



## Heat Stable Inhibitors of Protein Cross-linking from Sri Lankan Medicinal Plants

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### Authors' contributions

*This work was carried out in collaboration between both authors. Author HKIP concept and design of the study, obtaining grants, literature search, data collection, analysis and interpretation, manuscript preparation and critical revision of the manuscript. Author WKVKP performed the experiments. Both authors read and approved the final manuscript.*

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### ABSTRACT

**Aims:** Glycation induced protein cross-linking is recognized as the most damaging element of advanced glycation end product formation and is a key player of diabetic complications. Objectives of the present study were to assess the heat stability of potential glycation inhibitors of seven medicinal plants and to investigate whether the cross-linking is inhibited when the extracts are added after the commencement of glycation.

**Study Design:** Experimental.

**Place and Duration of Study:** Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka, from May 2013 to March 2014.

**Methodology:** Plant parts with promising *in vitro* cross-link inhibitory potential were analyzed. Lysozyme and fructose system was used as the glycation model. Effect of heated (at 95°C for 1 hour) and non heated samples on fructosamine formation and glycation induced protein cross-linking was compared using nitroblue tetrazolium method and sodium dodecyl sulfate

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polyacrylamide gel electrophoresis respectively. Effect of the addition of non heated samples on day 0, 1 and 2 on cross-linking was also compared.

**Results:** Both heated and non heated extracts of *Coriandrum sativum* seed, *Phyllanthus debilis* whole plant, *Phyllanthus emblica* fruit, *Syzygium aromaticum* flower and *Syzygium cumini* leaf showed reduction in the formation of fructosamine and lysozyme cross-links almost to the same extent. There was a reduction in the inhibitory effects of the heated samples of *Ficus racemosa* stem bark and *Pterocarpus marsupium* latex. All seven samples prevented further increase in cross-linking when added on day 1 of the incubation, but not when added on day 2 except for *S. cumini*.

**Conclusion:** *C. sativum*, *P. debilis*, *P. emblica*, *S. aromaticum* and *S. cumini* extracts possess thermo stable compounds that inhibit fructosamine and glycation induced protein cross-linking. Plants used are likely to have inhibitory effects on early or middle stages of glycation and did not demonstrate the ability of breaking cross-links.

**Keywords:** Heat stable; glycation; cross-linking; medicinal plants.

## 1. INTRODUCTION

Complications of diabetes mellitus are becoming the most significant cause of morbidity and mortality worldwide [1]. Advanced glycation end products (AGEs) formed as a result of protein glycation are key players of pathogenesis of chronic diabetic complications which include myocardial infarction, retinopathy, nephropathy and neuropathy [1-4]. A number of tissue proteins undergo non enzymatic glycation to the extent which depends on the severity and duration of hyperglycaemia. Proteins with long half lives such as collagen and elastin are more vulnerable for glycation induced changes as the period of the exposure is longer [1].

Non-enzymatic glycation of proteins is initiated with the condensation of free amine groups of proteins with a reducing sugar to yield Schiff-base intermediates. These intermediates undergo Amadori rearrangement to form stable ketoamine derivatives such as fructosamines which are early glycation products [4]. Free radicals are generated during the early events of glycation, accelerating the speed of the latter events. Early glycation process is monitored by measuring the concentration of the first stable glycation product fructosamine [4]. Fructosamines are subsequently oxidized, dehydrated and condensed to form AGEs.

Some AGEs lead to the formation of intermolecular cross-linking which subsequently change the properties and functions of the tissue proteins [5]. The occurrence of protein cross-links in extra cellular matrix proteins compromise the function of organs such as kidney leading to complications associated with diabetes [3]. Glycation induced protein cross-linking in long-

lived proteins is recognized as the most damaging element of AGE formation [6]. Cross-linked AGEs include pentosidine, crossline, glyoxal-derived lysine dimer (GOLD), methylglyoxal lysine dimer (MOLD) which are fluorescent AGEs and non-fluorescent AGEs such as arginine-lysine imidazole cross-links [1].

Antiglycating compounds include antioxidants that suppress generation of reactive carbonyl groups, anti AGE agents that can trap carbonyl groups, agents that suppress glycoxidation reactions such as metal ion chelators, agents that break AGE cross-links and block AGE receptors (RAGE) [7,8]. Plants have shown several of such mechanisms except the latter two effects that are usually demonstrated by synthetic inhibitors [7]. The determination of protein glycation inhibitory potential of medicinal plants is important to identify safe, effective and economically sustainable therapeutic alternatives in delaying or preventing the onset of diabetic complications. Even though there are studies reporting antiglycating effects of medicinal plants [7], attempts made to identify protein cross-link inhibitory effects are scanty.

In the system of traditional remedies, heat treatment is used frequently as a method to extract active compounds from medicinal plants. Decoctions are made often with hard parts such as bark, seed and root and hot infusions are prepared with soft parts such as flower and leaf [9]. Therefore it is important for the active ingredients to be heat stable. Scientific investigations carried out to prove the stability of glycation inhibitors after heat treatment is lacking, as the method of extraction in these studies are generally avoiding temperatures over 50°C.

Previously we have reported strong *in vitro* inhibitory effects of six medicinal plants on glycation induced protein cross-linking [10]. Major objective of the present study was to assess the heat stability of potential glycation inhibitors of seven medicinal plants. The other objective was to investigate whether the protein cross-linking is inhibited when the plant extracts are added after the commencement of glycation.

## 2. MATERIALS AND METHODS

### 2.1 Plant Extracts

Seven plant parts (from which, six have shown a strong inhibition on glycation induced cross-linking) [10] were used (Table 1). Methanol extracts were prepared from powdered dry plant parts. Dried latex of *Pterocarpus marsupium* was used without extraction. Plants were authenticated and the voucher samples were deposited at the Royal Botanical Gardens, Peradeniya, Sri Lanka.

### 2.2 Incubation of Reaction Mixtures to Determine the Heat Stability of Extracts

Six extracts and the dried *P. marsupium* latex were dissolved in 200 mM phosphate buffer (10 mg/ml) and 3 ml aliquots were heated at 95°C for 1 hour in sealed glass tubes. Total volumes of the heated samples were adjusted to the original volumes with phosphate buffer. Chicken egg lysozyme (Sigma) was incubated with 500 mM fructose [11] in the presence of heated or non heated extracts (50 and 250 µg/ml) for 3 weeks

at 37°C. Phosphate buffer (200 mM) with 0.02% sodium azide was used as the buffer (pH 7.4). Aminoguanidine (1 mg/ ml) was used as the positive control. Uninhibited control was prepared without a potential inhibitor.

### 2.3 Incubation of Reaction Mixtures to Assess Whether the Extracts Can Inhibit Cross-linking When Added after Commencement of Glycation

Chicken egg lysozyme was incubated with 500 mM fructose for 2 weeks at 37°C. Plant extracts (2.5 mg/ml) were added either on day 0, 1 or 2 of the incubation, to the lysozyme fructose mixture. Aliquots from the uninhibited control was collected on day 1 and day 2 and stored at -40°C for comparison.

### 2.4 Measurement of Fructosamine Concentration

Fructosamine concentration of the aliquots collected from incubation mixtures with heated and non heated extracts was measured using the method described by Meeprom et al. [12] with modifications using lysozyme instead of BSA. Test samples collected on day 9 were mixed with 0.1 M sodium carbonate buffer (pH 10.35) and nitroblue tetrazolium (0.5 M) in 0.1 M sodium carbonate buffer (pH 10.35). Reaction mixtures were incubated at 37°C for 15 minutes and the absorbance was measured at 530 nm. Percentage inhibition was calculated in terms of fructosamine concentration using the following formula.

$$\% \text{ Inhibition} = 100 - \frac{(\text{Absorbance of Test} - \text{Absorbance of Test Blank}) \times 100}{(\text{Absorbance of Uninhibited Control} - \text{Absorbance of Control Blank})}$$

**Table 1. Plant parts (or product)\* used**

| Plant species                             | Common name** | Plant part  |
|---|---------------|-------------|
| <i>Coriandrum sativum</i>                 | Kottamalli    | Seed        |
| <i>Ficus racemosa</i> L.                  | Attikka       | Stem bark   |
| <i>Phyllanthus debilis</i> Klein ex Willd | Pitawakka     | Whole plant |
| <i>Phyllanthus emblica</i> L.             | Nelli         | Fruit       |
| <i>Pterocarpus marsupium</i> Roxb.        | Gammalu       | Latex*      |
| <i>Syzygium aromaticum</i>                | Karabu        | Flower bud  |
| <i>Syzygium cumini</i> Skeels             | Madan         | Leaves      |

\*\*Commonly used Sri Lankan names of the plants are listed

## 2.5 Detection of Glycation Induced Protein Cross-links

Aliquots were collected from the reaction mixtures; day 6 and 15 from those with heated and non heated extracts and day 5 and 12 from those with the extracts added on day 0, 1 and 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to detect the extent of protein cross-linking [11]. SDS polyacrylamide gels (12%) were prepared and electrophoresis was conducted under denaturing conditions using Enduro Vertical Gel Electrophoresis system- E2010-P according to the standard Laemmli method [13]. Subsequently the gels were stained with Coomassie brilliant blue for 30 min and destained overnight to visualize the protein bands.

## 2.6 Statistical Analysis

Fructosamine inhibitory assay was performed three times. Each experiment was carried out in triplicates. Statistical analysis on the differences between relative fructosamine concentrations between two treatments was performed using analysis of variance (ANOVA).  $p < 0.05$  was considered as significant.

## 3. RESULTS AND DISCUSSION

### 3.1 RESULTS

#### 3.1.1 Effect of heat on the inhibitory effects of plant extracts on fructosamine formation

Fructosamine formation was compared using the aliquots collected on day 9 of the incubation with heated and non heated plant extracts. Difference between the absorbance at 530 nm of the test and blank with nitroblue tetrazolium was proportionate to the relative concentrations of fructosamine present in the aliquots. There was a significant reduction of fructosamine concentration in the presence of all seven non heated plant extracts at 50 and 250  $\mu\text{g/ml}$  ( $p < 0.01$ ) (Fig. 1). This reduction was comparatively lower with *F. racemosa* (Fig.1). There was a reduction of the inhibition on fructosamine formation when heated extracts of *F. racemosa* (50 and 250  $\mu\text{g/ml}$ ) and *P. marsupium* (50  $\mu\text{g/ml}$ ) were used (Fig. 1B). However at 250  $\mu\text{g/ml}$ , heated extract of *P. marsupium* resisted fructosamine production to the extent of the non heated extract (Fig.1A). The other five extracts did not show a significant increase in fructosamine when heated (Fig. 1) under the experimental conditions used.

#### 3.1.2 Effect of heat on the inhibitory effects of plant extracts on glycation induced protein cross-linking

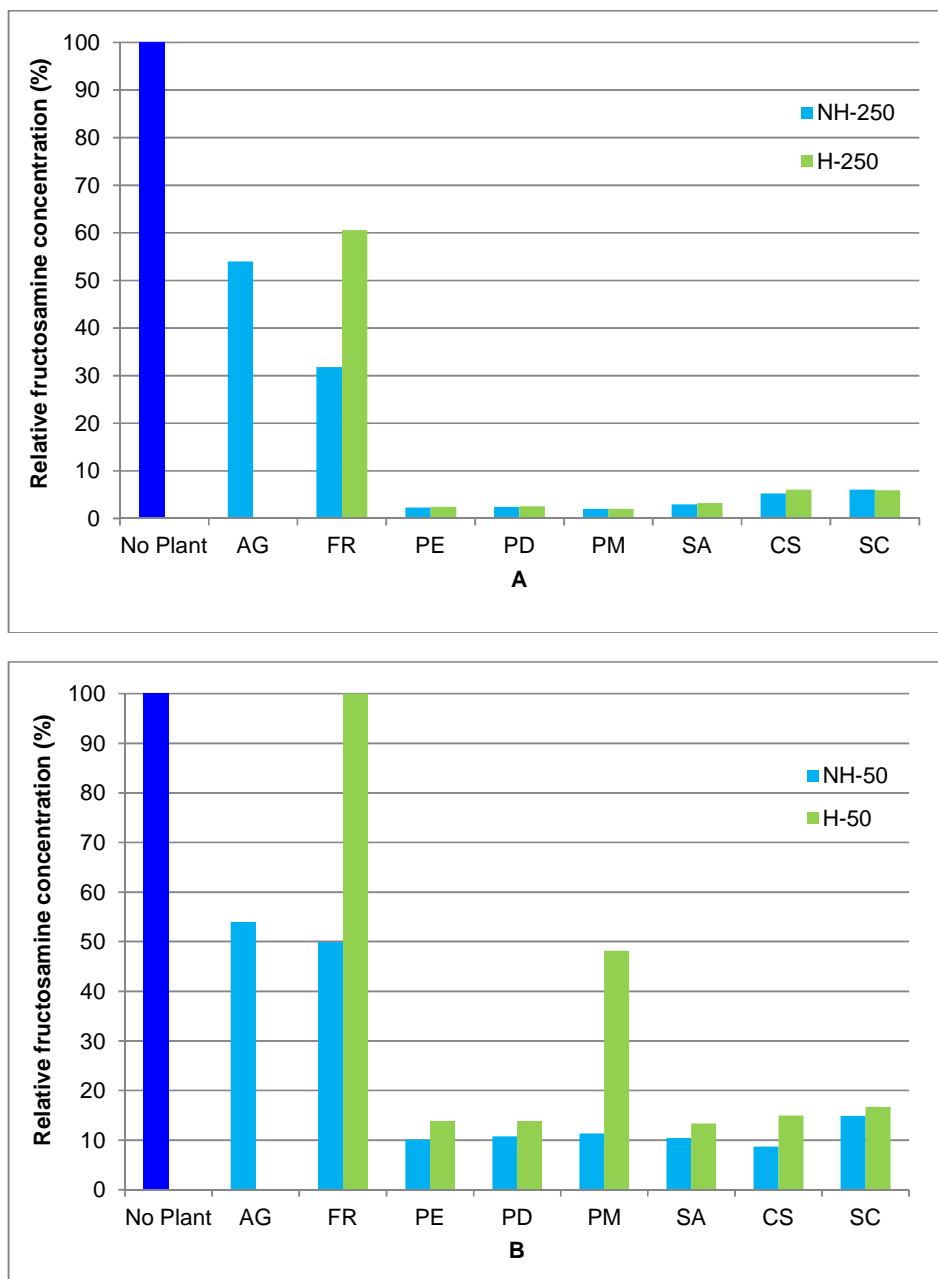
Lysozyme cross-linking was observed as high molecular weight products in the presence of fructose (Fig. 2 to 5). Those products represented the dimer, trimer and tetramer formed as a result of inter molecular cross-linking (Fig. 2). Cross-link formation was suppressed in the presence of AG (Fig. 2). Glycation induced cross-linking inhibitory effects of *S. cumini* leaf extracts was demonstrated for the first time. Cross-linking inhibition patterns of heated and non heated extracts of *C. sativum*, *P. debilis*, *P. emblica*, *S. aromaticum* and *S. cumini* in the presence of fructose were similar in the reaction mixtures incubated for 6 and 15 days with 250 (Fig. 3) and 50  $\mu\text{g/ml}$  extracts (Fig. 4). *F. racemosa* was found to have a comparatively lower efficacy in inhibiting cross-linking among the seven plants. It showed inhibitory effects on cross-linking at 250  $\mu\text{g/ml}$  (Fig. 3), but not at 50  $\mu\text{g/ml}$  (Fig. 4), when non heated extracts were added. *F. racemosa* showed some increase in the cross-link formation in the presence of the heated extracts by day 15 of the incubation period with 250  $\mu\text{g/ml}$  extract (Fig. 4B). *P. marsupium* also showed an increase in the cross-link formation with 50  $\mu\text{g/ml}$  heated extracts (Fig. 4B). However the findings indicated that *F. racemosa* and *P. marsupium* retain some inhibitory effects after heating, as seen in Fig. 3. Results revealed that the ability to inhibit glycation induced cross-linking remained in five medicinal plants of the seven tested at 50  $\mu\text{g/ml}$  after heating at 95°C for 1 hour.

#### 3.1.3 Effect of addition of plant extracts after commencement of glycation on the protein cross-linking

Addition of seven plant extracts on day 0 (at the commencement of incubation) showed almost complete inhibition of cross-linking (Fig. 5). Addition of the extracts after one day from the beginning of incubation also retarded further increase in protein cross-links (Fig. 5). There was no increase in the extent of cross-links observed from day 1 (Fig. 5 first lane) to day 5 when the extracts were added on day 1 (Fig. 5-lanes representing day 1). However the addition of extracts other than *S. cumini* on day 2 did not show a considerable retardation on the increase of protein cross-links. Extent of cross-links present on day 2 and 5 of the uninhibited control are shown in Fig. 5- second and third lanes

respectively. Extent of cross-links seen on day 5 when the extracts were added on day 2 (Fig. 5) was very similar to that of day 5 of the uninhibited control (Fig. 5- third lane). Among the seven extracts, *S. cumini* showed inhibitory

effects for a longer period of the glycation process (Fig. 5). Similar results were observed when the aliquots collected on day 12 were analyzed (data not shown).



**Fig. 1. Relative fructosamine concentrations detected in the presence of heated and non heated extracts**

Aliquots collected on day 9 from the incubation mixtures containing 250 µg/ml (A) and 50 µg/ml (B) of heated (H) and non heated (NH) extracts were used. No plant: uninhibited control with no extract, AG: aminoguanidine, FR: *F. racemosa*, PD: *P. debilis*, PE: *P. emblica*, PM: *P. marsupium*, CS: *C. sativum*, SA: *S. aromaticum*, SC: *S. cumini*

### 3.2 DISCUSSION

AGEs are key players of pathogenesis of chronic diabetic complications [1-4]. Glycation induced protein cross-linking in long-lived proteins such as collagen is recognized as the most damaging element of AGE formation [6]. Studies conducted on the inhibitory effects of plant extracts on glycation induced protein cross-linking are scarce. When the inhibitory effects are found it is important for the target compounds to be heat stable as most of the medicinal plants undergo heat treatment before consumption, to facilitate extraction of active compounds. The results of the current study revealed the presence of heat stable compounds that can inhibit protein cross-linking in *C. sativum*, *P. debilis*, *P. emblica*, *S. aromaticum* and *S. cumini*. According to up to date literature the current study has demonstrated the cross-link inhibitory effect of *S. cumini* leaves for the first time. Inhibitory compounds of *F. racemosa* and *P. marsupium* showed comparatively lower heat stability. Findings of the fructosamine assay which measured relative concentration of the early glycation product, also showed findings comparable with the protein cross-linking.

Previously we have reported strong glycation induced protein cross-linking inhibitory effects of *C. sativum*, *P. debilis*, *P. emblica*, *P. marsupium* and *S. aromaticum* extracts [10]. *In vitro* inhibition on the formation of fluorescent AGEs was revealed previously in the decoctions of *S. cumini* bark [14]. Another study by the same group demonstrated inhibition of fluorescent AGEs when the decoctions of *Osbeckia octandra* leaf were used [15]. The inhibitions observed in both studies with the decoctions were approximately two fold when compared with that of arbutin [14,15]. When decoction, water extract and ethanol extract of *Cerasus avium* stalk were investigated for antiglycation effects, decoction showed the highest inhibitory effects [16]. Findings with the *S. cumini* bark, *O. octandra* and *Cerasus avium* suggest the thermal stability of the effective compounds as those studies used decoctions [14-16]. According to the present study, the phytochemical/s contributing for the cross-link inhibition of *F. racemosa* and *P. marsupium* were relatively less heat stable as there was a reduction in the extent of inhibition to protein cross-linking (Fig. 4). However there was no complete loss of cross-link inhibition when the heated extracts of 250 µg/ ml *F. racemosa* and 50 µg/ ml *P. marsupium* were included in the reaction mixtures (Fig. 4). Higher concentration

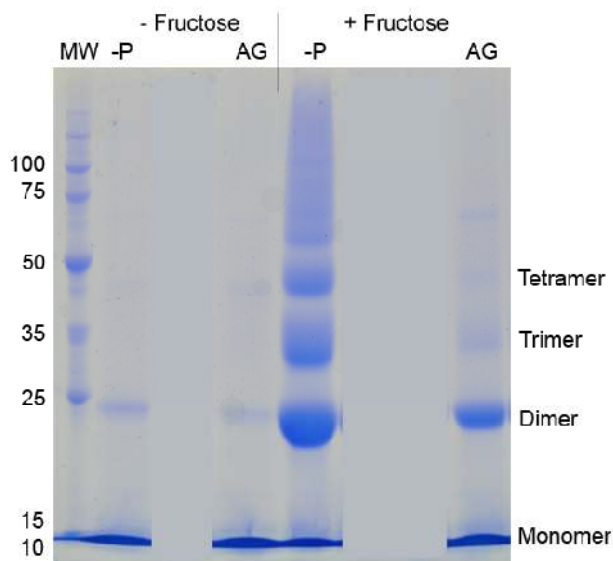
of these extracts demonstrated almost complete inhibition of protein cross-linking indicating the compounds are not completely heat labile (*P. marsupium* at 250 µg/ ml showed almost complete inhibition) (Fig. 3). All seven non heated extracts have shown an inhibition on the formation of fructosamine, indicating that the inhibitory compounds slow down early steps of glycation. There was a significant increase of fructosamine in the aliquots incubated with heated extracts of *F. racemosa* (250 µg/ ml) and *P. marsupium* (50 µg/ ml) (Fig.1) which tally with the findings on cross-linking (Fig. 4).

The antiglycation properties of several medicinal plants are known to be greater than that of the standard inhibitor of glycooxidation, aminoguanidine [17]. Scientific evidence demonstrates that the formation of glycooxidation products and cross-linking of collagen is inhibited under antioxidative conditions [18]. Various polyphenolics have proven the capacity to inhibit glycooxidation [12,17,19,20]. Phenolics are the most abundant antioxidants found in the diet and are known to act as chain breaking antioxidants. Several studies have revealed the correlation between antiglycation effect and the phenolic content of the extracts [20-22]. Phenolics may offer protection against AGE formation by scavenging reactive oxygen species produced during the glycation reaction thereby slowing the AGE formation [4]. Furthermore they provide "physical" protection to the protein molecule by non covalent binding, making the target protein inaccessible for glycation [4].

Previous studies have demonstrated free radical scavenging and antioxidant activities of ethanol extract of *F. racemosa* bark [23], ethanol extract of *P. emblica* fruit [24,25], aqueous extract of *P. marsupium* stem bark [26] and in 50% ethanol extract of *S. aromaticum* [21]. Some phenolics have shown inhibitory effects on the formation of cross-linking AGEs. Presence of polyphenolic compounds with antiglycation effects such as gallic acid, quercetin and ellagic acid in the extracts such as *S. aromaticum* was identified [27]. High level of total polyphenolics and antioxidants were found in *S. cumini* bark [14]. A phenolic acid, chlorogenic acid inhibits AGEs formation and collagen cross-linking apparently through its interactions with reactive dicarbonyl compounds, such as methylglyoxal [20]. Ability of ellagic acid to inhibit glycation induced cross-linking of lysozyme was identified [28]. Antiglycating effects of ellagic acid seem to be mediated through scavenging of the dicarbonyl

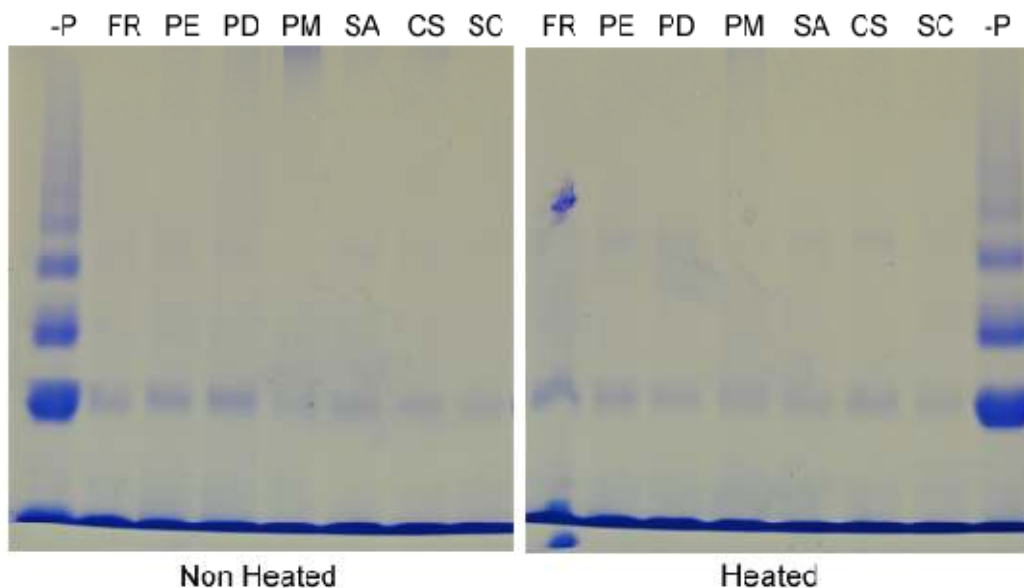
compounds [17]. When the effect of flavonoids on the formation of cross-linking AGE pentosidine, was measured in collagen, marked inhibitory effects were observed in the decreasing order as myricetin  $\geq$  quercetin > rutin > (+)-catechin > kaempferol [29]. Furthermore the

phenolic acids, cinnamic acid and its derivatives such as ferulic acid and isoferulic acid have demonstrated inhibitory effects on AGE formation *in vitro*. Isoferulic acid is identified to be a metal ion chelating agent [20].



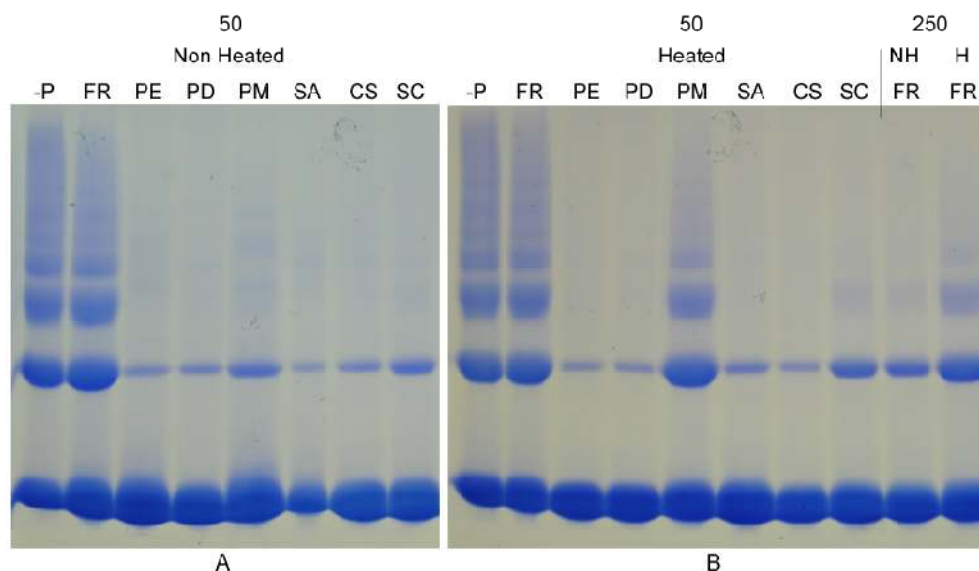
**Fig. 2. Effect of fructose on protein cross-linking**

SDS-PAGE was conducted with the aliquots collected on day 9. MW: Molecular weight markers, -P: In the absence of plant, AG: with 1 mg/ ml aminoguanidine. Experiment was performed three times

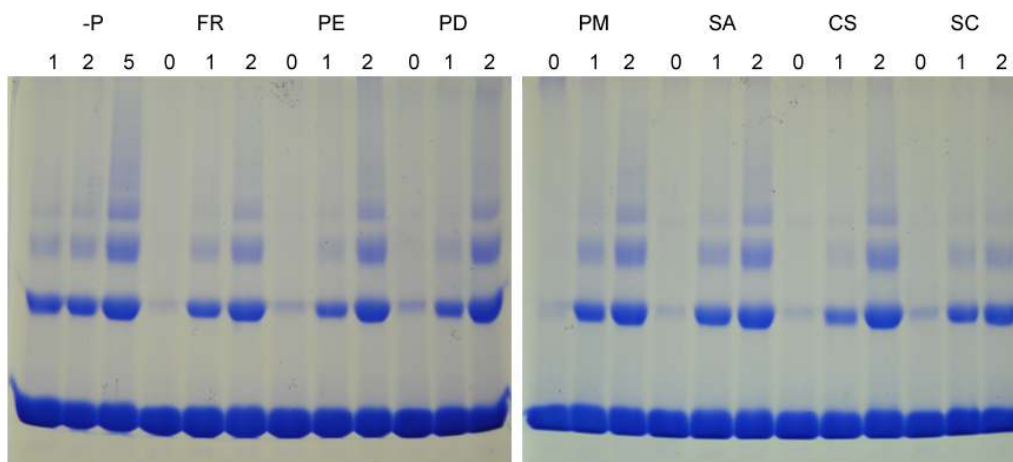


**Fig. 3. Effect of heat on cross-link inhibitory potential of 250 µg/ ml plant extracts**

SDS-PAGE was conducted with the aliquots collected on day 6. Incubations were carried out with 250 µg/ ml non heated (left) and heated (right) extracts. -P: in the absence of plant, others are in the presence of FR: *F. racemosa*, PD: *P. debilis*, PE: *P. emblica*, PM: *P. marsupium*, SA: *S. aromaticum*, CS: *C. sativum* and SC: *S. cumini*. Experiment was performed three times



**Fig. 4. Effect of heat on cross-link inhibitory potential of 50 µg/ ml plant extracts**  
 SDS-PAGE was conducted with aliquots collected on day 15. A: Non heated aliquots with 50 µg/ ml extracts (50). B: Heated aliquots with 50 µg/ ml extracts (50) or FR at 250 µg/ ml (250). -P: in the absence of plant, others are in the presence of FR: *F. racemosa*, PE: *P. emblica*, PD: *P. debilis*, PM: *P. marsupium*, CS: *C. sativum*, SA: *S. aromaticum*, SC: *S. cumini*, NH: Non heated extract, H: Heated extract. Experiment was performed three times



**Fig. 5. Effect of plant extracts on protein cross-linking when added on day 0, 1 and 2**  
 SDS-PAGE was conducted with aliquots collected on day 5 (except for the first two lanes which represent the aliquots taken on day 1 and 2 from the uninhibited control (-P)). Plant extracts (2.5 mg/ml) were added on Day 0 (0), day 1 (1) or day 2 (2) during the incubation period. Plant extracts added were FR: *F. racemosa*, PE: *P. emblica*, PD: *P. debilis*, PM: *P. marsupium*, CS: *C. sativum*, SA: *S. aromaticum*, SC: *S. cumini*. Experiment was performed three times

Even though the evidence on thermal stability of the compounds present in crude extracts is limited, there is evidence for the thermal stability of several pure phenolic compounds which are present in the plants tested. Phenolic antioxidants such as phenolic acids (gallic acid,

tannic acid, ellagic acid and caffeic acid) and flavonoids (quercetin and catechin) were investigated at temperatures as high as 180°C [30]. Quercetin and ellagic acid showed the highest thermal stability while gallic acid and caffeic acid showed the lowest thermal stability



[30]. The relatively lower thermal stability of the antiglycating compounds observed in *F. racemosa* and *P. marsupium* extracts in the current study may be due to the differences that may exist in the composition of different phenolics.

Antiglycating compounds are known to act at different stages of glycation such as early, middle and late stages. Plant extracts have shown mechanisms that suppress generation of reactive carbonyl groups, trap carbonyl groups and suppress glycoxidation reactions by chelating with transition metal ions [7]. All the extracts showed inhibition on the further cross-linking when added on day 1 as there was no further increase in cross-linking from day 1 to day 5 (Fig. 5). Based on the results obtained when the extracts were added on day 1 (Fig. 5), it is likely that the extracts studied do not have the ability to break the cross-links once formed. When added on day 2, the extracts other than *S. cumini* showed no detectable inhibition on the further increase of protein cross-linking (Fig. 5) suggesting that the active compounds are not effective after day 2. Results obtained when the extracts were added on day 0 indicate that the maximum beneficial effects are exerted when the extracts are present at the time of the commencement of glycation.

According to the findings of the present study, *C. sativum*, *P. debilis*, *P. emblica*, *S. aromaticum* and *S. cumini* contain heat stable phytochemicals which could be valuable in preventing diabetic complications. However, use of lower concentrations of those five extracts may be necessary to monitor the stability more closely. Further studies are required to identify the mode of inhibition of these extracts. Exposure of the extracts for a longer duration of heat may be required to match the preparation more closely with that of the decoctions. The stage of the glycation process on day 1 and 2 in the experimental set up need to be closely monitored as there was a clear distinction between the effects observed when the extracts were added on day 1 and day 2.

#### 4. CONCLUSION

The study demonstrated several novel findings including the evidence for inhibitory effects of *S. cumini* leaves on glycation induced protein cross-linking effects, the heat stability of the inhibitory effects of *C. sativum*, *P. debilis*, *P. emblica*, *S. aromaticum* and *S. cumini* on

fructosamine formation and protein cross-linking. Findings also revealed the relative instability of the inhibitors found in *F. racemosa* and *P. marsupium*. Study showed that the inhibitory effects of the plants investigated are likely to be exerted during early or middle stages of glycation. Results revealed that the extracts do not have the ability to break the cross-links once they are formed. Further studies are required to identify the mode of inhibition of these extracts.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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