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Protocol implementation for the detection of advanced glycation end products and screening of natural extracts involved in the prevention of diabetic complications

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Abstract
Advanced Glycated End products (AGEs) are involved in the pathogenesis of numerous affections. To improve a protocol for a rapid detection of glycation, in order to screen potential anti-glycation agents, bovine serum albumin (BSA) and methylglyoxal (MG) were incubated at 37 and 50 °C, hourly until 24 h. Electrophoresis and spectrophotometric analysis were used to measure glycated proteins and their aggregations. The anti-AGEs activity screening based on AGEs fluorescence, in 96- well plates, was conducted by incubating BSA and fructose at 50 °C overnight. Quantity of AGEs at 50 °C after 6h of incubation was similar to that at 37 °C after 24h. The screening test showed that four extracts had significant inhibitory effect on BSA glycation (between 40 and 50%). In contrast, some extracts activated this non-enzymatic reaction (between 45 and 60%). The fluorescence determination of BSA (20 mg/ml)-Fructose (0.5 M) system is a rapid and reliable way for anti-glycation phytochemicals screening.

Keywords: Advanced glycated end-product, screening, protocol, bovine serum albumin, electrophoresis, fluorescence

1. Introduction
Protein glycation is the non-enzymatic reaction between the a protein and a reducing sugar. In this reaction, carbonyl group of a sugar interacts with the nucleophilic amine group of the protein, producing an N-substituted glycosylamine (Schiff base) which is labile and can undergo two sequential rearrangements to yield a stable aminoketose - the product of Amadori [1]. Under physiological conditions, glucose reacts with a wide variety of proteins to form glycated products. These protein changes appear to contribute to the complications of patients with diabetes [2]. In addition to the *in vivo* non-enzymatic glycation process, reactive carbonyl species produced by auto-oxidation of sugar, lipid peroxidation and UV-photo damage can also contribute to the formation of AGEs. These AGEs cause various types of protein modifications, resulting in structural and functional deficiencies such as intra-and intermediate absorption, crosslinking, fluorescence at specific wavelengths and modification of enzyme activities [3].
It is very important to monitor the glycation process in order to predict potential complications. Thus, the discovery and study of compounds with an activity inhibiting the formation of AGEs will certainly offer a potential therapeutic approach for the prevention of diabetes or other pathogenic complications. To achieve this objective, a fast and reliable procedure for detection and screening of natural anti-glycative phytochemical compounds and other active compounds is required [4]. Two main objectives were fixed in this study: to increase the efficiency of the glycation reaction and develop a protocol that would allow for an effective and quicker pharmacological screening of natural compounds; On the other hand, to screen extracts in order to check for possible glycation inhibitors and to prove the effectiveness of our method.

2. Materials and Methods
2.1 Constitution of the sample bank
The essential oils, oils, aqueous extracts and vinegars used in this study were extracted from Moroccan plants. To these extracts four purified molecules were added (5 mM) (M1: Castanospermine, M2: Kifunensin, M3: Miglustat, M4: Swainsonine) (Table 1).

Table 1: Description of the plants used in the screening test

Scientific name	Family	Origin	Harvest time	Used part	Type of extract	Cultivated /Wild plant
Salvia officinalis. L	LAMIACEAE	Marrakech	March	Leaves	EO1 ; O1	Cultivated
Thymus satureioides	LAMIACEAE	Marrakech	March	Leaves	EO2 ; V2	Spontaneous
Rosmarinus officinalis. L	LAMIACEAE	Marrakech	June	Leaves	EO3 ; O3 ; V3	Spontaneous
Angelica archangelica	APIACEAE	France	August	Roots	EO4	Cultivated
Pelargonium asperum	GERANIACEAE	Taounate	March	Leaves	EO5	Cultivated
Mentha pulegium	LAMIACEAE	Marrakech	March	Leaves	EO6	Spontaneous
Citrus limetta Risso	RUTACEAE	Marrakech	March	Petals	EO7	Cultivated
Eucalyptus globulus	MYRTACEAE	Tanger	March	Leaves	EO8	Spontaneous
Pimpinella anisum. L	APIACEAE	Agouray	Septembr	Seeds	EO9	Cultivated
Nigella sativa. L	RANUNCULACEAE	Aghraisse	August	Seeds	O 2	Cultivated
Zingiber officinale	ZINGIBERACEAE	-	-	Roots	O4 ; V4 ; EO	Cultivated
Matricaria recutita	ASTERACEAE	Kenitra	-	Flowers	O5 ; EO	Spontaneous
Trigonella foenum grecum	FABACEAE	Settat	June	Seeds	O6	Cultivated
Vitis vinifera sativa	VITACEAE	Khémisset	June	Seeds	O7	Cultivated
Triticum sativum	POACEAE	Tanger	july	Seeds	O8	Cultivated
Cinnamomum zeylanicum	LAURACEAE	France	-	Barck	O9 ; AE2	Cultivated
Viscum album	VISCACEAE	France	June	Leaves	AE1	Spontaneous
Lavandula dentata	LAMIACEAE	Marrakech	March	Leaves	V1 ; EO	Spontaneous
Origanum compactum	LAMIACEAE	Marrakech	March	Leaves	EO	Spontaneous
Pinus pinaster	PINACEES	Chefchaouen	-	Resin	EO	Spontaneous
Citrus sinensis. L	RUTACEAE	Agadir	May	Petals	EO	Cultivated
Rosa damascena	ROSACEAE	Kallaât Maggouna	May	Petals	EO	Cultivated
Ricinus communis. L	EUPHORBIACEAE	Larache	August	Seeds	O	Spontaneous
Myrtus communis. L	MYRTACEAE	-	May	Leaves	EO	Spontaneous
Persea americana	LAURACEAE	Khémisset	May	Fruit	O	Cultivated
Sesamum indicum. L	PEDALIACEAE	Beni Mellal	Novembr	Seeds	O	Cultivated

EO; Essential Oils, O; Oils, AE; Aqueous Extracts, V; Vinegars

2.2 Chemicals

The reagents (methylglyoxal 40%, Ammonium persulfate, BSA, DMSO, D-Fructose, Aminoguanidin, acrylamide/bis-acrylamide, Tris-HCl, Glycerol, Glycine, SDS, Bromophenol Blue, Coomassie Blue R250) were from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and methanol were from Riedel-de Haen and TEMED was from Fluka. Metformin was purchased from the pharmacy (Glucophage 1000 mg, 78%).

3. Methods of study and dosages

3.1 The *in vitro* glycation of albumin

The BSA incubation was carried out according to the method of Matsuura *et al*., (2002) [5] with slight modification. BSA (5 mg/ml) was incubated with methylglyoxal (MG) (100 mM) and phosphate buffer (100 mM, pH 7.4) (Verlab S.A., Casablanca, Morocco). The reaction final volume was 250 µl. The solution was then incubated under stirring.

3.2 Glycation parameters

In order to fix the glycation parameters, the anti-glycation activity of methylglyoxal was tested for its capacity in the formation of glycated BSA at three temperatures (ambient temperature, 37 °C and 50 °C.). Then, glycation kinetics of BSA with three concentrations of MG (1, 5 and 10 mM) was carried out over 5 days. In parallel, levies were taken at different times (24 h, 48 h, 72 h, 96 h and 120 h). Also, kinetics over 24 h was carried out. An incubation with Metformin (Met) (100 mM), a known glycation inhibitor [6], was performed with three concentrations (5 mM, 10 mM, and 30 mM).

3.2.1 Screening of anti-glycation activity

The anti-glycation test was carried out by incubating 25 µl of the plant extract with BSA (5 mg/ml), MG (100 mM) and phosphate buffer (100 mM, pH 7.4). Two controls, one negative (BSA + MG) and the other positive (BSA + MG + Met), were also carried out. A volume of 5 and 10 µl of DMSO was added to dissolve essential oils and oils, respectively.

3.3 Measure of glycation inhibition

3.3.1 Measure glycation inhibition percentage on SDS PAGE and Native-PAGE gels

Glycation evaluation was first performed using SDS-PAGE gel electrophoresis [7]. Samples were denatured by adding 80 µl of the dissociation buffer (v/v) to 20 µl of the protein solution (0.5 mg/ml). This mixture was boiled for 3 minutes. The samples were then analyzed by PAGE-Native gel electrophoresis. For this, 80 µl of the load buffer (v/v) was added to 20 µl of the protein solution (0.5 mg/ml). Migration was carried out at room temperature for SDS-PAGE and at 4 °C for Native-PAGE.

The bands intensity of the scanned SDS-PAGE gels was measured by the "Image-J" software. The inhibition percentage was calculated by the equation *[(A-B) / A] x100*; A is the value of control density, and B is the value of after treatment with plant extract. The same software was used to measure the migration distance of the bands on Native-PAGE gels.

3.3.2 Measure by spectrophotometry

Trichloroacetic acid (TCA) at 1% (v/v) was added to the samples. After incubation for 10 min at 4 °C, the tubes were centrifuged at 10000 g for 10 minutes, and the supernatant was removed. The pellet was then dissolved in buffer at pH 10. Measure of glycated proteins aggregation was performed by reading the absorbance at two wavelengths: at 278 nm for proteins and at 260 nm for nucleic acids [8], using a UV-Visible spectrophotometer. These values have been integrated into the following formula:

[Proteins] (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$

The inhibition glycation percentage was calculated using the following formula :

% inhibition= *[1- (Absorbance of the solution with the inhibitor / Absorbance of the solution without inhibitor)] x100%.*

3.3.3 Measure by spectrofluorimetry

In this test, fructose was used as a glycation agent, with aminoguanidine as a glycation reference inhibitor. Incubation was carried out in 96-well plates, in order to increase the number of samples tested. BSA (60 mg/ml) and fructose (1.5 M) were prepared in phosphate buffer (0.2 M, pH 7.4) directly on the 96-well plate. Deionized water or inhibitor in deionized water (1/1/1) was then added. After incubation at 50 °C for 24 h, the TCA precipitation was carried out in the plate. After excitation at 360 nm, the fluorescence was read at 460 nm with Mithra LB 940 (Berthold). To verify if the fluorescence comes from AGEs or new interfering fluorescent substances, another plaque was prepared for the suspect samples, in the presence of aminoguanidine. If the fluorescence returns to the basal state, it can be deduced that it was really due to AGEs. The inhibitory activity of AGE formation for each extract was calculated using the following formula:

Relative anti-glycation activity (%) = *100- [(Sample fluorescence / Positive control fluorescence) *100*

Statistical analysis

GraphPad Prism, version 5.0 for Windows (GraphPad Software, Sandiego, CA) was used. The results were presented as mean ± SD of three replicates. The t test matched was applied to determine the extent of inhibitory activity. A $p<0.05$ value was considered statistically significant.

4. Results and Discussion

4.1 Improvements in albumin glycation protocol

4.1.1 Comparative kinetic analysis of the BSA glycation on polyacrylamide gel and by spectrophotometry

The demonstration of the *in vitro* BSA glycation and its inhibition by metformin was followed by various methods. First, analysis by Native-PAGE and SDS-PAGE by studying respectively the migration distance of bands and changes in bands inverse density of the (DMI). The second analysis was carried out by spectrophotometry (measure of DO at 278 nm).

The results obtained on Native-PAGE or SDS-PAGE gels showed that the glycation was visible after 24 h of incubation. During denaturing gel migration, the inverse density of the bands increased with the MG concentration (Fig. 1); The bands were more diffuse, wider and brighter than bands of the non-glycated BSA. Also, glycated BSA showed a higher apparent molecular weight than non-glycated BSA (Fig. 1).

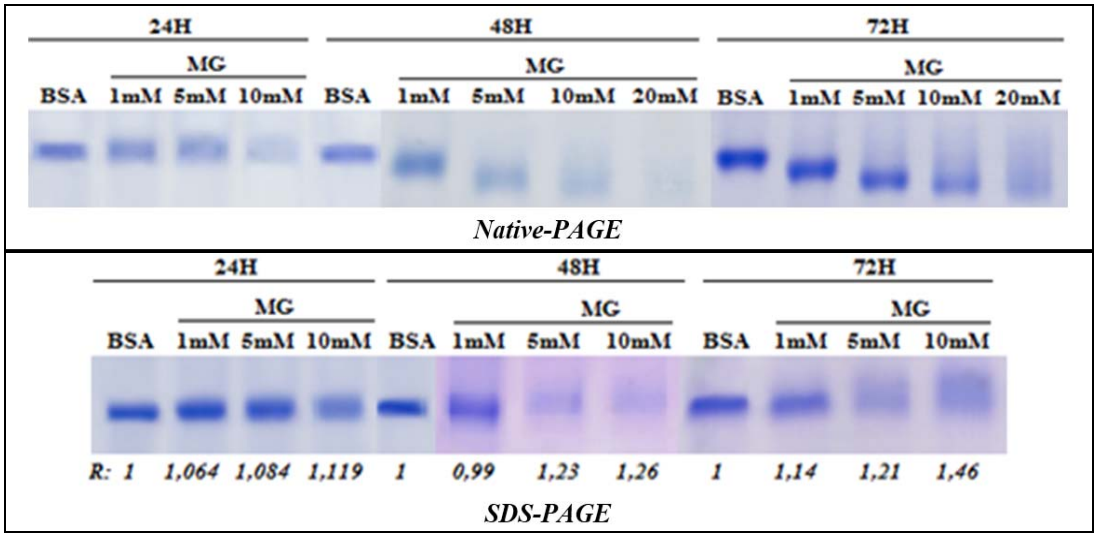

Fig 1: Electrophoretic profile of native BSA and BSA incubated for 1, 2, and 3 days with MG (1 mM, 5 mM and 10 mM). Samples were submitted to SDS-PAGE 10% and Native-PAGE 7%, for 2h, 85 V. R ; Ratio of inverse mean density of the sample/the inverse mean density of the BSA control.

The glycated BSA bands obtained after migration on Native-PAGE presented the same characteristics as those obtained on SDS-PAGE, with an important difference: When the MG concentration was high, the apparent molecular weight of the BSA glycated was low. This induced a forward shift which was characteristic of the glycated proteins migration on Native-PAGE gel (Fig. 1). This schift was therefore an indicator of the BSA glycation.

Concerning the effect of temperature, glycation at 37 °C was observed to be very similar to that observed at 50 °C with a slower rate. This suggests that the temperature does not affect the glycation reaction, but only accelerates it (Fig. 2). It has been reported in the study by Berthold *et al*. (2007) [9] that glycation was used to modify the functional properties of BSA. Other proteins, such as β-lactoglobulin, glycated at 60 °C to accelerate the Maillard reaction, showed an improvement in functional properties that were linked to the added sugar [10].

However, the inhibitory effect of metformin was less marked at 50 °C than at 37 °C (Fig. 2), suggesting that its concentration was insufficient to achieve complete inhibition at 50 °C and should therefore be rehabilitated. Even if the glycation was detectable only after 24 h, it was total after the 5th day of incubation, with a concentration of 10 mM in MG. It has been reported in the study of Weiss *et al*. (2003) [11] that the concentrations 5 and 10 mM allowed to have a molar excess in MG (respectively 3.79 and 7.59) relative to the number of glycation sites available. Whereas, 1 mM allowed to have 80% of the MG quantity necessary to glycate all the sites.

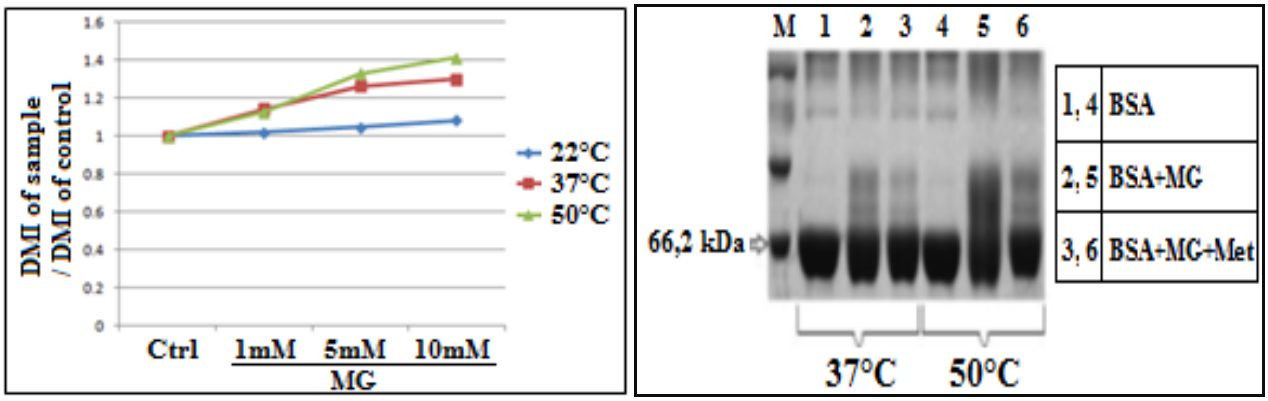

Fig 2: Effect of incubation temperature on BSA glycation. Samples were submitted to SDS-PAGE 10% and Native-PAGE 7%, for 2h, 85 V. BSA was incubated alone (1;4), with MG (10 mM) (2;5) or with MG (10 mM) and Met (10 µM) (3;6). M : Protein marker, DMI: The inverse mean density.

D'après la figure 3, la glycation augmente avec la durée d'incubation ce qui signifie une élévation progressive du taux de BSA glyquée, ceci est en concordance avec les études de Loske *et al.,* (1998) [12] qui montrent que la durée d'incubation du sucre réducteur avec une protéine a une corrélation directe avec la cytotoxicité des AGEs. Une autre recherche effectuée par Schmitt *et al.* [13] a montré qu'il existe une relation linéaire entre ces deux paramètres. Ils ont constaté que le pourcentage de lysines qui entre en réaction a augmenté rapidement jusqu'à 200 mM de glucose.

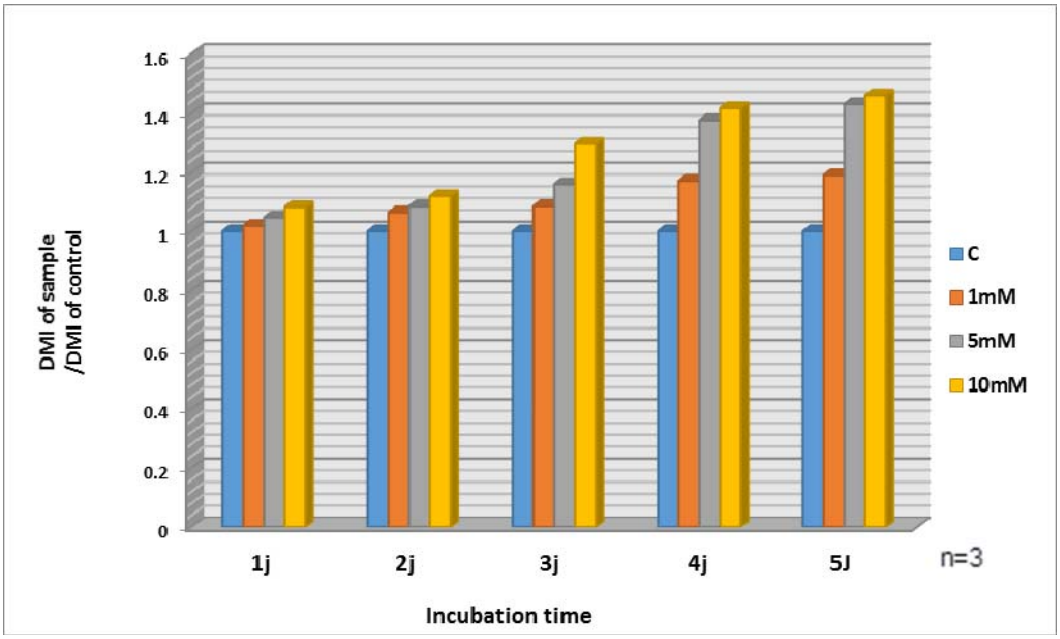

Fig 3: Effect of incubation time and methylglyoxal concentration on glycation of BSA. BSA was incubated à 37 °C with Three MG concentrations (1, 5 and 10 mM). Ctrl ; BSA alone at 1 mg/ml (control), DMI ; Inverse mean density.

Non-enzymatic glycosylation occurs at multiple residues such as arginine, lysine and cysteine. *In vivo* tests have shown that these residues are most subject to glycation mainly because of their high nucleophilic properties. The thiol group of cysteine residues is a potent nucleophile which can also be glycated *in vitro* by methylglyoxal to give rise to AGEs such as S-carboxymethylcysteine (CMC) [14].

4.1.2 Kinetic without metformin

The formation kinetics of glycated BSA were evaluated at 37 °C and 50 °C for 24 h to determine when optimal glycation occurred (Fig. 4). On the Native-PAGE gel, a slight shift was visible at 3 hours, but the apparent molecular weight of the band remained close to that of the unglycated BSA. After 6 hours of incubation, there was a clearly visible difference in the migration of the band from the control band, but the glycation is not total (Fig. 4).

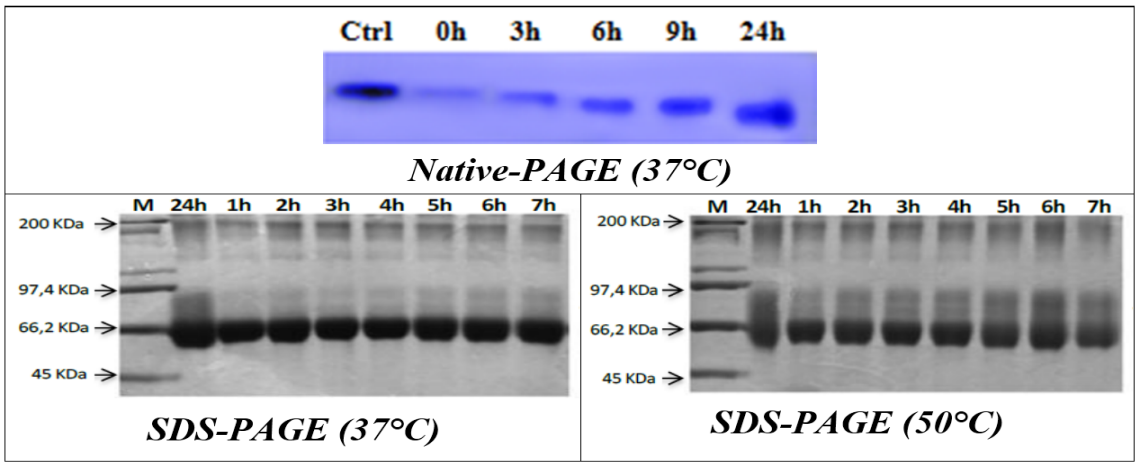

Fig 4: Kinetics of BSA glycation over 24 h of incubation. Samples were submitted to SDS-PAGE 10% and Native-PAGE 7%, for 2h, 85 V. BSA was incubated alone (1 ; 4), with MG (10 mM) (2 ; 5) or with MG (10 mM) and Met (10 µM) (3 ; 6). M : Protein marker. Ctrl ; BSA alone.

Visibly on SDS-PAGE gel, the intensity of glycated BSA was greater at 50 °C than at 37 °C. From 5 h of incubation, an optimal formation of glycated BSA at 50 °C was observed in comparison with 37 °C.

4.1.3 Kinetic with metformin

4.1.3.1 Electrophoretic analysis

In order to determine the incubation time from which an inhibitory effect of glycation is visible, kinetics with metformin have been carried out.

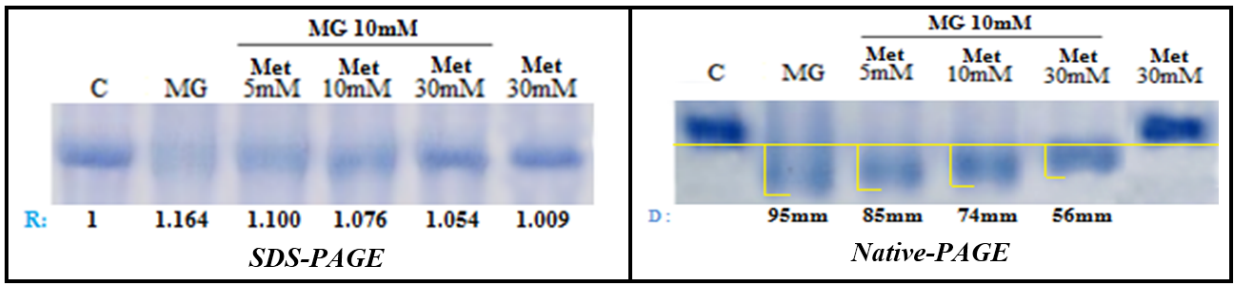

Fig 5: Electrophoretic profile of native BSA and BSA incubated with metformin. BSA (1 mg/ml) was incubated with MG (10 mM) and Met (5mM, 10mM and 30mM) for 5 days. Samples were submitted to SDS-PAGE 10% and Native-PAGE 7% for 2h, at 85V. C; control (BSA alone), MG; BSA + MG; of track 3 to 5; BSA+MG with Met (5mM, 10mM and 30mM), track 6 ; BSA + Met (30mM). R ; Report of the sample DMI on the control DMI. D; migration distance of the bands.

The increase of metformin concentration induced a decrease in the molecular weight of the bands which became close to that of unglycated BSA. In the absence of MG, it does not affect BSA migration (Fig. 5). The inhibitory effect of Met was also evident by measure of DMI (Fig. 6). In addition, it was evident that the effect of metformin was dose-dependent.

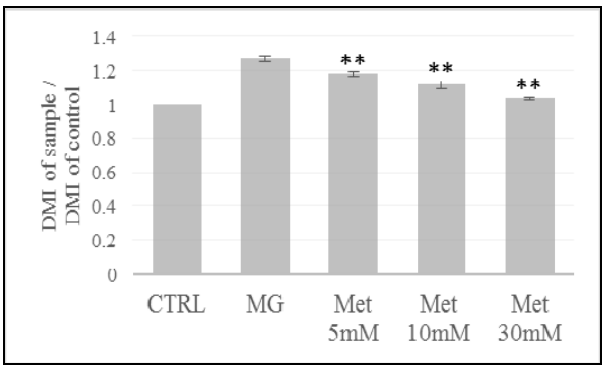

Fig 6: Inhibition effect of metformin on BSA glycation according to SDS-PAGE profile. C; control (BSA alone), MG; BSA + MG, Met ; BSA+MG with Met (5mM, 10mM and 30mM), ** Indicated a significant difference (p<0.01) compared to the MG control. DMI; inverse mean density.

On the SDS-PAGE profile, a band of glycated BSA greater than that of original BSA was observed. These results suggest that crosslinking between proteins or carbohydrates-proteins occurred during the formation of AGEs. This was confirmed by the study of Frye EB *et al*., (1998) [15], which detected cross-linking of RNase during incubation with MG by SDS-PAGE analysis. The intensity of the band with preliminary treatment with the inhibitor increased considerably indicating suppression of the crosslinks formation. Methylglyoxal irreversibly modifies proteins under physiological conditions, since glycated BSA is transformed into a less polar molecule due to the exposure of its hydrophobic sites relative to the native molecules and thus definitively loses its native secondary structure [16]. Therefore, to have inhibition, the extract should react with MG before reacting with protein [17].

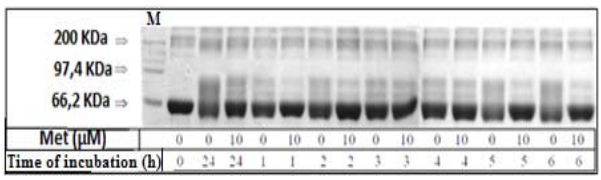

Fig 7: Kinetic of glycation at 50 °C with or without Met (10 µM). BSA (1 mg/ml) was incubated with MG (10 mM). Samples were submitted to SDS-PAGE 10%, for 2h, 85 V. M: Protein marker

It was observed that at 6 hours of incubation with Met, the level of glycated BSA decreased in comparison with the state at 24 h of incubation (Fig. 7). This result confirmed that the glycation at 50 °C for 6 hours of incubation was effective and can be used to test potential glycation inhibitors.

4.1.3.2 Spectrophotometric analysis

After the 5th day of incubation, the glycation caused a significant alteration of the colors in the albumin solution. In addition, during Maillard reaction, the intermediates reactions can generate dark brown aggregates [18]. Measure of the brownish color by Christian Warburg method allowed us to monitor the degree of BSA glycation. The results of this measure showed that the lowest absorbance was that of the tube which presented less coloring, indicating the presence of an inhibition.

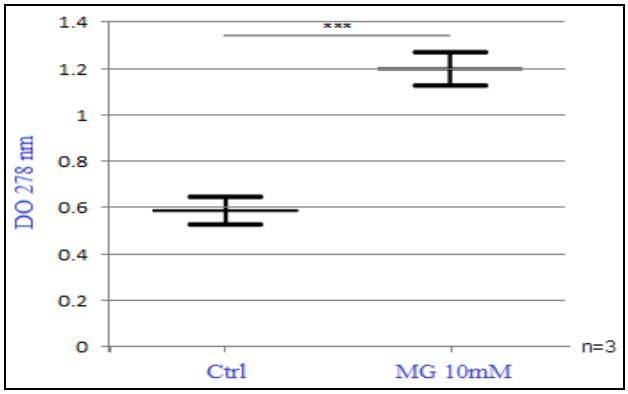

Fig 8: Measure of glycation of BSA by spectrophotometry. DO: mean at 278 nm of glycated and non-glycated BSA. *** $p<0.01$: non-parametric Mann-Whitney test.

On the other hand, there was a significant difference between the mean OD of control and glycated BSA (Fig. 8), confirming that spectrophotometry can be used for measuring BSA glycation.

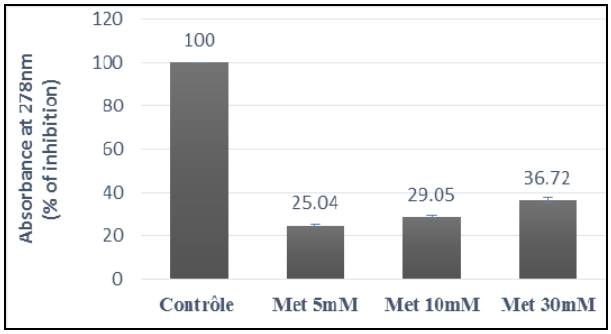

Fig 9: Measure of the metformin inhibitory effect on the BSA glycation by spectrophotometry. Control; Optical density of BSA, histogram 2, 3 and 4; BSA (1 mg/ml) + MG (10 mM) + Met (5 mM, 10 mM and 30 mM).

According to figure 9, it was found that the inhibition percentage significantly increased by incubating the reaction mixture with increasing concentrations of metformin, reaching about 35% at 30 mM of Met. This indicated a decrease in formation of glycated BSA, and a dose-dependent effect of the Met. This molecule is a well-known inhibitor of the *in vitro* and *in vivo* effects of glycation, partly due to inhibition of RAGE inhibitors expression [19].

Due to its clinical significance, studies of the bovine serum albumin (BSA) glycation process are of particular interest. This phenomenon has been studied by different techniques leading to characterization of structural modifications due to glycation or aggregation.

At the SDS-PAGE gel, the increase in inverse density of bands indicated that a large proportion of BSA was glycated and that the complexes have been trapped in the gel wells due to their large size obtained either by glycation or by crosslinking [20]. The aggregation induced by glycation is not necessarily associated with the modification of the secondary structure. In addition, the glycation process could result a stabilization in both the tertiary and secondary structure of the protein, which increases the stability of the protein and improves its lifetime [16]. Also, SDS-PAGE analysis has been reported to allow detection of RNase crosslinking during incubation with MG [15]. It has been reported that glycated proteins are capable of reacting with another sugar molecule. The reaction involves the addition of a reactive sugar carbonyl group to the secondary amine resulting from the Amadori rearrangement of the initial glycation [21].

While for Native-PAGE gel, bands with more glycation showed more shift to the positive pole of the gel. In fact, it has been reported in the study of Rondeau P. *et al*. (2008) [20] that glycation of albumin results in several structural changes, including an increase in the total molecular weight of the protein. While inhibition was present, the bands tend to regain the BSA control position and the shift was then minimized (Fig 5).

Concerning spectrophotometry measure, it has been reported in the study of Yeboah & Yaylayan, (2001) [22] that the increase in absorbance is proportional to the amount of AGEs in the reaction medium. This means that the inhibition percentage is inversely proportional to the absorbance of the reaction medium.

Briefly, the analysis of the BSA glycation on polyacrylamide gel maked it possible quantitative and qualitative measures by visualizing the presence of crosslinking. While the use of spectrophotometry allowed us to measure the proteins aggregation because of Schiff's bases, it does not directly detect the formation of these bases as does spectrofluorimetry.

In general, the BSA glycation affects the binding properties of this protein, which causes abnormal biological effects. During hemoglobin glycation (pH 7.4 and 24°C for 3 h), the availability of the amino acids to join the carbohydrates is a consequence of its three-dimensional structure rather than the amino acid sequence around the site of glycation [23]. In addition, the glycation conditions and accessibility of the amino acids involved in the reaction contribute to different sites and degrees of glycation.

4.2 Development of a spectrofluorimetry analysis protocol

The estimation of AGEs by determining the fluorescence is based in general on the use of a standard prepared by BSA and glucose at 37 °C for 60 days. Different sugars and glycation agents, such as MG, D-fructose, D-glucose and D-ribose, may undergo traditional Maillard reactions leading to the formation of various products [24]. It has been found that AGEs formed with MG, exclusively CEL and MOLD, do not possess intrinsic fluorescence. The use of fructose therefore offers many more possibilities [4]. In this protocol, the reagents incubation in oven with stirring and the reading of fluorescence were carried out directly on a 96-well plate. The tube-plate transfer step was avoided, thus limiting experimental errors.

4.2.1 Detection limits of fluorescence

The detection limit of fluorescence was verified by incubation

different concentrations of BSA (40, 30, 20, 15, 10 and 5 mg / ml) with fructose (1 to 0.125 M) (Fig. 10). It was observed that the AGEs fluorescence formed by glycated BSA was proportional to BSA and fructose concentrations. For the calculated ratios, an optimal difference between glycated and non-glycated BSA was observed at 20 mg/ml of BSA and 0.5 M of fructose. In fact, a previous study revealed a double *in vitro* formation of glycation products when the protein was incubated with appropriate proportions of sugars [25].

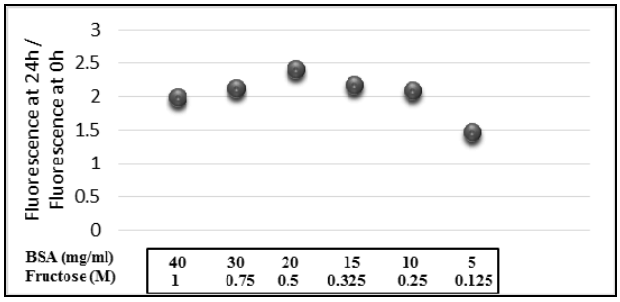

Fig 10: Ratios between fluorescence after 24 h of incubation and without incubation.

In the study of Mio H. *et al*., (2012) [26], fluorescence increased with time following incubation of glucose (2 M) with BSA. However, the same study showed that at 60 °C, the formation of fluorescent AGEs from fructose with collagen grew more rapidly compared to that with glucose. In another study, a simple temperature increase with addition of lysine (0.1 M) can accelerate the process of fluorescence induced by glycation [27]. Since fructose is present in solution in open linear form, in a much greater extent than glucose, a higher rate for the attachment of fructose to the secondary amine would be expected. This may explain, in part, the high rate of fluorescence generation during incubation with fructose [21].

4.2.2 Incubated volume test

Since the reagents were directly incubated on the plate, we wanted to evaluate whether, with a stirring of 120 rpm, a volume of 300 µl could have been excessive in generating overflows. In the two incubation volumes, it was observed that fluorescence was proportional to the BSA glycated (Fig. 13).

After incubation with aminoguanidine, the fluorescence reading was significantly much less important than the control. In addition, it has been reported in the study of Bhatwadekar A.D. *et al*., [27] that aminoguanidine reduces the intensity of fluorescence induced by glycation, while the addition of lysine intensifies the reaction.

Methods such as HPLC, ELISA, and immunohistochemistry were used to detect AGEs. Several studies have used non-fluorescent CML but also FIA, and have reported significant associations between AGE concentration and diabetic microvascular complications, aging and oxidative stress [28, 29]. However, these procedures are expensive and time-consuming, making them impossible for screening purposes as part of primary care. In the present study, we used a simple and rapid fluorometric assay, with a screening of 18 different products and 2 triple controls on the same plate.

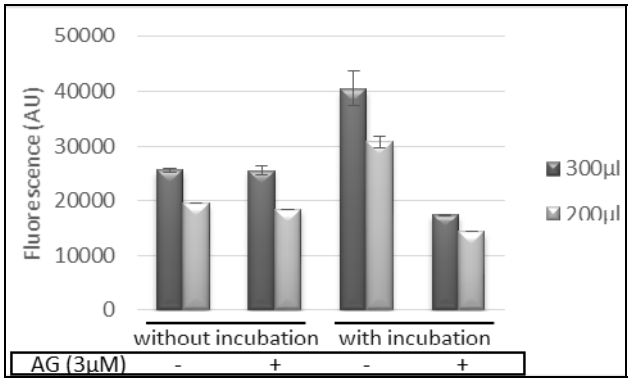

Fig 13: Fluorescence emitted by glycated BSA. BSA (20 mg/ml) and Fructose (0.5 M) were incubated for 24 h at 50°C with or without AG (3 µM) at a total volume of 300 and 200 µl.

4.3 Screening of the anti-glycation effect of plant extracts

4.3.1 Electrophoresis and spectrophotometric screening

This test was developed as a screening tool for the search for glycation inhibitors among natural extracts that often contain interfering substances such as fluorescence and extinguishing materials. These soluble substances do not pose a problem in this case since the relative quantification of the BSA glycated amount was not measured by fluorescence intensity. These vegetable extracts used are for the most part antibacterial or antimycotic, therefore no disinfection solution has been used. Also, the incubation of the natural extracts was carried out under physiological conditions. The incubation period was prolonged to the 5th day for total glycation, with 10 mM of MG and 30 mM of Met. For the same plant, several types of extracts were used. Anti-glycation tests revealed the existence of several extracts with an inhibitory effect. The most relevant results were obtained by treatment with essential oils (Fig. 14, 15 and 16), followed by crude oils and aqueous extract.

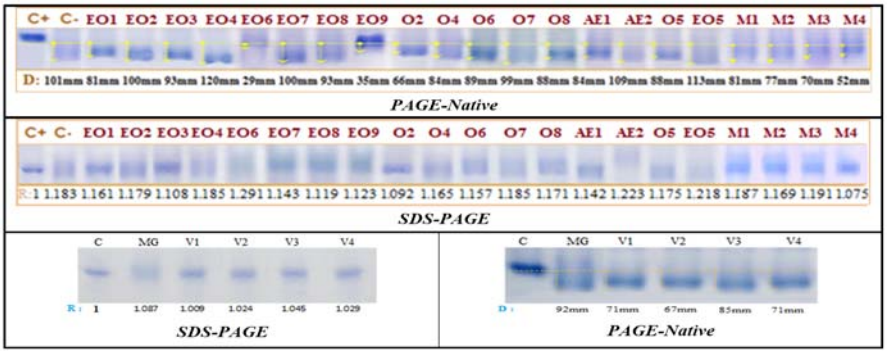

Fig 14: Profil électrophorétique de la BSA (1 mg/ml) incubée pendant 5 jours avec du MG (10 mM) et les différents extraits naturels. Les échantillons ont été soumis à une SDS-PAGE 10% et PAGE-Native pendant 2h à 3h, à 85V. Piste 1 ; C (contrôle (BSA seule)), piste 2 (MG) ; BSA + MG ; de la piste 3 à 6 ; BSA+ MG+ Extrait, R ; rapport de la DMI de l'échantillon sur la DMI du contrôle BSA. D; distance de migration des bandes par rapport à la bande contrôle.

After 5 days at 37 °C, the results showed that the extracts of *Angelica archangelica* (EO4), *Zingiber officinale* (O4) and *Vitis vinifera sativa* (O7) does not revealed any anti-glycation properties. While extracts of *Nigella sativa*. L (O2), *Viscum album* (AE1), *Rosmarinus officinalis*. L (EO3, O3) and *Eucalyptus globulus* (EO8), as well as Swainsonine (M4), showed a very significant inhibition of the glycated BSA formation. The inhibition rate of these samples varied between 38 and 50% (Fig. 14, 15 and 16).

It was also noted, on the one hand, that the extracts of *Triticum sativum* (O8) and *Matricaria recutita* (O5) respectively showed 34 and 35.48% of inhibition. On the other hand, extract of *Salvia officinalis*. L (EO1) had the same inhibitory effect compared to *Trigonella foenum grecum* (O6), *Citrus limetta risso* (EO7) and Kifunensin (M2); These effects were less important, but with a considerable inhibition rate which varied between 10 and 20% (Fig. 14, 15 and 16). These results were confirmed on Native-PAGE by a bands schift of these samples by several millimeters compared to the BSA control band.

Paradoxically, extracts of *Cinnamomum zeylanicum* (AE2 and O9) accelerated significantly the glycation reaction by more than 50%. This increase in glycation was clearly visible in the native and denaturing electrophoretic profiles; it was manifested by a very distinct shift towards the back in SDS-PAGE, and a forward shift in Native-PAGE. This was accompanied by a lower density of the two bands than that of control. This result was probably due to the formation of chemical bonds between the BSA and molecules present in the extract, which would have led to formation of aggregations [30]. There are several molecules with reducing functions that lead to glycation. The aldehydes resulting from the caramelization of sugars participate largely in the increase of this reaction, in particular in a fructose-glycine system. Also, peroxidized lipids form aldehydes capable of reacting with amino acids and gives Schiff bases.

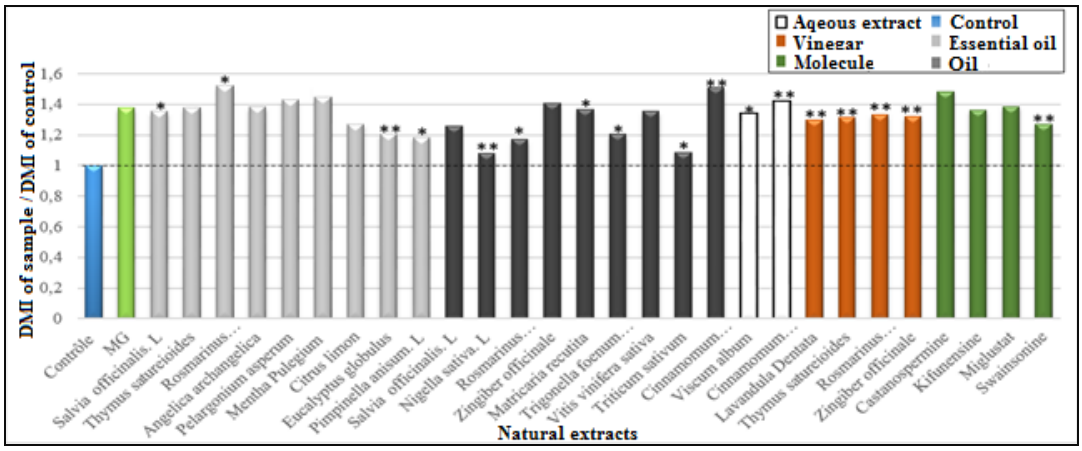

Fig 15: Effect of different types of extracts on glycated BSA formation. DMI: Reverse Mean Density. Inspection; BSA alone, * and **; a significant difference of $p<0.05$ and $p<0.01$ respectively.

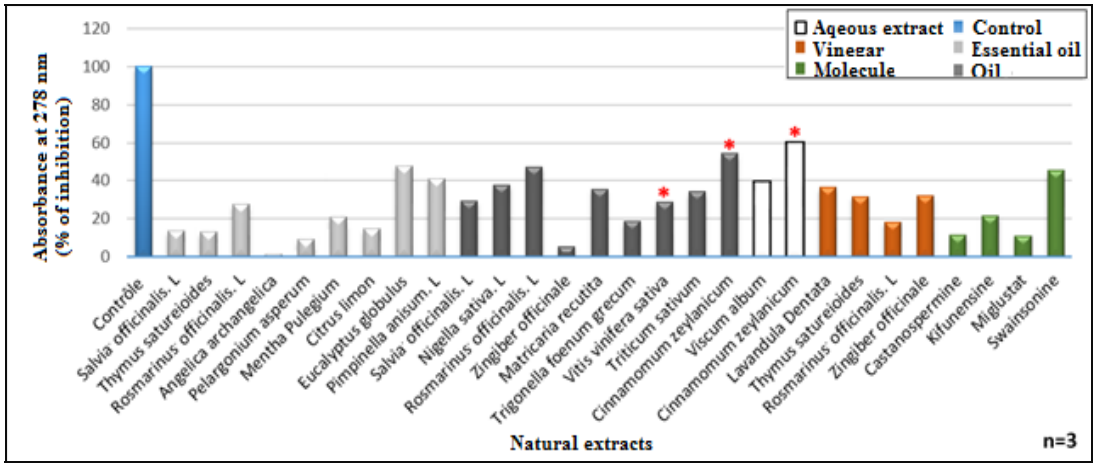

Fig 16: Inhibition percentage of BSA glycation by natural extracts. Control; BSA alone, ***** indicates an increase percentage in BSA glycation.

All vinegars samples tested inhibited the glycated BSA formation, with a slightly more marked activity in *Lavandula dentata* extract (V1) (Fig. 14, 15 and 16). The effect of *Zingiber officinale* vinegar has not been noticed in the oil of the same plant, this suggests that it was probably due to the pH of the extract which, according to Kanska U. and Boratynski J., 2002 [31], could affect the migration of glycated BSA. Thus, BSA incubation with MG and acetic acid or *Zingiber officinale* vinegar (V4) was performed.

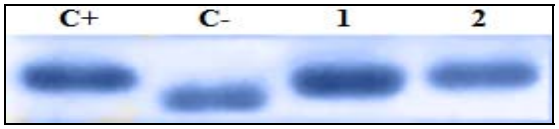

Fig 17 : Effect of acetic acid and V1 vinegar on glycation. Native-PAGE gel 7% obtained after migration of BSA (1 mg/ml) and MG (10 mM) in the presence of acetic acid (1) or Zingiber officinale vinegar (2), for 2h at 85V. C+ ; BSA alone, C- ; BSA+MG.

The glycated BSA migration in presence of *Zingiber officinale* vinegar was similar to that in presence of acetic acid (Figure 17). The effect observed previously would therefore be due to the acetic acid rather than to a molecule contained in this vinegar. These results were confirmed by the pH measure which was equal to 3.3. However, some recent articles showed that some extracts of *Zingiber officinale* have a hypoglycaemic and anti-glycant effect [32].

4.3.2 Screening by spectrofluorometry

A screening of 13 extracts was carried out to verify the relevance of AGEs fluorescence detection by the protocol developed (Fig. 18). After incubation, an increase in fluorescence was observed in almost all the extracts. This fluorescence was greater than the positive control (BSA + Fructose) except for *Origanum compactum* and *Mentha pulegium*. If this fluorescence was due to AGEs, this means that these extracts activated the glycation reaction, but this may also be due to formation of new fluorescent substances during incubation. To verify the origin of this fluorescence, aminoguanidine (AG) (3 µM) was added to each sample (Fig 18). A positive control was performed in parallel without AG. For *Zingiber officinale*, *Rosa damascena* and *Sesamum indicum* extracts, it is likely that their components were denatured and excessive amounts of fluorescent interfering substances were formed during the incubation, since even in the presence of AG, their fluorescence remains the same or increases. For these three extracts, it was not possible to conclude if they have an inhibitory effect on the AGEs formation (Fig. 18 and 19).

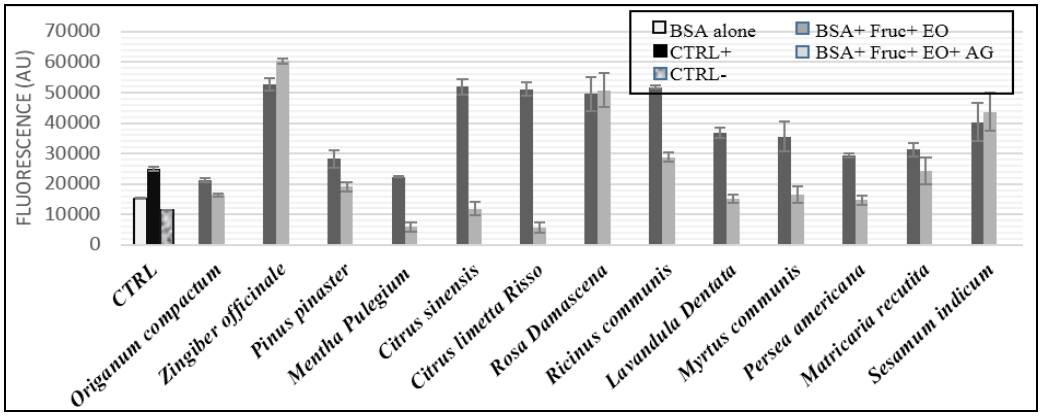

Fig 18: Screening of natural extracts. BSA (20 mg/ml) and Fructose (0.5 M) were incubated for 24 h at 50 °C with AG (3 µM) or with extracts (2 µl).

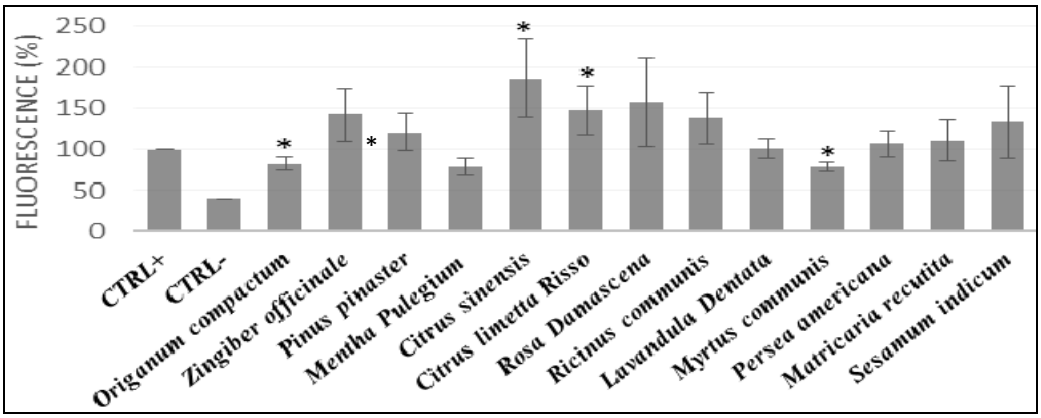

Fig 19: Increase percentage in fluorescence of extracts. BSA (20 mg/ml) and Fructose (0.5 M) were incubated for 24 h at 50 °C with AG (3 µM) or extract (2 µl). *: Significant difference of $p<0.05$.

For the other extracts which appear to be activators of the glycation reaction, part of the observed fluorescence was really due to an increase in AGEs, because the fluorescence decreased or returned to the basal state in the presence of AG. The *Ricinus communis* extract favored the highest level of AGEs formation, up to 38%. While the effect of *Citrus sinensis* and *Citrus limetta Risso* (86% and 56% respectively) may be due to the acidity of these two extracts which is respectively 3.35 and 3.05. It has been reported that an acidic pH promotes glycation [31].

After incubation, if some fluorescent substances appeared, precipitation of AGEs with trichloroacetic acid (TCA) could be performed in addition to the spectro fluorometric analysis, in order to measure the fluorescence intensity based only on AGEs [5]. In order to solve this problem, the ratios were calculated before and after incubation. Indeed, with the extract, the basal fluorescence (without incubation) was never the same of the control one. The ratios make it possible to avoid the differences due to the intrinsic fluorescence and thus a relative normalization of the data.

	CTRL+	CTRL-	O. C	Z. O	P. P	M. P	C. S	C. L	R. D	R. C	L. D	M. C	P. A	M. R	S. I
Ratio	1.7	0.7	1.8	2.8	2.1	1.7	3.8	3.1	2.4	3.2	1.9	1.8	0.8	2.1	2.8

(Ratio = Sample after incubation/ Sample without incubation)

These reports were used to calculate the relative anti-glycation activity. Thus, the results of figure 20 revealed that only the extract of *Mentha pulegium* has a weak inhibitory effect on the formation of AGEs.

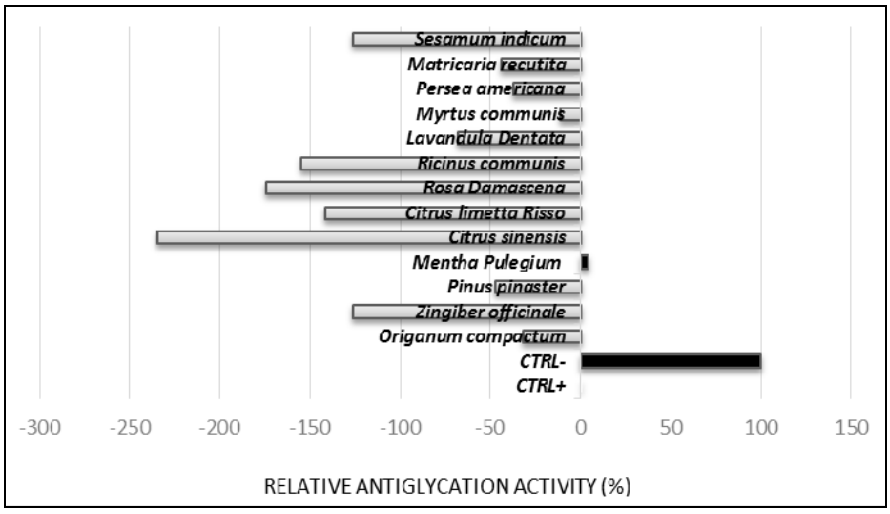

Fig 20: Percentage of relative anti-glycation activity.

In this study, we focused on the potential benefits of some plant species with antioxidant properties. Thus, the screening of inhibitors made it possible to analyze the effect of 26 natural extracts and four purified molecules on the BSA glycation. Several extracts have been shown to possess anti-glycation activity when tested by the MG-BSA system. However, this activity was relatively average and changes according to the nature of the extract.

Previous studies showed that these extracts with inhibitory activity were known for their effects on different organism parts. For example, *Nigella sativa* seeds have traditionally been used in the treatment of high blood pressure and diabetes with an hypoglycaemic activity [33], which makes this plant very attractive in the treatment of cardiovascular risk factors. In addition, leaves of *Eucalyptus globulus* have been used as traditional remedies for treatment of fungal infections and diabetes [34]. However, the anti-glycation activity of these two plants is little explored.

The study of Choudhary Iqbal M. *et al*. (2010) revealed a significant inhibition of the glycation in *Viscum album* extract, which was probably due to polyphenols of the plant. Moreover, this plant has biochemical side effects and may even be toxic at high doses, since previous studies have led to the isolation of viscotoxins in addition to lectins, alkaloids, flavonoids, amines, acids and Terpenoids [35]. On the other hand, lethality studies have shown that crude extract obtained from V. album leaves had a very wide margin of safety, and was probably non-toxic [36].

The extract of *R. officinalis* has been commonly used for the prevention of premature aging of the skin by protecting the cells against the aggressions of free radicals and this thanks to the antioxidant virtues [37]. The fact that this extract possesses an anti-glycation activity implies that it can, at a topical application, improve the elasticity of the skin by inhibiting the glycation of the collagen.

The major compounds of *N. sativa* EO can be the cause of its effects, since it contains about 46% of monoterpene hydrocarbons, the main ones were ρ-cymene (≈ 32%) and α-Pinene (≈ 9%), in addition to thymoquinone (24.5%) [38]. Whereas for *R. officinalis* we find: eucalyptol (35.20%), 1R-α-pinene (19.51%) and the (1R)-Camphor (7.32%). *Origanum compactum* Contained p-Thymol (63.60%), γ-Terpinene (17.2504%), p-Cymene (8.4457%) and α-Terpinen (2.1893%) as major compounds [39]. In fact, the acetone and ethyl acetate extracts of *M. recutita* possess a significant antioxidant effect [40]. Also, *R. officinalis* has an average anti-radical activity, while *O. compactum* has powerful anti-radical activity [39]. While, the antioxidant effect of N. sativa ethanolic extract was very good and higher than that of *R. officinalis* [41]. Due to their antioxidant activity, extracts of *E. globulus* were used as food additives [42].

It has been reported that free radicals have also contributed to formation of advanced glycated products (AGEs). Eventually, this can lead to oxidative stress and a variety of degenerative diseases, such as cancer, diabetes, and aging [43]. Elimination or inactivation of reactive oxygen species (ROS) is considered an instrumental approach to reduce the risk of these diseases [44]. Other studies have demonstrated the possibility of glycation inhibition induced by methylglyoxal and glyoxal by antioxidants [45]. Thus, like other natural compounds which have shown anti-glycation activity, it is very likely that these extracts possess active molecules which can exert their inhibitory effect on glycation by preventing the additional oxidation of glycated proteins as well as oxidation of glucose catalyzed by a metal and which leads to AGEs formation.

Various phenolic antioxidants extracted from plants have been found to inhibit the AGEs formation. Their inhibition of the free radicals production in the glycation process and a subsequent inhibition of protein modification were considered as the main mechanisms to arbitrate their anti-glycation activities. In similar studies, phenolic compounds have been reported to be the major contributors of anti-glycation activity in various plant extracts [46], because these compounds such as flavonoids, tannins and phenolic acids possess a strong antioxidant power due to their redox properties.

These antioxidant effects are believed to contribute to the subsequent inhibition of protein changes in the glycation process, so it was reasonable to expect phenolic antioxidants to inhibit AGEs formation. For the most synthetic AGE inhibitors, it is believed that direct trapping of MG is probably one of their major mechanisms of AGEs formation inhibition. In addition, it has been reported that some phenolic compounds trap reducing sugars under physiological conditions [47]. Also, it was demonstrated that the inhibitors effects were related to the number of phenols in the molecule structure, and to multiple sites of action. Apart ferulic acid, all

the phenolic constituents suppress clearly the formation of α-dicarbonyl compounds and AGEs. Therefore, the effects of our inhibitor extracts may be due to the different composition of the phenolic, non-phenolic compounds or other components.

5. Conclusion

A relatively large number of plant extract were systematically evaluated for their anti-glycation capacity using the novel *in vitro* model of BSA-MG and BSA-Fructose analysis. In fact, it has been shown qualitatively that the glycated BSA formation increased with temperature and time incubation, without inducing a change in the BSA conformation. This protocol could be applied in the screening of anti-glycation compounds and was effective. The extracts of *Nigella sativa*, *Viscum album*, *Rosmarinus officinalis*, *Eucalyptus globulus*, and the Swainsonine molecule had a significant inhibitory effect on glycation of albumin by MG, which varied between 40 and 50%. We have also found that the extracts of *Cinnamomum zeylanicum* acted as activator of the glycation reaction with a percentage greater than 50%. The anti-glycation properties of these plant species studied seem to be the basis of their potential usefulness as medicinal plants and offer remarkable prospects for the preventive treatment of pathogenesis under conditions associated with complications of diabetes, aging and a wide range of other conformational disorders.

6. Conflict of Interest

The authors declare that there is no conflict of interest.

7. Acknowledgment

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