[Chem. Pharm. Bull. 34(11)4718—4723(1986)]

Effect of Extracts Obtained from Rhubarb in Rats with Chronic Renal Failure

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(Received May 7, 1986)

An attempt was made to clarify the nature of the active components contained in rhubarb which show the improving effects on uremic symptoms. The progress of fractionation and purification was monitored by measuring the levels of blood urea nitrogen, creatinine, methylguanidine, and guanidinosuccinic acid after administration of each fraction to rats. Fraction II was found to have a blood urea nitrogen-decreasing effect among fractions I to V obtained by Sephadex LH-20 column chromatography. Fraction II-3, obtained by further fractionation of fraction II, was found to have a uremia-alleviating effect, causing significant decreases in blood urea nitrogen, creatinine, methylguanidine, and guanidinosuccinic acid levels. Purification of fraction II-3 by partition between ethyl acetate and H_2O resulted in recovery of the blood urea nitrogen-decreasing activity in the aqueous phase, which was found to contain mainly proanthocyanidin oligomers.

Keywords——rhei rhizoma; chronic renal failure; blood urea nitrogen; serum creatinine; methylguanidine; guanidinosuccinic acid; proanthocyanidin oligomer; rat

Rhubarb has been regarded as a laxative in Europe, and chemical and biological studies on rhubarb have been directed until recently to the elucidation of active components and the mechanism of the laxative activity.[1,2] On the other hand, a series of studies at our laboratory has shown that in uremic rats rhubarb causes a marked decrease of blood urea nitrogen and creatinine, as well as decreasing the levels of methylguanidine (MG) and guanidinosuccinic acid (GSA); hypocalcemia, hyperphosphatemia, and free amino acid patterns were also improved, suggesting an overall improvement of uremic symptoms.[3,4] It was suggested that rhubarb may be useful as a therapeutic agent for uremia in view of its pharmacological activity, that is, improvement of metabolic abnormalities such as urea cycle, amino acids, electrolytes, *etc.* The present study reports interesting findings obtained in the course of examination of the active components of rhubarb using blood urea nitrogen, creatinine, MG, and GSA as indices.

Materials and Methods

Animals and Treatment——Male rats of the JCL: Wistar strain, initially weighing 110—120 g, were used in this experiment. The animals were fed on commercial feed (CLEA Japan Inc., Tokyo, type CE-2) at a temperature of 25 ± 1 °C and with a 12-h dark-light cycle for a week. Then they were fed *ad libitum* on 18% casein diet containing 0.75% adenine. The 18% casein diet had the following composition (in 100 g): casein 18 g, α-cornstarch 57.9 g, sucrose 15 g, soybean oil 2 g, salt mixture[5] 4 g, vitamin mixture[5] 1 g, cellulose powder 2 g, and choline chloride 0.1 g. To this diet, adenine was added at the level of 0.75 g/100 g of the diet. The adenine feeding procedure produced experimental chronic renal failure, as reported previously.[6-9] During the adenine feeding period, each fraction from rhubarb extract was administered orally for 24 d to rats in drinking water, while control rats received tap water. Throughout

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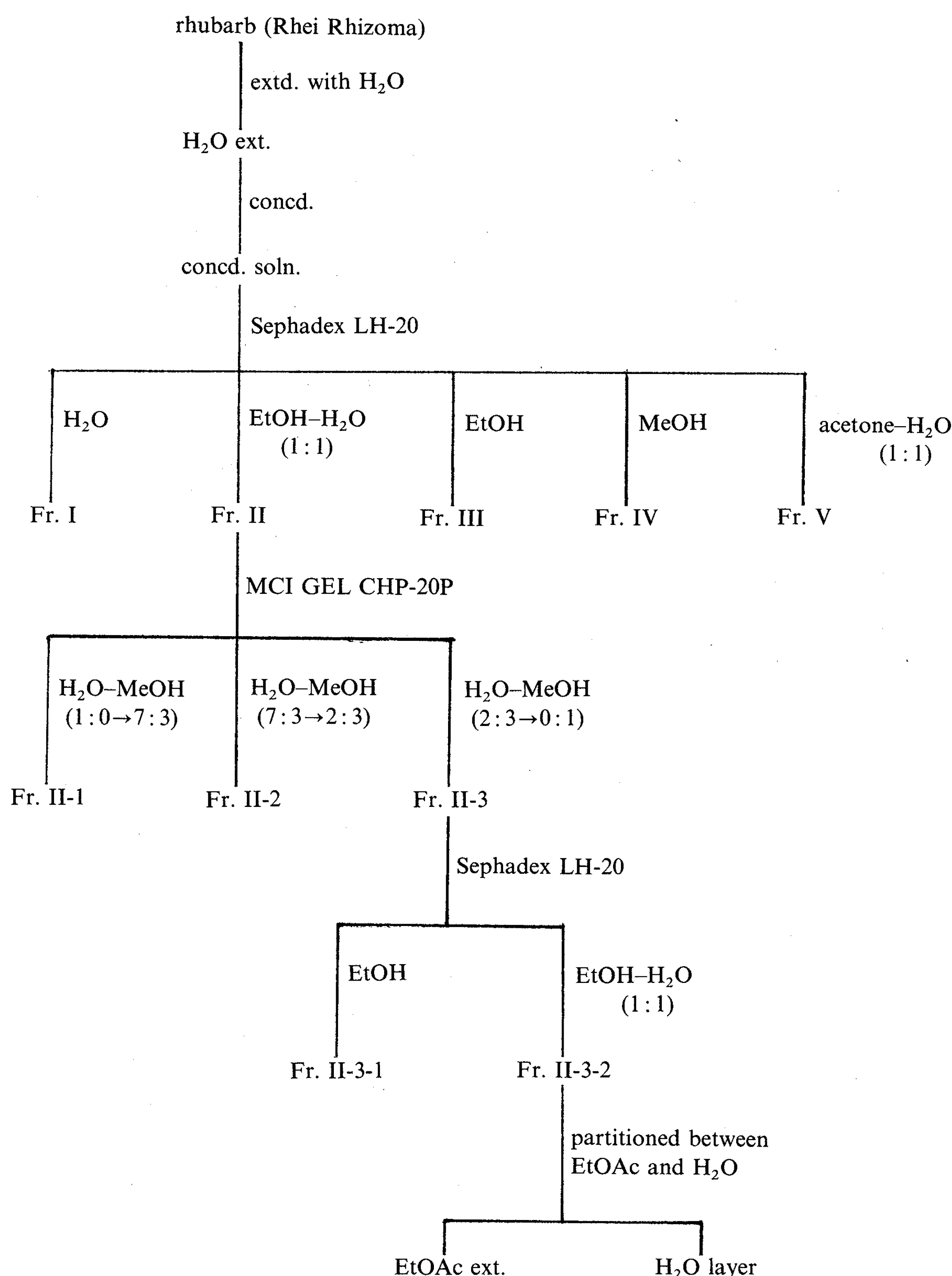

Chart 1. Extraction and Fractionation of Rhubarb

the experimental period, there was no statistically significant difference between the control and any group of rhubarb fraction-treated rats with regard to body weight. Food intake of each rat was essentially proportional to weight change. On the 6th, 12th, 18th, or 24th d of administration of a test fraction, blood samples were collected by heart puncture under sodium pentobarbital anesthesia. They were allowed to clot at room temperature and were then centrifuged. The sera obtained were used for the determination of urea nitrogen. On the last day of each treatment, rats were stunned by means of a sharp blow on the head and blood samples were collected in a conical centrifuge tube for the determination of creatinine and guanidino compounds.

Extraction and Purification of Rhei Rhizoma——Commercial rhubarb (root of *Rheum officinale* BAILLON) (3 kg) was ground into a fine powder and extracted with H_2O at room temperature (Chart 1). The filtrate was concentrated under reduced pressure and column-chromatographed on Sephadex LH-20. Successive elution with H_2O, EtOH–H_2O (1 : 1, v/v), EtOH, MeOH, and acetone–H_2O (1 : 1, v/v) afforded fractions I (525 g), II (193 g), III (67 g), IV (47 g), and V (11.5 g), respectively. Fraction II (150 g) was repeatedly chromatographed over MCI-GEL CHP-20P

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using H_2O–MeOH mixture as an eluate to afford fractions II-1 (81.5 g), II-2 (29.3 g), and II-3 (19.5 g). Fraction II-3 (17.6 g) was column-chromatographed on Sephadex LH-20, eluting with EtOH and then with an EtOH–H_2O mixture to give fractions II-3-1 (6.0 g) and II-3-2 (2.8 g). Fraction II-3-2 (2.8 g) was partitioned between ethyl acetate and H_2O, yielding the respective soluble portions (1.8 and 1.0 g).

Analyses——The level of urea nitrogen was determined by using a commercial reagent (Urea NB-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the urease–indophenol method.[10] Creatinine was determined by using a commercial reagent (Creatinine-Test Wako) based on the Folin–Wu method.[11] For the determination of MG and GSA levels, serum was deproteinized by addition of trichloroacetic acid (TCA) (final concentration, 10%). The supernatant obtained by centrifugation at 3000 rpm for 10 min was injected into a Shimadzu LC-5A liquid chromatograph using a step-gradient system. A fluorescence spectrometer, model RF-540 (excitation 395 nm, emission 500 nm; Shimadzu Co.) was used for detection of substances eluted from the column. Serum constituent levels of the normal rats were as follows: urea nitrogen 16.3 ± 1.1 mg/dl and creatinine 0.73 ± 0.01 mg/dl. MG and GSA were not detectable.

Statistics——The significance of differences between the control and the groups treated with the extracts from rhubarb was tested by the use of Student's *t*-test.

Results and Discussion

Fractionation was performed as shown in Chart 1, and fractions I, II, III, IV, and V thus obtained were each orally administered for 24 successive days in drinking water at a dose of 25—32 mg/rat/d. As shown in Fig. 1, significant reductions of blood urea nitrogen were found at 18 d (21%) and 24 d (33%) after the start of administration of fraction I. Fraction II showed a significant blood urea nitrogen-reducing effect from 12 d after the start of administration, and the effect lasted a further 12 d, with 29%—34% reduction. Fraction V showed a blood urea nitrogen-reducing tendency on days 12 and 18, and a significant reduction of 34% on day 24.

On the other hand, fractions I and II induced significant decreases of hepatic urea content (41% and 32%, respectively) on day 24. Serum phosphate level in the fraction I-treated group was decreased by 23% as compared with the control. Fraction II tended to decrease serum creatinine and showed a hyperphosphatemia-improving effect. Fractions III and IV did not cause any significant changes. Thus, fraction II was the most effective among

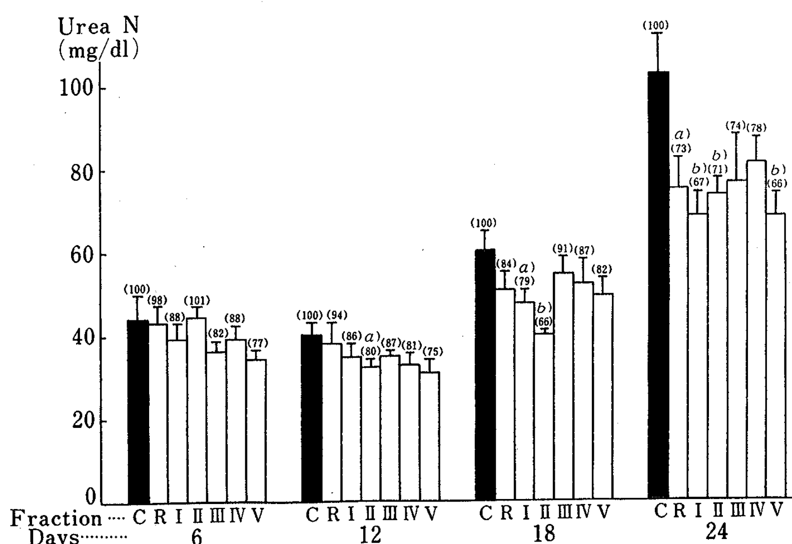

Fig. 1. Effect of Each Fraction on Levels of Urea Nitrogen in the Serum at 6 to 24 d after the Start of Oral Administration

C, control; R, rhubarb extract; I, fraction I; II, fraction II; III, fraction III; IV, fraction IV; V, fraction V. Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value. Significantly different from the control value, *a*) $p<0.05$, *b*) $p<0.01$.

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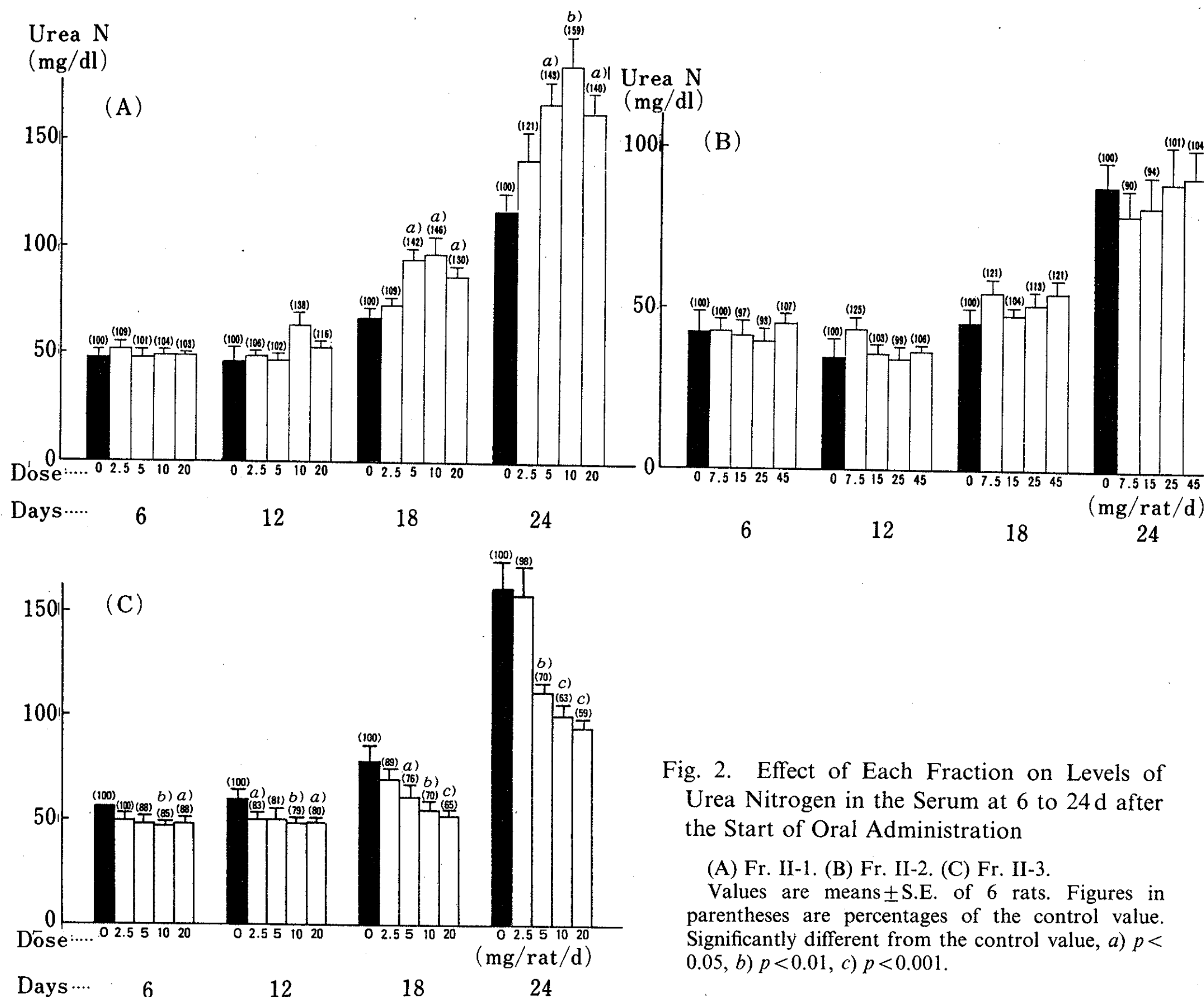

Fig. 2. Effect of Each Fraction on Levels of Urea Nitrogen in the Serum at 6 to 24 d after the Start of Oral Administration

(A) Fr. II-1. (B) Fr. II-2. (C) Fr. II-3.
Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value. Significantly different from the control value, *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

TABLE I. Effect of Each Fraction on Levels of Guanidino Compounds in the Serum at 24 d after the Start of Oral Administration

Material	Dose (mg/rat/d)	Cr (mg/dl)		MG (μg/dl)		GSA (μg/dl)	
Fraction II-1	0	2.90 ± 0.15	(100)	11.52 ± 1.23	(100)	142.33 ± 12.50	(100)
	2.5	3.21 ± 0.18	(111)	$16.03 \pm 1.85^{a)}$	(139)	168.85 ± 28.11	(119)
	5	$4.29 \pm 0.29^{b)}$	(148)	$26.76 \pm 1.80^{c)}$	(232)	$312.91 \pm 23.06^{c)}$	(220)
	10	$4.71 \pm 0.28^{c)}$	(163)	$29.28 \pm 4.36^{b)}$	(254)	228.70 ± 40.22	(161)
	20	$3.62 \pm 0.21^{a)}$	(125)	$22.66 \pm 1.71^{c)}$	(197)	$260.61 \pm 31.53^{a)}$	(183)
Fraction II-2	0	4.70 ± 0.37	(100)	10.35 ± 0.66	(100)	148.88 ± 14.72	(100)
	7.5	4.20 ± 0.30	(89)	10.50 ± 1.68	(101)	108.57 ± 13.34	(73)
	15	4.97 ± 0.38	(106)	9.69 ± 0.70	(94)	123.93 ± 6.00	(83)
	25	4.15 ± 0.39	(88)	8.74 ± 1.86	(84)	139.87 ± 24.45	(94)
	45	4.31 ± 0.15	(92)	11.38 ± 2.14	(110)	179.97 ± 11.63	(121)
Fraction II-3	0	3.60 ± 0.07	(100)	20.61 ± 2.67	(100)	183.43 ± 31.75	(100)
	2.5	$2.62 \pm 0.19^{c)}$	(73)	$11.37 \pm 0.87^{b)}$	(55)	$115.63 \pm 13.93^{a)}$	(63)
	5	$2.50 \pm 0.07^{c)}$	(69)	$6.79 \pm 0.46^{c)}$	(33)	$86.01 \pm 6.81^{b)}$	(47)
	10	$2.78 \pm 0.18^{b)}$	(77)	$5.78 \pm 0.61^{c)}$	(28)	$118.06 \pm 11.85^{a)}$	(64)
	20	$2.02 \pm 0.10^{c)}$	(56)	$3.89 \pm 0.23^{c)}$	(19)	$117.04 \pm 8.40^{a)}$	(64)

Cr, creatinine; MG, methylguanidine; GSA, guanidinosuccinic acid. Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value. Significantly different from the control value, *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

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fractions I to V.

Fraction II was therefore further separated in order to clarify the nature of the active components. As shown in Chart 1, the fraction was subjected to MCI-GEL CHP-20P chromatography with H_2O–MeOH mixture to give fractions II-1, II-2, and II-3.

The doses of 2.5, 5, 10, and 20 mg/rat/d of fraction II-1 were administered to rats to investigate the dose–response relationship. Administration of 5, 10, and 20 mg significantly elevated the blood urea nitrogen level by 30%—46% as compared with the control after 18 d. This tendency was more significant at day 24 with elevations of 43%, 59%, and 40% at the doses of 5, 10, and 20 mg, respectively (Fig. 2A). As shown in Table I, serum creatinine levels were significantly elevated by 48%, 63%, and 25% at the doses of 5, 10, and 20 mg, respectively. The MG level throughout the experiment was significantly higher in the fraction II-1-treated group than in control rats (16.03—29.28 μg/dl *vs.* 11.52 μg/dl, increase of 39%—154%). The GSA level at the doses of 5 and 20 mg of fraction II-1 also showed a significant increase (83%—120%) as compared with the control group. It is noteworthy that this fraction increased the accumulation of nitrogen compounds such as urea nitrogen and guanidino compounds in the body.

Administration of fraction II-2 at the doses of 7.5, 15, 25, and 45 mg/rat/d to rats resulted in no significant changes in blood urea nitrogen, creatinine, MG, and GSA levels as shown in Fig. 2B and Table. I.

Figure 2C illustrates blood urea nitrogen values obtained when fraction II-3 was given at doses ranging from 2.5 to 20 mg. It is clear that the renal failure in adenine-fed rats became more severe with increasing amount of adenine ingested. On day 6 and thereafter, a significant reduction in blood urea nitrogen was noted in the 10 and 20 mg groups and on day 12 in the 2.5 mg group (17%). From day 18 on, there was a significant dose-dependent reduction in all groups given 5 mg or more; the extent of the decrease was about 50 mg/dl (about 30%) in the 5 mg group on day 24. Serum creatinine, MG, and GSA were also significantly reduced. As shown in Table I, there was a 27% decrease of creatinine in the 2.5 mg group, 31% in the 5 mg group, and 44% in the 20 mg group on day 24. MG was reduced in a dose-dependent fashion (45% in the 2.5 mg group, 67% in the 5 mg group, 72% in the 10 mg group, and 81% in the 20 mg group), and GSA was significantly reduced (36%—53%) in the 2.5 to 20 mg groups.

The results obtained in this study were compared with those for rhubarb extract in a previous study.[3] The ability of fraction II-3 to reduce the levels of blood urea nitrogen, creatinine, MG, and GSA was markedly stronger than that of rhubarb extract, indicating that the uremia-alleviating compounds of rhubarb extract were concentrated in this fraction.

Furthermore, an attempt was made to isolate the active components from fraction II-3. As described in Chart 1, fraction II-3 was column-chromatographed on Sephadex LH-20. Fraction II-3-2 thus obtained was further partitioned between ethyl acetate and H_2O. The blood urea nitrogen-decreasing activity of each fraction was examined at 24 d after the start of

TABLE II. Effect of Each Extract of Fraction II-3-2 on Levels of Urea Nitrogen in the Serum at 24 d after the Start of Oral Administration

Material	Dose (mg/rat/d)	Urea nitrogen (mg/dl)
Control	0	122.4 ± 12.7 (100)
H_2O layer	2.5	83.7 ± 6.2[a] (68)
EtOAc extract	2.5	97.4 ± 10.5 (80)
EtOAc extract	5.0	106.6 ± 5.2 (87)

Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value. *a*) Significantly different from the control value, $p < 0.05$.

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oral administration. As shown in Table II, the blood urea nitrogen level was decreased by 32% as compared with the control value upon administration of 2.5 mg/rat/d of the H_2O layer, while the ethyl acetate extract showed no effect.

Analysis of the H_2O layer by means of high-performance liquid chromatography revealed that the aqueous layer consists mainly of oligomeric proanthocyanidins. Further studies on the mechanism of alleviation of the uremic state and a detailed structural examination of these proanthocyanidins are in progress.

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