ORIGINAL ARTICLE

Quality evaluation of *Kaempferia parviflora* rhizome with reference to 5,7-dimethoxyflavone

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J. Adv. Pharm. Technol. Res.

ABSTRACT

Kaempferia parviflora Wall. ex Baker is a medicinal plant found in the upper Northeastern regions of Thailand, which belongs to Zingiberaceae family. The present study aims to investigate the standardization parameters, to analyze chemical constituents of volatile oil by gas chromatography-mass spectrometry, and to determine the content of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by thin-layer chromatography (TLC)-densitometry compared to TLC image analysis. *K. parviflora* rhizomes from 15 different sources throughout Thailand were investigated for morphological and pharmacognostic parameters. 5,7-Dimethoxyflavone contents were determined by TLC-densitometry with winCATS software and TLC image analysis with ImageJ software. The mobile phase for TLC development consisted of toluene: chloroform: Acetone: formic acid (5: 4: 1: 0.2). For the Results, the pharmacognostic parameters of *K. parviflora* rhizome were demonstrated. The loss on drying, total ash, acid-insoluble ash, water content, volatile oil content, ethanol, and water-soluble extractive values were found to be 8.979 ± 0.041, 5.127 ± 0.060, 2.174 ± 0.092, 9.291 ± 0.458, 0.028 ± 0.003, 5.138 ± 0.092, and 8.254 ± 0.191 g/100 g of dry weight, respectively. *K. parviflora* volatile oil showed the major components of α-copaene, dauca-5, 8-diene, camphene, β-pinene, borneol, and linalool. The 5,7-dimethoxyflavone content of *K. parviflora* rhizomes determined by TLC-densitometry and TLC image analysis were found to be 2.15 ± 0.64 and 1.96 ± 0.51 g/100 g of dry rhizomes, respectively. The 5,7-dimethoxyflavone contents of both methods were not significantly different ($P > 0.05$) using paired *t*-test.

Key words: 5,7-dimethoxyflavone, *Kaempferia parviflora,* phamacognostic specification, quantitative analysis, thin-layer chromatography image analysis, thin-layer chromatography-densitometry

INTRODUCTION

Kaempferia parviflora Wall. ex Baker is known in common name as Krachai Dum, Thai Ginseng, Black Turmeric, and Black Galingale, which belongs to Zingiberaceae family. It is a herbaceous plant found in the upper Northeastern regions of Thailand. Since ancient time, *K. parviflora* has been used for medicinal purposes in Thailand. In herbal medicine, it is generally used to promote health and to cure gastrointestinal disorder and anti-inflammation.[1] It is also used as an aphrodisiac for stimulating sexual performance in male. It has traditionally been used to

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Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/japtr.JAPTR_147_17

How to cite this article: Pitakpawasutthi Y, Palanuvej C, Ruangrungsi N. Quality evaluation of *Kaempferia parviflora* rhizome with reference to 5,7-dimethoxyflavone. J Adv Pharm Technol Res 2018;9:26-31.

improve vitality and treat of metabolic ailments.[2] *K. parviflora* could be eaten either fresh or dry rhizome before physical performance to improve physical work capacity.[3] *K. parviflora* has been shown its pharmacological activities such as anti-inflammatory,[4] antioxidant,[5-7] aphrodisiac,[8-10] antigastric ulcer effect,[11] antiplasmodial, antifungal, and antibacterial activities.[12,13]

The previous quantitative analysis using gas chromatographic method revealed that *K. parviflora* had 11 flavonoid constituents which 5,7,4′-trimethoxyflavone and 5,7-dimethoxyflavone were considered to be main constituents.[1] Furthermore, the high-performance liquid chromatography (HPLC) analysis of methoxyflavones in *K. parviflora* ethanolic extract indicated that 5,7,4′-trimethoxyflavone, 5,7-dimethoxyflavone, and 3,5,7,3′,4′-pentamethoxyflavone were major components.[14] Therefore, 5,7-dimethoxyflavone [Figure 1], the main component in *K. parviflora* rhizomes was chosen as quantitative marker in this plant.

K. parviflora has been widely used in Thai traditional medicine for a long time, and nowadays, *K. parviflora* has been selected as one of the promoting herbal drugs in Thailand.[15] However, the quality parameters of *K. parviflora* crude drug in Thailand have never been established. Thin-layer chromatography (TLC)-densitometry is reliable and accurate for quantification of active compound in herbal material,[16] whereas image analysis is also capable to apply for alternatively quantitative TLC.[17] This study aimed to investigate the standardization parameters, to analyze chemical constituents of volatile oil by gas chromatography-mass spectrometry (GC-MS), and to determine the content of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC-densitometry compared to TLC image analysis.

MATERIALS AND METHODS

Plant materials

Fifteen samples of *K. parviflora* rhizomes were collected by Thai traditional practitioner from different Thai traditional drug stores in 15 provinces throughout Thailand and authenticated by Ruangrungsi N. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. After removal of any foreign matters, each authentic sample was air dried and pulverized into powders.

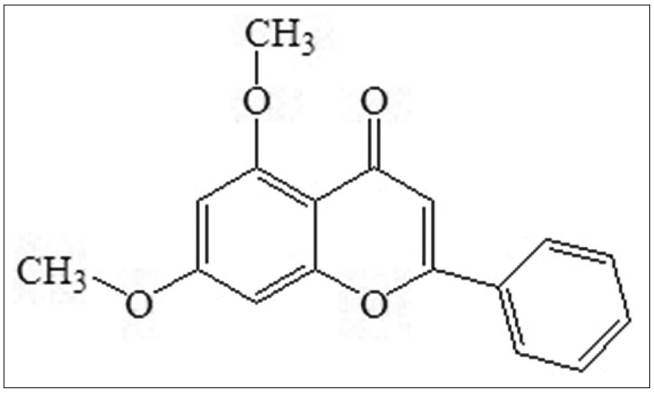

Figure 1: The structure of 5,7-dimethoxyflavone

Plant extraction

Plant materials were pulverized and exhaustively extracted with 95% ethanol by Soxhlet apparatus. The extract was filtered and evaporated to dryness *in vacuo*. The extract yields were weighed and recorded. The extract was dissolved with 95% ethanol to get the concentration of 2 mg/mL and further used for TLC-densitometry and TLC image analysis.

Preparation of standard 5,7-dimethoxyflavone

Standard 5,7-dimethoxyflavone was purchased from Sigma-Aldrich Co., USA. The stock solution was diluted to obtain the series of standard solution range from 0.2 to 1 mg/mL.

Determination of pharmacognostic specification

The pharmacognostic parameters including macroscopic characters, microscopic characters, determination of loss on drying, total ash, acid-insoluble ash, ethanol, and water extractive values, water content, and volatile oil content were examined by standard methods of World Health Organization.[18] Three grams of ground sample was dried at 105°C for 6 h until constant weight to determine loss on drying. Then, 3 g of ground sample was incinerated at 500°C until white to obtain the carbonless total ash. The ash was boiled with 25 mL of HCl (70 g/L); the insoluble matter was incinerated again at 500°C for 5 h to obtain the percentage of acid insoluble ash. Water content was conducted by azeotropic distillation with water-saturated toluene. Determinations of extractive matters were carried out with 95% ethanol and water as solvents. Ground sample (5 g) was macerated with 70 mL of the solvent under shaking for 6 h and standing for 18 h before filtration. The extract was filtered and adjusted to 100 mL after washing the marc. Twenty milliliters of the filtrate was evaporated to dryness and dried at 105°C until a constant weight was obtained. All samples were done in triplicate. The results were represented by grand mean ± pooled standard deviation. For the determination of TLC fingerprint, the ethanol extract was performed using TLC silica gel 60 GF_{254} plate as stationary phase and a mixture of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2) as mobile phase. The plate was examined under ultraviolet (UV) light (254, 365 nm) and detected by dipping in anisaldehyde reagent.

Gas chromatography-mass spectrometry analysis

The volatile oil was analyzed by a Finnigan Trace GC ultra with DSQ Quadrupole detector. BPX5 fuse silica column (30 mm × 0.25 mm, 0.25 μm film thicknesses) was used as stationary phase. The oven temperature started from 60° C

to 240° C with a constant rate of 3°C/min. The carrier gas was helium with the flow rate of 1 mL/min. One microliter of the oil (1:100 in HPLC grade methanol) was injected by Finnigan Autoinjector AI3000 with split ratio of 10:1. MS was performed by electron impact positive mode at 70 electron volts. The chemical constituents were identified by matching mass spectra and retention time indices with Adams Essential Oils Mass Spectral library and NIST05 Mass Spectral library. Peak area was shown in percentage.

Quantitative analysis of 5,7-dimethoxyflavone by thin-layer chromatography-densitometry

Three microliters of 15 ethanol extracts and standard solutions were applied onto the silica gel 60 GF_{254} TLC plate. The plate was developed in a TLC chamber that contained a mixture of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2), then the plate was removed and allowed to dry at room temperature. After that, the same TLC plate was developed again for two more times to increase the distance of the band. After development, the plate was dried and scanned with CAMAG TLC Scanner 4 (CAMAG, Switzerland) under wavelength of maximum absorbance (265 nm) and expressed as chromatographic peak by winCATS software (Camag, Switzerland).

Quantitative analysis of 5,7-dimethoxyflavone by thin-layer chromatography image analysis

The 5,7-dimethoxyflavone spots on the developed TLC plates were photographed under short wave UV (254 nm) by a digital camera. Peak area of each spot was quantitated using ImageJ free software (Department of Health and Human Services, National Institutes of Health (NIH) in the United State). The content of 5,7-dimethoxyflavone was determined by comparing peak area to the calibration curve obtained from the same TLC plate.

Method validation

According to the ICH guidelines, the method validation including calibration range, specificity, accuracy, precision, limit of detection, limit of quantitation, and robustness were performed.[19]

RESULTS AND DISCUSSION

Pharmacognostic specification

Macroscopic and microscopic examinations are the first process to determine the characteristics, identity and degree of purity of medicinal plant materials. The macroscopic and microscopic characteristics of *K. parviflora* rhizome were illustrated as the drawing of the plant by the author [Figure 2]. The herbal medicines need to provide the quality control evidences which indicate the quality evaluation of plant materials and make them more reliable. Pharmacognostic specification is primary important tool for identification, authentication, and standardization of herbal medicines.[18] The pharmacognostic specification of *K. parviflora* rhizome

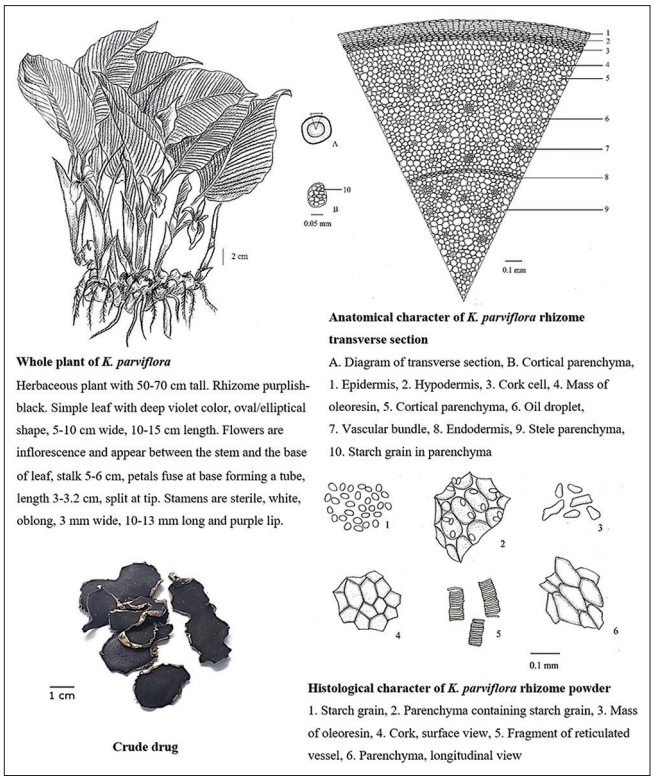

Figure 2: Pharmacognostic specification of *Kaempferia parviflora* rhizome: macroscopic and microscopic characteristics

were shown in Figures 2 and 3. For the pharmacognostic parameters, the loss on drying, total ash, acid-insoluble ash, water content, volatile oil content, ethanol, and water-soluble extractive values were found to be 8.979 ± 0.041, 5.127 ± 0.060, 2.174 ± 0.092, 9.291 ± 0.458, 0.028 ± 0.003, 5.138 ± 0.092, and 8.254 ± 0.191 g/100 g of dry weight, respectively. Furthermore, the quality control needs to measure the phytochemical compounds in medicinal plants for ensuring the quality reliability of natural products obtained from plant sources.[20] Thus, TLC fingerprint demonstrated the pattern of phytochemical characteristic constituents [Figure 3].

Gas chromatography-mass spectrometry analysis

The volatile oils of *K. parviflora* dried rhizomes consisted of at least 20 compounds as shown in Table 1. The major components of *K. parviflora* volatile oil were α-copaene (11.68%), dauca-5, 8-diene (11.17%), camphene (8.73%), β-pinene (7.18%), borneol (7.05%), and linalool (6.58%), respectively. The result was related to the previous studies of volatile oil in the hexane extract of *K. parviflora* rhizomes which reported the dominant components as borneol (10.24%), β-pinene (8.60%), camphene (7.62%), α-copaene (7.23%), and linalool (6.40%).[21]

Quantitative analysis of 5,7-dimethoxyflavone

The percent yield of ethanolic extracts of *K. parviflora* rhizomes was 9.57 ± 1.49 g/100 g by dry weight. The quantitative analysis of 5,7-dimethoxyflavone in the

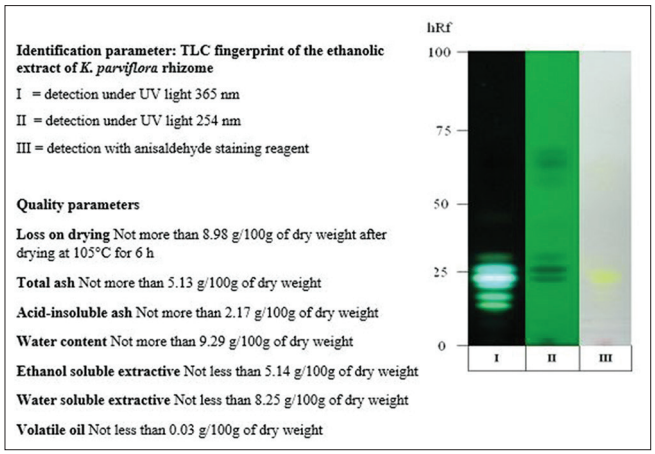

Figure 3: Pharmacognostic specification of *Kaempferia parviflora* rhizome: identification and quality parameters

Table 1: The chemical constituents of *Kaempferia parviflora* volatile oil

Retention time (min)	Compound name	Area percentage (mean±SD)	Kovat's index
6.67	α-pinene	5.51±2.75	939
7.12	Camphene	8.73±4.70	954
7.99	β-pinene	7.18±3.81	979
9.75	Limonene	1.80±0.16	1029
12.48	Linalool	6.58±4.64	1096
15.24	Borneol	7.05±2.70	1169
20.29	Bornyl acetate	3.75±2.18	1288
24.10	α-copaene	11.68±3.98	1376
24.74	β-elemene	5.83±2.86	1390
25.85	(E)-caryophyllene	6.03±2.33	1419
27.22	α-humulene	1.88±0.43	1454
28.34	Dauca-5,8-diene	11.17±5.21	1472
28.94	γ-gurjunene	2.78±1.13	1477
29.30	β-salinene	2.05±0.72	1490
29.96	Δ-cadinene	3.89±1.33	1523
32.05	Spathulenol	2.21±0.90	1578
32.25	Caryophyllene oxide	2.95±1.85	1583
34.45	Epi-α-muurolol	2.25±0.82	1642
34.90	α-cadinol	4.00±1.82	1654
35.56	Longiborneol acetate	1.93 ± 0.81	1685

SD: Standard deviation

extracts was performed by TLC-densitometry and TLC image analysis using toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2) as mobile phase. TLC chromatogram under UV 254 nm is shown in Figure 4. Densitometry is the quantitative and qualitative measurement of a reflection in absorbance or fluorescence mode with the optimal wavelength.[16] The compound separated by TLC are quantified using TLC densitometer with high reliability. In addition, ImageJ is a free software developed at the National Institutes of Health which can quantitate and calculate pixel intensity in digital image of TLC spot and

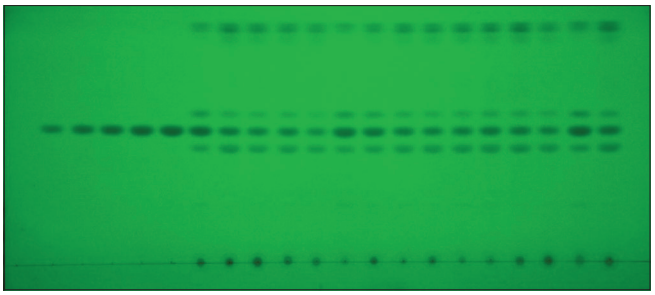

Figure 4: The thin-layer chromatography plate under ultraviolet 254 nm; standard 5,7-dimethoxyflavone (track 1–5), and *Kaempferia parviflora* rhizome extracts from 15 different sources (track 6–20)

transform to chromatographic peak.[22] TLC densitogram scanned in the range of 200–700 nm is shown in Figure 5. The 5,7-dimethoxyflavone content of *K. parviflora* rhizomes determined by TLC-densitometry and TLC image analysis were found to be 2.15 ± 0.64 and 1.96 ± 0.51 g/100 g of dry rhizomes, respectively. The 5,7-dimethoxyflavone contents of both methods were not significantly different ($P > 0.05$) using paired *t-test*. The results indicated that TLC image analysis was convenient and inexpensive technique which could be used as an alternative method to quantitate the 5,7-dimethoxyflavone contents in *K. parviflora* rhizomes.

Method validation

The specificity was confirmed by comparing UV spectrum of the peak among standard 5,7-dimethoxyflavone and all samples at 3 positions of the peak (apex, upslope, and down-slope). The maximum absorbance was at a wavelength of 265 nm [Figure 5]. The validity of TLC-densitometry and TLC image analysis were demonstrated in Table 2. The polynomial calibration curves ranged from 0.6 to 3 μg/spot [Figures 6 and 7]. The percent recovery was determined to evaluate the accuracy by spiking known three concentrations of 5,7-dimethoxyflavone in a sample. The recovery values of both methods were within acceptable limits (85.31%–100.56%). The repeatability and the intermediate precision were determined in the same day and in three different days. The repeatability and the intermediate precision of both methods were less than 6% relative standard deviation (RSD). The limit of detection and limit of quantitation of TLC-densitometry and TLC image analysis were calculated by the residual standard deviation of a regression line and found to be 0.03 and 0.10 μg/spot for TLC-densitometry, and 0.08 and 0.23 μg/spot for TLC image analysis, respectively. These values showed sufficient sensitivity of both methods. The robustness studied showed the values of 0.76% RSD for TLC-densitometry and 2.38% RSD for TLC image analysis. The result of robustness by changing the mobile phase ratio was not affected in both methods. The results from method validation indicated that TLC-densitometry, and TLC image analysis were efficient and reliable technique for quantitative analysis of 5,7-dimethoxyflavone in *K. parviflora* rhizomes.

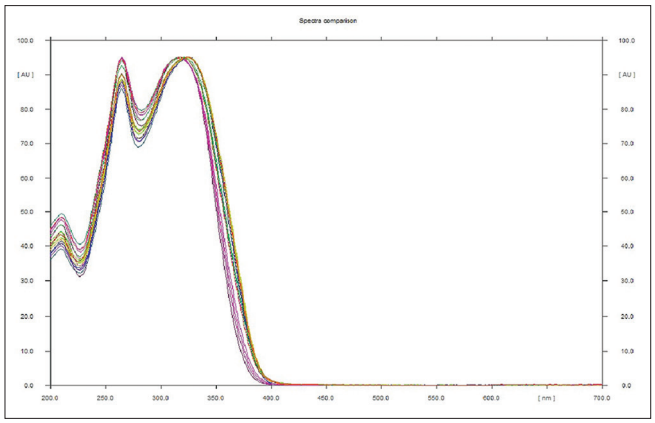

Figure 5: The absorption spectra of 5,7-dimethoxyflavone in standard and all sample bands

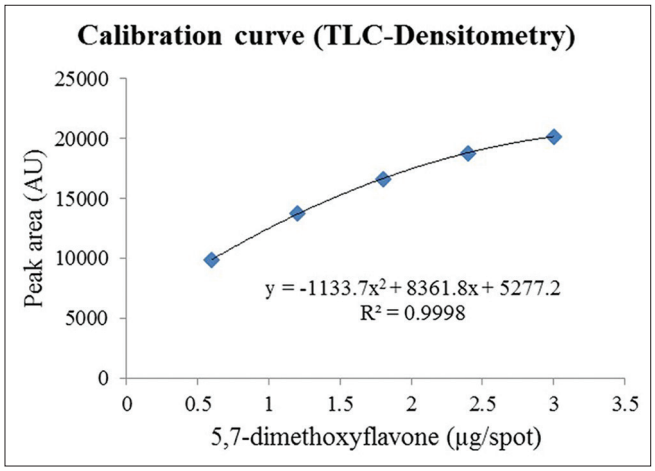

Figure 6: The calibration curve of 5,7-dimethoxyflavone by thin-layer chromatography-densitometry

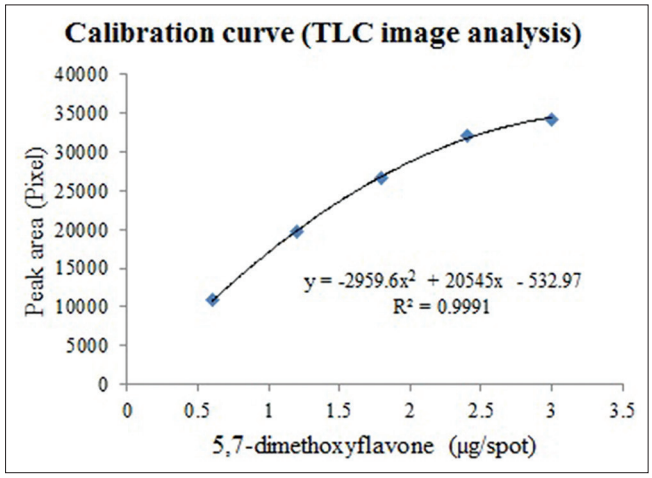

Figure 7: The calibration curve of 5,7-dimethoxyflavone by thin-layer chromatography image analysis

CONCLUSION

The pharmacognostic specification of *K. parviflora* rhizomes in Thailand was established. The chemical constituents of the volatile oil from *K. parviflora* dried rhizomes were clearly revealed. For quantitative analysis, TLC-densitometry as well as TLC image analysis of 5,7-dimethoxyflavone content of *K. parviflora* rhizomes were developed.

Table 2: Method validity of thin-layer chromatography-densitometry and thin-layer chromatography image analysis of 5,7-dimethoxyflavone in *Kaempferia parviflora* rhizome

Parameter	Validity	
	TLC-densitometry	TLC image analysis
Accuracy (percentage recovery)	97.89±1.28	94.02±7.86
Precision: Repeatability (%RSD)	2.29	3.26
Precision: Intermediate precision (%RSD)	2.72	4.49
Limit of detection (µg/spot)	0.03	0.08
Limit of quantitation (µg/spot)	0.10	0.23
Robustness (%RSD)	0.76	2.38

TLC: Thin-layer chromatography, RSD: Relative standard deviation

Acknowledgment

The authors are supported the scholarship from "The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship" and "The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund)." The authors wish to thank College of Public Health Sciences, Chulalongkorn University and all staff members for necessary assistance and instrument supports.

Financial support and sponsorship

This study was supported by "The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship" and "The 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund)."

Conflicts of interest

There are no conflicts of interest.

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