

Full Length Research Paper

Anti-hepatitis B virus activity of total saponins isolated from *Taraphochlamys affinis* *in vitro* and *in vivo*

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Accepted 26 April, 2013

Taraphochlamys affinis have been previously shown to possess anti-hepatitis B virus (HBV) activities. To identify the active ingredients, we isolated total saponins (TSTA) from the dried herb of *T. affinis* and examined the inhibitory effect of TSTA on HBV *in vitro* and *in vivo*. In human liver cell line HepG2.2.15 transected with HBV, the TC₅₀ of TSTA was 358.6 mg/ml and TC₀ was 77.6 mg/ml, which suggested that the inhibitory action of TSTA had no cytotoxicity. In nontoxic concentrations, TSTA effectively suppressed the secretion of HBV antigens from virus-infected HepG2.2.15 cells, achieving 77.2, 60.4, 55.3, 44.9 and 26.2% inhibition to the secretion of HBsAg, and 43.2, 26.5, 11.9, 2.5 and 19.2% inhibition to that of HBeAg, respectively, at 50, 25, 12.5, 6.25 and 3.125 mg/ml after 72 h of treatment. HBsAg and HBeAg values of treatment index (TI) were 33.6 and 4.1, respectively indicating that TSTA was effective and low in toxicity. In treatment of ducklings infected with duck hepatitis B virus (DHBV), the serum levels of DHBV-deoxyribonucleic acid (DNA) decreased in all 10 ducks treated with two doses of TSTA (1.0 and 2.0 g kg⁻¹ d⁻¹), respectively seven days after the cessation of treatment with TSTA, the effect of DHBV DNA inhibition persisted. Histopathological examination also confirmed the protective function of TSTA in the liver infected with DHBV. Together, our results demonstrate that TSTA possesses potent anti-HBV activity.

Key words: *Taraphochlamys affinis*; the total saponins of *Taraphochlamys affinis* (TSTA), anti-hepatitis B virus activity.

INTRODUCTION

Hepatitis B virus (HBV) infection is still a major global health problem. It is responsible for the delayed sequel of cirrhosis and hepatocellular carcinoma (HCC) (Park et al., 2006; Lee, 1997; Hantz et al., 1999; Liu et al., 2000). Approximately 2 billion people worldwide have been exposed to HBV, with approximately 360 million being chronically infected. Worldwide deaths from liver cancer caused by HBV infection probably exceed 1 million per year (Parkin et al., 1999). Beyond interferon- α (IFN- α),

only two drugs are currently approved for clinical use, namely lamivudine and adefovir. Interferon- α is only partially effective for clinical use and is limited by its side effects. Lamivudine suppresses HBV through inhibition of reverse transcriptase, but the treatment often fails due to the emergence of mutations within the catalytic site of HBV DNA polymerase, which leads to drug-resistance in patients (Buti et al., 2001; Lai et al., 2003). Therefore, it is highly desirable to search for new anti-HBV agents.

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Many screening efforts have been made to find antiviral agents from natural sources.

Plants have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of antiviral agents (Abad et al., 2000). In the last decade, as an alternative to conventional chemical agents, a large number of phytochemicals have been recognized as a way to control infections caused by viruses (Kalvatchev et al., 1997; Yamasaki et al., 1998; Abad et al., 1999; Abad et al., 2000). Natural compounds, due to their structural diversity, provide an ample large opportunity for screening anti-HBV agents with novel structure and distinct mechanism of action.

Taraphochlamys affinis, the dried herb of *T. affinis* (Giff) *Bremekhu* (*Strobilanthes affinis* (Griff) Y.C.Tang) has been widely used in traditional Chinese medicine since ancient times, with an excellent safety record and demonstrated efficacy in the improvement of immune disorders and liver diseases. The major active constituents of *T. affinis* are believed to be the total saponins (Xing et al., 2009a).

In this study, further to investigate this well-known Chinese medicine for its anti-HBV activity, we used 2.2.15 cell line and DHBV-infected duck model to observe and evaluate its anti-HBV activity both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents

Lamivudine (3TC) was purchased from GlaxoSmithKline. Minimum essential medium (MEM), fetal bovine serum (FBS) and G-418 were obtained from Gibco BRL (Gaithersburg, MD, USA). L-glutamine was obtained from Sigma (St Louis, MO, USA). HBsAg and HBeAg enzyme immunoassay (EIA) kits were purchased from Nanjing Jianchen Bioengineering Institute (Nanjing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA).

Preparation of the total saponins of *Taraphochlamys affinis* (TSTA)

Total saponins were prepared by the method described previously (Xi et al., 2008), with slight modification. Dried rhizomes (1 kg) of *T. affinis* collected in the Guangxi province of China and confirmed by the laboratory of traditional Chinese medicine of Guangxi Traditional Chinese Medicine University, was extracted three times with 80% (v/v) ethanol (herb:ethanol = 1:10, w/v) under reflux (85°C) for 90 min. The alcohol extract was concentrated, suspended in distilled water and then partitioned successively with chloroform (ratio 1:5, v/v) and n-butanol saturated with water (ratio 1:3, v/v, three times). The n-butanol extract was combined and evaporated using a rotary evaporator at 70°C to give a powder residue. The yield for the extract was 41.28 g (4.128%, w/w).

Determination of total saponins

The total saponins of *T. affinis* (TSTA) was determined according to

the method of Xi et al. (2008). TSTA was easily soluble in water. Then, TSTA 0.1 ml (0.418 g/ml) were mixed with the vanillin (8% w/v, 0.5 ml) and sulphuric acid (72% w/v, 5 ml). The mixture was incubated at 60°C for 10 min, cooled in an ice water bath for 15 min and the absorbance read at 538 nm. Oleanolic acid was used as a reference standard and the content of total saponins was expressed as oleanolic acid equivalents (OAE µg/mg extract). The total saponins content determination showed that TSTA content was 71.8% (w/w). It should be noted that the vanillin-sulphuric acid reagent gives only an approximate estimate of the total saponins content.

Cell line

HepG2.2.15 cells, a human HepG2 cell line transfected with HBV were provided by Viral Research Section of the First Affiliated Hospital of Beijing Medical University.

Experimental animals

Guangxi ducklings within 1 day of hatching were obtained from an animal breeding farm, The Center of Experimental Animal, Guangxi Medical University. The research was conducted in accordance with the US guidelines (NIH publication #85-23, revised in 1985) for laboratory animal use and care. The animals were cared under standard laboratory conditions of a 12 h light/dark cycle in room with controlled temperature and humidity. Food and water were available *ad libitum*.

Cell culture

The HepG2.2.15 cells were incubated in complete MEM (containing 10% FBS, 100 kU/L benzylpenicillin, streptomycin, G-418, L-glutamine 0.03%, pH 7.0) in 75 cm² tissue culture flasks at 37°C in a humidified 5% CO₂.

Toxicity measurements

The HepG2.2.15 cells were first seeded into 96-well plates at a density of 2×10^4 cells per ml and cultured in 100 µl complete MEM containing 10% FBS. After 24 h of incubation, cells were washed three times with phosphate-buffered saline (pH 7.0) and treated with different concentrations (62.5, 125, 250, 500 and 1000 mg/L) of TSTA in serum-free medium for 72 h. The medium was replaced every 24 h in MEM supplemented with various concentrations of TSTA. Untreated cells were used as a control. 10 µl MTT solution (5 g/ml) was then added to all the wells and incubation continued at 37°C for 4 h, followed by addition of acid-isopropanol (100 µl) to dissolve the dark blue crystals developed from MTT. Then, the plates were read directly by microplate reader at a wavelength of 490 nm (Wang et al., 2009).

Assays for HBsAg and HBeAg in the cell culture

The HepG2.2.15 cells were incubated in 24-well plates at a density of 2×10^4 cells per ml in 1 ml MEM medium containing 10% FBS. After 24 h, the 2.2.15 cells were treated with different concentrations of TSTA (3.125, 6.25, 12.5, 25 and 50 mg/l) in serum-free medium. Cells grew in the presence of drugs for 72 h with changes of medium every 24 h. After 72 h, supernatant was collected and performed at -20°C. The HBsAg and HBeAg in culture medium were simultaneously measured by enzyme immunoassay (EIA) kits (Li et al., 2005).

Table 1. Inhibitory effect of TSTA on HepG2.2.15 cell.

Group	Concentration (mg L ⁻¹)	OD ₄₉₀ (Mean±SD)	Inhibition ratio (%)
TSTA	1000	0.476±0.013	87.0
	500	1.269±0.089	60.9
	250	2.283±0.207	27.6
	125	2.687±0.189	14.3
	62.5	3.078±0.215	1.4
Cell control	-	3.121±0.327	-
3TC	1000	0.358±0.094	75.4
	500	0.928±0.113	61.7
	250	1.764±0.211	47.3
	125	2.889±0.269	12.1
	62.5	3.071±0.316	4.9
Cell control	-	3.274±0.328	-
Empty control	-	0.079±0.009	-

TSTA-induced cytotoxicity was observed by microscope. After 72 h of incubation with different concentrations (62.5, 125, 250, 500 and 1000 mg/L) of TSTA. When TSTA concentration increased, cell injury caused by TSTA was observed. The 50% toxic concentrations (TC₅₀) was 358.6 mg/ml and the maximum nontoxic concentrations (TC₀) was 77.6 mg/ml. n = 6.

Inhibition ratio % = [OD (control) - OD (sample)] / OD (control) × 100%

***In vivo* assays for anti-DHBV activity of TSTA in ducklings**

One-day-old ducklings were intravenously infected with a 5.7×10^6 viral DNA equivalent (VGE, 1 VGE = 3.3×10^6 pg) of duck hepatitis B virus (DHBV). Seven days after infection, ducklings were divided into five groups, with each consisting of 10 ducklings: the control group (normal saline), the positive drug group (3TC, 50×10^{-3} g kg⁻¹ d⁻¹), and the TSTA 0.5, 1.0, and 2.0 g kg⁻¹ d⁻¹ groups. Drugs were administered orally daily for 14 days. Serum samples were obtained at 0th, 7th, and 14th day during treatment, and withdrawn at 7th day. The serums were stored at -70°C for analysis.

Viremia analysis

Viremia was assessed throughout the treatment and follow-up period by a semi-quantitative detection of DHBV-DNA in duck serum using a dot-blot hybridization. Fifty microliters of serum was spotted directly onto nitrocellulose filters. After denaturation and neutralization, the filters were hybridized with a full-length DHBV genomic DNA probe labeled with ³²P. The filters were autoradiographed and the spots were counted in a scintillation counter (Guiqin et al., 2009).

Histopathological examination of hepatocytes

At the end of the experiment, each duckling was laparotomized to obtain the liver immediately after collecting blood from the leg vein. Fragments of the ducklings liver were fixed in 10% formalin solution, dehydrated with ethanol solution from 50 to 100%, embedded in paraffin and cut into 5 µm sections, and stained using

haematoxylin-eosin dye for photomicroscopic observations. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Statistics

The data were expressed as mean ± standard deviation (SD), and analyzed by one-way repeated-measure ANOVA and t-test for comparisons between groups. P < 0.05 was considered statistically significant.

RESULTS

***In vitro* Anti-HBV activity of TSTA in HepG2.2.15 cells**

The cytotoxicity of TSTA was measured in cultured HepG2.2.15 cells. It showed no inhibitory effect on cell proliferation at a concentration up to 77.6 mg/ml as analyzed by MTT assay. The 50% cytotoxic concentration (TC₅₀) was determined to be 358.6 mg/ml. These results were further used to determine the dose range of TSTA for the following experiments (Table 1). TSTA effectively suppressed secretion of HBV antigens from virus-infected HepG2.2.15 cells, achieving 77.2, 60.4, 55.3, 44.9 and 26.2% inhibition to the secretion of HBsAg, and 43.2, 26.5, 11.9, 2.5 and 19.2% inhibition to that of HBeAg, respectively, at 50, 25, 12.5, 6.25 and 3.125 mg/ml after 72 h of treatment (Table 2). HBsAg and HBeAg values of treatment index (TI) were 33.6 and 4.1,

Table 2. Inhibitory effect of TSTA on HBsAg and HBeAg (Mean±SD, n = 3).

Group	Concentration (mg L ⁻¹)	HBsAg OD ₄₉₀ (Mean±SD)	HBsAg inhibition ratio (%)	HBeAg OD ₄₉₀ (Mean±SD)	HBeAg inhibition ratio (%)
TSTA	50	0.195±0.041	77.2	1.538±0.193	43.2
	25	0.308±0.052	60.4	1.967±0.288	26.5
	12.5	0.342±0.046	55.3	2.341±0.385	11.9
	6.25	0.412±0.055	44.9	2.582±0.356	2.5
	3.125	0.537±0.039	26.2	2.639±0.398	0.2
3TC	50	0.298±0.043	61.8	1.442±0.278	47.0
	25	0.326±0.035	57.7	1.839±0.365	31.5
	12.5	0.401±0.040	46.5	2.471±0.408	6.8
	6.25	0.526±0.054	27.9	2.503±0.416	5.5
	3.125	0.633±0.062	11.9	2.598±0.453	1.8
Cell control	-	0.713±0.157	-	2.645±0.311	-
Empty control	-	0.042±0.008	-	0.083±0.009	-

TSTA had a remarkable inhibitory effect on secretion of HBsAg and HBeAg in 2.2.15 cell line, their values of treatment index (TI) were 33.6 and 4.1, respectively indicating that TSTA was effective and low toxic.

respectively indicating that TSTA was effective and low in toxicity.

***In vivo* anti-HBV activity of TSTA in ducks**

During the experiments, no obvious side effects were observed in animals receiving antiviral therapy or in control animals. The effects of TSTA and 3TC which were used for comparison on DHBV replication *in vivo* were determined by quantification of DHBV-DNA by dot-blot hybridization. The levels of serum viral DNA were recorded in the 5 groups before the experiment. During treatment, serum levels of DHBV-DNA decreased in all 10 ducks treated with TSTA 1.0 and 2.0 g kg⁻¹ d⁻¹ (Figure 1). But 7 days after the cessation of treatment with 3TC, the viral replication level returned to the pretreatment baseline. In ducks treated with TSTA, the effect of DHBV DNA inhibition lasted. No significant decrease of serum DHBV-DNA was observed during treatment with TSTA 0.5 g kg⁻¹ d⁻¹.

Histopathological features

Histopathological profiles of the liver from model group ducklings revealed necrosis, steatosis, and often swelling of the hepatic cytoplasm. The protective effect of TSTA was confirmed by histopathological examinations. Administration of TSTA to the experimental animals (2.0 g kg⁻¹ d⁻¹) showed a significant improvement of the hepatocellular architecture over the model group as evident from a considerable reduction in necrosis and vacuolation (Figure 2).

DISCUSSION

Many of natural the products extracted from Chinese traditional or folk medicines exhibit a variety of biological activities, including anti-HBV activity. For example, the polyphenolic extraction from *Geranium carolinianum* L. (PPGC) showed significant anti-HBV activities on HBV replication both *in vitro* and *in vivo* (Li et al., 2008). For another example, wogonin and ellagic acid extracted from *Scutellaria radix* and *Phyllanthus urinaria*, respectively possessed potent anti-HBV activity. Wogonin effectively suppressed the secretion of the HBV antigens and reduced HBV DNA level in a dose-dependent manner (Guo et al., 2007) while ellagic acid effectively blocked HBeAg secretion in HepG2 2.2.15 cells (Shin et al., 2005). Both wogonin and ellagic acid are under early development as anti-HBV drug candidates.

Since 2001, we have been investigating some traditional Chinese medicines which have long been used in the folk treatment of chronic hepatitis in China for novel anti-HBV agents. Laboratory work on collecting and screening hundreds of Chinese traditional herbs has been carried out, but unfortunately resulted in failure until we luckily found *T. affinis* which is promising in anti-inflammation, anti HBV (Xing et al., 2009b, c; Shi-jun et al., 2009) and anti-fibrosis resulting from liver damage. However, the active part of *T. affinis* against HBV is still unknown. Previously, we isolated the total saponins (TSTA) from *T. affinis*, and our studies showed that TSTA could markedly decrease alanine transaminase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA) content, increase superoxide dismutase (SOD), total antioxidant capacity (T-AOC) activity in serum and in

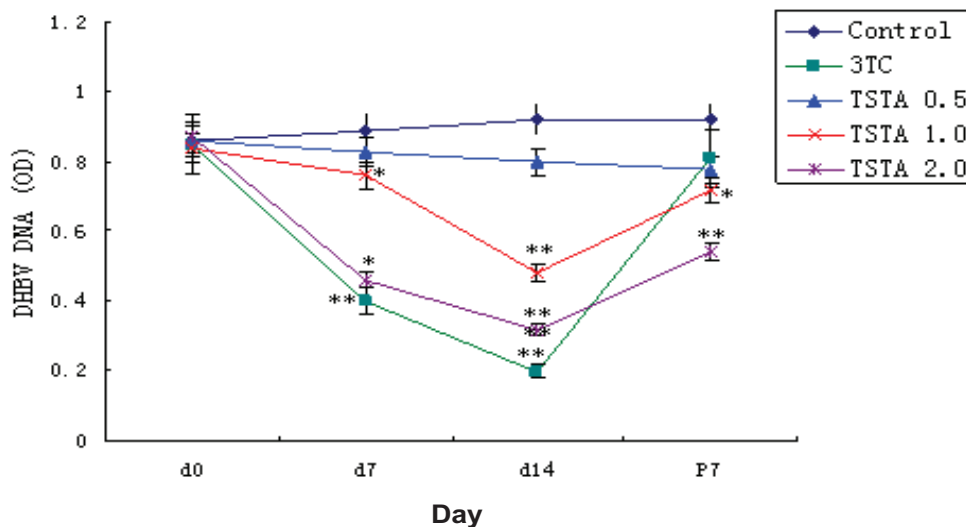


Figure 1. Mean changes of serum DHBV-DNA level in treated duck. (Control: DHBV model control; 3TC, 3TC ($50 \times 10^{-3} \text{g kg}^{-1} \text{d}^{-1}$); TSTA 0.5, TSTA ($0.5 \text{g kg}^{-1} \text{d}^{-1}$); TSTA 1.0, TSTA ($1.0 \text{g kg}^{-1} \text{d}^{-1}$); TSTA 2.0, TSTA ($2.0 \text{g kg}^{-1} \text{d}^{-1}$). Results are presented as the mean \pm SD (Copy/ml) ($n = 10$). * $p < 0.05$, ** $p < 0.01$ vs control. Compared with control group, serum levels of DHBV-DNA decreased in all ducks treated with TSTA (1.0 and $2.0 \text{g kg}^{-1} \text{d}^{-1}$) and 3TC ($50 \text{mg kg}^{-1} \text{d}^{-1}$) during treatment ($p < 0.05$), but 7 days after the cessation of treatment with 3TC, the viral replication level returned to the pretreatment baseline ($p > 0.05$). While in ducks treated with TSTA, the effect of DHBV DNA inhibition lasted ($p < 0.05$). No significant decrease of serum DHBV-DNA was observed during treatment with TSTA $0.5 \text{g kg}^{-1} \text{d}^{-1}$ ($P > 0.05$).

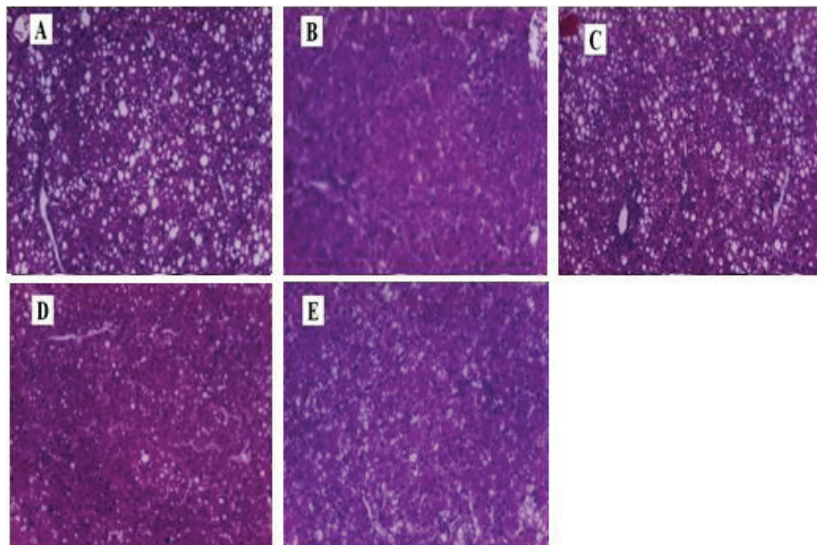


Figure 2. Histopathological examinations of liver in ducklings treated for 14d. (A) control; (B) 3TC group; (C,D,E) TSTA-treated group at a dose of 0.5, 1.0 and $2.0 \text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, respectively. Hematoxylineosin staining: A,B,C,D,E $\times 100$.

liver (Xing et al., 2009a), and could significantly increase percentage of $\text{CD}_3^+ \text{CD}_4^+$ cell and $\text{CD}_4^+/\text{CD}_8^+$, decrease the ratio of CD_8^+ (Xing et al., 2010), which indicated TSTA had a effect of anti-lipiperoxidation and a protective effect on immunological liver injury in mice.

As an extension of our previous investigation, total

saponins from *T. affinis* (TSTA) was extracted and tested in this study. The results demonstrated the inhibitory effect of TSTA on HBsAg and HBeAg secretion by human hepatoma 2.2.15 cells and on serum DHBV-DNA levels of ducklings infected with hepatitis B virus. TC_{50} of TSTA was 358.6mg/ml and TC_0 was 77.6mg/ml in

2.2.15 cells, which suggested that the inhibitory action of TSTA had no cytotoxicity. In nontoxic concentrations, TSTA significantly inhibited the secretion of HBsAg and HBeAg, with TSTA concentration increasing, a dose-dependent response was observed, and HBsAg and HBeAg values of treatment index (TI) were 33.6 and 4.1, respectively.

These results clearly illustrate an inhibitory effect of TSTA on HBsAg and HBeAg production in 2.2.15 cells, which provide strong evidence to evaluate the effect of drugs against HBV in a cellular model, but it is still necessary to verify this in an animal model. Therefore, the inhibitory effect of TSTA in the duck HBV model was investigated. Our experiments with TSTA (0.5, 1.0 and 2.0 g kg⁻¹ d⁻¹) in ducklings pointed to a suppressive action on DHBV replication *in vivo*. During treatment, serum levels of DHBV-DNA significantly decreased in all 10 ducks treated with TSTA 1.0 and 2.0 g kg⁻¹ d⁻¹. It was well known that most antiviral medicines had the inevitable rebound effect after drug cessation. This shortcoming had limited the therapy to those diseases infected by viruses such as HB or acquired immune deficiency syndrome (AIDS). The similar phenomena appeared in the positive control drug 3TC in the present study. TSTA showed therapeutic effects as well as 3TC, and no difference was observed after cessation of TSTA therapy compared to TSTA-treated animals. It suggested that TSTA could maintain for a long time in treating viremia of HBV, and the effect of DHBV-DNA inhibition showed a concentration-dependent response. Histopathological examination also confirmed the function of TSTA protecting the liver in DHBV-infected ducklings.

Conclusion

TSTA possessed the significant antiviral activity *in vitro* and *in vivo*, and it was one of the main active parts of *T. affinis* against HBV. Elucidation of the mechanism of its antiviral activity and identification of the active components in TSTA will greatly enhance the understanding of viral gene expressions and provide new clues to assist in the development of antiviral agents in the future.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support by the Science and Technology research development of Guangxi Province (10124008-6), Guangxi Key Laboratory for Prevention & Treatment of Regional High-Incidence Diseases (KFJJ2010-22) and the department of education of Guangxi Province (200710MS014; 200911MS28).

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