

# Mumefural, Citric Acid Derivative Improving Blood Fluidity from Fruit-Juice Concentrate of Japanese Apricot (*Prunus mume* Sieb. et Zucc)

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The effects of food components on blood fluidity were studied by in vitro assay using a dedicated microchannel instrument for model capillaries. We found that the fruit-juice concentrate of the Japanese apricot (*Prunus mume* Sieb. et Zucc), a traditional Japanese food, markedly improved the fluidity of human blood. Using HPLC, we isolated the active compounds and characterized them using UV, MS, IR, and NMR. They included a novel compound, 1-[5-(2-formylfuryl)methyl]dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate (mumefural), and a related compound, 5-hydroxymethyl-2-furfural (HMF). Mumefural markedly improved blood fluidity in all subjects, while HMF worked differently in different individuals. The flow rate of blood spiked with mumefural or HMF was compared to that of the two predominant organic acids in the fruit. Citric acid, malic acid, and furfuryl alcohol also improved fluidity in all subjects. The activity of *P. mume* is derived from not only artifacts produced during thermal processing, such as mumefural, but also from endogenous organic acids.

**Keywords:** *Prunus mume*; fruit-juice concentrate; blood fluidity; microchannel instrument; mumefural; HMF; citric acid

## INTRODUCTION

Elderly people comprise the fastest growing population segment in industrialized society, making the control of circulatory disease an important subject (Ettinger et al., 1994; Tamara et al., 1997). Increasing interest is being paid to the role of diet, particularly functional foods, sometimes called pharmafoods, designer foods, or nutraceuticals in the business press, because circulatory system function is closely linked to dietary factors. The relationship between blood flow, which is recognized as essential to good circulation, and food materials has been studied extensively. We studied the effects of traditional Japanese foods on blood fluidity (Kikuchi, 1995a) and found that the fruit-juice concentrate of Japanese apricot (scientific name: *Prunus mume* Sieb. et Zucc; Japanese name: Ume), markedly improves the fluidity of human blood.

The Japanese apricot, a deciduous tree of the genus *Rosaceae*, originated in the central China, and has more than 400 varieties worldwide. Some 113 000 tons of the fruits were produced in Japan in 1996 alone. The fruit has been used in folk medicine to alleviate fever, cough, and intestinal disorders. However, the raw fruit is poisonous due to two types of cyanogenic glucosides, i.e., prunasin and amygdalin (Terada and Sakabe, 1988; Ohtsubo and Ikeda, 1994), making it necessary to process it in ways such as pickling in vinegar, liquor, or syrup and making a fruit-juice concentrate.

In a continuation of studies using both chromatography and the assay of blood fluidity, we isolated the

active components from the fruit-juice concentrate. Here, we report the novel compound that improves blood fluidity and evaluate several compounds in the fruit-juice concentrate.

## MATERIALS AND METHODS

**Materials.** The fruit-juice concentrate of the Japanese apricot, a traditional Japanese food, was obtained from a food store in Osaka. The concentrate is made as follows: the fruit is minced and filtered through cloths, and the filtrate is boiled down with 100 °C. The fruit-juice concentrate is obtained from 50 times the amount of raw fruit.

Acetone-*d*<sub>6</sub> was obtained from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade. For the juice concentrate assay, 10 healthy male volunteers provided 20 mL of whole blood from an arm vein with anticoagulation by a heparin solution. For the compound assay, three subjects provided blood the same way as above. Diazomethane in diethyl ether was prepared from *p*-tolylsulfonfylmethylnitrosamide (Boer and Backer, 1963).

**Assay. Sample Solution.** The juice concentrate (3 g) was dissolved in 20 mL of water and centrifuged. To assay two isolated compounds and authentic standards such as malic acid, citric acid, and furfuryl alcohol, each was prepared in water at a concentration of 0.1 mmol/L.

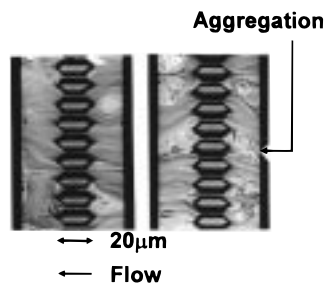
**Microchannel Instruments.** Blood fluidity measurement by a microchannel instrument was detailed by Kikuchi et al. (1994, 1995b). The microchannel array is shown in pictures taken by the microscope–video–recording system (Figure 1). Array microgrooves formed on the surface of a single-crystal silicon substrate were converted to leak-proof microchannels by tightly covering them with an optically flat glass plate.

**Method.** A 50  $\mu$ L portion of the sample solution was added to 1000  $\mu$ L of physiological saline solution, and then the 5  $\mu$ L aliquot of the solution was added to 500  $\mu$ L of blood. An aliquot of each suspension was transferred to the sample cylinder connected to the inlet of the chip holder and made to flow through channels when the solenoid valve connecting the

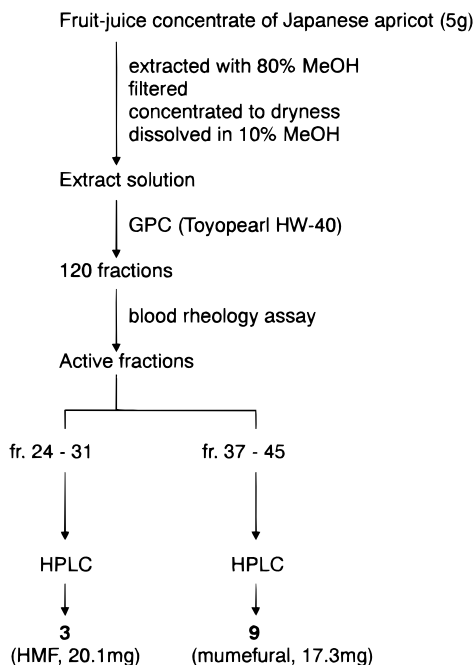
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**Figure 1.** Pictures of the system using the microchannel instrument. Control blood from volunteer 8 showed aggregation (right), and blood spiked with fruit-juice concentrate showed improvement (left).

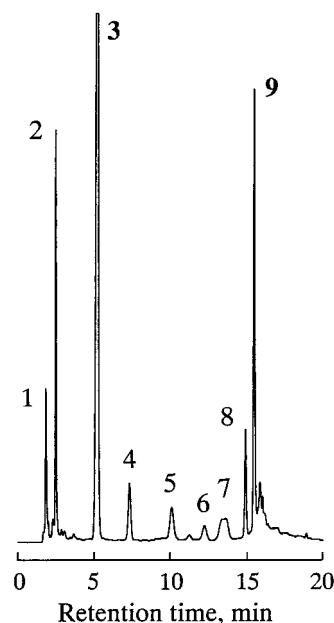


**Figure 2.** Schematic procedure for isolation of active compounds.

holder outlet was opened to a negative pressure of 20 cm height of  $H_2O$ . The time required for 100  $\mu L$  of blood to pass through a cylinder was recorded.

**HPLC Analysis.** The composition of the juice concentrate was determined by HPLC analysis using a 802-SC (Jasco) system controller equipped with an 851-AS intelligent sampler and a PU-980 (Jasco) pump system. The 875-UV detector was used to detect the analyte at 280 nm. The column (150 mm  $\times$  4.6 mm i.d., COSMOSIL 5C<sub>18</sub>-MS, Nacalai Tesque, Kyoto, Japan) was placed in a CO-965 (Jasco) thermostat-controlled system, operated at 40 °C. The following solvents and elution profiles were used: solvent A, 5% acetonitrile in water containing 0.2% formic acid; solvent B, 90% acetonitrile in water containing 0.2% formic acid; elution profiles: 0–10 min for 100% solvent A (isocratic) and 10–25 min for 100–20% solvent A (linear gradient); flow rate, 1.0 mL/min; injection volume, 20  $\mu L$ .

**Preparation. Crude Components.** Active compounds were isolated as shown in Figure 2. The juice concentrate (5 g) was extracted 3 times with 100 mL of 80% methanol (MeOH) in water. The solution was filtered through No. 5A filter paper and then analyzed by analytical high-performance liquid chromatography (HPLC) (Figure 3). The solution was concentrated to dryness in vacuo and dissolved in 30 mL of 10% methanol in water containing 0.2% formic acid. The solution was then subjected to gel permeation chromatography (GPC) [column, 450 mm  $\times$  40 mm i.d., Toyopearl HW-40 (Tosoh, Tokyo, Japan); 15 mL/fraction; elution, gradient of 10 to 100% of methanol in water containing 0.2% formic acid; total eluted



**Figure 3.** HPLC chromatogram of the total extract obtained from the fruit-juice concentrate of Japanese apricot. The fractions involving **3** (HMF) and **9** (mumefural) showed the activity.

fractions numbered, 120]. Eluted fractions were analyzed by HPLC, with the following results: in fraction (fr) 13, peak **1** in Figure 3 was the major component, **2** in fr 16, **3** in fr 26, **6** and **8** in fr 34, **7** and **9** in fr 37, **9** in fr 41, **5** in fr 51, and **4** in fr 77. Each fraction was concentrated to dryness in vacuo and dissolved in 1 mL water, and then the time for the individual-fractions-spiked blood to pass through the microchannel array was measured, as described above.

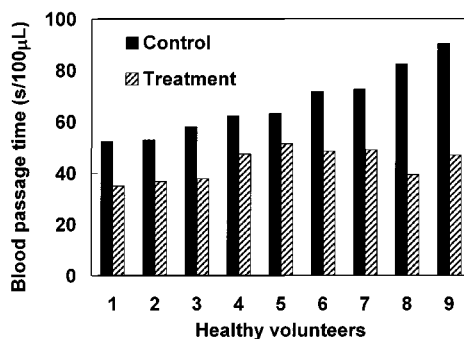
**Isolation of Active Compounds.** Two active compounds, **3** at 5.1 min and **9** at 15.4 min on the chromatogram, were isolated to be characterized. Fractions containing **3** from fr 24 to 31 were combined and further purified by preparative HPLC. Fractions containing **9** from fr 37 to 45 were treated in the same way.

**Preparative HPLC.** The preparative HPLC system consisted of an 880-PU (Jasco, Tokyo, Japan) pump, a manual injector (model 7125, Rheodyne, Cotati, CA) equipped with a 5.0 mL sample loop, and an 875-UV variable wavelength detector equipped with a preparative flow cell. Isolation was done using a C<sub>18</sub> coated silica gel column (250 mm  $\times$  20 mm i.d., Wakopak, Osaka, Japan) at 280 nm. The mobile phase was formic acid/water/acetonitrile (0.2/87/13), and the flow rate was 10 mL/min. Purity was checked by analytical HPLC, and compounds were rechromatographed if necessary.

**Acid Hydrolysis and Methylation.** Compound **9** was hydrolyzed by refluxing with 1 N HCl for 1 h, a 20  $\mu L$  aliquot was analyzed by HPLC, the remaining solutions were concentrated to dryness in vacuo, and then diazomethane was subsequently added to diethyl ether and let stand for 1 h at 0 °C with occasional shaking. The sample was added to 1 mL acetone and analyzed by GC/MS.

**Capillary GC/MS.** An HP 5890 gas chromatograph equipped with a splitless injector was coupled with an SX-102 (JEOL, Tokyo, Japan) mass spectrometer. A 10 m  $\times$  0.1 mm i.d. (df = 0.17  $\mu m$ ) HP-5 fused silica cross-linked 5% phenylmethyl silicone capillary column was used under the following temperature program: 50 °C (4 min isothermal) to 250 °C at 30 °C/min; the injection port and transfer line temperature = 200 °C; He carrier gas flow rate = 2.5 cm/s.

**Structural Identification.** UV absorption spectra were recorded on a UV-3100 spectrometer (Shimadzu, Kyoto, Japan). Mass spectra of compounds were obtained on an SX-102 (JEOL) spectrometer by fast atom bombardment (FAB) ionization using glycerol as a matrix in the negative-ion mode. IR spectra were measured on a Magna-IR 560 (Nicolet, Madison, WI) spectrophotometer using disposable IR cards (3M, St. Paul,



**Figure 4.** Effects on fluidity of blood spiked with the fruit-juice concentrate of Japanese apricot: control, whole blood spiked with saline only; treatment, whole blood spiked with fruit juice concentrate.

MN). NMR data for  $^1\text{H}$  at 300.13 MHz and  $^{13}\text{C}$  at 75.48 MHz were recorded on a DRX-300 (Bruker, Karlsruhe, Germany) spectrometer at room temperature in deuterated acetone. Chemical shifts were reported relative to tetramethylsilane as an internal standard. Optical rotation ( $[\alpha]_D$ ) was measured at room temperature (23 °C) on a DIP-181 (Jasco) spectropolarimeter.

## RESULTS AND DISCUSSION

**Fruit-Juice Concentrate Activity.** Figure 1 shows examples of blood flow through a microchannel array, with typical aggregation at right. At left is the blood-sample spiked with fruit-juice concentrate. Note the decrease in aggregation of blood cells and quick passage through the narrow channels. The effect mainly comes from increased red blood cell (RBC) deformability. The average diameter of a human capillary, about 5  $\mu\text{m}$ , is smaller than that of the RBC, about 8  $\mu\text{m}$ . RBCs must deform considerably to pass through microcirculation, and deformability is a prerequisite to good circulation (Celle, 1975; Usami et al., 1975; Hochmuth and Waugh, 1987). As mentioned, we measured the time it took for a certain amount of blood to pass through the filter. Under different conditions including pathological, the microchannel instrument provides more rapid quantitative analysis of blood fluidity *in vitro* than other techniques, such as measuring the negative pressure required to suck a portion of blood into a micropipet and elongation of RBCs adhering to the surface under the action of fluid shear stress (Bessis and Mohandas, 1975).

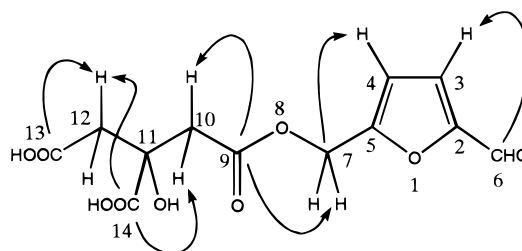
Figure 4 shows activity that improves blood fluidity. Control blood fluidity presented individual values, but improvement was marked for all subjects; the addition of fruit-juice concentrate to the blood samples reduced the blood passage time to 48–89% that of the control flow.

**Assay of Fractions from Crude Extract.** The activity of 120 fractions obtained by GPC from crude extracts was evaluated by the microchannel instrument, and the analysis of each fraction was performed by HPLC. It resulted in nine major peaks, each of which was evaluated. In contrast to the clearly negative activity in fraction 13 mainly containing **1**, positive activity was observed in 26 (**3**), 34 (**6**, **8**), 37 (**7**, **9**), and 41 (**9**). Positive activity was particularly strong in 26 (**3**) and 41 (**9**). Fraction 13 (**1**) induced aggregation and disturbed the blood flow. Other fractions showed no noticeable change. Both **3** and **9** showing clear positive activity were collected individually by preparative HPLC. Purified **3** contained 20.1 mg and **9** 17.3 mg.

**Structural Determination.** Physicochemical properties of the isolates **3** and **9** were as follows.

**Table 1.**  $^1\text{H}$ ,  $^{13}\text{C}$  NMR (ppm) Spectral Data for Compound **9**

	$^1\text{H}$ NMR		$J$ , Hz		$^{13}\text{C}$ NMR	
	Chemical shift	Multiplicity	Assignment	Value	Assignment	Chemical shift
3	7.37 d		$J_{3,4}$	3.5	2	154.0
4	6.76 d		$J_{10a,b}$	15.4	3	113.4
6	9.64 s		$J_{12a,b}$	15.9	4	110.0
7	5.19 s				5	156.4
10	2.93 d, 3.01 d				6	178.5
12	2.86 d, 2.97 d				7	58.6
					9	169.8
					10	43.2
					11	73.5
					12	43.5
					13	171.5
					14	174.7



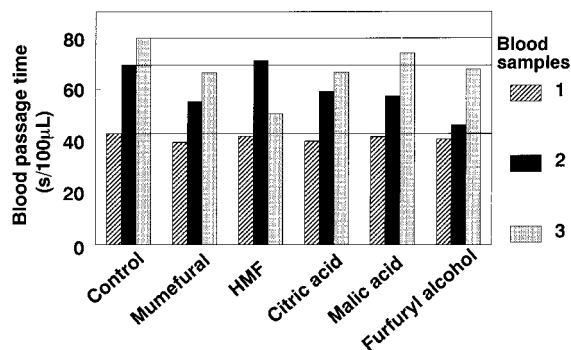
**Figure 5.** Chemical structure of mumefulral, 1-[5-(2-formylfuryl)methyl] dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate. The arrows represent cross-peaks observed in the HMBC experiment.

**Compound 3:** colorless syrup. NMR assignments are discussed below.

**Compound 9** (Figure 5): colorless viscous syrup; UV (water)  $\lambda_{\text{max}}$  282 nm ( $\epsilon$   $1.55 \times 10^4$ ), 229 ( $2.96 \times 10^3$ );  $[\alpha]_D$  0° (water,  $c$  13.83); IR  $\nu_{\text{max}}$  1739, 1675, 1523, 1405, 1340, 1193  $\text{cm}^{-1}$ ; FAB/MS,  $m/z$  299 ( $[\text{M} - \text{H}]^-$ ), 191. High-resolution FAB/MS: calcd for  $\text{C}_{12}\text{H}_{11}\text{O}_9$ , 299.0403; found, 299.0377. NMR assignments are shown in Table 1.

**Identification of Compound 3 by HPLC and NMR.** Compound **3** was attributed to 5-hydroxymethyl-2-furfural (HMF) because data for **3** agreed with that of  $^1\text{H}$  NMR and retention time (5.1 min) by HPLC of the authentic standard. Hexose, such as glucose, is known to be easily converted to HMF by heating under an acidic condition. According to Nakamura (1995), fresh mume contained 410 mg/kg glucose.

**Identification of Compound 9 by GC/MS and NMR.** The aqueous solution of compound **9** was acidic. The FAB mass spectrum obtained in the positive-ion mode did not show any peaks, but very intense peaks appeared in the negative-ion mode. These results suggested that the compound contained acidic functional groups. When **9** was treated with diazomethane and dimethyl sulfate, methylated products characterized by both NMR and MS were di- and trisubstituted. The  $^1\text{H}$  NMR spectrum in a chemical shift range of 2.86–3.01 ppm showed four doublets read as two pairs of an AB-spin system with geminal coupling ( $J = 15.4, 15.9$  Hz). Cross-peaks between carbonyl carbons and the geminal protons were observed in HMBC (Figure 5). So, **9** was thought to be a citric acid mono ester. Judging from the cross-peak in the 2D HMBC (heteronuclear multiple-bond correlation) spectrum, the position of the substituent group was at a terminus. We further concluded from 1D, 2D NMR results and negative FAB/MS fragments that the substituent group was HMF. This implied the structure of **9** to be 1-[5-(2-formylfuryl)methyl] dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate (Figure 5). All  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals were assigned and are shown in Table 1.



**Figure 6.** Effects on fluidity of blood spiked with various standard solutions (0.1 mmol/L).

This structure was confirmed by degradation of **9**. The retention time of acid hydrolysis products detected by HPLC at 280 nm was 5.3 min, in fairly good agreement with that of HMF. After methylation of products using diazomethane, the retention time of the derivative detected by GC/MS was 10.5 min, and the profile closely matched that of the trimethyl citrate standard.

Compound **9**, obtained as a pharmaceutically active compound from mume, was designated mumefural. Mumefural and HMF have not been detected in the fresh fruit by HPLC, which means that the both compounds are produced during food processing. Mumefural is asymmetric at C-11 (Figure 5), but mumefural isolated from the juice concentrate was racemate. This fact is consistent with its generation during food processing.

**Assay of Compounds.** The flow rate of blood spiked with mumefural or HMF was determined as detailed above. As shown in Figure 6, mumefural was an effective compound improving blood fluidity in all subjects. HMF was most effective in subject 3 but negative in subject 2. To determine the key structural factors in mumefural for stabilizing and enhancing activity, the assay was evaluated with related compounds of mumefural, i.e., citric acid, malic acid, and furfuryl alcohol. Citric acid is a component of mumefural, and furfuryl alcohol is a compound in which the aldehyde group of HMF is substituted by a hydrogen.

The relative positive activity (Figure 6) improving blood fluidity decreased in the sequence mumefural > citric acid > furfuryl alcohol > malic acid > HMF for subject 1; furfuryl alcohol > mumefural > malic acid > citric acid for subject 2; and HMF >> mumefural > citric acid > furfuryl alcohol > malic acid for subject 3. Mumefural, furfuryl alcohol, malic acid, and citric acid compounds other than HMF relatively effectively improved blood flow in all subjects, indicating that citric acid enables mumefural to exhibit activity and HMF enhances the citric acid activity.

With the guidance of a UV detector (280 nm), we isolated mumefural and HMF as active compounds, but neither citric nor malic acid was observable on the chromatogram. The juice concentrate has a large amount of citric and malic acid, however, and both contents are about 40% (w/w) in the fruit-juice concentrate. The blood-flow-improving activity in the concentrate is attributed not only to artifacts from the food processing but also to organic acids from fresh fruit.

The organic acids mentioned above and HMF are well-known as food components. The U.S. Food and Drug Administration lists citric acid as a multiple-purpose generally-recognized-as-safe (GRAS) food sub-

stance. Both citric and malic acid (Cherrington et al., 1991; Tamblyn and Conner, 1997) have pharmacological effects such as antimicrobial, antibacterial, and anti-oxidative activity. HMF is often used as an index of heat treatment and deteriorative changes in food such as tomato paste, infant milk, honey, and fruit juices (Hurtado et al., 1998; Vinas et al., 1992). In the case of mumefural found in this study, further research is needed to determine its bioavailability and metabolic fate in the human body.

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