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Enhancement of metformin bioavailability by *Tinospora cordifolia* extracts: Insights into pharmacokinetic interactions

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Tinospora cordifolia aqua-alcoholic extract (TCE) with metformin (MET) has been shown to be significantly more effective against clinical diabetes-associated disorders. Since there is insufficient clarity behind this, pharmacokinetic interaction was investigated to unravel the mechanism partly. A significant increase in area under the curve ($AUC_{0-\infty}$), half-life ($T_{1/2}$), and decrease in renal clearance (CLr) of MET was observed in animals dosed with TCE (100 mg/Kg) for 14 days. However, no significant change was observed in the time to reach maximum concentration (T_{max}), indicating that TCE selectively affects the CLr of MET. Many TCE's cationic components/metabolites showed a relatively higher potential affinity for organic cationic transporter 2 (OCT2), which partly justifies the selective pharmacokinetic interaction of MET with TCE. In conclusion, TCE enhances the bioavailability of MET by reducing its renal clearance. This partly explains the basis of TCE as a complementary therapy, which requires further validation.

Keywords: Herb-drug interactions, LC-MS, Metformin, Pharmacokinetic, *Tinospora cordifolia*, Type 2 diabetes

IPC code; Int. cl. (2021.01)– A61K 36/00, A61K 36/59, A61K 135/00, A61P 3/00

Introduction

Tinospora cordifolia (Willd.) Miers (Menispermaceae) is a large, glabrous, deciduous climbing shrub. The shrub is known as Guduchi/Giloy in Hindi and Amrita in Sanskrit. The traditional use of *T. cordifolia* (TC) in general debility, dyspepsia, fever, urinary diseases, jaundice, skin diseases, diabetes (madhumeha), and many other treatments continues to encourage research for exploration of its diverse pharmacology[1]. Its antidiabetic usages have been described in Ayurvedic Materia Medica in the names of pramehahara, pramehaghna, mehahara, and mehaghna[2]. This is also well supported by several pre-clinical[3] and clinical studies[4]. Although the mode of this antidiabetic efficacy is not clearly defined, some of its constituents, including berberine, palmatine, jatrorrhizine, and mangnoflorine, have been reported with insulin-mimicking and insulin-releasing effects[5]. Nevertheless, the antidiabetic efficacy is considered holistic with overlapping extrapancreatic and intrapancreatic mechanisms of action[6].

To address the challenges of effective glycemic control and disorders associated with it[7], alternative and complementary therapies have gained attention in recent years[8]. The ability of TC to manage diabetes and related disorders[9] has prompted the exploration of TC aqua-alcoholic extract (TCE) as a complementary therapy[10]. In a clinical trial involving diabetic patients with hypertriglyceridemia (HTG), TCE (3.0 g/per day) and metformin (MET, 850 mg/day) for 14 days have been shown to reduce the triglyceride, LDL, and VLDL levels while raising the HDL level significantly[11]. Since both TCE and MET[12,13] independently have the ability to significantly lower plasma triglycerides, the basis of complementary or supplementary effects is not very clear. Similarly, the combination of TCE and MET has been shown to reduce diabetes-induced nephrotoxicity[14], but it is known that both MET[15] and TCE independently have the potential to protect against diabetic nephropathy[16]. Thus, further investigations are necessary to demonstrate the relevance of complementary applications of TCE with MET. A pharmacokinetic interaction study between herbs and drugs is necessary to assess the relevance of complementary therapy[17]. However, no such study has been conducted on TCE and MET. So unravelling pharmacokinetic interactions between TCE and MET can further open up evaluations of complementary therapeutic strategies based on scientific validation.

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Supplementary tables and figures are available online only

Earlier, our findings have revealed the interaction of TCE with glibenclamide[18] through the inhibition of metabolic enzymes, leading to a significant increase in the bioavailability of glibenclamide. Unlike glibenclamide, MET is not metabolised and excreted unchanged through urine. Thus, very little metabolism-mediated drug interaction can be expected between TCE and MET. Interestingly, Liu *et al.* have reported a significant increase in the bioavailability of MET following pretreatment with a high dose (200 mg/kg) of berberine[19]. Although berberine is a component of TCE, it is present in a very low amount (approx. 0.3% w/w)[20] and is metabolised extensively[21]. Thus, interaction due to berberine in TC may be insignificant. However, holistic contributions from other components and metabolites that modulate the pharmacokinetics of MET cannot be ruled out.

Absorption and renal elimination of MET are known to be mediated through organic cation transporters (OCTs) that are saturable[22]. Many components of TCE containing nitrogen in their chemical structure (cationic) can also be likely substrates of these OCTs and saturate these transporters to affect the pharmacokinetics of MET. For example, components of TC like jatrorrhizine and berberine are reported to be transported by these OCTs[23,24]. Further, the affinity of these cationic components for different OCTs may not be the same. Moreover, the relative expression of these OCTs at the site of absorption and elimination may influence the extent of drug interaction[25]. For example, the Michaelis-Menten constant (Km) of berberine has been reported to be 14.8 µm for OCT1, whereas the same for OCT2 is 4.8 µm. Thus, berberine is a more efficient substrate of OCT2, and its influence on the pharmacokinetics of MET may depend on the relative expression of OCT2[26]. Similar phenomena may exist for each cationic component of TC. Since components of TC, like berberine, are extensively metabolised, the cationic metabolites may also compete for these saturable transporters and modulate the pharmacokinetics of MET[27].

Considering the very low levels of multiple TCE components and their metabolites, estimating their pharmacokinetics *in vivo* is a major hurdle. Nonetheless, the pharmacokinetics of MET can be estimated under the influence of TCE to assess the possibility of interaction. Further, computational tools can be used to investigate the relative affinity of components of TCE and their metabolites for OCTs to rationalise the pharmacokinetic interaction. Hence, in the current study, we evaluated the pharmacokinetic interaction of TCE with MET and subsequently investigated its mechanism through *in silico* analysis.

Materials and Methods

Chemicals and reagents

Authentic aqua-alcoholic (50% v/v) stem extract of *Tinospora cordifolia* was procured from Kisalaya Herbals Ltd., Indore, Madhya Pradesh (India), having batch no. KG/861 with 2.6% bitter content. Metformin hydrochloride (Fig. S1, 99.8% assay on the dried basis by potentiometry) was procured from Auro Laboratories Ltd, Mumbai, India. Acyclovir was procured from Sigma Aldrich, Mumbai, India. Methanol absolute ULC/MS grade was procured from Biosolve BV, Valkenswaard, Netherlands. Isoflurane was obtained from Primal Enterprises Ltd, Mumbai, India. Milli-Q water was prepared in-house using a Millipore Milli-Q water system, MA, USA. All procedures performed in this study involving animals complied with the ethical standards of the institution at which the studies were conducted. The animal study protocols were approved by the institutional animal ethical committee (Reg. No. 1246/09/ CPCSEA) of Piramal Enterprises Ltd, Goregaon, Mumbai, India and the experiments were conducted accordingly. This study was conducted in compliance with local, national, and international guidelines and legislation for studies involving plants.

Authentication of TCE

A 10 µg/mL solution of berberine, 1 mg/mL solution of TCE in Milli-Q water and blank solvent (Milli-Q water) were analysed using Thermo Q Exactive Plus high-resolution mass spectrometer (Thermo Fisher Scientific Inc. San Jose, CA USA) coupled with a Dionex UltiMate® 3000, LC system (Dionex Corporation, 1228 Titan Way P.O. Box 3603 Sunnyvale, CA 94088-3603) as a front end. The constituents of TCE were separated on an Eclipse XDB C18, 3.5 µ, 4.6×150 mm analytical column using 0.1% formic acid in water and 0.1% formic acid in methanol as mobile phases. A gradient elution program was employed with 80% aqueous at the beginning of the run. The mobile phase composition was held isocratic for two minutes, and then the aqueous concentration was lowered to 20% over 23 minutes. Again, an isocratic elution at 20% aqueous was performed for four minutes. The composition of the mobile phase was reversed back to

80% aqueous within 1 minute. The column was equilibrated at 80% aqueous composition for 10 minutes. The mass spectrometer was set to full scan mode to scan the m/z values from 150 Da to 1500 Da. The base peak chromatogram (BPC) of the berberine standard and TCE was superimposed for comparison. The accurate mass and fragmentation pattern of the berberine standard was compared with the accurate mass and fragmentation pattern of the principle eluting at the same retention time in TCE to authenticate the TCE.

Bioanalytical method

For quantification of MET from plasma, a highly sensitive fit-for-purpose Liquid chromatography-mass spectrometry (LC-MS/MS) method was developed using acyclovir as an internal standard (IS). The plasma sample (50 µL) and internal standard solution (100 µL, 1000 ng/mL acyclovir in methanol) were vortex-mixed. About 1.5 mL 0.5% ammonia in methanol was added to this and centrifuged (1968 g for 10 min at 4ºC) to collect the supernatant. Method 1: The supernatants of the single oral concomitant study were analysed by Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer coupled with a Dionex/Thermo Ultimate 3000 HPLC System as front end with a FORTIS Diphenyl-5 µm and 150 X 4.6 mm analytical column set at 40°C. The m/z values were scanned from 100 to 1000 m/z using full MS-SIM mode in positive polarity at a resolution setting of 70000. The chromatograms of MET and acyclovir (internal standard, IS) were constructed by extracting high-resolution exact m/z values 130.1087 (Rt = 4.59±0.04) and 226.0935 (Rt = 2.64±0.01) with a tolerance window of not more than 0.005 Da. Method 2: The supernatants of a 14-day recurring oral pretreatment study were analysed by Thermo TSQ Quantum ultra-triple quadrupole mass spectrometer coupled with a Dionex ultimate 3000 LC system as front end with a Chromolith® Speed Rod RP 18e 50 X 4.6 mm analytical column set at 40°C. MET and IS were detected by monitoring SRM transitions 130.1/71.1 and 226.03/152.08 for MET and IS, respectively. In both studies, MET and IS were eluted by using a mixture of methanol and 10 mM ammonium acetate in water (85:15 v/v) as the mobile phase. The mobile phase flow rate was set at 0.7 mL/min. The selectivity, sensitivity, linearity, recovery accuracy, and precision of both bioanalytical methods were calculated. The selectivity of the methods was evaluated by analysing blank plasma samples from six rats along with quality control at the lower limit of quantification (LLOQ-QC) samples. Sensitivity was evaluated in terms of accuracy and precision at the LLOQ level. The calibration curves (area ratio of analyte and IS vs concentration) were plotted to evaluate the linearity of the methods. Quality control samples at LLOQ-QC, low-quality control (LQC), middle-quality control (MQC), and high-quality control (HQC) levels were prepared within the dynamic range of the respective methods in 5 replicates and analysed to determine the accuracy and precision of the analytical runs.

Animal handling

Sprague Dawley (SD) rats weighing between 200 and 300 g were used to study pharmacokinetic interaction. The environmental conditions (Temperature 23±2ºC; relative humidity 50±10%; 12-h light/dark cycle) were controlled during the study duration. The rats were housed individually in polypropylene ventilated cages and fed with a standard rodent diet and water *ad libitum*. The oral dosing schedule was b.i.d (10:30 am and 06:30 pm) with an appropriate dose according to the study design.

Experimental design and pharmacokinetic interaction studies

Two independent pharmacokinetic studies were designed to investigate the effects of TCE on the pharmacokinetics of MET. In the single oral concomitant study, TCE and MET were coadministered, and plasma profiles of MET were determined. In another study, animals were pretreated with TCE for 13 days. On the 14th day, TCE and MET were concomitantly administered to study plasma levels of MET. 10 and 100 mg/kg b.i.d of TCE was selected as an experimental dose of herbal extract. The experimental dose of MET was kept at 30 mg/ kg (human equivalent of 250 mg) for all experiment groups for comparison. 2 and 20 mg/mL solutions of TCE were prepared by dilution with an appropriate volume of 0.9% saline. Suspension of MET (6 mg/mL) was prepared by using Metformin hydrochloride IP in a mixture of 2.5 µL/mL Tween 80 in 0.5 % w/v of methylcellulose. Appropriate volumes (10 mL/kg of body weight) of TCE mixture and MET suspension were administered by oral gavage.

In the single dose (oral) study, 18 rats weighing between 200 and 300 g were divided into 3 groups of 6 each and kept fasted overnight. Animal group 1 (Control) was treated with 0.9 % saline, whereas groups 2 and 3 were treated at 10 and 100 mg/kg of

TCE, respectively. A 30 mg/kg dose of MET in normal saline was administered to all three groups after 0.5 hours of TCE treatment.

In the 14-day recurring oral pretreatment study, 18 rats weighing between 200 and 300 g were divided into 3 groups of 6 each. Animal group 1 was treated with 0.9% saline, whereas group 2 and group 3 were treated with 10 and 100 mg/kg b.i.d of TCE, respectively, for 13 days. On the 14th day, after 0.5 hours of TCE treatment, a 30 mg/kg dose of MET was administered to all three groups.

In both pharmacokinetic studies, the treatments with TCE/vehicle and/or MET were always carried out in the morning between 8:30 am and 10:00 am, and serial blood sampling was followed. Retro-orbital bleeding (ROB) in a medial approach has been criticised for potential injury. Nonetheless, ROB with a lateral approach has been demonstrated to be humane and safe while providing blood samples of adequate volume and quality for analysis. Thus, this technique was used to collect about 0.2 mL of blood in Eppendorf tubes (0.5 mL) containing 10 µL of dipotassium ethylenediamine tetraacetic acid (K_2EDTA; 200 mM) at predose, 0.25, 0.5 1, 2, 4, 6, 8 and 24 h of MET administration under light isoflurane anaesthesia. Following centrifugation (1968 g for 10 min at 4ºC), the plasma was separated and stored at -80ºC for analysis.

Pharmacokinetic and statistical analysis

The concentrations higher than the LLOQ of the assay method were considered, and concentrations lower than the LLOQ were taken as zero. The Phoenix® WinNonlin®, version 4.1 (Pharsight Co., Mountain View, CA, USA) was used for non-compartmental pharmacokinetic analysis of MET. The highest concentrations of MET in plasma (C_{max}) and related time (T_{max}) were read from the plot. The linear trapezoidal rule was used to calculate the area under the concentration-time curve from time 0 to the last time point (AUC_{0-t}). From this, and using C_{last}/k_{el}, the area under the plasma level–time curve (AUC) from time zero to infinite ($AUC_{0-\infty}$) was calculated, where C_{last} is the last quantifiable concentration and k_{el} is the apparent terminal elimination rate constant. A one-way analysis of variance (ANOVA), followed by Dunnett's t-test, was used to compare the statistical significance of the mean results of the control and treatment groups. All values are represented in terms of Mean±SD.

***In silico* analysis of affinity of phytocomponents and their metabolites for OCTs**

The non-duplicate phytoconstituents of TCE were identified from published sources and databases[18,28]. The Canonical Simplified Molecular Input Line-Entry System (SMILES) of each cationic/nitrogen-containing active ingredient was retrieved. The classical interface of QSAR toolbox v4.6 (May 2023) was used to predict their metabolites[29]. Metabolism simulators, including microbial observed metabolism, microbial simulated metabolism, a rat *in vivo* observed metabolism, rat *in vivo* simulated metabolism, rat liver observed metabolism, rat liver S9 observed metabolism, and rat liver S9 simulated metabolism, were used to predict the metabolites.

The crystal structure of OCT1 (PDB code: 8ET8) co-crystallised with verapamil, OCT2 (PDB code: 8ET9) co-crystallised with 1-methyl-4-phenylpyridinium and OCT3 (PDB code: 7ZH6) co-crystallised with corticosterone was obtained from the Protein Data Bank (PDB, https://www.rcsb.org). Crystal structures were prepared using the Schrödinger suite's protein preparation wizard. Similarly, the collected cationic active ingredients of TCE and their metabolites were prepared using the LigPrep module of the Schrödinger suite to generate the correct energy-minimised 3D molecular stereoisomer. For docking metformin (control) and phytocompounds/metabolites into the active site of the OCTs, a receptor grid of 12 Å around the centroid of the crystallised ligand was generated. Further, the stereoisomers of phytocompounds/metabolites were flexibly docked using the Glide module of the Schrödinger suite in extra-precision (XP) mode. Further, the docked phytocompounds were re-ranked by estimating their relative binding free energy (ΔG_{bind}) using molecular mechanics generalised Born surface area (MM/GBSA) post-docking scoring protocol, where $\Delta G_{bind} = \Delta G_{solv} + \Delta EMM + \Delta GSA$. While the ΔG_{solv} term is related to the difference in solvation energy of the complex and unbound form, ΔEMM and ΔGSA present differences in minimised energies and surface area energies, respectively.

Results

Berberine is an established bioactive marker for *Tinospora cordifolia*[30] and is one of the phytocomponents that has been validated to interact with OCTs[26]. Thus, the TCE, supplied by an authentic vendor, was further verified with berberine as the marker. The chromatograms, high-resolution mass

spectra (HRMS), and product ion mass spectra of TCE were compared with those of standard berberine. Exact m/z at 17.24 minutes of chromatographic run in standard berberine and TCE were 336.12238 and 336.12207, respectively. Product ion spectra of berberine standards showed m/z values at 321.10, 320.09, 307.08, 306.08, 304.10, 292.10 and 270.08. This exactly matched the principle present in TCE (Fig. S2).

Validation of the bioanalytical method

The results of validation were tabulated in Table 1. The methods were found to be specific as no interference was observed with analyte and IS in blank plasma samples at the retention time of analyte and IS (Fig. S3 and Fig. S4). The method used to analyse MET from samples of a single oral dose study of TCE and MET was found to be linear (y = 0.0696x-0.0497, r^2=0.9982) from 3.083 ng/mL to 3054.94 ng/mL (Fig. S5a). Whereas the method used for analysis of samples from a 14-day recurring oral pretreatment study was linear (y = 0.0027x – 0.0006, r^2=0.9905) from 7.883 ng/mL to 7812.115 ng/mL of MET (Fig. S5b). Both methods were accurate and precise, with mean accuracy within 100±10% and relative standard deviation (RSD) below 10%. Recovery of the extraction method for the analyte and IS was evaluated by comparing the instrument response of unextracted (Neat) and extracted samples at the LQC level. The recovery of analyte and IS were 96.5±5.7 (n=3) and 93.7±7.2% (n=3) for MET and IS, respectively, during a single oral concomitant study. Similar recovery results (99.1±2.8% (n=3) and 100.0±6.3% (n=3) for the analyte and IS,

Table 1 — Validation parameters for bioanalytical methods quantifying metformin in rat plasma for single and recurring oral studies

Parameter	Result						
Selectivity	Method-1	No interference observed at retention time and m/z transition of analyte and IS in 6 lots of rat plasma from different animal					
	Method-2	No interference observed at retention time and m/z transition of analyte and IS in 6 lots of rat plasma from different animal					
Sensitivity	Method-1	LLOQ = 3.086 ng/mL with accuracy and precision 105.54±3.27% (Mean ± RSD)					
	Method-2	LLOQ = 7.779 ng/mL with accuracy and precision 104.23±1.41% (Mean ± RSD)					
Linearity (Calibration curve)	Method-1	The method was found linear from 3.083 ng/mL to 3054.94 ng/mL with a correlation coefficient > 0.99 (y = 0.0696x-0.0497, r^2=0.9982)					
	Method-2	The method was found linear from 7.883 ng/mL to 7812.115 ng/mL with a correlation coefficient > 0.99 (y = 0.0027x – 0.0006, r^2=0.9905)					
Accuracy and Precision		QC ID	Nominal concentration (ng/mL)	N	Mean	% Accuracy	% RSD (Precision)
	Intraday for Method-1	LLOQ-QC	3.086	5	3.257	105.54	3.27
		LQC	36.737	5	39.808	108.36	2.77
		MQC	1148.035	5	1172.105	102.1	4.64
		HQC	2296.07	5	2258.916	98.38	3.18
	Interday for Method-1	LLOQ-QC	3.086	10	3.196	103.56	9.25
		LQC	36.737	10	40.269	109.61	3.19
		MQC	1148.035	10	1206.327	105.08	4.61
		HQC	2296.07	10	2329.092	101.44	4.49
	Intraday for Method-2	LLOQ-QC	7.779	5	8.108	104.23	1.41
		LQC	111.128	5	110.937	99.83	4.82
		MQC	2778.201	5	2862.142	103.02	4.36
		HQC	3704.268	5	3477.891	93.89	7.07
	Interday forMethod-2	LLOQ-QC	7.779	10	7.828	100.63	3.93
		LQC	111.128	10	117.306	105.56	6.61
		MQC	2778.201	10	2995.959	107.84	5.6
		HQC	3704.268	10	3707.808	100.1	8.44
	Method 1:	Metformin: 96.5% ± 5.7 % (n=3) at LQC Level IS : 93.7% ± 7.2 % (n=3)					
	Method 2:	Metformin: 99.1% ± 2.8 % (n=3) at LQC Level IS : 100.0% ± 6.3 % (n=3)					

#Method 1: Used in single oral concomitant study; Method 2: Used in14-days recurring oral pre-treatment study

respectively) were obtained during the 14-day recurring oral pretreatment study because the extraction method was the same for both methods.

Pharmacokinetic analysis of a single oral concomitant study

Fig. 1 presents the mean plasma concentration-time profiles of MET following oral administration (30 mg/kg) to rats with vehicle (Control group, Group 1) and TCE (Group 2, 10 mg/kg; Group 3, 100 mg/kg). Following a non-compartmental analysis of individual concentration-time profiles, the mean plasma pharmacokinetic parameters of MET are shown in Table 2. The pharmacokinetic parameters obtained from groups 2 and 3, were statistically compared with the pharmacokinetic parameters obtained from group 1 (control group). No statistically significant difference was observed (p >0.05) between the pharmacokinetic parameters obtained from the control and the treatment groups. However, the $T_{1/2}$ obtained from group 3 (6.00 ± 1.43 h) seems to be delayed (p <0.05) as compared to the control group (2.14±1.46 h). This was supported by a decrease in renal clearance (CLr) (Table 2). This indicates that TCE can affect the CLr of MET in a negative direction.

Pharmacokinetic analysis of a 14-day recurring oral pretreatment study

Fig. 2 presents the mean plasma concentration-time profiles of MET following oral administration (30 mg/kg) to rats pretreated with vehicle control (Group 1) and TCE (Group 2, 10 mg/kg; Group 3, 100 mg/kg) for 14 days. Following a non-compartmental analysis of individual concentration-time profiles, the mean plasma pharmacokinetic parameters of MET are shown in Table 3. The pharmacokinetic parameters obtained from groups 2 and 3, are statistically compared with the pharmacokinetic parameters obtained from group 1 (Control group). There is no significant difference (P >0.05) in pharmacokinetic parameters observed between group 1 and group 2 (pretreated with 10 mg/ kg of TCE, p.o, b.i.d). Unlike in the single-dose study, the pharmacokinetic parameters of group 3 (Pretreated with 10 mg/ kg of TCE, p.o, b.i.d) animals showed significant changes. A significant increase in $AUC_{0-\infty}$ (p <0.01) and $T_{1/2}$ (p <0.01) with a decrease in CLr (p <0.01) was observed between group 1 and group 3 (Pretreated with 100 mg/ kg of TCE, p.o, b.i.d). The increase in $AUC_{0-\infty}$ in group 3 seems to be due to an increase in $T_{1/2}$ and a decrease in CLr of MET. This suggests constituents of TCE are decreasing the CLr of MET. There was an insignificant increase in the C_{max} of MET in group 3, whereas no changes were observed in T_{max} (Table 3).

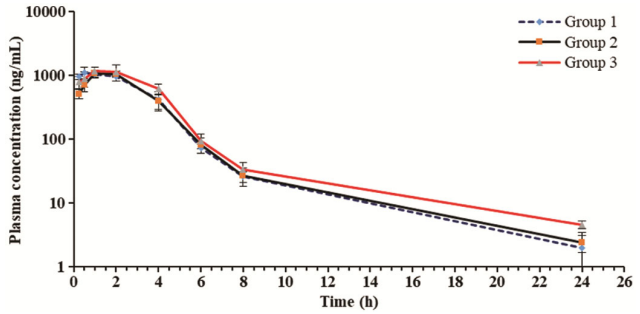

Fig. 1 — Plasma concentration-time profiles of metformin in rats following single-dose oral coadministration with *Tinospora cordifolia* extract (TCE). The plot shows the plasma concentration of metformin (ng/mL) over 24 hours in rats treated with a single oral dose of metformin (30 mg/kg) combined with vehicle (Group 1, blue dashed line), TCE at 10 mg/kg (Group 2, black solid line), or TCE at 100 mg/kg (Group 3, red solid line). Data points represent mean values, with error bars indicating standard deviation.

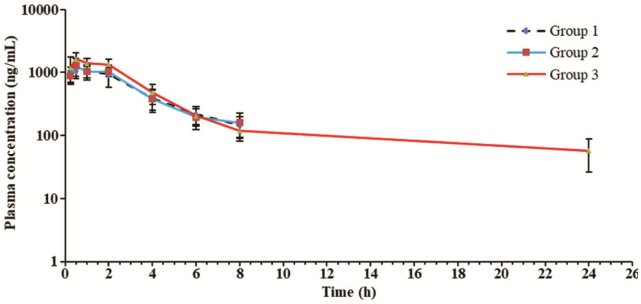

Fig. 2 — Plasma concentration-time profiles of MET from recurring dose study. Plasma level of MET over a period of 24 h, from rats simultaneously treated in a single dose of Metformin 30 mg/kg after recurring oral pretreatment of TCE for 14 days with vehicle (Group 1), TCE 10 mg/kg, p.o. (Group 2), and TCE 100 mg/kg, p.o. (Group 3) by oral gavage.

Table 2 — Mean plasma pharmacokinetic (PK) parameters of metformin after non-compartmental analysis of individual concentration-time profile from single dose oral concomitant study of *Tinospora cordifolia* extract and metformin

Animal Groups	C_{max} (ng/mL)	$AUC_{0-\infty}$ (ng x h/mL)	T_{max} (h)	$T_{1/2}$ (h)	CLr (mL/min/kg)
Group 1 (Control)	1185.84±178.28	3986.13±554.71	1.17±0.62	2.14±1.46	127.83±16.78
Group 2 (10 mg/kg TC)	1103.78±135.28	3861.53±362.88	1.68±0.47	2.15±1.60	130.64±11.50
Group 3 (100 mg/kg TC)	1280.58±186.93	5030.17±1154.52	1.33±0.47	6.00±1.34*	105.89±25.20

*p <0.01

Table 3 — Mean plasma pharmacokinetic (PK) parameters of metformin after non-compartmental analysis of individual concentration-time profile from recurring oral pretreatment for 14-days study with *Tinospora cordifolia* extract followed by metformin administration

Animal Groups	C_{max} (ng/mL)	$AUC_{0-\infty}$ (ng x h/mL)	T_{max} (h)	$T_{1/2}$ (h)	CLr (mL/min/kg)
Group 1	1155.11 ± 307.34	4802.00 ± 575.56	0.81 ± 0.80	2.56 ± 1.22	106.44 ± 12.01
Group 2	1370.24 ± 285.66	4881.02 ± 541.38	0.88 ± 0.75	2.23 ± 0.48	106.26 ± 13.56
Group 3	1659.71 ± 374.24*	7543.46 ± 757.64*	0.88 ± 0.75	7.98 ± 2.06*	67.74 ± 7.05*

*p <0.01

Table 4 — Relative Gibb's binding free energy (ΔG_{Bind}) of nitrogen containing phytoconstituents of TCE as compared to metformin against organic cation transporters (OCTs)

Title	PubChem CID	Generic Name	OCT1	OCT2	OCT3
T01	4091	Metformin (Control)	-183.05	-34.09	-71.74
M01	13387	1-Methyl-2-pyrrolidinone	230.69	53.31	-43.41
M02	305	Choline	200.48	-18.57	-89.64
M30	4840	Piperin	201.89	-139.16	-6.72
M33	23251787	N-Formylannonaine	276.99		-43.72
M34	125213	N-feruloyltyramine; Moupinamide	163.51	-36.89	-38.64
M35	550072	Dasycarpidan-1-methanol, acetate (ester)	138.21	-79.92	-96.83
M37	2353	Berberine	188.95	-160.11	-182.63
M38	72323	Jatrorrhizine	129.24	-62.20	-128.99
M39	48704	Luteanine	136.43		-35.74
M40	111119	(+/-)-Corydine	146.33		-63.75
M41	3999	(+)-Magnoflorine Iodide	99.09		-145.07
M42	4622630	Tembetarine	131.09	43.58	-53.28
M43	19009	Palmatine/Berbericinine	168.62	-182.20	-127.35
M44	5417	Rotundine	162.42	-65.24	-179.29

Thus, the absorption phase of MET seems to be unaffected by TCE.

***In silico* analysis of affinity of phytocomponents and their metabolites against OCTs**

From the published resources, 75 components were identified. Upon structural analysis, it was found that 14 of these compounds contained nitrogen in their structure and were thus included in the list of cationic phytocomponents of TCE (Table S1). These 14 compounds were used for metabolite prediction using the QSAR toolbox v4.6. By simulating *in vivo* rat metabolism, rat liver S9 metabolism, and microbial metabolism, a non-duplicate list of 141 metabolites was retrieved (Table S2).

Molecular docking and Prime's MM/ GBSA analysis of cationic phytocomponents of TCE generated binding affinities (XP GScore) and binding free energy (ΔG_{bind}) for OCT1, OCT2 and OCT3. It was revealed that ΔG_{bind} of MET in complexes with OCT2 and OCT3 are similar to those of the co-crystallised ligands (Table S3). Interestingly, this was higher compared to the co-crystallised ligand against OCT1, corroborating the fact that MET is a substrate of all these transporters[31]. Since MET is a common substrate of these transporters, to demonstrate the ability of components/ metabolites to saturate the OCTs or provide competition to MET, it is necessary to compare the ΔG_{bind} of their complex with that of MET[32] . Therefore, the ΔG_{bind} of the components was converted to relative ΔG_{bind} by subtracting the ΔG_{bind} value of MET from that of the phytocomponent (Table 4). This suggests that the components have a higher probability of competing with MET for OCT2 and OCT3.

The molecular docking analysis reveals that the majority of components/metabolites showed higher binding affinity than their co-crystallised ligand for OCT1 (n=127), OCT2 (n=97), and OCT3 (n=72). Thus, they are likely to saturate these transporters (Table S4). Further, Fig. 3a and Fig. 3b show that the binding affinity as well as binding free energy of the majority of component/metabolite is higher than average for OCT2. Compared to this, fewer components/metabolites were found to have higher than average binding affinity/free energy for OCT1 and OCT3 (Fig. 3c and Fig. 3d). This suggests that the components/metabolites are more likely to saturate OCT2 over other OCTS.

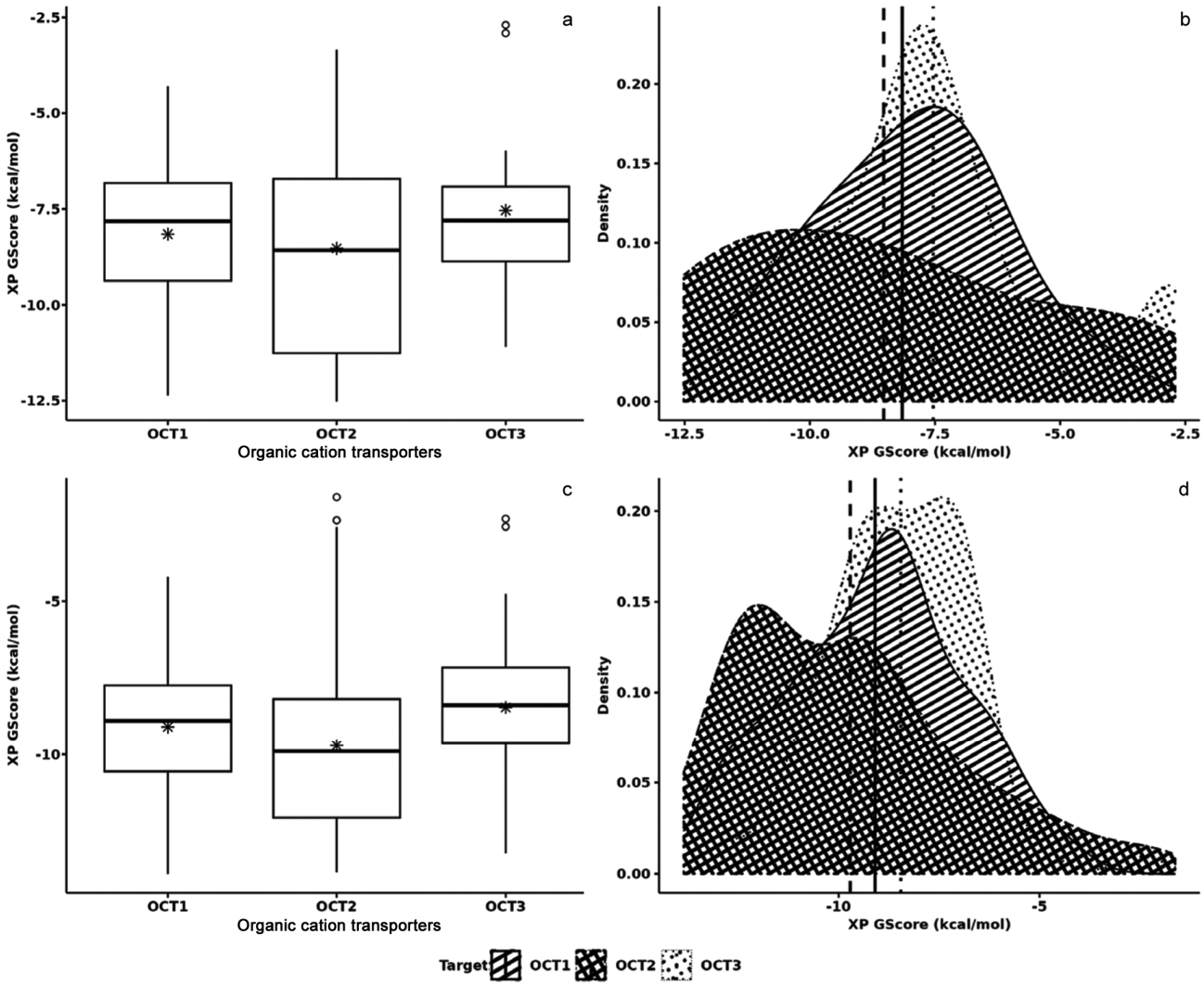

Fig. 3 — Comparative analysis of binding affinities (XP GScore) for *T. cordifolia* extract (TCE) phytoconstituents and their cationic metabolites. a–b) Box and density plots for TCE phytoconstituents: Panel (a) shows the XP GScore distribution (box plot) of 14 TCE phytoconstituents, with outliers as points; the star symbol and dashed line indicate the mean score. Panel (b) displays the corresponding density plot. c–d) Box and density plots for cationic metabolites: Panel (c) presents the XP GScore distribution (box plot) of cationic metabolites derived from the 14 phytoconstituents, with mean score marked (star and dashed line). Panel (d) shows the density plot for metabolites.

Molecular Docking Parameters and Binding Modes

Molecular docking analysis revealed that several phytocomponents and their metabolites exhibited superior binding affinities for OCTs compared to metformin and co-crystallised ligands (Table S3). The relative ΔG_{bind} values suggested that these phytocomponents have a higher probability of competing with metformin for OCT2 and OCT3 compared to OCT1, indicating potential for competitive inhibition of metformin transport across cellular membranes.

MET demonstrated varied binding affinities across the three OCT transporters, with ΔG_{bind} values of -183.05 kcal/mol for OCT1, -34.09 kcal/mol for OCT2, and -71.74 kcal/mol for OCT3 (Table S3). The interaction profile with OCT1 primarily involved electrostatic attractions with GLU386 and conventional hydrogen bonds with GLN241, forming a relatively simple interaction network (Fig. 4a; Table S5). For OCT2, metformin established salt bridges and attractive charge interactions with GLU387, conventional hydrogen bonds with THR444 and GLN242, and notable pi-cation interactions with TYR362 (Fig. 4b; Table S5). OCT3-metformin interactions featured an extensive hydrogen bonding network involving GLN247, THR447 and GLU390

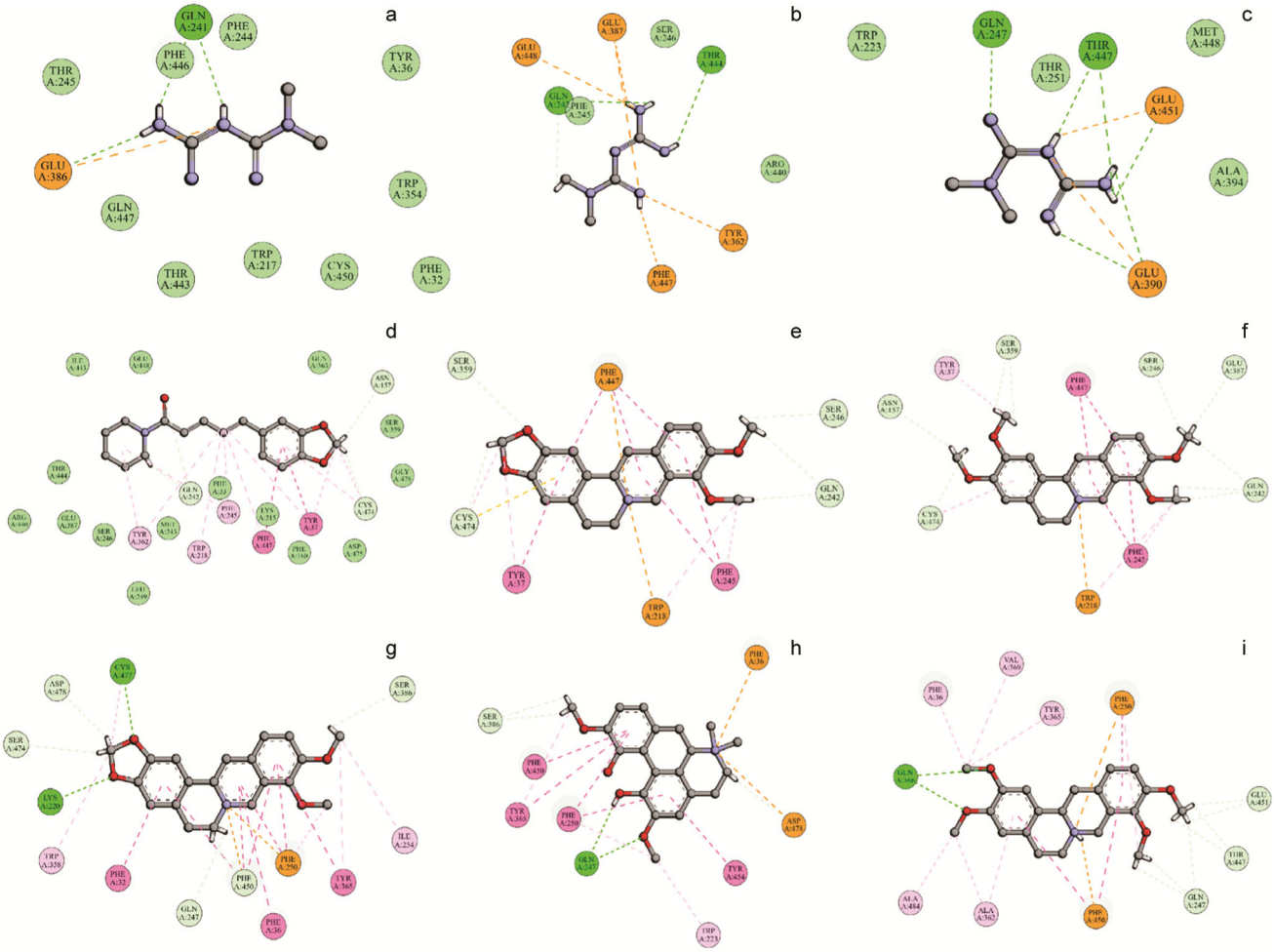

Fig. 4 — 2D Interaction diagram of OCTs with metformin (Control) and selected phytocomponents (Berberine, Piperin, Palmatine, Magnoflorine Iodide and Rotundine). The diagram illustrates the mechanism of organic cation transporters (OCT1, OCT2, OCT3) in facilitating the cellular uptake of metformin (MET) and cationic phytocomponents from TCE in key tissues (liver, kidney, muscle). 2D interaction of Metformin with a) OCT1, b) OCT2 and c) OCT3. 2D interaction of OCT2 with d) Piperin (M30), e) Berberine (M37) and f) Palmatine (M43). 2D interaction of OCT3 with g) Berberine (M37), h) Magnoflorine Iodide (M41) and i) Rotundine (M44).

alongside salt bridge formation with GLU451 (Fig. 4c; Table S5).

The phytocomponents demonstrated substantially more complex binding modes with OCT2. Piperin showed strong binding with a ΔG_{bind} of -173.25 kcal/mol, significantly stronger than metformin's -34.09 kcal/mol (Table S3). Piperin primarily formed carbon-hydrogen bonds with GLN242, ASN157, and CYS474 while establishing extensive hydrophobic interactions through pi-pi stacking with TYR37 and PHE447 (Fig. 4d; Table S5). Berberine exhibited even more remarkable binding with a ΔG_{bind} of -194.20 kcal/mol, creating carbon-hydrogen bonds with SER246 and GLN242, pi-cation interactions with TRP218 and PHE447, and unique pi-sulfur interactions with CYS474 (Fig. 4e; Table S5). Palmatine demonstrated the strongest binding to OCT2 with a ΔG_{bind} of -216.29 kcal/mol, forming an extensive hydrogen bonding network with residues including ASN157, CYS474, SER359, and GLN242, alongside pi-cation interaction with TRP218 and pi-pi stacked interactions with PHE245 and PHE447 (Fig. 4f; Table S5).

For OCT3, the selected phytocomponents exhibited exceptional binding affinities. Berberine (M37) demonstrated the strongest binding with a ΔG_{bind} of -254.37 kcal/mol, substantially higher than metformin's -71.74 kcal/mol (Table S3). Its binding involved conventional hydrogen bonds with LYS220 (2.49 Å) and CYS477 (2.68 Å), carbon-hydrogen bonds with SER386, GLN247, and PHE450, and extensive pi-pi stacking interactions with phenylalanine residues

(Fig. 4g; Table S5). Magnoflorine iodide showed remarkable affinity with a ΔG_{bind} of -216.81 kcal/mol, engaging in attractive charge interactions with ASP478, conventional hydrogen bonds with GLN247, and pi-cation interaction with PHE36, alongside pi-pi stacked interactions with PHE250, PHE450, and TYR454 (Fig. 4h; Table S5). Rotundine exhibited similarly strong binding to OCT3 with a ΔG_{bind} of -251.03 kcal/mol, forming conventional hydrogen bonds with GLN366, carbon-hydrogen bonds with THR447, GLN247, and GLU451, pi-cation interaction with PHE250, and pi-pi stacked interactions with PHE250 and PHE450 (Fig. 4i; Table S5). The structural complexity of these phytocomponents facilitated these extensive binding networks, suggesting they may effectively compete with metformin for transport via OCT2 and OCT3.

Discussion

Complementary therapy has been adopted for better glycemic control in diabetes[8]. Both traditional knowledge and scientific investigation support the use of TCE in diabetes[10]. Accordingly, TCE combination with MET has been demonstrated to ameliorate diabetes-associated hypertriglyceridemia and nephrotoxicity[14,33]. Since both MET and TCE have independent pleiotropic actions to manage these conditions, the basis of complementary or supplementary effects is not very clear. Knowledge of pharmacokinetic interaction between herbs and drugs is critical for the safety and effectiveness of therapeutic strategies. However, there is no such data on pharmacokinetic interaction between TCE and MET. Hence, a study on the influence of TCE on MET pharmacokinetics is necessary to open up studies for evaluating the merit of this complementary therapeutic approach. Earlier, we have demonstrated the ability of TCE to enhance the bioavailability of glibenclamide through metabolism-mediated interaction[18]. Although MET is not metabolised, it is absorbed and cleared through OCTs, which are also likely targets of components of TCE. Thus, the present study was undertaken to evaluate the possibility of pharmacokinetic-mediated interaction with MET. For this, TCE was authenticated based on a certificate of analysis provided by the vendor and the LC HRMS method. Berberine was chosen as a marker compound, as it is a well-known bitter principle that has the potential to produce drug-herb interaction mediated through OCTs with the antidiabetic drug MET[19]. Ideally, combinations of multiple bioactive markers are necessary to establish the authenticity of herbal drugs. Earlier, TCE from the same source has been authenticated based on berberine as a bioactive marker supported by the product ion spectra of TCE showing fragmentation attributed to other representative compounds, including jatrorrhizine and palmatine[30]. So, HRMS data and product ion spectra of standard berberine matching with those of TCE suggested its authenticity in support of the certificate of analysis.

Both the analytical methods used to analyse the study sample were found reliable in terms of specificity, accuracy, and precision. The sample pretreatment was found suitable using 0.5% ammonia in methanol, as the peak shapes of both analytes and IS were found to be better at the higher pH of the sample. The pH of the mobile phase was made acidic by using 0.1% formic acid to enhance the ionisation of the compounds in the mass spectrometer. The retention times of analyte and IS were different in both methods due to the difference in analytical columns used in both studies. In spite of the difference in elution patterns of the compounds among the methods, the accuracy and precision were found acceptable. Q Exactive Plus was found to be more sensitive with 3.083 ng/mL LOQ than the Triple quadrupole method with 7.883 ng/mL as LOQ due to the added selectivity of high-resolution mass over the unit resolution of the triple quadrupole type of mass analyser. With the limit of quantification of MET from plasma as the critical figure of merit, the methods were found to be comparable to earlier methods, which were reported to be in the range of 50 to 1 ng/mL[34–36]. Accordingly, the developed methods were considered suitable for monitoring MET concentration in rat plasma in the current study.

A single dose of 10 mg/kg of TCE showed no effect on the pharmacokinetics of MET, whereas a single dose of 100 mg/kg of TCE led to a minor increase in AUC and delayed half-life. These results inspired us to evaluate the effect of long-term treatment of TCE on the pharmacokinetics of MET. A significant increase in AUC, a decrease in CLr, and a delayed $T_{1/2}$ of MET at 100 mg/kg of TCE pretreatment for 14 days indicated a strong influence of TCE on the fate of coadministered MET. The elevated level of MET partly justifies the higher efficacy of its combination with TCE against hypertriglyceridemia and nephrotoxicity associated with diabetes[14,33].

Since metformin is not metabolised, is not significantly protein bound, and is excreted predominantly through the kidney[37] by the tubular secretion process, capitalising on OCTs[38], its clearance

from plasma has been primarily attributed to renal clearance[25]. Thus, a decrease in CLr suggests interference in renal clearance of MET. Although the exact mode of interference that leads to a reduction in MET CLr is not known, these findings support the hypothesis of competitive inhibition of OCTs by the constituents of TCE, leading to a decrease in the CLr of MET. While OCT3 is primarily involved in the intestinal absorption of MET[39], OCT1 is responsible for hepatic uptake. MET CLr is facilitated by renal tubular uptake primarily through OCT2[40]. An insignificant increase in C_{max} and no change in T_{max} suggest a lack of any interference on the absorption of MET by TCE. Whereas decreased CLr and delayed $T_{1/2}$ indicate interference in the CLr of MET. Thus, the TCE seems to selectively compete with MET for OCT2. This selectivity can be partly justified by the fact that MET is a superior substrate of OCT2 than other OCTs[40]. Besides, the selectivity due to interference from components of TCE cannot be ruled out.

Earlier, berberine has been shown to have selectivity towards OCT2[26]. However, this pharmacokinetic interaction cannot be explained by the selectivity of one component alone. To partly understand the potential holistic effects, the cationic components and their metabolites were docked with the resolved structures of these OCTs. It revealed that the components/metabolites have the potential to compete with MET for these transporters. Also, the majority of these components showed a relatively higher affinity for OCT2. Thus, the selectivity in binding affinity and free energy for OCT2 is in agreement with the *in vivo* data, which shows very little interference in absorption but a significant reduction in CLr. This partly suggests that the pharmacokinetic interaction is mediated through interference in the CLr of MET.

TCE has been shown to be effective against renal disorders[41,42] and is generally considered safe with an LD50 of >2000 mg/kg[43]. Thus, there is not enough data to indicate any toxicity due to the interaction of TCE with OCT2. However, there is a lack of adequate long-term studies to justify its chronic use. Since its complementary use in diabetes requires long-term administration and has the potential to saturate OCTs, this may have implications on the disposition of other drugs simultaneously administered with these antidiabetic regimens. Thus, more studies are necessary to validate the compatibility. Further, implications of elevated MET levels need investigations under preclinical and clinical settings. Moreover, pharmacokinetic interactions with other antidiabetic agents are not clear. Although prior research has shown the ability of TCE to elevate levels of glibenclamide through metabolism-mediated pharmacokinetic interaction[18], the same is not known for other antidiabetic drugs. So, a greater understanding of the potential of TCE to interfere with the absorption, distribution, metabolism, and excretion of other antidiabetic drugs is necessary to assess complementary compatibility.

Conclusion

Based on the potential of TCE as an alternative or complementary therapy for diabetes-related disorders, its combination with MET has been shown to be effective against hypertriglyceridemia and nephrotoxicity. The present investigation shows significant pharmacokinetic interaction between TCE (100 mg/kg) and MET (30 mg/kg) following coadministration over 2 weeks. This was associated with enhanced plasma levels of MET. While absorption of MET was not significantly affected, there was a significant reduction in clearance of MET, leading to a more than 2-fold increase in elimination half-life. The *in silico* analysis suggests the possibility of competitive saturation of OCTs involved in tubular secretions of MET as a plausible mode of this pharmacokinetic interaction. However, this mode of interaction needs further experimental validation. Also, prolonged use of TCE along with MET needs to be studied further to establish safety in preclinical and clinical conditions. Nonetheless, this study may partly explain the higher efficacy of the combination of TCE and MET against diabetes-related disorders. This may encourage further research on the use of TCE as a complementary therapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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