

In vitro* and *in vivo* evaluation of the anticarcinogenic and cancer chemopreventive potential of a flavonoid-rich fraction from a traditional Indian herb *Selaginella bryopteris

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Abstract

Prevention of cancer through nutritional intervention has gained significant recognition in recent years. Evidence revealed from mechanistic investigations coupled with molecular epidemiology show an inverse association of dietary flavonoids intake with cancer risk. The chemopreventive and anticarcinogenic potential of *Selaginella bryopteris*, a traditional Indian herb referred to as ‘Sanjeevani’ in the Ayurvedic system of medicine, was examined in the present study. Comprehensive *in vitro* and *in vivo* studies were conducted on the flavonoid-rich benzene fraction of the aqueous extract that demonstrated a significant cytoprotective activity. Biomarkers of chemoprevention such as proliferative index and status of cell-cycle regulatory proteins, antioxidant property, anti-inflammatory effect, reversal of stress-induced senescence and genoprotective effect were investigated in human and murine cell cultures. Chemopreventive potential was assessed in benzopyrene-induced lung carcinogenesis and 7,12-dimethyl benz(a)anthracene-mediated skin papillomagenesis test models. Inhibition of DNA fragmentation, unperturbed cell-cycle regulation, maintenance of intracellular antioxidant defence, anti-inflammatory activity, prevention of stress-induced senescence and genoprotective effects against methyl isocyanate carcinogenicity was observed. Medium-term anticarcinogenicity and two-stage skin papillomagenesis tests strongly substantiated our *in vitro* observations. Results from the present study provide evidence of anticarcinogenic and chemopreventive activities of *S. bryopteris* hitherto unreported and reaffirm the nutritional significance of flavonoids in cancer prevention.

Key words: Cancer therapy: Flavonoids: Translational oncology: Methyl isocyanate: Sanjeevani

Cancer results from a multifactorial and multi-stage process consisting of three distinguishable but closely related stages: initiation, promotion and progression phases. Apart from genetic factors, significant scientific efforts have been made recently in identifying the mechanistic facets of gene–environment interaction and increased incidence of human cancers^(1,2). The monographs programme on the evaluation of carcinogenesis risks to humans of the International Agency for Research on Cancer has currently identified eighty-eight human carcinogenic agents: sixty-four agents and groups of agents (twenty-two drugs; fourteen environmental chemicals; fourteen types of radiation; ten viruses, bacteria and parasites and four inorganic fibres), twelve mixtures and thirteen exposure circumstances⁽³⁾.

In spite of advances in therapeutic modalities, the overall mortality and morbidity rates of cancers have not changed significantly in the last decade⁽⁴⁾. Therefore, intervention to decelerate, arrest or reverse the process of carcinogenesis by the use of either natural or synthetic substances individually or in combination therapy has emerged as a promising and pragmatic medical approach to reduce cancer risk^(5–7). Mechanistic investigations coupled with dietary epidemiology, case–control, cohort and ecological observational studies have demonstrated that a regular intake of fruits and vegetables contributes to the prevention of cancer at multiple sites and that some of the foods and herbs contain a host of phytochemicals that confer cancer protection^(8–10). Plant-derived phytochemicals such as sulforaphane, resveratrol,

Abbreviations: 1 ×, 1 µg/µl; 10 ×, 10 µg/µl; 100 ×, 100 µg/µl; BP, benzo[a]pyrene; CM-H2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; DMBA, 7,12-dimethyl benz(a)anthracene; GR, glutathione reductase; MIC, methyl isocyanate; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; SOD, superoxide dismutase.

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geinsten, vincristine, vinblastine, curcumin, gingerol, diallyl sulfide, epigallocatechin-3-gallate, brassinin, 4'-bromoflavone, brusatol, etc have been employed for the control and restraintment of carcinogenesis^(11–13). Although several independent studies conducted on single phytochemical-based drugs have shown encouraging results *in vitro*, they have failed to demonstrate appreciable therapeutic efficacy when tested in pre-clinical animal trials and clinical conditions^(14–16). Therefore, an increasing trend towards the use of whole plant extracts is primarily advocated since they offer synergistic effects of myriad plant secondary metabolites and provide multiple points of intervention in cancer chemoprevention⁽¹⁷⁾. In addition, mixtures of compounds present in the plant extracts or their enriched fractions and metabolites may not only provide essential combinatorial approaches affecting multitude targets of cancer prevention, but also aid in achieving clinical efficacy beyond the scope of single compound-based moieties and alleviating potential toxicity, as low doses are sufficient⁽¹⁸⁾.

Selaginella bryopteris is a perennial, herbaceous lythophytic plant that grows in shallow soils on rocky outcrops of slopes of small hills in direct sunlight in humid tropical regions. It is a traditional herb that for centuries has occupied a prime place among the most sought-after herbs in Indian mythology as 'Sanjeevani' (one that infuses life) by virtue of its resurrection properties. The herb is popular among tribal people of India as a dietary supplement in treatment, signifying its role as a 'panacea' against varied maladies^(19,20). Several studies have explored the bioactive components contributing to the medicinal properties of varied species of *Selaginella*^(21–26). In particular, the flavonoid-rich contents of this herb have demonstrated numerous and varied biological activities^(27–30). In lieu of impressive epidemiological evidence for the cytoprotective effects of plant flavonoids and correlation of high flavonoid intake with a decreased risk of cancer, the present study aimed to extract an enriched flavonoid fraction from *S. bryopteris* and elucidate the molecular and synergistic mechanisms involving their potential anticancer property, which are still unknown.

In the present study, chromatographic fractions in order of polarity, namely petroleum ether, benzene, chloroform, ethyl acetate and methanol, were isolated from the aqueous extract of *S. bryopteris* using a standard protocol^(31,32). The cytoprotective potential of the obtained chromatographic fractions was evaluated on five different epithelial cell cultures treated with *N*-succinimidyl *N*-methylcarbamate (methyl isocyanate (MIC) analogue), a well-reported environmental and chemical carcinogen⁽³³⁾. Molecular mechanisms of the flavonoid-rich benzene fraction that exhibited a significant cytoprotective activity was studied for the determination of proliferative index and cell-cycle regulatory proteins, antioxidant property, anti-inflammatory effect, reversal of stress-induced senescence and genoprotective effect using *in vitro* culture model systems. The chemopreventive potential of the benzene fraction was also investigated *in vivo* using Yun's model of medium-term anticarcinogenicity and the two-stage skin papilloma-genesis test in mice.

Materials and methods

Test material

The plant material (*S. bryopteris*) was procured from Pachmarhi biosphere preserve located in the Satpura range of Madhya Pradesh state during the monsoon season, shade-dried, and coarsely powdered. Extraction was performed using the hot-soxhalation protocol^(31,32). The powdered material (100 g) was soxholated using 500 ml double-distilled water. The extract was filtered through a double layer of 100 μ m nylon wire mesh and concentrated under reduced pressure. The dried residue was free of solvent and weighed approximately 2 g. The resultant aqueous extract was subjected to silica gel column chromatography (60–120 mesh), eluted with petroleum ether, benzene, chloroform, ethyl acetate and methanol⁽³⁴⁾. Each chromatographic fraction of the aqueous extract was concentrated under reduced pressure to obtain a dried residue. For *in vitro* and *in vivo* studies, the dried residue dissolved (1 μ g/1 μ l) in PBS (pH 7.0) was used. Qualitative screening of flavonoids was carried out by treating different chromatographic fractions (1 g test material) of the aqueous extract with a few drops of concentrated HCl and Mg turnings (0.5 g). The presence of flavonoids was indicated if a pink or magenta-red colour developed within 3 min⁽³⁵⁾.

Chemicals

N-succinimidyl *N*-methylcarbamate, benzo[*a*]pyrene (BP) (97% purity HPLC) and 7,12-dimethyl benz[*a*]anthracene (DMBA) were obtained from Sigma-Aldrich (St Louis, MO, USA). High-purity-grade solvents (99.98%) procured from Merck Ltd (Mumbai, India) were used for the preparation of the test material. To quantify cytotoxicity, Annexin-V Fluos and an Apoptotic DNA ladder kit from Roche Applied Sciences (Mannheim, Germany) were used. For analysing nuclear DNA cell-cycle arrest, a BD CycleTEST PLUS DNA Reagent Kit (BD Immunocytometry Systems, San Jose, CA, USA) was used. Expression of p53 and p21 through Western blot was performed using antibodies obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). For relative gene expression analysis by quantitative real-time PCR, RNA isolation was done using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) and a Transcriptor One-step RT-PCR kit with SYBR green dye was used from the kit obtained from Roche Applied Sciences. Free radical-scavenging potential was determined through 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) fluochrome obtained from Molecular Probes (Invitrogen Co.). Quantification of antioxidative enzymes, glutathione reductase (GR) and superoxide dismutase (SOD) was performed by using GR and SOD ELISA assay kits (Trevigen Inc., Gaithersburg, MD, USA). A cellular senescence assay kit for β -galactosidase staining was procured from Millipore (Billerica, MA, USA). γ -H2AX immune labelling was performed using antibodies from Calbiochem (Nottingham, Notts, UK) with appropriate dilutions in 1 \times PBS before use. Analysis of secreted cytokine levels was performed using a Human inflammation multiplex Cytometric Bead Array assay kit (BD™ Biosciences, San Diego, CA, USA).

Experimental design

The study was comprised of two sections: (a) *in vitro* analysis involving evaluation of cytoprotective effect, proliferative index and cell-cycle regulatory proteins, antioxidant activity, anti-inflammatory and stress-reversal potential of the different fractions of a *S. bryopteris* aqueous extract; (b) *in vivo* analysis in Swiss albino mice to evaluate the chemopreventive effects of *S. bryopteris* against BP-induced lung adenoma and DMBA-induced skin papilloma.

In vitro study

Cell culture. Different normal human and mouse cell lines, namely, HEK-293 (human kidney epithelial cells, CRL-1573), FHC (human colon epithelial cells, CRL-1831), IMR-90 (human lung fibroblasts, CRL-1262), B/CMBA.Ov (mouse ovarian epithelial cells, CRL-6331), MM55.K (mouse kidney epithelial cells, CRL-6436) and GC-1 spg (mouse spermatogonia epithelial cells, CRL-2053) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and monolayer cultures of cells were maintained in 35 mm petri-dishes (BD Discovery Labware, Bedford, MA, USA) according to ATCC's catalogue instructions at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

We conducted three studies in two sections: dose-dependent and time-course kinetics. Dose-dependent responses on different cultures were conducted at 1 × (1 µg/µl), 10 × (10 µg/µl) and 100 × (100 µg/µl) concentrations, whereas time-course experiments were performed with a constant concentration of 10 × at time intervals ranging from 1 to 48 h. For all the investigations, cells were supplemented with or without the test material for 24 h followed by exposure to MIC, dissolved in 2 mM-dimethylsulfoxide with a final concentration of 0.005 µM. The selection of the 0.005 µM concentration was done on the basis of the authors' previous *in vitro* observations, as this concentration optimally induced DNA damage, apoptosis, oxidative stress and inflammation^(36,37).

Cytoprotective effect. Cytoprotection from MIC-induced apoptosis was carried out in HEK-293, FHC, B/CMBA.Ov, MM55.K and GC-1 spg cells, pretreated with aqueous extract and different chromatographic fractions isolated from it, along the concentration gradients of 1 ×, 10 ×, 100 × for 24 h. Apoptotic index was measured through flow cytometry using annexin-V fluorescein isothiocyanate–propidium iodide labelling after the exposure of chromatographic fraction-pretreated cells to MIC (concentration 0.005 µM) for 6 h⁽³⁶⁾. Examination of apoptotic DNA ladder pattern by agarose gel electrophoresis was studied following exposure to MIC (concentration 0.005 µM) for 6 and 12 h in cells before and following pretreatment with different chromatographic fractions of *S. bryopteris*⁽³⁶⁾.

Evaluation of proliferative index and cell-cycle regulatory proteins. In order to examine the molecular mechanism(s) and underlying changes in the cell cycle, investigations using the flavonoid-rich benzene chromatographic fraction of the *S. bryopteris* aqueous extract were conducted in HEK-293 cells at 3, 6, 12, 24, 48 and 72 h time intervals with

10 × concentration. DNA cell-cycle analysis was determined by flow cytometry using propidium iodide labelling⁽³⁸⁾. Analysis of p53 and p21 proteins through Western blot was performed as mentioned earlier⁽³⁹⁾. Relative gene expression analysis of proliferating cell nuclear antigen (PCNA) using one-step RT-PCR protocol was done as described in our previous study. The threshold cycle number (C_t value) for the gene was obtained by quantitative real-time PCR and was normalised to the C_t value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. The fold change in the expression was calculated using the 2^{-ΔΔC_t} method⁽⁴⁰⁾.

Antioxidant property. Antioxidant activity in HEK-293 cells pretreated with the benzene chromatographic fraction was determined at 3, 6, 12, 24 and 48 h time intervals with 10 × concentration. A fresh stock solution of CM-H2DCFDA (5 mM) was prepared in dimethylsulfoxide and diluted to a final concentration of 1 µM in 1 × PBS. The cells were washed with 1 × PBS followed by incubation with 50 µl of a working solution of fluorochrome marker CM-H2DCFDA (final working concentration adjusted to 2.5 µg/50 µl) for 2 h. The cells were harvested, washed in PBS, and cell-associated fluorescence was measured by flow cytometry in the FL1 channel. Levels of the antioxidant defence system enzyme GR were measured through a spectrophotometric ELISA assay in which the oxidation of NADPH to NADP⁺ is monitored by the decrease in absorbance at 340 nm, which is directly proportional to the GR activity in the sample. SOD in the cell extracts was also evaluated through ELISA, as reported previously. The percentage inhibition of the formation of nitroblue tetrazolium (NBT)–diformazan by SOD was converted to the relative activity of the sample, expressed as mU/ml⁽⁴¹⁾.

Anti-inflammatory effect. Supernatant fractions collected from cultured HEK-293 cells were used for measuring the inflammatory response by determining levels of the cytokines IL-8, IL-1β, IL-6, interferon-γ, TNF and IL-12p70; the assay was performed as detailed elsewhere^(42,43). Data acquisition and analysis were carried out on a flow cytometric platform using BD™ Cytometric Bead Array software (BD™ Biosciences).

Reversal of stress-induced senescence. HEK-293 cells pretreated with 10 × concentration of the flavonoid-rich benzene chromatographic fraction of the *S. bryopteris* aqueous extract for 24 h were exposed to 0.005 µM-MIC. Senescence-associated β-galactosidase staining was performed as detailed in Raghuram *et al.*⁽⁴⁴⁾.

Genoprotective effect. Persistence of DNA damage at 72 h was determined in IMR-90 cells through H2AX phosphorylation⁽⁴⁵⁾ after treatment with both 0.005 µM-MIC alone and after pretreatment with the flavonoid-rich benzene fraction (10 × concentration). Enumeration of cytogenetic anomalies was evaluated through conventional giemsa staining⁽⁴⁴⁾ in HEK-293 cells treated with 0.005 µM-MIC alone and cells pretreated with the flavonoid-rich benzene fraction (10 × concentration). Mean chromosomal aberrations per cell were calculated from a total of fifty cells, regarding each chromatid break as one break and other chromosomal

breaks or rearrangements. Spectral karyotyping to further discern any cryptic aberrations was performed in MM55.K cells all along passages 1 to 5, after treatment with both 0.005 μM -MIC alone and prior treatment with the flavonoid-rich benzene fraction (10 \times concentration)⁽⁴⁶⁾.

In vivo chemopreventive study. Swiss albino mice (aged 6–8 weeks) were procured from the Experimental Animal Facility of Hamdard University (New Delhi, India) and maintained as an inbred colony. Newborn mice (less than 24 h old) of both sexes were used for the experiments and fed on standard food pellets and water *ad libitum*. They were acclimatised to our laboratory conditions in polypropylene cages at a temperature of $24 \pm 2^\circ\text{C}$, relative humidity of 55 to 65% and 12 h light–12 h dark cycle after weaning at age 3 weeks according to institutional ethics committee regulations and in compliance with the Guidelines for Care and Use of Animals for Scientific Research⁽⁴⁷⁾.

Medium-term anticarcinogenicity test (Yun's model). Groups of thirty to sixty newborn Swiss albino mice (less than 24 h old) were given a single subcutaneous injection in the scapular region with 0.02 ml of BP (0.5 mg per mouse using a suspension of BP in 1% aqueous gelatin). After weaning at age 3 weeks, the test material (benzene chromatographic fraction of *S. bryopteris* aqueous extract) or control (double-distilled water) was administered in a volume of 0.1 ml for 6 weeks, once daily by oral administration. All mice were killed at the 9th week after birth⁽⁴⁸⁾. The study comprised of four groups of thirty mice each. Group I (no BP, no test material) was given double-distilled water for 6 weeks (after weaning) by oral administration. Oral administration was chosen as the route of exposure to carefully control the dose of the test material. Group II (test material alone) was given the test material (1 g/kg body weight) for 6 weeks (after weaning) by oral administration. Group III (BP alone) was subcutaneously injected once in the scapular region with 0.02 ml BP (0.5 mg per mouse using a suspension of BP in 1% aqueous gelatin). Group IV (BP + test material) was subcutaneously injected once in the scapular region with 0.02 ml BP (0.5 mg/mouse using a suspension of BP in 1% aqueous gelatin). After weaning, the test material (1 g/kg body weight) was administered for 6 weeks by oral administration. Upon killing, the lungs were excised and fixed in Tellyesniczky's solution (100 ml of 70% ethanol, 3 ml formalin and 5 ml glacial acetic acid), and the numbers of adenomas were counted by visual inspection. To obtain an index of tumour incidence, the percentage of tumour-bearing mice was calculated for each group. Tumour multiplicity was defined as the average number of tumours per tumour-bearing mouse and was obtained by dividing the total number of tumours by the total number of mice per group, including non-tumour-bearing animals.

Two-stage skin papillomagenesis test. For induction of tumours, a two-stage protocol consisting of initiation with a single topical application of DMBA followed by three-times-per-week treatment with a promoter (croton oil) were employed as described previously⁽⁴⁹⁾. Group I was a group of thirty animals; the animals of this group were treated with DMBA (100 $\mu\text{g}/50 \mu\text{l}$ acetone per animal). After 2 weeks,

0.1 ml croton oil was applied on to the shaved area; this was continued three times per week until the termination of the experiment. Group II was a group of thirty animals; they were administered the benzene chromatographic fraction of the *S. bryopteris* aqueous extract dissolved in double-distilled water (1 g/kg body weight) from 7 d before the application of DMBA (100 $\mu\text{g}/50 \mu\text{l}$ acetone per animal) followed by the application of croton oil (three times per week) and throughout the experiment. During the 16 weeks of experiments, mice were observed weekly and were weighed. The mice were carefully examined once per week for the presence of skin papillomas and these were recorded. Papillomas were defined as a lesion with a diameter greater than 1 mm that persisted for at least two consecutive observations.

Various parameters studied were tumour incidence (number of mice carrying at least one tumour expressed as percentage incidence), tumour yield (total number of tumour per growth and the mean number of tumours per mouse), tumour diameter and weight, tumour burden (total number of tumours per tumour-bearing mouse) at the end of each experiment. The body weight of each mouse was measured weekly. The average latency period – the time lag between the application of the promoting agent and the appearance of 50% tumours – was determined. The average latency period was calculated by multiplying the number of tumours appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by the total number of tumours. Average latency period = $\sum \text{FX}/n$ (where F is the number of tumours appearing in each week, X is the number of weeks and n is the total number of tumours).

Statistical analysis

Statistical analysis was performed by using the χ^2 test and ANOVA. The Statistical Package for Social Sciences software package (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis and $P \leq 0.001$ was considered to be significant.

Results

Cytoprotective effect

In the present investigation, a uniform induction of apoptosis was observed in HEK-293, FHC, B/CMBA.Ov, MM55.K and GC-1 spg cells following exposure to MIC for 6 h at 0.005 μM concentration. Morphological features typical of an apoptotic response, such as cell rounding, membrane blebbing, chromatin condensation and nuclear fragmentation, were observed, suggestive of MIC-induced genotoxicity. However, examination of the phenotypic characteristics of cells pretreated for 24 h with 1 \times , 10 \times and 100 \times concentrations of the aqueous extract of *S. bryopteris* and its various chromatographic fractions provided substantial evidence of cytoprotection from MIC-induced apoptosis (Fig. 1(a)). In the present study, flow cytometric evaluation of apoptotic index by fluorescently labelled annexin-V demonstrated significant cytoprotective activity ($P \leq 0.001$) from MIC-induced apoptosis in cells

pretreated with the aqueous extract of *S. bryopteris* and its various chromatographic fractions. The benzene fraction of *S. bryopteris* gave the maximum protection (up to 95%; $P \leq 0.001$) in comparison with the aqueous extract and other chromatographic fractions studied (Fig. 1(b)).

Expectedly, no time-dependent changes of DNA degradation were observed in cells pretreated with the aqueous extract of *S. bryopteris* and its various chromatographic

fractions on MIC exposure. Fig. 1(c) shows a representative photograph of the cytoprotective effect from MIC-induced inter-nucleosomal DNA fragmentation in cells pretreated with the benzene fraction (10 × concentration). Since the maximum cytoprotective effect was observed in the benzene fraction of *S. bryopteris*, molecular mechanisms of the flavonoid-rich benzene fraction were comprehensively investigated for downstream studies.

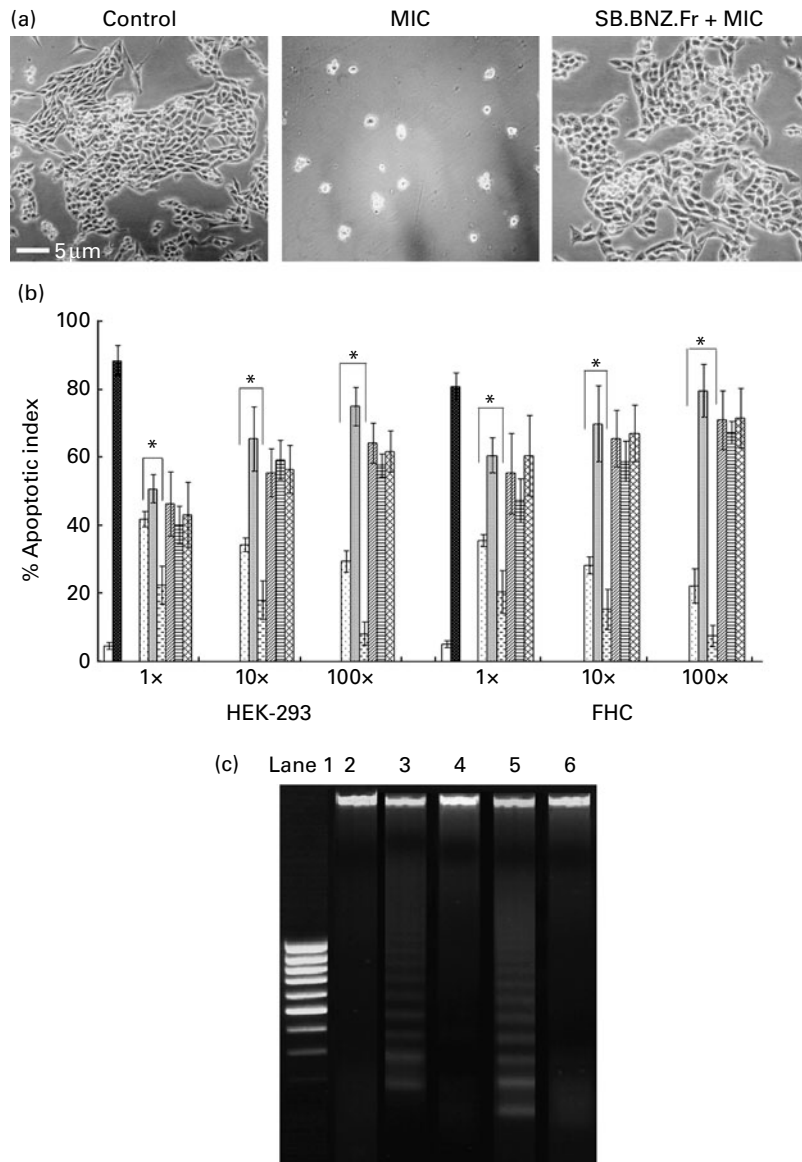


Fig. 1. (a) Protection of MM55.K cells from methyl isocyanate (MIC)-induced apoptosis by *Selaginella bryopteris* extract. MM55.K cells were plated at a density of 2×10^5 in 35 mm plates and maintained in culture for 18 h. A representative image for MM55.K cells exposed to $0.005 \mu\text{M}$ -MIC for 6 h and $10 \mu\text{g}/\mu\text{l}$ ($10 \times$) *S. bryopteris* benzene chromatographic fraction (SB.BNZ.Fr)-pretreated cells followed by $0.005 \mu\text{M}$ -MIC exposure is shown. Controls were the untreated cells neither exposed to MIC nor to SB.BNZ.Fr. (b) Prevention of apoptosis by *S. bryopteris* extract in human cell lines: human kidney epithelial cells (HEK-293) and human colon epithelial cells (FHC). Histograms depict the apoptotic index in cultured cells exposed to $0.005 \mu\text{M}$ -MIC for 6 h with and without (■) pretreatments with different fractions of *S. bryopteris* (petroleum ether (□), benzene (▨), chloroform (▩), ethyl acetate (▧) and methanol (▦) fractions and water extract (□)) at $1 \mu\text{g}/\mu\text{l}$ ($1 \times$), $10 \times$ and $100 \mu\text{g}/\mu\text{l}$ ($100 \times$) concentrations. (□), Control treatment. Values are the means of three independent experiments, with standard errors represented by vertical bars. * The benzene fraction of *S. bryopteris* aqueous extract demonstrated the maximum anti-apoptotic activity among all the cells studied ($P \leq 0.001$). (c) DNA fragmentation. Protective effect of *S. bryopteris* extract from MIC-induced DNA fragmentation in cultured HEK-293 cells. Representative image showing a time-dependent increase in DNA fragmentation of HEK-293 cells following $0.005 \mu\text{M}$ -MIC exposure, while pretreatment with $10 \times$ SB.BNZ.Fr protected cells from the toxic effect of MIC. Lane 1, molecular-weight marker; lane 2, control; lanes 3 and 5, cells treated with MIC alone for 6 and 12 h; lanes 4 and 6, cells pretreated with $10 \times$ SB.BNZ.Fr and exposed to MIC for 6 and 12 h, respectively.

Evaluation of proliferative index and cell-cycle regulatory proteins

Since induction of apoptosis is mediated through regulation of the cell cycle, effects of the flavonoid-rich benzene chromatographic fraction on proliferative index and cell-cycle regulatory proteins were investigated. Flow cytometric

analysis of the DNA cell cycle was performed at 3, 6, 12, 24 and 48 h in MIC-exposed HEK-293 cells pretreated with 10 × concentration of the benzene fraction. Compared with controls, MIC induced a significant G1 arrest (90%) in cells after 12 h, while 51% showed arrest in the G2/M phase after 48 h of exposure. Cells pretreated with the benzene fraction did not show blockage of DNA replication and inactivation

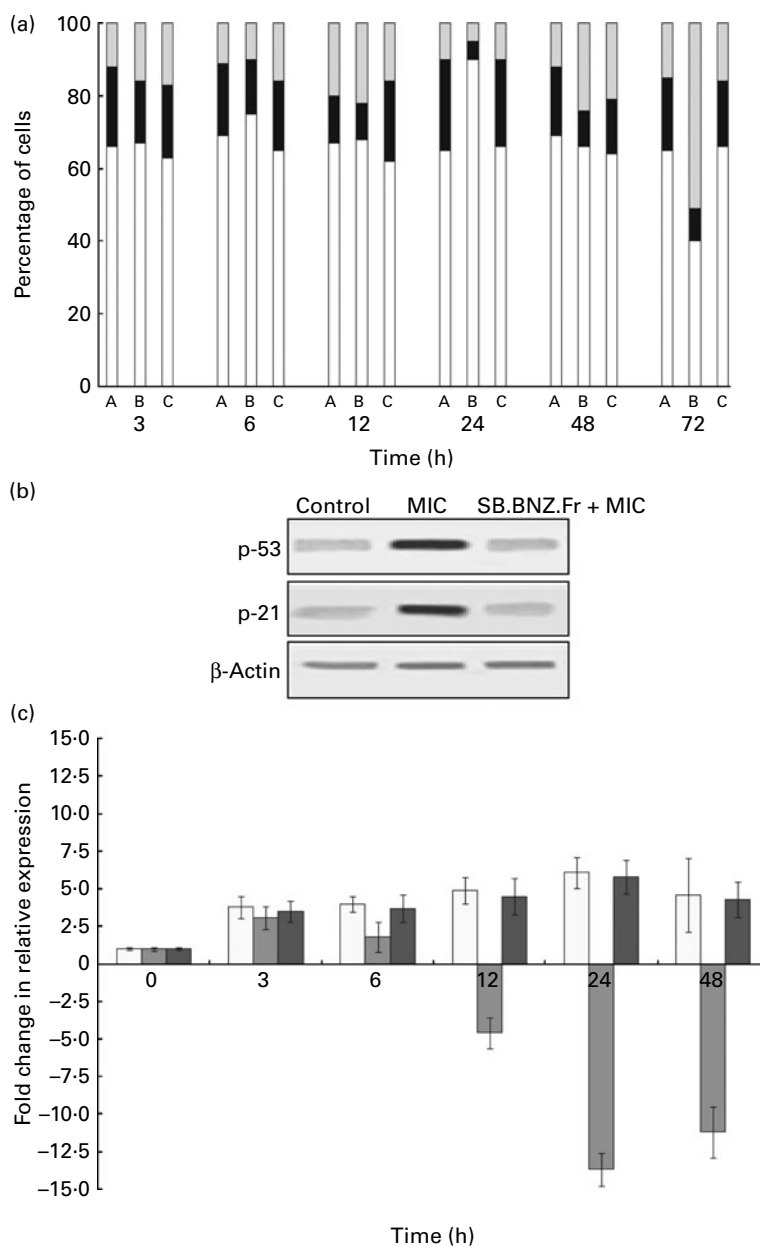


Fig. 2. (a) Cell-cycle progression. Effect of *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) (C) compared with 0.005 μM -methyl isocyanate (MIC) (B) and control (A) on HEK-293 cell-cycle progression. The cells were exposed to 0.005 μM -MIC with or without pretreatment with 10 $\mu\text{g}/\mu\text{l}$ (10 ×) SB.BNZ.Fr at varying time points of 3, 6, 12, 24, 48 and 72 h. The percentage of cells in the G1 (□), S (■) and G2/M (▨) phase was measured by flow cytometry after propidium iodide staining. Values are the means of three independent experiments ($n = 3$). (b) Induction of p53 and p21 protein expression. Immunoblots performed with anti-p53 antibody, anti-p21 antibody and anti- β -actin antibody (loading control) reported an altered expression of p53 and p21 proteins in 0.005 μM -MIC-exposed HEK-293 cells at 24 h. However, as compared with controls, cells pretreated with 10 × SB.BNZ.Fr for 24 h reported no such alterations in p53 and p21 expression. The blots represent one of three reproducible experiments ($n = 3$). (c) Effect of SB.BNZ.Fr on expression of the proliferating cell nuclear antigen (PCNA) gene. Quantitative real-time PCR analysis of PCNA gene expression in controls (HEK-293) (□), cells exposed to 0.005 μM -MIC (▨) and cells pretreated with SB.BNZ.Fr (■) for 3, 6, 12, 24 and 48 h. The threshold cycle number (C_t value) for the PCNA gene was obtained by quantitative real-time PCR and was normalised to the C_t value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. The fold change in the expression of the PCNA gene was obtained by using the $2^{-\Delta\Delta C_t}$ method. Values are means of all independent experiments ($n = 3$), with standard errors represented by vertical bars.

of cell-cycle progression up to the 48 h time period (Fig. 2(a)). In cells pretreated with the benzene fraction, protein levels of p53 and p21 were found unaltered, suggestive of uninterrupted cell-cycle progression (Fig. 2(b)).

Quantitative real-time PCR analyses for changes in PCNA gene expression were also conducted at 3, 6, 12, 24 and 48 h from cells exposed to MIC (alone) and pretreated with the benzene fraction. The relative expression of PCNA (fold changes; increase indicated by +, and decrease

indicated by - signs) as compared with controls was studied. The threshold cycle number (C_t value) for the gene was obtained by quantitative real-time PCR and was normalised to the C_t value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. The fold change in the expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Fig. 2(c) shows the mean of three independent experiments. Down-regulation of PCNA in MIC-exposed cells was recorded with folds of -4.6 at 12 h and -13.7 after 24 h, respectively.

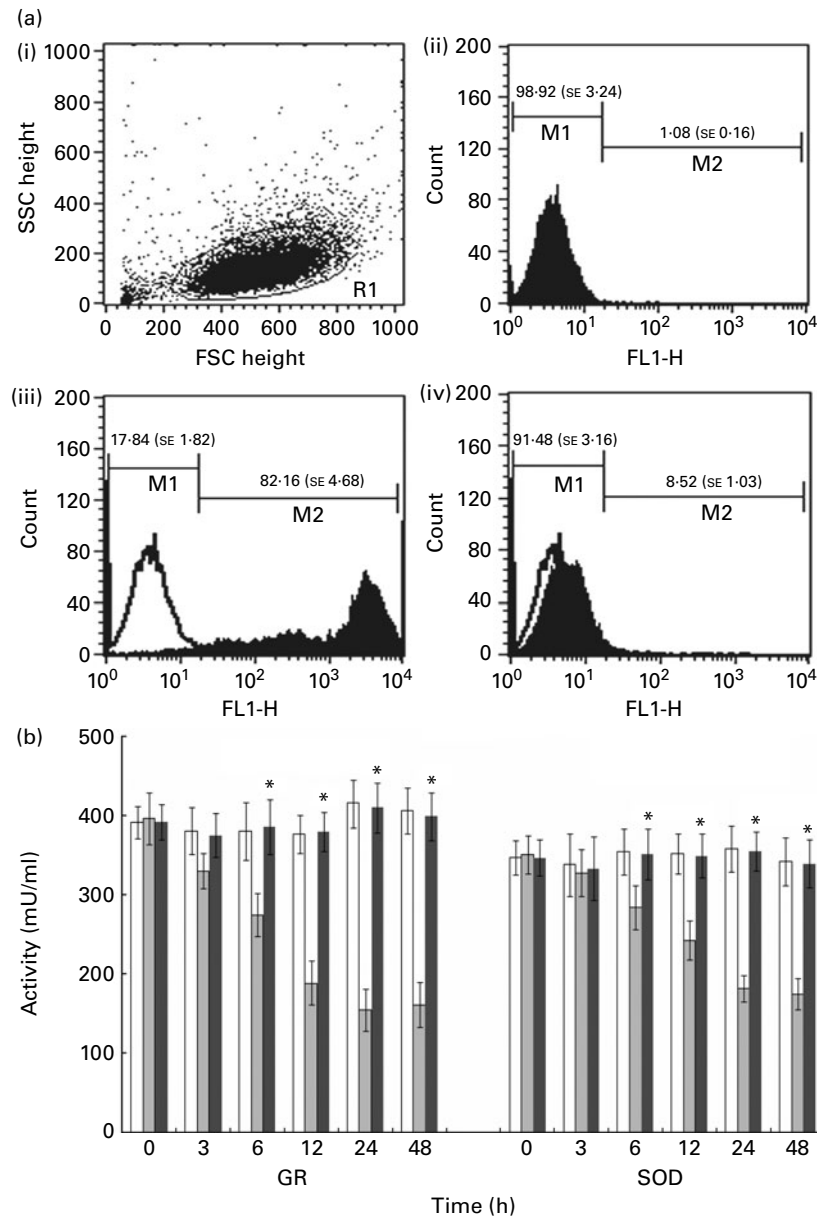


Fig. 3. (a) Inhibition of reactive oxygen species (ROS) generation. Flow cytometric evaluation for induction of ROS in cultured HEK-293 cells labelled with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA): (i) FSC/SSC (forward scatter/side scatter) plot showing the population of HEK-293 cells; (ii) control cells (M1 zone); (iii) cells treated with a 0.005 μM -methyl isocyanate (MIC) at 24 h showing increase in percentage induction of ROS within a population of cells (M2 zone); (iv) cells pretreated with a 10 $\mu\text{g}/\mu\text{l}$ ($10\times$) concentration of *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) at 24 h followed by MIC exposure showing abrogation in percentage induction of ROS within a population of cells analogous to control (M1 zone). FL1-H, fluorescence channel 1 height. (b) Sustained activities of antioxidant defence system enzymes glutathione reductase (GR) and superoxide dismutase (SOD). Effect of SB.BNZ.Fr on GR and SOD levels in 0.005 μM -MIC-treated HEK-293 cells (■) showing significant antioxidant activity along the time course in contrast to the MIC alone-treated cells (■) that displayed constant depletion of both GR and SOD. (□), Control. Values are means, with standard errors represented by vertical bars ($n\ 3$). * $P\leq 0.001$.

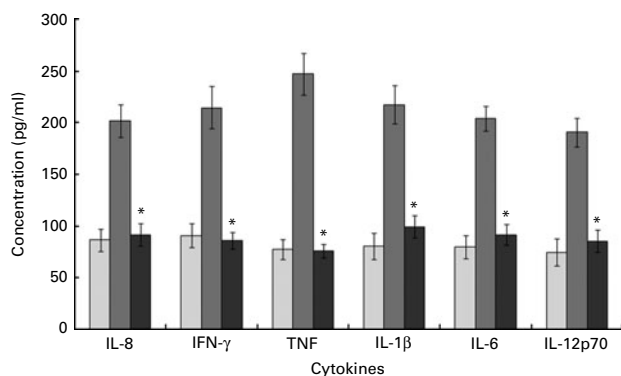


Fig. 4. Anti-inflammatory activity of *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) on HEK-293 cells. Histogram demonstrating the near static levels of inflammatory cytokines (IL-8, interferon- γ (IFN- γ), TNF, IL-1 β , IL-6, IL-12 p70) in HEK-293 cells pretreated with 10 $\mu\text{g}/\mu\text{l}$ ($10\times$) concentration of SB.BNZ.Fr (■) at 24 h. On the contrary, the levels of inflammatory cytokines were elevated in cells treated with 0.005 μM -methyl isocyanate (MIC) alone (■) as compared with controls (□). Values are means, with standard errors represented by vertical bars (n 3). * Mean value was significantly different from that for MIC treatment alone ($P \leq 0.001$).

The expression profile of PCNA did not show any appreciable alteration, suggestive of uninterrupted cell-cycle progression in cells exposed to MIC pretreated with the benzene fraction.

Antioxidant activity

The ability of the benzene fraction to scavenge the free radicals generated following treatment with MIC was studied in HEK-293 cells. A significant time-dependent increase in reactive oxygen species (ROS) generation was observed in cells treated with MIC alone, with levels attaining 82.16% at 24 h. However, in cells pretreated with the benzene fraction, levels of ROS generated were comparable with those in controls, providing strong evidence of the free radical-scavenging potential of the flavonoids present (Fig. 3(a)).

In the present study, the maximum inhibition of SOD and GR activity was observed at 24 h in cells incubated with MIC alone. However, unaltered enzyme activity throughout the time-course in HEK-293 cells previously treated with the benzene fraction suggested a significant antioxidant potential of the plant material studied ($P \leq 0.001$) (Fig. 3(b)).

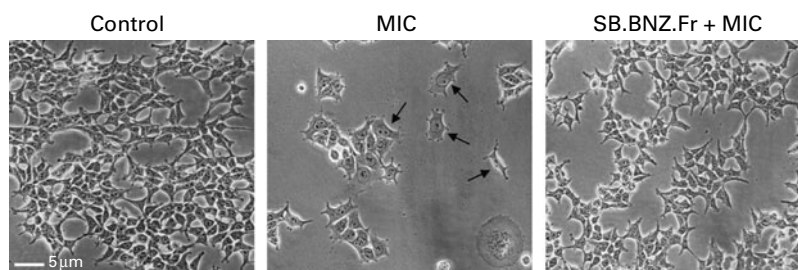


Fig. 5. Protection against stress-induced premature senescence. Representative phase contrast microphotographs ($\times 200$) showing HEK-293 control cells, cells treated with methyl isocyanate (MIC) (1, senescence cells) and cells pretreated with 10 $\mu\text{g}/\mu\text{l}$ ($10\times$) *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) at 72 h. Cells pretreated with the flavonoid-rich fraction show an apparent resistance to flattened senescence morphology and uptake of β -galactosidase stain along with a noticeable decrease in density of growth compared with that observed with MIC exposure alone (n 3).

Anti-inflammatory effect

Flow cytometric analysis at 24 h revealed a significant increase ($P \leq 0.001$) in the secreted levels of the inflammatory cytokines IL-8, IL-1 β , IL-6, interferon- γ , TNF and IL-12p70 in the cell culture supernatant fraction of the HEK-293 cells treated with 0.005 μM -MIC. On the contrary, the benzene fraction-pretreated cells did not show a marked increase with respect to parallel controls, implicating a potent anti-inflammatory activity of the benzene fraction (Fig. 4).

Reversal of stress-induced senescence

Stress-induced premature senescence primarily results from oncogenic activity or genotoxic stress independent of a change in telomere length. In the present investigation, 0.005 μM -MIC caused a gradual increase in senescence-associated β -galactosidase-staining activity, as judged by cytochemical staining. HEK-293 cells treated for 24 h displayed a perceptible increase in the absorption of the blue stain, indicating accumulation of lipofuscin granules associated with β -galactosidase activity, whereas untreated cells grown in parallel were negative. The majority of cells treated with MIC displayed a typical senescence-specific morphology with a 'flattened and enlarged' shape at 72 h. In contrast, no stress-induced premature senescence was observed in the cells pretreated with $10\times$ concentration of the benzene fraction, further strengthening our above observations and justifying the cytoprotective and antioxidant potential of the benzene fraction (Fig. 5).

Genoprotective effect

Phosphorylation of histone protein on serine 139 (γ H2AX) occurs at sites flanking DNA double-stranded breaks and can provide a measure of the number of DNA double-stranded breaks within a cell. Immunofluorescence analysis of IMR-90 cells treated with MIC showed elevated phosphorylation activity, suggestive of increased nuclear retention and foci formation damage in cells exposed to MIC for 72 h. However, at the same time point, H2AX fails to form foci in cells pretreated with $10\times$ concentration of the benzene fraction, indicating its protective action against the formation of DNA double-stranded breaks (Fig. 6(a)).

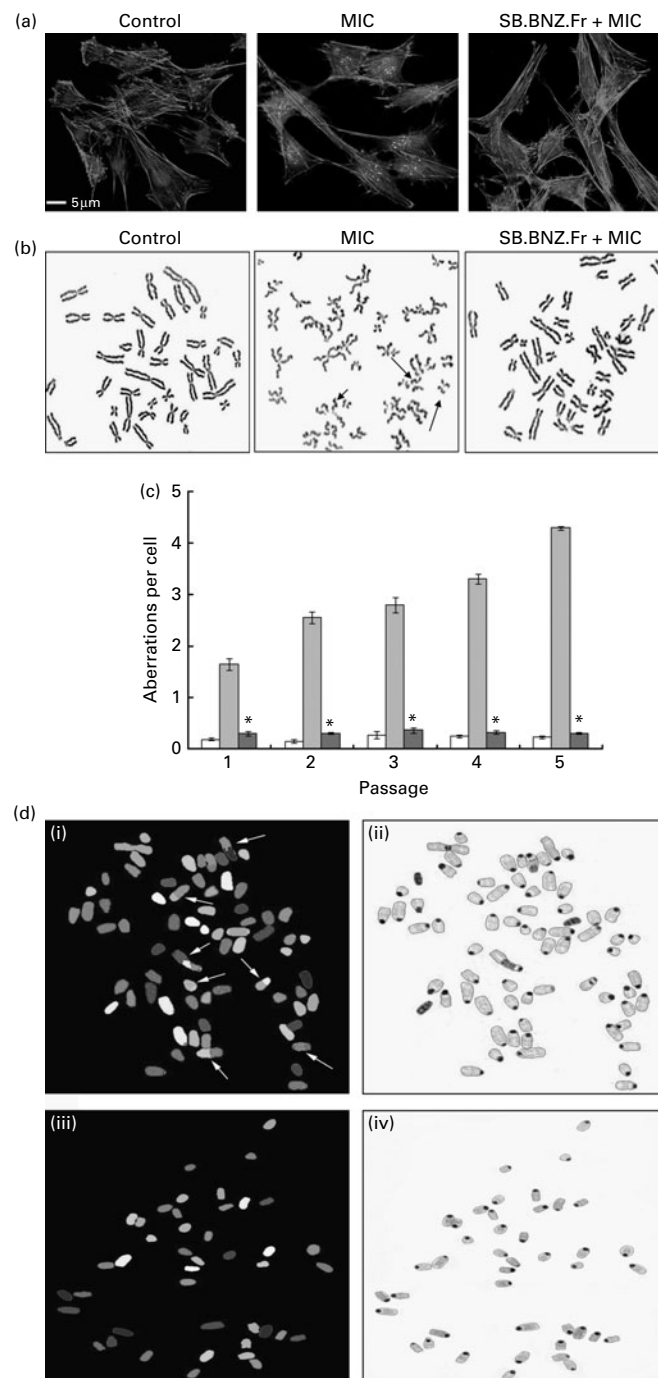


Fig. 6. (a) Genoprotection against DNA damage response. Representative microphotographs ($\times 200$) showing immunofluorescence analysis of H2AX phosphorylation with significant punctuated nuclear patterns of γ -H2AX foci in IMR-90 (human lung fibroblast) cells upon exposure to $0.005 \mu\text{M}$ -methyl isocyanate (MIC). However, pretreatment with the benzene fraction of *Selaginella bryopteris* (SB.BNZ.Fr) aqueous extract followed with MIC exposure shows an absence of γ -H2AX foci at 72 h in comparison with control. The nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI) while cytoskeletons are stained with phalloidin red. The signals of the γ -H2AX foci are stained with fluorescein isothiocyanate (FITC) (green). (b) Genoprotection against chromosomal aberrations. Representative partial metaphase spreads ($\times 630$) of HEK-293 cells showing control karyotype upon conventional cytogenetic analysis: cells exposed to MIC showing premature centromeric separation (\rightarrow); fragmentation (\dashrightarrow) where no noticeable chromosomal aberrations at passage 5 was observed after MIC exposure in cells pretreated with $10 \times$ SB.BNZ.Fr. (c) Anti-clastogenic activity of SB.BNZ.Fr in HEK-293 cells exposed to $0.005 \mu\text{M}$ -MIC with (▒) or without (■) SB.BNZ.Fr pretreatment during passages 1 to 5. Graph represents mean frequency (%) of total chromosomal structural aberrations (CSA) per cell observed in controls (□), MIC alone-treated cells and SB.BNZ.Fr-pretreated cells exposed to MIC. Total CSA considered were premature centromeric separation, dicentrics and chromosomal fragments. Values are means, with standard errors represented by vertical bars ($n = 3$). *Mean value was significantly different from that for MIC treatment alone ($P \leq 0.001$). (d) Spectral karyotyping (SKY) analysis depicting preventive effect of SB.BNZ.Fr against cryptic translocation in MM55.K cells. Photomicrographs show multiple translocations (\rightarrow) after treatment with $0.005 \mu\text{M}$ -MIC at passage 5 (SKY view image (ii) and inverted DAPI (i)), whereas no cryptic translocations in cells pretreated with SB.BNZ.Fr (SKY view image (iv) and inverted DAPI (iii)) were observed at passage 5.

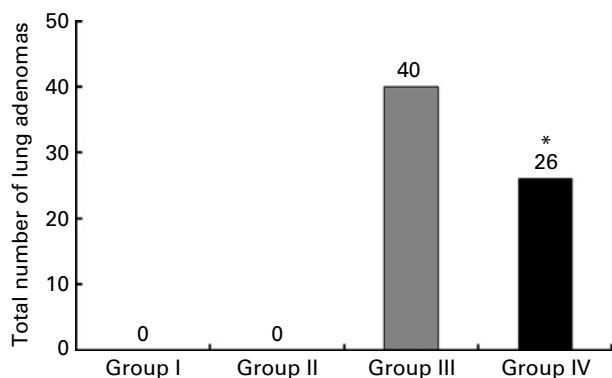


Fig. 7. Chemopreventive effect of *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) on benzo[*a*]pyrene (BP)-induced lung carcinogenicity in Swiss albino mice. Group I, mice neither treated with BP nor with SB.BNZ.Fr; group II, mice treated with SB.BNZ.Fr alone; group III, mice treated with BP alone; group IV, mice were treated with both BP + SB.BNZ.Fr. * Mean value was significantly different from that for group III ($P \leq 0.001$).

The formation of DNA double-stranded breaks has been known to compromise chromosome integrity; cytogenetic analysis of HEK-293 cells through conventional giemsa staining after exposure to MIC revealed chromosomal abnormalities in the form of fragments, chromatid breaks and premature centromeric separation with an incremental index of mean chromosomal aberrations per cell all through passages 1 to 5 (Fig. 6(b) and (c)). In addition, spectral karyotyping, which involves simultaneous labelling of all the pairs of chromosomes chromosome-specific DNA with different fluorophores, on metaphases of MIC-treated MM55.K cells displayed an array of hidden translocations (Fig. 6(d)) manifested during the five passages studied that further corroborated the genotoxicity of MIC ($P \leq 0.001$). In contrast, no such alterations in chromosomes were observed in the cells pretreated with $10 \times$ concentration of the benzene fraction, further strengthening our above observations and demonstrating the genoprotective potential of the benzene fraction of the *S. bryopteris* aqueous extract.

Medium-term anticarcinogenicity test

Treatment with BP alone resulted forty lung adenomas in total, with an incidence of 66%. A significant reduction ($P \leq 0.001$) in numbers to twenty-six (incidence 36.66%; inhibition rate 44.45%) was observed in animals administered with the benzene fraction after BP treatment (Fig. 7). Further, there was a significant increase in the average weight of mice and in the weight of the lungs of animals that were treated with the benzene fraction and BP in comparison with the average weight of mice and lungs in animals treated with BP alone. The tumour multiplicity was 0.80 in mice treated with BP alone, while BP treatment combined with the benzene fraction resulted in a significant reduction in tumour multiplicity to 0.43 ($P \leq 0.001$). The present study demonstrated that oral administration of the benzene fraction has chemopreventive effects against BP in Swiss albino mice. The benzene fraction produced significant reductions in lung adenoma incidence,

and significant increases in body weight and weight of the lungs in Swiss albino mice treated with BP as newborns.

Two-stage skin papillomagenesis test

In the control group (group I), in which a single topical application of DMBA was followed, 2 weeks later, by repeated application (three times per week) of croton oil, skin papillomas appeared in all the animals (100% tumour incidence). The cumulative number of papillomas as induced during the observation period of 16 weeks was 44.00 (SE 1.84) (Fig. 8). The tumour burden (mean number of tumours per effective mouse) was recorded as 4.82 (SE 0.10), with an average tumour weight of 268 (SE 14.22) mg. The average latency period was observed as 10.88 (SE 0.12) weeks in the control group (Table 1).

In the treatment group (group II, where 1 g/kg body weight benzene fraction was given orally), the animals showed a significant decrease in the number, size and weight of the papillomas compared with the control group (group I). In animals of the treatment group given a continuous treatment of the benzene fraction orally at the pre-, peri- and post-initiation phases, a significant reduction in tumour incidence (74.20 (SE 2.84) % at 1 g/kg body weight dose level) compared with 100% tumour incidence in the control group was observed. The cumulative number of papillomas during the observation period was 18 (SE 1.64). Tumour burden was recorded as 3.46 (SE 0.12) in this group. The average tumour weight was recorded as 188 (SE 10.48) mg. The average latency period in the treatment group was 11.82 (SE 0.18) weeks, significantly higher than in the control group (Table 1).

Discussion

In the present study, we examined the anticarcinogenic and chemopreventive activity of the flavonoid-enriched benzene fraction from an aqueous extract of *S. bryopteris*. The present results demonstrate that the flavonoid-enriched benzene fraction exerted its anticancer properties *in vitro* through

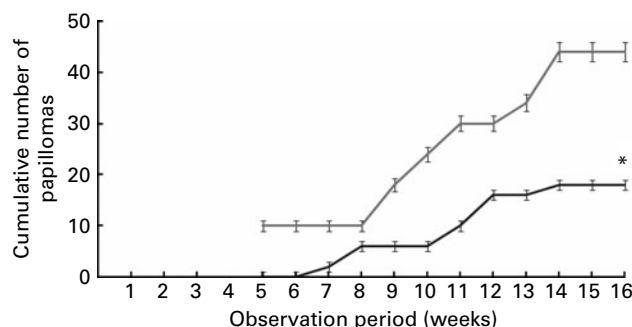


Fig. 8. Modulatory influence of *Selaginella bryopteris* benzene chromatographic fraction (SB.BNZ.Fr) on the cumulative number of papillomas after initiation by 7,12-dimethyl benz(a)anthracene (DMBA) followed by croton oil treatment in Swiss albino mice. (—), Group I mice treated with DMBA (100 μ g/50 μ l acetone) + croton oil (0.1 ml, 1% concentration); (—), group II mice treated with DMBA (100 μ g/50 μ l acetone) + croton oil (0.1 ml, 1% concentration) + SB.BNZ.Fr (1 g/kg body weight). Values are means, with standard errors represented by vertical bars. * $P \leq 0.001$.

Table 1. Average latency period, tumour burden, tumour incidence, tumour weight and size recorded after initiation by 7,12-dimethyl benz(a)anthracene (DMBA) followed by croton oil treatment with and without benzene chromatographic fraction of *Selaginella bryopteris* aqueous extract (SB.BNZ.Fr) treatment in Swiss albino mice (Mean values with their standard errors)

Group*	Treatment and dose	Average latency period (weeks)		Tumour burden		Tumour incidence (%)		Tumour weight (mg)		Tumour size (mm)						
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	< 2 mm	Mean	SE	2–6 mm	Mean	SE	6–10 mm
I	DMBA (100 µg/50 µl acetone) + croton oil (0.1 ml, 1% concentration)	10.88	0.12	4.82	0.10	100	0.00	268	14.22	32.20	1.10	8.60	0.84	3.40	0.48	
II	DMBA (100 µg/50 µl acetone) + croton oil (0.1 ml, 1% concentration) + SB.BNZ.Fr (1 g/kg body weight)	11.82	0.18	3.46	0.12	74.20	1.84	188	10.48	10.12	1.00	5.82	0.66	2.80	0.38	

* Group I, control group, 16 weeks duration; group II, SB.BNZ.Fr treatment at 1 g/kg body weight from day 7 before the application of DMBA and throughout the experiment.

cytoprotection, antioxidative and anti-inflammatory mechanisms and genoprotection in response to MIC-induced carcinogenicity. *In vivo* studies conducted on Swiss albino mice fed with the flavonoid-rich benzene fraction further provided evidence of its chemopreventive mechanism by causing a turnaround in BP- and DMBA-induced carcinogenesis.

The results of the present study have demonstrated that the aqueous extract of *S. bryopteris* and its various chromatographic petroleum ether, benzene, chloroform, ethyl acetate and methanol fractions demonstrated significant anti-apoptotic activity on HEK-293, FHC, B/CMBA.Ov, MM55.K and GC-1spg cultures of various lineages upon exposure to MIC. MIC was chosen on the basis of our data that it is known to react with exocyclic amino group of dNTP to form carbamoylated toxic DNA adducts contributing to cytotoxicity through apoptosis, oxidative stress, inflammation and genomic instability⁽³⁶⁾. Interestingly, of all the fractions tested, the benzene fraction conferred the maximum protection against apoptosis in pretreated cells through a minimal apoptotic index and inter-nucleosomal DNA fragmentation pattern, revealing a cytoprotective effect and indicating a plausible chemopreventive potential of the flavonoids present in the fraction (Fig. 1(a–c)).

The balance between cell proliferation and apoptosis is crucial for the healthy functioning of organisms. In the present study, the observed cytoprotective effects were further validated by the fact that cellular proliferation, under normal conditions, is a well-regulated process where proliferation signals interplay with cell-cycle checkpoint proteins. Cell-cycle arrest occurs in response to cellular stress through activation of regulatory checkpoints⁽⁷⁾. Concordantly, the benzene fraction of the *S. bryopteris* aqueous extract inhibited G1 and G2/M phase arrest, which was associated with a marked increase in the protein expression of p53 and p21 after MIC exposure (Fig. 2(a) and (b)). Also, the benzene fraction had a much reduced effect on the expression of the normal PCNA gene, implicating its critical role in the mechanisms underlying chemopreventive effects and a possible explanation for cytoprotective activity against MIC (Fig. 2(c)). Unperturbed p53 expression levels might also play a crucial role in chemoprevention due to close association with endogenous antioxidants such as glutathione and SOD⁽⁵⁰⁾.

Among several human disease states, carcinogenesis especially has been associated with oxidative stress. This condition arises in a cell or in a tissue when the concentration of the ROS generated exceeds its antioxidant capability, in turn contributing to the transcriptional activation of oncogene products and/or inactivating tumour-suppressor genes^(51–53). Dietary flavonoids with high antioxidant index in this regard have been shown to mediate and exert their anticarcinogenic effects by acting as free radical scavengers and inhibiting the initiation of lipoxygenation reactions through chelation with ROS-generating transition metals and thereby enhancing survival against cytotoxic compound exposure^(54,55). In an attempt to explain the observed cytoprotective effects, we looked at their effects on markers of cellular oxidative stress, such as CM-H2DCFDA, to measure ROS generation, and SOD and GR activity. There was no obvious increase in the generation

of ROS in cells treated with the benzene fraction of the *S. bryopteris* aqueous extract, as shown by a 2'-7'-dichlorofluorescein fluorescence comparable with that of control (Fig. 3(a)). Since SOD and GR play a crucial role in cell defence against ROS, free radicals and electrophilic metabolites, a severe depletion in SOD and GR levels allows the cells to be more vulnerable to oxidative damage by radicals and increasing protein thiolation or oxidation of thiol groups that may lead to alterations in cellular homeostasis⁽⁵⁶⁾. In line with this, the present results showed that treatment with the oxidative mutagen MIC, as expected, caused a significant increase in the concentration of total intracellular ROS levels. While pretreatment with the benzene fraction of the *S. bryopteris* aqueous extract was able to protect cells from the oxidative damage caused by MIC, as observed with no obvious change in GR levels, in the cells treated with the benzene fraction sustained GR activity was shown, contributing to dismutation of superoxide radicals. This is a possible explanation for the cytoprotective properties of *S. bryopteris*.

The significant link of inflammation with cancer points to the prospective and beneficial role of natural anti-inflammatory agents in chemoprevention against cancer⁽⁵⁷⁾. Incidentally, flavonoids have emerged as potential anti-inflammatory agents by their ability to selectively suppress the production of pro-inflammatory chemokines and cytokines^(58,59). Of late, extracts of *Selaginella* species have demonstrated to act as anti-inflammatory agents in response to various stimuli⁽⁶⁰⁾. The present study further provides evidence by the observation that pretreatment with a flavonoid-rich benzene fraction inhibited the expression of the inflammatory cytokines IL-8, IL-1 β , and TNF in MIC-stimulated HEK-293 cells at 24 h (Fig. 4). These data suggest that the benzene fraction contributed to the anti-inflammatory effect of the *S. bryopteris* aqueous extract in the present study.

Free radical generation, oncoproteins and tumour-suppressor proteins can accelerate the induction of senescence in early passage normal cells. This, in turn, leads to a proposed pro-tumoral role for senescence via oxidative damage affecting cancer-causing genes in some senescent cells, thereby promoting their evolution into tumour-initiating cells, as the first step towards carcinogenesis⁽⁶¹⁾. Recent studies with normal keratinocytes showed that partially transformed and neoplastic cells were generated from senescent cells, systematically and spontaneously, and ROS accumulation during senescence has been implicated as the driving force behind this process⁽⁶²⁾. Consistent with this, in the present study treatment with 0.005 μ M-MIC in HEK 293 cells promoted acceleration of senescence and the emergence of transformed cells. Lately, plant extracts and compounds have been demonstrated to protect cultured cells from stress-induced senescence^(63,64). Also, selaginellin, a component extracted from *Selaginella* species, has shown promising results against stress-induced senescence⁽⁶⁵⁾. Concomitantly, morphological transformation and senescence-associated (SA) β -galactosidase activity assessing premature senescence were inhibited upon pretreatment with the flavonoid-rich benzene fraction (10 \times concentration) of *S. bryopteris* in HEK-293 cells (Fig. 5). It can be speculated that the effect can be due to mechanisms related

to antioxidation via scavenging ROS and through reduction in nuclear DNA damage. Thereby, the potential of the benzene fraction to prevent senescence reasonably even after 72 h of exposure could be of great significance against MIC-induced toxicity.

At least two types of oxidative mutagenic damage, DNA strand breakage and base modifications, have been shown to occur in correlation with the increased ROS levels⁽⁶⁶⁾. Recently, we have demonstrated the incidence of DNA damage accumulated during senescence upon exposure to MIC⁽⁴³⁾. Indeed, in our present study MIC exposure was genotoxic and induced persistent DNA damage in IMR-90 cells after 72 h, with γ H2AX foci formation and instigated chromosomal aberrations in HEK-293, and as detected by spectral karyotyping (SKY) in MM55.K through all passages. Recently, the potential benefits of flavonoids against free radical-induced DNA damage and alterations at the chromosomal level have been well recognised by virtue of their ability to reduce the incidence of breaks in double-stranded DNA as well as residual base damage through fast chemical repair and protective effects in response to various mutagenic stimuli^(67,68). In the present study, pretreatment with the 10 \times dose of the benzene fraction of the *S. bryopteris* extract imparted a protective effect against DNA damage and attenuated the γ H2AX foci formation in IMR-90 cells (Fig. 6(a)). Cytogenetic analysis further substantiated that the benzene fraction of the *S. bryopteris* aqueous extract inhibited the genotoxicity of MIC at the doses studied, as the mean frequencies of chromosomal aberrations in pretreated cells did not differ significantly from those of the control (Fig. 6(b) and (c)). Absence of apparent chromosomal translocations in spectral karyotyping of pretreated MM55.K cells further confirmed its anti-clastogenic effect (Fig. 6(d)).

To examine the tumour-inhibitory effect of the flavonoid-rich benzene fraction *in vivo*, the chemopreventive action of the fraction was investigated in Swiss albino mice using BP and DMBA, two known carcinogens. Several reports have indicated polyphenolic flavonoids to be the inhibitors of carcinogenesis and especially of lung and oesophageal cancer *in vivo*⁽⁶⁹⁻⁷¹⁾. Consistent with these studies, the oral administration of the benzene fraction to mice pretreated with BP revealed a significant halt in tumour multiplicity through reduction in the incidence of lung adenomas, with gain in both body weight and the weight of lungs (Fig. 7). In a parallel study involving a two-stage protocol of DMBA-initiated and croton oil-promoted skin carcinogenesis as a model system, oral administration of the flavonoid-rich benzene fraction of *S. bryopteris* (1 g/kg body weight), from 7 d before the application of croton oil three times per week for 16 weeks, caused a significant reduction in the number of tumours per mouse and the percentage of tumour-bearing mice, with significant delay in the latency period for the appearance of the first tumour due to pretreatment, lending evidence to the anti-tumour-promoting effect of polyphenolic flavonoids as anticarcinogenic and/or anti-tumour-promoting agents⁽⁷²⁾ (Fig. 8; Table 1).

In conclusion, under the described experimental conditions, our studies demonstrate that the flavonoid-rich benzene

fraction of a *S. bryopteris* aqueous extract displayed optimum chemoprevention in mammalian epithelial cells through cytoprotective and genoprotective effects. Cytoprotective effects included inhibition of DNA fragmentation, normal expression of cell-cycle regulatory proteins p53, p21 and PCNA, antioxidative effects through the induction of SOD and GR activities, anti-inflammatory activity by mitigating pro-inflammatory cytokine levels and prevention of stress-induced senescence. Genoprotective effects included preventing DNA damage and abrogation of chromosomal aberrations, against the cytotoxicity, genotoxicity and mutagenicity induced by MIC *in vitro*. Also, *in vivo* administration of the benzene fraction of a *S. bryopteris* aqueous extract showed strong anti-cancer and chemopreventive activities through a medium-term anticarcinogenesis bioassay, at initiation as well as post-initiation phases of DMBA-induced mouse skin tumorigenesis. The present study provides evidence of anticarcinogenic and chemopreventive activity of *S. bryopteris* hitherto unreported. The observed effects may be attributed to a mixture of bioactive flavonoids present in the benzene fraction. It has been fairly well established that flavonoids offer high level of protection against a number of potential diseases including cancer. Often, described as biological response modifiers, flavonoids possess antioxidant activity helping the body to build immunity and fight off unhealthy free radical scavengers. In addition, they also possess anti-inflammatory properties and have a number of nutritional functions, as many of the medicinal actions of fruits, vegetables and herbs are directly related to their flavonoid content. Since it may not always be possible to obtain all the required amounts of flavonoids from these dietary sources, a nutritional supplement such as a flavonoid-enriched fraction from *S. bryopteris* reported in the present study might offer a decent alternative and the desired levels of cancer protection. Further studies to understand the subtle targets of intracellular signalling pathways, characterisation of individual flavonoids, pharmacological profile and toxicological safety of this bioactive fraction are essential to pave the way for successful translation of our findings to the clinic. The present study endorses and favours the inverse association between dietary flavonoid intake and cancer risk.

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P. K. M. and N. P. conceived the study, designed and coordinated and helped to draft the manuscript, equally. G. V. R. carried out the immunocytochemistry and cytogenetic studies. A. B. performed the flow-cytometric analysis. A. A. executed the Western blot experiments. R. S. conducted the animal studies. R. U. and S. K. J. helped in the collection, processing and phytochemical analysis of the plant material. G. V. R. and A. B. contributed equally in performing the statistical analysis and preparation of the manuscript. All authors read and approved the final manuscript.

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