



The Anti-oxidant and the Anti-diabetic Effects of *Terminalia chebula* and *Withania somnifera* in Subclinically Diabetic Dogs

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ABSTRACT

Background: The present study evaluated the antioxidant as well as the anti-diabetic potential of *Terminalia chebula* (Haritaki) and *Withania somnifera* (Ashwagandha) in subclinically diabetic dogs.

Methods: A total of thirty subclinically diabetic dogs were divided into five groups of six animals each; Group I (Positive control), Group II (*T. chebula* extract), Group III (*W. somnifera* extract), Group IV (*T. chebula* and *W. somnifera* extracts at 1:1 ratio) and Group V (N- acetylcysteine). Six apparently healthy dogs were kept as negative control (Group VI) for the study. The animals were evaluated based on diabetic biomarkers (Random blood glucose, fasting blood glucose, glycated hemoglobin, serum fructosamine and serum insulin) and oxidative stress markers (lipid peroxidation, reduced glutathione, superoxide dismutase and catalase activities) on days 0, 15 and 30 of the study.

Result: Significant oxidative stress was observed in the subclinically diabetic dogs and the supplementation of N- acetylcysteine, *T. chebula* and *W. somnifera* effectively curbed the oxidative impairments and further progression of diabetes. The improvement in oxidative, as well as diabetic biomarkers, was predominant in Group V animals, followed by Group IV, Group II and Group III. It may be concluded that therapeutic modulation of redox mechanisms may aid in the clinical management of subclinical diabetes mellitus, however, the mechanisms involved should be further explored.

Key words: Canine diabetes, Herbal, Oxidative stress, Subclinical, *Withania somnifera*.

INTRODUCTION

Diabetes mellitus (DM) is an emerging metabolic syndrome of greater importance in dogs because of its multi-systemic terminal effects and cachexia. The characteristic features of clinical canine diabetes mellitus are polydipsia, polyuria, polyphagia and weight loss similar to the human syndrome. The prime factors associated with the increased incidence of DM in dogs were obesity, altered food habits, lack of exercise, genetic manipulations and inbreeding (Temelkova-Kurktschiev and Stefanov, 2012; Raffan *et al.*, 2015). Earlier studies reported the prevalence of DM among dog populations in the range of 0.0005% to 1.5% (Wilkinson, 1960; Mattheuws *et al.* 1984) whereas, recent studies from India revealed the existence of clinical/subclinical DM among dog populations in alarming levels, that is 1.8% to 7.2% (Das and Lodh, 2015; Devi *et al.*, 2016). Geriatric female dogs were at high risk than their male counterparts may be due to their hormonal peculiarities leading to insulin resistance (Marmor *et al.*, 1982). In dogs, clinical DM was expressed as elevated blood glucose concentrations (180-220 mg/dl) which is above the renal threshold and the major contributing factor was untreated pancreatitis (Nelson and Reusch, 2014; Rand, 2020).

Prior to clinical manifestation of DM, there is a prediabetic stage often called as subclinical diabetes in humans as well as dogs. According to Buysschaert and Bergman (2011), prediabetes is the presence of Impaired Fasting Glucose (IFG) and/or Impaired Glucose Tolerance (IGT). Experimental studies on pre-diabetic dogs showed

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high subcutaneous fat deposits, diminished insulin sensitivity and impaired fasting glucose (Ionut *et al.*, 2010).

Insulin therapy is often essential for diabetic dogs since virtually all dogs have Insulin Dependent Diabetes Mellitus (IDDM). Also, oral hypoglycemic drugs such as

sulphonylurea, metformin *etc.* were not effective in dogs suffering from IDDM (Nelson, 2000).

In diabetes mellitus, chronic hyperglycemia lead to multiple biochemical damages. Oxidative stress may be the basis of pathogenesis and clinical manifestations of this multi-systemic syndrome (Giugliano *et al.*, 1996). Free radicals were synthesized in disproportionately higher rate and quantities in DM patients, by means of elevated glucose oxidation, non-enzymatic glycation of proteins followed by their oxidative degradation. Hence, the vital role of antioxidant therapy is significant. Antioxidants cannot be considered as a complete curative for DM. But intake of antioxidants can reduce the oxidative stress by interfering with the synthesis of free radicals or by inactivating them and thereby ameliorate the progression of pathological damages related to the disease (Cheng *et al.*, 2003). Earlier, the role and usage of natural antioxidants in prevention of oxidative damage associated with experimental diabetes in rats has been studied (Coldiron *et al.*, 2002; Vessal, *et al.* 2003). Strong natural antioxidants of plant origin included carotenoids, limonoids, tocopherols, ascorbates, and polyphenols (Bajpai *et al.*, 2005). Also, some plants showed hypoglycemic activity by interfering with insulin release/stimulation (Pari and Latha, 2002).

The development of new drugs and therapeutic strategies with least side effects were of greater interest to the scientific community at every point of time. Scientists were striving to evaluate antidiabetic activities of such natural products and its scientific validation (Florence *et al.*, 2014). The ethanolic extract of *T. chebula* fruit showed potent hypoglycemic activity in streptozotocin-induced diabetic rats and was found more efficacious than glibenclamide, a standard hypoglycemic drug in commercial use (Kumar *et al.*, 2006). Ethanolic extract of *W. somnifera* root have potent anti-inflammatory as well as anti-diabetic activity (Udayakumar *et al.*, 2009). Keeping this as background data, the present study was carried out to evaluate the ameliorative role of *T. chebula* and *W. somnifera* via redox modulation in subclinically diabetic dogs.

MATERIALS AND METHODS

Animal selection

The work was carried out at Division of Medicine, ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh during 2015-2016. Subclinically diabetic dogs were identified based on preliminary screening of random blood glucose, fasting blood glucose and Benedict's test for the presence of sugar in urine (Kaneko *et al.*, 2008). Animals selected *via* preliminary tests were further confirmed through glycated haemoglobin estimation by ion exchange chromatography using commercial kit (Coral clinical systems, India) as per Trivelli *et al.* (1971), serum fructosamine estimation by nitrobluetetrazolium reduction method (Sahu and Sarkar, 2008) and serum insulin estimation by ELISA (Bioassay Technology Laboratory, China).

Plant extract preparation

T. chebula fruits and *W. somnifera* roots were procured and later identified by Dr. R.K. Tiwari, Reader and Head, Department of Dravyaguna, SRM Govt. Ayurvedic College, Bareilly, Uttar Pradesh. *T. chebula* dried fruit was crushed to separate outer seed husk and the seed was ground into powder. Dried *W. somnifera* roots were also ground into powder form. Extraction of the coarse powder in 70% ethanol was done using Soxhlet extraction apparatus by running 20 cycles at 60°C. After filtration through Whatman No.1 filterpaper, the extract was then dried using evaporator. The dried extract was then crushed into fine powder and was filled in commercially available gelatin capsule covers at the rate of 700 mg powder in each capsule. Capsules were then preserved in a refrigerator at 4°C till usage.

Experimental study design

The study is approved by Institutional Animal Ethics Committee (IAEC) under institute project IVRI/MED/12-15/008. Thirty subclinically diabetic dogs were selected based on the diabetic biomarkers (Kaneko *et al.*, 2008) and were divided into five groups of six animals each; Group I was positive control without any treatment, Group II fed with *T. chebula* extract @ 100 mg/kg body weight, Group III fed with *W. somnifera* extract @ 100 mg/kg body weight, Group IV fed with both *T. chebula* extract and *W. somnifera* extract @ 100 mg/kg body weight each and Group V fed with standard antioxidant N-acetyl cysteine @ 10 mg/kg body weight. The antioxidants were administered for 30 days at the above mentioned daily dosages. Six apparently healthy dogs were kept without any treatment as negative control (Group VI). The animals were evaluated based on various diabetic biomarkers and oxidative stress markers on days 0, 15 and 30 of the study.

Blood sample processing

Blood samples were collected from the cephalic vein of the dogs in sterile vials with sodium fluoride (2 mg/ml) and heparin (0.2 mg/ml) as anticoagulants for the assessment of blood glucose and oxidative stress indices, respectively. About 2 ml of blood was collected into another clot activator vial and the serum was garnered for the evaluation of other diabetic biomarkers. Heparinised blood samples were centrifuged at 3000 rpm for 10 min to remove plasma and buffy coat and the red blood cells (RBCs) were harvested. The RBCs were washed thrice using normal saline solution and 10% hemolysate was prepared in chilled distilled water. This hemolysate was used for the estimation of oxidative stress indices namely LPO, SOD and Catalase activities. Reduced glutathione (GSH) level was estimated in 50% RBC suspension prepared by mixing with chilled normal saline (1:1).

Estimation of diabetic biomarkers

Random blood glucose level and fasting glucose level were estimated using portable glucometer (Accu-Chek, Roche

Diagnostics) and glucose oxidase and peroxidase (GOD-POD) method, respectively (Trinder 1969). Glycated haemoglobin (GHb), serum fructosamine and serum insulin concentration were estimated as per the method mentioned above.

Estimation of oxidative stress indices

Reduced glutathione (GSH) concentration in RBC suspension was estimated by 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) method as per Prins and Loos (1969). LPO level in the RBC hemolysate was estimated by the method of Placer (1967) based on the formation of a colored complex between the lipid peroxidation products and thiobarbituric acid (TBA). Superoxide dismutase (SOD) activity was estimated by the method described by Madesh and Balasubramanian (1998). And finally, catalase (CAT) activity in hemolysate was appraised by the method of Bergmayer (1983) using H_2O_2 as a substrate.

Statistical analysis

The values are expressed as mean $\pm$ S.E. Data were analyzed by analysis of variance followed by the Post-Hoc Tukey test using statistical software package, SPSS 16.0. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of different treatments on diabetic biomarkers and oxidative stress indices of subclinically diabetic dogs are presented on (Table 1). All the treatment groups showed significant ($P < 0.01$) reduction in random blood glucose, fasting blood glucose, glycated hemoglobin, serum fructosamine and serum insulin concentration than the positive control group after treatment. Among treatment groups, group V (N-acetylcysteine) showed better improvement in random blood glucose (15.19%), fasting blood glucose (15.23%), glycated haemoglobin (15.19%), serum fructosamine (33.5%) and Insulin (20%) levels followed by group IV (15.14%, 14.67%, 13%, 31.03%, 19.74%) treated with combination of *T. chebula* and *W. somnifera*, group II (14.09%, 13.66%, 6.6%, 26.22%, 16.13%) treated with *T. chebula* and group III (8.2%, 8.1%, 3.58%, 25.56%, 6.7%) treated with *W. somnifera*, respectively, on day 30 of the therapy (Fig 1-5). Significant improvement in oxidative biomarkers was noticed in all the four treatment groups on day 30. N-acetylcysteine treated group (group V) showed highest increment in antioxidant systems such as GSH (33.33%), SOD (59%) and CAT (69.57%), followed by group IV (20.45%, 45%, 50%) treated with combination of herbs, group II (8.5%, 36%, 31.96%) treated with *T. chebula* and group III (6.5%, 29.16%, 27.8%) treated with *W. somnifera*, respectively, after therapy (Fig 6-8). However, better reduction of LPO was exhibited by Group V (32.38%) followed by Group IV (29.94%), Group II (22.86%) and Group III (21.91%), respectively (Fig 9). No significant ($P > 0.01$) difference in diabetic as well as oxidative biomarkers were observed between Group IV and Group V

animals after treatment, unfolding the possibility of *W. somnifera* and *T. chebula* combination as an alternative to the standard antioxidant drug in management of subclinical diabetes mellitus in dogs.

Varied biological properties of *T. chebula* is attributed by its tannins, vitamin C, gallic acid, anthraquinones, triterpenoids and other miscellaneous compounds (Juang *et al.*, 2004). The antioxidant constituents are flavonol glycosides, triterpenoids and chebulin. *T. chebula* extract showed significant antioxidant and antidiabetic effect in alloxan-induced diabetic rats when administered at the dose rate 100 mg/kg body weight (Sabu and Kuttan, 2002). About thirty five pharmacologically important chemical constituents were reported from the root of *W. somnifera*. It includes various alkaloids, steroidal lactones, withanolides, withaferins, saponins *etc.* Among them, withaferin A and withanolide D were responsible for most of the pharmacological activity of *W. somnifera* (Mishra *et al.*, 2000). It is reported to reduce hepatic lipoperoxidation and stimulate thyroidal activity (Verma, 2010). Immunostimulant property of *Withania somnifera* has also been detected in poultry vaccinated against Newcastle's Disease (Mohanambal *et al.*, 2017). Udayakumar *et al.* (2009) demonstrated the hypoglycaemic effect of *W. somnifera* root ethanolic extract on experimentally induced diabetic rats at 100 mg/kg body weight.

N-acetyl cysteine has significant therapeutic potential in ameliorating oxidative stress by means of its GSH precursor effect as well as free radical scavenging ability (Gillissen *et al.*, 1997; Messonier, 2001). This antioxidant effect of N-acetylcysteine would have contributed in improving the diabetic biomarkers of the subclinically diabetic dogs by modulating redox mechanisms and preventing further progression of disease. Dried ripe fruit of *T. chebula* Retz (Combretaceae) is a vital constituent of Triphala, a traditional medicine in Asia (Perry and Metzger, 1980). Antidiabetic effect of chebulic acid derived from *T. chebula* has been reported by Lee *et al.* (2010). *W. somnifera* (Dunal), a well-known adaptogen, has proven hypocholesterolemic and antioxidant properties (Singh *et al.*, 2011). Also, it possesses anti-inflammatory, anticancer, antistress, immunomodulatory, rejuvenating properties along with endocrine, cardiopulmonary and central nervous system modulatory properties (Mishra *et al.*, 2000). *In vivo* and *In vitro* antioxidant potential of *T. chebula* and *W. somnifera* has already been reported (Singh, 2007; Deepa, 2014). Previous reports also suggest *W. somnifera* as an anti-diabetic agent (Udayakumar, 2009).

Subclinical diabetic/prediabetic animals were apparently healthy with persistently elevated fasting blood glucose concentrations above the reference range, but below the threshold concentration necessary for glycosuria (Rucinsky *et al.*, 2010). The present study reports elevated glycated haemoglobin, serum fructosamine and serum insulin level in prediabetic dogs which is in agreement with

Table 1: Effect of different treatments on diabetic biomarkers and oxidative stress indices (Mean±SE).

Parameters	Day	Group I positive control (n=6)	Group II <i>T. chebula</i> (n=6)	Group III <i>W. somnifera</i> (n=6)	Group IV <i>T. Chebula</i> and <i>W. Somnifera</i> (n=6)	Group V N-acetyl cysteine (n=6)	Group VI negative control (n=6)
Random glucose (mg/dl)	0	162.75±15.0 ^{aA}	168.50±6.18 ^{aA}	163.50±9.03 ^{aA}	158.50±6.99 ^{aA}	164.50±11.9 ^{aA}	83.75±6.00 ^{aA}
	15	167.00±15.8 ^{aA}	151.75±7.28 ^{abAB}	155.50±7.27 ^{abA}	138.00±1.47 ^{BB}	147.00±7.29 ^{abA}	82.12±6.00 ^{aA}
	30	157.50±12.8 ^{aA}	144.75±6.12 ^{abB}	150.00±3.93 ^{abA}	134.50±1.75 ^{BB}	139.50±4.29 ^{abB}	83.99±6.00 ^{aA}
Fasting glucose (mg/dl)	0	148.00±10.1 ^{aA}	155.50±6.18 ^{aA}	154.25±9.68 ^{aA}	146.25±9.83 ^{aA}	151.00±8.88 ^{aA}	70.5±3.88 ^{bA}
	15	149.00±10.5 ^{aA}	138.75±5.34 ^{abCB}	145.50±3.96 ^{abA}	126.50±3.12 ^{CB}	129.75±2.05 ^{bcB}	71.5±3.88 ^{dA}
	30	148.50±10.33 ^{aA}	134.25±3.75 ^{abCB}	141.75±3.25 ^{abA}	124.75±0.47 ^{CB}	128.00±2.27 ^{bcB}	70.8±3.88 ^{dA}
Glycated haemoglobin (%)	0	7.08±0.17 ^{aA}	7.02±0.23 ^{aA}	7.26±0.08 ^{aA}	7.15±0.06 ^{aA}	7.24±0.07 ^{aA}	5.02±0.05 ^{bA}
	15	7.21±0.09 ^{aA}	6.95±0.16 ^{aA}	7.09±0.05 ^{abB}	6.92±0.06 ^{aA}	7.08±0.08 ^{aA}	5.06±0.05 ^{bA}
	30	7.24±0.11 ^{aA}	6.55±0.26 ^{BB}	7.00±0.05 ^{BB}	6.22±0.12 ^{BB}	6.14±0.04 ^{CB}	4.99±0.04 ^{dA}
Serum fructosamine (mmol/L)	0	3.65±0.16 ^{aA}	3.89±0.11 ^{aA}	3.95±0.15 ^{aA}	3.77±0.06 ^{aA}	3.88±0.19 ^{aA}	1.92±0.03 ^{bA}
	15	3.69±0.18 ^{aA}	3.76±0.12 ^{aA}	3.82±0.17 ^{aA}	3.61±0.07 ^{aA}	3.66±0.20 ^{aA}	1.97±0.03 ^{bA}
	30	3.77±0.20 ^{aA}	2.87±0.16 ^{BB}	2.94±0.06 ^{BB}	2.60±0.08 ^{BB}	2.58±0.08 ^{BB}	1.96±0.03 ^{cA}
Insulin (IU/L)	0	33.18±0.94 ^{aA}	32.48±0.57 ^{aA}	34.17±0.82 ^{aA}	32.37±0.59 ^{aA}	33.09±0.97 ^{aA}	19.19±0.70 ^{aA}
	15	33.29±0.83 ^{aA}	29.53±0.61 ^{bcB}	33.10±0.76 ^{aA}	28.25±0.42 ^{CB}	30.33±0.65 ^{BB}	20.13±0.56 ^{dA}
	30	33.37±0.79 ^{aA}	27.24±0.20 ^{BC}	31.88±0.71 ^{aA}	25.98±0.21 ^{BC}	26.46±0.42 ^{BC}	19.57±0.55 ^{cA}
GSH (μmol/ml of packed RBC)	0	0.45±0.01 ^{bA}	0.47±0.02 ^{bA}	0.46±0.02 ^{bA}	0.44±0.02 ^{BB}	0.42±0.01 ^{BB}	0.61±0.01 ^{aA}
	15	0.43±0.01 ^{cA}	0.49±0.02 ^{bcA}	0.47±0.02 ^{bcA}	0.50±0.00 ^{bA}	0.51±0.02 ^{bA}	0.62±0.01 ^{aA}
	30	0.42±0.00 ^{dA}	0.51±0.01 ^{cdA}	0.49±0.02 ^{cdA}	0.53±0.01 ^{bcA}	0.56±0.00 ^{bA}	0.61±0.01 ^{aA}
LPO (nmol MDA/mg Hb)	0	5.92±0.20 ^{aA}	5.73±0.19 ^{aA}	5.43±0.43 ^{aA}	5.71±0.40 ^{aA}	5.99±0.40 ^{aA}	3.99±0.07 ^{bA}
	15	5.92±0.21 ^{aA}	5.22±0.15 ^{bA}	5.02±0.14 ^{bAB}	4.98±0.15 ^{bA}	4.77±0.15 ^{BB}	3.97±0.07 ^{cA}
	30	5.87±0.21 ^{aA}	4.42±0.16 ^{BB}	4.24±0.05 ^{bcB}	4.00±0.11 ^{CB}	4.05±0.08 ^{bcB}	3.95±0.07 ^{cA}
SOD (μmol MTT formazan/mg Hb)	0	0.23±0.01 ^{bA}	0.25±0.01 ^{BC}	0.24±0.01 ^{BB}	0.20±0.01 ^{BC}	0.22±0.02 ^{BB}	0.38±0.01 ^{aA}
	15	0.23±0.01 ^{dA}	0.30±0.00 ^{CB}	0.26±0.00 ^{DB}	0.25±0.00 ^{DB}	0.34±0.00 ^{bA}	0.36±0.01 ^{aA}
	30	0.22±0.00 ^{dA}	0.34±0.01 ^{abA}	0.31±0.01 ^{bcA}	0.29±0.01 ^{ca}	0.35±0.01 ^{aA}	0.37±0.01 ^{aA}
CAT (K/mg Hb)	0	3.97±0.14 ^{bA}	3.41±0.11 ^{BC}	3.99±0.32 ^{BB}	3.56±.40 ^{BC}	3.32±0.28 ^{BC}	6.93±0.10 ^{aA}
	15	4.04±0.09 ^{dA}	4.04±0.14 ^{DB}	4.35±0.21 ^{cdB}	4.52±0.12 ^{CB}	5.04±0.10 ^{BB}	6.92±0.10 ^{aA}
	30	3.88±0.11 ^{eA}	4.50±0.14 ^{cA}	5.10±0.07 ^{dA}	5.34±0.12 ^{bcA}	5.63±0.03 ^{bA}	6.92±0.10 ^{aA}

Values with superscript ^{a,b,c,d} differ significantly (P<0.01) in same row; Values with superscript ^{a,b,c,d} differ significantly (P<0.01) in same column.

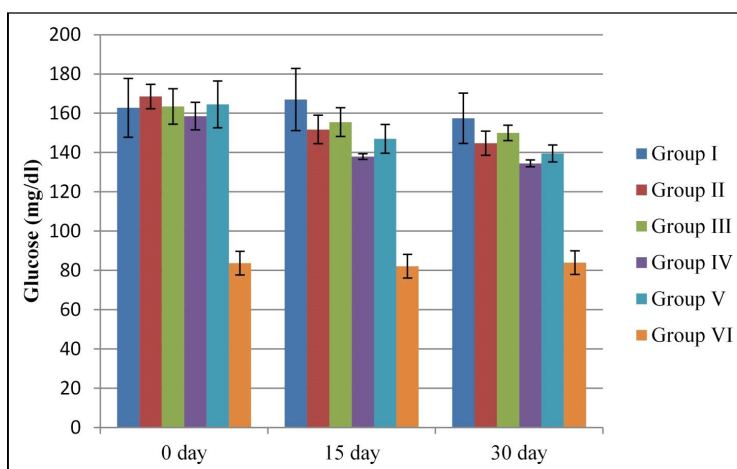


Fig 1: Effect of different treatments on random blood glucose (mg/dl).

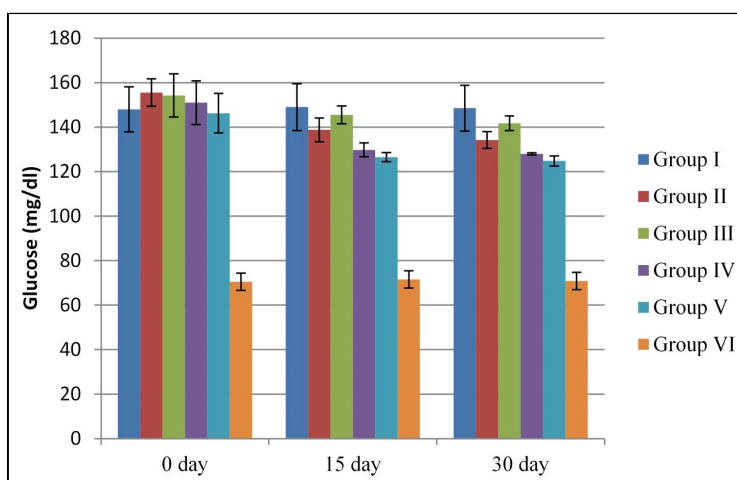


Fig 2: Effect of different treatments on Fasting blood glucose (mg/dl).

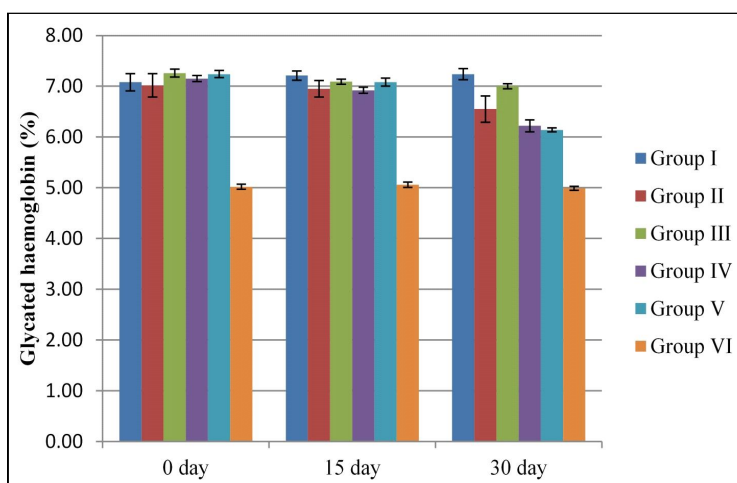


Fig 3: Effect of different treatments on glycated haemoglobin (%).

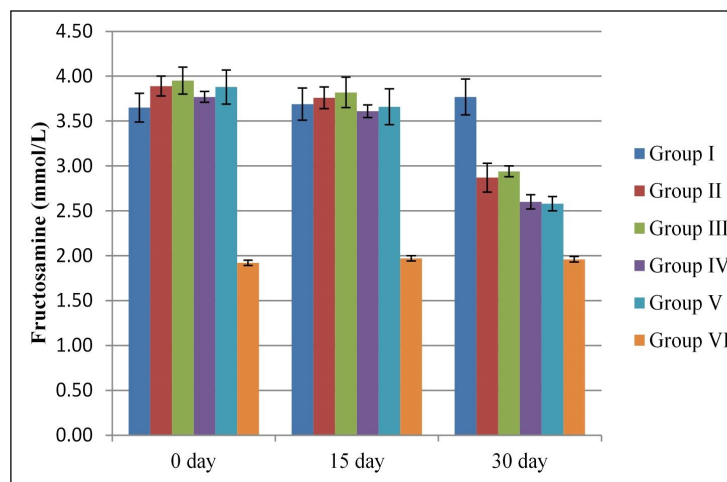


Fig 4: Effect of different treatments on serum fructosamine (mmol/L).

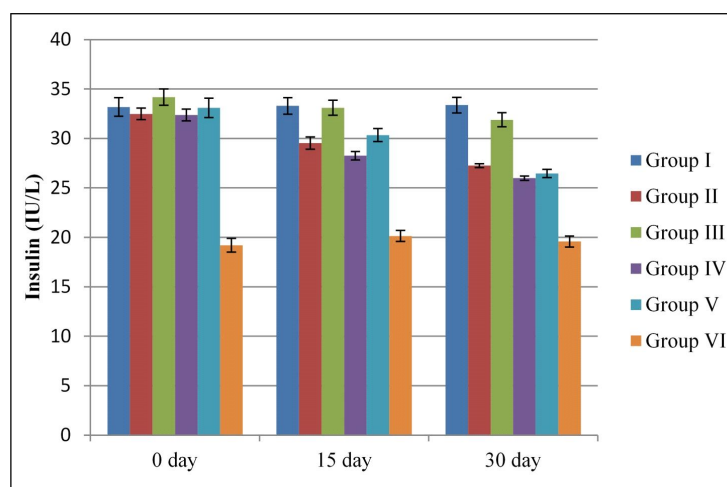


Fig 5: Effect of different treatments on serum Insulin (IU/L).

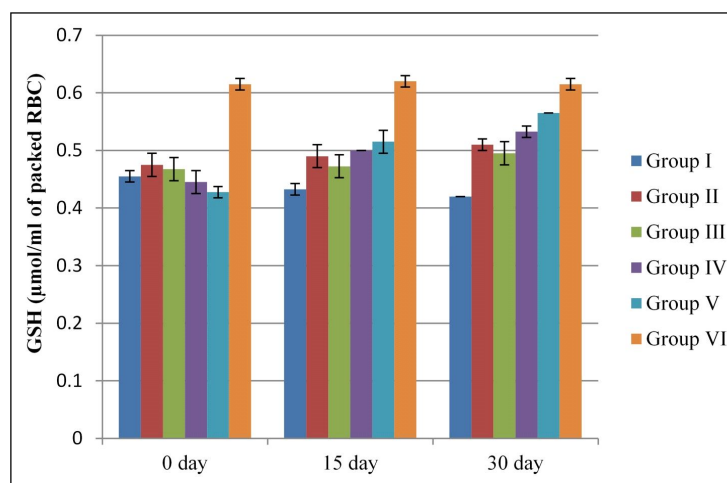


Fig 6: Effect of different treatments on GSH ($\mu\text{mol/ml}$ of packed RBC).

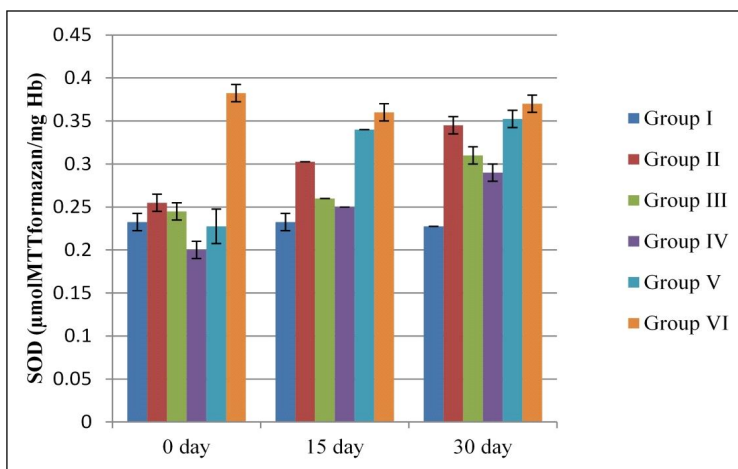


Fig 7: Effect of different treatments on SOD ($\mu\text{mol MTT formazan/mg Hb}$).

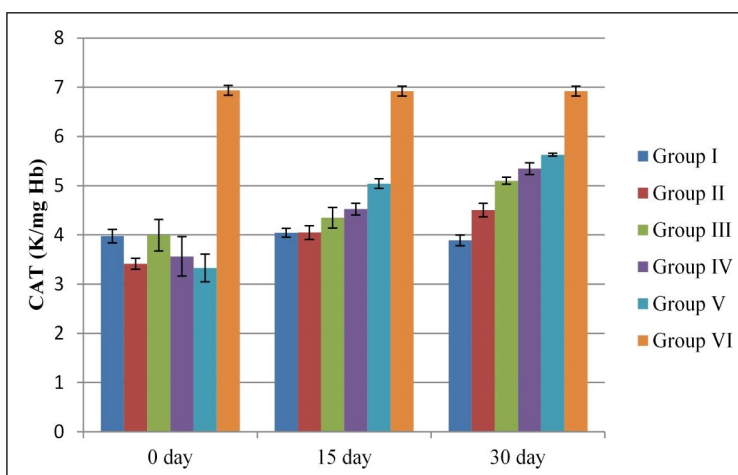


Fig 8: Effect of different treatments on CAT (K/mg Hb).

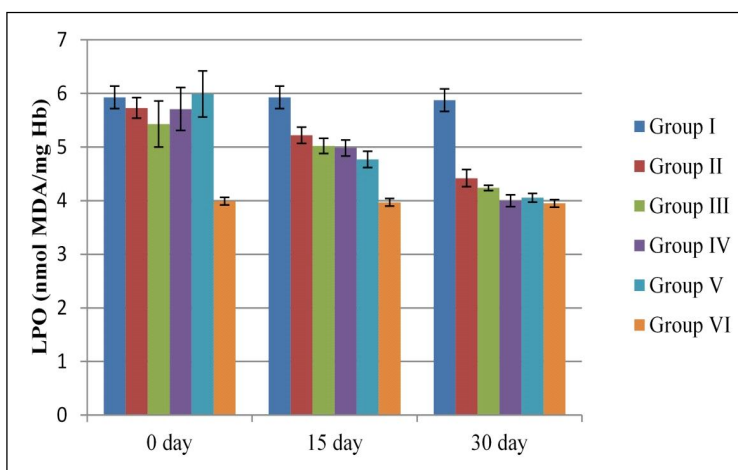


Fig 9: Effect of different treatments on LPO (nmol MDA/mg Hb).

Deepa (2014) and Kaneko *et al.* (2008). Elevated glycated hemoglobin (HbA1c) is important predictive biomarker of chronic hyperglycemia, whereas, the serum fructosamine provide the previous 2-3 weeks' estimate (Mared *et al.*, 2012). Recently canine HbA1c has shown least correlation with haematological parameters and therefore it can be suggested as a reliable biomarker of canine diabetes (Neumann, 2020). Canine diabetes mellitus is usually compared to latent autoimmune diabetes mellitus in human beings; initial hyperstimulation of pancreatic β cells followed by insulin deficient phase due to β cell exhaustion (Shanik *et al.*, 2008). The present study explains that the antioxidant therapy can curb this pathological mechanism by modulating oxidative metabolisms preventing exhaustive damage to β cells. Similarly, the modulation of host immune mechanisms by the antioxidant N-acetylcysteine in Th1 cytokine dominant disease conditions was previously suggested by Ajith *et al.* (2017). Also, the human form of IDDM is usually considered as a Th1 dominant condition with increased expression of Th1 cytokines (Eizirik *et al.*, 2009). Hence, early detection and strategic management of subclinical DM using better antioxidants is necessary for preventing its progression to clinical form, improving lifespan and ensuring better quality life.

CONCLUSION

Subclinical diabetes mellitus in dogs is a disease condition which needs more attention from clinical veterinary practitioners. Significant oxidative impairments were observed in even subclinical stages of diabetes mellitus and may be elaborated in the pathogenesis of clinical diabetes. N-acetylcysteine, *T. chebula* and *W. somnifera* ameliorate oxidative stress in subclinical diabetes mellitus and prevent further evolution of the disease. Hence, the current study established the antidiabetic potential of *W. somnifera* and *T. chebula* via improved oxidant-antioxidant profile. Further, they prevented damages on the body system resulted from appropriate glucose utilization, which itself is a triggering force for free radical generation and unstable body system. The present study emphasizes the early detection of subclinical form of diabetes and stress the need for antioxidant therapy in preventing progression of the clinical disease.

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