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EVALUATION OF GARLIC ORGANOSULFUR VOLATILES, DIALLYL DISULPHIDE AND DIALLYL SULPHIDE AS A NEW ANTI-GLYCATING COMPOUND BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY- AN INSULIN BASED GLYCATION ASSAY

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ABSTRACT

Glycated insulin could cause insulin resistance in type 2 diabetes mellitus. An in vitro insulin glycation assay was developed to screen organosulfur compounds of garlic for insulin glycation inhibitors by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. The percentage inhibition of glycated insulin was determined at different concentrations (0.5-5 mM) of diallyl disulfide and diallyl sulfide. Percentage inhibition of glycated insulin by diallyl sulfide was found to be 87.44±0.66 at 5 mM whereas for diallyl disulfide it was found to be at 76.76± 1.94. Furthermore, the IC_{50} of diallyl sulfide and diallyl disulfide was very less than standard inhibitors. These results showed that organosulfur compounds of garlic may increase insulin sensitivity and reduce metabolic complications in diabetic patients.

I. INTRODUCTION

Diabetes mellitus is a disorder characterized by chronic hyperglycemia due to deficiency of insulin or insulin resistance. Diabetic patients are more prone to many complications such as neuropathy, retinopathy, nephropathy associated with diabetes. Patients with uncontrolled blood glucose level are particularly at risk and hyperglycemia play important role in the pathogenesis of hinder complications **[1]**. Complications appear in the organ where uptake of glucose is independent of insulin as result intracellular accumulations of glucose in these cells are found to be more. Most of these complications are due to glycation of protein **[2]**. Glycated proteins

can undergo further reactions called advanced glycation end products (AGEs) which may result in alteration of protein structure and function **[3]**. Over the years, several *in vitro* and *in vivo* glycation and AGEs of model protein hemoglobin, BSA, HSA, IgG, collagen, insulin etc have been reported **[4]**.

Non-enzymatic glycation is a complex series of reactions which are initiated by nucleophilic addition reaction of amino groups of proteins and carbonyl groups in reducing sugars which leads to browning, fluorescence and cross-linking of proteins to form reversible Schiff's base. As a consequence of rearrangement reactions stable irreversible Amadori products are formed. Schiff bases formed over the period of hours whereas stable Amadori products occur in days. Glycated proteins can undergo further reactions through dicarbonyl intermediates to form AGEs.

In spite of advancement in the modern medicine, there is no single drug to prevent glycation and AGEs mediated diabetes complications. However, in the last few years it has been evident that glycated insulin could contribute to the development of insulin resistance in type 2 diabetes mellitus. Glycated insulin has been observed in the islets of Langerhans of both normal and diabetic animals **[5]**. Glycated insulin in pancreatic β cell lines depends on concentration and time of exposure to glucose **[6]**. Drugs such as aminoguanidine **[7]**, carnosine **[8]**, alagebrium chloride **[8]** and N-phenacyl thiazolium bromide **[8]** showed anti-glycation effect but not yet approved by US-FDA. However, some studies have indicated that aminoguanidine may have some toxicity in diabetic nephropathy patients **[9]**. Antidiabetic drug metformin showed anti-glycation activity but it is not an efficient inhibitor for anti-glycation. Therefore, it is very essential to find out new molecules for anti-glycation activity.

Several assays are reported for the determination and quantitation of anti-glycation effects of molecules. Glycated hemoglobin was determined by hemoglobin-δ gluconolactone assay **[10]** whereas fluorescence assay **[11]** method involves the measurement of fluorescence in presence and absence of inhibitor. Immunological assays based on ELISA [**12**]. AGEs specifically methylglyoxal and N-epsilon-(carboxymethyl) lysine were detected by polyclonal antibodies and monoclonal antibodies **[13]**. However, these techniques are not adequate enough to measure precisely glycated proteins due to their low sensitivity and do not provide specific site of glycation in protein. Interestingly, different mass spectrometric techniques have been increasingly used and found to be a promising to provide detailed information on protein glycation. Hence, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) based glycation assay has been used to screen anti-glycation activity of potential therapeutic molecules **[14-15]**.

Developments in the area of plant medicines have revealed their therapeutic benefits during last few decades **[16]**. Life style modification and dietary habits have been linked to the pathogenesis such as cardiovascular complications and cancer **[17].** However, diet rich in herbs, spices and fruits are associated with lower risk of disease complications. Allium species such as garlic, a well known medicinal and nutritional herb, has been used since ancient times for the treatment of various ailments. The healing efficacy of garlic in medical traditions of India like Tibbi, Unani and Ayurveda is well documented **[18].** Scientific reports describe the beneficial effect of garlic from animal and human studies in various diseases like hyperlipidemia, atherosclerosis, hypertension, microbial infection, cancer, diabetes, cardiac complications and immunomodulatory activities **[16].** Among all nutritional agents, garlic and its components play an important role in prevention of the diabetic complications. Organosulfur volatiles such as diallyl disulfide (DADS), diallyl

sulfide (DAS), diallyl trisulfide (DTS) and sulfur dioxide are formed by the decomposition of allicin. These products were found to have a higher antioxidant activity **[19]**. Intraperitoneal administration of DAS and DADS significantly decreased the cytochrome P-450, CYP2E1 activity **[20]** and increase the phase II detoxifying enzymes such as glutathione S-transferase, quinone reductase and glutathione peroxidase enzyme **[21]**, respectively. Raw garlic homogenate significantly decreased serum glycated hemoglobin and improved insulin sensitivity in fructose fed rats [**22**]. Therefore, the present study was designed to screen DADS and DAS molecules that inhibit insulin based glycation reaction by MALDI-TOF-MS. To the best of our knowledge; this is the first study on the evaluation of anti-glycation properties of organosulfur volatiles of garlic.

II. MATERIAL AND METHODS

2.1 Chemicals and Regents

DADS, DAS, insulin, glucose, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, aminoguanidine hydrochloride, pyridoxamine hydrochloride and sinapic acid were procured from Sigma-Aldrich.

2.2 Insulin Glycation Assay

Initially stock solution of 2 mg/mL of insulin, 0.5 M of glucose, O.2 M sodium phosphate buffer (pH 7.4), 20 mM of aminoguanidine hydrochloride, pyridoxamine hydrochloride, DADS and DAS were prepared. Serial dilutions were made, using stock solution, to prepare different concentration (10 mM, 5 mM and 2 mM) of DADS, DAS, aminoguanidine hydrochloride and pyridoxamine hydrochloride. For insulin glycation assay, 25 µL of insulin (0.5 mg/mL) was incubated at 37°C with 25µL of 0.5 M of glucose prepared in 0.2 M sodium phosphate buffer (pH 7.4) for fifteen days. 50 µL of different concentration of DADS, DAS, aminoguanidine hydrochloride and pyridoxamine hydrochloride were incubated at 37°C with 25µL of 0.5 M of glucose and 25 µL of insulin (0.5 mg/mL) for fifteen days in the ratio of 1:1:2 (insulin: glucose: inhibitors). Final concentration will be 5 mM, 2.5 mM and 1mM for each DADS, DAS, aminoguanidine hydrochloride and pyridoxamine hydrochloride.

2.3 Sample Preparation for MALDI-TOF-MS

Incubated reaction mixture each of control and inhibitors samples were removed after above specific time. Matrix was prepared by adding sinapic acid in mixture of 30% acetonitrile and 0.1% trifluroacetic acid. One µL of each assay sample and matrix were mixed together and spotted onto the stainless steel MALDI plate separately by dried-droplet method. Spotted plate was allowed to dry at 37°C before analysis.

III. MALDI-TOF-MS ANALYSIS

The mass spectral analysis was done on a MALDI-TOF, Shimadzu Biotech Axima Performance (Shimadzu Biotech). Analyses of each assay sample were carried out under identical experimental conditions. Mass spectra were acquired in positive ion reflectron mode using 337 nm pulsed nitrogen laser in the mass range of 1000 Da to 7500 Da. Typically, 200–250 laser shots were used for each mass spectrum. All spectra were obtained from at least two samples to verify the consistency of the results. Pulse extraction optimized at 5800 Da and the

experiment was repeated three times. The percentage inhibition of the glycated insulin peak by inhibitors was calculated by using formula: (1-(A/B)) × 100.

A- The intensity of glycated insulin in the presence of an inhibitor

B- The intensity of glycated insulin in the absence of an inhibitor

3.1 Determination of IC_{50} of inhibitors

The percentage inhibition of glycated insulin was determined at different concentrations of DADS (0.5-5 mM), DAS (0.5-5 mM), aminoguanidine hydrochloride (1-15 mM) and pyridoxamine hydrochloride (1-15 mM) in MALDI based insulin glycation assay. IC_{50} of DADS and DAS was determined by plotting % inhibition of glycated insulin to the concentration of inhibitors. Aminoguanidine hydrochloride and pyridoxamine hydrochloride were used as reference standards.

IV. RESULTS AND DISCUSSION

The present study has been undertaken to evaluate the anti-glycation properties of organosulfur volatiles of garlic following exposure of D-glucose to insulin in presence and absence of DADS and DAS by MALDI-TOF-MS analysis. The glycation reaction of insulin was monitored by measuring the intensity of glycated peak. The MALDI-TOF-MS spectrum of $[M+H]^+$ of unglycated insulin showed an ion at *m/z* 5808 Da **(Fig.1 (a))** whereas the glycated insulin at *m/z* 5970 Da **(Fig.1 (b))**. An increase of the *m/z* value by 162 Da compared to protonated insulin clearly indicates the glycation of insulin. The relative intensity of glycated insulin was found to be in the range of 30-50% compared to unglycated insulin. Inhibition of glycated insulin by the DADS and DAS were screened at 1mM, 2.5 mM and 5mM. Percentage inhibition of glycated insulin by DAS was found to be 87.44±0.66 (5 mM), 81.46± 1.54 (2.5 mM) and 32.00±1.35 (1mM). Similarly for DADS it was found to be at 76.76± 1.94 (5 mM), 75.10± 1.36 (2.5 mM) and 31.08 ±1.53 (1mM) **(Fig.2)**. Both, DADS and DAS, organosulfur volatiles of garlic, showed concentration dependent inhibition of insulin glycation. The standard inhibitors such as aminoguanidine hydrochloride and pyridoxamine hydrochloride showed less percentage inhibition of glycation when compared to organosulfur volatiles. DAS showed potent anti-glycation activity in comparison with some of the known antiglycating compounds such as aminoguanidine and pyridoxamine at 2.5 and 5 mM. Further, DAS showed higher anti-glycation activity even at concentration as low as 2.5 mM when compared to DADS.

Furthermore, the IC_{50} of DADS, DAS, aminoguanidine hydrochloride and pyridoxamine hydrochloride were determined by a MALDI-TOF-MS based insulin glycation assay. The IC_{50} of standards inhibitors such as aminoguanidine and pyridoxamine was found to be 4.6 mM and 3.06 mM, respectively (Fig.3 (a) and (b)). Whereas, the IC_{50} of DAS and DADS was found to 1.25 mM and 1.44 mM, respectively which suggest that both organosulfur volatiles are strong glycation inhibitors (Fig.3 (c) and (d)). The IC_{50} of organosulfur volatiles is very less than standard inhibitors.

Although the antidiabetic effect of raw garlic has been well established in the type I and type II diabetic animal model but only few studies have been conducted to evaluate the effect of garlic on insulin resistance in rats. This is the first report on strong anti-glycation activity of DADS and DAS at very low concentration. Further in vivo

studies need to be conducted on experimental animal models to elucidate the exact mechanism of DADS and DAS mediated glycation inhibition.

V. CONCLUSION

In conclusion, our study by MADI-TOF-MS insulin based glycation assay showed that organosulfur compounds of garlic such as DADS and DAS are strong glycation inhibitors at low concentration. This may increase insulin sensitivity and reduce metabolic complications in diabetic patients. Further *in vivo* studies are in progress on experimental animal models and in human to elucidate the exact mechanism of DADS and DAS mediated glycation inhibition.

VI. ACKNOWLEDGEMENT

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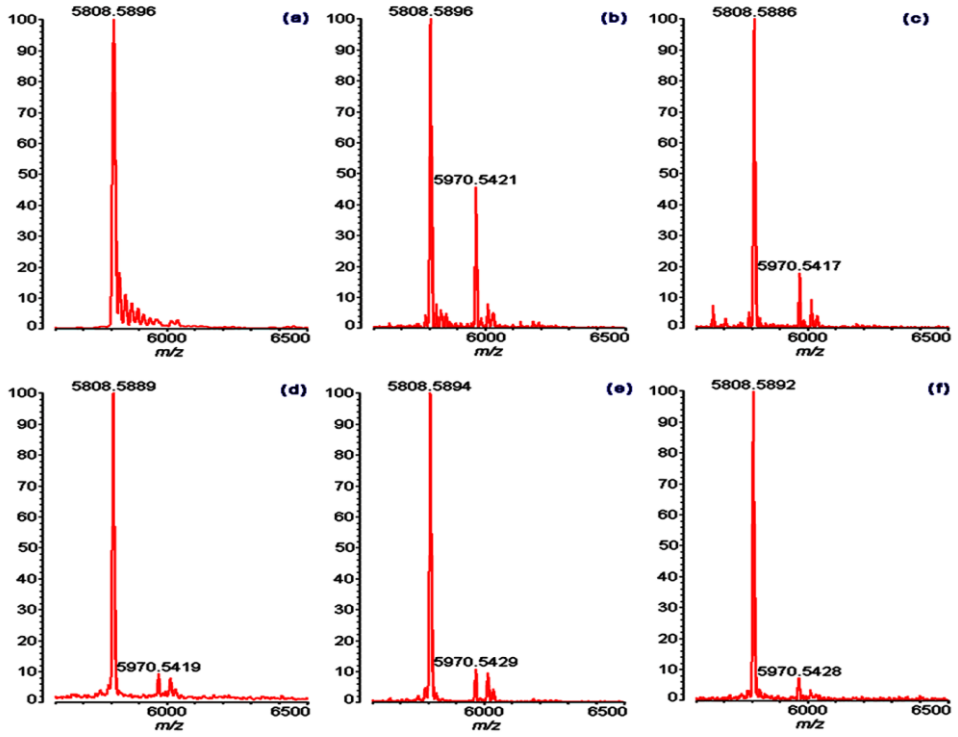

Fig 1. Insulin glycation assay by MALDI-TOF-MS (a) Control Insulin (0.5 mg/mL), (b) glycated insulin, Glycated insulin in presence (c) 5 mM aminoguanidine hydrochloride (d) 5mM pyridoxamine hydrochloride (e) 2.5 mM DADS and (f) 2.5 mM of DAS.

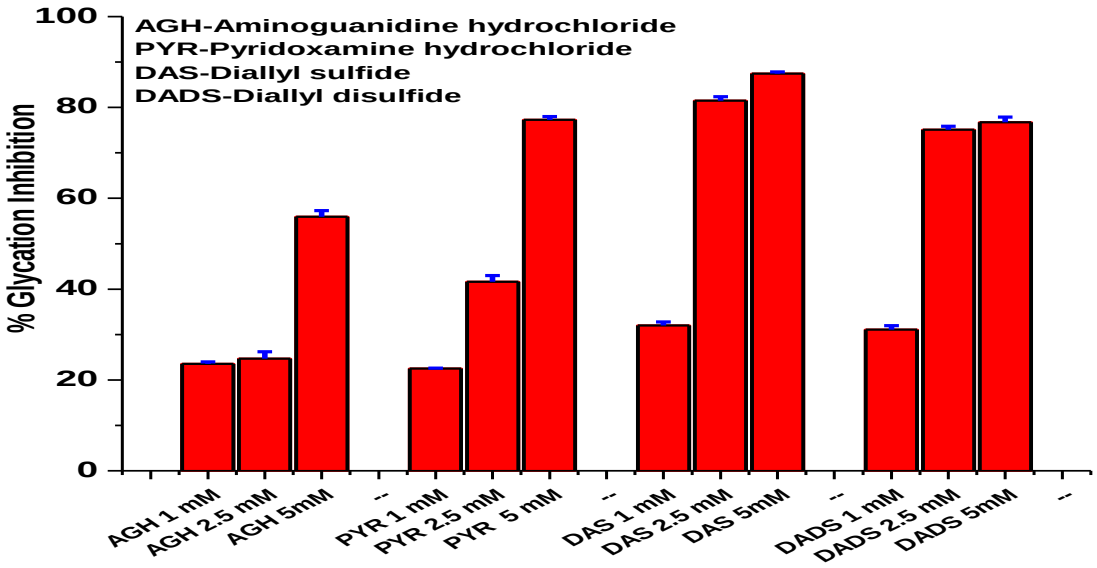

Fig.2. Percentage inhibition of glycated insulin at 1mM, 2.5mM and 5mM of aminoguanidine hydrochloride, pyridoxamine hydrochloride, diallyl sulfide and diallyl disulfide determined by insulin glycation assay by MALDI-TOF-MS. All values are mean ± SEM (*n=3*)

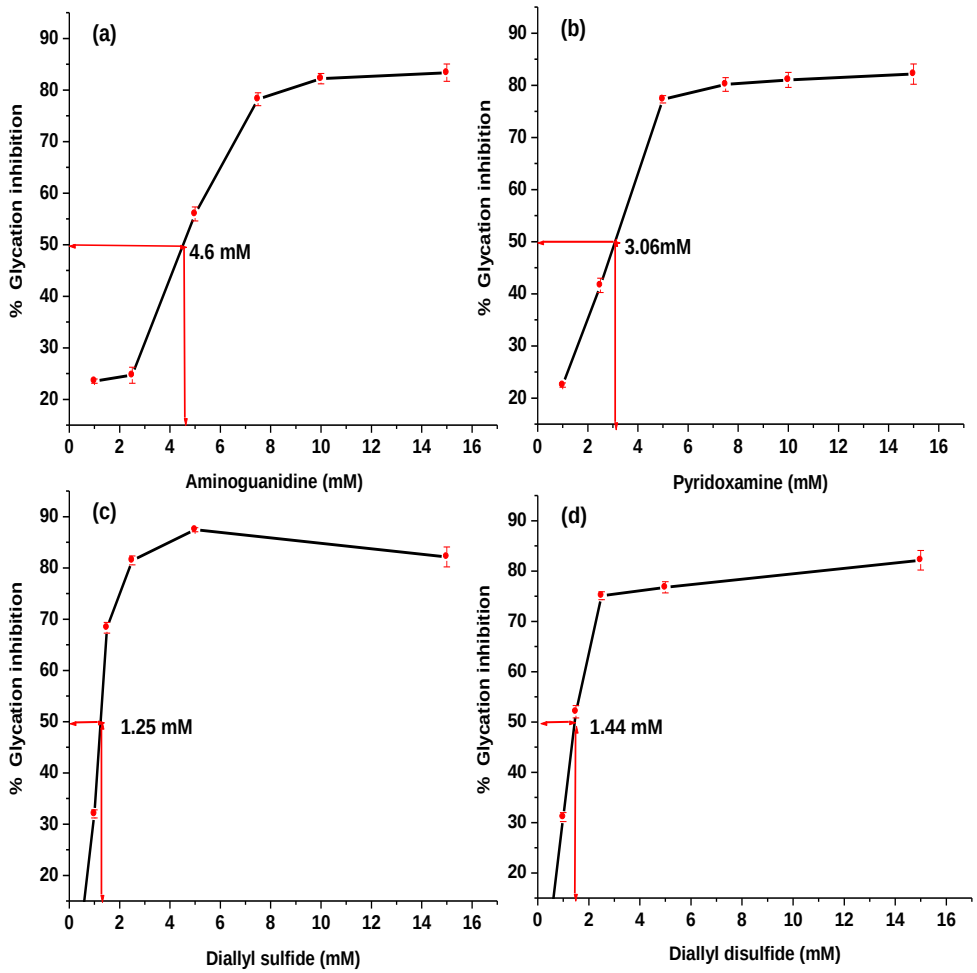

Fig. 3. IC_{50} values of (a) aminoguanidine hydrochloride (b) pyridoxamine hydrochloride (c) diallyl sulfide (d) diallyl disulfide .All values are mean ± SEM (*n=3*)

Figure Captions:

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