Medical University of Varna

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The abstract of the dissertation for the degree Doctor of science in "Pharmacology" of Assoc. prof. Kaloyan Dobrinov Georgiev, Ph.D.

“Identification, analysis and evaluation of pharmacokinetic and pharmacodynamics drug interactions”

Varna, 2020

Summary

The identification, analysis and evaluation of pharmacokinetic and pharmacodynamic drug interactions are of great importance at the preclinical stage in the development and implementation of new drugs. All new drug molecules, substrates, inhibitors or inducers of drug-metabolizing enzymes and/or drug-transporting proteins involved in their disposition should be tested to evaluate their risk of drug interactions. These tests are most commonly performed in vitro, and their risk of drug interactions is assessed using state-of-the-art simulation platforms (*in silico*). Today's computer technologies contribute to the high quality of these simulation programs that are able to simulate a clinical virtual study on a specific population group selected by age, gender, phenotype, co-morbidity, and more. It is for this reason that in silico and in vitro data are becoming increasingly important as an approach to identify likely, possible or unlikely drug interactions, thus allowing more informed planning of actual clinical trials or at low risk of simulations, the need to conduct such could be completely ignored. Pharmacodynamic drug interactions, most commonly evaluated in vitro, are a milestone in the development of combination drug therapies. Selecting those that increase the therapeutic effects on the one hand and reduce the side effects on the one hand is a real challenge for pharmaceutical science.

On the other hand, the identification, analysis and evaluation of pharmacokinetic and pharmacodynamic drug interactions is important in clinical practice as many infectious, cardiovascular, oncological and other diseases are treated by the simultaneous use of a large number of drugs - polypharmacy. Polypharmacy often leads to an increased risk of drug-related side effects. The older population is most affected by polypharmacy and its effects. The main cause factors behind this are the physiological changes associated with aging (decreased renal elimination, decreased liver function, decreased total body weight, etc.), the high incidence of comorbidities - polymorbidity, communication barriers, etc. Polypharmacy also leads to higher healthcare costs, increased risk of adverse drug events, and last but not least, it underlies the development of drug interactions. In addition to the risks it poses, using modern polypharmacy today, there is a significant improvement in the quality of life and the prevention of complications, including disability and unnecessary hospitalization. Significant progress has been made in understanding drug interactions and their successful use in clinical practice by introducing so-called pharmacokinetic boosters in anti-HIV and anti-hepatitis-C therapy. For this reason, balancing the risks and benefits of polypharmacy becomes a challenge for healthcare professionals. Strategies to help the healthcare practitioner succeed and avoid inappropriate polypharmacy are under development. The introduction into clinical practice of clinical pharmacists experienced in drug interactions, as well as improving physician-pharmacist communication, will increase the success of the therapy used and reduce the risk of adverse reactions for patients.

This dissertation aims to summarize the information available and obtained from the use of combined computational and experimental (in silico, in vitro, in vivo and in

populo) integrative pharmacometric models to support and enrich knowledge of drug interactions.

I. The Aim of the Study

The purpose of the dissertation is to study, analyze and evaluate drug interactions at the pharmacokinetic and pharmacodynamic level by applying modern and popular software platforms (*in silico*):

- Study and evaluation of pharmacokinetic interactions of novel synthetic oligopeptide molecules and plant fractions (*in vitro*)
- Study and evaluation of pharmacodynamic interactions (*in vitro*) and determination of organoprotective activity (*in vivo*)
- Detection of pharmacokinetic and pharmacodynamic interactions in clinical practice (*in populo*)

II. Tasks

On the basis of the objectives thus formulated, the following tasks were set:

1. Selection of plant fractions and oligopeptides for dissertation purposes:

a. Isolation and analysis of fractions with similar pharmacological characteristics from plant sources

- Isolation of polysaccharide fraction, polyphenolic fraction and total aqueous extract of *Lycium barbarum*
- Isolation of methylxanthine and catechin fraction from *Bancha* leaf and *Pu-erh* tea

b. Synthesis and analysis of peptidomimetics

- Synthesis and analysis of endomorphine-2 analogues
- Synthesis and analysis of RGD analogues

2. Determination of the inhibitory potential of in vitro experimental plant fractions and peptide analogues obtained from Task 1 on cytochrome P450 isoenzymes – *CYP3A4* and *CYP2C9*.

3. Selecting those who have shown potential for drug interactions and assessing the risk of drug interactions using basic static models.

4. Preparation of pharmacokinetic profile of Task 2 compounds and plant fractions using software platforms.

5. Simulation of possible pharmacokinetic drug interactions using modern simulation programs.

6. Determination of in vitro antiproliferative activity of the selected plant fractions on human tumor and non-tumor cell lines.

7. Determination of pharmacodynamic drug interactions (synergism, antagonism and additivity) in vitro on tumor cell lines of selected plant fractions when combined with standard therapeutic drugs (doxorubicin) using the most common methods for evaluating these interactions.

8. Determination of cardio- and nephroprotective effect of selected plant fractions in models of doxorubicin-induced cardio- and nephrotoxicity.

9. Study of potential drug interactions - pharmacokinetic and pharmacodynamic in clinical practice (in populo) using commonly used software programs (in silico).

10. Conclusion of the results obtained on the place of computer technology in the study and evaluation of drug interactions.

III. Materials and Methods

1. Isolation and analysis of fractions of *L.barbarum* (goji berry)
   - Isolation and analysis of safctin fraction - content of polyphenols (Singelton & Rosi), anthocyanins, phenolic acids and flavonoids (spectrophotometric)
   - Isolation and analysis of polysaccharide fraction – total uronic acids (Blumenkrantz & Asboe-Hansen), degree of esterification (Anthon & Baret), degree of acetylation (McComb & McCready) and protein content (Bradford)
2. Isolation and analysis of fractions of *Camellia sinensis* (*Pu-erh* and *Bancha*)
   - Catechin fraction isolation and analysis (spectrophotometric)
   - Isolation and analysis of methylxanthine fraction (HPLC)
3. Synthesis and analysis of endomorphine-2 and RGD analogues
   - Solid phase peptide synthesis
   - Analysis of synthesized oligopeptides – *HPLC*
4. *Vivid*® *CYP450* Screening Kits for *CYP3A4* and *CYP2C9*
5. Using simulators – *ADMEWORKS DDI* and *SimCYP*
6. Cell lines – *BJ, HT-29, MCF-10A, MCF-7, MDA-MB-231*
7. In vitro methods of antiproliferative activity – MTT-assay and neutral red assay
8. Methods used for determination of pharmacodynamics interactions – Bliss independence and Chou-Talalay combination index
9. Experimental animals – 72 Wistar rats, biochemical analysis and histopathological analysis
10. Selection of patients and analysis of potential DDI of prescribed pharmacotherapy with *Lexicomp* drug interactions checker

IV. Important results and discussion of pharmacokinetic drug interactions

For the needs of the dissertation, three fractions of *L. barbarum* fruits were isolated - total fraction (containing polyphenols and polysaccharides), polysaccharide fraction and pectin-free (polyphenolic) fraction. All fractions were analyzed spectroscopically and were determined the major content in them.

Determination of Inhibitory Potential of Fractions Isolated from L.barbarum fruits on CYP3A4 and CYP2C9 Activity

The both isolated fractions from *L.barbarum* were tested for inhibition on CYP3A4 and CYP2C9.

Assessment of CYP3A4 inhibition by L.barbarum fractions

To assess the inhibition of CYP3A4, we applied the polysaccharide and polyphenolic fractions from *L. barbarum* in following concentrations – 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml. Results are shown in Figure 1.

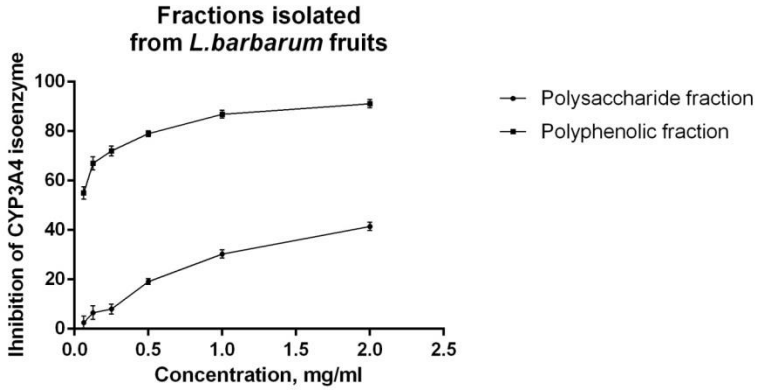


Figure 1

Assessment of CYP2C9 inhibition by L.barbarum fractions

The fractions isolated from *L.barbarum* were also tested for inhibition of *CYP2C9* in the same concentration manner. The results on *CYP2C9* were similar to those on *CYP3A4* (Figure 2).

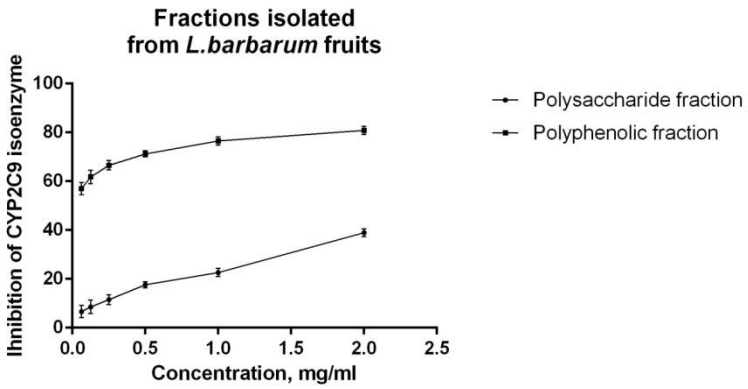


Figure 2

Determination of IC_{50} values

In order to determine the approximately IC_{50} values with 95% confidence interval (CI) of the individual fractions on the *CYP3A4* and *CYP2C9* isoenzymes; we have extrapolated the results in logarithmic manner. The results are summarized in Table 1.

	IC_{50}* values (95% CI) on *CYP3A4	***IC_{50}* values (95% CI) on *CYP2C9***
Polysaccharide fraction	2.244 mg/ml (1.593 to 3.161)	4.094 mg/ml (2.723 to 6.158)
Polyphenolic fraction	0.119 mg/ml (0.086 to 0.166)	0.048 mg/ml (0.036 to 0.063)

Table 1

As can be seen from the table, the polyphenol fraction is significantly more potent than the polysaccharide regards inhibition of *CYP3A4* and *CYP2C9*, ~ 20 folds and 85 folds, respectively.

Discussion

A “superfruit” or a “superfood”, as *Lycium barbarum* L. (Goji berry) is known in the Western countries, has many useful nutritional and medicinal properties such as antioxidant, antidiabetic, antihyperlipidemic, immunomodulatory, anticancer,

neuroprotective etc. Despite all the beneficial effects of consumption of *L.barbarum* fruits, there are many reports that announce for serious drug interactions with enzymes involved in the first phase of drug metabolism. Our results indicate that *L.barbarum* extracts have inhibitory effects on investigated CYPs – *CYP3A4* and *CYP2C9*. Moreover, the polyphenolic fraction has more pronounced effect than the polysaccharide fraction (table 1). This would presumably lead to possible herb-drug interactions (HDIs). These results are confirmed by other authors. No clinical interactions of *L.barbarum* extracts with *CYP3A4* substrate drugs have been reported in the literature. The major components in the individual fractions were not identified and at this stage it was impossible to build up simulation models to evaluate drug interactions. For this reason, basic prediction models were used to evaluate the risk of interactions converted to orally administered drugs (in this case herbal fractions) to evaluate the inhibition of intestinal *CYP3A4* and *CYP2C9* isoforms:

$$R_1 = \frac{AUC_i}{AUC_0} = 1 + \frac{[I_g]}{K_i}$$

The mechanism of inhibition is competitive, reversible, and according to the *Michaelis-Menten* kinetic equation, K_i would be $IC_{50}/2$, or in our case K_i, cyp3a4 = 2.244/2 = 1.122 mg/mL and 0.119/2 = 0.0595 mg/mL and *Ki*, cyp2c9 = 4.094/2 = 2.047 mg/mL and 0.048/2 = 0.024 mg/mL for the polysaccharide and pectin-free fraction, respectively. Due to the lack of the exact chemical composition in the two fractions used and also how much of them would be absorbed to reach the liver and interact with cytochromes, the worst possible assumptions are made. The maximum concentration in the intestinal lumen after an oral dose ([Ig]) is calculated as follows:

$$[I_g] = \frac{Dosage}{250\ ml}$$

The formula above assumes that 250 ml is the volume in which the entire dose is dissolved and this dissolution is immediate. Other assumptions have been made that with one standard oral dose of *L.barbarum* (15 g), about 0.75-1.2 g of polysaccharides (5-8% of dry weight) could be extracted [Cheng J et al., 2015] and about 0.005-0.05 g of polyphenols [Islam T et al., 2017]. The results are summarized in Table 2.

Fractions isolated from L.barbarum	*Ig, mg/mL*	*Ki, mg/mL*		*R_1*	
		CYP3A4	*CYP2C9*	*CYP3A4*	*CYP2C9*
Polysaccharide fraction 750 mg	3.0	1.1	2.1	3.73	2.43
Polysaccharide fraction 1200 mg	4.8	1.1	2.1	5.36	3.29
Polyphenolic fraction 5 mg	0.02	0.06	0.02	1.34	2.0
Polyphenolic fraction 50 mg	0.2	0.06	0.02	4.33	**11.0**

Table 2.

According to the *FDA* and *EMA* guidelines, if the calculated R value for an inhibitor is less than 1.1, it can be concluded that the drug being evaluated is not a "perpetrator" of drug interactions. Due to the higher concentrations of "perpetrators" in the small intestine, the limit values are different. For example, when [Ig] is calculated using equation 29, the limit value for R is 11 instead of 1.1.

The calculated GIT values of R_1 in the case of the polysaccharide fraction were 3.73 and 2.43 (at the 750 mg dose) and 5.36 and 3.29 (at the 1200 mg dose), and in the second case, the 5 mg polyphenolic fraction was 1.34 and 2.0 for *CYP3A4* and *CYP2C9*, respectively. According to the guidelines, as stated above, values of R_1 greater than 11 indicate a risk of drug interactions. In this case, the results obtained show that there is no risk of drug interactions when using separate fractions of *L. barbarum* with *CYP3A4* and *CYP2C9* substrates in enterocytes when co-administered with them. R_1 values were also calculated at a dose of 50 mg of polyphenols, which indicated that there was no risk with *CYP3A4* substrates but a potential risk with *CYP2C9* substrates - R1 = 11.

If such concentrations are able to reach the bloodstream and reach the liver enzymes, which is generally unlikely, then the risk of drug interactions would be high.

Determination of Inhibitory Potential of Methylxanthine Fractions Isolated from Pu-erh and Bancha tea leaves on CYP3A4 and CYP2C9 Activity

After isolation of the methylxanthine fractions from *Pu-erh* and *Bancha* tea leaves, *HPLC-UV* analysis showed that both fractions contain large amounts of caffeine (84.07% and 88.11%) and very small amounts of theobromine (0.16% and 0.11%, respectively). The results are summarized in Table 3 and Figure 3.

Methylxanthine	*Concentration (µg/mL) ± RSD**		*Concentration (%)*	
	Pu-erh	***Bancha***	***Pu-erh***	***Bancha***
Caffeine	525.45 ± 0.17%	220.28 ± 0.14%	~ 84.07	~ 88.11
Theophylline	0.0001 ± 0.098%	–	~ < 0.0001	–
Theobromine	3.88 ± 0.090%	1.06 ± 0.078%	~ 0.62	~ 0.42

- Below the quantification limit

Table 3

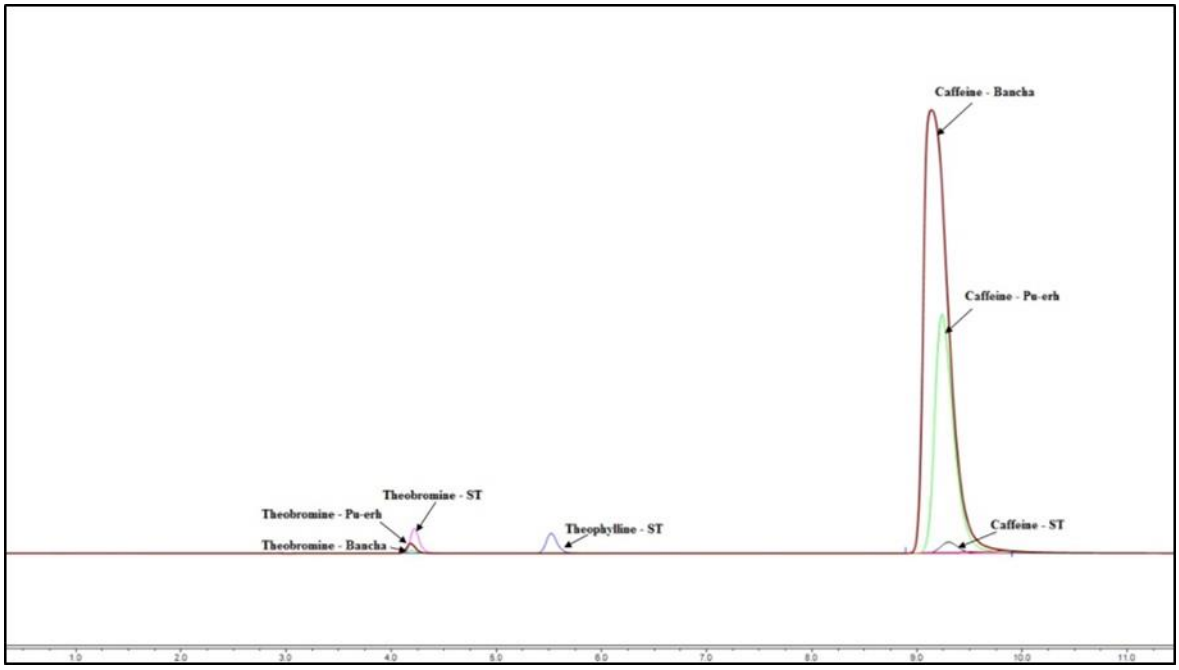


Figure 3

The effects of methylxanthines and caffeine alone on the activity of recombinant human CYP3A4 was analysed *in vitro* using a *Vivid*® *CYP3A4 Screening Kit*. The methylxanthine fractions inhibited *CYP3A4* (Figure 4) in a concentration-dependent manner.

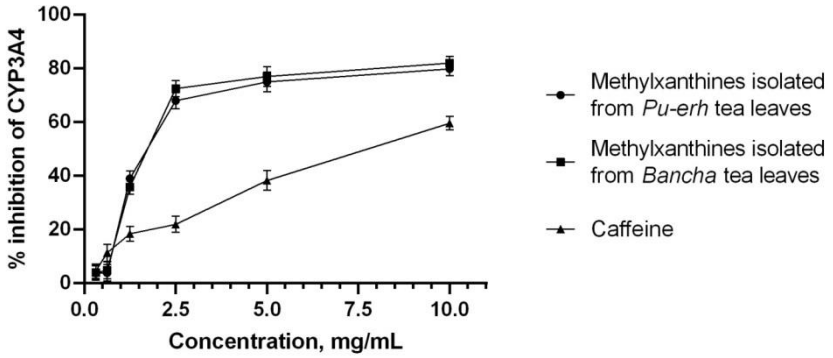


Figure 4

After analysing the results, we calculated the IC_{50} values for each fraction with a 95% confidence interval (CI). The IC_{50} value for the fraction isolated from *Pu-erh* was 1.283 mg/mL (CI: 0.9336 to 1.762 mg/mL), and that for the fraction isolated from *Bancha* was 1.351 mg/mL (CI: 1.119-1.631 mg/mL) (Table 4).

Test fractions and pure caffeine	*IC50 values with 95% CI on CYP3A4*
Methylxanthine fractions from Pu-erh tea leaves	1.28 mg/mL (CI 0.93 - 1.76 mg/mL)
Methylxanthine fractions from Bancha tea leaves	1.35 mg/mL (CI 1.11-1.63 mg/mL)
Caffeine	3,78 mg/mL (CI 1.59-8.99 mg/mL)

Table 4

To evaluate the risk of herb-drug interactions (HDI), we performed a simulations using ADMEWORKS DDI and *SimCYP* simulators with the methylxanthines isolated from *Pu-erh*. For the purpose of the study, we made some assumptions. First, we

assumed that the mechanism of inhibition is reversible and that it is generally competitive; therefore, according to the Michaelis-Menten Kinetic equation, Ki (the inhibition constant) would be $IC_{50}/2$, or in our case Ki = 1.28/2 = 0.64 mg/mL. Second, because it is the major component of the fraction, we used data from pure caffeine for the general information necessary for the simulation, and we only added the Ki values for inhibition of *CYP3A4*. For the substrate of *CYP3A4*, we chose midazolam because it is eliminated mainly through *CYP3A4*, and it is commonly used for evaluating CYP3A4 function. We simulated a single-dose administration of 7.5 mg of midazolam with concomitant consumption of 100, 250 and 500 mg of methylxanthines. For positive control we have used ketoconazole 400 mg single dose. The results performed by *ADMEWORSK DDI* are shown in Figure 5 and Table 5. The results performed by *SimCYP* are shown in Figure 6 and Table 6.

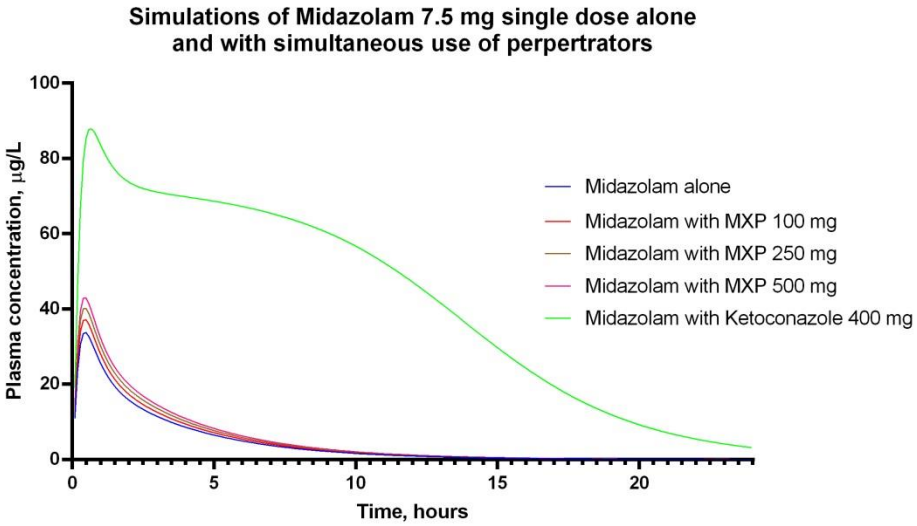


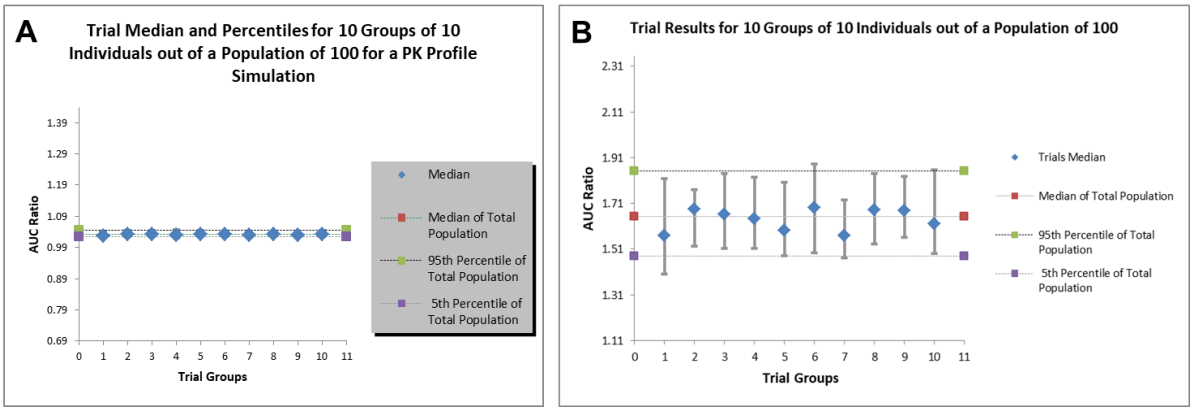
Figure 5

Different perpetrators	*Cmax ratio*	*AUCR*
MXP 100 mg single dose	1.103	1.099
MXP 250 mg single dose	1.194	1.187
MXP 500 mg single dose	1.276	1.27
Ketoconazole 400 mg single dose	2.618	11.438

Table 5

	$C_{max}R$ Dynamic	AUCR Dynamic	AUCR Static
***MXP* 100 mg**	1.0	1.0	1.14
***MXP* 250 mg**	1.01	1.01	1.34
***MXP* 500 mg**	1.02±0.01	1.03±0.01	1.65±0.12
***Ketoconazole* 200 mg**	3.36±1.08	9.73±3.80	17.14±6.94

Table 6



A. Dynamic B. Static

Figure 6

Discussion

The simulation of the simultaneous administration of the different doses of methylxanthine fraction from *Pu-erh* with midazolam resulted in a slight change in the time course curve, while ketoconazole used for positive control caused significant changes. According to the *EUFEPS* (European Federation for Pharmaceutical Sciences) conference report, AUCR values ≥ 2 indicate high risks of drug-drug interactions (DDIs), AUCR values ≤ 1.25 are associated with low risks, and AUCR values between 1.25 and 2 indicate moderate risks of DDIs. Therefore, in our case, the risk of clinically important herb-drug interactions (HDIs) is low. Although the changes observed in these simulations are insignificant, they are interesting, and some points have raised further questions.

In conclusion, it can be summarized that *in vitro* studies have suggested that methylxanthines in green tea, in this case isolated from *Pu-erh* and *Bancha*, can influence the activity of human *CYP3A4* isoenzymes. The modulation of CYPs has been well documented for green tea and its catechin fraction (described in more detail above). However, little is known about the involvement of methylxanthines, which may also be an important factor and most likely contributes to the biological activity of green tea. The concomitant administration of green tea, in the form of a dietary supplement or tea, and medicines metabolized predominantly by *CYP3A4* should be closely monitored for the potential risk of interactions. Using the two simulators, the negligible risk of potential interactions of the plant product, in this case the isolated methylxanthine fractions, and the drug was assessed. *ADMEWORKS DDI* software suggested a possible risk of using high doses of methylxanthines (500 mg), which was confirmed using a static model and rejected when performing dynamic simulations with *SimCYP* software. However, the interaction potential of methylxanthine fractions and their contribution to the observed effects of green tea use require further *in vivo* studies to confirm or reject these doubts.

Determination of CYP3A4 inhibitory potential of endomorphin-2 and RGD analogues

For the purpose of our study, we used four endomorphin-2 analogues, two of them were modified at third position - Phe(pF) (**1, Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine**) and conjugated at forth position with 1,2-ethylenediamine (**2, Tyr-Pro-Phe-Phe-1,2-ethylenediamine**). The other two, was conjugated at first position with deoxycholic acid and modified at third position - Phe(pF) (**3, Deoxycholic-Tyr-Pro-Phe(pF)-Phe-OH**) and Phe (pCl) (**4, Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH**) (Figure 7).

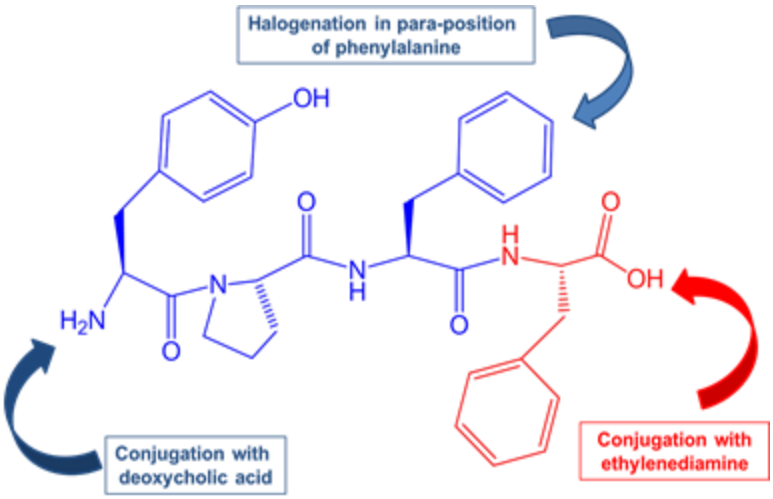


Figure 7

The four endomorphin-2 analogues we used showed potent and concentration-dependent inhibition of *CYP3A4* in concentrations of 6.25 to 100 μM. The observed effect of two of them (1 and 2) was more distinct. The other two analogues (3 and 4), have shown slightly less pronounced inhibition of *CYP3A4* (Figure 8). For comparison was used ketoconazole, classical inhibitor of *CYP3A4*, in concentration of 10 μM (90 % inhibition of *CYP3A4*).

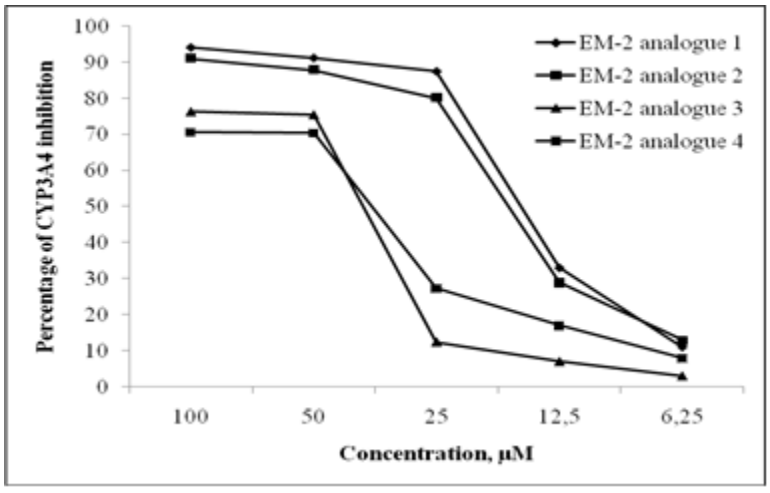


Figure 8

After obtaining the results and analyzing them, we calculated the following IC_{50} values for each analogue (Table 7).

EM-2 analogues	*IC_{50} values with 95% CI*
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine	15,00 μM (9,89-22,73) p<0,05
Tyr-Pro-Phe-Phe-1,2-ethylenediamine	16,42 μM (10,28-26,25) p<0,05
Deoxycholic-Tyr-Pro-Phe(pF)-Phe-OH	31,37 μM (20,02-54,07) p<0,05
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH	29,04 μM (21,33-32,96) p<0,05

Table 7

Based on *in vitro* results, some guidelines conclude, that if:

- 50 IC_{50}> 100 μM – low CYP inhibition
- 10 μM <IC_{50} <100 μM – moderate degree of CYP inhibition
- 50 IC_{50} <10 μM – high CYP inhibition

According to these criteria, all four endomorphin-2 analogs tested showed a moderate risk of *CYP3A4* inhibition. To date, no data have been found in the literature that have demonstrated the inhibitory capacity of endomorphins (endomorphin-1 and -2) or their analogs on cytochrome enzymes.

Simulation of pharmacokinetic behavior and drug interactions of endomorphine-2 analogues using ADMEWORKS DDI software

Because endomorphine-2 analogues are newly synthesized and there is no literature data yet available on their pharmacokinetic properties, appropriate software was needed to generate them based on their chemical structure. *ADMEWORKS DDI*® software was not provided with a modem capable of performing such a function. Therefore, two free software available on the web – *swissADME* and *pkCSM* – were used to predict the required physicochemical and some pharmacokinetic parameters. Because both software predict whether introduced molecules are substrates or inhibitors of the most common cytochrome isoforms, they have also been used to confirm or reject the observed inhibitory effects on the *CYP3A4* isoenzyme.

After receiving the *ADME* data from the two software used, pharmacokinetic behavior was simulated with the *ADMEWORKS DDI* and *SimCYP* softwares of two of the endomorphin-2 analogues – *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* and *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH*. The results are presented in Figure 9 and Table 8 and 9 for the 1 mg and the 10 mg dosage regimens.

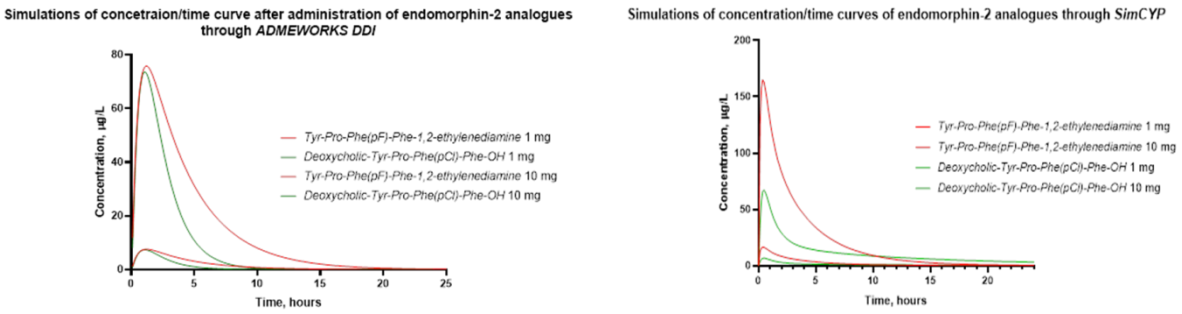


Figure 9

Endomorphin-2 analogues, dosage	*C_{max} (µg/L)*	*T_{max} (h)*	*AUC (µg/L.h)*
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine, 1 mg	7.58	1.09	38.23
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine, 10 mg	75.83	1.09	382.34
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH, 1 mg	7.368	1.08	22.056
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH, 10 mg	73.676	1.08	220.56

Table 8

Endomorphin-2 analogues, dosage	*C_{max} (µg/L) min PBPK/full PBPK*	*T_{max} (h) min PBPK/full PBPK*	*AUC (µg/L.h) min PBPK/full PBPK*
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine, 1 mg	12.83/16.5	0.98	53.4/53.14***
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine, 10 mg	128.35/165.3	0.98	533.53/535.75***
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH, 1 mg	2.81/6.72	1.7/0.49	28.69/27.88*
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH, 10 mg	28.11/67.18	1.7/0.49	286.85/278.65*

Table 9

To simulate drug interactions at the pharmacokinetic level, the two newly synthesized endomorphin-2 analogues, *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* and *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH*, were planned to be tested in 1 mg and 10 mg dosage regimens administered orally. The proven *in vitro* inhibition, as well as the

predicted *in silico* of the web platforms used (*swissADME* and *pkCSM*), were tested using *ADMEWORKS DDI* to predict the risk of drug interactions *in vivo*. The classical *CYP3A4* substrate and the positive control in the simulations were midazolam and ketoconazole, respectively. The results are presented in Figure 10 and Table 10, 11 and 12.

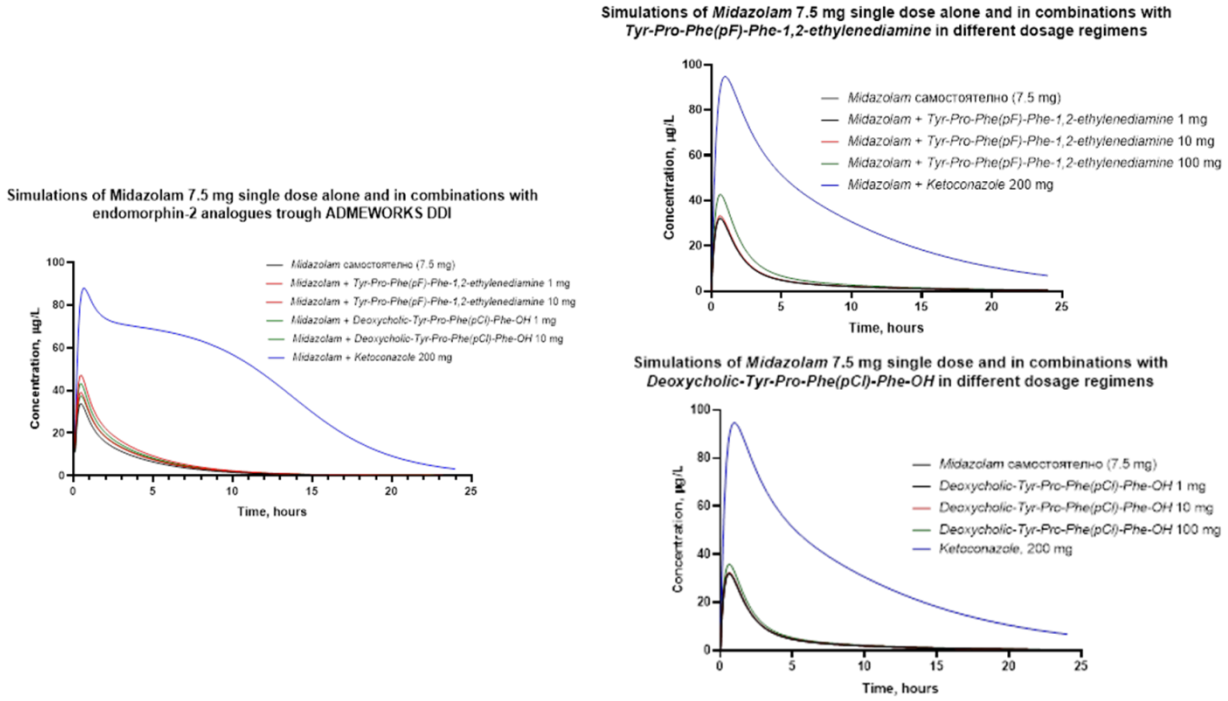


Figure 10

Endomorphin-2 analogues, dosage	***C_{max} ratio p.o.***	***AUCR p.o.***
***Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* 1 mg**	1.142	1.137
***Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* 10 mg**	1.38	1.375
***Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* 1 mg**	1.062	1.06
***Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* 10 mg**	1.281	1.275
***Ketoconazole* 200 mg**	2.602	10.13

Table 10

Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine	*C_{max}R/AUCR dynamic p.o.*	*C_{max}R/AUCR dynamic i.v. bolus*	*AUCR static p.o.*
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine 1 mg	1.0/1.0	1.0	1.01
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine 10 mg	1.02±0.01/1.03±0.01	1.04±0.01	1.14±0.04**
Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine 100 mg	1.36±0.11/1.42±0.12	1.18±0.06/1.22±0.07	1.67±0.26**
Ketoconazole 200 mg	3.68±1.08/9.83±3.8	10.03±4.18	17.99±9.54

Table 11

Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH	*C_{max}R/AUCR Динамичен p.o.*	*C_{max}R/AUCR Динамичен i.v.*	*AUCR Статичен p.o.*
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH 1 mg	1.0/1.0	1.0	1.0
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH 10 mg	1.01/1.01	1.0	1.04±0.01
Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH 100 mg	1.08±0.02/1.08±0.02	1.01/1.01	1.30±0.11**
Ketoconazole 200 mg	3.68±1.08/9.83±3.8	10.03±4.18	17.99±9.54

Table 12

Discussion

The construction of pharmacokinetic models of oligopeptides, as in our case, is extremely difficult, since they possess properties of large polypeptides/protein molecules and, small molecules at the same time. With respect to the newly synthesized peptide analogues of endomorphin-2, there was insufficient data to construct the simulation models. Therefore, two available in the internet software's were used – *swissADME* and *pkCSM*, which based on chemical structure, could predict pharmacokinetic parameters. Both platforms used to predict pharmacokinetic behavior, *swissADME* and *pkCSM*, predicted that the ethylenediamine analogs of enomorphine-2 – *Tyr-Pro-Phe-Phe-1,2-ethylenediamine* and *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* are substrates of *CYP2D6* and *CYP3A4*, and are also *CYP3A4* inhibitors. Moreover, *swissADME* predicts that *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* is an inhibitor of *CYP3A4*.

AUCi (with inhibitor)/AUCo (no inhibitor) (AUCR) values predicted by *ADMEWORKS DDI* software indicated a lack of possible interactions with *CYP3A4* substrates when administered at a low dose of 1 mg and a possible moderate risk of higher dose, in this case 10 mg of endomorphin-2 analogues.

Using a static model (Simcyp simulator) to evaluate the risk of drug interactions may in some cases lead to an overestimation of this risk due to the use of static inhibitor concentrations. In this case, an increase in the *AUCR* values was observed in the *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* analogue of endomorphine-2 using the static-mechanistic method, and the statistically significant values were in the 10 mg and the 100 mg. In the *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* analogue, statistically significant differences between the dynamic and static methods were observed at the 100 mg dose. The dynamic simulations of *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* with *SimCYP* software showed no risk of possible drug interactions by oral administration with CYP3A4 substrates (in this case midazolam) using doses of 1 mg and 10 mg. Only with the highest dose – 100 mg, there was a moderate risk of possible DDIs. The *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* analogue showed no risk of drug interactions at all three doses (1, 10 and 100 mg) used for the simulation. As mentioned above, the main use of endomorphine-2 and its analogues is parenteral. For this reason, parenteral drug interactions were simulated – intravenously, bolus at the same doses. With the *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* analogue, the change in the route of administration resulted in a decrease in the AUCR ratio and the risk accordingly. In the *Deoxycholic-Tyr-Pro-Phe (pCl)-Phe-OH* analogue, pathway change affected the *AUCR* ratio as well.

Conclusion

The dynamic simulations performed with the methylxanitine fractions showed a low risk of drug interactions with *CYP3A4* substrates, which, as recommended by regulatory agencies, does not require clinical studies for monitoring and evaluating if a new nutritional supplement will be developed and introduced to the market over time. Only the Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine analogue will be required to be

tested clinically for *DDIs* in doses of 100 mg or greater in the future. Of course, the simulations were performed with *in silico* data and if *in vivo* models are collected in the future, the situation may change.

This chapter illustrates only situations where the *CYP3A4* isoform of the cytochrome is involved, and fractions and oligopeptides are evaluated for their potential to influence it. Other isoforms of cytochromes, such as *CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19* and *CYP2D6*, glucuronyltransferases - UGT1A1 and UBT2B7, as well as transport proteins such as P-gp, BCRP, OATP1B1, OATP1B1, OATP1B1 K, should be evaluated in the same way if a risk of drug interactions is demonstrated.

V. Important results and discussion of pharmacodynamic drug interactions

In vitro evaluation of combination effects of doxorubicin with methylxanthine fractions isolated from Bancha and Pu-erh tea against breast cancer cells

To determine whether methylxanthine isolated from *Bancha* and *Pu-erh* could sensitizes *MCF-7* and MDA-MB-231 cells to treatment with doxorubicin, we cultured cells with doxorubicin at concentration range of 0.02 to 0,6 μM, alone or in combination with fixed 100 and 400 μg/ml methylxanthines from *Bancha*; 100 and 200 μg/ml methylxanthine from *Pu-erh*, for 72 hours. The cell proliferation was assessed using neutral red uptake assay. The results from treatment of *MCF-7* and MDA-MB-231 cells are shown in Figure 11.

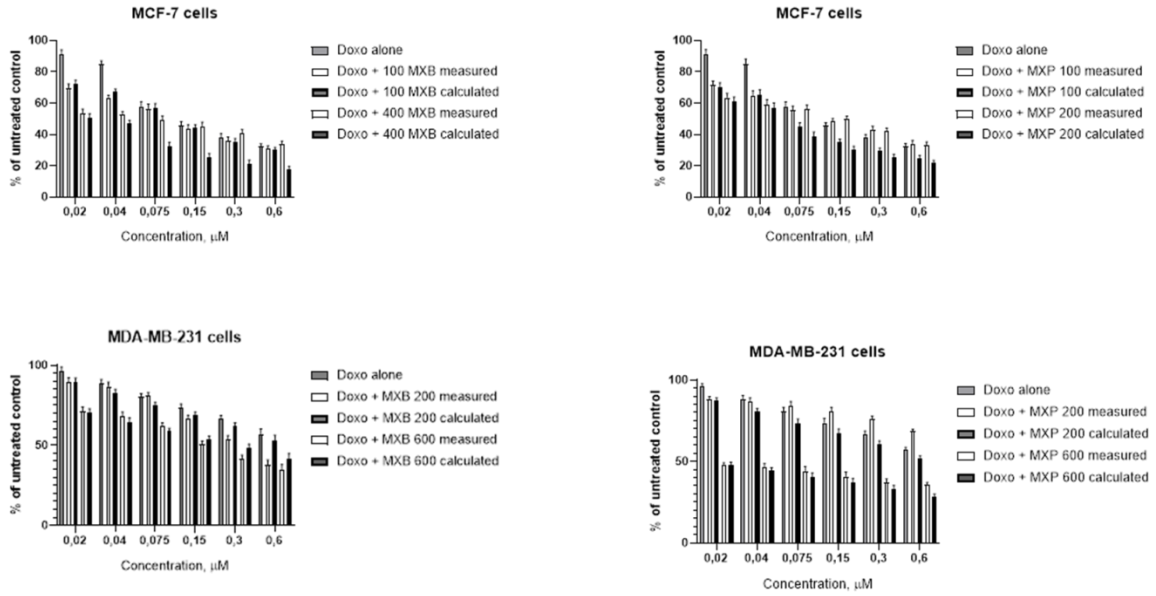


Figure 11

As can be seen from Figure 11, lower concentrations of doxorubicin (0.02-0.04 μM) with 100 μg/ml MXB have synergistic effects and with an increase in the concentrations of doxorubicin (0.075-0.6 μM) the effects become additive. The highest concentration of MXB – 400 μg/ml, leads to antagonistic effects. The situation with methylxanthine from *Pu-erh* was similar. We observe additive effects in lower concentrations of doxorubicin (0.02-0.04 μM) with both used concentrations of MXP. With the other used concentrations the effects were antagonistic. We tested combination effects of doxorubicin and methylxanthine isolated from *Bancha* and *Pu-erh* on *MDA-MB-231* cell line. Because *MDA-MB-231* cell line was more resistant to the action of doxorubicin, we used higher concentrations of methylxanthines – 200 and 600 μg/ml. The methylxanthine isolated from *Pu-erh* only at the lowest concentration of doxorubicin (0.02 μM) provided additive effects, all other concentrations showed antagonistic effects. The situation with methylxanthines isolated from Bancha was completely different. In the lower concentrations, doxorubicin (0.02-0.075 μM) and MXB (200 and 600 μg/ml) have additive effects, while in the highest concentrations (0.15-0.6 μM) the combination is with significantly expressed synergistic effect. To confirm the

observed synergistic effects, we used *CalcuSyn*® software, to calculate the combination index. The results are presented in table 13.

Doxorubicin concentration (µM)	*MXB concentration (µg/ml)*	*CI*	*MXB concentration (µg/ml)*	*CI*
0.02	200.0	1.20636	600.0	1.06166
0.04	200.0	1.24558	600.0	1.03197
0.075	200.0	1.21176	600.0	0.93512
0.15	200.0	0.81526	600.0	0.73723
0.3	200.0	0.76702	600.0	0.68348
0.6	200.0	0.59957	600.0	0.75423

Table 13

Cardio- and nephroprotective effects of methylxanthine fractions isolated from Bancha tea leaves against doxorubicin models of cardio- and nephrotoxicity in rats

Effects on biochemical parameters for cardio- and nephrotoxicity

Tables 14 and 15 and Figures 12 and 13 summarize the results showing the effect of metylxanthines isolated from *Bancha* tea leaves on serum levels of biochemical parameters for cardio- and nephrotoxicity.

Groups	*Creatine kinase (CK), U/L*	*Creatine kinase MB-fraction (CK-MB), U/L*	*Lactate dehydrogenase (LDH),U/L*	*Aspartate aminotransferase (ASAT), U/L*
Group 1 – control	324±14	22.3±1.6	209.63±19.6	89.87±9.87
Group 2 – 5 mg/kg (p.o.) MXB	304±9.7	24.7±3.6	155.63±7.6	78±5.7
Group 3 – 1 mg/kg (p.o.) MXB	311±9.6	23.7±3.6	164.7±5.8	69.7±6.7
Group 4 –5 mg/kg (p.o.) MXB + Doxo 20 mg/kg (i.p.)	856.8±69.4	34±5.7	325±32.6	254.8±46.9
Group 5 – 1 mg/kg (p.o.) MXB + Doxo 20 mg/kg (i.p.)	987.65±121.4	37±4.6	444±43.7	584±32.8
Group 6 – Doxo 20 mg/kg (i.p.)	1260±80.1	47.8±7.8	633.11±32.4	687.23±22.35

Table 14

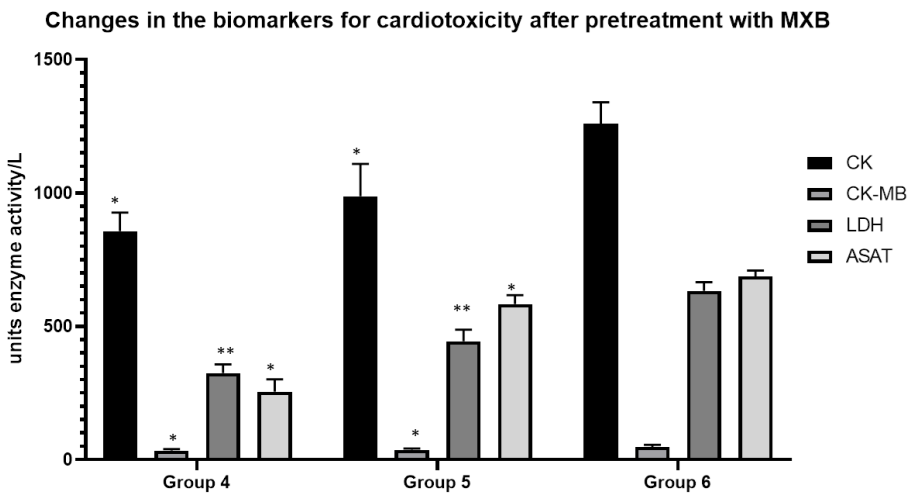


Figure 12

Groups	*Urea (BUN) mmol/L*	*Creatinine µmol/L*	*Uric acid µmol/L*
Group 1 – control	6.3±0.8	37.71±3.6	32.7±2.6
Group 2 – 5 mg/kg (p.o.) MXB	7.4±0.54	38.64±2.7	36.6±3.43
Group 3 – 1 mg/kg (p.o.) MXB	7.7±0.96	35.75±2.8	34.6±1.7
Group 4 –5 mg/kg (p.o.) MXB + Doxo 20 mg/kg (i.p.)	7.88±1.1	42.54±5.98	55.2±4.32
Group 5 – 1 mg/kg (p.o.) MXB + Doxo 20 mg/kg (i.p.)	9.87±1.6	59.3±2.8	98.8±6.5
Group 6 – Doxo 20 mg/kg (i.p.)	11.11±0.8	62.45±5.6	247.41±25.4

Table 15

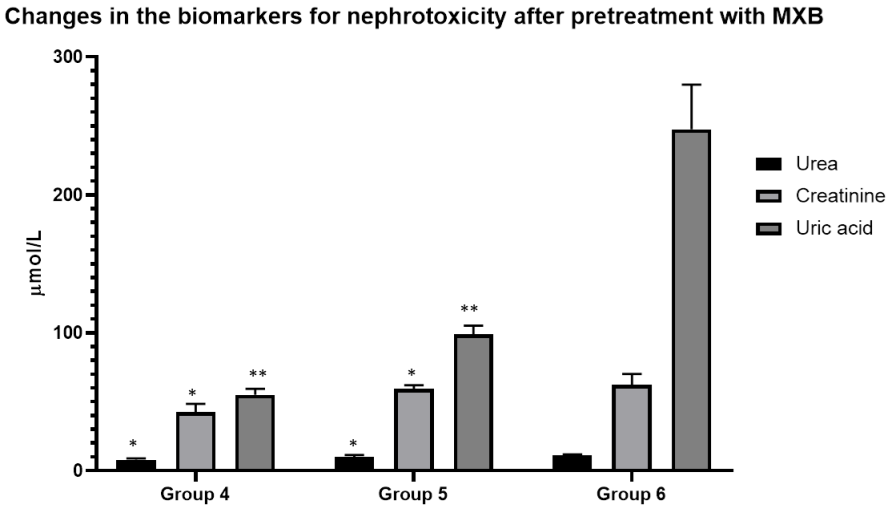


Figure 13

Cardio- and nephrotoxicity models induced by the cumulative dose of doxorubicin (20 mg/kg) show significant differences in the biochemical parameters (comparison results not shown in the figures) compared to control group ($p < 0.001$, receiving physiological solution only) indicating cardiac and renal tissue damage. Methylxanthines isolated from *Bancha* tea leaves did not lead to statistical changes in these parameters when applied alone compared to control group ($p = 0.573$, comparison results not shown in the figures). However, *Bancha* methylxanthines showed a dose-dependent decrease in serum levels of biochemical parameters when co-administered with doxorubicin, suggesting a decrease in cardiac ($p < 0.05$ in CK, CK-MB, and ASAT; $p < 0.01$ in LDH) and renal ($p < 0.01$ in uric acid; $p < 0.05$ in creatinine and BUN) tissue damage in comparison with the rats treated with doxorubicin alone.

Effects on histopathological parameters of cardio- and nephrotoxicity

Examination of hematoxylin-eosin (H&E) stained longitudinal sections of the control group (group 1) revealed a normal histological structure of the ventricular myocardium (Fig. 14A) - branched and transverse striated muscular fibers with central oval nuclei. In contrast, rats treated with doxorubicin alone (group 6), show significant histological changes (Fig. 14B) - disrupted myofibrils architecture, myocyte disorganization, cytoplasmic fading, and pyknotic nuclei formation are observed.

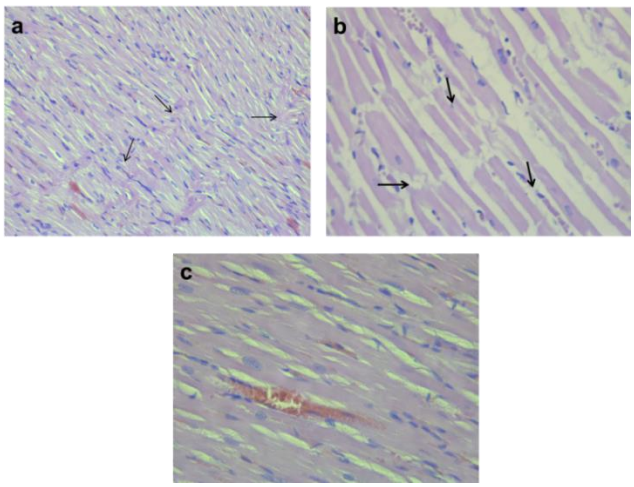


Figure 14

On the other hand, pretreatment with methylxanthines at dose of 5 mg/kg (Fig. 14C) results in an almost normal myocardial structure with very limited mononuclear infiltration of the interstitium and a marked reduction in collagen fibers between cardiac myocytes compared to the doxorubicin group.

Histological examination of the renal tissue in the control group rats showed normal architectonics of the renal glomeruli and tubules (Fig. 15A).

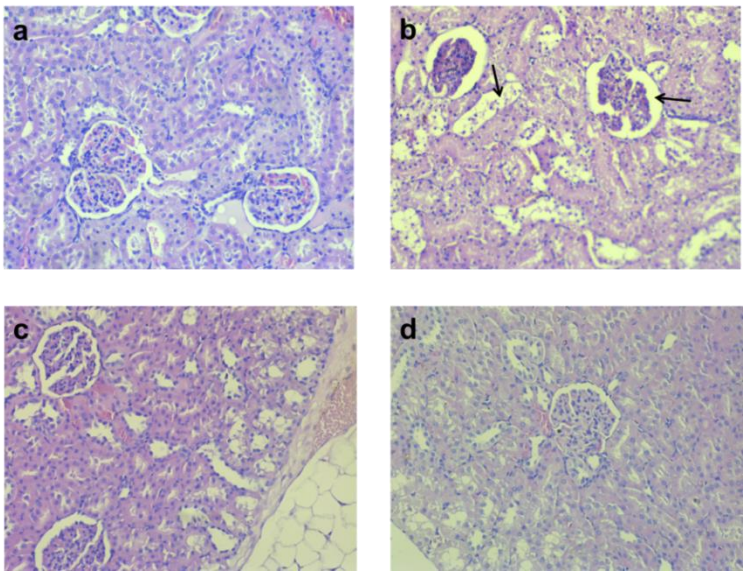


Figure 15

The doxorubicin group (Fig. 15B) showed significant glomerular and tubular disorders. We observed a total collapse of the glomeruli with necrotic changes in the epithelial tubular cells. Both groups pretreated with *Bancha* methylxanthines (5 mg/kg and 1 mg/kg) (Fig. 15C and 15D) showed significant resistance to the deleterious effects of doxorubicin. The lesions were mainly in the cortical area. At the higher doses of methylxanthines, there was a significant decrease in the morphological changes caused by doxorubicin exposure.

Conclusion

The methylxanthine fraction isolated from *Bancha* has shown encouraging results regarding the combined potential with conventional chemotherapeutic agents

such as doxorubicin. On the one hand, in vitro synergistic effects with doxorubicin have been demonstrated with respect to their co-administration to breast cancer cells, and on the other, they have shown the potential to reduce major limiting factors in the use of doxorubicin, namely cardio and nephrotoxicity, in animal models (*in vivo*) in rats.

Synergistic growth inhibitory effects of Lycium barbarum (Goji berry) extract with doxorubicin against human breast cancer cells

To determine whether *L. barbarum* extract (LBE) could sensitizes *MCF-7* and *MDA-MB-231* to treatment with doxorubicin, we cultured cells with doxorubicin at concentration range of 0.02 to 0.6 µM, alone or in combination with fixed 100, 120 or 140 µg/ml LBE concentrations, for 72 hours. The cell proliferation was assessed using MTT assay. The results showed that cell viability was much lower with the combination treatment than with doxorubicin alone. The calculated and measured combination effects, using the *Bliss* method, are presented in Figure 16 and 17.

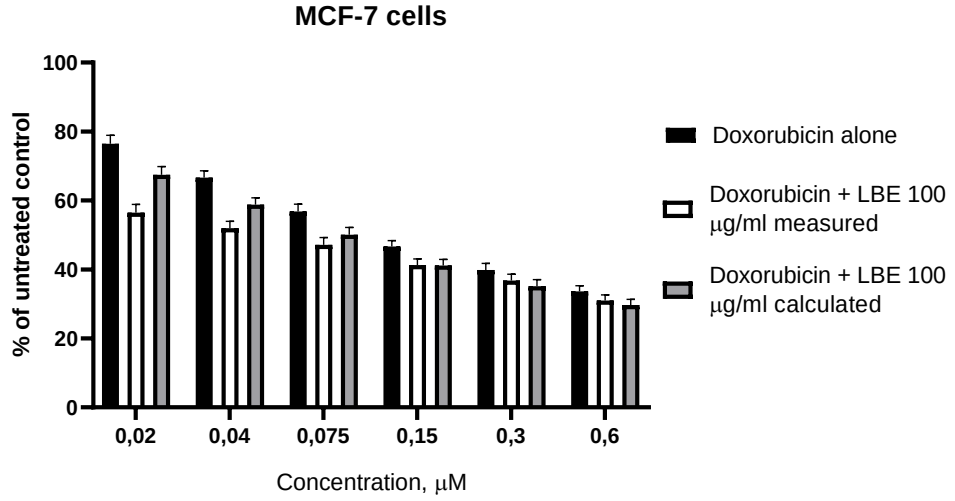


Figure 16

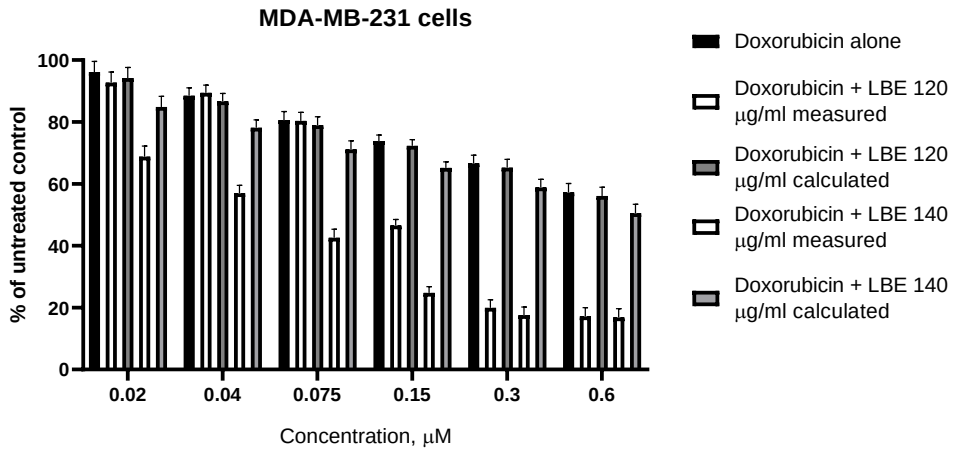


Figure 17

Using the same results and the *CalcuSyn*® software, we calculated combination index. The results are presented in table 16 and table 17.

Doxorubicin concentration (μM)	*LBE concentration (μg/ml)*	*CI*
0.02	100.0	0.75862
0.04	100.0	0.83698
0.075	100.0	0.89938
0.15	100.0	0.97530
0.3	100.0	1.19463
0.6	100.0	1.32293

Table 16

Doxorubicin concentration (μM)	*LBE concentration (μg/ml)*	*CI*	*LBE concentration (μg/ml)*	*CI*
0.02	120.0	1.51415	140.0	0.92343
0.04	120.0	1.66865	140.0	0.89044
0.075	120.0	1.42208	140.0	0.82990
0.15	120.0	0.84412	140.0	0.74387
0.3	120.0	0.64732	140.0	0.72160
0.6	120.0	0.69030	140.0	0.76843

Table 17

Cardio- and nephroprotective effects of fractions isolated from L.barbarum in doxorubicin-induced cardio- and nephrotoxicity models in rats

In a similar manner to the experimental design described above with methylxanthines, the study was performed with the individual fractions of *L. barbarum* - the polyphenolic fraction, the polysaccharide fraction and the combination. The doses used for the three fractions were 2 mg/kg - determined from the available polyphenols, polysaccharides and polyphenols/polysaccharides (mixed in a 1:1 ratio) respectively.

The results of the measured biochemical parameters are presented in the tables.

Groups	*Creatine kinase (CK), U/L*	*Creatine kinase MB-fraction (CK-MB), U/L*	*Lactate dehydrogenase (LDH), U/L*	*Aspartate aminotrasferase (ASAT), U/L*
Group 1 – control	324±14	22.3±1.6	209.63±19.6	89.87±9.87
Group 2 – 2 mg/kg (p.o.) polyphenolic fraction	302.5±103.4	23.32±3.2	199.98±22.6	92.42±22.9
Group 3 – 2 mg/kg (p.o.) polysaccharide fraction	334.75±56.5	22.9±4.8	194.43±17.5	81.65±19.7
Group 4 – 2 mg/kg (p.o.) combination	318.85±49.9	21.4±1.3	184.32±11.5	79.65±12.8
Group 5 – 2 mg/kg (p.o.) polyphenolic fraction + Doxo 20 mg/kg (i.p.)	457.5±98.4	31.98±5.5	209±11.7	112.5±22.9
Group 6 – 2 mg/kg (p.o.) polysaccharide fraction + Doxo 20 mg/kg (i.p.)	534.75±56.5	35.87±3.8	222±12.3	204±23.8
Group 7 – 2 mg/kg (p.o.) combination + Doxo 20 mg/kg (i.p.)	418.85±49.9	33.33±4.9	231±18.6	225±17.5
Group 8 – Doxo 20 mg/kg (i.p.)	1260±80.1	47.8±7.8	633.11±32.4	687.23±22.35

Table 18

Groups	*Urea (BUN) mmol/L*	*Creatinine µmol/L*	*Uric acid µmol/L*
Group 1 – control	6.3±0.8	37.71±3.6	32.7±2.6
Group 2 – 2 mg/kg (p.o.) polyphenolic fraction	6.43±1.1	38.54±6.5	30.56±5.4
Group 3 – 2 mg/kg (p.o.) polysaccharide fraction	6.23±1.3	41.34±4.6	29.9±7.7
Group 4 – 2 mg/kg (p.o.) combination	6.21±1.8	44.43±3.6	28.9±5.7
Group 5 – 2 mg/kg (p.o.) polyphenolic fraction + Doxo 20 mg/kg (i.p.)	7.25±0.65	41.98±3.6	31.38±2.7
Group 6 – 2 mg/kg (p.o.) polysaccharide fraction + Doxo 20 mg/kg (i.p.)	9.45±0.64	55.44±2.87	57.93±3.3
Group 7 – 2 mg/kg (p.o.) combination + Doxo 20 mg/kg (i.p.)	6.78±0.5	43.56±4.87	30.34±2.5
Group 8 – Doxo 20 mg/kg (i.p.)	11.11±0.8	62.45±5.6	247.41±25.4

Table 19

Effects on histopathological parameters of cardio- and nephrotoxicity

The study of longitudinal sections stained with H&E of the control group (group 1) and that of doxorubicin-treated rats only (group 8) are shown in Figure 14a and 14b, respectively. Figure 18 shows only the groups treated with the individual fractions of *L.barbarum*. The individual fractions of *L.barbarum* (Goji berry) significantly reduce the degree of cardiotoxicity, as can be seen from the taken histological images. In the pectin-free fraction (Figure 18a) and the total extract (Figure 18в), the histological analysis showed a structure similar to that of the controls. Fibrotic changes were observed in the polysaccharide fraction (Figure 18б), but no myofibrillar ruptures were observed.

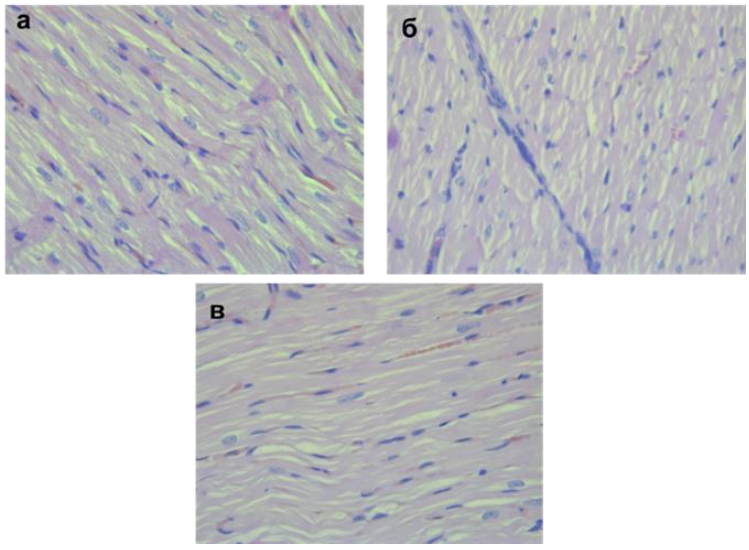


Figure 18

The control group and the model of doxorubicin nephrotoxicity are shown in Figures 15a and 15b. Figure 19 shows only the groups treated with the individual fractions of *L.barbarum*.

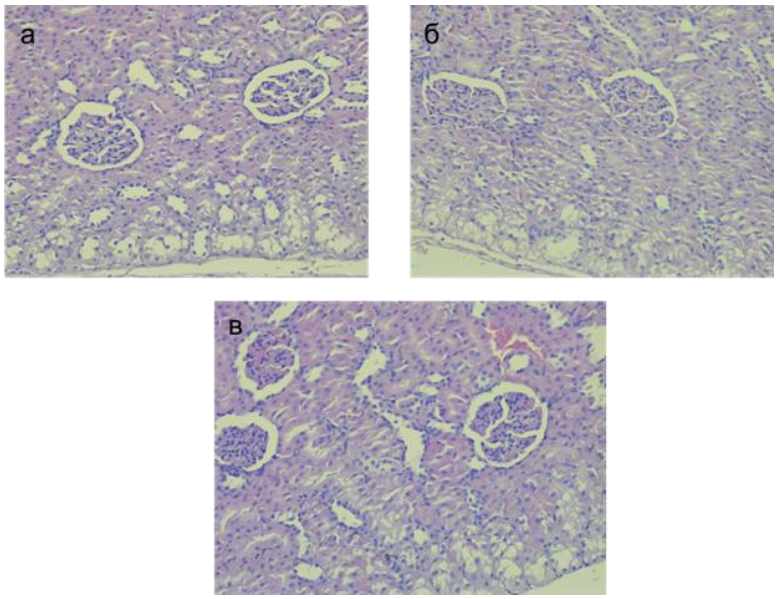


Figure 19

Here, renal histological incisions also show significantly less damage compared to the doxorubicin-treated groups alone. Minor damage in the cortical area is present, but significantly less pronounced than the model.

Conclusion

The fractions isolated from *L. barbarum* (Goji berry) and the methylxanthine fractions isolated from *Pu-erh* and *Bancha* have shown encouraging results regarding their combined potential with conventional chemotherapeutic agents such as doxorubicin. On the one hand, *in vitro* synergistic effects with doxorubicin have been demonstrated with respect to their co-administration to breast cancer cells, and on the other, they have shown the potential to reduce major limiting factors in the use of doxorubicin, namely cardio and nephrotoxicity, in rat's models (in vivo).

VI. Important results and discussion of pharmacokinetic and pharmacodynamic drug interactions in the clinical practice

After reviewing the medical records and the prescribed medications at discharge of 971 patients who have passed through the Cardiology Clinic at St. Marina University Hospital in Varna, Bulgaria, 248 patients were selected, based on the criteria mentioned above, and the risk of drug interactions was checked with the indicated software. The general patient characteristics are shown in Table 20.

Patients Characteristics	*Number (%); Mean ± standard deviation (SD) 2014*	*Number (%); Mean ± standard deviation (SD) 2015*
Gender		
Male	104 (43.5 %)	131 (52.8 %)
Female	135 (56.5 %)	117 (47.2 %)
Age		
≤ 40	2 (14.9%)	4 (14.9%)
41-60	38 (61.3 %)	33 (61.3 %)
≥ 60	199 (23.8 %)	211 (23.8 %)
Average age	70.34 (±10.16 SD)	72.35 (±10.16 SD)
Average age (males)	69.58 (±10.06 SD)	71.51 (±10.06 SD)
Average age (females)	70.93 (±10.24 SD)	73.29 (±10.24 SD)
Number of prescribed drugs		
≤5	11 (8.06%)	20 (8.06%)
5-7	129 (51.2%)	127 (51.2%)
≥ 7	99 (33.9%)	101 (33.9%)
Range	4-13	3-14
Average	7.27 (±2.07 SD)	7.12 (±2.07 SD)
Stage of heart failure (NYHA)		
2	15 (9.7%)	24 (9.7%)
3	222 (87.9%)	218 (87.9%)
4	2 (2.4%)	6 (2.4%)
Main comorbid condition		
Hypertension	239 (99.6%)	247 (99.6%)
Atrial fibrillation	99 (51.2%)	127 (51.2%)
Anemia	98 (40.7%)	99 (40.7%)
Diabetes mellitus	97 (39.9%)	101 (39.9%)
Kidney function		
eGFR<60 mL/min/m^2	87 (36.1%)	75 (30.2%)
eGFR<30 mL/min/m^2	23 (9.5%)	19 (7.8%)

Table 20

A comparison of the demographic characteristics of the two years showed no significant differences. In the study, 85% of the selected patients were elderly (median age> 70 years). The range of prescribed medicines is between three and fourteen, with 92% taking more than five medicines. On average, one patient received > 7

medications. In addition, > 35% of patients had chronic kidney disease and > 10% of patients had chronic kidney failure, another contributing factor to the increased risk of potential drug-drug interactions. The demographic characteristics we describe overlap with the previously published data. Straubhaar et al. reported an average of 79 years of age and an average of 8 medicines per patient at discharge of patients with CH after hospitalization [Straubhaar B et al., 2006]. All of these justify these patients being identified as high-risk for dangerous drug-drug interactions and the need for greater vigilance in their treatment. Patients with cardiovascular disease, especially those with HF, are more prone to potential drug-drug interactions. This is due to advanced age, complex drug regimens (polypharmacy) and, in addition, altered pharmacokinetics in these patients due to impaired hepatic and renal blood flow, leading to a change in drug elimination processes [Faulx MD & Francis GS, 2008].

DDIs Detected by Lexicomp®

In terms of the number of potential drug-drug interactions detected, during the two years (2014, 2015) they showed insignificant differences, with the average number of potential drug-drug interactions per patient being relatively constant - > 6 (6.2 ± 3.89 for 2014 and 6.28 ± 4.72 for 2015). Another risk factor indicating the need for increased alertness in these patients. The results obtained showed twice as many potential drug-drug interactions than studies by other colleagues [Straubhaar B et al., 2006], and are similar to those reported by Roblek et al. [Roblek T et al., 2014]. With regard to the risk category, most interactions were categorized into risk category C and, with respect to the severity of the interaction, a moderate interaction burden. These results overlap with other reports reported to date.

The most frequent drug interactions, with their possible mechanisms and results of interactions are presented in Table 21.

pDDI	Severity/Risk Category	Frequency	Result of Interaction	Possible Mechanism of DDI
ACE inhibitors				
ACE-inhibitors + Allopurinol	Major/D	6 (2.4 %)	↑risk of hypersensitivity	N/A*
Beta-blockers				
Beta-blocker + I_1-agonists	Moderate/D	16 (6.5 %)	↑risk of bradycardia	PD**
Beta-blocker + $Alfa_2$-agonist	Moderate/D	2 (0.8 %)	↑risk of bradycardia	PD
Carvedilol + Dabigatran	Major/D	4 (1.6 %)	↑risk of bleeding	P-gp***
Cardiac glycosides				
Digoxin + Amiodarone	Major/D	2 (0.8 %)	↑risk of digoxin toxicity	P-gp
Diuretics				
Loop diuretic + NSAIDs	Moderate/D	3 (1.2 %)	↑risk of nephrotoxicity/↓ effects of loop diuretics	PD
Loop diuretic + Desmopressin	Major/X	1 (0.4 %)	↑risk of hyponatremic effect of desmopressin	PD
Antiarrhythmics				
Amiodarone + Levofloxacin	Major/X	4 (1.6 %)	↑risk of QTc-prolonging effect	PD
The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins)				
Simvastatin + 1,4-DHP-CCB	Major/D	6 (2.4 %)	↑risk of statin toxicity	CYP3A4
Statin + Verapamil	Major/D	3 (1.2 %)	↑risk of statin toxicity	CYP3A4
Statin + Fenofibrate	Major/C	9 (3.6 %)	↑risk of statin toxicity	PD
Rosuvastatin + Amiodarone	Major/B	3 (1.2 %)	↑risk of statin toxicity	CYP2C9
Anticoagulants				
Acenocoumarol + Allopurinol	Moderate/D	2 (0.8 %)	↑risk of bleeding	CYP2C9?
Acenocoumarol + Fenofibrate	Major/D	4 (1.6 %)	↑risk of bleeding	N/A
Acenocoumarol + Amiodarone	Major/D	4 (1.6 %)	↑risk of bleeding	CYP2C9/PD?
Acenocoumarol + Thiamazole	Moderate/D	2 (0.8 %)	↓effect of acenocoumarol	PD
Acenocoumarol + NSAIDs	Moderate/D	3 (1.2 %)	↑risk of bleeding	N/A
Non-vitamin K oral anticoagulants (NOACs)				
Apixaban + Aspirin	Major/D	2 (0.8 %)	↑risk of bleeding	PD
Apixaban + Clopidogrel	Major/D	2 (0.8 %)	↑risk of bleeding	N/A
Dabigatran + Amiodarone	Major/D	2 (0.8 %)	↑risk of bleeding	P-gp
Dabigatran + Aspirin	Major/D	2 (0.8 %)	↑risk of bleeding	PD
Dabigatran + Carvedilol	Major/D	4 (1.6 %)	↑risk of bleeding	P-gp
Dabigatran + Fluconazole	Major/C	1 (0.4 %)	↑risk of bleeding	CYP3A4?, P-gp?
Antithrombotic drugs				
Clopidogrel + Proton-pump inhibitors	Moderate/D	24 (9.7 %)	↓effect of clopidogrel	CYP2C19
Clopidogrel + Apixaban	Major/D	2 (0.8 %)	↑risk of bleeding	N/A, PD?
Clopidogrel + Aspirin	Moderate/C	10 (4 %)	↑risk of bleeding	N/A, PD?
Ticagrelor + Aspirin	Major/D	1 (0.4 %)	↑risk of bleeding	N/A, PD?

Table 21

The results from the performed multivariate analysis have shown stronger association between the number of drugs taken and the occurrence of pDDIs – 37.84 (95%CI 9.012-158.896, P = <0.001). No statistically significant differences were found between age and occurrence of pDDIs – 1.008 (95%CI 0.441-2.308, P = 0.848) (Table 22).

Variable	*Total number of Patients (n=248)*		*Multivariate*	
	Detected pDDIs ≤ 9	Detected pDDIs > 10	OR (95% CI)	p-value
Patients' age				
≤ 60	29	8		
> 60	165	46	1.008 (0.441-2.308)	(P = 0.848)
Number of drugs				
≤ 7	135	2		
> 7	49	52	37.84 (9.012-158.896)	(P = <0.001)

Table 22

Simulation of pharmacokinetic drug interactions in low therapeutic width drugs using SimCYP software

As can be seen from Table 21, the most common pharmacokinetic mechanisms responsible for drug-drug interactions in selected drugs with small therapeutic widths included the cytochrome isoforms *CYP3A4* and *2C9*, as well as the efflux pump *P-gp*. Using the *Simcyp* software, examples of such interactions are illustrated in dissertation with the two cytochrome isoforms responsible mainly for the metabolism of statins and coumarin anticoagulants, as well as the transport protein *P-gp* responsible for the disposition of digoxin and dabigatran etexilate.

The wealth of data integrated into the *Simcyp* simulator allows to detect multiple drug interactions related to metabolizing enzymes and/or transport proteins and to simulate interactions that in many cases are as close as possible to those observed in clinical trials. The above examples have demonstrated some of the capabilities of the software, such as: 1) considering the possibility of incorporating alternative elimination pathways while inhibiting the primary drug elimination pathway; 2) identification of the most sensitive and most resistant to interactions in a population, depending on the genotype and phenotype manifestations in the population; 3) the ability to simulate drug interactions of major compounds and their active metabolites; 4) the ability to add several inhibitors simultaneously in the simulations; 5) the ability to change the route of administration, the dosage form used, and also change the timing of dosing around the clock.

Discussion

In the present study, 85% of the selected patients were elderly (median age 72). The range of prescribed drugs was between three and fourteen, 92% of patients have been taking more than five medicines with an average of 7.12 (±2.07 SD) per patient, and the average of pDDIs were 6 per one person (Table 1). The average age and the average number of drugs per patient were approximately overlapped with the data previously published. Straubhaar et al. have reported 79 years median age and medium 8 drugs per patient at discharging. The pDDI per patient at discharge they found to be 3. Our results have shown twice as many pDDIs, namely 6.28 (± 4.72 SD), and are similar to those reported by Roblek et al. Of course, it should be taken into account the fact, that the selection of the patients in the two cited studies are based primary on the diagnosis of HF, while our study is based on prescribed medications in HF patients with a high risk of interactions. Therefore we treated these patients, as patients more prone to hazardous drug interactions and in need of careful monitoring.

The most prevalent types of pDDIs were in category C - 1254 (81.85%) and moderate in severity - 1302 (84.99%) (Table 3). Both pDDI groups did not have serious and fatal consequences, but required careful monitoring of patients' symptoms. This result corresponds to the findings of similar reports. As can be seen from Table 4, drugs, which we have set as the main subject of the study – statins, anticoagulants and antithrombotics, cover ~ 37% of major DDIs and ~ 66% of pDDIs in category D.

In order to show the association of pDDIs with patients' age and number of drugs taken, we have performed a multivariate analysis. In our study, we found stronger association between the number of drugs taken (more than 7) and the occurrence of pDDI (more than 10) (Table 24). The association of pDDI and number of drugs is supported by other published studies. Ismail et al. and Murtaza G et al. demonstrated significant associations of pDDIs with old age, longer hospital stay, and increased number of prescribed medications. In our study, we didn't observe statistically significant association between old age and occurrence of pDDI (more than 10). Based on our study findings, we recommend an increased alertness in patients taking more than 10 drugs and closely monitoring for pDDIs.

In 2015, the Bulgarian Ministry of Health issued a regulation that demands hospitals with more than 400 hospital beds to appoint clinical pharmacists to track the medical care process. A grace period of three years has been envisaged for this regulation to enter into force. Such a policy aim to save up to 30% of the money for treatment and to prevent the side effects of certain medications. Reviewing the entire pharmacotherapy of the patients, the pharmacists can reduce hospital readmission and length of hospital stay. Therefore, a specialist, a clinical pharmacist, with a very good knowledge of the principles of pharmacology/pharmacotherapy should be involved for better outcome in the patient care. In order to keep a high professional level in clinical pharmacy it is necessary to innovate the training to meet the increased clinical requirements for the profession.

Conclusions

The incidence of drug interactions in HF patients is high. These patients should be carefully monitored for possible drug interactions. The clinical pharmacist, as a specialist in pharmacology/pharmacotherapy and drug-drug interactions, could contribute to the reduction of medication-related problems, such as drug interactions, and optimize the drug therapy by checking the prescribed treatment at the discharge of these patients.

VII. Conclusions of dissertation

Pharmacokinetic and pharmacodynamic interactions in methylxanitine fractions isolated from Bancha and Pu-erh

1. The main component in the isolated methylxanthine fractions from *Bancha* and *Pu-erh* tea leaves is caffeine – 88 % and 84 % respectively.
2. The methylxanthine fractions isolated from *Bancha* and *Pu-erh* only slightly inhibit the activity of *CYP3A4* isoenzymes but to a greater extent than pure caffeine, suggesting that other components are involved in the realization of this effect.
3. The calculated risk of drug interactions using basic equations to evaluate drug interactions does not imply such a risk when taking 100, 250 and 500 mg doses of methylxanthines.
4. In contrast, dynamic mechanistic models, using the *PBPK* models and using the software program (*ADMEWORKS DDI*), suggested a potential risk of drug interactions while using *CYP3A4* substrates and administering 500 mg doses of methylxanthines. Simulations performed with the *SimCYP* simulator negated this risk.
5. If a dosage form is created on the basis of the isolated fractions of methylxanthines from *Bancha* and *Pu-erh* after a time, it can be assumed that there will be no risk of drug interactions with *CYP3A4* substrates.
6. Methylxanthines isolated from *Bancha* and *Pu-erh* show mild antiproliferative effects on breast cancer cell lines.
7. Bancha-isolated methylxanthines show synergistic effects when combined with doxorubicin on breast cancer cell lines, whereas *Pu-erh*-isolated methylxanthines show rather antagonistic effects with doxorubicin.
8. *Bancha*-isolated methylxanthines show cardio- and nephroprotective effects in doxorubicin-induced cardio- and nephrotoxicity models.

9. *Bancha*-isolated methylxanthines are suitable partners of doxorubicin in the treatment of mammary gland tumors as they increase its antitumor activity and reduce its dose-limiting factors (cardio- and nephrotoxicity).

Pharmacokinetic interactions in newly synthesized endomorphin-2 and RGD analogues

1. The determined *in vitro* inhibitory potential (calculated values of IC_{50} and K_i) for the *CYP3A4* isoenzyme indicates a possible moderate risk of drug interactions of endomorphin-2 analogues – *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* and *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH*.
2. The established pharmacokinetic models and simulations of *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* at the used doses of 1 mg and 10 mg p.o. with *ADMEWORKS DDI* software indicated the danger of drug interactions (only with 10 mg) with classical *CYP3A4* substrates, which were subsequently rejected using the *SimCYP* software.
3. Simulations performed by the *SimCYP* software showed a moderate risk of drug interactions with the highest simulated dose of *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* – 100 mg, so if this oligopeptide is approved in the future administration at these doses *per os*, will require clinical studies.
4. Changing the application pathway – from *per os* to *i.v. bolus* of the highest dose of 100 mg *Tyr-Pro-Phe(pF)-Phe-1,2-ethylenediamine* reduced the risk of moderate to low.
5. *Deoxycholic-Tyr-Pro-Phe(pCl)-Phe-OH* analogue simulations, performed by the *ADMEWORKS DDI* software showed little risk of drug interactions with *CYP3A4* substrates. Static simulations performed by the *SimCYP* software, showed moderate risk only with the highest dose – 100 mg. Dynamic models rejected the risk of drug interactions in all three simulated doses – 1, 10 and 100 mg.
6. The calculated in vitro values of IC_{50} and K_i with respect to the *CYP3A4* isoenzyme in the *RGD* analogues showed no risk of drug interactions with *CYP3A4*.

Pharmacokinetic and pharmacodynamic interactions in isolated fractions of L.barbarum (goji berry)

1. The pectin-free fraction and total aqueous extract of *L.barbarum* (goji berry) showed high antioxidant potential.
2. The pectin-free fraction of *L.barbarum* (goji berry) showed a concentration-dependent antiproliferative effect on breast cancer cell lines.
3. This effect is potentiated by the addition of the polysaccharide fraction but it has a synergistic effect.
4. The pectin-free fraction of *L.barbarum* leads to a potent inhibition of *CYP3A4* and *CYP2C9* isoenzymes.
5. The polysaccharide fraction showed little effect on the activity of *CYP3A4* and *CYP2C9* isoenzymes compared to the pectin-free fraction, ~ 20x and ~ 100x respectively.
6. Basic drug interaction risk prediction models showed no risk when combining *L. barbarum* extracts with *CYP3A4* substrates, but suggested one when used concurrently with *CYP2C9* substrates in the presence of high content of polyphenols. Therefore, that patients receiving *L.barbarum*-based dietary supplements should be treated with caution when receiving concomitant *CYP2C9* substrates.
7. Total aqueous extract showed synergistic inhibitory effects when co-administered with doxorubicin on human breast cancer cell growth.
8. The pectin-free and polysaccharide fraction as well as the total aqueous extract of *L. barbarum* (goji berry) showed cardio- and nephroprotective activity on doxorubicin-induced cardio- and nephrotoxicity models.
9. Fractions isolated from *L.barbarum*, as well as total aqueous extract, are suitable partners of doxorubicin in the treatment of mammary gland tumors because they increase its antitumor activity and reduce its dose-limiting factors (cardio- and nephrotoxicity).

Drug interactions in patients with heart failure

1. Patients with heart failure are elderly, with much comorbidity, and take many medications (polypharmacy), which makes them more likely to develop drug interactions. The physicians of these patients should always be alert for potential drug interactions and check every new medicine for them.
2. Medicines with low therapeutic width, such as statins, anticoagulants, platelet antiplatelet agents, cardiac glycosides, are responsible for the greater number of potential drug interactions that are into risk group D (requiring change of therapy) and in severity group, depending on the reaction.
3. An increased number of drugs positively correlate with an increase in potential drug interactions about these patients.
4. The advanced age of these patients is not a prerequisite for an increased number of potential drug interactions.
5. In these patients, the pharmacokinetic to pharmacodynamic drug interactions predominate, making it difficult to detect and determine the mechanism of reaction. Using appropriate software, such as *SimCYP*, can contribute to greater clarity and understanding of these mechanisms.
6. Appropriate forecasting software should be used to reduce the risk of potential drug interactions. Another suitable option should be including a clinical pharmacist in the multidisciplinary team.