INFLUENCE OF TOAD PAROTID GLAND SECRETION FROM INDIAN TOAD (*BUFO MELANOSTICTUS)* IN DIABETIC RATS: AN EXPERIMENTAL EVIDENCE OF P-GLYCOPROTEIN INHIBITION

ABSTRACT:

The study was conducted to improve the bioavailability of glyburide(GLY) with Indian Toad Parotid Gland Secretions (TPGS).P-glycoproteinis an efflux transporter cellular protein and effluxes xenobiotics and drugsto the outside of cells, leads to decreased concentration of drugs at the target site. P-gp inhibitors essentially increase the levels and there is a need of new P-gp inhibitors to develop for the improvement of the oral bioavailability of P-gp substrate drugs because the existing inhibitors have serious side effects. This study was aimed to describe the P-gp inhibitory action from TPGS,*Bufo melanostictus*, in diabetic rats by using glyburide as p-gp substrate.Acute toxicity studies showed 300mg/kg as toxic dose and 50mg/kg was selected as study dose according to OECD 423. LCHRMS study conducted to identify the new compounds. Apparent permeability (P_{app}) was estimated by non-everted sac method (I*n Vitro*) with rat jejunum and ileum to confirm the P-gp inhibitory activity of TPGS. In *in-vivo*protocol rats grouped into 4 groups (n=6), the first one is normal, second diabetic, third GLY 30mg/kg, and fourth group GLY+ TPGS, 50mg/kg for single and multiple dose treatment study.Spectrometric analysisrevealed the new compounds, and TPGS P_{app}($X10^{-6}$ cm/s) in rat jejunum and ileum was significantly increased from 2.0±0.1 to 6.4±0.3 and 1.2±0.3 to 3.0±0.3 respectively. Blood glucose concentration in rats more than 250 mg/dl was considered as diabetic and in single, multiple dose interaction studies (SDI, MDI) the concentrations decreased from 140.0±2.0 and 122.0±2.2 µg/dl respectively. The pharmacokinetic parameters like C_{max}, Cl andin SDI, MDI andsignificant increase of C_{max} and AUC_t and decrease of Cl was observed.The above results conclude that TPGS had the potential P-gp inhibitory activity and improved the oral bioavailability of GLY significantly.Subsequent experimentation is warranted to chemicallycharacterizethe compounds from TPGS as potential new P-gp inhibitors.

Key words:ToxinousTPGS, LCHRMS,*Bufo melanostictus*, P-gp, Glyburide.

1. INTRODUCTION

Inoculation of venomsystem was not developed by the toads, even though they are treated as venomous animals as they secrete highly toxic venomous emanations from their skin.Toads contain alveolar mucus and acinar granular glands with different types of functions[1, 2].Frogs and toads skin, glandular secretions contain bioactive host-defensive molecules from Australian anurans and, thereare several types of glands available for the secretion of these compounds[3-5]. These natural compounds with biological activities like anti bacterial,anti inflammatory and anti cancer activities[6-11] are reported surprisingly there are no studies on Indian Toad secretions. Therefore, as these secretions are unique resources for novel drug development we conducted this study for the first time with Indian Toads. The secretions from mucus gland maintain to make the skin slippery as well as prevent its mechanical damageby some materials [12, 13]and mucus control the body-surface P^H and alsomaintain skin moisture [14]. These secretions are bacteriostatic, can trap microbial and fungal pathogens and also protect from adverse effect of prolonged contact with water and slow down evaporative loss of water. *Xenopuslaeris*, a type of African frog contain different types of mucins with different functions[15].The well developed parotid glands in toads are present in *Buffo* and some other species on the skin and secrets mucins[16].Surprisingly different types of compounds produced from toads and frogs like peptides, steroids alkaloids and many other uncharacterized toxins with variety of biological activities[17, 18]. Toxins from toads and frogs secretions proved to possess cardiotonic, micotoxic and neurotoxic, vascoconstrictive, hypotensive, hallucinogenic effects along with their harmful effects to their predators.Up on a systematic investigations of these secretions will be helpful to develop new chemical entities[19].Permeability glycoprotein (P-g)is aprotein that efflux outthe drugs and chemicals to the outside of cell likely to be presenton all cellsacts as a defense mechanism against harmful substances and is *ABCB1* geneencoded [20, 21].

Many of the drugs are P-gp substrates, because of its effect their concentrations inside the cell not maintained and if any P-gp inhibitor is given in combination, it will elevate the intracellular concentration. P-gp inhibiting drugs increase the concentration of the drugs those are substrates of

P-gp and thus enhance the pharmacological and/or toxicological effects of the substrate drugs. Glyburide (GLY) is a P-gp substrate, and Indian toad parotid gland secretion (TPGS) if exhibits P-gp inhibitory effect, there may bethe possibilityto alter the pharmacokinetics and pharmacodynamics and improve the oral bioavailability of GLY.

2. MATERIALS and METHODS

Acetonitrile (Merck, Mumbai), methanol (Merck, Mumbai), Glyburide (Sigma Aldrich, Bangalore), Potassium dihydrogen phosphate (Sigma Aldrich, Bangalore), Gliclazide (Aurobindo labs), Streptozotocin (Sigma Aldrich, Bangalore), Glucose kit (GOD-POD-KIT) (Viola, Mumbai). Equipments used are, HPLC (contains C18 column coated with 5micron particles), Biofuge (Heraeus instrument- Germany), Micropipettes (Torsons), Micro centrifuge tubes (Tarsons), Butterfly catheter, Ultra sonicator. LC HRMS (Instrument: Agilent Technologies, Modal: 0530).

2.1.**Animals**

Wistar rats procured from Mahaveer Enterprises, Hyderabad, acclimatized then used, with standard diet and water *ad libitum,* and 12 hours fasting was maintained before the start of the study. The protocol was approved by the Institutional Animal Ethical Committee, Kakatiya University, Warangal (IAEC/15/UCPSc/KU/2018).

2.2.**Sample collection and Preparation:**

Adult live Indian Toads (40-50 grams),Order– *Anura*, family – *Bufonidae*,genera – Bufo, sub genara–*melanostictus*were collected from the near vicinity of Kakatiya University, Warangal. *Bufonidae* includes more than 300 species the family includes 25 genera and the above selected species was authenticated by Zoology professor YV from our university. TPGS was freshly obtained by compressing the parotid glands by milking process (it oozes out as white mass) with the help of forceps and hands aseptically from living individual's secretions were collected on the surface of glass petri plates and its containing dried extract was used in the study[22-24] and the Toads were released to live.

2.3.**Spectrometric analysis study by LC HRMS:**

The aliquots of TPGS was dissolved in methanol and subjected for LCHRMS studymass spectra were recorded by Electron Spray Ionization-ESI[25]in the positive ion mode by direct infusion. The Q-TOF MS used a quadrupole (four parallel rods arranged in a square formation), a collision cell, and a time of light unit to produce spectra. Lighter ions accelerate faster down the flight tube to the detector thus determining the ions mass-to charge ratios.

2.4.**Toxicity assessment:**

TPGS suspended in water and toxicity findings were done in accordance with OECD guidelinesin female Wistar rats.Animalsseparated to(4 groups, n=3) normal salinetreated group, TPGS 5mg/ kg/p.o treated group, TPGS 50mg/kg/p.o treated group and TPGS 300 mg/ kg/p.o treated group, then observed for the possible toxicity up to 48 h. The mortality wasnoted to determine lethal dose of TGPS[26-28].

2.5.***In Vitro*** **study**

Non-everted sac method:

Rats were groupedup to 4 (n=4), after overnight fasting intestines were isolated under anesthesia by using thiopental sodium (50mg/kg/i.p), and ice-cold saline was used to flush. The rats were exsanguinated, the jejunum and ileum portions isolated and a segment of each was cut into a length of 10cm for preparation of the sac for the experiment. First group intestinal sacs were loaded with 500µg/mL of fexofenadine (FEX) aP-gp substrate alone and, second FEX+Verapamil(VER) as inhibitor, third FEX+TPGS 1000 µg/ml and fourth FEX+TPGS1500 µg/mlwas included in the sacs for the study protocol and apparent permeability coefficient (P_{app}) was calculated [29].

2.6.***In Vivo*** **Study:**

Rats were subjected for induction of diabetes and more than 200mg/dl glucose concentration was selected as diabetic [30].

Pharmacodynamic and Pharmacokinetic interaction study in diabetic rats:

Animals were grouped into four different sets like first as normal control, second set asdiabetic control,third set as glyburide treated and fourth set as GLY+ TPGS treated.The treatment is given for seven days; the interaction study was done with initial day as single dose and on seventh day as multiple dose study.At the pre set time points blood sampling was done from tail vein (0, 0.5,1, 2, 4, 6, 8, 12 and 24h). at predetermined time intervals between by using butterfly catheter(23GB). Serum was separated by centrifugation. Blood glucose concentrations were estimated by GOD-POD method [31], and pharmacokinetic parameters estimated.

2.7.**Pharmacokinetics evaluation:**

HPLC description:

Reverse Phase HPLC with LC-10AT 2 pumps, UV/VIS detector, CBML-20A, RP C-18 column by using LC solutions software. Statisticaldata was done with the help of WinNonlin latest software and Graph PadPrism software version 8.4.2 and the values are explainedas mean±SD.

3. RESULTS

3.1.**Spectrometric analysis study by LC HRMS:**

The presence of parotid gland is depicted in the Fg1A. The following mass followed by the proposed molecular formula was obtained from LCHRMS studies with TPGS. They are125.9864 ([C4 H N2 O S]+H)+,160.0752 ([C10 H9 N O]+H)+,219.1488 ([C13 H18 N2 O]+H)+,261.0430 ([C9 H6 N7 O S]+H)+,363.1107 ([C21 H16 N O5]+H)+, 437.2082 ([C15 H31 Cl N9 O2 S]+H)+,539.1929 ([C21 H24 N13 O3 S]+H)+,579.2921 ([C30 H38 N6 O6]+H)+,643.3939 ([C33 H46 N12 O2]+H)+,685.4066 ([C40 H59 Cl N O6]+H)+ Fig 1B-D.

Comment [WU1]: Please include a picture or graph of the results from the LC HRMS, the numbers in table form

3.2.T**oxicity Assessment:**

Themortalitywas observed withTPGS 300mg/kg and maximally tolerated dose (MTD) was decided as 50mg/kg/p.o. Table 1.

Table 1. Effect of TPGS of *Bufo melanostictus* for toxicity assessment.

Comment [WU2]: Please add the percentage of live or dead animals

Groups	Treatment	Sign of toxicity (TS/NS)	Mortality (D/S)
Normal control	Normal saline	0/3	0/3
Parotid gland extract	5mg/kg	0/3	0/3
Parotid gland extract	50mg/kg	0/3	0/3
Parotid gland extract	300mg/kg	2/3	2/3

TS= Toxicity Sign, NS=No sign of toxicity, D=Death, S= Survival (n=3).

3.3.***In Vitro* Study:**

TPGS P_{app}($X10^{-6}$ cm/s) in rat jejunum and ileum was significantly increased from 2.0±0.1 to 6.4±0.3 and 1.2±0.3 to 3.0±0.3 respectively Fig.2A.

Comment [WU3]: if you can add a histopathic picture

3.4.***In Vivo*Study:**

Blood glucose concentration in rats more than 250 mg/dl was considered as diabetic and in single, multiple dose interaction studies (SDI, MDI) the concentrations decreased from 140.0±2.0 and 122.0±2.2 µg/dl respectively Fig 2 B. The pharmacokinetic parameters like C_{max}, Cl andin SDI, MDI significantincrease ofC_{max} and AUC $_t$and decrease of Cl was noticed.**C_{max}** of Gly was increased from 1.725±0.299 µg/mL to 4.300±1.160 µg/mL, 7.600±1.071 µg/mL with Gly +TPGS single dose and multiple dose treatment.Clearance **of** Gly was decreased from 3536.9±277.72 mL/h/kg to 1130.409±311.1 mL/h/kg, 576.042±149.991 mL/h/kg with Gly +TPGS single dose and multiple dose treatment Table 2.

Comment [WU4]: if you can add statistical analysis, to find out the differences between groups

Table 2: GLY and GLYalong with TPGS pharmacokinetic parameters with diabetes.

Pk parameter	GLY (SDT)	GLY (MDT)	GLY + TPGS (SDT)	GLY + TPGS (MDT)
C_{max}(µg/mL)	1.725±0.299	3.750±0.810**	4.300±1.160	7.600±1.071**
T_{max}(h)	4.000±0.000	4.000±0.000	4.000±0.000	4.000±0.000
$T_{1/2}$(h)	2.028±0.327	3.951±2.420	2.953±1.270	3.612±1.316
AUC_t(h. µg /mL)	7.263±0.754	18.056±4.561	21.019±5.358**	36.044±3.549**
AUC_∞ (h. µg /mL)	8.519±0.626	28.081±7.665	27.850±6.421**	54.497±12.490**
CL (mL/h/kg)	3536.9±277.72	1146.5±387.9	1130.409±311.1***	576.042±149.991*
Vd (mL/kg)	10395.4±2158.065	5855.008±2245.70	4638.1±1692.73*	2804.3±612.884
Kel (h^{-1})	0.349±0.057	0.219±0.102	0.262±0.086	0.221±0.111
MRT (hr)	3.964±0.087	4.181±0.206	4.301±0.272	4.160±0.381

GLY= Glyburide, TPGS=Toad Parotid Glandular Secretion**.** Mean ±SD: *** p<0.001; ** p<0.01; * p<0.05 tested to GLY; SDT (Single dose treated); MDT (Multi dose treated). One way ANOVA (Dennett test) was used for statistical analysis.

4. DISCUSSION:

Diabetes needs lifelong treatment for the optimal blood glucose concentration to maintain and if not maintained, it may result inother several disorders which affect the major organ systems

in the body. Frog skin glands proved to have many potential compounds with pharmacological effects[32, 33]. LC HRMS study showed the presence of new chemical entities with vide range of mass starting from 100 -800. In *in-vitro* study FEX P-gp substrate[34],and TPGS as testing inhibitor were usedwith jejunum and ileum, in order to confirm the P-gp inhibitory potential.This study evidenced thatFEX, P_{app} was increased with VER as P-gp inhibitor [35] and also with different concentrations of TPGS on jejunum and ileumindicates the P-gp inhibitory effect of TPGS.Current study was undertaken to determine anti diabetic potential of compounds derived from TPGS by usingGLYas a P-gp substrate and to investigate the influence of TPGS on the absorption kinetics of GLY inSTZ induced diabetic model.Theenhanced absorption of GLY was due to P-gp inhibitionby TPGS.Enhanced oral bioavailability by TPGS is related to the inhibition of P-pg substrate drugs by P-gp inhibitors[36, 37].TPGS enhanced the oral pharmacokinetics of GLY suggesting that combined use may reduce the efflux of GLY.This indicates that natural compoundsfrom Indian Toad may inhibit the P-gp driven GLYpumping outside the cell to some extent.

Comment [WU5]: please compare the results of other studies

5. CONCLUSIONS

TPGS was found to be lethal at 300mg/kg. Shown to have P-gp inhibitory activity and enhanced the oral bioavailability of GLY. These results conclude that TPGS is a good source of the new chemical compounds of scientific interest to develop novel P-gp inhibitors. Further studies are warranted to isolate pure compounds as new chemical entities with potential pharmacological activities.

This study gives the scope for further exploring the systematic isolation of new chemical compounds to explore the mechanism of action of TPGS and also their interactions with other P-pg substrate drugs.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Barbosa C, Medeiros M, Riani Costa C, Camplesi A and Sakate M. Toad poisoning in three dogs: case reports. 2009;15:789-798.
2. Blaylock LA, Ruibal R and Platt-Aloia K. Skin Structure and Wiping Behavior of Phyllomedusine Frogs. Copeia. 1976;1976:283-295.
3. Bowie JH, Separovic F and Tyler MJ. Host-defense peptides of Australian anurans. Part 2. Structure, activity, mechanism of action, and evolutionary significance. Peptides. 2012;37:174-188.
4. Langowski JKA, Singla S, Nyarko A, Schipper H, van den Berg FT, Kaur S, Astley HC, Gussekloo SWS, Dhinojwala A and van Leeuwen JL. Comparative and functional analysis of the digital mucus glands and secretions of tree frogs. Frontiers in Zoology. 2019;16:19.
5. Mills JW and Prum BE. Morphology of the exocrine glands of the frog skin. Am J Anat. 1984;171:91-106.
6. Nalbantsoy A, Karis M, Yalcin HT and Gocmen B. Biological activities of skin and parotoid gland secretions of bufonid toads (Bufo bufo, Bufo verrucosissimus and Bufotes variabilis) from Turkey. Biomed Pharmacother. 2016;80:298-303.
7. Obert HJ and Schneider H. [Glands in the skin of the fire-bellied toad (Bombina bombina(L.); Discoglossidae, Anura): type, number, size and distribution under natural and experimental conditions]. Z Mikrosk Anat Forsch. 1978;92:241-72.
8. Qi J, Tan CK, Hashimi SM, Zulfiker AH, Good D and Wei MQ. Toad glandular secretions and skin extractions as anti-inflammatory and anticancer agents. Evid Based Complement Alternat Med. 2014;2014:312684.
9. Tatsunori S, Sakaé K and Noboru Y. Morphology of the Skin Glands of the Crab-eating Frog (Rana cancrivora). Zoological Science. 1995;12:623-626.
10. Toledo RC and Jared C. Cutaneous adaptations to water balance in amphibians. Comparative Biochemistry and Physiology Part A: Physiology. 1993;105:593-608.
11. Wang H, Ran R, Yu H, Yu Z, Hu Y, Zheng H, Wang D, Yang F, Liu R and Liu J. Identification and characterization of antimicrobial peptides from skin of Amolops ricketti (Anura: Ranidae). Peptides. 2012;33:27-34.
12. Welsch U, Storch V and Fuchs W. The fine structure of the digital pads of rhacophorid tree frogs. Cell Tissue Res. 1974;148:407-16.
13. Green DM. Adhesion and the Toe-Pads of Treefrogs. Copeia. 1981;1981:790-796.
14. Dominguez E, Navas P, Hidalgo J, Aijon J and Lopez-Campos JL. Mucous glands of the skin of Rana ridibunda. A histochemical and ultrastructural study. Basic Appl Histochem. 1981;25:15-22.
15. Schumacher U, Adam E, Hauser F, Probst JC and Hoffmann W. Molecular anatomy of a skin gland: histochemical and biochemical investigations on the mucous glands of Xenopus laevis. J Histochem Cytochem. 1994;42:57-65.
16. Sian Rutland C, Cigler P and Kubale V Reptilian Skin and Its Special Histological Structures. IntechOpen, 2019.
17. G. H. *Venomous Animals and Their Toxins*. Springer-Verlag Berlin Heidelberg, 1981.

18. Daly JW, Myers CW and Whittaker N. Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the amphibia. Toxicon. 1987;25:1023-95.
19. Cei JM, Erspamer V and Roseghini M. Taxonomic and evolutionary significance of biogenic amines and polypeptides occurring in amphibian skin. I. Neotropical leptodactylid frogs. Syst Zool. 1967;16:328-42.
20. Huret JL, Minor SL, Dorkeld F, Dessen P and Bernheim A. Atlas of genetics and cytogenetics in oncology and haematology, an interactive database. Nucleic Acids Res. 2000;28:349-51.
21. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL and Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science. 2009;323:1718-22.
22. Neerati P. Detection of antidiabetic activity by crude paratoid gland secretions from common Indian toad (bufomelano stictus). J Nat Sci Biol Med. 2015;6:429-433.
23. Rostelato-Ferreira S, Dal Belo CA, da Cruz-Höfling MA, Hyslop S and Rodrigues-Simioni L. Presynaptic effect of a methanolic extract of toad (Rhinella schneideri) poison in avian neuromuscular preparation. J Venom Res. 2011;2:32-36.
24. Perera Córdova WH, Leitão SG, Cunha-Filho G, Bosch RA, Alonso IP, Pereda-Miranda R, Gervou R, Touza NA, Quintas LE and Noël F. Bufadienolides from parotoid gland secretions of Cuban toad Peltophryne fustiger (Bufonidae): Inhibition of human kidney Na(+)/K(+)-ATPase activity. Toxicon. 2016;110:27-34.
25. Allen DR and McWhinney BC. Quadrupole Time-of-Flight Mass Spectrometry: A Paradigm Shift in Toxicology Screening Applications. Clin Biochem Rev. 2019;40:135-146.
26. Naveen KM and Prasad N. Toxinous Secretions from Indian Toad *(Bufo Meanostictus)* with Potential Antioxidant and Anti-Inflammatory Activities International Journal of Biology, Pharmacy and Allied Sciences. 2020;9:(In Press).
27. Padgaonkar AV, Suryavanshi SV, Londhe VY and Kulkarni YA. Acute toxicity study and anti-nociceptive activity of Bauhinia acuminata Linn. leaf extracts in experimental animal models. Biomedicine & Pharmacotherapy. 2018;97:60-66.
28. OECD. Acute Oral Toxicity-Acute Toxic Class Method, OECD Publishing [Web Page]. Test No. 423.
29. Wada S, Kano T, Mita S, Idota Y, Morimoto K, Yamashita F and Ogihara T. The role of inter-segmental differences in P-glycoprotein expression and activity along the rat small intestine in causing the double-peak phenomenon of substrate plasma concentration. Drug Metab Pharmacokinet. 2013;28:98-103.
30. Akbarzadeh A, Norouzian D, Mehrabi MR, Jamshidi S, Farhangi A, Verdi AA, Mofidian SMA and Rad BL. Induction of diabetes by Streptozotocin in rats. Indian J Clin Biochem. 2007;22:60-64.
31. Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol. 1969;22:158-61.
32. Demori I, Rashed ZE, Corradino V, Catalano A, Rovegno L, Queirolo L, Salvidio S, Biggi E, Zanotti-Russo M, Canesi L, Catenazzi A and Grasselli E. Peptides for Skin Protection and Healing in Amphibians. Molecules (Basel, Switzerland). 2019;24:347.
33. Rodríguez C, Rollins-Smith L, Ibáñez R, Durant-Archibold AA and Gutiérrez M. Toxins and pharmacologically active compounds from species of the family Bufonidae (Amphibia, Anura). J Ethnopharmacol. 2017;198:235-254.

34. Sakugawa T, Miura M, Hokama N, Suzuki T, Tateishi T and Uno T. Enantioselective disposition of fexofenadine with the P-glycoprotein inhibitor verapamil. Br J Clin Pharmacol. 2009;67:535-540.
35. Amin ML. P-glycoprotein Inhibition for Optimal Drug Delivery. Drug Target Insights. 2013;7:27-34.
36. Golstein PE, Boom A, van Geffel J, Jacobs P, Masereel B and Beauwens R. P-glycoprotein inhibition by glibenclamide and related compounds. Pflugers Arch. 1999;437:652-60.
37. Kalsi H and Grewal RK. Interaction of mouse intestinal P-glycoprotein with oral antidiabetic drugs and its inhibitors. Indian J Exp Biol. 2015;53:611-6.

Fig 1. A). Courtesy. NUS _Wiki.nusWeb page. Identified Mass and probable Molecular formula range of TPGS B). 100-300, C).300-600, D).600-800.

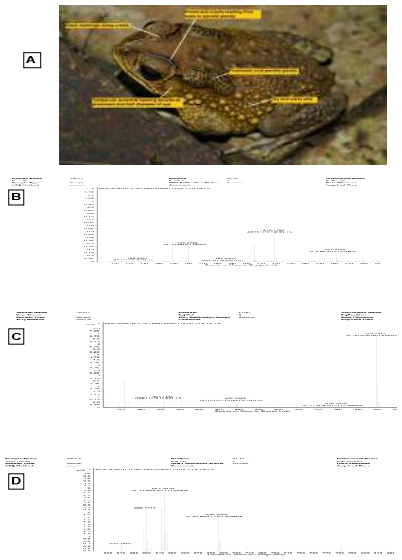

Fig.2 A. Effect of TPGS influence on P_{app} of FEX in rats and data represents (n=6). **B).** Estimation of blood glucose levels in diabetic rats with TPGS and GLY. SDT (single dose treated); MDT (multi dose treated) Statistical analysis was performed using Two way ANOVA (Bonferroni post test) with Mean±Standard deviation.

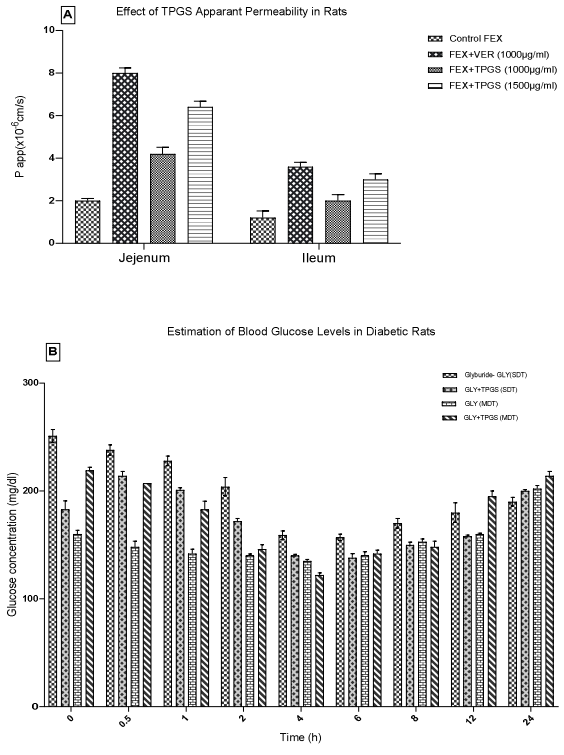

Fig. 3. A, **B**. Pharmacokinetic profiles of GLY and TPGS in rats for 7 days treatment. Each symbol with a bar represents the mean ± S.D (n=6).