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Development of a functional beverage enriched with statins from natural matrices

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List of abbreviations

24-DHCR	Hydroxy-steroid-24-reductase HMGCR
7-DHC	7-hydroxysteroid
7-DHCR	3-hydroxysteroid-7-reductase
ABCA1	ATP-binding cassette subfamily A member 1
ABCG1	ATP-binding cassette subfamily G member 1
ABCG5	ATP-binding cassette subfamily G member 5
ABCG8	ATP-binding cassette subfamily G member
ACAT	Acyl coenzyme A cholesterol acyltransferase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSH	Bile Salt Hydrolyse
CE	Cholesteryl Ester
CVDs	Cardiovascular Disease
DA	Disability-adjusted life yea
EtOH	Ethanol
FDA	Food and Drug Federation
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein Cholesterol
HMG-Co	Hydroxy methylglutaryl-CoA
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
MeOH	Methanol
NADH	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NPC1L1	Niemann–Pick type C1-like 1
PCSK9	Proprotein convertase subtilisin/kexin type
SM	monooxygenase
SREBP	sterol regulatory element-binding proteins
TC	total cholesterol
UV-B	Ultraviolet light B
VLDL	Very low-density lipoprotein
VLDL-C	Very low-density lipoprotein cholesterol
SRB	Sulforhodamine-B

IG_{50}	50% of growth inhibition
BB	Box Behnking
NF-B/AP-1	Nuclear factor κB
COX-2	Cyclogenase-2
UAE	Ultrasound assisted extraction
NCI-H460	non-small cell lung cancer
MCF-7	breast carcinoma
Caco	colon carcinoma
AGS	adenocarcinoma gastric cell
SRB	Sulforhodamine B
PLP2	porcine liver primary culture
RAW 264	mouse macrophage cell
ECAAC	European Collection of Authenticated Cell Cultures
DMEM	Dulbecco's Modified Eagle Medium

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Abstract

Dyslipidemia is a frequent clinical disorder that is a major risk factor for cardiovascular disease. Therefore, the common therapeutics is the use of statins, the first-line medications for treating lipid diseases. These compounds, which aroused to control and treat hypercholesterolemia and are known to be hydroxymethylglutaryl-CoA reductase (HMG-CoA) inhibitors, are usually well-tolerated, but in some cases, they can be associated with undesirableeffects, like myopathy, rhabdomyolysis, hepatotoxicity, and diabetes.

In the last years, several natural molecules with bioactive potential have been isolated from natural matrices, which can be included hypocholesterolemic agents such as polysaccharides, sterols, and statin-like compounds.

In this work, bio residues of the highly produced mushrooms *Agaricus bisporus* and *Pleurotus ostreatus* were explored for their richness in statin-like compounds and evaluated for their hypocholesterolemic activity. For this purpose the two mushrooms were subject to a preliminary extraction of statins by ultrasound-assisted extraction (UAE) , and a HPLC-UV identification and quantification , the highest amount of statin was detected in *Pleurotus ostreatus.* Afterward an optimization of the ultrasound-assisted extraction (UAE), through the Response Surface Methodology (RSM) is made to maximize the yield in pravastatin, at the optimal points of pravastatin ,ergosterol was quantified in advantage by HPLC-UV . furthermore the most promising extract , was evaluated in term of toxicity using the Sulforhodamine B (SRB) assay, yet the extract haven't shown any toxicity to the two kind of cells , also the anti-inflammatory potential was tested . The extract was also tested for hypocholesterolemic activity in CaCo2 cells. Finally the statin like enriched extract incorporated in a milk based beverage (chocolate milkshake) where it was lyophilized and subjected to nutritional profiling .the final product have shown a crude fat content higher in the control than in the functional beverage with the extract , which probably related to the fat-lowering efficiency of the bioactive molecules present in the extract of pravastatin and ergosterol.

Keywords: Dyslipidemia, cardiovasculardisease, statins, hypocholesterolemic, *A. bisporus, P. ostreatus.*

Resumo

A dislipidemia é um distúrbio clínico frequente, representando um importante fator de risco para doenças cardiovasculares. Neste sentido, a terapêutica comum consiste no uso de estatinas, consideradas medicamentos de primeira linha para o tratamento de doenças lipídicas. Esses compostos, que surgiram para controlar e tratar a hipercolesterolemia e conhecidos por serem inibidores da hidroximetilglutaril-CoA redutase (HMG-CoA), costumam ser bem tolerados, mas em alguns casos podem estar associados a efeitos indesejáveis, como miopatia, rabdomiólise, hepatotoxicidade, diabetes, entre outros.

Nos últimos anos, diversos agentes naturais com potencial bioativo têm sido isolados de matrizes naturais, nas quais podem ser incluídos agentes hipocolesterolémicos como polissacarídeos, esteróis e compostos similares às estatinas.

Neste trabalho, os bioresíduos dos cogumelos mais produzidos *Agaricus bisporus* e *Pleurotus ostreatus* foram explorados pela sua riqueza em compostos do tipo estatina e avaliados pela sua atividade hipocolesterolémica. Para este efeito, os dois cogumelos foram submetidos a uma extração preliminar de estatinas por extração assistida por ultrassom (UAE) e a uma identificação e quantificação por HPLC-UV, sendo que a maior quantidade de estatina foi detectada em *Pleurotus ostreatus*. Em seguida foi feita uma otimização da extração assistida por ultrassom (UAE), através da Metodologia de Superfície de Resposta (RSM) para maximizar o rendimento em pravastatina, nos pontos ótimos de pravastatina, o ergosterol foi quantificado com vantagem por HPLC-UV. Além disso, o extrato mais promissor foi avaliado em termos de toxicidade usando o ensaio da Sulforhodamine B (SRB), porém o extrato não apresentou toxicidade para os dois tipos de células. Também foi testado o potencial anti-inflamatório e a atividade hipocolesterolêmica em células CaCo-2.

Por fim, o extrato enriquecido tipo estatina foi incorporado numa bebida à base de leite (*milk shake* de chocolate) onde foi liofilizado e submetidoa avaliação do perfil nutricional, relacionado-o com a eficiência hipolipodémica das moléculas bioativas presentes no extrato de pravastatina e ergosterol.

Palavras-chave: Dislipidemia, doença cardiovascular, estatinas, hipocolesterolemia, *A. bisporus, P. ostreatus*

1. Introduction

According to World Health Organization, cardiovascular diseases (CVDs) are the major cause of mortality around the world. In 2019, about 17.9 million people died from CVDs, accounting for 32% of all global fatalities. Also, 85% of these fatalities were caused by a heart attack or a stroke. Several risk factors have been reported as being strongly linked to coronary heart diseases, being some habitssuch as smoking, high blood pressure, obesity, diabetes, lack of physical activity and highblood cholesterol levels, are the major causes of these diseases (WHO, 2021).

Besides the fact that dyslipidemia was a major risk factor for CVDs, it is also linked to many other diseases such as blood-brain barrier (BBB) dysfunction, peripheral inflammation, neuro-psychomotor alterations, amnesia, neuro-inflammation, Alzheimer's disease, and cancers especially pancreatic, colon, rectal, prostatic, testicular and breast (Rodrigues *et al*., 2021; Chu *et al*., 2015; Shibuya *et al*., 2015; Ding *et al.*, 2019).

Therefore, many cholesterol-lowering drugs are usually applied in therapeutics for people with high levels of this compound in the organism, mainly statins. Despite their widespread usage, there was always a controversial opinion about these therapeutic agents, dueto their side effects, such as the development of statin-associated muscular toxicity (Ward et al., 2019).

That’s why the development of alternative treatment with less hazardous side effects is crucial, mainly natural products with bioactive compounds including the capacity to decrease cholesterol levels. In this sense, mushrooms represent an excellent source of bioactive compounds such as lovastatin, pravastatin, polysaccharides-glucans (b-glucans), mycosterols such as ergosterol and oxysterol, among others, that have demonstrated strong hypocholesterolemic potential (Le et al., 2006; Close et al.,2019; Schneider et al. 2011). All of these micronutrients will be extracted but we will be focusing on the optimization of the extraction method of the statins with ultrasound-assisted extraction (UAE), which we will be incorporated in milk-based beverages to benefice the hypocholesterolemic effects and the advantages of the milk and cacao which will serve for the final product.

1.1. Cholesterol: problematic and common therapeutics

Cholesterol was first discovered in human blood in 1833 by Félix-Henri Boudet (Olsonet *al.*, 1998). The name of cholesterol came from the words chole (bile) and stereos (solid), followed by the chemical suffix -old for alcohol. The fundamental structure of this molecule (**Figure 1**) is a sterol nucleus formed from several molecules of acetyl-CoA, and it has a hydrophobic hydrocarbon and a hydrophilic hydroxyl head group (Hall *et al.*, 2011).

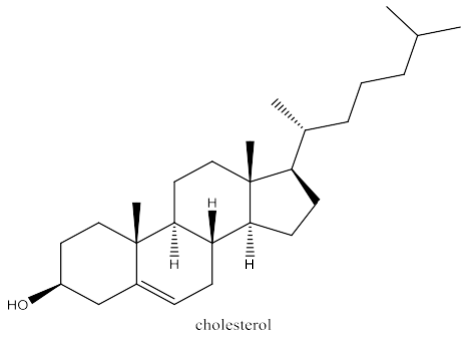

Figure 1: Chemical structure of cholesterol

Cholesterol performs various biological activities and is required for proper cellular homeostasis. It serves as a precursor to bile acids, helps in the formation of steroids such as androgens, estrogens, progesterone, glucocorticoids, mineralocorticoids, and vitamin D. Besides that, it plays an essential role in maintaining cellular membrane stiffness and fluidity and participates in the biosynthesis of bile salts (glycocholate). Thus, the lipidic cholesterol ($C_{27}H_{46}O$) has been a source of interest for scientists and physicians, for its important physiological and clinical significance; nevertheless, high cholesterol levels, have been importantly linked to many diseases (Luo *et al.*, 2020), in particular hypercholesterolemia, which defined as the high plasma cholesterol levels, mainly increasing of low-density lipoprotein (LDL) and normal plasma triglycerides. Hypercholesterolemia can be classified into two groups (Ramasamy *et al.*, 2016; Allan *et al.*, 2014):

Primary hypercholesterolemia: Familiar hypercholesterolemia including autosomal dominant hypercholesterolemia (heterozygous) and autosomal recessive hypercholesterolemia (homozygous), Polygenic hypercholesterolemia (PH) and Hyperlipoproteinemia (a).

Secondary hypercholesterolemia: Basically, caused by environmental elements like a high fat diet or lack of physical efforts or it can be caused by other diseases such ashypothyroidism, nephrotic syndrome, cholestasis, nervous anorexia, hepatoma, and various medicines such as

cyclosporine, progestogens, thiazide diuretics, and so on.

Furthermore, high cholesterol levels, engender a variety of illnesses and disorders other than cardiovascular diseases and atherosclerosis, including blood-brain barrier (BBB) dysfunction, peripheral inflammation, neuropsychomotor alterations, such as amnesia, neuro-inflammation, Alzheimer's disease, and cancers such as pancreatic, colon, rectal, prostatic, testicular and breast cancer (Rodrigues et *al.*, 2021; Chu et *al*., 2015; Shibuya et *al*., 2015; Ding et *al*., 2019). According to World Health Organization, a high amount of cholesterol level is projected to cause 2.6 million deaths (4.5 percent of all deaths) and 29.7 million DALYs (Disability-adjusted life year) (WHO, 2003).

As a result, scientists tried to develop new treatments over the years. Eight main kinds of medicines are available to decrease cholesterol levels (Feingold *et al*., 2021):

- **Statins**: HMG-CoA reductase inhibitors applied for decreasing cholesterol levels, they can reduce LDL-C levels by 60% and they represent first-line medications for treating lipid diseases.
- **Ezetimibe**: reduces LDL-C levels by around 20% by blocking cholesterol absorption in the intestines, resulting in less cholesterol delivered to the liver, and an up-regulation of hepatic LDL receptors. Usually, ezetimibe and statin are combined when the statin treatment is insufficient by itself or intolerable by the patient.
- **Proprotein convertase subtilisin/Kexin type 9 (PCSK9) inhibitors**: either monoclonal antibodies or small interfering RNA, reduce LDL-C by 50-60% via reducing PCSK9, which reduces LDL receptor degradation. PCSK9 inhibitors are extremely effective when maximally tolerated statin treatment fails to lower LDL adequately and in people who are statin resistant.
- **Bempedoicacid:** used in individuals with homozygous family hypercholesterolemia, who do not achieve their LDL-C targets with maximally tolerated statintreatment or who do not tolerate statins.
- **Mipomersen:** This compound is no longer available due to liver toxicity, it is a second-generation apolipoprotein anti-sense oligonucleotide that reduces apolipoprotein B synthesis, which engenders reducing in VLDL production.
- **Evinacumab**: a monoclonal antibody that inhibits angiopoietin-like protein3 activity,

which contributes to enhanced activity of lipoprotein lipase and endothelial cell lipaseand a reduction in LDL-C, HDL-C, and triglyceride levels.

- **Lomitapide:** inhibits an enzyme located in the endoplasmic reticulum of hepatocytes and enterocytes. This enzyme is responsible for the synthesis of very low-density lipoproteins in the liver (VLDL) it is described for patients with homozygous familiar hypercholesterolemia.
- **Bile Acid Sequestrants:** Often used in conjunction with statins, the addition of bile acid sequestrants to statin therapy results in an additional 10% to 25% reduction in LDL-C levels.

1.1.1. Synthesis and absorption of cholesterol

Almost all cells can produce cholesterol, and the liver accounts for almost half of total production in humans. Cholesterol production is a high-energy process that requires huge amounts of acetyl-CoA, ATP, oxygen, and the reducing agents NADPH and NADH. Moreover, a human adult requires around 1 g of cholesterol per day, which can be obtained by either *de novo* production (biosynthesis) or intestinal absorption (dietary source) (Repa *et al*., 2000).

Kandutsch-Russell and Bloch pathways are responsible for cholesterol production in cells. Those two mechanisms have the same basic stages, beginning with acetate and branching off at lanosterol. HMG-CoA reductase, which catalyzes the conversion of HMG-CoA into mevalonate and is a common step in both processes, is the first rate-determining enzyme in the cholesterol biosynthesis pathway. As a result, mevalonate is used in the production of both non-sterol isoprenoids and cholesterol. In the Kandutsch-Russell and Bloch routes, 7- dehydrocholesterol (7-DHC) and desmosterol are direct biosynthetic precursors of cholesterol, respectively.

The sole difference between 7-DHC and cholesterol is an additional double bondat the 7^{th} position in the sterol ring (Singh *et al*., 2007). Similarly, desmosterol possesses an additional double bond at the 24th position in the sterol's flexible alkyl side chain (Singh *et al*., 2009). In the final stage of the Kandutsch-Russell pathway, 3-hydroxysteroid-7-reductase (7-DHCR) catalyzes the conversion of 7-DHC to cholesterol. 3-Hydroxy-steroid-24-reductase (24-DHCR), on the flip side, catalyzes the conversion of desmosterol into cholesterol (the final stage in the Bloch route) by reducing instauration at the 24th position on desmosterol's flexible alkyl side chain (Singh *etal*., 2013).

To prevent toxicity caused by the excess of cholesterol, its production is controlled via two negative feedback pathways: sterol-induced degradation of HMGCR and sterol-regulated processing of sterol-regulatory element-binding proteins (SREBPs), which regulate the transcription of all cholesterogenic genes (DeBose-Boyd *et al*., 2018). The rate-limiting enzymes are monooxygenase (SM), and, in addition to the *de novo* manufacture, cholesterol delivered in the blood by low-density lipoprotein (LDL) particles can be taken up by the LDL receptor (LDLR) on the basal surface of polarized cells (such as enterocytes or hepatocytes). Free cholesterol can also be absorbed by enterocytes in the colon and hepatocytes in the liver from food sources (exogenous pathway) and bile in the biliary ducts. This absorption is mediated by Niemann–Pick type C1-like 1 (NPC1L1) and the accompanying flotillin. Surplus cholesterol is transferred to the blood via the ATP-binding cassette subfamily A member 1 (ABCA1) or the homodimer of ATP-binding cassette subfamily G member 1 (ABCG1), or to the intestinal lumen and bile ducts via the ABCG5 and ABCG8 heterodimer (**Figure 2**) (Luo *et al*., 2020).

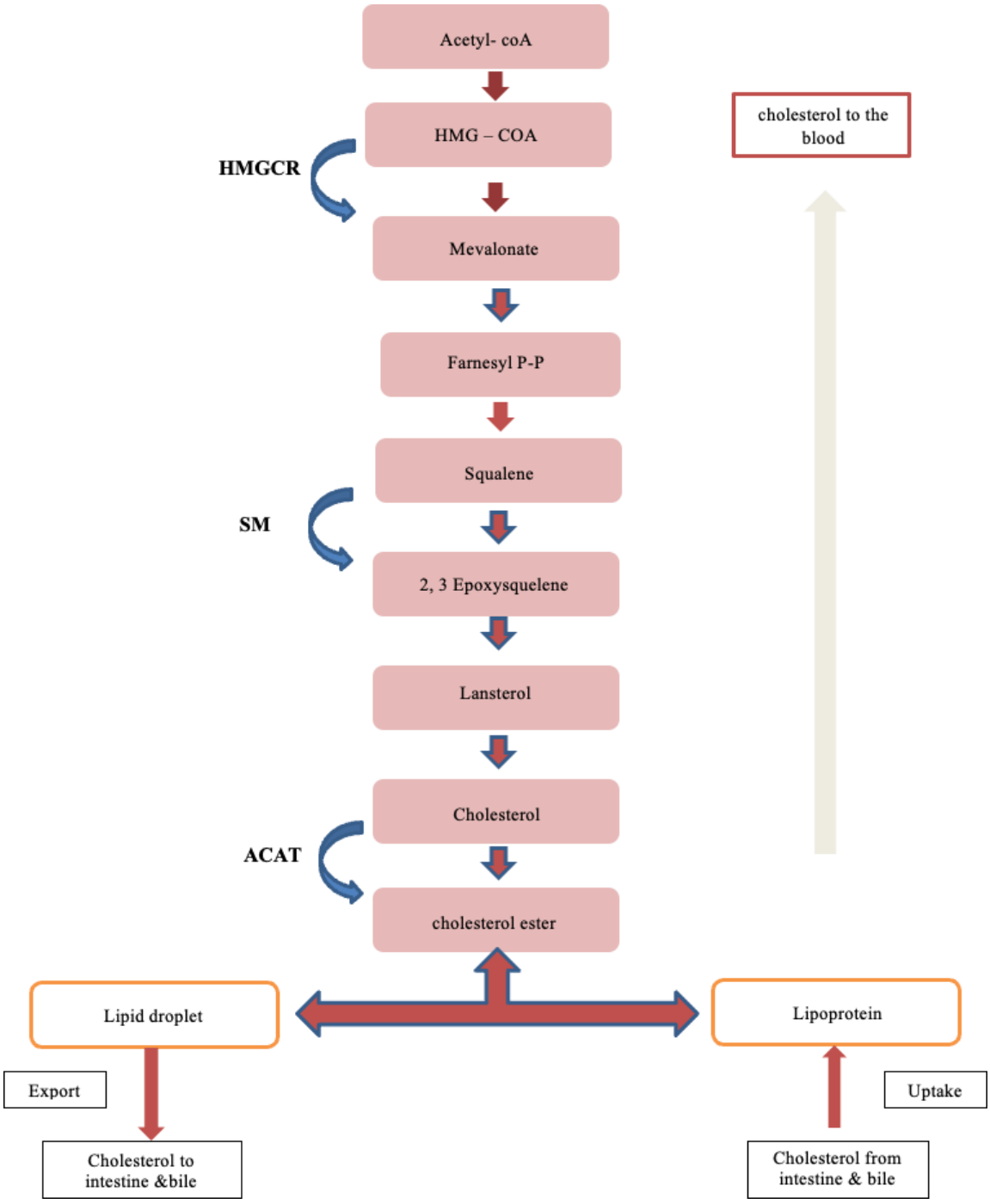

Figure 2: Mechanism of synthesis and absorption of cholesterol (Luo *et al*., 2020).

1.2. Statins

1.2.1. Definition

Statins are fungal-derived compounds that inhibit the hydroxymethyl glutaryl-CoA (HMG- CoA) reductase enzyme. This chemical is a strong cholesterol-lowering drug that works by blocking a crucial step in the sterol biosynthesis pathway. They have made significant advances in the prevention of cardiovascular disease. Their discovery in mycetes dates back over 40 years, and there were initially widely divergent opinions on their therapeutic potential. Since then, extensive pharmaceutical development has resulted in the clinical availability of seven statin compounds, each with unique bioavailability, lipo/hydrophilicity, cytochrome P-450 mediated metabolism, and cellular transport pathways. These distinctions are reflected in theirrelative potency (mg LDL-cholesterol decrease per mg dosage) and, presumably, in parenchymal or muscle toxicity (Sirtori *et al*., 2014).

Most of the statins in the market are generic medications (**Figure 3**) and are quite affordable. The kind of statin used, may be determined by the degree of cholesterol reduction required as well as the possibility of drug-drug interactions. Those cholesterol-lowering drugs are the first-line medications for treating lipid problems, making them one of the most extensively used pharmacological classes. Statins have transformed the area of preventive cardiology and contributed significantly to the decrease in atherosclerotic cardiovascular events (Feingold *et al*., 2021). Only lovastatin and simvastatin are lactone metabolites obtained from fungal fermentation. Fluvastatin, atorvastatin, rosuvastatin and pitavastatin are chemically synthesized compounds (Sirtori *et al*., 2014).

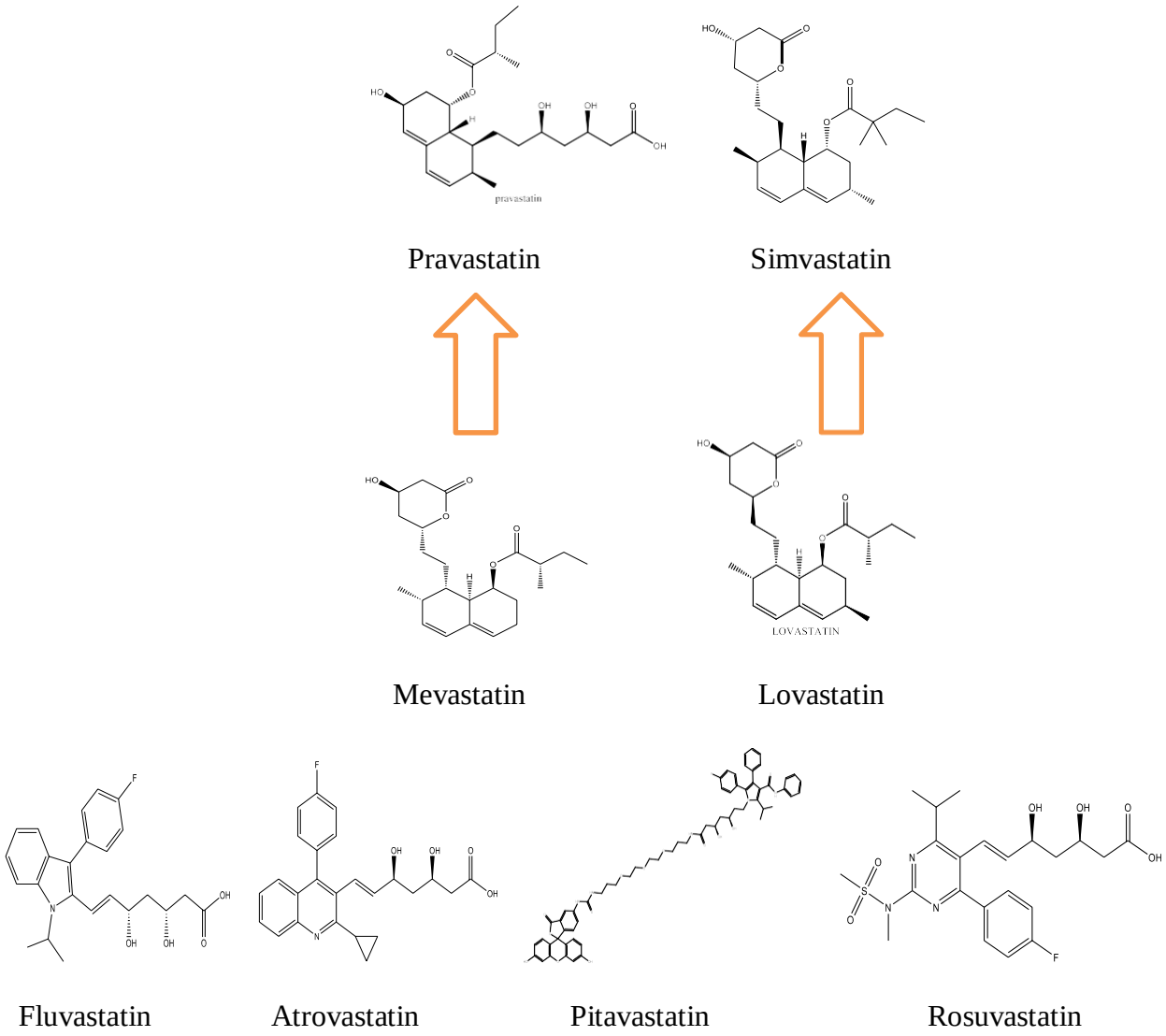

Figure 3: Chemical structures of the major statins presently available for human treatment.

1.2.2. Mechanism of action

Statins are selective, competitive inhibitors of the enzyme hydroxymethyl glutaryl-CoA (HMG-CoA) reductase, which converts HMG-CoA to mevalonate in the cholesterol production pathway. When hepatic cholesterol production is reduced, LDL receptors are upregulated, and hepatic absorption of LDL cholesterol from the circulation increases. (Sizar *et al.*, 2021). Statins do more than only compete with the typical substrate at the active region of the enzyme. When they attach to the enzyme's active site, they change its conformation. This prohibits HMG-CoA reductase from forming a functional structure. Because of the change in conformation at the active site, these medicines are extremely effective and targeted. Statins reversibly bind to HMG-CoA reductase, and their affinity for the enzyme is nanomolar, as opposed tothe natural substrate, which has a micromolar affinity (Corsini *et al.*, 1999).

Moreover, those drugs have been shown also to lower circulating LDL-C levels by up to 55% (Ward *et al*., 2019). Statins have demonstrated several non-lipid-lowering benefits, such as improved endothelial function, atherosclerotic plaque stabilization, and anti-inflammatory and immunomodulatory effects, due to the suppression of isoprenoid intermediates formation in the mevalonate pathway (Abdelmasih *et al*., 2021).

1.2.3. Effect and associated issues

Statin and myopathy-related symptoms are a common adverse effect of the medicine, with symptoms ranging from moderate myotoxicity to deadly rhabdomyolysis, and it can occur withor without an elevation in creatine kinase levels. Although statin-induced diplopia, and ptosis, are rare cases they have been documented as side effects on a few occasions. Myalgia is the most prevalent side effect of statin treatment, with reported rates ranging from 1 to 10%. Rhabdomyolysis is the most significant side effect of statin therapy, yet it happens less seldom (less than 0.1%) (Abdelmasih *et al.*, 2021).

Hypothyroidism, polypharmacy, and alcohol consumption are the most frequent risk factors for statin-related myopathy. Anomalies in liver function tests are prevalent, affecting up to 1% of individuals. However, the clinical relevance of this is uncertain. Some statin medicines have the potential to cause diabetes, and the risk appears to grow with increasing dosages. On the other hand, statins have several medication interactions, the majority of which involve the cytochrome p450 enzyme group (Ramkumar *et al*., 2016). Some other side effects are based on FDA reports, linked to the use of the statin, including unusual fatigue, weakness, lack of appetite, upper stomach discomfort, dark urine, yellowing of the skin, whites of the eyes, memory loss and confusion (FDA, 2016).

1.3. Mushrooms: source of alternative molecules

1.3.1. *Agaricus bisporus* and *Pleurotus ostreatus*

Agaricus bisporus L, commonly known as the button mushroom, belongs to the saprotrophic species. It is a Basidiomycota edible fungus that is one of the most popular mushrooms consumed globally, native to Europe and North America's grasslands. *A. bisporus* is rich in dietary fibers,polysaccharide cell walls, beta-glucans, homoglucans, heteroglucans, the minerals selenium, copper, potassium, and also vitamins, chitin, among others, all of which have

antioxidant and anticancer properties and are useful in the treatment of cardiovascular diseases (Xu *et al*., 2016; N *et al*., 2013; Kalaras *et al*., 2017; Conor Francis *et al*., 2017).

While *Pleurotus ostreatus* L. is a kind of wood-rotting mushroom (Cherno *et al*., 2013), it may grow on agri-food waste and might be utilized as an element in food fortification (Schneidera *et al*., 2011). According to the most recent research, *P. ostreatus* is a rich source of an active glucan, known as pleurant, which has a variety of health advantages (Close *et al*., 2020; Silveira *et al*., 2015; Close *et al*., 2019). These polysaccharides are also immunomodulatory agents, and have potential use in the therapy of cancer, infections, and immune system diseases (Close *et al.*, 2019). The chemical composition of both mushrooms is presented in **Table 1**.

Table 1 : Chemical composition of mushrooms morphological parts (% of dry weight) (Cherno et *al*., 2013)

Components	***A. bisporus***		***P. ostreatus***	
	Cap	**Stem**	**Cap**	**Stem**
Mono and oligo saccharides	1.2	1.4	16	11
Mannitol	30.4	28.5	7.9	5.2
Polysaccharides easily hydrolysable	13.7	21.9	25	27
Polysaccharides hardly hydrolysable	7.2	9.2	8.3	24
Polysaccharides including chitin	4	7.9	2.6	2.9
Total nitrogen	5.3	4.7	4.8	2.9
Non protein including nitrogen	2	1.7	1.9	1.1
Protein	18.9	15.3	17	10
Lipids	4	1.8	2.5	2
Phenolic compounds	8.3	4	4.9	3.2
Mineral elements	9	7.6	7.9	6.7

1.3.2. Statin-like compounds found in mushrooms

A research study by the Institute of Food Science and Human Nutrition of Leibniz University (2011) on a total of 20 subjects (9 male, 11 female; 20–34 years) were randomly assigned to either one dish of soup containing 30 g of dried oyster mushrooms or one part of a soup or a tomato soup as a placebo daily for 21 days. This study suggested that oyster mushrooms may improve blood lipid profile and lead to cardiovascular prevention (Schneider *et al*., 2011). In another study in China where mushroom's lovastatin was measured among fruiting bodies, *P. ostreatus* and *A. bisporus* had the most lovastatin contents (606.5 and 565.4 mg/kg, respectively), but among mycelia, *P. ostreatus* contained the lowest amount (606.5 mg/kg) (Shin-Yu *et al*., 2012). Lovastatin has been discovered in the fruiting bodies and mycelial cultures of edible mushrooms, particularly *Pleurotus* spp. (Alarcon *et al*., 2003; Lee *et al*., 2006). Lindequist, Niedermeyer and Jülich (2005) discovered that *P. ostreatus* had a hypocholesterolemic impact as well as the ability to reduce lipid peroxidation in rats and rabbits. Furthermore, an oyster mushroom diet containing 10% dry fruiting bodies significantly decreased the development and extent of atherosclerotic plaques in rabbits. Lovastatin with HMG-CoA reductase activity was found in this mushroom, and this may be the main component responsible for the reported effects.

1.3.3. Glucan in mushrooms

Glucans are unbranched linear polysaccharides found in a variety of natural sources, including mushrooms. They can interact with innate immune receptors due to their structure, but they also operate as dietary fibers in the digestive tract. Because glucans come in two varieties, insoluble and soluble, they can interact with lipids and bile salts in the colon, lowering cholesterol levels. As a result, because they are natural compounds with no substantial adverse effects, they may be developed as a feasible therapeutic alternative for treating individuals with dyslipidemia (Sima *et al*., 2018). This molecule has proven its ability to lower blood total cholesterol and low-density lipoprotein (LDL), improving high-density lipoprotein (HDL) cholesterol and blood lipid profiles (Daou *et al*., 2012).

Mushroom cell walls show an abundant amount of polysaccharides (glucans) which represent key elements of fungal cell walls. Their health-related effects have been explored in cellular and experimental models in recent years, to add scientifically based information to the long-lasting anecdotal therapeutic effects and to elucidate their processes. The immunomodulatory, anti-inflammatory and enhancement of the cardiovascular system by improving glucose, lipid metabolism, and blood pressure are effects that have been shown for oat and barley-glucans, which need to be confirmed in human trials with mushroom glucans (Cerletti *et al*., 2021).

1.3.4 Sterols in mushrooms

Sterols are a kind of lipidic molecule that includes cholesterol, oxysterols, and sterol esters. They serve as precursors to produce bile acids, steroid hormones, and vitamin D. They regulate gene expression in lipid metabolism and play a role in holding and storing cholesterol. Sterol esters serve a critical function in sterol homeostasis by packing into lipid droplets for cellular storage. Oxysterol esters are a kind of sterol ester, known to interact with a variety of enzymes, and disturbances in their interaction pathways have been linked to a variety of illnesses, including atherosclerosis (West *et al.*, 2021).

The most prevalent mycosterol in *A. bisporus* is ergosterol, which is a molecule with considerable commercial potential besides being equally efficient as some phytosterols such as sitosterol, in the displacement of cholesterol, when applied to the food matrix (Alicia *et al.*, 2014). Fruiting bodies of the oyster mushroom *P. ostreatus* exposed to UV-B light transformed the sterol in the onset of ergocalciferol (vitamin D_2) and the formation of this vitamin was immediate in fruiting bodies illuminated from the lamella side, in sliced fruiting bodies, and the stipes. Saturation concentrations above 100 μg/g of dry matter were reached after 1h (Krings *et al.*, 2014).

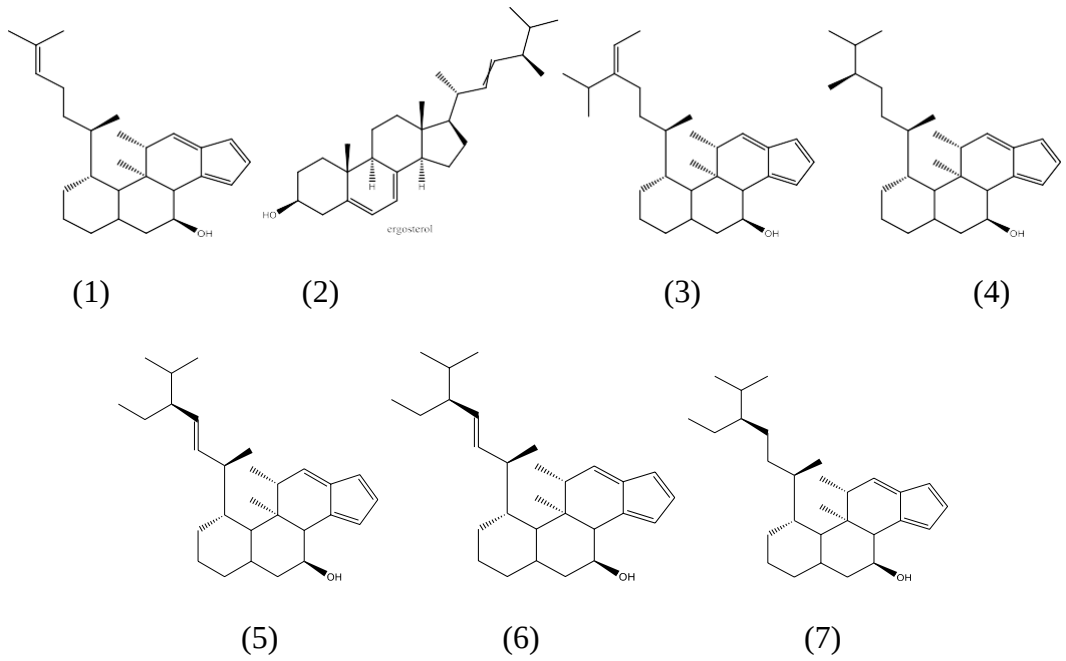

Figure 4: Chemical structures of sterols: desmosterol (1), ergosterol (2), fucosterol (3), campesterol (4), stigmasterol (5), and b-sitosterol (6)

1.4. Most applied extraction methods

The initial stage in separating desired bio compounds from basic materials is the extraction procedure (Zhang *et al*, 2018). The chemical properties of the target molecules are a crucial factor in determining the best extraction procedure. Polarity, molecular structure, and the number of hydroxyl groups are all important considerations (Pagano *et al*., 2021). The extraction methods are divided into two categories: the conventional methods or also called the classical procedures, the majority of these techniques rely on the extraction ability of different solvents in use, as well as the use of heat and/or mixing, moreover the choice of solvent is critical for this kind of extraction. When choosing a solvent, selectivity, solubility, cost, and safety should all be considered. According to the rule of resemblance and intermiscibility solvents with polarities close to the polarity of the solute will likely function better, and vice versa. Alcohols (EtOH and MeOH) are common solvents used in solvent extraction for phytochemical research. The most known conventional procedures are:

- **Maceration:** a conventional method of extraction of bioactive compounds that consists in stirring the samples for some time with the respective solvent;
- **Percolation:** This is a continuous process, in which the saturated solvent is continually replenished by a new solvent; it is more efficient than maceration;
- **Soxhlet extraction:** It employs the principles of reflux and siphoning to extract the herb with new solvent on a constant basis, with high-efficiency automated, continuous extraction technology, that takes less time and solvent than maceration or percolation;
- **Hydrodistillation:** Hydro distillation (HD) and steam distillation (SD) are two extensively utilized extraction processes for example to obtain essential oils.

  The second category of extraction methods is the non-conventional extraction techniques (Zhang *et al.*, 2018; Azmir *et al.*, 2013; Sania *et al.*,2020). They are defined as green techniques because they adhere to green chemistry objectives and the most often utilized are:

- **Supercritical fluid extraction (SFE):** The extraction solvent in supercritical fluid extraction (SFE) is supercritical fluid (SF). SF has a liquid-like solubility and a gas-like diffusivity, and it can dissolve a wide range of natural compounds;
- **Pressurized liquid extraction (PLE):** In the extraction process, PLE employs high pressure to extract bioactive compounds;

- **Ultrasound-assisted extraction (UAE):** Ultrasonic-assisted extraction (UAE), often known as ultrasonic extraction or sonication, uses ultrasonic wave energy;
- **Microwave-assisted extraction (MAE**): Microwaves create heat by interacting with polar chemicals in the plant matrix, such as water and certain organic components, via the ionicconduction and dipole rotation processes.
- **Pulsed electric field-assisted extraction:** By disrupting membrane structures, it improves mass transfer during extraction.

1.4.1. Main advantages of conventional and non-conventional methods

The conventional protocols of extraction, named also solvent extraction techniques, are still commonly utilized by industry, and researchers obtain large yields of chemicals while using very simple and low-cost equipment (Garcia *et al*., 2020). Besides the simplicity and the low cost of those techniques, they have been showing several advantages such as the capability of maceration to extract thermolabile constituents and the excellent extraction efficiency of the Soxhlet method which requires less time and solvent than maceration or percolation (Zhang *et al*., 2018).

On the other hand, the potential of the non-conventional approaches comes down with quick extraction periods and low amounts of toxic organic solvents. In addition, they are simple to implement and consume less energy and water; that's why they are called eco-friendly (Khoddami *et al*., 2013). For instance, PLE employs high pressure which preserves solvents in a liquid condition beyond their boiling point, resulting in high lipid solute solubility and diffusion rate in the solvent, as well as high solvent penetration in the matrix. When compared to other conventional techniques, PLE significantly reduced extraction time and solvent usage while improving reproducibility. Also, in MAE, heat and mass transfers occur in the same direction, resulting in a synergistic effect that speeds up extraction and improves extraction yield, as well as in the UAE (Zhang *et al*., 2018).

1.4.2. Main bottlenecks of conventional and non-conventional methods

The limitations of the conventional extraction procedures rely on the fact that they are time-consuming, labour-intensive, need huge volumes of water, and use toxic solvents sometimes, which may result in some target molecules degradation and a partial loss of volatilesforgetting to

mention the low extraction efficiency (Cravotto *et al*., 2011). However, the challenge of the non-conventional extraction methods is mainly due to the high operation costs, the complicated principles of use and the lack of efficiency for some compounds (Chemat *et al*., 2017). As an example, for ultrasound-assisted extraction (UAE), the active zone and distribution of ultrasonic power are difficulties that should be considered in the pharmaceutical sector (Alissandrakis *et al*., 2003), and for microwave assisted extraction (MAE), the high temperature needed of this method, may limit its use in the extraction of thermolabile chemicals, such as those present in bioactive materials and/or pharmaceutically relevant substances (Nyamayaro *et al*., 2020).

1.5. Extraction optimization through Response Surface Methodology (RSM)

Response Surface Methodology is a method that uses intricate calculations during the optimization process. This method creates an appropriate experimental plan that uses the data input from the experiment and integrates all the independent factors to arrive at a conclusion. Come up with a system of equations that can provide an output's theoretical value. The results are derived from a regression analysis with a good design that is based on the managed values of the independent variable (Mohamad *et al*., 2015). The initial research in this field, which was done in the 1950s, has been heavily utilized, particularly in the chemical and process sectors. RSM has been widely used for the past 15 years, and numerous significant breakthroughs have occurred (Myers *et al*., 2004).

The laboratory test step is made more effective by using the RSM approach in the optimization process, which reduces the amount of time needed to test the factors related to customer evaluation (Said *et al*., 2015). Additionally, parameter estimates can assist researchers to focus on the specific variables that directly affect the model's ability to predict consumer acceptance of a product (Schutz *et al*., 1983).

Two significant optimization techniques are used: BoxBehnken and central composite design. Additionally, each of these methodologies offers a distinct design of experiment that addresses various ways of data analysis (Mohamad *et al*., 2015).

1.5.1. Optimization using Central Composite Design (CCD)

One of the strategies used in the response surface methodology for designing the experimental protocols is Central Composite Design (CCD). A wide range of factors and the function of each

factor can be screened as part of optimization using CCD (Sahin *et al*., 2011). A single variable or the combined influence of the variables on the response can likewise be evaluated via CCD. Even while this ability is shared with other experimental design types like the complete factorial technique and partial factorial method, it differs in that the number of experimental runs is condensed. For instance, the full factorial technique will recommend at least 81 experimental trials plus replication when there are just four independent variables (Box *et al*., 1992).

All the variables must be coded according to the following equation:

$$\boldsymbol{xi} = (\mathbf{Xi} - \mathbf{X\,i})/\Delta\mathbf{Xi}$$

where xi is the coded level; Xi is the natural level for the independent variable; $\bar{X}i$ is the mean for the natural level of the independent variables; and ΔXi is the step change value (Mohamad *et al*., 2015).

1.5.2. Optimization using BoxBehnken (BB)

Another approach in the response surface methodology is called Box-Behnken (BB), and its goal is to identify the factors that will result in the best possible response or output. BB is a design without an embedded factorial or fractional factorial point that might be used to identify the variable condition at the midway edges as well as the centre of the variable's space (Ghorbani *et al*., 2013). The proportion of Box Behnken method experiments is calculated following this equation:

$$N = \mathbf{2}k(k - \mathbf{1}) + C\mathbf{0}$$

where k is the factorial number; $C0$ is the replicate number of the central point (Box *et al*., 1978; Ferreira *et al*., 2008; Mongomery *et al*., 2017).

1.6. Functional beverages

Modern life is not complete without functional beverages, which also improve nutritional health. There has been a surge in current efforts to comprehend and create functional beverages to support health and wellness. A detailed understanding is necessary to extract bioactive elements from food and incorporate them into beverages. Functional meals are suitable for this since they have fewer, or no side effects compared to the existing medication therapies. A complementary strategy for the prevention and treatment of various systemic disorders, functional dietary therapies are customized for each person's preference for increasing energy, losing weight, and improving

mental clarity (Yilmaz *et al*., 2019).

Additionally, it is feasible to add beneficial nutrients and bioactive substances to functional drinks, including vitamins, minerals, dietary fibers, prebiotics, proteins, peptides, unsaturated fatty acids, and antioxidants. As a result, there are now several novel beverages on the market that are intended to address health issues. Functional drinks come in a variety of forms, including dairy-based drinks, probiotic drinks, energy drinks, sports drinks, meal replacements, caffeinated drinks, and vegetable and fruit beverages. Besides their fundamental nutritional benefits, these functional drinks have positive effects on one or more bodily systems (Yilmaz *et al*., 2019).

Depending on their ingredients and manufacturing processes, functional beverages can lower the chance of developing cancer, strengthen the immune system, enhance physical and mental health, and possess anti-stress, anti-ageing, antioxidant, and anti-inflammatory effects. With the most recent advancements in the area, functional beverage classification is based most of all on fermented, dairy-, nondairy-, fruit-, and herbal-based functional beverages. Besides that, the functional food industry's largest and fastest-growing segment is the consumer demand for and market for this kind of product. Due to its crucial role in illness prevention and health promotion as mentioned before, the creation of functional beverages has attracted a lot of attention in recent years.

They create a great delivery system for different macro- and micronutrients, as well as bioactive substances like probiotics and prebiotics (Tolun *et al*., 2019).

As Li *et al.* (2016) reported, the market for functional beverages was worth over US$1347 billion in 2017. Rapid urbanization, growth of the middle class, rise in dual-income households, rising health concerns, and significant contributions to disease prevention and health promotion are all factors that have contributed to the enormous growth in the production and consumption of functional beverages.

1.6.1 . Milk-based products as a functional beverage

Dairy-based functional beverages are a growing segment in the sector of functional foods. Milk is an essential part of the daily diet of people all around the globe. Functional dairy-based beverages are exceedingly consumed by people of all age groups. The milk-based beverages include milk fortified with probiotics, prebiotics, phytosterols, antioxidant, bioactive peptides from milk, dietary fiber, minerals, vitamins, and colostral immunoglobulins, The above-mentioned bioactive components have been found to exert a profound effect on the overall health of the individual (Ozer *et al.*, 2010).

According to market trends, milk-based beverages are ideal vehicles for bioactive food ingredients that target lifestyle diseases. The number of dairy products with a "low" claim has skyrocketed in recent years. Different dairy beverage formulations tailored to different consumers, such as reduced sugar, reduced fat, mineral fortified beverages, probiotic milk, and whey beverages, are now available on the market. Functional milk and dairy beverages have been identified as ideal vehicles for the delivery of potential bioactive components such as "probiotics," which are live microorganisms that aid in the maintenance of good health via the intestine. Fermented milk beverages are the most common and preferred vehicle for probiotic delivery. Other trends in the present and future market of functional dairy beverages include the incorporation of omega-3 fatty acids, phytosterols, milk bioactive peptides, antioxidants, and dietary fiber. Even though milk is regarded as an excellent vehicle for the delivery of bioactive substances. However, when it comes to incorporating bioactive ingredients into dairy beverages, manufacturers face several challenges (Mudgil *et al*.,2019).

2. Objectives

The main objective of this work was the development of a functional formulation with statins-like compounds and ergosterol obtained from mushrooms, natural agents with hypocholesterolemic potential.

The specific objectives of this work are:

1. Extraction of statins from mushroom residues (*A. bisporus*, *P. ostreatus*), by conventional and emerging techniques such as maceration and ultrasound-assisted extraction.
2. Optimization of the statin with ultrasound-assisted extraction and Response Surface Methodologies.
3. Identification of statins by HPLC techniques.
4. Evaluation of the hypocholesterolemic activity of extracts obtained using the Caco2 cell line.
5. Evaluation of toxicity using normal cells by the sulforhodamine B method.
6. Incorporation of statins-like enriched extracts in a milk-based drink.
7. Evaluation of the nutritional parameters of the developed formulation.

3. Material and Methods

3.1. Mushroom samples

A. bisporus and *P. ostreatus* samples were provided by a local mushroom company named "Mogaricus-Sociedade Unipessoal Lda," and they were made up of bio-residues that had been discarded, specifically mushrooms that did not meet marketing standards (samples with broken shapes and misshapen physiognomy). The mushrooms were then frozen and lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). Finally, the samples were grinded with a coffee grind machine to obtain fine powder (**Figure 5**).

Figure 5: Mushroom´s preparation.

3.2. Extraction procedures

The statin extract was obtained by an ultrasonic system, according to Kala *et al*. (2020). Briefly, 2 g of lyophilized mushroom powder were extracted with 100 mL of methanol in the UAE (ultrasonic homogenizer 'CY-500') system for 20 min at a power of 375 W. The extracts obtained were filtered through a paper filter (Whatman nº 4) and the obtained filtrate was evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) with 40 °C temperature for 20 min. Afterwards, the volume of solvent (acetonitrile) added was calculated so that it was injected in the HPLC for quantitative and qualitative identification of statins. These standard parameters were used to do the optimization process through RSM (**Figure 6**).

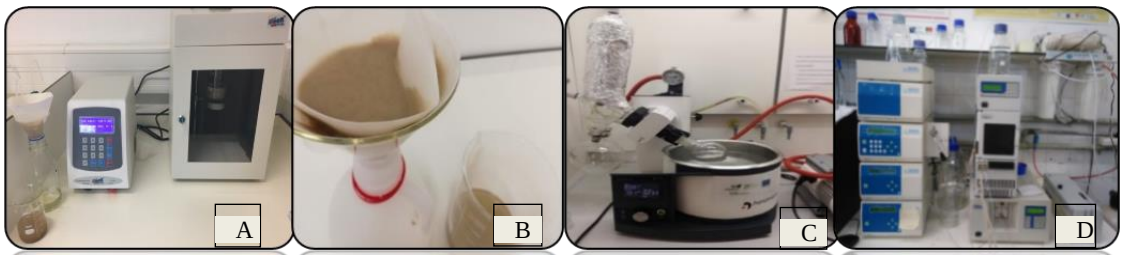

Figure 6: Extraction procedure ; **(A)** UAE, **(B)** filtration of the extract, **(C)** evaporation of the solvent, **(D)** HPLC identification

3.3. Statins profile

The statins were determined by HPLC coupled with a UV-Vis detector (HPLC-UV) (**Figure 7**) (Knauers, Smartline System 1000, Berlin, Germany) at 238 nm.

The obtained extracts were saluted in a solution containing acetonitrile/deionized water (70:30 *v/v*) to obtain a concentration of 10 mg/mL. After this process, the samples were filtered with 0.22 µm nylon filters and injected on the HPLC-UV.

For the determination of statins, a XBridge C18 column (4.6 x 250 mm, 5 µm, Waters) was used. The mobile phase used was acetonitrile/deionized water (70:30 *v/v*) operating at 40 ºC with a flow rate of 1 mL/min (oven 7971 R Grace, Berlin, Germany).

The identification and quantification of the statins (lovastatin and pravastatin) were performed using the retention times of commercial standards. The data were analyzed using Clarity 2.4 software (DataApex, Prague, Czech Republic). Finally, the results were expressed in g/100 g of dry weight (dw).

Figure 7: HPLC-UV

3.4. Optimization of the UAE using Response Surface Methodology (RSM)

The Design-Expert v.11 software was used to optimize the extraction parameters of the UAE from the two mushrooms using the Box-Benken design (Stat-Ease, Minneapolis, MN, USA).

A three-factor design was used, being the factors solid/liquid ratio (A), ultrasounic power (B), and time (C) ; Pravastatin yield (R), the most important response. For this, 17 runs of the varying conditions of the factors were used, in which their variation was as follows:

- A: Solid-liquid ratio S/L (5 - 30 g/L);
- B: The ultrasound's power (15 - 80%);

- C: Time (2 - 30min).

Before extraction, the mushroom´s material (*A. bisporus* and *P. ostreatus)* was powdered. Afterwards, 17 different samples were obtained with an ultrasonic homogenizer 'CY-500', under different extraction conditions. Then, after having the optimal predicted extraction conditions, samples extracted using these extraction values were prepared to validate the predictive mathematical model (**Figure 8)**.

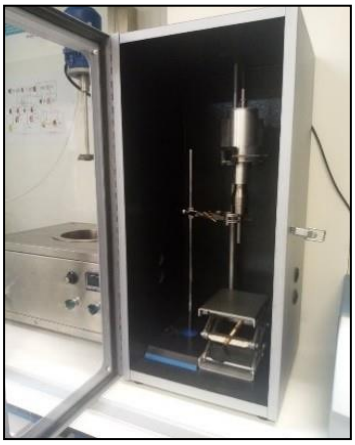

Figure 8: Ultrasound equipment used in the extractions

3.5. Ergosterol profile

As ergosterol is also an important molecule in terms of hypocolesterolemic effects, the extract enriched in statins (the one selected as being the richest one in this molecule, obtained under the conditions established by th RSM), was also analyzed for the presence of ergosterol.

Ergosterol were determined by HPLC coupled with a UV-Vis detector (HPLC-UV) (Knauer, Smartline System 1000, Berlin, Germany) set at 280 nm of wavelength. To the extract was added methanol until obtaining a concentration of 10 mg/mL. After this process, the samples were filtered with 0.22 µm nylon filters and injected on the HPLC-UV.

For the determination of ergosterol, a XBridge C18 column (4.6 x 250 mm, 5 µm, Waters) was used. The mobile phase used was acetonitrile/methanol (70:30 v/v) at 40 ºC with a flow rate of 1 mL/min (oven 7971 R Grace, Berlin, Germany). The identification and quantification of the ergosterol were performed using the retention times of commercial standards. The data were analyzed using Clarity 2.4 software (DataApex, Prague, Czech Republic). Finally, the results were expressed in g/100 g of dried weight (dw).

3.6. Bioactive Properties

3.6.1 Cytotoxicity

The Sulforhodamine B (SRB) assay was used to determine the cytotoxicity of the various extracts on human tumor cell lines and a non-tumor cell line. DSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen provided NCI-H460 (non-small cell lung cancer), MCF-7 (breast carcinoma), Caco (colon carcinoma), and AGS (adenocarcinoma gastric cell). According to an existing protocol, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse for hepatotoxicity testing, and it was designated as PLP2 (porcine liver primary culture). Briefly, both cell lines were routinely maintained as adherent cell cultures in RPMI-1640 containing heat-inactivated FBS (10%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) and were further incubated at 37 °C with humidified air and 5% CO_2.

To evaluate the cytotoxicity, the cell lines were plated in 96-well microplates (10000 cells/well), along with the different dilutions of the sample under analysis (6.25-500 µg/mL) and incubated for 72 hours at 37 ºC with 5% CO_2. After the incubation period, the adherent cells were fixed by the addition of 10% trichloroacetic acid previously refrigerated (100 µL) and incubated for 60 minutes at 4 °C. Afterwards, the microplates were washed with deionized water and dried. After this process, SRB (0.1% in 1% acetic acid, 100µL) was then added to the wells of the microplate and incubated for 30 minutes at room temperature. Subsequently, non-adhered SRB was removed by washing with 1% acetic acid solution and the plate was allowed to dry. The adhered SRB was solubilized with 10 mM Tris (200 µL) and the absorbance was read at a wavelength of 540 nm in the microplate reader (ELX800). The results were expressed as GI_{50} values (concentration of the sample, which inhibits 50% of cell growth). Ellipticine was used as a positive control (**Figure 9**).

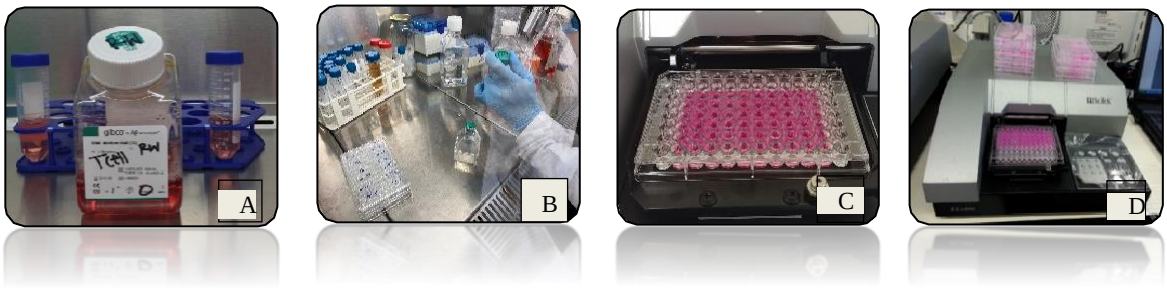

Figure 9: Cytoxicity assay;(**A)**Cell suspension, **(B)** Cell counting, **(C)** adding sample in cells**, (D)** absorbance at 540 m

3.6.2. Anti-inflammatory activity

RAW 264 was created using the procedure described by Taofiq et *al*. Anti-inflammatory activity was measured in 7 mouse macrophages. Cell cultures were collected in the European Collection

of Authenticated Cell Cultures (ECAAC) and grown in DMEM medium supplemented with 10% heat-inactivated bovine serum and L-glutamine at 37°C in humidified air with 5% CO_2 at 37°C. Cells with successful growth were scraped and balanced to an experimental density of $5x10^5$ cells/mL, with a dead cell ratio of less than 5%, using the Trypan Blue exclusion method. Cells were spread (300 L/well) into 96-well microplates and incubated for 24 hours at 37° C and 5% CO_2 to adhere and multiply. After that, they were incubated for 1 hour to determine the 50% output (EC_{50}, g/ml) with various extract solutions at final concentrations ranging from 400 to 1,56 µg/mL.. Lipopolysaccharides (LPS, 1 g/mL, 30 L) were given to them for 18 hours. Negative controls were created without the addition of LPS to see if they could induce changes in baseline nitric oxide levels (NO). As a positive control, 50 mM dexamethasone was used. To detect the presence of nitric oxide, a Griess Reagent Kit (Promega) containing sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride (NED), and nitrated solutions is used. The supernatant cell solution (100 L) was transferred to a microplate, along with the sulfanilamide and NED solutions, and mixed for 5 to 10 minutes each at room temperature. In a 96-well microplate, a reference curve for $NaNO_2$ (100 M at 1.6 M, $y = 0.0066x + 0.1349$; $R_2 = 0.9986$) was prepared.

By comparing the absorbance at 540 nm in an ELX800 Biotek microplate reader to the calibration curve, the amount of nitric oxide released was calculated. Finally, the extract concentration required to reduce NO output by 50% (EC_{50}, g/ml) was determined.

3.6.3 Evaluation of the hypocholesterolemic activity of the extracts obtained using the Caco-2 cell line

The Caco-2 cell line was maintained in RPMI-1640, without FBS, glutamine (2mM), penicillin (100U/ml) and streptomycin (100µg / ml), and were incubated with humidified air and 5% CO_2 at 37°C. The cell lines were then placed on 44 cm^2 insert membrane at a density of $5*10^5$ cells per insert, for a pore diameter of 0.4 µm. The culture medium was replaced every 3 days, and before further experimental procedures, cells were allowed to differentiate for 21 days, Measuring transepithelial electrical resistance was measured by evaluating the integrity of the cell layer. Only inserts above 400 Ω have been used. The mushroom extracts were applied to monolayers of Caco-2 cells at the upper compartment concentration of incomplete medium (medium without added cholesterol) in 975 µl. The microplate was incubated then with 5% CO_2 for 1h at 37 °C.

For the quantification of cholesterol, the upper solution as well as the solution underneath the cell monolayer were therefore collected to determine the cholesterol content absorbed by the Caco-2 cells and the extracts. The microplate was then incubated with 5% CO_2 at 37°C for one hour. Therefore, samples of the top solution and the solution below the cell monolayer were taken to

quantify the amount of cholesterol absorbed by the Caco-2 cells (**Figure 10**).

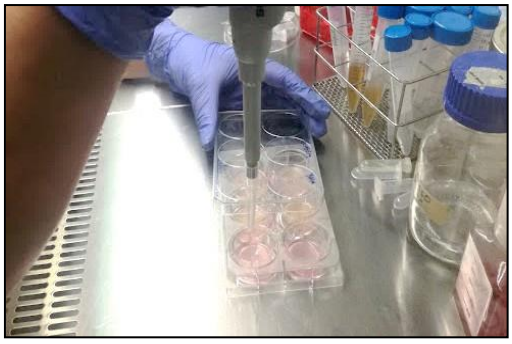

Figure 10: Addition of samples in Caco-2 cells for cholesterol absorption.

3.8. Cholesterol profile

To proceed with the cholesterol assessment, 1.5 mL of the sample (cholesterol + cell medium) was added with 1.5 mL of ethanol and 2.5 mL of hexane and centrifuged (500 g, 10 min). The supernatant phase was obtained and repeated the method. The hexane phase obtained after centrifugation was evaporated to dryness under nitrogen, and the dried residue was dissolved in 500 µL of methanol for further HPLC analysis. After this process, the samples were filtered with 0.22 µm nylon filters and injected into the HPLC-UV.

Cholesterol was determined by HPLC coupled with a UV-V is detector (HPLC-UV) (Knauer, Smartline System 1000, Berlin, Germany) set at 210 nm of wavelength and an XBridge C18 column (4.6 x 250 mm, 5 µm, Waters) was used. The mobile phase used was acetonitrile/methanol (70:30 v/v) at 40 ºC with a flow rate of 1 mL/min (oven 7971 R Grace, Berlin, Germany). The identification and quantification of the cholesterol were performed using the retention times of commercial standards. The data were analyzed using Clarity 2.4 software (DataApex, Prague, Czech Republic). Finally, the results were expressed in g/100 g of dry weight (dw).

3.9. Chocolate milkshake formulation and incorporation of the statin and ergosterol-enriched extract

The milkshake formulation followed a traditional recipe, in which 500 mL of fat milk with 60 g of cacao powder were mixed with 400ug/mL of *P. ostreatus* extract after ultrasound-assisted extraction optimization. After homogenization, the formulation was divided into small flasks of 100 ml each and lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). All the ingredients were sterilized in a UV chamber before utilization (**Figure 11)**.

The main objective was to develop a powdered formulation that could be prepared with water and consumed immediately, allowing an easy transport, and in line with the consumer´s demand for ready to eat and functional foods.

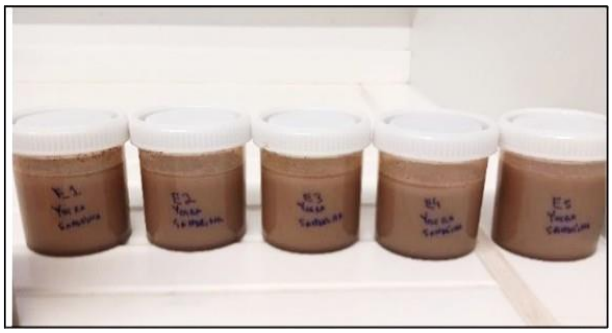

Figure 11: Chocolate milkshake in the flasks.

3.10. Nutritional value

Following the prescribed AOAC protocols, the nutritional value (moisture, proteins, fat, carbs, and ash) was assessed (AOAC, 2016). By using the macro-Kjeldahl method, the samples' crude protein content (N=6.38) was calculated. The crude fat content was calculated by extracting a known weight of powdered sample with petroleum ether using a Soxhlet apparatus. The ash content was calculated by incinerating the samples at 550 °C. By using the differential, total carbs were computed. The following formula was used to determine energy:

Energy (kcal)=4 (g protein + g carb) + 9 (g fat); KJ= Kcal*4.1868. Results were given as grams per 100 grams of fresh weight**.**

3.10.1. Moisture

As shown in **Figure 12**, the milkshake powder was placed on the metal plate and inserted into the balance moisture analyzer (Adam Equipment, PMB 163). To make the food's moisture evaporate, this equipment gradually raises the temperature to 105 °C. The sample is weighed once more once the weight remains constant and no evaporation has been noted. The following function was used to get the results:

(Mi-Mf)/Mi 100 = % Moisture. Where mf is the weight once it has reached a constant weight and mi is the weight at the beginning.

Figure 12: Moisture equipment

3.10.2. Fat

The crude fat was obtained from a Soxhlet extractor, as shown in **Figure 13**. It was used 1 g of the sample and petroleum ether as the extraction solvent. After the extraction, the sample was placed in the oven until the petroleum ether was evaporated, and the crude fat was obtained by the difference between the weight of the tube with the residue resulting from the extraction and the empty tube. The fat content was expressed in g/100 g of fresh weight.

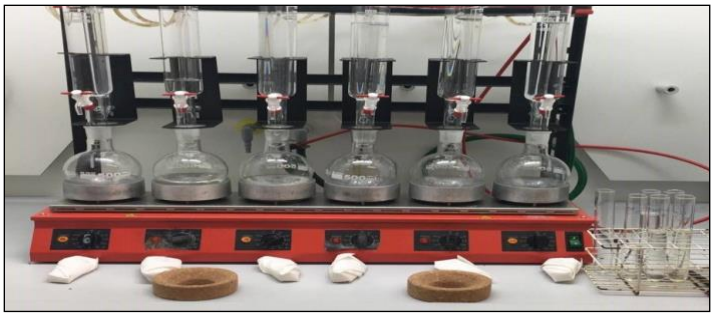

Figure 13: Soxhlet device.

3.10.3. Ashes

A crucible weighing 250 mg of the sample was placed in the muffle furnace (Lenton ECF 12/22, Hope Valley, UK) at a temperature of 550 °C for 5 hours (**Figure 14**). The ash content was determined by the difference between the weight of the incinerated sample and the empty crucible. The ash content was expressed in g/100 g of fresh weight.

Figure 14: Muffle for incineration.

3.10.4. Proteins

The Macro-Kjeldahl method, which quantifies the crude protein concentration as a function of the nitrogen content of the sample, was used to conduct the protein analysis. To speed up the digestion process, two selenium pellets and 15 mL of concentrated sulfuric acid were added to the digestion tube along with 500 mg of the milkshake samples.

For roughly 70 minutes at 400 °C, the tubes were placed in the digester (FossTM Digestor) (**Figure 15)**. The digesting tubes were put in the Kjeldahl device, which conducts distillation and titration automatically, after chilling. The number obtained for nitrogen was multiplied by a conversion factor chosen on the device to get the protein content. The protein content was expressed in g/100 g of fresh weight.

Figure 15: Kjeldahl device.

3.11. Statistical analysis

All analyses were performed at least in triplicate. Throughout the whole document, all data is expressed as mean±standard deviation. An analysis of variance (ANOVA) was used to analyse the samples, relying on a Student's T-Test for post-hoc classification, depending on Levene's test for Equality of Variances using the SPSS, version 28, software.

4. Results and Discussion

4.1. Debunking statin´s profile

An illustration of a chromatogram profile depicted in **Figures 16** and **17** where the two mushrooms' extracts exhibited a single peak similar to the standard chromatogram (**Figure 18**), at a retention time of respectively 1.4 and 1.3 minutes, with maximum UV spectrum absorbance at 238 nm is presented. These picks are consistent with findings for the commercially available standard compound of statins allowing the substance to be recognized as pravastatin.

When comparing the two peaks of pravastatin related to the two mushroom varieties, the one exhibited in *P. ostreatus* has a higher amplitude than the peak appearing in *A. bisporus*, which suggests that the amount of pravastatin in *P. ostreatus* is higher than in *A. bisporus*, as shown on the figures.

Those results are in concordance with Adler *et al.* (2022), Gil-Ramirez *et al.* (2011) and Mishra *et al.* (2018) that confirmed that mushrooms like *P. ostreatus* and *A. bisporus* contain HMG-CoA reductase inhibitors such as pravastatin and lovastatin, among other statins.

Using LC-MS/MS, Gil-Ramírez et *al.* (2013) didn't detect any peak at the retention time of pravastatin (3.82 min), atorvastatin (8.06 min), simvastatin (9.83 min) or lovastatin (20.26 min) which mention that statins were not found in the *A. bisporus* extracts with HMGCR inhibitory activity.

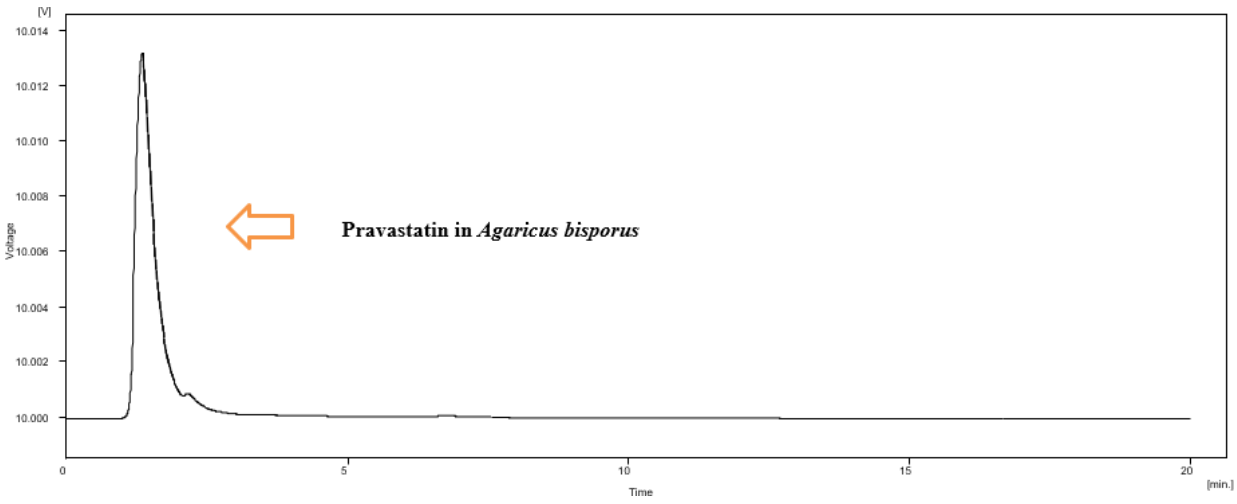

Figure 16: Exemplificative chromatogram profile recorded at 238 nm from *A. bisporus* extract.

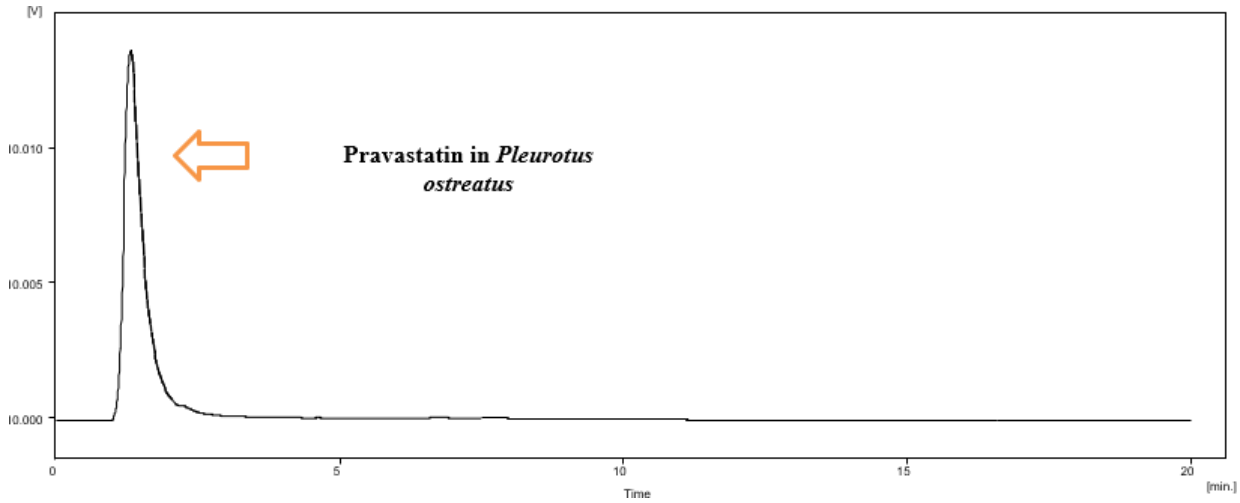

Figure 17: Exemplificative chromatogram profile recorded at 238 nm from *P. ostreatus* extract

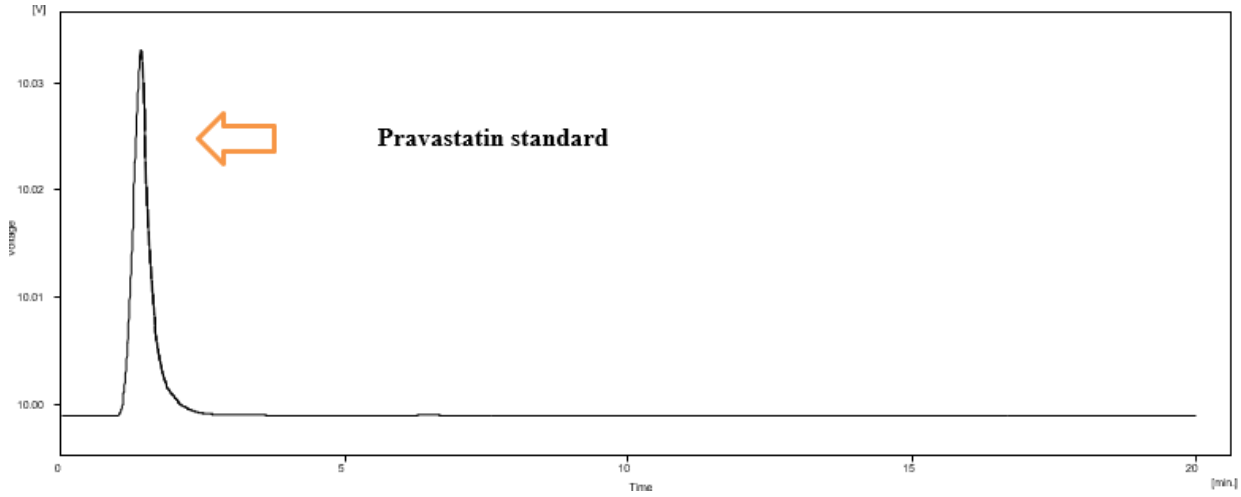

Figure 18: Chromatogram profile of pravastatin standard.

The chromatographic findings from HPLC-UV **Figures 19** and **20** have shown a retention time of 6.58 min, maximum UV absorption wavelengths at 238 nm.

Two samples of *P. ostreatus* extract and *A.* bisporus extract were subjected to ultrasound assisted extraction (UAE) condition qualified as hot (without putting ice bath on the ultrasound equipment), showed an identification of lovastatin related to two samples of *P. ostreatus* extract, however the two samples of *A.* bisporus didn't exhibit any peak of lovastatin .

The two peaks that appeared are identical to the peak of the lovastatin standard, chromatogram as shown in **Figure 20** and the exemplification of the chromatogram profile in **Figure 19**.

This result can be explained that the fungal strain and/or substrate, may have an impact on these differentiations, as lovastatin production is largely influenced by the carbon and nitrogen content

in the mushroom (Alarcón *et al*., 2006), as well as differences in gene adjustment and the bioavailability of substances that can activate lovastatin biosyntheses, such as methionine, glutamate, glycine, and histidine (Hajjaj *et al*., 2001).

Some studies found that overnight incubation (30 ºC) of the fruiting bodies with methanol: water (1:1) was the best method to extract lovastatin from fresh oyster mushrooms (*Gunde et al.*, 1995; Ramírez-Anguiano *et al.*, 2007; unde-Cimerman, 1993), while Gil-Ramírezno *et al*. (2011) didn't found any significant differences between the dried *P. ostreatus* mushroom powder or when the extracts were freshly prepared and applied, or after overnight incubation.

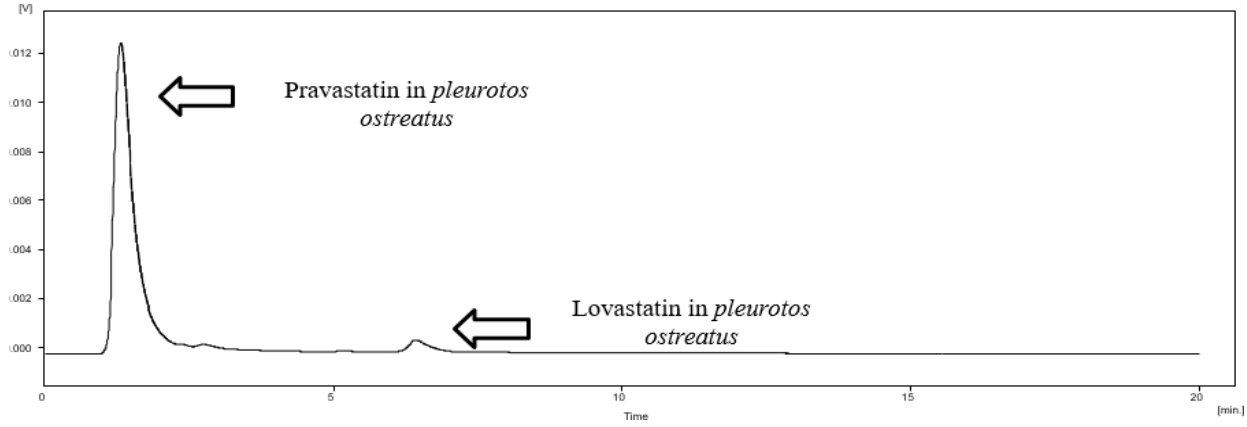

Figure 19: Exemplificative chromatogram profile recorded at 238 nm from *P. ostreatus* extract.

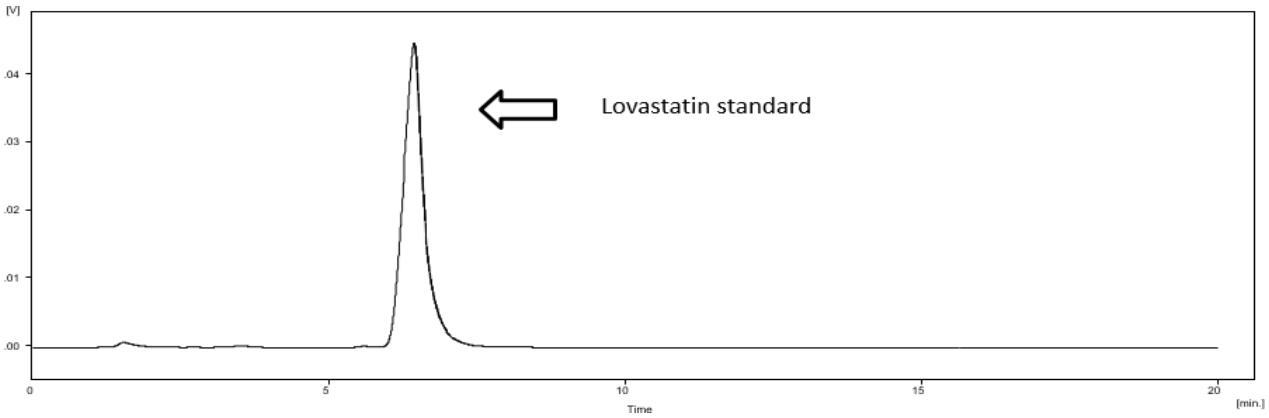

Figure 20: Chromatogram profile of lovastatin standard.

4.2. Optimization of the extraction conditions using ultrasound-assisted extraction, through the Response Surface Methodology (RSM)

In comparison to traditional one-factor-at-a-time approaches, which do not allow the evaluation of interactions between variables, RSM is a statistical tool suitable for optimizing extraction processes involving one or more response variables and allows for the determination of the optimal processing conditions with a reduced number of experimental trials (Pinela et *al*., 2016). Presented in **Table 2** are the conditions of the 17 individual extractions, as well as their response

(yield in Pravastatin).

Table 2: Experimental design for the optimization of Pravastatin (R) using the three individual factors A, B and C.

Run	S/L(g/L) (A)	Potential(%) (B)	Time(min) (C)	Pravastatin (mg/mL) (R)
1	17.5	47.5	16	0.3825
2	17.5	15	2	0.3375
3	17.5	47.5	16	0.3835
4	30	47.5	30	0.332
5	17.5	47.5	16	0.406
6	30	47.5	2	0.309
7	17.5	80	30	0.3895
8	5	47.5	30	0.2845
9	17.5	80	2	0.477
10	17.5	47.5	16	0.376
11	30	15	16	0.296
12	30	80	16	0.3535
13	5	47.5	2	0.408
14	5	15	16	0.2675
15	17.5	47.5	16	0.396
16	17.5	15	30	0.387
17	5	80	16	0.426

The individual results rendered a quadratic model with a significant fit and a non-significant lack of fit, as well as an adjusted R^2 of 0.917, as can be seen in **Table 3**. The quadratic equation is as follows:

$$R=0.3888-0.0119A+0.0448B-0.0173C-0.0253AB+0.0366AC-0.0343BC-0.0587A^2+0.0057B^2+0.0033C^2.$$

Table 3: Statistical parameters applied in the RSM

Statistics Model	
p-value	0,003
F-value	20.65
Lack of Fit	2.72
R ²	0.9637
R ²adj	0.9170
Adequate Precision	17.7965

R²: coefficient of determination; R²ajd: adjusted coefficient of determination.

The optimization of Pravastatin rendered three 3D plots showing the response surface of each pair of the individual factors **(Figure 21)**. Each plot is shown at the optimal point of the missing factor and thus the behavior of the two factors can be seen. On the left plot, which shows the response surface for S/L and power, it is clear that higher intensities promote the extraction of Pravastatin (shown by red zones) as well as lower S/L ratios.

In the middle plot, it is clear that lower rations of solid to liquid promote the yield of Pravastatin while lower extraction times also contribute to it positively. Finally, the right plot shows that, once again, higher extraction intensities and lower extractive time promote a higher yield.

Combining the three plots, the optimal conditions of Pravastatin are calculated, as shown in **figure 22.** The optimal extracting conditions are ultrasound power at 80% for 2 min using 16.55 g/L of mushroom extract in methanol which is predicted to render 0.477 mg/mL of Pravastatin extract was obtained, which was similar to the actual extracted amount. This result experimentally validates the extraction model developed.

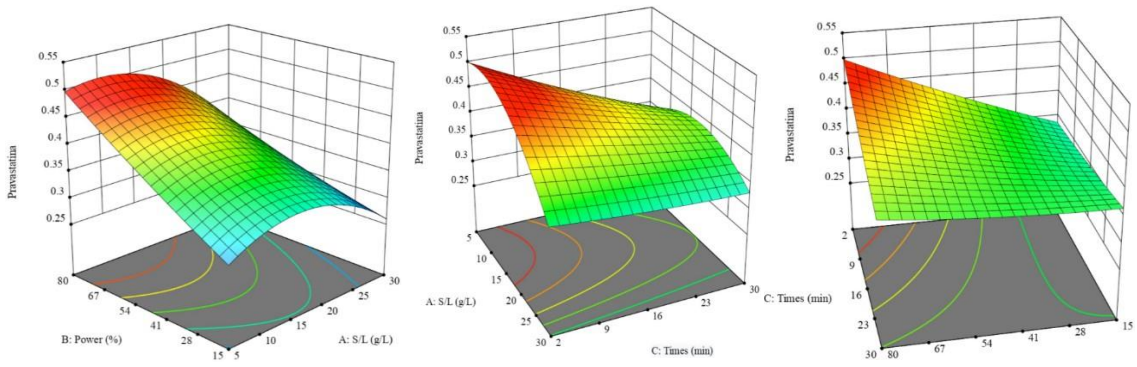

Figure 21: 3D plots of the pravastatin content (mg/mL extract) obtained from *P. ostreatus*.

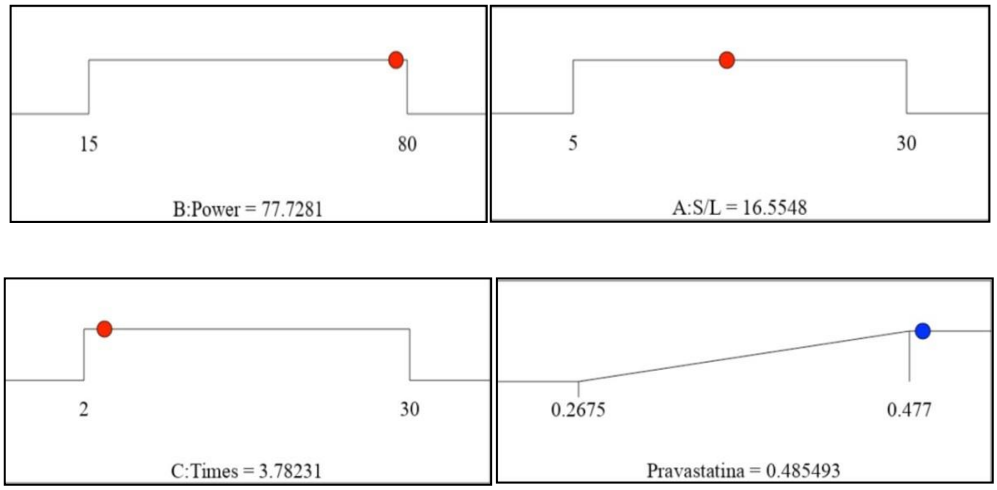

Figure 22: Graphical representation of the optimal point for the extraction of pravastatin using Ultrasound-Assisted Extraction (UAE).

4.3. Ergosterol profile

An illustration of a chromatogram profile in **Figure 23 and 24** was obtained, where the two mushrooms' extracts exhibited a single peak similar to the standard chromatogram (**Figure 25**), at a retention time of respectively 13.85 and 13.80 minutes, with maximum UV spectrum absorbance at 280 nm that is consistent with findings for the commercially available standard compound of ergosterol, allowing the substance to be recognized as ergosterol.

These findings are in confirmation with several authors, that state that ergosterol is an abundant secondary metabolite of mushrooms such as *Pleurotus ostreatus* and *Agaricus bisporus* (Nzekoue *et al*., 2022; Hu *et al*., 2021; Hu *et al*., 2020).

Gil-Ramírez *et al*. (2011) found out that *Boletus edulis* was the specie with the highest level of ergosterol and *Craterellus cornucopioides* the specie with the lowest concentration of total sterols, while *A. bisporus* and *P. ostreatus* had the amount of 7.8-4.4 mg/g.

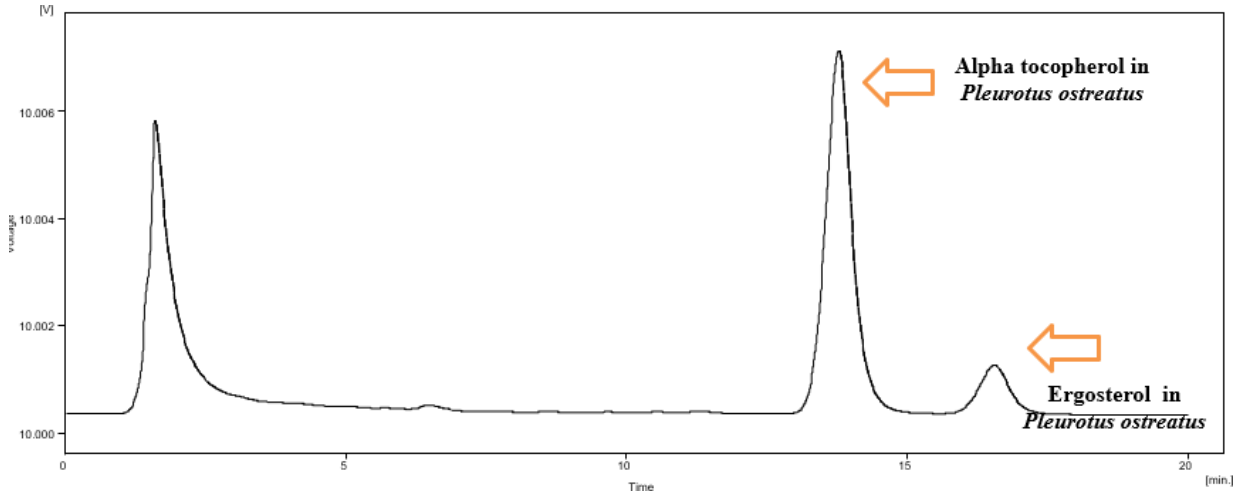

Figure 23: Exemplificative of ergosterol chromatogram profile recorded at 280 nm from *P. ostrautus* extract.

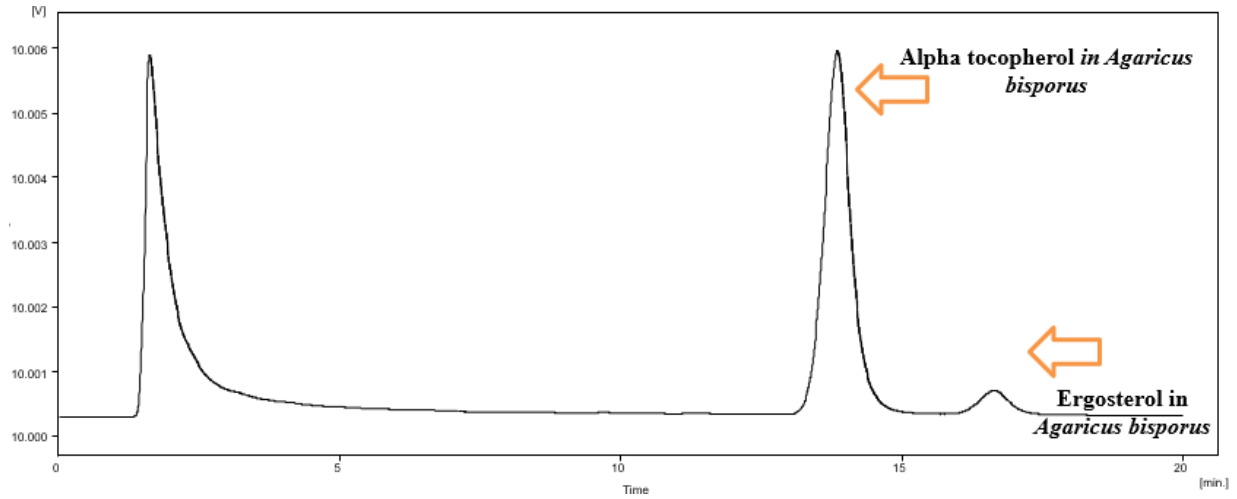

Figure 24: Exemplificative of ergosterol chromatogram profile recorded at 280 nm from *A. bisporus* extract.

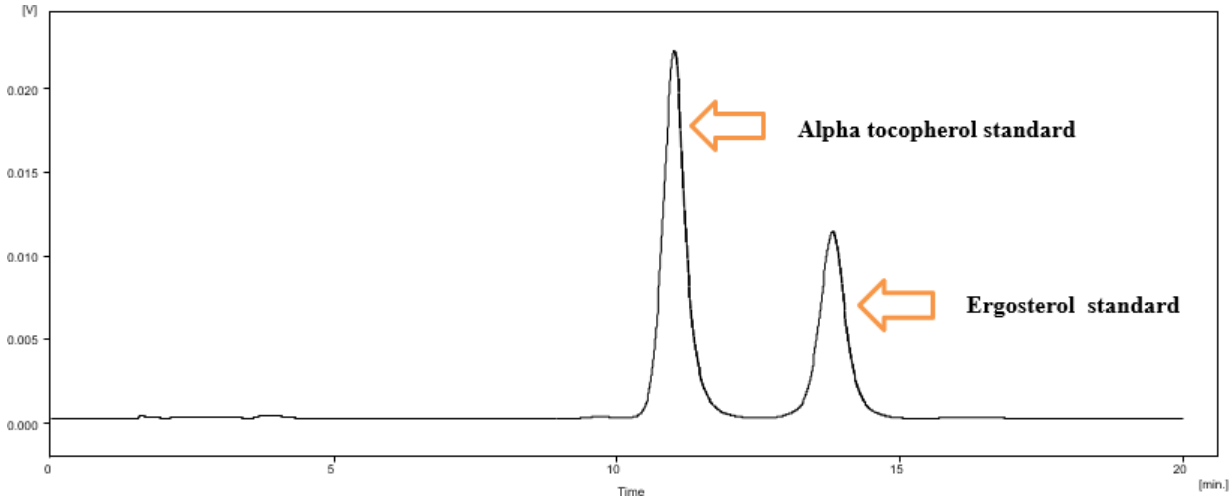

Figure 25: Chromatogram profile of alpha-tocopherol and ergosterol standards, respectively.

4.4. Bioactivity assays

The cytotoxic activity of the methanolic optimized extract of *P. ostreatus after* was tested against four human cancer cell lines: NCI-H460 (non-small cell lung cancer), MCF-7 (breast carcinoma), Caco2 (colon carcinoma), AGS (adenocarcinoma gastric cell); and non-tumour cell lines: PLP2 (porcine liver primary cell line), for those cells ellipticine was used a positive control. The extract was also tested for its anti-inflammatory activity using RAW 264.7 (mouse macrophage cell line), being dexamentose used as a positive control.

The cytotoxicity and anti-inflammatory activity of the sample analyzed are presented in **Table 4**. The hydromethanolic extract derived from the *P. ostreatus* extract did not exhibit any anti-proliferative activity in all tumour cell lines. However, the extract didn't have any cytotoxicity against PLP2 non-tumour cell lines.

In terms of anti-inflammatory activity, the extract didn't demonstrate anti-inflammatory potential in the RAW 264.7 mouse macrophage cell line (**Table 4**). Oyster mushroom extracts are high in polysaccharides such as beta-glucan and other macromolecules that have an anti-proliferative effect on cancer cell lines while not harming normal cells (Mishra *et al*., 2021).

According to Cao *et al.* (2015), polysaccharides extracted from the *P. ostreatus* mycelium component (polysaccharides POMP2), had a significant inhibitory effect on the BGC-823 human gastric cancer cell line *in vitro*.

Another study showed that the extracts of dark-grey and pink strains of oyster mushrooms inhibited the growth of the human colon cancer cell line HT-29 more effectively, with survival rates of 39.9 and 40.7%, respectively (Kim *et al.*, 2009).

According to Jedinak *et al*. (2011), oyster mushroom has anti-inflammatory properties and could be used as a dietary anti-inflammatory agent. In a more recent study by Stastny *et al.* (2022) where human recombinant cyclooxygenase-2 and nuclear factor κB were used in an *in vitro* enzymatic assay to test the anti-inflammatory activity of *Pleurotus spp.*, methanolic extract showed an 80% ability to inhibit COX-2 and NF-B/AP-1 activity which confirms the efficient anti-inflammatory activity of the mushroom extract. These findings were not in agreement with the ones presented in this study, maybe due to different extraction techniques or the presence of different molecules.

Table 4: Cytotoxic and anti-inflammatory activity of *P. ostreatus* extract.

Samples		Cytotoxicity					Anti-inflammatory (values IC_{50}, µg/mL)
		Tumor cell lines (values GI_{50}, µg/mL)				Non tumor cell lines (values GI_{50}, µg/mL)	
		AGS	**Caco2**	**MCF-7**	**NCI-H460**	**PLP2**	**RAW 264.7**
***P. ostreatus* extract**		>400	>400	>400	>400	>400	>400
Control	Ellipticine (µg/mL)	1.23 ± 0.03	1.21± 0.02	1,02 ± 0.02	1.01 ± 0.01	1.41 ± 0.1	
	Dexamethasone (µg/mL)						6.3 ± 0.4

Where GI_{50}: concentration that inhibited 50% of cell growth and IC_{50}: concentration of extract causing 50% inhibition of NO production.

4.5. Cholesterol profile

The chromatogram findings (**Figure 26**) in all the samples shows a retention time of 14.907 min, at a wavelength 210 nm of a possible derivative of cholesterol, that can result from an interaction of the cholesterol with one of the compounds present in the cell medium or with compounds from the extract, the statins. Ergosterol enriched extracts were previously perfomed in the research group and the cholesterol pick was easily detected and quantified. Therefore, the presence of statins could cause different interactions with cholesterol, making it impossible to detect.

The chromatogram profile of the cholesterol standard (**Figure 27**), recorded at 210 nm shows a retention time of 18.167 min. In fact, when comparing the two chromatograms. A significant difference was observed regarding the retention time (14.90) min for the cholesterol derivative and 18.167 min for cholesterol standard). This previous result limits the evaluation of the hypocholesterolemic effect of the extracts using the Caco-2 cells.

However different studies confirmed that CaCo-2 cell cultures isolated from a colon carcinoma were used as a model system to investigate the process of lipid metabolism (Iftikhar *et al.*, 2020; *Xu et al*., 2021; Bobin-Dubigeon *et al.*, 2020). *A. bisporus* ergosterol-enriched extracts mixed into lard, butter, and white chocolate clearly show the inhibition of cholesterol absorption in the intestinal epithelia using CaCo-2 studies (Gil-Ramírez *et al.*, 2014).

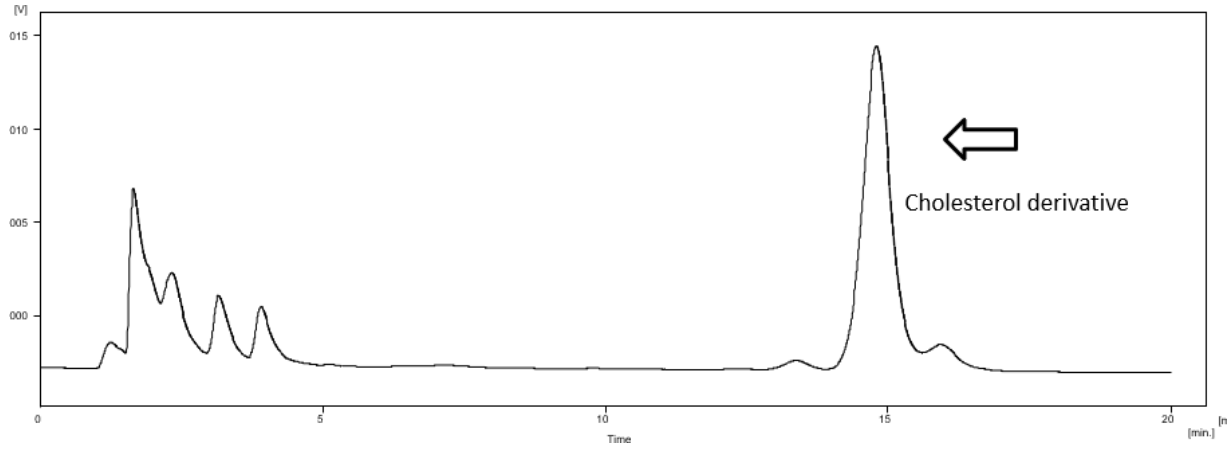

Figure 26: Exemplificative of a cholesterol derivative chromatogram profile recorded at 210 nm from extracts obtained using the Caco-2 cell line.

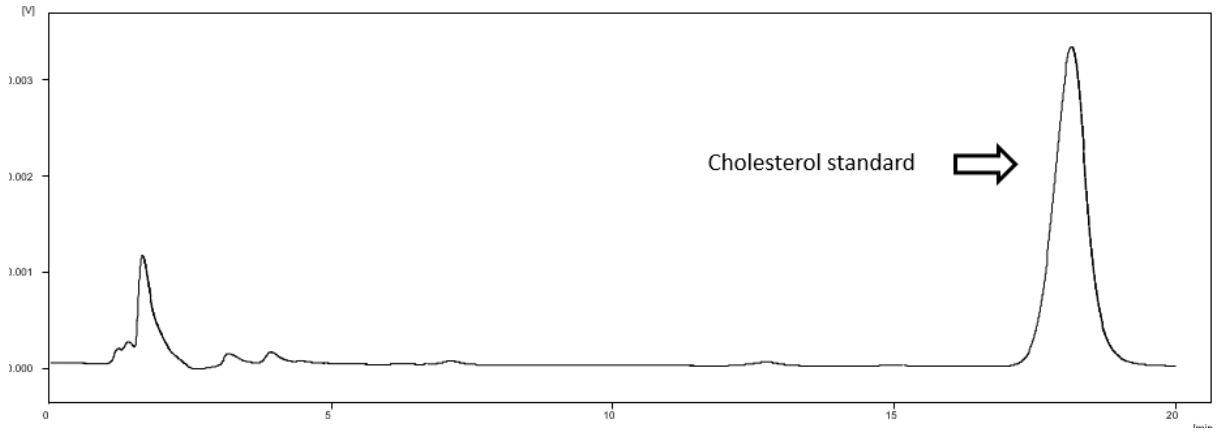

Figure 27: Chromatogram profile of cholesterol standard.

4.6. Nutritional profile of the milkshake

The nutritional profile was analyzed by evaluating the proximate composition, which is presented in **Table 5**. Macronutrients and their energy value, the content of crude fat, proteins, moisture, and ashes were determined.

Carbohydrates were the major macronutrients detected in the control and in the functional beverage enriched with the extract, presenting the following values: 12.53 ± 0.34 g/100 g fw in the control and 12.58 ± 0.27 g/100 fw in the functional beverage. For protein content, the control presented a value of 3.01 ± 0.02 g/100 g fw and 3.00 ± 0.15 g/100 g fw for the functional beverage with the extract.

The moisture content was higher in the control sample (82.70% ± 0.25%) than in the functional beverage with extract (82.80% ± 0.45%). Ashes were detected at lower concentrations, for the control (0.81 ± 0.18 g/100 g fw) and the beverage with extract (0.79 ± 0.13 g/100 g fw). Concerning the energetic value of the milkshakes, the results were as follows: 70.72 ± 1.34 Kcal for the control and 69.84 ± 2.15 Kcal for the extract incorporated in the beverage.

Excluding the crude fat, the statistical analysis has shown a *p-value* > 0.05 for all the previously mentioned results and therefore there were no significant differences between the control and the functional beverage with the extract.

While the crude fat content was higher in the control (0.95 ± 0.03 g/100g fw) than the functional beverage with the extract (0.83 ± 0.06 g/100 g fw), it is supposed that this difference is related to the fat-lowering efficiency of the bioactive molecules present in the extract of pravastatin and ergosterol, confirmed statistically since the *p-value* < 0.05.

Cilla et *al.* (2019) found that their phytosterol-enriched milk-based fruit beverages also presented a decrease in the fat amount with phytosterol (0.8 g/100 g) compared to the amount of fat without adding phytosterols (1.6 g/100 g), with non-significant changes in the other macronutrients and their energy value.

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Table 5 : Nutritional profile of the milkshake.

	Moisture (%)	**Crude fat (g/100 g fw)**	**Ashes (g/100 g fw)**	**Proteins (g/100 g fw)**	**Carbohydrates (g/100 g fw)**	**Energy (kcal)**	**Energy (kJ)**
Control	82.70 ± 0.25	0.95 ± 0.03 *	0.81 ± 0.18	3.01 ± 0.02	12.53 ± 0.34	70.72 ± 1.34	295.90 ± 5.61
Extract	82.80 ± 0.45	0.83 ± 0.06 *	0.79 ± 0.13	3.00 ± 0.15	12.58 ± 0.27	69.84 ± 2.15	292.22 ± 9.01
t-test *p-value* (n=6)	0.344	0.006	0.634	0.464	0.468	0.838	0.838

*Significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, but rather as the range of the recorded values.

5. Conclusion

Cardiovascular diseases are one of the leading causes of death and disability in the world, but it is often preventable with a healthy lifestyle. The most common health condition leading to CVD is hyperlipidemia, which is the high amount of cholesterol and triglyceride in the blood, the chemical treatment for this disease is the use of statin, this medicine can lower the cholesterol level in the blood by blocking the mevalonate pathway which by blocking the HMG-COA reductase, unfortunately, statins have shown multiple side effects after years of use mainly muscles toxicity, losing appetite and risk of diabetes among many other secondary effects.

That's why the need for the development of functional food/beverages is a promising and revolutionary trend in the agri-food industry due to the bioactive components incorporated in them and the raising of European consumer's awareness toward these kinds of products.

Currently, wide world research is focused on sustainable development and the demand for innovative clean technologies, nevertheless, natural potential reconsideration could represent a viable solution for the development of new pharmacological agents from renewable resources, bioactive molecules from natural sources are an excellent alternative, due to their benefits including antioxidants, anti-inflammatory, antifungal, antimicrobial, antiproliferative and hypocholesterolemic activities among many other properties, demonstrating how bioactive compounds are a living example of Hippocrates' concept of "let thy food be thy medicine."

These trends pose a challenge to food researchers, whose job it is to develop and identify raw food sources with properties that increase the level of health-improving ingredients. Mushrooms such as *A. bisporus* and *P. ostreatus* are rich in bioactive compounds, which make them an interesting source of functional food. Numerous studies have confirmed their nutritional value, and beta-glucans, statins and ergosterol are one of the active substances that, according to recent research, have a hypocholesterolemic effect.

This work aims to focus on the identification of the bioactive molecules such as statins and ergosterol and optimizing the number of statins that we could extract by UAE to incorporate the final extract in a milk based beverage (chocolate milkshake) where it was subjected to nutritional profiling after the formulation.

According to the findings, it is important to highlight that the mushroom material was mushroom bio-waste, which means that mushroom samples with no commercial value were used and valued. It was possible to extract high-value molecules from this residue, assisting the mushroom enterprise in finding a commercial use for this material.

It was confirmed that both mushrooms *A. bisporus* and *P. ostreatus* are a source of HMG Co-A reductase inhibitors, mainly pravastatin and ergosterol which are known for their competitivity

with cholesterol due to the structure similarity and knowing from the literature that other molecules such as beta-glucans and many other sterols as mushrooms bioactive compounds, with a high potential of lowering cholesterol.

However, for future perspectives, the aptitude of the extract to lower cholesterol should be studied with more efficient protocols and also studying other bioactive compounds in the mushrooms, knowing their ability to lower cholesterol and optimizing their extraction with submerging methods of extractions is also required to have an efficient functional food that contains a maximum of cholesterol-lowering bioactive compounds.

6. References

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