

Amomum subulatum seed extract exhibit antioxidant, cytotoxic and immune-suppressive effect

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Amomum subulatum was evaluated for its effect on the growth of concanavalin A stimulated peripheral blood mononuclear cells (PBMCs), human breast cancer (MCF-7) and cervical cancer (HeLa) cell line. It was observed that hexane and ethyl acetate *Amomum* extracts exhibit maximum cytotoxic activity against MCF-7 and HeLa cells. IC₅₀ value for hexane extract was 623 (MCF-7) and 510 (HeLa) µg/mL. In case of ethyl acetate, IC₅₀ value was 798 (MCF-7) and 793 (HeLa) µg/mL. Hexane and ethyl acetate extracts also inhibited the cell growth and tumour necrosis factor (TNF)-α production against PBMCs indicating its immunosuppressive potential. Free radical scavenging results show that ethyl acetate extract (IC₅₀ = 698 µg/mL) exhibits the antioxidant activity. Present results reveal that *Amomum* consumption is beneficial to health as it possesses antioxidant, cytotoxic and immunosuppressive properties.

Keywords: Cancer cell lines, DPPH assay, MTT assay, PBMCs, TNF-α

Herbs apart from their culinary usage play role in the prevention and treatment of many illnesses. On these lines, many scientists are working on herbal products from spices as potential therapeutic agents. Even though used in small amounts, spices come across as a potential source of future medicines. Spices have been used to enhance the sensory quality of food from ancient time across the world. In addition to being used as flavouring agents, they have been used for coloring, preservative and medicinal purposes^{1,2}. Studies have demonstrated that spices have antioxidant, anti-inflammatory, antimicrobial, anticancer and neuroprotective properties³⁻⁹. Spices are well known to possess several bioactive compounds such as eugenol, curcumin, thymoquinone, quercetin which have medicinal importance^{7,10-13}. Owing to their various beneficial effects on human health, spices are considered as functional foods¹⁴. The consumption of spices by humans since ages reveals that there are no considerable side effects of their moderate intake, which makes them ideal agents for exploring new bioactive molecules with a lesser concern for toxicological aspects. Animal studies were carried out with some spices to evaluate the safety aspect and it

was revealed that even at much higher dietary levels (up to 100 times the normal intake) have no adverse effect on growth and organ weight¹⁵.

Cardamom is one of the well-known aromatic spices which refer to *Elettaria* and *Amomum* genera of the Zingiberaceae family. *Elettaria* is commonly known as small or green cardamom, while *Amomum* is greater or large cardamom. *Amomum subulatum* (*A. subulatum*) is mainly distributed in tropical wet evergreen forests of Eastern Himalayas in India, Nepal, Bhutan, and Africa¹⁶. It is commonly used for the treatment of nausea, vomiting, cough, dyspepsia, and itching. Its seeds are used to treat jaundice and gonorrhoea¹⁷. Its dietary intake decreases low density lipoprotein profile, increases the fibrinolytic activity and total antioxidant capacity in patients with ischemic heart disease¹⁸. Reduction in cytochrome P450 level was reported when Swiss albino mice were fed with cardamom oil which suggests that cardamom oil can induce the host enzymes involved in detoxification of chemical carcinogens and mutagens¹⁹. Different phenolic compounds isolated from *Amomum xanthioides* suppress the histamine release and expressions of the pro-inflammatory cytokines in human mast cells²⁰. Several compounds such as limonene, linalool, methyl eugenol and geraniol which exhibit antioxidant and apoptotic activity have been reported to be present in Cardamom²¹. *Elettaria cardamomum* has shown to reduce the tumor incidence in chemically induced skin carcinogenesis in mice²².

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The biological benefits of *A. subulatum* are known but there are meagre scientific evidences to support its antioxidant, cytotoxic and immunosuppressive activities. Hence, in the present study, *A. subulatum* extracts prepared in different solvents of varying polarity were evaluated for their effect against concanavalin A stimulated peripheral blood mononuclear cells (PBMCs), human breast cancer (MCF-7) and cervical cancer (HeLa) cell line. Further, their antioxidant effect was evaluated by the ability to scavenge the free radical (DPPH: 2, 2-diphenyl-1-picrylhydrazyl D).

Material and Methods

Preparation of extracts

An authentic sample of *A. Subulatum* was obtained from GB Pant Institute of Himalayan Environment and Development, Sikkim, India. The dried fruits/seeds were ground to fine powder using porcelain pestle and mortar followed by sequential soxhlet extraction in six different solvents (hexane, diethylether, ethyl acetate, ethanol, acetone, and water) in order of increasing polarity. Finally, the solvents were removed under reduced pressure by rotary evaporator (Yamoto Scientific Co. Ltd, Japan) and extracts were reconstituted in dimethyl sulfoxide (DMSO).

Cell culture maintenance

MCF-7 and HeLa cell were procured from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM (Sigma, USA), containing 10% (v/v) foetal bovine serum (Gibco), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin.

Cell growth inhibition assay

The response of cancer cell lines to *A. subulatum* extracts was evaluated by cell growth inhibition studies using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay²³. 2×10^4 cells were seeded per well in a 96 well cell culture plate and incubated overnight. After 16h, sample extracts were added in varying final concentrations to the wells. The assay was carried out in triplicates. After 24, 48 and 72 h of incubation, 20 µL of MTT reagent (Sigma USA, 5mg/mL) was added to each well and the plate was again incubated for four hours. The formazan crystals thus formed were solubilized in 100 µL DMSO. Finally, the absorbance of each well was recorded at 570 nm, taking 630 nm as the reference wavelength, using the microplate reader (Tecan

infinite, Austria). Paclitaxel (20 µg/mL) was used as a positive control. Percentage of inhibition was calculated as following: $\{(\text{Mean OD of untreated cell} - \text{Mean OD of treated cell}) / \text{Mean OD of untreated cell}\} \times 100$

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) isolation was carried out by ficoll density gradient method. Heparinized venous blood (5 mL) was drawn from healthy volunteers after taking prior consent from each healthy volunteer. Blood was carefully layered onto the Histopaque 1077 (Sigma, USA) and centrifuged at $400 \times g$ for 30 min at room temperature. After centrifugation, plasma layer at the top was carefully removed with a pipette and opaque interface containing PBMCs was collected in a sterile conical centrifuge tube. The cells were washed twice with three volume of 1X PBS by centrifuging at $250 \times g$ for 10 min. The cell pellets were resuspended in RPMI-1640 media (Sigma, USA) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, 100 units/mL penicillin and 2.5 µg/mL amphotericin.

Lymphocyte proliferation assay

PBMCs were seeded at a density of 1×10^5 cells per well and then *A. subulatum* extracts were added in varying final concentrations. After 2h, concanavalin A (5 µg/mL), a mitogen was added in order to stimulate the PBMCs. The assay was carried out in triplicates. After 72h of incubation in the CO₂ incubator, MTT assay was carried out to measure the cell proliferation. MTT assay and calculation of inhibition in mitogen induced proliferation were done as described in case of cytotoxicity assay.

TNF- α measurement

In order to evaluate the production of TNF- α , concanavalin A stimulated PBMCs were incubated with varying concentrations of *A. subulatum* extract for 72 h as described in lymphocyte proliferation assay. After incubation, supernatants were collected and the secretion of TNF- α was measured using sandwich ELISA kit (Preprotech, USA) as per manufacturer's instruction. Inhibition in TNF- α production was calculated in the same manner as mentioned in the cell growth inhibition assay.

Antioxidant assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed to assess the antioxidant potential of *A. subulatum* hexane and ethyl acetate extract.

Hexane and ethyl acetate extract (50 μ L) with varying concentrations was mixed with 150 μ L of DPPH (100 μ M) in methanol, added to wells of a 96-well microtitre plate. Ascorbic acid (100 μ g/mL) was used as positive control. The plate was wrapped in aluminium foil and kept at 30°C for 30 min in dark. The change in color (from deep violet to light yellow) was measured at 517 nm using microplate reader (Tecan infinite, Austria). Free radical scavenging activity was expressed as the inhibition percentage calculated using formula, Free radical scavenging activity = $(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}} \times 100$

Statistical analysis

The data were expressed as the mean \pm standard deviation of three independent experiments. Data were analysed using analysis of variance⁷ and the means were compared by using Tukey's test at $P < 0.05$.

Results and Discussion

Effect of Amomum extracts on cancer cell lines

Although significant advancements have been achieved in the cancer treatment, cancer remains a serious threat to human life. Major limitations of the existing cancer therapy are potent side effects, high cost and an increased resistance toward anticancer drugs. Natural bioactive products are being explored to overcome bottlenecks associated with the currently used cancer therapy. Spices are one of the attractive options as they are known to have medicinal value in the traditional medicinal system. *A. subulatum*, a medicinally important spice was explored for its effect on the growth of cancer cell lines.

The crude extracts of whole fruit prepared in six solvents with different polarities (hexane, diethylether, ethyl acetate, ethanol, acetone, and water) were initially tested in order to assess the effect of extracts on cancer cell growth using MTT assay. Ethyl acetate and hexane fruits extracts showed highest cell growth inhibition (data not shown). Based on this preliminary result, ethyl acetate and hexane solvent were chosen to carry out for further studies. The most widely used part of *A. subulatum* is its seed and is extensively used in food preparation. So, we shifted our focus of further studies to the seeds which are also known to possess several bioactive compounds.

High activity with two solvent extracts of different polarity which indicates that two different compounds with varying polarity might be associated with the observed growth inhibition effect. To study the kinetics of cell growth inhibition, incubation of these

seed extracts with MCF-7 and HeLa cells was carried out for 24, 48 and 72h. Little or no cytotoxic effect of these extracts against cancer cells was observed after 24h, however, this effect became pronounced with an increase in time (Fig. 1). Further, the similar activity of both the extracts in two cell lines of different origin shows that the mechanism of cytotoxicity is general and not cell type specific.

The ethyl acetate and hexane seed extracts were evaluated for the effect of increasing concentration on MCF-7 and HeLa cells and thus, IC₅₀ was calculated. In these experiments, cells were exposed to the extracts for 72 h. IC₅₀ values of hexane extract were found to be 622 ± 115 and 500 ± 67 μ g/mL against MCF-7 and HeLa cells respectively. For ethyl acetate extract, IC₅₀ values were 778 ± 225 and 724 ± 223 μ g/mL against MCF-7 and HeLa cells respectively. However, hexane extracts showed lower IC₅₀ value in general for both the cell lines. Paclitaxel, (20 μ g/mL) an anticancer drug was used as a positive control which showed >90% inhibition in most of these experiments. The results thus obtained are in line with earlier reports of *Amomum villosum* and *Amomum kravanh* having cytotoxic properties against different cell lines²⁴. Another similar report showed that small pod cardamom reduces the size and number of 7,12-dimethylbenz[a]-anthracene (DMBA)-induced mice skin papilloma²⁵.

Effect of Amomum extract on immune cells

Spices are being used to promote the maintenance of a healthy immune system. Hence, the effect of *Amomum* extract was evaluated against peripheral blood mononuclear cells (PBMCs) based on the proliferative response and TNF- α production. It was observed that PBMCs did not show proliferating response against the mitogen concanavalin A in the presence of ethyl acetate and hexane seed extracts

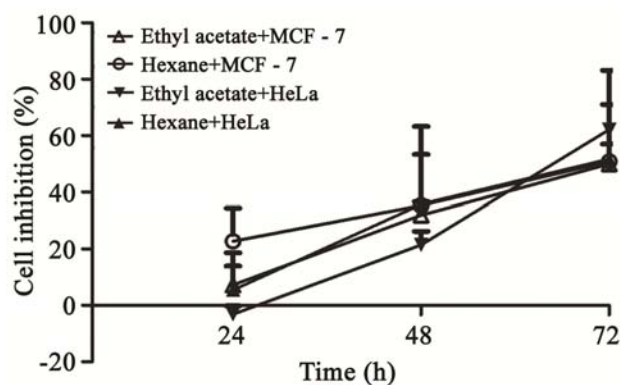


Fig.1— Kinetics of MCF-7 and HeLa cell growth inhibition by *Amomum subulatum*.

indicating their immunosuppressive property (Fig. 2). Cell inhibition obtained at 1 mg/mL were 95 ± 2 and $40 \pm 8\%$ for hexane and ethyl acetate extract respectively indicating a pronounced activity in hexane. The immunosuppressive effect of hexane extract increased significantly with concentration. The IC_{50} value obtained for hexane extract was $438 \pm 48 \mu\text{g/mL}$. Further, two different concentrations (250 and $500 \mu\text{g/mL}$) of *Amomum* extract were checked for their effect on TNF- α production in concanavalin A stimulated PBMCs. Inhibition of TNF- α production was also observed in both hexane and ethyl acetate extract though it was more pronounced in hexane (Fig. 3). These results are in concordance with earlier reports which have shown that different compounds isolated from *Amomum xanthioides* inhibit the mast cell degranulation and TNF- α production^{20,26}.

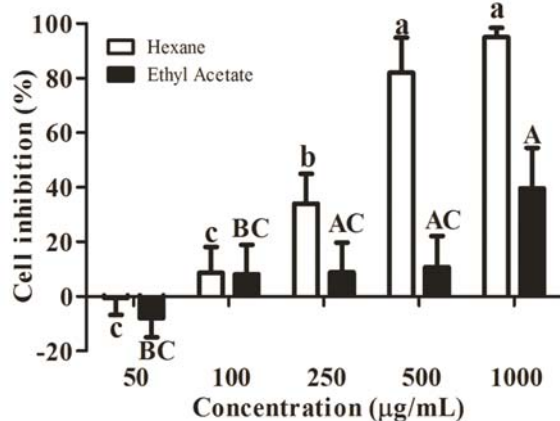


Fig. 2—Immunosuppressive effect of *Amomum subulatum* extract on concanavalin stimulated peripheral blood mononuclear cells (PBMCs). Means followed by the same letter are not significant at $P < 0.05$.

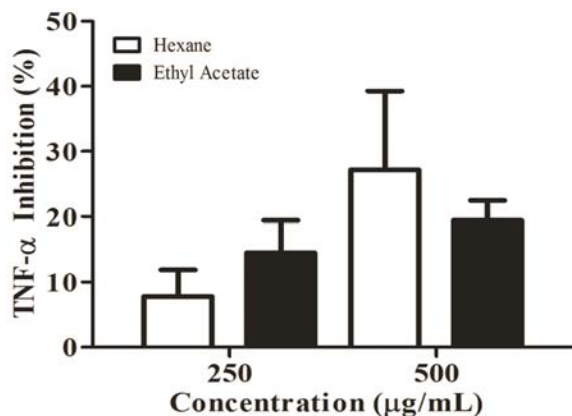


Fig. 3—Effect of *Amomum subulatum* extract on TNF- α production concanavalin A stimulated peripheral blood mononuclear cells (PBMCs).

Antioxidant activity of Amomum extract

Antioxidants neutralize the free radicals and hence protect the cells from damage. Free radical mediated reactions are found to be associated with various diseases such as cardiovascular disorder, diabetes, inflammatory diseases and cancer²⁷. Free radical scavenging activity was performed to evaluate the antioxidant effect of *Amomum* extract. Antioxidant effect of ethyl acetate extract was found to be significantly increased with concentration but hexane extract has shown little or no antioxidant effect (Fig. 4). The difference in the antioxidant effect of these two extracts indicates the involvement of different pharmacological active product. The IC_{50} value of scavenging activity of ethyl acetate extract was found to be $698 \pm 110 \mu\text{g/mL}$. Ascorbic acid used as positive control, showed $89 \pm 4\%$ antioxidant activity. In the earlier studies, leaves and fruit of *A. subulatum* were reported to exhibit antioxidant effect^{28,29}. In the present study, seeds were reported to have antioxidant properties. Apart from cardamom, other spices like turmeric, curcumin, red pepper, and fenugreek are source of antioxidants^{30,31}.

Spices are food adjuncts that have been used as taste enhancers and also known to possess medicinal properties as well. Present studies show that hexane and ethyl acetate seed extracts of *A. subulatum* reduce the growth of MCF-7 (breast cancer) and HeLa (cervical cancer) cells. Further, these solvents exhibit an immunosuppressive effect by suppressing the cell proliferation and TNF- α production in concanavalin A stimulated PBMCs. In addition to these activities, ethyl acetate extract has shown the antioxidant activity. Present results suggest that *A. subulatum* has potential preventive and therapeutics importance in maintaining good health. Further research is required

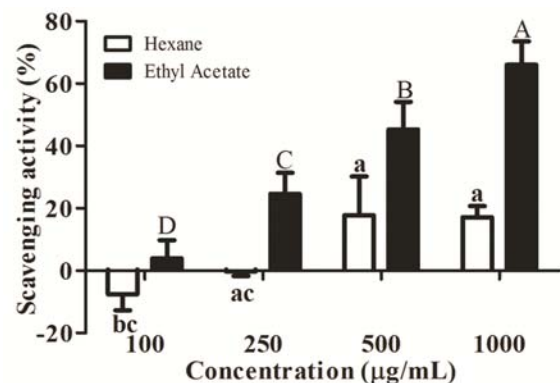


Fig. 4—Antioxidant effect of *Amomum subulatum* extracts based on free radical scavenging activity. Means followed by the same letter are not significant at $P < 0.05$.

to identify the potential bioactive compounds present in it and understand the underlying molecular mechanisms.

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