Indian herb '*Sanjeevani*' (*Selaginella bryopteris*) can promote growth and protect against heat shock and apoptotic activities of ultra violet and oxidative stress

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Selaginella bryopteris is a lithophyte with remarkable ressurection capabilities. It is full of medicinal properties, hence also known as '*Sanjeevani*' (one that infuses life). For lack of credible scientific evidence the plant is not in active use as a medicinal herb. We provide scientific evidence for why *S. bryopteris* is known as '*Sanjeevani*'.

The aqueous extract of *S. bryopteris* possesses growth-promoting activity as well as protective action against stress-induced cell death in a number of experimental cell systems including mammalian cells. Treatment of the cells in culture with 10% aqueous extract enhanced cell growth by about 41% in *Sf*9 cells and 78% in mammalian cells. Pre-treatment of cells with the Selaginella extract (SE) (1–2·5%) protected against oxidative stress (H_2O_2)-induced cell death. The killing potential of ultra violet (UV) was also significantly reduced when the cells were pre-treated with SE for 1 h. Thermal radiation suppressed cell growth by about 50%. Pre-treatment of cells with SE for 1 h afforded complete protection against heat-induced growth suppression. SE may possess anti-stress and antioxidant activities that could be responsible for the observed effects. Chemical analysis shows that SE contains hexoses and proteins. Taken together, *S. bryopteris* extract may help in stress-induced complications including those due to heat shock.

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1. Introduction

Selaginella bryopteris is a pteridophytic plant which is known for its remarkable resurrection capabilities. The dry plants have been used as a working remedy for several human health complications since centuries in India, particularly in the tribal areas. Briefly, the medicinal uses of *S. bryopteris* include: (i) relief from hot wave and burning sensation during urination; (ii) restoration to normalcy of menstrual irregularities and given externally to pregnant women for an easy delivery; and (iii) for curing jaundice.

Selaginella is a lithophytic xerophyte that grows on the hills of tropical areas, particularly the Arawali mountain

Keywords. Apoptosis; *Sanjeevani*; *Selaginella bryopteris*; thermal shock; UV

Abbreviations used: FBS, Foetal bovine serum; PECS, peritoneal exudate cells; ROIs, reactive oxygen intermediates; SE, Selaginella extract; UV, ultra violet.

terrains from east to west in India (figure 1a). The plants grow luxuriantly during rains exhibiting a lush green velvety landscape (figure 1b). During summer the plants undergo extreme desiccation. The fronds curl, become dry and virtually dead. In this condition they look like a closed fist hence often known in Unani as '*punjemariam*' or '*hathazori*'. The dry plants when left in water unfold their fronds, turn green and come back to active life. Several species of Selaginella have been used as potential herbal medicines (Silva *et al* 1995; Miao *et al* 1996; Lee *et al* 1999; Pandey *et al* 1999; Rojas *et al* 1999; Ma *et al* 2001; Goel and Sairam 2002).

Most of the medicinal properties of the different species of Selaginella have been examined under non-native conditions that include extraction of the plant content using organic solvents. Native methods of use of these plants include soaking them in water over night, preferably in an earthen pot. The plants are discarded and water containing aqueous leaching is filtered and taken orally to cure the above mentioned health complications. The primary objective of our endeavour is to produce an experimental evidence of medicinal efficacy of this herb in its native method as employed by the people. Because of the remarkable medicinal properties, *S. bryopteris* has also been known as '*Sanjeevani*'. It is, therefore, hypothesized that this herb possesses a growth-promoting activity as well as protective action against stress-induced cell death that play vital roles in organismal growth and development, tissue homeostasis, and maintenance of genomic integrity.

Inappropiate apoptosis has been conclusively shown to result in several human diseases including cancer, neurodegenerative diseases such as atherosclerosis, Alzheimers, Parkinsons, etc. (Campisi 2003; Friedlander 2003; Schmitt 2003; Zender and Kubicka 2004). We, therefore, wanted to use an experimental cell system containing *Sf9* insect cell and certain mammalian cells in culture to examine medicinal properties of the herb in its native form of use.

Our results demonstrate for the first time that aqueous extract of the herb possesses significant growth promoting and protective activities against several stress-induced apoptosis in experimental cell systems.

2. Materials and methods

2.1 *Cell culture of* Sf9 *cells and induction of apoptosis*

Spododptera frugiperda (Sf9) cells were cultured in TNMFH medium supplemented with 10% foetal bovine serum (FBS) and antibiotics in polystyrene tissue culture dishes. Cell viability was tested with trypan blue dye exclusion. *Sf9* cells were exposed to UVB radiation or H_2O_2 for induction of apoptosis.

2.1a *Cell culture of mammalian cells*: Rat peritoneal exudate cells (PECS) were induced by injection of thyoglycolate broth. The activated peritoneal macrophage cells were isolated from PECS (by plastic adherence, harvested by vigorous flushing with RPMI cell culture medium and maintained *in vitro* in RPMI supplemented with 10% FBS and 1% streptomycin and penicillin at 37°C in the presence of 5% CO_2.

2.1b *Experimental design*: For each treatment, 1×10^6 cells were taken (in triplicate) and treated with different concentrations of Selaginella aqueous extract (1–10%). The cells were incubated for 24 h at requisite temperature. The cells were harvested and counted by haemocytometer under microscope upon treatment with trypan blue.

In another set of experiments, cells were pre-treated with 1–10% aqueous extract for 1–6 h and were then exposed to ultra violet (UV), H_2O_2 or thermal radiation and incubated for another 15 h before scoring for apoptosis.

2.1c *Thermal radiation and induction of cell death*: The sub-confluent culture of *Sf9* cells in six well plates (1×10^6 cells/well) was exposed to thermal radiation in a water bath at 45°C for 30 min. The plates were made to float on the surface of water in a water bath. Then the plate was removed and incubated for 15 h at 27°C before harvest.

2.1d *Preparation of Selaginella extract*: Forty grams of the dry clean herb was soaked overnight in a minimal volume of distilled water (300 ml). Water containing aqueous leaching from the herb was filtered to remove macroscopic debris. The filtrate was then subjected to sterile filtration using 0·22 μ Millipore filters. The sterile filtrate was collected, aliquoted and kept at 4°C for experimental use.

2.1e *Treatment design*: The sub-confluent culture in 35 mm culture plates was pre-treated with 1–10% of the sterile extract of *S. bryopteris* for 1–6 h. The plates containing pre-treated cells were exposed to various stimuli (H_2O_2, UV, heat shock), followed by incubation for 15 h at requisite temperature. Cells were then harvested and subjected to examination for apoptosis following a method as previously described (Hasnain *et al* 1999).

2.1f *Induction of apoptosis by hydrogen peroxide*: Apoptosis was induced in *Sf9* insect cells by 0·5–1 mM concentration of H_2O_2. The sub-confluent culture of *Sf9* cells was treated with H_2O_2 in the absence of visible light in a 6-well plate, following which they were covered on all sides with aluminium foil and incubated at 27°C for 15 h.

2.2 *Induction of apoptosis by UVB*

One million sub-confluent culture cells of *Sf9* were exposed to UVB for the required period at room tempera-

ture in the absence of visible light. UV transilluminator (Ultra violet products, USA) equipped with 6-watt (312 nm) bulbs was used to generate UV rays. The exposure was given from the bottom of the dishes by putting them directly over the UV lamp in the trasilluminator. Following irradiation, the cells were incubated at 27°C for 15 h before harvesting for examination of apoptosis.

2.2a *Study of apoptosis*: Two methods were applied to study apoptosis which are based on our earlier publications (Hasnain *et al* 1999; Sah *et al* 1999; Sahdeo *et al* 2003). Trypan blue exclusion method was used to score apoptotic cells under a light microscope. Data were presented as percent cell growth or surviving cells.

2.2b *Fragmentation of genomic DNA*: We isolated total DNA by a standard method adapted from Hershberger *et al* (1994). Briefly, after treatment, the cells were harvested, pelletized and subjected to lysis using buffer containing 10 mM Tris-HCl, 10 mM EDTA, 0·2% Triton × 100, pH 7·5. DNA was isolated from the supernatant of the lysed cells by phenol, chloroform isoamyl (PCI) alcohol extraction procedure. DNA was ethanol-precipitated and the pellete dissolved in Tris EDTA buffer. Total DNA was run on a 2% TBE-agarose gel after treatment with 0·6 mg/ml RNase.

2.2c *Estimation of protein*: It was followed by the method of Bradford (1976). Briefly, a standard was prepared using 25–100 μg/ml of BSA, along with the unknown samples. To each of these was added diluted Bradford reagent which was procured as BioRad concentrate from BioRad (USA). Colour developed immediately. Optical density was measured at 595 nm wavelength of light. Concentration of protein in the aqueous extract was calculated from the standard graph.

2.2d *Gel electrophoresis of SE protein*: A 10% polyacrylamide denaturing resolving gel was prepared following a standard procedure. Briefly, a requisite volume of 30% polyacrylamide solution (29% acrylamide + 1% bisacrylamide) was taken in a buffer containing Tris.HCl (375 mM, pH 8·8), SDS, TEMED and ammonium persulphate. The gel was immediately cast in a pre-assembled electrophoresis apparatus and left for about 45 min for complete polymerization. On top of this, a stacking polyacrylamide gel (5%) containing requisite volume of 30% polyacrylamide solution, SDS, TEMED, ammonium persulphate in a Tris·Hcl buffer (125 mM, pH 6·8) was poured and a suitable comb was fixed. When this gel polymerized, protein samples prepared in a loading buffer (50 mM Tris·HCl, pH 6·8 + 100 mM dithiothreitol, + 2% SDS + 10% glycerol and 0·1% bromophenol blue) along with a suitable protein marker were loaded on suitable cleaned wells after removing the comb. The gel was run in a Tris-glycine buffer (25 mM Tris + 250 mM glycine, pH 8·3 + 0·1% SDS) at 100 V. The gel was then removed and stained in 0·25% Coommassie blue for 4 h at RT. The gel was then destained in a solution of methanol (50%), water (40%) and acetic acid (10%). Destaining was continued till the bands were clear.

2.2e *Estimation of carbohydrate*: Concentration of carbohydrate (hexoses) was estimated using Anthrone method. A standard glucose solution (100 μg/ml) was prepared. Solutions (0·1–1·0 ml) were taken in separate clean dry test tubes. Similarly, 0·2–1·0 ml aqueous extract of the herb was also taken. Total volume of the solutions was jacked up to 1·0 ml using double distilled water. To each of these samples was added 4 ml Anthrone reagent and incubated for 15 min in a boiling water bath, cooled to RT and abosbance recorded at 620 nm wavelength of light. Carbohydrate concentration was calculated from the standard curve.

2.2f *Computation of data*: The data obtained were subjected to statistical analysis and significance evaluated following students '*t*' test and presented as mean ± standard error (SE).

3. Results and discussion

In India, Ayurveda has often been proposed as a good alternative system of medicine. However, for lack of credible experimental evidences, it is not as popular as other systems. The need of the hour, therefore, is to subject the traditional system of medicine to vigorous experimental test and to fine tune treatment schedules for unambiguous results. We selected a herb which is often used by the tribal as well as native people for the treatment of several human complications. It was identified as *S. bryopetris* which is a xerophytic pteridophyte, and is often known as '*Sanjeevani*' (figure 1).

Following a native method of use, we sterilized the Selaginella extract and set-up treatment schedule as mentioned in the methods, and as legend to figures. As the name, '*Sanjeevani*' suggests the plant extract should have an ability to invigorate vital activities in cells/tissues. This led us to hypothesize that an aqueous extract of *S. bryopteris* may have a growth promoting and protective activity against certain stress-induced cell death. We carried out experiments on cultured insect *S. frugiperda* (*Sf9*) and mammalian mouse macrophage (BMC2) cells to test its potential. Results presented in figure 2 demonstrate that 5% herbal extract did not show a significant effect on the *Sf9*, but enhaced growth in BMC2 cells by about 75%. However, 10% extract in the cell culture medium enhanced number of cells by 41% in *Sf9* and about

78% in BMC2 cells. Although, the cultured cells grow under controlled conditions, yet they have to encounter oxygen present in the culture medium. It is well known that oxygen is essential for sustenance of life, but it may also produce significant detrimental effects that lead to aging and death, among others. There are numerous reports that show conclusively that oxidative stress can induce cell death in various experimental (cell systems) and *in vivo* systems (Halliwell 1988; Sah *et al* 1995, 1999; Thomson 1995; Buttke and Sandstorm 1995; Hasnain *et al* 1999, 2003; Sahdev *et al* 2003). Oxidative stress has been implicated in a number of human diseases, particularly those related to neurodegeneration, such as, Alzheimer's, Parkinson's, atherosclerosis, etc. (Nedeljkovic *et al* 2003; Stocker and Keaney 2004; Schultz *et al* 2005). Aqueous extract of *S. bryopteris* may contain some ingredients that detoxify the detrimental oxygen-derived species in a cell type-dependent manner. Mammalian cells (BMC2) respond well (75% enhancement in cell growth) even to 5% Selaginella extract suggesting that these cells are more sensitive than the insect cells. The observation that *S. bryopteris* possesses anti-stress and anti-oxidant activities supports our interpretation (Pandey *et al* 1999). Reduction of oxidative stress by certain ingredients of the Selaginella extract may be among the factors that help the stressed and even dying cells to recover and multiply to increase the number of cells. This interpretation is further supported by the observation that aqueous extract of *S. bryopteris* protects cells against oxidative stress-induced cell death, too. Results presented in figure 3 demonstrate that oxidative stress induced by H_2O_2 exerts a significant decrease (~ 60%) in cell survival. Pre-treatment of cells with 1–2·5% aqueous extract of the herb for 1 h enhanced cell survival by about 40–67%, which is highly significant (P = 0·01). This is like a typical dose-response histogram which is cell type-dependent. The differential response of the cells may be possible due to (i) varying sensitivity of the cells to stimuli, and (ii) cell type dendence. Higher concentrations of Selaginella extract do not support protection because the excess antioxidant molecules (i) may not have oxidants to interact with, (ii) or may be involved in the generation of more reactive secondary oxidants. Earlier, using an *in vitro* system (pBR 322 plasmid) we have shown that chemically generated superoxides can induce genetic damage and that antioxidants, such as, *b*-carotene, butein, canthaxanthine and *a*-tocopherol may suppress this damage (Sah *et al* 1995) to varying degrees. These observations are further supported by an earlier observation that H_2O_2 may induce apoptosis in *Sf*9 and BMC2 cells by releasing mitochondrial cytochrome *c* and that p35,

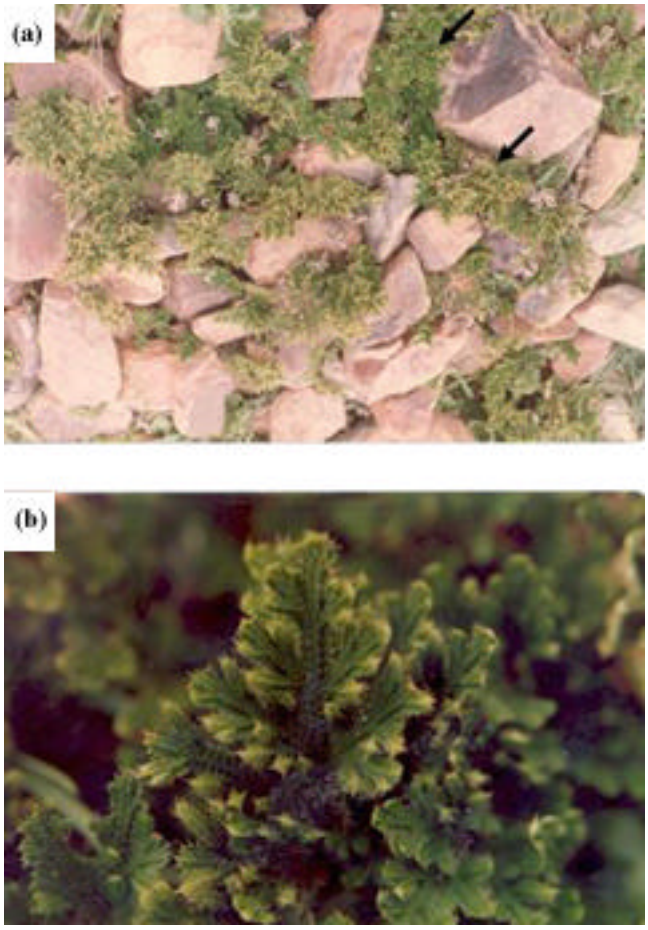

Figure 1. (**a**) *S. bryopteris* in its natural habitat. (**b**) A few fronds of the plant (closer view).

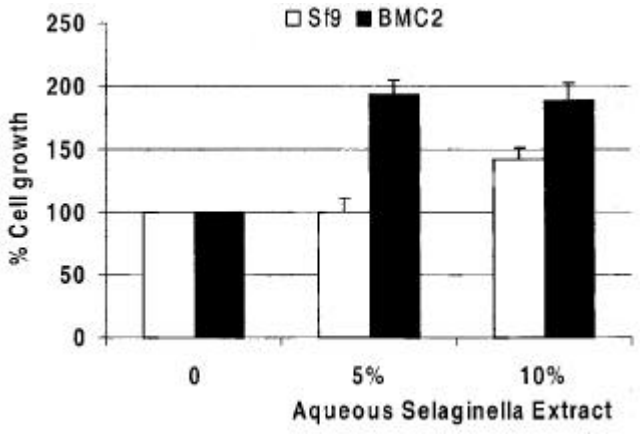

Figure 2. Growth promoting effects of the aqueous extract of *S. bryopteris* on insect (*S. frugiperda*, *Sf9*) and mammalian (BMC2) cell lines. Cells in culture were treated with the mentioned concentrations of the plant extract for 24 h and then cell number scored using haemocytometer.

which is a well known antiapoptotic protein, can suppress these activities as an antioxidant (Sahdev *et al* 2003). It is, therefore, likely that Selaginella extract contains some potent antioxidants that attenuate oxidative stress by scavenging reactive oxygen intermediates (ROIs) to protect the cells from death.

3.1 *UV-induced cell death is suppressed by Selaginella extract*

UV rays induce genetic damage (pyrimidine dimers); as well as oxidative stress mediated by hydroxyl radicals ($OH^{\bullet}$), superoxide radicals and hydrogen peroxide. This herbal extract may protect cells against UV-induced cell-killing also. Results presented in figure 4 shows that pre-treatment of cultured cells with the aqueous herbal extract for 1 h significantly suppressed the killing potential of UV, thus, increasing percent cell survival. Survival of *Sf*9 cells was enhanced from 40% to about 60% by 1–10% herbal extract. This observation is further supported by data in figure 3b on fragmented genomic DNA. Increase in cell survival is also reflected in terms of decrease in DNA fragmentation, which is a hallmark of programmed cell death. The observation that protection by the Selaginella extract is only about 20% is understandable because UV can cause both direct and indirect damage. Direct damage by UV is right on to the genetic material (pyrimidine dimers), whereas, indirect damage is mediated through UV-induced oxygen-dependent free radicals. Selaginella extract is expected to suppress only the indirect effects with the help of antioxidants. So, the level of protection is less. In *Sf*9 cells, UV-induced apoptosis has been shown to occur through release of cytochrome *c* from mitochondria and activation of *Sf*-caspase-1. Anti-oxidants, such as, butylated hydroxyanisole (BHA) and *a*-tocopherol acetate are able to suppress UV-induced mitochondrial cytochrome *c* release, activation of *Sf*-caspase-1 and apoptotic activities (Mohan *et al* 2003). These observations support our view that *S. bryopteris* extract may possess some active anti-oxidants that effectively attenuate H_2O_2- and UV-induced cell death.

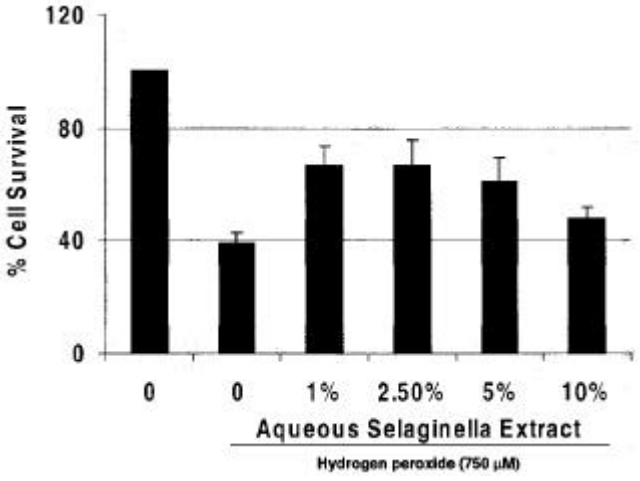

Figure 3. Aqueous extract of *S. bryopteris* protects against H_2O_2-induced apoptotic death in *Sf9* cells. Cells were pre-treated with the aqueous extract for 1 h followed by exposure to H_2O_2. Fifteen hours later, the cells were harvested and scored for apoptosis.

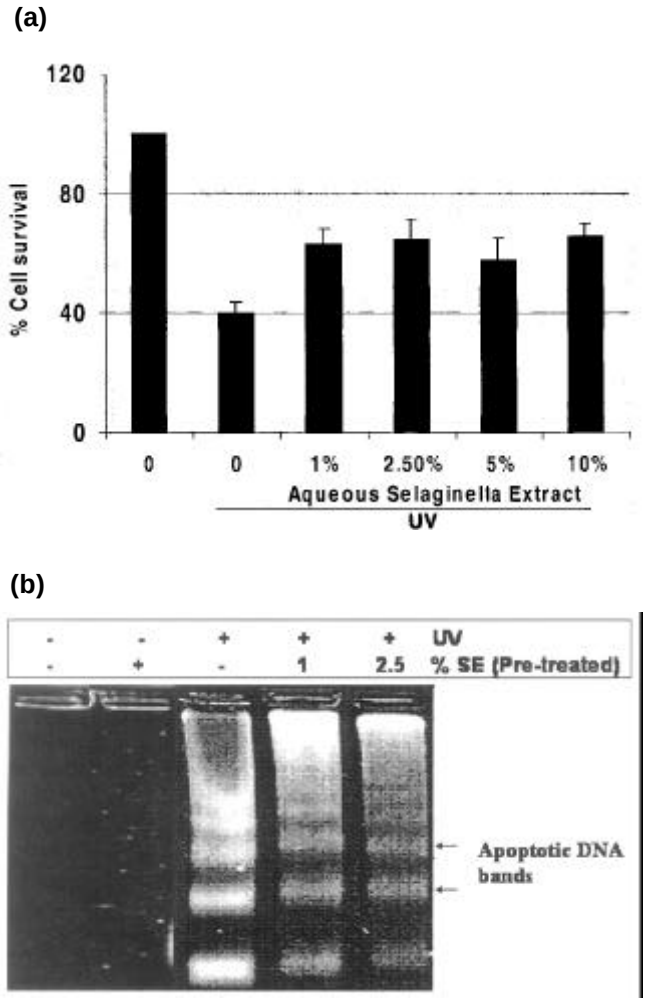

Figure 4. (**a**) *Sf9* cells are protected against UV-induced apoptotic death by the aqueous extract of *S. bryopteris*. *Sf9* cells were pre-treated with 1–10% aqueous extract of *S. bryopteris* for 6 h followed by exposure to UV radiation for 40 s in the absence of visible light. The cells were incubated for 15 h before harvesting for scoring apoptosis. (**b**) UV-radiation induces DNA fragmentation. Pre-treatment with aqueous extract of *S. bryopteris* suppresses DNA fragmentation. There is no fragmentation of DNA in the control and in the 2·5% SE-treated cells.

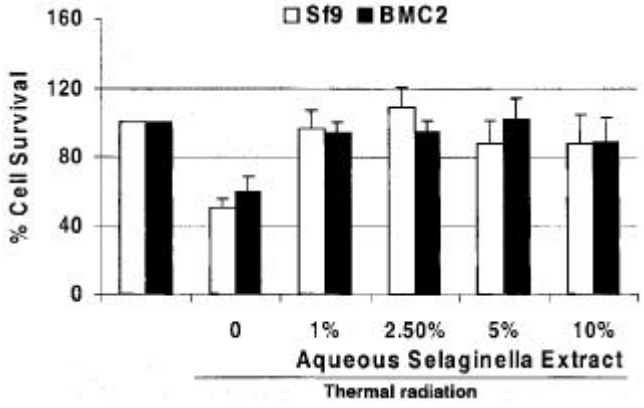

Figure 5. Thermal radiation-induced suppression of growth of cultured cells is completely reversed by pre-treatment with aqueous extract of *S. bryopteris* for 1 h. Cells in plates were exposed to thermal radiation at 45°C for 30 min. Cells were harvested for scoring apoptosis after 15 h incubation at optimal temperatures.

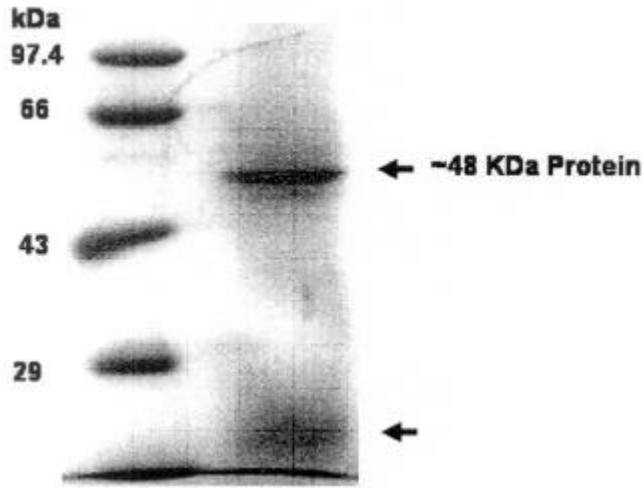

Figure 6. Polyacrylamide gel electrophoresis for identification of protein that are present in the aqueous extract of *S. bryopteris*. Proteins were precipitated using ammonium sulphate and were run on a 10% polyacrylamide gel.

Table 1. Some physico-chemical properties of aqueous extract of *S. bryopteris*.

Properties	Concentration/g plant tissue
Physical parameters	
Colour – Pale yellow	
Odour – Aromatic	
pH – 5·5–5·6	
Specific gravity – 1·01	
Chemical	
Protein	19·75 mg
Hexoses	61·88 mg

3.2 *Reversal of heat-induced suppression of cell growth by Selaginella extract*

According to an Indian mythological description, *S. bryopteris* was able to infuse life in 'Lakshman' (a legendary angel brother of Lord Ram) when he was fatally hit by a fierce fire-power (Shaktiwan). We examined if this herbal extract could protect against heat-induced cell death. Our result demonstrates (figure 5) that thermal radiation suppressed growth of cells by about 40–50% in *Sf9* as well as BMC2 cells. Pre-treatment of cells with 1–10% of the herbal extract for 1 h reversed suppression of growth completely. One of the important effects of heat shock is disruption of mitochondrial electron transfer pathways involving inner membrane bound complexes. The electrons from NADH generated by soluble and less temperature-sensitive Kreb's cycle enzymes are utilized to reduce O_2 into ROI by various components of the uncoupled electron transport chain resulting in build-up of oxidative stress (Davidson and Schiestl 2001). To counter these effects the organisms try to: (i) change the flow of the metabolites; (ii) suppress the pathways that get involved in the build-up of oxidative stress; and (iii) induce a battery of defense genes, such as, heat shock proteins (HSPs), activation of mitogen activated protein kinases (MAPKs) and ROI scavenging enzymes (Chen *et al* 1995; Zentella *et al* 1999; Dat *et al* 2000; Mittler 2002; Rizhsky *et al* 2002). How the herbal extract functions to show these effects is not clear. However, the presence of certain ingredient (s) with antioxidant and mitogenic properties is possible in the herbal extract. These ingredients may try to maintain the integrity of mitochondrial membrane by scavenging ROIs. This is supported by results of chemical analysis of the herbal extract that revealed presence of a number of components including sugar, phosphate and protein (table 1 and figure 6). Further, analysis of the protein that was precipitated with ammonium sulphate showed that it is of ~48 KDa. We are investigating whether these substances alone or in combination with others are responsible for reversal of the heat shock effects by Selaginella extract.

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