

Anti-tumor activity of *Micromelum hirsutum* extract

Satit Rodphukdeekul^{1*}, Tada Sueblinvong², Nijsiri Reungrungs³, Chaisak Chansriniyom³, Wacharee Limpanasithikul¹

¹ Department of Pharmacology, Faculty of Medicine, Chulalongkorn University

² Department of Biochemistry, Faculty of Medicine, Chulalongkorn University

³ Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University

* Presenting Author

Abstract

We investigated anti-tumor activity of the dichloromethane extract from branches of *Micromelum hirsutum* on human B lymphoma cells, Ramos. The extract exhibited Ramos cell cytotoxicity in a concentration-dependent manner with IC₅₀ 41.88 µg/ml at 24 h exposure. It induced cell death mainly by apoptosis at 12 h exposure. This apoptotic induction activity of the extract was markedly mediated by caspase activation. The extract induced Ramos cell accumulation at S and G2/M phase of the cell cycle. Our result demonstrated that the dichloromethane extract from branches of *M. hirsutum* has anti-tumor potential against B lymphoma.

Keywords: *Micromelum hirsutum*, cytotoxicity, apoptosis, cell cycle

Introduction

Micromelum hirsutum is a member of Rutaceae family. Plants in genus *Micromelum* are known to have carbazole alkaloids as parts of their active compounds. Mahanine is the well known carbazole alkaloid which is a major constituent in *M. minutum*. It exhibits many pharmacological activities including anti-mutagenic, antimicrobial, anti-oxidants, anti-inflammatory and anti-tumor activities (Ramsewak *et al.*, 1999; Tachibana *et al.*, 2001; Nakahara *et al.*, 2002; Roy *et al.*, 2004, 2005). It has been identified that the dichloromethane extract of the stem bark of *M. hirsutum* contains six carbazole alkaloids and one lactone derivative of oleic acid. There are one new carbazoles micromeline and five known carbazoles, lansine, 3-methylcarbazole, methyl carbazole-3-carboxylate, 3-formylcarbazole, and 3-formyl-6-methoxycarbazole. The lactone derivative of oleic acid is micromolide. This extract has been demonstrated to exhibit anti-tuberculosis activity (Ma *et al.*, 2000). All of these compounds have no reported on anti-tumor activities.

Methods

Plant extract: The precipitated from methanol extract from branches of *M. hirsutum* was partitioned in hexane. The precipitate from hexane was further partitioned in dichloromethane (CH₂Cl₂). The CH₂Cl₂ extract was dissolved in dimethyl sulfoxide (DMSO) and further diluted to various final concentrations at a constant concentration of DMSO (0.5 %).

Cells: Human B-Lymphoma cells, Ramos cells were from ATCC. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO₂.

Chemicals: Annexin V apoptosis detection kit was obtained from Santa Cruz Biotechnology, USA. The caspase inhibitor Z-VAD-FMK was from Promega, USA. Etoposide was obtained from Ebewe Pharma, Austria. Other chemicals were obtained from Sigma, Germany.

Cytotoxicity testing

Ramos cell at 1×10^6 cells/ml were treated with CH_2Cl_2 extract from branches of *M. hirsutum* at the concentrations 6.25-100 $\mu\text{g/ml}$ for 24 h. Viability of the treated cells were detected by staining with 1 mg/ml resazurin. Resorufin product in viable cells was determined by microplate reader at 570 and 600 nm. The percentage of cytotoxicity of the extract was calculated by comparing to the untreated control.

Determination of apoptosis

Ramos cells at 1×10^6 cells/ml were treated with 25, 50 and 100 $\mu\text{g/ml}$ extract for 12 h. The treated cells were stained annexin V-FITC and propidium iodide (PI) and the patterns of cell death were determined by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells which expose phosphatidylserine (PS) on the outer cell membrane. 0.5% DMSO solution was used as the negative control.

The caspase dependency of apoptotic induction activity of the extract was also determined. Ramos cells were pre-treated with 50 μM pan-caspase inhibitor (Z-VAD-FMK) for 1 h before adding the extract. The treated cells were performed as the above.

Cell cycle analysis

Ramos cells at 1×10^6 cells/ml were treated with 25, 50 and 100 $\mu\text{g/ml}$ extract for 1.5 h. The treated cells were washed twice with RPMI 1640 medium and further incubated in fresh complete RPMI 1640 medium for 48 h. The cells were collected, fixed with cold 70% ethanol, treated with 4 mg/ml RNaseA and finally stained with 0.05 $\mu\text{g/ml}$ PI. The DNA content of the fixed cells was determined by fluorescence flow cytometry.

Statistical analysis

All assays were performed in at least three independent experiments ($n=3$). The data were presented as mean \pm S.E. Data analysis was performed on SPSS 17.0. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. The p -value < 0.05 was considered statistically significant.

Results**Cytotoxicity of the extract**

The extract had cytotoxicity on Ramos cell in a concentration dependent manner with IC_{50} at 41.88 $\mu\text{g/ml}$ after 24 h exposure (Fig.1).

Apoptotic induction activity of the extract

The extract at the concentration of 25, 50 and 100 $\mu\text{g/ml}$ caused Ramos cell death mainly by apoptosis (Fig. 2). It induced apoptosis in a concentration-dependent manner. This apoptotic activity of the extract was markedly mediated by caspase activation. The pan caspase inhibitor Z-VAD-FMK inhibited the extract-induced Ramos cell apoptosis at all concentrations of the extract used in the study (Fig. 3).

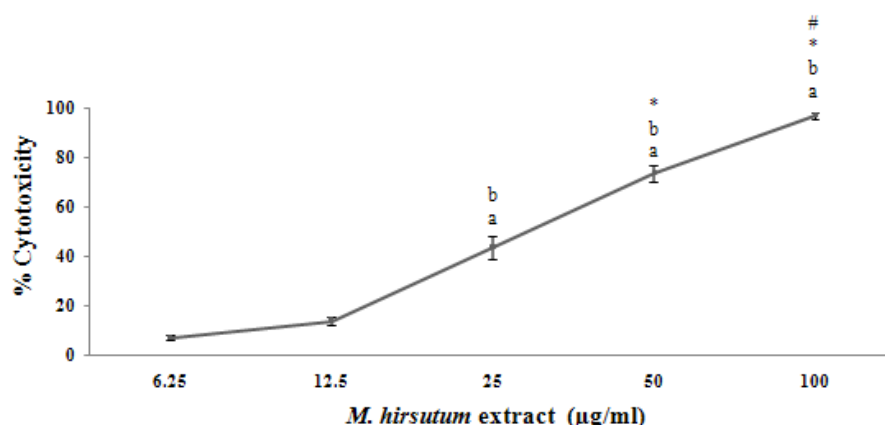


Figure 1: Cytotoxicity of the dichloromethane extract from branches of *M. hirsutum* on Ramos cells. The cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml for 24 hours. Viable cells were detected by staining with resazurin. The percentage of cytotoxicity was calculated by comparing with the untreated control. The data are expressed as mean \pm S.E. of four independent experiments (n=4). a $p < 0.05$ compared to 6.25 µg/ml extract, b $p < 0.05$ compared to 12.5 µg/ml extract, * $p < 0.05$ compared 50 and 100 µg/ml extract to 25 µg/ml extract, # $p < 0.05$ compared between 50 and 100 µg/ml extract.

Effect of the extract on the cell cycle

The extract had influence on the cell cycle of Ramos cells. It reduced the proportion of cells in G1 phase but the proportion of the treated cells was increase in S and G2/M phase (Fig. 4). These effects were clearly observed at 25 and 50 µg/ml extract. The 100 µg/ml extract-treated cells were not being able to detect the cell cycle in this study. One µg/ml etoposide induced cell accumulation in G2/M phase.

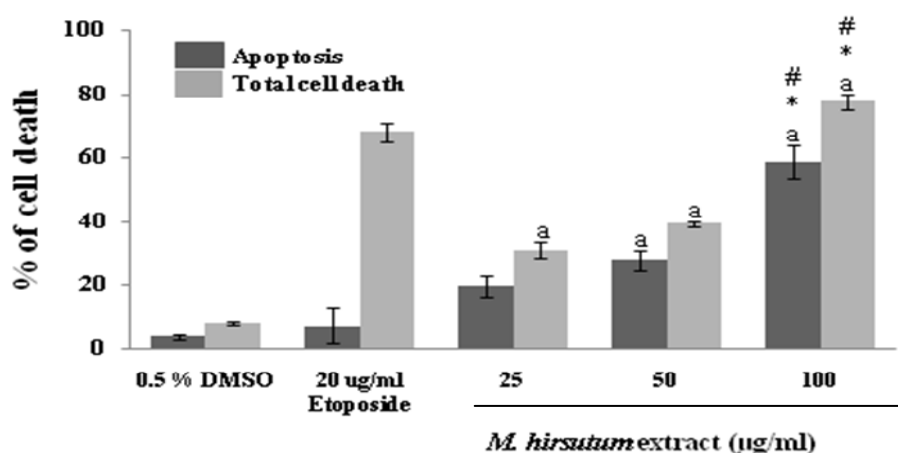


Figure 2: Effect of dichloromethane extract from branches of *M. hirsutum* on Ramos cell apoptosis. The cells were treated with 25, 50 and 100 µg/ml extract for 12 h. The treated cells were stained with annexin V-FITC and PI and the patterns of cell death were determined by fluorescence flow cytometer. Annexin V-FITC positive cells were determined as apoptotic cells. The data are represented as mean \pm S.E. of three independent experiments (n=3). a $p < 0.05$ compared to 0.5% DMSO, * $p < 0.05$ compared to 25 µg/ml extract, # $p < 0.05$ compared between 50 and 100 µg/ml of extract.

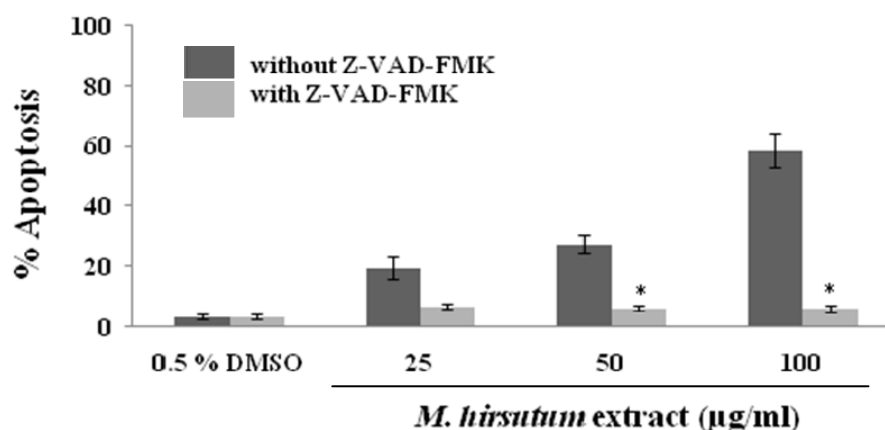


Figure 3: Effect of caspase inhibitor on the extract-induced Ramos cell apoptosis. The cell were pre-treated with or without Z-VAD-FMK for 1 h and then treated with 25, 50 and 100 µg/ml extract for 12 h. The percentage of apoptotic cells were determined by staining with annexin V-FITC/PI and detecting with fluorescence flow cytometer. Annexin V-FITC positive cells were determined as apoptotic cells. The data is expressed as mean \pm S.E. of three independent experiments (n=3).

* $p < 0.05$ compared between with and without Z-VAD-FMK.

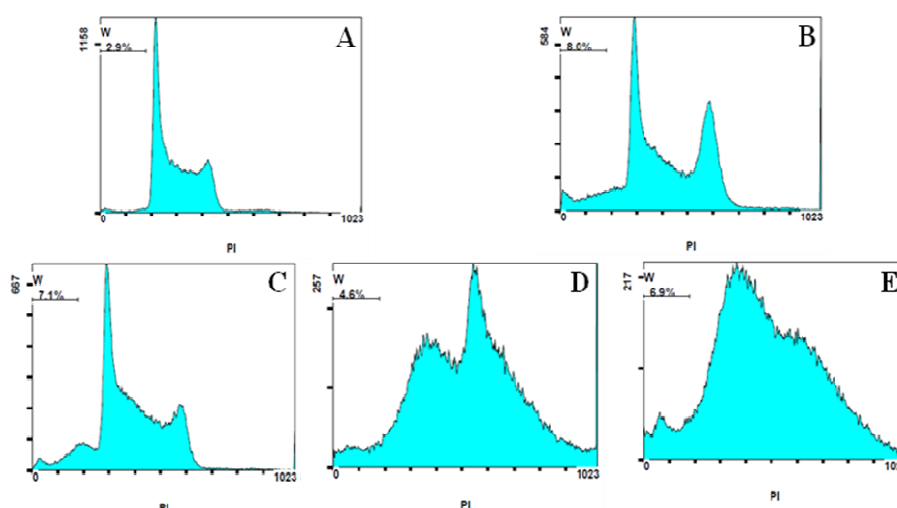


Figure 4: A representative histogram of the effect of dichloromethane extract from branches of *M. hirsutum* on Ramos cell cycle. The cells were treated with 12.5, 25 and 50 µg/ml extract for 1.5 h. The treated cells were washed twice and re-incubated without the extract for 48 h. the cells were fixed, treated with RNase. The cell cycle patterns were determined by fluorescence flow cytometer. (A) 0.5% DMSO; (B) 1 µg/ml etoposide; (C, D, E) 12.5, 25 and 50 µg/ml extract.

Discussion and Conclusion

Plants in genus *Micromelum* contain several carbazole alkaloids which have various pharmacological properties as well as anti-tumor activity. Mahanine is a well known carbazole form *M. minutum* that has been reported to exhibit various pharmacological activities including anti-oxidant, anti-inflammatory, antimutagenic and anti-tumor activities. Six carbazoles, including new carbazole, micromelin and other five known carbazoles were isolated from the CH_2Cl_2 extract from stem bark of *M. hirsutum*. In this study we investigated anti-tumor effect of the CH_2Cl_2 extract from branches of *M. hirsutum*. The extract had cytotoxic effect on human B lymphoma cells, Ramos, in a concentration-dependent manner with IC_{50} at 41.88 µg/ml. It induced Ramos cell death mainly by apoptosis (annexin V-FITC positive). The apoptotic effect of the extract was mediated by caspase activation. A pan

caspase inhibitor Z-VAD-FMK, which inhibits caspase 3, 6, 7, 8 and 9 markedly inhibited apoptotic activity of all concentrations of the extract used in the study (25-100 µg/ml). The extract also had effect on the cell cycle of Ramos cells. It induced accumulation of cell in S and G2/M phases. The effects of the extract on Ramos cells cyclins and cyclin-dependent kinase inhibitors (CKIs) are ongoing investigated. These results demonstrated that *M. hirsutum* may contain constituents which are possible candidates for anti-tumor agents.

Acknowledgment

This project was partly supported by the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund)

References

1. Ma C, Case RJ, Wang Y, Zhang HJ, Tan GT, Van HN, Cuong NM, Franzblau SG, Soejarto DD, Fong HH, Pauli GF. Antituberculosis constituents from the stem bark of *Micromelum hirsutum*. *Planta medica* 2005; 71: 261-7.
2. Nakahara K, Trakoontivakorn G, Alzoreky NS, Ono H, Onishi-kameyama M, Yoshida M. Antimutagenicity of some edible Thai plants, and a bioactive carbazole alkaloid, mahanine, isolated from *Micromelum minutum*. *Journal of Agricultural and Food Chemistry* 2002; 50: 4796-802.
3. Ramasewak RS, Nair MG, Strasburg GM, Dewitt DL, Nitiss JL. Biologically active carbazole alkaloids from *Murraya koenigii*. *Journal of Agricultural and Food Chemistry* 1999; 50: 444-7.
4. Roy MK, Thalang VN, Trakoontivakornb G, Nakahara K. Mechanism of mahanine-induced apoptosis in human leukemia cells (HL-60). *Biochemical Pharmacology* 2004; 67: 41-51.
5. Roy MK, Thalang VN, Trakoontivakorn G, Nakahara K. Mahanine, a carbazole alkaloid from *Micromelum minutum*, inhibits cell growth and induces apoptosis in U937 cells through a mitochondrial dependent pathway. *British Journal of Pharmacology* 2005; 145: 145-55.
6. Tachibana Y, Kikuzaki H, Lajis NH, Nakatani N. Antioxidative activity of carbazoles from *Murraya koenigii* leaves. *Journal of Agricultural and Food Chemistry* 2001; 49: 5589-94.