ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Anti-glycation study of hydro-alcohol and aqueous extracts of Moroccan plant species

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Article History:

Received on: 12.11.2018
Revised on: 21.02.2019
Accepted on: 24.02.2019

Keywords:

Anti-glycation,
Antioxidant,
Ethanol extracts,
Polyphenols,
Water Extracts

ABSTRACT

Inhibition of advanced glycation end products (AGEs) and free radicals generated during diabetes represents a major therapeutic target in the prevention and treatment of diabetic complications. Natural molecules present in fruits, vegetables and herbs and which are usually safe for human consumption, could represent a strong glycation inhibitor. Anti-glycation effect of nine plant species used in traditional medicine has been evaluated after extraction by hot (EAC) or cold (EAF) maceration and by ethanol (EE). Anti-glycation activity performed on a model system of bovine serum albumin, and methylglyoxal was measured by fluorescence and native electrophoresis. Total phenolic and flavonoid contents were assessed as well. Except for *Sesamum indicum*, all the species studied have an Anti-glycation effect. The highest effect was recorded in *Laurus nobilis* and was dose-dependent, inhibiting both formations of Amadori products and fluorescent AGEs. HPLC analysis revealed a richness of *Laurus nobilis* EE in phenolic compounds such as quercetin, vanillin and gallic acid. A strong correlation was registered between antioxidant power and phenolic/flavonoid content. In contrast, there was no correlation between antioxidant and anti-glycation power. Phenolic and flavonoid compounds were strongly involved in the observed anti-glycation effect. However, the anti-glycation activity obtained is probably attributed to non-antioxidant compounds.

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ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v10i2.259

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INTRODUCTION

The treatment of some lifestyle-diseases such as diabetes is often difficult; the inhibition of disease pathogenesis by daily diet is therefore expected to become a powerful way to prevent lifestyle-related diseases (Sugawa H *et al.,* 2016).

High levels of advanced glycation end products (AGEs) in diabetic patients promote the initiation and development of different diabetic complications such as stroke, neuropathy, cataract, coronary heart disease and atherosclerosis (Harris C.S. *et al.,* 2011). The formation of AGEs is accomplished by non-enzymatic reactions between proteins, lipids or nucleic acids and reducing carbohydrates such as glucose and fructose. Thereby, the formation of AGEs has been frequently considered as an efficient target for preventing lifestyle-related diseases such as diabetic complications (Jung H.A. *et al.,* 2009; Grzegorczyk-Karolak I *et al.,* 2016). To do so, different synthetic molecules approved inhibitory effect against AGEs formation;

however, they are not in clinical use because of their unsatisfactory safety effects (Grzegorczyk-Karolak I *et al.,* 2016). To avoid the problems related to the synthetic molecules, scientists resorted to natural molecules that may be present in fruits, vegetables and herbs and which are usually safe for human consumption and could be considered as strong glycation inhibitors (Pinto M.D.S. *et al.,* 2009).

Glycation acts synergistically with oxidative stress, thus generating diabetes complications. The mechanism of protein damage by glycation reactions is based on the production of ROS either from the auto-oxidation of glucose or in the process of oxidative degradation of intermediate Amadori products (Mossine V.V. *et al.,* 1999). On the other hand, Münch G *et al.,* 1998 reported that AGEs, in turn, can increase oxidative stress either by glucose oxidation and Amadori products, or by binding to specific receptors, or still by the production of oxygenated free radicals by indirect intervention in the process of the immune system. As a result, each secondary metabolite having an antioxidant effect will have an anti-glycation effect and vice versa.

Few studies have been carried out on the anti-glycation power of Moroccan plants. This country has a true plant-genetic reservoir, with about 4,500 species and sub-species of vascular plants (Benkhnigue O *et al.,* 2011). In this context, this study aimed to investigate the implication of antioxidants secondary metabolites in the anti-glycation effect and some of their working mechanisms. These compounds were extracted from plants selected because of their traditional use in medicine as an ingredient of many polyherbal formulations for the treatment of several pathologies (Baharvand-Ahmadi B *et al.,* 2015; Pusadkar P.P. *et al.,* 2015; Rahmani A.H. and Aly S.M., 2015; Bahmani M *et al.,* 2016; Raish M *et al.,* 2016; Vardapetyan H *et al.,* 2016; Özcan M.M. and Matthäus B, 2017; Sim M.O. *et al.,* 2017; Bahramsoltani R *et al.,* 2018).

MATERIAL AND METHODS

Chemicals: All reagents (Ethanol, Methanol, Sodium carbonate (Na_2CO_3), Sodium bicarbonate, Folin–Ciocalteau reagent, Gallic acid, Aluminum trichloride, Potassium acetate, Quercetin, Iron II ($FeCl_2$), Ferrozine, BSA, 2,2-diphenyl-1picrylhydrazyl (DPPH), methylglyoxal 40%, copper sulfate, sulfuric acid, anhydrous sodium sulfate, potassium sodium tartrate, arsenomolybdate and glucose) unless otherwise stated, were purchased from Sigma Chemical Co (St Louis, MO, USA). Metformin is commercialized as Glucophage 500 mg (purity 78%).

Plant collection and extract preparation

Plants were collected in March 2015, from different regions of Morocco (Table 1). The selected parts of the plants were dried at 40°C for 15 h. Samples were then ground into a fine powder that was passed through an 80-mesh sieve, collected and stored at 20°C until use. Aqueous extracts were obtained by extraction of samples (30 g) with distilled water (300 ml) for 45 min, three times, at 80°C (EAC) or 25°C (EAF). A hydroalcoholic extract was obtained by extraction of samples (20 g) with 200 ml of ethanol solution (70%) for 24h, three times. The macerates were filtered and centrifuged for 20 min (4000 t/min) at room temperature. After evaporation of supernatants, the dried extracts obtained were stored at 4°C away from light until use.

The following formula was used to determine the extracts yield (Harborne J.B., 1998):
R = (Px / Py) * 100 R: Extract yield (%), Px: Extract weight (g), Py: Plant weight (g).

Quantification of polyphenols, flavonoids and reducing sugars

Determination of Total Phenolic Contents (TPC)

Amount of TPC was determined by the Folin-Ciocalteu method (Boizot N and Charpentier J.P., 2006). Briefly, 100 µl of extracts (1 mg/ml) was added to 500 µl of 1:10 Folin–Ciocalteau reagent (prepared prior to use). After 4 min, 400 µl of sodium carbonate 7.5% (m/v: 75mg/ml) were added. After 30 min of incubation at room temperature, the optical density at 765 nm was measured by spectrophotometer type VARIAN Cary 50 UV/Vis. The standard range was prepared from a solution of gallic acid (GA) (5 mg/ml) with concentrations ranging from 0 to 150 µg/ml. The results were reported in Gallic Acids Equivalents (GAE) per g of sample.

Determination of Total Flavonoid Contents (TFC)

The TFC was determined by aluminium trichloride colorimetric method ($AlCl_3$) (Dehpour A.A. *et al.,* 2009), with modifications. Briefly, 250 µl of extracts (2 mg/ml) was added to 1.4 ml of deionized water, 50 µl of potassium acetate (1 M), 50 µl of aluminium trichloride 10% (m/v), and 750 µl of absolute ethanol. After 30 min of incubation at room temperature, the absorbance at 415 nm was measured (VARIAN Cary 50 UV-Vis). The standard range was prepared from a solution of quercetin (10 mg/ml of ethanol 80%), with concentrations ranging from 0 to 150 µg/ml. The results were reported in Quercetin Equivalents (QE) per g of sample.

Determination of Reducing Sugar Content

The reducing sugar content was determined by Nelson Somogyi method (Joslyn M.A., 1970), with modifications. Briefly, 100 µl of extracts (1 mg/ml) was added to 1.9 ml of ultrapure water, and 1 ml of alkaline copper tartrate reagent. After 10 min of incubation at 100 °C, the arsenomolybdic reagent was added. The volume was completed to 10 ml with distilled water, and the absorbance at 620 nm was measured. The standard range was prepared from a glucose solution (100 mg/ml), with concentrations ranging from 0 to 100 mg/ml. The results were reported in Glucose Equivalents (GE) per g of sample.

Determination of Total Tannin content (TTC)

The TTC was determined by the method of Folin-Denis (Glick Z and Joslyn M.A., 1970), with some modifications. A volume of 100 µl of the extract (1 mg/ml) was added to 500 µl of the Folin-Denis reagent (1:10). After 5 min, 400 µl of 7.5% Na_2CO_3 (w / v) was added followed by incubation for 30 min at room temperature. Absorbance was measured at 760 nm (VARIAN Cary 50 UV-Vis). A control with distilled water in place of the extract is carried out under the same conditions.

The standard range was prepared from a solution of tannic acid (TA) (10 mg/ml) with concentrations ranging from 0 to 150 µg/ml. The blank deprived of tannins was carried out as point 0 µg/ml of the range.

Qualitative analysis of the chemical composition by HPLC

The analysis was carried out by an HPLC (VP Shimadzu Liquid Chromatograph) at the biochemistry laboratory of the Pharmaceutical Department of Rabat, Morocco. After filtration through a membrane (pore size of 0.45 ml), 20 µl of each extract was injected onto a C18 reverse phase column (125 × 4.6 mm). The mobile phase consisted of two solvents: solvent A, water / formic acid (95: 5; v/v) and solvent B, acetonitrile/solvent A (60:40; v/v). The elution gradient applied was isocratic type spread over 10 min with 0% B, and gradient type from 0% to 5% B (30 min), from 5% to 15% B (18 min), from 15% To 25% B (14 min), from 25% to 50% B (31 min), from 50% to 100% B (3 min), followed by rinsing and reconditioning of the column. The flow rate was 1 ml/min at 25°C. Detection was performed by a UV-Vis detector at wavelengths equal to 280 and 350 nm (Hasim K *et al.,* 2009). Identification of phenolic compounds was obtained using different standards and by comparison of the retention time and the ultraviolet-visible spectra with those of the literature (Hasim K *et al.,* 2009).

Antioxidant activity

Radical DPPH-scavenging activity

Free radical-scavenging capacities of extracts were determined according to Braca A *et al*. (2002), with some modifications. For this, 4 mg of DPPH was dissolved in 100 ml of methanol and incubated in the dark for 3 h before use. Briefly, 250 µl of extract (0.06, 0.125, 0.25, 0.5, 1 and 10 mg/ml) were added to 750 µl of DPPH solution and then incubated for 30 min in the dark. The absorbance was measured at 517 nm against the control of methanol and DPPH solution (250 µl for 750 µl respectively). The standard range was prepared from a solution of ascorbic acid (5 mg/ml), with concentrations ranging from 0 to 500 µg/ml.

Fe^{++} chelating assay

The chelating capacity of extracts was measured according to Chiu *et al.* (2007) with modifications. Briefly, 250 µl of extracts (0.25, 0.5, 1, and 10 mg/ml) was added to 50 µl of $FeCl_2$ (0.6 mM) and 450 µl of methanol. After 5 min, 50 µl of Ferrozine (5 mM) was added. After 10 min of incubation at room temperature, the absorbance at 562 nm was measured (VARIAN Cary 50 UV-Vis). Methanol replaced the extract in the negative control. The standard range was prepared from a solution of quercetin (10 mg/ml of ethanol at 80%), with concentrations ranging from 0 to 150 µg/ml.

Antiglycation activity

In vitro glycation of bovine serum albumin

Bovine serum albumin (BSA 5 mg/ml, containing EDTA) was incubated with methylglyoxal (10 mM) and sodium azide (0.02%) in 0.1 M phosphate buffer (pH 7.4). Three concentrations of tested compounds (1.5, 3.5 at 10 mg/ml) were added to the reaction mixture then incubated for 24 h at 50°C away from light and stirred. Individual vials were removed at desired times and stored frozen at -20°C until analysed. Metformin (30 mM) was used as positive control.

Electrophoretic migration in native conditions

The mixture solution was applied to native polyacrylamide gel electrophoresis (Native-PAGE). The samples were separated on a 7% polyacrylamide gel. After migration, the gels were stained with Coomassie blue for 1 h. The destaining step was also conducted for 1 h.

Spectrofluorimetric measure

Fluorescence measure was performed in the chemistry laboratory of the National Office of food safety of Tangier (ONSSA), Morocco. Comparison of fluorescence spectrum (excitation at 370 nm) and change in fluorescence intensity (excitation at 370

nm and emission 423 nm) was performed through a spectrofluorimeter type (VARIAN Cary 50 UV-Vis).

Statistical analysis

The results of *in vitro* tests were expressed as mean ± SD. Difference between control and samples was determined by uni-varied ANOVA followed by Fischer's test. The p values of ≤0.05 were considered significant.

RESULTS AND DISCUSSION

Phytochemical composition

The results presented in tables 2 and 3 showed that total polyphenols and flavonoids varied according to the solvent used and the plant species. In fact, polyphenol concentrations ranged for EAC from 50.77±0.26 mg GA/g dry extract in *S. indicum* to 288.44±0.33 mg GA/g dry extract in *G. roseum*. Whereas for EAF, they varied between 33.58±0.36 mg GA/g dry extract in *L. nobilis* and 167.47±0.24 mg GA/g dry extract in *G. roseum*. For EE, values ranged from 55.51±0.34 mg GA/g dry extract in *T. foenum graecum* to 153.83±0.79 mg GA/g dry extract in *O. europaea*. For flavonoids, maximum levels were observed in EAC of *A. citrodora* (103.71 ± 0.29 mg Qu / g dry extract), in EAF of *N. sativa* (68.94 ± 0.24 mg Qu / g Ms) and in EE of *L. sativum* (76.55 ± 0.37 mg Qu / g Ms). Whereas, the minimum values were observed in EAC and EE of *S. indicum* and in EAF of *L. nobilis* (Table 2). Concerning tannins and sugars, we noted that several species had non-significant differences. *Trigonella foenum graecum* has the highest tannins concentration, with maximum levels in its EAC and EAF (Table 2 and 3).

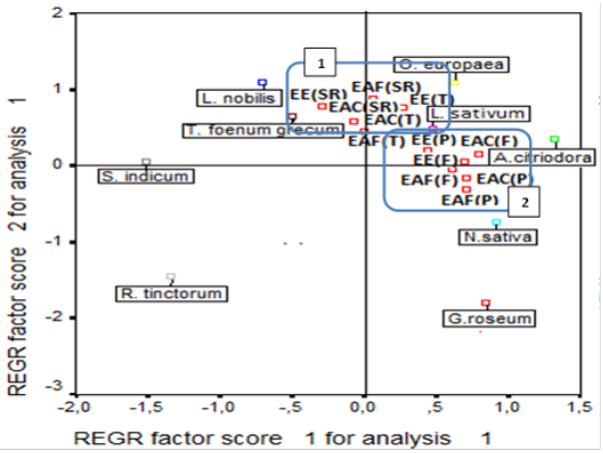

Figure 1: Projection of the phytochemical composition variables according to the two components; P:Polyphenols; F:Flavonoids; T: Tannins; SR: Reducing Sugars.

Variance analysis showed that only 55.17% of the components were absorbed by axis 2. This indicates a low homogeneity between the species.

According to the principal component analysis (PCA) of figure 1, the components were divided into two groups. Each group constituents were significantly correlated. Group 1 characterized *L. nobilis*, *T. foenum graecum* and *O. europaea*, whereas group 2 characterized *N. sativa* and *A. citrodora*.

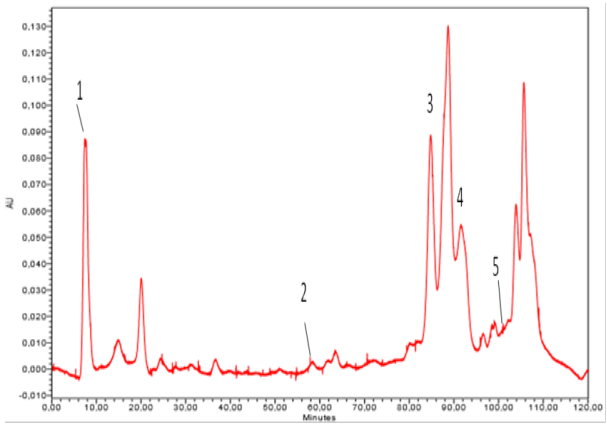

Figure 2: HPLC chromatogram of L. nobilis ethanolic extract recorded at 280 nm; 1: Gallic acid; 2: Vanillin; 3: Tannic acid; 4: Naringin; 5: Quercetin

The HPLC analysis was performed for *L. nobilis* which showed the best anti-glycation effect (Fig. 2). Comparison of the standards retention times (Table 4) with those recorded in the chromatograms allowed the identification of certain flavonoids as well as phenolic acids. Results of HPLC analysis showed a richness of this extract in flavonoids and phenolic acids, with the presence of quercetin, vanillin, tannic acid, gallic acid, and absence of hesperidin and ascorbic acid.

Different studies have outlined the importance of the phenolic acids in the prevention of different chronic diseases, including cancers, diabetes, cardiovascular diseases and gastrointestinal diseases. Among of these phenolic acids, gallic acid (GA) is reported to have a strong antioxidant and anticancer agent. GA can also be used to treat diabetes and to protect human cells against oxidative damage (Punithavathi V.R. *et al.,* 2011; Shahrzad S *et al.,* 2001). The protective role of GA against AGE-induced oxidative stress was also confirmed by Umadevi S *et al.* (2012). All these results indicate the important role of the GA as a potent inhibitor for AGEs formation and partially explain the result obtained with the *L. nobilis* extracts.

The antioxidant power of *R. tinctorum* is not surprising because of its high content of bioactive molecules such as alizarin, hydroxyl anthraquinones and rubiadin which are known for their strong antioxidant activity and which are used in various medicaments (Essaidi I *et al.,* 2017).

Table 1: Plants description

Botanical name	Family	Genus	Origin	Harvest time	Used part	Wild/cultivated
Geranium roseum	*Geraniaceae*	*Pelargonium*	Khmiss anjra	February	Leafed stems	Wild
Aloysia citrodora	*Verbenaceae*	*Aloysia*	Khmiss anjra	February	Leaves	Wild
Laurus nobilis	*Lauraceae*	*Laurus*	Khmiss anjra	March	Leaves	Wild
Lepidium sativum	*Brassicaceae*	*Lepidium*	Marrakech	July	Seeds	Cultivated
Nigella sativa .L	*Ranuncu-laceae*	*Nigella*	Aghraisse	August	Seeds	Cultivated
Olea europaea. L	*Oleaceae*	*Olea*	Aîn Bayda	April	Leaves	Wild
Rubia tinctorum L	*Rubiaceae*	*Rubia*	Meknès	March	Roots	Cultivated
Sesamum indicum. L	*Pedaliaceae*	*Sesamum*	Beni Mellal	November	Seeds	Cultivated
Trigonella foenum graecum	*Fabaceae*	*Trigonella*	Gharb	June	Seeds	Cultivated

Table 2: Comparative study of flavonoid and total polyphenol concentrations of EAC, EAF and EE

Species	Tannins (mg TAE/ g dry extract)		
	Mean EAC ± SD	Mean EAF ± SD	Mean EE ± SD
G. roseum	28,37±0,15 (d)	15,71±0,33 (b)	6,48±0,17 (a)
A. citrodora	30,39±0,40 (e)	26,40±0,23 (e)	37,08±0,38 (g)
L. Nobilis	65,85±0,24 (f)	23,18±0,23 (d)	32,50±0,23 (f)
L. sativum	18,47±0,23 (b)	10,80±0,25 (a)	14,48±0,25 (c)
N. sativa	23,92±0,28 (c)	21,22±0,22 (c)	18,61±0,32 (d)
O. europaea	18,96±0,27 (b)	30,35±1,73 (b)	49,05±2,42 (e)
R. tinctorum	12,51±0,18 (a)	11,55±0,21 (a)	14,60±0,26 (c)
S. indicum	13,53±0,28 (a)	11,78±0,25 (a)	15,65±0,28 (c)
T. foenum graecum	88,40±0,25 (g)	75,99±0,23 (f)	10,59±0,22 (b)
Total	36,18±2,57	33,74±2,60	22,23±1,42
Fisher	9532,22 (p<0,000) **	6758,56 (p<0,000) **	1498,74 (p<0,000) **

Table 2: Comparative study of flavonoid and total polyphenol concentrations of EAC, EAF and EE (Contd...)

Species	Flavonoids (mg QE/ g dry extract)		
	Mean EAC SD	Mean EAF SD	Mean EE ± SD
G. roseum	68,53±0,32 (f)	32,38±0,19 (c)	53,27±0,33 (f)
A. citrodora	103,71±0,29 (h)	65,41±0,23 (e)	44,14±0,47 (e)
L. Nobilis	48,51±0,36 (c)	15,36±0,20 (a)	33,70±0,42 (d)
L. sativum	72,73±0,19 (g)	23,46±0,30 (b)	76,55±0,37 (h)
N. sativa	52,67±0,20 (d)	68,94±0,24 (f)	71,34±0,30 (g)
O. europaea	56,42±0,27 (e)	32,21±0,21 (c)	70,42±0,16 (g)
R. tinctorum	29,02±0,23 (b)	15,12±0,17 (a)	31,52±0,23 (c)
S. indicum	25,46±0,23 (a)	22,59±0,32 (b)	14,28±0,25 (a)
T. foenum graecum	56,83±0,19 (e)	40,46±0,31 (d)	19,41±0,23 (b)
Total	57,00±2,32	45,94±2,29	46,11±2,32
Fisher	7577,91 (p<0,000) **	6340,63 (p<0,000) **	4962,64 (p<0,000) **

Table 2: Comparative study of flavonoid and total polyphenol concentrations of EAC, EAF and EE (Contd...)

Species	Correspondence in the literature		
	Polyphenols (mg AG/g Ms)	Flavonoids (mg Qu/g Ms)	Reference
G. roseum	-	-	-
A. citrodora	67±1 EE	-	[15]
L. Nobilis	-	-	-
L. sativum	122.67±3.03 EE 4	- 42	[16]
N. sativa	3.5 EE	-	[17]
O. europaea	13.37±0.47 EE 9.07±0.34 EAF	8.69±0.69 3.42±0.34	[18]
R. tinctorum	-	-	-
S. indicum	2.733 EE 1.287 EAC	1.137 0.515	[19]
T. foenum graecum	47.75 ± 0.53 EAF	9.59 ± 0.59 EAF	[20]
Total	-----		
Fisher			

Table 3: Comparative study of tannins and reducing sugars concentrations of EAC, EAF and EE

Species	Tannins (mg TAE/ g dry extract)		
	Mean EAC ± SD	Mean EAF ± SD	Mean EE ± SD
G. roseum	28,37±0,15 (d)	15,71±0,33 (b)	6,48±0,17 (a)
A. citrodora	30,39±0,40 (e)	26,40±0,23 (e)	37,08±0,38 (g)
L. Nobilis	65,85±0,24 (f)	23,18±0,23 (d)	32,50±0,23 (f)
L. sativum	18,47±0,23 (b)	10,80±0,25 (a)	14,48±0,25 (c)
N. sativa	23,92±0,28 (c)	21,22±0,22 (c)	18,61±0,32 (d)
O. europaea	18,96±0,27 (b)	30,35±1,73 (b)	49,05±2,42 (e)
R. tinctorum	12,51±0,18 (a)	11,55±0,21 (a)	14,60±0,26 (c)
S. indicum	13,53±0,28 (a)	11,78±0,25 (a)	15,65±0,28 (c)
T. foenum graecum	88,40±0,25 (g)	75,99±0,23 (f)	10,59±0,22 (b)
Total	36,18±2,57	33,74±2,60	22,23±1,42
Fisher	9532,22 (p<0,000) **	6758,56 (p<0,000) **	1498,74 (p<0,000) **

Table 3: Comparative study of tannins and reducing sugars concentrations of EAC, EAF and EE (Contd....)

Species	Reducing sugars (mg GE/ g dry extract)		
	Mean EAC ± SD	Mean EAF ± SD	Mean EE ± SD
G. roseum	11,62±0,19 (a)	10,61±0,23 (b)	6,54±0,22 (a)
A. citrodora	17,64±0,27 (c) (d)	12,31±0,25 (c)	13,45±0,24 (c) (d)
L. Nobilis	16,82±0,25 (c)	15,76±0,16 (f)	14,26±0,19 (d) (e)
L. sativum	18,57±0,29 (d)	14,19±0,20 (d) (e)	12,91±0,20 (c)
N. sativa	12,26±0,22 (a)	11,55±0,19 (b) (c)	11,31±0,18 (b)
O. europaea	16,48±0,33 (c)	14,63±0,20 (e)	12,63±0,29 (c)
R. tinctorum	11,47±0,27 (a)	9,41±0,11 (a)	10,31±0,24 (b)
S. indicum	18,26±0,26 (d)	12,34±0,26 (c)	15,26±0,23 (f)
T. foenum graecum	14,38±0,25 (b)	13,31±0,22 (d)	15,3±0,22 (g)
Total	15,29±0,29	12,71±0,21	12,46±0,28
Fisher	116,44 (p<0,000) **	92,57 (p<0,000) **	143,10 (p<0,000) **

Groups with the same letters do not differ significantly by the tukey test; Er.Std: Standard Error; **: very highly significant difference.

Study of the antioxidant activity

Screening for the antioxidant effect showed that *A. citrodora* has a promoter antioxidant power (Table 5). The EE of *R. tinctorum* had the strongest inhibitory effect of the DPPH radical. The EE also showed a chelating effect which was observed mainly in *G. roseum* and *T. foenum graecum* (P <0.05) (Table 5). From figure 3, it was noted that the inhibitory power was higher than the chelating one with a significant difference in the case of EE and EAC.

Table 5: DPPH Reducing and Chelating effects on ferrous ions of EAC, EAF and EE

Species	IC_{50} DPPH (mg/ml)		
	Mean EAC ± SD	Mean EAF ± SD	Mean EE ± SD
G. roseum	0,32±0,001 (c)	0,32±0,002 (a)	0,34±0,003 (c)
A. citrodora	0,068±0,001 (a)	0,36±0,003 (a)	0,56±0,003 (d)
L. nobilis	0,47±0,004 (d)	0,74±0,003 (c)	0,71±0,0004 (e)
L. sativum	0,18±0,002 (b)	0,93±0,005 (d)	0,33±0,004 (c)
N. sativa	0,26±0,003 (b) (c)	0,34±0,002 (a)	0,34±0,004 (c)
O. europaea	0,47±0,003 (d)	0,72±0,003 (b) (c)	0,24±0,003 (b)
R. tinctorum	0,86±0,002 (e)	0,93±0,007 (d)	0,14±0,05 (a)
S. indicum	1,07±0,004 (f)	0,87±0,04 (d)	0,75±0,002 (e)
T. foenum graecum	0,55±0,06 (d)	0,67±0,005 (b)	0,76±0,006 (e)
Total	0,47±0,03	0,65±0,02	0,47±0,02
Fisher	237,94(p<0,000) **	277,33(p<0,000) **	139,75(p<0,000) **

Groups with the same letters do not differ significantly by the tukey test; Er.Std: Standard Error; **: very highly significant difference.

Table 5: DPPH Reducing and Chelating effects on ferrous ions of EAC, EAF and EE (Contd…)

Species	I_{50} chelating effects (mg/l)		
	Mean EAC ± SD	Mean EAF ± SD	Mean EE ± SD
G. roseum	0,44±0,005 (c)	0,95±0,007 (d)	0,28±0,002 (a)
A. citrodora	0,26±0,007 (a)	0,45±0,004 (a)	0,62±0,0006 (c)
L. nobilis	0,66±0,003 (f)	1,87±0,003 (f)	2,06±0,008 (e)
L. sativum	0,37±0,003 (b)	0,97±0,01 (d) (e)	0,33±0,005 (b)
N. sativa	0,55±0,006 (d)	0,48±0,002 (a)	0,33±0,008 (b)
O. europaea	0,62±0,004 (e)	0,85±0,004 (c)	0,32±0,007 (b)
R. tinctorum	1,22±0,005 (g)	1,84±0,006 (f)	0,91±0,004 (d)
S. indicum	1,31±0,001 (h)	0,99±0,011 (e)	2,41±0,007 (f)
T. foenum graecum	0,55±0,003 (d)	0,65±0,002 (b)	0,26±0,003 (a)
Total	0,66±0,03	1,019±0,05	0,85±0,08
Fisher	5478,09 (p<0,000) **	5291,95 (p<0,000) **	19023,38 (p<0,000) **

Groups with the same letters do not differ significantly by the tukey test; Er.Std: Standard Error; **: very highly significant difference.

Table 6: Percentages of glycation inhibition after treatment with EAC, EAF and EE

		Concentration (mg/ml)	*G. roseum*	*A.citrodora*	*L. nobilis*	*L. sativum*
Fluorescence intensity	Mean EAC	1.5	65,03	47,78	60,26	30,66
		3.5	60,26	52,61	60,78	32,72
		10	57,58	53,77	61,80	34,69
	Mean EAF	1.5	66,53	53,97	50,24	22,40
		3.5	57,22	58,00	55,80	35,31
		10	55,68	61,76	61,43	36,25
	Mean EE	1.5	64,74	68,02	64,01	47,56
		3.5	61,80	69,65	64,15	49,05
		10	60,01	77,88	64,50	54,97
	BSA-MG-Met *control*		84,01			
Migration distance	Mean EAC	1.5	13,46	3,07	26,66	2,82
		3.5	16,92	16,79	29,10	1,79
		10	29,23	19,35	41,53	0,89
	Mean EAF	1.5	25	19,48	38,97	-0,12
		3.5	28,33	27,30	46,66	-1,28
		10	42,69	29,35	52,82	-1,66
	Mean EE	1.5	37,30	27,82	65,38	1,15
		3.5	36,53	30,64	67,69	-3,33
		10	45,76	30,12	70,38	-2,94
	BSA-MG-Met *control*		67,94			

Table 6: Percentages of glycation inhibition after treatment with EAC, EAF and EE (Contd...)

		Concentration(mg/ml)	*N. sativa*	*O. europaea*	*R. tinctorum*	*S. indicum*	*T. foenum graecum*
Fluorescence intensity	Mean EAC	1.5	27,96	42,71	43,71	-30,65	35,87
		3.5	27,63	45,14	35,80	-37,02	39,63
		10	19,62	47,06	28,23	-79,44	42,10
	Mean EAF	1.5	17,60	45,39	54,07	29,13	27,93
		3.5	16,09	46,25	39,48	22,57	27,99
		10	-0,43	47,34	34,23	21,39	62,14
	Mean EE	1.5	25,32	47,67	32,81	13,13	50,02
		3.5	28,83	54,14	25,34	2,83	52,15
		10	36,67	70,46	12,84	-18,41	71,22
	BSA-MG-Met *control*		84,01				
Migration distance	Mean EAC	1.5	-7,56	2,43	-5,12	-5,76	26,53
		3.5	1,53	13,97	1,79	0,89	33,07
		10	0,89	15,89	0,76	0,89	35,76
	Mean EAF	1.5	-1,53	0,64	7,69	1,79	11,41
		3.5	-1,02	9,87	-0,51	-1,15	28,58
		10	0,51	13,84	-1,41	1,41	31,53
	Mean EE	1.5	0,03	16,41	1,92	6,92	39,61
		3.5	1,02	29,35	1,41	1,92	45,89
		10	1,66	37,69	-2,17	1,15	45,89
	BSA-MG-Met *control*		67,94				

the result was consistent with those of Sultana B *et al.* (2009).

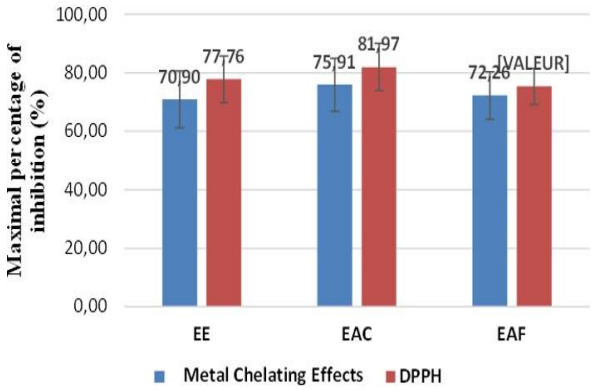

Figure 3: Graphical representation of the maximum inhibition percentages means for the DPPH and metal chelating tests

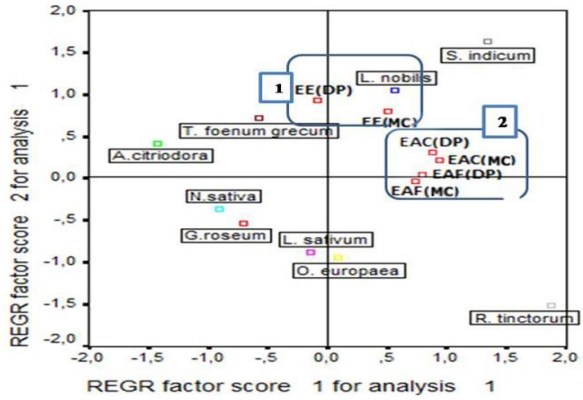

Figure 4: Projection of the anti-oxidation effect variables according to the two components; DP: DPPH test; MC: Metal chelating effects.

Table 4: Standards retention times

Standards	Retention time (min)
Ascorbic acid (Vit. C)	3.2
Gallic acid	8
Vanilline	58
Tannic acid	84
Naringin	92
Hesperidin	94.1
Quercetin	101.1

This analysis of the total variance showed that axis 2 absorbed 78.01% of the components. A highly significant correlation was observed in the case of EAF and EE with r=0.575 and r=0.535 respectively. The correlation was higher for EAC with r=0.942. The study of PCA showed that the components were divided into two groups (Fig. 4): the first characterised *L. sativum*, *O. europaea* and *G. roseum* and the second characterised *N. sativa* and *A Citrodora*. *Rubia tinctorum* was the least efficient in terms of antioxidant power.

The Fisher tables provided large critical values for a threshold α =5%. This meant that the antioxidant effect varies from one species to another.

Study of anti-glycation activity

From table 6, we observe that at 10 mg/ml, the highest percentages of the glycation inhibition in the fluorometric test were recorded in EEs, especially in *T. foenum graecum*, *A. citrodora* and *O. europaea* (Table 6). In the other hand, the Native-PAGE migration profile showed an inhibitory effect manifested as a reduction in the migration distance of the bands treated with the plant extracts

compared to the BSA-MG control band (C +) (Fig. 5). *Laurus nobilis* showed the strongest inhibitory effect of the early stages of the glycation process, with a non-significant difference from the BSA-MG-Met control, followed by *G. roseum* and *T. F. graecum*. However, *L. sativum*, *N. sativa*, *R. tinctorum* and *S. indicum* showed no significant inhibitory effect (p> 0.05). While the difference is highly significant between the fluorometric test values, the electrophoresis test showed no significant difference between some groups of plants such as *G. roseum* and *T. foenum graecum* and *N. sativa* and *S. indicum*.

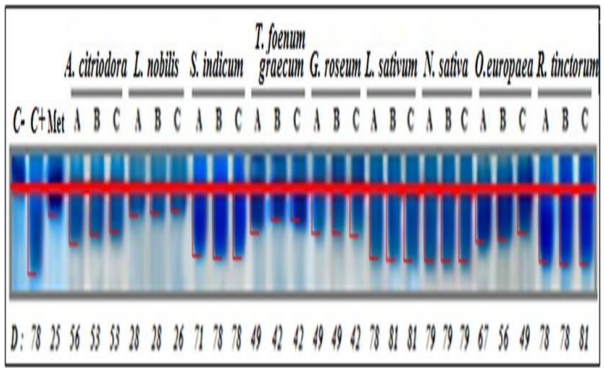

Figure 5: Effect of EE on glycated BSA migration; BSA (5 mg/ml) was incubated with MG (10 mM) in presence of the extract (1.5, 3.5 et 10 mg/ml) or metformin (30 mM). Samples were submitted to Native-PAGE electrophoresis 7%, 80 V during 2h. C-: BSA non glycated; C+: MG + BSA; Met: MG + BSA + Metformin; D: Migration distance in mm; A: 1.5 mg/ml; B: 3.5 mg/ml; C: 10 mg/ml

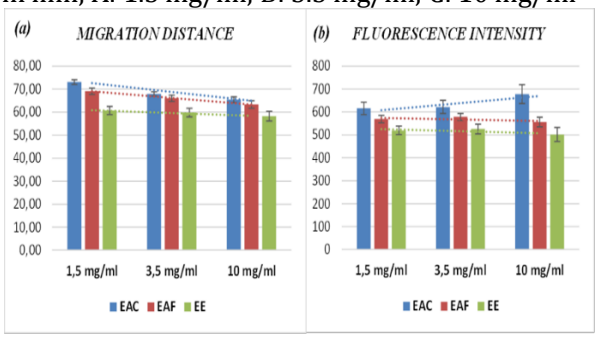

Figure 6: Representation of the anti-glycation effect at three mean concentrations for EAC, EAF and EE; *(a): EAC (y=-3,845x + 76,51; R^2= 0,963); EAF (y = -2,9x +71,975 ; R^2 = 0,9985); EE (y = -1,25x + 62,13; R^2 = 0,9773); (b): EAC (y = 30,94x + 576,87; R^2 = 0,824); EAF (y = 0,835x + 566,06; R^2 = 0,0491); EE (y = -9,005x + 533,89; R^2 = 0,5304)*

The elevated inhibition of AGEs observed for the *T. foenum graecum* in the fluorometric test was also obtained by Pradeep S.R. and Srinivasan K (2017) in a study of AGEs in circulation of diabetic rats. The effect of *T. foenum graecum* was also higher in the electrophoresis test. These effects are probably the result of an improved glycemic status and reduced hyperglycemia-induced protein glycation as proposed by by Pradeep S.R. and Srinivasan K (2017).

The graphic representation of the anti-glycation effect revealed a high inhibition in EE, whether during the first or last stages of glycation (Fig. 6). The observed effect was dose-dependent for the electrophoresis test.

A study of the linear correlation between the matched samples showed a positive and highly significant correlation in EAF and EAC between fluorescence intensity and migration distance; Which implies that these two extracts act simultaneously by the inhibition of the glycation during the first and the last stages, whereas at 10 mg/ml in EE, it was mainly an inhibition of fluorescent AGEs formation.

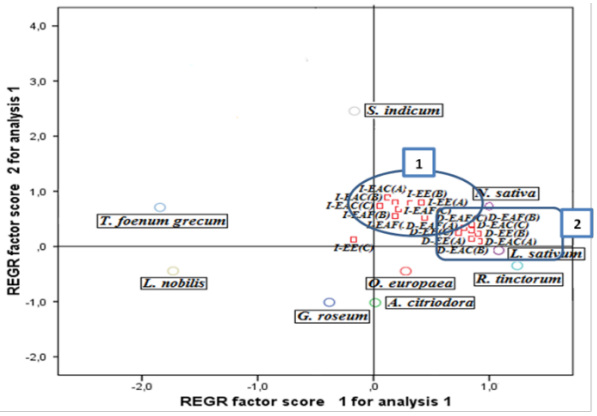

Figure 7: Projection of different variables of the Principal Component Analysis (PCA) on the factorial graph; I: Fluorescence intensity; D: Migration distance; A: 1.5 mg/ml; B: 3.5 mg/ml; C: 10 mg/ml

Analysis of the total variance showed that 80.92% of the variables were collected on axis 2 of the factorial plane (Fig. 7). These variables were divided into two groups.

Projection of the nine species studied showed that the anti-glycation effect of *L. nobilis* and *T. foenum graecum* was mainly due to their inhibition of the Amadori products formation during the early and reversible stages of glycation. The inhibitory effect of the Amadori products transformation into fluorescent AGEs was observed in *A. citrodora* and *G. roseum*. On the other hand, the EAC of *S. indicum* activated the formation of fluorescent AGEs significantly.

The literature shows that the seeds of *T. foenum graecum* L. are widely used for their hypoglycaemic effect. These seeds are an important source of protein, which includes albumin as the main fraction, followed by globulin, glutelin and prolamin (Feyzi S *et al.,* 2014). Phytochemical analysis of the hydro-alcoholic extract of the seeds revealed the presence of

alkaloids, steroids, flavonoids, tannins and saponins (Haeri M.R. *et al.,* 2009). In addition, oleuropein and its derivatives are the essential phenolic constituents of olive leaves and have shown a hypoglycemic effect (Dekanski D *et al.,* 2009). But, its effects on the degree of glycated haemoglobin need to be better investigated.

A correlation analysis was carried out to study the involvement of some chemical compounds previously quantified and the antioxidation power observed in the inhibitory effect of the glycation. In general, a contribution of tannins was observed, followed by total polyphenols and flavonoids in the anti-glycation effect ($p < 0.001$). It must be noted that the inhibition of glycation can be done at several levels: on the one hand, the cleavage of cross-linking products and the blocking of AGE receptors, on the other hand, the chelation of metals transition and trapping of free radicals (Rahbar S and Figarola J.L., 2003). As a result, the identified compounds can inhibit the formation of AGEs directly by their anti-glycation effect, or by reducing the formation of ROS, either by their chelating or reducing effect. A weak correlation was observed for the reducing sugars of EAF ($p < 0.05$). However, for EE, tannins were the only constituents responsible for the observed effect. Therefore, this effect may be due to other chemical compounds not quantified during this work, such as saponins and anthocyanins which have been described in the literature as effective glycation inhibitors (Harris C.S. *et al.,* 2014). In addition, the anti-oxidation effect was not implicated in the anti-glycation effect in these nine species studied. A single weak correlation ($r = -0.398$ **) was observed in the electrophoresis test in the case of EE, which implies that the antioxidant effect is partly responsible for the inhibition of the early glycation stages.

A high correlation was found between the total polyphenol, flavonoid and antioxidant levels, with highly significant correlations of up to 87% in EAC, up to 77% in EAF and up to 63% in EE. This is in agreement with the results of a research carried out on Vietnamese PAMs (Nguyen Q.T. *et al.,* 2011), which showed a good correlation between the values of IC_{50} DPPH and the polyphenol contents, but a low correlation between these contents and the chelation IC_{50} values. On the other hand, some authors have shown that there is no correlation between polyphenol contents and free radical scavenging (Kumaran A and Joel Karunakaran R, 2007).

CONCLUSION

Inhibitors of the AGEs formation have been considered in the treatment of complications of diabetes. Investigation of the anti-glycation effect of nine plant species showed that, with the exception of *S. indicum*, all these species had an average effect regardless of the extract type used. *Laurus nobilis* was characterised by the higher anti-glycation effect that was dose-dependent by both inhibiting the formation of the Amadori products and the fluorescent AGEs. HPLC analysis revealed a richness of the EE of this species in phenolic compounds, with the presence of quercetin, vanillin and gallic acid.

Extracts of *L. sativum*, *N. sativa*, *O. europaea* and *R. tinctorum* acted only as inhibitors of the fluorescent AGEs formation. It can be concluded that the inhibitory effect of these extracts was therefore mainly due to an inhibition of the second phase of the glycation reactions, namely the intervention of free radicals in the conversion of Amadori products to AGEs. This recorded that anti-glycation activity could be attributed to their antioxidant activity, as well as to their richness of active ingredients. The strong correlation that existed between the antioxidant power and the phenolic and flavonoid content suggests that these compounds were strongly involved in the observed effect. However, the lack of correlation between antioxidant and anti-glycation power is probably attributed to non-antioxidant compounds.

Conflict of interest

The authors have no conflict of interest to declare.

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