CHEMOPREVENTIVE ACTION OF *SYZYGIUM CUMMINI* SEED EXTRACT ON BENZOPYRENE-INDUCED GASTRIC CANCER

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Summary
The chemopreventive effect hydro alchoholic extract of *Syzygium cummini* extract (SCE) at a dose 125 mg/kg b.wt. was studied on the benzo(a)pyrene (BaP) induced fore stomach carcinoma in male mice. *Syzygium cummini* was used to explore antitumor promoting activity in a one-stage stomach carcinogenesis model. For this purpose, mice were administered with 1 mg of Bap in 100 µl sesame oil by oral gavage twice a week for 4 consecutive weeks and the animals were sacrificed 14 weeks after the last administration of BaP. Oral administration of the above extract in the Pre-post treated Group (i.e. such animals received the dose of SCE for 2 weeks before treatment with BaP followed by the concomitant treatment with SCE & BaP for 4 weeks during and 2 weeks after the last administration of BaP ), recorded a significant reduction in tumor incidence, tumor burden, cumulative number of papillomas along with a significant elevation of phase II detoxifying enzymes, and inhibition of lipid per oxidation in the stomach of the animals. The present data strongly suggests that the *Syzygium cummini* extract has anti-tumor potential against chemical induced gastric carcinogenesis.

Key Words: carcinogen, *Syzygium cummini*, chemoprevention, antioxidants, stomach papillomas, phase II enzymes.
Introduction

Environment pollutants are one of the main risk factors for the induction of cancer that is considered as a major public health concern and leading cause of death in both developing and developed countries. These pollutants include benzo(a)pyrene (BaP), which is a polycyclic aromatic hydrocarbon (PAH) formed by the pyrolytic process during smoking of cigarettes and other tobacco products as well as in combusted organic matter in automobile exhaust. BaP is frequently used as a representative indicator of total PAH levels. It is a complete carcinogen, as it produces initiation and promotion of carcinogenesis.

Cancer chemoprevention is a mean of cancer control by pharmacological intervention of the occurrence of the disease using chemical compounds. Recent events suggest that new emphasis in the development of medical treatment of human disease will be intimately connected to natural products. The use of medicinal plants in modern medicine for the prevention or treatment of cancer is an important aspect. For this reason, it is important to identify anti-tumor-promoting agents present in medicinal plants commonly used by the human population, which can inhibit the progression of tumor.

Consumption of fruits and vegetables is shown to lower the risk for chronic disorders such as cancer, cardiovascular diseases and stroke. The positive health effects may be due to high contents of certain phenolic compounds in plant-derived foods. Recently, phytochemicals and their effects on human health have been intensively studied. In particular, a search for antioxidants, hypoglycemic agents, and anticancer agents in vegetables, fruits, teas, spices and medicinal herbs has attracted great attention. Syzygium cumini, commonly known as Jamun has been attributed in the Indian folklore medicine system to possess several medicinal properties. The fruits and seeds are sweet, acidic, sour, tonic and these have many medicinal properties like anti-diabetic, anti-inflammatory, anti-diarrhea, anti-ringworm, anti-pharyngitis, anti-splenopathy. The leaves of such plants have been extensively used to treat diabetes, constipation, leucorrhoea, stomachalgia, fever, gastropathy, strangury and dermopathy, and to inhibit blood discharges in the faeces. The plant possesses acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercitin, quercetin, kaempferol and myricetin in different concentrations. Most of these compounds have been reported to possess antioxidant and free radical scavenging activities. The chemical composition and antioxidant activity of S. cumini fruits have been studied recently.

The present study has been undertaken to explore the possible anti-cancer and anti-oxidative potential of Syzygium cumini seed extract against chemical induced gastric carcinogenesis in mice.

Materials and Methods

Animal care and Handling

Random-bred Swiss albino mice (6-7 weeks old & weight 25±2 g) were used for the present experimentation. These animals were maintained in the animal house at room temperature of 24° ± 3° and 12 hrs light: 12 hrs dark periods. Such animals were housed in polypropylene cages and fed standard mice feed from Aashirvaad India Ltd., Chandigarh (India). Tap water was provided for drinking, and tetracycline once in a fortnight was given to prevent them against microbial infections. Animal care and handling were performed according to the guidelines set by the World Health Organization (WHO), Geneva, Switzerland, and the Indian National Science Academy (INSA), New Delhi, India. The departmental animal ethical committee has approved this study.
Chemical
Benzo(a)pyrene was procured from Sigma Chemical Company, St. Louis, MO, USA. The BaP-induced stomach tumorigenesis in mice was performed according to the method of Wattenberg et al.\textsuperscript{14} (1981) with minor modifications as suggested by Nagabhushan and Bhide\textsuperscript{15} (1987).

*Syzygium cummini* extract preparation

The fruits of *Syzygium cummini* were collected from local market after proper identification (Voucher No. RUBLR-20425) by a competent botanist in herbarium of the Department of Botany, University of Rajasthan, Jaipur (India). The pulp was removed from the fruit and the seed were washed properly and shade dried. After this, fruits were powdered in a mixture and the extract was prepared by refluxing with the double distilled water (DDW) for 36 (12×3) hrs at 40ºC. The extract was cooled and concentrated by evaporating its liquid contents. The required dose for treatment was prepared by dissolving the extract in DDW at a dose level of 125 mg/kg body weight.

Experimental Design

The Swiss albino mice of 6-8 weeks-old selected from random bred colony were divided into the following groups:

**Group I: Sterile tap water (STW) treated (Negative Control)**

The animals of this group received sterile tap water (STW) as drinking source throughout the study period.

**Group II: *Syzygium cumini* treated (Drug treated Control)**

Animals of this group were administered *Syzygium cumini* extract with the dose of 125 mg/kg/b.wt/day orally for 2 weeks.

**Group III: Sesame oil (SMO) treated (Vehicle treated Control)**

The animals of this group were injected with 100 µl/animal of sesame oil (SMO) by oral gavage twice a week for 4 consecutive weeks.

**Group IV: Carcinogen (BaP) treated (Positive Control)**

The animals of this group were administered with 1 mg of BaP in 100 µl sesame oil by oral gavage twice a week for 4 consecutive weeks (a total of eight administrations). These animals were sacrificed 14 week after the last administration of BaP.

**Group VII: SCE treated (Pre-post treatment group: Experimental)**

The animals of this group received the dose 125 mg/kg/b.wt./day of SCE for 2 weeks before treatment with BaP followed by the concomitant treatment of SCE and BaP for 4 weeks during and 2 weeks after the last dose of BaP in sesame oil.

The mice from all the groups were sacrificed at 14 weeks after the last dose of BaP for the following study:
1. Morphological
   a) **Tumor incidence.** This is the number of mice carrying at least 1 tumor expressed as percentage incidence.
   b) **Tumor yield.** This refers to the total number of tumors per group and the mean number of tumors per mouse.
   c) **Cumulative number of papillomas.** The total number of tumors appeared by the end of the experiment was considered the cumulative number of papillomas.
   d) **Tumor burden.** The average number of tumors per tumor bearing mouse was calculated as tumor burden.

2. Biochemical

Animals from all the above groups were necropsied at the end of the experiment i.e. 14 weeks and the whole stomach was taken for the study of following biochemical parameters were assayed.

**Lipid peroxidation (LPO)**

The level of LPO in stomach was measured in terms of thiobarbituric acid reactive substances by the method of Okhawa et al.16 (1979). Briefly, thiobarbituric acid (0.8%), sodium dodecyl sulfate (0.1%), and acetic acid (20%) were added to 100 ml. of the tissue homogenate for 60 min. It was cooled and extracted with N-butanolpyridine and the optical density of LPO was recorded at 532 nm. The content of LPO was expressed in nmol/mg.

**Glutathione (GSH)**

The level of reduced GSH was estimated by the method of Moron et al.17 (1979). The GSH content in the stomach was measured Spectrophotometrically using Ellman’s reagent with 5,5’-dithiobis 2-nitrobenzoic acid (DTNB) as a coloring agent, according to the method of Beutlar et al.18 (1963). The absorbance was recorded at 412 nm. The level of GSH was expressed as nmol/mg of protein.

**Catalase (CAT)**

Catalase activity was assayed in the stomach by the method of Aebi et al19 (1984). The content was estimated at 240 nm by monitoring the disappearance of H2O2

**Total Proteins**

Total proteins contents in stomach were measured by the method of Lowry et al.20 (1951). The absorbance was recorded at 680 nm.
Results

Morphological

Tumor incidence (Fig. 1)

Tumor incidence (number of mice carrying at least one tumor) is depicted in Fig. 1. In Group IV (Carcinogen-treated control), tumor incidence was found to be 100%. In the animals of Group V, tumor incidence was observed as 30%. Like this, experimental group, administered SCE, had considerably lower tumor incidences than the carcinogen treated control.

Tumor yield (Fig. 2)

Tumor yield (i.e. average number of papillomas per mouse), in Group IV (Carcinogen-treated control) was found to be 5.66, while in the animals of Group V, tumor yield was observed only 1.2.

Cumulative number of papillomas (Fig. 3)

No tumor was observed in the animals of Groups I, II and III. Cumulative number of papillomas in carcinogen treated control was recorded as 34. The counts of cumulative number of such papillomas were highly reduced (i.e. 10) in the experimental animals (Group V) as compared with carcinogen treated control (Group IV).

Tumor Burden (Fig. 4)

In Group IV (Carcinogen-treated control), tumor burden was found to be 5.65. On the other hand, the same was recorded as 1.69 in the animals which were treated with SCE along with carcinogen (Group V).

Biochemical

Lipid peroxidation (Fig. 5)

A significant increase ($P < .001$) in the level of LPO was recorded in Group IV (Carcinogen treated control) as compared with Groups I and II. Administration of SCE significantly ($P < .001$) reduced the level of lipid peroxidation in all the SCE treated experimental mice (Groups V) in comparison to the carcinogen treated control (Group IV).

Reduced glutathione (Fig. 6)

Treatment with Syzygium cummini extract resulted in an enhanced level of the non enzymatic antioxidant protein GSH in stomach of animals in group V as compared with the carcinogen treated control (Group IV). On contrary, a significant decrease ($P < .001$) in the level of GSH was recorded in Group IV (carcinogen treated control) animals as compared with the Groups I, II and III.

Catalase (Fig. 7)

The activity of catalase was observed as significantly ($P < .001$) declined in carcinogen treated control (Group IV) as compared to normal (Groups I), drug treated control (Group II) and sesame oil treated (Group III). A significantly increased ($P < .001$) CAT activity was recorded in the stomach of SCE-treated experimental animal (Groups V) than the carcinogen treated control (Group IV).
**Total Proteins (Fig. 8)**

A significantly increase ($P < .001$) in total proteins activity was recorded in the stomach of SCE-treated experimental animal (Groups V) as compared with the carcinogen treated control (Group IV).
Fig. 3: Variations in cumulative number of papillomas during BaP-induced gastric carcinogenesis with or without SCE administration

Fig. 4: Variations in tumor burden during BaP-induced gastric carcinogenesis with or without SCE administration
Fig. 7: Variations in catalase (CAT) level during BaP-induced gastric carcinogenesis with or without SCE administration

Significant level: Normal vs Carcinogen treated Control; Carcinogen treated Control vs Experimental
P value: b ≤ 0.01, c ≤ 0.001

Legend:
- Normal
- Sesame oil treated
- SCE treated
- Carcinogen treated Control
- SCE treated Experimental

Fig. 8: Variations in total proteins level during BaP-induced gastric carcinogenesis with or without SCE administration

Significant level: Normal vs Carcinogen treated Control; Carcinogen treated Control vs Experimental
P value: a ≤ 0.05 b ≤ 0.01, c ≤ 0.001

Legend:
- Normal
- Sesame oil treated
- SCE treated
- Carcinogen treated Control
- SCE treated Experimental
Benzo[\(a\)]pyrene is a polycyclic aromatic hydrocarbon (PAH) that can be derived from coal tar occurring ubiquitously in products of incomplete combustion of fossil fuels and it has been identified in ambient air, surface water, drinking water, waste water and in charbroiled foods (IARC, 1983). Benzo[\(a\)]pyrene is primarily released to the air and removed from the atmosphere by photochemical oxidation and dry deposition to land or water. Biodegradation is the most important transformation process in soil or sediment (ATSDR, 1990). Benzo[\(a\)]pyrene is readily absorbed following inhalation, oral and dermal routes of administration (ATSDR, 1990). Following inhalation exposure, benzo[\(a\)]pyrene is rapidly distributed to several tissues in rats (Sun et al. 1982; Weyand and Bevan, 1986). The metabolism of benzo[\(a\)]pyrene is complex and includes the formation of a proposed ultimate carcinogen, benzo[\(a\)]pyrene 7,8 diol-9,10-epoxide (IARC, 1983). The major route of its excretion is hepatobiliary followed by elimination in the feces (U.S. EPA, 1991).

The exposure of mice to the BaP in Group IV (carcinogen treated control) caused significantly high tumor incidence, tumor yield, cumulative number of papillomas and tumor burden, but all these parameters were found to be greatly decreased in the experimental mice (Group VII) in which SCE was administered. This fall may be due to factors such as inhibition of B(a)P metabolism to its active form or delay in the promotion phase of tumorigenesis via down regulation in the production of reactive oxygen species (ROS). A similar effect of BaP in tumor induction has been reported earlier by the others (Wattenberg et al., 1980; Nagabhushan and Bhide, 1987; Azuine and Bhide, 1992; Deshpande et al., 1997; Agha et al., 2001).

There was a significant increase in lipid peroxidation while the specific activity of CAT, reduced GSH, total proteins were decreased in BaP induced mice as compared with corresponding experimental group. This may be due to the adverse effect of B(a)P. B(a)P, an extremely potent pro-carcinogen, which is metabolized by biotransformation enzymes to a variety of metabolites that are responsible for initiating carcinogenesis (Choi et al.). Biotransformation enzymes have broadly been divided into two categories namely phase-I and phase-II. The former constitutes cytochrome P-450 based mono-oxygenase system which is responsible for initiating conversion of pro-carcinogens to several of their metabolites including ultimate carcinogens. Glutathione-S-transferase (GST) is a major phase II detoxifying enzyme that primarily functions in catalyzing the active carcinogenic metabolites to endogenous ligand-reduced glutathione (GSH) favoring their elimination from the body of the organisms (Hartman et al.). The balance between the phase-I carcinogen-activating enzymes and the phase-II detoxifying enzymes is critical to determining an individual's risk for cancer (Wilkinson et al.). There is substantial evidence that chemopreventive agents including medicinal plants exert their anti-carcinogenic effects by modulation of phase-I and phase-II xenobiotic biotransformation enzymes (Subapriya et al.).

Syzygium cumini seed extract treated mice showed a significant increase in the level of CAT, reduced GSH, total proteins in the stomach as compared to carcinogen control treated. It may be due to the singlet oxygen quenching ability of phenolic compounds present in such seed extract. The antioxidant properties of flavanoids from different plant sources have been reported by earlier workers (Husain et al, 1987; Robak & Gryglewski, 1988; Rios et al, 1992; Calomme et al, 1995). Plant extract treatment increases the activity of SOD and CAT, and it scavenges superoxide radicals and reduces cellular damage caused by free radicals (Prince et al, 1998; Chaurasia et al, 2000; Lee and Shibamoto, 2001). From the present results, it is evident that induced SOD serves to remove O' by accelerating the formation of H2O2. Since, H2O2 is harmful to cells, CAT, GSH and GPx levels decreased in carcinogen treated control mice. The increased level of CAT and GSH and GPx in experimental mice (Syzygium cumini treated) would help to metabolize H2O2 to water. GSH reeducates helps GSH, GPx by way of action as GSH reducing agent and also increased...
to protect the cells from pro-oxidant. Lipid peroxidation was significantly decreased in the extract treated group in the present study. The observed reduction in the level of lipid peroxidation may be due to the presence of anti oxidant compound such as β-carotene and eugenol in Syzygium cumini. It has been reported that eugenol plays an important role in the reduction of lipid peroxidation\(^40\) (Lee and Shibamoto, 2001) by scavenging superoxide anion radicals. Decrease in lipid peroxides could also be due to the reduction of free fatty acids and increased level of free radicals scavenging enzymes\(^41\) (Chithra & Leelamma, 1999). In the others finding, alcoholic extract of the shoot Hypericum perforatum inhibited lipid peroxidation partly by scavenging the OH\(^-\) radicals and chelating of Fe\(^{2+}\)/Fe\(^{3+}\) iron and thereby inhibiting the production of super oxide anions\(^42\) (Tripathi and Pandey, 1999). Similarly, ethyl acetate and methanol extracts of Pleurotus florida showed a significant lipid peroxidation inhibition activity\(^43\) (Nayana Jose and Janardhanan, 2000).

In the present investigation, inhibition of the lipid peroxidation, tumors incidence, tumor yield, cumulative number of papillomas and tumor burden, and enhancement of the antioxidant enzymes level could be due to the seed extract of Syzygium cumini which possess a strong free radical scavenging properties. These results demonstrate the possible use of such plant extract in chemoprevention of chemical induced carcinogenesis in mammals.

References

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