

Chemical constituents with free-radical-scavenging activities from the stem of *Fissistigma polyanthum*

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ABSTRACT

Background: *Fissistigma polyanthum* is a liane belonging to the Annonaceae family and it is one of the most important crude drugs in traditional Chinese medicine. **Objective:** The objective was to describe the structural elucidation and the free-radical-scavenging activities of the isolated compounds from *Fissistigma polyanthum*. **Material and Methods:** The chemical constituents were isolated and purified by normal, reverse column chromatography and HPLC. Their structures were identified by spectroscopic methods (¹H NMR and ¹³C NMR) and by comparison with literature values, and the free-radical-scavenging activities of these two compounds were also evaluated through three *in vitro* model systems (DPPH, trolox equivalent antioxidant capacity (TEAC) and Co (II) EDTA-induced luminol chemiluminescence by flow injection). **Results:** Two known compounds, named kanakugiol (1) and teutenone A (2), were isolated from the stem of *Fissistigma polyanthum* for the first time, and compound **1** exhibited moderate free-radical-scavenging activity. **Conclusion:** *Fissistigma polyanthum*, which has traditionally been used as an important Chinese medicine, showed a certain free-radical-scavenging activity.

Key words: Co (II) EDTA-induced luminol chemiluminescence by flow injection, DPPH, *Fissistigma polyanthum*, free-radical-scavenging activities, TEAC

INTRODUCTION

The genus *Fissistigma*, belonging to the family Annonaceae, comprises almost 75 species worldwide, spreading across Africa, Oceania, and Asia. In China, there are 23 species. Previous phytochemical investigations revealed in the genus the presence of alkaloids,^[1-4] cyclopentenones,^[5] furanone,^[6] and flavones,^[7] which exerted diverse bioactivities such as liver protection, anti-inflammatory, anti-arthritic, and anti-tumor effects.^[1] *Fissistigma polyanthum* is a liane that is abundant in China, India, Burma, and Vietnam. It has been traditionally used to prepare herbal medicines, while limited numbers of reports concerning the chemical constituents and biological activities of *Fissistigma polyanthum* have appeared in the literature.

Reactive oxygen species, such as peroxy radical, superoxide

anion radical, and hydroxyl radical, are constantly generated *in vivo* both by aerobic metabolism and exogenous sources such as UV radiation, environmental pollution, and the diet. The formation of reactive oxygen species may cause oxidative stress and destruction of unsaturated lipids, DNA, proteins, and other essential molecules, which leads to aging and the pathogenesis of such degenerative or chronic diseases as arteriosclerosis and cancer.^[8,9] Therefore, the antioxidants which can prevent oxidative reactions in biological tissues against molecular targets are essential for the normal metabolism of human body. However the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are widely used nowadays in processed food products, is now in doubt due to safety concerns about their potential toxicity and unwanted side effects.^[10,11] Thus, more and more attention is increasingly being focused on the development and utilization of natural sources as antioxidants.^[12,13] To date, there have been few reports addressing the evaluation of the free-radical-scavenging activities of the stem of *F. polyanthum*. Thus, it is necessary to conduct further research on the chemical constituents with free-radical-scavenging activities from this plant.

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MATERIALS AND METHODS

Plant material

Fissistigma polyanthum were collected from Xishuangbanna prefecture, Yunnan province, P. R. China and identified by Xishuangbanna prefecture National medicine Research Institute. The voucher specimen was deposited in the Herbarium of the College of Pharmacy, South Central University for Nationalities.

General

The ^1H - and ^{13}C -NMR spectra were measured on a Bruker-AM-400 NMR spectrometer at room temperature, using TMS as an internal standard. Chemical shifts (δ) were expressed in parts per million (ppm), with the coupling constants (J) reported in Hertz (Hz). Column chromatographies were carried out with silica gel 60 H (200-300 mesh, Qingdao Haiyang Chemical Group Co., China), C18 reversed-phase silica gel (YMC Co., Ltd., Japan) and Sephadex LH-20 (Pharmacia), TLC was performed on a precoated silica gel GF254 plate (Qingdao Haiyang Chemical Group Co., China), with spots detected by UV254 and anisaldehyde/ H_2SO_4 (10%). 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich. Gallic acid and ascorbic acid (V_C) were purchased from the Chemical Company, Shanghai, P. R. China. Other chemicals and solvents in this experiment were all analytical grade or higher and from Shanghai Chemical Reagent Co.

Extraction procedures

The dried and powered stems of *Fissistigma polyanthum* (876 g) were extracted with 95% methanol (MeOH) three times at room temperature. The MeOH extract was evaporated under vacuum to dryness as a dark brown mass (103 g) and then the concentrated MeOH extract was suspended in 90% H_2O /methanol (MeOH). The solution was successively partitioned with petroleum ether (P.E.), ethyl acetate (EtOAc), and *n*-BuOH.

Isolation procedures

The combined P.E. and EtOAc extracts (26 g) were chromatographed on a silica gel with P.E.- acetone (Me_2CO) (95:5, 9:1, 8:2, 7:3, 6:4, 0:1, v/v) to provide eleven fractions (Fr.1-Fr.11). Fr.7 (2.067 g) was subjected to column chromatography (silica gel, cyclohexane/EtOAc 95:5, 9:1, 8:2, v/v) to give eleven fractions (Fr.7.1-Fr.7.11). Fr.7.6 (336 mg) was subjected to column chromatography (ODS, H_2O /MeOH 7:3, 6:4, 1:1, 3:7) to give compound **1** (10 mg). Fr.7.10 (226.2 mg) was subjected to column chromatography (ODS, H_2O /MeOH 7:3, 6:4, 1:1, 4:6, 3:7, 2:8) to give compound **2** (14.3 mg).

Spectral data of the purified compounds isolated from *Fissistigma polyanthum* [Figure 1].

Compound **1**, yellow oil, ^1H -NMR (400 MHz, CD_3OD): δ 8.22 (1H, d, $J = 16$ Hz, H- α), 8.14 (1H, d, $J = 16$ Hz, H- β), 8.01 (1H, d, $J = 7.6$ Hz, H-2), 7.54 (1H, t, $J = 7.6$ Hz, H-3), 7.63 (1H, m, H-4), 7.54 (1H, t, $J = 7.6$ Hz, H-5), 8.01 (1H, d, $J = 7.6$ Hz, H-6), 3.84 (6H, s, $2 \times \text{OCH}_3$), 4.18 (3H, s, $-\text{OCH}_3$), 3.90 (3H, s, $-\text{OCH}_3$); ^{13}C -NMR (100 MHz, CD_3OD) δ 138.8 (C-1), 128.0 (C-2), 128.3 (C-3), 132.4 (C-4), 128.3 (C-5), 128.0 (C-6), 111.6 (C-1'), 149.0 (C-2'), 137.3 (C-3'), 149.5 (C-4'), 137.1 (C-5'), 150.2 (C-6'), 123.0 (C- α), 138.7 (C- β), 192.8 (C=O), 60.3 ($2 \times \text{OCH}_3$), 60.4 ($-\text{OCH}_3$), 60.6 ($-\text{OCH}_3$), ^1H and ^{13}C -NMR data were identical to those recorded in reference.^[14]

Compound **2**, colorless needle, ^1H -NMR (400 MHz, CD_3OD): δ 1.31 (3H, s), 1.42 (3H, s), 5.95 (1H, s, H-6); ^{13}C -NMR (100 MHz, CD_3OD): δ 39.8 (C-1), 17.0 (C-2), 40.0 (C-3), 70.2 (C-4), 171.6 (C-5), 122.4 (C-6), 202.3 (C-7), 41.0 (C-8), 33.5 (C-9), 35.7 (C-10), 23.3 (C-14), 27.5 (C-15), ^1H and ^{13}C -NMR data were identical to those recorded in reference.^[15]

The free-radical-scavenging activities of the purified compounds were evaluated through 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method

Scavenging activities of the purified compounds from *F. polyanthum* toward DPPH radical were assessed by using the method described by Scherer and Godoy with a slight modification.^[16,17] Briefly, a 0.08 mM solution of DPPH radical solution in methanol was prepared and then the purified compounds at different concentrations (0.1 ml) were added to the prepared DPPH radical solution (3.9 ml); the mixture was shaken vigorously, after a 30-minute incubation period at 37°C in the dark, the absorbance was measured at 517 nm by using a UV-

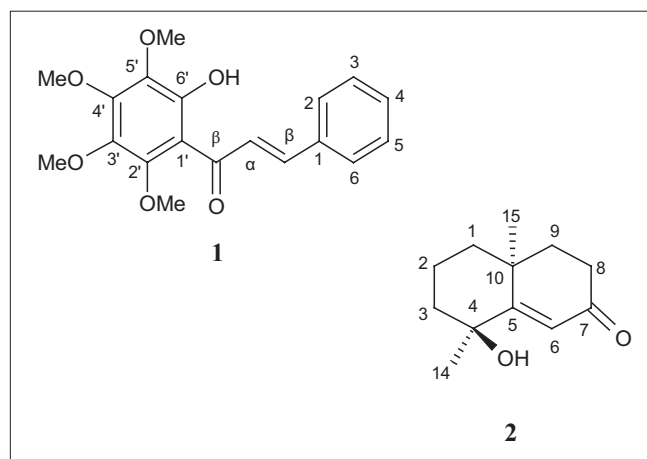


Figure 1: Structures of the compounds (1 and 2) isolated from *Fissistigma polyanthum*

visible spectrophotometer. Obviously, decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. The radical scavenging activity was given as DPPH radical scavenging effect that is calculated in equation (1):

$$\text{DPPH radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad [1]$$

where A_0 was the absorbance of control and A_1 was the absorbance in the presence of the standard or purified compounds at different concentrations. Ascorbic acid (V_C) and gallic acid served as positive controls, respectively. All the tests were performed in triplicate. The scavenging activities of the purified compounds toward DPPH radical were expressed as IC_{50} , which was determined to be effective concentration at which DPPH radical was scavenged by 50%. The IC_{50} value was obtained by interpolation from linear regression analysis.

The free-radical-scavenging activities of the purified compounds were evaluated through TEAC method

Scavenging activities of the purified compounds from *F. polyanthum* toward ABTS radical were also measured.^[18-20] Briefly, a stock solution of ABTS radical cation was prepared by dissolving ABTS (7 mM, 25 ml in deionised water) with potassium persulfate ($K_2S_2O_8$) (140 mM, 440 μ l). The mixture was left to stand in the dark at room temperature for 15-16 hours (the time required for formation of the radical) before use. For the evaluation of ABTS radical scavenging activity, the working solution was prepared by the previous solution and diluting it in ethanol to obtain the absorbency of 0.700 ± 0.02 at 734 nm (ABTS working solution should be replaced every 5 days at least because the free radical is easy to degrade). The purified compounds (0.1 ml) at different concentrations were mixed with the ABTS working solution (1.9 ml) and the reaction mixture was allowed to stand at 30°C for 6 minutes; then the absorbance was measured by using a UV-visible spectrophotometer at 734 nm, at which point the antioxidants present in the purified compounds began to inhibit the radical, producing a reduction in absorbance, with a quantitative relationship between the reduction and the concentration of antioxidants present in the tested sample. The radical scavenging activity was given as ABTS radical scavenging effect that is calculated in the equation (2):

$$\text{ABTS radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad [2]$$

At the same time a standard curve was obtained using trolox standard solution at various concentrations (ranging from 0 to 100 μ g/ml) in 95% ethanol. Scavenging activities of the purified compounds toward ABTS radical were expressed as TEAC (trolox equivalent antioxidant capacity). Different

concentrations of each purified compound were chosen to test the ABTS radical scavenging activity. The results were compared with the standard curve for calculation of TEAC.

Ascorbic acid (V_C) and gallic acid were used for positive controls, respectively. All the tests were performed in triplicate.

The free-radical-scavenging activities of the purified compounds were evaluated through Co (II) EDTA-induced luminol chemiluminescence by flow injection method

Scavenging activities of the purified compounds from *F. polyanthum* toward hydroxyl radical were assessed by Co (II) EDTA-induced luminol chemiluminescence by the flow injection method described by Giokas, Vlessidis, and Evmiridis with some modifications.^[21-23] Briefly, the FIA (flow injection analysis) manifold was designed. Once switching on the pump, the Co (II)-stream (7.12×10^{-4} mol/l, pH = 9.0 ± 0.03), luminol-stream (2.28×10^{-4} mol/l, pH = 9.0 ± 0.03) and carrier-stream were initially mixed in order to reach a stable background. A stable baseline was obtained by mixing the H_2O_2 -regent stream (0.8×10^{-3} mol/l, pH = 9.0 ± 0.03) into the system when Cobalt (II) ion increased the chemiluminescence signal of luminol- H_2O_2 system. A loss of signal (negative peak) was observed which corresponded to the hydroxyl radical scavenging activity of the antioxidant when the antioxidant was injected to the system. Thus, the effect of antioxidant was measured by the depression of the signal from its initial level (uninhibited) and expressed as hydroxyl radical scavenging effect, calculated as follows:

$$\text{Hydroxyl radical scavenging effect (\%)} = [\text{height of negative peak} / (\text{baseline} - \text{background})] \times 100 \quad [2]$$

Each purified compound was injected three times on time intervals of 40 seconds at least. Ascorbic acid (V_C) and gallic acid were used for positive controls, respectively. The scavenging activities of the purified compounds toward hydroxyl radical were also expressed as IC_{50} .

Statistical analyses of results of activity studies

The results were performed as mean \pm standard deviation (SD) of three determinations. Analysis of significance differences among means were tested by one-way analysis of variance. The IC_{50} values were calculated by linear regression analysis.

RESULTS AND DISCUSSION

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and hydroxyl radical-scavenging activities of the purified compounds of *F. polyanthum* are shown in Table 1.

Table 1: *In vitro* antioxidant activities of the purified compounds (1-2) from *Fissistigma polyanthum*

Compound	DPPH radical-scavenging effects (IC ₅₀ , μM) ^a	ABTS radical-scavenging effects (TEAC, μM) ^b	Hydroxyl radical-scavenging effects (IC ₅₀ , μM) ^a
1	90.69 ± 3.78	0.38 ± 0.01	3.85 ± 0.13
2	ND ^c	ND ^c	ND ^c
Ascorbic acid (V _C) ^d	13.16 ± 0.03	0.67 ± 0.01	0.76 ± 0.03
Gallic acid ^d	5.86 ± 0.03	2.78 ± 0.08	0.67 ± 0.03

^aIC₅₀ (μM) values were obtained by interpolation from linear regression analysis. Values were performed as means of three replicate determinations ± standard deviation (SD); ^bTEAC (μM) values were performed as means of three replicate determinations ± standard deviation (SD). Values were expressed as μg trolox equivalents/μg tested compounds; ^cND = not detected; ^dascorbic acid (V_C) and gallic acid served as positive controls, respectively

The IC₅₀ values of DPPH radical scavenging activity decreased as follows: gallic acid > V_C > **1** > **2**. The DPPH radical is considered to be a model of lipophilic radical in this study. In the presence of an antioxidant, this can donate an electron to DPPH radical, the purple color typical of free DPPH radical decay. Thereafter the DPPH radical receives a proton from the antioxidant and become a protonated DPPH species, which can be followed spectrophotometrically in absorbance at 517 nm.

The trolox equivalent antioxidant capacity (TEAC) assay is widely used applied to assess the amount of radicals that can be scavenged by an antioxidant, which is based on the antioxidant ability (in terms of radical-scavenging capacity) to react with ABTS⁺ generated in the system. The TEAC value is assigned by comparing the scavenging capacity of an antioxidant to that of trolox and a high TEAC value indicated a high level of antioxidant activity.^[24] From the TEAC values listed in Table 1, the ABTS radical scavenging activity of the tested compounds was in a decreasing order: gallic acid > V_C > **1** > **2**, which was consistent with the results of the DPPH assay.

Scavenging activities of the purified compounds toward hydroxyl radical was measured by Co (II) EDTA-induced luminol chemiluminescence by flow injection method which based on the catalytic oxidation of hydrogen peroxide by luminol-H₂O₂-Co (II)-EDTA system, forming a hydroxyl radical flux that can produce a stable chemiluminescence signal which was attenuated in the presence of antioxidants. As shown in Table 1, the hydroxyl radical scavenging activity of the tested compounds decreased in the order: gallic acid > V_C > **1** > **2**, which correlated highly with the results measured by the DPPH and TEAC assays.

It was observed that radical-scavenging activity of the

purified compounds of *F. polyanthum* decreased with the order: gallic acid > V_C > **1** > **2** in all three tests mentioned above, although the potency of the compounds was quite different in the three assays. Among the tested compounds, compound **1** with a phenolic hydroxyl group exerted moderate antioxidant activity, while compound **2** with no phenolic hydroxyl group showed a very weak capacity. These results supported the idea that free-radical-scavenging activity can be attributed to the number of protons available for donation by free hydroxyl groups, and the phenolic hydroxyl structural group in benzene ring contributes much to the free-radical-scavenging activity.^[25]

CONCLUSION

Chromatographic separation of the P.E. and EtOAc extracts of *F. polyanthum*, using a normal-phase and reverse-phase silica gel column chromatography, yielded two natural products: kanakugiol (**1**) and Teutenone A (**2**), which were isolated from *F. polyanthum* for the first time. The compounds were identified by spectroscopic methods (¹H NMR and ¹³C NMR) and by comparison with literature values. The free-radical-scavenging activities of these two compounds were also determined by comparing their free-radical-scavenging effects through not only DPPH and TEAC, but also through the more sensitive and convenient Co (II) EDTA-induced luminol chemiluminescence by flow injection test. Among them, compound **1** with a phenolic hydroxyl group exhibited moderate antioxidant activity (TEAC = 0.38 ± 0.01) against DPPH and hydroxyl radicals with IC₅₀ values of 90.69 μM and 3.85 μM, respectively and therefore may be a promising natural antioxidant. Further research on isolation and identification of more bioactive compounds from *F. polyanthum* will be helpful to understand this traditional herbal medicine.

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