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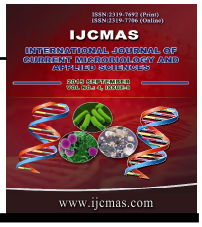

Original Research Article

An anti-glycating property roots ethanol and methanol extracts of *Withania somnifera* on in-vitro glycation of elastin protein

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Keywords

Native elastin, inhibition, NBT reduction assay, m-PAGE, AGEs, Inflection point

A B S T R A C T

To investigate the inhibitory and crosslink breaking antiglycating effects of Withania phytochemicals on the elastin protein glycation in-vitro. Using NBT (Nitrobluetetrazolium assay) assay, and combine use of m-PAGE and HPLC protein mapping, anti-glycating effects of Withania were investigated. Glycation was inhibited and break crosslinking by ethanolic and methanolic extracts prepared from *Withania somnifera* roots. as potential inhibitors of glycation and AGE formation. Roots extracts of etanolic and methanolic also showed "AGE-breaking" activities in vitro by asses sing biochemical methods. Current evidence is that non - enzymatic glycation form dimer trimer polymer of protein and/or trapping or non specificity of native protein. Here, we investigate the inhibitors of glycation and AGE-breakers published to date and present the results of our in vitro investigations on native protein reformation or level of inhibition and crosslink breaking. These glycation-inhibitors and glycation-breakers may find therapeutic use in the treatment of diseases that AGE formation and accumulation may be responsible for their pathogenesis such as diabetes ,Alzheimer_s, rheumatoid arthritis, atherosclerosis and diabetic later complication .

Introduction

The high blood sugar concentration that is found during diabetes is related to either insufficient insulin production (i.e., Type-1 diabetes) or resistance to insulin (i.e., Type-2 diabetes) (Vorum, *et al.,* 1995).This increased blood glucose concentration has a number of effects in the body. Many of these complications are due to protein glycation and the formation of advanced glycation end products (Armbuster, 1987).

Glycation (also called as non-enzymatic glycosylation) is the result of a sugar molecule, such as fructose , glucose, bonded to a protein or lipid molecule without controlling action of an enzyme. . Glycation has the potential to alter the biological structure and function of the protein Moreover, glycation end products (AGE) result in abnormal biological effects that leads to tissue damage via alteration of the

structure and functions of tissue proteins(Mendez, et al . ,2003) .Elastin protein have the backbone for architectural and functional stability of tissues cell and organs. When AGEs accumulations particularly high in elastin, proteins are result in intra and inter molecular cross-linking and later has been hypotized to stiffening of this protein and believed to play an important role in the process depends on altering extensions and contractions during physiological functions. It concerns the blood vessel walls, lung parenchyma, ligaments, skin and elastic cartilage (Mecham, 2008). Change in elastin contents or defects in the structure of elastin or micro-fibrils results in etiology of various AGEs related diseases (Aronson, 2003).

An alternative strategy for diabetes treatment is the use of medicinal plants as a useful source for the development of new pharmaceuticals, as well as, dietary supplements to existing therapies (Bailey and Day, 1989). Ashwagandha is also known by the names Winter Cherry, Indian Ginseng, and Withania. The herb is prevalent in India, Pakistan, Sri Lanka, and Africa. Ashwagandha is an important herb used in Ayurveda, Siddha and Unani(Qumrudin, *et al* 2012). The use of ashwagandha in Ayurvedic medicine extends back over 3000 to 4000 years to the teachings of an esteemed Rishi (sage) Punarvasu Atriya. It has been described in the sacred texts of Ayurveda,including the Chakra and Sushrutha Samhitas where it is widely extolled as a tonic especially for emaciation in people of all ages including babies, enhancing the reproductive function of both men and women(Sangwan, 2004). The extracts as well as different isolated bioactive constituents of *W. somnifera* have been reported to possess adaptogenic, anticancer, anti-convulsant, immunomodulatory, antioxidative and neurological effects (Gupta , *et al* 2005).. Its increasing therapeutic benefits continuously attract the attention of pharmacologists for biomedical investigations on plant extracts and isolated phytochemicals (Chatterjee *et al*., 2010).The goal of this proposed research has been to investigate the effect of *Withania somnifera* (Solanaceae) in non enzymatic glycation inhibition and crosslink breaker reaction for isolated elastin protein.

Materials and methods

Preparation of roots (*Withania somnifera*) extracts: Dried and powered root rhizomes was extracted by Soxhelt method with 1:8 dry feed solvent ratio of absolute ethanol and methanol at 37°C for 32 hours and then filtered and stored at 4°C for further experiment.

Sample Recovery: Rotary Evaporator was used for recovery of sample by ethanol evaporation. Obtained semisolid and ground sample was dissolved in 25^{-ml} of DMSO (Dimethoxysulphoxide) and stored at 4°C for further use.

Isolation and characterization of elastin protein from chickens:

Connective tissues came from 36-d-old broiler chickens and were mechanically separated in a local slaughterhouse. Approximately 4 kg of tissue were minced in an American Eagle4 laboratory meat grinder (model AE-G22S, 5-mm plate). The ground tissue was then homogenized for5 min in an Omni5 homogenizer (model 17105, 2-mmplate).Elastin isolated by Starcher method (Work, 1969). The isolated proteins will be characterized and quantified by Laemmli SDS-PAGE method (Laemmle 1970), and Bradford method (Sheikh *et al* 2004)

***In vitro* glycation of protein with Withania root and leaves (*Withania somnifera*) extracts:** Glucose, proteins with or without inhibitor (plant extracts in PBS pH 7.4) were prepared and their mixture was incubated at 37°C for 1 to 5 weeks. During this, samples were drawn for glycation inhibition activity after 1st, 3rd and 5th week of incubation (Yadav, *et al* ,2012). For crosslink breaker study the reaction mixtures were incubated at 37°c for 1-5 weeks. After over of the incubation time precipitated the glycated E.C.M. proteins and incubated at 37°C for 1 week. The samples kept at 4^{o}C until analysis.

Nitro blue tetrazolium reduction assay (NBT assay) method for antiglycating (inhibition and crosslink breaker) activity of Withania root and leaves (*Withania somnifera*) extracts:

After over of the incubation time use of TCA enables the removal of any soluble interfering substances prior to the measurement of anti-glycation assay .Extent of glycation of protein under the influence of test samples was analyzed by measuring formation of fructosamines as described by Wu, *et al*., (2009).Briefly, 200 µl glycated material and 800 µl of NBT reagent and 300 µM in sodium carbonate buffer (100mM, pH 10.35) was incubated at ambient temperature for 15 min, and absorbance was measured spectrophotometer (D750DU, BioTek Instruments, Inc. USA) at 530 nm against a blank.

Methacrylamido phenylboronate acrylamide gel electrophoresis

Preparation of methacrylamido phenylboronic acid (MPBA) polyacrylamide gels using Jackson ,*et al.,* 2008 methods. In mP-AGE, acrylamide gels incorporating phenylboronatewere easily prepared by adding a small percentage (0–1%) of MPBA to the electrophoresis gel preparation solution prior to polymerization. Polyacrylamide resolving gels were polymerized in the absence or presence of MPBA in concentrations ranging from 0–1% by mixing MPBA powder with an 8% acrylamide solution (from 40% stock solution of acrylamide:bis-acrylamide, 29:1 in 40mM Tris buffer at pH 8.8and cast in a gel casting cassette (height: 100mm_width:100mm_thickness: 0.75 mm). After polymerization of the resolving gel, using 10%ammonium persulfate (Sigma Aldrich) and TEMED (SigmaAldrich) the stacking gel, containing no boronic acid, was prepared with 10% acrylamide (from 40% stock solution of acrylamide:bis-acrylamide, 29:1; Fisher Scientific) in 10mM Tris buffer pH 6.5 and cast on top the resolving gel. The protein samples where applied to the stacking gel in sample buffer (2% w/v SDS, 2mM DTT, 15% glycerol, 100mM TrispH 6.8 and bromophenol blue) and gels were electrophoresed at 60mA for 60 min in glycine buffer (25mM Tris,pH 8.3, 250mM glycine and 0.1% SDS) at room temperature..

High performance liquid chromatography(HPLC)

Cut to m-PAGE gel in separate lane and centrifuge with PBS (pH7.4) in 5000rpm at 5 min. and filter using 0.22u syringe filter. The 20 µl of the aqueous layer was injected into the flow system (JASCO, 2017, HPLC system, USA) Water flow rate was 0.5 ml^{-}_{min} (deionized water, HPLC grade, Labconco); spectroscopy detector was set at 206-280 nm for detection of peptides. Four pooled glycation inhibition and crosslink breaker sample were prepared and mixing. These pools were diluted with HPLC grade

water and, diluted (pools 1–3 times, pool) samples are presented (Wrobel, *et al.*, 1997).

Statistical analysis

All experiments were performed in triplicate (n=3) and results were expressed as mean ± SEM. Statistical analysis was carried out with (SPSS package version 10.0). HPLC signal peak width determined by mean±SD and compare mean significancy by (ANOVA). A $P<0.05$ has been considered statistically significant.

Result and Discussion

The amount of respective dried powder was extracted by using ethanol polar organic solvent and rotary evaporator used to recover the sample. The Starcher method combines autoclave treatment with extraction using reducing and chaotropic agents and enzymatic digestion. Because elastin lacks methionine, cyanogen bromide is used to cleave non-elastin proteins (Soskel and Sandburg,1983). The purified tropoelastin is extremely ‘‘sticky” and significant loses can occur when tropoelastin was stored in solution. For this reason, purified tropoelastin should be kept as small aliquots of lyophilized protein and stored frozen. R_f value was determined and indicated purified protein elastin was a chain of elastin ≈70 kDa (fig-1). The extracted proteins were purified (polishing) by size exclusion chromatography. After polishing step 4.8 mg^{-ml} elastin content was obtained form chick tissues. The term non-enzymatic glycation of proteins refers to a wide variety of spontaneous reactions between reducing sugars and protein-bound amines. The non-enzymatic glycation reaction with elastin increased significantly upon incubation with glucose by 1:1-fold, respectively.. Each extract along with glucose and elastin protein were incubated at 37°C for 1-5 weeks to monitored glycation and inhibitory activity. In crosslink breaker activity was done with already glycated reaction mixtures. Use to the methods reported by Matsuura and colleagues with slight modification (Matsuura, *et al.*, 2002).precipitated to 100%and 10% TCA and each extracts with (($0.1mg^{-ml}$, $0.2\ mg^{-ml}$, $1mg^{-ml}$) concentrations(Table-1). This gave the apparent inhibitory and crosslink breaker anti-glycating activity. The results were obtained after the measurement of glycation level with NBT assay in *in-vitro* glycation reaction in different incubation period in static condition. The maximum optical density (λ^{530}) was found in (0.082) in 5th week. Fig.2 clearly indicated the increase in incubation was correspondent to maximum adducts formation and step of AGEs formation or polymerization of protein over the time. The level of glycation inhibition was least to 2.36% in I_1 in 3rd week incubation When glycation level was measured by NBT reduction assay glycation level inhibition was increased 85.42% in 1st week at I_3 (ethanol) concentration. In most of the experiments glycation level was lowered in 1st week of incubation. here it was seen that the 3nd week inhibition level was low in I_1 (ethanol) . Table -2 inspected and , it was found that level of glycation was reduced by I_3 ($1mg^{-ml}$) and I_2 ($0.2mg^{-ml}$) as compared to I_1 ($0.1mg^{-ml}$) . It is cleared from results given, with I_3 ($1mg^{-ml}$) and I_2 ($0.2mg^{-ml}$) give better response while with I_1 ($0.1\ mg^{-ml}$) minimum response. These finding are in accordance with Sheikh,*et al*,(2004) who used garlic (*Allium Sativum)* on albumin glycation *in-vitro*. In the presence of various concentrations of garlic, albumin was glycated. The results showed that garlic has a statistically significant ($P<0.05$) effect in inhibiting or decreasing the reaction of albumin glycation. The findings of this research show that garlic probably inhibits

the reaction of glycation. McInty, et al., (2009) revealed that anti-glycation effects of V. angustifolium leaf extract, relative to the leaf extract, the stem was a more potent inhibitor of AGE formation, which could be a result of the unique phytochemistry of leaf . This observed effect might be attributed by the presence of bioactive compounds in the plant extract like flavonoids, alkaloids, phenols, tannins, trepnoids and sterols (Sun, *et al.,* 2011).The level of breaking the crosslink of proteins obtained mostly negative results showed in table 2, which directly reflects that suspended extracts are already formed AGEs in collagen protein and can incubated several days. The presence of free sugar, glycosides, flavinoids attach in pre-accumulated AGEs and increase the level of glycation or increase the rate of polymerization of pre-accumulated AGEs.Extracts from methanol, were also given results but at very low percentage. The level of glycation and protein modification pattern (dimer, trimer ,oligomer and polymer formation) and understanding the glycation inhibition and cross linking breaker assaying . What level of protein transform after the treatment with drugs that means which level of protein recover in inhibitory action and crosslink breaking. The non glycated protein separation and glycated protein separation in electrophoresis were compared the elactrogram in fig-3 showed the clear difference between native protein and glycated protein. SDS-PAGE elactrogram clearly evidenced that the glycated protein lost its character in glycated form like its mobility in respect of its charge and mass ratio and lost the resolution . The mP-AGE have been employed for characterization of maximum and minimum inhibition elastin adducts and was compared to the mobility of positive (glycated, elastin) and negative control (native, elastin). The mP-PAGE results partially indicate the inhibition pattern of collagen protein but it is not shown the crosslinking pattern of pre-accumulation dimer, trimer , oligomer or polymer reverting to monomer or dimer level . The results indicated that mP-AGE is an ideal technique to identify and separate early and late glycation product especially the proteins containing glucose adducts (McPherson, *et al.,*1988). The mP-AGE technique is an easy-to-use and cost-effective method that can complement available A maximum band shift in the mPAGE gels is achieved with the linear gluconoyl adducts followed by glucose induced AGE. After comparative analysis of m-P-AGE gel cut and centrifuged to remove the dye and acrylamide , and filter to 0.22µm filter. Four pooled glycation inhibition and crosslink breaker sample were prepared by mixing (20 µL) .These pools were diluted with HPLC grade water and, diluted (pools 1–3 times, pool) samples are presented. The absorption peaks for protein and AGE-protein with absorbance at 280 nm appeared in signal spectra of hydrolyzed calibrators at 2.18 min. retention time and its peak width is 0.97(Wi) (Fig-15), confirming that this peak corresponded to native proteins. Moreover, significant differences can be observed (plate 5) between relative elastin glycation inhibition signals in roots extracts treated pools, which were in agreement with expected inhibition to protein-protein interaction with glucose, The root extracts collagen and elastin showed increase level of inhibition activity compared to leaf extracts treated sample. The Peak characters directly correlate to mP-AGE results. It seems possible that the spectra observed the less inhibitory activity showed by high peak width by high degree of glycation or inhibit only trimer or dimer level protein crosslinking. Indeed, the lowest relative minimum peak width in root extracts was observed in collagen pooled from I_3(1mg^{-ml})

concentration, whose pool contained protein in dimer or monomer were accumulated because of impaired glycation level. In Crosslink breaking of elastin glycated protein from roots extracts maximum breaking activity was shown in the sample selected pooled(fig-4). The progressive width of glycated towards the position of minimum on the crosslink breaker -derived AGE spectra can be observed with the expected increase of AGE content in glycated pool with respect to that of negative control. These results confirmed that after dispensing the roots methnolic and ethanolic extracts a certain unknown phytoconstituents many break the protein crosslinking , the protein aggregates get removed and polymerized protein remodel into trimer dimer or monomer . In the present work, UV detector was connected on-line in a flow system to measure simultaneous signals corresponding to AGEs and to native protein in the sample. In fig:-5 the example of measurement record for native elastin samples injected in triplicate was presented. The signal found in elastin inhibition from collagen protein was more sharp and peak width narrow 0.135(Wi) compared to 0.218(Wi) glycated peak. All peptide mapping observed HPLC signal mean peak width (Wi) was summarized in table -3. It was evidenced that the coupled to molecular techniques m-PAGE had given full proof estimation to protein glycation as well as protein specificity. Furthermore, in such experimental conditions, AGE content in four pools prepared from *in-vitro* glycation evidenced clear differentian of different groups of extracts. In the previous reports, The mean value of the peak width relative absorbance found statistically significant..Molecular sieve HPLC confirmed the inhibition of formation of larger.

The present study concluded that Withania roots both extracts have ability to inhibit Maillard products that ultimately lead to AGEs production. It was also cleared that I_3 ($1mg^{-ml}$) concentration of extracts was more active towards inhibition of elastin glycation. However, crosslink breaking activity of elastin in all three concentration of Withania root extract was very least able to produce effect. So it is suggested that more study required of Withania root extract should be complete anti-glycating activity in case of diabetes mellitus and persistent hyperglycemia which is major cause of loose the elasticity of connective tissue specially stiff to elastin protein .

Table.1 Constituents and concentration of in-vitro glycation reaction

S.No.	Components	Concentration
1	B --- BUFFER(PBS)	0.01mM
2	G ---GLUCOSE	250mM
4	P_1 ---PROTEIN (Elastin)	20mg/ml
5	I_1 --- WSE	0.1 mg^{-ml}
6	I_2 --- WSE	0.2 mg^{-ml}
7	I_3 --- WSE	1 mg^{-ml}

WSE= withania extract concentration

Table.2 Comparison of the effect of extracts of *W. somnifera* roots on inhibition and crosslink breaking of elastin glycation with reference to different concentrations

S.No.	Plant Extracts	Extracts Concentration (mg_ml)	Level of %inhibiting of glycation (Mean±SEM)			Level of %Breaking of crosslink glycation of protein (Mean±SEM)		
			NBT tests			NBT tests		
			1st week	3rd week	5th week	1st week	3rd week	5th week
1	Ethanol	I_1	10.85±0.881	2.36±2.666	33.55±1.33	**-179.3±0.47**	-86.04±0.33	-208.34±1.0
		I_2	45.19±1.856	35.09±1.99	31.72±0.33	**-100.1±0.08**	-56.5±0.619	-156.2±0.92
		I_3	85.42±2.666	84.38±0.95	77.58±1.66	**-58.3±1.175**	-28.9±0.074	-4.16±6.822
2	Methano	I_1	-4.76±3.631	14.84±0.33	27.73±1.32	**-256.6±2.35**	-110.9±3.33	-156.2±0.51
		I_2	51.50±1,500	22.91±0.33	49.41±2.13	**00.00±0.00**	-42.7±0.128	-66.6±5.221
		I_3	67.09±4.333	81.59±0.66	72.58±0.36	**08.14±0.387**	15.52±0.968	12.50±0.666

Table.3 Protein mapping results for AGE in four groups of sample pool

S.N.	sample	Peak width(mean±SD)(wi)
1	Negavtive control(Elastin)	0.134±0.031805*
2	Glycated (collagen)	0.22875±0.034288*
3	Maximum collagen inhibition(root) pool	0.117333±0.024214*
4	Maximum collagen inhibition(leaf) pool	0.129±0.05299*
5	Maximum collagen crosslink (root) pool	0.112333±0.017858*
6	Maximum elastin crosslink(root) pool	0.125667±0.068398*

Fig.1.Elactrogram of isolated elastin protein after polishing step with size exclusion chromatography

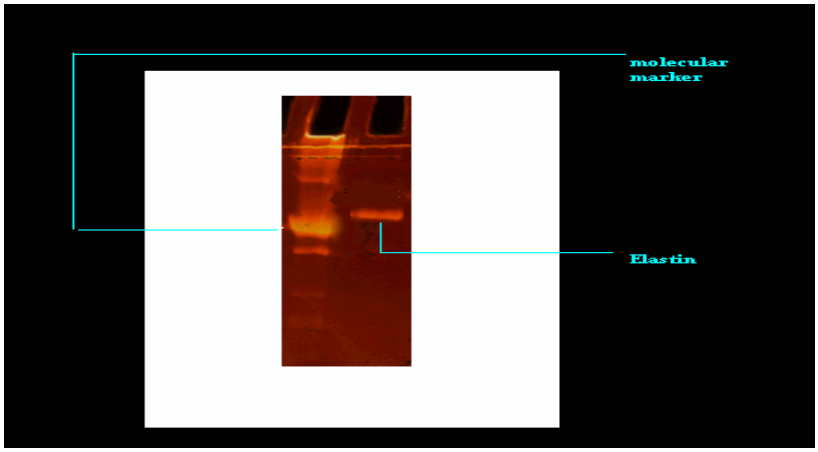


Fig.2 Glycation level in different incubation period

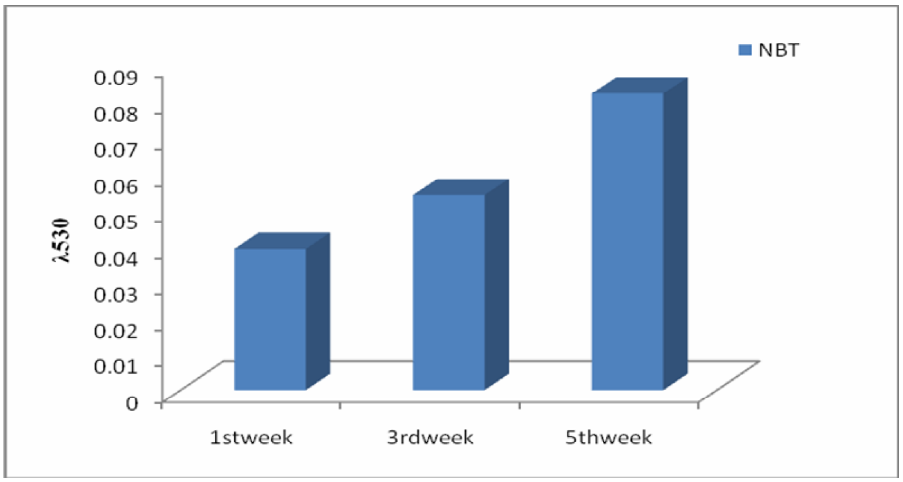


Fig.3 m-PAGE –elactrogram

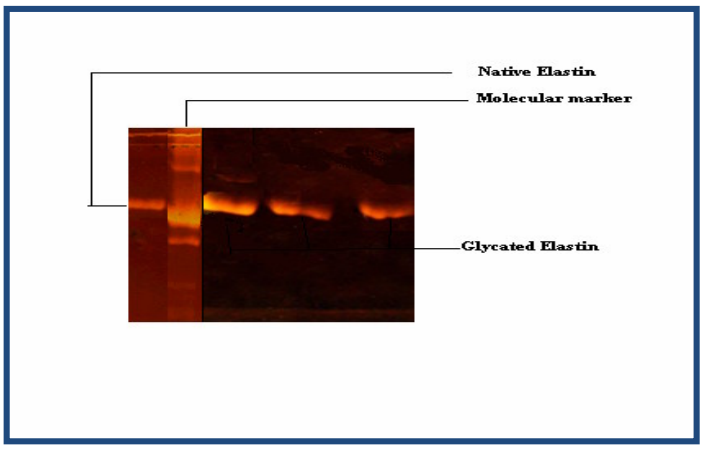


Fig.4 m-PAGE elactrogram profiling to roots extracts in elastin protein reformation

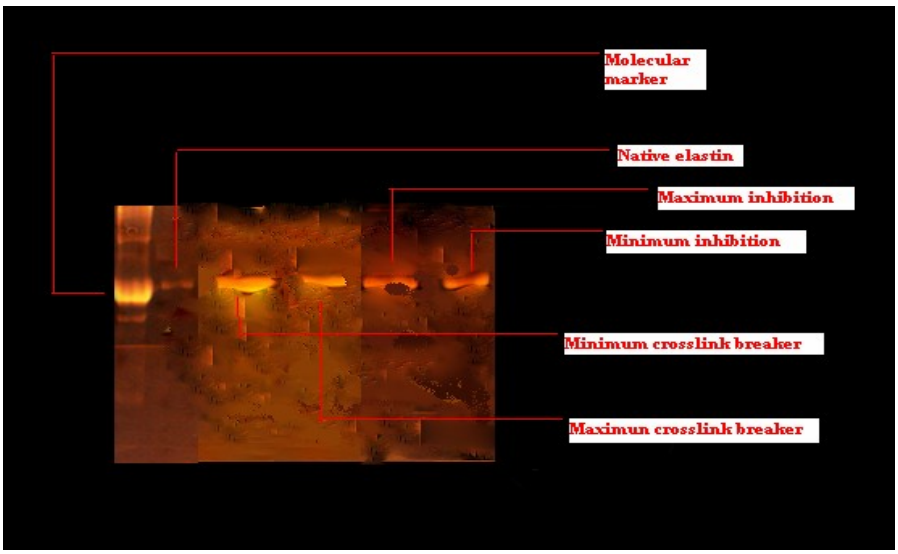
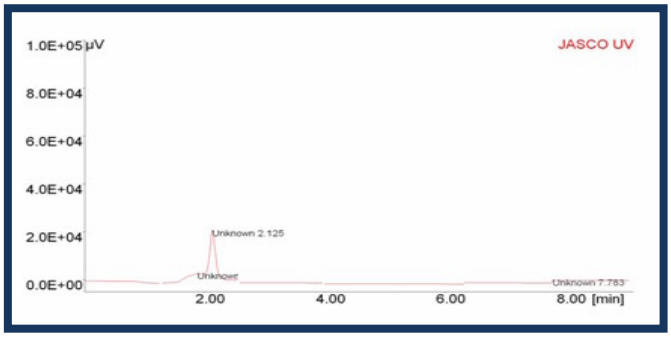


Fig.5 Calibration peak for Elastin (protein)



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