

Identification of an Anti-Plant-Virus Molecule in *Alpinia Zerumbet*

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Abstract

In plants, viral diseases are second only to fungal diseases in terms of occurrence, causing substantial damage to agricultural crops. The aqueous extract of *Alpinia zerumbet* exhibits inhibitory effect against virus infection in Solanaceae members. In this study, we isolated an anti-plant-virus molecule from the extract using a conventional method of a combination of reversed phase column chromatography, dialysis, and lyophilization. The anti-plant-virus molecule was identified as proanthocyanidin, which mostly consisted of epicatechin and showed more than 40 degrees of polymerization.

Introduction

Recent advances in biotechnology and chemistry of natural products have enabled to make substantial progress in bio-pesticide development. Bio-pesticides, which are environmentally safe owing to their low residue, have garnered attention. The production of bio-pesticides is currently growing at a rate of 16% per year, approximately three-times higher than that of conventional agro-chemicals, at 5.5% per year (Marrone 2013).

In plants, viral diseases are second only to fungal diseases in terms of occurrence, causing substantial damage to agricultural crops. Globally, plant viruses cause economic losses of approximately \$60 billion annually, and the loss of food crops alone account for \$20 billion (Xie et al. 2009).

More than 500 compounds have been isolated from 35 *Alpinia* species, and the major compounds are terpenoids and diaryheptanoids. The crude extracts and identified compounds exhibit a broad spectrum of bioactivities including anti-emetic, anti-ulcer, anti-bacterial, anti-inflammatory, anti-amnesic, and anti-cancer activities (Ma et al. 2017). *Alpinia zerumbet* (Pers.) B.L. Burt & R.M. Smith, a member of the family Zingiberaceae (Teschke and Xuan 2018), grows widely in subtropical and tropical regions in East Asia, including the Okinawa Islands in Japan. It is commonly used in traditional Okinawa cuisine and as a herbal medicine. Bioactive compounds have been isolated and identified from crude *A. zerumbet* extracts (Tawata et al. 2008). A recent experimental study demonstrated that *A. zerumbet* substantially increased the lifespan of animals (Upadhyay et al. 2013).

In our previous study, we found that an aqueous extract of *A. zerumbet* exerted inhibitory effect against virus infection in Solanaceae members (Narusaka et al. 2020). In this study, we isolated and identified an anti-plant-virus molecule, proanthocyanidin (PAC), in *A. zerumbet*.

Methods

Materials

Procyanidins A2 and B1 were purchased from Funakoshi (Japan). 4-Dimethylaminocinnamaldehyde (DMAC) was obtained from Sigma-Aldrich (Japan).

Plant Material

We used *A. zerumbet* plants, known as “*Gettou*” or “*Shima-gettou*,” collected in Nakijin Village, Okinawa Prefecture, Japan, to identify an anti-plant-viral molecule.

Purification of the anti-plant-viral molecule

An aqueous extract of *A. zerumbet* was obtained by squeezing its leaves and stems using a sugar cane squeezer (YBK-2; Yabiku, Japan). The extract was centrifuged ($3,260 \times g$) for 10 min, and the supernatant was filtered to remove debris. One hundred milliliters of the filtrate was applied to C18 Sep-Pak® (10g (35cc); Nihon-Waters, Japan), which was equilibrated with distilled water before use. Thereafter, the column was washed with 100 mL of distilled water and eluted with 100 mL of 5%, 10%, 20%, and 40% acetonitrile. The fraction rich in anti-plant-viral molecules was obtained by elution with 100 mL of 20% acetonitrile. The fraction was evaporated to remove acetonitrile under reduced pressure at 50°C, and the resultant solution was dialyzed using a dialysis membrane (cutoff 10 kDa) against distilled water (4 L $\times$ 4 times), and the dialysate was lyophilized. The resultant powder was used to characterize the purified anti-plant-viral molecule. The anti-plant-viral molecule was characterized by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, ^{13}C nuclear magnetic resonance (NMR) analysis, gel permeation chromatography (GPC), and spectrophotometric assay using DMAC.

Anti-viral assay

The anti-viral assay was performed following a method described in our study (Narusaka et al. 2020). To evaluate the inhibitory effect of purified sample, the sample solution was applied on *Nicotiana benthamiana* plants (in the third true leaf stage) as foliar sprays; 3 days post treatment, the plants were inoculated with a plant virus. Inoculation of tomato mosaic virus (ToMV) was performed as described with some modifications using pTL-derived plasmids (pTLBN.G3), which contain a full-length ToMV cDNA and a gene encoding green fluorescent protein (GFP) (Kubota et al. 2003). Briefly, linearized pTLBN.G3 was used as a template for in vitro transcription by AmpliCap-Max T7 High Yield Message Maker Kit (CELLSCRIPT, USA). The transcription mixture was diluted and mechanically inoculated to the third true leaves of *N. benthamiana* that had been treated with the sample solution or distilled water as the control. GFP foci were used to detect virus infection, and they were observed under blue light irradiation at 3 days post inoculation (dpi). The anti-viral activity was assessed based on the number of GFP foci formed on the control and the treated *N. benthamiana* leaves.

MALDI-TOF-MS analysis

The purified sample (1 mg) was dissolved in 1 mL of distilled water. 2,5-Dihydroxy benzoic acid was used as a matrix in the positive-ion mode. To enhance ion formation, sodium chloride was added to the matrix. A MALDI-TOF-MS spectrum was acquired using a Shimadzu MALDI-8020 MALDI-TOF-MS apparatus.

DMAC assay

This method has been described previously (Wang et al. 2016). Methanol-based DMAC reagent was prepared by adding 50 mg of DMAC to 17.5 mL of HCl (37%), and then making up the volume to 50 mL with methanol. The purified sample stock solution (2.5 mg/mL) was diluted with methanol to 0.25 mg/mL. A mixture of 0.125 mL of sample solution with 0.875 mL of DMAC reagent were prepared in cuvettes, with a 1-cm pass length. The spectrum (400–700 nm) of the DMAC conjugate was measured using an SH-8000 spectrometer (CORONA electric, Japan).

GPC analysis

The GPC analysis was performed by Torey Research Center (Japan). The purified sample (1 mg) was dissolved in 1 mL of dimethyl formamide (DMF) containing 50 mM lithium chloride, and 200 μ L of the sample was subjected to GPC. The molecular mass was estimated using a high-performance liquid chromatograph equipped with a refractive index detector (RI-8020; TOSOH, Japan), and TSK gel α -4000 and TSK gel α -2500 columns (7.8 mm $\times$ 30 cm; TOSOH), which were connected directly. DMF containing 50 mM lithium chloride was used as the solvent at a flow rate of 0.7 mL/min and the column temperature was maintained at 23°C. The eluted sample was used for molecular mass determination, with a calibration curve obtained using standard polystyrene kits (PSt Quick E and F; TOSOH).

¹³C NMR analysis

We used a Varian VNMRs (600MHz) spectrometer (Varian, USA) for the analysis. The purified sample was dissolved in acetone-d₆/D₂O = 1/1.

Results And Discussion

Purification of an anti-viral molecule in *A. zerumbet*

When the aqueous extract of *A. zerumbet* was fractioned by a reverse phase chromatography and anti-viral assay was performed as shown in Fig. 1, the anti-viral molecule was concentrated in the fraction by elution with 20% acetonitrile. The fraction was then evaporated, dialyzed, and lyophilized. The resultant sample was used as the purified sample. The anti-viral activity of the purified sample was confirmed by the inhibition against growth of GFP-tagged ToMV (Fig. 2). The protect value (%) of the purified sample was increased as the final concentration of the sample treated to the leaves increased. Thus, we successfully purified an anti-plant-virus molecule from the aqueous extract of *A. zerumbet* using a conventional method. To estimate material balance in each fraction in the purification process, aliquots of each fraction were lyophilized (Table 1). The yield of the final fraction of the water extract was approximately 12% (w/v). This fraction was rich in the active molecule, accounting for ca. 10% (w/v) of the water extract of *A. zerumbet*.

Table 1
Material balance of each fraction in the purification process

Fraction	Lyophilized material (mg)	Yield (%)
Water extracts (100 mL)	4,321	100.0
Pass & wash	3,332	77.1
20% acetonitril eluate	989	22.9
Dialysate	504	11.7

MALDI-TOF-MS analysis

As presented in Fig. 3, a series of peaks with ions corresponding to sodium adducts $[M + Na]^+$ distributed from m/z 889.5 to 6089.6 were observed in the mass spectrum of the purified sample by MALDI-TOF-MS. The distance between the adjacent ion peaks was 288 Da ($-C_{15}H_{12}O_6^-$), corresponding to one catechin or epicatechin monomer. The results indicate that the active molecule is a polymer consisted of catechin or epicatechin. The mass intensity of the sample decreased with increasing degrees of polymerization (DP). The lower sensitivity of the large ions may be reasonable due to collisional fragmentations during the time of flight or when the ions are extracted from the matrix (Flamini 2003; Perret et al. 2003). MALDI-TOF-MS analysis enables the determination of the absolute molecular weight of individual chains with high accuracy, as long as the polymer is not too large (approximately < 10 kDa) (Koster et al. 2000). If intact molecular adducts are produced in the gas phase and the adducts are clearly identified, the analysis allows to confirm the nature of end-groups based on the sum of mass of adducted monomer units and both end groups (Li et al. 2010; Charles 2014). From the data of the series of peaks of sodium adducts, we estimated the end structure of PAC. As shown in Table 2, both end structures were protons because the average molecular mass of both end groups was 3.6 (ca. 2.0). Thus, the anti-viral molecule in *A. zerumbet* is a PAC polymer, consisted of a simple condensation of catechin or epicatechin monomer.

Table 2
Estimation of end structures of PAC from *A. zerumbet*

Observed molecular mass (A)	Cation (Na ⁺) molecular mass (B)	Exact molecular mass (A) – (B)	Unit molecular mass (D)	Degree of polymerization (C)÷(D) = (E)	Estimated end molecular masses (C) – ((D)×(E))
889.50	22.99	866.51	288	3	2.5
1177.78	22.99	1154.79	288	4	2.8
1466.26	22.99	1443.27	288	5	3.3
1754.55	22.99	1731.56	288	6	3.6
2043.43	22.99	2020.44	288	7	4.4
2332.06	22.99	2309.07	288	8	5.1
					Average
					3.6

DMAC assay

The polymeric nature of PACs results in structural variations, including DP, linkage type, and position between constituent units. Catechin and epicatechin are the two most common flavan-3-ol units present in PAC polymers. The most common linkage of the flavan-3-ol units is a single C-C bond (B-type), between the C4 of one flavan-3-ol unit (referred to as upper) and C8 or C6 of another (lower) unit. Wang et al. reported that in the absorption spectra, B-type dimers including procyanidin B1 showed a secondary 440-nm absorption peak, besides the primary 640-nm peak, which is typically measured using the DMAC assay (Wang et al. 2006). The A-type dimers, with double interflavan linkage, showed only a 640-nm absorbance peak in the assay (Fig. 4 (A)). PAC in *A. zerumbet*-DMAC conjugate also showed a secondary 440 nm peak, as shown in Fig. 4 (B). Wang et al. have described that all B-type cocoa PACs exhibited a secondary 440 nm absorbance peak that declined in intensity when DP increased (Wang et al. 2006). Based on the results, PAC from *A. zerumbet* was categorized as a B-type.

GPC analysis

Figure 5 shows the molecular mass of PAC from *A. zerumbet*. The weight average molecular weight (Mw), number average molecular weight (Mn), polydispersity (Mw/Mn), and DP of the PAC were 12,400, 5,640, 2.2, and 43, respectively.

¹³C NMR analysis

The spectrum of PAC from *A. zerumbet* is shown in Fig. 5. Resonance assignments were observed according to a previous study (Eberhardt and Young 1994). All assignments were consistent with those

reported in the previous study. With respect to the aromatic B-ring attached at C-2, the orientation of the hydroxyl group at C-3 can be either cis or trans as shown in Fig. 6. The relative amounts of the two stereochemical configurations (2,3-cis or trans) in PAC from *A. zerumbet* were estimated from ^{13}C NMR spectra by integrating the resonances at 76.0 and 82.5 ppm corresponding to C-2 in 2,3-cis and 2,3-trans chain extender units, respectively. The results revealed that PAC in *A. zerumbet* consists of epicatechin (i.e., 2,3-cis configuration) because there was a low-intensity signal at 82.5 ppm, which was assigned a 2,3-trans configuration (Eberhardt and Young 1994). To the best of our knowledge, for the first time, we report the identification of an anti-plant-virus molecule, PAC, in *A. zerumbet*. Further studies are necessary to determine the mechanism of anti-plant-virus activity of PAC.

Conclusions

In this study, we identified the anti-plant-virus molecule in *A. zerumbet* as PAC. PAC, as the active molecule, was observed at a high level in the 10% (w/v) water extract. In addition, PAC was categorized as B-type, with more than 40 DP, and consisted of epicatechin. Thus, *A. zerumbet* is a potential bioresource of pesticides against viral diseases in plants.

Abbreviations

DMAC

4-dimethylaminocinnamaldehyde; PAC:proanthocyanidin; MALDI-TOF:matrix-assisted laser desorption/ionization time of flight; MS:mass; nuclear magnetic resonance:NMR; GPC:gel permeation chromatography; ToMV:tomato mosaic virus; GFP:green fluorescent protein; DMF:dimethyl formamide; DP:degree of polymerization; Mw:weight-average molecular weight; Mn:number average molecular weight

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

MU and TH purified the anti-plant-virus molecule from *A. zerumbet*. YY, YN, and MN conducted the anti-viral assay. TH identified the anti-plant-virus molecule and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

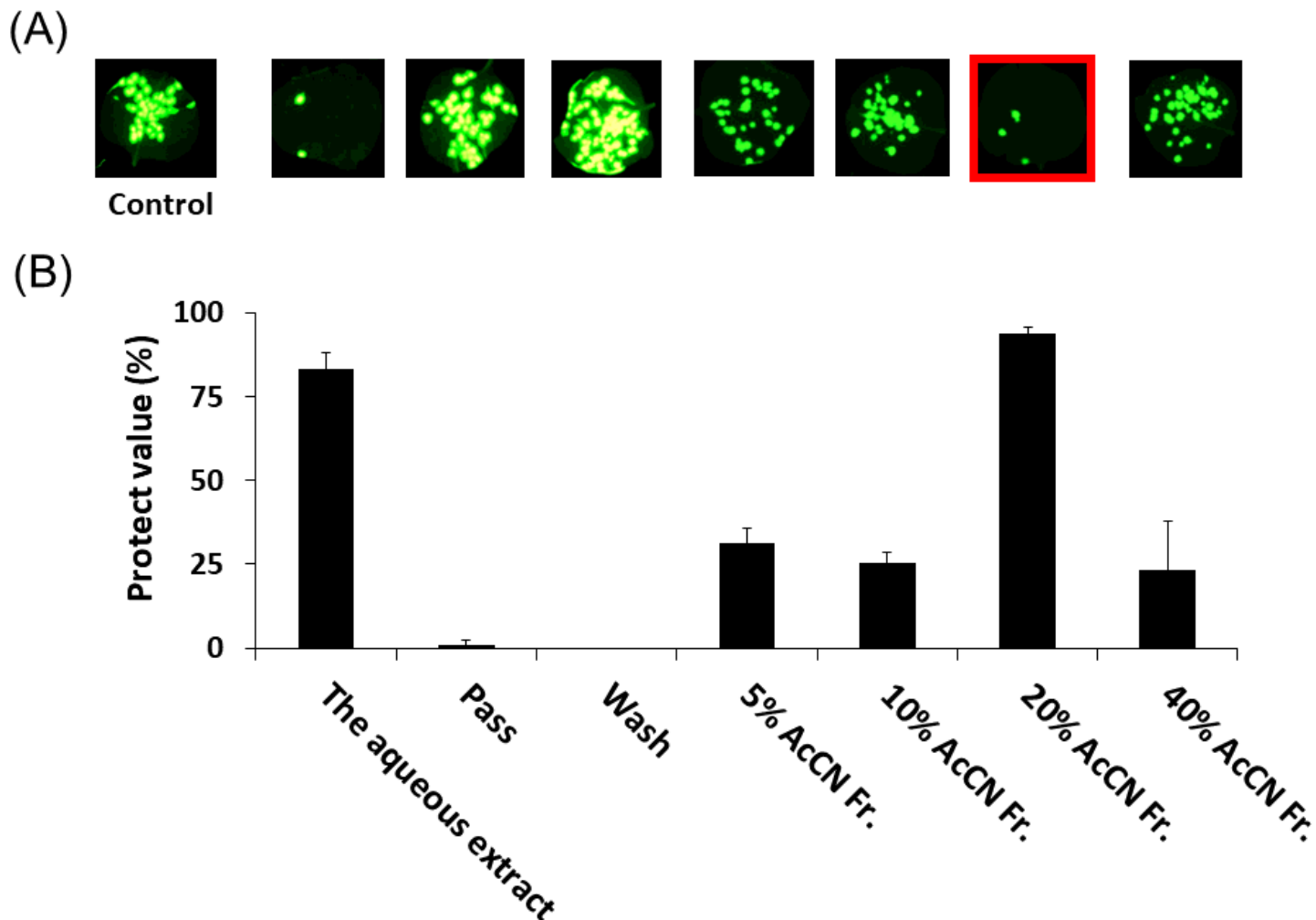


Fig.1

Figure 1

Anti-viral activities of fractions of *A. zerumbet* extract separated by reverse phase chromatography using C18 Sep-Pak®. GFP-tagged ToMV was inoculated to *N. benthamiana* leaves treated with indicated sample or fractions separated by C18 Sep-Pak® from *A. zerumbet* extract. (a) GFP foci formed at 3dpi. Images were taken under blue-light irradiation using the ChemiDoc™ MP Imaging System (Bio-rad, USA). (b) The GFP spots formed on the inoculated leaves were counted at 3 dpi. Protective value (%) = (1 - number of GFP spots on the treated plants / number of GFP spots on the control plants) × 100.

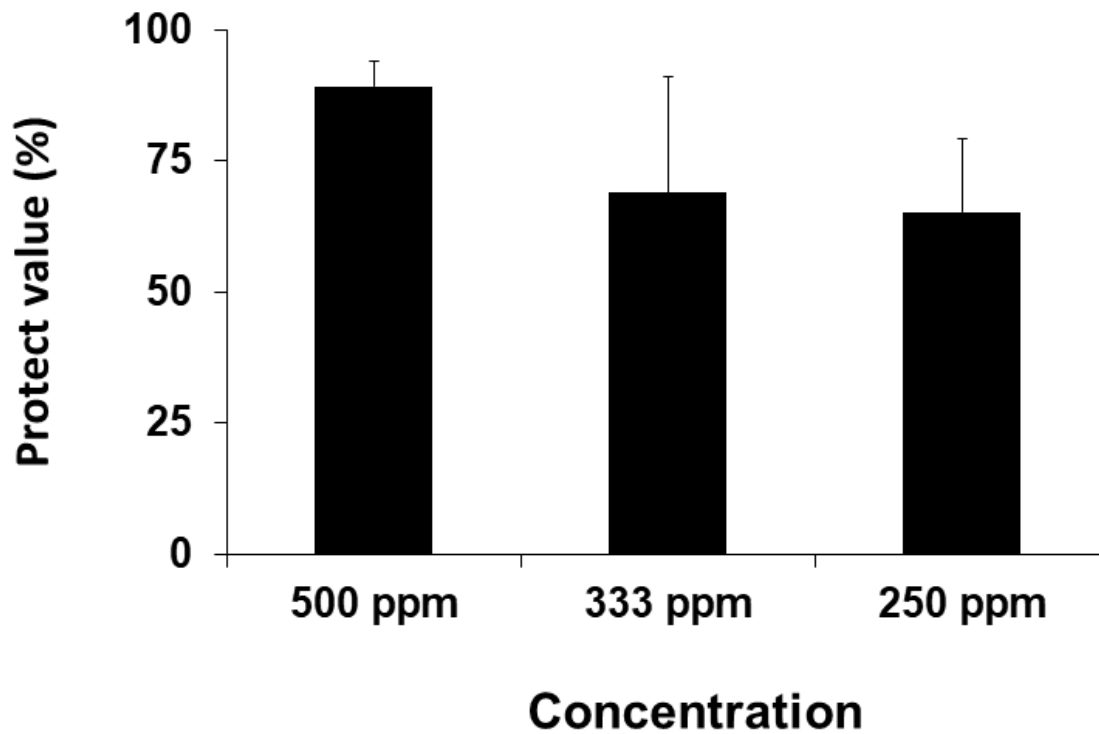


Fig.2

Figure 2

Antiviral activities of the purified sample of *A. zerumbet* against GFP-tagged ToMV virions. *Nicotiana benthamiana* plants were treated with water (control) and different concentrations of the purified samples from the aqueous extract of *A. zerumbet*, and then inoculated with GFP-tagged ToMV at 3 days post treatment. The GFP spots formed on the inoculated leaves were counted at 3 dpi. Protective value (%) = $(1 - \text{number of GFP spots on the treated plants} / \text{number of GFP spots on the control plants}) \times 100$.

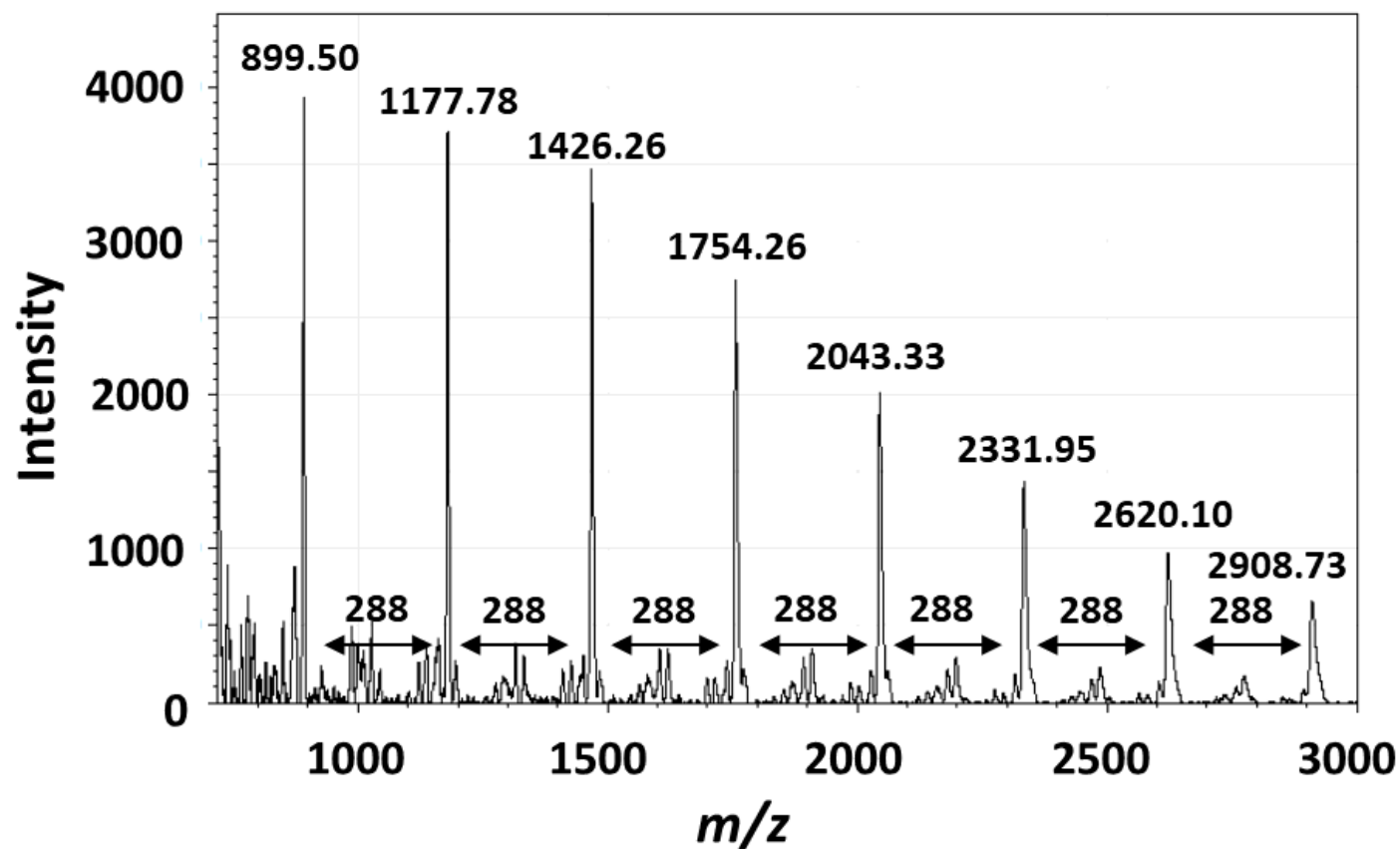


Fig.3

Figure 3

MALDI-TOF MS spectrum of the purified sample from the aqueous extract of *A. zerumbet*.

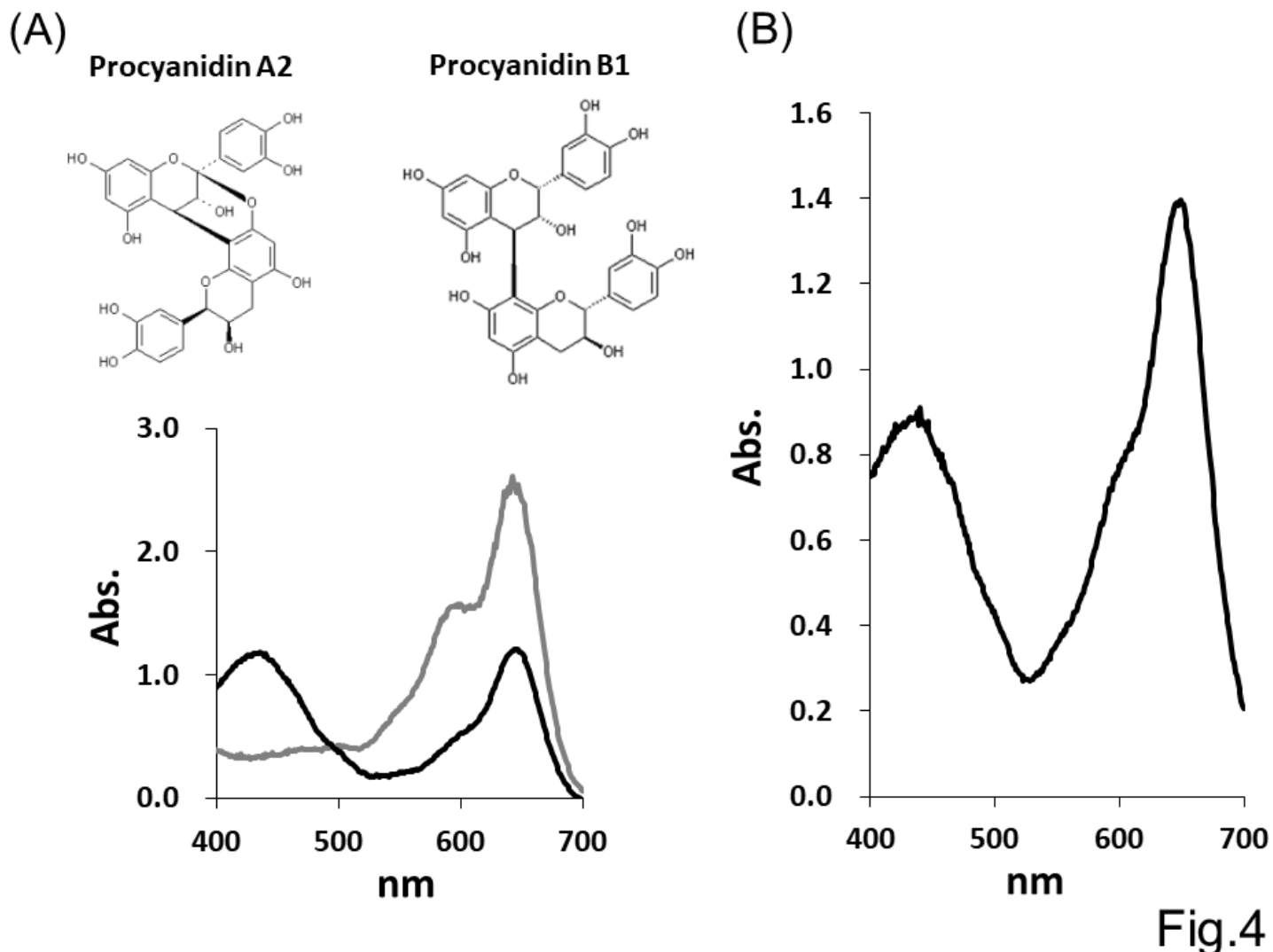


Figure 4

Visible spectra of procyanidins and the purified sample from A. zerumbet aqueous extract. (a) Chemical structure of procyanidins A2 and B1 (upper), the spectra of procyanidin A2 (gray line) and procyanidin B1 (black line), determined using the DMAC assay (lower). (b) The spectrum of the purified sample from the aqueous extract of A. zerumbet, obtained using the assay.

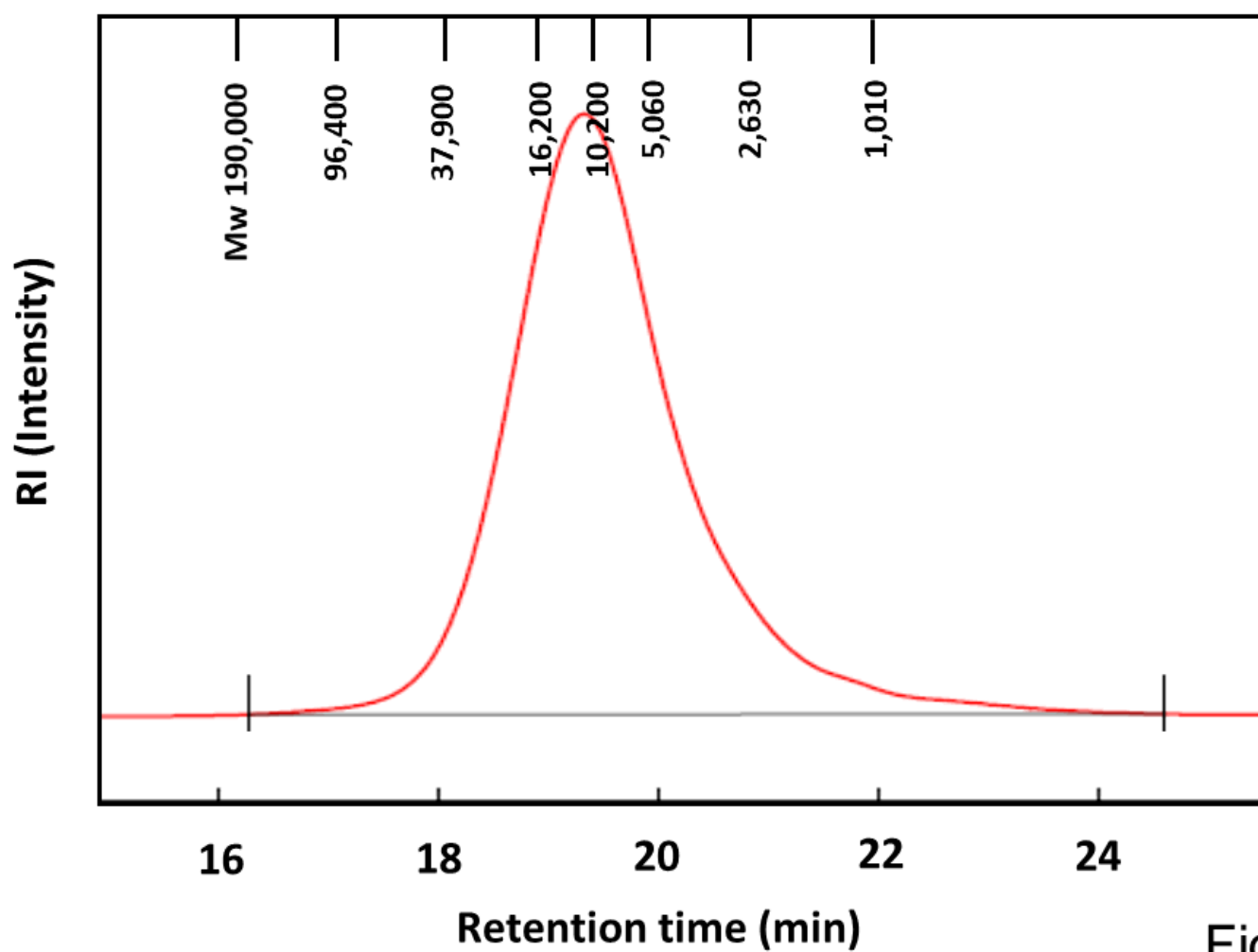


Fig.5

Figure 5

Molecular mass distribution of the purified sample from *A. zerumbet* aqueous extract (red line).

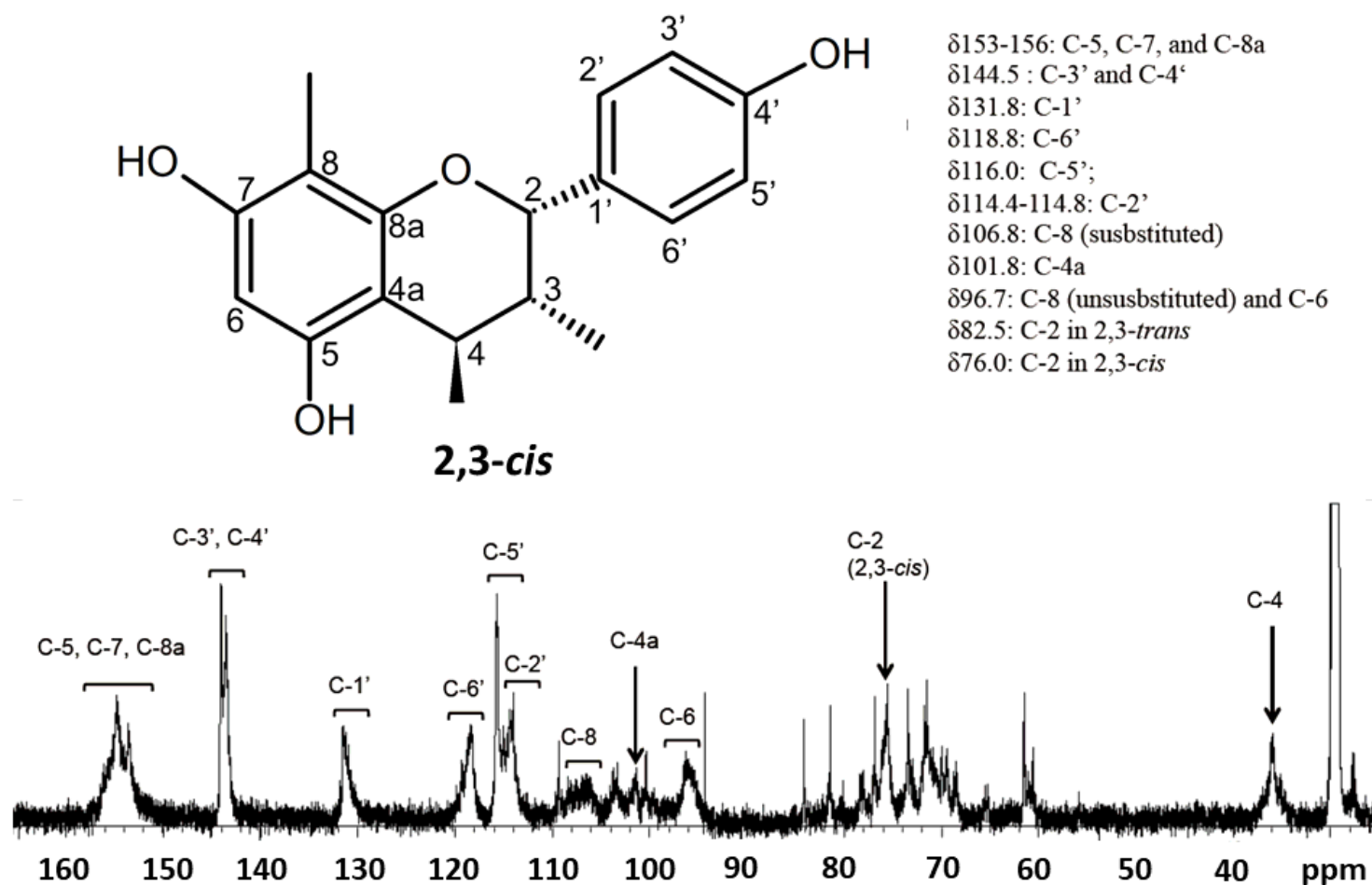


Fig.6

Figure 6

Solution-state ^{13}C -NMR spectrum of proanthocyanidin polymer preparation of the aqueous extract of A. zerumbet.

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