SUPPLEMENTARY MATERIAL

The active fraction from the tuber of *Bolboschoenus yagara* inhibits melanoma B16 cells metastasis LPS-induced *in vitro* and *in vivo*

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ABSTRACT

This study was to identify anti-metastatic active fractions and compounds of *Bolboschoenus yagara* (*B. yagara*). The results indicated that 50 µg/mL ethyl acetate fraction (Et) can dramatically inhibit mouse melanoma B16 cells migration and invasion *in vitro*. In B16 cells pulmonary and hepatic metastasis assays, 50 µg/mL Et alleviated mouse lung and liver weights, the number of metastatic nodules and the levels of TNF-α and IL-6 in mouse serum and organs. Histological studies showed that Et fraction was able to prevent liver and lung metastasis. And the inhibition of 50 µg/mL Et fraction against hepatic metastasis was almost equivalent to that of 1 µM TAK242. In addition, fourteen compounds of Et were quantified by HPLC analysis, in which, isocoumarins, stilbenes and xanthones obviously abated LPS- modulated B16 cells migration and invasion.

Keywords: *Bolboschoenus yagara* (*Scirpus yagara*); melanoma; metastasis; LPS; isocoumarins

1. Experimental

1.1 Cell culture, chemicals and biochemicals

Mouse melanoma cells B16 was purchased from the Cell Bank of the Chinese Academy of Sciences. B16 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. B16 cells were incubated at 37°C in a humidified incubator with 5% CO\(_2\). TAK-242
lipopolysaccharide (LPS) (Escherichia coli 055: B5) and MTT were purchased from Sigma (St. Louis, MO, USA); Matrigel was purchased from Becton Dickinson (Biosciences, San Jose, California); methanol (purity >99% by HPLC) was purchased from Tedia (USA), and formic acid (purity >99% by HPLC) was obtained from Sinopharm Chemical Reagent Co., Ltd. (China); ELISA kits for TNF-α and IL-6 were provided by eBioscience (San Diego, CA); RPMI-1640 medium and fetal bovine serum were provided by Wisent Biotechnology Co., Ltd. (Nanjing, China).

1.2 Plant materials

The tubers of Bolboschoenus yagara (Ohwi) Y.C.Yang & M.Zhan (i.e Scirpus yagara) were collected from the medicinal material market in Yangzhou City in September 2015, Yangzhou, Jiangsu Province, China. The plant material was authenticated by Prof. Qiao-Li Liang. A voucher specimen (Jingsl 20150917) had been deposited in Herbarium of the Department of Chinese Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China.

1.3 Extraction and isolation

Dried tubers of B. yagara (25 kg) were crushed and extracted with 75% ethanol (120 L) three times (2 h each) under reflux at 80°C. After filtration and concentration under vacuum, the EtOH extract (527 g) was suspended with water (15 L) and then fractionated with ethyl acetate (50 L) and n-butyl alcohol (50 L). The resulting solutions were concentrated and dried in vacuum, to give ethyl acetate (Et) (79.2 g) and n-butanol (Bu) (107.6 g) fractions, respectively.

1.4 Animals

BALB/c female mice (20 ± 2 g) were purchased from the Shanghai Jiesijie Laboratory Animal Center (certificate No.2010002608097 Shanghai, China). The animals were maintained at 25°C on a 12 h light/dark cycle, and provided standard commercial diet and water ad libitum. The animals were acclimated in rooms for 7 days before the initiation of the experiment. The mouse experiments were approved by the committee for Animal Care and use of Laboratory Animals, College of Pharmacy, Nanjing University of Chinese Medicine (approved on December 8, 2016, NO. NJACLA-20161108) for minimizing animal suffering.

1.5 Cell viability
Cell viability was determined using MTT assay. Cells were seeded at $1 \times 10^5$ cells/well in 96-well plates and then treated with tested samples in different concentrations for 24 h. MTT (5 mg/mL in the medium) was then added to the medium, and the cells were incubated for an additional 4 h. The formazan crystals in each well were solubilized in dimethyl sulfoxide (DMSO, Sigma, Steinheim, Germany), and the absorbance was measured at 490 nm on a microplate reader.

1.6 Wound-healing assay

Wound-healing assay was used to evaluate the activity of tested samples suppressing the migration of B16 cells. In brief, B16 cells ($8 \times 10^5$ cells/well) were seeded in a 6-well culture plate and grown to 80-90% confluence. After aspiration of the medium, the center of the cell monolayers was scraped with a yellow pipette tip to create a denuded zone of constant width. Subsequently, B16 cells were exposed to various concentrations of tested samples. Wound closure was photographed at 0 h and 24 h with a Nikon inverted microscope (Nikon, Tokyo Japan), and cells migration into the scratch area was analyzed using Image-Pro Plus 6.0 software.

1.7 Matrigel invasion analysis

The invasion assays were carried out using transwell chamber with 10 mm diameter and 8 µm pore size polycarbonate membrane (Corning Costar, Cambridge, Massachusetts) coated with matrigel. B16 cells were trypsinized and resuspended in serum-free medium. 200 µL cell suspension ($10^5$ cells) with different concentrations of tested samples were added to the upper chamber of each well. The bottom chambers were filled with 500 µL RPMI 1640 medium supplemented with 20% fetal bovine serum. The chamber was incubated for 24 h at 37°C. At the end of incubation, these cells in the upper surface of the membrane were carefully removed with a cotton swab. Cells invading across the matrigel to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The invading cells on the lower surface of the membrane filter were counted with a light microscope.

1.8 Lung metastasis assay

In order to evaluate lung metastasis of Et against B16 cells, four groups were considered as Untreated group (B16 cells untreated), LPS group (B16 cells pre-treated with LPS for 4 h),
10 µg/mL Et group (B16 cells pre-treated with LPS and 10 µg/mL Et for 4 h) and 50 µg/mL Et group (B16 cells pre-treated with LPS and 10 µg/mL Et for 4 h). Then, these B16 cells (3×10^5 cells suspended in 200 µL physiological saline) were injected through the caudal vein into BALB/c female mouse. Subsequently, the mice were sacrificed 21 days later and lung metastatic foci were identified. Half of the lung tissue was fixed with 10% neutral-buffered formalin, embedded in paraffin medium and sectioned (4 µm), and then stained with hematoxylineosin. Pathological changes were evaluated under a light microscope and digital images were obtained at magnifications of 200. The other half of the lung tissue were placed in homogenization buffer (0°C), centrifuged at 12000 rpm, 4°C for 10 min and frozen at -80°C until use. The blood samples of four groups were collected by retroorbital bleeding. Serum was separated by centrifugation at 3000 rpm for 15 min at 4°C and frozen at -70°C until use. The TNF-α and IL-6 concentrations in the lung tissue supernatant and serum were detected using ELISA according to the manufacturer's instruction.

1.9 Hepatic metastasis assay

Eritoran and TAK242, TLR4 antagonists, decreased the metastatic potential of HT-29 and HCC inflammation-induced, respectively (Rich et al. 2011). Mice grouping was the same as the above besides the positive control TAK242 group (B16 cells pre-treated with LPS and TAK242 for 4 h). After BALB/c mice were anesthetized using 4% Chloral hydrate, spleens were exposed through a small abdominal incision. 2.5×10^5 cells resuspended in 100 µL 0.9% physiological saline were injected intrasplenically and the mice were splenectomized 1 minute post injection. Mice were sacrificed 9 days later. The liver from each animal was removed and weighed individually and surface liver metastases were counted. Liver tissue preservation is similar to that of lung tissue. Pathological changes were evaluated by histopathological examination. The TNF-α and IL-6 concentrations in the liver supernatant were detected using ELISA assay.

1.10 HPLC analyze the main compounds in Et

Authentic compounds with 95% purity were isolated from the tubers of B. yagara in our lab. Stock solutions were prepared in DMSO and stored at -20°C. HPLC system was consisted of Waters 2695 HPLC System coupled with a 2998 UV detector (Waters, USA). The separation was performed in an C18 column (Dikma Technologies, 4.6 mm × 250 mm, 5
μm, China) with a gradient of solvent A (acetonitrile) and solvent B (0.20% formic acid) at a flow rate of 1.0 mL/min as follows: 0-5 min, 5% A; 5-12 min, 5%-20% A; 12-27 min, 20%-25% A; 27-42 min, 25%-38% A; 42-50 min, 38%-40% A; 50-70 min, 40%-60% A; 70-85 min, 60%-100% A; 85-90 min, 100%-5% A. The chromatograms were recorded at 300 nm while the UV spectra were monitored over a range of 200-450 nm.

1.11 Statistical analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used to carry out all statistical analysis. One-way analysis of variance was used for multiple group comparison. When only two groups were compared, Student's t test was performed. P-values of less than 0.05 (P<0.05) were considered to be significant. All quantitative data were expressed as the mean ± S.E.M.

Figure S1: Effects of Et and Bu fractions on the cell vitality of B16 cells. B16 cells were treated with different concentrations of Et and Bu for 24 h and then assayed by MTT. (*P<0.05 vs. control, n = 3).
Figure S2: Et and Bu fractions suppressed LPS-induced B16 cells migration and invasion (A) Black lines indicate the wound edge. Migrated cells across the black lines were counted in nine random fields from each treatment. The photographs were taken at the magnification of ×40. Quantitative results for wound healing data have been shown. (B) The invasion ability of cells was quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane. The photographs were taken at the magnification of ×200. Quantitative results for invasion data have been shown. The data were presented as mean ± S.E.M (n=3). *P<0.05 vs. control group, †P<0.05 vs. LPS group, ▲P<0.05 vs. drug treatment groups.
Figure S3: Et fraction reduced metastasis in experimental lung metastasis assay. Cells were exposed to 1 μg/mL LPS or Et (10 μg/mL or 50 μg/mL) for 4 h. The suspension of B16 cells (3×10⁵ cells in 0.2 mL per mouse) were injected through the tail vein into mice. All mice were sacrificed 21 days after the injection of the tumor cells. (A) The photograph of lung tissue of B16 experimental metastasis model. (B) The number of lung metastases foci in each group in experimental lung metastasis model. (C) A representative histological view of the lung sections (100 ×) was photographed, Scale bar: 100 µm (D) Lung weight was counted. Levels of TNF-α and IL-6 in serum (E) and lung (F) were tested by ELISA. The data were presented as mean ± S.E.M (n=3). #P<0.05 vs. control group, *P<0.05 vs. LPS group.
Figure S4: Et fraction decreased metastasis in experimental hepatic metastasis test. B16 cells were incubated with 1 μg/mL LPS 4 h in the presence or absence of Et (10 μg/mL or 50 μg/mL). The suspension of B16 cells (2.5×10^5 cells in 0.1 mL per mouse) were injected intrasplenically into anesthetized mice. Mice were sacrificed 9 days later. (A) The photograph of hepatic tissue of B16 experimental metastasis model. (B) The number of liver metastases foci in each group in experimental liver metastasis model. (C) A representative histological view of the liver sections (100 ×) was photographed, Scale bar: 100 μm (D) Liver weight was counted. Levels of TNF-α and IL-6 in liver (E) were tested by ELISA. The data were presented as mean ± S.E.M (n=3). #P<0.05 vs. control group, *P<0.05 vs. LPS group.
Figure S5: Analysis of the main compounds in Et fraction by HPLC. Sample was detected through UV absorption at 300 nm. HPLC chromatograms of (A) the standards and (B) Et. (C) Structures of compounds indentified from Et.
Figure S6: Effects of compounds on the migration and invasion of B16 cells LPS-stimulated. (A) Part of compounds structure indentified from Et. (B) Cells were treated with various concentrations of compounds for 24 h. The data showed the inhibitory effect of
compounds on LPS-induced cells migration. (C) The data indicated the inhibitory effect of compounds on LPS-stimulated cells invasion. The data were presented as mean ± S.E.M (n=3). #P<0.05 vs. control group, *P<0.05 vs. LPS group.

Reference

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