BRNO UNIVERSITY OF TECHNOLOGY

Faculty of Chemistry

DOCTORAL THESIS

Brno, 2023 Ing. Marie Vysoká

BRNO UNIVERSITY OF TECHNOLOGY

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY

FAKULTA CHEMICKÁ

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

PREPARATION AND CHARACTERIZATION OF NATURAL EXTRACTS WITH ANTIMICROBIAL EFFECT

PŘÍPRAVA A CHARAKTERIZACE PŘÍRODNÍCH EXTRAKTŮ S ANTIMIKROBIÁLNÍM ÚČINKEM

DOCTORAL THESIS

DIZERTAČNÍ PRÁCE

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ŠKOLITEL

BRNO 2023

BRNO UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMISTRY

Assignment Doctoral Thesis

Department:	Institute of Food Science and Biotechnology	Academic year: 2022/23
Student:	**Ing. Marie Vysoká**	
Study programme:	Chemistry and Technology of Foodstuffs	
Field of study:	Food Science	
Head of thesis:	**prof. RNDr. Ivana Márová, CSc.**	

Title of Doctoral Thesis:

PREPARATION AND CHARACTERIZATION OF NATURAL EXTRACTS WITH ANTIMICROBIAL EFFECT

Doctoral Thesis:

Cílem práce je příprava a charakterizace různých typů přírodních extraktů s antioxidačním a antimikrobiálním účinkem.
Dílčí cíle:
1. Litrární rešerše – přehled léčivých rostlin a screening kvasinek s vysokou antioxidační, antimikrobiální aktivitou a významnými nutričními vlastnostmi.
2. Optimalizace metod pro charakterizaci a složení rostlinných extraktů
3. Produkce a charakterizace mikrobiální biomasy a extraktů z vybraných druhů kvasinek
4. Analýza aktivvních složek, antioxidačního a antimikrobiálního účinku rostlinných a mikrobiálních extraktů a jejich směsí
5. Testování biologických účinků a bezpečnosti přírodních extraktů s využitím testů cytotoxicity a apoptózy na humánních buněčných liniích
6. Diskuse a možná aplikace výsledků.

Deadline for Doctoral Thesis delivery: 31.5.2023:

Ing. Marie Vysoká student	prof. RNDr. Ivana Márová, CSc. Head of thesis	prof. RNDr. Ivana Márová, CSc. Head of department

In Brno dated 10.6.2017

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ABSTRACT

Worldwide consumption of antibiotics has increased drastically in the past few decades. The application of antibiotics has led to the production of antibiotic resistance genes (ARGs) which represent a growing and serious human health threat worldwide. The use of natural or alternative medicines increased markedly over the last few years. Therefore, much attention is paid to natural products, which could be used as effective drugs to treat human diseases, with high efficiency against pathogens and negligible side effects. Yeast biomass that contains valuable fatty acids, carbohydrates, nucleic acids, vitamins, and minerals was tested in the potential combination with plant extracts as a food supplement.

This doctoral thesis is focused on impact assessment of selected plant extracts such as acai, clove, turmeric, ginger, ginseng and oregano and their antimicrobial activity, antioxidant activity, cytotoxicity and important nutritional benefits. Subsequently, the biological effects of yeast biomass of the strains *Metschnikowia pulcherrima* CCY 029-002-145, C*ystofilobasidium infirmominiatum* CCY 17-18-4, *Phaffia rhodozyma* CCY 77-1, *Rhodotorula kratochvilovae* CCY 20-2-26 were tested. These yeasts were chosen especially for the high content of active substances in the produced biomass. In conclusion, the potential synergistic effect of plant extracts in combination with yeast biomass to increase the overall antimicrobial effect, antioxidant activity and effect on the growth of Caco-2, B16F1 and HaCaT cell lines was assessed. Considering the results achieved, it is appropriate to include immortalized yeast biomass in combination with natural extracts in food supplements in an appropriate form, as it is clear that there is some direct relationship between cell viability and apoptosis. All microbial extracts showed some positive results for the apoptosis of cancer cell lines and at the same time no cytotoxic effects on non-cancer cell lines at identical concentrations.

KEY WORDS:

antioxidants, antimicrobials, plants, yeasts, apoptosis

ABSTRAKT

Celosvětová spotřeba antibiotik v posledních několika desetiletích významně vzrostla, což vedlo ke zvýšenému výskytu genů antibiotické rezistence (ARG), které představují rostoucí vážnou hrozbu pro lidské zdraví na celém světě. Používání přírodních nebo alternativních léčivých přípravků tudíž v posledních několika letech výrazně vzrostlo. Velká pozornost je nyní věnována přírodním extraktům, které by mohly být v jisté formě použity jako jedna z možností léčby bakteriálního onemocnění, a to s vysokou účinností proti patogenům a se zanedbatelnými vedlejšími účinky. Biomasa kvasinek, která obsahuje cenné mastné kyseliny, sacharidy, nukleové kyseliny, vitamíny a minerály, byla testována jako možná alternativa, a to samotná i v kombinaci s rostlinnými extrakty ve formě doplňku stravy.

Tato disertační práce je zaměřena na studium antimikrobiální aktivity a nutriční přínosy vybraných rostlinných extraktů, jako je acai, hřebíček, kurkuma, zázvor, ženšen a oregano. Následně byly testovány biologické účinky kvasinkové biomasy rodu *Metschnikowia pulcherrima* CCY 029-002-145, *Cystofilobasidium infirmominiatum* CCY 17-18-4, P*haffia rhodozyma* CCY 77-1, *Rhodotorula kratochvilovae* CCY 20-2-26. Tyto kvasinky byly vybrány zejména pro vysoký obsah aktivních látek v produkované biomase. Na závěr byl posouzen potenciální synergický efekt rostlinných extraktů ve spojení s kvasinkovou biomasou ke zvýšení celkového antimikrobiálního účinku, antioxidační aktivity a vlivu na růst buněčných linií Caco-2, B16F1 a HaCaT. Vzhledem k dosaženým výsledkům lze konstatovat, že zařazení imortalizované kvasinkové biomasy v kombinaci s přírodními extrakty do doplňků stravy ve vhodné formě může být prospěšné lidskému zdraví. Existuje přímý vztah mezi životaschopností buněk a apoptózou. Všechny mikrobiální extrakty vykazovaly určité pozitivní výsledky na apoptózu u rakovinných buněčných linií a současně nevykazovaly žádné cytotoxické účinky na nezhoubné buněčné linie při stejných koncentracích.

KLÍČOVÁ SLOVA:

antioxidanty, antimikrobialní látky, rostliny, kvasinky, apoptóza

CITATION

VYSOKÁ, Marie. *Příprava a charakterizace přírodních extraktů s antimikrobiálním účinkem.* Brno, 2023. Dostupné také z: https://www.vut.cz/studenti/zav-prace/detail/153410. Dizertační práce. Vysoké učení technické v Brně, Fakulta chemická, Ústav chemie potravin a biotechnologií. Vedoucí práce Ivana Márová.

DECLARATION

I declare that the doctoral thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the doctoral thesis is the property of the Faculty of Chemistry of Brno University of Technology, and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

Acknowledgment:

At this point I would like to thank mainly the supervisor of the doctoral thesis, prof. RNDr. Ivana Márová, CSc., for professional guidance and valuable advice during the entire doctoral study. I would like to thank my colleagues, especially Ing. Dana Byrtusová Ph.D., my family and closest friends for their continues support and patience throughout the study.

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1 INTRODUCTION

The use of natural products or alternative medicaments has increased rapidly over the last few years. More and more older adults (i.e., baby boomers) are using dietary supplements without advice from a physician on the assumption that these substances will have a beneficial effect. Natural products, as substitutes of synthetic chemical preservatives, are increasingly accepted because they are innately better tolerated in human body. It is necessary to study their antimicrobial activity for their further use in food and pharmaceutical industry.

Nowadays, antimicrobial resistant microorganisms are increasing the mortal rates due to the capability of surviving the exposure of antibiotics, which function as the cure to infectious diseases [1]. Antibiotic resistant bacteria decreasing the antibiotic effectiveness and limit the therapeutic effect even for common infections [2]. Therefore, much attention is paid to natural products, which could be used as effective drugs to treat human diseases, with high efficiency against pathogens and negligible side effects.

Spices have been used for flavouring the food since ancient times [3], and as medicine and food preservatives as well [4,5]. Many spices - such as clove, oregano, thyme and cinnamon have been applied to treat infectious diseases or protect food due to their antimicrobial activities against pathogenic fungi and bacteria [3,6]. Their secondary metabolites are already known as antimicrobial agents and are generally recognized as safe materials for food with insignificant side effects [4]. Therefore, spices could be candidates to discover and develop new antimicrobial agents against human pathogens and as food preservatives.

In fungal, or plant sources, β-glucans are naturally occurring polysaccharides of D-glucose monomers linked by β-glycosidic bonds. They serve as energy stores and structural components. Nowadays, β-glucans are produced commercially mainly from *Saccharomyces cerevisiae* and higher fungi. Due to its hydroxyl groups, yeast β-glucan is insoluble in water, which limits its biological functions. Many physiological functions, such as anti-obesity effects, reductions in postprandial glucose increases, and the normalization of serum cholesterol levels have been reported. The recent interest in barley and oat β-glucans has been sparked by reports discussing their prebiotic action, which is dependent on molecular weight [7,8,9,10,11].

Acai, clove, curcuma, ginger, ginseng and oregano were chosen for its source diversity representatives of natural extracts. Oregano as leaf, ginger and ginseng and turmeric as root, acai as fruit and clove flower. All chosen samples showed strong antioxidant and antimicrobial effects and has a potential to influence human health.

In this thesis, *Metschnikowia pulcherrima* CCY 029-002-145, *Cystofilobasidium infirmominiatum* CCY 17-18-4, *Phaffia rhodozyma* CCY 77-1, *Rhodotorula kratochvilovae* CCY 20-2-26, yeast strains were studied to produce biomass containing many biologically active components including vitamins, provitamins and of beta-glucans. Some synergy effect of yeast extracts with selected plant extracts to increase antimicrobial effect, antioxidant activity and last, but not least, to effect cell growth of Caco-2, B16F1 and HaCaT cell lines was tested.

2 THEORETICAL PART

The natural antimicrobials are readily available from various natural sources, such as plants, animals, and microorganisms, in which they constitute part of host defense mechanisms against microbial infections. Natural antimicrobials, such as essential oils and herbs, are traditionally known for their antimicrobial properties and used in different indigenous practices [12].

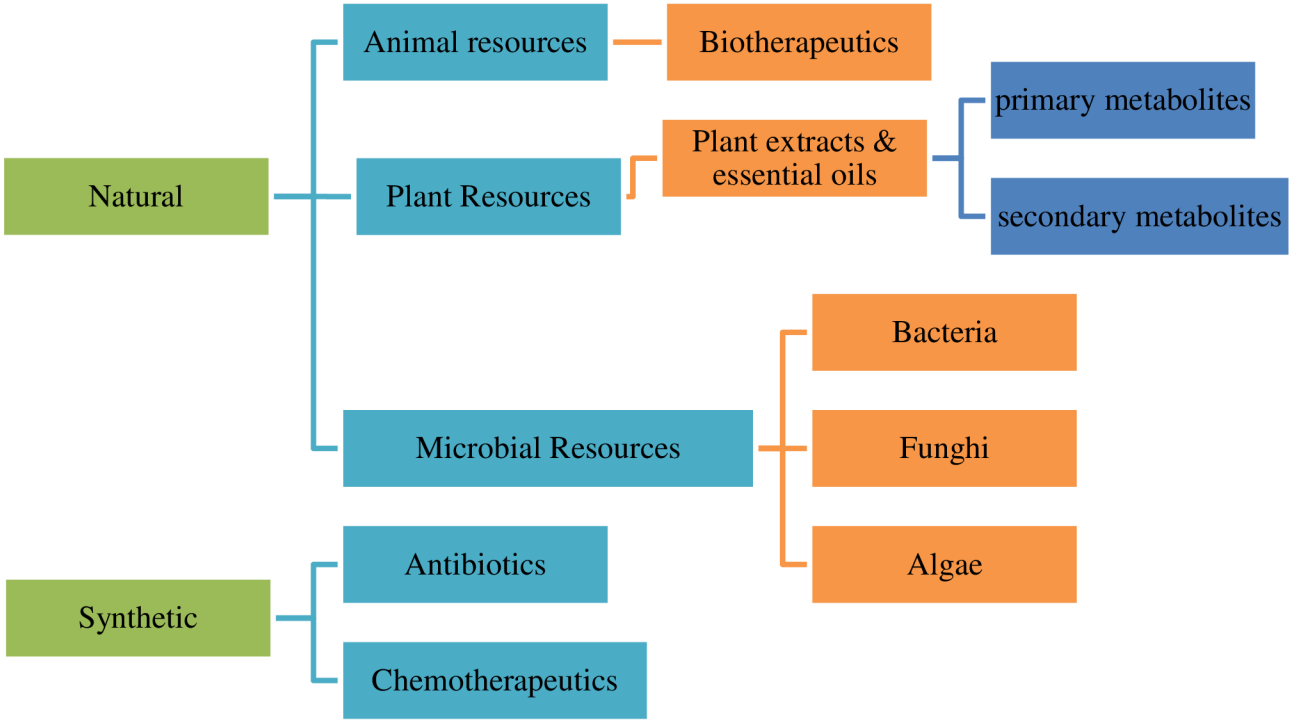

***Figure 1**: Natural and synthetic antimicrobial producents*

2.1 ANTIMICROBIALS OF ANIMAL ORIGIN

Natural defense systems are reported to exist in animal products, such as milk and eggs, that exhibit strong antimicrobial properties due to well-characterized compounds, such as lactoferrin, lactoperoxidase, and lysozyme. Several polypeptides originating from various animal sources, such as chitosan, megainin, pleurocidin, curvacin A, and spheniscin are reported to exhibit antimicrobial activity. Many of these polypeptides have been studied for their potential application as food preservatives. In this section, some of the most well-characterized antimicrobials of animal origin are described.

Lysozyme is a bacteriolytic enzyme widely reported for its application as an antimicrobial in food products, and it is nontoxic to humans. Lysozymes from different sources, such as hens' eggs, have been extensively studied for their potential as natural antimicrobials for food applications. Lysozyme has the ability to hydrolyze the β-1,4 linkage between N-acetylmuramic acid and N-acetyl-glucosamine in the peptidoglycan of microbial cell wall [12].

Lactoferrin, an iron-binding glycoprotein present in milk, is reported to possess antimicrobial activity against a wide range of bacteria and viruses in addition to other activities,

such as regulation of immune function, stimulation of intestinal proliferation, and differentiation and facilitation of iron absorption. Lactoferrin has been recently approved for application on beef in the United States and applied as an antimicrobial in a variety of meat products. The antimicrobial functionality of lactoferrin is attributed to its protein structural conformation. Lactoferrin binds with specific targets on the cell surface of gram-negative bacteria leading to outer membrane damage. Hence, the antibacterial activity of lactoferrin is dependent on tight binding with the bacterial cell surface [12].

Ovotransferrin or conalbumin is a glycoprotein of egg white albumin that contributes to antimicrobial defense systems of hens' eggs. Ovotransferrin belongs to a group of proteins known as metalloproteinases and has a high affinity for iron, thereby rendering iron unavailable for bacterial metabolism. This subsequently inhibits bacterial growth due to iron deprivation. The peptide OTAP-92 is responsible for the bactericidal activity of ovotransferrin. The purified form of this peptide has strong bactericidal activity against gram-positive and gram-negative bacteria, such as *Staphylococcus aureus* and *E. coli* [12].

Protamine is composed of cationic antimicrobial peptides naturally present in salmon and has broad antimicrobial activity against gram-positive bacteria, gram-negative bacteria, and fungi. The antimicrobial activity of protamine is likely due to its electrostatic affinity to the negatively charged cell envelopes of actively growing bacteria [12].

Chitosan (CH) is a polycationic biopolymer naturally present in the exoskeletons of crustaceans and arthropods. A collective group of all partially and fully deacetylated chitinous compounds is known as CH [poly (β-1, 4)-2-amino-2-deoxy-D-glucopyranose]. CH has a wide spectrum of antimicrobial activity against bacteria and fungi, and is safe for antimicrobial applications because of its low toxicity against mammalian cells (Franklin & Snow 1981, Kong et al. 2010). CH has become increasingly popular for its applicability in innovative food processing techniques and considered safe as a food additive by countries such as Korea and Japan (KFDA 1995, Kong et al. 2010). Owing to low water solubility, CH is solubilized in organic acids, such as acetic or lactic acid, for use in food processing applications (No et al. 2007). However, the acidic solution may cause hydrolysis and chain depolymerization of CH molecules, thereby diminishing its antimicrobial activity. Modified water-soluble derivatives that constitute a stable CH structure in different solvents have been described. For example, a water-soluble CH derivative known as glucosyloxyethyl acrylated CH has been synthesized using the Michael Addition reaction of CH with glucosyloxyethyl acrylate (Wang et al. 2011). Low-molecular-weight CHs at a pH less than 6.0 can provide effective antimicrobial preservative activities in liquid and solid foods (Friedman & Juneja 2010). CH can be used to improve both the quality and shelf life of food. For example, adding CH to freshly made noodles extended their shelf life by six days at 4°C. Further, when Maillard reaction products prepared from CH and xylose were used, the shelf life was extended to 14 days at 4°C (Huang et al. 2007). Similarly, a CH coating can be used to reduce bacterial contamination of egg contents resulting from trans-shell penetration by S. Enteritidis and other bacteria, such as *Pseudomonas sp., E. coli,* and *L. monocytogenes* [12].

2.2 ANTIMICROBIALS OF PLANT ORIGIN

Traditionally, medicinal plants are an important source of compounds with therapeutic potential, and still represent a significant pool for the discovery of new drugs. The medicinal properties of plants are caused mainly due to the presence of active secondary metabolites. Although medicinal plants continue to be an important source of new therapeutic leads, its scientific validation remains inadequate due to the lack of adequate plant material, choice and execution of suitable high-throughput screening bioassays, availability of bioactive compounds in large quantities, and finally their regulatory approval and clinical trials. Although the inherent complication of natural product-based pharmaceutical invention demands integrative research and protocol; the systemic study, latest scientific approaches, and regulatory approvals, but scientific developments unmistakably showed that plant-based products will be the significant upcoming resources of novel therapeutic compounds. [13].

2.2.1 Major components of natural substances

Plants are sunlight-based biochemical manufacturing units, which produce a large collection of bioactive compounds that are collected and extracted. Important phyto-compounds and their biosynthetic pathways are shown in the figure 1. Plant-based molecules are classified as primary or secondary compounds. Primary compounds are extensively distributed in the environment and are required for normal growth and development of plants. In contrast, secondary compounds are biosynthetic derivative of primary compounds but are present in restricted numbers and small quantities among plants. They regularly take part in ecologically important role in the interactions of plants with their environment and help in the continued existence of plants [14].

The important secondary metabolites found in higher plants include alkaloids, flavonoids, phenols, glycosides, saponins, tannins, volatile oils, gums, and resins. Plants-derived molecules are known to have beneficial activities. Some might slow down microbial growth, block metabolic pathways, or alter gene expression and signal transduction (Manson, 2003; Surh, 2003; KrisEtherton et al., 2002). Phyto-compounds can also act as chemotherapeutics or chemopreventives (compounds that slow down, annul, or delay tumorigenesis), and can be used for cancer treatment (Sarkar and Li, 2006; D'Incalci et al., 2005). Many herbal extracts and essential oils revealed diverse antibacterial activity, like the interaction with phospholipids bilayer of the cell membrane resulting in enhanced cell permeability and cell damage, loss of the enzymes associated with cellular respiration and manufacturing of different cellular constituents along with the damage to genetic components of the cell [15,16,17,18,20].

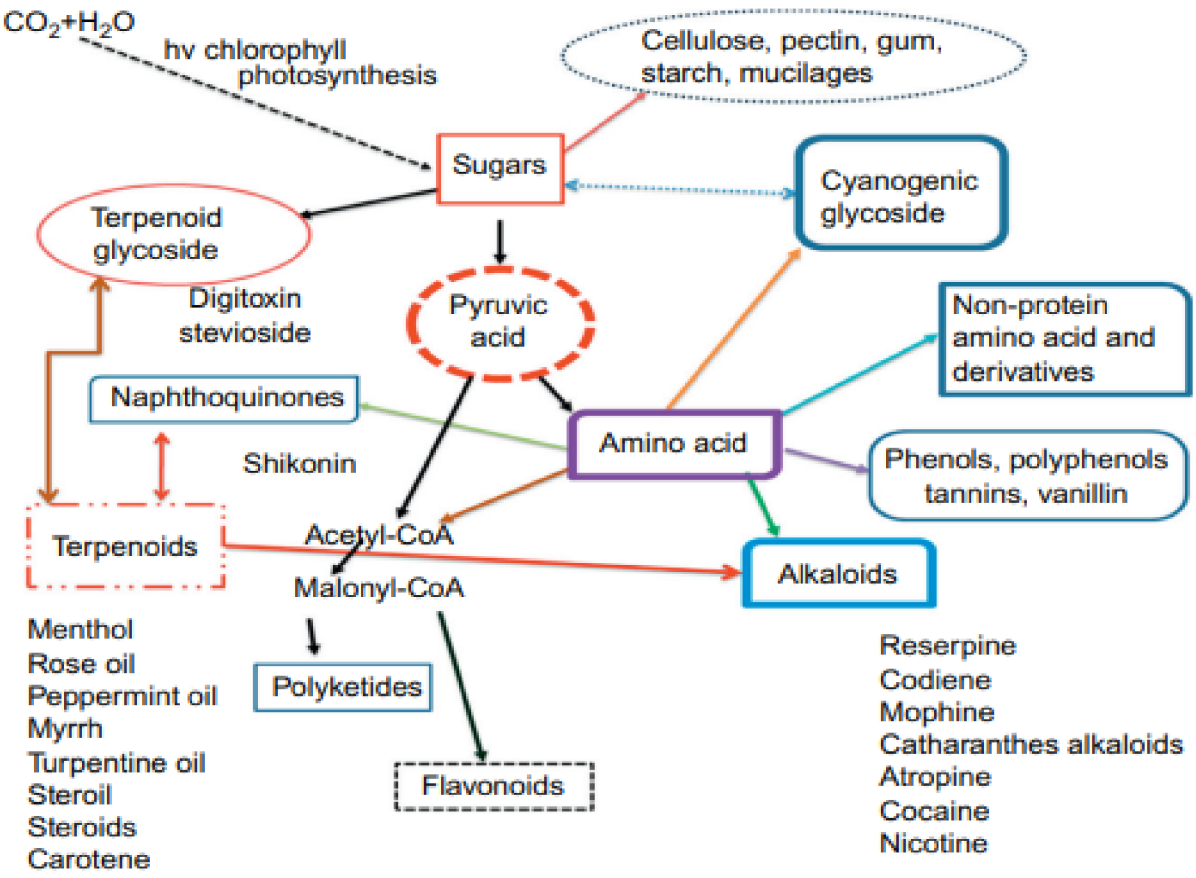

Figure 2: *Biosynthetic pathway of important phyto-compounds. [14]*

The therapeutic actions depend fundamentally on age, geographical location, and parts of the plant species used (Firenzuoli and Gori, 2007). The variability in chemical composition of herbal products from the same plant species leads to differences in pharmacological activity. Also, the timing of harvesting process and incidents of adulterations with microorganisms affects in attaining the absolute standards of herbal medicines globally. [19]

2.2.2 Biologically active compounds in plants

Among the most important antimicrobial substances are, for example, bacteriostatics, which block the function of macromolecules (proteins, nucleic acids). They stop physiological functions by damaging the cellular components and, thus, stop the growth of the microorganism.

2.2.2.1 Mechanism of action

a) Antimicrobials

In the pharmaceutical or food industry, antimicrobials are used as preservatives because they limit the proliferation of undesirable microorganisms. Examples of their use in everyday life can be found, for example, in sterile products such as eye drops (killing of unwanted microorganisms that have penetrated the packaging during use). Preservatives must be able to act in very small amounts as opposed to sterilizers [14].

Weak organic acids are among the most common preservatives. Molecules of these acids inhibit the growth of bacterial, fungal and yeast cells. These preservatives have an optimal inhibitory effect at low pH values, which promote the undissociated state of the molecule. The undissociated molecule is freely permeable across the plasma membrane and, thus, able to enter the cell. Consequently, at higher pH inside the cell, the molecule dissociates, resulting in the release of charged anions and cations that cannot pass through the plasma membrane. Another examples of antimicrobials are alcohol (ethanol, isopropanol, benzyl alcohol), aldehydes (glutaraldehyde, formaldehyde), halogens, metals, surfactants, and dyes. Next are bacteriocins, antibacterial proteins produced by bacteria that stop or inhibit the growth of other bacteria. Many lactic acid bacteria produce a high number of diverse bacteriocins. These bacteriocins-are found in numerous fermented and non-fermented foods. Here, nisin in particular is widely used as a preservative today. Bacteriocins are usually classified into 3 or 4 groups, which are divided on the basis of similar or different properties and also according to the composition of peptides and some amino acids (AMK) [21,22,23].

b) Phytoncides

Phytoncides are an essential part of the essential oils from plants. They are obtained from plant materials by pressing, fermentation, extraction and most commonly steam distillation. The essential oil can contain more than 60 individual components. The main component can represent up to 85 % of the essential oil, while other substances can only be present in trace amounts. Some of these minor components may play an important role in the antibacterial effect, presumably through synergistic effects with other components of the essential oil. Such is the case with sage, some species of sage and oregano. Many phenolic compounds, terpenoids, nitrogen heterocyclic compounds and many others may be responsible for antimicrobial activity in food-important plants. The best-known active ingredients are allicin in garlic, eugenol in cloves, cinnamaldehyde and eugenol in cinnamon, carvacrol and thymol in oregano and thyme and vanillin in vanilla pods [24].

Phytoncides also exhibit other beneficial effects, such as anti-fungal, anti-inflammatory, anti-stress, analgesic, and anti-spoilage activities, and they can be used as food preservatives. They also exhibit anti-mycoplasma activity, anti-larvicidal activity against malaria and dengue, anti-septic activity, and anthelminthic activity; in addition, they facilitate wound healing, can act as cholesterol inhibitors, can enhance sleep, and even enhance bacterial susceptibility to antibiotics. Phytoncides exert allelopathy effects, where these secondary metabolites from plants affect microorganisms, acting as a defence mechanism for plants. Hence, phytoncides are recognized as allelochemicals. Phytoncides can prevent mucosal damage in the digestive tract and have been proven to have anti-inflammatory effects in the stomach, colon, and other digestive parts such as the aesophagus, small intestine and duodenum [24].

c) Antioxidants

Antioxidants defend cells against the harmful result of reactive oxygen species known as free radicals (like singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxynite) which increase oxidative stress and cause cell injury. Natural antioxidants, such as vitamin C

and E and polyphenols, can be found in fruits and vegetables, and are generally considered to be beneficial in human diet. Important antioxidants are also carotenoids that are described in chapter below.

Oxidative stress

Oxidative stress is an imbalance between (increased) production of reactive oxygen species (ROS) and oxidized metabolites and the limited capacity of antioxidant mechanisms. Stress can be caused by ionization, UV radiation, smoking, smog, inflammation etc. The most common endogenous causes are electron leakage during the respiratory chain, breakdown of phagocytes and macrophages (inflammation, burns, sepsis), NADPH oxidase, uric acid formation (injuries, necrosis, postoperative conditions), methaemoglobin formation (oxidized form of Fe^{3+} in Hb). Superoxide and peroxide are not very reactive forms of oxygen, the main danger is the conversion to a hydroxyl radical [25].

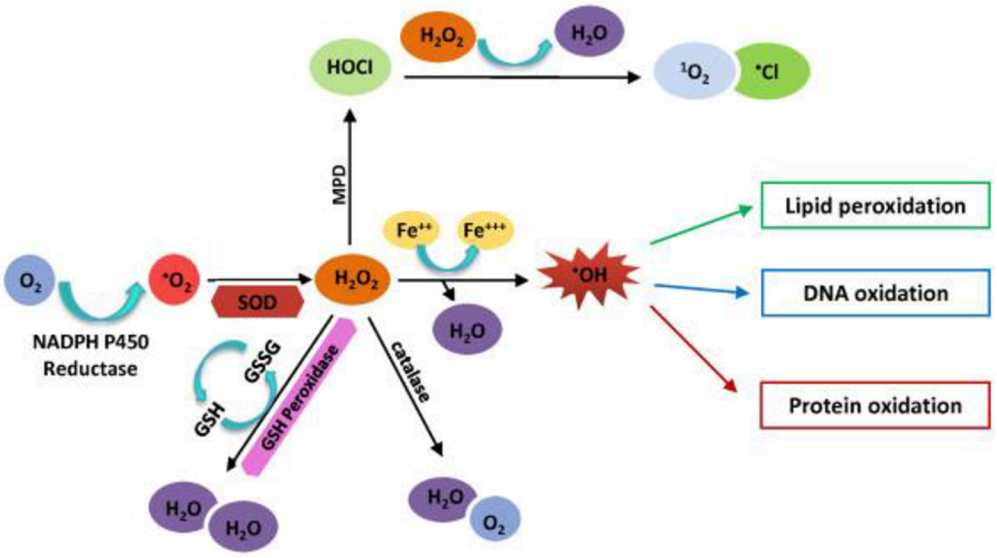

Figure 3: *Redox reaction in cell [26]*

Free radicals that are form in the human body can be eliminated by a natural antioxidant mechanism. If the capacity of endogenous antioxidants is insufficient, their deficiency must be supplemented from external natural sources containing natural antioxidants such as vitamin C, vitamin E, flavones, and carotenes [26].

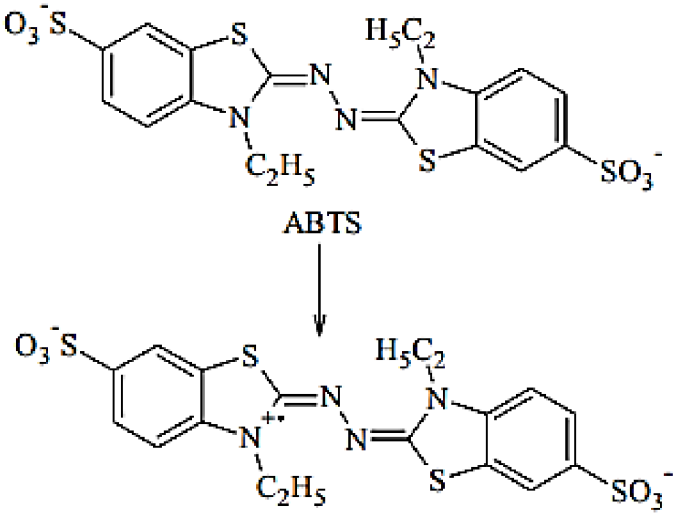

Figure 4: *Electron oxidation ABTS*

2.2.2.2 Selected biological active compounds of plant origin

a) ***Phenols and polyphenols***

Phenolic compounds are widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light [26,27]. These compounds include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins [28,29].

Polyphenols

Polyphenols are natural antioxidants in plants, especially fruits and vegetables, and play a vital role in human health as antioxidants, enzyme cofactors, or heavy metal scavengers. Epidemiological studies show positive correlations between increased fruit and vegetable intake and decreased incidence of cardiovascular disease, aging and other degenerative diseases associated with oxidative stress [30].

Polyphenolic compounds contain two or more hydroxyl groups attached to the aromatic nucleus in their molecule. They are present in almost all plants, where they perform various functions, as protects plants from oxidative stress, UV radiation and pathogens, tannins protect plants from being eaten by herbivores, lignans form the mechanical reinforcement of the plant body and other polyphenols can function as signalling molecules [30].

The mechanism of action of individual polyphenols depends in most cases on their chemical structure, which is very diverse, and therefore there are several possibilities by which polyphenols protect the organism against tumors. Based on the number of protective properties and mixtures of polyphenols present in the diet, it can be assumed that their action against carcinogenesis is probably complex in nature, consisting of the contributions of individual substances. The mechanism of action of polyphenols as inhibitors of angiogenesis is different. Some inhibit the growth and division of the endothelium, while others prevent the activation of

receptors by growth signals, inhibit protein kinase C, prevent endothelial cell migration or inhibit the production of angiogenic factors. Reliene et al. in their study deal with nanoencapsulation of bioactive components of pomegranate as a possible prevention of breast cancer. Pomegranates contain many polyphenolic substances with high antioxidant activity, free radicals, tannins and fiber [31,32,33].

Flavonoids

Flavonoids are an important class of natural products; particularly, they belong to a class of plant secondary metabolites having a polyphenolic structure, widely found in fruits, vegetables, and certain beverages. They have miscellaneous favourable biochemical and antioxidant effects (shown in figure 5) associated with various diseases such as cancer, Alzheimer's disease. In nature, flavonoid compounds are products extracted from plants and they are found in several parts of the plant. Flavonoids are used by vegetables for their growth and defence against plaques. [34].

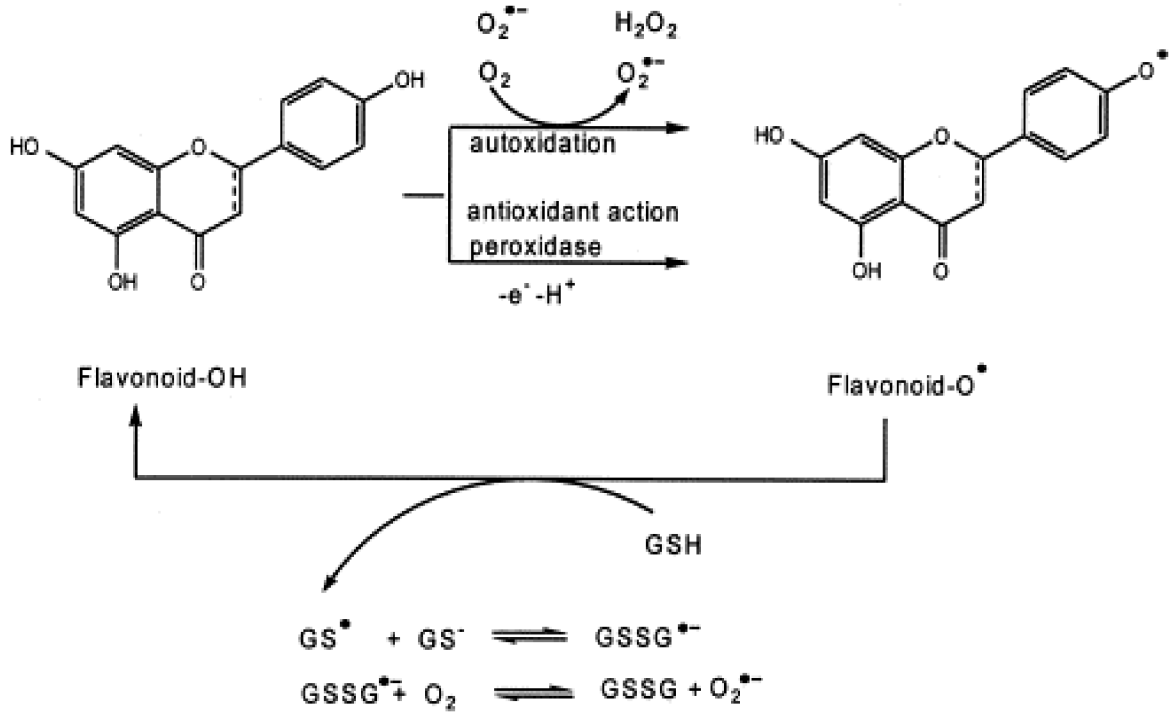

Figure 5: *Antioxidant effect of flavonoids [34]*

2.2.3 Spices with antibacterial activity

The most common reasons for using traditional medicine are that it is more affordable, more closely corresponds to the patient's ideology, allays concerns about the adverse effects of chemical (synthetic) medicines, satisfies a desire for more personalized health care, and allows greater public access to health information. The major use of herbal medicines is for health promotion and therapy for chronic, as opposed to life-threatening, conditions. Spices have been used as food and flavouring since ancient times, and as medicine and food preservatives in recent decades. Many spices such as clove, oregano, thyme, cinnamon, and cumin have been applied to treat infectious diseases or protect food because they were experimentally proved to

possess antimicrobial activities against pathogenic and spoilage fungi and bacteria. Moreover, the secondary metabolites of these spices are known as antimicrobial agents, the majority of which are generally recognized as safe materials for food with insignificant adverse effects. Therefore, spices could be candidates to discover and develop new antimicrobial agents against foodborne and human pathogens [35].

Thyme (*Thymus vulgaris*), belonging to family Lamiaceae, is a subshrub native to the western Mediterranean region. Thyme is widely used as a spice to add special flavor to foods. In recent studies, thyme was found to possess efficient antimicrobial activities and was used in some foods to extend the shelf-life. Thyme EO showed effective bactericidal and antifungal activities against tested microorganism strains with MICs ranging from 75 to 1100 μg/mL for bacteria, and from 80 to 97 μg/mL for fungi. In another study, EOs obtained from thyme harvested at four ontogenetic stages were tested for their antibacterial activities against nine strains of Gram-negative bacteria and six strains of Gram-positive bacteria using the bioimpedance method to test the bacteriostatic activities and plate counting technique to study the inhibitory effects by direct contact. The major active compound of thyme is thymol, which exerted its antimicrobial action through binding to membrane proteins by hydrophobic bonding and hydrogen bonding, and then changing the permeability of the membranes. Thymol also decreased intracellular adenosine triphosphate (ATP) content of *E. coli* and increased extracellular ATP, which could disrupt the function of plasma membranes. As thymol was proved to act differently against Gram-positive and Gram-negative bacteria, the exact mechanisms of antimicrobial action should be further studied [35].

Cinnamon (*Cinnamomum zeylanicum*), belonging to family Lauraceae, is widely applied in savory dishes, pickles, and soups. Cinnamaldehyde, cinnamyl acetate, and cinnamyl alcohol are the three main compounds of cinnamon. Due to its antimicrobial activities, cinnamon is also used in cosmetics or food products, and also used as health-promoting agents to treat diseases like inflammation, gastrointestinal disorders, and urinary infections.

The antimicrobial activities of cinnamon were evaluated in some studies. Gupta et al. compared the antimicrobial activities of cinnamon extract (50 % ethanol) and EO against 10 bacteria and 7 fungi by the agar well diffusion method. Cinnamon EO was more effective than cinnamon extract against tested microorganisms, with the MICs ranging from 1.25% to 5% v/v. Cinnamon EO exerted the strongest effect on *B. cereus* among bacteria, and *Rhizomucor* sp. among fungi. Cinnamon extract showed the highest activities against *B. cereus* among bacteria, and *Penicillium* sp. among fungi. Ceylan et al tested the antibacterial effects of cinnamon, sodium benzoate, potassium sorbate, and their combinations on *E. coli* at 8 and 25 °C in apple juice. The results showed that 0.3% w/v cinnamon provided 1.6 log CFU/mL reduction on *E. coli* at 8 °C and 2.0 log CFU/mL reduction at 25°C. Cinnamon had synergistic effects with sodium benzoate and potassium sorbate on *E. coli* at 8 and 25°C. Recently, the anti-biofilm effects of cinnamon EO and liposome-encapsulated cinnamon EO on methicillin resistant *S. aureus* (MRSA) were evaluated in a study by scanning electron microscopy and laser scanning confocal microscopy analyses. Cinnamon EO possessed effective antibacterialactivity and prominent anti-biofilm activity against MRSA. In the presence of liposomes, the stability

and the acting time of cinnamon EO were further improved. The major component of cinnamon, cinnamaldehyde, possesses antimicrobial effects on microorganisms, as it inhibited cell wall biosynthesis, membrane function, and specific enzyme activities. More specific cellular targets of cinnamaldehyde are still required to be studied in detail [35].

Cumin (*Cuminum cyminum*) is an aromatic plant belonging to the Apiaceae family. Cumin has been used since ancient time as an ingredient in foods in Middle East, and cumin seeds have long been used as antiseptic and disinfectant in India. Cuminaldehyde, cymene, and terpenoids are the major bioactive constituents of cumin EOs [35].

In a previous research, the antimicrobial activities of cumin EO against *E. coli, S. aureus, S. faecalis, P. aeruginosa,* and *K. pneumoniae* were investigated by agar diffusion and dilution method. *E. coli*, *S. aureus*, and *S. faecalis* were susceptive to various cumin EO dilutions while P. aeruginosa and *K. pneumoniae* were resistant. In another study, the antifungal activities of cumin seeds EO against 1230 fungi isolated from food samples were tested. The EO was fungicidal to most of the fungal species and exerted a broad spectrum of fungal toxicity at MIC (0.6 μL/mL) against all 19 foodborne fungi strains except *R. stolonifer*. Furthermore, Abd El Mageed et al. explored the effects of microwaves on EO of cumin seeds on its antimicrobial activities against *E. coli*, *S. aureus*, *P. aeruginosa*, *A. niger*, *A. parasiticus*, and *C. albicans* using the disk diffusion method. Both microwave and conventionally (oven) roasted cumin oils had similar antimicrobial effects on microorganisms tested and were more effective than those of raw oils. Reza et al. studied the effects of ã-irradiation (10 and 25 kGy) on the antibacterial activities of cumin against *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus*, by the agar well diffusion method and disk diffusion method. The results indicated that cumin EO exerted antibacterial effects on bacteria tested, and irradiation (10 and 25 kGy) to cumin seeds had no significant effects on the antimicrobial activities of cumin [35].

Rosemary (*Rosmarinus officinalis*), belonging to the Lamiaceae family, is a perennial shrub with pleasant smell and grows all over the world. Rosemary has been used in pharmaceutical products and traditional medicine, and also used as a flavoring agent in food products due to its desirable flavor, antioxidant activities, and antimicrobial activities.

Tavassoli et al. reported rosemary EO suppressed *Leuconostoc mesenteroides*, *Lactobacillus delbruekii*, *S. cerevisia*, and *C. krusei*. The results indicated that rosemary EO showed higher inhibitory effects on bacteria (MICs: 0.5–1.5 mg/mL) tested than on yeasts. Bozin et al. identified the antimicrobial activities of EOs of rosemary and sage against 13 bacterial strains and 6 fungi by the microdilution technique. Compared with bifonazole, rosemary EO showed better antifungal activities especially against *C. albicans*, *Trichophyton tonsurans* and *Trichophyton rubrum* at lower MICs (15.0–30.2 μL). Rosemary EO also expressed important antibacterial activities on *E. coli*, *S. typhimurium*, *S. enteritidis*, and *Shigella sonei*. Weerakkody et al. compared the antibacterial effects of extracts from seven spices and herbs on *E. coli*, *S. typhimurium*, *L. monocytogenes*, and *S. aureus* by the agar disc diffusion and broth dilution assay. The results of both methods indicated that hexane extract of rosemary exhibited significantly higher antibacterial activities than ethanol and water extracts

against all bacteria tested except *S. typhimurium* with the MICs ranging from 1.25 to 5.0 mg/mL [35].

Garlic (*Allium sativum*) belongs to the Liliaceae family. The antimicrobial activities of garlic have been recognized for many years, and the active component was identified as allicin, a diallyl thiosulfinate (2-propenyl-2-propenethiol sulfonate).

In a study, Sallam et al. examined the antimicrobial effects of fresh garlic, garlic powder, and garlic oil on microorganisms in raw chicken sausage by aerobic plate count. Garlic materials showed antimicrobial activities in such an order: fresh garlic > garlic powder > garlic oil > butylated hydroxyanisole. Another study also assessed the antimicrobial activities of dried garlic powders made by different drying methods against S. aureus, E. coli, S. typhimurium, *B. cereus* and a mixed lactic culture containing *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*. Fresh garlic exhibited the highest activities followed by freeze-dried powder. The retaining of active components responsible for antimicrobial activities was mainly affected by both drying temperature and time. Chopped garlic at concentrations from 0% to 10% were investigated for the antimicrobial effects in ground beef (stored at refrigerator and ambient temperatures) and raw meatballs (stored at room temperature) by determining the colony counts of total aerobic mesophilic bacteria, yeast, and molds at 2, 6, 12, and 24 h after storage. The results indicated that chopped garlic delayed the growth of microorganisms in ground meat, which depended on the garlic concentrations. The addition of garlic (5% or 10%) to the raw meatball mix reduced the microorganism counting, in terms of total aerobic mesophilic bacteria, yeast, and mold counts. Garlic EO penetrated the cellular membranes and even the menbranes of organelles like mitochondria, damaged organelles, and resulted in the death of *C. albicans*. Furthermore,garlic EO induced differential expression of several critical genes including those involved in oxidation-reduction processes, and cellular response to drugs and starvation [35].

Basil (*Ocimum basilicum*) is one of the oldest spices, which is widely used in the flavoring of confectionary, baked goods, condiments, etc. Basil oil was also used in perfumery, as well as in dental and oral products. Basil is a natural spice which possesses antimicrobial activities as many studies have reported.

In a study, the antimicrobial activities of EOs from aerial parts of basil (collected at full flowering stage during summer, autumn, winter, and spring) against *S. aureus, E. coli, B. subtilis*, and *Pasteurella multocida*, as well as pathogenic fungi *A. niger, Mucor mucedo, Fusarium solani, Botryodiplodia theobromae*, and *R. solani* were assessed by the disc diffusion method and the MICs were determined by a microdilution broth susceptibility assay. The results indicated that basil Eos possessed antimicrobial activities against all tested microorganisms. Antimicrobial activities of the EOs varied significantly as seasons changed, and EOs from winter and autumn crops exhibited greater antimicrobial activities. In another study, the antimicrobial activities of chloroform, acetone and 2 different concentrations of methanol extracts of basil against 10 bacteria and 4 yeasts were determined by the disc diffusion assay. Methanol extracts provided inhibition zones on *P. aeruginosa*, *Shigella sp.*, *L. monocytogenes*,

S. aureus, and two strains of *E. coli*, but the chloroform and acetone extracts exhibited no effects. Kocic-Tanackov et al. reported the antifungal effects of basil extract on *Fusarium* species (*Fusarium oxysporum, Fusarium proliferatum, Fusarium subglutinans,* and *Fusarium verticillioides* isolated from cakes), by the agar plate test. Basil extract showed significant activities against *F. proliferatum* and *F. subglutinans* at the concentration of 0.35 and 0.70% v/v, but showed lower activities against other tested *Fusarium* species. Basil extract 100% inhibited aerial mycelium of all tested *Fusarium* spp. at 1.50% v/v. Beatovic et al. investigated the antimicrobial activities of EOs of 12 basil cultivars against 8 bacterial species (*B. cereus*, *Micrococcus flavus*, *S. aureus* and *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *L. monocytogenes*) and 7 fungi *Aspergillus fumigatus*, *A. niger, Aspergillus versicolor, Aspergillus ochraceus*, *Penicillium funiculosum, Penicillium ochrochloron*, and *Trichoderma viride* by a modified microdilution technique. All basil EOs tested showed significant antimicrobial activities, with MICs ranging from 0.009 to 23.48 µg/mL for bacteria and 0.08–5.00 µg/mL for fungi. All the EOs showed 100-fold higher antibacterial activities than ampicillin for some bacteria, and 10- to 100-fold higher antifungal activities than the commercial antifungal agents, e.g., ketoconazole and bifonazole [35].

Fennel (*Foeniculum vulgare*), belonging to family Umbellifarae, is widely planted in temperate zones and the tropical belt for its aromatic fruits, and is used as an ingredient in the cooking. The EO of fennel seeds has been reported with significant antifungal activities and antibacterial activities.

In a study, the antibacterial activities of fennel seeds EO against *Streptococcus* strains were tested. The results showed that growths of all *Streptococcus* mutans strains tested were completely inhibited by fennel seeds EOs at concentrations higher than 80 ppm. Diao et al. also determined the antibacterial activities of EO from fennel seeds against several foodborne pathogens by the kill-time curve assay method. The results showed that fennel seeds EO exerted antibacterial effects on *Streptomyces albus, B. subtilis, S. typhimurium, Shigella dysenteriae.* and *E. coli*, among which *S. dysenteriae* was the most sensitive with the lowest MIC (0.125 mg/mL) and MBC (0.25 mg/mL). In another study, the antimicrobial activities of crude extract of fennel was determined using the agar diffusion method against *E. coli, S. blanc, P. merabilis, P. vulgaris, S. epidemidis, S. saprophyticus, A. versicolor, A. fumigates,* and *Penicilium camembertis*. The results indicated that the crude extract of fennel had a great potential as an antimicrobial material against all the nine microorganisms tested, especially fungal strains. Some studies also tested the methanol, ethanol, and acetone extracts of fennel. In a study, the antifungal activities of EO and acetone extract of fennel against 10 fungi were assessed by the inverted petriplate method. The results showed that fennel EO completely inhibited *A. niger, A. flavus, F. graminearum,* and *Fusarium moniliforme* at 6 µL (in 20 mL culture medium), and it was effective on *A. niger* even at 4 µL. Fennel seed EO could break the permeability of cell membrane of *S. dysenteriae* and result in the leakage of electrolytes, losses of proteins, reducing sugars, etc., and eventually lead to the decomposition and death of cells [35].

2.2.4 Sellected plant extacts used in this thesis

In presented thesis several plants from different origin were used to evaluate its antimicrobial effect. Plant selection was based on previous results.

2.2.4.1 Acai

The açaí berry is a small round berry (size of a grape) that is green when immature and ripens to a dark purple colour. Açaí pulp (*Euterpe oleracea*) has received much attention in recent years as one of the new 'superfruits'. As a food, açaí berry is consumed raw and as juice. The juice is used commercially in jelly, syrup, ice cream, liquors, energy drinks and a variety of other beverages, and can be used as a natural food colorant. Açaí juice is viscous and contains about 2.4 % protein and 5.9 % lipids. The fruit pulp contains about 4 % protein and 12 % lipids. Nutrients include vitamins A, C, and E, calcium, phosphorus, iron, thiamine, polyphenols, and anthocyanins. The consumption of açaí from the Amazon region has been increasing, mainly owing to the benefits reported in the scientific literature. Açaí fruit possesses two predominant anthocyanins, cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R). The *Euterpe* genus has high economic potential, since it is used to prepare açaí beverages which are exported all over the world as energy drinks [36].

2.2.4.2 Clove

Clove is derived from flower buds of the *Eugenia caryophyllata* tree. Several bioactive components are found in clove, including tannins, terpenoids, eugenol, and acetyleugenol. While no studies have been conducted in humans to date to evaluate use of cloves in cancer prevention, a few studies conducted in mice suggest its effectiveness, especially in modifying cellular detoxification processes. Antimicrobial activities of clove water extract were studied in vitro and in vivo against pathogenic microorganisms (*S. aureus* and *E. coli*, in a model of pyelonephritis) [37]. An in vitro study was conducted with the agar well diffusion method, and the results suggested that clove water extract showed antibacterial activity against S. aureus (minimum inhibitory concentration (MIC: 2 mg/mL) and E. coli (MIC: 2.5 mg/mL). While in vivo, the study was conducted in 40 adult male albino rats, and the results confirmed the efficacy of clove extract as natural antimicrobials. The direct antimicrobial activities of ultra-fine powders of ball-milled cinnamon and clove were tested by Kuang et al. [38,39,40].

The major component of clove oil is usually considered to be eugenol, with β-caryophyllene and lesser amounts of other components such as benzyl alcohol, but the proportions vary widely. Clove essential oil, used as an antiseptic in oral infections, inhibits Gram-negative and Gram-positive bacteria as well as yeast [41].

2.2.4.3 Curcuma

C. longa is a common spice in the United States and Asian countries which is known to be useful for health. Turmeric has been used to treat coughs, jaundice and other common diseases in Indian Ayurvedic and traditional Chinese medicines. Curcumin, as a member of curcuminoids, is a natural nonflavonoid phenol. The main components of the *Curcuma longa* extract are curcumin (2269.2 ± 12.3 mg/100 g), bisdemethoxycurcumin

(1283.5 ± 8.5 mg/100 g), and demethoxycurcumin (1284.6 ± 7.0 mg/100 g), as identified using high-performance liquid chromatography (HPLC) analysis [42].

Turmeric rhizome contains two major classes of secondary metabolites: phenolic curcuminoids and essential oil. These metabolites are largely responsible for the pharmacological effects of turmeric. The composition of both metabolites depends on a genotype, the environment, harvest season, dry process, and storage conditions. Curcuminoids are responsible for the yellow color of the turmeric, and the essential oil that it contains for its aroma and taste. The major and the most studied curcuminoid found in turmeric is curcumin, which is recognized as the most responsible compound for most beneficial effects which this miraculous plant exhibits [43].

Turmeric is used as a dietary spice, coloring agent in foods and textiles, and a treatment for a wide variety of ailments. It is widely used in traditional Indian medicine to cure biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis. There is also significant effect in health benefits with psoriasis, cardiovascular disease, chemopreventive, liver injury, lung fibrosis and etc.

Anticancer Properties: Curcumin has been shown to block transformation, tumor initiation, tumor promotion, invasion, angiogenesis, and metastasis. *In vivo*, curcumin suppresses carcinogenesis of the skin, forestomach, colon, and liver in mice.

Skin Diseases: Curcumin has been shown to be effective against different skin diseases including skin carcinogenesis, psoriasis, scleroderma, and dermatitis [44,45].

2.2.4.4 Ginger

Ginger is a member of a plant family that includes cardamom and turmeric. Its spicy aroma is mainly due to presence of ketones, especially the gingerols, which appear to be the primary component of ginger studied in much of the health-related scientific research. The rhizome, which is the horizontal stem from which the roots grow, is the main portion of ginger that is consumed.

Although many food-derived supplements are consumed today with little knowledge of their activity or safety, more attention is beginning to be given to addressing these issues. The most well-studied bioactive component of ginger is probably [6] - gingerol.

Although the medicinal properties of ginger have been known for thousands of years, a significant number of *in vitro*, *in vivo*, and epidemiological studies further provide substantial evidence that ginger, and its active compounds are effective against wide variety of human diseases including GI cancer. Ginger has been found to be effective against various GI cancers such as gastric cancer, pancreatic cancer, liver cancer, colorectal cancer, and cholangiocarcinoma. However, its anticancer effects on other GI cancers like duodenal, oesophageal, anal, GI carcinoid tumor and pancreatic islet cell cancer have yet not been established. Therefore, efficacy of such potent agents on these cancers is warranted. Ginger and its polyphenols have been shown to target multiple signalling molecules that provide a basis for its use against multifactorial human diseases [46].

Ginger was proved to possess antimicrobial activities in several studies. Singh et al. determined the antifungal activities of EO and oleoresin of ginger against *Aspergillus terrus*, *A. niger*, *Aspergillus flavus*, *Trichothecium roseum*, *Fusarium graminearum*, *Fusarium oxysporum* and *Curvularia palliscens*, by food poison and inverted petri-plate technique. The results showed that the EO 100% inhibited *F. oxysporum*, while the oleoresin 100% inhibited *A. niger*. [47] Park et al. compared the ethanol and n-hexane extracts of ginger and five ginger constituents against three anaerobic Gram-negative bacteria, *Porphyromonas, Porphyromonas endodontalis* and *Prevotella intermedia*. The results indicated that ginger extracts exhibited antibacterial activities against three tested bacteria. Two highly alkylated gingerols showed significant inhibition against the growth of these oral pathogens with the MICs ranging from 6 to 30 μg/mL, and also killed the oral pathogens at a MBC range of 4–20 μg/mL. Sa-Nguanpuag et al. evaluated the in vitro and in vivo antimicrobial activities of ginger oils which were obtained by hydrodistillation and solvent extraction method [48] The results showed that the oils extracted by both methods possessed antimicrobial activities against *B. subtilis*, *Bacillus nutto*, *P. aerugenosa*, *Rhodoturola sp*., *Samonella newport*, *S. enteritidis*, and *Fu*sarium sp.; except *E. coli*, *Campylobactor coli*, and *Campylobactor jejuni* (C. jejuni) *in vitro*. In the case of shredded green papaya, when the package was added with 5 and 10 μL ginger oils the growth of microorganisms was inhibited well, while with 15 μL ginger oil a reduction in growth rate was observed [35]

2.2.4.5 Ginseng

Ginseng is a medicinal plant widely used for the treatment of various conditions. The pharmacological effects of ginseng have been demonstrated in cancer, diabetes, cardiovascular diseases and have been used for promoting immune function, central nervous system (CNS) function, relieving stress, and for its antioxidant activities (Jung and Jin 1996). Korean ginseng contains various functional constituents in addition to saponin, such as phenolic compounds with antioxidant activity, polyacetylene, which exhibits cytotoxicity to cancer cells, sesquiterpene, an essential oil, and acidic polysaccharides, which are currently studied actively in relation to immune response [49,50].

2.2.4.6 Oregano

Oregano belonging to family *Lamiaceae*, has been used as food seasoning and flavouring for a long time. Origanum species are a wide group of plants that are rich in several compounds, such as monoterpene hydrocarbons, oxygenated monoterpenes, phenolic monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and phenolics. Origanum presents a broad variety of properties and uses, such as antifungal, insecticidal, antibacterial, antiparasitic, and antioxidant activities. Babacan et al. [51] evaluated the antimicrobial activities of oregano extract against various Salmonella serotypes by evaluating the bacterial growth with disc diffusion method. The results showed that DIZs of oregano were 15, 19, and 16 mm for *Salmonella gallinarum* (*S. gallinarum*), *Salmonella enteritidis* (*S. enteritidis*), and *S. typhimurium*, respectively. Santoyo et al. [51] observed the antimicrobial activities of EO-rich fractions of oregano which were selectively precipitated in the second separator in different conditions against six microorganism strains (*S. aureus, Bacillus subtilis, E. coli, P. aeruginosa,*

C. albicans, and Aspergillus niger) using the disk diffusion and broth dilution methods. [52,54,53,54].

2.3 ANTIMICROBIALS OF MICROBIAL ORIGIN

Plant metabolites and bioactive compounds from chemical synthesis have found their efficacy to be dwindling, despite some of them being developed as drugs and used to treat human infections for several decades. Microorganisms are considered untapped reservoirs for promising biomolecules with varying structural and functional antimicrobial activity.

2.3.1 Antibiotics

Worldwide consumption of antibiotics has increased drastically in the past few decades. The application of antibiotics has led to the production of antibiotic resistance genes (ARGs) which represent a growing and serious human health threat worldwide.

The discovery of first antibiotic occurred by chance when a staphylococci agar plate inoculated by Sir Alexander Fleming got contaminated by a mold. The mold colony displayed a clear zone of bacterial inhibition around itself (Fleming 1980). The compound which was suppressing bacterial growth was identified as penicillin, which was then used as antibiotics to cure many infections and diseases caused by bacteria. Discovery of sulfonamides and β-lactam antibiotics in 1930s leads to an immense improvement in health and medicine services as diseases and bacterial infections which were fatal earlier became curable. Introduction of streptomycin in 1944 was another achievement for the cure of tuberculosis. The golden era for antibiotic discovery was marked till 1970, up to where maximum classes of antibiotics were discovered [55].

2.3.1.1 Mechanism of action

An ideal antibiotic should not interfere with the normal functioning of the host cell but should bring about inhibition of target microbe by disturbing its metabolism. This disturbance can be interference or inhibition of any biomolecule such as enzyme, nucleic acid, polysaccharides, or metabolites critical for survival [55].

Replication (of Genetic Material) and Transcription Inhibitors. DNA replication is one of the essential functions of growing cells and hence inhibition of this process results in inhibition of cell division. Antibiotics which bind to form complex with important components of DNA replication such as topoisomerase can cause bacterial death Similarly, all kinds of RNA are synthesized by the action of DNA-dependent RNA polymerase through transcription, which is again essential for growth. Rifamycins bind with the β subunit of DNA-dependent RNA polymerase and inhibit the initiation and ribonucleotide chain growth in transcription. Acridines disturb the phosphate backbone of DNA helix and actinomycin D binds with DNA double helix to hamper the movement of RNA polymerase across the DNA chain to inhibit transcription and hence cause bactericidal effect [55].

Protein Synthesis Inhibitors. Synthesis of mRNA (messenger ribonucleic acid) involves the role of ribosomes (30S and 50S) and its interaction with other components, which is the target of antibiotics inhibiting translation. Streptomycin and tetracycline disrupt the 30S subunit

of ribosome to block its interaction with amino acyl tRNA (transfer ribonucleic acid) and discontinuing protein synthesis. Macrolides and chloramphenicol act by blocking the 50S subunit whereas kanamycin and gentamicin act at the 16S rRNA (ribosomal ribonucleic acid) of the 30S ribosome to inhibit protein synthesis [55].

Cell Wall Synthesis Inhibitors. Both the Gram-positive and Gram-negative bacterial cell walls are composed of variable amount of peptidoglycan which maintains the integrity of cell wall and helps the bacteria to survive extreme environmental conditions. Antibiotics such as penicillin, cycloserine, and vancomycin either inhibit the synthesis of peptidoglycan or its assembly with other components to form an intact and functional cell wall, which then leads to bacterial cell wall lysis [55].

Inhibitors of Cytoplasmic Membrane Function. Lipids, proteins, and lipoproteins are the major constituents to form cytoplasmic membrane which acts as a differentially permeable barrier for exchange of ions, nutrients, and water in a cell. Antibiotics like polymyxin, tyrocidin, valinomycin, and amphotericin B disrupt the structure of the membrane and cause unwanted exchange or leak of cellular components to outside (Ca^{2+}, K^{+}, Mg^{2+} ions, metabolites, and nutrients). These components are involved in various essential processes for survival such as maintaining osmotic pressure of cell, oxidative phosphorylation, and protein biosynthesis and hence cause bacterial cell death [55].

Antibiotics are produced by several groups of microbes such as bacteria, fungi, and actinomycetes as their natural defense system against other microbes living in their vicinity. Soil microorganism had always been the primary source for the production of antibiotics and still continues to maintain its significance. But indiscriminate use of antibiotics and disinfectants in medicine, agriculture, and fish culture and their release in environment have given birth to another critical problem of multidrug-resistant pathogenic microbes, and hence, we are still in need of more and more effective metabolites that can be used to combat these resistant strains. Isolation of antibiotics from microorganism is much easier than chemical synthesis of these compounds and hence has resulted in discovery of countless novel antibiotics till date. Fungal antibiotics such as penicillins, cephalosporin, fusidic acid griseofulvin, and fumagillin have been obtained by fungal species *Penicillium*, *Cephalosporium*, and *Aspergillus*. Several *Pseudomonas* species and *Bacillus* species are among the soil bacteria which have been exploited for the production. of antibiotics like gramicidin, bacitracin, tyrothricin, pyocyanin, and pyrrolnitrin *Streptomyces* species is one of the soil actinomycetes which have provided the highest number of commercial antibiotics such as tetracyclines, streptomycin, viomycin, and kanamycin. Several other commonly used antibiotics, gentamicin and rifamycin, have been isolated from actinomycetes like *Micromonospora*, *Actinomadura*, and *Nocardia* species. [55]

2.3.2 Antimicrobial peptides

Many microbial metabolic products act as growth inhibitors against other microorganisms, including spoilage and pathogenic bacteria. Many gram-positive bacteria often produce cationic, amphiphilic, membrane-permeabilizing peptides that are smaller in size and have antimicrobial activity against a wide range of microorganisms. For example, *Lactobacillus spp.*

produce one of the groups of antimicrobial peptides known as bacteriocins that can exhibit potent antimicrobial activity. The antimicrobial activities of bacteriocins are due to a heterologous subgroup of ribosomally synthesized cationic peptides (de Vugst & Vandamme 1994a). Various classification schemes have been proposed for bacteriocins (Tiwari et al. 2009). For example, in one such classification, bacteriocins are named after the genus, species,or family of bacteria producing them, such as lantibiotics for the bacteriocins produced by *Lactobacillus spp.* and colicins for bacteriocins from *E. coli* (Riley & Chavan 2006) [71].

Nisin is a ribosomally synthesized polycyclic peptide that contains unusual amino acids, including lanthionine, methyllanthionine, didehydroalanine, and didehydroaminobutyric acid (Hansen 1994). Nisin is commercially produced by fermentation of modified milk medium using several strains of nisin-producing L. lactis and is an effective antimicrobial against many grampositive bacteria. The antibacterial activity of nisin is attributed to its interaction with phospholipids in the cytoplasmic membrane of bacterial cells resulting in disruption of normal membrane function [71].

2.3.2.1 Classification of AMPs Based on Sources

The sources of AMPs can be divided into mammals (human host defense peptides account for a large proportion), amphibians, microorganisms, and insects according to statistical data in APD3. The AMPs found in oceans have also attracted widespread attention.

Mammalian Antimicrobial Peptides. Mammalian antimicrobial peptides are found in human, sheep, cattle, and other vertebrates. Cathelicidins and defensins are the main families of AMPs. Defensins can be divided into α-, β-, and θ-defensins depending on the position of disulfide bonds. Human host defense peptides can protect human from microbial infections but show different expressions in every stage of human growth. For example, cathelicidin LL-37, a famous AMP derived from the human body, is usually detected in the skin of newborn infants, whereas human beta-defensin 2 is often expressed in the elderly instead of the young. HDPs can be identified in many parts of the body such as skin, eyes, ears, mouth, respiratory tract, lung, intestine, and urethra. Besides, AMPs in human breast milk also play an important role in breastfeeding because it can decrease the morbidity and mortality of breast-feeding infants. What's interesting is that Casein (peptide derived from β-Casein 201–220 aa), identified in colostrum, shows different levels in preterm human colostrum and term human colostrums. Dairy is an important source of AMPs, which are generated through milk enzymatic hydrolysis. Several AMPs have been identified from α-lactalbumin, β-lactoglobulin, lactoferrin, and casein fractions, and the most famous peptide obtained is lactoferricin B. Furthermore, whether the AMPs derived from dairy products can be used for dairy preservation is also an interesting subject to develop [56,57].

Amphibian-Derived Antimicrobial Peptides. Antimicrobial peptides from amphibians play an important role in the protection of amphibians from the pathogens that have induced the global amphibian population decline. Frogs are the main source of amphibian AMPs and the most famous AMP from frogs is magainin; the skin secretions of frogs from general

Xenopus, Silurana, Hymenochirus, and *Pseudhymenochirus* under the *Pipidae* family are rich in AMPs [58,59].

Insect-Derived Antimicrobial Peptides. Antimicrobial peptides are mainly synthesized in fat bodies and blood cells of insects, which is one of the main reasons for insects' strong adaptability to survival. Cecropin is the most famous family of AMPs from insects, and it can be found in guppy silkworm, bees, *Drosophila*. Cecropin A shows activity against different inflammatory diseases and cancer. What should be known is that the number of AMPs varies greatly between species, for example, invasive harlequin ladybird (*Harmonia axyridis*) and black soldier fly (*Hermetia illucens*) have up to 50 AMPs, while pea aphid (*Acyrthosiphon pisum*) lacks AMPs. Jellein, a peptide derived from bee royal jelly, shows promising effects on several bacteria and fungi, and its lauric acid-conjugated form can inhibit the parasite *Leishmania major* [60,61].

Microorganisms-Derived Antimicrobial Peptides. Antimicrobial peptides can be obtained from microorganisms like bacteria and fungi, and some famous peptides are nisin, gramicidin from *Lactococcus lactis*, *Bacillus subtilis*, and *Bacillus brevis*. Due to the high price of chemical synthesis of AMPs, the biological expression has attracted the increase of attention. Specific yeast species like Pichia pastoris, Saccharomyces cerevisiae, and bacteria like Escherichia coli, B. subtilis, and plants have been used for expression systems, but it should be noticed that because of the toxicity, proteolytic degradation, and purification, AMPs are difficult to be produced in E. coli, which is necessary to take advantage of fusion tags [62,63]

Besides, numerous AMPs have also been extracted and isolated from the stems, seeds, and leaves of plants, and they are classified into several groups, including thionins, defensins and snakins [64]. More marine-derived AMPs have been reported to have given the increasing value allotted by people to marine resources. Although most of the reported marine AMPs have been tested in vitro, several of these AMPs have shown promising results in vivo, for example, As-CATH4 shows an immunity-stimulating effect in vivo and can enhance the anti-infective capability of drugs used in combination with it. Myticusin-beta is an immune-related AMP of Mytilus coruscus and a promising alternative to antibiotics. Moreover, GE33, known as pardaxin, is a marine AMP and the GE33-based vaccine has shown the ability to enhance antitumor immunity in mice [65].

2.3.3 Other antimicrobial substances of microbial origin

Organisms, such as algae, bryozoans, corals, molluscs, sponges, tunicates, and viruses, are considered potential sources of novel antimicrobials. Their external body structures could serve as an avenue for new bioactive compounds. Additionally, the internal enzymatic machinery of some of these microorganisms enables them to produce secondary metabolites with antimicrobial properties. For example, *Pseudovibrios* species, a marine bacterium of the order Rhodobacterales and class alphaproteobacteria, has bioactive structural composition coupled with harbored polyketide synthases, non-ribosomal peptide synthases, or hybrid enzyme systems that putatively aid them to produce secondary metabolites and new bioactive compounds with antimicrobial activity against varying clinical strains, notably *Staphylococcus*

aureus, Bacillus subtilis, and *Escherichia coli* [66]. Psychrophiles, extremophilic organisms that tolerate very low temperatures, were also investigated as a source of new antimicrobials. Given the varying environmental conditions between psychrophiles and temperate regional dwellers and their adaptive evolution, the bioactive compounds produced by the former might presumably differ from the latter, and that merits its consideration as an antimicrobial source. Tadesse et al. [67] identified synoxazolidinones A and B, oxazolidinone derivative antimicrobial isolates from the Norwegian sea squirt, which showed antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Sanchez et al. also reported the bacteriocin properties of Serraticin A, a bioactive compound produced by *Serratia proteomaculans* and with antimicrobial activity against *Escherichia coli* and *Salmonella enterica*. [68] This compound is putatively considered to exhibit such activity by inhibiting DNA synthesis. [69] Similarly, Phelan et al [70] found subtilomycin, a lantibiotic from the marine sponge *Haliclona simulans*, known to exhibit polymyxin B activity (cell membrane inhibition or pore formation) against strains of *Bacillus cereus, Bacillus megaterium, Clostridium sporogenes, Listeria monocytogenes, Listeria innocua, Staphylococcus aureus, MRSA*, and vancomycin-resistant *Staphylococcus aureus* [71].

2.3.4 Oleaginous yeasts as a producers of microbial active compounds

It has been well documented that yeasts have many applications in fermentation, food, feed, agricultural, biofuel, medical, and chemical industries, as well as environmental protection (Adedayo et al., 2011; Kurtzman and Fell, 2000). They have been used for production of fermented food since 7000 BC (Reed and Nagoda-withana, 1988). Yeast biomass from so-called nutritional yeasts is widely used as a source of nutritional components, such as single-cell protein (SCP). Dried and killed *Y. lipolytica* protein - rich biomass is recognized as safe for human and animal nutrition in accordance with the current food and nutrition safety law. It is worth emphasizing that *Y. lipolytica* is is not causing any allergic reactions to humans. It's a valuable source of bioactive compounds such as proteins, trace minerals, vitamins, and other valuable compounds such as yeast biomass itself [72]. The natural occurrence of *Y. lipolytica* in food, particularly in cheese, other dairy products, and meat, is an additional argument in favor of its safety. The occasional occurrence of opportunistic infections of *Y. lipolytica* in immunocompromised and catheterized patients does not differ from other microorganisms with a history of safe use, such as *S. cerevisiae* [73]. *Rhodotorula* produces pink to red colonies and blastoconidia that are unicellular, lacking pseudohyphae and hyphae. Several authors have isolated *Rhodotorula* in different ecosystems and environments. Previously considered non-pathogenic, *Rhodotorula* species have emerged as opportunistic pathogens with the ability to colonise and infect susceptible patients. Recent studies have demonstrated that the incidence of fungemia caused by *Rhodotorula* was between 0.5% and 2.3% in the USA and Europe. Most cases of infection with *Rhodotorula fungemia* are associated with central catheters in patients with haematologic malignancies [74].

In general yeast biomass contains valuable fatty acids, carbohydrates, nucleic acids, vitamins, and minerals. It is rich in certain essential amino acids, such as lysine and methionine, which are limited in most plant and animal foods. Some of them, so-called "red" yeasts, can produce carotenoids pigments. *Phaffia rhodozyma*, another red yeast, produces in large

scale the carotenoid pigment astaxanthin, but this yeast has also the ability to produce torulene and torularhodin in its metabolic pathway. Torularhodin and torulene are two widespread microbial carotenoids. Torularhodin shows a considerable antioxidant activity that helps the stabilization of membranes under stress conditions. These carotenoids are beneficial because they are precursors of vitamin A and hormones, and they have antiaging and antioxidant capacity. They may also prevent certain types of cancer and enhance the immune system. These possibilities make torularhodin and torulene a hot research topic in carotenoid biotechnology [74].

2.3.5 Carotenoids

Carotenoids are among the most widely distributed pigments and naturally exhibit red, orange and yellow colors. Carotenoids are lipid-soluble pigments, which can be found in many kinds of fruit, vegetables, fungi, flowers, and some kinds of animals (Ötles and Cagindi, 2008) [77]. Photosynthetic bacteria, algae, fungi, and plants can produce carotenoids through biosynthesis, whereas carotenoids found in humans as well as animal cells (e.g., lutein and zeaxanthin in human eyes and astaxanthin in salmon) are only from diets (Ellison, 2016; Kaczor et al., 2016) [78]. More than 750 different structures of carotenoids have so far been isolated from natural sources; about 500 structures have been fully characterized (Rodriguez-Amaya, 2016) [79]. Based on their structures, carotenes and xanthophylls are two main subclasses of carotenoids (NgamwonglumLert et al., 2017) [80]. Different carotenoids structures naturally possess different physical, chemical, and functional properties as well as stabilities. Consumption of carotenoids-rich foods as well as carotenoids supplements has become more popular as several researchers have reported that carotenoids can help reduce the risks of several degenerative disorders, including cancer, cardiovascular and ophthalmological diseases due to their antioxidant properties (Stahl and Sies, 2003). The most approved health-promoting attributes of carotenoids for the individuals include their pro-vitamin A function, their crucial role in improving the eye and heart health, and enhancing the brain cognitive functions, prevention of skin cancer by its protection against UV light, contribution to the maternal and infant nutrition, immune modulation/stimulation, and genomic effects on transcription/translation. Systematically, the principal advantages of carotenoids have been ascribed to their potent antioxidant properties [81,82,83,84].

Some study results supported a protective effect of beta-carotene, shown in the Figure 6, supplementation against sunburn in humans, suggesting a potential benefit toward skin cancer risk. However, in a randomized controlled trial investigating the effect of an antioxidant supplementation containing beta-carotene, supplemented women had a significantly increased risk of skin cancer compared to women in the placebo group [83,84, 85].

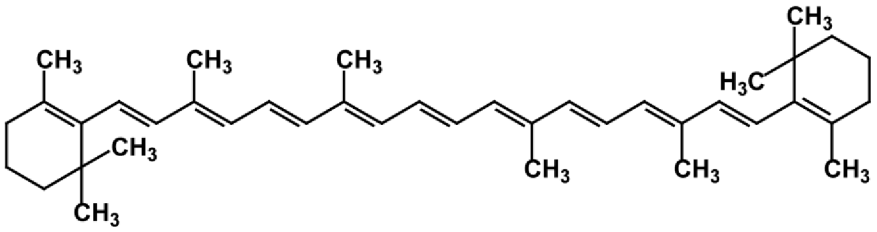

***Figure 6**: β-carotene*

2.3.6 Other biologically active substances of oleaginous yeasts

Some microorganisms are defined oleaginous as a result of their ability to accumulate lipids by as much as 20% of their dry cellular weight. This group includes several eukaryotic microorganisms (such as fungi, yeasts and algae) and some species of autotrophic and heterotrophic bacteria able to accumulate lipids in the form of triglycerides (TAGs) and free fatty acids (FA).

2.3.6.1 Fatty acids

As lipids constitute a major portion of the majority membranes suggest that the presence of massive concentrations of unsaturated FA within membranous structures. In addition, well recognized that the PUFA are bioactive mediators of diverse pathways involved in cellular homeostasis or, in some cases, interact with cellular macromolecules resulting in cell death. These cellular responses may be a consequence of the vulnerability of unsaturated FA to diverse oxidation reactions, or radical reactions, or both. Reactive oxygen species readily bind to unsaturated FA in lipids that contain multiple double bonds (DB), “steal” electrons, and trigger a free radical chain reaction. This oxidative process usually consists of initiation (production of a FA radical), propagation (creation of a peroxyl-FA radical), and termination (production of electrophilic carbonyls. The free radical mediated production of electrophilic products of PUFA proceeds by autocatalysis and is, as a result, not well regulated. SFA are resistant to oxidation. The cellular membranes and lipoproteins containing large amounts of SFA are less active functionally [86,87].

Omega-9 fatty acids exhibit essential myriad of pharmacological activities that pose them as potential candidate to alleviate many pathological conditions. The observed anti-inflammatory effects reported for oleic acid, mead acid, and erucic acid were directed to attenuate inflammation in several physiological and pathological conditions such as wound healing and eye inflammation by altering the production of inflammatory mediators, modulating neutrophils infiltration, and altering VEGF effector pathway. The anti-neoplastic action of omega-9 fatty acids is though controversial compared to its anti-inflammatory actions, with the effect varies with the type of cancerous tissue and the effector pathway. Most documented anti-neoplastic action of omega-9 is evidenced in case of olive oil-rich diets. These diets enriched in oleic acid content are believed to possess chemo preventive effect against breast cancer. Oleic acid antitumor action is mediated via multiple mechanisms including suppression of proliferation and migration and breast cancer cells, as well activation of tumor suppressor genes. [88]

Palmitic acid (C16:0) is one of the important constituent acids of TG in adipose tissue. In this regard, palmitic acid content of blood has the greatest diagnostic and prognostic significance. It is considered that palmitic acid is linked with adverse cardiovascular events.

Stearic acid (C18:0). In contrast to palmitic acid, which correlates with hypercholesterolemia (HC), it is considered that stearic SFA does not have a significant influence on lipid metabolism.

Palmitoleic acid (C16:1) is one of the main MUFA of the omega-7 (ω-7) FA family. There is evidence that the C16:1 acid is a major product of endogenous lipogenesis.

Oleic acid (C18:1) is the most prevalent MUFA in human food. It constitutes approximately half of FA content of TG in adipose tissue and has turned out to be the main acceptor of reactive oxygen species (ROS) in models of oxidative stress. The strongest oxidants for oleic FA are the superoxide anion-radical, nitrogen dioxide, and ozone. Various animal and plant sources including olive oil, cod oil, corn oil and palm oil are rich in OA (Oleic Acid). OA has been shown in numerous reports to inhibit cellular proliferation in several tumor cell lines. OA inhibited HER2 overexpression, a well-known oncogene involved in the development, and metastasis of numerous human cancers. In carcinoma cells, OA also plays a significant role in the intracellular calcium signalling pathways related to apoptosis and growth induction. The mechanisms underlying the apoptotic event caused by OA are linked to the rise in intracellular caspase 3 activity and the development of ROS.

Linoleic acid (LA) (C18:2) is the most prevalent PUFA in phospholipids, in cardiolipins. It constitutes ~10% of FA content of adipose tissue and more than 20% of all FA in human blood. LA is not synthesized in the mammalian (human) body. Substantial amounts are present in plant oils [86].

2.3.6.2 β-D-glucans

Glucans constitute the cell wall of fungi and yeast, and are the main polysaccharides present in mushrooms, in addition to water (90 %), proteins and amino acids, antioxidants and others. β-Glucans from yeast have similar structures to those from fungi. The biological activities of β-glucans and their applications in healthcare have been investigated for many years [87]. Within the β-glucan health-related effects, the most studied and characterized are the modulation of the immune system and metabolic and gastrointestinal effects [88]. carefully reviewed the information on structure–function relationship of β-glucans in relation to cellular immune modulation and antitumor activities: molecular weight, degree of branching, length of the sidechains, conformation of sugar residues, and consequent tridimensional structure, degree of solubility, and particulate size appear to be the most important features of B -glucans immune recognition, although some literature data are often inconsistent or contradictory [88].

Microbial β-glucan is a component of cell walls or is secreted by microorganisms in the growing medium, as in the case of lactic acid bacteria that are living and growing on plants, often under harsh conditions, such as *Pediococcus damnosus* [88,90].

2.4 METHODS OF EXTRACTION AND DETERMINATION OF PLANT ACTIVE COMPOUNDS

Based on the bioactive properties of certain compounds, such as antioxidant and anti-inflammatory activities, an interesting subject of research are natural substances present in various parts of plants. The choice of the most appropriate method for separation and quantification of biologically active compounds from plants and natural products is a crucial step of any analytical procedure.

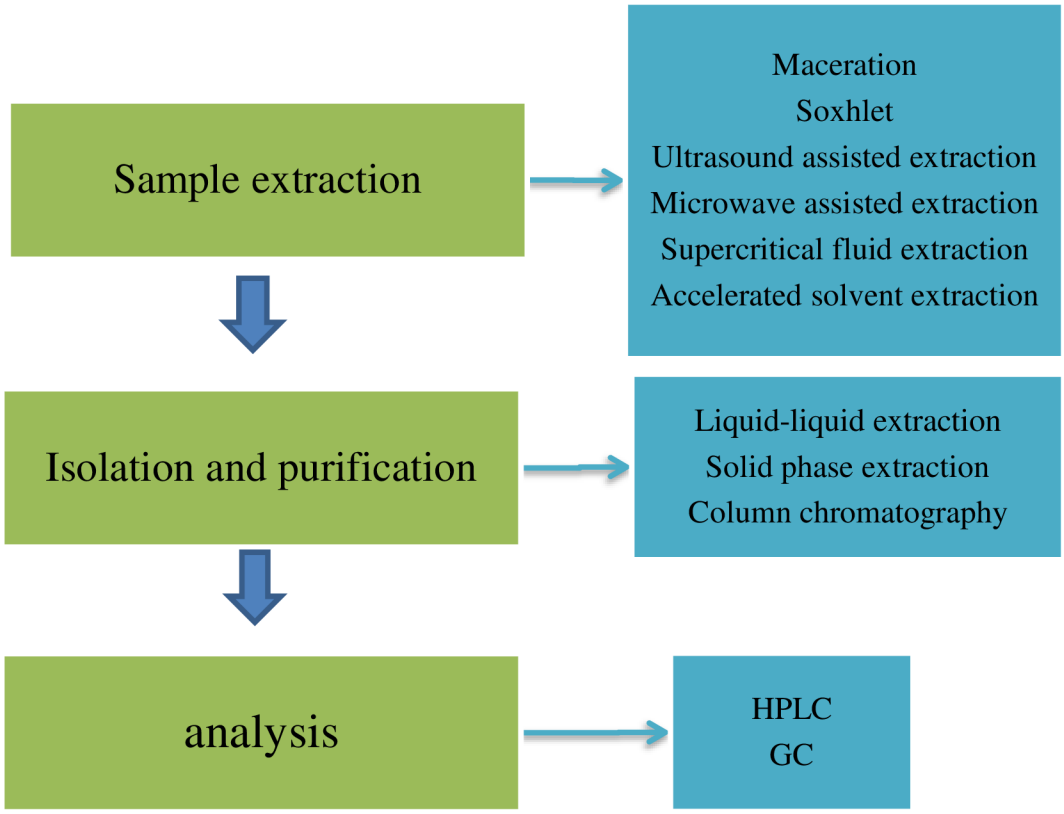

***Figure 7**: Steps required and available options for identification of biologically active compounds from plant material.*

2.4.1 Water extracts

Maceration is a conventional and very simple extraction method which uses different solvents for extraction of components from plant material. The principle is a very simple one: plant material is left together with the solvent – from a couple of hours up to several days – and after that filtration procedures will clean off the extract from solid suspensions. Even if maceration implies the use of relatively high amounts of solvents, it presents the advantage to be a very suitable technique for thermally labile components.

It can be also performed on shakers. Various orbital or tilting shakers are used. Due to the shaking, sufficient contact between the sample and the solvent is ensured and the mass is transferred. The separated substance passes from the solid phase to the liquid phase. It must be

ensured that the separated substance passes rapidly from one phase to another, which is related to the contact area of the individual phases. It is therefore necessary to increase this area as much as possible [91].

2.4.2 Soxhlet extraction of essential oils

Soxhlet extraction has traditionally been used for a solid sample with limited solubility in a solvent in the presence of insoluble impurities. A porous thimble loaded with a solid sample is placed inside the main chamber of the Soxhlet extractor. By refluxing the solvent through the thimble using a condenser and a siphon side arm, the extraction cycle is typically repeated many times. Soxhlet extraction is a rugged, well-established technique and permits unattended extraction. However, it requires a long extraction time and the consumption of a large amount of solvent. [91].

2.4.3 Ultrasound-assisted extraction (UAE)

UAE is an efficient extraction method placed at the border between classical and modern techniques and used for a variety of bioactive compounds. Compared with maceration and Soxhlet procedures, UAE possesses several strategic advantages; shorter extraction times, a reduced amount of necessary solvent and lower energy consumption are the most important benefits of UAE compared with conventional methods. The operating principle of UAE is the utilization of ultrasounds (waves with a frequency between 20 - 100 MHz) to develop bubbles inside the solvent. These bubbles, once created by cavitation phenomenon will induce plant wall cells' disruption and will speed-up the penetration of the solvent into the plant materiál [92].

2.4.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction is a valuable and environmentally friendly extraction technique used for extracting a large variety of bioactive compounds, presenting the advantages to be fast, selective and solvent saving. Supercritical state occurs when the temperature and pressure of the fluid are raised above its critical point. Carbon dioxide is the most used solvent in SFE being very efficient for extraction of fat, lipids and other non-polar compounds. For extraction of polar substances, a polar modifier called co-solvent (for example methanol, ethanol, acetonitrile, acetone, water, ethyl ether or dichloromethane) is necessary be added to the supercritical fluid in order to increase the solubility. SFE was used for D-pinitol extraction from carob pods and the best parameters were optimized in order to obtain the highest possible yields involving the most advantageous costs [92].

2.4.5 Accelerated solvent extraction (ASE)

Accelerated solvent extraction is a modern extraction technique used for the recovery of bioactive compounds involving solvents under high temperature and pressure, but without reaching the critical point. Also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), or high-pressure solvent extraction (HPSE), in this extraction method different solvents are required; the most popular are methanol, ethanol, or mixtures of other solvents as well [92].

2.4.6 Determination of total phenol content

The determination of the total phenol content in natural products is most performed spectrophotometrically using the Folin-Ciocaltau reagent. This is a reproducible and simple method. The method is based on the color reaction of the Folin-Ciocaltau reagent with the hydroxyl groups of the substances in the sample solution. The sample reacts with Folin-Ciocaltau reagent to produce a blue color. The intensity of the blue coloration depends on the concentration of polyphenols present in the sample. The polyphenol content is then determined spectrophotometrically as the absorbance of the resulting solutions at 750 nm. Gallic acid is commonly used as a standard [26].

2.4.7 Determination of flavonoides

One of the methods for determination total amount of flavonoids is based on changing the color of the reaction mixture in the presence of flavonoids. The intensity of the coloration depends on the concentration of flavonoids in the sample. The content is measured spectrophotometrically at 510 nm and catechin is used as a standard [26].

2.4.8 Total antioxdiant activity

Total antioxidant activity (TAA) is a term that describes the total concentration of all substances with antioxidant effects in a sample. Methods for the determination of TAA tend to be very diverse due to the various reaction mechanisms of low molecular weight antioxidants. In general, they can be divided into two groups: methods for assessing the ability to eliminate radicals and methods for assessing the redox properties of substances.

One of the spectrophotometric methods for determining the total antioxidant activity is TEAC (Trolox-Equivalent Antioxidant Capacity Assay). The TEAC assay is based on the reaction with ABTS which is a peroxidase substrate that reacts with peroxyl radicals or other oxidants and forms in the presence of H_2O_2 the metastable radical cation ABTS $\cdot^+$. The reaction is shown in the figure 4. ABTS $\cdot^+$ has intensely green-blue color and can be monitored spectrophotometrically in the range 600 - 750 nm. The TEAC parameter is used to evaluate the total antioxidant activity of the samples compared to a defined amount of a synthetic derivative of Trolox (a water-soluble vitamin E analogue). ABTS $\cdot$ in solution is not affected by ionic forces and can be dissolved in both, water, and organic solvent. For this reason, we can measure hydrophilic and lipophilic antioxidants. The method of determining TAA samples using ABTS is simple, fast in implementation and has a wide range of applications [93].

2.5 DETERMINATION OF BIOLOGICAL ACTIVITY OF NATURAL COMPOUNDS

The methods by which the possible antimicrobial effect of polyphenolic and other natural substances can be monitored. It can be divided into two groups, diffusion and dilution methods. Diffusion methods are mostly used as screening methods due to their simplicity and speed of implementation. Dilution methods are also suitable for quantitative assessment of the degree of sensitivity and for determining the level of MIT (minimum inhibitory concentration), which is the lowest concentration that visibly inhibits the growth of a given microorganism. The minimum inhibitory concentration is the most common expression of the degree of

antimicrobial action. In some studies, the term minimum bactericidal concentration (MBC) also occurs, which can be used as the lowest substance needed to kill a given microorganism.

Testing of different microbial cultures is performed in suitable culture media under optimal conditions, with respect to the individual strains tested. Microorganisms that are used for testing are obtained from microorganism collections [94].

2.5.1 Antimicrobial activity - Dilution test

Antibacterial dilution tests can be used to determine a particular drug's minimal inhibitory concentration (MIC), the lowest concentration of drug that inhibits visible bacterial growth, and minimal bactericidal concentration (MBC), the lowest drug concentration that kills ≥ 99.9 % of the starting inoculum. Determining these concentrations helps identify the correct drug for a particular pathogen. For the macrobroth dilution assay, a dilution series of the drug in broth is made in test tubes and the same number of cells of a test bacterial strain is added to each tube. The MIC is determined by examining the tubes to find the lowest drug concentration that inhibits visible growth; this is observed as turbidity (cloudiness) in the broth. Tubes with no visible growth are then inoculated onto agar media without antibiotic to determine the MBC [95].

2.5.2 Antimicrobial activity - Diffusion test

Filter paper disks impregnated with known amounts of antibacterial drugs to be tested are then placed on the agar plate or the other method is to fill the whole in agar with plant extract. As the bacterial inoculum grows, antibiotic diffuses from the circular disk into the agar and interacts with the growing bacteria. Antibacterial activity is observed as a clear circular zone of inhibition around the drug-impregnated disk or around the hole [95].

2.5.3 Time-kill test

ATP bioluminescence assay and flow-cytofluorometric method are all techniques used to screen and determine the susceptibility of microbes to antimicrobial compounds. ATP bioluminescence has been used to estimate the amount of ATP present in different cell types. The luciferin–luciferase bioluminescent assay method is mostly preferred due to its high sensitivity. In this method, $MgATP^{2+}$ changes luciferin into a state that can be catalytically oxidized by the luciferase in high quantum yield chemiluminescent reaction. There is a relationship between light intensity and ATP concentration under the right conditions. Cellular ATP can be measured when free ATP released from broken down cell is made to react with the luciferin–luciferase resulting in light emission. The amount of light emitted is measured by a luminometer [95].

The time-kill test on the other hand, is suitable for evaluating bactericidal and fungicidal activity. It provides information about the relationship between the antimicrobial agent and the microbial strain depending on the time taken for the action to occur and the concentration of the antimicrobial agent [95].

2.5.4 Human cells-based methods for determining the biological effects

Cells, tissues, and even whole organs (or parts of organs) can be kept (grown) alive outside the body in a buffered solution with nutrients, i.e.. under conditions that faithfully mimic their physiological situation. In medicine, biology and related fields, the term in vitro (from Latin "in glass") is used in connection with the cultivation of organisms under artificial conditions in test tubes or other laboratory glassware. In contrast, the term in vivo (from Latin "in living") means the occurrence of organisms (or their organs or their simpler components and substances) in natural conditions (in the case of components or organs, this means on / in a living organism). The primary tissues used to establish cultures in laboratories are often obtained from animals, but human tissues (e.g., blood cells, umbilical cord cells, placentas, and cartilage) can also be used.

The cells are separated (mechanically or enzymatically) into a suspension, which can then be cultured in one layer on a solid support or as a suspension in the culture medium. Cell lines need immortalization, or cell immortality, to make the culture permanent. This occurs in cells naturally (somatic mutation), viral transformation or hybridization (linking a host cell to an "immortal cell line").

Today, cell and tissue culture methods are important, for example, in the development and validation of new drugs, in the production of vaccines, in toxicity testing, in diagnostics, are used for culturing and long-term passage of intracellular parasites or viruses or in gene manipulation. The use of cell and tissue cultures thus contributes greatly to the reduction of animal experimentation [95].

2.6 CELL CYCLE AND ITS REGULATION

The cell cycle is one of the fastest growing areas of modern biology. Most of our current knowledge of the cell cycle has been made possible by the production of conditioned cell cycle mutants, which were created during the 1970s by Leland Hartwell in *Saccharomyces cerevisiae* and later by Paul Nurse in *Schizosaccharomyces pombe*. The course of cell division is well characterized in yeast and higher eukaryotes. In plants and especially in algae, less regulation of cell division is known. Green algae are often divided in a special way called multiple division. This type of cell cycle is characterized by a long growth phase, during which the cell doubles its volume several times. Subsequently, either during or at the end of the growth phase, several DNA replications and nuclear and cell divisions take place. Maternal cells are commonly divided into four, eight or more daughter cells [96,97].

Many enzymatic and non-enzymatic substances (cyclins and CDK protein kinases) are responsible for cell cycle regulation. In general, there are factors that accelerate cell division and factors that, in turn, slow down or stop cell division. In a complex multicellular organism (such as a human), strict regulation of cell division is necessary, as this is the only way to achieve a harmonious functioning of the organism, where only those cells that are currently needed are divided. Uncontrolled cell division is found in various cancers. Conversely, a reduction in the ability to divide can have serious consequences (for example, reduced production of blood elements in patients after cytostatic treatment).

G0 phase - the phase when the cell no longer divides, stopping the cell cycle. We meet in differentiated cells. Its onset is influenced by the control node located at the beginning of the G1 phase. If the cell no longer divides, it enters the G0 phase, instead of the G1 phase. Fully differentiated cells (e.g., neurons) no longer divide. Conversely, some other cells (e.g., liver cells - hepatocytes) are able, if necessary, to switch from the G0 phase to the G1 phase and begin to divide again.
G1 phase - Also called postmitotic. Cell growth period, preparatory phase for further division. DNA is inspected and repaired here, before its future replication in the next phase. It takes about 10 - 12 hours.
S phase - DNA replicates in double amount. Each chromosome has since been doubled, made up of a pair of sister chromatids. It takes about 6-8 hours.
G2 phase - Doubling of organelles, creation of structures needed for cell division. It takes about 2-4 hours.
M phase - It consists of nuclear division (mitosis or meiosis) and cytokinesis itself. It takes about
1 - 2 hours.
Cell division consists of two phases - karyokinesis (nuclear division) and cytokinesis (whole cell division).

2.6.1 Cytotoxicity testing

Cytotoxicity is defined as the toxicity caused due to the action of chemotherapeutic agents on living cells. Cytotoxicity tests are very important as they help in the determination of the proposed biomedical use. The method for determination of cytotoxicity and cell viability involves dyes, such as Trypan Blue, Alamar Blue, neutral red, and Coomassie Blue. The method differentiates the various cells in terms of colors. The cells are differentiated based on the ratio of the color uptake of both living and dead cells. The other methods for assaying cytotoxicity include tritium-labelled thymidine uptake assay, the MTT method, WST assay, and dehydrogenase-based assay (Li et al., 2012) [96].

2.6.1.1 MTT test

MTT assay is the first high throughput screening cell viability assay developed for a 96-well format. Solid materials are extracted in cell culture medium and multiple dilutions of the extract are prepared and added into each well containing 1×10^4 cells. After 24 h incubation, the water soluble MTT substrate (yellowish solution when prepared in media or salt solutions
lacking phenol red) is added and incubated with Raji cells for 3 h, and then MTT is converted to an insoluble formazan. Since formazan must be solubilized prior to absorbance readings, methods have been developed to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. The purple formazan can be quantified at 540 nm by spectrophotometer. Cytotoxicity is calculated based on the formazan signal generated which has been shown to have good linearity up to 10^6 cells per well and is dependent on the MTT concentration, the incubation time, and metabolically active viable cells. If the mean viability of Raji cells is reduced to < 50% of the blank control, the sample is considered potentially cytotoxic. The exact mechanism of MTT

reduction is not well understood but is believed to involve NADH or similar reducing molecules that transfer electrons to MTT [98].

2.6.1.2 Alamar Blue

AlamarBlue cell viability assay has been used for years in biological and environmental studies. Furthermore, analysis of cell proliferation and cytotoxicity is a vital step in evaluating cellular health and in the drug discovery process. alamarBlue is a proven cell viability indicator that uses the natural reducing power of live cells to convert resazurin to the fluorescent molecule, resorufin. Analysis can be evaluated quantitatively on an absorbance- or fluorescence-based microplate reader; while qualitative analysis can be evaluated by the visual color change of the solution which is indicative of metabolically active cells. The active ingredient of alamarBlue (resazurin) is a non-toxic cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces a very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity [99].

2.6.1.3 ATP

ATP determination offers a convenient bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its substrate D-luciferin. The assay is based on luciferase's absolute requirement for ATP in producing light (emission maximum ~560 nm at pH 7.8). This assay is extremely sensitive, and its sensitivity has led to numerous applications for detecting ATP production in various enzymatic reactions, including ATPase and NADPH oxidase, as well as for detecting low-level bacterial contamination in samples such as blood, milk, urine, soil, and sludge. The luciferin–luciferase bioluminescence assay has also been used successfully to study the effects of antibiotics on bacterial populations, to determine cell proliferation and cytotoxicity in both bacterial and mammalian cells, and to distinguish cytostatic versus cytocidal potential of anticancer drugs on malignant cell growth [100].

2.6.1.4 LDH

LDH is a cytosolic enzyme present in many different cell types and is a well-established and reliable indicator of cellular toxicity. Damage of the plasma membrane results in a release of LDH into the surrounding cell culture medium. This extracellular LDH can be quantified by a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490 nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium [101].

2.6.2 Apoptosis

Apoptosis is a process of programmed cell death occurring in multicellular organisms in whom development, maintenance and sculpturing organs and tissues.

Taken together, apoptotic processes are of widespread biological significance; being involved in e.g., development, differentiation, proliferation/homoeostasis, regulation, and function of the immune system and in the removal of defected harmful cells. Dysregulation of apoptosis can play a primary or secondary role leading to cancer whereas excessive apoptosis

contributes to neuro degeneration, autoimmunity, AIDS, and ischemia. Gaining insight into the techniques for detecting apoptotic cells will allow the development of more effective, higher specific and therefore better-tolerable therapeutic approaches [102].

Annexin V staining

Reorganization of the plasma membrane (shown in figure 7) occurs early in the apoptotic process disrupting phospholipid asymmetry and leading to the exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane. In the presence of calcium, Annexin V binds to phosphatidylserine allowing apoptotic cells to be easily identified by flow cytometry or fluorescence microscopy.

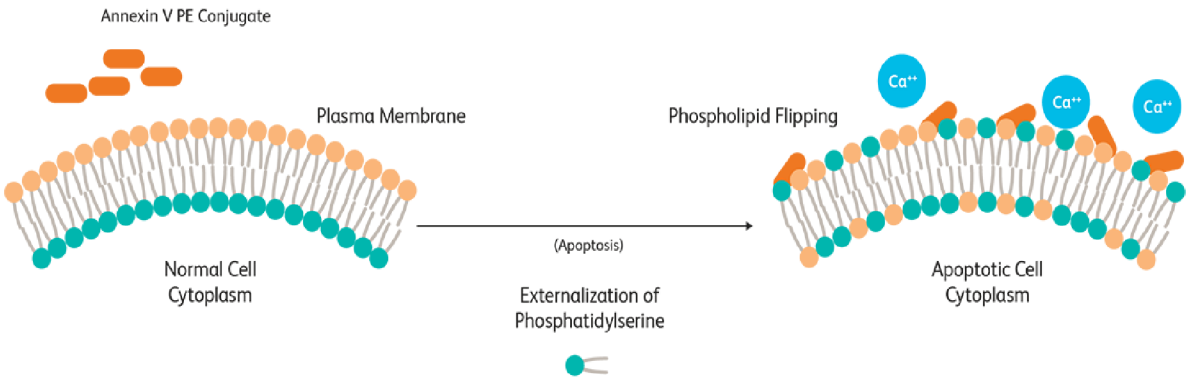

Figure 8: *Schematic Representation of Apoptosis-Induced Membrane Changes Recognized by Annexin V[103]*

In a normal cell these residues are on the inner surface of the membrane and therefore inaccessible to annexin V unless the cell membrane becomes permeable. At an early stage of apoptosis the phosphatidylserine residues are translocated to the outside of the cell. This is considered irreversible at this point; the cell is now committed to death by apoptosis. By allowing annexin V to react with these cells, any cell that has been committed to apoptosis will have exposed phosphatidylserine residues and thus will bind annexin V. If the annexin V is conjugated to FITC, the proportion of cells giving a positive FITC signal will provide a measure of the proportion of the population undergoing apoptosis. An important consideration is the fact that if the cell membrane is compromised, the phosphatidylserine residues within the cell will also stain and thus affect the result of the assay. One way around this problem is to incorporate a propidium iodide (PI) staining step in the protocol. If the cell membrane is compromised, the propidium iodide will be able to stain the DNA in the nucleus and thus can be used to discriminate the apoptotic cell from the damaged or necrotic cell.

2.6.3 Human Cell Lines and their use in biological tests

The skin is a continuously self-renewing organ that dynamically manages the outside-inside-outside relationships of the human body and actively participates in the host defences. Keratinocytes (KCs) represent 95% of the epidermal cells. Primarily, they play the structural and barrier function of the epidermis, but their role in the initiation and perpetuation of skin inflammatory and immunological responses, and wound repair, is also well recognized.

Spontaneously immortalized human keratinocytes cell line **HaCaT** from skin has been used as a model for the study of keratinocytes functions. HaCaT is a nontumorigenic monoclonal cell line, adapted to long-term growth without feed-layer or supplemented growth factors [105].

B16F1 is a melanoma murine tumor cell line used for research as a model for human skin cancers. Melanin is responsible for the human skin color, which is variable from pale to nigra, and also is responsible for human protection against sun/UV radiation. Despite its protective role, excessive melanin synthesis, and skin pigmentation due to hormonal changes, UV exposure or chronic inflammation may cause unpleasant aesthetic problems such as melasma, freckles, and spots. Therefore, controlling melanin levels and skin pigmentation has become a great interest in the cosmetic industries. Melanin is synthesized in melanosomes in two forms including black-to-brown eumelanin and red-to-yellow pheomelanin during the melanogenesis process [107].

The human epithelial cell line **Caco-2** has been widely used as a model of the intestinal epithelial barrier. The Caco-2 cell line is originally derived from a colon carcinoma. However, one of its most advantageous properties is its ability to spontaneously differentiate into a monolayer of cells with many properties typical of absorptive enterocytes with brush border layer as found in the small intestine. The Caco-2 cell line is heterogeneous and contains cells with slightly different properties. Thus, cultivation conditions can be expected to select for the growth of subpopulations of cells resulting in a cellular model system with properties that may differ from the original cell line. [108]

Raji is the first continuous human cell line of hematopoietic origin and was derived 45 years ago from a Nigerian patient with Burkitt's lymphoma (BL). Historically, these cells have proven to be convenient target cells in studies assessing the effector functions mediated by monoclonal antibodies [109]

2.6.4 Flow cytometry

Flow cytometry is a technology that rapidly analyses single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analysed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two different directions, the forward direction (Forward Scatter or FSC) which can indicate the relative size of the cell and at 90° (Side Scatter or SSC) which indicates the internal complexity or granularity of the cell. Light scatter is independent of fluorescence. Samples are prepared for fluorescence measurement through transfection and expression of fluorescent proteins (ex. Green Fluorescent Protein, GFP), staining with fluorescent dyes (e.g.,

Propidium Iodide, DNA) or staining with fluorescently conjugated antibodies (e.g., CD3 FITC). [103,104].

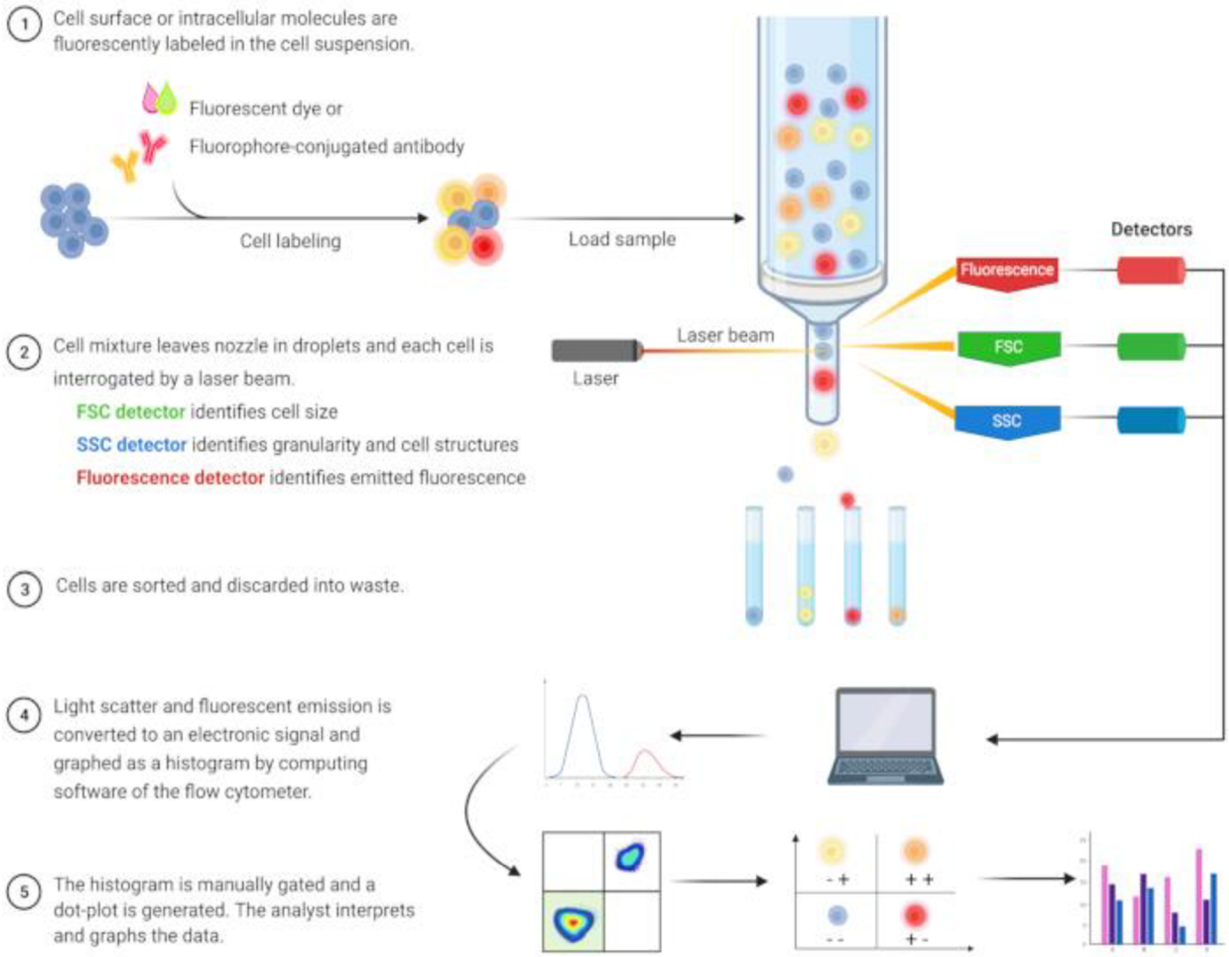

Figure 9: *Flow cytometry description [105].*

Traditional Flow Cytometers consist of three systems: fluidics, optics, and electronics. The fluidics system consists of sheath fluid (usually a buffered saline solution) that is pressurized to deliver and focus the sample to the laser intercept or interrogation point where the sample is analysed. The optical system consists of excitation optics (lasers) and collection optics (photomultiplier tubes or PMTs and photodiodes) that generate the visible and fluorescent light signals used to analyse the sample. A series of dichroic filters steer the fluorescent light to specific detectors and bandpass filters determine the wavelengths of light that are read so that each individual fluorochrome can be detected and measured. More specifically, dichroic filters are filters that pass light through that is either shorter or longer in wavelength and reflect the remaining light at an angle [104,105].

2.7 POSSIBLE APPLICATIONS OF NATURAL EXTRACTS

Plants due to the high content of various bioactive compounds are the main raw material for production of valuable, and useful bio-products (e.g., food, cosmetics, medicines, biostimulants, biopesticides, and feed). Natural origin of plant extracts make an excellent candidates to replace synthetic molecules, which are generally considered to have toxicological and carcinogenic effects.

2.7.1 Cosmetics

The use of EOs and herbal extracts as cosmetics has attracted peoples' attention for a long time, and active substances obtained from plants were used as perfumes or cosmetics. Historical findings obtained from the countries of origin of these plants, such as India, China, Egypt, and Iran, confirm the long-time usage of medicinal plants as cosmetic products [110].

It is estimated that more than 80% of the world's population uses conventional medicine (plant extracts or their active compounds) to meet their health needs. For example, in ancient Egypt, the oil of medicinal plants was extracted by steaming, while the Romans and Greeks used the distillation method for extraction. Also, with the advent of Islamic civilization, the use of medicinal plants accelerated with innovative approaches, and the methods of extracting oil, EO, and their substance took a practical step in the path of evolution. In addition, the EO obtained from species like *Cedrus libani, Ocimum kilimandscharicum, Artemisia annua, Acacia vestita, Piper angustifolium, Sassafras albidum, and Rosmarinus officinalis* contains camphor, which has been identified as an aromatic substance for centuries, and in ancient Japan and China. In other Asian and European countries, it has been used medicinally as well as in culinary and cosmetic applications [110].

Currently, consumers' tendency to purchase herbal cosmetics, which is both environmentally friendly and renewable, has increased. In recent years, "natural cosmetics" recorded a large quota of cosmetics (about $40 billion by 2021, which is about 10% of the global cosmetics market). Due to their anti-inflammatory, antimicrobial, and antioxidant properties, plant EOs, either as active ingredients or as preservatives, are used in various cosmetic products such as moisturizers, lotions, and cleansers in skin care. Cosmetics, conditioners, masks, or anti-dandruff products are used in hair care products, lipsticks or perfumes in perfumery.

By adding rosemary and chamomile EOs to shampoo, EOs can quickly penetrate the scalp, feed the hair follicles, moisturize the hair, strengthen the hair, and eliminate the adverse molecules that block the pores of the skin. EOs have a major impact on stimulating hair growth and preventing hair loss. It was reported that topical mint oil extracted from *Mentha piperita* with a relatively low dose (3% weight on weight) is typically used to stimulate hair growth. One of the most common applications of EOs in skin care is to prevent acne from appearing, using the ability of EOs to inhibit *Propionibacterium acnes*, which *Citronella'*s EO has the ability to do. EOs can be used in sunscreen creams because they can absorb most ultraviolet rays (in the wavelength range of 290 to 400 nm), prevent aging, sunburn, wrinkles, and other skin damage. A cream formulation that contains EOs of *Calendula officinalis* was studied in

laboratory conditions, and the results confirmed that the prepared formula had good properties for protecting the skin from exposure to sunlight [110].

Preservatives are added to cosmetic products to prevent microbial spoilage and thus increase the shelf life of the products. It is also necessary to protect the consumer against potential infections. Although chemical preservatives prevent microbial growth, their safety is questioned by consumers. Therefore, there is a considerable interest in producing preservative-free or self-preservative cosmetics. Therefore, the use of essences and extracts derived from plants in the production of cosmetics as multipurpose antimicrobial compounds, both as an alternative or natural preservatives and with anti-pathogenic properties, can help to improve the quality of these products. Some species from *Nepeta*, for example *Nepeta cataria* var (*Citriodora*), *Nepeta cataria,* and *Nepeta grandiflora*, are used in herbal cosmetics. In these three species, the major compounds were nepetalactone and geraniol in *N. cataria*; citronellol and geraniol in *N. citriodora*; and o-cymene, c-terpinene, carvacrol and p-cymene in *N. grandiflora* [27].

2.7.2 Food chemistry

Spices and herbs (phytoncides) have become increasingly important in recent years as potential sources of natural food preservatives due to the growing interest in the development of safe and effective natural food preservatives. The amount of phenolic compounds plays a major role in their antimicrobial effects, but also depends on a wide range of internal and external factors that must be characterized before using herbs and spices as ingredients. This leads to the need for high concentrations of these plant extracts to inhibit microbial growth in foods, which cause negative organoleptic effects and limit their use. Synergistic interactions with physical treatments or various natural additives can increase their antibacterial efficacy at a sufficiently low concentration, which can reduce their adverse effects and facilitate their use in a food preservation system [111].

2.7.2.1 The use of plant extracts for food packaging

Extracts that are rich in bioactive compounds can also be used to improve food packaging features. It should be mentioned that a significant process of active antioxidant packaging for meat replaces artificial additives with natural bioactive compounds. The most typical natural antioxidants employed in these innovative packaging technologies are tocopherols, EOs, and plant extracts such as rosemary, oregano, tea, etc. Accordingly, green tea extract combined in films made from dried protein seeds has been successfully used in the classification of pork, which has reduced lipid oxidation compared to control samples [112]. Studies have shown that a combination of green tea extract in potato starch film significantly improves the mechanical features of meat and reduces the oxidation of packaged fresh beef [113]. In another study, researchers examined the effect of green tea extract on the shelf life of cooked ham (21 days at 2 °C), and reported that the active packaging formulated with green tea extract (1%) was effective in preserving meat color. In another study on lamb ham, it was indicated that the use of oregano extract, without the need for phenol terpene compounds, prevented the oxidation of the bioprotein part compared with the control samples during 120 days of storage at 18 °C [114,119].

2.7.3 Application of essential oils and plant extracts in the griculture industry

Different plant parts, for instance: seeds, fruits, flowers, stems, leaves, and roots can be used for their manufacture. Nowadays, there is a clear need to develop new, efficient, and environmentally safe methods of stimulation of plant, growth and crop protection.

2.7.3.1 Application of essential oils and plant extracts as insecticides

Plants are a rich source of defensive chemicals. These substances may exhibit insecticidal, repellent, attractant, antinutritional, and growth-regulating effects on insects and usually have minor adverse effects on non-target organisms (parasites and predators) and the environment. Traditionally, synthetic insecticides have been used for many years to control postharvest diseases. Along with the benefits of these insecticides, the expansion of their use has caused consumers to worry about the possible dangers of toxic substances in crop residues. This issue has challenged the usefulness of pesticides and created the need for developing other methods to reduce the usage of chemicals. Controlling and reducing the damage of pathogenic pathogens after harvest using plant products such as plant extracts and essences in recent years could be a suitable alternative to reduce or eliminate the use of chemicals to control postharvest diseases of fruits and vegetables. Currently, there are products based on neem tree extract (neem) and EOs of cloves, rosemary, mint, cinnamon, lemon, thyme, etc., produced commercially for sanitary, agricultural, and greenhouse pest management [119].

Plant extracts as biological stimulants and plant protection products play an essential role for modernizing agriculture. Semerdjieva et al. studied the biological activity of four juniper species EOs as biopesticides and their results demonstrated that all tested EOs have significant insecticidal and repellent activity against two aphid species *Sitobion avenae* (English seed aphid) and *Rhopalosiphum padi* (bird oat cherry aphid) at 1%, 2.5% and 5% EO concentrations in solutions [115]. Turmeric (*Curcuma longa*), in addition to the antibacterial properties mentioned above, produces biological activities such as insect repellant and anti-snake venom activity that can be used as bioinsecticide [116]. Also, various studies have shown that *J. virginiana* EO have activities against insects and pathogens. Yohana et al [117] studied the important anti-mosquito properties of *Juniperus virginiana* (*Cupressaceae*) EOs against dominant malaria vectors, and their findings demonstrated that its EO has the potential for the development of new, efficient, safe, and affordable agents for mosquito control. Also, Semerdjieva et al. [115] reported that *Satureja pilosa* EO has larvicidal and mosquito repellent activities against *Aedes aegypti* that can be utilized for the development of new mosquito management control products [119].

2.7.3.2 Application of essential oils and plant extracts as herbicides

A biological method that uses allelochemicals for controlling weeds is an excellent alternative in organic systems. Most allelochemicals are used as secondary metabolites in plants, like EOs, tannins, alkaloids, and glycosides, as alternative strategies for weed management. For example, the findings of Semerdjieva et al. showed that *J. sabina* and *J. exselsa* EOs could manage the control of weed seeds [119].

2.7.3.3 Application of allelopathy, essential oils and plant extracts effects on the germination of seeds

The term “allelopathy” in plants is a non-resource-based beneficial or harmful interaction among plants and is more related to allelochemical release. Allelopathy with the help of plant EOs can be practical in modern agriculture [118]. One of its applications in modern agriculture is preventing preharvest sprouting (vivipary) in plants such as wheat and barley. In corn plants, seed germination in the cob head, which can happen due to rain at the time of ripening and before it is harvested due to adverse weather conditions, causes a significant decrease in yield and quality. Of course, preharvest germination depends on many other factors, such as seed maturity stage, dormancy, the cultivar’s genetic resistance, the amount and duration of rainfall, and environmental temperature [119].

2.7.4 Use of nanotechnologies in the application of natural substances

From nanotechnological processes, the encapsulation of active substances into some form of particles, fibres or gels is most often used. Several techniques are used to prepare functionalized materials, the most common of which is to enclose the active ingredient in a suitable particle. Encapsulation is defined as the encapsulation or entrapment of droplets or solid particles of a substance by natural or synthetic material, the size of the capsules usually being a few hundreds of micrometres. It is therefore possible to encapsulate solid, liquid, or gaseous substances. The term bioencapsulation refers to the capture of biologically active substances (or tissues) in a semipermeable membrane. Encapsulation has begun to be used in biotechnology and pharmacy. In the 1960s. It is now widely used in the food industry to transfer bioactive molecules into food. The encapsulation of biologically active substances prevents its rapid decomposition, reactivity with the surrounding environment, thus achieving a gradual release of the substance from the packaging, we can mask the undesirable odour or taste. The particle thus consists of a shell and a core. The coating or coating of the particle has a certain shelf life depending on the type of particles used for encapsulation. After a certain time, the packaging is partially degraded, and the encapsulated substance is gradually released into the environment. The particle size ranges from nanometres to millimetres [111,120,121].

2.7.4.1 Nanoparticles - targeted distribution and stabilization

The use of liposomes as drug carriers is still the most common way of their medical use. They can serve as a controlled release drug reservoir or as a carrier system for the targeted delivery of encapsulated drug to appropriate tissues and structures. Liposomal drugs penetrate the target cells more easily due to their high phospholipid content. Drugs often have low stability or solubility. The encapsulation of the drug in nanoparticles protects the drug during transport to the site of action. Due to the targeted transport of drugs, it is possible to reduce doses and thus protect the body from the adverse effects of their action. The main goal is to achieve long-term and permanent release with a minimum probability of premature disruption of the packaging. A similar application can be found, for example, in the food industry when encapsulating vitamins that dissolve in a hydrophobic environment, such as vitamin A, D, E, K, or carotenoids, which can be very well encapsulated in liposomes, usually more effectively compared to water-soluble vitamins [111,120,121].

2.7.4.2 Nanotechnology in cosmetics

Cosmetics use nanotechnology mainly in sunscreens, hair care products and moisturizers. The advantage of nanoparticles in creams and shampoos is that they can transfer the active ingredients deeper into the skin or hair. Sunscreens may contain zinc oxide and titanium dioxide nanoparticles. Older creams used larger particles that provided sun protection but left a whitish color. The nanoparticles are less visible and do not leave a whitish tinge on the skin when spread. Less well known is the use in perfumes. Nanotechnology helps to achieve a cleaner odour, gradual release, odour absorption and, finally, price reduction. However, if the perfume does not contain nanoparticles, it does not mean that it is bad or outdated. In medicine, antimicrobial or antibacterial dressings with silver nanoparticles are used, which create a very inhospitable environment for bacteria, thus preventing their multiplication and slowly destroying them. The use of nano silver is also considered in the production of wound sewing threads [123].

2.7.4.3 Nanotechnologies in the food industry

Applications of nanotechnologies in the food industry include encapsulation and delivery of substances to target sites, increase of taste, introduction of antibacterial nanoparticles into food, increase of shelf life, detection of contamination, better food storage, monitoring and brand protection. Nano-processing of food and products can change colour, taste, or sensory properties; it also alters nutritional functionality, removes chemicals or pathogens from food. Nanomaterials for food packaging can prolong food life, improve food safety, alert consumers that food is contaminated or spoiled, or gradually release preservatives to extend the life of food in packaging [124].

3 AIM OF THESIS

The aim of presented thesis was to prepare and characterize natural extracts with antioxidant and antimicrobial effect.

The thesis is focused on following partial goals:

1. Literary review-overview of medicinal plants and screening of yeast strains with high antimicrobial activity, antioxidant activity and important nutritional benefits.
2. Optimization of methods of characterization and composition of plant extracts
3. Production and characterization of microbial extracts from selected yeast strains.
4. Analysis of active substances, antioxidant and antimicrobial effect of plant extracts, microbial extracts and their mixtures.
5. Testing biological effects and safety of natural extracts by using cytotoxicity and apoptosis tests on human cell lines.
6. Discussion of results and possible applications.

4 MATERIALS AND METHODS

4.1 MATERIALS

Acai, Iswari, CZE
Clove, Sonnentor, CZE
Curcuma, Sonnentor, CZE
Ginger, awashop. CZE
Ginseng, ebio24, CZE
Oregano, lumigreen, CZE

4.2 CHEMICALS

ABTS, (Sigma-Aldrich SVK)
Acetonitril for HPLC, (Lach-Ner Ltd., Czech Republic)
Aluminium chloride, (Sigma-Aldrich, Germany)
Annexin V, (Sigma-Aldrich, Germany)
Antibiotics, Antomycotic 100X (Biosera), Biotech (SRN)
Bacteriological agar, HiMedia CZE)
Camptothecin, (Sigma-Aldrich, GER)
Carotenoids standard, (Sigma-Aldrich, GER)
Catechin, (Sigma-Aldrich SVK)
Chloroform p.a., (Lach-Ner Ltd. Czech Republic)
Cholesterol- Sigma-Aldrich (SRN) Lecithin, Sigma-Aldrich (SVK)
DMEM, (Sigma-Aldrich, UK)
Ergosterol (Sigma-Aldrich, Germany)
Ethylacetate for HPLC (Lach-Ner Ltd., CZE)
Ferrous sulfate heptahydrate p.a., (Lach-Ner Ltd., CZE)
Fetal bovine serum, (HyClone USA)
Folin- Ciocalteau - p.a., (LachNer, CZE)
Gallic acid- (Sigma-Aldrich, SVK)
Glucose, (LachNer, CZE)
Hexan for HPLC (Lach-Ner Ltd., CZE)
KH_2PO_4 p.a., (Lach-Ner Ltd., CZE)
LB- Luria broth, (Himedia, India)
Methanol for HPLC (Lach-Ner Ltd., CZE)
Methanol p.a., (Lach-Ner Ltd., CZE)
Methylene Blue, (Sigma-Aldrich, UK)
NB- Nutrient Broth, (Himedia, India)
Pepton, HiMedia (CZE)
Phosphate-buffered saline, VWR (USA)
Potassium nitrate p.a., (Lach-Ner Ltd., CZE)
Propidium Iodide, (Sigma-Aldrich, UK)
RPMI, (Thermo Fisher, IT)

SDS- Sodium dodecyl sulphate (SRN)
Sodium carbonate p.a., (Lach-Ner Ltd., CZE)
Sodium chloride p.a., (Lach-Ner Ltd., CZE)
Sodium hydroxide p.a., (Lach-Ner Ltd., CZE)
Sodium nitrate p.a., (Lach-Ner Ltd., CZE)
Sulphuric acid 96 %, (Lach-Ner Ltd., CZE)
Thiazolyl blue formazan, (Duchefa Biochemistry, NL)
Tris HCl, (Lach-Ner Ltd., CZE)
Trolox- (Sigma-Aldrich, SVK)
Tween, (Sigma-Aldrich, UK)
Trypsin, (Versene EDTA, P-Lab, CZE)
Yeast and mushroom Beta-glucan Assay Kit, (Megazyme, PL)
Yeast extract, (HiMedia, India)

4.3 INSTRUMENTS

Analytical weight, Boeco (GER)
Biohazard box, model Airstream, class II -ESCO, Biotech (CZE)
Cell Culture CO_2 Incubator, ESCO (SRN)
Centrifuge Boeco U-32R, Hettich Zentrifugen (GER)
Centrifuge Z 366 – HermLe, HermLe (GER)
Centrifuge, Sartorius, Biotech (CZE)
Column Kinetex, EVO coreshell c 18, 150 x 4.6 mm, 2.6 µm, Chromservis (CZE)
Column Zorbax ZB-FAME 30 m x 0.25 mm x 0.2 µm
DLS and Zetasizer ZS, Malvern (UK)
ELISA Reader BioTek ELx808, Biotek (DE)
Flow cytometer, Cytek Aurora, Cytek (USA)
Freeze-dryer Labconco FreeZone 4.5 Freeze Dryer (USA)
HPLC Thermo Fischer Scientific Dionex UltiMate 3000 series (USA)
Inverz biological microscope I-101 L-Scientific, Laboserv (CZE)
Magnetic stirrer, Lavat – Verkon (CZE)
Orbital shaker WiseSHake SHO (USA)
Pre-weight Kern 440-43, Kern & Sohn GmbH (DE)
Soxtherm, SOX 412, Gerhardt (DE) • Exsikátor, Kavalier (CZE)
Termoblock VWR, (CZE)
Thermo Scientific TRACETM Gas Chromatograph (Thermo Fischer Scientific, USA)
Thermostat shaker Heidolph Unimax 1010, Labicom (CZE)
Ultrasound homogenizator Sonoplus HS 3200, Bandeline, Verkon (CZE)
Vortex, TK3S, Kartel spa (USA)

4.4 MICROORGANISMS AND CELL LINES

B16F1- CRL-632 - Murine melanoma cell line
Caco-2 – CRL-2102- Human adenocarcinoma cell line
HaCaT - Human keratinocytes cell line
Raji - CCL-86- Human lymphoblast cell line
Micrococus Luteus, CCM 1569
Candida Rugosa
Cystofilobasidium infirmominiatum CCY 17-18-4
Escherichia Coli CCM 3954
Metschnikowia pulcherrima CCY 029-002-145
Phaffia rhodozyma CCY 77-1
Rhodotorula kratochvilovae CCY 20-2-26
Saccharomyces cerevisiae CCY 6646
Serratia Marcescens, CCM 8587
Stafylococus Aureus CCM 299
Stafylococus dermatitis CCM 4418

4.5 PROCESSING OF PLANT MATERIALS

All the dry samples were pulverized to obtain large reaction surface and has all the samples at the same condition (root, fruit, leaves). Acai and curcuma were already bought as a powder. Clove, oregano, and ginseng root was pulverized for 30 s. Ginger was firstly dried and then pulverized for 30s.

4.5.1 Preparation of water plant extracts

10 grams of dried sample was mixed with 100mL of distilled water and heat at 80 °C for 24 hours, at magnetic stirrer (120 rpm). After 24 hours the samples were multiply centrifuged (6000 rpm) to obtain clear extract. All the samples were frozen at – 80°C and lyophilized to measure the exacts weight of dry extract. Samples (0,1 g) were hydrated with 1 mL of distilled water directly before use and filtrated (nylon filters, pore size 0.22 μm, diam. 47 mm). Characterization of plant extracts is in chapter about spectrophotometric methods.

4.5.2 Preparation of oil extracts

Oil extracts were prepared using a Soxtherm. Hexane was used as an extraction agent. 10 g of dry pulverized sample was weighed into a cartridge that was blocked with cotton wool. The cartridge with sample was placed in a glass container and poured over with 100 mL of hexane. A boiling stone was placed in the glass container and then the container was inserted into the Soxtherm apparatus. The settings of the extraction program can be found in table below. Whole process takes 3 hours. After the extraction was completed, the oil with the remaining hexane was poured into a pre-weighed evaporating flask. Hexane was evaporated from the oil using a vacuum evaporator. After evaporation, the flask with the oil was left overnight in a desiccator. The next day, the oil was weighed and stored in a freezer box for further use.

Table 1: *Extraction parameters for Soxtherm extractor*

Extraction temperature	170 °C
Reduction interval	3 min 3 s
Reduction pulse	3 s
Hot extraction	1 h 30 min
Evaporation A	5x interval
Extraction time	1 h
Evaporation B	2x interval
Evaporation C	10 min

4.6 PREPARATION OF LIPOSOMES

Preparation of particles by ultrasound. 450 mg of soy lecithin and 50 mg of cholesterol were added to 20 mL of aqueous extract. The mixture was then sonicated to form liposomes.

4.6.1 Determination of encapsulation efficiency

Encapsulation efficiency was measured by determining the content of total phenols. To determine encapsulation efficiency, the concentration of phenols in the extract was first measured before encapsulation. After encapsulation, the concentration of phenols in the supernatant was measured. To determine the encapsulation efficiency using this method, the concentration of total polyphenols in the extracts before and after encapsulation was calculated. After subtracting these values, the encapsulated amount of phenols in the particles was calculated and the encapsulation efficiency was given as a percentage.

4.6.2 Determination of particle size and distribution using DLS

The particle solutions were diluted 100x with distilled water. 1 mL of the diluted particle solution was pipetted into the cuvette. The cuvette was placed in a DLS Malvern Zetasizer ZS and the particle size distribution, average size and polydispersity were determined.

4.6.3 Determination of particle stability using zeta potential.

The particle solutions were diluted 100x with distilled water. 1 mL of the particle solution was pipetted into the cuvette and the electrode adapter was inserted. The cuvette was placed in a DLS Malvern Zetasizer ZS and the zeta potential was measured. Particle stability was derived from the zeta potential value. Stable particles are those with a zeta potential value above ± 30 mV.

4.7 CHROMATOGRAPHIC METHODS

High-performance liquid chromatography (HPLC) is a separation technique, which can be applied to analyse compounds of different samples. Depending on the nature, chemical structure, and molecular weight of the analytes, it is possible to select the type of HPLC. In this sense, different kinds of HPLC have emerged to allow qualitative and quantitative information regarding individual components of the sample under study. Gas chromatography (GC) is a separation technique capable of separating highly complex mixtures based primarily upon differences of boiling point/ vapor pressure and of polarity [124,126].

4.7.1 Determination of carotenoids by HPLC

Into plastic extraction tubes 15 ± 5 mg of freeze-dried biomass was weight and rehydrated by addition of 1 mL distilled water. The water was removed by centrifugation and to the pellet was added 250 ± 50 mg acid-washed glass beads (250 – 500 μm diameter, Roth, Germany) and 1 mL of methanol. To obtain biomass with ruptured cell wall for analysis, it was necessary to vortex for 10 minutes. The content of the PP tube was transferred into a glass reaction tube by washing it with a 2,000 μl of chloroform and the glass tube was vortexed for 10 min. Then, 1 mL of distilled water was added for the phase separation. After centrifugation (3,000 rpm/5 min/4 °C), the separated bottom chloroform phase with extracted pigments was evaporated under nitrogen at 25 °C followed by the addition of 1 mL of mixture ethylacetate:acetonitrile (20:60). The ethylacetate:acetonitrile mixture containing extracted pigments were filtered through syringe filter (0.45 μm, PTFE membrane, 13 mm) and transferred into glass vials for further HPLC analysis.

The conditions of HPLC separation are presented below. Contents of individual pigments were calculated according to calibration standards (beta-carotene, astaxanthin, lycopene, lutein, violaxanthin, neoxanthin, chlorophyll a, chlorophyll b, ergosterol) from regress equation. Composition of mobile phase A is: acetonitrile 840 mL, methanol 20 mL, 0.1M Tris-HCl (pH=8) 140 mL), and mobile phase B: methanol 680 mL, ethylacetate 320 mL.

Table 2: *Conditions for HPLC analysis of carotenoids*

Volume of sample	20 μL
Column	Kinetex, EVO 150 x 4.6 mm, 2.6 μm
Elution	Gradient 0-13 min: from 100% A to 100% B linearly 13-19 min: 100% B 19-20 min: from 100% B to 100% A linearly 20-25 min: 100% A
PDA	285, 435, 450 and 680 nm
Temperature	25 °C
Time of analysis	25 min

4.7.2 Lipid content and fatty acids profile by Gas chromatography

20 mg of biomass was weighed into crimp vials. Subsequently, 1.8 mL of a transesterification mixture containing 0.5 mg/mL of dissolved C17:0 internal standard in 15 % sulfuric acid in methanol was added. The vials were heated to 85 ° C for 2 hours. After cooling, the entire content of the vial was transferred to a 4 mL vial with 0.5 mL of 0.05 M NaOH. 1 mL of HPLC hexane was pipetted into the sample. The resulting mixture was then vortexed vigorously for 5 minutes. After phase separation, 100 μl of the upper hexane phase was collected in a glass vial for HPLC / GC with pre-pipetted 900 μl of HPLC hexane. Samples with fatty acid methyl esters were then analyzed by a gas chromatograph Thermo Scientific TRACETM Gas Chromatograph with a Thermo Scientific A1 1310 autosampler, Zebron ZB-FAME column (30 m x 0.25 mm x 0.25 μm) and a flame ionization detector (FID) was used.

Table 3: *Mobile phase and temperature program for GC analysis*

Separation parameters	Hydrogen flow	1 mL/min
	sample injection volume	1 µl
	Injector temperature	250 °C with flow divider ratio 10
	Detector temperature	260 °C (air flow 350 mL/min, hydrogen flow 25 mL/min, nitrogen flow 30 mL/min)
Temperature program	80 °C 1 minute	
	140 ° C with a temperature gradient of 15 ° C per minute (t_R = 5 minutes)	
	190 ° C with a temperature gradient of 3 ° C per minute (t_R = 21.7 minutes)	
	260 ° C with a temperature gradient of 25 ° C / min (t_R = 24.5 minutes)	
	Maintaining the temperature at 260 ° C for 1 minute (t_R= 25.5minutes)	

4.8 SPECTROMETRIC METHODS

4.8.1 Total phenolic content

The reaction of the Folin-Ciocalteu reagent with polyphenols forms a blue complex.1mL of 10x diluted Folin-Ciocalteu reagent with 10 mL of distilled water and 50 µL of sample. Left the mixture for 5 minutes. Then add 1 mL of saturated sodium carbonate. After 15 minutes measure the absorbance at 750 nm. Each sample was measured in triplicates. The concentration of the unknown sample is calculated from the equation from the graph of absorbance versus concentration of the standard, which is gallic acid.

$$y = 1.412 \cdot x\ mg \cdot mL^{-1}$$

4.8.2 Flavonoids

The reaction starts with 0,5 mL of sample, 1,5 mL of water and 0,2 mL of sodium nitrite After 5 minutes add 0.2 mL of aluminum chloride. After another 5 minutes add to the solution 1,5 mL of sodium hydroxide and 1 mL of water. After 15 minutes measure the absorbance at 510 nm.

of the red complex at 510 nm. The concentration of the unknown sample is calculated from the equation from the graph of absorbance versus concentration of the standard, which is catechin

$$y = 2.874 \cdot x\ mg \cdot mL^{-1}$$

4.8.3 Antioxidant activity

ABTS was dissolved in distilled water in concentration of 7 mmol / l. Radical anion ABTS · + was obtained by reaction with 2.45 mmol / l potassium persulphate. The solution was left in dark for 12 hours at room temperature. Before the measurement the ABTS · + was diluted in ethanol to obtain absorbance 0.700 at λ = 734 nm. Then the reaction starts with 1 mL of

ABTS · + and 10 µl of extract. Absorbance was measured in 0 and 10 minutes. As a blank, distilled water was used. For calibration was prepared Trolox solution in the concentration range 50 - 400 µg/mL.

$$y = 1.5388 \cdot x\ mg \cdot mL^{-1}$$

4.8.4 Isolation and determination of β-glucans from yeasts

Dried biomass was weight (25 mg) to a glass centrifuge tube and wetted with 0.2 mL of ethanol (50 % v/v). 4 mL of sodium phosphate buffer (20 mM, pH 6.5) were added, and the content was stirred on vortex mixer. The tube was placed into a boiling water bath and incubated for 3 min. Next, the temperature was decreased to 50 °C and the sample was hydrolysed one hour by lichenase (0.2 mL, 10 U, 50 °C). 5 mL of sodium acetate buffer were added into tube (200 mM, pH 4.0) and the sample was left to equilibrate to room temperature (5 min) and then centrifuge (1,000 g, 10 min). Aliquots of the sample (0.1 mL) were dispensed into the bottom of three test tubes and βglucosidase (0.1 mL, 0.2 U) in 50 mM sodium acetate buffer (pH 4.0) was added to two of these tubes (the reaction). To the third (the reaction blank), 50 mM acetate buffer (0.1 mL, pH 4.0) was pipetted. The samples were incubated at 50 °C for 10 min. In the end, 3 mL of GOPOD Reagent was added to each tube and incubated at 50 °C for a further 20 min. The absorbance was measured at 510 nm against reagent blank. [127]

4.9 CULTIVATION OF MICROORGANISMS

4.9.1 Cultivation for antimicrobial tests

Plant extracts were prepared by serial one to two dilutions in Luria broth (LB) liquid medium (in the case of fungal strains in, RPMI-1640) with 0.5% Tween 80 at V / V concentration from 10 % to 5 %. The assay was performed in 96-well plates in a final volume of 200 µl per well: 100 µl of each plant extract dilution and 100 µl of microbial suspension at a concentration of 10^6 CFU/mL, were added to each well and then incubated at 37 °C for 24 hours (24/48 for fungal strains).

To determine the MBC value, 10 µL were seeded on solid media, the plates were incubated for 24-48 h at the temperature of 37°C. MBC was considered as the lowest concentration inhibiting bacterial growth. Each strain was tested in duplicate and a positive growth control (the test strain without PE) and a negative one (medium only) is included in each test. The plates were incubated at 37° C for 24/48 h and monitored to detect microbial growth

Different option to determinate MBC is to was perform only in 96-well plates in a final volume of 200 µl per well: 100 µl of each plant/microbial extract dilution and 100 µl of microbial suspension at a concentration of 10^6 CFU/mL, added to each well and then incubated at 37 °C for 24 hours (24/48 for fungal strains). Then read the absorbance at 540 nm of blank (only media), cell control (200 µl of microbial extract) and samples. The decrease in absorbance compared to control samples is equivalent to growth inhibition of a microorganisms

Microorganisms used for the tests: *Candida Rugosa, Stafylococus Aureus, Stafylococus dermatitis, Escherichia coli, Serratia Marcescence, Bacteriococus Luteus.*

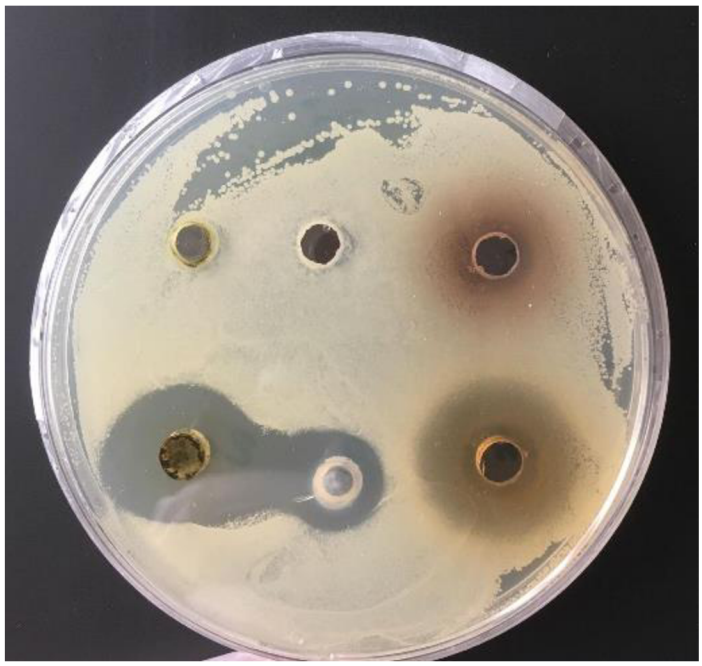

Figure 10: *Example of zone inhibition of Candida*

4.9.2 Cultivation of yeasts strains

Yeast strains of *Metschnikowia pulcherrima* CCY 029-002-145, *Cystofilobasidium infirmominiatum* CCY 17-18-4, *Phaffia rhodozyma* CCY 77-1, *Rhodotorula kratochvilovae* CCY 20-2-26, and *Saccharomyces cerevisiae* CCY 6646 yeast strains were obtained from Culture Collection of Yeast (Institute of Chemistry, Slovak Academy of Science, Bratislava, Slovakia). First, the yeast from frozen stock were cultured on YPD agar plates (composition see Table 4) for 72 hours at laboratory temperature. Next, the inoculum was prepared by transfering of one biological loop from agar plate into 100 mL of sterile YPD medium (yeast extract, 10 $g \cdot l^{-1}$; peptone, 20 $g \cdot l^{-1}$, glucose, 20 $g \cdot l^{-1}$) in Erlenmeyer flask (250 mL) and cultured under constant shaking regime (120 rpm) for 24 hours. The yeast were then inoculated directly into the production medium in a ratio of 1:5 (inoculum: production medium) and cultivated for 96h at laboratory temperature. Composition of production medium is in Table 5. Before inoculation, each medium was sterilized for 45 minutes at 120 ° C and then cooled to room temperature.

Table 4 *:Composition of YPD agar media*

	Concentration [g/l]
Glucose	20
Yeast extract	10
Pepton	20
Agar	20

Table 5: *Composition of production media*

	concentration [g/l]
Glucose	69,29
KNO_3	1,52
KH_2PO_4	4
$MgSO_4 \cdot 7H_2O$	0,7

4.10 CELL CULTURES CULTIVATION AND TESTS

Working with cell cultures and their cultivation is specific due to its high demands. They demand precision, some experience of the staff and sterility of all laboratory equipment and laboratories. Cell cultures have many uses and in research they serve primarily as a source of biological material for experiments.

B16F1 is a mouse melanocyte cell line. These are skin cancer cells that produce melanin. Melanin is a substance responsible for skin color and protects against the effects of solar UV radiation.

HaCaT a human keratinocyte cell line that has been immortalized. The advantage of this line is easier culture and slower cell aging compared to normal keratinocytes. The HaCaT cell line is used to test skin sensitivity and cytotoxicity.

Caco-2 are epithelial cells isolated from colon tissue derived from a 72-year-old, White, male with colorectal adenocarcinoma. This cell line is a suitable transfection host and has applications in cancer and toxicology research.

Raji cell line of lymphoblast-like cells was established by R.J.V. Pulvertaft in 1963 from a Burkitt's lymphoma of the left maxilla of an 11-year-old black male. This cell line can be used in immunology research.

Figure 11: *Cultivation of cell cultures and preparation for subculturing*

4.10.1 Cryopreserved cells

The cells come deep-frozen shipped on dry ice. The cryovials were stored below -80 °C. For culturing the cryopreserved cells were quickly thaw by rapid agitation in a 37 °C water bath within 40 - 60 seconds. As soon as the sample has thawed, cryovial were removed from the water bath. A small ice clump should still remain, and the vial was cold. From now on, all operations were carried out under aseptic conditions. Cryovials were transferred to a sterile flow cabinet and wipe with 70 % alcohol. Resuspend cryovial in 15 mL centrifuge tube containing 8 mL of culture medium (room temperature). Centrifuge at 300 rcf for 3 min and discard the supernatant. The centrifugation step may be omitted, but in this case the remains of the freeze medium have to be removed 24 hours later. Cells were resuspended carefully in 10mL fresh cell culture medium and transfer them into two T25 cell culture flasks.

4.10.2 Subculturing

To subculture the cells firstly the medium was removed and adherent cells were rinsed using PBS without calcium and magnesium (3-5 mL PBS for T 25, 5-10mL for T 75 cell culture flasks). Exact volume of trypsin was added (1-2mL per T 25, 2.5mL per T 75 cell culture flask), the cell sheet must be covered completely. Cells were incubated at ambient temperature for 8-10 minutes. Carefully resuspend the cells with medium (10 mL), centrifuge for 3 min at 300rcf, resuspend cells in fresh medium and dispense into new flasks which contain fresh medium.

Seeding density: 1-2x10^4 cells/cm^2

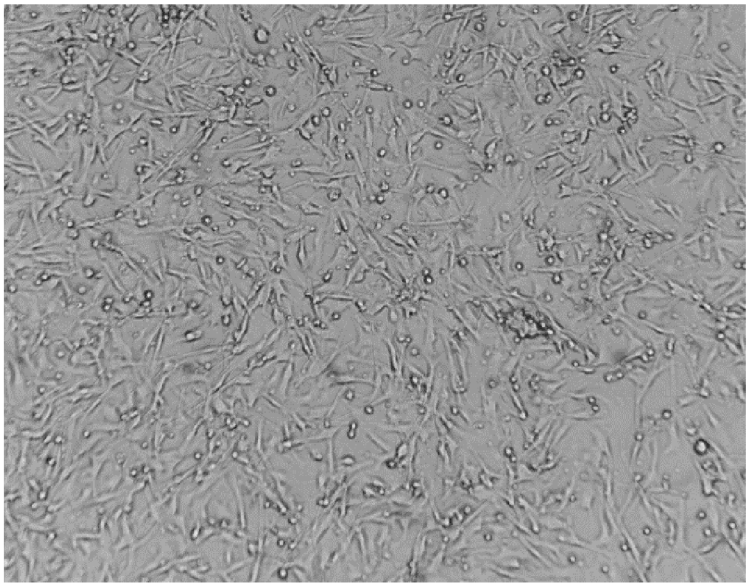

Figure 12: 90 % confluence of B16F1 cell line, ready to subculture.

4.10.3 Cultivation of Cell lines

B16F1, *HaCaT* and *CaCO-2* cell lines were cultivated in DMEM, high glucose medium with 1 % of antibiotics and 10 % of FBS (Fetal Bovine serum), at 37 °C and 5 % of CO_2 atmosphere. *Raji* Cell lines were cultivated in RPMI medium with 1 % of antibiotics and 10 % of FBS (Fetal Bovine serum), at 37 °C and 5 % of CO_2 atmosphere.

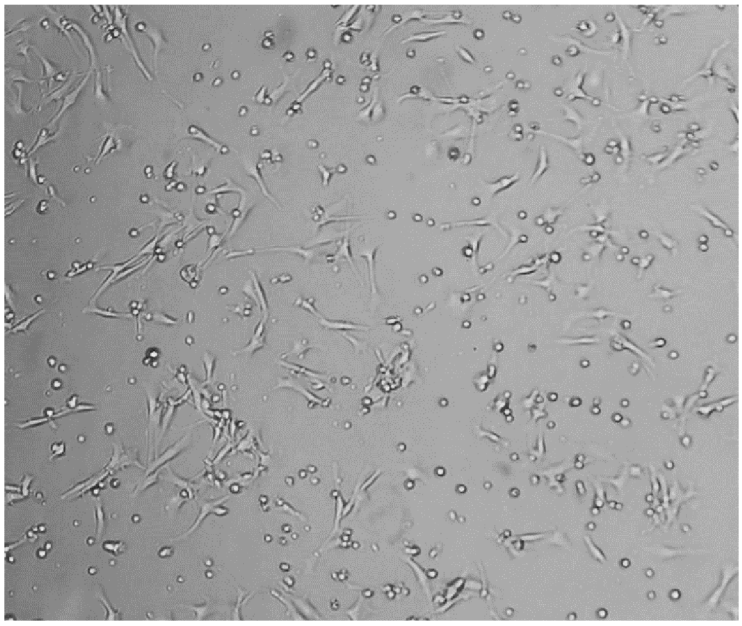

Figure 13: Cell line *B16F1* after 6 hours of cultivation from cryovial.

4.10.4 Determination of the growth curve of cell lines

The procedure for determining the growth curve of the cell lines was initially identical to the subculturing procedure described in the previous chapter. After centrifugation and resuspension of the cell pellet in the medium, the cell concentration was count using a Bürker chamber. According to their number, the suspension was suitably diluted with medium and pipetted into a 6-well plate of 2 mL. After every 24 hours, the number of viable cells in a single well of the plate was counted. After each count, the plate was returned to the incubation box. The growth curve of the cultures used was constructed from the number of cells in the wells. The growth curve is mainly used to determine the time schedule of the experiment (here specifically the MTT test). It is recommended to start the experiment in the so-called log phase of the growth curve, i.e., in the phase when the number of cells grows exponentially, the cells use all nutrients for their metabolism and are suitable objects for testing.

4.10.5 MTT cytotoxicity test

Generally, cells are plated in triplicates to minimize the variability of the results. The volume of cells depends on the type of plate used. Each plate should contain control wells (without extract) and blank wells (without cells). For some extract that also show absorbance at the given wavelengths an additional control is required of wells with medium (without cells) including the range of extract used. The number of plates needed depends on the specific experiment. A common MTT assay experiment requires a testing plate for the OD of the extract, a testing plate to determine the growth curve for the starting amount of cells seeded per well, and a broad

dilution range to determine the dilution range for the experiments. The OD is measured at 540 nm. The measured data are copied into an excel sheet and with the use of the following formula, the percentage of living cells can be determined: The average OD of the blank control wells (without cells and if the extract has no specific OD without extract as well) is subtracted from the average OD of the control wells and the wells containing the extract.

Cells should be cultured for a relevant time to be able to demonstrate the effect of the drug. For cell lines this should only be during the log phase, while for primary cells the MTT assay must be completed before all untreated cells are dead. Only for cell lines, a day 0 plate is used to precisely measure the activity of the starting cell dilution at day 0 without the extract's effects.

To account for possible influences of the dissolvent of the extract on the background OD, the control medium should contain the concentrations of dissolvent of extract.

Solid materials are extracted in cell culture medium and multiple dilutions of the extract are prepared and added into each well containing 1×10^4 cells. After 24 h incubation, the water soluble MTT substrate (yellowish solution when prepared in media or salt solutions lacking phenol red) is added and incubated with cells for 3 h, and then MTT is converted to an insoluble formazan.

Since formazan must be solubilized prior to absorbance readings, methods have been developed to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components.

Cytotoxicity is calculated based on the formazan signal generated which has been shown to have good linearity up to 10^6 cells per well and is dependent on the MTT concentration, the incubation time, and metabolically active viable cells. If the mean viability of cells is reduced to < 50 % of the blank control, the sample is considered potentially cytotoxic. The exact mechanism of MTT reduction is not well understood but is believed to involve NADH or similar reducing molecules that transfer electrons to MTT.

4.10.5.1 MTT Test procedure:

1 DAY: seed $1*10^4$ cell/100µL/ 1 well in 96 well plate, incubate for 24 h

2 DAY: add 20 µL of extract, filtrated and mixed with medium for specific concentration

3 DAY: add 20 µL of MTT (concentration 2.5 mg/ mL PBS)

→ After 3 hours add stop solution which is 10 % SDS in PBS (10g/100mL PBS), 100µL in each, leave it in dark, at room temperature for 20 hours.

4 DAY: Elisa reader 540 nm

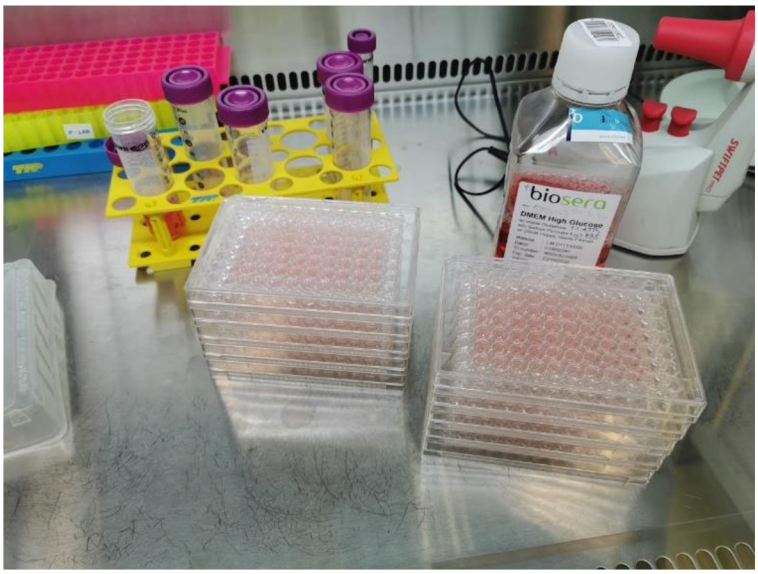

Figure 14: *MTT test in 96 well plates*

4.10.6 Apoptosis with B16F1 cell lines

Murine melanoma cell lines B16F1 were seeded ($5 \cdot 10^4$ cell/500µL/ 1 well in 6 well plate), incubate for 24 h. Samples were prepared, mixed with media and placed into a wells for 24 hours (to have comparable results according to the cytotoxicity test procedure). Then the samples were proceeded according to the instructions in ExBio kit [128].

4.10.6.1 Reagents

- Annexin V-FITC in stabilizing phosphate buffered saline (PBS) solution containing 15mM sodium azide.
- Propidium Iodide solution in deionized water (0.1 mg/mL).
- Annexin V Binding Buffer filter-sterilized solution (0.1M HEPES/NaOH, pH 7.4, 1.4M NaCl, 25mM $CaCl_2$).

4.10.6.2 Procedure

- Harvest cells intended for analysis by centrifugation (different cells may need different centrifugation conditions), discard supernatant. Resuspend cell pellet in cold PBS and wash cells by gentle shaking or by up and down mixing with a pipette tip. Recentrifuge washed cells again and discard supernatant.
- Resuspend cell pellet in Annexin V Binding Buffer and adjust cell density to 2 – 5x 10^5 cells/ mL, preparing a sufficient volume of cell suspension
- Add 5 µl of Annexin V - FITC and 5 µl of Propidium Iodide to each 100 µl of cell suspension and mix gently.
- Incubate for 15 minutes at room temperature in the dark.

- Centrifuge cells and resuspend pellet in 100 µl of 1x Annexin V Binding Buffer (or in an appropriate volume according to a method of sample acquisition)
- Analyse the stained cells by flow cytometry as soon as possible.

4.10.7 Apoptosis with Raji cell lines

Raji cell were seeded ($5 \cdot 10^4$ cell/500µL/ 1 well in 6 well plate), incubated for 24 hours. Next day samples were prepared, mixed with media for exact concentrations and placed into a wells for 24 hours (to have comparable results according to the cytotoxicity test procedure). Then the samples were proceeded according to the instructions below.

- Cultures, maintained as cell suspension, were harvested by centrifugation, and fixed with 70 % ethanol for 30 min on ice.
- After fixation samples were placed at -20°C until cytofluorometric analysis.
- For cytofluorometric analysis, the cells were harvested by centrifugation and cell pellet resuspended in sodium citrate buffer.
- Fluorochromization was performed by using **Propidium Iodide** and **Fluorescein isothiocyanate** (**FITC**).
- Propidium iodide was used alone for DNA staining or simultaneously with **Fluorescein isothiocyanate** (**FITC**) for the analysis of the DNA/Protein ratio; FITC is reactive towards nucleophiles groups including amine and sulfhydryl groups on proteins.

4.10.7.1 DNA - Propidium Iodide Staining

The cell pellet was gently resuspended in about 5 mL of a solution of propidium iodide (50 µg/mL) containing 4 mM sodium citrate (pH 7.8), 30 units/ mL of (DNAse-free) RNAse, and 0.1% TX-100. After an incubation period of 30 min at 37°C, NaCl was added to a final concentration of 0.15-0.25 M. Flow cytometry fluorescence of individual nuclei was measured on a Flow Cytometer CyFlow Space (Partec), equipped with an argon ion laser tuned to 488 nm at 500 mW, was used to excite the DNA-dye complex.

4.10.7.2 DNA - Propidium Iodide /Protein - FITC - Staining

For bi-parametric analysis (simultaneous labelling with Propidium Iodide and Fluorescein isothiocyanate), after staining with Propidium Iodide, the cells, after centrifugation, were incubated in a FITC solution (20 µg/mL) for 30 min at 37°C.
Fluorescence emissions, at 590 nm (Propidium Iodide) and at 520 nm (FITC), were collected, amplified, and displayed.

5 RESULTS

Presented work is focused on preparation of selected natural extract, namely plant and microbial extract. Both of the extract types were prepared as water-based extracts and organic oil extracts. Plant organic extracts were prepared by Soxtherm. Microbial water extracts were prepared by rehydration of biomass and oil extracts were prepared by Folch extraction. For evaluation of the extracts was necessary to test the set of metabolic parameters and biologically active substances (such as total phenolic content and antioxidant activity) and then choose the most suitable and representative samples for the tests.

5.1 PLANT EXTRACTS CHARACTERIZATION

Firstly, it was important to measure total phenol content, flavonoids and antioxidant activity of selected plant extracts to see potential of health benefits. From water extracts Curcuma and Clove has the highest amount of phenolic compounds. Curcuma and Ginseng has the highest antioxidant activity compared to Trolox equivalent.

5.1.1 Characterization of plant extracts

Natural plant extracts were characterized for antioxidant activity, total phenolic and flavonoids content. In the case of plant extracts, content of value-added substances was measured by procedures given in chapter 4.8. The preparation of individual extracts proceeded according to the procedure mentioned in chapter 4.5.

Table 6: *Natural plant extracts characterization*

Extracts	Plants	Polyphenols [mg/g]	Flavonoides [mg/g]	Antioxidant activity [mg/g]
Water	Acai	1.943 ± 0.08	1.082 ± 0.02	1.041 ± 0.04
	Clove	2.724 ± 0.07	1.705 ± 0.03	0.801 ± 0.02
	Curcuma	4.723 ± 0.07	3.656 ± 0.01	5.260 ± 0.06
	Ginger	0.959 ± 0.13	0.600 ± 0.01	1.135 ± 0.01
	Ginseng	1.906 ± 0.02	0.306 ± 0.01	2.260 ± 0.02
	Oregano	0.887 ± 0.09	0.778 ± 0.02	0.760 ± 0.01
Oil	Acai	1.418 ± 0.02	0.822 ± 0.03	0.987 ± 0.04
	Clove	2.181 ± 0.08	1.205 ± 0.03	0.652 ± 0.02
	Curcuma	2.176 ± 0.09	1.589 ± 0.02	3.258 ± 0.04
	Ginger	0.781 ± 0.01	0.560 ± 0.01	1.112 ± 0.01
	Ginseng	0.623 ± 0.02	0.215 ± 0.01	1.152 ± 0.02
	Oregano	0.533 ± 0.03	0.258 ± 0.02	0.650 ± 0.01

The antioxidant effect of the tested natural water extracts. as well as the total contents of phenolic substances and flavonoids are given in the *Table 6*. The highest content of phenolic substances was determined in turmeric extract (4.723 mg/g). while the lowest content was detected in oregano (0.887 mg /g). Turmeric also showed the highest antioxidant effect (5.26 mg /g). For the Oil extracts the best results were obtained from acai, clove and curcuma extracts for total phenol account, for acai 1.418 mg/g, for clove 2.181 mg/g and curcuma 2.176 mg/g.

Antioxidant activity of ginseng oil 1.152 mg/g was comparable with ginger 1.112 mg/g and higher result was obtained from curcuma 3.258 mg/g.

5.1.2 Characterization of liposomes

Particle size and size distribution are key parameters used to evaluate the physical stability of nanoparticles. Dynamic light scattering (DLS) is the most commonly used technique for measuring particle size. DLS is a widely used method for determining the size distribution. especially of small particles suspended in a liquid medium. Another important monitored parameter. except average size and particle size. distribution is the polydispersity index (PDI). PDI values o f 0.1 to 0.25 indicate a narrow size distribution, while PDIs greater than 0.5 indicate a wide distribution particle size.

In this work. the prepared particles were analysed on a colloidal analyser Malvern Zetasizer Nano ZS, which provided basic data on particles such as particle size distribution. average particle size and polydispersity index. The zeta potential was also measured using an electrode adapter. from which the approximate stability of the particles was directly estimated. Zeta potential (ZP) evaluates electrophoretic mobility suspended particles in the medium. Generally an absolute ZP value above 60 mV indicates excellent particle stability, a value of 30 mV generally indicates good stability. 20 mV is acceptable in the case of short-term stability. less than 5 mV indicates very fast aggregation and particle instability

Table 7: *Polydispersity and size of prepared nanoparticles*

	Water extracts		Oil extracts	
	PdI	Ø size [nm]	PdI	Ø size [nm]
Acai berry	0.201 ± 0.019	182.9 ± 0.9	0.131 ± 0.010	151.37 ± 1.07
Clove	0.489 ± 0.014	332.1 ± 7.3	0.115 ± 0.019	149.4 ± 1.3
Curcuma	0.438 ± 0.004	272 ± 3	0.175 ± 0.013	161.7 ± 2.4
Ginger	0.227 ± 0.014	187 ± 1	0.167 ± 0.014	150.5 ± 3.6
Ginseng	0.241 ±0.0012	227.0 ± 0.9	0.184 ± 0.006	158.0 ± 0.6
Oregano	0.47 ± 0.03	187.3 ± 1.4	0.116 ± 0.023	151.2 ± 1.05

Table 8: *Stability of liposomes*

	Water extracts	Oil extracts
	ZP [mV]	ZP [mV]
Acai berry	-44 ± 4	-37.7 ± 2.1
Clove	-43 ± 4	-34.6 ± 1.6
Curcuma	-39 ± 3	-40 ± 3
Ginger	-39 ± 3	-39 ± 3
Ginseng	-43 ± 4	-35.6 ± 2.6
Oregano	-41 ± 2	-37 ± 1.1

According to the value of the Zeta potential shown in table 8 the liposomes of the prepared particles showed very good stability. on average around -40 mV. The average size of the

liposomes in the aqueous extracts was 200 nm, only the clove extract had a particle size of about 330 nm. Liposomes prepared from oil extracts were smaller. about 150 nm. The polydispersity indices of the particles prepared from the aqueous extracts were also higher compared to the oil extracts and ranged from 0.2-0.5. The lowest polydispersity indices 0.1 were measured for oil extracts.

5.1.2.1 Encapsulation efficiency

The encapsulation efficiency (EE)was determined according to the procedure in Chapter 2.4.6. which was determined for liposomes from water and oil extracts. Efficiency was determined in % as the difference between phenolics in the supernatant after centrifugation at 14.800 rpm and the water extract. Each measurement was repeated. and the average obtained from the values obtained was used for subsequent calculations.

Table 9: *Encapsulation efficiency of liposomes in percent and concentration of encapsulated phenolic substances*

	Water extracts		Oil extracts	
	c [mg/g]	EE [%]	c [mg/g]	EE [%]
Acai berry	0.4	43.6	0.17	40.7
Clove	0.96	35.6	1.3	59.6
Curcuma	1.88	40.6	1.7	78.14
Ginger	0.38	39.9	0.8	90.8
Ginseng	1.01	53.11	0.9	97.5
Oregano	0.29	33.09	0.4	75.11

According to the given table it's obvious that oil extracts had higher efficiency of encapsulation. at ginseng oil extract it was 97.5 % which is about 0.9 mg/g of phenolic compounds. The lower efficiency of oils had acai oil. only 40.7 % which is 0.17 mg/g of phenolic compounds. At water extracts the higher encapsulation efficiency was measured at ginseng 53 % which is 1.01 mg/g of phenolic compounds that is comparable with oil because ginseng oil has only half amount of phenolic compounds compare to the water extract. The lower encapsulation efficiency of water extract was measured in clove. only 35.6 % which is 0.96 mg/g phenolic compounds.

5.1.2.2 Antioxidant activity

The antioxidant activity of liposomes was determined by method described in chapter 4.8.3

Table 10: *The amount of antioxidant activity of liposomes to Trolox equivalent*

	Water extracts c [mg/g]	Oil extracts c [mg/g]
Acai berry	0.19 ± 0.02	0.03 ± 0.03
Clove	0.78 ± 0.24	0.68 ± 0.13
Curcuma	0.24 ± 0.08	0.23 ± 0.13
Ginger	0.06 ± 0.02	0.8 ± 0.3
Ginseng	0.05 ± 0.02	0.3 ± 0.2
Oregano	0.34 ± 0.21	0.39 ± 0.07

According to the results of antioxidant activity compared with water extract. For example. water extract from curcuma contained 5.2 mg/g antioxidants and in liposomes only 0.24 mg/g. Therefore. it was not effective to continue preparing nanoparticles and the work was focused more on natural water and oil extracts.

5.1.3 Antibacterial activity. dilution method

Antimicrobial activity is tested using standard antimicrobial assays. Testing is performed on microorganisms in suitable culture media and under optimal conditions. Antimicrobial tests are divided into diffusion and dilution. Both types of tests were used in presented study. Thus, various methods for determining inhibitory measures have been tested properties including both visual detection of inhibition zones on solid agar plates and measurements changes in culture concentration during cultivation in liquid media. The selected samples were also monitored for antimicrobial efficacy by changing density during cell lysis. The antimicrobial efficacy of the test substances and extracts was monitored against the bacterial of gram-positive strains - *Micrococcus luteus* and gram-negative strains - *Serratia marcescens*.

Previously this method was performed in a series of test tubes. Today, microtiter plates are mainly used. Test cultures of microorganisms are inoculated with test substances. After 24h incubation the inhibition effect is measured. The evaluation can be performed visually, turbidimetrically, by measurement absorbance or by direct determination of the number of cells in the counting chambers or by flow cytometry. The antimicrobial activity of all tested plant extracts was tested using this method.

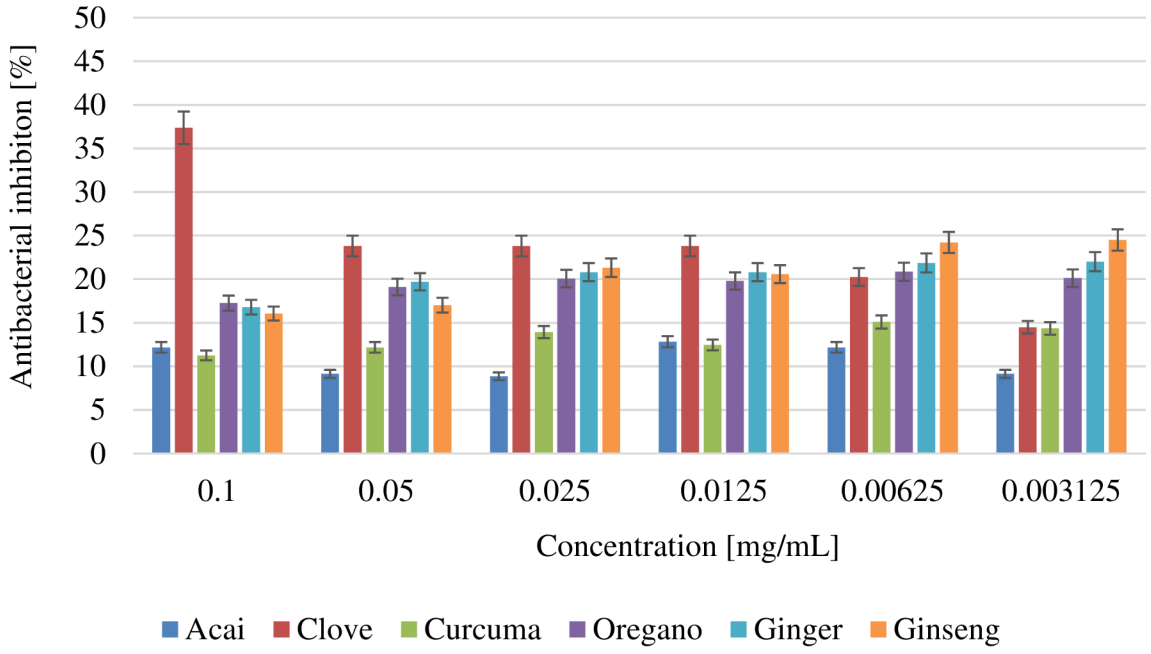

Figure 15: *Antibacterial inhibition of Serratia marcescens with water plant extracts [%]*

In monitoring the growth inhibition of *Serratia marcescence*, antibacterial activity of selected plant extracts was measured. Most of the samples showed similar results in all concentration range from 0.1 mg/mL to 0.003 mg/mL of dry materiál diluted in water. Clove extract showed highest antibacterial effect against gram-negative strains.

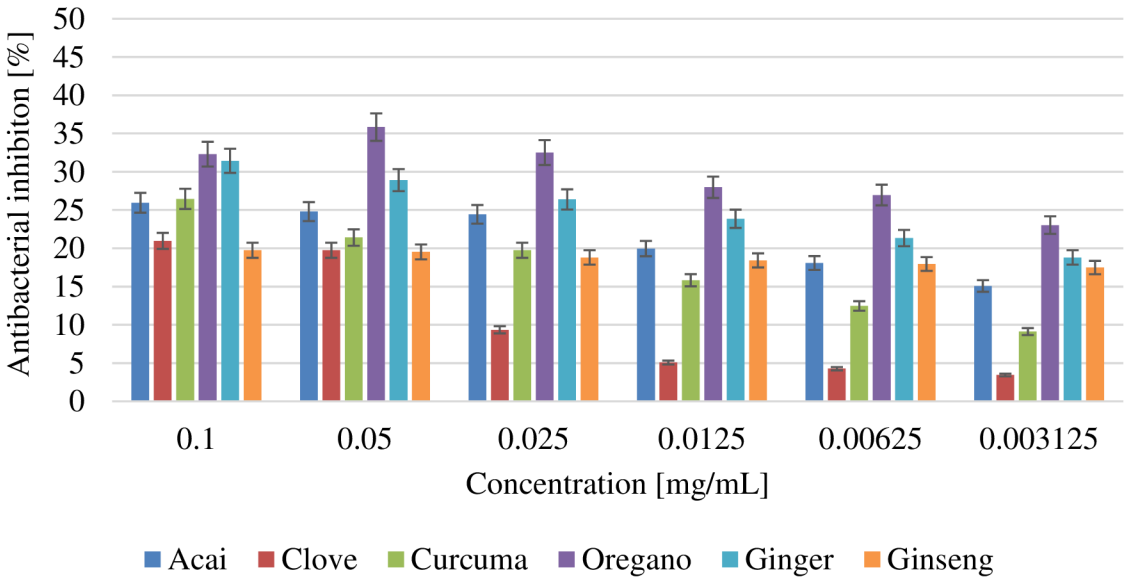

Figure 16: *Antibacterial inhibion of Micrococus luteus with water plant extracts [%]*

For gram- positive strains the most effective was extract from oregano. On the other hand. clove extract wasn't so effective and also showed concentration dependent effect. same as curcuma and ginger.

5.1.4 Antimicrobial activity. dilution method

For better clarity. the following microorganisms were compared with each other in a single table. The percentage efficacy of 2.5 % and 5 % of the extracts was monitored on 3 strains. *Candida glabrata*. *Staphylococcus aureus* and *E. coli*. There were significant differences in the individual extracts when measuring their antimicrobial activity. Water extracts were established 2.5 % and 5 % according to the following tests with oils. Oil extracts were diluted in DMSO and then mixed with media due to a solubility of oils in media.

Table 11: *Antimicrobial activity of selected plant extracts*

Microorganisms		***Candida glabrata***		***Staphylococcus aureus***		***Escherichia coli***			
Concentration [%]		5	2.5	5	2.5	5	2.5		
Plant Extracts	**Acai**	-	-	-	-	-	-	**+**	**Inhibition 1 - 10 %**
	Clove	+++	+++	+++	++	+	++	**++**	**11 - 25 %**
	Curcuma	+	-	+	-	+	-	**+++**	**≥ 26 %**
	Ginger	++	+	-	-	-	-	**-**	**≤ 1 %**
	Ginseng	-	-	-	-	-	-		
	Oregano	++	-	++	+	+	-		

When measuring antimicrobial activity of selected 2.5 % a 5 % water plants extracts is significant that clove extract showed the highest potential of growth inhibition against microorganisms. On the other hand. ginseng extract was not effective at all.

5.1.5 Tests of plant extracts on the cell lines

The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals. which can be solubilised for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 nm. Concentration is calculated in percentage that correspond 1:1 to mg/g of dry sample.

B16F1, Raji, HaCaT and Caco-2 cells were treated with different plant extracts in concentration range between 4-28 mg/g for 24h. A considerable reduction in the cell viability was observed in a concentration dependent when compared with the control treated cells.

5.1.5.1 Keratinocytes HaCaT

Immortalized human keratinocytes cell line **HaCaT** from skin has been used as a model for the study of keratinocytes functions. MTT test was performed as described in chapter 4.10.5.

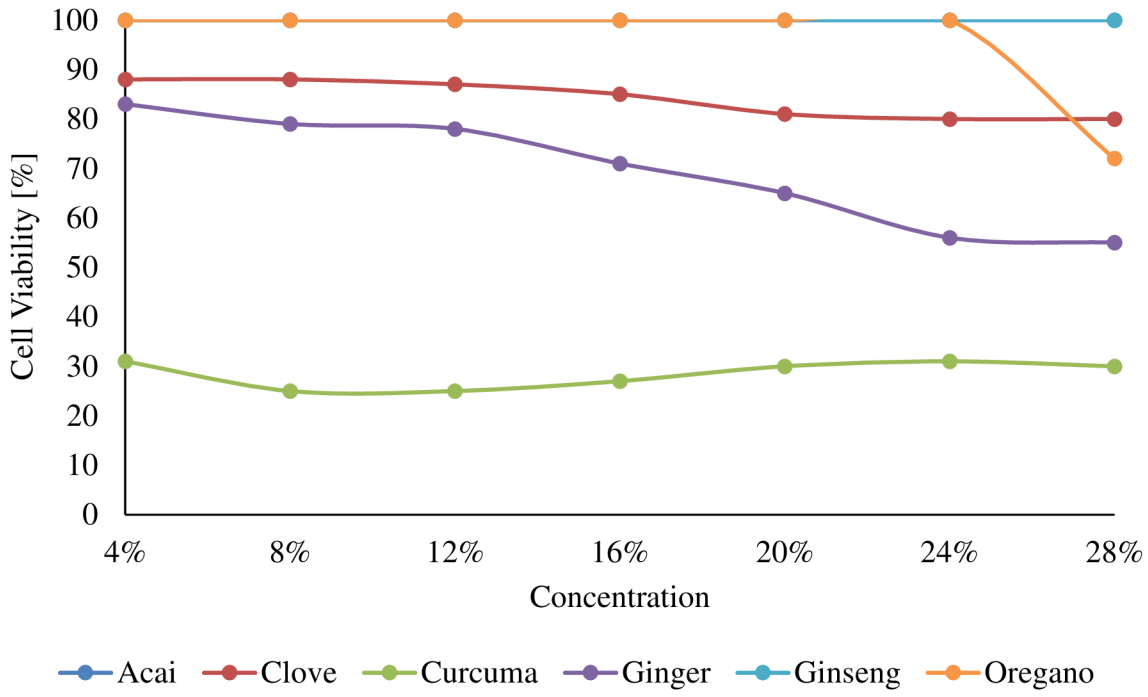

***Figure 17**: Cytotoxicity of plant extracts tested on HaCaT cell lines*

Firstly was HaCaT cell line exposed to plant water extracts. In the figure 17 we can see that only curcuma extract was below the 50 % of cell viability and therefore was toxic for the HaCaT cell line. Curcuma was measured in lower concentration ratio and its toxicity was concentration dependent. Below 1 % volume extract which correspond to 1 mg/mL was not toxic. Ginger and oregano extract also showed concentration dependent toxicity and over 30 % which correspond to 30 mg/g were toxic for HaCaT cell line. On the other hand. ginseng and acai. which is hidden behind sample of ginseng were not toxic at all. Clove extract was in the all-concentration range around 85 - 80 % of viability.

5.1.5.2 Melanoma murine tumor cell line B16F1

B16F1 cell line is usually used for research as a model for human skin cancers. Therefore. it's interesting the correlation between analysis of HaCaT and B16F1. It's required that some extracts with toxic effect on B16F1 would be also safe in the same concentration range for HaCaT cell lines.

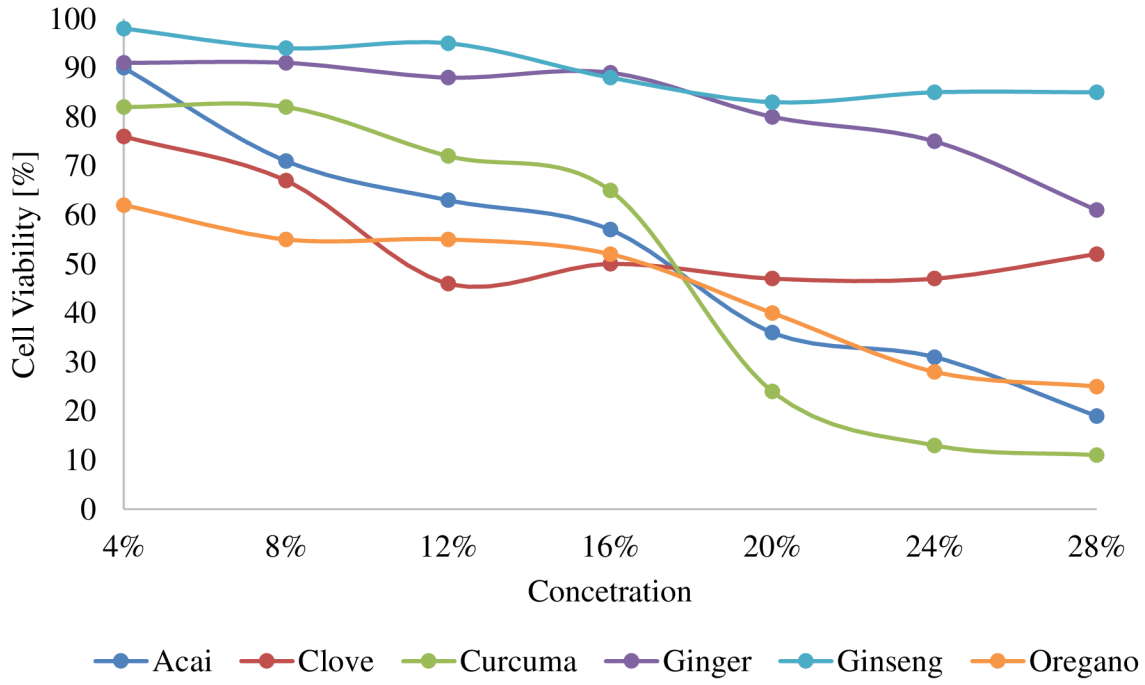

***Figure 18**: Cytotoxocity of plant extracts tested on B16F1 cell lines*

Firstly, was B16F1 cell line exposed to plant water extracts. In figure 18 we can see that Ginseng and Ginger extracts were not toxic in all concentration range. but the ginger extract showed concentration depend on trend between 24 % a 28 % so we can expect toxicity at higher concentrations. All the other extracts showed toxicity at higher concentrations. Especially in concentration range from 16 % to 20 % all of the samples except ginger and ginseng have reached the toxicity limit 50 % of viable cells.

5.1.5.3 Raji cell line

These cells have proven to be convenient target cells in studies assessing the effector functions mediated by monoclonal antibodies.

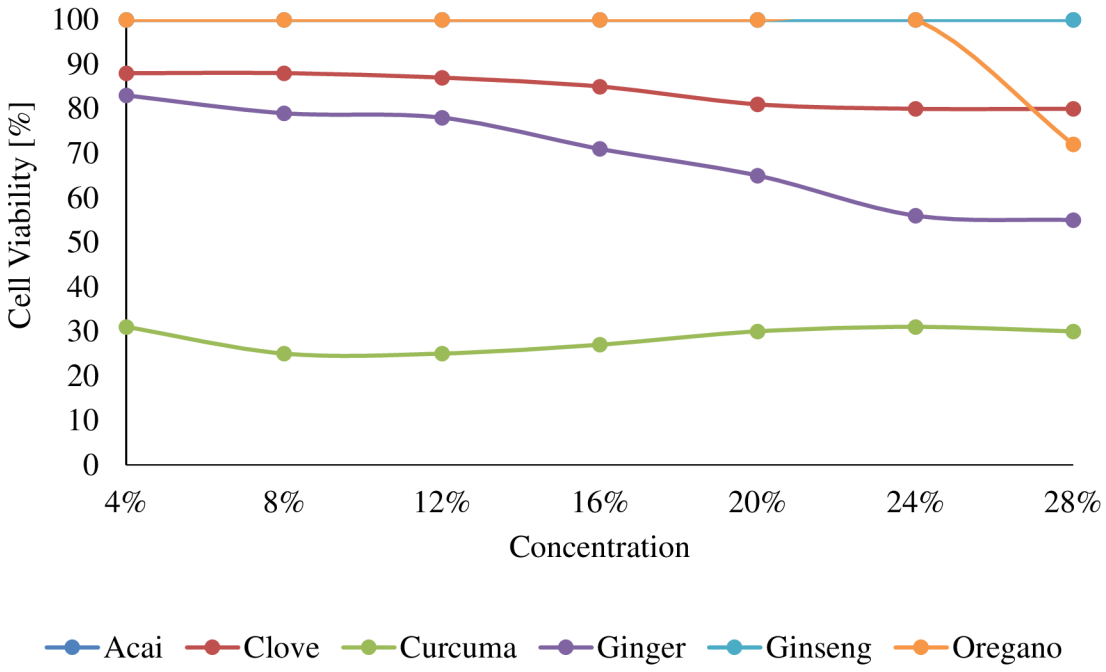

Figure 19: *Cytotoxicity of plant extracts tested on Raji cell lines*

Firstly, was Raji cell line exposed to plant water extracts. In the Figure 19 we can see that Acai extract that is hidden behind Ginseng. because is was not toxic in all concentration range. Clove and Oregano extracts were not toxic in our concentration range. but the oregano extract showed concentration depend on trend between 24 % a 28 % so we can expect toxicity at higher concentrations. Curcuma was measured in lower concentration ratio and its toxicity was concentration dependent. Below 1 % volume extract which correspond to 1 mg/g was not toxic. Toxicity of ginger extract was concentration dependent especially from 24 %.

5.1.5.4 Caco-2

The Caco-2 cells isolated from human colorectal adenocarcinoma are widely used to screen for absorption rate of new compounds in the initial stage of drug discovery.

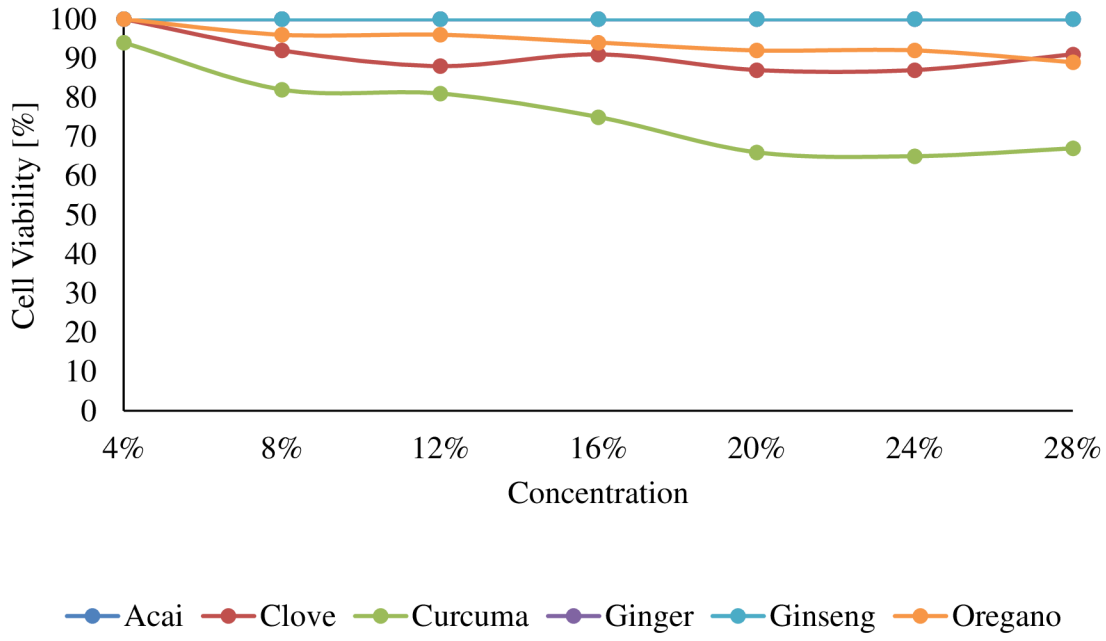

Figure 20: *Cytotoxicity of plant extracts tested on Caco-2 cell lines*

Firstly, was Caco-2 cell line exposed to plant water extracts. In the Figure 20 we can see that acai and ginger extracts that are hidden behind ginseng, because it was not toxic in all concentration range. Clove and oregano extracts were not toxic in our concentration range, but the extracts showed concentration depend on trend so we can expect toxicity at higher concentrations. Curcuma was concentration dependent and showed highest toxicity.

5.1.6 Apoptosis

Apoptosis was tested on two types of cell lines. Raji cell lines were used in combination with plant extracts of oregano. clove. curcuma and ginger. Melanoma murine tumor cell line B16F1 was used in combination with both. plant and microbial extracts.

5.1.7 Apoptosis of Raji Cell lines

General evaluation of the results obtained. allows us to underline that treatment of *Raji* cell cultures with plant extracts induces effects on cell distribution in the various phases of the cell cycle (G1-S-G2/M). This is observed in all the experimental conditions with the exception of sample named *Origano 1*.

Percentage values of cell distribution in the cell cycle phases are shown in the table below. in which the more significant changes. in comparison with control condition. are highlighted in red. In green the values less different from control are shown.

Control reference histogram was obtained from Raji cell cultures maintained in our laboratory. separately.

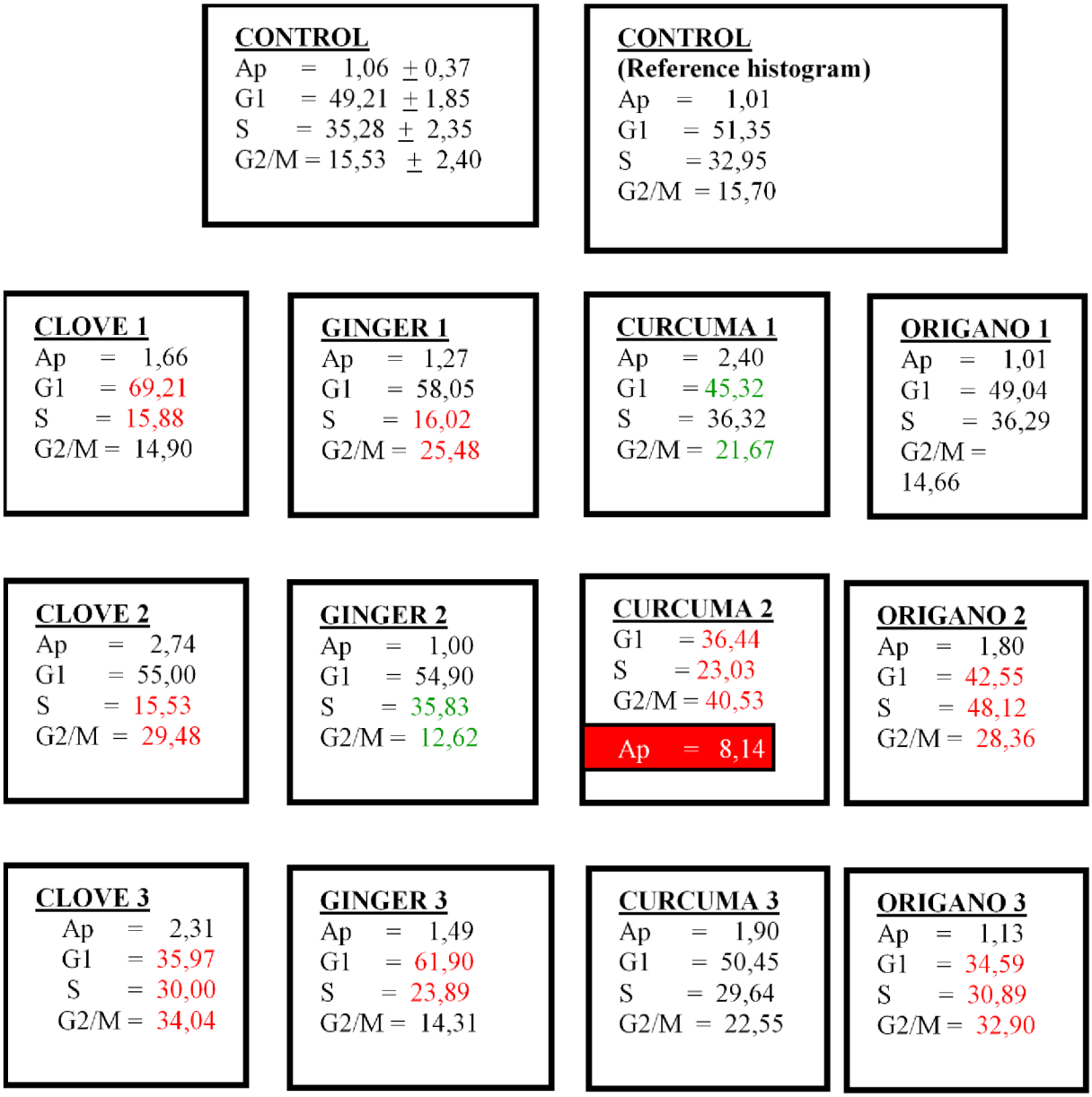

Figure 21: *Percentage values of cell distribution i the cell cycle phases*

In the Figure 21, cell cycle analyses are reported. Showing the major effects induced by natural plant extracts: *curcuma 2* represents the only condition in which an apoptotic incidence significantly different (even though limited) from controls is revealed.

In general. plant extract treatment (at the conditions here used) do not seem to induce apoptotic effects. Instead. in some conditions particularly cytostatic effects (partly different). caused by a slow-down of the transition of the cells through the cell cycle phases. were

observed. Percentage of the cells in the cell cycle phases (G1-S-G2/M) and in apoptosis (Ap). In control. the values ± SD are reported.

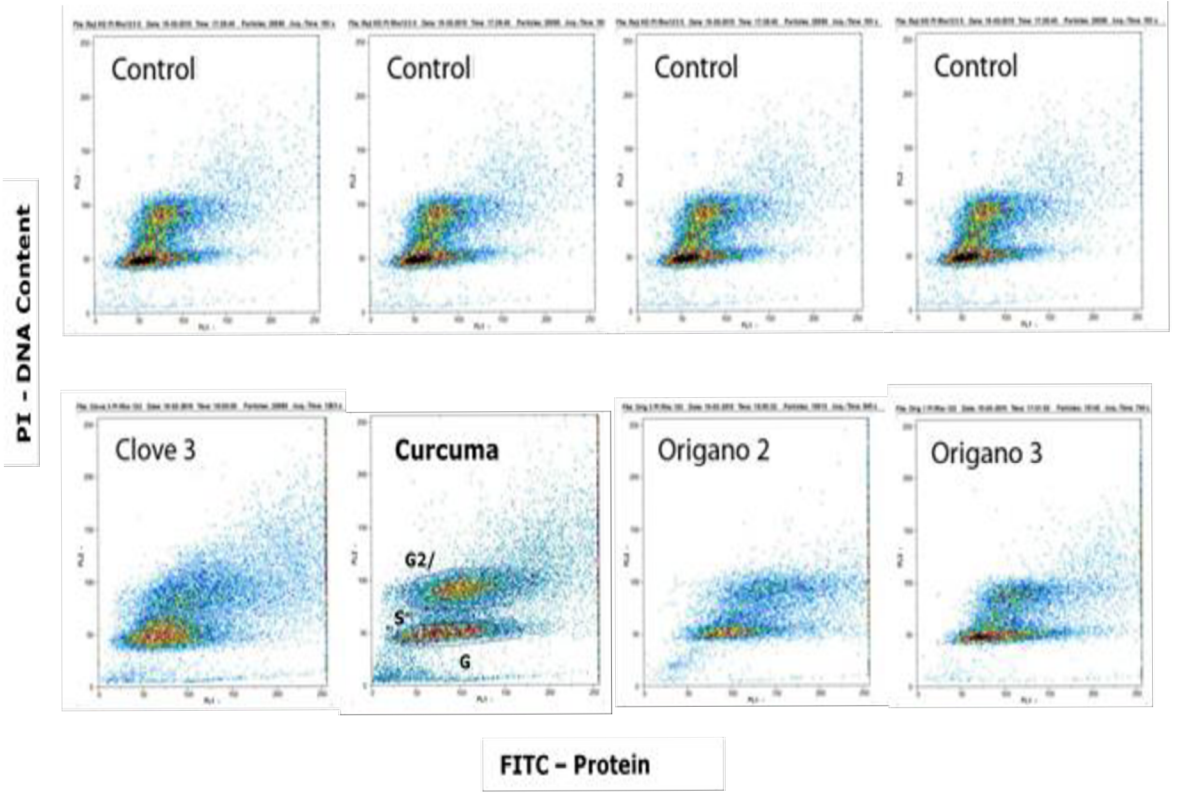

Figure 22: *Delimitation of cells*

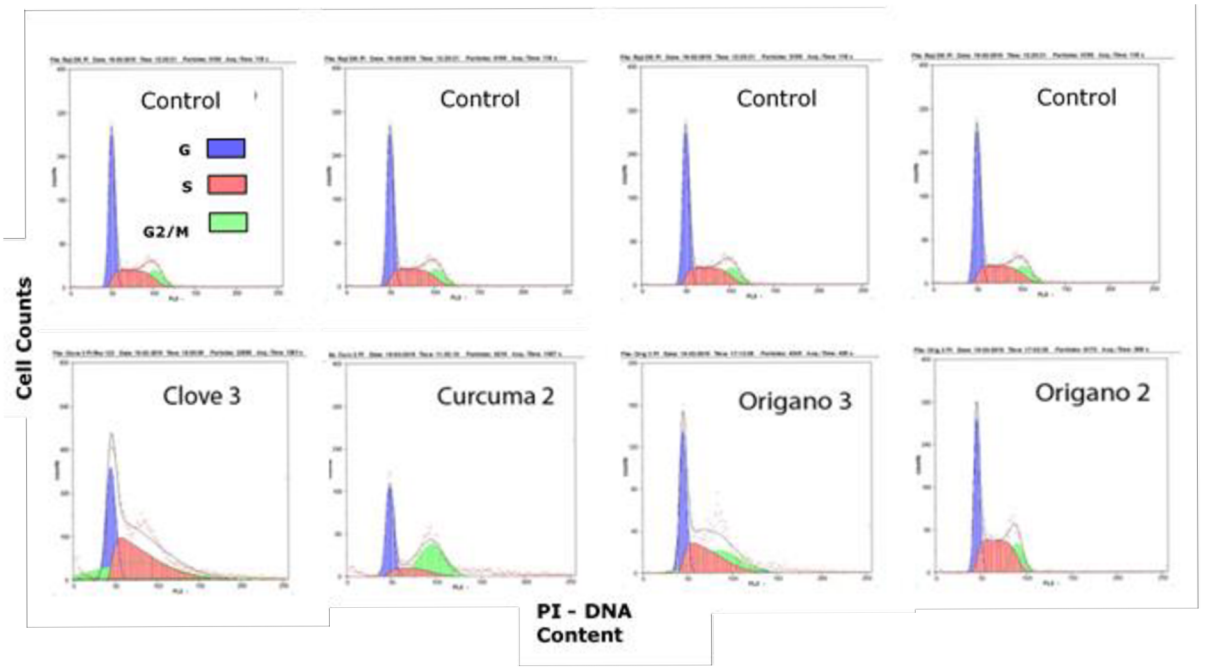

Figure 23: *Histograms of of cell distribution i the cell cycle phases*

5.1.8 Apoptosis of B16F1

The purpose of the viability experiments was to observe and compare the impact of selected plant and microbial extracts.

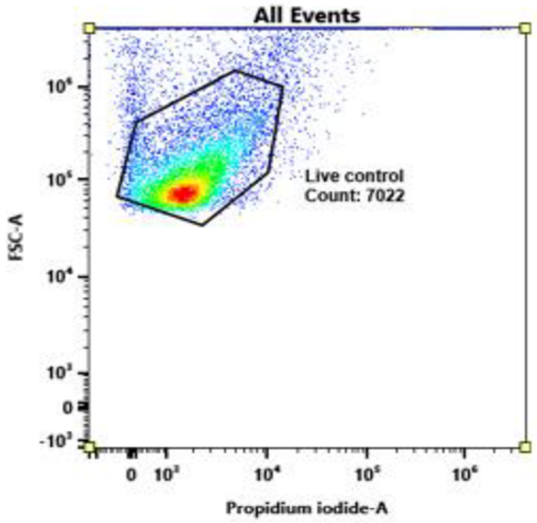

Figure 24: *Delimitation of untreated control cells*

Untreated control cells visualized in the forward-scatter (FSC) versus Propidium iodide dotplot shown in Figure 24 were used as functional control of the method.

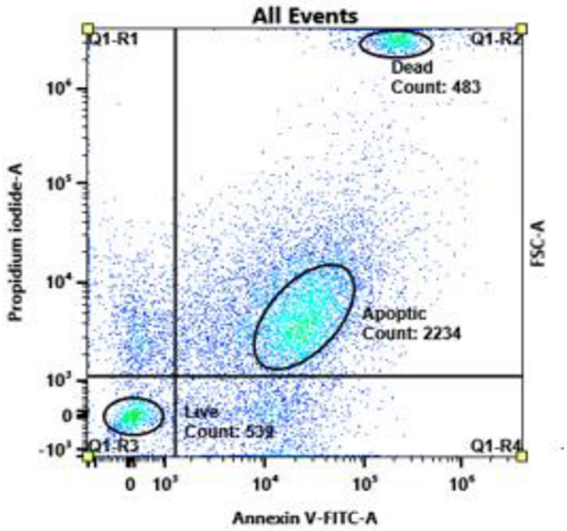

Figure 25: *Stained apoptotic cells (camptothecin treated)*

Camptothecin treated cells visualized in the Propidium iodide versus Annexin V dotplot shown in Figure 25 were used as positive control of apoptotic cells. For negative control we used cell treated with 70 % of ethanol.

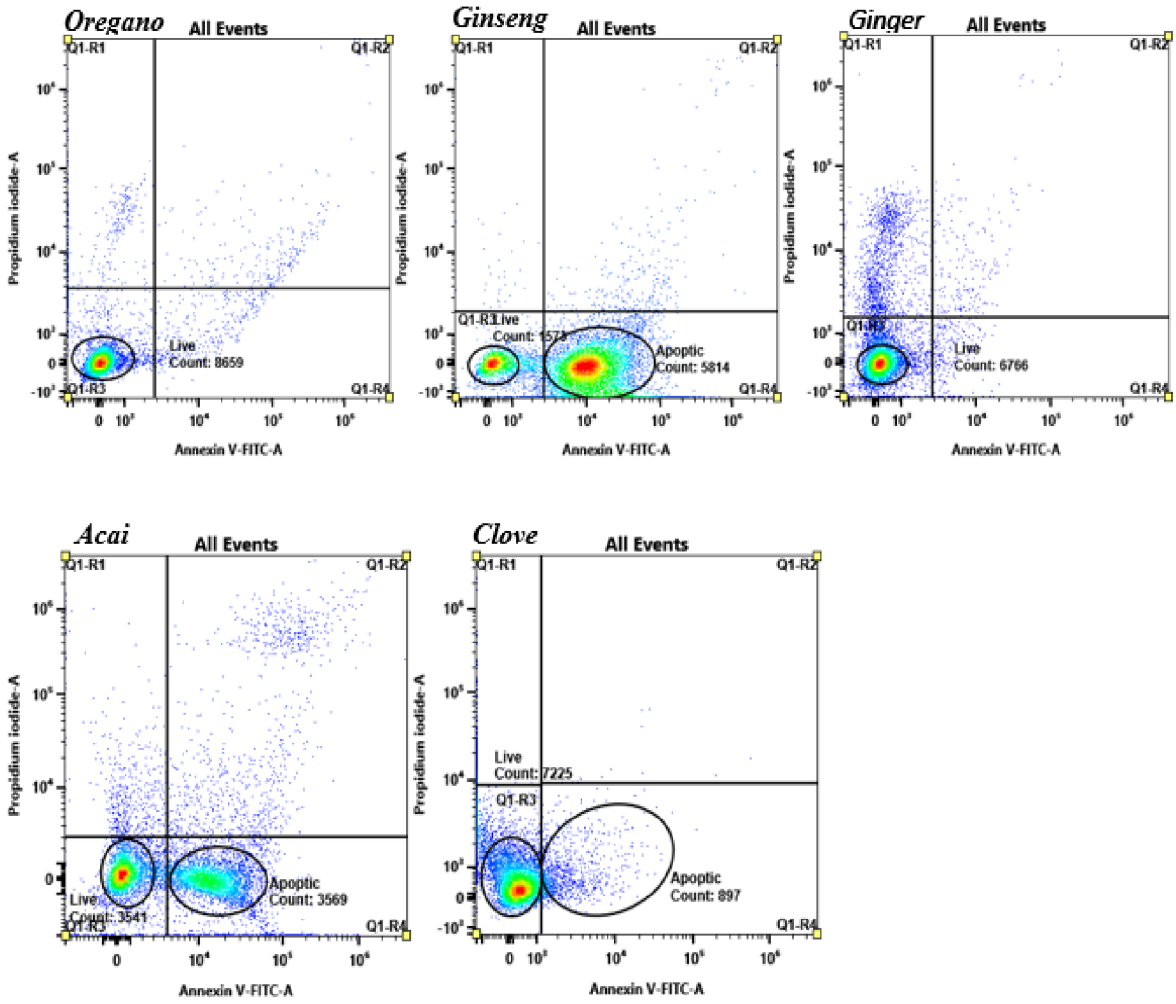

Figure 26: *Stained apoptotic cells (plant extracts treated)*

The cytotoxic effect of selected plant extracts on Raji and murine cancer cell lines was evaluated using MTT assay. We found that plant extract treatment was particularly effective against B16F1 melanoma cells. When measuring apoptosis. in the cytograms we can observe that cells treats with oregano and ginger do not seem to induce apoptotic effects. Instead. treatment with acai. clove and ginseng incidence significantly apoptotic cells in cytograms. Curcumin showed high autofluorescence of itself in cytograms. It was necessary to measure curcumin without PI and Annexin V to obtain better results. Generally, as compared to control cells. there was a significant reduction in cell viability in plant extracts-treated cells. Effect of plant extracts on cell viability was confirmed by crystal violet staining. From these data. we concluded that the cytotoxicity of plant extracts was dose dependent in B16F1 cells and Raji cells. The toxic effects of plant extracts can be correlated to the antioxidant activity and antimicrobial effect. It's obvious that there is correlation between cell viability and the apoptosis.

5.2 PRODUCTION OF MICROBIAL EXTRACTS FROM SELECTED YEAST STRAINS

Yeast strains of *Metschnikowia pulcherrima* CCY 029-002-145. *Cystofilobasidium infirmominiatum* CCY 17-18-4. *Phaffia rhodozyma* CCY 77-1. *Rhodotorula kratochvilovae* CCY 20-2-26. and *Saccharomyces cerevisiae* CCY 6646 were cultivated in Erlenmeyer flasks to obtain biomasses for all the tests below. *S. cerevisiae* CCY 6646 was used as a control strain due to its commercial use as the sole representative of yeast to produce β-D-glucans.

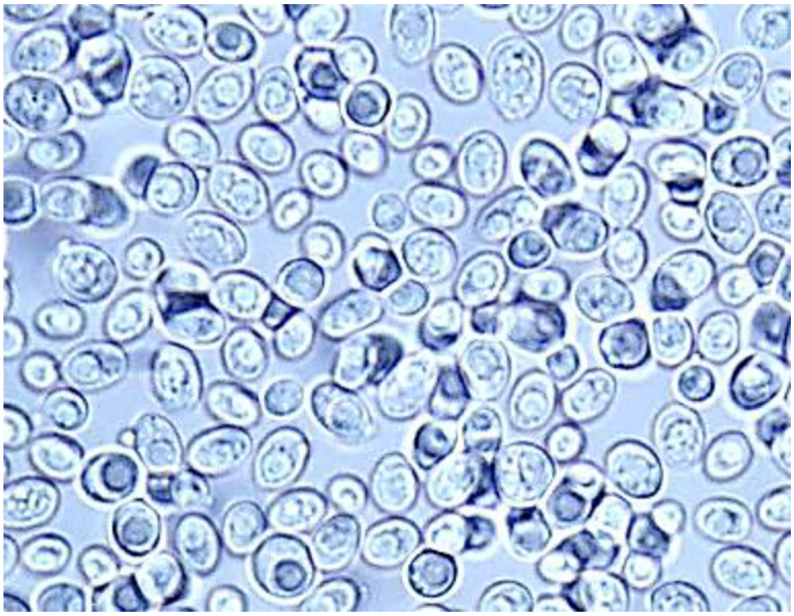

Figure 27: *Biomass cultivation of Rhodotorula kratochvilovae in YPD medium*

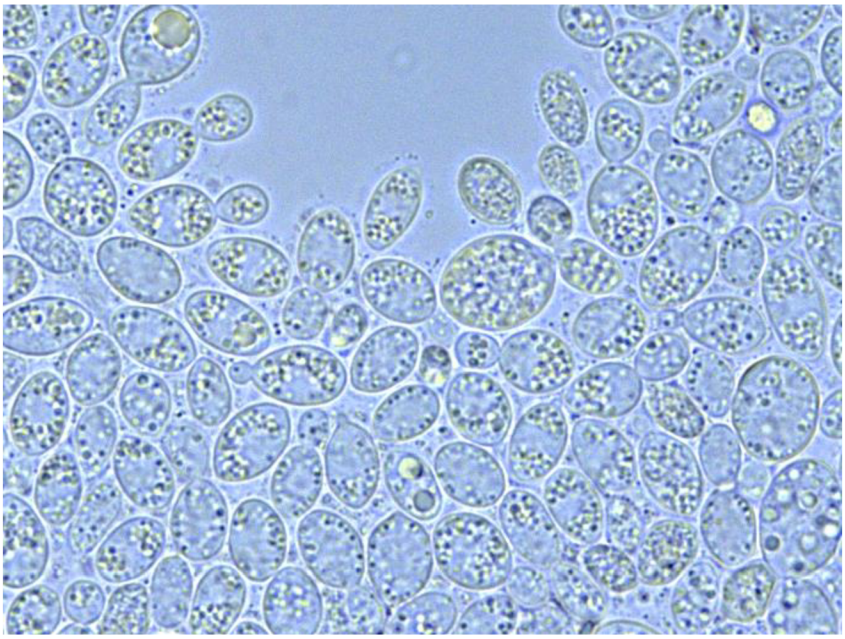

Figure 28: *Biomass cultivation of Phaffia rhodozyma in YPD medium*

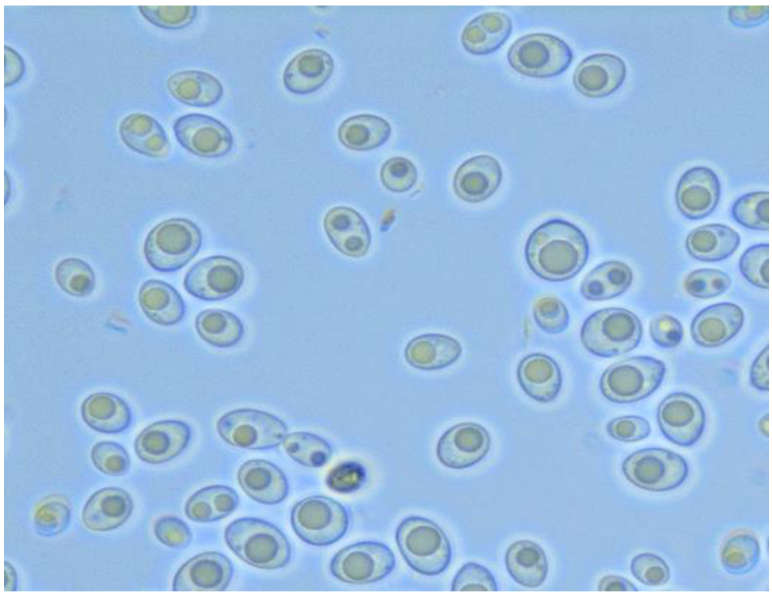

Figure 29: *Biomass cultivation of Cystofilobasidium informominiatum in YPD medium*

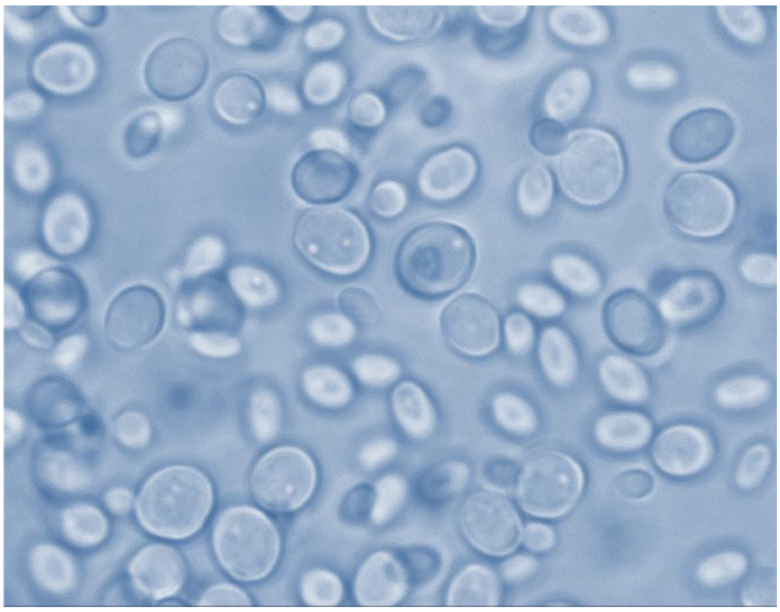

Figure 30: *Biomass cultivation of Metschnikowia pulcherrima in YPD medium*

All yeast strains were cultivated under the same conditions. The absence of pathogens was checked microscopically. When viewing the specimen under high magnification yeasts appears as an egg-shaped organism. It is also possible to observe the buds. which can be seen on some of the yeast cells. After 96 hours biomass was centrifuged and prepared for tests.

5.2.1 Characterization of microbial extracts

Health benefits are the most important uptake for this research. Firstly. we measured antioxidant activity. antimicrobial and antibacterial effect for comparison with plant extracts and then prepare some mixture of those extracts. Microbial extract was tested as biomass itself and then

as organic Folch extract to measure benefits from carotenoids and other organic substances in biomass.

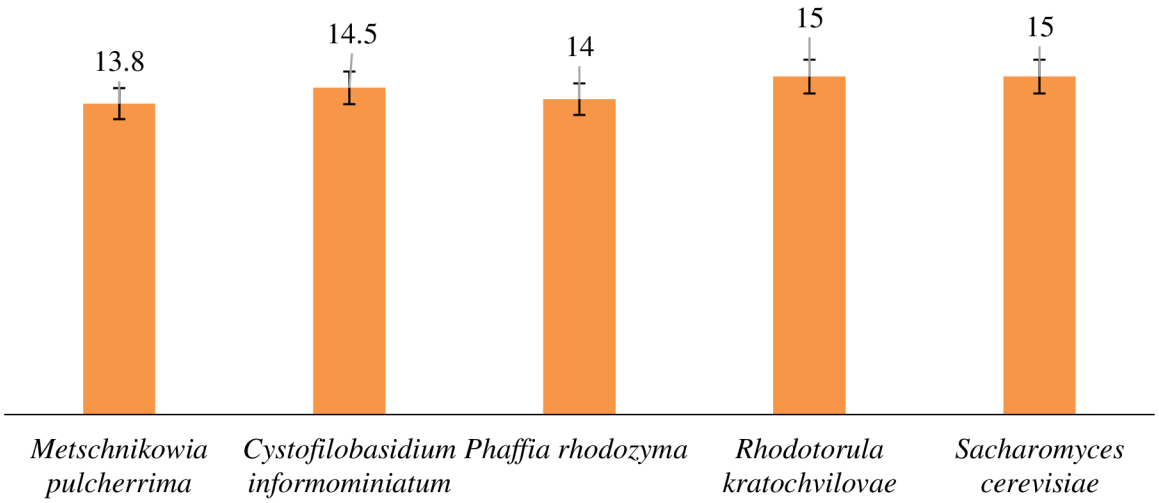

Figure 31: *Biomass yield (g/L) cultivated in Erlenymeyer flasks after 96 hours.*

The biomass yield after the cultivation on the control YPD broth production medium for 96 h in Erlenmeyer flasks. All the strains showed comparable biomass yield from 13.8 g/l to 15 g/l. The biomass was 3x washed with distilled water after cultivation and centrifuged. Biomass was placed in 50mL tubes and store at -80 °C for 48 hours and then lyophilized. After the lyophilization the dry among of biomass was weighted and after rehydration was tested and used for all of the procedures below.

5.2.2 Determination of carotenoids

Carotenoids are an essential group of compounds produced by red yeasts. They are important antioxidants. food colorants. cosmetic ingredients. and feed additives.

Total carotenoids content in presented carotenogenic yeast strain *Rhodotorula kratochvilovae* and *Phaffia rhodozyma* is around 1.3 mg/g of dry biomass. The main carotenoids occurred in yeast biomass are beta-carotene. thorulene and small amount of lycopene.

Table 12: *Content of carotenoids in biomass [mg/g]*

	CCY 029-002-145	***CCY 17-18-4***	***CCY 77-1***	***CCY 20-2-26***
Ergosterol	0.210	0.940	1.121	2.955
Ubiquinon	0.469	1.350	1.548	2.335
Torularhodin	-	-	0.856	1.063
Lycopene	-	-	-	0.051
Torulen	-	-	0.058	0.103
β- carotene	-	0.006	0.024	0.066
Total carotenoids	-	0.007	1.250	1.374

Content of carotenoids was measured by HPLC method described in chapter 4.7.1

5.2.3 Yeasts as a fatty acids producents

The amount of lipids and the fatty acid content of lyophilized yeast biomass was determined by gas chromatography in parallel with the production of glucans. Fatty acids were divided into three groups. polyunsaturated fatty acids (PUFA). monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). The results were graphically evaluated by showing the individual ratios of PUFA. MUFA and SFA in the total amount of fatty acids in the biomass. The total percentage of fatty acids in the biomass feed was also evaluated.

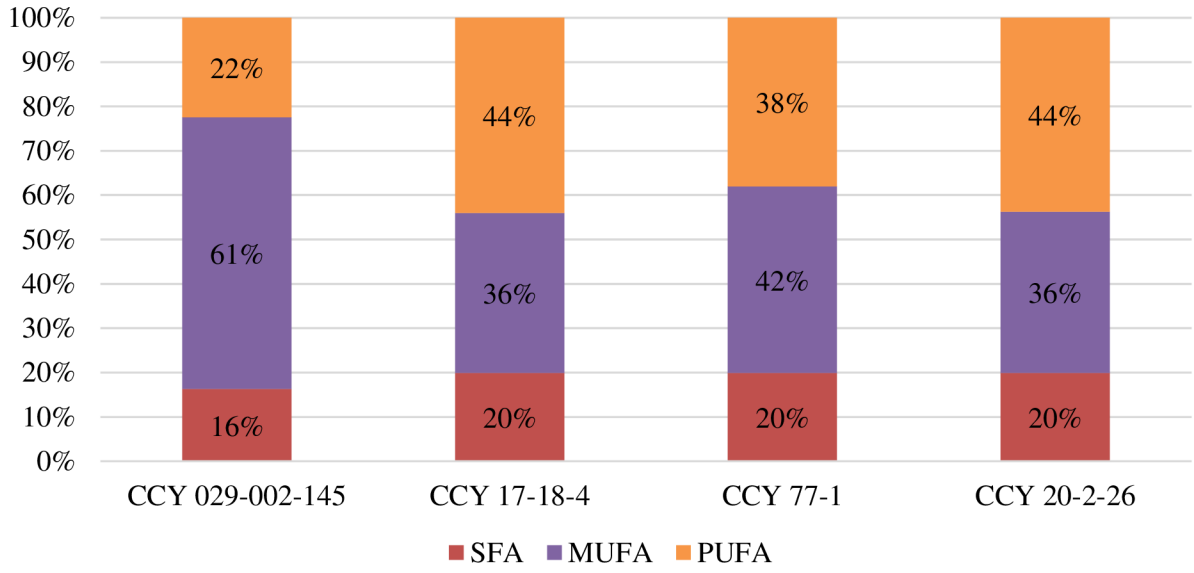

***Figure 32**: Production of fatty acids by selected yeast strains*

From the graph shown in figure 32 it's obvious that there is a big difference in amount of individual ratios between yeast that were used in this thesis. Yeast strain of *Metschnikowia pulcherrima* has the highest amount of monounsaturated fatty acids.

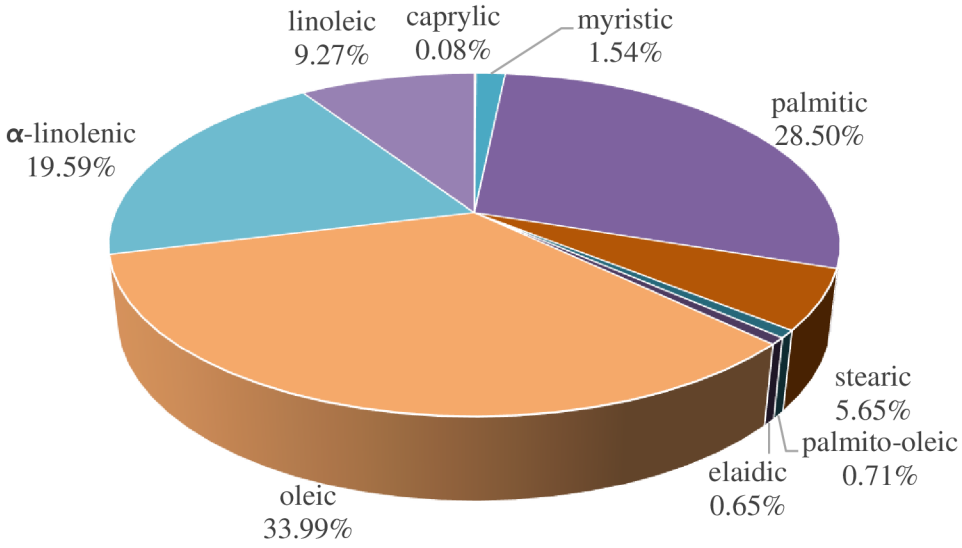

Figure 33: *Percentage of fatty acids in strain Rhodotorula kratochvilovae*

The graph in the figure 33 shows percentage of determinated fatty acids in strain *Rhodotorula kratochvilovae*. Significantly highest amount is of oleic acid (33.99 %), palmitic acid (28.50 %), myristic acid (19.59 %) and α-linolenic acid.

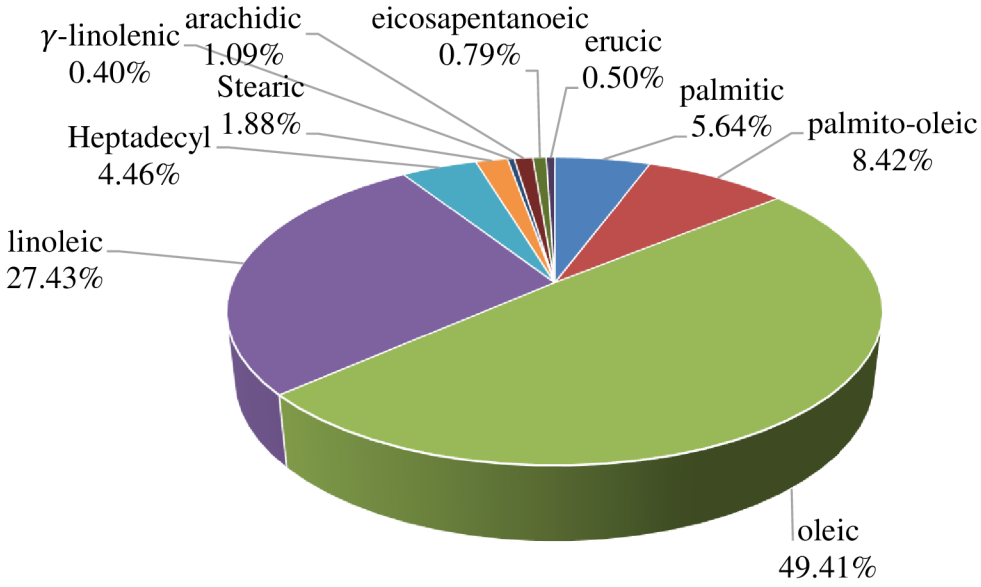

Figure 34: *Percentage of fatty acids in strain Metschnikowia pulcherrima*

The graph in the figure 34 shows percentage of determinated fatty acids in strain *Metschnikowia pulcherrima*. Significantly highest amount is of oleic acid (49.41 %), linolic acid (27.43 %), palmito-oleic acid (8.42 %) and palmitic acid.

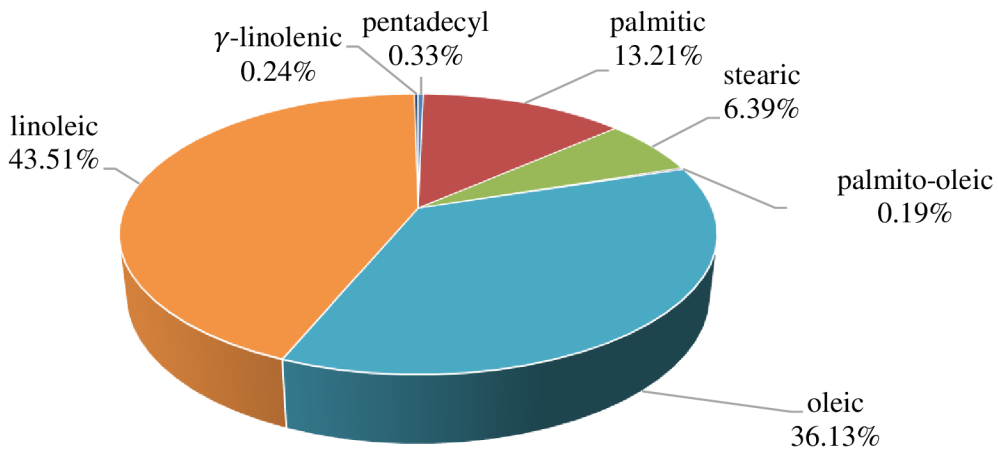

Figure 35: *Percentage of fatty acids in strain Cystofilobasidium informominiatum*

The graph in the figure 35 shows percentage of determinated fatty acids in strain *Cystofilobasidium informominiatum*. Significantly highest amount is of linoleic acid (43.51 %), oleic acid (36.13 %), palmitic acid (13.21 %) and stearic acid.

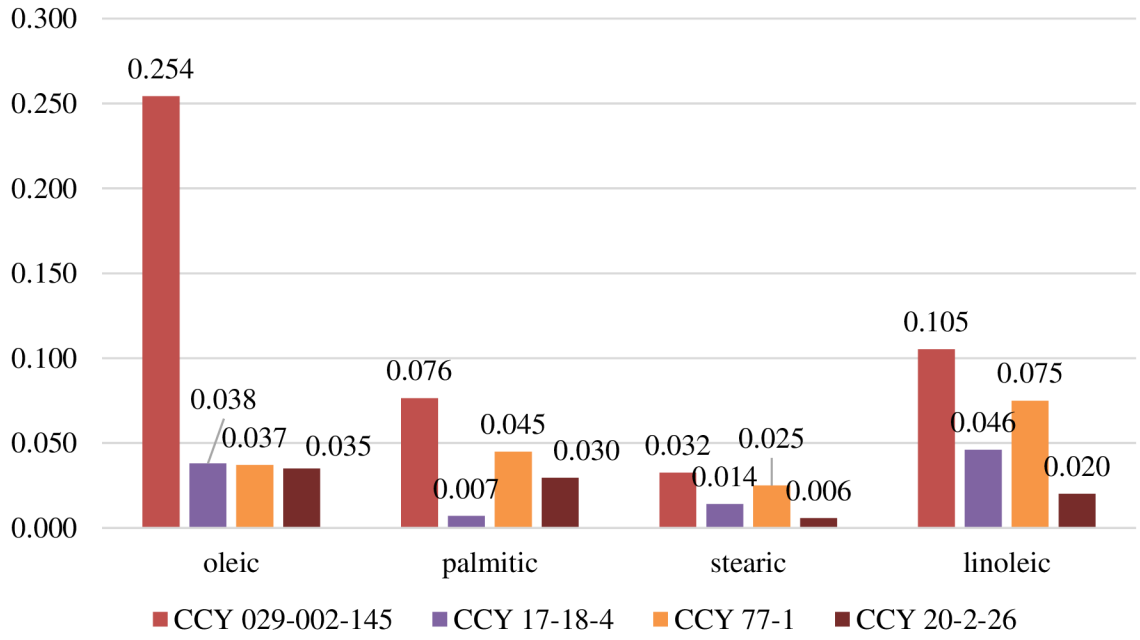

Figure 36: *Volume of the main fatty acids in biomass [mg/g]*

Volume of fatty acids was also measured by gas chromatography. From the figure 36 we can see that yeast strain of *Metschnikowia pulcherrima* has the highest amount of oleic, palmitic, stearic and linoleic acid.

5.2.4 Isolation and determination of β glucans

Several studies have shown a baker's yeast beta-1.3/1.6-D-glucan. extracted from Saccharomyces cerevisiae. is effective in reducing the incidence of cold and flu symptoms. Isolation and determination of beta-glucans in cultivated biomass was described in chapter 4.8.4.

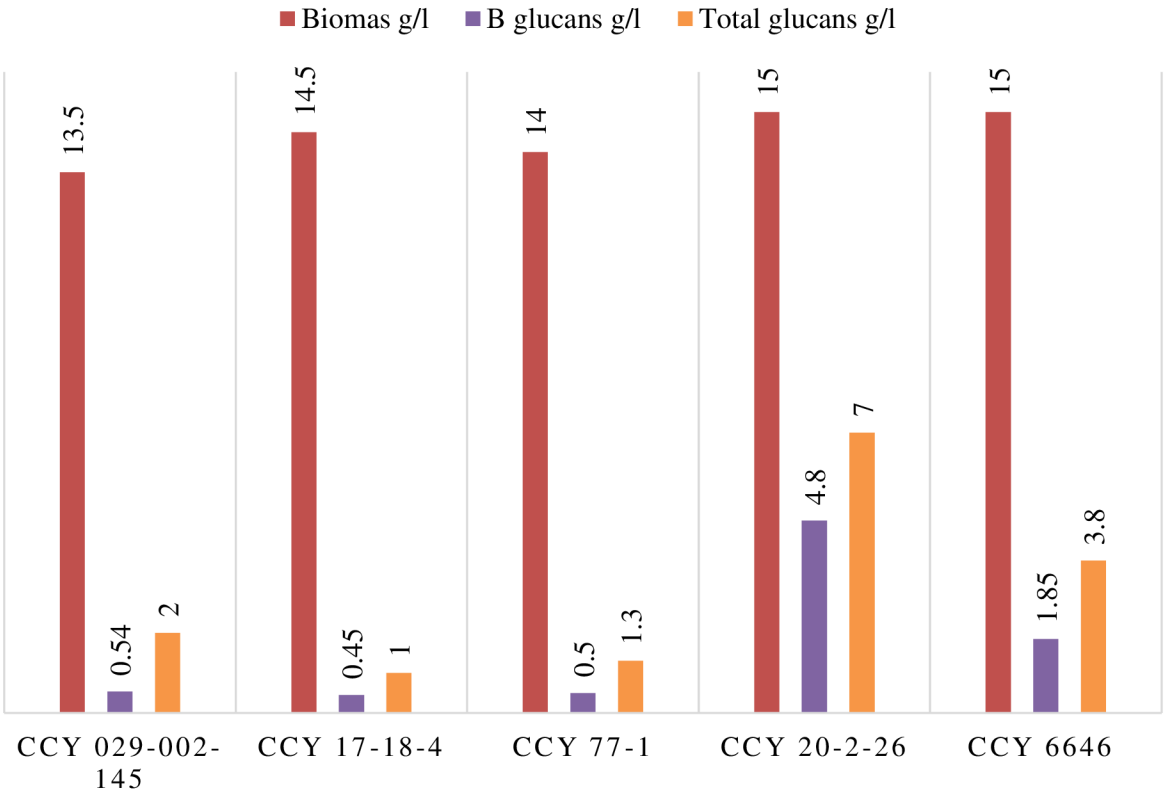

Figure 37: *Production of glucans by selected yeasts strains*

In the Figure 37 we can see the yields of glucans and biomass of all tested yeast strains. From *Rhodotorula katochvilovae* we obtain the highest amount of total glucans which was 7.12 g/L of biomass and from 7.12 of total glucans we obtained 4.75 g/L of beta- glucans. From *Metschnikowia pulcherrima* we obtain 1.92 g/L of total glucans and 0.405 g/L of beta-glucans. From *Cystofilobasidium informominiatum* we obtain 1.12 g/L of total glucans and 0.475 g/L beta-glucans. From *Phaffia rhodozyma* we obtain 1.42 g/L of total glucans and 0.654 g/L beta-glucans

5.2.5 Antioxidant activity

Microbial extracts were characterized for antioxidant activity which is described in 4.8.3 The preparation of microbial extracts proceeded according to the procedure mentioned in chapter 4.9.2.

Table 13: *Antioxidant activity of rehydrated biomass and Folch extract*

	Antioxidant activity [mg/g]	
	Rehydrated biomass	Folch extract
CCY 029-002-145	4.435 ± 0.05	4.107 ± 0.01
CCY 20-2-26	5.807 ± 0.08	5.002 ± 0.02
CCY 77-1	4.215 ± 0.02	4.321 ± 0.03
CCY 17-18-4	4.536 ± 0.07	4.612 ± 0.01

According to the results in Table 13 we can compare antioxidant activity of rehydrated biomass and of folch extract of rehydrated biomass. All the samples showed high potential to be strong antioxidants comparable for example with water extract of curcuma.

Antimicrobial activity is tested using standard antimicrobial assays. Testing is performed on microorganisms in suitable culture media and under optimal conditions. Antimicrobial tests are divided into diffusion and dilution. Both types of tests were used in the work. Thus. various methods for determining inhibitory measures have been tested properties including both visual detection of inhibition zones on solid agar plates and measurements changes in culture concentration during cultivation in liquid media. The antimicrobial efficacy of the test substances and extracts was monitored against the bacterial of gram-positive strains - *Micrococcus luteus* and gram-negative strains - *Serratia marcescens.* To compare plant extracts and microbial extracts. was also prepared biomass of *Rhodotorula kratochvilovae* and mixture in the ration of 1:1 with plant extracts.

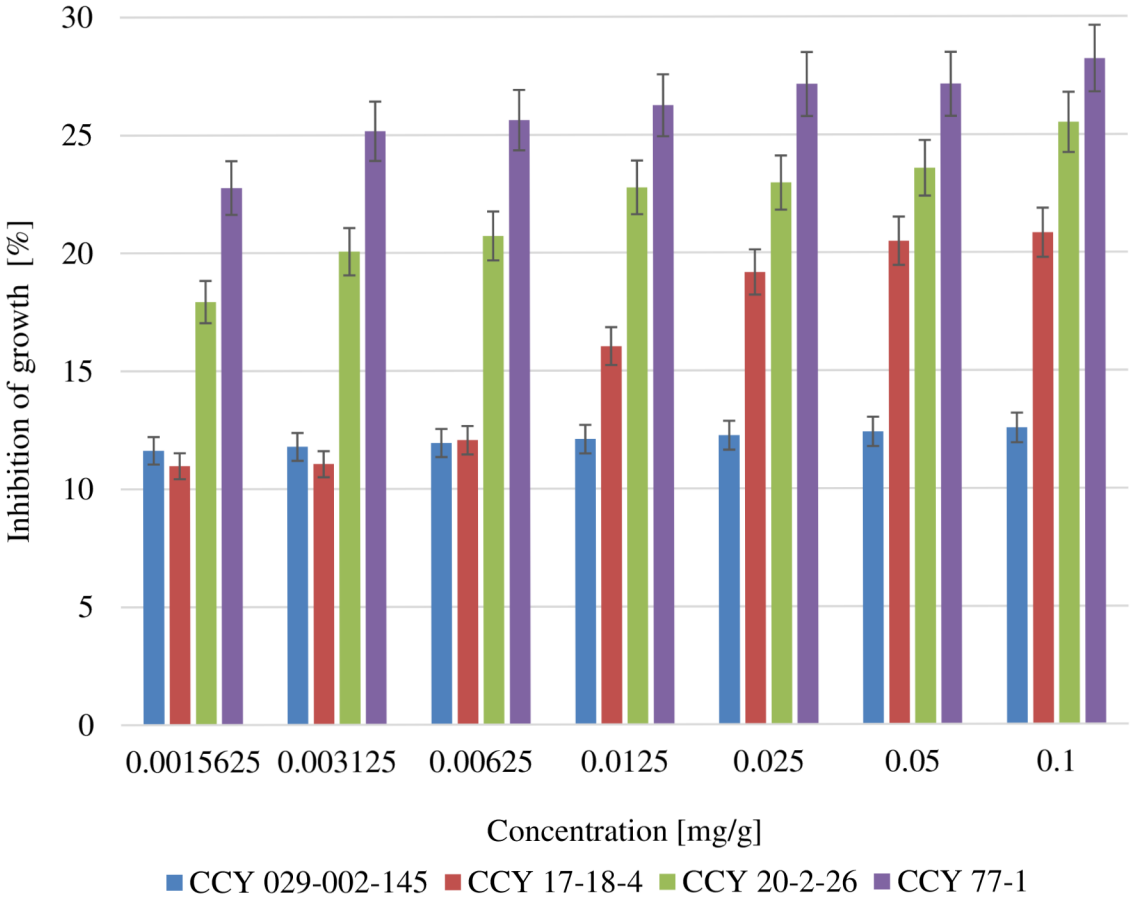

Figure 38: *Antibacterial inhibition of microbial extracts and mixture Serratia marcescens [%]*

According to the test it's obvious that microbial extracts are antimicrobial active substances. The highest potential showed extract of *Phaffia rhodozyma* in all concentration range. Inhibiton of microbial growth showed concentration dependent trend.

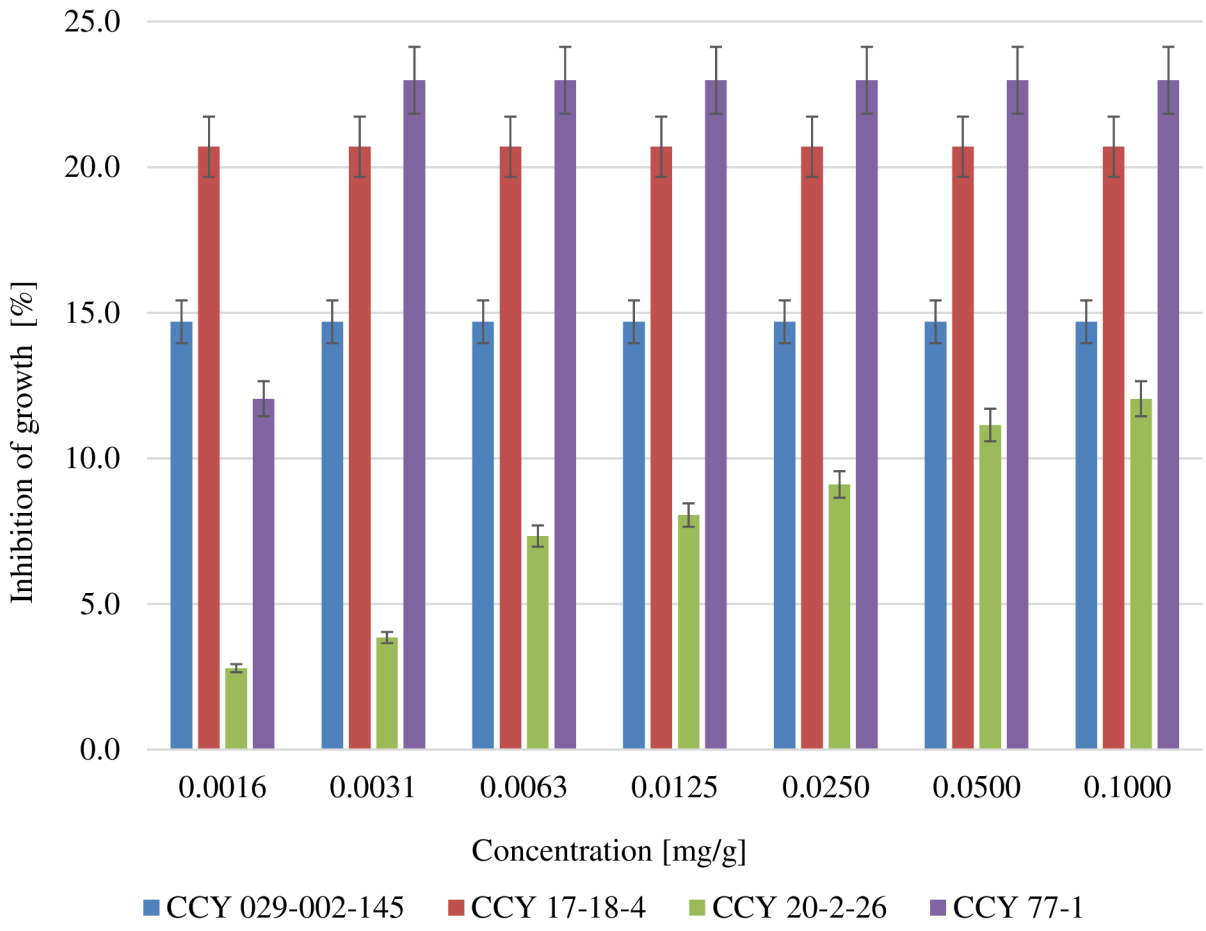

Figure 39: *Antibacterial inhibition of microbial extracts and mixture Micrococcus Luteus [%]*

According to the test it's obvious that microbial extracts are antimicrobial active substances. The highest potential showed extract of *Phaffia rhodozyma* in all concentration range. Inhibiton of microbial growth showed concentration dependent trend. On the other hand extract from *Rhodotorula kratochvilovae* showed less potential as antimicrobial agent.

5.2.6 Tests on cell lines

The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals. which can be solubilised for homogenous measurement. Thus. any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 nm.

B16F1, HaCaT and Caco-2 cells were treated with different microbial extracts in concentration range between 4-28 mg/g for 24h. A considerable reduction in the cell viability was observed in a concentration dependent when compared with the control treated cells.

5.2.6.1 Keratinocytes HaCaT

Immortalized human keratinocytes cell line **HaCaT** from skin has been used as a model for the study of keratinocytes functions. MTT test was performed as described in chapter 4.10.5. Microbial extracts were tested on keratinocytes. According to the results of antimicrobial activity and antioxidant activity it was assumed on slightly higher toxicity than at plant extracts.

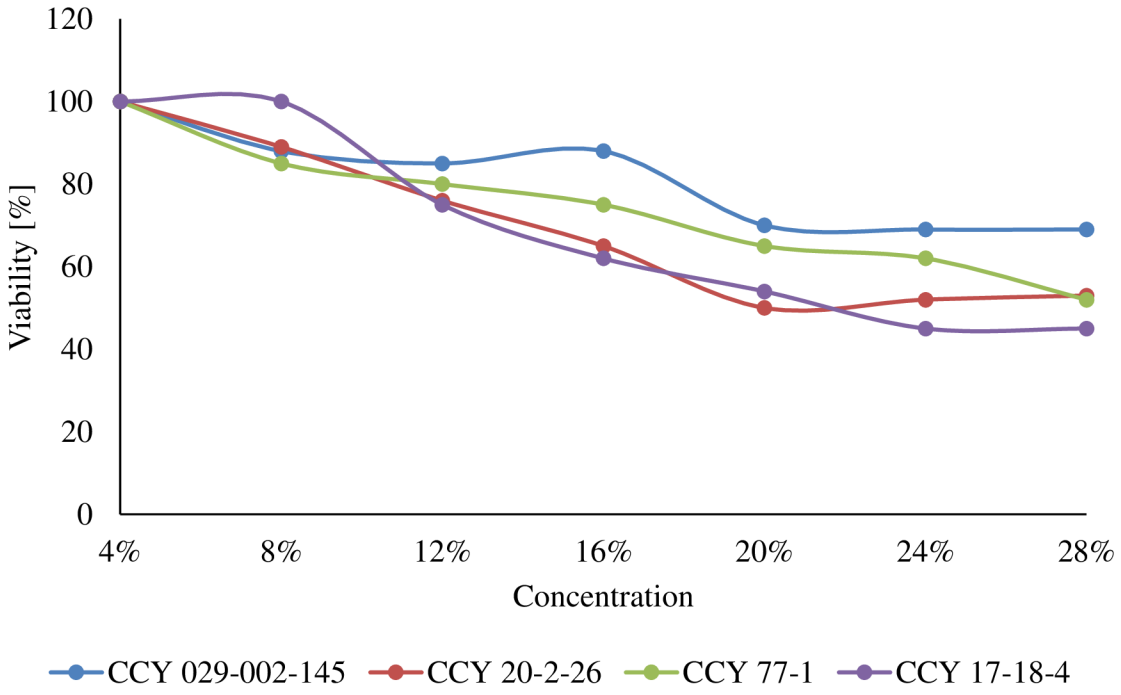

Figure 40: *Cytotoxicity of microbial extracts tested on HaCaT cell lines*

In the Figure 40 we can see that toxicity of the microbial extracts was concentration dependent and in low concentration it was not toxic at all. From 20 % which correspond to 20 mg of dry biomass diluted in 1 mL of DMEM media. was extract of *Cystofilobasidium informominiatum* and *Rhodotorula kratochvilovae* on the edge of toxicity. On the other hand. extract of *Metschnikowia pulcherrima* and *Phaffia rhodozyma* was in all concentration range above the edge of toxicity.

5.2.6.2 Melanoma murine tumor cell line B16F1

B16F1 cell line is usually used for research as a model for human skin cancers. Therefore, it's interesting the correlation between analysis of HaCaT and B16F1. It's required that some extracts with toxic effect on B16F1 would be also safe in the same concentration range for HaCaT cell lines.

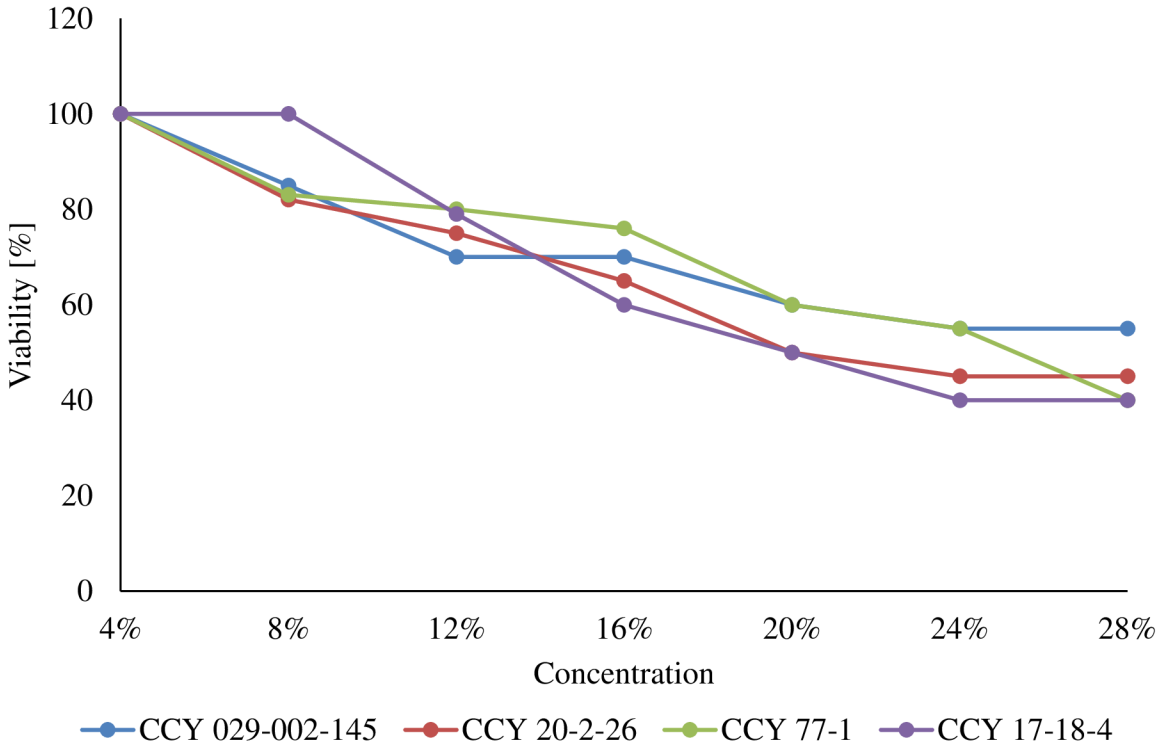

Figure 41: *Cytotoxicity of microbial extracts tested on B16F1 cell lines*

In the Figure 41 we can see that toxicity of the microbial extracts was concentration dependent in all concentration range. Viability of mouse melanoma cells with microbial extracts in lower concentration about 4 % which correspond to 4 mg of dry lyophilized biomass diluted in 1 mL DMEM media was 100 % or slightly above. From 20 % which correspond to 20 mg of dry lyophilized biomass diulted in 1 mL of DMEM media. were all extracts on the edge of toxicity.

After comparing the cytotoxic effect of natural plant extract and microbial extract on HaCaT cell lines and B16F1 cell lines. we can conclude slightly antitumor effect of microbial extracts in concentrations from 16 % which is on the edge between toxicity for B16F1 and non-toxic for HaCaT. Natural plant extracts showed slightly antitumor effect on the concentration range from 16 % to 28 % except ginger and ginseng. Especially acai extract was remarkable result according to microbial effect. curcuma showed the toxicity from 16 % exponentially. Also oregano extract was toxic for B16F1 but that's what we expect according to the antimicrobial activity and antioxidant activity.

To evaluate possibility of apoptotic effect on B16F1 cell line was necessary prepare organic extract of dry lyophilized biomass. Biomass was rehydrated and then Folch extraction was used to extract all the active compounds from biomass. The organic solvent was evaporated under nitrogen and then dissolved in 1 mL of DMSO. Before testing the toxicity, the sample was diluted in DMEM media. The highest acceptable concentration of DMSO for cell lines was 5%.

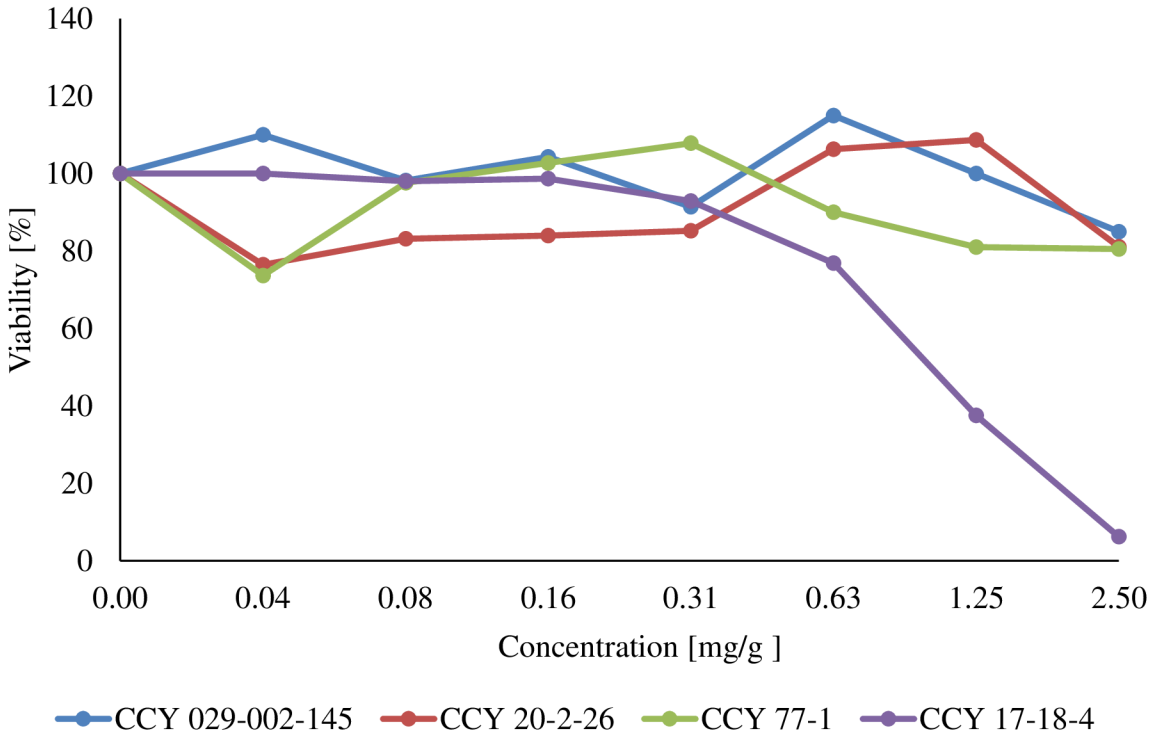

Figure 42: *Cytotoxicity of Folch extracts from lyophilized biomass on B16F1 cell lines*

In the Figure 42 we can see that all the extracts were concentration dependent. Especially from 0.31 mg/g of dry biomass. All of the extracts were toxic at concentration of 5 mg/g of dry biomass and the blank sample of 5 % DMSO was tested too to avoid false positive results.

5.2.6.3 Caco-2

The Caco-2 cells isolated from human colorectal adenocarcinoma are widely used to screen for absorption rate of new compounds in the initial stage of drug discovery.

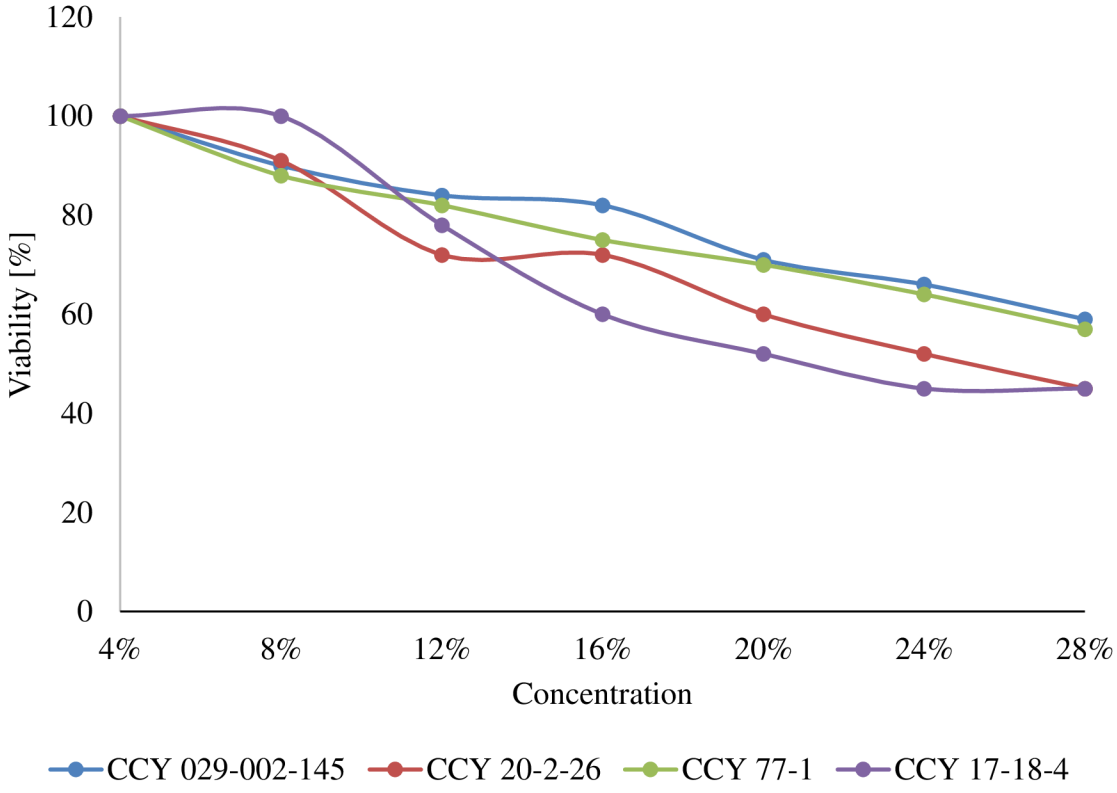

Figure 43: *Cytotoxicity of microbial extracts tested on Caco-2 cell lines*

In the Figure 43 we can see that toxicity of the microbial extracts was concentration dependent and in low concentration it was not toxic at all. From 16 % which correspond to 16 mg of dry biomass diluted in 1 mL of DMEM media. was extract of *Cystofilobasidium informominiatum* on the edge of toxicity. Extracts of *Metschnikowia pulcherrima*, *Phaffia rhodozyma* and *Rhodotorula kratochviloave* were slightly toxic from higher concentrations about 24 % – 28 %.

5.2.7 Apoptosis

Apoptosis was tested on 2 cell lines. Raji cell lines were used in combination with plant extracts of oregano. clove. curcuma and ginger. Melanoma murine tumor cell line B16F1 was used in combination with both. plant and microbial extracts.

5.2.7.1 Apoptosis of B16F1

The purpose of the viability experiments was to observe and compare the impact of selected plant and microbial extracts.

From the microbial extracts. oils were prepared by Folch extraction described in Chapter 4.7.1.

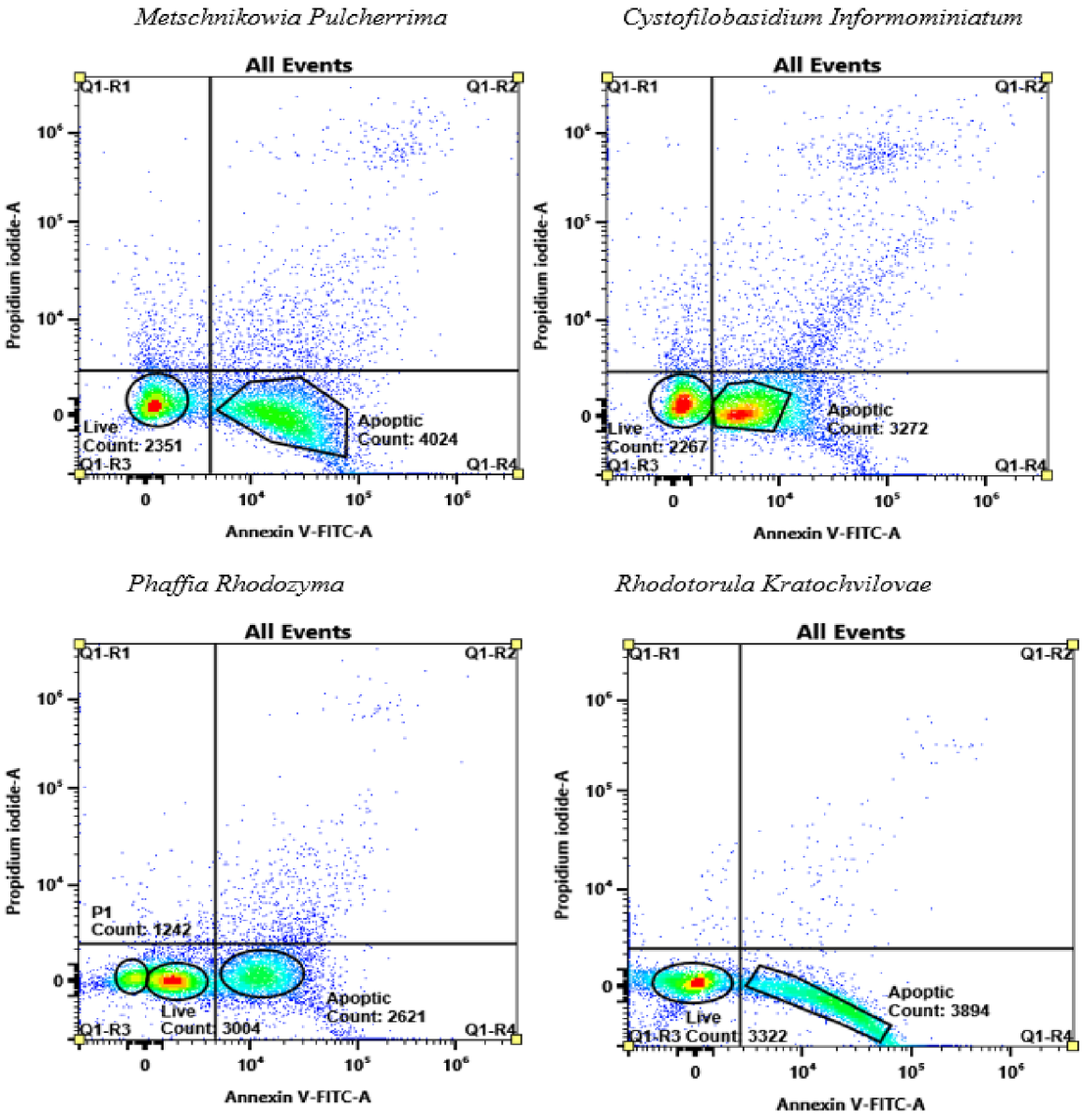

Figure 44: *Stained apoptotic cells (microbial extracts treated)*

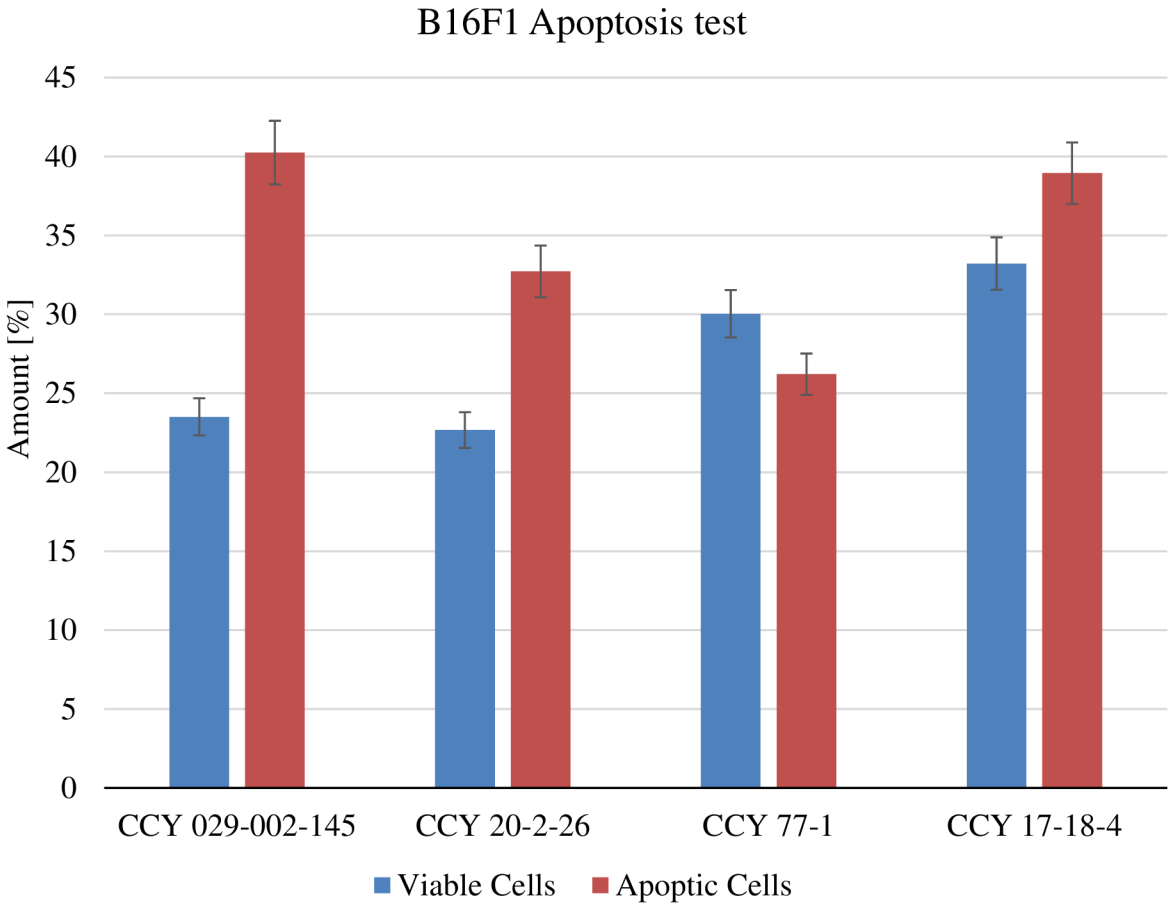

Figure 45: *Amounts of stained apoptotic cells (microbial extracts treated)*

As compared to control cells. there was a significant reduction in cell viability in microbial extracts-treated cells. Effect of microbial extracts on cell viability was confirmed by crystal violet staining. From these data. we concluded that the cytotoxicity of microbial extracts was dose dependent in B16F1 cells. The toxic effects of microbial extracts can be correlated to the antioxidant activity and antimicrobial effect. It's obvious that there is correlation between cell viability and the apoptosis. All of the microbial extracts showed some positive results for apoptosis and also in sample of *Phaffia rhodozyma* there are 3 populations of cells.

Cancer development and progression in most cases is mediated by the suppression of apoptosis. PI/Annexin V detects cellular apoptosis because of differential nuclear staining. The normal and early apoptotic cells are characterized by intact membranes; the plasma membrane undergoes structural changes that include translocation of phosphatidylserine from the inner to the outer leaflet (extracellular side) of the plasma membrane. It has been reported that the translocated phosphatidylserine on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages.

5.3 SYNERGIC EFFECT

One of the goals of this work was to evaluate the synergistic effect of plant and microbial extracts. The well-known strain of yeast *Rhodotorula kratochvilovae* was selected from the microbial extracts.

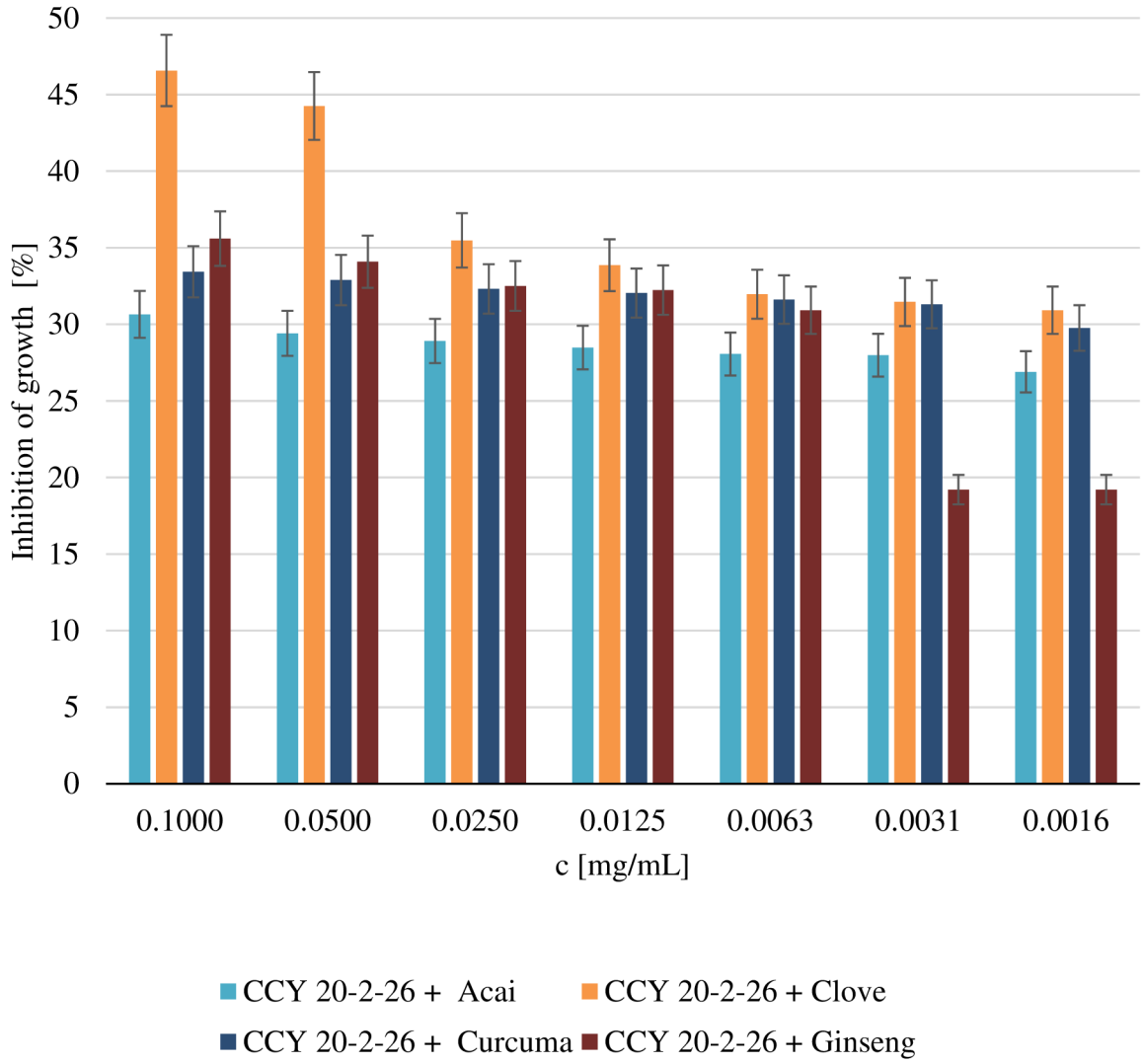

Figure 46: *Antibacterial inhibition of Serratia marcescens by Rhodotorula kratochvilovae extract in combination with plant extracts [%]*

According to the graph in the figure 46 it's obvious that combination of plant extracts and biomass highly increased antimicrobial activity. The highest potential showed extract of *Rhodotorula kratochvilovae* with mixture of clove extract where at 0.1 mg/mL was 48 % of inhibition and at 0.0016 mg/mL was inhibition still around 30 %.

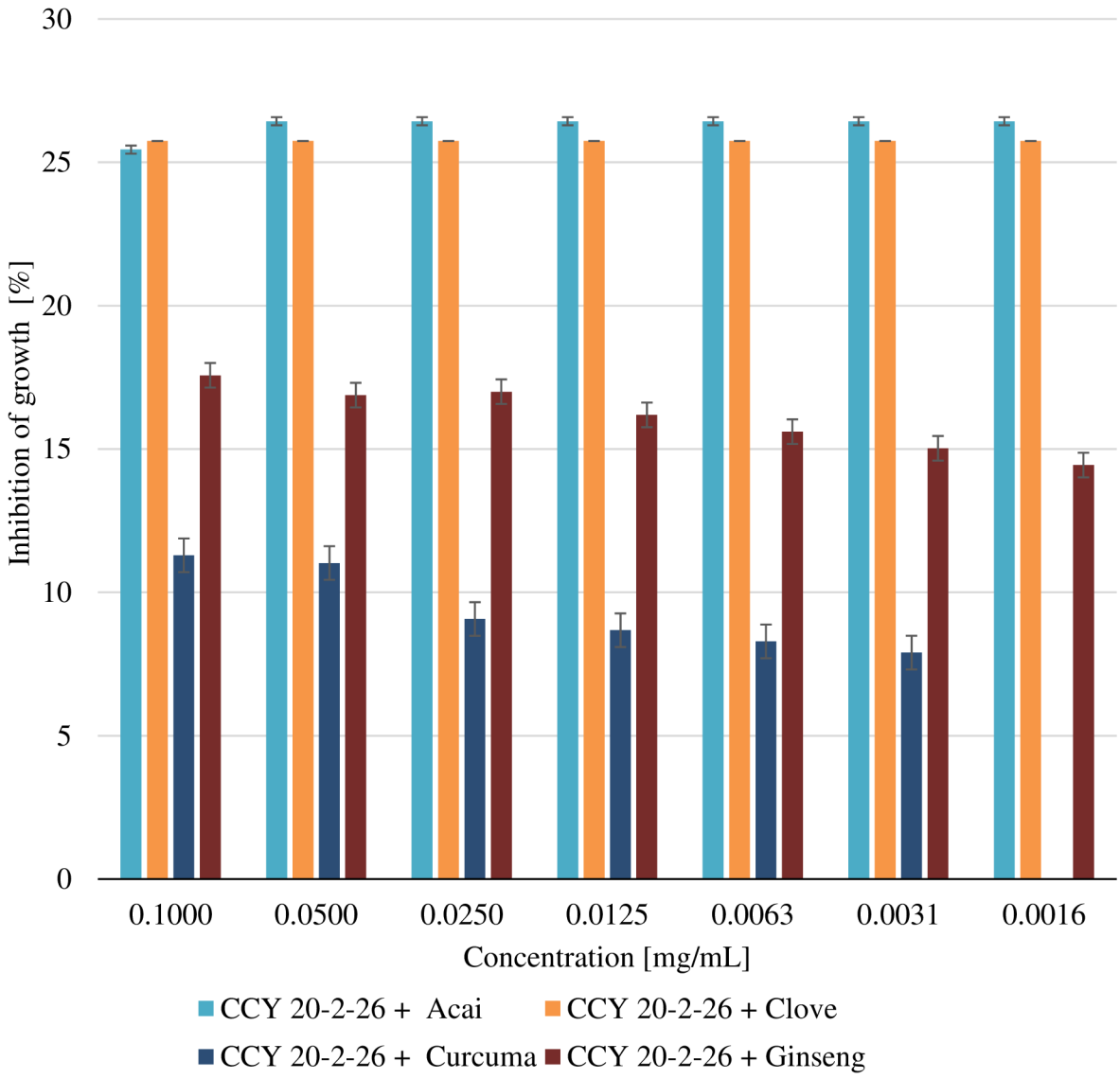

Figure 47: *Antibacterial inhibition of Micrococus luteus by Rhodotorula kratochvilovae extract in combination with plant extracts [%]*

In the figure 47 we can see that combination of plant extracts of acai or clove with *Rhodotorula kratochvilovae and* highly increased antimicrobial activity. Inhibition was around 25 % in all concentration range (0,1 - 0,0016 mg/mL). When we compare the separate results of microbial and plant extracts. we can infer a mutually beneficial increase in microbial activity.

6 DISCUSSION

The presented doctoral thesis is focused on the study of possible synergic effect of natural and microbial extracts on human health. From natural extracts. acai. clove. curcuma. ginger. ginseng and oregano were chosen. prepared and tested. These extracts were prepared both. aqueous (stirring for 24 h in 70° C) and oily (soxtherm extraction with hexane). Microbial extracts were produced by yeast strain of *Rhodotorula kratochvilovae*. *Metschnikowia pulcherrima. Cystofilobasidium informominiatum* and *Phaffia rhodozyma*. The microorganisms were cultured under conditions suitable for beta–glucan production. The biomass was also rich in carotenoids and fatty acids that has high benefit in supplementary diet because mammals cannot synthesize carotenoids de novo, and therefore, carotenoids must be obtained from diet. Higher consumption of carotenoid-containing fruits and vegetables and higher plasma concentrations of several carotenoids, including β-carotene. are associated with a lower risk of many different cancers. As lipids constitute a major portion of the majority membranes suggest that the presence of massive concentrations of unsaturated FA within membranous structures. In addition, well recognized that the PUFA are bioactive mediators of diverse pathways involved in cellular homeostasis or, in some cases, interact with cellular macromolecules resulting in cell death, these cellular responses may be a consequence of the vulnerability of unsaturated FA to diverse oxidation reactions, or radical reactions. or both.

First part of the work was focused on the preparation and characterization of natural extracts. Subsequently. their antimicrobial efficacy and influence on mitochondrial activity. The highest content of phenolic substances was determined in curcuma extract (4.723 mg/g), while the lowest content was detected in oregano (0.887 mg/g). Turmeric also showed the highest antioxidant effect (5.26 mg/g). For the oil extracts the best results were obtained from acai, clove, and curcuma extracts for total phenol account, from acai 1.418 mg/g, for clove 2.181 mg/g and curcuma 2.176 mg/g. Antioxidant activity of ginseng oil 1.152 mg/g was comparable with ginger 1.112 mg/g and higher result was obtained from curcuma 3.258 mg/g.

Natural plant extracts were encapsulated into lipidic nanoparticles. The value of the zeta potential prepared nanoparticles showed very good stability. on average around -40 mV. The average size of the liposomes in the aqueous extracts was 200 nm. Liposomes prepared from oil extracts were smaller. about 150 nm. The polydispersity indices of the particles prepared from the aqueous extracts were also higher compared to the oil extracts and ranged from 0.2-0.5. The lowest polydispersity indices 0.1 were measured for oil extracts. According to the results of antioxidant activity of nanoparticles compared with water extract. For example, water extract from curcuma contained 5.2 mg/g antioxidants and in liposomes only 0.24 mg/g. Therefore, it was not effective to continue preparing nanoparticles and the work was focused more on natural water and oil extracts and its possible combination with yeast biomass.

In monitoring the growth inhibition of *Serratia marcescence*. antibacterial activity of selected plant extracts was measured. Most of the samples showed similar results in all concentration range from 0.1 mg/mL to 0.003 mg/mL of dry material diluted in water. Clove extract showed highest antibacterial effect against gram-negative strains. When measuring inhibiton of gram- positive strains *Micrococus luteus*. the most effective was oregano extract.

On the other hand, clove extract wasn't so effective and also showed concentration dependent effect. same as curcuma and ginger.

Subsequently, attention was paid to the reactions of plant extracts with cell lines, especially its possibility to induced apoptosis in cancer cell lines. When comparing the cytotoxic effect of natural plant extract on HaCaT cell lines and B16F1 cell lines. we can conclude slightly antitumor effect of natural plant extracts on the concentration range from 16 mg/g to 28 mg/g except ginger and ginseng. Especially Acai extract was remarkable result. Curcuma showed the toxicity from 16 mg/g exponentially. Also, oregano extract was toxic for B16F1 but that's what we expect according to the antimicrobial activity and antioxidant activity.

Raji cell line showed significant toxicity only with curcuma extract. Therefore. this extract was used for cell cycle analysis and also apoptosis. Results showed that curcuma represents the only condition in which an apoptotic incidence significantly different (even though limited) from controls was revealed at Raji cell line. In general. plant extract treatment (at the conditions here used) do not seem to induce apoptotic effects. Instead, in some conditions particularly cytostatic effects (partly different), caused by a slow-down of the transition of the cells through the cell cycle phases are observed. For Caco-2 cells also only curcuma extract showed cytotoxic effect. Curcuma was concentration dependent and showed highest toxicity. We found that plant extract treatment was particularly effective against B16F1 melanoma cells. In the cytograms we can observe that cells treated with oregano and ginger do not seem to induce apoptotic effects. Instead, treatment with acai, clove and ginseng incidence significantly apoptotic cells in cytograms. Curcumin showed high autofluorescence of itself in cytograms. It was necessary to measure curcumin without PI and Annexin V to obtain better results. As compared to control cells. there was a significant reduction in cell viability in plant extracts-treated cells. Effect of plant extracts on cell viability was confirmed by crystal violet staining. From these data. we concluded that the cytotoxicity of plant extracts was dose dependent in B16F1 cells and Raji cells. The toxic effects of plant extracts can be correlated to the antioxidant activity and antimicrobial effect. It's obvious that there is correlation between cell viability and the apoptosis.

The second part of the work was focused on the preparation and characterization of biomass that was cultivated from 4 different strains and was tested for antioxidant activity and antimicrobial activity. Volume of fatty acids was also measured by gas chromatography. *Metschnikowia pulcherrima* has the highest amount of oleic, palmitic, stearic and linolic acid. Volume of beta glucans was tested by enzymatic kit from Megazyme. From the yeast strain *Rhodotorula katochvilovae* we obtained the highest ammount of total glucans which was 7.12 g/L of biomass and from which 4.75 g/L was beta- glucans. From *Metschnikowia pulcherrima* we obtain 1.92 g/L of total glucans from which 0.405 g/L was beta-glucans. From *Cystofilobasidium informominiatum* we obtain 1.12 g/L of total glucans and 0.475 g/L was beta-glucans. From *Phaffia rhodozyma* we obtain 1.42 g/L of total glucans from which 0.654 g/L was beta-glucans. All of the microbial extracts showed some positive results for apoptosis on cancer cell lines. On the other hand, at the same concentration range there was not cytotoxic effect on HaCaT cell lines that is not carcinogenic.

Positive biological effects of pigmented yeast biomass can be related to the content of valuable fatty acids, sterols, beta-glucans and other carbohydrates, nucleic acids, vitamins and provitamins, minerals, quinones, certain essential amino acids and other components. Red yeasts can produce carotenoids pigments. such as astaxanthin. torulene and torularhodin. whose show a considerable antioxidant and antibacterial activity and may also prevent certain types of cancer and enhance the immune system. Another component of oleaginous yeast biomass are unsaturated fatty acids – mainly high portion of oleic acid and up to 40% of PUFA. Recently it was found that both oleic acid and alpha-linoleic acid significantly down-regulated cell proliferation, adhesion and/or migration. Also, it was observed that both these fatty acids positively cross-regulates the expression levels of AMPK/S6 axis. Moreover, they up-regulated tumor suppressor genes (p53, p21, and p27), have an important role in oesophageal cancer and thus might be useful agents in the management or chemoprevention of oesophageal cancer.

Recent evidence suggests that patients nutritional status plays a major role in immunotherapy outcome. Fatty acids are essential in a balanced diet and well-known to influence the immune response. Moreover, short-chain fatty acids (SCFAs) show beneficial effects in metabolic disorders as well as in cancer and polyunsaturated fatty acids (PUFAs) contribute to body weight and fat free mass preservation in cancer patients. In line with these data. several studies imply a role for SCFAs and PUFAs in boosting the outcome of immunotherapy. Potential roles of omega-9 fatty acids in inflammation and cancer management were discussed. Preclinical and clinical evidence indicating that SCFAs and PUFAs may have the potential to boost immunotherapy efficacy was demon-strated and opportunities for successful application of nutritional interventions focusing on SCFAs and PUFAs to increase the therapeutic potential of immunotherapeutic approaches for cancer was addressed as well.

Last part of this thesis was focused on combination of plant extracts and biomass from *Rhodotorula kratochvilovae* yeast strain. Combinations highly increased antimicrobial activity, the highest potential showed extract with mixture of clove extract where at 0.1 mg/mL was 48 % of inhibition and at 0.0015 mg/mL was inhibition still around 30 %. On the other hand, yeast strain *Metschnikowia pulcherrima* was effective only around 12 % against gram-negative growth. Mixtures of *Rhodotorula kratochvilovae* and acai or curcuma showed the highest potential in inhibition of growth of gram-positive microorganisms, around 30 % in higher concentration extracts. The inhibition was concentration dependent for all extracts.

As part of the work, various projects were solved. one of which was. for example, a project with the company Vincentka. The goal of this collaboration was to develop a new type of energy drink based on Vincentka natural mineral water from Luhačovice. Various natural extracts in several forms were used for this. Lyophilized, filtered, macerated, essential oils or concentrated tinctures.

7 CONCLUSION

The main results could be summarized as follows:

- The highest content of phenolic substances (4.723 mg/g) and antioxidant effect (5.26 mg /g) was determined in turmeric extract.
- Clove extract showed the highest antibacterial effect to Serratia marcescence cells. When measuring the inhibition of *Micrococcus luteus*, the most effective was oregano extract.
- Cytotoxic effect of natural plant extract was tested at HaCaT and B16F1 cell lines. Acai extract was toxic for B16F1 and, at the same time, not toxic for the HaCaT cell line at all tested concentrations. There was no cytotoxicity observed in Caco-2 cell lines.
- Curcuma affected the cell cycle of Raji cell lines by slow-down of the transition of the cells through the cell cycle phases.
- 4 yeast strains were cultivated for biomass and lipids production. *Metschnikowia pulcherrima* has the highest amount of oleic, palmitic, stearic, and linolic acid
- Rhodotorula katochvilovae has the highest amount of total glucans, 7.12 g/L of biomass and from which 4.75 g/L was beta-glucans.
- Apoptotic effect of natural extracts was measured. When comparing viable/apoptotic cells treated with microbial extracts, *Phaffia rhodozyma* effected B16F1 cells in ratio (30:25 viable:apoptotic). The only sample with higher ration of viable cells than apoptotic.
- When mixing biomass of *Rhodotorula katochvilovae* with clove extract, the concentration at 0.1 mg/mL caused 48 % inhibition, and at 0.0015 mg/mL the inhibition was still around 30 % for gram-negative strain.
- Mixtures of *Rhodotorula kratochvilovae* and acai or curcuma showed the highest potential in inhibition of the growth of gram-positive microorganisms.

In order to conclude results from the presented thesis it should be taken into account that to complex activity contribute not only the biological effects of individual natural or microbiological extracts, but also their harmlessness for healthy cell lines. It is advisable to include immortalized yeast biomass in combination with natural extracts in food supplements in a suitable form. Ideally. tablet or capsule form colud be recommended due to the possible taste of biomass or coated microtablets in gel capsules.

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9 LIST OF ABBREVIATIONS

DLS	DYNAMIC LIGHT SCATTERING
DMEM	DULBECCO'S MODIFIED EAGLE MEDIUM
FA	FATTY ACIDES
FC	FLOW CYTOMETRY
FID	FLAME IONIZATION DETECTOR
GC	GASS CHROMATOGRAPHY
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
MUFA	MONOUNSATURATED FATTY ACIDES
PBS	PHOSPHATE-BUFFERED SALINE
PDA	PHOTO DIODE ARRAY
PDI	POLYDISPERSITY INDEX
PI	PROPIDIUM IODID
PUFA	POLYUNSATURATED FATTY ACIDES
RPMI	ROSWELL PARK MEMORIAL INSTITUTE
SDS	SODIUM DODECYL SLPHATE
UV-VIS	ULTRAVIOLET AND VISIBLE SPECTROMETRY
ZP	ZETA POTENTIAL

10 CURRICULUM VITAE

Name: Marie Vysoká
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Education:

2006-2012	Secondary grammar school Komenského in Třinec. Matriculation exam subjects: Biology. Chemistry. English. Czech. CZ
2012-2015	Bc.in Chemistry for Medical Applications Brno University of Technology. Faculty of Chemistry. CZ Subject: Using of Buchwald-Hartwig reaction in the synthesis of aromatic tertiary amines
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Time:	1. 7. 2016 - 1. 8. 2016
Subject:	Pharmaceuticals Development and Research Division. Advanced Chromatography
Place:	*Vri: Veterinary Research Institute*
Time:	1. 4. 2018 - 1. 5. 2018
Subject:	Guidelines for the use of cell lines in biomedical research
Place:	*UNISS: University of Sassari*
Time:	20. 10. 2018 – 20. 2. 2019
Subject:	Cell lines in biomedical research. cytotoxicity. genotoxicity. apoptosis tests

11 PUBLICATIONS

PAPERS

SZOTKOWSKI. M.; BYRTUSOVÁ. D.; NĚMCOVÁ. A.; **VYSOKÁ. M.;** RAPTA. M.; SHAPAVAL. V.; MÁROVÁ. I. Study of Metabolic Adaptation of Red Yeasts to Waste Animal Fat Substrate. Microorganisms. 2019. 7.11 (Jimp. Q2)

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CONFERENCE ABSTRACTS

VYSOKÁ. M.; MAZZARELLO. V..; SCIOLA. L; MÁROVÁ. I. BIOLOGICAL EFFECTS OF SELECTED PLANT EXTRACTS. 2019.

HOOVÁ. J.; **VYSOKÁ. M.;** DZURICKÁ. L.; MATOUŠKOVÁ. P.; MÁROVÁ. I. THE USE OF BIOACTIVE COMPOUNDS AND THEIR ENCAPSULATION INTO LIPOSOMES TO INCREASE THE EFFECTIVENESS AND CONTROL RELEASE. In NANOCON 2018. 10TH ANNIVERSARY INTERNATIONAL CONFERENCE ON NANOMATERIALS – RESEARCH & APPLICATION. Ostrava. Czech republic: TANGER. 2019. s. 386-391. ISBN: 978-80-87294-89-5.

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PROJECTS

CMV: „udržitelnost a rozvoj“ LO1211

LipofungI „Bioconversion of low-cost fat materials into high-value PUFA-Carotenoid-rich biomass“ 268305

Vincentka „ preparation of new type of energy drink with mineral water Vincentka“

Aplikace moderních postupů při výrobě, zpracování a analýze potravin, jejich složek a surovin a při valorizaci odpadů. FCH-S-18-5334

Využití pokročilých metod a postupů v rámci moderních potravinářských věd FCH-S-19-5983

Výzkum a vývoj nové generace inkontinenční pomůcky FW01010649

Aplikace progresivních metod a postupů v rámci moderních potravinářských věd FCH-S-20-6316

Vývoj a využití progresivních postupů při zpracování, analýze a hodnocení potravin, potravinářských surovin a odpadů. FCH-S-21-7483

Příspěvek k rozvoji moderních potravinářských věd FCH-S-22-7961