Article

Changes in Free Amino Acid, Protein, and Flavonoid Content in Jujube (*Ziziphus jujube*) Fruit during Eight Stages of Growth and Antioxidative and Cancer Cell Inhibitory Effects by Extracts

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ABSTRACT: Jujube (*Ziziphus jujube*) was analyzed at eight stages of ripeness (S1−8) for protein, by HPLC and mass spectroscopy for free amino acids and flavonoids, and by colorimetry for total flavonoids and antioxidative activity. The ripe fruit had lower levels of protein, flavonoids, and antioxidative activity than that of the unripe fruit. Free amino acids levels peaked at S5, due mainly to an increase in free asparagine. Extracts were also tested against four cell lines using the MTT cell viability assay. All growth stages dose-dependently inhibited HeLa cervical cancer cells, whereas the inhibition of Hel299 normal lung and A549 lung cancer cells decreased as the fruit matured and was well correlated with the flavonoid content and antioxidative activity. Chang normal liver cells were inhibited by only the S5 extract. U937 lymphoma cells were unaffected by the extracts. These results show the effect of fruit maturity on nutritional and health-promoting components.

KEYWORDS: *jujube, antioxidative, anticancer, MTT assay, flavonoids, phenolics, maturity, ABTS, DPPH, Folin−Ciocalteu, FRAP*

■ INTRODUCTION

Jujube fruits are widely consumed in Asian countries and have the potential to serve as a health-promoting functional food with numerous beneficial effects. Recent studies describe anticancer,[1,2] antiepileptic,[3] anti-inflammatory,[4] anti-insomnia,[5] and neuroprotective effects[6] in cells, animals, and humans.

In previous publications, we describe the determination of the content of adverse and beneficial bioactive compounds in cucumbers,[7] jujubes,[8] mushrooms,[9] meat,[10] onions,[11] peppers,[12,13] potatoes,[14,15] rice hull liquid smoke,[16] sweet potatoes,[17] teas,[18] tomatoes,[19] and toxic weed seeds.[20,21] With respect to jujube, we measured free amino acid, individual and total phenolic content, and antioxidative activities in three jujube fruit pulp and two seed extracts.[8] The distribution of the individual flavonoids among the different samples varied widely. Data determined by the ferric reducing/antioxidant power (FRAP) antioxidative assay were well correlated with total phenolic content. Because individual jujube flavonoids are reported to exhibit different health-promoting effects, knowledge of the composition and concentration of bioactive compounds of jujube products can benefit consumers.

Bioactive compounds can be both synthesized and degraded as the plant matures, thus there is a need to define the content at different stages of maturity. For example, in the case of tomatoes, the content of the glycoalkaloid α-tomatine decreases and that of the antioxidant lycopene increases during the maturation of the tomato fruit on the vine.[19,22]

To facilitate selection of the maturity stage of the jujube fruit that can provide optimum benefits as a functional food, the
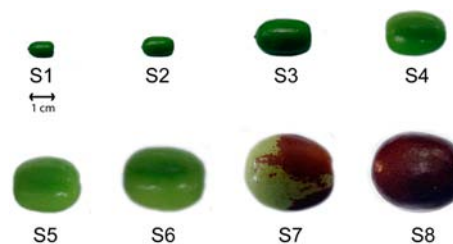

Figure 1. Photographs of eight stages of growth (S1−S8) of Korean Boen-daechu jujube fruit variety.

main objectives of this study were (a) to determine protein, free amino acid, and total and individual flavonoid content of the fruit during eight stages of field-grown plants, (b) to compare antioxidative and cancer cell inhibiting activities of extracts of the fruits, and (c) to correlate the flavonoid content and antioxidative activities with the anticancer activities. Individual flavonoids were determined by characterizing the structures of the phenolic compounds present in the extracts by HPLC-MS, and by quantification by HPLC. Protein ($N \times 16$) was determined by the Kjeldahl method, and free amino acids by HPLC. Total flavonoids were determined by a chelation method described

Table 1. Dimension, Weights, and Water and Protein ($N \times 6.25$) Contents of Eight Growth Stages (S1–S8) of Jujube Fruits[a]

stage	days after flowering	color	length (mm)	width (mm)	weight (g)/fruits	water (%)	protein (g/100 g dry wt)
S1	10	G	10.3 ± 0.6	6.5 ± 0.4	0.4 ± 0.0[1]	93.5	
S2	17	G	14.4 ± 1.0	8.9 ± 1.0	0.7 ± 0.1[1]	95	42.1 ± 1.9[1]
S3	24	G	21.0 ± 0.7	13.9 ± 0.6	2.1 ± 0.1	93.6	41.8 ± 1.1[1]
S4	38	G	24.2 ± 0.4	18.3 ± 1.0	4.1 ± 0.4	93.4	20.2 ± 1.5[2]
S5	52	G	30.5 ± 1.4	21.8 ± 1.4	7.3 ± 1.1	93.5	27.4 ± 3.1
S6	80	G	34.1 ± 0.4	26.8 ± 0.7	11.3 ± 0.7	90.3	21.9 ± 1.2[2]
S7	101	RB (1/2)	36.8 ± 2.2	29.4 ± 0.8	16.2 ± 0.4	77.8	6.8 ± 0.1[3]
S8	115	RB	40.2 ± 0.7	31.5 ± 1.1	19.0 ± 0.8	73.5	4.6 ± 0.6[3]

[a]Jujube variety: Boeun-daechu fruit (*Ziziphus jujuba* forma hoonensis C. S. Yook); The flowering date was July 5, 2010; G = green; RB = reddish brown; length, width, and weight are average ± SD ($n = 4$); protein value is average ± SD ($n = 3$).

Table 2. Concentration of Free Amino Acids (FAA) and Amino Acid Metabolites in Pulp of Eight Growth Stages (S1–S8) of Jujube Fruits[a]

amino acid	growth stage							
	S1	S2	S3	S4	S5	S6	S7	S8
p-Ser	227.1 ± 37.1	280.4 ± 4.0	87.5 ± 0.3	87.3 ± 0.6	102.6 ± 6.1	124.0 ± 6.6	51.4 ± 0.4	29.6 ± 0.6
o-Pea	nd	nd	11.7 ± 1.6	10.4 ± 0.5	14.9 ± 4.3	nd	nd	nd
L-Asp	30.9 ± 0.5	28.5 ± 0.4	37.1 ± 0.3	45.0 ± 0.8	87.3 ± 0.2	87.0 ± 0.5	76.6 ± 0.6	51.5 ± 0.5
L-Thr	27.4 ± 1.7	36.0 ± 0.2	63.5 ± 1.0	49.9 ± 0.8	81.0 ± 0.6	22.4 ± 0.1	10.9 ± 0.3	12.8 ± 0.2
L-Ser	64.0 ± 1.1	104.8 ± 0.4	58.5 ± 0.8	49.6 ± 0.6	62.8 ± 0.2	60.5 ± 0.5	44.3 ± 0.5	37.9 ± 0.5
L-Asn	173.5 ± 1.2	163.6 ± 0.8	682.8 ± 12.8	738.7 ± 5.5	3184.7 ± 28.9	2015.6 ± 30.4	1468.5 ± 13.1	915.0 ± 9.8
L-Glu	55.0 ± 0.6	63.6 ± 0.4	700.5 ± 5.9	372.4 ± 3.2	119.1 ± 10.9	84.7 ± 0.1	106.1 ± 2.0	93.6 ± 0.8
L-Gln	115.2 ± 1.4	148.7 ± 3.2	58.0 ± 0.5	343.8 ± 2.9	28.9 ± 0.3	14.7 ± 0.5	15.8 ± 0.1	32.6 ± 0.4
L-Pro	nd	nd	nd	nd	nd	113.9 ± 3.5	715.8 ± 3.0	1593.4 ± 19.9
L-Gly	9.8 ± 0	10.3 ± 0.79	6.4 ± 0.2	5.3 ± 0.2	8.7 ± 0.5	9.3 ± 0.1	3.7 ± 0.2	3.4 ± 0
L-Ala	52.22 ± 0	87.5 ± 3.0	54.5 ± 1.7	44.0 ± 1.7	45.5 ± 0.5	53.8 ± 0.3	24.3 ± 0.2	8.6 ± 0.2
L-Cit	nd	nd	3.4 ± 0.2	2.9 ± 0.2	10.7 ± 0.2	3.9 ± 0.1	0.9 ± 0.1	0.8 ± 0
L-Cys	109.2 ± 3.1	130.7 ± 0.4	114.5 ± 3.9	73.41 ± 5.2	95.7 ± 4.1	31.2 ± 17.0	19.3 ± 3.4	27.2 ± 0.4
L-Val	17.9 ± 0.3	21.2 ± 0.2	7.8 ± 0.2	7.4 ± 0	8.6 ± 0.2	6.9 ± 0.4	4.5 ± 0.4	6.3 ± 0.1
L-Met	nd	nd	nd	nd	nd	nd	nd	nd
L-Ile	8.7 ± 0.8	8.9 ± 0.2	nd	nd	nd	3.5 ± 1.8	1.3 ± 0	1.8 ± 0
L-Leu	10.3 ± 0.5	12.7 ± 0.4	2.7 ± 0	3.5 ± 1.5	2.9 ± 0.2	5.3 ± 1.9	2.4 ± 0	2.0 ± 0.2
L-Tyr	8.0 ± 0	12.1 ± 0.2	4.4 ± 0.6	nd	3.5 ± 0.6	6.4 ± 2.5	nd	nd
L-Phe	5.2 ± 0.2	8.9 ± 0.2	nd	nd	1.4 ± 0	3.8 ± 0	0.9 ± 0.1	nd
β-Ala	nd	nd	nd	nd	4.4 ± 0.3	5.1 ± 0.3	2.8 ± 0.1	2.4 ± 0
4Abu	195.9 ± 0.9	244.6 ± 1.0	88.1 ± 0.6	79.5 ± 0.9	115.3 ± 0.5	103.4 ± 1.4	30.4 ± 0.1	16.9 ± 0.2
Trp	nd	nd	nd	nd	nd	nd	nd	nd
EtNH$_2$	7.5 ± 0.9	43.4 ± 0.4	25.4 ± 0.8	20.2 ± 0	21.9 ± 0.2	15.2 ± 0.2	6.8 ± 0.1	5.8 ± 0.1
Hyl	44.7 ± 0.3	27.5 ± 18.0	39.3 ± 0.3	36.1 ± 1.1	38.7 ± 1.7	22.7 ± 0	9.4 ± 0.5	8.9 ± 0.2
L-Lys	3.4 ± 0	3.2 ± 0.2	2.7 ± 0.2	2.3 ± 0	3.1 ± 0.3	5.7 ± 0.1	3.4 ± 0.1	1.5 ± 0
MetHis	nd	nd	nd	nd	nd	nd	nd	nd
L-His	4.1 ± 0.5	3.6 ± 0.2	nd	nd	3.5 ± 0.2	6.2 ± 0.1	7.4 ± 0.1	11.1 ± 0.3
L-Car	58.5 ± 14.4	nd	14.5 ± 4.8	15.0 ± 1.2	nd	nd	nd	nd
L-Arg	nd	nd	5.3 ± 0.2	7.4 ± 0	22.2 ± 0.2	72.7 ± 0.3	70.3 ± 0.8	6.7 ± 0.3
TFAA[b]	1228 ± 40	1440 ± 19	2069 ± 16	1994 ± 9	4068 ± 32	2878 ± 36[1]	2677 ± 16	2870 ± 22[1]
essential[c] TFAA	77.0 ± 2.0[1]	94.4 ± 0.6[2]	76.6 ± 1.0[1]	63.0 ± 1.7[3]	100.5 ± 0.7[2]	53.7 ± 2.6[3]	30.7 ± 7.4[4]	35.5 ± 0.4[4]
Asn/TFAA (%)	14.1 ± 0.5	11.4 ± 0.2	33.0 ± 0.7[1]	37.0 ± 0.3	78.3 ± 0.9	70.0 ± 1.4	54.9 ± 0.6	31.9 ± 0.4[1]
TFFA/protein (%)		3.4 ± 0.2[1]	4.9 ± 0.1[1]	9.9 ± 0.7[1]	14.8 ± 1.7[1]	13.2 ± 0.8[1]	39.6 + 0.7	62.4 ± 8.5

[a]Amino acid abbreviations follow IUPAC standard; values are averages of duplicate determinations (mg/100 g dry wt) ± SD ($n = 2$); values with the same superscript within rows are not significantly different ($p < 0.05$). nd, not detected. [b]TFAA = sum of all free amino acids. [c]Essential TFAA = sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

previously.[23] Four antioxidative tests were used: ABTS, DPPH, Folin−Ciocalteu, and FRAP. Cell viability was determined by the MTT assay.

The results suggest that the maturity stage at which the jujube fruits are harvested strongly influences the content of bioactive compounds and antioxidative activities as well as protein content and free asparagine content, which can affect product quality during postharvest processing. The effect of maturity on the content and quality of pectins in jujube fruit has recently been evaluated.[24] To our knowledge, this is the first report on the dynamics of the biosynthesis of jujube bioactive components at different stages of maturity of the fruit harvested from plants grown under the same environmental conditions.

■ MATERIALS AND METHODS

Materials. Quercetin-3-O-galactose (lot no. 0001438413, ≥97.0%), (−)-epicatechin (lot no.0001423660, ≥90%), quercetin-3-O-rutinoside (lot no. BCBB6172), tannic acid (lot no. 082K0037, ≥98%), quercetin (lot no. 113K1051, ≥98%), 2,2-diphenyl-1-picrylhydrazyl (DPPH, catalogue no. D9132, lot no. 12K1944, ≥90%), 2(3)-t-butyl-4-hydroxyanisole (BHA, catalogue no. B1253, lot no. 098K0242, ≥95%), potassium persulfate (lot no. 216224, ≥99%), and 2,2′-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS, lot no. A1888, ≥98%) were purchased from Sigma (St. Louis, MO). Folin−Ciocalteu phenol reagent (lot no. OF1181) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Tripyridyltriazine (TPTZ, lot no. FHL01, ≥98%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Procyanidin dimer B2 (Fluka no. 42157, lot no. BCBB3135, ≥90%) was purchased from Aldrich (Milwaukee, WI). All other reagents (analytical grade) were obtained from commercial sources. HPLC grade acetonitrile and formic acid were purchased from J.T. Baker (Phillipsburg, NJ) and Aldrich (Milwaukee, WI). The solvents were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA) and degassed in an ultrasonic bath before use.

Human cervical carcinoma (HeLa), histiocytic lymphoma (U937), lung cancer (A549), and normal human liver (Chang) and lung cell lines (Hel299) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in an MEM medium supplemented with fetal bovine serum (10%), penicillin (50 units/mL), and streptomycin (50 mg/mL), at 37 °C in a 5% CO_2 incubator. Cell culture reagents were obtained from GibcoBRL (Life Technologies, Cergy-Pontoise, France). Each sample was dissolved in DMSO (2 mg/200 μL) and stored at −4 °C.

Sampling of Jujube Fruits. Boeun-deachu (Ziziphus jujuba forma hoonensis C.S.Yook) used in this experiment is an improved variety widely cultivated in Korea (Figure 1). This variety contains no seeds in the shell. The plants were grown in a field station in Boeun-gun, Chungbuk, Korea, and the fruits were harvested from July 8 to October 12, 2010 in eight stages (S1−S8) of maturity, from 10 to 115 days after flowering (Table 1). The fruits were collected, weighed, and measured for size (Table 1).

Extraction of Amino Acids and Phenolic Compounds from Jujube Fruit. Triplicate extractions were performed for each growth stage except for S1 ($n = 1$) and S2 ($n = 2$) due to limited sample availability. Where triplicate extractions were not possible, additional analytical analyses were performed to $n = 3$. Uniform-sized fresh jujube fruits (10−20) were selected for analysis. The fruits were divided into pulps and shells with a knife. The pulps were then cut with a knife to thin strips (2 mm × 2 mm) and mixed well. A sample of the pulp mixture (7 ± 1 g) was macerated in a glass mortar to which was added 80% methanol/water (20 mL). The suspension was then centrifuged at 12000g for 10 min at 10 °C. The residue was re-extracted three times with 80% methanol (20 mL) and centrifuged. The combined supernatants were diluted to 100 mL with 80% methanol. An aliquot (20 mL) was concentrated in a rotary evaporator at 30 °C. The residue was then dissolved in 80% ethanol (2.0 mL), and used for analysis of free amino acids and flavonoids by HPLC and inhibition of cancer cells by the MTT assay. For the antioxidative assays and determination of total flavonoids, these extracts were redissolved in dilute DMSO as follows. Each jujube extract (500 μL) was placed into a 10 mL vial and then dried completely at 30 °C under reduced pressure. The residue was weighed and then dissolved in 10% DMSO in water (10 mL).

Analysis of Crude Protein Content. The Kjeldahl N content of dry samples of jujube fruit of seven maturity stages of jujube fruit (S2−S8), weighing 1.0−1.5 g, was determined in triplicate analyses by the Tecator digestion system and the Kjeltec 2300 autoanalyzer (Foss Tecator, Inc., Eden Prairie, MN) according the manufacturer's instructions. Protein content (g/100 g dry wt) was calculated by the formula $N \times 6.25$. There was insufficient material to analyze the first stage (S1).

Analysis of Free Amino Acids. Free amino acid analysis was carried out by ion-exchange chromatography using methods adapted from the literature.[19,25] Briefly, the jujube extract (10 μL) was injected into an Hitachi model L-8800 amino acid analyzer (Hitachi Co. Ltd., Tokyo, Japan) with a column packed with Hitachi custom ion-exchange resin 2622 (4.6 mm i.d. × 60 mm, particle size 5 μm). Lithium citrate buffer and ninhydrin flow rate were 0.35 and 0.30 mL/min, respectively. The column temperature was 30−70 °C, and the reaction coil temperature was 135 °C.

HPLC and LC/MS Analysis of Flavonoids. For quantification of the flavonoids, HPLC-diode array detection (HPLC-DAD) analysis was performed on a liquid chromatography system (Agilent 1200 series, Agilent Technologies, Santa Clara, CA). Data were acquired and processed with Analyst software (Applied Biosystems Inc., Foster City, CA). Extract (20 μL) was injected into the HPLC column [5 μm, 4.6 mm × 250 mm Inertsil ODS-3 V (GL Science Inc., Tokyo, Japan)]. The mobile phase consisted of the following gradient: acetonitrile (A) and 0.5% formic acid (B); (A) = 5% (0−5 min), 18% (5.1−30 min), 70% (30.1−90 min), 90% (90.1−100 min), and 5% (100.1−120 min). The flow rate was 0.8 mL/min at 30 °C. Peaks were monitored at 340 nm, and UV spectra were recorded.

For identification of the flavonoids, HPLC/MS experiments were performed with the 3200 Q TRAP LC/MS/MS system (Applied Biosystems Inc., Foster City, CA) equipped with an HPLC system and using the same methods as above. The HPLC eluate was introduced into the mass spectrometer from 5 to 40 min. MS and tandem mass spectrometry (MS/MS) were operated in the negative-ion mode in the mass range of m/z 1601−200. Helium was used as the collision gas for the MS/MS spectrometric procedures, followed by the isolation of ions over a selected mass window of 2 Da. MS/MS represents multiple stages of precursor ion m/z selection followed by product ion detection for successive progeny ions. Mass selection of the analyte by m/z was followed by fragmentation and analysis of the fragments. For quantification, integrated chromatographic peak areas from each jujube sample were compared to peak areas of known amounts of standard sample.

Determination of Total Flavonoids (TF). Flavonoids are strong chelators. Chelation with aluminum causes a bathochromic shift in the absorption bands of flavonoids yielding a highly colored product.[26] TF content was determined following the procedure adapted from Dewanto et al.[23] Aliquots (1 mL) of the DMSO extract were placed in a 10 mL volumetric flask. To the flask was then added ethanol (60%, 8 mL) followed by $NaNO_2$ solution (5%, 0.2 mL). A solution of $AlCl_3$ (10%, 0.2 mL) was then added after 6 min and NaOH (4%, 0.6 mL) after another 6 min. The total volume was then adjusted with water to 10 mL. The solution was mixed and the absorbance was measured at 415 nm. The TF content from triplicate analyses was expressed as quercetin equivalents (g/100 g dry wt) based on a standard curve of quercetin.

Determination of Antioxidative Capacity. We employed the following four methods that assess antioxidant capacity of the extracts primarily by electron transfer measurement:[27] Folin−Ciocalteu, DPPH, ABTS•+, and FRAP.

Folin−Ciocalteu Reducing Capacity (F−C) Assay. The reduction of complexes of phosphomolybdic/phosphotungstic acids by sample antioxidants, including but not limited to phenolics, was measured by the method of Chew et al., with some modification.[28] Aliquots of the DMSO extract (1.0 mL) were mixed with 10% Na_2CO_3 (1.5 mL) and incubated at room temperature for 2 min. After the addition of 50% Folin−Ciocalteu reagent (500 μL) and water (7 mL), the reaction tube was further incubated for 1 h at room temperature. The absorbance was then read at 700 nm. F−C values were expressed as gallic acid equivalents (g/100g dry wt), based on a standard curve of gallic acid.

DPPH Radical Scavenging Assay. The reduction of the purple-colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to the yellow form by sample antioxidants was measured by the method of Brand-Williams et al.,[29] with some modifications. An aliquot (0.8 mL) of a dilution series of the DMSO jujube extract was added to 0.15 mM DPPH (0.2 mL). The antioxidant BHA was used as a positive control. After a 30 min incubation period at room temperature, the absorbance was read at 517 nm against a blank. The percent reduction

in absorbance was plotted against concentration to determine the EC$_{50}$, defined as the concentration of extract (in μg/mg) that reduced the DPPH radicals by 50%.

ABTS Radical Scavenging Assay. A variation of the Trolox equivalent antioxidant capacity assay (TEAC), the reduction of the blue 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) radical to an uncolored form by sample antioxidants was measured by the method of Re et al.[30] ABTS$^{\bullet+}$ reagent was generated by the reaction of ABTS (7 mM) in H$_2$O with potassium persulfate (2.45 mM) for 24 h in the dark at room temperature. The ABTS$^{\bullet+}$ reagent was then diluted to an absorbance of 0.70 ± 0.02 at 732 nm with phosphate buffer (0.1 M, pH 7.4). The resulting ABTS$^{\bullet+}$ solution (990 μL) was added to a dilution series of the sample (10 μL). After 1 min, the absorbance was read at 732 nm against a blank. The percent reduction in absorbance was plott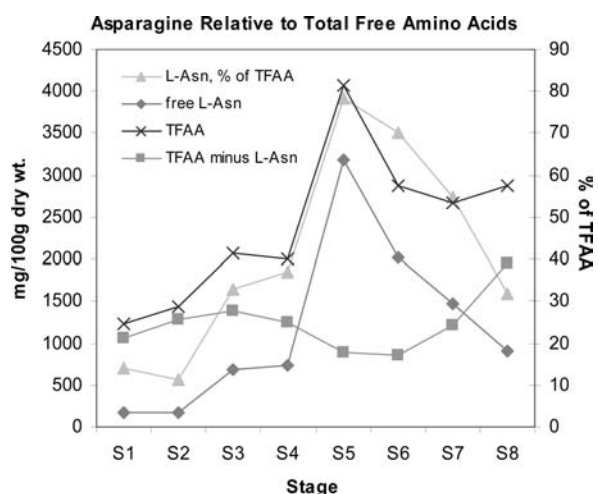ed against concentration to determine the efficient concentration (EC$_{50}$), defined as the concentration of extract (in μg/mg) that reduced the ABTS$^{\bullet+}$ radicals by 50%.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) by sample antioxidants was determined by the method of Benzie and Strain.[31] The FRAP reagent was freshly prepared by mixing acetate buffer (100 mL, 300 mM, pH 3.6), TPTZ solution (10 mL, 10 mM TPTZ in 40 mM/HCl), and FeCl$_3$·6H$_2$O solution (10 mL, 20 nM) in a ratio of 10:1:1 and then adding distilled water (12 mL) at 37 °C. To perform the assay, FRAP reagent (1.8 mL), deionized water (180 μL), and sample (60 μL) were added to a test tube and incubated at 37 °C for 4 min. Absorbance was measured at 593 nm, using FRAP working solution as blank. Sample was diluted when the resulting relative absorbance was outside the range 0−2.0. The antioxidant potential was expressed as moles Fe reduced/100 g dry wt based on a standard curve of reacted FeSO$_4$·7H$_2$O.

MTT Assay for in Vitro Growth Inhibition of Cells. The MTT assay for cell viability, in which the yellow tetrazole, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is reduced to purple formazan in living cells was adapted from the literature.[32] The following reagents and instruments were used: MTT reagent, 5 mg/mL in phosphate-buffered saline (PBS), protected from light, and stored at 20 °C; MEM cell medium (containing 10% fetal bovine serum, 1% penicillin/streptomycin); and microplate reader (Bio-Rad Co., Hercules, CA). Cell lines were seeded into a 96-well microplate (1 × 10^4 cells/well) and incubated for 24 h. Next, cells were treated with four concentrations (1, 10, 50, and 100 μg/mL) of extract for 48 h. The MTT solution (0.1 mg/mL) was then added to each well. After 4 h incubation at 37 °C, DMSO (200 μL) was added to each well. The absorbance (A) was then read at 540 nm. A decrease in A$_{540}$ indicates a decrease in the number of viable cells, reported as percentage inhibition.

Statistical Analysis. Pearson correlation coefficients (r values) between measured parameters were determined and ANOVA tests between the growth stages and extract doses were run with the aid of SigmaPlot 11 (Systat Software, Inc., San Jose, CA).
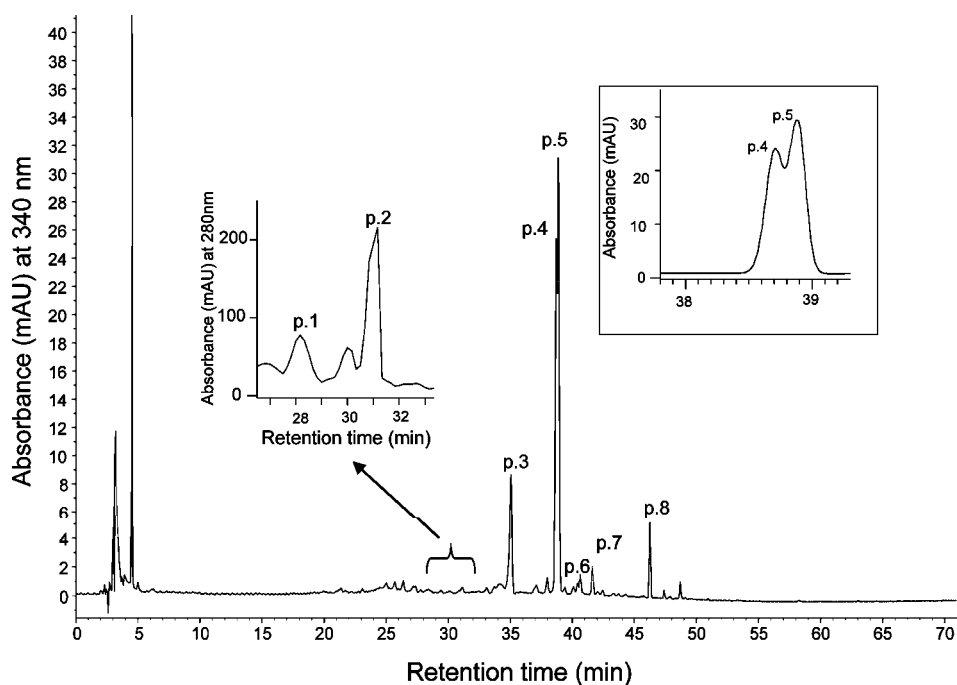

Figure 2. Percent of free L-asparagine relative to total free amino acids (TFAA) in jujube fruits harvested during eight stages (S1−S8) of growth.

Figure 3. HPLC chromatograms of flavonoid compounds of Boeun-deachu jujube pulp (S6). Conditions: column, Inertsil ODS 3v (5 μm, 4.5 mm × 250 mm); column temperature, 30 °C; mobile phase, acetonitrile/0.5% formic acid (gradient mode); flow rate, 0.8 mL/min; detector, 280 and 340 nm.
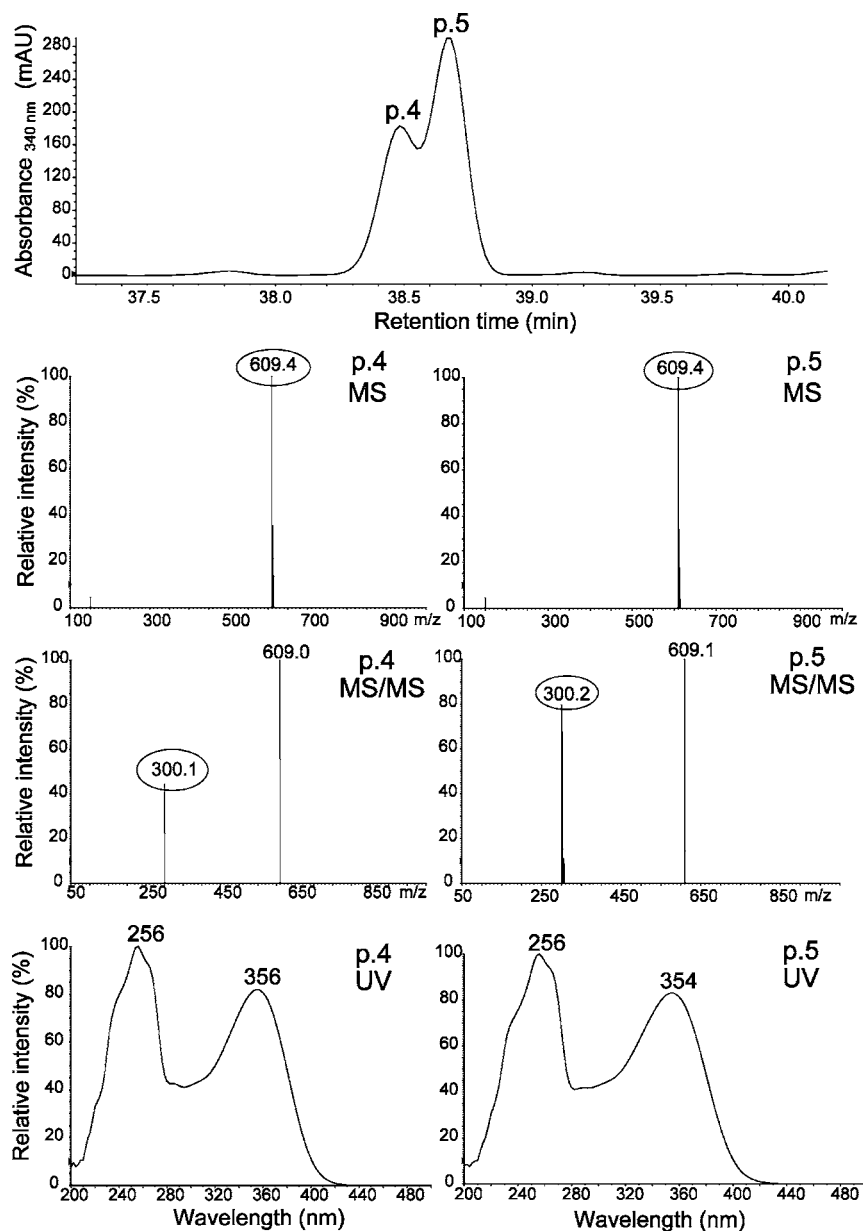
Figure 4. HPLC chromatogram, MS, MS/MS mass spectra, and UV spectra of peaks 4 and 5 of jujube pulp extract from S6.

■ RESULTS AND DISCUSSION

Changes in the Composition of Jujube Fruit during Ripening. *Color, Dimensions, Weights, Moisture, and Protein Content.* Figure 1 shows photographs of eight jujube fruit harvested from 10 to 115 days after flowering of the plant. Table 1 lists the changes in fruit color, size and weight, moisture, and crude protein content during the different stages of growth and maturation of the fruit. Only S7 and S8 are suitable for market, the other stages being too immature. During maturation, the fruits increased in size, water and protein content decreased, and the fruits changed from green to red. These results indicate that the dimensions of the fruit continuously increase and the water and protein content per unit wt continuously decrease during the 115-day growth period of the plant.

Free Amino Acids and Amino Acid Metabolites. Table 2 shows the free amino acid composition in all eight growth stages of jujube. The combined (total) free amino acid and other nitrogen-containing compounds (in mg/100 g dry wt) ranged between 1228.5 (S1) and 4067.5 (S5), a 3.3-fold variation from lowest to highest value. The content increased, peaked, and then decreased again in S6–S8 to ~2800.

Table 2 shows that the jujube fruit contains an exceptionally high content of free Asn, with a maximum value of 3184.7 at the S5 stage. When calculated as percentage of total free amino acids (penultimate row in Table 2), it is clear that L-Asn is a major contributor to free amino acid content, as high as 78.3% at S5. Figure 2 shows that L-Asn levels parallel total free amino acids. When the contribution of L-Asn is subtracted from the total, the free amino acid content shows only minor changes throughout the growing period, except for a significant increase found at the last stage (S8). Free L-Asn decreases during the last three stages relative to its peak level at S5. Because L-Asn serves as a precursor for the potentially toxic acrylamide formed during heat-processing of plant foods,[33] when the fruit is processed, it may be important to select fruit containing a low amount of this amino acid.

The data also show that none of the samples contained free L-Met, and only the last three growth stages contained free L-Pro, which rapidly increased from 113.9 (S6) to 1593.4 (S8). The content of the total free essential amino acid (third row from the bottom in Table 2) paralleled that of total free amino acids (fourth to last row in Table 2).

The extracts also contained several nitrogen-containing amino acid metabolites. These include o-Pea, which was present only in three samples (S4−S6); L-Cit, present in S3−S8, with a maximum at S5; β-Ala, which was present in four samples (S5−S8); EtNH$_2$, which was present in all samples and the content of which continually decreased from S2 (43.4) to S8 (5.77); and Hyl, which was present in all samples and the content of which continually decreased from 44.7 (S1) to 8.91 (S8).

We calculated the contribution of total free amino acids to the total protein determined from Kjeldahl nitrogen, reported in Table 1. The percentage of total free amino acid values shown in the last row of Table 2 increased dramatically from 3.4 (S2) to 62.4 (S8). In the fully ripe fruit, free amino acids are the major contributor, more than protein, to nitrogen content.

Table 3. Flavonoid Compounds Identified by LC-PDA, MS, and MS/MS in the Pulp Extracts from Jujube Fruits

HPLC peak no.	retention time (min)	UV/vis (nm)	$[M - H]^-$ (m/z)	MS/MS fragments	identification
1	28.15	280, 242	577.1	451.1, 425.2, 407.2	procyanidin dimer B2 (PCDB2)
2	31.07	280, 242	289.3	136.0	epicatechin (EP)
3	35.04	354, 254	741.7		unidentified substance (UIS-I)
4	38.70	356, 256	609.4	300.1	quercetin-3-robinobioside (Q-3-RB)
5	38.87	354, 256	609.1	300.2	quercetin-3-rutinoside (Q-3-R)
6	40.44	354, 254	463.6	300.1	quercetin-3-galactoside (Q-3-G)
7	41.62	346, 266, 248	593.7	284.3	kaempferol-glucosyl-rhamnoside
8	46.26	334, 278, 248	716.8	698.4, 641.4, 615.4	unidentified substance (UIS-II)

This could increase the digestibility of the nitrogen present but also could contribute to reactions such as Maillard browning.

Phenolic Content of Jujube Fruit. Jujube fruit is an excellent source of flavonoids, but variation in the content of these compounds by maturity, variety, origin from different geographic locations and soil and climate environments,[34−36] and postharvest changes[37,38] are not well documented. In the present study, we investigated the composition and content in fruits harvested at eight growth stages from the same plant grown under known environmental conditions.

Identification of Individual Flavonoids. Figure 3 shows a chromatogram of the jujube flavonoids separated by HPLC. Peaks 1, 2, 3, 5, 6, 7, and 8 in Figure 3 exhibited the same chromatographic and mass spectrophotometric properties as those precharacterized in our previous study.[8] In that study, the chromatogram of peak 4 was unresolved to peak 5. With some fine-tuning of the HPLC conditions, we succeeded in separating these two peaks. For peak 4, the mass spectra (Figure 4) showed a $[M - H]^-$ ion of m/z= 609.4 and the chromatogram a retention time at 38.70 min. The fragment ion of m/z = 300.1 (quercetin moiety) in the MS/MS spectra was produced by loss of hexose−rhamnose [m/z = 609−300 = 309 = hexose moiety (163) + rhamnose moiety (146)]. From these results and other studies,[39−41] peak 4 was identified as quercetin-3-robinobioside (Q-3-RB). Peak 5 matched the standard quercetin-3-rutinoside with respect to HPLC retention time and UV−vis, MS, and MS/MS spectrum, and thus was identified as such. Figure 4 shows the unique spectra (MS and UV) for peaks 4 and 5. Table 3 lists the HPLC, UV−vis spectra, and mass spectral parameters used to assign structures to the eight compounds. On the basis of these data, procyanidin B2, epicatechin, quercetin-3-O-robinobioside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, and kaempferol-glucosyl-rhamnoside were identified in the extracts. The chromatograms also showed two unidentified flavonoids, labeled UIS I and UIS II. Figure 5 depicts the structures of characterized jujube flavonoids.

Flavonoid Content of Jujube Fruits. Table 4 lists the content of individual flavonoids in jujube determined by UV

epicatechin   procyanidin B2   kaempferol-glucosyl-rhamnoside

quercetin-3-galactoside   quercetin-3-robinobioside   quercetin-3-rutinoside
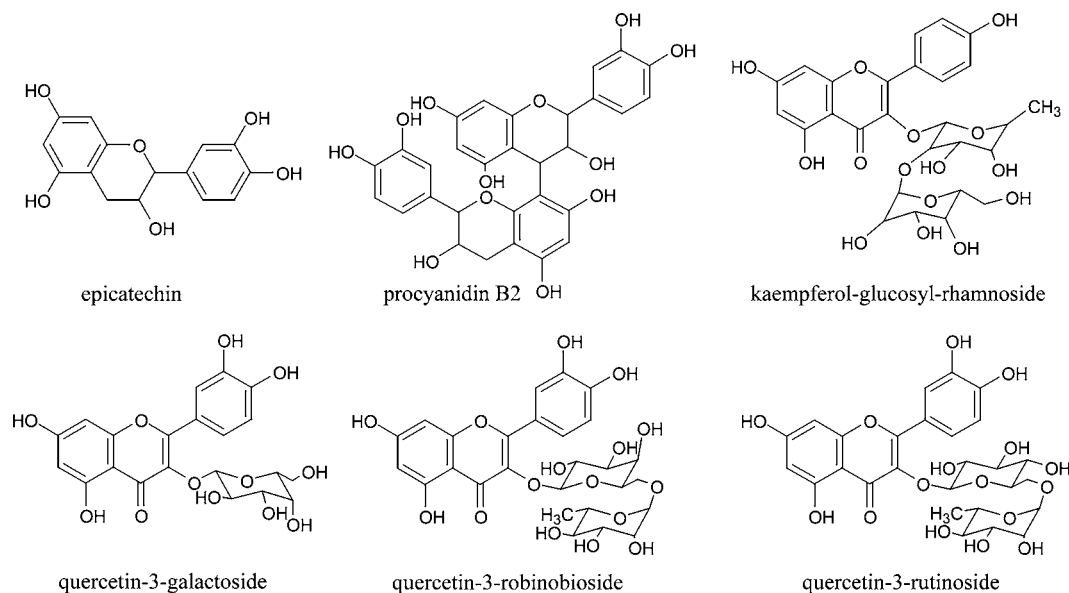
Figure 5. Structure of flavonoids in pulp of jujube fruits evaluated in the present study.

absorption-HPLC (Figure 3). Levels of nearly all the flavonoids, except epicatechin, continuously decreased as the fruits matured, especially in the later stages. Epicatechin levels increased to very high levels during the early stages of maturation from S1 to S6, becoming the most abundant flavonoid in S6 by nearly 2 orders of magnitude. Levels then rapidly decreased to 1/100 of that amount in the fully ripe fruit, S8. The sum of flavonoid values, which appears to change little until S7, is largely governed by these epicatechin levels. The consistent decrease of all other flavonoids during ripening is masked by increasing epicatechin levels during midripening. Thus, total flavonoids (in g/100 g dry wt) ranged from 19.14 to 26.52 in the first six stages, then dropped to 6.46 (S7) and 0.35 (S8) in the later stages, whereas epicatechin, consistently the most prevalent flavonoid in S3–S8, increased from 6.83 (S1) to 22.67 (S6), then decreased to 0.20 (S8). The second most prevalent flavonoid was quercetin-3-rutinoside (Q-3-R), followed closely by quercetin-3-robino-bioside (Q-3-RB). In the immature fruit, S1 and S2, Q-3-R was the most prevalent flavonoid. In the mature fruit, Q-3-R decreased to ∼60 mg/100 g dry wt, which corresponds to ∼16 mg/100 g fresh wt. This is more than 4× higher than previously reported in ripe jujube by San et al.[42] but less than was found in three different varieties in our previous study (296−1147 mg/100g dry wt).[8] San et al. also found catechin, whereas we did not, and found lower levels of epicatechin than that reported here. Because the present study shows that flavonoid content dramatically changes during the ripening process, maturity level would need to be carefully controlled for useful comparisons between laboratories.

Total flavonoid content determined by colorimetry[23] gave lower values than the HPLC sum. But when epicatechin was excluded from the sum, the values were much closer and were well correlated ($r = 0.987$). It appears that some flavonoids are better chelators than others. The benzoyl moiety, which epicatechin is lacking, is a major site of chelation.[26] Thus, the chelation method may provide better comparative results for structurally similar flavonoids.

The last column in Table 4 shows the flavonoid content by HPLC per unit fruit using the HPLC sum of flavonoids and the data in Table 1 on the weight and moisture content of the fruit. These values (in mg/unit fruit) increased from 7.6 (S1) to 264.5 (S6) then dropped to 17.8 (S8). S7 contained only about 12% less than S6 (231.5), whereas the value for S8 was 93% lower. Thus for similar size of the fruits and therefore similar serving size, based on flavonoid content, S7 is a more appealing choice for consumers than S8.

Antioxidative Activities. Flavonoids are phenolic compounds that act as antioxidants primarily by radical scavenging via electron transfer mechanisms and by chelation with transition metals involved in generating free radicals.[26,43] In the present study, we determined antioxidative activity by four independent methods: F−C, FRAP, ABTS, and DPPH. Table 5 shows that all four measures record a decrease (a high EC_{50} value indicates low activity) of antioxidative activity per unit dry wt as the fruit matures although at somewhat different rates. The F−C and FRAP assays were highly correlated with each other ($r = 0.985$), with the total colorimetric flavonoids ($r > 0.97$), and with all the individual flavonoids by HPLC except for epicatechin and UIS II ($r > 0.92$). The DPPH assay was more weakly correlated with the other methods ($r \sim 0.8$), but was better correlated with the HPLC sum of flavonoids ($r = -0.981$), although surprisingly it was not well correlated with any of the individual flavonoids. The ABTS assay gave very

Table 4. Concentration of Flavonoid Compounds in the Pulp Extracts from Eight Growth Stages of Jujube Fruits Determined by HPLC[a]

| growth stage | HPLC peak number | | | | | | | | HPLC sum | colorimetric flavonoids[b] | HPLC sum/unit fruit |
| | p.1 | p.2 | p.3 | p.4 | p.5 | p.6 | p.7 | p.8 | | | |
	PCDB2	EP	UIS-I	Q-3-RB	Q-3-R	Q-3-G	KGR	UIS-II			
S1	0.69 ± 0.037[1]	6.83 ± 0.15[1]	1.63 ± 0.03	6.44 ± 0.23	10.41 ± 0.27	0.05 ± 0.001	0.32 ± 0.004	0.16 ± 0	26.52	14.92 ± 0.12	7.6
S2	0.78 ± 0.13[1]	6.35 ± 0.24[1,2]	1.74 ± 0.05	5.05 ± 0.06	9.99 ± 0.12	0.04 ± 0.001	0.42 ± 0.003	0.19 ± 0.002	24.56	12.65 ± 0.24	8.2
S3	0.40 ± 0.001[2]	14.33 ± 0.23	0.69 ± 0.004	1.85 ± 0.01	2.24 ± 0.01	0.009 ± 0	0.12 ± 0.0002	0.21 ± 0.001	19.84	5.38 ± 0.12	26.3
S4	0.32 ± 0.01[2,3]	16.74 ± 0.02[3]	0.36 ± 0.01[1]	0.85 ± 0.01	1.14 ± 0.01	0.01 ± 0.0002	0.06 ± 0.002	0.17 ± 0.001	19.65	4.78 ± 0.35	52.8
S5	0.25 ± 0.0002[3,4]	17.11 ± 0.06[3]	0.32 ± 0.04[1]	0.53 ± 0.01[1]	0.73 ± 0.02	0.004 ± 0.0003	0.05 ± 0.0002	0.14 ± 0.0008	19.14	2.62 ± 0.33	91.5
S6	0.18 ± 0.0001[4]	22.67 ± 0.66	0.22 ± 0.02	0.41 ± 0.002[1]	0.48 ± 0.004	nd[1]	0.03 ± 0.0001	0.06 ± 0.0002	24.06	1.69 ± 0.25	264.5
S7	0.05 ± 0.001[5]	6.13 ± 0.003[2]	0.05 ± 0.002[2]	0.08 ± 0.001[2]	0.13 ± 0.001[1]	nd[1]	0.008 ± 0.0002[1]	0.02 ± 0.0001	6.46	0.58 ± 0.01	231.5
S8	0.008 ± 0.0002[5]	0.20 ± 0.01	0.02 ± 0.0004[2]	0.05 ± 0.01[2]	0.06 ± 0.002[1]	0.0002 ± 0[1]	0.01 ± 0.0002[1]	0.02 ± 0	0.35	0.31 ± 0.07	17.8

[a]Listed values are average (g/100 g dry wt) ± SD ($n = 3$); nd = not detected. [b]Flavonoids by the chelation method. Shared superscripts within a column mean not significantly different ($p < 0.005$). The value of UIS-I, Q-3-RB, KGR, and UIS-II is expressed as Q-3-R content. Abbreviations: PCDB2, procyanidin dimer B2; EP, epicatechin; UIS-I and -II, unidentified substance-I and -II; Q-3-R, quercetin-3-rutinoside; Q-3-RB, quercetin-3-robinobioside; Q-3-G, quercetin-3-galactoside; KGR, kaempferol–glucosyl–rhamnoside.

Table 5. Antioxidant Activity by F−C, FRAP, DPPH, and ABTS Methods in Extracts of Jujube Fruits[a]

growth stage	F−C value[b] (g/100 g dry wt)	FRAP value (mol Fe^{2+}/100g dry wt)	ABTS value EC_{50} (μg/g)	DPPH value EC_{50} (μg/g)
S1	23.39 ± 2.34[1]	125.95 ± 18.03[1]	25.56 ± 0.20[1]	51.46 ± 0.48
S2	23.33 ± 2.22[1]	117.53 ± 13.67[1,2]	25.89 ± 0.21[1]	64.53 ± 0.93
S3	13.59 ± 1.37[2]	88.73 ± 16.12[1,3]	50.84 ± 1.22[1,2]	154.65 ± 1.34
S4	10.89 ± 1.22[2,3]	73.13 ± 15.56[3]	65.35 ± 1.24[2]	170.47 ± 1.31[1]
S5	9.85 ± 0.78[3]	75.67 ± 14.24[2,3]	61.29 ± 0.67[2]	173.99 ± 1.75[1]
S6	8.11 ± 0.68[3]	54.88 ± 0.07[3]	467.82 ± 3.4[3]	179.11 ± 3.35
S7	3.00 ± 0.29[4]	49.76 ± 17.65[3]	497.14 ± 3.77[3]	627.51 ± 0.99
S8	2.11 ± 0.14[4]	45.78 ± 18.03[3]	2114.02 ± 34.51	846.08 ± 5.25

[a]Listed values are average ± SD ($n = 3$). [b]Value is expressed as gallic acid equivalent. Values with the same superscript are not significantly different ($p < 0.05$).

Table 6. Inhibitory Effects of Jujube Fruit Extracts against Normal Liver (Chang), Normal Lung (Hel299), Cervical Carcinoma (HeLa), Lung Cancer (A549), and Histiocytic Lymphoma (U937) Cells Determined by the MTT Assay[a]

growth stage	dose (μg/mL)	Chang	Hel299	HeLa	A549	U937
S1	1	−0.23 ± 0.21[a]	0.23 ± 0.50	9.91 ± 0.47	7.82 ± 0.17[a]	0.37 ± 0.35[a]
	10	0.02 ± 0.14[a]	12.39 ± 4.75	13.13 ± 0.76	8.14 ± 4.27[a]	0.49 ± 0.60[a]
	50	0.68 ± 0.57[a]	23.08 ± 0.64[a]	22.55 ± 0.18	17.44 ± 0.72[b]	2.94 ± 0.55[b]
	100	0.82 ± 1.11[a,1]	25.35 ± 2.34[a,1]	27.74 ± 2.18[1]	20.50 ± 1.53[b,1]	2.47 ± 0.29[b,1]
S2	1	−0.68 ± 0.18[a]	0.37 ± 1.28[a]	11.96 ± 0.77[a]	0.13 ± 0.30	−0.25 ± 0.11[a]
	10	−0.43 ± 0.27[a]	1.90 ± 0.83[a]	12.30 ± 1.74[a]	10.48 ± 4.77[a]	−0.14 ± 0.20[a]
	50	−1.45 ± 1.19[a]	16.28 ± 2.57	13.05 ± 1.88[a]	18.17 ± 1.97[b]	1.64 ± 0.38[b]
	100	−0.68 ± 0.39[a,1]	25.54 ± 2.48[1]	26.00 ± 0.40[1,2]	14.33 ± 1.22[a,b,2]	2.25 ± 0.61[b,1,2]
S3	1	−0.79 ± 0.16[a]	1.77 ± 1.14[a]	15.80 ± 1.55	0.86 ± 0.96[a]	−0.44 ± 0.78[a]
	10	−0.86 ± 0.35[a]	2.27 ± 0.89[a]	20.84 ± 0.44	1.90 ± 0.59[a]	0.01 ± 0.25[a,b]
	50	−0.49 ± 0.66[a]	6.89 ± 3.35	25.81 ± 0.44[a]	15.12 ± 2.07[b]	0.67 ± 0.22[a,b]
	100	−1.08 ± 1.19[a,1]	16.71 ± 0.27[2]	26.74 ± 2.58[a,1,2]	15.43 ± 3.57[b,1,2]	1.03 ± 0.29[b,3,4]
S4	1	−3.01 ± 1.98[a]	0.72 ± 0.44	12.34 ± 3.40[a]	0.02 ± 0.92[a]	−0.07 ± 0.27[a]
	10	−0.85 ± 0.56[a]	3.78 ± 1.63	15.06 ± 0.28[a]	0.41 ± 0.25[a]	0.07 ± 0.16[a]
	50	−0.39 ± 0.42[a]	8.55 ± 0.89	23.74 ± 1.30[b]	2.39 ± 0.59	0.22 ± 0.14[a]
	100	−0.91 ± 1.36[a,1]	12.91 ± 1.68[2,3]	25.81 ± 3.59[b,1,2]	11.77 ± 0.69[2]	0.86 ± 0.33[3,4]
S5	1	−5.13 ± 4.82[a]	−3.33 ± 1.69	14.88 ± 0.74[a]	0.03 ± 0.19[a]	−0.46 ± 0.42[a]
	10	0.72 ± 1.02[a]	−0.48 ± 1.30[a]	21.03 ± 0.80[a]	2.03 ± 2.24[a,b]	−0.01 ± 0.31[a,b]
	50	11.88 ± 4.31[b]	2.43 ± 1.03[a]	22.33 ± 6.06[a]	4.67 ± 0.67[a,b]	0.03 ± 0.21[a,b]
	100	14.83 ± 3.06[b]	10.39 ± 1.72[3]	23.76 ± 4.80[a,1,2]	5.96 ± 3.37[b,3]	0.42 ± 0.11[b,3,4]
S6	1	−0.24 ± 0.52[a]	−6.04 ± 2.71	12.05 ± 1.55	−0.46 ± 0.45[a]	−0.17 ± 0.19[a]
	10	−0.21 ± 0.58[a]	0.26 ± 1.54	17.88 ± 0.32	−0.02 ± 0.17[a]	0.03 ± 0.16[a]
	50	−0.15 ± 0.52[a]	4.53 ± 1.10[a]	21.87 ± 0.97[a]	0.04 ± 0.74[a]	0.05 ± 0.36[a]
	100	−0.14 ± 0.14[a,1]	6.19 ± 0.62[a,4]	22.73 ± 2.96[a,1,2]	1.13 ± 1.69[a,3,4]	0.07 ± 0.30[a,3]
S7	1	−0.48 ± 0.43[a]	−5.97 ± 2.33	4.83 ± 3.48	0.21 ± 0.06[a]	0.06 ± 0.14[a]
	10	−0.52 ± 0.45[a]	0.00 ± 1.48[a]	11.58 ± 2.26	0.70 ± 0.45[a,b]	0.07 ± 0.13[a]
	50	−0.11 ± 0.53[a]	3.54 ± 2.17[a,b]	18.70 ± 1.00[a]	0.85 ± 0.25[a,b]	0.31 ± 0.29[a,b]
	100	0.14 ± 0.18[a,1]	4.55 ± 0.82[b,4]	20.07 ± 0.70[a,1,2]	0.95 ± 0.07[b,3,4]	0.58 ± 0.11[b,3,4]
S8	1	−0.46 ± 0.70[a]	−4.23 ± 1.30	2.08 ± 1.61[a]	−0.28 ± 0.19[a]	−0.03 ± 0.45[a]
	10	−0.13 ± 0.47[a]	0.50 ± 0.72[a]	2.40 ± 1.95[a]	0.06 ± 0.09[a]	0.13 ± 0.62[a]
	50	0.08 ± 0.27[a]	2.43 ± 1.27[a,b]	12.30 ± 3.07	0.33 ± 0.32[a]	0.25 ± 0.36[a]
	100	0.11 ± 0.05[a,1]	5.12 ± 1.73[b,4]	18.72 ± 1.27[2]	0.40 ± 0.48[a,4]	1.23 ± 0.66[a,2,4]

[a]Listed values are average ± SD ($n = 3$). ANOVA statistical analysis was run within a cell line for 100 μg/mL doses between extract stages (superscript numbers) and for the four different doses by an extract (superscript letters). Values with the same superscript are not significantly different ($p < 0.05$).

different results than the other methods in that it showed a large decrease in antioxidative activity between S5 and S6 that was not reflected in the other methods. This coincides with the maximum level of epicatechin at S6. We do not know the reason for this behavior. Perhaps ABTS is less sensitive to epicatechin than the other flavonoids. At S6, there is a spike in the level of free asparagine, possibly interfering with the assay.

FRAP and Folin−Ciocalteu assays detect only electron transfer antioxidants, whereas ABTS and DPPH are neutralized by both electron transfer (reduction) and hydrogen transfer (quenching) antioxidants.[43] The larger dyes are susceptible to steric hindrance, and Folin−Ciocalteu is susceptible to interference from small reducing molecules.[43] Brand-Williams[29] found that different antioxidant molecules affected the stoichiometry of the DPPH reaction differently. These observations

suggest that the assays might have somewhat different sensitivities to different antioxidative molecules.

Inhibition of Cancer Cells. Normal liver (Chang) and lung (Hel299) cells and three cancer cell lines (HeLa cervical, A549 lung, and U937 lymphoma) were treated with four concentrations (1, 10, 50, and 100 μg/mL) of the S1–S8 jujube extracts, followed by the determination of cell viability by the MTT assay. Because the highest concentration tested inhibited less than 50% of the cells, statistical analysis was performed on the observed percentage inhibition of the highest concentration tested (100 μg/mL) instead of the commonly used IC_{50} parameter.

Table 6 shows that the cell lines HeLa, Hel299, and A549 were all dose-dependently inhibited to a similar degree by extracts of S1. The dose-dependency of the inhibition was determined graphically (results not shown). To prove dose-dependence, analysis of variance (ANOVA) was applied to the dose levels. Because of the low sampling rate, the accuracy of the tests (the power) was below desirable levels; the results must therefore be viewed with caution.

As maturity increased, the rate of cell inhibition progressively decreased for Hel299 and A549 but decreased very little for HeLa. HeLa cell rate of inhibition decreased only from 27.74 (S1) to 18.72 (S8). This inhibition was well-correlated with DPPH ($r = -0.915$). The inhibition of Hel299 and A549 was well-correlated with FRAP, F–C, and total colorimetric flavonoids ($r \geq 0.9$). Chang cells were only dose-dependently inhibited by S5. U937 cells were mostly unaffected by the extracts. The susceptibility appears different for HeLa than for A539 and Hel299 because the HeLa cells were not as affected by maturation or the phenolic concentration, suggesting that jujube extracts may be acting by a different mode against HeLa cells.

Because they were correlated with the DPPH antioxidative measure, there may be an undiscovered nonphenolic antioxidant responsible for the anti-HeLa activity. A related study[44] reported that an extract of the Indian jujube *Ziziphus mauritiana* seeds inhibited Hel-60 Ehrlich ascite carcinoma cells in vitro and in mice. It is unclear why the normal Chang cell line was inhibited by S5. We did not find anything extraordinary in our analysis of the S5 extract. Because triplicate samples (extracts) were analyzed, the observed response is probably not an artifact.

Dietary Significance. Jujube fruits have been shown here to contain six known and two unknown flavonoids. Flavonoid content by HPLC decreased from 26.7 (g/100 g dry wt) in the most immature fruit to 0.35 in the most mature. The antioxidant levels decrease rapidly during ripening, indicating that fruits should be eaten at the earliest time of palatability; however, this may also need to be balanced against the high levels of free Asn, a precursor of acrylamide, found in midmaturity fruits. It would seem that consuming the fruit at the S7 stage might have the greatest benefit on the basis of the following facts: (a) S7 and S8 are the only stages that would normally be considered for market, (b) the flavonoid content of S7 is 13× higher than that of S8, and (c) the Asn content of S7 has dropped by 54% from the maximum at S5.

With respect to bioavailability and plasma levels of ingested phenolics, it has been established that continued consumption of teas results in increases in the concentration of flavonoids in different organs, reviewed in Friedman.[45] Other phenolic compounds are also transformed (conjugated, metabolized, and catabolized) and seem to be sequestered in different organs.[46–48] We therefore do not know whether jujube metabolites would be more or less bioactive in vivo. These considerations suggest that the absorption, metabolism, and anticarcinogenic effects of jujube compounds and extracts from different stages of maturity merit further study in animals and humans.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

A, absorbance; AA, amino acid (standard amino acid abbreviations were used); ABTS, 2,2′-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid); BHA, 2(3)-*t*-butyl-4-hydroxyanisole; DMSO, dimethylsulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC_{50}, median effective dose; EP, epicatechin; FAA, free amino acids; FRAP, ferric reducing/antioxidant power; HPLC, high performance liquid chromatography; HPLC-DAD, high performance liquid chromatography diode array detection; IC_{50}, dose-dependent concentration that inhibited 50% of the cells; KGR, kaempferol–glucosyl–rhamnoside; MEM, Eagle's Minimum Essential Medium; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N, nitrogen; PCDB2, procyanidin dimer B2; Q-3-G, quercetin-3-galactoside; Q-3-R, quercetin-3-*O*-rutinoside; Q-3-RB, quercetin-3-*O*-robinobioside; *r*, Pearson correlation coefficient; ROS, reactive oxygen species; rt, retention time; TEAC, Trolox equivalent antioxidant capacity assay; TF, total flavonoids; TFAA, total free amino acids; (FC, total phenolics; TPTZ, tripyridyltriazine; UIS, unidentified substance; UV–vis, ultraviolet–visible light

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