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In Vitro Antioxidant Activity of Chufa Tubers (*Cyperus esculentus* L.) Extracts in Liposome Peroxidation Systems

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THE ANTIOXIDANT activity of hexane, ethanol or water extracts of chufa tubers (*Cyperus esculentus* L.) were investigated *in vitro* using liposome peroxidation systems. Incorporation of 1 mg/ml of chufa oil, water or ethanol extracts of the tubers to phosphatidylcholine (PtdCho) liposomes displayed different antioxidative activity. All fractions possessed antiperoxidation activity in ferrous ions dependent peroxidation stress and in free radical mediated peroxidation by the water soluble initiator 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH), while the water extract possessed the strongest effect. However, in the lipid soluble free radicals initiator 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) mediated peroxidation, the antioxidant activity was found only by the oil fraction. The antioxidant activity of chufa oil may be attributed to its fatty acid profile and vitamin E content. Chufa oil contains about 72% of monounsaturated fatty acids, mainly oleic acid and 18.6 mg/100g of vitamin E. The resistance of chufa water and ethanol extracts may be attributed in part to its isoflavones content (20.9µg/g).

Keywords: Chufa, olive oil, Anhydrous milk fat, Liposome, Antioxidant activity, Lipoperoxidation, Ferrous ions, AAPH, AMVN

Introduction

Chufa plant is a member of the grass family Cyperaceae to which nutsedge weeds also belong. Chufa tubers are daily ingredients of the diet of many people in North Africa and Spain. In North Africa the tubers are consumed in their natural form or after being soaked in water for some hours. In Spain, the tubers are consumed mainly as a drink called locally "horchata de chufa" (chufa milk). The use of chufa tubers in Egypt is limited to be eaten as a nut on certain occasions or as a coffee adulterant. The tubers of this plant are known by several names, such as tiger nut, earth nut, earth almond, rush nut and chufa. Several studies demonstrated the high nutritional quality of chufa tubers (Arafat et al., 2009, Adejuyitan 2011 and Codina-Torrella et al., 2015). It provides about 400-450 kcal/100g tubers due to its high oil content (~25%) and carbohydrates (~50%). It also contains considerable amounts of protein (~8%), dietary fibers (~6%), minerals (~4%) i.e. calcium, sodium, potassium, magnesium and phosphorus and several enzymes in particular, catalase, lipase and amylase (Adejuyitan, 2011). According to its nutritional value, chufa tubers are considered as an interesting solution for the protein-calorie malnutrition in the developed countries, and a promising material for the production of gluten free bread for people with celiac disease (Aguilar et al., 2015). Chufa can be eaten raw, baked, roasted or processed into drink. It can also be used as a flavoring agent for ice cream and biscuits (Cantatejo, 1997).

Dietary nutrients with antioxidant properties are of great significance in the pathogenesis of many disorders related to free radical damage (Mancini et al., 1995, Owen et al., 2000 and Dipartimento, 2000). Therefore, the research regarding the use of the antioxidant rich ingredients in food production take a special interest. Chufa oil contains about 70% of oleic acid. Oleic acid was reported to have a protective

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effect against peroxidation stress either in vitro when incorporated to liposomes (Lee et al., 1998) or in vivo when supplied to a diet (Bonanome et al., 1992 and Sola et al., 1997). In a preliminary note, Mohamed and El-Fors (1959) have reported that a small amount of chufa oil (6%) protected linseed oil against peroxidation stress in in vitro. The aim of the present study is to explore the antioxidant activity of extracts of chufa tubers by hexane (oil fraction), ethanol or water in transition metal ions-dependent peroxidation or in free radicals mediated peroxidation stress by the aqueous- or lipids-phase, azo-derived peroxyl radicals AAPH and AMVN,respectively when incorporated to multi-lamellar liposomes.

Materials and Methods

Chufa tubers were purchased from the local market at Kafr El-Sheikh city, Egypt. The tubers were sorted for stone and other physical defects, washed and dried overnight at 60°C as described by Amankwah et al. (2009). The tubers were allowed to cool, and then milled using a laboratory miller to pass through 40 mesh sieve.

Chemical composition of chufa tubers

Chemical composition of chufa tubers was determined according to the methods described by the AOAC (1995).

Analysis of fatty acid composition

Fatty acid composition of chufa oil, olive oil and anhydrous cow milk fat was determined using GLC as described by Imaizumi et al. (1993).

Determination of vitamin E content

The concentration of vitamin E in chufa oil was determined by high-performance liquid chromatography (HPLC) (Waters 600E, Japan Millipore, Tokyo) according to the method described by Zommara et al. (1998).

Determination of isoflavones

Chufa isoflavones were extracted with 70% aqueous ethanol at room temperature and determined by HPLC on a CAPCELL PAK C18 AG120 column (Shiseido, Tokyo, Japan) using a Waters 600 multi-solvent delivery system, Waters 486UV detector and Waters 741 data module (Millipore Corporation, Milford, MA) according to the method described by Kudou et al. (1991).

Preparation of chufa extracts

The milled chufa tubers were extracted by shaking it with 3 volumes of hexane for one hour. The mixture was centrifuged at 3000 rpm for 5 min and hexane layer was removed. Extraction was repeated 3 times with the solid residue and hexane layers were combined. The hexane extractable material (oil) was obtained by removing hexane under reduced pressure in a rotary evaporator and kept under argon gas at minus 30°C until to be used. The defatted milled tubers were stirred for 12 h with 7 volumes of distilled water at room temperature. The suspension was centrifuged at 3000 rpm for 5 min and the supernatant was filtered through cotton cloth. The extraction was repeated twice and the supernatant were combined as water extract (WE). An ethanolic extract (EE) was prepared by shaking the defatted tubers powder in 5 volumes of 80% ethanol for 2 h at room temperature. The suspension was allowed to stand for 15 min and filtered through cotton cloth. Extraction was repeated twice with the solid residue and combined filtrate was centrifuged at 3000 rpm for 5 min. The supernatant was concentrated by removing ethanol under reduced pressure in a rotary evaporator at 40°C. The water and ethanolic extracts were freeze-dried and solutions (0.1% w/v) were prepared from the freeze-dried extracts. The pH values of these solutions were measured at 25 °C using pH-meter (Beckman, Instrument Company, Inc., USA). The color of the extracts was visually estimated.

Preparation of liposomes

Multil-amellar liposomes were prepared according to the method described by Yoshida et al. (1991).Briefly, egg yolk PtdCho (PC-98HC, QP. Corp., Tokyo) in chloroform solution was evaporated to dryness. The dried lipid film was dispersed in 10 mMTris-HCl buffer (pH 7.4) by vigorous shaking on a vortex mixer followed by ultrasonication under argon gas in ice bath for 2 min. Final concentration in the liposomal solution was 10 mMPtdCho and 1000 ppm of chufa oil, WE or EE. A positive control was prepared by incorporation of 10 ppm of Butylatedhydroxyltoluene (BHT) (NacalaiTesque Inc., Kyoto, Japan) to the liposomal solution instead of the chufa fractions. A negative control liposome sample was run simultaneously free of the investigated chufa extract or BHT.

Evaluation of antioxidant activity

The antioxidant activity of the investigated

extracts was performed using three oxidation systems, transition metal ion-dependent peroxidation, water soluble free radical initiator and lipid soluble free radical initiator. Transition metal ion dependent peroxidation was induced by the addition of 0.1 mM FeSO4, 1 mM ascorbic acid and 0.04 mM cumen hydroperoxides (CuOOH) (NacalaiTesque Inc., Kyoto, Japan). Free radical mediated peroxidation was induced by the addition of 20 mM 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) or 2 mM 2,2'-azobis (2,4- dimethylvaleronitrile) (AMVN) (Wako Pure Chemicals Co., Osaka, Japan). AAPH and AMVN are water and lipid-soluble azo compounds that thermally decomposes and thereby generates radicals at known and constant rates (Ingold et al., 1993). Since the concentration of AMVN has to be kept low to avoid disrupting the membrane structure, the incubation temperature has to be high (i.e. 50^{o}C). The high incubation temperature may affect the antioxidant active component(s) in the extracts. For that, the liposomes were incubated at 37^{o}C and accordingly the Ptd Cho-hydroperoxides increased slowly. Incubation was carried out in water bath at 37^{o}C with constant shaking. The samples were removed at appropriate intervals for lipid peroxide assays. The transition metal ion dependent oxidation was terminated by adding final concentration of 20 µM BHT and 2mM disodium ethylene diamine tetraacetic acid (Na2-EDTA). Free radical mediated peroxidation was terminated by immersing the samples in liquid nitrogen. All samples were kept at -30^{o}C until to be assayed within few hours. Lipid peroxidation was evaluated by thiobarbituric acid (TBA) assay (Jiang et al., 1992).

Statistical analyses

Statistical analysis was carried out by Duncans new multiple range test (Duncan, 1955).

Results and Discussion

Chemical composition of chufa tubers

The average chemical composition of chufa tubers were as follows: 34% starch, 25% oil, 8% protein, 16% sucrose, 10% fibers and 7% others. Also, chufa tubers contained 12.38µg/g and 8.46µg/g of the isoflavones daidzein and genistein, respectively. Chufa tubers were extracted to three extracts using hexane, ethanol and water. The hexane fraction (oil) has a golden yellow color, pleasant odor and acceptable flavor. The fatty acid composition of the oil is shown in Table 1. The predominant fatty acid in the oil is oleic acid (about 70%). The oil contains also about 10% of polyunsaturated fatty acids with a small amount of α-linoleic acid and about 20% of saturated fatty acids.The oil content of vitamin E is about 18.6 mg/100 g oil.The obtained chemical composition and fatty acid profile of chufa tubers was in consistence with that obtained by Mokady and Dolev (1970) and Oladele and Aina (2007). However, the proximate composition of chufa is quite variable and mainly dependable on their geographical origin (Codina-Torrella et al., 2015).

In vitro antioxidant properties of chufa extracts

Some characteristics of the ethanolic and water extracts were obtained. The yield of the ethanolic extract (EE) was 30.4% with a yellow color while the figure was 37.4% for the water extract (WE) with a light grey color. The pH of 0.1%, (w/v)solution of the ethanolic and the water extracts was 4.8 and 6.2, respectively. The effect of incorporation of 1000 ppm of the chufa extracts to Ptd Cho liposomes on iron-mediated peroxidation is shown in Fig. 1. The oil, EE and WE extracts markedly inhibited the lipid peroxide formation and resulted in significantly lower thiobarbituric acid reactive substances (TBARS) values than the control sample throughout the incubation period. The order of the antioxidant activity of the chufa extracts was WE>EE>oil. The addition of 10 ppm of BHT to the liposomes completely inhibited the peroxidation progress during the incubation time. The effect of the chufa extracts in the water soluble free radical

TABLE 1. Fatty acid composition of chufa oil

Fatty Acid	Content (mol %)
Palmitic (16:0)	13.9 ±0.38
Palmitoleic (16:1)	0.38±0.01
Stearic (18:0)	4.24±0.08
Oleic (18:1)	71.1 ±0.71
Linoleic (18:2)	9.48±0.17
α-Linoleic (18:3,n-3)	0.39±0.04
SFA	18.15
MUFA	71.49
PUFA	9.87

Data are mean ± SE of 3 oil extracts.
SFA=Saturated fatty acids.
MUFA=Monounsaturated fatty acids
UFA=Polyunsaturated fatty acids

mediated peroxidation induced by AAPH is shown in Fig. 2. The peroxidation rate was slow as the TBARS increased slowly during the incubation time. The liposomes added with the chufa extracts significantly reduced peroxidation progress and resulted in lower TBARS than the control sample throughout the incubation period. The WE was more effective to suppress the peroxidation than the other extracts. The BHT-liposomes was more resistant to peroxidation stress and exhibited the lowest TBARS values.

Figure 3 shows the effect of incorporation the chufa extracts on the lipid soluble free radical mediated peroxidation stress induced by AMVN. Chufa oil possessed the highest antioxidantactivity as the oil added liposomes resulted in lower TBARS values than the other treatments. The peroxidation profiles of the liposomes added with the EE and WE were equivalent to that of the control samples with a slight antioxidant activity after 10 and 12 hr of incubation. The BHT added liposomes was less susceptible to peroxidation and resulted in the lowest TBARS values.

The obtained data show that, all examined chufa fractions exhibited a marked antioxidantactivity in ferrous ions and AAPH mediated peroxidation stress, although this effect was predominant in ferrous ions dependent peroxidation. On the other hand, chufa water extract showed stronger antioxidantactivity than that of the ethanolic and hexane extracted fractions. In AMVN mediated peroxidation, liposomes added with chufa oil were less susceptible to peroxidation stress however, the other extracts did not exhibit any antioxidant activity. In our previous study (Zommara and El-Shaer 2001), chufa oil exhibited superior lipoperoxidation resistance against peroxidation stress initiated by transition metal ferrous ions, AAPH or AMVN compared to several vegetable oils and anhydrous milk fat incorporated to liposomes. Also, in an early investigation, Mohamed and El-Fors (1959) found that, the addition of 6% (v/v) of chufa oil to linseed oil resulted in a mixture which was strongly resistant against peroxidation stress when exposed to a stream of oxygen compared to linseed oil alone. The resistance of chufa oil to peroxidation stress may be related in part, to its content of vitamin E and oleic acid. Lee et al. (1998) demonstrated that, liposomes enriched in oleic acid are shown to be less susceptible to oxidation and have less pro-inflammatory activity when exposed to oxidizing condition. Also, chufa oil was found to contain remarkable concentration of essential oils (Hassanein et al., 2014). Essential oils were reported to have a powerful antioxidant capacity (Amorati et al., 2013).

The antioxidant activity of the water extract fraction (WE) may be related to tannins (Chung et al., 1998), organic acids (Niki, 1991 and Crackel et al., 1998) and water soluble proteins (Al-Saikhan, et al., 1995). In fact, Emmanuel and Edward (1984) demonstrated the presence of 0.16% of tannins in chufa tubers. The yellow color found in the ethanolic extract of chufa tubers suggests the presences of polyphenol compounds, flavonoide pigments and carotenoids (Endo et al. 1985a, b, Olson 1999) which acts as scavengers for free radicals and active oxygen (Pietta 2000, Wand et al., 2000). in this respect, our results showed that chufa tubers contains a moderate amount of the isoflavones, daidzein and genistein (12.4 and 8.5 µg/g, respectively) which come in the ethanolic extract and may participate in the observed antioxidantactivity of this fraction. In fact, isoflavones showed antiperoxidation activity either in vitro when incorporated to liposomes (Arora et al., 1998) or in vivo when fed to experimental animals (Yousef et al., 2004).

The addition of 10 ppm of BHT to liposomes was more effective to suppress the peroxidation stress than did 1000 ppm of chufa tubers extracts. The BHT is a synthetic antioxidant compound and may have harmful properties to health when added to foodsatinappropriate concentration, however chufa tubers are a natural and edible plant which already used as a food ingredient in several societies without any health risk. In conclusion, chufa tubers exhibited an antioxidantactivity in in vitro peroxidation stress. Therefore, the inclusion of chufa tubers, its hexane, water or ethanol extracts in a diet may have beneficial effects on free radicals accelerated disorders such as cancer and atherosclerosis. Further studies are needed to explore this effect using laboratory animals.

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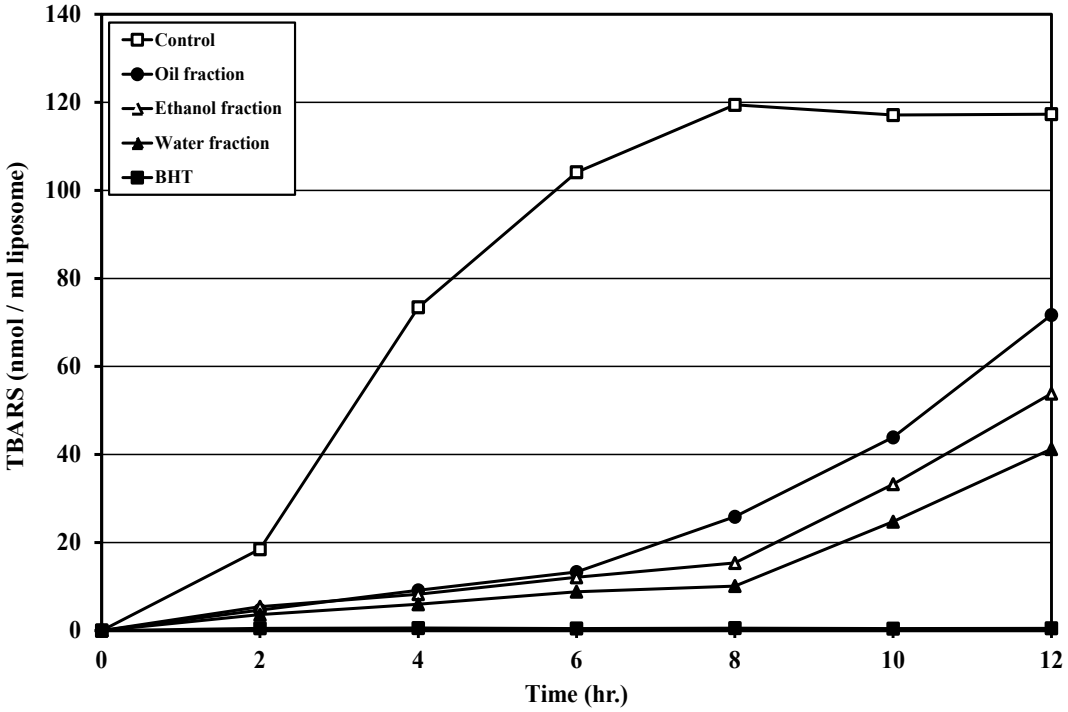

Fig. 1. ***In vitro*** **ferrous ions-dependent peroxidation of PtdCho liposomes contains 1000 ppm of oil, ethanolic extract, water extract of chufa tubers or 10 ppm of BHT, Data are mean ± SE of three replicates**

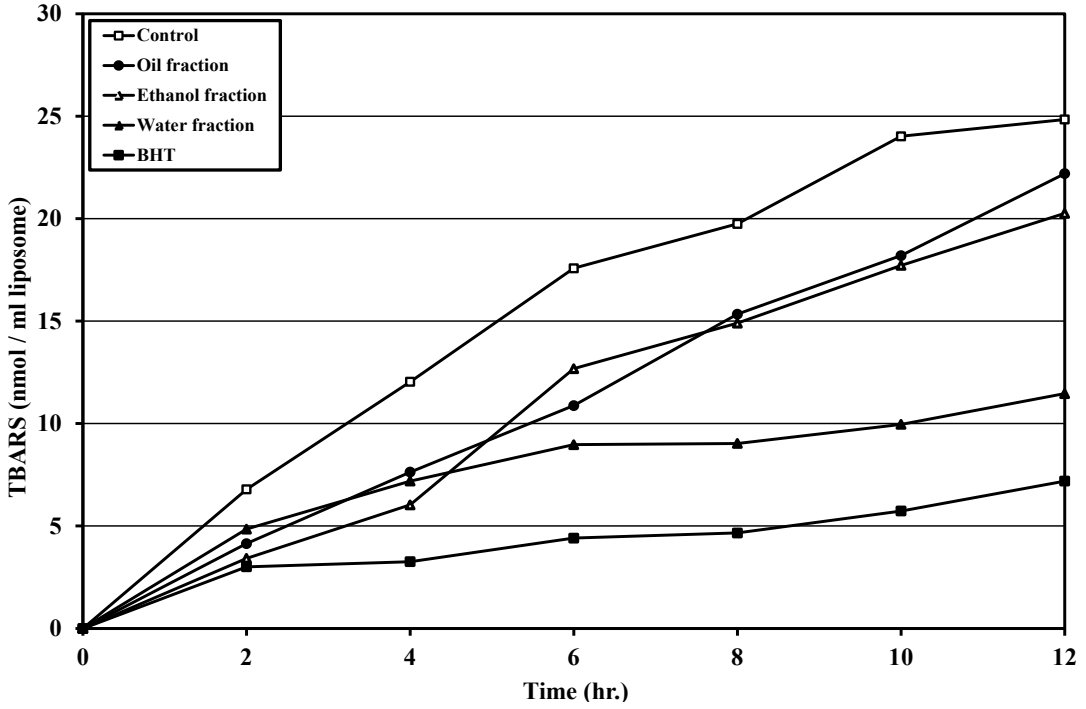

Fig. 2. ***In vitro*** **AAPH peroxidation of PtdCho liposomes contains 1000 ppm of oil, ethanolic extract, water extract of chufa tubers or 10 ppm of BHT, Data are mean ± SE of three replicates**

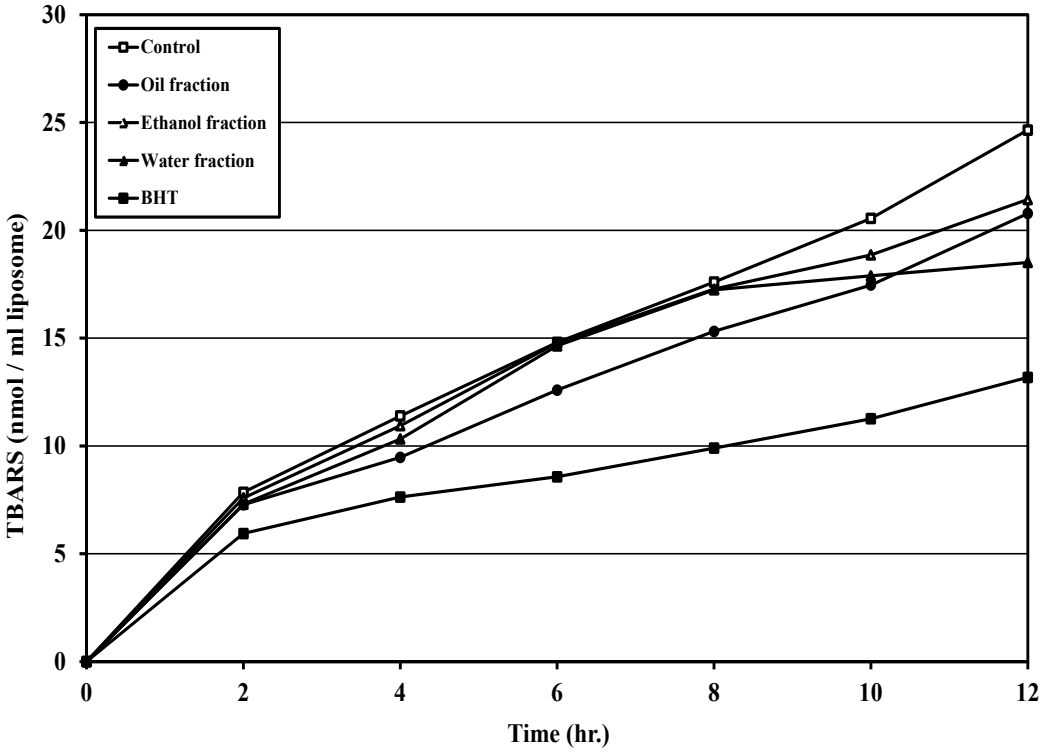

Fig. 3. *In vitro* AMVN peroxidation of PtdCho liposomes contains 1000 ppm of oil, ethanolic extract, water extract of chufa tubers or 10 ppm of BHT, Data are mean ± SE of three replicates

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