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Isolation and analysis of methylxanthine fraction, catechin fraction and total Bancha green tea extract and study of their influence on the pharmacokinetics of sildenafil in rats

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Internal members:

1. *Assoc. Prof. Evgeni Evgeniev Grigorov, MScPharm, PhD – Chair (standpoint)*
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The dissertation contains a total of 139 pages, illustrated with 27 figures and 14 tables. The bibliography includes 392 sources, of which 7 in Cyrillic and 385 in Latin.

The public defense of the dissertation will take place on 29.04.2022 from …… in ……

The materials on the defense are published on the website of Medical University "Prof. Dr. Paraskev Stoyanov " – Varna.

CONTENT

ИЗПОЛЗВАНИ СЪКРАЩЕНИЯ

AUC	Area under the curve
BAS	Biologically active substances
b. w.	Body weight
CF	Catechin fraction
cGMP	Cyclic guanosine monophosphate
Cl/F	Clearance
C_{max}	Maximum plasma concentration
CNS	Central nervous system
CYP450	Cytochrome P450
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EC	Epicatechin
ECG	Epicatechin-3-gallate
GIT	Gastrointestinal tract
HED	Human equivalent dose
HPLC	High performance liquid chromatography
ICH	International conference on harmonization
LOQ	Limit of quantification
MATE	Multidrug and toxin extrusion
MF	Methylxanthine fraction
NO	Nitric oxide
OATP	Organic-anion-transporting polypeptides
OCT	Organic cation transporter

PBPK	Physiologically based pharmacokinetic
PDE5	Phosphodiesterase 5
PXR	Pregnane X receptor
P-gp	P-glycoprotein
SD	Standard deviation
TE	Total extract
T_{max}	Time to reach maximum plasma concentration
$t_{1/2}$	Plasma half life
UV	Ultraviolet
Vz/F	Volume of distribution
w/w	weight/weight

I. INTRODUCTION

Green tea is one of the most consumed beverages in the world. It is produced from the leaves of the tea plant *Camellia sinensis* (L.) Kuntze. Green tea is known for a number of health benefits such as antioxidant, anti-inflammatory and antimicrobial activity, antitumor effect, as well as preventive action in cancer, weight loss, slowing aging and others, which have been observed in a number of in vitro and in vivo studies. Tea leaves contain a wide variety of biologically active substances (BAS) such as polyphenolic compounds, polysaccharides, alkaloids, saponins, free fatty acids, vitamins, minerals and others.

The polyphenols, in particular catechins, are considered to be essential for the described beneficial effects of green tea. The four main catechins that usually occur in green tea are - (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC). EGCG is the predominant catechin in tea leaves and usually accounts for 50 to 70% of the catechins contained. In addition, EGCG is listed as the most active compound in the catechin group, which is responsible for most of their biological effects. Different mechanisms of action of catechins and EGCG are discussed, but many of their effects are thought to be due to modulation of key signaling pathways and transcription factors, which affect gene expression and protein expression in cells. However, despite the reported beneficial effects, the use of catechins as potential therapeutic agents in various diseases is limited due to their low stability as well as low oral bioavailability, resulting in very low plasma concentrations.

Another important group of BAS in green tea is the group of methylxanthines. Natural methylxanthines include caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine). Among them, caffeine is found in the largest amounts in tea leaves. Theobromine is found in very low amounts, and theophylline is not usually detected. Caffeine is popular for its central nervous system (CNS) stimulant effect, making it the most widely used and readily available psychostimulant worldwide. This effect is most likely due to the blockade of adenosine receptors in the CNS, which is considered to be the main mechanism of action of caffeine, responsible for most of its pharmacological effects. Other important therapeutic effects of caffeine are

its use in the treatment of apnea in premature infants, as well as an adjuvant in combination with non-opioid analgesics and nonsteroidal anti-inflammatory drugs to potentiate their analgesic effect.

Interestingly, the composition of green tea can vary widely, depending on a number of factors such as the conditions under which tea plants are grown, the time of leaf collection, post-harvest processing and storage conditions. The differences in the content of BAS is also related to different organoleptic properties of the tea leaves and respectively of the tea drink after preparation. In addition, different types of green tea are available, such as Chinese, Japanese, Korean and others, depending on the geographical conditions under which the tea plants were grown. It was found that this also affects the qualitative and quantitative content of BAS. For example, one of the most commonly consumed types of green tea in Japan is called Bancha. It is obtained from tea leaves, which are collected in late summer or early autumn, the so-called late seasonal picking, while the first and second harvest leaves yield a tea called Sencha. There is evidence that Bancha green tea contains less BAS than other popular Japanese green teas, such as Sencha and Matcha, but there is limited information in the scientific literature.

A number of studies have reported potential interactions when green tea is taken concomitantly with conventional drugs, especially drugs used in the treatment of cardiovascular and tumor diseases. Changes in plasma concentrations of drugs may affect their therapeutic potential or safety. Although green tea extract contains a large number of components that can interact with drug molecules, catechins are considered to be the main compounds that, in addition to being carriers of biological activity, are also the cause of interactions. A number of preclinical and clinical studies have been performed to evaluate the potential of green tea extract and / or catechins to modulate the activity of drug-metabolizing enzymes as well as some transmembrane transporters. The results of *in vitro* studies indicate that green tea extract and / or EGCG inhibit the activity of cytochromes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4) as well as some transmembrane transporters (P-glycoprotein (P-gp), organic anion-transporting polypeptides (OATP1A2, OATP1B1, OATP1B3, OATP2B1), organic cationic transporters (OCT1, OCT2) and multidrug and toxic compound extrusion (MATE1/ MATE2-K). In experimental animals, green tea extract as well as EGCG have been shown

to increase plasma levels of CYP3A4 and P-gp substrates and to decrease plasma concentrations of drugs, substrates of organic anionic transporters, possibly due to inhibition of their activity. However, in clinical trials, the results are contradictory. On the other hand, caffeine as a major representative of methylxanthines may also interact with certain drugs. Interactions at the level of absorption and metabolism, mainly with CYP1A2 substrates, have been reported. In addition, in a previous study, we observed that methylxanthine fractions isolated from Bancha and Pu-erh tea leaves, as well as pure caffeine, inhibited CYP3A4 activity *in vitro*. Caffeine has also been reported in the literature to inhibit the activity of the enzyme CYP3A4. In addition, caffeine has been observed to inhibit the metabolism of dextromethorphan by CYP2D6 *in vitro*. However, the available information on potential interactions between caffeine and drugs, substrates of enzymes other than CYP1A2 is limited.

Therefore, considering the currently reported interactions between green tea extract and / or EGCG with CYP3A4 substrates, it can be concluded that further studies are needed to identify potential interactions. Sildenafil is a selective inhibitor of the enzyme phosphodiesterase type 5 (PDE5), used in the treatment of erectile dysfunction and pulmonary arterial hypertension. It is an example of a drug that is almost completely metabolized, mainly by CYP3A4, and is eliminated mainly in the form of metabolites. In addition, sildenafil is a Class II medicinal product (low solubility and high permeability) according to the Biopharmaceutical Classification System (BCS). In addition, there is evidence that sildenafil is a substrate of P-gp. Efflux transport mediated by this transmembrane protein is thought to contribute to the low oral bioavailability of the drug. It is known that high-permeability drugs that undergo significant metabolism, such as sildenafil, are often involved in drug interactions. Moreover, due to the significant contribution of CYP3A4 to the metabolism of sildenafil, co-administration with drugs, foods, beverages or herbal extracts that affect the activity of this enzyme may lead to pharmacokinetic interactions. These, in turn, may lead to increased sildenafil toxicity or treatment failure. Interactions between sildenafil and CYP3A4 inhibitors are known to increase the risk of adverse reactions. At the same time, interactions between sildenafil and plant extracts have been reported, leading to changes in the pharmacokinetics of the drug. In addition, sildenafil is used by men without medical indications to increase

sexual endurance, which can lead to improper use of the drug and an increased risk of side effects, especially if used concomitantly with other drugs and / or herbal medicines. Even more, there is evidence that sildenafil has been illegally added to the composition of some herbal preparations and alcoholic beverages. The possibility of being purchased and used without a prescription or accidentally taken further exacerbates the problem of its irrational use and increases the likelihood of drug interactions, including interactions with plant components.

Therefore, based on the above, there is a good reason to suggest that interactions may occur when sildenafil is co-administered with green tea extract and / or EGCG. In addition, considering the observed *in vitro* inhibition of CYP3A4 by methylxanthine fractions isolated from Bancha and Pu-erh tea and pure caffeine, we suppose that an *in vivo* pharmacokinetic interaction may occur after co-administration of sildenafil and methylxanthines, in particular caffeine. The present dissertation aims to test this hypothesis, as we have chosen to use a specific type of green tea, namely Japanese green tea Bancha. The reason is that information in the scientific literature on the content of active substances, potential therapeutic and toxic effects of this type of Japanese green tea, as well as the potential to participate in drug interactions is quite scarce, although Bancha is one of the most widely used teas in Japan.

II. AIM AND OBJECTIVES

1. Aim

The aim of the present dissertation is to evaluate the influence of methylxanthine fraction, catechin fraction and total extract of Japanese Bancha green tea on the pharmacokinetics of sildenafil.

2. Objectives

2.1. Obtaining plant extracts of Japanese Bancha green tea:

2.1.1. Preparation of total extract (TE);
2.1.2. Isolation of catechin fraction (CF);
2.1.3. Isolation of methylxanthine fraction (MF);

2.2. Qualitative and quantitative analysis of the obtained extracts:

2.2.1. Qualitative and quantitative analysis of catechins, gallic acid and caffeine in samples of TE and CF obtained from Japanese Bancha green tea:

- ❖ Development and optimization of reverse phase HPLC-UV method for analysis of EGCG, (+)-catechin, gallic acid and caffeine in standard sample solutions;
- ❖ Validation of the liquid chromatographic method;
- ❖ Approbation of the liquid chromatographic method - analysis of the EGCG, (+) - catechin, gallic acid and caffeine content in test solutions of TE and CF.

2.2.2. Qualitative and quantitative analysis of methylxanthines (caffeine, theophylline, theobromine) in samples from the isolated MF.

2.3. Qualitative and quantitative determination of sildenafil in plasma samples from experimental animals:

2.3.1. Development and optimization of reverse phase HPLC-UV method for analysis of sildenafil in rat plasma samples;
2.3.2. Validation of the liquid chromatographic method;
2.3.3. Development of an efficient method for extraction of sildenafil from plasma samples;

2.3.4. Approbation of the liquid chromatographic method - analysis of plasma samples from experimental animals treated with sildenafil.

2.4. Animal studies for evaluating the influence of the obtained extracts on the pharmacokinetics of sildenafil:

2.4.1. Development of a protocol for conducting each of the experiments with laboratory animals;

2.4.2. Conducting the two planned experiments separately and treating the experimental animals with the substances used (sildenafil, ketoconazole, TE, CF and MF from Japanese Bancha green tea);

2.4.3. Plasma samples collection and sample preparation;

2.4.4. Application of the developed HPLC-UV method for the analysis of sildenafil in plasma samples from experimental animals;

2.4.5. Analysis and evaluation of changes in sildenafil pharmacokinetic parameters in rats after repeated administration of TE, CF and MF from Japanese Bancha green tea;

2.4.6. Analysis and evaluation of changes in sildenafil pharmacokinetic parameters in rats after a single dose of MF isolated from Japanese Bancha green tea;

2.5. Development of PBPK models for predicting potential interactions between sildenafil and TE, CF or MF from green tea in humans:

2.5.1. Construction of PBPK models of the obtained extracts using Simcyp® computer software;

2.5.2. Evaluation of potential drug interactions by applying mechanistic-static and mechanistic-dynamic PBPK models.

2.6. Statistical processing of the obtained results.

III. MATERIALS AND METHODS

1. Materials

1.1. Chemicals and reagents

- ❖ Japanese Bancha green tea, purchased from a local herbal store;
- ❖ Sildenafil citrate (≥ 98 % (HPLC), Sigma-Aldrich GmbH); Ketoconazole (≥ 98 % (HPLC), Sigma-Aldrich GmbH); Caffeine (pharmaceutical secondary standard, certified reference material, Sigma-Aldrich GmbH); Theophylline (pharmaceutical secondary standard, certified reference material, Sigma-Aldrich GmbH); Theobromine (analytical standard, ≥ 99 % (HPLC), Sigma-Aldrich GmbH); Epigallocatechin gallate (pharmaceutical secondary standard, certified reference material, Sigma-Aldrich GmbH); (+)-catechin (analytical standard, ≥ 99 % (HPLC), Sigma-Aldrich GmbH); Gallic acid (certified reference material, Sigma-Aldrich GmbH); Methanol (≥ 99.8 % (HPLC), Fisher Chemicals, UK); Ethanol, absolute (99.99% (HPLC), Fisher Chemicals, UK); Sulfuric acid (Sigma-Aldrich GmbH); Phosphoric acid (Sigma-Aldrich GmbH); Chloroform (Sigma-Aldrich GmbH); Ethyl acetate (Sigma-Aldrich GmbH); Sodium hydroxide (Sigma-Aldrich GmbH); Sodium carboxymethyl cellulose (Sigma-Aldrich GmbH); Saline (0.9%, 500 mL, B. Braun Melsungen AG, Germany); Double distilled water (obtained in laboratory conditions);

1.2. Laboratory and medical supplies

- ❖ Filters Nylone white membranes (0.45 µm, d = 47.0 mm, filtraTECH, France);
- ❖ Vials (brown glass, 2 ml., ND 8 mm, Thermo Scientific ™, USA);
- ❖ Inserts (200 µL, 02-NV, Thermo Scientific™, USA);
- ❖ Polypropylene plastic feeding tubes for rodents FTP-18-75 (18ga, 75 mm x 1.2 mm, 30 µL; Instech Laboratories, Inc., USA);
- ❖ Vacutainers with EDTA.K3 (Cat.№ 630903, Boen Healthcare Co., Ltd.)

1.3. Equipment and tools

- ❖ Analytical balance Ohaus Explorer Analytical – SmarText™ software 2.0 (USA);
- ❖ Distiller Gesellschaft für labortechnik mbH (Germany);
- ❖ Vacuum evaporator (Büchi Labortechnik, Switzerland)
- ❖ pH meter METTLER TOLEDO, SevenCompact™ S210
- ❖ Heating chamber with natural convection (Model ED 56, Binder GmbH, Germany)
- ❖ Eppendorf pipettes 1000 μL и 100 μL;
- ❖ Vortex-mixer ZX3 Advanced (Italy);
- ❖ Ultrasonic bath (Advantage Lab, Belgium)
- ❖ Benchtop multi-function centrifuge Ohaus Frontier FC5706 (USA);
- ❖ Stuart SBHCONC / 1 concentration system, equipped with Stuart SBH130D / 3 heating unit (UK);
- ❖ High-efficiency liquid chromatographic system Thermo Scientific UltiMate 3000 Analytical LC System (USA), equipped with a quaternary pump (Thermo Scientific Dionex UltiMate 3000 LPG-3400SD Quaternary Pump, USA), automatic injector (Thermo Scientific Dionex UltiMate 3000 Autosampler, USA /, variant) VIS detector (Thermo Scientific Dionex UltiMate 3000 VWD-3100 Variable Wavelength Detector / VWD, USA) and diode array detector (Thermo Scientific Dionex UltiMate 3000 DAD-3000 Diode Array Detectors, USA); The high-performance liquid chromatographic system is equipped with Thermo Scientific ™ Chromeleon ™ 7.2 Chromatography Data System software for processing and analysis of the obtained results;

1.4. Laboratory animals

The studies were performed on 198 white male Wistar rats weighing 220 - 250 g, provided by the Vivarium to the Medical University - Varna. Among them, 192 rats were treated and participated in pharmacokinetic studies, while 6 of the animals were not treated and were used for plasma collection. Rats were housed in cages (6 animals per cage) at room temperature (23 ± 2 ° C) in a well-ventilated room for a 12-hour light / dark cycle with free access to standard rat chow and pure drinking water. The humidity of the room was 50 ± 10%. Before the start of the experiments, the

animals were acclimatized for a period of one week. Computer-generated random numbers were used to ensure random distribution of the animals in the different groups. Rodents were treated at the same time and in the same order each day during the experiments to minimize potential confusing factors. In addition, the location of the animals during the experiments was not changed.

1.5. Computer software

Simcyp® software simulator, version 20.1 (Certara® UK Limited) was used to build PBPK models for green tea extracts, as well as to evaluate potential drug interactions with CYP3A4 substrates (sildenafil) in humans, after obtaining an annual license. The program has the ability to predict the pharmacokinetic behavior of a compound in humans and to build a complete and minimal distribution PBPK model. At the same time, the platform includes extensive data on demographic characteristics, disease states, anatomical, physiological, genetic and biochemical variables in various pathological and physiological conditions.

2. Experimental methods

The experimental work was performed entirely in the Department of Pharmacology, Toxicology and Pharmacotherapy at the Faculty of Pharmacy, with the exception of animal experiments, which are conducted in the Vivarium at the Medical University - Varna.

2.1. Extraction methods

For the aim of the experiment, a total extract (TE), catechin fraction (CF) and methylxanthine fraction (MF) of Japanese Bancha green tea were prepared.

2.1.1. Preparation of total aqueous extract of Japanese Bancha green tea

100.0 g of unsaturated tea leaves of Japanese Bancha green tea ape brewed for 3 minutes with 1.0 L of hot double-distilled water (80 °C). The resultant extract was filtered through gauze and evaporated under vacuum to obtain a thick resinous mass, which was then dried in an oven at 25 °C to dryness. The dry extract was stored in a refrigerator at 4 °C. Figure 1 shows

a photo of Bancha tea leaves, as well as a photo of the obtained aqueous extract before drying.

Figure 1. Photos of the used Bancha tea leaves and the prepared infusion..

2.1.2. Extraction of catechin fraction from Japanese Bancha green tea

50.0 g of dried Bancha green tea leaves were accurately weighed and then extracted with 100.0 mL of methanol for 60 minutes. The obtained extract is evaporated in a water bath. To the resultant dry extract was added 10.0 mL of double distilled water, sodium chloride and phosphoric acid to obtain a pH 3.5. The resultant mixture was then placed in a separatory funnel with 10.0 mL of ethyl acetate and the combined ethyl acetate extracts were washed twice with double distilled water until neutral reaction (pH 7.0). The catechin fraction was obtained after evaporation of the organic solvent and then stored in a refrigerator at 4 °C.

2.1.3. Extraction of methylxanthine fraction from Japanese Bancha green tea

An accurately weighed amount of dried Bancha tea leaves (50.0 g) was extracted under reflux with 250.0 mL of hot double distilled water for 60 minutes, then filtered through a Buchner funnel. The resultant aqueous extract was acidified with 25% sulfuric acid (5.0 mL) and concentrated to half of its original volume. The hot solution was then filtered and extracted four times with 50.0 mL of chloroform in a separatory funnel, and the obtained chloroform extracts were combined in a glass flask. This was followed by washing the chloroform extract (200.0 mL) twice with 5% sodium hydroxide solution (50.0 mL) and twice with double distilled water

(50.0 mL). The methylxanthine fraction is obtained after evaporation of the chloroform. The dry mass is stored in a refrigerator at 4 °C.

2.2. Methods for qualitative and quantitative determination of methylxanthines and catechins in the extract and fractions isolated from Japanese Bancha green tea

Two liquid chromatographic methods were used for the analysis of the obtained plant extracts, one of which was developed and validated in connection with the present dissertation.

2.2.1. Liquid chromatographic method for qualitative and quantitative determination of epigallocatechin-3-gallate, (+)-catechin, gallic acid and caffeine in TE and CF of Bancha green tea

In order to fulfill the tasks set in the dissertation, a selective and reliable reverse phase HPLC-UV method for analysis of EGCG, (+)-catechin, gallic acid and caffeine in samples of plant origin has been developed.

❖ **Preparation of standard stock and working solutions of EGCG, (+)-catechin, gallic acid and caffeine**

- The standard stock solutions of EGCG, (+)-catechin and caffeine are prepared by dissolving 50.0 mg of the corresponding standard substances in 50.0 mL of double distilled water. The standard stock solution of gallic acid was prepared by dissolving 50.0 mg of gallic acid (standard substance) in 50.0 mL of methanol.
- The working standard solutions are prepared by sequentially diluting the respective stock solutions with the solvent used to obtain concentrations of 1.0, 10.0, 50.0, 100.0, 150.0 and 200.0 μg/mL for each analyte.

All standard solutions were prepared immediately before the analysis and stored in dark glass flasks at 4 °C.

❖ **Chromatographic conditions**

HPLC analysis was performed with a Thermo Scientific UltiMate 3000 Analytical LC System. The analytes were divided into a Hypersil GOLD aQ C18 analytical column (150.0 x 4.6 mm, 5 μm, Thermo Scientific™), protected by a Hypersil GOLD aQ C18 precolumn (10.0 x 4.0

mm, 5 µm, Thermo Scientific™). The mobile phase consisted of acetonitrile / acetic acid / bi-distilled water in a ratio of 8: 1: 91 v/v/v. Elution was performed in an isocratic mode at a flow rate of 1.2 mL/min. The injection volume was 20.0 µL and the wavelength of the UV detector was 275 nm. The temperature of the chromatographic column and the injector was maintained at 20 °C. The total duration of the analysis was 25 minutes.

❖ **Quantitative determination of epigallocatechin-3-gallate, (+)-catechin, gallic acid and caffeine in the prepared standard solutions**

Each of the prepared standard working solutions of the four test substances (with concentrations in the range of 1.0 to 200.0 µg/mL) was analyzed six times to construct calibration curves and to evaluate the linearity of the proposed method. The calibration curves are constructed on the basis of the ratio between the area and the height of the peak from the chromatograms of the tested standard solutions for each analyte. The calibration curves are constructed by linear regression analysis using the method of least squares.

❖ **Validation of the liquid chromatographic method**

The results obtained from the qualitative and quantitative analysis of the prepared working solutions of EGCG, (+)-catechin, gallic acid and caffeine are used to validate the liquid chromatographic method. The HPLC-UV method has been validated according to the recommendations of the International Conference on Harmonization (ICH) Q2 (R1) for validation of analytical procedures: text and methodology.

❖ **Sample preparation**

The TE and CF test solutions were prepared separately after weighing 50.0 mg of each substance. The weighed quantities are transferred into various dark glass flasks with a volume of 50.0 mL and diluted with double distilled water to the mark. The resultant aqueous solutions had a final concentration of 1.0 mg/mL. After degassing and homogenizing in an ultrasonic bath for 10 minutes, all samples were centrifuged for 5 minutes at 5000 x g. Six aliquots of 20.0 µL of each sample were injected into the HPLC system.

❖ **Qualitative and quantitative determination of epigallocatechin-3-gallate, (+)-catechin, gallic acid and caffeine in test solutions of TE and CF**

The identification of EGCG, (+)-catechin, gallic acid and caffeine is performed according to the ultraviolet (UV) absorption spectrum and the retention time of each substance from the chromatograms of the standard solutions. Quantitative analysis is performed by the method of absolute calibration.

2.2.2. Liquid chromatographic method for qualitative and quantitative determination of methylxanthines (caffeine, theophylline, theobromine) in MF isolated from Bancha green tea

The liquid chromatographic method for analysis of methylxanthines was developed and validated in a previous study in the Department of Pharmacology, toxicology and pharmacotherapy and was applied for the analysis of the isolated MF used for the aim of the dissertation. Standard solutions of caffeine, theophylline and theobromine were prepared and analyzed again before the analysis of the methylxanthine fraction.

❖ **Preparation of standard stock and working solutions of caffeine, theophylline and theobromine**

- For the preparation of standard solutions of caffeine, theophylline and theobromine, it was used a double distilled water with pH 8, obtained by adding 250.0 µl of freshly prepared 0.1M NaOH solution to 500.0 ml of double distilled and filtered water. The pH value of the solution was determined using a pH meter. Standard stock solutions of caffeine (1.0 mg/mL final concentration) and theophylline (1.0 mg/mL final concentration) were prepared separately by accurate weighing of 50.0 mg of each standard substance and subsequent dissolution in 50.0 mL of hot, double distilled water (~ 60 ºC) with pH 8. The standard stock solution of theobromine was prepared by dissolving 10.0 mg theobromine (standard substance) in 100.0 mL hot, double distilled water (~ 60 ºC) with pH 8 (0.1 mg/mL final concentration).
- Standard working solutions are prepared by serial dilutions of each standard stock solution with double distilled water to obtain

concentrations in the range 1.0 - 750.0 µg / mL. All standard stock and working solutions were freshly prepared immediately before analysis.

❖ **Chromatographic conditions**

HPLC analysis was performed with a liquid chromatographic system Termo Scientifc UltiMate 3000, equipped with a variable UV/Vis detector. HPLC separation was performed on an analytical column (Termo Scientifc AQUASIL C18, 150.0 mm × 4.6 mm, 5 µm), protected by an AQUASIL C18 precolumn (10.0 mm × 4.6 mm, 5 µm) with a flow rate of 0.8 mL/min and UV detection at 274 nm. The injection volume was 20.0 µL. The mobile phase was a mixture of double distilled and filtered water with acetonitrile in a ratio of 90:10%, v/v. The analysis was performed in an isocratic mode with a total duration of 12 minutes. The column temperature was maintained at 30 °C.

❖ **Validation of the liquid chromatographic method**

The HPLC-UV method was validated according to the guidelines of the International Conference on Harmonization (ICH) Q2 (R1) for validation of analytical procedures: text and methodology. A calibration curve was constructed for each standard substance (caffeine, theophylline and theobromine) using working standard solutions with eight different concentrations ranging from 1.0 to 750.0 µg/mL. The reported correlation coefficient is $R^2 = 0.9995$.

❖ **Sample preparation**

The test solution of MF isolated from Japanese Bancha green tea was prepared by accurate weighing of 25.0 mg of the powdered MF and subsequent dissolution in hot, double distilled water (~ 60 °C) with pH 8 to obtain a solution with a final concentration of 2.5 mg/mL. All samples were prepared immediately before the analysis and filtered through a 0.45 µm Sartorius RC membrane filter before injection into the HPLC system.

❖ **Qualitative and quantitative determination of caffeine, theophylline and theobromine in the isolated MF**

The identification of caffeine, theophylline and theobromine is performed according to the UV absorption spectrum and the retention time

of each compound from the chromatograms of the standard solutions. The concentrations of caffeine, theophylline and theobromine in the isolated MF were calculated from the constructed calibration curves.

2.3. Liquid chromatographic method for the qualitative and quantitative determination of sildenafil in biological samples from experimental animals:

2.3.1. Preparation of standard stock and working solutions of sildenafil

- A standard stock solution of sildenafil (final concentration 0.05 mg/mL) was prepared by precise weighing of 7.0 mg sildenafil citrate (standard substance) equivalent to 5.0 mg sildenafil and subsequent dissolution in 100.0 mL methanol (140.45 mg sildenafil citrate equivalent to 100.0 mg sildenafil).
- Standard working solutions were prepared by serial dilution of the standard stock solution with methanol to obtain concentration levels in the range of 5.0 to 100.0 ng/mL.

All standard stock and working solutions were freshly prepared immediately before analysis and stored in a refrigerator at 4 ºC.

2.3.2. Chromatographic conditions

HPLC analysis was performed on an analytical column Thermo science AQUASIL C18 (150.0 mm x 4.6 mm, 5.0 µm), protected by a precolumn AQUASIL C18 (10.0 mm x 4.6 mm, 5.0 µm) with a flow rate of 0.6 ml/min and UV detection at a wavelength of 228 nm. The injection volume was 20.0 µL. The separation was carried out in isocratic mode with mobile phase, a mixture of methanol and double distilled water in a ratio of 85:15%, v/v. The temperature of the chromatographic column and the injector was maintained at 25 °C. The duration of the analysis was 10 minutes.

2.3.3. Quantitative determination of sildenafil in the prepared standard solutions

The sildenafil concentration is calculated from the calibration curve using the external standardization method. The calibration curve was

constructed by six-fold analysis of standard sildenafil solutions at ten different concentrations ranging from 5.0 to 100.0 ng/mL.

2.3.4. Validation of the liquid chromatographic method

The results of the qualitative and quantitative analysis of the prepared working solutions of sildenafil serve to validate the liquid chromatographic method. The HPLC method has been validated according to the recommendations of the International Conference on Harmonization (ICH) Q2 (R1) for the validation of analytical procedures: text and methodology.

2.3.5. Sample preparation

- The prepared standard working solutions of sildenafil with concentrations ranging from 5.0 to 100.0 ng / mL were added to plasma samples from untreated rats. Each of the samples was prepared by adding 100.0 µL of standard sildenafil solution of various concentrations to 100.0 µL of rat plasma. All samples were homogenized on a vortex mixer at 6000 x g for 30 seconds. The resultant plasma samples are subjected to a subsequent procedure for extraction of the introduced analyte.
- The protein precipitation technique was used to extract sildenafil from plasma samples. Aliquots of 100.0 µL plasma were placed separately in glass tubes with a volume of 10.0 mL with a screw cap. Each was then treated with 1.0 mL of the precipitating agent (methanol) and stirred on a vortex mixer at 6000 x g for 30 seconds. The obtained precipitates are solidified by centrifugation at 5000 x g for 5 minutes. Subsequently, each supernatant was transferred to a clean glass tube (10.0 mL) with a screw cap. 300.0 µL of methanol is added to each of the precipitates. The latter were then stirred again at 6000 x g for 30 seconds and centrifuged at 5000 x g for 5 minutes. The second supernatant was combined with the first one and the resultant samples were evaporated to dryness under a stream of nitrogen at 40 °C. The dry residue was dissolved in 200.0 µL of double distilled water, stirred in a vortex mixer for 30 seconds and centrifuged at 5000 x g for 5 minutes. 20.0 µL of each supernatant was then injected into the HPLC-UV system.

2.3.6. Qualitative and quantitative determination of sildenafil in biological samples

Qualitative analysis of sildenafil in plasma samples was performed according to the UV absorption spectrum and retention time of the analyte from chromatograms of the standard solutions. The quantification of sildenafil in the tested plasma samples was determined on the basis of the constructed calibration curve.

2.4. Pharmacokinetic studies in experimental animals

Two separate experiments were performed. In the first experiment sildenafil was administered after pretreatment of the animals with TE, CF or MF isolated from Bancha green tea for one week, and in the second experiment sildenafil was administered after a single dose of the isolated MF.

The animal experiments were conducted in accordance with the national (Ordinance № 20 of 01.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for sites for use, breeding and/or delivery) and international requirements for protection and the welfare of laboratory animals as defined by Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The performed experimental procedures were also approved by the Bulgarian Food Safety Agency at the Ministry of Agriculture (permit with registration № 175, valid until 15.09.2022).

2.4.1. Determination of the dose of each substance used

The choice of the dose to be administered for each of the substances used (sildenafil, ketoconazole, TE, CF, MF) in both experiments was made on the basis of a literature study on the doses used of sildenafil, ketoconazole, green tea extract, EGCG and caffeine in preclinical trials as well as by calculating the human equivalent dose (HED) for some of the substances, using the following formula:

$$\mathbf{HED = animal\ dose\ in\ mg/kg \times (animal\ weight\ in\ kg / human\ weight\ in\ kg)^{0.33}},$$

Doses of all substances used in both experiments were calculated in mg/kg body weight (b.w.).

- **Determination of sildenafil dose in both experiments**

In the first experiment, which aimed to evaluate the effect of repeated intake of TE, CF and MF from Bancha green tea on the pharmacokinetic parameters of sildenafil in rats, a dose of sildenafil equal to 2.5 mg/kg was selected. The selection was made on the basis of literature data for pharmacokinetic experiments in rats as well as using the formula for calculating HED. A single dose of 2.5 mg/kg sildenafil in rats was estimated to be equivalent to a dose of 0.39 mg/kg or approximately 27.0 mg in humans (for a patient weighing 70.0 kg).

In the second experiment, a dose of 60.0 mg/kg sildenafil was administered, based on the literature review as well as toxicological studies. This is the highest dose of sildenafil administered orally to rats that does not cause toxic effects.

- **Determination of ketoconazole dose**

The dose of 10.0 mg/kg ketoconazole was used in both experiments. Ketoconazole has been used as a positive control in the studies due to its well-known inhibitory effect on CYP450 activity.

- **Determination of the doses of TE, CF and MF from Bancha green tea**

The doses of TE (100.0 mg/kg) and CF (30.0 mg/kg) from Bancha green tea were selected after a careful study of the scientific literature on the doses of green tea extract and EGCG used in rat studies. In addition, the HED of EGCG and caffeine were calculated after administration of 100.0 mg/kg TE and 30.0 mg/kg CF from Bancha in rats. After performing the necessary calculations, we found that 100.0 mg/kg of TE provide 4.12 mg/kg EGCG and 4.19 mg/kg caffeine, while 30.0 mg/kg of CF are equivalent to 5.0 mg/kg EGCG and 0.8 mg/kg caffeine. Therefore, after substitution in the HED calculation formula, we found that the amount of EGCG and caffeine that rats received after ingestion of 100.0 mg/kg TE from Bancha was equal to HED of ~ 45.0 mg EGCG and 46.0 mg caffeine. Such amounts of EGCG and caffeine can be administered with a cup of tea prepared at home. The amount of EGCG and caffeine administered after ingestion of 30.0 mg/kg CF in rats was equivalent to a HED of ~ 54.0 mg EGCG and 8.7 mg caffeine.

The dose of MF (5.7 mg/kg) was selected after reviewing the literature on caffeine doses used in rats, as caffeine is the major component in the isolated fraction. At the same time, the formula for calculating HED was used. The MF administered at a dose of 5.7 mg/kg is equivalent to caffeine at a dose of 5.0 mg/kg. According to the HED calculation formula, the single dose of caffeine that rats received after 5.7 mg/kg MF was equivalent to a single dose of 0.78 mg/kg caffeine in humans or approximately 54.0 mg caffeine (in human weighing 70 kg). The same dose of methylxanthine fraction was used in both experiments.

2.4.2. Preparation of solutions / suspensions for oral administration to experimental animals

All substances used were dissolved or suspended before use in experimental animals. Sildenafil and ketoconazole are used as suspensions while the plant extracts are dissolved in water. In the first experiment, TE, CF and MF isolated from Bancha tea leaves were administered to rats twice daily for 7 consecutive days before taking sildenafil. For the aim of the experiment, the solutions of the herbal extracts are prepared every day immediately before the first intake of the day, in the amount necessary for the treatments throughout the day. During the day, the solutions are stored in a refrigerator at a temperature of 4 ºC and tempered before the next administration to animals. Suspensions containing sildenafil and ketoconazole were freshly prepared immediately before administration to rats. In the second experiment, animals were given a single dose of MF, which was prepared immediately prior to administration.

All solutions / suspensions of the substances used were administered to the animals in an amount calculated according to their body weight so that all animals received the same dose (mg/kg b.w.) of the drugs and/or herbal substance.

❖ **Preparation of suspensions containing sildenafil**

To prepare suspensions for each of the experiments, a 1% aqueous solution of sodium carboxymethyl cellulose was first prepared by dissolving 2.0 g of sodium carboxymethyl cellulose in 200.0 mL of double distilled water. After reconstitution, the solution is stored in a refrigerator for several

hours until a viscous solution is obtained.Then it is used to prepare suspensions for the treatment of experimental animals.

- First experiment - assessment of the influence of repeated intake of TE, CF and MF isolated from Bancha green tea

Accurately weighed amount of sildenafil citrate (standard substance) was suspended in a certain amount of the obtained 1% sodium carboxymethyl cellulose solution to obtain a suspension with a final concentration of 0.625 mg/mL sildenafil. Each animal received an amount equivalent to 2.5 mg/kg (b.w.) sildenafil (approximately 1.0 mL) from the resultant suspension.

- Second experiment - assessment of the influence of a single dose of MF isolated from Bancha green tea

Accurately weighed amount of sildenafil citrate (standard substance) was suspended in a certain amount of the obtained 1% sodium carboxymethyl cellulose solution to obtain a suspension with a final concentration of 15.0 mg/mL sildenafil. Each animal received an amount equivalent to 60.0 mg/kg (b.w.) sildenafil (approximately 1.0 mL) from the obtained suspension.

The reconstituted suspensions have a white color and look similar (fig. 2).

❖ **Preparation of a suspension containing ketoconazole**

Accurately weighed amount of ketoconazole (standard substance) was suspended in a certain amount of 1% sodium carboxymethylcellulose solution to obtain a suspension with a final ketoconazole concentration of 2.5 mg/mL. A certain amount of the prepared suspension is administered to each animal, which provides a dose of 10.0 mg/kg (b.w.) ketoconazole (fig. 2). In both experiments, a suspension of ketoconazole was prepared as described.

❖ **Preparation of aqueous solutions of TE, CF and MF isolated from Bancha green tea**

After weighing of the TE and CF, they were dissolved in double distilled water (~ 30 °C) and homogenized on a vortex mixer at 6000 x g for 30 seconds. The MF was dissolved in hot double distilled water (~ 60 °C). Photographs of the resultant aqueous solutions are shown in figure 2.

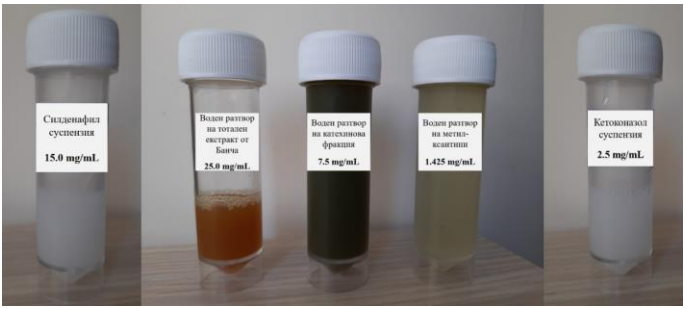


Figure 2. Photographs of the prepared suspensions of sildenafil (15.0 mg/mL) and ketoconazole (2.5 mg/mL) and aqueous solutions of TE, CF and MF.

The obtained aqueous solutions had a final concentration of 25.0 mg/mL for TE, 7.5 mg/mL for CF and 1.425 mg/mL for MF. The prepared solutions have a characteristic color - red-brown for TE, dark green for CF and pale green for MF.

In both experiments, before sildenafil administration the animals were left without food overnight (approximately 12 hours) but with free access to pure drinking water. The reconstituted solutions and suspensions are administered by an oro-gastric feeding tube for rodents. Figure 3 shows photos of the orogastric administration of the substances in experimental animals.

Figure 3. Photos of the orogastric administration of the substances in experimental animals.

2.4.3. Study of the pharmacokinetics of sildenafil after repeated administration of TE, CF and MF isolated from Bancha green tea

All experimental procedures were performed twice daily between 8 and 10 o'clock in the morning and between 16 and 17 o'clock in the afternoon. The rats were randomly divided into five groups (24 animals in each group (n = 24), of which 6 animals per time point to ensure statistical significance of the results obtained):

- Group 1 (control group) - each rat received 1.0 mL of saline twice daily for 7 consecutive days. On the 8th day in the morning all animals received 1.0 mL of saline and a certain amount of sildenafil, equivalent to a dose of 2.5 mg/kg (about 1.0 mL of the reconstituted suspension), 30 minutes after saline administration.
- Group 2 (positive control) - each animal received 1.0 mL of saline twice daily for 7 consecutive days. On the 8th day in the morning each animal received 10.0 mg/kg ketoconazole (approximately 1.0 mL of the reconstituted suspension) and 2.5 mg/kg sildenafil, 30 minutes after ketoconazole administration.
- Group 3 (total green tea extract) - each rat received 100.0 mg/kg aqueous solution of TE from Bancha green tea twice daily (approximately 1.0 mL per intake) for 7 consecutive days. On the 8th day in the morning all animals received 100.0 mg/kg aqueous solution of the extract and 2.5 mg/kg sildenafil 30 minutes later.
- Group 4 (catechin fraction) - each rat received a certain amount of aqueous CF solution, equivalent to a dose of 30.0 mg/kg twice daily (approximately 1.0 mL each) for 7 consecutive days. On the 8th day in the morning all animals received 30.0 mg/kg catechins and 2.5 mg/kg sildenafil, 30 minutes after CF administration.
- Group 5 (methylxanthine fraction) - each animal received 5.7 mg/kg aqueous solution of MF twice daily (approximately 1.0 mL per intake) for 7 consecutive days. On the 8th day in the morning all rats received 5.7 mg/kg of aqueous solution of MF and 2.5 mg/kg of sildenafil, 30 minutes after methylxanthine administration.

Blood samples were taken at the following time points: 0.5, 1, 2 and 3 hours after sildenafil administration.

2.4.4. Study of the pharmacokinetics of sildenafil after a single administration of MF from Bancha green tea

Rats were randomly divided into three groups (n = 24, of which 6 animals per time point):

- Group 1 (control group) - each rat received a certain amount of freshly prepared sildenafil suspension, equivalent to 60.0 mg/kg (approximately 1.0 mL of reconstituted suspension).
- Group 2 (positive control) - each rat received 10.0 mg/kg ketoconazole (approximately 1.0 mL of reconstituted suspension) and 60.0 mg/kg sildenafil, 30 minutes after ketoconazole intake.
- Group 3 (methylxanthine fraction) - each animal received 5.7 mg/kg aqueous solution of the extracted MF (approximately 1.0 mL) and 60.0 mg/kg sildenafil, 30 minutes after methylxanthines administration.

All substances were administered once, orally. Blood samples were taken at 0.5, 1, 3 and 6 hours after sildenafil administration.

2.4.5. Sample collection and sample preparation

At the end of both experiments, blood was collected from the sublingual vein of each animal. This blood collection technique was chosen because it is easy to perform and less stressful for the animals. Blood was taken at specific time points to determine the plasma concentration-time profile of sildenafil. Blood samples from each experimental animal were collected separately in vacutainers designed for plasma separation. Immediately after collecting the required amount of blood, the liver and kidneys of all rats were removed and washed with ice-cold saline. The organs were separated for subsequent analysis of sildenafil concentration in tissues and frozen at -80 °C. Blood samples were centrifuged at 5000 x g for 5 minutes at room temperature, after which the plasma was separated and frozen immediately at -20 °C. All rats were euthanized by cervical dislocation under diethyl ether anesthesia.

2.4.6. Pharmacokinetic analysis

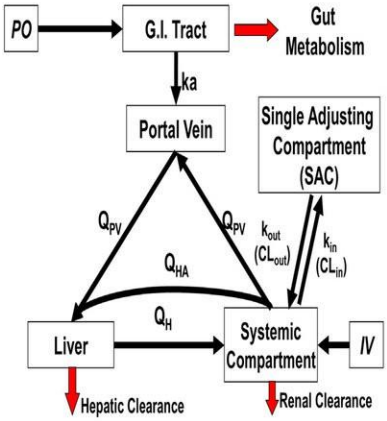
Plasma concentration-time profiles of sildenafil were analyzed by non-compartmental analysis using PKSolver, version 2.0 (a freely available program as an add-in for Microsoft Excel). The following pharmacokinetic

parameters were calculated: maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), area under the curve from time zero to the last measured concentration (AUC_{0-t}), area under the curve from time zero to infinity (AUC_{0-inf}), apparent volume of distribution (Vz/F) and apparent clearance (Cl/F). C_{max} and T_{max} values were obtained directly from the plasma concentration-time curves. The values of AUC_{0-t} and AUC_{0-inf} were determined by the linear trapezoidal method for the measured values and by subsequent extrapolation to infinity to calculate AUC_{0-inf}.

2.5. PBPK models for predicting potential interactions between sildenafil and TE, CF and MF from Bancha green tea in humans

For the simulations described in the present dissertation, a study on a virtual population of healthy volunteers, men aged 60 to 65 years (10 groups of 10 people) is performed.

For the purpose of the study, a mechanistic-static and a mechanistic-dynamic PBPK model were used. To the latter it was performed a minimum PBPK model with a single added compartment (figure 4).



Фигура 4. Minimum PBPK model with a single added compartment. QH, QPV and QHA are blood flows to the liver, portal vein and hepatic artery, respectively; k_{in} and k_{out} are first order rate constants; IV and PO are intravenous and oral routes of administration, respectively; k_a is the first-order rate of absorption.

For the construction of PBPK models of the catechin and methylxanthine fractions isolated from Bancha green tea, it was taken into account that the main proven components in them are EGCG in CF and caffeine in MF. Therefore, it has been suggested that the pharmacokinetic behavior of these fractions would be similar to their major components. For EGCG it was used the available information on physicochemical and pharmacokinetic properties in the global database, while for caffeine it was used the data available in the software program and the inhibitory constant on CYP3A4 determined by us in previous studies. The required pharmacokinetic parameters of sildenafil (a drug substrate for the enzyme CYP3A4) used to predict potential interactions were available in the program. The classic CYP3A4 inhibitor ketoconazole (also available in the software database) was used as a positive control.

2.6. Methods for statistical analysis

Statistical analysis was performed using GraphPad Prism, version 9.2.0 (332). Results are expressed as mean ± standard deviation (SD). Statistical comparisons between the calculated pharmacokinetic parameters for the different groups in the two experiments were performed using analysis of variance (ANOVA), followed by Dunnett's test. A p-value ≤ 0.05 (two tailed test) is considered statistically significant.

IV. RESULTS

1. Isolation of TE, CF and MF from Japanese green tea Bancha

1.1. Isolation of total extract

The obtained total extract from Bancha green tea is a solid glassy mass with a reddish-brown color. It has a sweet aroma and is easily soluble in lukewarm water. In fig. 5 is presented a photograph of the extract. The percentage yield is 16.37 % (w/w).

Figure 5. A photo of the obtained total extract of Bancha green tea.

1.2. Isolation of catechins

The isolated catechin fraction is a solid mass with dark green to black color. It has no characteristic odor and dissolves in lukewarm double distilled water after vigorous shaking. A photo of the obtained fraction is shown in figure 6. The calculated percentage yield is 3.85 % (w/w).

Figure 6. A photo of the isolated catechin fraction.

1.3. Isolation of methylxanthines

The isolated MF has a light yellow-green color and a specific odor. It dissolves very quickly in hot water. Figure 7 shows a photograph of the methylxanthine fraction. The percentage yield of methylxanthines is 0.95 % (w/w).

Figure 7. A photo of the isolated methylxanthine fraction.

2. Qualitative and quantitative analysis of TE, CF and MF isolated from Bancha green tea

2.1. Analysis of TE and CF from Bancha green tea

2.1.1. Method validation

In connection with the present dissertation, a reliable HPLC-UV method for analysis of EGCG, (+)-catechin, gallic acid and caffeine was developed and validated. The ability of the analytical procedure to reproduce results that are directly proportional to the amount of the standard substances tested has been demonstrated. Linearity in the range of 1.0 to 200.0 μg/mL was estimated for the four substances. In all cases the correlation coefficient (R^2) is greater than or equal to 0.9998. The LOQ of EGCG, (+)-catechin, gallic acid and caffeine were determined by serial dilution of the standard solution with the lowest concentration (1.0 μg/mL) involved in the construction of the corresponding calibration curve. As a result, LOQ values of 0.50, 0.30, 0.03 and 0.01 μg/mL, respectively, were found.

2.1.2. HPLC-UV analysis of the TE and CF

The developed method is applied for qualitative and quantitative determination of EGCG, (+)-catechin, gallic acid and caffeine in TE and CF

of Bancha green tea. Equivalent amounts of EGCG and caffeine and a significantly lower amount of (+)-catechin were found in the total extract. All four compounds were found in CF, with EGCG in the largest amount, as expected. The results are shown in tables 1 and 2, and the chromatograms of TE and CF are presented in figures 8 and 9, respectively. Based on the results of the analysis of the TE, we calculated that 1.0 g of dried tea leaves contained 6.86 mg caffeine, 6.74 mg EGCG and 0.43 mg (+)-catechin while according to the analysis of CF we calculated that 1.0 g of dried tea leaves contains 6.46 mg EGCG, 0.78 mg (+)-catechin, 0.07 mg gallic acid and 1.03 mg caffeine.

Table 1. Calculated concentrations of EGCG, (+)-catechin and caffeine in the tested aqueous solution of TE from Bancha green tea (1.0 mg/mL).

	Concentration, µg/mL	**Concentration, % ± SD**
EGCG	41.2	4.12 % ± 0.44
(+)-catechin	2.6	0.26 % ± 0.28
caffeine	41.9	4.19 % ± 0.14

Table 2. Calculated concentrations of EGCG, (+)-catechin, gallic acid and caffeine in the tested aqueous solution of CF from Bancha green tea (1.0 mg/mL).

	Concentration, µg/mL	**Concentration, % ± SD**
EGCG	167.8	16.78 ± 0.54
(+)-catechin	20.2	2.02 ± 0.37
gallic acid	1.7	0.17 ± 0.24
caffeine	26.7	2.67 ± 0.19

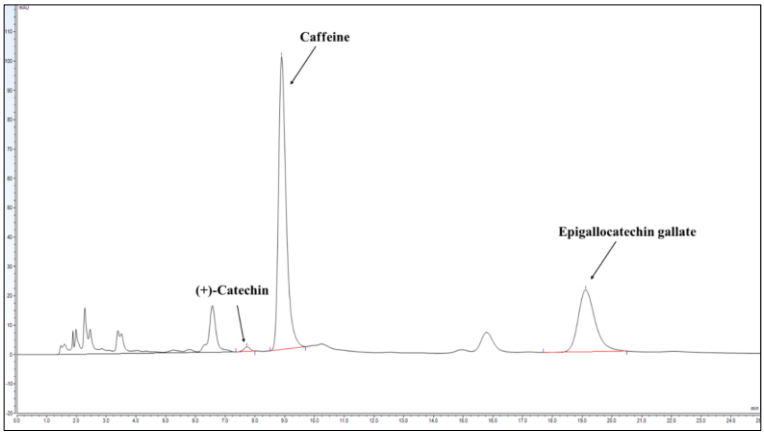


Figure 8. Chromatogram of the tested aqueous solution of TE from Bancha (1.0 mg/mL).

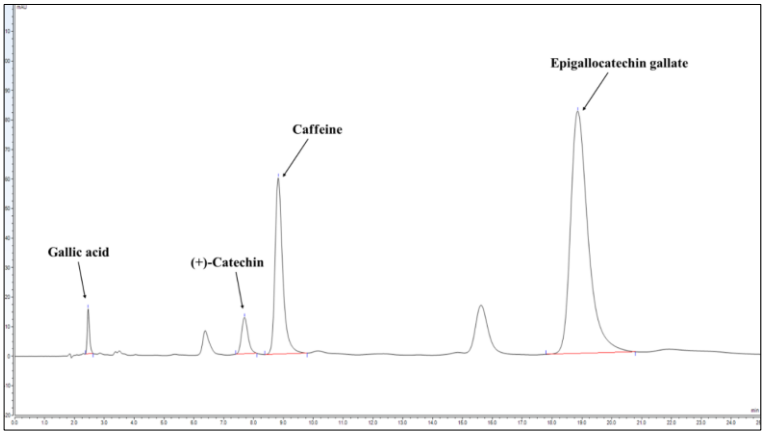


Figure 9. Chromatogram of a sample of CF (1.0 mg/mL).

2.2. Analysis of the isolated methylxanthine fraction

The isolated MF was analyzed by a previously developed HPLC-UV method for qualitative and quantitative determination of caffeine, theophylline and theobromine. The isolated fraction contained a large amount of caffeine and a significantly lower amount of theobromine, while

theophylline was not detected. The results are shown in table 3, and figure 10 shows a chromatogram of MF isolated from Bancha tea leaves. According to the obtained results, we calculated that 1.0 g of dried Bancha tea leaves contains 8.34 mg of caffeine and 0.009 mg of theobromine.

Table 3. Calculated concentrations of caffeine, theophylline and theobromine in the tested aqueous solution of MF (2.5 mg/mL).

	Concentration, µg/mL	**Concentration, % ± SD**
caffeine	2193.5	87.74 ± 0.83
theophylline	-	-
theobromine	2.3756	0.095 ± 0.02

- below the limit of quantification

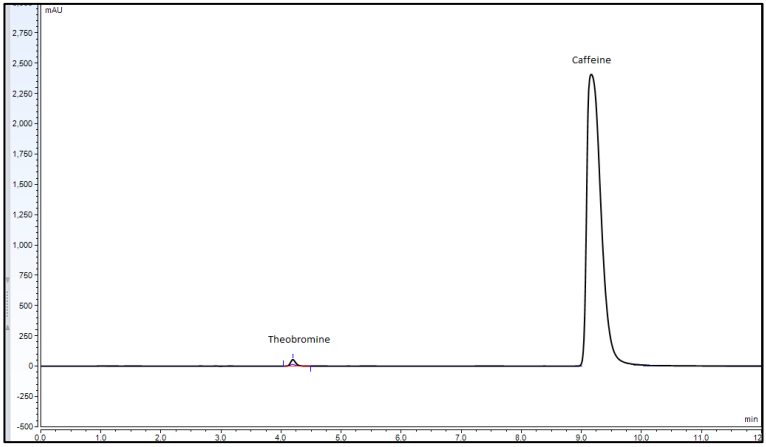


Figure 10. Chromatogram of a sample of MF (2.5 mg/mL).

The developed HPLC-UV method for determination of EGCG, (+)-catechin, gallic acid and caffeine was also used to determine the content of catechins in the isolated MF. The liquid chromatographic analysis confirmed the high content of caffeine in the MF, while EGCG, (+)-catechin and gallic acid were not detected.

3. Qualitative and quantitative analysis of sildenafil in plasma samples from experimental animals

3.1. Method validation

For the aim of the dissertation, a validated HPLC-UV method for qualitative and quantitative analysis of sildenafil in rat plasma was developed and applied. The observed accuracy and precision meet the ICH criteria. The linearity of the method was confirmed by the correlation coefficient (R^2> 0.998). In addition, a high sensitivity of the method was achieved as the LOQ of sildenafil was 5.0 ng/mL.

3.2. HPLC-UV analysis of biological samples containing sildenafil

The proposed protein precipitation method provides ≥ 93.0% recovery of sildenafil at all concentrations used (± 1.89% for samples to which sildenafil was added). In addition, no other components were eluted in the retention time of the analyte, proving the suitability of the sample preparation technique. A chromatogram of a test sample is shown in figure 11.

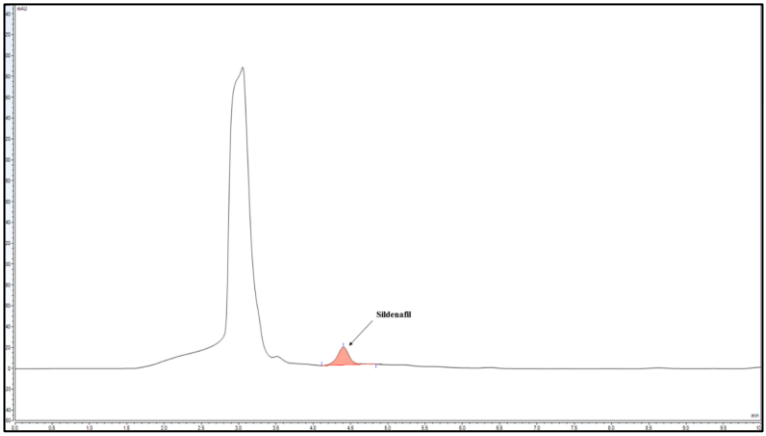


Figure 11. Chromatogram of a rat plasma sample obtained after a single oral administration of sildenafil (2.5 mg/kg)

4. Pharmacokinetic studies in experimental animals

Repeated administration of TE, CF or MF from Japanese Bancha green tea, as well as co-administration of a single dose of methylxanthines with sildenafil affected the pharmacokinetics of the drug in rats.

4.1. Study of the pharmacokinetics of sildenafil after repeated administration of TE, CF and MF from Bancha green tea

The measured maximum plasma concentration of sildenafil (C_{max}) after a single oral dose of 2.5 mg/kg in rats was 15.96 ± 2.45 ng/mL, the plasma half life ($t_{1/2}$) was average 0.98 hours (0.91 – 1.02), and T_{max} was 0.5 hours. In group 2, after co-administration of sildenafil and ketoconazole (enzyme inhibitor) an increase in C_{max} ($p < 0.001$), AUC_{0-3} ($p < 0.001$) and AUC_{0-inf} ($p < 0.001$) of sildenafil was observed, as well as an increase in the time to reach maximum plasma concentration (T_{max} = 1 h). In order to facilitate the interpretation of the results, as well as to compare the effect of single and repeated administration of MF on the pharmacokinetics of sildenafil, the results of repeated administration of TE and CF were considered separately from those after repeated administration of MF.

4.1.1. Influence of repeated administration of TE and CF from Bancha green tea on the pharmacokinetics of sildenafil in rats

Significant changes in the pharmacokinetic parameters of sildenafil were observed when it was taken after repeated administration of TE and CF from Bancha green tea to rats. Table 4 presents the main pharmacokinetic parameters of sildenafil calculated for each group, and figure 12 shows the plasma concentration-time curves of sildenafil alone and in combination. When sildenafil was administered after repeated treatment of animals with TE or CF from Japanese Bancha green tea, a statistically significant increase in C_{max} ($p < 0.001$ for TE; $p < 0.0001$ for CF), AUC_{0-3} ($p < 0.001$) and AUC_{0-inf} ($p < 0.01$ for TE; $p < 0.001$ for CF) of sildenafil were observed, as well as a significant decrease in the volume of distribution (Vz/F) and a decrease in clearance (Cl/F) compared to the control group. No change in the time to reach maximum plasma concentration of sildenafil (T_{max} = 0.5 h) was observed. The observed C_{max}, AUC_{0-3} and AUC_{0-inf}, Vz/F and Cl/F values of sildenafil administered after repeated pre-treatment of animals with TE and CF were close to those observed with co-administration of sildenafil with

ketoconazole (increased C_{max}, AUC_{0-3} and AUC_{0-inf} by more than 100 % compared to the control group). Figures 13, 14 and 15 show comparative graphs of the indicated pharmacokinetic parameters in the different groups. Also, no significant change in plasma half life ($t_{1/2}$) and mean residence time (MRT) of sildenafil was observed in animals that received multiple TE and CF of Bancha green tea.

Table 4. Pharmacokinetic parameters of sildenafil after a single oral dose of 2.5 mg/kg in rats alone, in combination with a single dose of ketoconazole and after pre-treatment of animals with TE or CF from Bancha

Pharmaco-kinetic parameter	**Sildenafil (control group)**	**Sildenafil + ketoconazole**	**Sildenafil + TE from Bancha tea**	**Sildenafil + CF from Bancha tea**
C_{max} (ng/mL)	15.96 ± 2.45	42.75 ± 6.81 ***	66.15 ± 12.30 ***	64.74 ± 9.95 ****
T_{max} (h)	0.5	1	0.5	0.5
AUC_{0-3} (ng*h/mL)	16.20 ± 2.73	64.31 ± 12.92 ***	72.90 ± 15.20 ***	66.66 ± 12.33 ***
AUC_{0-inf} (ng*h/mL)	23.28 ± 4.17	70.20 ± 14.59 ***	86.23 ± 19.84 **	77.99 ± 15.93 ***
Vz/F (L/kg)	0.15 ± 0.02	0.04 ± 0.01 ****	0.05 ± 0.01 ****	0.05 ± 0.01 ****
Cl/F ((mg/kg)/ (ng/ml)/h)	0.11 ± 0.02	0.04 ± 0.01 ***	0.03 ± 0.01 ****	0.03 ± 0.01 ***

Values are expressed as mean ± SD, n=24. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared to the control group.

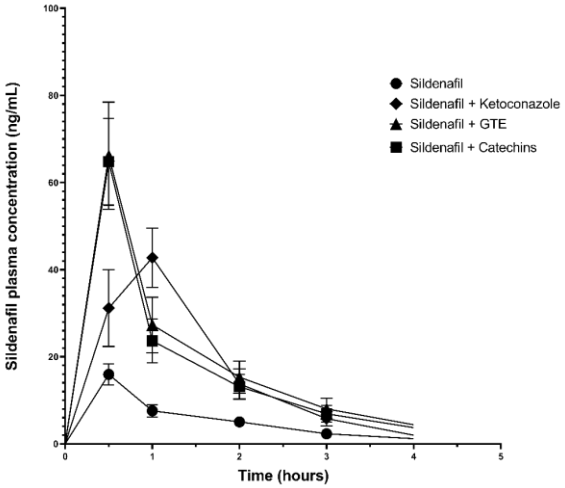


Figure 12. Plasma concentration-time curve (mean ± SD) of sildenafil after a single oral dose of 2.5 mg/kg in rats alone, in combination with a single dose of ketoconazole and after repeated administration of TE or CF from Bancha.

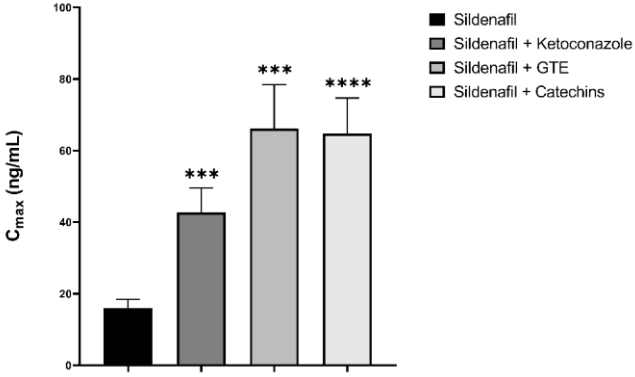


Figure 13. Maximum plasma concentration (Cmax) of sildenafil when used alone (2.5 mg / kg), in the presence of ketoconazole or after repeated repeated treatment with TE or CF in rats. Values are expressed as mean ± SD, *** $p \leq 0.001$, **** $p \leq 0.0001$, compared to the control group.

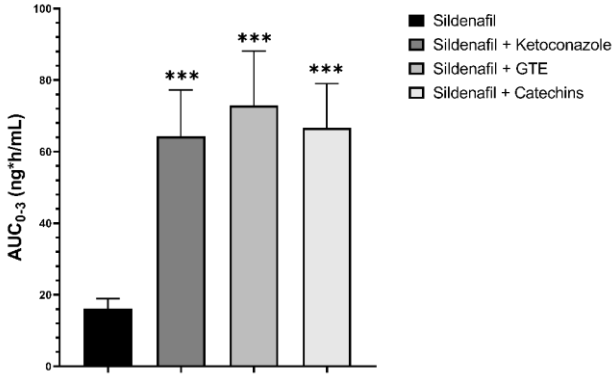


Figure 14. Area under the plasma concentration-time curve from time zero to the last measured value (AUC_{0-3}) of sildenafil administered alone (2.5 mg/kg) in combination with ketoconazole or after repeated administration of TE or CF to rats. Values are expressed as mean ± SD, *** $p \leq 0.001$, compared to the control group.

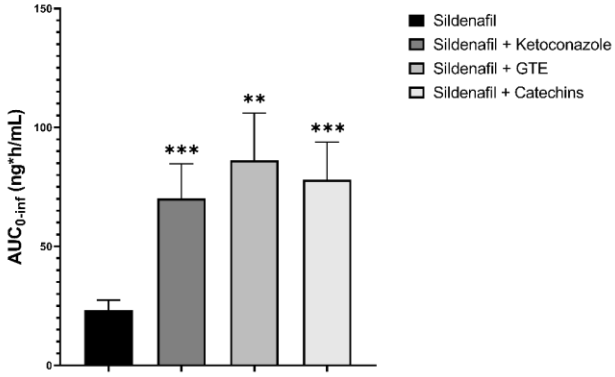


Figure 15. Area under the plasma concentration-time curve from time zero to infinity (AUC_{0-inf}) of sildenafil administered alone (2.5 mg/kg) in combination with ketoconazole or after repeated administration of TE or CF to rats. Values are expressed as mean ± SD, ** $p < 0.01$, *** $p \leq 0.001$, compared to the control group.

4.1.2. Influence of repeated administration of MF from Bancha green tea on the pharmacokinetics of sildenafil in rats

Plasma concentration-time curves of sildenafil alone, after administration with ketoconazole or after multiple doses of methylxanthines are shown in fig. 16. Administration of sildenafil after pretreatment of rats with MF from Bancha green tea resulted in a statistically significant increase in C_{max} ($p < 0.0001$), AUC_{0-3} ($p < 0.0001$) and AUC_{0-inf} ($p < 0.001$) and decrease in Vz/F and Cl/F, compared to the control group. In addition, methylxanthines delayed the time to peak plasma concentration of sildenafil (T_{max} = 1 h) and reduced its plasma half life ($t_{1/2}$ = 0.77 часа (0.67-0.93), $p <$ 0.01). The main pharmacokinetic parameters of sildenafil in each group are shown in table 5. The observed values of C_{max}, AUC_{0-3} and AUC_{0-inf}, Vz/F and Cl/F of sildenafil after pretreatment with MF are close to those observed after co-administration of sildenafil with ketoconazole (increased by more than 100% compared to control group). Fig. 17, 18 and 19 present a comparison of sildenafil C_{max}, AUC_{0-3} and AUC_{0-inf} between groups 1, 2 and 5. Methylxanthines did not lead to a significant change in the mean residence time (MRT) of sildenafil in rats.

Table 5. Pharmacokinetic parameters of sildenafil after a single oral dose of 2.5 mg/kg in rats alone, in combination with a single dose of ketoconazole or after pretreatment of the animals with MF from Bancha green tea

Pharmaco-kinetic parameter	**Sildenafil (control group)**	**Sildenafil + ketoconazole**	**Sildenafil + MF from Bancha tea**
C_{max} (ng/mL)	15.96 ± 2.45	42.75 ± 6.81 ***	37.42 ± 3.44 ****
T_{max} (h)	0.5	1	1
AUC_{0-3} (ng*h/mL)	16.20 ± 2.73	64.31 ± 12.92 ***	56.27 ± 8.83 ****
AUC_{0-inf} (ng*h/mL)	23.28 ± 4.17	70.20 ± 14.59 ***	63.91 ± 11.56 ***
Vz/F (L/kg)	0.15 ± 0.02	0.04 ± 0.01 ****	0.05 ± 0.004 ***

Cl/F ((mg/kg)/ (ng/ml)/h)	0.11 ± 0.02	0.04 ± 0.01 ***	0.04 ± 0.01 ***

Values are expressed as mean ± SD, n=24. *** $p < 0.001$, **** $p < 0.0001$, compared to the control group.

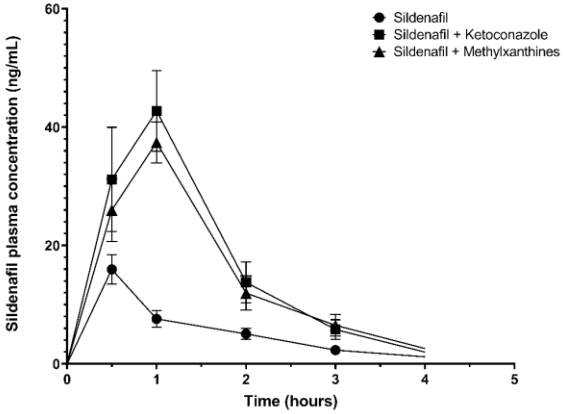


Figure 16. Plasma concentration-time curve (mean ± SD) of sildenafil after a single oral dose of 2.5 mg/kg in rats alone, in combination with a single dose of ketoconazole or after pretreatment with MF from Bancha green tea

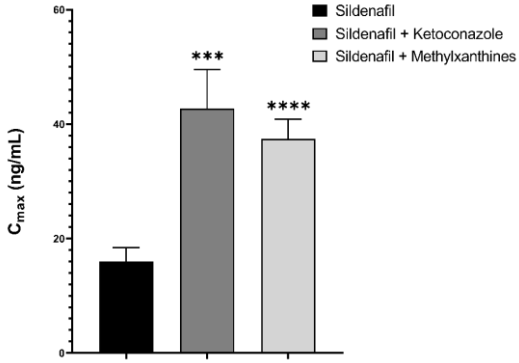


Figure 17. Maximum plasma concentration (C_{max}) of sildenafil alone (2.5 mg/kg), in the presence of ketoconazole or after pretreatment with methylxanthines in rats. Values are expressed as mean ± SD, *** $p \leq 0.001$, **** $p \leq 0.0001$, compared to the control group.

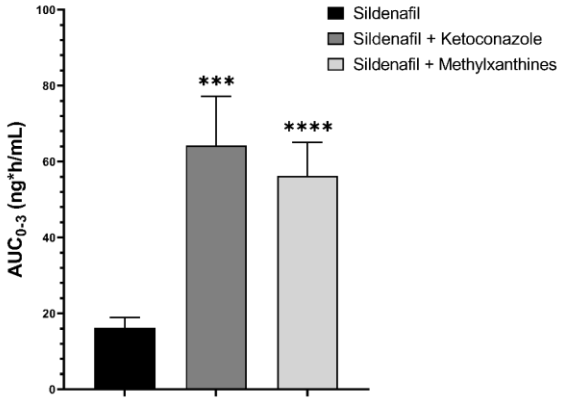


Figure 18. Area under the plasma concentration-time curve from time zero to the last measured value ($AUC_{0\text{-}3}$) of sildenafil administered alone (2.5 mg/kg) in combination with ketoconazole or after repeated administration of MF to rats. Values are expressed as mean ± SD, *** $p \leq 0.001$, **** $p \leq 0.0001$, compared to the control group.

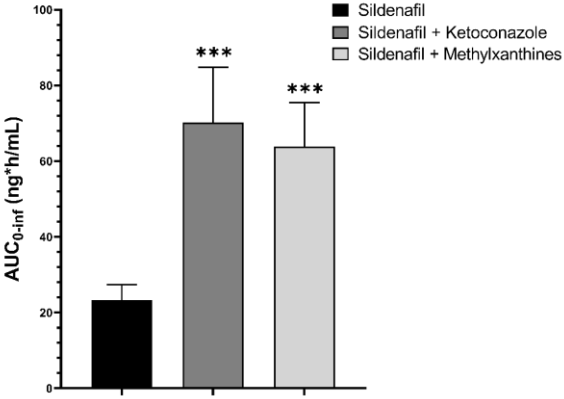


Figure 19. Area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}inf}$) of sildenafil administered alone (2.5 mg/kg) in combination with ketoconazole or after repeated administration of MF to rats. Values are expressed as mean ± SD, *** $p \leq 0.001$, compared to the control group.

4.2. Study of the pharmacokinetics of sildenafil after a single administration of MF from Bancha green tea

Co-administration of sildenafil with a single oral dose of Bancha green tea methylxanthines (5.7 mg/kg) showed changes in some of the pharmacokinetic parameters of sildenafil compared to the sildenafil group alone. When sildenafil was co-administered with ketoconazole, a significant increase in sildenafil C_{max} ($p < 0.01$), AUC_{0-6} and AUC_{0-inf} ($p < 0.05$) was observed, compared to the control group. The plasma concentration-time curves of sildenafil administered alone and with ketoconazole or MF from Bancha green tea are shown in fig. 20. Following sildenafil intake alone at a dose of 60.0 mg/kg in rats, the mean maximum plasma concentration (C_{max}) was 152.82 ± 31.41 ng/mL. The main pharmacokinetic parameters of sildenafil in each group are presented in table 6. Oral administration of sildenafil with a single dose of methylxanthines resulted in decreased C_{max} (29.44%), AUC_{0-6} (33.06%) and AUC_{0-inf} (29.60%), compared to the control group. The decrease in C_{max} was not statistically significant ($p > 0.05$) in contrast to the decrease in AUC_{0-6} and AUC_{0-inf} ($p < 0.05$). The C_{max}, AUC_{0-6} и AUC_{0-inf} values of sildenafil were increased by 61.97%, 49.84% and 43.45% respectively. T_{max} values were the same in all groups (1 h). No statistically significant differences in clearance (Cl/F), plasma half life ($t_{1/2}$) and MRT of sildenafil were found between the groups. The Vz/F value of sildenafil was increased in the MF group and decreased in the ketoconazole group, compared to the control group. The differences in sildenafil C_{max}, AUC_{0-6} and AUC_{0-inf} between the groups are shown in fig. 21, 22 and 23 respectively.

Table 6. Pharmacokinetic parameters of sildenafil after a single oral dose of 60.0 mg/kg in rats alone and in combination with a single dose of ketoconazole or MF from Bancha green tea

Pharmaco-kinetic parameter	**Sildenafil (control group)**	**Sildenafil + ketoconazole**	**Sildenafil + MF from Bancha tea**
C_{max} (ng/mL)	152.82 ± 31.41	247.53 ± 48.80 **	107.83 ± 32.42

T_{max} (h)	1	1	1
AUC_{0-6} (ng*h/mL)	626.83 ± 125.77	939.23 ± 190.64 *	419.60 ± 97.31 *
AUC_{0-inf} (ng*h/mL)	1042.94 ± 227.53	1496.11 ± 332.03 *	734.20 ± 145.33 *
Vz/F (L/kg)	0.37 ± 0.08	0.23 ± 0.06 *	0.55 ± 0.13 *
Cl/F ((mg/kg)/ (ng/ml)/h)	0.06 ± 0.02	0.04 ± 0.01	0.09 ± 0.02

Values are expressed as mean ± SD, n=24. * $p < 0.05$, ** $p < 0.01$, compared to the control group.

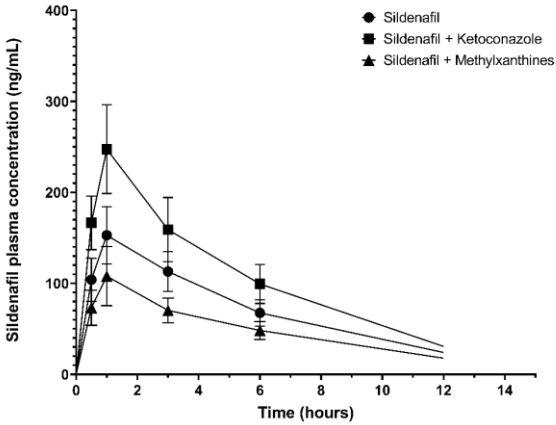


Figure 20. Sildenafil plasma concentration-time curve (mean ± SD) after a single oral dose of 60.0 mg/kg in rats alone and in combination with a single dose of ketoconazole or MF from Bancha green tea.

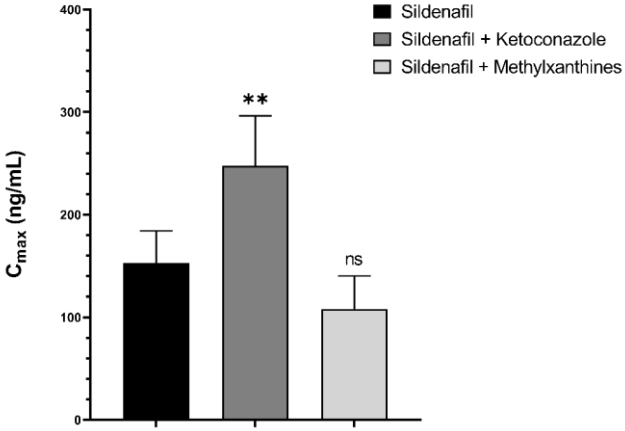


Figure 21. Maximum plasma concentration (C_{max}) of sildenafil alone (60.0 mg/kg) and in the presence of ketoconazole or MF from Bancha green tea in rats. Values are expressed as mean ± SD, ** $p \leq 0.01$, ns – non-significant, compared to the control group.

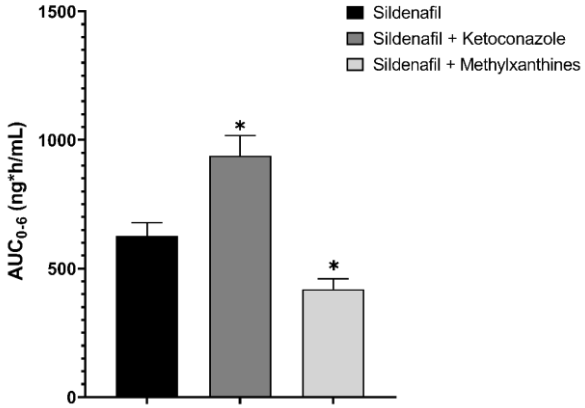


Figure 22. Area under the plasma concentration-time curve from time zero to the last measured value (AUC_{0-6}) of sildenafil administered alone (60.0 mg/kg) and in combination with ketoconazole or methylxanthines in rats. Values are expressed as mean ± SD, * $p \leq 0.05$, compared to the control group.

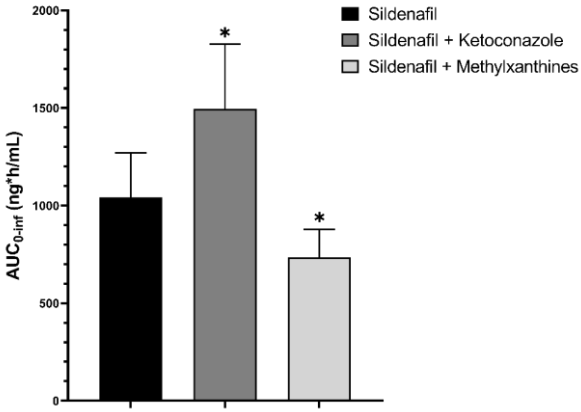


Figure 23. Area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}inf}$) of sildenafil administered alone (60.0 mg/kg) and in combination with ketoconazole or methylxanthines in rats. Values are expressed as mean ± SD, * $p \leq 0.05$, compared to the control group.

5. Development of PBPK models

10 experiments were conducted with 10 subjects or a total of 100 analyzed virtual samples. A single oral dose of ketoconazole (100.0 mg) was used as a positive control. The following experimental settings are simulated:

- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after administration of a single dose of ketoconazole (100.0 mg);
- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after administration of a single dose of CF (400.0 or 600.0 mg);
- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after administration of a single dose of MF (250.0 or 500.0 mg);
- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after a single administration of CF (400.0 mg) and MF (250.0 mg) simultaneously.

- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after a single administration of CF (400.0 mg) and MF (500.0 mg) simultaneously.
- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after a single administration of CF (600.0 mg) and MF (250.0 mg) simultaneously.
- ❖ Administration of the substrate (sildenafil) in a single oral dose of 50.0 mg, 30 minutes after a single administration of CF (600.0 mg) and MF (500.0 mg) simultaneously.

The results of the simulations are presented in Table 7.

Table 7. Changes in the AUC ratio values and in the ratios of Cmax and AUC in the presence and absence of inhibitor, using a static and dynamic model.

Sildenafil 50.0 mg +	**AUCR Mechanistic static model**	**CmaxR/AUCR Mechanistic dynamic model**
Ketoconazole 100.0 mg	7.61 (4.38-14.77)	2.78±0.92/ 4.93±2.09
CF 400.0 mg	**1.10 (1.03-1.20)**	1.02±0.01/ 1.03±0.01
CF 600.0 mg	**1.14 (1.05-1.28)**	1.03±0.01/ 1.04±0.01
MF 250.0 mg	1.03 (1.01-1.05)	1.01±0.01/ 1.01±0.01
MF 500.0 mg	1.05 (1.03-1.11)	1.01±0.01/ 1.02±0.01
CF 400.0 mg/ MF 250.0 mg	**1.12 (1.05-1.22)**	1.03±0.01/ 1.04±0.01
CF 400.0 mg/ MF 500.0 mg	**1.15 (1.06-1.25)**	1.04±0.01/ 1.04±0.01
CF 600.0 mg/ MF 250.0 mg	**1.17 (1.07-1.31)**	1.04±0.01/ 1.05±0.01
CF 600.0 mg/ MF 500.0 mg	**1.19 (1.08-1.33)**	1.05±0.01/ 1.06±0.01

The simulations conducted with the mechanistic-static model showed a moderate risk of interactions when sildenafil is used with CF at doses of 400.0 or 600.0 mg. The combined use of sildenafil and MF (250.0 mg) showed a low risk, while at the higher dose of 500.0 mg, a moderate risk of drug interactions was reported in some of the virtual population. Co-

administration of sildenafil with CF and MF in all simulated doses – CF 400.0 mg / MF 250.0 mg; CF 400.0 mg / MF 500.0 mg; CF 600.0 mg / MF 250.0 mg and CF 600.0 mg / MF 500.0 mg have been reported in the summation of effects with respect to AUCR, but still relate to those with a moderate risk of drug interactions. The simulations performed with the mechanistic-dynamic PBPK model showed a low risk of interactions between sildenafil and CF or MF even at the highest simulated doses, both alone and in combination. The classic CYP3A4 inhibitor ketoconazole was used to verify the simulation model.

V. DISCUSSION

Interactions between drugs and plant extracts can be potentially dangerous and may lead to serious adverse effects. Due to the frequent use of medicines and phytopreparations in recent years, it is necessary to conduct more in-depth preclinical and/or clinical trials to assess the likelihood of clinically significant interactions when taking certain conventional drugs and daily consumed plant extracts and/or their active components. Thus, some interactions may be anticipated and avoided, and this will increase patient safety when taking phytopreparations. The confirmation of a specific interaction requires research under different conditions and using different methods, including a study on potential pharmacokinetic and pharmacodynamic interactions in humans, if applicable. This is the only way to get a complete picture of the impact of drug interactions on the therapeutic effect and/or safety of the drugs.

The present dissertation aims to investigate and evaluate the potential interactions that may occur with the simultaneous use of sildenafil and an extract isolated from Japanese Bancha green tea or its BAS (catechins and methylxanthines). As a result of the literature review, it is noteworthy that some types of Japanese green tea, such as Sencha and Matcha, are better studied, compared to others such as Bancha green tea. In the scientific literature in recent years it has been found a limited information on the composition and beneficial effects of Japanese Bancha green tea, although it is quite popular among the Japanese population, and probably in other parts of the world. This requires a thorough study of the qualitative and quantitative content of this type of green tea, as well as an assessment of its therapeutic potential *in vivo* and the potential to participate in drug interactions, considering the reported differences in BAS content compared to other types of green tea. In a previous study, we investigated the effect of MF isolated from Japanese Bancha green tea on drug-induced cardiotoxicity and nephrotoxicity and methylxanthines showed cardioprotective and nephroprotective effects, proven biochemically and histologically. The *in vitro* antioxidant activity of the isolated MF was also studied by two different methods (DPPH and ABTS radical-binding activity) and methylxanthines were found to show significant antioxidant activity. Therefore, the health benefits as well as the potential risks associated with regular consumption of

Japanese Bancha green tea need to be clarified due to its frequent use. This is the reason to chose Bancha tea leaves for the aim of the present work.

To achieve this goal, it is first necessary to extract the BAS of tea leaves. After careful consideration and planning of the experimental design of the studies in laboratory animals, we decided to prepare and use three plant extracts - TE, CF and MF from Japanese Bancha green tea. The total extract contains the whole complex of BAS, which are found in green tea, while the isolation of CF and MF aims to extract mainly catechins and methylxanthines, respectively. These are the two main groups of BAS contained in green tea, with EGCG and caffeine as the main representatives, respectively. Thus, we could assess whether the observed effects of total green tea extract are mainly due to the catechins or methylxanthines. After preparation of the plant extracts it is necessary to perform qualitative and quantitative determination of the BAS in each of them.

1. Isolation of TE, CF and MF from Bancha green tea

Already proven extraction procedures have been used to isolate the three plant extracts.

1.1. Isolation of TE from Bancha green tea

The isolation of the total extract from Japanese Bancha green tea was carried out in such a way as to be as close as possible to the preparation of tea at home. Green tea is usually prepared by brewing tea leaves with hot water (80-90 °C) for a 3 to 4 minutes. Therefore, hot double distilled water with a temperature of ~ 80 ºC was used as an extractant, and the duration of the infusion was 3 minutes. The extraction method described by Chakraborty et al. (2014) was applied, but some changes have been made. The ratio between the amount of tea leaves and the extractant (100.0 g tea leaves to 1.0 L hot water) was the same, as well as the subsequent filtration and evaporation under vacuum to obtain a thick sticky mass. The infusion time is reduced from 10 to 3 minutes and the resulting thick mass is subjected to additional drying in an oven to obtain a solid and dry mass. It was found that water temperature and infusion time have a significant impact on the efficiency of extraction of BAS such as polyphenols and methylxanthines, as much of the contained EGCG and caffeine are extracted from the leaves at a water temperature of 80 ºC. In addition, differences in the rate of extraction

and the content of BAS in the infusion of loose tea leaves compared to the powdered substance can be observed. In terms of extraction time, some studies have shown that the total amount of extracted polyphenols increases with increasing infusion time (≥ 10 minutes), but at the same time increases the astringency and bitterness affecting the organoleptic properties of the tea beverage. A study conducted with Japanese Bancha green tea also found that increasing the brewing time of tea leaves (5 to 30 minutes) lead to extraction of more polyphenols, as well as more tannins, which are usually the cause. for the unpleasant astringent taste of brewed tea. At the same time, as the infusion time increases and high water temperature is used, catechins may undergo chemical changes such as epimerization, whereby epicatechins are converted to the corresponding catechins. It was found that the EGCG epimerizes in the GCG at temperatures above 85 ºC. Unlike catechins, caffeine has a stable molecule that is easily soluble in hot water and its concentration in tea is relatively stable. In addition, Saklar et al (2015) reported that the largest amounts of EGCG and caffeine were extracted at a water temperature of about 85 ºC during a 3-minute infusion. Despite the data for extracting more BAS with a longer infusion time, we believe that we have chosen an optimal conditions for extracting the maximum amount of EGCG and caffeine from Bancha tea leaves. In addition, there is an evidence in the literature that prepared tea drinks may contain many toxic elements such as aluminum, arsenic, lead, cadmium, manganese and others. Prolonged steaming of tea leaves has been reported to be associated with the extraction of larger amounts of toxic metals, with the lowest concentration of listed toxic compounds being found in beverages prepared with a 3 minute infusion of the tea leaves. Therefore, it is recommended to avoid steaming the leaves for more than 3 minutes when making green tea. It was also found that the average amount of aluminum and some heavy metals such as manganese, lead, cadmium, arsenic, mercury and chromium was higher in mature tea leaves than in young leaves. These results support the benefits of a 3-minute infusion, considering the use of mature tea leaves in the present study. Additional drying of the extract in an oven was performed at a temperature of 25 ºC until a solid dry mass was obtained, because of the instability of the catechins.

In conclusion, we can say that the obtained percentage yield of BAS (16.37% w/w) after extraction of the total extract is lower than that reported

by Chakraborty et al. (2014) (24.76% w/w), whose extraction method we applied. This is probably due to the use of Bancha green tea leaves, the shorter infusion time, the brewing of the tea leaves without prior chopping, as well as the additional drying of the extract to obtain a dry mass.

1.2. Isolation of CF from Bancha green tea

The method proposed by Georgiev, Iliev and Zhelev (2015) was applied for the isolation of CF. The use of ethyl acetate to extract catechins from the aqueous phase is a well-known technique that has been used by other authors. Acidification of the extract with phosphoric acid is used to increase the stability of catechins and is importance for increasing the percentage yield.

1.3. Isolation of MF from Bancha green tea

The isolation of MF was performed according to the method reported by Georgiev, Iliev and Zhelev (2015). The use of chloroform in the process of extraction of methylxanthines is considered to be one of the most suitable methods for extraction of these compounds from plant extracts. Caffeine, theophylline and theobromine pass into the chloroform extract, while other BAS, such as catechins remain in the aqueous phase.

2. Qualitative and quantitative analysis of TE, CF and MF isolated from Bancha green tea

Despite the large number of HPLC methods reported for the analysis of green tea beverages, the protocols for the analysis of Bancha green tea to date are extremely insufficient. In this regard, we have developed and validated two liquid chromatographic methods, which were subsequently applied to perform qualitative and quantitative determination of the isolated plant extracts.

2.1. Liquid chromatographic analysis of TE and CF from Bancha green tea

2.1.1. Development of HPLC-UV method

An easily feasible and environmentally friendly method for the simultaneous determination of EGCG, (+)-catechin, gallic acid and caffeine has been developed. As the quality and antioxidant potential of green tea are

thought to be related to the catechin content, the quantification of EGCG in Bancha TE and CF is essential for subsequent pharmacological studies. EGCG is expected to be in the highest concentration in TE and CF, as it is the dominant catechin in the tea leaves. In addition, the developed method will be used to analyze the methylxanthine fraction to determine if it also contains catechins, in particular EGCG. At the same time, this method will determine the qualitative and quantitative content of caffeine in TE and CF. In general, the amount of caffeine is relatively stable and remains unchanged during the processing of tea leaves, although some microorganisms may stimulate its breakdown. Therefore, an important priority of the present work is to ensure the stability of the four analytes, especially of EGCG. As mentioned, catechins and especially EGCG are unstable compounds and are easily degraded under the action of light, as well as at certain pH and ambient temperature values. For this reason, some authors recommend the addition of stabilizers such as ascorbic acid to the samples. On the other hand, a review of the literature shows that under such chromatographic conditions, ascorbic acid tends to elute at the beginning of the chromatogram, close to gallic acid, so its use in the present work is not appropriate. In order to avoid the addition of ascorbic acid, which may compromise the identification of gallic acid, certain gentle conditions have been chosen for the analysis and storage of the samples in order to ensure the stability of the EGCG. The effectiveness of the chromatographic conditions was confirmed by the analysis of series of samples for quality control. Of course, the importance of the approach to the addition of stabilizers should not be ruled out in cases where degradation of the EGCG is unavoidable (e.g. in bioassay).

The optimization of the HPLC method developed by Fernando and Soysa (2016) allowed the simultaneous and accurate quantification of the four BAS in the samples of Bancha green tea. The reported method uses an internal standard that can be eluted with some of the sample components (theophylline, catechins, etc.). For this reason, the standard additive method was used for quantification in the present study in order to minimize the matrix effects that could interfere with the analyte measurement signals. As a result of the applied modifications, a higher sensitivity of the method has been achieved. This is confirmed by the determined LOQ values of the EGCG, (+)-catechin, gallic acid and caffeine, despite the smaller injection volume. In connection with the objectives of the present study, the achieved

sensitivity can be considered completely sufficient. An even more significant contribution of the present work is the ability to qualitatively and quantitatively determine (+)-catechin – an analyte, which is not included in the work of Fernando and Soysa (2016). In addition, the proposed method could be considered suitable for the analysis of other types of green tea in which the content of the four substances is expected.

2.1.2. BAS content

Following the liquid chromatographic analysis of the TE from Bancha green tea, we calculated that 1.0 g of dried Bancha tea leaves contained 6.74 mg EGCG, 0.43 mg (+)-catechin and 6.86 mg caffeine. In addition, the percentage of EGCG, (+)-catechin and caffeine in the prepared aqueous extract was 0.674%, 0.043% and 0.686%, respectively. Therefore, we can assume that a cup of green tea made from 3.0 g of Bancha tea leaves will contain approximately 20.0 mg of EGCG, 1.0 mg of (+)-catechin and 20.0 mg of caffeine. From the analysis of the isolated CF we calculate that 1.0 g of dried Bancha tea leaves contains 6.46 mg EGCG, 0.78 mg (+)-catechin, 0.07 mg gallic acid and 1.03 mg caffeine, which corresponds to a percentage of 0.646%, 0.078%, 0.007 % and 0.103% respectively. The obtained values are lower than the average quantitative content of catechins, including EGCG and caffeine in green tea extracts, which is reported in the literature. On the other hand, our study confirms the results reported by other authors about the lower content of catechins and caffeine in Japanese Bancha green tea. The lower catechin content in Bancha tea leaves probably contributes to the lower antioxidant activity observed by Kodama et al. (2010).

There are several possible reasons for the lower quantitative content of EGCG and caffeine in Bancha tea leaves. The processing of tea leaves (pasteurization, sterilization, etc.) as well as their storage can lead to changes in the structure of tea polyphenols, including catechins, and reduce their quantitative content in the final product. Also, different studies use different methods of extraction of tea leaves (different extractants, different duration of infusion time, different water temperature in the preparation of aqueous extract, etc.), which affects the percentage yield of BAS. It should be borne in mind that in the preparation of an aqueous extract, the use of high temperature water (> 85 ºC) in combination with prolonged infusion time may lead to degradation of some of the catechins contained, including EGCG.

However, the method of extracting BAS from tea leaves using hot water has been used in a number of studies with different types of green tea and is probably not the main reason for the lower catechin content in Bancha green tea. In addition, extracting caffeine from tea leaves is more effective if hot water is used. The pH of the water used in the brewing of tea leaves may also contribute to the degradation of some of the catechins, which are sensitive to high pH values of the solution. As mentioned, catechins, including EGCG, are most stable at acidic pH but unstable at neutral or alkaline pH. At the same time, the pH of the drinking water used for making tea at home is in the range between 6.5 and 8.5, while the pH of the double distilled water used in the present work is ~ 7.0. It was determined by using a pH meter. Thus, there is a good reason to believe that even in the process of preparing a tea drink at home or in a laboratory, it is possible that the structure of some catechins may be changed. In addition, as mentioned, the chopping of tea leaves affects the rate of extraction and the content of BAS, both in the preparation of extracts in the laboratory and in the preparation of tea at home. In our opinion, the period of collecting tea leaves is of major importance for the lower concentrations of catechins and caffeine in Bancha green tea. As mentioned, Bancha green tea is made from leaves that are picked in late summer and/or early autumn when they are more mature and larger. It has been found that the amount of BAS (catechins, caffeine and L-theanine) in tea leaves varies from season to season. Gong et al. (2020) reported that the highest catechin content is found in summer (June), compared to spring (April) and autumn (September). This is thought to be due to the most sunlight hours during the summer months, which play a key role in catechin biosynthesis. In all seasons, the EGCG content is highest compared to other catechins. The amount of L-theanine is highest in the spring, while in terms of the amount of caffeine, there is no significant difference in the different seasons. However, other studies report that the amount of caffeine is higher in younger leaves than in more mature ones. In addition, the geographical location is also reported to have a significant impact on the total catechin content of tea leaves, which highlights the impact of the environment on the amount of BAS formed. Another important factor that determines the differences in the content of BAS is the storage of tea leaves after their collection and processing. It has been observed that when green tea is stored at a low temperature (4 ºC), catechins and caffeine retain their structure for 8 weeks,

and catechins do not undergo significant degradation for a period of 6 months. However, at a storage temperature of about 25 ºC, the amount of catechins decreased significantly, with the amount of EGCG decreasing by 68% after a storage period of 90 days. The optimal storage temperature for green tea seems to be around 4 ºC, but products containing green tea that are available in herbal stores, drugstores and pharmacies are not stored under refrigerated conditions. Therefore, long-term storage of tea leaves at room temperature (about 25 ºC) is associated with a gradual reduction in the amount of BAS. This again raises the question of the effectiveness and safety of the tea products available on the market and raises fears that the consumption of green tea may not lead to the beneficial effects we expect. At the same time, it is possible to use different methods for the preparation of green tea at home, which probably leads to large differences in the quantitative content of catechins in the final products. The lack of standardization of extracts in some of the food supplements available on the market further exacerbates concerns about their efficacy and safety, which are directly related to the qualitative and quantitative composition of the included extract.

In conclusion, it can be said that a number of factors affect the quantitative content of BAS in green tea leaves. Therefore, the composition of the different types of green tea available on the market can vary significantly. Thus, it would be appropriate to test the quantitative content of BAS (total catechin content, caffeine, L-theanine) before packing the tea leaves and to indicate it on the packaging to inform consumers about the composition of the purchased product. The high content of catechins is usually associated with high quality tea, but it is also a reason for the bitter or astringent taste, which not all consumers like. The caffeine content can also influence the choice of a certain type of green tea, especially if the tea drink is consumed in the evening. In this case, it will be appropriate to consume green tea with low caffeine content, such as Japanese Bancha green tea.

2.2. Liquid chromatographic analysis of MF from Bancha green tea

2.2.1. HPLC-UV methods for analysis

For the qualitative and quantitative determination of methylxanthines (caffeine, theophylline and theobromine) in the isolated MF, the sensitive liquid chromatographic method developed earlier was applied. During the development of the method, different mobile phases were tested and it was

found that the best separation of the analytes was observed in an isocratic mode using a mobile phase composed of 90% H_2O / 10% acetonitrile. In the sample preparation process, a hot double distilled water (~ 80 ºC) with alkaline pH was used to prepare standard solutions and test samples from the isolated MF, due to the lower solubility of methylxanthines in cold water. Caffeine is easily soluble in hot water (1:2). The solubility of theophylline and theobromine increases with the use of hot water, but is significantly lower than that of caffeine. These compounds dissolve well in alkaline solutions. Therefore, hot double distilled water with pH 8 is chosen as a solvent, in which all three methylxanthine derivatives are expected to dissolve easily.

Subsequently, MF was analyzed by the liquid chromatographic method for determination of EGCG, (+)-catechin, gallic acid and caffeine. This analysis was performed mainly to determine if the MF contains catechins (EGCG and (+)-catechin), which could compromise the results of the experiments with laboratory animals, as there is an evidence that catechins affect the pharmacokinetics of drugs, substrates of CYP3A4, such as sildenafil.

2.2.2. BAS content

Following the qualitative and quantitative determination of caffeine, theophylline and theobromine in the isolated MF, it was found that caffeine is the dominant methylxanthine in the isolated fraction and represents 87.74% ± 0.83 of its composition. Theobromine was detected in a very low amount (0.095% ± 0.02), while theophylline was not detected, probably because its concentration in the samples was below the LOQ. Based on these results, we calculated that 1.0 g of dried tea leaves contained 8.34 mg of caffeine and 0.009 mg of theobromine, which corresponds to 0.834% caffeine and 0.0009% theobromine. Our results on the content of individual methylxanthines in green tea confirm those reported by other authors according to which caffeine is the main methylxanthine in tea leaves.

In conclusion, the high amount of methylxanthines, in particular caffeine in the isolated MF confirms the efficacy of the applied method for extraction of caffeine, theophylline and theobromine from tea leaves. However, no catechins were detected in the isolated fraction. Therefore, the described extraction procedure achieves a good separation of the two main groups of BAS in green tea - methylxanthines and catechins.

3. Qualitative and quantitative analysis of sildenafil in plasma samples from experimental animals

3.1. Sample preparation

The sample preparation process is very important for the accurate quantification of the compounds contained in the blood sample. The applied extraction method should ensure the highest possible percentage yield of sildenafil citrate. Most of the reviewed literature sources use the methods of liquid-liquid extraction or solid-phase extraction to extract the test substance from blood plasma. These methods are more complex and time consuming. Therefore, a protocol for the extraction of sildenafil citrate from plasma samples based on the protein precipitation method has been developed in the present work. Although it does not surpass solid-phase extraction in terms of purification capacity, protein precipitation is highly efficient, easy to perform and does not require the use of specific laboratory consumables. In support of the approach used is the lack of isolated components in the analyzed samples. Methanol was chosen as a precipitating agent because it does not react with sildenafil citrate and is relatively safe to work with. Thus, it was obtained a percentage yield of sildenafil citrate exceeding 93.0%.

3.2. HPLC analysis

In order to develop and optimize a liquid chromatographic method for the analysis of sildenafil, a thorough review of the literature on the applied chromatographic methods for qualitative and quantitative determination of the target analyte in biological samples was performed. In connection with the optimization of the developed method, different chromatographic conditions (flow rate, mobile phase composition, wavelength, etc.) were tested. Under the conditions of the present experiment, the most pronounced chromatographic peak of sildenafil citrate was observed at a wavelength of 228 nm, a mobile phase from methanol / double distilled water (85: 15%) and a flow rate of 0.6 mL/min. The selected column temperature was 25 ºC, as with most reported methods of analysis of sildenafil. In addition, a relatively short analysis time (10.0 minutes) was achieved. The developed method is highly sensitive, which is confirmed by the LOQ of sildenafil (5.0 ng/mL). Achieving high sensitivity of the method is essential considering the low plasma concentration of sildenafil in male rats.

Based on the obtained results, we believe that the presented procedures for sample preparation and analysis are effective and easy to perform. Thus, they can be successfully implemented in the routine analysis of biological samples containing sildenafil.

4. Pharmacokinetic studies in experimental animals

Nowadays, many herbs, beverages and supplements are used daily in physiological doses for the prevention and/or treatment of diseases. Some of them have been shown to inhibit or induce the activity of CYP450 enzymes *in vitro* and *in vivo*. Therefore, clinically significant interactions may occur if co-administered with certain drugs. As mentioned, biotransformation of CYP3A4 drugs is the major metabolic pathway for more than half of the prescribed drugs. Therefore, drug substrates of this enzyme are expected to have the highest risk of drug interactions when taken concomitantly with other drugs, foods, beverages or phytopreparations.

Green tea and coffee are some of the most popular and daily consumed beverages worldwide. However, there is an evidence that both beverages may affect the pharmacokinetics of some co-administered drugs. Both green tea and coffee contain a variety of bioactive compounds, including methylxanthines, mainly caffeine, which has certain pharmacological effects. In addition to caffeine, green tea also contains polyphenols, of which catechins are considered the main carriers of its biological activity. In addition, green tea extract, as well as the containing EGCG and caffeine are also available in the form of dietary supplements.

Caffeine is mainly metabolised by the enzyme CYP1A2 in humans and its concomitant use with CYP1A2 substrates may lead to an inhibition of their biotransformation and an increased risk of adverse reactions. Caffeine has also been reported to be a substrate for CYP3A4, but the available information on potential interactions between caffeine and drugs that are primarily metabolised by CYP3A4 is very limited. In a previous study, we observed that MF isolated from Bancha green tea showed strong inhibition of CYP3A4 activity *in vitro*, while pure caffeine also inhibited the enzyme activity, but to a lesser extent. A special Vivid® CYP3A4 Green Screening Kit, based on a recombinant human isoenzyme, was used to evaluate the *in vitro* potential of Bancha green tea methylxanthines and pure caffeine to inhibit CYP3A4 activity. Different concentrations of MF and caffeine in the

range of 10.0 mg/mL to 0.3125 mg/mL obtained by serial dilution were used. HPLC-UV analysis found that the concentrations of MF used contained 8.8 mg/mL to 0.275 mg/mL caffeine, respectively. Ketoconazole (10.0 μM) was used as a positive control. As a result of the study, we observed that MF at a concentration of 10.0 mg/mL inhibited CYP3A4 enzyme activity by 72%, while pure caffeine showed less inhibition (approximately 60%). Moreover, the percentage inhibition of enzyme activity from MF is close to that observed with ketoconazole. Considering the obtained results, in our opinion, the observed inhibitory effect is not only due to the caffeine, but probably to other components contained in the MF, and there is a possibility for mutually potentiation of the effects of some compounds. In addition, the inhibitory effect of MF on CYP2C9 enzyme activity was investigated *in vitro*, but little inhibitory activity was observed. Kovacić et al. (2009) also reported that caffeine inhibited CYP3A4. However, Dresser et al (2017) reported that coffee bean extracts with varying degrees of roasting inhibited CYP3A4 activity *in vitro*, but *in vivo* only one of the participants in the experiment showed a slight increase in C_{max} and AUC of felodipine used as substrate for CYP3A4. Therefore, the authors concluded that coffee did not significantly affect the pharmacokinetics of felodipine in humans, despite the small number of participants in the experiment. They also do not discuss the components that are responsible for the observed effects. This necessitates further studies of the potential interactions between methylxanthines and caffeine with CYP3A4 substrates, considering the results of reported *in vitro* studies.

A number of studies have been performed to determine the effect of green tea extract and/or EGCG on the activity of drug-metabolizing enzymes and transmembrane transporters. Most *in vitro* studies have reported inhibition of drug-metabolizing enzymes and some transporter proteins. However, the observed results in experimental animals and humans are contradictory, so further research is needed in this direction. In order to more accurately predict the potential interactions between green tea and/or its main components with co-administered medications, more *in vivo* studies are needed in both experimental animals and humans.

The PDE5 inhibitor sildenafil was used in the present work as a drug whose pharmacokinetics to be monitored and analyzed in experimental animals. The choice was made due to the fact that sildenafil is a drug which

poses a relatively high risk of participating in drug interactions, because of its significant biotransformation by cytochromes and especially CYP3A4. It has also been suggested that it may be a substrate for transmembrane transporters. In addition, sildenafil is often used without consulting a doctor, probably because patients feel uncomfortable to discuss sexual issues with their doctors. This may increase the risk for co-administration of sildenafil with other drugs and/or herbal preparations and further increases the risk of interactions. However, drug interactions are more predictable because their pharmacokinetics and pharmacodynamics are studied in the process of drug development and the potential to participate in drug interactions as a victim or a perpetrator is assessed. However, there are relatively few studies in the literature that investigate and evaluate the potential interactions between sildenafil and foods, beverages and food supplements containing one or more plant extracts, so more research is needed in this regard, in connection with the widespread use of sildenafil and its pharmacokinetic profile.

Based on the results reported by *in vitro* and *in vivo* studies on the effect of green tea and its main components on the pharmacokinetics of other drugs, as well as the observed inhibitory effect of MF on CYP3A4 activity, we suggest that a pharmacokinetic interaction may occur when taking sildenafil with TE, CF or MF from Bancha green tea. On the other hand, sildenafil is a selective inhibitor of PDE5 and caffeine is a non-selective inhibitor of phosphodiesterases. However, caffeine has been shown to improve erectile function in diabetic rats, possibly due to phosphodiesterase inhibition. Lopez et al (2015) reported that caffeine intake with 2-3 cups of coffee (170-375 mg/day) reduces the risk of erectile dysfunction in men, including those with weight gain, obesity or hypertension, but not in men with diabetes. Therefore, caffeine could potentiate the effect of other drugs used in the treatment of erectile dysfunction, although epidemiological studies have shown conflicting results. In addition, Chen et al. (2017) reported that EGCG dose-dependently improved erectile function in adult rats by regulating PRMT1/ DDAH/ ADMA/ NOS metabolic pathways, including influence on the NO/ cGMP signaling pathway, resulting in increased NO production and cGMP levels in corpus cavernosum. In another study conducted by Rai et al (2020), a significant improvement in sexual and behavioral parameters was observed in male rats after administration of (+)-catechin. According to the authors, the effect may be due to the established

in silico agonism of D_2-receptors and inhibition of PDE5. Therefore, there is a good reason to believe that co-administration of sildenafil with green tea, green tea extracts, EGCG and caffeine is likely to result in both pharmacokinetic and pharmacodynamic interactions.

It was found one study in the literature that investigated the effect of aqueous green tea extract on the pharmacokinetics of sildenafil in healthy male volunteers. Although various sources reported that an interaction has been observed between green tea and sildenafil, it appears that they all cite the same study. Hegazy et al. (2014) conducted a study in healthy men, which they divided into two groups. The volunteers in the first group took a single dose of 250.0 mL of water, while in the other group men received 250.0 mL of freshly prepared green tea extract (prepared from 2.0 g of dried green tea leaves), after which all participants took one tablet of sildenafil (50.0 mg) and one tablet of midazolam (7.5 mg) orally. An increase in sildenafil $AUC_{0\text{-}inf}$ and C_{max} of about 30% was observed when administered after green tea. Also, green tea prolongs the elimination half life of sildenafil by about 36%. According to the authors, the increased bioavailability of sildenafil after green tea intake may be due to catechins, which may alter the spontaneous activity of the small intestine or affect the activity of transmembrane transporters. The green tea used is in a package (2.0 g) and according to the manufacturer contains 3.0 g catechins/100.0 g green tea. Therefore, the prepared green tea extract received by the study participants contained no more than 60.0 mg of catechins. However, there is no information about the preparation of the extract itself (water temperature, duration of infusion, etc.), as well as conducting qualitative and quantitative analysis of the prepared extract. In addition, in that study, sildenafil was taken at the same time with midazolam as a test drug for CYP3A4 activity. However, it should be borne in mind that midazolam may have influenced the observed results and may compensate to some extent for the change in sildenafil plasma levels following green tea extract administration, as both drugs are primarily metabolised by CYP3A4. These concerns are shared by other authors.

For this reason, our experiments aimed to evaluate the effect of TE, CF and MF from Bancha green tea on the pharmacokinetics of sildenafil administered alone without a probe drug for CYP3A4 activity. In the first experiment, the isolated TE, CF and MF from Bancha were administered repeatedly before taking sildenafil while in the second experiment, only MF

was administered once. In both experiments, changes in the pharmacokinetic parameters of sildenafil were observed when co-administered with ketoconazole, a well-known inhibitor of the CYP3A4 activity.

4.1. Preparation of solutions/suspensions for use in animals in both experiments

The choice for the preparation of a solution or suspension depends on the water solubility of drugs and herbal substances. Sildenafil is slightly soluble in water, which is why its citrate salt, sildenafil citrate, is included in the dosage forms. The latter has a better water solubility (4.1 mg/mL). In experimental animals, sildenafil is administered as a solution and suspension, which are prepared after dissolving/suspending sildenafil citrate in 1% aqueous sodium carboxymethylcellulose solution. The reconstituted solution and suspension had a final concentration of sildenafil of 0.625 mg/mL and 15.0 mg/mL, respectively. In the first case, the citrate salt of sildenafil will dissolve well in water, but in the second it will not dissolve completely and a suspension will form. Due to the rapid precipitation of drug particles in the aqueous suspension and the risk of administering different doses of sildenafil to experimental animals, we decided to prepare and use a 1.0% aqueous solution of sodium carboxymethylcellulose. This compound is a high molecular weight cellulosic polymer that dissolves in water to form viscous colloidal solutions. Aqueous solutions of sodium carboxymethylcellulose with a concentration of 0.5% to 2.0% have been used for the suspension of medicinal and herbal substances in a number of studies due to the increased viscosity of the solution and the significantly slower precipitation of particles. At a concentration of 0.1% to 1.0%, sodium carboxymethylcellulose is used mainly as a viscosity enhancer for oral or topical formulations in which medicinal substances are suspended. For this purpose, we prepared aqueous solutions with different concentrations of sodium carboxymethylcellulose (0.5%, 1.0% and 2.0%), of which we chose the 1.0% solution, due to achieving optimal viscosity, allowing application by orogastric feeding tube and at the same time slow precipitation of suspended solids. In order to perform the experiments under the same conditions, 1.0% aqueous sodium carboxymethylcellulose solution was used as a carrier for sildenafil in both experiments.

The antifungal agent ketoconazole has a very low water solubility, so it is also administered to experimental animals as a suspension in 1% aqueous sodium carboxymethylcellulose solution. This method of oral administration of ketoconazole to rats has been reported by other authors.

The isolated TE, CF and MF dissolve well in water, so they are used as aqueous solutions. The total extract and CF were dissolved in lukewarm water and then homogenized on a vortex mixer to dissolve completely. This avoids the use of hot water, which which increased the risk for degradation of some of the catechins. MF was dissolved in hot double distilled water due to the lower solubility of caffeine and other methylxanthines in cold water.

4.2. Influence of repeated administration of TE, CF and MF on the pharmacokinetics of sildenafil

In this study, sildenafil was administered to male rats after pretreatment of rats with TE, CF and MF isolated from Bancha twice daily for one week. The application of herbal substances is carried out in the morning and in the afternoon, in order to mimic the daily intake of green tea. The choice of twice daily use of plant extracts was made on the basis of data from the review of systematic reviews and meta-analyzes on the beneficial effects associated with green tea consumption, according to which the greatest reduction in the risk of a number of health problems (overall mortality, cardiac death, coronary heart disease, stroke and type 2 diabetes mellitus) is observed when taking two to three cups of green tea daily. The calculated pharmacokinetic parameters of sildenafil after combined administration were compared with those after single administration and after co-administration with ketoconazole.

4.2.1. Determination of the doses used in the present experiment

The dose of sildenafil (2.5 mg/kg) was selected after reviewing the literature as well as using the HED calculation formula, according to which the dose of 2.5 mg/kg sildenafil in rats was equivalent to a single dose of approximately 27.0 mg in humans. This dose is very close to the lowest dose of sildenafil in humans (25.0 mg) and is recommended as a starting dose, especially if the patient is taking other medicines that have been reported or are expected to interact with sildenafil or substrates of CYP3A4 (CYP3A4 inhibitors; alpha-blockers).

The total extract was administered as a single dose of 100.0 mg/kg, which provides EGCG and caffeine in doses of 4.12 mg/kg and 4.19 mg/kg, respectively. The dose of the TE administered is consistent with the doses used in other pharmacokinetic studies. The dose of CF (30.0 mg/kg) was selected to be equivalent to a dose of 5.0 mg/kg EGCG, the major compound in this fraction, and the most active catechin. It should be noted that the reported in the literature doses of green tea extract and EGCG, which are used in different studies, vary widely. In addition, after calculating the EGCG and caffeine quantity that animals receive through the TE, it became clear that the dose we chose to administer to in rats provided such amounts of EGCG and caffeine that a person could take with a cup of tea (approximately 45 mg EGCG and 46 mg caffeine). Accordingly, with the intake of CF experimental animals received 5.0 mg/kg EGCG and 0.8 mg/kg caffeine, which is equivalent to a dose of approximately 54.0 mg EGCG and 8.7 mg caffeine in humans.

The single dose of caffeine that rats received after intake of 5.7 mg/kg of MF was equivalent to a HED of approximately 54.0 mg of caffeine (for a 70 kg person). This amount of caffeine can be obtained after drinking a cup of tea.

The dose of ketoconazole (10.0 mg/kg) was selected following a literature study on the use of the drug in pharmacokinetic studies in rats.

4.2.2. Changes in the pharmacokinetics of sildenafil after repeated administration of TE and CF from Bancha green tea

In the present experiment, a relatively low plasma concentration of sildenafil (C_{max} = 15.96 ± 2.45 ng/mL) was observed after administration of 2.5 mg/kg to rats. However, similar C_{max} values have also been reported by other authors following the administration of sildenafil in male rats due to significantly poorer absorption. Administration of sildenafil after pretreatment of the animals with TE and CF from Bancha green tea leads to a statistically significant increase in C_{max}, AUC_{0-3} and AUC_{0-inf} and a decrease in the volume of distribution and clearance of sildenafil, as the values of these pharmacokinetic parameters are close to those after co-administration of sildenafil with ketoconazole. Elevated plasma concentrations of sildenafil, concomitant with no change in the time to peak plasma concentrations of the drug when co-administered with TE and CF (T_{max} = 0.5 h), suggest a possible

increase in the extent of absorption of sildenafil but not in the rate of absorption. Therefore, no delay in the onset of action of sildenafil is expected after repeated intake of Bancha green tea. Also, no significant change in the plasma half life of sildenafil was observed after taking TE and CF from Bancha, therefore we can assume that the observed effects are not due to effect on sildenafil excretion. The increased bioavailability of sildenafil may be explained by an increase in its absorption and/or inhibition of its metabolism due to inhibition of the activity of CYP3A4 in the intestinal epithelial cells, which is responsible for the presystemic metabolism of sildenafil at this level. Inhibition of CYP2C9 may also be achieved, contributing to the observed results, but this is not expected to be the main reason. In addition, mainly CYP3A isoforms mediate presystemic drug metabolism in intestinal and liver cells after oral administration, and as mentioned earlier, sildenafil undergoes significant first-pass metabolism, which is the reason for its low oral bioavailability. Considering the large number of studies that reported inhibition of drug-metabolizing enzymes by catechins, we can assume that the observed effects are due to these compounds and in particular to EGCG, which dominates in TE and CF. According to some authors, green tea catechins are more likely to inhibit drug-metabolizing enzymes in intestinal cells than in hepatocytes due to their poor absorption and low oral bioavailability. Catechins plasma concentrations achieved after oral administration are significantly lower than those used in a number of *in vitro* studies, which supports the theory that their intestinal concentrations are significantly higher and this is probably the site where they affect cytochrome activity. Plasma concentrations of EGCG are proportional to the administered dose, but show high interindividual variability in studies in healthy volunteers. In addition, studies examining the daily administration of EGCG or green tea extract for up to two weeks showed almost no accumulation of catechins in the blood plasma. Therefore, we can conclude that the inhibition of CYP3A4 in intestinal cells by catechins reduces the presystemic metabolism of sildenafil in the intestinal wall and explains its increased bioavailability after taking TE or KF from Bancha green tea. At the same time, according to Ikarashi et al. (2017) oral food containing 1.5% EGCG resulted in a decreased expression of CYP3A enzymes in the liver of mice. The authors of the study suggested that the effect of EGCG on cytochromes was indirect, as no significant change in

CYP3A expression in hepatocytes was observed with peritoneal administration of EGCG (20.0 mg/kg). The authors explain the observed effects with the suppressive effect of EGCG on the growth of bacteria of the genus *Clostridium*, part of the normal bacterial flora involved in the production of secondary bile acids (lithocholic acid, deoxycholic acid). Very low concentrations of lithocholic and deoxycholic acid in the colon of mice were observed after intake of oral EGCG. These secondary bile acids are ligands for the PXR receptor, which is involved in regulating the expression of cytochromes and P-gp. The authors hypothesize that reduced concentrations of lithocholic and deoxycholic acid in the gut will lead to lower concentrations of these bile acids in the liver, resulting in suppression of nuclear translocation of the PXR receptor and reduced expression of CYP3A enzymes in the liver.

Catechins have also been reported to inhibit the activity of some transmembrane transporters such as OATP1A2, OATP1B1, OATP1B3, OATP2B1, OCT1, OCT2, MATE1, MATE2-K and P-gp, which may affect drug absorption, distribution and/or excretion. However, there is an evidence that sildenafil is a substrate of P-gp and efflux transport mediated by this protein is likely to contribute to the low oral bioavailability of the drug. Therefore, the increased oral bioavailability of sildenafil after ingestion of TE and CF from Bancha green tea may be due to inhibition of P-gp activity in intestinal cells, resulting in decreased intestinal efflux of sildenafil and increased extent of absorption. However, due to the significantly reduced clearance of sildenafil when co-administered with TE and CF, we believe that the increase in the extent of absorption of sildenafil is not the main reason for the observed increase in plasma levels of the drug.

Our results are in line with those reported by Hegazy et al. (2014). However, the results cannot be compared, as the studies were conducted in different species, with different dosage regimens of green tea, as well as intake of green tea with different BAS content. Also, the results are consistent with those published by other authors about the effect of green tea extract and/or EGCG on the pharmacokinetics of drugs, substrates of CYP3A4 in rats. Misaka et al. (2013) reported that green tea extract (400.0 mg/kg) increased simvastatin $AUC_{0\text{-}6}$ and C_{max} in rats, possibly due to suppression of the intestinal metabolism by CYP3A4 and intestinal efflux by P-gp. Several studies have shown an increase in the bioavailability of calcium channel

blockers and tamoxifen following EGCG in rats, suggesting inhibition of the intestinal metabolism of drugs by CYP3A4 and/or intestinal efflux by P-gp. In addition, in the cited studies monitoring the pharmacokinetics of verapamil, diltiazem and nicardipine in rats, the EGCG doses administered to rats were in the range from 1.0 to 10.0 mg/kg and were close to the EGCG dose received by the animals with the intake of TE and CF in the present work.

The effect of repeated use of green tea on the pharmacokinetics of simvastatin was also studied in two studies in healthy volunteers, resulting in an increase in AUC_{0-6} of the simvastatin active metabolite (simvastatinic acid) but a slight increase in simvastatin AUC_{0-6}. However, large variations in the pharmacokinetics of simvastatin were observed in individuals in both studies. The authors assumed an inhibition of CYP3A4-mediated intestinal metabolism of simvastatin and/or intestinal efflux by P-gp. Moreover, a twofold increase in tacrolimus plasma concentrations has been reported following green tea intake in a kidney transplant patient, but the authors do not specify the amount of green tea administered or its composition. At the same time, Abdelkawy et al (2020) reported that green tea extract reduced the bioavailability of atorvastatin, a drug substrate of CYP3A4, in humans. The probable cause, according to the authors, is inhibition of the OATP1A2 transporter in the GIT and decreased intestinal absorption of atorvastatin. A number of studies have reported a decrease in the oral bioavailability of certain drugs (nadolol, rosuvastatin, imatinib, digoxin, etc.) after taking green tea extract and/or EGCG, and indicate that the most likely cause is inhibition of SLC or ABC transmembrane proteins involved in drug absorption. On the other hand, concomitant use of the tyrosine kinase inhibitor sunitinib, a substrate of CYP3A4 with EGCG resulted in a decrease in the plasma concentration of the antitumor agent. The authors of the study suggest that a complex is formed between the two compounds, resulting in a decrease in the extent of absorption of sunitinib.

In conclusion, further research is needed to clarify the mechanism responsible for the observed interactions. Based on the current knowledge of the reported inhibition of CYP3A4 and P-gp activity by catechins, as well as the involvement of these proteins in sildenafil metabolism and intestinal efflux, respectively, we hypothesize that the most likely cause of the elevated plasma concentrations of sildenafil in rats was inhibition of CYP3A4 activity

in intestinal cells and/or intestinal efflux by P-gp. Moreover, the content of EGCG, considered to be the most active catechin, in TE and CF is very close, and the observed changes in the values of the pharmacokinetic parameters of sildenafil in both groups are also close.

4.2.3. Changes in the pharmacokinetics of sildenafil after repeated administration of MF from Bancha green tea

The isolated MF contains mainly caffeine, which is why we assume that it is the main active substance responsible for the observed effects. Daily administration of caffeinated beverages achieves higher plasma concentrations of caffeine than a single intake and the accumulation of its major pharmacologically active metabolite (paraxanthine), which is likely to contribute to the pharmacological effects of caffeine and the development of caffeine tolerance and withdrawal syndrome.

The methylxanthine fraction administered repeatedly before sildenafil resulted in an increase in sildenafil C_{max}, AUC_{0-3} and AUC_{0-inf} and decreased Vz/F and Cl/F compared to the control group. The pharmacokinetic parameters of sildenafil in the MF group were close to those observed with ketoconazole. The observed results can be explained by an inhibition of CYP450 enzymes involved in sildenafil metabolism and are consistent with the observed *in vitro* inhibition of CYP3A4 by MF isolated from Bancha green tea. The biotransformation of sildenafil in male rats was mediated mainly by CYP3A4 and CYP2C11. However, the enzymes CYP1A2, CYP2C11 and CYP3A4 have been reported to be involved in caffeine metabolism in rats. In addition, caffeine undergoes similar metabolic processes in humans and rodents, but there are some quantitative differences in the metabolites that are produced. In humans, caffeine undergoes mainly N-demethylation to paraxanthine, theobromine and theophylline, with a very small portion of the dose undergoing C-8 hydroxylation (approximately 6%). In rodents, about 40% of the caffeine ingested undergoes C-8 hydroxylation to trimethyl derivatives, and the main products of N-demethylation are theophylline and theobromine. Due to the involvement of the same drug-metabolising enzymes in the metabolism of sildenafil and caffeine, a major component in the administered MF, it is possible that both drugs compete for the same enzyme (CYP3A4 and/or CYP2C11), which may lead to suppression of sildenafil biotransformation. There is an evidence in the

literature that green tea extract as well as catechins inhibit the activity of CYP3A4, CYP2D6, P-gp, organic anionic transporters and others, but very little information is available about the effects of methylxanthines on these enzymes and transporters. One study reported that decaffeinated green tea extract was unlikely to affect the pharmacokinetics of drug substrates of CYP3A4 and CYP2D6, supporting the hypothesis that caffeine may modulate CYP3A4 activity and contribute to reported inhibition of cytochromes by green tea extract.

Another possible explanation for the increased plasma levels of sildenafil is the inhibition of transmembrane transporters involved in its absorption. As mentioned, there is evidence that sildenafil is a substrate of P-gp. However, caffeine is not a substrate of P-gp, so it is not expected to interact with it and to inhibit its activity. In addition, increasing the rate of absorption of sildenafil may also lead to increased oral bioavailability, but considering the observed changes in clearance, we do not assume this to be the main reason for the observed effects.

Methylxanthines also lead to an increase in the T_{max} of sildenafil, which is probably related to a decrease in the rate of absorption of sildenafil. The results are consistent with data published by Halim et al (2013), who developed orally disintegrating tablets containing sildenafil citrate to achieve higher bioavailability and to reduce the T_{max} of sildenafil. After selecting the appropriate composition of the dosage form, the authors add caffeine, which aims to avoid lowering blood pressure after taking sildenafil. They examined the pharmacokinetics of manufactured disintegrating tablets containing sildenafil or sildenafil plus caffeine after a single dose in six healthy volunteers, comparing the calculated pharmacokinetic parameters of sildenafil with those after taking the commercial oral product Viagra®. From the published results it is noteworthy that after taking a formulation containing sildenafil plus caffeine, a slightly higher value of T_{max} (0.88 ± 0.12) was reported compared to that calculated after taking a tablet containing only sildenafil citrate (0.63 ± 0.10). The prolongation of T_{max} could be explained by the formation of a complex between sildenafil and caffeine or another component contained in the isolated MF, as well as by delaying gastric emptying, which usually leads to delayed absorption of drugs. Caffeine can reduce the absorption of drugs by forming a complex mainly with drugs that have acidic properties through forming hydrogen bonds or

dipole-dipole interactions. However, in the present experiment, the likelihood of complex formation is low, considering the observed changes in the other pharmacokinetic parameters of sildenafil (C_{max}, AUC, Cl/F). Regarding gastric emptying, there is evidence that coffee accelerates gastric emptying and may increase the rate of drug absorption. In a clinical study, caffeine was found to increase the bioavailability of felodipine, with the authors suggesting that this may be due to an increase in gastric emptying rate. At the same time, Gounaris et al. (2020) observed that caffeine significantly delayed gastric emptying time in newborns. Delayed gastric emptying by caffeine was also observed in newborn rats. There are a number of studies investigating the effect of coffee on GIT, but information on the effect of caffeine on gastric emptying is limited. Therefore, the change in gastric emptying rate by caffeine cannot be ruled out as a possible reason for the prolongation of sildenafil T_{max} in the experiment.

In conclusion, based on the calculated pharmacokinetic parameters of sildenafil after co-administration with MF isolated from Bancha green tea in rats, we believe that the most likely cause of elevated plasma levels of the drug is modulation of enzymes involved in sildenafil biotransformation.

4.3. Influence of a single dose of MF from Bancha green tea on the pharmacokinetics of sildenafil

During the experiment, the animals received single doses of the administered substances at 30-minute intervals. The experiment was performed to evaluate the effect of a single dose of methylxanthines isolated from Bancha on the pharmacokinetic parameters of sildenafil. Ketoconazole was used as a positive control again.

4.3.1. Determination of the doses used in the present experiment

In this experiment, sildenafil was administered at a higher dose due to significantly lower oral bioavailability, shorter plasma half life and higher metabolic clearance in male rats than in humans. Oral bioavailability in male rats is ~ 15%, while in humans it is about 40%. The dose of 60.0 mg/kg was the maximum safe dose of sildenafil in rats determined in toxicological studies. This dose is preferred in order to achieve higher plasma concentrations that can be detected and monitored, considering the relatively low plasma levels of sildenafil that were detected in the previous experiment.

Ketoconazole and the MF were administered as single doses of 10.0 mg/kg and 5.7 mg/kg b.w., respectively, similar to the previous experiment.

4.3.2. Changes in the pharmacokinetics of sildenafil following single administration of MF from Bancha green tea

The pharmacokinetic parameters of sildenafil after co-administration with methylxanthines were compared with those observed after sildenafil administration alone and after co-administration with ketoconazole. A single dose of MF resulted in a slight decrease in sildenafil C_{max} and a statistically significant decrease in AUC_{0-6} and AUC_{0-inf}, compared to control group. Co-administration of ketoconazole resulted in a statistically significant increase in sildenafil C_{max}, AUC_{0-6} and AUC_{0-inf}, as expected and previously reported. The observed decrease in sildenafil plasma levels may be due to several reasons. It is possible that the extent of absorption of sildenafil is reduced by MF and in particular by the caffeine content. Caffeine stimulates the secretion of gastric juice with high acidity and lowers gastric pH, which may affect the absorption of weak base drugs such as midazolam. At the same time, sildenafil is administered in the form of sildenafil citrate, which is a weak base and its absorption may be reduced by lowering gastric pH, similar to midazolam. On the other hand, as mentioned, the solubility of sildenafil citrate in GIT is pH dependent and may therefore vary due to changes in gastric acidity. Decreased AUC and C_{max}, together with acceleration of sildenafil clearance, indicate a possible effect of MF on the elimination process of sildenafil. A possible reason for the lower oral bioavailability of the drug is enzyme induction. Caffeine has been reported to induce the expression of CYP1A2 and sulfotransferases in rats. On the other hand, there are no available data about the impact of these enzymes on sildenafil metabolism. Therefore, there is no reason to believe that the observed results are due to induction of drug-metabolizing enzymes. Furthermore, as mentioned, the induction of drug-metabolizing enzymes is a process that usually takes place after prolonged use of the inducer, as opposed to inhibition. On the other hand, sildenafil is a drug that is metabolised almost entirely by the liver. Therefore, the decreased plasma concentration of sildenafil may be explained by the fact that caffeine increases heart rate and blood pressure and may also increase cardiac output, leading to increased blood flow to the liver, which may result in an accelerated drug

biotransformation. Sildenafil is a drug with an intermediate to high extraction ratio, therefore its clearance will increase with increasing in hepatic blood flow and therefore its plasma concentrations will decrease. Our results are consistent with those reported by Abdulla et al. (2018), who investigated the effect of a single intake of Red Bull® energy drink on the pharmacokinetics of sildenafil in rats. The authors observed a significant reduction in plasma levels of sildenafil when co-administered with Red Bull®. One of the main ingredients in this energy drink are caffeine and taurine. According to the authors, the interaction may occur at any pharmacokinetic level, but is likely to be due to accelerated metabolism of sildenafil by taurine, which has been shown to induce CYP3A4 and/or caffeine activity, which increases hepatic blood flow. However, the hypothesis of induction of cytochromes from taurine contradicts the available information on the time required to increase the expression of drug-metabolizing enzymes, as in the study the energy drink was taken only twice – in the day before the study and in the day of the study, concomitantly with sildenafil. In addition, caffeine increases glomerular pressure and may lead to increased renal excretion of drugs.

A possible limitation of the experiments may be the variability in drug pharmacokinetics between humans and rats. Despite potential species differences, rats were reported to be an appropriate model for studying the pharmacokinetics of sildenafil, whose biotransformation is mainly mediated by CYP3A and CYP2C isoforms in humans and rats. The isoforms of these enzymes are very similar in both species and show more than 70% homology. In addition, the major metabolite of sildenafil is the same in humans and rats. Therefore, the obtained results should be taken into account when considering co-administration of sildenafil with green tea, coffee, herbal preparations containing green tea extract, EGCG or caffeine.

In summary, interactions were observed when sildenafil was administered after long-term use of TE, CF and MF from Bancha green tea, in rats as well as when sildenafil was administered after a single dose of MF. In our opinion, the observed changes in the pharmacokinetic parameters of sildenafil are mainly due to the action of EGCG contained in TE and CF and caffeine, which is the main component in MF. The possibility that caffeine may contribute to the effects observed after intake of TE and CF, as well as other components contained in TE, CF and MF, cannot be ruled out. In addition, the observed changes in sildenafil C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$

indicate that the duration of methylxanthine intake, and in particular caffeine, may be essential for drug pharmacokinetics. However, further *in vivo* studies are needed to elucidate the mechanism underlying the observed interactions and to assess the risk of these interactions in humans. In addition, our experiments followed a change in the pharmacokinetics of a single dose of sildenafil, and it should be borne in mind that sildenafil is sometimes dosed daily for an extended period of time. Therefore, the potential for interactions should be considered when both sildenafil and green tea/coffee/green tea extract are taken repeatedly. In conclusion, based on current knowledge, it is recommended to avoid the simultaneous use of sildenafil with beverages such as green tea and coffee, as well as with phytopreparations that contain large amounts of green tea extract, EGCG and caffeine.

5. Development of PBPK models

The development of PBPK models for plant extracts is extremely difficult and poses a huge challenge for researchers. The reasons are that these extracts contain a very large set of BAS (sometimes more than 200), which may interact with each other and/or with other co-administered drugs, both pharmacokinetically and pharmacodynamically. The idea to take up this challenge was to assess the risk of potential interactions between sildenafil and green tea extracts in humans. In the presented simulations, high doses of CF and MF were used, which can be obtained by taking food supplements containing catechins and methylxanthines, respectively, but not with a cup of tea. The use of catechins and caffeine in the form of phytopreparations is widespread due to their various beneficial effects, which were discussed above. The simulations were performed in a virtual population of healthy male volunteers aged 60 to 65 years, because the use of sildenafil is more common among men of this age in connection with its main indication - erectile dysfunction. The performed simulations used mechanistic-static and mechanistic-dynamic minimum PBPK models, which are built into the Simcyp® software.

A consensus adopted in 2001 determined the assignment of drug interactions to three groups, according to the ratio between the AUC values of the drug (AUCR) in the presence and absence of inhibitor:

- AUCR > 2 – high risk of drug interactions;
- 1.1 < AUCR < 2 – moderate risk of drug interactions;

❖ AUCR < 1.1 – low risk of drug interactions.

Based on this consensus, the results of mechanistic-static simulations when sildenafil is taken with CF and with combinations of CF and MF show a moderate risk of drug interactions. The reasons for this are that in static models the worst case scenario is taken into account - the highest achievable values of plasma concentrations are taken in the simulated interactions, without taking into account the dynamic changes in plasma concentrations. Therefore, there is an overestimation of the possible risk of drug interactions. However, it should be borne in mind that many assumptions have been made in the construction of these models and the pharmacokinetic parameters of EGCG and caffeine have been used as the main representatives of CF and MF, respectively. However, as we know, these fractions also contain other components that could also contribute to interactions, but in this case are not taken into account.

The establishment of a moderate risk (using a mechanistic-static model) for the occurrence of an interaction when sildenafil is co-administered with CF or with a combination of CF and MF through simulations, together with the results of animal studies, require their use with caution in patients as well as careful monitoring to avoid serious side effects. Conducting additional *in vivo* studies will help to clarify the mechanisms responsible for the observed interactions.

VI. CONCLUSIONS

1. Isolation of TE, CF and MF from Japanese Bancha green tea

- A satisfactory percentage yield was achieved through the applied methods for extraction of TE, CF and MF, considering the type of tea leaves used and the reported lower content of BAS.

2. Qualitative and quantitative determination of the isolated TE, CF and MF of Japanese Bancha green tea

- The isolated TE contains less EGCG and caffeine than other types of green tea.
- The developed method for determination of EGCG, (+)-catechin, gallic acid and caffeine could be considered suitable for analysis of other types of green tea, which are expected to contain the four substances.
- The applied methods for extraction of CF and MF achieve a higher percentage of catechins and methylxanthines in the obtained fractions, respectively, compared to the total aqueous extract of Bancha green tea.

3. Qualitative and quantitative determination of sildenafil in biological samples

- The developed liquid chromatographic method for analysis of sildenafil is characterized by high analytical reliability and sensitivity.
- The protein precipitation method is suitable for extraction of sildenafil from plasma samples, extracting more than 93.0% of the drug contained.
- The presented HPLC-UV method as well as the protein precipitation method can be used in routine practice for qualitative and quantitative determination of sildenafil in plasma samples.

4. Pharmacokinetic studies in experimental animals

- Bancha green tea extract has a significant effect on the pharmacokinetics of sildenafil in rats, despite the lower content of catechins and caffeine compared to other types of green tea.

- ❖ Increased plasma concentrations of sildenafil in rats have been observed after repeated administration of TE or CF from Bancha green tea.
- ❖ Depending on the duration of administration of MF isolated from Bancha green tea, various changes in the pharmacokinetic parameters of sildenafil were observed in rats - a single dose of MF decreased plasma concentrations of the drug while an increase in plasma levels was observed after repeated administration of MF.

5. Development of PBPK models

- ❖ When sildenafil is co-administered with green tea extract, EGCG or caffeine, there is a certain risk of pharmacokinetic interactions in humans. Further studies are needed to evaluate the potential interactions between sildenafil and green tea in humans, and to clarify the mechanisms responsible for their occurrence.
- ❖ Based on the current knowledge, it is recommended that concomitant use of green tea extract, EGCG or caffeine with sildenafil and other CYP3A4 substrates to be performed after consultation and monitoring of patients for potential interactions.

VII. CONTRIBUTIONS

ORIGINAL CONTRIBUTIONS:

- ❖ TE, CF and MF from Japanese Bancha green tea have been isolated and analyzed for the first time in Bulgaria.
- ❖ For the first time it was performed a pharmacokinetic study to assess potential interactions when sildenafil is taken after repeated administration of TE, CF and MF from Japanese Bancha green tea in rats and it was proven their influence on sildenafil pharmacokinetics.
- ❖ For the first time it was performed a pharmacokinetic study to assess potential interactions when sildenafil is taken after a single dose of MF from Japanese Bancha green tea in rats and it was proven its influence on sildenafil pharmacokinetics.
- ❖ For the first time, static and dynamic PBPK models have been developed and implemented to predict potential interactions between sildenafil and CF or MF from green tea, both alone and in combination in humans.

CONTRIBUTIONS OF SCIENTIFIC AND APPLIED NATURE:

- ❖ A selective and reliable HPLC-UV method for qualitative and quantitative determination of EGCG, (+)-catechin, gallic acid and caffeine in samples of Japanese Bancha green tea has been developed and validated. The proposed analytical approach can be used in the analysis of other types of tea, as well as other plant extracts that are believed to contain these compounds.
- ❖ A sensitive and reliable HPLC-UV method for qualitative and quantitative determination of sildenafil in plasma samples from experimental animals has been developed and validated. The proposed analytical method can be used in the analysis of sildenafil in health facilities or other institutions performing an analysis of drugs in biological samples.

CONFIRMATIVE CONTRIBUTIONS:

- ❖ The lower quantitative content of EGCG and caffeine in Bancha green tea has been confirmed.

- ❖ The efficacy of protein precipitation technique in the plasma sample preparation from experimental animals treated with sildenafil has been confirmed.

VIII. LIST OF PUBLICATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION

PUBLICATIONS IN SCIENTIFIC JOURNALS

1. **Radeva-Ilieva M,** Stoeva S, Hvarchanova N, Zhelev I, Georgiev KD. Influence of methylxanthines isolated from Bancha green tea on the pharmacokinetics of sildenafil in rats. *DARU J. Pharm. Sci*. 2022 Feb. doi:10.1007/s40199-022-00433-z **(IF: 3.117)**
2. **Radeva-Ilieva MP**, Georgiev KD, Hvarchanova NR, et al. Protective effect of methylxanthine fractions isolated from Bancha tea leaves against doxorubicin-induced cardio- and nephrotoxicities in rats. *Biomed Res Int*. 2020 Aug; 2020: 4018412. doi:10.1155/2020/4018412 (**IF: 2.276**)
3. **Radeva-Ilieva M,** Georgiev K. Application of the physiologically based pharmacokinetic (PBPK) model in the prediction of drug interactions. *Annual for Hospital Pharmacy*. 2020 Oct; 6(1): 25-32.

PARTICIPATIONS RELATED TO THE DISSERTATION WORK PRESENTED AT NATIONAL AND INTERNATIONAL FORUMS

1. **Radeva-Ilieva, M.**, Georgiev, K. Potential drug-drug and herb-drug interactions of PDE5 inhibitors. 11-th South-East European Conference on Chemotherapy, Infections and Cancer and 31st Annual Assembly of the International Medical Association Bulgaria (IMAB), 28-31 October 2021 (poster)
2. Mihalev, K., **Radeva-Ilieva, M.**, Stoeva, S., Hvarchanova, N., Zhelev, I., Georgiev, K. Literary review of potential interactions between green tea and drugs. Seventh Pharmaceutical Business Forum and Scientific and Practical Conference, Varna, 2021 October 22-23 (poster)
3. Kirilova, S., **Radeva-Ilieva, M.**, Stoeva, S., Hvarchanova, N., Zhelev, I., Georgiev, K. HPLC-UV analysis of various extracts of Bancha green tea. Seventh Pharmaceutical Business Forum and Scientific and Practical Conference, Varna, 2021 October 22-23 (poster)

IX. PARTICIPATION IN RESEARCH PROJECTS RELATED TO THE DISSERTATION

1. "Pharmacological study of methylxanthine fraction isolated from green tea", 2020 - today
Scientific supervisor of the project: Assoc. Prof. Kaloyan Georgiev, PhD, DSc
Institutional funding: Science Fund at the Medical University "Prof. Dr. Paraskev Stoyanov ", Varna

2. „Study of drug interactions at a biotransformation level“, 2016 – 2019
Scientific supervisor of the project: Assoc. Prof. Kaloyan Georgiev, PhD, DSc
Institutional funding: Science Fund at the Medical University "Prof. Dr. Paraskev Stoyanov ", Varna

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