaram and Thyagarajan, 1996). Recently we have reported the anti- carcinogenic potential of its extract in animal models. Simultaneous administration of *P. amarus* extract along with carcinogen has been reported to inhibit the hepatocellular carcinoma development induced by N-nitrosodiethylamine (NDEA) (Jose et al., 1999; Rajeshkumar and Kuttan, 2000). *P. amarus* extract administration also inhibited the sarcoma development induced by 20-methyl cholanthrene (MC) (Rajeshkumar et al., 2002) and growth of transplanted ascites tumours in mice. Anti-mutagenic activity of *P. amarus* extract to Salmonella typhimurium strains has also been reported (Regi et al., 2002; Sripanidkulchai et al., 2002), along with inhibition of P-450 enzymes responsible for the activation of carcinogens (Rajeshkumar and Kuttan, 2000).

Despite the decrease in incidence, gastric cancer remains the second leading cause of cancer-related death worldwide (Nardone and Rocco, 2004) and it is one of the major causes of mortality in the Southern part of India. Exposure to environmental nitrite and nitrosation of smoked foods has been found to be associated with an increased risk of stomach cancer (Bartsch et al., 1992). Although the key nitrosating agent is nitrite, the situation with regard to the formation of carcinogenic nitrosocompounds is greatly complicated by the presence of other chemicals in the environment.

The present study was designed to assess the anti-carcinogenic activity of *P. amarus* against MNNG induced gastric cancer in Wistar rats.

Materials and Methods

Animals

Male Wistar rats (160-180g) purchased from the Small Animal Breeding Station, Veterinary College, Mannuthy, Kerala were used for the studies. They were housed in ventilated cages and fed with pelleted diet (Sai Durga Feeds and Foods, Bangalore) and water ad libitum. The animal experiments were conducted after obtaining prior permission

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from Institutional Animal Ethics Committee (IAEC) and was done as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Chemicals

NADP, 5'-5'dithiobis (2-nitro benzoic acid) (DTNB), reduced glutathione (GSH), and 1-chloro 2, 4 dinitro benzene (CDNB) were purchased from SRL Pvt. Ltd. Mumbai. The source of Folin Ciocalteu's reagent was Nice Chemicals Pvt. Ltd. Cochin. N-methyl N'-nitro-N-nitrosoguanidine (MNNG), gelatin and neutral red were obtained from Sigma Chemicals, St. Louis. Haematoxylin, eosin and silver nitrate were procured from E- Merck India. All other chemicals used were of analytical reagent grade.

Extraction of Phyllanthus amarus

Aerial parts of Phyllanthus amarus were collected from Thrissur district of Kerala and dried at 500C. A voucher specimen of the plant was identified by Regi Raphael K, Botanist, Amala Cancer Research Centre, Thrissur, Kerala (Voucher No: EUP. 9) and has been kept in the herbarium of Amala Ayurvedic Hospital and Research Centre, Thrissur.

Dried aerial parts of *Phyllanthus amarus* were powdered and the powder was extracted twice in 5 volumes of 75% methanol by stirring overnight and centrifuged at room temperature. This supernatant was evaporated to dryness at 50 0C under reduced pressure using a rotary evaporator. The yield of the extract was 8%. The extract was resuspended in double distilled water and used for the experiments.

Determination of the effect of P. amarus on N-methyl N'-nitro N-nitrosoguanidine (MNNG) induced stomach cancer

Treating animals with MNNG has been shown to result in the formation of preneoplastic cells. Further treatment of MNNG causes conversion of preneoplastic cells to neoplastic cells. Anti-neoplastic activity was evaluated by administering P. amarus extract during MNNG treatment. Male Wistar rats (10 animals / group) were used for the study. They were divided into four groups:

Group I : Normal, untreated
Group II : MNNG treated + Distilled water
Group III : MNNG + *P. amarus* 150mg/kg. b. wt
Group IV : MNNG + *P. amarus* 750mg/ kg. b. wt.

MNNG induced stomach cancer in rats was produced by the method described by Yamane et al (1995). MNNG was dissolved in water at a concentration of 1mg/ ml. This solution was kept in the freezer as aliquots and 1ml of the solution (1mg) was given to each rat by oral gavage every day for the 28 weeks. This procedure has been shown to induce gastric cancer in all untreated control animals (Yamane et al., 1995) (Group II), which received the vehicle i.e. distilled water. *P.amarus* was given orally starting from the day of MNNG administration and continued for 20 weeks. All the rats were sacrificed at the 44th week of the experiment as given in the original procedure. The oesophagus, forestomach, glandular stomach and duodenum were excised. The stomach was opened along the greater curvature and examined the gastric mucosa grossly. The location and size of tumours were recorded.

Biochemical analysis

The mucosa of the glandular stomach was removed by scraping with a blunt knife and 10% homogenate was prepared. Total protein was estimated by the method of Lowry et al (1951). The following biochemical parameters were assessed in the homogenized stomach mucosa: 1) γ-glutamyl transpeptidase (Tate and Meister, 1974); 2) cytosolic glutathione-S-transferase (Habig et al., 1974), a detoxifying enzyme generally increased in response to carcinogen administration; 3) tissue glutathione (Moron et al., 1979), generally decreased during carcinogen administration because of increased utilization for detoxification; 4) cytosolic glutathione reductase activity (Racker, 1955), increased to replace reduced cellular glutathione level during carcinogen administration.

Histopathological analysis

Tumour and normal appearing stomach sections were stained with haematoxylin and eosin and were examined under microscope (10x). Nucleolar organizer region associated proteins (AgNORs) were studied in paraffin sections of background stomach mucosa after staining with silver nitrate (Murray et al., 1989) as described by Lakshmi et al (1993).

Statistical analysis

The values are expressed as mean ± standard deviations. The results were analyzed statistically by one-way ANOVA followed by Kruskal Wallis test. Values for p less than 5% ($p < 0.05$) were considered to be indicative of statistical significance.

Results

Effects of P. amarus extract on MNNG induced carcinogenesis

The rats in the control and MNNG treated group did not show any significant variation in body weights during the experiment and there was no mortality. All the animals in the MNNG alone group demonstrated tumours at the non-glandular region of stomach. None were found in the controls. Administration of *P. amarus* inhibited stomach tumour development (Table 1). Nearly all lesions were typical adenomatous tumours. Numbers of tumours developed in P. amarus treated group was found to be significantly less, as reflected in the stomach weights (Table 1).

The treatment with *P. amarus* extract effectively lowered γ-GT, a marker of neoplasms (Hanigan and Pitot, 1985) in the stomach mucosa, from 20.3 ± 6.7 to 2.8 ± 0.9 mmol/ min/mg protein (Table 2) which is almost same as the normal level. MNNG administration increased mucosal GST and

Table 1. Effects of *P. amarus* on MNNG-induced Rat Gastric Carcinogenesis

Group	Treatment	No of tumour bearing rats	Stomach weight[1]
I	Normal Control	Nil	0.53 ± 0.05
II	MNNG Control	9/9	1.06 ± 0.10
III	MNNG+P.amarus 150mg	6/9	0.89 ± 0.08*
IV	MNNG+P.amarus 750mg	4/9	0.71 ± 0.06*

[1]Relative value g/ 100gb. wt *P< 0.05 as compared with group II

Table 2. Effects of *P. amarus* on γ-GT, GST, GSH and Glutathione Reductase Levels in the Stomach Mucosa of Rats Treated with MNNG

Group	g-GT[1]	GST[2]	GSH[2]	GR[2]
I	1.6 ± 4.6	344.9 ± 22	9.8 ± 1.2	129 ± 24
II	20.3 ± 6.7	1317.6 ± 211	4.6 ± 0.9	368 ± 66
III	10.5 ± 1.4*	779.8 ± 144*	5.5 ± 1.3*	286 ± 41*
IV	2.8 ± 0.9*	494.8 ± 76*	8.5 ± 1.4*	192 ± 45*

* P< 0.05 as compared with group II [1]mmol/min/mgprotein
[2]nmol/min/mgprotein

Table 3. Effects of *P. amarus* on Mean Frequency of AgNOR Dots and Clusters in Rat Stomach

Group	AgNOR dots	AgNOR clusters
I	1.5 ± 0.29	0.4 ± 0.11
II	3.9 ± 0.98	1.5 ± 0.45
III	2.9 ± 0.30*	1.0 ± 0.47*
IV	1.8 ± 0.51*	0.7 ± 0.37*

*P< 0.05 as compared with group II

P. amarus significantly reduced the elevation. Similarly trends were observed for stomach mucosal glutathione reductase. MNNG administration decreased GSH and this was partially reversed by administration of P. amarus.

Effect of P. amarus on AgNOR counts

The normal rats showed very low AgNOR values. This is to be expected since in the normal cells, hypertrascriptional activity of rDNA genes is not necessary. AgNOR dots and clusters of MNNG administered rats were found to be increased significantly (Table 3) in MNNG treated animals as compared to normal rats and this was partially reversed by *P. amarus*.

Discussion

The present study showed that *P. amarus* administration inhibits MNNG induced stomach cancers in rats in terms of both incidence and multiplicity. In addition, γ-GT, GST and GR, which were elevated in response to MNNG treatment , were almost normalized by *P. amarus* extract. MNNG treatment was found to suppress the level of GSH and this was found to be significantly increased by *P. amarus*.

Nucleolar organizer regions (NORs) are loops of DNA located on acrocentric chromosomes in the nuclei of normal and abnormal cells (Sirsat and Khanolar, 1962; Massimo et al., 1990). They encode for ribosomal RNA (rRNA) and the associated proteins are argyrophilic (Newbold et al., 1989) hence the term AgNOR. Since RNA are the sites of protein synthesis, the number of AgNORs per nucleus has a direct relationship to the cellular activity. It has been shown recently that the increase in the number of AgNORs in a proliferating cell is due to a wider dispersal of otherwise compact clusters of NOR associated proteins (Crocker et al., 1988).

AgNOR staining has now been recommended as a prognostic (Simha et al., 1996) and diagnostic tool in human (Anon, 1987) and canine studies (Bostock et al., 1989). Tumours having less than four AgNOR counts and a low proliferative index are generally benign, while greater counts indicate tumours of a malignant nature (Mehrotra and Chandra, 1998). *P. amarus* extract also produced significant decrease in AgNOR counts indicating that the extract decreases proliferative activity within the cell.

Hydrolysable tannins, namely amariin and geraniin, corilagin, 1,6-digalloylucopyranoside, rutin and quercetin–3-0- glucopyranoside, have been isolated from polar fractions of a methanolic extract of aerial parts of *P. amarus* (Foo, 1993). Some of the hydrolysable tannins were found to be potent inhibitors of wheat embryo Ca2+ dependent protein kinase (CDPK), rat brain Ca2+ protein kinase and phospholipid dependent protein kinase (PKC) and Ca2+-calmodulin dependent myosin light chain kinase (Polya et al., 1995). How this activity is related with the anti-carcinogenic activity of the extract is not known at present. Earlier reports from our laboratory showed that *P. amarus* could inhibit cdc 25 tyrosine phosphatase and cdc 2 kinase, topoisomerase II. It was also found to inhibit cytochrome P450 enzymes needed in the activation of carcinogen (Rajeshkumar and Kuttan, 2000) and further showed anti-oxidant activity and scavenged free radicals generated in vitro.

In summary, *P. amarus* has anti-carcinogenic action in the rat stomach which could be due to: a) alteration in cell signaling mechanisms; b) inhibition of cell cycling; c) inhibition of topoisomerase; d) inhibition of carcinogen activation; e) increased carcinogen detoxification; f) scavenging of carcinogen induced free radicals. Combined actions presumably contribute to overall activity of the extract against carcinogenesis.

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