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Inhibitory activity of chalcones on key digestive enzymes for the management of Diabetes *mellitus*

Sónia Aguiar da Rocha

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Sónia Aguiar da Rocha

FACULDADE DE FARMÁCIA

Departamento de Ciências Químicas
Laboratório de Química Aplicada

Inhibitory activity of chalcones on key digestive enzymes for the management of Diabetes *mellitus*

Atividade inibitória de chalconas em enzimas digestivas chave para o tratamento da Diabetes mellitus

Dissertação do 2.º Ciclo de Estudos Conducente ao Grau de Mestre em Controlo de Qualidade

Especialização em Fármacos e Plantas Medicinais

Sónia Aguiar da Rocha

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Abstract

Chalcones, with a 1,3-diarylprop-2-en-1-one framework, are secondary metabolites of terrestrial plants and precursors of the flavonoids biosynthesis. Scientific community is currently interested in chalcones, due to their simple chemistry, ease of synthesis, diversity of substituents, safety, and a vast number of recognized biological activities, from which the anti-diabetic activity stands out. Diabetes *mellitus* (DM) is one of the biggest epidemics worldwide, characterized by deficiency in insulin secretion and/or action, leading to chronic elevated levels of glucose in blood, known as hyperglycaemia. The use of anti-diabetic drugs has been increasing worldwide. However, new interventions are required. The inhibition of α-amylase and α-glucosidase, which are carbohydrate-hydrolysing enzymes, is one of the major therapeutic strategies for the treatment of type 2 DM. The inhibition of these two enzymes delays the absorption of monosaccharides on the gastrointestinal tract, preventing the rise of postprandial hyperglycaemia. The currently commercial available inhibitors are associated with gastrointestinal side effects, due to the non-specific inhibition of α-amylase. Low to moderate α-amylase inhibition with potent α-glucosidase inhibition might overcome the inconvenient of the adverse effects. In the present work, a panel of forty-one chalcones with hydroxyl, methoxy, methyl, nitro, chlorine, fluorine and bromine substituent groups were evaluated, most of which for the first time, against α-amylase and α-glucosidase activities. The inhibitory kinetic analysis was also performed for the most active compounds. From the obtained results, chalcone **4** (butein) was the most promising compound, followed by chalcone **21**. The presence of hydroxyl groups at 2' and 4' positions of the A ring and at 3 and 4 positions of the B ring on chalcone **4** (butein), and the presence of a hydroxyl group at 2' position of the A ring and a nitro group at 4 position of the B ring on chalcone **21**, seems to be essential for the inhibitory activity of the chalcones. Chalcone **4** (butein) displayed a competitive type of inhibition for both enzymes. Chalcone **21** presented a non-competitive type of inhibition for α-glucosidase. In conclusion, this study provided potentially anti-diabetic compounds that should serve to the basis for the design and synthesis of more structures, in order to obtain the most potent chalcone' scaffold for the intended activity.

Keywords: *Chalcones; diabetes mellitus; α-amylase; α-glucosidase.*

Resumo

Chalconas, com a estrutura de 1,3-diarilprop-2-en-1-ona, são metabolitos secundários das plantas terrestres e são precursores da biossíntese dos flavonoides. A comunidade científica tem revelado um crescente interesse por estes compostos, devido à sua simplicidade química, facilidade de síntese, diversidade de substituintes, segurança, e o vasto número de reconhecidas atividades biológicas, das quais a atividade antidiabética se tem destacado. A diabetes *mellitus* (DM) é uma das maiores epidemias em todo o mundo, caracterizada por uma deficiência na secreção de insulina e/ou na sua ação, levando a níveis elevados de glicose no sangue, conhecido como um estado de hiperglicemia. O uso de fármacos antidiabéticos tem aumentado em todo o mundo, contudo, novas intervenções são necessárias. A inibição da α-amilase e da α-glicosidase, que são enzimas que hidrolisam hidratos de carbono, é uma das maiores estratégias terapêuticas para o tratamento da DM tipo 2. A inibição destas duas enzimas atrasa a absorção de monossacarídeos no trato gastrointestinal, prevenindo o aumento da hiperglicemia pós-prandial. Os inibidores atualmente comercializados estão associados com efeitos secundários a nível gastrointestinal, essencialmente devido à inibição não especifica da α-amilase. Uma inibição reduzida ou moderada da α-amilase e uma potente inibição da α-glicosidase pode evitar o aparecimento de efeitos adversos. No presente trabalho, o efeito inibitório de um painel de quarenta e uma chalconas, com grupos substituintes hidroxilo, metoxilo, metilo, nitro, cloro, fluor e bromo, foi avaliado, em muitas das quais pela primeira vez, sobre a atividade da α-amilase e da α-glicosidase. O estudo da cinética enzimática e do tipo de inibição foi efetuado para os compostos mais ativos. Segundo os resultados obtidos, a chalcona **4** (buteína) foi o composto mais ativo, seguido pela chalcona **21**. A presença de hidroxilos nas posições 2' e 4' do anel A e nas posições 3 e 4 do anel B da chalcona **4** (buteína), e a presença de um grupo hidroxilo na posição 2' do anel A e um grupo nitro na posição 4 do anel B da chalcona **21**, parecem ser essenciais para a atividade inibitória das chalconas. A chalcona **4** (buteína) demonstrou um tipo de inibição competitiva para as duas enzimas. A chalcona **21** mostrou um tipo de inibição não-competitiva para a α-glicosidase. Em conclusão, este estudo permitiu encontrar potenciais compostos antidiabéticos que podem servir como base para o *design* e síntese de mais estruturas, de modo a encontrar a estrutura da chalcona ideal para a atividade antidiabética.

Palavras-chave: *Chalconas; diabetes mellitus; α-amilase; α-glicosidase.*

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List of Abbreviations

[S]	Concentration of the substrate
ADA	American Diabetes Association
AIC	Akaike information criterion
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
CHR	Chalcone reductase
CHS	Chalcone synthase
CNP	2-Chloro-*p*-nitrophenol
CNPG3	2-Chloro-*p*-nitrophenyl-α-D-maltotrioside
DM	Diabetes *mellitus*
DMSO	Dimethyl sulfoxide
DPP-4	Dipeptidyl peptidase 4
GDM	Gestational diabetes *mellitus*
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
HIV	Human immunodeficiency virus
IC_{50}	Concentration of the inhibitor required to produce 50% inhibition
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
Ki	Inhibitory constant
K_{ic}	Inhibitor dissociation constant of enzyme-inhibitor
K_{iu}	Inhibitor dissociation constant of enzyme-substrate-inhibitor complex
K_m	Michaelis-Menten constant
MGAM	Maltase-glucoamylase
***p*NPG**	*p*-Nitrophenyl-α-D-glucopyranoside
SEM	Standard error of mean
SGLT	Sodium/glucose co-transporter
SI	Sucrase-isomaltase
V_{max}	Maximal velocity
WHO	World Health Organization

General considerations

This work was carried out in the context of the Master's Degree in Quality Control of Faculty of Pharmacy of University of Porto. The theme was chosen based on the growing concern around DM, a chronic, progressive, incompletely understood metabolic condition of the twenty-first century, which is mainly characterized by hyperglycaemia. The anti-diabetic drugs currently used in the management of DM have been showing reduced efficacy and adverse side effects. Therefore, new effective interventions to control hyperglycaemia are required. Chalcones have been recognized for multiple biological activities, including anti-diabetic efficacy. Thus, the aim of this thesis was to compare the inhibitory effect of a panel of chalcones, against the activity of two carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase. For this purpose, the evaluation of α-amylase and α-glucosidase activity was performed using *in vitro* microanalysis methodologies.

Structure of the dissertation

This dissertation is divided in six chapters. The first one, **Introduction,** refers to a theoretical framework with the characterization of chalcones, its chemistry and classification, occurrence, biosynthesis, chemical synthesis and biological activities. In this chapter, it is also possible to find the classification of DM, the risk factors associated to the disease, the pathophysiology, symptoms, complications and treatment of DM. This chapter also includes the definition of the dietary carbohydrates, the description of α-amylase and α-glucosidase and the action of the inhibitors of these enzymes. The general and specific objectives of the dissertation are also present in this section. The second chapter, **Materials and Methods**, described all the experimental aspects related to the *in vitro* inhibitory activity of chalcones against α-amylase and α-glucosidase activity and the corresponding inhibitory kinetic analysis. In the third chapter, **Results**, all the results related to the enzymatic inhibition assays and the subsequent kinetic analysis were described. The chapter four, **Discussion**, contains the integrated discussion of these results. Chapter five, **Conclusions**, summarizes the main conclusions of the performed work. Lastly, chapter six, **References**, has the bibliographical references that supported the development of this work.

1. Introduction

1.1 Chalcones

The medicinal use of natural products, namely compounds that are derived from natural sources such as plants, animals or microorganisms, has been largely applied since the primordial human history. As example, our earliest ancestors chewed on certain herbs to relieve pain, or wrapped leaves around wounds to improve healing. Currently, in less developed countries, natural products constitute almost the sole means to treat diseases and injuries (1).

The modern tools of chemistry and biology have allowed the isolation and the structure elucidation of complex natural products, contributing for the improvement of the drug discovery and development of new pharmacological drugs with therapeutic value. Together, the safety, easy access, and chemical diversity, make these compounds attractive as potential therapeutic agents (1, 2).

Studies on plant secondary metabolites have been increasing over the last years, with special attention to their health benefits and modulatory effects against several human diseases. As natural phytochemicals, polyphenols are among the most abundant and widely distributed secondary metabolites of plants, being important constituents of the human diet (3, 4). These compounds are largely found in fruits, vegetables, tea, coffee, chocolate, legumes, cereals, and beverages. As example, 100 g of fruits, including grapes, apple, pear, cherries, and berries, contain 200-300 mg of polyphenols. Moreover, a cup of tea, coffee or a glass of red wine, contain more than 100 mg (5, 6). Polyphenols comprises a heterogeneous group of molecules, where more than 8000 phenolic structures have been already identified (5). These compounds contain at least one aromatic ring with one or more hydroxyl groups in *ortho*, *meta*, and/or *para* positions (Fig. 1) (7, 8). The distribution and diversity of polyphenols originate different types of classification based on: their sources, biological activities and chemical structures (8). The most important polyphenol subgroups may be classified, based on the number of phenol rings and structural elements that bind theses rings, into: phenolic acids (include the benzoic acids and cinnamic acids, based on C1-C6 and C3-C6 skeletons, respectively); stilbenes (C6-C2-C6 structure); flavonoids (C6-C3-C6 structure) and lignans (C6-C4-C6 structure) (Fig. 1) (4, 5, 9, 10).

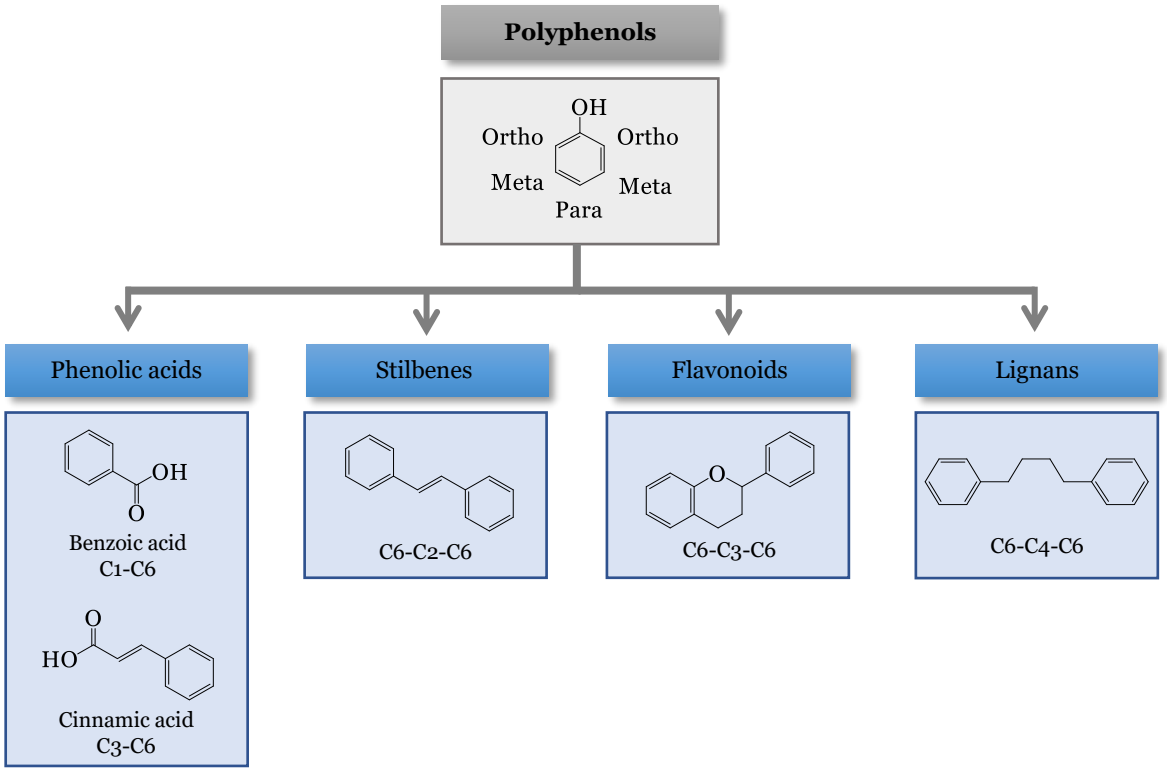


Fig. 1: Main polyphenol subgroups and their respective chemical structure backbone, based on the number of phenol rings and structural elements that bind these rings (4, 10).

Flavonoids are the most abundant polyphenols in the human diet (representing 60% of the polyphenolic compounds) and are distributed in a wide variety of plants (4, 7, 11). These compounds play several important functions in plants, such as protection against pathogens and ultraviolet radiation and are also responsible for a wide range of plant pigmentation patterns (12, 13). In addition, flavonoids are associated with a wide variety of health-promoting effects and biological activities, due to their antioxidant, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties (13). Their biochemical actions depend on the presence and position of substituent groups (12). Chemically, flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B) linked through three carbons, that form the C6-C3-C6 backbone (Fig. 2). The C3 intermediate chain can assume different configurations, ranging from open to heterocyclic chains, condensed with the aromatic A ring. Most of the flavonoids are phenyl benzopyran derivatives, in which the three carbons form an oxygenated heterocycle ring (C ring) (8, 14). Flavonoids can be classified based on the C ring hydroxylation and opening, into their main subclasses, namely: isoflavones, anthocyanins, dihydroflavonols, flavanones, flavones, flavan-3-ols, flavonols, dihydrochalcones and chalcones (Fig. 2) (14-16).

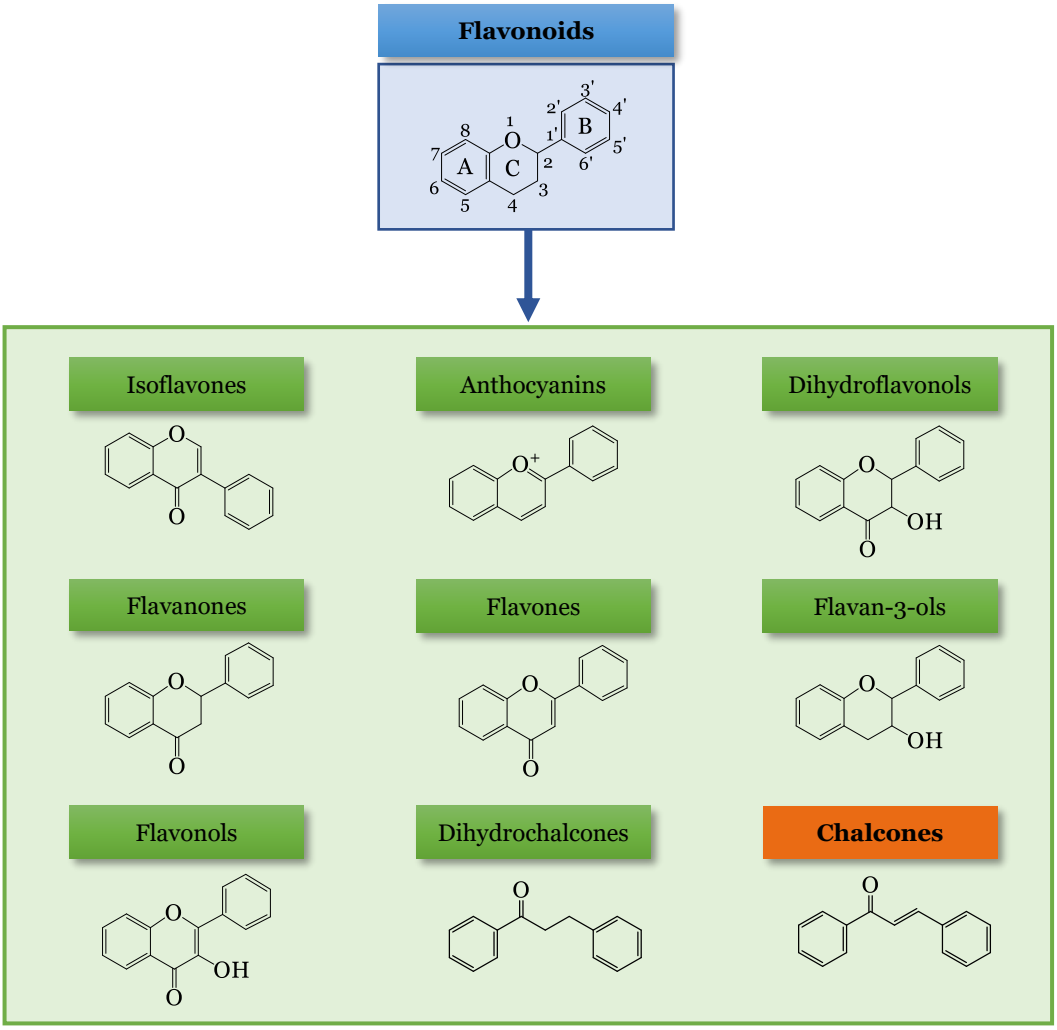


Fig. 2: Main flavonoid subclasses and their respective chemical structure backbone, based on the C ring hydroxylation and opening (14-16).

Chalcones are considered one of the most important subclasses of flavonoids, characterized as the open-chain precursors of flavonoids (17). They also contain a C6-C3-C6 backbone, with an unsaturated C3 chain structure linking the A and B rings, instead of a heterocycle C ring (Fig. 2) (13, 18, 19). Naturally occurring chalcones are biosynthesized through the polyketide and phenylpropanoid pathways and have been isolated from different parts of plants (20). Over the last years, increasing attention has been dedicated to chalcones, due to their simple chemistry, ease of synthesis, diversity of substituents, safety, and a vast number of recognized biological activities (21-23) (Fig. 3) that will be discussed in the section 1.1.5.

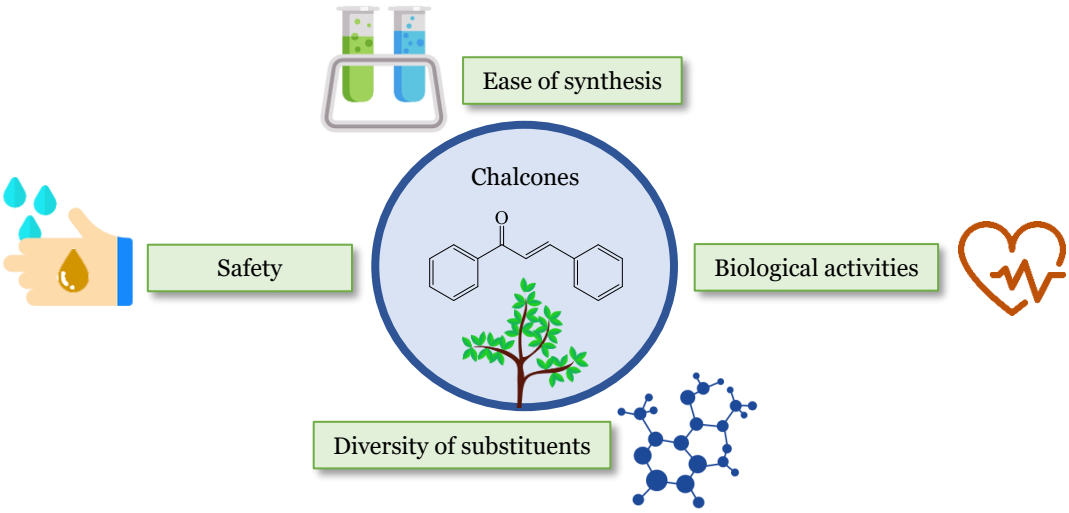


Fig. 3: The characteristics that contribute for the importance of chalcones on drug discovery.

This interest by the scientific community is represented in the Fig. 4, which show the increasing number of published papers in the Pubmed database in the last 17 years.

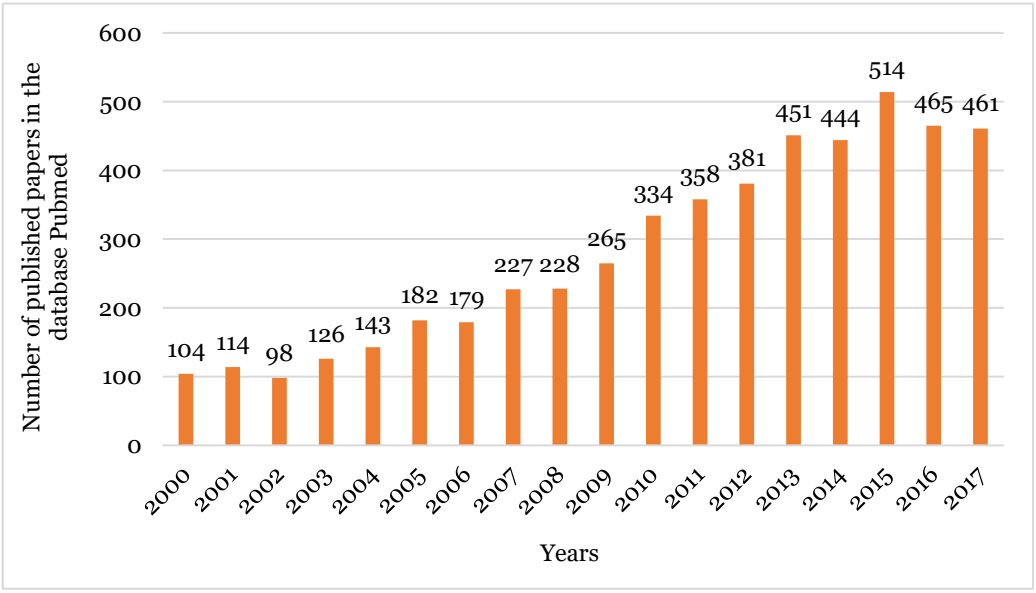


Fig. 4: Number of publications with the word “chalcones” or “chalcone” on the PubMed database, from 2000-2017.

The diversity of chalcones found in the Plantae Kingdom and the above mentioned peculiar characteristics, have inspired the synthesis of different new chalcone derivatives (24), in order to obtain more effective chemical structures in the management and/or treatment of several diseases, such as DM, obesity, tuberculosis and cancer (25). A growing number of new techniques and synthetic routes have been reported for the synthesis of chalcones. The development and isolation of novel chalcones constitute a promising approach for the design and discovery of new chemicals as potential pharmacological drugs (26). Their chemistry and classification, occurrence and distribution on nature, biosynthesis, chemical synthesis and biological activities will be described in the next sections.

1.1.1 Chemistry / Classification

Carthamin was the first chalcone described as a naturally occurring compound from *Carthamus tinctorius,* known as safflower, a dye plant widely used in India (27).

In 1899, Kostanecki together with Josef Tambor, introduced the term "chalcone" (28). "Chalcone" is a generic term used to describe compounds with a 1,3-diarylprop-2-en-1-one framework (Fig. 5). These compounds are also known as benzalacetophenone or benzylidene acetophenone. The term chalcone is derived from the Greek word "chalcos", meaning "bronze", which results from the colour of the majority of chalcones, that is due to the presence of the chromophore -CO-CH=CH-, or to other auxochromes (18, 29).

The existence of a double bond between α and β carbons, results in either *cis* (*Z*) and *trans* (*E*) (Fig. 5) isomeric forms, of which the *trans* is the most thermodynamically stable (21, 24, 30), making it the most common configuration among chalcones (17), being this representation system followed throughout this work. In what concerns the nomenclature, the phenyl ring linked to the carbonyl group is defined as A ring, numbered from 1' to 6', and the B ring is numbered from 1 to 6, and this numeration system is used throughout this thesis (Fig. 5) (18, 29, 31).

Chalcones' properties depend on the ring substitutions and on the presence of the α, β-unsaturated ketone moiety (31). The α, β-unsaturated ketone moiety makes chalcones biologically active, and the exclusion of the carbonyl system, makes them biologically inactive, ensuring stability in both *cis* and *trans* forms. Chalcones display conjugated double bonds with absolute delocalization and two aromatic rings with π bonds, which confers moderately low redox potential and the possibility of occurring electron transfer reactions (18).

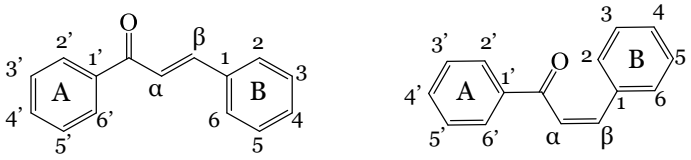


Fig. 5: Structural and numerical representations of chalcone isomeric forms (17).

As above mentioned, chalcones are secondary metabolites of terrestrial plants and are considered precursors of the biosynthesis of flavonoids (32, 33). Indeed, chalcones are one of the most structurally diverse group of flavonoids (34), due to the number and positions of the several substituents, including hydroxyl, methoxy, methyl, prenyl, geranyl, lavandulyl, pyran, furan and glycosyl derivatives (Fig. 6) (23, 32).

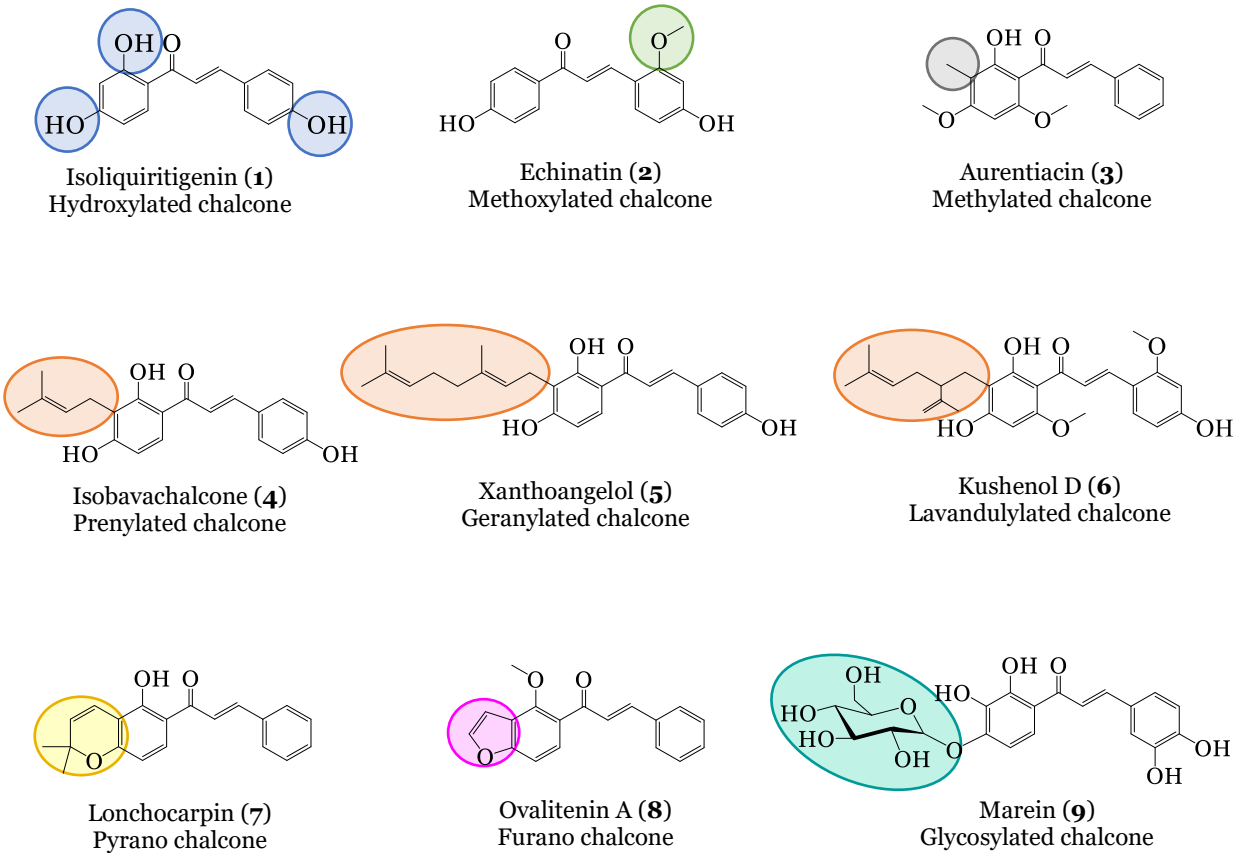


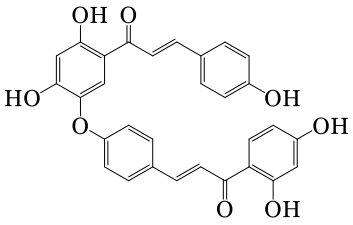
Fig. 6: Examples of naturally occurring chalcones with hydroxyl (**1**), methoxy (**2**), methyl (**3**), prenyl (**4**), geranyl (**5**), lavandulyl (**6**), pyran (**7**), furan (**8**) and glycosyl (**9**) substituents.

Rozmer and Perjési (23) reported the existence of more than 400 naturally occurring chalcones (a research from 1975 until 2014). Hydroxylated (**1**, Fig. 6) and methoxylated (**2**, Fig. 6) are the most commonly chalcones found in nature (35). In the

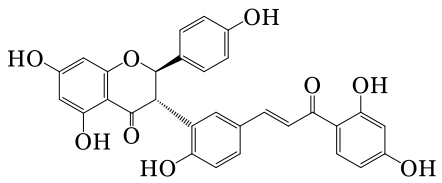
Rozmer and Perjési's study (23), among the 400 identified natural chalcones, almost 100 were hydroxyl and/or methoxy substituted in both A and/or B rings. Also, the number of hydroxyl and methoxy moieties may vary between 1 and 6, being the tri, tetra and penta-substituted the most commonly found. Chalcones with the same substituent groups in A and/or B rings are uncommon, as example, only 13 compounds presented only hydroxyl group substituents and 3 had only methoxy substitutions (23).

An important and common group within the natural occurring chalcones is the prenyl one. The term 'prenylchalcones' includes the compounds with prenyl (**4**, Fig. 6), geranyl (**5**, Fig. 6) and farnesyl (lavandulyl-groups) (**6**, Fig. 6) side attachments. The most frequent type of prenylation is represented by the 3,3-dimethylallyl substitution (prenyl group). The prenylated chalcones can also have hydroxyl and/or methoxy groups. Generally, most chalcones are *C*-prenylated being the *O*-prenylated chalcones the most uncommon type (23, 32).

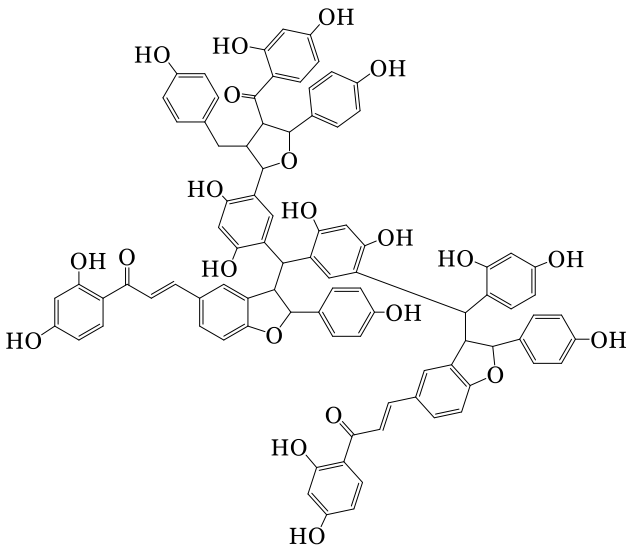
Besides the above mentioned substitutions (Fig. 6), and despite the majority of natural chalcones being found in monomeric form (23), chalcones can also occur as dimers (**10**, Fig. 7), heterodimers (**11**, Fig. 7) and oligomers (**12**, Fig. 7) (32, 36).



Rhuschalcone II (**10**)
Dimeric chalcone



6"'- Hydroxylophirone B (**11**)
Heterodimeric chalcone



Azobechalcone (**12**)
Oligomeric chalcone

Fig. 7: Examples of natural chalcones occurring as dimers (**10**), heterodimers (**11**) and oligomers (**12**).

1.1.2 Occurrence

Chalcones were originally discovered as yellow flower pigments in the Asteraceae family (also known as Compositae), which includes a very large and widespread family of flowering plants (23). Thus, chalcones are the secondary metabolites responsible for the yellow pigments found in certain flowers, crucial for the attraction of pollinators such as insects and birds. Along with the other flavonoids subclasses, chalcones also play a central role in plant radiation protection, pathogen defence, seed dispersal and enzyme inhibition (32). However, these natural compounds are not restricted to flowers. Chalcones are also widely distributed in other parts of the plants, namely in leaves, fruits, roots stem, resin and seeds (30, 32). A high number of chalcones has been identified in the Leguminosae (Fabaceae) and Moraceae families (23). In the Leguminosae family, chalcones are mostly obtained from the root (23) and have been found mainly in the genera *Glycyrrhiza, Crotalaria, Derris, Flemingia, Lonchocarpus, Milletia, Sophora* and *Tephrosia*. The genus *Glycyrrhiza*, known as licorice, includes around 30 species, being *Glycyrrhiza inflata* one of the species with the highest number of isolated chalcones in nature, namely isoliquiritigenin (**1**, Fig. 8) and licochalcone A (**13**, Fig. 8) (26, 37). Another relevant specie from Leguminosae family is *Psoralea corylifolia,* which contains a large amount of chalcones, and is particularly rich in isobavachalcone (**4**, Fig. 6) (26, 38).

In Moraceae family, chalcones are well distributed among the different plant parts, namely in leaves, twigs and roots. This family is well-known for its high content in chalcones, namely in the genera *Artocarpus*, *Dorstenia* and *Morus*. The species *Artocarpus communis, Dorstenia barteri* and *Morus nigra* contain a large amount of chalcones (23).

In the Asteraceae family, chalcones are usually distributed in the aerial part, leaves, flowers and stem. Various chalcones have also been isolated from the *Helichrysum* genera, in which the specie *Helichrysum forskahlii* has the higher quantity. The specie *Metalasia cymbifolia* also exhibit a considering variety of chalcones (23).

Along with *Glycyrrhiza inflata* (Leguminosae), *Humulus lupulus* and *Angelica keiskei* are the species with the greatest number of isolated chalcones (23, 39). The specie *Humulus lupulus*, from Cannabinaceae family, known as hop plant, contains xanthohumol (**14**, Fig. 8), a prenylated chalcone. This plant is commonly used in beer production, to add bitterness and flavour. Consequently, beer is one of the major dietary source of xanthohumol (40).

Angelica keiskei (from Umbelliferae family) is an edible plant known in Japan as "ashitaba", which has various types of chalcones in its constitution (41). *Angelica keiskei* has been consumed as a healthy food with recognized biological activities, such as anti-

inflammatory, anti-obesity and anti-tumor, particularly attributed to the presence of xanthoangelol (**5**, Fig. 6) (42).

Some identified natural occurring chalcones and its distribution in nature are summarized in Fig. 8.

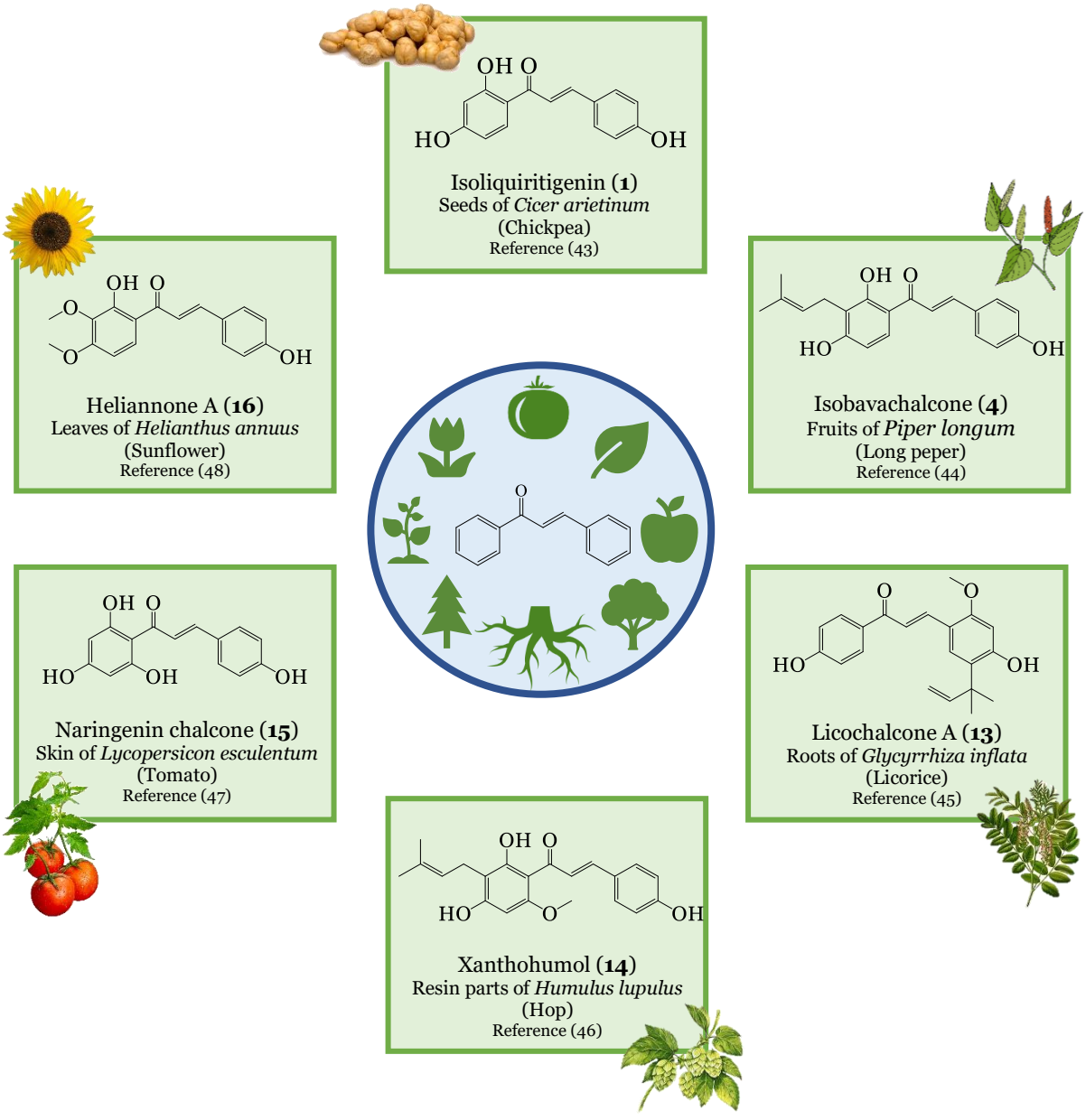


Fig. 8: Some examples of natural occurring chalcones, their chemical structures and distribution in nature.

1.1.3 Biosynthesis

Chalcones can be biosynthesized in specific plant tissues in response to various internal and external factors, such as the plant development, the nutrient status, environmental variations and stress induction (32).

The chalcone biosynthesis consists in the conjunction of two biosynthetic pathways, the polyketide and the phenylpropanoid, responsible for the formation of A and B rings, respectively (Fig. 9). In the phenylpropanoid pathway, the enzyme phenylalanine ammonia-lyase (PAL) is responsible for the removal of an amino group of the amino acid phenylalanine (key phenylpropanoid precursor), originating cinnamic acid. The cinnamic acid follows two stepwise enzymatic conversions, catalysed by cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA-ligase (4CL), leading to the formation of 4-coumaroyl-CoA, the main precursor of chalcones (23, 32). Simultaneously, in the polyketide pathway, the reaction of acetyl-coA with acetyl-coA carboxylase (ACC) results in the production of malonyl-CoA (32). The production of chalcones results from the condensation of one molecule of 4-coumaroyl-CoA with three malonyl-CoA molecules, which is catalysed by the chalcone synthase (CHS) (49). Besides 4-coumaroyl-CoA, CHS can also use caffeoyl-CoA, hexanoyl-CoA, benzoyl-CoA or feruloyl-CoA. However, coumaroyl-CoA is the most efficiently used and, in some cases, the exclusive substrate of these enzyme (23, 36).

CHS is a key enzyme responsible for the biosynthesis of chalcones. CHS is a representative member of type III polyketide synthases (PKS), responsible for the biosynthesis of different metabolites in plants. CHS is activated by environmental and developmental stimuli, depending on the stress conditions, such as UV light, fungal and/or bacterial infections (23, 50). Besides CHS, a growing number of functionally divergent CHS-like type III PKS have been identified (51). In general, these enzymes originate a complete series of decarboxylation, condensation, cyclisation and aromatization reactions with a single active site, which can result in the formation of two main types of chalcones, differing on the presence of a hydroxyl group at the 6' position. The condensation of malonyl-CoA with 4-coumaroyl-CoA, catalysed by CHS, result in the formation of 6'- hydroxychalcones, as naringenin chalcone (**15**, Fig. 9). Plants generally produce chalcones with a hydroxyl group at the 6' position, however, in some plants, such as in Leguminoseae family, the presence of a second polyketide reductase leads to the formation of other chalcones. If chalcone reductase (CHR) is simultaneously active with CHS (CHS/CHR), 6'-deoxychalcones are produced, as isoliquiritigenin (**1**, Fig. 9). In both cases, 6'-hydroxychalcones or 6'- deoxychalcones, are substrates and key intermediates for the synthesis of other flavonoids (23, 32).

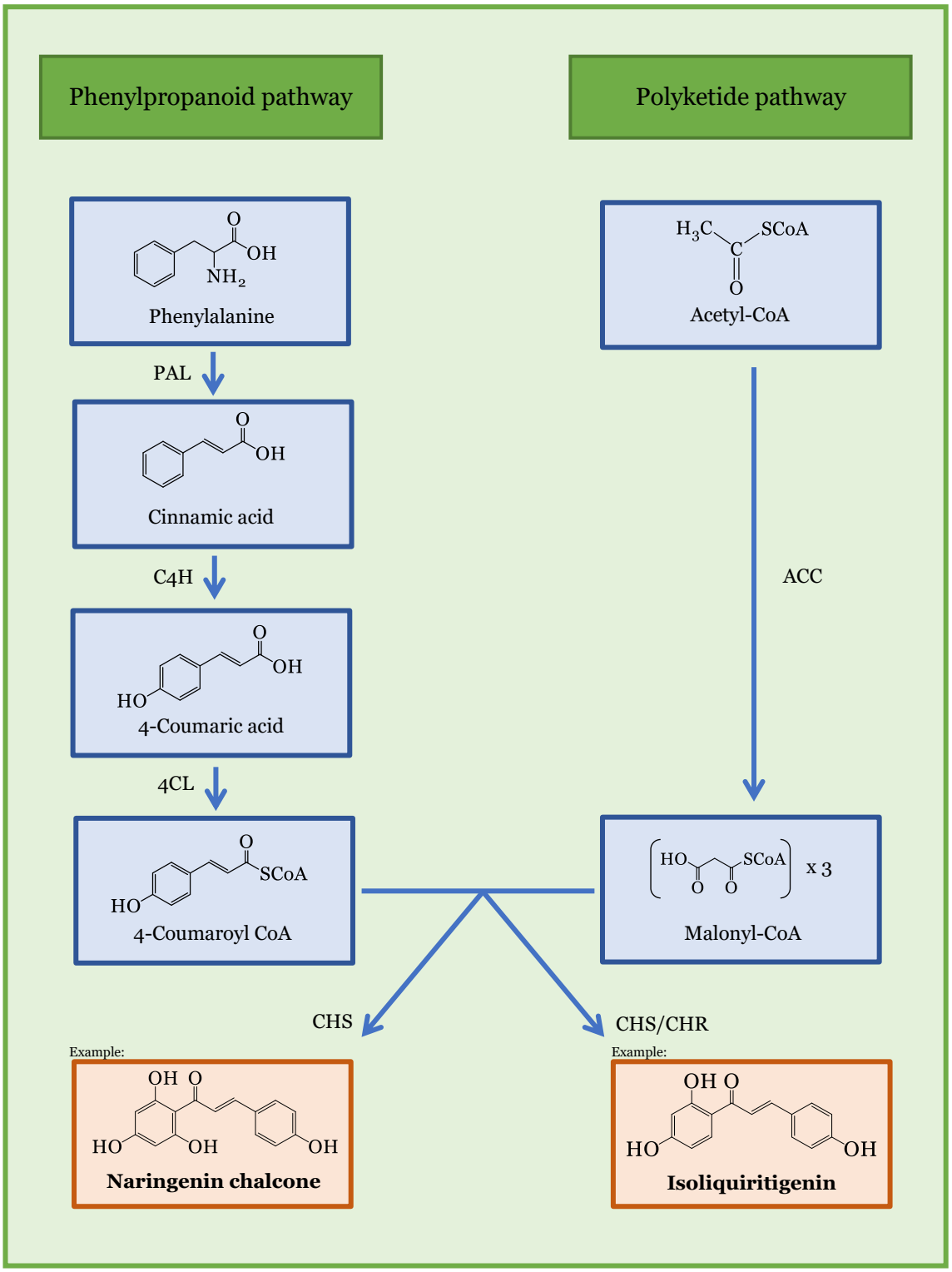


Fig. 9: Summary of the biosynthesis pathway of chalcones.

4CL, 4-coumarate CoA-ligase; ACC, acetyl-CoA carboxylase; C4H, cinnamate 4-hydroxylase; CHR, chalcone reductase; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase.

1.1.4 Chemical synthesis

The privileged chalcone scaffold remained a fascination among researchers in the 21st century, due to the great number of replaceable hydrogens, which allow the synthesis of a large number of derivatives (17). Although chalcones occur naturally, their isolation from natural sources involves extended procedures. Due to the time-consuming and intensive processes of isolation techniques, synthetic chalcones appeal the attention of the researchers. Therefore, it is possible, through an efficient and simple synthesis, to obtain, in large amounts, chalcone derivatives with various functional groups (52). Recent literature describes several procedures for the synthesis of chalcones and several researchers search for alternative routes for the synthesis of this type of compounds and new derivatives with improved properties (31). In each of these procedures, the most important part is the condensation of two aromatic systems (with nucleophilic and electrophilic groups) to form the chalcone structure (17). Several techniques have been explored for the synthesis of chalcones. The most commonly used are Claisen–Schmidt condensation, Suzuki coupling reaction, Carbonylative Heck coupling reaction, Julia–Kocienski olefination, Friedel–Crafts acylation reaction (26, 29), that are summarized below:

- **Claisen–Schmidt condensation**

Among all methods, the Claisen–Schmidt condensation, also known as Aldol Condensation, is the most used technique and is one of the most classical reactions in organic chemistry, because of the accessibility of the preliminary materials, such as acetophenones and benzaldehydes (31, 53, 54). Claisen–Schmidt condensation (Fig. 10) consists in a condensation of acetophenone and benzaldehyde derivatives, in the presence of alkaline or acid catalysts in liquid solvent, for several hours. The resulting chalcone comprise the A ring substituents provided by the acetophenone and B ring substituents supplied by the benzaldehyde. Some improvements to this technique have been done, that include solvent-free conditions, microwave and ultrasound irradiation, which results in reduced synthesis time and in the increased yield of the reaction (17, 26, 31).

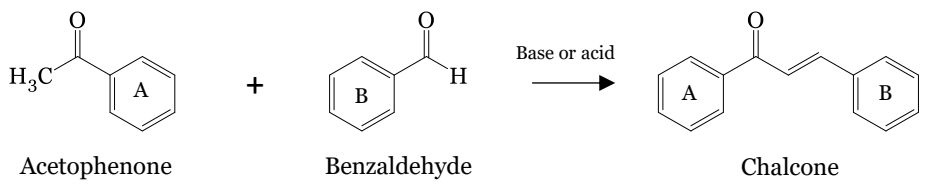


Fig. 10: Classical Claisen–Schmidt condensation (17).

- **Suzuki coupling**

In the Suzuki coupling reaction (Fig. 11), two possible approaches are possible for the chalcone synthesis: one that involves the coupling of benzoyl chloride with styrylboronic acid (Fig. 11 A), and another that involves the coupling between phenylboronic acid and cinnamoyl chloride (Fig. 11 B). Both reactions occur in the presence of palladium catalysts, base and anhydrous toluene (17, 29).

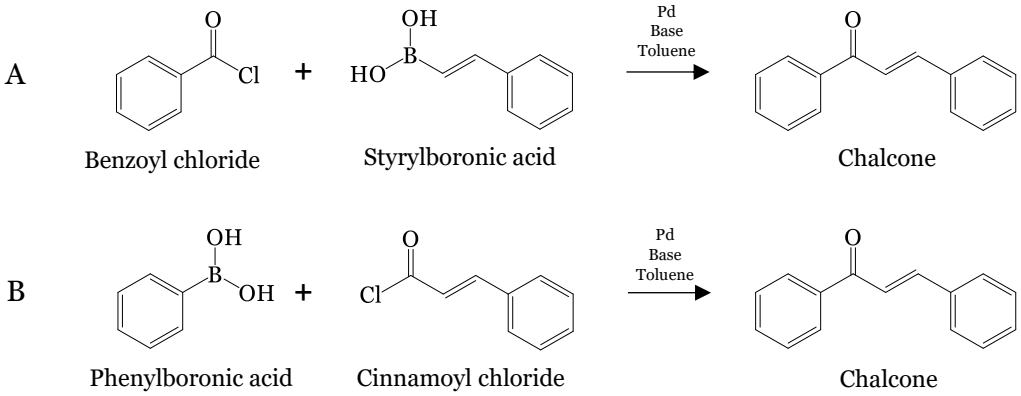


Fig. 11: Suzuki coupling reaction (17).

- **Carbonylative Heck coupling**

Carbonylative Heck coupling reaction (Fig. 12) involves the carbonylative vinylation between a phenyl halide and a styrene, in the presence of carbon monoxide and a palladium catalyst (17).

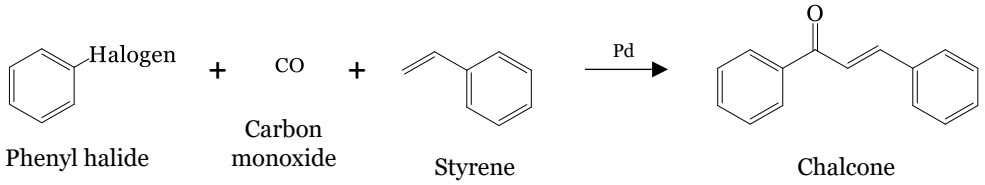
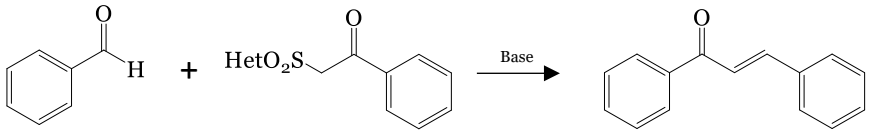


Fig. 12: Carbonylative Heck coupling (17).

- **Julia–Kocienski olefination**

Julia–Kocienski olefination (Fig. 13), that is a modification of the classical Julia olefination reaction, is based in the coupling between heteroarylsulfones and carbonyl compounds, in basic media, in a one-step protocol to produce chalcones (55).

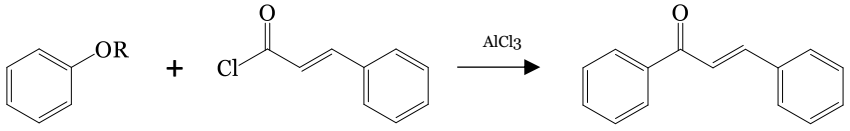


Benaldehyde Heteroarylsulfone Chalcone

Fig. 13: Julia–Kocienski olefination (29).

- **Friedel–Crafts acylation**

Chalcones can be synthesized by the Friedel–Crafts acylation reaction (Fig. 14) of an aromatic ether and a cinnamoyl chloride, in the presence of a strong Lewis acid catalyst, such as $AlCl_3$, that is essential to increase the polarity (29).



Aromatic ether Cinnamoyl chloride Chalcone

Fig. 14: Friedel–Crafts acylation (29).

1.1.5 Biological activities

Over the years, naturally occurring chalcones, isolated from different plants, and various compounds inspired on the chalcone skeleton, have attracted much interest, not only due to the above mentioned synthetic and biosynthetic perspectives, but also due to the promising biological activities (Fig. 15) (21). There are several reviews about the biological activities of natural and synthetic chalcones, highlighting their pharmacological potential (23, 26, 34).

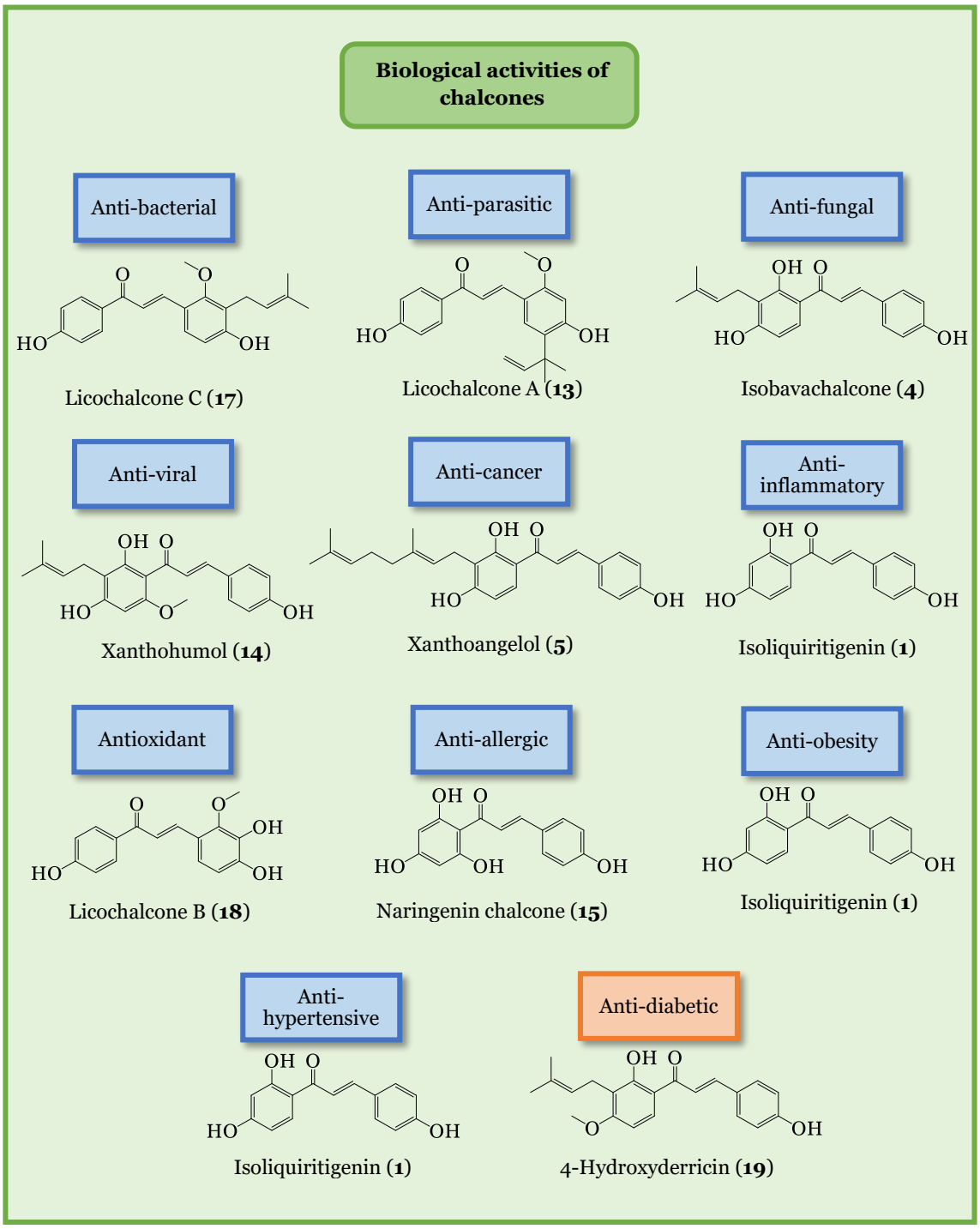
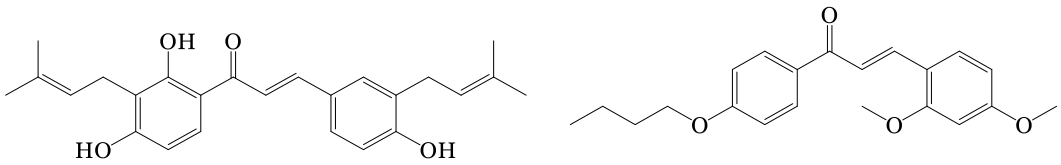


Fig. 15: Biological activities of chalcones, followed by some examples of chalcones displaying the respective activity (23, 29).

As displayed in Fig. 15, chalcones exhibit a wide range of biological activities, including anti-bacterial activity (56, 57). This activity has been related to the ability of the unsaturated ketone of chalcones to react with the nucleophilic group of essential proteins of bacteria. As example, licochalcone A (**13**, Fig. 15) and licochalcone C (**17**, Fig. 15) showed

effective anti-bacterial activity, especially against *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* (30). Tuberculosis, caused by *Mycobacterium tuberculosis*, is a global health problem, being one of the top causes of death worldwide, alongside with the human immunodeficiency virus (HIV) (58). *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium kansasii*, *Mycobacterium xenophii* and *Mycobacterium marinum* were also inactivated by licochalcone A (**13**, Fig. 15) (30), indicating that this chalcone might be of interest as an anti-bacterial drug (23). Nielsen *et al.* (59) studied A ring substituted 4'-carboxy chalcones and demonstrated that these compounds were interesting as anti-bacterial compounds.

Chalcones have also revealed anti-parasitic properties, namely against *Leishmania* protozoan parasite, acting as anti-leishmanial agents. Licochalcone A (**13,** Fig. 15) and kanzonol C (**20,** Fig. 16) exhibited potent anti-leishmanial activity, mainly by the interference with the mitochondria activity of the parasite (60) (61, 62). Zhai *et al.* (63) tested new oxygenated chalcones that inhibited the *in vitro* growth of parasites, which might be also a consequence of the interference with the function of the mitochondria of the parasite. Chalcones are also described as anti-malarial agents. Malaria parasites are micro-organisms that belong to the genus *Plasmodium* (64, 65). Licochalcone A (**13**, Fig. 15) is one of the most promising compounds. Xanthohumol (**14**, Fig. 15), was also found to be active as an anti-malarial agent (23). 2,4-Dimethoxy-4′-butoxychalcone (**21**, Fig. 16) (66), an analogue of licochalcone A, exhibited potent anti-malarial activity and might lead to a novel anti-malarial drug.



Kanzonol C (**20**) 2,4-Dimethoxy-4'-butoxychalcone (**21**)

Fig. 16: Kanzonol C and 2,4-dimethoxy-4'-butoxychalcone, examples of an effective anti-leishmanial agent and an anti-malarial agent, respectively.

Anti-fungal activity of chalcones has also been described (67, 68). Isobavachalcone (**4**, Fig. 15) showed inhibitory activity against *Candida albicans* and *Cryptococcus neoformans* (23). Gupta and Jain (67) synthesized new derivatives of chalcones and showed their anti-fungal activity against *Microsporum gypseum*.

Chalcones have also anti-viral (69, 70) properties that depend on their substitution patterns. Xanthohumol (**14**, Fig. 15) may represent some potential as a novel therapeutic agent for acquired immunodeficiency syndrome (AIDS), caused by HIV (30).

Several natural and synthetic chalcones revealed anti-cancer activity (71), which is one of the most studied biological activities of these compounds (26). Chalcones display anti-cancer activity through multiple mechanisms, including cell cycle disruption, angiogenesis inhibition, apoptosis induction, tubulin polymerization inhibition, etc (72). As example, xanthoangelol (**5**, Fig. 6) induces apoptotic cell death in neuroblastoma and leukemia cells (73). Xanthohumol (**14**, Fig. 15) may be an interesting chemopreventive agent against prostate carcinogenesis, by induction of apoptosis and down-regulation of the nuclear factor kappa B (NK-κB), a transcription factor involved in inflammatory diseases and cancer (21). In Syam's study (74) some chalcones were synthesized and tested against human cancer cell lines, including human breast, lung, prostate, colorectal and liver cells. Most of the compounds were very active cytotoxic agents and induced apoptosis in the studied cancer cell lines.

The anti-inflammatory (75, 76) and antioxidant (77, 78) activities of chalcones are also well known. Inflammation is a biological response comprising complex cytological and chemical reactions, in response to an injury or harmful stimuli that together protect our organism. However, the persistence of inflammation in the body leads to various complications and diseases, such as atherosclerosis, diabetes and cancer. Chalcones have been reported as good anti-inflammatory agents, namely isoliquiritigenin (**1**, Fig. 15) and naringenin chalcone (**15**, Fig. 15). The antioxidant activity of natural compounds is also relevant. In chalcones this activity is associated with the hydroxyl and methoxy substitutions, as demonstrated by the activities of isoliquiritigenin (**1**, Fig. 15), licochalcone B (**18,** Fig. 15) and by other hydroxylated chalcones (23).

Chalcones were also found to act as anti-allergic compounds, through the inhibition of histamine release. Naringenin chalcone (**15**, Fig. 15) is one of the most effective chalcones in reducing the allergic symptoms (79).

Anti-obesity properties have also been studied, and isoliquiritigenin (**1**, Fig. 15) demonstrated some potential in the inhibition of triacylglycerol acyl hydrolase, the main lipolytic enzyme, which is one of the approaches for the development of novel anti-obesity drugs (80).

Chalcones also display anti-hypertensive activity (81, 82). Hypertension is associated with an increased cardiovascular risk, constituting one of the major public health problems. Isoliquiritigenin (**1**, Fig. 15) display cardioprotective effects, due to the protection against myocardial ischemia and the decrease of the plasma total cholesterol and triglycerides levels (23).

DM is one of the major public health problems with epidemic proportions, thereby, the anti-diabetic potential of chalcones, one of the most relevant biological activity of these compounds, will be the focus of this work.

1.2 Diabetes *mellitus*

DM is one of the main worldwide emergency issues of the 21st. century. It is, perhaps, one of the oldest diseases, first described in Egyptian manuscripts about 3000 years ago (83). The term "diabetes" is derived from the Greek word "Diab" and means "pass through heavy thirst and frequent urination", and "*mellitus*" is derived from the Latin and means "sweetened with honey", and refers to the presence of glucose in urine (84). DM is a group of metabolic disorders, with abnormalities in the metabolism of carbohydrates, lipids and proteins, and is characterized by the presence of chronic elevated levels of glucose in blood, known as hyperglycaemia (83, 85). DM occurs when β cells of the pancreatic islets of Langerhans do not produce sufficient insulin (Fig. 17), a peptide hormone that regulates the glucose levels in blood, or when cells do not respond properly to the insulin that is produced, or even in both situations. In non-diabetic subjects, insulin binds to specific receptors present on many cells, including fat, liver and muscle cells, stimulating glucose uptake and balancing blood glucose levels (Fig. 18) (86).

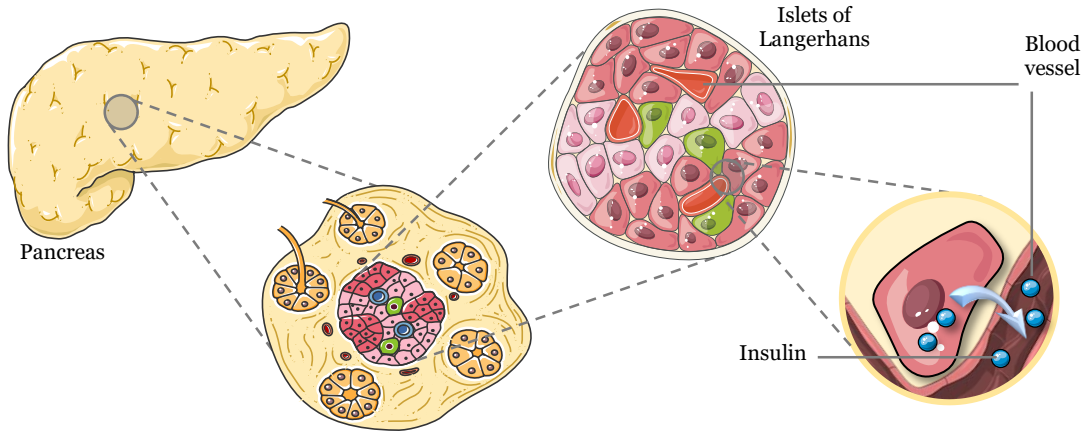


Fig. 17: Pancreatic islets of Langerhans and insulin release. Adapted from (87).

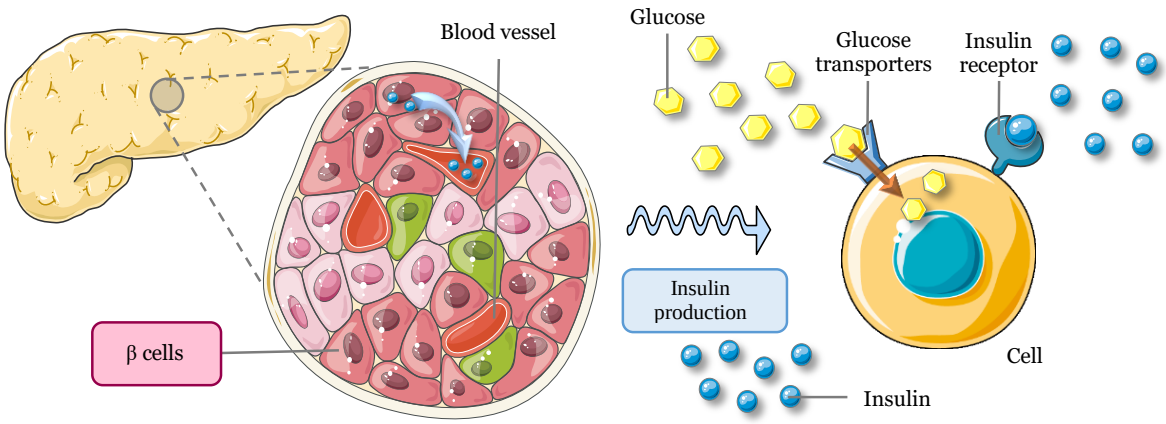


Fig. 18: Schematic representation of insulin release and glucose uptake in non-diabetic subjects. Adapted from (88).

International Diabetes Federation (IDF) (89) reported that, in 2017, DM caused almost 4.0 million deaths, which corresponds to 1 death at every eight seconds. It was estimated that 425 million people have DM and 352 million have impaired glucose tolerance (IGT), which is characterized by high glucose levels after a meal, with a consequent high risk of developing DM in the future. It is important to note that 212.4 million (50%) of the people with DM are undiagnosed. Furthermore, it was estimated that, by 2045, the number of patients with DM will increase to 629 million (Fig. 19) (89). The global estimative of the number of diabetic patients per globe region, in 2017 and 2045, is represented in Fig. 19. Particularly in Europe, in 2017, the estimated number of people with DM is 58 million, including 22 million of undiagnosed cases. 36 Million people was estimated to have IGT. Europe has the highest prevalence of children and adolescents with type 1 DM, near 286,000 children, with a growth of 28,200 new cases per year. In 2017, it was estimated that in Portugal the prevalence of DM was 13.9%, representing more than 1 million of adults, from whom, near 460,000 were undiagnosed. During this year, it was also estimated that, in Portugal, almost 8000 deaths are due to DM (89).

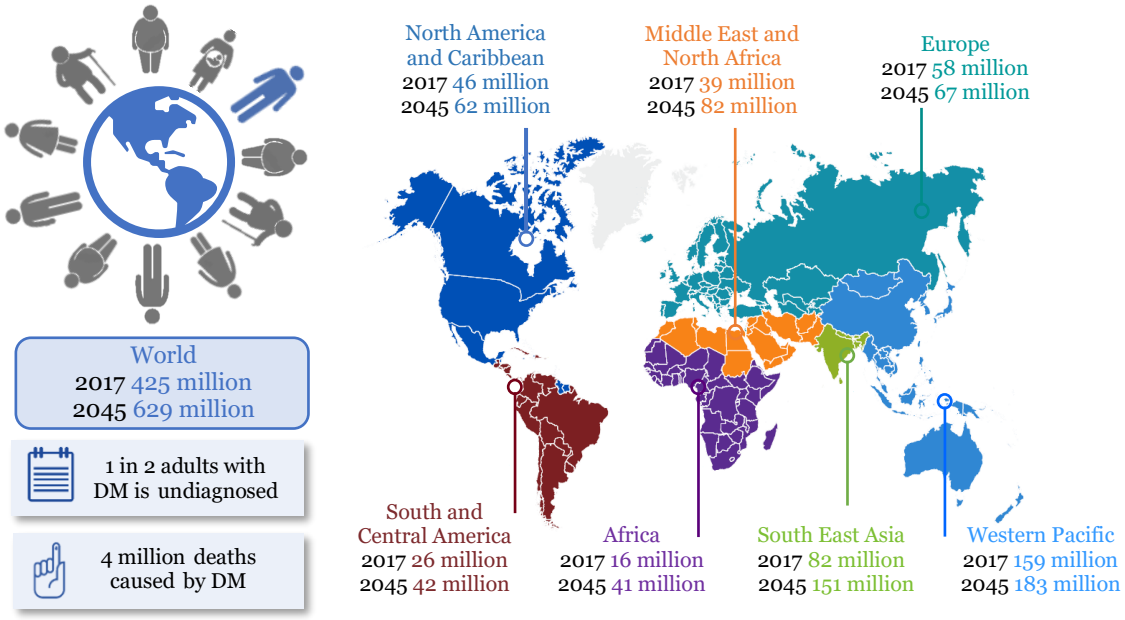


Fig. 19: Estimated number of people living with DM worldwide and per region, in 2017 and 2045, according to the last estimative by IDF (89).

1.2.1 Classification of Diabetes *mellitus*

The classification of DM has been a continuous subject of debate and revision over many decades (89). The American Diabetes Association (ADA) (90) classifies DM into four main types: type 1 DM, type 2 DM, gestational DM (GDM) and other currently accepted

specific types. ADA (90) also classifies an intermediate form between normal glucose levels and DM, characterized as “prediabetes”.

- **Type 1 Diabetes *mellitus***

Type 1 DM represents around 7-12% of all cases of DM (89) and includes two forms, idiopathic and autoimmune DM. Idiopathic type 1 DM is an unusual form with unknown cause, with almost complete insulin deficiency and no evidence of autoimmunity. Although being a rare form, most patients are of African or Asian descent. This category of type 1 DM exhibits a strong hereditary component and lacks immunological evidence for β cell autoimmunity (91, 92).

Autoimmune type 1 DM (Fig. 20) (70-90% of the patients with type 1 DM), previously known as insulin-dependent, juvenile or childhood-onset DM, involves an autoimmune destruction of the insulin-producing β cells in the pancreas, which results in a deficient insulin production (86). Autoimmune type 1 DM is associated with the formation of autoantibodies that can occur years before the onset of any clinical symptoms, serving as biomarkers of the development of autoimmunity. The cause of β cell autoimmunity remains unknown. However, it is believed that is related to a combination of specific genetic and environmental factors. The disease starts with the appearance of autoantibodies, with consequent pancreatic β cell destruction. It results in insulin insufficiency, and patients develop life-threatening hyperglycaemia. With the disease progression, symptoms such as thirst, weight loss and frequent urination appear (88). These symptoms often appear in children and adolescents, however, can also appear in adults. The progression of the disease is extremely individualized (93). Almost all patients require daily administration of insulin to regulate the amount of glucose in blood, in order to achieve normoglycaemia (91).

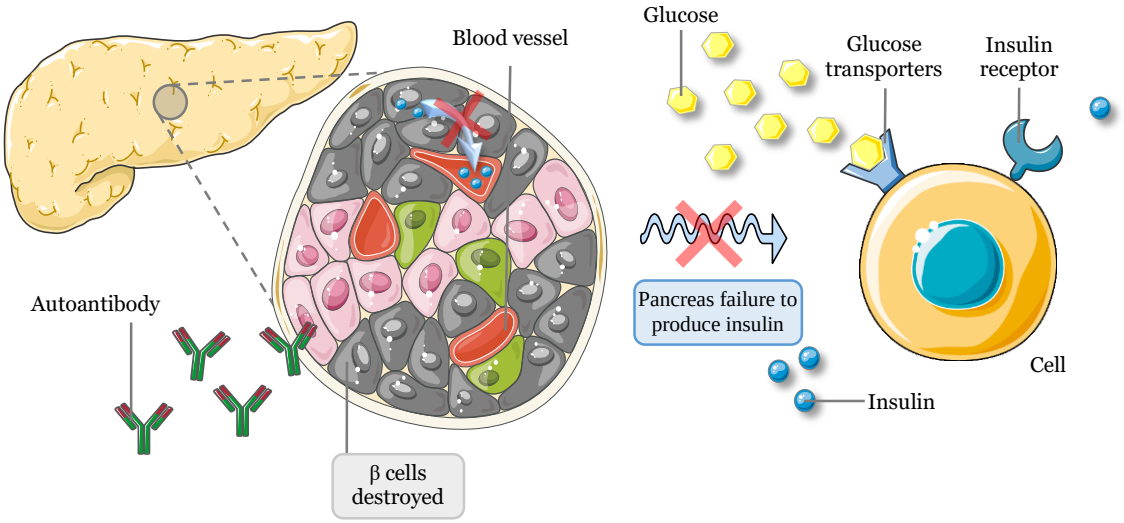


Fig. 20: Schematic representation of autoimmune type 1 DM. Adapted from (88).

- **Type 2 Diabetes *mellitus***

Type 2 DM (Fig. 21) is an expanding global health problem and is the predominant form of DM, representing 87-91% of all cases. This condition was previously designated as non-insulin-dependent or adult DM (89). Type 2 DM is characterized by impaired insulin secretion from pancreatic β cells, insulin resistance in target tissues, such as muscle, liver and adipose tissue, or a combination of both. Hyperglycaemia occurs when insulin secretion fails to compensate the insulin resistance (94, 95). Insulin resistance, the pathologic term for reduced insulin sensitivity, is the suppression or delay of an organ or tissue to respond to insulin's hormonal signal (96). Although the specific cause remains unclear, it did not seem to be related with the autoimmune destruction of β cells. Commonly, type 2 DM remain undiagnosed for several years, because the hyperglycaemia develops gradually, and patients are asymptomatic (91). However, the disease progression in patients can vary considerably (95). In type 2 DM patients, postprandial hyperglycaemia, which refers to plasma glucose concentrations after meals, is one of the initial abnormalities in glucose homeostasis. Insulin levels are insufficient to control postprandial hyperglycaemia. Consequently, higher and prolonged postprandial hyperglycaemia levels are detected in patients with type 2 DM, than in nondiabetic individuals. The prevalence and incidence of various complications, such as heart attack, stroke, kidney failure, vision loss and nerve damage, have been correlated with postprandial hyperglycaemia (97). Type 2 DM, as type 1 DM, are multifactorial diseases. Type 2 DM has been related with older onset (often >35 years old, however recently it has occurring more often in youth) and overweight and/or obesity, and with genetic factors (83, 86). This condition can be managed with lifestyle adjustments and/or oral hypoglycaemic agents and, in most cases, insulin therapy is not needed (98).

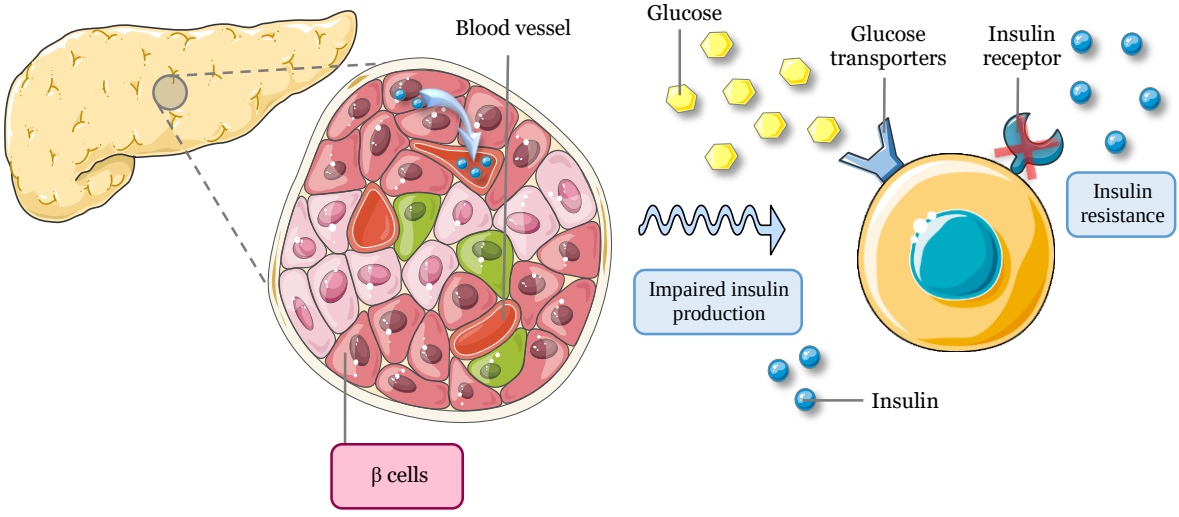


Fig. 21: Schematic representation of type 2 DM. Adapted from (95).

- **Gestational Diabetes *mellitus***

GDM is defined as a glucose intolerance, with onset or first recognition during pregnancy that can increase the risk of some complications for both the mother (e.g. preeclampsia and caesarean section) and the foetus (e.g. hypoglycaemia, obesity, hyperbilirubinemia, or DM later in life) (99). Similarly to the other types of DM, GDM is characterized by an insufficient response of the pancreatic β cells, which are unable to compensate the extra demand for insulin production during the pregnancy, resulting in high blood glucose levels. It is estimated that almost 10% of women diagnosed with GDM have DM after delivery (100). As with the other types of DM, the factors associated with GDM include obesity, age and genetic factors. Almost 70-85% of the cases are controlled with nutritional and physical counselling (99). However, if this multidisciplinary management fails, pharmacotherapy might be necessary, namely insulin treatment or oral anti-diabetic agents can be used if maternal glucose levels and/or fetal growth parameters demonstrate increased risk of perinatal complications (100).

- **Other specific types**

There are other, most uncommon types of DM (around 1-3% of all DM cases), as monogenic and secondary DM. Monogenic DM, in contrast with the other types of DM, that require a combination of genetic and environmental factors, is characterized as a single genetic mutation in an autosomal dominant gene. Examples of monogenic DM include neonatal DM and maturity-onset diabetes of the young (MODY) (86, 89, 91).

Secondary DM arises as a consequence of other diseases, including genetic defects in the insulin action, associated with mutations of the insulin receptor; diseases of the exocrine pancreas such as pancreatitis; dysfunctions associated with other endocrinopathies; dysfunctions caused by drugs or chemicals; infections; uncommon forms of immune-mediated DM and other genetic syndromes associated with DM, as Down and Turner syndromes (86, 91).

- **Prediabetes**

Without fulfilling the standards for the diagnosis of DM, an intermediate form can occur, characterized by the transition among normal blood glucose levels and DM, which is known as “prediabetes”, “intermediate hyperglycaemia” or “high risk state of developing diabetes”. Prediabetes is characterized by impaired fasting glucose (IFG) and/or IGT (90). Individuals with IFG levels are characterized by fasting glucose levels that are too high to

consider normal, however, do not reach the criteria value for DM diagnosis. Individuals with IGT display higher glucose levels than normal individuals after a meal. However, it is not considered enough to be diagnosed as DM (89, 95). Prediabetes is the precursor stage before DM, especially type 2 DM (86), in which, every year, 5–10% of individuals become diabetic, and the same proportion return to normoglycaemia. Near to 80% of the individuals remain in their abnormal glycaemic state (101). Lifestyle modifications are the main factor to prediabetic individuals return to normal blood glucose levels (90).

1.2.2 Risk factors

There are many factors that can influence the progress of DM, and the most significant are lifestyle behaviours, related with urbanization, which include a diet constituted by processed foods (with a high fat content and refined carbohydrates) and physical inactivity. Together, these factors increase the risk of occurrence of obesity and/or overweight and consequently the development of type 2 DM (89). Some of the risk factors of DM, such as genetics, ethnicity and age cannot be modified. However, there are other risk factors, as overweight, obesity, unhealthy diet, smoking and insufficient physical activity that can be altered, resulting in significant disease improvements (86). Fig 22 summarizes the most commonly described risk factors of DM and its incidence.

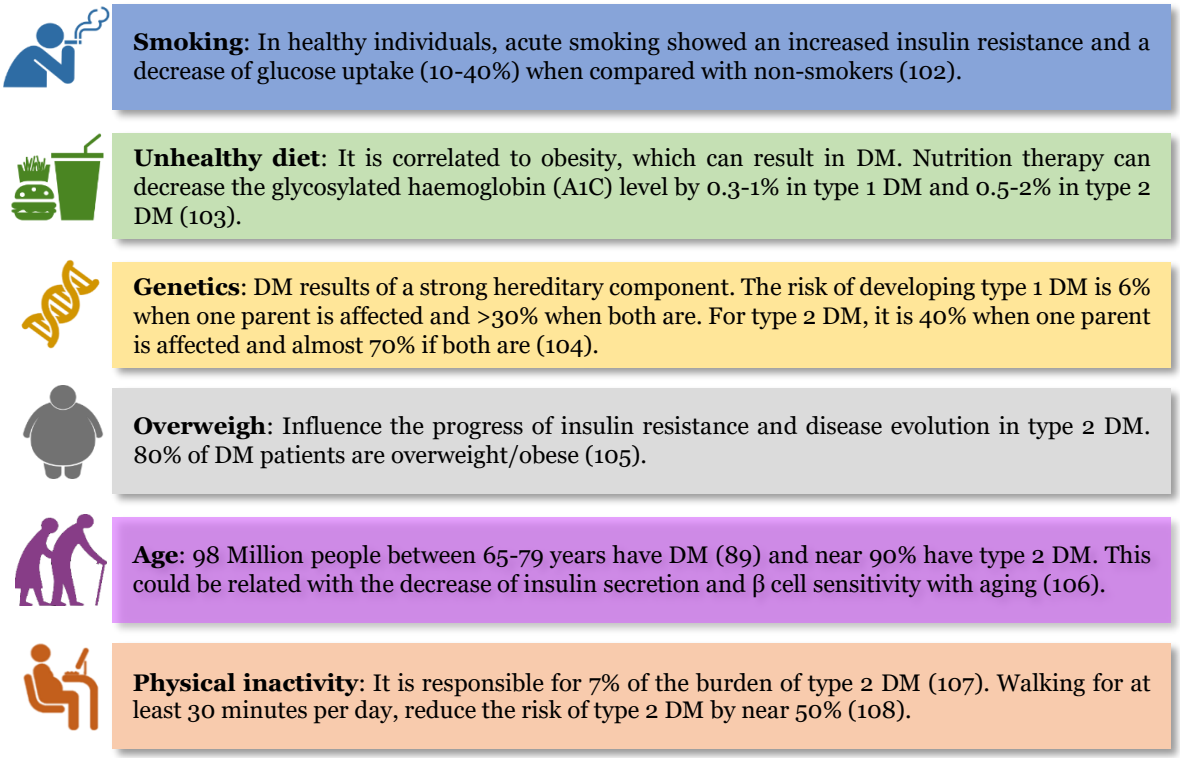


Fig. 22: Risk factors of DM.

1.2.3 Pathophysiology

In order to maintain normal body functions, the human body require a continuous balance of the blood glucose levels (109). Glucose is an essential source of cellular energy, and although all cells require glucose, 50% of the body's glucose is used by the brain, 25% by the liver and gastrointestinal tissue, and the remaining 25% by the insulin-dependent tissues, including muscle and adipose tissue. Insulin and glucagon are the key regulatory hormones, responsible for the glucose homeostasis, secreted by the β and α cells, respectively, of the pancreatic islets of Langerhans (110). Under normal circumstances, after dietary glucose ingestion, the increase of plasma glucose levels activates insulin release (Fig. 23), which stimulates the glucose uptake in the tissues and suppresses endogenous glucose production, mainly by the liver. Consequently, the blood glucose levels decrease by the removal of exogenous glucose from the blood. In contrast, glucagon, an antagonist of insulin, is secreted in response to low blood glucose levels (Fig. 23), accelerating glucose production by the liver. After a high ingestion of glucose, glucagon secretion is inhibited by hyperinsulinemia, thus, suppressing the hepatic glucose production (109).

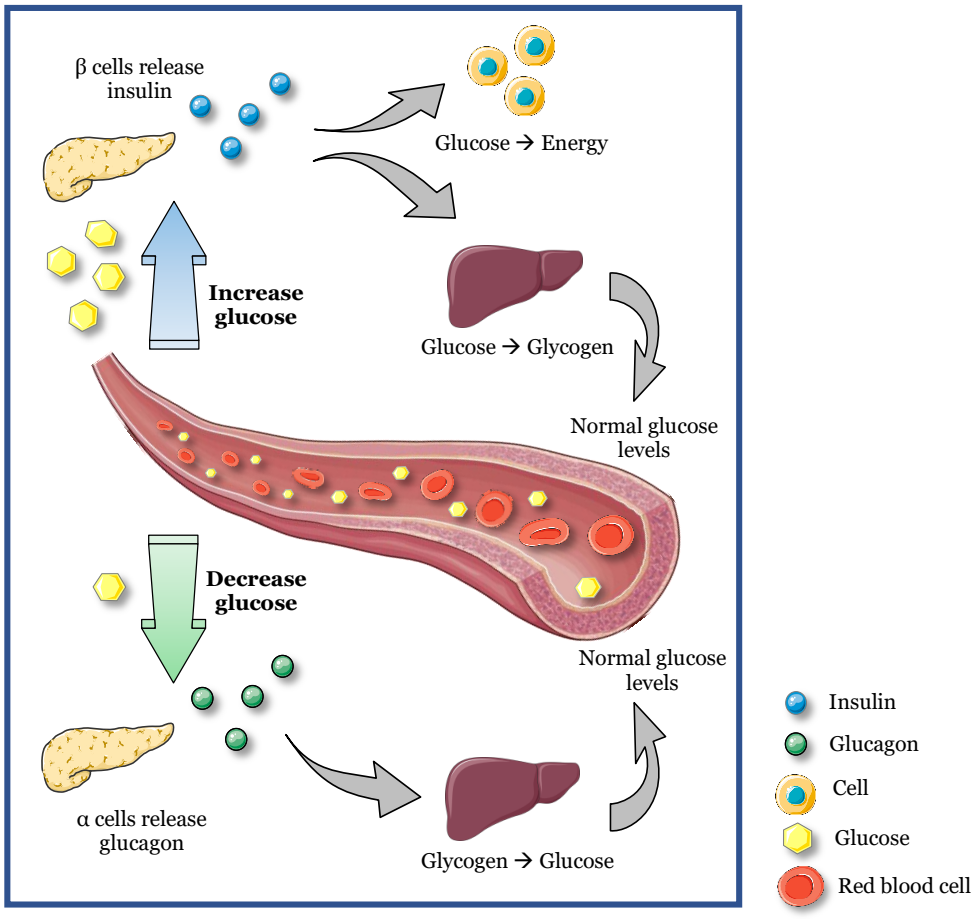


Fig. 23: Insulin and glucagon action in the regulation of glucose levels. Adapted from (111).

As mentioned above, the main stimulus for insulin release is the increase of the plasma glucose levels after a meal ingestion. Exogenous glucose is taken up by the glucose transporter type 2 (GLUT2), on the surface of the pancreatic β cells (Fig. 24). Once inside the β cell, glucose undergoes glycolysis, the metabolic pathway that converts glucose into pyruvate, producing adenosine triphosphate (ATP). The increase in glucose metabolism leads to the increase of the intracellular ATP/ADP ratio, which promotes the closure of ATP-sensitive K^+-channels (K_{ATP}-channels) and membrane depolarization. This depolarization consequently activates the voltage-dependent Ca^{2+}-channels, increasing the Ca^{2+} levels, which stimulates insulin release (109). The distribution of glucose among the various tissues of the body is mediated by the translocation of the glucose transporter type 4 (GLUT4). When the insulin levels increase, intracellular GLUT4 is translocated and fused with the plasma membrane, increasing the glucose uptake by cells, maintaining normoglycaemia (Fig. 24) (112).

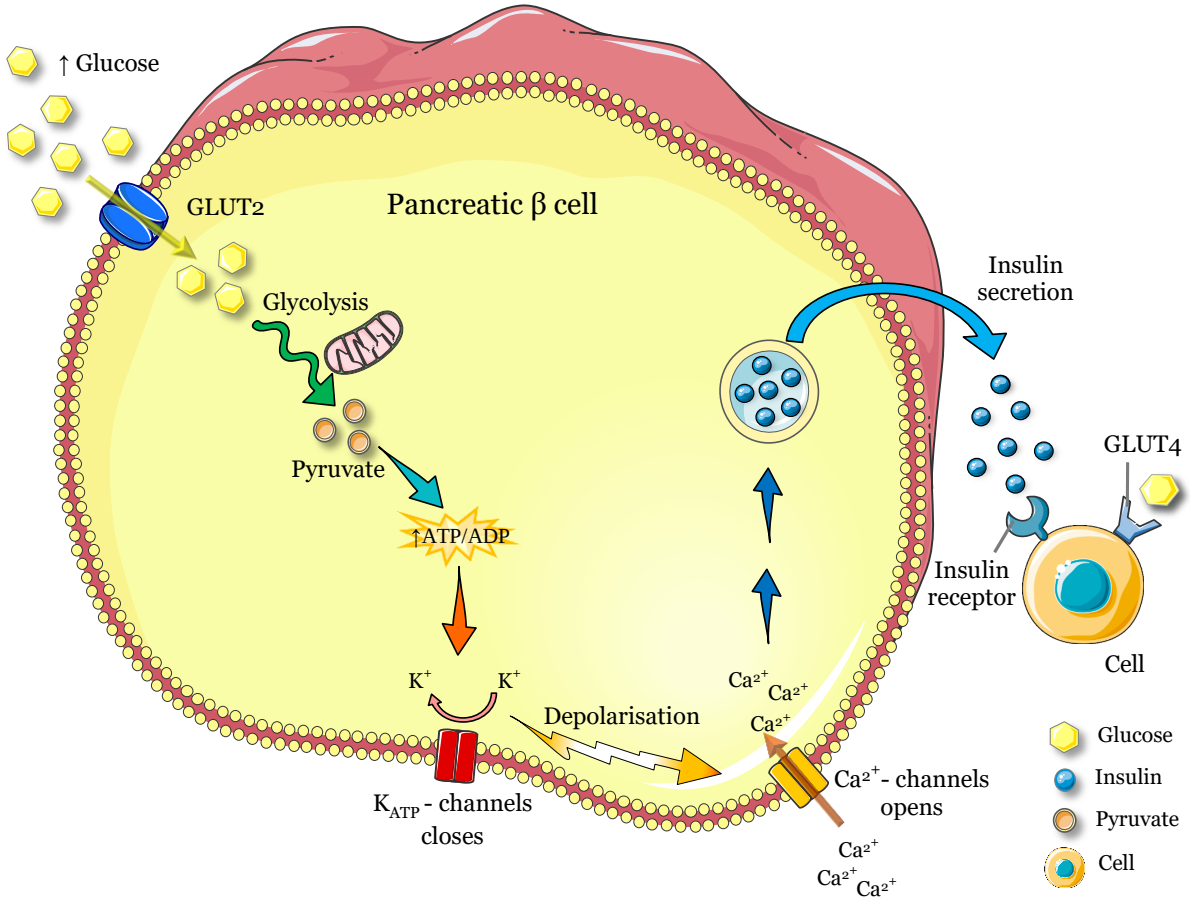


Fig. 24: The role of pancreatic K_{ATP} - channel in insulin secretion. Adapted from (113).

Focusing on type 2 DM, the impairment of insulin secretion and/or insulin resistance remain the main dysfunctions associated to this disease. Insulin resistance in muscle and liver is the initial detectable defect in individuals which may develop type 2 DM. However, type 2 DM only develops if pancreatic β cells are incapable of release sufficient insulin to compensate the insulin resistance. *Post-mortem* analysis of subjects with type 2 DM has shown that β cell mass is reduced by 30–40%, when compared with non-diabetic subjects (95). In addition to muscle, liver and β cells, adipocytes, gastrointestinal tract, pancreatic α cells, kidney, brain, the vascular insulin resistance and the inflammatory process, also contribute to the development of type 2 DM. Together, they comprise the "decadent decoplet" of hyperglycaemia in type 2 DM, a model created by DeFronzo in 2009 (Fig. 25) (114) and updated in 2015 (95). In adipocytes of healthy individuals, insulin inhibits lipolysis. However, in type 2 DM patients, the insulin resistance in adipocytes results in an increase of lipolysis, releasing free fatty acids into the blood stream, which elevates toxic lipids metabolites, aggravating insulin resistance and β cell failure. Additionally, two hormones known as incretins, due to their capacity to stimulate insulin release, glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are secreted in the gastrointestinal tract. However, because of the severe pancreatic β cell resistance to its stimulatory effects, insulin secretion is reduced. Also, in type 2 DM patients, glucagon production by pancreatic α-cells is increased and glucagon is not suppressed appropriately, contributing to the excessive glucose production by the liver. In the kidney, the sodium/glucose co-transporter 2 (SGLT2) is responsible for approximately 90% of the glucose filtered to the blood stream. In type 2 DM, due to the high glucose levels, the expression of SGLT2 is increased, consequently, enhances the renal glucose reabsorption and hyperglycaemia levels. Also, in type 2 DM, brain cells are resistant to insulin and dysfunctions are noticed in neurotransmitters, such low brain dopamine and increased brain serotonin levels, contributing to appetite dysregulation, weight gain, and insulin resistance in muscle and liver. High levels of inflammatory cells are observed in adipose tissue, liver and serum of patients with type 2 DM. Inflammation is also a contributor to insulin resistance, due to the increased levels of macrophages and other inflammatory cells with subsequent production of pro-inflammatory cytokines. Insulin induces microvascular vasodilation and capillary recruitment. In type 2 DM it is known that an impaired insulin-mediated vasodilation also contributes to insulin resistance (95, 114-116).

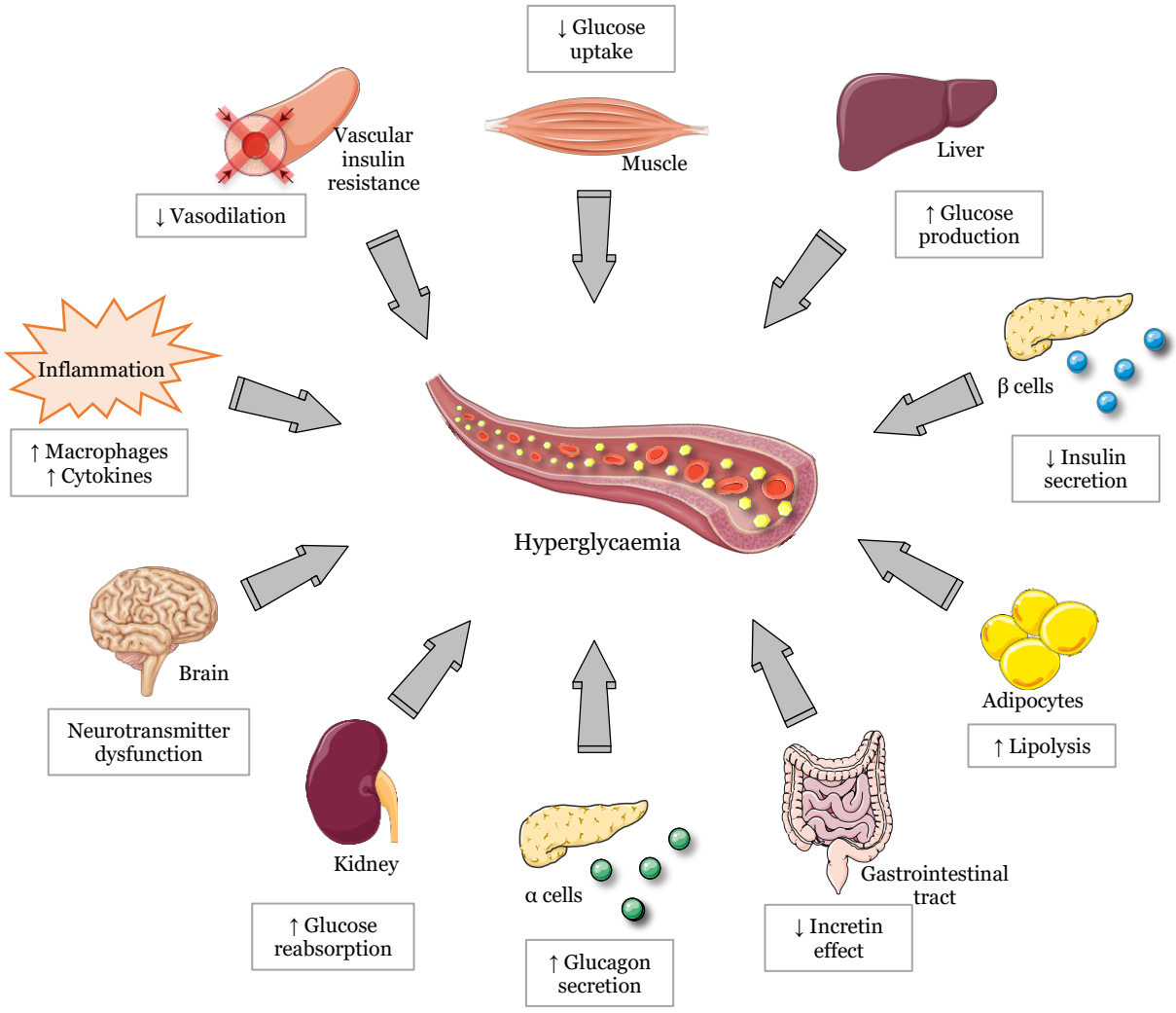


Fig. 25: The "decadent decoplet" of hyperglycaemia in type 2 DM. Adapted from (95).

1.2.4 Symptoms and complications

The usual symptoms of DM (Fig. 26), which can alert patients to contact health services are polyphagia (excessive hunger), polyuria (excessive urination), polydipsia (excessive thirsty) and glycosuria (glucose in urine). These symptoms occur more commonly in type 1 DM, which has a rapid development of severe hyperglycaemia, but can also appear in type 2 DM. Unexplained weight loss is also a common symptom of DM (89, 117).

Hyperglycaemia can lead to serious complications (Fig. 26) that can be divided into microvascular diseases (retinopathy, nephropathy, neuropathy), related to damage in small blood vessels, and macrovascular diseases (cardiovascular disease), related to damage in

arteries (118). Pregnancy complications can occur due to high blood glucose levels in pregnancy, leading to excessive size and weight of the foetus and complications during delivery (100). Diabetic ketoacidosis is a major acute metabolic problem, with associated risk of morbidity and mortality, which is due to relative or absolute deficiency of insulin and extra counter-regulatory hormones, such as glucagon, responsible for the increase in hyperglycaemia, glycosuria and dehydration. Ketoacidosis can be smelled on individuals breath (119). "Diabetic foot" is related to neuropathy, that interfere with the sensory nerves in feet, leading to pain and loss of sensation, and allow injuries to go unnoticed, and therefore leading to ulcerations, infections and amputations (89).

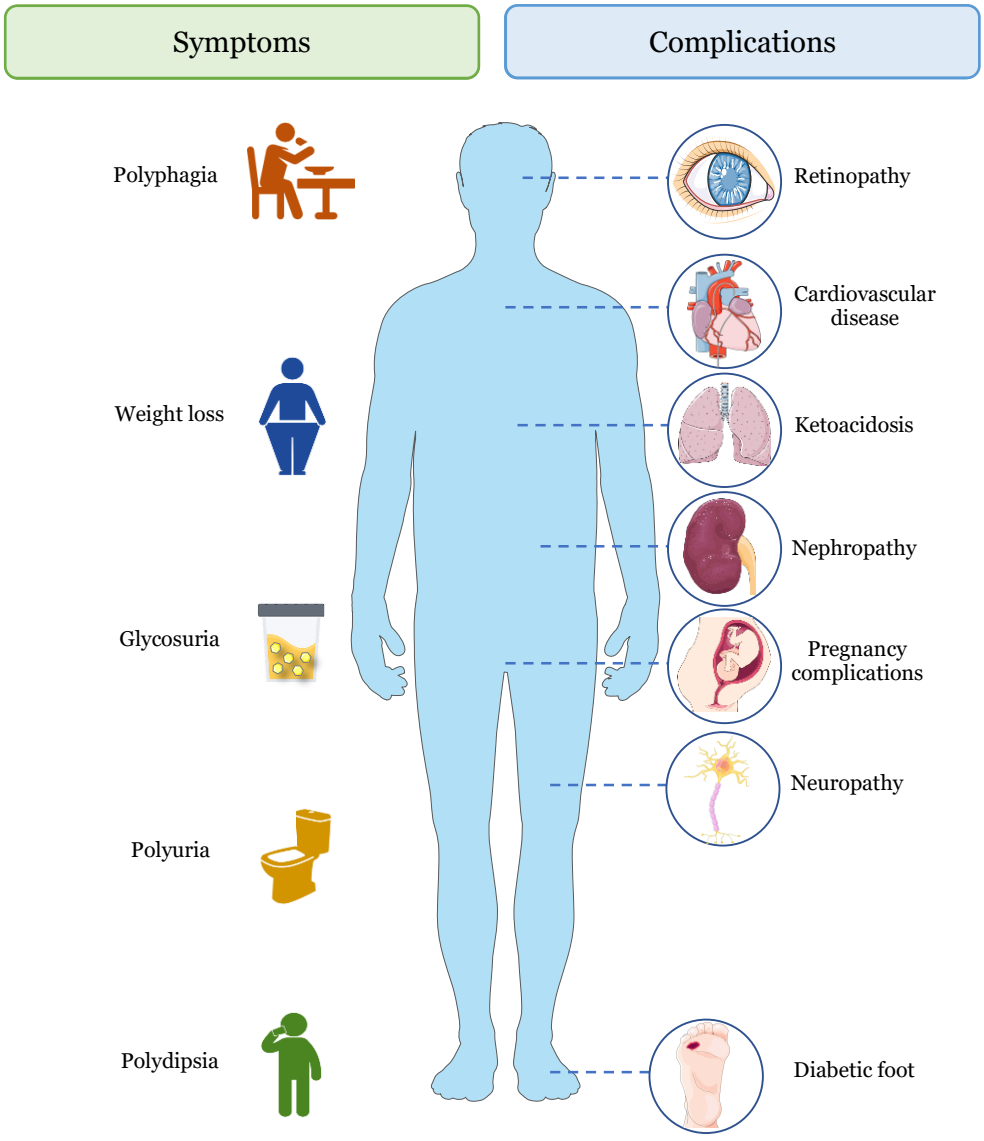


Fig. 26: Symptoms and complications in DM. Adapted from (89, 120).

1.2.5 Treatment

The main goal of DM management is to re-establish the blood glucose levels and consequently to decrease the incidence of complications. Insulin is the main therapy for patients with type 1 DM. This therapy allows the reduction of hyperglycaemia and the improvement of the quality of life by reducing the diabetic complications. However, the main barrier to reach normal glucose levels is the risk of hypoglycaemia (121). The symptoms of hypoglycaemia include shakiness, irritability, confusion, tachycardia and hunger, that can even culminate in loss of consciousness, coma or death (122). In addition to hypoglycaemia, insulin therapy is also associated to weight gain, and has the inconvenient to be an injectable therapy that can be painful and restrict some daily activities (123). Despite its importance in the management of type 1 DM, insulin therapy is not the first-line treatment for type 2 DM. Lifestyle change is the new trend for managing patients with type 2 DM. When lifestyle efforts are not enough to control the blood glucose levels, a pharmacological therapy is adopted. Pharmacological therapy should be individualized to each patient, taking into consideration the efficacy, cost, potential side effects, weigh gain, comorbidities, hypoglycaemia risk and patient preferences (124).

Currently available anti-diabetic therapy for type 2 DM, target one or more of the main pathways of the "decadent decoplet" (Fig. 25). The 12 classes of oral and injectable anti-diabetic medications (biguanides, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, dipeptidyl peptidase 4 (DPP-4) inhibitors, bile acid sequestrants, dopamine-2 agonists, SGLT2 inhibitors, GLP-1 receptor agonists, amylin mimetics and insulins) are currently used in the United States and some of them in Europe. These classes are used as monotherapy or in combination, to reach an individualized treatment in patients with type 2 DM. Single medication is frequently unable to reverse the multiple abnormalities in type 2 DM, thus, combination therapy has increased widespread acceptance. Most of these classes act by lowering blood glucose levels through the increase of insulin secretion or sensitivity. Nonetheless, α-glucosidase, DPP-4 and the recent SGLT2 inhibitors display a different mechanism of action (95, 124, 125).

The inhibition of α-amylase and α-glucosidase, enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial hyperglycaemia and retard the absorption of glucose. Consequently, both enzymes play a central role in the management of blood glucose levels and represent an important strategy in the management of type 2 DM (126). For that reason, this thesis focus in the inhibitory effect of chalcones against α-amylase and α-glucosidase activities, being their characterization discussed below.

1.3 Dietary carbohydrate digestion

Dietary carbohydrates, the principal substrates for energy metabolism, include the main macronutrient in human diet and should provide 45–65% of the total energy intake (127). Based on Food and Agriculture Organization (FAO) and World Health Organization (WHO) classification (128), dietary carbohydrates may be classified according to their degree of polymerization (number of monomeric sugar) into sugars (1-2 monomers), oligosaccharides (3-9 monomers) and polysaccharides (≥10 monomers). Sugars include monosaccharides (such as glucose, fructose and galactose), disaccharides (such as sucrose, lactose, maltose) and polyols (such as sorbitol, mannitol, lactitol). Oligosaccharides include malto-oligosaccharides (malto-dextrins) and other oligosaccharides (such as fructo- and galacto-oligosaccharides). In the polysaccharides are included the starch (such as amylose, amylopectin) and non-starch polysaccharides (such as cellulose, hemicellulose, pectins) (127, 128). Some important examples of dietary carbohydrates are represented in Fig. 27.

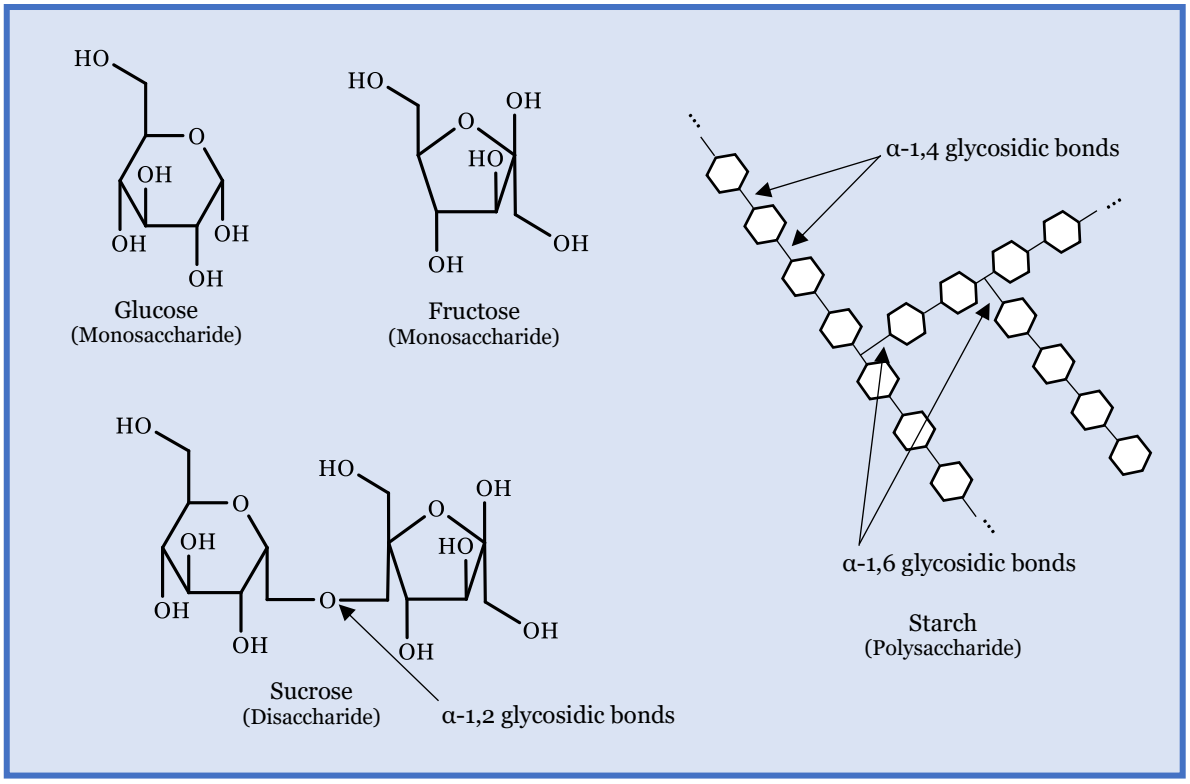


Fig. 27: Structure and classification of some important dietary carbohydrates. Adapted from (129).

Dietary starch is a plant storage polysaccharide and is the main source of carbohydrates in the human diet, and therefore it is the major source of blood glucose following carbohydrate digestion (130, 131). Starch is a junction of two glucose polymers,

amylose and amylopectin. Amylose is a straight chain molecule, with several α-1,4 linked units of glucose, while amylopectin is highly divided, comprising several α-1,6 linkages of glucose and also α-1,4 linkages (130, 132). Glycogen is the polysaccharide storage molecule found in animal cells, with a structure similar to the amylopectin (133).

Dietary carbohydrates, such as starch, are digested into their component monosaccharides by hydrolysis of glycosidic bonds, before the absorption. Monosaccharides, such as glucose, known as absorbable carbohydrates, do not need to be digested to enter into the cells. In turn, carbohydrates that include two or more units, need to be enzymatically hydrolysed, in order to be absorbed (134). The hydrolysis of starch requires multiple enzymatic reactions. This hydrolysis starts in the mouth with mastication, which allows the breakdown of the macroscopic structure of foods. During mastication and swallowing, human salivary α-amylase starts the enzyme digestion into smaller chains. The digestion of carbohydrates continues in stomach, however, occurs mainly in the upper part of the small intestine, in which the pH permits the activity of specific digestive enzymes, pancreatic α-amylase, secreted into the intestinal lumen, and α-glucosidase. Some polysaccharides, such as cellulose, are resistant to the hydrolysis in the human intestine, thereby, polysaccharides follow through the gut and are excreted undigested (129, 130).

1.3.1 α-Amylase

The enzyme α-amylase (1,4-α-D-glucan-glucanohydrolase, EC 3.2.1.1) (Fig. 28) catalysis the first step of the digestion of starch (Fig. 29). This enzyme is present in animals, plants, bacteria and fungi (135). The word “amylase” is derived from the Greek word “amylone”, which means starch (136). In humans, two different, but closely related genes code for amylase: AMY1, the salivary α-amylase gene, responsible for the presence of the enzyme in saliva; and AMY2, the pancreatic α-amylase gene, which encodes the enzyme produced in pancreas and secreted into the duodenum (131). These two forms of amylases share a similarity of 97% on amino acid sequence and 92% in the catalytic domains (137). α-Amylase contains three structural domains, A, B and C, (Fig. 28) in which the A domain is the largest, with about 280-300 residues, and contains important active site residues, including two aspartate and one of glutamate residue, known as the catalytic triad (Asp, Asp, Glu). The B domain is inserted between A and C domains, linked with A by a disulphide bond. C domain is attached to A domain by a simple polypeptide chain and seems to be an independent domain with unknown function (137, 138).

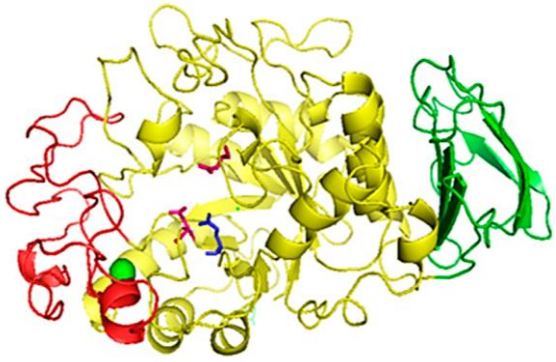


Fig. 28: Three-dimensional structure of pancreatic α-amylase. A, B, and C domains are presented in yellow, red and green colors, respectively. Adapted from (138).

α-Amylase is the most abundant enzyme in human saliva, accounting for 40 to 50% of salivary protein, and has the capacity to rapidly alter the physical properties of starch within the oral cavity (139). Human salivary amylase is mostly formed in the parotid gland, but also in the sublingual glands. Salivary α-amylase cleaves the α-(1,4)-glycosidic bonds of polysaccharides, which facilitate the carbohydrate metabolism (Fig. 29) (140). This partial digestion by salivary α-amylase results in the degradation of the polymeric substrate into shorter oligomers (135). This enzyme is neutralized by acid pH, thus, remains active in the stomach only while protected from stomach acid, as it happens when it is surrounded within a big bolus of food, continuing able to digest complex carbohydrates. Before food reach to small intestine, almost 30-40% of the carbohydrates are already hydrolysed into small molecules (133).

The pancreas is a mixed exocrine-endocrine gland organ, in which the exocrine tissues constitute the major part of the organ volume. As an exocrine gland, pancreas produces digestive enzymes into the duodenum, and as an endocrine organ, secretes hormones into the blood, such as insulin. Acinar cells, which form the exocrine tissues, upon stimulation, produce and discharge digestive enzymes, namely pancreatic α-amylase, proteases and lipases, into a system of intercalated ducts by exocytosis, to help in the digestion (136, 141, 142). Inside the small intestine, pancreatic juice enters into the lumen through the hepatopancreatic sphincter (also known as sphincter of Oddi or Glisson's sphincter). Thus, pancreatic α-amylase reaches into the intestinal lumen and provides a more extensive hydrolysis of the resultant mixture of carbohydrates, into maltose, maltotriose, trisaccharides and other larger oligosaccharides (Fig. 29). Since di-, tri- and oligosaccharides are the result of the hydrolysis of carbohydrates by α-amylase, an additional digestion is required to release absorbable monosaccharides, namely glucose (133).

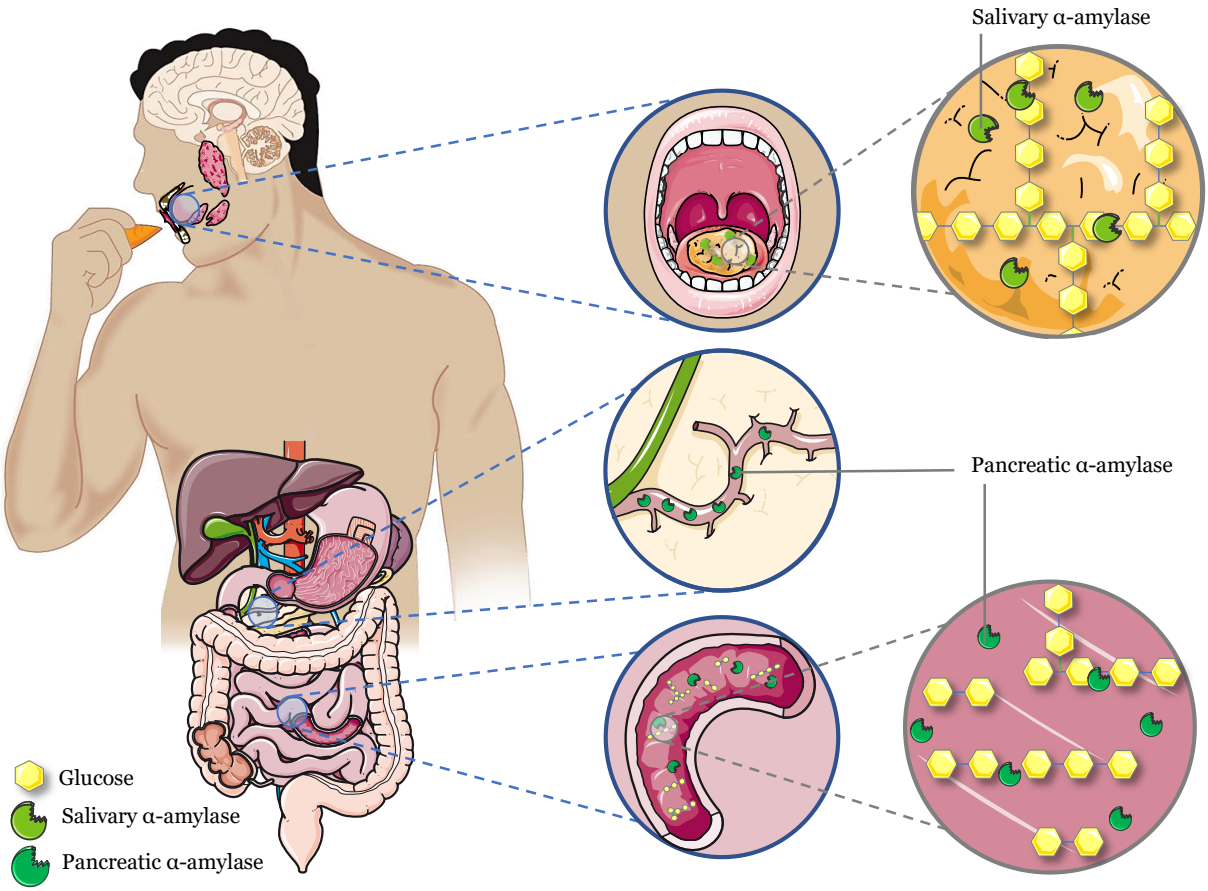


Fig. 29: α-Amylase activity.

1.3.2 α-Glucosidase

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), enzyme located in the brush borders of the enterocytes of the jejunum, catalyses the final step of the digestive process of carbohydrates, by the hydrolysis of glycosidic bonds from non-reducing ends of substrates (Fig. 30) (120, 126, 135). Based on the sequence homology, α-glucosidases are mainly classified into two groups, glycoside hydrolase family 13 and glycoside hydrolase family 31. α-Glucosidases from different origins display different substrate recognitions, which divide α-glucosidases into 3 groups, type I, II and III. α-Glucosidase type I, which belong to glycoside hydrolase family 13, and can be found in bacteria, yeast *Saccharomyces cerevisiae* and insects. This enzyme type presents higher tendency to hydrolyse heterogeneous substrates, such as sucrose (Fig. 27) than homogeneous substrates, such as glycogen. α-Glucosidases type II (can be found in mold) and type III (can be found in animals and plants) are members of glycoside hydrolase family 31 and have more affinity to homogeneous substrates (143, 144).

In general, glycoside hydrolase family 31 are closely related with complete digestion of starch into glucose. In the mammalian small intestine, the two membrane-bound glucosidases involved in the degradation of dietary starch are maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI). MGAM and SI are responsible for the hydrolysis of oligosaccharides, resultant from α-amylase activity, into glucose. MGAM and SI are composed by duplicated catalytic subunits, the *N*-terminal subunit (NtMGAM and NtSI) and the *C*-terminal subunit (CtMGAM and CtSI) (Fig. 30). The four domains exhibit exo-glucosidase activities against α-1,4 glycosidic linkages. However, these catalytic subunits display discriminative substrate selectivity. NtMGAM and CtMGAM are mainly responsible for the hydrolysis of α-1,4 glycosidic linkages, yet with different affinities for the substrate's degree of polymerization, since CtMGAM hydrolyse substrates with higher degree of polymerization than NtMGAM. NtSI have additional activity in the hydrolysis of α-1,6 glycosidic linkages and CtSI with the α-1,2 linkages (Fig. 30) (143, 145). The amino acid sequence homology between NtMGAM and NtSI is about 60%, higher than the percentage of identity between CtMGAM and CtSI (40%). The three-dimensional structures of NtMGAM and NtSI are known and show highly similarity in their overall folding pattern (146). The CtMGAM structure is also known, being CtSI still under study (143). CtMGAM is the subunit with the highest activity among the four subunits, thus, inhibitors of CtMGAM have revealed to be an efficient treatment for type 2 DM (138, 146). CtMGAM contains five domains, the trefoil type-P domain, *N*-terminal domain, the catalytic domain (which includes the catalytic (β/α)8 domain, catalytic domain insert 1, catalytic domain insert 2), proximal *C*-terminal domain and the distal *C*-terminal domain, which are represented in Fig. 31 (146).

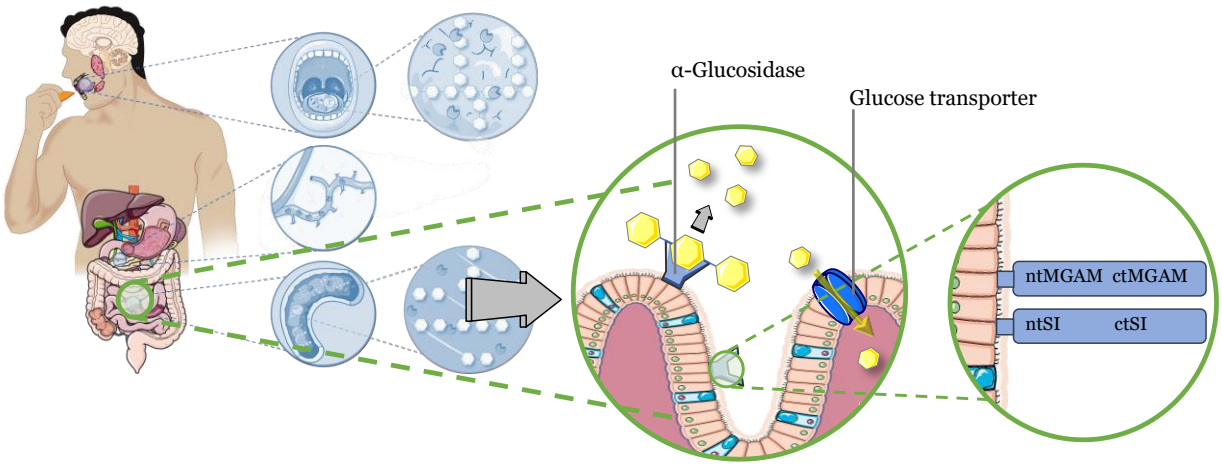


Fig. 30: α-Glucosidase activity.

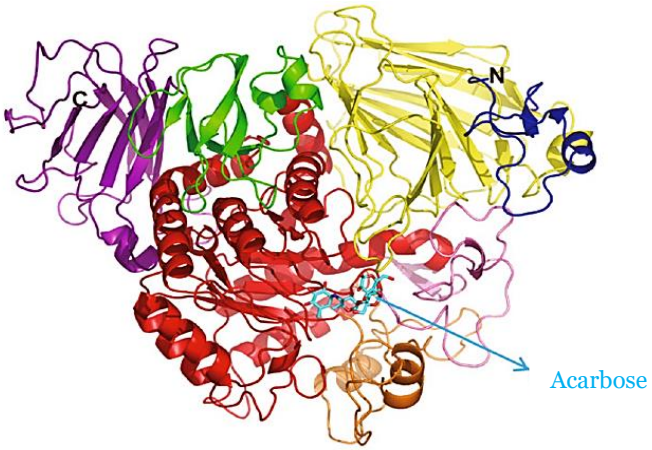


Fig. 31: Three-dimensional structure of CtMGAM domains linked with an α-glucosidase inhibitor, acarbose. Trefoil type-P domain is represented in blue, *N*-terminal domain in yellow, catalytic (β/α)8 domain in red, catalytic domain insert 1 in orange, catalytic domain insert 2 in pink, proximal *C*-terminal domain in green and distal *C*-terminal domain in purple. Adapted from (146).

After the α-glucosidase action, monosaccharides are transported across the intestinal mucosa to blood vessels and distributed to cells and tissues (Fig. 32). Glucose absorption into the epithelial cells is mediated by SGLT1, a membrane protein that couples two molecules of Na^+ together with one molecule of glucose. Additionally, the transport from enterocytes into blood is mediated by GLUT2 (147, 148).

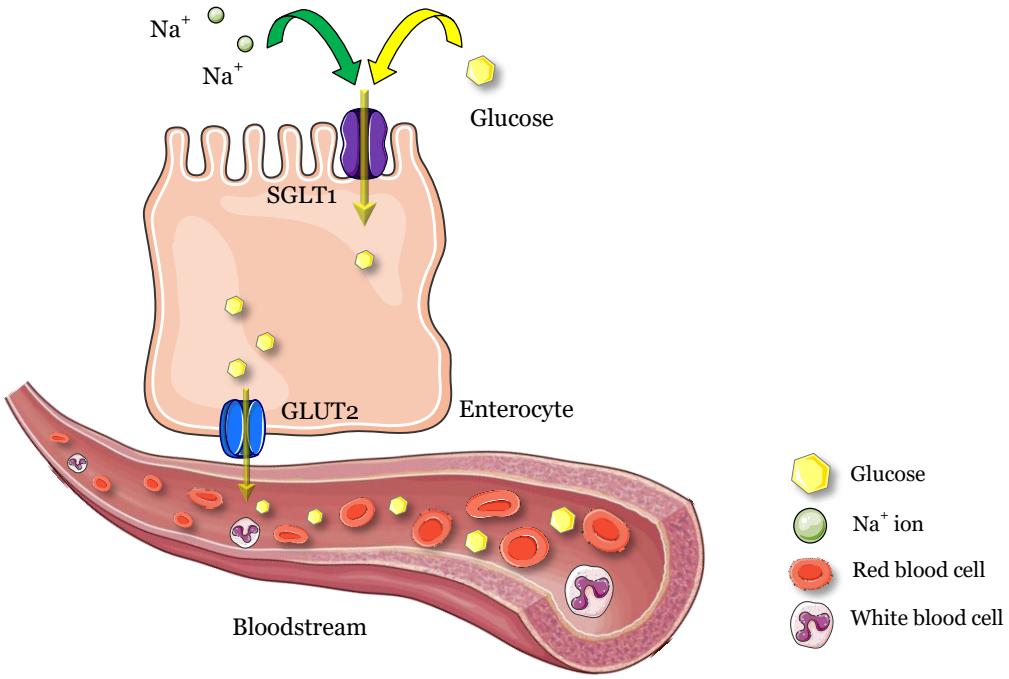


Fig. 32: Transport of glucose by SGLT1 from the intestinal lumen to the cytosol of enterocytes and from the cytosol to blood by GLUT2.

1.3.3 Inhibitors of α-amylase and α-glucosidase

Avoiding wide oscillations in blood glucose levels and glycaemic control are the cornerstone for the management of type 2 DM (149, 150). As such, there is an urge need to control the postprandial hyperglycaemia by the use of α-amylase and α-glucosidase inhibition therapies (Fig. 33). Such inhibitors avoid the increase of plasma glucose levels after meals, by delaying the hydrolysis of dietary complex carbohydrate and the consequent absorption of monosaccharides on the gastrointestinal tract, resulting in a reduction of plasma glucose. This type of therapy is currently used to modulate type 2 DM and prevent its complications, known as α-glucosidase inhibitors, which are oral anti-diabetic drugs (97, 150).

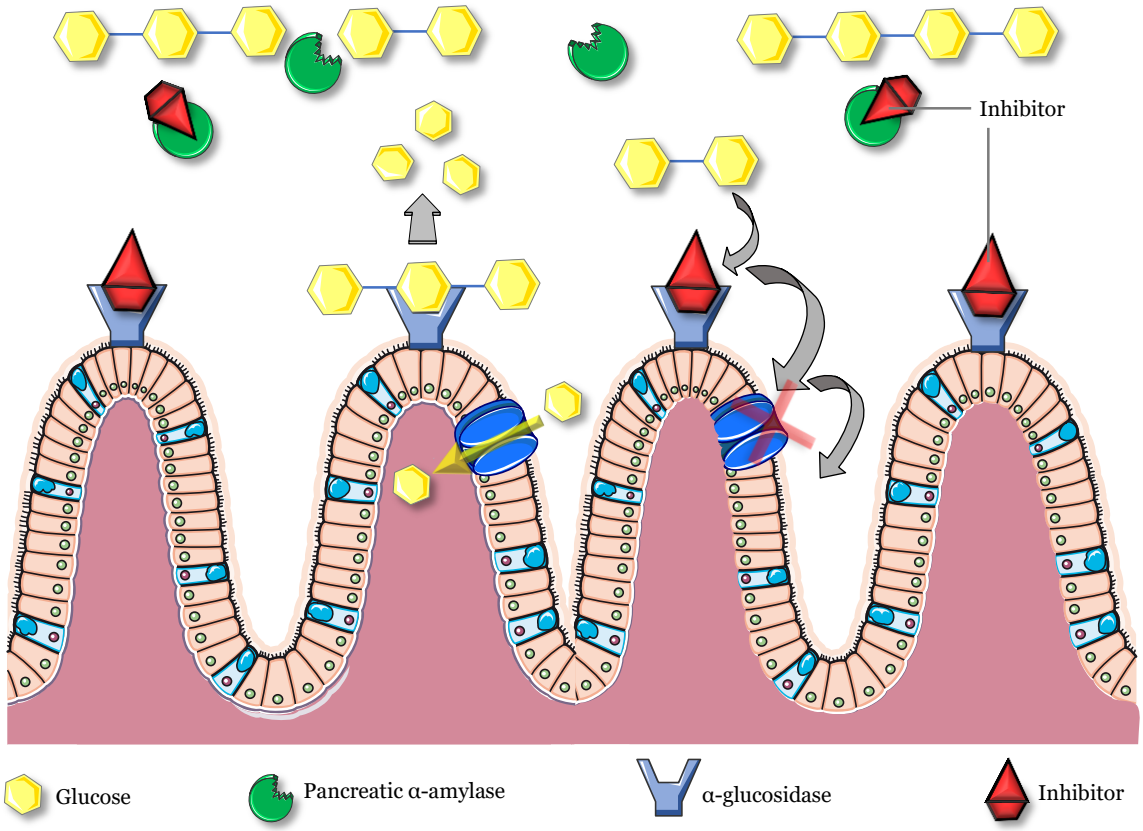


Fig. 33: Inhibition of α-amylase and α-glucosidase activities.

α-Glucosidase inhibitors are recommended as first, second and third line treatment options as monotherapy or in combination with metformin, sulfonylureas and insulin, in patients which display uncontrolled hyperglycaemia. These inhibitors have been available for clinical use in type 2 DM for almost three decades and have revealed efficacy, long-term safety, low price, reduced risk of hypoglycaemia and no increase in body weight (150).

Currently, three types of α-glucosidase inhibitors are available for the treatment of type 2 DM: acarbose, miglitol and voglibose (Fig. 34). Acarbose is the most used drug of this class for over 20 years. It is a pseudotetrasaccharide with a nitrogen bound between the first and second molecule of glucose, resultant from a fermentation process of a microorganism, *Actinoplanes utahensis*. This modification of a natural tetrasaccharide is crucial for the stability and affinity with the active centers of α-glucosidase. Acarbose binds to α-glucosidase with 10^4 - 10^5 - fold higher affinity than dietary carbohydrates. Miglitol is a derivative from 1-deoxynojirimycin and voglibose is formed by reductive alkylation of valiolamine, a compound derived from antibiotic validamycin C (150, 151).

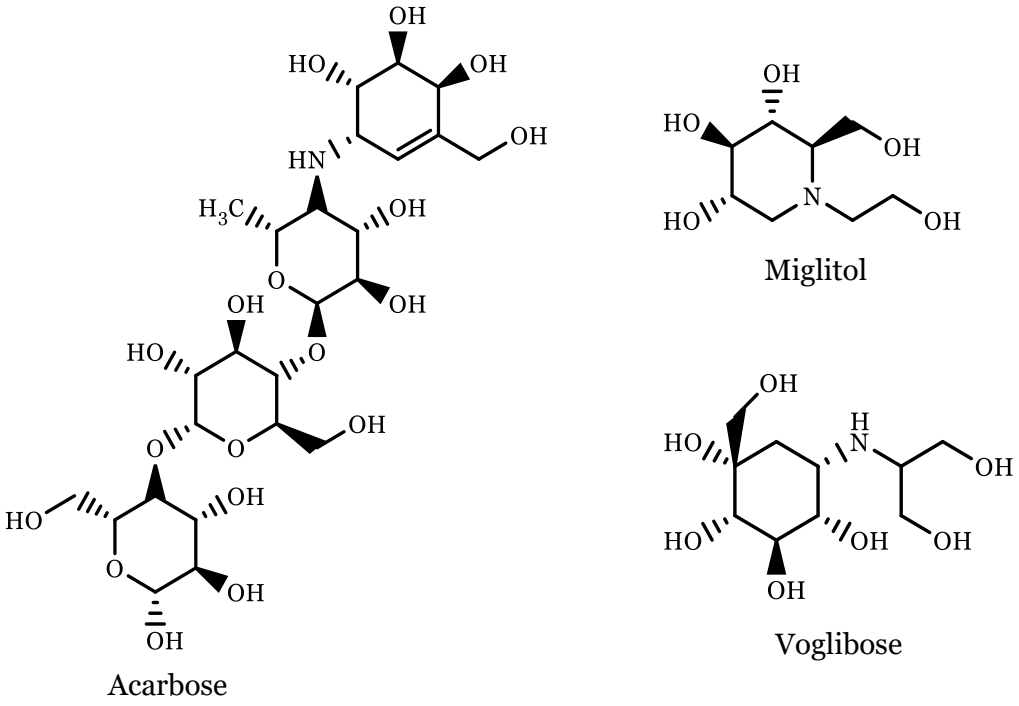


Fig. 34: Chemical structures of acarbose, miglitol and voglibose.

Acarbose was approved for the treatment of type 2 DM in 1990 and is now used in 117 countries. Acarbose is the α-glucosidase inhibitor prescribed in Portugal. Miglitol was accepted in 1996 in 10 countries, whereas voglibose is only commercially available in Japan, since 1994, and is approved in 3 countries (150, 152).

In general, acarbose, miglitol and voglibose show the same mechanism of action, acting as pseudocarbohydrates in the intestine (153), binding with the active center of α-glucosidase in the small intestine, altering the intestinal digestion and absorption of dietary carbohydrates and reducing the postprandial glycaemia peaks. Nevertheless, these inhibitors display slight differences in the mechanism of action. Acarbose competes with polysaccharides and oligosaccharides, whereas miglitol and voglibose competes with

disaccharides at the active site of the enzyme. Acarbose also inhibits salivary and pancreatic α-amylase, however, miglitol and voglibose do not display activity on this enzyme (150). Since acarbose, miglitol and voglibose prevent the hydrolysis of complex carbohydrates, these inhibitors should be taken at the start of main meals, with the first bite of the food. The efficacy of α-glucosidase inhibitors in reducing the postprandial hyperglycaemia depends on the amount of dietary carbohydrates (151).

α-Glucosidase inhibitors are associated with very few serious adverse effects. However, gastrointestinal side effects, including flatulence, soft stools, abdominal discomfort and diarrhoea, which are generally slight to moderate, have been discussed as a limiting factor (150). These adverse effects might occur due to the undigested complex carbohydrates that reach to the large intestine following the use of α-glucosidase inhibitors. The undigested carbohydrates get fermented by gut microbiota, which results in intestinal gas production (150, 154). The non-specific inhibition of α-amylase is also correlated with the excessive accumulation of undigested carbohydrates in the large intestine, resulting in gastrointestinal side effects. Thus, a moderate α-amylase inhibition with high α-glucosidase inhibitory activity might overcome the inconvenient adverse effects (149). Meanwhile, the commonly used α-glucosidase inhibitors were also reported to be related with several rare adverse hepatic events (155). In the past three decades, the scientific community have been focused in the discovery and development of novel active intestinal inhibitors with reduced adverse side effects (149).

1.4 General and specific objectives of the dissertation

The main objective of this dissertation was to evaluate the anti-diabetic effect of a panel of chalcones with hydroxyl, methoxy, methyl, nitro, chlorine, fluorine and bromine substituent groups, in order to contribute for the design of novel effective molecules for the treatment of DM.

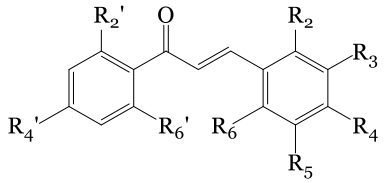
The following specific objectives were established:

- ✓ Evaluation of the inhibitory effect of chalcones against the activity of α-amylase;
- ✓ Determination of the inhibition type of the most active compounds and the positive control;
- ✓ Evaluation of the inhibitory effect of chalcones against the activity α-glucosidase;
- ✓ Determination of the inhibition type of the most active compounds and the positive control.

2. Materials and Methods

2.1 Chemicals

The following reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO): α-amylase from porcine pancreas, 2-chloro-*p*-nitrophenyl-α-D-maltotrioside (CNPG3), α-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), dimethyl sulfoxide (DMSO), Na_2HPO4 and acarbose. Compounds **1-3**, **7-13**, **16**, **17** (Fig. 35) were obtained from Indofine Chemical Company, Inc. (Hillsborough, NJ). Compounds **4** and **5** (Fig. 35) were purchased from Extrasynthese (Z.I Lyon Nord). Compounds **6**, **14**, **15**, **18-41** (Fig. 35) were synthesized as previously described (156-159).



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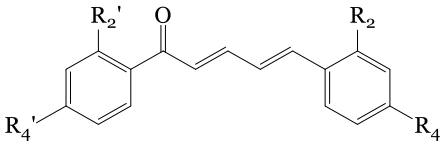


29-41

1: $R_{2'}=R_{4'}=R_{6'}=R_2=R_3=R_4=R_5=R_6=H$
2: $R_{2'}=OH$, $R_{4'}=R_{6'}=R_2=R_3=R_4=R_5=R_6=H$
3: $R_{2'}=R_{4'}=OH$, $R_{6'}=R_2=R_3=R_4=R_5=R_6=H$
4: $R_{2'}=R_{4'}=R_3=R_4=OH$, $R_{6'}=R_2=R_5=R_6=H$ (Butein)
5: $R_{2'}=R_{4'}=R_{6'}=R_3=R_4=OH$, $R_2=R_5=R_6=H$
6: $R_{2'}=OH$, $R_2=OMe$, $R_{4'}=R_{6'}=R_3=R_4=R_5=R_6=H$
7: $R_{2'}=OH$, $R_3=OMe$, $R_{4'}=R_{6'}=R_2=R_4=R_5=R_6=H$
8: $R_{2'}=OH$, $R_4=OMe$, $R_{4'}=R_{6'}=R_2=R_3=R_5=R_6=H$
9: $R_{2'}=R_4=OH$, $R_3=OMe$, $R_{4'}=R_{6'}=R_2=R_5=R_6=H$
10: $R_{2'}=OH$, $R_3=R_4=OMe$, $R_{4'}=R_{6'}=R_2=R_5=R_6=H$
11: $R_{2'}=OH$, $R_3=R_4=R_5=OMe$, $R_{4'}=R_{6'}=R_2=R_6=H$
12: $R_{2'}=R_{4'}=OH$, $R_4=OMe$, $R_{6'}=R_2=R_3=R_5=R_6=H$
13: $R_{2'}=R_{4'}=OH$, $R_3=R_4=OMe$, $R_{6'}=R_2=R_5=R_6=H$
14: $R_{2'}=OH$, $R_{4'}=R_{6'}=R_4=OMe$, $R_2=R_3=R_5=R_6=H$
15: $R_{2'}=OH$, $R_{4'}=R_{6'}=R_3=R_4=R_5=OMe$, $R_2=R_6=H$
16: $R_{2'}=R_{4'}=R_{6'}=R_3=R_4=OMe$, $R_2=R_5=R_6=H$
17: $R_3=R_4=OMe$, $R_{2'}=R_{4'}=R_{6'}=R_2=R_5=R_6=H$
18: $R_{2'}=OH$, $R_3=Me$, $R_{4'}=R_{6'}=R_2=R_4=R_5=R_6=H$
19: $R_{2'}=OH$, $R_2=R_4=R_6=Me$, $R_{4'}=R_{6'}=R_3=R_5=H$
20: $R_4=NO_2$, $R_{2'}=R_{4'}=R_{6'}=R_2=R_3=R_5=R_6=H$
21: $R_{2'}=OH$, $R_4=NO_2$, $R_{4'}=R_{6'}=R_2=R_3=R_5=R_6=H$
22: $R_4=Cl$, $R_{2'}=R_{4'}=R_{6'}=R_2=R_3=R_5=R_6=H$
23: $R_{2'}=OH$, $R_2=Cl$, $R_{4'}=R_{6'}=R_3=R_4=R_5=R_6=H$
24: $R_{2'}=OH$, $R_3=Cl$, $R_{4'}=R_{6'}=R_2=R_4=R_5=R_6=H$
25: $R_{2'}=OH$, $R_4=Cl$, $R_{4'}=R_{6'}=R_2=R_3=R_5=R_6=H$
26: $R_{2'}=OH$, $R_2=R_4=Cl$, $R_{4'}=R_{6'}=R_3=R_5=R_6=H$
27: $R_{2'}=OH$, $R_2=R_6=Cl$, $R_{4'}=R_{6'}=R_3=R_4=R_5=H$
28: $R_{2'}=OH$, $R_3=R_4=Cl$, $R_{4'}=R_{6'}=R_2=R_5=R_6=H$

29: $R_{2'}=R_{4'}=R_2=R_4=H$
30: $R_{4'}=Me$, $R_{2'}=R_2=R_4=H$
31: $R_{4'}=OMe$, $R_{2'}=R_2=R_4=H$
32: $R_{4'}=Cl$, $R_{2'}=R_2=R_4=H$
33: $R_{4'}=F$, $R_{2'}=R_2=R_4=H$
34: $R_{4'}=Br$, $R_{2'}=R_2=R_4=H$
35: $R_{4'}=NO_2$, $R_{2'}=R_2=R_4=H$
36: $R_4=Me$, $R_{2'}=R_{4'}=R_2=H$
37: $R_4=OMe$, $R_{2'}=R_{4'}=R_2=H$
38: $R_4=NO_2$, $R_{2'}=R_{4'}=R_2=H$
39: $R_2=NO_2$, $R_{2'}=R_{4'}=R_4=H$
40: $R_{2'}=OH$, $R_{4'}=R_2=R_4=H$
41: $R_{2'}=OH$, $R_2=NO_2$, $R_{4'}=R_4=H$

Fig. 35: Chemical structures of the tested chalcones (**1-28**) and chalcone analogues (**29-41**).

2.2 *In vitro* α-amylase inhibition assay

2.2.1 Principle of the method

The α-amylase activity was measured using a method of Trinh *et al.* (160), slightly modified. The assay was carried out by measuring the α-amylase-mediated transformation of CNPG3 into 2-chloro-*p*-nitrophenol (CNP), 2-chloro-*p*-nitrophenyl-α-D-maltoside (CNPG2), maltotriose and glucose (Fig. 36), using an *in vitro* spectrophotometric technique. The formation of the yellow product, CNP, was monitored spectrophotometrically by measuring the absorbance at 405 nm. CNP is proportional to the concentration of the α-amylase, as such, higher absorbance values indicate higher activity of α-amylase, thus, higher conversation of CNPG3 into CNP. On the other hand, reduced values of absorbance mean less conversion of CNPG3, thus, inhibition of the enzyme.

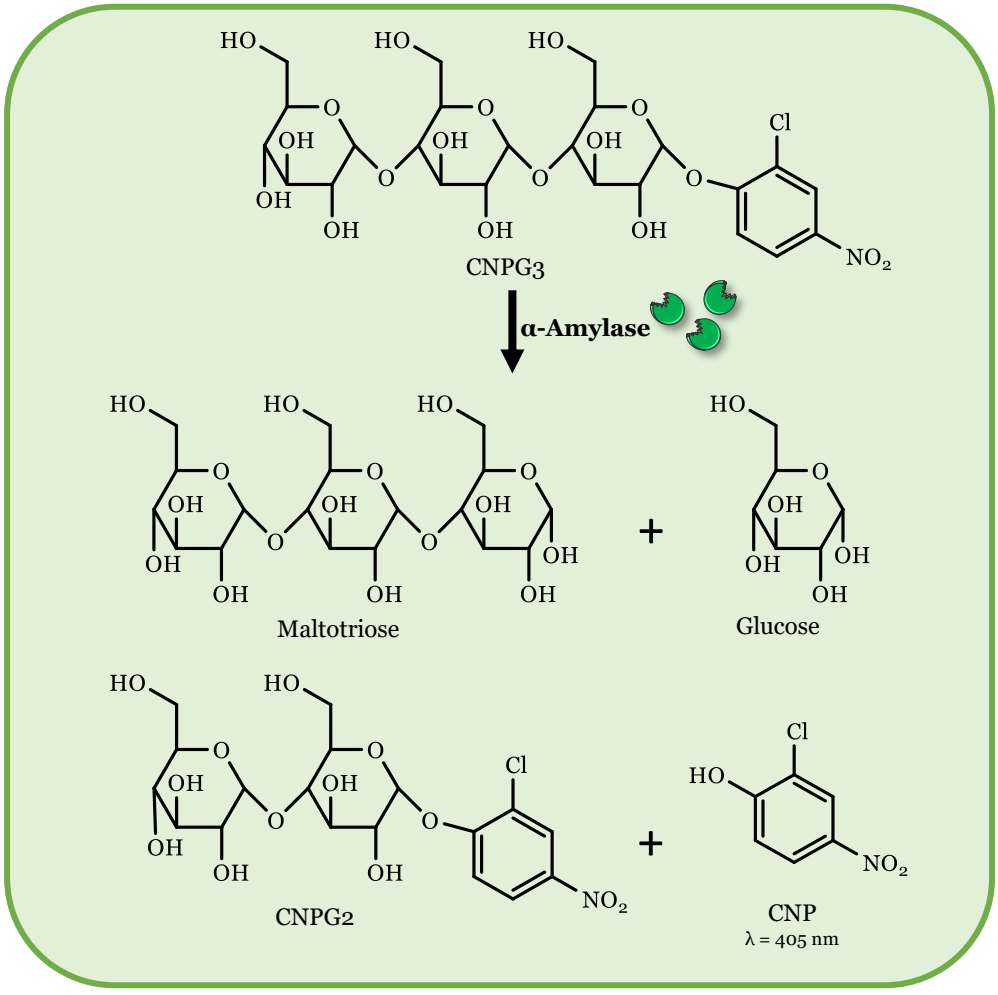


Fig. 36: Mechanism of hydrolysis of CNPG3 by α-amylase.

CNPG3, 2-chloro-*p*-nitrophenyl-α-D-maltotrioside; CNPG2, 2-chloro-*p*-nitrophenyl-α-D-maltoside; CNP, 2-chloro-*p*-nitrophenol.

2.2.2 *In vitro* assay

In a 96-well plate, the enzyme α-amylase (0.1 U/mL) dissolved in 20 mM phosphate buffer (pH 6.8), was pre-incubated at 37 ºC for 10 minutes, with the chalcones under study (0-100 µM) dissolved in DMSO. After incubation, the substrate, CNPG3 (500 µM, final concentration in the assay) dissolved in phosphate buffer, was added, followed by another incubation, at 37 ºC, for 30 minutes. The resultant yellow color formed in the enzymatic reaction was monitored spectrophotometrically, in a microplate reader (Synergy HT, BIO-TEK), by measuring the absorbance at 405 nm (Fig. 37). Acarbose (0-10 µM, final concentration in the assay) was used as positive control. The obtained values correspond to the slope of the kinetic reaction, measured between 5 and 30 minutes. The results were expressed as the % of inhibition of α-amylase activity, calculated using equation (1), and represent at least three independent experiments.

(1) $$\% \text{ Inhibition: } 100 - \frac{[V_{(A)} - V_{(B)}] \times 100}{V_{(C)} - V_{(B)}}$$

$V_{(A)}$= Velocity of the reaction (ΔAbsorbance/minutes) in the presence of the compounds under study

$V_{(B)}$= Velocity of the reaction (ΔAbsorbance/minutes) in the absence of α-amylase and the compounds under study

$V_{(C)}$= Velocity of the reaction (ΔAbsorbance/minutes) of control (in absence of the compounds under study)

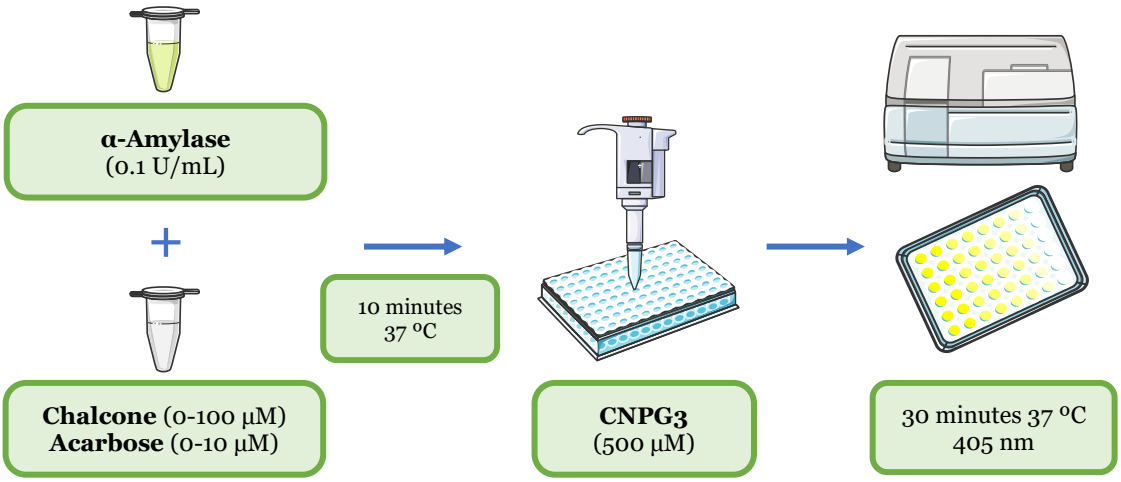


Fig. 37: Schematic representation of the *in vitro* α-amylase inhibition assay.

2.3 *In vitro* α-glucosidase inhibition assay

2.3.1 Principle of the method

The α-glucosidase activity was based on the method of Tadera *et al.* (161) that was slightly modified. The assay was performed *in vitro,* by monitoring the α-glucosidase-mediated transformation of the substrate *p*NPG into glucose and *p*-nitrophenol (Fig. 38). The formation of the yellow product, *p*-nitrophenol, was monitored spectrophotometrically at 405 nm. The formation of *p*-nitrophenol is proportional to the concentration of α-glucosidase present. As such, higher absorbance values indicate higher activity of α-glucosidase, thus, higher conversation of *p*NPG into *p*-nitrophenol.

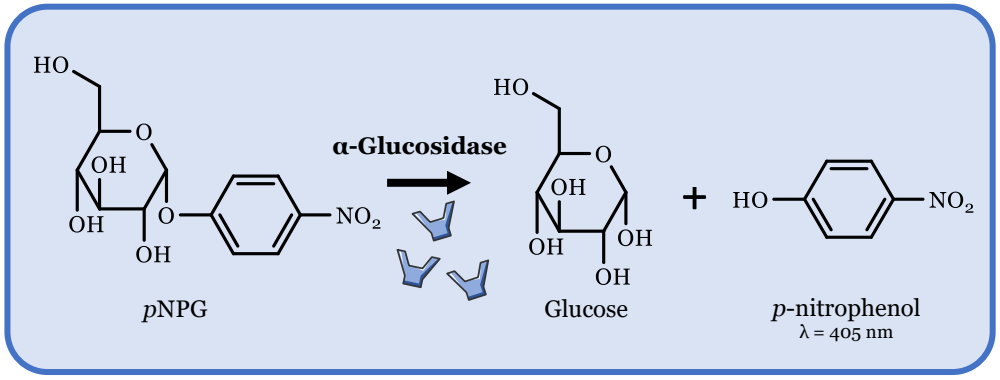


Fig. 38: Mechanism of hydrolysis of *p*NPG by α-glucosidase.
*p*NPG, *p*-nitrophenyl-α-D-glucopyranoside.

2.3.2 *In vitro* assay

In a 96-well plate, the enzyme α-glucosidase (0.04 U/mL) dissolved in 100 mM phosphate buffer (pH 6.8), was pre-incubated with the chalcones under study (0-150 μM) dissolved in DMSO. After a pre-incubation of 5 minutes at 37 ºC, the reaction was started by the addition of the substrate *p*NPG (600 μM, final concentration in the assay) dissolved in phosphate buffer, that was incubated in the reaction mixture at 37 ºC for 30 minutes. The enzymatic reaction was monitored spectrophotometrically at 405 nm, by measuring the resultant yellow color formed (Fig. 39). Acarbose (0-2285 μM) was used as positive control. The obtained values correspond to the slope of the kinetic reaction, measured between 5 and 30 minutes. The obtained results were expressed as the % of inhibition of α-glucosidase activity, calculated using equation (2), and represent at least three independent experiments.

(2) % Inhibition: $100 - \frac{[V_{(A)} - V_{(B)}] \times 100}{V_{(C)} - V_{(B)}}$

$V_{(A)}$= Velocity of the reaction (ΔAbsorbance/minutes) with the tested compounds

$V_{(B)}$= Velocity of the reaction (ΔAbsorbance/minutes) on white (without α-glucosidase and tested compounds)

$V_{(C)}$= Velocity of the reaction (ΔAbsorbance/minutes) on control (without the tested compound and in the presence of α-glucosidase)

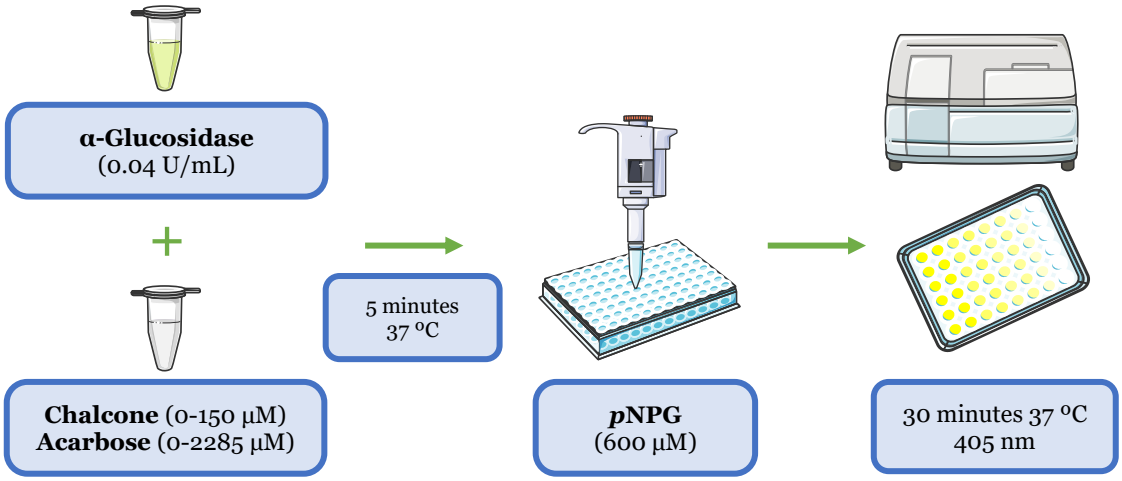


Fig. 39: Schematic representation of the *in vitro* α-glucosidase inhibition assay.

2.4 Inhibitory kinetic analysis

2.4.1 Principle of the method

The Michaelis-Menten equation, represented at equation (3), is one of the most recognized models used to characterize the enzyme kinetics. In this equation, V represent the velocity of the reaction, V_{max} the maximal velocity, [S] is the concentration of the substrate and K_m is the Michaelis constant. K_m is equal to the concentration of the substrate at half V_{max}, and is also known as a measure of binding affinity of an enzyme with its substrate. An equation with low K_m value shows a large binding affinity, thus, the reaction will reach faster to V_{max}. On the other hand, a high K_m indicates that the enzyme does not bind efficiently with the substrate, and the V_{max} is reached only at higher concentrations of substrate (162).

(3) Michaelis-Menten equation: $V = \frac{V_{max}[S]}{K_m + [S]}$

V = Velocity of the reaction

V_{max} = Maximal velocity

K_m = Michaelis constant

[S] = Concentration of the substrate

The Michaelis-Menten equation describes a hyperbolic function, as represented at Fig. 40 A. It is difficult to precisely determine the V_{max} and K_m in a hyperbola. The Lineweaver-Burk plot, equation (4), is a linearization method which transform the original nonlinear Michaelis-Menten equation into a linear one, thus, the data is fited by a linear regression exhibited as a straight line in a 2-dimensional graph, as displayed in Fig. 40 B. In this equation, K_m/V_{max} is the slope of the graph, $1/V_{max}$ is the y-intercept and $-1/K_m$ is the x-intercept (162, 163).

(4) Lineweaver-Burk plot: $\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$

V = Velocity of the reaction

V_{max} = Maximal velocity

K_m = Michaelis constant

[S] = Concentration of the substrate

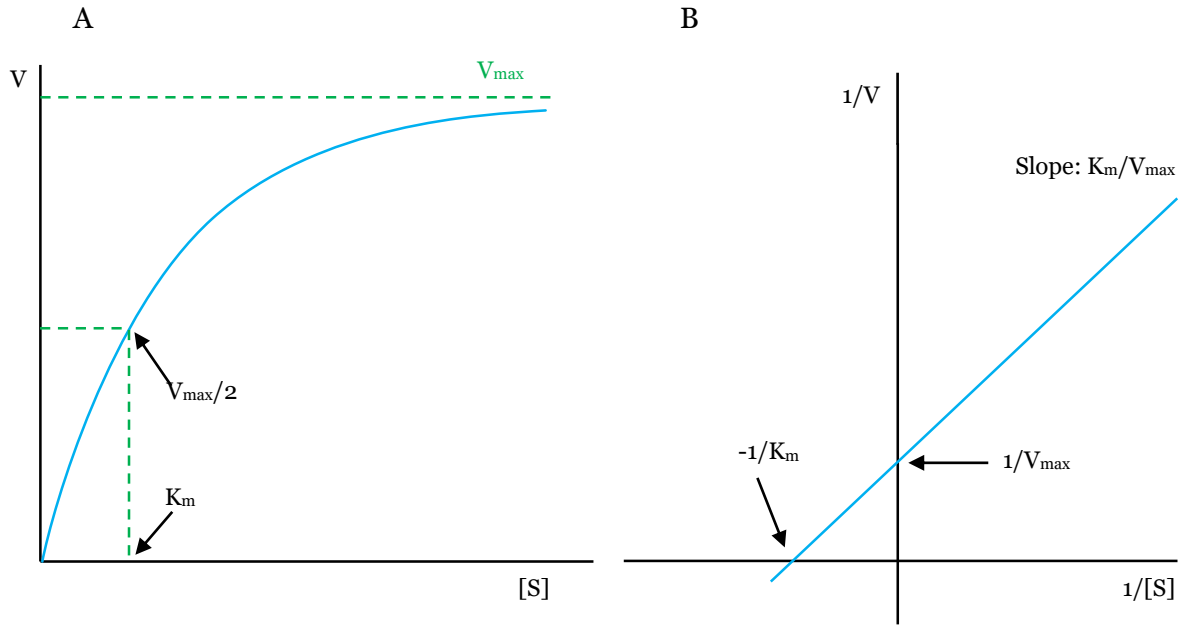


Fig. 40: Graphical representation of the Michaelis-Menten equation (A) and the Lineweaver-Burk plot (B). Adapted from (163).

The study of enzyme inhibitors and the interaction between enzymes and inhibitors is an essential part of the development of drugs. The Lineweaver-Burk equation constitutes an easy method to study the type of inhibition of a compound (Fig. 41), by fixing the concentrations of the enzyme, altering the substrate concentrations, in the absence and in the presence of different concentrations of inhibitor. Enzyme inhibitors are divided into irreversible, when the inhibitors bind covalently to their target enzymes and modify them chemically, and reversible, when inhibitors bind to enzymes without establishing a covalent bond (163). Reversible inhibitors are divided into competitive, uncompetitive, non-competitive and mixed. In the competitive inhibition, the inhibitor binds to the same site as the substrate, often in the active site of enzyme. In an uncompetitive inhibition type, the inhibitor combines only to the enzyme-substrate complex at a location outside the active site, forming an enzyme-substrate inhibitor complex. In non-competitive inhibition type, the inhibitor is able to bind equally well with the free enzyme and the enzyme-substrate complex, outside the active site. In a mixed inhibition type, inhibitors can bind to both the free enzyme and the enzyme-substrate complex, thus displaying characteristics of both competitive and uncompetitive inhibition types, however, has a higher affinity for one state or the other, therefore, K_m can increase or decrease (164).

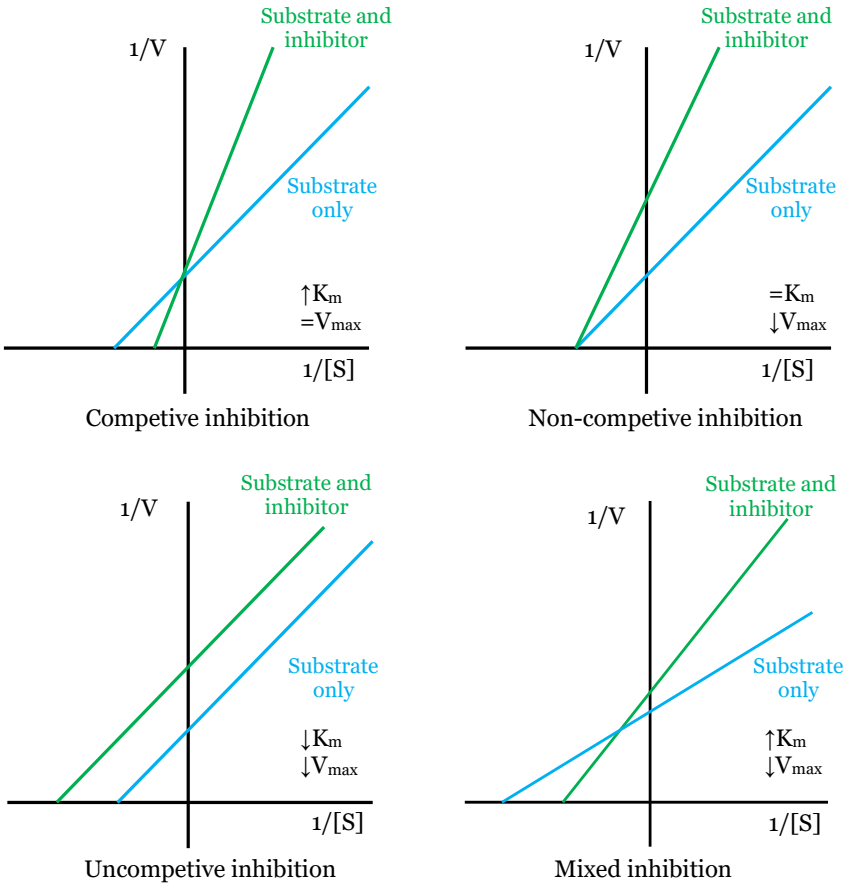


Fig. 41: Graphical representation of the types of inhibition according to the Lineweaver-Burk plot. Adapted from (164).

Despite linear transformations are widely used, it is described that in enzyme kinetic analysis, linear transformations of the Michaelis-Menten equation, such as the Lineweaver-Burk plots, present some limitations. Linear transformations are less accurate than the use of the nonlinear regression, since it distorts the experimental error and the relationship between x and y axes. Until recently, the analysis of kinetic parameters using nonlinear equations was a difficult task. Nowadays, this is no longer a problem, as it is possible to use software applications, such as the Solver supplement of Excel Microsoft Office™, to obtain the kinetic constants for an enzyme catalysed reaction and distinguish between the different kinetic models (165).

2.4.2 Inhibitory kinetic analysis of α-amylase

Enzymatic kinetics of α-amylase inhibition was performed for chalcone **4** (butein) (0-50 μM) and the positive control acarbose (0-0.75 μM). Briefly, in a 96-well plate, the compounds, dissolved in DMSO, were pre-incubated with the enzyme α-amylase (0.1 U/mL), dissolved in 20 mM phosphate buffer (pH 6.8), for 10 minutes, at 37 ºC. In sequence, the substrate CNPG3 (250, 500, 1000 μM) was added and incubated in the reaction mixture for 30 minutes at 37 ºC. The obtained values correspond to the slope measured between 5 and 30 minutes of kinetic reaction.

The type of inhibition (competitive, non-competitive, uncompetitive or mixed) of the tested compounds was determined graphically from the nonlinear regression Michaelis-Menten equation and the corresponding Lineweaver-Burk plots for each concentration of the inhibitor and substrate. The obtained results represent at least five independent experiments.

Using the same experimental values, the type of inhibition was estimated using the Solver supplement of Excel Microsoft Office™, according to Bezerra *et al.* (166) and Dias *et al.* (165). Therefore, the kinetic parameters of the conversion of CNPG3 by α-amylase was evaluated by nonlinear least squares regression using the general model for mixed inhibition translated by the equation (5), and each one of its simplifications regarding the different types of inhibition:

(5) $$V_{inic} = \frac{V_{máx}(S)}{K_m(1+\frac{|I|}{K_{ic}})+(S)(1+\frac{|I|}{K_{iu}})}$$

V_{inic} = Initial velocity of formation of absorbing CNP in ΔAbsorbance/minutes

V_{max} = Maximum achievable velocity when for the 0.1 U/mL of enzyme used all catalytic sites are saturated by the substrate

S = CNPG3 concentration in mM

K_m = Michaelis-Menten constant in mM

K_{ic} = Inhibitor dissociation constant of enzyme-inhibitor expressed in μM^{-1}

K_{iu} = Inhibitor dissociation constant of enzyme-substrate-inhibitor complex expressed in μM^{-1}

For each tested condition, the equation parameters were estimated by Solver. The obtained values for the parameters of the simplest model (without inhibition) were used as initial values, then were calculated the other inhibition types, competitive, non-competitive, uncompetitive, finishing with the more complex model, mixed inhibition. The actual mechanism of inhibition was established by comparison between models, applying the extra sum-of-square F test and the Akaike information criterion (AIC) test. In order to determine the error of the kinetic constants values, the jackknife procedure was applied, which consisted on the calculation of standard deviation of all estimations by Solver when each experimental data point was removed from the initial set.

2.4.3 Inhibitory kinetic analysis of α-glucosidase

Enzymatic kinetics of α-glucosidase inhibition was performed for the most active chalcones, **4** (butein) (0-75 μM), **21** (0-45 μM), **26** (0-70 μM), **41** (0-40 μM) and the positive control, acarbose (0-2285 μM).

Similarly, with the enzymatic kinetics of α-amylase inhibition, the enzyme α-glucosidase (0.04 U/mL) dissolved in 100 mM phosphate buffer (pH 6.8) was pre-incubated with the compounds under study, dissolved in DMSO, in a 96-well plate, for 5 minutes at 37 ºC. Then, the substrate *p*NPG (300, 600 and 1200 μM) was added and incubated in the reaction mixture for 30 minutes at 37 ºC. The obtained values correspond to the slope of the enzymatic reaction measured between 5 and 30 minutes.

The study of the inhibition type was performed as above mentioned in 2.4.2, for α-amylase inhibition, and the results represent at least five independent experiments.

2.5 Statistical analysis

The results of the *in vitro* inhibitory activity of chalcones against the pancreatic α-amylase and α-glucosidase activity are expressed as mean±standard error of mean (SEM). Statistical comparison between the active chalcones was estimated applying the one-way analysis of variance (ANOVA). Differences among the groups were compared by Tukey test with a p value < 0.05 considered statistically significant. All the statistical analysis was performed using GraphPad Prism™ (version 5.0; GraphPad Software).

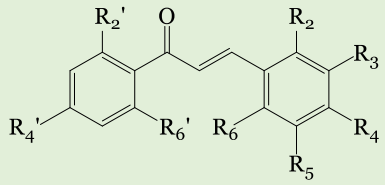
The type of inhibition using Solver was established by comparison among models, applying the extra sum-of-square F test and AIC test. ANOVA was applied to evaluate the precision of the method.

3. Results

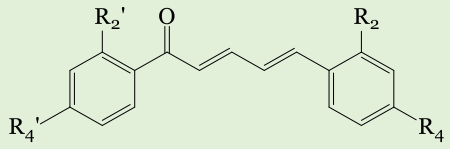
3.1 *In vitro* α-amylase inhibition assay

The inhibitory effect against pancreatic α-amylase activity of the compounds **1-41** and the positive control, acarbose, are shown in Table 1. The compounds were divided in chalcones **1-28**, with the 1,3-diarylprop-2-en-1-one framework, and chalcone analogues **29-41**, known as cinnamylideneacetophenones, which hold two double bonds linking the A and B rings. Among chalcones (**1-28**), one is unsubstituted (**1**), four are hydroxylated (**2-5**), ten are hydroxylated and methoxylated (**6-15**), two are only methoxylated (**16**, **17**), two chalcones display methyl substitutions (**18**, **19**), two chalcones with nitro groups (**20**, **21**) and seven chalcones with chlorine substitutions (**22-28**). Among chalcone analogues (**29-41**), one is unsubstituted (**29**), eleven with a single substitution, including methyl (**30, 36**), methoxy (**31, 37**), chlorine (**32**), fluorine (**33**), bromine (**34**), nitro group (**35, 38, 39**), hydroxyl (**40**), and one is di-substituted with a hydroxyl and a nitro group (**41**).

Table 1: Structures and *in vitro* α-amylase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript).



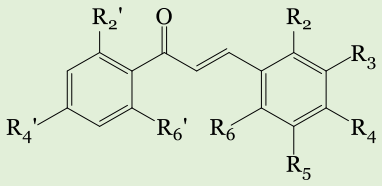
1-28



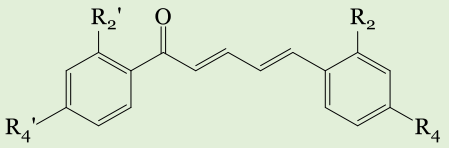
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Amylase inhibitory activity (%)
1	-	-	-	-	-	-	-	-	$<20^{100\ \mu M}$
2	OH	-	-	-	-	-	-	-	$25.9\pm0.8^{100\ \mu M\ (a)}$
3	OH	OH	-	-	-	-	-	-	$<20^{100\ \mu M}$
4 (Butein)	OH	OH	-	-	OH	OH	-	-	$59.6\pm3.5^{100\ \mu M\ (b)}$
5	OH	OH	OH	-	OH	OH	-	-	ND
6	OH	-	-	OMe	-	-	-	-	$<20^{25\ \mu M}$

Table 1: Structures and *in vitro* α-amylase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



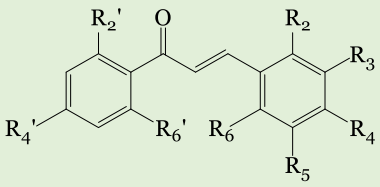
1-28



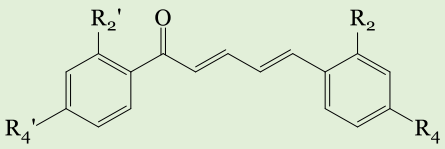
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Amylase inhibitory activity (%)
7	OH	-	-	-	OMe	-	-	-	$<20^{50\ \mu M}$
8	OH	-	-	-	-	OMe	-	-	$<20^{50\ \mu M}$
9	OH	-	-	-	OMe	OH	-	-	$28.9\pm1.8^{100\ \mu M\ (a)}$
10	OH	-	-	-	OMe	OMe	-	-	$<20^{50\ \mu M}$
11	OH	-	-	-	OMe	OMe	OMe	-	$36.2\pm3.2^{100\ \mu M\ (a)(d)}$
12	OH	OH	-	-	-	OMe	-	-	$<20^{100\ \mu M}$
13	OH	OH	-	-	OMe	OMe	-	-	$<20^{50\ \mu M}$
14	OH	OMe	OMe	-	-	OMe	-	-	$<20^{25\ \mu M}$
15	OH	OMe	OMe	-	OMe	OMe	OMe	-	$<20^{50\ \mu M}$
16	OMe	OMe	OMe	-	OMe	OMe	-	-	$<20^{100\ \mu M}$
17	-	-	-	-	OMe	OMe	-	-	$<20^{100\ \mu M}$
18	OH	-	-	-	Me	-	-	-	$<20^{50\ \mu M}$
19	OH	-	-	Me	-	Me	-	Me	$<20^{25\ \mu M}$
20	-	-	-	-	-	NO_2	-	-	$<20^{50\ \mu M}$

Table 1: Structures and *in vitro* α-amylase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



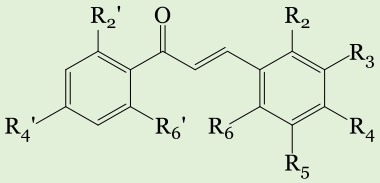
1-28



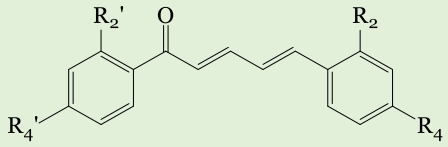
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Amylase inhibitory activity (%)
21	OH	-	-	-	-	NO_2	-	-	$24.5\pm2.6^{100\ \mu M\ (a)}$
22	-	-	-	-	-	Cl	-	-	$<20^{50\ \mu M}$
23	OH	-	-	Cl	-	-	-	-	$<20^{50\ \mu M}$
24	OH	-	-	-	Cl	-	-	-	$21.7\pm3.1^{50\ \mu M\ (a)(e)}$
25	OH	-	-	-	-	Cl	-	-	$<20^{50\ \mu M}$
26	OH	-	-	Cl	-	Cl	-	-	$<20^{50\ \mu M}$
27	OH	-	-	Cl	-	-	-	Cl	$<20^{50\ \mu M}$
28	OH	-	-	-	Cl	Cl	-	-	$<20^{25\ \mu M}$
29	-	-	-	-	-	-	-	-	$<20^{50\ \mu M}$
30	-	Me	-	-	-	-	-	-	$<20^{25\ \mu M}$
31	-	OMe	-	-	-	-	-	-	$<20^{25\ \mu M}$
32	-	Cl	-	-	-	-	-	-	$<20^{25\ \mu M}$
33	-	F	-	-	-	-	-	-	$<20^{50\ \mu M}$
34	-	Br	-	-	-	-	-	-	$<20^{50\ \mu M}$

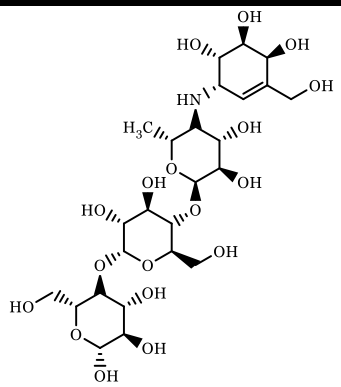
Table 1: Structures and *in vitro* α-amylase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



1-28



29-41

Compound	R_2'	R_4'	R_6'	R_2	R_3	R_4	R_5	R_6	α-Amylase inhibitory activity (%)
35	-	NO_2	-	-	-	-	-	-	$<20^{25\ \mu M}$
36	-	-	-	-	-	Me	-	-	$<20^{25\ \mu M}$
37	-	-	-	-	-	OMe	-	-	$<20^{25\ \mu M}$
38	-	-	-	-	-	NO_2	-	-	$<20^{25\ \mu M}$
39	-	-	-	NO_2	-	-	-	-	$<20^{25\ \mu M}$
40	OH	-	-	-	-	-	-	-	$<20^{50\ \mu M}$
41	OH	-	-	NO_2	-	-	-	-	$<20^{50\ \mu M}$
Positive control: **Acarbose**									$97.5\pm0.4^{10\ \mu M\ (c)}$

ND: Not determined due to interference with the methodology
% Inhibition with different superscript letters are significantly different from each other ($p<0.05$)

No relevant inhibitory activity was observed, up to the highest concentration, possible to be tested in these experimental conditions (indicated, in superscript, in the Table 1 for each chalcone). Among all the tested compounds, chalcone **4** (butein), with hydroxyl

groups at 2' and 4' positions of the A ring and 3 and 4 positions of the B ring, was the compound with the highest inhibitory activity, 59.6±3.5% at 100 µM. The results may indicate that the presence of hydroxyl groups is important for the inhibitory activity of the compounds against α-amylase. From all the hydroxylated compounds (**2**-**5**), chalcone **4** (butein) was the most active one, followed by chalcone **2** (25.92±0.8% at 100 µM), with hydroxyl groups at 2' position of the A ring. Comparing with acarbose, the positive control (IC_{50}=0.7±0.1 µM) (Fig. 42 B), the IC_{50} obtained for chalcone **4** (butein) (IC_{50}=82.7±7.1 µM) (Fig. 42 A) is considerably higher.

From the hydroxylated and/or methoxylated chalcones (**6**-**17**), low inhibitory activities were observed, ranging from 28.9±1.8% at 100 µM (chalcone **9**) and 36.2±3.2 at 100 µM (chalcone **11**). In general, substitutions with methoxy groups seems to be disadvantageous for α-amylase inhibitory activity.

Similarly, the studied chalcones with methyl substitutions (**18**, **19**) were not able to inhibit α-amylase.

Chalcones holding nitro groups (**20**, **21**) also showed reduced activity. Chalcone **21,** holding a hydroxyl group at 2' position of the A ring and a nitro group at 4 position of the B ring, showed the higher inhibitory activity, 24.5±2.6% at 100 µM.

In what concerns the chalcones with chlorine substitutions (**22**-**28**), also low α-amylase inhibition was observed. Among these seven compounds, only one has activity superior to 20%, chalcone **24** (21.7±3.1% at 50 µM), which has a hydroxyl group at 2' position of the A ring and a chlorine at 3 position of the B ring.

All the tested compounds from the group of chalcone analogues (**29**-**41**), which hold two double bonds linking the A and B rings, were not able to inhibit the α-amylase activity, at the maximum tested concentrations.

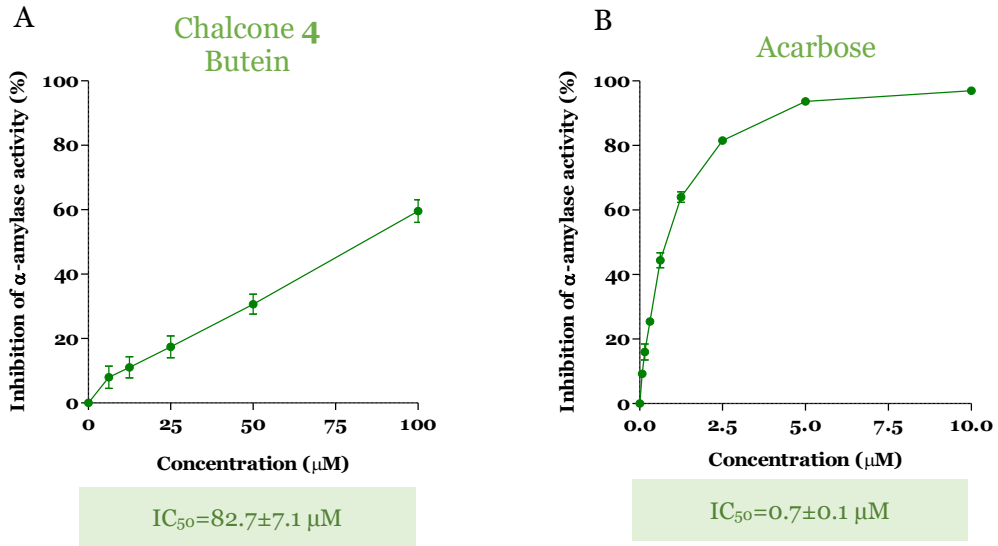
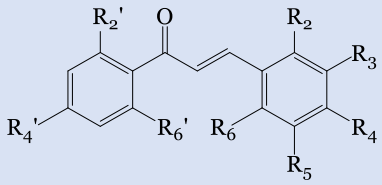


Fig. 42: α-Amylase inhibition by chalcone **4** (butein) (A) and the positive control acarbose (B). Each value represents mean±SEM of at least three experiments.

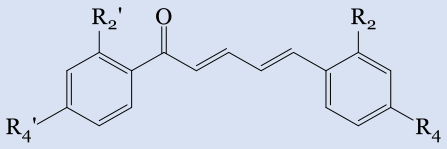
3.2 *In vitro* α-glucosidase inhibition assay

The inhibitory effect of chalcones **1-28**, chalcone analogues **29-41** and the positive control, acarbose, against α-glucosidase activity are shown in Table 2.

Table 2: Structures and *in vitro* α-glucosidase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript).



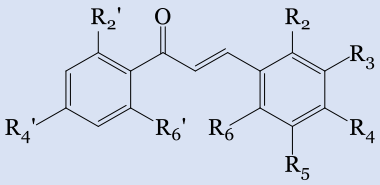
1-28



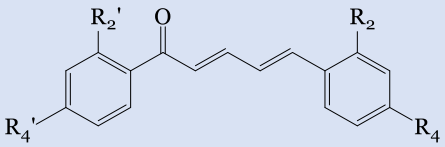
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Glucosidase inhibitory activity (%)
1	-	-	-	-	-	-	-	-	$<20^{150\ \mu M}$
2	OH	-	-	-	-	-	-	-	$<20^{75\ \mu M}$
3	OH	OH	-	-	-	-	-	-	$<20^{150\ \mu M}$
4 Butein	OH	OH	-	-	OH	OH	-	-	$86.3\pm2.2^{150\ \mu M\ (a)}$
5	OH	OH	OH	-	OH	OH	-	-	ND
6	OH	-	-	OMe	-	-	-	-	$<20^{40\ \mu M}$
7	OH	-	-	-	OMe	-	-	-	$<20^{40\ \mu M}$
8	OH	-	-	-	-	OMe	-	-	$<20^{40\ \mu M}$
9	OH	-	-	-	OMe	OH	-	-	$33.6\pm3.5^{150\ \mu M\ (b)}$
10	OH	-	-	-	OMe	OMe	-	-	$<20^{40\ \mu M}$
11	OH	-	-	-	OMe	OMe	OMe	-	$<20^{40\ \mu M}$

Table 2: Structures and *in vitro* α-glucosidase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



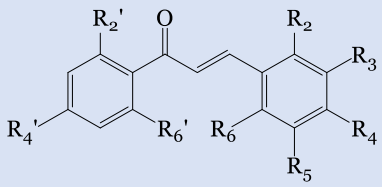
1-28



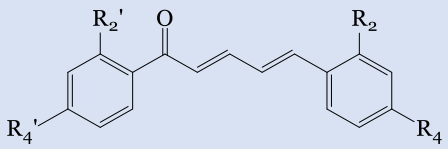
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Glucosidase inhibitory activity (%)
12	OH	OH	-	-	-	OMe	-	-	<20[150 µM]
13	OH	OH	-	-	OMe	OMe	-	-	<20[150 µM]
14	OH	OMe	OMe	-	-	OMe	-	-	<20[40 µM]
15	OH	OMe	OMe	-	OMe	OMe	OMe	-	<20[40 µM]
16	OMe	OMe	OMe	-	OMe	OMe	-	-	<20[150 µM]
17	-	-	-	-	OMe	OMe	-	-	<20[150 µM]
18	OH	-	-	-	Me	-	-	-	<20[40 µM]
19	OH	-	-	Me	-	Me	-	Me	<20[40 µM]
20	-	-	-	-	-	NO_2	-	-	<20[75 µM]
21	OH	-	-	-	-	NO_2	-	-	95.6±1.8[75 µM (a)]
22	-	-	-	-	-	Cl	-	-	<20[40 µM]
23	OH	-	-	Cl	-	-	-	-	<20[40 µM]
24	OH	-	-	-	Cl	-	-	-	<20[40 µM]
25	OH	-	-	-	-	Cl	-	-	<20[40 µM]

Table 2: Structures and *in vitro* α-glucosidase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



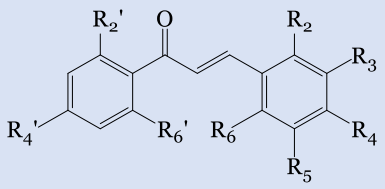
1-28



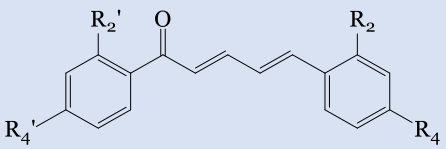
29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Glucosidase inhibitory activity (%)
26	OH	-	-	Cl	-	Cl	-	-	$96.3 \pm 2.2^{150\ \mu M\ (a)}$
27	OH	-	-	Cl	-	-	-	Cl	$<20^{40\ \mu M}$
28	OH	-	-	-	Cl	Cl	-	-	$<20^{40\ \mu M}$
29	-	-	-	-	-	-	-	-	$<20^{40\ \mu M}$
30	-	Me	-	-	-	-	-	-	$<20^{40\ \mu M}$
31	-	OMe	-	-	-	-	-	-	$<20^{40\ \mu M}$
32	-	Cl	-	-	-	-	-	-	$<20^{40\ \mu M}$
33	-	F	-	-	-	-	-	-	$<20^{40\ \mu M}$
34	-	Br	-	-	-	-	-	-	$<20^{40\ \mu M}$
35	-	NO_2	-	-	-	-	-	-	$<20^{40\ \mu M}$
36	-	-	-	-	-	Me	-	-	$<20^{40\ \mu M}$
37	-	-	-	-	-	OMe	-	-	$<20^{40\ \mu M}$
38	-	-	-	-	-	NO_2	-	-	$<20^{40\ \mu M}$
39	-	-	-	NO_2	-	-	-	-	$<20^{40\ \mu M}$

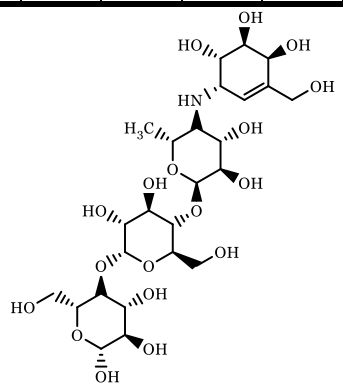
Table 2: Structures and *in vitro* α-glucosidase inhibition by chalcones (% inhibition at the highest tested concentration, indicated in superscript) (continued).



1-28



29-41

Compound	$R_{2'}$	$R_{4'}$	$R_{6'}$	R_2	R_3	R_4	R_5	R_6	α-Glucosidase inhibitory activity (%)
40	OH	-	-	-	-	-	-	-	$<20^{40\ \mu M}$
41	OH	-	-	NO_2	-	-	-	-	$95.7\pm0.6^{75\ \mu M\ (a)}$
Positive control: **Acarbose**									$81.6\pm1.2^{2285\ \mu M\ (a)}$

ND: Not determined due to interference with the methodology

% Inhibition with different superscript letters are significantly different from each other ($p<0.05$)

The results presented in Table 2, showed that some chalcones have interesting inhibitory activities against α-glucosidase activity. The most promising inhibitors were chalcone **4** (butein), chalcone **21**, chalcone **26** and chalcone analogue **41**, with the IC_{50} values of 32.9±0.9 μM, 47.3±0.3 μM, 83.8±1.4 μM, 36.1±1.0 μM, respectively (Fig. 43).

Among the hydroxylated compounds (**2-5**), chalcone **4** (butein), with hydroxyl groups at 2' and 4' positions of the A ring and 3 and 4 positions of the B ring, was the most active compound, with an IC_{50} value of 32.9±0.9 μM, which is almost sixteen times lower than the IC_{50} found for acarbose, the positive control (IC_{50}=517.9±24.3 μM). Chalcone **2**, with a hydroxyl group at 2' position of the A ring and no substitutions at the B ring, and chalcone **3**, with hydroxyl groups at 2' and 4' positions of the A ring and without substitutions at the B ring, showed no inhibitory activity. According to the results, hydroxyl

substitutions seems to have an important function in the inhibitory activity of these compounds.

Considering the hydroxylated and methoxylated chalcones (**6**-**15**) and the methoxylated chalcones (**16**, **17**), no relevant inhibitory activities were showed up to the highest tested concentration. Chalcone **9**, with a hydroxyl group at 2' position of the A ring, a methoxy substitution at 3 position and a hydroxyl group at 4 position of the B ring, showed an inhibitory activity of 33.6±3.5 at 150 μM, despite the low activity, was the highest inhibitory activity found among these group of chalcones. The results indicate that the presence of methoxy groups did not favoured the inhibitory activity against α-glucosidase.

In what concerns the studied chalcones with methyl substitutions (**18**, **19**), no inhibitory activity against α-glucosidase activity was observed.

From the group of chalcones holding nitro groups (**20**, **21**), chalcone **21** (IC_{50}=47.3±0.3 μM), with a hydroxyl group at 2' position of the A ring and a nitro group at 4 position of the B ring, showed almost eleven times more activity than the positive control, acarbose (IC_{50}=517.9±24.3 μM) (Fig. 43). Notably, the presence of a nitro group together with a hydroxyl group, increased the inhibitory activity, as observed comparing chalcone **21** and chalcone **20** (<20% at 75 μM), which has no substitutions at the A ring and a nitro group at the same position of the B ring.

Examining the results of the chlorinated chalcones (**22**-**28**), chalcone **26,** with a hydroxyl group at 2' position of the A ring and a chlorine at 2 and 4 positions of the B ring, was the most active compound, with an IC_{50} value of 83.8±1.4 μM, which is six times higher than acarbose (IC_{50}=517.9±24.3 μM) (Fig. 43). Chalcones **22-25,** chalcone **27** and chalcone **28** had no activity against the enzyme.

Among the chalcone analogues (**29**-**41**), which hold two double bonds linking the A and B rings, chalcone **41,** with a hydroxyl group at 2' position of the A ring and a nitro group at 2 position of the B ring, showed a high inhibitory activity, with an IC_{50} value of 36.1±1.0 μM, almost fifteen times more activity than the positive control, acarbose (IC_{50}=517.9±24.3 μM) (Fig. 43). In turn, the presence of the nitro group seems to be essential for the inhibitory activity of the compounds. The tested chalcone analogues **29**-**40** showed an inhibitory activity inferior to 20%, up to the highest tested concentrations.

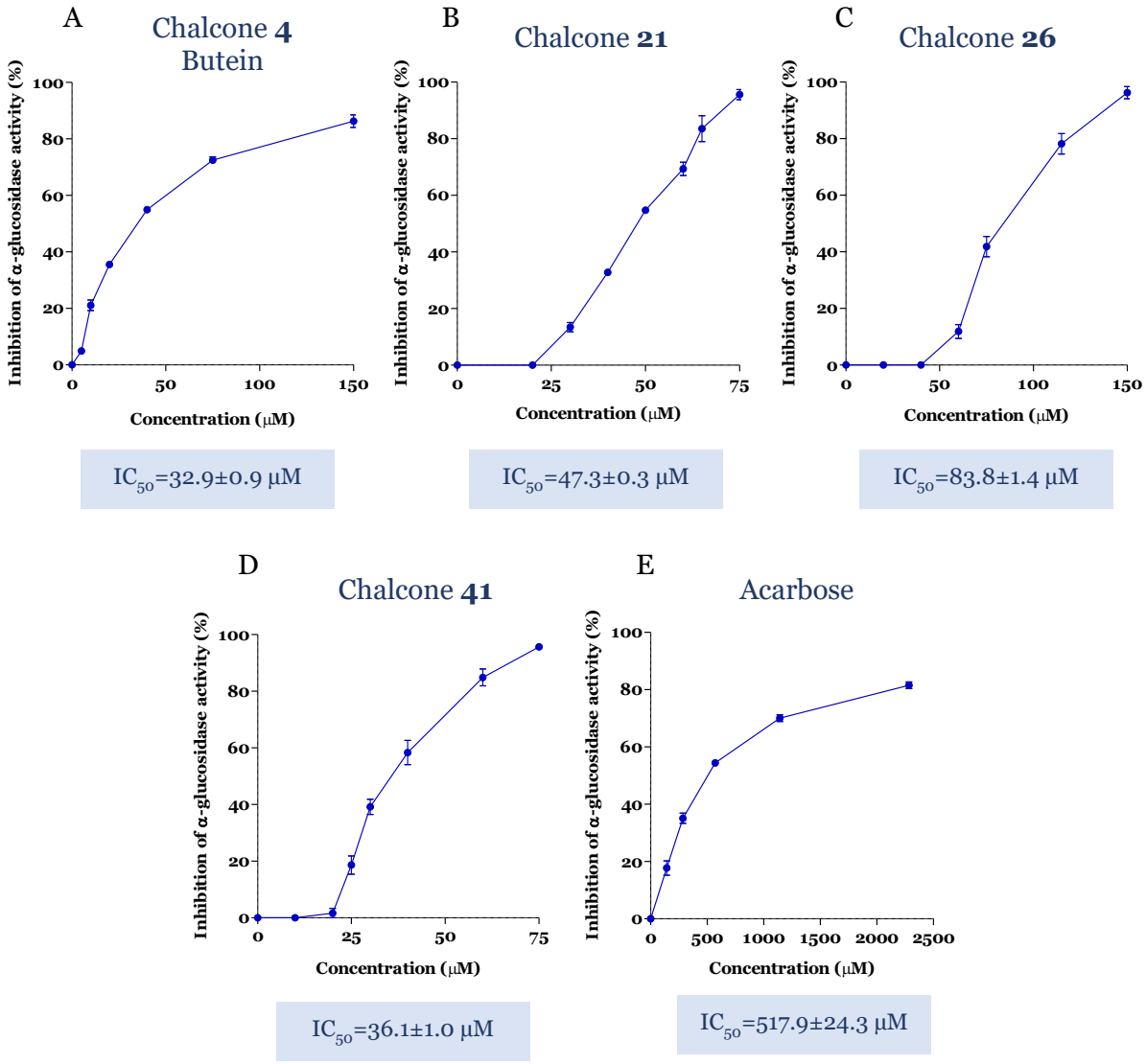


Fig. 43: α-Glucosidase inhibition by chalcone **4** (butein) (A), chalcone **21** (B), chalcone **26** (C), chalcone analogue **41** (D) and acarbose (E), the positive control. Each value represents mean±SEM of at least three experiments.

3.3 Inhibitory kinetic analysis

3.3.1 Inhibitory kinetic analysis of α-amylase

In accordance with the described results, the type of inhibition of the chalcone **4** (butein), which was the most active chalcone to inhibit α-amylase activity, and the positive control acarbose, were obtained by the nonlinear regression of Michaelis-Menten and Lineweaver-Burk plots, with the estimation of K_m and V_{max}. As observed in Fig. 44, chalcone

4 (butein) showed a competitive inhibition, since the K_m value increased (ϰ-intercept moves closer to the origin with increasing inhibitor concentrations) and V_{max} value remained constant (same y-intercept). Acarbose have shown a mixed inhibition type, in which the K_m and V_{max} value decreased (ϰ-intercept and y-intercept deviate from the origin with increasing inhibitor concentrations).

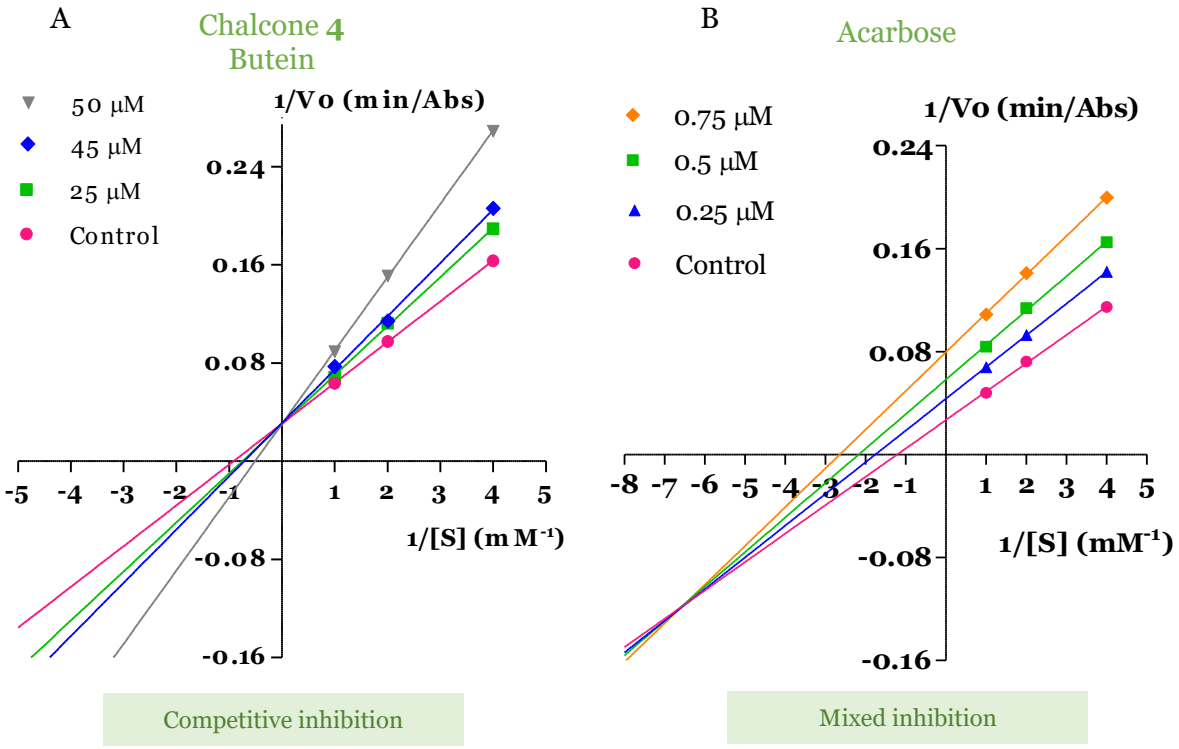


Fig. 44: Lineweaver-Burk plots of α-amylase inhibition by chalcone **4** (butein) and acarbose, the positive control.

It was possible to select the best inhibition model using Solver, by comparing the sum of the square errors values among the different enzyme inhibition models. The best kinetic model corresponds to the model with the lowest sum of the square error value. The results for the type of inhibition and the kinetic constants values (V_{max}, K_m, K_{ic} and/or K_{iu}) for chalcone **4** (butein) and acarbose, estimated using Solver are showed in Table 3. The obtained results corroborate that chalcone **4** (butein) is a competitive inhibitor and acarbose is a mixed inhibitor. The analysis by ANOVA one-factor showed that the procedure adopted had a precision better than 2.2 Δabsorbance/minutes and 1.4 Δabsorbance/minutes for chalcone **4** (butein) and acarbose, respectively, as calculated from the within-groups mean square. The ANOVA also showed a F value for the assays with chalcone **4** (butein) and acarbose, of 18.0 and 59.9, respectively, higher than the F critical value (1.9 and 1.4, respectively), corresponding to a *p* value < 0.05, as consequence of its significant effect on the enzymatic activity.

Table 3: Type of inhibition (using Solver supplement) of chalcone **4** (butein) and acarbose against α-amylase activity and respective kinetic parameters values: V_{max}, K_m, K_{ic} and K_{iu} (mean±SEM).

Compound	Type of inhibition	V_{max}	K_m	K_{ic}	K_{iu}
Chalcone **4** Butein	Competitive	34.7±1.5	1.3±0.1	122.6±4.7	-
Acarbose	Mixed	40.5±0.6	0.9±0.02	2.6±0.2	0.4±0.01

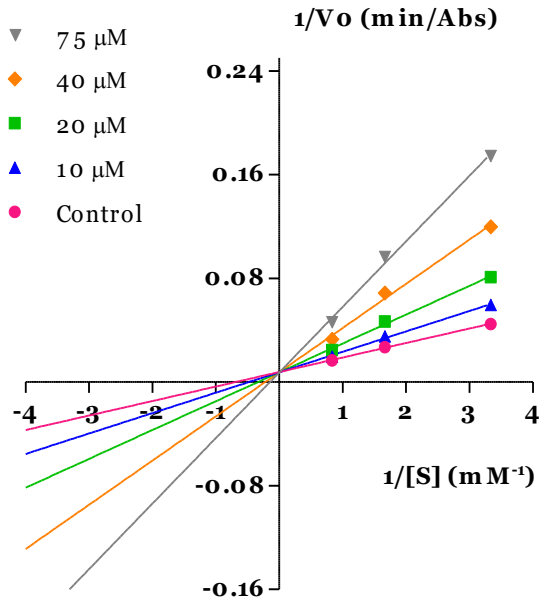
3.3.2 Inhibitory kinetic analysis of α-glucosidase

The type of inhibition of the most active compounds, chalcone **4** (butein), chalcone **21**, chalcone **26**, chalcone analogue **41** and acarbose, against α-glucosidase activity, were deducted by the nonlinear regression of Michaelis-Menten and Lineweaver-Burk plots. Fig. 45 shows the Lineweaver-Burk plots of α-glucosidase activity. Chalcone **4** (butein), chalcone **21** and acarbose showed a competitive inhibition, since the K_m value increased and V_{max} value remained constant. Chalcone **26** and chalcone **41** showed a non-competitive inhibition, which the K_m value remained constant (same $\varkappa$-intercept) and V_{max} value decreased (y-intercept deviate from the origin).

Additionally, was used the nonlinear regression to determine the kinetic parameters and the type of inhibition using Solver supplement. The obtained results are showed in Table 4. The results confirmed the inhibition type obtained by the Lineweaver-Burk plots, with the exception of chalcone **21**, which exhibited a non-competitive inhibition. The analysis by ANOVA one-factor enabled to conclude that the procedure adopted had a precision better than 5.7 Δabsorbance/minutes, 4.7 Δabsorbance/minutes, 7.2 Δabsorbance/minutes, 4.4 Δabsorbance/minutes, 2.4 Δabsorbance/minutes for chalcone **4** (butein), chalcone **21**, chalcone **26**, chalcone analogue **41** and acarbose, respectively, as calculated from the within-groups mean square. The ANOVA also showed a F value for the assays with chalcone **4** (butein), chalcone **21**, chalcone **26**, chalcone analogue **41** and acarbose of 35.8, 77.7, 24.6, 77.5, 238.2, respectively, higher than the F critical value (1.9, 1.8, 1.8, 1.8 and 1.9 respectively), corresponding to a p value < 0.05, as consequence of its significant effect on the enzymatic activity.

A

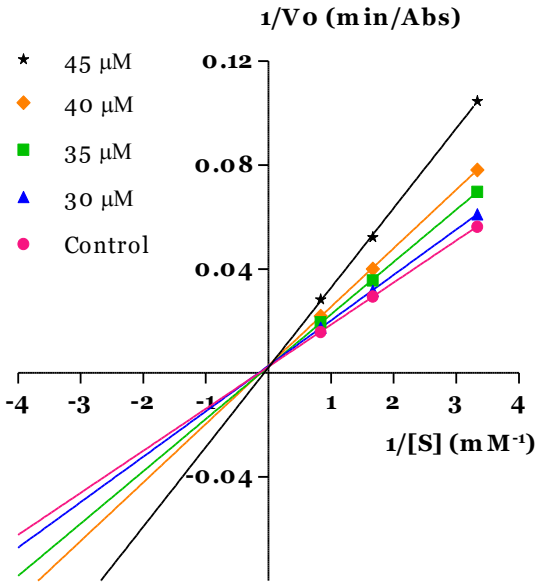
Chalcone **4**
Butein



Competitive inhibition

B

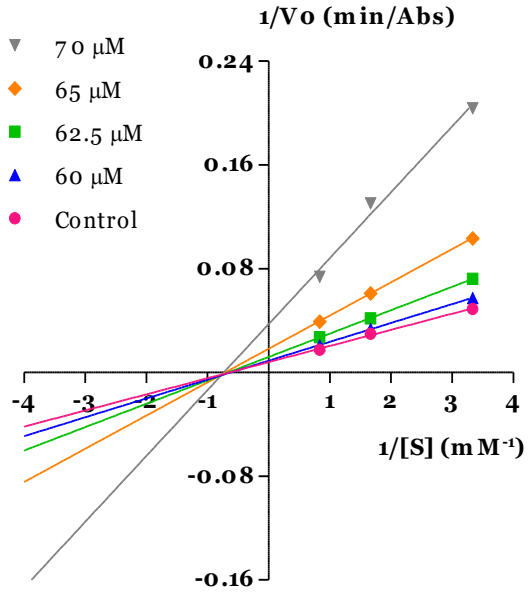
Chalcone **21**



Competitive inhibition

C

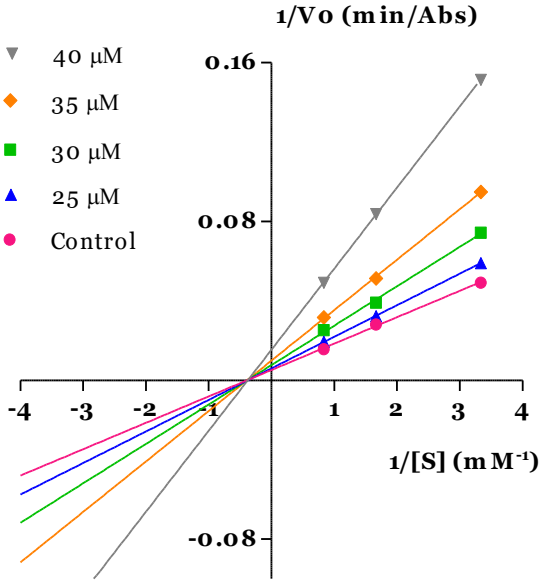
Chalcone **26**



Non-competitive inhibition

D

Chalcone **41**



Non-competitive inhibition

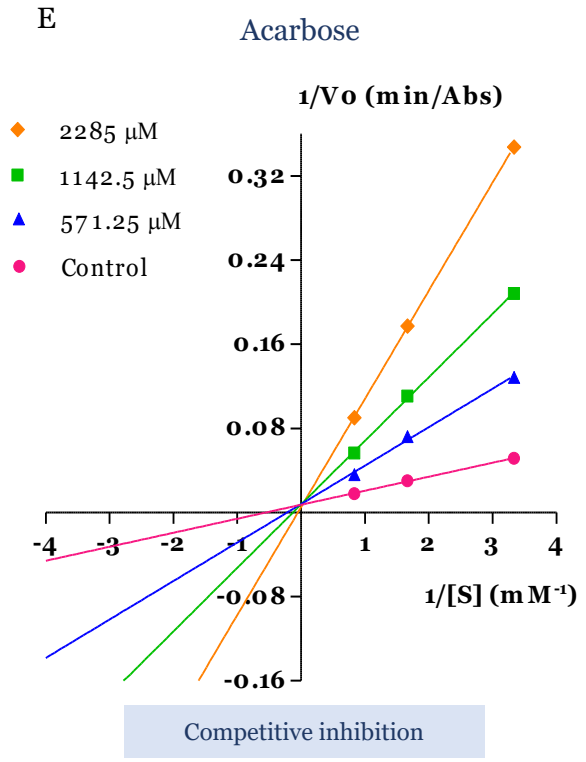


Fig. 45: Lineweaver-Burk plots of α-glucosidase inhibition by chalcone **4** (butein) (A), chalcone **21** (B), chalcone **26** (C), chalcone **41** (D) and acarbose (E), the positive control.

Table 4: Type of inhibition (using Solver supplement) of chalcone **4** (butein), chalcone **21**, chalcone **26**, chalcone analogue **41** and acarbose against α-glucosidase activity and respective kinetic parameters values: V_{max}, K_m, K_{ic} and K_{iu} (mean±SEM).

Compound	**Type of inhibition**	**V_{max}**	**K_m**	**K_{ic}**	**K_{iu}**
Chalcone **4** Butein	Competitive	135.4±2.5	1.6±0.04	22.3±0.3	-
Chalcone **21**	Non-competitive	458.6±31.8	7.3±0.9	101.5±1.8	101.5±1.8
Chalcone **26**	Non-competitive	154.9±9.8	2.1±0.2	82.7±3.2	82.7±3.2
Chalcone analogue **41**	Non-competitive	216.6±11.7	2.8±0.3	42.1±1.1	42.1±1.1
Acarbose	Competitive	165.5±3.2	2.4±0.1	372.1±3.3	-

4. Discussion

The currently available pharmacological therapy used in the management of DM has not been totally effective, showing some undesirable side effects. Therefore, it is essential to find safer, more effective and specific hypoglycaemic drugs to control this disease. The enzymes α-amylase and α-glucosidase play a central role in the breakdown of carbohydrates before the intestinal absorption of monosaccharides, thus, display an important role in controlling the postprandial blood glucose levels. Inhibition of these two enzymes is one of the therapeutic approaches used to modulate DM and its complications (149, 150).

Chalcones, abundant in edible plants, display several biological activities, including the anti-diabetic (120), which is the focus of the present thesis. The effect of some natural and synthetic chalcones have already been studied against α-amylase and α-glucosidase activities (167). However, the comparison of the results found in the literature is difficult due to several experimental variables: 1) the *in vitro* model used: cellular model with different types of cells, or enzymatic model, with different origin of enzymes and substrates; 2) the origin of chalcones: chemical synthesis, isolated from natural sources, or commercially acquired; 3) the tested concentrations of chalcones, and 3) time of exposure. These differences lead to discrepancies and difficulties in the comparison among the studies. Furthermore, the published works and the panel of chalcones studied regarding their inhibitory effect against digestive enzymes are scarce. Thus, in the present work it was evaluated the inhibitory effect of a panel of twenty-eight chalcones and thirteen chalcone analogues, in a total of forty-one structurally related scaffolds, against α-amylase and α-glucosidase activities.

This work started with the study of an unsubstituted chalcone (**1**), which allow the understanding about the impact of different substituents in the aryl rings in the inhibitory activity of these compounds. Chalcone **1** was unable to inhibit α-amylase at the maximum tested concentration (100 μM). In turn, contrary to our findings, Najafian *et al.* (168) also studied this compound, and reported a reversible partial inhibitory effect, with an IC_{50}=96.44 μM and a competitive behaviour. In order to validate their results, the authors studied α-amylase inhibitory activity of chalcone **1** in an *in vivo* model (169). The authors observed that, at 8 mg/kg, a significant reduction of hyperglycaemia, normalization of lipid profile, along with a reduction of water intake and urine volume, was found, showing that chalcone **1** is an effective agent for the treatment of type 2 DM and also in the prevention of obesity, mainly through the inhibition of α-amylase. These different results could be explained by the differences found in the experimental conditions, such as the use of starch as substrate and the measure of maltose to determine the inhibition of α-amylase.

Different substitutions in the main chalcone scaffold were tested against α-amylase inhibitory activity. The most active one was the hydroxylated chalcone **4** (butein)

(IC_{50}=82.7±7.1 μM). The chalcones **1**, **2**, and **3**, studied here for the first time, did not shown any activity against α-amylase. These results demonstrate that, the presence of hydroxyl groups at 2' and 4' positions of the A ring and 3 and 4 positions of the B ring (**4**, butein), seems to contribute to the inhibitory effect. As example, chalcone **3** which has hydroxyl groups at 2' and 4' positions of the A ring and no substitutions at B ring, showed no inhibitory activity, suggesting that the presence of the catechol group was crucial for the activity of chalcone **4** (butein). A similar conclusion was drawn by Seo *et al.* (170) which studied the inhibitory effect of chalcones substituted with hydroxyl, amine and sulfonamide groups against α-amylase. The authors report that the most active compound was a sulfonamide chalcone with a catechol group in 3 and 4 positions at the B ring. By the comparison with the same chalcone with just one hydroxyl group at 4 position of the B ring, which presented the double of the IC_{50}, the authors concluded that the catechol group is essential for the inhibition of α-amylase (170).

Accordingly, it was already described that the presence of the catechol group on other polyphenol scaffold, as flavonoids, is essential for the inhibitory activity against α-amylase (171, 172).

In general, substitutions with methoxy groups were not favourable for the inhibitory activity towards α-amylase. To the best of our knowledge, chalcones **6-17** were studied here for the first time. Chalcone **13** differ from the chalcone **4** (butein), due the presence of the two methoxy groups in the B ring (**13**) instead the two hydroxyl groups present in chalcone **4**. Analysing these two chalcones, it is possible to conclude that the presence of methoxy groups was not favourable for the inhibitory activity, as shown by the loss of activity of chalcone **13.** Comparing chalcone **3**, with hydroxyl groups at 2' and 4' positions of the A ring, and chalcone **12** with the same hydroxyl substitutions and an additional methoxy substitutions at the B ring, it is possible to see, that the addition of the methoxy group did not improve the inhibitory activity of the chalcone. These results are in accordance with Yang *et al.* (173), which studied one hydroxylated chalcone and two chalcones with a *O*-glucopyranoside at the A ring. Among chalcones with a *O*-glucopyranoside, one chalcone has hydroxyl substitutions at 3 and 4 positions of the B ring, and when the hydroxyl at 4 position is substituted by a methoxy, the activity decreased from 41% to 15%, at the concentration of 556 μg/mL (173). This is consistent with what has been found by Bale *et al.* (174), which showed that the substitution with methoxy groups also decreased the inhibitory activity of the most active compound. In this study, the authors tested two groups of chalcones, six chalcones with a methyl group at 4' position of the A ring, and the other group of seven chalcones with a methoxy substitution at the same position, both with different substitutions at the B ring. The most active compound of each

group has a SCH_3 at the 4 position of the B ring. However, when this group is substituted by a methoxy group, the IC_{50} value increased (174).

Nevertheless, the presence of three methoxy groups at 3, 4 and 5 positions of the B ring, originated a slight improvement of the inhibitory effect, since chalcone **11** showed 36.2±3.2% of inhibition at 100 μM. Despite the slight inhibitory activity, it is suggested that the presence of a hydroxyl group at 2' position of the A ring, together with methoxy substitutions at 3, 4 and 5 positions of the B ring, seems to benefit the inhibitory activity.

The methyl group is not favourable for the inhibitory effect of chalcones, as it is possible to see by the lack of activity observed for chalcone **18** and chalcone **19**. As far as we know, these structures were also studied here for the first time. However, Hu *et al.* (175) studied a chalcone with methyl substitutions at 3' and 5' positions of the A ring, together with hydroxyl groups at 2' and 4' positions and a methoxy substitution at 6' position of the A ring, and no substitutions at the B ring. This chalcone inhibited the α-amylase in a dose-dependent and in a non-competitive manner, with an IC_{50}=43 μM. Also, Bale *et al.* (174) studied compounds with methyl substitutions, at 4' positions of the A ring, with different attachments at the B ring. In their study, the tested chalcones showed significant *in vitro* α-amylase inhibitory activities, presenting IC_{50} values from 1.27±0.7 to 2.26±0.07 μM. These differences among studies clearly shown that the pattern of substitutions can alter the activity of the compounds.

In what concerns the chalcones holding nitro groups (**20**, **21**), there are no reports in literature about their effect against α-amylase. Indeed, these chalcones shown low or none activity.

In what concerns the group of chalcones with chlorine substitutions (**22-28**), also reduced α-amylase inhibition was observed. As far as we know, chalcones **22-28** were also studied here for the first time. The presence or absence of the hydroxyl group at 2' position of the A ring is indifferent for the intended effect, as it is shown comparing chalcone **22**, with no substitutions at the A ring and a chlorine at 3 position of the B ring, with chalcone **25**, with the hydroxyl group at the A ring and a chlorine at the same position in the B ring. Increasing chlorine substitutions at the B ring was also indifferent for the activity. This is clear by comparing chalcone **23**, which has a hydroxyl group at 2' position of the A ring and chlorine substitution at 2 position of the B ring, with chalcone **26**, with the same substitutions and a second chlorine at 4 position of the B ring. A similar pattern of results was obtained by Bale *et al.* (174), which also studied chalcones with chlorine substitutions at the B ring. Despite the significant inhibitory activity of the studied compounds, when at the most active chalcone, the SCH_3 at the B ring was substituted by a chlorine group, the IC_{50} value increased. The same effect was observed with other tested chalcone, in which the addition of two chlorine groups at the B ring, also increased the IC_{50} value (174).

All the tested new chalcone analogues (**29**-**41**), showed an inhibitory activity inferior to 20%, up to the highest tested concentrations. These compounds have two double bonds linking the A and B rings, and different compounds were tested, including an unsubstituted chalcone (**29**), substitutions at 4' positions of the A ring with methyl (**30**), methoxy (**31**), chlorine (**32**), fluorine (**33**), bromine (**34**), nitro group (**35**); substitutions at 4 positions of the B ring with methyl (**36**), methoxy (**37**), nitro group (**38**); substitutions at 2 position of the B ring with a nitro group (**38**), substitutions at 2' position of the A ring with hydroxyl (**40**), and a is di-substituted with a hydroxyl and a nitro group (**41**). The tested substitutions at these positions did not favoured the indeed effect. Bale *et al.* (174) studied similar chalcone analogues against α-amylase activity, also holding two double bonds linking the A and B rings, and with different substitutions, including chlorine, bromine, methoxy, ethoxy and methyl-sulfane, mainly at 4' position of the A ring and at 4 position of the B ring. However, the ketone is located in the middle of the two double bonds. The tested compounds demonstrated α-amylase inhibitory potential, in the range of IC_{50}=1.63±0.18–2.40±0.09 μM. The most active compound was a chalcone holding methoxy groups at 4' position of the A ring and at 4 position of the B ring. Once again, the differences in the pattern of substitutions and/or the variables found in the experimental conditions, could dictate different results.

After the study of the effectiveness of the tested chalcones on α-amylase inhibition, the most active chalcone (chalcone **4**, butein) and the positive control acarbose, were selected to study their type of inhibition. The nonlinear regression of Michaelis-Menten with the complement Lineweaver-Burk plots were used in order to determinate their inhibitory kinetics. Since the linear transformations of the Michaelis-Menten equation are less accurate, the type of inhibition and the kinetic constants were also evaluated using Solver supplement of Excel Microsoft Office™. The two methods are in accordance and showed that chalcone **4** (butein) is a competitive inhibitor, due to the increase of K_m value and the constant value of $V_{max,}$ with increasing inhibitor concentrations. This means that chalcone **4** (butein) competes with the substrate for the active site of the enzyme, thus, it is required more concentration of CNPG3 to generate the reaction product. Acarbose have shown a mixed type of inhibition, in which the K_m and V_{max} value decreased with increasing inhibitor concentrations. This means that acarbose can bind to both the free enzyme and the enzyme-substrate complex. This finding is corroborated by other authors (176, 177). The analysis by ANOVA also showed that chalcone **4** (butein) and acarbose have a F value higher than the F critical value, which allowed the rejection of the null hypothesis, reinforcing that the compounds exhibited an inhibitory effect on the enzymatic activity.

Analysing the results of the α-glucosidase inhibition by chalcones it is possible to observe that the most active compounds were chalcone **4** (butein), chalcone **21**, chalcone

26 and chalcone analogue **41**, with IC_{50} values of 32.9±0.9 μM, 47.3±0.3 μM, 83.8±1.4 μM, 36.1±1.0 μM, respectively. Chalcone **4** (butein), with hydroxyl groups at 2' and 4' positions of the A ring and 3 and 4 positions of the B ring, was the most active compound, followed by the chalcone analogue **41**, which has two double bonds linking the A and B rings, and a hydroxyl group at 2' position of the A ring and a nitro group at 2 position of the B ring, chalcone **21**, with a hydroxyl group at 2' position of the A ring and a nitro group at 4 position of the B ring, and finally chalcone **26**, which has a hydroxyl group at 2' position of the A ring and a chlorine at 2 and 4 positions of the B ring.

In what concerns the studies with compounds holding hydroxyl groups (**2**-**5**) and the hydroxylated and/or methoxylated compounds (**6**-**17**), chalcone **4** (butein) was the only chalcone that showed a potent inhibitory activity, with an IC_{50} almost sixteen times lower than the IC_{50} found for acarbose, the positive control. No relevant activity was observed for the other hydroxylated and/or methoxylated chalcones (**2**,**3**, **5**-**17**), except chalcone **9**, which has a hydroxyl group at 2' position of the A ring, a methoxy substitution at 3 position and a hydroxyl group at 4 position of the B ring, which showed an inhibitory activity of 33.6±3.5 at 150 μM. These results suggest that the presence of the hydroxyl groups is crucial for the inhibition of α-glucosidase activity. Comparing chalcone **3**, with hydroxyl groups at 2' and 4' positions of the A ring, with chalcone **4** (butein), it is possible to conclude that the presence of the catechol group at 3 and 4 positions of the B ring favoured the inhibitory activity effect against α-glucosidase activity. On the other hand, the comparison of chalcone **4** (butein) and chalcone **13**, which has hydroxyl groups in the same positions of the A ring and methoxy groups at 3 and 4 positions of the B ring, it is possible to conclude that the presence of methoxy substitutions in the chalcone scaffold, did not bring any advantage for the intended effect. Moreover, increasing the number of methoxy groups in the B ring, in contrast with was obtained for α-amylase, did not favour the inhibitory activity, as it is possible to see comparing chalcone **10** with chalcone **11**. A similar pattern of results was obtained by Cai *et al.* (178), which tested a series of hydroxyl and methoxy chalcones against α-glucosidase activity. Comparing the tested compounds, in general, chalcones holding methoxy groups, showed much lower inhibitory activities than chalcones with hydroxyl groups, at the same positions. As example, the authors tested the inhibitory activity of one chalcone which has methoxy groups at 2' and 4' positions at the A ring and a hydroxyl group at 4 position at the B ring, and when compared with one chalcone which has hydroxyl groups in the same positions of the A and B ring, the IC_{50} decreased. Taking into account these results, the authors reported that the presence of a H-bond donor is the key for a better interaction of these compounds with the enzyme. In line with our rationale, the authors also described that the number and position of the hydroxyl groups in chalcone is an important factor for their inhibitory activity. The authors concluded that the presence of a catechol

group in the A and B rings was relevant for the inhibitory activity (178). Similarly to our results, Ansari *et al.* (179) tested one chalcone with a methoxy group at the B ring, and when a hydroxyl group at 2' position of the A ring was added to the chalcone, the IC_{50} value decreased (179). Sun *et al.* (180) evaluated the inhibitory activity of hydroxylated, prenylated and/or geranylated chalcones, and the results showed that the presence of prenyl and geranyl substitutions were essential for the inhibitory activity. Besides that, the substitution of the hydroxyl for methoxy groups at one prenylated chalcone diminished the activity (180) corroborating our results. Ryu *et al.* (181) tested another group of chalcones with different substitutions. The prenylated chalcone with hydroxyl groups at 2' and 4' positions of the A ring, together with a 3,4-di-hydroxyl group at the B ring was the most active compound. The catechol was important for the activity, since when a hydroxyl was removed from the B ring, the IC_{50} increase for the double (181). Seo *et al.* (170) also showed the importance of the catechol group at the B ring. The authors studied hydroxylated, aminated and sulfonamide chalcones. Among the tested chalcones, the sulfonamide chalcone with a catechol at 3 and 4 positions of the B ring was the most potent inhibitor of α-glucosidase activity.

The presence of a nitro group in the chalcone scaffold increased the inhibitory activity of chalcones against α-glucosidase activity. Chalcone analogue **41**, with an IC_{50} value of 36.1±1.0 μM, showed almost fifteen times more activity than the positive control, acarbose. Chalcone **21** exhibited eleven times more activity than acarbose. The addition of the nitro groups at chalcone **2** and chalcone analogue **40**, both with a single hydroxyl group at 2' position of the A ring, resulted in strong inhibitory activities. Moreover, the presence of a hydroxyl group was crucial for the α-glucosidase inhibition. This is evident by comparing the result obtained for chalcone **20**, with a nitro group at 4 position of the B ring, which has no activity, and chalcone **21**, with an additional hydroxyl group at 2' position of the A ring, that presented an IC_{50}=47.3±0.3 μM. The same effect was observed in chalcone analogues, as example, comparing chalcone analogue **39**, with a nitro group at 2 position of the B ring, with chalcone analogue **41**, with an additional hydroxyl group at 2' position of the A ring. In turn, changing the position of the nitro group in chalcone **39**, to 2' position of the A ring (chalcone analogue **35**) and to 4 position of the B ring (chalcone analogue **38**) did not improve the inhibition of the compounds. As far as we know, chalcone **21** and chalcone analogues with nitro groups (**35**, **38**, **39**, **41**) was studied here for the first time. Chalcone **20** was already evaluated by Ansari *et al.* (179) and Jabeen *et al.* (182). In both studies, the authors evaluated the inhibitory activity of different compounds with different substitutions, including hydroxylated, methoxylated, aminated and sulfonamide chalcones. In these studies, chalcone **20**, was the less active compound.

From all the chalcones containing chlorine (**22** – **28**), the most active compound was chalcone **26**, with an IC_{50} value six times higher than acarbose. To the best of our knowledge, this was the first study with chalcones holding chlorine substitutions for the α-glucosidase activity. Recently, Proença *et al.* (183) studied a series of chlorinated flavonoids. The flavonoid holding a chlorine group at the C ring and hydroxyl substitutions at the A and B ring was one of the most active compounds. The same group has shown that chlorinated flavonoids display potential anti-inflammatory activity (184), essential to avoid damages induced by inflammatory processes, which is exacerbated under diabetic conditions, and consequently there is an excessive production of reactive species. These together with our results indicate that the chlorine is a substituent that should be explored with possibilities to originate promising chemical structures for the intended effect.

In what concerns the other tested compounds, chalcones substituted with methyl groups (**18**, **19**) were not able to inhibit α-glucosidase activity, at the maximum tested concentration. The same conclusion was drawn by Hu *et al.* (175), that evaluated a chalcone with methyl substitutions at 3' and 5' positions of the A ring, together with hydroxyl and methoxy substitutions at the A ring, and no substitutions at the B ring, which did not present any inhibitory activity against α-glucosidase activity.

The chalcone analogues with methyl (**30, 36**), methoxy (**31, 37**), chlorine (**32**), fluorine (**33**) and bromine (**34**) substitutions were not able to inhibit α-glucosidase activity, at the maximum tested concentration of 40 μM. As far as we know, these chalcone analogues with two double bonds between the A and B rings were evaluated here for the first time. Cai *et al.* (178) studied a series of hydroxylated and methoxylated chalcones and bis-chalcones, which contain two chalcone moieties in a single structure, thus, also display two double bond in its structure. The authors reported that the introduction of hydroxyl groups in bis-chalcones were determinant to increase the inhibitory activity (178).

The type of inhibition of the most active compounds were studied using Lineweaver-Burk plots and Solver supplement. Both methods shown that chalcone **4** (butein) and acarbose have a competitive inhibition type. The competitive inhibition means that these compounds competes with the substrate to the active site of the enzyme. The K_m value increased, and the V_{max} value remained constant with increasing inhibitor concentrations. In what concerns chalcone **21**, a difference between both methods was observed. Lineweaver-Burk plots showed a competitive inhibition, while in the Solver supplement the type of inhibition was non-competitive. It is known that the Lineweaver-Burk plots are less accurate than the use of nonlinear regression calculated with the Solver supplement (166). Moreover, using the Solver supplement, the definition of the type of inhibition was performed according to the F test and the AIC test, allowing us to conclude that the inhibition type of chalcone **21** is non-competitive. In turn, chalcone **26** and

chalcone **41** also showed a non-competitive inhibition. As non-competitive inhibitors, these chalcones can bind with equal affinity to both the free enzyme and the enzyme-substrate complex, outside of the active site. In what concerns acarbose, the competitive type of inhibition was already reported by other authors (176, 183, 185). However, Son *et al.* (186) reported that acarbose is a mixed type inhibitor. These different results may be due to differences in experimental conditions. The analysis by ANOVA showed that the method has a precision better than 5.7 Δabsorbance/minutes, 4.7 Δabsorbance/minutes, 7.2 Δabsorbance/minutes, 4.4 Δabsorbance/minutes, 2.4 Δabsorbance/minutes for chalcone **4** (butein), chalcone **21**, chalcone **26**, chalcone analogue **41** and acarbose, respectively. The analysis by ANOVA also showed that the compounds have a F value higher than the F critical value, which means that, despite the variability found in the method precision, the compounds exhibited inhibitory effect against the enzyme activity.

The commercially available α-glucosidase inhibitors induce an excessive increase of undigested carbohydrates in the intestine, mainly due to the non-specific inhibition of α-amylase, leading to gastrointestinal side effects. A strong α-amylase inhibitory activity, lead to prolonged inhibition of starch hydrolysis and the accumulation of undigested carbohydrates in the colon, which result in excessive bacterial fermentation of carbohydrates, that are than used as substrate by bacterias. The consequences for the patients are severe gastrointestinal complications such as diarrhoea, flatulence and abdominal distention. Therefore, inhibitors of α-glucosidase with mild inhibitory activity against α-amylase, constitute one of major therapeutic strategies for the treatment of type 2 DM. Acarbose is one of the mostly prescribed α-glucosidase inhibitor in type 2 DM. However, its use is associated with severe gastrointestinal side effects thus, is essential to find therapeutic strategies to overcome this inconvenient and search for more effective and safer inhibitors (149, 187).

Chalcone **4**, butein, is an important natural occurring chalcone. Butein is present in several plants, such as in *Semecarpus anacardium*, and in herbs including *Caragana jubata*, *Rhus verniciflua*, and *Dalbergia odorifera*. All these plants have been showing antioxidant, anti-inflammatory, anticancer, antinephritic, antibacterial and anti-diabetic properties (188, 189). The anti-diabetic activity of butein was already evaluated *in vitro* and *in vivo* against aldose reductase, the responsible reductase for polyol pathway, the alternative route of glucose metabolism, which converts glucose into sorbitol, that accumulates in tissues causing several complications. Butein was shown to significantly reduce the activation of aldose reductase, preventing those complications (190, 191). It is known that inflammation contribute to the development of insulin resistance and disfunction of pancreatic β cells. Butein was also shown to be a potential therapeutic anti-

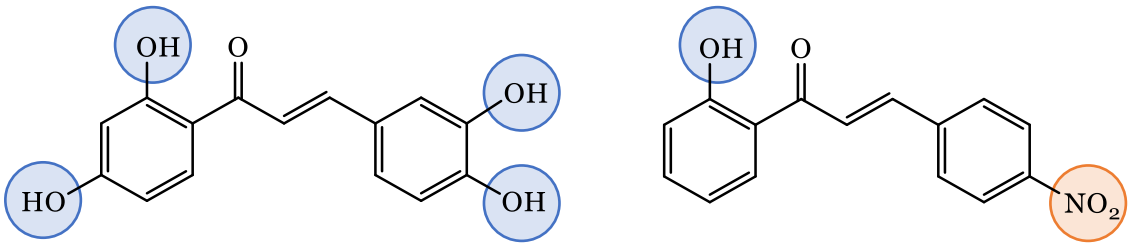
inflammatory agent (188). To the best of our knowledge, the inhibitory activity of butein against α-amylase and α-glucosidase, was studied here for the first time.

Since the most recent recommendations of the scientific community is that it should be found a compound with a mild inhibition of α-amylase and potent inhibition of α-glucosidase activity, among the tested chalcones, chalcone **4** (butein) and chalcone **21** constitute the promising molecules to address type 2 DM control and/or treatment.

5. Conclusions

Natural chalcones and their derivatives, have been shown to be bioactive molecules with great interest for the scientific community, due to their putative anti-diabetic properties. Few studies have been published in order to stablish the structure-activity relationship of chalcones against the inhibitory activity of the digestive enzymes, α-amylase and α-glucosidase. Therefore, in the present study, a panel of forty-one chalcones was evaluated for this effect, most of them for the first time. It was possible to conclude that the substitution pattern influences the inhibitory activity of the compounds, being the presence of hydroxyl groups favourable for the intended effect. From the obtained results, chalcone **4** (butein) and chalcone **21** were the most active compounds, showing a moderate action on α-amylase and a potent α-glucosidase inhibition. Therefore, the presence of hydroxyl groups at 2' and 4' positions of the A ring and at 3 and 4 positions of the B ring on chalcone **4** (butein) and hydroxyl groups at 2' position of the A ring and a nitro group at 4 position of the B ring, on chalcone **21**, seems to be essential for the inhibitory activity of the chalcones (Fig. 46). Moreover, it was proved that chalcone **4** (butein) displayed a competitive type of inhibition for both enzymes and chalcone **21** presented a non-competitive type of inhibition for α-glucosidase.

This study provided important considerations about the chalcones' scaffold and their anti-diabetic effect. These results also contribute for the design of novel molecules that can be useful in the management of type 2 DM.



Chalcone **4** (butein) Chalcone **21**

Fig. 46: Potential promising substitutions of chalcones which contributes to α-amylase and α-glucosidase inhibition.

6. References

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