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Chemical constituents of *Piper wallichii* (Miq.) Hand.-Mazz. and inhibitory effects on Tca83 cells.

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Abstract

To study the chemical constituents of *Piper wallichii* (Miq.) Hand.-Mazz., and to explore the effects of extractive on proliferation and telomerase activity of human tongue squamous cell carcinoma Tca83 cells and its anti-cancer mechanisms. Sephadex LH-20 and several other column chromatography techniques were employed for separation of chemical constituents from Piper wallichii (Miq.) Hand.-Mazz., and for structure elucidation. Effects of different concentrations of extractive on Tca-83 cell proliferation were detected by MTT assay; meanwhile, changes in telomerase activity of Tca83 cells were determined by TRAP-ELISA. Data were statistically analyzed using SPSS 13.0. Five compounds were isolated and identified from ethyl acetate fraction of Piper wallichii (Miq.) Hand.-Mazz. Extractive could markedly inhibit Tca-83 cell proliferation in a dose and time dependent manner, and lower the telomerase activity. Extractive can markedly inhibit the proliferation and telomerase activity of Tca83 cells. Inhibition of telomerase activity may be one of the anti-tongue carcinoma mechanisms of Piper wallichii (Miq.) Hand.-Mazz.

Keywords: *Piper wallichii* (Miq.) Hand.-Mazz, Chemical constituent, Tca83, Telomerase.

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Introduction

As a plant in the genus Piper of the family Piperaceae with vine as the medicinal part, *Piper wallichii* (Miq.) Hand.-Mazz. has wind-cold dispelling, waist and knee strengthening and kidney-yang invigorating functions, which is traditionally used for rheumatic arthralgia, lumbocrural pain, etc. [1]. *Piper wallichii* (Miq.) Hand.-Mazz. contains a variety of chemical constituents, including lignins, amide alkaloids, organic acids, sterols, etc. [2-4]. According to current findings, *Piper wallichii* (Miq.) Hand.-Mazz. possesses multiple activities, such as hepatoprotective, antioxidant, vasodilator, antiarrhythmic and anticancer effects [5-7].

To further reveal the material basis for its efficacy and facilitate its development and utilization, we separated the chemical constituents of *Piper wallichii* (Miq.) Hand.-Mazz. extract to obtain five compounds, which were identified as: futoenone (1), futoquinol (2), isofutoquinol A (3), futoamide (4) and dihydropiperlonguminine (5). Degree of change in telomerase activity is associated with cellular senescence, immortalization and carcinogenesis; enhancement of telomerase activity is an important aspect of malignant cellular transformation [8-10]. According to statistics, over 85% of malignant tumors have telomerase activity. This paper studies the effect of *Piper wallichii* (Miq.) Hand.-Mazz. extract on telomerase activity, and explores its antitumor mechanisms.

Instruments and Materials

AVANCE 600 NMR spectrometer (TMS as internal standard, Bruker); API4000 LC-MS system (Applied Biosystems); Sephadex LH-20 gel (GE); ODS-A reversed phase silica gel (YMC), silica gel (Qingdao Haiyang Chemical Plant); reagents (AR, Nanjing Chengci Chemicals Co., Ltd.). Medicinal material was purchased from Hebei Anguo Medicinal Material Company, which was identified by Professor Fang Wenqi at the China Medicine University as the dried vine of the piperaceous plant *Piper wallichii* (Miq.) Hand.-Mazz. The collection date is 2015.06.20, collector is Ting HUYAN, Specimen number is 201506-28.

Piper wallichii (Miq.) Hand.-Mazz. extract (ethyl acetate fraction) was self-prepared, and stored below -20, which was diluted to the desired concentrations before use. TRAP-ELISA reagents: telomerase detection kit S7750-KIT was purchased from Chemicon, USA. SYBR Green I nucleic acid gel stain was purchased from invitrogen, USA. Bradford working solution and serum albumin used for protein determination were purchased from Sigma, USA.

Methods

Extraction and separation

10 kg of *Piper wallichii* (Miq.) Hand.-Mazz. medicinal material was extracted under reflux with an 8-fold amount of

water for 2 h three times, and then the extracts were concentrated into a gummy extract. A portion of the gummy extract was dissolved in water, and extracted sequentially with petroleum ether, ethyl acetate and n-butanol to give respective fractions. Afterwards, the ethyl acetate fraction was separated by chromatography on repeated positive phase silica gel, reversed phase silica gel and Sephadex LH-20 columns and recrystallized to give five compounds.

Cell cultivation

Human tongue squamous cell carcinoma Tca83 cells were from the Stomatological Laboratory, Chengdu University of TCM, which were cultured in an incubator at 37, 5% CO2 with saturated humidity with 10% FBS-containing RPMI 1640 medium.

MTT assay of cell proliferation inhibition

Cell proliferative activity was determined by MTT assay. 180 μl of single cell suspension of logarithmic phase Tca83 cells was seeded in each well of 96-well plates at 1×104 cells/well. Meanwhile, control wells containing blank medium only were set up. Each well was added with 20 μl of *Piper wallichii* (Miq.) Hand.-Mazz. extract until final concentrations of 0.200, 0.100, 0.050, 0.025 g/L were achieved. Each concentration had three replicate wells. Meanwhile, a group of wells was added with 20 μl of blank medium only to serve as a control. 24, 48, 72 h after medication, respectively, 5 g/L MTT solution was added at 20 μl/well; and 4 h later, 200 μl of DMSO was added. Then, absorbances of samples were measured at A490 nm with a microplate reader, and averaged by group. Finally, growth inhibition rate was calculated according to the following formula: growth inhibition rate=[1- (treatment - background) / (control - background)] × 100%.

Telomerase activity detection

PCR-ELISA assay: Telomeric repeats (TTAGGG) were added to the 3' end of biotin-labeled synthetic PI(TS) primer by telomerase. Through repeated PCR amplification of specific product between PI(TS) and P(RP) primers, PCR product containing telomerase-specific 6-nucleotide was generated. After denaturation, the PCR product was hybridized to the probe for digoxin-labeled telomerase-specific repetitive sequence, and the resulting hybrid was immobilized onto biotin-affinity protein-coated microplate via biotin-labeled probes. Afterwards, the immobilized PCR product was detected with a peroxidase-conjugated antibody (Anti-DIG-POD). Finally, peroxidase substrate TMB produced colored reaction to visualize the probes.

Telomerase extraction: 106 cells were placed into a sterile reaction tube containing 200 μl of chilled lysate. After ice bathing for 30 min, the cells were centrifuged at 12,000 g at 4 for 20 min. Then, supernatant was carefully removed into a DEPC-treated tube, protein content in the extraction liquid was determined by Bradford assay, and each specimen was adjusted for protein concentration to 1 g/L and cryopreserved below -80 for later use.

TRAP assay: 50 μl reaction system included dNTP, Taq polymerase, internal standard primer, biotinylated primer (TS) 5'-AATCCGTCGAGCACAGTT-3' and biotinylated primer (RP). Primers were extended with 1 μl of extraction liquid: 1 cycle at 30 for 30 min; PCR amplification: 33 cycles at 94 for 30 s and at 55 for 30 s.

Electrophoresis: 10% non-denaturing polyacrylamide gel and SYBR Green I nucleic acid gel stain were used. Electrophoresis conditions: vertical electrophoresis tank; 0.5 × TBE (Tris-Borate-EDTA) buffer; gel electrophoresis apparatus; voltage of 400 V; 90 min. Gels were imaged with GDS-800 gel imaging system.

Hybridization and ELISA procedures of PCR product: 5 μl of PCR amplification product and 20 μl of denaturant were incubated at room temperature for 10 min, added with 225 μl of hybridizing buffer, and mix well. 100 μl of the mixture was then placed onto avidin-coated microplates, and incubated in a 37, 300 r/min shaker for 2 h. After washing, 100 μl of anti-DIG-POD was added for reaction at 18~22 for 30 mim. Finally, 100 μl of POD substrate TMB was added for color development for 10 min, and then the reaction was terminated by adding stop buffer. Absorbance was measured at 450 nm and 690 nm with a microplate reader, and A=A450-A690 value was calculated for each sample. Absorbance difference ΔA was judged as positive if higher than 0.150. ΔA=A sample - A inactivated sample.

Statistical analysis

Experimental data obtained were expressed as x ± s, and all data were statistically processed using SPSS 13.0 for windows software. Differences between groups were compared by one-way ANOVA, and $P<0.05$ was considered statistically significant.

Results

Structure elucidation

Compound 1: white needle crystals (methanol). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.78 (^{1}H, d, J = 7.8 Hz, H-17), 6.78 (^{1}H, d, J=1.8 Hz, H-14), 6.75 (^{1}H, dd, J=7.8, 1.8 Hz, H-18), 6.02 (^{2}H, s, H-19), 5.87 (^{1}H, s, H-2), 5.51 (^{1}H, s, H-5), 5.09 (^{1}H, m, H-8), 3.72 (^{3}H, s, OCH_3), 2.59 (^{1}H, dt, J=12.0, 6.0 Hz, H-10), 2.43 (^{1}H, m, H-7β), 2.31 (^{1}H, m, H-9β), 2.22 (^{1}H, m, H-7α), 2.12 (^{1}H, m, J=14.3, 6.6 Hz, H-11), 1.76 (^{1}H, dd, J=14.4, 12.0 Hz, H-9α), 0.64 (^{3}H, d, J = 6.0 Hz, 12-CH_3); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 183.2 (C-3), 180.2 (C-1), 154.7 (C-4), 147.7 (C-15), 146.7 (C-16), 137.3 (C-13), 123.0 (C-18), 109.2 (C-17), 108.7 (C-14), 107.9 (C-2), 101.5 (C-5), 101.2 (C-19), 81.7 (C-8), 55.3 (C-7), 50.3 (-OMe), 46.5 (C-6), 45.6 (C-10), 43.7 (C-9), 38.1 (C-11), 14.6 (C-12). The above data were consistent with the reports in literature [11], so compound 1 was identified as futoenone.

Compound 2: white crystals (methanol). ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.71 (^{1}H, s, H-7'), 6.87~6.74 (3H, m, H-2', 5', 6'), 6.16 (^{1}H, s, H-2), 5.99 (^{2}H, s, -OCH_2O-), 5.89 (^{1}H, m, J = 15.6, 6.6 Hz, H-8), 5.89 (^{1}H, s, H-5), 5.15 (^{2}H, m, H-9), 3.81 (^{3}H, s, 4-OCH_3), 3.29 (^{3}Hs, 3-OCH_3), 3.16 (^{2}H, m, H-7), 1.69 (^{3}H, d, J = 1.2 Hz, 8'-CH_3); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 188.2 (C-6), 172.5 (C-4), 147.5 (C-3'), 147.3 (C-4'), 142.1 (C-2), 139.9 (C-1), 135.2 (C-8), 133.5 (C-8'), 131.7 (C-1'), 128.2 (C-7'), 123.2 (C-6'), 117.3 (C-9), 110.2 (C-2'), 109.1 (C-5'), 105.9 (C-5), 103.1 (-OCH_2O-), 80.1 (C-3), 56.3 (4-OMe), 52.4 (3-OMe), 31.9 (C-7), 14.1 (8'-CH_3). The above data were consistent with the reports in literature [12], so compound 2 was identified as futoquinol.

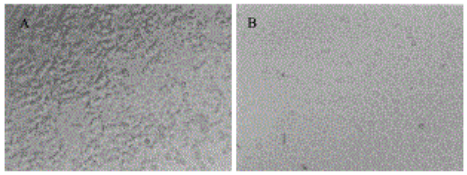
Compound 3: colorless oil. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.64 (1H, d, J=7.8 Hz, H-5'), 6.42 (^{2}H, m, H-2',6'), 6.12 (^{1}H, m, H-2″), 5.78 (^{2}H, s, -OCH_2O-), 5.31 (^{2}H, m H-3″), 4.96 (^{1}H, s, H-4), 3.87 (^{1}H, s, H-7), 3.57 (^{3}H, s, 3-OMe), 3.41 (^{3}H, s, 2-OMe), 2.89 (^{1}H, dd, J=14.4, 6.6 Hz, Ha-1″), 2.57 (^{1}H, dd, J=14.4, 7.8 Hz, Hb-1″), 2.29 (^{1}H, s, H-1), 1.62 (^{3}H, s, H-9); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 196.1 (C-5), 173.2 (C-3), 147.6 (C-3'), 146.3 (C-4'), 134.7 (C-2″), 133.2 (C-1'), 119.4 (C-6'), 118.7 (C-3″), 107.7 (C-5'), 106.8 (C-2'), 104.4 (C-4), 101.2 (-OCH_2O-), 67.8 (C-2), 56.9 (2-OMe), 56.5 (3-OMe), 53.9 (C-6), 51.1 (C-7), 39.6 (C-1″), 36.3 (C-8), 35.8 (C-1), 12.9 (8-Me). The above data were basically consistent with the reports in literature [13], so compound 3 was identified as isofutoquinol A.

Compound 4: white crystals (methanol). Dark spots by 10% phosphomolybdic acid ethanol solution, and under 254 nm UV light. ESI-MS m/z: 302.5, [M+H]+. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.92~6.75 (^{4}H, m, H-3,2″,5″,6″), 6.34 (^{1}H, d, J=15.6 Hz, H-7), 6.02 (^{1}H, m, H-6), 5.92 (^{2}H, s, -OCH_2O-), 5.82 (^{1}H, d, J = 15.6 Hz, H-2), 5.43 (^{1}H, brs, -NH), 3.17 (^{2}H, t, J=7.2 Hz, H-1'), 2.35 (^{4}H, m, H-4,5), 1.82 (^{1}H, m, H-2'), 0.96 (^{6}H, d, J=6.6 Hz, H-3'); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 166.5 (C-1), 147. 1 (C-3″), 147.7 (C-4″), 143.7 (C-3), 131.6 (C-1″), 132.1 (C-7), 127.0 (C-6), 124.3 (C-2), 120.4 (C-6″), 107.1 (C-5″), 105.9 (C-2″), 101.2 (-OCH_2O-), 46.3 (C-1'), 32.1 (C-5), 31.7 (C-4), 28.9 (C-2'), 19.0 (C-3'). The above data were basically consistent with the reports in literature [14], so compound 4 was identified as futoamide.

Compound 5: white crystals (methanol). Dark spots by 10% phosphomolybdic acid ethanol solution, and under 254 nm UV light. ESI-MS m/z: 276.2 [M+H]+. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.79 (^{1}H, m, H-3), 6.75 (^{1}H, d, J=7.8 Hz, H-5″), 6.67 (^{1}H, d, J=1.8 Hz, H-2″), 6.62 (^{1}H, d, J=7.8 Hz, H-6″), 5.92 (^{2}H, s, -OCH_2O-), 5.76 (^{1}H, d, J = 15.6 Hz, H-2), 5.45 (^{1}H, brs, -NH), 3.14 (^{2}H, t, J=7.8 Hz, H-1'), 2.69 (^{2}H, t, J = 7.2 Hz, H-5), 2.45 (^{2}H, m, H-4), 1.81 (^{1}H, m, H-2'), 0.92 (^{6}H, d, J=6.6 Hz, 2'-Me); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 165.9 (C-1), 146.9 (C-3″), 145.5 (C-4″), 143.3 (C-3), 134.4 (C-1″), 124.2 (C-2), 121.2 (C-6″), 108.9 (C-5″), 108.6 (C-2″), 101.7 (-OCH2O-), 46.6 (C-1'), 34.5 (C-5), 34.3 (C-4), 28.7 (C-2'), 21.0 (C-3'). Its ^{1}H-NMR data were basically consistent with the literature [15], so compound 5 was identified as dihydropiperlonguminine.

Antiproliferative effect of Piper wallichii (Miq.) Hand.-Mazz. extract on Tca83 cells

After treating Tca83 cells with Piper wallichii (Miq.) Hand.-Mazz. extract for 72 h, adherent cells in the treatment group presented apparent apoptotic features such as shrinkage, rounding and shedding under an inverted fluorescence microscope (Figure 1). MTT assay found that the proliferation inhibition rates in the 0.4, 0.2, 0.1 and 0.05 g/L *Piper wallichii* (Miq.) Hand.-Mazz. extract groups were (34.9 ± 4.1)%, (24.7 ± 3.8)%, (21.4 ± 2.1)% and (14.5 ± 2.9)%, respectively, at 24 h; (42.1 ± 2.4)%, (33.7 ± 4.1)%, (25.9 ± 1.9)% and (17.2 ± 2.3)%, respectively, at 48 h; and (65.9 ± 3.7)%, (51.4 ± 2.9)%, (47.1 ± 3.2)% and (22.9 ± 2.1)%, respectively, at 72 h. Compared with the control group, the proliferation activity significantly decreased in various experimental groups at corresponding time periods ($P<0.05$). For the same concentration group, antiproliferative action increased with prolonging time ($P<0.05$), indicating the Tca83 cell proliferation inhibiting effect of Piper wallichii (Miq.) Hand.-Mazz. extracts *in vitro* in a time- and concentration-dependent manner (Figure 2).



A: Control Group B: Treatment group

Figure 1: *Tca83 cells in the control and treatment groups after treating with Piper wallichii (Miq.) Hand.-Mazz. extract (0.025 g/L) for 72 h.*

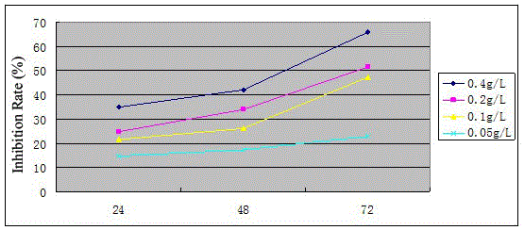


Figure 2: *Antiproliferative effect of Piper wallichii (Miq.) Hand.-Mazz. extract on Tca83 cells.*

Telomerase activity expression

TRAP assay of sample extract showed an internal standard band at 36 bp, and appearance of bands at a 6 bp interval from 50 bp, forming alternately dark and bright ladder bands, which indicated positive telomerase activity. Presence of internal standard only would indicate negative telomerase activity. Gel electrophoresis demonstrated that the alternately dark and bright bands darkened with increasing drug concentration and prolonging action time, especially for the 0.2 g/L group, where 72 h sample showed markedly darker bands than the positive control. This suggested that YHL-TA had a certain inhibitory effect on telomerase activity of Tca83 cells.

ELISA semi-quantification of telomerase activity

Expression of telomerase activity decreased gradually in the control group, as well as in the 0.1 and 0.2 g/L *Piper wallichii* (Miq.) Hand.-Mazz. extract groups. Telomerase activity was already inhibited for the 0.2 g/L *Piper wallichii* (Miq.) Hand.-Mazz. extract group at 24 h; and at 72 h, telomerase activity was significantly lower for the *Piper wallichii* (Miq.) Hand.-Mazz. extract groups than the control group ($P<0.05$). At the same concentrations, enhanced inhibition of telomerase activity was noted with prolonging action time (Table 1, Figure 3).

Table 1: *Telomerase activity expression of Tca83 cells at different times and concentrations of YHL-TA ($x \pm s$).*

Action time (h)	Concentration of extract		Control group (0 g/L)
	0.2 g/L	0.1 g/L	
24	1.72 ± 0.03*	1.81 ± 0.04	1.90 ± 0.02
48	1.46 ± 0.03*	1.75 ± 0.03*	1.92 ± 0.03
72	1.31 ± 0.04*	1.62 ± 0.03*	1.93 ± 0.03

Note: *Comparison with the blank control group of the same time, $P<0.05$; Comparison with the same concentration group at 24 h, $P<0.05$

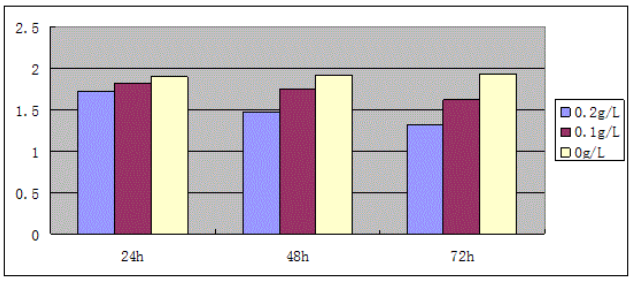


Figure 3: *TRAP-ELISA detection of the telomerase activity expression in Tca83 cells.*

Discussion

Telomerase is present in most tumor cells, while in normal cells, its activity is undetectable. Telomerase activation is a must way to cell immortalization, and immortalization is an important step in malignant transformation of tumors. Therefore, some scholars have proposed the telomerase theory of carcinogenesis [16].

Anti-tumor constituents from TCM extracts have been receiving increasing attention. Recent studies have found that a lot of chemical constituents from plants have anti-tumor activities, which can be categorized into the following: lignins, neoliginins, amide alkaloids, organic acids, sterols, etc. [17] With the deepening of research on telomere and telomerase, oncotherapy studies targeting the inhibition of telomerase activity have been increasing, and it has been confirmed that many natural herbal ingredients have an anti-telomerase action [18]. Inhibition of telomerase activity by natural small molecules is expected to become a more effective and safer novel antineoplastic protocol since it differs from the ordinary cytotoxic antitumor therapies.

Piper wallichii (Miq.) Hand.-Mazz. extract, which contains active constituents of the plant, has hepatoprotective, analgetic, sedative, immunoenhancing and antioxidant effects. *Piper wallichii* (Miq.) Hand.-Mazz. has definite clinical efficacy, which is used in combination with other drugs in most cases. Anticancer activity of *Piper wallichii* (Miq.) Hand.-Mazz. extract has been scarcely studied. To this end, this study reported the effects of *Piper wallichii* (Miq.) Hand.-Mazz. extract on proliferation and telomerase activity of human tongue squamous cell carcinoma Tca83 cells and its anticancer mechanisms.

Conclusion

Piper wallichii (Miq.) Hand.-Mazz. extract used in this experiment is self-prepared by recrystallization technique based on the systematic research of chemical constituents. The extract mainly contains five compounds: futoenone (1), futoquinol (2), isofutoquinol A (3), futoamide (4) and dihydropiperlonguminine (5).

Results of this study show that the *Piper wallichii* (Miq.) Hand.-Mazz. extract can inhibit the proliferation of tongue carcinoma cells, and suppress telomerase activity with increasing time and concentration. Thus, interruption of tongue carcinoma genesis and progression is expected by inhibiting telomerase activity to cause lost infinite proliferative capacity of cells. For telomerase, this molecular biological target of oncotherapy, *Piper wallichii* (Miq.) Hand.-Mazz. is a good candidate drug.

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