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**THE CYTOTOXIC AND APOPTOTIC EFFECTS OF 1'S-1'-  
ACETOXYCHAVICOL ACETATE FROM *ALPINIA CONCHIGERA* GRIFF., ON  
VARIOUS HUMAN TUMOUR CELL LINES**

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### INTRODUCTION

Apoptosis, also commonly known as programmed cell death, describes the genetically regulated and orchestrated breakdown of a cell. It is normally characterized by membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA followed by the rapid engulfment of the cellular corpse by macrophages. This process occurs especially during embryogenesis, in tumour regression and in the control of an immune response. Some types of cancer are characterized by defects in apoptosis and apoptotic regulatory pathways. Today, cancer is the second leading cause of death worldwide behind cardiovascular related diseases and fourth in Malaysia (Chin, 2002).

Traditional medicine from various plant types containing active natural compounds such as chalcones (Hsu *et al*, 2006), xanthoangelol (Tabata *et al*, 2005) and licochalcone-A (Fu *et al*, 2004) have been used as an alternative treatment for cancer patients in many parts of the world including Asia (Itharat *et al*, 2004). More than 70% of all anti-cancer drugs approved by the FDA within the past 20 years have been based on natural products (Newman *et al*, 2003). One traditional Asian natural compound, 1'S-1'-acetoxychavicol acetate (ACA), derived from the rhizomes of a subtropical ginger, *Languas galanga* (Zingiberaceae), has been shown to exhibit antitumor properties against a wide variety of cancers (Itharat *et al*, 2004). The pro-apoptotic effects of ACA from the Thai ginger isolate, has previously been documented in human breast carcinoma cells (Campbell *et al*, 2007), human T cell lymphoma (Ichikawa *et al*, 2005) and in inhibition of tumour-promoter-induced Epstein-Barr virus (Kondo *et al*, 1993).

In this study, the natural compound, 1'S-1'-acetoxychavicol acetate (ACA), extracted from rhizomes of the Malaysian ethno-medicinal plant *Alpinia conchigera* Griff., was investigated for its potential as an anti-cancer drug.

### METHODOLOGY

#### **Materials and Compounds**

DMEM, RPMI-1640, FBS and antibiotics were purchased from Lonza Inc. (USA). MTT solution, RNase A, Annexin V-FITC apoptosis detection kit, propidium iodide (PI) and Suicide Track™ DNA Ladder Isolation Kit were purchased from EMD Chemicals Inc. (Calbiochem, San Diego, CA, USA).

### **Cell Culture**

Human breast adenocarcinoma (MCF7), human oral squamous carcinoma (HSC-2 and HSC-4) were obtained from Cancer Research Initiative Foundation (CARIF, Malaysia), while human hepatocyte carcinoma (HepG2) and human epidermoid cervical carcinoma (CaSki) cells were obtained from University Malaya Medical Center (UMMC, Malaysia). All cells were cultured in DMEM except for MCF-7 cells which were cultured in RPMI 1640 and both supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin.

### **Cell Viability Assay**

Cell viability was determined by the MTT assay which measures mitochondrial activity of viable cells. ACA was dissolved in DMSO to a final working concentration of 5.0mM. Briefly, cells were treated with ACA at concentrations of 0.0, 5.0, 10.0, 20.0, 40.0 and 80.0µM for 0, 1, 3, 6, 12, 18 and 24hrs respectively. Final DMSO concentration in each well did not exceed 0.5% (v/v) to prevent solvent induced cytotoxicity. A microtiter plate reader (Tecan Sunrise<sup>®</sup>, Switzerland) was used to detect absorbance at a test wavelength of 570 nm, with a reference wavelength of 650 nm.

### **Cell Cycle Analysis**

Cell cycle analysis was performed using PI based staining methods. Briefly, ACA-treated and untreated cells were fixed in ice-cold 70% (v/v) ethanol and kept at -30°C. Staining of DNA content was conducted by adding PI (50µg/ml) and RNase A (10mg/ml). Fluorescence from a population of 10,000 cells was detected using a flow cytometer (BD FACSCalibur<sup>™</sup>, USA) at 488 nm wavelength.

### **Annexin-V Apoptosis Assay**

Detection of the various stages of apoptosis was conducted using the Annexin V FITC Apoptosis Detection Kit according to manufacturer's protocol. Briefly, media binding reagent containing FITC-conjugated Annexin-V anticoagulant (200µg/mg) was added to ACA treated and untreated cells. All tubes were then centrifuged and re-suspended in 1X cold binding buffer and PI (30µg/ml). Detection of signals from a 10,000 cell population was obtained using a flow cytometer (BD FACSCalibur<sup>™</sup>, USA) and analyzed using the BD CellQuest Pro Software (IVD) (Becton Dickenson, USA).

### **DNA Fragmentation Assay**

Total DNA was extracted from both untreated cells and cells treated with ACA for 0, 6 and 12hrs. Isolated DNA was analysed on a 1.0% agarose gel electrophoresis and stained with ethidium bromide. Fragmentation of DNA was observed under UV illumination and visualized using a gel documentation system (Alpha Inotech, USA).

### **Statistical Analysis**

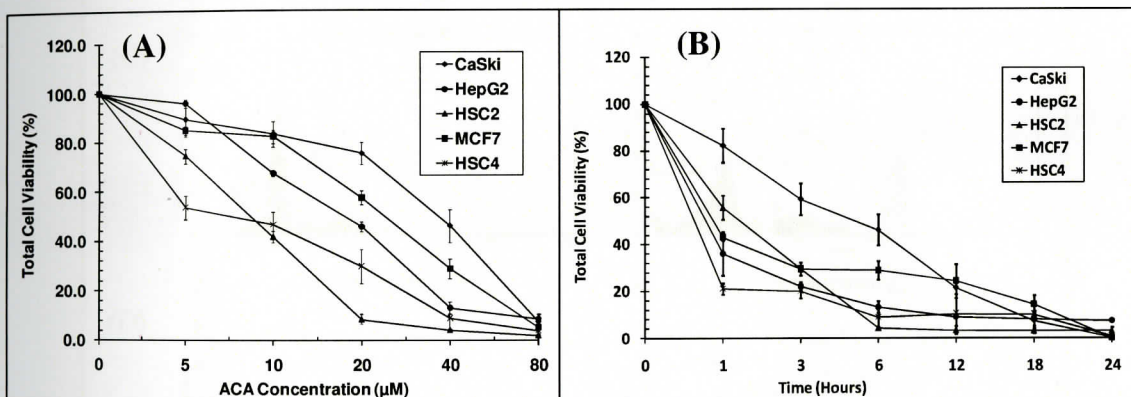
Results are expressed as the mean  $\pm$  standard error of the mean (SEM). All data were performed in triplicates and analyzed using one-way ANOVA, differences were considered significant at  $p \leq 0.05$ .



## RESULTS AND DISCUSSION

### *ACA induces dose and time dependant cytotoxicity in tumour cells*

To determine if cytotoxicity was dose and time dependant, the MTT cell viability assay was conducted. Results indicated that cells treated with ACA induced cytotoxicity in a dose dependant manner. Highest levels of cytotoxicity were observed in oral squamous carcinoma cells (HSC-4 and HSC-2) with  $IC_{50}$  values of  $7.0\mu M$  and  $8.0\mu M$  respectively (Fig. 1A). At  $80.0\mu M$  ACA concentration, 80% to 100% of all tumour cells had died after 12hrs exposure to ACA (Fig. 1A). MTT assay data in Fig. 1B demonstrated that the effects of ACA on tumour cell lines were also time dependant. At  $40.0\mu M$  ACA concentration, all cell lines were reportedly killed after 24hrs post-treatment time and the viability level of all cell lines were reduced below 40% after 12hrs of ACA treatment (Fig. 1B). The reduction of cellular viability was once again found to be greatest in HSC-4 and HSC-2 cells, indicating that ACA induced cell death most efficiently in oral squamous carcinoma cell lines (Fig. 1B). Viability of cells treated with DMSO without ACA were insignificantly affected ( $<10.0\%$ ) even at the highest DMSO concentrations used (data not shown), indicating that cytotoxicity was induced by ACA and not the DMSO solvent, which could be cytotoxic at high concentrations (Violante *et al.* 2002).

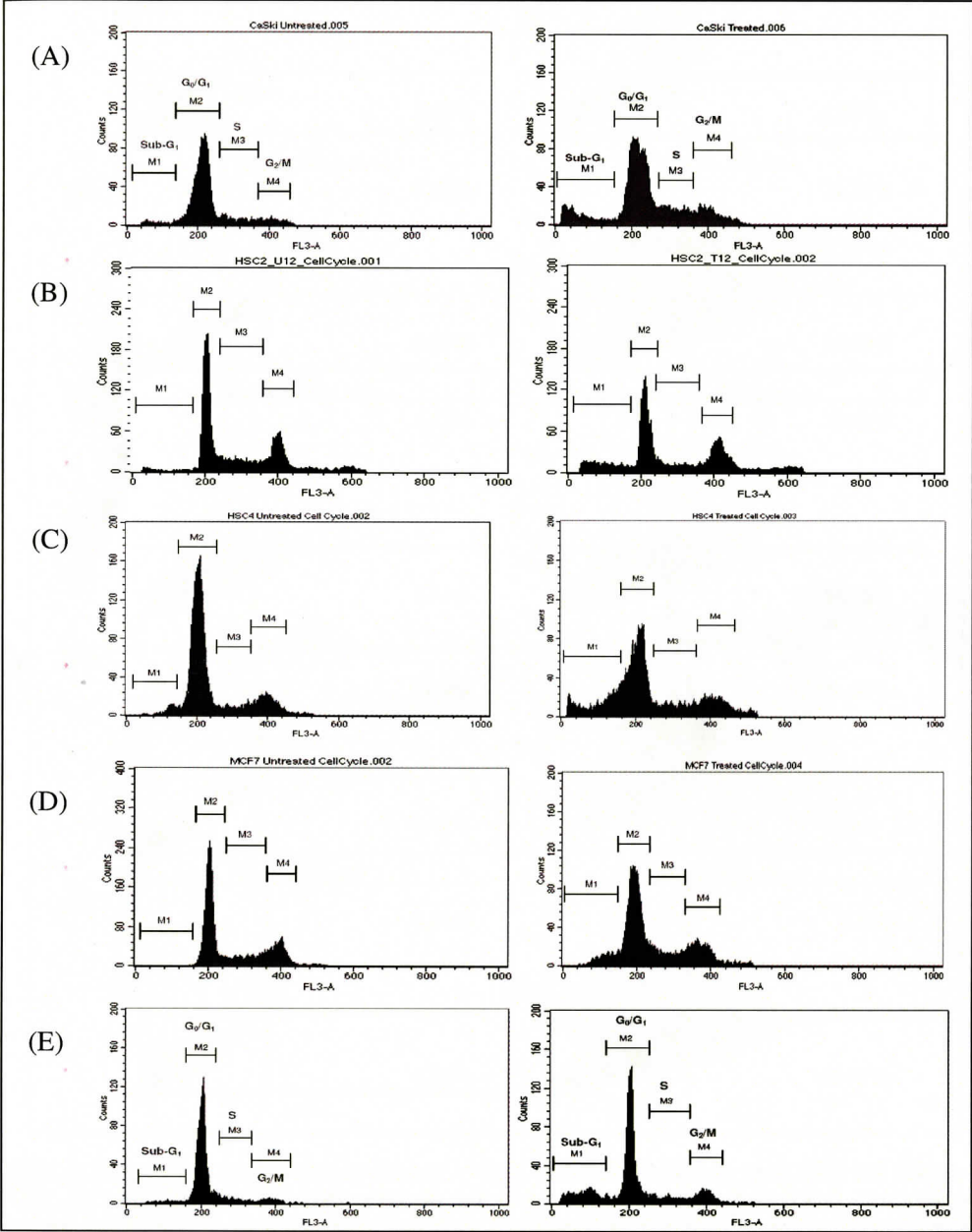


**Figure 1:** The cytotoxic effects of ACA on tumour cell lines were assessed using the MTT cell viability assay. (A) Comparison of total cell viability (%) between various tumor cell lines after treatment with ACA at different concentrations (0 –  $80\mu M$ ) at 12 hours post-treatment time. (B) Comparison of total cell viability (%) between various tumor cell lines after treatment with  $40.0\mu M$  ACA from 0 to 24 hours. All experiments are plotted as mean values ( $n = 3$ ).

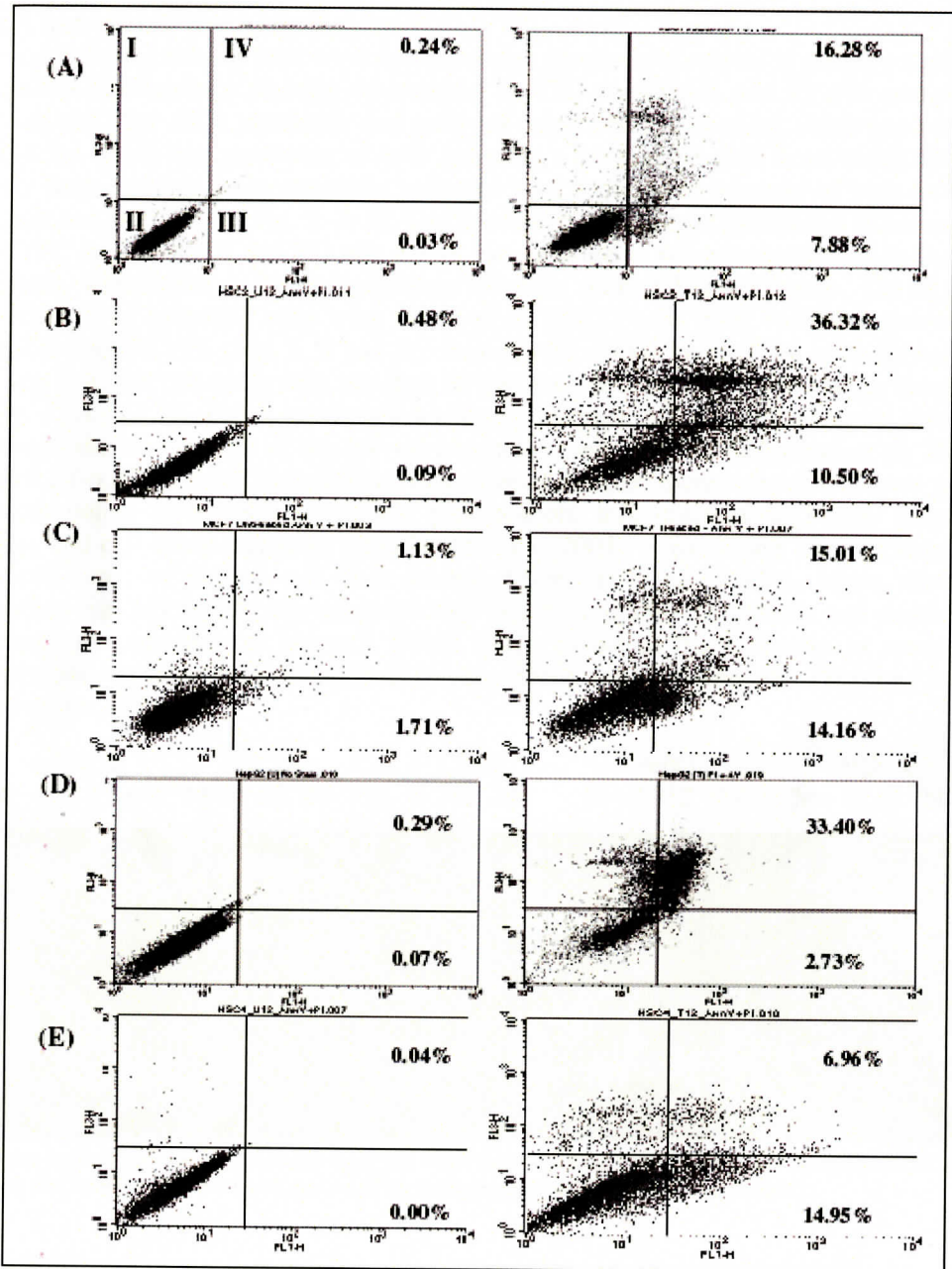
### *ACA induces potential cell cycle arrest at different stages within different cell lines*

To compare and quantify cell cycle distributions before and after ACA treatment, both attached and floating tumor cells were stained with PI and analyzed by flow cytometry. Cultivation with ACA significantly increased the population of CaSki and HepG2 cells in the  $G_2/M$  phase indicating a potential arrest during the  $G_2$  and mitotic phase (Fig. 2: A and E). HSC-2, HSC-4 and MCF7 cells on the other hand, displayed a reduction of cells within the  $G_0/G_1$  phase concurrent with an increase in S phase, possibly indicating cell cycle arrest during the DNA replication (Fig. 2: B, C and D). All tumour cell lines

demonstrated an indication of apoptosis by the appearance of a hypodiploid DNA peak within the sub-G<sub>1</sub> phase 12 hours after ACA treatment (Figure 2: A to E), which was a specific marker of apoptosis. These observations strongly suggest that ACA may have both anti-proliferative and apoptotic properties on tumour cells due to its ability to cause cell cycle arrest at various stages, followed by death via apoptosis.



**Figure 2:** Cell cycle distribution of tumor cell lines before (left panel) and after treatment with ACA for 12 hours (right panel). (A) CaSki; (B) HSC-2; (C) HSC-4; (D) MCF7; (E) HepG2. DNA content was stained with PI and 10,000 cells were analyzed using a flow cytometer.



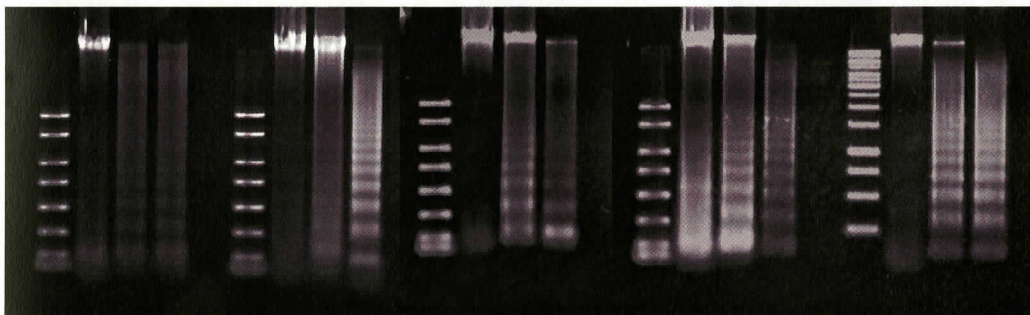
**Figure 3:** Detection of apoptosis using annexin V-FITC and PI dual staining. (A) CaSki; (B) HSC-2; (C) MCF7; (D) HepG2; (E) HSC-4. Untreated cells (left panel) and treated cells (right panel). Quadrants were designed as follows – I: PI stained cells indicating 2<sup>n</sup>-necrosis; II: non-stained cells indicating live cells; III: annexin V stained cells indicating early apoptosis; and IV: annexin V and PI stained cells indicating late apoptosis. All dot plots are a representation of an equal cell population (n = 10,000).



### ***ACA induces cell death via apoptosis***

To confirm that tumour cells were dying through apoptosis in oppose to primary necrosis, a double fluorescence staining of annexin V-FITC conjugated and PI was performed before and after ACA treatment, and analyzed using a flow cytometer. After exposure to ACA for 12hrs, the population of cells indicated a progressive shift from viable cells to early stage and late stage apoptosis indicated by a decrease in intensity of both forward scatter and side scatter (Fig. 3: A-E). The increase in cell percentage stained with annexin V-FITC (quadrants III and IV) was due to the exposure of phosphatidylserine on the cell surface, which was a strong indication of cells undergoing apoptosis. The highest percentage of apoptotic cells were observed in HSC-2 cells with 46.82% followed by HepG2 with 36.13% (Fig. 3: B and D), respectively. CaSki and HSC-4 cells showed the lowest levels of cell death with less than 25% of the entire cell population being apoptotic (Fig. 3: A and E). It has previously been reported that ACA inhibits cellular invasion through the suppression of the NF- $\kappa$ B regulated gene products (Ichikawa *et al*, 2005). Since NF- $\kappa$ B is a transcription factor that regulates the transcription of numerous genes corresponding to immune responses, proliferation, inflammation, anti-/pro- apoptotic genes and cell cycle regulators (Beinke and Ley, 2004), it would not be surprising that various types of tumour cell lines reacted differently towards ACA, hence allowing multiple means of inducing apoptotic cell death. Apoptotic cell death was confirmed through the laddering of genomic DNA, which is one of the hallmarks of apoptosis. Partial and complete fragmentation of genomic DNA was observed after 12hrs and 24 hrs respectively (Fig. 4).

HSC2				MCF7				CaSki				HepG2				HSC4			
M	0	12	24	M	0	12	24	M	0	12	24	M	0	12	24	M	0	12	24



**Figure 4:** Confirmation of apoptosis using the DNA fragmentation assay. Cells were treated with ACA for 12hrs and 24hrs to observe its effects on DNA laddering, (M: 100bp DNA Size Marker).

### **CONCLUSION**

In summary, this study provides evidence of the tropical Malaysian ginger compound, 1'S-1'-acetoxychavicol acetate of possessing anti-cancer properties which triggers the induction of cell cycle arrest and apoptosis in five tumour cell lines, namely, cervical, oral, liver and breast carcinoma cells. Further in-depth studies on various cell signaling pathways is required to elucidate its precise molecular course of action.

## ACKNOWLEDGEMENT

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## REFERENCES

- Beinke, S. and Ley, S. C. 2004.** Review Article: Functions of NF- $\kappa$ B1 and NF- $\kappa$ B2 in immune cell biology. *Journal of Biochemistry*, 382, 393-409.
- Chin, G. C. L. (2002).** Overview of cancers in Malaysia. *Journal of Clinical Oncology*, 32(Supp. 1): 37-42.
- Fu, Y., Hsieh, T. C., Kunicki, J., Lee, M. Y., Darzynkiewicz, Z., and Wu, J. M. (2004).** Licochalcone-A, a novel flavonoid isolated from licorice root (*Glycyrrhiza glabra*), causes G2 and late-G1 arrest in androgen-independent PC-3 prostate cancer cells. *Journal of Biochemistry and Biophysical Research Communications*, 322: 263-270.
- Hsu, Y. L., Kuo, P. L., Tzeng, W. S. and Lin, C. C. (2006).** Chalcone inhibits the proliferation of human breast cancer cell by blocking cell cycle progression and inducing apoptosis. *Journal of Food and Chemical Toxicology*, 44: 704-713.
- Ichikawa, H., Takada, Y., Murakami, A., Aggarwal, B. B. 2005.** Identification of a novel blocker of I $\kappa$ B $\alpha$  kinase that enhances cellular apoptosis and inhibits cellular invasion through suppression of NF- $\kappa$ B regulated gene products. *Journal of Immunology*, 174, 7383-7392.
- Itharat, A., Houghton, P. J., Eno-Amooquaye, E., Burke, P. J., Sampson, J. H., Raman A. 2004.** *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of Ethnopharmacology*, 90, 33-38.
- Kondo, A., Ohigashi, H., Murakami, A. 1993.** A potent inhibitor of tumor-promoter-induced Epstein Barr virus activation, 1'-acetoxychavicol acetate from *Languas galanga*, a traditional Thai condiment. *Biosci. Biotechnol. Biochem.*, 57, 1344-1345.
- Newman, D. J., Cragg, G. M., Snader, K. M. 2003.** Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.*, 66, 1022-1037.
- Tabata, K., Motani, K., Takayanagi, N., Nishimura, R., Asami, S., Kimura, Y., Ukiya, M., Hasegawa, D., Akihisa, T. and Suzuki, T. (2005).** Xanthoangelol, a major chalcone constituent of *Angelica keiskei*, induces apoptosis in neuroblastoma and leukemic cells. *Biological and Pharmacological Bulletin*, 28: 1404-1407.
- Violante, G. D., Zerrouk, N, Richard, I., Provot, G., Chaumeil, J. C., Arnaud, P. 2002.** Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures. *Biol. Pharm. Bull.*, 25(12), 1600-1603.