Octa Journal of Biosciences
International peer-reviewed journal
Octa. J. Biosci. Vol. 2(1):13-17

ISSN 2321 – 3663
June 2014

Octa Journal of Biosciences

journal homepage: www.sciencebeingjournal.com

Evaluate the Effect of *Withania Somnifera* Methanolic Extracts as *In-Vitro* Anti-Glycating Agents

Nadeem Khan[1*], Ragini Gothalwal[1] .N.M. Shrivastava[2] and Savita Shrivastava[3]
1. Department of Biotechnology, Barkatullah University, Bhopal (M.P.) 462026
2. Department of Pharmacology, Gandhi Medical College, Bhopal (M.P.) 462026
3. Department of Chemistry, MLB, Girl (PG) College, Bhopal (M.P.) 462026

ARTICLE INFO

Received 24 Feb.2014
Revised 16 Mar. 2014
Accepted 10 May 2014
Available online 16 Jun. 2014

Keywords:
non-enzymatic glycation , inhibition , nitroblue tetrazolium reduction assay , diabetic complication

Email:nadeem_khan620@yahoo.com

ABSTRACT

Various diabetes complications are due to protein glycation and the formation of advanced glycation end products When AGEs accumulations particularly high in E.C.M., proteins are result in intra and inter molecular cross-linking and later has been hypotized to stiffening of these proteins and believed to play an important role in etiology of various AGEs related diseases. The anti-glycation activity of W. somnifera phytoconstituents which target the essential stages of glycation through-(i) inhibition (ii) reduction of AGEs cross- linking. The inhibitory and Crosslink breaker activity of root and leaf extracts of W. somnifera showed the best results obtained by colorimetric methods selected for molecular level. The peak width of native protein collagen/elastin (0.114, 0.102Wi) was compared with glycated (0.215, 0.198 Wi) at inflation points and significant difference were observed. The peak width was the indication of shape of the protein and clearly distinguishes the results of inhibition as well as crosslink breaking activity, which is helpful in prophylaxis of aging, overall diabetes management strategy and also reduced the risk of AGEs related disease (retinopathy, neuropathy, nephropathy).

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder that is alarmingly increasing with more than 381 million people affected worldwide as recorded by International Diabetes Federation in 2013 and its incidence is increasing rapidly (William, 2013). Diabetes in urban Indians is reaching an epidemic and the prevalence of type- 2 diabetes mellitus in Indians ranges from 2.7% in rural India to 14% in urban India. 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, which include an increased risk of heart disease (Peters, 1996), kidney disease (Stewart, et al.,2001), blindness (Schnolzer, et al.,2005), and Alzheimer (Wa, et al.,2006). Many of these complications are due to protein glycation and the formation of advanced glycation end products (Armbuster, 1987).

E.C.M. (Extra cellular matrix), proteins such as collagen, elastin, actin, and myosin are the backbone for architectural and functional stability of tissues cell and organs. When AGEs accumulations particularly high in E.C.M., proteins are result in intra and inter molecular cross-linking and later has been hypotized to stiffening of these proteins and believed to play an important role 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 Charak 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).. The goal of this research were to investigate the effect of Withania somnifera (Solanaceae) in non enzymatic glycation inhibition and crosslink breaker reaction for isolated E.C.M. proteins.

MATERIALS & METHODS

Preparation of Ashwagandha root & leaves (*Withania somnifera*) extracts:

Dried and powered root rhizomes and levees (100g) was extracted by Soxhelt method with 600mL of absolute methanol at 37°C for 48 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 of E.C.M. proteins from chickens:

Extra cellular matrix proteins collagen was isolated by Acid-soluble method (Sato *et al* 1987), and Elastin isolated by Starcher method (Work, 1969). The isolated proteins were characterized and quantified by Laemmli SDS-PAGE method (Laemmle 1970), and Bradford method (Sheikh *et al* 2004)

***In vitro* glycation of E.C.M. proteins 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 5th week. After over of the incubation time precipitated the glycated E.C.M. proteins and incubated with different concentration of extracts at 37°C for 1 week. The samples kept at 4°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.

High performance liquid chromatography (HPLC)

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

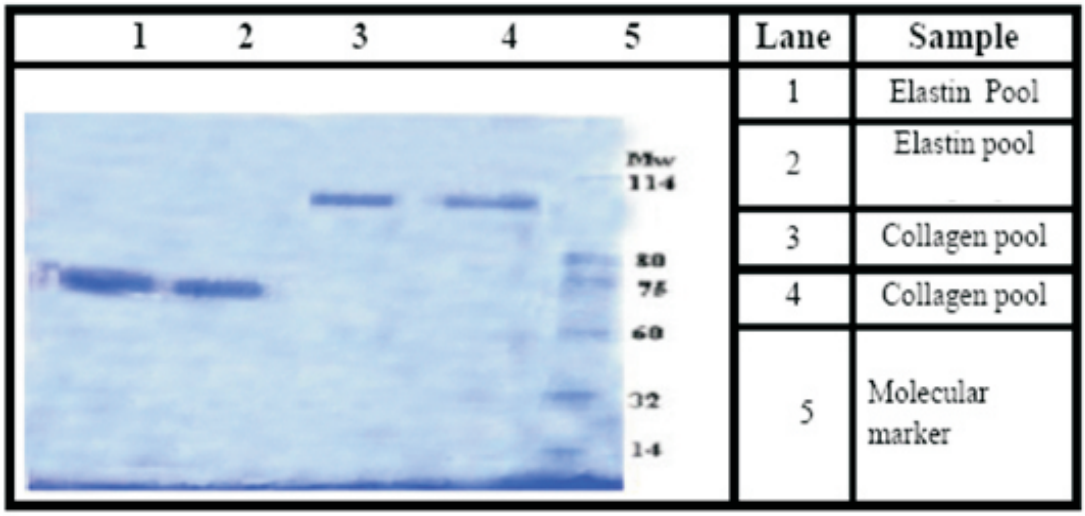
All experiments were performed in triplicate (n=3) and results were expressed as mean ± SEM. Statistical analysis was carried out with (SPSS package version 10.0). HPLC signal peak width determined by mean±SD and compare mean significancy by (ANOVA). A $P<0.05$ has considered statistically significant.

RESULTS & DISCUSSION

Withania root rhizomes and leaves were dried in hot air oven at 37°C. After drying, ground to powder form and kept in absolute methanol for extraction. Rotary evaporator was used to recover the sample. The isolated protein was characterized by SDS-PAGE gel profile (fig:1). R_f value was determined and indicated purified protein collagen was a chain of ≈ 94 kDa and elastin ≈ 70 kDa. Glucose concentration 250 mM was cleared that a hyperglycaemic condition as a normal glucose level. Isolated E.C.M. protein was used as protein for glycation and its concentration was 20mg^{-ml}. Withania root and leaves extracts (0.1mg^{-ml} , 0.2 mg^{-ml} , 1mg^{-ml}) in phosphate buffer were prepared. Each extract along with glucose and E.C.M. proteins were incubated at 37°C for five weeks to monitored glycation and Millard reaction 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. This gave the apparent inhibitory and crosslink breaker anti-glycating activity.

Maillard reaction inhibitory and crosslink breaker activity by Withania root and leaves (*Withania somnifera*) extract:

The results obtained after the measurement of the glycation level with NBT reduction assay in in-vitro glycation inhibition by root extracts of W. somnifera have presented in table 1. The % inhibition was maximum (82.73,85.42 %) by I_3 concentration (collagen/elastin) after 1st week of incubation and minimum (29.62,2.36%) inhibition by I_1 concentration (collagen/ elastin) after 3rd week in



Lane	Sample
1	Elastin Pool
2	Elastin pool
3	Collagen pool
4	Collagen pool
5	Molecular marker

Fig:-1: SDS-PAGE profile of purified collagen and elastin

Table 1 Comparison of the effect of methanol extract of *W. somnifera* roots and leaves on inhibition of collagen glycation with reference to different concentrations

S.No.	Extracts	Extracts Concentration (mg_ml)	Level of %inhibiting of glycation (Mean±SEM)					
			Collagen			Elastin		
			1st week	3rd week	5th week	1st week	3rd week	5th week
1	Roots	I_1	38.30±4.96	29.62±8.33	45.42±2.13	10.85±0.881	2.36±2.666	33.55±1.33
		I_2	64.62±1.98	54.51±2.66	65.08±2.08	45.19±1.856	35.09±1.99	31.72±0.33
		I_3	82.73±0.65	74.25±1.66	76.36±0.56	85.42±2.666	84.38±0.95	77.58±1.66
2	Leaves	I_1	24.97±0.54	42.11±0.77	56.9±0.33	22.09±0.564	33.31±2.68	8.87±0.298
		I_2	56.64±1.20	27.44±0.23	45.67±0.67	54.09±0.666	47.72±0.56	42.68±0.33
		I_3	83.61±0.99	83.85±1.34	85.91±0.88	82.93±0.307	81.92±0.33	77.04±0.58

tests. The effect of *W. somnifera* leaves extract with different concentration of extracts were tested , it was found that level of glycation inhibition was increased 85.91% in 5th week at I_3 (1 mg^{-ml}) collagen protein and elastin maximum %inhibition(82.93) in 1st week at same concentration . In most of the experiments glycation level was lowered in 1st week of incubation and it was seen that the 3rd week inhibition level was low in I_1 (collagen & elastin). Table:-1 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 ($1mg^{-ml}$) and I_2 ($0.2mg^{-ml}$) give better response. while with I_1 ($0.1\ mg^{-ml}$) minimum response . 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 . In line with our results Babu, et al.,(2004) also studied the in-vitro inhibitory effect of W. somnifera against the formation of non-enzymatic glycation . 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 measurement of NBT reduction assay in in-vitro crosslink breaking activity roots and leaves extracts presented in table 2. The breaking activity was maximum with elastin I_3 (7.71%) and minimum with I_3 (collagen) 4.16% of incubation sample.

In leaf extracts elastin (6.25%) give maximum and collagen (1.80%) give minimum of breaking activity in I_3 concentration. Mostly value obtained in crosslink breaking study was negative. Which directly reflects that suspended extracts are already formed AGEs in collagen/elastin and can incubated several days. The presence of free sugar, glycosides, flavinoids, alkaloids, phenols, tannins,

Table 2: Comparison of the effect of methanol extracts of W. somnifera root and leaves on breaking crosslink of collagen glycation reaction in reference to different concentrations

S.N0.	Plant Extracts	Extracts Concentration (mg ml)	Level of %Breaking of crosslink glycation of protein (Mean±SEM)	
			Collagen	Elastin
1	Roots	I_1	-208.34±1.0	-175.0±0.04
		I_2	-156.2±0.92	-59.3±0.017
		I_3	4.16±6.822	7.71±0.102
2	leaves	I_1	-136.6±10.2	-62.50±0.77
		I_2	-45.2±1.667	-12.5±1.26
		I_3	1.80±0.82	6.25±6.280

trepnoids and sterols (Sun, et al., 2011).The measurement of NBT reduction assay in in-vitro crosslink breaking activity roots and leaves extracts presented in table 2. The breaking activity was maximum with elastin I_3 (7.71%) and minimum with I_3 (collagen) 4.16% of incubation sample. In leaf extracts elastin (6.25%) give maximum and collagen (1.80%) give minimum of breaking activity in I_3 concentration. Mostly value obtained in crosslink breaking study was negative. Which directly reflects that suspended extracts are already formed AGEs in collagen/elastin 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 .This type of modification of proteins already reported by formation of free radicals It can be assumed that the positive anti-glycation activity of these compounds may be at least partly attributed to their scavenging ability.

On the other hand, flavonoids baicalin and baicalein exerted overall negative influence on the catalytic activity of AST alone and in the combination with fructose (Boušová, et al., 2012) . This anti-AGE agent chemically breaks α-dicarbonyl compounds by cleaving the carbon-carbon bond between the carbonyls. Alagebrium chloride (ALT-711), a highly potent cross-link breaker with higher stability, has been discovered. This compound successfully completed preclinical studies and Phase II clinical study on healthy volunteers (Yamagishi , 2013). This finding will help in the prediction of phytochemical of root and leaf extract of *w. somnifera*. has potential to inhibit as well as crosslink breaker activity .

Glycated protein (collagen/elastin) profiling by HPLC:

Four pooled glycation inhibition and crosslink breaker sample were prepared by mixing (20 µL) . The results obtained by these methods are explained below. 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-2a), confirming that this peak corresponded to native proteins. Moreover, significant differences can be observed (fig:-2a.2b) between relative signals in four pools in glycated and native protein. The signal found in collagen and elastin inhibition from collagen protein were more sharp and peak width narrow (0.112 Wi) as compared to glycated peak. Summarized the crosslink breaker sample peak and that collagen was given good results compared to elastin or glycated and native collagen. Protein aggregation is a common issue encountered during non-enzymatic glycation /AGEs formation. Even small amounts of aggregates can alter the proteins basic structure and function. Inter- and intra molecular cross-links with protein to protein were formed as a result of

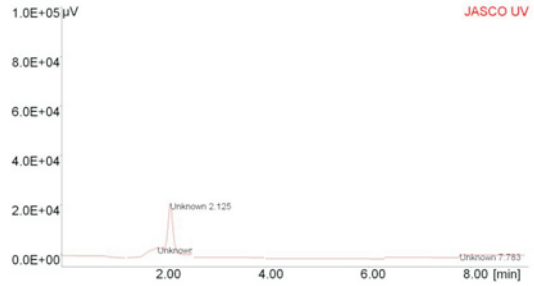


Fig.(a): Calibration peak for BSA (protein)

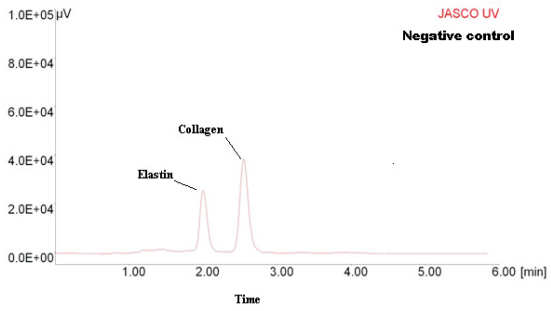


Fig.(b): Signal peak to native proteins(collagen/elastin)

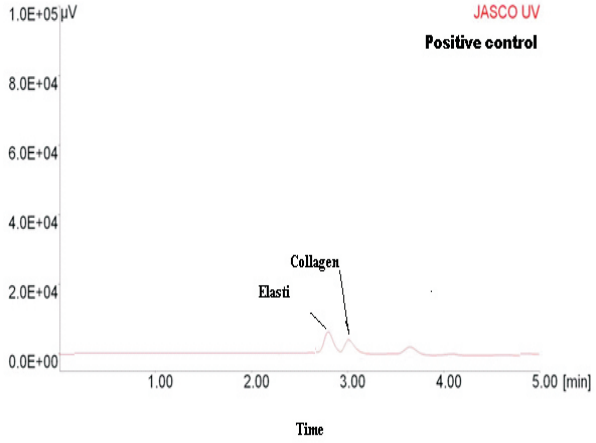


Fig.(C): Signal peak to glycated proteins (collagen/elastin

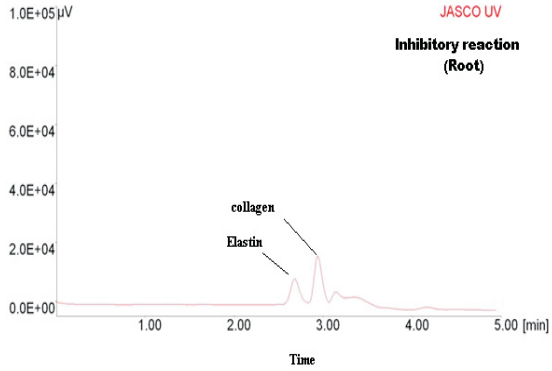


Fig.(d): Inhibitroy effeats of root extracts on glycation inhibiting signal peak of collagen and elastin

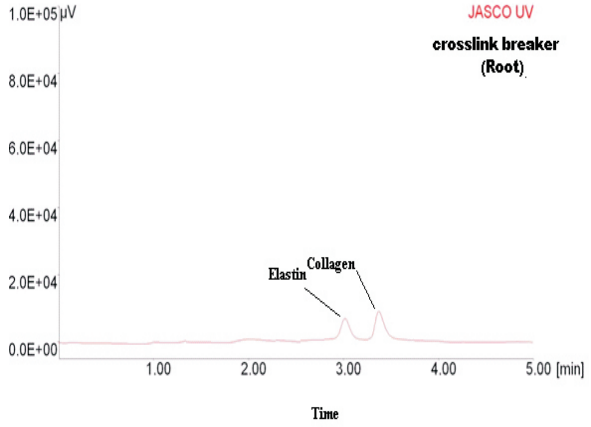


Fig.(e): Inhibitroy effeats of leaf extracts on

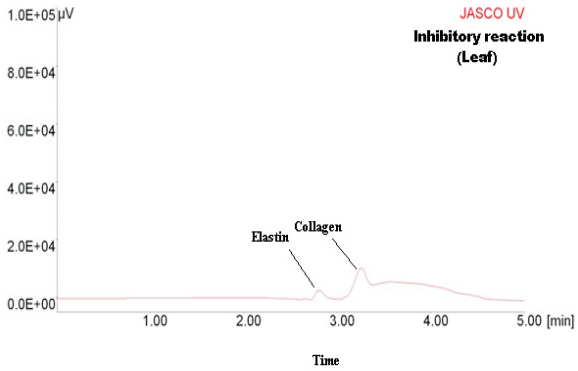


Fig.(f): Signal peak for determination of crosslink breaker

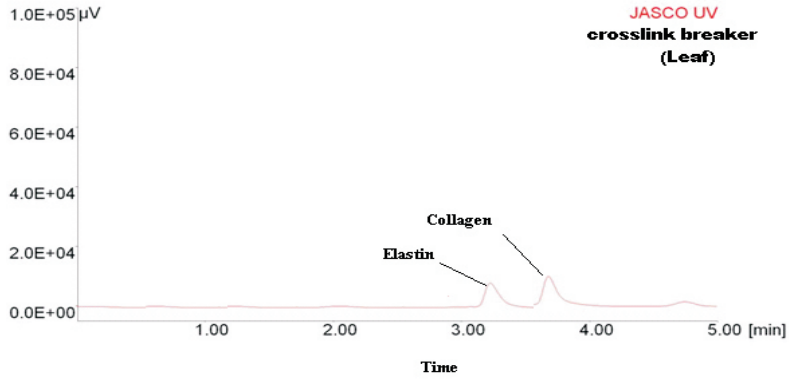


Fig.(g): SIgnal peak for determination of crosslink breaker activity of collagen and elastin by leaf extracts

Table-3: Analytical results for AGE in four groups of sample pool.

S.No.	Sample	Peak width (mean±SD) (wi)
1	Negavtive control(collagen)	0.114±0.03*
2	Negative control(elastin)	0.102±0.002*
3	Glycated (collagen)	0.215±0.03*
4	Glycated (elastin)	0.198±0.004*
5	Maximum collagen inhibition(root) pool	0.111±0.024*
6	Maximum elastin inhibition (root)pool	0.128±0.068*
7	Maximum collagen inhibition(leaf) pool	0.112±0.052*
8	Maximum elastin inhibition(leaf) pool	0.133±0.103*
9	Maximum collagen crosslink (root) pool	0.103±0.017*
10	Maximum elastin crosslink(root) pool	0.128±0.068*
11	Maximum collagen crosslink(leaf) pool	0.118±0.105*
12	Maximum elastin crosslink(leaf) pool	0.123±0.052*

glycation which are responsible for resistance to its functions. 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. (fig:-2b,c,d,e).In Crosslink breaking of collagen and elastin glycated protein from root and leaf extracts maximum breaking activity was shown in the sample selected pooled and presented with (fig:-2f,g). These results confirmed that after dispensing the root and leaf extracts a certain unknown phytoconstituents many break the protein crosslinking , the protein aggregates get removed and polymerized protein remodel into trimer dimer connected on-line in a flow system to measure simultaneous signals corresponding to AGEs and to native protein in the sample. In table-3 the example of measurement record for 12 samples injected in triplicate was presented. The mean value of the peak width relative absorbance found statistically significant.. Caengprasath, *et al.,*(2013) characterized the active components in the pomelo (*Citrus grandis* L) extract by using (HPLC), whose findings showed that signal peak characteristics changes glycated and un-glycated proteins .

CONCLUSION

These studies were explored that in natural crude phytochemicals found in *W. somnifera* has the powerful potential of inhibiting non-enzymatic glycation /AGEs formation not only in antioxidant phytochemicals but also some other phytochemical have a potential to inhibit the glycation or AGEs molecule. This gave the apparent inhibitory and crosslink breaker anti-glycating activity. Particularly for those who are at risk of developing diabetic complications. However, additional research in animal models and other advance techniques are required to clarify anti-glycation effects.

REFERENCES

1. Armbuster, D. (1987) A Fructosaminase: Structure, analysis and clinical usefulness.*Clin. Chem. , 33*; 2153-2163.
2. Aronson, D. (2003) Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *Journal of Hypertension*, *21*, Pg. 3-12
3. Babu, P.V.; Dhandayuthbani, G.A.; Dowlath, R.A.; Kumar,C.V.; Iqbal M.D. and Ahamed. N. (1994) Protective effect of *Withania somnifera* (Solanaceae) on collagen glycation and cross-linking.: Comparative biochemistry and physiology. Part B. Biochemistry & molecular biology. ISSN 1096-4959, *Elsevier, Amsterdam, PAYS-BAS*.127-138.
4. Boušová, I.; Srbov, L. and Dršata, J.,(2012) Non-Enzymatic Glycation of Aminotransferases and the Possibilities of Its Modulation. *InTech,* ISBN 978-953;581-585.
5. Caengprasath,N.;Ngamukote,S.;Mäkynen,K and Adisakwattana,S.(2013) The proteactive effects of pomelo extract (*Citrus grandis)* against fructose mediated protein oxidation and glycation. *EXCLI Journal, 12; 491-502.*
6. Gupta, R.K.; Kesari, A.N.; Murthy, P.S.; Chandra, R.; Tandon, V. and Watal, G.(2005) Hypoglycemic and anti-diabetic effect of ethanolic extract of leaves of *Annona squamosa* L. in experimental animals. *J. Ethnopharmacol.*, 99; 75–84.
7. Hollenbach,S.; Thampi,P.; Viswanathan, T. and Edathara C. (2003). Cleavage of *in vitro* and *in vivo* formed lens protein cross-links by a novel cross-link breaker. *Mole. Cellular Bioch.,* 243;73–80.
8. Laemmle, U.K. (1970) Nature (London.) UK, 227, Pg. 845-647.
9. Peters, T.(1996) In *All About Albumin: Biochemistry, Genetics, and MedicalApplications. Academic Press: San Diego*, 12; 102-126.
10. Qamaruddin, L.; Samiulla, V.; Singh, K. and Jamil, S.S. (2012) Phytochemical and Pharmacological Profile of *Withania somnifera* Dunal: *A Review J. of A. Phar. Scie.*,02 ; 170-175.

11. Sangwan, R.S.)(2004)Photochemical variability in commercial herbal products and preparations of *Withania somnifera'*,*Current Science*,23,pg.461.
12. Sato, K.; Yoshinaka, R.; Sato M., and Shimizu Y. (1987). Isolation of native acid soluble collegen from fish muscle. Nippon suisan Gakkaishi, 53, Pg. 1431-1436
13. Schnolzer, M.; Jedrzejewski, P. and Lehmann, W.D.(2005)Protease-catalyzedincorporation of 18O intopeptide fragments and its application for protein225sequencing by electrospray and matrix-assisted laser desorption/ionization massspectrometry. *Electrophoresis. , 17;* 945-953.
14. Sheikh, N.; Safari, M.R.; Kashani M.; Araghchian M., and Zeraat F. (2004). Study on the effect of garlic on the in-vitro albumin glycation reaction. *Acta Medica Iranica,* 42, Pg. 16-18.
15. Stewart, I. I.; Thomson, T. and Figeys, D. (2001).$_{18}$O labeling: A tool for proteomics. *Rapid.J., 34 ;12-18.*
16. Vorum, H.; Fisker, K.; Otagiri, M.; Pedersen, A. O. and Hansen, U. K.(1995) Calcium ion binding to clinically relevant chemical modifications of human serum albumin. *Clin. Chem. , 41;*1654-1661
17. Wa, C.; Cerny, L. and Hage, D. S. (2006)Identification and quantitative studies of protein immobilization sites by stable isotope labeling and mass spectrometry. *Anal.Chem., 78;* 7967-7977.
18. *Williams textbook of endocrinology* (12th ed.). Philadelphia: Elsevier/Saunders. pp. 1371–1435. ISBN 978-1-4377-0324-5.
19. Work, T.S., and Work E. (1969). Laboratory techniques in biochemistry and molecular biology. Appleton and Lange, Amsterdam, Pg. 423-428.
20. Wrobel, K.;Eugenia M.; Garay ,S., Eugenia, L. and Malacara ,J.M.(1997)Novel analytical approach to monitoring advanced glycosylation end products in human serum withon-line spectrophotometric and spectrofluorometric detection in a flow system *Clinical Chemistry* ,43(91);563–1569.
21. Wu, J.W.; Hsieh, C.L.; Wang, H.Y. and Chen, H.Y. (2009).Inhibitory effect of guava (*Psidium guajava*) leafextracts and its active compounds on the glycation process of protein. *Food Chemistry*, 113; 78-84
22. Yadav, D.P.; Chhipa, R.C. and Singh, B.(2012). Anti-glycation effect of Whitten root(*E, nuda*) *in-vitro* condition. *IJPSR*, 3(9); 3502-3506.
23. Yamagishi, S. H. (2013) Potential Clinical Utility of Advanced Glycation End Product Cross-Link Breakers in Age- and Diabetes-Associated Disorders. *Rejuv. Research*, 15; 364-572