Valorisation of wild mushrooms as functional foods: chemoinformatic studies

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ABSTRACT

Intermolecular interactions play essential roles in several life processes and understanding these interactions is critical for pharmaceutical and functional foods industries. Mushrooms represent an unlimited source of compounds with antitumor and immunostimulating properties and mushroom intake has been shown to reduce the risk of breast cancer. In this work, two *in silico* studies were performed in an attempt to elucidate potential mechanisms of mushroom bioactivity. First, a QCAR (Quantitative Composition-Activity Relationships) modelling approach was used to study and predict mushroom antioxidant activity. Next, molecular docking and virtual ligand screening (VLS) studies were performed in an attempt to elucidate possible mechanisms of mushroom anti-breast cancer activity.

For the initial QCAR study a PLS (Partial Least Square) statistical technique was applied to evaluate the relationship between antioxidant potential (scavenging effect on free radicals and reducing power) and chemical composition of twenty three samples from seventeen Portuguese wild mushroom species. A wide range of analytical parameters including ash, carbohydrates, proteins, fat, monounsaturated fatty acids, polyunsaturated fatty acids, saturated fatty acids, phenolics, flavonoids, ascorbic acid and β-carotene was studied and the data was analyzed by the PLS regression analysis to find correlations between all the parameters. Antioxidant activity correlated well with phenolic and flavonoid contents. A QCAR model was constructed, and its robustness and predictability was verified by internal and external cross-validation methods. This model proved to be a useful tool in the prediction of mushrooms reducing power.

For the VLS study, molecular docking software AutoDock 4 was used in order to evaluate which wild mushroom low molecular weight (LMW) compounds, including antioxidants, could be involved in anti-breast cancer activity. A representative dataset of 43 LMW compounds (individual phenolic acids, flavonoids, tocopherols, carotenoids, sugars and fatty acids) was selected and molecular docking was carried out against three known protein targets involved in breast cancer (Aromatase, Estrone Sulfatase and 17-β-hydroxysteroid dehydrogenase 1). The top ranked LMW compounds with breast cancer inhibition activity was predicted and the information provided showed several interesting starting points for further development of inhibitors of the mentioned

proteins. 4-*O*-caffeoylquinic acid, naringin and lycopene stand out as the top ranked potential inhibitors for Aromatase, Estrone Sulfatase and 17β-HSD-1, respectively.

The performed chemoinformatic studies allowed valorisation of mushrooms as functional foods and could be used in Industries focused on developing new nutraceuticals or functional foods.

RESUMO

As interacções intermoleculares desempenham um papel essencial nos diversos processos biológicos, sendo fundamental a compreensão destas interacções nos Sectores das Indústrias Farmacêuticas e de Alimentos Funcionais. Os cogumelos representam uma fonte ilimitada de compostos com propriedades antitumorais e imunoestimulantes, e o seu consumo foi já relacionado com a redução do risco de cancro da mama. No presente trabalho, foram desenvolvidos dois estudos *in silico* com o intuito de melhor compreender quais os mecanismos moleculares responsáveis por diferentes propriedades bioactivas dos cogumelos. Primeiro utilizou-se uma metodologia de modelação QCAR (Relações Quantitativas Composição − Actividade) para estudar e prever a actividade antioxidante de cogumelos. Num segundo estudo utilizaram-se ferramentas de "docking" molecular e "virtual ligand screening" (VLS) para tentar elucidar possíveis mecanismos de actividade dos cogumelos contra o cancro da mama.

No estudo QCAR inicial foi utilizada a técnica estatística dos Mínimos Quadrados Parciais (PLS) para avaliar a relação entre o potencial antioxidante (efeitos bloqueadores de radicais livres e poder redutor) e a composição química de vinte e três amostras de dezassete espécies de cogumelos silvestres Portugueses. Estudaram-se vários parâmetros analíticos tais como cinzas, hidratos de carbono, proteínas, gorduras, ácidos gordos monoinsaturados, ácidos gordos polinsaturados, ácidos gordos saturados, fenóis, flavonóides, ácido ascórbico e β-caroteno, e os seus resultados foram analisados por PLS de forma a estabelecer correlações entre todos os parâmetros. A actividade antioxidante mostrou estar correlacionada com o teor em fenóis e flavonóides. Foi construído um modelo QCAR, cuja robustez e capacidade de previsão foram verificadas por métodos de validação cruzada internos e externos. Finalmente, este modelo provou ser uma ferramenta útil na previsão do poder redutor de cogumelos.

Nos estudos de VLS foi utilizado o software de "docking" molecular Autodock 4 com o objectivo de identificar compostos de baixo peso molecular (LMW), incluindo antioxidantes, presentes em cogumelos e potencialmente envolvidos na actividade contra o cancro da mama. Foi seleccionado um grupo representativo de 43 compostos de LMW (ácidos fenólicos, flavonóides, tocoferóis, carotenóides, açúcares e ácidos gordos) e efectuou-se "docking" molecular usando como alvo três proteínas envolvidas no cancro da mama (Aromatase, Esterona Sulfatase e 17-β-hidroxi-esteróide

desidrogenase 1). Os compostos LMW foram classificados quanto à sua capacidade de inibição do cancro da mama. A informação obtida estabelece um bom ponto de partida para o desenvolvimento de inibidores das proteínas mencionadas. O ácido 4-*o*-cafeoilquínico, a naringina e o licopeno revelaram-se, respectivamente, os melhores inibidores para Aromatase, Esterona Sulfatase e 17β-HSD1.

Os estudos de Química Computacional realizados permitiram a valorização dos cogumelos como alimentos funcionais, podendo ser muito úteis para Indústrias que visem o desenvolvimento de novos nutracêuticos ou alimentos funcionais.

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ABREVIATIONS

17β-HSD-1 17-β-hydroxysteroid dehydrogenase 1.

ΔG Gibbs free energy.

ρ Significance.

Ǻ Ångströms.

A Androstenedione.

CAT Catalase.

CYP19A1 Aromatase gene.

DNA Deoxyribonucleic acid.

DPPH 1,1-Diphenyl-2-picrylhydrazyl.

E1 Estrone.

E2 Estradiol.

E1S Estrone sulfate.

E2S Estradiol sulfate.

ETS Electron transport system.

F Fisher ratio value.

FuFoSE European Commission's Concerted Action on Functional Food Science in Europe.

GC-FID Gas Chromatography-Flame Ionization Detector.

GPx Glutathione peroxidases.

Gred Glutathione redutase.

GSH Glutathione.

GSNO S-nitrosoglutathione.

GST Glutathione-S-transferases.

GS-SG Glutathione disulphide

HPLC High Performance Liquid Chromatography

HPLC/DAD-ESI/MS HPLC/Diode Array Detector- Electron Spray Ionization/Mass Spectrometry.

HPLC-RI HPLC-Refraction Index Detector.

HTS High-Throughput Screening.

ILSI International Life Science Institute

Ki Inhibition constant.

Km Michaelis-Menten Constant.

LMW Low Molecular Weight.

M Molar

MUFA Monounsaturated Fatty Acids.

NADP+ Nicotidamide adenine dinucleotide phosphate: oxidized.

NADPH Nicotidamide adenine dinucleotide phosphate reduced.

NOS Nitric oxide synthase.

PDB Protein Data Bank.

PLS Partial Least Square.

PRESS Predictive Error Sum of Squares

PSK Polysaccharide Krestin.

PUFA Polyunsaturated Fatty Acids.

Q^2 Chi-square test.

Q^2_{LOO} Q^2 Leave One Out

QCAR Quantitative Composition-Activity Relationships

QSAR Quantitative Structure-Activity Relationships

R^2 Squared correlation coefficient.

Rcal Gas constant for calories/K/mol.

RMSE Root Mean Squared Errors.

RNS Reactive Nitrogen Species.

ROS Reactive Oxygen Species.

RP Reducing Power.

RSA Radical Scavenging Activity.

S Standard deviation of regression.

SFA Saturated Fatty Acids.

SOD Superoxide dismutase.

STS Estrone sulfatase.

T Testosterone.

TK Temperature in Kelvin.

TSS Total Sum of Squares

VLS Virtual Ligand Screening

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I. INTRODUCTION

1. Functional foods

In the last decades consumer demands in the field of food production has changed considerably. Consumers more and more believe that foods contribute directly to their health. Today foods are not intended to only satisfy hunger and to provide necessary nutrients for humans but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumers. In this regard, functional foods play an outstanding role. The increasing demand on such foods can be explained by the rapid advances in science and technology, increasing cost of healthcare, changes in food laws affecting label and product claims, the steady increase in life expectancy, the desire of older people for improved quality of their later years, and rising interest in attaining wellness through diet (Siro et al., 2008).

According to the Institute of Medicine's Food and Nutrition Board "Functional Foods" are foods or dietary components that may provide a health benefit beyond basic nutrition. We can take greater control of our health through the food choices we make, knowing that some food can provide specific health benefits (Hasler, 1998). The European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE), coordinated by International Life Science Institute (ILSI) Europe defined functional food as follows: ''a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule just as normal food form'' (Siro et al., 2008).

Functional food could not exist without nutraceutical compounds, the bioactive compounds that give functional properties to food. A nutraceutical can be defined as a substance that may be considered a food or part of a food and provides medical or health benefits like the prevention and treatment of disease. Nutraceuticals may range from isolated nutrients and dietary supplements to genetically engineered "designer"

foods, herbal products and processed products such as cereals, soups and beverages (Andlauer and Furst, 2002).

The term ''functional food'' itself was first used in Japan, in the 80s, for food products fortified with special constituents that possess advantageous physiological effects (Hasler, 1998; Siro et al., 2008). Functional foods may improve the general conditions of the body (e.g. pre- and probiotics), decrease the risk of some diseases (e.g. cholesterol-lowering products), and could even be used for curing some illnesses. It was recognized that there is a demand for these products as different demographical studies revealed that the medical service of the aging population is rather expensive. The European market for functional foods was estimated to be between 4 and 8 billion US$ in 2003 depending which foods are regarded as functional. This value has increased to around 15 billion US$ by 2006. The current market share of functional food is still below 1% of the total food and drink market. Germany, France, the United Kingdom and the Netherlands represent the most important countries within the functional food market in Europe (Siro et al., 2008).

The design and development of functional foods is a scientific challenge that should rely on a stepwise process. The process begins with basic scientific knowledge relevant to functions that are sensitive to modulation by food components, which are pivotal to maintenance of well-being and health, and that, when altered, may be linked to a change in the risk of a disease. The exploitation of this knowledge in the development of markers that can be shown to be relevant to the key functions is the second step. Next is a new generation of hypothesis-driven human intervention studies that will include the use of these validated, relevant markers and allow the establishment of effective and safe intakes. Last is the development of advanced techniques for human studies that, preferably, are minimally invasive and applicable on a large scale. The targets for functional food science may include: Gastrointestinal functions; Redox and antioxidant systems; Metabolism of the macronutrients; Development in fetal and early life; Xenobiotic metabolism and its modulation by non-nutritive dietary components; Mood and behaviour or cognition and physical performance (Roberfroid, 2000).

The development of functional foods provides a unique opportunity to contribute to improvement of the quality of the food offered to consumers who want to benefit their health and well-being. Only a rigorous scientific approach producing highly significant results will guarantee the success of this new discipline of nutrition. It is clearly a challenge for the food industry (Roberfroid, 2000).

2. Mushrooms as Functional Foods

Mushrooms are something special in the living world, being neither plant nor animal. They have been placed in a kingdom, called Myceteaea. The word mushroom may mean different things to different people and countries. In a broad definition "mushrooms are macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous and large enough to be seen with naked eye and to be picked by hand". Thus, mushrooms can be Ascomycetes that can grow underground and have a non-fleshy texture and need not be edible (Miles and Chang, 1997).

Edible mushrooms have been widely used as human food for centuries and have been appreciated for texture and flavours as well as some medicinal and tonic attributes. However, the awareness of mushrooms as a healthy food and as an important source of biological active substances with medicinal value has only recently emerged. Various activities of mushrooms have been studied which includes antibacterial, antifungal, antioxidant, antiviral, antitumor, cytostatic, immunosuppressive, antiallergic, antiatherogenic hypoglycaemic, anti-inflammatory and hepatoprotective activities (Lindequist et al., 2005). In the present work we will focus on antioxidant and antitumor activities.

2.1 Nutritional Value

Mushrooms are considered a healthy food because they are low in calories and fat but rich in protein and dietary fibers (Cheung et al., 2003; Manzi et al., 1999).

The crude protein content of edible mushrooms is usually high, but varies greatly and is affected by factors such as species and stage of development. The crude protein content of some common edible mushrooms varies from 15.0 to 23.2% w/w. Albumins (24.8%), globulins (11.5%), glutelin-like material (7.4%), glutelins (11.5%), prolamins (5.7%) and prolamine-like material (5.3%) are the main proteins present in mushrooms. Several authors referred mushrooms as a good source of essential amino acids such as: leucine, valine, threonine, lysine, methionine and tryptophan. Leucine and valine were found to be the most abundant essential amino acids, comprising 25–40% of the total amino acid content. Wild mushroom proteins also contain considerable amounts of non-essential amino acids such as alanine, arginine, glycine, glutamic acid,

aspartic acid, proline and serine. They are important in providing structure to cells, tissues and organs and therefore essential for growth and repair (Diez and Alvarez, 2001; Kalac, 2009; Manzi et al., 1999).

Mushrooms are recognized as an excellent choice for low energy diets, as they have high water and low fat content (average of 2–6% of dry weight). Fat in mushrooms contains all classes of lipid compounds including free fatty acids, mono-, di-, and triglycerides, sterols, sterol esters and phospholipids.. Within fatty acid composition, polyunsaturated linoleic acid (C18:2n-6), monounsaturated oleic acid (C18:1n-9) and nutritionally undesirable saturated palmitic acid (C16:0) prevail. The proportions of nutritionally neutral saturated stearic acid (C18:0), and especially of desirable α-linolenic acid (C18:3n-3), are low. Other fatty acids are present at only low levels (Heleno et al., 2009a). Contents of odd- and branched-chain acids and hydroxy fatty acids are negligible. The occurrence of *trans* fatty acids in mushrooms has not been reported and it is not expected. Phosphatidylcholine was the major phospholipid present in 55 of 58 wild growing mushroom species of several families. The nutritional value of wild growing mushroom lipids is thus limited, due to low total lipid content and a low proportion of desirable n-3 fatty acids (Kalac, 2009).

Cultivated mushrooms are a good source of several vitamins, such as riboflavin, niacin, and folates, with concentrations that vary within the range of 1.8-5.1, 31-65, and 0.30-0.64 mg/100g dry weight, respectively, depending on the species. The vitamin B2 content in mushrooms is higher than than generally founds in vegetables, and in some varieties even at a level found in egg and cheese. Mushrooms contain moderately high amounts of folates at concentrations that are of the same magnitude as is generally found in vegetables. In addition to riboflavin, niacin and folates, cultivated mushrooms also contain small amounts of vitamin C and vitamin B1 and traces of vitamins B12 and D2 (Clifford et al., 1991; Mattila et al., 2001).

The carbohydrate content of edible mushrooms varies with species and ranges from 3 to 65% dry weight. Glucose, mannitol and trehalose are the main representatives of monosaccharides, their derivatives and oligosaccharide groups, respectively. Usual contents of glucose and trehalose are low; the content of mannitol, which participates in volume growth and firmness of fruiting bodies, differs widely. Reducing sugars are only a small part of carbohydrates content since wild edible mushrooms are rich in non-starch polysaccharides (dietary fiber, 3–32% dry weight), such as glycogen (animal and fungi reserve polysaccharide), β-glucan and chitin (structural polymers). Edible

mushrooms are believed to contain a high levels of oligosaccharides and only a low levels of total soluble sugars (Bano and Rajarathnam, 1988; Kalac, 2009).

2.2 Antioxidant activity

2.2.1 Oxidative stress

Free radicals are produced in the normal natural metabolism of aerobic cells, mostly in the form of reactive oxygen species (ROS). Once produced, most of the free radicals are neutralized by cellular antioxidant defences (enzymes and non-enzymatic molecules). Beneficial effects of ROS occur at low or moderate concentrations and involve cellular physiological roles of signalization and regulation. Nevertheless, the equilibrium between ROS production and antioxidant defences might be displaced either by the overproduction of ROS or by the loss of the cell antioxidant defences. This disequilibrium is known as oxidative stress (Ferreira et al., 2009).

Oxidative stress might have natural causes such as extreme exercise or inflammation processes, or non-natural causes such as the presence of xenobiotics in the organism or situations related to several diseases (**Figure 1**).
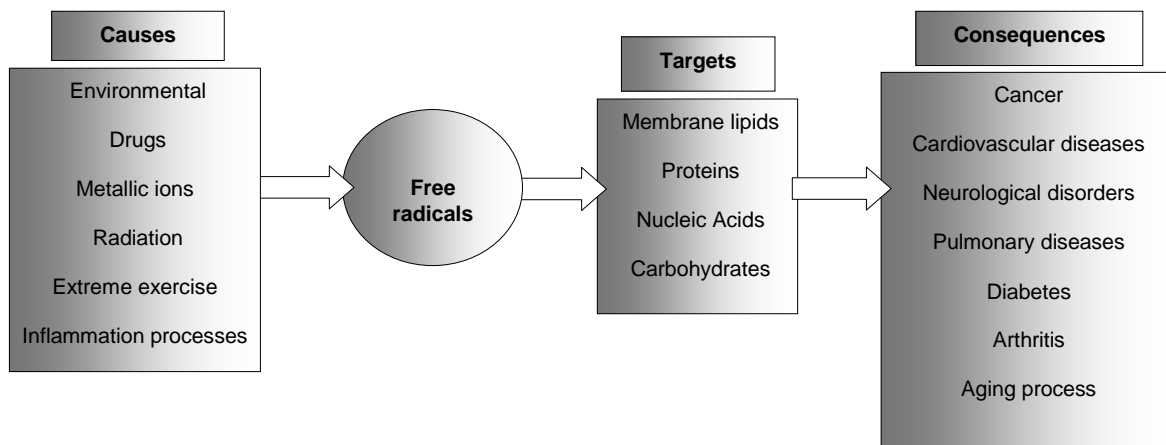
Figure 1. Major causes for over production of free radicals (oxidative stress), possible cellular targets and conditions associated to oxidative stress. Source: Ferreira et al., 2009.

In fact, the non-controlled production of free radicals has been related to more than one hundred diseases including several kinds of cancer, diabetes, cirrhoses, cardiovascular diseases, neurological disorders, among others. The overproduction of ROS has also been related to the aging process.

Several ROS production pathways and the main endogenous antioxidant defences of the cell are described in **Figure 2**.
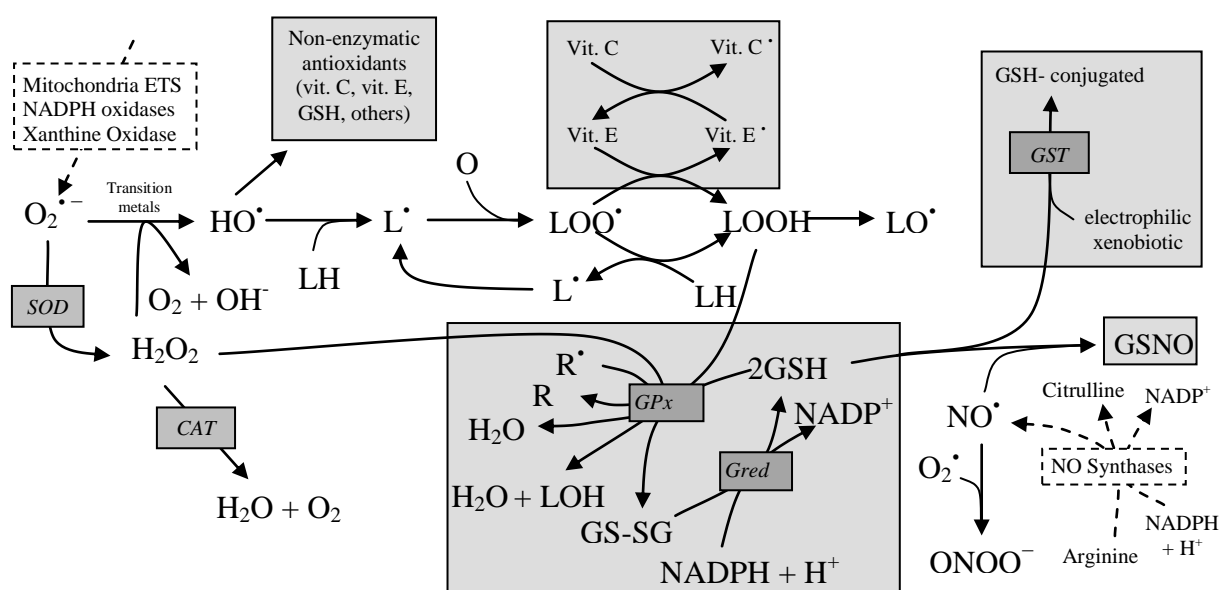
Figure 2. Overview of the main reactions involving reactive Oxygen species (ROS) / reactive Nitrogen species (RNS), and major endogenous enzymatic and non-enzymatic antioxidant defences in the cell. The most representative endogenous sources (traced rectangles) of ROS/RNS are presented and include: Mitochondrial ETS (Electron transport system), NADPH oxidases, Xanthine oxidase for ROS and NO synthases for RNS. The main antioxidant defences are presented in shaded rectangles and the enzymes involved are presented in italic. Molecular Oxygen (O_2), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hydroxide ion (HO^-) membrane lipids (LH), lipid radical ($L^{\bullet}$), peroxyl radical ($LOO^{\bullet}$), hydroperoxide lipid (LOOH), lipid alkoxyl radical ($LO^{\bullet}$), nitric oxide ($NO^{\bullet}$), radicals ($R^{\bullet}$), non-radicals (R), alcohols (LOH), glutathione (GSH), glutathione disulphide (GS-SG), α-tocopherol or vitamin E (vit. E), vitamin E radical (vit. $E^{\bullet}$), vitamin C (vit. C), vitamin C radical (vit. $C^{\bullet}$), S-nitrosoglutathione (GSNO), nicotidamide adenine dinucleotide phosphate: oxidized ($NADP^+$), reduced (NADPH). Enzymes: Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione redutase (Gred), glutathione-S-transferases (GST), Mitochondrial ETS (electron transport system), nitric oxide synthase (NOS). Adapted from Ferreira et al., 2009.

Superoxide anion ($O_2{}^{\bullet-}$ - "primary" ROS) is mostly produced in mitochondria, due to a small but continuous "leak" of the electrons in the mitochondrial electron transport system (ETS). Superoxide anion can also be produced by different endogenous enzymatic systems present in the cell like NADPH oxidases and xanthine oxidase. Even though $O_2{}^{\bullet-}$ is not a very active radical, it can interact with other molecules generating what are considered as "secondary" ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). Hydroxyl radical has a very short life time but is considered to be the most toxic among all ROS, being responsible for the attack to DNA molecules, damaging purins and pyrimidines and the structure of desoxyribose DNA. Mitochondria are the most important source of ROS, but they are also the first targets of these radicals because ROS have an easy access to the membrane lipids, which are susceptible to free radicals attack. This attack is called lipid peroxidation and promotes the production of different types of ROS (**Figure 2**). The lipid peroxidation usually begins with the extraction of a hydrogen atom from a polyunsaturated lipid (LH) chain, through the action of reactive species such as $HO^{\bullet}$. This generates a highly reactive lipid radical ($L^{\bullet}$) that can react with O_2 to form a peroxyl radical ($LOO^{\bullet}$). If not neutralized by antioxidants defences, the peroxyl radical will react with other adjacent lipids producing hydroperoxides lipids (LOOH) that can easily be decomposed to form new $L^{\bullet}$ radicals, initiating a process that is known as chain propagation reactions. This process when not stopped, can lead to much superior damage than the ROS that started the reaction. It is also important to notice the existence of radicals with nitrogen called Reactive Nitrogen species (RNS). The principal RNS is nitric oxide ($NO^{\bullet}$) and it is generated in biological tissues by specific nitric oxide synthases (NOS), which metabolise arginine to citrulline (**Figure 2**) (Ferreira et al., 2009).

Exposure to free radicals from a variety of sources has led organisms to the development of a series of defence mechanisms (**Figure 2**). These defences were the evolution response to the inevitability of the existence of oxygen radicals in aerobic life conditions, and can be classified into enzymatic and non-enzymatic. There are many different endogenous enzymatic antioxidant defences in the organism, either in intracellular or extracellular medium. Examples of these defences include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), and glutathione redutase (Gred) among others. The endogenous non-enzymatic antioxidant defences include glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C), lipoic acid, and other antioxidants (Ferreira et al., 2009).

The implication of oxidative and nitrosative stress in the etiology and progression of several acute and chronic clinical disorders has led to the suggestion that antioxidants can have health benefits as prophylactic agents. This suggests that changes in dietary behaviour, increasing consumption of plant-based foods, which contain significant amounts of bioactive phytochemicals, may provide desirable health benefits, beyond basic nutrition, to reduce the risk of chronic diseases.

2.2.2. Contribution of mushrooms against oxidative stress

Natural products with antioxidant activity may help the endogenous defence system. In this perspective, the antioxidants present in the diet assume a major importance as possible protector agents reducing oxidative damage.

Many studies have concluded that edible mushrooms possess potent antioxidants. Research conducted in Japan showed the antioxidant activity of the crude ethanol extract of 150 Japanese mushrooms using the peroxide value in the methyl linoleate system (Cheung, 2009). It showed that many mushrooms, especially those belonging to the *Suillus* genus, had a peroxide value 80% lower than the control. A study of methanol extracts from black (*Auricularia mensenterica*), red (*Auricularia polytricha*) and snow (*Auricularia fuscosuccinea*) ear mushrooms found that they had an inhibitory effect on lipid peroxidation, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydroxyl radical scavenging, strong reducing power and ability to chelate ferrous ions. Similar studies of other mushrooms including, *Dictyophora indusiata*, *Grifola frondosa*, *Hericium erinaceus*, *Tricholoma giganteum, Lentinula edodes, Pleurotus cystidiosus,* and *Pleurotus ostreatus*, showed that these mushrooms also possess the afore mentioned antioxidant properties. It is therefore likely that most mushrooms possess hydroxyl and DPPH radical scavenging effects, inhibit lipid peroxidation, chelate metals, and has a strong reducing effect (Mau et al., 2001; Mau et al., 2002; Yang et al., 2002). Similar antioxidant properties have also been reported for other edible mushrooms, including *Agrocybe cylindracea* and *Hypsizigus marmoreus*, both of which belong to the Tricholomataceae family (Lee et al., 2007; Tsai et al., 2006).

Furthermore, several other mushrooms from Portugal (*Lactarius deliciosus, Lactarius piperatus, Macrolepiota mastoidea, Macrolepiota procera, Sarcodon imbricatus, Agaricus arvensis, Agaricus bisporus, Agaricus silvicola, Agaricus silvaticus, Agaricus romagnesii, Leucopaxillus giganteus, Sarcodon imbricatus,*

Cantharellus cibarius, Hypholoma fasciculare, Lepista nuda, Lycoperdon molle, Lycoperdon perlatum, Ramaria botrytis and *Tricholoma* acerbum), Korea (*Grifola frondosa* and *Lentinus* edodes), China (*Lentinus edodes, Volvariella volvacea* and *Agrocybe* aegerita), Taiwan (*Grifola frondosa, Morchella esculenta, Termitomyces albuminosus, Dictophora indusiata, Grifola frondosa, Hericium erinaceus, Trichloma giganteum, Ganoderma lucidum, Ganoderma tsugae, Coriolus versicolor, Armillariella mellea, from India Termitomyces heimii, Helvella crispa, Termitomyces tylerance, Lactarius sanguifluus, Morchella conica, Termitomyces mummiformis, Pleurotus sajor-caju, Termitomyces shimperi, Lentinus squarrulosus, Boletus edulis, Pleurotus djamor, Macrolepiota procera, Cantharellus clavatus, Morchella angusticeps, Termitomyces microcarpus, Lactarius deliciosus, Geastrum arinarius, Hydnum repandum, Lentius sajor-caju, Sparassis crispa, Russula brevepis, Auricularia polytricha* and *Cantharellus cibarius*), Turkey (*Agaricus bisporus, Polyporus squamosus, Pleurotus ostreatus, Lepista nuda, Russula delica, Boletus badius, Verpa conica* and *Lactarius deterrimus*) and Brazil (*Lentinula edodes* and *Agaricus blazei*) were also reported to have antioxidant activity, which was mainly related to their phenolic content (Barros et al., 2007b; Barros et al., 2007d; Barros et al., 2008c; Barros et al., 2007f; Barros et al., 2008d; Cheung and Cheung, 2005; Cheung et al., 2003; Choi et al., 2006; Elmastas et al., 2007; Ferreira et al., 2007; Kitzberger et al., 2007; Lee et al., 2008; Lo and Cheung, 2005; Mau et al., 2004; Mau et al., 2002; Ng et al., 2007; Puttaraju et al., 2006; Sarikurkcu et al., 2008; Soares et al., 2009; Song et al., 2003; Tsai et al., 2007; Turkoglu et al., 2007; Yang et al., 2002).

The antioxidants found in mushrooms are mainly phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids. These molecules were quantified in tens of different species mainly from Finland, India, Korea, Poland, Portugal, Taiwan and Turkey. The values are available in literature, but expressed in different basis (dry weight, fresh weight and extract) (Ferreira et al., 2009).

In the present work, the antioxidant potential of mushrooms was correlated to their chemical composition, including nutrients and antioxidants, using chemoinformatic tools.

2.3 Anticancer activity

2.3.1. Breast Cancer

Breast cancer is the most common type of cancer among women worldwide and its rate is increasing in both developed and developing countries. The burden is not evenly distributed and there are large variations in the incidence rates of breast cancer between different countries (Parkin et al., 2005).

Most breast cancers (about 95%), whether in pre- or postmenopausal women, are initially hormone-dependent (Pasqualini and Chetrite, 2005). The majority of breast cancers occur during the postmenopausal period when the ovaries have ceased to be functional. Despite the low levels of circulating estrogens, the tissular concentrations of estrone (E1), estradiol (E2) and their sulfates (E1S, E2S) in breast tumours are several times higher than those found in the plasma or in the area of the breast considered as normal tissue, suggesting a specific tumoral biosynthesis and accumulation of these hormones (Pasqualini, 2004).

Figure 3 shows the biosynthesis of estrogens from steroid precursors via the aromatization of androstenedione to estrone by aromatase or via the hydrolysis of estrone sulfate by Estrone sulfatase (STS).
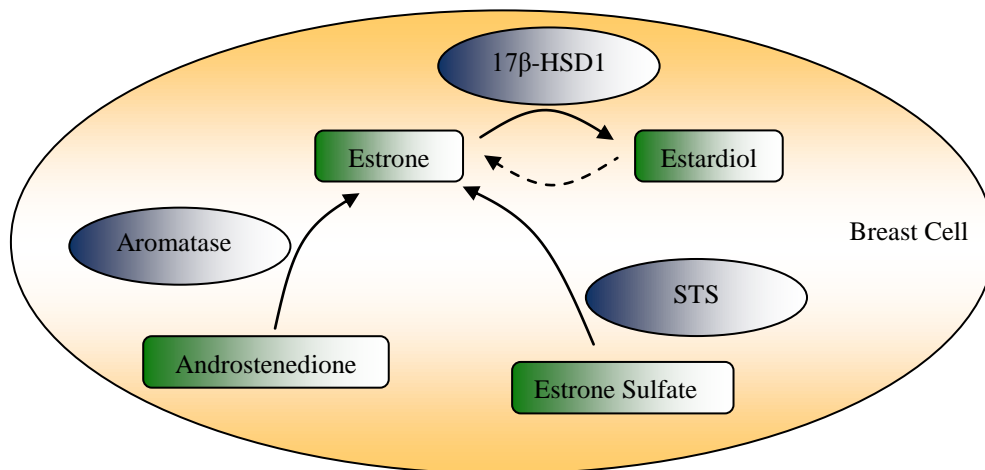
Figure 3. Estrogens pathway in breast cell. Estrone sulfatase (STS), 17-β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and Aromatase.

There are three enzymes that are directly involved in the production of estrone and estradiol in the breast cell, that are Estrone sulfatase (STS), 17-β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and Aromatase. These proteins were used as targets in the present work.

Estrone sulfatase is a microsomal enzyme and is an integral membrane protein of the Endoplasmic Reticulum (**Figure 4**). It is most active at or near neutral pH and can be solubilised only in the presence of detergents (Ghosh, 2007).
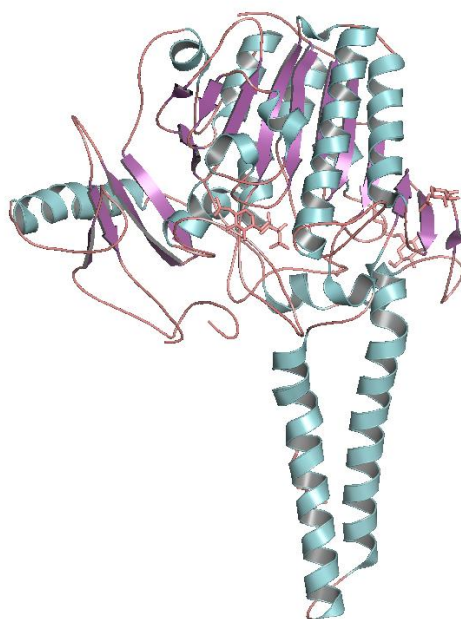
Figure 4. Estrone sulfatase X-ray protein structure (PDB: 1P49) represented in cartoon format; docked natural ligands are represented in wire format (pink).

STS is an alternative source of sex-steroid precursors for the local biosynthesis of active estrogens and androgens. STS catalyzes the hydrolysis of E1S to unconjugated E1, which is subsequently reduced to estradiol (E2) by 17-β-hydroxysteroid dehydrogenase 1 (17β-HSD1). Androstenedione (A) to E1 and testosterone (T) to E2 aromatization steps are catalyzed by aromatase (**Figure 3**). However, local biosynthesis of E2 from E1S has been proposed to be the major cause of high levels of active estrogens in the breast for post-menopausal women. The presence of STS in breast carcinomas and STS dependent proliferation of breast cancer cells have been demonstrated. STS immunoreactivity was detected in 84 out of 113 breast carcinoma cases and was significantly associated with their mRNA levels as well as enzymatic activities (Ghosh, 2007).

There are fifteen discovered 17β-HSD enzymes in mammals (Jansson, 2009). The common feature for this enzymes is the possibility to catalyze oxidation or reduce the carbon at position 17 in steroids. The enzymes have different preferences for substrates such as estrone, estradiol, testosterone, androstenedione, and dihydrotestosterone, are expressed in different parts of the cell, and in diverse tissues. This shows that the enzymes have separate physiological functions (Jansson, 2009). 17β-HSD1 (**Figure 5**) known to be of main importance in breast tissue (Pasqualini, 2004).
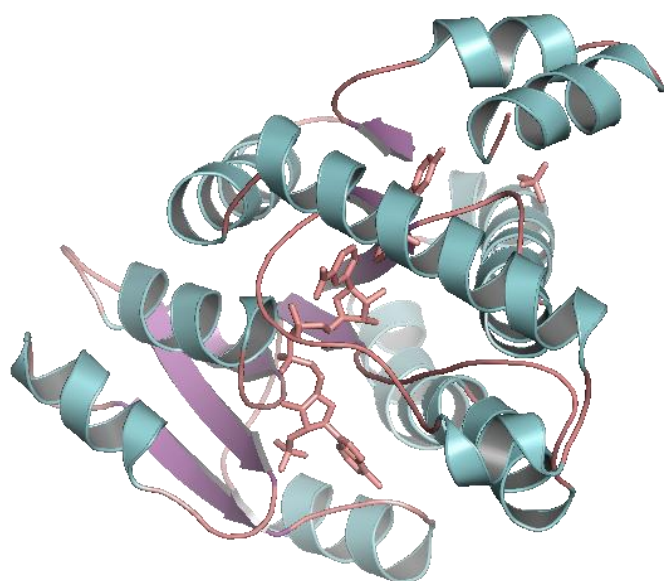
Figure 5. 17β-HSD1 X-ray protein structure (PDB: 1FDT) represented in cartoon format; docked natural ligands represented in wire format (pink).

17β-HSD1 predominantly catalyzes reduction of estrone to estradiol using NAD(H) or NADP(H) as co-factor (**Figures 3** and **5**). The expression is low in normal breast epithelium but increases in a large proportion on breast tumours. Highly variable amounts of this enzyme have been detected in benign and malign breast tissue. In some studies it was detected in all the analysed tumours, but others detected expression in only 20%. In invasive breast tumours 17β-HSD1 protein expression was detected in approximately 60% (Jansson, 2009).

Aromatase (**Figure 6**), an enzyme of the cytochrome P450 (CYP450) subfamily and the product of the CYP19A1 gene, is highly expressed in the placenta and in the granulose cells of ovarian follicles in premenopausal women. In menopause, androstenedione produced in the adrenals and, to a small extent, testosterone produced in the ovaries are released to the circulation and then sequestered to nonglandular tissues (e.g., liver and breast cells), where they are converted to estrone and estradiol, respectively, by aromatase located in these tissues (Desta et al., 2009).
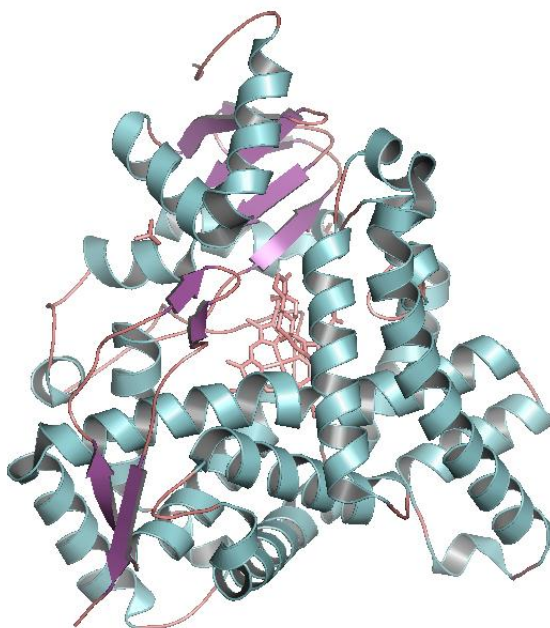
Figure 6. Aromatase (PDB: 3EQM) represented in cartoon format; docked natural ligands in wire format (pink).

Drugs that effectively inhibit the aromatase-mediated synthesis of estrogens in peripheral tissues including the breast, thus depriving the system of estrogens, are widely used in the treatment of breast cancer. These drugs include the nonsteroidal triazole derivatives anastrozole and letrozole and the steroidal exemestane (Desta et al., 2009). Aromatase inhibitors are used widely as second-line therapy in breast cancer; and there is now evidence for a chemopreventive role for these agents (Zaidman et al., 2005).

The present work explores mushrooms as sources of potential inhibitors of the three enzymes (estrone sulfatase, 17β- and aromatase) involved in breast cancer.

2.3.2 Contribution of mushrooms against cancer

The mushroom *Cordyceps militaris* has been use for a long time in eastern Asia as a nutraceutical and in traditional Chinese medicine as a treatment for cancer patients. *Cordyceps militaris* proteins exerted strong antifungal effect against the growth of the fungus *Fusarium oxysporum*, and exhibited cytotoxicity against human breast and bladder cancer cells. New discoveries in molecular oncology along with rapid expansion of our knowledge concerning the processes that govern differentiation, apoptosis, immune surveillance, angiogenesis, metastasis, cell cycle, and signal transduction control have unveiled an abundance of specific molecular targets for cancer therapy, including a variety of small-molecule compounds that inhibit or stimulate these molecular targets (Park et al., 2009).

In a recent study, it was found that *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus florida* and *Pleurotus pulmonaris* possessed profound antioxidant and antitumor activities. This indicated that these mushrooms would be valuable sources of antitumor and antioxidant compounds. Extracts of fruiting bodies of *Boletus edulis* and other Basidiomycetes also revealed antitumor activity against Sarcoma 180 line in mice. In the 1960s, calvacin was the most commonly cited natural product isolated from the medicinal mushroom *Calvatia gigantean* and was broadly used in many laboratories as an antitumor agent (Ajith and Janardhanan, 2007; Lucas et al., 1957).

In Eastern Europe, the fruiting bodies of *Ionotus obliquus* have been used as a folk medicine for cancer and stomach diseases since the 16th or 17th century. antitumor effects of several extracts and isolated compounds from mushrooms could be demonstrated in tumour cell systems and in animal assays (Burczyk et al., 1996; Molitoris, 1994).

Several phytochemicals have been isolated from medicinal mushrooms and three of these, which are carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are "Krestin" (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), "Lentinan" from the fruiting bodies of Shiitake (*Lentinus edodes*) and "Schizophyllan" (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*)(Mizuno, 1993). Lentinan and schizophyllan are pure β-glucans, whereas PSK is a protein bound polysaccharide (Larone, 2002). The biological activity of these three products is related to their immunomodulating properties, which enhance the host's defence against various forms of infectious disease. These

immunopotentiators, or immunoinitiators, are also referred as "biological response modifiers" (Zaidman et al., 2005; Zjawiony, 2004).

A recent clinical study suggests that higher dietary intakes of fresh and dried mushrooms are associated with a reduced breast cancer risk with a dose–response relationship in both pre- and postmenopausal Chinese women. The combination of dietary intake of mushrooms and green tea drinking decreased breast cancer risk with an additional reduced effect on the malignance (Zhang et al., 2009)

Searching for new antitumor and other medical substances from mushrooms and studying their medical value has become a matter of great significance. In the present work, virtual ligand screening was performed using estrone sulfatase, 17β-HSD1 and aromatase as targets, in order to evaluate which mushrooms compounds may be potentially involved in anti-breast cancer activity.

3. Chemoinformatics methodologies

The use of bioinformatic tools is widespread in all areas of basic and applied scientific knowledge. These tools include chemoinformatics methods that are used in the design, creation, organization, management, retrieval, analysis, dissemination, visualization and use of chemical information. In this work, we selected the appropriate chemoinformatic tool for each study performed: QCAR for mushroom antioxidant activity prediction and molecular docking for potential mushroom anti-breast cancer activity against selected protein targets.

3.1 QCAR - Quantitative Composition-Activity Relationships

Because most sets of biological observations, particularly those produced by testing different chemical structures in the same biological system, cannot be adequately described by existing theory, researchers often seek semi empirical models in which the changes in observed values are predicted as a mathematical function of properties which are better understood (Cramer, 1993).

In modern pharmaceutical industry, computer-aided drug design methods, such as quantitative structure–activity relationship (QSAR) study has greatly accelerated the pace of drug discovery in recent decade. The underlying assumption behind QSAR analysis is that the variation of biologic activity within a group of compounds can be correlated with the variation of their respective structural and chemical features (Wang et al., 2006).

Because detailed information about the antioxidant activity of each one of the compounds present in mushrooms are not available (antioxidant activity EC_{50} values correspond to extracts and not individual compounds), QSAR method cannot be directly used to predict bioactivity of mushrooms. However, variation of antioxidant activity of mushrooms is tightly associated with the variation of their chemical composition. Such relationship between chemical composition and biological activity (antioxidant activity) is termed Quantitative Composition–Activity Relationship (QCAR), and is a sister method of the well-known QSAR methodology. With QCAR instead of QSAR, compound composition is used instead of compound structure as studied variable, but the statistical analysis and tools used are essentially the same.

To develop the QCAR model, the PLS (Partial Least Squares) statistical method was used. PLS is an important technique for producing a linear equation to describe or predict differences in the values of one or more properties from differences in the values of other properties (Cramer, 1993). In composition–bioactivity studies, such a linear equation is usually called a QCAR model. The described or predicted properties (in this case, antioxidant activity) are called the 'dependent variables' or, in the PLS literature, the 'Y-block'. The describing or predicting properties (chemical composition) are called the 'independent variables' or the 'X-block'. To implement PLS technique and build a QCAR model we used SIMCA-P software a user-friendly software program (Fernandez et al., 2005).

By quantitatively analyzing the chemical composition–bioactivity relationship, a QCAR mathematical model was established that is able to successfully predict antioxidant activity of mushrooms. The QCAR approach is very recent and, when used to study complex biological matrixes (like mushrooms), is a very promising methodology, better suitable for biological activity predictions than the more widely used QSAR methods.

3.2 Molecular Docking and Virtual Ligand Screening

Structure based drug design is now an established approach in drug discovery. Computational methodologies are used to facilitate structure based drug design at various stages of the process. One of the most important and routinely adopted methods is molecular docking (or just docking) and refers to the prediction of the binding mode of a specified compound within the active site of the protein target of interest (Congreve et al., 2005; Verdonk et al., 2007).

Selecting (or designing) compounds *in silico* that bind to a protein active site is difficult. First, the *in silico* method must solve the docking problem by finding the optimum binding orientation for the compound in the active site of the protein. This means that it must predict the correct ligand conformation and orientation, in addition to any protein movement that is induced by the ligand, although for most applications of docking the protein is assumed rigid. Many methods and programs have been developed and tested in docking applications. Docking accuracy is usually assessed by the ability to reproduce the experimentally determined binding mode of a ligand as the highest-ranking solution starting from a random ligand geometry but using the correct

conformation of the protein. Currently, state-of-the-art docking programs correctly dock ~70–80% of ligands when tested on large sets of protein–ligand complexes (Congreve et al., 2005).

The second challenge is that the *in silico* method must score the compound so that its relative affinity can be judged versus other compounds.

The use of molecular docking to search large databases of compounds for possible ligands of a protein receptor is usually termed virtual ligand screening (VLS) and has been successfully applied in several therapeutic programs at the lead discovery stage (Ghosh et al., 2006). Rapid accumulation of high-resolution three-dimensional structures, further accelerated by the structural proteomics initiative and the improvements of docking and scoring technology, are making VLS an attractive alternative to the traditional methods of lead discovery. VLS can sample a virtually infinite chemical diversity of drug-like molecules without synthesizing and experimentally testing every screened molecule. Typically, a corporate high-throughput screening (HTS)-ready compound library ranges from 200,000 to 1,000,000 molecules. The high cost of such massive experimental testing and its technical complexity are further motivation for the theoretical alternative. Finally, the virtual experiment, as opposed to a high-throughput assay, can be easily designed to select for a particular binding site or receptor specificity (Abagyan and Totrov, 2001).

In the present work, VLS was performed using estrone sulfatase, 17β-HSD1 and aromatase as protein targets, in order to evaluate the potential of mushrooms as sources of compounds with anti-breast cancer activity. We used AutoDock 4 (Morris et al., 1998), acknowledged to be one of the most reliable and broadly used molecular docking tool (Sousa et al., 2006), with several examples of accurate docking predictions already published (Chen et al., 2007; Li et al., 2004).

II RESULTS

1. A QCAR model for predicting antioxidant activity of wild mushrooms

1.1 Introduction

Free radicals play important roles in many physiological and pathological conditions (Valko et al., 2007). In general, excess of free radicals caused by the imbalance between free radical generation and scavenging may contribute to disease development. Free radicals can damage membranes, proteins, enzymes and DNA, increasing the risk of diseases such as cancer, Alzheimer's, Parkinson's, angiocardiopathy, arthritis, asthma, diabetes, and degenerative eye disease (Machlin and Bendich, 1987; Valko et al., 2007).

Mushrooms have become attractive as functional foods and as a source of physiologically beneficial compounds including antioxidants (Lindequist et al., 2005; Wasser, 1999). Different wild mushroom species were reported to have antioxidant activity, which was mainly related to their phenolic content (Cheung et al., 2003; Elmastas et al., 2007; Kim et al., 2008; Lee et al., 2008; Mau et al., 2004; Soares et al., 2009; Tsai et al., 2007). Nevertheless, none of the available reports present a quantitative study to obtain a predict model for antioxidant potential. Furthermore, other chemical substances in mushrooms including proteins, carbohydrates, vitamins and fibers could also contribute to the antioxidant capacity (Maisuthisakul et al., 2008).

Our recent investigation provided an insight to the chemical composition, nutritional value and antioxidant properties of several wild mushroom species from Northeast of Portugal, one of the European regions with higher wild edible mushrooms diversity, valorising mushrooms as a source of nutrients and nutraceuticals (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2007c; Barros et al., 2008b; Barros et al., 2008d). The analysis of nutrients included determination of proteins, fats, ash, and carbohydrates, particularly sugars by High Performance Liquid Chromatography coupled to a refraction index detector (HPLC-RI). The analysis of nutraceuticals included determination of fatty acids by Gas-Chromatography with a flame ionization detector (GC-FID), and other phytochemicals such as phenolic compounds by HPLC

coupled to a diode array detector and mass spectrometry (HPLC/DAD-ESI/MS), carotenoids and ascorbic acid, by spectrophotometric techniques. The antioxidant activity was screened through chemical and biochemical assays (Barros et al., 2007b; Barros et al., 2007d; Barros et al., 2007f; Barros et al., 2008c; Barros et al., 2008d). Numerous tests have been used for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively (Prior et al., 2005). DPPH radical scavenging activity (RSA) and reducing power (RP) assays are two of the most widely used methods for antioxidant activity screening.

The relationship between chemical substances including phenolic compounds and antioxidant properties may be complex, and there is very little data to elucidate the relationship between chemical composition and antioxidant capacity of wild mushrooms. Herein, the antioxidant potential (RSA and RP) and chemical composition of some Portuguese wild mushrooms were evaluated using linear regression analysis (Partial Least Square, PLS), in order to find possible relationships between those parameters. Furthermore, a Quantitative Composition-Activity Relationships (QCAR) model was constructed in order to predict the reducing power of mushrooms.

1.2. Methods

1.2.1. Data set

A total of twenty three samples from seventeen Portuguese wild mushroom species were used in this study (**Table 1**). The samples were selected using the following criteria: wild mushrooms studied by our research group using the same methodologies; results of chemical composition and antioxidant activity available on the same sample. In some species, results considering different stages of fruiting body growth (SI, SII and SIII) and different conservation conditions (frozen and dehydrated) were available, and therefore used in this study.

Table 1. Chemical composition and antioxidant activity (reducing power, RP and radical scavenging activity, RSA) values of Portuguese wild mushrooms.

Species	Ash (g/100g)	Carbohydrates (g/100g)	Proteins (g/100g)	Fat (g/100g)	PUFA (%)	MUFA (%)	SFA (%)	Phenolics (mg/g ext)	Flavonoids (mg/g ext)	Ascorbic acid (mg/g ext)	ß-Carotene (µg/g ext)	EC_{50} (RP) (mg/ml)	EC_{50} (RSA) (mg/ml)	References
Agaricus arvensis	3.53	37.45	56.27	2.75	-	-	-	2.72	1.65	0.02	8.52	4.2	15.85	(Barros et al., 2007a; Barros et al., 2008c)
Agaricus bisporus	9.9	8.25	80.93	0.92	76.41	1.52	22.1	4.49	1.73	0.03	1.95	3.63	9.61	(Barros et al., 2008b; Barros et al., 2008c)
Agaricus romagnesii (a)	-	-	-	-	-	-	-	6.18	2.87	0.04	1.32	2.23	6.22	(Barros et al., 2008c)
Agaricus silvaticus	16.48	9.49	71.99	2.05	75.23	7.67	17.1	8.95	3.88	0.04	5.42	2.08	5.37	(Barros et al., 2008b; Barros et al., 2008c)
Agaricus silvicola	14.93	12.18	70.47	2.43	76.95	4.25	18.8	6.4	3.4	0.04	3.02	3.24	6.39	(Barros et al., 2008b; Barros et al., 2008c)
Cantharellus cibarius	12.12	14.25	69.14	4.49	54.08	23.29	22.6	0.88	0.67	0.86	13.56	5.89	7.41	(Barros et al., 2008b)
Hypholoma fasciculare (a)	-	-	-	-	-	-	-	17.67	5.09	0.09	24.62	0.95	1.13	(Barros et al., 2008d)
Lactarius deliciosus (F)	9.53	57.68	24.33	8.45	21.68	24.69	53.5	2.95	1.91	-	-	4.65	20.54	(Barros et al., 2007b; Barros et al., 2007e)
Lactarius deliciosus (D)	14.28	60.3	17.87	6.47	23.65	21.85	54.4	3.4	2.71	0.24	90.1	4.98	16.31	(Barros et al., 2007b; Barros et al., 2007e)
Lactarius piperatus (SIII)	9.8	42.48	40.79	6.93	8.64	27.33	64	3.09	0.35	0.13	17.22	5.4	20.24	(Barros et al., 2007c; Barros et al., 2007d)
Lactarius piperatus (SII)	8.77	70.06	14.74	6.44	6.94	11.51	81.5	5.76	1.58	0.16	33.78	2.29	5.19	(Barros et al., 2007c; Barros et al., 2007d)
Lactarius piperatus (SI)	6.94	78.18	6.86	8.11	6.74	6.63	86.6	5.52	1.26	0.15	26.08	2.83	12.92	(Barros et al., 2007c; Barros et al., 2007d)
Lepista nuda	18.46	24.88	59.39	1.77	52.1	30.32	17.6	6.31	3.36	0.23	2.52	3.53	4.41	(Barros et al., 2008d)
Lycoperdon molle	20.16	62.33	16.77	0.73	65.92	9.04	25	11.48	2.45	0.34	4.48	2.27	3.23	(Barros et al., 2008d)
Lycoperdon perlatum	31.89	50.57	17.09	0.44	71.52	4.91	23.6	10.57	2.1	0.21	12.5	2.96	3.95	(Barros et al., 2008d)
Macrolepiota mastoidea (D)	7.96	67.6	21.89	2.55	60.76	19.04	19.9	3.08	2.1	-	-	4.35	8.18	(Barros et al., 2007b)
Macrolepiota mastoidea (F)	11.76	60.68	24.51	3.05	60.89	20.27	18.6	2.69	1.56	-	-	4.44	8.49	(Barros et al., 2007b)
Macrolepiota procera (D)	9.86	80.38	7.62	1.45	64.72	10.17	24.6	3.17	0.99	-	-	4.18	5.38	(Barros et al., 2007b)
Macrolepiota procera (F)	916	79.28	9.36	2.18	62.31	15.23	22.3	2.59	0.9	-	-	4.49	6.95	(Barros et al., 2007b)
Ramaria botrytis	8.8	50.05	39.89	1.37	38.91	44.69	16.4	20.32	16.56	0.27	10.41	0.68	0.66	(Barros et al., 2008d)
Sarcodon imbricatus (D)	12.14	54.43	29.98	3.45	28.69	50.46	20.7	3.06	1.52	0.16	2.53	4.41	5.82	(Barros et al., 2007b; Barros et al., 2007f)
Sarcodon imbricatus (F)	8.31	55.98	25.71	8.94	25.37	52.88	21.6	2.22	1.12	-	-	5.94	10.98	(Barros et al., 2007b; Barros et al., 2007f)
Tricholoma acerbum (a)	-	-	-	-	-	-	-	5.53	1.87	0.22	75.48	3.27	3.6	(Barros et al., 2008d)

(a) More that 50% of missing data; F- frozen mushrooms; D- dehydrated mushrooms; SI immature (cap diameter less than 4.5 cm); SII mature (cap diameter between 4.5 and 7 cm) with immature spores; SIII mature (cap diameter higher than 7 cm) with mature spores. All the other samples were lyophilized and in SII.

The samples were collected in Bragança (Northeast of Portugal), in autumns of 2005 and 2006. Different chemical parameters (ash, carbohydrates, proteins, fat, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), phenolics, flavonoids, ascorbic acid and β-carotene), radical scavenging activity (RSA) and reducing power (RP) values were obtained from previous reports of our research group (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2007c; Barros et al., 2007d; Barros et al., 2007e; Barros et al., 2007f; Barros et al., 2008b; Barros et al., 2008c; Barros et al., 2008d).

Ash was determined by incineration at 600±15 ºC; the proteins content (N × 4.38) was estimated by the macroKjeldahl method; fat was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus; carbohydrates were calculated by difference; saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were determined by GC-FID after a trans-esterification procedure. Phenolics, flavonoids, ascorbic acid and β-carotene were determined by spectrophotometer assays. These phytochemicals are frequently analyzed in mushrooms and reported in literature.

For antioxidant activity data, we used the results of two *in vitro* chemical assays, previously reported by us: reducing power - RP (measures the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form) and scavenging activity on DPPH radicals-RSA (measures the decrease in DPPH radical absorption after exposure to radical scavengers). These assays are the most commonly used methods for assessment of the antioxidant properties of natural products. Both assays are suitable for solvent extracts and as rapid assays, they can be applied for monitoring the activity of numerous samples over a limited period of time (Amarowicz et al., 2004; Maisuthisakul et al., 2008). Moreover, they are reproducible and strongly correlated with phenolic compounds (Maisuthisakul et al., 2008). The RSA was calculated as a percentage of DPPH discolouration using the equation: % RSA = [(A_{DPPH}-A_S)/A_{DPPH}] × 100, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The RP was obtained by measuring the absorbance of the product obtained by the reduction of the ferric ion to the ferrous form; a higher absorbance indicates higher RP.

To make the RSA and RP data homogenous and directly comparable, all the values were reported as EC_{50}, (expressed in mg/mL, concentration required to achieve 50% of RSA or 0.5 of absorbance in RP).

1.2.2. Statistical Analysis

The relationships between antioxidant activity (RP and RSA) and the different chemical composition parameters were studied using PLS (Wold et al., 2001) method implemented in SIMCA-P v12 statistics software (Fernandez et al., 2005), and using NIPALS algorithm for missing data (A.B. Umetrics, 2008). Because *Agaricus romagnesii*, *Hypholoma fasciculare and Tricholoma acerbum* had more than 50% of missing data, these observations were not used in the models.

The goodness of fit of the model was evaluated using the following statistical parameters: squared correlation coefficient (R^2), standard deviation of regression (S), significance of the model (ρ) and Fisher ratio value (F).

The predictive stability and robustness of the model was first verified by internal cross-validation calculating the following parameters: Q^2_{LOO} ("Leave-One-Out"; 1-PRESS/TSS were PRESS is the Predictive Error Sum of Squares and TSS the Total Sum of Squares) and RMSE $_{(training\ set)}$ (Root Mean Squared Errors for the training set) (Gramatica, 2007; Gramatica and Papa, 2005).

1.2.3. QCAR model

To build the QCAR model the complete data set was used (table 1) and the Partial Least Square (PLS) method implemented in SIMCA-P v12 statistics software was used. The twenty three samples were first divided in two groups: training and test sets. The training set, representing about 3/4 of the total number of samples (17 samples), was used to build the QCAR model. The remaining 1/4 (6 samples) was assigned to the test set and used to validate the model. The division was made to cover all the antioxidant activity scale (Farkas et al., 2004; Saiz-Urra et al., 2007) and the samples included on the training set were randomly selected within each group (Durand et al., 2007).

The goodness of fit of the models was evaluated using the following statistical parameters: squared correlation coefficient (R^2), standard deviation of regression (S), significance of the model (ρ) and Fisher ratio value (F).

The predictive stability and robustness of the model was first verified by internal cross-validation calculating the following parameters: Q^2_{LOO} ("Leave-One-Out"; 1-PRESS/TSS were PRESS is the Predictive Error Sum of Squares and TSS the Total Sum of Squares), permutation test of SIMCa-p software and RMSE $_{(training\ set)}$ (Root Mean Squared Errors for the training set) (Eriksson et al., 1997; Gramatica, 2007; Gramatica and Papa, 2005). Using the test set, the model was further checked by external cross-validation by calculating parameters: Q^2_{ext} (External, 1-PRESS/SD) and RMSE $_{(test\ set)}$ (Root Mean Squared Errors for the test set). PRESS is defined as the sum of the squared difference between the observed value and the predicted value for each compound in the test set, and SD is defined as the sum of the squared deviation between the observed value and the mean measured value of the training test (Gramatica, 2007).

1.3. Results and Discussion

1.3.1. Relationships between antioxidant activity and chemical composition

The relationships between antioxidant activity (RP and RSA) and several chemical components from various species of Portuguese wild mushrooms were evaluated using PLS regression (**Figure 7**). This approach has been adopted because PLS can predict possible relationships using the chemical variables as explicative ones. PLS extracts a few linear combinations (PLS factors) of the chemical and antioxidant data that predict as much of the systematic variation in the sample data as possible. Standarlized regression coefficients for the relationships between variables revealed by the PLS were estimated by cross-validation and were used to evaluate the importance of each chemical component on antioxidant activity (**Figure 7**).
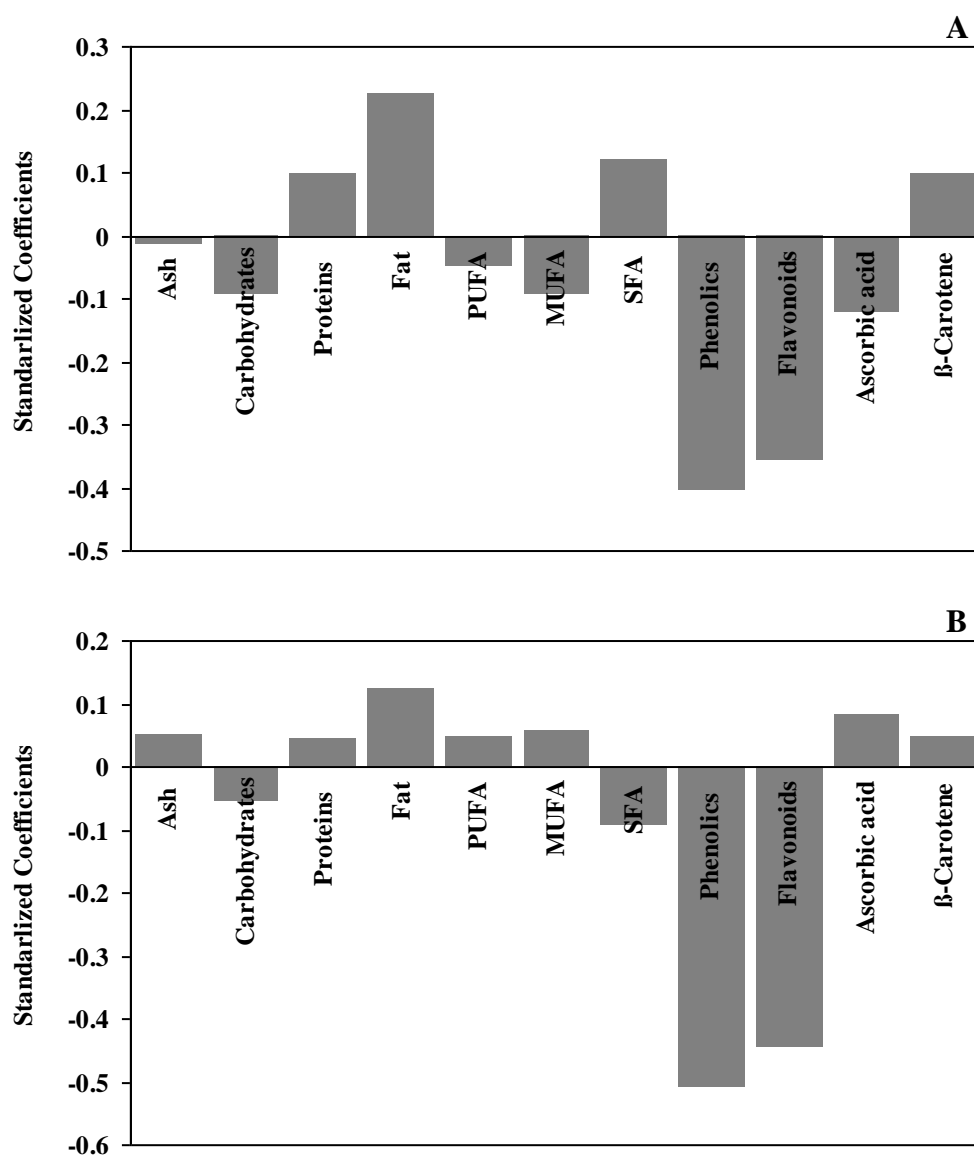
Figure 7. Standardized coefficients of the chemicals compounds, Ash, Carbohydrates, Proteins, Fat, Polyunsaturated fatty acids (PUFA), Monounsaturated fatty acids (MUFA), Saturated fatty acids (SFA), Phenolics, Flavonoids, Ascorbic acid and β-Carotene, used in the in the approached model for RSA (A) and RP (B) .

This analysis presented good statistical parameters for both RP and RSA, as summarized in **Table 2**. Information about some chemical compounds was not available (**Table 1**). To overcome this lack of information, we used a specific statistic algorithm available on SIMCA-P (A.B. Umetrics, 2008). This algorithm dynamically selects samples with more than 50% of the chemical compound results. From the data set available four mushroom species did not meet this criterion and were not considered in this initial analysis.

Table 2. Statistical parameters of the models radical scavenging activity (RSA) and reducing power (RP) using PLS method.

Parameter	RSA	RP
N	20	20
S	2.985	0.488
R^2	0.84	0,92
ρ	0,011	0.023
F	4.74	3.87
Q^2_{LOO}	0.59	0,70
RMSE	3,1942	0,5162

N- number of samples, S- standard deviation, R^2- squared correlation coefficient, ρ- significance, F- Fisher ratio, Q^2_{LOO}- "Leave-One-Out" correlation coefficient and RMSE$_{(training\ set)}$- Root Mean Squared Errors for the training set.

A similar relationship between chemical composition and antioxidant activity was observed for RSA (**Figure 7A**) and RP (**Figure 7B**), with the exception of fatty acids and ascorbic acid. A close observation of the standarlized coefficients of the analysed chemical parameters shows that antioxidant activity is strongly positively related to phenolics and flavonoids contents (**Figure 7**). This is in agreement with several manuscripts reporting phenolic compounds as the main antioxidant substances in mushrooms, particularly phenolic acids and flavonoids (Ferreira et al., 2009). Phenolic substances (ArOH) serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals (Rice-Evans et al., 1997), according to:

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet}$$
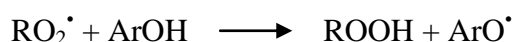
Ascorbic acid is a well-known powerful antioxidant, reported in mushrooms in lower amounts than phenolics (Ferreira et al., 2009). Accordingly, it seems to contribute positively to the RSA but with less significance. Otherwise, it gives a negative contribution to the reducing power of the samples. In fact, ascorbic acid mechanism of action has been related to free radicals scavenging effects and not to reducing processes with electrons transference (which is present in RP assay- reducing Fe^{3+} to Fe^{2+}); ascorbic acid protect biomembranes against lipid peroxidation damage by eliminating peroxyl radicals in the aqueous phase before the latter can initiate lipid peroxidation (Davey et al., 2000). Carbohydrates gave a small positive contribution possible due to the presence of mannitol (reducing sugar), a very

abundant sugar in mushrooms which functions to provide support and expansion of the fruit body (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2007c; Barros et al., 2008b; Barros et al., 2008d). Unsaturated fatty acids (MUFA and PUFA) show also a small positive contribution to RSA but a negative contribution to RP; the presence of double bonds makes them susceptible to oxidation; they can react with free radicals and become radicals themselves. Therefore, they act mostly as free radical scavengers. Particularly, oleic (C18:1) and linoleic acid (C18:2), abundant in mushrooms (Barros et al., 2007a; Barros et al., 2007b; Barros et al., 2007c; Barros et al., 2008b; Barros et al., 2008d) proved to have more than 80% of RSA (Henry et al., 2002). Nevertheless, it should be noticed that fatty acids exist in mushrooms in a very low concentration.

Surprisingly, β-carotene gave a small negative contribution to the antioxidant activity. This can be explained by the fact that this compound is present is mushrooms only in vestigial amounts (Ferreira et al., 2009). The negative correlation between the fat and the antioxidant properties was expectable since total fat obtained by soxhlet extraction include linked compounds, not free fatty acids; to obtain fatty acids, a derivatization process should be done. Saturated fatty acids (SFA) are not antioxidants and therefore, seem not to contribute to the scavenging effects. Particularly, stearic acid (C18:0), a fatty acid abundant in mushrooms proved to have less than 20% of RSA (Henry et al., 2002). Surprisingly, SFA show a slightly positive contribution to the RP of samples, probably through the reducing properties of the carboxylic moiety. The biosynthesis of phenolic compounds is derived from some amino acids, including tyrosine and tryptophan, in the shikimic acid pathway. A possible explanation for the negative contribution of proteins to the antioxidant properties is that they might be used as a source of amino acids to obtain phenolics, decreasing proteins content. The negative correlation between the ash and the reducing properties can also be explained as ash contains minerals and heavy metals (including iron) which can act as pro-oxidants (Maisuthisakul et al., 2008).

1.3.2. QCAR model

Based on the preliminary analysis we then set out to develop a QCAR model that could be used for predictive purposes. As phenolics and flavonoids contents were the parameters that had better correlate to antioxidant activity they were chosen to build the model. Also RP analysis consistently gave better statistical parameters when compared to

RSA analysis (**Table 2**) and thus we selected RP values to build the predictive QCAR model (**Table 3**).

Table 3. Phenolics, flavonoids, experimental and predicted EC_{50} reducing power values of Portuguese wild mushrooms.

Species	Phenolics (mg/g ext)	Flavonoids (mg/g ext)	Experimental EC_{50} RP	Predicted EC_{50} RP	Residues
Agaricus arvensis	2.72	1.65	4.20	4.26	-0.06
Agaricus bisporus	4.49	1.73	3.63	3.84	-0.21
Agaricus romagnesii	6.18	2.87	2.23	3.25	-1.02
Agaricus silvaticus	8.95	3.88	2.08	2.62	-0.54
Agaricus silvicola (a)	6.40	3.40	3.24	3.11	0.13
Cantharellus cibarius	0.88	0.67	5.89	5.02	0.87
Hypholoma fasciculare (a)	17.67	5.09	0.95	1.50	-0.55
Lactarius deliciosus F	2.95	1.91	4.65	3.83	0.82
Lactarius deliciosus D	3.40	2.71	4.98	4.13	0.85
Lactarius piperatus (SIII) (a)	3.09	0.35	5.40	3.74	1.66
Lactarius piperatus (SII)	5.76	1.58	2.29	3.62	-1.33
Lactarius piperatus (SI)	5.52	1.26	2.83	4.54	-1.71
Lepista nuda	6.31	3.36	3.53	3.13	0.40
Lycoperdon molle	11.48	2.45	2.27	2.49	-0.22
Lycoperdon perlatum	10.57	2.10	2.96	2.68	0.28
Macrolepiota mastoidea D (a)	3.08	2.10	4.35	4.06	0.29
Macrolepiota mastoidea F (a)	2.69	1.56	4.44	4.29	0.15
Macrolepiota procera D	3.17	0.99	4.18	4.33	-0.15
Macrolepiota procera F	2.59	0.90	4.49	4.50	-0.01
Ramaria botrytis	20.32	16.56	0.68	0.62	0.06
Sarcodon imbricatus D	3.06	1.52	4.41	4.21	0.20
Sarcodon imbricatus F	2.22	1.12	5.94	4.53	1.41
Tricholoma acerbum (a)	5.53	1.87	3.27	3.60	-0.33

(a) test set observations; F- frozen mushrooms; D- dehydrated mushrooms; SI immature (cap diameter less than 4.5 cm); SII mature (cap diameter between 4.5 and 7 cm) with immature spores; SIII mature (cap diameter higher than 7 cm) with mature spores. All the other samples were lyophilized and in SII.

Figure 8A shows the number of samples assigned to the three groups of antioxidant activity: 0–3, 3–4.5 and 4.5–6 according to EC_{50} values.

The QCAR model equation obtained and the statistical parameters were the following:

EC_{50} RP = $10^{(-0.0239517 \text{ x Phenolics (mg/g ext)} - 0.027891 \text{ x Flavonoids (mg/g ext)} + 0.740363)}$

N=17; R^2=0,84; F=37.53 ρ=2.37x10^6; S=0.8114 ; Q^2_{LOO}=0.83; RMSE $_{(training\ set)}$ = 0.7507; Q^2_{ext} =0,95; RMSE$_{(test\ set)}$ = 0.4979

were N is the number of samples used, R^2 is the squared correlation coefficient, ρ is the significance of the model, F is the Fisher ratio, Q^2_{LOO} is the "Leave-One-Out" correlation coefficient and RMSE(training set) and RMSE(test set) are Root Mean Squared Errors for the training and test sets, respectively.
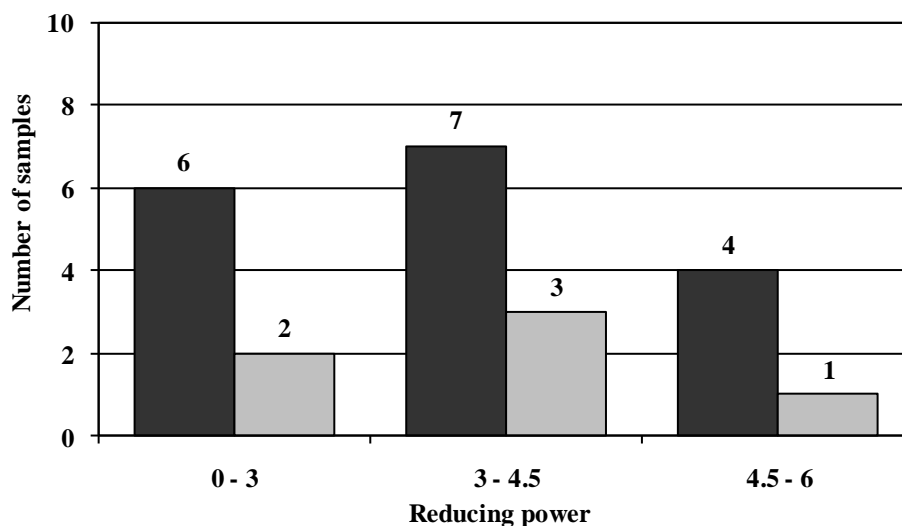
Figure 8. Distribution of reducing power (EC_{50} RP) versus number of samples for the training set (black) and test set (grey) of the QCAR model.

The model was evaluated for its robustness and predictive power by internal leave-one-out (LOO) validation, as demonstrated by R^2 and Q^2 values, and by external validation as demonstrated by Q^2_{ext} value. Also RMSE values, for both the training and test sets, validate the model by presenting low and similar values. External validation is acknowledged to be the best method to validate a model as it is usually immune to overfitness and overprediction.

To validate even further the model, a permutation test was performed (Gramatica, 2007). In this test the model is recalculated for randomly reordered response data (EC_{50} RP) and these permuted EC_{50} RP values are related to intact predictor data by refitting the model and including cross-validation. When $R^2_{permutation\ test}$ and $Q^2_{permutation\ test}$ are plotted as a function of the correlation coefficient between the original values and the predicted values, the intercept with the Y axis express to which degree these values rely on chance. **Figure 9** shows the results obtained from 100 permutations for each of the samples under study. The intercepts of the two regression lines (for $R^2_{permutation\ test}$ and $Q^2_{permutation\ test}$) indicate the degree

of overfit and overprediction. Intercepts for $R^2_{\text{permutation test}}$ and $Q^2_{\text{permutation test}}$ below 0.30 indicate a valid model, as is the case.
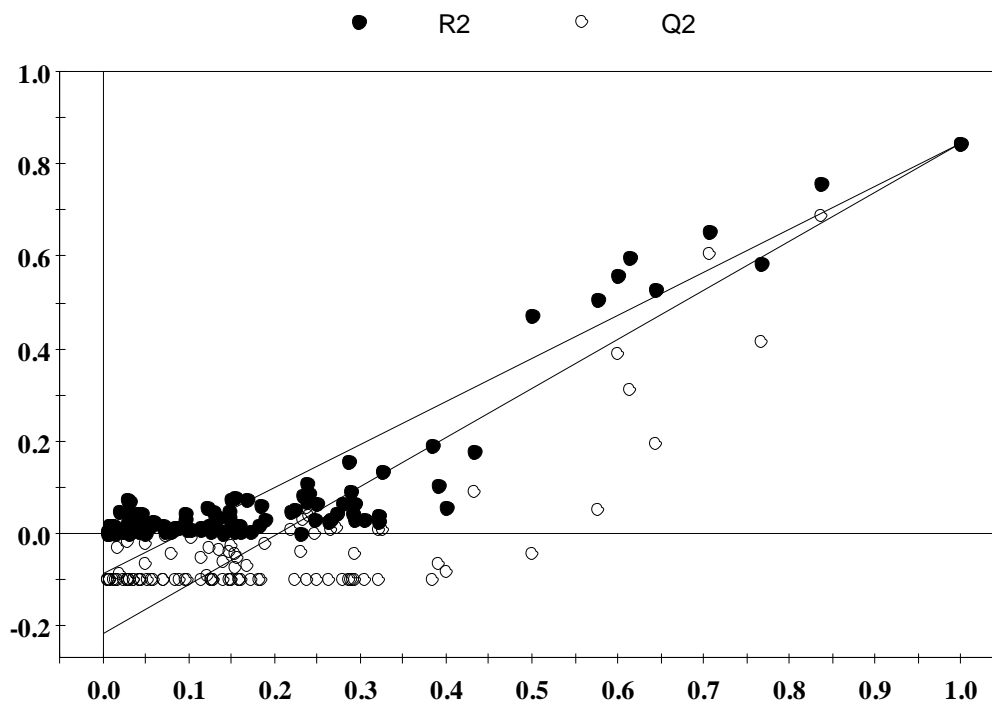
Figure 9. Results of the permutation test. The R^2 and Q^2 values were obtained from 100 permutations for the four developed PLS models. Intercepts: (R^2) $R^2_{\text{permutation test}}$=0, -0.0818, (Q^2) $Q^2_{\text{permutation test}}$ =0, -0.244

A plot of predicted EC_{50} RP versus experimental EC_{50} RP values, for both the training and test sets, is shown on **Figure 10**. The agreement observed between the predicted and experimental values confirmed the predictability of this QCAR model. Also plot of the residuals (predicted EC_{50} RP–experimental EC_{50} RP) versus experimental EC_{50} RP, for both the training and test sets is shown on **Figure 11**. A random distribution of the residuals about zero was observed for both sets. Because of this, no sample was considered a possible outliers as residuals were all within the standard deviation interval (-3S to 3S) usually considered the limit line for spotting outliers. The relation between RP and phenolic and flavonoids content was not linear rather exponential. This is an indication that higher contents of these compounds will elevate by order of magnitudes RP (lower RP values means better antioxidant activity). Overall, as far as we know, this is the first report of a QCAR model to predict reducing power using the chemical composition of mushrooms.
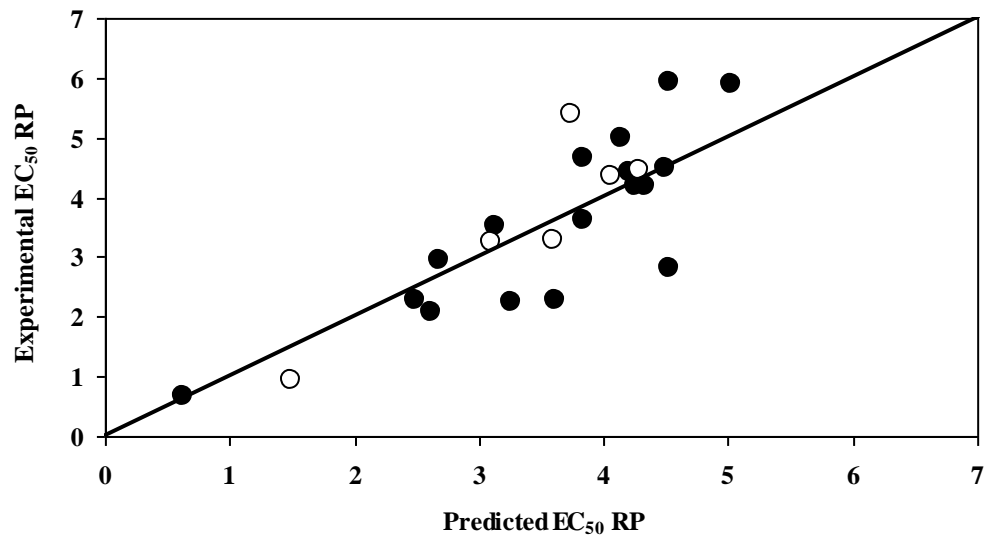
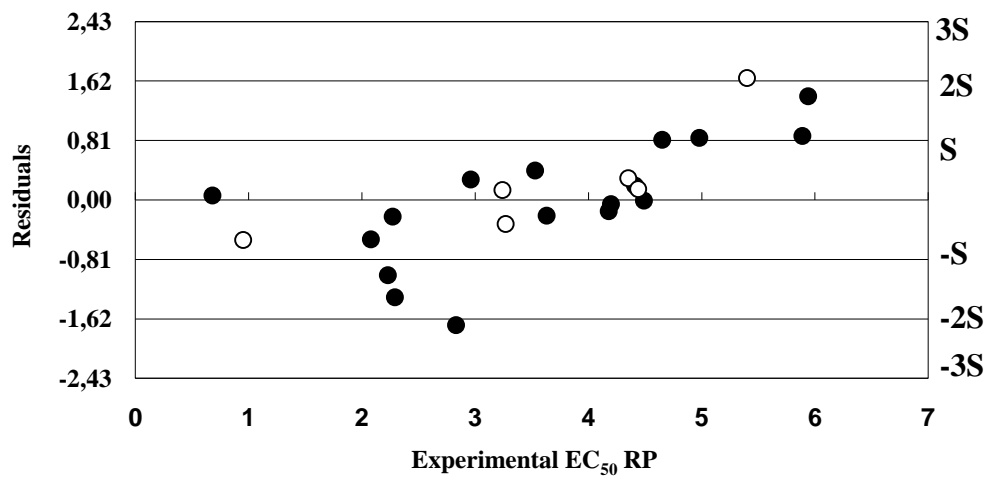**Figure 10.** Predicted versus experimental EC$_{50}$ RP for for the training (●) and test sets (○).,

Figure 11. Residual versus experimental log EC$_{50}$ RP for the training (●) and test sets (○).-

2. Insights on wild mushrooms anti-breast cancer activity by Virtual Ligand Screening of low molecular weight compounds

2.1. Introduction

Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In particular, and most importantly for modern medicine, they represent an unlimited source of compounds with antitumor and immunostimulating properties (Wasser, 2002; Zaidman et al., 2005; Zjawiony, 2004). Mushrooms contain compounds known as long-chain, large-molecular weight polysaccharides which, when present in specific configurations or linkages (beta, 1-3 glucan and beta, 1-6 glucan), have strong effects on the immune system of humans (Borchers et al., 2004; Mattila et al., 2000; Zhang et al., 2006). Several phytochemicals have been isolated from medicinal mushrooms and three of these, which are carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are "Krestin" (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), "Lentinan" from the fruiting bodies of Shiitake (*Lentinus edodes*) and "Schizophyllan" (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*) (Larone, 2002; Mizuno, 1993). More importantly extracts from wild mushrooms species have been shown to reduce the risk of breast cancer in Chinese women (Zhang et al., 2009) and in breast cancer cell lines (Grube et al., 2001). Mushrooms are also rich sources of low molecular weight (LMW) antioxidant compounds mainly phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids as described by our research group (Ferreira et al., 2009). In fact, in the last years tens of different mushroom species from Northeast of Portugal, one of the European regions with higher wild edible mushrooms diversity, were evaluated by us, for their composition on those LMW compounds (Barros et al., 2007f; Barros et al., 2008a; Barros et al., 2008b; Barros et al., 2008c; Barros et al., 2008d; Barros et al., 2009; Heleno et al., 2009b). Since the non-controlled production of free radicals has been related to more than one hundred diseases including several kinds of cancer, it was our goal to evaluate the potential properties of the antioxidants found in mushrooms against some proteins identified as targets in breast cancer.

Most breast cancers (about 95%), whether in pre- or postmenopausal women, are initially hormone-dependent and it is well accepted that estradiol plays an important role in

their development and progression. Estradiol in complex with their receptor can mediate the activation of proto-oncogenes or oncogenes (e.g. c-fos, c-myc), nuclear proteins, as well as other target genes. Consequently, processes that modulate the intracellular concentrations of active estrogens can have the ability to affect the etiology of this disease. It is known that that mammary cancer tissue contains all the enzymes responsible for the local biosynthesis of estradiol from circulating precursors (Pasqualini and Chetrite, 2005). Two principal pathways are implicated in the last steps of estradiol formation in breast cancer tissues: the 'aromatase pathway', with Aromatase (EC: 1.14.14.1) that forms androgens into estrogens and the 'sulfatase pathway' which converts estrone sulfate into estrone by the Estrone Sulfatase (EC: 3.1.6.2). The final step of steroidogenesis is the conversion of the weak estrone to the potent biologically active estradiol by the action of a reductive 17β-hydroxysteroid dehydrogenase type 1 activity (17β-HSD-1; EC: 1.1.1.62) (Pasqualini and Chetrite, 2005). **Figure 12** gives a general view of estrogen formation and transformation in human breast cancer.
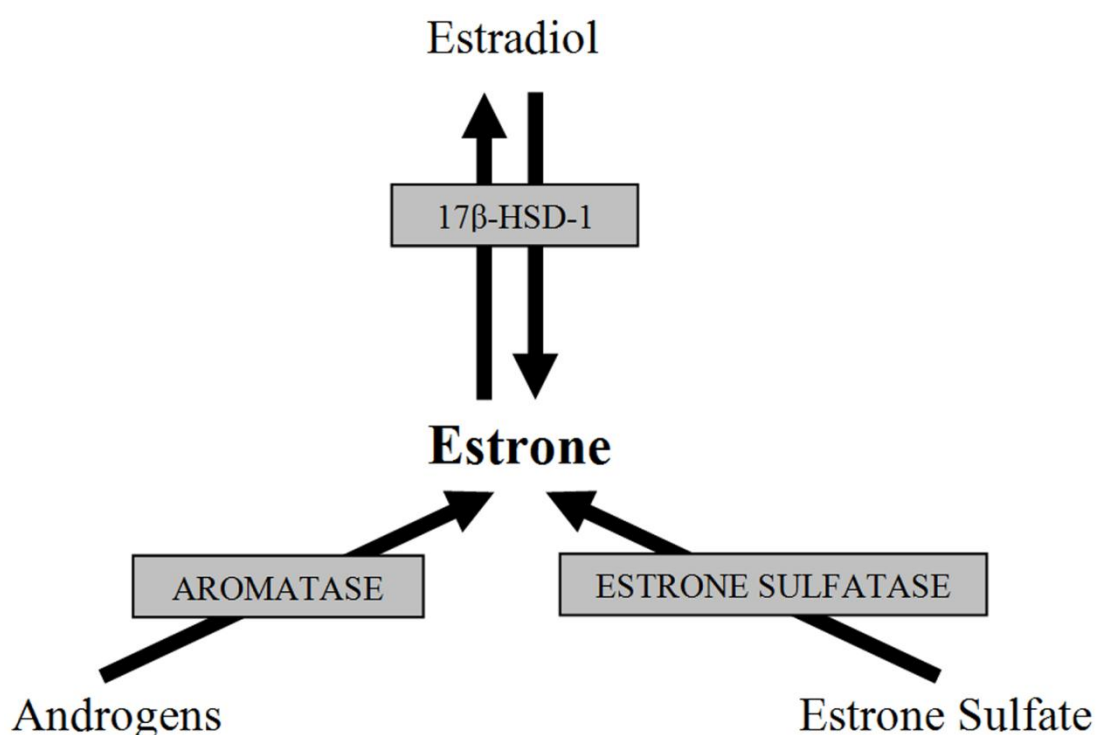
Figure 12. General view of estrogen formation and transformation of estrogens in human breast cancer: aromatase and sulfatase pathways.

Intermolecular interactions between proteins and small ligands play essential roles in several life processes and understanding these interactions is critical for pharmaceutical and functional food industries (Vaque et al., 2008). Molecular docking is an *in silico* tool that predicts how a ligand (substrate or drug candidate) interacts with a receptor usually by predicting the ligand binding free energy and the three-dimensional structure of the ligand-receptor complex. The use of molecular docking to search large databases of compounds for possible ligands of a protein receptor is usually termed virtual ligand screening (VLS) and has been successfully applied in several therapeutic programs at the lead discovery stage (Ghosh et al., 2006). We have used AutoDock 4 (Morris et al., 1998), acknowledged to be one of the most reliable and broadly used molecular docking tool (Sousa et al., 2006), with several examples of accurate docking predictions already published(Chen et al., 2007; Li et al., 2004). In the present study, we performed VLS using 3-D structures of Aromatase, Estrone Sulfatase and 17β-HSD-1 as targets and phenolic acids, flavonoids, tocopherols, carotenoids, sugars and fatty acids as ligands. The main goal was to identify which mushrooms LMW antioxidant compounds may be active against human breast cancer by identifying the potential protein targets. In addition, the molecular basis of the interaction between the best LMW compounds identified and the protein target is discussed. This study suggests what type of LMW compounds should be looking for in wild mushrooms that present activity against human breast cancer.

2.2. Methods

2.2.1 Ligand dataset

The ligand dataset used was composed of 43 LMW compounds that are representative of the chemical composition of wild mushrooms. The 2-D structure of the ligand dataset was constructed using MDL Isis/Draw 2.5 (http://www.symyx.com). We then used the software VegaZZ 2.3.1 (Pedretti et al., 2004) to: convert all ligands to 3-D, perform energy minimization and record files in pdb format. Next, AutoDockTools1.5.2 (ADT) (Sanner, 2005) was used to: merge nonpolar hydrogens, add Gasteiger charges, and set up rotatable bonds through AutoTors (Gasteiger and Marsili, 1980). Finally, all ligands were recorded in pdbqt format, a file format used by AutoDock 4.

2.2.2 Protein structures preparation

The X-ray 3-D structures of the protein targets used were extracted from the Protein Data Bank (PDB) (http://www.rcsb.org) including: Aromatase (PDB: 3EQM), Estrone Sulfatase (PDB: 1P49) and 17β-HSD1 (PDB: 1FDT).

For 3EQM and 1FDT the co-crystallized ligand (Androstenedione and Estrone respectively) was extracted from the PDB file (**Table 4**). This procedure was not done with 1P49 structure because this structure was determined without a co-crystallized ligand. ADT was then used to assign polar hydrogens and Gasteiger charges to the protein structures and the structure was in pdbqt format, a format needed for docking with AutoDock 4 (Morris et al., 1998).

Table 4. Comparison of estimated and experimental values of Km (ηM) and ΔG (Kcal/mol) values.

Enzyme	Ligand	Experimental Km (ηM)	Estimated Km (ηM)	Experimental ΔG (Kcal/mol)	Estimated ΔG (Kcal/mol)	Mean RMSD
Aromatase (PDB: 3EQM)	Androstenedione	20 [a]	4	-10,51	-11.46	0.08 Å
Estrone Sulfatase (PDB: 1P49)	Estrone Sulfate	6850 [b]	416	-7,04	-8.70	-
17β-HSD1 (PDB: 1FDT)	Estrone	124 [c]	238	-9.44	-9.02	0.66 Å

RMSD- Root Mean Square Deviation; [a] Numazawa et al., 1996; [b] Ishida et al., 2008; [c] Mendozahernandez et al., 1984..

For each protein structure, AutoGrid 4 (Morris et al., 1998) was used to create affinity grid maps for all the atoms present on the protein and ligands used. We used ADT to choose the correct parameters before using AutoGrid 4. All affinity grid maps were centred on the active site and coordinates were selected in order to encompass all the active site for each protein. 3EQM affinity grids enclosed an area of 100 Å by 100 Å by 100 Å with 0.375 Å spacing, centred on the coordinates x=86.312 y=51.204 z=48.26, 1P49 affinity grids maps enclosed an area of 80 Å by 80 Å by 80 Å with 0.375 Å spacing, centred on the coordinates x=71.9 y=-5.072 z=30.368 and 1FDT affinity grids enclosed an area of 80 Å by 110 Å by 110 Å with 0.375 Å spacing, centred on the coordinates x=39.685 y=1.159 z=37.333.

2.2.3 Virtual Ligand Screening using AutoDock 4

AutoDock 4 (version 4.0.1) with the Lamarckian genetic algorithm was used to simulate ligand-receptor molecular docking (Morris et al., 1998). Docking parameters selected for AutoDock 4 runs were as follows: 50 docking runs, population size of 200, random starting position and conformation, translation step ranges of 2.0 Å, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 2.5 million energy evaluations. Docked conformations were clustered using a tolerance of 2.0 Å RMSD (Root Mean Square Deviation). All 3 crystal structures are used as receptors for VLS against the ligand dataset. The entire VLS experiment was performed on a cluster of 8 Intel Dual-Core 2.8 GHz computers using custom designed software called MOLA (Abreu et al., submitted). Inhibition constant (Ki) for all ligands was calculated by AutoDock 4 as follows: Ki = exp((ΔG*1000.)/(Rcal*TK)) where ΔG is the binding energy, Rcal is 1.98719 and TK is 298.15. The Michaelis-Menten constant (Km) for natural ligands presented on **table 4** were calculated by AutoDock 4 using the same equation indicated above. The 3-D ligand-protein docking pose was analysed manually using ADT and the images presented on figures 2 and 4 were prepared using the software PyMOL 0.99r6 (http://pymol.sourceforge.net/).

2.3. Results and Discussion

2.3.1 Docking validation

In order to validate the molecular docking approach the respective ligand (natural substrate) was docked to the active site of the proteins (from which the natural ligand was previously removed).

Then the estimated Km obtained was compared with experimental Km values (**Table 4**) and the estimated 3-D binding mode was compared with the experimentally (X-ray crystallography) determined co-crystallized binding mode (**Figure 13**). For the proteins studied, when comparing experimental and estimated Km values, we observe that values fall in the same order of magnitude with estimated Km being 5 times lower for Aromatase, 20 times lower for Estrone Sulfatase and 2 times higher for 17β-HSD1 (**Table 4**). Km values were calculated from the binding energy (ΔG) and the difference between experimental and estimated ΔG were 0.95, 1.60 and 0.42 Kcal/mol for Aromatase, Estrone Sulfatase and 17β-HSD1, respectively. This variation is well within the standard error for binding energy (ΔG)

previously reported for AutoDock 4 that is around 2.5 Kcal/mol (Morris et al., 1998) corresponding to a difference between estimated and experimental Km of more than 2 orders of magnitudes. Also the binding mode of the docked ligands for Aromatase and 17β-HSD1 corresponded well with the binding mode of the co-crystallized ligands, with RMSD values of 0.08 Å and 0.66 Å, respectively (**Figure 13**).
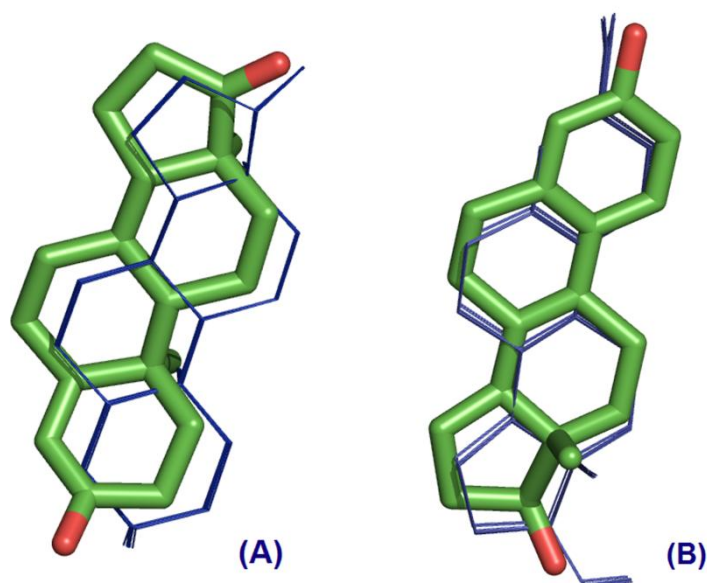
Figure 13. Superimposition of X-ray (sticks and balls) and docked configurations (wire) for: (A) Androstenedione in Aromatase and (B) Estrone in 17β-HSD1. The 10 best docked configurations are represented for easier inspection.

These values shows that the difference between the X-ray conformation and the predicted docked conformations of the ligands was very small (**Figure 13**) thus validating the protein structures for VLS with the LMW dataset. This comparison is not possible for Estrone Sulfatase as there is no experimentally determined structure with a co-crystallized ligand, only the non bounded protein structure. This probably explains the higher different between estimated and experimental Km for Estrone Sulfatase (1.60 Kcal/mol) although well within the expected error for docking using AutoDock 4.

2.3.2 Mushrooms LMW Virtual Ligand Screening

After validation of the 3 protein targets for molecular docking with AutoDock 4 we performed the VLS of the selected wild mushroom LMW dataset against the 3 target structures. The ligand dataset used is not exhaustive but is a good representation of the different LMW families of compounds that can be found in wild mushrooms. The results will be discussed for each family of compounds: phenolic compounds (benzoic acid and cinnamic acid derivatives, and flavonoids), vitamins (tocopherols and ascorbic acid), carotenoids, sugars and fatty acids.

The results obtained using phenolic acids (**Table 5**) revealed that benzoic acid derivatives appear to have no significant inhibitory activity for the 3 enzymes studied with all values well above 1 μM. Cinnamic acid derivatives also had no significant inhibitory activity except for 4-*O*-caffeylquinic and 5-*O*-caffeylquinic which presented moderate inhibition activity for the enzymes with values in the hundreds of ηM (**Figure 14** and **Table 5**). The presence of quinic acid seems to be an essential condition for phenolic acid inhibition. Also the fact that both compounds present activity against all the 3 enzymes probably results from the fact that the natural ligands have similar structures. This simultaneous inhibition activity may result in a synergistic inhibition of overproduction of Estrone in breast cancer by inhibiting both "Aromatase" and "Sulfatase" pathway as well as inhibiting estradiol to estrogen conversion by 17β-HSD1.

Table 5. Docking studies with phenolic acids found in mushrooms as ligands.

<div align="center">
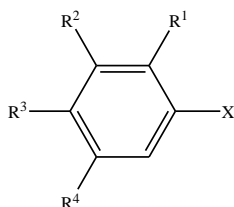
Benzoic acids   **Cinnamic acids**

</div>
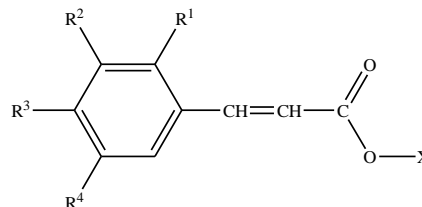
Benzoic acid Derivatives	Substitution					Estimated Ki (µM)		
	X	R^1	R^2	R^3	R^4	Aromatase	Estrone Sulfatase	17β-HSD1
p-Hydroxibenzoic	COOH	H	H	H	OH	607.4	278.3	101.6
Protocatechuic	COOH	H	H	OH	OH	365.9	155.0	102.2
Gallic	COOH	H	OH	OH	OH	358.3	129.2	100.6
Gentisic	COOH	OH	H	H	OH	546.1	599.0	578.3
Homogentisic	CH_2COOH	OH	H	H	OH	939.4	583.9	116.4
Vanillic	COOH	H	OCH_3	OH	H	227.0	98.08	89.70
5-Sulphosalicylic	COOH	OH	H	H	HSO_3	219.7	381.8	365.5
Syringic	COOH	H	OCH_3	OH	OCH_3	239.9	212.4	93.68
Veratric	COOH	H	OCH_3	OCH_3	H	309.4	98.04	98.60
Vanillin	CHO*	H	OCH_3	OH	H	213.5	299.4	>1 000

Cinnamic acid derivatives	Substitutions					Estimated Ki (µM)		
	X	R^1	R^2	R^3	R^4	Aromatase	Estrone Sulfatase	17β-HSD1
p-Coumaric	H	H	H	OH	H	80.91	28.81	124.6
o-Coumaric	H	OH	H	H	H	78.17	27.27	118.2
Caffeic	H	H	OH	OH	H	57.28	19.48	73.97
Ferulic	H	H	CH_3O	OH	H	26.63	105.5	91.29
Sinapic	CH_3O	H	CH_3O	OH	CH_3O	8.260	122.5	27.10
4-O-caffeoylquinic	*	H	OH	OH	H	0.317	0.474	0.289
5-O-caffeoylquinic	*	H	OH	OH	H	0.760	3.990	0.255

 * The carboxylic group is esterified with quinic acid.

LMW dataset compounds			Aromatase	Estrone Sulfatase	17β-HSD1
Phenolic acids	Benzoic acid	p-Hydroxibenzoic			
		Protocatechuic			
		Gallic			
		Gentisic			
		Homogentisic			
		Vanillic			
		5-Sulphosalicylic			
		Syringic			
		Veratric			
		Vanillin			
	Cinnamic acid derivatives	p-Coumaric			
		o-Coumaric			
		Caffeic			
		Ferulic			
		Sinapic			
		4-O-caffeoylquinic	2	2	13
		5-O-caffeoylquinic	8		12
Flavonoids	Flavonols	Quercetin	1		9
		Rutin		3	10
		Kaempferol			11
		Myricetin	9		8
	Flavones	Chrysin	5		15
	Flavan-3-ols	Catechin			
	Flavanones	Hesperetin			
		Naringenin	3		14
		Naringin	7	1	2
	Isoflavones	Formonetim	4		16
		Biochanin	6		18
Vitamins and Carotenoids	Tocopherols	α-tocopherol		5	3
		β-tocopherol			4
		γ-tocopherol		4	7
		δ-tocopherol		6	6
	Ascorbic acid	Ascorbic acid			
	Carotenoids	β-Carotene			7
		Lycopene			1
Sugars	Sugars	Maltose			17
		Trehalose			
		Melezitose			
Fatty acid	Fatty acids	Myristic acid (C14:0)			
		Palmitic acid (C16:0)			
		Stearic acid (C18:0)			
		Oleic acid (C18:1n9c)			
		Linoleic acid (C18:2n6c)			

Figure 14. Colour coded representation of the best results obtained by virtual ligand screening of the LMW dataset against the 3 protein targets. Colours used are green for good inhibiton activity (< 0.1 μM), yellow for moderate inhibition activity (0.1 μM > Ki > 1 μM) and red for weak or no inhibition activity (Ki > 1 μM). Green and yellow tagged ligands are ordered from best to worst Ki value.

The results obtained using flavonoids (**Table 6**) as ligands showed that naringenin was the best compound to inhibit Aromatase, while naringin proved to be better for Estrone Sulfatase and 17β-HSD1. Interestingly, the substitution of the hydroxyl group for the disaccharide rutinose increases Ki values for Aromatase and decreases the corresponding values for the other two enzymes (see in **Table 6** and **Figure 14** rutin relative to quercetin, and naringin relative to naringenin).

Table 6. Docking studies with flavonoids found in mushrooms as ligands.

	Substitution			Estimated Ki (µM)		
Flavonols	**R'$_1$**	**R'$_2$**	**R'$_3$**	**Aromatase**	**Estrone Sulfatase**	**17β-HSD1**
Quercetin	OH	OH	H	0.316	4.560	0.092
Rutin*	OH	OH	H	29.80	0.488	0.094
Kaempferol	H	OH	H	1.090	8.960	0.249
Myricetin	OH	OH	OH	0.790	5.620	0.091
Flavones Chrysin	H	H	H	0.610	15.03	0.467
Flavan-3-ols Catechin	H	OH	OH	11.76	11.54	3.210
Flavanones	**R'$_1$**	**R'$_2$**	**R'$_3$**	**Aromatase**	**Estrone Sulfatase**	**17β-HSD1**
Hesperetin	OH	H	H	5.410	7.970	2.310
Naringenin	H	OH	H	0.342	10.67	0.413
Naringin *	H	OH	H	0.743	0.206	0.001
Isoflavones	**R'$_1$**	**R'$_2$**	**R'$_3$**	**Aromatase**	**Estrone Sulfatase**	**17β-HSD1**
Formonetim	H	OCH$_3$	H	0.590	17.40	0.571
Biochanin**	H	OCH$_3$	H	0.710	11.14	0.771

* OH in position-3 is substituted with the disaccharide rutinose; ** OH in position-5.

The presence of rutinose in those compounds might increase the stereochemical hindrance of the molecules decreasing their binding capacity to Aromatase, which contains a heme group. In general, it was observed that a good number of the flavonoids from different groups present inhibition activity. This is probably because, from the ligand dataset used, flavonoids have more similar structures with the natural ligands.

The results obtained using vitamins and carotenoids as ligands are given in **Table 7**. Vitamin E (tocopherols) proved to have better binding capacity than vitamin C (ascorbic acid). The four isoforms of vitamin E (α, β, δ, and γ-tocopherol) revealed very good inhibition properties for 17β-HSD1 with Ki in the nanomolar range. Furthermore, β-carotene and lycopene revealed excellent properties for inhibition of 17β-HSD1 with lycopene reacting on the subnanomolar range (0.2 ηM), the best result in all the VLS. Also, α, δ, and γ-tocopherol showed moderate inhibition activity against Estrone Sulfatase.

Table 7. Docking studies with vitamins and carotenoids found in mushrooms as ligands.
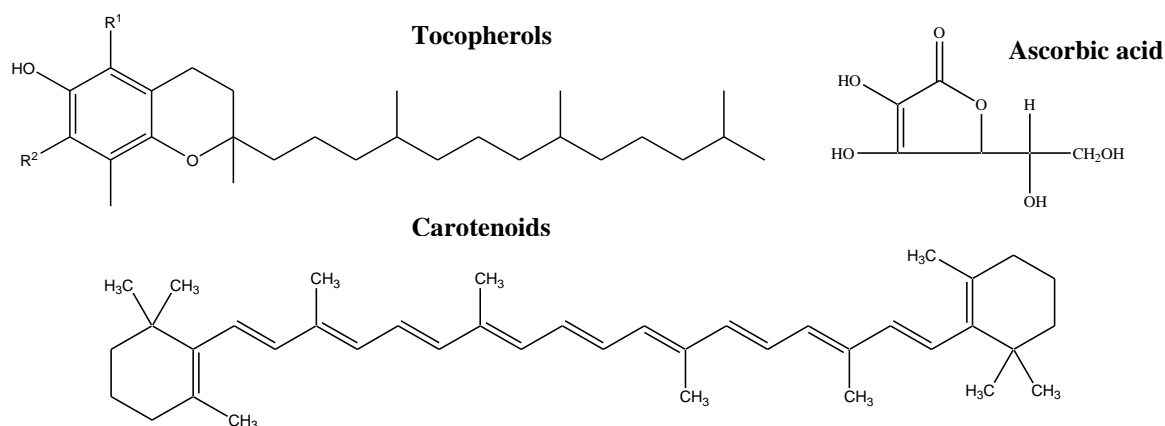
Tocopherols	Substitution		Estimated Ki (µM)		
	R_1	R_2	Aromatase	Estrone Sulfatase	17β-HSD1
α-tocopherol	CH_3	CH_3	41.23	0.672	0.002
β-tocopherol	CH_3	H	35.38	1.510	0.009
γ-tocopherol	H	CH_3	61.36	0.505	0.010
δ-tocopherol	H	H	59.68	0.882	0.012
Ascorbic acid	-		277.7	85.87	268.8
Carotenoids					
β-Carotene	-		16.27	> 1 000	0.016
Lycopene*	-		> 1 000	5.100	0.0002

*The rings are opened.

In relation to the results obtained using sugars and fatty acids (**Table 8**) it was not observed any significant inhibition activity in any of the studied targets. Only maltose showed a very moderate activity 17β-HSD1 but with a relatively high Ki value (0.605 μM); interestingly maltose is the only reducing sugar which may be an important fact as 17β-HSD1 is a dehydrogenase enzyme. Nevertheless sugar and fatty acids do not seem implicated in anti-breast cancer activity.

Table 8. Docking studies with sugars and fatty acids found in mushrooms as ligands.

	Estimated Ki (μM)		
Sugars	**Aromatase**	**Estrone Sulfatase**	**17β-HSD1**
Maltose	1.520	3.340	0.605
Trehalose	9.080	5.650	12.23
Melezitose	> 1 000	> 1 000	> 1 000
Fatty acids			
Myristic acid (C14:0)	10.59	283.8	56.01
Palmitic acid (C16:0)	6.940	157.0	12.04
Stearic acid (C18:0)	2.770	108.6	7.810
Oleic acid (C18:1n9c)	6.070	84.64	5.070
Linoleic acid (C18:2n6c)	1.450	28.18	3.730

2.3.3 Structure analysis of the best docked conformations

The docked binding mode of all the compounds with good Ki values was manually inspected in order to verify that they effectively bind to the catalytic site in a structurally viable conformation.

For Aromatase the top ranked compound was 4-*O*-caffeoylquinic acid and the docked structure occupies the space where the natural ligand Androstenedione binds (**Figure 15A**).
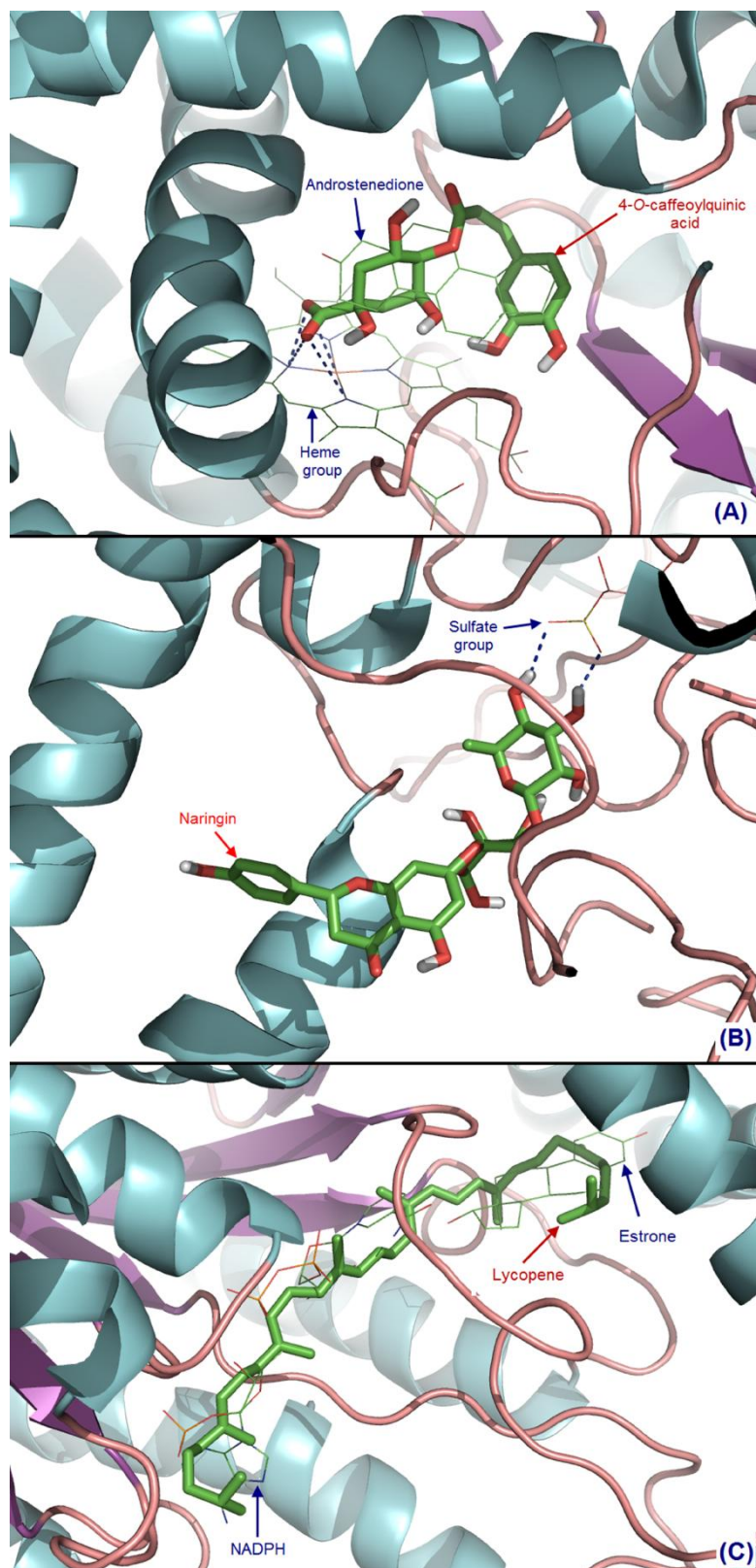
Figure 15. Docking of the top ranked inhibitor for each of the studied protein targets. Figure shows (A) Aromatase, (B) Estrone Sulfatase and (C) 17β-HSD1 docked with 4-*O*-caffeoylquinic, naringin and lycopene, respectively. Protein target are represented in cartoon format, docked inhibitor in sticks and balls format (red) and natural X-ray ligands in wire format (blue).

The aromatic rings from 4-*O*-caffeoylquinic occupies the space of the aromatic rings of Androstenedione and the quinic acid seems to be the key element in the binding mode with the carboxylic acid stabilized by polar contacts with the Heme group. It's important to note that all the estimated Ki values (317 ηM for 4-*O*-caffeoylquinic) obtained with Aromatase as the protein target was at least one order of magnitude higher than the experimental Km value obtained with Androstenedione (20 ηM). This fact indicates that Aromatase is probably not the most important target for LMW compounds in wild mushrooms.

For Estrone Sulfatase inhibition the top ranked compound was the flavanone naringin (**Figure 15B**; **Table 6**). The disaccharide rutinose seems to play a pivotal role in naringin inhibition by promoting hydrogen bonds with the sulfate group present. The X-ray structure used for docking had no co-crystallized ligand. The Ki value obtained for naringin (206 ηM) was more than one order of magnitude lower than the value obtained for estrone sulfate (6850 ηM), the natural ligand of the enzyme. Also several compounds have Ki values below 6850 ηM. This indicates that probably the "sulfatase" pathway is the most likely target for LMW wild mushrooms compounds inhibition. This is even more interesting in view of recent findings in human breast cancer that point towards "sulfatase" pathway as the more likely path for estradiol production with "aromatase" pathway playing a secondary role (Pasqualini and Chetrite, 2005).

Finally 17β-HSD1 was the most susceptible protein target to the studied ligands with the lowest Ki of all the dataset. Lycopene (**Figure 15C; Table 7**) presented the best Ki value (0.2 ηM) about three orders of magnitude lower than the Ki value for the natural ligand Estrone (124 ηM). Analysing the docked structure (**Figure 15C**) we can see that lycopene "fits" exactly on the binding pocket occupied by the coenzyme NADPH and the natural ligand estrone. Because lycopene is composed only of carbon and hydrogen, its structure is stabilized by van der Walls interactions. It is important to note that, although the estimated Ki value was very low, its inhibition ability is probably 'balanced by the difficulty of lycopene to reach the binding site of 17β-HSD1 due to his insolubility in water.

III. CONCLUSION

In a first step, the information about chemical compounds found in wild mushrooms was collected. Then, chemical parameters, including primary and secondary metabolites, were correlated to bioactive properties such as antioxidant and antitumor activities.

Several relationships were established between antioxidant activity and chemical composition (ash, carbohydrates, proteins, fat, monounsaturated fatty acids, polyunsaturated fatty acids, saturated fatty acids, phenolics, flavonoids, ascorbic acid and β-carotene) that provide a better understanding of the complex mechanisms of antioxidant activity of wild mushrooms. The positive effect of phenolics and particularly flavonoids, was clearly established and other possible correlations with different composition parameters were discussed. This information allowed the development of a predictive QCAR model using mushroom flavonoids and phenolic composition. Several internal and external validation protocols were used and confirm the predictive power of the QCAR model. Especially external validation is acknowledged to be the best rationale protocol to avoid overprediction and overfitting. This model could be a useful tool in the study and prediction of antioxidant activity of more mushroom species.

Since the non-controlled production of free-radicals has been related to more than one hundred diseases including several kinds of cancer, it was our goal to evaluate the potential properties of antioxidants found in mushrooms against some proteins identified as targets in breast cancer. In fact, this study highlighted several low molecular weight compounds from wild mushrooms that may act against breast cancer by inhibiting several proteins involved in overproduction of estrone and estradiol. From the studied phenolic acids, cinnamic acid derivatives esterified with quinic acid (4 and 5-*O*-caffeylquinic acid) were the only ones with significant inhibition against the three protein targets studied, specially 4-*O*- caffeoylquinic acid that presented the best ki against Aromatase. Among flavonoids, several compounds presented moderate to good inhibition ability with flavanones (naranigenin and naringin) and flavonols (quercetin and rutin) as the best compounds. Naringin was the top ranked inhibitor against Estrone Sulfatase indicating that the presence of the disaccharide rutinose may be a key element for active compounds against breast cancer. Vitamins and carotenoids were target specific showing very good inhibition ability only against 17β-HSD1, with Lycopene as the top ranked inhibitor. Sugar and fatty acids did not show any significant inhibition ability. The

highlighted compounds are the ones to look for in wild mushrooms when searching for species with anti-breast cancer activity. Furthermore, the information provided shows several interesting starting points for further development of Aromatase, Estrone Sulfatase and 17β-HSD1 inhibitors.

The identification of the active natural compounds against cancer, and the understanding of the molecular basis of the interaction between the best compounds and the protein targets are key factors for development of nutraceuticals or functional foods. The present work raises the possibility of using chemoinformatic tehniques as a part of the development process of new nutraceuticals or functional foods.

The present work gave two international publications (correspondent to the results section):

[1] Hugo J.C. Froufe, Rui M.V. Abreu, Isabel C.R.R. Ferreira, "A QCAR model for predicting antioxidant activity of wild mushrooms", SAR and QSAR Environmental Research, 2009, accepted (corrected proofs).

[2] Hugo J.C. Froufe, Rui M.V. Abreu, Isabel C.R.R. Ferreira, "Insights on wild mushrooms anti-breast cancer activity by Virtual Ligand Screening of low molecular weight compounds", Journal of Molecular Graphics and Modelling, 2009, submitted on October 2009.

IV. REFERENCES

A.B. Umetrics (2008). SIMCA-P 12+, User Guide. Umetrics Inc. 17 Kiel Ave, Kinnelon NJ 07405, USA.

Abagyan, R., and Totrov, M. (2001). High-throughput docking for lead generation. Current Opinion in Chemical Biology 5, 375-382.

Abreu R.M.V., Froufe, H., Queiroz, M.J.R.P., Ferreira, I.C.F.R..(submitted on June 2009). MOLA: a method for automation of parallel virtual screening on AutoDock using a diverse set of computers

Ajith, T.A., and Janardhanan, K.K. (2007). Indian medicinal mushrooms as a source of antioxidant and antitumor agents. Journal of Clinical Biochemistry and Nutrition 40, 157-162.

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B., and Weil, J.A. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chemistry 84, 551-562.

Andlauer, W., and Furst, P. (2002). Nutraceuticals: a piece of history, present status and outlook. Food Research International 35, 171-176.

Bano, Z., and Rajarathnam, S. (1988). Pleurotus Mushrooms .2. Chemical-composition, nutritional-value, post-harvest physiology, preservation, and role as human food. Critical Reviews in Food Science and Nutrition 27, 87-158.

Barros, L., Baptista, P., Correia, D.M., Casal, S., Oliveira, B., and Ferreira, I.C.F.R. (2007a). Fatty acid and sugar compositions, and nutritional value of five wild edible mushrooms from Northeast Portugal. Food Chemistry 105, 140-145.

Barros, L., Baptista, P., Correia, D.M., Morais, J.S., and Ferreira, I.C.F.R. (2007b). Effects of conservation treatment and cooking on the chemical composition and antioxidant activity of Portuguese wild edible mushrooms. Journal of Agricultural and Food Chemistry 55, 4781-4788.

Barros, L., Baptista, P., Estevinho, L.M., and Ferreira, I.C.F.R. (2007c). Effect of fruiting body maturity stage, on chemical composition and antimicrobial activity of Lactarius sp mushrooms. Journal of Agricultural and Food Chemistry 55, 8766-8771.

Barros, L., Baptista, P., and Ferreira, I.C.F.R. (2007d). Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays. Food and Chemical Toxicology 45, 1731-1737.

Barros, L., Calhelha, R.C., Vaz, J.A., Ferreira, I.C.F.R., Baptista, P., and Estevinho, L.M. (2007e). Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts. European Food Research and Technology 225, 151-156.

Barros, L., Correia, D.M., Ferreira, I.C.F.R., Baptista, P., and Santos-Buelga, C. (2008a). Optimization of the determination of tocopherols in Agaricus sp edible mushrooms by a normal phase liquid chromatographic method. Food Chemistry 110, 1046-1050.

Barros, L., Cruz, T., Baptista, P., Estevinho, L.M., and Ferreira, I.C.F.R. (2008b). Wild and commercial mushrooms as source of nutrients and nutraceuticals. Food and Chemical Toxicology 46, 2742-2747.

Barros, L., Duenas, M., Ferreira, I.C.F.R., Baptista, P., and Santos-Buelga, C. (2009). Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species. Food and Chemical Toxicology 47, 1076-1079.

Barros, L., Falcão, S., Baptista, P., Freire, C., Vilas-Boas, M., and Ferreira, I.C.F.R. (2008c). Antioxidant activity of *Agaricus* sp. mushrooms by chemical, biochemical and electrochemical assays. Food Chemistry 111, 61-66.

Barros, L., Ferreira, M.J., Queiros, B., Ferreira, I.C.F.R., and Baptista, P. (2007f). Total phenols, ascorbic acid, beta-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chemistry 103, 413-419.

Barros, L., Venturini, B.A., Baptista, P., Estevinho, L.M., and Ferreira, I.C.F.R. (2008d). Chemical composition and biological properties of Portuguese wild mushrooms: A comprehensive study. Journal of Agricultural and Food Chemistry 56, 3856-3862.

Borchers, A.T., Keen, C.L., and Gershwin, M.E. (2004). Mushrooms, tumors, and immunity: An update. Experimental Biology and Medicine 229, 393-406.

Burczyk, J., Gawron, A., Slotwinska, M., Smietana, B., and Terminska, K. (1996). Antimitotic activity of aqueous extracts of *Inonotus obliquus*. Bollettino Chimico Farmaceutico 135, 306-309.

Chen, K., Al Aowad, A.F., Adelstein, S.J., and Kassis, A.I. (2007). Molecular-docking-guided design, synthesis, and biologic evaluation of radioiodinated quinazolinone prodrugs. Journal of Medicinal Chemistry 50, 663-673.

Cheung, L.M., and Cheung, P.C.K. (2005). Mushroom extracts with antioxidant activity against lipid peroxidation. Food Chemistry 89, 403-409.

Cheung, L.M., Cheung, P.C.K., and Ooi, V.E.C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. Food Chemistry 81, 249-255.

Cheung, P.C.K. (2009). Nutritional Value and Health Benefits of Mushrooms. In Mushrooms as Functional Foods, P.C.K. Cheung, ed., pp. 71-109.

Choi, Y., Lee, S.M., Chun, J., Lee, H.B., and Lee, J. (2006). Influence of heat treatment on the antioxidant activities and polyphenolic compounds of Shiitake (*Lentinus edodes*) mushroom. Food Chemistry 99, 381-387.

Clifford, A.J., Heid, M.K., Peerson, J.M., and Bills, N.D. (1991). Bioavailability of food folates and evaluation of food matrix effects with a rat bioassay. Journal of Nutrition 121, 445-453.

Congreve, M., Murray, C.W., and Blundell, T.L. (2005). Keynote review: Structural biology and drug discovery. Drug Discovery Today 10, 895-907.

Cramer, R.D. (1993). Partial Least Squares (PLS): Its strengths and limitations. Perspectives in Drug Discovery and Design 1, 269-278.

Davey, M.W., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I.J.J., Strain, J.J., Favell, D., and Fletcher, J. (2000). Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. Journal of the Science of Food and Agriculture 80, 825-860.

Desta, Z., Modak, A., Nguyen, P.D., Lemler, S.M., Kurogi, Y., Li, L., and Flockhart, D.A. (2009). Rapid identification of the hepatic cytochrome P450 2C19 activity using a novel and noninvasive [C-13]pantoprazole breath test. Journal of Pharmacology and Experimental Therapeutics 329, 297-305.

Diez, V.A., and Alvarez, A. (2001). Compositional and nutritional studies on two wild edible mushrooms from northwest Spain. Food Chemistry 75, 417-422.

Durand, A.C., Farce, A., Carato, P., Dilly, S., Yous, S., Berthelot, P., and Chavatte, P. (2007). Quantitative structure-activity relationships studies of antioxidant hexahydropyridoindoles and flavonoid derivatives. Journal of Enzyme Inhibition and Medicinal Chemistry 22, 556-562.

Elmastas, M., Isildak, O., Turkekul, I., and Temur, N. (2007). Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. Journal of Food Composition and Analysis 20, 337-345.

Eriksson, L., Johansson, E., Muller, M., and Wold, S. (1997). Cluster-based design in environmental QSAR. Quantitative Structure-Activity Relationships 16, 383-390.

Farkas, O., Jakus, J., and Heberger, K. (2004). Quantitative structure - Antioxidant activity relationships of flavonoid compounds. Molecules 9, 1079-1088.

Fernandez, M., Caballero, J., Helguera, A.M., Castro, E.A., and Gonzalez, M.N. (2005). Quantitative structure-activity relationship to predict differential inhibition of aldose reductase by flavonoid compounds. Bioorganic and Medicinal Chemistry 13, 3269-3277.

Ferreira, I.C.F.R., Baptista, P., Vilas-Boas, M., and Barros, L. (2007). Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. Food Chemistry 100, 1511-1516.

Ferreira, I.C.F.R., Barros, L., and Abreu, R.M.V. (2009). Antioxidants in Wild Mushrooms. Current Medicinal Chemistry 16, 1543-1560.

Gasteiger, J., and Marsili, M. (1980). Iterative Partial Equalization of Orbital Electronegativity - a Rapid Access to Atomic Charges. Tetrahedron 36, 3219-3228.

Ghosh, D. (2007). Human sulfatases: A structural perspective to catalysis. Cellular and Molecular Life Sciences 64, 2013-2022.

Ghosh, S., Nie, A.H., An, J., and Huang, Z.W. (2006). Structure-based virtual screening of chemical libraries for drug discovery. Current Opinion in Chemical Biology 10, 194-202.

Gramatica, P. (2007). Principles of QSAR models validation: internal and external. QSAR and Combinatorial Science 26, 694-701.

Gramatica, P., and Papa, E. (2005). An update of the BCF QSAR model based on theoretical molecular descriptors. QSAR and Combinatorial Science 24, 953-960.

Grube, S.J., Eng, E.T., Kao, Y.C., Kwon, A., and Chen, S. (2001). White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation. Journal of Nutrition 131, 3288-3293.

Hasler, C.M. (1998). Functional foods: Their role in disease prevention and health promotion. Food Technology 52, 63-70.

Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., and Ferreira, I.C.F.R. (2009a). Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. Microchemical Journal 93, 195-199.

Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., and Ferreira, I.C.F.R. (2009b). Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. Food Chemistry doi. 10.1016/j.foodchem2009.09.25.

Henry, G.E., Momin, R.A., Nair, M.G., and Dewitt, D.L. (2002). Antioxidant and cyclooxygenase activities of fatty acids found in food. Journal of Agricultural and Food Chemistry 50, 2231-2234.

Ishida, H., Sato, N., Hosogi, J., Tanaka, H., and Kuwabara, T. (2008). Inactivation of recombinant human steroid sulfatase by KW-2581. Journal of Steroid Biochemistry and Molecular Biology 108, 17-22.

Jansson, A. (2009). 17Beta-hydroxysteroid dehydrogenase enzymes and breast cancer. Journal of Steroid Biochemistry and Molecular Biology 114, 64-67.

Kalac, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. Food Chemistry 113, 9-16.

Kim, M.Y., Seguin, P., Ahn, J.K., Kim, J.J., Chun, S.C., Kim, E.H., Seo, S.H., Kang, E.Y., Kim, S.L., Park, Y.J., et al. (2008). Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. Journal of Agricultural and Food Chemistry 56, 7265-7270.

Kitzberger, C.S.G., Smania, A., Pedrosa, R.C., and Ferreira, S.R.S. (2007). Antioxidant and antimicrobial activities of shiitake (*Lentinula edodes*) extracts obtained by organic solvents and supercritical fluids. Journal of Food Engineering 80, 631-638.

Larone, D.H. (2002). Medically Important Fungi. (USA).

Lee, Y.L., Han, S.Y., Lian, P.Y., and Mau, J.L. (2008). Antioxidant properties of extracts from a white mutant of the mushroom *Hypsizigus marmoreus*. Journal of Food Composition and Analysis 21, 116-124.

Lee, Y.L., Yen, M.T., and Mau, J.L. (2007). Antioxidant properties of various extracts from *Hypsizigus marmoreus*. Food Chemistry 104, 1-9.

Li, C.L., Xu, L., Wolan, D.W., Wilson, I.A., and Olson, A.J. (2004). Virtual screening of human 5-aminoimidazole-4-carboxamide ribonucleotide transformylase against the NCI diversity set by use of AutoDock to identify novel nonfolate inhibitors. Journal of Medicinal Chemistry 47, 6681-6690.

Lindequist, U., Niedermeyer, T.H.J., and Julich, W.D. (2005). The pharmacological potential of mushrooms. Evidence-Based Complementary and Alternative Medicine 2, 285-299.

Lo, K.M., and Cheung, P.C.K. (2005). Antioxidant activity of extracts from the fruiting bodies of *Agrocybe aegerita* var. alba. Food Chemistry 89, 533-539.

Lucas, E.H., Ringler, R.L., Byerrum, R.u., Stevens, J.A., and Stock, C.C. (1957). Tumor inhibitors in *Boletus edulis* and other holobasidiomycetes. Antibiotics Chemistry 7, 1-4.

Machlin, L.J., and Bendich, A. (1987). Free-Radical Tissue-Damage - Protective Role of Antioxidant Nutrients. Faseb Journal 1, 441-445.

Maisuthisakul, P., Pasuk, S., and Ritthiruangdej, P. (2008). Relationship between antioxidant properties and chemical composition of some Thai plants. Journal of Food Composition and Analysis 21, 229-240.

Manzi, P., Gambelli, L., Marconi, S., Vivanti, V., and Pizzoferrato, L. (1999). Nutrients in edible mushrooms: an inter-species comparative study. Food Chemistry 65, 477-482.

Mattila, P., Konko, K., Eurola, M., Pihlava, J.M., Astola, J., Vahteristo, L., Hietaniemi, V., Kumpulainen, J., Valtonen, M., and Piironen, V. (2001). Contents of vitamins, mineral elements, sand some phenolic compounds in cultivated mushrooms. Journal of Agricultural and Food Chemistry 49, 2343-2348.

Mattila, P., Suonpaa, K., and Piironen, V. (2000). Functional properties of edible mushrooms. Nutrition 16, 694-696.

Mau, J.L., Chang, C.N., Huang, S.J., and Chen, C.C. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. Food Chemistry 87, 111-118.

Mau, J.L., Chao, G.R., and Wu, K.T. (2001). Antioxidant properties of methanolic extracts from several ear mushrooms. Journal of Agricultural and Food Chemistry 49, 5461-5467.

Mau, J.L., Lin, H.C., and Song, S.F. (2002). Antioxidant properties of several specialty mushrooms. Food Research International 35, 519-526.

Mendozahernandez, G., Calcagno, M., Sancheznuncio, H.R., and Diazzagoya, J.C. (1984). Dehydroepiandrosterone Is a substrate for estradiol 17 beta-dehydrogenase from human-placenta. Biochemical and Biophysical Research Communications 119, 83-87.

Miles, P.G., and Chang, S.T. (1997). Mushroom biology: concise basics and current developments World Scientific, 144.

Mizuno, T. (1993). Food function and medicinal effect of mushroom fungi. Foods and Food Ingredients Journal 158, 55-70.

Molitoris, H.P. (1994). Mushrooms in medicine. Folia Microbiologica 39, 91-98.

Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., and Olson, A.J. (1998). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. Journal of Computational Chemistry 19, 1639-1662.

Ng, L.T., Wu, S.J., Tsai, J.Y., and Lai, M.N. (2007). Antioxidant activities of cultured *Armillariella mellea*. Prikl Biokhim Mikrobiol 43, 495-500.

Numazawa, M., Oshibe, M., Yamaguchi, S., and Tachibana, M. (1996). Time-dependent inactivation of aromatase by 6-alkylandrosta-1,4-diene-3,17-diones. Effects of length and configuration of the 6-alkyl group. Journal of Medicinal Chemistry 39, 1033-1038.

Park, B.T., Na, K.H., Jung, E.C., Park, J.W., and Kim, H.H. (2009). Antifungal and anticancer activities of a protein from the mushroom *Cordyceps militaris*. Korean Journal of Physiology & Pharmacology 13, 49-54.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics, 2002. Cancer Journal for Clinicians 55, 74-108.

Pasqualini, J.R. (2004). The selective estrogen enzyme modulators in breast cancer: a review. Biochimica Et Biophysica Acta-Reviews on Cancer 1654, 123-143.

Pasqualini, J.R., and Chetrite, G.S. (2005). Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. Journal of Steroid Biochemistry and Molecular Biology 93, 221-236.

Pedretti, A., Villa, L., and Vistoli, G. (2004). VEGA - An open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming. Journal of Computer-Aided Molecular Design 18, 167-173.

Prior, R.L., Wu, X.L., and Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53, 4290-4302.

Puttaraju, N.G., Venkateshaiah, S.U., Dharmesh, S.M., Urs, S.M., and Somasundaram, R. (2006). Antioxidant activity of indigenous edible mushrooms. Journal of Agriculural and Food Chemistry 54, 9764-9772.

Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1997). Antioxidant properties of plant-derived compounds. Trends on Plant Science 2, 152-159.

Roberfroid, M.B. (2000). Concepts and strategy of functional food science: the European perspective. American Journal of Clinical Nutrition 71, 1660s-1664s.

Saiz-Urra, L., Gonzalez, M.P., and Teijeira, M. (2007). 2D-autocorrelation descriptors for predicting cytotoxicity of naphthoquinone ester derivatives against oral human epidermoid carcinoma. Bioorganic and Medicinal Chemistry 15, 3565-3571.

Sanner, M.F. (2005). A component-based software environment for visualizing large macromolecular assemblies. Structure 13, 447-462.

Sarikurkcu, C., Tepe, B., and Yamac, M. (2008). Evaluation of the antioxidant activity of four edible mushrooms from the Central Anatolia, Eskisehir - Turkey: *Lactarius deterrimus*, *Suillus collitinus*, *Boletus edulis*, *Xerocomus chrysenteron*. Bioresource Technology 99, 6651-6655.

Siro, I., Kapolna, E., Kapolna, B., and Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance-A review. Appetite 51, 456-467.

Soares, A.A., de Souza, C.G.M., Daniel, F.M., Ferrari, G.P., da Costa, S.M.G., and Peralta, R.M. (2009). Antioxidant activity and total phenolic content of Agaricus brasiliensis (*Agaricus blazei* Murril) in two stages of maturity. Food Chemistry 112, 775-781.

Song, Y.S., Kim, S.H., Sa, J.H., Jin, C., Lim, C.J., and Park, E.H. (2003). Anti-angiogenic, antioxidant and xanthine oxidase inhibition activities of the mushroom *Phellinus linteus*. Journal of Ethnopharmacology 88, 113-116.

Sousa, S.F., Fernandes, P.A., and Ramos, M.J. (2006). Protein-ligand docking: Current status and future challenges. Proteins-Structure Function and Bioinformatics 65, 15-26.

Tsai, S.Y., Huang, S.J., and Mau, J.L. (2006). Antioxidant properties of hot water extracts from *Agrocybe cylindracea*. Food Chemistry 98, 670-677.

Tsai, S.Y., Tsai, H.L., and Mau, J.L. (2007). Antioxidant properties of *Agaricus blazei*, *Agrocybe cylindracea*, and *Boletus edulis*. Lwt-Food Science and Technology 40, 1392-1402.

Turkoglu, A., Duru, M.E., Mercan, N., Kivrak, I., and Gezer, K. (2007). Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. Food Chemistry 101, 267-273.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. International Journal of Biochemistry and Cell Biology 39, 44-84.

Vaque, M., Ardrevol, A., Blade, C., Salvado, M.J., Blay, M., Fernandez-Larrea, J., Arola, L., and Pujadas, G. (2008). Protein-ligand docking: A review of recent advances and future perspectives. Current Pharmaceutical Analysis 4, 1-19.

Verdonk, M.L., Taylor, R.D., Chessari, G., and Murray, C.W. (2007). Illustration of current challenges in molecular docking: Subtitle An application of docking and virtual screening to thymidine cinase. In Jhoti, H., Leach, A.R.,editors. Structure-Based Drug Discovery. Springer Netherlands.

Wang, Y., Wang, X.W., and Cheng, Y.Y. (2006). A computational approach to botanical drug design by modeling quantitative composition-activity relationship. Chemical Biology and Drug Design 68, 166-172.

Wasser, S.P. (1999). Medicinal properties of substances occurring in higher Basidiomycete mushrooms: current perspective (review). International Journal of Medicinal Mushrooms 1, 31-62.

Wasser, S.P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Applied Microbiology and Biotechnology 60, 258-274.

Wold, S., Sjostrom, M., and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems 58, 109-130.

Yang, J.H., Lin, H.C., and Mau, J.L. (2002). Antioxidant properties of several commercial mushrooms. Food Chemistry 77, 229-235.

Zaidman, B., Yassin, M., Mahajana, J., and Wasser, S.P. (2005). Medicinal mushrooms modulators of molecular targets as cancer therapeutics. Applied Microbiology and Biotechnology 67, 453-468.

Zhang, M., Cui, S.W., Cheung, P.C.K., and Wang, Q. (2006). Polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. Trends in Food Science and Technology 18, 4-19.

Zhang, M., Huang, J., Xie, X., and Holman, C.D.J. (2009). Dietary intakes of mushrooms and green tea combine to reduce the risk of breast cancer in Chinese women. International Journal of Cancer 124, 1404-1408.

Zjawiony, J.K. (2004). Biologically active compounds from aphyllophorales (polypore) fungi. Journal of Natural Products 67, 300-310.