

ANTI-HEPATITIS B VIRUS OF SEVEN COMPOUNDS ISOLATED FROM *PIPER* *KADSURA* (CHOISY) OHWI

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In Vitro anti-human hepatitis B virus effects of Chinese and folk herbal drugs on the HBV producing cell line (MS-G2) were studied. Among seven compounds isolated from *Piper kadsura* (Choisy) Ohwi, we found that futoquinol, (-)-galbelgin, and meso-galgravin at concentration of 25 μ M can effectively suppress both HBV surface and e antigen productions, and high concentration (50 μ M) of piperenone can only suppress HBV e antigen productions, while piperlactam S even depicts significant cytotoxic effects. In contrast, futoenone and (+)-crotepoide do not show any noticeable anti-viral effects.

Key words: Human hepatitis B virus, HBsAg, HBeAg, *Piper kadsura*, Piperaceae.

INTRODUCTION

Hepatitis B virus (HBV) is one of the causative agents of viral hepatitis. Infection with HBV can cause acute, chronic and fulminant hepatitis. Some chronic hepatitis patients subsequently suffer from cirrhosis and liver failure, or even develop hepatocellular carcinoma (HCC)¹⁻⁴. Although clinical treatments with interferon, antiviral agents such as ribavirin, lamivudine, acyclovir, adenine arabinoside, dideoxy-nucleoside, and immunomodulatory drugs, employed either alone or in combination, have been reported with various degrees of success⁵⁻⁷, to-date a comprehensive and effective antiviral treatment for patients already chronically infected with HBV is still lacking. Thus, it is important to search for more effective anti-HBV drugs.

Piper kadsura (Choisy) Ohwi, also named *Piper futokadsura* Sieb. Et Zucc.⁸ (Piperaceae) is a medicinal plant naturally inhabiting the forests at low to medium altitudes throughout Taiwan⁹. The plant has a fragrant odor. Its stem part, haifengteng, has long been used as an indigenous medicine for the treatment of asthma and arthritis¹⁰⁻¹¹. Several lignans and neolignans have been isolated from the genus *Piper*, and have been shown to possess anti-tumor, antiviral, inhibition of cAMP phosphodiesterase, and anti-microbial activities¹²⁻¹⁴.

In our laboratory we have been systematically studying the antiviral properties of various natural compounds, using HBV producing cell line MS-G2¹⁵. In this study, the antiviral effects of seven pure components isolated from the acetone extract of the stems of *P. kadsura* (i.e., futoquinol, futoenone, (+)-crotepoixide, (-)-galbelgin, meso-galgravin, piperlactam S, and piperenone), by using ELISA assay, to see the inhibitory effect on HBsAg and HBeAg levels as anti-viral indicators were reported.

MATERIALS AND METHODS

Plant Material: The stems of *Piper kadsura* (Choisy) Ohwi were collected in August 1997, in Taipei, Taiwan. Identification of plant materials were confirmed by comparing with a voucher specimen (TAI 222523), that had been deposited at the Herbarium of the Department of Botany at National Taiwan University.

Test compounds: The seven test compounds were isolated from acetone extract of the stem of *Piper kadsura*. These were futoquinol (**1**)^{16, 17}, futoenone (**2**)¹⁸, (+)-crotepoixide (**3**)¹⁹, meso-galgravin (**4**)^{20, 21}, (-)-galbelgin (**5**)²², piperlactam S (**6**)²³, and piperenone (**7**)²⁴ (see Fig. 1 for structures). The structures of these compounds were identified by comparing with existing data (¹H-NMR, ¹³C-NMR, EIMS, and IR) from the literature. The purities of these compounds were determined to be more than 99% by HPLC analyses. Each compound was dissolved in dimethyl sulfoxide (DMSO) for the bioassay.

Anti-hepatitis B virus: Antiviral analyses were performed as previously described¹⁵. Briefly, the HBV-producing cell line MS-G2 was plated onto 24-well flat-bottomed tissue culture plates at a density of 3×10⁵ cells/ml-well. After an overnight stay to ensure that the cell had been properly attached, the cells were challenged by the test compounds. DMSO alone was added to each culture as solvent control. All test compounds were dissolved in DMSO at concentrations of 1, 5, 10, 25, and 50 µM, respectively. The concentration of DMSO in the media was maintained at not more than 2.5 µl/ml to ensure that it did not affect the growth of MS-G2 cells. Subsequently, the culture media were collected at day 3 for anti-viral assay.

We then evaluated their anti-viral activity by determining the change of HBsAg and HBeAg levels in the presence or absence of the test compounds as analyzed by ELISA assay. The percentage inhibition (%) was calculated by comparing with the control group. Inhibition between 25 - 35% was defined as slight inhibition, 35 - 50% as medium inhibition, 50 - 65% as strong inhibition, while that over 65% was defined as very strong inhibition.

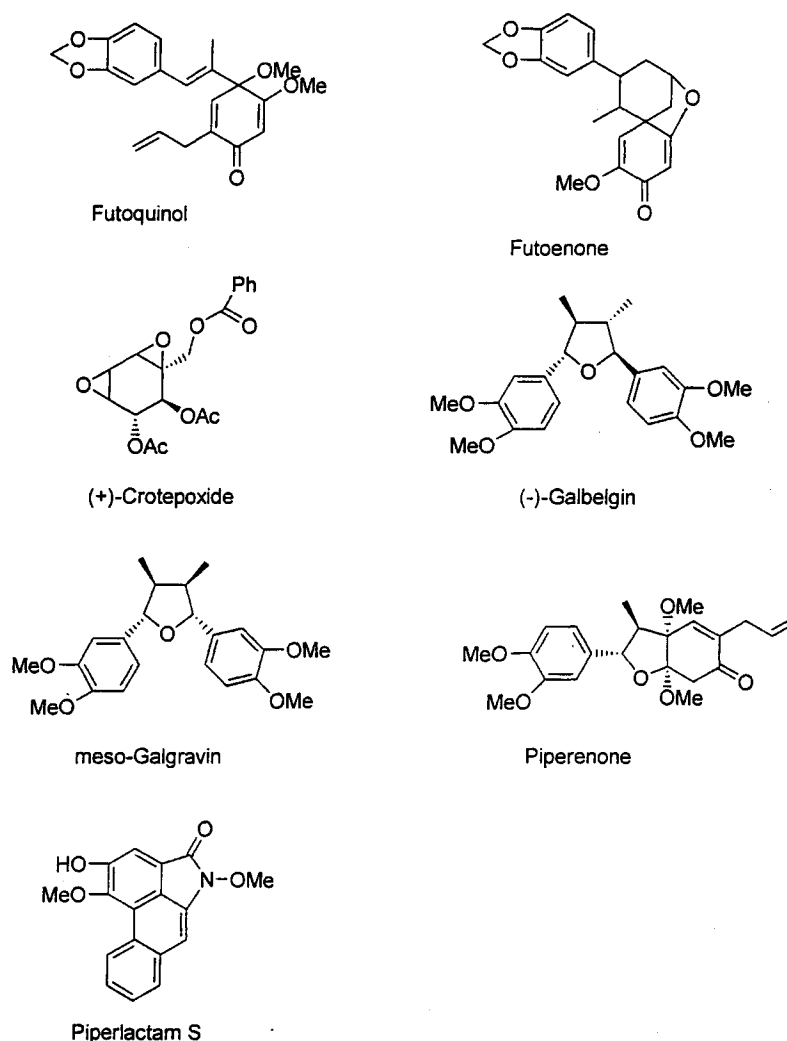


Fig.1. Structures and names of components isolated from *Piper kadsura*.

Cytotoxic assay: Cell damage was tested by AST (aspartate transaminase) Fuji kit. AST values higher than 25 I.U./L served as an indication of cell damage. MTT assay was further conducted for determining cytotoxicity²⁵, and calculating the IC₅₀ values²⁶.

Statistics: The results were expressed as the mean \pm standard deviation of the mean (SDM). The significance of the difference between the DMSO control and the test compound groups was analyzed by Student's t-test.

RESULTS

The results are shown in Table 1. It can be seen that futoquinol, (-)-galbelgin, and meso-galgravin compounds exhibited effective anti-HBV effects at non-toxic concentration of 50 μ M, showing very strong anti-HBsAg (> 65 %) and anti-HBeAg (> 65 %) effects. Specifically, for the anti-HBsAg effects which are also plotted in Fig. 2, futoquinol,

Table 1. Anti-HBsAg and Anti-HBeAg Effects of futoquinol, futoenone, (+)-crotepoide, (-)-galbelgin, meso-galgravin, piperlactam S, piperenone.

Treatments for 3 days	μM	HBsAg (Inhibition %)	HBeAg (Inhibition %)	AST (I.U./L)
Control	0	0	0	< 25
DMSO	2.5 $\mu\text{l/ml}$	16.4 \pm 3.1	16.6 \pm 2.4	15.7 \pm 2.1
Futoquinol	50	80.6 \pm 3.7***	69.4 \pm 12.4**	20.0 \pm 1.0
MW354	25	62.9 \pm 2.4***	33.9 \pm 9.8*	16.3 \pm 1.5
	10	19.7 \pm 3.8	-1.6 \pm 10.7	17.7 \pm 3.2
	5	3.0 \pm 4.1	0.7 \pm 4.8	17.0 \pm 1.7
	1	5.3 \pm 2.7	1.9 \pm 1.4	18.0 \pm 2.6
Futoenone	50	9.4 \pm 2.7	17.8 \pm 1.5	21.3 \pm 1.5
MW340	25	0.1 \pm 3.1	5.7 \pm 3.9	17.7 \pm 1.5
	10	1.0 \pm 2.9	-10.3 \pm 1.1	21.3 \pm 4.2
	5	1.1 \pm 4.1	-10.3 \pm 3.7	18.3 \pm 1.2
	1	-9.3 \pm 2.8	-14.8 \pm 3.8	20.0 \pm 2.0
(+)-Crotepoide	50	-12.0 \pm 2.8	12.3 \pm 2.0	20.7 \pm 0.6
MW362	25	-16.7 \pm 1.2	8.0 \pm 1.6	18.7 \pm 2.1
	10	-13.1 \pm 2.0	-1.9 \pm 2.0	20.0 \pm 1.0
	5	-12.6 \pm 3.0	-3.9 \pm 1.3	21.0 \pm 4.6
	1	-3.6 \pm 5.7	-3.9 \pm 2.8	17.3 \pm 2.1
(-)-Galbelgin	50	81.9 \pm 2.8***	70.9 \pm 7.1***	19.7 \pm 0.6
MW372	25	68.5 \pm 11.5**	65.1 \pm 8.4***	15.7 \pm 1.5
	10	29.3 \pm 15.8	33.6 \pm 9.6*	16.7 \pm 1.2
	5	-1.0 \pm 3.3	3.8 \pm 2.4	16.7 \pm 1.5
	1	-0.5 \pm 4.4	-2.0 \pm 5.0	18.7 \pm 3.1
Meso-Galgravin	50	82.2 \pm 7.5***	70.2 \pm 5.6***	20.7 \pm 2.1
MW372	25	76.0 \pm 5.4***	70.1 \pm 9.8***	16.3 \pm 2.1
	10	25.6 \pm 3.0*	30.7 \pm 6.4*	15.0 \pm 1.0
	5	6.1 \pm 1.3	11.2 \pm 1.9	18.0 \pm 1.0
	1	-1.9 \pm 6.1	-1.5 \pm 5.8	16.7 \pm 5.8
Piperlactam S	50	78.5 \pm 1.8***	6.9 \pm 2.5	59.3 \pm 2.5
MW295	25	57.8 \pm 4.5***	6.8 \pm 0.6	37.7 \pm 2.1
	10	22.2 \pm 1.6*	5.6 \pm 0.6	29.0 \pm 2.0
	5	8.4 \pm 1.3	1.7 \pm 0.9	22.3 \pm 1.5
	1	0.4 \pm 4.4	1.0 \pm 1.1	19.3 \pm 1.5
Piperenone	50	17.4 \pm 1.0	28.1 \pm 1.3**	18.3 \pm 1.5
MW388	25	4.9 \pm 2.1	21.5 \pm 1.4*	18.0 \pm 1.7
	10	-0.6 \pm 3.5	16.1 \pm 1.5	17.3 \pm 2.1
	5	2.2 \pm 1.3	12.6 \pm 2.4	16.7 \pm 2.1
	1	-1.9 \pm 5.0	12.9 \pm 1.4	16.3 \pm 2.3

Values are based on three experiments performed in triplicate. Values significantly different from DMSO group are indicated by Student's t-test. * : $P < 0.05$; ** : $P < 0.01$; *** : $P < 0.001$.

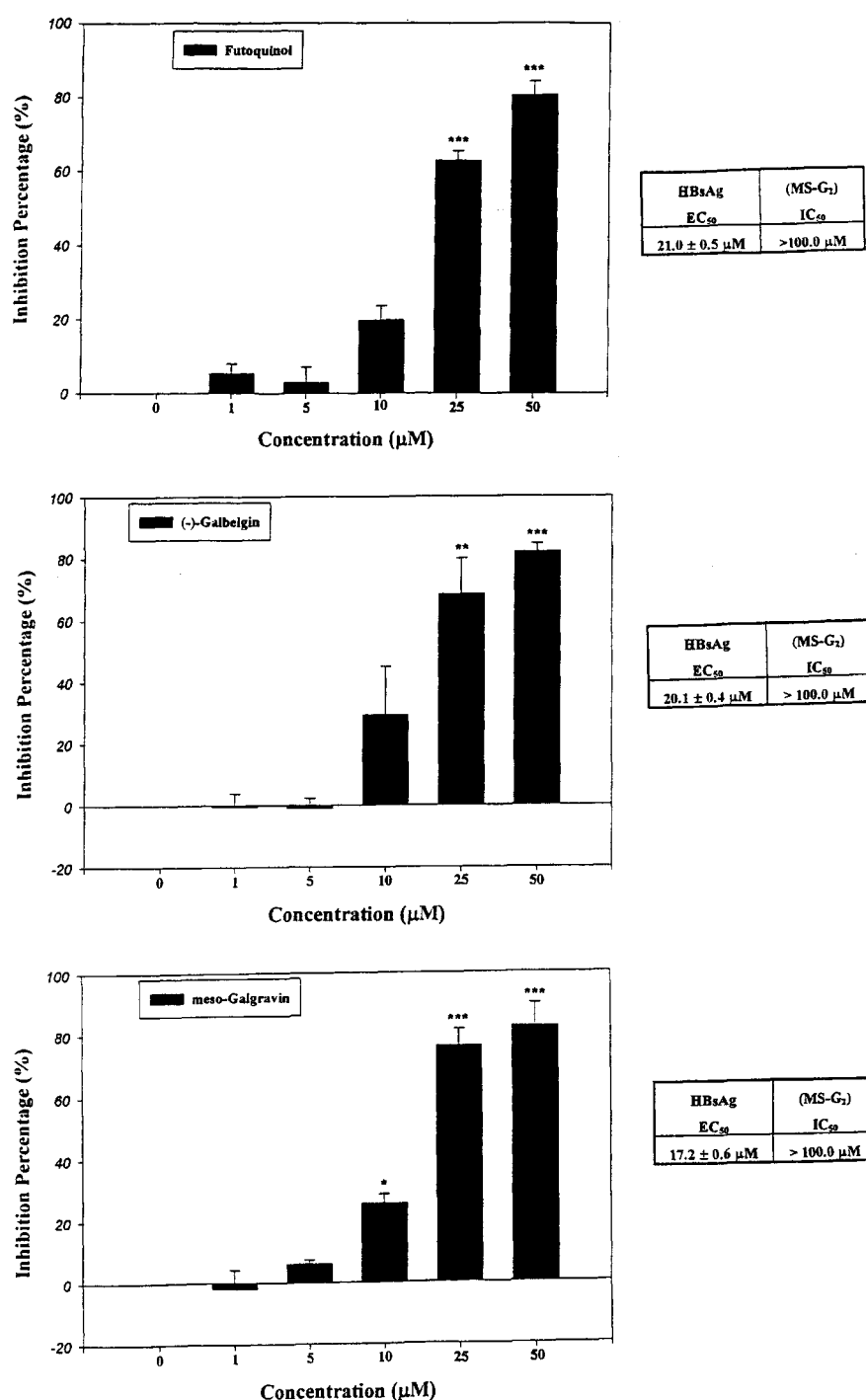


Fig. 2. Anti-HBsAg Effect of Futoquinol, (-)-Galbelgin, and meso-Galgravin Isolated from *Piper kadsura*.

(-)-galbelgin, and meso-galgravin exhibited an anti-HBsAg inhibition percentage of 80.6 ± 3.7 ($P < 0.001$), 81.9 ± 2.8 ($P < 0.001$), and 82.2 ± 7.5 % ($P < 0.001$), respectively. While for the anti-HBeAg effects which are also plotted in Fig. 3, futoquinol, (-)-galbelgin, and meso-galgravin exhibited anti-HBeAg inhibition percentages of 69.4 ± 12.4 ($P < 0.01$),

70.9 ± 7.1 ($P < 0.001$), and 70.2 ± 5.6 % ($P < 0.001$), respectively. In contrast, futoenone and (+)-crotepoxyde did not

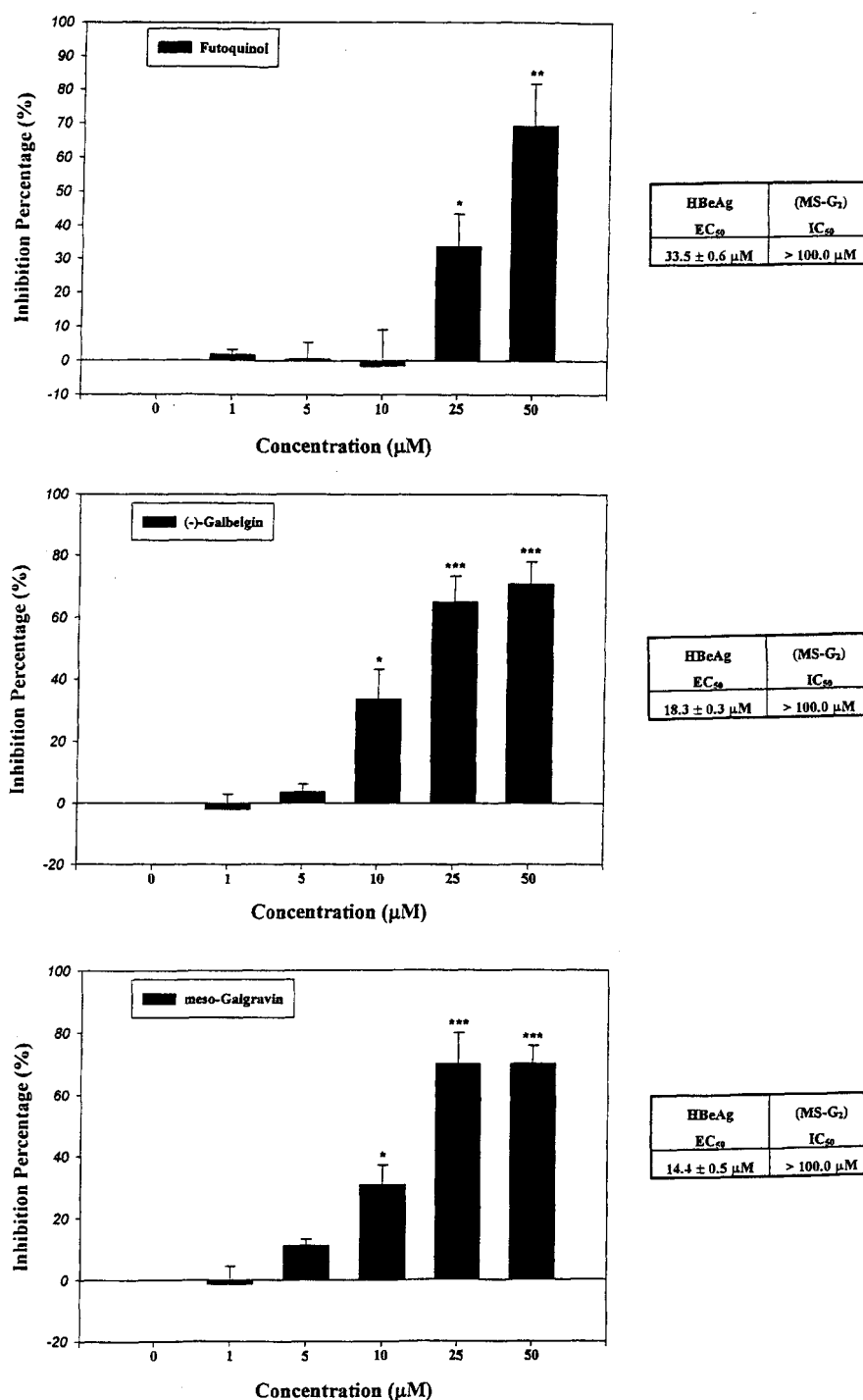


Fig. 3. Anti-HBeAg Effect of Futoquinol, (-)-Galbelgin, and meso-Galgravin Isolated from *Piper kadsura*.

show any noticeable anti-viral effect (also from Table 1). As for piperlactam S, although it showed strong anti-HBsAg effects with an inhibition percentage of $78.5 \pm 1.8\%$ ($P < 0.001$) at 50 μM, it also exhibited cytotoxic effects. Specifically, for the AST levels which are also plotted in Fig. 4, cultured MS-G2 cells depicted normal AST level of < 25 I.U./L, while the AST level of piperlactam S-treated group was found to be 59.3 I.U./L, indicating cytotoxic effects.

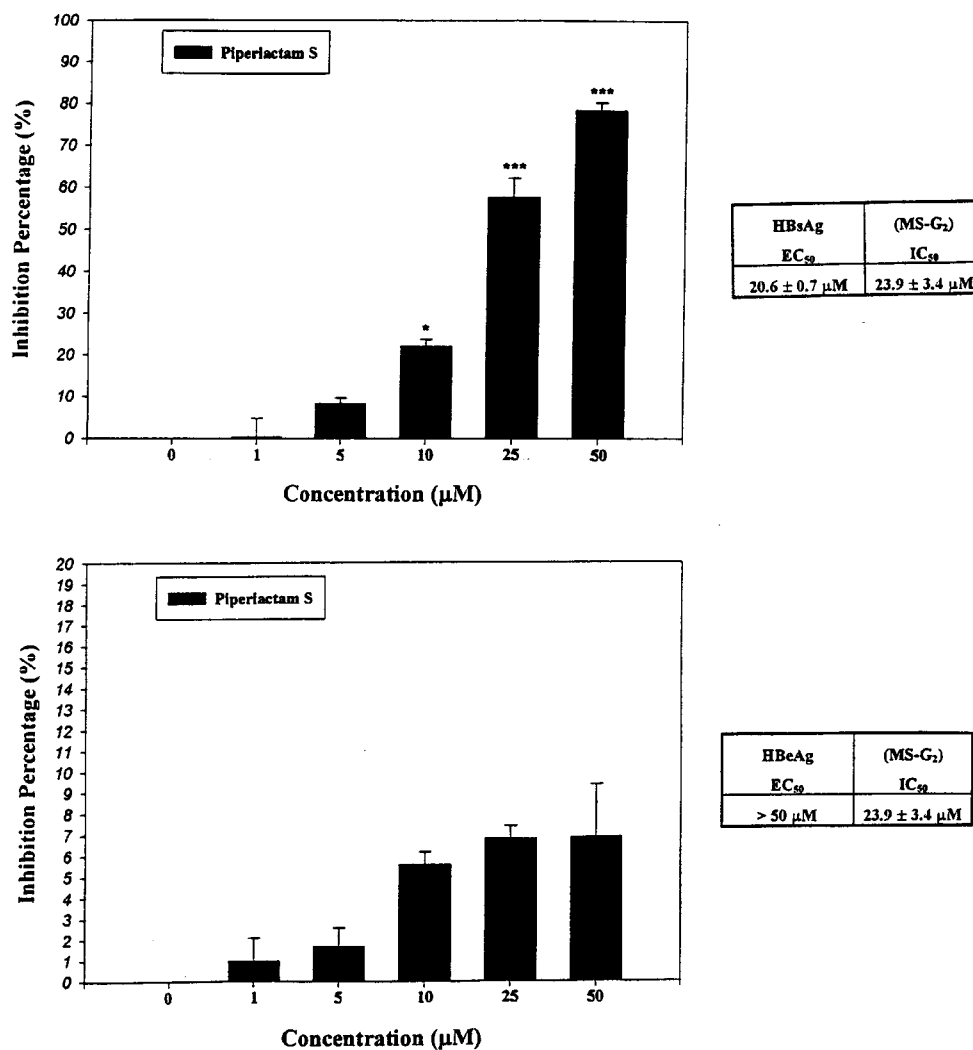


Fig. 4. Anti-HBsAg and Anti-HBeAg Effects of Piperlactam S Isolated from *Piper kadsura*.

Finally, as can be seen in Table 1 and also plotted in detail in Fig. 5, piperenone only slightly suppressed HBV e antigen production even at the high concentration of 50 μM (i.e., 25 - 35 %). As shown in Fig. 5, the anti-HBeAg inhibition percentage is 28.1 ± 1.3 % ($P < 0.01$), while showing essentially no anti-HBsAg activity at all. The effective concentration fifty (EC₅₀) of anti-HBsAg and anti-HBeAg for the seven test compounds is tabulated in Table 2. The effective anti-HBsAg concentration fifty (EC₅₀) of futoquinol, futoenone, (+)-crotopoxide, (-)-galbelgin, meso-galgravin, piperlactam S, and piperenone are 21.0 ± 0.5 , > 50, > 50, 20.1 ± 0.4 , 17.2 ± 0.6 , 20.6 ± 0.7 , and > 50 μM, respectively. In addition, the anti-HBeAg effective concentration fifty (EC₅₀) of futoquinol, futoenone, (+)-crotopoxide, (-)-galbelgin, meso-galgravin, piperlactam S, and piperenone are 33.5 ± 0.6 , > 50, > 50, 18.3 ± 0.3 , 14.4 ± 0.5 , > 50, and > 50 μM, respectively.

By using the MTT method, the cytotoxic effects of the seven test compounds were assayed in parallel with antiviral activity. The concentration required to inhibit cell viability by 50 % (the IC_{50}) was determined and also

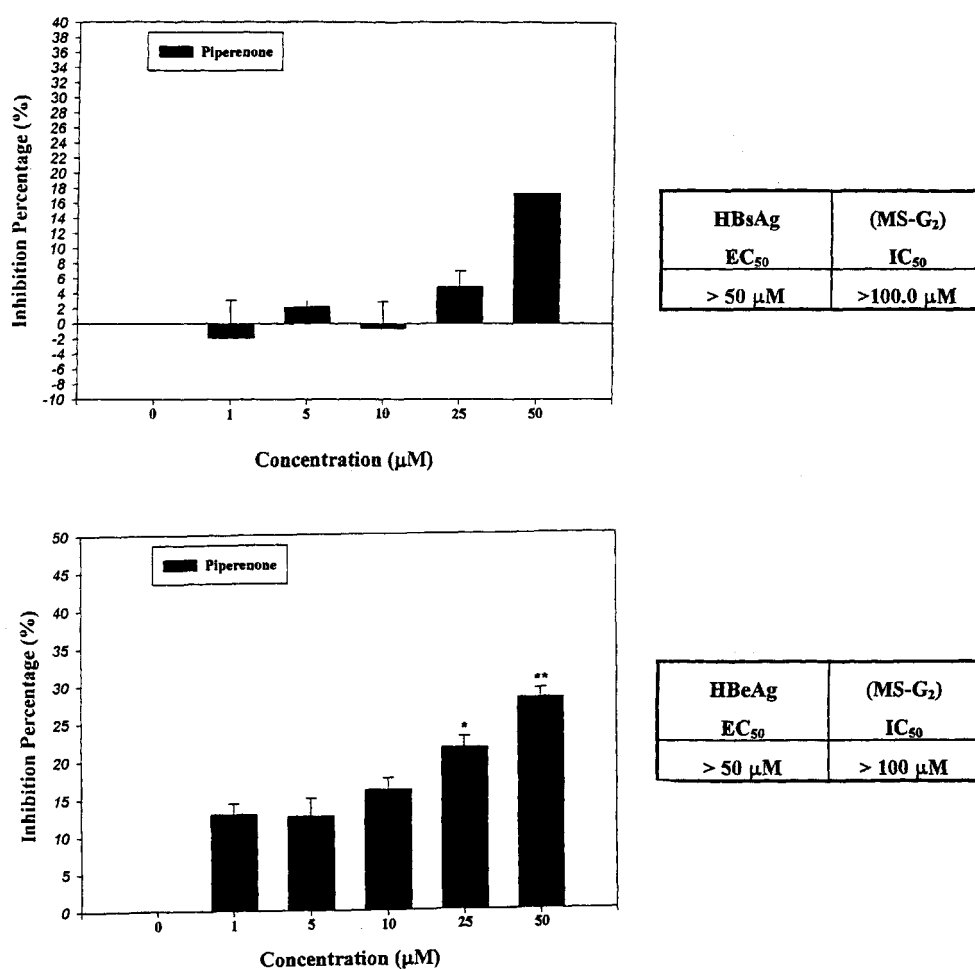


Fig. 5. Anti-HBsAg and Anti-HBeAg Effects of Piperenone Isolated from *Piper kadsura*.

Table 2. Effective Concentration Fifty (EC₅₀) and Inhibition Concentration Fifty (IC₅₀) of Seven Test Compounds.

Compounds	HBsAg (EC ₅₀ , μM)	HBeAg (EC ₅₀ , μM)	IC ₅₀ , μM
Futoquinol	21.0 ± 0.5	33.5 ± 0.6	> 100
Futoenone	> 50	> 50	> 100
(+)-Crotepoxide	> 50	> 50	> 100
(-)-Galbelgin	20.1 ± 0.4	18.3 ± 0.3	> 100
meso-Galgravin	17.2 ± 0.6	14.4 ± 0.5	> 100
Piperlactam S	20.6 ± 0.7	> 50	23.9 ± 3.4
Piperenone	> 50	> 50	> 100

summarized in Table 2. The IC₅₀ of futoquinol, futoenone, (+)-crotepoxide, (-)-galbelgin, meso-galgravin, and piperenone were all > 100 μM and showed non-cytotoxic effects. Only piperlactam S showed cytotoxic effects. In

particular, the IC_{50} of piperlactam S is $23.9 \pm 3.4 \mu M$, which was very close to its anti-HBsAg effective concentration fifty (EC_{50}) $20.6 \pm 0.7 \mu M$, thus further confirmed its cytotoxicity.

DISCUSSION

The HBV producing cell line MS-G2 is derived from human hepatoblastoma (HepG2) in which the HBV genome is stably incorporated into the host genome where HBV is replicated, causing the production of viral particles which leak to culture medium²⁷. HBV is known to consist of 42-nm Dane particles and 22-nm subviral particles (which contain both spherical and filamentous shell), both having the surface envelope. Serological markers are used routinely as diagnostic and prognostic indicators of acute and chronic HBV infections. Presence of HBsAg is the most common marker of HBV infection whereas HBeAg is used as an ancillary marker, primarily to indicate active HBV replication and associated progressive liver disease.

In the present study, we observed that futoquinol, (-)-galbelgin, and meso-galgravin can effectively suppress both HBV surface and e antigen productions. These results suggest that some natural products are potent agents against HBV. Further study of the action mechanisms of these compounds is necessary. In addition, piperlactam S proved to be toxic because none of the test concentrations of piperlactam S that showed activity against HBV were nontoxic for the cell.

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REFERENCES

1. Szmunes W. Hepatocellular carcinoma and hepatitis B virus: evidence for a causal association. *Prog Med Virol* 24: 40-69, 1978.
2. Beasley RP, Huang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. *Lancet* 2: 1129-1133, 1981.
3. Beasley RP, Hwang LY. Epidemiology of hepatocellular carcinoma. In Vyas, G. N. (eds). *Viral Hepatitis and Liver Disease*. Grune & Stratton, New York, pp.209-224, 1984.
4. Tong M.J, Sun SC, Schaeffer BT, Chang NK, Lo KJ, Peters RL. Hepatitis-associated antigen and hepatocellular carcinoma in Taiwan. *Ann Intern Med* 75: 687-691, 1971.

5. Mutchnick MG, Ehrinpreis MN, Kinzie JL, Peleman RR. Prospective on the treatment of chronic hepatitis B and chronic hepatitis C with thymic peptides and antiviral agents. *Antiviral Res.* 24: 245-257, 1994.
6. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 119: 312-323, 1993.
7. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. Preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 333: 1657-1661, 1995.
8. Takahashi S. The presence of the tumor inhibitor crotepoixide (futoxide) in *Piper futokadzura*. *Phytochem* 8: 321-322, 1969.
9. Lin TT, Lu SY. Piperaceae, In *Flora of Taiwan*: 2nd ed.; Editorial Committee of the Flora of Taiwan; Taipei, Vol. II, pp. 624-631, 1996.
10. Han GQ. PAF receptor antagonistic principles from Chinese traditional drugs. *Prog Nat Sci* 5: 299-306, 1995. *Chem Abstr* 123:305964r, 1995.
11. Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jain A, Tyagi OD, Prasad AK, Wengel J, Olsen CE, Boll PM. Phytochemistry of the genus *Piper*. *Phytochem* 46: 597- 673, 1997.
12. Chang MN, Han GQ, Arison BH, Springer JP, Hwang SB, Shen TY. Neolignans from *Piper futokadzura*. *Phytochem* 24: 2079-2082. 1985.
13. MacRae AD, Towers GHN. Biological activities of lignans. *Phytochem* 23: 1207-1220, 1988.
14. Neerja J, Garg HS, Bhakuni DS. Chemical constituents of *Piper schmidtii*: Structure of a new neolignan schmidtin. *J Nat Prod* 53: 479-482, 1990.
15. Huang RL, Chen CC, Huang YL, Hsieh DJ, Hu CP, Chen CF, Chang CM. Osthole increases glycosylation of hepatitis B surface antigen and suppresses the secretion of hepatitis B virus in vitro. *Hepatology* 24: 508-515, 1996.
16. Takahashi S, Ogiiso A. The structure of futoquinol, a constituent of *Piper futokadzura* Siebet Zucc. *Chem Pharm Bull* 18: 100-104, 1970.
17. Tyagi OD, Jensen S, Boll PM, Sharma NK, Bisht KS, Parmar VS. Lignans and neolignans from *Piper schmidtii*. *Phytochem* 32: 445-448, 1993.
18. Angle SR, Turnbull KD. Synthesis of neolignans via a proposed biosynthetic intermediate. Total synthesis of (±)-futoenone. *J Org Chem* 58: 5360- 5369, 1993.
19. Shing YKM, Tam EKW. Enantiospecific syntheses of (+)-crotepoixide, (+)-boesenoxide, (+)-β-senepoxide, (+)-pipoxide acetate, (-)-iso-crotepoixide, (-)-senepoxide, and (-)-tingtanoxide from (-)-quinic acid. *J Org Chem* 63: 1547-1554, 1998.
20. Takeya T, Matsumoto H, Kotani E, Tobinaga S. New reagent systems containing CrO₃ provide precursors for syntheses of neo-lignans. *Chem Pharm Bull* 31: 4364-4367, 1983.

21. Holloway D, Scheinmann, F. Two lignans from *Litsea grandis* and *L. gracilipes*. *Phytochem* 13: 1233-1236, 1974.
22. Sumathykutti MA, Rao JM. 8-Hentriacontanol and other constituents from *Piper attenuatum*. *Phytochem* 30: 2075-2076, 1991.
23. Wu QL, Wang SP, Tu GZ, Feng YX, Yang JS. Alkaloids from *Piper puberulum*. *Phytochem* 44: 727-730, 1997.
24. Koul JL, Koul SK, Taneja SC, Dhar KL. Oxygenated cyclohexanes from *Piper cubeb*. *Phytochem* 41: 1097-1099, 1996.
25. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JD. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* 47: 936-942, 1987.
26. Huang RL, Chen CC, Huang YL, Ou JC, Hu CP, Chen CF, Chungming Chang. Anti-tumor effects of *d*-dicentrine from the root of *Lindera megaphylla* Hemsl. *Planta Medica* 64: 212-215, 1998.
27. Sureau, C., Romet-Lemonne JL, Mullins JI, Essex M. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell* 47: 37-47, 1986.

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風藤抗 B 型肝炎病毒活性成分之研究

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以具有 B 型肝炎病毒基因體嵌入其內之人類肝癌細胞株 MS-G2，進行中草藥抗病毒之篩檢。Futoquinol, Futoenone, (+)-Crotopoxide, (-)-Galbelgin, meso-Galgravin, Piperlactam S 及 Piperenone 為七個分離自胡椒科 (Piperaceae) 植物風藤 *Piper kadsura* (Choisy) 之純化合物，實驗結果顯示：Futoquinol, (-)-Galbelgin 及 meso-Galgravin 三個成分，均於 25 μM 濃度具有顯著抑制 B 型肝炎病毒表面抗原及 e 抗原之活性，Piperenone 對 B 型肝炎病毒表面抗原無抑制作用，須要到高劑量 50 μM 才對 e 抗原具輕度抑制作用，至於 Piperlactam S 其對 B 型肝炎病毒的有效作用濃度 (EC_{50}) 為 $20.6 \pm 0.7 \mu\text{M}$ ，對 MS-G2 細胞生長抑制濃度 (IC_{50}) 為 $23.9 \pm 3.4 \mu\text{M}$ ，顯示 Piperlactam S 無抑制病毒的作用，而具細胞毒性作用，而其他二個成分 Futoenone 及 (+)-Crotopoxide 則無抑制 B 型肝炎病毒表面抗原及 e 抗原之活性。

關鍵詞：B 型肝炎病毒，表面抗原，e 抗原，風藤，胡椒科。