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All Res. J. Biol., **2013**, 4, 19-23

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Issue 3, Vol 4, 2013, 19-23

Low effects on cytotoxicity against neuroblastoma cancer cell line (SK-N-SH) and free-radical scavenging activity of *Stemona* alkaloids

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Abstract: *Stemona* plants contain alkaloids with different skeletal types. They are well-characterized and are commonly used for insecticide, killing head lice, treating skin diseases, antitussive, and anticancer treatment. Eleven *Stemona* alkaloids of three skeletal types, protestemonine-, croomine-, and stichoneurine-type, i.e. protostemonine-type (didehydrostemofoline, stemofoline, stemocurtisine, stemocurtisinol, stemokerrine, oxystemokerrine), croomine-type (croomine), and stichoneurine-type (tuberostemonine, tuberostemonine A, tuberostemonine N, neotuberostemonine) were isolated from various *Stemona* roots and investigated for their cytotoxicity and free radical scavenging activity, which have not yet been reported. Cytotoxicity on neuroblastoma cancer cell (SK-N-SH) was determined by MTT assay, while free radical scavenging activity was investigated using the DPPH radical scavenging method. Protostemonine type alkaloids possessed insignificant effect on the cytotoxicity and free radical scavenging activity, while croomine and stichoneurine type alkaloids showed weak to moderate effects.

Keywords: anticancer; antioxidant; MTT assay; stemofoline; *Stemona*; tuberostemonine

Introduction

Stemona spp. of the family Stemonaceae are commonly known in Thailand under the name of "Non Tai Yak." These plants have been used as a natural insecticide against head lice, scabicide, anticancer agent, and for treatment of skin and respiratory diseases[1-3]. *Stemona* alkaloids represent a unique chemical character of the family Stemonaceae[4,5]. *Stemona* plants contain various amounts of these alkaloids[6,7], which can be divided into 3 main skeletal types[4], i.e. protostemonine-type, croomine-type, and stichoneurine-type (Fig. 1). The major alkaloids of the first skeletal type includes didehydrostemofoline, stemofoline, stemocurtisine, stemocurtisinol, oxystemokerrine, and stemokerrine, while the second type includes croomine, and the last type includes tuberostemonine, tuberostemonine A, tuberostemonine N, and neotuberostemonine. The reported biological activities of these *Stemona* alkaloids are insect toxicity[8,9], antitussive[10,11], oxytocin antagonist[12], nitric oxide inhibition[13], and increasing chemosensitivity via P-glycoprotein-mediated multidrug resistance[14-16]. Cytotoxicity of *Stemona* extracts or their isolated alkaloids has been reported against several human cancer cell lines such as medullary thyroid carcinoma[17,18], human fibroblast[18], leukemia carcinoma[19], nasopharyngeal[19], gastric carcinoma[19], breast adenocarcinoma[20]. However, free radical scavenging activity and cytotoxicity of these alkaloids on neuroblastoma cancer cells has not been reported.

Oxidative stress can lead to numerous pathophysiological conditions, including cancer. Antioxidants can terminate or retard the oxidation process by scavenging and sequestering the free radicals that are implicated in many chronic diseases. In the present study, eleven *Stemona* alkaloids (Fig. 1), which were isolated from underground parts of various *Stemona* plants in our previous works[6], were investigated for their free radical scavenging activity and cytotoxicity against neuroblastoma cell line (SK-N-SH).

Materials and methods

Chemical and reagents

RPMI 1640 medium and fetal calf serum (FCS) were obtained from Biochrom (Berlin, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma (St. Louis, MO).

Eleven *Stemona* alkaloids of three skeletal types, i.e. didehydrostemofoline, stemofoline, stemocurtisine, stemocurtisinol, stemokerrine, oxystemokerrine of protostemonine-type; croomine of croomine-type; and tuberostemonine, tuberostemonine A, tuberostemonine N, neotuberostemonine of stichoneurine-type were isolated from the roots of various *Stemona* plants and identified in our previous work[6].

Cell culture

SK-N-SH cell line was obtained from the American Type Culture Collection (Rockville, MD) was cultured in RPMI 1640 medium supplemented with 5% (v/v) FSC, 100 mg/l of streptomycin and 100,000 U/l of penicillin G, at 37°C in 5% CO_2 incubator.

Cell viability evaluation by MTT assay

MTT assay was a conventional method to assess the number of viable cells growing in microtiter plate after treatment with various substances. Serial dilutions of samples (50 µl) were added into each of 96-well plates. The cells were plated at a density of 1×10^4 cells/well and incubated for 48 hrs. After incubation, the medium was removed and the cells in each well were incubated with phosphate buffered saline (PBS) containing 1 mg/ml MTT for 2 hrs at 37°C in 5% CO_2 incubator. MTT solution was then discarded and 50 µl of isopropanol was added into each well to dissolve insoluble formazan crystals. Plates were then agitated for 5 min at room temperature to complete the solubilization. The color of formazan derivative was analyzed on a microplate reader (Molecular Devices, CA) at a wavelength of 590 nm. Each concentration was done in triplicate. The percentage of cell viability was calculated according to the following equation:

cell viability (%) = (OD of treated cells / OD of control cells) × 100%.

Determination of free radical scavenging activity

The free radical scavenging activity of *Stemona* alkaloids was investigated using DPPH radical scavenging assay. The solution of DPPH· was prepared at concentration of 60 mg/l (152 µM) in methanol, while stock solution of the sample (1 mg/ml) was serially diluted between 12.5 and 800 µg/ml. The DPPH· scavenging reaction was performed by adding DPPH· solution (100 µl) to the sample solution of the same volume. The mixture was incubated for 30 min at room temperature and measured for absorbance at 517 nm by a microplate reader. The corresponding blank sample was also taken and percent inhibition was then calculated as follows:

Scavenging activity (%) = $[1 - (A_1 - A_2) / A_0] \times 100\%$ where A_0 was the absorbance of control (DPPH· solution without sample), A_1 was the absorbance of DPPH· solution in the presence of the sample and A_2 was the absorbance of the sample without DPPH· solution.

Statistical analysis

The experiments were repeated three times and the results were expressed as mean ± S.D. Statistical analysis was done using two-tailed Student's *t* test and *P* values at a level of 95% confidence limit.

Results

Cytotoxicity on neuroblastoma cell line SK-N-SH

The cytotoxic effect of *Stemona* alkaloids on neuroblastoma cell line SK-N-SH was investigated by MTT assay. Cells were treated with *Stemona* alkaloids at concentrations ranging from 0 to 200 µg/ml for 48 hours and the percentage of cell viability was analysed. The EC_{50} was then calculated as shown in Table 1. Croomine and stichoneurine derivatives, i.e. tuberostemonine, tuberostemonine A, tuberostemonine N, and neotuberostemonine inhibited the proliferation of SK-N-SH cells in a dose-dependent manner. A similar result was observed when quercetin was used as a positive control.

Free radical scavenging activity

The free radical scavenging activity of *Stemona* alkaloids was determined by the DPPH· free radical scavenging assay. At a concentration of 400 µg/ml, protostemonine alkaloid derivatives - didehydrostemofoline, stemofoline, stemocurtisine, stemocurtisinol, stemokerrine, and oxystemokerrine - increased free radical scavenging activity by only 8.81%, 9.92%, 45.03%, 8.55%, 8.52%, and 59.27%, respectively, whereas croomine and the stichoneurine derivatives - tuberostemonine, tuberostemonine A, tuberostemonine N, and neotuberostemonine - increased scavenging activity by 88.84%, 88.76%, 79.98%, 84.81%, and 84.73%, respectively. Serial dilutions of each sample were also measured for their free radical scavenging activity. The EC_{50} of each alkaloid was then calculated (Table 1).

Croomine and stichoneurine derivatives possessed free radical scavenging activity in a dose-dependent manner. Similar results were observed when quercetin and trolox were used as positive control.

Discussion

Stemona alkaloids could be classified, based on biosynthetic hypothesis, into three skeletal types: protostemonine-, stichoneurine-, and croomine-type. Protostemonine type alkaloids, which include didehydrostemofoline, stemofoline, stemocurtisine, stemocurtisinol, stemokerrine, and oxystemokerrine, possessed insignificant cytotoxicity on SK-N-SH cells and low free radical scavenging activity, while croomine and the stichoneurine-type alkaloids, which are tuberostemonine, tuberostemonine A, tuberostemonine N, and neotuberostemonine, showed weak to moderate activities compared to the positive control (Table 1).

Although *Stemona* roots have been traditionally used as an ingredient in several anti-cancer preparations, only a few scientific studies on their anti-cancer properties were reported. Only medullary thyroid carcinoma cell lines gave positive results when treated with *Stemona* extracts[17,18] whereas other cell lines showed weak responses or no cytotoxicity activity[15,19,20]. Although those compounds do not seem to exhibit a strong cytotoxicity, they might still be acting to potentiate the effect of anti-neoplastics. For example, stemofoline, stemocurtisine, and stemocurtisinol were reported to sensitize the multidrug resistant cancer cell to putative chemotherapeutic agents including vinblastine, paclitaxel and colchicine via P-glycoprotein mediated pathway[14-16].

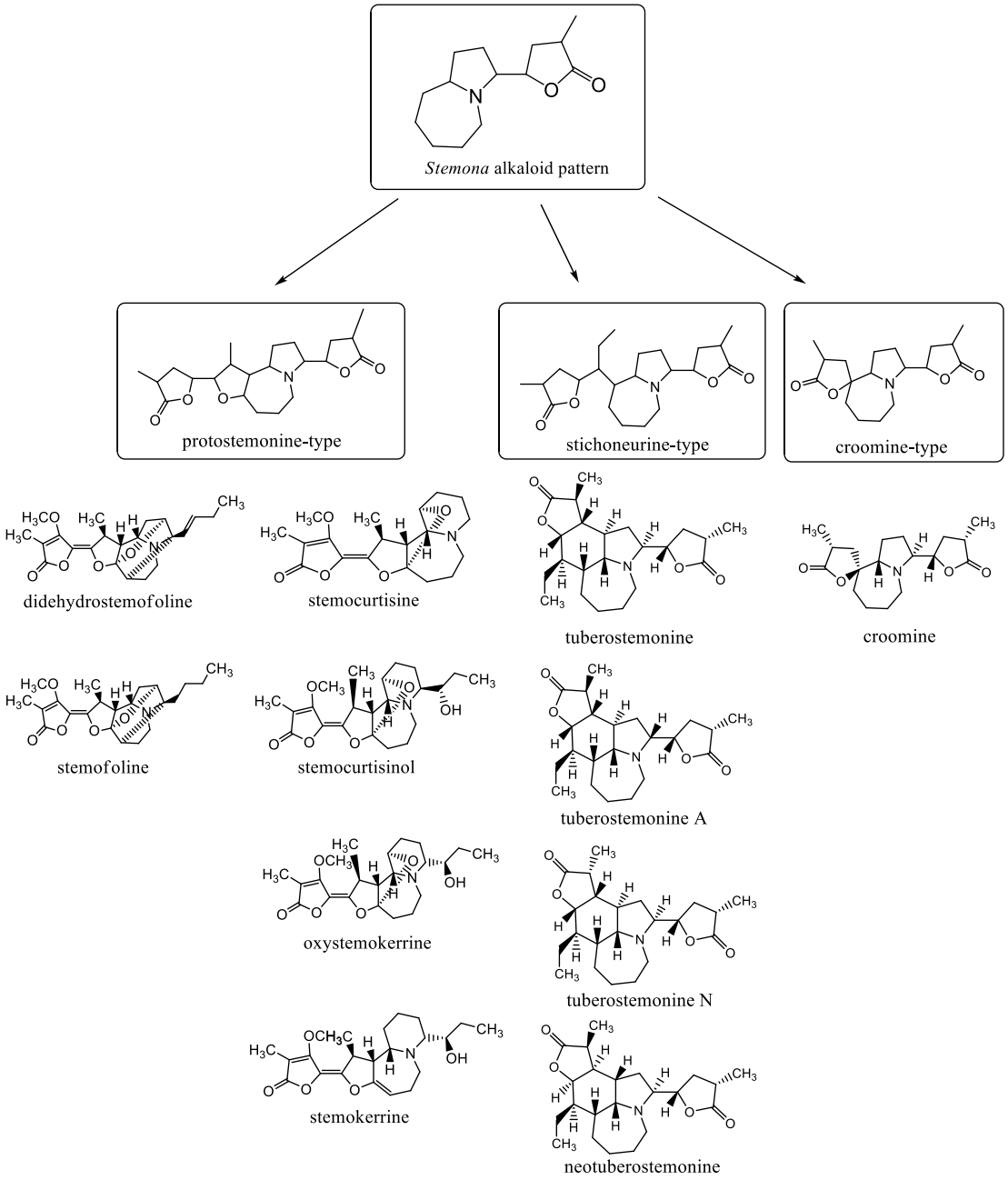

Figure 1 Structure of *Stemona* alkaloids used in this study.

Table 1 Cytotoxicity and free radical scavenging activity of *Stemona* alkaloids

Type[a]	Compounds	EC_{50} (µg/ml)	
		Cytotoxicity[b]	Free radical scavenging activity[b]
I	Didehydrostemofoline	> 200	>> 400
	Stemofoline	> 200	>> 400
	Stemocurtisine	> 200	> 400
	Stemocurtisinol	> 200	>> 400
	Stemokerrine	> 200	> 400
	Oxystemokerrine	> 200	156.68 ± 2.72
II	Tuberostemonine	140.49 ± 7.92	34.88 ± 1.92
	Tuberostemonine A	144.58 ± 10.39	218.33 ± 2.00
	Tuberostemonine N	193.44 ± 10.57	57.03 ± 0.68
	Neotuberostemonine	71.27 ± 2.52	66.74 ± 1.56
III	Croomine	198.42 ± 5.48	107.74 ± 0.33
Postive control	Trolox	-	2.62 ± 0.05
	Quercetin	36.67 ± 4.39	2.29 ± 0.02

[a] I = protostemonine-type alkaloid, II = stichoneurine-type alkaloid, III = croomine-type alkaloid
[b] Results expressed as mean ± SD (n = 3)

Conclusion

Protostemonine alkaloid derivatives showed an insignificant effect on both cytotoxicity of SK-N-SH cells and free radical scavenging activity, while stichoneurine alkaloid derivatives and croomine alkaloid demonstrated these activities at low to moderate levels. The highest cytotoxic effect was observed in neotuberostemonine with an EC_{50} of 71.27 µg/ml, which was 2-fold lower activity than that of quercetin (EC_{50} 36.67 µg/ml). The highest free radical scavenging activity was observed in tuberostemonine with EC_{50} of 34.88 µg/ml, which is about 15 times lower than those observed with trolox and quercetin (EC_{50} 2.62 and 2.29 µg/ml, respectively). These results demonstrate that *Stemona* alkaloids, the major contents in the roots of *Stemona* species, may not directly possess cytotoxicity on cancer cells. When included in traditional anti-cancer preparations, *Stemona* possibly synergizes and enhances the activity of other active compounds through increasing chemosensitivity.

Acknowledgements : This study is a part of a Ph.D. thesis at Mahidol University, financially supported by Thailand Research Fund and Mahidol University (Royal Golden Jubilee Ph.D. Program Grant No. PHD/0139/2550). The project is also supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative. We thank Mr. Panupon Khumsupan for his kind help in proofreading the manuscript.

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