Chemomodulatory Influence of *Cissampelos pareira* (L.) Hirsuta on Gastric Cancer and Antioxidant System in Experimental Animal

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Abstract

The objective of the present study was to investigate the protective effect of ethanolic extract of *Cissampelos pareira* (CPE) against gastric cancer and its mode of action. The bioassay-guided fractionation of the CPE yielded a compound, identified as 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Quercetin) with the help of NMR and various spectrophotometer methods as U.V., I.R. and other chemical tests. The protective effects of *C. pareira* were studied against Benzo(a)pyrene (B(a)P) induced gastric cancer, tumor multiplicity, micronucleus polychromatic erythrocytes (MnPCEs) in mice. The effect of CPE on SOD, CAT, LPO and GST, GPx, GSH was also studied.

**Keywords:** *Cissampelos pareira,* Quercetin, Gastric cancer, Micronucleus polychromatic erythrocytes

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Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in plant origin. Over 4000 different flavonoids have been described (Hollman and Batan, 1997), and they are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. Flavonoids have a variety of biological effects on numerous mammalian cell systems, *in vitro* as well as *in vivo*. They have been shown to exert antimicrobial, antiviral, antiulcerogenic, cytotoxic, antineoplastic, mutagenic, anti-inflammatory, antioxidant, antihepatotoxic, antihypertensive, hypolipidemic and antiplatelet activities (Formica and Regelson, 1995).

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Natural products represent a reservoir of diverse templates and increasingly, medicinal plants are being tapped to outsource novel anti-cancer agents (Harvey, 2000). Gastric cancer may be regarded as a series of malignant diseases characterized by abnormal growth of cells into neoplasm, ability to invade adjacent and even distant tissues and eventually the death of the patient. Therefore, the cancer chemotherapeutic agents that are currently being used are cytotoxic drugs that kill malignant cells or modify their growth pattern. Benzo(a)pyrene (B(a)P) is a polycyclic aromatic hydrocarbon and is proved to be an effective carcinogen in producing lung and fore-stomach neoplasia, in many animal model systems (Badary et al., 1999). Since B(a)P is a widespread environmental pollutant and is believed to be a risk factor in human chemical carcinogenesis, it becomes increasingly important to identify the naturally occurring/synthetic compounds that can interfere with B(a)P-induced carcinogenesis (Halliwelland Gutteridge, 1989).

Cissampelos pareira (L.) Hiruta (family: Menispermaceae) is a wound healer and antidote, paste of roots are used in fistula, pruritis, skin disorders and snake poison externally. Internally it is useful in anorexia, indigestion, abdominal pain, diarrhoea and dysentery (Anonymous, 1992). It is also used in cough and as it purifies breast milk, is used in various disorders of breast milk secretion. They are frequently prescribed for cough, dyspepsia, dropsy, urino-genital troubles such as prolapus uteri, cystitis, haemorrhage and menorrhagia, and calcular nephritis (Kirtikar and Basu, 2001). Cissampareine, a bis-benzyl-isoquinoline alkaloid, showed a significant and reproducible inhibitory activity against human carcinoma cells of the naso-pharynx in cell culture (Morita et al., 1993). The present experiment was designed to study the efficacy of CPE against Benzo(a)pyrene induced forestomach cancer, genotoxicity including the biochemical parameters.

Materials and Methods

Plant material and preparation of extracts

Root of Cissampelos pareira (L.) Hiruta (family: Menispermaceae) were collected locally in September 2004 and were identified and authenticated taxonomically at National Botanical Research Institute, Lucknow. They were washed with distilled water to remove dirt and soil, and shade dried. The dried materials were powdered and passed through a 10-mesh sieve. The coarsely powdered material (2.5 Kg) was extracted thrice with ethanol (50% v/v). The extracts were filtered, pooled and concentrated at reduced temperature (-5°C) on a rotary evaporator (Buchi, USA) and then freeze-dried (Freezone® 4.5, Labconco, USA) at high vacuum and at temperature -40 ± 2°C (yield 3.4%, w/w). The dry extracts (CPE) were subjected to various chemical tests to detect the presence of different phytoconstituents (Amresh et al., 2003). Total phenol present in the extract was calculated by the method of Taga et al. (1984).

The extract (CPE; 75g) was suspended in distilled water (1,500 ml) and then extracted exhaustively with hexane (8 X 500 ml), chloroform (8 X 500 ml) and methanol (8 X 500 ml) in succession and solvents were removed. The methanolic
fraction was saponified with 20% ethanolic KOH (300ml) for 2h. The contents of the flask were then evaporated to remove all traces of EtOH, the lost volume being replaced by water from time to time. The unsaponifiable portion was then extracted with ether (5 X 300ml). All the ethereal fractions were combined, dried over anhydrous sodium sulphate and the solvent was evaporated to afford a yellow residue.

The yellow residue obtained was dissolved in CHCl₃ and adsorbed over neutral alumina. After evaporation of the solvent it was loaded onto a neutral alumina column prepared in hexane. Column chromatography was carried out on Silica gel and neutral alumina. The column was eluted first with hexane, then with graded mixtures of hexane: toluene (95:5, 90:10, 80:20 and 50:50), then with toluene followed by graded mixtures of toluene: chloroform (95:5, 90:10, 80:20 and 50:50), chloroform and finally chloroform: methanol (95:5, 90:10, 80:20 and 50:50). The elution was monitored by TLC (visualization: vanillin-sulphuric acid). Each time 5 ml elutes were collected and identical elutes (TLC monitored) were combined and concentrated under reduced pressure.

Elutions carried out with chloroform: methanol (80:20) resulted in deposition of yellow solid which on recrystallization from toluene gave a compound, M.P. 312°C, Rf 0.51 (toluene: ethyl acetate 85:15) designated as compound I (93mg).

Selection of animals and ethics

Albino mice (18 – 24 g) of either sex were purchased from the animal house of the Central Drug Research Institute, Lucknow. They were kept in departmental animal house in well cross ventilated room at 27 ± 2°C, and relative humidity of 44 - 56%, light and dark cycles of 10 and 14h respectively for one week before and during the experiments. Animals were provided with standard rodent pellet diet (amrut, India) and the food was withdrawn 18 - 24h before the experiment thought, water was allowed ad-libitum. All the experiments were performed in the morning according to current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals (Amresh et al., 2004).

Treatment protocol

All the chemicals used were of analytical grade from standard companies and were purified before use. The water represents the double distilled water. The standard orogastric cannula was used for oral drug administration. C. pareira extract given orally showed no gross evidence of any abnormalities or mortality in mice at a dose of 2 g/kg body wt. up to the end of the observation period. The doses were fixed according to on our earlier studies on the ethanolic extract of C. pareira (Amresh et al, 2004). For the pharmacological tests the extract was suspended in carboxy methyl cellulose (1% w/v, CMC) in double distilled water.

B(a)P induced Gastric cancer

Benzo(a)pyrene was procured from Sigma Chemical Company, St. Louis, USA. The B(a)P induced stomach tumor in mice was performed according to Wattenberg
et al. (1980) with minor modifications (Nagabhushan and Bhide, 1987). Group 1 received basal diet and tap water along with 1% CMC throughout the experiment and served as the untreated control. The animals in group 2 were administered 1mg of B(a)P in 100 μl sesame oil p.o. gavage twice a week for 4 weeks (a total of eight administrations) to induce cancer. Two different doses of CPE (50, 100 mg/kg, body wt.) were administered p.o. gavage twice a day to group 3 and group 4 animals for 6 weeks; 2 weeks before the treatment with B(a)P and 4 weeks along with the B(a)P. The experiment was terminated 14 weeks after the last administration of B(a)P and all the animals were sacrificed by cervical dislocation after an overnight fast.

10% phosphate buffered formalin was immediately injected into the stomach, so that it would be distended and fixed. The forestomach was cut open longitudinally and kept in 10% buffered formalin for 24 h for fixation. The stomach papillomas that were 1mm or larger in diameter were counted under a stereozoom microscope. Formalin fixed forestomach was embedded in paraffin wax and processed for histology. The hematoxylin and eosin stained slides were scored in blind fashion.

The chemopreventive tumor response was assessed on the basis of tumor incidence, mean and multiplicity of tumors as follows:

Tumor incidence = Number of animals having tumors

Mean = (Lowest number of tumors + Highest number of tumors)/2

Tumor Multiplicity = Total number of tumors scored/Total number of animals with tumors

**Micronucleus test and Cytogenetic analysis**

The bone marrow micronucleus test was carried out according to Schmid (1975) for evaluating chromosomal damage in experimental animals. The bone marrow from the femur was flushed in the form of a fine cell suspension into a centrifuge tube containing fetal calf serum. The cell suspension was centrifuged at 500 × g for 10 min and the supernatant was discarded. The pellet was resuspended in a drop of serum and used for preparing slides. The air-dried slides were stained with May-Grünwald and Giemsa. A total of 2,500 polychromatic erythrocytes (PCEs) were scored per animal from a single slide to determine the frequency of micronucleus polychromatic erythrocytes (MnPCEs).

Bone marrow cells from control and experimental animals were processed for analysis of chromosomal aberrations by the method of Sharma and Sharma (1994). The bone marrow from the femurs was flushed into a centrifuge tube containing 0.9% saline and centrifuged at 500 × g for 5 min. The supernatant was removed and hypotonic KCl was added to the sediment. After incubation for 20 min at 37°C, the contents were centrifuged for 5 min and the sediment was fixed in methanol-acetic acid (3:1v/v). Three changes of fixative were given prior to slide preparation. The slides were air-dried, stained in Giemsa solution and scored blindly. One hundred well-scattered metaphase plates were scored for each animal. All the slides were scored by the same observer.
Biochemical analysis

In order to detect the influence of CPE on the antioxidant status of B(a)P bearing mice, the stomach tissue were used to study the various biochemical parameters. The stomach tissue of the mice treated with B(a)P was homogenized (5%) in ice-cold 0.9% NaCl with a Potter-Elvehjem glass homogenizer for 30 seconds. The homogenate was centrifuged at 800 x g for 10 min and the supernatant was again centrifuged at 12,000 x g for 15 min. The resultant mitochondrial pellet was then washed and resuspended in 0.25 M sucrose. The purity of the mitochondria were assessed by assaying the activity of succinate dehydrogenase (SDH) and used for further analyses. Superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) were estimated by the established methods in our lab already described in Rao et al. (2004). The SOD enzyme has been expressed as units (U) of SOD activity/mg protein, Catalase was expressed as units (U) of CAT activity/mg protein, while the LPO was expressed as nmoles/mg protein using 1,1,3,3-Tetraethoxypropan as standard for calibration of the curve. Reduced glutathione (GSH) was determined by the method of Anderson (1985), glutathione (GPx) by that Rottruckand Pope (1973) while Glutathione S-transferase (GST) activity by Habig et al. (1981). GSH and GST activities were expressed as nanomole per minute per milligram protein (nmol/min/mg protein) while GPx activity was determined by determining the amount of H₂O₂ by estimating GSH content.

Statistical analysis

All the data were presented as mean ± SEM and analysed by Wilcoxon Sum Rank Test and unpaired Student’s t-test for the possible significant interrelation between the various groups. A value of \textit{P}<0.05 was considered statistically significant.

Results

The preliminary phytochemical screening revealed the presence of flavonoids, saponins, triterpenoids, alkaloids, proteins, gums, fatty oils, mucilage, carbohydrates, resins and tannins. The isolated compound I was identified with the help of \textsuperscript{1}H and \textsuperscript{13}C NMR spectra and various other chemical tests as Shinoda’s test (Magenta color), Neutral lead acetate (Bright orange precipitate), Ferric chloride (Green color), Aqueous sodium hydroxide (Deep yellow solution), Wilson’s boric-citric acid reagent (yellow solution). The comparison of UV and IR spectra of compound I with literature data (Kim \textit{et al.}, 2006), showed that all the spectra of compound I have matched with that of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Quercetin). \textsuperscript{1}H NMR spectra of the compound was taken on varian EM- 360 (270MHz) NMR spectrometer using CDCl₃ as solvent. Mass spectra were recorded on a spectrophotometer and FAB–MS (positive) data on JEOL SX 102/DA–600, which further supported the findings. The CPE was also found to contain 337 ± 15.4 mg/g total polyphenolics expressed as gallic acid equivalent (GAE, mg/g of GAE).

75
In the present study, the protective effects of CPE (50, and 100 mg/kg body wt.) against B(a)P induced gastric cancer in mice were studied. The mean body weight of different groups was also affected by the use of the CPE. There was a dose dependent and significant body weight gain in the CPE treated groups compared to the B(a)P treated group (Figure 1). The 50 and 100 mg/kg body wt. doses of CPE showed protective effect against B(a)P induced gastric cancer. The tumor incidence was reduced while the mean number of tumor was reduced significantly and dose dependently. The tumor multiplicity was also reduced significantly at 100 mg/kg (Figure 1).

![Graph showing tumor incidence, number of tumor, tumor multiplicity, and body wt variation across different groups.](image)

Values are mean ± SEM; n = 6 mice

P: \(^a<0.01\), \(^b<0.02\); and \(^c<0.001\) \(^d<\)not significant compared to respective B(a)P group

**Figure 1.** Chemopreventive effect of *Cissampelos pareira* extract (CPE) on Benzo(a)pyrene (B(a)P) induced gastric cancer in mice

Figure 2 shows the antigenotoxic effects of CPE on the frequency of B(a)P induced MnPCEs and chromosomal aberrations. A significant increase (P<0.001) in the frequency of MnPCEs and chromosomal aberrations was found in the group of B(a)P treated mice as compared to control group. Treatment of animals with B(a)P and CPE significantly reduced the incidence of MnPCEs (P<0.001) at 100 mg/kg and chromosomal aberrations (P<0.01, and P<0.001) compared to B(a)P treated group dose dependently.
Values are mean ± SEM; n = 6 mice
P: *<0.001 compared to control group
P: a<0.01; and b<0.001 compared to respective B(a)P group.

**Figure 2.** Inhibitory effect of *Cissampelos pareira* extract (CPE) on Benzo(a)pyrene (B(a)P) induced bone marrow micronucleated polychromatic erythrocyted (MnPCEs) and chromosomal aberration in mice

Figures 3 and 4 illustrate the effect of treatment of CPE on stomach weight, SOD, CAT, LPO and GST, GPx, GSH respectively. Relative stomach weight at the termination of the experiment has not significantly varied. Administration of B(a)P significantly increased SOD (P<0.001) and LPO (P<0.001) while decreased CAT (P<0.001) when compared to control group. Further treatment with CPE decreased the LPO and SOD significantly and dose dependently while the catalase level was significantly and dose dependently increased (Figure 3).

The level of GSH and the activities of GPx and GST were significantly (P<0.01, P<0.05 and P<0.001, respectively) reduced in B(a)P treated animals compared to control. Treatment of animals with CPE increased GSH, GPx and GST significantly and dose dependently (Figure 4).
Values are mean ± SEM; n = 6 mice
P: x<0.001 compared to control group
P: a<0.05, b<0.01 and c<0.001 compared to respective B(a)P group

Figure 3. Effect of *Cissampelos pareira* extract (CPE) on lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) in gastric tissue on Benzo(a)pyrene (B(a)P) induced gastric cancer in mice.
Values are mean ± SEM; n = 6 mice.
P: a<0.05, b<0.01 and c<0.001 compared to respective control group.
P: a<0.05, b<0.01 and c<0.001 compared to respective B(a)P group.

**Figure 4.** Effect of *Cissampelos pareira* extract (CPE) on Reduced Glutathione (GSH), Glutathione Peroxidase (GPx), Glutathione S-transferase (GST) activity in gastric tissue on Benzo(a)pyrene (B(a)P) induced gastric cancer in mice.
Discussion

Chemopreventive agents can be targeted by intervention at the initiation, promotion, or progression stage of multistage carcinogenesis (Stoner and Mukhtar, 1995). The intrusion of cancer at the promotion stage, however, seems to be the most appropriate and practical. The present experiment was designed to study the potential of *Cissampelos pareira* root extract on antioxidant enzymes to check its efficacy against benzo(a)pyrene induced forestomach carcinogenesis.

The administration of the carcinogen B(a)P caused 100% incidence of forestomach tumors, while the control did not induce any tumors in the recipient animals. The tumor multiplicity in the B(a)P alone group was also high. A similar effect of B(a)P in tumor induction has been reported earlier (Wattenberg et al., 1980; Nagabhushan and Bhide, 1987). The administration of *Cissampelos pareira* extract (CPE) before and during the carcinogen treatment suppressed the B(a)P induced forestomach tumorigenesis. There was a dose dependent decrease in the multiplicity of the tumors and the significant effect was observed at 100 mg/kg CPE.

In recent studies, it has been shown that pro-oxidants expeditiously block the antioxidant systems of mucosal cells, which cause ROS formation. As a consequence of this process, oxidative damages occur. Antioxidants have various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Rao et al., 2004).

Under the present experimental conditions, CPE may provide sufficient protection against any pro-oxidant-mediated injury, including tissue damage, owing to intracellular and/or extracellular hydrogen peroxide accumulation. The activity of another antioxidant enzyme, SOD that has been found to be augmented by CPE, accelerates dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Hydrogen peroxide produced is further removed by catalase (Das and Banerjee, 1993). Therefore, CPE induced SOD activity, in conjunction with catalase, antagonizes any free radical induced injury (Sairam et al., 2002). CPE significantly reduces lipid peroxidation, as measured by MDA production, and eliminates the possibility of oxidative stress due to the administration of extract to mice which causes mucosal tissue damage, and the lipid peroxidation product malondialdehyde in an indicator of ROS generation in the tissue breaking of DNA strands and denaturing cellular proteins (Halliwell and Gutteridge, 1989), which is being protected by CPE.

Enhanced lipid peroxidation also coupled with depletion of GSH and GSH dependent enzymes in the stomach of B(a)P treated mice may shift the redox status, with adverse effect on -SH groups of functional proteins resulting in oxidative stress. The increase frequency of bone marrow micronuclei seen in the present study may have been due to B(a)P induced oxidative stress. Oxidative stress arising due to ROS overproduction and break down of antioxidant defenses has been documented to induce chromosomal damage and formation of micronuclei (Simic, 1994). Enhanced bone marrow micronuclei in B(a)P treated mice in the present study confirm results by other workers on the genotoxic effect of B(a)P (Salmenova et al., 1997).

Treatment with CPE significantly decrease the frequencies of B(a)P induced MnPCEs. The enhanced activity of the GSH/GST detoxification systems seen in the present study may attenuate B(a)P induced oxidative stress and the frequency of bone marrow micronuclei. GSH, an important non-protein thiol, in conjugation with GPx and GST, plays a central role in
maintaining the integrity of the gastric mucosa, when challenged by the toxic agents as B(a)P (Hayes and Pulford, 1995).

It has been shown that many agents as 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Quercetin) isolated from numerous plant species control lipid peroxidation in tissues by reducing the levels of lipid peroxides (Formica and Regelson, 1995), and also have catalyzing effects on glutathione (GSH). GSH and GSH-bound enzymes in tissues, especially, glutathione peroxidase (GPx) and GST, have been proposed as potential chemopreventive agents for their antioxidant and detoxification properties (Hayes and Pulford, 1995). GSH is supplied to stomach tissue from liver. GPx uses GSH as a substrate to catalyze the reduction of organic hydroperoxide and hydrogen peroxides (Meister, 1994).

Phenolic constituents have been studied extensively as important contributors to the anticancerous and antioxidant activity in plants. There are reports in the literature which correlate the total phenolics content of a plant extract with its antioxidant activity and this was also the case in the present study. The root extracts with high phenolics content has also high antioxidant and lipid peroxidation inhibition capacities. Quercetin appears to have many beneficial effects on human health, including cardiovascular protection, anti-cancer activity, anti-ulcer effects, anti-allergic activity, cataract prevention, antiviral activity, and anti-inflammatory effects (Guardia et al., 2001). Quercetin may also increase the effectiveness of chemotherapeutic agents which has been investigated in a number of animal models and human cancer cell lines, and has been found to have antiproliferative effects. (Scambia et al., 1990; Scambia et al., 1992). The protective effect of the Cissampelos pareira extract against benzo(a)pyrene induced gastric cancer may therefore be due to the quercetin present therein, which act as an anti-neoplastic agent.

To conclude, our studies have shown that the root of Cissampelos pareira possess a marked anticancerous agent and could therefore have a promising role in the treatment of forestomach cancer. Our future studies will be therefore focused on the isolation and characterization of other active-marker compounds in the crude extracts of the CPE responsible for the anti-cancerous activities.

References


Received: 13.07.2006
Accepted: 27.11.2006