Research Article

Effect of Standardized Polyherbal Formulations on Blood Glucose, Body Weight, Food and Water Consumption of Rat

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A B S T R A C T

Background: Standardization of polyherbal formulation is mandatory to evaluate the best quality of drugs for therapeutic value. The main objective of this study was to develop a standardized, safe and effective polyherbal juice with significant anti-oxidant and anti-diabetic activity. **Methods:** Polyherbal juice was formulated using cold maceration method and was evaluated for Total phenolic content, Total flavonoid content, Antioxidant activity, Particle size, IR, HPTLC and Heavy metal analysis. **Results:** Total phenolic content was found to be 139.23 ± 0.072 mg/g gallic acid equivalent and Total flavonoid content was 62.01± 0.063 mg/g Catechin equivalent. In DPPH scavenging assay the RC50 value of polyherbal formulation was found to be 6.0 μg/ml. Preliminary phytochemical tests and HPTLC was performed for Quantitative Analysis of Phytoconstituents in formulation. **Conclusion:** In-vivo study shows significant anti-diabetic activity as compared with marketed formulations. Thus polyherbal formulation possesses good anti-diabetic and antioxidant activity.

Introduction

Ayurveda is one of the world's oldest alternative systems of medicine. It was originated and evolved in India before over thousands of years. It deals with both the prevention and cures the diseases of human beings in most systematic way and presents a close similarity to WHOs concept of health put forwarded in the modern era.[1,2] The polyherbal formulation contains combination of herbs and plant material. Many of traditional medicines have been tested for their anti-oxidant property. Free radicals are responsible for lots of disorders in humans including hyperlipidaemia, atherosclerosis, diabetes, arthritis, ischemia and repercussion injury of many tissues, gastritis and cancer. The factor cause depletion of the immune system are change in gene expression and induce abnormal proteins, environmental pollutants, radiation, chemicals, toxins, deep fries, physical stress and free radicals. The oxidation process is the most prominent routes for generation of free radicals in food, drugs, and even living systems. Antioxidants are main components which have the capacity of protecting organisms from damage caused by free radical generated oxidative stress. The antioxidant property of phenolics is majorly contributed to their redox properties, which facilitates them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators.[1]

Collection, Authentication and Identification of Plant Material

Neem (*Azadirachta indica*), Triphala (*Emblica officinalis, Terminalia belerica, Terminalia belerica),* Tulsi (*Ocimum santum*), Ginger (*Zingiber officinalis*), Cinnamon (*Cinnamonum zeylanicum*), Liquorice (*Glycyrrhiza glabra*) was purchased from local market of kopargaon. Identification and authentication was carried out in S.S.G.M.College Kopargaon. Fresh plant material was washed with tap water, air dried and then homogenized to fine powder and stored in container.

Chemicals

Alloxan (Loba Chemie Pvt. Ltd. Mumbai), Glimiperide (Ipca Laboratories Ltd., Mumbai), Diagnostic kit for analysis of glucose was procured from Merck limited, Mumbai. DPPH reagent, Research lab Fine chem. industry Ltd, Mumbai. Ascorbic acid, Research lab Fine chem. industry Ltd, Mumbai.

Plant Material Analysis As Per WHO Guidelines
Preliminary phytochemical screening

Polyherbal juice was subjected to preliminary phytochemical screening for detection of various constituents. Test for alkaloids, flavonoids, glycosides, saponin and tannins were performed to estimate certain phytochemicals.[3-4]

Physicochemical parameters for standardization as per WHO guidelines

Ash values, Extractive value, Moisture content, Loss on drying help in detection of adulteration and thus establish standards for herbal drugs.[5-6]

Table 1. Formulation of polyherbal Juice.

Sr. No	Ingredients	Quantity (g)
1	Neem	167
2	Triphala	66.66
3	Jestamadh	66.66
4	Cinnamon	66.66
5	Tulsi	66.66
6	Ginger	66.66
7	Vehicle	q.s 1000ml

Formulation of Polyherbal Juice

All the plants were powdered and subjected to cold maceration for duration of 72 hours using distilled water as a solvent. It was followed by filteration and addition of sodium benzoate as a preservative. The formulation was then stored in container.[7]

Composition of Marketed Anti-diabetic Formulation A and Marketed Formulation B is as follows:

Marketed Formulation A:

Neem (10%),Giloy(5%), Gudmar(10%), Karela(10%), Kutki(1%),Jamun(10%), Methi (5%),Sagargota(10%), Jangli halad(6%), Tulsi(5%), Saptrangi(5%), Gorakhmundi(5%), Dalchini(5%), Chandraprabha vati (2%), Shilajit(1%), Triphala(120%)

Marketed Formulation B:

Neem (10%), Giloy(5%), Gudmar(10%), Karela(10%), Kutki(1%),Jamun(10%), Methi (5%), halad(6%), Tulsi(5%), Trivang bhasma(1%), Yashad Bhasma, Sarpagandha (2%)

Materials and Methods:

Estimation of Total phenolic content

The total phenolic content was estimated by Folin-Ciocalteau reagent as method described by Singleton and Rossi (1965). Gallic acid stock solution (1000µg/ml) was prepared by dissolving 100 mg of gallic acid in 100ml of ethanol. Various dilutions of standard gallic acid were prepared from this stock solution. Folin-Ciocalteau reagent was prepared by mixing Folin reagent with Phenol reagent (1:1), and diluted 1:1 in distilled water, before use.Calibration curve was plotted by mixing 1ml aliquots of 20, 40, 60, 80, 100, 120 and 140µg/ml of gallic acid solution with 5.0 ml of Folin-Ciocalteu reagent (diluted tenfold) and 4.0 ml of sodium carbonate solution (75g/l). The absorbance was measured after 30 min at 20 degree at 765nm. One ml of extract was mixed separately, with the same reagents as did in construction of calibration curve, and after 30 min, the absorbance was measured for the determination of total phenolic compound using formula:

$$C = C1 * \frac{V^9}{M} \qquad \text{Eq.(1)}$$

Where, C= Total content of phenolic compounds in mg/g, in GAE (gallic acid equivalent);
C1= the concentration of gallic acid established from calibration curve in mg/ml; V= Volume of extract in ml; M= The weight of plant extract in g.[9]

Total Flavonoid Content

Total flavonoid content was determined by colorimetric method. 0.25ml aliquot of methanolic extract was diluted with 1.2ml of distilled water, 1ml of 5% $NaNO_2$ solution was added to the mixture and after 6 min 1ml 10 $AlCl_3$ H_2O solution was added .The mixture was allowed to stand for 5 min and next 0.5 ml of 1 M NaOH was added and total volume make up to 5ml with distilled water. The solution was mixed well and absorbance was measured immediately against the blank at 510 nm. The result may express as mg catechine equivalent.[9-10]

Anti oxidant activity by DPPH radical scavenging assay

The effect on DPPH radical was estimated using the method of Liyana-Pathiranan and Shahidi. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0ml of juice in methanol. The reaction mixture was left in the dark at room temperature for 30min. The absorbance of the mixture was measured spectrophotometrically at 517nm. Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abscontrol - Abssample)]/ (Abscontrol)] x 100 where Abscontrol is the absorbance of DPPH radical + methanol; Abssample is the absorbance of DPPH radical + sample extract /standard.[11-12]

HPTLC

Preparation of Standard Gallic acid solution

10mg of Gallic acid was accurately weighed into 10ml of volumetric flask and then solution was made upto 10ml with methanol. From this stock solution, 1ml was pipette out and further diluted upto 10ml to obtain final concentration of 100µg/ml.[13-14]

Method

Polyherbal juice and standard gallic acid were spotted on pre-coated silica gel 60 F plates (10×10cm, 0.2mm thickness) as 8mm wide band width by using automatic TLC Applicator Linomat V, 10mm from bottom. Toluene: Ethyl acetate: Glacial acetic acid: Formic acid (20:45:20:05) was used as mobile phase. The plates were kept for saturation in twin chamber for 15min. After development the plates were dried in air and scanned at 280nm for gallic acid using CAMAG Scanner III.[15]

Infrared Spectroscopy

The formulation was sandwiched between polished sodium chloride plates, so as to spread the liquid in a thin layer between the plates, and clamp the plates together. The spectrum was measured and peaks were identified using FT-IR.[14,16]

Particle size analysis

The particle size of formulation was determined by Malvern Zetasizer (Nano ZS 90, UK) using acetone as dispersion medium. This technique yeilds the mean particle diameter and the range of particle size distribution. (Polydispersity index, PDI)[15]

Heavy metal analysis

Analysis of heavy metals like Lead, Cadmium, Arsenic and Mercury was outsourced from IIT POWAI using Inductively Coupled Plasma Atomic Emission Spectroscopy.[17]

In-vivo Anti-diabetic Activity
Experimental Animals

Wistar rats weighing 150 – 180 g of either sex were procured from the Laboratory Animal Resource Section of Institute of Pharmaceutical Education and Research, Wardha, India. The animals were housed in 37cm x 23cm x 16cm polypropylene cages with maximum 3 animals per cage and acclimatized for a period of 7 days. Individual animal was identified by a mark on tail with permanent marker and cages were identified with label pasted on cages with relevant information. Animals were housed at a temperature of 24 ± 2 ºC and relative humidity of 30 to 70 %. A 12:12 light: dark cycle was followed. All animals had free access to water and standard pelleted laboratory animal diet. The experimental protocol was approved by the Institutional Animal Ethics Committee.[18]

Induction of Experimental Diabetes

Rats were made diabetic by single administration of Alloxan monohydrate (120 mg/kg/i.p) dissolved in normal saline. Forty-eight hours later, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only those animals which showed hyperglycemia (blood glucose levels>250 mg/dl) were considered as diabetic and used in the experiment.[19]

Formulation Dosing

Formulations were administered in the form of suspension. Animals were deprived of food for 2 hrs before dosing. All formulations were administered in a single dose orally by gavage using a syringe fitted with suitable sized canula. Volume of formulation was 0.2 ml for 150 g rat. Actual amount was decided on body weight basis. After administration animals were fasted for 1hr.

Groups of animal

Diabetic rats were randomly divided in five groups (Group 2 to 6) and group 1 was of normal animals. These animals were given treatment as follows:
Group 1: Normal animals
Group 2: Control treated with vehicle (Distilled water)
Group 3: Standard treated with Glimiperide (8 mg/kg)
Group 4: Sample 1 treated with polyherbal formulation (1 ml/kg)
Group 5: Sample 2 treated with Marketed formulation A (1ml/kg)
Group 6: Sample 3 treated with Marketed formulation B (1 ml/kg)
Treatment was given once daily for 28 days.

Determination of blood glucose

Blood samples were collected 1h after the last dose administration through the retro-orbital plexus. Chloroform was used to create drowsiness in experimental animals to ease the blood withdrawal. Blood glucose was analyzed by Autoanalyzer (Microlab 2000) using standard diagnostic kits.

Statistical Analysis

The results were expressed as mean±S.D. All the data were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's t-test. A value of $P < 0.05$ was considered statistically significant.[20]

Results and Discussion
Preliminary Phytochemical Screening

Polyherbal juice was evaluated for presence of various phyto-constituents. Phytochemical analysis shows the presence of flavonoids, phenols, alkaloids, carbohydrates, saponins and therapeutically active phyto-constituent.

Physicochemical parameters for standardization

Physiochemical parameters including Loss on drying- 7.6%, Total Ash- 7.2%, Water soluble ash- 28.5%, Alcohol soluble extractive- 31.05% complies with the standards.

Quantitative Phytochemical Screening
Estimation of Total phenolic content (TPC)

Total Phenolic Content was calculated as a gallic acid equivalent and was found to be 139.23 ± 0.072 mg/ml. The relatively good amount of Total Phenolic Content in formulation indicates the good anti oxidant property of the formulation.

Total Flavonoid Content (TFC)

Total flavonoid content was calculated as a catechin equivalent and was found to be 62.01±0.063 mg/ml. The flavonoids also have significant anti-oxidant activity. The strong correlation between antioxidant properties and TPC/TFC in polyherbal formulation indicates that polyphenols and flavonoids are major components which are fundamentally responsible for the significant antioxidant potential of the polyherbal

formulation. Both of the components are known for their beneficial effects on health of human being.

DPPH radical scavenging activity

In DPPH scavenging assay, the RC50 value was found to be 6.0μg/ml whereas % DPPH radical scavenging activity for polyherbal juice was found to be 66.63% and that of marketed polyherbal formulation A and B was 53.04% and 55.95% respectively.
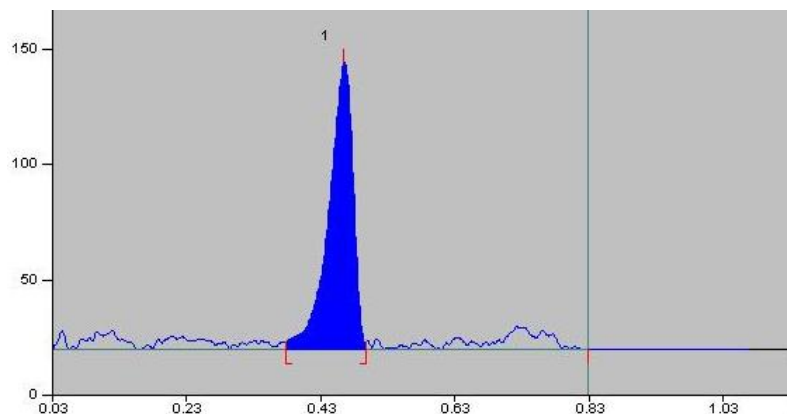
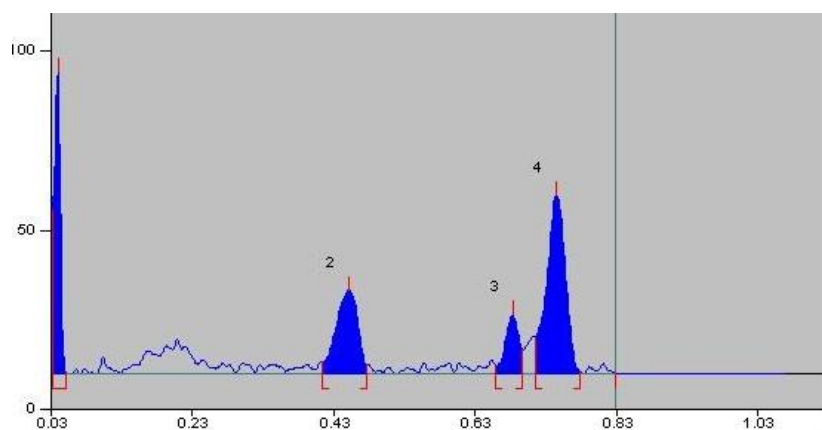**Figure 1.** HPTLC Chromatogram of standard gallic acid.

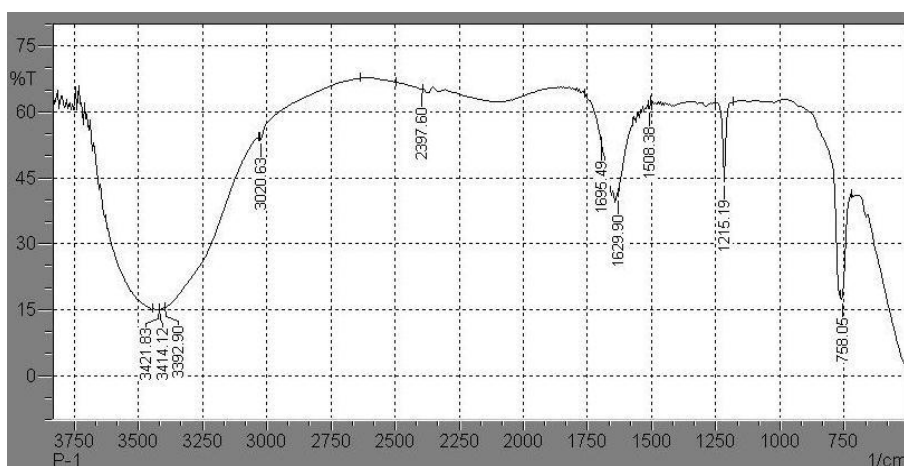**Figure 2.** HPTLC Chromatogram of polyherbal formulation.

Figure 3. FT-IR Spectra of polyherbal formulation.

HPTLC

HPTLC was performed using Toluene:Ethyl acetate:Gallic acid:Formic acid (20: 45: 20: 05) as a mobile phase. Rf value of marker and formulation was found to be 0.48 which indicates significant presence of gallic acid in formulation. Chromatogram was plotted as Absorbance on x-axis against retention time on y-axis.(Figure 1-2)

FT-IR Spectroscopy

For confirmation of Gallic acid present in polyherbal juice, the IR spectrum was recorded by applying sample spot on KBr film and major peak in spectrum was observed.(Figure 3)

Interpretation of FT-IR Spectra of Gallic acid gives following observations-

3414- O-H strech of carboxylic acid, 3020- C-H strech of alkene (out-of-plane bend), 1629- C=C strech of aromatic ring, 758- C-H strech in armoatic ring ((out-of-plane bend), 1695- C=O of carboxylic acid. The Infrared spectra was plotted % Transmittance against wavelength.
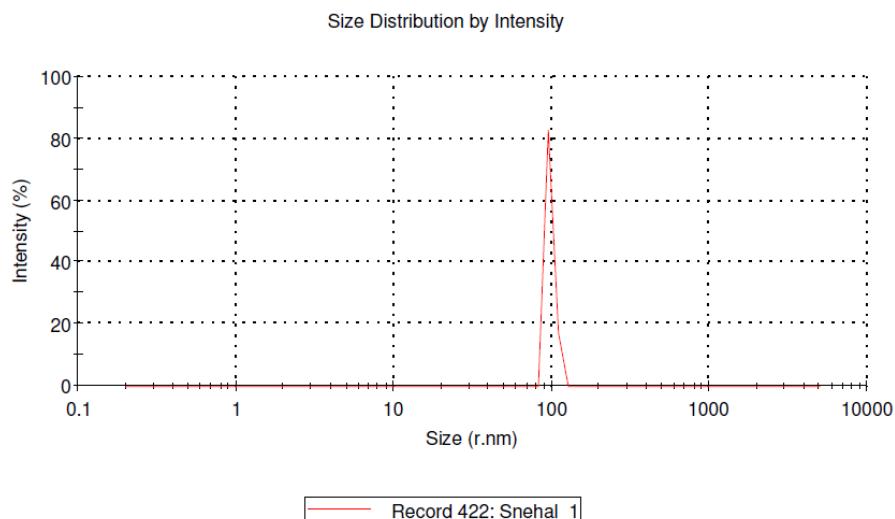

Figure 4. Particle Size analysis of polyherbal formulation.

Particle Size Analysis

The PDI of formulation was found to be 0.940 and particle size was 1131nm. The particle size determines the contact surface available for interaction with the solvent used to obtain the plant derivative. It is a preliminary and important parameter for choosing the appropriate extraction process, as it has a direct influence on its efficiency. The less particle size of polyherbal formulation facilitates more permeation of active principles in to different part of body.(Figure 4)

Heavy Metal Analysis

Polyherbal formulation was analysed by ICP-AES for estimation of Lead, Cadmium, Arsenic and Mercury. No significant concentration (less than 0.01ppm) of heavy metals was found. The levels of heavy metals, such as arsenic, cadmium, chromium, lead and mercury in the polyherbal formulation was within the World Health Organization (WHO) limits

In-vivo anti-diabetic activity
Effects on blood glucose

Table 2 show the blood glucose level that was measured in normal and experimental rats at 0, 7th, 14th, 21st, and 28th day of administration. Alloxan-treated diabetic rats showed significant increase in the levels of blood glucose when compared to normal rats ($p<0.01$).

Oral administration of Glimeperide (8 mg/kg), Polyherbal formulation (1 ml/kg), Marketed formulation A (1 ml/kg), and Marketed formulation B (1 ml/kg) dose once daily for 28 days showed significant decrease in blood glucose level when compared with Diabetic control group ($p<0.01$). However mean decrease in blood glucose level was more in Polyherbal treated group compared to both marketed formulations. The observed effects might be achieved by stimulating insulin release from pancreatic β-cells, inhibiting glucose absorption in the gut, stimulating glycogenesis in the liver, and/or it may be due to increasing glucose utilization by the rat body. The polyherbal formulation also exhibited significant antioxidant activity, which may restore the enzymatic functions, and help in repair and regeneration of pancreatic islets and the alleviation of liver and renal damage. The observed antidiabetic activity might be due to various types of medicinally active components from various plants and its related mechanism of action. Therefore, polyherbal formulation may be beneficial to treat metabolic disorders.

Effects on body weight, food and water intake

Table 3 shows the result of body weight in diabetic rats. There was significant reduction ($p<0.01$) in body weight of diabetic animals on day 14, 21 and 28 when

compared with normal rats. Treatment of animal with Glimeperide (8 mg/kg) shown significant (p<0.01) increased body weight in diabetic animals on day 7, 14, 21 and 28. Treatment of animal with Polyherbal formulation (1 ml/kg) shown significant (p<0.01) increased body weight in diabetic animals on day 21 and 28. Treatment of animal with Marketed formulation A (1 ml/kg) shown significant (p<0.01) increased body weight in diabetic animals on day 28 and treatment of animal with Marketed formulation B (1ml/kg) shown significant (p<0.01) increased body weight in diabetic animals on day 14, 21 and 28 compared to Diabetic control group.

Table 4 and 5 shows the result of effects food and water intake in diabetic animals. Average food and water intake in diabetic animals was increased compared to normal animals. All treatment groups shown decrease in food and water intake. The Body weight, food intake, water intake are improved in polyherbal formulation treated rats compared to diabetic rats indicating the returning of glucose uptake utilization back to normal levels. This observed effects might be due increase in hepatic antioxidant enzyme (SOD, CAT and GSH) levels with a significant reduction of LPO on treatment.

Table 2. Effect of polyherbal formulations on the blood glucose in Alloxan induced diabetic rats.

Groups (n=5)	Treatment	Blood glucose concentration (mg/dl)	
		Initial Day 0	Final Day 28
1	Normal(Vehicle)	80.20 ±10.44	85.30 ±7.75
2	Control(Vehicle)	269.05 ±13.66$^{\#}$	263.40 ±22.62$^{\#}$
3	Standard(Gli. 8 mg/kg)	271.70 ±9.86	111.68 ±9.07*
4	Polyherbal formulation(1 ml/kg)	270.79 ±12.07	147.57 ±16.09*
5	Marketed formulation A (1 ml/kg)	265.97 ±7.41	204.80 ±9.58*
6	Marketed formulation B (1 ml/kg)	273.18 ±12.66	191.38 ±8.81*

Readings are mean values ± S.D. n =5, $^{\#}$ p < 0.01 vs. Normal; * p < 0.01 vs. diabetic control.

Table 3. Effect of polyherbal formulations on the body weight in Alloxan induced diabetic rats.

Groups (n=5)	Treatment	Body weight (g)				
		Day 1	Day 7	Day 14	Day 21	Day 28
1	Normal(Vehicle)	164.2±8.87	170.8±6.97	177.2±7.59	182.8±4.20	187.8±6.76
2	Control(Vehicle)	159.8±13.06	152.8±14.58	142.2$^{\#}$±18.61	137.6$^{\#}$±18.50	131$^{\#}$±16.91
3	Standard(Gli. 8 mg/kg)	178.4±4.03	175.8*±3.34	178.2*±6.72	185.6*±4.33	188*±2.44
4	Polyherbal formulation(1 ml/kg)	168.4±10.35	165.6±10.78	162.4±12.73	166.8*±12.37	171*±11.76
5	Marketed formulation A (1ml/kg)	157.8±12.87	154.4±13.75	149.8±13.27	152.2±11.90	159.4*±11.45
6	Marketed formulation B (1ml/kg)	174.2±8.52	170±9.92	171.8*±9.83	175.4*±10.01	177*±9.38

Readings are mean values ± S.D. n =5
$^{\#}$ p < 0.01 vs. Normal
* p < 0.01 vs. diabetic control.

Table 4. Effect of polyherbal formulations on Food intake in Alloxan induced diabetic rats.

Groups (n=5)	Treatment	AVERAGE FOOD INTAKE (g)				
		Day 0	Day 7	Day 14	Day 21	Day 28
1	Normal(Vehicle)	7.5	9.3	8.5	11.3	9.6
2	Control(Vehicle)	8.3	10.2	16.4	17.4	16.2
3	Standard(Gli. 8 mg/kg)	8.6	12.4	14.3	11.8	11.9
4	Polyherbal formulation(1 ml/kg)	7.3	9.2	12.6	14.7	13.1
5	Marketed formulation A (1 ml/kg)	6.9	12.2	14.1	13.2	15.4
6	Marketed formulation B (1 ml/kg)	9.5	8.4	10.6	12.8	14.3

Readings are average food consumed by rats in g.

Table 5. Effect of polyherbal formulations on Water intake in Alloxan induced diabetic rats.

Groups (n=5)	Treatment	Average Water Intake (ml)				
		Day 0	Day 7	Day 14	Day 21	Day 28
1	Normal(Vehicle)	16.3	15.3	17.5	16.8	18.4
2	Control(Vehicle)	27.3	21.4	24.6	19.3	26.7
3	Standard(Gli. 8 mg/kg)	21.1	18.6	20.2	16.3	14.8
4	Polyherbal formulation(1 ml/kg)	15.5	15.3	18.7	17.2	16.1
5	Marketed formulation A (1 ml/kg)	18.4	16.1	18.6	17.3	17.5
6	Marketed formulation B (1 ml/kg)	16.6	15.9	18.6	14.3	13.2

Readings are average water consumed by rats in ml.

Conclusion

The present investigations revealed that the polyherbal formulation possesses excellent anti-oxidant and anti-diabetic activity. Anti-oxidant activity observed may be due to presence of phenolic and flavonoid components present in prepared polyherbal formulation. The polyherbal formulation when compared with marketed formulations A and B, it revealed that it has more prominent antidiabetic and antioxidant potential than the former two. In animal study it has been observed that the polyherbal formulation may be responsible for increase in body weight (p< 0.01) and average food and water intake in alloxan induced diabetic rats. Thus, polyherbal formulation possesses significant anti-diabetic and anti-oxidant properties and it may be useful in the treatment of diabetes.

Conflict of Interest

The authors report no conflicts of interest.

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