ท/0245/48

INHIBITORY ACTIVITY ON HEPATITIS B SURFACE ANTIGEN SECRETION AND ANTIBACTERIAL ACTIVITY OF THAI MEDICINAL PLANT EXTRACTS

Sattaporn Sirotamarat[1,*] *Thaveechai Vachirayonstien*[2] *Kruavon Balachandra*[2] *Ekarin Saifah*[3]

Abstract

Eleven ethanol extracts of Thai medicinal plant were investigated for inhibitory activity on hepatitis B surface antigen (HBsAg) secreted from PLC/PRF/5 cells and antibacterial activity. All samples inhibited HBsAg secretion from PLC/PRF/5 cells, calculated by IC_{50}. The toxicity of ethanol extracts was performed and calculated by CC_{50}. The selectivity index (SI, CC_{50}/ IC_{50}) of the extracts were evaluated. Two ethanol extracts, *Derris scandens* and *Rhinacanthus nasutus*, were selected for further purification with other solvents including hexane, chloroform, ethyl acetate and butanol, respectively. These fractions were further investigated for inhibitory activity and cytotoxicity. The best activity were attained by butanol extract of *D. scandens* (IC_{50} 54.82 μg/ml, CC_{50} 126.53 μg/ml, SI 2.31 and 0.09% of yield) and hexane extract of *R. nasutus* (IC_{50} 126.12 μg/ml, CC_{50} 257.87 μg/ml, SI 2.04 and 0.38% of yield). For antibacterial activity against test organisms, ethanol extracts of *Caesalpinia sappan*, *Santalum album* and *R. nasutus* (50 mg/ml) could inhibit the growth of *Staphylococcus aureus* ATCC 6538P. Hexane, chloroform and butanol extract of *D. scandens* (50 mg/ml) exhibited inhibition zone on *S. aureus* ATCC 6538P (10, 14, 11 mm) and *Bacillus cereus* ATCC 6548 (12, 12, 12 mm). Hexane, ethyl acetate and butanol extracts of *R. nasutus* (50 mg/ml) could exhibit inhibition zone on *S. aureus* ATCC 6538P (15, 9, 13 mm) and the extracts of hexane and ethyl acetate showed the same effect on *B. cereus* ATCC 6548(12, 11 mm).

Key words: Medicinal plant extracts, Hepatitis B virus (HBV), HBsAg, Inhibitory activity, Antibacterial activity

[1]Department of Microbiology and [3]Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok

[2]Medical Biotechnology Center, Department of Medical Sciences, Ministry of Public Health, Nonthaburi

*To whom correspondence should be addressed: e-mail address sattaporn.s@chula.ac.th, Tel: 0 2218 8377, Fax: 0 2254 5195

นิพนธ์ต้นฉบับ

ฤทธิ์ยับยั้งการสร้างแอนติเจนชนิดผิวของไวรัสตับอักเสบบี และฤทธิ์ต้านแบคทีเรียของสารสกัดสมุนไพรไทย

สัตถาพร สิโรตมรัตน์[1,] ทวีชัย วชิรยนเสถียร[2] เครือวัลย์ พลจันทร[2] เอกรินทร์ สายฟ้า[3]*

บทคัดย่อ

นำสมุนไพรไทย 11 ชนิดมาสกัดด้วยเอทานอล ทดสอบฤทธิ์ยับยั้งการสร้างแอนติเจนชนิดผิวของไวรัสตับอักเสบบี (IC_{50}) ซึ่งหลั่งออกมาจากเซลล์เพาะเลี้ยง PLC/PRF/5 และความเป็นพิษของสารสกัด (CC_{50}) คำนวณหา selectivity index (SI, CC_{50}/ IC_{50}) ของทุกตัวอย่าง เลือกสารสกัดสมุนไพร 2 ชนิด คือ เถาวัลย์เปรียง (*Derris scandens*) กับทองพันชั่ง (*Rhinacanthus nasutus*) มาทำให้บริสุทธิ์มากขึ้นด้วยตัวทำละลายเฮ็กเซน คลอโรฟอร์ม เอทิลอะซิเตต และบิวทานอล ตามลำดับ พบว่าสารสกัดที่มีฤทธิ์ยับยั้งดีที่สุด คือ สารสกัดบิวทานอลของเถาวัลย์เปรียง (IC_{50} 54.82 μg/ml, CC_{50} 126.54 μg/ml, SI 2.31, 0.09 % yield) และสารสกัดเฮ็กเซนของทองพันชั่ง (IC_{50} 126.12 μg/ml, CC_{50} 257.87 μg/ml, SI 2.04, 0.38 % yield) เมื่อทดสอบฤทธิ์ของสารสกัดสมุนไพรต่อการยับยั้งการเจริญของแบคทีเรียทดสอบ พบว่าสารสกัดเอทานอลของฝาง แก่นจันทน์ และทองพันชั่งเข้มข้น 50 มก./มล. มีฤทธิ์ยับยั้ง *Staphylococcus aureus* ATCC 6538P ส่วนสารสกัดเฮ็กเซน คลอโรฟอร์ม และบิวทานอลของเถาวัลย์เปรียง (50 มก./มล.) มีฤทธิ์ยับยั้ง *Staphylococcus aureus* ATCC 6538P เกิดโซนใส 10, 14 และ 11 มม. และยับยั้ง *Bacillus cereus* ATCC 6548 (โซนใส 12, 12 และ 12 มม.) สารสกัดเฮ็กเซน เอทิลอะซิเตต และบิวทานอล ของทองพันชั่ง (50 มก./มล.) มีฤทธิ์ยับยั้ง *Staphylococcus aureus* ATCC 6538P (15, 9 และ 13 มม.) และสารสกัดเฮ็กเซนกับเอทิลอะซิเตตของทองพันชั่งมีฤทธิ์ยับยั้ง *Bacillus cereus* ATCC 6548 (12 และ 11 มม.) ตามลำดับ

คำสำคัญ : สารสกัดสมุนไพร, ไวรัสตับอักเสบบี, แอนติเจนชนิดผิวไวรัสตับอักเสบบี, ฤทธิ์ยับยั้ง, ฤทธิ์ต้านแบคทีเรีย

[1]ภาควิชาจุลชีววิทยา [3]ภาควิชาเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ
[2]ศูนย์เทคโนโลยีชีวภาพทางการแพทย์และสาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข นนทบุรี
*ติดต่อได้ที่ รศ. สัตถาพร สิโรตมรัตน์ e-mail address: sattaporn.s@chula.ac.th โทรศัพท์ 0 2218 8377 โทรสาร 0 2254 5195

Introduction

There is currently a large and ever-expanding global population base that prefers the use of natural products in treating and preventing medicinal problems. Fortunately, many traditional medicinal plants and herbs were reported to have potential as antiviral and antibacterial activity and some of them have already been used to treat people and animals that suffered from the infections. Thai medicinal plants have been used in treating diseases for a long time in Thailand[1]. However reports from research laboratory presenting their responses and effectiveness are still few. Infectious diseases are medicinal problems for Thailand public health and hepatitis B viral infection is one of the most important diseases. It is a leading cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for million of deaths annually. In addition, chronic hepatitis B infection is the most important risk factors, which lead to liver cancer. The distribution of hepatitis B infection varies greatly throughout the world. More than 350 million individuals were infected worldwide[2]. In Thailand, hepatitis B virus (HBV) carriers were about 4.36-10 percent of the patients[3] and especially about 6.3 percent were pregnant women[4]. HBV is transmitted in 2 modes. The high distribution, vertical transmission, is from mother to her child and the lower, horizontal transmission, is through parenteral or percutaneous contact with infected blood, body fluid or sexual intercourse[2]. Nowadays, the treatment of chronic hepatitis B was aimed at the elimination of the infectivity and transmission by the use of interferon[5] and antiviral drugs, namely lamivudine[6] and famciclovir[7]. However the curing responses from patients treated by interferon were different. Recovery from hepatitis B viral infection was estimated about 25-40 percent and absolutely curing from the disease was about 8 percent[8]. Moreover the cost of interferon and antiviral drugs were high and their limitations in treatment were founded[9]. Thus, the use of medicinal plants becomes an interesting target for research to substitute the conventional drugs and chemicals. The purpose of this research was to screen for inhibitory activity on HBsAg secretion from PLC/PRF/5 cells and antibacterial activity in some Thai medicinal plants. Usefulness of this research was to decrease dependency on import drugs.

Materials and Methods

1. Plant materials

Eleven varieties of medicinal plants were bough from local markets. Each of them was identified by comparing with crude drug specimens in Museum of Natural Medicine of

Faculty of Pharmaceutical Sciences, Chulalongkorn University and from the recommendation of two associate professors in Department of Pharmaceutical Botany (Dr. Rapepol Bavovada and Dr. Ekarin Saifah), Chulalongkorn university. Then these plants were extracted twice with 95% ethanol, filtered and evaporated at 70 °C. To increase their solubility, the extracts were subjected to complex form by treating with polyvinylpyrrolidone (1:4-1:6 w/w, PVP K30, Fluka)[10, 11].

The ethanol extract of *D. scandens* and *R. nasutus* were partitioned by hexane and evaporated at 50 °C and then, further partitioned by chloroform, ethyl acetate and butanol, respectively. Each fraction was subjected to complex form by PVP to become hexane, chloroform, ethyl acetate and butanol extracts.

2. Cell Culture

Human hepatocellular carcinoma cell line, PLC/PRF/5cells, was provided from Medical Biotechnology Center, Department of Medical Sciences, Ministry of Public Health, Thailand. One milliliter of PLC/PRF/5 seed culture was thawed at 37°C and mixed in 10 ml maintenance medium, Minimal essential medium (Eagle's MEM, Nissui, Japan) with 2% fetal bovine serum albumin (FBS, HyClone^{R}, USA). The cell suspension was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and added 15 ml of growth medium, 10% FBS in MEM. Cell suspension was cultured in 75 cm^2 tissue culture flask and incubated in 5% CO_2 incubator at 37°C until the cell monolayers were confluent.

3. Cytotoxicity by MTT assay

The cells were washed twice with phosphate buffered solution (PBS) and 2.5 ml of 0.04% trypsin (GIBCO^{TM}) was added to detach the cells. Trypsin was discarded and the growth medium was added to the cells. Viable cells were counted by staining with 0.2% trypan blue (Fluka). Then, the cells were diluted with growth medium to a concentration of $2x10^5$cell/ml and distributed to each well of 96 well flatbottomed tissue culture plate. (Costar^{R}, Corning, USA) and incubated in CO_2 incubator at 37°C for 24 hrs. Medicinal plant extracts were diluted as serial two-fold dilution with the maintenance medium. Each solution was sterilized by filtration with 0.2 μm filter membrane (Minisart^{R}, Sartorius, Germany). Then the culture medium in 96 well plate was discarded and 100 μl of test sample was added into each well. For control well, only 100 μl of maintenance medium was

added. Each experiment was performed in triplicate and incubated in CO_2 incubator at 37°C for 24 hrs.

To perform MTT assay, 12 3-(4,5-dimethyl-thiazoyl-2-yl) 2,5-diphenyl tetrazolium bromide, (USBTM, USA) , culture medium was discarded and 50 µl of MTT in PBS (2 mg/ml) was added. The cells were incubated for 3 hrs. and 100 µl of dimethyl sulfoxide (DMSO, Merck, Germany) was added to dissolve the formazan crystal. Then absorbance reading (optical density, OD) was performed at 570 nm by spectrophotometer (BIO-RAD Model 550 Microplate reader). Thirteen percent viability and 50% cytotoxic concentration (CC_{50}) were calculated.

4. Inhibitory activity (Anti HBsAg) assay by ELISA

The cell was prepared with the same method as that in cytotoxic assay but used 24 well plate (CostarR, USA). After discarding culture medium from plate, 1 ml of test sample was added to each well. For control, only maintenance medium was added and each experiment was performed in duplicate and incubated in CO_2 incubator at 37°C for 24 hrs.

To determine anti-HBsAg activity, culture medium was harvested and the amounts of HBsAg were assessed by the enzyme linked immunosorbent assay (ELISA, Murex HBsAg Verion 3, Abbott, Thailand). Absorbance reading on each well was performed at 450/620 nm by spectrophotometer (Lucy 2 ELISA reader, Lucysoft). Percentage inhibition as 50% inhibition concentration (IC_{50}) was calculated.

5. Antibacterial activity

Eleven ethanol extracts of medicinal plants and hexane, chloroform, ethyl acetate and butanol extracts of *D. scandens* and *R. nasutus* were diluted in sterilized water to concentration 50 mg/ml. The antibacterial activity of plant extracts against test organisms, namely *Staphylococcus aureus* ATTCC 6538P, *Bacillus cereus* ATTCC 6548, *Escherichia coli* ATTCC 25922, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, were performed by agar diffusion method. Each of test organisms was prepared to approximately $1x10^8$ cells/ml in sterilized normal saline comparing turbitdity with 0.5 McFarland no.1 and then spread on Muller Hinton agar (MHA, Difco) plate by sterilized cotton swab. The stainless cups, 200 µl by volume, were placed on the surface of MHA plate and filled with the extract solutions. Each experiment was performed in triplicate and incubated at 37°C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones

and the mean of the diameters of the inhibition zones was calculated. Diameter of stainless cup was 7 mm.

Results

Table 1 showed types of medicinal plant and their parts to be used and ratios of ethanol extracts and PVP. The result showed that PVP had no effect on viable of cell culture at concentration 5-20 mg/ml.

Table 1 Plants, parts of plant and polyvinylpyrrolidone (PVP) ratio

No.	Plants	Parts of Plant	Ethanol extract : PVP (w/w)
1	*Caesalpinia sappan* Linn.(ฝาง)	Leaves and stem	1 : 4
2	*Derris scandens* Benth.(เถาวัลย์เปรียง)	Leaves and stem	1 : 4
3	*Duranta repens* Linn.(เทียนหยด)	Leaves	1 : 4
4	*Gossypium herbaceum* Linn.((ฝ้าย)	Leaves and stem	1 : 4
5	*Homalomena aromatica* Schott. (เต่าเกียด)	Dried root	1 : 4
6	*Houttuynia cordata* Thunb (พลูคาว)	Whole stem	1 : 4
7	*Santalum album* Linn. (แก่นจันทน์)	Heart wood	1 : 4
8	*Loranthus pentandrus* Linn. (กาฝากมะม่วง)	Leaves	1 : 6
9	*Phyllanthus emblica* Linn. (มะขามป้อม)	Fruit	1 : 4
10	*Phyllanthus amarus* Schum&Thonn. (ลูกใต้ใบ)	Whole stem	1 : 5
11	*Rhinacanthus nasutus* (Linn.) Kurz (ทองพันชั่ง)	Leaves and stem	1 : 4

Eleven ethanol extracts of medicinal plants had inhibitory activity to suppress the secretion of HBsAg from PLC/PRF/5 cells (Table 2). *P. amarus* extract showed the highest inhibitory activity followed by *D. scandens* and *R. nasutus* as calculated in IC_{50} values of 46.72, 138.32 and 140.48 μg/ml, respectively. The cytotoxicity by MTT staining calculated in CC_{50}, showed that the three top most highest toxicity were the extracts from *Duranta repens*, *Santalum album* and *D. scandens*, respectively (CC_{50} 166.33, 172.15, 186.72 μg/ml). But the low toxicity was the extracts from *R. nasutus*, *Homalomena aromatica*, and *Caesalpinia sappan*, respectively (CC_{50} 541.00, 379.75, 325.99 μg/ml). The extracts that had high SI (CC_{50}/ IC_{50}) were *P. amarus*, *R. nasutus*, *C. sappan* and *D. scandens*, respectively (4.67, 3.85, 1.45 and 1.35).

Table 2 Fifty percent of inhibition concentration (IC_{50}), cytotoxicity concentration (CC_{50}) and selectivity index (SI, CC_{50} /IC_{50}) of ethanol extracts

No.	Plant	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
1	*C. sappan*	225.06	325.99	1.45
2	*D. scandens*	138.32	186.72	1.35
3	*D. repens*	159.07	166.33	1.14
4	*G. herbaceum*	221.31	281.00	1.27
5	*H. aromatica*	335.97	379.75	1.13
6	*H. cordata*	202.44	238.95	1.18
7	*S. album*	155.88	172.15	1.10
8	*L. pentandrus*	486.75	>2000	>4.11
9	*P. emblica*	555.26	>2000	>3.66
10	*P. amarus*	46.72	218.27	4.67
11	*R. nasutus*	140.48	541.00	3.85

The extracts of *R. nasutus* (SI 3.85, IC_{50} 140.48 μg/ml, CC_{50} 541.00 μg/ml) and *D. scandens* (SI 1.35, IC_{50} 138.32 μg/ml, 186.72 μg/ml) were selected to purify by hexane, chloroform, ethyl acetate and butanol and investigated their inhibitory activity and cytoxicity. The results showed that the highest activity of *D. scandens* extract was butanol (IC_{50} 54.82 μg/ml, CC_{50} 126.53 μg/ml, SI 2.31) and those of *R. nasutus* extract was hexane (IC_{50} 126.12 μg/ml, CC_{50} 257.87 μg/ml, SI 2.04) (Table 3). It was found that chloroform provided the highest percentage of *D. scandens* yield extracted (1.11%) and for *R. nasutus,* it was hexane (1.69%) (Table 4).

Table 3 *In vitro* CC_{50}, IC_{50} and SI of various solvent extracts

No.	Plant	Fraction	CC_{50} (μg/ml)	IC_{50} (μg/ml)	SI
1	*D. scandens*	Hexane	163.61	119.67	1.34
		Chloroform	674.99	498.88	1.35
		Ethyl acetate	158.34	68.68	2.31
		Butanol	126.53	54.82	2.31
2	*R.nasutus*	Hexane	257.87	126.12	2.04
		Chloroform	215.58	134.25	1.61
		Ethyl acetate	456.25	465.80	0.98
		Butanol	>1000	>1000	UD

UD = undetermined

Table 4 Percent yield of plant extracts in various solvents

No.	Plant	Dried Weight (g)	Fraction	Weight of extracts (g)	% yield
1	*D. scandens*	1188	Hexane	10.7975	0.91
			Chloroform	13.2230	1.11
			Ethyl acetate	2.9322	0.25
			Butanol	1.0741	0.09
2	*R. nasutus*	2000	Hexane	33.8651	1.69
			Chloroform	3.4284	0.17
			Ethyl acetate	2.5288	0.13
			Butanol	3.5412	0.18

Investigation of antibacterial activity of ethanol extracts (50 mg/ml concentrated in sterilized water) was found that extracts of *C. sappan, R. nasutus and S. album* could inhibit the growth of only *S. aureus* ATCC 6538P, the diameter of inhibition zone were 13.5, 12.5, 9 mm, respectively. In addition, hexane, chloroform and butanol extracts of *D. scandens* (50 mg/ml) exhibited antibacterial activity against *S. aureus* ATCC 6538P (10, 14, 11 mm of inhibition zone) and *B. cereus* ATCC 6548 (12, 12, 12 mm of inhibition zone). The hexane, ethyl acetate and butanol extract (50 mg/ml) of *R. nasutus* exhibited antibacterial activity against *S. aureus* ATCC 6538P (15, 9, 13 mm of inhibition zone) and hexane extract and ethyl acetate extract of *R. nasutus* inhibited the growth of *B. cereus* ATCC 6548 (11, 12 mm of inhibition zone) (Table 5). None of all ethanol extracts and various solvent extracts of *D. scandens* and *R. nasutus* could inhibit the growth of *E. coli* ATCC 25922, *Ps. aeruginosa* and *S. typhimurium.*

Table 5 Antibacterial activity of plant extracts against test organisms

Plant	Extract	Inhibition zone (mm)	
		S. aureus ATCC 6538P	*B. cereus* ATCC 6548
C. sappan	ethanol	13.5	-
S. album	ethanol	12.5	-
R. nasutus	ethanol	9.0	-
	hexane	15.0	12.0
	ethyl acetate	9.0	11.0
	butanol	13.0	-
D. scandens	hexane	10.0	12.0
	chloroform	14.0	12.0
	butanol	11.0	12.0

PVP had no antibacterial activity at concentration 5-20 mg/ml

Discussion

Aims of this research were to screen medicinal plants that had inhibitory activity against HBsAg secreted from PLC/PRF/5 cells, and then test for antibacterial activity. There are reports that many kind of medicinal plants and herbs had antiviral activity[14], especially against hepatitis B virus such as *P. amarus*[15], *P. urinaria*[16], *Glycyrrhiza species*[17,18] and *Gossypium herbaceum*[19]. Some researchers reported that the active compounds from *P. amarus* and *P. urinaria* had anti HBV activity[20-23]. Thyagarajan and colleagues reported that dried powder of *P. amarus was* effective in clearing HBsAg from blood of positive carriers in India[24]. However, a number of researches reported that *P. amarus* extract had no anti HBV activity not only *in vitro* but also in clinical treatment with patients in New Zealand using dried *P. amarus* extract in the form of capsules[25]. Narrendranathan and colleagues reported that *P. amarus* powder did not significantly reduce the duration of jaundice in HBV persons[26]. The subsequent clinical result concerning the use of *Phyllanthus* species for hepatitis had been contradicting. This probably caused by the extract standardization, species used, the location they were harvested. These resulted in different levels of active constituents in samples used. Our investigation (*in vitro*) was found that extract from *P. amarus* had the highest inhibitory activity followed by *D. scandens, R. nasutus* and *S. album*, respectively (IC_{50} were 46.72, 138.32, 140.48, 155.88 μg/ml). *P.amarus* was not selected for further investigation since numerous researches of antiviral activity and active compounds of this plant has already been widely studied and published[20-22]. *D. scandens* and *R. nasutus* were interesting for investigation of inhibitory (anti HBsAg) activity because none of documents about this activity from these plants was reported. However, *D. scandens* was reported that it had *in vitro* effect on normal lymphocyte proliferation and its activity on natural killer cells in normal and HIV-1 infected patients[27]. In addition, *R. nasutus* was reported that it had anti-fungal and antibacterial activities[28].

Furthermore, extracts were investigated for antibacterial activity against test organisms, namely *S. aureus* ATCC 6538P, *B. cereus* ATCC 6548, *E. coli* ATCC 25922, *Ps. aeruginosa* and *S. typhimurium*. Results showed that ethanol extracts of *C. sappan, S. album* and *R. nasutus* had antibacterial activity against *S. aureus* ATCC 6538P, a Gram positive strain, but could not inhibit Gram negative strains. This result was related with other reports, *C. sappan* could inhibit on growth of methicillin-resistant *S. aureus*[29]. Moreover, *S. album* or sandalwood oil was used traditionally by herbalists to treat skin diseases, acne, dysentery and gonorrhea[30]. There was reported that this volatile oil contained high amounts of

alpha- and beta-santalol. According to a test tube study, these small molecules possess antibacterial properties[31]. Internal use of sandalwood was approved by the German Commission E for the supportive treatment of infections of the lower urinary tract (usually the urinary bladder). However, clinical trials are lacking to support this use[32]. In addition, various solvent extracts of *D. scandens* and *R.nasutus* were also inhibited Gram positive strains such as *S. aureus* ATCC 6538P and *B. cereus* ATCC 6548 but none of them could inhibit Gram negative strains. This investigation gave result in line with other investigations on the antibacterial activity of *R. nasutus* against only Gram positive strains[33,34].

In conclusion, *in vitro D. scandens* and *R.nasutus* had inhibitory potential on HBsAg secreted from PLC/PRF/5cells and could inhibit the growth of Gram positive bacteria. However, this investigation suggests that *in vivo* testing should be conducted to confirm the plants anti-hepatitis B viral activity. Improvement on the extraction and purification technique should be sought out to ensure a better result.

Acknowledgements

We would like to thank Faculty of Pharmaceutical Sciences, Chulalongkorn University for funding research and Medical Biotechnology Center, Department of Medical Sciences, Ministry of Public Health, Nonthaburi for research supports and excellent cooperation.

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