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## SHORT COMMUNICATION

# A FLT3-inhibitory constituent from the rhizomes of *Anemarrhena asphodeloides*

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### Abstract

Bioactivity-guided investigation for the rhizomes of *Anemarrhena asphodeloides* using the Fms-like tyrosine kinase 3 (FLT3) inhibition assay led to the identification of an active xanthone, mangiferin. Mangiferin was found to inhibit activity of the FLT3 wild type and a mutated form of FLT3 with IC<sub>50</sub> values of 0.7 and 1.2  $\mu$ M, respectively. Furthermore, this compound was assessed with a small panel of select kinases anaplastic lymphoma kinase (ALK), insulin receptor, and epidermal growth factor receptor) and was also found to be active in ALK assay.

**Keywords:** *Anemarrhena asphodeloides*, mangiferin, FLT3, ALK, xanthone

## Introduction

The Fms-like tyrosine kinase 3 (FLT3), a receptor tyrosine kinase, is involved in the proliferation, differentiation, and apoptosis of haematopoietic cells. FLT3 is expressed in acute myeloid leukaemia (AML) in the majority of patients, and activating mutations of FLT3, the FLT3 internal tandem duplication mutation and point mutations (tyrosine kinase domain) which are located in the juxtamembrane domain and the intracellular tyrosine kinase domain, respectively, are frequently found in many AML cases<sup>1</sup>. Therefore, inhibition of FLT3 kinase activity has been proposed as a novel therapy for AML patients. Several small-molecule FLT3 inhibitors, such as lestaurtinib (CEP-701), midostaurin (PKC-412), tandutinib (MNL-518), sorafenib, KW-2449, and AC-220 are currently under clinical trials<sup>2</sup>.

As a part of discovering small-molecule inhibitors of plant origin, the rhizomes of *Anemarrhena asphodeloides* Bunge (Anthericaceae) were selected for further investigation due to their inhibitory effect against FLT3 activity in the initial screening. The rhizomes of *A. asphodeloides* have been used as an anodyne, an antidiabetic, an antiphlogistic, an antipyretic, a diuretic, and a sedative in mainland China, Japan, and Korea<sup>3</sup>. Previous

investigations on this species have led to the identification of norlignans, phenolics, steroidal saponins, and xanthenes, exhibiting various biological activities including antiadipogenic, antidepressant, antidiabetic, antifungal, antioxidant, and antiproliferative effects<sup>4</sup>. The present study describes the isolation of mangiferin from *A. asphodeloides* rhizomes and the evaluation of its inhibitory activity against the FLT3 wild type and an FLT3 mutant in an *in vitro* assay as well as in a small panel of select kinases.

## Material and methods

### General experimental procedures

UV spectra were obtained with a Shimadzu UV-1601 spectrophotometer. Nuclear magnetic resonance (NMR) spectroscopic data were recorded at room temperature on a Varian Unity 400 FT-NMR spectrometer with tetramethylsilane as the internal standard. Electrospray ionization (ESI) mass spectrometric analysis was performed with a Waters Q-ToF Premier mass spectrometer. A Perfectsil ODS column [150  $\times$  4.6 mm internal diameter (i.d.), 3  $\mu$ m] was used for high performance liquid chromatography (HPLC) separation on a Finnigan ChromQuest model

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## Abbreviations

NMR	Nuclear magnetic resonance	FLT3	Fms-like tyrosine kinase 3
HPLC	High performance liquid chromatography	AML	Acute myeloid leukaemia
HR-ESI-MS	High resolution electrospray ionization mass spectrometry	ALK	Anaplastic lymphoma kinase
HSCCC	High-speed counter-current chromatography	EGFR	Epidermal growth factor receptor

instrument (Thermo Electron). Column chromatography was performed using RP-18 resin (Cosmosil 75C<sub>18</sub>-PREP, Kyoto, Japan). Thin-layer chromatography was carried out on pre-coated Silica gel 60 F<sub>254</sub> (0.25 mm, Merck, Darmstadt, Germany) and RP-18 plates (0.25 mm, Merck, Darmstadt, Germany). Preparative high-speed counter-current chromatography (HSCCC) was carried out with a model TBE-1000A (Shanghai Tauto Biotech Co. Ltd, Shanghai, China) with three serially connected multi-layer coil separation columns (i.d. of the tubing = 1.6 mm; total volume = 1000 mL) and an 80 mL sample loop. This system was equipped with TBP5002 LPLC pumps (Shanghai Tauto Biotech Co. Ltd, Shanghai, China), a Thermo Finnigan SSI 500 UV Detector (Thermo Electron Co., San Jose, CA), and Autochro-WIN software (version 2.0, Youngin-Tech, Korea). The upper (stationary) and lower phase (mobile) were simultaneously pumped into the multilayer-coiled column in a 3:1 volumetric ratio. When the column was completely filled with the two phases, the apparatus was rotated at 500 rpm; simultaneously, the mobile phase was pumped through the column at a flow-rate of 5.0 mL/min. The temperature was set at 25°C. The sample solution (15 mL) containing the crude BuOH extract was loaded onto the HSCCC and the effluent monitored at 254 nm.

## Plant material

A dried powder of the rhizomes of *A. asphodeloides* (Anthericaceae), purchased in a Korean oriental market, was used for the present study and identified by Dr. Joongku Lee, International Biological Material Research Center, Korea Research Institute of Bioscience & Biotechnology. A representative sample (KRIBBCYW-0002) has been deposited as a powder in the Korean Research Institute of Bioscience & Technology, ChungBuk, Korea.

## Extraction and isolation

The powder of the rhizomes of *A. asphodeloides* (600 g) was extracted by maceration with MeOH three times (1000 mL each) at room temperature, for up to 1 day each repetition, with the extractives pooled and then evaporated *in vacuo*. The dried MeOH extract (80 g) was suspended with H<sub>2</sub>O (500 mL) and partitioned sequentially with hexanes, CHCl<sub>3</sub> (3 × 500 mL), EtOAc (3 × 500 mL) and *n*-BuOH (3 × 500 mL). Of these, the BuOH-soluble partition (23 g) exhibited inhibitory activity against FLT3 (62% inhibition at 40 µg/mL), using the protocol indicated below. For preliminary screening of the active partition, a portion of the BuOH-soluble fraction (250 mg)

was subjected to HSCCC, eluting the lower phase of the two-phase solvent system composed of ethyl acetate/isopropanol/water (3:2:5, v/v), and pooled into six sub-fractions (ABF1-ABF6). Of these sub-fractions, ABF4 (*t*<sub>R</sub> 212–260 min, 18 mg) was found to be active (95% at 40 µg/mL) in FLT3 kinase inhibition assay. This sub-fraction was further purified by HPLC separation using a Perfectsil ODS column (25% MeOH, 1 mL/min) and yielded mangiferin (*t*<sub>R</sub> 11.5 min, 14 mg).

## Mangiferin

Yellow powder. HR-ESI-MS *m/z* 423.0938 [M + H]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>19</sub>O<sub>11</sub>, 423.0927). The spectroscopic data (<sup>1</sup>H and <sup>13</sup>C NMR) of this compound were consistent with published values<sup>5</sup>.

## In vitro kinase assay

Inhibition of kinase activity against a variety of recombinant kinases [FLT3, FLT3 D835Y, anaplastic lymphoma kinase (ALK), insulin receptor, and epidermal growth factor receptor (EGFR)] was measured using homogeneous time-resolved fluorescence (HTRF) assays<sup>6</sup>. In brief, the assays are based on the phosphorylation of peptide substrates in the presence of adenosine triphosphate (ATP). Resulting phosphorylated substrates are detected by a time resolved-fluorescence resonance energy transfer (TR-FRET) signal. Recombinant proteins containing a kinase domain were purchased from Millipore (Billerica, MA). Optimal enzyme, ATP, and substrate concentrations were established for each enzyme using an HTRF KinEASE kit (Cisbio, France) according to the manufacturer's instructions. Assays consist of enzymes mixed with serially diluted compounds and peptide substrates in a kinase reaction buffer (250 mM HEPES (pH 7.0), 0.5 mM orthovanadate, 0.05% bovine serum albumin, 0.1% NaN<sub>3</sub>). Following the addition of reagents for detection, the TR-FRET signal was measured using an EnVision multi-label reader (Perkin Elmer, Waltham, MA). IC<sub>50</sub> was calculated by a non-linear regression using Prism version 5.01 (GraphPad, La Jolla, CA). Each partition and chromatographic fraction was tested against FLT3 and the isolated compound was tested in FLT3, FLT3 D835Y, ALK, insulin receptor and EGFR.

## Results and discussion

The preliminary screening results of the rhizomes of *A. asphodeloides* demonstrated that a BuOH-soluble

partition exhibited the inhibitory activity (62% inhibition at 40  $\mu\text{g/mL}$ ) against FLT3 in an *in vitro* kinase assay. Fractionation of the BuOH-soluble extract using HSCCC and evaluation of the sub-fractions thereof afforded an active sub-fraction ( $\text{IC}_{50}$  4.2  $\mu\text{g/mL}$ ) containing mangiferin as a major constituent. The structure of mangiferin, xanthone C-glycoside (1,3,6,7-tetrahydroxyxanthone-2-C-glucopyranoside), was confirmed by comparison of the measured  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and high resolution electrospray ionization mass spectrometry with published values (Figure 1). To assess direct inhibition of kinase activity by mangiferin, purified recombinant FLT3 protein was pre-treated with the aforementioned compound and subjected to kinase reaction by adding substrates and ATP. As shown in Figure 2, mangiferin inhibited FLT3 activity with an  $\text{IC}_{50}$  value of 0.7  $\mu\text{M}$ , as compared with the positive control, lestaurtinib ( $\text{IC}_{50}$  1.2 nM), used in this assay system. Moreover, mangiferin exhibited equivalent inhibitory activity ( $\text{IC}_{50}$  1.2  $\mu\text{M}$ ) against FLT3 D835Y, an activating mutation of FLT3 found in AML<sup>7</sup>. In addition, in order to give more information on FLT3-inhibitory activity of

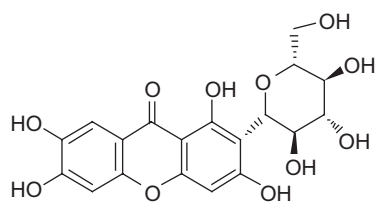


Figure 1. Structure of mangiferin.

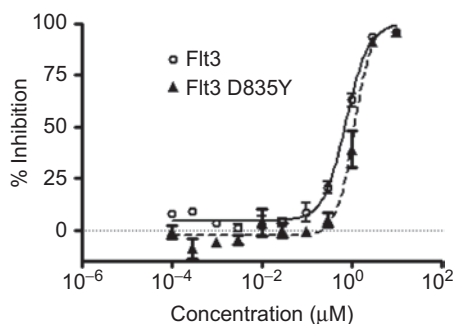


Figure 2. Effects of mangiferin on the kinase activity of FLT3 and FLT 3 D835Y. Positive control, lestaurtinib, was used.

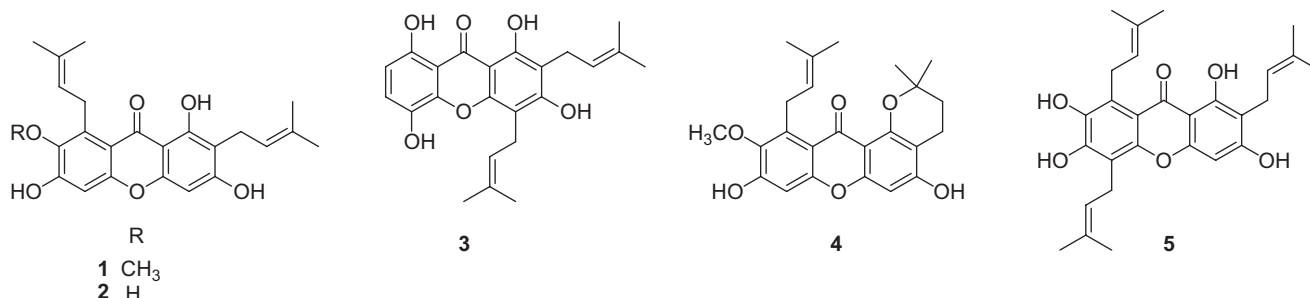


Figure 3. Structures of compounds 1–5 from *Garcinia mangostana* fruits  $\alpha$ -mangostin (1),  $\gamma$ -mangostin (2), gartanin (3), 1-isomangostin (4) and garcinone E (5).

xanthenes, five prenylated xanthenes [ $\alpha$ -mangostin (1),  $\gamma$ -mangostin (2), gartanin (3), 1-isomangostanin (4), and garcinone E (5)] previously isolated from *Garcinia mangostana* (Figure 3) were tested in the same assay system and it was found that none of them inhibited FLT3 activity ( $\text{IC}_{50} > 10 \mu\text{M}$ ). Even though  $\gamma$ -mangostin (2) and garcinone E (5) have the same 1,3,6,7-tetrahydroxyxanthone-type skeleton as mangiferin, these compounds were found to be inactive in this assay system, which may be due to the prenyl substituents in these compounds. Furthermore, mangiferin was tested against ALK, insulin receptor, and EGFR, for kinase selectivity and found to inhibit ALK activity with an  $\text{IC}_{50}$  value of 0.8  $\mu\text{M}$ , whereas it was found inactive against insulin receptor ( $> 10 \mu\text{M}$ ), and EGFR ( $> 10 \mu\text{M}$ ; Table 1). As described elsewhere<sup>6,8,9</sup>, small-molecule FLT3 inhibitors in clinical development, midostaurin, lestaurtinib, tandutinib and linifanib, are known to target multi-tyrosine kinase. This xanthone used in the current study was also found to display a non-selective inhibitory effect against FLT3 and ALK. Given that FLT3 is highly activated in AML and acute lymphocytic leukaemia, and given that small-molecule FLT3 kinase inhibitors are under clinical trials, inhibition against FLT3 and mutant FLT3 activity is recognized as an important process for cancer therapy. Thus, FLT3-inhibitory activity of mangiferin in the present study, which has not been reported in this xanthone-type structure, was deemed to demonstrate this structure to be worthy of exploiting as a potential FLT3 kinase inhibitor. To date, mangiferin isolated from several plant sources has been known to possess biological activities relevant to cancer intervention, such as apoptosis<sup>10</sup>, modulation of nuclear factor- $\kappa\text{B}$  signal transduction<sup>11</sup> and cancer chemoprevention<sup>12</sup>. There has been no previous report of xanthone with inhibitory activity against FLT3 tyrosine kinase. Current finding in the present study seems to disclose

Table 1. *In vitro* activity of mangiferin against select kinases ( $\mu\text{M}$ ).

Kinase	Mangiferin
ALK	0.81
Insulin receptor	$> 10$
EGFR	$> 10$

ALK, anaplastic lymphoma kinase, EGFR, epidermal growth factor receptor.

a new mode of action of this compound germane to cancer intervention. In this regard, this xanthone may merit further work including chemical modifications and mechanistic study.

## Declaration of interest

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