Processing of *Nux Vomica*. VII. Antinociceptive Effects of Crude Alkaloids from the Processed and Unprocessed Seeds of *Strychnos nux-vomica* in Mice

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We examined the antinociceptive effects of the crude alkaloid fractions (CAF) of *nux vomica* (the dried seeds of *Strychnos nux-vomica* L.) and the influences of various processing methods upon their antinociception in three analgesic tests in mice. In the tail-pressure test, the CAF (0.01—1 $\mu\text{g/kg}$, i.p.) of *nux vomica* that was unprocessed or treated with sand-, licorice-, oil- or vinegar and sand-processing showed clear antinociception. The CAF (1 $\mu\text{g/kg}$, i.p.) of vinegar-processed *nux vomica* showed antinociception, without effects at lower doses of 0.01 and 0.1 $\mu\text{g/kg}$ and those treated with urine- or urine and sand-processing were without effects at doses of 0.01—1 $\mu\text{g/kg}$. Morphine (2 mg/kg, s.c.) showed short-lasting antinociception, without effects at a dose of 1 $\mu\text{g/kg}$. In the hot-plate test, the CAF (100 $\mu\text{g/kg}$, i.p.) of *nux vomica* having undergone sand-processing produced a significant antinociception, without effects at lower doses of 0.01 and 1 $\mu\text{g/kg}$. The CAF (0.01—100 $\mu\text{g/kg}$, i.p.) of *nux vomica* that was unprocessed or treated with oil- or vinegar and sand-processing and morphine (1 and 100 $\mu\text{g/kg}$, s.c.) were without effects. In the acetic acid-induced writhing test, the CAF (1 $\mu\text{g/kg}$, i.p.) of *nux vomica* that was treated with sand-processing significantly inhibited the writhing behavior, while those of *nux vomica* that was unprocessed or treated with oil- or vinegar and sand-processing and morphine were without effects at a dose of 1 $\mu\text{g/kg}$. The present results demonstrate the antinociceptive effects of the CAF of *nux vomica* and suggest that sand-processing is good for the analgesic potency of *nux vomica*. It is also suggested that the CAF of *nux vomica* has distinct antinociceptive potency, even after treatment with licorice-, oil-, vinegar and sand-processing.

Key words *Strychnos nux-vomica*; drug-processing; strychnos alkaloid; morphine; antinociception

Nux vomica, the dried seed of *Strychnos nux-vomica* L., is used clinically for relieving pain, promoting blood circulation, alleviating blood stasis and curing indigestion.¹⁾ It is included as an ingredient in many analgesic prescriptions of traditional Chinese medicine such as “maqian-zi-san,” “shu-feng-ding-tong-pain,” “fei-bu-wan,” etc. For administration to internal medicine patients, *nux vomica* is generally used after appropriate processing because it is extremely poisonous. For example, when taken orally, 7 raw seeds of *S. nux-vomica* could be lethal for adult humans.²⁾ Processing methods such as the parching of *nux vomica* in a sand bath is described in the Pharmacopoeia of the People’s Republic of China (P.R.C.) and that of frying it in sesame oil was described in the Pharmacopoeia before 1963. Processing methods such as boiling it in a licorice decoction, storage in vinegar and storage in urine have been traditionally carried out in various provinces and cities in China.^{3,4)}

Alkaloids are the major indicative components in the processed and unprocessed seeds of *S. nux-vomica*, as described in the Pharmacopoeia of P.R.C. In our previous papers,⁵⁾ we reported the structures of strychnos alkaloids isolated from the seeds processed with a sand bath, and the contents of 12 alkaloids and an irridoid glycoside in the seeds processed by seven different methods. Furthermore, we compared the acute toxicities of the crude alkaloid fractions from the processed seeds with that of the unprocessed seeds. LD₅₀ values of the seeds treated with seven different processing methods ranged from 2.18—2.57 mg/kg in the mouse, while that of the unprocessed seeds was 1.21 mg/kg. Thus, the processing of

nux vomica decreased the toxicity down to one-half that of the unprocessed substance. However, it was not known whether the pharmacological activities of *nux vomica* were altered by the processing. Since analgesic action is one of the characteristic clinical effects of *nux vomica* in traditional Chinese medicine, and strychnos alkaloids are major constituents in the seeds, we investigated the antinociceptive effects of the crude alkaloid fractions from processed or unprocessed *nux vomica*.

MATERIALS AND METHODS

Materials The seeds of *S. nux-vomica* were supplied by Nanjing Company of Chinese Herbal Drugs (Nanjing, China). Urine was collected at 8:00 a.m. from five male kindergarten children. Their health conditions had been previously examined by a medical doctor. The vinegar was purchased from a food store and morphine hydrochloride from Sankyo Co., (Tokyo).

Animals Male ddY mice, 4—5 weeks of age, weighing 21—28 g, were used in the experiments. They were housed under controlled temperature (23—25 °C) and light (lights on from 0800 to 2000). Food and water were freely available.

Processing of Seeds of *S. nux-vomica* The methods of drug-processing, which were carried out on a laboratory scale using the same batch of raw seeds of *S. nux-vomica*, were described in detail in the previous paper.⁵⁾ The methods are briefly mentioned as follows: 1) Sand-processing: the raw seeds were heated in a sand bath up to 230 °C for 3 min. 2) Licorice-processing: the raw seeds

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were boiled in a licorice decoction for 4 h. 3) Oil-processing: the raw seeds were heated in sesame oil at 230 °C for 3 min. 4) Vinegar-processing: the raw seeds were kept in water for 2 d, cut into 1.5 mm-thick slices, and then kept in vinegar for 5 d at room temperature. 5) Vinegar and sand-processing: the raw seeds were kept in water for 2 d, sliced, and put into vinegar, in which they were boiled for 10 min and then kept for 12 h at room temperature. The seeds were further processed using the same procedures as the sand-processing. 6) Urine-processing: the raw seeds were kept in fresh urine, from healthy children, in a cool place for 49 d. 7) Urine and sand-processing: the raw seeds were kept in the fresh urine for 7 d and processed using the same procedures as the sand-processing. The raw seeds (unprocessed seeds) and processed seeds were cut into slices and then dried at 80 °C.

Preparation of Crude Alkaloid Fraction Slices of processed or unprocessed seeds of *S. nux-vomica* were ground into a fine powder, which was passed through a sieve (20 mesh). Each powder sample (200 g) was put into a conical flask with a plug, moistened with 15% aqueous ammonia (20 ml) and kept for 2 h. The moistened powder was soaked in chloroform (600 ml), thoroughly stirred, and then kept at room temperature for 24 h with occasional stirring. The solution was filtered and the residue was re-extracted with chloroform (600 ml) in a similar manner. The resulting residue was further re-extracted three times with chloroform (50 ml each). The combined solution was concentrated *in vacuo* to a volume of 40 ml. The solution was extracted six times with 1 N HCl (50, 50, 35, 35, 20 and 20 ml each). The combined acidic solutions were adjusted to pH 11–12 with 40% NaOH and extracted six times with chloroform (100 ml each). The combined chloroform solutions were evaporated *in vacuo* to give a crude alkaloid fraction. The compositions of 12 alkaloids in each crude alkaloid fraction were determined by thin-layer chromatography-densitometry according to the previously reported method.⁵⁾ The percentages of the respective alkaloids were as follows: strychnine, 53.7–55.0% of each alkaloid fraction; brucine, 21.2–27.4%; colubrine, 2.8–5.0%; vomicine, 2.1–4.2%; pseudo-strychnine, 1.1–1.8%; brucine *N*-oxide, 0.25–3.03%. Detailed analytical procedures and results will be described in the next paper. The yields of crude alkaloid fractions were described in the previous paper.⁵⁾

Drug Administration The crude alkaloid fractions were dissolved in a small amount of 0.5 M H₃PO₄ and then adjusted to pH 6.5 with 0.5 M NaOH and 0.5 M H₃PO₄. After dilution to an appropriate concentration with 0.1 M phosphate buffer (pH 6.5), they were i.p. administered in a volume of 0.01 ml/g body weight. Morphine hydrochloride was dissolved in 0.9% saline and s.c. administered in a volume of 0.01 ml/g.

Tail-Pressure Test⁶⁾ Male ddY mice (5 weeks old) were used. The nociceptive threshold of the tail for mechanical stimulation was measured using a pressure analgesimeter (Ugo Basile, Milan, Italy) with a loading rate of 48 g/s. The nociceptive response was defined as the elicitation of either a struggle or of turning to bite. Prior to the experiments, the mouse was exposed to the procedure three times a day for 2 d before the test. The cutoff threshold

was 600 g and the mice that had immediately responded to a pressure of 300 g were used in the experiments. Before injection, the nociceptive threshold was determined three times at 20-min intervals and their average served as a pre-injection value.

Hot-Plate Test⁷⁾ Male ddY mice (4 weeks old) were used. The animal was placed on a hot plate (Ugo Basile, Milan, Italy) maintained at a temperature of 53 ± 0.2 °C. Latency of nociceptive responses, such as licking of either hind paw or jumping, was measured. This test was done twice a day for 2 d before the experiments for acclimation. The mice that did not show any nociceptive responses within 45 s were discarded from experiments. On the day of the experiment, nociceptive latency was determined three times at 20-min intervals, and the average of the last two determinations served as a pre-injection value.

Acetic Acid-Induced Writhing Test⁸⁾ Male ddY mice (5 weeks old) were given an i.p. injection of 0.6% acetic acid at a volume of 0.01 ml/g 15 min after the drug administration. The number of writhing behaviors was counted for 20 min after the injection of acetic acid. The number of writhings of the vehicle-treated group was taken as control and the percentage inhibition in each animal was calculated.

Statistical Analysis Data was presented as means and S.E. Statistical analysis was performed using Bonferroni multiple comparisons. Differences were considered significant at $p < 0.05$.

RESULTS

Antinociceptive Effects in Tail-Pressure Test Although i.p. administration of the vehicle was without effects on the nociceptive threshold, the crude alkaloid fraction from the unprocessed seeds produced a dose-dependent antinociception at i.p. doses of 0.01 and 0.1 µg/kg (Figs. 1a and 2a). The effect of a dose of 1 µg/kg was not larger than that of 0.1 µg/kg (Figs. 1a and 2a). These effects peaked at 15 or 30 min and almost subsided at 60 min after administration (Fig. 1a). The crude alkaloid fraction from the seeds treated with the sand-processing produced a dose-dependent antinociception at i.p. doses of 0.01–1 µg/kg (Fig. 1b) and the potency was similar to that of the unprocessed seeds (Fig. 2a). These effects peaked at 15 or 30 min and almost subsided at 120 min after administration (Fig. 1b). Higher doses of 23 and 230 µg/kg did not produce larger effects than that of 1 µg/kg (data not shown). The crude alkaloid fraction from the seeds treated with licorice-processing produced apparent antinociceptive effects at i.p. doses of 0.1 and 1 µg/kg, although a dose of 0.01 µg/kg was without significant effects (Figs. 1c and 2b). The effects peaked at 15 min after administration and almost subsided at 120 min (Fig. 1c). The crude alkaloid fraction from the seeds treated with oil-processing produced significant antinociception at i.p. doses of 0.01–1 µg/kg, although the peak effects observed at 30 min were not apparently different among the doses tested (Figs. 1d and 2b). The crude alkaloid fraction from the seeds treated with vinegar and sand-processing produced a dose-dependent antinociception at i.p. doses of 0.01–1 µg/kg (Figs. 1e and 2c). The effects peaked at 15 or 30 min after

Fig. 1. Effects of Crude Alkaloid Fractions of Nux Vomica and Morphine on the Nociceptive Threshold of the Mouse in the Tail-Pressure Test

The mouse was given an i.p. injection of crude alkaloid fractions of nux vomica that was (a) unprocessed, or treated with (b) sand-, (c) licorice-, (d) oil- or (e) vinegar and sand-processing or an s.c. injection of morphine. ○, vehicle ($n=12$); ●, 0.01 µg/kg ($n=8$); ▲, 0.1 µg/kg ($n=8$); ■, 1 µg/kg ($n=8$); □, 2 mg/kg ($n=8$). The values are means and S.E. * $p < 0.05$ when compared with vehicle.

administration and subsided at 120 min (Fig. 1e). The peak effect of 1 µg/kg was most apparent among the crude alkaloid fractions examined. The crude alkaloid fraction from the seeds treated with vinegar-processing produced significant antinociception at an i.p. dose of 1 µg/kg, without effects at doses of 0.01 and 0.1 µg/kg (Fig. 2c). The crude alkaloid fractions from the seeds treated with urine-processing or urine and sand-processing were without effects at i.p. doses of 0.01–1 µg/kg (Fig. 2d). None of these crude alkaloid fractions at doses of 0.01–1 µg/kg produced apparent alterations in gross behaviors of the mouse. Morphine at an s.c. dose of 2 mg/kg produced a short-lasting antinociception without effects at a dose of 1 µg/kg (Figs. 1f and 2a).

Antinociceptive Effects in Hot-Plate Test The sand-processing is a processing method described in the present

Pharmacopoeia of P.R.C. and oil-processing was described in the Pharmacopoeia before 1963. The crude alkaloid fraction from the seeds treated with vinegar and sand-processing showed the most potent antinociceptive effect in the tail-pressure test. Therefore, we compared the antinociceptive effects of crude alkaloid fractions from the processed and unprocessed seeds in the hot-plate test. The crude alkaloid fraction from the seeds treated by the sand-processing elicited significant antinociception at an i.p. dose of 100 µg/kg without effects at lower doses of 0.01 and 1 µg/kg (Fig. 3). Crude alkaloid fractions from the unprocessed seeds and the seeds treated with oil-processing or vinegar and sand-processing were without effects at i.p. doses of 0.01–100 µg/kg (Fig. 3). The crude alkaloid fractions from these seeds did not produce apparent alterations in gross behaviors of the mouse at a

Fig. 2. Dose-Response Curves for Antinociceptive Effects of Crude Alkaloid Fractions of Nux Vomica in the Tail-Pressure Test

The mouse was given an i.p. injection of crude alkaloid fractions of nux vomica that was unprocessed or treated with sand-, licorice-, oil-, vinegar and sand-, vinegar-, urine and sand- or urine-processing or an s.c. injection of morphine. The percentage increase in the nociceptive threshold for 15 min after injection was plotted against the dose. The values are means and S.E. of 12 (vehicle: VEH) or 8 (the others) experiments. * $p < 0.05$ when compared with VEH.

Fig. 3. Effects of crude Alkaloid Fractions of Nux Vomica and Morphine on Nociceptive Latency of the Mouse in the Hot-Plate Test

The mouse was given an i.p. injection of vehicle or crude alkaloid fractions of nux vomica that was unprocessed or treated with sand-, oil- or vinegar and sand-processing or an s.c. injection of morphine. The ordinate represents the percentage increase in the nociceptive latency for 30 min after injection. The values given are means and S.E. of 15 (vehicle) or 8 (the others) experiments. * $p < 0.05$ when compared with vehicle.

dose of 100 µg/kg. Morphine was also without effects at subcutaneous doses of 1 and 100 µg/kg (Fig. 3).

Antinociceptive Effects in Acetic Acid-Induced Writhing Test The crude alkaloid fraction from the seeds treated with the sand-processing (1 µg/kg, i.p.) produced a significant inhibition of writhing behaviors induced by

Fig. 4. Effects of Crude Alkaloid Fractions of Nux Vomica and Morphine on the Writhing Behaviors Induced by Acetic Acid

The mouse was given an i.p. injection of vehicle or crude alkaloid fractions (1 µg/kg) of nux vomica that was unprocessed or treated with sand-, oil- or vinegar and sand-processing or an s.c. injection of morphine (1 µg/kg) 15 min before the injection of acetic acid. The ordinate represents the percentage inhibition of writhing behavior as compared with vehicle-treated group. The values given are means and S.E. of 15 (morphine) or 8 (the others) experiments. * $p < 0.05$ when compared with vehicle.

acetic acid (Fig. 4). There was a tendency toward inhibition of writhing behavior following administration of the crude alkaloid fractions from the unprocessed seeds and the seeds treated with oil-processing or vinegar and sand-processing at a dose of 1 µg/kg (Fig. 4). Morphine was without effect at a dose of 1 µg/kg (Fig. 4).

DISCUSSION

The crude alkaloid fractions from the sand-processed seeds of *S. nux-vomica* at doses of 0.01–1 µg/kg produced a dose-dependent antinociceptive effect in the tail-pressure test in mice. In contrast to this crude alkaloid fraction from nux vomica, morphine at a dose of 1 µg/kg was without effects, although a higher dose of 2 mg/kg produced short-lasting antinociception. This crude alkaloid fraction at a dose of 1 µg/kg produced an increase in the nociceptive threshold similar to that induced by morphine at a dose of 2 mg/kg. Thus, this crude alkaloid fraction might be about 1000 times more potent than morphine, although higher doses did not produce larger effects.

The antinociceptive potency of the crude alkaloid fraction of the sand-processed seeds was similar to that of the unprocessed seeds. On the other hand, the LD₅₀ value of the former (2.35 mg/kg) is about twice that of the latter (1.21 mg/kg).⁵⁾ Therefore, it is suggested that the sand-processing doubled the therapeutic index (to the suppression of mechanical nociception) of the crude alkaloid fraction of nux vomica. In this context, it should be noted that although the crude alkaloid fraction from the unprocessed seeds was without significant effects at doses examined in the hot-plate and acetic acid-induced writhing tests, that of sand-processed seeds showed significant antinociception in either analgesic test. Thus, sand-processing, which is mentioned in the present Pharmacopoeia of P.R.C., may be good for reducing the toxicity.

With regard to the effect of drug processing on the antinociceptive potency in the tail-pressure test of crude alkaloid fraction, the crude alkaloid fractions from the seeds treated with licorice-processing or vinegar and sand-processing also produced distinct antinociception. The crude alkaloid fractions from the seeds treated with oil-processing or vinegar-processing showed antinociception only at the highest dose tested (1 µg/kg). On the other hand, the crude alkaloid fractions from the seeds treated with urine-processing or urine and sand-processing were without effects at doses tested. Urine-processing and urine and sand-processing have been used for a long time as traditional processing methods in China. Although these processing methods reduce the toxicity of the crude alkaloid fraction of nux vomica,⁵⁾ the present results suggest the possibility that these processing methods also reduce the pharmacological potency. It is unclear why these seven kinds of processing altered the antinociceptive potency of the strychnos alkaloid fraction. However, these processing methods altered the composition of strychnos alkaloids^{5,9)} and produced novel alkaloids.^{10,11)} Thus, it is conceivable that alterations in composing alkaloids were responsible for changes in antinociceptive potency. To test this issue, we are examining the antinociceptive effect of each alkaloid contained in the crude alkaloid fractions of nux vomica.

The crude alkaloid fractions of nux vomica unprocessed or treated with sand-, oil- or vinegar and sand-processing were more effective in the tail-pressure test (mechanical noxious test) than in either the hot-plate test (thermal noxious test) and acetic acid-induced writhing test

(chemical noxious test). The reasons for these differences are unclear. Although some neuropeptides, such as substance P, somatostatin, galanin and calcitonin gene-related peptide, are involved in nociceptive transmission and regulation in the spinal dorsal horn, substance P^{12–14)} and galanin^{14,15)} are more closely associated with mechanical nociception than with thermal nociception. In addition, the descending noradrenergic systems play a more important role in mechanical antinociception than in thermal antinociception.^{16–18)} Therefore, if an inhibition in nociceptive transmission mediated by substance P or galanin and the enhancement of the descending noradrenergic system are involved in the antinociception of the crude alkaloid fractions of nux vomica, this antinociception might be more apparent in mechanical nociception than in thermal nociception. Investigation into the antinociceptive effects of each strychnos alkaloid may also be needed to examine this issue.

In summary, we demonstrated the antinociceptive effects of crude alkaloid fractions of processed and unprocessed nux vomica. Sand-processing may be good for the analgesic potency of nux vomica, because the crude alkaloid fraction of nux vomica treated with such processing showed distinct antinociceptive effects in all analgesic tests examined.

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