EFFECT OF INGANEN ANTICANCER PROPERTIES ON MICROTOBULE ORGANIZATION

ALI KHALEGHIAN[a, b], GHOLAM HOSSEIN RIAZI[b], MAHMOUD GHAFARI[b], MARZIEH REZAIE[a], AKIRA TAKAHASHI[c], YUTAKA NAKAYA[c] AND HOSSAIN NAZARI[a,c]*

[a]*Department of Biochemistry and Hematology, Semnan University of medical science, Semnan, Iran*
[b]*Department of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*
[c]*Department of Nutrition and Metabolism, University of Tokushima, Tokushima, Japan*

ABSTRACT

Euphorbia tirucalli (Euphorbiaceae family) an environmental risk factor for Burkitt's lymphoma also has pharmacological activities. In the northeast of region in Brazil its latex is used as an antimicrobial, antiparasitic in the treatment of coughs, rheumatism, cancer and other disease as folk treatment. The prevalent constituents of this plant latex are diterpenes from the Inganen types (ingenol esters) as well as the tigliane (phorbol esters). Scientifically, there is not any data till now about anticancer effects of the Euphorbia tirucalli Linn. , since the Ingenol esters have already presented tumor-promoting ability. Microtubules (MTs), and cytoskeletal proteins are essential in eukaryotic cells for a variety of functions, such as cellular transport, cell motility and mitosis. Single Inganen in cytoplasm can interact with these proteins and affect on their crucial functions. In this study, we showed the effects of Inganen on MT organization using ultraviolet spectrophotometer and fluorometry. The fluorescent spectroscopy showed a significant tubulin conformational change at the presence of Inganen which decrease polymerization of tubulin as well as the ultraviolet spectroscopy results. The aim of this study is to find the potential function of Inganen for treatment of cancer in cells and human organs.

Keywords: Inganen, microtobule, anticancer, polymerization.

INTRODUCTION

Euphorbia tirucalli L. (Euphorbiaceae family), is originally from Africa and America, was brought to tropical countries, including Brazil and is found in the northeast of region located in the state of Amazonas and in some coastal of areas of Iran. Euphorbia tirucalli as an important environmental risk factor for African Burkitt's lymphoma has been suggested. There is a coincidence between endemic Burkitt's lymphoma (Bl) and human exposure to *Euphorbia tirucalli,* particularly observed in the lymphoma belt in Africa (MacNeil, 2003; Van den Bosch, 1993; Imai, 1994).

Euphorbia tirucalli latex seems to reduce the specific cellular immunity associated with the virus Epstein-Barr injection by activating the virus lytic cycle (Jurberg, 1985; Tiwari, 2003). The bark/latex of *Euphorbia tirucalli* presents pharmacological activities as an antibacterial, molluscicide, antiherpetic (Betancur *et al*., 2002) and anti-mutagenic (Rezende *et al*., 2004). It also shows co-carcinogenic and anticarcinogenic activities (Gscwhenot, 1969; Hecker, 1968). Inganen is a part of the active ingredients which found in Kansui, a traditional Chinese herbal remedy. It is known as a protein kinase C (PKC) activator as well as phorbol class, has a three-fused-ring structural core, with a seven transmembered ring in the center. Although Ingenol derivatives are potent inhibitors of acute HIV-1 infection, as well as potent activators of HIV-1 transcription in chronically infected cells (Fujiwara *et al*., 1996).

Valadares demonstrated a myelomodulating activity of this species and the inhibition of the ascitic tumor in mice (Valadares *et al*., 2006). In the northeast of region in Brazil, the latex of *Euphorbia tirucalli* is used as a folk medicine against syphilis. As an antimicrobial; a laxative agent to control intestinal parasites to treat asthma, cough, earache, rheumatism, verrucae, cancer, epithelioma, sarcoma and skin tumors (Costa, 2002; Almeida, 1993). *Euphorbia tirucalli* contains a large quantity of terpenes and sterols among its constituent and the following substances which have been isolated; alcohol eufol, alfa-euforbol and taraxasterol e tirucallol (Imai, 1994; Costa, 2002). The main constituents of the latex of Euphorbia tirucalli's stem are water (53.8–79.9%), tigliane (phorbol esters) and Inganen (ingenol esters). Fresh latex contains terpenic alcohol, taraxasterol and tirucallol (Cataluna *et al*, 1999). Some of the studies show the toxicity activity of Euphorbia tirucalli on the uterus of pregnant rats (Osore *et al.*, 1984). There is no any information about its acute, chronic or reproductive toxicity. Considering the therapeutically potential of the latex of *Euphorbia tirucalli*, this study was developed in the Antibiotic Department of Pernambuco Federal University (UFPE) to investigate its general and reproductive toxicology (Yasukawa *et al*, 2000).

Tubulin is an important protein with a variety of functions; signal transduction, neurotransmitters trans-

**Corresponding author:* e-mail: hossen253@yahoo.co.uk

porting in nerve cells for cell division and cell shape maintenance in other organs (Robinson, 1995; Desai, 1997). Inganen can enter and persist in cells, where their concentration increases and they interact with cytoplasmic proteins (Itokawa, 1989; Kupchan, 1976). The aim of this study is to survey the effect of Inganen on microtubule dynamic which polymerization and depolymerization of them can be referred to its anticancer property.

MATERIALS AND METHODS

Plant material

Euphorbia tirucalli L. was collected on the campus of IBB, at Tehran University, identified by Dr. Ghafari and Ebrahimi, it was placed in the IBB Herbarium of the Tehran University. An amount of 0.05mL of latex was obtained from Euphorbia tirucalli's shaft, which was diluted in 100mL of distilled water, according to folkloric anti-inflammatory use. The final solution had a latex concentration of 0.050%.

Ultrafine Inganen was a gift from Dr. Moradi (biochemistry Laboratory from Pastur Institute, Tehran, Iran). The Inganen was resolved in piperazine-1,4-bis (2-Ethanesulfonic acid) (PIPES) buffer (Merck, Darmstadt, Germany) to obtain an 4-mg/ml final concentration. Before use, KOH was added to adjust the pH of the colloidal solution to 6.9. Particles were then sonicated with a Bandelin sonicator (Bandelin, Berlin, Germany) for 3 mins and immediately added to the protein solution. EGTA, guanosine-5′-triphosphate (GTP), ATP, glycerol and MgSO4 were prepared from Sigma (Dorset, England). Phosphocellulose P11 was obtained from Whatman (Florham Park, USA). All other chemicals; NaCl, KOH and ANS (Merck) were of analytical grade and used without further purification. All solutions were also prepared with double distilled water and were kept at 4ºC before use.

Purification of Tubulin

Before homogenization in PEM buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 2 mM MgSO4) and 1 mM MgATP, followed by two cycles of temperature-dependent assembly and disassembly. MT proteins was prepared from sheep brains (Valiron, 2001; Downing, 2000). PMG (100 mM PIPES, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA and 3.4 M glycerol) was used as polymerization buffer. Microtubule-associated proteins and free tubulin were confirmed by chromatography on phosphocellulose P11 with a slight modification of the method used by Weingarten *et al* (Weingarten *et al*, 1974). Eluted tubulin fractions were stored at -70ºC for further study. The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, USA) with bovine serum albumin as standard (Marshal *et al*, 1993).

UV Spectroscopy

Turbidimetric assay of MT and tubulin were carried out by incubating the protein in PIPES buffer (with the final concentration of 2 mg/ml) in cuvettes at 37ºC in a thermostatically controlled UV spectrophotometer (Varian, Melbourne, Australia). Turbidity change was measured at 350 nm. To examine the effect of Inganen on MT organization, the MT and tubulin proteins were pre-incubated with NPs at 4ºC for 30 min. Polymerization of tubulin was initiated with adding one mM GTP in control group but was inhibited in the presence of Inganen. The mixture was heated to 37ºC.

Fluorescence spectroscopy

All fluorescence experiments were carried out by using a Varian eclipse spectrofluorometer equipped with a computer to add and subtract spectra. Denaturation of tubulin was measured as tryptophan emission after excitation at 295 nm (Bhattacharya *et al*, 1996). Interactions in the presence of increasing concentrations of Inganen were carried out at 25ºC. To test conformational changes, 8-anilino-1-naphthalenesulfonic acid (ANS) was used to detect whether the Inganen - treated tubulin had an exposed hydrophobic surface area (Sarkar *et al*, 1995). The excitation wavelength was 380 nm, and emission was monitored between 450-550 nm. All measurements used 2 µM tubulin, and all experiments were carried out at 25ºC.

RESULTS

Inhibition of MT Polymerization by Inganen

The effect of Inganen on the polymerization of tubulin was measured as shown in fig. 2. MT assembly was clearly inhibited by different concentrations of Inganen in 2 mg/ml MT solution compared with control groups. Specifically, Inganen inhibited both the rate and the extent of MT assembling, and it had influence on the rate of MT nucleation by which delaying the formation of central nucleation in increasing the time.

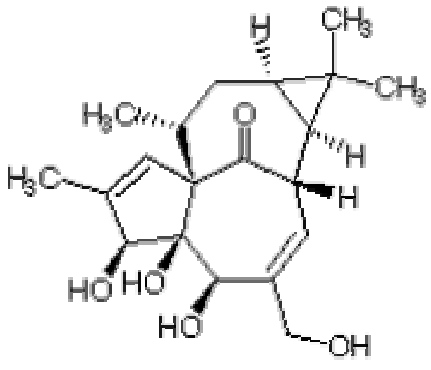

Fig. 1: Structure of INGANEN

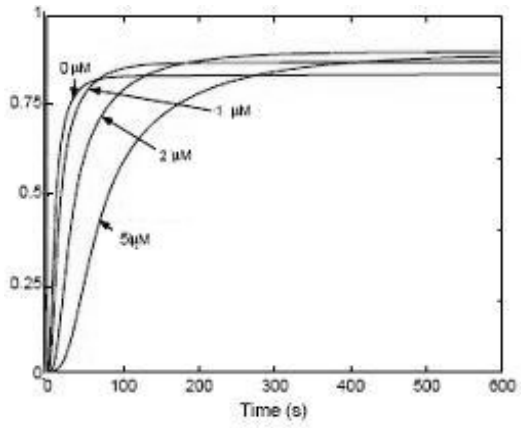

Fig. 2: Effect of Inganen (INGENOL) on microtubule (MT) assembly.

MT proteins (2 mg/ml) were preincubated at 4ºC in piperazine-1, 4-bis (2-ethanesulfonic acid) buffer with different concentration of INGANEN. Polymerization was initiated by adding 1 mM guanosine-5′-triphosphate. The turbidity was monitored at 350 nm at 37ºC. Representative images were taken at selected concentration points (0, 1, 2 and 5 µM/ml Inganen). Similar results were observed in four additional experiments.

MT repolymerization assay

Repolymerization assays showed that the assembled MTs were disassembled by cooling to 4ºC; re-warming the solution to 37 ºC induced assembling of tubulin as a control. Depolymerized MTs organized after 30 min incubation at 4ºC at the presence of all ingredients (fig. 2). Fig. 3 shows the inhibitory effect of Inganen on MT dynamics. It was observed a decrease in turbidity when the reaction was reached a new equilibrium in the presence of Inganen as well as equilibrium at zero time in the absence of Inganen (fig. 3).

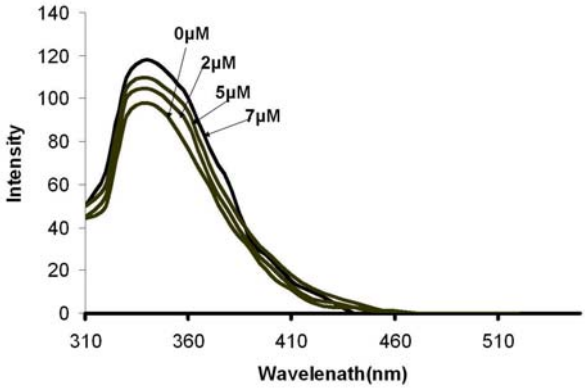

Fig. 3: Effect of Inganen on Emission Spectra (Excited at 280 nm).

The solution conditions were 1 M piperazine-1, 4-bis (2-ethanesulfonic acid), pH 6.9, containing 1 mM EGTA and 2 mM MgSO4. The excitation and emission band passes were 5 nm. Data show emission spectra are decreased at the presence of Inganen. Representative fluorescence patterns were taken at selected concentration points (0, 2, 5 and 7 µM/ml Inganen). Results were obtained from five separate experiments.

Intrinsic fluorescence spectra

To obtain structural information at the tertiary level, intrinsic (tryptophan) fluorescence spectrum of tubulin in the presence of different concentrations of Inganen was measured. Fluorescence analysis indicated that the interaction of Inganen with tubulin resulted in changing fluorescence quenching of surface-exposed tryptophans in tubulin. Fig. 3 shows that the fluorescence intensity was increased with increasing of Inganen.

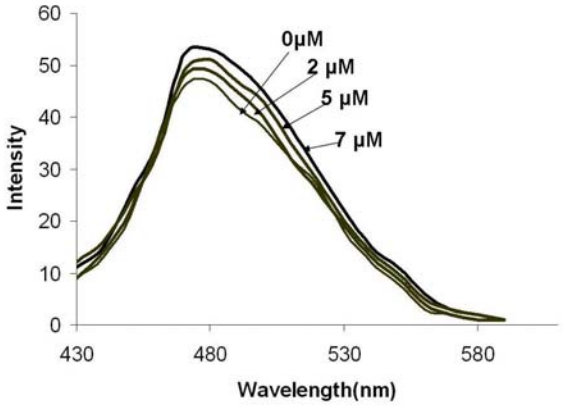

Fig. 4: Effect of Inganen on Emission Spectra (excited at 380 nm).

Tubulin was mixed with 0 µg/ml Inganen (curve 1), 2 µg/ml Inganen (curve 2), 5 µg/ml Inganen (curve 3) and 7 µg/ml Inganen (curve 4) for 10 min at 4 °C. The 8-anilino- 1-naphthalenesulfonic acid (7 µM final concentration) was added and after 7 minutes fluorescence was measured. The solution conditions were 1 M piperazine-1, 4-bis (2- Ethanesulfonic acid), pH 6.9, containing 1 mM EGTA and 2 mM MgSO4. The excitation and emission band passes were 5 nm. Tubulin-bis-ANS complexes decreased fluorescence spectra by Inganen. Similar results were observed in at least four additional experiments. (a.u. arbitrary unit).

Increasing of Tubulin-bis-ANS Fluorescence by Inganen

There are several low affinity sites and one high affinity site for the polar molecule ANS in tubulin. Tubulin-ANS complex has a strong fluorescence and is extremely environmentally sensitive. Therefore, it is a useful tool for probing the conformational state of the tubulin dimer. Tubulin-ANS fluorescence has been used to determine the nature of interactions. Tubulin (2 µM) was incubated at the presence of various concentrations of Inganen for 10 mins at 4ºC. ANS (50 µM final concentration) was added to the tubulin-Inganen solution and incubated again for 7 mins. Data showed that Inganen causes increase in tubulin-ANS fluorescence with a concentration-dependent manner (fig. 4). Furthermore, incubation of tubulin with ANS before adding Inganen generated similar results like control (data not shown). Next we do experiment for

confirm inhibitory effect of Inganen on tubulin organization. Cholshisin as well as Inganen caused tubulin depolimerization and increased the intensity of dimmer tubulin in comparison with taxol which causes tubulin polymerization (fig. 5).

Tubulin was mixed with 0 µg/ml Inganen (curve 1), 2 µg/ml Inganen (curve 2), 5 µg/ml Inganen (curve 3) and 7 µg/ml Inganen (curve 4) for 10 min at 4°C. The 8-anilino-1-naphthalenesulfonic acid (7 µM final concentration) was added and after 7 minutes fluorescence was measured. The solution conditions were 1 M piperazine-1, 4-bis (2- Ethanesulfonic acid), pH 6.9, containing 1 mM EGTA and 2 mM $MgSO_4$. The excitation and emission band passes were 5 nm. Tubulin-bis-ANS complexes decreased fluorescence spectra by Inganen. Similar results were observed in at least four additional experiments. (a.u. arbitrary unit).

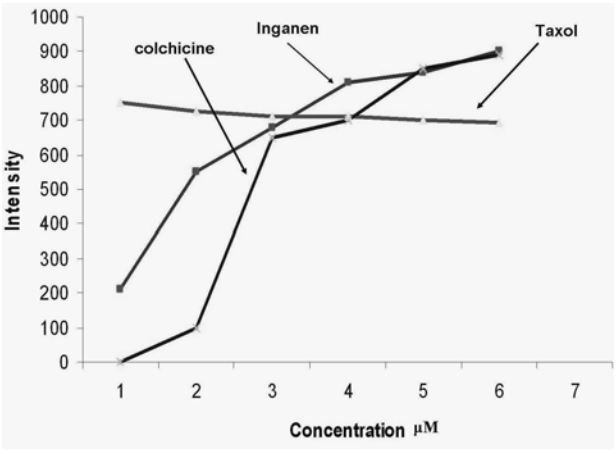

Fig. 5: Inganen increased intensity of tubulin.
Before exposure of medium included tubulin to Colchisin and Inganen, we do experiments for MTs formation. Then, add different concentration of inhibitory reagent on MTs. Polymerization of tubulin is stopped and their depolymerization is increased at the presence of Inganen and Colchisin in comparison with Taxol as a control reagent.

DISCUSSION

Tubulin is an important protein with a variety of functions; signal transduction, neurotransmitters transporting in nerve cells for cell division and cell shape maintenance in other organs (Desai *et al*, 1997). Inganen can enter and persist in cells, where their concentration is increased and they interact with cytoplasm proteins. Some studies have shown an interaction between Inganen and some proteins, such as human plasma fibrinogen. However, there are few reports about the cytotoxic and genotoxic effects of Inganen (Falsone, 1982; Itokawa, 1989). Studies have also shown that some neurons exposed to Inganen initiate a cellular process that can ultimately lead to cell death, which known, Inganen induces apoptosis (Noack *et al*, 1980), but the toxic effect of Inganen on MT organization has not yet been elucidated. In this study, we showed that Inganen disestablish tubulin polymerization and formation of MT nucleation. The disorganization of tubulin depends on the concentration of Inganen (fig. 2), which our data show that destabilizing and depolymerization of MTs structure were increased at the presence of 15 µm/ml Inganen as well as colchicine treatment in comparison with control sample or those MTs treated to Taxol which causes the increase and stabilized. Suggesting that Inganen is affected both soluble tubulin and tubulin in MT structure, tubulin conformational change is led to decrease tubulin stabilizing ability. We identified changes in protein conformation which involved in protein functional alteration by using intrinsic fluorescent spectroscopy. Inganen modified the polarity in the vicinity of tryptophan residues causes to observe fluorescence quenching and the maximum blue shift of the emission wavelength (fig. 2). Fluorescence experiments with ANS demonstrated that Inganen induces some increase in fluorescence emission (fig. 3). The tubulin- ANS complexes more increase in fluorescence, it may result from contracting some of the intrinstic tubulin hydrophobic pockets with ANS. Alternatively, binding may induce a conformational change in tubulin leading to increased ANS binding which causes to increase tubulin-ANS fluorescence (Bhattacharya *et al*, 1996). These results indicated that Inganen induces conformational changes in tubulin that cause changes in tryptophan position, moving them towards guanosine-5'-diphosphate (GTP) binding sites in protein structures (Solomaha *et al*, 2005). GTP has the main role in fluorescent quenching ability. Treatment MT with ANS causes hydrophobic pockets to be reached in protein structure which causes decreeing tubulin-ANS fluorescence. Both GTP and its binding site in tubulin have a crucial role in tubulin organization. GTP should be hydrolyzed to GDP when tubulin soluble polymerized to MT (Solomaha, 2005; O'Brien, 1980). Turbidimetric assays demonstrated that conformational changes in protein suppressed tubulin polymerization and stabilization (Downing, 2000; Weingarten, 1974).

In conclusion, our data has shown that Inganen as well as colchicine have an inhibitory effect on tubulin polymerization, instable of MT nucleation and formation. Tubulin organization decreased in the presence of Inganen comparison with Taxol which causes more instability on cytoskeleton structure. Low stability of microtubules inhibits cell cycle mechanisms, so cells are stopped in the end of G phase. On the other hand, there is some chemical and herbal drugs causes arrangement, polymerization of tubulin and microtubule stability which inhibits cell growth in cancer situation (Ito *et al*, 1981). Ultimately, long-term exposure to Ingenol esters (Inganen) can be dangerous, and needs further research to

detect the suitable concentration clinically and side effects. However Inganen are widely used in various commercial products, there is insufficient knowledge about their side effects. Also this data help to do understand the mechanisms of anticancer effect of Euphorbia tirucalli.

REFERENCES

Almeida ER (1993). Plantas medicinais brasileiras. Conhecimentos populares e científicos, Hermus Editora., *Rio de Janeiro*, 70-71.

Betancur-Galvis LA, Morales GE, Forero JE and Roldan J (2002). Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the Euphorbia genus. *Memórias do Instituto Oswaldo Cruz*., **97**: 541-546.

Bhattacharya A, Bhattacharyya B and Roy S (1996). Fluorescence energy transfer measurement of distances between ligand binding sites of tubulin and its implication for protein-protein interaction. *Protein. Sci*., **5**: 2029-2036.

Cataluna P and Taxa SMK (1999). The traditional use of the latex from Euphorbia tirucalli Linnaeus (Euphorbiaceae) in the treatment of cancer in South Brazil. *Acta Horticulturae.,* **501**: 289-296.

Costa AF (2002). Farmacognosia, Fundação Calouste Gulbenkian. *J. Lisboa,*788-790.

Desai A and Mitchison TJ (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell .Dev Biol*., **13**: 83-117.

Downing KH (2000). Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. *Annu. Rev. Cell. Dev. Biol.,* **16**: 89-111.

Falsone G, Crea AE and Noack EA (1982). Constituents of Euphorbiaceae, 7. 20-Deoxyingenol Monoesters and ingenol diesters from Euphorbia biglandulosa Desf. *Arch. Pharm. (Weinheim)*, **12**: 1026-1032.

Fujiwara M, Ijichi K, Tokuhisa K, Katsuura K, Shigeta S, Konno K, Wang GYS and Uemura D (1996). Mechanism of selective inhibition of human immunodeficiency virus by ingenol triacetate. *Antimicrob Agents Chemother*., **40**: 271-273.

Gscwhenot M and Hecker E (1969). Tumor promoting compounds from E. triangular. *Tetrahedron Letters*, **40**: 3509-3512.

Hecker E (1968). Co-carcinogenic principles from seed oil of Croton tiglium and other Euphorbiaceae. *Cancer Research*, **28**: 2338-2349.

Imai S, Sugiura M, Mizuno F, Ohigashi H, Koshimizu K, Chiba S and Osato T (1994). African Burkitt's lymphoma: A plant, Euphorbia tirucalli, reduces Epstein-Barr virus-specific cellular immunity. *Anti-cancer Research*, **14**: 933-936.

Ito Y, Kawanishi M, Harayama T and Takabayashi S (1981). Combined effect of the extracts from Croton tiglium, Euphorbia lathyris or Euphobia tirucalli and n-butyrate on Epstein – Barr virus expression in human lymphoblastoid P3HR-1 and Raji cells. *Cancer Lett*., **12**: 175-180.

Itokawa H, Ichihara Y, Watanabe K and Takeya K (1989). An antitumor principle from Euphorbia lathyris. *Planta Med*., **55**: 271-272.

Itokawa H, Ichihara Y, Watanabe K and Takeya K (1989). Anantitumor principle from Euphorbia lathyris. *Planta Med.*, **55**: 271-72.

Jurberg P, Cabral Neto JB and Schall VT (1985). Molluscicide activity of the 'avelós' plant (Euphorbia tirucalli, L.) on Biomphalaria glabrata, the mollusk vector of schistosomiasis. *Memórias do Instituto Oswaldo Cruz*., **80**: 423-427.

Kupchan SM, Uchida I, Branfman AR, Dailey RGJr and Yu Fei B (1976). Antileukemic principles isolated from Euphorbiaceae plants. *Science*, **191**: 571-572.

MacNeil A, Sumba OP, Lutzke ML, Moormann A and Rochford R (2003). Activation of the Epstein-Barr virus lytic cycle by the latex of the plant Euphorbia tirucalli. *British Journal of Cancer,* **88**: 1566-1569.

Marshal T and William KM (1993). Bradford protein assay and the transition from an insoluble to soluble dye complex: Effect of sodium dodecyl sulphate and other additives. *Biochem. Biophys. Methods*, **26**: 237-240.

Noack EA, Crea AE and Falsone G (1980). Inhibition of mitochondrial oxidative phosporylation by 4-deoxyphorbol triester, a poisonous constituent of the latex of Euphorbia biglandulosa Desf. *Toxicon*., **18**: 165-174.

O'Brien TG, Saladik D and Diamond L (1980). Metabolism of tumor-promoting phorbol diesters by cells in culture. *Proc. Am. Assoc. Cancer Res*., **21**: 71.

Osore H (1984). Oxytoxic properties of the latex of *Euphorbia tirucalli* (Euphorbiaceae) on the gravid rat uterus. *Indian Journal of Pharmacology*, **16**: 241-242.

Rezende JR, Rodrigues SB, Jabor IAS, Pamphile JA and Rocha CLMSC (2004). Efeito antimutagênico do látex de Euphorbia tirucalli no sistema metionina em Aspergillus nidulans. *J. Acta Scientiarum Biological Sciences,* **26**: 481-484.

Robinson J and Vandre D (1995). Stimulus-dependent alterations in macrophage microtubules: increased tubulin polymerization and detyrosination. *Cell Sci.*, **108**: 645-655.

Sarkar N, Mukhopadhyay K, Parrack PK and Bhattacharyya B (1995). Aging of tubulin monomers using 5,5'-bis(8-anilino-1-naphthalenesulfonate) as a probe. *Biochemistry*, **34**: 13367-13373.

Solomaha E and Palfrey HC (2005). Conformational changes in dynamin on GTP binding and oligomerization reported by intrinsic and extrinsic fluorescence. *Biochem.*, **391**: 601-611.

Tiwari S, Singh P and Singh A (2003). Toxicity of Euphorbia tirucalli plant against freshwater target and

non-target organisms. *Pakistan Journal of Biological Sciences*, **6**: 1423-1429.

Valadares MC, Carrucha SG, Accorsi W and Qquiroz MLS (2006). *Euphorbia tirucalli* L. modulates myelopoiesis and enhances the resistance of tumour-bearing mice. *International Immonopharmacology*, **6**: 294-299.

Valiron O, Caudron N and Job D (2001). Microtubule dynamics. *Cell. Mol. Life. Sci.,* **58**: 2069-2084.

Van den Bosch C, Griffin BE, Kazembe P, Dziweni C and Kadzamira L (1993). Are plant factors a missing link in the evolution of endemic Burkitt's lymphoma? *British Journal of Cancer*, **68**: 1232-1235.

Weingarten MD, Suter MM, Littman DR and Kirschner MW (1974). Properties of the depolymerization products of microtubules from mammalian brain. *Biochemistry*, **27**: 5529-5537.

Yasukawa K, Akihisa T, Yoshida ZY and Takido M (2000). Inhibitory effect of euphol, a triterpene alcohol from the roots of Euphorbia kansui, on tumour promotion by 12-O tetradecanoylphorbol-13-acetate in two-stage carcinogenesis inmouse skin. *Pharm. Pharmacol*., **52**: 119-124.