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COMBINED ANTI-AGES AND ANTIOXIDANT ACTIVITIES OF DIFFERENT SOLVENT EXTRACTS OF *SOLANUM ELAEAGNIFOLIUM* CAV (SOLANACEA) FRUITS DURING RIPENING AND RELATED TO THEIR PHYTOCHEMICAL COMPOSITIONS

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ABSTRACT

Oxidative stress and advanced glycation end products (AGEs) are known as key factors for the development of diabetic complications such as retinopathy, cataract as well as atherosclerosis and neurodegenerative diseases, including Alzheimer's diseases. In this context, natural products have been previously identified as promising sources for antioxidant and anti-glycation compounds. The current study focuses on the evaluation of antioxidant and glycation inhibitory activities of different solvent extracts of *Solanum elaeagnifolium* Cav (Solanaceae) fruits at different ripening stages. The results showed that antioxidant and anti-AGEs activities were significantly influenced by solvents polarities and ripening stages of *S. elaeagnifolium* Cav. With one exception, methanolic extract of overripe *S. elaeagnifolium* Cav fruit showed important protective effects against cellular oxidative stress. The aqueous extract showed the highest $ABTS^{+}$ scavenging ability. Principal component analysis showed that total phenolic and flavonoid contents correlated well with observed antioxidants and anti-glycation activities. These results bring attention to the possible use of *S. elaeagnifolium* Cav as a valuable source of bioactive compounds exhibiting antioxidant effects and potentially alleviating diabetic complications.

Keywords: *Solanum elaeagnifolium* Cav, advanced glycation end-product (AGEs), in vitro and ex-vivo antioxidant activities, phenolic compounds

INTRODUCTION

Glycation or the Maillard reaction is the non-enzymatic adduct formation between proteins and reducing sugars, resulting in formation of Amadori products (Odjakova et al., 2012; Poulsen et al., 2013). Over time, these products are transformed through a series of complex reaction, leading to the formation of advanced glycation end products

(AGEs). These compounds are causative factors for the many pathogenesis of diabetes complications including retinopathy, cataracts, atherosclerosis, and neurodegenerative diseases, including Alzheimer's diseases (Boulanger et al., 2007; Deetae et al., 2012). Also, AGEs have the propensity to generate reactive oxygen species (ROS) in addition to auto-oxidation reactions yielding radicals and other reactive intermediates. Furthermore, oxidative stress has been postulated to contribute significantly to the accelerated accumulation of advanced glycation end products (AGEs). Therefore inhibition of their formation and/or deleterious effects has been searched for as well as increasing their breakdown and/or elimination.

Numerous synthetic drugs have previously been evaluated as inhibitors against the formation of advanced glycation end products (AGEs); however their practical applications are limited in clinical trials due to relatively low efficacies, poor pharmacokinetics, and the unsatisfactory safety. For example aminoguanidine (AG), despite their efficiency to ameliorated diabetic complications in an animal model, it was withdrawn from the crucial phase III of clinical trials because of potential toxicity and side effects (Peyroux and Sternberg, 2006). Recently, the scientific community tried to identify and develop new natural compounds that offer combined antioxidant and anti-AGEs properties and that can protect the human body against glycation-derived free radicals damage.

In fact, many plant extracts known to possess antioxidative and pharmacological properties have been proven to possess anti-AGEs activity in vivo and in vitro (Jin and Cho, 2011). Several studies have demonstrated that various phenolic compounds, especially flavonoids, can inhibit AGEs formation by acting not only as radical scavengers and metal chelators but also as carbonyl trapping agents (Dugé de Bernonville et al., 2010; Ferchichi et al., 2012; Morel et al., 2013). Moreover, since an oxidative reaction is involved in the formation of AGEs and in AGEs-induced cell damage, compounds with both anti-glycation and antioxidant proprieties have been proposed as potential therapeutic agents (Ho et al., 2010)

Solanum elaeagnifolium belongs to the Solanaceae family, commonly called silverleaf nightshade, bitter apple and tomato weed. This species is widely distributed in America and propagated in Australia, Egypt, Greece, India, Israel, Zimbabwe, Sicily, Greece, South Africa, the Maghreb countries and Spain (Sforza and Jones, 2007). Traditionally indigenous medicine used this plant for many purpose, such as the treatment of sore throats, an antiseptic agent, toothaches, and gastrointestinal disorders (Boyd et al., 1984). In recent years, despite its reputation as a weed, *S. elaeagnifolium* undeniably has attracted increasing interests for their appreciable and medicinal values. Phytochemical analysis of berries extracts revealed the presence of kaempferol 8-C-β-galactoside that possess medicinal proprieties including hepatoprotective and curative effects against histopathological and histochemical damage induced by paracetamol in liver. Steroidal glyoalkaloids (solanidine) in the root and fruit part of the plant are characterized, and have been shown to be effective in variety of medical applications, including limiting growth of certain cancer cells, treating herpes complex viruses and commercially used in the preparation of contraceptive and corticosteroid drugs. Recent studies have shown that berries of bitter apple possess not only mollucicidal activity but also nutritional. Moreover, berries of bitter apple possess not only mollucicidal activity but also nutritional (Mellado et al., 2008; Larhsini et al., 2010) and ecologic.

To our knowledge, no information is available about the antioxidant proprieties of *S. elaegnifolium* in the literature, except its phytochemical constituents. The aim of this work was to evaluate the *in vitro* antioxidant and glycation inhibitory activity of different solvent extracts of *S. elaeagnifolium* Cav fruit during ripening stage. Their polyphenol and flavonoid contents were also investigated. Protective effects of *S. elaeagnifolium*

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Cav extracts fruit against cellular oxidative stress were also determined.

MATERIALS AND METHODS

Chemicals and reagents

Folin–Ciocalteu's phenol reagents, gallic acid, vanillin reagent, trichloroacetic acid (TCA), iron (III) chloride anhydrous (FeCl3), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate anhydrous (Na2CO3), sodium nitrite (NaNO2), aluminum chloride hexahydrate (AlCl3, 6H2O), sodium hydroxide (NaOH), potassium ferricyanide (K3Fe (CN)6), quercetin and ascorbic acid (Vit C) were obtained from Fluka (Buchs, Switzerland). Hank's balanced salt solution (HBSS), fluorescein sodium salt (FL), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), 2,7-dichlorofluorescein (DCFH), 2',7'-dichlorofluorescein (DCF), tert-butyl hydroperoxide (t-BH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), quercetin, 2,2-azobis (2-methyl-propionamidine) dihydrochloride (AAPH) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Oakville, ON). Bovine serum albumin (BSA, fraction V), potassium phosphate monobasic, potassium phosphate dibasic trihydrate, sodium azide, aminoguanidine hydrochloride were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Ribose was from Alfa Aesar (Schiltigheim, France). Commercial natural products were purchased from Sigma-Aldrich or Extrasynthèse (Genay, France).

Ninety-six well black bottom plates and their silicone lids were from Greiner Bio One (Fisher Scientific, Illkirch, France). The automated 96-well microtiter plate assay was conducted on a Freedom Evo® 100 liquid handling workstation (Tecan, Lyon, France). The liquid handling (LiHa) arm was equipped with four LiHA standard fixed washable tips (Teflon®-coated stainless steel, resistant to DMSO, Tecan). Dispensing steps, i.e., liquid class parameters, were optimized and programmed using Evoware software. AGE fluorescence was measured using a microplate spectrofluorometer infinite M200 (Tecan, Lyon, France) and Magellan software (Tecan).

Preparation of plant extract

Solanum elaeagnifolium fruits were collected at different ripening stages in September from Jediada (5.5 km North of Tunis) in Tunisia. Authentication was performed by Pr. A. Samoui, (Biotechnology Center at the Technopark of Borj-Cedria and according to "Fore de la Tunisie", Le Floc'h and Boulous, 2008). The voucher (CBBC-LSBA 06/03/10/2011) was deposited at the Herbarium of the Laboratory of Bioactive Substances at the Biotechnology Center. Lyophilized powder of the *Solanum elaegnifolium* fruits was successively Soxhlet-extracted during 3 h with cyclohexane, dichloromethane, ethyl acetate and methanol. Between each step, solvent extracts were filtrated with a Whatman filter paper (N°4) and concentrated under rotary vaccum evaporator (Rotovapor-El, Labortechnik AG, Büchi, Switzerland) at 40 °C. Finally, the crud extracts obtained were mixed with DMSO and stored at 4 °C until further analysis. For the aqueous extract, the powdered *Solanum elaeagnifolium* fruits were macerated with distilled water at ambient temperature using a shaker during 24 h. Afterwards, the filtrates were lyophilised (Christ, Alpha 2-4LSC, 102042) and were then mixed with DMSO.

Cell culture

Cell lines WS-1, Human Skin Fibroblast (ATCC # CRL-1502) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). They were cultured in Dulbecco's modification Eagle's medium (DMEM, Mediatech Cellgro®, Herndon, USA) containing sodium bicarbonate L-glucose and L-glutamine, to which were added 10 % fetal bovine serum (Hyclone, Logan, USA), vitamins (IX), penicillin (100 I.U.

mL-1) and streptomycin (100 µg mL-1), essential amino acids (IX) and sodium pyruvate (IX) (Mediatech Cellgro, VA) and grown cells in a humidified environment at 37 °C containing 5 % CO_2.

Phytochemical analysis

Preliminary qualitative phytochemical screening

Principal chemical constituents like flavonoids, tannins, saponins, alkaloids, steroids and triterpens present in different fruit extracts were characterized with the colorful reaction test using standard procedures described by Oleszek (2002).

Determination of total polyphenols contents

Total polyphenols contents in the fruit extracts were determined by method of Dewanto et al. (2002) involving Folin-Ciocalteu reagent and gallic acid as a standard. The reaction mixture contained 125 µL of diluted extract solution, 125 µL of Folin–Ciocalteu reagent, 1250 L of 7 % Na_2CO_3 solution and 1.5 mL of distilled water. After standing for 90 min, the absorbance of resulting bleu color was measured at 760 nm by Optizen 2120 UV-visible spectrophotometer (Optizen 2120 UV plus, Mecasys, Korea) and reported as milligrams of gallic acid equivalents per gram of extract (mg Eq GA/g extract). Quantification of total phenolic was done on the basis of the standard curve of gallic acid (concentration range: 50-200 µg/mL).

Determination of total flavonoids contents

Aluminum chloride method introduced by Heimler et al. (2006) was used to determine flavonoid contents and results were expressed as milligrams of quercetin equivalent per gram extract (mg Eq Q/g extract). 250 µL of the diluted sample was added to 75 µL of a 7 % $NaNO_2$ solution. After 5min, 150 µL of a freshly prepared 10 % $AlCl_3$-$6H_2O$ solution added, and finally 500 µL of 1 M NaOH solution added after in. The final volume was adjusted to 2.5 mL with deionized water. The mixture was allowed to stand for 5 min and the absorbance was measured at 510 nm. Total flavonoid contents were calculated with respect to quercetin standard curve (concentration range: 125-1000 µg/mL).

In vitro antioxidant properties of bitter apple fruit extracts

Numerous techniques are available to evaluate the antioxidant activities of compounds or complex mixtures, such as plant extracts. Despite the various methods, just one procedure cannot give a reductive suggestion of the antioxidant properties of the extracts. For that reason, bitter apple fruit extracts were screened for their possible antioxidant activities by using three complementary in vitro assays: ORAC, ferric reducting power, chelating activity, inhibition of DPPH and ABTS free radicals, anti-AGEs and *ex vivo* cell assay using 2'-7'-dichlorofluorescin-diacetate (DCFH-DA).

ORACFL assay

The ability of anti-oxidant compounds to inhibit the decline in fluorescein (FL) fluorescence induced by a peroxyl radical generator-AAPH was determined using the method described in Morel et al. (2011). Briefly, the ORAC assay was performed out on an Infinite® M200 Magellan injector plate reader (Tecan). Trolox was used as a control standard. The experiment was conducted at 37.5 °C and pH 7.4, with a blank sample in parallel. Trolox standards (12.5-75 µM), fluorescein (0.01 µM), and AAPH solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.4), blanks solutions in triplicate were also prepared using corresponding solvents to serve as controls, then 20 µL of sample extracts and blanks were placed in the well of a Costar 3631 assay plate (Corning Incorporated, Corning, NY) and fluorescein (100 µL) was added to each well, subsequently, AAPH (50 µL) was added. The plate was top read at excitation and emission wavelengths of 485 and 520 nm, respectively, at 37.5 °C. The fluorimeter was programmed to record the fluorescence of fluo-

rescein every 40 min after addition of 2,2-azobis (2-amidinopropane) dihydrochloride. The final results were calculated by comparing the net areas under the fluorescein decay curves between the blank and the samples. ORAC values were expressed in micromoles of Trolox equivalents (TE) per milligram (µmol TE/mg).

DPPH radical scavenging assay

Free radical scavenging capacity of the *S. elaeagnifolium* fruits extracts against a stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was evaluated by the method of Morel et al. (2011). Acid chlorogenic (Acros Organics, 109240010) was used for comparison. 25 µL of freshly prepared DPPH solution and 75 µL of absolute ethanol were added to 100 µL of the samples containing various concentrations using microplate reader injector (Infinite® 200, Tecan, France). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm. Trolox (12.5 µM to 75 µM) was used for calibration to compare the DPPH quenching capacity of the sample and the results were expressed in micromoles of Trolox equivalents (TE) per gram (µM TE/g dry extract).

ABTS radical scavenging assay

A modification of the original method of Re et al. (1999) was applied to assess the scavenging capacity of *S. elaeagnifolium* fruit extracts in a reaction with the ABTS+ radical cation. ABTS•+ radical solution was generated by oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt stock solution with potassium persulfate (K2S2O8). Stock solutions of ABTS (5 mM) and potassium persulfate (2.7 mM) in water were prepared, and ABTS•+ radical solution was produced by reacting 5 mL of the ABTS stock solution with 5 mL of potassium persulfate solution. The mixture was left to stand in the dark at room temperature for 12–16 hours before use. For the evaluation of antioxidant capacity, the ABTS•+ solution was diluted with phosphate buffer (20 mM, pH 7.4) to obtain the absorbance of 0.700 ± 0.020 at 660 nm. 1980 µL of ABTS•+ solution were mixed with 20 µL of sample solution and the decrease in the absorbance was measured at 734 nm after 15 min. The reagent blank was prepared by adding 20 µL of solvent instead of the sample. Trolox concentration was used as the standard. The percentage of inhibition of ABTS•+ was calculated with the following formula:

$$I\ (\%) = [(A_{control} - A_{sample}) / A_{control}] * 100$$

The concentration of sample necessary to decrease the absorbance ABTS•+ by 50 % (IC50, µg/mL) was calculated graphically for inhibition percentage against extract concentration.

Ferric reducing power assay

The ferric reducing capacities of *S. elaeagnifolium* fruits extracts was estimated by the modified method of Oyaizu (1986) and compared against ascorbic acid. Various concentrations of fruit extracts (20 µL) were mixed with phosphate buffer (50 µL, 0.2 mM, pH 6.6) and 1 % of K_3Fe (CN)6 aqueous solution (50 µL), The reaction mixtures were then incubated at 50 ºC for 20 min. Afterwards, 50 µL of 10 % trichloroacetic acid was added, to the mixture which was then mixed with 50 µL distilled water and 10 µL of 0.1 % $FeCl_3$ solution. The intensity of the blue-green color appeared was measured at 630 nm using an automated 96-well plate reader (ELx 800 Biotek, Instruments, Highland Prk, Box 998 Winnooski, vt 05404-0998 SN 215537, USA). Higher absorbance indicated greater reducing capacity. The effective concentration providing 0.5 nm of absorbance (EC50, µg/mL) was calculated from the graph of absorbance at 630 nm against concentration. Ascorbic acid was used a positive control.

Chelating activity on Fe^{2+}

The chelating ability of ferrous ions by the extracts from *S. elaeagnifolium* fruits

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was estimated by the method of Dinis et al. (1994). Extracts of different concentrations were added to a solution of 2 mM $FeCl_2.4H_2O$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The mixture was incubated in the dark at room temperature for 10 min. and the absorbance was then measured at 562 nm. EDTA served as the positive control and the results were expressed as percentage of inhibition of ferrozine-Fe^{2+} complex formation according to the formula:

Chelating activity (%) = [1- (abs sample / abs control)] ×100.

Ex-vivo antioxidant cell assay using 2'-7'-dichlorofluorescin-diacetate (DCFH-DA)

Intracellular formation of ROS was evaluated using the DCFH-DA assay as described by Girard-Lalancette et al. (2009), with some modifications. Briefly, human skin fibroblast cells were plated in 96-microwell plates (BD Flacon) at 10.000 cells per well and incubated for 48 h at 37 °C and 5 % CO_2. The cells were washed with 150 µL Hank's balanced salt solution at pH 7.4 and incubated for 90 min with 100 µL HBSS (pH 7.4) containing 5 µM DCFH-DA. The cells were then washed again with 150 µL HBSS. To assess antioxidant activity, the cells were incubated either with different concentrations of extract from *S. elaeagnifolium*, or trolox and quercetin (both positive standards), in the absence or presence of 200 µM tert-butyl hydroperoxyde (t-BuOH). Fluorescence was measured immediately after t-BuOH administration and again 120 min later using an automated 96-well plate reader (Fluoroskan Ascent FLTM, Thermo-Labsystems) using 485 nm for excitation and 530 nm for emission. Antioxidant activity is expressed as the concentration of extract inhibiting 50 % of DCFH oxidation induced by t-BuOH. IC_{50} were calculated using the logarithmic regression of the dose-response curve after subtraction of both blank and intrinsic sample fluorescence values.

Anti-AGEs activity

Glycation inhibiting properties of *S. elaeagnifolium* fruit extracts were evaluated by a glucose-bovine serum albumin (BSA) based on AG fluorescence. The assay involved incubating BSA (10 mg/mL) with D-ribose (0.5 M) and the tested compound (3 µM to 3 mM) or extract (1 µM to 1 mM) in 50 mM phosphate buffer at pH 7.4 (NaN_3 0.02 %). Solutions were incubated in 96-well microtiter plates at 37 °C for 24 h in a closed system before AGE fluorescence measurement. Fluorescence resulting from the incubation, under the same BSA (10 mg/mL) and tested compound (3 µM to 3 mM) or extract (1 µM to 1 mM) conditions, was subtracted for each measurement. A control, i.e., no inhibition of AGE formation, consisted of wells with BSA (10 mg/mL) and D-ribose (0.5 M). A blank of control, i.e., 100 % inhibition of AGE formation, consisted of wells with only BSA. The final assay volume was 100 µL. Both vesperlysines-like (λexc 370 nm; λem 440 nm) and pentosidine-like (λexc 335 nm; λem 385 nm) AGE fluorescence were measured using a microplate spectrofluorometer.

The percentage of AGE formation was calculated as follows for each compound/ extract concentration:

AGEs (%) = [fluorescence intensity (sample) − fluorescence intensity (blank of sample)] / [fluorescence intensity (control) − fluorescence intensity (blank of control)] × 100.

Dose-effect curves were best fit with a sigmoidal dose-response equation using Sigma Plot 12.0 Software, which enabled calculation of the IC_{50} values.

Statistical analyses

Data were subjected to statistical analysis by using statistical program package STATISTICA. Chemical compositions and antioxidant activity of each were the mean ± S.D. of three experiments. The one-way analysis of variance (ANOVA) followed by Tukey HSD test were employed and the differences between individual means were deemed to be significant at $p<0.05$.

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RESULTS AND DISCUSSION

Total phenolic and flavonoid contents of various extracts

Total phenol contents of *S. elaeagnifolium* fruit extracts at different maturity stage by using different extraction solvents, are presented in Table 1. Our results were in agreement with previous reports (Sultana et al., 2009; Ghasemzadeh et al., 2011; Edziri et al., 2012) and confirmed the effect of the used solvent on the total polyphenol extract capacities. In unripe and overripe fruit, methanol extracts has been shown to have significantly higher contents of total phenol, which approximately were 2 fold more than aqueous extracts and 4 fold more than the ethyl acetate extracts. In ripe fruit ethyl acetate and methanol extracts showed the highest polyphenol contents.

The contents of total flavonoid in fruit extracts exhibited also significant ($p<0.001$) variation depending on the used solvent (Table 1). For example, in ripe fruit, the highest content of flavonoid was found with ethyl acetate extracts (61.09 ± 1.92 mg eq Q/g extract), followed by dichloromethane (34.17 ± 0.22 mg eq Q/g extract), methanol (17.62 ± 0.42 mg eq Q/g extract) and aqueous extracts (15.14 ± 2.74 mg eq Q/g extract). Other authors have been reported that ethyl acetate is more efficient for recovering the higher amounts of flavonoids (Jayaprakasha et al., 2008).

In addition, our analysis revealed that the concentration of total phenols and total flavonoid from bitter apple fruits was also significantly ($p<0.001$) influenced by the maturity stage (Table 1). Total polyphenol contents firstly decreased from unripe (2.29-12.79 mg/g extract) to ripe fruits (1.92-9.04 mg/g extract), and then subsequently increased to attain their maximum level in overripe fruits (2.04-12.66 mg/g extract). No significant differences were found between unripe and overripe fruits ($p>0.05$). A similar trend is found in different fruits such as tomatoes, strawberry and mulberry, as reported by Miletić et al. (2012) and Tahir et al. (2012). Total flavonoid in fruit extracts exhibited also significant ($p<0.001$) variation depending on the used solvent (Table 1). For example, in ripe fruit, the highest content of flavonoid was found with ethyl acetate extracts (61.09 mg QE/g extract), followed by dichloromethane (34.17 mg QE/g extract), methanol (17.62 mg QE/g extract) and aqueous extracts (15.14 mg QE/g extract). It is reported that ethyl acetate solvent is more efficient for recovering a higher amounts of flavonoids (Jayaprakasha et al., 2008). Firstly, total flavonoid reached a maximum in ripe fruits, and afterwards decreased with advancing maturity. Similar results are observed for two varieties of Citrus (Moulehi et al., 2011).

Table 1: Total phenol contents (expressed as mg GAE/g extract) and total flavonoid contents (expressed as mg QE/g extract) of different extraction solvents of *Solanum elaeagnifolium* fruit collected at different stages of ripening

Solvents	Total phenols			Total flavonoids		
	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe
Dichloro-methane	2.29 ± 0.25^{cA}	1.92 ± 0.29^{cA}	2.04 ± 0.10^{cA}	12.48 ± 0.16^{aB}	45.22 ± 0.22^{aA}	29.25 ± 0.86^{aB}
Ethyl acetate	3.00 ± 0.27^{bA}	8.23 ± 0.44^{bA}	3.71 ± 0.39^{bA}	22.07 ± 0.30^{abB}	61.09 ± 1.92^{abA}	5.89 ± 0.29^{abB}
Methanol	12.79 ± 0.83^{aA}	9.04 ± 0.08^{aA}	12.66 ± 2.54^{aA}	10.24 ± 0.31^{bcB}	17.62 ± 0.42^{bcA}	12.74 ± 1.10^{bcB}
Aqueous	5.09 ± 0.16^{bA}	6.56 ± 0.19^{bA}	6.21 ± 0.07^{bA}	3.00 ± 0.31^{cB}	15.14 ± 2.74^{cA}	5.73 ± 0.01^{cB}

The values are expressed as means ± SD of triplicate tests. Superscript letters (a, b, c) within the same line indicate significant ($P < 0.05$) differences with the extracting solvent; Superscript letters (A, B, C) within the same column indicate significant ($P < 0.05$) differences with the maturity stage according to the Tukey HSD test.

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Antioxidant proprieties of Solanum eleaegnifolium fruit extracts

The results of the antioxidant activity of the different solvent extracts were summarized in Table 2 and 3. It was found that the antioxidant activity of bitter apple fruit extracts depended mostly on both used solvent and ripening stages, and their interaction. In DPPH assay, for instance, methanol extracts of overripe fruit were able to effectively reduce the stable free radical DPPH (7203 µM TE/g dry extract), when compared to unripe and ripe extracts (4220 and 1592 µM TE/g dry extract, respectively). Cyclohexane, dichloromethane and ethyl acetate extracts did not show DPPH radical scavenging activity. So, the antioxidant effectiveness of bitter apple fruit extracts decreased in following order:

methanol > chlorogenic acid ≥ aqueous, and the ranking order of three maturity stages of fruit was as follows: overripe > ripe > unripe

In accordance with radical quenching activity, reducing power of these different solvents extracts is presented in the following descending order:

methanol > ascorbic acid > aqueous > ethyl acetate > dichloromethane > cyclohexane

Methanol extracts of fruit at different stages exhibited also higher reducing power towards the Fe^{3+}/ferricyanide complex (EC_{50} values ranged from 0.39 to 0.53 µg/mL) (Table 3). No significant differences were found at stages unripe, ripe and overripe. Generally, the reducing power of the methanol extracts was approximately 2 or 3 times more potent than ascorbic acid, as positive control (EC_{50} = 1.56 µg/mL).

In addition, on the basis of ORAC values, the strength of peroxyl radical scavenging activity was in the order:

chlorogenic acid (13738 µM TE/g) > methanol (1615-4087 TE/g) ≥ aqueous (2752 TE/g)

Methanol extracts of unripe fruits showed the highest antioxidant activity (4087 ± 151 µM TE/g). It was also encouraging to note that most of the fruit extracts studied exhibited ORAC values compared to the ethanolic extract of rosemary (2640 µM TE/g).

Different solvent extracts produce an antioxidant efficacy with the radical ABTS. The values were ranged from 2.5 ± 0.1 µg/mL (overripe aqueous extract) to 4.2 ± 2.6 µg/mL (unripe aqueous extract). This efficiency of solvent extracts was better compared to the synthetic standard trolox (IC50 = 3.47 ± 0.51 µg/mL).

Another interesting observation in this study was the ability of the aqueous extract to inhibit the chelated ferrous ions (15.79-21.90 %). No significant difference on chelating activity between unripe and ripe fruits was observed.

In the cell-based assay using DCFH-DH, the anti- and pro-oxidant properties of antioxidant compounds in the extract was tested by measuring their ability to scavenge ROS produced by normal metabolism by cells, and then to inhibit the oxidation of DCFH to DCF. Results presented in Table 3 show that all solvent extracts tested were significantly able to inhibit the oxidation of DCFH in a dose-dependent manner and consequently reduce the oxidative stress observed intracellular. IC_{50} values calculated from logarithmic curves indicated that the methanol extracts obtained in overripe fruit exhibited a potent antioxidant activity that could inhibit the t-BH-induced oxidation of DCFH with an IC_{50} equal to 0.6 ± 0.1 µg/mL and similar to those of standard phenolic compound (quercetin). In addition, this value is higher than those found with other plants from Tunisia (Oueslati et al., 2012) and from Canada (Girard-Lalancette et al., 2009) suggesting the strongly pro-oxidation effects of overripe fruits.

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Table 2: Antioxidant capacities of different solvent extracts of *S. elaeagnifolium* fruit collected at different maturity stages

Solvent	Antioxidant cell assay (IC_{50}. µg/mL)			$ABTS^+$ radicals (IC_{50}. µg/mL)			DPPH radicals (µmol TE /g extract)			ORAC (µmol TE /g extract)		
	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe
Cyclo-hexane	20 ± 6^{cA}	100 ± 25^{cA}	>100	NA	NA	NA	NA	NA	NA	NA	NA	NA
Dichloro-methane	>100	>100	2.9 ± 0.4^{cA}	24.33 ± 2.5^{c}	NA	NA	NA	NA	NA	NA	NA	NA
Ethyl acetate	4.4 ± 0.4^{bA}	8 ± 4^{bA}	3.5 ± 0.4^{bA}	16.8 ± 0.8^{b}	7.3 ± 0.2^{b}	10.5 ± 5.2^{b}	1666 ± 98^{bA}	NA	NA	NA	NA	NA
Methanol	7 ± 0.01^{aA}	>100	0.6 ± 0.1^{aA}	6 ± 0.5^{aA}	5.4 ± 0.2^{aA}	5 ± 0.05^{aA}	1592 ± 83^{aA}	4220 ± 83^{aA}	7203 ± 290^{aA}	4087 ± 151^{cA}	2890 ± 159^{cA}	1615 ± 317^{cA}
Aqueous	5.6 ± 0.1^{abA}	4 ± 1^{abA}	26 ± 14^{abA}	4.2 ± 2.6^{aA}	2.5 ± 0.1^{aA}	2.4 ± 0.1^{aA}	NA	3594 ± 198^{ab}	NA	NA	2752 ± 130^{b}	NA
Standards												
Quercetin	0.54±0.03			-			-			-		
Trolox	-			3.47±0.51			-			-		
Chloro-genic acid	-			-			3530±84			13738		
Ethanolic extract of rosemary	-			-			-			2640		

NA indicates no antioxidant activity. The values are expressed as means ± SD of triplicate tests. Superscript letters (a, b, c) within the same line indicate significant ($P < 0.05$) differences with the extracting solvent; Superscript letters (A, B, C) within the same column indicate significant ($P < 0.05$) differences with the maturity stage according to the Tukey HSD test.

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Table 3: Antioxidant capacities of different solvent extracts of *S. elaeagnifolium* fruit collected at different maturity stages

Solvent	Inhibition of iron Fe^{2+} (%)			Reducing power EC_{50} (µg/mL)		
	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe
Cyclo-hexane	NA	NA	NA	72.1 ± 0.17^{cA}	166 ± 25^{cA}	242.66 ± 2.34^{cA}
Dichloro-chloro-methane	NA	NA	NA	163.5 ± 2.12^{cA}	119 ± 0.07^{cA}	119 ± 1.41^{cA}
Ethyl acetate	NA	NA	NA	72 ± 1.63^{bA}	32.45 ± 0.47^{bA}	81.5 ± 1.52^{bA}
Methanol	NA	NA	NA	0.39 ± 0.01^{aA}	0.53 ± 0.02^{aA}	0.43 ± 0.01^{aA}
Aqueous	21.81 ± 2.2^{aA}	21.90 ± 2.6^{aA}	15.79 ± 1.58^{aA}	37.06 ± 1.43^{abA}	19.66 ± 0.97^{abA}	28.5 ± 0.57^{abA}
Standard						
EDTA	98±5.2					
Ascorbic acid				9.30±0.43		

NA indicates no antioxidant activity. The values are expressed as means ± SD of triplicate tests. Superscript letters (a, b, c) within the same line indicate significant ($P < 0.05$) differences with the extracting solvent; Superscript letters (A, B, C) within the same column indicate significant ($P < 0.05$) differences with the maturity stage according to the Tukey HSD test.

In this study, the presence of phenolic compounds was detected in four extracts of *S. elaeagnifolium* fruit (Table 1). Furthermore, total phenols were present on considerable amounts in methanol extracts compared to dichloromethane, ethyl acetate and aqueous extracts, which might be responsible for their higher scavenging abilities on DPPH and peroxyl radicals and reducing power, and for the potent pro-oxidant proprieties. The strong ABTS radical scavenging capacity and the noticeable chelating iron activity of aqueous extracts could be due, in part, to the presence of flavonoid contents which are known to possess efficient antioxidant activity. In addition, the phytochemical screening revealed the presence of alkaloid and steroidal glycosides saponins compounds. These compounds are widely reported in the species Solanum species displaying significant antioxidant activity.

Nevertheless, cyclohexane fruit extracts did not display noteworthy antioxidant ability. This result was expected because the chemical composition of cyclohexane extract did not show the presence of phenolic compounds (Table 1).

Anti-AGEs capacities of S. eleaegnifolium fruit extracts

The anti-glycation capacities of *S. elaeagnifolium* fruit extracts evaluated by their inhibition of the formation of global fluorescent AGEs in the BSA/glucose system is depicted in Table 4. Overripe fruits demonstrated a dose-response inhibition of the vesperlysines-like AGEs formation (AGE-IC_{50}; 0.5, 0.7 and 0.65 mM for ethyl acetate, methanol and aqueous extracts, respectively). Also, this inhibiting effect of overripe fruit extracts was higher than that of amminoguanidine, as positive control (IC50 = 0.9 mM). Moreover, our results indicated that the overripe fruit extracts (ethyl acetate and methanol) were more efficient in inhibiting the glucose-mediated formation of pentozidine-like AGEs, which were almost similar to those of amminoguanidine (IC_{50} = 0.3 mM). Unripe fruits with methanol (IC_{50} = 0.9 mM) and aqueous extracts (IC_{50} = 0.7 mM) were the second most active extract followed by ripe fruits. When comparing the IC_{50} values of our sample and some food and medicinal plants (Séro et al., 2013), fruits of *S. ealeagnifolium* can potentially exert an anti-AGEs effect.

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Statistical analysis

Statistical correlations have been studied, as summarized in Table 5. Total phenols contents (TPC) were shown to provide the highest correlation with ORAC (r = 0.73), DPPH (r = 0.68), ABTS (r = 0.76) and reducing power (r = 0.80) assays, and anti-glycation activities (r = 0.66 and r = 0.36 for Anti-AGEs vesperlysine and Anti-AGEs pentosidine, respectively), indicating that phenolic content present in the extracts was the more effective component. Our results corroborated with other researchers (Ho et al., 2010; Deetae et al., 2012) who have reported that the amount of phenolics present in the natural extracts is a key determinant of their antioxidant and anti-glycation proprieties. However, the comparative analysis demonstrated that significant correlation with total flavonoid content and anti-AGEs activity of the extracts (r = 0.37 and r = 0.34, respectively), suggesting that the flavonoid contents are probably the main compounds that are responsible for the anti-glycation capacity of *S. elaeagnifolium* fruit extracts. Ramkisson et al. (2013) showed also positive correlation existing between total flavonoid content and anti-glycation activity of Thymus vulgaris. On the other hand, the anti-AGEs formation capacity was correlated to antioxidant activity determined using DPPH, ABTS, ORAC, reducing power and *ex vivo* cell assays (Table 5). These analytical results imply that the potency of anti-AGEs formation depends on the capacities of primary antioxidants.

Cluster analysis (CA) was carried out in order to evaluate the influence of tested parameters in the categorization and differentiation of the fruits studies. One cluster can be observed in the dendrogram (Figure 1A). Unripe fruit was categorized in the same cluster of overripe fruit, indicating that the properties of unripe fruit were similar to overripe fruit.

Regarding the classification of the extracts, the cyclohexane and dichloromethane, as a primary group, were discriminated in the ethyl acetate group (Figure 1B). Methanol and ethyl acetate, which were found as second group, were characterized by the highest antioxidant and anti-AGEs activity related with their higher contents of total phenolic compounds and total flavonoid. Aqueous extracts were characterized by the moderate polyphenols content, presented the highest antioxidant capacity to quenching free radical ABTS+, as compared with two groups.

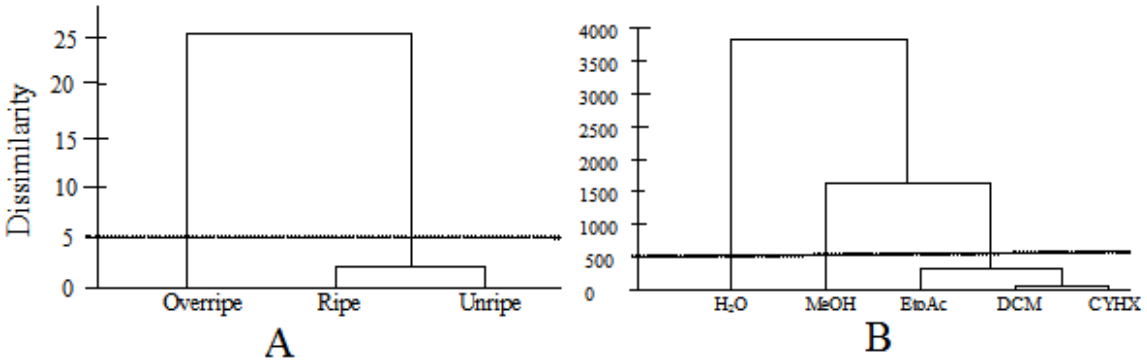

Figure 1: Dendrogram obtained with cluster analysis of **A:** three fruits collected at different maturity stages; **B:** extracting solvent used including cyclohexane (CYHX), dichloromethane (DCM), ethyl acetate (EtoAC), methanol (MeOH) and water (H_2O)

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Table 4: Effect of solvents polarities of *S. elaeagnifolium* fruits extracts on vesperlysines and pentosidine-like AGE formation expressed as IC_{50} (mM)

Solvents	Effect on vesperlysines-like AGE formation (IC_{50}. mM)			Effect on pentosidine-like AGE formation (IC_{50}. mM)		
	Unripe	Ripe	Overripe	Unripe	Ripe	Overripe
Cyclohexane	>1	>1	>1	0.5	>1	>1
Dichloromethane	>1	>1	>1	>1	>1	0.6
Ethyl acetate	>1	>1	0.5	0.5	>1	0.25
Methanol	0.9	>1	0.75	0.8	0.7	0.3
Aqueous	0.7	0.5	0.65	0.8	1.0	0.9
Natural product						
Aminoguanidine	0.9			0.15		

The values are expressed as means ± SD of triplicate tests. Superscript letters (a, b, c) within the same line indicate significant differences with the extracting solvent; Superscript letters (A, B, C) within the same column indicate significant ($P < 0.05$) differences with the maturity stage according to the Tukey HSD test.

Table 5: Correlation coefficient, R, for relationship between values obtained from total phenol content, anti-glycation activity and antioxidant activity determined by different assays

	TPC	TFC	ABTS	Reducing power	DPPH	ORAC	Cell assay	Anti-AGEs vesperlysine	Anti-AGEs pentosidine
TPC	1	0.14	0.76	0.80	0.68	0.73	0.00	0.66	0.36
TFC	0.14	1	-0.01	-0.12	-0.05	-0.07	-0.23	0.77	0.34
ABTS	0.76*	-0.01	1	0.81	0.45	0.46	-0.17	0.64	0.34
Reducing power	0.80*	-0.12	0.81*	1	0.55	0.58	0.32	0.59	0.53
DPPH	0.68*	-0.05	0.45*	0.55*	1	0.97	-0.28	0.37	0.22
ORAC	0.73*	-0.07	0.46*	0.58*	0.97*	1	-0.26	0.43	0.35
Cell assay	0.00	-0.23	-0.17	0.32*	-0.28	-0.26	1	0.24	0.38
Anti-AGEs vesperlysine	0.66*	0.77*	0.64*	0.59*	0.37*	0.43*	0.24	1	0.56
Anti-AGEs pentosidine	0.36*	0.34*	0.34*	0.53*	0.22	0.35*	0.38*	0.56*	1

*: significant correlation ($P < 0.05$. $n = 44$)

CONCLUSION

The goal of this work is the evaluation of antioxidant and glycation inhibitory activities of *Solanum elaeagnifolium* Cav (Solanaceae) fruit extracts using different solvents at different maturity stages. Results showed solvents polarities and maturity stages affects significantly the phenolic content and the antioxidant and anti-AGEs activities. Statistical analysis showed that total phenolic and flavonoid contents correlated well with antioxidants and anti-glycation activities. In conclusion, *S. ealaegnifolium* possessed combined anti-glycation and antioxidant proprieties which correlated with their phytochemical composition, suggesting their application for the management of diabetic complications.

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