Acta Alimentaria 51 (2022) 1, 105–114
DOI: 10.1556/066.2021.00186

AKADÉMIAI KIADÓ

Evaluation of the influence of *in vitro* human digestion simulation on the chemical composition and bioactivities of *Ziziphus jujuba* Mill.

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ORIGINAL RESEARCH PAPER

Received: August 19, 2021 • Accepted: December 11, 2021
Published online: February 10, 2022

ABSTRACT

Fruit of *Ziziphus jujuba* Mill. are used as functional foods for centuries due to their rich content and bioactivities. Although *in vitro* antioxidant and hypoglycaemic activity of jujube fruit were investigated previously, the bioavailability phenomenon has been disregarded so far. For this study, 80% ethanol extract of *Ziziphus jujuba* fruit (ZJE) was investigated for its *in vitro* hypoglycaemic and antioxidant potentials, before and after the interaction with simulated human digestion. DPPH scavenging activity, FRAP, CUPRAC, and TOAC assays were used for this purpose. Moreover, inhibition potentials of α-amylase and α-glucosidase enzymes and advanced glycation end products (AGE) were examined for the hypoglycaemic effect. Results indicated that ZJE showed significant antioxidant and dose dependent enzyme and AGE inhibition activity. Nonetheless, subsequent to simulated human digestion *in vitro* bioactivities of ZJE were significantly lowered for bioavailable fraction (IN). Protocatechuic acid (PA) (major phenolic compound of the fruit) contents of the extract and fractions were measured via HPTLC for more accurate understanding of the effects of human digestion and bioavailability profile.

KEYWORDS

Ziziphus jujuba, protocatechuic acid, HPTLC, antioxidant, hypoglycaemic

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1. INTRODUCTION

The genus *Ziziphus* (Rhamnaceae) is widely used in traditional medicine for the treatment of various health problems. This genus is known for its edible fruit with their nutritional and biologically active compounds. Thus, dried fruit has been used as food and food additive for thousands of years (Gao et al., 2013). Additionally, this edible fruit is traditionally used against conditions such as diabetes, blood-borne diseases, tuberculosis, etc. (Hossain, 2019; Mongalo et al., 2020). This has led to many pharmacological activity studies and some utilisations have been scientifically confirmed. The plant has been found to have antioxidant, anti-inflammatory, antimicrobial, cytotoxic, neuroprotective, sedative, and hypoglycaemic activities in previous studies (Zhang et al., 2010; Hossain, 2019). Furthermore, studies on chemical composition of the fruit indicated the presence of various secondary metabolites such as flavonoids, phenolic acids, and saponins, which were reported to be responsible for the attributed bioactivities (Hossain, 2019; Mongalo et al., 2020).

Hyperglycaemia is one of the major symptoms of diabetes and causes lipid peroxidation and membrane damage by producing reactive oxygen species (ROS). Oxidative stress is known to be involved in the etiology of diabetes (Asmat et al., 2016). Additionally, hyperglycaemia may accelerate the formation of AGEs, which are considered to play a pivotal role in the development of diabetic complications. Inhibition of the accumulation of AGE is recognised as a convincing approach in the management of diabetic complications (Peppa and Vlassara, 2005). Besides, intestinal α-glucosidase and pancreatic α-amylase are fundamental enzymes of carbohydrate digestion and their inhibition may be effective in delaying the glucose absorption to restrain postprandial hyperglycaemia (Tadera et al., 2006). Currently-available oral anti-hyperglycaemic drugs may be unsatisfactory due to their possible side effects. Therefore, there is growing interest in discovering safe and effective sources of functional foods in order to tackle the limitations and complications of diabetes. Research on the use of natural antioxidants against hyperglycaemia is becoming important in this perspective (Upendra Rao et al., 2010).

Although there are several *in vitro* studies regarding antidiabetic and antioxidant activities of the jujube, there is limited information about the physicochemical changes during the digestion process. Assessment of bioavailability is an essential step in evaluating the actual biological activity after oral consumption (Barak et al., 2020). *In vitro* gastrointestinal (GI) digestion simulation model has been extensively carried out to estimate the bioavailability of such samples. Although *in vivo* bioavailability tests have better accuracy, *in vitro* model is regarded as an easily-applicable, well-correlated alternative in terms of ethical considerations and time advantages (Barak et al., 2019). Considering all these factors, this study is aimed to evaluate *in vitro* enzyme inhibitory potential and antioxidant activity of 80% ethanolic extract of *Ziziphus jujuba* fruit. Moreover, AGEs inhibitory activity of each fraction was conducted by protein glycation model. In addition, the major phenolic component of the fruit was analysed by high performance thin layer chromatography (HPTLC) method. Furthermore, alterations in chemical composition and biological activities before and after digestion were monitored by *in vitro* GI human digestion simulation method. Thus, the pharmacological activities of the fruit were evaluated in terms of pharmacokinetic parameters and bioavailability data.

2. MATERIALS AND METHODS

2.1. Plant samples

The fruit of *Ziziphus jujuba* was collected in September 2019 from Muğla province (Turkey). After harvesting, the fruit pieces were frozen at −20 °C until extraction process. The authenticity of the plant material was confirmed by H. Bardakci, and a voucher specimen was deposited at Acibadem University Faculty of Pharmacy Herbarium (ACUPH 00001).

2.2. Preparation of extracts

Fresh fruit of *Ziziphus jujuba* (1 kg) was cut into slices, seeds were removed and the fleshy portion was mashed with an electrical blender, then extracted with 80% ethanol for 1 h under sonicator and 3 h on shaker (120 r.p.m.) with overnight maceration at dark, room temperature (25 ± 2 °C). The extraction process was repeated three times, and the extracts were filtered using a Whatman filter paper each time. The filtrates were concentrated in rotavapor 80% ethanolic extract of *Z. jujuba* (ZJE) yielded 31% of fresh weight. Extracts were lyophilised and stored at –20 °C until further use. The lyophilised ZJE was dissolved in water prior to the experiment as non-digested (ND) sample.

2.3. *In vitro* human digestion simulation procedure

The simulation of human GI digestion model was operated as previously indicated (Barak et al., 2019). The sample solution was added to 20 mL of gastric environment, and peristaltic movement was imitated by incubating the sample in a shaking water bath for 2 h. The mixture was reserved to represent the post gastric (PG) sample. After 2 h incubation in intestinal medium with cellulose dialysis tubing with sufficient amount of powdered $NaHCO_3$, the content that diffused into the dialysis membrane was referred to as IN (i.e., modelling the absorption) and the solution remaining outside the membrane was named as OUT (i.e., modelling the intestine).

2.4. Quantification of the major bioactive metabolite by HPTLC

Protocatechuic acid (PA) contents in plant extract, PG, and IN fractions of *Z. jujuba* were determined by using the previously described method of Bardakci et al. (2020). A stock standard solution (1 mg mL^{-1}) of PA was prepared in MeOH. Standard contents were obtained by comparing AUCs with the calibration curve of standards at 254 nm. The presence of standards in extracts was ensured by comparison of both retention factors (R*f*) and overlaying UV spectra of each extract and standards. Quantity of the PA was determined by comparison of the intensity of diffusely reflected light of the extract and fractions with the standard compound.

2.5. Estimation of *in vitro* phenolic profile

2.5.1. Total phenolic content assay. The evaluation of total phenolic contents of the samples was executed according to a previously applied method (Bardakci et al., 2020). FCR (Folin–Ciocalteu reagent) was used, and absorbance was read in 765 nm.

2.5.2. Total flavonoid content assay. Total flavonoid contents of the fractions were measured as a method previously described by Barak et al. (2020). After the incubation period, measurement of the absorbance was conducted at 415 nm.

2.6. Estimation of *in vitro* antioxidant activity

2.6.1. DPPH radical-scavenging activity. The scavenging of DPPH radicals was assessed determined according to the method described previously (Barak et al., 2019). Butylated hydroxytoluene (BHT) was used as the reference, and the activity was expressed by the EC_{50} value defining the concentration showing 50% activity.

2.6.2. Cupric reducing antioxidant capacity (CUPRAC). CUPRAC activity of the samples were measured according to the method specified before by Bardakci et al. (2020). After the incubation period, the absorbance was measured at 450 nm in a microplate spectrophotometer.

2.6.3. Ferric reducing antioxidant power (FRAP). The redox-linked colorimetric method described by Kurt-Celep et al. (2020) was used to estimate FRAP activity of the samples. After the incubation period, the absorbance of the mixture was read at 593 nm using the plate reader.

2.6.4. Determination of total antioxidant capacity. Total antioxidant capacities of the extracts were determined according to the method described by Kurt-Celep et al. (2020). Following the 90 min incubation period, absorbance was measured at 695 nm.

2.7. *In vitro* hypoglycaemic activity assays

2.7.1. α-Amylase inhibition assay. The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA), according to the method of İnan et al. (2020).

2.7.2. α-Glucosidase inhibitory activity. The α-amylase inhibition assay was carried out spectrophotometrically as described previously by İnan et al. (2020).

2.7.3. Inhibition activity of the advanced glycation end-products. The AGE inhibition activities of the different concentrations of plant extracts were measured according to the method described by İnan et al. (2020).

2.8. Statistics

All experiments and tests were performed in triplicate. GraphPad Prism (6.1) software program was used for all data. 1-way ANOVA Tukey's Multiple comparisons analysis section was used for the results of antioxidant experiments. 2-way ANOVA Sidak's Multiple comparisons analysis section was used for the results of anti-diabetic assays. $P < 0.05$ was set as statistically significant difference for all tests.

3. RESULTS AND DISCUSSION

3.1. *In vitro* phenolic content and antioxidant activity assays

Two different assays were conducted on ZJE for evaluating total amounts of phenolics and flavonoids and their variation after the steps of simulated *in vitro* digestion. Results given in Table 1 showed that total amounts of phenolics and flavonoids slightly increased after the gastric phase, nonetheless, amounts significantly decreased in the bioavailable part. In addition, four different studies were conducted to determine *in vitro* antioxidant potential of fruit and variations subsequent to digestion simulation. Results from Table 1 revealed that ZJE has notable metal reducing capacity, however, after digestion, bioavailable counterparts have significantly lower bioactivities. The same phenomenon was observed for free radical scavenging potential of ZJE (Table 1).

3.2. HPTLC analysis

For this study, the amount of protocatechuic acid (PA), known major metabolite of ZJE, was measured before and after simulated digestion via HPTLC. Results demonstrated that PA content of the extract slightly diminished after gastric digestion, decrement was also determined for the bioavailable fraction. Quantitative results are given in Table 2 and chromatogram and UV spectra are shown in Fig. 1.

3.3. *In vitro* hypoglycaemic activity assays

Determination of *in vitro* hypoglycaemic potential of ZJE was done via α-amylase and α-glucosidase assays, and also by measuring AGE inhibition capacity. Results indicated that, likewise *in vitro* antioxidant bioactivity, hypoglycaemic potential significantly declined for all assays after simulated human digestion. Prior to digestion simulation, ZJE showed significant

Table 1. In vitro antioxidant activity of ZJE before and after simulated human digestion

	ND	PG	IN
DPPH	$2300^{a} \pm 11.7$	$2160^{b} \pm 9.58$	$12000^{c} \pm 21.9$
FRAP	$1.06^{a} \pm 0.01$	$1.06^{a} \pm 0.01$	$0.55^{b} \pm 0.01$
CUPRAC	$38.8^{a} \pm 2.06$	$47.9^{b} \pm 2.83$	$34.1^{a} \pm 2.15$
TOAC	$86.3^{a} \pm 0.04$	$85.6^{b} \pm 0.01$	$62.9^{c} \pm 0.03$

ND: non-digested; PG: post-gastric; IN: bioavailable.
DPPH: 2,2-diphenyl-1-picrylhydrazyl; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and EC_{50} value of the reference compound (BHT).
FRAP: Ferric reducing antioxidant power; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and as mM $FeSO_4$ equivalents in 1 g sample.
CUPRAC: Cupric reducing antioxidant capacity; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.
TOAC: Total Antioxidant Capacity; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.
[a, b, c]: Different letters in the same row indicate significant difference ($P < 0.05$).

Table 2. HPTLC quantification data for protocatechuic acid and *in vitro* phenolic profile

	Protocatechuic acid* (w/w%)	CV (%)	TPC$^{A\#}$	TFC$^{B\#\#}$
ND	0.0956	1.32	44.08^{a} ± 0.12	19.86^{a} ± 0.36
PG	0.0807	0.38	45.18^{b} ± 0.68	21.41^{b} ± 0.44
IN	0.0665	0.45	24.46^{c} ± 0.21	11.71^{c} ± 0.19

* Samples and standard compounds were applied in triplicate.
ND: non-digested; PG: post-gastric; IN: bioavailable.
$^{a, b, c}$: Different letters in the same row indicate significant difference ($P < 0.05$).
TPC: Total phenolic content; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.
TFC: Total flavonoids content; Results are expressed as the mean of triplicates ± standard deviation (S.D.) and as mg quercetin equivalents (QE) in 1 g sample.

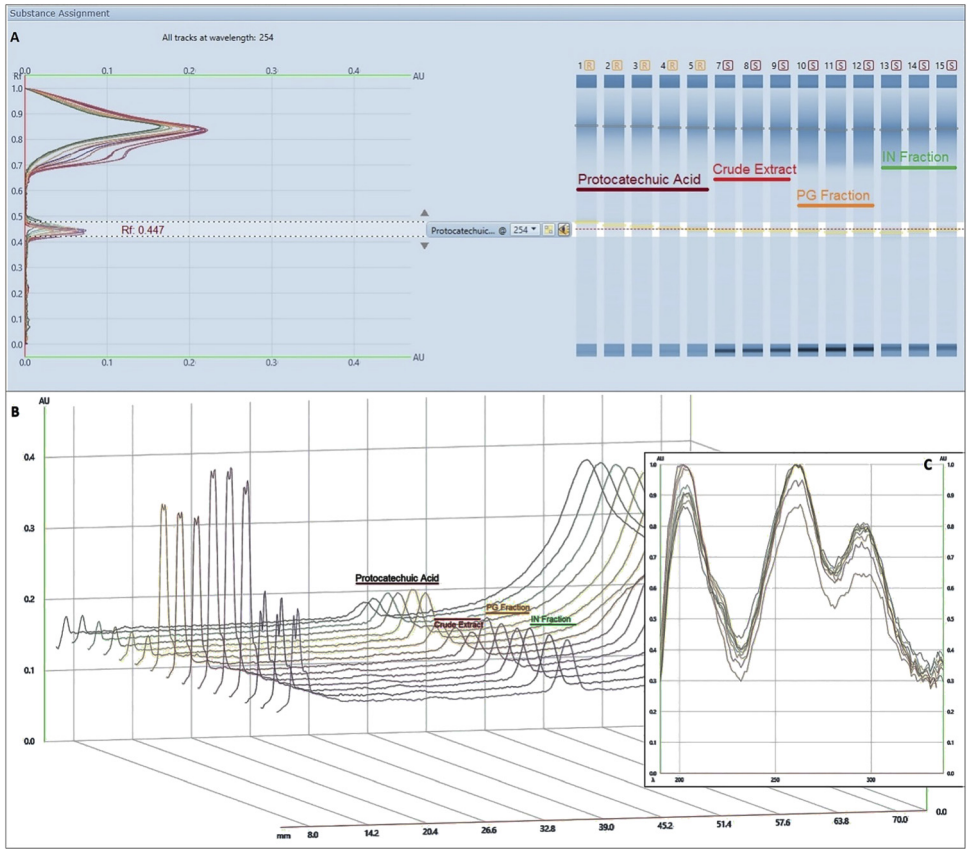

Fig. 1. A) HPTLC chromatogram of standard protocatechuic acid and test samples at 254 nm. B) HPTLC chromatogram of standard protocatechuic acid and test samples at 254 nm. C) Overlapped UV spectra standard protocatechuic acid and test samples

dose dependent enzyme and AGE inhibitory potentials though lower than reference substances (Table 3).

The use of jujube fruit both as medicine and food dates back to nearly 4,000 years ago. Also, jujube has been traditionally used against a panel of health problems (Gao et al., 2013). Along with being grown naturally, jujube is cultivated in a number of regions in Turkey (particularly, the coastal regions) (Bilgin, 2020).

Most of the previous studies overlooked the bioavailability phenomenon and its indisputable influence on the activity potential. After being ingested, the bioactive compounds become susceptible to the harsh conditions of the GI tract including body temperature, pH changes, enzymatic activity, etc. Besides, interactions of bioactive molecules with macromolecules present in the plant matrix (i.e. hemicelluloses, fibres, etc.) also affect bioavailability (Barak et al., 2019; İnan et al., 2020).

The current study was designed to assess the role of bioavailability on the bioactivity of Turkish jujube fruit. Miscellaneous studies investigated the phenolic content of Turkish jujube in terms of both total content and molecules individually (Tepe and Doyuk, 2020). Their results concluded that jujube fruit is an excellent source of phenolic compounds. However, they did not take the bioavailability phenomenon into consideration. The current study seems to be the first one delving into the bioavailability of phenolic content and its effect on certain bioactivities. Total phenolic and flavonoid contents of both non-digested and digested fractions of jujube fruit are presented in Table 2. Several studies reported the same downward trend with the phenolic content (Barak et al., 2019; İnan et al., 2020), indicating that specific GI conditions affect these molecules significantly. Likewise, jujube was noted to contain a substantial amount of various phenolic acids. Protocatechuic acid was reported to be the predominant (Zhang et al., 2010) or one of the major (Tepe and Doyuk, 2020) phenolic acids in the fruit. Previous studies have explicitly shown that protocatechuic acid has a marked antidiabetic effect (Kakkar and Souravh, 2014). Considering that the major component of *Ziziphus jujuba* is protocatechuic acid, it can be assumed that the antidiabetic activity is due to this compound. Results in Table 2 show that,

Table 3. Enzyme and AGE inhibitory activities of the samples

	ND	PG	IN
α-Amylase^{A} (2.5 mg mL^{-1})	65.41^{a} ± 2.89	74.01^{b} ± 1.16	39.43^{c} ± 5.28
α-Amylase^{A} (5 mg mL^{-1})	77.19^{a} ± 2.60	96.19^{b} ± 3.70	54.32^{c} ± 0.97
α-Glucosidase^{B} (2.5 mg mL^{-1})	45.98^{a} ± 1.28	72.30^{b} ± 0.24	40.35^{c} ± 0.38
α-Glucosidase^{B} (5 mg mL^{-1})	64.93^{a} ± 3.11	81.98^{b} ± 0.67	43.90^{c} ± 0.17
AGE^{C} (2.5 mg mL^{-1})	76.54^{a} ± 2.06	95.54^{b} ± 1.34	54.28^{c} ± 2.33
AGE^{C} (5 mg mL^{-1})	88.24^{a} ± 1.90	105.88^{b} ± 3.74	68.76^{c} ± 0.84

AGEs: Advanced glycation end products; ND: non-digested; PG: post-gastric; IN: bioavailable.
^{A}: Acarbose was used as control group with 74.14 ± 4.94% inhibition at 1 mg mL^{-1}, 57.55 ± 8.10% inhibition at 0.5 mg mL^{-1}.
^{B}: Quercetin was used as control group with 79.94% inhibition at 1 mg mL^{-1}, 56.36 ± 0.03% inhibition at 0.5 mg mL^{-1}.
^{C}: EGCG was used as control group with 97.19 ± 0.78% inhibition at 1 mg mL^{-1}, 87.10 ± 1.01% inhibition at 0.5 mg mL^{-1}.
$^{a, b, c}$: Different letters in the same row indicate significant difference ($P < 0.05$).

even though amount of protocatechuic acid decreased in bioavailable fraction, there is still a significant level of bioavailability. A similar drop in the amount of this molecule was seen in the intestinal phase, compared to the gastric counterpart (Wang et al., 2019). Such declines in the amounts are expected, since mono-ring phenolics were previously documented to be negatively affected by alkaline pH (Friedman and Jürgens, 2000).

Various studies, albeit without mentioning bioavailability, indicated that fruit extracts of jujube exert a considerable antioxidant activity (Rajaei et al., 2020). Lately, Turkish jujube was shown to be a potent scavenger of NO and DPPH radicals (Tepe and Doyuk, 2020). Similarly, jujube fruit was shown to have high potential of metal reducing activity as well (Gündüz and Saraçoğlu, 2014). This high activity is expected given the antioxidant-rich content of the jujube fruit. Comparative studies illustrated that each tissue of the fruit possesses high antioxidant capacity, with the peels being the most effective, since they had the highest total phenolic and flavonoid contents (Zhang et al., 2010). Our results in Table 1 revealed the same activity potential of the jujube fruit. The bioavailable IN fraction was predictably found to be less active, given that this fraction was also detected to contain lower amounts of antioxidant phenolics together with the major phenolic acid, protocatechuic acid. These data reflect that bioactivity is as susceptible to the effects of digestion as the active components.

WHO estimates that there will be more than half a billion people with type II diabetes by 2030 (Yazdanpanah et al., 2017). Given these data, new strategies are necessary to fight diabetes. The decoction prepared from jujube fruit was reported to be used against hyperglycaemia in Turkey (Polat and Satıl, 2012). Besides, a randomised clinical trial on 116 diabetic participants revealed that the application of jujube fruit infusions significantly improved HbA1c levels and lipid profiles (Yazdanpanah et al., 2017). Similarly, another recent randomised clinical trial indicated that whole-dried fruit consumption for 12 weeks ameliorated insulin resistance (Irannejad Niri et al., 2021). Tackling postprandial hyperglycaemia is also regarded as an important approach to diabetes. α-amylase and α-glucosidase are important enzymes having role in the digestion process of sugar molecules. Table 3 reveals that the samples considerably suppressed both mentioned enzymes. Besides, it should be noted that a decrease in the inhibition activity of the bioavailable IN fraction was observed. Given the decreases in the phenolic contents of the IN fraction, as shown in Table 2, such reductions are predictable. Similarly, the methanolic extract of jujube fruit from Iran significantly inhibited α-amylase (Afrisham et al., 2015). Table 3 demonstrated that jujube fruit also inhibits AGE-formation dose-dependently, in spite of the decrease in the IN fraction. Likewise, plants rich in phenolic antioxidants were previously reported to block AGE-formation and possess high antiglycation potential (İnan et al., 2020). These data suggest the potential effect of jujube against both postprandial hyperglycaemia and its complications.

4. CONCLUSIONS

In conclusion, *in vitro* antioxidant and antidiabetic activities of *Ziziphus jujuba* fruit extract were investigated with several assays. Also, *in vitro* human digestion simulation was employed to further understand the effect of bioavailability on the bioactivity. HPTLC analysis was employed before and after digestion procedure to determine the amount and variation of the known major metabolite, protocatechuic acid. Results of the study demonstrated that *Ziziphus jujuba* fruit extract has significant antidiabetic potential even though it is affected by human digestion.

ACKNOWLEDGMENTS

This study was supported by Acıbadem Mehmet Ali Aydınlar University Commision of Scientific Projects. (No: 2020/02/04).

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