

Full Length Research Paper

Effects of *Allium hirtifolium* (Iranian shallot) and its allicin on microtubule and cancer cell lines

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Allium hirtifolium Boiss. (Iranian Shallot) belongs to *Allium* genus (*Alliaceae* family). Microtubule proteins (MTs) are crucial in maintenance of cell shape as well as cell division and mitosis. The present study aims at defining the anti-microtubule activities of *A. hirtifolium* and its allicin and examining its effects on nerve cell microtubules. MTs were prepared from sheep brain through two cycles of polymerization and depolymerization. The cell growth inhibition was measured by 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) after treatment with *A. hirtifolium* and its allicin on HeLa and MCF-7 and L-929 cell lines. *A. hirtifolium* displayed growth inhibitory activity against HeLa and MCF-7 cells with IC (50) value of 20 and 24 mg/L, respectively, for 72 h and obviously showed cell growth inhibition on these cell lines at non-toxic concentration (lower than 1 g/L). Inhibition of MTs polymerization induced by *A. hirtifolium* and its ability to bind to tubulin as a ligand was tested through turbidimetry assay then investigated by Transmission Electron Microscopy (TEM). The concentration of *A. hirtifolium* necessary to inhibit the assembly of MTs by 50% was 1.2 g/L, while an inhibition higher than 80% was observed in the presence of 4 g/L of *A. hirtifolium*. This plant decreased MTs polymerization; therefore we suggest *A. hirtifolium* can be an effective ligand for cancer therapy.

Key words: *Allium hirtifolium*, cell proliferation, microtubule assembly dynamic, tubulin, SH containing protein.

INTRODUCTION

Microtubules represent the best target for cancer chemotherapy and will remain a promising target for new chemotherapeutic agents. Microtubules are essential proteins in cell transport in all eukaryotes (Nogales, 2001; Allan and Vale, 1991; Karki and Holzbaur, 1999; Zhai et al., 1995). MTs, long hollow tubes about 25 nm in diameter and microns in length, play important roles in supporting cell structures (Stracke et al., 2000; Zhou et al., 2007). Mitotic block by drugs at concentrations that suppress microtubule dynamics or alter microtubule mass induces apoptosis (Wilson and Jordan, 1995; Jordan and Wilson, 1998) and they are effective targets for a number

of anti-cancer drugs (Downing and Nogales, 1998). In recent years numerous tubulin ligands with anti-mitotic properties and anti-cancer potential have been discovered (Hamel, 1998). Some of these drugs play seminal roles in experiments probing the basic mechanism of mitosis such as paclitaxel (Taxol) which inhibit cancer cell proliferation by stabilizing the microtubules (Bogdan and Ding, 1992) or vinblastine and their analogs inhibit cell proliferation by depolymerizing the microtubules (Dhamodharan et al., 1995).

Many of these anti-microtubule drugs are found to be less toxic toward normal cells, signifying their potential utility in clinical settings (Polizzi et al., 1999) and some of them like Taxol and vinblastine analogs have clinical application (Kruczynski et al., 1998; Verdier et al., 1999). The success of microtubule-targeted drugs in cancer chemotherapy largely depends on their preferential targeting of cancer cells and tolerable side effects on patients.

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Differential dynamic behavior and isotype composition of microtubules in cancer cells make them selective targets for microtubule-targeting anti-cancer agents (Attard et al., 2006; Jordan and Wilson, 2004; Deplanque and Harris, 2000). During the past ten years more attention has been given to finding the benefits of garlic sulfur compounds in relation to cancer (Milner, 1996; Singh et al., 1996). It is believed that the products resulting from the breakdown of alliin are responsible for the antiproliferative effects of Alliaceae family (Knowles and Milner, 2001).

Allium hirtifolium (Iranian shallot) has a completely smooth sheet in the living state, bulb tunics disintegrating in fibre-like parts (Jafarian et al., 2003). This taxon belongs to the *Alliaceae* family and it is an Iranian traditional native herb which is used as a condiment spice. It is well known in Iranian folk medicine and its bulbs have been widely used for treating rheumatic and inflammatory disorders (Jafarian et al., 2003; Barile et al., 2005).

A. hirtifolium contains some useful biological secondary metabolites, which include alliin, alliinase, alliin, S-allyl-cysteine (SAC), diallyldisulphide (DADS), diallyltrisulphide (DATS), and methylallyltrisulphide. Alliin is converted to alliin when its bulbs are crushed (Block et al., 1992). Each of these secondary metabolites is capable of disrupting microtubule proteins (Khouhorsky et al., 2007). Alliin is a precursor of several sulfur-containing compounds that are responsible for the flavour, odour and pharmacological properties of *A. hirtifolium* (Jafarian et al., 2003). Alliin has been reported in treatment of cancer (Knowles and Milner, 2001). Both α and β tubulin contain cysteine residues bearing SH-groups that could potentially interact with alliin. The inhibitory effect of alliin on tubulin polymerization *in vitro* can be partially abolished by SH-reducing reagents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME); it means that alliin interferes with microtubule assembly by modifying SH groups in both α and β subunits (Xiao et al., 2003).

A great potential has been shown in enhancing the anticancer activity of the conventional microtubule-targeting anti-cancer drugs, which does not harm regular cells. These intrinsic characteristics make these compounds attractive as research tools and experimental drug candidates. Hence, we were interested in investigating anti-proliferative activity of *A. hirtifolium* extracts involved interference with the microtubule cytoskeleton. The present study focused on comparative effects of water and chloroform-soluble *A. hirtifolium* extracts on tubulin function in the assembly of microtubule dynamic. Although there are some MT targeting agents for clinical uses, many more remain to be discovered.

MATERIALS AND METHODS

Collection of the plant

The bulbs of *A. hirtifolium* were collected from plants growing wild in Dare bid mountain area of Khansar in Isfahan province, center of

Iran, in June, 2006. The plant specimen was identified by the Iranian Research Institute of Forests and Rangelands, Tehran, Iran.

Preparation of Persian shallot chloroformic and aqueous extracts (PSCE and PSAE)

Collected Persian shallot bulbs, were washed with tap water and cut into small slices then, the slices were macerated in chloroform for 24 h at cool temperature (10°C). After that the extracts were filtered through cotton cloth and air dried at 10°C, then they were frozen dried. After that dried extracts were collected and preserved at -4°C for later uses. For preparation of PSAE, all above steps were done except water was used instead of chloroform (Lawson et al., 1991).

Preparation of Allicin from *A. hirtifolium*

Alliin was isolated from chloroformic and aqueous extracts of *A. hirtifolium* by column chromatography; then the compound was detected by silica gel thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Ogra and Ishiwata, 2005; Miron et al., 2002). The existence of alliin was determined and confirmed by UV-spectroscopic analysis with absorption maximum at around 240 nm. The frozen dried mass of extracts was dissolved in water or PEM buffer before utilize in turbidity tests.

Solutions and reagents

0.3 L Homogenization buffer (freshly prepared), 1 L [piperazine-N, N'-bis 2-ethanesulfonic acid (PIPES), EGTA, MgCl₂] (PEM) buffer, 100 mmol/L MgATP, 1L PMG Buffer, 100mmol/LMgGTP, sodium salt. Unless otherwise stated, all compounds were obtained from Sigma Chemical Co., St Louis, MO, USA.

Cell culture

The human cervical carcinoma cell line (HeLa, NCBI C-115), estrogen receptor-positive human breast cancer cell line (MCF-7 NCBI C135), and mouse fibroblast cell line (L929 NCBI C161) were purchased from the National Cell Bank of Pasteur Institute of Iran. All the cell lines were grown at 37°C and in the presence of 5% CO₂, in RPMI-1640 medium supplemented by 10% heat-inactivated fetal bovine serum (FBS), antibiotics (penicillin 0.100 IU/L, streptomycin 100 mg/L) in culture flasks. The cells were fed until confluence (2×10^6) and expanded by trypsinization and subculture at lower numbers in new culture flasks.

The cultured cells were treated with alliin and *A. hirtifolium* extracts; we also examined the effects of them in different concentrations. Evaluation of cells was performed at 24, 48 and 72 h after treatment.

Assay of cytotoxicity (MTT and gel electrophoresis and DNA fragmentation)

RPMI medium was used to solve the frozen dried form of chloroform-soluble extract of *A. hirtifolium* and alliin. The effects of *A. hirtifolium* and its alliin on cell proliferation were determined by using standard MTT based colorimetric assay (Mosmann, 1983).

For DNA fragmentation analysis, the DNA from cultivated cells was isolated (Herrmann and Lorenz, 1994). Cells (2×10^6) were treated with the extracts and Alliin and then collected by centrifugation (2000 g, 10 min). The samples were analyzed by electrophoresis on a 1.5% agarose gel and were assessed under UV illumination.

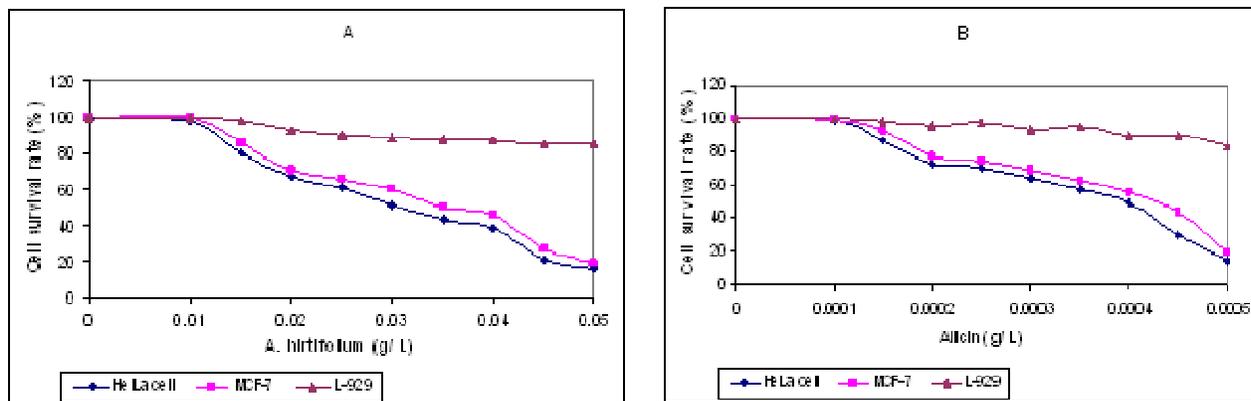


Figure 1. The effect of the extent of confluence on cell survival after treatment with *A. hirtifolium* extract (A) and Allicin (B). The cells were seeded on a 96-well plate. The cells were then treated with *A. hirtifolium* and Allicin for 72 hours and cell survival was measured by MTT assay. Percentage of surviving HeLa and MCF-7 cells in all concentrations of Allicin and *A. hirtifolium* extract was significantly decreased when compared with L929 cells ($p < 0.001$). *A. hirtifolium* had less cytotoxic effect on L929 as a normal cell. Each point represents the Mean \pm SD ($n = 3$).

Microtubule assembly

Twice cycled tubulins were isolated from sheep brain tissue through two cycles of assembly/disassembly (Castoldi and Popov, 2003; Ihara et al., 1975; Williams and Lee, 1982). Concentration of the tubulins varied from 1.5-3 g/L depending upon the batch preparation. The protein was stored in liquid nitrogen. Prior to use, aliquots were thawed and centrifuged for 5 min at 21,000 g. *In vitro* microtubule assembly or disassembly was followed by quantitating the variations of absorption at 350 nm using a spectrophotometer (Cary - 100) equipped with thermostatically controlled cuvettes (Ihara et al., 1975). The microtubular protein preparations initially at 0°C with or without the tested ligands (*A. hirtifolium* extracts and allicin) were warmed to 37°C in order to initiate microtubule assembly. After 10 min at 37°C, temperature was decreased to 0°C to induce disassembly.

Electron microscopy

Tubulins (treated and untreated with *A. hirtifolium* (2.4g/L) and allicin (0.030g/L) for TEM were prepared by negative staining using uranyl acetate on Formvar/carbon coated copper grids (Bozzoll, 2001; Karjala et al., 2005) and observed by a transmission LEO 912AB electron microscope operating at 75 KV.

Statistical analysis

Computer program (Graph Pad Prism) was used to calculate the IC₅₀ (50% inhibition of cell proliferation) values. Student's t-test was used for statistical analyses while P-values < 0.05 were considered to be statistically significant.

RESULTS

Effects of PSCE and PSAE on cell lines

Most anti-microtubule agents target microtubules directly

and either inhibit or stimulate microtubule polymerization *in vitro* (Jordan and Wilson, 2004). To determine if *A. hirtifolium* extracts had any effect on different cell lines, its water and chloroform-soluble extracts were prepared according to the previous method (Williams and Lee, 1982). For cytotoxic experiments, RPMI was used as a solvent and for turbidity investigation; PEM buffer and water were used to dissolve solid masses.

For cell culture, HeLa, MCF-7 and L929 cell lines were chosen to determine the cytotoxic activity of *A. hirtifolium* extracts. A typical dose-dependent inhibition of cell growth was seen documenting the cell survival rate plotted versus *A. hirtifolium* and allicin concentration respectively (Figure 1). After 72 h incubation, increasing concentrations of *A. hirtifolium* (0-0.05g/L) and allicin (0 - 0.0005 g/L) led to a gradual decrease of the fraction of viable cells. On the contrary, L929 cells were more resistant to the cytotoxic effect of this plant. With respect to the cell lines, we observed that HeLa cell was more sensitive to *A. hirtifolium* and allicin than MCF-7 and L929 cell lines. The results with water-soluble extract of *A. hirtifolium* and allicin were less than these (not shown).

IC₅₀ values were varying from 20 mg/L (HaLa) to 24 mg/L (MCF-7) and 250 mg/L (L929) (Table 1).

DNA laddering fragmentation assay

Incubation of MCF-7 and HeLa cells with *A. hirtifolium* showed typical DNA ladder by electrophoresis after 72 h. We did not find the DNA ladder shape with L929 cell line (Figure 2).

Determination of yield of Allicin in *A. hirtifolium*

Based on our TLC results, the R_f of allicin was 0.4. After

Table 1. Growth-inhibitory effect of Allicin and *A. hirtifolium* extract on HeLa, MCF-7 and L929 cells.

| Cell line | IC50 (mg/L) allicin | IC50 (mg/L) <i>A. hirtifolium</i> |
|-----------|---------------------|-----------------------------------|
| HeLa | 0.15 | 20 |
| MCF-7 | 0.20 | 24 |
| L929 | 1.25 | 250 |

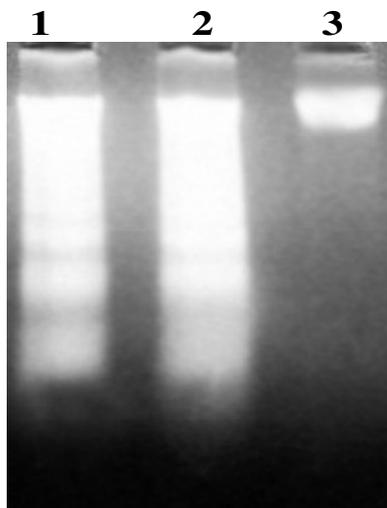


Figure 2. Agarose gel electrophoresis of DNA extracted from cells treated with *A. hirtifolium* 2.4 g/L. DNA laddering, typical for apoptotic cells, was visible for treated HeLa (lane 1) and MCF-7 cells (lane 2), but it was not found for L929 cells (lane 3).

treatment with the reagent, allicin was distinguished by observing the characteristic violet-brown zone in *A. hirtifolium*. The yield of allicin in bulbs of *A. hirtifolium* was 0.34%.

Effect of *A. hirtifolium* extracts on assembly and disassembly of MTs

SDS-PAGE showed that the microtubule protein preparations contained 60% tubulin. Tubulin polymerization tests were conducted to determine *A. hirtifolium* effect on polymerization of pure tubulin into microtubules *in vitro* and it was shown that the extracts disrupted microtubule assembly *in vitro*, and induced tubulin aggregation.

Then Purified brain tubulin (2.5 g/L) was pre-incubated with PEM buffer with MgGTP 1 mM on ice and the temperature was raised to 37°C. Microtubules polymerized spontaneously in the presence of MgGTP, where as 2.4 g/L *A. hirtifolium* or 0.030 g/L of allicin prevented the assembly of microtubules (Figures 3A and 3B). However, *A. hirtifolium* and allicin did not inhibit polymeriza-

tion at any concentration tested up to 0.5 and 0.005 g/L, respectively. These effects were much less when water was solvent (Figures 3C and 3D). The ability of *A. hirtifolium* to inhibit tubulin assembly *in vitro* was determined by monitoring turbidity changes over time, using 2.5 g/L of microtubular proteins. Microtubule assembly was induced by increasing the temperature from 0-37°C and was detected in 350 nm. To determine whether the effects of *A. hirtifolium* and allicin can inhibit microtubule polymerization or its nucleation, tubulin (2.5 g/L) was preincubated with PEM Buffer again, and then *A. hirtifolium* and Allicin were added, *in vitro* assays of microtubule assembly/disassembly showed that 1.2 g/L *A. hirtifolium* and 0.015 g/L allicin inhibited tubulin polymerization by 50%, suggesting that tubulin or microtubules was likely a target of these compounds. Microtubule filament was shown to be very different before and after treatment with *A. hirtifolium*. Our tubulin protein preparation contained at least 40% of microtubule associated proteins (according to SDS-PAGE); therefore, it was not excluded that other proteins could also be involved.

Turbidity was measured by UV- spectrometry (350 nm), at 0 and 37°C, in the absence or in the presence of 1.2 and 2.4 g/L of *A. hirtifolium* or 0.015 and 0.030 g/L of allicin. Represented curves were typical of one experiment repeated at least three times.

Exposure to 2.4g/L of *A. hirtifolium* and 0.030g/L of allicin decreased the plateau in all tests but the effects of *A. hirtifolium* and allicin in PEM with all concentrations on microtubule assembly were more than them in water.

Turbidity was followed by the ability of *A. hirtifolium* and allicin to alter the microtubule assembly by TEM and the effect of *A. hirtifolium* and allicin on microtubule morphology was observed. Microtubules treated with or without 2.4 g/L *A. hirtifolium* or 0.030 g/L allicin were prepared for TEM. The *A. hirtifolium* caused noticeable decrease in the polymerization of microtubule. Microtubule disassembly was observed when incubated with 2.4 g/L *A. hirtifolium* or 0.030 g/L of allicin while entire assembly was observed in the absence of these ligands. The percentages of tubulins aggregation were higher with *A. hirtifolium* and allicin than in untreated pure microtubule (Figure 3).

Electron photomicrographs showed the structures of microtubule protein polymer that treated by *A. hirtifolium* (A), and allicin (B) in presence of MgGTP(C) 1 mM at 37°C were different. In the case of *A. hirtifolium* and Allicin treated samples, we observed that the assembled microtubules were shorter and thinner than normal ones. The shape, elongation and nucleation of normal microtubule, *A. hirtifolium* and Allicin treated ones were investigated.

Our results showed the concentration-dependent inhibition of microtubule assembly induced by *A. hirtifolium* and allicin. Although the latent phase of microtubule assembly was not significantly modified, the inhibition was attested by a decrease of both the rate and the plateau of assembly (Figure 3). Allicin and *A. hirtifolium* induced a dose-

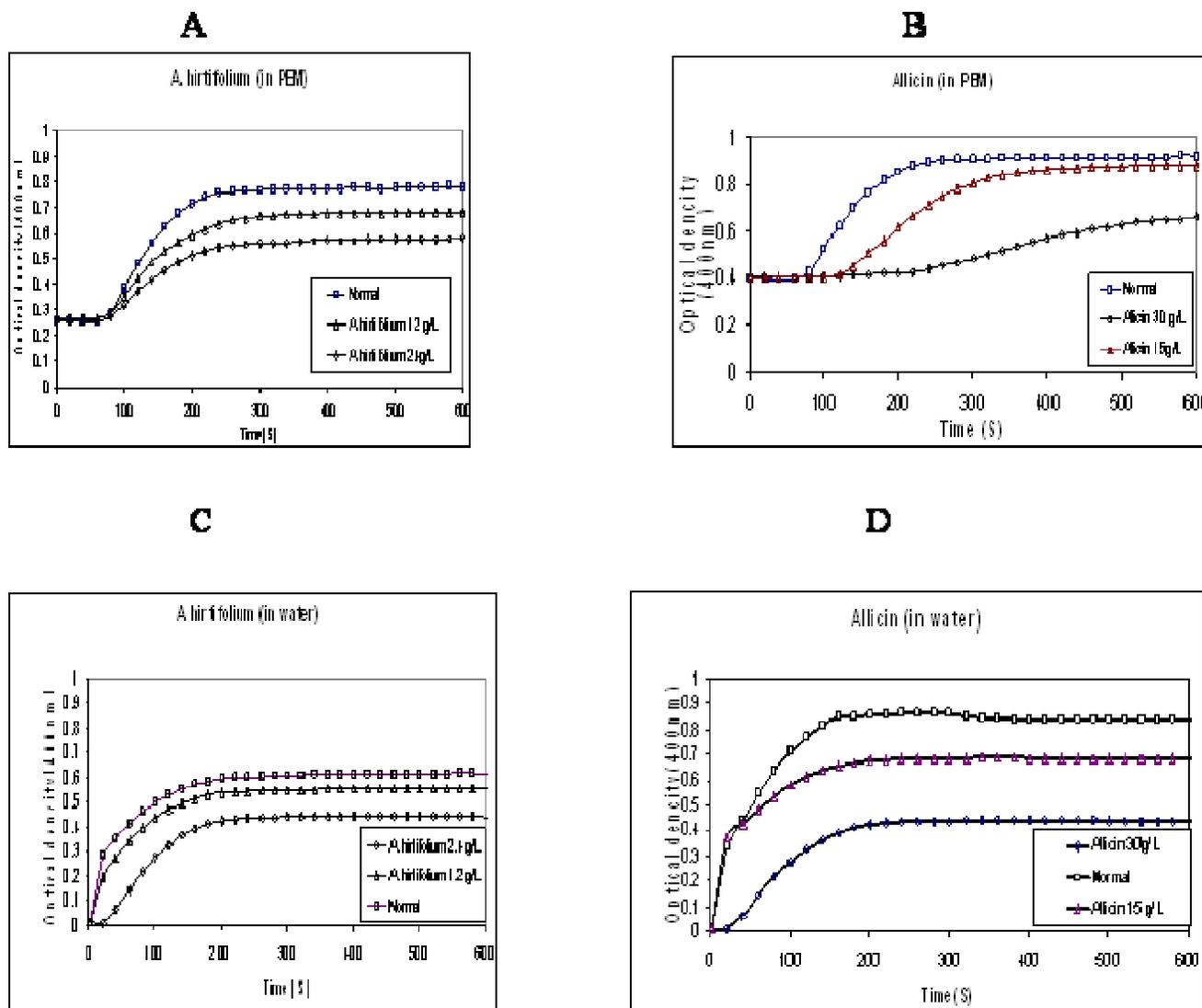


Figure 3. (A) Effect of *A. hirtifolium* extract on assembly and disassembly of MT. (B) Effect of Alliin on assembly and disassembly of MT. (C) Effect of *A. hirtifolium* extract on assembly and disassembly of MT. (D) Effect of alliin on assembly and disassembly of MT (In turbidity tests: chloroform from samples was removed and the solid mass solved in PEM).

dependent reduction of the microtubule network not only in length but also in shape. At higher concentration of this plant (2.4 g/L), aggregates of tubulin could be observed (Figure 4A and 4B). Interestingly, tubulin assemblies could be reversibly dissociated by decreasing the temperature to 0°C.

DISCUSSION

While Hela and MCF-7 cells were sensitive to *A. hirtifolium*, the cell survival rate was almost unchanged in L929 cell line. It means that *A. hirtifolium* did not affect the normal L-929 cell; it only decreased cancer cells population (Figure 1). Several pieces of evidences sug-

gested that allyl sulfides, found in processed Alliaceae family, possess anticancer properties as shown by their ability to suppress tumor proliferation *in vitro* (Milner, 1996; Singh et al., 1996). Concentration and duration of the exposure to *A. hirtifolium* increased the anti-proliferative effects. Anti-cancer effect was greater for chloroform-soluble than for water-soluble *A. hirtifolium*.

It was reported that 65% protein and 35% microtubule associated proteins (MAPs) in MTP structure (Nickerson and Wells, 1984; Gaskin et al., 1974); however, SDS-PAGE in our study showed that the microtubule protein preparations contained 60% tubulin.

The present study showed the *A. hirtifolium* and alliin affect on MT. Our observation indicated different structures other than normal structure of MT polymers in pre-

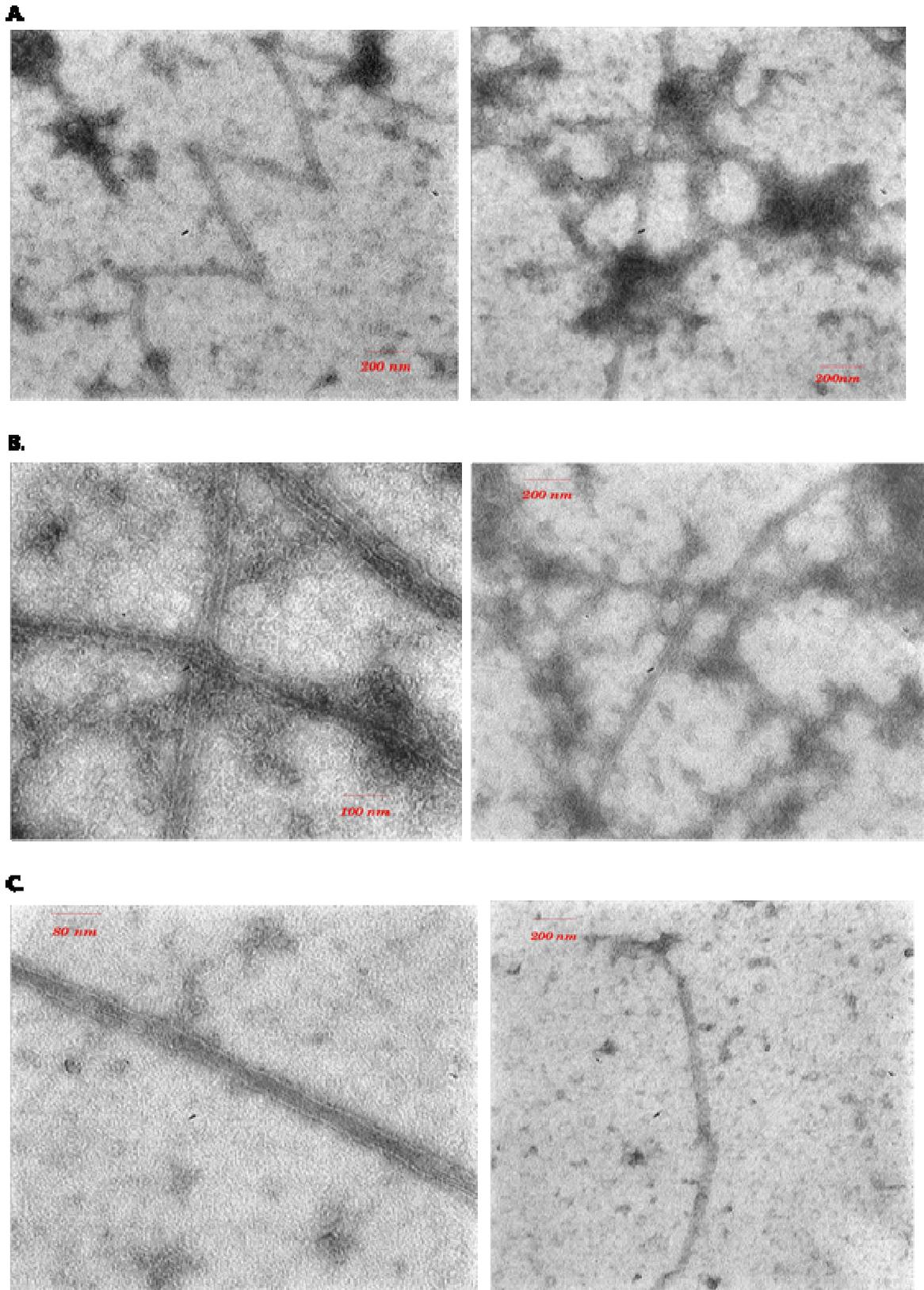


Figure 4. Demonstration of MT disassembly by TEM. Microtubules were treated with (A) 2.4 g/L *A. hirtifolium* or (B) 0.030 g/L of allacin or (C) without them (On the Left: Sample with water as a solvent; on the right: Sample with PEM as a solvent).

sence of the extracts. We may connect *in vitro* inhibitory character of *A. hirtifolium* on tubulin to the evidence we found in cell culture.

Conclusion

Our data clearly indicate that a series of antimetabolic agents would be synthesized based on purified components of *A. hirtifolium* extracts which would not affect normal cells but stop the tumor cells in cancer patients. Due to significant effect of *A. hirtifolium* on microtubule and cancer cell lines, efforts are going on in our laboratory to investigate primarily the phytochemical composition of *A. hirtifolium*. The ability of *A. hirtifolium* to preferentially suppress the growth of neoplastic over non-neoplastic cells provides interesting possibilities for the development of new anticancer strategies in humans. Elucidation of the mechanism involved in *A. hirtifolium* cytotoxicity *in vitro* and in tumor growth inhibition in animals is an important step for a better understanding of the antitumor activity of allium compounds.

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