Protection against azaserine induced pancreatic cancer in rats by
Phyllanthus amarus: a preliminary study

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Abstract
Pancreatic cancer is a major cause of morbidity and mortality in
developed and developing countries. We studied the
chemopreventive effect of Phyllanthus amarus on tissue lipid
peroxidation and antioxidant status, which is used as biomarkers in
azaserine induced pancreatic carcinogenesis in wistar rats. Male
albino rats were randomized into 8 groups of 10 animals each. Rats
in group 1 received 1.0 ml of 0.5% carboxyl methyl cellulose
(CMC) everyday via intragastric intubation and served as an
untreated control. Groups 2- 4 rats received Phyllanthus amarus via
intragastric intubation (p.o) at a daily dose of (100, 150, 200 mg/kg
body weight). The rats in groups 5-8 received azaserine (5 mg/kg
body weight) injection once in a week intraperitonially (i.p) for 3
weeks. In addition, groups 6 - 8 received Phyllanthus amarus as in
groups 2-4 respectively and continued till the end of the
experimental period. The animals were sacrificed at the end of 3
weeks. In the presence of azaserine, relative to the results for the
control rats, there were increased levels of lipid peroxidation, as
denoted by thiobarbituric acid reactive substances and decreased
activities of the enzymatic antioxidants (superoxide dismutase
(SOD), catalase (CAT), glutathione peroxidase (GPx), reduced
 glutathione (GSH)). Our results indicate that intragastric
administration of Phyllanthus amarus inhibits pancreatic
carcinogenesis, not only by modulating lipid peroxidation and
antioxidant status, but also by preventing azaserine induced
histopathological changes. Our results thus indicate that Phyllanthus
amarus may act as a chemopreventive agent for pancreatic
carcinogenesis.

Keywords: azaserine, antioxidants, lipid peroxidation, Phyllanthus
amarus.

Introduction
Pancreatic cancer is the fourth leading cause of cancer death in both
men and women (American Cancer Society 2009). It is usually
diagnosed late thus precluding effective treatment; and, with the
exception of an association with the smoking of cigarettes, the
etiology of this cancer is largely unknown (Mack 1982). Azaserine
(o-diazoacetyl-L- serine) is a bacterial mutagen and a pancreatic
carcinogen and serves as a standard model to study the health-
promoting effects of various substances in pancreatic carcinoma.
(Longeecter and Curphey 1975).

Azaserine is known to induce DNA damage in vivo (Cox et al
1973; Damjanov et al. 1973; Goodman et al. 1974; Sarma et al.
1975). One type of DNA damage observed is the alklylation of
purine and pyrimidine bases (Cleaver 1975; Sarma et al. 1975), a
characteristic action of monofunctional alkylating agents (Sarma et
al. 1975; Strauss 1974). Alkylated bases can be removed
enzymatically by endonucleases and/or by glycosidases (Lindahl
1976). In addition, purine alkylation can result in destabilization of
the N-glycosidic linkage between the base and its deoxyribose,
leading to spontaneous depurination (Lawley et al 1963) causing
DNA damage that eventually leads to neoplastic transformation of
pancreatic cells.

Pancreatic cancer is a pathological consequence of persisting
oxidative stress leading to DNA damage and mutations in cancer
related genes. (Cercutti 1994). All the experimental, clinical and
epidemiological studies suggest that reactive oxygen species (ROS)
are involved in the initiation and progression phases of pancreatic
cancer (Oberley 1988).

Now-a-days, medicinal plants play an important role in the
chemoprevention of pancreatic cancer. Herbs have recently
attracted attention as health beneficial foods and as source materials
for drug development. In addition, they are increasingly utilized in
treating a wide variety of diseases including cancer (Chattopadhyay
2003). Phyllanthus amarus is commercially known as bhumi amla
and has traditionally been used to treat flu, dyspsy, diabetes and
jaundice (Chopra et al. 1986).

The use of medicinal plants or their crude extracts in the prevention
and/or treatment of several chronic diseases has been traditionally
practiced in various ethnic societies worldwide. Phyllanthus
amarus has a long history of use in herbal medicine. It is well

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known for the biologically active compounds it possesses (Rizk 1987) including alkaloids, astragalins, brevifolin, carboxylic acids, corilagin, cymene, ellagic acid, ellagitannins, gallo catechins, geraniin, hypophyllanthin, phyllanthin, lignans, linetralins, lupeols, methyl salicylate, phyllanthine, phyllanthenol, phyllochrysine, phytetralin, repandusinic acids, quercetin, quercetol, quercirin, rutin, saponins, tricitranal and tricitonald (Khanna et al. 2002).

However, no studies have been undertaken to assess the effect of Phyllanthus amarus on the biochemical and histopathological changes occurring in experimentally-induced pancreatic cancer in rats. We therefore examined the protective effect of Phyllanthus amarus on azaserine-induced pancreatic carcinogenesis using lipid peroxidation and antioxidant levels as biomarkers.

The aim of this work was to evaluate the levels of antioxidants enzymes and thiobarbituric acid reactive substances (TBARS) formed during lipid peroxidation on pancreatic tumor-bearing rats. This article is the report on inhibitory and chemopreventive effect of Phyllanthus amarus on pancreatic carcinogenesis in rats.

Materials and Methods

Chemicals

Azaserine was obtained from the Sigma chemical company, St. Louis, USA. All the other chemicals and reagents used were of analytical grade.

Formulation and administration of Phyllanthus amarus

Phyllanthus amarus plant powder was suspended in 0.5% carboxy methyl cellulose (CMC) and each animal belonging to three different groups received 1.0 mL of Phyllanthus amarus suspension at three different dose of 100, 150 and 200 mg/kg body weight every day respectively by intragastric intubation.

Tumour induction

Azaserine was dissolved in 0.9 Nacl solutions. The rats were given a weekly intraperitoneal injection of azaserine at a dose of 5mg/kg body weight for three weeks (Longnecker D and Curphey T 1975).

Experimental animals

Eighty male albino wistar rats weighing 110-150 g were selected for the study. They were housed in plastic cages with filter tops under controlled conditions of 12h light and 12h dark cycles, 50% humidity at 28°C. Standard pellet diet was fed to rats throughout the experimental period and water was given ad libitum. The study protocol was carried out as per the rules and regulation of the institutional animal ethical committee (IAEC).

Treatment schedule

The rats were randomly assigned to 8 groups of ten animals each. The rats in group 1 received 1.0 mL of 0.5% CMC everyday via intragastric intubation and served as the untreated control. The rats in groups 2-4 received Phyllanthus amarus via intragastric incubation (p.o) at a daily dose of (100, 150 and 200 mg/kg body weight). The rats in groups 5-8 received azaserine (5 mg/kg body weight) injection once a week intraperitoneally (i.p) 3 weeks. In addition, groups 6-8 received Phyllanthus amarus in groups 2-4 respectively and continued till end of the experimental period. The experiment was terminated at the end of three weeks and all animals were killed by cervical dislocation after an overnight fast.

Preparation of tissue homogenate

Tissue samples were immediately transferred to ice-cold containers weighed and homogenized prior to biochemical assessments.

Biochemical assessments

Lipid peroxidation was estimated by measuring the level of thiobarbituric acid reactive substances (TBARS) in tissues via the method of (Ohkawa 1979). The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation was measured at 532nm. The values are expressed as nmole/100 g tissue. Reduced glutathione (GSH) content was determined in tissue via the method of Ellman (1959). The values are expressed as µmoles of GSH utilized/min/mg protein. Superoxide dismutase (SOD, EC.1.15.1.1) was assayed using the method of (Kakkar et al. 1984) based on the 50% inhibition of the formation of NADH-phenazine methosulfate-nitroblue tetrazolium formazan at 520nm. One unit of the enzyme was taken as the amount of enzyme required for 50% inhibition of NBT reduction/min/mg protein. The activity of catalase (CAT, EC.1.11.16) was determined via the method of (Sinha 1972). Dichromate in acidic acetate was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H2O2), with the formation of perchromic acid as an unstable CAT intermediate. The chromic acetate formed was measured at 590nm. Catalase was allowed to split H2O2 for different periods of time. The reaction stopped at different time intervals via the addition of a dichromate-acetic acid mixture, and heating the reaction mixture and measuring chromic acetate colorimetrically determined the remaining H2O2. The values were expressed as µmoles of H2O2 utilized/min/mg protein.

Histopathology

Part of the tissue was immediately fixed in 10% formalin for 24 hours, then the tissue was cut open at the antimesentric border and embedded on paraffin wax 3-5 µm sections, were sliced, stained with hematoxylin and eosin and the cell morphology as a whole was studied, following the standard micro technique (Carson 1990).

Statistical analysis

The results presented here are the means ± SD of 10 rats in each group. The results were analyzed using a one-way analysis of variance [ANOVA] and the group means were compared using Duncan’s multiple range test [DMRT] using SPSS version 12 for Windows. The findings were considered statistically significant if p<0.05 (Duncan 1957).

Results

Macroscopic observations

Figure 1 shows representative examples of the histopathological changes of the pancreas of the rats in the various experimental groups, as observed with a light microscope. The pancreas of the control rats (group 1) showed normal architecture. (Fig. 1A) which
Figure 1: show representative examples of the histopathological changes of the pancreas of the rats in various groups. (A) Control: shows normal architecture of pancreatic cells. (B-D) Phyllanthus amarus shows normal architecture of pancreatic cells with no pathological alterations. (E) Azaserine: shows macrovesicular fatty change in cirrhotic nodules, proliferated bile ducts and lymphocytic infiltrate. (F-H) Phyllanthus amarus + Azaserine: shows normal architecture of pancreatic cells.

was also the case when Phyllanthus amarus was administered at three different doses (100, 150 and 200 mg/kg body weight) throughout this study (Fig. 1B, 1C and 1D). The pancreatic architecture of the Phyllanthus amarus treated rats (groups 2-4) and control rats (group 1) was also observed to be similar. The pancreas of the azaserine treated rats (group 5) showed macrovesicular fatty change in cirrhotic nodules, proliferated bile ducts and lymphocytic infiltrate. In addition with this moderately differentiated adenocarcinoma, a small lobule of necrotic cells, focal necrosis of tumour cells and a desmoplastic stroma were also observed. (Fig. 1E). Finally, the phyllanthus amarus (100, 150 and 200 mg/kg body weight) + azaserine (5 mg/kg body weight) treated rats (groups 6-8) also showed normal architecture of pancreatic cells (Fig 1F, 1G and 1H).

Changes in the tissue levels of lipid peroxidation in the pancreas

Figure 2 shows tissue levels of TBARS in the control and experimental rats to be significantly increased in azaserine treated rats (group 5) as compared to the control rats (group 1). Phyllanthus amarus supplementation to azaserine treated rats (groups 6-8) significantly inhibited TBARS formation as compared to the un-supplemented azaserine treated rats (group 5).

Changes in the tissue levels of antioxidants in the pancreas

Figure 3 shows tissue levels of antioxidants (SOD, CAT, GPx and GSH) in control and experimental rats. The tissue levels of antioxidants (SOD, CAT, GPx and GSH) were significantly decreased in azaserine treated rats (group 5) as compared to the control rats (group 1). Phyllanthus amarus administration to azaserine treated rats (groups 6-8) significantly increased antioxidant levels (SOD, CAT, GPx and GSH) as compared to the un-supplemented azaserine treated rats (group 5).
Discussion

The search of new chemopreventive and antitumour agents that are more effective and less toxic has generated a great interest in activities such as antiviral and anticarcinogenic. Previously, it has been reported that *Phyllanthus amarus* prevents the induction of tumor in rats by inhibition of N-methyl N-nitro -N-nitrosooguanidine (MNNG) induced gastric carcinogenesis.(Regi Raphael et al. 2006).

Lipid peroxidation is a free radical -based mechanism of cellular damage. Azaserine is an important environmental carcinogen with the ability to interact with cellular biomolecules and also induce free radical generation (Dawid et al. 1963). On the other hand, free radicals can react with lipids causing lipid peroxidation and thus resulting in the release of major lipid peroxidation by-products such as malondialdehyde, 4-hydroxynonenal, etc. . An increase in lipid peroxide levels indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function and cell death (Pomppella et al. 1991).

In our study, we observed increased lipid peroxidation (TBARS) and decreased levels of antioxidant status in azaserine-induced rat pancreatic carcinoma . Tumor cells generally display high levels of lipid peroxidation, which in turn can stimulate cell division and promote tumor growth (Hammer et al. 1997; Dreher et al. 1996) primarily by setting up an oxidant-antioxidant imbalance that favours neoplastic transformation (Slater et al. 1984; McCord 2000).

*Phyllanthus amarus* administration to azaserine treated rats (groups 6-8) restored lipid peroxidation levels to near control levels (group1). *Phyllanthus amarus* exhibits anti-proliferative activity against various types of cancer (Primchanien et al. 2004) and thus suggesting that it may be able to regulate cell proliferation. In addition, *phyllanthus amarus* possess an antioxidant activity that may be responsible for its free radical-scavenging ability and thus preventing tumor promotion (Kumar and Kuttan 2005). Furthermore, it is a rich source of phytochemicals like flavonoids, phenols tannins and polyphenols (Chevallier 2002) two of which (e.g. quercecin and astragalin) have been found to prevent lipid peroxidation and enhance antioxidant defences in azaserine induced pancreatic cancer (Ledwozyw et al. 1986).

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide are produced under normal conditions and the body scavenges them through endogenous enzymatic (SOD, GPxs, Catalase) as well as non-enzymatic antioxidants (glutathione, bilirubin and vitamins). However, increased concentrations of circulating lipid peroxidation by-products (e.g. MDA) have been implicated in carcinogenesis (Manju and Nalini 2005; Chung et al. 2001; Ledwozyw et al. 1986; Cerutti, 1994). Our study does not only rests on endogenous antioxidant systems in order to scavenge free radicals but also on exogenous antioxidants such as vitamins, flavonoids and polyphenols derived from plants. Thus, plants might have critically important components for cancer prevention because of their ability to inhibit cell growth, induce apoptosis and scavenge free radicals (Surh 1999; Lambert et al. 2005).

Superoxide dismutase (SOD) is a major cellular antioxidant enzyme that plays an important role in cellular protection against the potentially harmful effects of superoxide anion formed during various biological processes (Zelko et al. 2002). On the other hand, catalase (CAT) is an enzyme found in all living organisms exposed to oxygen and catalyzes the decomposition of hydrogen peroxide to water and oxygen (Maehly and Chance, 1954). Finally, glutathione peroxidase (GPxs) is an important antioxidant enzyme that protects against free radical-induced cellular and tissue damage. It is a selenium-dependent enzyme that has high potency in scavenging superoxide ion and hydrogen peroxide radicals (Muller 2007). Glutathione (GSH) is a tripeptide that is utilized as substrate for GPxs and GST and constitutes an important source of reducing equivalents during oxidative stress (Fairlamb & Cerami 1992).In our study, we observed decreased levels of GSH, GPxs, SOD and CAT in azaserine-treated rats.. Increased lipid peroxidation associated with depletion of GSH and GPxs is a well known phenomenon in carcinogenesis. Supplementation with *phyllanthus amarus* increased the levels of GSH, GPxs SOD and CAT in azaserine-treated rats and could potentially inhibit cancer cell growth.

Our results reveal that supplementation with *phyllanthus amarus* induces the levels of the antioxidant molecules GSH, GPxs, SOD and CAT and thus reduces free radical generation-induced pancreatic damage. Furthermore, the protective antioxidant role of *phyllanthus amarus* may be due to its high content in flavonoids and tannins, both of which can act as strong superoxide radical and singlet oxygen quenchers (Jung 2007).

Conclusions

Our study provides evidence that administration of *phyllanthus amarus* significantly inhibits azaserine - induced pancreatic cellular damage by inhibiting lipid peroxidation formation and restoring azaserine-depleted antioxidant levels back to control levels. However, the usefulness of *phyllanthus amarus* supplementation as a chemoprevention agent against pancreatic cancer formation needs further research.

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