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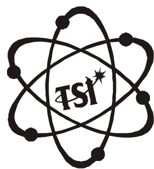

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Cytotoxicity evaluation and antioxidant activity of date seed oil from "Deglet-nour Tunisian cultivar" (Phoenix dactylifera L.)

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ABSTRACT

Date seed oil extract has been reported to be a rich source of natural antioxidant compounds. The aim of this study was to evaluate the cytotoxicity and the protective effect of date seed oil against oxidative stress. The antioxidant activity of this oil was evaluated by its scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH). Its potency to protect human epithelial cells against Fe^{2+} induced oxidative stress was assessed on the HeLa cell line. The Cytotoxic effect of this oil was evaluated using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Results showed that DPPH radicals scavenging activity of date seed oil was better than that of olive oil (IC50: 40, 54±4 mg/ml vs 60, 36±2, 56 mg/ml). The addition of different concentrations of date seed oil (1%, 5%, 10%) resulted in a significant protection of HeLa cells against the oxidative stress induced by Fe^{2+}, evidenced by a decreased MDA level. On the other hand, date seed oil was showed to be devoided of any cytotoxic activity. Our data suggest that date seed oil can be potentially used in food, cosmetics and/or pharmaceutics applications because of its high antioxidant capacity. © 2011 Trade Science Inc. - INDIA

KEYWORDS

Oxidative stress;
Date seeds oil;
TBARs;
Antioxidant capacity;
Emulsion;
MDA.

INTRODUCTION

The date is a staple food in the arid and semiarid regions of the world. It is composed of a fleshy pericarp and seed. Tunisia is considered as one of the most important producers. The number of date palm (Phoenix dactylifera L.) trees is estimated to be over 4 millions and around 100, 000 tons of dates are produced annually, from which, about 10-15% represent the seeds[1,2]. This fraction is a by-product generated during technological or biological transformation of date flesh[3,4]. Therefore, a large quantity of date seeds could be easily collected from the date processing industries or from the waste product. However, presently, very little use is made of this compound: seeds are discarded or used as fodder for domestic farm animal.

Because synthetic antioxidants such as butylated hydroxytoluene or butylated hydroxyanysol could promote cancer development in rats[5], dietary components with antioxidant activity have received particular attention. Plant phenolics have been reported to have an in vitro antioxidant activity, even higher than

the common antioxidants vitamin C and E[6,7]. Antioxidant compounds play an important role in counteracting oxidative stress, which plays an important role in the etiology of many pathological disorders such as atherosclerosis and related cardiovascular diseases, diabetes and cancer.

Previous studies reported that date seeds from Deglet Nour cultivar have extractable components especially in the lipid fraction that may have high added-value[8]. Indeed, date seed oil has a better oxidative stability than that of the most vegetable oils[9], due to its high antioxidant content mainly phenols and tocopherols[10].

The aims of this study were to evaluate (i) the cytotoxic effect of this date seed oil by using the MTT cytotoxic test (ii) to compare with olive oil, a rich Mediterranean source of phenolic compounds, the *in vitro* antioxidant capacity of date seed oil extracted from Deglet Nour cultivar. The trolox was used as a positive control.

EXPERIMENTAL

Seed material

Date palm fruits (Phoenix dactylifera L.) were obtained from the National Institute of Arid Zone (Degach, Tunisia). The seeds were directly isolated from 50 kg of date fruit having the same origin, collected at the "Tamr stage" (full ripness) and kept at 10°C for a week. The seeds were soaked in water, washed to get rid of any adhering date flesh, and then air-dried. Their relative percentage weight compared with the weight of the fresh fruits was about 11.32%. Then, they were further dried at about 50°C. Date pits were milled in a heavy-duty grinder to pass 1-2 mm screens and then preserved at -20°C until use. One day before lipid extraction, an appropriate quantity of powdered date seed was kept at 5°C.

Date seed oil extraction and preservation

Lipid extraction was carried out as described by Besbes *et al.*[8]. Briefly, about 15g of powdered date seeds were used for oil extraction with petroleum ether at 40-50°C (Merk, for analysis), in Soxhlet post. The extraction was repeated at least 12 times. The operational conditions were 30 mn with thimble immersed in boiling solvent (immersion time) and 60 mn of reflux washing (washing time). After removing solvent, using a Rotavapor apparatus, the seed oil drained under a stream of nitrogen and then stored at -20°C until use.

DPPH radical-scavenging assay

The hydrogen atom or electron donation ability of date seed oil was measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometer assay (Biochrom, Libra S32) uses stable radical diphenylpicrylhydrazyl (DPPH) (Sigma, Aldrich) as a reagent. Aliquots (1 ml) of various concentrations of the test compound in methanol (0-0,5- 1- 5- 10- 20- 30%) were added to 2 ml of a 0.004% methanol solution of DPPH. After a 30 min of incubation period at room temperature in dark, the absorbance was determined against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in the following way: I% = [(Ablank-Asample)/Ablank]*100, where Ablank is the absorbance of the control reaction (containing all reagents except the test compound), and Asample is the absorbance of the test compound. Test compound concentration providing 50% inhibition (IC50, expressed in mg ml^{-1}) was calculated from the graph plotted inhibition percentage against extract concentration.

Preparation of the emulsion

Because lipids are not miscible in an aqueous medium, emulsions are prepared in 10% (V/W) arabic gum solution. Oil was added in Arabic gum solution in equal volume and sonicated using SONIC vibra cell sonicator under the following conditions: 5x20s at 35T2/mn.

HeLa cell culture

The continuous human cell lines HeLa (epithelial cervical cancer cell line) was investigated for cytotoxicity and antioxidant effect of date seed oil. This adherent cell line was grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamin in tissue culture flasks (Nunc). It was passed twice a week and kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Induction of oxidative stress

Cells were adjusted to 5 10^5 cells/ ml in 25 cm^2 flasks, and incubated at 37 °C. Oxidative stress was induced, after 72 h, by addition of Fe^{2+} (as Fe_2SO_4) to the cells at a final concentration of 100 µM, for 1 h. The oxidation was performed in phosphate buffered saline solution (PBS).

MDA determination

For evaluation of MDA production rate, thiobarbituric acid-reactive species (TBARs) assay was used. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 500 μL of deionized water and lysed by five cycles of sonication during 20 s at 35% (Sonisc, vibracell). One millilitre of TBA solution (15% trichloroacetic acid, 0.8% thiobarbituric acid, 0.25 N HCl) was added. The mixture was heated at 95°C for 15 min to form MDA–TBA adduct. Optical density (OD) was measured by a spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane (1.1.3.3 TEP).

Diene conjugate determination

The evaluation of diene conjugate production rate was performed on cells previously treated as described above. The diene conjugates were extracted by adding 3 ml of chloroform/methanol (75:25) V/V. After centrifugation at 3000 rpm for 10 minutes, 2 ml of supernatant were dried at 45 °C. Then, 2ml of methanol were added and absorbance was measured at 232nm.

Antioxidant effect

To assay the capacity of date seed oil to protect HeLa cells from ROS-mediated oxidative injury, cells were preincubated for 72 h in the presence of water/ date seed oil emulsion (1%, 5% and 10%). At the end of the preincubation time, the medium was changed before the addition of the oxidative stress-inducing agent. Finally, the above mentioned markers were evaluated. A comparative study was conducted using olive oil at the same concentrations.

Different controls were used: (i) HeLa cells without any treatment; (ii) HeLa cells with 100μM Fe^{2+}; (iii) HeLa cells with 100μM Trolox.

MTT cell proliferation assay

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assay measures the cell proliferation rate and conversely, the reduction in cell viability when metabolic events lead to apoptosis or necrosis. The yellow compound MTT (Sigma) is reduced by mitochondrial dehydrogenases to the water insoluble blue compound formazan, depending on the viability of the cells.

3 10^4 cells/ml were grown on microtiter plates (200 μl of cell suspension/well) in 96 well microplates with serial dilutions of water/ date seed oil emulsion on arabic gum (AG) only. 72 h later, a 20 μl of a solution of MTT (5mg/ ml in PBS) was added per well. The plate was incubated for 4 h at 37°C in a CO_2 incubator. After incubation, 180 μl of medium were removed from each well. 180μl of DMSO/methanol (50:50) were added to each sample, and the preparations were mixed thoroughly on a plate shaker with the cells containing formazan crystals. After all of the crystals were dissolved, absorbance was measured at 570nm with a microplate reader (Elx 800 microplate reader).

Statistical analysis

Each value is the mean of three replications. Values of different parameters were expressed as the mean ± standard deviation (x±SD). The one-way analysis of variance (ANOVA) was performed at the level of $p<0.05$ to evaluate the significance of differences between mean values. Statistical analysis was performed using SPSS (SPSS 13 for Windows) statistical software.

RESULTS

Antioxidant potential by DPPH radicals scavenging assay

The date seed oil was screened for its antioxidant capacity by DPPH radicals scavenging assays (Figure 1). Results were compared with those of olive oil. Date seed oil displayed an antioxidant activity (IC50: 40,54± 4mg/ml), which was significantly higher than olive oil one (IC50 =60,36± 2,56 mg/ml) (p=0,0137).

Biological antioxidant activity in human cell cultures

The investigation of the biological antioxidant activity of date seed oil was carried out on the HeLa cell line. The cells were cultured with or without addition of date seed oil used at different concentrations (1, 5 and 10%). The cell viability or morphology was not affected by this oil addition. After 72 hours of culture, the oxidative stress was induced by adding 100 μM Fe^{2+} solution (as Fe_2SO_4) in PBS for 1 h. Indeed, ferrous iron is known to readily induce cell-damaging compounds by triggering the production of noxious ROS, especially hydroxyl radicals ($OH^{\cdot}$) (Pereira *et al.* 2006; Parinos *et al.* 2007). Malondialdehyde (MDA) and diene conjugate (DC), two lipid peroxidation markers, were evaluated.

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The oxidative treatment resulted in an increase in MDA and DC concentrations compared to control cells. As shown in Figure (2.A) and (2.B), a protection against oxidative stress was obtained with the three date seed oil concentrations. However, the lipid peroxidation inhibition was statistically significant only for MDA levels (p=0,004). This effect is better than that obtained with 100µM trolox used as antioxidant control. Interestingly, the date seed oil exhibited an antioxidant activity comparable to olive oil one except for the concentration of 1%, for which date seed oil exhibited a significant higher antioxidant activity (p=0,011). No correlation was obtained between the antioxidant effect and the concentrations of date seed oil emulsion used.

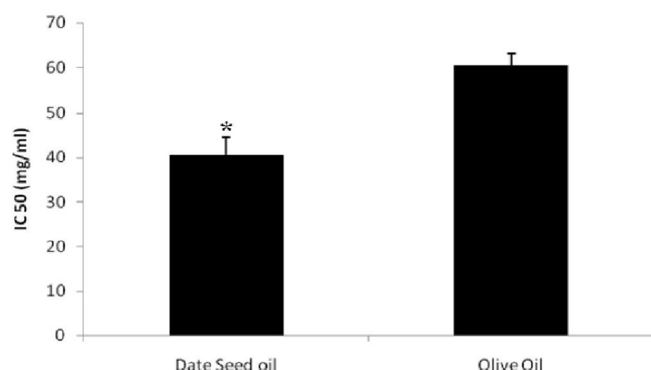

Figure 1 : DPPH antioxidant scavenging activity of date seed oil and olive oil.

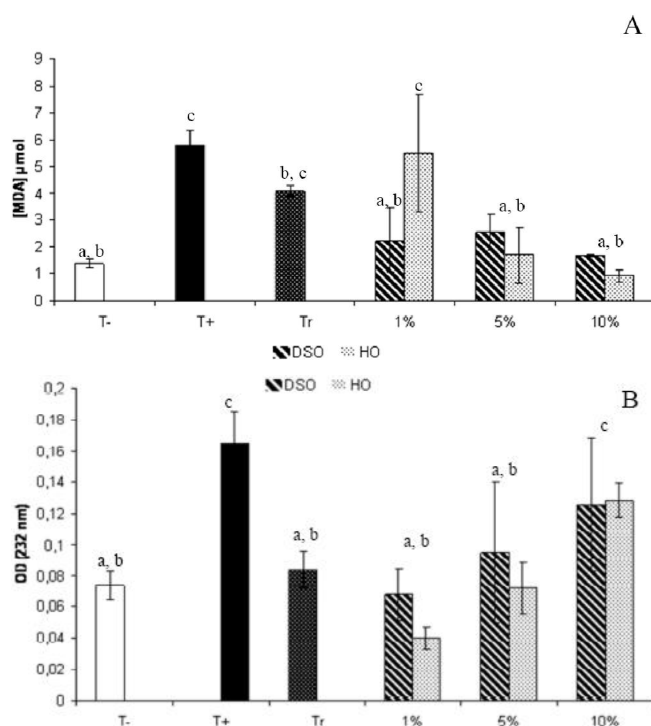

Figure 2 : MDA and DC levels in date seed oil and olive oil supplemented Hela cell line.

CYTOTOXICITY ASSAY

To investigate its cytotoxic effects on HeLa human cell line, various concentrations of date seed oil were used to treat cells for 72 h, that where then submitted to the MTT test. As far as we know, no data are available in the literature concerning the study of cytotoxic properties of date seed oil using the cell culture model system. As shown in Figure 3, no cytotoxic effect of this emulsion was noticed for all the added percentages in the culture medium, with a cell viability exceeding 95%.

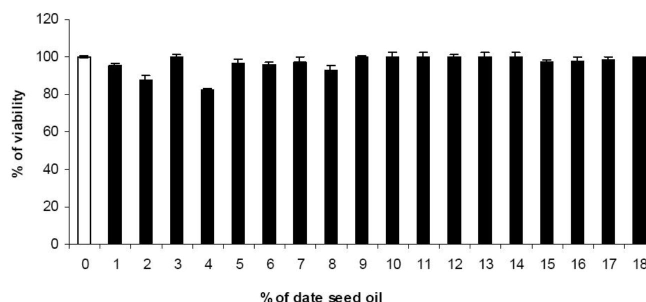

Figure 3 : Cytotoxic effect of date seed oil on HeLa cultured cells.

DISCUSSION

A number of plant oils have been tested and described for their antioxidant effect[11-13]. In this study, a new source of antioxidants: date seed oil from Deglet nour Tunisian cultivar was investigated. The antioxidant effect was measured first by (DPPH) scavenging test and then, in HeLa cells subjected to oxidation by Fe^{2+} solution (Fe_2SO_4.).

Date seed oil displayed a strong antioxidant activity (IC50 =40,54± 4mg/ml), which was higher than that of olive oil (IC50 =60,36± 2,56mg/ml). These results are in agreement with those previously reported by Besbes et al[8,9] who showned that date seed oil has a better oxidative stability when evaluated by the rancimat method. According to these authors, the oxidative stability of date seed oil was higher than that of most vegetable oils.

When conducted on Fe^{2+} treated HeLa cells, our study showed that malondialdehyde (MDA) and diene conjugates production rates decreased in date seed oil treated cells. Moreover, date seed oil exhibited a better antioxidant potency than olive oil at the lowest concentration used (1%) (p=0,011), which is in correlation with the lower IC50 of date seed oil

compared to olive oil.

Previous studies have shown an antioxidant effect of date seed oil on humain skin organ culture. This oil limited the oxidative injuries induced by hydrogen peroxide by preserving the dermal-epidermal junction, promoting cell survival and preventing epidermal necrosis[14]. A recent study showed that date seed oil provided an efficient complement to photoprotective measures and seemed to contribute to the reduction of the DNA damage[15]. Ben abdallah et al[16] reported that date seed oil improved the epididymal sperm quality and ameliorated the testicular strategy defences.

The antioxidant efficiency of date seed oil is attributed to the presence of high antioxidant molecules such as tocopherol and phenolic compounds[8]. Indeed, the Deglet nour variety has higher phenolic compounds when compared to other date varieties such as "Allig".

Besides, date seed oil has a higher phenol content compared to most edible oils except olive oil, which is considered as a rich source of phenolic compounds in the Mediteranean diet. Polyphenols belong to the natural antioxidants compounds and are the most abundant ones in our diet. They play an important role in human nutrition as preventative agents against several diseases through their protection of the body tissues against oxidative stress[18-22]. Hydroxythyrosol (HT) is the highest polyphenol content in Deglet nour seed oil. It is also the main natural antioxidant in olive oil. This compound is attracting distinct attention because of its ortho-diphenolic structure. Many in vitro studies indicate that HT is a powerful antioxidant that exerts different biological activities, e.g. anti-inflammatory and anti-thrombotic ones that are being investigated in vivo.

Considering that the identification of date seed oil compounds is still incomplete and 58% of phenolics are not yet identified[8] we investigated the toxic effect of date seed oil on human cells. The results showed that this oil didn't exhibit any significant cytotoxic effect on HeLa cells for all the concentrations used.

According to these results, date seed oil from Deglet nour cultivar may be used as an additive to improve oxidative stability and to increase the nutrition value of some foods containing low antioxidant molecule content. It could be also incorporated into cosmetic, pharmaceuticals, soaps and detergents. Its high antioxidant activity, observed at low concentration can compensate the low oil yield of date seeds (10%).

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