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Research Article

Screening of Efficacy of P-gp Inhibitors on Ex-vivo Permeation of Metformin

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ABSTRACT

P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) transporter superfamily that acts as a physiological barrier by removing harmful and foreign substances from cells. Distinct synthetic and natural origin compounds have shown the potential to inhibit P-gp transport activity, leading to increased intracellular drug accumulation, MDR reversal, and improvement of the pharmacokinetic and pharmacodynamic profiles of various challenging molecules. Metformin hydrochloride is a biguanide derivative that is commonly used to treat type 2 diabetes. Metformin has a low oral bioavailability of 50% to 60 %. To overcome these challenges, metformin was used as a Pgp substrate in this research work and used in conjunction with natural P-gp inhibitors. After evaluating the effectiveness of different P-gp inhibitors at different concentration concentrations i.e. Piperine, Ginger, Drumstick, and Verapamil (standard) at (2mg/ml, 4mg/ml and 6mg/ml) by non-everted gut sac study. Piperine was found to be the most potent inhibitor of all because it shows complete release with higher permeation in less time than ginger and drumstick when given in conjunction with Metformin than Metformin alone. The study also revealed that there is no significant difference between the drug permeation of metformin with Piperine and verapamil, where verapamil was used as standard Pgp inhibitor. It was concluded from this research work that Piperine shows significant improvement in percentage drug permeation when compared using the F2 similarity index and its formulation with metformin may offer a simple and safe approach to enhance the pharmacological profile of metformin for effective anti-diabetic therapy in humans.

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INTRODUCTION

P-glycoprotein (P-gp) (P is permeability) is a member of the ATP-binding cassette (ABC) transporter superfamily that acts as a physiological barrier by removing harmful and foreign substances from cells. In humans, P-gp is a small gene family with two isoforms. The MDR1/ABCB1 isoform transports drugs, while the MDR2/3/ABCB4 isoform exports phosphatidylcholine into the bile (Sharom FJ, 2011). P-gp was isolated in Colchicine-resistant Chinese hamster ovary cells in 1976, where it modified drug permeability and showed anticancer resistance (Juliano RL & Ling V, 1976). The research data demonstrated that P-gp has the potential to develop resistance to cytotoxic agents. P-glycoprotein is present in cells other than tumors. It is also expressed in various normal, non-cancerous epithelial and endothelial cells, including the adrenal cortex, the brush border of the proximal renal tubule epithelium, the luminal surface of biliary hepatocytes, pancreatic ductules, and small and large intestine mucosa. The presence of p-

glycoprotein in both the small and large intestines is particularly intriguing.

P-gp is unique in its potential to recognize substrate molecules and rapidly expel them from the gastrointestinal lumen, restricting absorption into the systemic circulation and actively enhancing excretion from the body via biliary and urinary routes. Such studies have facilitated research to inhibit P-gp activities in-order to increase drug delivery and combat drug resistance. Distinct synthetic and natural origin compounds have shown the potential to inhibit P-gp transport activity, leading to increased intracellular drug accumulation, MDR reversal, (Ambudkar SV et al, 2008), and improvement of the pharmacokinetic and pharmacodynamic profiles of various challenging molecules, beneficial in designing clinically useful oral formulations of drugs that, due to poor oral absorption, are administered only via parenteral routes, also affect absorption, distribution, metabolism, and elimination of P-gp substrates in the process of improving pharmacokinetics. Metformin hydrochloride is a biguanide derivative that is commonly used to treat type 2 diabetes. Metformin is absorbed primarily from the upper small intestine after oral administration and has poor bioavailability. Metformin has an absolute bioavailability of 50% to 60%. Its bioavailability problem is attributed to the presence of an intestinal efflux transporter, P-glycoprotein (P-gp), an ATP binding protein (Baetlett MC et al, 2009).

It has a biological half-life ($t_{1/2}$) of 0.9–2.6h. For better treatment outcomes, high dosages of metformin (500 mg two or three times every day, or 850 mg either once or twice every day with or after meals) must be administered repeatedly. As a result, patient compliance decreases, and adverse effects such as nausea, anorexia, diarrhea, weight loss, vomiting, and taste disturbance become more common. Furthermore, biguanides have been associated with lactic acidosis, which can be lethal (Sweetman S ed., 2007). To overcome these challenges, metformin is used as a Pgp substrate in this research and can be used in conjunction with natural P-gp inhibitors. During oral absorption, the rate and amount of drug diffused over the basolateral membrane to reach the systemic circulation are determined by drug characteristics (solubility and permeability) and P-gp efflux through the intestine apical membrane (Choonara Y.E et al, 2015). As a result, P-gp efflux screening is an important phase in the drug development process. (Sparreboom et al, 1997) identified the function of P-gp in reducing Paclitaxel (PTX) oral absorption and facilitating direct excretion of the drug from the systemic circulation into the intestinal lumen. The results demonstrate that the oral bioavailability of Paclitaxel (PTX) increased from 11 % in wild-type mice to 35 % in mdr1a (−/−) mice, while the cumulative fecal excretion decreased from 87 % of the given dosage in wild-type mice to 3 % in mdr1a (−/−) animals. As a result, P-gp efflux is characterized as one of the aspects of drug oral bioavailability and intestinal efflux (Murakami T et al, 2006). Because oral administration is among the most significant and preferred routes of drug delivery, it is significant to overcome the absorption barrier imposed by P-gp.

For oral absorption analysis, the Caco-2 cell line is employed; however, it is very expensive and must be cultivated for many weeks before it can be used, as well as a cell-culture infrastructure is required.

In general, drug transport over the layer mimics drug oral bioavailability in humans (Artursson P & Karlson J, 1991); however the rate of transport of molecules across the cell layer is extremely slow. Because it is widely assumed that the small intestine is the primary site of drug absorption for orally delivered pharmaceuticals, reliable ex-vivo research for examining drug transport across the small intestinal epithelium will be beneficial. The gut sac model has been widely studied for pharmacokinetic studies on drug absorption, drug metabolism or pro-drug conversion in gastrointestinal segments, efflux transport, multidrug resistance, and the effect of efflux transport modulators on drug absorption. The existence of a mucus layer and a relatively high surface area suitable for absorption are benefits of this model (Barthe L et al, 1998). In this study, a chicken ileum gut sac from the intestine was used.

MATERIAL AND METHOD

Material

Metformin HCL was obtained from Aarti Drugs Limited, Sarigam, Gujarat, Verapamil was obtained from Aurore Pharmaceuticals Private Limited Hyderabad, Telangana, India, and Piperine, Drumstick, Ginger, were obtained in standard packs from the local market and other materials for buffer was obtained from S.D Fine Chem Ltd., Mumbai.

Method

Ex-vivo gut sac study (Non-everted)

To evaluate drug transport from the mucosal to the serosal surface, the ex-vivo sac method was utilized. In this research, a small portion of a non-everted intestinal sac of chicken was utilized to demonstrate the efflux mechanism of an anti-diabetic agent. The ileum was collected and sectioned off (10 cm each). The sections were then rinsed with a physiological solution (such as oxygenated tyrode's solution). The ileum was then clamped at 37°C before being injected with a drug solution. The filled intestinal section was then closed by clamping the other end. The filled intestinal sac was placed in a beaker containing 200 ml oxygenated medium at 37°C after another end was clamped. An oxygen supply was provided by the use of an aeration tube. The sampling was performed at different time

intervals and analyzed using UV spectroscopy (Shimadzu) at 234nm.

Fig. 1: Assembly of Non-everted Gut Sac Study

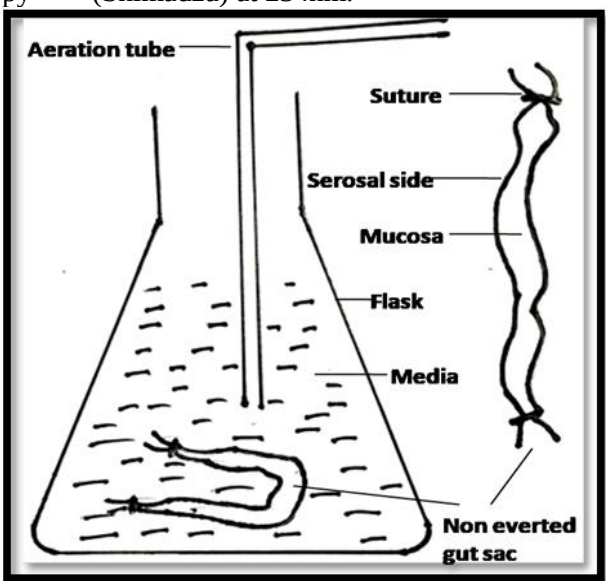

Fig. 2: Schematic Diagram of Non-everted Gut Sac Model

Extraction Method

Ginger

Ginger rhizome was cleaned, rinsed under running water, sliced into little pieces, and dried in the open air. The powdered dried rhizomes were kept at lab temperature (20–23 °C). 0.2g of this powder was macerated in 100 ml of distilled water at room temperature for 12 hours before being filtered. The concentrate had a concentration of 2 mg/ml (Dimo T et al, 2002). Different concentrations were prepared similarly.

Pepper

Piperine was extracted from black pepper seeds utilizing ethanol as a solvent. For 2 - 3 hours, powdered black pepper (0.2 g) was macerated to obtain in 100ml of 20% ethanol at room temperature before being filtered (N. Sreevidya S, 2003). The concentrate had a concentration of 2mg/ml. Different concentrations were prepared similarly.

Drumstick

Dried powder of pods of drumstick (*Moringa oleifera*) (0.2 g) was macerated with 100 ml of a hydro alcoholic solution containing 20% v/v ethanol for 24 hours. The extract was then filtered and used (Sadek KM, 2014).

RESULT

Permeation study

Three natural inhibitors (piperine, ginger, and drumstick) and Verapamil (standard) were studied to check their P-gp inhibition capacity by non-everted gut sac study with drug concentration maintained at 2mg/ml and percentage drug permeation was studied. The data in table 1 reveals that Verapamil and piperine show significant improvement in percentage drug permeation when compared using the *f2* similarity index. At the same time, ginger and drumstick could not show any significant improvement.

Table 1: Metformin Transport in the Non-everted ileum of Chicken in the Presence and Absence of P-gp inhibitors at 2mg/ml Concentration

Time (min)	Met (2mg/ml)	Met+Ver (2mg/ml)	Met+Pip (2mg/ml)	Met+Gin (2mg/ml)	Met+Ds (2mg/ml)
15	9.21	21.22	18.36	10.25	10.02
30	20.35	44.21	36.57	24.34	23.12
45	32.33	68.43	55.64	40.14	39.79
60	47.48	79.86	75.17	58.04	57.66
75	63.34	90.41	84.91	74.12	73.88
90	80.43	99.73	92.50	88.22	87.71
120	90.24	-	99.01	99.16	99.13
180	99.80	-	-	-	-
***f2* value**		**32.07**	**38.32**	**56.34**	**57.24**

Note: Met=Metformin, Ver=Verapamil, Pip=Piperine, Gin=Ginger, Ds=Drumstick

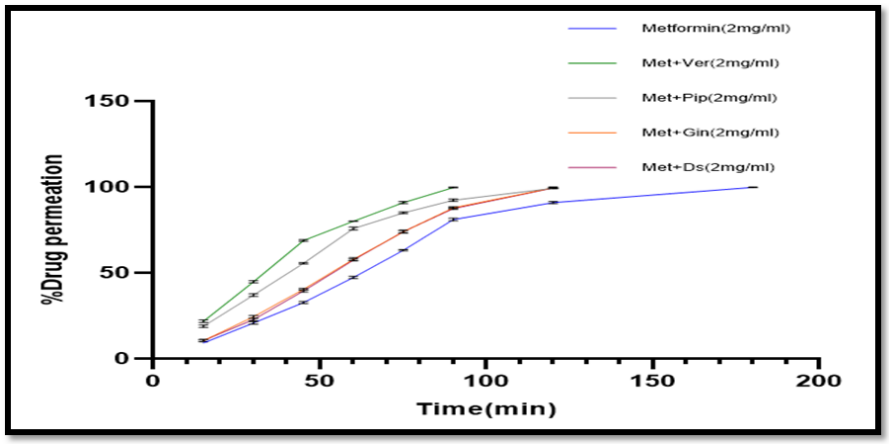

Fig. 3: Permeation Study of Metformin in the Presence and Absence of P-gp Inhibitors at 2mg/ml Concentration

Table 2: Metformin Transport in the Non-everted ileum of Chicken in the Presence and Absence of P-gp Inhibitors at 4mg/ml Concentration

Time (min)	Met (2mg/ml)	Met+Ver (4mg/ml)	Met+Pip (4mg/ml)	Met+Gin (4mg/ml)	Met+Ds (4mg/ml)
15	9.21	23.21	21.05	11.98	10.09
30	20.35	48.47	44.84	25.45	23.32
45	32.33	74.25	69.71	43.21	41.76
60	47.48	88.21	82.66	63.76	61.79
75	63.34	99.80	92.78	78.41	76.23
90	80.43	-	99.05	89.23	88.35
120	90.24	-	-	99.20	99.16
180	99.80	-	-	-	-
***f2* value**		**28.62**	**31.04**	**49.97**	**52.91**

Three natural inhibitors (piperine, ginger, and drumstick) and Verapamil (standard) were studied to check their P-gp inhibition capacity by non-everted gut sac study with drug concentration maintained at 2mg/ml and percentage drug permeation was studied. The data in table 2 reveals that Verapamil and piperine and Verapamil and Ginger show significant improvement in percentage drug permeation when compared using the *f2* similarity index. At the same time, the drumstick could not show any significant improvement at this concentration.

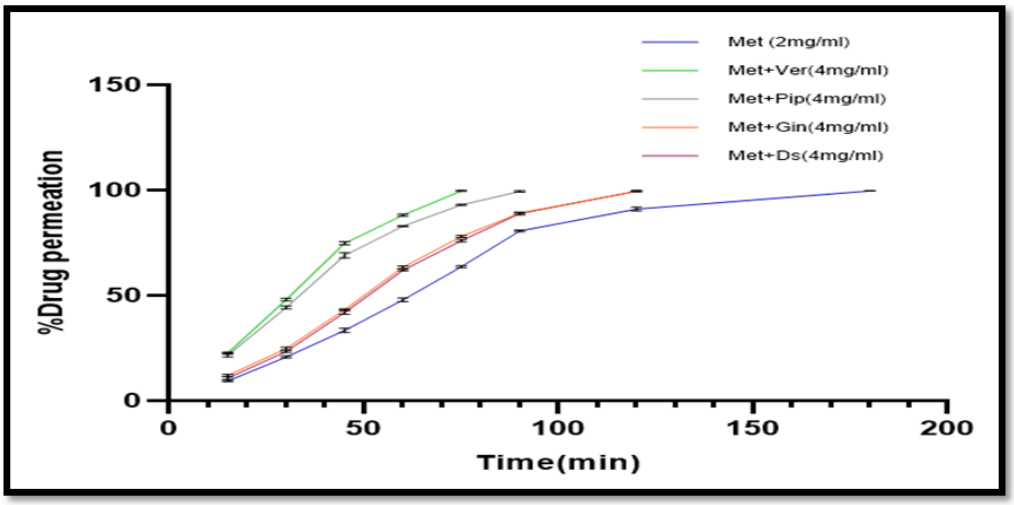

Fig. 4: Permeation Study of Metformin in the Presence And Absence of P-Gp Inhibitors At 4mg/Ml Concentration.

Table 3: Metformin Transport in The Non-Everted Ileum of Chicken In The Presence And Absence of P-Gp Inhibitors At 6mg/Ml Concentration

Time (min)	Met (2mg/ml)	Met+Ver (6mg/ml)	Met+Pip (6mg/ml)	Met+Gin (6mg/ml)	Met+Ds (6mg/ml)
15	9.21	25.33	23.33	12.45	10.22
30	20.35	54.22	52.46	29.22	27.32
45	32.33	79.34	71.22	50.25	48.67
60	47.48	99.87	86.23	75.83	73.56
75	63.34	-	100.1	90.76	90.49
90	80.43	-	-	99.22	99.20
120	90.24	-	-	-	-
180	99.80	-	-	-	-
***f2* value**		**27.49**	**28.88**	**38.44**	**39.59**

Three natural inhibitors (piperine, ginger, and drumstick) and Verapamil (standard) were studied to check their P-gp inhibition capacity by non-everted gut sac study with drug concentration maintained at 2mg/ml and percentage drug permeation was studied. The data in table 3 reveals that all three natural inhibitors show a significant difference in percentage drug permeation when compared using the *f2* similarity index. But Piperine was found to be the most potent of all 3 inhibitors because it shows complete release with higher permeation in less time than Ginger and Drumstick when given in conjunction with Metformin.

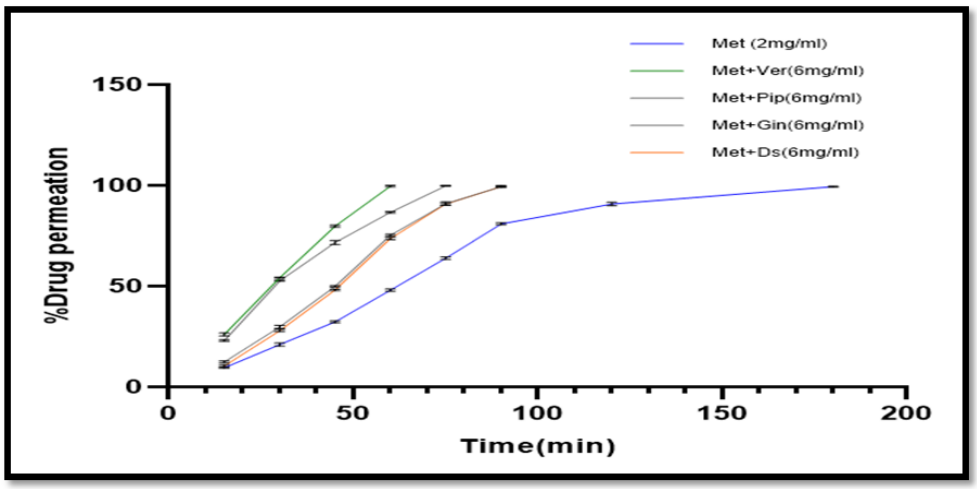

Fig. 5: Permeation Study of Metformin in the Presence and Absence of P-gp Inhibitors at 6mg/ml Concentration

Table 4: Comparative Study of the Efficacy of Piperine at (2mg/ml, 4mg/ml, 6mg/ml) Concentration

Time (min)	Met+Pip (2mg/ml)	Met+Pip (4mg/ml)	Met+Pip (6mg/ml)
15	18.36	21.05	23.33
30	36.57	44.84	52.46
45	55.64	69.71	71.22
60	75.17	82.66	86.23
75	84.91	92.78	100.1
90	92.50	99.05	-
120	99.01	-	-

Comparative permeation study of different concentrations of P-gp inhibitors

It was observed in the above data, using the *f2* similarity parameter, that there is no significant difference in the percentage drug permeation of Metformin in the presence of 2mg/ml of Piperine versus 4mg/ml piperine. The same is with 4mg/ml of piperine versus 6mg/ml of piperine.

However, when the percentage drug permeation of Metformin in the presence of 2mg/ml piperine was compared to 6mg/ml piperine, a significant difference was observed.

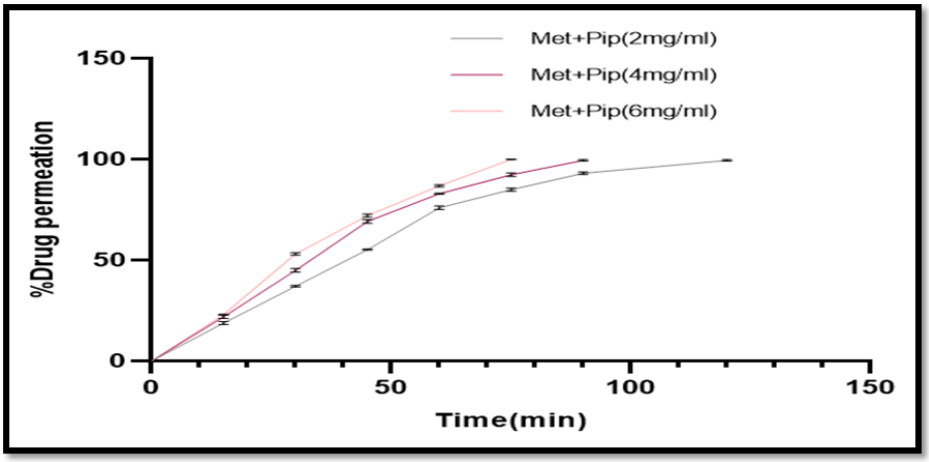

Fig. 6: Permeation Study of Metformin in the Presence of Piperine at 2, 4, and 6mg/ml Concentration

Table 5: Comparative Study of the Efficacy of Ginger at (2mg/ml, 4mg/ml, 6mg/ml) Concentration

Time (min)	Met+Gin (2mg/ml)	Met+Gin (4mg/ml)	Met+Gin (6mg/ml)
15	10.25	11.98	12.45
30	24.34	25.45	29.22
45	40.14	43.21	50.25
60	58.04	63.76	75.83
75	74.12	78.41	90.76
90	88.22	89.23	99.22
120	99.16	99.20	-

It was observed in the above data, using the *f2* similarity parameter, that there is no significant difference in the percentage drug permeation of Metformin in the presence of 2mg/ml of ginger versus 4mg/ml of ginger. The same is with 4mg/ml of ginger versus 6mg/ml of ginger. However, when the percentage of drug permeation of Metformin in the presence of 2mg/ml of ginger was compared with 6mg/ml of ginger, a significant difference was observed.

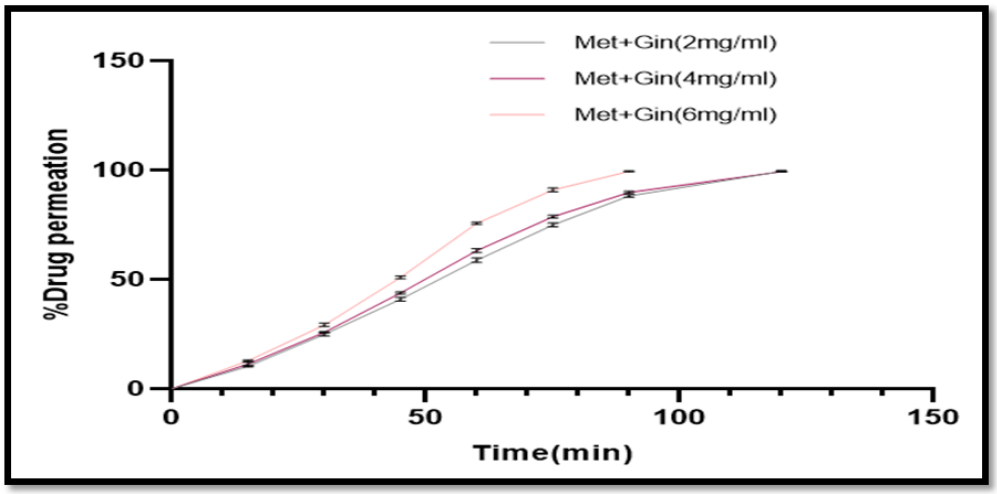

Fig. 7: Permeation Study of Metformin in the Presence of Ginger at 2, 4, and 6mg/ml Concentration.

Table 6: Comparative Study of the Efficacy of Drumstick at (2mg/ml, 4mg/ml, 6mg/ml) Concentration

Time (min)	Met+Ds (2mg/ml)	Met+Ds (4mg/ml)	Met+Ds (6mg/ml)
15	10.02	10.09	10.22
30	23.12	23.32	27.32
45	39.79	41.76	48.67
60	57.66	61.79	73.56
75	73.88	76.23	90.49
90	87.71	88.35	99.20
120	99.13	99.16	-

It was observed in the above data, using the *f2* similarity parameter, that there is no significant difference in the percentage drug permeation of Metformin in the presence of 2mg/ml of drumstick versus 4mg/ml ginger. The same is with 4mg/ml of drumstick versus 6mg/ml of a drumstick. However, when the percentage drug permeation of Metformin in the presence of 2mg/ml of drumstick was compared with 6mg/ml drumstick, a significant difference was observed.

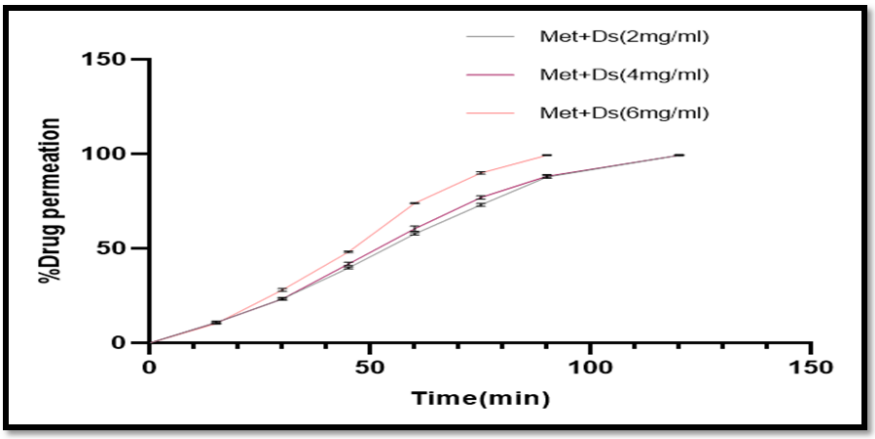

Fig. 8: Permeation Study of Metformin in the Presence of Drumstick at 2, 4, and 6mg/ml Concentration

DISCUSSION

This research work was carried out to determine the efficacy of co-administered P-gp inhibitors on Metformin permeation. The study commenced with a chicken non-everted gut sac model that closely resembled in vivo intestinal transport processes. The effect of different P-gp inhibitors on Metformin intestinal permeability was examined in this study to fully recognize the potential significance of Pgp and intestinal metabolism. Out of all the three selected P-gp inhibitors, i.e. piperine, ginger, and drumstick; Piperine showed a significant increase in Metformin permeability over non-everted gut sac tissue. It is assumed that the increase in permeability of Metformin at 6mg/ml concentration of piperine is due to significant inhibition of Pgp-mediated efflux. The extent of the bio-enhancement of Metformin achieved with piperine in the current study suggests that piperine should be investigated further in the in-vivo study for its pharmacokinetic enhancing effects. According to the study's outcomes, piperine can increase Metformin absorption by blocking P-glycoprotein in a concentration-dependent manner.

CONCLUSION

The purpose of this research work was to determine the efficacy of co-administered P-gp inhibitors, piperine, ginger, and drumstick, on Metformin absorption in an ex-vivo chicken non-everted gut sac model. In the current study, piperine has shown significant enhancement in the drug permeation when compared with ginger and drumstick. Piperine at 6mg/ml has proven effective in inhibiting the Pgp efflux pump significantly and enhancing Metformin permeability. The current study reveals that piperine in a suitable proportion can be highly effective in increasing the oral

bioavailability of Metformin. This approach may provide a simple and safe alternative to enhance the pharmacological profile of Metformin for effective anti-diabetic therapy in humans.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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