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Nine Representative Phytochemicals Occurring in Leaves and Rhizomes of *Alpinia oxyphylla* Different to Those of Fruits Determined Using LC-MS/MS

YONG-HUI LI[1,2,#], FENG CHEN[1,2,#], HAI-LONG LI[1,2], YIN-FENG TAN[1,2] and JUN-QING ZHANG[1,2,*]

[1]School of Pharmacy, Hainan Medical University, Haikou, P.R. China
[2]Hainan Provincial Key Laboratory of R&D of Tropical Herbs, Haikou, P.R. China
[#]These authors contributed equally to this work

*Corresponding author: Fax: +86 898 66893460; Tel: +86 898 66895337; E-mail: jqzhang2011@163.com

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Alpinia oxyphylla is commonly used in traditional East Asian medicine. Usually, only its fruits are regarded as an important medicinal plant part. However, the plant resource of *A. oxyphylla* is insufficient. Therefore, we question whether the leaves or rhizomes can be used as alternative parts for this genus based on phytochemical composition. In the present study, nine chemical components (*i.e.*, tectochrysin, izalpinin, chrysin, apigenin-4',7-dimethyl ether, kaempferide, yakuchinone A, yakuchinone B, oxyphyllacinol and nootkatone) were regarded as representative constitutes of fruits. The abundance of the nine constituents in leaves and rhizomes was evaluated by a validated LC-MS/MS method and then compared with that of fruits. The results revealed that the monitoring of the nine phytochemicals were also made in leaves or rhizomes samples as fruits. The content levels of these compounds were comparable between leaves and rhizomes, but both were significantly less than fruits. As the fruits ripening (from day 10 to day 50), the content levels of flavonoid were increased with fluctuation in rhizomes. In contrast, the concentrations measured in leaves decreased. The content changes of nootkatone and diarylheptanoid with growth time were similar between rhizomes and leaves. Additional work is required to assess the bioactive properties of the fruits, leaves or rhizomes and make the best use of these valuable medicinal plant resources.

Keywords: *A. oxyphylla* rhizomes and leaves, Harvest times, Diarylheptanoids, Flavonoids, LC-MS/MS.

INTRODUCTION

Fructus *Alpinia oxyphylla* (ginger family, Zingiberaceae) is mainly used in traditional East Asian medicine and also as plant food. As herbal medicine, it has been used for treating diarrhea with splenic cold, polyuria, gastralgia, spontaneous salivation, kidney asthenia with enuresis, spermatorrhea and turbid urine[1]. Recently, there has been increasing evidence of the beneficial effects of *A. oxyphylla* fruits such as anti-inflammatory[2], anti-allergy[3], anti-ulcer[4] and neuroprotective activities[5-7]. It also has a long history as a component of a formula which has been used to control frequent urination and loss of bladder control[8].

Alpinia oxyphylla is an herbaceous perennial plant and its fruits have been used as an important medicinal part of this plant. As one of four najor south China medicinal plants, *A. oxyphylla* fruits are mainly produced in Hainan province, China, accounting for > 95 % total outputs. The annual total outputs of the fruits are around 800-900 tons and the supply of this plant resource is insufficient[9]. Therefore, we question whether the leaves or rhizomes of *A. oxyphylla* can be used alternative parts for this genus. If this were true, we would better make use of this valuable tropical plant.

In order to better explore this hypothesis, the chemical composition and content levels in fruits, leaves and rhizomes should be firstly evaluated. As well known, *A. oxyphylla* fruits contain flavonoid (*e.g.*, tectochrysin, izalpinin, chrysin, apigenin-4',7-dimethyl ether and kaempferide), diarylheptanoid (*e.g.*, yakuchinone A and B and oxyphyllacinol), sesquiterpenes (*e.g.*, nootkatone), volatile oil, steroids and their glycosides, *etc*[10,11]. Recently, we reported the content levels of nine representative components occurring in the fruits of *A. oxyphylla* harvested at different times[12,13]. In present study, we monitored the changes of the main components present in the fruits along with different growth period[12]. In present study, we aimed to assess content levels the nine representative components in the leaves and rhizomes collected at the same harvest times as fruits, using a validated method[12].

EXPERIMENTAL

Reference standard of nootkatone (purity, 98 %; similarly hereinafter) was purchased from Sigma-Aldrich (St Louis, MO,

USA). Yakuchinone A (98 %), Yakuchinone B (98 %) and oxyphyllacinol (98 %) were purchased from Chenfun Medical Technology (Shanghai) Co., Ltd. (Shanghai, China). Tectochrysin, izalpinin, chrysin, kaempferide and apigenin-7,4'-dimethyl ether were separated and identified from *A. Oxyphylla* by Prof. Zhang (Hainan Provincial Key Laboratory of R&D on Tropical Medicinal Plants, Haikou, China). On the basis of UV, NMR and MS analysis, the structures of isolated reference standards were confirmed and their purities determined using HPLC-PDA-MS were over 98 %. HPLC-grade methanol and acetonitrile were products of Merck (Darmstadt, Germany). HPLC-grade formic acid was purchased from Aladdin Industrial Inc. (Shanghai, China). HPLC-grade water was prepared by double-distillation of deionized water. The other chemical reagents of analytical grade or better were obtained from Hainan YiGao Instrument Co., Ltd (Haikou, China).

The rhizomes and leaves of *A. oxyphylla* of different harvest times (10, 15, 20, 25, 27, 30, 35, 40, 45, 50, 55, 60 and 65 day) were collected and dried in the shade from Qiongzhong county, Hainan province, China. These materials were identified by Prof. Jian-ping Tian at Hainan Medical University. The specimen was deposited in the Hainan Provincial Key Laboratory of R&D of Tropical Herbs, Haikou 571101, Hainan province, China.

Preparation of sample solutions: The leaves and rhizomes of *A. oxyphylla* were ground into powder with 40 mesh, respectively. An aliquot (0.5 g) was weighed precisely and refluxed with 10 mL of 70 % ethanol for 0.5 h at 60 °C and finally made to a volume of 10 mL using 70 % ethanol. Three replicates of the extraction process were carried out on each sample. The solution was filtered through 0.22 μm membrane prior to use. The filtrates were diluted to 1:100 with methanol and a 5 μL aliquot was injected into the UFLC-MS/MS system for final analysis.

Analysis of nine phytochemicals occurring in roots and leaves of *A. oxyphylla*: The analytical method validation was partly described by Li *et al.*[12]. Briefly, an AB-SCIEX API 4000+ mass spectrometer (Toronto, Canada) interfaced *via* a Turbo V ion source with a Shimadzu Prominence UFLC chromatographic system (Shimadzu Corporation, Kyoto, Japan), which is equipped with two LC-20AD pumps, a model DGU-20A3R degasser unit, a SIL-20A HT autosampler and a CTO-20A column oven. The AB-SCIEX Analyst software packages were used to control the LC-MS/MS system, as well as for data acquisition and processing. Mass calibration of the mass spectrometer is performed every one month using polytyrosine glycol as standard in our laboratory.

Chromatographic separations of prepared samples were achieved using a Shim-pack XR-ODS column (2 mm i.d × 100 mm) maintained at 35 ºC. The LC mobile phases included H_2O containing 0.04 ‰ formic acid for solvent A and methanol containing 0.04 ‰ formic acid for solvent B. A specially designed LC binary gradient program (2 % B at 0 min to 100 % B at 10 min) was used to separate the nine phytochemicals and the effluent was delivered at 0.3 mL/min throughout the gradient program.

The mass spectrometer was operated in the ESI positive ion mode with multiple reaction monitoring (MRM) for all the analytes. The pneumatically nebulized ESI spraying was achieved by using inner coaxial nebulizer N_2 gas of 55 psi through a Turbo V ion Spray probe, a high voltage of + 5.5 kV applied to the sprayer tip and heated dry N2 gas of 55 psi at 550 ºC from two turbo heaters adjacent to the probe. To prevent solvent droplets from entering and contaminating the ion optics, a curtain N_2 gas of 25 psi was applied between the curtain plate and the orifice. The collision gas flow was set at level 4. The precursor-to-product ion pairs used for multiple reaction monitoring of nootkatone, Yakuchinone A and Yakuchinone B, oxyphyllacinol, tecto-chrysin, izalpinin, chrysin, kaempferide and apigenin-7,4'-dimethyl ether were *m/z* 219.2 → 163.0 (the optimal collision energy, 22 V), 313.2 → 136.9 (13 V), 311.2 → 117.0 (30 V), 315.3 → 137.0 (22 V), 269.1 → 226.0 (43.5 V), 285.0 → 242.0 (43 V), 255.1 → 152.9 (42 V), 301.1 → 286.0 (37 V) and 299.2 → 256.0 (45 V), respectively, with a scan time of 25 ms for each ion pair.

Method Validation: A full validated method was described by Li *et al.*[12] for determination of nine compounds from *A. oxyphylla* fruits at different harvest times. In the present study, we applied the same method to measure the same compounds occurring in leaves and rhizomes of *A. oxyphylla*. Therefore, an additional partial validation was conducted focusing on the method precision and accuracy.

RESULTS AND DISCUSSION

Optimization of extraction conditions: Because the rhizomes or leaves are different plant organs compared with fruits, we applied the orthogonal ($L_{16}\,4^3 \times 2^1$) design to optimize the extraction conditions. The factors including solvent-to-sample ratio, solvent composition, extraction time and extraction temperature were assessed through quantitatively analyzing the indices k and R values. The similar results as fruits were obtained for leaves and rhizomes. The factors were ranked by importance for the monitored ingredients occurring in rhizomes as follows: solvent-to-sample ratio > solvent > extraction time > extraction temperature. At last, the 20:1 of 70 % ethanol (v/v) for 0.5 h at 60 °C was chosen as the optimized condition for the extraction of nine phytochemicals from *A. oxyphylla* leaves or rhizomes.

Analytical method validation: The precision and accuracy were validated and the results are shown in Table-1. The RSD values of intra- and inter-day variations of the nine analytes occurring in leaves or rhizomes were almost less than 5 %. The overall recoveries lay between 95.0% and 105% with RSD less than 4.80 and 6.90 % for leaves and rhizomes, respectively. These results indicated that the established method was accurate and reliable.

Quantitative analysis of leaves and rhizomes samples: The validated UFLC-MS/MS method was subsequently applied to qualify and quantify the nine representative phytochemicals present in leaves or rhizomes of *A. oxyphylla*. Identification of the nine compounds in leaves and rhizomes were achieved through comparing the *m/z* ion pairs and retention times with those of standards (Fig. 1). The quantitative analysis results are shown in Table-2 and Fig. 2 for the leaves and Table-3 and Fig. 3 for rhizomes.

The content levels of nootkatone and diarylheptanoids (*e.g.*, Yakuchinone A and B and oxyphyllacinol) in leaves (Fig. 2)

TABLE-1
INTRA-DAY PRECISION, INTER-DAY PRECISION AND RECOVERY TEST FOR THE CURRENT UFLC-MS/MS METHOD

Compound	Nootkatone	Yakuchinone A	Yakuchinone B	Oxyphyllacinol	Tectochrysin	Izalpinin	Chrysin	Kaempferide	Apigenin-7,4'-dimethylether
Leaves of *A. oxyphylla*									
Precision (RSD, %)									
Intraday (n = 6)	0.98	1.7	3.65	4.82	2.75	1.58	3.9	2.1	2.37
Interday (n = 18)	2.62	4.52	4.02	4.52	4.22	2.11	4.69	2.85	2.98
Recovery									
Mean ± SD, %, n = 6	104 ± 4	100 ± 4	97.9 ± 4.3	105 ± 3	95.0 ± 3.1	98.5 ± 4.8	99.2 ± 4.8	101 ± 3	102 ± 5
RSD (%)	3.60	3.66	4.35	3.32	3.26	4.87	4.80	2.82	4.62
Rhizomes A. oxyphylla									
Intraday (n = 6)	1.07	3.9	2.26	3.1	3.55	2.98	1.72	2.67	2.66
Interday (n = 18)	2.03	5.31	1.85	2.69	4.48	3.09	3.5	3.59	2.18
Recovery									
Mean ± SD, %, n = 6	105 ± 6	96.4 ± 2.8	97.7 ± 6.1	96.1 ± 2.4	99.4 ± 6.5	99.4 ± 3.5	95.7 ± 6.6	97.3 ± 4.7	101 ± 4
RSD (%)	6.07	2.96	6.28	2.55	6.57	3.53	6.90	4.79	3.89

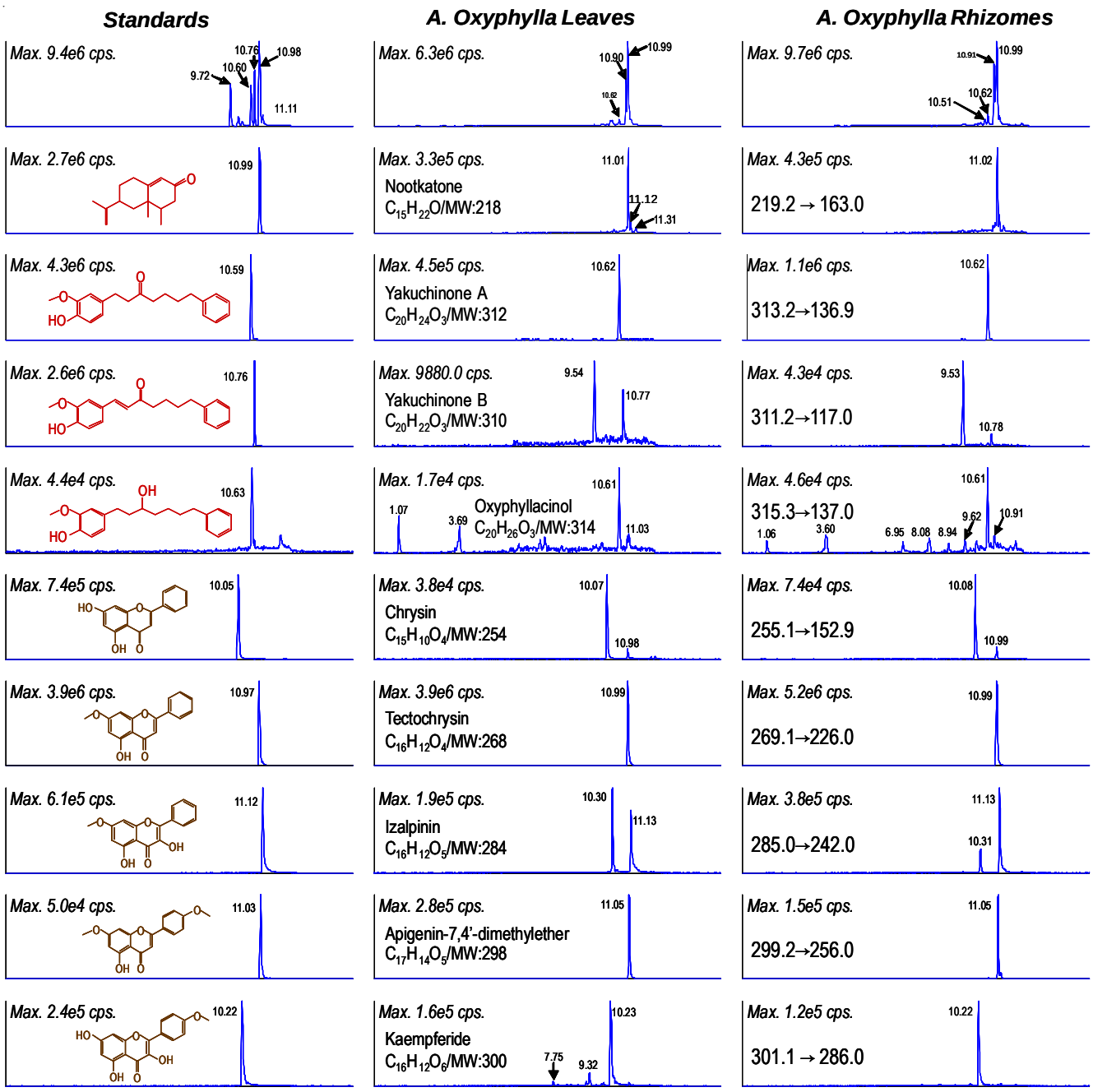

Fig. 1. Representative UFLC-MS/MS chromatograms. Left panel mixed nine standard compounds solution (1 µg/mL); Middle panel leaves sample solution of *A. oxyphylla* (harvested at day 20); Right panel rhizomes sample solution of *A. oxyphylla* (harvested at day 20)

TABLE-2
CHEMICAL CONTENTS (µg/g, mean ± SD) IN LEAVES OF *A. oxyphylla* HARVESTED AT DIFFERENT TIMES

Harves time (day)	Nootkatone	Yakuchinone A	Yakuchinone B	Oxyphyllacinol	Tectochrysin	Izalpinin	Chrysin	Kaempferide	Apigenin-7,4'-dimethylether
10	1.47 ± 0.06	1.28 ± 0.04	0.0645 ± 0.0021	2.67 ± 0.13	62.1 ± 2.4	8.98 ± 0.17	2.15 ± 0.03	69.3 ± 1.7	164 ± 7
15	1.83 ± 0.07	1.5 ± 0.02	0.0308 ± 0.0024	2.44 ± 0.12	24.3 ± 1.4	4.72 ± 0.23	0.69 ± 0.04	29.9 ± 1.5	86 ± 4.1
20	6.11 ± 0.28	7.97 ± 0.22	0.2301 ± 0.0098	9.68 ± 0.62	40.4 ± 1.9	6.98 ± 0.2	0.8 ± 0.01	22.3 ± 0.3	86.2 ± 1.4
25	1.84 ± 0.08	0.86 ± 0.04	0.0261 ± 0.0008	3.58 ± 0.24	20.9 ± 1.0	3.52 ± 0.12	0.26 ± 0.02	24.6 ± 1.2	70.8 ± 2.2
27	3.00 ± 0.12	0.41 ± 0.03	0.0124 ± 0.0006	1.09 ± 0.07	8.6 ± 0.55	1.67 ± 0.06	0.11 ± 0.01	12.9 ± 0.9	70.4 ± 5.6
30	1.24 ± 0.03	0.66 ± 0.04	0.0155 ± 0.0007	2.06 ± 0.08	44.4 ± 3.7	7.02 ± 0.61	0.97 ± 0.05	19.4 ± 1.2	258 ± 11
35	3.12 ± 0.05	0.96 ± 0.05	0.011 ± 0.0006	2.06 ± 0.04	23.7 ± 1.2	3.96 ± 0.16	0.34 ± 0.01	15.2 ± 0.7	194 ± 9
40	1.07 ± 0.05	0.14 ± 0.01	0.0041 ± 0.0002	0.93 ± 0.05	19.0 ± 1.7	3.64 ± 0.1	0.25 ± 0.01	16.6 ± 0.4	194 ± 9
45	1.15 ± 0.05	0.73 ± 0.04	0.0267 ± 0.0016	1.95 ± 0.04	18.2 ± 1.1	3.28 ± 0.09	0.33 ± 0.01	20.8 ± 0.9	168 ± 8
50	3.38 ± 0.05	3.62 ± 0.07	0.0673 ± 0.0019	5.12 ± 0.18	23.8 ± 0.4	4.88 ± 0.14	0.45 ± 0.01	27.3 ± 1.2	230 ± 10
55	2.27 ± 0.02	2.18 ± 0.06	0.022 ± 0.0012	3.91 ± 0.23	12.5 ± 0	2.32 ± 0.06	0.21 ± 0.01	16.5 ± 0.7	95.3 ± 4.4
60	0.93 ± 0.04	0.26 ± 0.01	0.0105 ± 0.0003	1.19 ± 0.08	10.7 ± 0.3	1.97 ± 0.01	0.25 ± 0.01	14.6 ± 0.9	181 ± 6
65	3.08 ± 0.05	0.36 ± 0.02	0.0097 ± 0.0002	1.42 ± 0.07	11.7 ± 0.3	2.02 ± 0.09	0.24 ± 0.02	14.4 ± 0.6	193 ± 5

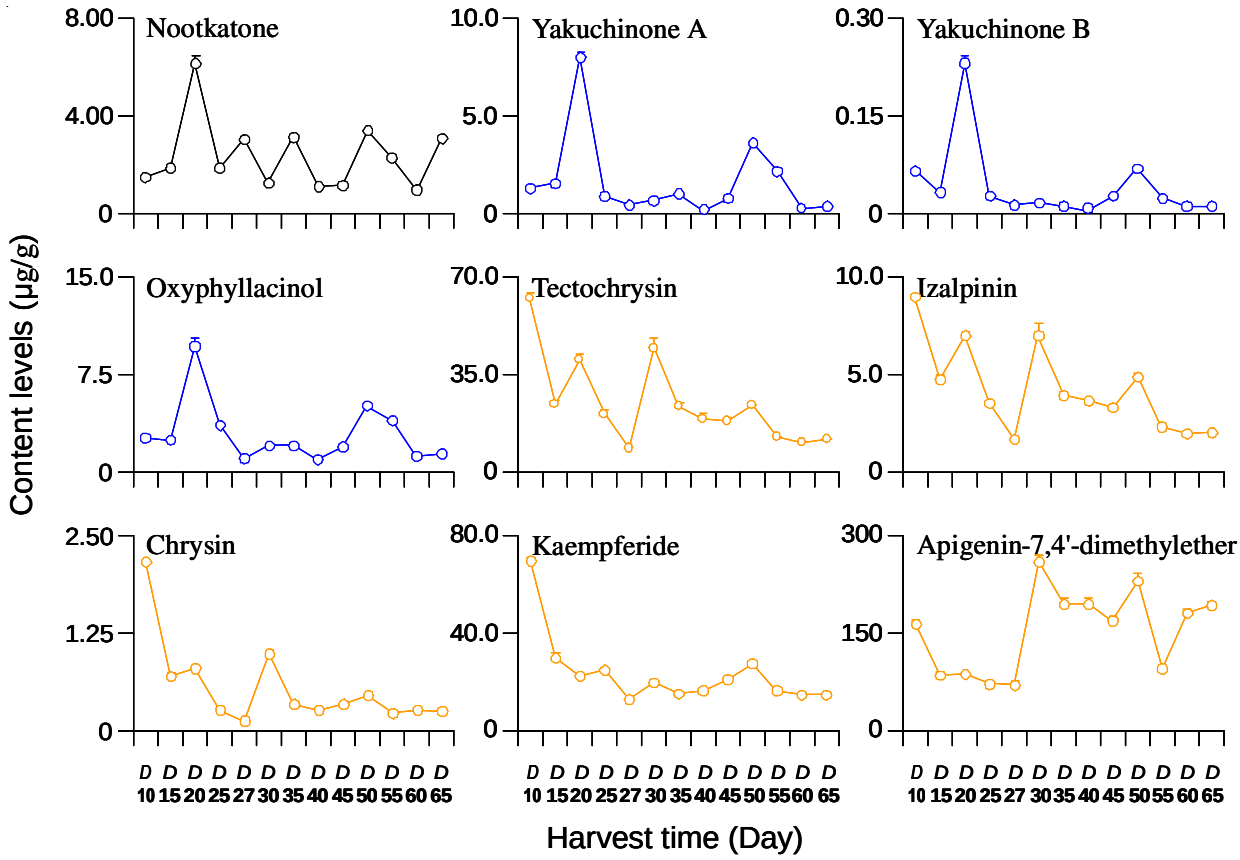

Fig. 2. Content levels of nine representative compounds occurring in leaves of *A. oxyphylla* harvested at different times

TABLE-3
CHEMICAL CONTENTS (µg/g, MEAN ± SD) IN RHIZOMES OF *A. oxyphylla* HARVESTED AT DIFFERENT TIMES

Harves time (day)	Nootkatone	Yakuchinone A	Yakuchinone B	Oxyphyllacinol	Tectochrysin	Izalpinin	Chrysin	Kaempferide	Apigenin-7,4'-dimethylether
10	0.51 ± 0.01	2.25 ± 0.14	0.0054 ± 0.0001	4.10 ± 0.05	83.1 ± 3.9	13.9 ± 0.4	1.61 ± 0.01	35.1 ± 1.8	96.0 ± 1.0
15	0.56 ± 0.01	1.81 ± 0.06	0.0055 ± 0.0001	6.25 ± 0.1	44.0 ± 0.8	8.15 ± 0.31	1.57 ± 0.06	44.9 ± 1.8	92.2 ± 6.6
20	0.47 ± 0.01	19.8 ± 0.4	0.045 ± 0.0014	26.7 ± 0.99	53.0 ± 1.6	8.47 ± 0.51	0.96 ± 0.02	15.2 ± 0.6	101 ± 6
25	0.56 ± 0.01	3.58 ± 0.17	0.025 ± 0.0011	4.91 ± 0.17	66.3 ± 2.5	10.3 ± 0.3	1.16 ± 0.06	20.3 ± 1	69.7 ± 2.4
27	0.42 ± 0.04	2.14 ± 0.14	0.0047 ± 0.0002	3.08 ± 0.17	30.3 ± 0.4	4.84 ± 0.32	0.41 ± 0.02	17.9 ± 0.7	115 ± 8
30	0.58 ± 0.03	1.37 ± 0.09	0.0057 ± 0.0001	2.2 ± 0.09	47.9 ± 1.1	6.58 ± 0.26	0.38 ± 0.02	15.6 ± 0.8	128 ± 6
35	0.63 ± 0.02	1.38 ± 0.10	0.0139 ± 0.0001	3.78 ± 0.13	46.0 ± 1.5	6.53 ± 0.2	0.33 ± 0.01	13 ± 0.8	101 ± 2
40	0.53 ± 0.04	8.06 ± 0.29	0.03 ± 0.0004	10.1 ± 0.26	42.7 ± 1.1	6.39 ± 0.23	0.39 ± 0.03	15.7 ± 0.7	118 ± 1
45	2.77 ± 0.11	4.37 ± 0.30	0.0286 ± 0.0012	5.10 ± 0.34	93.4 ± 0.8	12.3 ± 0.4	1.41 ± 0.02	27 ± 1.5	80.9 ± 3.4
50	0.26 ± 0.01	6.09 ± 0.45	0.0213 ± 0.0005	7.17 ± 0.18	100 ± 1	15.5 ± 1.2	2.13 ± 0.06	38.9 ± 0.4	165 ± 12
55	0.39 ± 0.02	5.78 ± 0.22	0.0191 ± 0.0007	7.35 ± 0.40	45.2 ± 1.6	5.71 ± 0.11	0.29 ± 0.02	13 ± 0.4	136 ± 4
60	0.27 ± 0.00	2.71 ± 0.24	0.0042 ± 0.0002	3.68 ± 0.17	41.3 ± 0.4	6.12 ± 0.01	0.57 ± 0.03	20.4 ± 0.3	156 ± 12
65	0.52 ± 0.02	1.12 ± 0.02	0.0048 ± 0.0001	1.62 ± 0.02	21.5 ± 0.6	3.21 ± 0.05	0.23 ± 0.01	9.85 ± 0.39	97.6 ± 3.0

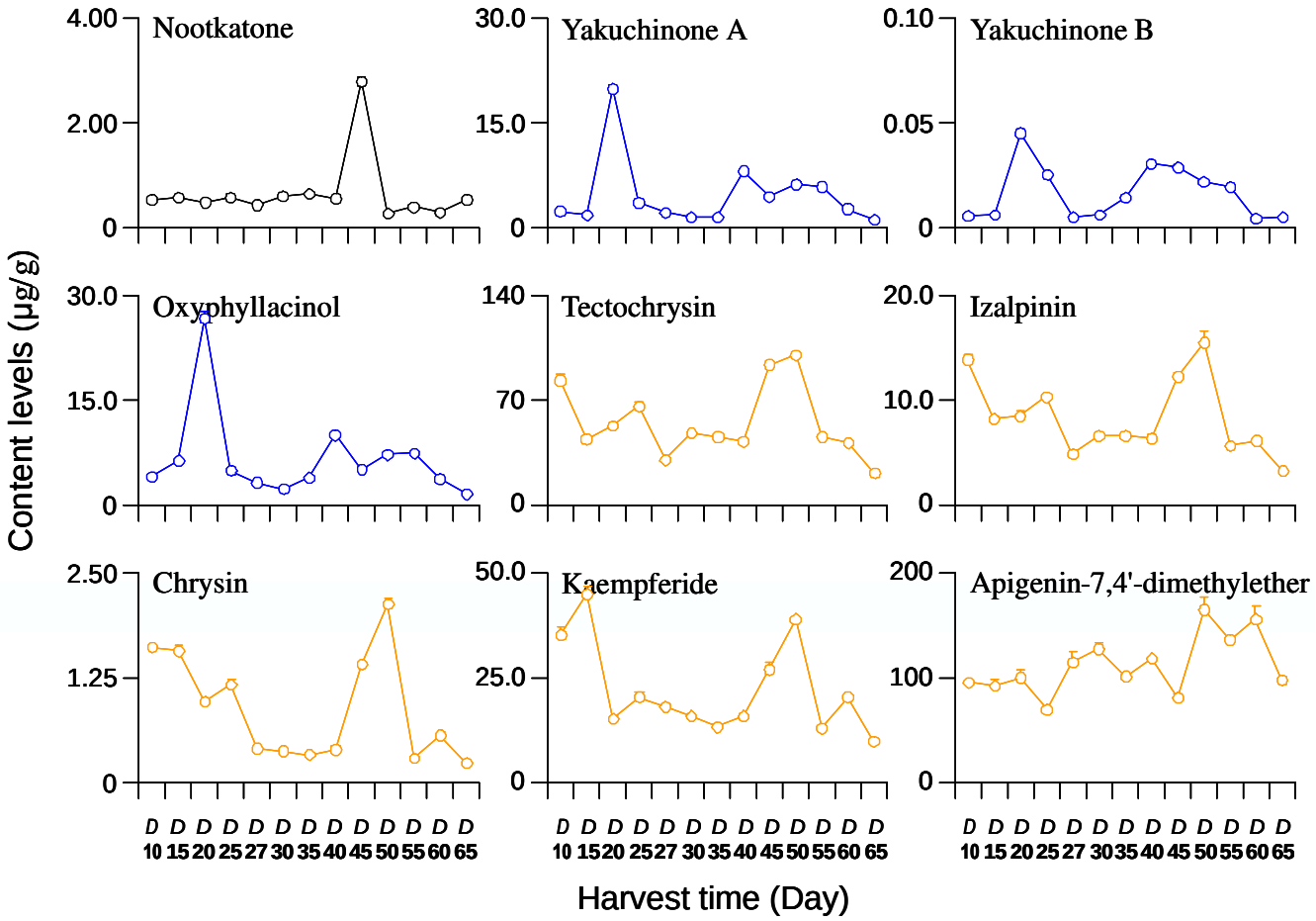

Fig. 3. Content levels of nine representative compounds occurring in rhizomes of *A. oxyphylla* harvested at different times

or rhizomes (Fig. 3) samples were no greater than 30 μg/g, which were significantly less than the concentrations in fruits samples[12]. In addition, the amounts of nootkatone and diarylheptanoids both in leaves and rhizomes decreased with the growth time. Nootkatone has anti-platelet effects[14] and anti-inflammation roles *via* heme oxygenase-1 induction[15]. Yakuchinone A and B exhibit anti-inflammatory, anti-tumor, antibacterial and gastric protective activities[16-19]. It appears that leaves or rhizomes could not be used as alternative medicinal plant parts for fruits of *A. oxyphylla* only based on the content differences of the active diarylheptanoid and sesquiterpene constituents.

The content levels of five flavonoid constituents present in leaves or rhizomes samples were also less than those in *A. oxyphylla* fruits[12], especially for chrysin. As shown in Fig. 2, a decreasing trend of phytochemical content levels with growth time in leaves was observed, which was generally similar to that of fruits. However, an almost opposite trend was observed in rhizomes (Fig. 3). The content levels of flavonoid were fluctuated varied. Currently, it is not clear how to explain the differences of flavonoid content variations between leaves and rhizomes. It is worth noting that the contents of tectochrysin and apigenin-7,4'-dimethyl ether occurring in leaves, rhizomes and fruits were comparable. This observation indicates that leaves and rhizomes of *A. oxyphylla* may be alternatives to fruits when being used to cure diseases which can be treated by tectochrysin and apigenin-4',7-dimethyl ether. It has been reported that tectochrysin is proposed to be the active constituent responsible for the anti-diarrheal effects of the *A. oxyphylla* fruits[20]. Therefore, further studies to evaluate the pharmacological activities of tecto-chrysin and apigenin-4',7-dimethyl ether would be beneficial to making full use of the leaves and rhizomes resources of *A. oxyphylla*.

Conclusion

In summary, the validated LC-MS/MS approach was successfully employed to analyze the nine phytochemicals present in leaves and rhizomes of *A. oxyphylla*. The results showed that (1) the monitored nine phytochemicals were also measurable in rhizomes or leaves samples as fruits; (2) the content levels of these compounds were comparable between rhizomes and leaves, but both were significantly less than fruits; (3) as the fruits ripening, the content levels of flavonoids were increased with fluctuation in rhizomes; meanwhile, the concentrations measured in leaves decreased; (4) the content changes of nootkatone and diarylheptanoid with growth time were similar between rhizomes and leaves; and (5) contents of tectochrysin and apigenin-7,4'-dimethyl ether occurring in leaves, rhizomes and fruits were comparable. It appears that in general leaves or rhizomes could not be used as alternative medicinal plant parts to fruits of *A. oxyphylla*, based only on the photochemical composition.

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