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Therapeutic role of *Rauwolfia serpentina* in minimizing the risk of glycosylation and associated biomarkers in experimentally induced type 1 diabetic mice

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Abstract: Present work investigates the effects of hydro-methanolic roots extract (HyMREt) of *Rauwolfia serpentina* in type 1 diabetic mice. Mice were divided into normal, diabetic, negative and positive controls (I-IV) and three test (HyMREt doses) groups (V-VII - 50, 100, &150mg/kg). Allocated treatment of each group was given orally for 14 days in overnight fasted state. Percent change in fasting blood glucose (FBG), body weights, body tissue weights, hepatic glycogen, total lipids, glycosylated hemoglobin (HbA_{1c}), complete blood profile and antioxidant enzymes including catalase (CAT) and superoxide dismutase (SOD) were estimated. HyMREt doses produced meaningful (p<0.0001) reduction (-39 to -53%) in FBG. Hemoglobin (Hb) levels were raised, HbA_{1c} were considerably decreased (4.5-3.77%) and glycosylation (HbA_{1c} to Hb) ratio was expressively (p<0.0001) improved in test groups. Dose-wise improvement (p< 0.05) in total glycogen and decrement (p<0.05) in lipids were observed in livers of test groups. HyMREt significantly decreased (p<0.05) percent inhibition of SOD and CAT. HyMREt doses progressively (p<0.05) improved RBC and other hematological parameters while decrement was only noticed in leucocyte counts. Administration of test doses of HyMREt were significantly reduced the glycosylation, oxidative stress and anemia caused by alloxan intoxication in mice.

Keywords: Alloxan, anaemia, catalase, glycosylation, *Rauwolfia serpentina,* superoxide dismutase.

INTRODUCTION

Molecules with single or multiple unpaired electron(s) in their outermost shell are classified as free radicals (Blois, 1958). At cellular level, oxidation associated with abnormal pathway(s) lead to the generation of free (reactive) radicals and these radicals essentially formed by the relocation of electrons (or protons) with other cellular substances (Davies, 2000). The entire set of macromolecules including carbohydrates, lipids, nucleic acids and proteins are potential targets (resultant of intermediary metabolic routes) of reactive oxygen and nitrogen species and this may possibly result in deleterious biochemical consequences (Davies, 2000). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two prevalent and reported classes of cellular free radicals. Entities regarded as reactive oxidants include, hydroxyl (OH•), superoxide (O_2•$^-$), nitric oxide (NO•), nitrogen dioxide (NO_2•), peroxyl group (ROO•) and lipid peroxyl (LOO•) group. Alternatively, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and lipid peroxide (LOOH) are examples of non-reactive oxidant entities (Pham-Huy *et al.,* 2008).

Reactive oxidants within the cell emerges from electron transport chain (mitochondrial pool), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, neutrophilic myeloperoxidase (MPO) and endothelial cell xanthine oxidase (XO) (Seifu *et al.,* 2012). Degenerative changes, thrombus formation, carcinogenesis, diseases of heart and vessels, diabetes and inflammation are linked with the effects of ROS (Ames *et al.,* 1993; Hasani-Ranjbar *et al.,* 2009; Roberts & Sindhu, 2009). Substances including enzymes and organic compounds that are capable of antagonizing disadvantageous effects of oxidant referred to as antioxidants (Tiwari, 2001). Oxidative effects are manifestations of an imbalance between free radical generation through biological processes leading to the condition recognized as "oxidative stress" (Seifu *et al.,* 2012).

Practice of developing formulations and preparation from medicinal plants for the purpose of therapeutics is thought to have been employed since the origin of human race. Many previously reported such formulations from

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medicinal origin described that antioxidant based phyto-metabolites are also substantially related with the antidiabetic potential (Rahimi *et al.,* 2005). Researches reported that such medicinal plant possess the scavenging potential of free radical (Seifu *et al.,* 2012). Free radical detoxification was mentioned through variety of mechanism (s) including those involving enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR) and non-enzymes like albumin, ceruloplasmin, ferritin, ascorbic acid, α-tocopherol, β-carotene, glutathione (GSH) and uric acid (Pham-Huy *et al.,* 2008; Seifu *et al.,* 2012). *Rauwolfia serpentina* Benth (family *Apocynaceae*) is recognized phytomedicine for its potential effectiveness in the cure from venom (snake sources), gastric and intestinal disorders, cancer of mammary cells, skin (dermis) problems, etc (Qureshi *et al.,* 2009; Azmi and Qureshi 2012). From last decades, its antidiabetic potential has been reported in animal models of diabetes (Qureshi *et al.,* 2009; Azmi and Qureshi 2012). It was also reported to improve the cardioprotective indices as well as reducing the oxidative stress and maintaining the haematinic potential through its pure methanolic form (Azmi *et al.,* 2012; Azmi and Qureshi, 2013; Azmi *et al.,* 2015).

In this study the protective role of hydro-methanolic (HyMREt) extract from roots of *Rauwolfia serpentina* in anemia, oxidative stress and glycosylation in mice intoxicated with pyrimidine (alloxan) derivative was investigated.

MATERIALS AND METHODS

Collection of plant material and preparation of aqueous methanolic extract

The roots of *R. serpentina* were procured from Hamdard Dawakhana Sadar, Karachi-Pakistan in 2010. The plant specimen was verified and authenticated from Specialist of Botany Department, University of Karachi, Pakistan, before use. The extract i.e., HyMREt used for this study was prepared according to the method described earlier by Azmi *et al.,* 2018.

Experimental mice and ethical approval

Male albino mice (weight between 25-35 g) were purchased from breeding house of Dow University of Health Sciences (DUHS), Karachi, Pakistan. The mice were acclimatized and maintained individually in cages in an air conditioned room of 12 hours light/ dark cycles with temperature 23±2^{o}C (Relative humidity 55%) for one week prior to the experiment in the conventional animal house of the same university. Mice were given standard laboratory diet with free access to water *ad libitum* and no physical stress was provided (Qureshi *et al.,* 2009). During the entire experimental period, the care and handling of these mice were in accordance with internationally accepted standard guidelines. The experimental protocol was approved by the Institutional Ethical Review Board (IERB) of Dow University of Health Sciences, Karachi (Letter Ref. No: IRB-186/ DUHS-10).

Chemicals, reagent and medicine used

Alloxan monohydrate (2,4,5,6-tetraoxypyrimidine,5,6-dioxyuracil) was purchased from AppliChem GmbH Darmstadt, Germany. Single intraperitoneal injection of alloxan 150mg/kg was used to induce type 1 diabetes in overnight fasted mice. After 72 hours of this injection fasting blood glucose levels were monitored from tail vein of mice with the help of glucometer (Optium Xceed, DiabetesMonitoring system by Abbott), mice showed glucose level ≥190 mg/dL were selected for investigating antidiabetic activity of HyMREt. Antidiabetic medicine 'glibenclamide' as product name *Doanil* (5mg/kg) of Sanofi-aventis Pakistan Ltd, were used as positive control. All other reagents and chemicals used were of analytical grade and purchased from authentic dealer of Department of Biochemistry, University of Karachi.

Grouping of mice

The mice were divided into seven groups (six animals each). The elaborative detail of each animal group is mentioned in fig. 1. Each treatment was given to its respective group orally once in a day for 14 days consecutively. At the end of animal trial, mice were decapitated to collect whole blood, and visceral organs (kidney, liver, spleen and heart) for analytical purpose. Serum and liver tissues were also used to analyze hematological, biochemical and antioxidant parameters (Azmi *et al.,* 2015).

Determination of wet organs weight, their relative weights (percent body weight) and other Biochemical Parameters

On final day (14th day) of animal trial, after sacrificing mice, body organs including heart, kidneys, liver and spleen were dissected out carefully, tabbed on filter paper and their weights were recorded in grams (g) by using digital weighing balance and finally their relative weights (percent body weight) were calculated with the help of following formula (Adaramoye *et al.,* 2012; Azmi *et al.,* 2015).

$$\text{Relative weight (\% body weight)} = \left(\frac{\text{Wet organ weight}}{\text{Body weighton final day}}\right)\times 100$$

Fasting blood glucose levels were monitored by pricking the tail vein of each mice of each group by using glucometer at initial (0) and final (14th) day of trial and recorded as milligram per deciliter (mg/dL). To determine percent glycemic change, following formula was used (Perfumi and Tacconi, 1996; Azmi and Qureshi, 2012).

$$\text{Glycemic change (\%)} = \left(\frac{FBG_x - FBG_o}{FBG_o}\right)\times 100$$

Where, FBG_o = blood glucose level at 0 day and FBG_x = blood glucose level at 14 day.

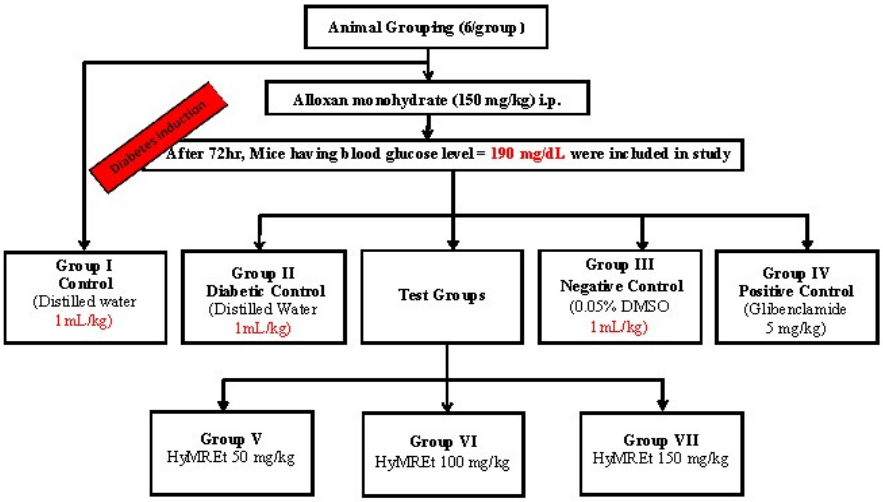

Fig. 1: Animal Grouping

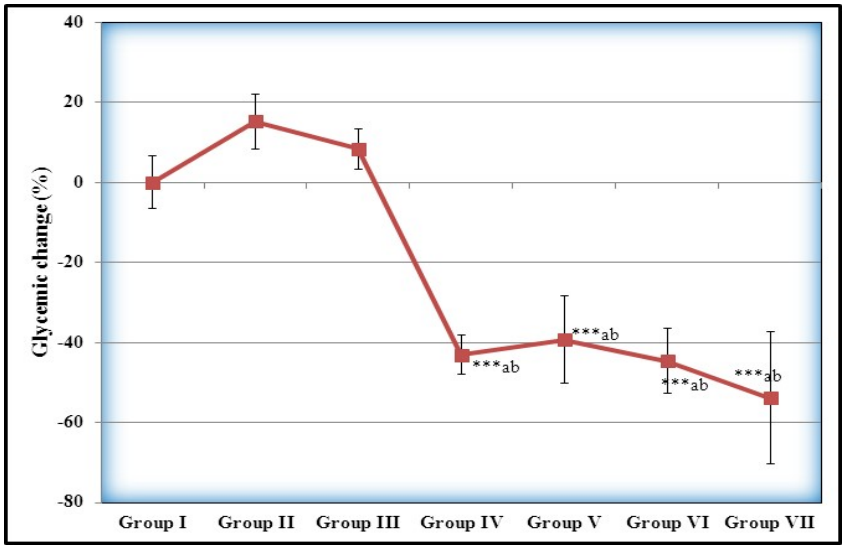

Fig. 2: Effect of HyMREt on Percent (%) Glycemic Change of Alloxan-Induced Diabetic Mice
Values are expressed as mean ± SD (n = 6). *p<0.0001 when compared with group II (a) and III (b).

Determination of antioxidant enzymes and hepatic biomarkers

Antioxidant enzymes viz., catalase (CAT) and superoxide dismutase (SOD) were measured in liver homogenate by the methods previously reported by Azmi and Qureshi, 2013. Estimation of total lipids and glycogen contents in liver homogenate was done by gravemetric and colorimetric methods, respectively (Azmi and Qureshi, 2012). Quantification of Alanine transaminase (ALT) was done in serum by using commercially available assay kits of Randox, United Kingdom (Azmi and Qureshi, 2012).

Determination of hematological parameters

Glycosylated hemoglobin (HbA1c) level was evaluated by commercially available Kit (Nycocard Kit, USA) (Azmi and Qureshi, 2012). Complete blood profile (CBP) including total hemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cell volume or hematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration were estimated by Sysmex (XS-1000i) automated hematology analyzer.

STATISTICAL ANALYSIS

Results of the present study are expressed as mean ± SD (standard deviation). The data were analyzed with Statistical Package for Social Sciences (SPSS version 18) by using one-way ANOVA followed by LSD (least significant difference) test at $p<0.05$. The differences were considered significant at $p<0.05$, $p < 0.01$, $p<0.001$, and $p< 0.0001$ when compared with respective controls.

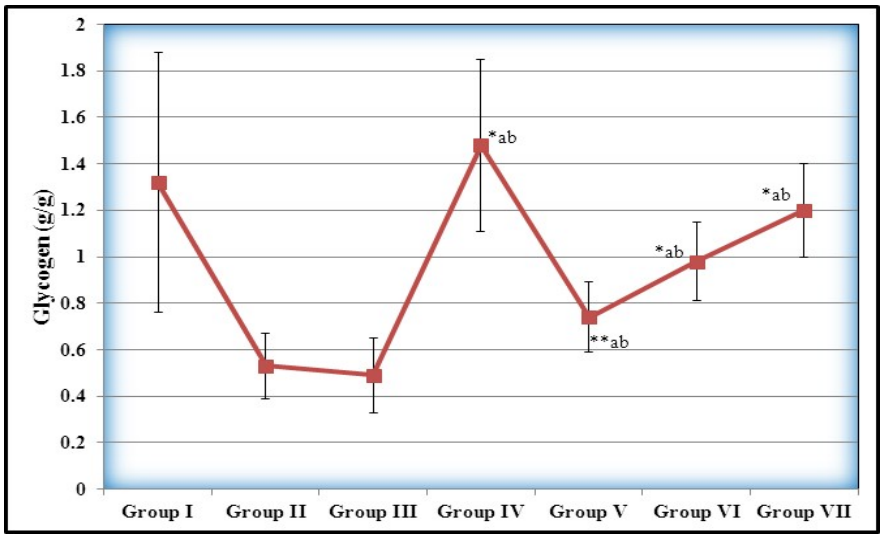

Fig. 3: Effect of HyMREt on Glycogen in Liver Tissues of Alloxan-Induced Diabetic Mice
Values are expressed as mean ± SD (n = 6). *p<0.05 and **p<0.01 when compared with group II (a) and III (b)

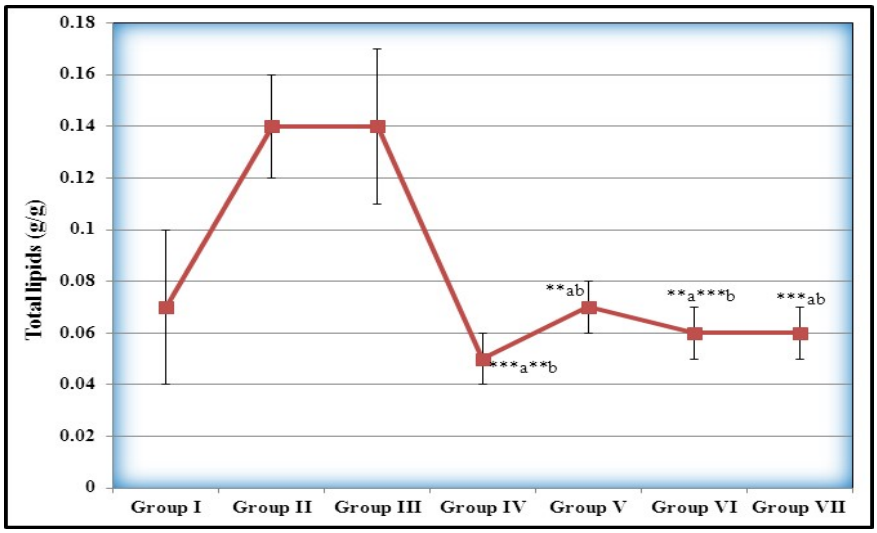

Fig. 4: Effect of HyMREt on Total Lipids in Liver Tissues of Alloxan-Induced Diabetic Mice
Values are expressed as mean ± SD (n = 6). **p < 0.01 and ***p < 0.0001, when compared with group II (a) and III (b)

RESULTS

Effects on Wet, percent body (relative) organs weights and percent glycemic reduction in type 1 diabetic mice

After the repeated respective treatments, the wet organs weights have no significant difference in all seven groups (table 1). Similarly, dose magnitudes of HyMR*Et* also have no major impact in terms of percent body (relative) organs weights when compared with normal controls and other (table 1).

In respective treatment groups, dose-wise magnitude of HyMREt showed prominent (p<0.0001) decrement in percent glycemic change, which was much elevated in two important control groups i.e., diabetic (group II) and negative (group III) (fig. 2).

Effect on glycosylated (HbA1C) hemoglobin, hepatic glycogen and total lipid in type 1 diabetic mice

Gradual improvement in hemoglobin levels was found in all three doses of HyMREt (i.e., 50-150mg/kg) treated groups when compared with respective controls of mice. Opposite to this, prominent (p<0.0001) reduction in glycosylated hemoglobin levels was also found in all three treatments groups of HyMREt treated groups, when compared with markedly elevated levels of glycosylated (HbA1C) hemoglobin in diabetic (group II) and negative (group III) controls mice (table 2). Decreased in HbA1c to Hb ratio was also confirmed the improvement of glycosylation in in all three doses of HyMREt (i.e., 50-150 mg/kg) treated groups (table 2).

Table 1: Effect of HyMREt on wet and relative organs body weights of alloxan-induced diabetic mice

Group	Wet organ weight (g)				Relative Weight (% body weight)			
	Kidney	Liver	Spleen	Heart	Kidney	Liver	Spleen	Heart
Group I	0.43±0.07	1.44±0.13	0.08±0.02	0.09±0.01	1.40±0.24	4.62±0.23	0.27±0.04	0.29±0.05
Group II	0.42±0.06	1.42±0.09	0.10±0.03	0.11±0.01	1.60±0.30	5.33±0.58	0.36±0.09	0.40±0.07
Group III	0.43±0.05	1.39±0.07	0.09±0.02	0.09±0.01	1.63±0.16	5.34±0.51	0.36±0.10	0.33±0.02
Group IV	0.42±0.10	1.39±0.10	0.10±0.02	0.10±0.02	1.32±0.31	4.40±0.32^{**ab}	0.31±0.04	0.32±0.07
Group V	0.46±0.03	1.42±0.06	0.10±0.02	0.10±0.02	1.67±0.22	5.09±0.32	0.34±0.07	0.34±0.09
Group VI	0.48±0.02	1.35±0.11	0.09±0.01	0.09±0.02	1.64±0.06	4.63±0.38^{*ab}	0.31±0.04	0.30±0.06^{*a}
Group VII	0.42±0.08	1.39±0.06	0.10±0.02	0.10±0.02	1.36±0.24	4.52±0.21^{**ab}	0.31±0.08	0.33 ± 0.06

Values are expressed as mean ± SD (n = 6). *p<0.05 and **p<0.01 when compared with group II (a) and III (b)

Table 2: Effect of HyMREt on Hb, HbA_{1c} and HbA_{1c} to Hb ratio in alloxan-induced diabetic mice

Groups	Hb (%)	HbA_1c (%)	HbA_{1c} / Hb ratio
Group I	13.67 ± 0.70	5.99 ± 1.16	0.44 ± 0.09
Group II	10.14 ± 0.26	10.84 ± 0.69	1.07 ± 0.07
Group III	9.93 ± 0.53	10.55 ± 0.68	1.07 ± 0.10
Group IV	12.36 ± 0.59^{**ab}	6.12 ± 0.80^{***ab}	0.50 ± 0.07^{***ab}
Group V	7.25 ± 0.61^{***ab}	4.52 ± 0.42^{***ab}	0.62 ± 0.03^{***ab}
Group VI	10.43 ± 0.85	3.89 ± 0.63^{***ab}	0.37 ± 0.04^{***ab}
Group VII	10.91 ± 1.75	3.77 ± 0.72^{***ab}	0.36 ± 0.10^{***ab}

Values are expressed as mean ± SD (n = 6). **p < 0.01 and ***p < 0.0001, when compared with group II (a) and III (b)

Table 3: Effect of HyMREt on liver specific and antioxidant enzymes of alloxan-induced diabetic mice

Groups	Treatment	ALT (U/L)	Percent Inhibition (%)	
			CAT	SOD
Group I	Distilled water (1 ml/kg)	28.25±14.81	35.30±4.56	42.68 ± 11.91
Group II	Alloxan (150 mg/kg)	61.25±11.15	83.65±5.47	78 ± 14.19
Group III	Alloxan (150 mg/kg) + 0.05% DMSO (1 ml/kg)	65.50±5.07	82.12±5.72	73.15 ± 11.42
Group IV	Alloxan (150 mg/kg) + Glibenclamide (5 mg/kg)	41.25±10.73^{*a}	44.16±15.21^{**ab}	29.27 ± 12.03^{***ab}
Group V	Alloxan (150 mg/kg) + HyMREt (50 mg/kg)	48.67±10	42.73±12.85^{**ab}	39.33 ± 4.5^{***a**b}
Group VI	Alloxan (150 mg/kg) + HyMREt (100 mg/kg)	44.75±10.62^{*b}	31.88±10.47^{***ab}	54.26 ± 1.23^{*ab}
Group VII	Alloxan (150 mg/kg) + HyMREt (150 mg/kg)	40.25±10.45^{*a**b}	44.12±9.19^{**ab}	30.61 ± 3.26^{***ab}

Values are expressed as mean ± SD (n = 6). *p<0.05,**p<0.01, and ***p<0.0001, when compared with group II (a) and III (b)

Table 4: Effect of HyMREt on Blood Profile of Alloxan-Induced Diabetic Mice

Groups	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCV (fl)	MCH (Pg)	MCHC (g/dl)
Group I	4.59±0.54	3.67±0.75	23.80±8.77	51.87±10.86	14.99±3.80	35.43±6.62
Group II	2.99±0.51	5.11±1.04	15.82±3.93	30.96±4.75	10.78±0.80	22.89±9.30
Group III	2.78±0.68	5.30±0.95	16.58±9.07	34.37±7.43	10.54±3.08	21.54±7.20
Group IV	3.80±0.28^{*b}	3.22±0.85^{**ab}	24.19±6.12	44.91±11.92^{*a}	17.02±4.26^{**ab}	33.74±5.11^{*a**b}
Group V	4.60±0.73^{**ab}	3.15±1.22^{**ab}	24.90±1.15	54.85±6.18^{***a**b}	16.30±0.92^{**ab}	29.90±1.62^{*b}
Group VI	6.20±0.41^{***ab}	3.35±0.40^{**ab}	32.10±0.69^{**ab}	51.90±2.31^{**ab}	15.80±0.12^{*a**b}	30.50±1.15^{*b}
Group VII	6.08±1.19^{***ab}	2.90±0.35^{**ab}	31.70±8.89^{**ab}	51.45±4.56^{**ab}	15.40±1.04^{*ab}	29.90±0.58^{*b}

Values are expressed as mean ± SD (n = 6). *p < 0.05, **p<0.01, and ***p<0.0001, when compared with group II (a) and III (b).

Significant impact (p<0.05 & p<0.01) of improvement was estimated in hepatic glycogen contents in HyMREt treated mice whereas, prominently ($p<0.05$) suppressed levels of hepatic total lipids were found in these treated groups (figs. 3-4) of HyMREt doses.

Effect on liver specific and antioxidant enzymes in type 1 diabetic mice

ALT was observed up to normal levels in all three treatment groups which showed the safest impact of HyMREt on liver of mice.

However, significantly ($p<0.05$, $p<0.01$ & $p<0.0001$) reduced values of percent inhibition of SOD and CAT was also found in all HyMREt treated groups when compared with two important control groups i.e., diabetic (group II) and negative (group III) controls (table 3).

Effect on blood profile in type 1 diabetic mice

Test doses (i.e., 50-150mg/Kg) of HyMREt progressively ($p<0.05$) improved RBC and other hematological parameters like PCV, MCV, MCH and MCHC while significant ($p<0.0001$) decrement was only noticed in leucocyte counts (table 4).

DISCUSSION

Researches often used wet and percent body (relative) organs weights because of their supportive part in evaluating the condition when chemically linked variation are expected (Peters and Boyd, 1966; Pfeiffer, 1968). Successively, after the 14 days of administration of HyMREt in alloxan-induced diabetic mice, indicated the non-hazardous and non-deleterious influence of test extract (table 1). Any unusual increase or decrements in these variables have extensively been used as a sign of treatment based effects on visceral organs (Michael *et al.,* 2007). Liver is the principal body organ, mostly involved in the detoxification of major harmful substances (Azmi and Qureshi, 2012). Beside this, kidney, heart and spleen also serves as sensitive organs to assess the biological as well as metabolically linked alteration or increment in percent body (relative) organs or any impression of immune associated toxicities or any other kind of cellular damages, after the repeated administration of test substance. Therefore, present outcomes assured the safest impact of HyMREt on these body organs.

Characteristic hyperglycemia was commonly linked when oxygenated pyrimidine analogue i.e., alloxan was injected in animals (Sun *et al*., 2008; Azmi and Qureshi, 2012). This may be due to metabolic impairment and disruption in the uptake of sugar (glucose) due to the deficiency in insulin's availability towards the target cells and receptor (Daneman, 2006). Mice intoxicated with pyrimidine (alloxan) derivative showed perpetual destruction in the insulin synthesizing pancreatic (β-cells) area resulting insulin's shortage in terms of its bioavailability (Daneman, 2006; Azmi and Qureshi, 2012). Presently, the repeated use of HyMREt in treatment groups indicated a good glycemic reduction which may be attributed with the previously reported hypoglycemic potential of this medicinal substance (Azmi and Qureshi, 2012).

Diabetes is a fatal metabolic disorder (in chronic state), which was characteristically assessed with different biochemical markers, among which an increase in glycosylated (HbA1c) hemoglobin indicates excess glucose in blood and is non-enzymatically conjugate with Hb, resulting into glycosylation of heme residues i.e., HbA1c (Davis, 2006; Azmi *et al.,* 2015). Greater than 6 % HbA1c was reported or linked with diabetic state (Azmi *et al.,* 2015). Treatments with HyMREt showed a prominent reduction in HbA1c levels in all test groups which may be due to the protective role of this root extract as lowering of glucose level in blood relate with its less interaction with hemoglobin, hence marked decrement reported (Azmi and Qureshi, 2012; Azmi *et al.,* 2015).

The above mentioned mechanism of lowering of hyperglycemic state was properly attributed with improved storage of glucose in hepatic tissues in its polymeric form (glycogen) and reduced hepatic lipids contents (Vats *et al.,* 2004; Azmi and Qureshi, 2012). This could be one of associated factors which strengthen the hypoglycemic potential of HyMREt in type 1 diabetic mice.

The normal (around 37 U/L) values of ALT in serum reported the normal physiological impact of liver. However, it's higher value represents hepatic injury or damage (Moss and Henderson, 1996; Reitman and Frankel, 1957). Consequently, all three doses of HyMREt found nontoxic to hepatic function.

Beside this continued hyperglycemic state also relates with the development and accumulation of advance glycated end products (AGES) which correlate with several severities for example formation of free (reactive) radicals or oxygenated species (ROS), renal issues, development atherosclerosis, cataract formation etc (Lorenzi, 2007; Rabbani *et al*., 2011). Less percent inhibition of both antioxidant (CAT & SOD) enzymes in test groups was mainly due to reduction in hyperglycemia caused by the repeated administration of HyMREt and less accumulation of AGES due to decrement in glycosylated hemoglobin. Hence, the test doses of HyMREt improved the physiological role of SOD, which transforms superoxide based anion into hydrogen peroxide radical (Seifu *et al*., 2012) and promoted the role of CAT, which hydrolyzes hydrogen peroxide radicals into oxygen and water, thus HyMREt shielded the tissues from oxidative destruction induced by free radicals (Mao *et al*., 1993).

Persistent hyperglycemia based glycosylation and free radicals induced oxidative stress not only disrupts the RBC's osmotic fragility but also decreases the half ($t_{1/2}$) life resulting in the progress of hemolysis (Saba *et al.,* 2010). In this work, three doses of HyMREt showed dose-

dependent and gradual increase ($p< 0.05$) in RBC's count when matched diabetic control group and established the hematopoietic potential of *R. serpentina* (Azmi and Qureshi, 2013). Contrary to this, the increased leucocyte count was observed in diabetic (group II) and negative (group III) control mice that signify the toxic assault stab in the pancreatic normal functioning by alloxan (Tanko *et al.,* 2011). HyMREt significantly ($p<0.05$) stabilized the leucocyte count in all treatment groups, therefore, lowers the alloxan based inflammation in pancreas. Others parameters (i.e., PCV, MCH, MCV and MCHC) were also recovered ($p<0.05$) in treatment groups of HyMREt as compared in diabetic (group II) control and negative (group III) control mice. Mild anemia was mainly associated in diabetes hence present findings emphasized and validated the potential feature of *R.serpentina* that it may have hematinic potential in type 1 diabetic mice.

CONCLUSION

Therefore, results concluded the therapeutic role of HyMREt of *R. serpentina* by possessing hematinic, antiglycation and antioxidant effects in mice intoxicated with alloxan monhydrate.

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