Pergamon

PII: S0031-9422(97)01102-3

Phytochemistry, Vol. 48, No. 1, pp. 119–124, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

FORMATION OF PAVINE ALKALOIDS BY CALLUS CULTURE OF *CRYPTOCARYA CHINENSIS*

WEN-TE CHANG, SHOEI-SHENG LEE, FU-SHIN CHUEH and KARIN C. S. LIU*

School of Pharmacy, College of Medicine, National Taiwan University, 1 Jen-Ai Road, Sec. 1, Taipei 100, Taiwan, Republic of China

(*Received in revised form* 17 *November* 1997)

Key Word Index—*Cryptocarya chinensis*; Lauraceae; callus culture; pavine alkaloid; neocaryachine *N*-metho salt.

Abstract—Three quaternary pavine alkaloids, caryachine *N*-metho salt, neocaryachine *N*-metho salt and crychine *N*-metho salt were isolated from a callus culture of *Cryptocarya chinensis*. Neocaryachine *N*-metho salt is reported for the first time as a natural product. The effect of various culture media components on the growth of callus, and the production of caryachine *N*-metho salt were also investigated.

INTRODUCTION

Cryptocarya chinensis Hemsl. is a perennial woody plant widely distributed in Taiwan [1]. Past studies on the constituents of this species revealed that it is rich in pavine alkaloids [2–6]. So far, four tertiary and one quaternary pavine alkaloid, namely, crychine, (−)-caryachine, (+)-O-methylcaryachine, (−)-neocaryachine, and (−)-caryachine *N*-metho salt have been isolated and identified from this species which is the only pavine-containing lauraceous plant. These types of alkaloid have been reported to possess biological activities such as behavioural [7] and antitumour effects [8]. The electrophysiological effect and antiarrhythmic potential of (−)-caryachine on rat cardiac tissues were reported recently [9, 10]. These results prompted us to look for a source of pavine more advanced pharmacological studies. Here we report on the first establishment of callus cultures of this species and the study of their secondary metabolites.

RESULTS AND DISCUSSION

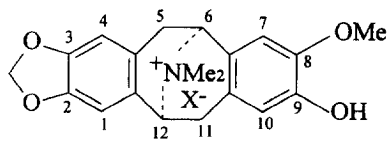
It has been reported that the establishment of callus for woody plants is more difficult than for herbal plants. Induction of callus was attempted by the use of leaflets cultured on MS medium supplemented with 2,4-D (2 mg/l) and kinetin (0.5 mg/l). Callus was induced along the midrib after 40 days. It was dark brown-colored, textured and hard, but not differentiated. The basal media (MS or B_5), growth regulators (IAA, NAA, 2,4-D, BA, kinetin) and organic nitrogen source (casein hydrolysate) were varied in order to optimize the growth condition (to be discussed later). 1/2 MS basal solid medium containing NAA (8 mg/l), BA (1 mg/l), casein hydrolysate (2 g/l) and sucrose (3%) was found to support good growth of callus; the doubling time of which was ca 30 days.

The alcohol extract of calli obtained from the 1/2 MS culture medium was triturated with $CHCl_3$ and MeOH, successively. The methanol-soluble fraction being positive to Dragendorff reagent was fractionated by centrifugal partition chromatography (CPC), followed by CC to yield compounds **1**–**3**.

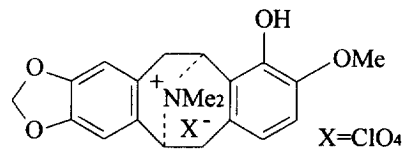
Compound **1** was identified as caryachine *N*-metho salt from its physical data, including ^{1}H NMR: four aromatic singlets at 6.82, 6.78, 6.59 and 6.53, an AB system at δ 5.94 and 5.91 (*d*, J_{AB} = 0.8 Hz) for $OC\underline{H}_2O$, an OMe at δ 3.85, and N^+Me_2 at 3.24; and characteristic mass fragments at *m/z* 188 (**A**) and 190 (**B**) [4].

Compounds **2** and **3** were separated by prep HPLC. The ^{1}H NMR spectra of **2** exhibited an AX system at δ 6.90 and 6.62 (J_{AX} = 8.4 Hz), two singlets at δ 6.79 and 6.59 in the aromatic region, an AB system for $OC\underline{H}_2O$ at δ 5.94 and 5.90 (*d*, J_{AB} = 0.8 Hz), an OMe at δ 3.83, and N^+Me_2 at δ 3.24 in the aliphatic region. The EIMS spectrum showed [M-X]+ at *m/z* 339, and characteristic fragments at *m/z* 188 (**A**) and 190 (**C**). These data established that compound **2** was neocaryachine *N*-metho perchlorate. The ^{1}H NMR spectrum of **3** contained four aromatic proton signals at δ 6.78 (*s*, 2H) and 6.61 (*s*, 2H). The aliphatic region displayed an AB system for $OC\underline{H}_2O$ at δ 5.95 and 5.92

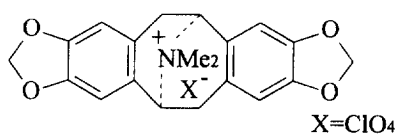
* Author to whom correspondence should be addressed.



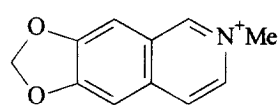
1



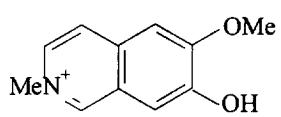
2



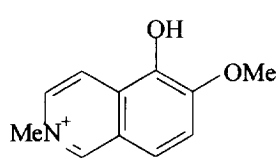
3



A *m/z* 188



B *m/z* 190



C *m/z* 190

(d, J_{AB} = 1.2 Hz), and N^+Me_2 at δ 3.25. These findings established that compound **3** was crychine *N*-metho perchlorate. This structure was supported by the mass spectrum which showed [M-MeX]$^+$ at *m/z* 323, and a characteristic fragment at *m/z* 188 (**A**). To confirm these structural assignments for **2** and **3**, authentic samples of neocaryachine *N*-metho perchlorate and crychine *N*-metho perchlorate were prepared from the corresponding tertiary base by reaction with methyl iodide, followed by treatment of the salts with sodium perchlorate. The ^{1}H NMR spectrum, and R_f on HPLC (to be discussed) of each natural compound was found to be identical to the corresponding chemically prepared sample and proved unambiguously the structures of **2** and **3**. To our knowledge, this is the first report of compound **2** as a natural product.

Quantitative analysis of the pavine alkaloids produced by the callus cultures was carried out by RP-HPLC. A good separation of compounds **1**–**3** was achieved by the use of a μ-Bondapak C-18 column, eluted with 0.5% TEA-MeCN-MeOH (48:12:1, TEA in 0.05 M KH_2PO_4, pH 4.0 adjusted with H_3PO_4), with detection at 290 nm. The results indicated that *C. chinensis* callus cultured on the above mentioned medium produced compounds **1**–**3** in yields (in terms of callus dry wt) of 0.27±0.06% (R_t 8.3 min), 0.024±0.003% (R_t 16.0 min) and 0.042±0.006% (R_t 19.1 min).

The optimal conditions for callus growth and production of **1** were also investigated. The effects of auxins (2,4-D, NAA and IAA) on callus growth and compound **1** production are shown in Fig. 1. A higher concentration of NAA (8 mg/l) increased the growth of callus some 2.8-fold by weight relative to the control medium. IAA (4 mg/l) was found to be most effective for the production of **1**; however, it was not superior to NAA (8 mg/l) in terms of overall yield of cells. The combination of auxin and kinetin or BA in the medium did not enhance the production of **1** significantly (data not shown), although the optimum condition for callus growth was best with NAA (8 mg/l) and BA (1 mg/l). On varying the sucrose concentration (Fig. 2), it was found that 3% to 5% of sucrose favoured callus growth, whereas the formation of **1** was reduced. 1% sucrose was found to be optimal for the biosynthesis of **1** (up to 0.46% yield), although callus growth rate was relatively low.

The use of casein hydrolysate as organic nitrogen source in the culture medium has been found to improve the growth of some tissues, such as *Taxus* spp. [11]. In this study, the addition of casein hydrolysate (2 g/l) promoted both callus growth and the production of **1** some 2.5 and 1.5 fold, respectively (Fig. 3). However, higher concentrations of casein hydrolysate (4–8 g/l) were found to retard callus growth and the production of **1** as well.

The present study has demonstrated the possibility of obtaining quaternary pavine alkaloids from plant tissue culture. In callus cultures of *C. chinensis*, the best yield of caryachine *N*-metho salt was achieved using 1/2MS basal medium containing IAA (4 mg/l) or a low concentration (1%) of sucrose.

EXPERIMENTAL

Plant material and callus culture

The plant, *Cryptocarya chinensis* Hemsl., was collected in Mt Wu-Lai, Taiwan. A voucher specimen

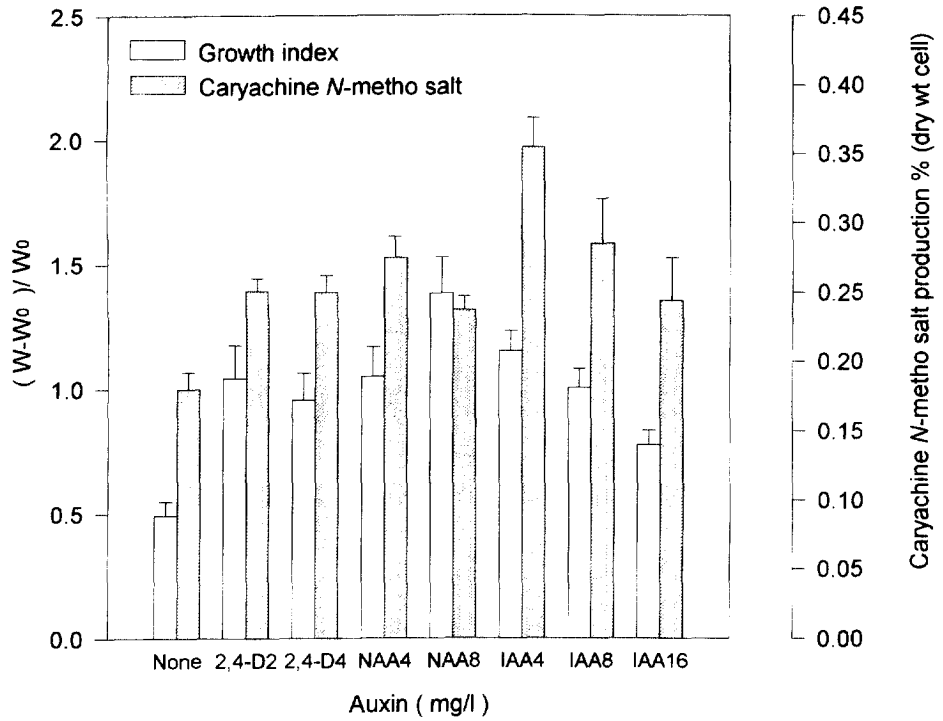


Fig. 1. Effect of auxins on callus growth and caryachine *N*-metho salt production of *C. chinensis*. *W*: wt of callus obtained after 30 days; W_0: wt of callus obtained initially. Mean values of at least three replicate determinations.

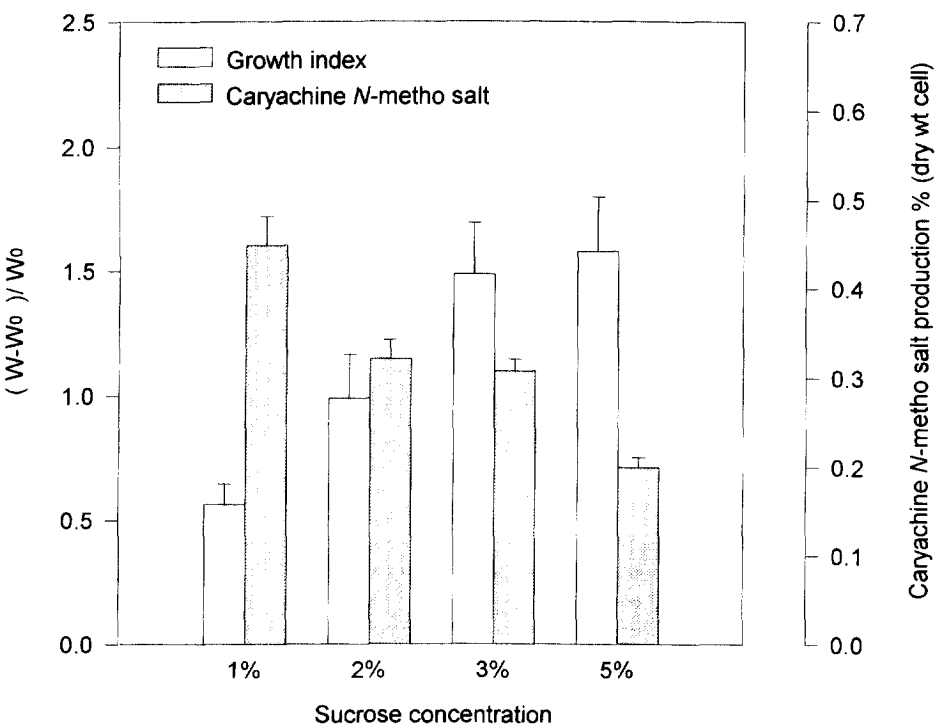


Fig. 2. Effect of sucrose on callus growth and caryachine *N*-metho salt production of *C. chinensis*. *W*: wt of callus obtained after 30 days; W_0: wt of callus obtained initially. Mean values of at least three replicate determinations.

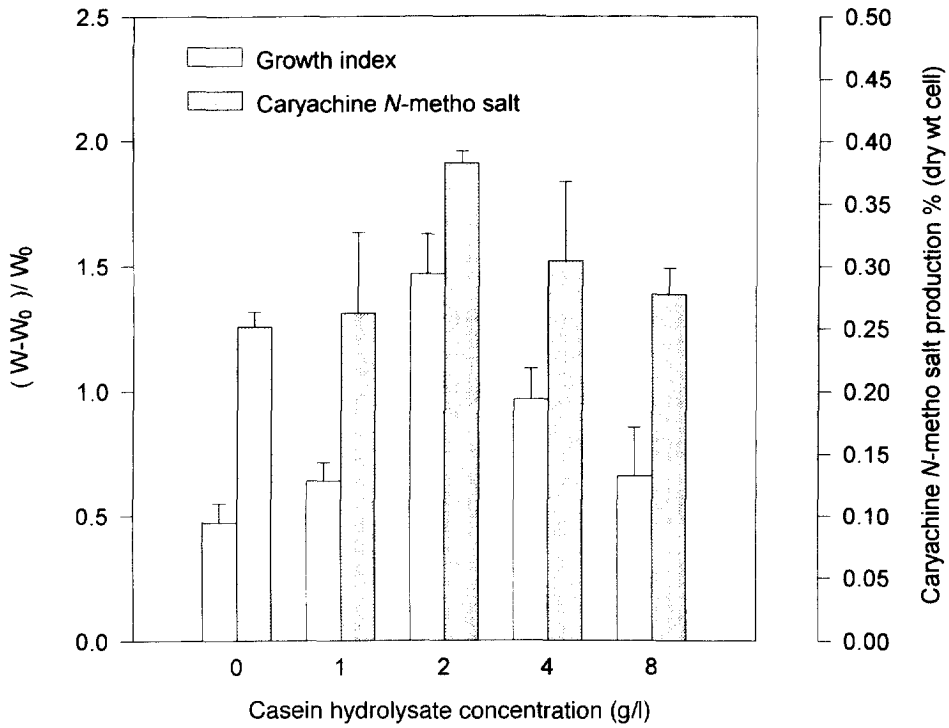


Fig. 3. Effect of casein hydrolysate on callus growth and caryachine *N*-metho salt production of *C. chinensis*. *W*: wt of callus obtained after 30 days; W_0: wt of callus obtained initially. Mean values of at least three replicate determinations.

(LA-820612) was deposited in the Herbarium of the School of Pharmacy, National Taiwan University. For callus culture induction, leaflets were washed with H_2O, surface sterilized by immersing in 70% EtOH for 5 min, then in 1% and 2% Na hypochlorite containing several drops of Triton X100 for 30 and 20 min under ultrasonication, respectively. After rinsing with sterile H_2O and the removal of damage tissue, the explant was cultured on a medium containing Murashige and Skoog (MS) basic salts supplemented with 2,4-D (2 mg/l), kinetin (0.5 mg/l), sucrose (3%) and agar (0.9%, pH 5.7). The cultures were maintained at 25° ± 1° in the dark. The initial callus was cut into small pieces and subcultured on 1/2 strength MS basic salts supplemented with NAA (8 mg/l), BA (1 mg/l), casein hydrolysate (2 g/l), sucrose (3%) and agar (0.9%, pH 5.7), again at 25° ± 1° in the dark. Callus was routinely transferred at 4 week intervals over a period of 5 months.

Extraction and isolation

Dried callus samples (16.47 g) were pulverized and extracted with 95% EtOH at room temp. Conc. of the EtOH extract afforded a residue (3.64 g) that was triturated with $CHCl_3$ and filtered. The $CHCl_3$-insoluble ppt (3.28 g) was then dissolved in MeOH and the MeOH soln fractionated by Sanki CPC (LIN type, 1000E cartridge, flow rate 3.5 ml min^{-1}, 1000 rpm, pressure 30 kg/cm^2), using the organic and aq layers of the solvent system $CHCl_3$–MeOH–0.5% aq. HOAc (2:2:1) as stationary and mobile phase, respectively. This reversed-partition chromatography yielded 10 frs which were combined after Silica gel TLC analysis using the above stationary phase and HOAc (47:3).

Fr. 6 (572.0 mg) was further fractionated on a Sephadex LH-20 column (ca 200 g) eluted with MeOH to yield 4 subfrs. Subfr. 2 (443.9 mg) was further purified by Sephadex LH-20 (90 g) CC eluted with 50%–100% MeOH to give caryachine *N*-metho salt (**1**) (12.9 mg). Fr. 7 (162.4 mg) was fractionated on the same Sephadex LH-20 column eluted with MeOH to give 2 subfrs. Subfr. 1 (121.1 mg) was further fractionated on a Sephadex LH-20 column (90 g) eluted with 50%–100% MeOH to give 5 frs. The quaternary alkaloids in fr. 3 (34.1 mg) were collected as a perchlorate ppt. by adding satd $NaClO_4$ to the aq. soln of this fr. The perchlorate salts were sepd with repeated Lobar RP-18 (240 × 10 mm) CC [MeOH–0.1 M $HClO_{4(aq.)}$(13:27)] to give a fr. containing compounds **2** and **3** (2.3 mg). This fr. was worked up as follows: after removal of the MeOH *in vacuo*, the $HClO_4$ soln was neutralized with Na_2CO_3 and the neutral solution passed through an Amberlite XAD-2 column eluting with H_2O (to remove the $NaClO_4$) and then MeOH. The neocaryachine *N*-metho salt (**2**) (1.1mg) and crychine *N*-metho salt (**3**) (0.7 mg), were finally sepd by prep. HPLC on a μ-Bondapak C-18 column (300 × 19 mm; Waters) with MeOH–0.5% $HOAc_{(aq.)}$ (31:19) as the mobile phase and a flow rate of 9 ml min^{-1}, with UV detection at 290 nm.

Caryachine N-*metho salt* (**1**)

$[\alpha]_D^{25}$ −224° (MeOH; c 0.13); UV λ_{max}^{MeOH} nm (log ε): 291.5 (3.95), 225 (4.07); ^{1}H NMR (400 MHz, CD_3OD): δ 6.82, 6.78, 6.59 and 6.53 (4 s, 4 ArH), 5.94 and 5.91 (*d*, J = 0.8 Hz, $OC\underline{H}_2O$), 3.85 (*s*, 8-OMe), 3.24 (*s*, N^+Me_2); EIMS 70 eV, *m/z* (rel. int.): $[M\text{-}1]^+$ 339 (19), 325 (35), 309 (7), 204 (23), 190 (**B**, 57), 188 (**A**, 100), 175 (6).

Neocaryachine N-*metho perchlorate* (**2**)

$[\alpha]_D^{25}$ −40.1° (MeOH; c 0.011); UV λ_{max}^{MeOH} nm (log ε): 288.0 (3.64), 206.5 (4.53); ^{1}H NMR (400 MHz, CD_3OD): δ 6.90 (*d*, H-7), 6.79 (*s*, H-1), 6.62 (*d*, H-10), 6.59 (*s*, H-4), 5.94 and 5.90 (*d*, J = 0.8 Hz, $OC\underline{H}_2O$), 3.83 (*s*, 8-OMe), 3.24 (*s*, N^+Me_2); EIMS 70 eV, *m/z* (rel. int.) $[M^+\text{-}X]$ 339 (7), 325 (17), 204 (6), 190 (**C**, 14), 188 (**A**, 100), 175 (5).

Crychine N-*metho perchlorate* (**3**)

$[\alpha]_D^{20}$ +50.0° (MeOH; c 0.007); UV λ_{max}^{MeOH} nm (log ε): 287.5 (3.16); ^{1}H NMR (400 MHz, CD_3OD): δ 6.78 (*s*, H-1 and H-7), 6.60 (*s*, H-4 and H-10), 5.92 and 9.95 (*d*, J = 1.2 Hz, $OC\underline{H}_2O$), 3.25 (*s*, N^+Me_2); EIMS 70 eV, *m/z* (rel. int.) $[M^+\text{-}X]$ 337 (7), 323 (12), 188 (**A**, 100).

Preparation of **2**

A soln of 20 mg neocaryachine in MeOH was mixed with MeI (2 ml). The resultant soln was refluxed for 3 h and then evaporated under red. pres. to give a pale brown residue. The residue was dissolved in H_2O and treated with satd aq. $NaClO_4$ to give a white ppt, neocaryachine *N*-metho perchlorate (**2**). $[\alpha]_D^{26}$ −93.3° (MeOH; c 0.6); UV λ_{max}^{MeOH} nm (log ε) 288.0 (3.64), 206.5 (4.53); ^{1}H NMR (400 MHz, CD_3OD): δ 6.89 (*d*, H-9), 6.78 (*s*, H-1), 6.58 (*d*, H-10 and H-4), 5.93 and 5.90 (*d*, J = 0.8 Hz, $OC\underline{H}_2O$), 3.82 (*s*, 8-OMe) and 3.29 (*s*, N^+Me_2).

Preparation of **3**

Treatment of crychine with CH_3I and $NaClO_4$ in the same manner as described above for **2** yielded crychine *N*-metho salt.

Establishment of callus cultures on different auxins, casein hydrolysate and sucrose

Small pieces of callus (ca 250 mg fr. wt) were transferred onto 1/2 MS solid medium containing different concs of auxins: (I) either 2,4-D (2 and 4 mg/l) or NAA (4 and 8 mg/l) or IAA (4, 8 and 16 mg/l) with BA (1 mg/l) and sucrose (3%); (II) casein hydrolysate (0, 2, 4 and 8 g/l) with NAA (8 mg/l), BA (1 mg/l) and sucrose (3%); (III) sucrose (0, 1, 3 and 5%) with NAA (8 mg/l) and BA (1 mg/l). The cultures were maintained under identical conditions as described and the callus was harvested after 30 days, freeze-dried and weighed.

Sample preparation for HPLC analysis

The dried callus samples were collected, pulverized, and extracted with MeOH under ultrasonication for 30 min (×3). The total MeOH extracts were concd under red. pres. to give a dry residue which was dissolved in MeOH (10 ml) for HPLC analysis.

Conditions for HPLC analysis

μ-Bondapak C-18 column (Waters, 300 × 3.9 mm) with 0.5% TEA–MeCN–MeOH (48:12:1, TEA in 0.05 M KH_2PO_4, pH 4 adjusted with H_3PO_4) as the mobile phase and a flow rate of 1 ml min^{-1}. UV detection was performed at 290 nm with a diode array detector. R_fs **1**, **2** and **3** = 8.3, 16.0 and 19.1 min, respectively.

Quantitation of pavine alkaloids

The content of **1** in the callus from various cultures was determined by HPLC. The HPLC conditions were the same as described above. Boldine was utilized as an int standard. The R_fs boldine and **1** were 5.7 min and 8.3 min, respectively. The results were from at least three replicate determinations. To establish the calibration curve, five standard caryachine *N*-metho salt solns of different concs were prepared and analysed. The injected amount (mg) of **1** (X) was found to be proportional to its peak height ratio (Y). The regression equation was $Y = -0.0375 + (0.00605 \times X)$, r = 0.998. For the determination of **1**, 10 μl was injected onto the HPLC column. The content of **1** in each sample was calculated from the height of the peak and the regression equation. The contents of compound **2** and **3** were estimated by reference to compound **1**, based on the peak-area ratios.

Acknowledgement—This work was supported by a grant from the National Science Council of the Republic of China.

REFERENCES

1. Li, H. L., Liu, T. S., Huang, T. C., Koyama, T. and DeVol, C. E., (ed.) *Flora of Taiwan*, Vol. II, Epoch Publishing, Taipei, 1976, p. 422.
2. Lu, S. T. and Lan, P. K., *Yakugaku Zasshi*, 1966, **86**, 177.
3. Lu, S. T., *Yakugaku Zasshi*, 1966, **86**, 296.
4. Chen, C. H., Lee, S. S., Lai, C. F., Wu, J. and Beal, J. L., *Journal of Natural Products*, 1979, **42**, 163.

5. Lee, S. S., Liu, Y. C. and Chen, C. H., *Journal of Natural Products*, 1990, **53**, 1267.
6. Lee, S. S., Chen, C. H. and Liu, Y. C., *Journal of Natural Products*, 1993, **56**, 227.
7. Meisenberg, G., Simmons, W. H. and Collins, M. A., *Pharmacology, Biochemistry and Behavior*, 1984, **20**, 355.
8. Wu, Y. C., Liou, Y. F., Lu, S. T., Chen, C. H., Chang, J. J. and Lee, K. H., *Planta Medica*, 1989, **55**, 163.
9. Wu, M. H., Su, M. J., Lee, S. S., Lin, L. T. and Young, M. L., *British Journal of Pharmacology*, 1995, **116**, 3211.
10. Chen, L., Su, M. J., Wu, M. H. and Lee, S. S., *Journal Cardiovascular Pharmacology*, 1996, **27**, 740.
11. Gibson, D. M. and Ketchum, R. E. B., *Plant Cell Reports*, 1993, **12**, 429.