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ARTICLE

Cell Viability Effects and Antioxidant and Antimicrobial Activities of Tunisian Date Syrup (Rub El Tamer) Polyphenolic Extracts

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The aqueous−acetone polyphenolic extract of the traditionally derived date syrup, known as "Rub El Tamer", was analyzed using RP-HPLC-DAD and ESI-MS. The phenolic content of the extract was 394.53 ± 1.13 mg per 100 g of syrup with caffeoylsinapylquinic acid as the most abundant compound (72.23%). The extract exhibited strong antioxidant activities as evaluated using the ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) methods. The extract antimicrobial potential against a range of microorganism strains showed that *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus cereus* were the most sensitive bacteria with MBC in the range of 0.5−0.05 mg/mL. Furthermore, in the presence of the syrup extract (8.18−131 μg/mL), the Human SH-SY5Y neuroblastoma and the 3T3 fibroblast cell lines showed dissimilar reduction of viability suggesting a higher cytotoxic effect against tumorigenic cells. Our results provide new insights into date syrup characterization which should stimulate further studies of this hot desert resource.

KEYWORDS: Antioxidant; antimicrobial; antiradical; cell viability; date syrup; polyphenols; RP-HPLC−MS

INTRODUCTION

Date syrup, namely "Rub el Tamer", is a common traditionally derived date product generally used for softening and preserving dates in jars and in homemade confectionery. It is widely consumed in Tunisia, especially, during the cold periods of the year. This syrup is mainly used for the preparation of cakes, cookies and homemade confectionery. Particularly, in many Arabic countries, it is mainly poured on cooked dough (asseeda) on specific occasions, such as the celebration of the Muslim's Prophet Mohamed's birthday (*1*). Early records revealed that date syrup, even more concentrated than the date itself and easily turned into a nutritious drink, may have been an overland export article between the Gulf area and China in the past (*1*). Even though a different syrup type could be obtained from date palm sap, locally called "Legmi" syrup (*2*), the preparation of the common fruit syrup "Rub el Tamer" is more frequent. The latter is prepared by mixing the fruits with water (ratio 1:1) and boiling the mixture for about 1.5 h. After a filtration through a cloth, the juice is collected. The presscake was supplemented by water and boiled again. Following four times repeated extraction with water, the combined collected juice is filtered and boiled down to obtain the syrup (*1*). Dates (*Phoenix dactylifera* L.) are produced largely in the hot desert regions of the world (*3*). Although Tunisia accounts for only 2% of world date production, it is the world-leading producer of *Deglet Nour* variety (*1*). In addition, Tunisia contributes with 55% of total quantities of dates imported in the European Union. This fruit is listed in folk remedies for the treatment of various infectious diseases (*4*). Particularly, it is a highly nutritious food product, exceptionally rich in potassium and extremely low in sodium, thus desirable for hypertensive persons (*5*, *6*).

During recent decades, evidence for the role of polyphenols in the prevention of degenerative diseases such as cancer and cardiovascular diseases is well reported (*7*, *8*). Therefore, plant-derived polyphenolic substances have attracted a great deal of attention for their health promoting properties. Particularly, extensive studies have been focused on the positive role of fruit and vegetable polyphenols as free radical scavengers and antimicrobials and to control human and animal cell proliferation (*9*, *10*). Recently, the limited scientific works on date fruit reported its richness in polyphenolic compounds (*11*, *12*). In addition, to our knowledge, there is no work on the derived date syrup product concerning its polyphenolic fraction and biological potentials. Thus, herein, the objective of the study is to identify and quantify the phenolic content (LC-DAD−MS) and to evaluate the antiradical (DPPH, ABTS) and the capacity to reduced Fe(III) at neutral pH (FRAP assay) as well as antimicrobial activities of the aqueous−acetone extract obtained from Tunisian date syrup. In addition, the effect of the extract on the cell viability using human tumorigenic SH-SY5Y neuroblastoma

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and nontumorigenic 3T3 fibroblast cells was investigated. The authors believe that the present work will contribute to the scientific knowledge about date syrup and may stimulate further studies into this byproduct of a hot desert noble natural resource.

MATERIALS AND METHODS

Standards, Solvents, and Reagents. Polyphenolic standards, solvents, $K_2S_2O_8$, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the nutrient mixture F-12 HAM were from Sigma-Aldrich (France). Folin−Ciocalteu reagent, $FeCl_3$-($6H_2O$) and 2,4,6-tripyridyl-*s*-triazine were from Fluka (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Standard stock and working (100 mg/L) gallic acid solutions were prepared in methanol. Water was distilled and filtered through a Milli-Q apparatus before use.

Sample Preparation. Three homemade date syrup samples were kindly provided by Tunisian families (Kebili and Tozeur regions) in 2009. In the laboratory, samples were divided in aliquots (100 g) and frozen at −20 °C until used.

Polyphenol Extraction. The sample (1 g) was mixed with 3 mL of water and sonicated for 20 min. The mixture was supplemented by 7 mL of cold acetone (−20 °C), centrifuged at 10000*g* for 15 min at room temperature. The supernatant was collected, pooled and concentrated using a rotary evaporator (60 °C) to a final volume of 3 mL (*9*). To prevent oxidation of the polyphenols, extraction was rapidly achieved and extracts were immediately used or stored at −20 °C until further use.

Colorimetric Estimation of Total Phenolic Content. Prior to HPLC analysis, the total phenolic content (TPC) was estimated spectrometrically using the Folin−Ciocalteu essay described by Singleton et al. (*13*) using gallic acid as a standard (*14*). TPC was expressed as gallic acid equivalents (GAE) per 100 g of fresh weight (fw).

Reversed Phase HPLC. Phenolic compounds were analyzed using the previously described liquid chromatography technique (*15*) with slight modifications. A HPLC system (Bruker Daltonics, GmbH, Germany), having a diode array detector model D166, an L-7200 autosampler and an L-7100 pump (Merck), was used. Data were processed with Merck-Hitachi D-7000 chromatography data station software. The separation was achieved on a Merck LiChrospher 100 RP-18 (5 μm, 25 × 4 mm) column at room temperature. The mobile phase comprised (A) 1% formic acid in water and (B) methanol, which were previously degassed with a sonicator system. The solvent gradient started at 95% A and 5% B, reaching 75% A at 10 min, 65% A at 30 min, 55% A at 35 min, 55% A at 40 min, 50% A at 45 min, 45% A at 50 min, 30% A at 53 min, 25% A at 56 min, and 20% A at 60 min, followed by a post-time isocratic plateau for 10 min at 95% A before the next injection. The flow rate was 0.8 mL/min, and the injection volume was 20 μL. The monitoring wavelengths were 280 and 350 nm (*9*, *15*). Quantification of the identified phenolic compounds was performed by correlating the measured peak area with the calibration curves obtained with reference compounds. For gallic acid and vanillic acid esters, HPLC quantification was based on free gallic and vanillic acids calibration curves.

LC−Electrospray Ionization (ESI)-MS. The LC−ESI-MS system consisted of an Agilent LC 1100 series (Agilent Technologies, Inc., CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion trap mass analyzer. The ESI was operated in the positive mode with ESI source probe at 250 °C; CDL at 250 °C, block at 240 °C, flow gas (N_2) at 4.5 L/min, probe voltage 4.5 kV, fragmentor voltage 20 V, and a nominal mass range up to *m*/*z* 800.

Antioxidant Activity. The antiradical activity of the phenolic extracts was evaluated as the scavenging of the free anionic $DPPH^{\bullet}$ radical as described by Tuberoso et al. (*16*). The Trolox equivalent antioxidant capacity (TEAC) was calculated from the equation determined from linear regression after plotting known solutions of Trolox (0.05 to 0.8 μM).

A second functional assay was also used based on the cationic $ABTS^{\bullet+}$ radical-scavenging activity (*17*). The radical was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate for 16 h at room temperature in the dark, then diluted to get an absorbance of 0.70 ± 0.02 at 734 nm. 50 μL of the sample was added to 950 μL of the ABTS solution and incubated for 10 min. The absorbance recorded at 734 nm against a methanol blank was compared to the antiradical potency of Trolox, used as a reference.

In order to determine the capacity to reduce Fe(III) at neutral pH, the FRAP method of Hang et al. (*18*) with slight modifications was used. The FRAP reagent contained 2.5 mL of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer at pH 3.6. Freshly prepared FRAP reagent (250 μL) was mixed with 40 μL of the sample and incubated for 10 min at room temperature. Absorbance at 593 nm was read against a reagent blank containing distilled water. Aqueous solutions of known Fe(II) concentrations in the range of 25−200 μM ($FeSO_4 \cdot 7H_2O$) were used for calibration. The change of absorbance ($\Delta A = A_{10min} - A_{0min}$) is calculated and related to ΔA of an Fe(II) standard solution. A is linearly proportional to the concentration of antioxidant.

Antimicrobial Tests. To assess the antimicrobial activity of the extracts, the following microorganisms were used: Gram positive *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 106510), *Bacillus cereus* (ATCC11778), Gram negative *Pseudomonas aerogenosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), a *Salmonella* sp. strain isolated from food and the yeast *Candida albicans* (ATCC 14053). Susceptibility of the test organism to the extract was determined by employing the standard disk diffusion technique as described by Fattouch et al. (*15*). Inhibited microorganisms were also tested using the broth microdilution method (*19*) to determine the minimum inhibitory and bactericidal concentrations (MIC and MBC). A 100 μL amount of the diluted working extract or pure phenolic solution and 100 μL of the bacterial suspensions (5×10^5 cfu/mL) were added in the microwells. After incubation at 37 °C for 24 h, bacterial growth was revealed by the presence of turbidity and a "pellet" on the well bottom. MICs were determined as the first well in ascending order that did not produce a pellet. To confirm MIC and establish MBC, 25 μL of the broth was removed from each well and inoculated on Muller Hinton (MH) plates. After overnight incubation at 37 °C, the number of surviving organisms was determined; MBC was determined when 99.9% of bacteria were dead.

Human Cell Culture and Viability. Human tumorigenic neuroblastoma SH-SY5Y cells and nontumorigenic 3T3 fibroblast cells were obtained from the European cell collection. Neuroblastoma cells were cultivated in DMEM:F-12 HAM (1:1 v/v) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in the presence of 1% penicillin/streptomycin. Fibroblast cells were cultured in DMEM containing L-glutamine, 1 g/L of D-glucose, 10% heat-inactivated FBS and 1% of mixed of antibiotics (streptomycin, penicillin and neomycin). Cells were grown in 75 cm^2 cell culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2 in air (*20*) and then cultivated in 60 mL flasks until 90−95% confluence. After trypsin treatment, cells were resuspended in medium at 3×10^6 cells/mL. Cell viability was measured in 96-well plates based on the mitochondrial metabolic activity using the MTT colorimetric assay (*21*). Briefly, cells having viability higher than 95% (*22*) were supplemented by 200 μL of diluted date syrup extract (8.18.−131 μg phenolics/mL) and incubated for 1, 3, 6, 24 h at 37 °C. Following extract removal, 200 μL of MTT (1 mg/mL) was added to the cells and incubated at 37 °C for 3 h. Then, MTT was removed and the colored formazan was dissolved in DMSO (*23*). Using a Bio-Rad 3350 microplate reader, the absorbance (Abs) was determined at 540 and 690 nm as a background. Cell viability (%) was expressed as percentage of the untreated control as follows:

$$\%\ \text{cell viability} = (\text{Abs of treated cells}/\text{Abs of untreated cells}) \times 100$$

Statistical Analysis. All tests and analyses were run in triplicate and averaged. Quantitative presented data are means ± standard deviations ($n = 3$). One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Differences of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) Estimation. Folin−Ciocalteu is a widely used and reported method for the simple and rapid estimation of total phenolics. The Tunisian *Deglet Nour* derived

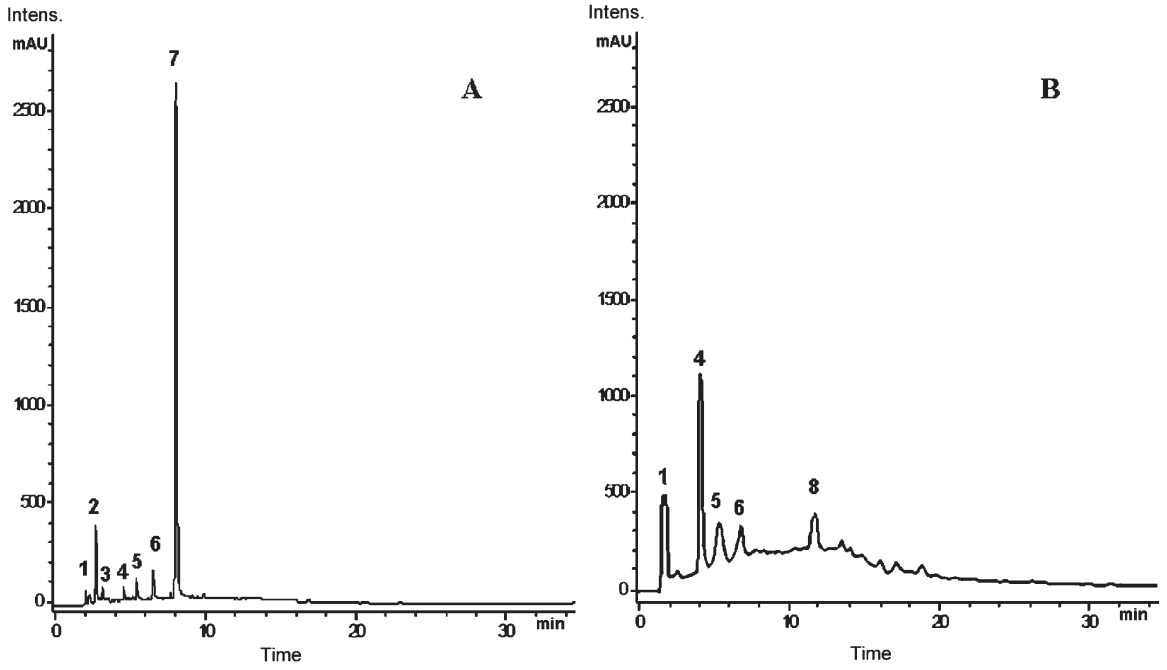

Figure 1. Typical HPLC profile of date syrup aqueous−acetone extracts with detection at 280 (**A**) and 350 nm (**B**). Peaks: (1) coumaric acid, (2) ester of gallic acid, (3) ester of vanillic acid, (4) cinnamic acid, (5) 3,4-dicaffeoylquinic acid, (6) 5-*o*-caffeoylshikimic acid, (7) caffeoylsinapylquinic acid, (8) caffeic acid.

Table 1. LC−ESI-MS Characteristics of the Identified Polyphenols in Date Syrup Aqueous−Acetone Extract[a]

peak no.	t_R (min)	λ_{max}	$[M + H]^+$	phenolic compound	content (mg/100 g fw)	SD
1	2.2	310	165	coumaric acid	23.03	1.23
2	2.8	270	277	ester of gallic acid	6.79	0.05
3	3.6	254−294	355	ester of vanillic acid	2.55	0.03
4	4.5	322	373	cinnamic acid	35.79	2.97
5	5.2	296−326	517	3,4-dicaffeoylquinic acid	13.86	0.53
6	6.9	274	337	5-*O*-caffeoylshikimic acid	17.70	0.92
7	8.2	296	561	caffeoylsinapylquinic acid	285.00	2.30
8	11.8	318	181	caffeic acid	9.81	1.02
total					394.53	1.13

[a] Data presented are means ± standard deviation (n = 3).

syrup showed high TPC of about 548 ± 55 mg of GAE/100 g fw suggesting the richness of this highly appreciated local product. Almost no significant ($P > 0.05$) TPCs were obtained for the different syrup samples provided by the Tunisian families suggesting a similar local traditional preparation mode. Recently, Al-Farsi et al. (*14*) comparatively analyzed the composition of different date varieties grown in Oman, as well as their syrups, and found dissimilar TPCs with the highest values were of the Mabseeli date flesh and its derived syrup of about 246 ± 8.3 and 162 ± 10.4 mg of GAE/100 g fw, respectively. Wu et al. (*24*) reported higher TPCs of 661 and 572 mg of GAE/100 g fw of *Deglet Noor* and *Medjoul* varieties, respectively, which are comparable to the Tunisian *Deglet Nour* derived syrup TPC determined in this work.

Phenolic Profile Analysis. The phenolic compounds of date syrup extracts were fractionated and analyzed by reversed phase HPLC-DAD, and the corresponding chromatograms obtained at 280 and 350 nm are presented in **Figure 1**. The repeatability of the method was high, with respect to both retention times and peak areas. The ESI-MS analysis resulted in protonated ions ($[M + H]^+$) at m/z ratios similar to those obtained with authentic standards. The ESI-MS technique was useful to confirm and complete the identification of the phenolic compounds based on their specific and characteristic molecular ions described in the literature (**Table 1**). The eight main peaks identified in this work, namely, peaks 1−8, were attributed to coumaric acid, ester of gallic acid, ester of vanillic acid, cinnamic acid, 3,4-dicaffeoylquinic acid, caffeoylshikimic acid, caffeoylsinapylquinic acid, and caffeic acid. The HPLC-DAD−MS analysis showed that the major phenolic compounds of the date fruit are caffeoylsinapylquinic acid (72%), cinnamic acid (9%) and coumaric acid (6%). Our results are in agreement with those reported by Mansouri et al. (*11*) for the date fruit extracts. The particular high free cinnamic acid content is not frequently encountered in fruits. According to Regnault et al. (*25*), only date fruit seems to display high free coumaric acid content. Macheix et al. (*26*) reported that the presence of coumaric acid is probably associated with the original maturation of the fruit and the intense extraction conditions. The presence of 5-*O*-caffeoylshikimic acid (dactyliferic acid), a shikimic ester, was also observed and suggested to be a contributor to the browning reactions which take place during the maturation of the fruit (*27*).

Antioxidant Activities. Date syrup extract exhibited a strong $DPPH^•$ scavenging effect of about 101.27 ± 7.8 μmol of TEAC/100 g fw. The free radical $DPPH^•$ is known to involve electron transfer reaction (*18*). DPPH is believed to accept an electron and become a stable diamagnetic molecule (*27*). It has been shown that many antioxidant molecules such as ascorbic acid (vitamin C), tocopherol (vitamin E), and flavonoids reduced DPPH due to their hydrogen donating ability (*28*), a property that may share

Table 2. Antibacterial Activity of The Date Syrup Aqueous−Acetone Extracts[a]

	S. aureus- (ATCC6538)	*S. epidermidis*- (CIP 106510)	*B. cereus*- (ATCC11778)	*P. aeruginosa*- (ATCC9027)	*E. coli*- (ATCC8739)	*Salmonella* sp.	*C. albicans*- (ATCC14053)
syrup extract[b]	16.0 ± 1.0	20.0 ± 1.0	18.3 ± 0.56	15.2 ± 0.4	n	12.0 ± 1.0	10.3 ± 1.4
Amp[b]	15.2 ± 0.5	16.3 ± 0.36	19.2 ± 0.7	17.5 ± 0.5	16.5 ± 1.5	15.7 ± 0.3	n
Tet[b]	17.5 ± 1.3	19.5 ± 1.3	18.7 ± 0.6	19.8 ± 0.7	14.0 ± 0.5	13.5 ± 0.2	n
MIC[c]	50	50	10^2	2.5×10^2	>	5×10^2	3.75×10^2
MBC[c]	50	50	10^2	2.5×10^2	>	5×10^2	3.75×10^2

[a] (n): No antimicrobial activity (∅ = 6 mm). (>): No inhibition at the highest reached concentration (5×10^2 μg/mL). AMP: ampicillin. TET: oxytetracyclin. [b] Inhibition zone diameter (mm) including the disk; values are mean ± SD of two separate experiments done in triplicate. [c] Phenolic concentration (μg/mL).

the date syrup extract. Our results are comparable to those found by Al-Farsi et al. (*14*) who analyzed different date syrups and found that antioxidant activities ranged from 84 to 106 μmol of TEAC/100 g fw of syrup.

The free radical scavenging potential of date syrup extracts was also assessed by their ability to quench the cationic $ABTS^+$ radical generated in the assay system. Tunisian date syrup extract exhibited 520.47 ± 15 μmol of TEAC/100 g fw based on the ABTS method. The difference between ABTS/DPPH based antioxidant activities has been observed for other plant extracts and thought to be correlated with the concentration and chemical structure of individual polyphenolic compounds which could selectively scavenge anionic or cationic free radicals (*29*).

Moreover, the capacity of the Tunisian *Deglet Nour* date syrup extract to reduce Fe(III) was assessed using the FRAP method and found equivalent to 382.87 ± 23.2 μg/100 g fw. For all the antioxidant evaluation tests, the high repeatability of the experience was verified, and no significant difference ($P > 0.05$) has been found between the different syrup extracts.

In Vitro Antimicrobial Tests. The consistent and reproducible results obtained using the standard disk diffusion technique showed that Tunisian date syrup extract exhibited strong antimicrobial potential (**Table 2**). Varying degree of bacteria sensitivity was observed, suggesting a differential intrinsic tolerance of microorganisms and/or the particular nature and combination of the phenolic compounds present in the date syrup extract. The strongest antibacterial activity of date syrup was recorded against *S. epidermidis*, and the lowest activity was observed against *Salmonella* sp. while no inhibitory effect was noticed against *E. coli*. In addition, the extract did not show antifungal activity using the yeast *Candida albicans*. Furthermore, the extracts showed both bacteriostatic and bactericide activities with MIC and MBC values found equal and ranged from 0.05 to 0.5 mg/mL except for *E. coli*, for which neither bactericide nor bacteriostatic effect was observed even at the highest phenolic concentrations used in our experiments (5×10^2 μg/mL).

The obtained results suggest that aqueous−acetonic extract of date syrup was more efficient to inhibit Gram+ than Gram− bacteria. Several studies attributed the inhibitory effect of plant extracts against bacterial pathogens to their phenolic composition (*30*, *31*). The antimicrobial effect of these phenolics could be explained by bacterial growth inhibition as they adsorb to cell membranes, interaction with enzymes and effectors, or deprivation of substrates and metal ions (*32*), we can assume that structural diversity of the bioavailable phenolics in the date syrup extracts will influence their exhibited antimicrobial potentials.

Cell Viability Assay. When the SH-SY5Y neuroblastoma and 3T3 fibroblast cells were treated for 1, 3, 6, and 24 h with different concentrations of date syrup extract, their viability significantly decreased in a dose/time dependent manner compared to the control cells. As shown in **Figure 2**, concentrations of syrup extract more than 45 μg/mL decreased the cell viability to 70−80% in 1−3 h with no marked differences for both cell lines; while

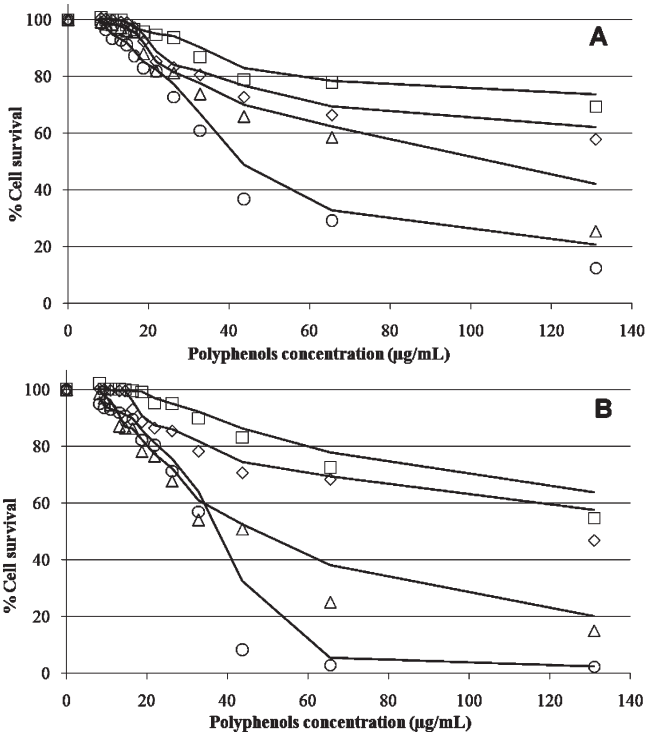

Figure 2. Viability of the 3T3 fibroblast (**A**) and SH-SY5Y neuroblastoma (**B**) cells treated with different concentrations (8.18−131 μg phenolics/mL) of date syrup extract and incubated for 1 (square), 3 (lozenge), 6 (triangle) and 24 (circle) hours. Cell viability was measured by the MTT assay. Data are presented as means of three independent experiments and are different from the untreated control cells at a level of $P < 0.05$.

at 6 h neuroblastoma cell viability (**Figure 2B**) was significantly decreased much more than that of fibroblast (**Figure 2A**). After 24 h of incubation, the cell viability of the tumorigenic neuroblastoma decreases drastically compared to fibroblast cells. At 65 μg/mL and after 1, 3, or 6 h incubation, fibroblast cells maintain a viability ≥60%; in contrast, the viability of neuroblastoma cells reached 23% after 6 h incubation and practically no viability was detected after 24 h (**Figure 2B**). Higher concentrations of the syrup extract decreased progressively more the viability of both cell lines. The obtained data illustrated a distinct behavior of the tumorigenic and nontumorigenic cells in the presence of increased concentrations of syrup extract in the medium. The susceptibility of the neuroblastoma cells was significantly higher ($P < 0.05$) than that of fibroblast cells, suggesting a pronounced cytotoxic effect particularly against tumorigenic cells. This distinction was central in many scientific studies to selectively target cancer cells in a therapeutic treatment. The obtained results emphasize the potential use of the date syrup extract for curative applications. Similar results were obtained by Lantto et al. (*20*) using SH-SY5Y neuroblastoma and CV1-P fibroblast cells. These authors found

that curcumin decreased the cell viability and increased the amount of p53 in SH-SY5Y cells more effectively than in CV1-P cells. Our results indicate a potential role of the studied date syrup extract as health-promoting food constituents, as well as candidates for further drug development. This study should be followed by a deeper investigation to elucidate the cytotoxic mechanism and to identify the active compounds.

In conclusion, this work contributes to the knowledge of the beneficial properties of the phenolic extracts prepared from date syrup. This local date derived product could potentially be a good source of natural phenolics with antioxidant/antimicrobial activities and health-promoting effects. The obtained results are the first steps for large scale implementation process.

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