ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

In vitro Antiglycation Activity of Isorhamnetin on Bovine Serum Albumin with different sugars using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sindhuja A[1], Vimalavathini R[2]*, Kuzhandai Velu V[3], Vickneshwaran V[4]

[1,2]Department of Pharmacology, College of Pharmacy, Mother Theresa Post Graduate and Research Institute of Health Sciences, Gorimedu, Puducherry, India - 605006.

[3,4]Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Pillaiyarkuppam, Puducherry, India - 607402

*Corresponding Author E-mail: **vimalavathini@gmail.com**

ABSTRACT:

Advanced glycation end products (AGEs) resulting from glycation of proteins, lipids and nucleic acids has several pathophysiological manifestations by altering the structure and functions of molecular proteins. Isorhamnetin is a flavonoid with anti-inflammatory, anti-oxidant, anti-obesity, anticancer, antidiabetic and anti-atherosclerosis activity. Based on the structure activity relationship and our insilico antiglycation study of isorhamnetin, we hypothised that isorhamnetin may have antiglycation activity by inhibiting protein glycation on sugar molecules due to its antioxidant and free radical scavenging activity. Hence our aim of the study was to determine the glycation level of bovine serum albumin (BSA) with varying sugar concentration of glucose, fructose and ribose on 14th and 21st day of incubation. Our second objective of the study was to determine the antiglycation activity of isorhamnetin on BSA using all the sugars at 14th and 21st day of incubation using SDS - PAGE. Our study showed that increase in concentration of glucose, fructose and ribose (0 – 50mM) showed a dose dependent decrease in migration of protein implying increased glycation of BSA. Isorhamnetin (100µM) exhibited antiglycation activity for fructose (30mM) at 14th day onwards and for glucose (30mM) was at 21st day onwards. But isorhamnetin did not exert antiglycation activity for ribose (30mM) on both 14th and 21st day of incubation. Our study establishes the antiglycation activity of isorhamnetin however further invivo studies are necessary to warrant this activity.

KEYWORDS: AGE, isorhamnetin, SDS – PAGE, glycation, sugars.

INTRODUCTION:

AGEs are formed when the reducing sugars are non-enzymatically glycated and oxidised to proteins, lipids or nucleic acids[1] at three different phases. Initially it leads to the formation of Schiff bases which undergo further oxidation, dehydration, polymerisation or oxidative breakdown forming amadori products at the propagation phase and moves on to the advanced phase by forming non cross linking, fluorescent and non fluorescent AGEs[2]. AGE disturbs numerous molecular signalling cascades associated with inflammation and oxidative stress[3] such as nuclear factor-kB (NF-kB), protein kinase B (Akt/PKB), Janus kinase – signal transducer and activator of transcription (JAK-STAT), mitogen activated protein kinase (MAPK), c- Jun N- terminal kinase (JNK), cAMP response element-binding protein (CREB) and extracellular signal regulated kinase (ERK) pathways[4]. AGE and its derivatives serve as biomarker of various diseases such as diabetes mellitus[5,6], neurodegenerative disorders[7], atherosclerosis[8], cardiovascular diseases[9], ageing and cancer[10,11].

There are several screening methods to detect AGEs to manage various pathological conditions. Recently, an increasing number of immunotechniques as well as bio-analytical techniques have been developed to efficiently measure the levels of AGEs qualitatively as well as quantitatively[12] such as spectrofluorimetry (for measuring amadori products), ELISA, western blotting and HPLC (for detecting anti –AGE antibodies against glycated proteins). In most of the methods, BSA is used

as a targeting protein, aminoguanidine (AG) is used as a standard inhibitor of AGE formation which was studied extensively for its antiglycation activity against AGE associated disease[13] and sugars such as glucose, ribose and fructose are used as glycating agents[14].

Therapeutic intervention of protein glycation plays an important role in the prevention of clinical complications. The currently available clinical options to reduce AGEs accumulation are AGE cross-link breakers, vitamins, tyrosin phosphorylation inhibitor and clinically approved anti-diabetic and anti-hypertensive drugs[15]. However, designing a new drug with anti-AGE activity is a challenge due to the complexity of reactions involved in AGE formation at different stages. But the above mentioned clinical drugs with anti-AGE effect including AG had failed in numerous clinical trials due to their side-effects, low safety and efficacy[14]. Therefore, discovering a new drug from natural origin with antiglycation property with maximum safety and efficacy is very much needed to ameliorate those chronic diseases and it was reported that the secondary metabolite, flavonoid (quercetin)may have antiglycation activity against AGEs and its derivatives due to its anti-oxidant effect[16,17] by targeting signalling pathways[18].

Isorhamnetin is a naturally occurring o- methylated flavonoid present in various parts of the plants such as Ginkgo biloba[19], goldenrod, red turnip, sea buckthorn, true indigo and maindenhair tree[20,21]. It is a versatile molecule with many pharmacological importance such as antimicrobial, anti-oxidant, anticancer, anti-inflammatory, anti-obesity, antidiabetic, anti-atherosclerosis, antituberculosis, anti-osteoporosis, anti-hypoxia, antiviral, anti-hyperuricemia, antivitiligo, cardio-cerebrovascular and nerve protection, lung protection and heptoprotective activity[20,22,23]. However the mode of action of isorhamnetin is poorly established. Structure activity studies of isorhamnetin reveals the presence of hydroxyl group in the B-ring of the isorhamnetin may contribute to antiglycation activity[24]. Insilico docking studies of isorhamnetin done by us on 17 molecular proteins of AGE pathway (yet to be published) suggests antigylcation activity by modulating these proteins. Based on the structure activity and insilico docking studies, we proposed that isorhamnetin may exhibit anticancer, anti-obesity, antidiabetic, anti-atherosclerosis activity by modulating AGE pathway[25]. Thus the objectives of the present study was to determine the level of glycation of BSA with different sugars in varying sugar concentration at 14th and 21st day of incubation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and to detect the antiglycation potential of isorhamnetin on sugar protein crosslinks due to its antioxidant activity[19] using SDS-PAGE.

MATERIAL:

D-glucose, D-fructose, D-ribose and Aminoguanidine were procured from Avra (Hyderabad, India). BSA was purchased from SRL (Maharashtra, India) and isorhamnetin was from TCI (Tokyo, Japan). Na_2HPO_4, NaH_2PO_4, NaCl, KCl, Sodium azide.

METHODS:

The samples were prepared by mixing BSA (1mg/mL) with varying concentrations of three different sugars such as glucose, fructose and ribose (10-50mM) in phosphate buffer (PB) at pH 7.4 containing 0.02% sodium azide as a preservative to prevent microbial growth during incubation[6]. The protein sugar mixtures were incubated at 37^{0}C for 21 days in the sealed glass tubes.

The effect of different sugars on protein glycation was studied by using AG (10µM) was used as a positive control. Corresponding blank was prepared in the absence of sugars containing BSA (1 mg/mL) in PB (pH 7.4). Isorhamnetin (100µM) was incubated with 30mM of D-glucose, D-fructose and D-ribose separately in BSA buffer (pH 7.4) solution containing 0.02% of Sodium azide. The test solutions were incubated at 37^{0}C for 21 days in the closed glass tubes. Aliquots were collected at the end of 14th and 21th day and stored at -20 ^{0}C until further analysis by SDS-PAGE to identify the inhibitory potential of isorhamnetin against protein glycation of all the three sugars. SDS polyacrylamide gels (12%) were prepared according to the standard Laemmli procedure[14]. The collected aliquot mixtures were heated with the SDS buffer at 95°C for 3 min, before loading the samples to the SDS gel. The separation of protein crosslinks was done at pH 8.6 to obtain protein bands which were visualized by staining with Coomassie brilliant blue. At the end, the changes in the migration position and the intensity of the BSA bands for all the three different sugars were compared to conclude the results for its antigycation activity against AGEs.

RESULT:

Our study showed that an increase in concentration of glucose, fructose and ribose (Figure 1) had decreased the migration of BSA. Also the BSA bands exhibited broader migration with increasing concentration of all three sugars on 21st day of incubation. To add on the colour intensity of glucose in later stages was high and this finding was consistent with the previous study[14]. Thus under non denaturing conditions, migration of BSA band (SDS-PAGE) towards the anode showed detectable variation but was not significant. Decrease in BSA migration seemed to be proportionate to the degree of protein glycation. The rate of BSA migration at 14th day of incubation was slower than that of 21st day of migration for all three sugars and did not show

detectable changes in migration.

In presence of isorhamnetin (100µM), the transit of BSA along with fructose increased on both 14th and 21st day of incubation (Figure 2) and this was comparable to that of AG. The migration of BSA with glucose was increased in presence of isorhamnetin only on 21st day of incubation and was similar to that of AG. Isorhamnetin (100µM) did not improve the migration of BSA with ribose. Thus isorhamnetin (100µM) exhibited invitro antiglycation activity at the concentration of 30mM for fructose and glucose on 14th and 21st day of incubation respectively and this inhibition was on par with AG.

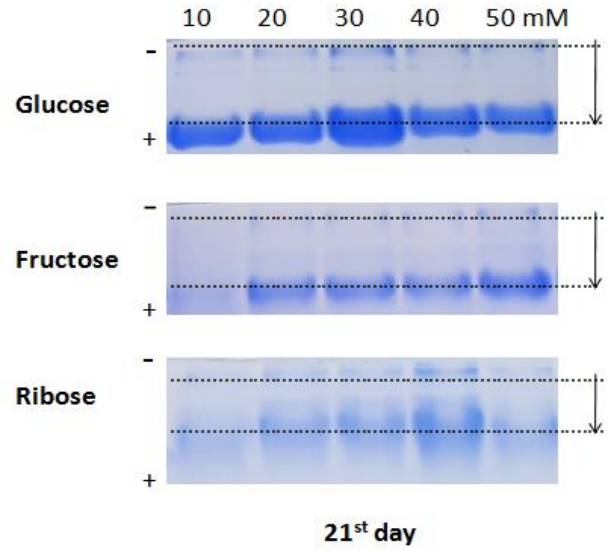

Figure 1: Effect of glucose, fructose and ribose in different concentrations (0 – 50 mM) on BSA migration on 21st day of incubation using SDS - PAGE.

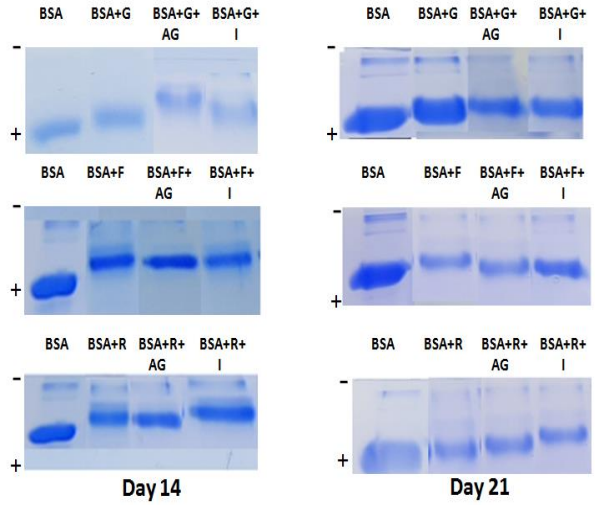

Figure 2: Effect of isorhamnetin on BSA migration on 14th and 21st day of incubation of BSA with all three sugars using SDS-PAGE (G – glucose, F – fructose, R- ribose, BSA – Bovine serum albumin, AG- aminoguanidine, I - isorhamnetin).

DISCUSSION:

Glycation[26,27] is the key molecular mechanism for various ailments and hence targeting this will pave way for new therapeutic strategies for these ailments[14]. In our study, an increase in concentration of glucose, fructose and ribose (0 – 50mM) decreased the migration of BSA (Figure 1) implying glycation of these proteins. This effect was more profound on the 21st day of incubation but not on 14th day of incubation. This is consistent to the previous studies suggesting that the decrease in migration observed was due to the increase in molecular weight and net negative charge of BSA due to glycation[28].

Isorhamnetin (100µM) exhibited invitro antiglycation activity at the concentration of 30mM for fructose on 14th and 21st day and for glucose on 21st day of incubation. The antiglycation activity of isorhamnetin was on par to the antiglycation activity of AG (10µM) (Figure 2) when compared with glucose and fructose. AG exhibits antiglycation activity by inhibiting reactive carbonyl groups in glycation products[29].However isorhamnetin did not exhibit antiglycation activity of BSA in presence of ribose (30mM) on both 14th and 21st day of incubation. Fructose forms glycation products that are highly reactive and has different structure and reactivity when compared to the glycation products of glucose. Though tissue level of fructose does not rise, it can have a detrimental effect in tissues that operate by the polyol pathway such as ocular lens, peripheral nerves and erythrocytes[6,9,30]. Thus isorhamnetin (100 µM) is found to be efficient in arresting glycation by fructose on the 14th day itself suggesting its efficacy as an antiglycation agent. But isorhamnetin (100µM) exhibited antiglycation activity for glucose on 21st day of incubation and further studies on dose increment of isorhamnetin are necessary to get clear cut knowledge on this perspective. Isorhamnetin (100µM) did not shown antiglycation activity for ribose on 14th or 21st day of incubation hence further studies are required to cast light on this aspect of the study.

Based on our insilico study of isorhamnetin on molecular proteins of AGE pathway[31,32,33,34] (yet to be published), we found that isorhamnetin exhibited antiglycation activity by modulating RAGE, PKB/Akt2, ATF4, CREB, ERK and STAT molecular proteins in this pathway. Isorhamnetin (3, 5, 7-Trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) is a quercetin derivative[21] and consists of three carbon aromatic rings and many phenolic hydroxyl groups. Presence of ortho hydroxyl structure in B ring and a C2-C3 double bond in conjugation with a 4-oxo function[22] in the C ring (benzopyran-4-one) are mandatory for free radical scavenging[35]. The ortho-dihydroxy structures in the B ring provide stability for the flavonoid. Phenoxyl

radicals contribute hydrogen atom release[36], electron delocalization and hydrogen bonding ability. The C2-C3 structure in conjugation with a 4-oxo function in the C ring provides co-planarity of the hetero ring and increases radical stability by electron delocalization over all three-ring system. The hydroxyl groups at positions 3 and 5 in the A and C ring respectively, contributes to free radical scavenging activity by providing hydrogen bonding to the oxo-group[37]. Presence of hydroxyl groups on B-ring of isorhamnetin may be one of the key factors for its antiglycation activity[24]. Thus our study showed that all three sugars on increasing concentration exerted a decreased migration of protein depending on its dose due to increased glycation of BSA. Isorhamnetin exhibited antiglycation activity for fructose on 14th day itself whereas for glucose it was on 21st day. Isorhamnetin did not inhibit glycation for ribose on 14th or 21st day.

CONCLUSION:

In our study, we concluded that glucose, fructose and ribose on increasing concentration exhibited a dose dependent decrease in migration of protein implying increased glycation of BSA. Isorhamnetin exhibited AGE inhibition in presence of fructose and glucose but not for ribose.

CONFLICT OF INTEREST:

There is no conflict of interest.

ACKNOWLEDGEMENT:

There is no acknowledgement.

REFERENCES:

1. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products, sparking the development of diabetic vascular injury. Circulation. 2006 Aug 8; 114(6): 597-605. doi: 10.1161/CIRCULATIONAHA.106.621854.
2. Gkogkolou P and Bohm M. Advanced glycation end products, Key players in skin aging?. Dermato-Endocrinology. 2012 Jul 1; 4(3): 259-70. doi: 10.4161/derm.22028
3. Prasad K, Mishra M. AGE–RAGE Stress, Stressors, and Antistressors in Health and Disease. International Journal of Angiology. 2018 Mar; 27(1):1-12. doi: 10.1055/s-0037-1613678.
4. Athanasios P, Goumenos A, Adamopoulos C, Papavassiliou AG. AGE/RAGE signalling regulation by miRNAs: Associations with diabetic complications and therapeutic. The International Journal of Biochemistry & Cell Biology. 2015 Mar; 60: 197-201. doi: 10.1016/j.biocel.2015.01.009.
5. Miller AG, Zhu T, Berka JLW. The rennin angiotensin system and advanced glycation end-products in diabetic retinopathy: impacts and synergies. Current Clinical Pharmacology. 2013 Nov; 8(4): 285-96. doi: 10.2174/1574884711308040004.
6. Gaviraj EN, Ramarao A, Veeresham C, Shivakumar B, Kalyane NV, Biradar SM. Inhibitory activities of some Folklore remedies on Aldose reductase of rat lens and generation of advanced glycation end products. Research Journal of Pharmacy and Technology. 2019; 12(4):1947-1952. doi: 10.5958/0974-360X.2019.00326.3.
7. Sasaki N, Fukatsu R, Tsuzuki K, Hayashi Y, Yoshida T, Fujii N, Koike T, Wakayama I, Yanagihara R, Garruto R, Amano N, Makita Z. Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. The American Journal of Pathology. 1998 Oct; 153(4):1149-55. doi: 10.1016/S0002-9440(10)65659-3
8. Lindsey JL, Cipollone F, Abdullah SM, McGuire DK. Receptor for advanced glycation end-products (RAGE) and soluble RAGE (sRAGE): cardiovascular implications. Diabetes and Vascular Disease Research. 2009 Jan; 6(1): 7-14. doi: 10.3132/dvdr.2009.002.
9. Hegab Z, Gibbons S, Neyses L, Mamas MA. Role of advanced glycation end products in cardiovascular disease. World Journal of Cardiology. 2012 Apr 26; 4(4): 90-102. doi: 10.4330/wjc.v4.i4.90.
10. Schroter D, Hohn A. Role of Advanced Glycation End Products in Carcinogenesis and their Therapeutic Implications. Current Pharmaceutical Design. 2018; 24(44): 5245-5251. doi: 10.2174/1381612825666190130145549.
11. Vani M, Latha KP, Malyada AA, Harathi P, Anusha M, Beulah J, Soujaya R, Bindu PN. Pharmacognostical, Phytochemical and In Vitro antiglycation studies on the leaf extracts of Hibiscus micranthus Linn. Research Journal of Pharmacy and Technology. 2017; 10(3): 663-673. doi: 10.5958/0974-360X.2017.00124.X.
12. Ashraf JM, Ahmad S, Choi I, Ahmad N, Farhan M, Tatyana G, Shahab U. Recent Advances in Detection of AGEs: Immunochemical, Bioanalytical and Biochemical Approaches. International Union of Biochemistry and Molecular Biology, 2015 Dec; 67(12): 897-913. doi: 10.1002/iub.1450.
13. Perera HKI, Handuwalage CS. Analysis of glycation induced protein cross-linking inhibitory effects of some antidiabetic plants and spices. . BMC Complementary medicine and therapies. 2015 Jun 9; 15: 175. doi: 10.1186/s12906-015-0689-1.
14. Perera K, Wijetunge D. A novel in vitro method to detect inhibitors of protein glycation. Asian Journal of Medical Sciences, 2014 Feb; 5(3): 15-21. doi:10.3126/ajms.v5i3.8670
15. Jud P, Sourij H .Therapeutic options to reduce advanced glycation end products in patients with diabetes mellitus: A review. Diabetes Reserach and Clinical Practice. 2019 Feb; 148: 54-63. doi: 10.1016/j.diabres.2018.11.016.
16. Chakraborthy P, Kumar S, Dutta D, Gupta V. Role of antioxidants in Common Health Disease. Research Journal of Pharmacy and Technology. 2009 April- June; 2(2); 238-244.
17. Thennarasu A. Quercetin in Health and Disease. Research Journal of Pharmacy and Technology. 6(12): Dec. 2013; Page 1397-1399.
18. Hanaa H. Ahmed, Hadeer A. Aglan, Ghada H. Elsayed, Hebatallah G. Hafez, Emad F. Eskander. Quercetin Offers Chemopreventive Potential against Breast Cancer by Targeting a Network of Signalling Pathways. Research Journal of Pharmacy and Technology. 2021; 14(5). doi: 10.52711/0974-360X.2021.00499.
19. Kale MK, Patil MP, Bhusari KP. Evaluation of Ginkgo biloba in Diabetic Nephrotoxicity. Research Journal of Pharmacognosy and Phytochemistry 2011; 3(6): 286-288.
20. Gong G, Guan Y, Zhang Z, Raman K, Wang S, Zhou S, Luan X and Zhang H. Isorhamnetin: A review of Pharmacological effects. Biomedicine and pharmacotherapy. 2020 Aug; 128: 110301. doi: 10.1016/j.biopha.2020.110301
21. Kandakumar S, Manju V. Pharmacological applications of isorhamnetin: A short review. International Journal of Trend in Scientific Research and Development. 2017 may- jun; 1(4); 2456-6470.
22. Sahu P, Sahu K, Dubey RD, Chatterjee S, Chatterjee T. Herbal Antioxidants: A Review. Research Journal of Pharmacognosy and Phytochemistry. 2010; 2(6): 427-434.
23. Prabakaran K, Sathiyaseelan M. Evaluation of Anti-inflammatory activites of Isorhamnetin 3-O-α-L- (6"-E-p-coumaroyl)-rhamnoside isolated from Indigofera tinctoria. Research Journal of Pharmacy and Technology. 2020; 13(5): 2187-2191. doi: 10.5958/0974-360X.2020.00393.5.
24. Ronsisvalle S, Panarello F, Longhitano G, Siciliano EA, Montenegro L, Panico A. Natural Flavones and Flavonols: Relationships among Antioxidant Activity, Glycation, and Metalloproteinase Inhibition. Cosmetics .2020, 7(3), 71.

doi:10.3390/cosmetics7030071.
25. Hyun CY. The cytoprotective effect of isorhamnetin against oxidative stress is mediated by the upregulation of the Nrf2-dependent HO-1 expression in C2C12 myoblasts through scavenging reactive oxygen species and ERK inactivation. General Physiology and Biophysics. 2016 Apr; 35(2): 145-54. doi: 10.4149/gpb_2015034.
26. Kim CS, Park S, and Kim J. The role of glycation in the pathogenesis of aging and its prevention through herbal products and physical exercise. Journal of Exercise Nutrition and Biochemistry. .Sep 30 2017; 21(3): 55–61. doi: 10.20463/jenb.2017.0027.
27. Milordini G, Zacco E, Percival M, Puglisi R, Piaz FD, Temussi P and Pastore A. The Role of Glycation on the Aggregation Properties of IAPP. Frontiers in Molecular Biosciences. 03 June 2020; 7: 104. doi: 10.3389/fmolb.2020.00104.
28. Ledesma-Osuna AI, Ramos-Clamont G, Vázquez-Moreno L. Characterization of bovine serum albumin glycated with glucose, galactose and lactose. Acta Biochimica Polonica. 2008 Sep; 55(3):491-7.
29. Rahbar S and Figarola JL. Novel inhibitors of advanced glycation end products. Archives of Biochemistry and Biophysics. 2003 Nov 1; 419(1): 63-79. doi: 10.1016/j.abb.2003.08.009.
30. Amani S, Fatima S. Glycation with fructose: the bitter side of nature's own sweetener. Current Diabetes Reviews. 2020 Nov; 16(9): 962-970. doi: 10.2174/1389450121666200204115751.
31. Nedic O, Rattan SIS, Grune T, Trougakos IP. Molecular effects of advanced glycation end products on cell signalling pathways, ageing and pathophysiology. Free Radical Research. Aug 2013; 47 (1): 28-38. doi: 10.3109/10715762.2013.806798.
32. Ulrich P, Cerami A. Protein glycation, diabetes, and aging. Recent Progress in Hormone Research. 2001; 56: 1–21. doi: 10.1210/rp.56.1.1.
33. Cho SJ, Roman G, Yeboah F, Konishi Y. The road to advanced glycation end products: a mechanistic perspective. Current Medicinal Chemistry. 2007; 14: 1653–1671. doi: 10.2174/092986707780830989.
34. Takeuchi M, Yamagishi S. Involvement of toxic AGEs (TAGE) in the pathogenesis of diabetic vascular complications and Alzheimer's disease. J. Alzheimers Disease. 2009; 16: 845–858. doi: 10.3233/JAD-2009-0974.
35. Nayak Y, Jayashree BS, Unnikrishnan MK. Benzopyran-4-one is an Ideal Pharmacophore for Lead Optimisation in Antidiabetic Drug Discovery. Research Journal of Pharmacy and Technology. Aug 2012; 5(8); 1025-1033.
36. Marref SE, Benkiki N, Melakhessou MK. In vitro Antioxidant Activity, Total Phenolics and Flavonoids Contents of Gladiolus segetum Extracts. Research Journal of Pharmacy and Technology. 2018; 11(11): 5017-5023. doi: 10.5958/0974-360X.2018.00915.0.
37. Tapas A, Sakarkar D, Kakde R. The chemistry and Biology of Bioflavonoids. Research Journal of Pharmacy and Technology. July- Sept 2008; 1(3): 132-143.