

HPLC PROFILING AND ANTIHYPERGLYCEMIC EVALUATION OF PHE (POLYHERBAL EXTRACT) OF SELECTED INDIAN MEDICINAL HERBS IN ALLOXAN DIABETIC RATS

P. Manorama¹, Valmiki Aruna¹, D. Geetha¹ and Gangadhara Angajala^{1, 2, ✉}

¹Department of Chemistry, Kalasalingam Academy of Research and Education, Anand Nagar,
Krishnankoil-626126, Tamilnadu, India.

²Department of Humanities and Sciences, Vallurupalli Nageswara Rao Vignana Jyothi Institute
of Engineering & Technology, Hyderabad, 500090, Telangana, India.

✉ Corresponding Author: gangadharaangajala@gmail.com

ABSTRACT

In the present work antihyperglycemic screening of the polyherbal powder was carried out by oral administration of an aqueous extract of polyherbal powder on alloxan-induced diabetic rats. The polyherbal powder displayed a substantial increase in hypoglycemic efficacy at a dose of 100 mg/kg and 200 mg/kg in both Alloxan-induced diabetic rats and normal rats. The powder also exhibited enhancement in various parameters like the glycogen content of the liver, restoration of pancreatic β -cells, body weight, and carbohydrate metabolizing enzymes. Results obtained showed that the Alloxan diabetic rats on treatment with polyherbal powder at various doses of 100 mg/kg and 200 mg/kg considerably improved their body weights to 222.30 ± 3.42 g and 221.6 ± 3.60 g when compared to non-diabetic rats respectively. In addition to this, the histopathological analysis demonstrated that polyherbal powder enhanced the histological architecture of the islets of Langerhans significantly which is on par when compared to the standard Glibenclamide.

Keywords: Polyherbal Powder, Antihyperglycemic, Medicinal Plants, Histopathology

RASAYAN J. Chem., Vol. 15, No. 4, 2022

INTRODUCTION

The importance of traditional medicine and its consumption is growing intensely throughout the world mainly due to its ability to allow extreme public usage of health information especially when conventional treatment is ineffective in the treatment of new infectious diseases and some advanced cancer.¹⁻³ Currently medicines derived from plants are applied in the treatment of various ailments such as inflammation, depression, and cardiovascular problems. Herbal medicines are also playing a crucial role as starting materials in the synthesis of potent pharmaceutical drugs, especially in drug discovery and development.⁴⁻⁶ Large proportion of natural products in drug discovery shows additional drug likeliness and greater biological compatibility in comparison to synthetic drugs which further explores the possibility of converting them as potential candidates for the drug discovery process.⁷⁻⁹ The separation and identification of phytochemicals is always a unique challenge because the multiple active compounds present in the plant extract can deliver a synergistic effect that may not be possible for any single-component drug.^{10,11} Diabetes mellitus is the largest health issue of the present century and the leading cause of 4 million death per year.¹² This become a very big challenge to control its prevalence level. Over the centuries, various types of research have been ongoing for finding suitable therapy for replenishing the destroyed beta cells in the pancreas and reducing its secondary complications.¹³ Herbal formulations for antidiabetes along with multi-beneficiary effects are a promising economically viable alternative as they eliminate the adverse side effects and low cost.¹⁴⁻¹⁷ Even though numerous drugs are available in the market for the treatment of diabetes, there is a need for effective therapy as most of the drugs possess limitations mainly due to their higher cost and severe side effects associated with them such as an increase in weight, toxicity to the liver, hypoglycemia, heart-related diseases development of gastrointestinal disorders.¹⁸ By considering the role

of oxidative stress and its influence in worsening diabetes mellitus, extensive research is being carried out to explore appropriate antidiabetic and antioxidant therapy.

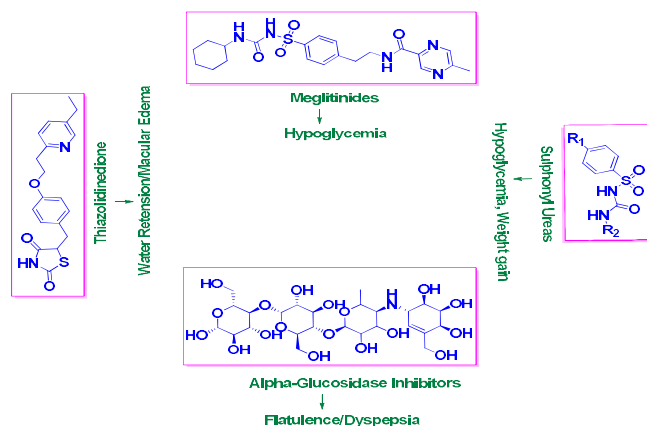


Fig.-1: Various Side Effects of Antihyperglycemic Drugs

The main limitation associated with the incorporation of phytomedicines in present clinical therapies is the absence of proper systematic scientific and clinical data showing their effectiveness and safety.¹⁹ The current research focuses on antihyperglycemic screening of the aqueous extract of polyherbal powder. The rationale of this work is to find out novel herbal formulations to be utilized in the management of diabetes mellitus a major chronic metabolic disease that is causing more deaths throughout the globe.

EXPERIMENTAL

Chemicals

Alloxan monohydrate was obtained from S.D Fine. Chem. Ltd, Mumbai, India. Glibenclamide was obtained from Micro Labs, Hosur, India.

Collection of Plant Materials

Seven folk Indian medicinal plants namely *Eugenia-jambolana* (seeds), *Trigonella-foenum-graecum* (whole plant), *Aegle-marmelos* (leaves), *Cassia-auriculata* (flowers), *Marsilea-quadrifolia* (Whole Plant), *Mangifera-indica* (leaves) and *Musa-paradisiaca* (flower) were collected from Madurai district, Tamilnadu, India. The collected plants were authenticated by Dr. M. Shanthi, HoD, Department of Botany, S.F.R College, Sivakasi, Tamilnadu, India.

Preparation of Plant Extract

The shade-dried herbal powder of 100 g of each of all eight samples was taken and subjected to methanol extraction using the Soxhlet apparatus. After the completion, the extract was filtered off and further distilled in a vacuum under pressure. It was dried and stored in the refrigerator at 4 °C until required for the determination of antihyperglycemic activity Fig.-2. The Phytochemical screening and spectroscopic investigation of the polyherbal extract have been reported in our previous work.²⁰

HPLC Analysis of PHE

HPLC is a versatile, reproducible chromatographic technique for the identification of secondary metabolites in plants. 500 mg of the methanolic extract was treated with 10 mL of the reagent acetonitrile and water in a ratio of 30:70. The methanolic extract of the PHE sample was subjected to HPLC analysis in HPLC SHIMADZU, LC-10 ATVP. 0.02 mL of the diluted sample was injected into the system under optimized chromatographic conditions. A chromatogram was recorded and studied for various compounds present in the polyherbal extract.

Selection of Animals

Male Wistar rats weighing 180-200 g were selected and used for this study. The animals were housed under standard husbandry conditions maintaining a temperature of (22 ± 5 °C) and relative humidity (55 ± 5 %) with 12 h each of day and night cycles. Rats were fed with standard pellets and water ad libitum. The animal

experiments were approved by the Institutional animal ethical committee. IAEC Approval No. assigned is AKCP/IAEC/003/16-17.

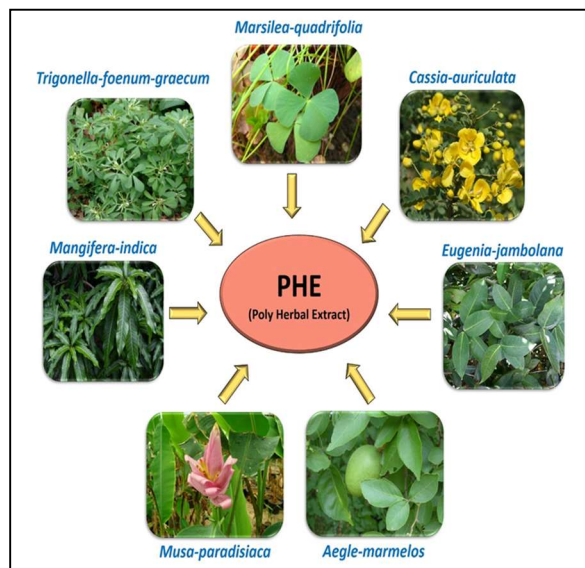


Fig.-2: Preparation of PHE (polyherbal extract) from Selected Medicinal Herbs

Acute Oral Toxicity Studies of Poly Herbal Powder

The acute oral toxicity studies of an aqueous solution of Poly Herbal Powder were carried out as per the OECD guidelines. Oral stepwise doses of an aqueous solution of polyherbal powder ranging from 5 mg/Kg, 50 mg/Kg, 300 mg/Kg, 600 mg/Kg, and 1000 mg/Kg of body weight were administered through the oral route and daily observed for 24 hours. Nonsignificant signs of toxicity or mortality in the tested rats were noted. One-tenth of the upper limit dose was selected to evaluate the experimental design on antidiabetic activity. Hence the experimental dosage was fixed as 100 mg/Kg and 200 mg/Kg of body weight.

Induction of Diabetes Mellitus

In male Wistar rats, diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of Alloxan monohydrate (150 mg/kg Body Weight) in 0.1 M citrate buffer (pH 5.5) to the overnight fasted diabetic rats whereas the normal rats were injected with citrate buffer as the vehicle.²¹ After 3 to 4 days of Alloxan monohydrate injection blood glucose levels were estimated and subsequently rats having blood glucose levels between 200-260 mg/dl were selected for further study.

Experimental Procedure

In the current research, antidiabetic activity was monitored in 25 rats (20 diabetic & 5 normal rats). The rats were divided into groups as follows

Group-I: Treated with 1 % Tween 80 in water orally after intraperitoneal injection of citrate buffer (0.1 M) - Normal control

Group II: Treated with 1 % Tween 80 in water orally after intraperitoneal administration of Alloxan monohydrate (150 mg/kg body wt) - Diabetic control

Group-III: Diabetic rat administered orally with standard Glibenclamide (10 mg/Kg) through intragastric tube after 48 h of Alloxan monohydrate injection for a period of 28 days – Positive control

Group-IV: Diabetic rat administered with 2 ml containing (100 mg/Kg) PHP daily through an intra-gastric tube for a period of 28 days – Treatment group [low dose]

Group-V: Diabetic rat administered with 2 ml containing (200 mg/Kg) PHP daily through an intra-gastric tube for a period of 28 days – Treatment group [high dose].

Biochemical Analysis

Estimation of Blood Glucose

Blood glucose levels were measured by using single-touch glucometers (easy glucose monitoring kit - One Touch Ultra by Johnson & Johnson).²²

Hexokinase and Glucokinase Activity

The excised liver parts taken for analysis were perfused with ice-cold perfusion solution [0.15 M KCl, 2mM EDTA solution]. The liver tissues were further homogenized using 0.1 ml Tris-HCl buffer (50 mM, pH 7.4) and the homogenates were subjected to centrifugation for 30 min at 4 °C. By using glucose-6-phosphate dependent spectrophotometric analysis the glucose phosphorylation was investigated.

Glucose-6-phosphatase Activity

The excised liver homogenates were incubated in the MOPS-KCl buffer at a temperature of 37 °C in the presence of 10 mM glucose-6-phosphate for 10 min. The reaction was terminated by heat denaturation and further centrifuged for 20 min at 4 °C. The phosphate content of the supernatants was analyzed and glucose-6-phosphatase activity was determined as per the reported method.²⁴

Glycogen Content

Liver glycogen content was determined as per the method described by morales *et al.*, (1950). The method involves the digestion of liver tissue in hot 30 % KOH followed by precipitation of the glycogen with ethanol. The glycogen content is treated with anthrone reagent and calorimetrically estimated for glucose content.²⁵

Histopathological Examination

The pancreas of male Wistar rats treated with PHP and control were isolated for histopathological analysis. The isolated pancreas was stored in 10 % formalin after washing it in phosphate buffer saline. The corresponding pancreatic tissue was stained in eosin and haematoxylin to evaluate pancreatic β cells under a light microscope.²⁷

Statistical Analysis

The results are expressed as mean $\pm$ S.D. The data obtained in various biochemical experiments were investigated using a one-way analysis of variance (ANOVA). A difference in the mean P value < 0.001 was considered significant.

RESULTS AND DISCUSSION

HPLC Analysis

The purpose of this HPLC profiling method is to quickly confirm and identify the compounds present in PHE. Using Acetonitrile and water as a more discriminating solvent system, appropriate temperature and gradient elution allows about seven peaks well separated being resolved. Each peak is actually a cluster of phenolic acids and flavanoids that makes interpretation based on retention time easy. Results were presented in Fig.-3-4 and the corresponding compounds resolved were recorded in Table-1. The absorption peak with RT (Retention time) of 1.840 was identified as protocatechuic acid. The peak with RT of 2.10 was referred to as Gallic acid. The compound epicatechin (-) showed the characteristic absorption peak with RT of 2.477. The peak with RT 2.677 attributed to the presence of para-anisic acid. The peak with RT value 2.870 assigned the presence of catechin. The peak with RT value 3.330 was reported as quercetin. The absorption peak with RT 4.600 located the presence of the compound glycollic acid. The compounds proto-catechuic acid, gallic acid, epicatechin (-) and catechin located are polyphenols. Quercetin and para-anisic acid identified are flavanoids.

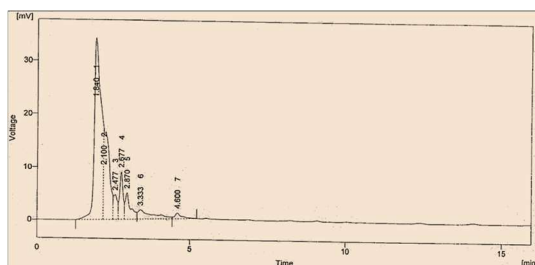


Fig.-3: HPLC Chromatogram of PHE

Thus PHE, a rich source of polyphenols and flavanoids are significant antioxidants, capable of reducing oxidative stress, eliminate the free radicals mediated toxicity effectively, increases the metabolic rate,

speeds up fat oxidation, inhibit carbohydrate hydrolyzing enzymes, enhance insulin activity and prevents hyperglycemia.

Table-1: HPLC Analysis of PHE

Peak No	RT	Compound Name
1	1.840	Proto Catechuic acid
2	2.100	Gallic acid
3	2.477	Epi Catechin(-)
4	2.677	Para Anisic acid
5	2.870	Catechin
6	3.333	Quercetin
7	4.600	Glycolic acid

^a Retention time

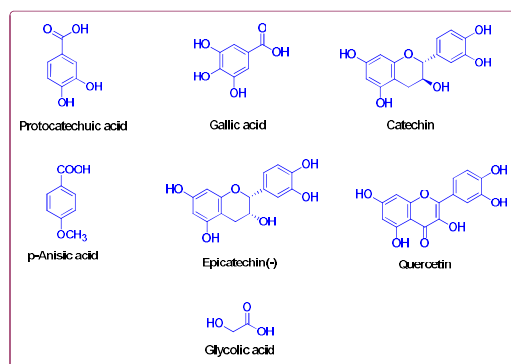


Fig.-4: Bioactive Compounds Identified from the Methanolic Extract of PHP

Effect of PHP on Body Weight

The results obtained from effect of PHP on body weight analysis on normal and diabetic rats clearly depicts considerable decrease in mean body weight of diabetic rats (178.42 ± 2.40) when compared to normal rats (211.40 ± 3.15). Treatment with poly herbal powder at a dose of 100 mg/kg and 200 mg/kg to the diabetic rats significantly improved the body weights (222.30 ± 3.42 and 221.6 ± 3.60 g) which is higher when compared to diabetic rats which are not treated with PHP [Group-II] and also in par with the positive control [Group-III]. (Table-2).

Table-2: PHP Effect on Body Weight of Normal and Diabetic Rats in Each Group

Groups	Initial Body Weight (g)	Final Body Weight (g)
Group I	204.12 ± 4.15	211.40 ± 3.15
Group II	181.75 ± 4.75	178.42 ± 2.40
Group III	202.12 ± 4.75	224.30 ± 4.55
Group IV	219.60 ± 5.52	222.30 ± 3.42
Group V	209.42 ± 4.16	221.60 ± 3.60

Effect of PHP on Plasma Glucose Levels

Treating diabetic rats with poly herbal powder significantly reduces blood glucose levels. In diabetic control [Group-II] at the end of 2nd and 3rd week of treatment there was a marked increments in the blood glucose levels (175.05 ± 3.30 and 215.6 ± 4.45) as compared to normal rats. Subsequently treatment with PHP at a dose of 100 mg/kg and 200 mg/kg reveals a marked decrease in elevated blood glucose levels (115.35 ± 4.92 and 101.55 ± 3.40) [Group-IV] (88.56 ± 4.46 and 86.30 ± 3.20) [Group-V] with $p < 0.001$ is considered as significant. The effect of PHP was found to be maximum at the end of 28th day and also on par to that of standard Glibenclamide (74.62 ± 4.70) respectively (Table-3).

Table-3: Effect of PHP on Blood Glucose Levels of Normal and Diabetic Rats

Groups	Initial	14 th Day	28 th Day
Group I	72.60 ± 3.72	77.80 ± 3.2	70.12 ± 3.33
Group II	154.60 ± 4.60	175.05 ± 3.30	215.60 ± 4.45
Group III	87.90 ± 4.20	84.35 ± 3.08	74.62 ± 4.70
Group IV	125.28 ± 4.05	115.35 ± 4.92	101.55 ± 3.40
Group V	123.60 ± 4.08	88.56 ± 4.46	86.30 ± 3.20

Effect of PHP on Glycogen Content

Table-4 depicts the values of liver glycogen content in experimental rat groups. At the end of 4th week when compared to normal control [Group-I] a decrease in glycogen content (8.25 ± 0.52 mg/g) was noticed in diabetic control group [Group-II]. On the other side treatment of diabetic rats with PHP at two doses (100 mg/kg and 200 mg/kg) demonstrated an elevated glycogen content respectively which was on par to that of standard Glibenclamide. This elevated levels of glycogen was mainly attributed due to increase in peripheral uptake of glucose by the stimulated insulin released from pancreatic β -cells. The phytoconstituents present in the PHP plays a major role in mimicking the insulin action thereby promoting the entry of glucose into the tissues.

Table-4: Effect of PHP on Glycogen Content (mg/g tissue)

Groups	Glycogen Content (mg/g tissue)
Group I	40.30 ± 2.36
Group II	08.25 ± 0.52
Group III	30.50 ± 1.60
Group IV	22.40 ± 1.12
Group V	24.85 ± 1.30

To control blood glucose homeostasis hepatic enzymes plays a crucial role in the management of Type-2 diabetes. They predominantly phosphorylate glucose to glucose-6-phosphate thereby blocking glucose inside the cell. So an elevated levels of hepatic enzymes is very essential in inducing antihyperglycemic activity. In the present work when compared to diabetic control [Group-II] there was a substantial increase in mean level of enzymes ($p < 0.001$) Glucokinase, Hexokinase and substrate Glucose-6-phosphate in experimental groups [Group-III, IV & V] when treated with PHP and standard Glibenclamide (Table-5).

Table-5: Effect of PHP on Enzymes Involved in Carbohydrate Metabolism in Rats

Groups	Hexokinase ($\mu\text{g}/\text{mg}$)	Glucose-6-Phosphate ($\mu\text{g}/\text{mg}$)	Glucokinase ($\mu\text{g}/\text{mg}$)
Group I	0.278 ± 0.01	0.392 ± 0.01	23.90 ± 0.15
Group II	0.080 ± 0.04	0.118 ± 0.07	04.80 ± 0.18
Group III	0.176 ± 0.02	0.281 ± 0.01	16.20 ± 0.92
Group IV	0.130 ± 0.05	0.232 ± 0.07	12.15 ± 0.50
Group V	0.131 ± 0.06	0.242 ± 0.08	16.16 ± 0.90

Histopathological Studies

Degradation of the pancreatic β -cells due to the production of free radicals is a crucial feature involved in the Alloxan induced diabetes. The PHP helps to neutralize the free radicals generated in the pancreas and thus decreases the toxicity of Alloxan and its associated secondary complications.²⁸⁻³⁴

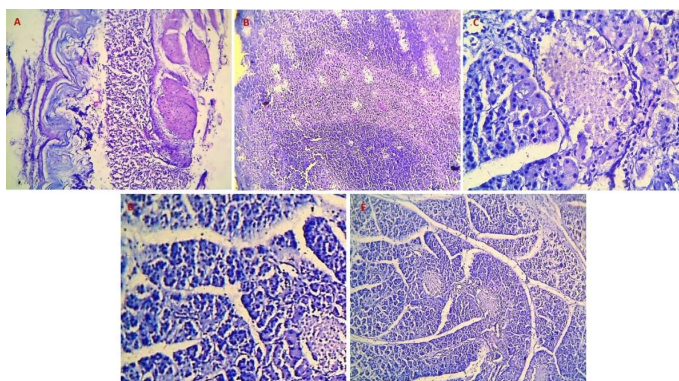


Fig.-5: Histopathological Studies of PHP [A] Normal [B] Diabetic [C] Glibenclamide (Std) treated [D] Low dose PHP treated [E] High Dose PHP Treated

Histopathological studies of male wistar rats from various treated groups clearly reveal that the PHP possesses the ability to increase the pancreatic β -cells in diabetic rats when compared to non-diabetic rats. Figure-5A represents the normal pancreatic cells in standard quantities. The regular acinar cells are oriented

in lobules with prominent nuclei. Figure-5B depicts the reduced number of acinar cells around the islets which are mostly surrounded by eosinophilic material and few atrophic cells. Figure-5C illustrates regeneration of acinar cells around the islets with small inflammatory cell infiltration. Besides there is no accumulation of eosinophilic material. Figure-5D and 5E represents renewal of acinar cells around the islets of Langerhans similar to standard Glibenclamide signifying the existence of stable cells in the islets with the capacity of restoring. The important phytochemicals of PHP [flavonoids and phenolic compounds] may have potentially inhibited further damage to the remaining β -cells in the islets of Langerhans by wiping up the circulating reactive oxygen species induced by the Alloxan intended to destroy the β -cells and thereby permitting remaining phytochemicals of the plant to perform regenerative activities.

CONCLUSION

In conclusion the results obtained from experimental studies provide pharmacological insights into seven folk Indian medicinal plants possessing antihyperglycemic activity. The research outcomes encourage the utilization of traditional herbal medicinal practices for the management of diabetes mellitus. The antihyperglycemic activity of PHP is mainly attributed to the synergistic efficacy of the various phytoconstituents present in the prepared polyherbal formulation. In the future additional toxicological and pharmacological investigations need to be considered to further characterize and prove the safety of bioactive constituents acquired from these medicinal plants.

ACKNOWLEDGEMENT

The authors sincerely thank Arulmigu Kalasalingam College of Pharmacy for their extended support and for providing facilities for completing this research work.

REFERENCES

1. T. Hartmann, *Phytochemistry*, **68(22-24)**, 2831(2007), <https://doi.org/10.1016/j.phytochem.2007.09.017>
2. J.W.H. Li and J.C. Vederas, *Science*, **325 (5937)**, 161(2009), <https://doi.org/10.1126/science.1168243>
3. G. Angajala and R. Subashini, *Biocatalysis & Agricultural Biotechnology*, **16**, 586(2018), <https://doi.org/10.1016/j.bcab.2018.10.005>
4. H. Jenke-Kodama, R. Muller and E. Dittmann, *Progress in Drug Research*, **65(119)**, 121(2008), https://doi.org/10.1007/978-3-7643-8117-2_3
5. World Health Organization (WHO), National Policy on Traditional Medicine and Regulation of Herbal Medicines. Geneva: 2005. Report of WHO global survey.
6. J. Engebretson, *Complementary Therapies in Nursing & Midwifery*, **8(4)**, 177(2002), <https://doi.org/10.1054/ctnm.2002.0638>
7. G.M. Rishton, *American Journal of Cardiology*, **101(10A)**, 43D(2008), <https://doi.org/10.1016/j.amjcard.2008.02.007>
8. L. Conboy, T.J. Kaptchuk, D.M. Eisenberg, B. Gottlieb and D. Acevedo-Garcia, *Complementary Therapies in Clinical Practice*, **13(3)**, 146 (2007). <https://doi.org/10.1016/j.ctcp.2006.12.003>
9. B. Schmidt, D.M. Ribnicky, A. Poulev, S. Logendra, W.T. Cefalu and I. Raskin, *Metabolism*, **57(7)**, S3(2008), <https://doi.org/10.1016/j.metabol.2008.03.001>
10. G. Angajala, R. Ramya and R. Subashini, *Acta Tropica*, **135**, 19(2014), <https://doi.org/10.1016/j.actatropica.2014.03.012>
11. E. Martins, *Frontiers in Pharmacology*, **4**, 177(2014), <https://doi.org/10.3389/fphar.2013.00177>
12. A. Ramachandran, C. Snehalatha, A.S. Shetty and A. Nanditha 2012. *World Journal of Diabetes*, **3(6)**, 110 (2012), <https://doi.org/10.4239/wjd.v3.i6.110>
13. J.K. Grover, S. Yadav and V. Vats, *Journal of Ethnopharmacology*, **81(1)**, 81(2002), [https://doi.org/10.1016/s0378-8741\(02\)00059-4](https://doi.org/10.1016/s0378-8741(02)00059-4)
14. M. Modak, P. Dixit, J. Londhe, S. Ghaskadbi, T. Paul and A. Devasagayam, *Journal of Clinical Biochemistry and Nutrition*, **40 (3)**, 163(2007), <https://doi.org/10.3164/jcbrn.40.163>
15. D. Glugliano, A. Ceriello and G. Paolisso. *Diabetes Care*, **19(3)**, 257(1996), <https://doi.org/10.2337/diacare.19.3.257>

16. J.W. Baynes, S.R. Thorpe, *Current Opinion in Endocrinology & Diabetes*, **3(4)**, 277(1997), <https://doi.org/10.1385/CBB:43:2:289>
17. B. Lipinski, *Journal of Diabetes and its Complications*, **15(4)**, 203(2001), [https://doi.org/10.1016/s1056-8727\(01\)00143-x](https://doi.org/10.1016/s1056-8727(01)00143-x)
18. P. Scartezzini and E. Sproni, *Journal of Ethnopharmacology*, **71(1-2)**, 23(2000), [https://doi.org/10.1016/s0378-8741\(00\)00213-0](https://doi.org/10.1016/s0378-8741(00)00213-0)
19. L.W. Oberlay, *Free Radical Biology and Medicine*, **5(2)**, 113(1988), [https://doi.org/10.1016/0891-5849\(88\)90036-6](https://doi.org/10.1016/0891-5849(88)90036-6)
20. P. Manorama, G. Angajala and G. Das, *International Journal of Innovative Technology and Exploring Engineering*, **9(2S2)**, 2278(2019), <https://doi.org/10.35940/ijitee.B1173.1292S219>
21. L. Al-Shamaony, S.M. Al-Khazraji and H.A. Twaiji, *Journal of Ethnopharmacology*, **43(3)**, 167(1994), [https://doi.org/10.1016/0378-8741\(94\)90038-8](https://doi.org/10.1016/0378-8741(94)90038-8)
22. P. Trinder, *Journal of Clinical Pathology*, **22(2)**, 158(1969), <https://doi.org/10.1136/jcp.22.2.158>
23. R.K. Crane and A. Sols, Animal tissue hexokinase. In: Colowick S P. Kaplan (eds) *Methods in Enzymology*, 2nd edition, Academic Press, New York, 277(1955).
24. C.H. Fiske and Y. Subbaraw, *Journal of Biological Chemistry*, **66(2)**, 375(1925), [https://doi.org/10.1016/S0021-9258\(18\)84756-1](https://doi.org/10.1016/S0021-9258(18)84756-1)
25. M.A. Morales, A.J. Jabbagy and H.P. Terenzi, *Neurospora News Letter*, **20**, 24(1975), <https://doi.org/10.4148/1941-4765.1830>
26. L.Q. Tang, W. Wei, L.M. Chen and S. Liu, *Journal of Ethnopharmacology*, **108(1)**, 109(2006), <https://doi.org/10.1016/j.jep.2006.04.019>
27. Q.G. Li, R. Sun and F.Z. Gao, *China journal of Chinese Materia Medica*, **26**, 488 (2001).
28. G. Knatterud, C.R. Klimt, M.E. Levin, M.E. Jacobsen and M.G. Goldner. *Journal of American Medical Association*, **240(1)**, 37(1978), <https://doi.org/10.1001/jama.1978.03290010041020>
29. A. Aloulou, K. Hamden and D. Elloumi, *Complementary Alternative Medicine*, **12(63)**, 1(2012), <https://doi.org/10.1186/1472-6882-12-63>
30. J.R. Colca, N. Kotagel, C.L. Brooks, P.E. Lacy, M. Landt and M.L. McDaniel, *Journal of Biological Chemistry*, **25**, 7260(1983), [https://doi.org/10.1016/S0021-9258\(18\)32168-9](https://doi.org/10.1016/S0021-9258(18)32168-9)
31. H.B. Akpan, A.K. Adefule, F.A. Fakoya and E.A. Caxton-Martins, *Journal of Analytical Sciences*, **1**, 21(2007).
32. J. Welihinda and E.H. Karunanayake, *Journal of Ethnopharmacology*, **17**, 247(1986), [https://doi.org/10.1016/0378-8741\(86\)90112-1](https://doi.org/10.1016/0378-8741(86)90112-1)
33. M.M. Lolitkar and M.R.R. Rao, *Indian Journal of Pharmacy*, **28**, 129(1966), <https://doi.org/10.1007/BF02871000>
34. H. Hikino, M. Kobayashi, Y. Suzuki and C. Konno, *Journal of Ethnopharmacology*, **25**, 295(1989), [https://doi.org/10.1016/0378-8741\(89\)90035-4](https://doi.org/10.1016/0378-8741(89)90035-4)

[RJC-6498/2021]