Bioavailability enhancement of Pterocarpus marsupiumby Phospholipids-Complexation of Pterostilbene

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Abstract -

The purpose of research work was oriented on development of effective formulation for diabetic management. Pterocarpus marsupium was reported as plant which extensively used in traditional system of medicine for cure of diabetes. The secondary metabolites which are responsible for pharmacological activity are Phenolics out of which Pterostilbene was reported marker compound. By study it was found that the bioavailability of phytoconstituent was limited. During study the constituent was isolated from plant source and standardized with various spectroscopic techniques. The isolate Pterostilbene was formulated in the form of Phospholipids complex and intended in vesicles carrier system for oral delivery. Phospholipids- complex was characterized by spectrophotometric analysis, melting point determination and solubility data. The formulated vesicles were characterized by TEM (Transmission Electron Microscopy) for size and morphology. Also the entrapment efficiency was determined for various formulation codes.

Pharmacological study performed in vivo on albino diabetic rats for Antidiabetic and hypolipidemic potential evaluation taking Glipizide as standard drug. Pterostilbene and its vesicle are administered in equimoler dose. The biochemical parameter glucose, LDL-C, HDL-C, VLDL-C and TG are determined for comparison between treated and untreated animals. Out of pure Pterostilbene, Ptero-PC complex vesicle and Glipizide, the vesicle shows positive improvement in diabetic symptoms more prominent cooperative to others treatments.

Key words : Ptero-PC complex (Pterosome), Pterocarpus marsupium, Bioavailability

Introduction -

Disease diabetes mellitus is increasing in human population extensively. But no reliable and economic formulation is developed for complete cure of disease. In the Indian traditional system of medicine the heart wood of Bijasar (Latin: Pterocarpus marsupium; Family: Leguminaceae) has been extensively used to treat diabetes mellitus (API, Vol. I). Several polyherbal formulations for diabetes (Diabecon Tab, Diabetomed, D-Fit) contains extract of Heartwood known as Kino extract (Mallavadhani et al. 2003).

Several Polyphenolics and their derivatives have been isolated from the various part of P. Marsupium. (Mourya et al., 2004). It is a rich source of polyphenolic compounds. The key compound includes diarylpropylderivatives, Proterol, the stilbene, Pterostilbene, hydrochalcone, Pterosupine. The phenolics Pterostilbene, Pterosupin, and Marsupine have been identified as the blood sugar lowering components of the heartwood (Manikam et al., 1997). While (-)-epicatechin has been identified as the blood sugar lowering compound in the bark (Chakaravarthy et al.,1985).

The heartwood of P. marsupium contain maximum amount of Pterostilbene (tero-STILL-bean) reported as marker compound (Mallavadhani et al., 2003) along with other phenolic glycosides Isoaurone C- glucoside named as pterocarposide (Handa et al., 2000) and a novel C-glucoside, 1-(2', 6'-dihydroxyphenyl)-ß-D-glucopyranoside present in aqueous decoction of heartwood of (Suri et al., 2003).

Chemically Pterostilbene (4-(2-(3,5 dimethoxyphenyl)ethenyl)phenol) is methoxy analog of Reservtrol (Mikstacka et al., 2010). The compound Pterostilbene is known for multiple activities like antidiabetic (Manikam et al., 1997), hypolipidemic (Pari et al., 2006), anticancer (Mannal et al., 2007;Rimando et al., 2002) anti-inflammatory, antimicrobial, antioxidant (Mikstacka et al., 2010).

Preliminary pharmacokinetic studies revealed poor oral bioavailability of Pterostilbene due to less absorption and decomposition in stomach (Lin et al., 2009). Pterostilbene and its derivatives are Phenolics (lipophilic) in nature and do not solubilize into the intestinal fluid, the systemic availability is limited. That's why their therapeutic activity is reduced and for effective antidiabetic potential higher dose is required.

The Phospholipids complex formulation is used as novel approach for targeted delivery of Pterostilbene and along with dose reduction strategy. This approach is useful

to improve amphiphilicity of phytoconstituent which help us to formulate it as miscible entity in aqueous environment of intestine. Along with that encapsulation by lipid protect it from decomposition in GIT environment. (Bombardelli et al., 1993; Yanyu et al., 2006; Maiti et al., 2007)

Material and Method -

Heartwood of Pterocarpus marsupium tree(Bijasar) is collected from crude drug supplier Jevan Herbals Sagar (M. P.) India. Identification and authentication was done by taxonomist Department of Botany, Dr. H. S. Gour Central University, Sagar (M. P.), India with Herbarium No.- Bot/Her.1913. The soy phosphatidyl choline (Lipoid S 100) was obtained as gift sample from Lipoid (Ludwigshafen, Germany). Alloxan monohydrate was purchased from HIMEDIA laboratory Mumbai. Glipizide was received as gift sample from Ranbaxy Pharmaceutical, Devas, India. All other chemicals were used of analytical grade

Extraction and Isolation -

Powdered and dry heartwood (3.2 kg) was initially defatted by extraction with petroleum ether (600-800) (5 liter) in Soxhlet apparatus. Defatted drug was dried and subjected to subsequent extraction with Ethyl acetate (7 liter) in Soxhlet apparatus. Extraction was done till the whole drug was exhausted then collected extract was filtered and concentration.

Column fractionation, purification, and structural elucidation -

A column was packed using silica gel 60-120 mesh size. The ratio of material ethyl acetate extract and silica gel loaded was 1:20. Column was continuously eluted with Benzene (3 liter) fractions (20ml each) of eluent collected. After complete elution with benzene the solvent consistency was modified to Benzene: Ethyl acetate (19:1) further fractions are collected. Fractions are concentrated and TLC analysis was performed for all collected fractions. Fractions with similar Rf were pooled and concentrated. The collected eluted solution was then evaporated to give yellowish white compound which was recrystalized by Hexane-chloroform to give powdered pure isolated Pterostilbene (Maurya et al. 1984, Mallavdhani et al., 2003).

Characterization of Isolated Pterostilbene -

Characterization of isolated Pterostilbene was done by TLC chromatography

and melting point study. Spectrophotometric analysis was carried out by using double beam U. V. visible spectrophotometer (Cintra-CII-70), FT-I R (SHIMADZU R-8400 S), 1H-NMR spectroscopy on BRUKER AVANCE II 400 NMR spectrophotometer at SAIF Punjab University, by dissolving in CDCl3. All the resulted were compared with the standard Pterostilbene spectrophotometric data (Mallavdhani et al., 2003andMei Lu et al.,2019).

Pterostilbene R_1= CH_3, R_2=CH_3

Reservatrol R_1= R_2= H

Figure 1: Structure of Pterostilbene

Preparation of Pterostilbene-phospholipid complex -

Soya phosphatidylcholine and Pterostilbene in molar ration (1:1) were reacted in 20 ml of anhydrous acetone in round bottom flask which is attached to a reflux cylinder. Solution continuously stirred under mild reflux below 37°C for 3hr. The resulting solution was then concentrated to 5ml. The complex solution was then poured in to hexane for washing and precipitation. Complexed drug with phophotidylcholine settled at the bottom which was dried in vacuum and complex was recovered. Unbounded phospholipids and drug separated with n-hexane (Piffari et al., 2004).

Characterization of Pterostilbene-PC complex

TLC

Pterostilbene and Ptero-PC complex dissolved in methanol and TLC was performed using solvent system Hexane: Ethyl acetate: methanol (7: 3: 1) and 10% sulphuric acid in methanol as spraying reagent. Spots were observed under visible lamp.

Melting Point Study

Melting point of pure Pterostilbene and vacuum dried Ptero-PC complex was determinate by automatic digital melting point apparatus.

Pterostilbene (pure): 84-90°C

Ptero-PC complex: 140-144°C

Ultraviolet spectroscopic analysis

U. V. scan of Pterostilbene, phosphatidylcholine, and Pter-PC complex was performed in between range 200-400nm by dissolving in methanol using double beam

U. V. visible spectrophotometer (Cintra-CII-70).

Infrared spectroscopic analysis

The FTIR spectrum of Pterostilbene, Phosphatidylcholine, physical mixture of Pterostilbene -phosphatidylcholine and Ptero-PC Complex were taken in KBr pellet using Shimadzu Fourier transformed infrared (FT-IR) spectrophotometer (R-8400 S) instrument in the department laboratory.

Nuclear magnetic Resonance spectroscopic analysis

1H-NMR (400 MHZ CDCl3, δ in ppm) spectroscopy of phosphatidylcholine, Pterostilbene and Ptero-P Complex was carried out by using BRUKER AVANCE II 400 NMR (400 MHZ FT-NMR with low and high temperature facility, -90°C to +80°C) at SAIF Punjab University, Chandigarh, India.

Solubility study

Solubility studies were performed by adding an excess amount of the Pterostilbene, soy-PC, and Ptero-PC complex to 10 ml of different solvents and shaking the contents in volumetric flasks.

Conversion of Pterostilbene-Phospholipid complex to Vesicle (Ptero-some)

Ptero-PC complex and Cholesterol were taken in 7:3 ratios in round bottom flask containing Chloroform: Methanol (3:2) as organic solvent. The components were dissolved gently and the flask was attached to rotator vacuum evaporator for the formation of thin film. The rotator evaporator operates at 60rpm (35±20C) solvent evaporated and collected by vacuum. The film formation was done until whole solvent was removed by vacuum and a thin uniform film is formed. The dry film was then hydrated by PBS pH 7.4 with continuous rotation at 50±20C until the whole film dispersed in buffer solution and form milky suspension.

Formed vesicles (Ptero-some) were seen dispersed in the milky suspension when observed under light microscope (40X).

The entrapment efficiency of Ptero-some was calculated as follows:

PDE (Percentage drug entrapped) = [(T-C)/T] ×100

Where T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant.

Table 1:- Entrapment efficiency and Particle size of optimized Vesicle (Ptero-some)

Formulation Code	Molar Ratio of Pterostilbene: PC	% Drug Entrapped	Vesicle Size (µm)
P_4	5:4	61 %	4.45
P_5	5:5	82 %	5.68
P_6	5:6	80 %	4.56

P_4 to P_6 = Optimized Vesicle formulations

Particle Morphology study of Prepared Vesicle (Ptero-some).

Transmission electron microscope (TEM) was used as a visualizing aid for studying the particle morphology. The sample (10µL) was placed on the grid and allowed to stand at room temperature for 90 sec. Excess fluid was removed by touching the edge with filter paper. The samples were examined under a transmission electron microscope (Philips Morgagni 268, Eindhoven, Netherlands) on acceleration voltage of 100 kV, and photomicrographs were taken at suitable magnification, at AIIMS, New Delhi.

In vitro Bioavailability study

In vitro absorption study was perform using everted small intestine sac method Intestine of goat (2-2.5 inch piece) was taken, washed, and freed of intestinal contents and everted using a glass rod. One end of the intestine was fastened using thread while a thread-tied cannula was fitted at another end, and kept in PBS solution (pH 7.4). Two flasks were taken, one of which was filled with 50 ml phosphate buffer saline containing drug (A) and another one with the 50 ml of phosphate buffer saline containing Ptero-PC complex (B); 2.0 ml of mammalian ringer's solution was injected in each of the two intestine pieces and immersed in the separate flasks containing drug (A) and complex (B) solutions. After the specified time interval the serosal fluid of each intestine fragment was assayed for drug content using a UV spectrophotometer by taking absorbance at 308 nm.

In vivo antidiabetic study of Pterostilbene-PC complex

Experimental diabetes was induced in rats by injecting alloxan monohydrate intraperitoneally (i. p.) at a dose of 120 mg/kg body weight in Normal Saline (0.9%). After 72 hr of administration blood was collected from the tail vein under mild ether anesthesia of all surviving rats and blood glucose levels were determined using automatic glucometer (Accu-chek). Rats with blood sugar levels of 200-350 mg/dL were considered as diabetic and were employed in the study (Chakrabarthy et al., 1981, Khosla et al., 1995, Dhanbal et al., 2006, Raphael et al., 2002).

In study - Five groups (contains 6 rats each) of fasted rats were taken: Ist group treated as normal control, 2nd as Diabetic control, 3rd diabetic group received Glipizide (0.05 mg /kg p. o.), 4th diabetic group received Pure Pterostilbene (40mg/kg p. o. as suspension in CMC), 5th diabetic group receives Ptero-PC complex vesicle (equivalent 40mg/kg p. o. in PBS). For fifteen days and after that biochemical parameters such as Glucose level, Lipid profile and creatinin level determined in blood by using span diagnostic kit (GOD-POD and CHOD-POD) (Trinder et al. 1969; Burstein et al., 1970) and auto analyzer respectively.

In statistical analysis, all data are expressed as mean± SEM and difference between groups were considered to be significant using one way ANOVA paired't' test (Analysis of variance) software.

Result and Discussion

TLC study of isolated Pterostilbene compared with standard compound shows similar Rf 0.92 in Toluene: Ethyl acetate: Methanol: Acetic Acid (2.7: 5.0: 2.0: 0.3) as solvent system. Melting point study of isolated Pterostilbene found in rage 84-90 0C. The U. V. spectrum of isolate gives intense absorption at 308nm due to parent ring (trans-Stilbene) absorption. In the spectra low intensity absorption band also found around 219 nm, which is characteristic of n-?* transition, indicating presence of hetero-atom oxygen having unshared pair of electron(Silverstein et al., 1998).

The FTIR spectrum indicates characteristic band at 3201 cm-1 is due to O-H stretching indicating the presence of hydroxyl (O-H) groups in Pterostilbene. The band at 1296cm-1 is due to presence of C-O stretch in compound. The band observed at

1589.40cm-1 and 1458.23 cm-1 are given by aromatic ring due to C-C=C symmetric stretching and C-C=C asymmetric stretching respectively. The band at 3032cm-1 due to C=C-C indicate presence of alkene in between the two aromatic rings. Presence of various distinct peaks at 2831.60cm-1 and 2939.61cm-1 are proof of presence of H-C-H asymmetric, symmetric stretching and C-H aliphatic stretching (Silverstein et al., 1998)Figure 1.

The H1 NMR of isolate shows ? (6.8042, 6.7992) and (6.8158, 6.8209) splited peaks due to benzyl proton at 3, 5 positions are symmetrically equal and a small peak observed at ? 5.5016 due to hydroxyl group proton. Peaks at ? (7.3649, 7.3696) and (7.3816, 7.3863) due to ethylene bond proton (2, 6) indicate both attaches closer to benzene ring. Peak at ? 7.0277 and ? 6.8934 may be due to ? - H and ? -H proton of ethylene bond. Peak at ? 6.6499 and 6.6453 up field due to 2'6' proton and 4' given splited peak (6.3775, 6.3831, and 6.3887). Intense peak observed at 3.8157 due to -OCH3 (2) protons. 1H-NMR study was performed in agreement with the reported literature (Silverstein et al., 1998, Mallavadhani et al., 2003)(Figure 2).

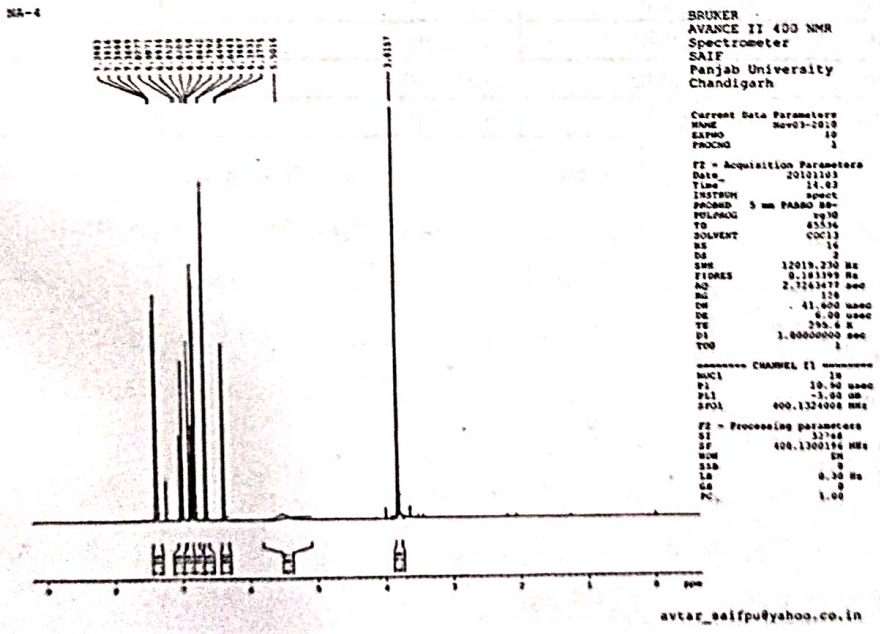


Figure 2: H1NMR Spectrum of Pterostilbene

Characterization of Complex

Formulation of complex done in various ration (Table 1) of Pterostilbene and phosphatidylcholine the optimized formulation contain both of them in 1:1 molar ration. The binding between Pterostilbene and phosphatidylcholine was characterized by TLC analysis using solvent system Hexane: Ethyl acetate: methanol (7: 3: 1) in which Pterostilbene, PC and complex shows 0.86, 0.21, 0.52 different Rf values respectively.

Melting point study shows that complex has higher melting temperature (140-1440C) comperative to pure Pterostilbene (84-900C).

Solubility study Preformed in different solvent verioussolubiliy changes are observed. The hydrophilicity of Ptero-PC complex improved comperative pure drug (Table 2)

Table 2:- Solubility Profile of pure Pterostilbene and Ptero-PC complex

Solvent	Pterostilbene	Phosphotidylcholin	Ptero-PC Complex
Distilled Water	Insoluble	Form Micelle Solution	Form Micelle Solution
Hexane	Soluble	Soluble	Insoluble
Methanol	Soluble	Soluble	Soluble
Ethanol	Soluble	Soluble	Soluble
Acetone	Soluble	Soluble	Soluble
Chloroform	Soluble	Soluble	Partially soluble

The complex was characterized by U. V. , NMR and FTIR spectroscopy

The max of Ptero-PC complex was found in between Pterostilbene and Phosphatidylcholine, showed absorption at 216 nm, representing a significant change in feature of that of the drug as well as phosphatidylcholine due to complexation. Also the blue-Shift (to lower wavelength) in the ?maxof the compound indicates intermolecular hydrogen bonding.

Infrared spectroscopic characterization was done the band at 3201 and 3232 cm-1 in Pterostilbene disappears and band broadening is seen in that range. A newer band appears at 1885 cm-1 due to some stretching and bond formation. The band at 1242.3 cm-1 as shown in soya phosphatidylcholine (Figure 8) due to P=O disappears indicating that an interaction between Pterostilbene and phosphate group of choline

take place in the complex (Figure 3).

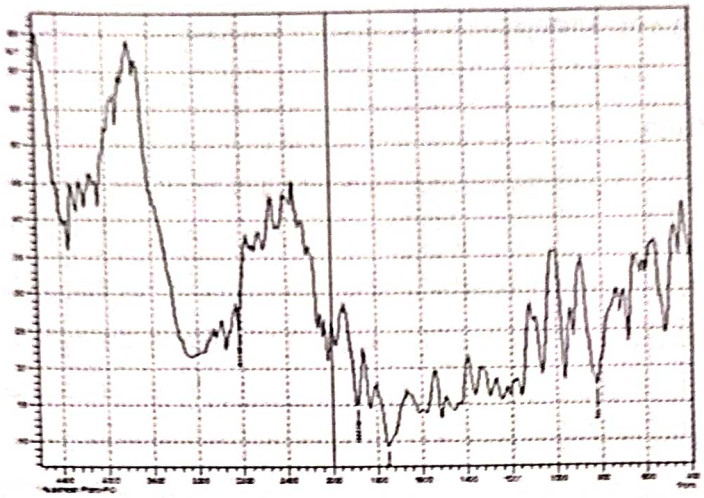
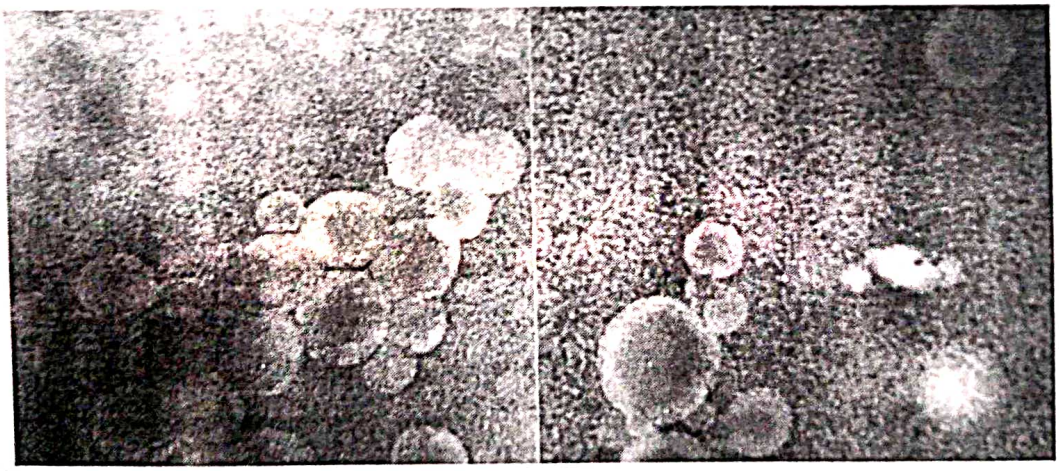


Figure 3: IR spectrum of Ptero-PC complex

Formulation of vesicle was done using phospholipids complex (1:1) and size of the vesicle determined by microscopy at (40X), size found to 5.64 µm. The entrapment efficiency of prepared vesicle was determined which is found significant (82 %) in formulation code P5.

Particle Morphology study of Prepared Vesicle (Ptero-some) performed using TEM photograph taken at optimum magnification which shows that the prepared vesicles are spherical in shape and arranged in group and also individually (Figure 4).



(A) (B)

Figure 4:- Photomicrograph of Ptero-PC complex vesicles (Ptero-Somes).

(A) Group of vesicles are visible in Photograph. (B) Individual vesicle can be observed with drug entrapped

Antidiabetic study

Hypoglycemic activity

Glucose level of normal rats was found 97.89±14.95 on 15th day. Rats treated with alloxan monohydrate (120mg/kg, i. p.) had higher glucose level 288.92±4.93mg/dl. In group treated with standard antidiabetic drug (Glipizide) level was 163.78±4.97 .Pure Pterostilbene administered p. o. reduced glucose level diabetic animal up to some extant (191.51±20.21 mg/dl). Pterostilbene-phospholipid complex (Ptero-some) equivalent p. o. once daily maintain glucose level (150.14±26.78) on 15th day. These values were significantly lower (P<0.001) when compared to the serum glucose level in diabetic control rats (288.92±4.93mg/dl) shown in Table 3 and graphically represent in figure 5.

Table 3:- Study of blood Glucose Level in rats.

Group	Treatment	Glucose Level (mg/dl)			
		Normal	After Alloxan (after 72hrs)	After Drug Treatment	Decrease (%)
1	Normal Control	83.65± 4.54	-	97.89± 14.95	-
2	Diabetic control (Alloxan)	80.36± 5.12	283.74± 15.48	288.92± 4.93*	+1.82
3	Standard Drug (Glipizide)	81.82± 3.01	263.86± 18.78	163.78± 4.97$	-37.92
4	Pterostilbene	82.75± 4.33	285.45± 20.13	191.51± 20.21$	-32.29
5	Ptero-PC complex (Ptero-somes)	97.25± 5.14	272.49± 17.53	151.19± 26.78$	-44.51

Data are expressed as standard mean ± SEM of six rats.

+Denotes increase and - denotes decrease in hyperglycemic activity in comparison with diabetic control groups.

* Values are Statistical significance P<0.01 comparable to normal group.

$ Values are Statistical significance P<0.001 comparable to diabetic control group

Statistical analysis was done by ANOVA paired 't' test on software Graph pad prism 5.

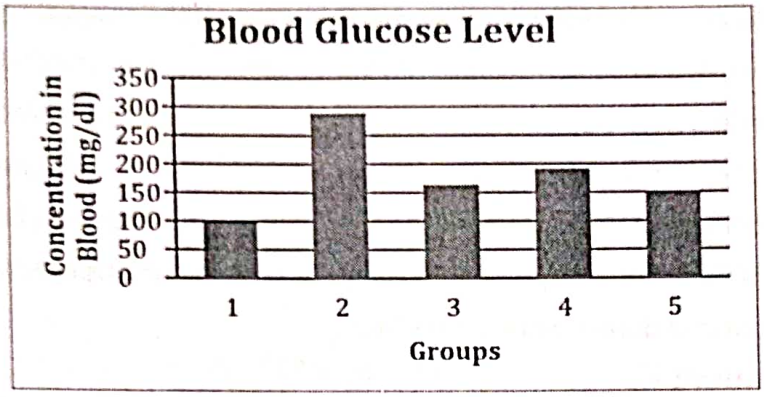


Figure 5: Graphical representation of glucose level in various groups of rats.

Lipid Profile study

The rats of normal group had serum level of triglyceride 76.25±2.97, total cholesterol 100.26±17.08 and HDL 25.71±1.44 measured on 15th day. Diabetic animals have significantly higher (P<0.01) level TG (147.86±4.84), TC (178.51±14.68) due to sub-acute toxicity of alloxan as seen and lower HDL level (15.72±2.78) due to Dyslipidemia.

The isolated Pterostilbene was administered in CMC suspension (40mg/dl, p.o.) has shows lipid profile as TG (114.53±7.32), TC (140.87±6.12) and HDL (22.09±2.79). While Pterostilbene-phospholipid complex (Ptero-some) shows grater improvement in misbalanced lipid profile of diabetic animals. The Ptero-PC complex treated animals has higher level of HDL (28.69±3.82), lowered TG (98.21±4.89) and TC (126.46±5.78) comparative to normal group (P<0.001) Figure 6.

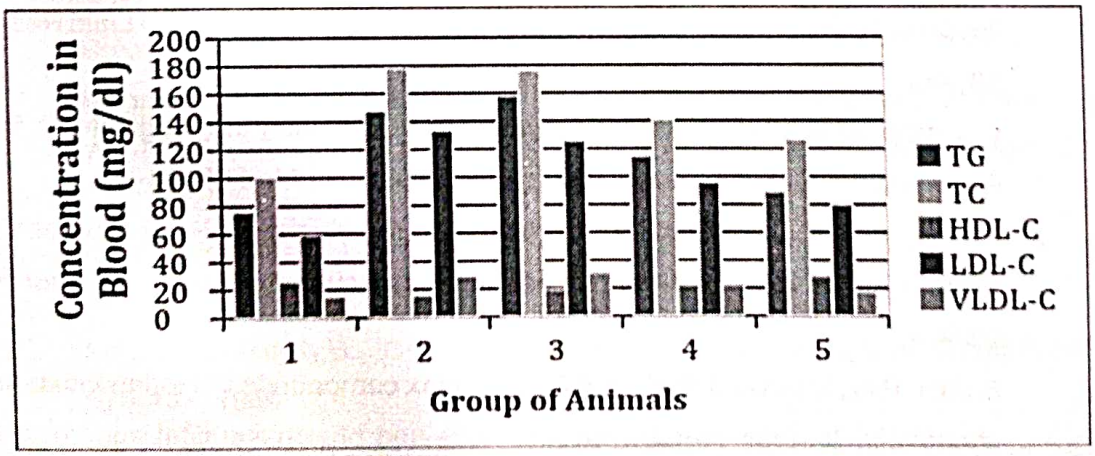


Figure 6: Graphical representation of Lipid profile in experimental rats.

Conclusion

Ptero-PC complex formulated can work as carrier for Pterostilbene to the blood circulation. The vesicle system prepared with the complex has added effect of protection from degradation in GIT, which result in improved absorbtion and bioavilability. The lesser dose of Pterostilbene can be deliver in the form of complex which can reach up to therapeutic concentration. The prepared Ptero-PC complex formulation can prove good for management of diabetic complication like hyperglycemia, hyperlipidemia and diabetic nephropathy produced during diabetes mellitus.

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