

Cytotoxicity Assessment of Ma-huang (*Ephedra*) under Different Conditions of Preparation

M. K. Lee,* B. W. H. Cheng,† C. T. Che,† and D. P. H. Hsieh*¹

Departments of *Biology and †Chemistry, The Hong Kong University of Science and Technology, Hong Kong

Received March 6, 2000; accepted May 8, 2000

Ma-huang is a traditional Chinese medicinal herb derived from *Ephedra sinica* Stapf and other *Ephedra* species, used to treat asthma, nose and lung congestion, and fever with anhidrosis. It contains 0.5–2.5% by weight of total alkaloids, of which ephedrine accounts for 30 to 90%. Recently, large amounts of ma-huang were used as a source of ephedrine in many dietary supplements formulated for weight reduction, because ephedrine has been found effective in inducing weight loss in diet-restricted obese patients. However, indiscriminate consumption of ma-huang-containing products has resulted in many cases of poisoning, some of which were fatal. The objective of this study is to investigate the relative toxicity of ma-huang extracted under different conditions. The toxicities of various extracts were assayed using MTT colorimetry on a battery of cell lines, while ephedrine alkaloids were analyzed with HPLC. The results are summarized as follows. (1) The cytotoxicity of all ma-huang extracts could not be totally accounted for by their ephedrine contents, suggesting the presence of other toxins in the extracts. (2) Grinding was a significant condition enhancing the toxicity of the extracts. (3) The relatively high sensitivity of the Neuro-2a cell line to the toxicity of ma-huang extracts suggests that the toxic principles were acting on neuronal cells. (4) One condition to produce a ma-huang extract with high ephedrine-to-toxins ratio would be to boil the whole herb for two h.

Key Words: cytotoxicity; *Ephedra*; ephedrine; ephedrine-to-toxins ratio; herb preparations; ma-huang; MTT colorimetry.

Ma-huang (*Ephedra*) is a traditional Chinese medicinal herb derived from the stems and branches of *Ephedra sinica* Stapf and other *Ephedra* species (Chung-hua Jen Min Kung Ho Kuo Wei Sheng Pu Yao Tien Wei Yuan Hui, 1995). It has been used to treat asthma, nose and lung congestion, and fever with anhidrosis for centuries (Blumenthal and King, 1995; Huang, 1993; Lou *et al.*, 1995; Yang, 1993). In recent years, ma-huang was sold in large quantities worldwide. About 100,000 kg of ma-huang powders and extracts were imported into the United States in 1993 alone (Hurlbut *et al.*, 1998).

¹ To whom correspondence should be addressed at the Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, SAR, The People's Republic of China. Fax: (852) 2358-1559. E-mail: dphsieh@ust.hk.

Ephedrine-type alkaloids (ETA) are the active principles of ma-huang. Within these alkaloids, (-)-ephedrine (E) is the most abundant, constituting between 30 and 90% of the total alkaloid content (Tyler, 1993). Other ETA include (+)-pseudoephedrine (PE), (-)-N-methylephedrine (NME), (+)-N-methylpseudoephedrine (MPE), (-)-norephedrine (NE), and (+)-norpseudoephedrine (NPE). The acute thermogenic and lipolytic effects of E on basal metabolism and/or on diet-induced thermogenesis have now been confirmed in lean, obese and post-obese humans. The chronic administration of E in clinical trials has been reported to induce weight loss in diet-restricted obese patients (Astrup *et al.*, 1992; Dulloo and Stock, 1993). Large amounts of ma-huang have been recently used as a source of E in numerous dietary supplements formulated for weight reduction. However, the U.S. Food and Drug Administration has received many reports of poisoning and other serious side effects caused by the indiscriminate consumption of these ma-huang-containing products (FDA, 1997).

As ETA are the active principles, the quality of ma-huang is determined by the contents of total ETA, with higher contents indicating better quality. However, this practice of grading using only the total ETA contents as sole parameter is inadequate. The profile of alkaloids is also important, because although individual ETA has similar pharmacological activity, they vary significantly in potency (Cetaruk and Aaron, 1994; Dollery, 1991). Both the contents and the profile of ETA in ma-huang vary with plant species and varieties (Cui *et al.*, 1991; Liu *et al.*, 1993; Zhang *et al.*, 1989), plant parts, (Chen and Schmidt, 1930; Liu *et al.*, 1993), sex, seasons of harvest (Chen and Schmidt, 1930; Kasahara *et al.*, 1986) and geographical origins (Zhang *et al.*, 1989). In order to accurately evaluate the quality of the crude drug, the quantitative analysis of individual ETA in ma-huang is urgently needed.

In addition to ETA, ma-huang also contains other phytoconstituents, which may modify its pharmacological and toxicological activities. Therefore, the toxicity of ma-huang, and hence the more than 800 reports of adverse effects received by the U.S. FDA associated with the use of ma-huang-containing dietary supplements, cannot be totally accounted for by its ETA contents alone (FDA, 1997). A bioassay is needed to

determine the total toxicity of ma-huang due to the combined effect of the alkaloids and other constituents.

In all of the published studies reviewed, the ma-huang samples were ground before extraction. This preparation method is similar to that used in preparing the marketed forms of ma-huang as they are exported from China to foreign countries, either as powder or as concentrated extract. However, these practices are different from the traditional use of ma-huang as documented in the Chinese literature (e.g., Chung-hua Jen Min Kung Ho Kuo Wei Sheng Pu Yao Tien Wei Yuan Hui, 1995). Traditionally, ma-huang was known to be used in whole-herb form and to be extracted for a longer period of time than other herbs in a prescription (Jan, 1993; Yang, 1993). Also, with reference to *Shang Han Lun*, a classical Chinese pharmacopoeia, all herbs were only to be extracted once and then the extract was taken, without extracting the herbs any further (Wang, 1988). However, the current practice is that all herbs are extracted twice and pooled together before taking. There has been no information found regarding the effects of these different ways of preparation on the biological activities of ma-huang.

In the present study, ma-huang was extracted with water under different conditions to examine the effect of several parameters, including grinding, boiling for 0.5 or 2 h, and extracting once or twice, on toxicity and drug extraction efficiency. For each extract, cytotoxicity to a battery of cell lines was measured using MTT colorimetry, and the contents of E, PE, NME, NE, and NPE were analyzed by HPLC. Having both the E (drug) contents and the cytotoxicity data, an optimal extraction process was identified in which a high drug-to-toxin ratio (DTR) was achieved.

MATERIALS AND METHODS

Materials. Ma-huang, originating from the Hu-pei Province of China, was obtained from local vendors. (-)-Ephedrine hydrochloride, PE, NME, (-)-norephedrine hydrochloride and (+)-norpseudoephedrine hydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (TLC grade), acetonitrile (HPLC grade), dimethylsulfoxide (DMSO) (reagent grade), phosphoric acid (AR grade), and sodium dodecylsulfate (electrophoresis reagent grade) were from Sigma (St Louis, MO, USA). Materials for tissue cultures were from Gibco-BRL (Grand Island, NY, USA). ELISA reader MR5000 was from Dynatech (Denkendorf, Germany). MTT powder was dissolved in Dulbecco's PBS to form a stock solution of MTT (5 mg/mL). The stock solution was filter-sterilized through a 0.20 μm filter and stored at -20°C .

Sample preparation. The whole herb of ma-huang was ground into powdered form using a laboratory mill. Each 20.0 g aliquot of ma-huang, powdered or whole herb, was refluxed in 200 mL of double-distilled water for 0.5 h or 2 h. The extracts were collected and centrifuged twice at 4000 rpm at 20°C for 20 min to obtain the supernatant. These supernatants were named G1, G3, NG1, and NG3, respectively. Subsequently, the residue of each extraction was refluxed in another 200 mL of double-distilled water for another 0.5 h or 2 h. The extracts were collected and centrifuged twice at 4000 rpm at 20°C for 20 min to obtain the supernatant. These supernatants were named G2, G4, NG2 and NG4, respectively. All eight extracts were evaporated to dryness by rotary evaporation at 58°C and 0.125 atm, and reconstituted in Dulbecco's PBS to 90% of the final volume. These reconstituted extracts were neutralized to pH

TABLE 1
Ma-huang Extracts Prepared under Different Conditions

Sample	Extraction parameters
G1	G + 0.5 h ^a + 1st ^b
G2	G + 0.5 h + 2nd ^c
G3	G + 2 h ^d + 1st
G4	G + 2 h + 2nd
NG1	NG + 0.5 h + 1st
NG2	NG + 0.5 h + 2nd
NG3	NG + 2 h + 1st
NG4	NG + 2 h + 2nd

Note. G, ground; NG, not ground (whole herb).

^aBoiled for 0.5 h.

^bFirst extract.

^cSecond extract.

^dBoiled for 2 h.

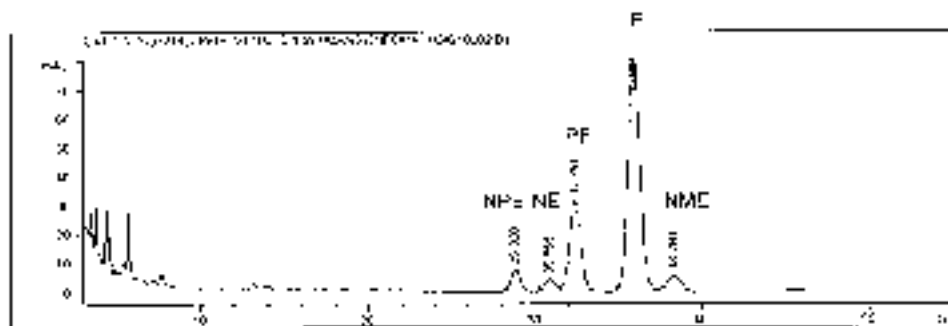
7.4 and centrifuged twice at 4000 rpm at 20°C for 20 min to obtain the supernatant. Finally, all extracts were adjusted to a final volume of 20 mL (to attain a 1-g herb equivalent/mL) and stored at -20°C . At use, the frozen extracts were thawed until they reached room temperature. Table 1 shows the nomenclature of ma-huang extracts prepared under different conditions.

Apparatus and conditions. The HPLC instrument used was a Hewlett-Packard HPLC Model 1100 (Palo Alto, CA, USA) equipped with a Hewlett-Packard 1100 vacuum de-gasser, quaternary pump, autosampler, column compartment, and diode-array detector. Separation was performed on a Restek Inertsil[®] ODS-2 column (4.6 \times 150 mm, 5 μm particle size; FOB Bellefonte, PA) equipped with Hewlett-Packard Zorbax[®] ODS (C18) guard column (4.6 \times 12.5 mm, 5 μm particle size). The HPLC column was equilibrated with a mobile phase containing acetonitrile-water-sodium dodecylsulfate-phosphoric acid (350 mL:650 mL:5 g:1 mL), at pH 2.42. The wavelength of the UV detector was set at 214 nm. After filtering with 0.2 μm PTFE pore filters, 1 μL of each ma-huang extract was injected into the HPLC system. The column temperature was held at 30.0°C and isocratic flow rate was maintained at 1 mL/min.

Cell cultures. Human hepatoblastoma cell line (HepG2) (ATCC# HB8065) and mouse neuroblastoma cell line (Neuro-2a) (ATCC# CCL131) were cultured in RPMI Medium 1640. Mouse fibroblastoma cell line LM(TK-) expressing human $\alpha 1\text{b}$ and human $\alpha 2\text{A}$ adrenergic receptors (L-alpha-1b and L-alpha-2A) (ATCC# CRL11139 and CRL11180) were cultured in Dulbecco's Modified Eagle Medium (DMEM). Chinese hamster ovary cell line CHO-K1 expressing rat $\beta 3$ adrenergic receptors (CHO-beta3) (ATCC# CRL11628) was cultured in F12 Nutrient Mixture (F12). All culture media were supplemented with 10% fetal bovine serum, 1% antibiotic and antimycotic solution (50,000 units/L of penicillin and 50 mg/L of streptomycin), and 2mM glutamine. Cells were incubated at 37°C in 5% carbon dioxide at 95% humidity.

MTT assay. Cytotoxicity was determined by the MTT dye-reduction assay. The methodology described is a modification of the original MTT colorimetric assay developed by Mosmann (1983). Cells were harvested from maintenance cultures in the exponential phase and counted by a hemocytometer using trypan blue solution. The cell suspensions were dispensed (75 μL) in triplicate into 96-well culture plates at optimized concentrations of 1.5×10^5 cells/mL for HepG2, 2×10^4 cells/mL for L-alpha-1b and L-alpha-2A, 5×10^3 cells/mL for CHO-beta3, and 8×10^4 cells/mL for Neuro-2a, respectively. After a 48-h recovery period, the ETA standards or ma-huang extracts diluted with medium (25 μL) were added. For median inhibition concentration (IC_{50}) determination, dose-response curves were conducted with a series of different concentrations of alkaloids or ma-huang extracts that were approximately equal to the IC_{50} . To control wells, only culture medium (15

FIG. 1. The HPLC profile of ephedrine-type alkaloids in a typical ma-huang extracts. E: (-)-ephedrine, PE: (+)-pseudoephedrine, NME: (-)-N-methylephedrine, NE: (-)-norephedrine, and NPE: (+)-norpseudoephedrine.



μL) with vehicle (10 μL PBS) was added. After an additional 72-h incubation period, the medium in each well was aspirated and replaced with 110 μL of MTT working solution (MTT stock solution mixed with medium to attain a final concentration of 0.5 mg/mL). The cells were incubated at 37°C for 4 h, and then the medium was aspirated and replaced with 100 μL DMSO to dissolve the formazan crystals formed. The culture plates were shaken for 5 min and the absorbance of each well was read at 570 nm with 750 nm as the reference wavelength.

The relative viability of the treated cells as compared to the control cells was expressed as the % cytoviability, using the following formula:

$$\% \text{ cytoviability} = [A_{570} \text{ of treated cells}] \times 100\% / [A_{570} \text{ of control cells}].$$

IC_{50} was then determined by nonlinear regression analysis of the corresponding dose response curve.

Statistical analysis. Data is presented as either means \pm standard deviation (SD) or means \pm standard error of the mean (SEM). Statistical analysis was performed using either paired *t*-test or analysis of variance (ANOVA). For ANOVA, pairwise comparisons between treatments were made using Tukey's Multiple Test Comparison.

RESULTS AND DISCUSSION

ETA in Ma-huang Extracts Prepared under Different Conditions

The HPLC profile of ETA in a typical ma-huang extract is shown in Figure 1. All 5 ETA, which were detectable in the water extract of ma-huang, could be resolved within 40 min. The detection limit of our HPLC system for each individual ETA was 4 ng. After substitution of peak areas for the corresponding regression equations of the ETA standards, the E, PE, NME, NE, and NPE contents of G1 to G4 and NG1 to NG4 were quantified (Fig. 2). It was found that the contents of all ETA in G1, G3, and NG3 were the highest among the 8 different preparations. For the ground ma-huang extracts (G1 through G4), the majority of ETA were extracted in the first extraction, except for NME which was fully extracted in the first extraction. Boiling the whole herb of ma-huang for 0.5 h (NG1 and NG2) could only extract a portion of ETA. When the boiling time was extended to 2 h (NG3 and NG4), much greater amounts of ETA were extracted. The results indicate that boiling the unground whole herb of ma-huang for a longer period could yield comparable amounts of ETA, as produced from the ground herb.

When the first and second extracts were pooled together, the yields of total ETA from the 4 preparation conditions were within 15% of difference, with the process of boiling the whole NG3+4 herb for 2 h containing the highest amount of alkaloids (0.902% of herb) (Fig. 3). This alkaloid content was close to that required of a good-grade ma-huang, which should yield 1–2% by weight, of total alkaloids (Lundstrom, 1989).

For chemical analysis of ETA in ma-huang, ethanol, which yields greater amounts of ETA, was usually used as the solvent. The solubility of E in water is 50 g/L, while it is completely soluble in ethanol (Richardson and Gangolli, 1992). Sagara *et al.* (1983) have shown that an extracting solvent made up of acetonitrile-water-sodium dodecylsulfate-phosphoric acid (35:65:0.5:0.1) was more efficient in extracting ETA from ma-huang than either water or methanol alone, with water being the least efficient. Therefore, if our ma-huang samples were extracted with other organic solvents, the yields of ETA might have been higher. However, in this study, ma-huang was extracted with water to simulate the traditional use of the herb as a TCM constituent. In addition, water is non-toxic to cells used in the MTT assays while organic solvents are mostly toxic.

In terms of E alone, the total amount obtained in 2 extractions ranged from 69.1 (NG3+4) to 69.9% (NG1+2) of the total ETA. This amount was within the range reported in the literature (30 to 90%) (Tyler, 1993). In a study by Tyler (1994), a tea prepared by steeping 2 g of ma-huang in 240 mL of boiling water for 10 min yielded a dose of 15 to 30 mg of E. In our study, 20.0 g of ma-huang was boiled in 200 mL of water, equivalent to steeping 2.0 g of ma-huang in 20 mL of water. The total amount of E obtained in two extractions ranged from 5.37 (NG1+2) to 6.23 (NG3+4) mg E/g herb. Hence, in our case, 2 g of ma-huang yielded 10.7 to 12.5 mg of E, which is less than those found by Tyler (1994), probably due to the smaller volume of water used in extraction in our study.

Cytotoxicity of E and Ma-huang Extracts Prepared under Different Conditions

Cytotoxicity to a battery of cells was evaluated by determining the IC_{50} values of different preparations to the cells. Using

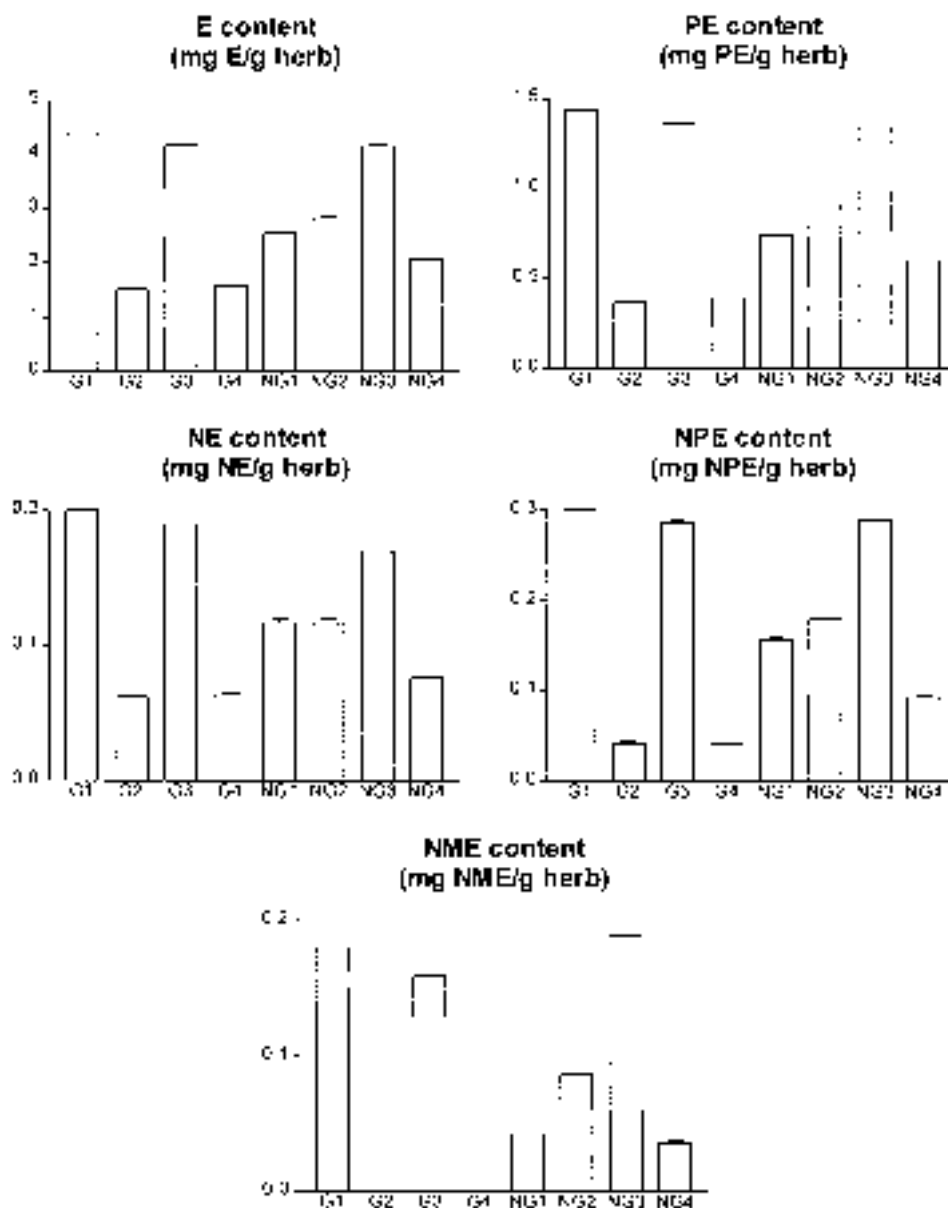


FIG. 2. The contents of ephedrine-type alkaloids in ma-huang extracts prepared under different conditions. Values are means and S. D. obtained from three replicate injections (some S. D.'s are too small to see). E: (-)-ephedrine, PE: (+)-pseudoephedrine, NME: (-)-N-methylephedrine, NE: (-)-norephedrine, and NPE: (+)-norpseudoephedrine.

nonlinear regression analysis of the corresponding dose-response curves, the IC_{50} values of E and ma-huang extracts prepared under different conditions for a battery of cell lines were obtained and are shown in Figure 4. Tukey's Multiple Test Comparison showed that the IC_{50} values of E and ground ma-huang extracts (G1 through G4) were significantly lower than those of whole herb extracts (NG1 through NG4), with $p < 0.001$ for L-alpha-2A, $p < 0.01$ for HepG2, and $p < 0.05$ for L-alpha-1b, CHO-beta3, and Neuro-2a. The results indicate that E and ground ma-huang extracts were significantly more cytotoxic than the unground preparations. Grinding was found to be a crucial condition for increased toxicity.

With reference to the adverse events associated with ma-huang-containing dietary supplements, approximately 60% of the adverse events were characterized as general stimulatory effects on the cardiovascular and nervous systems of a "less clinically serious" nature, including anxiety, nervousness, hyperactivity, tremor, insomnia, and altered heart rate or rhythms (FDA, 1997). Approximately 16% of the reports mentioned serious nervous system effects, including seizure, psychosis, mania, severe depression, vestibular disturbances, and loss of consciousness (Capwell, 1995; FDA, 1997). Therefore, Neuro-2a was used in the cytotoxicity assay. Neuro-2a is also frequently used in the cytotoxicity test for other chemicals with

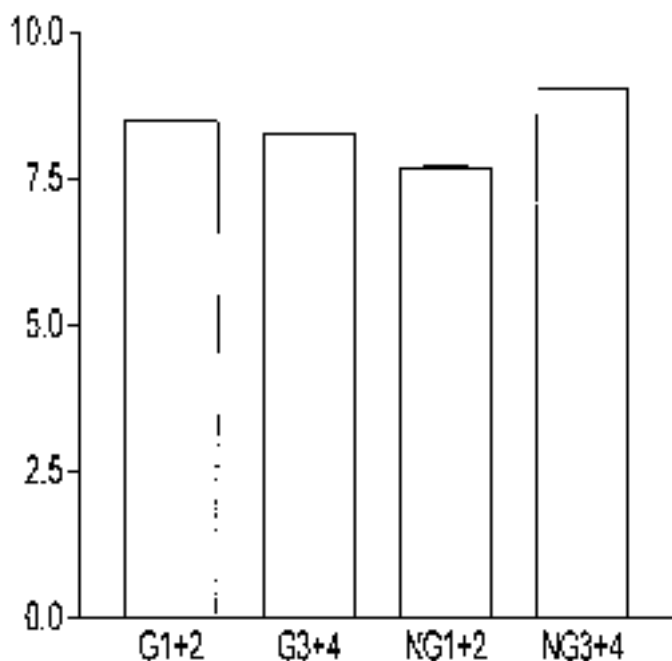


FIG. 3. The total combined ephedrine-type alkaloids contents (mg E alkaloid/g herb) of first and second extracts of ma-huang prepared under different conditions. Values are means and S. D. obtained from three replicate injections.

neurological effects (De Ferrari *et al.*, 1998; Manger *et al.*, 1993; Walker *et al.*, 1995).

Among the adverse events associated with ma-huang-containing dietary supplements, 4 percent of the reports mentioned overt hepatitis (FDA, 1997; Nadir *et al.*, 1996). This observation prompted us to include HepG2 in the cytotoxicity assay, a substance widely used in cytotoxicity testing. Comparison with freshly isolated human adult hepatocytes has revealed that activities of cytochrome P-450-dependent, mixed-function oxidase (MFO) of HepG2 are 5–10-fold lower than in primary hepatocytes (Grant *et al.*, 1988). However, the activities of their conjugating enzymes, UDP-glucuronyltransferase (GT) and glutathione-S-transferase (GST), were similar (Duthie *et al.*, 1988). It was found that HepG2 retains many of the specialized functions normally lost in established hepatocytes in culture (Knowles *et al.*, 1980).

The cell lines L-alpha-1b, L-alpha-2A and CHO-beta3 were included in the MTT assay, as each of them expresses alpha-1, alpha-2, and beta-3 adrenergic receptors respectively, on which E basically acts.

While there were no significant differences in the IC_{50} values of E for different cell lines, the IC_{50} values of ma-huang extracts prepared under different conditions for Neuro-2a were significantly lower than those for L-alpha-1b and L-alpha-2A (both with $p < 0.05$), indicating that Neuro-2a cells were more sensitive to ma-huang extracts when compared with mouse fibroblastoma cells. This suggests that the toxins in ma-huang extracts may be more specific to neuronal cells. This result was

consistent with the adverse events associated with dietary supplements containing ma-huang, as mentioned in the foregoing paragraphs. This difference in response to the same herbal extract suggests that using a battery of cell lines from different origins is of significance in the cellular approach to toxicity assessment of herbal products.

In our MTT assay, the IC_{50} value of bromobenzene on HepG2 was 0.5542 ± 0.0006 mM, which was 20-fold lower than that determined by previous investigators (10 mM) using a different assay protocol (Thabrew *et al.*, 1997), indicating that our assay was considerably more sensitive than theirs. Because of their status as controlled substances (class IV), NME, MPE, NE, and NPE were not available for cytotoxicity determination. Cytotoxicity was therefore determined for only E and PE in HepG2. This is appropriate, as E and PE are the major alkaloids in ma-huang. The % cytoviability of equimolar concentrations (3.0 mM) of E and PE for HepG2 was 30.83% and 89.43% respectively (data not shown). A paired *t*-test showed that the cytotoxicity of E to HepG2 was significantly higher than that of PE ($p = 0.0013$).

In view of the abundance and the high potency of E, we used it as the reference compound in comparing cytotoxicity among ma-huang extracts prepared under different conditions. Indeed, when E is used chronically, it can cause cardiomyopathy,

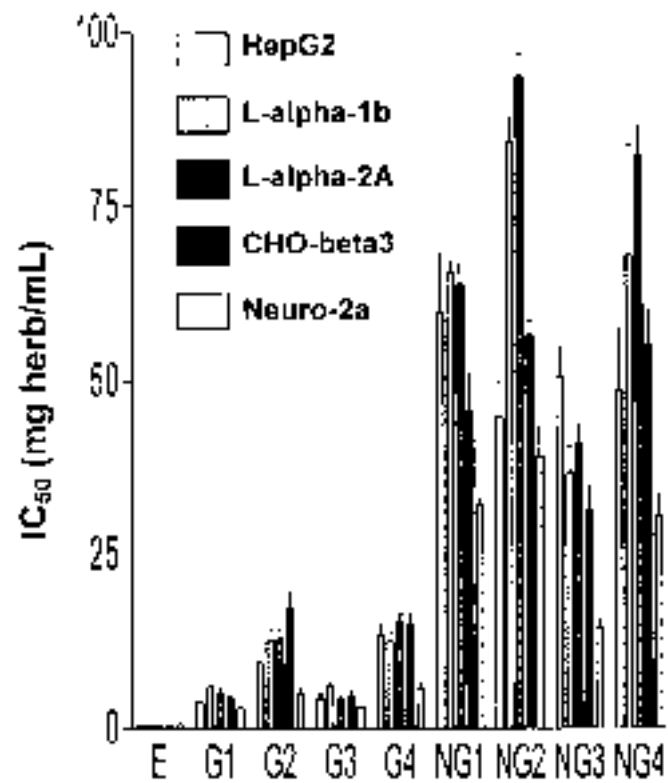


FIG. 4. The IC_{50} values of (-)-ephedrine (mg/mL) and ma-huang extracts (mg herb/mL) prepared under different conditions for a battery of cell lines. Values are means and S. D. obtained from three experiments. IC_{50} : concentration of test sample to inhibit cytoviability by 50%.

which is related to catecholamine-mediated cytotoxicity (Gualtieri and Harris, 1996; To *et al.*, 1980; Van Mieghem *et al.*, 1978).

The MTT Assay—Pros and Cons

The results of cytotoxicity testing can be determined by several endpoints, such as counting cells that include/exclude a dye, measuring the release of ^{51}Cr -labeled protein after cell lysis, measuring the incorporation of radioisotopes ($[^3\text{H}]$ thymidine, $[^3\text{H}]$ uridine, $[^{125}\text{I}]$ iododeoxyuridine, and $[^3\text{H}]$ amino acid) during cell proliferation, and measuring the colorimetric changes of tetrazolium salts by metabolically active cells (McAteer and Davis, 1994; Wilson, 1986). Among these assays, the radioisotope incorporation and colorimetric assays are most suitable for handling a large number of samples. However, radioisotope incorporation assays suffer from the hazards of handling and disposal of radioactive materials. For colorimetric assays using tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), apart from not involving radioactive materials, this salt is only cleaved by metabolically active cells, and therefore only living cells are assayed. The amount of formazan generated is directly proportional to the cell number over a wide range. These features permit micro-scale testing which reduces the amount of sample required and other resources needed. The results of the MTT assays can be optically visualized, and thus it is very useful if a rapid qualitative check is desired. Finally, the results obtained from MTT assays agree closely with those of $[^3\text{H}]$ thymidine incorporation assays (Mosmann, 1983). A drawback of the colorimetric assay is that a few parameters, such as duration of MTT treatment, concentration of MTT used, and the number of test cells used have to be optimized. This drawback makes the direct inter-laboratory comparison of results difficult. It should be noted that the MTT assay measures the effects on the changes of enzyme activities that are affected by culture conditions. Therefore, standardized culture conditions must be maintained throughout the tests (Doostdar *et al.*, 1988).

Drug-to-Toxin Ratio (DTR)

The IC_{50} values of ma-huang extracts, normalized by their E contents, were calculated by multiplying the IC_{50} values of ma-huang extracts determined by MTT assay (in mg herb/mL) by their E contents obtained from HPLC analyses (in mg E/mg herb). They are shown in Figure 5. The IC_{50} values of all ma-huang extracts normalized by their E contents were lower than those of the pure E, indicating that E did not account for the entire toxicity and that there were other toxins present in the extracts. Tukey's Multiple Test Comparison showed that the IC_{50} values of ground ma-huang extracts normalized by their E (G1 through G4) were significantly lower than those of whole herb extracts (NG1 through NG4), with $p < 0.001$ for L-alpha-2A, CHO-beta3 and Neuro-2a, and $p < 0.01$ for HepG2 and L-alpha-1b. This showed that grinding significantly increased the extraction of toxins.

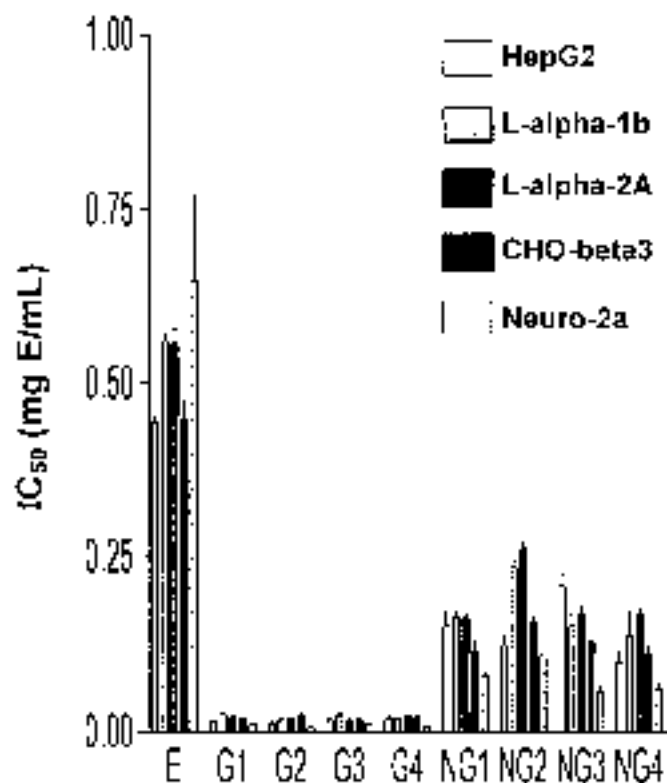


FIG. 5. The IC_{50} values (mg E/mL) of (-)-ephedrine and ma-huang extracts normalized by E for a battery of cell lines ($=\text{IC}_{50}$ (mg herb/mL) \times E content (mg E/mg herb)).

Boiling the whole herb for 2 h represented the best, among the conditions used in this study, for preparing ma-huang extracts with the highest drug-to-toxin ratio (DTR). Under this condition, the E content was relatively high (high drug content), the cytotoxicity was relatively low (low toxin content), and the total E obtained in 2 extractions combined was the highest in quantity compared with the extractions under other conditions.

CONCLUSIONS

- (1) The IC_{50} values of E and ground ma-huang extracts were significantly lower than those of whole herb extracts, indicating that E and ground ma-huang extracts were more cytotoxic. Grinding was a crucial condition for increased toxicity.
- (2) The Neuro-2a cell line was significantly more sensitive to the cytotoxicity of ma-huang extracts than cells from other origins, suggesting that the toxic principles might be acting on neuronal cells.
- (3) The IC_{50} values of all ma-huang extracts normalized by their E contents were significantly lower than pure E, indicating the presence of toxins other than E in the extracts.
- (4) Boiling the whole herb for 2 h represented the best condition for preparing ma-huang extracts, with the highest drug-to-toxin ratio (DTR).

ACKNOWLEDGMENTS

The study was supported in part by the Hong Kong University Grant Council and the Industry Service Fund (AF-178-97). E was a generous gift from Dr. Paul R Carlier of The Hong Kong University of Science and Technology, Hong Kong. PE, NME, and (-)-norephedrine hydrochloride and (+)-norpseudoephedrine hydrochloride were kindly provided by Dr. Chuang of Brion Research Institute of Taiwan. We thank Dr. Susan Huxtable and Mr. Garry H Chang for their help in experimental work and their comments on this manuscript.

REFERENCES

- Astrup, A., Toubro, S., Christensen, N. J., and Quaade, F. (1992). Pharmacology of thermogenic drugs. *Am. J. Clin. Nutr.* **55**, 246S–248S.
- Blumenthal, M., and King, P. (1995). Ma-huang: Ancient herb, modern medicine, regulatory dilemma. *Herbalgram* **34**, 22–26, 43, 56–57.
- Capwell, R. R. (1995). Ephedrine-induced mania from an herbal diet supplement. *Am. J. Psychiatr.* **152**, 647.
- Cetaruk, E. W., and Aaron, C. K. (1994). Hazards of nonprescription medications. *Emerg. Med. Clin. North Am.* **12**, 483–510.
- Chen, K. K., and Schmidt, C. F. (1930). *Ephedrine and Related Substances*, pp. 1–117. The Williams and Wilkins Company, Baltimore.
- Chung-hua Jen Min Kung Ho Kuo Wei Sheng Pu Yao Tien Wei Yuan Hui (1995). *Chung-hua Jen Min Kung Ho Kuo Yao Tien [PRC Medicinal Dictionary]*, pp. 285–286. Kuang-chou Ko Chi Chu Pan She, Kuang-chou Shih; Hua Hsueh Kung Yeh Chu Pan She, Pei-ching Shih.
- Cui, J. F., Niu, C. Q., and Zhang, J. S. (1991). Determination of six *Ephedra* alkaloids in Chinese *Ephedra* (ma huang) by gas chromatography. *Acta Pharmaceutica Sinica* **26**, 852–857.
- De Ferrari, G. V., von Bernhardt, R., Calderon, F. H., Luza, S. C., and Inestrosa, N. C. (1998). Responses induced by tacrine in neuronal and non-neuronal cell lines. *J. Neurosci. Res.* **52**, 435–444.
- Dollery, S. C. (1991). *Therapeutic Drugs*, pp. E26–29, P91–93, P297–299. Churchill Livingstone, Edinburgh.
- Doostdar, H., Duthie, S. J., Burke, M. D., Melvin, W. T., and Grant, M. H. (1988). The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived HepG2 cell line. *FEBS Lett.* **241**, 15–18.
- Dulloo, A. G., and Stock, M. J. (1993). Ephedrine as a thermogenic drug. *Int. J. Obes.* **17**(Suppl. 1), S1–S2.
- Duthie, S. J., Coleman, C. S., and Grant, M. H. (1988). Status of reduced glutathione in the human hepatoma cell line, Hep G2. *Biochem. Pharmacol.* **37**, 3365–3368.
- FDA (Food and Drug Administration) (1997). Dietary supplements containing ephedrine alkaloids; proposed rule. *Federal Register* **62**, 30678–30724.
- Grant, M. H., Duthie, S. J., Gray, A. G., and Burke, M. D. (1988). Mixed function oxidase and UDP-glucuronyltransferase activities in the human Hep G2 hepatoma cell line. *Biochem. Pharmacol.* **37**, 4111–4116.
- Gualtieri, J., and Harris, C. (1996). Dilated cardiomyopathy in a heavy ephedrine abuser. *J. Toxicol. Clin. Toxicol.* **34**, 581–582.
- Huang, K. C. (1993). *The Pharmacology of Chinese Herbs*, pp. 229–232. CRC Press, London.
- Hurlbut, J. A., Carr, J. R., Singleton, E. R., Faul, K. C., Madson, M. R., Storey, J. M., and Thomas, T. L. (1998). Solid-phase extraction cleanup and liquid chromatography with ultraviolet detection of ephedrine alkaloids in herbal products. *J. AOAC Inst.* **81**, 1121–1127.
- Jan, H. T. (1993). *Chung-hua Yao Hai*, pp. 102–106. Ha-erh-pin Chu Pan She, Ha-erh-pin.
- Kasahara, Y., Hayasaka, H., Oba, K., and Hikino, H. (1986). Seasonal dynamics of the accumulation of ephedrine alkaloids in *Ephedra distachya* herbs. *Shoyakugaku Zasshi* **40**, 390–392.
- Knowles, B. B., Howe, C. C., and Aden, D. P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209**, 497–499.
- Liu, Y. M., Sheu, S. J., Chiou, S. H., Chnag, H. C., and Chen, Y. P. (1993). A comparative study on commercial samples of *Ephedrae Herba*. *Planta Med.* **59**, 376–378.
- Lou, Z. T., Zhang, J. S., Li, S. H., Tian, Z., and Qi, Z. (1995). Research on ma-huang. In *Species Systematization and Quality Evaluation of Commonly Used Chinese Traditional Drugs, North-China Edition* (C. T. Lou and P. Chin, Eds.), pp. 39–121. Pei-ching I Ko Ta Hsueh and Chung-kuo Hsieh Ho I Ko Ta Hsueh, Pei-ching.
- Lundstrom, J. (1989). β -Phenethylamines and ephedrine of plant origin. In *The Alkaloids. Chemistry and Pharmacology* (A. Brossi, Ed.), Vol. 35, pp. 77–154. Academic Press, San Diego.
- Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M., and Wekell, M. M. (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: Semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Anal. Biochem.* **214**, 190–194.
- McAteer, J. A., and Davis, J. (1994). Basic cell culture technique and the maintenance of cell lines. In: *Basic Cell Culture: A Practical Approach* (J. M. Davis, Ed.), pp. 122–132. IRL Press, Oxford, U.K.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63.
- Nadir, A., Agrawal, S., King, P. D., and Marshall, J. B. (1996). Acute hepatitis associated with the use of a Chinese herbal product, ma-huang. *Am. J. Gastro.* **91**, 1436–1438.
- Richardson, M. L., and Gangolli, S. (1992). *The Dictionary of Substances and Their Effects*, Vol 4, pp. 237–238. The Royal Society of Chemistry, England.
- Sagara, K., Oshima, T., and Masaki, T. (1983). A simultaneous determination of norephedrine, pseudoephedrine, ephedrine, and methylephedrine in *Ephedrae Herba* and oriental pharmaceutical preparations by ion-pair high-performance liquid chromatography. *Chem. Pharm. Bull.* **31**, 2359–2365.
- Thabrew, M. I., Hughes, R. D., and McFarlane, I. G. (1997). Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. *J. Pharm. Pharmacol.* **49**, 1132–1135.
- To, L. B., Sangster, J. F., Rampling, D., and Cammens, I. (1980). Ephedrine-induced cardiomyopathy. *Med. J. Aust.* **2**, 35–36.
- Tyler, V. E. (1993). *The Honest Herbal: A Sensible Guide to the Use of Herbs and Related Remedies*, pp. 119–121. Pharmaceutical Products Press, New York.
- Tyler, V. E. (1994). *Herbs of Choice: The Therapeutic Use of Phytochemicals*, pp. 88–90. Pharmaceutical Products Press, New York.
- Van Mieghem, W., Stevens, E., and Cosemans, J. (1978). Ephedrine-induced cardiomyopathy. *Br. Med. J.* **1**, 816.
- Walker, T. M., Starr, B., Dewhurst, B. B., and Atterwill, C. (1995). Potential neurotoxicity of a novel aminoacridine analogue. *Human and Experimental Toxicology* **14**, 469–474.
- Wang, C. (1988). *Shang Han Lun Chiang Chieh*, pp. 418. Ho-nan Ko Hsueh Chi Shu Chu Pan She, Cheng-chou.
- Wilson, A. P. (1986). Cytotoxicity and viability assays. In *Animal Cell Culture: A Practical Approach* (R. I. Freshney, Ed.), pp. 183–216. IRL Press, Oxford, U.K.
- Yang, T. L. (1993). *Tu Yao Pen Tsao*, pp. 178–186. Chung-kuo Chung I Yao Chu Pan She, Pei-ching Shih.
- Zhang, J. S., Tian, Z., and Lou, Z. C. (1989). Quality evaluation of twelve species of Chinese *Ephedra* (ma-huang). *Acta Pharmaceutica Sinica* **24**, 865–871.