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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.03.012>***In vivo* anticancer activity of maesopsin 4-*O*- β -glucoside isolated from leaves of *Artocarpus tonkinensis* A. Chev. Ex Gagnep**Trinh Thi Thuy^{1*}, Dao Duc Thien¹, Tran Quang Hung¹, Nguyen Thanh Tam¹, Nguyen Thi Hoang Anh¹, Nguyen Thi Nga², Nguyen Thi Cuc², Le Phuong Mai³, Tran Van Sung¹, Domenico V. Delfino⁴, Do Thi Thao^{2*}¹Institute of Chemistry, Vietnam Academy of Science and Technology, 18-Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Viet Nam²Institute of Biotechnology, Vietnam Academy of Science and Technology, 18-Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Viet Nam³National Research Council Canada, Measurement Science and Standards, 1200 Montreal Road, Building M-12, Ottawa, Ontario K1A 0R6, Canada⁴Department of Medicine, University of Perugia, Piazzale Gambuli, S.Andrea delle Fratte, 06132 Perugia, Italy

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ABSTRACT

Objective: To investigate the antitumor effect of maesopsin 4-*O*- β -glucoside (TAT2) isolated from the leaves of *Artocarpus tonkinensis* (*A. tonkinensis*) A. Chev. ex Gagnep. **Methods:** The antitumor activity of TAT2 was evaluated in Lewis lung carcinoma (LLC) tumor-bearing mice. BALB/c mice had tumors induced by implantation with 2×10^6 LLC cells into the subcutaneous right posterior flank. Tumor-bearing mice were treated orally with a range of doses of TAT2 and a standard drug, doxorubicin. Animals were observed for tumor growth and mortality rate. Blood was collected to determine hematological and biochemical parameters.

Results: TAT2 was isolated from an ethanolic extract of *A. tonkinensis* leaves. Its structure was determined by MS and NMR spectroscopy, and identified as TAT2. The compound did not show acute toxicity at the highest dose tested (2000 mg/kg body weight). TAT2 exhibited antitumor activity by decreasing tumor growth, increasing the survival rate, and ameliorating some hematological and biochemical parameters at doses of 100 and 200 mg/kg body weight ($P < 0.05$).

Conclusions: These results indicate that TAT2 possesses clear antitumor activity. Due to its bioavailability and low toxicity, and the fact that it could be isolated in a large scale from *A. tonkinensis* leaves, the compound shows promise as a potential anticancer drug.

1. Introduction

Artocarpus tonkinensis (*A. tonkinensis*) A. Chev. ex Gagnep. is usually found in North Vietnam. The decoction of its leaves and roots is used in folk medicine to treat backache, rheumatism and joint disorders [1]. A crude extract from *A. tonkinensis* leaves has been used successfully in Vietnam as an immunosuppressive agent for skin transplantation in mice [2]. In recent years, our group has focused on the chemical identification of the active ingredients in *A. tonkinensis* leaves. We demonstrated that

flavonoid glycosides such as maesopsin 4-*O*- β -glucoside (hovetrichoside C, TAT2), alphonin-*O*- β -glucoside, artonkin-4'-*O*- β -glucopyranosid, and astragaloside strongly contributed to its immunosuppressive and anti-inflammatory activities [3,4]. These compounds have potent anti-proliferative and anti-inflammatory effects both *in vitro* and *in vivo* [4,5]. The anti-inflammatory effects demonstrated in a rat model of arthritis correlated well with the inhibition of mitogen-induced T-cell proliferation [6]. Furthermore, the main compound, TAT2, inhibited the growth of OCI-AML cells (and additional acute myeloid leukemia cells) but not Jurkat cells, a T lymphoma. Moreover, gene expression profiling identified 19 genes modulated by TAT2 and, among them, indicated HMOX1 and SRXN1 as consistently and highly upregulated [5]. Although TAT2 is a major component (yield 0.2%) of *A. tonkinensis* leaves, its antitumor activity has not yet been studied *in vivo*. Due to its promising pharmacological properties, this study focused on the large-scale isolation of TAT2 and its potential anticancer effect *in vivo* for possible use in alternative medicine.

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2. Materials and methods

2.1. Chemicals

Fractionation was performed over DIAION HP20 resin (Supelco, Bellefonte, PA, USA), Sephadex[®] LH20 (Sigma, St. Louis, MO, USA); silica gel (Merck). Dulbecco's modified Eagle's medium and MEM nonessential amino acid were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum and antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO., USA).

2.2. Plant material, extraction and isolation

Leaves of *A. tonkinensis* were collected in Hanoi, Vietnam (December 2013) and identified by the taxonomist -Ngo Van Trai (Institute of Materia Medica, Hanoi, Vietnam). The voucher specimen (Nr. 1482-AT-2013) was deposited in the Institute of Chemistry, Vietnam Academy of Science and Technology.

Dried ground leaves of *A. tonkinensis* (12 kg) were extracted with 70% EtOH (each 36 L, overnight, ×3 times) at room temperature. The extract was filtered and concentrated *in vacuo* (temperature 45–60 °C) to remove EtOH affording an aqueous solution, and partitioned with *n*-hexan. The aqueous phase was dissolved in water and loading over Diaion HP20 column, washed with water, 50% aq. MeOH and MeOH to give 4 fractions (Fr1 → Fr4). Fr3 (eluting with 50% MeOH, 740 g) was chromatographed over Sephadex LH20 eluted with MeOH to give 4 sub-fractions (Fr3.1 → Fr3.4). Fraction 3 (Fr3.3, 350 g) was further chromatographed over silica gel, eluted with EtOAc-MeOH-H₂O (75:25:1 → 70:30:2 and then 65:35:5) to afford 5 sub-fractions (Fr3.3.1 → Fr3.3.5). Sub-fraction 4 (Fr3.3.4, 40 g) was further purified by CC (silica gel, EtOAc-MeOH-H₂O, 4:1:0.1) to afford compound TAT2 (24 g, yield 0.2%, compared with dried material).

2.3. Identification of purity and characterization of compound TAT2

The purity of compound TAT2 was determined by HPLC-DAD (HPCHEM) using a Synergi Fusion RP80A column (250 mm × 2.0 mm i.d.) at 25 °C. UV spectrum was recorded from 190 nm to 400 nm. The mobile phase consisted of A, water containing 0.1% formic acid, and B, acetonitrile containing 0.1% formic acid. The elution gradient was performed using a pump from 10% to 35% B for 50 min; 35%–80% B from 50 to 55 min at a flow rate of 0.25 mL/min. Detection was carried over 60 min at a flow rate of 0.25 mL/min. Detection was carried out at 290 and 294 nm. The injection volume was 5 µL. The HPLC chromatogram of TAT2 showed major peak with retention time (Rt) at 20.39 min, percentage 95.8%.

HR-ESI MS spectrum was obtained on QStar Pulsar (Applied Biosystems). ¹H NMR (500.13 MHz) and ¹³C NMR (125.77 MHz) spectral data were measured in methanol-d₄ (CD₃OD) on a Bruker Avance 500 NMR spectrometer, at 25 °C. Chemical shifts were expressed in δ (ppm) downfield from TMS as an internal standard, and coupling constants were reported in Hertz.

2.4. Animals

Male and female albino BALB/c mice (8–10 weeks old) were obtained from the Institute of Biotechnology, Vietnam Academy

of Science and Technology (Hanoi, Vietnam). All mice were housed in a temperature-controlled room on a 12-h light/12-h dark cycle with food and water provided *ad libitum*. Experiments were performed in accordance with Vietnamese ethical laws and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

2.5. Acute toxicity study

The acute oral toxicity of the compound was tested using the OECD GUIDELINE 420 [7]. Mice were administered TAT2 orally by using a stomach tube. Animals received the compound in serial doses of 2000 mg/kg; 1500 mg/kg; 1000 mg/kg; and 500 mg/kg body weight (bw) after 4 h diet. They were observed for 14 d to monitor survival and toxic symptoms.

2.6. Antitumor study

The tumor cell line Lewis lung carcinoma (LLC) was kindly provided by Dr. Jeanette Maier, University of Milan, Italy. The cells were maintained in 75 cm² tissue culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% non-essential amino acids and 1% antibiotics (Sigma, St. Louis, MO, USA) at 37 °C in a humidified 5% CO₂ atmosphere. Cells were subcultured every 2–3 d.

2.7. Treatment schedule

Mice were tumorized by implantation with 2 × 10⁶ LLC cells (in 0.2 mL serum-free DMEM) into the subcutaneous right posterior flank. Drug treatment began when the tumor volume reached approximately 100 mm³. The day the LLC cells were implanted was day 0 of the experiment. Tumor-bearing mice were divided into six groups of ten: Group I received distilled water orally and served as a negative control; Group II received a standard drug (doxorubicin) at a dose of 5 mg/kg/day; animals in Group III, IV and V were treated with TAT2 at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg bw, respectively. All compounds were administered orally once daily. The animals were observed every day for tumor growth. Body weight, tumor width and length were assessed every 7 d.

2.8. Determination of tumor volume

Based on tumor size, which was measured by calipers, tumor volume was calculated using the following formula [8,9]: volume (cm³) = W² × L/2, where W is the tumor width and L is the tumor length. The percent of tumor growth inhibition was determined using the following equation: % of tumor inhibition = 100% - (100 × MT × MC⁻¹), where MC is the mean tumor volume of the control group and MT is the mean tumor volume of the treated group.

2.9. Measurement of hematological parameters

Blood from the control and TAT2-treated groups were collected to determine hematological parameters. Blood was obtained from the tail vein and used for the detection of hemoglobin (Hb), red blood cells (RBC), white blood cells

(WBC), monocytes, lymphocytes (LYM), platelets (PLT), mean corpuscular (cell) volume, and mean corpuscular hemoglobin concentration using a blood automatic analyzer (AU680, Beckman Coulter). Levels of other enzymes in the sera such as serum glutamate pyruvate transaminase (SGPT), serum aspartate aminotransferase (SGOT) and creatinine were also measured.

2.10. Statistical analysis

Data are expressed as the mean \pm SD. Statistical analyses and significance, as measured by one-way analysis of variance (ANOVA), were performed using GraphPad PRISM software version 4.0 (GraphPad Software, USA). In all comparisons, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chemical analysis

In extraction experiment, 70% ethanol was used, as TAT2 is soluble in both ethanol and water. TAT2 was isolated as a

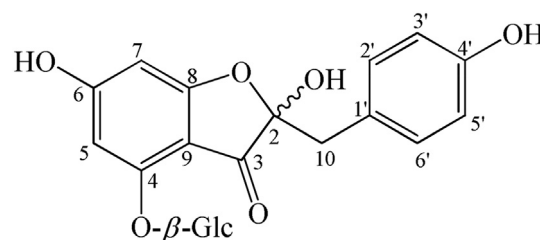


Figure 2. Structure of compound TAT2 isolated from the leaves of *A. tonkinensis*.

yellow amorphous powder from the EtOH extract by repeated column chromatography over DIAION HP20, sephadex LH-20 (MeOH) using appropriate solvent systems. An ion exchange resin (Diaion HP20) column and gel-filtrate (Sephadex LH20) chromatography with appropriate solvent systems were effective for preparing the compound on a large scale. The purity of TAT2 was determined to be 95.8% by HPLC. HR-ESI-TOF-MS (m/z): 473.1053 [$M + Na$] $^+$ ($C_{21}H_{22}O_{11}Na$ requires 473.1054). ESI-MS (negative ions): 449 [$M-H$] $^-$. 1H and ^{13}C NMR spectral data are shown in Table 1. The HPLC chromatogram of TAT2 is shown in Figure 1 and its structure is shown in Figure 2.

3.2. In vivo antitumor activity of TAT2

3.2.1. Acute toxicity studies

Results from the acute toxicity study showed that TAT2 did not cause toxicity in mice even at the highest dose of 2000 mg/kg body weight. All mice treated with TAT2 survived and did not show any toxic symptoms or abnormal behavior at the tested doses over the 14 d of observation (data not shown). Therefore, TAT2 was considered safe to use to determine the antitumor activity at lower doses (200 mg/kg, 100 mg/kg and 50 mg/kg bw).

3.2.2. Tumor growth and survival indices

In the *in vivo* antitumor experiment, TAT2 suppressed solid tumor growth in a dose-dependent manner (Table 2). Although the tumor volumes were the same for the first 14 d between the control group and the TAT2-treated groups, TAT2 increased at a much slower speed than the negative control (vehicle-treated group) over the same time period. The data collected at the end of the study demonstrated that TAT2 showed significant activity

Table 1

^{13}C and 1H NMR data of compound TAT2 [CD_3OD , 125/500 MHz, δ (ppm)] (shifts of the minor compound in brackets, if different).

C	δ_C	δ_H (J in Hz)
2	107.59 [107.64]	–
3	196.88 [196.98]	–
4	158.42 [158.30]	–
5	97.59 [97.26]	6.07 <i>br s</i>
6	171.41	–
7	93.17 ^c [93.26] ^c	5.96 <i>br s</i>
8	174.53 [174.55]	–
9	103.48 [103.67]	–
10	41.94 [42.11]	3.10 <i>s</i> ^c [3.11 <i>d</i> ^a (14)] ^c
1'	125.58 [125.56]	–
2'/6'	132.50	7.00 <i>"br d"</i> ^b (8.3)
3'/5'	115.75 [115.79]	6.59 <i>"d"</i> ^b (8.3) [6.58 <i>"d"</i> ^b (8.3)]
4'	157.25 ^c [157.22] ^c	–
1''	101.70 [101.67]	4.89 <i>d</i> (7.5) [4.87 <i>d</i> (7.5)]
2''	74.00 [74.06]	3.56 <i>m</i> ^d
3''	77.38 [77.31]	3.48 <i>m</i> ^d
4''	71.17	3.43 <i>m</i> ^d
5''	78.39 ^c [78.32] ^c	3.43 <i>m</i> ^d
6''	62.35	3.89 <i>br d</i> (12), 3.70 <i>ddd</i> (2, 7, 12)

^a Very strong AB-effect. ^b AA'XX' system. ^c Exchangeable.

^d Overlapped.

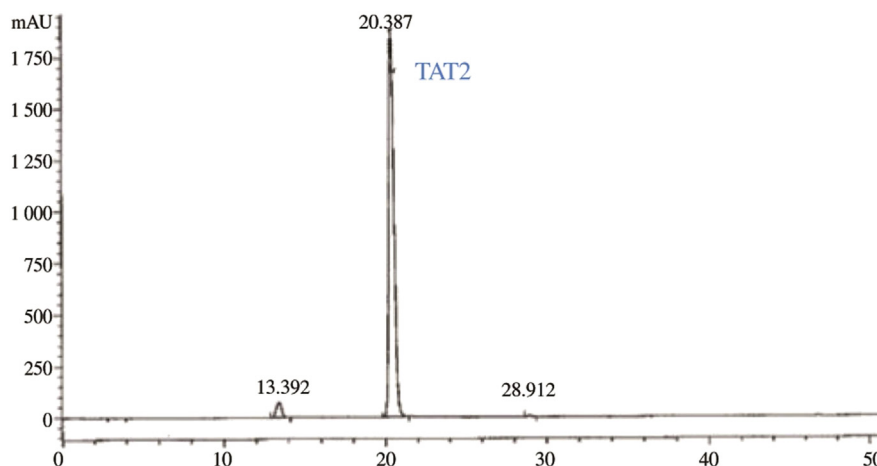
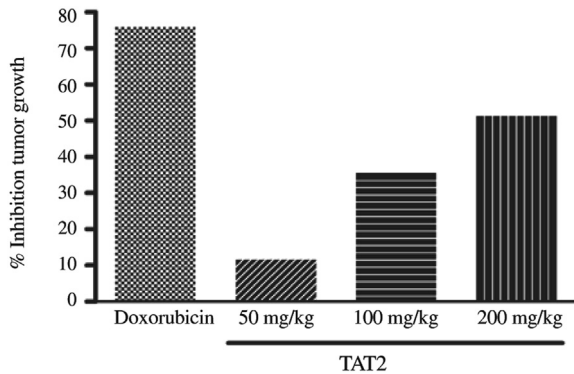


Figure 1. Analytical HPLC chromatograms of compound TAT2 (retention time 20.4 min, purity 95.8%).

Table 2

Effect of TAT2 on tumor growth in LLC tumor-bearing mice.

Groups	Tumor volumes (cm ³)					
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Negative control	0.124 ± 0.037	1.264 ± 0.519	4.445 ± 1.994	6.331 ± 2.639	7.197 ± .884	10.715 ± 0.594
Doxorubicin	0.095 ± 0.009	0.469 ± 0.107	0.938 ± 0.109	1.318 ± 0.088	1.818 ± 0.072	2.586 ± 0.161
TAT2: 50 mg/kg	0.122 ± 0.009	1.214 ± 0.248	4.118 ± 1.917	6.188 ± 2.988	6.636 ± 0.798	9.477 ± 0.709
TAT2: 100 mg/kg	0.119 ± 0.017	1.181 ± 0.399	2.074 ± 0.164	4.123 ± 1.163	5.172 ± 1.359	6.915 ± 0.602
TAT2: 200 mg/kg	0.111 ± 0.008	1.193 ± 0.514	2.028 ± 0.814	3.594 ± 1.251	4.496 ± 2.289	5.224 ± 1.300

**Figure 3.** Percentage of tumor growth inhibition by TAT2.**Table 3**

Effects of TAT2 on body weight and tumor weight in LLC-bearing mice.

Group	Body weight (g)		Mortality rate	Tumor weight (g)
	Pre-treated	After-treated		
Negative control	22.25 ± 2.06	28.37 ± 1.00	4/6	11.95 ± 0.72
Doxorubicin	22.00 ± 2.00	27.03 ± 1.36	0/6	2.19 ± 0.43
TAT2: 50 mg/kg	22.50 ± 1.29	27.68 ± 0.95	2/6	10.88 ± 1.10
TAT2: 100 mg/kg	22.13 ± 1.65	26.88 ± 1.11	1/6	8.38 ± 1.05
TAT2: 200 mg/kg	22.55 ± 1.86	26.33 ± 2.57	1/6	6.36 ± 1.08

at doses of 100 mg/kg and 200 mg/kg body weight, with tumor inhibition being 35.47% and 51.24%, respectively (Figure 3).

Tumor weights are shown in Table 3, which indicated that growth was clearly reduced in the TAT2-treated groups at

doses of 100 mg/kg and 200 mg/kg body weight compared with the negative control ($P < 0.05$). The mean tumor weight of the negative control group was (11.95 ± 0.72) g, while the means of the TAT2 – administered groups at doses of 100 mg/kg and 200 mg/kg body weight decreased to (8.38 ± 1.05) g and (6.36 ± 1.08) g, respectively. There was no significant difference between the body weights of the tumor-bearing mice treated with TAT2 and the control group at the end of the experiment ($P > 0.05$). The antitumor activity of the compound was also measured through survival; the survival rate improved considerably in the TAT2-treated groups.

3.2.3. Hematological parameters

Hematological parameters are presented in Table 4. The WBC and LYM counts were higher in the LLC tumor-bearing mice and lower in the doxorubicin-treated group. TAT2 administration (200 mg/kg body weight) also significantly reduced the number of WBC and LYM compared with the negative control group ($P < 0.05$). In contrast to WBC, the number of RBC and PLT and Hb content were improved in tumor-bearing mice treated with TAT2, but only at doses of 100 and 200 mg/kg body weight.

During the evaluation, the activities of some functional enzymes of the liver and kidney were also measured (Table 4). Although there were no differences in SGOT or creatinine levels between groups, the mean SGPT level was (43.05 ± 4.45) UI/L in the group treated with the highest dose of TAT2 and was significantly decreased in comparison with the negative control group (51.73 ± 1.40 UI/L) ($P < 0.05$). These results indicate that TAT2 could ameliorate several hematological and biochemical parameters in LLC-induced tumor-bearing mice.

Table 4

Effect of TAT2 on hematological and biochemical parameters of tumor-bearing mice.

Parameters	Groups				
	Negative control	Doxorubicin	TAT2 50 mg	TAT2 100 mg	TAT2 200 mg
WBC (10 ⁹ /L)	14.40 ± 0.14	9.65 ± 0.38	13.08 ± 0.95	12.50 ± 0.99	11.30 ± 0.42
LYMLymphocyte (%)	82.20 ± 2.40	68.34 ± 2.45	73.54 ± 23.85	65.35 ± 2.62	70.10 ± 8.06
Monocyte (%)	9.45 ± 1.48	12.12 ± 3.67	12.36 ± 4.21	14.50 ± 1.84	16.55 ± 5.44
Other WBC (%)	6.25 ± 0.78	19.54 ± 12.64	14.10 ± 2.58	19.65 ± 1.48	18.35 ± 9.68
RBC (× 10 ¹² /L)	7.49 ± 0.17	10.38 ± 0.56	7.68 ± 0.83	9.68 ± 0.30	9.62 ± 1.53
PLT (× 10 ⁹ /L)	616.50 ± 16.26	721.15 ± 13.46	677.84 ± 15.90	720.00 ± 38.18	714.00 ± 43.84
Hb (g/L)	116.00 ± 2.83	138.01 ± 3.57	122.23 ± 4.38	136.50 ± 6.30	137.00 ± 25.45
MCV (fg)	42.60 ± 0.95	44.06 ± 2.12	42.35 ± 4.02	41.77 ± 3.06	43.95 ± 3.75
MCH (pg)	15.16 ± 1.65	14.39 ± 1.35	13.95 ± 2.65	13.33 ± 0.74	14.15 ± 0.35
MCHC (g/L)	338.00 ± 10.52	325.86 ± 1.72	324.60 ± 6.54	321.33 ± 9.86	323.50 ± 9.09
SGOT (UI/L)	501.60 ± 81.19	302.16 ± 13.64	504.63 ± 45.98	517.05 ± 55.79	563.55 ± 62.01
SGPT (UI/L)	51.73 ± 1.40	41.55 ± 3.63	48.36 ± 7.83	5760 ± 1.27	43.05 ± 4.45
Creatinine (UI/L)	30.26 ± 0.64	30.10 ± 1.02	30.41 ± 0.62	30.33 ± 1.15	29.95 ± 0.35

MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

4. Discussion

In traditional medicine, *A. tonkinensis* leaves are used as immunosuppressive drugs for the treatment of arthritis. To the best of our knowledge, our study is the first to report phytochemical investigations of the leaves of this plant. Compound TAT2 (yield 0.2%, purity 95.8%) was isolated from a 70% EtOH extract of *A. tonkinensis* leaves by repeated column chromatography. The molecular formula of TAT2 was determined to be $C_{21}H_{22}O_{11}$ by HR ESI MS (m/z 473.1053 $[M + Na]^+$, $C_{21}H_{22}O_{11}Na$ requires 473.1054). The ^{13}C NMR spectrum revealed 21 carbons as two sets of signals due to an isomeric hemiketal at C-2. On the basis of the MS results, NMR spectroscopic data and comparison with previously reported data [4,10], its structure was confirmed as TAT2, the rare auronol glycoside in nature [11].

In recent years, the cancer rate has increased, especially in less developed countries. Identification and development of new chemotherapy drugs has been critical for cancer treatment. Plants have been a rich source of natural drugs [12,13]. In addition, compounds derived from plants are diverse in structure and bioactivity and exhibit low toxicity, therefore they play an important role in pharmaceutical research [14]. In cancer therapy, many plant-derived drugs such as vincristine, paclitaxel and taxol have been identified and developed. As reported, TAT2 isolated from *A. tonkinensis* showed promising anticancer properties *in vitro* [5]. Therefore, we assessed the antitumor activity of this compound *in vivo*. Because of its high tumorigenic properties, the LLC cell line was selected to create a tumor model for this study. TAT2 caused a significant decrease in tumor growth at various doses, being the most effective at a dose of 200 mg/kg body weight.

Inflammatory responses play critical roles in the evolution of cancer from initiation to metastasis. It has become evident that the inflammatory microenvironment is an essential component of all tumors [15]. Solid tumor growth induces a region of hypoxia, stimulating necrosis in the tumor's core. In addition, malignant cells also produce many cytokines and chemokines that attract leukocytes [15,16]. Today, inflammation has become a target for cancer prevention and therapy [17]. In this study, the decreased WBC count including LYM indicated that inflammation was reduced in mice administrated TAT2. This result was in accordance with other reported effects that *A. tonkinensis* exerted immunomodulatory activity against disorders of chronic inflammation such as rheumatoid arthritis [3,6]. Some compounds isolated from this plant, including TAT2, were found to cause an anti-inflammatory effect due to the inhibition of T-cell proliferation at high concentrations [3]. This explains why the number of LYM was reduced at the highest dose of TAT2 but not at the lowest dose (50 mg/kg body weight) compared to the negative control.

In addition to inflammation, anemia is a common phenomenon in patients with cancer and impacts survival and quality of life [18]. Anemia causes multiple function disturbances of various organs and tissues [19]. The management of anemia in cancer patient has to improve treatment efficacy and increase survival [20]. In cancer, anemia occurs due to the reduction of RBC count and Hb levels. Increasing the RBC count and Hb content in TAT2-administrated mice indicated that this compound may improve the hematopoietic system and survival rate.

Further, TAT2 had low toxicity when studied in mice. According to these results, TAT2 has potential as an antitumor agent.

In conclusion, the results of the present study indicate that TAT2 can be isolated from *A. tonkinensis* leaves in large quantities (0.2%). Furthermore, *in vivo* tests demonstrated a significant inhibition of tumor growth at 100 and 200 mg/kg body weight. These results indicate that TAT2, a potential antitumor agent from *A. tonkinensis*, may be valuable for preparing functional foods and pharmaceutical medicines.

Conflict of interest statement

We declare that we have no conflict of interest.

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