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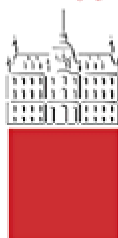

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EVALUATION OF A METHOD FOR MEASUREMENT OF INHIBITORY ACTIVITY OF PLANT PREPARATIONS AGAINST ALPHA-GLUCOSIDASE

VREDNOTENJE METODE ZA MERJENJE ZAVIRALNEGA UČINKA RASTLINSKIH PRIPRAVKOV NA ALFA-GLUKOZIDAZO

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Plagiarism statement

I, Martina Tomažin, hereby confirm that the work submitted in this thesis is my own. Any ideas, quotations, and paraphrasing, from other people's work and publications have been appropriately referenced.

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Abstract

Diabetes mellitus is a chronic metabolic disorder characterized by elevated levels of blood glucose. According to the International Diabetes Federation there are 415 million adults aged 20-79 with diabetes worldwide, great majority with type 2, and by 2040 there will be 642 million people living with the disease (data from 2015). Diabetes mellitus type 1 is treated with insulin. Treatment of Diabetes mellitus type 2 in Western medicine includes drugs which are frequently related with severe adverse drug reactions and high costs. For this reasons new remedies are a great demand. Potential source of new medications can be found in Traditional Chinese Medicine.

In the course of this master's thesis the α-glucosidase inhibitory test was evaluated for its suitability for the screening of potential α-glucosidase inhibitors. α-glucosidase can be found in the small intestine where it breaks disaccharides to α-D-glucose units. In the presence of an inhibitor such as the anti-diabetic drug acarbose, the postprandial rise in blood glucose is diminished. In the *in vitro* test *p*-nitrophenol-D-glucopyranoside is used as a substrate, which undergoes enzymatic hydrolysis and results in the yellow colored product *p*-nitrophenol. Its absorbance was measured spectrophotometrically at 405 nm. Acarbose was used as positive control which almost completely inhibits the activity of the enzyme.

In the process of evaluation, the precision, repeatability, intermediate precision and the robustness of the test were investigated, which showed the test is suitable for usage. The inhibitory activity was measured under different temperatures (37 °C, room temperature), incubation conditions (direct use, 2h-preincubation of reagents), and enzyme solution storage (freshly prepared, in refrigerator for 24 h). None of these changes showed a significant decrease in enzyme activity. The effect of different concentrations of DMSO and acarbose was also tested. Concentrations of DMSO above 6 % had an inhibitory effect on the enzyme and therefore cannot be used. Acarbose in concentration lower than 8 mg/ml cannot be applied because the inhibition is too low. Finally, inhibitory activity of three herbal formulas used in Traditional Chinese Medicine was measured: Fang Feng Tong Sheng Wan, Ermiao Wan and Si Miao Wan. The highest inhibitory activities were 77.9 ± 2.6 %, 62.2 ± 4.5 % and 62.0 ± 0.8 %, respectively. All three herbal formulas can potentially be used in the treatment of Diabetes mellitus type 2.

Keywords: Alpha-glucosidase inhibitors, Traditional Chinese Medicine, Diabetes mellitus type 2, α-glucosidase inhibitory test

Razširjeni povzetek

Diabetes mellitus je kronična presnovna motnja, za katero je značilna povišana vsebnost glukoze v krvi. Po podatkih Mednarodne federacije diabetikov iz leta 2015 je na svetu 415 milijonov odraslih ljudi, starih med 20-79 let, obolelih za diabetesom, večina za tipom 2. Do leta 2040 se bo ta številka povzpela na 642 milijonov. Cilj zdravljenja je uravnavanje vrednosti glukoze v krvi in s tem preprečitev zapletov, ki se pojavljajo pri diabetesu. Pred začetkom farmakološkega zdravljenja se uvede sprememba življenjskega sloga, kar vključuje zdravo prehrano, zmerno telesno aktivnost, zmanjšanje telesne mase in podučitev o bolezni. Nadaljnji korak je zdravljenje z zdravili. Najpogosteje se uporablja metformin. Če z uvedbo le tega ne dosegamo ciljnih vrednosti, kar se običajno zgodi z napredovanjem bolezni, se uvede dvo- ali celo trotirna terapija. Metforminu se doda eno (oz. dve) izmed naslednjih zdravil: sulfonilsečnino, tiazolidindion, zaviralec DPP-4 (dipeptidil peptidaza-4), zaviralec SGLT-2 (natrijev glukozni prenašalec-2), agonist GLP-1 (glukagonu podoben peptid-1) ali bazalni inzulin. V določenih okoliščinah se uporabljajo tudi druga zdravila kot so zaviralci α-glukozidaze (akarboza, miglitiol), kolesevelam, bromokriptin in pramlintid.

Vsa ta zdravila, ki se uporabljajo v zahodni medicini, pogosto povzročajo resne neželene učinke in so draga. Ta razloga vplivata na nenehno iskanje novih zdravil. Potencialen vir se skriva v tradicionalni kitajski medicini (TKM). TKM je sistem zdravljenja, ki se je skozi več tisočletij razvijal na območju današnje Kitajske. Bistvo TKM je celosten (holističen) pristop k razumevanju človeškega telesa, ki se odraža tudi pri reševanju zdravstvenih težav. Pomembno je odpraviti vzroke nastanka bolezni in ne samo zdraviti simptomov. Cilj je, da ponovno uravnotežimo delovanje celotnega človeškega sistema, saj pri bolezni pride do porušenja le-tega. Najpogosteje uporabljene terapevtske metode so akupunktura, moksibustija, zeliščna medicina, dieta, miselne in telesne vaje (*qi gong in tai chi)* ter masaža (*tui na*). V naši nalogi smo se osredotočili na zeliščne mešanice, in sicer smo želeli ovrednotiti antidiabetični učinek treh zeliščnih formul. To so Fang Feng Tong Sheng San, Er Miao Wan in Si Miao Wan. Z metodo, ki smo jo morali prvo ovrednotiti, smo merili zaviralen učinek omenjenih formul na encim α-glukozidaza.

Namen magistrske naloge je ovrednotenje metode za merjenje zaviralnega učinka na alfa-glukozidazo, ki se uporablja za presajanje potencialnih zaviralcev α-glukozidaze. Uporabljen encim α-glukozidaza se *in vivo* nahaja v tankem črevesju, kjer skrbi za razgradnjo disaharidov na α-D-glukozo. Ob prisotnosti zaviralca, kot je anti-diabetično zdravilo akarboza, je ta proces zavrt. Posledično je dvig glukoze v krvi po obroku zmanjšan. V naši metodi smo za substrat uporabili *p*-nitrofenol-D-glukopiranozid. Po encimski hidrolizi nastane rumeno obarvan produkt *p*-nitrofenol katerega absorbanco merimo pri 405 nm. Za pozitivno kontrolo se uporablja akarboza, ki skoraj 100 % zavre aktivnost encima, kar se kaže v zmanjšanem nastajanju produkta in s tem nižji absorbanci. Rezultati meritev so izraženi v obliki deleža inhibicije.

Za izvedbo testa smo vsakodnevno pripravili potrebne reagente, saj so le-ti morali biti sveži. Liofiliziran encim in substrat smo raztopili v fosfatnem pufru (PBS), akarbozo v DMSO. Vzorce (zeliščne formule) smo prvo raztapljali 12 ur v vodi. Usedlino smo zavrgli, supernatant pa večkrat centrifugirali, da smo se znebili nečistot. Za meritev smo vzorce ustrezno razredčili z DMSO. Na mikrotitrsko ploščo smo pipetirali po sledečem vrstnem redu: prvo smo nanesli PBS, nato DMSO ali akarbozo ali vzorec, na koncu pa smo dodali še encim. Temu je sledila 2-minutna inkubacija na 37 °C v čitalcu mikrotitrskih plošč. Nato smo dodali raztopino substrata in merili absorbanco pri 405 nm vsakih 30 sekund v obdobju petih minut pri temperaturi 37 °C. Vsak standard, vzorec in meritev z DMSO smo naredili v triplikatu. Izmerjene podatke smo prenesli in obdelali s programom Excel 2010.

V samem procesu ovrednotenja smo preverili natančnost metode s študijo ponovljivosti znotraj istega dne in med različnimi dnevi. Znotraj istega dne smo opravili meritev z 10 ponovitvami znotraj ene meritve. Za ponovljivost med različnimi dnevi smo opravili 20 meritev v različnih dnevih. Na osnovi dobljenih meritev smo izračunali povprečne vrednosti deleža inhibicije za 10 ponovitev oziroma 20 meritev. Iz izračunanih standardnih odklonov lahko zaključimo, da se je metoda izkazala z nizko stopnjo razpršenosti rezultatov. Spremenili smo običajne merilne pogoje in opravili naslednje meritve: z znižano inkubacijsko in merilno temperaturo, z reagenti, ki so 2 uri stali na sobni temperaturi, z reagenti, ki so 2 uri stali na sobni temperaturi in pri znižani inkubacijski in merilni temperaturi ter s 24 ur staro raztopino encima. V nobenem primeru ni prišlo do znižanja encimske aktivnosti. Preverili smo tudi vpliv različnih koncentracij DMSO in akarboze. Raztopine DMSO s končnimi koncentracijami, višjimi od 6 %, so delno zavrle encimsko

aktivnost, zato jih ni priporočljivo uporabljati v tej metodi. Prav tako za uporabo niso primerne koncentracije akarboze, ki so nižje od 8 mg/ml, saj ne dobimo zadostnega odziva. Izmerili smo tudi zaviralno aktivnost treh zeliščnih formul, ki se uporabljajo v tradicionalni kitajski medicini. To so Fang Feng Tong Sheng San, Er Miao Wan in Si Miao Wan. Najvišji izmerjeni zaviralni učinki za vsako izmed formul si sledijo tako: 77,9 ± 2,6 %, 62,2 ± 4,5 % in 62,0 ± 0,8 %. Vse tri formule so potencialni kandidati za zdravilo proti diabetesu tipa 2.

Ključne besede: zaviralci α-glukozidaze, tradicionalna kitajska medicina, diabetes mellitus tip 2, α-glukozidaza zaviralni test

List of abbreviations

DM – Diabetes mellitus

DMSO – dimethyl sulfoxide

DPP – Diabetes Prevention Program

DPS – Diabetes Prevention Study

EMW – Ermiao Wan herbal preparation

FFTS – Fang Feng Tong Sheng herbal preparation

GDM – gestational diabetes mellitus

HbA1c – glycated haemoglobin

IDF – International Diabetes Federation

IFG – impaired fasting glycaemia

IGT – impaired glucose tolerance

NIDDM ¬ non-insulin-dependent-diabetes mellitus

PBS - phosphate buffer saline

pNP – p-nitrophenol

pNPG – p-nitrophenyl-α-D-glucopyranoside

SD – standard deviation

SMW – Si Miao Wan herbal preparation

T1DM – Diabetes mellitus type 1

T2DM – Diabetes mellitus type 2

TCM – Traditional Chinese Medicine

WHO – World Health Organization

1. INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a group of chronic and progressive metabolic disorders of multiple etiologies characterized by hyperglycemia (elevated levels of glucose in the blood) in which the pancreatic β-cells do not produce enough insulin or when the body cells cannot effectively use the insulin it produced, or the combination of both (1). The outcome is abnormal metabolism of carbohydrates, fats and proteins. As a result, various organs are subjected to long-term damage, dysfunction and failure, especially the eyes, nerves, kidneys, blood vessels, heart and brain.

The life expectancy is reduced. Specific diabetes related microvascular complications (retinopathy, nephropathy and neuropathy) contribute to significant morbidity. There is increased risk of macrovascular complications (ischemic heart disease, stroke and peripheral vascular disease). In general, the quality of life of diabetic patients is diminished.

DM is one of the largest and most challenging health problems in our century. There are 415 million adults or 8.8% of people aged 20-79 with DM worldwide, including 193 million who are undiagnosed, according to the last data of the International Diabetes Federation from 2015. In addition, 318 million adults are supposed to have impaired glucose tolerance and with that higher risk to develop the disease in the future. The major cause of death in most countries is DM and its complications. In 2015, 5 million adults died from DM. With increasing prevalence of both DM type 1 (T1DM) and type 2 (T2DM), it is estimated that by 2040 there will be 642 million people living with the disease. The contributing lifestyle factors that increase the risk of developing T2DM are urbanization, unhealthy diets, and reduced physical activity, a trend seen all over the world. The cause of the increase in T1DM incidence in children is currently unknown. Besides having a huge impact on a patient's quality of life it also represents a large economic burden for afflicted patients and countries. Globally, 12% of the health expenditures are already intended for DM treatment and related complications. With the growth of population and consequently higher prevalence of DM, the healthcare costs will be even higher.

There are several types of diabetes and intermediate conditions of impaired glucose metabolism (intermediate hyperglycemia), which vary depending on the etiopathogenesis, clinical picture, course and prognosis. The most common type is T2DM, which affects approximately 87% to 91% of all diabetic patients, followed by T1DM with 7% to 12% and 1% to 3% of other types of diabetes such as gestational diabetes (GDM).

T1DM is characterized by deficient insulin production in the body due to a cell-mediated autoimmune destruction of the β-cells of the pancreas. People with T1DM need daily administration of insulin otherwise they cannot survive. The disease typically occurs in children and young adults, although it can appear at any age. The cause of T1DM is not known and the disease is currently incurable.

GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy and usually disappears after giving birth. It carries long-term risk of T2DM for the mother and child. If untreated, women and their infants are at great risk of some complications during pregnancy and delivery (2).

Prediabetes indicates an intermediate condition in the transition between normal blood glucose levels and diabetes, more known as impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG). People with these two conditions are more likely to develop T2DM and have a heart attack and stroke. Several studies, presented in Table I, had shown that changes in lifestyle such as incorporating physical activity in your daily routine, eating healthy foods and maintaining a healthy weight can lead to normalization of glucose tolerance and as such preventing development of T2DM. The subjects in all researches were people with IGT subjected to lifestyle modification or treatment with metformin or acarbose. Da Qing showed in a Diabetes Prevention Study (DPS) and Diabetes Prevention Program (DPP) that modification of lifestyle is a highly effective way of delaying or preventing T2DM. As seen from DPP and from Stop– non-insulin-dependent-diabetes mellitus study (Stop NIDDM), metformin and acarbose could also be used, either as an alternative or in addition to changes in lifestyle (3,4,5,6).

Table I: Studies about effects of changed lifestyle and some drugs on risk reduction for development of T2DM

Study	Diagnosis	Intervention	Duration (years)	Risk reduction in %
Da Qing	IGT	Lifestyle*	6 years	42%
DPS	IGT	Lifestyle*	6 years	58%
DPP	IGT	Lifestyle*	3 years	58%
		metformin		31%
Stop NIDDM	IGT	acarbose	3 years	36%

*Legend: Da Qing – the name of prefecture in China; DPS – Diabetes Prevention Study; DPP – Diabetes Prevention Program; NIDDM – non-insulin-dependent-diabetes mellitus; *lifestyle intervention includes diet, exercise and weight loss*

The basis of diagnostic criteria to define DM is plasma glucose measurement. The World Health Organization (WHO) gives guidelines for the diagnostic criteria for DM and intermediate hyperglycemia (Table II). DM is diagnosed if one or both of criteria are fulfilled (fasting plasma glucose ≥7.0 mmol/l and two-hour plasma glucose ≥11.0 mmol/l following a 75 g oral glucose load). The diagnosis of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are confirmed only if both criteria are met. Any test for diagnosis of DM requires confirmation with a second measurement in the elevated range unless there are clear symptoms of diabetes (e.g. polyuria, unexplained weight loss polydipsia, and blurred vision). Recent recommendation by WHO is to use HbA1c (glycated hemoglobin) for diagnosis of DM. If HbA1c levels are ≥6.5% the diagnosis of DM is confirmed. Levels of HbA1c just below 6.5% may indicate the presence of intermediate hyperglycemia (8). Initially, HbA1c was and still is used as a marker of glycemic control, because it reflects the average blood glucose level over the last 2 to 3 months. The normal HbA1c level is less than 6%, the therapeutic goal for non-pregnant adults in general is less than 7%. According to the European Association for the Study of Diabetes (EASD) and International Diabetes Federation (IDF) guidelines the goal for HbA1c is < 6.5% (9).

Table II: WHO recommendations for the diagnostic criteria for DM and intermediate hyperglycemia (7)

Condition	Fasting plasma glucose	2-hour glucose (OGTT)*
Unit	mmol/l	mmol/l
Normal	<6.1	<7.8
IFG	6.1 - 6.9	<7.8 (if measured)
IGT	<7.1	≥7.7
DM	≥7.0	≥11.1

*Legend: OGTT – oral glucose tolerance test; IFG – impaired fasting glucose; IGT – impaired glucose tolerance; *venous plasma glucose 2-h after ingestion of 75g oral glucose load*

1.2 DIABETES MELLITUS TYPE 2

Diabetes mellitus type 2 (T2DM) was formerly known as non-insulin-dependent or adult onset diabetes due to its occurrence mainly in adults. However, these are outdated expressions because T2DM is increasingly seen in children and adolescents. The hyperglycemia develops because of increased resistance of adipocytes, hepatocytes and myocytes so that the insulin is ineffective. To overcome this abnormality, the insulin is overproduced, which results in mild hyperglycemia in early stages of T2DM. As the disease progresses, pancreatic *β-cells* get worn out and fail to produce adequate amount of insulin (10).

T2DM is a result of the interaction between many risk factors which fall in three categories: a genetic predisposition, behavioral and environmental risk factors. Based on a variety of population, family and twin-based studies, there is 20-80% hereditary risk of developing the disease, e.g. individuals who have one parent with T2DM have 40% chance of developing T2DM and 70% if both are affected. In addition, the concordance rate of T2DM in monozygotic twins is about 70% whereas the concordance in dizygotic twins is only 20%–30% (11). The strongest risk factors are overweight and obesity which are closely related with physical inactivity and poor nutrition. About 80 % of the patients are overweight or obese. Other risk factors include earlier GDM, advancing age, ethnicity, low birth weight, socioeconomic status and smoking.

The first step in treatment of T2DM is the implementation of increased physical activity, a healthy diet and maintenance of a normal body weight. However, it is often insufficient to maintain long-term glycemic control just with lifestyle modification. In such cases,

pharmacological intervention is required. The most commonly used drug is metformin, unless there are contraindications. After a while, when this approach is not sufficient enough to maintain HbA1c levels under 6.5%, a combination of drugs is used. Possible options are: sulfonylurea, thiazolidinedione, dipeptidyl peptidase-4 inhibitor (DPP-4 inhibitor), sodium-glucose co-transporter-2 inhibitor (SGLT2 inhibitor), glucagon-like peptide-1 receptor agonist (GLP-1-RA), or basal insulin. Less common drugs used in specific situations are: α-glucosidase inhibitors (acarbose, miglitol), dopamine-2 agonist (bromocriptine), amylin mimetic (pramlintide) and bile acid sequestrant (colesevelam). They are usually not favored due to their modest efficacy, the frequency of administration, and/or limiting side effects (12).

1.3 TRADITIONAL CHINESE MEDICINE

1.3.1 Introduction

Traditional Chinese Medicine (TCM) is a system of health care originating from China and is based on the theories, beliefs and experiences that are thousands of years old. In several Asian countries TCM is an essential part of the healthcare system and is finding its way as complementary or alternative medicine to North America and Europe. To promote health and treat disease, a variety of techniques are used. The most frequently used therapeutic methods are acupuncture, moxibustion (burning a herb above the skin to apply heat to acupuncture points), herbal medicine, diet therapy, mind and body exercises (*qi gong* and *tai chi*), and *tui na* (Chinese massage). The core of TCM is a holistic approach to health that tries to bring the body, mind and spirit into harmony. No single sign, symptom, or body part can be understood except within the context of the whole body. The yin-yang theory and the five phases theory or five element theory are two basic TCM theories explaining the body's structure, physiological functions, pathological changes and also guide clinical diagnosis and treatment. Yin and yang describes how seemingly opposite forces or objects may actually be complementary and how things function in relation to each other and to the universe. Yin is cold, wet and female. Yang is hot, dry and male. Disease results from an imbalance between yin and yang and is treated by re-establishing the balance. The theory of five elements, *wu xing*, presumes that substances can be divided into one of five basic elements: wood, fire, earth, metal and water, which contain their own specific characteristics and properties. Within this model, each element is associated with an organ and other natural

phenomena such as weather, seasons, cardinal directions, emotions and color. Fire is linked to the heart, water to the kidneys, metal to the lungs, wood to the liver and earth to the spleen-pancreas-stomach. Between these elements a dynamic balance and relationship exists. Pathological changes may occur, if the balance is interrupted or destroyed. Another important concept in TCM is *qi* or *chi*, the life force that is constantly flowing and circulating throughout the body through channels, called meridians and organ systems. Chi provides life-force energy crucial for the development and growth of the human body and to perform the physiological functions of organ, meridians and tissues. When a person is ill, chi is weakened or blocked. To make a diagnosis, there are four examination methods used: questioning/history taking, listening and smelling, inspection, and palpation. Internal pathological (disease) changes are diagnosed by a TCM practitioner through observation and analysis of external signs. First, the practitioner asks a patient many questions about his personal and family history, and the symptoms he experiences. Examination focuses on the color, shape and coating of the tongue, and the color and expression of the face. Then the practitioner checks if there are any changes in the patient's voice and odor of the breath and body. Finally, the strength, rhythm, and quality of the pulse is checked. Once a diagnosis is established, therapy aims at restoring the body's balance and therefore allowing the body to repair itself. Therapy includes one or a combination of already mentioned therapeutic methods. It treats the root cause of the disease and consequently the manifested symptoms. In addition, it treats the whole person, taking into account not only the physical aspects of a patient, but also the emotional and spiritual (13).

1.3.2 Traditional Chinese medicine view on Diabetes mellitus

TCM categorizes DM as "wasting and thirsting" disease (*xiao-ke*, in Chinese). The prevailing pathological mechanism is dysfunction in the transportation and distribution of body fluids resulting in yin deficiency with dryness and heat. The main organs affected are lungs, stomach and kidneys. DM can be sorted into three groups: upper, middle and lower xiao-ke, each with characteristic symptoms. The upper type is defined with lung heat drying up body fluids resulting in excessive thirst, the middle with stomach fire damaging fluids causing excessive hunger, and the lower with kidney yin deficiency and consequently excessive urination. Over time, with the progress of disease, most people with DM manifest symptoms of all three groups. As stated by TCM, xiao-ke is an outcome of improper diet (over-consumption of greasy, fatty, sweet and pungent food, hot drinks and alcohol), a

constitutional yin deficiency, overwork, emotional disturbances (anger, over-thinking, hate and frustration), prolonged stress or illness, excessive sexual activity and pregnancy. Treatment is adjusted to specific needs of the individual patient based on a disharmony patterns that occur in DM. It focuses on the organ which is most yin deficient. The treatment functions so that it strengthens the function of the affected organ, clears heat and dryness, and nourishes yin. Various methods may be used for treatment including acupuncture, Chinese herbal medicine, lifestyle/dietary changes and physical exercise (14). Clinical and experimental studies have showed that acupuncture and electro acupuncture have a favorable hypoglycemic effect reached by increasing insulin levels and/or elevating insulin sensitivity. These methods can also lower free fatty acids and lipid levels of blood plasma. There are more than 800 plants used to treat and prevent T2DM as single herbal extracts or in complex formulas, and more than 400 have been demonstrated experimentally to be effective *in vitro* or *in vivo*. Usually, a combination of herbs is prescribed. Prepared formulas contain numerous extracts and compounds with multiple mechanisms of action. These pharmacological mechanisms can be classified as reducing carbohydrate absorption, such as inhibition of α-glucosidase, α-amylase and aldose reductase, stimulating insulin secretion, increasing insulin sensitivity, increasing glucose uptake, potentiating endogenous incretins, being antioxidant and decreasing β-cell apoptosis, and inhibiting hepatic glycogenolysis (15).

1.4 Acarbose

Acarbose (known as **Glucobay®** in Europe) is classified as non-insulinotropic oral antidiabetic drugs (OAD) called **α-glucosidase inhibitors**. It is an oligosaccharide, originating from microbes. Its function is to competitively, selectively and reversibly inhibit the α-glucosidase activity in the brush border membrane of the small intestine and as such is used as a treatment for T2DM (16). For commercial usage it is obtained from a fermentation process of a microorganism from the genera Actinoplanes and Streptomyces. It is composed of an acarviosyl moiety with a maltose at the reducing terminus as shown in Figure 1 (17).

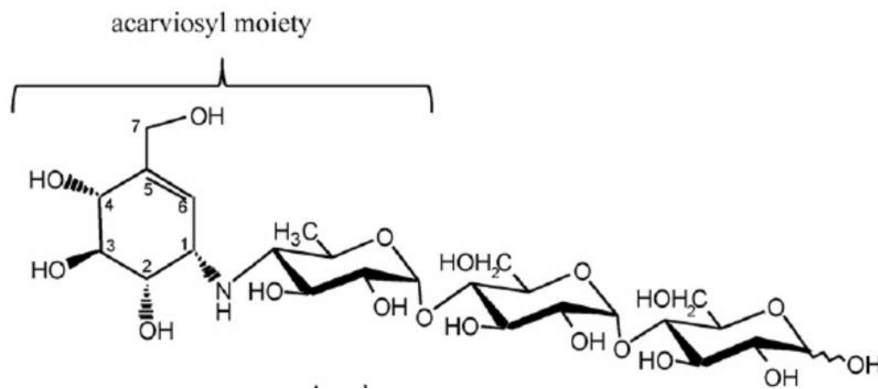

Figure 1: Chemical structure of acarbose (17)

Acarbose molecules attach to the carbohydrate binding sites of α-glucosidase with an affinity constant that is much higher (10^4 to 10^5 times) than that of the natural substrates such as sucrose, maltose, maltotriose and dextrins (18). This results from the nitrogen between the first and second glucose molecule. The inhibition capacity and the binding affinity of acarbose for various glucosidases are listed in descending order: maltase-glucoamylase, followed by sucrase and dextranase. Compared to lactase, acarbose does not inhibit β-glucosidases. It weakly inhibits α-amylase (19). Under normal circumstances, the membrane-bound intestinal α-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the brush border membranes of enterocytes in the small intestine, while pancreatic alpha-amylase hydrolyzes complex starches to oligosaccharides in the lumen of the small intestine. Only monosaccharides can be absorbed by enterocyte and transferred into the bloodstream. Acarbose tablets are administered orally together with a meal, which slows down the digestion of carbohydrates and extends the period over which glucose is absorbed into the blood stream. The consequence is dose-dependently decreased postprandial rise in blood glucose and the glucose-induced insulin secretion is weakened (16). Short-term effect of acarbose is reduction of the fasting plasma glucose for 1.1 mmol/l, and postprandial glycemia for about 2.3 mmol/l. A long-term effect is seen in a reduction of HbA1c for 0.5 to 1% (19). Acarbose is not widely used due to its moderate effect in combination with Western diet (high intake of red meat, processed meat, high-fat dairy products, butter, eggs, and refined grains), frequent dosage (3 times daily) and side effects (flatulence, diarrhea and abdominal pain) (20).

1.5 α-glucosidase

α-glucosidase (EC number 3.2.1.20, systematic name: α-D-glucoside glucohydrolase, other names: maltase, glucoinvertase, maltase-glucoamylase, α-glucopyranosidase, etc.) belongs to a class of glycosidases, i.e. enzymes that hydrolyze O- and S-glycosyl compounds. More specifically, it presents a group of enzymes which are specialized for the exohydrolysis of (1→4)-α-glycosidic linkages. The released product is a single α-D-glucose molecule, which detaches after hydrolysis of terminal non-reducing (1→4)-linked α-D-glucose residues. This reaction takes place in the brush-border surface membrane of enterocytes, where the enzyme is located (21). There are two proposed mechanisms of enzyme action. One includes a nucleophilic displacement and the other an oxocarbenium ion intermediate (22). It is composed of two subunits with differing substrate specificity. The C-terminal domain has a broader substrate specificity and activity against glucose oligomers, while the N-terminal catalytic domain has highest activity against maltose (23).

1.6 TRADITIONAL CHINESE HERBAL PREPARATIONS

Traditional Chinese herbal therapy accounts for the majority of treatments in TCM. A combination of several herbs is usually prescribed. The principal herb is the one with therapeutic properties matching the syndrome of the disease. To reach a synergistic effect, supportive herbs are added to the principal one. To alleviate the side effects or sometimes to improve the overall taste, other herbs may be added. Occasionally, substances of animal origin or minerals are also present in the combination (15).

1.6.1 FANG FENG TONG SHENG HERBAL PREPARATION

Fang Feng Tong Sheng (FFTS), also known as Siler and Platycodon formula, or if translated into English Ledebouriella powder that sagely unblocks. It was formulated over 800 years ago in China as a herbal medicine. It comes in the form of pills (wan) or granules (keli). It is used to purge away the dampness heat from the interior, disperse pathogenic wind (heat) from the body surface and unblocks the bowels. Many diseases can be treated with it, such as influenza, acne, obesity, asthma, dizziness. It is a mixture of 18 components: *Herba Saposhnikoviae (Ledebouriella), Herba Ephedrae, Wine-treated Radix et Rhizoma Rhei, Natrii Sulfas, Herba Schizonepetae, Herba Menthae, Fructus Gardeniae, Talcum, Gypsum, Fructus Forsythiae, Radix Scutellariae, Radix Platycodi, Rhizoma Chuanxiong, Radix*

Angelicae Sinensis, Radix Paeoniae Alba, Rhizoma Atractylodis Macrocephalae, Radix. Glycyrrhizae and *Rhizoma Zingiberis Recens* (24).

1.6.2 ERMIAO WAN HERBAL PREPARATION

Ermiao Wan, also known as Powder of two effective ingredients, or if translated into English Two-marvel powder, is another Chinese herbal medicine that consists of two ingredients: *Cortex Phellodendri and Rhizoma Atractylodis.* It is used to eliminate heat and dampness. Many conditions can be treated with it, such as arthritis, eczema, low back pain, knee pain, dermatitis, weakness. (25).

1.6.3 SI MIAO WAN HERBAL PREPARATION

Si Miao San, also known as Pill of four effective ingredients, or if translated into English Four-marvel pill, is a pill used in TCM to eliminate heat and resolve dampness. It is a modification of the classic formula Ermiao Wan and contains besides *Cortex Phellodendri* and *Rhizoma. Atractylodis* 2 additional herbs, *Radix Achyranthis Bidentatae* and *Semen Coicis*. It was created to expand the application of the formula Ermiao Wan to treat atrophic syndromes in the lower body. Other clinical applications are the same as in Ermiao Wan (26).

2. WORK PLAN

The purpose of this thesis is to evaluate the α-glucosidase inhibitory test developed at the Department of Pharmacognosy (Institute of Pharmaceutical Sciences, University of Graz). The test is intended to be used for the screening of extracts from plants and herbal mixtures or pure compounds for their α-glucosidase inhibitory activity.

The aim is to confirm that this test fits its purpose of use. To determine the test suitability, several evaluation parameters will be examined (test repeatability, intermediate precision). For the purpose of optimization, test parameters including the incubation and measurement temperature and preincubation of reagents will be assessed and enzyme stability will be determined. The effect of different concentrations of DMSO will be determined and the minimal acarbose concentration causing a 100 % inhibition will also be determined. Finally, the inhibitory activity of seven traditional Chinese herbal samples towards α-glucosidase will be measured.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

The reagents used in the test are listed in Table III. All the reagents and samples were prepared at the Department of Pharmacognosy. The measurement of enzyme inhibition took place at the Department of Pharmacology.

Table III: list of used reagents

Name	**Art. No**	**Company**	**Storage**
Acarbose ≥95%	A8980	Sigma-Aldrich	RT
Sodium Azide NaN3 ReagentPlus®, 99.5%	S2002	Sigma-Aldrich	RT
Albumin from bovine serum	A7906	Sigma-Aldrich	2 - 8°C
4-Nitrophenyl α-D-glucopyranoside, ≥99%	N1377	Sigma-Aldrich	-20°C
α-Glucosidase from *Saccharomyces cerevisiae*	G5003	Sigma-Aldrich	-20°C
Dimethyl sulfoxide (DMSO), ≥99,8%	4720.3	Carl Roth GmbH	RT
$NaH_2PO_4 x 2H_2O$, ≥98%	2370.1	Carl Roth GmbH	RT
$Na_2HPO_4 x 2H_2O$, ≥99,5%	4984.1	Carl Roth GmbH	RT

Legend: RT = room temperature

a) Enzyme α-glucosidase from *Saccharomyces cerevisiae* (Type 1) solution (1 unit/ml)

For the enzyme solution 0.1 mg of lyophilized powder (19.3 units/mg solid) was dissolved in 2 ml PBS. The final concentration of the enzyme solution was 1 unit/ml. The enzyme solution had to be prepared freshly and kept at 4 °C in the refrigerator no longer than 2 days.

b) Substrate 4-nitrophenyl α-D-glucopyranoside solution (4 mM)

3.615 mg of 4-nitrophenyl α-D-glucopyranoside (pNPG) (Figure 2) was dissolved in 3 ml PBS to give a final concentration of 4 mM. The solution was vortexed until it was clear. The substrate solution had to be prepared freshly.

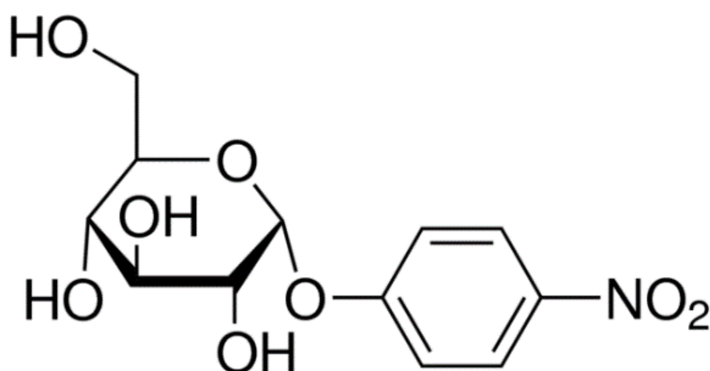

Figure 2: 4-Nitrophenyl α-D-glucopyranoside.

c) Acarbose solution (8 mg/ml)

3.2 mg of the α-glucosidase inhibitor acarbose were dissolved in 80 µl DMSO and 320 µl dH_20 to give a final concentration of 8 mg/ml. The solution was vortexed until it was clear. The solution had to be prepared freshly.

d) Dimethylsulfoxid (DMSO) solution (20% v/v)

640 µl DMSO was diluted with 2560 µl dH_20 to yield a 20% v/v DMSO. The solution had to be prepared freshly.

e) Phosphate buffer saline (PBS) solution (10 mM, pH = 7,0)

A 20 mM solution of Na_2HPO_4 and a 20 mM solution of NaH_2PO_4 were prepared. 177.99 mg $Na_2HPO_4x2H_2O$ and 156.01 mg $NaH_2PO_4x2H_2O$ were dissolved respectively in 50 ml distilled water (dH_20). The two solutions were mixed together until the mixture reached a

pH value of 7. Then the solution was diluted 1:1 with dH_20 to a final concentration of 10 mM. The phosphate buffer was kept in the refrigerator at 4°C.

Right before use, 19.2 mg bovine serum albumin and 1.92 mg NaN_3 (sodium azide) were dissolved in 9.6 ml of PBS. The solution had to be prepared freshly.

f) Herbal samples solutions (8 mg/ml)

The herbal samples tested were as follows (Figure 3):

1. Fang Feng Tong Sheng Wan®/Keli® (FFTS): five samples of the same mixture from different Chinese manufacturers. Four of them were in pills and one in granulated form (China Beijing Tong Ren Tang Technologies Co., LTD; Jiangsu Pingguang Xinyi (Jiaozuo) Chinese Medicine Co., LTD; Changchun Overseas Pharmaceutical Group LTD; Shangquin City, Kinmen and Matsu Pharmaceutical Co., LTD; Yantai Bohai Pharmaceutical Group Co., LTD).
2. Si Miao Wan® (SMW): one sample in pill form (Jilin Zixin Pharmaceutical Industrial Co., LTD)
3. Ermiao Wan® (EMW): one sample in pill form (Beijing Tong Ren Tang Pharmaceutical Co., LTD)

32 µl of the previous prepared samples with a nominal concentration of 100 mg/ml were diluted with 368 µl 20% v/v DMSO to reach a final concentration of 8 mg/ml.

Figure 3: Herbal preparations of FFTS (five different samples 1.1 - 1.5), SMW (one sample) and EMW (one sample). FFTS = Fang Feng Tong Sheng herbal preparation, SMW = Si Miao Wan herbal preparation, EMW = Ermiao Wan herbal preparation.

3.1.2. Laboratory equipment

Various laboratory equipment such as measuring flasks, volumetric pipettes, micropipettes, Vortex mixer, analytical balance (Sartorius CPA225D-0CE), microcentrifuge, magnetic stirrer, Eppendorf centrifuge (5810 R) and centrifuge tubes were used for the preparations of reagents. 96 micro titer plates (flat bottom), Eppendorf Repeater® plus pipettes and Eppendorf combitips pipettes (5 ml) were used for the enzyme reaction. The measurements of the reaction end products were performed with a Microplate reader Rosys anthos ht3 (Anthos Labtec Instruments).

3.2. Method

3.2.1 Enzymatic reaction

The enzyme α-glucosidase hydrolyzes the substrate p-nitrophenyl-α-D-glucopyranoside (pNPG) to α-D-glucose and p-nitrophenol (pNP) (Figure 4). The yellow colored pNP product is than detected at 405 nm. The amount of the product formed is proportional to the enzyme activity. In the presence of α-glucosidase inhibitors, there is less product resulting in lower absorbance.

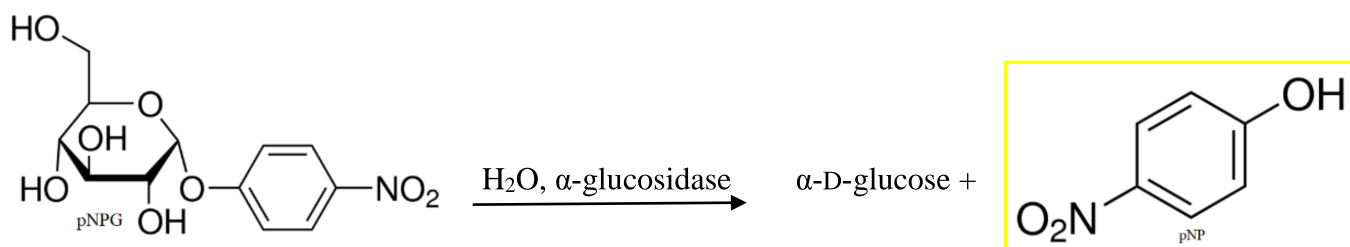

Figure 4: Scheme of catalytic activity of α-glucosidase.

4. EXPERIMENTAL WORK

4.1. Enzyme inhibitory test

The enzyme inhibitory test was performed in phosphate buffer saline (PBS) solution. The samples and reagents except the substrate solution were transferred into 96 microwell plate following the pipetting scheme described in Table IV. Each cell in the Table IV presents one of the possible reaction taking place in a single well on 96 microwell plate. The pipetting order was as follows: 1. PBS, 2. herbal sample /acarbose standard/DMSO and 3. enzyme. The mixture was incubated for 2 min at 37 °C in the microplate reader. Then 50 µl of the substrate were added and the microtiter plate was put into the microplate reader. The absorbance of the released pNP was measured at 405 nm every 30 seconds for 5 minutes at 37 °C. The tests were performed in triplicates along with appropriate blanks and the measurement data was exported and processed with Excel 2010.

Table IV: Pipetting scheme

	Solvent control well	**Acarbose standard well**	**Sample well**
Determination	50 µl DMSO solution 50 µl PBS 50 µl enzyme solution	50 µl standard acarbose solution 50 µl PBS 50 µl enzyme solution	50 µl herbal sample 50 µl PBS 50 µl enzyme solution
Blank (without enzyme)	50 µl DMSO solution 100 µl PBS	50 µl standard acarbose solution 100 µl PBS	50 µl sample 100 µl PBS

4.2. Effect of temperature and reagent preincubation

We conducted an experiment where the temperature at which the incubation and measurement were performed, was changed. Instead of 37 °C, room temperature (23 °C) was used. The second variation that may impact the enzymatic reaction rate was the introduction of a 2 hour-preincubation of freshly prepared reagents and enzyme, before being mixed in the microtiter plate. The solvent control and acarbose standard solutions were incubated for 2 hours at room temperature (RT, 23 °C) while the enzyme solution was stored for 2 hours in a refrigerator (4 °C). The 2 hour-preincubation was followed by the reaction performed at 37 °C and RT.

4.3 Evaluation of 24-hour enzyme stability

The enzymatic test was performed immediately, after the preparation of the enzyme solution and after a 24-hour storage of the enzyme solution in the refrigerator at 4 °C in order to estimate the stability of the enzyme in solution.

4.4 Effects of DMSO (solvent control)

To check the effects of DMSO on the enzymatic reaction ten solvent controls of DMSO (concentrations ranging from 0% to 60%) were prepared. A final volume of 400 µl for each DMSO concentration was prepared by mixing DMSO with the appropriate amount of dH_20 (Table V).

Table V: Preparation of different DMSO solutions with dH_2O used as solvent controls.

[%]	**0**	**1.5**	**3**	**6**	**12**	**20**	**24**	**30**	**45**	**60**
DMSO (µl)	0	6	12	24	48	80	96	120	180	240
dH_2O (µl)	400	394	388	376	352	320	304	280	220	160

4.5 Effects of acarbose (acarbose standard)

Different solutions of acarbose with concentrations ranging from 0.125 to 16 mg/ml, were prepared to find out the relationship between various acarbose concentrations and the rate of enzymatic reaction. A 16 mg/ml stock solution of acarbose was prepared by dissolving 0.0224 g of acarbose in 1400 µl 20% v/v DMSO. Initially, three dilutions (8, 10 and 12 mg/ml) of acarbose were prepared from the stock solution by adding 20% v/v DMSO as represented in Table VI.

Table VI: Preparation of acarbose solutions with concentrations of 12, 10 and 8 mg/ml

Acarbose concentration (mg/ml)	Volume of acarbose stock solution (µl)	Volume of 20% v/v DMSO (µl)	Volume of final solution (µl)	Dilution factor
12	300	100	400	1.3333
10	250	150	400	1.6
8	400	400	800	2

Further acarbose solutions of **4**, **2**, **1**, **0.5**, **0.25**, and **0.125 mg/ml** were prepared from the 8 mg/ml solution using serial 1:1 dilution. The final volume of each acarbose solution was 400 µl.

4.6 Herbal sample preparation

2.5 g of each herbal sample was initially dissolved in 25 ml of dH_2O (at a nominal concentration of 100 mg/ml) by stirring with a magnetic stirrer at RT for 12 hours. The sample solutions were purified by two purification methods. The first method comprised a 3 time-centrifugation of 1 ml of the 100 mg/ml solution for 4 minutes at 2000 x g. After every centrifugation the supernatant was transferred to a new tube and the pellet was discarded. The inhibitory activity of the samples was measured immediately after centrifugation and the remaining supernatant was stored in the refrigerator at 4 °C. The remaining 24 ml of the 100 mg/ml solution were stored in the refrigerator while waiting to

be purified by in a different way. The second purification method comprised double centrifugation. The first centrifugation was performed for 30 minutes at 1800 x g at RT. The supernatant was transferred into a new tube and centrifuged for another 18 minutes at 3200 x g, at -5 °C. The pellet was discarded and the supernatant was used immediately for the enzymatic test. The remaining supernatant was stored in the refrigerator at 4 °C for later usage.

4.7 Calculation of inhibitory activity

Each herbal sample, solvent control and acarbose standard were measured in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula:

$$\boldsymbol{Inhibitory\ activity}\ (\%) = \left(\mathbf{1} - \left(\frac{\boldsymbol{As - Asb}}{\boldsymbol{Ac - Acb}}\right)\right) * \mathbf{100}$$

where, ***As*** is the absorbance in the presence of herbal sample or acarbose standard, ***Asb*** is the absorbance of the appropriate blank (i.e. blank of herbal sample or blank of acarbose standard without enzyme), ***Ac*** is the absorbance of the solvent control and ***Acb*** is the absorbance of solvent control blank (solvent control without enzyme).

5. RESULTS AND DISCUSSION

5.1 Precision

Precision is a statistical measure of the variation between a series of measurements of the same sample under the same conditions. Usually it is expressed as the variance (s^2), standard deviation (SD) or coefficient of variation (CV) of a series of measurements. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The acceptance criteria for precision was chosen based on FDA Guidance for Industry (Bioanalytical method validation). It is expressed as RSD and should be less than 15 % (27).

5.1.1 Repeatability within a single test run (repeatability)

Repeatability expresses the precision under the same operating conditions (same sample, same measurement procedure, same operators, same measuring system, same operating conditions and same location) over a short interval of time (one day or one analytical run).

In this study the repeatability was determined by measuring the inhibitory activity of acarbose (final concentration is 8 mg/ml) ten times within a single test run. Then the percentage of inhibition for each 30 s time point over 5 minutes was calculated and the results were expressed in terms of mean ± RSD (Table VII).

Table VII: Inhibition of ten measurements within a single test run (mean ± SD, max, min, range)

Time [s]	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**
Mean [%]	93.8	92.4	91.7	91.2	90.7	90.3	89.3	88.9	89.0	88.1
RSD [%]	0.4	0.3	0.3	0.4	0.4	0.6	0.6	0.8	0.8	0.8
Max. [%]	94.5	93.0	92.4	92.0	91.6	91.3	90.2	89.9	90.1	89.2
Min. [%]	93.3	91.9	91.3	90.6	90.1	89.6	88.3	87.7	87.6	86.8
Range [%]	1.2	1.1	1.1	1.4	1.5	1.7	1.9	2.1	2.5	2.4

The RSDs of ten measurements at each time point are ranging from 0.3 to 0.8 %, which meets our accepted criteria for repeatability (RSD <15%). The lowest RSDs are observed in the beginning of the reaction (30 - 150 s). Along with the low RSDs, the ranges (difference between the largest and smallest values) are suitable as well. The results in Table VII are graphically presented in Figure 5. A slow decrease in enzyme inhibition can be seen after time. The inhibition decreases from 93.8 % to 88.1 % during the 5 min measurement.

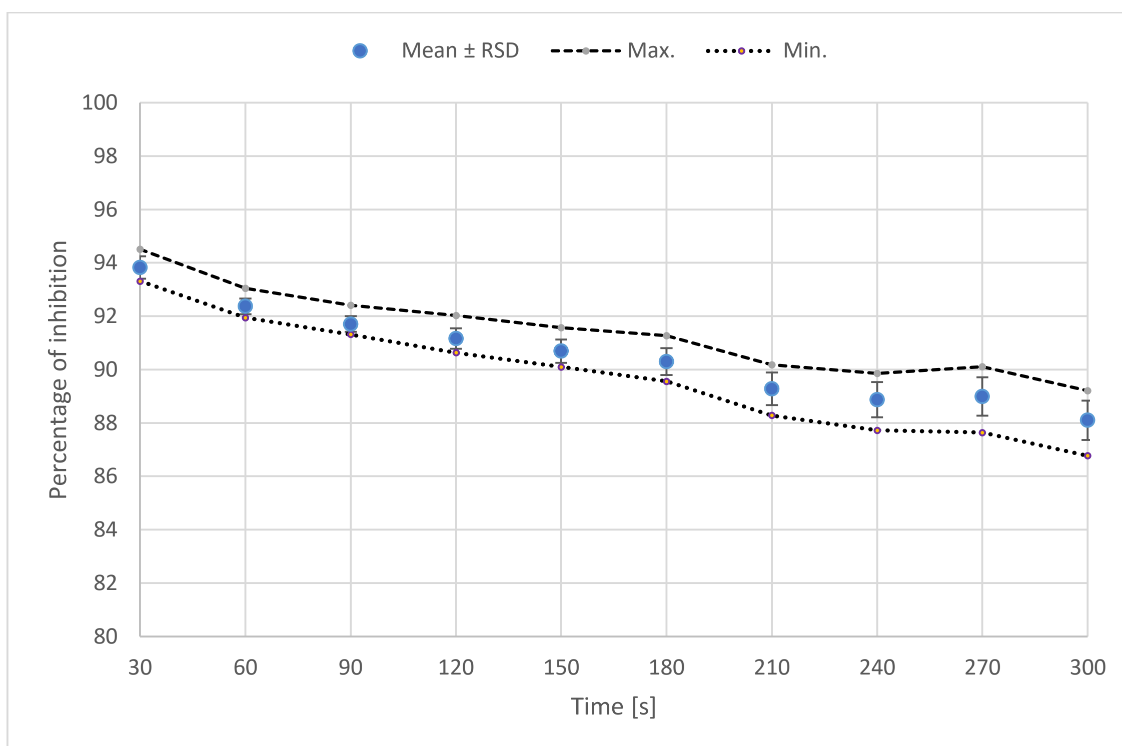

Figure 5: Mean % inhibition within-run for each time point (mean ± RSD) with maximum and minimum values. RSD = relative standard deviation.

5.1.2 Repeatability between days (intermediate precision)

The intermediate precision obtained from measurements performed in 20 different days, was determined by calculating the mean percentage of inhibition of acarbose (working concentration is 8 mg/ml) for each time point. The results are expressed in terms of mean ± RSD and are presented in Table VIII. Standard deviations are higher compared to RSDs within a single test run. This was expected, because more effects are reflected by the intermediate precision compared to repeatability. The lowest RSDs are observed in the time interval between 120 - 210 s and are 1.8. During this interval the product formation seems to be the most constant.

Table VIII: inhibition of 20 measurements for each time point (mean ± RSD, max, min, range)

Time [s]	30	60	90	120	150	180	210	240	270	300
Mean [%]	90.8	90.5	90.5	90.6	90.4	90.0	89.7	89.4	89.3	88.7
RSD [%]	2.9	2.3	2.2	1.8	1.8	1.8	1.8	1.9	2.1	2.1
Max. [%]	94.0	93.2	92.9	92.6	92.4	92.2	91.9	91.6	92.5	91.2
Min. [%]	86.7	85.0	84.8	87.4	86.7	86.2	85.8	85.2	84.7	84.0
Range [%]	7.3	8.2	8.1	5.2	5.7	6	6.1	6.4	7.8	7.2

Results from Table VIII are graphically presented in Figure 6. A decrease in the enzyme inhibition is not as evident as during the single run test. The inhibition decreases from 90.8 % to 88.7 %.

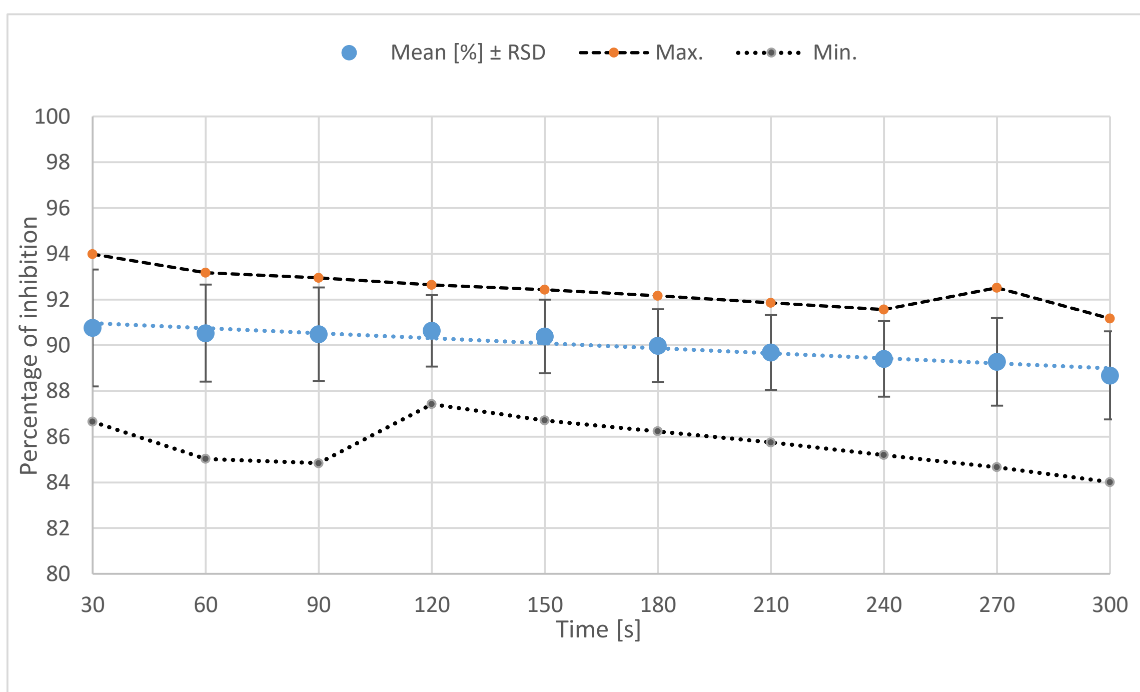

Figure 6: Mean % inhibition of 20 measurements for each time point (mean ± RSD) with maximum and minimum values. RSD = relative standard deviation.

Factors that account for higher SD of intermediate precision compared to repeatability can be different batches of reagents and pipetting, which is usually a major source of error. There

are many steps in preparation of reagents, where random mistakes can be made, especially weighing small quantities of enzyme. This problem can be avoided by preparing larger amounts of enzyme solution and planning of enzyme test with more samples at once. Pipetting steps should be reduced as much as possible to avoid pipetting mistakes. This is important particularly when pipetting small volumes of reagents to a microtiter plate. In order to minimize this effect, a test mixture for the whole test series could be prepared instead of individual solution preparation. A solution would also be the usage of a multichannel micropipette or an Eppendorf Repeater® plus, as it was in our case.

5.2 Robustness of the test (evaluation of stability)

Robustness is a measure of the test capacity to remain unaffected by small variations in method parameters (incubation time, temperature, sample preparation, buffer pH) and other environmental factors (laboratory temperature, air humidity, etc.). By determining the robustness, we characterize the reliability of the test during normal usage.

5.2.1 Effect of temperature, effect of waiting with measurement and both together

The rate of any chemical reaction increases with temperature to reach the maximum rate at the optimum temperature. Increasing the temperature even more has an adverse effect due to enzyme destabilization and denaturation. Another very important parameter in bioanalytics is the stability of reagents. Over time, spontaneous hydrolysis of reagents can occur, which results in a decreased of reagent concentration.

The enzyme inhibition by acarbose was measured under modified temperature conditions (RT instead of 37 °C) and incubation conditions (2h-preincubation of reagents instead of direct use), followed by the reaction performed at 37 °C and RT. The changes did not affect the enzyme activity (Figure 7). The enzyme inhibition by acarbose was in the range of inhibition by acarbose (20 measurements between days) measured at 37 °C without preincubation of reagents. The small differences are likely due to random variations in the test rather than different measurement conditions. Nevertheless, the absorbance values of both blanks (0.037 and 0.032 for solvent control and acarbose standard, respectively) were higher in case RT and preincubation were used compared to the cases measured at RT (0.025) or with preincubation (0.024). There are practical reasons behind changing these conditions. In case if we forget to set the temperature to 37 °C or we cannot perform the test

immediately after the reagents preparation, we wanted to see the possible influence on final result. Even though the final results (percentage of inhibition) are similar, we continued to perform the reaction under usual conditions (measurement and incubation at 37 °C and no preincubation).

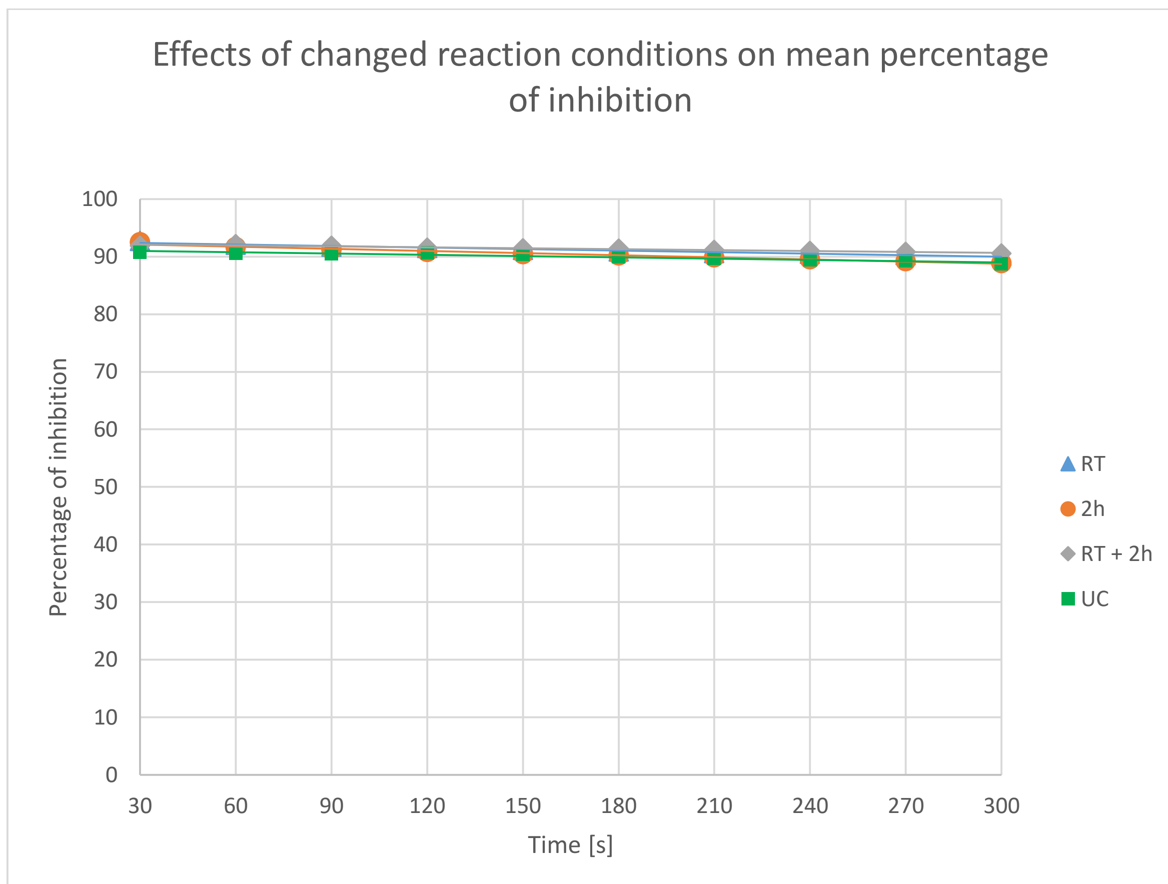

Figure 7: Effects of changed reaction conditions on percentage of inhibition; **RT** measurement at room temperature (23°C) and no preincubation; **2h** measurement at 37 °C and with reagents preincubated for two hours; **RT + 2h** measurement at RT with a two hour preincubation, **UC** measurement under usual conditions (measurement and incubation at 37 °C with no preincubation). RT = room temperature.

5.2.2 Evaluation of 24-hour enzyme stability

We presumed that storage of the enzyme solution at 4 °C for 24 hours would decrease the enzyme activity. Nevertheless, this will have no effect on percentage of inhibition, because the same enzyme is used in reaction with solvent control and in acarbose standard. As seen from the histogram (Figure 8), the absorbance of product formed in enzyme reaction without acarbose with 24 hours old enzyme is lower than the absorbance of fresh enzyme, but this is more likely due to daily variations. In the graph are also included percentages of inhibition for both enzyme reactions. The results show a similar percentage of inhibition between the freshly prepared enzyme and the one stored for 24 h in the refrigerator decreasing over time

from 91.8 % to 88.8 and from 92.0 % to 85.6 %, respectively. We can conclude that the enzyme solution can still be used after 24 hours at 4 °C.

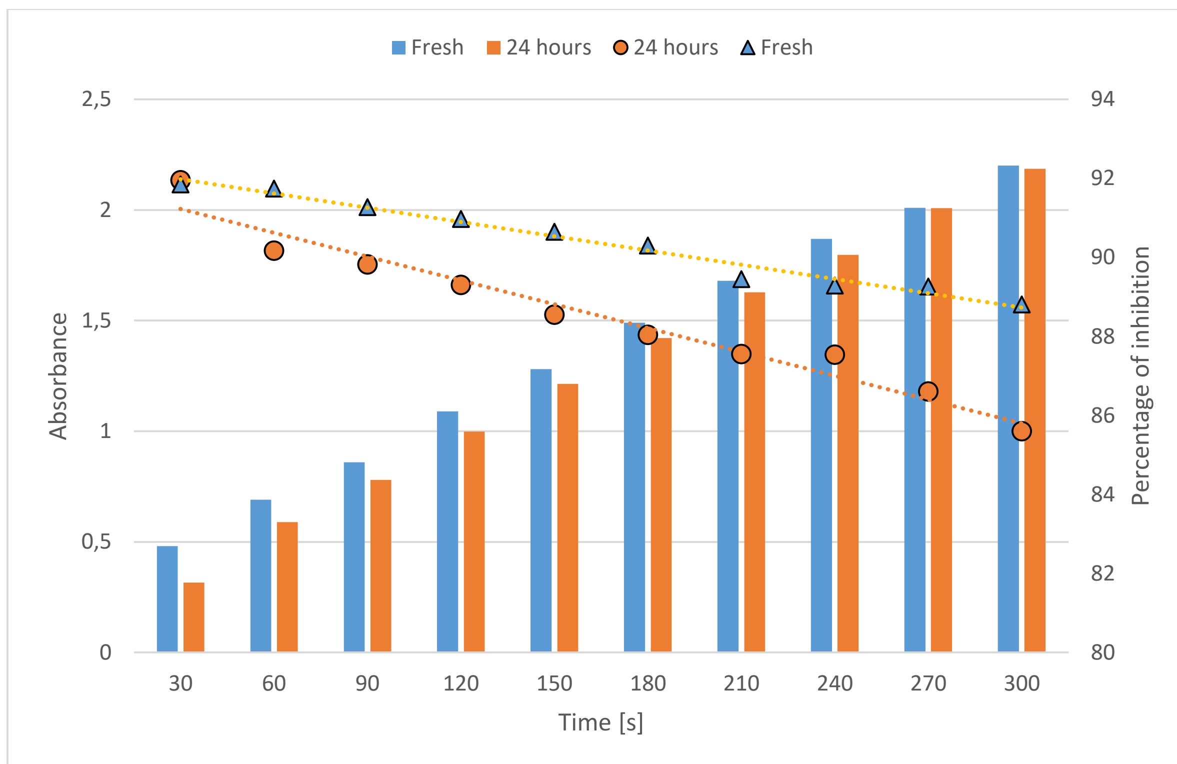

Figure 8: Effects of 24 hour storage of enzyme solution at 4 °C (orange columns and circles) compared to freshly prepared enzyme solution (blue columns and triangles). Columns represent absorbance values, triangle and circle represent percentage of inhibition.

5.3 Effects of different DMSO concentrations

DMSO is a colorless liquid used as polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible with a wide range of organic solvents as well as with water. It is involved in the oxidation of some compounds and can change conformation of biomolecules, which in case of enzymes could lead to a drop of their activity.

The initially prepared concentrations of DMSO were diluted 4-times in the final solution on the microtiter plate (Table IX).

Table IX: Concentration of DMSO before and after dilution

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Initial conc. [%]	0	1.5	3	6	12	20	24	30	45	60

Final diluted conc. [%]	0	0.375	0.75	1.5	3	5	6	7.5	11.25	15

The effects of different DMSO concentrations on enzyme activity are presented in Figure 9. A histogram shows relationship between absorbance of product formed in enzyme reaction without acarbose and different DMSO concentrations in first 2 minutes of measurement. The absorbance increase is gradually diminishing, proportionally to increasing DMSO concentration. This effect is also observed when calculating percentage of inhibition, as can be seen in a line graph (Figure 9). With higher DMSO concentrations ranging from 7.5 to 15 % a lower inhibition at the beginning of the reaction can be observed compared to the lower value from the intermediate precision experiment (86.7 %). The values at 30 s are 84.1 %, 80.6 % and 76.7 % for 7.5 %, 11.25 % and 15 % DMSO, respectively. Based on the lower value from the intermediate precision, it is recommended to avoid using concentrations of DMSO higher than 6 %.

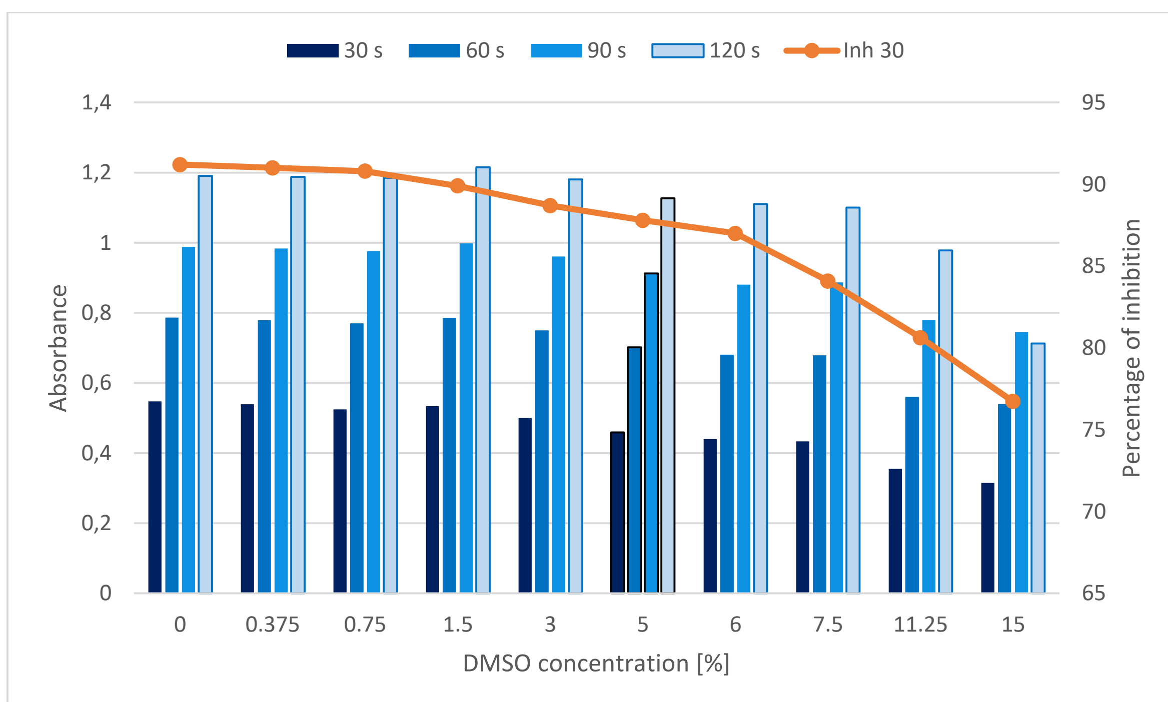

Figure 9: Effects of solvent control with different DMSO concentrations; blue colored columns present absorbance for following time intervals: first 30, 60, 90 and 120 seconds of measurement; orange line presents percentage of inhibition at first 30 seconds of measurement. DMSO = dimethyl sulfoxide.

5.4 Effect of different acarbose concentrations

Enzyme inhibition was measured using different acarbose standard solutions in the range from 0.125 mg/ml to 16 mg/ml (from 0.19 mM to 24.8 mM). The highest inhibition is achieved with 16 mg/ml (95.7 % at 30 s), and is gradually decreasing with the lowest inhibition at 0.125 mg/ml (68.6 % at 30 s) (Figure 10). The concentration of the acarbose standard solution has to be high enough to give a respond close to 100 % inhibition over the whole time of measurement (5 min). In our experiment this was true for concentrations higher than 8 mg/ml. With lower concentrations, especially from 1 mg/ml to 0.125 mg/ml, we can see a drastic drop in inhibition over time, because the concentration of the substrate (4 mM) exceeds the concentration of the inhibitor (1.5 mM to 0.19 mM).

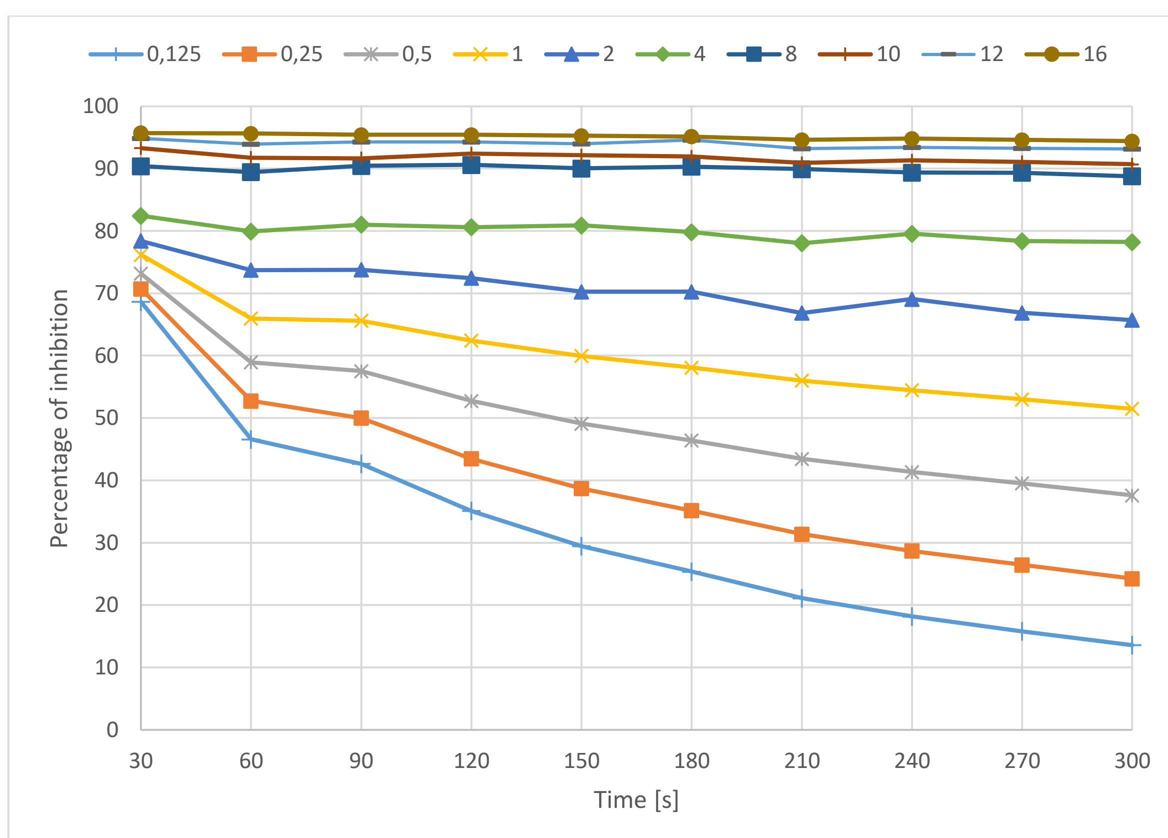

Figure 10: Effects of different acarbose concentrations on the enzyme inhibition; all concentrations are in mg/ml.

5.5 Inhibitory activity of different herbal preparation samples

The inhibitory effect of seven samples of complex traditional TCM herbal preparations on α-glucosidase was investigated. Samples with same herbal composition (FFTS 1 - FFTS 5) from different producers were compared. Two other TCM herbal preparations, EMW and

SMW, were also investigated for their ability to inhibit α-glucosidase. The herbal preparations were dissolved in 20 % v/v DMSO and four different purification strategies were employed for supernatant purification of each herbal preparation (denoted M1 - M4). M1 represents supernatants that were centrifuged three times and measured immediately, M2 supernatants were centrifuged twice and used right away, M3 were centrifuged like M2 supernatants and refrigerated for one day, while M4 were centrifuged as M1 and refrigerated for seven days (maximum possible days left in our time frame for evaluation of a method). Different concentration of acarbose standards were used (S1, S2 and S3 with 8 mg/ml, 0.25 mg/ml and 0.125 mg/ml concentrations, respectively).

5.5.1 Fang Feng Tong Sheng Wan (Keli)

The highest inhibition was achieved with the M4 supernatant of FFTS 5 (77.9 ± 2.6 %), for the other four herbal preparations the maximum inhibition was ranging from 65.9 ± 1.5 % to 71.1 ± 1.0 %. An average of 56.5 ± 11.1 % inhibition of α-glycosidase was achieved with all five FFTS preparations included compared to a 92.8 ± 1.2 % of the 8 mg/ml acarbose standard. In another research the results for all 5 herbal preparations measured with the same method and equipment but with different sample preparation and analyst were for FFTS 1 - FFTS 5 59.0 ± 1.5, 48.6 ± 1.6, 58.3 ± 2.0, 56.9 ± 3.2 and 78.2 ± 9.8 %, respectively (28). The most comparable results with this research were achieved with the M1 supernatant. A significant difference is seen between FFTS 5 and the other four samples in both researches. This is probably due to the different drug form; FFTS 5 is granulated meanwhile the other four are in the form of pills. The different purification methods used for supernatant purification (M1-M4) results in different inhibitory activities of the supernatants. Contrary to our expectations the inhibition was higher with M4 compared to M1, while the lower inhibitory activity of M3 compared to M2, matched (Table X). This could not be a consequence of variations in test performance (e.g. preparation of reagents) as standard acarbose solutions gave proper response every time, and also results do not differ significantly within measurements. Maybe it has to do something with the solubility of samples and formation of precipitates. Therefore, more attention should be paid to thorough mixing of the samples right before pipetting them on the microwell titer plate.

Table X: Mean inhibitory activity ± RSD [%] of the herbal preparations FFTS 1 - FFTS 5, and standard acarbose solution 8 mg/ml at first 30 s of measurement. M1 - M4: supernatants purified using four different purification methods.

	FFTS 1	FFTS 2	FFTS 3	FFTS 4	FFTS 5	Acarbose standard
M1	57.5 ± 2.6	46.2 ± 4.3	56.1 ± 4.6	56.3 ± 2.3	73.8 ± 12.2	92.6 ± 1.0
M2	65.7 ± 0.9	61.7 ± 2.1	65.2 ± 1.7	61.9 ± 0.6	75.2 ± 5.8	91.3 ± 0.3
M3	38.6 ± 2.6	26.9 ± 2.6	30.6 ± 3.2	22.5 ± 17.8	39.2 ± 11.9	93.6 ± 0.3
M4	68.1 ± 1.0	65.9 ± 1.5	71.1 ± 1.0	68.9 ± 0.7	77.9 ± 2.6	93.6 ± 0.3
Average (M1-M4)	57.5 ± 23.3	50.2 ± 35.3	55.8 ± 32.1	52.4 ± 39.3	66.5 + 27.5	92.8 ± 1.2

Figure 11 presents the mean percent of inhibition for all four supernatants (M1 –M4) for randomly used representative FFTS herbal preparation, that is FFT3. For other four preparations Table X can be checked. The acarbose standards are also included for comparison. The highest inhibition of 71.1 % (for M4) correlates with the inhibitory activity of S2 acarbose standard (0.25 mg/ml). The inhibitory activity for the other three supernatants (M1 - M3) could not be evaluated as their inhibitory activity was lower than the inhibitory activity of the lowest acarbose standard concentration used (0.125 mg/ml).

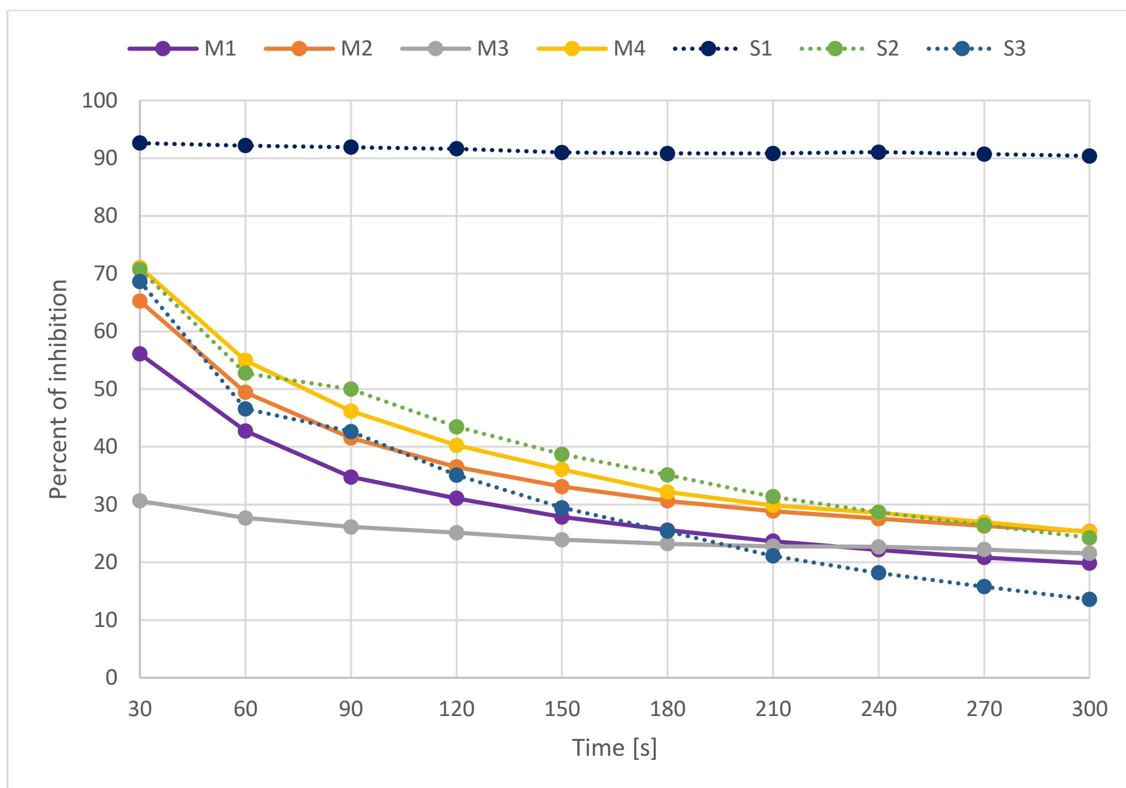

Figure 11: Inhibitory activity of herbal preparation FFTS 3. FFTS = Fang Feng Tong Sheng herbal preparation.

5.5.2 EMW and SMW

The highest inhibition was achieved with M1 supernatants, namely 62.2 ± 4.5 % by EMW and 62.0 ± 0.8 % by SMW. An average of 47.5 ± 37.0 % and 45.6 ± 38.6 was achieved with EMW and SMW, respectively, in comparison with the acarbose standard (91.4 ± 2.5 %) which is lower than the inhibitory activity of FFTS herbal preparations. The average inhibitory activities of the herbal preparations EMW and SMW for all four purification methods are seen in Table XI. There were some differences between individual supernatants (M1-M4). As with FFTS samples, it would be expected that the inhibitory activity of M4 would be lower compared to M1, which was the case, and that of M3 lower compared to M2, which was not the case. A possible explanation is the same as for the FFTS herbal preparations.

Table XI: Mean values ± RSD [%] of samples EMW and SMW, and acarbose standard measured at four different times at first 30 s of measurement.

	EMW	SMW	Acarbose standard
M1	62.2 ± 4.5	62.0 ± 0.8	92.6 ± 1.0
M2	22.0 ± 23.6	20.6 ± 16.9	90.9 ± 0.3
M3	53.7 ± 1.9	50.1 ± 0.8	93.6 ± 0.3
M4	52.2 ± 2.7	49.5 ± 9.5	88.4 ± 0.2
Average (M1-M4)	47.5 ± 37.0	45.6 ± 38.6	91.4 ± 2.5

The mean percentage of inhibition for herbal preparation SMW is shown in Figure 12. Other sample has nearly identical curves. The exact inhibitory activity of M1-M4 could not be determined due to their lower inhibition compared to the acarbose standard 0.125 mg/ml.

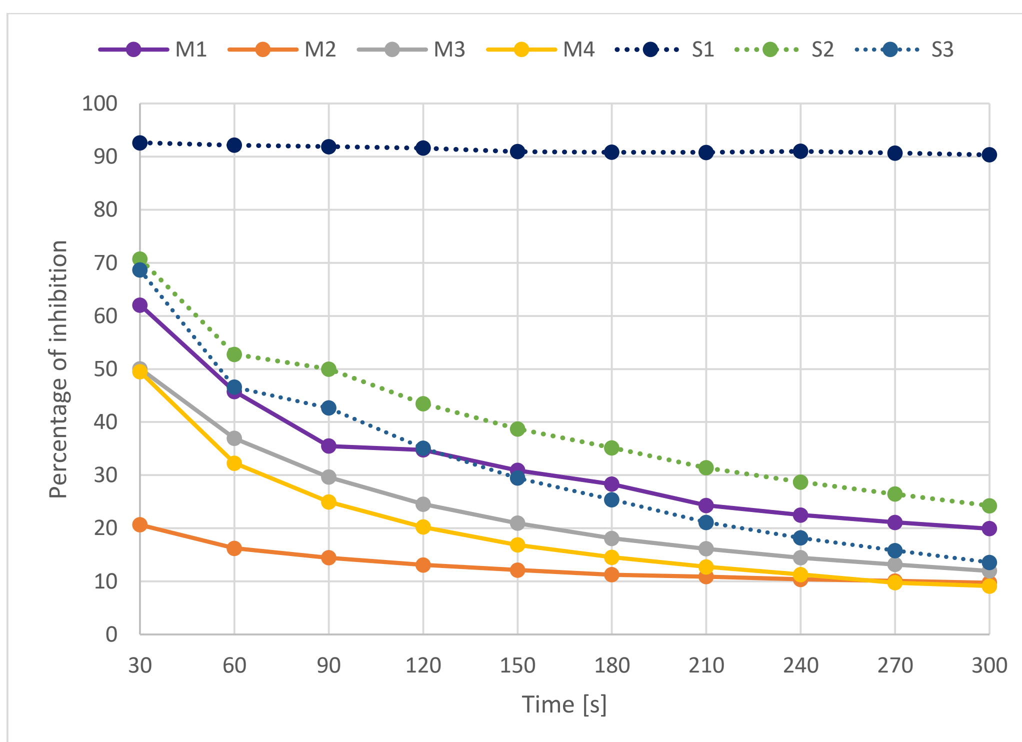

Figure 12: Inhibitory activity of sample SMW. SMW = Si Miao Wan herbal preparation.

6. CONCLUSION

The goal of the work presented in this master's thesis was the evaluation of the enzymatic α-glucosidase inhibitory test. Initially, we examined how precise the test is by determining the repeatability and the intermediate precision. The test fits the criteria for precision, because both RSDs for repeatability and intermediate precision are lower than 15 % (RSD from 0.3 – 0.8 % for repeatability and 1.8 – 2.9 % for intermediate precision). The conditions of the test were changed to check the robustness of the test. The following criteria were chosen as variables: incubation and measurement at room temperature (RT) instead of 37 °C and/or the usage of freshly prepared reagents or stored for 2 hours at RT. The enzyme solution was left for 24 hours at 4 °C to see the stability of the enzyme. In all cases there was no decrease in the inhibitory activity of acarbose standards. The effect of different concentrations of DMSO and different concentration of acarbose standards were tested. We observed that concentrations of DMSO above 6 % show an inhibitory effect on the enzyme and therefore cannot be used in the test. Acarbose concentrations lower than 8 mg/ml should not be utilized because the inhibition is not strong enough. After testing several parameters, it can be concluded that this test remains unaffected by small variations in method conditions and is precise enough to be used for the investigation of potential α-glucosidase inhibitors. The α-glucosidase inhibitory activity of seven TCM preparations was measured and compared. The complex TCM formulation Fang Feng Tong Sheng (FFTS 1-FFTS 5) consists of 18 components. Samples of 5 different suppliers were included in the investigations. Additionally, Ermiao Wan (EMW), a formula containing two herbs and Si Miao Wan (SMW) with four herbs were tested. The highest inhibition was achieved by sample FFTS 5 (77.9 ± 2.6 %), while the other four FFTS samples inhibited the enzyme from 65.9 ± 1.5 % to 71.1 ± 1.0 %. An average of 56.5 ± 11.1 % inhibition of α-glycosidase was achieved including all five FFTS samples with all four supernatants (M1-M4) in comparison to the acarbose standard (92.8 ± 1.2 %). EMW and SMW resulted in a lower inhibition compared to the FFTS samples with an average of 47.5 ± 37.0 % and 45.6 ± 38.6, respectively, in comparison to the acarbose standard (91.4 ± 2.5 %). The highest inhibition of 62.2 ± 4.5 % for EMW and of 62.0 ± 0.8 % for SMW was achieved with supernatant M1. High variations between the different measurements of the same sample could be observed. We excluded possible variation due to test performance, or different preparations and their storage conditions. The reason therefore might be the solubility of samples and the forming

of precipitates. This problem can be solved by paying more attention to the mixing of samples before pipetting them on the microtiter plate or the solubility can be increased by changing the solvent. Regarding to effects of these three herbal formulations on α-glucosidase inhibition *in vitro* all of them could be potentially used in management of T2DM by decreasing postprandial blood glucose level through α-glucosidase inhibition. To confirm this assumption, a clinical trial should be conducted. Further studies should be employed to identify active ingredients and molecular mechanisms of action in each individual plant used and to optimize the herbal formulas to pursue maximum treatment efficiency.

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