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Cytotoxicities of alkaloids from processed and unprocessed seeds of *Strychnos nux-vomica*[1]

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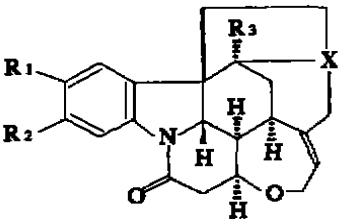
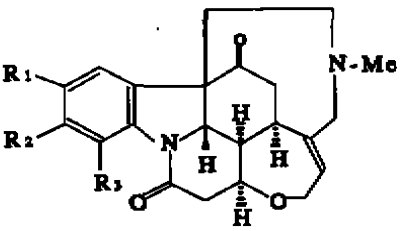
KEY WORDS *Strychnos nux-vomica*; strychnine; alkaloids; immunologic cytotoxicity; cell division; thymidine; DNA; Vero cells

AIM: To examine the cytotoxicities of 6 crude *Strychnos* alkaloid fractions from the seeds of *Strychnos nux-vomica* unprocessed or processed with various traditional processing methods and 13 pure *Strychnos* alkaloids from the fractions. **METHODS**: Using cell culture, their inhibitory effects on Vero cell growth-inhibition assay, and host cell DNA synthesis by [^{3}H]thymidine ([^{3}H]TdR) uptake assay. **RESULTS**: The IC_{50} of processed seeds were 155 % and 212 % of unprocessed ones in cell growth-inhibition assay and in [^{3}H]TdR uptake assay, respectively. The IC_{50} of 13 compounds were 0.45 – 0.80 mmol · L^{-1} and 0.50 – 12 mmol · L^{-1}, respectively. The processing method with sand bath exhibited a wide safety margin compared with other traditional processing methods or no processing. The isomers of *Strychnos* alkaloids and their *N*-oxides showed much lower cytotoxicities among these alkaloids. Isobrucine *N*-oxide showed the lowest cytotoxicity. The contents of isomers and *N*-oxides of *Strychnos* alkaloids were the highest in the sand processing. **CONCLUSION**: Processing of nux vomica plays a critical role in its toxicity.

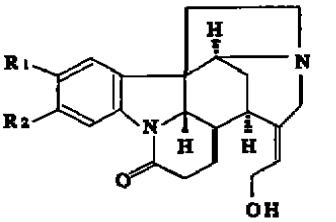
The seeds of *Strychnos nux-vomica* L are clinically used for relieving pain and promoting blood circulation. The seeds have to be processed by parching in a sand bath, frying in an oil bath, treating with a licorice decoction, submerging in urine from healthy children, treating with vinegar, *etc*, due to high toxicity.



1: $R_1 = R_2 = R_3 = H$, X = N
2: $R_1 = R_2 = OMe$, $R_3 = H$, X = N
3: $R_1 = OMe$, $R_2 = R_3 = H$, X = N
5: $R_1 = R_2 = H$, $R_3 = OH$, X = N
6: $R_1 = R_2 = OMe$, $R_3 = OH$, X = N
8: $R_1 = R_2 = R_3 = H$, X = N→O
9: $R_1 = R_2 = OMe$, $R_3 = H$, X = N→O
12: $R_1 = OH$, $R_2 = OMe$, $R_3 = H$, X = N



4: $R_1 = R_2 = H$, $R_3 = OH$
7: $R_1 = R_2 = OMe$, $R_3 = H$



10: $R_1 = R_2 = H$
11: $R_1 = R_2 = OMe$
13: $R_1 = R_2 = OMe$, N→O

***Strychnos* alkaloids**

The analgesic potency of the crude alkaloid fraction of the sand-processed seeds was higher than that of the unprocessed seeds in animal experiments[1]. The LD_{50} values of crude

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alkaloids from processed seeds of *S nux-vomica* in mice were in a narrow range of 2.18 – 2.57 $mg \cdot kg^{-1}$, while that of the unprocessed seeds was 1.21 $mg \cdot kg^{-1}$[2]. Strychnine, strychnine *N*-oxide, brucine, and brucine *N*-oxide are major components in *S nux-vomica*. Their LD_{50} were 1.10, 10.92, 50.10, and 766.70 $mg \cdot kg^{-1}$, respectively[2]. The content of *N*-oxide alkaloids in the seed processed with a sand bath was higher than unprocessed ones. On the other hand, the LD_{50} value of the former (2.35 $mg \cdot kg^{-1}$) was about twice that of the latter (1.21 $mg \cdot kg^{-1}$)[3]. Therefore, the sand-processing doubled the therapeutic index. Although the toxicities of the seeds with 5 different processing methods were decreased to about 50 % compared with the unprocessed one, it was not known how each alkaloid was in crude alkaloid fractions on cytotoxicity. In this paper, cytotoxicity of 6 crude *Strychnos* alkaloid fractions from the seeds of *S nux-vomica* unprocessed or processed with various traditional processing methods and 13 pure *Strychnos* alkaloids from the fractions were examined for their inhibitory effects on cell growth and host cell DNA synthesis by [^{3}H]TdR uptake assay.

MATERIALS AND METHODS

Preparation of test drugs Traditional drug-processings were carried out on a laboratory scale using the same batch of the raw seeds of *S nux vomica*[4]. Ground powder of the unprocessed and processed seeds were moistened with 15 % ammonia water, extracted with chloroform, further re-extracted with HCl 1 $mol \cdot L^{-1}$. The combined acidic solutions were adjusted to pH 11 – 12 with 40 % NaOH solution and extracted with chloroform. The combined chloroform solutions were evaporated *in vacuo* to give a crude alkaloid fraction. For content of major *Strychnos* alkaloids from various processings of nux vomica, we analyzed the composition of crude alkaloid fractions by thin-layer chromatography-densitometry (Tab 1).

Strychnine (1), brucine (2), β-colubrine (3), vomicine (4), pseudostrychnine (5), pseudobrucine (6), novacine (7), strychnine *N*-oxide (8), brucine *N*-oxide (9), isostrychnine (10), isobrucine (11), 2-hydroxy-3-methoxystrychnine (12) and isobrucine *N*-oxide (13) were isolated from the crude *Strychnos* alkaloid fractions[5]. Compounds 1 – 10 were isolated from the unprocessed and processed seeds of *S nux-vomica*, whereas 11 – 13 were isolated from only the processed ones.

Each crude alkaloid fraction and 13 *Strychnos* alkaloids were dissolved in 0.2 mL Me_2SO to a solution of 2 $g \cdot L^{-1}$ concentration for experiments. All reagents used were of AR grade.

Cells Vero cells (Africa green monkey kidney cell line) were grown as a monolayer

Tab 1. Alkaloid compositions in the crude alkaloids from the processed and unprocessed seeds of *S nux-vomica*. $n = 4$ (test times), $\bar{x} \pm s$. TLC scanner ($\lambda_S = 260$ nm, $\lambda_R = 360$ nm).

Compounds	Alkaloid compositions/%					
	A	B	C	D	E	F
Strychnine	54.9 ± 2.9	52.3 ± 3.8	52.7 ± 3.7	53.3 ± 2.8	53.7 ± 2.9	52.5 ± 3.8
Brucine	27.4 ± 2.2	22.6 ± 2.0	21.5 ± 1.3	24.2 ± 2.2	22.5 ± 2.1	20.6 ± 2.8
β-Colubrine	5.0 ± 1.0	3.3 ± 0.3	4.1 ± 0.7	3.3 ± 0.4	4.2 ± 0.3	4.8 ± 0.4
Strychnine *N*-oxide	0.9 ± 0.4	2.1 ± 0.5	2.1 ± 1.2	1.6 ± 0.9	1.5 ± 1.0	1.5 ± 0.7
Brucine *N*-oxide	0.3 ± 0.2	2.4 ± 0.7	1.7 ± 0.5	3.0 ± 1.1	1.0 ± 0.2	2.4 ± 1.2
Isostrychnine	0.9 ± 0.4	1.6 ± 1.1	0.8 ± 0.7	1.1 ± 1.1	1.5 ± 0.7	1.1 ± 0.8
Isobrucine	Faint	2.7 ± 1.0	2.2 ± 1.6	1.8 ± 2.0	1.4 ± 1.0	2.4 ± 1.3
2-Hydroxy-3-methoxystrychnine	Faint	0.53 ± 0.28	0.33 ± 0.17	0.22 ± 0.08	0.24 ± 0.07	0.42 ± 0.37
Isobrucine *N*-oxide	Faint	0.13 ± 0.06	0.14 ± 0.05	0.13 ± 0.04	0.14 ± 0.07	0.09 ± 0.03

A) That was prepared by slicing the raw seeds and drying at 80 ℃. B) The raw seeds were put into a sand bath, heated up to 230 ℃ for 3 min. C) The raw seeds were put into fresh urine from healthy children for 7 d, followed by treatment (B). D) The raw seeds were submerged in water for 2 d, cut into 1.5 mm thick slices, put into vinegar, boiled for 10 min, submerged in vinegar for 12 h, and then treated with (B). E) The raw seeds were put into sesame oil, heated to 230 ℃ for 3 min. F) Slices of licorice roots were boiled twice in water. The raw seeds were put into the filtrate and boiled for 4 h.

cultured and maintained in Eagle's minimum essential medium (MEM) supplemented with 5 % and 2 % calf serum. The cultures were maintained at 37 ℃ in a humidified 5 % CO_2 serum.

Apparatus A dual-wavelength chromatoscanner (CS-910, Shimadzu, Kyoto, Japan). The content of [^{3}H]TdR uptake into DNA was measured with a liquid scintillation counter (LSC-5100, Aloka Co, Tokyo, Japan).

Cytotoxicity experiments Cytotoxicity of *Strychnos* alkaloids was monitored by measuring their effects on the growth of Vero cells[6]. Vero cells were seeded 2.5×10^4 cells/well in 24-well plates and grown at 37 ℃ for 2 d. The culture medium was replaced by fresh medium containing *Strychnos* alkaloids 100, 200, and 300 mg·L^{-1} and then cells were further grown for 2 d. Eight replicated wells containing no test solution were served as control. The monolayer cells were treated with trypsin and the cell number was determined with a hemocytometer by Trypan blue exclusion test. The growth rate of each test sample was determined as living cell number of control as: % Growth rate = Number of living cells (test sample)/Number of living cells (control)

The IC_{50} was defined as the concentration of alkaloids required to reduce growth rate of cells by 50 %. The computer Bliss analysis was applied to determine the IC_{50} and its 95 % confidence limits (CL_{95}).

As a second marker for cytotoxicity, *Strychnos* alkaloids were evaluated for their inhibitory effects on host cell DNA synthesis. The Vero cells were grown in 24-well plates for [^{3}H] TdR-uptake assay. The culture medium was replaced with fresh medium containing [*methyl*-^{3}H]TdR 37 kBq (3.1 TBq/mmol·L^{-1}, Amersham) and 3 concentrations of each alkaloid sample. After a 22-h exponential growth period, the cells were lysed with Tris 20 mmol·L^{-1}, (pH 8.0), edetic acid 5 mmol·L^{-1}, 0.5 % (wt/vol) SDS and proteinase K 100 mg·L^{-1} at 37 ℃ for 3 h. The lysates were spotted on filter of 2 cm × 2 cm in size (514 A paper filters, Toyo Roshi Co, Japan) and absorbed into the filter. The filter was immediately put into cold 5 % trichloroacetic acid (TCA) for fixing DNA. After 20 min, the filters were washed 3 times with cold 5 % TCA and then once with 95 % EtOH. The dried filter was put into a liquid scintillation counter. The content of [^{3}H]TdR uptake into DNA of alkaloids-treated cells to that of untreated cells was shown in percent. The IC_{50} for DNA synthesis was defined as the concentration of alkaloids required to reduce [^{3}H]TdR incorporation by 50 %.

RESULTS AND DISCUSSION

In the processed seeds by a sand bath (B), the contents of isostrychnine, isobrucine, strychnine *N*-oxide, brucine *N*-oxide, and 2-hydroxy-methoxystrychnine analyzed comparably by TLC-densitometry were higher than those by the other processing methods. Strychnine and brucine *N*-oxide were formed by oxidation of strychnine and brucine during heating, the amounts of transformed *N*-oxide in the processed (B) were 2.3-fold and 9.7-fold of unprocessing (A).

In the cytotoxicity experiments, the IC_{50} value of the processed (B) was 155 % of the unprocessed (A) in cell growth-inhibition assay and was 212 % of A in [^{3}H]TdR uptake assay. Two assays taken together, the processed (B) showed the lowest cytotoxicity among various processing methods. Most alkaloids by *N*-oxidation and/or ring-opening reaction of *Strychnos* alkaloids and 2-hydroxy-3-methoxy-strychnine showed comparatively low cytotoxicity in [^{3}H]TdR uptake assay (Tab 2).

Tab 2. Cytotoxicity of crude *Strychnos* alkaloid fractions from the unprocessed and processed seeds of *S nux-vomica*.

Processing type	IC_{50} of cell growth (95 % confidence limits) /mg·L^{-1}	IC_{50} of DNA synthesis (95 % confidence limits) /mg·L^{-1}
A	110 (87 - 132)	131 (107 - 156)
B	170 (148 - 237)	277 (233 - 323)
C	145 (119 - 191)	242 (217 - 316)
D	184 (142 - 254)	233 (200 - 298)
E	101 (78 - 166)	199 (163 - 290)
F	131 (103 - 187)	131 (113 - 188)

Processing type A - F were shown in Tab 1.

Isobrucine *N*-oxide showed the lowest cytotoxicity among these alkaloids tested (Tab 3).

Tab 3. Cytotoxicity of *Strychnos* alkaloids isolated from the unprocessed and processed seeds of *S nux-vomica*.

Compounds	IC_{50} of cell growth (95 % confidence limit) /μmol·L^{-1}	IC_{50} of DNA synthesis (95 % confidence limit) /μmol·L^{-1}
1	444 (349 – 492)	790 (512 – 881)
2	622 (560 – 896)	850 (716 – 1 356)
3	453 (399 – 639)	630 (568 – 1 196)
4	494 (439 – 612)	530 (481 – 762)
5	720 (608 – 984)	830 (739 – 1 196)
6	579 (451 – 784)	960 (864 – 1 056)
7	782 (705 – 957)	700 (616 – 998)
8	807 (718 – 1 284)	1 500 (1 000 – 2 000)
9	467 (395 – 856)	2 600 (2 100 – 3 400)
10	459 (404 – 695)	1 100 (800 – 1 600)
11	607 (553 – 876)	910 (746 – 1 419)
12	758 (548 – 1256)	6 000 (5 100 – 7 700)
13	760 (684 – 978)	11 900 (9 100 – 16 400)

Because most of these alkaloids were formed during processing, this indicated that the toxicity of the seeds of *S nux-vomica* was decreased by processing. That was why the processed seed must be used in traditional Chinese medicine therapy and the processing mechanisms may be explained in a series of our studies. The reason that the crude *Strychnos* alkaloid fractions from the processed (B) showed the lowest cytotoxicity may be associated with change of these constituents described above, for the contents of these *Strychnos* alkaloids in the processed (B) were higher than other processing methods. These results demonstrated that the processing method with sand bath had a wide safety margin comparing with other traditional processing methods or unprocessing one. On the other hand, as was stated above in the test of acute toxicity, the LD_{50} value of the sand-processed seeds (2.35 mg·kg^{-1}) was 192 % of that of the unprocessed seeds (1.21 mg·kg^{-1}), which were in accord with the cytotoxicity tests.

Sand-processing is a good processing method. The crude alkaloid fraction of *S nux-vomica* treated with such processing showed lower toxic and distinct analgesic effects in all tests stated above. Besides, the processed (B) seems to be more convenient and adequate in view of reducing toxicity, it is desirable to evaluate the processing in view of the therapeutic efficacies in traditional Chinese medicine.

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φ25-φ28 (7)

马钱子生品及炮制品中马钱子类生物碱的细胞毒性[1]

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关键词 马钱; 士的宁; 生物碱; 免疫细胞毒性; 细胞分裂; 胸苷; DNA; 非洲绿毛猴肾细胞

目的: 测定 6 种中药马钱子生品及不同炮制品的总生物碱以及 13 个单体生物碱的细胞毒性. **方法**: 采用细胞培养法观察马钱子生物碱对 Vero-细胞增殖抑制和[^{3}H]胸腺嘧啶核苷酸参入法对宿主细胞内 DNA 合成的影响. **结果**: 砂炒炮制品的 IC_{50}值分别是生品的 155 % 和 212 %. 13 个化合物的 IC_{50}值分别是 0.45 – 0.80 mmol·L^{-1}和 0.50 – 12 mmol·L^{-1}. 马钱子中异型生物碱及氮氧化合物在砂炒炮制品中含量最高, 但这些生物碱显示出较小的细胞毒性. 其中异马钱子碱氮氧化物毒性最低. 砂炒炮制品与其它传统炮制品及生品比较具有更宽的安全范围. **结论**: 马钱子的炮制对其毒性起关键性作用.