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ORIGINAL ARTICLE

Components with Lifespan-prolonging Effects in the Fibrous Roots of *Anemarrhena asphodeloides* Characterized by Ultra-High Performance

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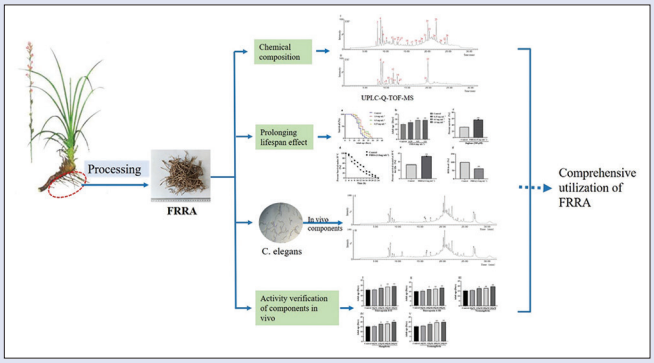
ABSTRACT

Background/Context: The fibrous roots of Rhizoma *Anemarrhena* (FRRA) are produced in the primary processing of Rhizoma *Anemarrhena* (RA). The lifespan-prolonging effect and the bioactive compounds of FRRA were rarely reported. **Objectives:** To study the lifespan-prolonging effects and reveal the potential bioactive components of FRRA. **Materials and Methods:** *Caenorhabditis elegans* were incubated with FRRA extracts (0.25, 0.5, 1.0 mg/mL). The number of live worms was recorded till all worms were dead. The components in FRRA or *in vivo* in *Caenorhabditis elegans* were detected and identified via UHPLC-Q-TOF-MS and Unify Software. The lifespan-prolonging effect of the components detected *in vivo* in *Caenorhabditis elegans* was verified. The contents of the components detected *in vivo* in *Caenorhabditis elegans* in FRRA and RA were determined and compared using UHPLC-MS. **Results:** FRRA extracts (0.5 mg/mL) significantly (**$P < 0.01$) extended the lifespan of the *Caenorhabditis elegans* (mean value from 15.07 to 19.10 days). A total of 28 components were detected and identified in FRRA, and 4 components were first screened in FRRA. A total of 5 components including neomangiferin, mangiferin, isomangiferin, timosaponin B-II and timosaponin A-III were detected *in vivo* in *Caenorhabditis elegans* and the effective concentration for the 5 components were 100, 100, 100, 120 and 120 μM, respectively. The average contents of neomangiferin, mangiferin, isomangiferin, timosaponin B-II and timosaponin A-III in FRRA were 0.80, 1.44, 0.50, 3.62 and 0.72%, respectively. **Conclusion:** FRRA is rich in lifespan-prolonging compounds and could be used as a potential drug resource.

Key words: Bioactive components, *caenorhabditis elegans*, fibrous roots of rhizoma *anemarrhena*, lifespan-prolonging, UHPLC-Q-TOF-MS

SUMMARY

- Rhizoma *Anemarrhena* (RA) is used as a folk medicine to slow ageing. The fibrous roots of RA (FRRA) are produced in the process of origin processing of RA and still contain many active compounds, but few studies have been reported. The anti-ageing effect of FRRA was studied in *Caenorhabditis elegans* (*C. elegans*). Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) was applied to detect and identify the components in FRRA and to screen the components *in vivo* in *C. elegans*. Components of FRRA and those of *C. elegans in vivo* were identified by using Unify Software or according to MS/MS fragments and reference substances. The lifespan-prolonging effects of the five components detected in the worm were verified. The contents of the components detected *in vivo* in *C. elegans* in FRRA and RA were determined and compared using UHPLC-MS. All FRRA concentrations were found to prolong the lifespan of *C. elegans*, and 1.0 mg/mL FRRA extract showed the best effect (**$P < 0.01$). A total of 28 components were identified in FRRA, and 4 components, namely, smilageninoside, 6-methyl-4′,5,7-trihydroxy-homoisflavone, tetrahydroberberine and rosmarinic acid, were first screened in FRRA. Neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III were detected *in vivo* in *C. elegans* and found to exert anti-ageing effects. According to the results from UHPLC-MS analysis, the average contents of mangiferin and timosaponin B-II in FRRA were higher than those in RA. The present study provides a scientific basis for further development and comprehensive utilization of FRRA.



Abbreviations used:
Alzheimer's disease: AD *Caenorhabditis elegans*: *C. elegans Escherichia coli* strain OP50: *E. coli* OP50 The fibrous roots of Rhizoma *Anemarrhenae* FRRA limits of detection: LODs limits of quantification: LOQs Nematode growth medium: NGM Rhizoma *Anemarrhena*: RA Reactive oxygen species: ROS Wild-type *C. elegans*: N_2 2′,7′-dichlorofluorescein diacetate: H_2DCF-DA

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INTRODUCTION

Ageing is a global problem in modern times. Many pathological or physiological changes are associated with ageing such as neuronal injury,[1] a decline in cognitive functions or even Alzheimer's disease (AD).[2,3] It has become important to screen the anti-ageing chemical components in pharmaceutical research, but there are few natural drugs that can prolong the lifespan.

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Anemarrhena asphodeloides Bunge. is a traditional Chinese medicine belonging to the Liliaceae family.[4] Many bioactive compounds including steroidal saponins and flavonoids have been isolated from Rhizoma *Anemarrhena* (RA) and have shown good therapeutic effects in ameliorating cognitive decline and exhibiting antidepressant activity.[5,6] In the clinic, the fibrous roots of Rhizoma *Anemarrhena* (FRRA) must be cut off before further processing. The output of FRRA accounts for more than one-third of RA, and abandoned FRRA can lead to environmental pollution and wasted resources. Previous studies have showed that an extract of FRRA had protective effects on PC12 cells injured by OGD and SH-SY5Y cells damaged by H_2O_2,[7,8] which indicate that FRRA shows neuroprotective effects. However, there have been few reports on the effect of FRRA on prolonging the lifespan.

Caenorhabditis elegans (*C. elegans*) has been a popular model organism in the study of ageing in recent years.[9] It has characteristics including transparent bodies, short lifespans, easy cultivation, and simple nervous systems and has been selected as a model for investigating bioactive components related to human life.[10] Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) has been used as an effective method for detecting chemical components due to its sensitivity, high resolution and accuracy.[11] Compounds in herbal medicine can be detected and identified via UHPLC-Q-TOF-MS.[12]

In the present study, the prolonging lifespan effects of FRRA on *C. elegans* were first studied. In addition, components in FRRA and *in vivo* in *C. elegans* were detected and identified by using UHPLC-Q-TOF-MS and the reference substances. Then the anti-ageing effect of components detected *in vivo* in *C. elegans* was studied. The differences between RA and FRRA in terms of the content of two steroidal saponins and three flavonoids were determined and compared using UHPLC-MS. The present research provides a scientific basis for the comprehensive development and utilization of FRRA.

MATERIALS AND METHODS

Instruments and materials

The equipment includes a Waters Acquity Ultra-High Performance Liquid phase system, Waters Xevo Q-TOF MS system (Waters, USA); BS-300$^+$ Electronic Balance (Shanghai Yousheng Weighing Instrument); and MS detector (Waters, USA).

The reference substances of neomangiferin (No: MUST-17042232), mangiferin (No: MUST-18040123), isomangiferin (No: MUST-18072905), timosaponin B-II (No: MUST-18052605) and timosaponin A-III (No: MUST-19040108) were all purchased from Munster Biotechnology (Chengdu, China). The purity of all reference substances was not lower than 98%. HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher (USA). Formic acid was purchased from Siyou Tianjin (Tianjin, China). Distilled water was acquired by using a Milli-Q Advantage A10 (Millipore company, USA).

C. elegans Strain N_2 (wild type, *C. elegans* Genetic Center, USA), *Escherichia coli* strain OP50 (OP50) was kindly supplied by the University of Science and Technology of China, nematode growth medium (NGM) plates (50 mM NaCl, 2.0% agar, 2.325 mg/mL peptone, 20 mM K_2HPO_4, 80 mM KH_2PO_4, 5 μg/mL cholesterol, 0.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$), and M9 buffer solution (3.0% KH_2PO_4, 6.0% Na_2HPO_4, 5.0% NaCl) were also purchased. All wild-type *C. elegans* were maintained at 16°C on NGM plates and fed with *E. coli* OP50.

Plant materials

Ten batches of RA were collected in January 2019 in Bozhou City (Anhui Province, China) and identified as *Anemarrhena asphodeloides* Bunge. (Liliaceae) by Professor Peng HS (School of Pharmacy, Anhui University of Chinese Medicine). The fibrous roots were segregated from RA after removing the soil and aerial parts with the guidance of the Chinese pharmacopoeia (2020). The voucher specimen (ID: FRRA-190101) were stored in the medicine specimen room, School of Pharmacy, Anhui University of Chinese Medicine (Hefei, China).

Sample preparation

Sample preparation for the assessment of C. elegans lifespan

FRRA powder (10 g) was weighed and extracted under ultrasonication (40 kHz, 600 W) with 100 mL of 50% ethanol twice, each time for 20 min. Then, the filtrate was vaporized with a rotary evaporator to yield the extract. The residue was dried at 50°C to obtain the powder of the extract, with a yield of 26.7% (w/w). The extract powder was precisely weighed and dissolved in distilled water. The FRRA solution concentrations for low, medium and high doses were 0.25, 0.5 and 1.0 mg/mL, respectively.

Sample preparation for UHPLC-ESI-Q-TOF-MS analysis

The dried FRRA powder (60-mesh) was accurately weighed (0.1 g) and extracted with 50% methanol (10 mL) in an ultrasonic bath for 25 min at 30°C (40 kHz, 120 W). The supernatant was filtered through a 0.22 μm membrane prior to injection.

Lifespan assay, stress resistance assay and the measurement of ROS

Lifespan assay

The experiment was carried out with Song's method with some modifications.[13] Wild-type *C. elegans* (N_2) in L4 was transferred to nematode growth medium (NGM) plates with or without FRRA extracts. The FRRA extract concentrations at low, medium and high doses were 0.25, 0.5 and 1.0 mg/mL, respectively. The NGM plate without FRRA extracts was selected as the control group. 5-Fluorouracil (50 mg/mL) was added to all plates, and all nematodes were incubated at 20°C for 48 hr before the experiment. The growing states of nematodes were observed under a microscope at 8:00 a.m. every day, and live nematodes were transferred to another NGM plate with or without FRRA extracts until all of the nematodes were dead. The live nematodes were recorded every day, and the survival curve was depicted. There were 60 nematodes on each plate, and each experiment was repeated three times.

Stress resistance assay

The stress resistance assay was carried out by applying oxidative and temperature stress.[14] Worms in L4 were age-synchronized and transferred to NGM plates with or without the FRRA extracts. For the oxidative stress assay, all worms in each plate were exposed to juglone (200 μM) for 2 hr, transferred to a fresh NGM plate and then incubated for 2 hr at 20°C. Next, worms in the normal state were scored for FRRA-treated and untreated conditions, and the percentage survival was calculated. The temperature stress assay was conducted according to Sonani's methods.[15] FRRA-treated and untreated worms were shifted from 20°C to 35°C. The number of worms was recorded every 2 hr until all worms died.

Measurement of reactive oxygen species (ROS)

Endogenous ROS were detected by using 2',7'-dichlorofluorescein diacetate (H_2DCF-DA).[16] Worms were incubated in NGM plates pre-treated with 1.0 mg/mL FRRA extracts at 20°C for 6 days. Then, the

worms were washed with phosphate-buffered saline (PBS) three times and treated with 10 mM H_2DCF-DA for 3 hr at 35°C. The fluorescence intensity was determined at excitation and emission wavelengths of 485 and 535 nm, respectively. The experiment was conducted three times in parallel.

Pharyngeal pumping and food intake assay

The frequency of pharynx contractions was selected to assay the pharyngeal pumping. Worms were treated with or without 0.5 mg/mL FRRA extracts and cultured in NGM plates without food for two or five days. The pharynx contractions were counted for 30 sec by observation. There were 30 worms in each group and the assay was conducted three times.[17]

Food intake assay was conducted in a liquid medium. A 96-well plate (optically clear) with 120 μL total volume per well contained S-complete, *E. coli* OP50 and 10 FRRA extract-treated worms per well. A total of 6 wells containing S-complete, *E. coli* OP50 and FRRA extract was selected as the control group. Each plate was shaken with a plate shaker for 1 min before detection. The absorbance of each well was determined at 600 nm. The assay was taken on the days 2 and 5, and each assay was conducted three times.[18,19]

UHPLC-Q-TOF-MS conditions

UHPLC conditions

UHPLC analysis was performed using a Waters Acquity TM system. An Agilent SB-C_{18} column (2.1 mm × 100 mm, 1.8 μm) was selected to separate the components of FRRA. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid water (B) with gradient elution (0–5 min, 4%–15% A; 5–8 min, 15%–31% A; 8–19 min, 31%–46% A; 19–25 min, 46%–90% A; 25–30 min, 90%–100% A). The column temperature was 30°C, and the flow rate and injection volume were 0.2 mL/min and 2 μL, respectively.

MS conditions

The sample cone voltage for both ESI + and ESI − was 40 V, and the ion source temperature and the desolvation gas temperature were 120°C and 450°C, respectively. The volume flow rate of the desolvation gas was set at 800 L/h, and the scan range (*m/z*) was 50 ~ 1200 Da. MSE acquisition mode was applied in the MS analysis for 30 min, and the scanning interval was 0.1 sec. Leucine enkephalin was selected as the lock mass with a mass-to-charge ratio (*m/z*) of 554.2615. The data acquisition system was supplied by MassLynx version 4.1 software. The collision low energy was 6 V, and the collision high energy was 10~35 V. The collision gas was high-purity He gas, and the atomization gas was high-purity N_2 gas.

Components detected in vivo in C. elegans

A total of 600 adult nematodes were cultured with 0.5 mg/mL FRRA extracts for five days combined with *E. coli*. All nematodes were fasted for 12 hr, washed from the NGM plate with M9 buffer solution, transferred to a flask, and then centrifuged at 4000 rpm for 3 min. The lower part was collected and washed with 10 mL of 70% methanol (V: V) three times for 10 min each time. All the nematodes were transferred into a flask, suspended in 350 μL of buffer solution and then homogenized by ultrasound for 10 min. Methanol (10 mL) was added to the flask, and the solution was centrifuged at 2000 rpm for 2 min. The supernatant solution was collected and filtered through a 0.22-μm membrane and injected into the UHPLC-Q-TOF-MS system; the injection volume was 2 μL.[20]

Effects on prolonging lifespan of compounds detected in worms

The worms were incubated in NGM plates. as described in the "Lifespan assay" section above, and various concentrations of timosaponin B-II, timosaponin A-III, neomangiferin, mangiferin and isomangiferin were prepared and added to different plates. Worms were scored every day until all worms were dead, and the percentage survival of worms in each plate was calculated. All experiments were carried out in parallel three times.

UHPLC-MS analysis for FRRA and RA

Chromatographic conditions

The equipment, stationary phase, mobile phase gradient and injection volume were the same as in the "UHPLC conditions" section. A mass spectrometer equipped with an electrospray ionization source set at positive (ESI^+) and negative (ESI^-) polarity and multi-reaction ion monitoring (MRM) were used for detection. The optimized precursor/product ion pairs were 583.1 → 493.1 for neomangiferin, 421.1 → 331.0 for mangiferin, 421.1 → 301.0 for isomangiferin, 921.5 → 741.4 for timosaponin B-II, and 741.4 → 579.4 for timosaponin A-III. The ion spray voltages for ESI + and ESI − were 5.5 kV and − 4.5 kV, respectively. Nitrogen was selected as the nebulizer gas and drying gas at a flow rate of 3 mL/min and 15 L/min, respectively; the iron source temperature was 400°C. The scan range for the MS spectra was set between 50 Da and 1200 Da for positive and negative modes.

Preparation of reference substance solution and sample solution

Standard substances including neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III were precisely weighed and dissolved in 50% methanol in the same 100 mL volumetric flask to prepare stock solutions. The concentrations of neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III were 45.2, 39.2, 40.8, 40.1, and 39.6 μg/mL, respectively. Both RA and FRRA powders (0.1 g) were precisely weighed and extracted in an ultrasonic bath (40 kHz, 120 W) with 50% methanol (25 mL) for 25 min. The supernatant was filtered through a 0.22-μm membrane before injection into the UHPLC system.

Method validation

A series of working solutions were diluted with fresh methanol to obtain calibration curves. The calibration curves were calculated by plotting the peak areas and the concentrations of the standard solutions. Y = aX + b was used for the form of the regression equations, where X and Y represent the injected concentration and response signal value, respectively. The limits of detection (LODs) and limits of quantification (LOQs) were measured with signal-to-noise ratios of 3 and 10, respectively. The intraday precision was determined by analyzing six replicate samples within 1 day, and the inter-day precision test was conducted by analyzing the same analyte on consecutive days. The stability test was evaluated by injecting the same sample solution into the UHPLC system six times (n = 6) within 24 hr. FRRA samples from the same batch were divided into six parts on average, and the content of each compound was determined. A total of 0.1 g of FRRA sample (precisely weighted) was spiked with the mixed standard solution and prepared as mentioned in the "Preparation of reference substance solution and sample solution" section. The average recoveries were estimated in the form of recovery (%) = (the amount found − original amount)/(amount spiked × 100%), and RSD = (S.D./mean) × 100%. All RSD percentage values for each test were used to validate the method.

Data analyses

The chemical components in FRRA detected by UHPLC-Q-TOF-MS were identified by Unify software. The survival curve for *C. elegans* was depicted using GraphPad Prism 8.0 (USA). One-way analysis of variance (ANOVA)

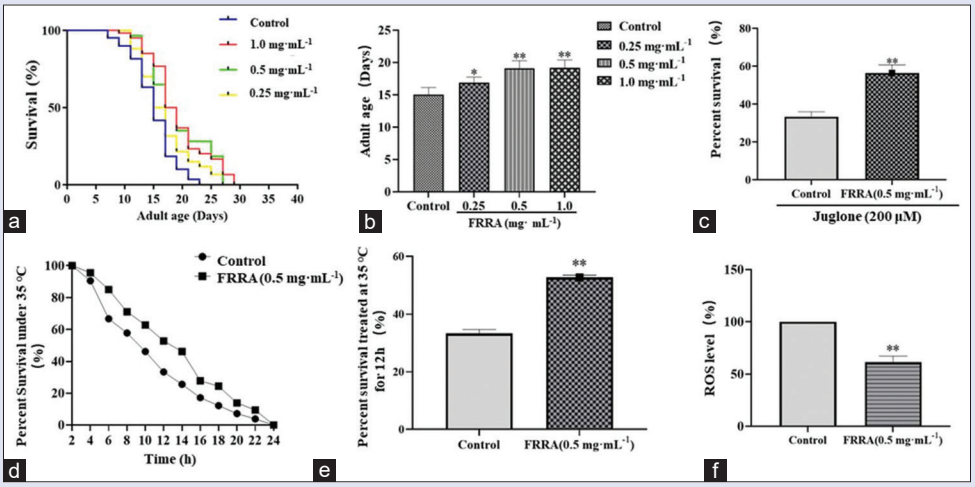

Figure 1: Lifespan-prolonging effects of FRRA on *C. elegans*. a) Effect of different concentrations of FRRA on the percentage survival of nematodes; b) Effect of different concentrations of FRRA on the mean adult age of nematodes; c) FRRA extracts significantly increased the percentage survival of nematodes after being exposed to juglone (200 μM); the average survival was 33.40 ± 2.61 and 56.50 ± 4.30 for the control and FRRA extract groups, respectively; d) FRRA extract treatment enhanced the thermal stress tolerance of worms; e) FRRA extracts significantly increased the percentage survival of nematodes after being treated at 35°C, and the average survival was 33.33 ± 1.36 and 52.78 ± 1.79 for the control and FRRA extract groups, respectively; f) FRRA extracts significantly decreased the *in vivo* ROS level of worms. Comparing with control group, $^{*}P < 0.05$, $^{**}P < 0.01$.

was used as the statistical analysis, and the significance of the difference between groups was determined using LSD tests. Differences were considered statistically significant at $P < 0.05$ or 0.01.

RESULTS

Anti-ageing effects of FRRA on *C. elegans*

FRRA extracts extended the lifespan of C. elegans

To investigate the effect of FRRA extracts on the lifespan of *C. elegans*, three concentrations of FRRA extracts were tested. As shown in Figures 1a and 1b, the lifespan of the nematodes were prolonged by all groups of FRRA extracts, and the mean lifespans of the nematodes for the control group and the 0.25, 0.5, and 1.0 mg/mL FRRA-treated groups were 15.07 ± 0.75, 16.90 ± 1.56, 19.10 ± 1.17, and 19.37 ± 0.95, respectively. The lifespan of *C. elegans* was significantly prolonged by using 0.25, 0.5, and 1.0 mg/mL FRRA extracts, and there were no significant differences between the 0.5 mg/mL group and the 1.0 mg/mL group. Therefore, the 0.5 mg/mL FRRA extract was chosen for further experiments.

FRRA extracts enhance stress tolerance in *C. elegans*

After exposure to juglone (200 μM) for 2 hr, the percentage survival of worms in the FRRA extract-treated groups was 156.5 ± 4.30, which increased significantly compared with that of the control group (33.4 ± 2.60) [Figure 1c]. As shown in Figures 1d and 1e, the percentage survival of worms at 35°C in the 0.5 mg/mL FRRA extract group increased significantly compared with that in the control group. In addition, the FRRA extract treatment decreased ROS accumulation in the worms by approximately 40%, compared with the control group [Figure 1f], which indicated that the prolongation effect of FRRA was associated with eliminating ROS.

Pharyngeal pumping and food intake assay

According to Figure 2a, on the second day of the culture of the worms, there was no significant difference in the pharyngeal pumping rate between the control and FRRA-treated groups. While on the fifth day of the culture of worms, the pharyngeal pumping rate in the FRRA-treated group significantly increased ($P < 0.05$) compared with the control group, which suggested that the feeding behaviors of *C. elegans* could be affected by FRRA extracts. As shown in Figure 2b, the OD value in the FRRA-treated group significantly decreased ($P < 0.05$) compared with the FRRA-untreated group, which suggested that FRRA could prolong the life span of worms by improving their feeding ability.

UHPLC-Q-TOF-MS analysis of FRRA

As shown in Figure 3 and Table 1, a total of 28 compounds were detected in the FRRA extracts via UHPLC-Q-TOF-MS. Twenty-five and 14 compounds were detected in the positive and negative models, respectively. Among these, 14 saponins (including steroidal saponin and steroidal sapogenin), 5 flavonoids, 3 fatty acids, 2 benzophenones, 1 alkaloid, 1 organic acid, and 4 other types of chemical compounds were detected and tentatively identified using the Unify software and the reference substances. Peak 2, 4, 5, 22, and 23 were identified as neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III, respectively.

Identification of FRRA constituents *in vivo* in *C. elegans*

According to Figure 4 and Table 2, the chemical constituents of FRRA *in vivo* in *C. elegans* were detected via UHPLC-Q-TOF-MS in both positive and negative modes. According to the base peak chromatogram, five compounds, namely, neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III, were detected and identified according to the Unify software database, MS/MS fragments and the standard references. Other peaks were not identified due to a lack of standard substances or databases.

Prolonging lifespan effect of compounds detected *in vivo* in *C. elegans*

According to Figure 5a and 5b, both timosaponin B-II and timosaponin A-III significantly extended ($P < 0.05$) the lifespan of worms at an initial

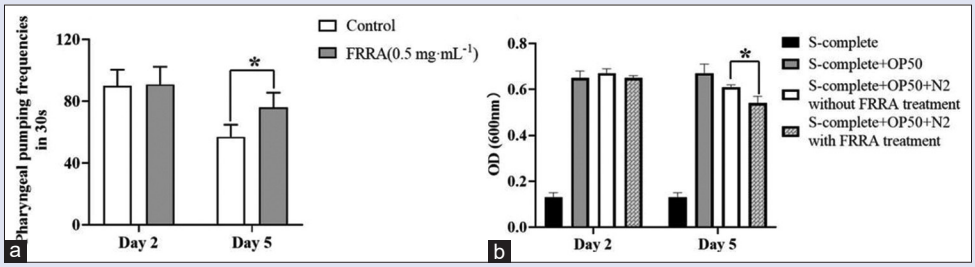

Figure 2: FRRA extracts on feeding behaviors of *C. elegans*. a) Comparing with control group, FRRA extracts significantly increased (*$P < 0.05$) the pharyngeal pumping frequencies of *C. elegans* after being cultured for 5 days; b) The OD value in worms of the FRRA-treated group significantly decreased (*$P < 0.05$) compared with that of the FRRA-untreated group

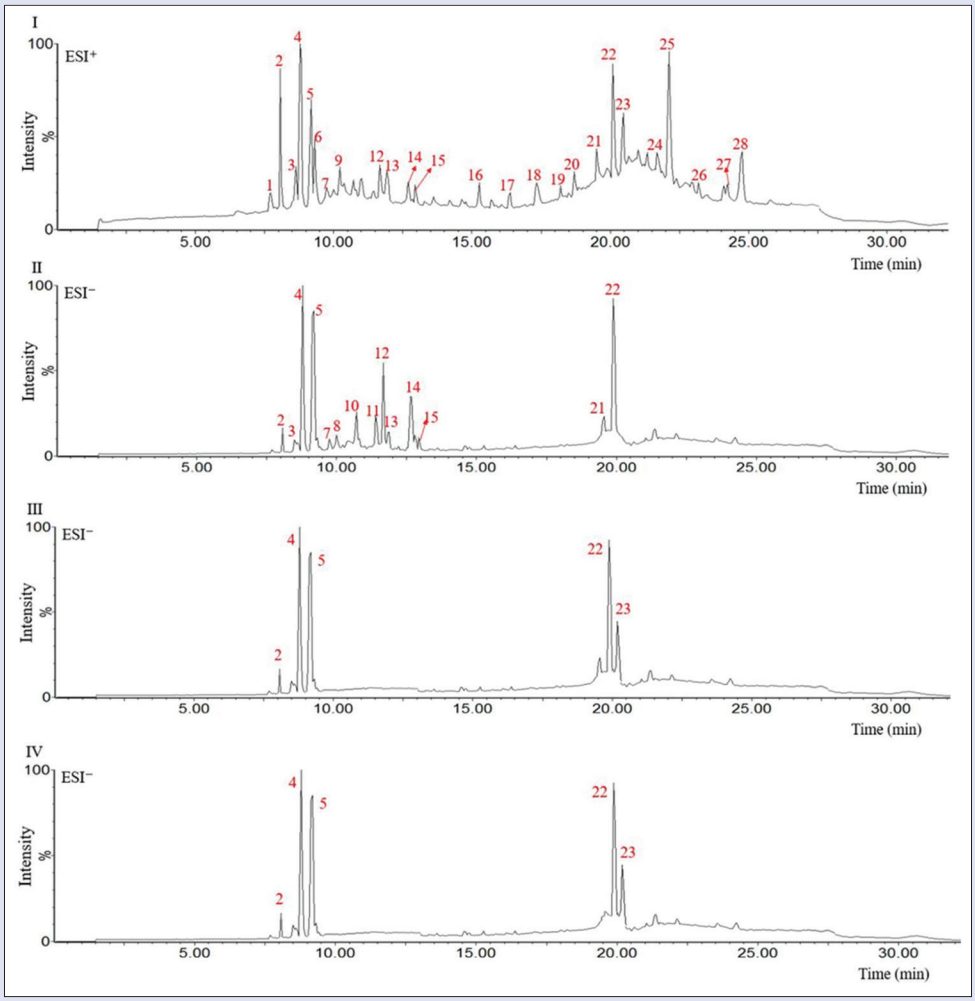

Figure 3: UHPLC-Q-TOF-MS base peak chromatography of FRRA. I: FRRA extracts in negative mode; II: FRRA extracts in positive mode; III: Standard references detected under negative mode, IV: Standard references detected under positive mode

concentration of 100 μM. Neomangiferin, mangiferin and isomangiferin significantly extended the lifespan of worms starting at a concentration of 120 μM [Figure 5c, 5d and 5e]. The results showed that the chemical components in FRRA detected *in vivo* in *C. elegans* could prolong the lifespan of nematodes and that FRRA could be used as an anti-ageing resource.

Determination of five components both in FRRA and RA

Methodological Studies for UHPLC-MS

The UHPLC-MS chromatogram of the FRRA sample and reference substances are shown in Figure 6. The standard curves and linear ranges for the five compounds are listed in Table 3, which shows that

Table 1: Identification and analysis of FRRA compounds via UHPLC-Q-TOF-MS.

$t_{R/min}$	Identification	Selected Ion	Measured mass (*m/z*)	Error (ppm)	Fragments (*m/z*)	Formula	Category
7.76	Timosaponin A-IV	$[M+HCOO]^+$	785.4338	−1.15	739.4319, 593.3927, 313.2933, 183.1397, 146.9656	$C_{39}H_{64}O_{13}$	Steroidal saponin
8.34	Neomangiferin	$[M-H]^-$	583.1334	−0.51	565.1189, 493.0977, 463.0862, 421.0776, 331.0448, 301.0360	$C_{25}H_{28}O_{16}$	Flavonoids
8.46	Catechin-7-O-β-D-Glucopyranoside	$[M-H]^-$	451.3289	−0.60	99.9244	$C_{21}H_{24}O_{11}$	Polyphenols
8.63	Mangiferin	$[M-H]^-$	421.0776	−0.71	331.0448, 301.0360, 259.0233	$C_{19}H_{18}O_{11}$	Flavonoids
8.85	Isomangiferin	$[M+H]^-$	423.0932	−0.72	387.0734, 327.0948, 303.0497, 273.0397	$C_{19}H_{18}O_{11}$	Flavonoids
9.16	Diosgenin	$[M-H]^-$	415.6345	−0.39	273.2190, 198.1809, 119.0864	$C_{27}H_{42}O_3$	Steroidal sapogenin
9.35	Caffeic acid	$[M+H]^+$	181.0494	−0.58	163.0432	$C_9H_8O_4$	Organic acids
9.51	Timosaponin C	$[M+H]^-$	903.4951	−1.79	741.4402, 579.3908, 417.3381, 399.3271, 273.2224	$C_{45}H_{74}O_{18}$	Steroidal saponin
9.87	Timosaponin I	$[M+HCOO]^-$	803.4469	0.50	758.4504, 595.3833	$C_{39}H_{66}O_{14}$	Steroidal saponin
10.26	Timosaponin F (C50)	$[M+HCOO]^-$	1095.5337	−0.91	918.4852, 899.4702, 772.4233, 755.4270, 595.3883, 460.1711, 423.1083, 329.2340, 271.0628, 229.144	$C_{50}H_{82}O_{23}$	Steroidal saponin
10.73	Timosaponin A-I	$[M+H]^+$	579.3908	−0.48	435.2765, 273.2224, 255.2103	$C_{33}H_{54}O_8$	Steroidal saponin
11.13	Timosaponin A-II	$[M+HCOO]^-$	801.4354	−0.25	755.427	$C_{39}H_{64}O_{14}$	Steroidal saponin
11.47	6-methyl-4',5,7-trihydroxy-homoisflavone*	$[M+H]^+$	301.0397	−0.35	176.9630, 149.0222, 121.0304	$C_{17}H_{16}O_5$	Flavonoids
11.53	Timosaponin B-III	$[M+HCOO]^-$	947.494	−0.53	901.4844, 755.421, 623.2400, 593.3677, 432.1814, 329.1040, 174.9556, 146.9656, 129.9749	$C_{45}H_{74}O_{18}$	Steroidal saponin
11.98	Timosaponin III	$[M+HCOO]^-$	801.4354	−1.5	755.427, 575.1749, 529.1702, 367.1185	$C_{39}H_{64}O_{14}$	Steroidal saponin
12.36	Tetrahydroberberine*	$[M+H]^+$	340.2586	−0.37	679.5100, 227.1303, 213.1298, 182.1148, 163.6062	$C_{20}H_{21}O_4N$	Alkaloids
12.67	F-gitonin	$[M+HCOO]^-$	1095.5337	−0.91	1049.5278, 887.4750, 755.4270, 405.0986, 311.0562	$C_{50}H_{82}O_{23}$	Steroidal saponin
12.74	Sarsasapogenin	$[M+H]^+$	417.3	−0.31	273.2021, 285.4135	$C_{27}H_{44}O_3$	Steroidal sapogenin
12.83	Baohuoside I	$[M-H]^-$	513.1838	−0.19	366.1115, 351.088, 323.0916	$C_{27}H_{30}O_{10}$	Flavonoids
12.98	Michehedyoside D	$[M+H]^+$	343.9102	0.03	180.2356, 153.3427	$C_{16}H_{22}O_8$	Phenylpropane
15.34	Smilageninoside*	$[M-H]^-$	739.4319	1.53	593.3927, 313.2933, 183.1397, 146.9656	$C_{39}H_{64}O_{13}$	Steroidal saponin
16.47	Timosaponin B-II	$[M+H]^+$	921.5018	1.45	903.4951, 741.4458, 579.3958, 417.3381, 399.3271, 273.2224	$C_{45}H_{76}O_{19}$	Steroidal saponin
17.34	Timosaponin A-III	$[M+H]^+$	741.4458	−0.25	579.3908, 417.2765, 275.2224, 257.2103	$C_{39}H_{64}O_{13}$	Steroidal saponin
20.13	Monomethyl-Z-(-) hinokiresinol	$[M-H]^-$	265.1488	0.32	183.0107, 146.9633, 96.9582	$C_{18}H1_8O_2$	Benzophenone
20.63	Heneicosanoic acid	$[M-H]^-$	325.1851	0.25	311.1696, 183.0107, 99.9244	$C_{21}H_{42}O_2$	Fatty acids
22.24	Rosmarinic acid*	$[M-H]^-$	359.0774	0.26	197.2367, 177.3453, 161.6778	$C_{18}H_{16}O_8$	fatty acids
24.35	7-hydroxy-3-(4-hydroxybenzyl) chroman	$[M-H]^-$	255.2332	0.30	248.9720, 125.9288	$C_{16}H_{16}O_3$	Benzophenone
24.96	(Z) - 6-octadecanoic acid	$[M-H]^-$	296.2715	−0.09	277.2159, 195.1387, 146.9656, 99.9261	$C_{19}H_{36}O_2$	Fatty acids

*: 4 components were first screened in FRRA

there is a good linear relationship among the components in their respective ranges. In addition, the RSD percentage values for the precision test, stability test and repeatability test were all less than 3%, and the values of all recovery tests were between 95% and 105%. All the results showed that the method developed in this study is feasible, stable and accurate.

Comparison of Contents between FRRA and RA

The contents of the compounds in FRRA and RA were determined and compared by UHPLC-MS. According to Table 4, the content of timosaponin A-III in FRRA was close to that of timosaponin A-III in RA. Moreover, the average content of mangiferin and timosaponin B-II in FRRA was higher than that in RA, which was 1.44% for mangiferin and 3.62% for timosaponin B-II.

Table 2: Detection and Identification FRRA compounds *in vivo* of *C. elegans* by UHPLC-ESI-Q-TOF-MS

$t_{R/min}$	Identification	Selected Ion	Measured mass (*m/z*)	Error (ppm)	Fragments (*m/z*)	Formula	Category
8.36	Neomangiferin	$[M-H]^-$	583.1334	−0.51	565.1189, 493.0977, 463.0862, 421.0776, 331.0448, 301.0360	$C_{25}H_{28}O_{16}$	Flavonoid
8.68	Mangiferin	$[M-H]^-$	421.0776	−0.71	331.0448, 301.0360, 259.0233	$C_{19}H_{18}O_{11}$	Flavonoid
8.91	Isomangiferin	$[M-H]^-$	421.0776	−0.72	331.0448, 301.0360	$C_{19}H_{18}O_{11}$	Flavonoid
16.50	Timosaponin B-II	$[M+H]^+$	921.5018	1.44	903.4951, 741.4458, 579.3958, 417.3381, 399.3271, 273.2224	$C_{45}H_{76}O_{19}$	Steroidal saponin
17.53	Timosaponin A-III	$[M+H]^+$	741.4458	−0.25	579.3908,435.2765, 417.2765,399.3271, 273.2224, 255.2103	$C_{39}H_{64}O_{13}$	Steroidal saponin

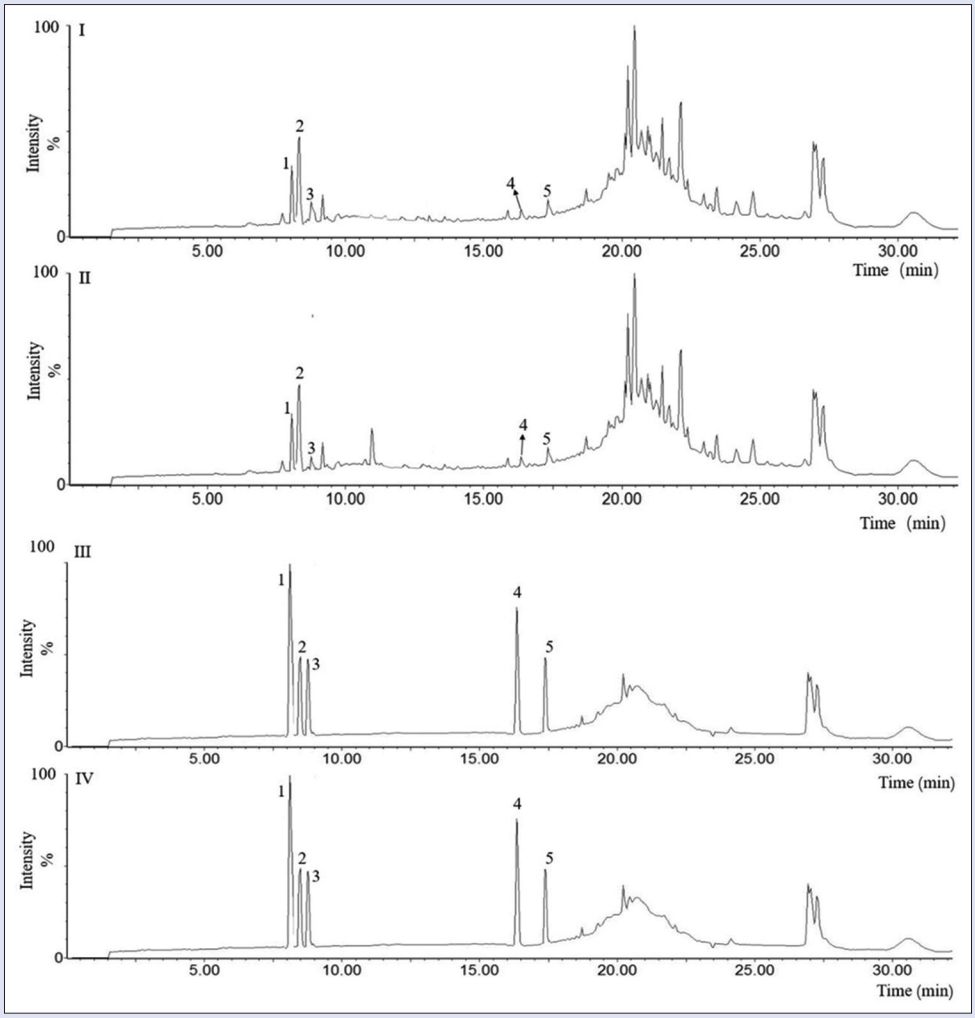

Figure 4: UHPLC-Q-TOF-MS base peak chromatography of components *in vivo* of *C. elegans*. I: Components detected *in vivo* of nematodes under negative mode; II: Components detected *in vivo* of nematodes under positive mode, III: Standard references detected under negative mode, IV: Standard references detected under positive mode

DISCUSSION

Survival under environmental stress is considered to be related to life expectancy.[21] Tolerance to oxidative stress and heat declines gradually and weaken during the process of ageing.[22] What's more, the level of endogenic ROS increases with the accumulation of age-related oxidative damage.[23] Besides, the pumping rate and predatory ability of *C. elegans* can be used as a predictor of lifespan and food intake ability.[24,25] The results of the present study suggested that the FRRA extracts prolonged the lifespan of *C. elegans* by reducing the endogenic ROS level and improving their feeding ability.

According to the UHPLC-Q-TOF-MS analysis, saponins and flavonoids accounted for 67.8% of the detected compounds in FRRA, which indicated that saponins and flavonoids were the main components in FRRA. Compounds including saponins and flavonoids have hypoglycemic and extending lifespan effects,[26,27] which indicates that FRRA can also be used as a potential drug resource. Moreover, smilageninoside, 6-methyl-4',5,7-trihydroxy-homoisflavone, tetrahydroberberine and

Table 3: The results of method validation

Compound	Curves	r	LOD ($\mu g \cdot L^{-1}$)	LOQ ($\mu g \cdot L^{-1}$)	Precision (RSD%)		Stability	Repeatability	Recovery	
					Intra-day	Inter-day	(RSD%)	(RSD%)	Mean (%)	RSD (%)
Neomangiferin	Y=801138X+571265	0.999 2	2.62	5.43	0.45	1.54	1.27	1.07	99.67	2.01
Mangiferin	Y=16763X+17240	0.999 6	1.08	6.54	1.64	0.87	2.01	1.21	101.35	1.65
Isomangiferin	Y=9000314X+4051070	0.999 7	2.36	6.43	1.07	1.25	1.87	1.66	102.34	1.73
Timosaponin B-II	Y=125230X+78757	0.999 5	1.35	5.43	2.44	2.35	1.23	1.59	96.76	1.09
Timosaponin A-III	Y=12565X-4574.8	0.999 3	2.25	7.35	0.56	1.32	1.23	0.67	98.88	2.47

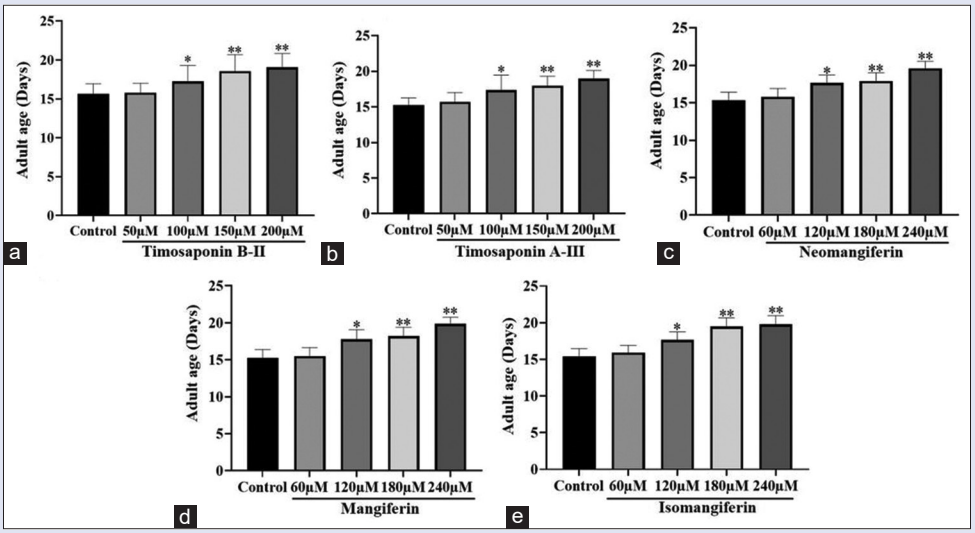

Figure 5: Lifespan-prolonging effects of compounds detected *in vivo* in *C. elegans*. a) Lifespan-prolonging effect of timosaponin B-II, b) Lifespan-prolonging effect of timosaponin A-III, c) Lifespan--prolonging effect of neomangiferin, d) Lifespan-prolonging effect of mangiferin, e) Lifespan-prolonging effect of isomangiferin. Comparing with control group *$P < 0.05$, **$P < 0.01$

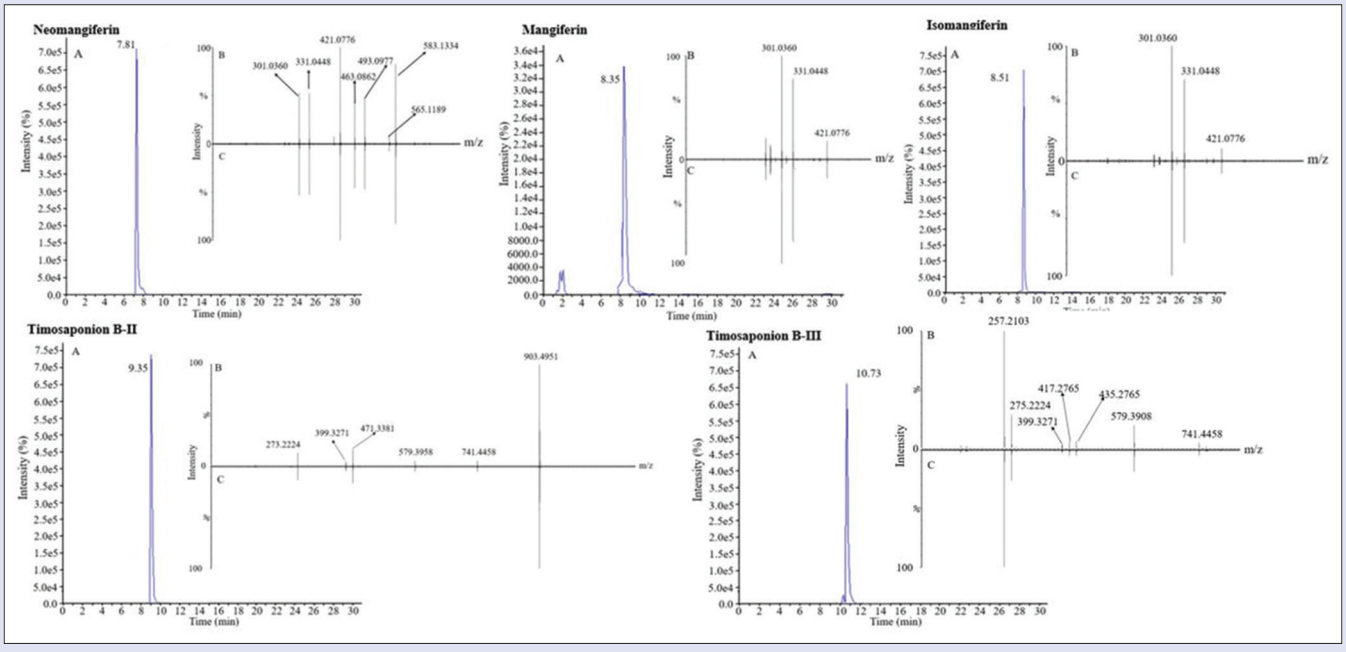

Figure 6: The MS/MS spectro grams of 5 compounds detected *in vivo* of *C. elegans*. a) LC-MS chromatogram; b) MS/MS spectrometry; c) MS/MS spectrometry of reference substance

rosmarinic acid were first detected in FRRA, but the effect of these compounds requires further study.

Neomangiferin presented the formula of $C_{25}H_{28}O_{16}$, and the retention time was 8.36 min [Figure 5, Table 2]. The fragment

Table 4: Comparison of the contents of 5 compounds in FRRA and RA

FRRA					RA				
Neomangiferin	Mangiferin	Isomangiferin	Timosaponin B-II	Timosaponin A-III	Neomangiferin	Mangiferin	Isomangiferin	Timosaponin B-II	Timosaponin A-III
0.87	1.32	0.54	3.51	0.76	0.58	1.26	0.74	3.46	0.73
0.75	1.34	0.44	3.56	0.64	0.54	1.19	0.75	3.26	0.71
0.82	1.42	0.51	3.65	0.61	0.56	1.28	0.74	3.52	0.77
0.91	1.54	0.43	3.54	0.71	0.61	1.31	0.81	3.19	0.82
0.89	1.36	0.41	3.78	0.73	0.65	1.27	0.72	2.96	0.65
0.76	1.47	0.48	3.12	0.81	0.54	1.16	0.71	3.11	0.86
0.85	1.38	0.57	3.16	0.77	0.55	1.25	0.66	2.87	0.79
0.65	1.43	0.54	3.87	0.69	0.64	1.30	0.68	3.42	0.68
0.78	1.55	0.52	4.02	0.78	0.58	1.27	0.72	3.17	0.94
0.73	1.61	0.54	3.95	0.65	0.65	1.25	0.63	3.37	0.72
0.80	1.44	0.50	3.62	0.72	0.59	1.25	0.72	3.23	0.76

at *m/z* 583.1334 ([M-H]$^-$) was the molecular ion detected in negative mode (ESI$^-$). Fragment ions of *m/z* 565.1189 ([M-H_2O]$^-$), 493.0977 ([M-$C_3H_6O_3$]$^-$), 463.0862 ([M-$C_4H_8O_4$]$^-$), and 421.0776 ([M-Glc]$^-$) were detected. Commonly, neutral fragments at *m/z* 18, 90 and 120 are easily lost in negative mode[28] and O-glycoside is less stable than C-glycoside. The fragment at *m/z* 421.0776 was produced by the loss of glucose (163 Da), and mangiferin was temporarily identified.

Isomangiferin is the isomeric compound of mangiferin, and they exhibit the same molecular formula and ion fragments of the same molecular weight.[29] It was difficult to identify the two compounds according to the ion fragments. However, isomangiferin and mangiferin were identified according to the retention times of the reference substance, which were 8.68 and 8.91 min, respectively. Timosaponin B-II and timosaponin A-III are steroidal saponins.[30,31] The sugar chains are easily lost. The fragments at *m/z* 903.4951, 741.4458, 579.3958, and 417.2765 occurred in the cleavage of timosaponin B-II, and fragments at *m/z* 579.3908 and 417.2765 were observed in the MS spectrum of timosaponin A-III. Moreover, the loss of $C_8H_{16}O_2$ (144 Da) was due to the cleavage of E rings.

Drugs entering the body are responsible for their therapeutic effects. Neomangiferin, mangiferin, isomangiferin, timosaponin B-II and timosaponin A-III were detected *in vivo* in *C. elegans*, which indicated that saponin and flavonoids were the main active compounds in FRRA and prolonged the lifespan of the worms. To validate the effects of these compounds, a series of concentrations of all compounds were prepared and added into NGM plates with nematodes. The results showed that the lifespan of nematodes were prolonged by the compounds detected in the worms.

It can be inferred that saponins and flavonoids in FRRA have some merits such as neuron protection and anti-ageing activity. Timosaponin B-II can alleviate oxidative stress damage in PC12 cells caused by H_2O_2.[32] Skin ageing can be suppressed by timosaponin A-III,[33] and memory deficits in mice caused by cerebral ischemia reperfusion can be improved by mangiferin.[34] If FRRA can be collected and extracted reasonably, it can have a positive impact on the comprehensive utilization of traditional Chinese medicine resources.

CONCLUSION

In the present study, the lifespan-prolonging effect of FRRA on *C. elegans* was studied, and the FRRA components were profiled via UHPLC-Q-TOF-MS. A total of 28 compounds including 13 steroidal saponins and 5 flavonoids were detected in FRRA. Four compounds were first screened in FRRA, which need to be separated and identified in future research. Five compounds, namely, neomangiferin, mangiferin, isomangiferin, timosaponin B-II, and timosaponin A-III, were detected *in vivo* in *C. elegans*, and the effect of these compounds on prolonging the lifespan was validated. The present study suggests that FRRA can be further utilized and used as a potential medicinal plant resource.

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Authorship contribution statement

Jianhong Li and Rumin Zong: Data curation, Writing an original draft. Zhiyuan Shao, Yu Meng and Qianqian He : Methodology,

Investigation. Qi Huang and Deling Wu: Project administration, Funding acquisition.

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Conflicts of interest

There are no conflicts of interest.

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