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Technological separation and analysis of flavanones from different plants and their microbiological activity

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Thank you from all my heart for your Love,
Love which was a source of everything
and brought me everything!!!

All wisdom comes from the LORD and with him it remains forever.
The sand of the seashore, the drops of rain, the days of eternity: who can number these? Heaven's height, earth's breadth, the depths of the abyss: who can explore these? Before all things else wisdom was created; and prudent understanding, from eternity. To whom has wisdom's root been revealed? Who knows her subtleties?
There is but one, wise and truly awe-inspiring, seated upon his throne:
It is the LORD; he created her, has seen her and taken note of her. He has poured her forth upon all his works, upon every living thing according to his bounty;
he has lavished her upon his friends....

Sir 1, 1-10

Dedicated to

my wonderful Family

Contents

List of figures

List of Tables

List of Abbreviations

$[\alpha]_D$	Optical Rotation at 589 Nanometer
µL	Micro Liter
µm	Micrometer
AAc.	Acetic Acid
ACN	Acetonitrile
AIDS	Acquired Immunodeficiency Syndrome
APCI	Atmospheric Pressure Chemical Ionization
ATCC	American Type Culture Collection
AV	Average
a_w	Water Potential
B. subtilis	*Bacillus subtilis*
BHI	Brain Heart Infusion
c	Concentration
C. glutamicum	*Corynebacterium glutamicum*
CD	Circular Dichroism
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
CF	Cystic Fibrosis
cfu	Colony-Forming Units
CHD	Coronary Heart Disease
Chiralcel OD-H	Cellulose Tris-3,5-dimethylphenylcarbamate
Chiralpak AS-H	Cellulose Tris(*S*)-1-phenylethylcarbamate
cm	Centimeter
CSP	Chiral Stationary Phase
CYP 450	Cytochrom P450
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EAEC	Enteroaggregative *E. coli*

EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
Eq.	Equation
ESI	Electrospray Ionization
ETEC	Enterotoxigenic *E. coli*
FAB	Fast Atom Bombardment
g	Gram
GC	Gas Chromatography
GLC	Gas-Liquid Chromatography
h	Hour
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSV	Human Simplex Virus
L	Liter
L. monocytogenes	*Listeria monocytogenes*
LDL	Low Density Lipoproteins
M. luteus	*Micrococcus luteus*
m/z	Mass to Charge Ratio
m^3/h	Cubic Meter pro Hour
mAU	Milli-Absorpance-Units
MCCTA	Microcrystalline Cellulose Triacetate
MeOH	Methanol
mg	Milligram
mg/d	Milligram per day
mg/kg	Milligram per Kilogram
min	Minute
mL	Milliliter
MLCK	Myosin Light Chain Kinase
mm	Millimeter
M_r	Molecular mass
MS	Mass Spectrometry
mV	Millivolt

nm	Nanometer
No.	Number
^{o}C	Degree Celsius
OD	Optical Density
OD_{600}	Optical density at 600 nm
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
PKC	Protein Kinase C
ppm	Parts per Million
psi	Pound per Square Inch
QqQ	Triple Quadrupol
RP	Reversed Phase
rpm	Rounds per Minute
s	Second
S. aureus	*Staphylococcus aureus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. enteridis	*Salmonella enteridis*
S. epidermis	*Salmonella epidermis*
S. pasteurianus	*Saccharomyces pasteurianus*
SD	Standard Deviation
SFC	Supercritical Fluid Chromatography
SPE	Solid Phase Extraction
T	Temperature
TLC	Thin Layer Chromatography
UV	Ultra Violet
V	Volt
v/v	Volume per Volume
Vis	Visible
VTEC	Vero cytotoxic *E. coli*
WHO	World Health Organization
YNB	Yeast Nitrogen Base
λ	Wavelength

1. Introduction

In ancient times, when human beings were gatherers and hunters, living in the sense of the expression "from hand to mouth", and nature constantly offered fresh and high quality nutrition, it was not necessary to have methods of food preservation available. In the New Stone Age when mankind changed lifestyle from gathering and hunting to tilling the soil and animal-keeping, people were forced to store food and to protect it from theft and external natural influences. During the past decade, people were more and more inventing methods to harmonize food safety and to satisfy consumers' needs. [LÜCK 1995]

To date, a consumer by selecting food is guided by the following criteria [CZAPSKI 1996]:

- a real diet as a condition for keeping up health
- food should be easy to prepare
- traditional methods of food production increase consumers' confidence
- eating is a big part in lifestyle, then it should be a pleasure.

The consumers nowadays expect from the food industry that their high requirements about the products' quality will be met by as far as possible unprocessed food, which means it should be fresh and natural, moderately cheap, additive-free, of high nutritional value, of good texture and of natural flavor and taste [GOULD 1996]. This further goes along with the need for a long shelf life and microbiological safety when buying products and also with the simplicity of preparation.

As to the subject of prevention of food-spoilage, over a longer time period, food products are exposed to various kinds of chemicals as well as physical and biological processes, however, many of these techniques have been associated with adverse changes in organoleptic characteristics and loss of nutrients [VALERO & FRANCÉS 2006]. The products are sold day by day in areas far remote from their production places. Besides, there is still a very important and global problem because of various resistances of bacteria and food borne pathogenic microorganisms against antimicrobial processes and agents. These kinds of problems need to be dealt with on a daily basis, so that food should be more and more processed. On the other hand the consumers are increasingly avoiding these highly processed food stuffs and food prepared with chemically-synthesized preservatives. There is a pressure on manufactures and a worldwide effort to minimize the use, or completely remove, preservatives of chemical origin contained in food. Therefore, the consumers' requirements lead to the need for a

provision of more "natural" and safe food with a longer shelf live. The food industry has to again develop new and alternative processes and come up with respective solutions for production; on one hand the producers need to satisfy the consumers' expectations and on the other hand to attend competition. [HOLLEY 2005, PROCZEK 2006, SCHÖBERL ET AL. 1999, TERNES ET AL. 1993]

Nature comprises plenty of compounds with antimicrobial characteristics (phytoalexins) playing an important role in the natural host defense mechanisms against all kinds of living organisms. Medicinal plants have been used for centuries as remedies for human diseases. In the last few decades there have been reports that different compounds from herbs and spices, fruits and vegetables, leaves and bark, stems, various animal tissues and microorganisms possess antimicrobial properties. Currently, numerous of valuable plants ingredients are used in the food industry as various additives of food-products and in medicine as medicaments. They are already used as a source particularly rich in famous antibiotics, e.g. the penicillin in 1940, the tetracycline in 1948 and glycopeptides in 1955 and also most of them are well-known in the science for their antioxidant and antimicrobial activity, e.g. essential oils, alkaloids, organic acids, various polyphenols with a group of flavonoids. [AL-BAKRI ET AL. 2007, CUSHINE 2005, RAUHA ET AL. 2000, ROLLER 1995, SERRA ET AL. 2008]

A first study about preservation activity of spices was made by HOFFMAN & EVANS (1911). During the 20th century, many researchers have already studied a large number of various plants extracts in context of their antioxidative, antiviral and antimicrobial activities, but the spoilage and poisoning of foods by microorganisms is still a big problem and until now is not under adequate control, despite the huge number of preservation techniques available. The food additives may play an important role in the safety of food and in spoilage, but it is very important as well that they could be metabolized and excreted by human body without any problems [DAVIDSON 2005]. There are many well-known natural antimicrobial compounds, but only a few of them have been exploited in food technology as preservatives [DAVIDSON & HARRISON 2002, HOLLEY & PATEL 2005, SOFOS ET AL. 1998]. The most famous group of antimicrobial plant extracts are essential oils. However, these compounds when added to the food products undergo changes in their taste and flavor, which may not always be desirable [SOFOS ET AL. 1998, ZAIKA 1987].

Another huge group, which has raised considerable interest recently because of its potential beneficial effects on human health, is flavonoids. They are flavor-less and have been reported many times for their properties and activities [BUHLER & MIRANDA 2000].

1.1. The topic of the thesis

Although the problem of food spoilage and poisoning has been solved during the centuries, food industry still has to worry about the longer shelf life of food and about the demands of the consumer with growing interest in so-called "natural food". Therefore, researchers still search for new naturally occurring substances which could have antimicrobial properties and be a natural preservative.

This thesis focused on the antimicrobial activity of the chiral substances with no taste and flavor, extracted from ubiquitous plants. Based on previous literature [E.G. BENAVENTE-GARCÍA ET AL. 1997, MANTHEY & GROHMANN 1996, US PATENT 6096364, YÁÑEZ ET AL. 2007], the substances chosen for the extraction in this work were plant materials that are normally consumed by humans, such as various citrus fruits, tomatoes, thyme or peanuts.

It is well-known that compounds containing phenolic rings exhibit antimicrobial properties. For centuries, the physicians and lay healers have used flavonoids as the principal physiological active component of medicinal mixtures against different human diseases, because of their desired properties [CUHNIE & LAMB 2005]. Previous studies, including for examaple HARBONE & WILLIAMS (2000), PROESTOS ET AL. (2006), RAUHA ET AL. (2000), TERESCHUK ET AL. (1997), have demonstrated that many compounds from the family of flavonoids are antimicrobials. However, the antimicrobial analysis was often performed using plant extracts as mixtures but to a less extent using single and pure substances. Therefore, in this work we extracted pure phenolic compounds from plant materials, which belonged to the group of flavanones (as a family of flavonoids) and checked for their antimicrobial properties. The five chosen flavanones were naringenin, isosakuranetin, eriodictyol, homoeriodictyol and hesperetin, which possess one centre of asymmetry at C-2 and what makes them optically active. As an example for studying antimicrobial differences between pure flavanone and flavanone-glycoside, hesperidin was chosen, which possesses a sugar molecule in its structure.

It is known that two enantiomers of one chiral molecule may have totally different effects on cells. Often only one of them can be of interest and the other one may be even harmful. This was the case with the drug thalidomide, which in the 1960s was sold in a medicament called Contergan as an effective tranquilizer and painkiller for pregnant women. One of the enantiomers of thalidomide helped against nausea, but the other one could cause fatality [WWW.NOBELPRIZE.ORG, SCHMAL 1987, WWW.ROEMPP.COM].

This issue leads to the next objective of the thesis, which was to separate the enantiomers from the four substances as naringenin, isosakuranetin, eriodictyol and homoeriodictyol, and to examine the antimicrobial effectiveness of each form of the substances. The differences between the (+)- and (–)-enantiomers and their racemates should be revealed. There are still only a few found papers about the antimicrobial differences of enantiomeric compounds, i.e. the antimicrobial activity of N-(3-oxo-octanoyl)-HSL against *B. substilis* [POMINI & MARSAIOLI 2008].

2. Theoretical Background

In food preservation all possibilities against any spoilage of food, especially against microbial action, are taken into consideration. There are three fundamental types of methods used, pertaining to physical, biological and chemical. To the physical group belong well-known methods such as:

- heat-treatment – sterilization, pasteurization,
- refrigeration – cooling and freezing
- dehydratation – drying
- irradiation
- high pressure.

To the biological group belong the safe and harmless microbial cultures, named "protective cultures", which are added to food and which are known for their activity against others spoilage microorganisms. Very interesting, but still unknown to a large part are the group of chemical methods, especially with the use of natural plant extracts instead of chemically-synthesized preservatives.

To naturally occurring, safe and healthy substances belongs a group of organic compounds which are known as flavonoids. This chapter is designed to describe the natural substances that have been used in this work, and which belong to the group of flavonoids, to describe the theoretical backgrounds of their extraction from different plants including their chiral separation, as well as to describe the methods for determining their antimicrobial activity and the food spoilage microorganisms used in this work.

2.1. Flavonoids

The term flavonoids (Lat Flavus = yellow) was first used for the family of yellow-colored compounds containing a flavones moiety (2-phenyl-chromone). Later, the name was extended to various polyphenols and now flavonoid is a term used to describe one of the more numerous groups of organic molecules and natural products. [NAIDU 2000]

2.1.1 Structure and nomenclature of flavonoids

The basic chemical structures of the compounds contain the flavan nucleus (Fig.), which consists of 15 carbon atoms and is based on a C_6-C_3-C_6 carbon framework or more specifically on a phenylbenzopyran-functionality. The skeleton consists of two benzene rings (A and B) which are connected through a heterocyclicpyrane ring (C) [GROTEWOLD 2008, DAS 2006, ERLUND 2004].

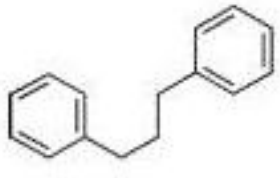


Figure 1. Skeleton of the flavan.

Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, these natural products may be classified into:

- flavonoids (2-phenylbenzopyrans)
- isoflavonoids (3-benzopyrans)
- neoflavonoids (4-benzopyrans).

Additionally, they differ in saturation of the heteroatomic ring C, in the place of the aromatic ring B at the position C-2 or C-3 of ring C, and in the overall hydroxylation patterns. The flavonoids may be modified by hydroxylation, methoxylation, or O-glycoxylation of hydroxyl groups as well as by C-glycosylation directly to the carbon atom of the flavonoid skeleton. [GROTEWOLD 2008, DAS 2006, HARNLY ET AL. 2006, NAIDU 2000, SIMONS ET AL. 2009, HARBORNE 1975, BROWN 1980]

Depending on the classification, there are more categorizations of flavonoids [BEECHER 2003, BUHLER & MIRANDA 2000, GROTEWOLD 2008, HARBORNE 1975]. Based on their skeleton, flavonoids are divided into eight groups [HAVSTEEN 2002, HODEK 2002]:

- flavans
- flavanones – 2-phenyl-3-dihydro-chromones, 2-phenyl-flavanones
- isoflavanones – 3-phenyl-2-dihydro-chromones
- flavones – 2-phenyl-chromones
- isoflavones – 3-phenyl-chromones

- anthocyanidines – hydroxyl-4-dihydroflavonoles
- chalcones
- flavonoligans.

The structure of these compounds is derived from a heterocyclic hydrocarbon, chromane and by an oxo-group in the position 4 forms flavanones and isoflavanones [HAVSTEEN 2002, HODEK 2002].

2.1.2. Occurrence of flavonoids

The flavonoids play important biochemical and physiological roles in plant tissues (e.g. protection against fungal pathogens). They occur ubiquitously as white and yellow pigments in all parts of a plant, for instance inside the photosynthesizing cells or on the surfaces of the plant organs (flowers, seed, stems, roots, sapwood, bark, green parts and fruit). They are secondary metabolites that are formed in plants. They are biosynthesized via a confluence of the acetate/malonate and shikimate pathways from the aromatic amino acids [GATTUSO ET AL. 2007, O'CONNELL & FOX 2001], phenylalanine and tyrosine, together with acetate units. [CUSHINE & LAMB 2005, MIDDLETON ET AL. 2000, NAIDU 2000]

Flavonoids participate in the light-dependent phase of photosynthesis during which they catalyze the electron transport. Because of their favorable UV-absorbing properties they provide protection from harmful UV-sun-radiation [CUSHNIE & LAMB 2005, NAIDU 2000]. The flavonoids occur in all soil-based green plants. They are not produced by animals, although due to their accumulation from plants as food sources, they may occasionally be found in animal tissues as well [CUSHNIE & LAMB 2005].

In different plant families, different combinations of flavonoids can be found. Most of them occur in the form of glycosides, e.g. glucosides, rhamnoglucosides and rutinosides. To date, over 8,000 individual compounds of the flavonoids group have been identified and described [HODEK ET AL 2002]. They are suggested to be used as nutracuetical ingredients for reducing the possibility of coronary heart and liver diseases [HODEK 2002]. "Nutraceutical" is a term defined as food or parts of food that provide medical or health benefits, including the prevention and treatment of diseases [NAIDU 2000]. They can act as potent antioxidants and metal chelators. The flavonoids appear to be effective at influencing the risk of cancer. Overall, several of these flavonoids appear to be effective as anticancer promoters and cancer chemopreventive agents. The next subchapters are designed to give an understanding of the

biological and molecular role of the plant-flavonoids. [CUSHNIE & LAMB 2005, DAS 2006, NAIDU 2000, GROTEWOLD 2008]

2.1.3. Medicinal properties of flavonoids

Flavonoids, because of their many useful properties, have been used for centuries by physicians and lay healers as the principal compound of medicinal mixtures, and now, have increasingly become of importance in the medicine as a treatment against human diseases, for instance propolis. In 1936 Albert Szent-Györgyi (Nobel Prize Laureate) proved that a mixture of two flavanones decreased capillary permeability and fragility in humans.

Many of *in vitro* and animal experimental studies describe that flavonoids can inhibit and sometimes induce a large variety of mammalian enzyme systems. Some of these enzymes are responsible for regulation of cell division and proliferation, platelet aggregation, detoxification, inflammatory and immune response. [HOLLMAN & KATAN 1997, WELLMANN 2002]

They exert a highly specific effect on a huge number and variety of receptors in organisms and of eukaryotic and circular regulatory enzymes as phospholipase A_2, which is an important intra- and extracellular mediator of inflammation, DNA synthetases, RNA polymerases, hydrolases, oxidoreductases, oxygenase, lipooxygenase, cyclooxygenase, monooxygenase, xantine oxidase, as well as mitochondrial ATPase, HIV-1 proteinase, HIV-1 intefrasee, NADH-oxidase, the cyclic nucleotide phosphidiesterase, reverse transcriptase, and many others [HODEK 2002, HAVSTEEN 2002, MIDDLETON 2000]. Flavonoids are also able to inhibit the protein kinases, e.g. a partially purified rat brain protein kinase C (PKC), or myosin light chain kinase (MLCK), by competing with ATP for the binding to the catalytic site, which inspires an explanation for a molecular basis of flavonoid anti-inflammatory effects [DAS 2006, MIDDLETON ET AL. 2000].

It is currently unknown how they can enter the cells and whether they could accumulate in certain organ cells. It is supposed that the inhibition of enzymes is possible due to the interaction between them and different parts of flavonoid molecules, including carbohydrate, the phenyl ring, phenol, and the benzopyrone ring [HODEK 2002]. In the case of kinases there is possibly, that they don't have any activity on these enzymes, but only interfere with the ATP. The type of inhibition, in some cases, is competitive, but more often it is allosteric. The molecular basis is still unknown [HAVSTEEN 2002]. Besides these effects, they possess a wide

range of activities including estrogenic, antimicrobial, antiallergic, antioxidant, vascular and antitumor activities. [CUSHNIE & LAMB 2005, HODEK 2002]

SCHAMALLE and coworkers (1986) reviewed flavonoids as non-potent contact allergens in food sources investigated in Europe. However, some highly toxic flavonoids were found in Africa and in Australian blackwood, hydroxyflavans [HAVSTEEN 2002, MIDDLETON ET AL. 2000].

2.1.4. Human therapeutic significance of flavonoids

Flavonoids are present in the plant kingdom, in foods and beverages derived from plants and, therefore, they are also important constituents of the non-energetic part of human diet and thus connected to human life [CUSHINE & LAMB 2005, GROTEWOLD 2008, NAIDU ET AL. 2000]. According to HERTOG and coworkers (1993b), the dietary intake of mixed flavonoids is not, as previously estimated, within the range of 500–1000 mg per day in USA [E.G. CUSHINE & LAMB 2005], which was based on limited analyses of only a few foods. The real consumption ranges from 20 and 170 mg/d in USA, Denmark and Finland to 70 mg/d in Holland [BEECHER 2003, COOK & SAMMAN 1996]. Moreover, it can vary appreciably in different countries. The consumption of flavonoids can be higher in Mediterranean diet, which is richer in olive oil, citrus fruits and greens [MIDDLETON ET AL. 2000].

Many people have a high intake of saturated fat, which is related, however, to high mortality by coronary heart disease (CHD) [FERGUSON 2001, GORINSTEIN ET AL. 2006, ROSS & KASUM 2002, HUANG ET AL. 2007, LEE & REIDENBERG 1998]. The mortality rate from cardiovascular disease in France is much lower that for example in USA, Great Britain or Germany (MONICA PROJECT – WHO, 1989), although with comparable intake of saturated fat, smoking tendencies and cholesterol level. Several epidemiological studies pointed out that a correlation exists between intake of flavonoids and diseases risk; for example, the publication of HERTOG and coworkers (1993) on cardiovascular diseases risk [HERTOG ET AL. 1993A]. This shows the difference in the type of diet in France and other Mediterranean countries, which is higher in fruits, vegetables or red wine consumption (French paradox) [HOLLMANN & KATAN 1997, RENAUD & DE LORGERI 1992, RICE-EVANS ET AL. 1996].

Several flavonoids protect α-tocopherol and possibly other endogenous antioxidants. They possess also the ability to inhibit the cell-free oxidation of LDL mediated by $CuSO_4$, and the modification of LDL by mouse macrophages, which are risk factors in coronary artery

disease (CAD) [MIDDLETON ET AL. 2000, ERLUND 2004]. Some flavonoids glycosides in orange were reported to possess also a vasodilatory activity. [MIDDLETON ET AL. 2000]

2.1.5. Absorption and metabolism of flavonoids

Flavonoids present in food are usually bound to saccharides as beta-glycosides and, therefore, are not able to absorb through the cell walls. The molecules without sugar, the aglycones, can be absorbed by the passive diffusion. The glycosides (with a sugar molecule) are hydrolyzed into the free flavonoids, aglycones, by intestinal microorganisms contained in the colon, and it is assumed that this hydrolysis allows the absorption of liberated aglycones [MANTHEY ET AL. 2001], although the bacteria in the colon may also degrade the flavonoid moiety by cleavage of the heterocyclic ring, depending on the ring structure. The metabolism of these phenolic compounds can run over two major pathways, with micro-flora in the colon, which degrades the flavonoids into phenolic acids. Flavonoids can undergo oxidation and reduction reactions, as well as methylation, glucuronidation and sulfation in animal species. DAS ET AL. (1971) have demonstrated the rapid absorption and metabolism of 83 mg/kg of (+)-catechins in humans. After excretion within 24 h, eleven metabolites were detected in urine [MIDDLETON ET AL. 2000]. The studies of HOLLMAN and KATAN (1999) showed that quercetin glycosides from onions were easier absorbed (52%) than the pure aglycones (24%), and quercetin was slowly eliminated from the blood. This suggests an effectiveness of enterohepatic circulation. [MIDDLETON ET AL. 2000, HOLLAMAN & KATAN 1999, PROESTOS ET AL. 2006, ROSS & KASUM 2002]

HAVSTEEN (2002) showed that the lymph with flavonoids enters the portal blood near the liver. Probably in the first pass, the majority of substances (80%) are absorbed. One part is attached to serum albumin and another part is found in the conjugates. Flavonoids are transported by hepatocytes to the Golgi apparatus and probably to the peroxisomes as well, in which they degrade oxidatively. Also in the intestine they may degrade by bacterial enzymes that can cleave the C-ring. [HAVSTEEN 2002]

It is, however, still unclear, whether flavonoids are more effective in the human body as whole molecules or as free aglycones. Most likely, it depends on the particular flavonoid and on its biological activity. Recent studies also suggest that certain flavonoid glycosides can be absorbed by active transport in the small intestine. [HODEK ET AL. 2002, HOLLMAN & KATAN 1997, WELLMANN 2002]

2.1.6. Antioxidant properties of flavonoids

Antioxidant compounds by definition protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite [BUHLER & MIRANDA 2000].

A polyphenol to be defined as an antioxidant has to fulfill the following conditions [RICE – EVANS 1996]:

- when present in low concentration relative to the substrate to be oxidized, it can delay, retard or prevent autooxidation or free radical-mediated oxidation
- the resulting radical formed after scavenging must be stable against further oxidation through intramolecular hydrogen bonding.

Flavonoids are powerful chain-breaking antioxidants in both lipophilic and hydrophilic systems [RICE–EVANS ET AL. 1996]. Their antioxidant properties may protect tissues against oxygen free radicals and lipid peroxidation. The antioxidative- and lipid-peroxidation inhibiting potential of flavonoids predominantly resides in their radical scavenging capacity rather than in their metal-chelating potential [GORINSTEIN ET AL. 2006, HEO ET AL. 2004]. Some flavonoids are capable of chelating bivalent metals such as iron and copper to make them unavailable for redox cycling reactions [CHENG & BREEN 2000, DAS 2006, HODEK 2002, WELLMANN 2002]. The radical scavenging activity is important, because the reduction potential of flavonoid radicals are lower than those of alkyl peroxyl radicals and superoxide radicals, which means that the flavonoids may inactivate these oxyl species and prevent the deleterious consequences of their reactions. They interrupt radical chain reactions. [RICE–EVANS ET AL. 1996, VAN ACKER ET AL. 1996, BENAVENTE-GARCIA ET AL. 1997, FERGUSON 2001, HERTOG ET AL. 1993, HOLLMAN & KATAN 1997]

Other studies [E.G. BENAVENTE-GARCIA ET AL. 1997, BUHLER & MIRANDA 2003, FERGUSON 2001, HOLLMAN & KATAN 1997] suggested that all substances containing the above structural features possess a higher redox potential than ascorbate and should be capable of oxidizing it to an ascorbyl radical. To this group belongs the compound quercetin, that also, along with some others flavonoids, can protect low density lipoproteins (LDL) from oxidation, induced by macrophages or catalyzed by metal ions like copper. Taxifolin has a lower redox potential than the ascorbyl radical and it can be expected that naringenin and hesperetin belong to this group as well [RICE – EVANS 1996, BENAVENTE-GARCIA 1997].

The antioxidant activity of flavonoids depends on their molecular structure, in which for example the prenyl group plays an important role in the antioxidative capacity of flavonoids. A comparison of a range of flavanones and flavones in their capacity to increase the induction period to autoxidation of fats has led to the conclusion that optimum antioxidant activity is associated with structural features such as multiple phenolic groups, especially the 3',4'-orthodihydroxy configuration in the B ring, and the 4-carbonyl group in the C ring. In contrary with aqueous phase interactions, the 2,3-double bond is deemed to be less important because taxifolin is more effective than its unsaturated analog quercetin. [RICE-EVANS 1996]

Flavonoids containing a phenol B ring, like naringenin or apigenin, were shown to be prooxidants that deplete NADH and generate NAD radicals when metabolized *in vitro* by peroxidase [HODEK 2002]. Flavanones that only possess one hydroxyl group in the B ring, such as naringenin or hesperetin, have been suggested to exhibit little antioxidant activity within a lipid system [RICE – EVANS 1996].

Flavonoids can therefore react as [MIDDLETON ET AL. 2000, PROESTOS ET AL. 2006]:

- metal chelators and reducing agents,
- scavengers for ROS,
- chain-breaking antioxidants,
- quenchers of the formation of singlet oxygen, and
- protectors of ascorbic acid.

2.1.7. Tumors protective activity of flavonoids

On the basis of redox capacity, flavonoids might prevent a damage of the DNA. In animal experiments, the anticancer capacity of these plant compounds were detected, such as against breast, colon, skin and stomach cancer, as well as oral cancer forms.

Flavonoids modulate an activity of cytochrom P450 (CYP 450). The inhibition of such enzymes from the first metabolism phase from xenobiotica might prevent the cancer activity. Benzo[a]pyren through Cyp1A1 is oxidized to mutagens and through the arylhydrocarbon receptor is an inductor of Cyp1A1 transcription. Some of the flavonoids, because of their structural similarity to nucleotides might stimulate the DNA repair. [WELLMANN 2002, FERGUSON 2001, GAO ET AL. 2006, BENAVENTE-GARCIA 1997, IBRAHIM 1990, SIMONS 2009]

It has been proved as well that various flavonoids have an effect in inhibiting DNA topoisomerases. The induction of apoptose has been seen a therapeutic aim for the active

tumor therapy. Moreover, flavonoids inhibit the *in vitro* proliferation of cancer cells by reducing the expression of protoonkogenes as for example Ki-ras and c-myc.

Some flavonoids can bind estrogen receptors and with that modulate the activity or, by inhibition of aromatase, influence the estrogen mirror. On this basis they apply to a potential cancer therapeutic against breast and prostate cancer. [FERGUSON 2001, MANTHEY ET AL. 2001, IBRAHIM 1990, SIMONS 2009, WELLMANN 2002]

Citrus flavonoids can inhibit the invasion of chick heart fragments and synergic mice liver by malignant mouse [BENAVENTE-GARCIA ET AL. 1997].

Flavonoids are capable to inhibit carcinogenesis by possibly the following mechanisms:

- inhibiting the metabolic activation of the carcinogen to its reactive intermediates
- inducing the enzymes involved in the detoxification of the carcinogen
- binding to reactive forms of carcinogens, and thereby preventing their interaction with critical cellular targets such as DNA, RNA, and protein.

For this reason, flavonoids seem to be some of the most promising anticancer natural products that have been investigated [HAVSTEEN 2002]. The YAÑEZ ET AL. (2007) report, that the pharmacokinetics, anticancer and antiinflamatory activity of the individual enantiomers has been only studied as an influence of S and R naringenins over cyclosporine A oxidase activity in human liver microsomes, which depends on the activity of the cytochrome P450 3A4 [YAÑEZ ET AL. 2007, CACCAMESE ET AL. 2005].

2.1.8. Flavonoids as immune modulators

A complex group of cells that are responsible for health of every living organism is the basis of an immune system. These cells can interact with each other in a manner or respond to intercellular messages with hormones, cytokines and autacoids (histamine, kinins, leukotrienes, prostaglandins and serotonin). The immune system can be modified by pharmacologic agents, environmental factors, pollutants and diet with naturally occurring food chemicals such as vitamins or flavonoids, which can significantly affect the function of this system and of inflammatory cells. [MIDDLETON 2000]

The *in vitro* and *in vivo* observation shows that the flavonoids are immune modulators. They are able to bind to one or more of the plasma proteins. They are only weakly antigenic. Dose-dependent, they inhibit also the lymphocytes B and T proliferation, disturb the antigen presentation through macrophages and the mitogen-stimulated immunoglobulin secretion of

IgG, IgM, and IgA isotypes, as well as exhibited antitumor activity against certain solid tumors in mice [MIDDLETON ET AL. 2000, FERGUSON 2001, WELLMANN 2002]. Many flavonoids stimulate the production of interferon (INF-α, INF-β), which activates a different part of the immune system [HAVSTEEN 2002].

The flavonoids, because of the inhibition of a generation of lipid hydroperoxides, modulate the macrophage stimulated LDL-oxidation. They show activity in conserving the α-tocopherol content of LDL and delay the beginning of measurable lipid peroxidation. [MIDDLETON ET AL. 2000]

2.1.9. Antiviral activity of flavonoids

Since years now, flavonoids are also well known for their antiviral properties both upon *in vitro* and *in vivo* analysis. Several groups have been reported to exhibit inhibitory activity against human immunodeficiency virus (HIV), as the causative agent of AIDS [CUSHINE & LAMB 2005, HARBORNE & WILLIAMS 2000]. Some of them showed to have virucidal activity against enveloped viruses, e.g., the herpes simplex virus (HSV), respiratory syncytial virus, poliovirus (e.g. quercetin and hesperetin) and Sindbis virus, but they did not possess any activity against non-enveloped viruses [BENAVENTE-GARCIA 1997, CUSHINE & LAMB 2005]. Rutin, hesperidin and citrus bioflavonoids complexes are utilized in the therapy of viral diseases. Several flavonoids showed the ability to inhibit the replication of picornaviruses and some chalcones and flavans to inhibit selectively a variety of serotypes of rhino- and poliomyelitis viruses [MIDDLETON ET AL. 2000, NAIDU ET AL. 2000]. These compounds showed synergism between each other and other antiviral agents [CUSHINE & LAMB 2005].

The sensitivity of a virus depends on its serotype and the kind of flavonoid compound [NAIDU ET AL. 2000], whereas the antiviral activity of the flavonoid compounds depends on its structure [MIDDLETON ET AL. 2000]. It seems that the 4'-hydroxyl and 3'-metoxyl groups as a substitute in the 5th position and a poly-substituted A ring are the most important in antiviral potent and the presence of substitution of hydroxyl group with a sugar moiety decrease or completely abolish this effect [MIDDLETON ET AL. 2000, NAIDU ET AL. 2000].

2.1.10. Antimicrobial activity of flavonoids

Flavonoids are known to play a role in protecting plants against microorganisms. The antimicrobial activity of flavonoids has been thoroughly documented, and is also the main aim of this study.

As compounds for preparations used in medicinal treatments, flavonoids and their antimicrobial activities have been screened by many researchers. They examined numerous plant extracts for their content of flavonoids, or as pure commercially available substances. This includes substances such as: apigenin, galangin, pinocembrin, ponciretin, genkwanin, sophoraflavanone G and its derivatives, naringin, epigallocatechin gallate and its derivatives, luteolin and luteolin 7-glucoside, quercetin, 3-O-methylquercetin and various quercetin glicosides, kaempferol and its derivatives. [HARBORNE & WILLIAMS 2000, NAIDU 2000]

The researchers reported antifungal and antibacterial activities of flavonoids. The majority of them with any antifungal activities are isoflavonoids, flavans and flavanones [HARBORNE & WILLIAMS 2000]. Examples of antifungal activity can be flavonol galangin, which commonly occurs in propolis, showing inhibitory activity against *Aspergillus tamarii*, *Aspergillus flavus*, *Cladosporium sphaerospermum*, *Penicillium digitatum Penicillium italicum* and *Candida* spp [CUSHINE & LAMB 2005, NAIDU ET AL. 2000]. Unsubstituted flavones and flavanone were highly active against 5 storage fungi of Aspergillus, while the catechins showed only weak effects. Flavanones can also inhibit spore germination of Helmithosporum oryzae, Rhizopus artocarpi and Fusarium oxysporum ciceri [NAIDU ET AL. 2000].

5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two or three additional hydroxyl groups at position 7, 2’ and 4’ inhibited the growth of *Streptococcus mutans* and *Streptococcus sobrinus*, but did not exhibit inhibitory activity with additional hydroxyl groups at positions 7 and 4’. In general, the potent antifungal activity of flavones seems to depend on the absence of polar groups in the molecule. [NAIDU ET AL. 2000]

Some studies have shown a synergy between naturally occurring flavonoids and other antimicrobial agents against resistant strains of bacteria, for example synergy between epicatechin gallate and sophoraflavanone G [CUSHINE & LAMB 2005].

2.1.11. Toxicity of flavonoids

Currently, there are no doubts about the toxicological effect of flavonoids contained in food. It is assumed, however, that the toxicity of them is minimal, because of their wide occurrence in vegetables, fruits and beverages, and also because of their use in traditional medicine since years, as well as due to other characteristics such as low solubility in water, short residence time in the intestine and low absorption coefficient [CUSHNIE & LAMB 2005, HAVSTEEN 2002, WELLMANN 2002]. GARG ET AL. (2001), however, announced that citrus flavonoids appear to be extremely safe and without side effects even during pregnancy.

The Ames test, which indicates the potential of mutagenicity in human, did not confirm the mutagenicity of flavonoids, but due to the test being expensive, only few animals have been tested [HAVSTEEN 2002]. HOLLMAN & KATAN (1997) reported that mutagenicity of flavonoids *in vivo* in mammals was never found. However, it has also been published that they possess a range of activities in mammalian cells, and that quercetin showed some mutagenic activity [FERGUSON 2001, WELLMANN 2002].

In human blood of some individuals, antibodies to flavonoids were recognized, and it was also discovered that about 3–5% of the population reacts allergic to these compounds [HAVSTEEN 2002].

Of clinical significance are interactions of flavonoids with the cytochrom P450 depended enzyme CYP3A4, which plays an important role in the metabolism of medicaments. Thereby, food containing these phenolic compounds, as e.g. naringenin in grapefruit juice, can slow down the degradation of a medicinal drug.

The question, whether and in what amounts the absorption of flavonoids is healthy for the human body remains still unanswered and needs an *in vivo* confirmation of their side effects, although, as HAVSTEEN (2002) has noted, flavonoids that have normally been absorbed are probably the safest drugs ever known. [CUSHNIE & LAMB 2005, FERGUSON 2001, IBRAHIM 1990, SIMONS 2009, WELLMANN 2002]

2.2. Flavanone

The name of the group of flavanones derives from flavanone as the parent compound. The flavanones are constructed upon the same fundamental structure based on 2-phenylbenzopyran-4-one (Fig. 2). They present themselves in the following families: leguminosae, acanhaceae, tutaceae (primarily in citrus fruits), asteraceae, theaceae, compositae, myrtaceae, cruciferae, balanophoraceae, fabaceae, eucryphiaceae, anacardiaceae, and gymnospermae, as contained in peels but also in the fruit pulp. They are slightly water soluble. [HARBORNE, 1975, HARBORNE 1994, WWW.ROEMPP.COM, DE NYSSCHEN ET AL. 1996, SUDJAROEN ET AL. 2005]

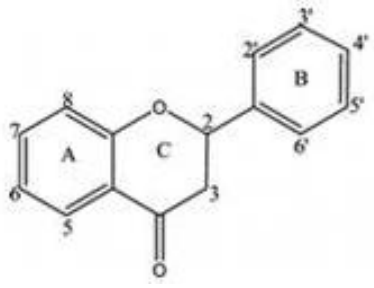


Figure 2. Structure and numbering of flavanone [IBRAHIM, ABUL-HAJJ 1990]

Flavanones (isosakuranetin, naringenin, hesperitin, and eriodictyol) occur mostly as glycosides in citrus fruits such as in Table 1. The non-bitter isomer, hesperetin-7-rutinoside (hesperidin) occurs in oranges (*Citrus sinensis*). [BELITZ ET AL. 2004]

Table 1. The occurrence of flavanones-glycosides in citrus fruits [BELITZ ET AL. 2004]

Fruit	**Compound**
Orange: flesh peel	 hesperetin-7-rutinoside hesperetin-7-rutinoside, nobiletin, isosakuranetin-7-rhamnoside-glucoside
Bitter orange	hesperetin-7-neohesperidoside
Grapefruit	naringenin-7-neohesperidoside
Lemon - peel	hesperetin-7-rutinoside, diosmetin-7-rutinoside, luetolin, limocitrin, eriodictyol-7-rutinoside, limocitrol, apigenin, chrysoeriol, quercetin, isorhamnetin

2.2.1. Naringenin

One of the most widely occurring flavanones is an aglycon of naringin, 4',5,7-trihhydroxyflavanone (naringenin or naringetol) (Fig. 3), with the following physico-chemical characteristics: molecular weight M_r = 272.25, melting point at T = 251ºC, and optical rotation $[\alpha]_D^{27}$ = –22.5 in methanol. Naringenin is, like every flavanone, only weakly soluble in water [PATENT DE 69817862(T2) 2004, GROTEWOLD 2008, WWW.ROEMPP.COM].

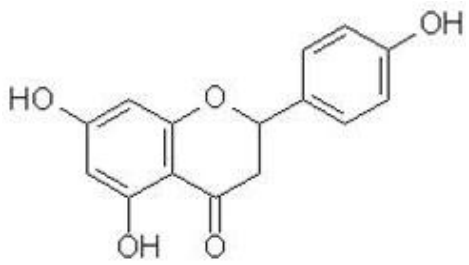


Figure 3. Structure of naringenin – 4',5,7 – trihhydroxyflavanone [WWW.EXTRASYNTHESE.COM]

There are different forms of naringenin, which has two possible B-ring positional isomers. The rutinoside and neohesperidoside are responsible for taste characteristics in citrus fruits.

The main sources of naringenin are tomatoes, *Lycopersicum esculentum* (until 3 mg/kg) [HERRMANN 1979, KRAUSE & GALENSA 1992] and tomato-based products, peels and fruit pulp of citrus fruits, including lemons, grapefruits, tangerines, lime and oranges (*Citrus sinensis*) [BUGIANESI ET AL. 2002, ERLUND 2004, PATENT DE 69817862(T2) 200].

Naringenin shows a protective effect against UV-induced DNA damage [GAO ET AL. 2006, BENAVENTE-GARCIA ET AL. 1997] and displays no toxicity *in vivo* upon the oral dosage of 1000 mg/kg in a mouse, which is equivalent to 50–100 g/kg in human, related to a person weighing 50 kg [NAHMIAS ET AL. 2008, PATENT DE 69817862(T2) 2004, VILA-REAL ET AL. 2007].

Naringenin inhibits the human cytochrom P-450 isoform, CYP 1A2, the CYP450-enzyms, CYP 3A4 [FUHR ET AL. 1993, PARL AND GNANASOUNDARL 2006], and aflotoxin B1. In the epithelial cells of the intestine, it activates phosphoglycoprotein and suppresses the expression of the cytochrome P450 3A4 gene [HAVSTEEN 2002].

It was reported that this flavanone shows biological effects such as antioxidant, anti-ulcer, anti-mutagenic and anti-inflammatory, as well as possessing antiviral, antiallergic, anticancer, antiestrogenic activities, through inhibiting the proliferation of breast cancer and delaying mammary tumorigenesis [BUGIANESI ET AL. 2002, PATENT DE 69817862(T2) 2004, ABBATE ET AL. 2009, PARL AND GNANASOUNDARL 2006, YAMAMOTO ET AL. 2004, RUSSO ET AL. 2007, US PATENT 6221357, WWW.ROEMPP.COM, VILA-REAL ET AL. 2007, HEO ET AL. 2004]. It has also an effect by improving lipid metabolism, so to prevent cardio-circulatory diseases. Without prenyl groups, it acts as a pro-oxidants and promotes rather than limits the oxidation of LDL by copper and decreases cholesterol [PATENT DE 69817862(T2) 2004, BUHLER & MIRANDA 2003]. Naringenin can also prevent, or can be used in medical treatment of hepatitis, fatty liver and liver cirrhosis [PATENT DE 69817862(T2) 2004, FELGINES ET AL. 2000].

Naringenin dissolve in ethanol shows cytoprotective properties on mucosal injury in rats. This flavanone was studied against DPPH radical and exhibits no activity [MIDDLETON ET AL. 2000]. As an antimicrobial, naringenin was mildly active against fungi of the *Aspergillus glaucus* group, showing highest inhibition of 20.7% by using the test microorganism *Aspergillus chevalieri*. The low antifungal activity is possible due to the partially substituted ring A and the absence of methoxy groups. [NAIDU ET AL. 2000]

2.2.2. Isosakuranetin

Isosakuranetin is the 4’-methyl isomer of sakuranetin. (Fig. 4)

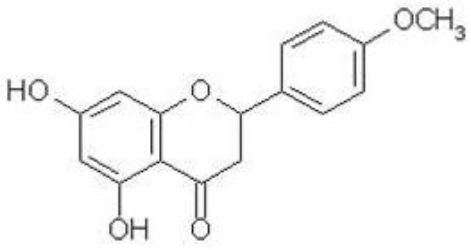


Figure 4. Structure of isosakuranetin – 5,7-Dihydroxy-4'-methoxyflavanone [WWW.EXTRASYNTHESE.COM]

Isosakuranetin und naringenin belong to the flavanones having one B-ring hydroxyl.

2.2.3. Eriodictyol

To the group of flavanones that have two B-ring hydroxyls belong substances such as eriodictyol, homoeriodictyol, hesperetin and hesperidin. Eriodictyol (Fig. 5), with a molecular mass of M_r = 288 [GEISSMAN ET AL. 1967], is the parent compound of several natural flavanones and possesses also B-ring positional isomers, but they are much less common [GROTEWOLD 2008, HARBORNE 1994]. Eriodictyol naturally occurs in peanut hull (*Arachis hypogaea*), in the gaviota tarplant (*Hemizonia increscens*) and in thyme (*Thymus vulgaris*), with predominantly in the S(-) configuration. In lemons, limes and yerba santa, it was determined as a minor compound [LEY ET AL. 2005, YAÑEZ ET AL. 2007].

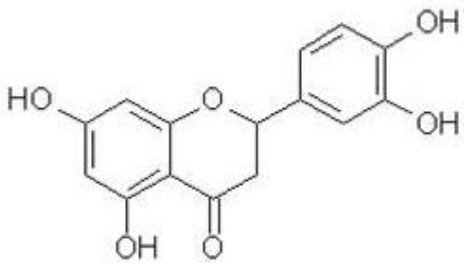


Figure 5. Structure of eriodictyol – 3',4',5,7-Tetrahydroxyflavanone [WWW.EXTRASYNTHESE.COM]

Eriodictyol was reported to possess antioxidant activity in lipid peroxidation [MIDDLETON ET AL. 2000] and showed the most remarkable masking effects from the flavanones against bitter taste of caffeine [LEY ET AL. 2005].

2.2.4. Homoeriodictyol

Homoeriodictyol is the 3'-methyl ether of eriodictyol (Fig. 6) with a molecular mass M_r = 302 [HARBORNE 1994, GEISSMAN ET AL. 1967]. It is the most important compound of the plant dry material of yerba santa (*Eriodictyon glutinosum* and *Eriodictyon californicum*), with predominantly in the S-(-) configuration [LEY ET AL. 2005, YAÑEZ ET AL. 2007].

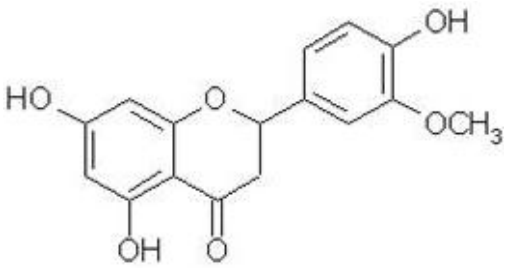


Figure 6. Structure of homoeriodictyol – 4',5,7-Trihydroxy-3'-methoxyflavanone [WWW.EXTRASYNTHESE.COM]

It seems that homoeriodictyol can protect plant tissues against damages caused by UV-light [LEY ET AL. 2005] and homoeriodictyol-7-O-β-D-Glccopyranoside inhibits *Cladosporium cucumerinum* and CYP1B1, which activates carcinogens [ZHAO ET AL. 2007].

2.2.5. Hesperetin

Hesperetin is the 4'-methyl ether of eriodictyol (Fig. 7) and is a well known ingredient of citrus fruits, where it was found as 7-O-rutinoside (hesperidin), and/or as 7-O-neohesperidoside (neohesperidin). It occurs as well in *Anthurium* (*Araceae*) and *Zanthoxylum* (*Rutaceae*). [HARBORNE 1994]

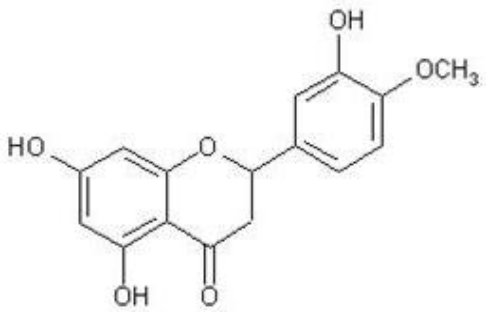


Figure 7. Structure of hesperetin 3',5,7-Trihydroxy-4'-methoxyflavanone [WWW.EXTRASYNTHESE.COM]

Hesperetin can actively inhibit the infectivity and/or replication of HSV-1, the polio viruses, the parainfluenza type viruses, and the syncytial viruses [MIDDLETON ET AL. 2000]. It improves the lipid metabolism in order to prevent cardio-circulatory diseases, and possesses also anticancer and antiviral activities [US PATENT 6221357 2001]. Hesperetin shows some

antioxidant activities, although in poorer capacities than compared to many other polyphenols, and also has effects on lipid metabolism [ERLUND 2004]. Hesperetin and hesperidin both possess capillary-enhancing, permeability-reducing, and anti-inflammation activities. Obtainable from citrus peels, hesperetin can decrease blood pressure and is effective against cholesterol [US PATENT 6221357 2001]. It has also been shown to inhibit chemically induced mammary, urinary bladder and colon carcinogenesis in laboratory animals [ERLUND 2004]. It has shown antimutagenic effect against aflatoxin B1 [GARG ET AL. 2001]. Hesperetin, as well as in the same mode as naringenin, was only mildly active against fungi of the *Aspergillus glaucus* group [NAIDU ET AL. 2000].

2.2.6. Hesperidin

Hesperidin is a flavanone glycoside, composed of an aglycone, hesperetin, or methyl eriodictyol and an attached disaccharide, rutinose (Fig. 8). The disaccharide comprises of one molecule of rahmnose and one of glucose. Hence, the molecule of glucose is attached directly to hesperetin and rahmnose to the glucose. [GARG ET AL. 2001]

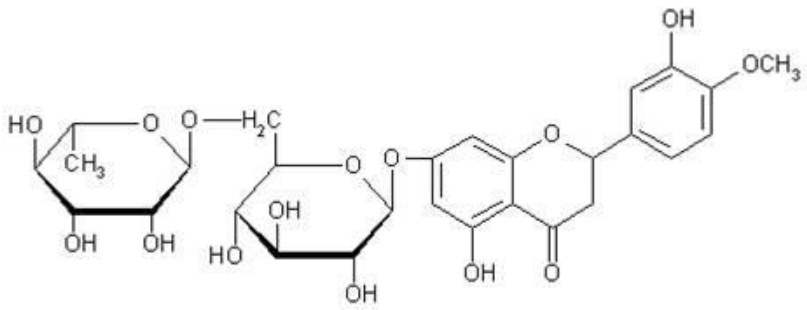


Figure 8. Structure of hesperidin [WWW.EXTRASYNTHESE.COM]

Hesperidin is a yellow, tasteless and water insoluble flavanone-glycoside with a molecular weight M_r = 610.57, and a melting point at T = 251ºC [BENAVENTE-GARCIA 1997, MANTHEY & GROHMANN 1996, WWW.ROEMPP.COM]. Hesperidin is one of the most consumed polyphenols from citrus fruits and respective juices [NAIDU ET AL. 2000, NIELSEN ET AL. 2006] and is a mayor, abundant and inexpensive by-product in the citrus industry [GALATI 1994, LOSCALZO ET AL. 2008]. It was found in oranges (19,000 – 21,000 ppm in orange peel), in sweet lemons and in tangerines [US PATENT 6096364, 2000]. It is usually found in association with vitamin C [GARG ET AL. 2001].

Hesperidin is of historical importance. It was found that when in mixture with citrin, it possesses vitamin-like activity and the mixture was shortly called vitamin P. In study experimental animals, it was proven that the both compounds had the capacity of decreasing capillary permeability and fragility, prolonging the life of marginally scorbutic guinea pigs and reducing the signs of hypovitaminosis C. Both flavonoids had potent antioxidant-dependent and vitamin C-sparing activity. [MIDDLETON ET AL. 2000]

Hesperidin can prevent against cardio-circulatory diseases through improving the lipid metabolism [US PATENT 6221357 2001]. It prevents the progression of atherosclerosis, decreases cancer risk [CHIBA ET AL. 2003] and shows inhibitory activities against hypotension and analgesia [KAWAGUCHI ET AL. 2004]. Hesperidin possesses some antiviral activity against 11 types of viruses [MIDDLETON ET AL. 2000, US PATENT 6221357 2001], but shows inactivity against HIV-virus, pseudorabies virus, rhinovirus and herpes simplex virus [GARG ET AL. 2001]. It can be used as an inexpensive and mild anti-inflammatory agent [GALATI 1994, HARBORNE & WILLIAMS 2000, LOSCALZO ET AL. 2008].

Hesperidin was studied as a chain-breaking antioxidant for the oxidation of linoleic acid in acetyl trimethylamonium bromide micelles, and appreciably did not show oxidation [MIDDLETON ET AL. 2000]. In a test with rats, it could be demonstrated that hesperidin has a possibility of increase HDL and low cholesterol LDL, plasma triglycerides and the total lipids [GORINSTEIN ET AL. 2007]. Hesperidin is capable of enhancing the reduction of dehydroascorbic acid by glutathione [MIDDLETON ET AL. 2000, MONFORTE ET AL. 1995].

2.3. Methods of extraction and identyfication of flavonoids

The method of isolation depends to some extent both on the source material and the type of flavonoid being isolated [HARBORNE 1975]. To resolve and identify phenolic compounds many techniques can be used including capillary electrophoresis (CE) and different types of chromatography [PROESTOS ET AL. 2006, GEL-MORETO ET AL. 2001]. CE separation is easy to carry out and to quantify the flavonoids; it allows for a rapid monitoring [GARG ET AL. 2001]. However, the most popular and powerful method of separation is a chromatography, which was developed for extraction and purification of various plant extracts [GUIOCHON 2002, BRANDT 2002]. This method was applied more to prepare the compounds than to analyze them [SCHULTE & STRUBE 2001, GUIOCHON 2002]. It can, however, as well be used for identification and quantification of separated compounds. Chromatography, especially, was

developed for analytical purposes, but now it is used also for preparative analyses. There may exist more distinguished types of chromatography compared to, e.g., thin-layer chromatography, gas-liquid and gas-solid chromatography, and low- and high performance column liquid chromatography [HUANG ET AL. 2007, GUIOCHON 2002, PROESTOS ET AL. 2006, PENG ET AL. 2006, NEUE ET AL. 2003, HAGEN ET AL. 1965, MIZELLE ET AL. 1965].

In earlier times, thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis were the major separation techniques used for phenolics. From these methods, still TLC is the workhorse for flavonoid analysis. It is used as a rapid, simple and versatile method for following polyphenolics in plant extracts and in fractionation works. However, the majority of published work now refers to qualitative and quantitative applications of high-performance liquid chromatography (HPLC) used for analysis. Flavonoids can be separated, because with the information from the UV spectrum, it may be possible to identify the compound subclass or perhaps even the compound itself. The typical wavelength for various flavonoid groups are: 270 and 330 to 365 nm for flavones and flavonols, at 280-290 nm for flavanones, at 236 or 260 nm for isoflavones, at 340 to 360 nm for chalcones, at 280 nm for dihydrochalcones, at 502 or 520 nm for anthocyanins, and at 210 or 280 nm for catechins [BELITZ ET AL. 2004, GATTUSO ET AL. 2007].

The chromatography, however, often does not give satisfactory results and the UV-Vis spectrum does not provide for a safe identification. Therefore, chromatography is often coupled with mass spectrometry (e.g. HPLC-MS), which gives an alternative and powerful technique in order to obtain full structural information [PROESTOS ET AL. 2006, GATTUSO ET AL. 2007]. Mass spectrometry (MS) is one of the physico-chemical and analytical methods applied to qualitative and quantitative determination of organic compounds [MATA BILBAO ET AL. 2007, STOBIECKI 2000]. To analysis, MS uses different physical principles, as for example ionization and separation of the ions generated according to their mass (m) to charge (z) ratio (m/z) [STOBIECKI 2000]. MS can be carried out using fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS) and atmospheric pressure chemical ionization (APCI-MS) [GATTUSO ET AL. 2007]. Flavonoids are a group of polar, non volatile and thermally labile compounds [STOBIECKI 2000].

2.4. Chirality of flavanones and their separation methodes

The term "chiral" comes from the Greek word "cheir" and means "hand" [WWW.NOBELPRIZE.ORG]. Our both hands are chiral, because the right hand is a mirror image of the left. The same occurs as well with most of the molecules and the two mirror images of substances are called enantiomers. In the nature exists a huge number of chiral substances. As reports show, it is evident that nature mainly uses only one of the two enantiomers and that these two forms of one molecule show often different effects on cells [WWW.NOBELPRIZE.ORG, NAKANISHI ET AL. 1994, VOLLHARDT & SCHORE 2000].

The flavanones present a unique structural feature known as chirality (Fig. 9). This can distinguish them from all other classes of flavonoids. They possess one asymmetric centre in position C-2, which means that these naturally occurring substances are also optically active [KWON ET AL. 2007, HARBORNE 1975, HARBORNE & WILLIAMS 2001, YAÑEZ ET AL. 2007, WISTUBA ET AL. 2006].

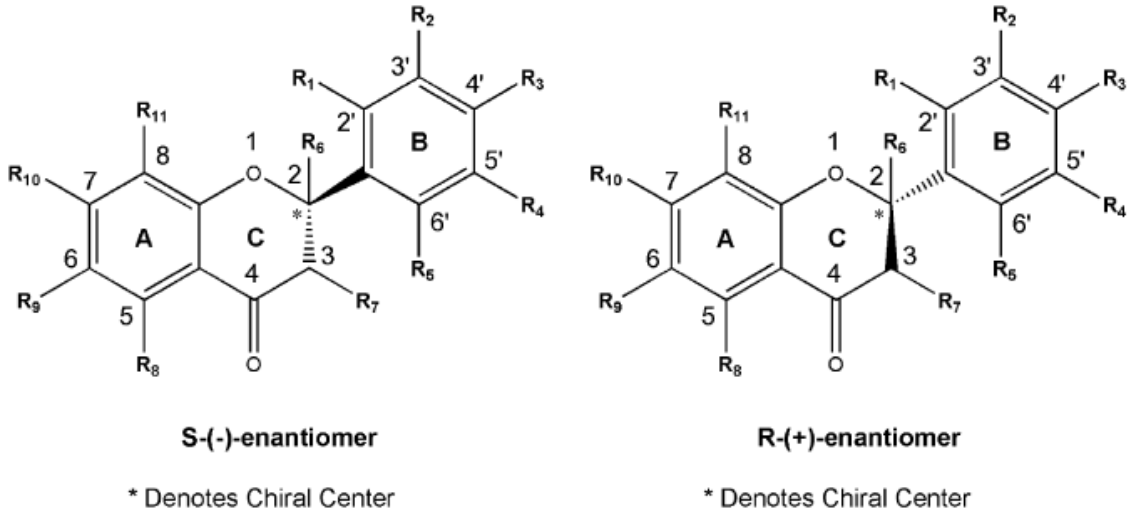


Figure 9. Spatial disposition of the enantiomers of chiral flavanones [YAÑEZ ET AL. 2007]

Chiral substances can undergo changes as a process of racemization, in which enantiomers form a racemate, or as to enantiomerization, when a racemate is interconvert to its single enantiomer. The racemization process among other parameters depends on temperature, moisture, solvent and pH. The reports show that depending on the variety of substitution around the stereogenic center, some chiral flavanones are stereochemically unstable. The enantiomers of flavanones with a free hydroxyl group in the position 4', e.g.

naringenin and eriodictyol, racemize easier than the compounds with a methoxy group on this position, as hesperetin or isosakuranetin. [YAÑEZ ET AL. 2007]

There are many methods available for the chiral separation of flavonoids, including capillary electrophoresis (CE) [KWON ET AL., 2007], capillary electrochromatogrpahy (CEC) [CHEN ET AL. 2004], micellar electrokinetic chromatography (MEC) [ASZTEMBORSKA ET AL. 2003, PARK & JUNG 2005], super and sub-critical fluid chromatography (SFC), gas chromatography (GC) and high performance chromatography (HPLC). The last two methods historically were developed at first [GEL-MORETO ET AL. 2003, YAÑEZ & DAVIES 2005, CHANKVETADZE ET AL. 1996, CHANKVETADZE ET AL. 2004, WISTUBA ET AL. 2006, FANALI ET AL. 2001]. CEC unites the characteristics of high efficiency of CE and the high selectivity of HPLC [CHEN ET AL. 2004]. However, one of the most important and essential methods in the analytical level of enantioseparation is HPLC [SUBRAMANIAN 2007, FRANCO ET AL. 2004].

HPLC can be used as indirect or direct enantio-separation. The indirect separation is more flexible. It is carried out on an achiral stationary phases, which avoids the costs of expensive columns and is based on the use of chiral derivatization reagents from diastereomeric derivatives. The chiral derivatization reagent has to be of high enantiomeric purity and posses derivatizable groups in the analyte. The direct separation is more convenient but requires the use of expensive columns with chiral stationary phases. [GÜBITZ & SCHMID 2001]

There are many commercially available chiral stationary phases (CSPs) [SUBRAMANIAN 2007], which is based on different chiral principles, as for example chiral π-donor and π-acceptor phases, phases based on multiple hydrogen bonds, and CSPs based on polysaccharides or cyclodextrin phases [GÜBITZ & SCHMID 2001].

In 1980, flavanones were separated using HPLC chiral columns that used polysaccharide derivatives; cellulose trans-tris (4-phenylazaphenylcarbamate). Afterwards a cellulose tris (3,5-dimethylphenylcarbamate) column was used. Cellulose mono- and disubstituted carbamates including cellulose-4-substituted triphenylcarbamate dervivatives, cellulose chloro-substituted tripenyl carbamate and cellulose methyl-substituted triphenylcarbamate supported in silica gel, were utilized for the separation of unsubstituted flavanones. A variety of reports demonstrated many possibilities to resolve the flavanone enantiomers. For example, enantiomeric separation of hesperetin worked successful on the commercially available Chiralpak AD-RH tris (3,5-dimethylphenylcarbamate) derivative of amylase column. Macroporous silica gel coated with cellulose tris (3,5-dimethylphenylcarbamate) separated a variety of flavanone derivatives and exists as Chiralcel OD column. Chiralcel OD-RH (tris-

3,4-dimethylphenylcarbamate) possesses the ability to resolve naringenin enantiomers in isocratic reverse phase in a validated assay in biological matrices. Homoeriodictyol can be separated also on the Chiralcel OC column (tris-phenylcarbamate), while eriodictyol and hesperetin on the Chiralcel OJ column (tris-4-methylphenyl0benzoate ester). The Chiralpak AS-H (tris (S)-1-phenylethylcarbamate) is able to resolve naringenin, eriodictyol and hesperetin. Commercially available Chiralcel OA (the microcrystalline cellulose triacetate) demonstrates the ability to separate naringenin, hesperetin, eriodictyol, homoeriodictyol and isosakuranetin. In enantiomeric separations of flavanones are also used as a CSP materials cyclodextrin and "mixed" cyclodextrin. [YAÑEZ ET AL. 2007, SUBRAMANIAN 2007, KRAUSE & GALENSA 1990, ASZTEMBORSKA ET AL. 2003, YAÑEZ & DAVIES 2005, YAÑEZ ET AL. 2008, WISTUBA ET AL. 2006, CHANKVETADZE ET AL. 1996, KRAUSE & GALENSA 1988, KRAUSE & GALENSA 1990, GIORGIO ET AL. 2004]

All these methods have some advantages but also disadvantages. Many of these columns and methods are no longer commercially available. There are also new columns available that can be used for enantiomeric separations. They vary in costs and come with various run times, at which a longer run time is not desirable. The addition of cyclodextrins to the mobile phase can improve the effectiveness of separation on CSP cyclodextrin columns [YAÑEZ ET AL. 2007].

Chromatography is also an effective preparative method [FRANCO ET AL. 2004]. A chiral separation can be scaled up depending on the CSP, but some of them are not feasible for preparative purposes [SUBRAMANIAN 2007].

2.5. Food safety and microbiology

The food we eat needs to be nutritious, metabolizable and safe. Food, however, depending on the kind, is in fact never really sterile. It carries various types of microorganisms, and its composition depends on which microorganisms can gain access and how they can grow, survive and interact with the food matrix over time. These microorganisms have their sources from the natural micro-flora of the raw material. The numerical difference between the various types of microorganisms in food is determined by the characteristics of the food, the storage environment, by their own biological characteristics and their mode of actions. [ADAMS & MOSS 2008 & 1995, JAY ET AL. 2005]

In most cases this micro-flora has no discernible effect and the food is consumed without objection and with no adverse consequences, they however sometimes show their presence in

several ways such as food spoilage and food borne illness, and, beneficially, food fermentation [ADAMS & MOSS 2008].

As has been stated by the WHO, "food borne disease is the most widespread health problem in the world and an important cause of reduced economic productivity", there is no doubt that food has a big influence in the transmission of diseases. It is evident that microbiological contaminants (between 60% and 90%) are the major cause in this respect [ADAMS & MOSS 2008 & 1995, BELITZ ET AL. 2004].

The food poisoning can be a cause of [BELITZ ET AL. 2004]:

- intoxication, which means poisoning by for example *Clostridium botulinum* and *Staphylococcus aureus*
- diseases caused by massive infection with *Clostridium perfringens* and *Bacillus cereus*
- infections by *Salmonella* spp., *Shigella* spp., and *Escherichia coli*
- diseases of unclear etiology, such as those from *Proteus* spp. and *Pseudomonas* spp.

2.6. Microbiological methods

There exist many various methods used to determinate antimicrobial activity of natural substances, including the following [CUSHINE & LAMB 2005, RIOS ET AL. 1988, ZAIKA 1987, WERK & KNOTHE 1984]:

- the agar dilution technique – an antimicrobial substance is mixed with medium and the growth is compared with a control sample [HAUSER ET AL. 1975, RIOS ET AL. 1988]
- the liquid dilution technique – turbidity of samples is taken as an indicator of bacterial growth such as with the macro-and micro dilution techniques [PUJOL ET AL. 1996, RIOS ET AL. 1988]
- the paper disk diffusion assay – a substance is deposited on a small filter paper disk (Ø = 0.5 cm), which is placed in the center of a Petri dish containing agar growth medium and inoculated with a test microorganism (after incubation the zone of inhibition is measured and recorded in mm) [ZAIKA 1987, LIN ET AL. 2004]
- the hole-plate diffusion method – in this method, holes (Ø = 12,7 mm) are made in the agar with a sterile cork borer and loaded with a substance [FYHRQUIST ET AL. 2002]
- the cylinder diffusion method – an antimicrobial substance is added to cylinders placed on an agar surface [RAUHA ET AL. 2000].

2.6.1. *Bacillus subtilis*

The genus of *Bacillus* belongs to the order of *Bacillales* and the prokaryotic family of *Bacillaceae*. This heterogeneous group of bacteria is gram-positive, rod-shaped, catalase- and occasionally oxidase-positive. Depending on the species, they can grow under aerobe or anaerobe conditions. Under stress condition, which is generally the case upon depletion of some essential nutrients in the milieu of growth, they can form a spore (endospore). Spores are dormant structures with highly protected genetic elements of the cells, and with the enzymes necessary for the germination and initial outgrow of the spore. These abilities allow the microorganism to survive under extreme environmental conditions. *Bacillus* are extremely resistant to environmental factors such as chemical and radiation treatments, as well as high temperatures, dry conditions, and UV exposure. They can persist in the environment for a long time, which provides difficulties for the safe production of food.

Many species of *Bacillus* are able to produce extra cellular enzymes that are responsible for the degradation of carbohydrates, proteins and fats. Numerous of the *Bacillus* species have been associated with food poisoning, including *Bacillus cereus* and *Bacillus subtilis* (Fig. 11).

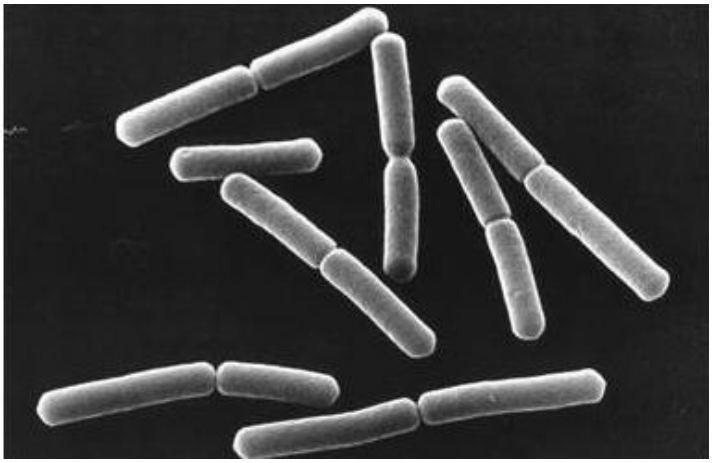


Figure 10. *Bacillus subtilis* cells [WWW.NASA.GOV/IMAGES/CONTENT/177389MAIN_POEMS1.JPG].

Bacillus subtilis is one of the most studied gram-positive bacteria and the best understood prokaryote in the science of molecular and cell biology. *B. subtilis* species are able to move very quickly and can divide symmetrically, making two cells or asymmetrically, producing single endospores. This microorganism can usually be found in soil and rotting plants. Because it is rarely a cause for human illness, it belongs to non pathogenic bacteria. It is responsible, however, for the poisoning of baked goods, such as bread (ropey bread) and

crumpets. In this case, spores that survived the baking process degrade the loaf's internal structure and produce a sticky and stringy slime, due to the bacterial production of polysaccharides. [ADAMS & MOSS 2008 & 1995, WWW.ROEMPP.COM, BLACKBURN & MCCLURE 2002, HARRIGAN 1999, JAY ET AL. 2005, KEWELOH 2008, WWW.NCBI.NLM.NIH.GOV/GENOMEPRJ/17579]

2.6.2. *Corynebacterium glutamicum*

The term *Corynebacterium* comes from Greek coryne, which means knotted rod and bacterion-rod. Their cells are rod-shaped. The genus of *Corynebacterium* belongs phylogenetically to the actinomycetes, and *Corynebacterium glutamicum*, in particular, to the mycolic acid-containing actinomycestes, to the family of *Corynebacteriaceae*. They are aerobic, gram-positive and catalase positive bacteria, which are straight or slightly curved (Fig. 10). They are immovable, non-sporulating and fast growing bacteria. Besides of normal cell division, also multiple divisions can occur, whereby many cells out of one cell can be created in form of many short sticks.

Corynebacterium is found in dairy products, in soil, air and as parasites and pathogens in humans, animals and plants. *Corynebacteriaceae* can be divided into three groups like the following:

1) Parasite and pathogen in human and animal such as *C. diphtheria*
2) Plant pathogenic forms such as *C. michiganese* and *C. fascians*
3) Non- pathogenic forms such as *C. glutamicum, C. herculis* or *C. acetophilum*

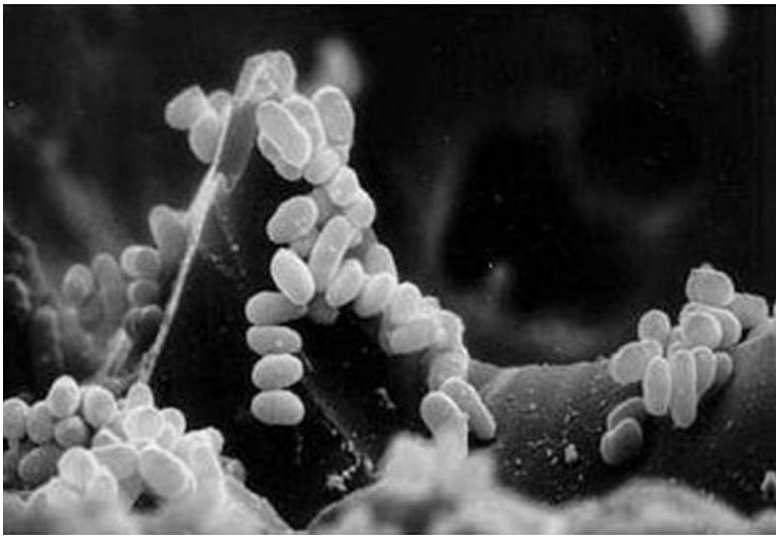


Figure 11. *Corynebacterium glutamicum* cells [WWW.FZ-JUELICH.DE/IBT/CORYNE.HTML].

The highly toxicity of the diphtheria comes from pathogen *Corynebacterium diphtheria* and is based on an exotoxin, which affects the heart muscle, kidneys and nerves and is classified to second risk group. The kinds that are biotechnologically, such as for example *Corynebacterium glutamicum*, are short ones and non-pathogenic, and are classified to first risk group. The *C. glutamicum* bacterium is able to use n-alkans, and can metabolize a variety of carbon and energy sources such as carbohydrates, organic acids or alcohols, and under optimal conditions is capable to convert glucose into high yields of L-glutamic acids. Currently, it possesses a high economic value, because it is used on industrial scale for the production of the amino acids, L-glutamine and L-lysine, as well as in smaller amounts for L-alanine, L-isoleucine, L-proline, L-tryptophan and L-homoserine.

In food industry, *Corynebacterium* is sometimes involved in the spoilage of vegetables and meat products. [FANOUS 2007, HARRIGAN 1999, HERMANN ET AL. 1998, HERMANN ET AL. 2001, JAY ET AL. 2005, KALINOWSKI ET AL. 2003, RÖMPP ONLINE 2010, SILBERBACH & BURKOVSKI 2006]

2.6.3. *Micrococcus luteus*

The term *Micrococcus* comes from Greek where micros means small and coccus pip, or beery. They belong to the order of *Actinomycetales*, and the family of *Micrococcaceae*. The genus *Micrococcus* is gram-positive, is a nitrite and catalase positive bacterium with proteolysis activity. The spherical cells have a diameter between 0.5 and 2.0 µm, and typically appear in pairs, tetrads or accumulations. They mainly are immovable and do not form spores. Several species of these bacteria are strictly aerobic. The bacterial wall does not contain teichoic acid, which is often covalently bound to the peptidoglycan layer.

Micrococci can grow well in the temperature range between zero and 37 °C, with less water in the environment, and under high salt concentration as well as at pH values between 5.6 and 8.1. Particularly, they occur on human and mammalian skin and in many environmental compartments such as soil, dust and water. In foodstuffs they can be found in meat and dairy products. Due to their capability of synthesizing long-chain alkenes and their ability to concentrate heavy metals from low-grade ores, *Micrococci* are interesting in terms of biotechnological applications.

Micrococcus luteus (Fig. 12) is a saprotrophic and obligate aerobic bacterium, which forms bright, yellow colonies, when grown on nutrient agar.

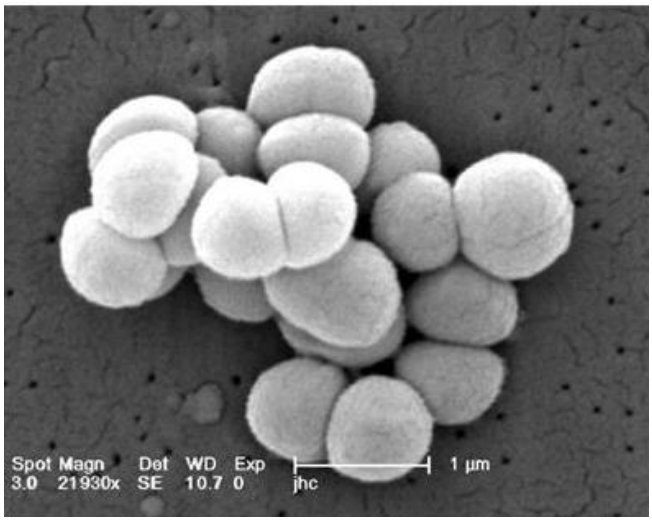


Figure 12. *Micrococcus luteus* spherical cells [HTTP://CELLBIOLOGY.MED.UNSW.EDU.AU/UNITS/IMAGES/GRAM-POSITIVE%20MICROCOCCUS%20LUTEUS%20BACTERIA.JPG].

M. luteus can colonize in the human mouth, mucosae, oropharynx and the upper respiratory tract. It cannot form spores as a surviving structure, although it is able to survive under certain stress conditions such as for example low temperatures over a long time period. It belongs to the non-pathogenic group of microorganisms but it can cause undesirable effects in immune-deficient persons. These species can grow under reduced water conditions, high salt concentrations and can survive drying. It has been associated with spoilage of fish products. Degrading compounds in sweat can produce an unpleasant odor. [GREENBLATT ET AL. 2004, HARRIGAN 1999, HOERR ET AL. 2004, JAY ET AL. 2005, WWW.ROEMPP.COM, YOUNG ET AL. 2010]

2.6.4. *Escherichia coli*

Escherichia coli belong to the order *Enterobacteriales*, family *Enterobacteriaceae*. *Escherichia coli* are described as gram-negative, aerobe or facultative anaerobe, often motile and non sporulating bacteria. *E.coli* is the best molekularbiologically and genetically investigated organism. Their cells are typically rod-shaped and the cell walls are in many species thickened. They are short about 2 µm and 5 µm in diameter. All species can ferment glucose both under aerobic and anaerobic conditions, with the formation of acid or of acid and

gas. They are fermentative, catalase positive and oxidase negative microorganisms, which can also reduce nitrates to nitrites. *E. coli* (Fig. 13) occurs in the colon of humans and actually in all animals, where they are typically intestinal parasites. Some species can occur also in other parts of the human body, as well as on plants and in the soil.

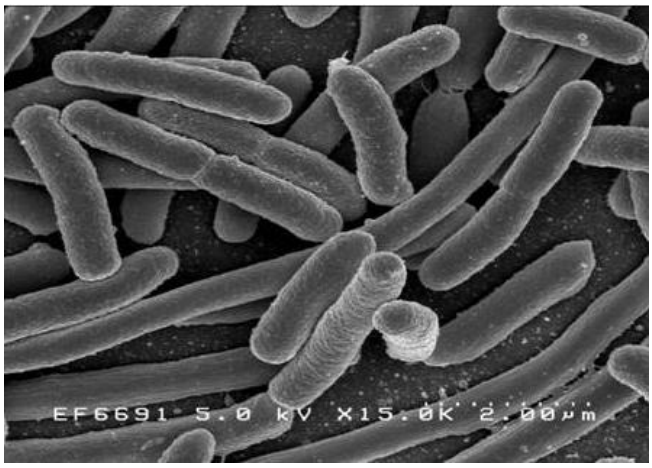


Figure 13. *Escherichia coli* cells [HTTP://WWW.PUBLIC.IASTATE.EDU/~EEVANS/ESCHERICHIACOLI_NIAID.JPG].

This group of microorganism can multiply at temperatures between 2.5 and 50 °C, with an optimum around 37 °C. They can grow between pH values of 4.0 and 9.5 on a wide variety of substrates. *E. coli* plays a role in outbreaks of human diseases because of contaminated food and water. Many of the *E. coli* species are pathogenic or produce Vero cytotoxin (VTEC).

The genus *E. coli* is subdivided into serotypes. There has been a correlation established between serogroup and virulence of these microorganisms that is a basis of following *E. coli* classification:

- enteropathogenic *E. coli* (EPEC) – cause of diarrhea in humans, rabbits, dogs, cats and horses
- enterotoxigenic *E. coli* (ETEC) – cause of diarrhea in humans, pigs, sheep, goats, dogs, cattle and horses
- enteroinvasive *E. coli* (EIEC) – found only in humans
- enterohaemorrhagic *E. coli* (EHEC) – found in humans, cattle and goats. It is the most frequent cause of diarrhea. The most common EHEC serotype reported is *E. coli O157:H7*
- enteroaggregative *E. coli* (EAEC) – found only in humans.

The different virulence factors show the ability to invade epithelial cells of the small intestine and to produce haemolysin and toxins, which can lead to various types of diseases. In developed countries they are not very common causes of food-borne diseases, but in less developed countries they are a problem of the childhood diarrheas. *E. coli* plays a very important role in the food industry as an indicator for the hygienic status of raw materials, of processed and finished food, and of the water supply in a company. *E. coli* can occur in food matrices such as:

- raw material or product exposed to contamination from bovine origin (meat or faeces)
- manufactured products with no processing stage capable of destroying the organism, e.g. cooking
- products exposed to post-process contamination,
- products sold as ready to eat
- in contact with an infected individual or animals.

To avoid *E. coli* in a final product, the industry has to perform quality controls of raw materials, as well as of process condition, post-process conditions and retail or catering practices, because humans as consumers are still prone to infections with *E. coli*.

The most common reason why *E. coli* can occur in food is still contaminated raw material. For instance, raw milk becomes contaminated from the faeces of the cow, and raw meat through the transfer of feacal pathogens from the intestine to the muscle tissues. Vegetables, fruits and freshly pressed unpasteurized fruit juice are contaminated from the soil, where animal manure has been applied. In addition, many wild animals and birds can transfer the VTECs pathogenic to humans. [ADAMS & MOSS 2008 & 1995, BLACKBURN &MCCLURE 2002, HARRIGAN 1999, JAY ET AL. 2005, KEWELOH 2008, WWW.ROEMPP.COM]

2.6.5. *Enterococcus faecalis*

The term *Enterococcus* comes from Greek, where enteron means intestine and coccus pip or beery. They belong to the order of *Lactobacillales* and family *Enterococcaceae*. The bacteria of the genus *Enterococcus*, including *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus avium* and *Enterococcus durans*, were assigned before to the genus *Streptococcus*.

Enterococci are gram-positive, lactic acid bacteria and facultative anaerobic. They do not need oxygen to metabolize, but can tolerate it in their environment for growing. Often, they form pairs (diplococci) or short chains. This genus of the microorganism does not form a spore, but they can survive at pH values between 4.5 and 10, and under high sodium chloride concentrations (< 6.5%). It is one of the most resistant non-sporulating microorganisms that can grow at temperatures between 10 and 50 °C, with an optimum at 37 °C, and can survive for 30 min at 60 °C.

As suggested by the name, they are common inhabitants of the human gastrointestinal and genitourinary tracts. They are known as a cause of infections, such as enterococcal bacteraemia, heart illness, bacterial endocarditis and urinary tract infections. Recently [JAY ET AL. 2005], they have been recognized as the leading cause of hospital-acquired infections, in parallel to increased antimicrobial resistance to most currently drugs. *Enterococcus faecalis* is the most common species of *Enterococci*. It is an immovable bacterium that is capable of fermenting glucose without gas production and is unable to produce catalase. It is found in the gastrointestinal tracts of humans and other mammals and can cause endocarditis, and infections of the bladder, prostate and the epididymal. These microorganisms show resistance against some antibiotics such as cephalosporins, clindamycin and aminoglycosides.

Next to *E. coli*, *Enterococcus faecalis* (Fig. 14) plays an important role as hygiene indicator in food. The presence of the *E. faecium* or *E. faecalis* in food products or water indicates a faecel contamination.

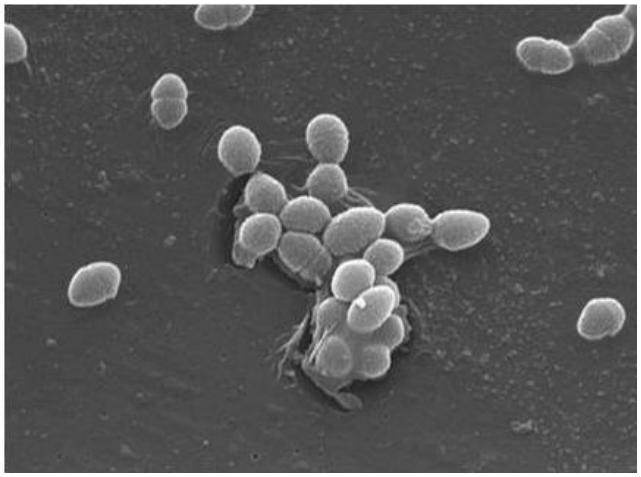


Figure 14. Enterococcus faecalis cells
[HTTP://WWW.GENOME.GOV/IMAGES/PRESS_PHOTOS/LOWRES/20024-72.JPG].

The *Enterococcus* spp. took the place of fecal coliform as the new federal standard for water quality at public beaches. In food industry, they play different roles. They are desirable in dairy technology, e.g. in cheese production, but very undesirable in the meat industry. [ADAMS & MOSS 2008 & 1995, HARRIGAN 1999, JAY ET AL. 2005, KEWELOH 2008, PIHEIRO ET AL. 2004, WWW.ROEMPP.COM]

2.6.6. *Pseudomonas aeruginosa*

Pseudomonas comes from Greek, with pseudo meaning false and monas meaning a single unit. It is a genus of γ-proteobacteria and belongs to the order *Pseudomonadales* and the family of *Pseudomonadaceae*. *Pseudomonades* are gram-negative obligate aerobic and catalase, protease and lipase positive. They are rod shaped bacteria with a size between 0.5 and 1 x 1 and 4 µm. They are not fermentative, able to move and able to reduce nitrate to nitrite. They do not form a spore. They often build water soluble and fluorescent pigments (e.g. *Pseudomonas aeruginosa*) (Fig. 15). Normally, they are mesophile, but some of them are psychrophile such as *P. aeruginosa* and *P. fluorescens*.

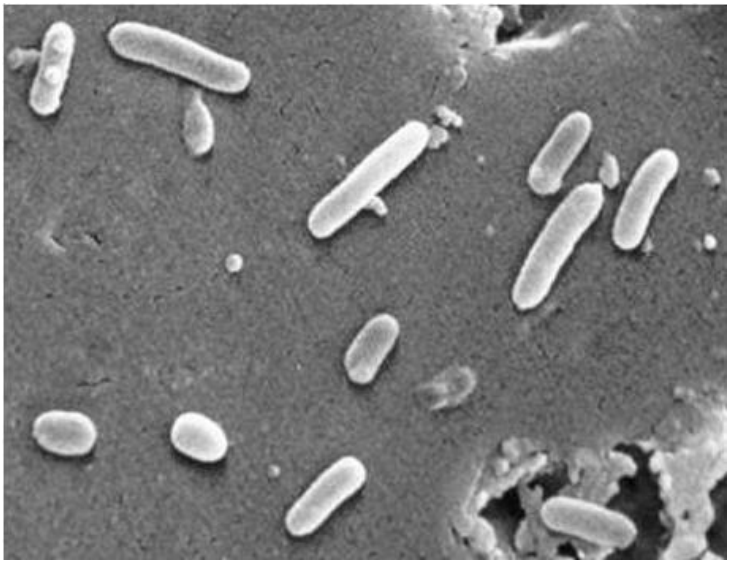


Figure 15. Pseudomonas aeruginosa cells

[HTTP://WWW.TEXTBOOKOFBACTERIOLOGY.NET/IMAGES/P.AERUGINOSASEM.JPG].

Some of the Pseudomonades are pathogenic for plants, animals and humans. *P. aeruginosa* is an opportunistic pathogen and one of the strongest for human that can cause a

variety of infections such as nosocomial, skin and pneumonia infections, urinary tract infections, surgical wound and bloodstream infections, especially for patients who are immune deficient or otherwise compromised. It is the single most important pathogen for cystic fibrosis (CF) and the most important cause of morbidity and mortality for humans that suffer of CF. The virulence factors of *P. aeruginosa* are exotoxin A, elastase, and phospholipase C.

P. aeruginosa is a facultative anaerobe that obtains energy via aerobic respiration and can well adapt to conditions of limited oxygen supply. It grows anaerobically with nitrate as a terminal electron acceptor and in the absence of nitrate can ferment arginine to generate ATP by substrate-level phosphorylation. Pseudomonades are typical soil and water bacteria and are widely distributed on the surface of fresh food, especially plants, fruits, vegetables, meats, poultry, seafood products, in raw milk and in butter. *P. aeruginosa* can multiply at temperatures between 2 and 42 °C, at pH values between 5.5 and 8.1 and by a water potential $a_w = 0.97$. [COLLINS 1955, COOPER ET AL. 2003, HARRIGAN 1999, JAY ET AL. 2005, KERR & SNELLING 2009, RAHME ET AL. 1997, WILLIAMS ET AL. 2006, WORLITZSCH ET AL. 2002, WWW.ROEMPP.COM]

2.6.7. *Sacharomyces pasteurianus*

The term *Saccharomyces* in Latin means sugar fungi (sarkara, a sugar from bamboo). *Saccharomyces* belongs to the order of *Saccharomycetales* and the family of *Saccharomycetaceae*. They are species of yeast with spherical, ellipsoidal or cylindrical cells. They multiply vegetatively and multilaterally and grow rapidly. They are flat, smooth and creamy in color.

They predominantly live in the nature on fruits and in plant juices, and are non-pathogenic. They are able to ferment various sugars, including glucose, maltose, galactose and raffinose, into ethanol and are used in the production of wine, bread, beer and ethanol. *Saccharomyces sensu stricto* yeasts include the four sibling species: *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces paradoxus*, and *Saccharomyces pasrtorianus (carlsbergensis)*.

Saccharomyces pastorianus (Fig. 16) is synonymous with *S. carlsbergensis* and is a natural hybrid of *S. cerevisiae* and *S. bayanus*. *S. pastorianus* includes the group of bottom fermenting species, which are most commonly used as brewing yeasts in the production of bottom-fermented beer.

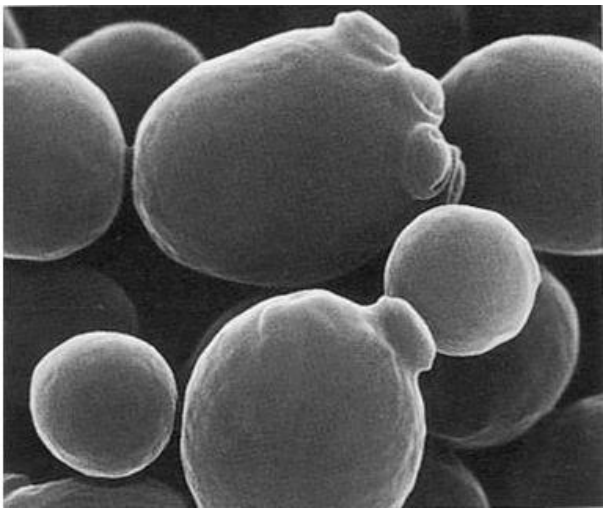


Figure 16. *Saccharomyces* cells [HTTP://WWW.BATH.AC.UK/BIO-SCI/IMAGES/PROFILES/WHEALS2.GIF].

They are non-mater, sporulate poorly and have very low spore viability. The strains *S. bayanus* and *S. bayanus* var. *uvarum* are known to be more cold-resistant than *S. cerevisiae*. Thereby, they better carry out fermentation at cold temperatures (between 8 and 12 °C) than *S. cerevisiae* alone. *S. pastorianus* never grows below 34 °C, whereas *S. cerevisiae* can still grow at 37 °C. *Saccharomyces cerevisiae* and *S. carlsbergensis* are unable to utilize lysine as a sole nitrogen source, whereas other types of yeast can exploit this amino acid. A differentiation can be made between yeast for baking, brewing, wine and champagne making. They rarely cause spoilage. [CASAREGOLA ET AL. 2001, DUNN & SCHERLOCK 2008, HARRIGAN 1999, JAY ET AL. 2005, MONTROCHER R ET AL. 1998, NAKAO ET AL. 2009, NGUYEN & GAILLARDIN 2005, TAMAI ET AL. 1998, TOSCH ET AL. 2006]

3. Material Equipments and Methods

3.1. Materials

3.1.1. Samples of plants materials

All citrus fruits used to researches were purchased from a local supermarket.

1) Grapefruits – *Citrus paradisi* –from Spain

2) Oranges (Navelinas) – *Citrus sinensis* –from Greece

3) Mandarins (Clementins) – *Citrus clementina* –from Spain.

All fruits had a first quality class.

Ketchup – Tomatoes (*Lycopersicum esculentum*) – Chez Pierre was purchased from local supermarket.

Thyme (*Thymus vulgaris*) dried ground leaves – Raps GmbH & Co. KG, Kulmbach, Germany

Raw Peanuts (*Arachis hypogea*) – Sandos Naturkost, Berlin, Germany

3.1.2. Chemicals and solvents

Methanol (MeOH) – for HPLC, Gradient Grade UN1230	VWR International
Dimethylsulfoxide (DMSO) – ≥ 99,0% 7033	Backer, Deventer, Holland
Acetonitrile – for HPLC, Gradient Grade UN1648	VWR International
Ethanol 96% – UN1170	VWR International
***2*-Propanol** – Rotisolv® HPLC	Roth, Karlsruhe, Germany
Acetonitrile – ROTISOLV® HPLC, Gradient Grade UN1648	Roth, Karlsruhe, Germany
Acetic acid – glacial Rotipuran® 100% p.a. UN 2789	Roth, Karlsruhe, Germany
***n*-Hexane** – Rotisolv® HPLC UN1208,	Roth, Karlsruhe, Germany
***tert*-Butyl methyl ether** – for synthesis, ≥ 99,0% UN 2398	Merck, Germany
Distilled water	
Nitrogen Gas 5,0 – ≥ 99,999 Vol.%	Air Liquid, Germany

3.1.3. Standards of flavanones

(+/-) Naringenin – $C_{15}H_{12}O_5$ – 4',5,7-Trihydroxyflavanone

- beige-colored powder
- wavelength λ = 289, 335 (± 2 nm) sh in ethanol (UV/Visible spectrum)

Sigma Aldrich Chemie GmbH, Taufkirchen, Germany

Isosakuranetin – $C_{16}H_{14}O_5$ – 5,7-Dihydroxy-4'methoxyflavanone

- white to whitish powder
- wavelength λ = 290, 329 (± 2 nm) sh in ethanol (UV/Visible spectrum)

Extrasynthese, Genay, France

Eriodictyol – $C_{15}H_{12}O_6$ – 3',4',5,7-Tetrahydroxyflavanone

- slightly beige-colored powder
- wavelength λ = 288, 330 (± 2 nm) sh in methanol (UV/Visible spectrum)

Extrasynthese, Genay, France

Homoeriodictyol – $C_{16}H_{14}O_6$ – 4',5,7-Trihydroxy-3'-methoxyflavanone

- white to beige colored powder
- wavelength λ = 287, 340 (± 2 nm) in ethanol (UV/Visible spectrum)
- $[\alpha]_D = 0°$ in ethanol

Extrasynthese, Genay, France

(–) Homoeriodictyol – 4',5,7-Trihydroxy-3'-methoxyflavanone, $C_{16}H_{14}O_6$

- slightly yellow powder
- wavelength λ = 287, 330 (± 2 nm) sh in ethanol (UV/Visible spectrum)
- $[\alpha]_D = - (16 \pm 5)°$, c = 0,5 in ethanol

Extrasynthese, Genay, France

Hesperetin, 3',5,7-Trihydroxy-4'-methoxyflavanone, $C_{16}H_{14}O_6$

- beige-colored powder
- wavelength λ = 287, 333 (± 2 nm) in methanol (UV/Visible spectrum)

Extrasynthese, Genay, France

Hesperidin, 4',5,7-Trihydroxy-3'-methoxyflavanone, $C28H_{34}O_{15}$

- white to slightly yellow powder
- wavelength λ = 284, 330 (± 2 nm) sh in ethanol + 0,25% dimethylsulfoxide (UV/Visible spectrum)

Extrasynthese, Genay, France
[WWW.EXTRASYNTHESE.COM]

3.1.3.1. Standards of Antibiotics:

Tetracyclin hydrochloride ≥ 95% for Biochemistry – Roth, Karlsruhe, Germany

Natamax – Natural Antimicrobial Material for Use in Food (E235 Natamycin, Lactose) – Danisco A/S, Grindsted, Denmark

3.1.4. Bacteria strains, media and growth conditions

3.1.4.1. Bacteria Strains

For the thesis, following bacteria strains were used:

***Corynebacterium glutamicum* ATCC 13032** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Bacillus subtilis* ATCC 6633** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Micrococcus luteus* ATCC 10240** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Escherichia coli* ATCC 23716** – vacuum dried culture from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Escherichia coli ATCC 25922* (Pathogen – Risk Group 2)** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Enterococcus faecalis* ATCC 19433 (Pathogen – Risk Group 2)** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Pseudomonas aeruginosa* ATCC 10145 (Pathogen – Risk Group 2)** – vacuum dried culture – German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany

***Saccharomyces pastorianus ssp. carlsbergensis* W 34/70 Weihenstephan –** Technical University Munich, Freising – Weihenstephan, Germany

3.1.4.2. Media

Wort – Gelatine Medium:

Wort Preparation

Hopped and casted wort (Original Weihenstephaner) was subjected to filter aid (Fimacel 3, Seitz) and afterwards autoclaved for 10 min at 121 °C. The wort then was filtered, using a pressure filter with CO2-inlet, through a coarse filter layer (deep filter) (HS 800, Pall Seitz-Schenk Filtersystems), and afterwards freed from the filter aid. The wort was sterilized at 100 °C for 45 min, stored as such afterwards.

Filling into Flasks and Tubules

The filtered wort was again filtered through a fine filter layer (deep filter) (Seitz-EK, Pall Seitz-Schenk Filter Systems) and freed from the sludge. Then the wort is filled into sterilized flasks (50 ml) and tubules (10 ml) and sterilized for 45 min at 100 °C.

Wort – Gelatine Medium

The hopped and casted wort is filtered through a thick filter layer and mixed with 150 g gelatine per litre of wort. The wort then was left to swell for 1 h and dissolved at 100 °C using a pressure cooker. As thereby sludge is again produced, the wort-gelatine mixture is filtered through a thick filter and filled in hot condition into 50 ml flasks. These flasks are then sterilized at 100 °C for 30 min.

BHI Medium

Bacto™ Brain Heart Infusion Medium (BHI-medium) – Becton, Dickinson and Company, Sparks, USA

Agar – Agar for bacteriology, powder – VWR/ BDH Prolabo International

BHI media were prepared by suspending 37 g of the powder in 1 L of purified water. After agitation, were sterilized by autoclaving at 121 °C for 15 minutes.

Content per liter:

Calf Brains, Infusion from 200 g	7.7 g
Beef Heart, Infusion from 250 g	9.8 g
Proteose Peptone	10.0 g

Dextrose	2.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g

Final pH-value: 7.4 ± 0.2

YNB – medium

Yeast Nitrogen Base for microbiology (YNB-substratum) – Fluka / BioChemika, Buchs, Switzerland

Sodium hydroxide – pellets GR for analysis – Merck, Darmstsadt, Germany

Sodium succinate dibasic anhydrous, purum ≥ 98,0% (NT) – Fluka / BioChemika, Buchs, Switzerland

D (+) Glucose monohydrate for microbiology – Merck, Darmstadt, Germany

Adenine, minimum 99% – Sigma-Aldrich, Steinheim, Germany

L-Histidine for biochemistry – Merck, Darmstadt, Germany

L-Leucine for biochemistry – Merck, Darmstadt, Germany

L-Tryptophan for biochemistry – Merck, Darmstadt, Germany

Uracil, minimum 99% – Sigma-Aldrich, Steinheim, Germany

6.0 g of sodium hydroxide (NaOH) and 10 g of sodium succinate were suspended in ca. 800 mL of purified water and filtered. The pH-value was adjusted to 5.8 with succinate and/or NaOH and with purified water filled up to 1 L.

A mixture containing 20 g of glucose, 6.7 g of YNB-substratum, 200 mg of leucine, 100 mg of histidine, 100 mg of tryptophan, 100 mg of adenine and 100 mg of uracil was prepared and added to the cooled succinate/NaOH solution. The resulting mixture was again filtered and afterwards autoclaved at 121 °C for 15 min.

YNB – Substratum – Content per liter:

Vitamins:

Biotin	2 µg
Calcium pantothenate	400 µg
Folic acid	2 µg
Inositol	2000 µg

Niacin	400 µg
p-aminobenzoic acid	200 µg
Pyridoxin Hydrochloride	400 µg
Riboflavin	200 µg
Thiamin Hydrochloride	400 µg

Trace elements:

Boric acid	500 µg
Copper sulfate	40 µg
Potassium iodide	100 µg
Iron chloride	200 µg
Manganese sulfate	400 µg
Sodium molybdate	200 µg
Zinc sulfate	400 µg

Macro-elements:

Potassium dihydrogenphosphate	1.0 g
Magnesium sulfate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g

Final pH-value: 4.5

3.1.4.3. Grow conditions

Corynebacterium glutamicum **ATCC 13032**	was grown aerobically on BHI medium at 30 ºC on a shaking platform by rpm = 172.
Bacillus subtilis **ATCC 6633**	was grown aerobically on BHI medium at 30 ºC on a shaking platform by rpm = 185.
Micrococcus luteus **ATCC 10240**	was grown aerobically on BHI medium with addition of glucose (10 g/L) at 30 ºC on a shaking platform by rpm = 210.
Escherichia coli **ATCC 23176**	was grown aerobically on BHI medium at 37 ºC on a shaking platform by rpm = 110.

Escherichia coli **ATCC 25922**	was grown aerobically on BHI medium with addition of glucose (10 g/L) at 37 ºC on a shaking platform by rpm = 210.
Enterococcus faecalis **ATCC 19433**	was grown aerobically on BHI medium with addition of glucose (10 g/L) at 37 ºC on a shaking platform by rpm = 210.
Pseudomonas aeruginosa **ATCC 10145**	was grown aerobically on BHI medium with addition of glucose (10 g/L) in 37 ºC on a shaking platform by rpm = 210.
Saccharomyces pastorianus	1) was grown on Petri dishes on Wort-Gelatine Medium at 26 ºC for 4 days (Agar Inhibitory Test) 2) was grown aerobically on YNB medium at 30 ºC in a Tecan SunRise.

3.1.5. Miscellaneous materials

- Centrifuge Tubes, Gamma-Sterilized, Freedom from pyrogenics, Freedom from RNA, DNA, RNases and DNases – TPP Switzerland
- Disposal Bags, Plastibrand – Brand, Wertheim, Germany
- Sterile Inokulation Loop – Greiner, Frickenhausen, Germany
- Laboratory Film, Parafilm "M" – American National Can, Chicago, USA
- Micro Test Tubes with safety lid lock and scale graduation 1.5 mL – Eppendorf – Netheler – Hinz GmbH, Hamburg, Germany
- Pipette tips in racks, Plastibrand TIP-SET – Brand, Wertheim, Germany
- Polyalcohol Hands Antisepticum – Antiseptica, Pulheim / Brauweiler, Germany
- Single-use syringes 2-piece, Injekt 20 mL – Braun, Melsungen, Germany
- Tissue Tucher – Roth, Karlsruhe, Germany
- Weighing Paper MN 226.9 x 11.5 cm – Macherey – Nagel, Düren, Germany
- Nunclon Surface – Nagle Nunc, Brand Products, Denmark, VWR Bruchsal, Germany (Petrischalen)
- Pipette Eppendorf – Reference 10 –100 µL – Eppendorf, Hamburg, Germany
- Pipette Eppendorf – Reference 100 –1000 µL – Eppendorf, Hamburg, Germany
- Folded Filters 595 ½ Ø = 90 mm, Whatman® Schleicher & Schuell, Dassel, Germany
- Syringe Filter 25 mm, w/ 0.45 µm Polypropylene Membrane, VWR, USA
- Single-use Syringe Without needle, 2 mL, non pyrogenic – Terumo Europe, Leuven, Belgium
- Labor's glass

3.1.6. Solid phase extraction (SPE)

SPE-Instrument:

Lichrolut™ Vacuum Manifold, Merck, Darmstadt, Germany

Extraction Columns:

Strata C-18-E (55 um, 70A), 500 mg / 3 mL, Phenomenex, Aschaffenburg, Germany

3.1.7. High performance liquid chromatography

High Pressure Pump:

Gynkotek High Precision Pump, Model 480GT, Germering, Germany

Manual Injection:

Microliter Syringes 100 µL, Hamilton, Bonaduz, Switzerland

HPLC – Column:

Column 150 x 4,60 mm, 5 micron, LiChrospher 5u, RP-18e – Phenomenex, Aschaffenburg, Germany

Detectors:

LDC / Milton Roy SpectroMonitor™ D variable wavelength detector, Riviera Beach, Florida, USA

Merck – Hitachi L-4000 A, UV Detector, Darmstadt, Germany

Software:

Chromeleon Version 6.70, Dionex, Idstein, Germany

3.1.7.1. Chiral separation technique

High Pressure Pump:

Gynkotek High Precision Pump, Model 480GT, Germering, Germany

Manual Injection:

Microliter Syringes 100 µL, Hamilton, Bonaduz, Switzerland

Security Grad Column:

Vertex-Column 5 x 4 mm, Europak 01, 5 m

HPLC – Chiral Column:

Chiral Vertex Column 250 x 4.6 mm, Europak 1000 - 5 01, 5 µm, WG 113 – Knauer, Berlin, Germany

Detector:

Merck – Hitachi L-4000A, UV Detector, Darmstadt, Germany

Software:

Chromeleon Version 6.70, Dionex, Idstein, Germany

3.1.8. Mass spectrometry

Mass Spectrometer:

Agilent Technologies, 6410 Triple Quad LC/MS, Böblingen, Germany

Injection Pump:

Harvard Apparatus Model 11 Plus, Holliston, USA

Injection:

Microliter Syringes 1710 RNR 100 µL, Hamilton, Bonaduz, Switzerland

3.1.9. Circular dichroism

Spectropolarimeter:

Jasco J-710 – Jasco Labor und Datentechnik GmbH, Groß-Umstadt, Germany

Cell:

Quartz Suprasi cell with a thickness of 1 mm, Hellma, Müllheim, Germany

3.1.10 SunRise Tecan

SunRise Remote, Tecan, Männedorf, Switzerland

Sofware:

Makro für Excel Tecan X Fluor, Version 4.51

Plate:

Multiple Well Plate 96-Well, Flat Bottom with Lid – Sarstedt Inc, Newton, USA

3.1.11. Other instruments

Autoclave:

Table Autoclave – Systec, Wettenberg, Germany

Autoclave for pathogens:

Viroclav – Dampfsterilizatoren – H+P Labortechnik GmbH, Oberschleißheim (Munich), Germany

Drying Oven:

Modell T6120 – Heraeus – Electronic, Hanau, Germany

Incubator:

Incubatora Friocell – MMM – Group, Medcenter Einrichtungen GmbH, Planegg/Munich, Germany

Optical Microscope:

Light Microscope – E. Leitz GmbH, Wetzlar, Germany

Thoma-Objectnetzmicrometer:

Objektnetzmikrometer 436963 (depth 0.100 mm) – Carl Zeiss, Jena, Germany

Stereo Microscope:

Wild M75 – Heerbrugg, Switzerland

pH meter:

inoLab pH Level 1, (with meter electrode – SenTix 41, Basis pH-meter chain) – WTW GmbH (Wissenschaftlich-Technische Werkstätten), Weilheim, Germany

Rotary Evaporator:

Laborota 4003 control – Heidolph, Germany

Vacuum controller, VAC Senso T – Heidolph, Germany

Shaking Platform:

Infors AG – Bottmingen, Switzerland

Sonicator:

Typ Sonorex Super RK 510 H, Bandelin electronic GmbH & Co. KG, Berlin

Spectrophotometer:

UV-VIS Recording Spectrophotometer UV-2401 PC – Shimadzu Corporation, Kyoto, Japan

Software:

UV Probe Version 1.0

Cell:

Single-use cell from Polystyrol 1.5 mL – Roth, Karlsruhe, Germany

Sterile Box:

UniFlow UVUB 1200 Biohazard, KR-125 Safety, Air Flow 1250 m^3/h, UniEquip, Martinsried, Germany

Vortex:

Vortex-Genie 2 – Scientific Industries INC., Bohemia, USA

Weighs:

- Analytical Weigh AUW120D – Shimadzu Corporation, Kyoto, Japan
- Precisa 40 SM-2001 (Precisa Balances) – PAG Oerlikon AG, Zürich, Switzerland
- Precisa 2200C (Precisa Balances) – PAG Oerlikon AG, Zürich, Switzerland

Water Preparation:

Mili-Q Plus 185 System, Serie MembraPure – Milipore GmbH, Schwalbach, Germany

3.2. Methods

3.2.1. Flavonoids extraction from plants

The citrus fruits were peeled and their fresh peel was homogenized using a blender and left in the drying oven at 40 ºC for 2 days. Contamination of the peel with juice and citrus segment membranes was avoided.

Peanuts (*Arachis hypogea*) were peeled and the hulls were used for analysis.

One gram (g) of dried peel of citrus fruits, tomatoes ketchup, peanut hulls (*Arachis hypogea*) was extracted with 20 mL HPLC-grade methanol (MeOH), thyme (*Thymus vulgaris*) was extracted with 20 mL *tert*-butyl methyl ether at ambient temperature in sonicator. The extracts were dried in vacuum at 40 °C. The residues were dissolved in 5 mL of 10% MeOH.

3.2.2. Solid phase extraction

For the analysis a C-18 cartridge was used. After preconditioning with 3 mL MeOH and 3 mL 10% MeOH, the solution of the extract in 10% MeOH was applied and washed with 4 ml 10% MeOH. The flavonoids were eluted with 5 mL 70% MeOH. The eluate was evaporated to dryness in vacuum at 40 °C and the residue re-dissolved in pure MeOH. This solution was filtered using a filter membrane (0.45 μm) prior to analysis and injection to HPLC.

3.2.2.1. Preparative extraction of (–) eriodictyol

For the preparative extraction of (–) eriodictyol, 2.5 g of peanut hulls was taken and extracted with 40 ml of HPLC-grade MeOH and sonicated at room temperature. The extracts were dried in vacuum at 40 °C. The residues were dissolved in 10 mL 10% MeOH and applied to a preconditioned (3 mL MeOH and 3 mL 10% MeOH) C-18 cartridge. After washing with 4 mL 10% MeOH, the flavonoids were eluted with 10 mL 70% MeOH and evaporated to dryness at 40 ºC in vacuum. The dried extract was re-dissolved in pure MeOH. This solvent was filtered through a filter membrane (0.45 μm) prior to analysis and injected to the HPLC.

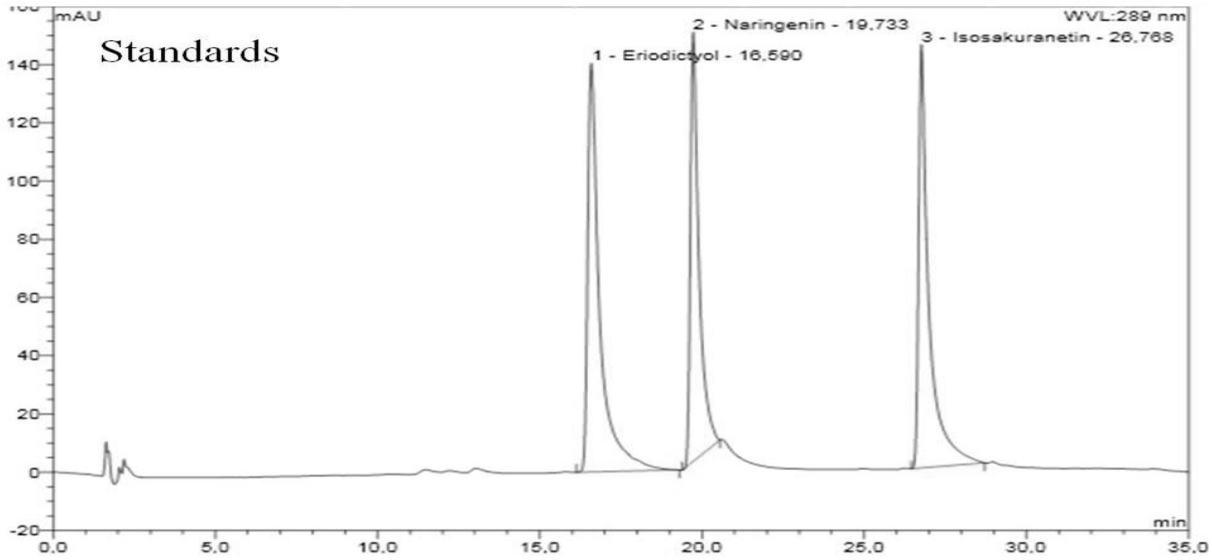
Every preparative extraction of (–) eriodictyol was carried out under analytical HPLC conditions. Peak fractions of multiple injections were collected and combined to yield an extract containing pure (–) eriodictyol.

3.2.3. High performance liquid chromatography conditions

The flavonoids from every plant were analyzed using HPLC with exactly the technical conditions given in chapter 3.1.7. To separate the flavonoids, a mobile phase was used, which was composed of solvent A – acetonitrile (ACN), and solvent B – 2% acetic acid (v/v). The initial solvents were 10% of A and 90% of B. Over the first 30 min the solvent A linearly increased to 60% and then decreased down to 10% over the last 5 min. The whole program lasted for over 35 min with a flow rate of 1.0 mL/min. The injection volume was 20 μL. The column was operated at room temperature and the flavonoids were detected at 289 nm.

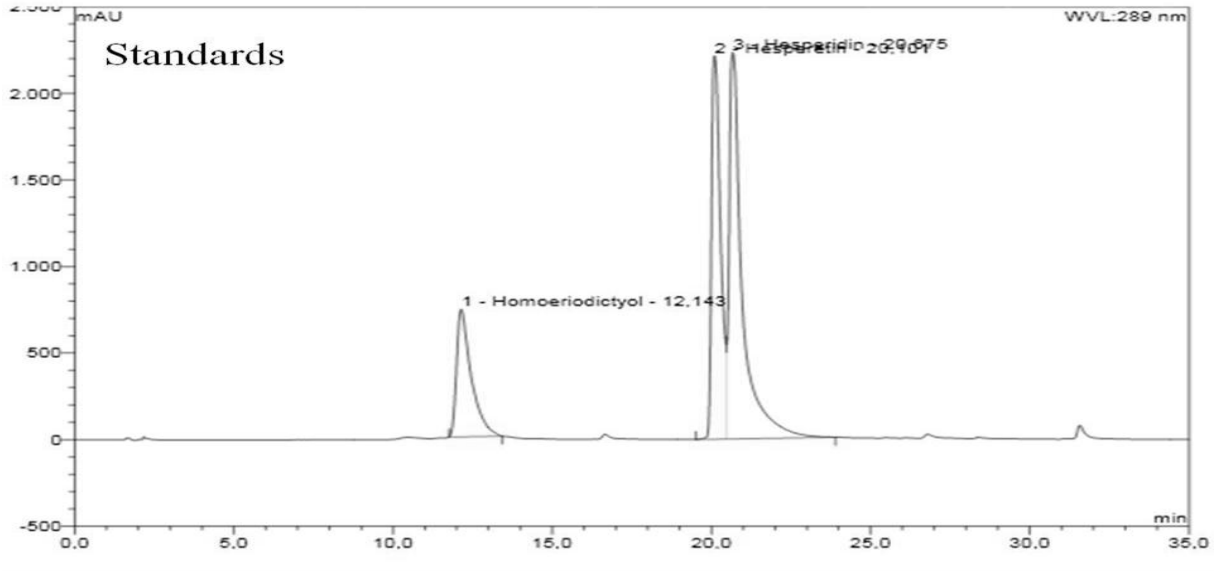
The next two figures (Fig. 17 and Fig. 18) present the HPLC chromatograms of standards of the chosen flavanones. Figure 17 shows the retention times for 1 mg/mL of eriodictyol,

naringenin and isosakuranetin, and Figure 18 those of the other three flavanones, homoeriodictyol, hesperetin and hesperidin. Afterwards, the peaks from the extraction of individual plants were collected and verified by comparison with mass spectrum (MS spectra) of the corresponding flavanone standards.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	16,59	Eriodictyol	140.41	60.80	37.99
2.	19,73	Naringenin	147.38	45.35	28.34
3.	26,77	Isosakuranetin	145.47	53.89	33.67
Total:			433.26	160.05	100.00

Figure 17. HPLC Chromatogram and retention times of eriodictyol, naringenin and isosakuranetin standards



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	12,14	Homoeriodictyol	736.67	391.25	17.78
2.	20,10	Hesperetin	2213.26	730.29	33.19
3.	20,68	Hesperidin	2233.06	1078.69	49.03
Total:			5183.00	2200.23	100.00

Figure 18. HPLC chromatogram and retention times of homoeriodictyol, hesperetin and hesperidin standards

3.2.4. Mass spectrometry

The collected HPLC peaks were injected to the triple quadrupol (QqQ) mass spectrometry (exact dates chapter 3.1.8). The measurements were carried out by electrospray ionization (ESI), with 250 ºC drying gas temperature, 5 mL/min drying gas flow and 30 psi nebulizer gas pressure. The MS detection was simultaneously performed in positive detection mode with use a capillary voltage 4000 V and fragmentor voltage of 150 V. The flow of samples of the collected HPLC peaks was set to 10 µL and the mass range from 250 to 310 for low molecular weight samples – naringenin, isosakuranetin, eriodictyol, homoeriodictyol, hesperetin, and to 610 for hesperidin. The follwoing six figures (Fig. 19, Fig. 20, Fig. 21, Fig.22, Fig. 23, Fig. 24) show the spectra of the individual flavanone standards. The masses of

the individual compounds were used to verify the occurrence of the flavanones contained in the plant extracts.

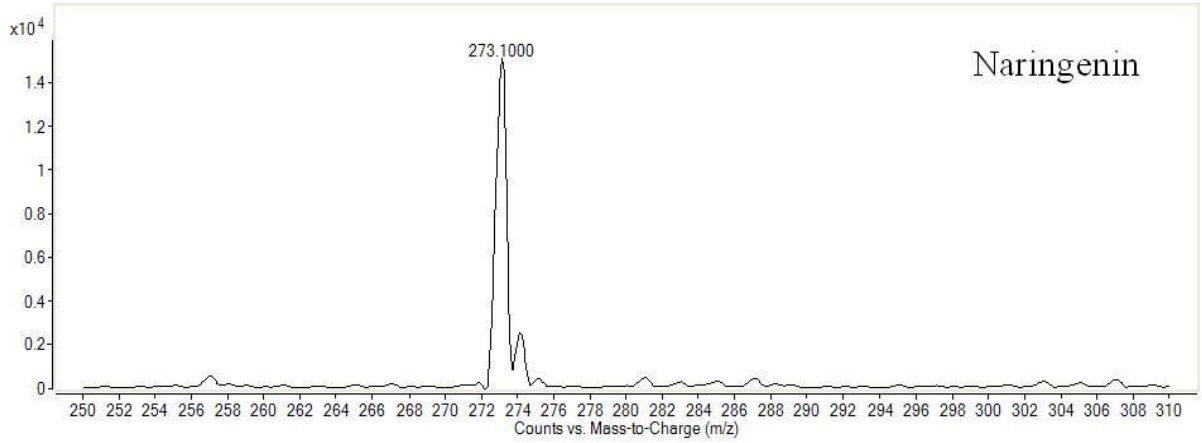


Figure 19. Mass spectrum of naringenin – standard

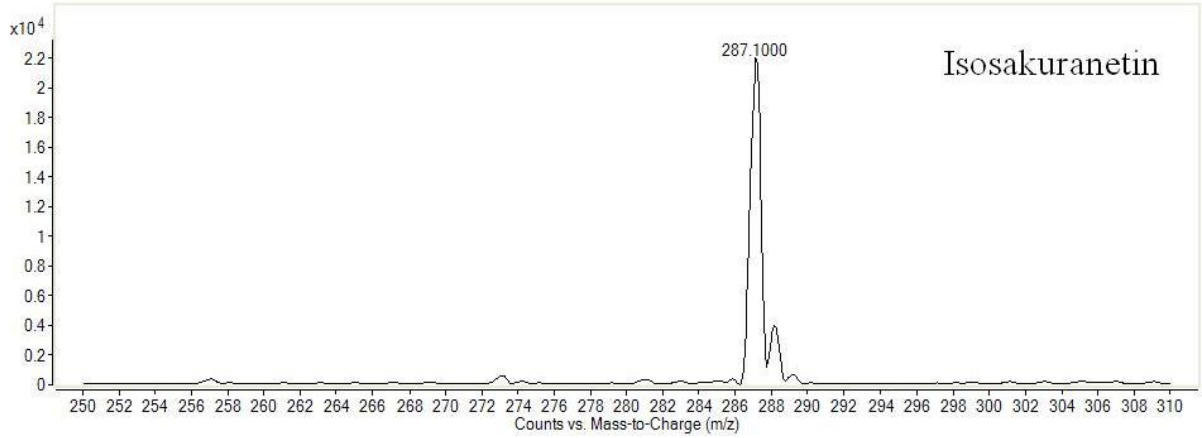


Figure 20. Mass spectrum of isosakuranetin – standard

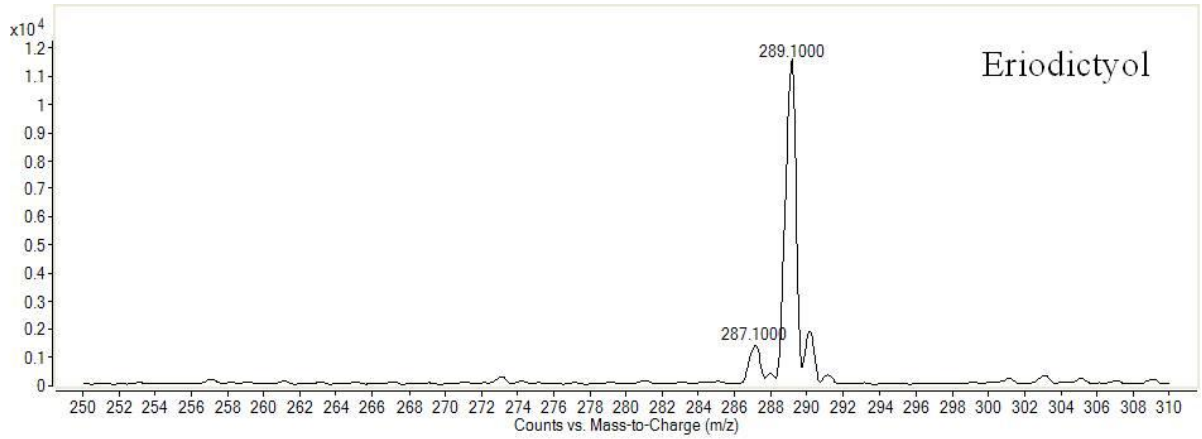


Figure 21. Mass spectrum of eriodictyol – standard

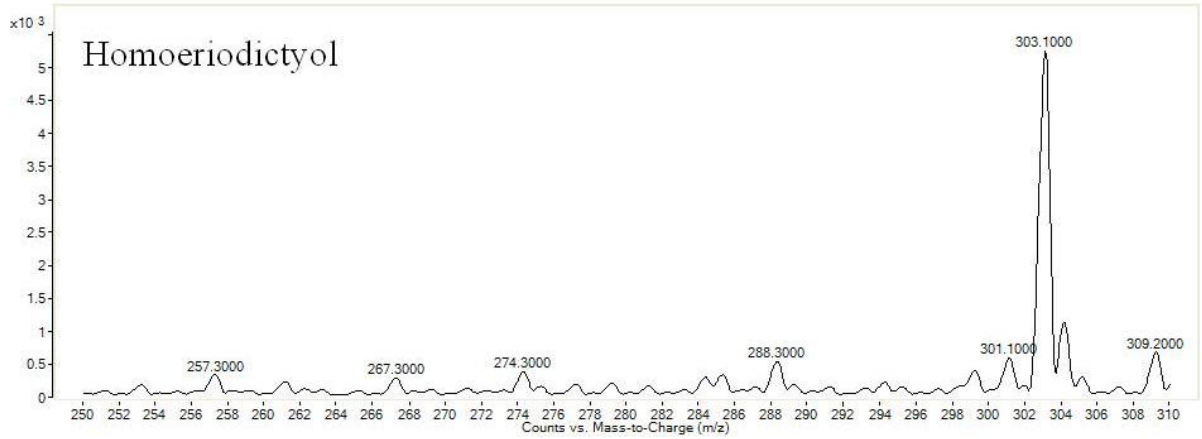
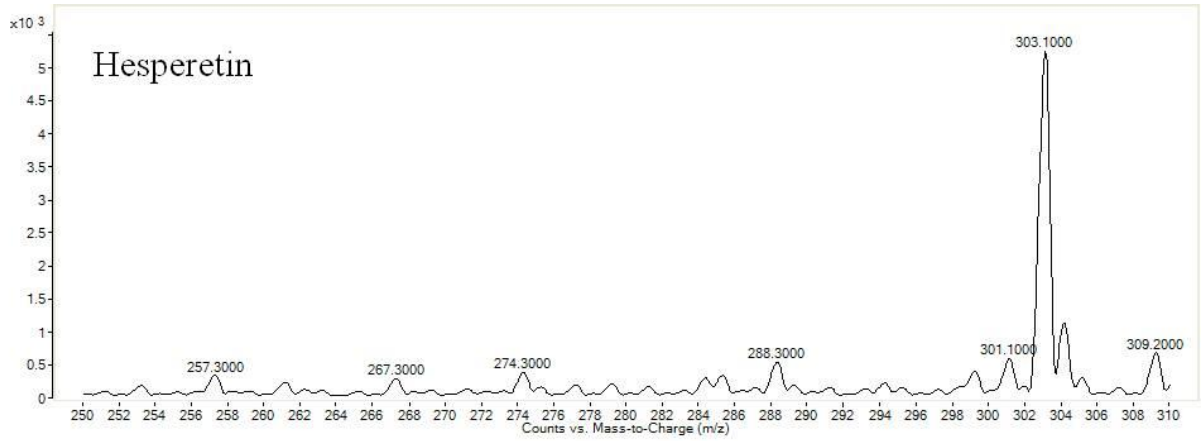


Figure 22. Mass spectrum of homoeriodictyol – standard



ss

Figure 23. Mass spectrum of hesperetin – standard

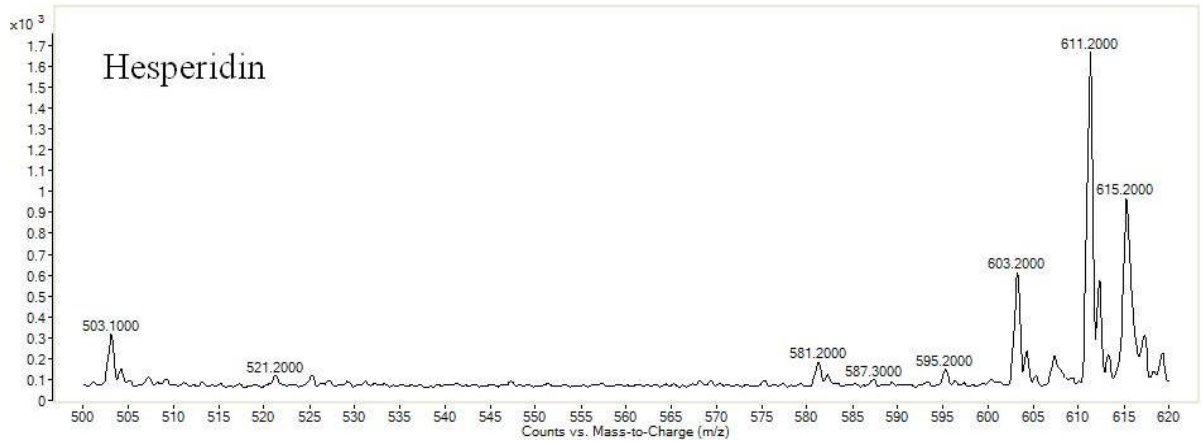


Figure 24. Mass spectrum of hesperidin – standard

3.2.5. Conditions of chiral separation

For the chiral separations were used HPLC with a chiral stationary phase column (exact technical conditions are given in chapter 3.1.7.1). The substances were dissolved in concentration of 1 mg/mL in MeOH and 20 µL was injected into HPLC. All separations were carried out in isocratic modus and at room temperature. The enantiomers were detected at 289 nm. The optical activities of the peaks were determined by circular dichroism (CD).

3.2.5.1. Naringenin

chiral HPLC – conditions:

Mobile Phase:	MeOH
Flow Rate:	1.0 mL/min
Temperature:	ambient
Detection:	UV, 289 nm
Duration:	20 min

3.2.5.2. Isosakuranetin

chiral HPLC – conditions:

Mobile Phase:	MeOH
Flow Rate:	1.0 mL/min
Temperature:	ambient
Detection:	UV, 289 nm
Duration:	35 min

3.2.5.3. Eriodictyol

chiral HPLC – conditions:

Mobile Phase:	MeOH / Water (95 / 5)
Flow Rate:	1.0 mL/min
Temperature:	ambient
Detection:	UV, 289 nm
Duration:	20 min

3.2.5.4. Homoeriodictyol

chiral HPLC – conditions:

Mobile Phase:	MeOH / Water (92 / 8)
Flow Rate:	1.0 mL/min
Temperature:	ambient
Detection:	UV, 289 nm
Duration:	15 min

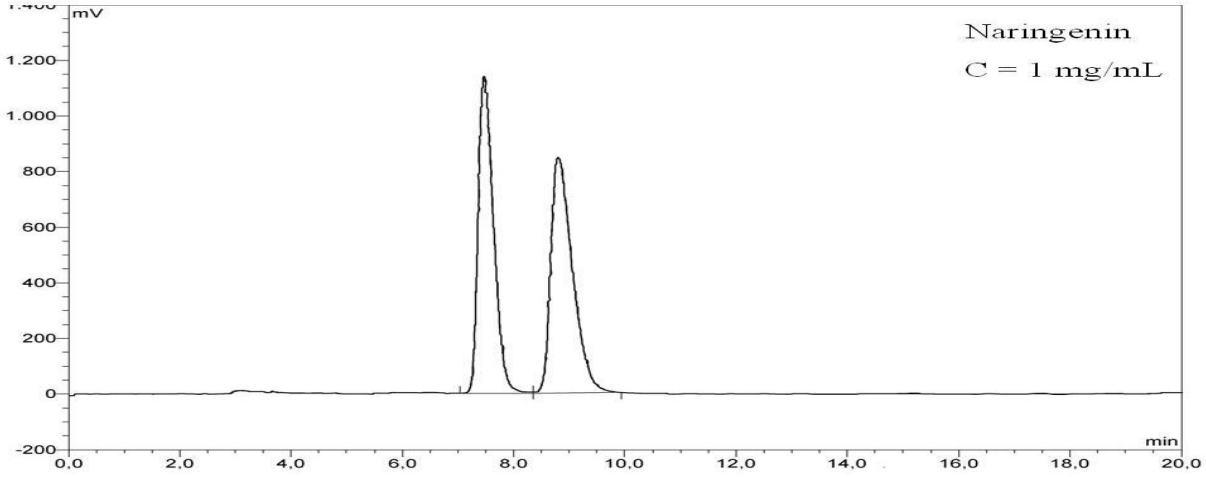
3.2.5.5. Hesperetin

chiral HPLC – conditions:

Mobile Phase:	MeOH
Flow Rate:	1.0 mL/min
Temperature:	ambient
Detection:	UV, 289 nm
Duration:	35 min

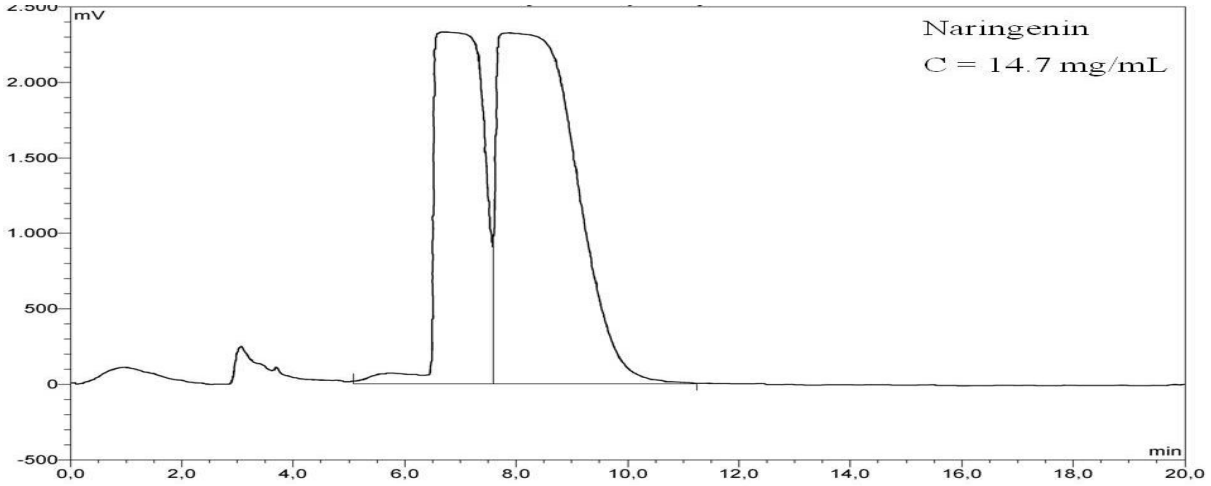
3.2.6. Conditions of chiral preparative separation

The enantiomers of naringenin and isosakuranetin were separated preparatively by analytical HPLC with the chiral stationary phase column (exact technical conditions are given in chapter 3.1.7.1). Before the preparative chiral separation was performed, the full capacity of the chiral stationary phase column was checked. The concentrations for injection into the HPLC were between 1 mg/mL (very good separation) and around 26 mg/mL for naringenin, or 25 mg/mL for isosakuranetin (almost no separation) with a mid-concentration of around 14 – 15 mg/mL (50% of separation). The results of the full capacity are shown in the figures (Fig. 25, Fig. 26, Fig. 27) for naringenin and in the figures (Fig. 28, Fig. 29, Fig. 30) for isosakuranetin.



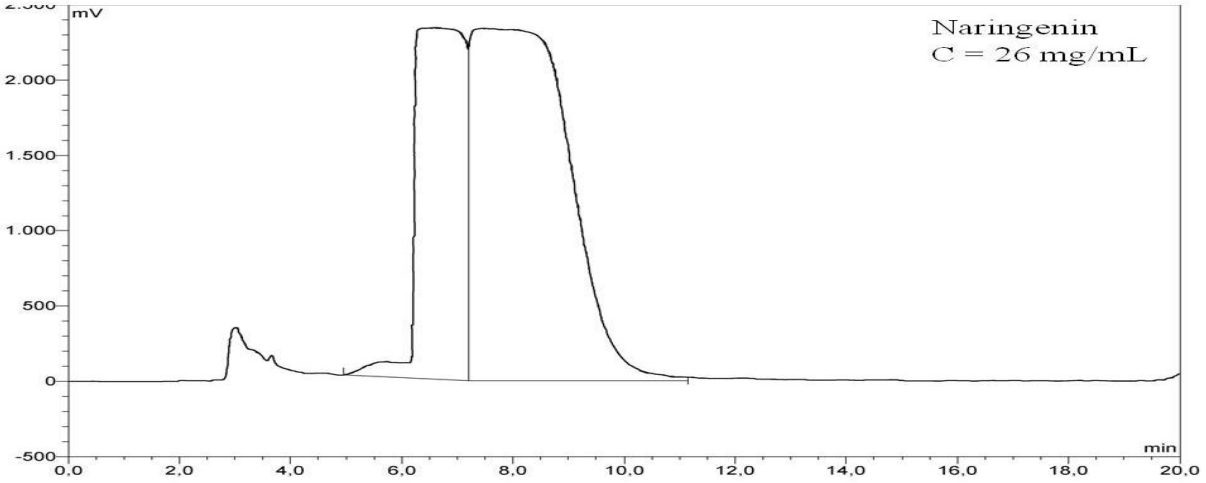
No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	7.59	(–) Naringenin	954.02	377.28	48.70
2.	9.10	(+) Naringenin	703.97	397.43	51.30
Total:			1657.99	774.72	100.00

Figure 25. Chiral separation of naringenin – standard at the concentration of 1 mg/mL, on the Europak column



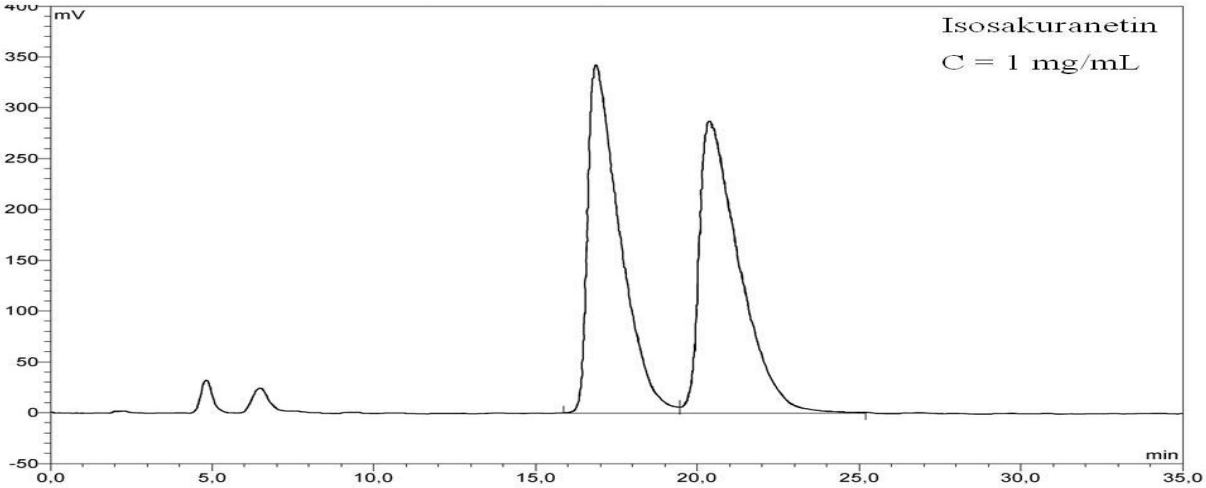
No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	6.69	(–) Naringenin	2331.90	2350.75	38.54
2.	7.81	(+) Naringenin	2324.34	3748.60	61.46
Total:			4656.24	6099.35	100.00

Figure 26. Chiral separation of naringenin – standard at the concentration of 14,7 mg/mL, on the Europak column



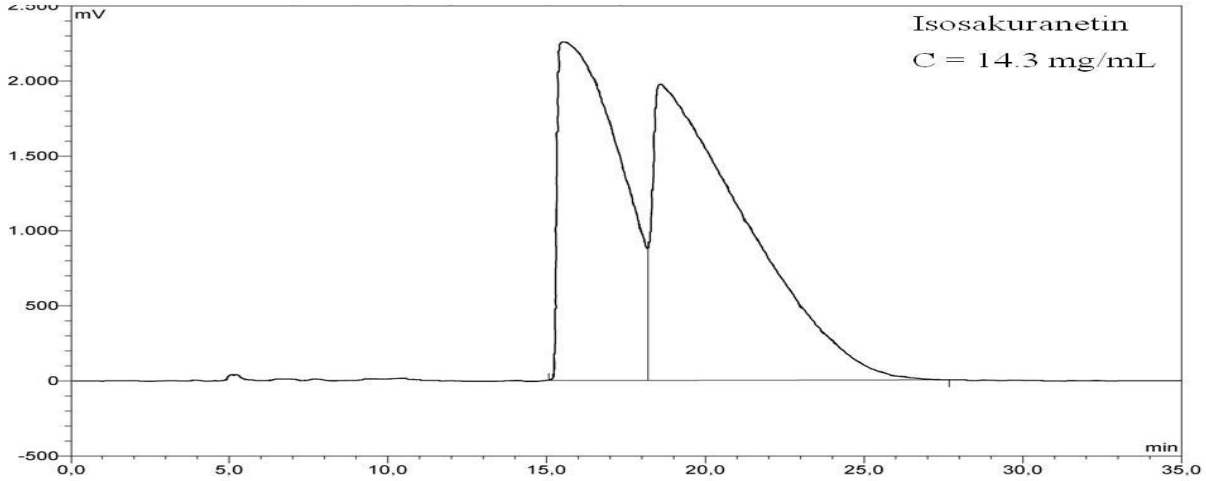
No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	6.67	(–) Naringenin	2334.16	2370.01	33.42
2.	7.47	(+) Naringenin	2340.70	4721.12	66.58
Total:			4674.87	7091.13	100.00

Figure 27. Chiral separation of naringenin – standard at the concentration of 26 mg/mL, on the Europak column



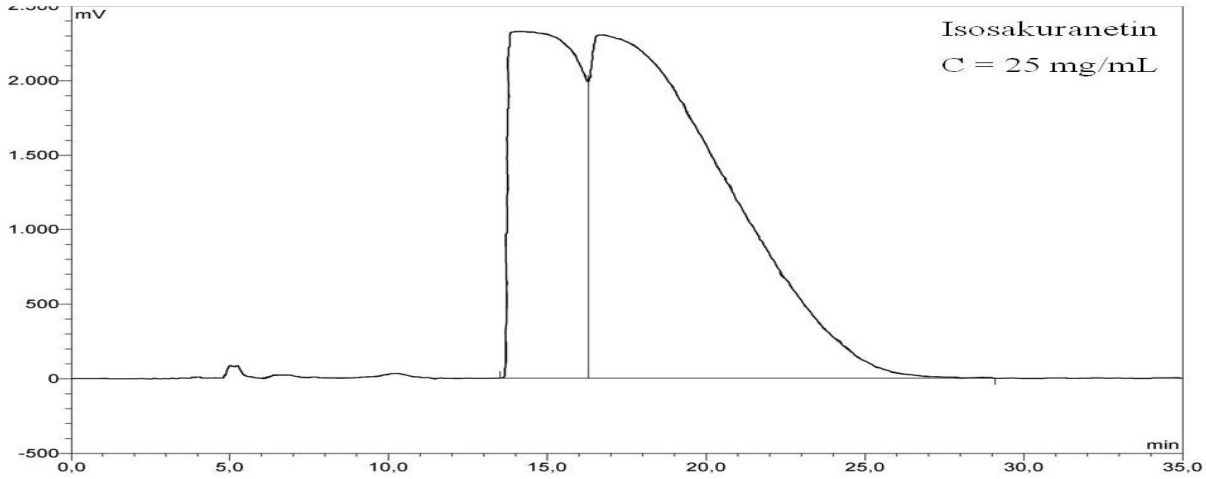
No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	16.86	(–) Isosakuranetin	342.69	394.75	49.05
2.	20.38	(+) Isosakuranetin	287.74	410.02	50.95
Total:			630.44	804.77	100.00

Figure 28. Chiral separation of isosakuranetin – standard at the concentration of 1 mg/mL, on the Europak column



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	15.52	(–) Isosakuranetin	2259.65	5046.54	42.83
2.	18.58	(+) Isosakuranetin	1977.63	6735.92	57.17
Total:			4237.28	11782.46	100.00

Figure 29. Chiral separation of isosakuranetin – standard at the concentration of 14,3 mg/mL, on the Europak column



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	14.04	(–) Isosakuranetin	2327.12	5755.14	33.47
2.	16.64	(+) Isosakuranetin	2304.28	11437.50	66.53
Total:			4631.40	17192.64	100.00

Figure 30. Chiral separation of isosakuranetin – standard at the concentration of 25 mg/mL, on the Europak column

Afterwards, we repeatedly injected 20 µL of around 15 mg/mL of every racemate, collected and combined fractions with the separated enantiomers. The other conditions were used the same as for the analytical separation, which are described in previous chapter (3.2.5.). After the separation, the enantiomers were evaporated to dryness at 40 ºC in vacuum and were stored at 0 ºC.

3.2.7. Circular dichroism conditions

Circular dichroism spectra (CD spectra) of the separated enantiomers dissolved in MeOH were obtained on a Jasco Spectropolarimeter J-710 CD (exact technical information in chapter 3.1.9.). The spectrum of between 200 and 400 nm was recorded at a temperature of 10 ºC using 1 mm quartz cell Suprasi. There have been 10 accumulation performed. Prior to the measurement, a spectrum of the MeOH was recorded, in which the enantiomers were dissolved. Thereby, the measured values of the enantiomers' solution were MeOH corrected.

3.2.8. Antimicrobial assay

3.2.8.1. Agar inhibition test for *Saccharomyces pasteurianus*

Pre-culturing

Bottom fermented yeast, strain 34/70, was syringed (using a sterile inoculation loop) out of a slope culture and placed into a sterilized 250 ml Erlenmeyer flask, then inoculated with 50 ml of wort, and sealed by cotton stuff. The aerobic culture was then stored in incubator for 24 h at 26 °C.

Determination of the cell count

The total cell count (living and died-off) in the pre-culture was determined microscopically with the help of the THOMA counting chamber. Before counting, the cell concentration of the suspension was estimated. Suspensions counting more than 3×10^8 cells/mL should be diluted prior the real counting. The THOMA counting chamber was filled the yeast suspension using a Pasteur pipette. The counting occurred over 16 small squares using Hellfeld microscope at 400-fold magnification. The counting should be repeated at least one-fold upon beginning.

The cell count (*Z*) was determined through the mean value according to the following equation (Eq.):

$$Z[\text{L/mL}] = \text{Total cell count} / 256 \times 4 \times 10^6 \times \text{dilution factor}$$

"*256*" is the number of small squares counted out and multiplies the measured chamber deepness (in μm).

Dilution and sample preparation

Depending on the cell count, the suspension was diluted with sterilized water until 10 and 10^2 cells/mL, and these series of dilution were then analyzed. For this, 1 mL of the diluted suspension was poured into Petri dishes and mixed with 1 mL of the substance (of each concentration and each flavonoid). Into the Petri dishes, a mixture of wort-gelatine as a nutrition medium was added, and the dishes were stored to breed for 4 days at 26 °C. Also blank sample was prepared for controlling purposes, which did not contain the substance but was prepared with 1 mL of DMSO as the solvent.

3.2.8.2. Turbidity inhibition test – Macrobroth dilution assay

Bacteria

Rehydration of dried cultures

The vacuum dried cultures of bacteria purchased from DSMZ Germany were first rehydrated (according to DSMZ instructions) and then were grown in Petri dishes on BHI-agar medium for 24 h at 30 °C or 37 ºC, respectively (see chapter 3.1.4.3). Thereafter they were stored at 4 ºC and prepared for analysis.

Pre-culture preparation of bacteria

In order to obtain the needed reproducibility, at first a pre-culture is prepared. The flasks were sterilized before the analysis in the autoclave at 121 ºC for 20 min and the media at 121 ºC for 15 min.

With the help of a sterile inoculating loop, the cell material is syringed out of the Petri dish, and then spiked into 100 mL of BHI medium (or BHI with glucose medium), which beforehand was placed into a 500 mL sterilized Erlenmeyer flask. The flasks were sealed using an aluminum foil. The aerobic culture was left over night to breed on a shaking platform at adequate temperature. The turbidity of the suspension (OD between 0.5–2) was measured

threefold, using a spectrophotometer (at 600 nm). Sterilized BHI culture medium served as blank sample.

Pre-culture preparation of yeast

In order to obtain the needed reproducibility, at first a pre-culture was prepared. For this, yeast cells (TUM collection) were syringed out of the slope culture using a sterile inoculation loop, placed into 50 mL of YNB culture medium, contained in a 250 mL Erlenmeyer flask, which was then closed with cotton stuff. The aerobic culture was left over night on the shaking platform (with 172 rpm), at 30 °C for breeding. The turbidity of the yeast suspension (with OD between 0.7–2) was measured threefold using a spectrophotometer (at 590 nm). Sterilized YNB medium was serving as a blank sample.

Preparation of main culture and the microbiological tests

The optical density of the pre-culture was measured and the volume of the pre-culture used for preparing the main culture (*V*) was calculated using the following Eq.:

$$V = \frac{50 \times 1}{OD - 1}$$

where *OD* is the optical density of the pre-culture.

The appropriate volume of pre-culture was mixed up until 50 mL with the freshly sterilized BHI medium (OD ≈ 0.1), with both being placed in a 500 mL Erlenmeyer flasks. As a control sample was served 50 mL of inoculated medium with 1 mL of water. The MeOH or DMSO samples were prepared by mixing 50 mL of inoculated medium with 1 mL of MeOH or DMSO, respectively. The test samples were prepared by mixing 50 mL of inoculated medium with addition 1.25, 2.5, 5, 10 and 20 mg of each substance dissolved in 1 mL of MeOH or DMSO. For obtaining the initial bacterial concentration, ODs were measured immediately after inoculation (marked as 0 h on the graph). All samples were cultivated on the shaking platform at temperature depending on the bacteria. Afterwards, 0.1 mL of samples were usually taken after every hour and diluted until 1 mL of the fresh medium. The growth of microorganisms was manifested by the turbidity of the suspension and was followed by measuring the OD using the spectrophotometer at 600 nm. The ODs of every bacteria suspension were compared to the pure liquid media.

The ODs of media containing substances were compared to the OD of the media containing only a solvent (MeOH or DMSO). Each test was run in triplicate and averages were calculated. The values obtained were taken for drawing the growth curves which then were compared to each other.

The antibacterial activity was defined as an average of percentage inhibition calculated by the following Eq.:

$$\text{Inhibition (\%)} = [OD_S - OD_X] / OD_S \times 100$$

where OD_S is the optical density of solvent at a certain time between second hour and achievement of the summit level, and OD_X is the optical density of sample at a certain time between second hour and achievement of the summit level. The OD results were expressed as means ± standard deviation (SD) of three parallel measurements.

Liquid micro-dilution technique – Micro-plate photometer test

The micro-plate photometer method was used to determine the antimicrobial activity of the substances and their enantiomers. The 96-well plates were filled with 276 µL of the growing culture (OD = 0.2) and mixed with 24 µL of various concentrations of the substances and their enantiomers. The control sample was prepared by addition 24 µL of water; the MeOH and DMSO sample was prepared by addition 24 µL of the solvent. The assay was performed in BHI Medium for every bacterium and YNB medium for the yeasts. The plates were incubated at specific, appropriate conditions (37 ºC for *E. coli*, *E. faecalis*, *P. aeruginosa*, and 30 ºC for *C. glutamicum*, *B. subtilis*, *M. luteus* and *S. cerevisiae*). After each hour, the main culture, prior to the measurement, was automatically shaken for 5 s, and the absorbance was read out at 590 nm. The plates were then agitated for 24 h. Each test was run in triplicate and averages were calculated. The values obtained were taken for drawing the growth curves which then were compared to each other.

The antibacterial activity was defined as an average of percentage inhibition calculated by the following Eq.:

$$\text{Inhibition (\%)} = [OD_S - OD_X] / OD_S \times 100$$

where OD_S is the optical density of solvent at a certain time between second hour and achievement of the summit level, and OD_X is the optical density of sample a the certain time between second hour and achievement of the summit level. The OD results were expressed as means ± standard deviation (SD) of three repetitions. Standard deviation calculations and graph design were carried out with Microsoft Excel.

4. Results

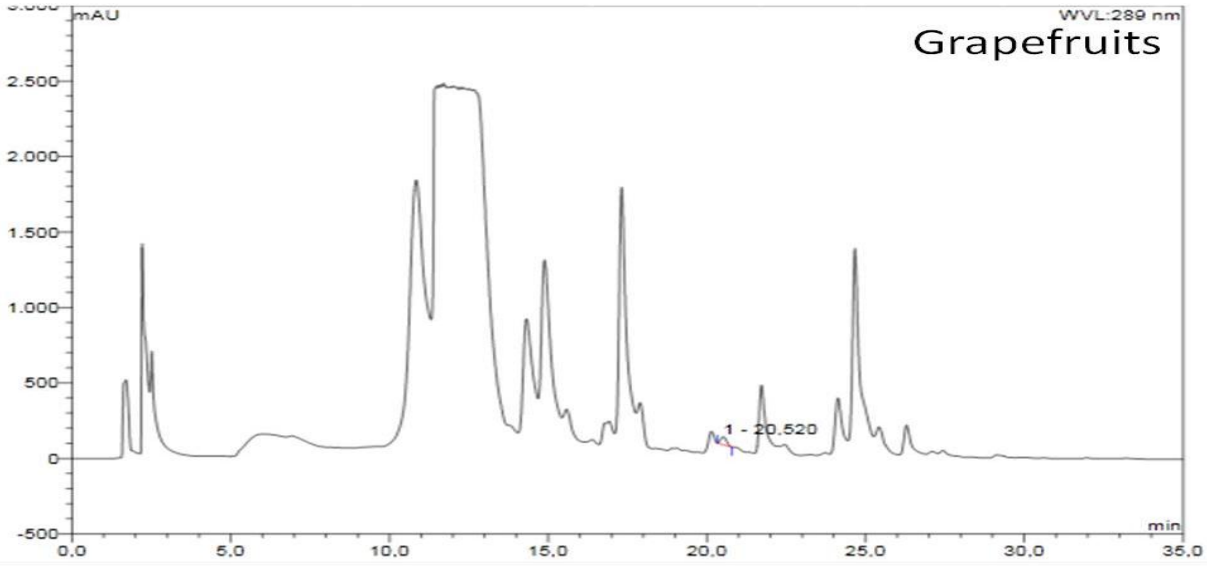
This chapter is divided into four parts as the main aims of the thesis. At first, the extraction of flavanones from naturally occurring plants is described. The second part is devoted to the optical activity of the substances, in which the results about analytical and preparative chiral separation and circular dichroism are demonstrated. The last two parts are dedicated to the antimicrobial activity of the flavanones. The penultimate shows the antimicrobial effects of the racemates and the last, one those, of their enantiomers.

4.1. Analytical characterization and quantification of extraction from plants

This subchapter presents results of the extraction of flavanones from various plants including citrus fruits (grapefruits, mandarins and oranges), tomatoes, thyme and peanut hulls. For the isolation of pure substances from crude extracts in this work, the HPLC device was utilized. The separations were carried out in reversed phase, which means, that we used non-polar stationary phase and polar mobile phase (mixture of ACN and 2% AAc., according to chapter 3.2.3). In every chromatogram, only the peaks of which the retention time corresponded to the retention times of flavanone standards used in this work was labeled. To confirm exactly the flavanone compound, mass spectroscopy was also utilized (exact information in the chapter 3.2.4.) and the corresponded m/z values to those of our flavanones are marked with red circles.

4.1.1. Extraction and identification of flavanone from grapefruits

Figure 31 shows the HPLC-chromatogram received from the crude extract from grapefruits peel. In this Figure there are more peaks, but only one of them, of which the retention time (20.52 min) was similar to the retention time of the naringenin standard, were marked, and analyzed in the MS as presented below.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	20.52	Naringenin	53.27	11.58	100.00
Total:			53.27	11.58	100.00

Figure 31. HPLC chromatogram of extraction of flavanones from grapefruit

The occurrence of naringenin in grapefruits peel was confirmed with the data obtained from the MS spectra. The MS spectrum of this peak (Fig. 32) showed the presence of m/z = 273 corresponding to this flavanone.

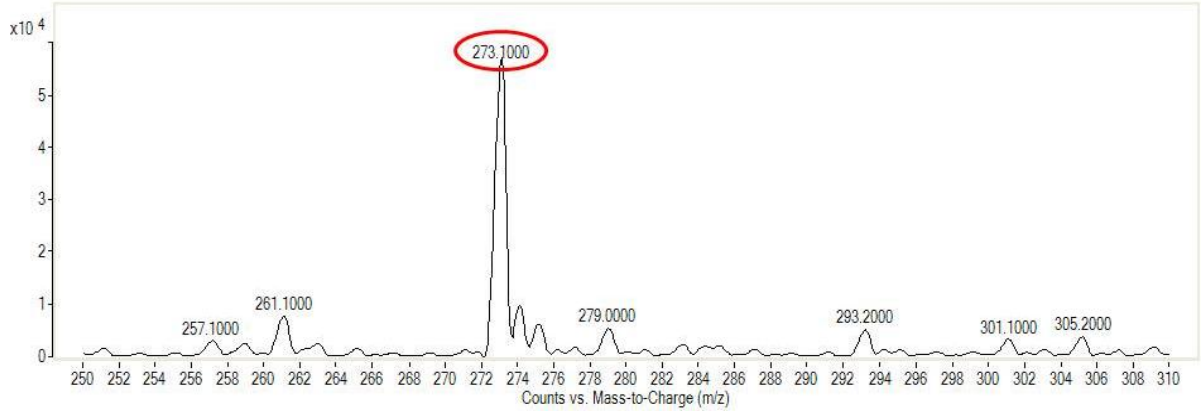
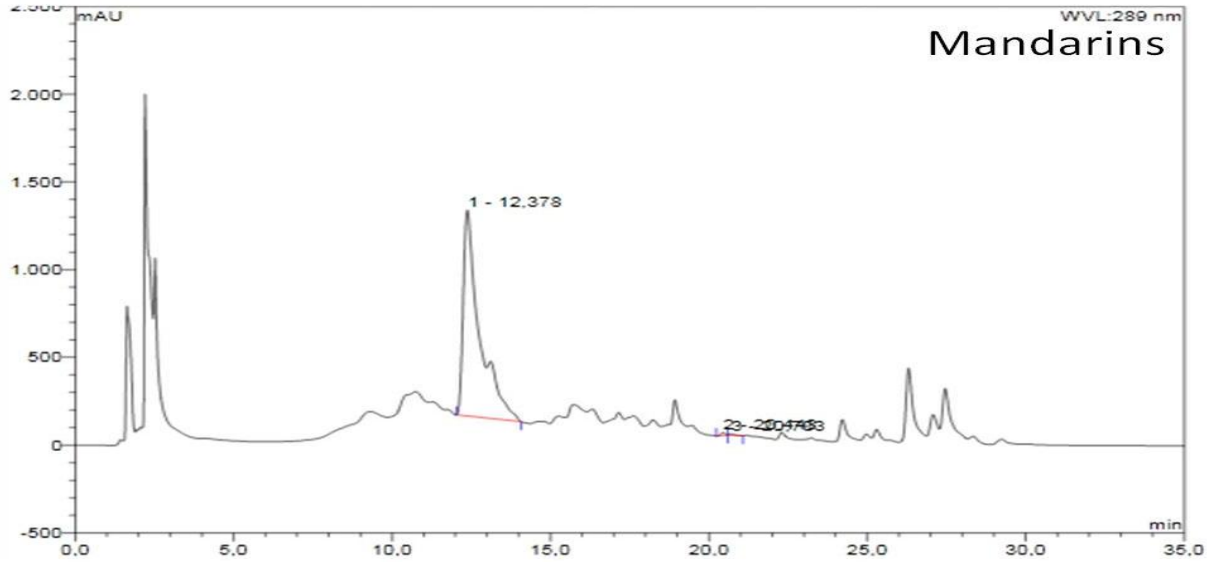


Figure 32. Mass spectrum of the peak with the retention time 20.52 min – extraction from grapefruits

4.1.2. Extraction and identification of flavanone from mandarins

The HPLC-chromatogram presented in Fig. 33 showed the presence of flavanones in mandarins peel. There was three retention times found that corresponded to the standards of homoeriodictyol, naringenin and hesperetin. The occurrence of the flavanones demonstrated by the presence of the peaks in this Figure was also confirmed with the data obtained from the MS spectra.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	12.38	Homoeriodictyol	1172.32	698.65	99.48
2.	20.45	Naringenin	14.81	2.45	0.35
3.	20.70	Hesperetin	7.18	1.21	0.17
Total:			1194.31	702.31	100.00

Figure 33. HPLC chromatogram of extraction of flavanones from mandarins

The first peak given in Figure 33 shows the similar retention time to this of the homoeriodictyol standard. This relationship is confirmed by the MS spectrum presented in Figure 34.

The occurrence of homoeriodictyol in mandarins peel was indicated by the presence of the peak with m/z 303.

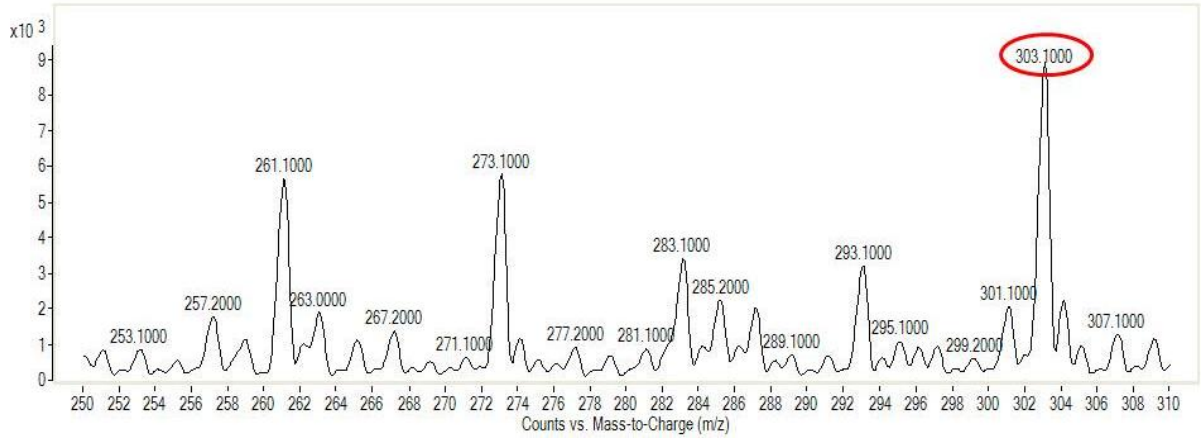


Figure 34. Mass spectrum of the peak with the retention time 12.38 min – extraction from mandarins

Figure 35 presents the MS spectrum which refers to the second peak with the retention time of 20.45 min, corresponding to naringenin. This MS spectrum indicates the occurrence of this compound in the peels of mandarin. Due to the very similar retention times of naringenin and hesperetin, the presence of m/z of hesperetin can also be observed (m/z 303).

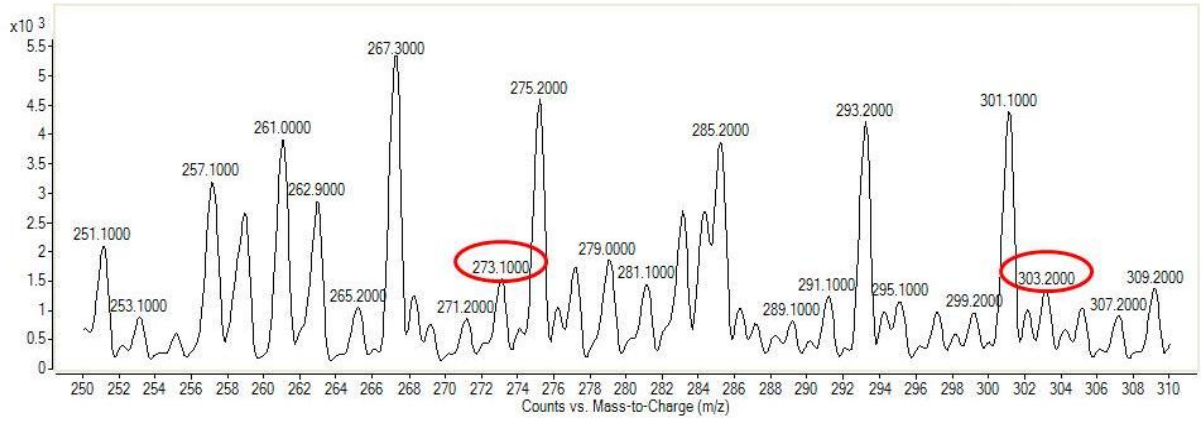


Figure 35. Mass spectrum of the peak with the retention time 20.45min – extraction from mandarins

The MS spectrum presented in Figure 36 is similar to the previous one, but with the difference of the intensity between naringenin and hesperetin. This Figure shows a little bit lower intensity of naringenin than this of hesperetin while the previous MS spectrum presented properly the higher content of naringenin.

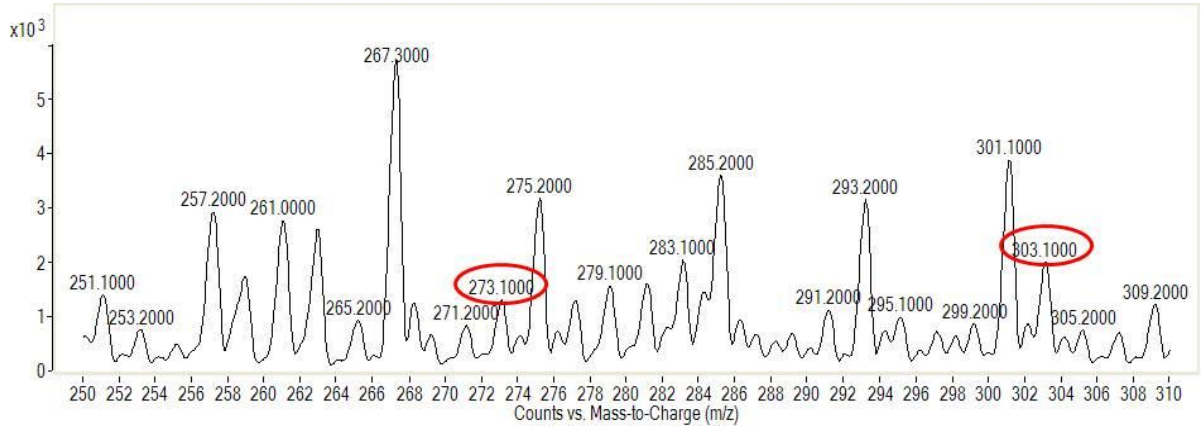
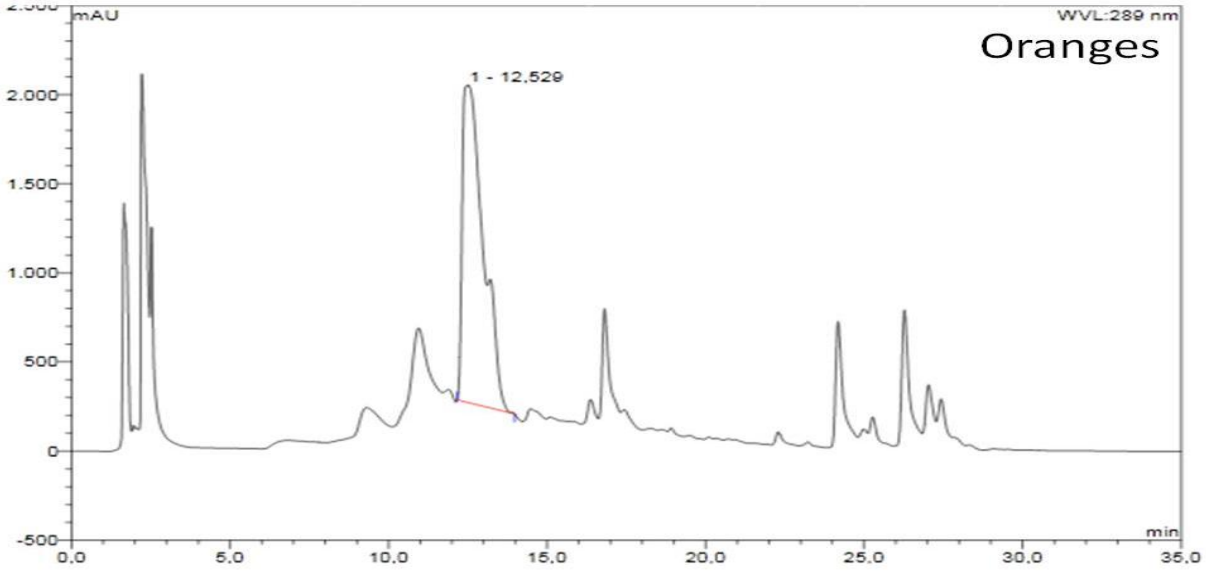


Figure 36. Mass spectrum of the peak with the retention time 20.70 min – extraction from mandarins

4.1.3. Extraction and identification of flavanone from oranges

Figure 37 shows the HPLC-chromatogram received from the crude extract from oranges peel. Only one peak with the retention time of 12.53 presented in the chromatogram corresponded to the flavanone standards and demonstrated the occurrence of homoeriodictyol.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	12.53	Homoeriodictyol	1782.50	1361.04	100.00
Total:			1782.50	1361.04	100.00

Figure 37. HPLC chromatogram of extraction of flavanone from oranges

The occurrence of homoeriodictyol in oranges peel is also confirmed by the MS spectrum of the peak with the retention time of 12.53 and the results are presented in Figure 38.

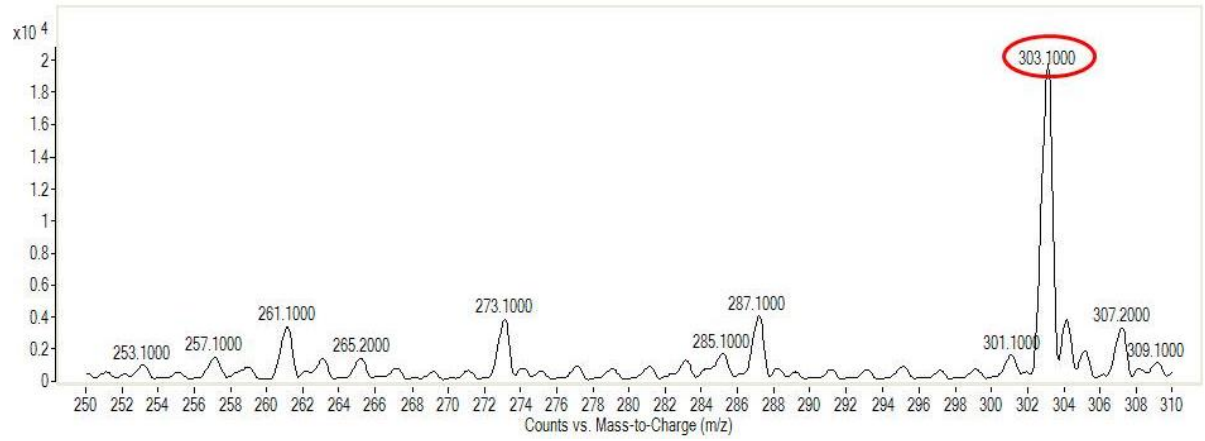
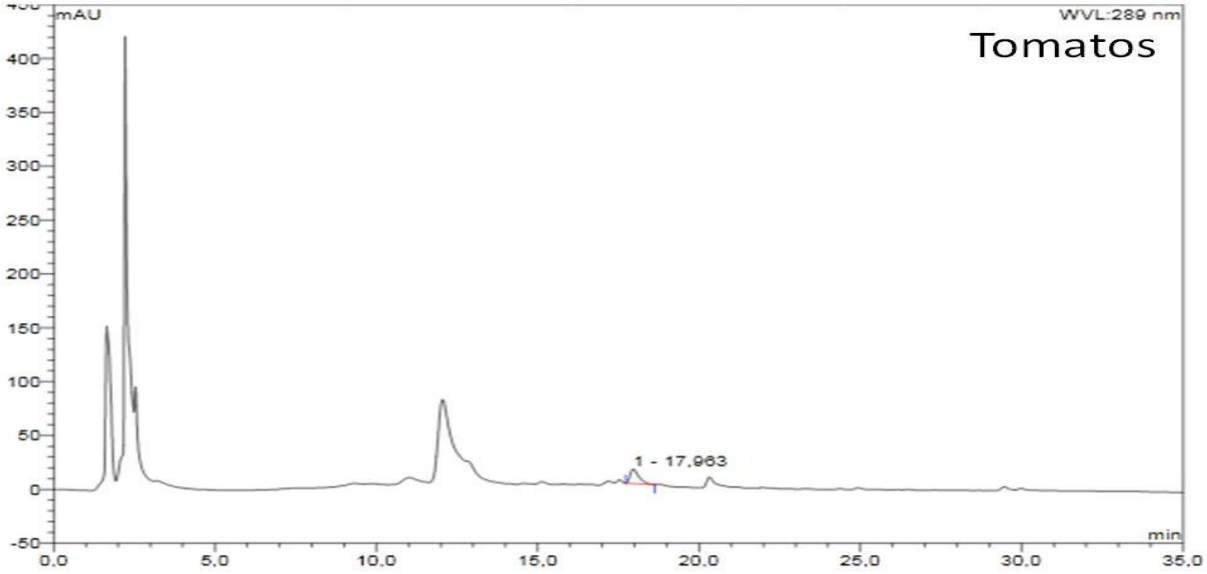


Figure 38. Mass spectrum of the peak with the retention time 12.53 min – extraction from oranges

4.1.4. Extraction and identification of flavanone from tomatoes

Figure 39 presents the HPLC chromatogram of flavanones contained in tomatoes. As before, we collected the peaks with the retention times similar to the retention times of our standards and checked the real presence of the substance by using MS.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	17.96	Naringenin	13.49	4.24	100.00
Total:			13.49	4.24	100.00

Figure 39. HPLC chromatogram of extraction of flavanone from tomatoes

The peak with a retention time of 17.96 min shown in Figure.39 referred to the occurrence of naringenin in tomatoes. Also this received HPLC peak was confirmed on the MS and the data are demonstrated in Figure 40. The MS spectrum confirms the occurrence of naringenin.

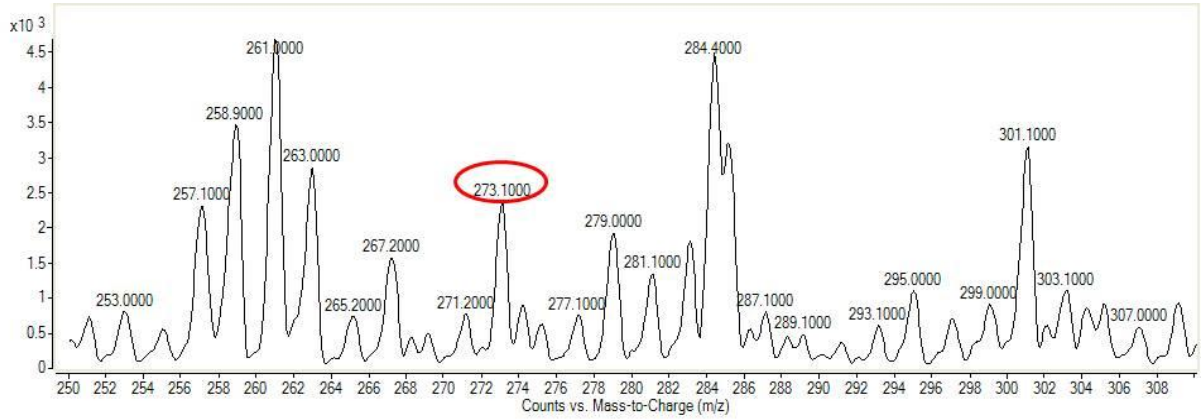
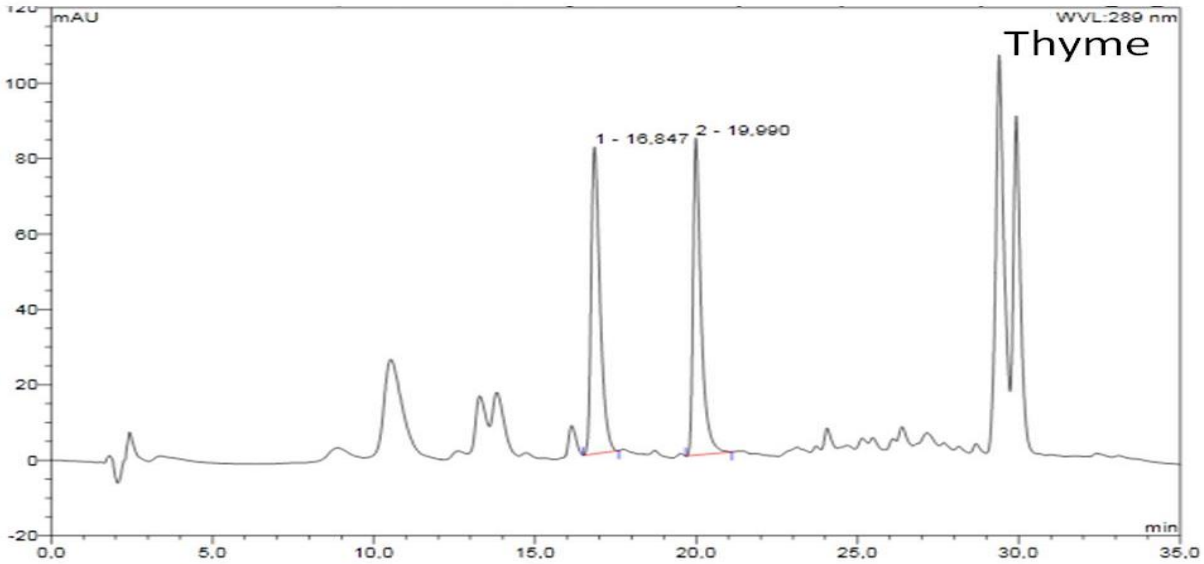


Figure 40. Mass spectrum of the peak with the retention time 17.96 min – extraction from tomatoes

4.1.5. Extraction and identification of flavanone from thyme

The data presented in Figure 41 show contents of flavanones in thyme. The received retention times were compared with the results received for the standards and the four peaks obtained were collected and analyzed afterwards by MS.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	16.85	Eriodictyol	81.35	25.82	51.73
2.	19.99	Naringenin	84.15	24.10	48.27
Total:			165.50	49.92	100.00

Figure 41. HPLC chromatogram of extraction of flavanone from thyme

The first peak from this chromatogram with the retention time of 16.85 min, without a doubt, corresponds to the retention time of eriodictyol and is also confirmed by the MS spectrum presented in Figure 42.

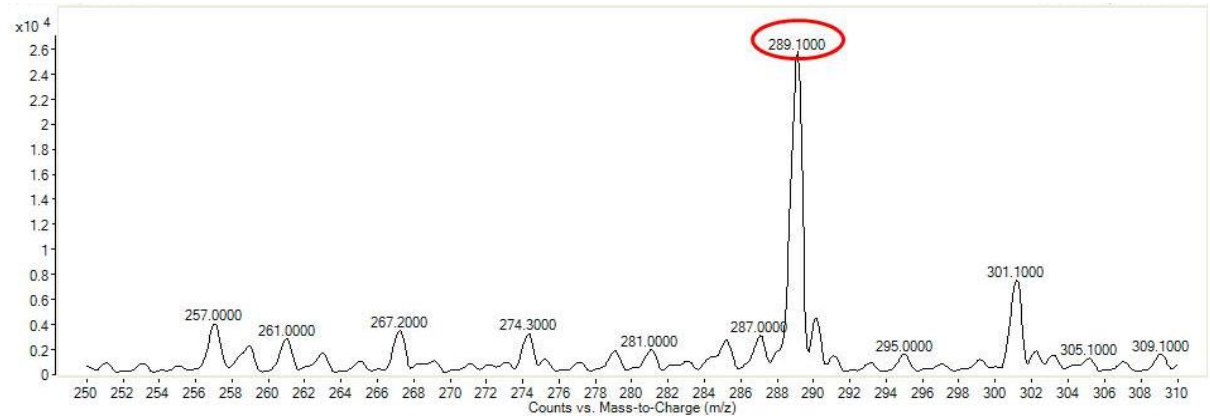


Figure 42. Mass spectrum of the peak with the retention time 16.85 min – extraction from thyme

Figure 43 shows the MS spectrum of the second peak from the chromatogram (Fig. 41) and confirms the presence of naringenin in thyme.

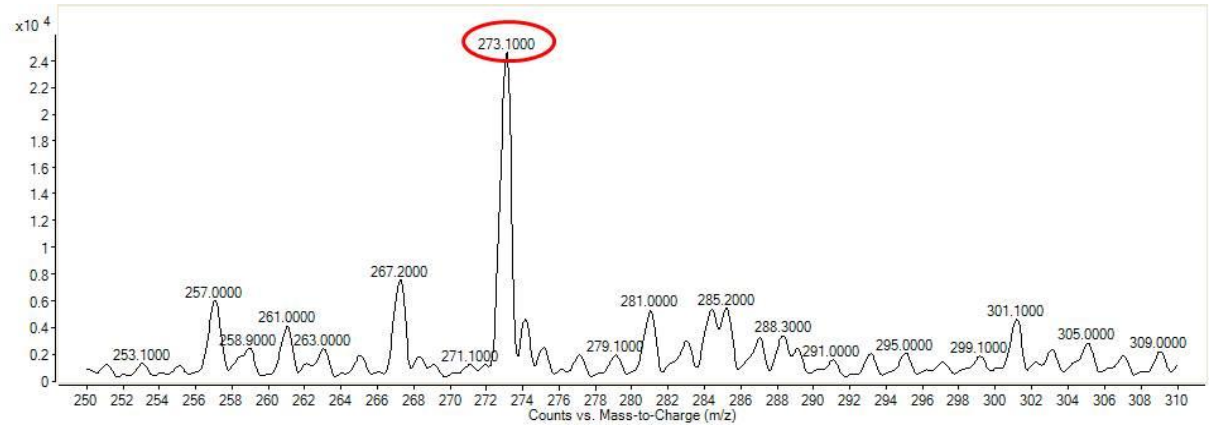
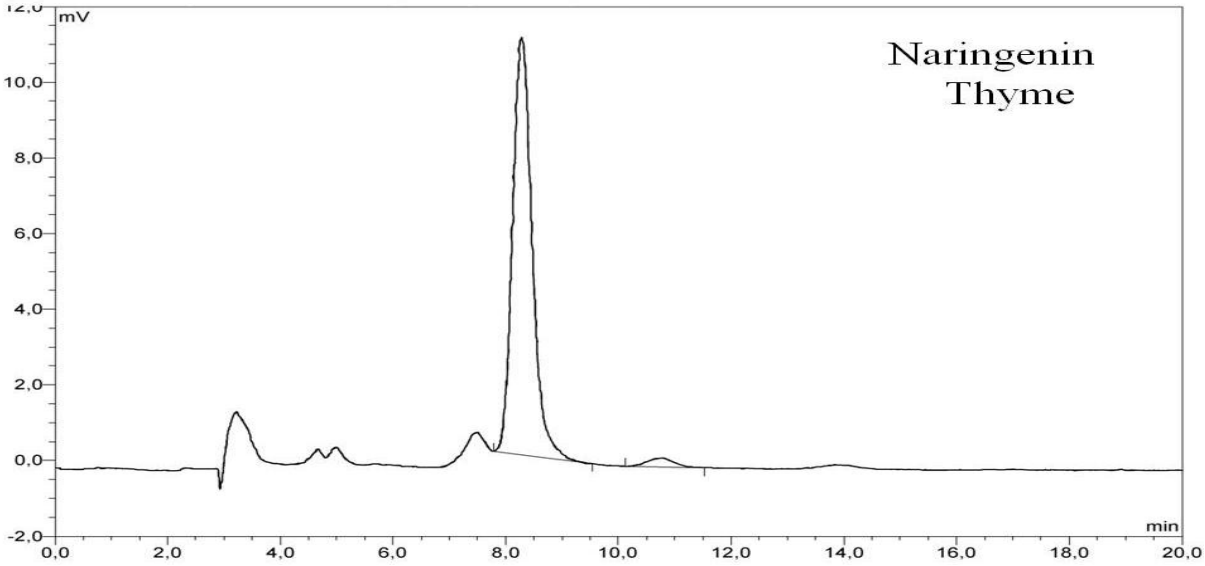


Figure 43. Mass spectrum of the peak with the retention time 19.90 min – extraction from thyme

4.1.5.1 Chiral separation of naringenin extracted from thyme

In Figure 44, the data of the chiral separation of naringenin extracted from thyme are shown. In the chromatogram is presented that the second peak is much bigger than the first one. It shows that the contents of (+) and (–) enantiomers in this plant are very different, because above 97% of naringenin from thyme extract fell to *S*-(–) enantiomer and only 2.9% to *R*-(+) naringenin.

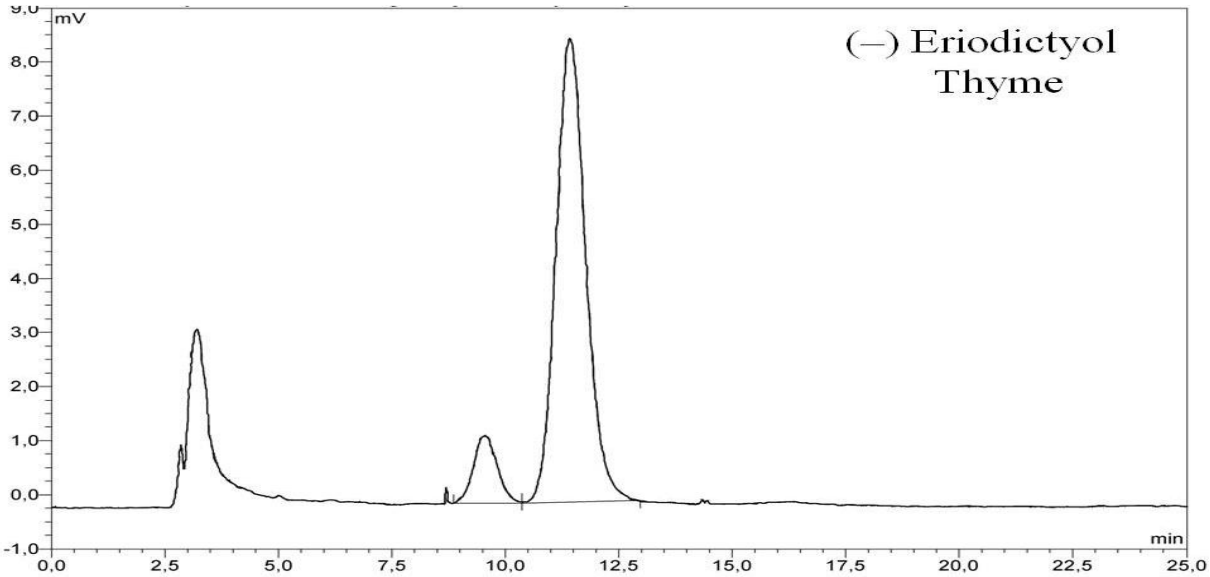


No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	8.28	(–) Naringenin	11.05	4.42	97.12
2.	10.72	(+) Naringenin	0.24	0.13	2.88
Total:			11.29	4.55	100.00

Figure 44. Chiral HPLC chromatogram of naringenin extracted from thyme

4.1.5.2. Chiral separation of eriodictyol extracted from thyme

Due to the high content of eriodictyol in thyme, we were able to test also in this case the differences in the content of enantiomers of this flavanone. Figure 45 shows that in thyme occurs almost 90% of *S*-(–) and only around 10% of *R*-(+) eriodictyol.

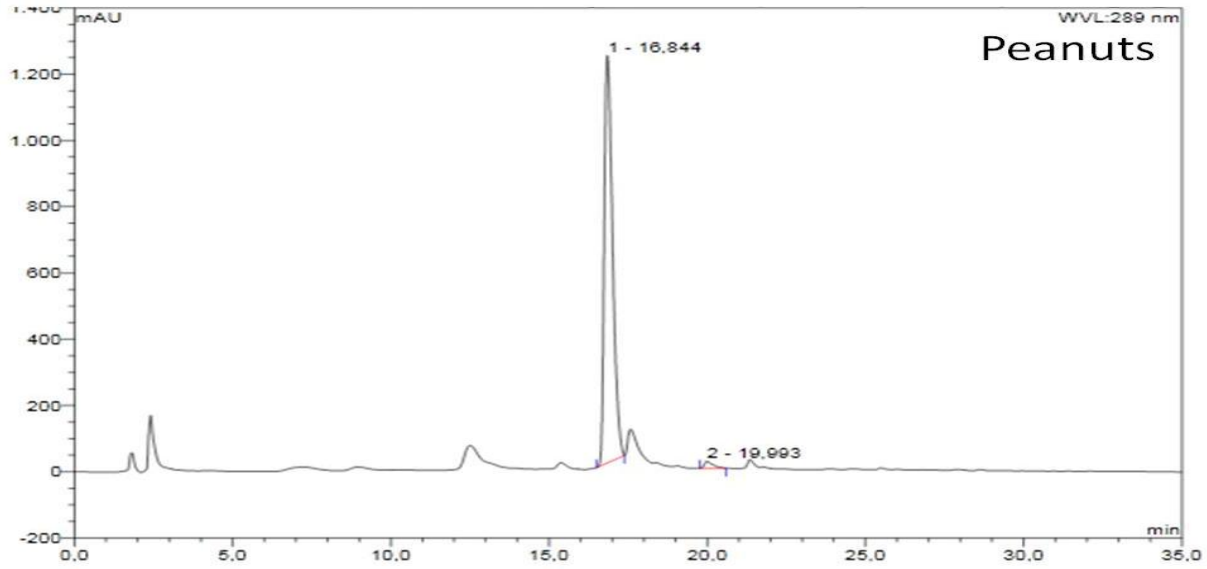


No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	9.55	(+) Eriodictyol	1.26	0.77	10.56
2.	11.42	(–) Eriodictyol	8.58	6.54	89.44
Total:			9.84	7.31	100.00

Figure 45. Chiral HPLC chromatogram of eriodictyol extracted from thyme

4.1.6. Extraction and identification of flavanones from peanut hulls

Figure 46 shows the occurrence of flavanones in peanut hulls. Based on the comparison of retention times it can be assumed that peanut hulls contain naringenin, and a high amount of eriodictyol. The peaks of individual flavanones were collected and confirmed by MS.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	16.84	Eriodictyol	1229.85	375.21	98.30
2.	19.99	Naringenin	20.58	6.47	1.70
Total:			1250.44	381.69	100.00

Figure 46. HPLC chromatogram of extraction of flavanone from peanut hulls (*Arachis hypogea*)

The first peak with the retention time of 16.84 min was analyzed by MS and the data are shown in Figure 47. The high intensity of the peak confirmed the high content of eriodictyol in peanut hulls (m/z 289).

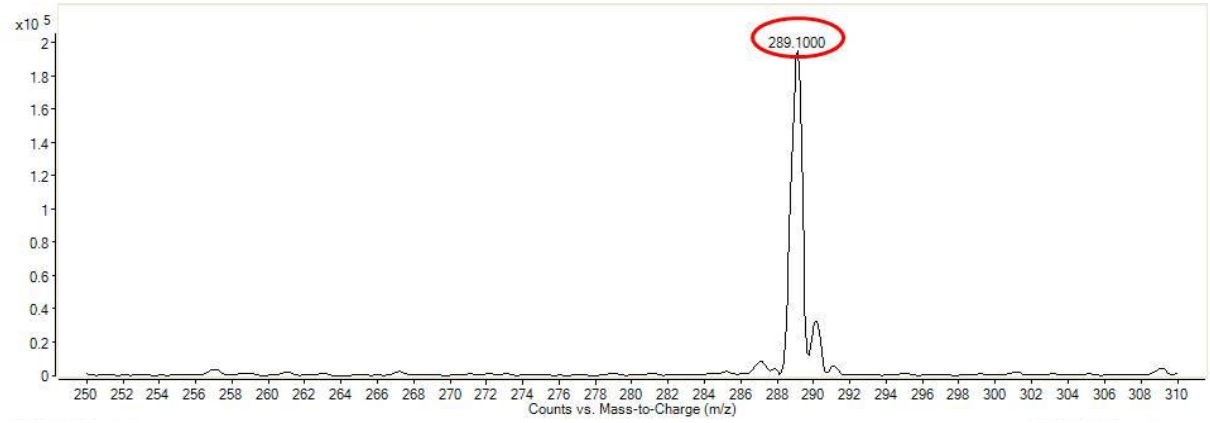


Figure 47. Mass spectrum of the peak with the retention time 16.84 min – extraction from peanut hulls

The second peak with the retention time of 19.99 min was also analysed by MS and confirmed the content of naringenin which provided the presence of the compounds with m/z 273 (Fig. 48).

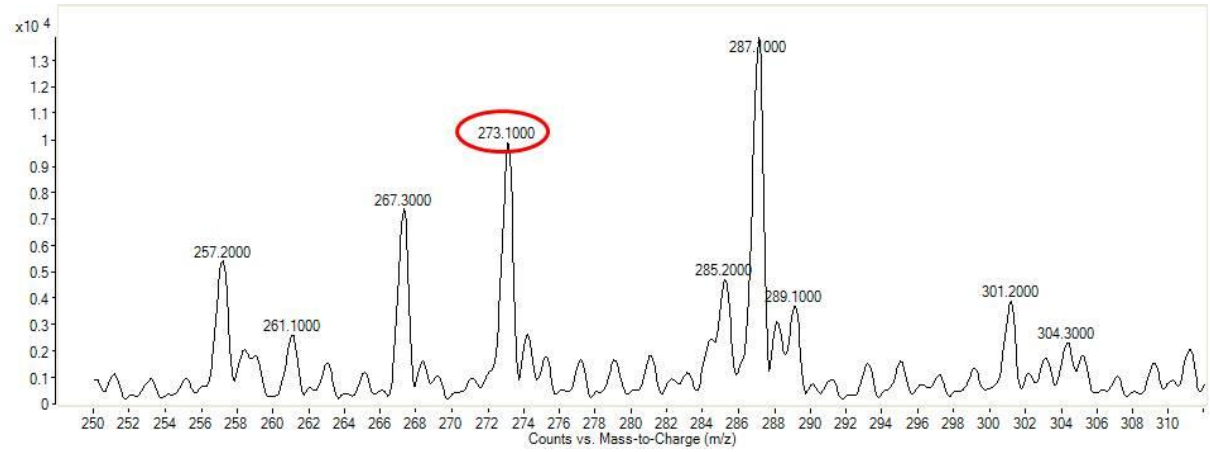
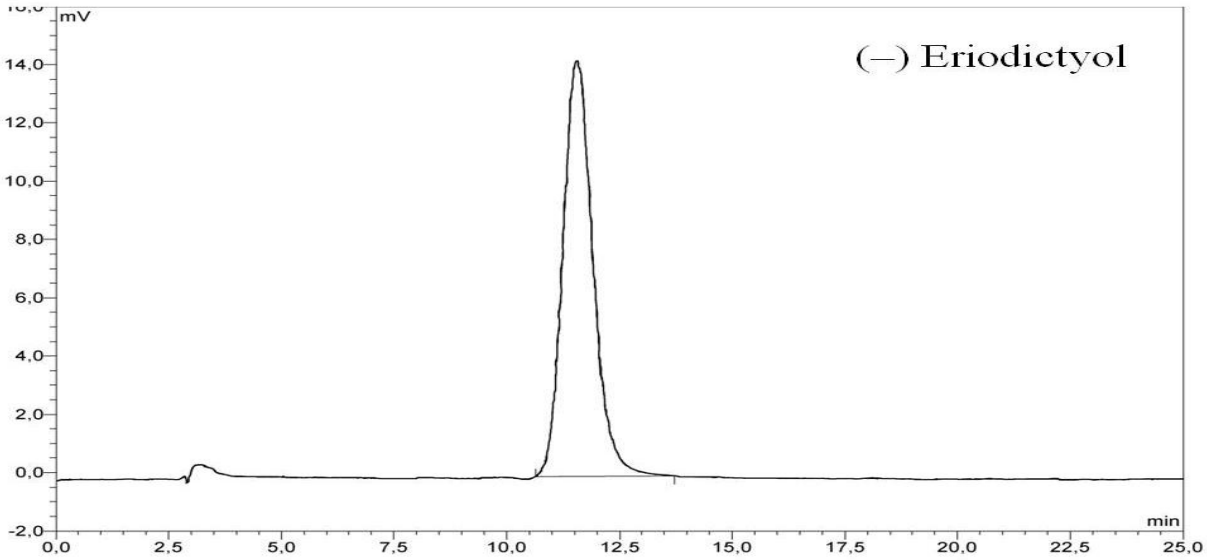


Figure 48. Mass spectrum of the peak with the retention time 19.99 min – extraction from peanut hulls

4.1.6.1. Chiral separation of eriodictyol extracted from peanut hulls

Figure 49 refers to the content of individual enantiomers of eriodictyol extracted from peanut hulls. It demonstrates that peanut hulls, only the *S*-(–) configuration of this flavanone was found. Afterwards, this (–) enantiomer was preparative extracted from the peanut hulls and used for further analysis.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	11.56	(–) Eriodictyol	14.28	11.02	100.00
Total:			14.28	11.02	100.00

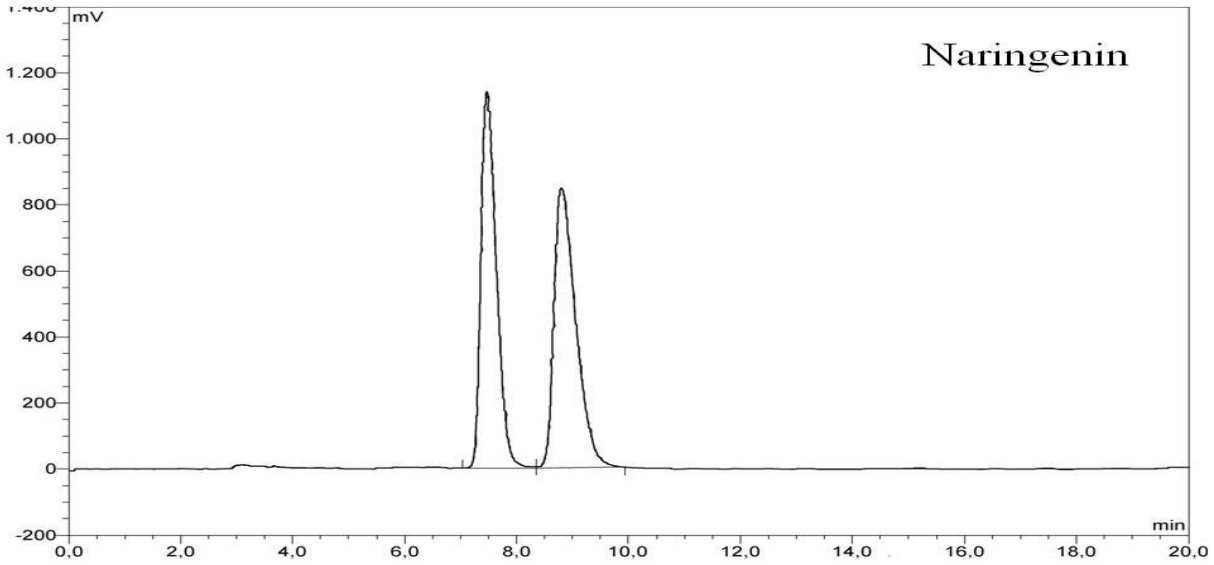
Figure 49 Chiral HPLC chromatogram of eriodictyol extracted from peanut hulls (*Arachis hypogea*)

4.2. Chiral separation and circular dichroism

The five analyzed flavanones, naringenin, isosakuranetin, eriodictyol, homoeriodictyol and hesperetin, possess one chiral center in the carbon atom on the second position (C-2) (Fig. 9, chapter 2.4). They are optical active and hence, their racemates consist of two enantiomers. The chiral separations of the substances were carried using HPLC with the chiral Europak column from Knauer (Germany) as it was described in chapter 3.2.5. The results are shown in HPLC chromatograms which also confirmed the presence of one chiral center in form of two peaks, as presented in the Figure 50. These two peaks correspond to the occurrence of two enantiomers, (+) and (–).

4.2.1. Naringenin – chiral activity

Figure 50 presents two peaks, with different retention times, 7.47 min for the first peak, assigned later as Peak 1, and 8.80 min for the second one, assigned as Peak 2. The occurrence of two peaks means that the purchased standard of naringenin was a racemate consisting of two enantiomers (+) and (–). However, the chromatogram does not conclude which peak is the *R*-(+) and *S*-(–) enantiomer. In order to obtain this information, it was necessary to analyze the optical activity of both peaks. The purchased racemate was separated preparative and both peaks were collected separately in flasks. Afterwards, they were analyzed on the spectrophotometer as explained chapter 3.2.6. The results of the optical activity of both naringenin peaks are shown in the CD spectrum (Fig. 51). The spectrum shows that the first peak, Peak 1 in the CD spectrum (black line) exposes a negative spectrum. It means that Peak 1 turned the polarized light towards left and, thus is the negative enantiomer, *S*-(–) naringenin. The second peak presents a positive spectrum and, thus corresponds to the positive enantiomer, *R*-(+) naringenin, which turns the polarized light to the right. Figure 50 shows as well that the second peak possesses a slightly larger area (below 51%) from the total real area. This on the other hand meant that the purchased standard was not a pure racemate and did not consist of 50% *R*-(+) and 50% of *S*-(–) configuration), but contained more (+) naringenin. However, the green line in the middle of the CD spectrum (baseline) corresponds to the optical activity of the racemate of naringenin and oscillated around zero. Thereby, the small domination of the *R*-(+) enantiomer showed no influence on the optical activity of the racemate.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	7.47	(–) Naringenin	1141.27	362.36	48.79
2.	8.80	(+) Naringenin	848.28	380.29	51.21
Total:			1989.55	742.65	100.00

Figure 50. Chiral separation of naringenin using HPLC with the chiral column, Europak

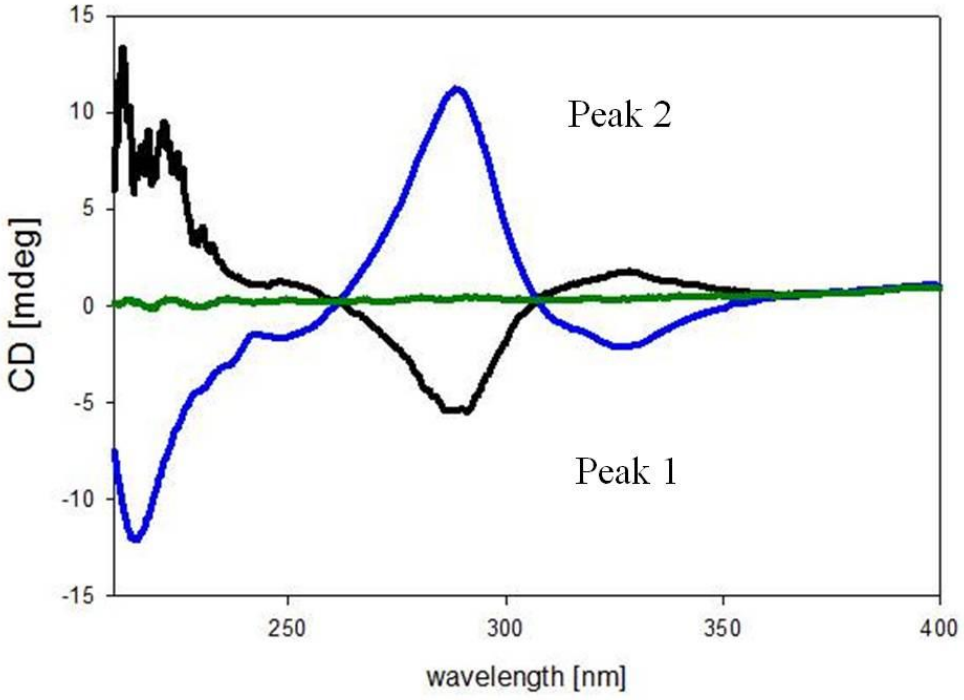
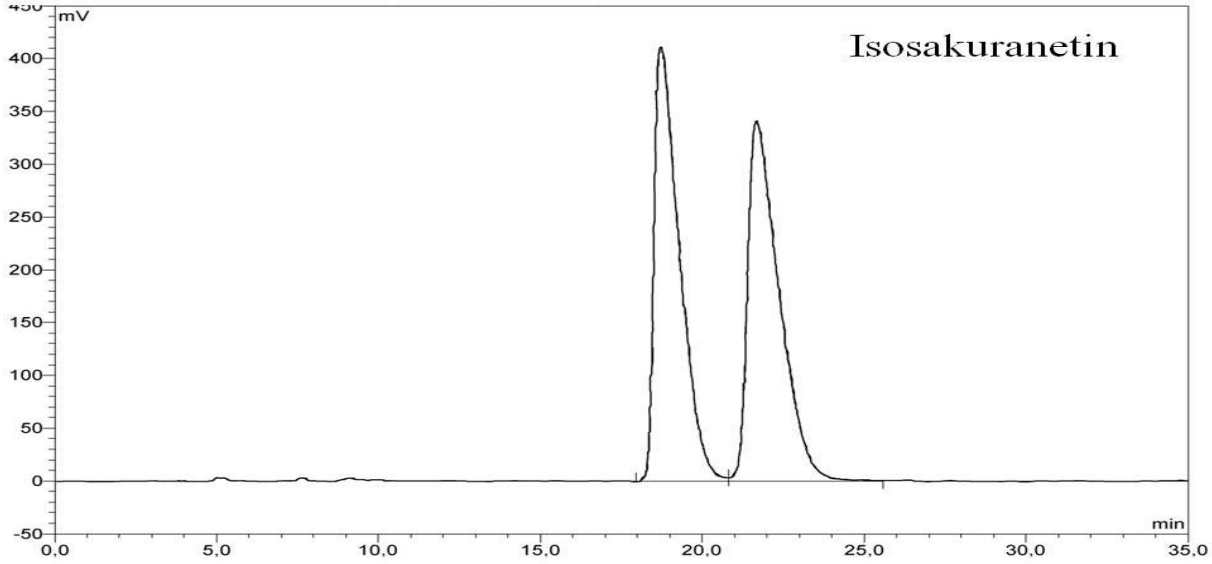


Figure 51. Spectrum of circular dichroism of naringenin

4.2.2. Isosakuranetin– chiral activity

The results of the chiral separation of isosakuranetin in gave also two peaks with a very small dominance of the second peak (49% : 51% ratio) (Fig. 52). There was no significant difference in the CD spectrum of the racemate of isosakuranetin (green line) (Fig. 53). The first peak (black line) is presented in the negative area of optical activity and corresponds to *S*-(–) isosakuranetin. The second peak, as the *R*-(+) enantiomer turned the polarized light to the right and exposes the positive spectrum (red line).



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	18.72	(–) Isosakuranetin	411.34	371.16	49.26
2.	21.68	(+) Isosakuranetin	341.32	382.25	50.74
Total:			752.66	753.40	100.00

Figure 52. Chiral separation of isosakuranetin using HPLC with the chiral column, Europak

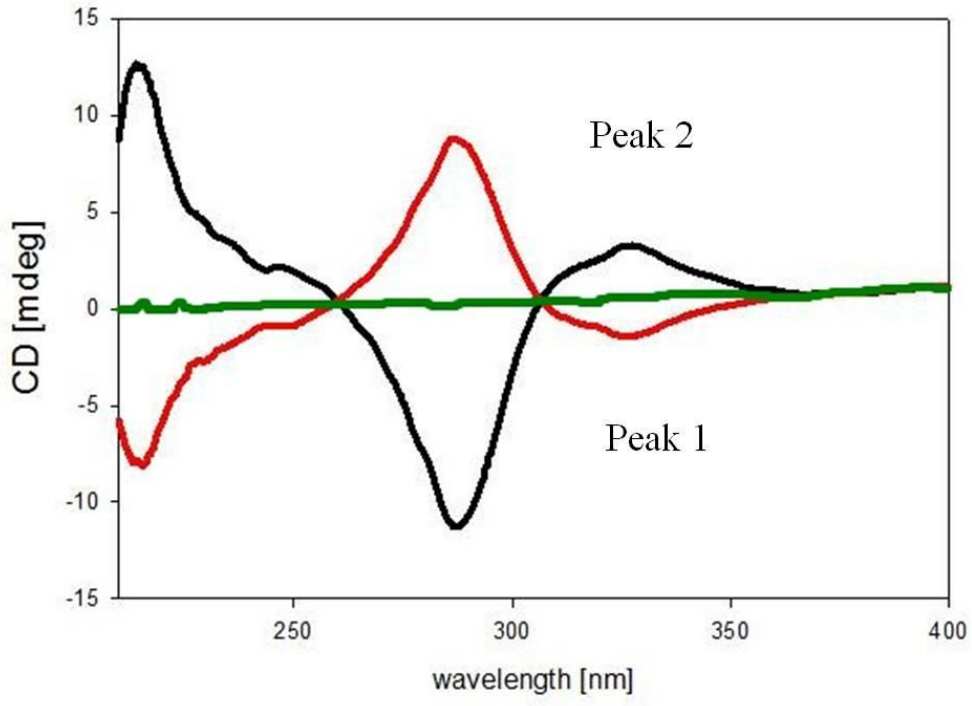
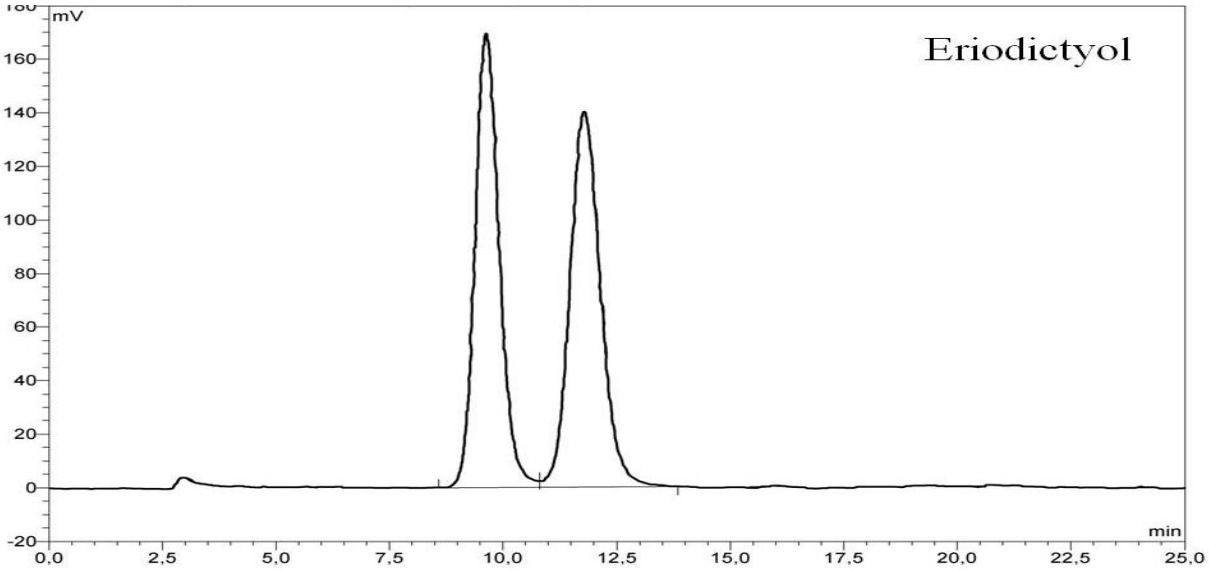


Figure 53. Spectrum circular dichroism of isosakuranetin

4.2.3. Eriodictyol – chiral activity

Eriodictyol, as a member of the family of flavanone, exhibited also optical activity with one chiral center (Fig. 54). However, in comparison with naringenin and isosakuranetin there are some differences. The change was already observed at chiral separation. It was necessary to add 5% of water to the mobile phase which consisted of MeOH. Besides, Figure 55 shows that the second peak corresponds to the negative, and not the positive spectrum, as it was for the previous described flavanones. In this case, Peak 2 turned the polarized light to the left and occurred as *S*-(–) eriodictyol, while Peak 2 of naringenin and isosakuranetin showed the opposite turns and corresponded to *R*-(+) enantiomers. Also this time, Figure 54 shows a small dominance of (–) eriodictyol (by 51%) in the purchased standard. However, there are no changes observable in the CD spectrum of the racemate (red line) (Fig. 55). Unfortunately, the (+) eriodictyol was not available for the analyses.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	9.63	(+) Eriodictyol	169.70	105.18	49.04
2.	11.79	(–) Eriodictyol	140.31	109.30	50.96
Total:			310.01	214.48	100.00

Figure 54. Chiral separation of eriodictyol using HPLC with the chiral column, Europak

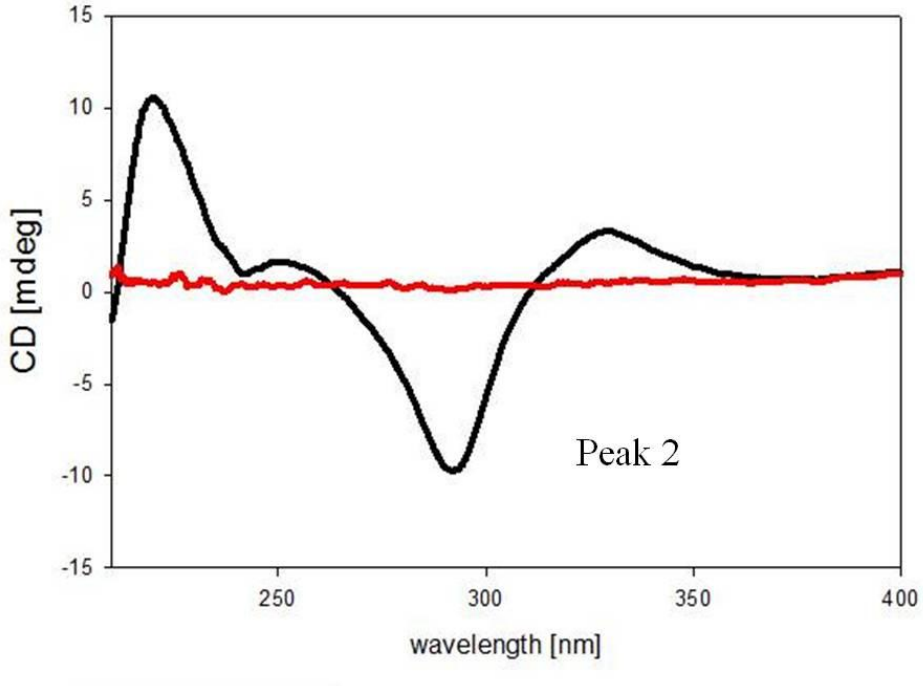
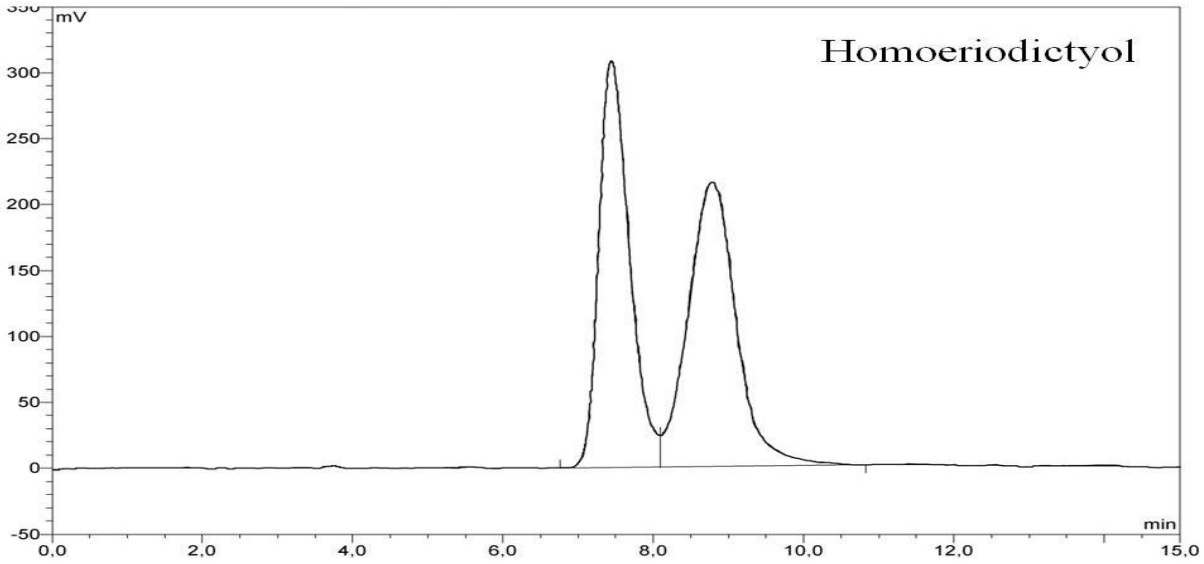


Figure 55. Spectrum of circular dichroism of eriodictyol

4.2.4. Homoeriodictyol – chiral activity

The structure of homoeriodictyol is similar to this of eriodictyol and exhibits also similar properties in the discussed analysis. Figure 56 exposes enantio-separation of homoeriodictyol. The second peak with the larger real area (52%) corresponds to the *S*-(–) homoeriodictyol (Fig. 57). Here as well, we did not observe any difference in the CD spectrum of the racemate, that could be caused by the domination of (–) homoeriodictyol. Similar to the eriodictyol, *R*-(+) homoeriodictyol could not be analyzed too.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	7.44	(+) Homoeriodictyol	308.57	144.96	47.64
2.	8.78	(–) Homoeriodictyol	215.79	159.33	52.36
Total:			524.36	304.30	100.00

Figure 56. Chiral separation of homoeriodictyol using HPLC with the chiral column, Europak

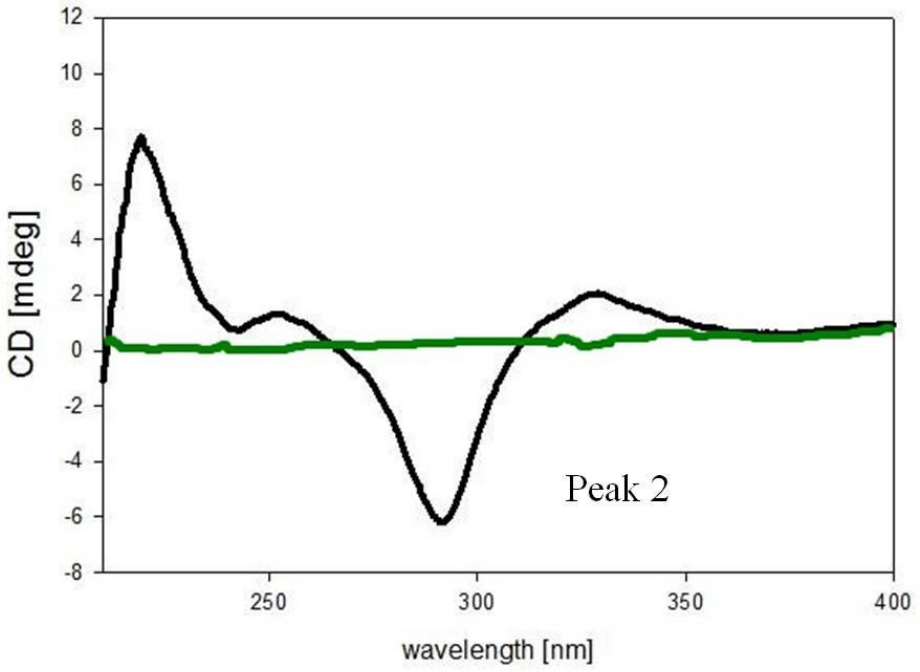
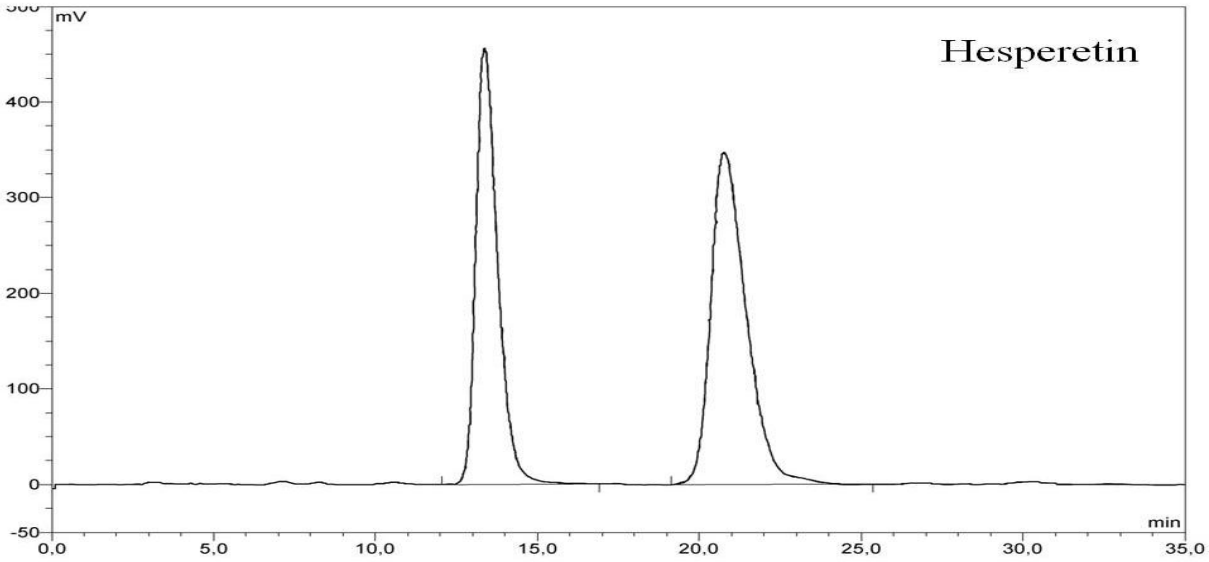


Figure 57. Spectrum of circular dichroism of homoeriodictyol

4.2.5. Hesperetin – chiral activity

Hesperetin was separated in this work only in analytical form (Fig. 58) and none of its enantiomers were analyzed. The chromatogram of hesperetin shows the biggest difference between Peak 1 and Peak 2 compared to all analyzed flavanones with areas amounting to 45% and 55% of the real area.



No.	Retention Time [min]	Peak Name	Height [mV]	Area [mV*min]	Real Area [%]
1.	13.36	(+) Hesperetin	456.50	352.83	44.57
2.	20.77	(–) Hesperetin	347.68	438.72	55.43
Total:			804.18	791.55	100.00

Figure 58. Chiral separation of hesperetin using HPLC with the chiral column, Europak

4.3. Antimicrobial activity of analyzed racemates

The second main aim of this thesis was to demonstrate antimicrobial activities of the naturally occurring substances. These antimicrobial effects were studied by carrying out two microbiological methods, including agar and liquid dilution technique. This subchapter is divided between the results of the two methods and describes the antimicrobial activities of the chosen flavanones against eight various microorganisms, including pathogens, that are important for the food industry.

4.3.1. Agar dilution technique

The agar dilution technique was carried out by using Petri dishes and the growth of the *S. pasteurianus* with the addition of various concentrations of flavanones was compared with the growth of a control sample (without any substances). As an example, in Table 2 are shown the results of the antimicrobial activity of naringenin against *S. pasteurianus*. Each test was performed six times and the averages of the colony forming units (cfu) and standard deviations (SD) are presented.

The first test (No. 1. in the Table) exhibits a control sample which consisted of between 126 and 132 cfu per Petri dish ($1{:}10^5$ dilution serie). The sample with addition of 0.5 mg/mL of naringenin exposed a range between 72 and 156 cfu per Petri dish, while the presence of 1 mg/mL of naringenin showed significant antimicrobial activity and the values are between 29 and 73 cfu per Petri dish. The second test (No. 2) that was carried out in the same way as the previous one and showed totally different results. The control sample showed between 177 and 283 cfu per Petri dish, the sample with addition of 0.5 mg/mL of naringenin, between 198 and 254 cfu, and the sample which contained 1 mg/mL of the flavanone presented the growth between 191 and 223 cfu/Petri dish. It is clear to see that there was no difference between the control sample and the samples containing naringenin. Both tests, No. 1 and No. 2, were not compatible. The test No. 3 showed similar results to the test No. 2. However, in every test the SD was very high. Due to the particular results from the individual tests that were unfortunately fairly inconclusive, this method was not used for the further analyses. The results with the other substance were also incompatible and, hence, are not presented in this thesis.

Table 2. Inhibitory effect of naringenin against *S. pasteurianus* using the agar dilution technique; AV – average, SD – standard deviation

No.	Dilution Series	Number of colonies in Petri dish [cfu]						
		Control		DMSO	Naringenin			
					0.5 mg/mL		1 mg/mL	
		AV ± SD	Area	AV	AV ± SD	Area	AV ± SD	Area
1.	$1 : 10^5$	129 ± 2.19%	126 - 132	123	114 ± 36.7%	72 - 156	51 ± 43.4%	29 - 73
	$1 : 10^6$	7 ± 30.3%	5 - 9	8	16 ± 9.55%	14 - 18	10 ± 47.3%	5 - 15
2.	$1 : 10^5$	230 ± 23%	177 - 283	245	226 ± 12.6%	198 - 254	207 ± 7.8%	191 - 223
	$1 : 10^6$	15 ± 18.9%	12 - 18	14	21 ± 20.8%	17 - 25	21 ± 20.8%	17 - 25
3.	$1 : 10^5$	192 ± 6.6%	179 - 205	186	199 ± 6%	187 - 211	188 ± 8%	173 - 20
	$1 : 10^6$	17 ± 22%	13 - 21	12	19 ± 30%	13 - 25	13 ± 25%	10 - 16

4.3.2. The liquid dilution technique - turbidity test

The turbidity was taken as an indicator of bacterial density. Changes of turbidity by the growth of every microorganism can spectrophotometrically be measured and afterwards recorded by following the bacteria growth with time in form of growth curve. It corresponds to the main principle of another antimicrobial method, called liquid dilution technique. These analyses were carried out according to the macro-dilution technique on the BHI medium for every bacterium and to the micro-dilution technique on the YNB medium for the yeast. The tests, depending on the growth rate of the microorganisms, lasted 8, 10 or 24 h (chapter 3.2.8.2). In every case the growth curves were compared with those obtained in a medium without flavanones, but containing MeOH or DMSO.

4.3.2.1. Naringenin

To demonstrate the main data about the growth curve, the graph of activity of naringenin against *B. subtilis* ATCC 6633 was chosen. Similar graphs and tables relate to every substance and every microorganism investigated in this work and have all been made available in the Annex (chapter 9).

In Figure 59, eight growth curves are shown, which correspond to the control sample (blue line), the sample containing a solvent (in this case MeOH, pink curve), tetracycline as

an antibiotic (orange line), and samples with six various concentrations of naringenin. The measurement of turbidity shows very clearly, which antimicrobial activity the solvent and every concentration of naringenin possesses against *B. subtilis*. The MeOH solvent showed a small inhibitory effect against this bacterium. The concentration of 0.025 mg/mL of naringenin (green line in the graph) presents only a slight drop of the growth curve between 2 and 5 h in comparison to the activity of MeOH. Afterwards, this concentration of the substance showed the same activity as the sample containing the solvent and reached OD 6.5. The increase of the concentration of the substance caused a rise of the inhibitory effect of naringenin. The concentration of 0.05 mg/mL of the flavanone showed lower turbidity during 8 h than the previous concentration and amounted to 5.7. The highest increase of the growth inhibition of *B. subtilis* was observed between the concentrations of 0.05 and 0.1 mg/mL. Without a doubt, the best antimicrobial activity of naringenin against *B. subtilis* was observed in the sample with the highest content of this flavanone, 0.4 mg/mL, and the OD in the last hour amounted only to 1.05. It suggests that there was no growth recorded and the inhibitory capacity was compatible to the activity of the antibiotic tetracycline at the concentration of 0.2 mg/mL.

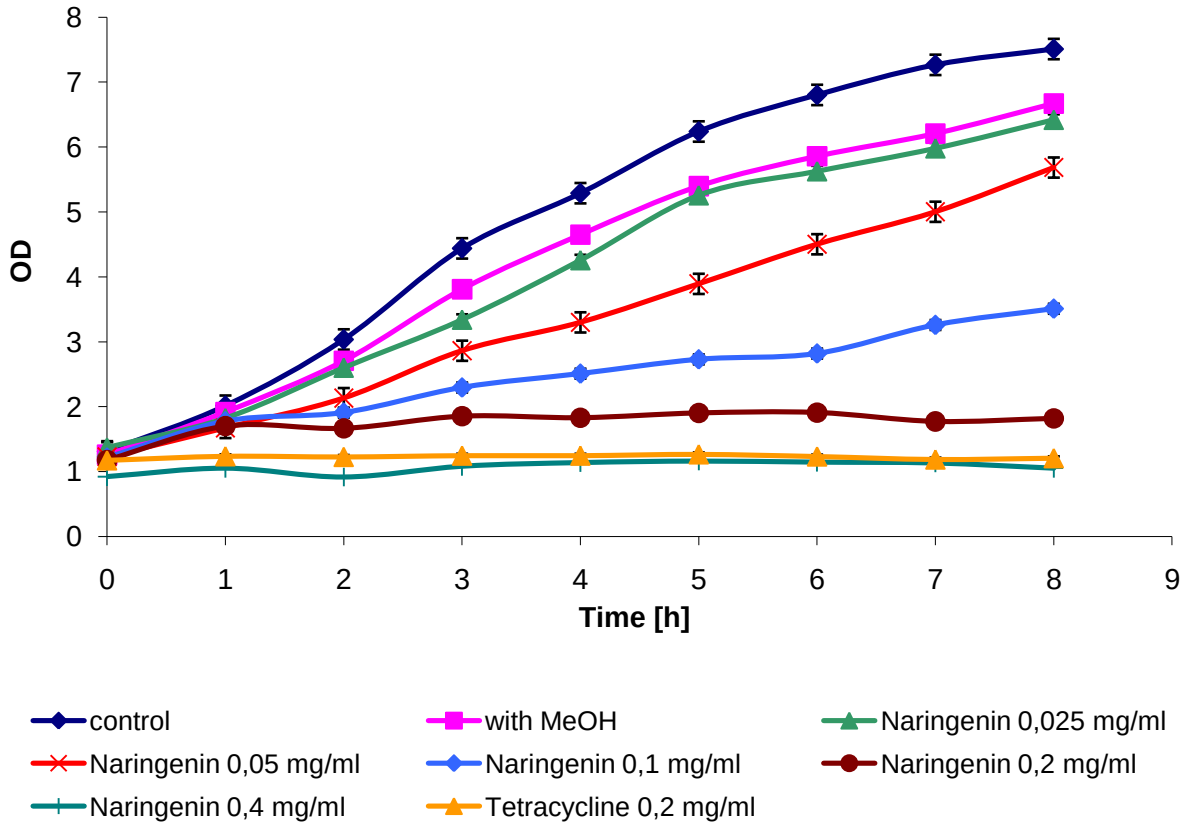
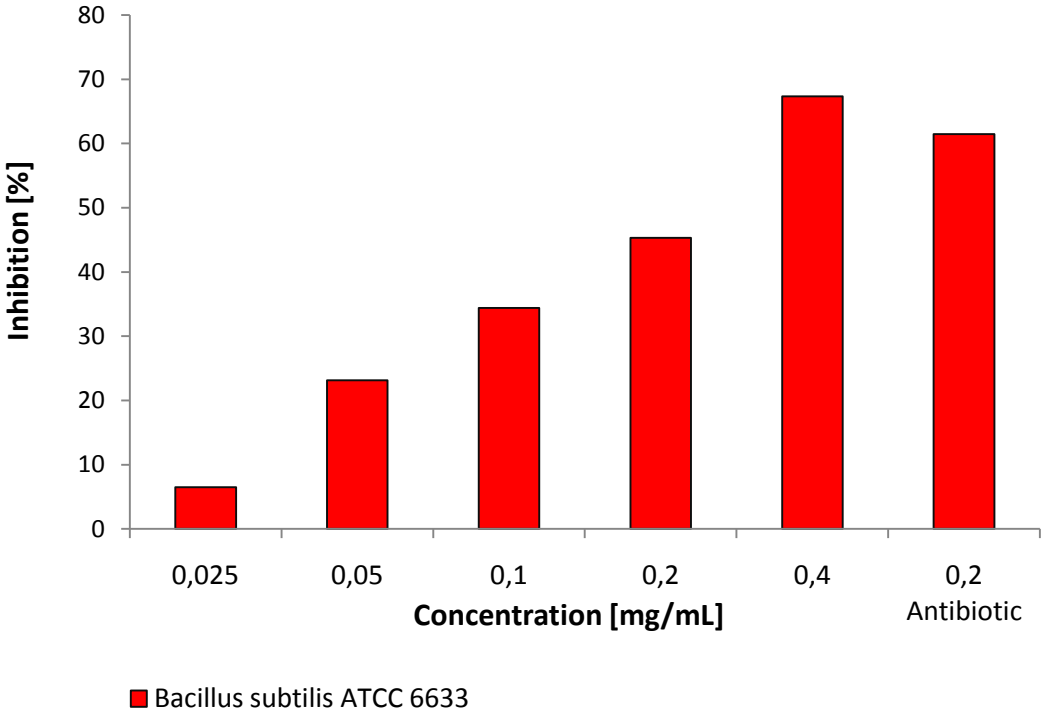


Figure 59. Growth curves of *B. subtilis* ATCC 6633 with inhibitory effect of methanol (MeOH) and various concentration of naringenin; OD – optical density

Table 3. Growth data of *B. subtilis* with presences of methanol (MeOH), tetracycline and various concentration of naringenin; OD – optical density, SD – standard deviation

Time [h]	OD							
	Control	MeOH	Tetracycline 0.2 mg/mL	Naringenin				
				0.025mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.31	1.26	1.17	1.37	1.23	1.22	1.18	0.92
1	2.02	1.92	1.24	1.82	1.67	1.78	1.70	1.05
2	3.04	2.71	1.23	2.60	2.13	1.91	1.67	0.92
3	4.44	3.81	1.25	3.34	2.86	2.30	1.85	1.08
4	5.29	4.65	1.25	4.26	3.30	2.51	1.83	1.14
5	6.24	5.40	1.27	5.26	3.89	2.73	1.90	1.16
6	6.80	5.86	1.23	5.63	4.50	2.82	1.91	1.15
7	7.27	6.21	1.19	5.98	5.00	3.26	1.77	1.13
8	7.51	6.67	1.21	6.42	5.69	3.51	1.82	1.05
SD	0,15699779	0,10953883	0,030295216	0,0845851	0,15546267	0,076341864	0,02809366	0,03662827

The next graph (Fig 60) shows the growth inhibitory effect of naringenin against *B. subtilis*, in percentage. This figure presents clearly the linear increase of the inhibitory effect of naringenin driven by the linear increase of the concentration of the substance in the samples. The content of 0.2 mg/mL of the analyzed substance was not as active as 0.2 mg/mL of the commonly known antibiotic, tetracycline. However, the concentration of 0.2 mg/mL of tetracycline showed, in this case, lower inhibitory effect than 0.4 mg/mL of naringenin.



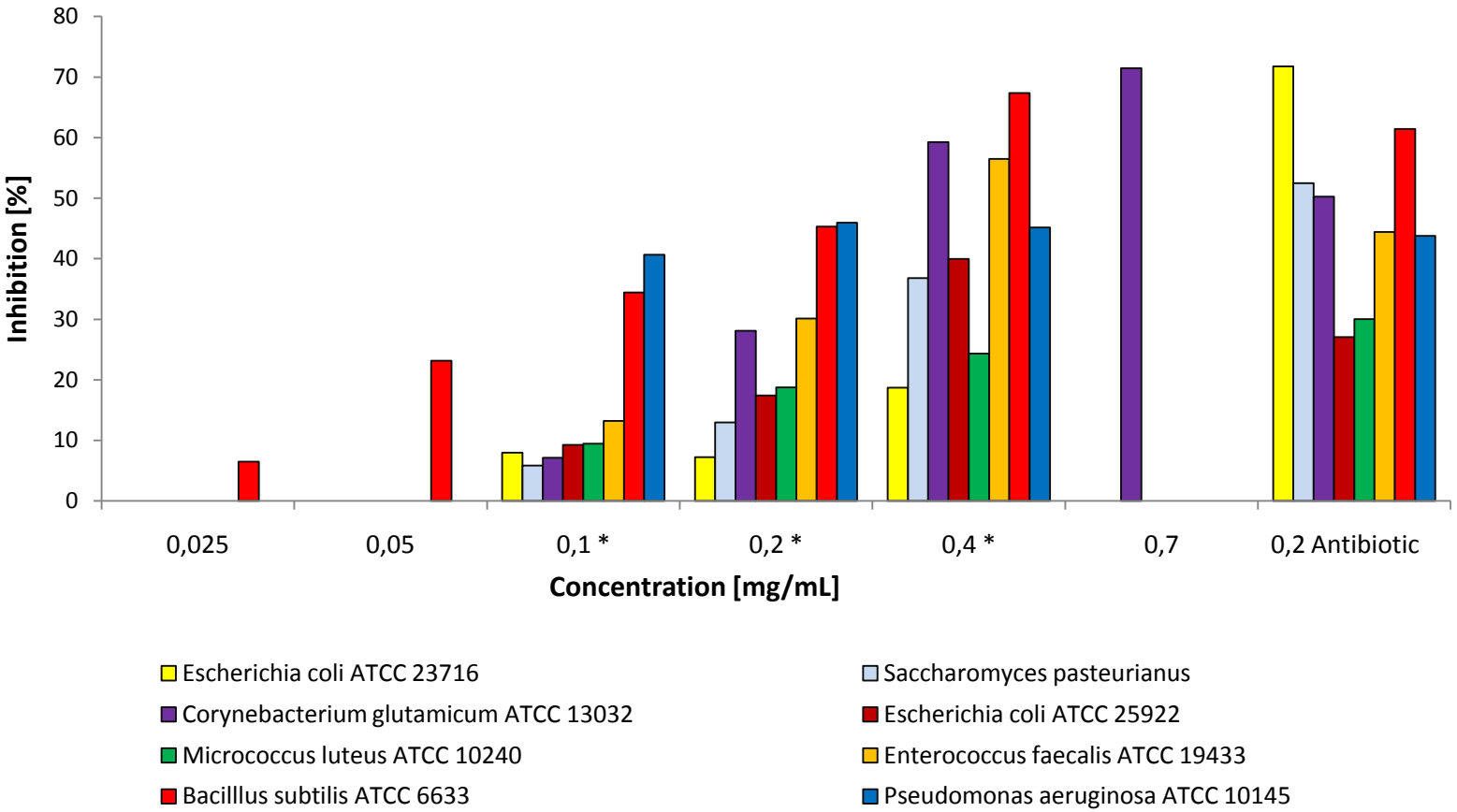
Naringenin [mg/mL]	Growth Inhibitory Effect of *B. subtilis* [%]
0.025	6.47
0.05	23.15
0.1	34.41
0.2	45.32
0.4	67.35
Antibiotic 0.2 [mg/mL]	61.45

Figure 60. Percentage of growth inhibitory effect of various concentration of naringenin against *B. subtilis* ATCC 6633 (acquired from Figure 59)

Figure 61 shows the inhibitory effect of naringenin with various concentrations against eight microorganisms chosen for this work and that are important for the food industry. The x-axis presents six concentrations of naringenin (0.025, 0.05, 0.1, 0.2, 0.4, 0.7 mg/mL, with a small change for *C. glutamicum*) and one concentration of the antibiotic, 0.2 mg/mL. Not every microorganism was investigated in every concentration (Table 4). Due to *B. subtilis* having already shown a high sensibility to the concentration of 0.1 mg/mL of the described flavanone, it was also studied with lower concentrations of naringenin, including 0.025 and 0.05 mg/mL. However, the high inhibitory effect against *P. aeruginosa* was also observed at the concentration of 0.1 mg/mL, but because of growth problems, this bacterium was only investigated with three concentrations, including 0.1, 0.2, and 0.4 mg/mL.

The strongest antimicrobial activity (below 70%) was exhibited at the highest concentration of 0.7 mg/mL against *C. glutamicum*, but only this bacterium was examined at such a high concentration. The three bacteria, *B. subtilis*, *C. glutamicum* and *E. faecalis*, in the presence of 0.4 mg/mL of naringenin, showed the highest inhibitory effects with 67%, 60% and 56%, respectively (Table 4). *B. subtilis*, *E. faecalis* and *P .aeruginosa* proved to be the most sensitive bacteria at lower concentrations, including 0.2 and 0.1 mg/mL. However, 0.1 mg/mL of naringenin caused 40% and 34% of growth inhibition of *B. subtilis* and *E. faecalis*, respectively, but only 13% to *P. aeruginosa*. The inhibitory effects on the other microorganisms were situated between 5.8% for *S. pasteurianus* and 9.5% for *M. luteus*, at the concentration of 0.1 mg/mL. The non-pathogenic strain of *E. coli* (ATCC 23716) turned out to be the most resistant microorganism and the inhibitory effect amounted to 7%. The concentration of 0.4 mg/mL showed similar but properly higher antimicrobial effects in comparison to the previous concentration, and for example, the growth inhibition to *E. coli* augmented to 18.7%.

In contrast to the antimicrobial activity of naringenin, the strongest inhibitory effect of the antibiotic was observed against *E. coli* ATCC 23716 (71%), and next against *B. subtilis* (61%). The most resistant microorganism against tetracycline proved to be the pathogenic strain of *E. coli* (ATCC 25922) with the inhibitory effect of 27%. However, it is worth noting that the concentration of 0.4 mg/mL of naringenin inhibited the growth of this bacterium to 40%. Although to various extents, every bacterium exhibited certain sensibilities against naringenin.



* By *Corynebacterium glutamicum* instead of 0.1 – 0.08 mg/mL; 0.2 – 0.17 mg/mL; 0.4 – 0.33 mg/mL

Figure 61. Inhibitory effect of naringenin against all chosen microorganisms; Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Table 4. Inhibitory effect of Naringenin against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Naringenin [mg/mL]	Growth Inhibitory effect of Naringenin [%]							
	E. coli **ATCC 23716**	***S. pasteurianus***	***C. glutamicum*** **ATCC 13032**	***E. coli*** **ATCC 25922**	***M. luteus*** **ATCC 10240**	***E. faecalis*** **ATCC 19433**	***B. subtilis*** **ATCC 6633**	***P. aeruginosa*** **ATCC 10145**
0.025	**	**	**	**	**	**	6.47	**
0.05	**	**	**	**	**	**	23.15	**
0.1 *	7.96	5.83	7.11	9.26	9.45	13.21	34.41	40.68
0.2 *	7.23	12.98	28.10	17.42	18.76	30.13	45.32	45.95
0.4 *	18.68	36.80	59.23	39.96	24.34	56.48	67.35	45.18
0.7	**	**	71.48	**	**	**	**	**
Antibiotic 0.2 mg/mL	71.74	52.46	50.26	27.06	30.04	44.44	61.45	43.77

* By *Corynebacterium glutamicum* instead of 0.1 – 0.08 mg/mL; 0.2 – 0.17 mg/mL; 0.4 – 0.33 mg/mL

** Concentration was not investigated

4.3.2.2. Isosakuranetin

The antimicrobial activity of isosakuranetin was investigated in the same way as naringenin. The results of the inhibitory effect of this flavanone are shown in Figure 62 The sample with 0.006 mg/mL of isosakuranetin was studied only against the yeast *S. pasteurianus* and showed no antimicrobial activity, while the presence of 0.012 mg/mL inhibited the growth by almost 7% (Table 5). The same concentration inhibited only 2% the growth of *B. subtilis* which, therefore, proved to be the weakest bacterium in general. The analyses of the inhibitory effect on *S. pasteurianus* were only possible until 0.05 mg/mL. Higher concentrations rendered the solution turbid and the analysis could not be properly performed anymore. The presence of 0.05 mg/mL of isosakuranetin indicated a 10% stronger growth inhibition of *B. subtilis* than of *S. pasteurianus* (30% and 20%, respectively). At the highest measured concentration of 0.2 mg/mL, *B. subtilis* exhibited the strongest sensitivity to isosakuranetin, with almost 50% of growth inhibition.

The other microorganisms were investigated only at three concentrations, including 0.1, 0.2 and 0.4 mg/mL. The bacteria, *C. glutamicum*, *P. aeruginosa* and *M. luteus* showed a higher sensibility against isosakuranetin. Their growth inhibition was between 30% and 50%. Both *E. coli* and also *E. faecalis* have been recorded as the most resistant microorganisms to isosakuranetin and presented less than 5% of growth inhibition at every measured concentration. An increase of the concentration of the flavanone exhibited no changes in the growth of *E. coli* ATCC 23716. The pathogenic strain of *E. coli* showed the strongest resistance to isosakuranetin with a growth inhibition amounting only to 1.75%, using a concentration of 0.2 mg/mL. The two other concentrations did not expose any changes in the growth of this bacterium during 8 h. The highest inhibitory effect of isosakuranetin was observed at the concentration of 0.2 mg/mL against *C. glutamicum*, which was still not as effective as the antimicrobial activity of 0.2 mg/mL of tetracycline (around 60%).

All microorganisms were also tested for susceptibility to antibiotics. Beyond *M. luteus*, all other microorganisms presented in the graph (Figure 62) showed a higher sensitivity to antibiotics than to isosakuranetin. The highest difference between the flavanone and antibiotics was observed for *E. coli* ATCC 23716, and amounted to almost 62% (5.04% for isosakuranetin and 67% for tetracycline). Also the pathogenic strain of *E. coli*, ATCC 25922, showed a high difference between the inhibitory effect of antibiotic and of the flavanone, which amounted to 58%. The lowest differences are reported by the growth inhibition of *C. glutamicum* and *B. subtilis*, which is around 8% and 13%, respectively.

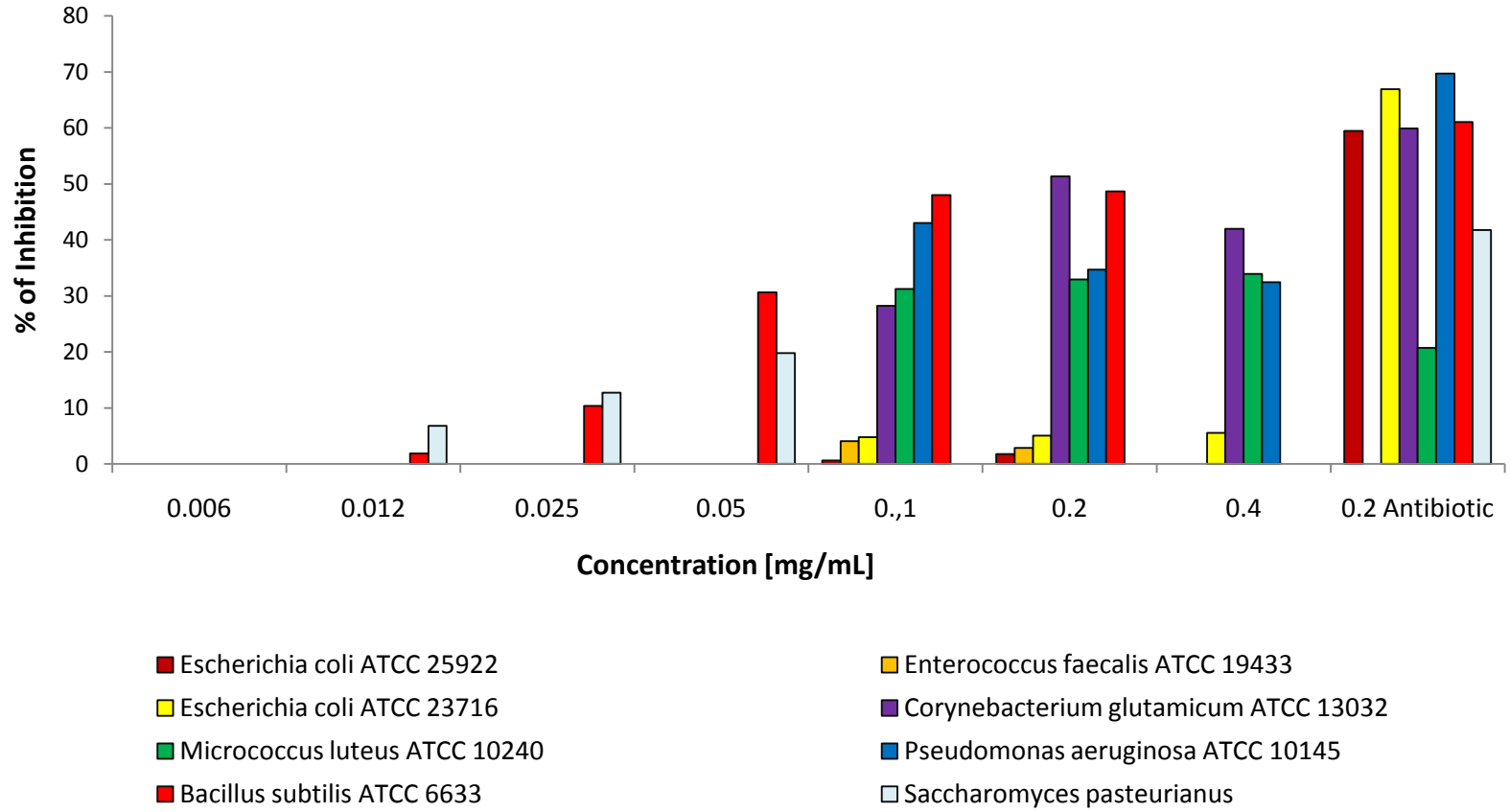


Figure 62. Inhibitory effect of Isosakuranetin against all Chosen Microorganisms; Antibiotic – tetracycline for every bacterium and natamax for the yeast, *S. pasteurianus*

Table 5. Inhibitory effect of Isosakuranetin against all Chosen Microorganisms; Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Isosakuranetin [mg/mL]	Growth Inhibitory effect of Isosakuranetin [%]							
	E. coli ATCC 25922	**E. faecalis** ATCC 19433	**E. coli** ATCC 23716	**C. glutamicum** ATCC 13032	**M. luteus** ATCC 10240	**P. aeruginosa** ATCC 10145	**S. pasteurianus**	**B. subtilis** ATCC 6633
0.006	**	**	**	**	**	**	0.00	**
0.012	**	**	**	**	**	**	6.79	1.85
0.025	**	**	**	**	**	**	12.73	10.36
0.05	**	**	**	**	**	**	19.77	30.65
0.1	0.59	4.06	4.78	28.21	31.25	43.00	turbid	48.03
0.2	1.75	2.88	5.04	51.34	32.95	34.68	turbid	48.68
0.4	0.00	0.00	5.55	41.97	33.94	32.45	turbid	**
Antibiotic 0.2 mg/mL	59.45	44.44	66.91	59.93	20.71	69.70	41.74	61.06

** Concentration was not investigated

4.3.2.3. Eriodictyol

Figure 63 presents the results of the antimicrobial activity of eriodictyol. By applying this flavanone at the concentration of 0.025 mg/mL, *B. subtilis* showed 10% growth inhibition (Table 6). A twofold increase of concentration (from 0.025 to 0.05 mg/mL and after that from 0.05 mg/mL to 0.1 mg/mL) caused exactly a doubled higher inhibitory effect on this bacterium. However, the concentrations of 0.2 and 0.4 mg/mL did not present such a linear increase of growth inhibition of *B. subtilis*. The linear increase of antimicrobial activity with the increase of concentration was observed also for *E. faecalis*. The highest inhibitory effect against this bacterium amounted to almost 50%. A good antimicrobial activity of eriodictyol was also recorded for *P. aeruginosa* with an inhibitory effect oscillating between 33% and 39%. *E. coli* ATCC 25922 and *C. glutamicum* at the eriodictyol concentration of 0.4 mg/mL were also inhibited with higher percentage values, amounting to 35% and 37%, respectively. It is important to note that eriodictyol exhibited a stronger inhibitory effect on the pathogenic strain of *E. coli* than on *E. coli* ATCC 23716, which belongs to the first risk group of microorganisms. The maximum of growth inhibition of *E. coli* ATCC 25922 was presented at the concentration of 0.4 mg/mL and amounted to almost 7%. Eriodictyol exhibited a slight antimicrobial effect against *S. pasteurianus*, which was around 13% at the concentration of 0.2 mg/mL. Because of the slight solubility of flavanones in water, it was difficult to study the antimicrobial capacity at higher concentrations of eriodictyol against *S. pasteurianus*. With the higher percentage of water, the substance precipitated which made the solution turbid.

The antimicrobial activity of eriodictyol was also compared to the capacity of commonly occurring antibiotics. We observed in more cases that the antibiotics exhibited a stronger inhibitory effect than the flavanone. The strongest antimicrobial activity showed tetracycline with a value of 71% against *B. subtilis*. It indicates that the inhibitory effect of tetracycline at the concentration of 0.2 mg/mL was almost 20% higher compared to 0.4 mg/mL of eriodictyol. The highest difference of antimicrobial activity of the antibiotic and the flavanone was observed for *E. coli* ATCC 23716, amounting to 55% (7% of inhibitory effect of eriodictyol and 62% of tetracycline). However, the antibiotic exhibited a very slight activity against *M. luteus* and *E. coli* ATCC 25922, with only around 3% and 22%, respectively.

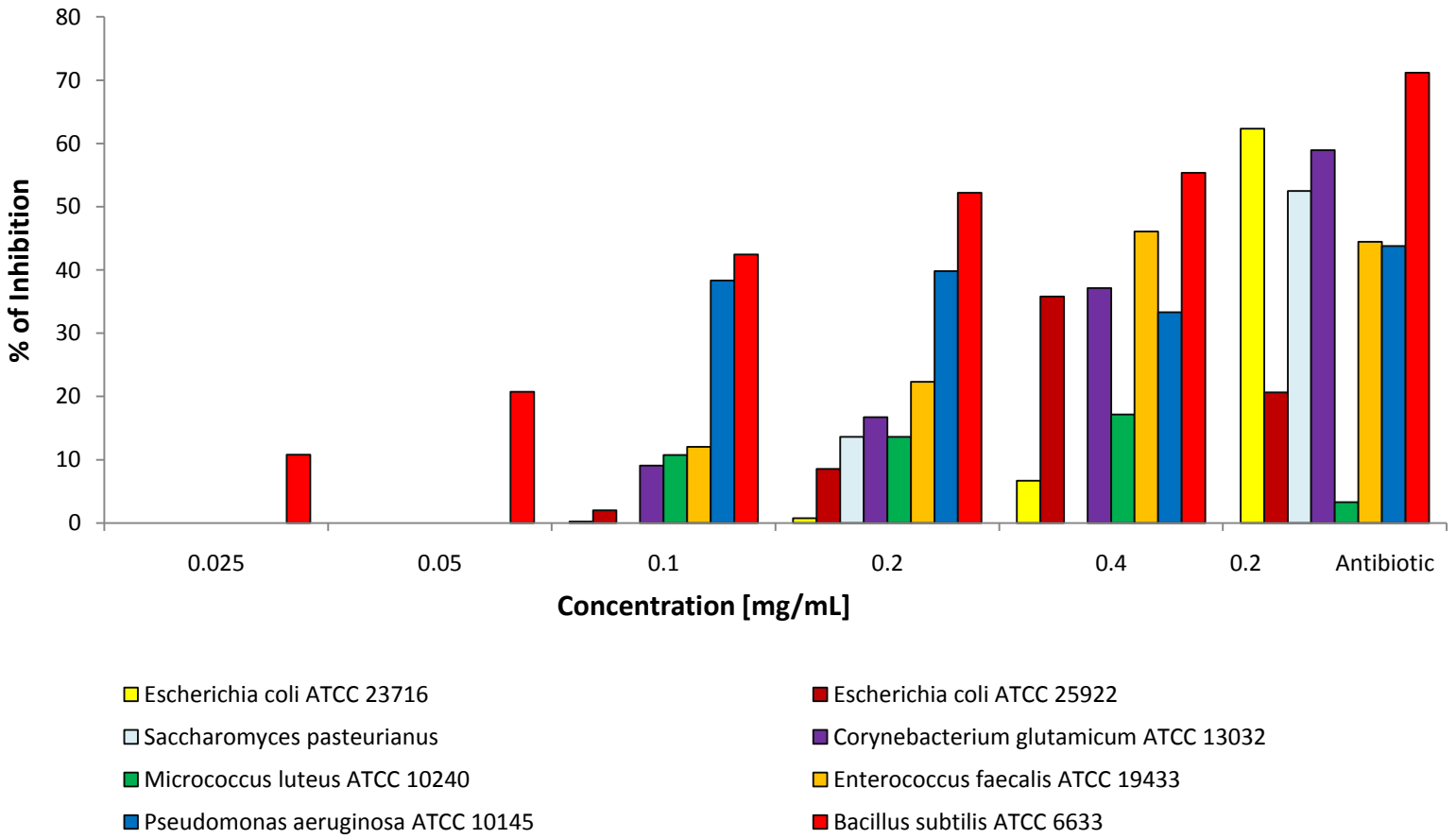


Figure 63. Inhibitory effect of Eriodictyol against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Table 6. Inhibitory effect of Eriodictyol against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Eriodictyol [mg/mL]	Growth Inhibitory effect of Eriodictyol [%]							
	E. coli ATCC 23716	**E. coli** ATCC 25922	**S. pasteurianus**	**C. glutamicum** ATCC 13032	**M. luteus** ATCC 10240	**E. faecalis** ATCC 19433	**P. aeruginosa** ATCC 10145	**B. subtilis** ATCC 6633
0.025	**	**	**	**	**	**	**	10.77
0.05	**	**	**	**	**	**	**	20.75
0.1	0.21	2.02	0.00	9.06	10.75	12.04	38.35	42.45
0.2	0.75	8.54	13.63	16.71	13.64	22.32	39.82	52.21
0.4	6.68	35.78	turbid	37.13	17.15	46.08	33.29	55.34
Antibiotic 0.2 [mg/mL]	62.32	20.66	52.46	58.94	***	44.44	43.77	71.17

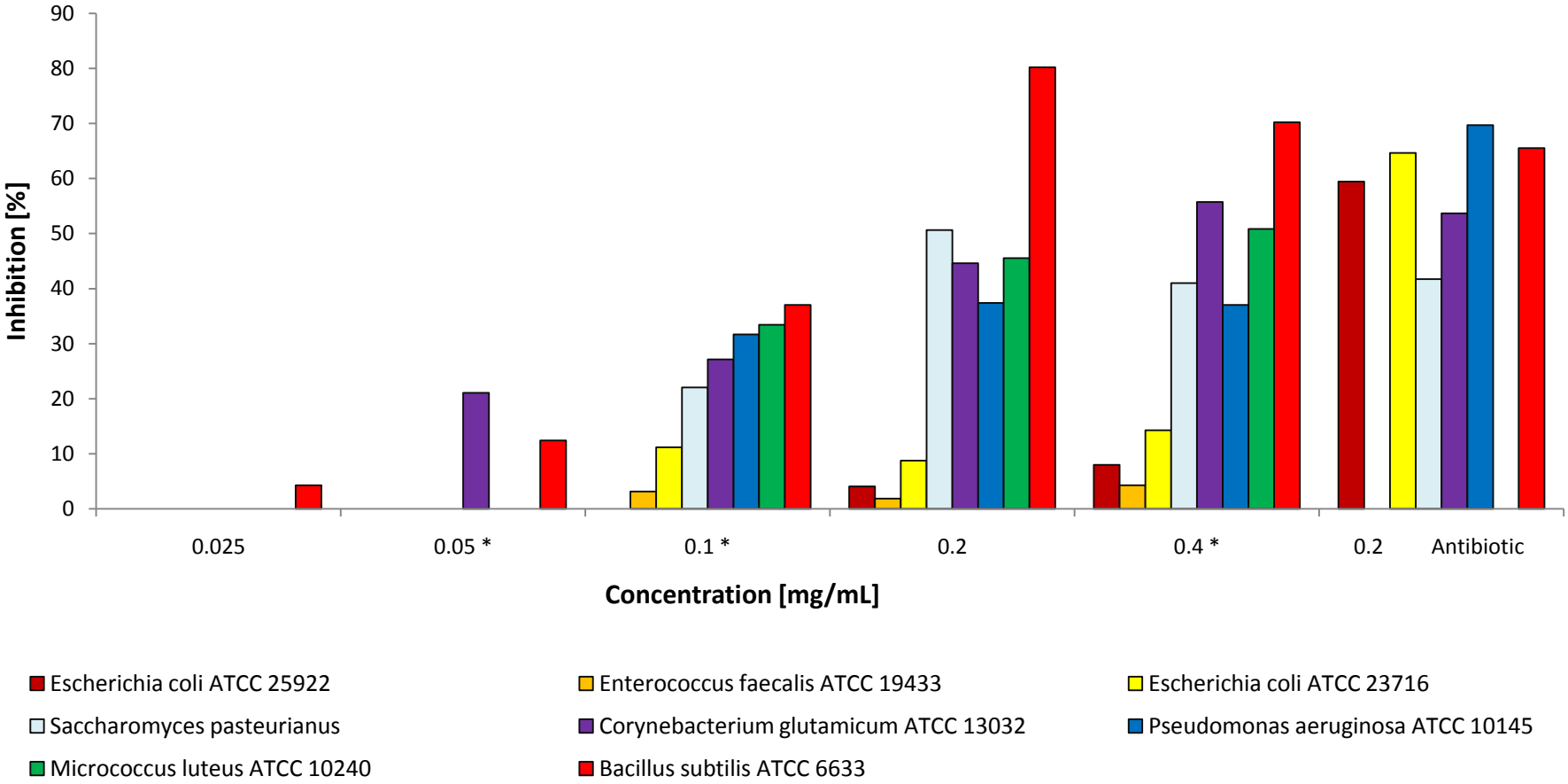
** Concentration was not investigated

*** Error of analysis

4.3.2.4. Homoeriodictyol

The results of the antimicrobial activity of homoeriodictyol are presented in Figure 64. In the presence of this flavanone, *B. subtilis* proved also to be the most sensitive microorganism, which was investigated in lower concentrations of homoeriodictyol compared to the others. The concentration of 0.025 mg/mL of homoeriodictyol showed 4% of inhibitory effect against this bacterium, while the 0.05 mg/mL exhibited a three-time higher antimicrobial effect in comparison to the previous concentration and amounted to around 12.5% (Table 7). The investigation of 0.1 mg/mL of homoeriodictyol showed again a triple increase of inhibitory effect and reached 37%. However, the next doubling of concentration presented only a two-fold increase of growth inhibition. The highest inhibitory effect on *B. subtilis* with 80% was observed at the concentration of 0.2 mg/mL. It was also recorded that 0.4 mg/mL of homoeriodictyol exposed a lower antimicrobial effect than compared to 0.2 mg/mL. This flavanone showed a good antimicrobial activity against *C. glutamicum* and against *S. pasteurianus* as well. The highest inhibitory effects of these two microorganisms amounted to 56% at 0.4 mg/mL of homoeriodictyol for the bacterium and to 52% at 0.2 mg/mL for the yeast. 50% of growth inhibition was recorded at the concentration of 0.4 mg/mL for *M. luteus*. In the presence of 0.2 and 0.4 mg/mL of the described flavanone, there was still a good inhibitory effect observed for *P. aeruginosa*, which amounted to 37% at both concentrations. Lower antimicrobial activity showed homoeriodictyol against all of the faecal bacteria. At the highest investigated concentration of 0.4 mg/mL, there the inhibitory effect was observed with 4% for *E. faecalis* and with 14% for the non pathogenic strain of *Escherichia*. The presence of 0.1 mg/mL of homoeriodictyol showed no activity against *E. coli* ATCC 25922.

The comparison between antibiotics and homoeriodictyol demonstrates that the concentration of 0.2 mg/mL of the flavanone inhibited stronger the growth of *B. subtilis* than compared to the same content of tetracycline. The concentration of 0.4 mg/mL of the flavanone exhibited also better activity against *C. glutamicum* than the presence of 0.2 mg/mL of the antibiotic. The highest difference between the inhibitory effects of the antibiotic and homoeriodictyol was observed with the growth of *E. coli* ATCC 25922. Due to some growth problems by the performance of the analyses, the data of antimicrobial activity of tetracycline against *E. faecalis* and *M. luteus* are not shown.



* By *Corynebacterium glutamicum* instead of 0.05 mg/mL was placed 0.1 mg/mL; instead of 0.1 - 0.17 mg/mL; and 0.4 – 0.33 mg/mL

Figure 64. Inhibitory effect of Homoeriodictyol against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Table 7. Inhibitory effect of Homoeriodictyol against all Chosen Microorganisms; Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Homoeriodictyol [mg/mL]	Growth Inhibitory effect of Homoeriodictyol [%]							
	E. coli ATCC 25922	E. faecalis ATCC 19433	E. coli ATCC 23716	S. pasteurianus	C. glutamicum ATCC 13032	P. aeruginosa ATCC 10145	Mc. luteus ATCC 10240	B. subtilis ATCC 6633
0.025	**	**	**	**	**	**	**	4.31
0.05 *	**	**	**	**	21.08	**	**	12.44
0.1 *	0.00	3.13	11.21	22.08	27.17	31.71	33.44	37.04
0.2	4.10	1.85	8.77	50.62	44.62	37.41	45.56	80.17
0.4 *	8.00	4.27	14.27	41.01	55.72	37.02	50.85	70.21
Antibiotic 0.2 [mg/mL]	59.45	44.44	64.63	41.74	53.69	69.70	***	65.50

* By *Corynebacterium glutamicum* instead of 0.05 – 0.1 mg/mL; 0.1 – 0.17 mg/mL; 0.4 – 0.33 mg/mL

** Concentration was not investigated

*** Error of analysis

4.3.2.5. Hesperetin

Hesperetin was the last chosen substance from the flavanone group and its microbiological results are presented in Figure 65 and Table 8. Hesperetin showed the lowest antimicrobial activity in comparison to all other tested flavanones. *B. subtilis* proved again to be the most sensitive bacterium and the hesperetin concentration of 0.4 mg/mL. The lowest concentration used for the analysis against *B. subtilis* was 0.025 mg/mL and exhibited 5% of growth inhibition. Good inhibitory effect, although slightly lower than the activities of the other analyzed flavanones, showed hesperetin against *C. glutamicum*, *M. luteus* and *P. aeruginosa*. The highest inhibitory effects against these bacteria were observed at the concentrations of 0.4 mg/mL and amounted to 34% for *Pseudomonas*, to 35% for *Corynebacterium* and to 37% for *Micrococcus*. *S. pasteurianus* showed a higher susceptibility to the presence of hesperetin in terms of growth inhibition. The three faecal bacteria showed also the strongest resistances. The non-pathogenic strain of *E. coli* presented the highest inhibitory effect by value of 8.5% at the concentration of 0.2 mg/mL, while the second strain of this bacterium showed only 5% inhibition in the presence of 0.4 mg/mL of hesperetin. The antimicrobial activity of this flavanone against *E. faecalis* oscillated between 3% and 7%.

The concentration of 0.2 mg/mL of antibiotics showed stronger inhibitory effects against all microorganisms than compared to the presence of 0.4 mg/mL of hesperetin. The highest difference between the inhibitory effect of antibiotics and the flavanone was recorded for the growth of the non-pathogenic strain of *E. coli*. The antimicrobial activity of tetracycline against this bacterium amounted to almost 69%, while the inhibitory effect of hesperetin reached only 8.5%. A very high difference was also shown when comparing the growths of the strain from second risk group of *E. coli* (5% to 59%) in the presence of flavanone and the antibiotic.

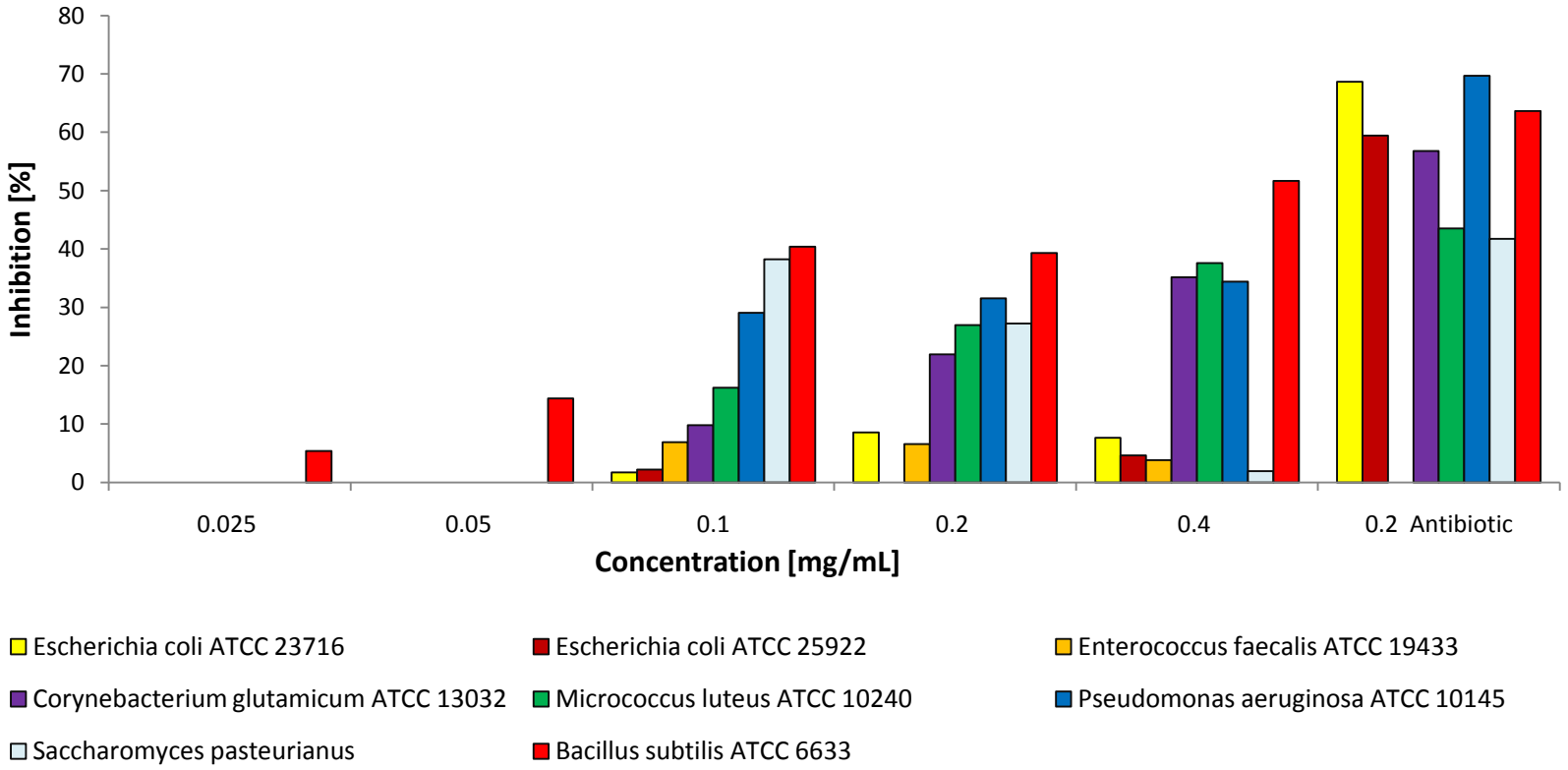


Figure 65. Inhibitory effect of Hesperetin against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Table 8. Growth Inhibitory effect of Hesperetin against all Chosen Microorganisms; Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*.

Hesperetin [mg/mL]	Growth Inhibitory effects of Hesperetin [%]							
	E. coli ATCC 23716	**E. coli** ATCC 25922	**E. faecalis** ATCC 19433	**C. glutamicum** ATCC 13032	**Mc. luteus** ATCC 10240	**P. aeruginosa** ATCC 10145	**S. pasteurianus**	**B. subtilis** ATCC 6633
0.025	**	**	**	**	**	**	**	5.41
0.05	**	**	**	**	**	**	**	14.40
0.1	1.73	2.19	6.91	9.80	16.22	29.05	38.22	40.39
0.2	8.55	0.00	6.56	21.96	26.94	31.52	27.22	39.32
0.4	7.63	4.63	3.81	35.15	37.61	34.38	1.95	51.68
Antibiotic 0.2 [mg/mL]	68.66	59.45	44.44	56.79	43.52	69.70	41.74	63.62

** Concentration was not investigated

4.3.2.6. Hesperidin

Hesperidin belongs to the group of flavanone glycoside and possesses a sugar in its molecule. In Figure 66 the antimicrobial activity of hesperidin is presented. Nevertheless, the results of hesperidin differ from the previously reported ones. Most of them are located below zero, and therefore in the negative area of the diagram. In the presence of hesperidin, except for the antibiotics, only one microorganism showed a slight growth inhibition and that is *E. coli* ATCC 23716. The strongest inhibitory effect of hesperidin was observed at the highest tested concentration of 0.4 mg/mL and achieved almost 10% against the non pathogenic strain of *Escherichia* (Table 9) At the concentration of 0.1 mg/mL, both *M. luteus* and *C. glutamicum* exhibited 1% growth inhibition. However, the increase of concentration of hesperidin up to 0.2 and to 0.4 mg/mL, respectively, did not lead to a growth inhibition but to growth stimulation, hence, the negative results shown in Figure 66,amounting to -7% and -12% for *M. luteus* and -2%, -17% for *C. glutamicum*, respectively.

The other investigated microorganisms showed no inhibition. On the contrary, the negative values of inhibition efficiency suggest that hesperidin stimulates the microbial growth. The strongest stimulation effect of almost 50% was recognized for *S. pasteurianus* at the lowest hesperidin concentration of 0.1 mg/mL. Due to the low solubility of hesperidin in MeOH, it was not possible to analyze the activity of higher concentrations against the yeast. Similar effect of growth increase, around 45%, was observed in the presence of 0.4 mg/mL of the flavanone glycoside against *P. aeruginosa*. The concentrations of 0.1 and of 0.2 mg/mL of hesperidin stimulated the growth of this pathogenic bacterium with 17% and 20%. The growth of the strain of *E. coli* ATCC 25922 was also slightly stimulated and at the concentration of 0.2 mg/mL the stimulation amounted to 11%. The presence of 0.1 and 0.2 mg/mL of hesperidin showed only 1% of growth increase for *E. faecalis* in comparison to the control sample. The stimulation effect of hesperidin on the growth of other microorganisms was measured with below 15%. The samples with the addition of antibiotics showed the expected inhibitory effect and thereby confirmed the accuracy of the analyses performance.

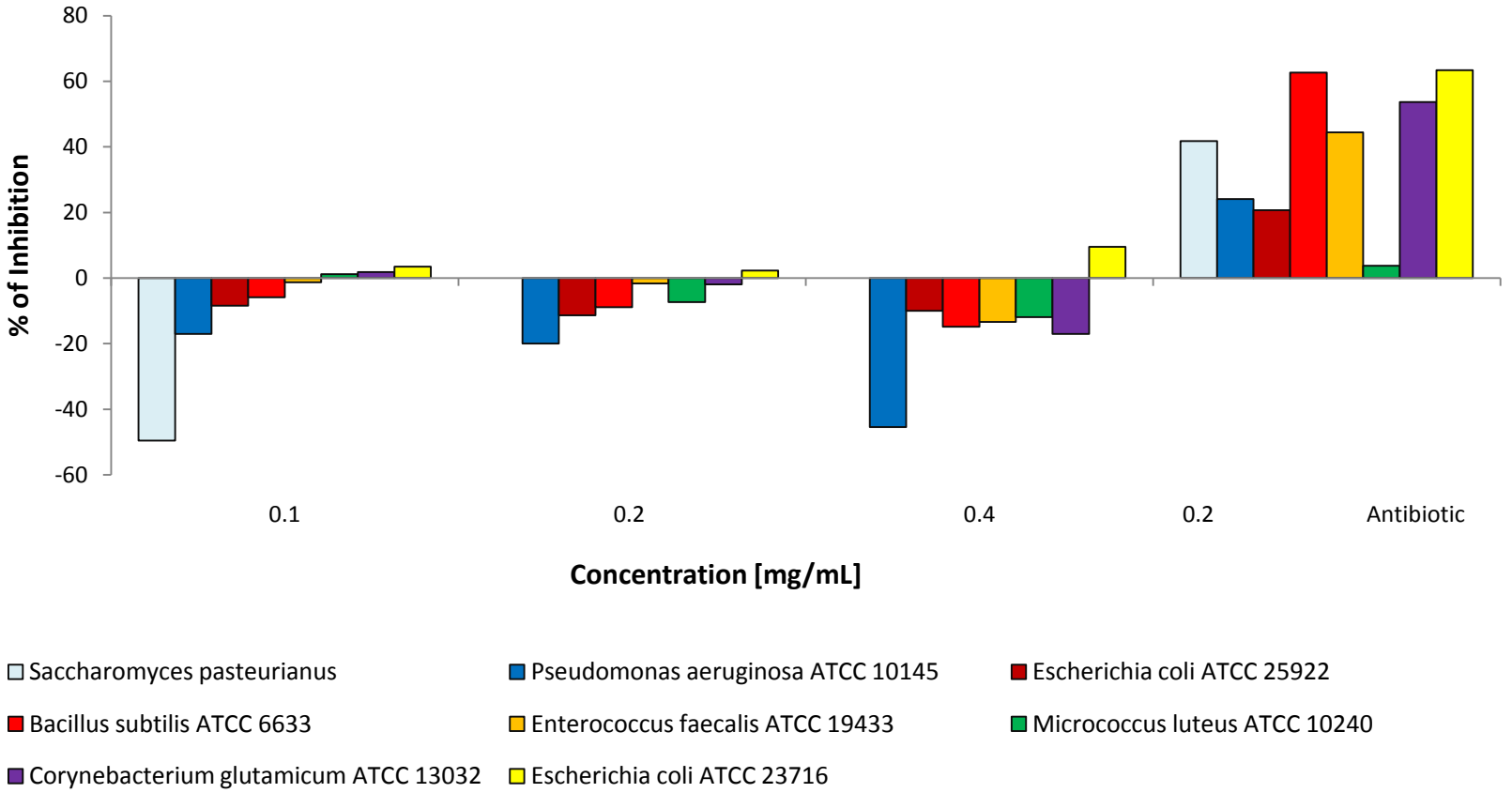


Figure 66. Inhibitory effect of Hesperidin against all Chosen Microorganisms, Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*

Table 9. Growth inhibitory effect of hesperetin against all chosen microorganisms; Antibiotic – tetracycline for every bacterium, and natamax for the yeast, *S. pasteurianus*.

Hesperidin [mg/mL]	**Growth inhibitory effect of hesperidin [%]**							
	S. pasteurianus	***P. aeruginosa*** **ATCC 10145**	***E. coli*** **ATCC 25922**	***B. subtilis*** **ATCC 6633**	***E. faecalis*** **ATCC 19433**	***M. luteus*** **ATCC 10240**	***C. glutamicum*** **ATCC 13032**	***E. coli*** **ATCC 23716**
0.1	-49,59	-17,09	-8,43	-5,84	-1,28	1,14	1,81	3,48
0.2		-19,99	-11,40	-8,85	-1,63	-7,34	-1,93	2,30
0.4		-45,49	-9,98	-14,81	-13,37	-11,92	-17,09	9,56
Antibiotic 0.2 [mg/mL]	41,74	24,05	20,66	62,69	44,44	***	53,69	63,37

*** Error of analysis

4.4. Antimicrobial activity of analyzed enantiomers

The next main aim of the thesis was the examination and presentation of the differences between the antimicrobial activity of the individual enantiomers and racemates of flavanones by using the same concentrations. As already mentioned, it was possible to investigate the inhibitory effects of both enantiomers and racemates of naringenin and isosakuranetin but only the racemates and the *S*-(–) enantiomers of eriodictyol and homoeriodictyol were compared. The enantiomers of hesperetin were not available for this analysis. To study the antimicrobial activity of enantiomers, liquid micro-dilution technique was used. The microorganisms grew also on the BHI or on the YNB medium, for 24 h (according to the chapter 3.2.8.2). The growth of every microorganism was also recorded by the development of growth curves.

4.4.1. Naringenin – comparison of enantiomers and racemate

To demonstrate and to explain the main data about the growth curve, the graph of antimicrobial activity of naringenin against *E. faecalis* ATCC 19433 was chosen. This kind of graph and table relates to every enantio-separated substance and every microorganism investigated in this work. This chapter presents only an example of one substance and one microorganism – the graphs of other substances and microorganisms are presented in Annex (chapter 9).

Figure 67 shows the antimicrobial activity of individual enantiomers and of the racemate of naringenin. In this analysis, the flavanone was studied at the concentration of 0.2 mg/mL in all samples. The turbidity changes were measured by Tecan SunRise during 24 h and subsequently, the growth curves were determined. The figure presents five growth curves. The blue curve corresponds to the control sample (blank), the pink one is the sample containing a solvent (in this case, MeOH), and the three other lines correspond to the samples with addition of enantiomers and naringenin racemate. The green curve determines the results of the antimicrobial activity of *S*-(–) naringenin, the red one of the *R*-(+) naringenin and the blue one of the naringenin racemate (+/–) naringenin.

This figure shows that the sample containing MeOH presented a slight inhibitory effect on *E. faecalis*. The (–) naringenin concentration of 0.2 mg/mL exposed a significant inhibitory effect and in the last hour, the OD amounted to 0.7 at 590 nm (Table 10). A

stronger inhibitory effect with the same concentration was presented by (+) naringenin and the OD reached around 0.6. The figure shows that from 2 h until 10 h, the inhibitory effect of (+) naringenin in comparison to (–) naringenin was increasing. A similar effect was observed by the antimicrobial activity of the naringenin racemate in comparison to both enantiomers. It is clear to see that the *S*-(–) configuration of naringenin in Figure 67 caused the weakest growth inhibitory effect against *E. faecalis*. However, it is not so clear to see the difference between the *R*-(+) naringenin and naringenin racemate because of overlapping deviations. Both of them exhibited similar inhibitory effect against this bacterium. The SD of these two growth curves agree with each other. The OD measured in the last hour amounted to almost the same value of around 0.6. However, the growth curve of the naringenin racemate presents a slight increase of inhibitory effect, especially at the beginning of the analysis.

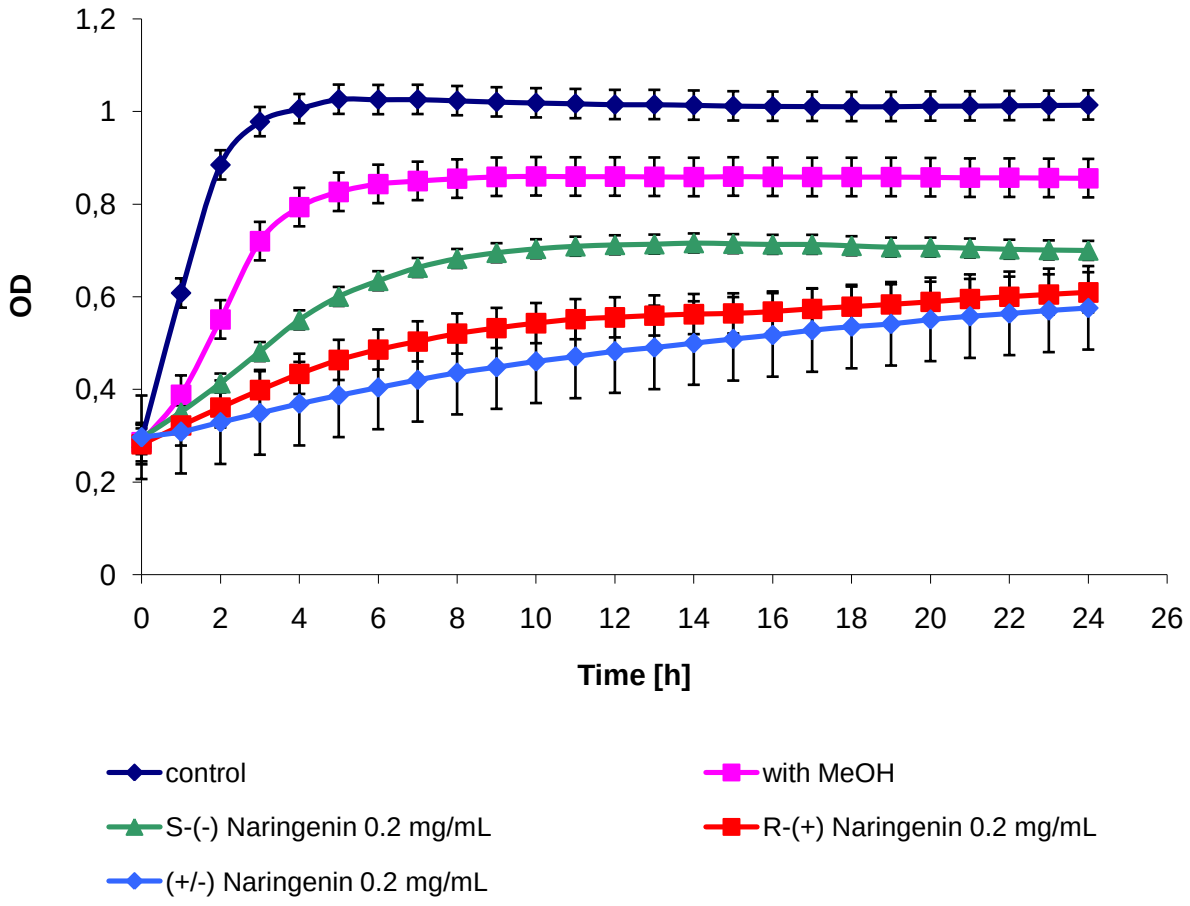
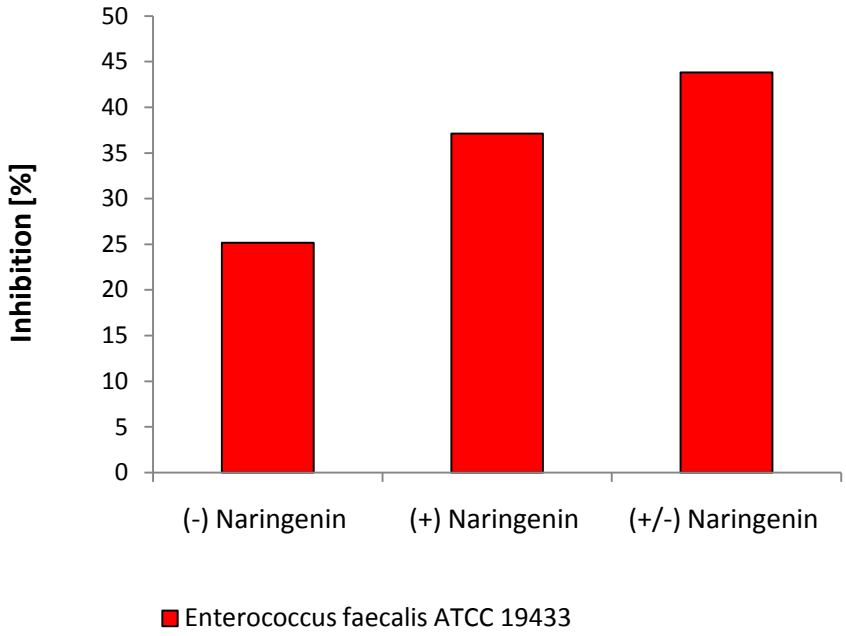


Figure 67. Growth curves of *E. faecalis* ATCC 19433 with the presence of methanol (MeOH) and enantiomers and racemate of naringenin; OD – optical density

Table 10. Growth date of *E. faecalis* ATCC 19433 with the presence of methanol (MeOH) and enantiomers and racemate of naringenin; OD – optical density, SD – standard deviation

Time [h]	OD				
	Control	MeOH	(+) Naringenin 0.2 mg/mL	(–) Naringenin 0.2 mg/mL	(+/-) Naringenin 0.2 mg/mL
0	0.2917	0.2860	0.2950	0.2815	0.2965
1	0.6080	0.3885	0.3510	0.3220	0.3085
2	0.8847	0.5510	0.4135	0.3610	0.3290
3	0.9780	0.7200	0.4815	0.3985	0.3490
4	1.0060	0.7935	0.5500	0.4335	0.3690
5	1.0263	0.8265	0.6005	0.4635	0.3870
6	1.0257	0.8435	0.6345	0.4860	0.4040
7	1.0260	0.8500	0.6630	0.5035	0.4205
8	1.0233	0.8550	0.6825	0.5205	0.4360
9	1.0207	0.8590	0.6950	0.5325	0.4480
10	1.0187	0.8600	0.7035	0.5430	0.4605
11	1.0170	0.8595	0.7090	0.5515	0.4710
12	1.0150	0.8595	0.7120	0.5555	0.4825
13	1.0150	0.8590	0.7135	0.5595	0.4905
14	1.0137	0.8585	0.7160	0.5625	0.5000
15	1.0120	0.8595	0.7145	0.5640	0.5090
16	1.0113	0.8590	0.7130	0.5680	0.5175
17	1.0110	0.8585	0.7130	0.5740	0.5280
18	1.0107	0.8585	0.7100	0.5785	0.5355
19	1.0107	0.8585	0.7070	0.5835	0.5415
20	1.0117	0.8580	0.7070	0.5890	0.5510
21	1.0120	0.8570	0.7050	0.5950	0.5580
22	1.0127	0.8570	0.7025	0.6000	0.5640
23	1.0133	0.8565	0.7010	0.6050	0.5705
24	1.0140	0.8560	0.7000	0.6095	0.5760
SD	0.03162462	0.04157788	0.02056267	0.04338807	0.09017026

Figure 68 shows the percentage of inhibitory effect of naringenin enantiomers and of its racemate against *E. faecalis* ATCC 19433. The graph demonstrates a linear increase of antimicrobial activity depending on the form of the substance. These results present clearly the lowest inhibitory ability of (–) naringenin against *E. faecalis* amounting around 25%. However, the (+) naringenin showed higher inhibitory effect than the other enantiomers, it was still almost 7% lower than the antimicrobial activity of the racemate (43%). The naringenin racemate exhibited the best antimicrobial capacity against *E. faecalis*.



Naringenin	Growth inhibitory effect of *E. faecalis* [%]
(-) Naringenin	25.15
(+) Naringenin	37.11
(+/-) Naringenin	43.82

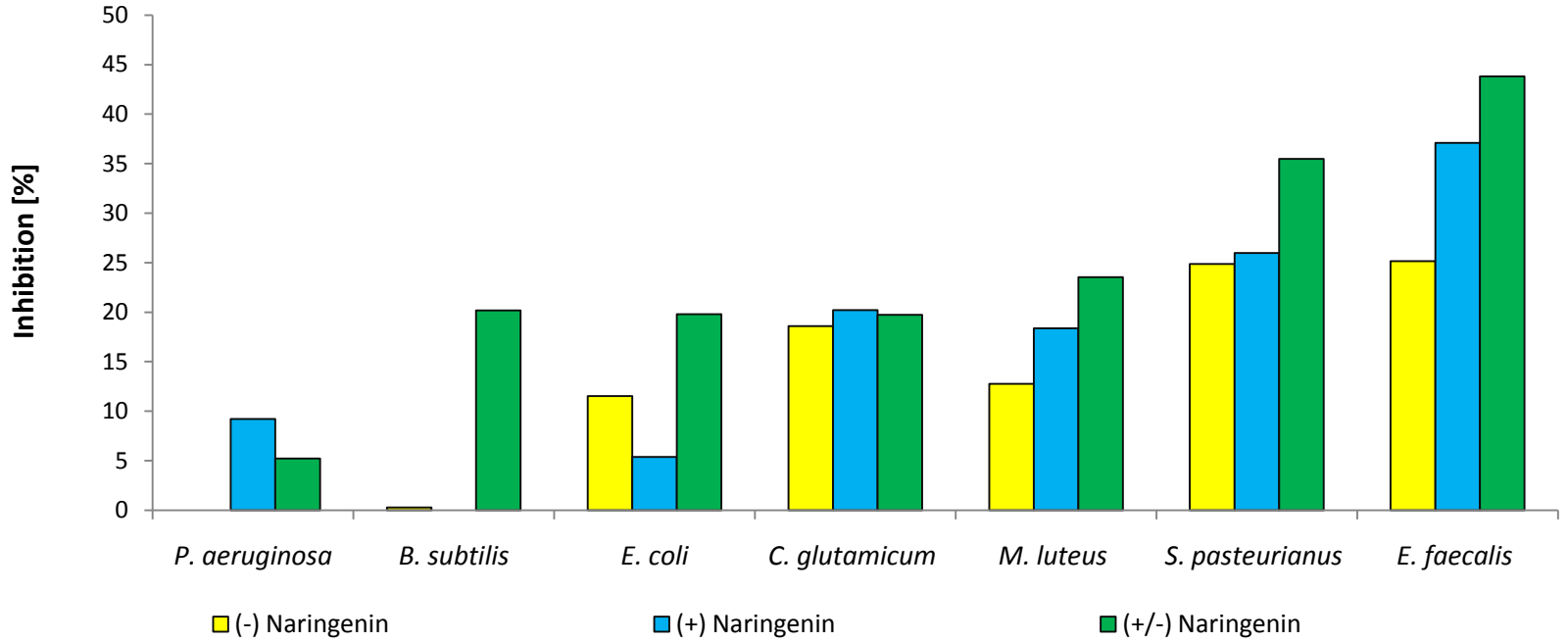
Figure 68. Growth inhibitory effect of naringenin racemate and its enantiomers against *E. faecalis* ATCC 19433

Figure 69 shows the growth inhibitory effect of the naringenin racemate and its enantiomers against seven various and important microorganisms. The analyses were carried out in different concentrations, however, the concentration of enantiomers and racemate from

one substance to another, used for one analysis and against one microorganism, was always the same. In the tests for *P. aeruginosa*, with the concentration of 0.2 mg/mL, the *R*-(+) enantiomer presented the strongest inhibitory effect (below 9%), while the racemate caused only around 5% growth inhibition. The *S*-(–) configuration showed no antimicrobial capacity against this pathogen. Around 20% of growth inhibition was observed for the naringenin racemate with a concentration of 0.05 mg/mL against *B. subtilis*. However, both of its enantiomers exhibited almost no antimicrobial activity against this bacterium. The inhibitory effects of the enantiomers against *B. subtilis* were measured with 0.28% for the *R*-(+), while no effect was observed for the *S*-(–) configuration. Thereby, this test presented the highest difference of the inhibitory effect between the racemate and the enantiomers of naringenin.

The growth of the pathogenic strain *E. coli* was investigated in the presence of 0.2 mg/mL naringenin. The racemate inhibited this microorganism with almost 20%. The (–) enantiomer exhibited half of the activity of the racemate with around 11%, while the inhibitory effect of (+) naringenin amounted to only 5%. The racemate and the enantiomers of the described flavanone showed compatible antimicrobial capacities against *C. glutamicum* and amounted to around 19% for these three configurations. The strongest effect was observed for the *R*-(+) enantiomer with 20.21%. The inhibitory effect against *M. luteus* increased linearly. The highest antimicrobial capacity exhibited the racemate, and the lowest was recorded in the sample containing *S*-(–) naringenin. A similar effect showed the analyses for *E. faecalis*, which showed almost 45% inhibition with the naringenin racemate, 37% for the *R*-(+) and only 25% for the *S*-(–) enantiomer. Compatible effects of the enantiomers, however lower than racemate, were also observed for naringenin against *S pasteurianus*.

The data show that the strongest inhibitory effect on most of the investigated microorganisms was observed for the naringenin racemate. Only the tests with *P. aeruginosa* and *C. glutamicum* showed a higher activity of the *R*-(+) enantiomer than of the racemate. The *S*-(–) configuration showed definitely the lowest inhibitory effect except for the analyses with *B. subtilis* and *E. coli*.

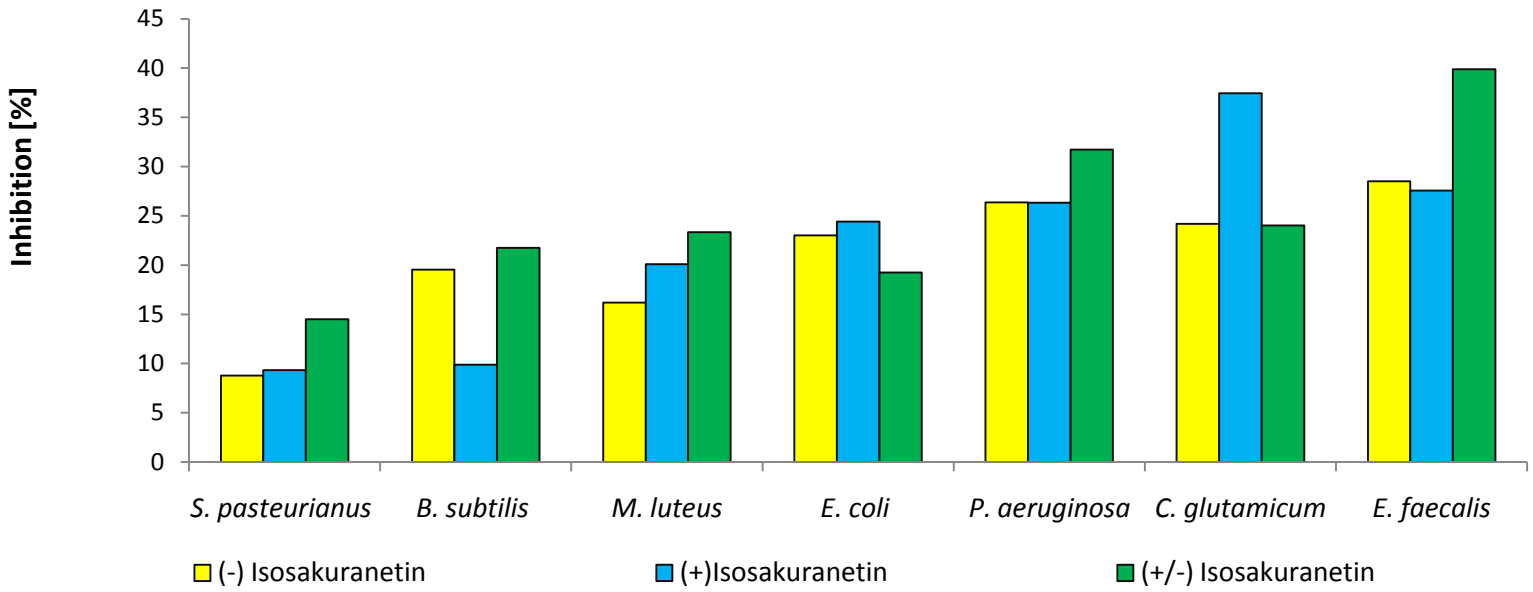


Naringenin [mg/mL]	Growth inhibitory effect of naringenin [%]						
	P. aeruginosa **ATCC 10145**	***B. subtilis*** **ATCC 6633**	***E. coli*** **ATCC 25922**	***C. glutamicum*** **ATCC 13032**	***M. luteus*** **ATCC 10240**	***S. pasteurianus***	***E. faecalis*** **ATCC 19433**
(-) Naringenin	0.00	0.28	11.52	18.58	12.75	24.88	25.15
(+) Naringenin	9.20	0.00	5.38	20.21	18.38	25.96	37.11
(+/-) Naringenin	5.22	20.19	19.78	19.73	23.52	35.49	43.82
Concentration [mg/mL]	0.2	0.05	0.2	0.2	0.1	0.2	0.2

Figure 69. Growth Inhibitory effect of Naringenin Racemate and Its Enantiomers against Seven Chosen Microorganisms

4.4.2. Isosakuranetin

The results of antimicrobial activity of the isosakuranetin racemate and its enantiomers compiled with various microorganisms are presented in Figure 70. This flavanone exhibited also a linear increase of inhibitory effect against *S. pasteurianus*. The strongest antimicrobial capacity against the yeast was observed using the isosakuranetin racemate and the lowest in the presence of the *S*-(–) enantiomer. The *S*-(–) and not the *R*-(+) enantiomer inhibited the growth of *B. subtilis* with around 20% and 10%, respectively. The highest inhibitory effect was observed in the sample containing the racemate (21%). The bacteria, including *M luteus*, *P. aeruginosa* and *E. faecalis*, were most strongly inhibited in the presence of isosakuranetin racemate with amounting to 23%, 31% and 40%, respectively. The weakest antimicrobial activity was exhibited against *M. luteus* using the (–) enantiomer and reached only around 16%, while the inhibitory effects of *R*-(+) and *S*-(–) configurations of isosakuranetin against *P. aeruginosa* and *E. faecalis* were very compatible. There was almost no difference observed between the antimicrobial activity of the enantiomers against *P. aeruginosa* (26.37% for the *S*-(–) and 26.34% for the *R*-(+) enantiomer), and also against *E. faecalis*, which amounted to 27.56% for the (+) and 28.52% for the (–) isosakuranetin. The strongest antimicrobial activity against the growth of *E. coli* and *C. glutamicum* was observed in the presence of *R*-(+) isosakuranetin, while the weakest effect for these two bacteria was exhibited in the sample with the isosakuranetin racemate. However, in case of *C. glutamicum* the difference was not so significant than by the growth of the pathogenic strain of *E. coli*.



Isosakuranetin [mg/mL]	Growth inhibitory effect of isosakuranetin [%]						
	S. pasteurianus	*B. subtilis* ATCC 6633	*M. luteus* ATCC 10240	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 10145	*C. glutamicum* ATCC 13032	*E. faecalis* ATCC 19433
(-) Isosakuranetin	8.79	19.53	16.20	23.01	26.37	24.18	28.52
(+)Isosakuranetin	9.33	9.87	20.10	24.43	26.34	37.44	27.56
(+/-) Isosakuranetin	14.50	21.74	23.35	19.23	31.71	24.03	39.89
Concentration [mg/mL]	0.025	0.1	0.5	0.2	0.1	0.2	0.1

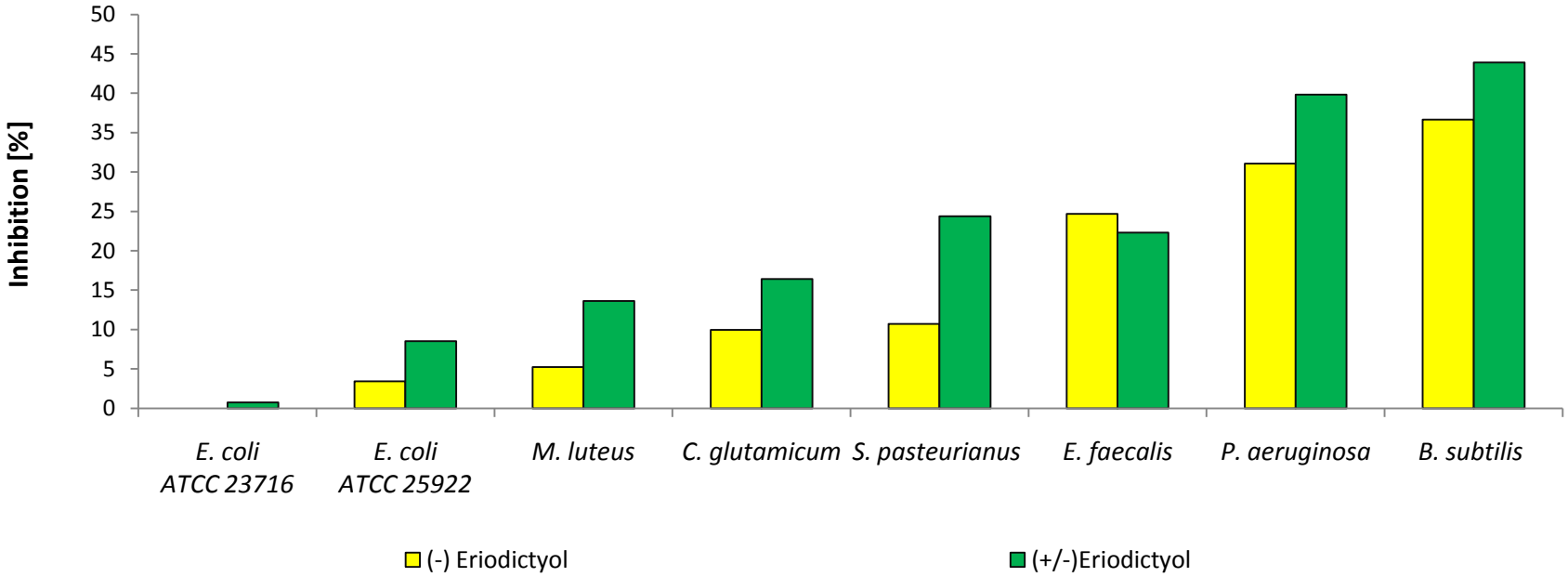
Figure 70. Growth inhibitory effect of isosakuranetin racemate and its enantiomers against seven chosen microorganisms

4.4.3. Eriodictyol

The flavanone eriodictyol was investigated against eight microorganisms and compared between the racemate and its (–) enantiomer. The results presented in Figure 71 show that only against *E. faecalis*, the (–) eriodictyol exhibited a slightly higher growth inhibitory effect than the racemate, amounting to 25% and 22%, respectively. All other microorganisms showed stronger sensibility to the eriodictyol racemate than to the (–) enantiomer. The results, however, demonstrate various differences between the inhibitory effect of the racemate and of (–) eriodictyol against the individual microorganisms. The smallest difference was presented against *E. coli* ATCC 23716. The *S*-(–) enantiomer exhibited no antimicrobial activity, and the eriodictyol racemate inhibited the growth of this microorganism only to 0.75%. The highest difference was observed in the tests against *S. pasteurianus*. The antimicrobial activity of the racemate for the microorganism amounted to 24%, while (–) eriodictyol inhibited the yeast only to 11%. The other bacteria strains showed 5% of inhibitory effect on *E. coli* ATCC 25922, 7% for *C. glutamicum* and *B. subtilis*, and until around 8% for *M. luteus* and *P. aeruginosa*.

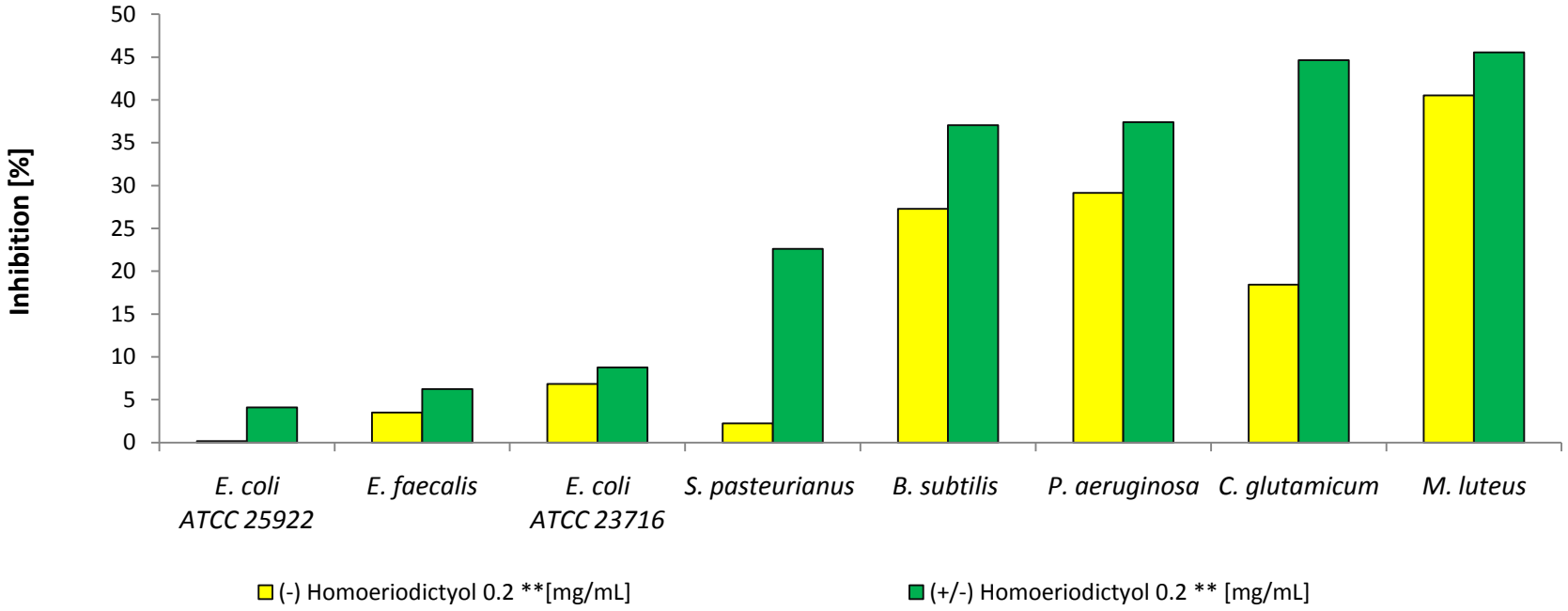
4.4.4. Homoeriodictyol

A comparison of the homoeriodictyol racemate and its (–) enantiomer on the inhibitory effect against the eight chosen microorganisms is presented in Figure 72. The results were compatible to the results of eriodictyol. However, the flavanone homoeriodictyol showed a higher inhibitory effect by its racemate against every microorganism. The analyses of the racemate and (–) homoeriodictyol against *S. pasteurianus* showed a higher difference, which amounted to 20% (2% of inhibitory effect of the *S*-(–) enantiomer and 22% of the racemate). The highest difference (26%) was observed against the growth of *C. glutamicum*, while the racemate showed 44% of the inhibitory effect and the *S*-(–) enantiomer reached only 18%. The lowest difference was observed against *E. coli* ATCC 23716, amounting to 2% of the inhibitory effect. Three bacteria, including *E. faecalis*, *E. coli* ATCC 25922 and *M. luteus*, showed lower differences between the inhibitory effects as to the racemate and *S*-(–) homoeriodictyol, which amounted to 3%, 4% and 5%, respectively. *P. aeruginosa* and *B. subtilis* exposed 8% and 10% of the differences of the antimicrobial activities between the homoeriodictyol racemate and its (–) enantiomer.



Eriodictyol [mg/mL]	Growth inhibitory effect of eriodictyol [%]							
	E. coli **ATCC 23716**	***E. coli*** **ATCC 25922**	***M. luteus*** **ATCC 10240**	***C. glutamicum*** **ATCC 13032**	***S. pasteurianus***	***E. faecalis*** **ATCC 19433**	***P. aeruginosa*** **ATCC 10145**	***B. subtilis*** **ATCC 6633**
(-) Eriodictyol	0.00	3.42	5.24	9.95	10.69	24.68	31.05	36.66
(+/-)Eriodictyol	0.75	8.54	13.64	16.41	24.40	22.32	39.82	43.89
Concentration [mg/mL]	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2

Figure 71. Growth inhibitory effect of eriodictyol racemate and its enantiomers against eight chosen microorganisms



** For *B. subtilis* 0.1 mg/mL

Homoeriodictyol [mg/mL]	**Growth inhibitory effect of homoeriodictyol [%]**							
	E. coli **ATCC 25922**	***E. faecalis*** **ATCC 19433**	***E. coli*** **ATCC 23716**	***S. pasteurianus***	***B. subtilis*** **ATCC 6633**	***P. aeruginosa*** **ATCC 10145**	***C. glutamicum*** **ATCC 13032**	***M. luteus*** **ATCC 10240**
(-) Homoeriodictyol	0.15	3.48	6.85	2.25	27.27	29.15	18.44	40.53
(+/-) Homoeriodictyol	4.10	6.24	8.77	22.62	37.04	37.41	44.62	45.56
Concentration [mg/mL]	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2

Figure 72. Growth inhibitory effect of homoeriodictyol racemate and its enantiomers against eight chosen microorganisms

5. Discussion

This chapter is presented in a manner similar than chapter 4, the results chapter, along with an arrangement into several subchapters. In the first subchapter the results are discussed in the context of published data in regards to the extraction of flavanones from various plants. The following two parts are devoted to the discussion on antimicrobial activities of racemates and then regarding the enantiomers with respect to various substances and microorganisms, involving available literature.

5.1. Extraction of flavanone from various plants

A lot of plants are rich in many useful compounds, which also possess antimicrobial capacity. It is well known that many fruits and vegetables, especially citrus fruits, are rich in flavonoids. For the extraction of the flavanones considered for this thesis, three citrus fruits, including grapefruit, mandarins and oranges, were chosen. The content of flavanones was also reported in tomatoes, thyme and peanuts. The literature, as well as analyses from this thesis (data not shown herein) confirms that the highest concentration of flavanones is contained in the peel of the fruits and of vegetables [E.G. MANTHEY & GROHMANN 1996].

Chapter 4.1 presents the results of the extraction of flavanones from the chosen plants and demonstrates the differences in their prevalence and content. In this work, in the peels of grapefruit the presence of naringenin was recorded. The authors of the US PATENT 6096364, YAÑEZ ET AL. (2007B) stated also the occurrence of naringenin, as well as the presence of isosakuranetin, eriodictyol, hesperetin and hesperidin in this citrus fruit. The results of this thesis showed also that the peels of mandarin contained naringenin, homoeriodictyol and hesperetin and in the peels of orange only homoeriodictyol was recognized. However, the reports of EL GHARRAS (2009), ERLUND (2004), GATTUSO ET AL. (2007) and HO ET AL. (2000), as well as the US PATENT 6096364 (2000) stated that in both mandarins and oranges the occurrence of naringenin, hesperetin and hesperidin was recorded. No data was found about the presence of homoeriodictyol in these both described citrus fruits. According to the results from this thesis and from the indicated literature, it can be concluded that these small differences in the content of the chosen flavanones are presumably caused by the use of different fruit species.

KRAUSE & GALENSA (1992), JUSTESEN ET AL. (1998), BUGIANESI ET AL. (2002), ERLUND (2004) and YAÑEZ ET AL. (2005) demonstrated that the extract from tomatoes contains naringenin. According to the analysis from the report of KRAUSE AND GALENSA (1992), the tomato ketchup was examined in this thesis. The analyses of this work confirmed the occurrence of naringenin.

MARTIN ET AL. 2007 and YAÑEZ ET AL. 2007B stated that various species of thyme contain especially naringenin and eriodictyol. This thesis presents, and at the same time confirms, the occurrence of these both flavanones in thyme and showed also, that the extract of peanut hulls contained the same flavanones, including naringenin and eriodictyol. While the content of eriodictyol in peanut hulls was widely studied [E.G. WEE ET AL. 2007, YAÑEZ ET AL. 2007B], the occurrence of the other flavanone was not reported in the available literature.

There were also differences observed in the occurrence of individual enantiomers contained in the plant extracts of thyme and peanut hulls. The higher contents of naringenin and eriodictyol in thyme and of eriodictyol in peanut hulls allowed for examining the prevalence and content of the individual enantiomers of these substances. The analyses from the present thesis confirmed the report of YAÑEZ and coworkers (2007), which stated that naringenin found in thyme consisted of nearly 97% of the *S*-(–) configuration, while the eriodictyol occured in this plant with 90% of the *S*-(–) enantiomer. The same authors reported that also eriodictyol extracted from peanut hulls, consisted in this fruits only as the (–) enantiomer, which could also be confirmed by this thesis work (chapters 4.1.5 and 4.1.6).

5.2. Chiral separation technique

During the past years, the flavanones have been separated in various modes using different CSPs. For the enantio-separation of all of the flavanone aglycones in this thesis, one chiral column, named Europak was used, which contains amylose 3,5-dimethylcarbamate as the active separation medium. The separation of each substance was well achieved, with the exception only of homoeriodictyol, which enantiomers could not be separated until the baseline. During the development of the method for chiral separation for this work, it was observed that the compounds with a substitution at the 3' and 4' position (both hydroxyl or methoxy group), such as naringenin, isosakuranetin and hesperetin, could be resolved by using pure MeOH as the mobile phase. The compounds with the substitution at 4' and 5' positions, besides of MeOH, needed a small portion of water, with 5% for eriodictyol and 8%

for homoeriodictyol. This suggests a relationship between polarities of the substances and of the eluents. On the basis of the separations of eriodictyol (containing two hydroxyl groups) and homoeriodictyol (with hydroxyl and methoxy groups), it was demonstrated that with increased polarity of the compound, the polarity of eluent has to be decreased. The substitution of a less polar methoxy group for a more polar hydroxyl group increases the retention time [e.g. KUZNETSOVA 1970]. This relationship was observed for the separation on the Europak of naringenin (with an OH group) and of isosakuranetin or hesperetin (both containing OCH_3). However, homoeriodictyol (OCH_3) eluted faster than the enantiomers of eriodictyol containing two hydroxyl groups. The same observation was made by DOWD & PELITIRE (2008) by performing the chiral separation of other substances.

YAÑEZ ET AL. in their review from 2007 stated that although naringenin, eriodictyol and homoeriodictyol could be separated under reverse and normal phase conditions on modified MCCTA (microcrystalline cellulose triacetate, including CTA I, CTA II, or CTA III, according to KRAUSE & GALENSA (1988), and KRAUSE & GALENSA (1990)), the enantiomers of isosakuranetin were successfully resolved only on the CTA II column [YAÑEZ ET AL. 2007]. CACCAMESE ET AL. (2005) presented also CD spectra of the chiral separation of naringenin on the Chiralcel OD-H column, and of eriodictyol and hesperetin separation on the Chiralpak AS-H column. The authors reported that the first peak of naringenin separated on the Chiralcel OD-H column presented the maximum within the positive spectrum and thereby proved to be the *R*-(+) enantiomer. However, the CD spectrum from the separation on the Europak column purchased from Knauer showed that the S-(–) naringenin enantiomer eluted at first. Such a noticeable difference was not observed for the chiral separation of eriodictyol on the Chiralpak AS-H column. The changes in the elution of enantiomers were driven by the differences in kind of the filling of the columns. The Chialcel OD-H column consists of cellulose tris-3,5-dimethylphenylcarbamate, the Chiralpak AS-H of tris(*S*)-1-phenylethylcarbamate, and the column used in this thesis, Europak, consists of amylose 3,5-dimethylcarbamate coated on silica gel. Moreover, it was observed that by using the Europak column the retention times for all substances were shorter in comparison to previous studies using the other CSP-columns [e.g. KRAUSE & GALENSA 1988, CACCAMESE ET AL. 2005]. For example, the elution for both enantiomers of eriodictyol on the Chiralpak AS-H column lasted for around 35 min [CACCAMESE ET AL. 2005], while on the Europak column the elution time was around 12 min. Furthermore, with only a few changes in the polarity of mobile phase, the Europak column used in this work was able to resolve all of the tested flavanone aglycones.

This involves saving of time and the use of eluents, which is more economical and can be recommended for the separations of flavanones.

5.3. Antimicrobial activity of analyzed racemates

The fruits and vegetables used during industrial production generate a large number of byproducts, including peel, when utilizated brings a marginal profit for the business. In addition, it has long been recognized and this work also confirms it that flavanones, predominantly, occur in the peel of the fruits and vegetables. Furthermore, there is an increased effort in trying to avoid chemical preservatives and to develop naturally occurring substances as antimicrobials in foods because of the growing interest in so-called natural foods. Therefore, in the present study the antimicrobial activities of selected flavanones, which were extracted from the citrus and vegetable peel, were evaluated.

5.3.1. General antimicrobial activity of flavanone racemates

Antimicrobial properties of five flavanones and one flavanone glycoside were measured against selected Gram-positive and Gram-negative bacteria and yeast. The results showed that the different investigated bacterial species exhibited significant different sensitivities towards these substances. In addition, different strains of the same bacterial species (*E. coli*) showed differences in sensitivity against the same flavanone. Furthermore, various applied concentrations of the flavanones inhibited the growth of individual microorganisms to varying degrees. For instance, 0.1 mg/mL of naringenin showed 7% of inhibitory effect against *C. glutamicum* and 9% against *M. luteus*, while 0.4 mg/mL of the flavanone showed 59% and only 24% of growth inhibition, respectively, for the same microorganisms. It seems to be logical that increasing concentration also increase the degree of growth inhibition. However, each flavonoid showed its own strength of inhibition which was demonstrated as various degree of slope (more explanation in the chapter 5.3.2).

In general, there was no variation observed between the permeability of cell walls of Gram-positive and Gram-negative bacteria for flavanones. However, the differences in the cell walls of each microorganism and differences in the structure of the substances showed in turn some differences in the antimicrobial activity of individual flavanones, which will be explained below. To demonstrate clearly the differences between the antimicrobial properties of individual flavanones against all microorganisms, the concentration of 0.2 mg/mL was

chosen as a representative concentration upon which Figure 73 (next page) was created for discussing the subject further.

When considering all inhibitory tests, the strongest inhibitory effect against *B. subtilis* showed homoeriodictyol, amounting to 80%, at the concentration of 0.2 mg/mL (Fig. 73). This microorganism proved also to be the most sensitive one in regards to all flavanones tested in this work. The highest concentration of 0.7 mg/mL of naringenin showed also a strong activity against *C. glutamicum*, which reached more than 70%. In general, the strongest antimicrobial activity exposed naringenin, showing the highest inhibitory effects against five bacteria, including *C. glutamicum*, *E. faecalis*, *P. aeruginosa* and both strains of *E. coli* (Fig. 61, chapter 4.3.2.1).

On the basis of the presented results, the various antimicrobial properties of the substances with similar structure can suggest that the degree of the hydroxylation and methoxylation in the molecule may have an effect on the antimicrobial capacity of flavanones. Consistent to this observations, the study of TSUCHIYA ET AL. (1996) presents that 5,7-dihydroxylation of the A ring and 2',4'- or 2',6'-dihydroxylation of the B ring in the flavanone structure was responsible for the inhibition of the methicilin-resistant *S. aureus*. However, the 2',4'-hxdroxylation was more important in the growth inhibition than the 2',6'-dihydroxylation. CUSHINE & LAMB (2007B) reported also that one, two or three additional hydroxyl groups at the 7, 2', 4' positions in 5-hydroxyflavanones and 5-hydroxyisoflavanones exhibited the inhibitory effect against *S. mutans* and *Streptococcus sobrinus*. Furthermore, they indicated the presence of hydroxyl groups at the 7 and 4' positions in 5-hydroxyflavones and 5-hydroxyisoflavones, which did not show this antimicrobial activity. In addition, two isoflavones with hydroxylation at the 5, 2', 4' positions exhibited an intensive inhibitory effect against more strains of *Streptococcus*. This may suggest that the substitution of the hydroxyl group at the 2' position is important for antimicrobial activity. However, more researchers noted also the importance of hydroxyl group at the 5 position in regards to the inhibitory effect of flavanones and flavones. [CUSHINE & LAMB 2005, XU & LEE 2001, SUKSAMRARN ET AL. 2004, NAIDU 2000]

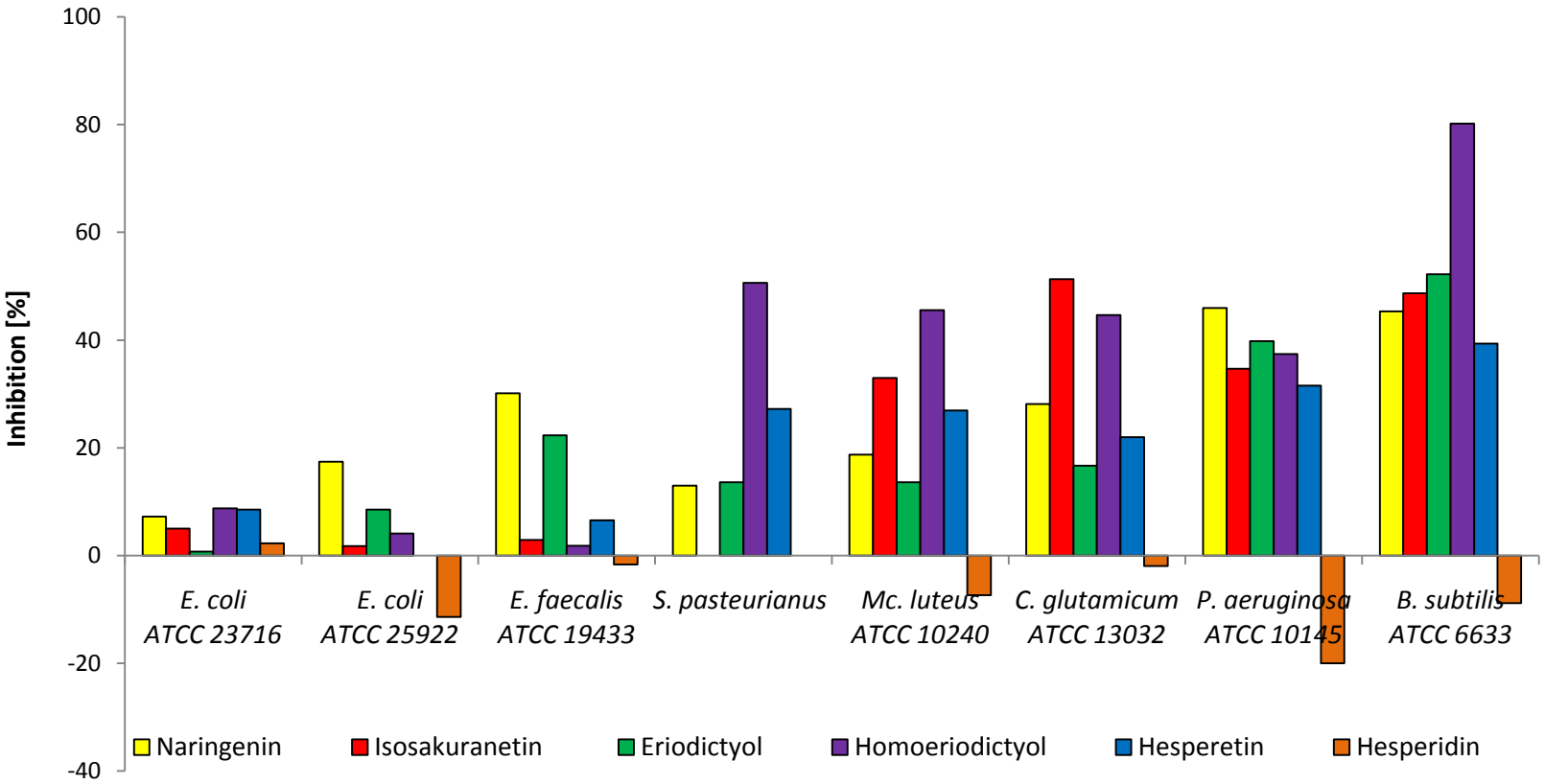


Figure 73. Comparison of inhibitory effects of all used substances at the concentration of 0.2 mg/mL against eight chosen microorganism; the negative values on the graph indicate the growth stimulation

Consequently to these previous observations, our findings also indicate this correlation. All flavanones chosen for this work contain the hydroxylation at the 5 and 7 position in the A ring. Except from hesperidin and although in different degrees, all of the aglycones exhibited an inhibitory effect against various microorganisms. As it has already been mentioned, the highest inhibitory effect in comparison to other tested flavanones, in general, exhibited naringenin having the hydroxylation at the 4' position. In addition, but more slightly than naringin, a good antimicrobial capacity showed eriodictyol against all microorganisms with the hydroxylation at the 4' and 5' position. It suggests that the additional hydroxylation at the 5' position decreases the antimicrobial properties of the flavanone. The 0.4 mg/mL concentration of eriodictyol showed also a higher inhibition of the growth of *B. subtilis* than naringenin. Homoeriodictyol containing also one hydroxyl group in the 4' position and a methoxy group at the 5' position presented the best antimicrobial activity against *B. subtilis*, *M. luteus* and *S. pasteurianus*. The inhibitory effect against *C. glutamicum* exhibited as high as thus of naringenin.

CUSHINE & LAMB (2005) and also XU & LEE (2001) indicated that the presence of methoxy groups in the structure of flavonoids decreases the antimicrobial activity drastically. However, NAIDU (2000) reported that flavanones with the A ring fully substituted or at least containing two methoxy groups showed an antifungal activity. Based on the results of this thesis (cf. Fig. 73) it is noticeable that the best activity against the yeast *S. pasteurianus* was shown by homoeriodictyol and hesperetin at the concentration of 0.2 mg/mL. Both compounds contain one methoxy group at 5' and 4' position, respectively. Furthermore, isosakuranetin, which possesses one methoxy group at the 4' position instead of the hydroxyl group in naringenin, exhibited a good inhibitory effect against *S. pasteurianus* too (Fig. 62, chapter 4.3.2.2). However, except for *M. luteus* and *B. subtilis*, isosakuranetin showed a slighter antibacterial activity than naringenin. Hesperetin in comparison to the structure of isosakuranetin possesses an additional hydroxyl group at position 3'. However, both exhibited quite similar antimicrobial activities against the tested microorganisms.

The antimicrobial analyses described in this work confirms the previous reports from the literature that in general, the presence of methoxy groups leads to a higher antifungal activity and the occurrence of hydroxyl group in the structure intensifies the antibacterial capacity. However, this observation depends on the strains. On the basis of the presented results, it can be concluded that *M. luteus* and *B. subtilis* are more sensitive to flavanones containing a

methoxy group in their structure (homoeriodictyol, isosakuranetin, hesperetin), rather than a hydroxyl molecule, such as naringenin.

Furthermore, in this thesis it was observed that all flavanones containing a methoxy group, such as homoeriodictyol, isosakuranetin, and hesperetin, showed only a very slight activity against all faecal bacteria. In addition, *E. coli* proved to be the most resistant bacterium. ALVAREZ ET AL. (2008) reported that *E. coli* in the outer membrane possesses proteins called porins, which form a large water-filled pore as a pathway for the exchange between external environment and the interior of the cell. This allows for the diffusion of ions and hydrophilic molecules of low molar mass. From the report of ALVAREZ ET AL. (2008) it can be assumed that the high resistance of *E. coli* was caused by the slight water solubility of the flavanones and the permeability of the porins. Moreover, the authors stated that most of the strains from the *Enterobacteriaceae* family possess also nonspecific porins for the entrance of small hydrophilic molecules and can, furthermore, moderate the porins content, which can show an additional resistance mechanism. This may explain the differences in sensibility between both strains of *E. coli* examined in this thesis with the presence of flavanones.

In previous literature the antimicrobial properties of various flavonoids was reported. RAUHA ET AL. (2000) presented their results of inhibitory activity of Finnish plant extracts containing flavonoids. The authors also analyzed naringenin as a pure substance against nine bacteria, one mould and two yeasts. The results showed that this flavanone in the concentration of 500 µg per Petri dish, exhibited strong antimicrobial effects against *S. aureus*, *M. luteus*, *B. subtilis* ATCC 9372 and two strains of *Salmonella epidermis*. A clear antimicrobial ability of naringenin was presented against *E. coli* ATCC 11775 and *B. subtilis* ATCC 6633 (also tested in this thesis). They reported also a slight antimicrobial capacity against *S. cerevisiae*, although there was no activity observed against *Aspergillus niger* and *Candida albicans*. In contrast to these described results, those from this work showed that naringenin, however used at higher concentrations than by RAUHA ET AL (2000), exhibited the strongest activity in comparison to all of the tested flavanones.

PROTOES ET AL. (2006) studied the antimicrobial properties of flavonoids in the Greek aromatic plants. They showed that the extract from the plant *Astanea vulgaris* containing naringenin, quercetin, apigenin and rutin, exhibited a clear antimicrobial capacity against the food-borne pathogen *Listeria monocytogenes* and a slight activity against *E. coli* O157:H7, *B. cereus* and *Pseudomonas putida*. The extract did not present any inhibitory effect against *Salmonella enteridis* and *S. aureus*. However, they stated as well that the extract from *Styrax*

officinalis consisting of naringenin, quercetin, (+) catechins hydrated and (–) epicatechin, showed no activity against the pathogens, including *L. monocytogenes*, *S. enteridis* and *S. aureus*. This mixture exhibited only a slight antimicrobial activity against *E. coli*, *B cereus* and *P. putida*. However, MANDALARI ET AL (2010) indicated that naringenin possesses a good activity against *S. aureus*, *L. monocytogenes* and *S. enterica* in the range between 250–500µg/mL. In addition, ULANOWSKA ET AL. (2007) showed that by estimation of generation times in liquid bacterial cultures, naringenin in various concentrations did not significantly influence the growth rate of *B. subtilis*, but exhibited a significant effect on *E. coli* and the strain of *M. luteus* by showing complete growth inhibition. MANDALARI ET AL. (2007) presented the antimicrobial activity of hesperetin, eriodictyol and naringenin against *E. coli*, *B. subtilis* and *S. cerevisiae*. In contrast to the results from the present thesis, the strongest inhibitory effect against all bacteria was observed for eriodictyol. Eriodictyol was also reported in the mixture with quercetin as an extract from *Humulus lupulus* and presented clearly an inhibition of the growth of *L. monocytogenes* and moderately of *P. putida* [PROTEOS ET AL. 2006], while isosakuranetin was stated as active against *Cryptococcus neoformans* [DA SILVA FILHO ET AL 2008]. These presented variations of the antimicrobial activity from different studies may be reflected due to differences in the methods used for the analyses. Similar conclusions have been drawn also by CUSHINE & LAMB (2007B) in their review.

5.3.2. Antimicrobial mechanisms of the action of flavonoids

There are a few research works that dealt with the mechanisms underlying the antimicrobial activities of flavonoids. The literature indicates that different compounds within one group may have an effect on different components and functions of microbial cells. It is also possible that various flavonoids may act on more than one specific spot in the bacterial cell.

CUSHINE & LAMB (2007) described that it is possible that the flavonoids strongly inhibited the DNA in certain microorganisms, while in the others inhibit the RNA synthesis. PLAPER ET AL. (2003) reported that the compounds with the B-ring hydroxylation caused the inhibition of DNA gyrase which further inhibited the enzyme's ATPase activity. Other suggested mechanisms were described as an inhibition of topoisomerase or even as a damage of the bacterial membranes which causes an increase of permeability of the inner bacterial

membrane and a dissipation of the membrane potential. Although most researchers worked on the antimicrobial mechanism of action of flavanones, and dealt with the question whether flavonoid activity is bacteriostatic or bactericidal, both issues are still unclear. [CUSHINE & LAMB 2005, SOUSA ET AL. 2009, ULANOWSKA ET AL. 2007, PIIPPONEN-PIMIÄ ET AL. 2001, XU & LEE 2001, LIN ET AL. 2005, FISHER & PHILLIPS 2008, HAVESTEEN 2002]

5.3.3. Linear relationship between increase of the concentration and growth inhibition

Every substance was analyzed in various concentrations against each microorganism. A relationship between the increase of the substances' concentration and growth inhibitory effects was observed. This relationship is presented as an example of various concentrations of naringenin subjected against *B. subtilis* (Fig. 74). It shows that the increase in concentration of naringenin [mg/mL] caused a linear increase of the growth inhibition of *B. subtilis* (%).

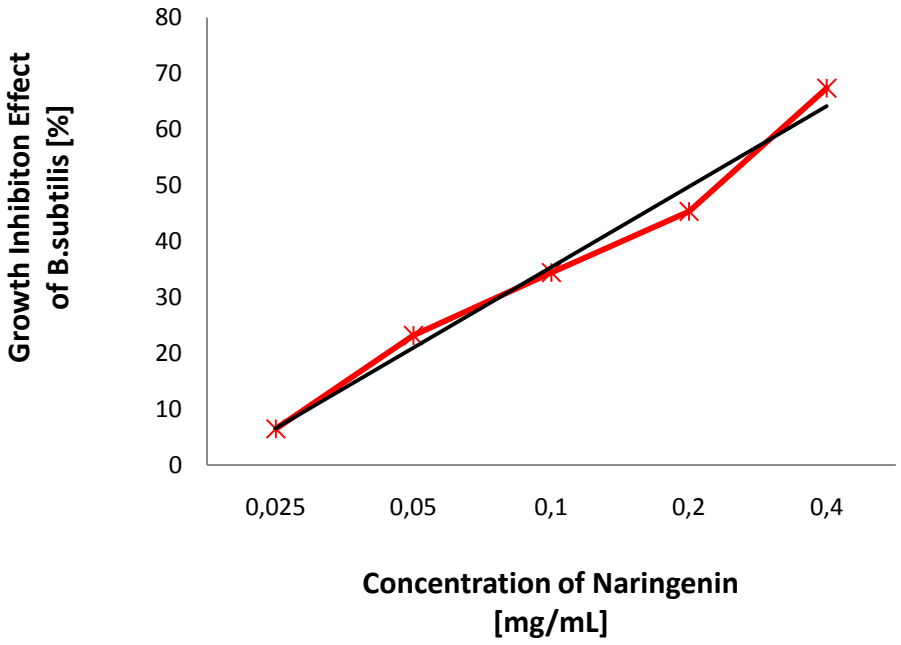


Figure 74. Linear Relationship between Concentrations of Naringenin and Inhibitory Effect of *Bacillus subtilis*, where the Red Line is the Line of Relationship and the Black is the Trend Line

It is logically understandable that an increase in concentration causes an increase of the inhibitory effect. However, as it has been already mentioned, each flavanone possesses an

individual capacity to inhibit the growth of microorganisms. These differences in inhibitory strength between the various flavanones are displayed by the degree of slope of the lines.

Furthermore, this linear relationship was not observed for every flavanone against the tested microorganisms. An increase of the naringenin concentration caused a linear increase of growth inhibition for all microorganisms, except for *P. aeruginosa* and *E. coli* ATCC 23716. This linear correlation was also presented for isosakuranetin against *E. coli* ATCC 23716, as well as against *M. luteus*, *B. subtilis* and *S. pasteurianus*. Besides *P. aeruginosa*, with the increase of concentration of eriodictyol, every other microorganism showed the linear increase of inhibitory effect. The data of homoeriodictyol showed a linear relationship for the growth inhibition of the pathogenic strain of *E. coli*, *C. glutamicum*, *M. luteus* and *B. subtilis*. However, for the last bacteria, the highest concentration of homoeriodictyol (0.4 mg/mL) showed a lower inhibitory effect than the concentration of 0.2 mg/mL. Hesperetin demonstrated only partially the linear relationship against the investigated microorganisms, while hesperidin, showed almost no antimicrobial activity. However, the growth stimulation caused by the flavanone glycoside was also mostly recorded in the form of linear relationship along with the increase of concentration. The non-linear increase of the inhibitory effect of *P. aeruginosa*, observed with the increase of concentration, could be related to growth problems while performing the experiment. However, for the other microorganisms, a reasonable explanation of non-linear increase could not be given.

5.3.4. Hesperetin and hesperidin – the differences

It is important to bring into focus a comparison of the antimicrobial activities of two substances, i.e. hesperetin and hesperidin. Hesperetin, when considering all tested flavanones, did not show the highest antimicrobial activity, but still a good inhibitory effect (up to 50%). Its flavanone glycoside, hesperidin, presented, however, almost no antimicrobial capacity against the tested microorganisms but even stimulated their growth (Fig. 73).

Hesperidin, as it was described in the chapter 2.2.6., is comprised of hesperetin and an attached disaccharide, rutinose, which consists of one glucose molecule and of one rahmnose molecule. Hence, the molecule of the flavanone glycoside, as well as its molecular weight is twice as much as the molecule of the other tested substances, especially hesperetin. It suggests the following explanations as to why the antimicrobial activity of these two compounds showed such significant differences. Firstly, the sugar in the structure and thereby bigger size

of the molecule and higher molecular weight of hesperidin shows that the same concentrations of hesperetin and hesperidin gave different amounts of the antimicrobial active structure. Hesperetin was consumed by microorganisms in its entirety as an antimicrobial agent, while hesperidin consisted of the hesperetin molecule and a small amount of sugar. Therefore, the inhibitory effect of hesperidin was also less active than the effect of its aglycone. On the other hand, it seems that the microorganisms were able to break the bond between the molecule of flavanone and rutinose. Moreover, this disaccharide may have been broken down into two monosaccharides and may have been utilized by the microorganisms as building material.

It can be assumed that also the shape of the structure and the size of the molecule might cause some problems by the diffusion through the cell membrane, and thereby, influence on the antimicrobial activity (the steric hindrance). NAIDU (2000) noted as well that the substitution of the hydroxyl group with a sugar moiety at the 7 position may also decrease or even completely diminish the inhibitory effectiveness. Furthermore, GARG and colleagues (2001) informed that hesperidin was not only inactive against the following bacteria, *S. aureus*, *Streptococcus hemolyticus*, *Klebsiella* species, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexneri* and *Vibrio cholerae*, but also showed no antifungal effect against *Trichoderma glaucum*, *Aspergillus flumigatus* and *A. niger*. All of these facts may clarify why hesperidin did not show almost any inhibitory effect but even intensified the microbial growth rate of the tested microorganisms.

It is also interesting that the non-pathogenic strain of *E. coli* showed the highest and similar resistance against both hesperetin and hesperidin. It can be assumed that this bacterium does not possess the ability to degrade the disaccharide into monosaccharides. Consistent with this observation, MANDALARI ET AL. (2007) stated that except for neoeriocitrin against *E. coli*, no inhibition was evident with any of the flavonoid glycosides, including neohesperedin, neoeriocitrin and naringin. The exception of *E. coli* might confirm the diversified permeability of the membranes for this bacterium. It is also worthy to note that the concentration of 0.1 mg/mL of hesperidin showed an inhibitory effect against *C. glutamicum* and *M. luteus*, while the higher concentrations exhibited only growth stimulations. However, regarding currently available facts, no explanation for this observation can be given.

5.3.5. Comparison to antibiotics

The extracts with different degrees of antimicrobial activity were compared with the reference standard (according to Fig. 61, Fig. 62, Fig. 63, Fig. 64, Fig. 65, Fig. 66, chapter 4.3.2). For the bacteria, the concentration of 0.2 mg/mL of tetracycline was investigated and for the yeast the same concentration of the antibiotic, natamax. The comparison with the antibiotic showed that at the same concentration of naringenin, a higher inhibitory effect was only observed against *P. aeruginosa*, amounting to 44% and 46%, respectively. Isosakuranetin in comparison to tetracycline presented higher antimicrobial activity against *M. luteus* (33% and 21% for the antibiotic). Homoeriodictyol showed a stronger inhibitory effect than the tetracycline against *B. subtilis* (80% and 65% respectively) and also against *S. pasteurianus*, 51% for the flavanone and 42% for natamax. A similar behavior but with slighter antimicrobial capacity in comparison to tetracycline, showed isosakuranetin against *C. glutamicum* (51% for the flavanone and 60% for the antibiotic) and eriodictyol against *P. aeruginosa* with 40% for the flavanone and 44% for tetracycline. By an increase of concentration up to 0.4 mg/mL, several substances presented a higher percentage of inhibition than with 0.2 mg/mL of the antibiotic. However, due to the difference of the concentrations, they cannot be exactly compared to each other.

Overall, the tested flavanones appear to act on some of the selected bacteria more specifically than tetracycline, and therefore further studies should be continued in order to extend the possibility of finding natural antibiotics.

5.4. Antimicrobial activities of analyzed enantiomers

Nature produces a huge number of chiral compounds. It has also been confirmed by this work that several plants contain only one of the enantiomers. It is also well known that the enantiomers differ from each other in terms of some biological activities. The objective of this thesis was also to determine whether the enantiomers could exhibit better antimicrobial activities than their racemates.

In this work, the enantiomers and the racemates of four flavanones were tested against seven microorganisms. The results presented in chapter 4.4 showed the comparison between the antimicrobial properties of both the enantiomers and racemates for naringenin and isosakuranetin as well as between the (–) enantiomer and racemate for eriodictyol and

homoeriodictyol. The figures (Fig. 67, Fig. 68, Fig. 69, Fig. 70, chapter 4.4) present many differences in terms of the activity against various microorganisms between the individual enantiomers and racemates. The *R*-(+) configuration of naringenin exhibited the highest antimicrobial activity against *P. aeruginosa* and *C. glutamicum* and the same enantiomer of isosakuranetin against *C. glutamicum* and *E. coli*. The exception were isosakuranetin and eriodictyol against *E. faecalis*, as well as for isosakuranetin and naringenin against *B. subtilis* and *E. coli*, the *S*-(–) configuration showed the slightest antimicrobial ability. Only in case of isosakuranetin against *E. coli*, both enantiomers showed slightly stronger antimicrobial activity than the racemate. A comparison between the (–) enantiomer and the homoeriodictyol racemate showed, although in very different percentage values, but evidently, a stronger growth inhibition of the microorganisms by the racemate. In general, the phenomenon of synergism of the presence of both enantiomers was observed, since the racemates showed definitely the strongest antimicrobial properties. It is also clear to see that each chiral structure possess a different and individual inhibitory capacity against various microorganisms. Moreover, due to the differences in the activity of the individual enantiomers, it can be speculated that a model exists with two independent bacterial receptors for *R*- and *S*-configuration, each with a specific affinity to one of them.

Consistent to the results obtained in this thesis, the AGGARWAL ET AL. (2002) observed that the *R*-(+) carvone and the *R*-(+) limonene were more active than their (–) optical forms, while the racemates were not investigated. However, the study of VUUREN & VILJOEN (2007) reported that the highest inhibitory effect against more bacteria was demonstrated by the (–) limonene in comparison to its (+) configuration and the racemate. POMINI & MARSAIOLI (2008) compared the antimicrobial activities of the enantiomers and the racemates of *N*-(3-oxo-octanoyl)-HSL against *B. cereus*. The results demonstrated that the *R*- and *S*-configurations exhibited similar inhibitory effects, while the racemate presented the lowest antimicrobial activity against this bacterium. Based on all of the results it can only be assumed that the different chiral configurations from various substances exhibit also diversified antimicrobial properties.

6. Summary

The microbial quality of food products has a high importance and a broad influence on their shelf life. The term, food preservation refers to all treatments taken against any spoilage of food. Many of techniques used in food industry have been associated with adverse changes in organoleptic characteristics and loss of nutrients. Furthermore, day by day, the food stuffs are also sold in areas far remote from their production places. Besides, microorganisms, including food borne pathogens, acquire a new resistance to used antimicrobial processes and agents.

The consumers nowadays expect also from the food industry additive-free, fresh, natural and high nutritional value food products. The food stuffs should be moderately cheap, of good texture, natural flavor and taste. There is also a demand for the simplicity of preparation, adequate durability and microbiological safety of the food. The consumers are also increasingly avoiding these highly processed food stuffs and food prepared with chemically-synthesized preservatives. They hope that the high standards for product quality will be met as far as possible minimum processing of their food. These demands, as well as the greater consumer awareness and concerns regarding the use of synthetic chemical additives has lead to greater interest in natural preservatives.

Naturally occurring antimicrobials are abundant in the environment. Many of the plant ingredients are organic substances that play a role in the protection of plants because of their antimicrobial activities. It is also well-known that compounds containing phenolic rings exhibits antimicrobial properties. The group of organic compounds and plant extracts – flavonoids with the subgroup of flavanones is naturally and overall, safe and healthy for the human body. They can also act as potent antioxidants, metal chelators and antimicrobial compounds.

The objective of this study was focused on the determination whether the chiral flavanones, including naringenin, isosakuranetin, eriodictyol, homoeriodictyol, hesperetin and hesperidin, occurring and extracted from citrus fruits, tomatoes, thyme and peanuts, could affect the food antimicrobial protection against microorganisms. These may influence the deterioration or even spoilage of food and cause the human diseases. For the purpose of the thesis the following bacteria were selected, *C. glutamicum*, *B. subtilis*, *M. luteus*, *E. coli*, *E. faecalis* and *P. aeruginosa*, as well as yeast, *S. pasteurianus*. Furthermore, four of the

flavanones, naringenin, isosakuranetin, eriodictyol and homoeriodictyol were chiraly separated and analyzed in the order to determine the antimicrobial activities of each enantiomer and to compare among each other and to the racemate.

The flavanones extraction from the plants was carried out using the HPLC technique and the collected peaks were identified on the MS. The HPLC devices was also utilized for the enantio-separation of the substances, however, coupled with the chiral column consisting of amylose-3,5-dimethylcarbamate (Europak). The optical activity of the compounds was obtained using the CD. The antimicrobial properties of the flavanones were investigated using three microbiological methods, including, agar dilution technique as well as macro- and micro-dilution techniques on the BHI medium for all of the bacteria and YNB for the yeast. The inhibitory effect of each substance on the food important microorganisms was studied in the various concentrations.

The flavanones are mostly concentrated in the peel of fruits and vegetables. The extraction from grapefruit showed contents of one compound from the group, naringenin. The assay of mandarins indicated the occurrence of flavanones naringenin, homoeriodictyol and hesperetin. The content of homoeriodictyol was recorded for the peels of orange. According to the results obtained during the tests in this study, naringenin was presented also in tomatoes. The results obtained from the extractions from thyme and peanut hulls showed that they contain naringenin and eriodictyol. Furthermore, it was investigated, that the naringenin and eriodictyol extracted from thyme consisted of the *S*-(–) configuration in 97% and 90%, respectively and eriodictyol obtained from peanut hulls was recorded in 100% as the *S*-(–) enantiomer.

The chiral separation carried out using the Europak column showed very good enantio-resolutions for all five tested flavanones containing one chiral center. The retention times of the compounds were shorter in comparison to the previously analyzed kinds of CSP columns. The agar dilution test did not give satisfactory results and was considered inaccurate. Hence, the antimicrobial activity was further investigated using two other techniques, macro- and micro-dilution. The results presented that except for hesperidin, all of the flavanones racemates possess the ability to inhibit growth of investigated microorganisms, however, with a different potency. In addition, various concentrations inhibited the growth of individual microorganisms to varying degrees. It was also observed that with the increase of concentration of the flavanones in the sample an increase of the inhibitory effect was recorded.

The best inhibitory effect showed the homoeriodictyol concentration of 0.2 mg/mL against *B. subtilis* amounted to 80%. In general, the best acting substance proved to be naringenin which showed the highest inhibitory effects against five bacteria, including *C. glutamicum*, *E. faecalis*, *P. aeruginosa* and both strains of *E. coli*. Homoeriodictyol exhibited also a strong antimicrobial activity and presented the highest inhibition to the growth of *S. pasteurianus*, *M. luteus* and *B. subtilis*. The lowest antimicrobial activity from the flavanone aglycones showed hesperetin. Hesperidin exhibited only a slightly inhibitory effect against the non-pathogenic strain of *E. coli* ATCC 23716. The reason for low antimicrobial activity of hesperidin might be owing to the presence of the disaccharide in its molecule, which can even cause the growth stimulation. All the extracts have also been compared to the reference standards, tetracycline for bacteria and natamax for yeast, investigated at the concentration of 0.2 mg/mL.

There was no correlation observed between the antibacterial properties of flavanones against the gram-negative or gram-positive bacteria. It is suggested that the presence of the hydroxyl and methoxy group showed an importance in the antimicrobial activity of investigated substances. Previous researchers stated that the hydroxylation at the 5, 2' and 4' position could be important for the antimicrobial ability. The results from this thesis confirmed this suggestion and showed as well that the flavanones containing one methoxy group exhibited the strongest antifungal effect in comparison to substances a with hydroxyl group. They presented also a very good activity against some bacteria strains. The exact mechanism of action of flavanones is still unknown.

As far as it is known, this is the first report of the evaluation of the antimicrobial activity of the individual enantiomers of flavanones. The results from this thesis showed that in the majority of the tests the racemate was the most effective form against the microorganism. It can suggest a kind of synergism between the enantiomers. However the previous literature stated very diverse data about the antimicrobial activity of the enantiomers in comparison to themselves and their racemates.

Because of legislations governing the use of current preservatives, there is an increasing demand for natural and minimally processed ingredients that might sufficiently extend the shelf life of food products and guarantee a high degree of safety. The present study has demonstrated that flavanones present in plants are non toxic and active against a range of food-borne microorganisms, including pathogens. Flavanones may be used as natural antibacterial agents in food systems, thus extending the shelf life of food. It suggests that many by-products of the fruits and vegetables processing industry are a potential and

inexpensive source of natural antimicrobials. However, further studies need to be performed to understand the precise mechanisms responsible for their interactions and then, perhaps due to any combination of different flavanones, flavonoids or other natural substances, it may be found to be a much more effective alternative in the protection of food.

7. Zusammenfassung

Die mikrobielle Qualität von Lebensmitteln hat eine hohe Wichtigkeit und einen breiten Einfluss auf ihre Haltbarkeit. Der Ausdruck Lebensmittelkonservierung bezieht sich auf alle gegen Verderb von Nahrung vorgenommenen Behandlungen. Die Lebensmittelindustrie verwendet viele Techniken um den Verderb zu verhindert, jedoch oft mit ungünstigen Änderungen in den organoleptischen Eigenschaften und einem Nährstoffverlust. Zudem werden Nahrungsmittel auch weit entfernt von ihren Produktionsstätten verkauft. Darüber hinaus erwerben Mikroorganismen in der Nahrung, einschließlich Krankheitserregern, eine Resistenz gegenüber gebräuchlichen antimikrobiellen Prozessen und Mitteln.

Heutzutage erwarten die Verbraucher von der Lebensmittelindustrie zusatzstofffreie, frische, und natürliche Lebensmittel mit hohem Nährwertgehalt. Die Nahrungsmittel sollten einigermaßen preiswert, von guter Konsistenz und mit natürlichem Aroma und Geschmack sein, dabei weiterhin einfach zubereitbar, mit adäquater Haltbarkeit und mikrobiologischer Sicherheit. In zunehmendem Maße vermeiden die Konsumenten stark behandelte Nahrungsprodukte und Nahrung mit chemisch synthetisierten Konservierungsmitteln. Sie wünschen, dass ihren hohen Ansprüchen bezüglich der Produktqualität bei minimaler Behandlung der Nahrung entsprochen wird. All diese Ansprüche, das größere Verbraucherbewusstsein, sowie die Sorge um die Verwendung synthetischer Konservierungsstoffe, führen zu einem gesteigerten Interesse an natürlichen Konservierungsmitteln.

Die Natur ist reich an antimikrobiellen Substanzen. Viele pflanzliche Zutaten sind organische Substanzen, welche die Pflanze vor schädlichen Mikroorganismen schützen. Zudem ist bekannt, dass Substanzen, die Phenolringe enthalten, eine gewisse antimikrobielle Aktivität zeigen. Zu dieser Gruppe gehören Flavonoide mit der Untergruppe von Flavanonen – organische Verbindungen und Pflanzenextrakte, die natürlich vorkommend, sowie sicher und gesund für den menschlichen Körper sind. Sie können auch als starke Antioxidationsmittel, Metall Chelator und antimikrobielle Substanzen wirken.

Ziel dieser Arbeit war es zu untersuchen welche antimikrobiellen Eigenschaften die chiralen Flavanone, einschließlich Naringenin, Isosakuranetin, Eriodictyol, Homoeriodictyol, Hesperetin und Hesperidin, extrahiert aus Zitrusfrüchten, Tomaten, Thymian und Erdnüssen, gegen Mikroorganismen, die die Verschlechterung oder sogar den Verderb der Nahrung

beeinflussen, haben. Hierfür verwendet wurden sowohl Bakterien – *C. glutamicum*, *B. subtilis*, *M. luteus*, *E. coli*, *E. faecalis* und *P. aeruginosa*, als auch Bierhefe – *S. pasteurianus*. Weiterhin wurden vier der Flavanonen: Naringenin, Isosakuranetin, Eriodictyol und Homoeriodictyol, chiral getrennt und im Hinblick auf die antimikrobiellen Eigenschaften der einzelnen Enantiomeren im Vergleich zu den entsprechenden Racematen analysiert.

Der Roh-Extrakt gelöst in MeOH wurde mittels HPLC zur Gewinnung von Einzelsubstanzen fraktioniert und die verzeichneten Peaks wurden auf dem Massenspektrometer identifiziert. Die HPLC wurde auch für die chirale Trennung verwendet. Jedoch wurde dieses Mal die HPLC mit einer chiralen Säule (Europak) verbunden, die aus Amylose 3,5-Dimethylcarbamate besteht. Die optische Aktivität der Substanzen wurde mit Hilfe der CD ermittelt. Die antimikrobiellen Eigenschaften der Flavanonen wurden basierend auf drei mikrobiologischen Methoden untersucht, einschließlich sowohl Agar-Verdünnungstechnik als auch Makro- und Mikro-Verdünnungstechniken auf dem BHI-Medium für alle Bakterien und dem YNB-Medium für die Bierhefe. Die hemmende Wirkung jeder Substanz auf wichtige Mikroorganismen in der Nahrung wurde in den verschiedenen Konzentrationen überprüft.

Die Flavanone sind hauptsächlich in der Schale von Früchten und Gemüse konzentriert. Der Extrakt aus Grapefruit beinhaltete eine Substanz aus der Gruppe der Flavanonen, Naringenin. Die Untersuchung von Mandarinen wies auf das Vorkommen von Naringenin, Homoeriodictyol und Hesperetin hin. Das Vorhandensein des Homoeriodictyols wurde in der Orangenschale nachgewiesen. Entsprechend den während der Versuche in dieser Arbeit erhaltenen Ergebnissen, trit Naringenin in Tomaten auf. Die Extraktionen aus Thymian und Erdnusshülsen ergaben, dass sie Naringenin, und Eriodictyol enthalten. Weiterhin wurde festgestellt, dass das aus Thymian extrahierte Naringenin und Eriodictyol zu 97% und 90% in der *S*-(–) Konfiguration vorlag, das Eriodictyol aus den Erdnusshülsen dagegen zu 100% als *S*-(–) Enantiomer.

Mit Hilfe der Europak-Säule wurde eine sehr gute chirale Trennung für alle fünf getesteten Flavanone mit einem chiralen Zentrum erhalten. Die Retentionszeiten der getrennten Enantiomere waren kürzer im Vergleich zu den zuvor bei der Analyse verwendeten Arten von chiralen Säulen.

Der Agar-Verdünnungstest brachte keine zufrieden stellenden Ergebnisse und wurde als nicht aussagekräftig betrachtet. Daher wurde die antimikrobielle Aktivität im Weiteren basierend auf zwei anderen Techniken, Makro- und Micro-Verdünnung untersucht. Die

Ergebnisse zeigten, dass außer Hesperidin, alle Racemate der Flavanone eine wachstumshemmende Wirkung auf die überprüften Mikroorganismen besitzen, jedoch mit unterschiedlicher Stärke. Außerdem hemmten verschiedene Konzentrationen das Wachstum einzelner Mikroorganismen in unterschiedlichem Maße: grundsätzlich stieg mit Zunahme der Konzentration der Flavanone in der Probe auch die hemmende Wirkung.

Die am besten hemmende Wirkung zeigte die Homoeriodictyol Konzentration von 0,2 mg/ml gegen *B. subtilis*, und betrug 80%. Als die am besten wirkende Substanz zeigte sich Naringenin, das die höchste hemmende Aktivität gegen fünf Bakterien, einschließlich *C. glutamicum*, *E. faecalis*, *P. aeruginosa* und beider Stämme von *E. coli* aufwies. Homoeriodictyol zeigte auch eine starke antimikrobielle Wirkung und die höchste Wachstumshemmung von *S. pasteurianus*, *M. luteus* und *B. subtilis*. Die niedrigste antimikrobielle Aktivität der Flavanonen-Aglykonen zeigte Hesperetin. Bei Hesperidin war nur eine leicht hemmende Wirkung gegen den nicht-pathogenischen Stamm von *E. coli* ATCC 23716 festzustellen. Der Grund der niedrigen antimikrobiellen Aktivität des Hesperidins könnte die Anwesenheit des Disaccharides in seinem Molekül sein, das die Wachstumsstimulierung sogar verursachen kann. Alle antimikrobiellen Eigenschaften der Flavanonen sind auch mit den Referenzstandards, Tetracyklin für Bakterien und Natamax für Hefe (beide in der Konzentration von 0,2 mg/ml) verglichen worden.

Es wurde keine Korrelation zwischen den antibakteriellen Eigenschaften der Flavanonen gegen die gramnegativen oder grampositiven Bakterien beobachtet. Darüber hinaus kann man vermuten, dass die Anwesenheit der Hydroxyl- und Methoxy-Gruppe eine wichtige Rolle in der antimikrobiellen Aktivität der untersuchten Substanzen spielt. Vorhergehende Forschungsergebnisse legen nahe, dass die Hydroxylierung an der 5, 2’ und 4’ Position für die antimikrobielle Fähigkeit wichtig sein könnte. Die Ergebnisse dieser Arbeit bestätigten diese Vermutung und zeigten auch, dass Flavanonen, die eine Methoxy-Gruppe enthalten, die stärkste Wirkung gegen Hefe, im Vergleich zu den Substanzen mit Hydroxylgruppe zeigten. Auch eine sehr gute Aktivität gegen einige Stämme getesteter Bakterien konnte nachgewiesen werden. Dagegen ist der genaue Mechanismus der antimikrobiellen Wirkung von Flavanonen immer noch unbekannt.

Soweit bekannt, ist dies die erste Arbeit über die Auswertung der antimikrobiellen Aktivität der einzelnen Enantiomere der Flavanone. Die Ergebnisse dieser Arbeit zeigen, dass in der Mehrzahl der Tests das Racemat die wirksamste Form gegen den Mikroorganismus ist. Es konnte eine Art der Synergie zwischen den Enantiomeren vorgeschlagen werden. Die

Literatur gab jedoch sehr unterschiedliche Daten über die antimikrobielle Aktivität der Enantiomere an, sowohl im Vergleich zueinander als auch mit ihren Racematen.

Auf Grund geltender gesetzlicher Bestimmungen zur Lebensmittelkonservierung gibt es eine wachsende Forderung nach natürlichen, und minimal behandelten Zutaten, die die Haltbarkeit der Lebensmittelprodukte verlängern und eine hohe Sicherheit garantieren könnten. Diese Arbeit hat gezeigt, dass Flavanone die in Pflanzen vorkommen und aktiv gegen eine Auswahl von Mikroorganismen, einschließlich Krankheitserregern sind. Flavanone könnten als natürliche antibakterielle Mittel bei der Nahrungsproduktion verwendet werden und die Haltbarkeit der Nahrung auf diese Art verlängern. Denkbar ist, dass Abfallprodukte der Früchte- und Gemüseindustrie als potentielle und preisgünstige Quelle von natürlichen Konservierungsmitteln genutzt werden könnten. Um allerdings die verantwortlichen Mechanismen genau zu verstehen und eventuell Kombinationen verschiedener Flavanone, Flavonoiden oder anderer natürlichen Substanzen zu finden, welche eine wirksamere Alternative bei der Lebensmittelkonservierung sein könnten, müssen in Zukunft noch weitere Studien auf diesem Gebiet durchgeführt werden.

8. Literature

Abbate S., Burgi L.F., Castiglioni E., Lebon F., Longhi G., Toscano E., Caccamese S., 2009, Assessment of Configuration and Conformational Properties of Naringenin by Vibrational Circular Dichroism, 21, 436-441

Adams M.R., 1995, Food microbiology, The Royal Society of Chemistry, Cambridge UK

Adams M.R., 2008, Food microbiology, The Royal Society of Chemistry, Cambridge UK

Aggarwal K.K., Khanuja S.P.S., Ahmad A., Santha Kumar T.R., Gupta V.K., Kumar S., 2002, Antimicrobial Activity Profiles of the Two Enantiomers of Limonene and Carvone Isolated from the Oils of *Mentha spicata* and *Anethum sowa*, Flavour and Fragrance Journal, 17, 59-63

Al-Bakri A.G., Afifi F.U., 2007, Evaluation of Antimicrobial Activity of Selected Plant Extracts by Rapid XTT Colorimetry and Bacterial Enumeration, Journal of Microbiological Methods, 68, 19-25

Al-Fatimi M., Wurster M., Schröder G., Lindequist U., 2007, Antioxidant, Antimicrobial and Cytotoxic Activities of Selected Medicinal Plants from Yemen, Journal of Ethnopharmacology, 111(3), 657-666

Alvarez M.A., Debattista N.B., Pappano N.B., 2008, Antimicrobial Activity and Synergism of Some Substituted Flavonoids, Folia Microbiologica, 53, 1, 23-28s

Asztemborska M., Miśkiewicz M., Sybilksa D., 2003, Separation of Some Chiral Flavanones by Micellar Electrokinetic Chromatography, Electrophoresis, 24, 2527-2531

Atlas R.M., 1995, Handbook of Microbiological Media for the Examination of Food, CRC Press, Inc

Atlas R.M., 2006, Handbook of Microbiological Media for the Examination of Food, Boca Raton

Bast E., 1999, Mikrobiologische Methoden, Spectrum, Akademischer Verlag, Heidelberg

Baumgart J., 1993, Mikrobiologische Untersuchung von Lebensmitteln, Behr's Verlag, Hamburg***Beecher G.R., 2003***, Proceedings of the Third International Scientific Symposium on Tea and Human Health: Role of Flavonoids in the Diet, Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake, The Journal of Nutrition, 3248S-3254S

Belitz H-D., Grosch W., 1999, Food Chemistry, Springer – Verlag, Berlin Heidelberg

Belitz H-D., Grosch W., Schieberle P., 2004, Food Chemistry, Springer – Verlag Berlin Heidelberg

Benakmoum A., Abbeddou S., Ammouche A., Kefalas P., Gerasopoulos D., 2008, Valorisation of Low Quality Edible Oil with Tomato Peel Waste, Food Chemistry, 110, 3, 684-690

Benavente-García O., Castillo J., Marin F.R., Ortuño A., Del Río J.A., 1997, Reviews: Uses and Properties of Citrus Flavonoids, Journal of Agricultural and Food Chemistry, 45, 12, 4505-4515

Benkeblia N., 2004, Antimicrobial Activity of Essential Oil Extracts of Various Onions (*Allium cepa*) and garlic (*Allium sativum*), Lebensmittel-Wissenschaft und Technologie, 37, 263-268

Berkemeyer C., Letzel T., 2007, LC-API-MS(-MS) im Einsatz bei Biologischen Proben: Renaissance der Komplementären Parameter Hydrophobizität, Molekulare Masse und Molekülstruktur, LC-GC Ausgabe in deutscher Sprache, Dezember 2007, 36-45

Biddle M.M., Lin M., Scheidt K.A., 2007, Catalytic Enantioselective Synthesis of Falvanones and Chromanones, Journal American Chemical Society, 129, 3830-3831

Blackburn C.W., McClure P.J., 2002, Foodborne Pathogens, Woodheadd Publishing Ltd and CRC Press LLC, USA

Brandt A., Kueppers S., Majors R.E., 2002, Practical Aspects of Preparative HPLC in Pharmaceutical and Developmnet Production, LC-GC Europe, March, 2-5

Brown J.P., 1980, A Review of the Genetic Effects of Naturally Occurring Flavonoids, Anthraquinones and Related Compounds, Mutation Research, 75, 243-277

Buer C.S., Muday G.K., Djordjevic M.A., 2007, Flavonoids are Differentially Taken Up and Transported Long Distances in Arabidopsis, Plant Physiology, 145, 478-490

Bugianesi R., Catasta G., Spigno P., D'Uva A., Maiani G., 2002, Naringenin from Cooked Tomato Paste is Bioavailable in Men, The Journal of Nutrition, 132, 3349-3352

Buhler D.R., Miranda C., 2000, Antioxidant Activities of Flavonoids, http://lpi.oregonstate.edu/f-w00/flavonoid.html

Bülle J., Hüttermann A., 2000, Das Basiswissen der Organischen Chemie – Die Wichtigsten Organischen Reaktionen im Labor und in der Natur, Georg Thieme Verlag, Stuttgart

Caccamese S., Caruso C., Parrinello N., Savarino A., 2005, High-Performance Liquid Chromatography Separation and Chiroptical Properties of the Enantiomers of Naringenin and Other Flavanones, Journal of Chromatography A, 1076, 155-162

Caccamese S., Manna L., Scivoli G., 2003, Chiral HPLC Separation and CD Spectra of the C-2 Diasteromers of Naringin in Grapefruit during Maturation, Chirality, 15, 661-667

Casaregola S., Nguyen H.-V., Lapathitis G., Kotyk A., Gaillardin C., 2001, Analysis of the Constitution of the Beer Yeast Genome by PCR, Sequencing and Subtelomeric Sequence Hybridization, International Journal of Systematic and Evolutionary Microbiology, 51, 1607-1618

Casteele K.V., Geiger H., Van Sumere C.F., 1982, Separation of Flavonoids by Reversed – Phase High Performance Liquid Chromatography, Journal of Chromatography, 240, 81-94

Chankvetadze B., Yamamoto C., Tanaka N., Nakanishi K., Okamoto Y., 2004, High-Performance Liquid Chromatographic Enantioseparation on Capillary Columns Containing Monolithic Silica Modified with Cellulose Tris (3,5-Dimethylphenylcarbamate), Journal of Separation Science, 27, 905-911

Chankvetadze B., Yashima E., Okamoto Y., 1996, Dichloro-, Dimethyl-, and Chloromethylphenylcarbamate Derivatives of Cyclodextrins as Chiral Stationary Phases for High-Performance Liquid Choramtography, Chirality, 8, 402-407

Chen S., Qin F., Liu Y., Kong L., Zou H., 2004, Preparation of a Positively Charged Cellulose Derivative Chiral Stationary Phase with Copolymerization Reaction for Capillary Electrochromatographic Separation of Enantiomers, Electrophoresis, 25, 2817-2824

Cheng I.F., Breen K., 2000, On the Ability of Four Flavonoids, Baicilein, Luteolin, Naringenin, and Quercetin, to Suppress the Fenton Reaction of the Iron-ATP Complex, BioMetals, 13, 77-83

Chiba H., Uehara M., Wu J., Wang X., Masuyama R., Suzuki K., Kanzawa K., Ishimi Y., 2003, Hesperidin, a Citrus Flavonoid, Inhibits Bone Loss and Decreases Serum and Hepatic Lipids in Ovariectomized Mice, The Journal of Nutrition, 133, 1892-1897

Choudhury R., Chowrimootoo G., Srai K., Debnam E., Rice-Evans A., 1999, Interaction of the Flavonoid Naringenin in the Gastrointestinal Tract and the Influence of Glycosylation, Biochemical and Biophysical Research Communications, 265, 410-415

Cieśla Ł., Waksmundzka-Hajnos M., 2009, Two-Dimensional Thin-Layer Chromatography in the Analysis of Secondary Plant Metabolites, Journal of Chromatography A, 1216, 1035-1052

Clavin M., Gorzalczany S., Macho A., Muñoz E., Ferraro G., Acevedo C., Martino V., 2007, Anti-inflammatry Activity of Flavonoids from *Eupatorium arnottianum*, Journal of Ethnopharmacology, 112, 3, 585-589

Collins F.M., 1955, Effect of Aeration on the Formation of Nitate-Reducing Enzymes by *Ps. aeruginosa*, Nature, 4447, 173-174

Cook N.C., Samman S., 1996, Flavnoids – Chemistry, Metabolism, Cardioprotective Effects, and Dietary Sources, Nutritional Biochemistry, 7, 66-76

Cooper M., Tavankar G.R., Williams H.D., 2003, Regulation of Expression of the Cyanide-Insensitive Terminal Oxidase in *Pseudomonas aeruginosa*, Microbiology, 149, 1275-1284

Coudron P.E., Markowitz S.M., Wong E.S., 1992, Isolation of a β-Lactamase-Producing, Aminoglycoside-Resistant Strain of *Enterococcus faecium*, Antimicrobial Agents and Chemotherapy, 36, 5, 1125-1126

Cowan M.M., 1999, Plant Products as Antimicrobial Agents, Clinical Microbiology Reviews, 12, 4, 564-582

Cushine T.P.T., Lamb A.J., 2005, Antimicrobial Activity of Flavonoids, International Journal of Antimicrobial Agents, 26, 343-356

Czapski J., Milanówka-Jacygrad D., 1996, Nietermiczne Metody Przedłużania Trwałości Żywności o Małym Stopniu Przetworzenia, Przemysł Spożywczy, 3, 27-30

Da Silva Filho A.A., de Sousa J.P.B., Soares S., Furtado N.A.J.C., Silva M.L.A., Cunha W.R., Gregório L.E., Nanayakkara N.P.D., Bastos J.K., 2008, Antimicrobial Activity of the Extract and Isolated Compounds from *Baccharis dracunculifolia* D.C. (*Asteraceae*), Zeitschrift für Naturforschung, 63c, 40-46

Das S., Rosazza J.P.N., 2006, Microbial and Enzymatic Transformations of Flavonoids, Journal of Natural Products, 69, 499-508

Davidson M., Sofos J.N., Branen A.L., 2005, Antimicrobials in Food, Tylor & Francis Group, LLC, USA

Davidson P.M., Branen A.L., 1993, Antimicrobials in Foods, Marcel Dekker, Inc., New York

Davidson P.M., Harrison M.A., 2002, Resistance and Adaptation of Food Antimicrobials, Sanitizers, and Other Process Controls (Scientific Status Summary), Foodtechnology, 56, 11, 69-78

De Nysschen A.M., Van Wyk B.-E., Van Heerden F.R., Schutte A.L., 1996, The Major Phenolic Compounds in the Leaves of *Cyclopia* Species (Honeybush Tea), Biochemical Systematics and Ecology, 24, 3, 243-246

Dowd, M.K., Pelitire, S.M., 2008, HPLC Preparation of the Chiral Forms of 6-Methoxy-Gossypol and 6,6'-Dimethoxy-Gossypol, Journal of Chromatography B, 867, 69-77

Dunn B., Sherlock G., 2008, Reconstruction of the Genome Origins and Evolution of the Hybrid Lager Yeast *Saccharomyces pastorianus*, Genome Research, 18, 1610-1623

Ebermann R., 2008, Lehrbuch Lebensmittelchemie und Ernährung, Springer, Wien

Eisgruber H., 2003, Mikrobiologische Kriterien für Lebensmittel, Behr's Verlag, Hamburg

El Gharras H., 2009, Polyphenols: Food Sources, Properties and Applications – a Review, International Journal of Food Science and Technology, 44, 2512-2518

Eliel E.L., Wilen S.H. Mander L.N., 1994, Stereochemistry of Organic Compounds, John Wiley & Sons, Inc, New York, USA

Endres H., Hörmann H., 1963, Präparative und Analytische Trennung Organischer Verbindung durch Chromatographie an Polyamid, Angewandte Chemie, 6, 288-294

Erlund I., 2004, Review of the Flavonoids Quercetin, Hesperetin, and Naringenin. Dietary Sources, Bioaktivities, Bioavaliability, and Epidemiology, Nutrition Research, 24, 851-874

Erlund I., Meririnne E., Alfthan G., Aro A., 2001, Plasma Kinetics and Urinary Excretion of the Flavanones Naringenin and Hesperetin in Humans after Igestion of Orange Juice and Grapefruit Juice, Journal of Nutrition, 131, 325-241

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000, Determination of Minimum Inhibitory Concentrations (MICs) of Antibacterial Agents by Agar Dilution - Eucast Definitive Dokument, E.Def 3.1 June 2000, Clinical Microbiology and Infection, 6, 9, 509-515

Fanali S., Catarcini P., Blaschke G., Chankvetadze B., 2001, Enantioseparations by Capillary Electrochromatography, Electrophoresis, 22, 3131-3151

Fanous A., Weiland F., Lück C., Görg A., Friess A., Parlar H., 2007, A Proteome Analysis of *Corynebacterium glutamicum* After Exposure to the Herbicide 2,4-dichlorophenoxz Acetic Acid (2,4-D), Chemosphere, 69, 25-31

Felgines C., Texier O., Morand C., Manach C., Scalbert A., Régerat F., Rémésy C., 2000, Bioavailability of the Flavanone Naringenin and ist Glycosides in Rats, American Journal of Physiological Gastrointestinal Liver Physiology, 279, G1148-G1154

Ferguson L.R., 2001, Role of Plant Polyphenols in Genomic Stability, Mutation Research, 475, 89-111

Ferreira D., Kamara B.I., Brandt E.V., Joubert E., 1998, Phenolic Compounds from *Cyclopia intermedia* (Honeybush Tea), Journal of Agricultural and Food Chemistry, 46, 3406-3410

Fisher K., Phillips C., 2008, Potential Antimicrobial Uses of Essential Oils in Food: is Citrus the Answer?, Trends in Food Science & Technology, 19,156-164

Fonseca A.P., Extremina C., Fonseca A.F., Sousa J.C., 2004, Effect of Subinhibitory Concentration of Piperacillin/Tazobactam on *Pseudomonas aeruginosa*, Journal of Medical Microbiology, 53, 903-910

Franco P., Schaeffer M., Zhanf T., Heckmann D., 2004, The Impact of Productivity in the Preparative Separation of Enantiomers by Chromatography, Chemistry Today, March/April, 28-30

Franzke C., 1996, Allgemeines Lehrbuch der Lebensmittelchemie, B. Behr's Verlag GmbH & Co., Hamburg

Fuhr U., Klittich K., Staib A.H., 1993, Inhibitory Effect of Grapefruit Juice and its Bitter Principal, Naringenin, on CYP1A2 Dependent Metabolism of Caffeine in Man, British Journal Clinical Pharmacology, 35, 431-436

Fukui H., Katsumi G., Mamoru T., 1988, Two Antimicrobial Flavanones from the Leaves of *Glycyrrhiza glabra*, Chemical and Pharmaceutical Bulletin, 36(10), 4174-4176

Fyhrquist P., Mwasumbi L., Hæggström C.-A., Vuorela H., Hiltunen R., Vuorela P., 2002, Ethnobotanical and Antimicrobial Investigation on Some Species of *Terminalia* and *Combretum* (*Combretaceae*) Growing in Tanzania, Journal of Ethnopharmacology 79, 169-177

Gaffield W, Lundin R.E., Gentili B., Horowitz R.M., 1975, C-2 Stereochemistry of Naringin and Its Relation to Taste and Biosynthesis in Maturing Grapefruit, Bioorganic Chemistry, 4, 259-269

Gaffield W., 1970, Circular Dichroism, Optical Rotary Dispersion and Absolute Configuration of Flavanones, 3-Hydroxyflavanones and their Glycosides – Determination of Aglycone Chirality in Flavanone Glycosides, Tetrahedron, 26, 4093-4108

Galati E.M., Monforte M.T., Kirjavainen S., Forestieri A.M., Trovato A., Tripodo M.M., 1994, Biological Effects of Hesperidin, a Citrus Flavonoid. (Note I): Antiinflammatory and Analgesic Activity, Farmacology, 40, 11, 709-712

Gao K., Henning S.M., Niu Y., Youssefian A.A., Seeram N.P., Xu A., Heber D., 2006, The Citrus Flavonoid Naringenin Stimulates DNA Repair in Prostate Cancer Cells, Journal of Nutritional Biochemistry, 17, 89-95

Garg A., Garg S., Zaneveld L.J.D., Singla A.K., 2001, Chemistry and Pharmacology of the Citrus Bioflavonoid Hesperidin, Phytotherapy Research, 15, 655-669

Garo E., Maillard M., Antus S., Mavi S., Hostettmann K., 1996, Five Flavans from Mariscus Psilostachys, Phytochemistry, 43, 6, 1265-1269

Gattuso G., Barreca D., Gargiulli C., Luezzi U., Caristi C., 2007, Flavonoid Composition of Citrus Juices, Review, Molucules, 12, 1641-1673

Geissman T.A., Mukherjee R., Sim K.Y., 1967, Constituents of *Helichrysum viscosum* var. *bracteatum* DC, Phytochemistry, 6, 1575-1581

Gel-Moreto N., Streich R., Galensa R., 2001, Chiral Separation of Six Diastereomeric Flavanone-7-O-Glycosides by Capillary Electrophoresis and Analysis of Lemon Juice, Journal of Chromatography A, 925, 279-289

Gel-Moreto N., Streich R., Galensa R., 2003, Chiral Separation of Diastereomeric Flavanone-7-O-glycosides in Citrus by Capillary Electrophoresis, Electrophoresis, 24, 2716-2722

Giorgio E., Parrinello N., Caccamese S., Rosini C., 2004, Non-Empirical Assignment of the Absolute Configuration of (-) Naringenin, by Coupling the Exciton Analysis of the Circular Dichroism Spectrum and the ab initio Calculation of the Optical Rotatory Power, Organic and Biomolecular Chemistry, 2, 3602-3607

Gorinstein S., Caspi A., Libman I., Lerner H.T., Huang D., Leontowicz H., Leontowicz M., Tashma Z., Katrich E., Feng S., Trakhtenberg S., 2006, Red Grapefruit Positively Influences Serum Triglyceride Level in Patients Suffering from Coronary Atherosclerosis: Studies in Vitro and in Humans, Journal of Agricultural and Food Chemistry, 54, 1887-1892

Gorinstein S., Leontowicz H., Leontowicz M., Krzemiński R., Gralak M., Jastrzębski Z., Park Y.-S., Jung S.-T., Kang S.-G., Trakhtenberg S., 2007, Effect of Hesperidin and Naringin on the Plasma Lipid Profile and Plasma Antioxidant Activity in Rats Fed a Cholesterol-Containing Diet, Journal of the Science of Food and Agriculture, 87, 7, 1257-1262

Gould G.W., 1996, Industry Perspectives on the Use of Natural Antimicrobials and Inhibitors for Food Applications, Journal of Food Protection Supplement, 82-86

Greenblatt C.L., Baum J., Klein B.Y., Nachshon S., Koltunov V., Cano R.J., 2004, *Micrococcus luteus* – Survival in Amber, Microbial Ecology, 48, 120-127

Grosse S., Letzel T., 2007, Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry with Post-Column Liquid Mixing for the Efficient Determination of Partially Oxidized Polycyclic Aromatic Hydrocarbons, Journal of Chromatography A, 1139, 75-83

Grotewold E., 2008, The Science of Flavonoids, Springer Science + Business Media

Gübitz G., Schmid M.G., 2001, Chiral Separation by Chromatographic and Electromigration Techniques, A Review, Biopharmaceutics & Drugs Disposition, 22, 291-336

Guiochon G., 2002, Preparative Liquid Chromatography, Journal of Chromatography A, 965, 129-161

Hagen R.E., Dunlap W.J., Mizelle J.W., Wender S.H., Lime B.J., Albach R.F., Griffiths F.P., 1965, Chroamtographic-Fluorometric Method for Determination of Narinin, Naringenin Rutinoside, and Related Flavanone Glycosides in Grapefruit Juice and Juice Sacs, Analytical Biochemistry, 12, 472-482

Harbone J.B., 1994, The Flavonoids – Advances in Research Since 1986, Chapman and Hall London

Harbone J.B., Mabry T.J., Mabry H., 1975, The Flavonoids, Chapman and Hall London

Harbone J.B., Williams C.A., 2001, Anthocyanins and Other Flavonoids, The Royal Society of Chemistry, 18, 310-333

Harborne J.B., Williams C.A., 2000, Advances in Flavonoid Research since 1992, Phytochemistry, 55, 481-504

Harrigan W.F., 1999, Laboratory Methods in Food Microbiology, WBC Book Manufactures, Bridgend, Mid-Glamorgan, Great Britain

Hauser K.J., Johnston J.A., Zabransky R.J., 1975, Economical Agar Dilution Technique for Susceptibility Testing of Anaerobes, Antimicrobial Agnets and Chemotherapy, 7, 5, 712-714

Havsteen B.H., 2002, The Biochemistry and Medical Significance of the Flavonoids, Pharmacology & Therapeutics, 96, 67-202

Henke H., 1999, Flüβig-Chromatographie, Vogel Buchverlag, Würzburg

Heo H.J., Kim D.-O., Shin S.C., Kim M.J., Kim B.G., Shin D.H., 2004a, Effect of Antioxidant Flavanone, Naringenin, from *Citrus junos* on Neuroprotection, Journal of Agricultural and Food Chemistry, 52, 1520-1525

Heo H.J., Kim M.-J., Lee J.-M., Choi S.J., Cho H.-Y., Hong B., Kim H.-K., Kim E., 2004b, Naringenin from *Citrus junos* has an Inhibitory Effect on Acetylcholinesterase and a Mitigating Effect on Amnesia, Dementia and Geriatric Cognitive Disorders, 17, 151-157

Hermann T., Pfefferle W., Baumann C., Busker E., Schaffer S., Bott M., Sahm H., Dusch N., Kalinowski J., Pühler A., Bendt A.K., Krämer R., Burkovski A., 2001, Proteome Analysis of *Corynebacterium glutamicum*, Electrophoresis, 22, 1712-1723

Hermann T., Wersch G., Uhlemann E-M., Schmid R., Burkovski A., 1998, Mapping and Identification of *Corynebacterium glutamicum* Proteins by Two-Dimensional Gel Electrophoresis and Microsequencing, Electrophoresis, 19, 3217-3221

Herrmann K., 1979, Übersicht über die Inhaltsstoffe der Tomaten, Zeitschrift für Lebensmittel- Unterschuchung und – Forschung, 169, 179-200

Hertog M.G.L., Feskens E.J.M., Hollman P.C.H., Katan M.B., Kromhout D., 1993a, Dietary Antioxidant Flavonoids and Risk of Coronary Heart Disease: the Zutphen Elderly Study, The Lancet, 342, 1007-1011

Hertog M.G.L., Hollman P.C.H., Katan M.B., 1992, Content of Potentially Anticarcinogenic Flavonoids of 28 Vegetables and 9 Fruits Commonly Consumed in The Netherlands, Journal of Agricultural and Food Chemistry, 40, 2379-2383

Hertog M.G.L., Hollman P.C.H., Katan M.B., Kromhout D., 1993b, Intake of Potentially Anticarcinogenic Flavonoids and Their Determinants in Adults in The Netherlands, Nutrition and Cancer, 20, 1, 21-9

Ho P.C., Saville D.J., Coville P.F., Wanwimolruk S., 2000, Content of CYP3A4 Inhibitors, Naringin, Naringenin and Bergapten in Grapefruit and Grapefruit Juice Products, Pharmaceutica Acta Helvetiae, 74, 379-385

Hodek P., Trefil P., Stiborová M., 2002, Flavonoids-potent and Versatile Biologically Active Compounds Interacting with Cytochromes P450, Chemico-Biological Interactions, 139, 1-21

Hoerr V., Stich A., Holzgrabe U., 2004, Critical Aspects of Analysis of *Micrococcus luteus*, *Neisseria cinerea*, and *Pseudomonas fluorescens* by Means of Capillary Electrophoresis, Electrophoresis, 25, 3132-3138

Hoffmann T., Troup B., Szabo A., Hungerer C., Jahn D., 1995, The anaerobic life of *Bacillus subtilis*: Cloning of the Genes Encoding the Respiratory Nitrate Reductase System, FEMS Microbiology Letters, 131, 219-225

Holley R.A., Patel D., 2005, Improvement in Shelf-Life and Safety of Perishable Foods by Plant Essential Oils and Smoke Antimicrobials, Food Microbiology, 22, 273-292

Hollman P.C.H., Katan M.B., 1997, Absorption, Metabolism and Health Effects of Dietary Flavonoids in Man, Biomed & Pharmacother, 51, 305-310

Hoshino Y., Mukai A., Yazawa K., Uno J., Ishikawa J., Ando A., Fukai T., Mikami Y., 2004, Transvalencin A, a Thiazolidine Zinc Complex Antibiotic Produced by a Clinical Isolate of Nocardia transvalensis – I. Taxonomy, Fermentation, Isolation and Biological Activities, The Journal of Antibiotics, 57, 12, 797-802

Hsieh P.-C., Mau J.-L., Huang S.-H., 2001, Antimicrobial Effect of Various Combination of Plant Extracts, Food Microbiology, 18, 35-43

http://cellbiology.med.unsw.edu.au/units/images/Gram-positive%20Micrococcus%20luteus%20bacteria.jpg

http://www.codexalimentarius.net/web/index_en.jsp

http://www.dsmz.de

http://www.ebi.ac.uk

http://www.eufic.org/article/en/page/BARCHIVE/expid/basics-food-additives/

http://www.eufic.org/article/pl/1/8/artid/57/

http://www.extrasynthese.com

http://www.nasa.gov/images/content/177389main_POEMS1.jpg

http://www.ncbi.nlm.nih.gov/genomeprj/17579

http://www.phytochemicals.info

http://www.roempp.com

http://www.sigmaaldrich.com

Huang Z., Wang B., Eaves D.H., Shikany J.M., Pace R.D., 2007, Phenolic Compound Profile of Selected Vegetables Frequently Consumed by African Americans in the Southeast United States, Food Chemistry, 103, 1395-1402

Hvattum E., 2002, Determination of Phenolic Compounds in Rose Hip (*Rosa canina*) Using Liquid Chromatography Coupled to Electrospray Ionisation Tandem Mass Spectrometry and Diode-Array Detection, Rapid Communications in Mass Spectrometry, 16, 655-662

Ibrahim A-R., Abul-Hajj Y.J., 1990, Microbiological Transformation of (±)-Flavanone and (±) Isoflavanone, Journal of Natural Products, Vol 53, No. 3, 644-656

Jay J.M., 2005, Modern Food Microbiology, Springer Science + Business Media, Inc., USA

Justesen U., Knuthsen P., Leth T., 1997, Quantitative Analysis of Flavonols, Flavones, and Flavonones in Fruits, Vegetables and Beverages by High-Performance Liquid

Chromatography with Photo-Diode Array and Mass Spectrometric Detection, Journal of Chormatography A, 799, 101-110

Kalinowski J., Bathe B., Bartels D., Bischoff N., Bott M., Burkovski A., Dusch N., Eggeling L., Eikmanns B.J., Gaigalat L., Goesmann A., Hartmann M., Huthmacher K., Krämer R., Linke B., McHardy A.C., Meyer F., Möckel B., Pfefferle W., Pühler A., Rey D.A., Rückert C., Rupp O., Sahm H., Wendisch V.F., Wiegräbe I., Tauch A., 2003, The Complete *Corynebacterium glutamicum* ATCC 13032 Genome Sequence and its Impact on the Production of L-Aspartate-Derived Amino Acids and Vitamins, Journal of Biotechnology, 104, 5-25

Kawaguchi K., Kikuchi S., Hasunuma R., Maruyama H., Yoshikawa T., Kumazawa Y., 2004, A Citrus Flavonoid Hesperidin Suppresses Infection-Induced Endotoxin Shock in Mice, Biological & Pharmaceutical Bulletin, 27, 5 679-683

Kawaii S., Tomono Y., Katase E., Ogawa K., Yano M., 1999, Quantitation of Flavonoid Constituents in Citrus Fruits, Journal of Agricultural and Food Chemistry, 47, 3565-3571

Kerr K.G., Snelling A.M., 2009, *Pseudomonas aeruginosa*: a Formidable and Ever-Present Adversary, Journal of Hospital Infection, 73, 338-344

Keweloh H., 2008, Mikroorganismen in Lebensmitteln, Fachbuchverlag, Pfanneberg

Klajnert B., Janiszewska J., Urbanczyk-Lipkowska Z., Bryszewska M., Shcharbin D., Labieniec M., 2006, Biological Properties of Low Molecular Mass Peptide Dendrimers, International Journal of Pharmaceutics, 309, 208-217

Knowles W.S., Noyori R., Sharpless K.B., 2001, The Nobel Prize in Chemistry 2001, http://nobelprize.org/nobel_prizes/chemistry/laureates/2001/public.html, Information for the Public

Kobilinsky A., Nazer A.I., Dubois-Brissonnet F., 2007, Modeling the Inhibition of *Salmonella typhimurium* Growth by Combination of Food Antimicrobials, International Journal of Food Microbiology, 115 (1), 95-109

Kotzekidou P., Giannakidis P., Boulamatsis A., 2007, Antimicrobial Activity of Some Plant Extracts and Essential Oils Against Foodborne Pathogens In Vitro and on the Fate of Inoculated Pathogens in Chocolate, LTW – Food Science and Technology, 41, 1, 119-127

Krämer J., 2007, Lebensmittel-Mikrobiologie, Verlag Eugen Ulmer KG, Stuttgart

Krause M., Galensa R., 1988, Direct Enantiomeric Separation of Racemic Flavanones by High Performance Liquid Chromatography Using Cellulose Triacetate as a Chiral Stationary Phase, Journal of Chromatography, 441, 417-422

Krause M., Galensa R., 1990a, Improved Chiral Stationary Phase Based on Callulose Triacetate Supported on Non-Macroporous Silica Gel Diol for the High-Performance Liquid Chromatographic Separation of Racemic Flavanones and Diastereomeric Flavanone Glycosides, Journal of Chromatography, 502, 287-296

Krause M., Galensa R., 1990b, Optical Resolution of Flavanones by High Performance Liquid Chromatography on Various Chiral Stationary Phases, Journal of Chromatography, 514, 147-159

Krause M., Galensa R., 1991, Analysis of Enantiomeric Flavanones in Plant Extracts by High Performance Liquid Chromatography on a Cellulose Triacetate Based Chiral Stationary Phase, Chromatographia, 32, ½, 69-72

Krause M., Galensa R., 1992, Bestimmung von Naringenin und Naringenin–Chalkon in Tomatenschalen mit RP-HPLC nach Festphasenextraktion, Zeitschrift für Lebensmittel-Untersuchung und –Forschung, 194, 29-32

Kromidas S., 2006, HPLC Made to Measure, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

Kuznetsova G.A., 1970, The Gas-Liquid Chromatography of Some Natural Coumarins and Coumarin Fractions from Plants, Khimiya Prirodnykh Soedinenii, 6, 4, 406-412

Kwon C., Park H., Jung S., 2007, Enantioseparation of Some Chiral Flavanones Using Microbial Cyclic β-(1→3),(1→6)-glucans as Novel Chiral Additives in Capillary Electrophoresis, Carbohydrate Research, 342, 762-766

Lee S.H., Park Y.B., Bae K.H., Bok S.H., Kwon Y.K., Lee E.S., Choi M.S., 1999, Cholersterol-Lowering Activity of Naringenin via Inhibition of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase an Acyl Coenzyme A: Cholesterol Acyltransferase in Rats, Annals of Nutrition & Metabolism, 43, 173-180

Lee Y.S., Reidenberg M.M., 1998, A Method for Measuring Naringenin in Biological Fluids and its Disposition from Grapefruit Juice by Man, Pharmacology, 56, 314-317

Leone L., Ferri D., Manfredi C., Persson P., Shchukarev A., Sjöberg, Loring J., 2007, Modeling the Acid – Based Properties of Bacterial Surfaces: A Combined Spectroscopic and Potentiomeric Study of the Gram-Positive Bacterium *Bacillus subtilis*, Environmental Science & Technology, 41, 6465-6471

Ley J.P., Krammer G., Reinders G., Gatfield I.L., Bertram H-J., 2005, Evaluation of Bitter Masking Flavanones from Herba Santa (*Eriodictyon californicum* (H. & A.) Torr., *Hydrophyllaceae*), Journal of Agricultural and Food Chemistry, 53, 6061-6066

Lin L-Z., Mukhopadhyay S., Robbins R.J., Harnly J.M., 2007, Identification and Quantification of Flavonoids of Mexican Oregano (*Lippia graveolens*) by LC-DAD-ESI/MS Analysis, Journal of Food composition and Analysis, 20, 5, 361-369

Lin Y.T., Labbe R.G., Shetty K., 2004, Inhibition of *Listeria monocytogenes* in Fish and Meat Systems by Use of Oregano and Cranberry Phytochemical Synergies, Applied and Environmental Microbiology, 70, 9, 5672-5678

Lin Y.T., Labbe R.G., Shetty K., 2005, Inhibition of *Vibrio parahaemolyticus* in Seafood System Using Oregano and Cranberry Phytochemical Synergies and Lactic Acid, Innovative Food Science and Emerging Technologies, 6, 453-458

Loscalzo L.M., Wasowski C., Paladini A.C., Marder M., 2008, Opioid Receptors are Involved in the Sedative and Antinociceptive Effects of Hesperidin as well as in its Potentiation with Benzodiazepines, European Journal of Pharmacology, 580, 306-313

Lottspeich F., Engels (Hrsg.) J.W., 2009, Bioanalityk, Spectrum Akademischer Verlag, Heidelberg

Lück E, Jager M., 1995, Antimicrobial Food Additives: characteristics, uses, effects, Springer Verlag, Berlin Heidelberg New York

Majhenič L., Škerget M., Knez Ž., 2007, Antioxidant and Antimicrobial Activity of Guarana Seed Extracts, Food Chemistry, 104, 1258-1268

Mandalari G., Bennett R.N., Bisignano G., Trombetta D., Saija A., Faulds C.B., Gasson M.J., Narbad A., 2007, Antimicrobial Activity of Flavonoids Extracted from Bergamot (*Ctirus bergamina* Risso) peel, a Byproduct of the Essential Oil Industry, Journal of Applied Microbiology 103, 2056-2064

Mandalari G., Bisignano C., D'Arrigo M., Ginestra G., Arena A., Tomaino A., 2010, Antimicrobial Potential of Polyphenols Extracted from Almond Skins, Letters in Applied Microbiology, 51, 83-89

Manthey J.A., Grohmann K., 1996, Concentrations of Hesperidin and Other Orange Peel Flavonoids in Citrus Processing Byproducts, Journal of Agricultural and Food Chemistry, 44, 811-814

Manthey J.A., Guthrie N., Grohmann K., 2001, Biological Properties of Citrus Flavonoids Pertaining to Cancer and Inflammation, Current Medicinal Chemistry, 8, 135-153

Marin P.D., Grayer R.J., Kite G.C., Matevski V., 2003, External Leaf Flavonoids of *Thymus* Species from Macedonia, Biochemical Systematics Ecology, 31, 1291-1307

Marino M., Bersani C., Comi G., 2001, Impedance Measurements to Study the Antimicrobial Activity of Essential Oils from *Lamiaceae* and *Compositae*, International Journal of Food Microbiology 67, 187-195

Mata Bilbao M. de L. M., Andrés-Lacueva, Jáuregui O., Lamuela-Raventós R.M., 2007, Determination of Flavonoids in a Citrus Fruit Extract by LC-DAD and LC-MS, Food Chemistry, 101, 1742-1747

Mc Caleb R.S., 1994, Food Ingredient Safety Evaluation, G. Charalambous (Ed.), Spices, Herbs and Edible Fungi, 131-136

Meyer V.R., 1993, Praxis der Hochleistungs-Flüssigchromatographie, Otto Salle Verlag, GmbH & Co., Frankfurt am Main

Middleton E.JR., Kandaswami C., Theoharide T.C., 2000, The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease and Cancer, The American Society for Pharmacology and Experimental Therapeutics, Pharmacological Reviews, 53, 673-751

Mizelle J.W., Dunlap W.J., Hagen R.E., Wender S.H., Lime B.J., Albach R.F., Griffiths F.P., 1965, Isolation and Identification of Some Flavanone Rutinosides of the Grapefruit, Analytical Biochemistry, 12, 316-324

Molina-Torres J., García-Chávez A., Ramírez-Chávez E., 1999, Antimicrobial Properties of Alkamides Present in Flavouring Plants Traditionally Used in Mesoamerica: affinin and capsaicin, Journal of Ethnopharmacology, 64, 241-248

Monforte M.T., Trovato A., Kirjavainen S., Forestieri A.M., Galati E.M., Lo Curto R.B., 1995, Biological Effects of Hesperidin, a Citrus Flavonoid, (Note II): Hypolipidemic activity on experimental Hypercholesterolemia in Rat, Farmacology, 50, 9, 595-599

Montrocher R., Verner M.-C., Briolay J., Gautier C., Marmeisse R., 1998, Phylogenetic Analysis of the *Saccharomyces cerevisiae* Group Based on Polymorphisms of rDNA Spacer Sequences, International Journal of Systematic Bacteriology, 48, 295-303

Mori A., Nishino C., Enoki N., Tawata S., 1987, Antibacterial Activity and Mode of Action of Plant Flavonoids Against *Proteus vulgaris* and *Staphylococcus aureus*, Phytochemistry, 26, 8, 2231-2234

Nahmias Y., Goldwasser J., Casali M., van Poll D., Wakita T., Chung R.T., Yarmush M.L., 2008, Apolipoprotein B-Dependent Hepatitis C Virus Secretion Is Inhibited by the Grapefruit Flavonoids Naringenin, Hepatology, 47, 5, 1437-1445

Naidu A.S., 2000, Natural Food Antimicrobials System, CRC Press LLC, Florida

Nakanishi K., Berova N., Woody R.W., 1994, Circular Dichroism – Principles and Applications, VCH Publisher, Inc

Nakao Y., Kanamori T., Itoh T., Kodama Y., Rainieri S., Nakamura N., Shimonaga T., Hattori M., Ashikari T., 2009, Genome Sequence of the Lager Brewing Yest, and Interspecies Hybrid, DNA Research, 16, 115-129

Nakatani N., 1994, Antioxidative and Antimicrobial Constituents of Herbs and Spices, G. Charalambous (Ed.), Spices, Herbs and Edible Fungi, 251-271

Ndi C.P., Semple S.J., Griesser H.J., Pyke S.M., Barton M.D., 2007, Antimicrobial Compounds from the Australian Desert Plant *Eremophila neglecta*, Journal of Natural Products, 70, 1439-1443

Neue U.D., Mazza C.B., Cavanaugh J.Y., Lu Z., Wheat T.E., 2003, At-Column Dilution for Improved Loading in Preparative Chromatography, Chromatographia Supplement, 57, S121-S126

Nguyen H.-V., Gaillardin C., 2005, Evolutionary Relationships between the Former Species *Saccharomyces uvarum* and the Hybrids Saccharomyces bayanus and *Saccharomyces pastorianus*; Reinstatement of *Saccharomyces uvarum* (Beijerinck) as a Distinct Species, FEMS Yeast Research, 5, 471-483

Nielsen I.L.F., Chee W.S.S., Poulsen L., Offord-Cavin E., Rasmussen S.E., Frederiksen H., Enslen M., Barron D., Horcajada M.-N., Williamson G., 2006, Bioavailability is improved by Enzymatic Modification of the Citrus Flavonoid Hesperidin in Humans: A Randomized, Double-Bind, Crossover Trial, The Journal of Nutrition, 136, 404-408

Nijveldt R.J., van Nood E., van Hoorn D.E.C., Boelens P.G., van Norren K., van Leeuwen P.A.M., 2001, Flavonoids: a Review of Probable Mechanisms of Action and Potential Applications, American Journal of Clinical Nutrition, 74, 418-425

Nishino C., Enoki N., Tawata S., Mori A., Kobayashi K., Fukushima M., 1987, Antibacterial Activity of Flavonoids Against *Staphylococcus epidermidis*, a Skin Bacterium, Agricultural Biology and Chemistry , 139-143

Nogata Y., Ohta H., Yoza K.-I., Berhow M., Hasegawa S., 1994, High Performance Liquid Chromatographic Determination of Naturally Occurring Flavonoids in Citrus with a Photodiode-Array Detector, Journal of Chromatography A, 667, 59-66

O'Connell J.E., Fox P.F., 2001, Significance and Application of Phenolic Compounds in the Products and Quality of Milk and Dairy Products: A Review, International Dairy Journal, 11, 103-120

Özcan M., Boyraz N., 2000, Antifungal Properties of Some Herb Decoctions, European Food Research and Technology, 212, 86-88

Özçdlik B., Orhan I., Toker G., 2006, Antiviral and Antimicrobial Assessment of Some Selected Flavonoids, Zeitschrift für Naturforschung, 61c, 632-638

Özer H., Sökmen M., Güllüce M., Adigüzel A., Kiliç H., Sahin F., Sökmen A., Baris O., 2006, In Vitro Antimicrobial and Antioxidant Activities of the Essential Oils and Methanol Extracts of *Hyssopus officinalis* L. ssp. *angustifolius*, Italian Journal of Food Science, 18, 1, 73-83

Park H., Jung S., 2005, Separation of Some Chiral Flavonoids by Microbial Cyclosophoraoses and Their Sulfated Derivatives in Micellar Electrokinetic Chromatography, Electrophoresis, 26, 3833-3838

Parl L., Gnanasoundarl M., 2006, Influence of Naringenin on Oxytetracycline Mediated Oxidative Damage in Rat Liver, Basic & Clinical Pharmacology & Toxicology, 98, 456-461

Patent DE69817862(T2), 2004, Naringin und Naringenin als Mittel zur Vorbeugung oder Behandlung von Leber-Erkrankungen, Vertreter: Boehmert & Boehmert, Bremen

Peng Y., Liu F., Ye J., 2006, Quantitative and Qualitative Analysis of Flavonoid Markers in *Frucus aurantii* of Different Geographical Origin by Capillary Electrophoresis with Electrochemical Detection, Journal of Chromatography B., 830, 224-230

Pepper K., Horaud T., Le Bouguenec C., De Cespedes G., 1987, Location of Antibiotic Resistance Markers in Clinical Isolates of *Enterococcus faecalis* with Similar Antibiotypes, Antimicrobial Agents and Chemotherapy, 31, 9, 1394-1402

Peterson J.J., Beecher G.R., Bhagwat S.A., Dwyer T., Gebhardt S.E., Haytowitz D.B., Holden J.M., 2006, Flavanones in Grapefruit, Lemons, and Limes: A Compilation and Review of the Data from the Analytical Literature, Journal of Food Composition and Analysis, 19, S74-S80

Pinheiro E.T., Gomes B.P.F.A., Drucker D.B., Zaia A.A., Ferraz C.C.R., Souza-Filho F.J., 2004, Antimicrobial Susceptibility of *Enterococcus faecalis* Isolated from Canals of Root Filled Teeth with Periapical Lesions, International Endodontic Journal, 37, 756-763

Pitchhardt K., 1998, Lebensmittelmikrobiologie, Springer - Verlag Berlin, Heidelberg

Plaper A., Golob M., Hafner I., Oblak M., Šolmajer T., Jerala R., 2003, Characterization of Quercetin Binding Site on DNA Gyrase, Biochemical and Biophysical Research Communications, 306, 530-536

Pomini A.M., Marsaioli A.J., 2008, Absolute Configuration and Antimicrobial Activity of Acylhomoserine Lactones, Journal of Natural Products, 71, 1032-1036

Proczek I., 2006, Estimation of usefulness of high pressure technology for preservation of raspberry, Master Thesis at University of Warmia and Mazury, Poland

Proestos C., Boziaris I.S., Nychas G-J.E., Komaitis M., 2006, Analysis of Flavonoids and Phenolic Acids in Greek Aromatic Plants: Investigation of Their Antioxidant Capacity and Antimicrobial Activity, Food Chemistry, 95, 664-671

Pujol I., Guarro J., Llop C., Soler L., Fernández-Ballart J., 1996, Comparison Study of Broth Macrodilution and Microdilution Antifungal Susceptibility Tests for the Filamentous Fungi, Antimicrobial Agents and Chemotherapy, 40, 9, 2106-2110

Puupponen-Pimiä R., Nohynek L., Meier C., Kähkönen M., Heinonen M., Hopia A., Oksman-Caldentey K.-M., 2001, Antimicrobial Properties of Phenolic Compounds from Berries, Journal of Applied Microbiology, 90, 494-507

Rahme L.G., Tan M.-W., Le L., Wong S.M., Tompkins R.G., Calderwood S.B., Ausbel F.M., 1997, Use of Model Plant Hosts to Identify *Pseudomonas aeruginosa* Virulece Factors, Proceeding of the National Academy of Sciences of the United States of America, 94, 13245-13250

Rauha J.-P., Remes S., Heinonen M., Hopia A., Kähkönen M., Kujala T., Pihlaja K., Vuorela H., Vuorela P., 2000, Antimicrobial Effects of Finnish Plant Extracts Containing Flavonoids and Other Phenolic Compounds, International Journal of Food Microbiology, 56, 3-12

Rehm H., Letzel T., 2010, Proteinbiochemie/Proteomics – Der Experimentator, Spectrum Akademischer Verlag, Heidelberg

Renaud S., de Lorgeril M., 1992, Wine, Alcohol, Platelets, and the French Paradox for Coronary Heart Disease, The Lancet, 339, 1523-1526

Ribeiro S.F.F., Agizzio A.P., Machado O.L.T., Neves-Ferreira A.G.C., Oliveira M.A., Fernandes K.V.S., Carvalho A.O., Perales J., Gomes V.M., 2007, A New Peptide of Melon Seeds which Shows Sequence Homology with Vicilin: Partial Characterization and Antifungal Activity, Scientia Horticulturae, 111, 399-405

Rice-Evans C.A., Miller N.J., Paganga G., 1996, Structure – Antioxidany Activity Relationship of Flavonoids and Phenolic Acids, Free Radical Biology & Medicine, 20, 933-956

Rice-Evans C.A., Packer L., 1998, Flavonoids in Health and Disease, Marcel Dekker, Inc., New York

Rios J.L., Recio M.C., Villar A., 1988, Screaning Methods for Natural Products with Antimicrobial Activity: A Rewiew of the Literature, Journal of Ethnopharmacology, 23, 127-149

Robards K., Prenzler P.D., Trucker G., Swatsitang P., Glover W., 1999, Phenolic Compounds and Their Role in Oxidative Processes in Fruits, Food Chemistry, 66, 401-436

Roller S., 1995, The Quest for Natural Antimicrobials as Novel Means of Food Preservation: Status Report on a European Research Project, International Biodeterioration & Biodegradation, 333-345

Ross J.A., Kasum C.M., 2002, Dietary Flavonoids: Bioavailability, Metabolic Effects, and Safety, Annual Review of Nutrition, 22, 19-34

Rupasinghe H.P.V., Boulter-Bitzer J., Ahn T., Odumeru J.A., 2006, Vanilin Inhibits Pathogenic and Spoilage Microorganisms In vitro and Aerobic Microbial Growth in Fresh-Cut Apples, Food Research International, 39, 575-580

Russell N.J., Gould G.W., 2003, Food Preservatives, Kluwer Academic/Plenum Publishers, New York

Russo J., Barreiro M.I., Vila-Real H., Ribeiro M.H.L., 2007, Immobilization of Naringinase by Selective Adsorption and Covalent Binding to Microstructured Particles, Abstracts / Journal of Biotechnology, 131S, S74-S97

Saeed S., Ahmad S., Rasool S.A., 2004, Antimicrobial Spectrum, Production and Mode of Action of Staphylococcin 188 Produced by *Staphylococcus aureus*, Pakistan Journal of Pharmaceutical Sciences, 17, 1, 1-8

Schepers S., 2001, Antimicrobial Activity of Rooibos Tea (*Aspalathus linearis*) on Food Spoilage Organisms and Potential Pathogens, Master Thesis in University of Stellenbosch

Schlegel H.G., 1992, Allgemeine Mikrobiologie, Georg Thieme Verlag, Stuttgart

Schmahl H.-J., 1987, Präparative und Analytische Trennung der Enantiomeren des Tratogenen Racemats EM 12 [2-(2,6-Dioxopiperidin-2-yl)-phthalimidin] Mittels Niederdruck-Säulenchromatographie (LC) und HPLC, Fresenius Journal of Analytical Chemistry, 326, 150-152

Schmidt-Traub H., 2006, Preparative Chromatography of Fine Chemicals and Pharmaceutical Agents, Whiley-Vch Verlag GmbH & Co. KGaA

Schöberl H., 1999, Physikalisch-chemische und Strukturelle Auswirkungen einer Hydrostatischen Hochdruckbehandlung auf Lebensmittel, Herbert Utz Verlag, Wissenschaft, München

Schröder G., Wehinger E., Lukačin R., Wellmann F., Seefelder W., Schwab W., Schröder J., 2004, Flavonoid Methylation: a Novel 4‘-O-methyltransferase from *Catharanthus roseus*, and Evidence that Partially Mehtylated Flavanones are Substrates of Four Different Flavonoid Dioxygenases, Phytochemistry, 65, 1085-1094

Schulte M., Strube J., 2001, Preparative Enantioseparation by Simulated Moving Bed Chromatography, Journal of Chromatography A., 906, 399-416

Schwedt G., 1994, Chromatographische Trennmethoden – Theoretische Grundlagen, Techniken und Analytische Anwendungen, Georg Thieme Verlag, Stuttgart

Schwedt G., 2008, Analytische Chemie – Grundlagen, Methoden und Praxis, Wiley-Vch Verlag GmbH & Co. KGaA, Weinheim

Serra A.T., Matias A.A., Nunes A.V.M., Leitão M.C., Brito D., Bronze R., Silva S., Pires A., Crespo M.T., San Romão M.V., Duarte C.M., 2008, In vitro Evaluation of Olive- and Grape-Based Natural Extracts as Potential Preservatives for Food, Innovative Food Science and Emerging Technologies, 9 (3), 311-319

Sezonov G., Joseleau-Petit D., D'Ari R., 2007, *Escherichia coli* Physiology in Luria-Bertani Broth, Journal of Bacteriology, 189, 23, 8746-8749

Si W., Gong J., Tsao R., Kalab M., Yang R., Yin Y., 2006, Bioassay-Guided Purification and Identification of Antimicrobial Components in Chinese Green Tea Extract, Journal of Chromatography A, 1125, 204-210

Silberbach M., Burkovski A., 2006, Application of Global Analysis Techniques to *Corynebacterium glutamicum*: New Insights into Nitrogen Regulation, Journal of Biotechnology, 126, 101-110

Simons A.L., Renouf M., Murphy P.A., Hendrich S., 2009, Greater Apparent Absorption of Flavonoids is Associated with Lesser Human Fecal Flavonoid Dissappearance Rates, Journal of Agricultural and Food Chemistry, Article ASAP

Slade D., Ferreira D., Marais J.P.J., 2005, Circular Dichroism, a Powerful Tool for the Assessment of Absolute Configuration of Flavonoids, Phytochemistry 66, 2177-2215

Smith M.C.M, Young D.I., Greenblatt C.L., 2010, Genome Sequence of the Fleming Strain of *Micrococcus luteus*, a Simple Free-Living Actinobacterium, Journal of Bacteriology, 192, 3, 841-860

Socha R., Juszczak L., Pietrzyk S., Fortuna T., 2009, Antioxidant Activity and Phenolic Composition of Herbhoneys, Food Chemistry, 113, 568-574

Sofos J.N., Beuchat L.R., Davidson P.M., Johnoson E.A., 1998, Naturally Occurring Antimicrobials in Food, Regulatory Toxicology and Pharmacology, 28, 71-72

Sousa F., Guebitz G.M., Kokol V., 2009, Antimicrobial and Antioxidant Properties of Chitosan Enzymatically Functionalized with Flavonoids, Process Biochemistry, 44, 749-756

Stobiecki M., 2000, Application of Mass Spectrometry for Identification and Structural Studies of Flavonoid Glycosides, Phytochemistry, 54, 237-256

Stojanović G., Radulović N., Hashimoto T., Palić R., 2005, In vitro Antimicrobial Activity of Extracts of Four *Achillea* species: The composition of *Achillea clavennae* L (*Asteraceae*) extract, Journal of Ethnopharmacology, 101, 185-190

Subramanian G., 2007, Chirale Separation Techniques, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

Sud I.J., Feingold D.S., 1981, Heterogeneity of Action Mechanisms among Antimycotic Imidazoles, Antimicrobial Agents and Chemotherapy, 20, 1, 71-74

Sudjaroen Y., Haubner R., Würtele G., Hull W.E., Erben G., Spiegelhalder B., Changbumrung S., Bartsch H., Owen R.W., 2005, Isolation and Structure Elucidation of Phenolic Antioxidants from Tamarind (*Tamarindus indica* L.) Seeds and Pericarp, Food and chemical Toxicology, 43, 1673-1682

Suksamrarn A., Chotipong A., Suavansri T., Boongird S., Timsuksai P., Vimuttipong S., Chuaynugul A., 2004, Antimycobacterial Activity and Cytotoxicity of Flavonoids from the Flowers of *Chrolaena odorata*, Archives of Pharmacal Research, 27, 5, 507-511

Tamai Y., Momma T., Yoshimoto H., Kaneko Y., 1998, Co-Existence of Two Types of Chromosome in the Bottom Fermenting Yeast, *Saccharomyces pastorianus*, Yeast, 14, 923-933

Tereschuk M.L., Riera M.V.Q., Castro G.R., Abdala L.R., 1997, Antimicrobial Activity of Flavonoids from Leaves of *Tagetes minuta*, Journal of Ethnopharmacology, 56, 227-232

Ternes, Täufel, Tunger, Zobel, 1993, Lebensmittel – Lexikon A-Z, Behr's Verlag

Tosch W., Lanthaler K., Boote V., Stretz D., Robson G.D., Geiger E., Drucker D.B., 2006, Molecular Species of Phosphatidylethanolamine from Continuous Cultures of *Saccharomyces pasteurianus* syn. *carlsbergensis* Strains, Yeast, 23, 75-82

Tsuchiya H., Sato M., Miyazaki T., Fujiwara S., Tanigaki S., Ohyama M., Tanaka T., Iinuma M., 1996, Comparative Study on the Antibacterial Activity of Phytochemical Flavanones against Methicillin-Resistant *Staphylococcus aureus*, Journal of Ethnopharmacology, 50, 27-34

Tyukavkina M.A., Dem'yanovich V.M., Kolesnik Y.A., Ruchkon V.E., Rulenko I.A., Litvinenko V.I., 1989, Configuration of Stereoisomers of Naringenin 5-Glucoside, Plenum Publishing Corporation, 2, 185-188

Ulanowska K., Majchrzyk A., Moskot M., Jakóbkiewicz-Banecka J., Węgrzyn G., 2007, Assessment of Antibacterial Effects of Flavonoids by Estimation of Generation Times in Liquid Bacterial Cultures, Biologia, Bratislava, 62, 2, 132-135

US Patent 6096364, 2000, Method for Lowering Blood Glucose Level by the Administration of Bioflavonoid

US Patent 6221357, 2001, Flavonoids Derived From Citrus Peels as Collagen-Induced Paltelet Aggregation Inhibitor

Vági E., Simándi B., Suhajda A., Héthelyi E., 2005, Essential Oil Composition and Antimicrobial Activity of *Origanum majorana* L. Extracts Obtained with Ethyl Alcohol and Supercritical Carbon Dioxide, Food Research International, 38, 51-57

Valero M., Francés E., 2006, Synergistic Bactericidal Effect of Carvacrol, Cinnamaldehyde or Thymol and Refrigeration to Inhibit *Bacillus cereus* in Carrot Broth, Food Microbiology, 68-73

Valero M., Ginger M.J., 2006, Effect of Antimicrobial Components of Essential Oils on Growth of *Bacillus cereus* INRA L2104 in and the Sensory Qualities of Carrot Broth, International Journal of Food Microbiology, 106, 90-94

Van Acker S.A.B.E., Van den Berg D.-J., Tromp M.N.J.L., Griffioen D.H., Van Bennekom W.P., Van der Vijgh W.J.F., Bast A., 1996, Structural Aspects of Antioxidant Activity of Flavonoids, Free Radical Biology & Medicine, 20, 3, 331-342

Van Vuuren S.F., Viljoen A.M., 2007, Antimicrobial Activity of Limonene Enantiomers and 1,8-Cineole Alone and in Combination, Flavour and Fragrance Journal, 22, 540-544

Vega-Villa K.R., Remsberg C.M., Podelnyk K.L., Davies N.M., 2008, Stereospecific High-Performance Liquid Chromatographic Assay of Isosakuranetin in Rat Urine, Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Science, 875, 1, 142-147

Vega-Villa K.R., Yañez J.A., Remsberg C.M., Ohgami Y., Davies N.M., 2008, Stereospecific High-Performance Liquid Chromatographic Validation of Homoeriodictyol in Serum and Yerba Santa (*Eriodictyon glutinosum*), Journal of Pharmaceutical and Biomedical Analysis, 46, 971-974

Veldhuizen E.J.A., Rijnders M., Claassen E.A., van Dijk A., Haagsman H.P., 2008, Porcine β-Defensin 2 Displays Broad Antimicrobial Activity Against Pathogenic Intestinal Bacteria, Molecular Immunology, 45, 2, 386-394

Verástegui M.A., Sánchez C.A., Heredia N.L., García-Alvarado J.S., 1996, Antimicrobial Activity of Extracts of Three Major Plants from the *Chihuahuan desert*, Journal of Ethnopharmacology, 52, 175-177

Vila-Real H., Alfaia A.J., Calado A.R.T., Ribeiro M.H.L., 2007, Development of Co-Solvent System in the Biocoversion of Naringin to Naringenin by Naringinase, Abstracts / Journal of Biotechnology, 131S, S74-S97

Vollhardt K.P.C., Schore N.E., 2000, Organische Chemie, Wiley-VCH Verlag GmbH, Weinheim, Germany

Wang S.-P., Haung K.-J., 2004, Determination of Flavonoids by High-Performance Liquid Chromatography and Capillary Electrophoresis, Journal of Chromatography A, 1032, 273-279

Wee J.-H., Moon J.-H., Eun J.-B., Chung J.H., Kim Y.G., Park K.-H., 2007, Isolation and Identification of Antioxidants from Peanut Shells and the Relationship between Structure and Antioxidant Activity, Food Science and Biotechnology, 16, 1, 116-122

Wellmann Frank, 2002, Molekulare und strukturelle Charakterisierung der Flavonolsynthase aus *Citrus unshiu*, Universität Marburg

Werk R., Knothe H., 1984, Comparison of Agar Dilution Test, Broth Dilution Test, and Broth Elution Test for Assaying Susceptibility of *Candida* spp., Current Microbiology, 10, 173-176

Williams H.D., Zlosnik J.E.A., Ryall B., 2006, Oxygen, Cyanide and Energy Generation on the Cystic Fibrosis Pathogen *Pseudomonas aeruginosa*, Advances in Microbial Physiology, 52, 1-71

Wistuba D., Trapp O., Gel-Moreto N., Galensa R., Schurig V., 2006, Stereoisomeric Separation of Flavanones and Flavanone-7-O-glycosides by Capillary Electrophoresis and Determination of Interconversion Barriers, Analitycal Chemistry, 78, 10, 3424-3433

Won D., Shin B-K., Han J., 2008, Synthesis and the Absolute Configuration of Isoflavanone Enantiomers, Journal of Applied Chemistry, 51 (1), 17-19

Worlitzsch D., Tarran R., Ulrich M., Schwab U., Cekici A., Meyer K.C., Birrer P., Bellon G., Berger J., Weiss T., Botzenhart K., Yankaskas J.R., Randell S., Boucher R.C., Döring G., 2002, Effects of Reduced Mucus Oxygen Concentration in Airway *Pseudomonas* Infections of Cystic Fibrosis Patients, The Journal of Clinical Ivestigation, 109, 3, 317-325

Yamamoto T., Yoshimura M., Yamaguchi F., Kouchi T., Tsuji R., Saito M., Obata A., Kikuchi M., 2004, Anti-allergic Activity of Naringenin Chalcone from a Tomato Skin Extract, Bioscience, Biotechnology and Biochemistry, 68, 8, 1706-1711

Yan Y., Kohli A., Koffas M.A.G., 2005, Biosynthesis of Natural Flavanones in *Saccharomyces cerevisiae*, Applied and Environmental Microbiology, 71, 9, 5610-5613

Yáñez J.A., Andrews P.K., Davies N.M., 2007a, Methods of Analysis and Separation of Chiral Flavonoids, Journal of Chromatography B, 848, 159-181

Yáñez J.A., Davies N.M., 2005, Stereospecific High-Performance Liquid Chromatographic Analysis of Naringenin in Urine, Journal of Phanrmaceutical and Biomedical Analysis, 39, 164-169

Yañez J.A., Miranda N.D., Remsberg C.M., Ohgami Y., Davies N.M., 2007b, Stereospecific High-Performance Liquid Chromatographic Analysis of Eriodictyol in Urine, Journal of Pharmaceutical and Biomedical Analysis, 43, 255-262

Yáñez J.A., Remsberg C.M., Mirande N.D., Vega-Villa K.R., Andrews P.K., Davies N.M., 2008, Pharmacokinetics of Selected Chiral Flavonoids: Hesperetin, Naringenin, Eriodictyol in Rats and their Content in Friut Juices, Biopharmaceutics & Drug Disposition, 29, 63-82

Yano Y., Satomi M., Oikawa H., 2006, Antimicrobial Effect of Spices and Herbs on *Vibrio parahaemolyticus*, International Journal of Food Microbiology, 111, 6-11

Yesil Celiktas O., Hames Kocabas E.E., Bedir E., Vardar Sukan F., Ozek T., Baser K.H.C., 2007, Antimicrobial Activities of Methanol Extracts and Essential Oils of *Rosmarinus officinalis*, Depending on Location and Seasonal Variations, Food Chemistry, 100, 553-559

Yoshimura K., Miyazaki T., Nakahama K., Kikuchi M., 1986, *Bacillus subtilis* Secretes a Foreign Protein by the Signal Sequence of *Bacillus amyloliquefaciens* Neutral Protease, Applied Microbiology and Biotechnology, 23, 250-256

Youdim K.A., Dobbie M.S., Kuhnle G., Proteggente A.R., Abbott N.J., Rice-Evans C., 2003, Interaction between Flavonoids and the Blood-Brain Barrier: In Vitro Studies, Journal of Neurochemistry, 85, 180-192

Young M., Artsatbanov V., Beller H.R., Chandra G., Chater K.F., Dover L.G., Goh E-B., Kahan T., Kaprelyants A.S., Kyrpides N., Lapidus A., Lowry S.L., Lykidis A., Mahillon J., Markowitz V., Mavromatis K., Mukamolova G.V., Oren A., Rokem J.S., Zaika L.L., 1987, Spices and Herbs: Their Antimicrobial Activity and Its Determination, Journal of Food Safety, 9, 97-118

Zhang J., Brodbelt J.S., 2004, Screening Flavonoid Metabolites of Naringin and Narirutin in Urine after Human Consumption of Grapefruit Juice by LC-MS and LC-MS/MS, The Royal Society of Chemistry, 129, 1227-1233

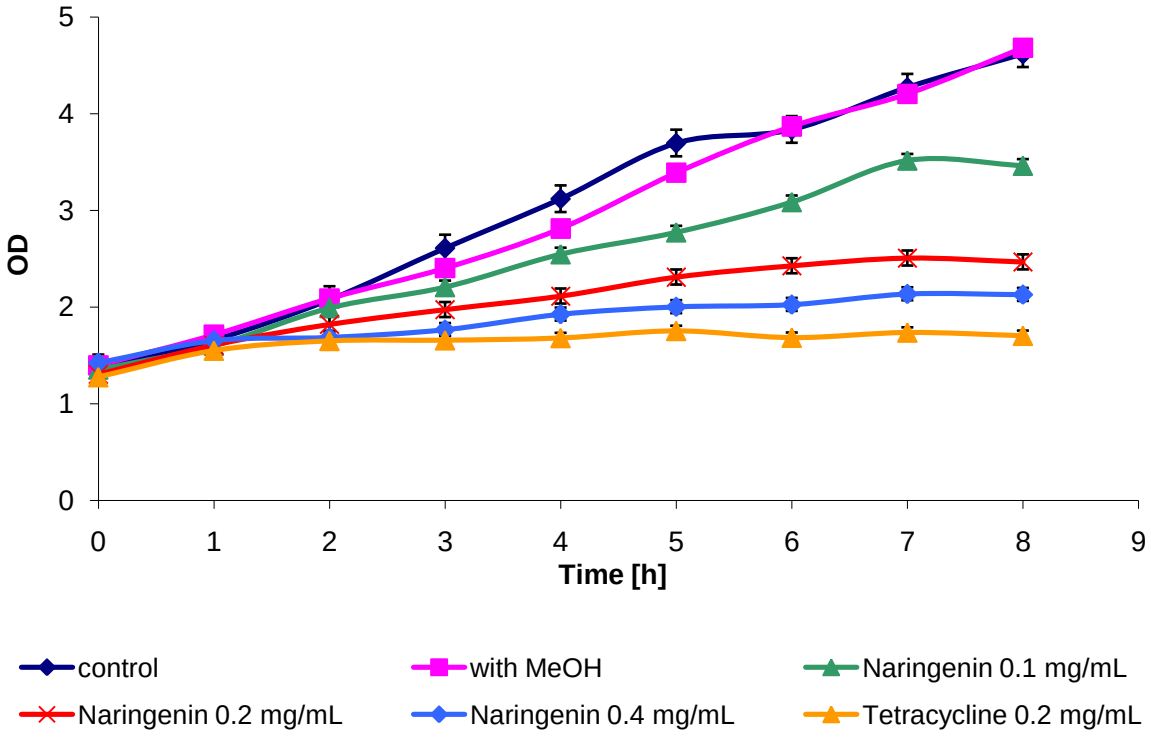
Zhao Z., Yu Z., Zhang L., Zhou D., Chen X., Bi K., 2007, Simultaneous Determination of Homoeriodictyol-7-O-β-D-Glccopyranoside and its Metabolite Homoeriodictyol In Rat Tissues and Urine by Liquid Chromatography – Mass Spectrometry, Journal of Pharmaceutical and Biomedical Analysis, 44, 293-300

9. Annexes

Annex I. Growth curves of the racemates in various concentrations

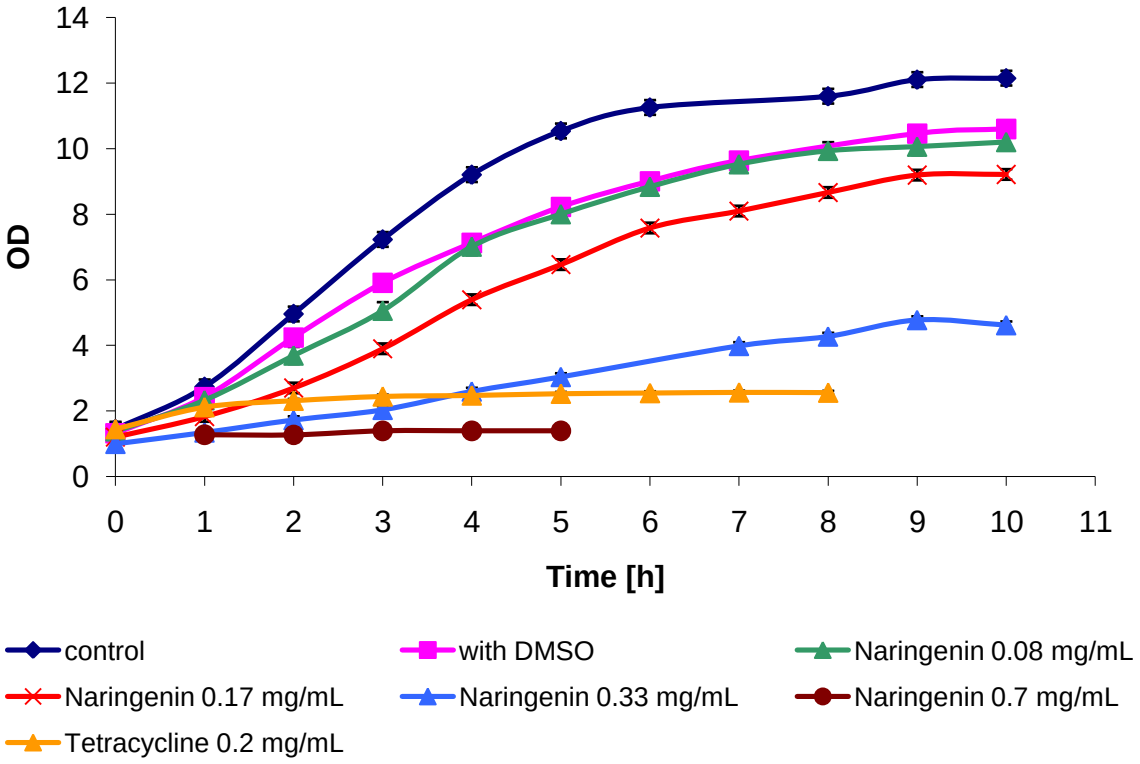
Naringenin

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of naringenin; OD – optical density, SD – standard deviation.



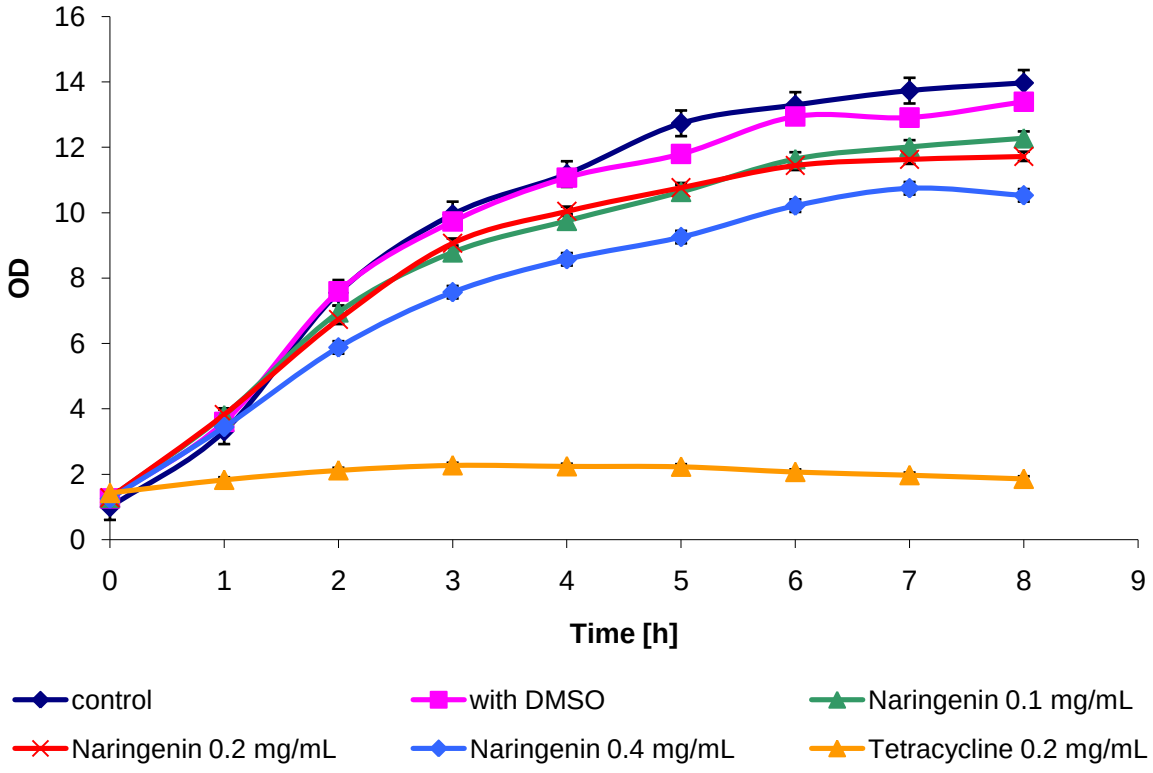
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Naringenin 0.1 mg/mL	Naringenin 0.2 mg/mL	Naringenin 0.4 mg/mL
0	1.37	1.40	8.59	3.10	6.68	0.00
1	1.66	1.71	9.53	6.61	6.23	3.50
2	2.08	2.09	21.05	4.78	12.92	19.30
3	2.61	2.40	31.07	8.18	17.89	26.49
4	3.12	2.81	40.28	9.48	24.88	31.52
5	3.70	3.39	48.28	18.19	31.86	40.90
6	3.84	3.87	56.50	20.24	37.30	47.63
7	4.27	4.21	58.72	16.40	40.41	49.21
8	4.62	4.68	63.63	26.05	47.33	54.52
SD	0.13783456	0.06044968	0.05256633	0.06651422	0.07787079	0.06711052

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of naringenin; OD – optical density, SD – standard deviation.



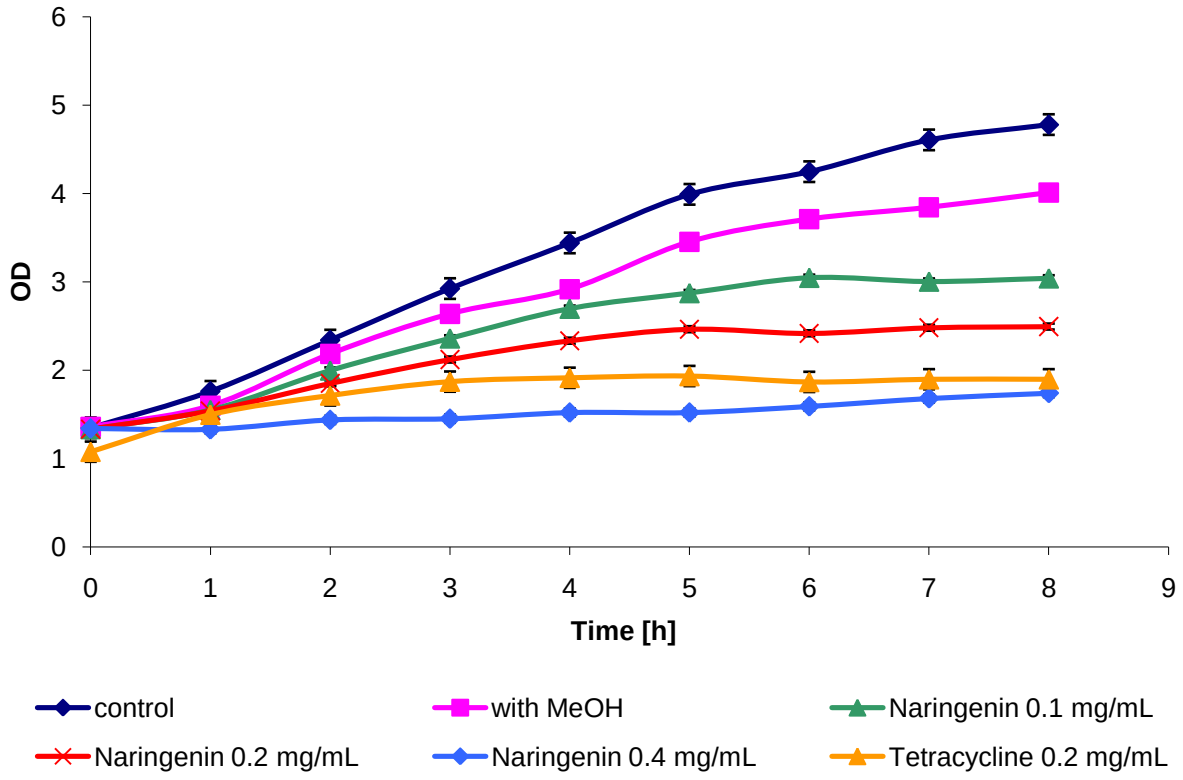
Time [h]	OD						
	Control	DMSO	Tetracycline 0.2 mg/mL	Naringenin			
				0.08 mg/mL	0.17 mg/mL	0.33 mg/mL	0.7 mg/mL
0	1.44	1.32	1.45	1.38	1.20	1.00	--
1	2.73	2.42	2.12	2.33	1.83	1.34	1.27
2	4.96	4.24	2.31	3.69	2.70	1.72	1.27
3	7.23	5.91	2.44	5.06	3.90	2.03	1.39
4	9.21	7.12	2.47	7.01	5.39	2.59	1.39
5	10.54	8.22	2.53	8.00	6.46	3.04	1.39
6	11.26	9.01	2.55	8.84	7.58	2.97	--
7	10.90	9.63	2.57	9.53	8.10	3.98	--
8	11.60	9.59	2.56	9.94	8.66	4.27	--
9	12.11	10.46	--	10.06	9.19	4.77	--
10	12.15	10.60	--	10.20	9.21	4.62	--
SD	0.22410244	0.21951887	0.05198393	0.2612201	0.1605452	0.1116528	0.0696241

Growth curve of *Escherichia coli ATCC 23716* on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of naringenin; OD – optical density, SD – standard deviation.



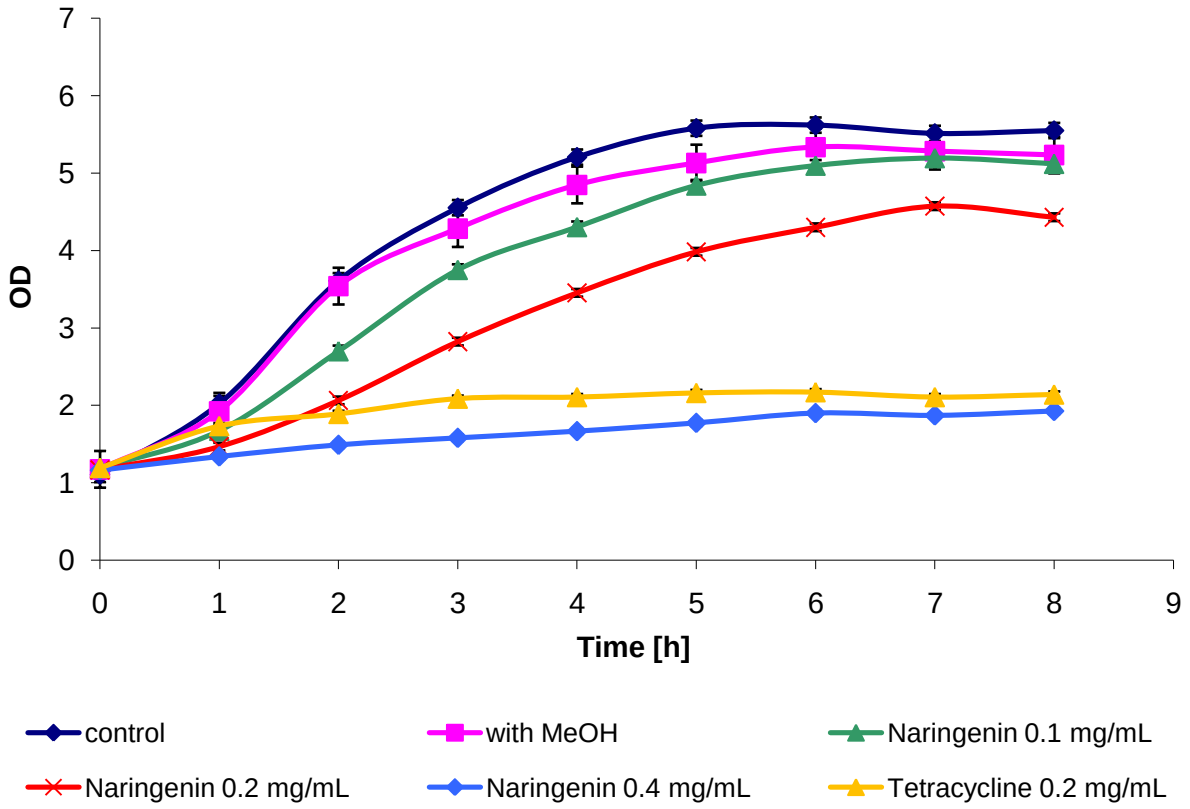
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Nairngenin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.99	1.26	1.43	1.28	1.28	1.25
1	3.31	3.60	1.83	3.81	3.83	3.44
2	7.55	7.59	2.12	6.95	6.73	5.88
3	9.94	9.73	2.27	8.79	9.07	7.57
4	11.18	11.07	2.24	9.75	10.04	8.57
5	12.73	11.80	2.23	10.64	10.77	9.25
6	13.29	12.94	2.07	11.64	11.45	10.21
7	13.73	12.91	1.97	12.01	11.63	10.75
8	13.97	13.39	1.86	12.28	11.72	10.53
SD	0.39311795	0.22442825	0.07569628	0.20458383	0.14190797	0.19109016

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of naringenin; OD – optical density, SD – standard deviation.



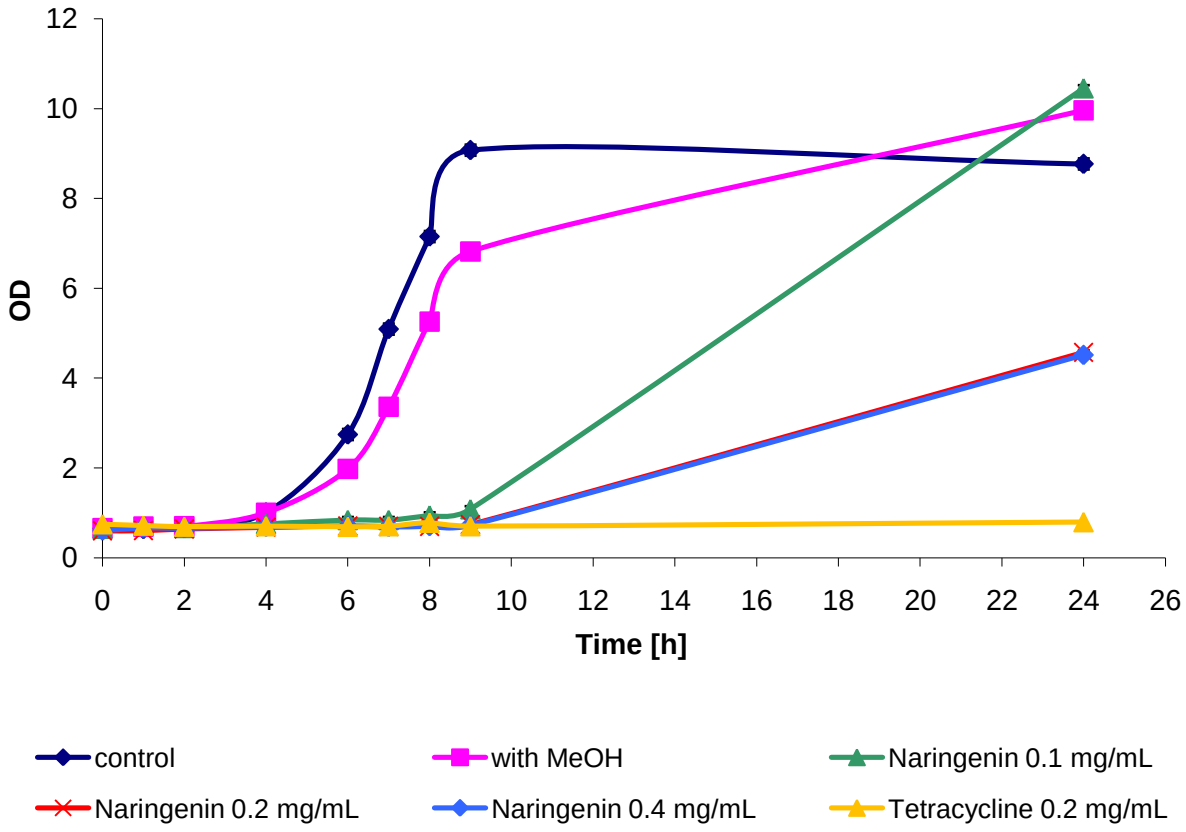
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Naringenin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.35	1.36	1.08	1.33	1.33	1.34
1	1.76	1.60	1.50	1.55	1.54	1.33
2	2.34	2.18	1.71	2.00	1.85	1.44
3	2.92	2.64	1.87	2.36	2.12	1.45
4	3.44	2.92	1.91	2.70	2.33	1.52
5	3.99	3.45	1.93	2.87	2.46	1.52
6	4.25	3.71	1.87	3.05	2.42	1.59
7	4.61	3.84	1.90	3.00	2.48	1.68
8	4.78	4.01	1.90	3.04	2.49	1.74
SD	0.11657707	0.07658852	0.11445771	0.03417139	0.03373248	0.04566597

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of naringenin; OD – optical density, SD – standard deviation.



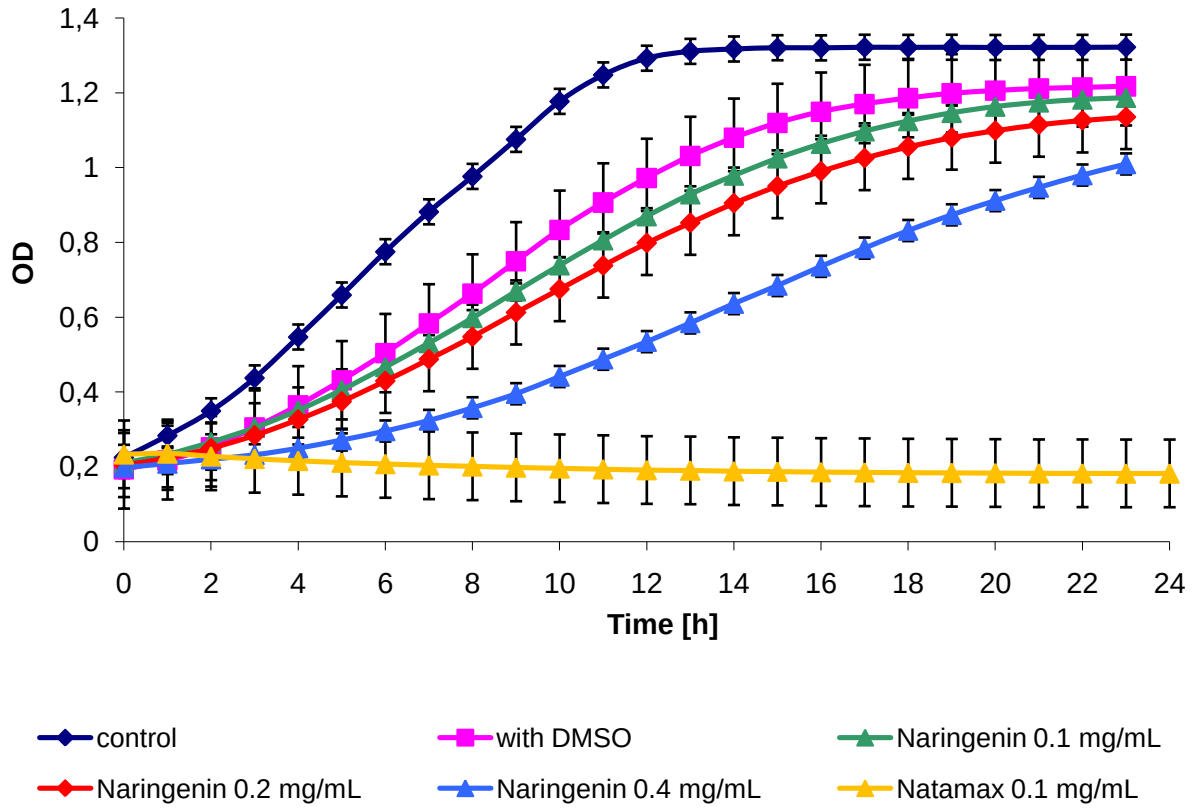
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Naringenin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.11	1.17	1.19	1.19	1.17	1.16
1	2.02	1.92	1.73	1.67	1.47	1.34
2	3.61	3.54	1.89	2.70	2.06	1.49
3	4.55	4.28	2.09	3.75	2.82	1.58
4	5.21	4.85	2.11	4.30	3.45	1.67
5	5.58	5.13	2.16	4.84	3.98	1.77
6	5.62	5.34	2.17	5.10	4.30	1.90
7	5.51	5.28	2.11	5.19	4.57	1.87
8	5.55	5.23	2.14	5.12	4.43	1.93
SD	0.09848988	0.23713693	0.03921362	0.07163363	0.04954568	0.03921362

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of naringenin; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Naringenin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.65	0.66	0.75	0.65	0.60	0.61
1	0.64	0.69	0.72	0.71	0.61	0.67
2	0.69	0.70	0.69	0.66	0.64	0.66
4	1.01	1.00	0.70	0.75	0.68	0.69
6	2.74	1.98	0.69	0.84	0.71	0.71
7	5.09	3.36	0.70	0.84	0.70	0.69
8	7.15	5.26	0.78	0.94	0.70	0.71
9	9.07	6.82	0.70	1.08	0.74	0.72
24	8.77	9.96	0.79	10.45	4.57	4.52
SD	0.12316401	0.10533159	0.05209636	0.06711505	0.0411364	0.04094696

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of naringenin; OD – optical density, SD – standard deviation.

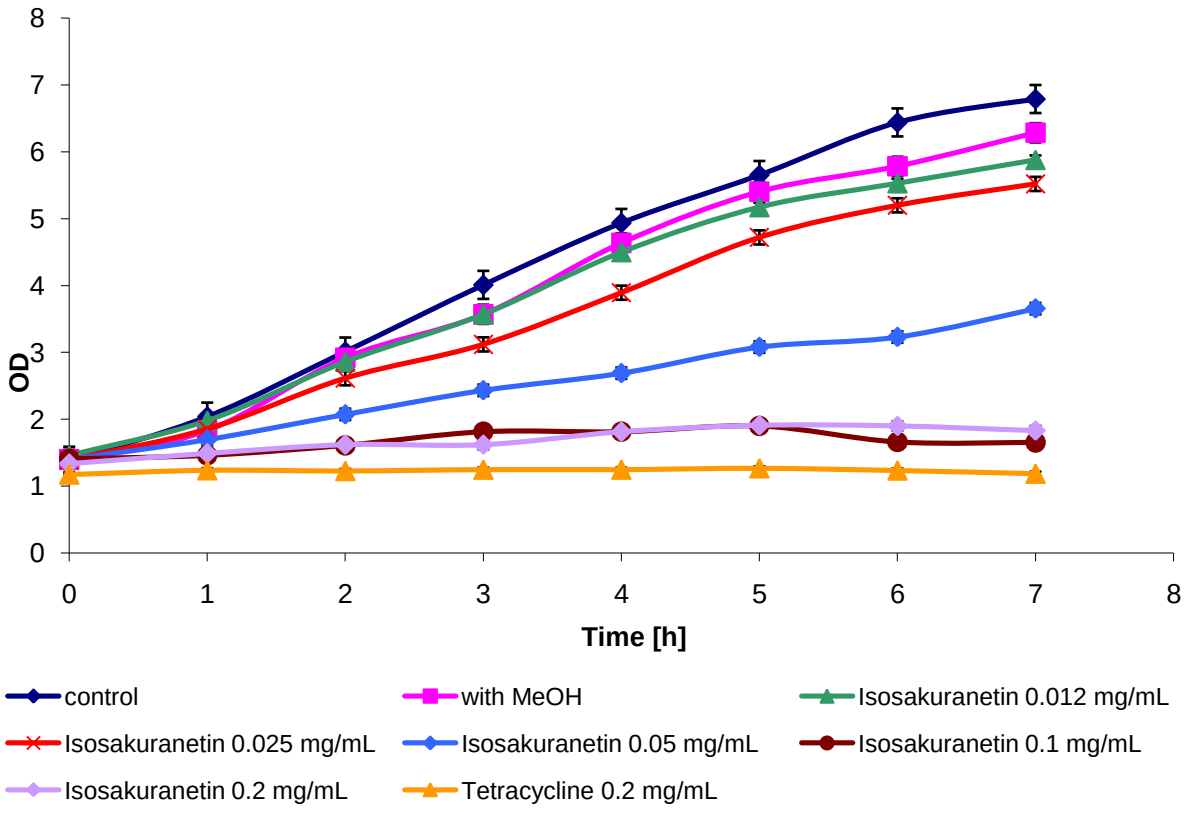


Time [h]	OD					
	Control	DMSO	Natamax 0.2 mg/mL	Naringenin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.2253	0.1930	0.2333	0.2103	0.2047	0.1983
1	0.2837	0.2173	0.2357	0.2333	0.2240	0.2087
2	0.3497	0.2520	0.2280	0.2660	0.2500	0.2203
3	0.4377	0.3050	0.2213	0.3043	0.2843	0.2320
4	0.5470	0.3643	0.2160	0.3513	0.3267	0.2497
5	0.6593	0.4313	0.2113	0.4057	0.3750	0.2717
6	0.7750	0.5040	0.2077	0.4667	0.4297	0.2957
7	0.8817	0.5833	0.2040	0.5310	0.4877	0.3240
8	0.9763	0.6633	0.2013	0.5980	0.5477	0.3577
9	1.0753	0.7493	0.1983	0.6693	0.6127	0.3953
10	1.1770	0.8337	0.1960	0.7390	0.6750	0.4413
11	1.2477	0.9067	0.1937	0.8060	0.7380	0.4877
12	1.2923	0.9723	0.1913	0.8703	0.7983	0.5347

13	1.3107	1.0310	0.1903	0.9290	0.8523	0.5847
14	1.3170	1.0797	0.1883	0.9790	0.9047	0.6363
15	1.3203	1.1193	0.1873	1.0250	0.9503	0.6847
16	1.3200	1.1493	0.1860	1.0640	0.9900	0.7360
17	1.3217	1.1700	0.1853	1.0973	1.0253	0.7847
18	1.3213	1.1857	0.1843	1.1247	1.0553	0.8317
19	1.3217	1.1983	0.1840	1.1467	1.0800	0.8737
20	1.3210	1.2057	0.1833	1.1633	1.0987	0.9117
21	1.3213	1.2113	0.1827	1.1747	1.1147	0.9470
22	1.3213	1.2140	0.1827	1.1823	1.1260	0.9800
23	1.3220	1.2173	0.1823	1.1870	1.1350	1.0097
SD	0.03347273	0.10475891	0.09044731	0.02094417	0.08564982	0.02822055

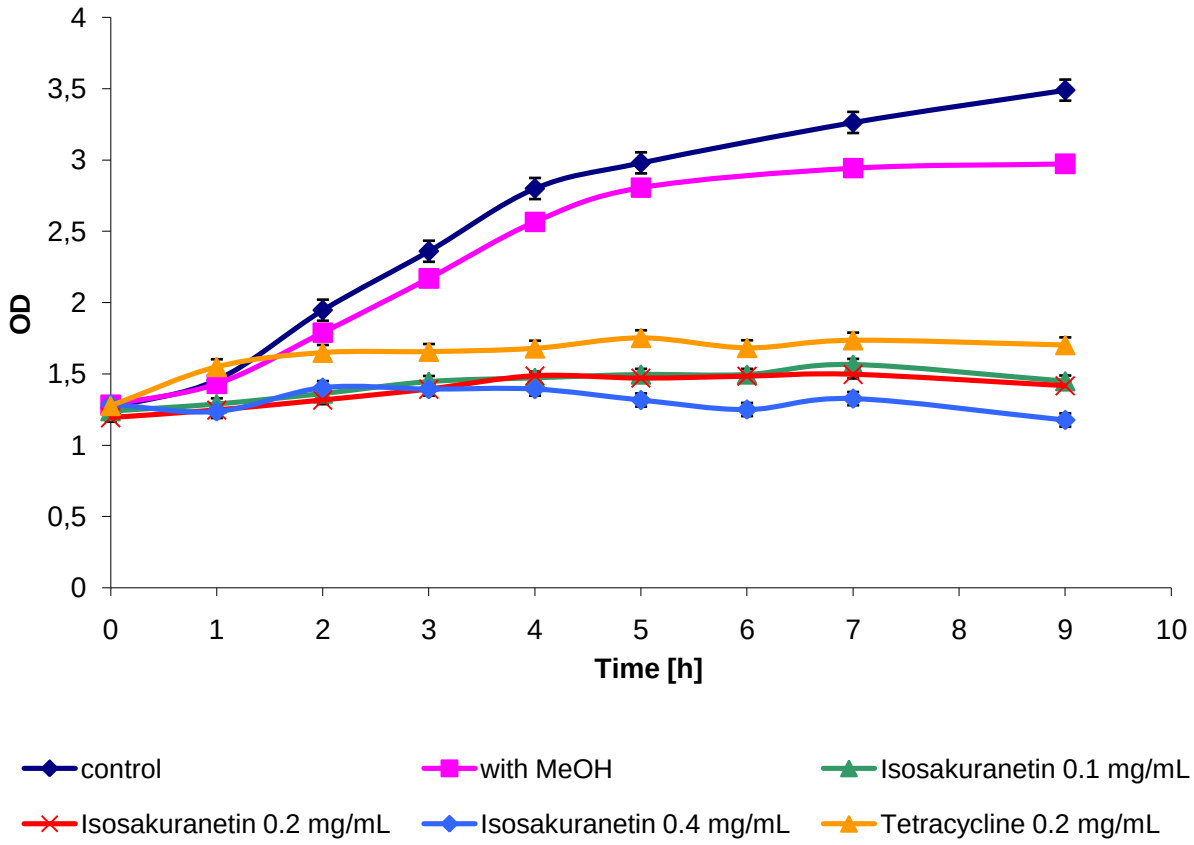
Isosakuranetin

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



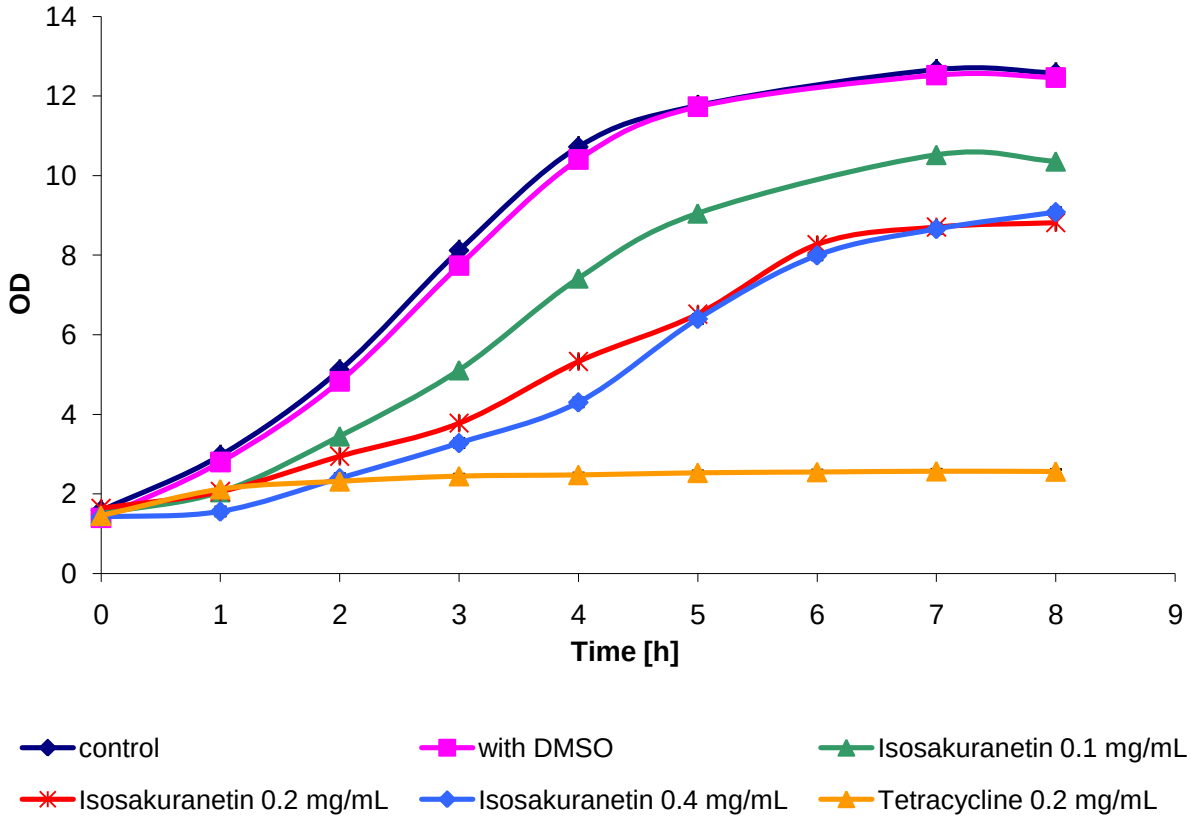
Time [h]	OD							
	Control	MeOH	Tetracycline 0.2 mg/mL	Isosakuranetin				
				0.012 mg/mL	0.025 mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL
0	1.38	1.40	1.17	1.46	1.39	1.40	1.41	1.34
1	2.04	1.83	1.24	1.98	1.85	1.70	1.46	1.49
2	3.01	2.92	1.23	2.86	2.61	2.07	1.61	1.62
3	4.01	3.57	1.25	3.57	3.12	2.43	1.81	1.62
4	4.94	4.64	1.25	4.50	3.89	2.69	1.81	1.81
5	5.65	5.40	1.27	5.17	4.72	3.08	1.90	1.91
6	6.44	5.78	1.23	5.53	5.20	3.23	1.66	1.90
7	6.79	6.29	1.19	5.88	5.52	3.66	1.66	1.83
SD	0.209594	0.143647	0.030295	0.066198	0.105792	0.085186	0.051125	0.069175

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



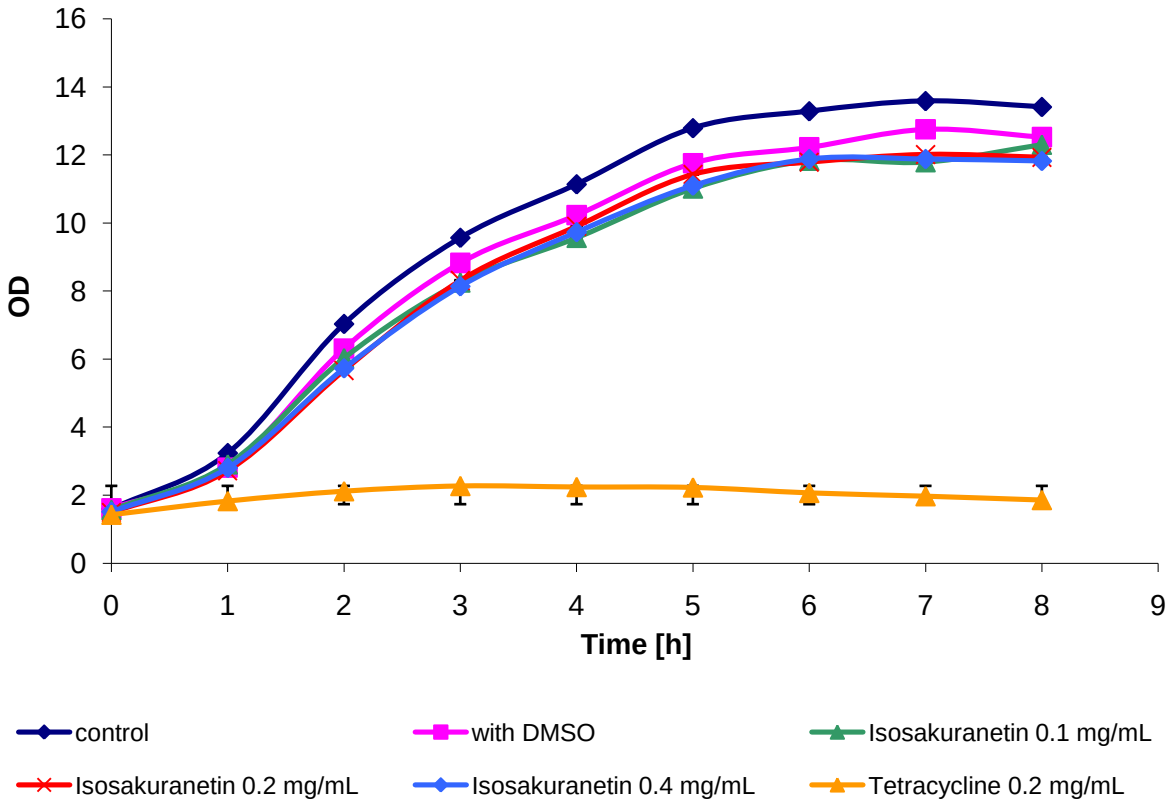
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Isosakuranetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.26	1.28	1.28	1.24	1.19	1.29
1	1.46	1.43	1.55	1.29	1.25	1.24
2	1.95	1.79	1.65	1.36	1.32	1.40
3	2.36	2.17	1.66	1.45	1.39	1.39
4	2.80	2.57	1.68	1.47	1.49	1.39
5	2.98	2.81	1.75	1.50	1.47	1.32
6	2.88	2.41	1.68	1.50	1.48	1.25
7	3.26	2.94	1.74	1.57	1.50	1.33
9	3.49	2.97	1.70	1.45	1.42	1.18
SD	0.07394904	0.04844493	0.05256633	0.03850894	0.02891076	0.04629028

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



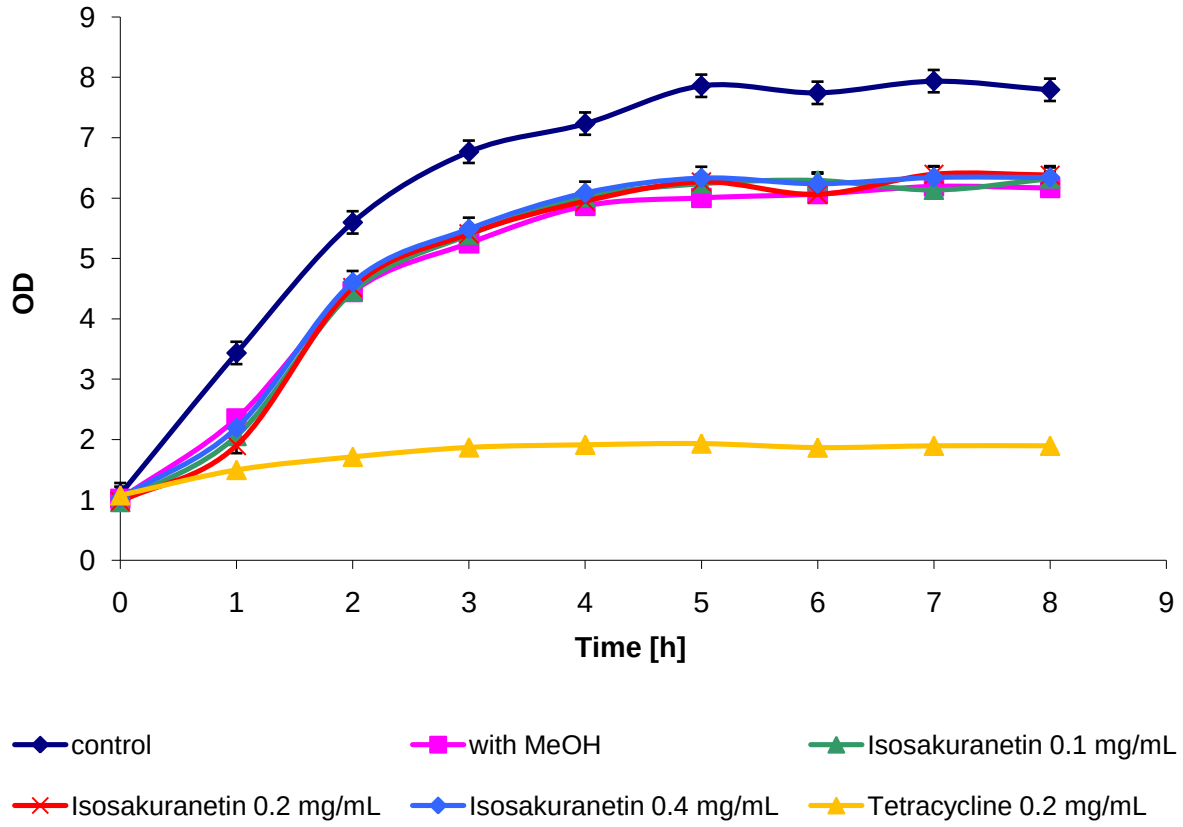
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Isosakuranetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.58	1.40	1.45	1.51	1.42	1.64
1	2.97	2.80	2.12	2.05	1.56	2.06
2	5.11	4.83	2.31	3.45	2.39	2.95
3	8.12	7.74	2.44	5.11	3.28	3.78
4	10.72	10.41	2.47	7.42	4.30	5.33
5	11.76	11.73	2.53	9.05	6.40	6.52
6	11.78	11.85	2.55	9.43	7.99	8.27
7	12.66	12.53	2.57	10.52	8.66	8.71
8	12.58	12.47	2.56	10.36	9.08	8.82
SD	0.2676481	0.16493159	0.05198393	0.11700406	0.11597931	0.20483945

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



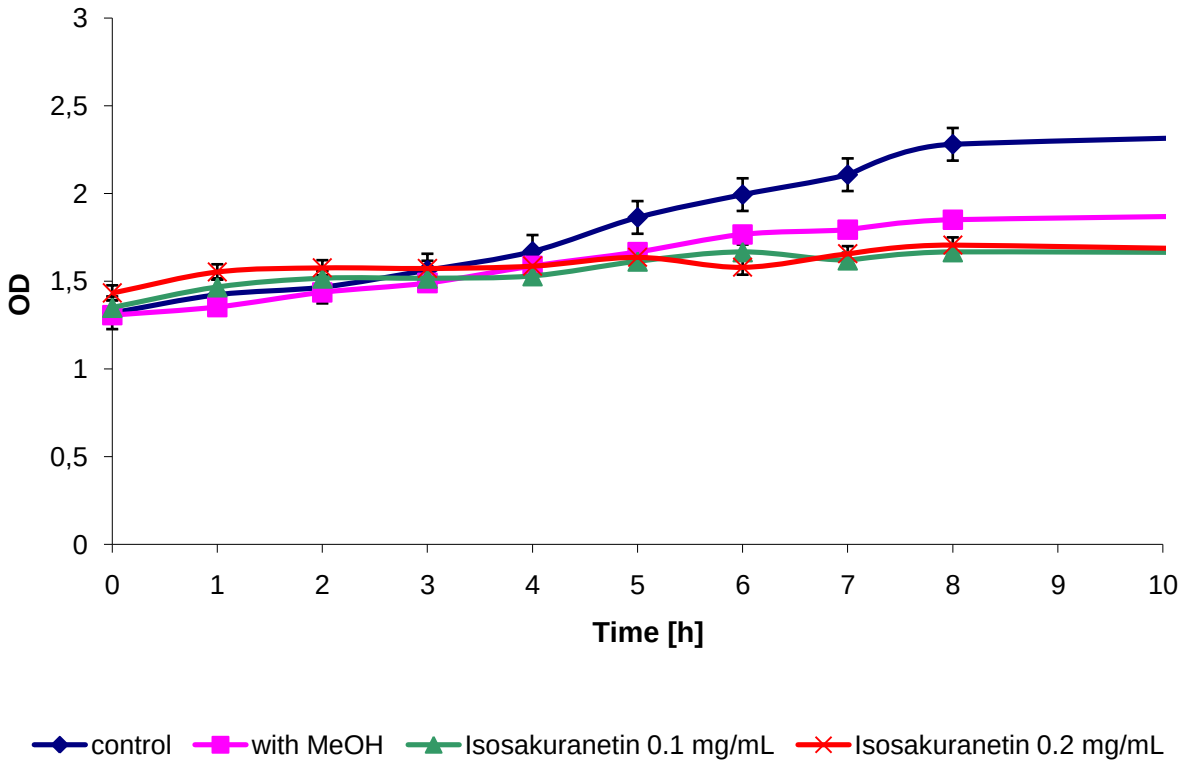
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Isosakuranetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.60	1.62	1.43	1.57	1.49	1.51
1	3.24	2.81	1.83	2.90	2.72	2.80
2	7.03	6.31	2.12	6.03	5.67	5.73
3	9.57	8.83	2.27	8.25	8.31	8.14
4	11.14	10.23	2.24	9.56	9.90	9.73
5	12.79	11.76	2.23	11.01	11.43	11.10
6	13.29	12.22	2.07	11.83	11.79	11.88
7	13.59	12.75	1.97	11.79	12.01	11.88
8	13.41	12.53	1.86	12.30	11.93	11.83
SD	0.04626025	0.01494254	0.07569628	0.02047316	0.01736914	0.02145879

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



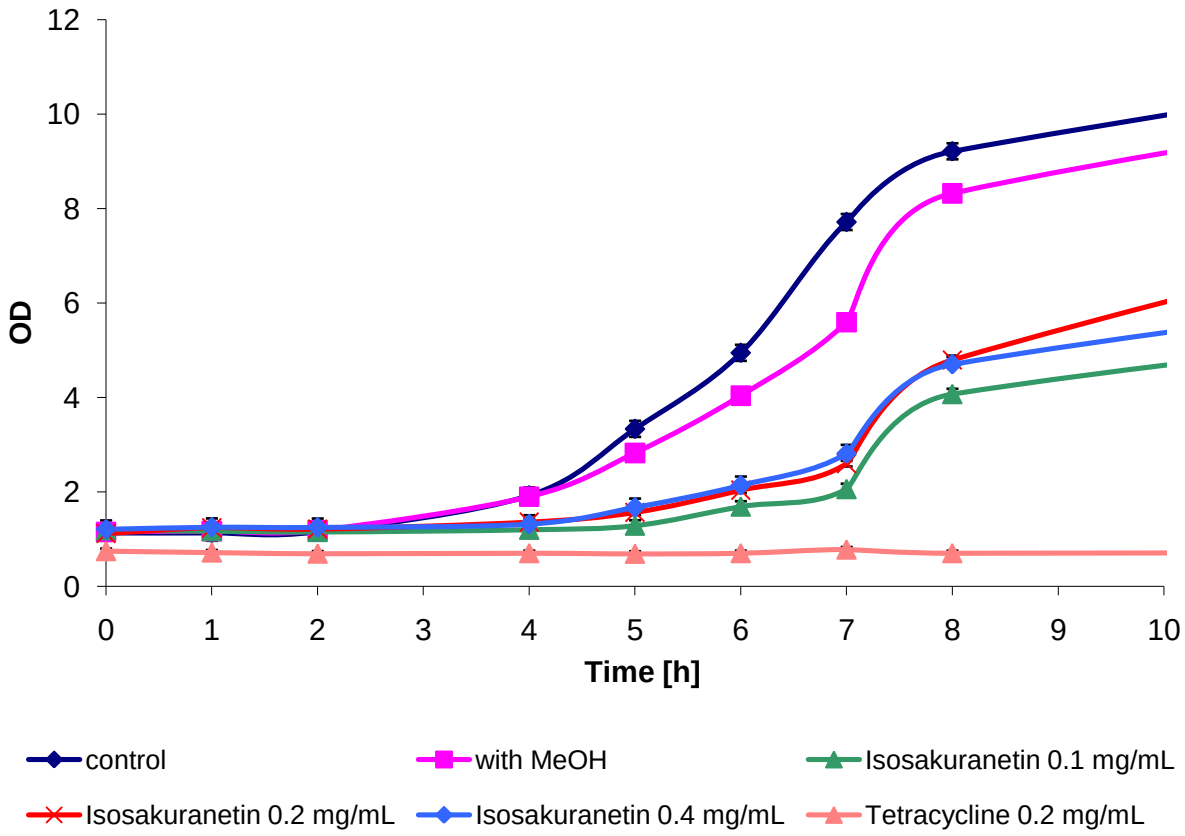
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Isosakuranetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.09	1.02	1.08	0.97	0.97	1.03
1	3.43	2.35	1.50	2.06	1.90	2.19
2	5.60	4.44	1.71	4.45	4.52	4.61
3	6.77	5.25	1.87	5.39	5.41	5.49
4	7.23	5.87	1.91	6.02	5.95	6.09
5	7.86	6.00	1.93	6.24	6.27	6.33
6	7.74	6.07	1.87	6.29	6.06	6.24
7	7.94	6.19	1.90	6.14	6.39	6.34
8	7.79	6.17	1.90	6.32	6.38	6.34
SD	0.18522006	0.12798383	0.02695436	0.11054529	0.12662967	0.18472093

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



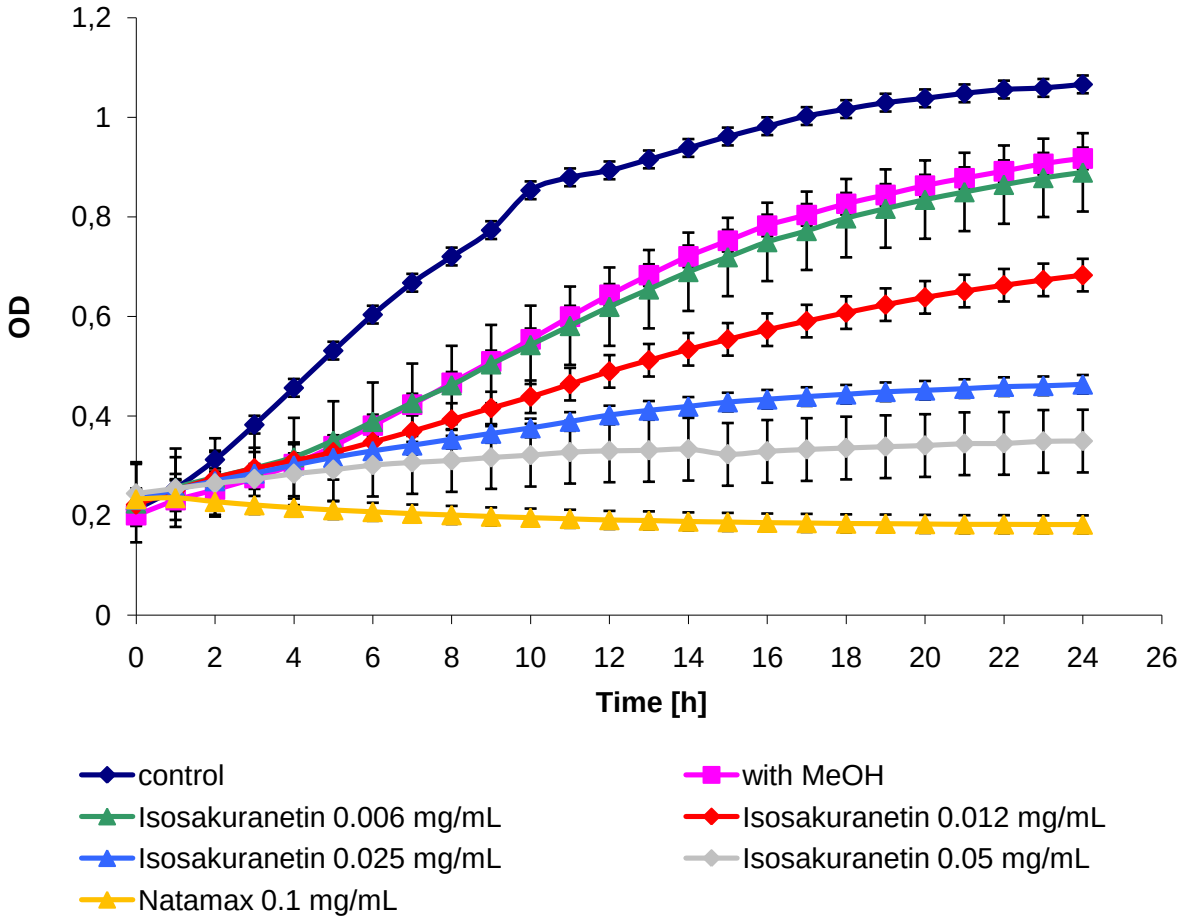
Time [h]	OD				
	Control	MeOH	Isosakuranetin		
			0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.32	1.31	1.35	1.43	1.44
1	1.42	1.35	1.47	1.55	1.56
2	1.47	1.44	1.52	1.58	1.65
3	1.56	1.49	1.52	1.57	1.56
4	1.67	1.59	1.53	1.59	1.76
5	1.86	1.67	1.61	1.64	1.70
6	1.99	1.77	1.67	1.58	1.70
7	2.11	1.79	1.62	1.66	1.71
8	2.28	1.85	1.67	1.71	1.83
SD	0.09303908	0.04113348	0.04225569	0.04293357	0.07829021

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Isosakuranetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.13	1.15	0.75	1.19	1.12	1.21
1	1.13	1.17	0.72	1.17	1.24	1.25
2	1.16	1.18	0.69	1.15	1.21	1.25
4	1.92	1.90	0.70	1.20	1.36	1.32
6	3.33	2.82	0.69	1.29	1.57	1.67
7	4.94	4.04	0.70	1.69	2.04	2.14
8	7.71	5.59	0.78	2.06	2.60	2.81
9	9.21	8.32	0.70	4.07	4.79	4.70
SD	0.16959777	0.1625339	0.05209636	0.11137448	0.05939814	0.18408121

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of methanol (MeOH) and various concentration of isosakuranetin; OD – optical density, SD – standard deviation.

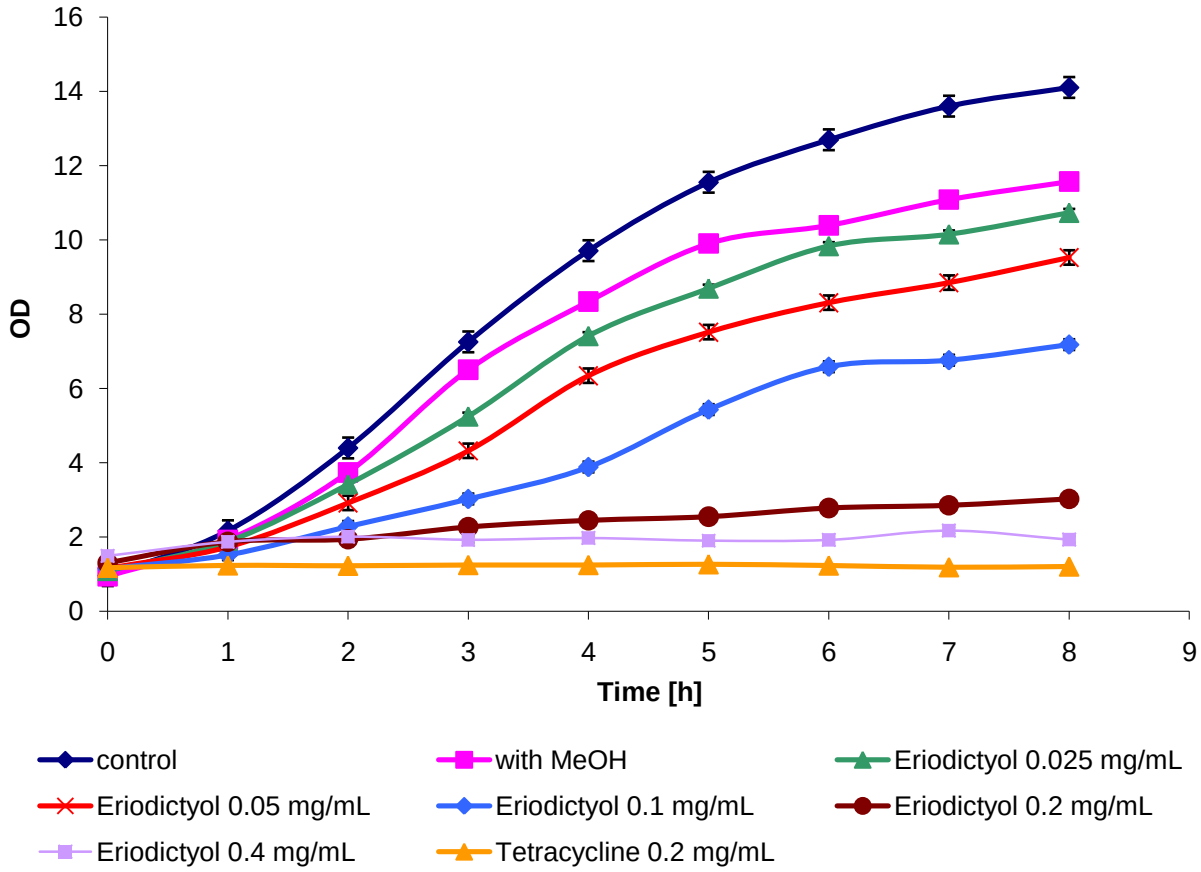


Time [h]	OD									
	Control	MeOH	Natamax 0.2 mg/mL	Isosakuranetin						
				0.006 mg/mL	0.0125 mg/mL	0.025 mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.2127	0.2010	0.2333	0.2250	0.2220	0.2320	0.2443	0.2867	0.3510	0.5997
1	0.2553	0.2310	0.2357	0.2560	0.2510	0.2520	0.2543	0.3090	0.3710	0.7077
2	0.3127	0.2523	0.2280	0.2767	0.2757	0.2697	0.2647	0.3217	0.3760	0.7890
3	0.3827	0.2753	0.2213	0.2957	0.2950	0.2850	0.2733	0.3393	0.3740	0.7820
4	0.4567	0.3033	0.2160	0.3177	0.3107	0.3013	0.2840	0.3440	0.3757	0.7830
5	0.5313	0.3397	0.2113	0.3510	0.3273	0.3167	0.2920	0.3477	0.3787	0.7920
6	0.6037	0.3810	0.2077	0.3887	0.3480	0.3300	0.3013	0.3537	0.3813	0.7870
7	0.6677	0.4230	0.2040	0.4267	0.3697	0.3413	0.3067	0.3597	0.3843	0.7923
8	0.7203	0.4667	0.2013	0.4623	0.3930	0.3533	0.3107	0.3703	0.3880	0.7873

9	0.7733	0.5097	0.1983	0.5043	0.4160	0.3650	0.3167	0.3740	0.3933	0.7810
10	0.8533	0.5540	0.1960	0.5430	0.4387	0.3760	0.3213	0.3700	0.4013	0.7837
11	0.8793	0.5997	0.1937	0.5813	0.4640	0.3890	0.3273	0.3747	0.4060	0.7797
12	0.8933	0.6433	0.1913	0.6197	0.4897	0.4020	0.3300	0.3657	0.4087	0.7733
13	0.9153	0.6830	0.1903	0.6547	0.5120	0.4113	0.3310	0.3813	0.4077	0.7683
14	0.9383	0.7207	0.1883	0.6897	0.5340	0.4193	0.3333	0.3763	0.4063	0.7643
15	0.9613	0.7520	0.1873	0.7193	0.5540	0.4280	0.3230	0.3570	0.4073	0.7733
16	0.9820	0.7827	0.1860	0.7497	0.5733	0.4337	0.3290	0.3437	0.4073	0.7637
17	1.0023	0.8040	0.1853	0.7720	0.5907	0.4390	0.3327	0.3137	0.4030	0.7643
18	1.0163	0.8263	0.1843	0.7973	0.6077	0.4437	0.3357	0.3220	0.4010	0.7637
19	1.0293	0.8440	0.1840	0.8167	0.6237	0.4487	0.3383	0.2830	0.3960	0.7540
20	1.0380	0.8627	0.1833	0.8347	0.6383	0.4517	0.3407	0.2543	0.3927	0.7607
21	1.0480	0.8777	0.1827	0.8500	0.6510	0.4550	0.3443	0.2490	0.3910	0.7647
22	1.0557	0.8917	0.1827	0.8647	0.6627	0.4590	0.3450	0.2270	0.3843	0.7533
23	1.0590	0.9067	0.1823	0.8783	0.6733	0.4607	0.3490	0.2697	0.3820	0.7590
24	1.0660	0.9173	0.1823	0.8893	0.6830	0.4637	0.3497	0.2657	0.3797	0.7510
SD	0.01786	0.02171	0.01817	0.07869	0.03270	0.01875	0.06298	0.05842	0.06259	0.11214

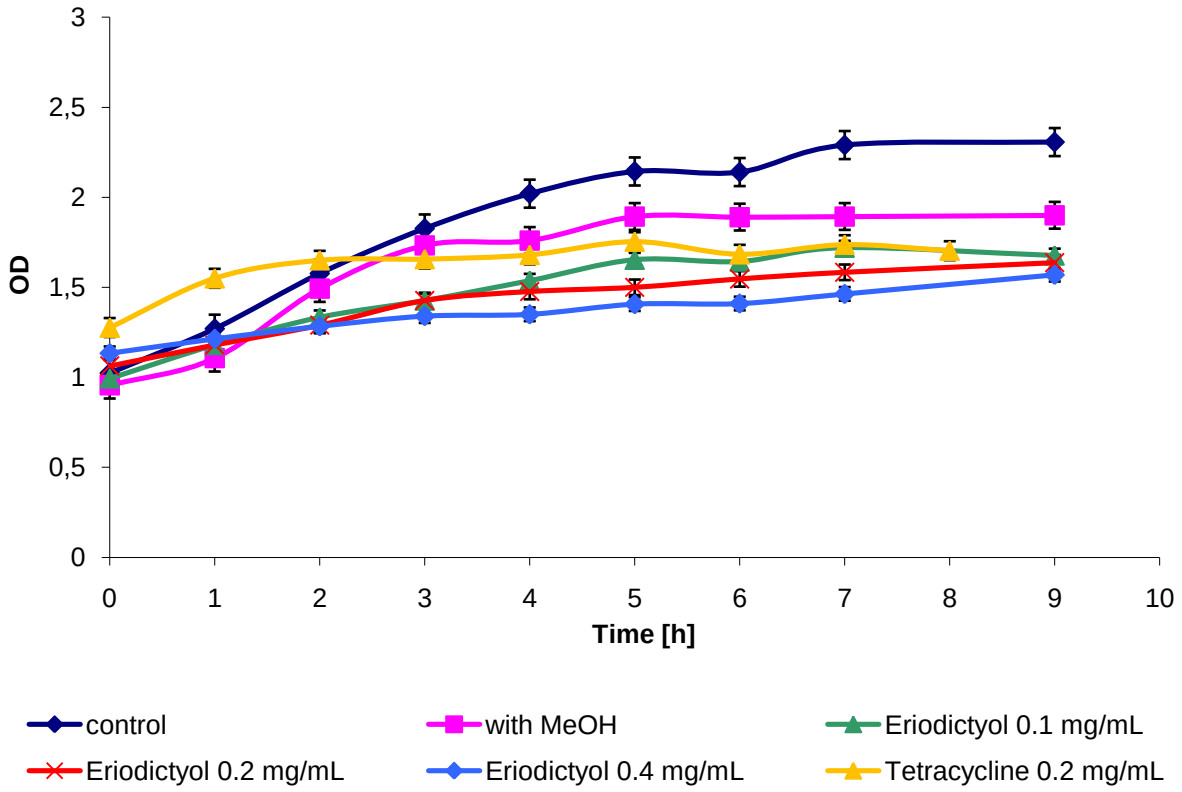
Eriodictyol

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



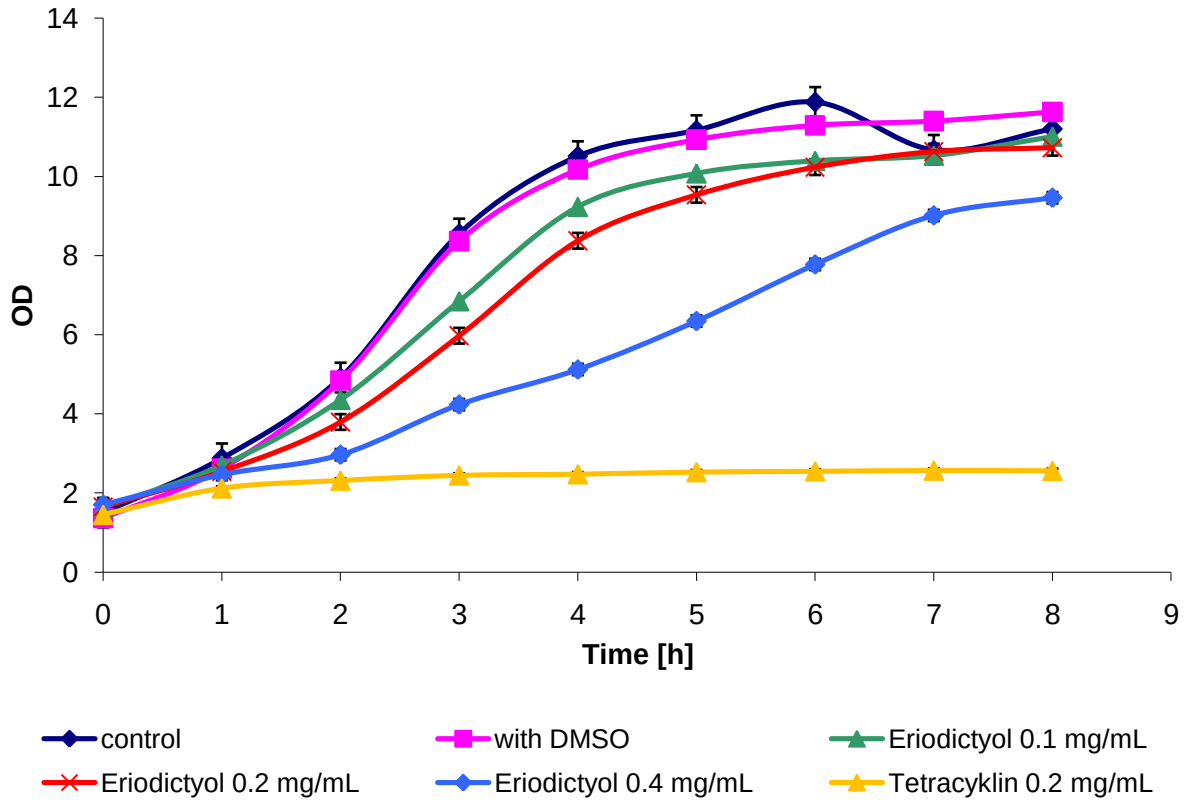
Time [h]	OD							
	Control	MeOH	Tetracycline 0.2 mg/mL	Eriodictyol				
				0.025 mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.95	0.94	1.17	1.08	1.15	1.15	1.30	1.49
1	2.16	1.92	1.24	1.87	1.73	1.52	1.87	1.87
2	4.39	3.74	1.23	3.41	2.91	2.28	1.94	2.01
3	7.25	6.50	1.25	5.24	4.32	3.02	2.27	1.92
4	9.71	8.34	1.25	7.41	6.34	3.88	2.45	1.97
5	11.55	9.90	1.27	8.69	7.51	5.43	2.55	1.90
6	12.69	10.39	1.23	9.83	8.31	6.58	2.78	1.92
7	13.60	11.09	1.19	10.15	8.85	6.76	2.85	2.17
8	14.10	11.57	1.21	10.73	9.52	7.18	3.03	1.93
SD	0.279514	0.137805	0.030295	0.101013	0.193934	0.144037	0.128538	0.085878

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



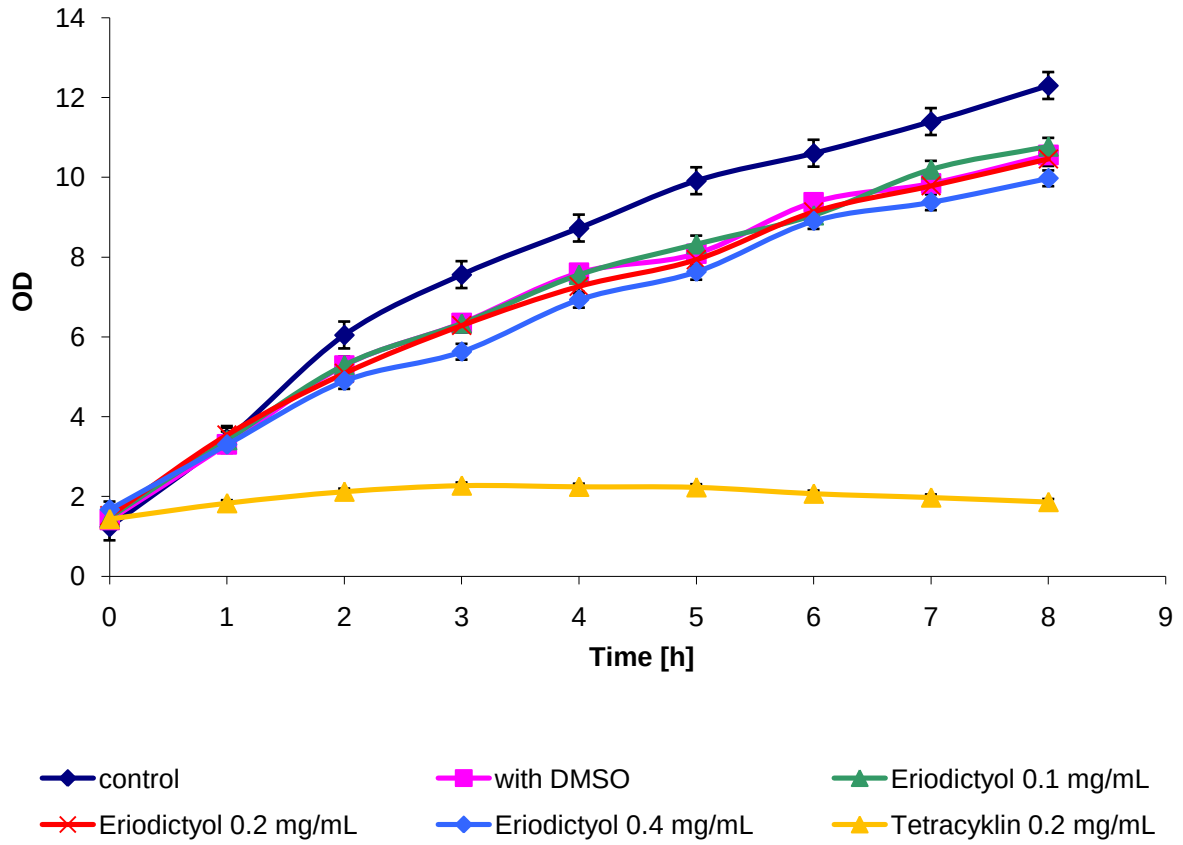
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Eriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.02	0.96	1.28	0.99	1.06	1.13
1	1.27	1.11	1.55	1.18	1.18	1.21
2	1.58	1.49	1.65	1.33	1.29	1.28
3	1.83	1.73	1.66	1.43	1.43	1.34
4	2.02	1.76	1.68	1.54	1.48	1.35
5	2.14	1.89	1.75	1.65	1.50	1.41
6	2.14	1.89	1.68	1.64	1.55	1.41
7	2.29	1.89	1.74	1.72	1.58	1.46
9	2.31	1.90	1.70	1.68	1.64	1.57
SD	0.07790883	0.07439556	0.05256633	0.03800476	0.04317725	0.03789231

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



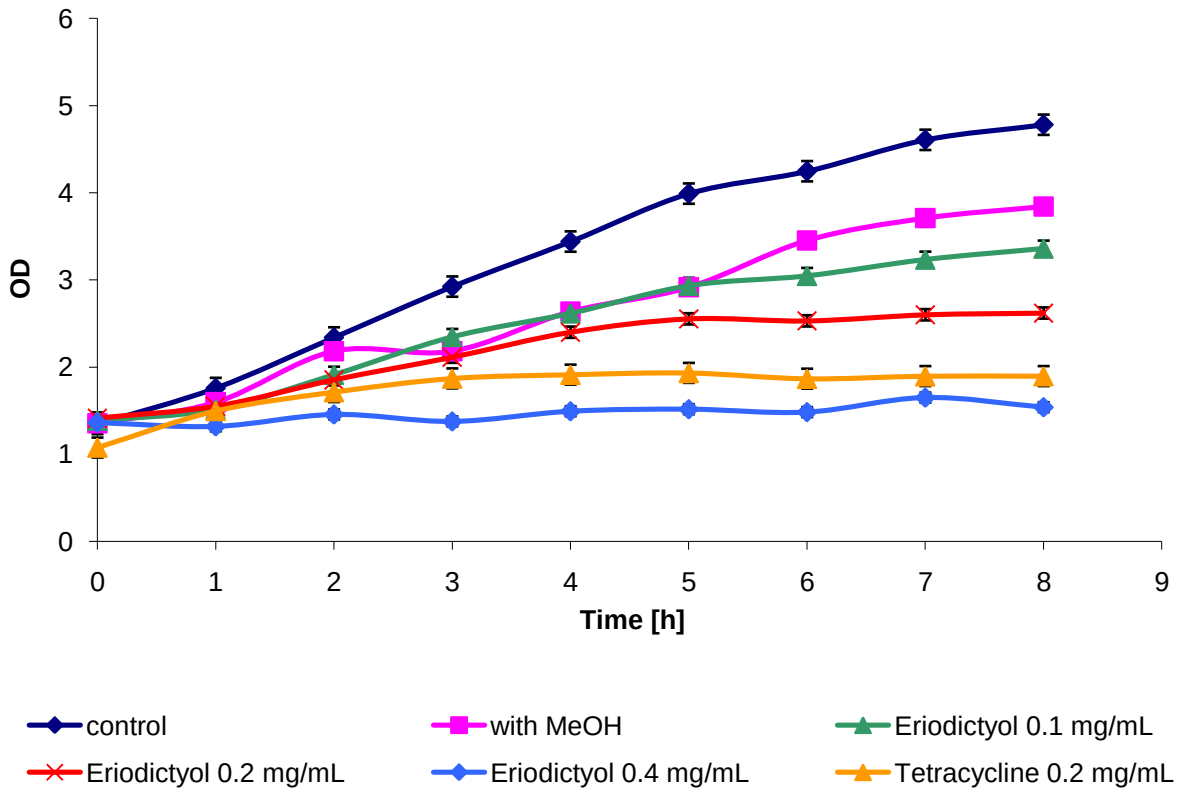
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Eriodictyol 0.1 mg/mL	Eriodictyol 0.2 mg/mL	Eriodictyol 0.4 mg/mL
0	1.50	1.37	1.45	1.64	1.65	1.71
1	2.88	2.62	2.12	2.70	2.54	2.46
2	4.92	4.84	2.31	4.35	3.79	2.97
3	8.56	8.37	2.44	6.85	5.97	4.23
4	10.51	10.17	2.47	9.23	8.37	5.12
5	11.17	10.93	2.53	10.08	9.53	6.34
6	11.88	11.29	2.55	10.40	10.23	7.77
7	10.67	11.40	2.57	10.53	10.63	9.01
8	11.20	11.63	2.56	11.01	10.72	9.46
SD	0.37356718	0.12541885	0.05198393	0.13342932	0.19818992	0.14327482

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



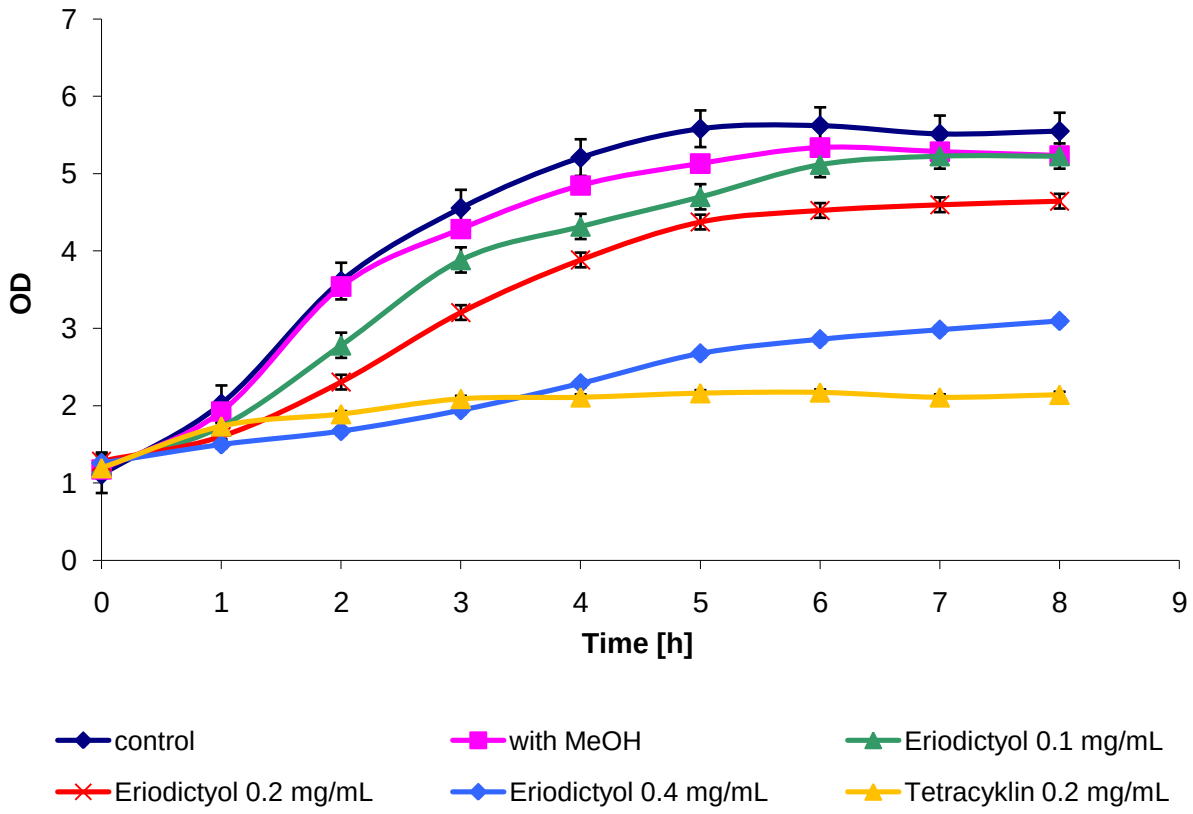
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Eriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.24	1.41	1.43	1.47	1.52	1.67
1	3.43	3.31	1.83	3.41	3.54	3.30
2	6.05	5.28	2.12	5.29	5.09	4.89
3	7.56	6.35	2.27	6.33	6.29	5.63
4	8.73	7.61	2.24	7.55	7.27	6.93
5	9.91	8.09	2.23	8.32	7.94	7.63
6	10.60	9.38	2.07	9.05	9.13	8.90
7	11.40	9.85	1.97	10.19	9.78	9.37
8	12.30	10.56	1.86	10.77	10.46	9.97
SD	0.33715781	0.16619333	0.07569628	0.21787569	0.17614307	0.19766904

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



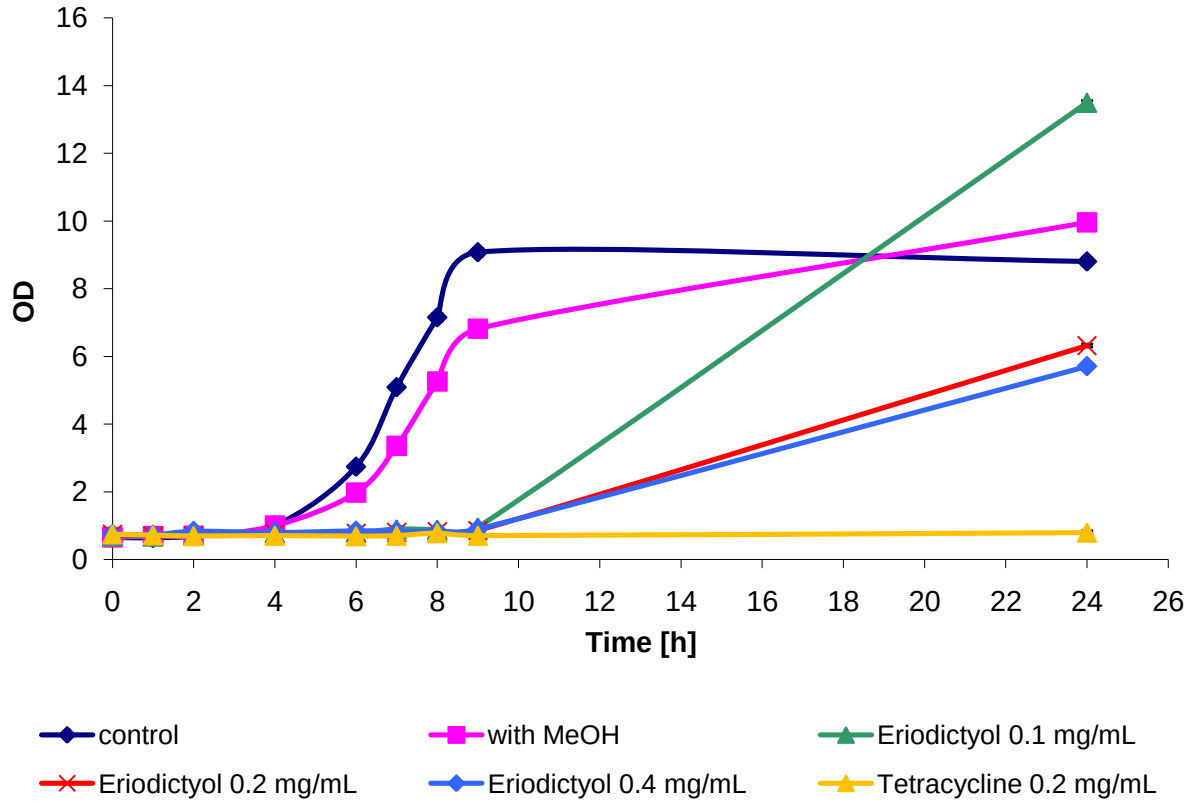
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Eriodictyol 0.1 mg/mL	Eriodictyol 0.2 mg/mL	Eriodictyol 0.4 mg/mL
0	1.35	1.36	1.08	1.39	1.41	1.36
1	1.76	1.60	1.50	1.52	1.55	1.32
2	2.34	2.18	1.71	1.91	1.85	1.46
3	2.92	2.18	1.87	2.35	2.11	1.38
4	3.44	2.64	1.91	2.62	2.40	1.49
5	3.99	2.92	1.93	2.93	2.55	1.52
6	4.25	3.45	1.87	3.05	2.53	1.48
7	4.61	3.71	1.90	3.23	2.60	1.65
8	4.78	3.84	1.90	3.36	2.62	1.54
SD	0.11657707	0.07600719	0.11445771	0.09067677	0.06442774	0.05695954

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



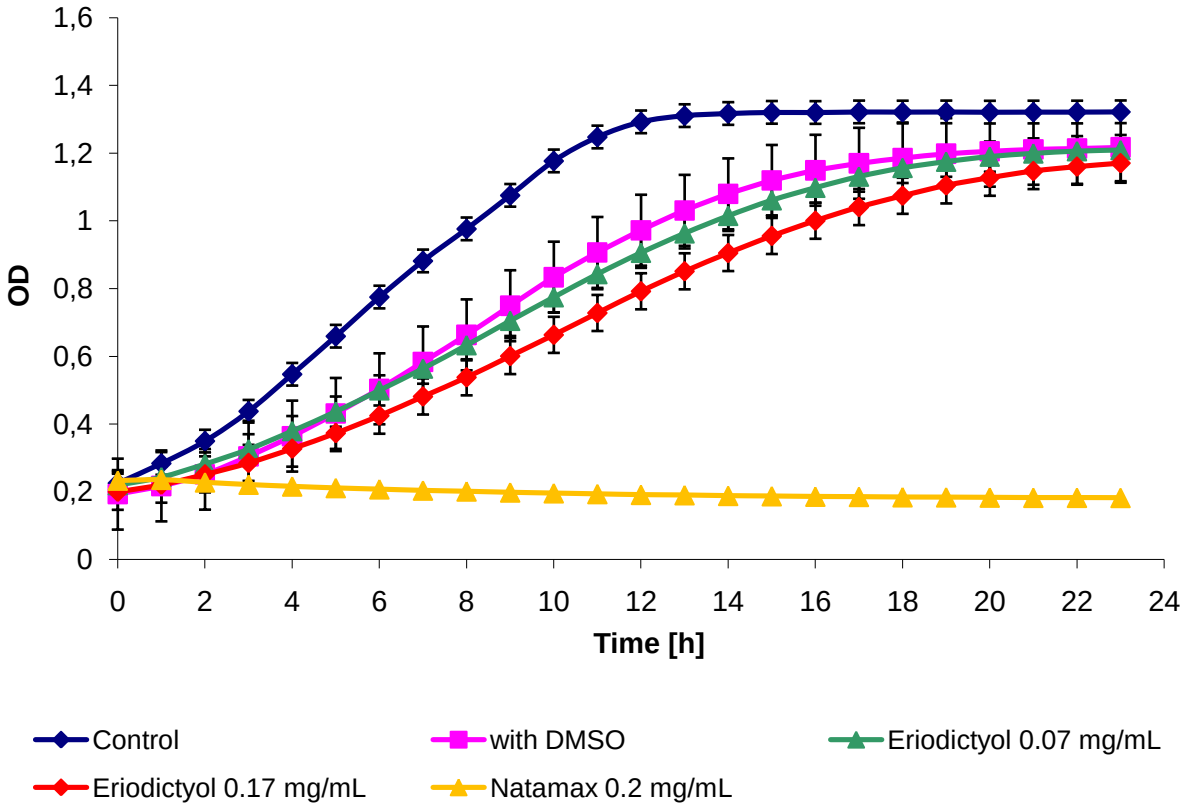
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Eriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.11	1.17	1.19	1.23	1.28	1.26
1	2.02	1.92	1.73	1.73	1.60	1.50
2	3.61	3.54	1.89	2.78	2.30	1.67
3	4.55	4.28	2.09	3.88	3.20	1.94
4	5.21	4.85	2.11	4.32	3.88	2.29
5	5.58	5.13	2.16	4.70	4.37	2.67
6	5.62	5.34	2.17	5.12	4.52	2.86
7	5.51	5.28	2.11	5.23	4.60	2.98
8	5.55	5.23	2.14	5.23	4.64	3.09
SD	0.23713693	0.08796835	0.03921362	0.16313065	0.09513479	0.04740686

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Eriodictyol 0.1 mg/mL	Eriodictyol 0.2 mg/mL	Eriodictyol 0.4 mg/mL
0	0.65	0.66	0.75	0.70	0.75	0.75
1	0.64	0.69	0.72	0.69	0.69	0.73
2	0.69	0.70	0.69	0.71	0.71	0.84
4	1.01	1.00	0.70	0.78	0.77	0.80
6	2.74	1.98	0.69	0.81	0.77	0.85
7	5.09	3.36	0.70	0.90	0.80	0.83
8	7.15	5.26	0.78	0.89	0.82	0.84
9	9.07	6.82	0.70	0.95	0.85	0.90
24	8.80	9.96	0.79	13.49	6.32	5.71
SD	0.11157338	0.09769516	0.05209636	0.06341818	0.04471735	0.03953425

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of eriodictyol; OD – optical density, SD – standard deviation.

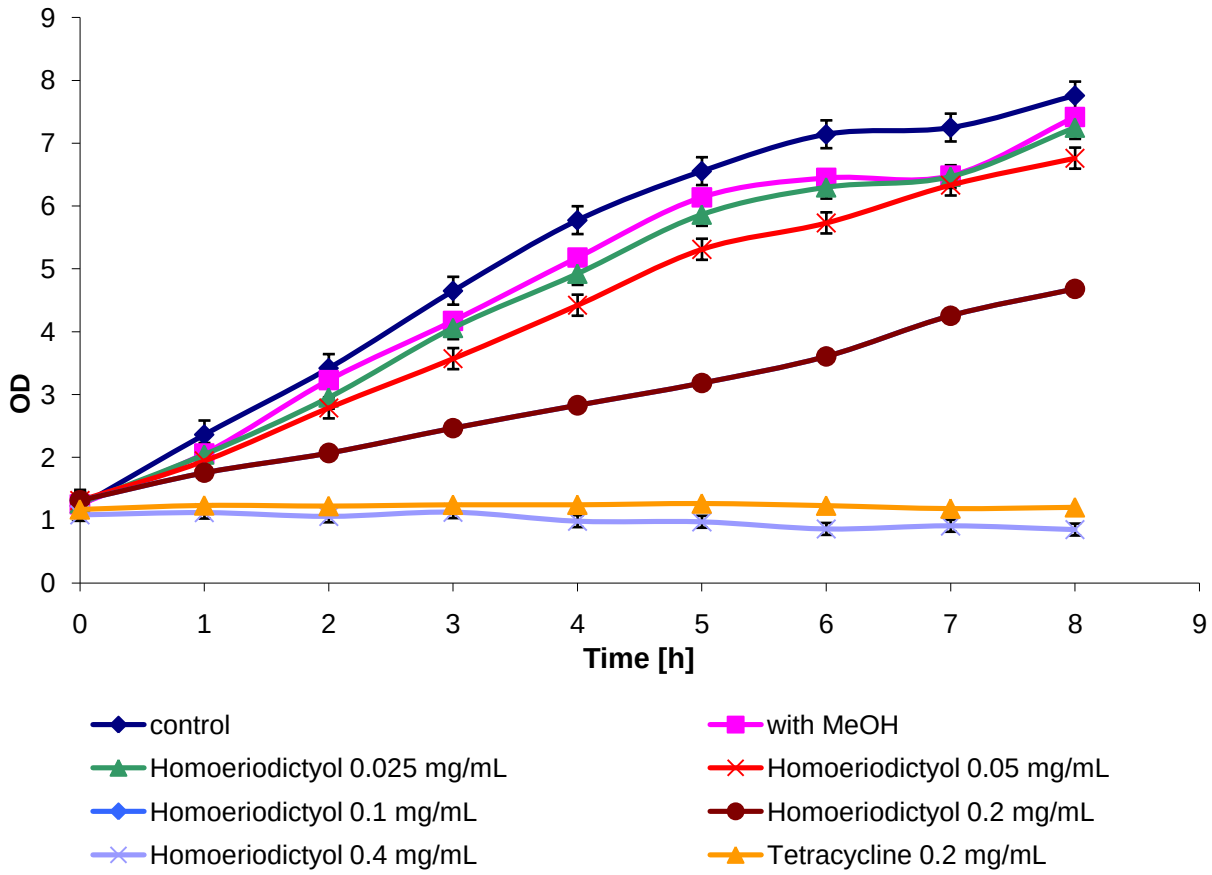


Time [h]	OD				
	Control	DMSO	Natamax 0.2 mg/mL	Eriodictyol 0.07 mg/mL	Eriodictyol 00.17 mg/mL
0	0.2253	0.1930	0.2333	0.2193	0.2000
1	0.2837	0.2173	0.2357	0.2430	0.2210
2	0.3497	0.2520	0.2280	0.2817	0.2513
3	0.4377	0.3050	0.2213	0.3253	0.2853
4	0.5470	0.3643	0.2160	0.3790	0.3273
5	0.6593	0.4313	0.2113	0.4367	0.3733
6	0.7750	0.5040	0.2077	0.4993	0.4247
7	0.8817	0.5833	0.2040	0.5637	0.4813
8	0.9763	0.6633	0.2013	0.6327	0.5380
9	1.0753	0.7493	0.1983	0.7047	0.6007
10	1.1770	0.8337	0.1960	0.7743	0.6633
11	1.2477	0.9067	0.1937	0.8427	0.7280
12	1.2923	0.9723	0.1913	0.9057	0.7920

13	1.3107	1.0310	0.1903	0.9630	0.8510
14	1.3170	1.0797	0.1883	1.0147	0.9050
15	1.3203	1.1193	0.1873	1.0607	0.9553
16	1.3200	1.1493	0.1860	1.0977	1.0003
17	1.3217	1.1700	0.1853	1.1307	1.0407
18	1.3213	1.1857	0.1843	1.1563	1.0740
19	1.3217	1.1983	0.1840	1.1747	1.1047
20	1.3210	1.2057	0.1833	1.1897	1.1273
21	1.3213	1.2113	0.1827	1.1990	1.1470
22	1.3213	1.2140	0.1827	1.2057	1.1600
23	1.3220	1.2173	0.1823	1.2090	1.1703
SD	0.03347273	0.10475891	0.09044731	0.04458107	0.05326358

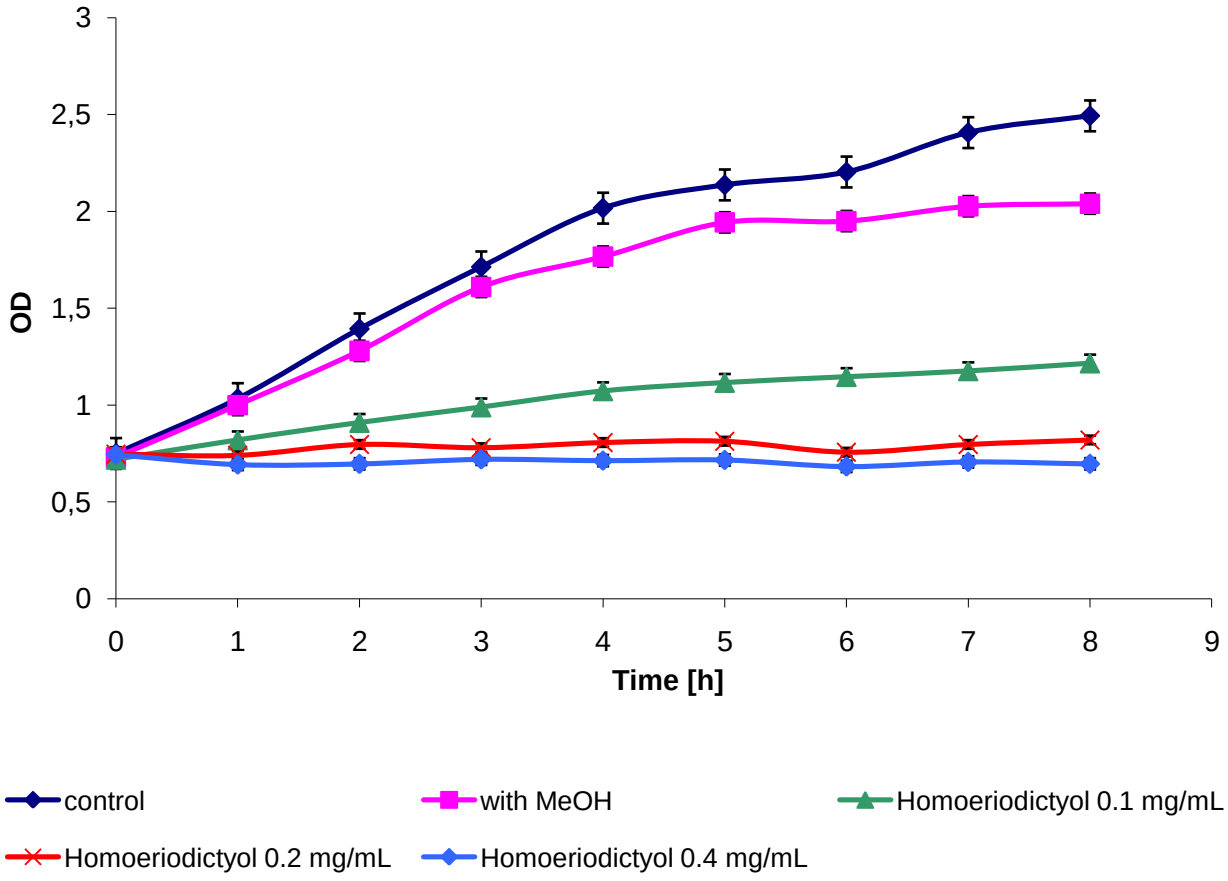
Homoeriodictyol

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density. SD – standard deviation



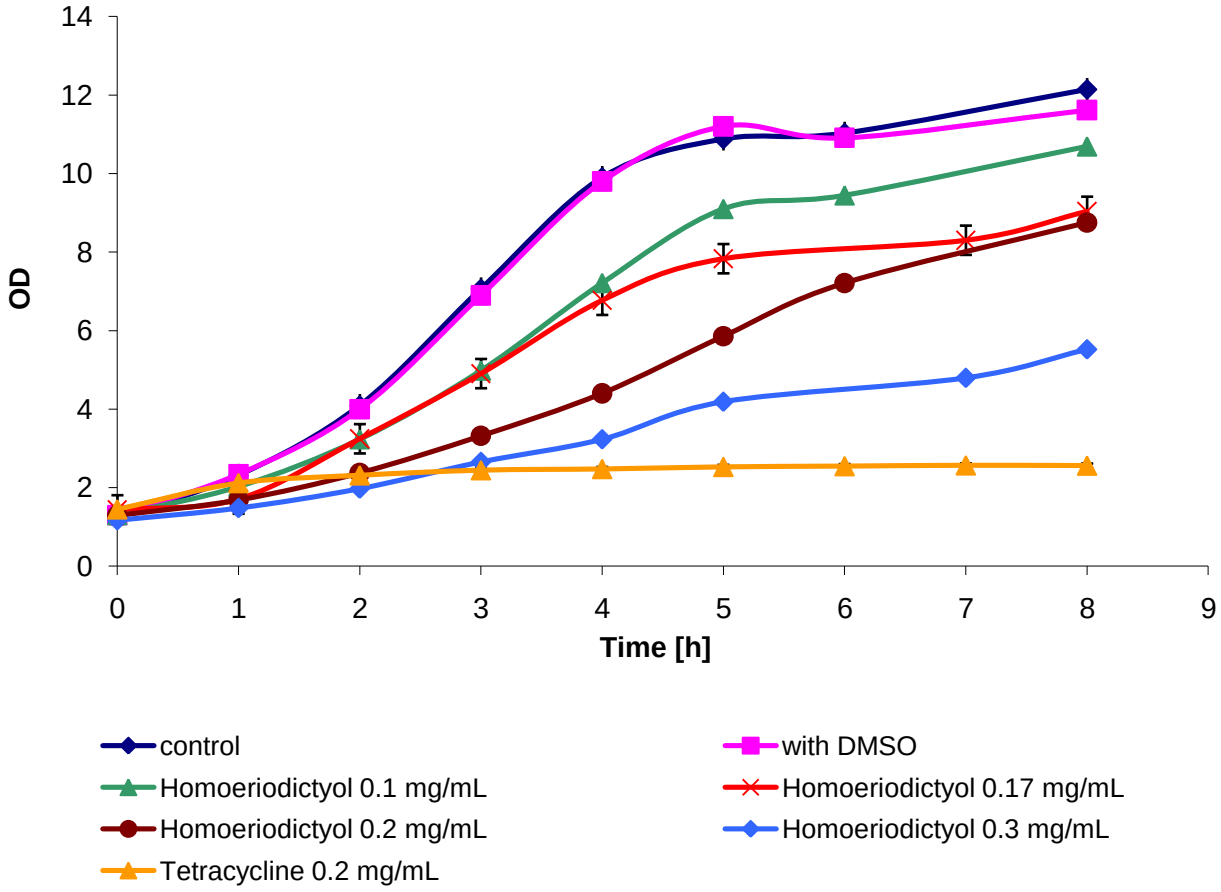
Time [h]	OD							
	Control	MeOH	Tetracycline 0.2 mg/mL	Homoeriodictyol				
				0.025 mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.23	1.25	1.17	1.27	1.31	1.32	0.73	1.08
1	2.36	2.06	1.24	2.05	1.94	1.76	0.72	1.12
2	3.42	3.23	1.23	2.95	2.79	2.07	0.73	1.06
3	4.65	4.17	1.25	4.06	3.57	2.47	0.71	1.13
4	5.77	5.18	1.25	4.92	4.42	2.83	0.69	0.98
5	6.55	6.14	1.27	5.86	5.31	3.18	0.71	0.97
6	7.14	6.45	1.23	6.30	5.73	3.61	0.61	0.86
7	7.25	6.48	1.19	6.47	6.33	4.25	0.63	0.91
8	7.76	7.42	1.21	7.25	6.76	4.68	0.63	0.85
SD	0.2215765	0.1237365	0.0302952	0.18034	0.167854	0.100789	0.020033	0.004981

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



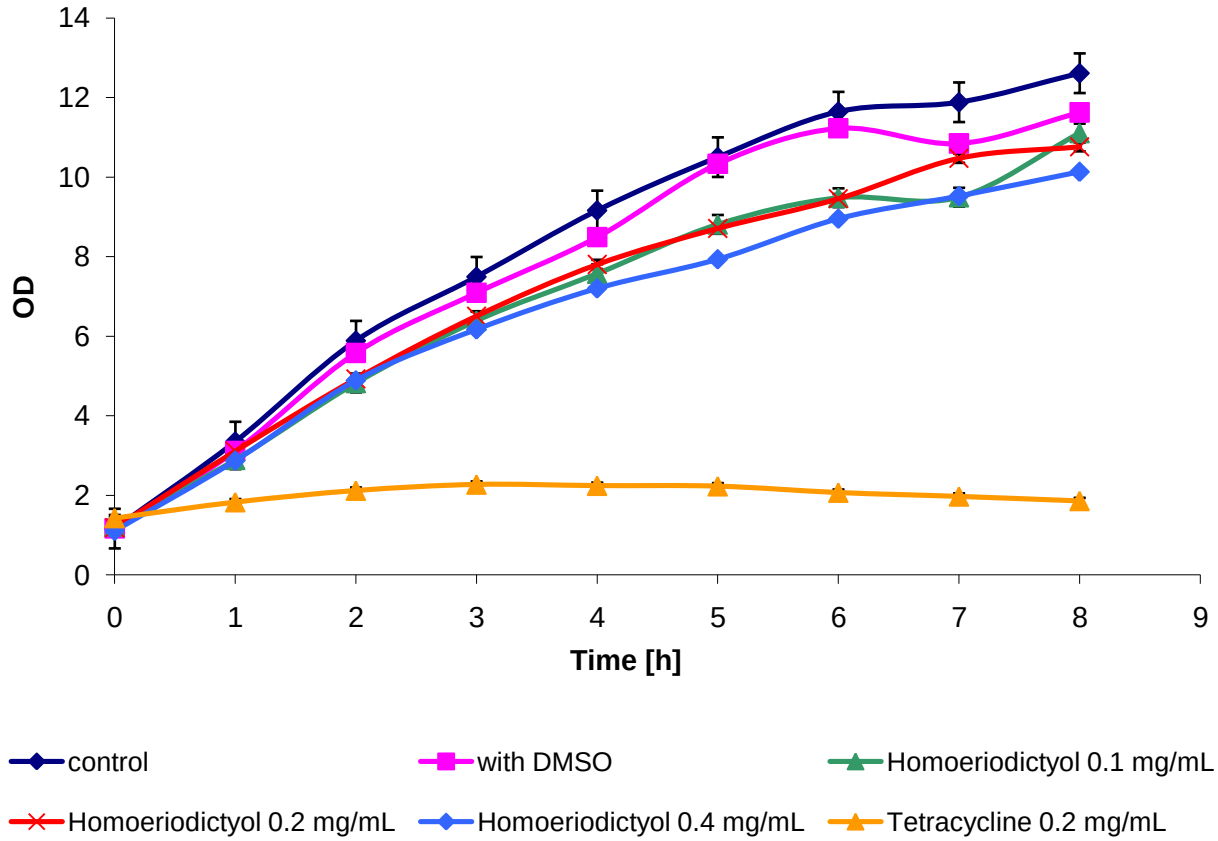
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Homoeriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.75	0.73	1.28	0.72	0.75	0.74
1	1.03	1.00	1.55	0.82	0.74	0.69
2	1.39	1.28	1.65	0.91	0.80	0.70
3	1.71	1.61	1.66	0.99	0.78	0.72
4	2.02	1.77	1.68	1.07	0.81	0.71
5	2.14	1.94	1.75	1.12	0.81	0.72
6	2.20	1.95	1.68	1.15	0.76	0.68
7	2.41	2.03	1.74	1.18	0.80	0.71
8	2.49	2.04	1.70	1.22	0.82	0.70
SD	0.07961781	0.05228394	0.05256633	0.04380187	0.0222552	0.02918528

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



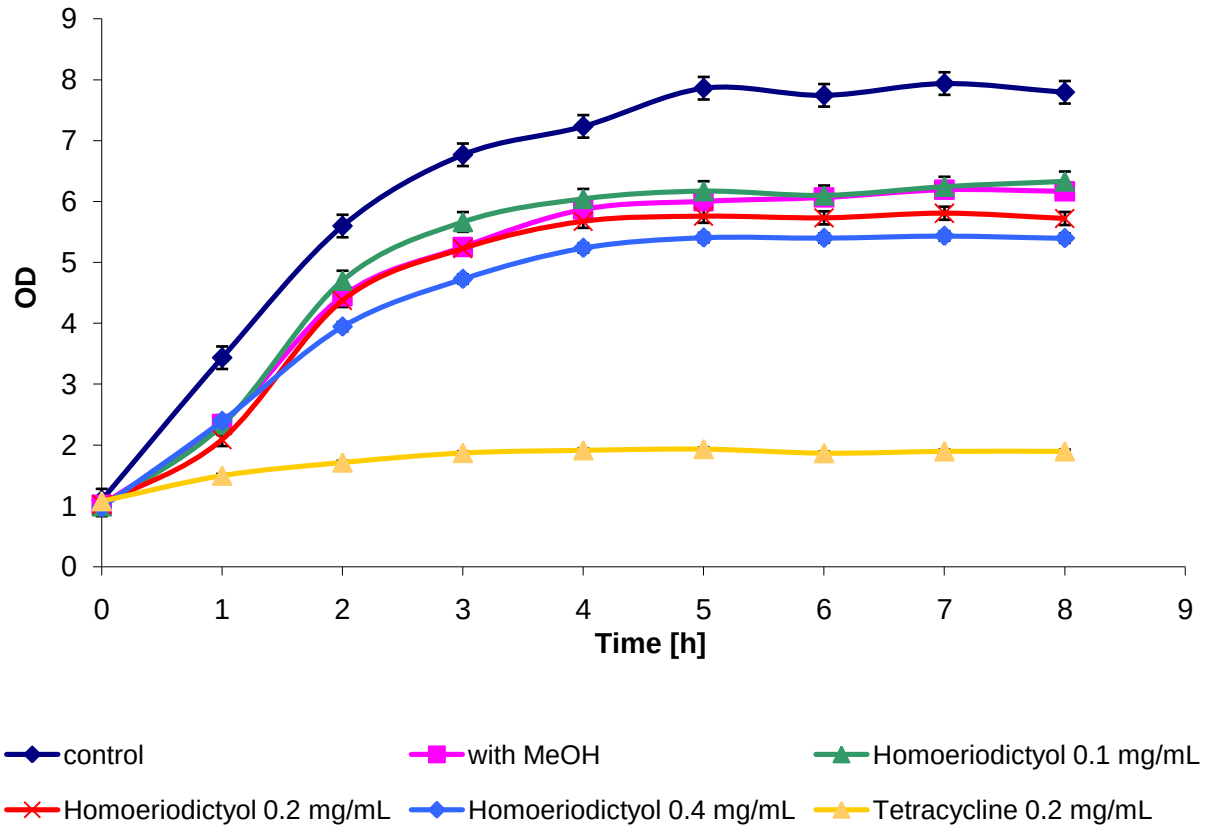
Time [h]	OD						
	Control	DMSO	Tetracycline 0.2 mg/mL	Homoeriodictyol			
				0.1 mg/mL	0.17 mg/mL	0.2 mg/mL	0.3 mg/mL
0	1.24	1.30	1.45	1.30	1.43	1.29	1.17
1	2.31	2.34	2.12	2.03	1.71	1.69	1.48
2	4.09	4.00	2.31	3.23	3.24	2.38	1.97
3	7.06	6.90	2.44	4.98	4.90	3.32	2.65
4	9.90	9.80	2.47	7.21	6.77	4.40	3.23
5	10.88	11.20	2.53	9.10	7.83	5.86	4.19
6	11.03	10.91	2.55	9.45	6.39	7.21	3.51
8	12.14	11.62	2.57	10.69	8.30	8.75	4.79
SD	0.29079465	0.21741951	0.05198393	0.16514506	0.37290415	0.16190746	0.15676561

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



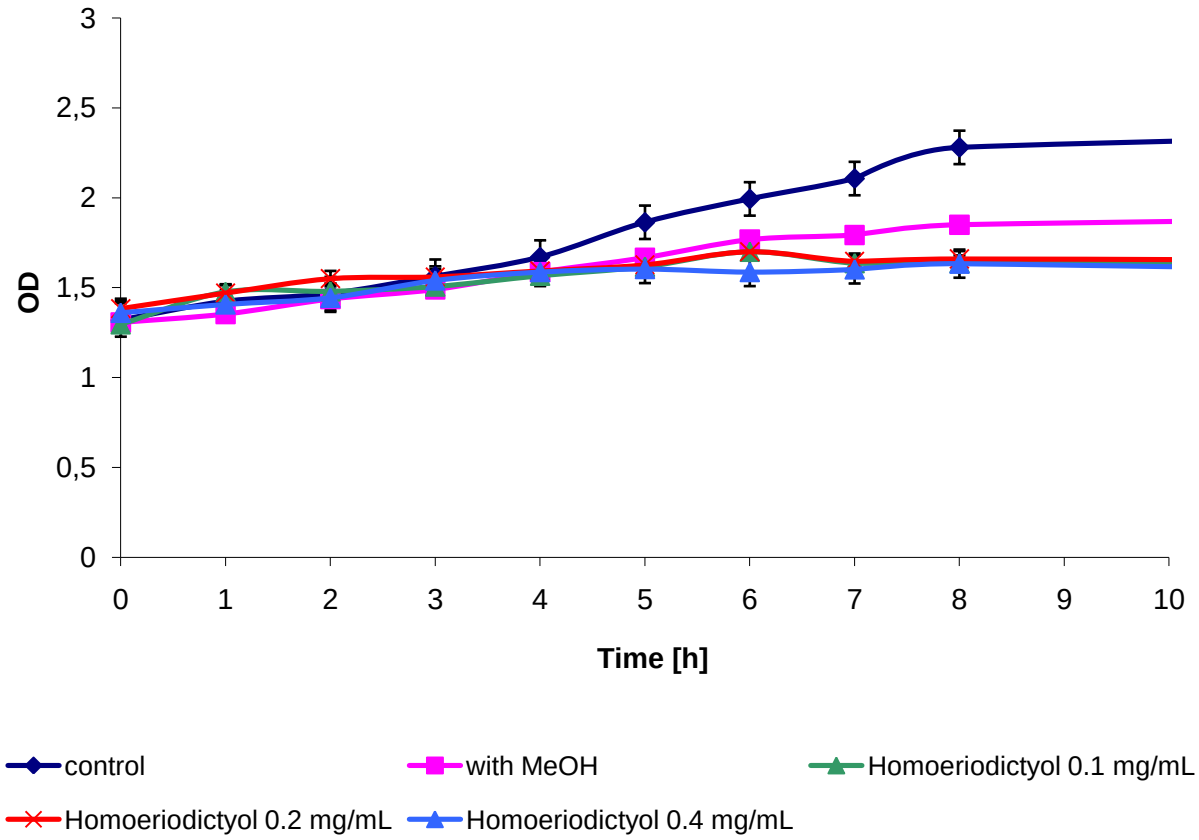
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Homoeriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.16	1.17	1.43	1.22	1.19	1.11
1	3.35	3.11	1.83	2.89	3.11	2.87
2	5.89	5.58	2.12	4.82	4.93	4.89
3	7.49	7.09	2.27	6.39	6.51	6.17
4	9.16	8.49	2.24	7.57	7.80	7.20
5	10.50	10.33	2.23	8.81	8.71	7.94
6	11.64	11.23	2.07	9.48	9.46	8.96
7	11.88	10.85	1.97	9.49	10.47	9.53
8	12.61	11.63	1.86	11.11	10.76	10.14
SD	0.49803199	0.19264049	0.07569628	0.2363242	0.11667008	0.21947701

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



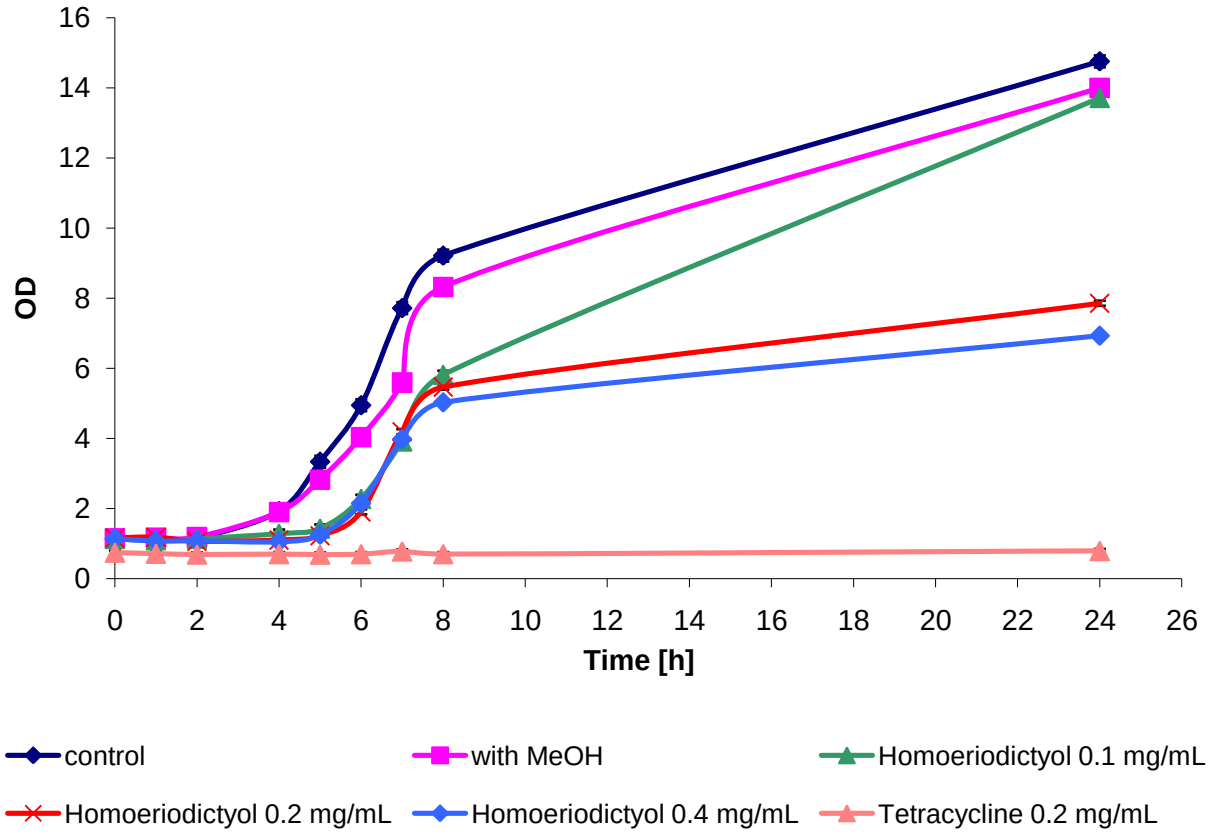
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Homoeriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.09	1.02	1.08	0.99	1.02	0.98
1	3.43	2.35	1.50	2.33	2.09	2.39
2	5.60	4.44	1.71	4.70	4.37	3.94
3	6.77	5.25	1.87	5.66	5.23	4.73
4	7.23	5.87	1.91	6.04	5.67	5.24
5	7.86	6.00	1.93	6.17	5.76	5.41
6	7.74	6.07	1.87	6.10	5.73	5.40
7	7.94	6.19	1.90	6.24	5.81	5.43
8	7.79	6.17	1.90	6.33	5.72	5.40
SD	0.18522006	0.11445771	0.11445771	0.16213004	0.10739067	0.07696203

Growth curve of *Enterococcus faecalis* ATCC 19433on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



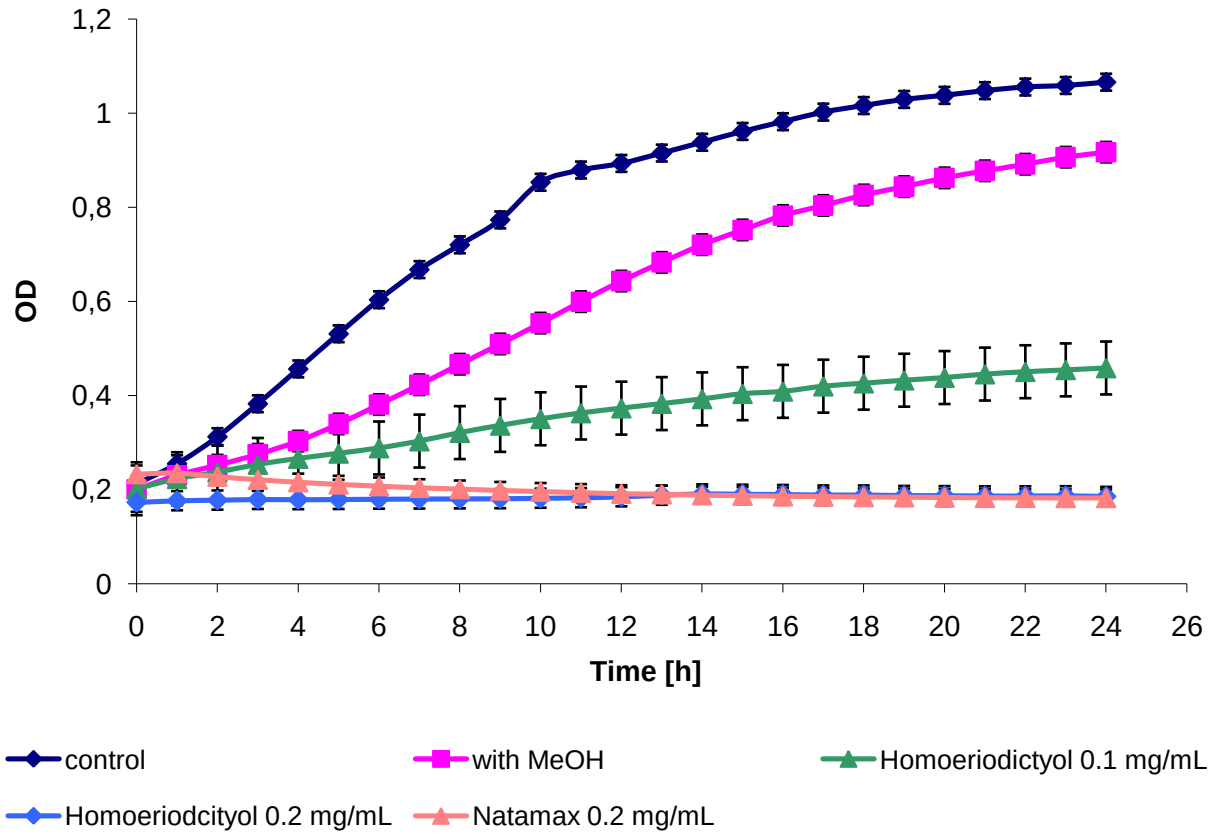
Time [h]	OD				
	Control	MeOH	Homoeriodictyol		
			0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.32	1.31	1.30	1.38	1.36
1	1.42	1.35	1.48	1.47	1.41
2	1.47	1.44	1.48	1.55	1.44
3	1.56	1.49	1.51	1.56	1.54
4	1.67	1.59	1.57	1.59	1.59
5	1.86	1.67	1.62	1.63	1.60
6	1.99	1.77	1.70	1.70	1.59
7	2.11	1.79	1.64	1.65	1.60
8	2.28	1.85	1.64	1.66	1.63
SD	0.09303908	0.04113348	0.04225569	0.04293357	0.07829021

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Homoeriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.13	1.15	0.75	1.14	1.16	1.14
1	1.13	1.17	0.72	1.10	1.18	1.07
2	1.16	1.18	0.69	1.14	1.06	1.08
4	1.92	1.90	0.70	1.29	1.10	1.05
5	3.33	2.82	0.69	1.43	1.22	1.25
6	4.94	4.04	0.70	2.28	1.90	2.15
7	7.71	5.59	0.78	3.92	4.19	3.98
8	9.21	8.32	0.70	5.82	5.46	5.03
24	14.75	14.00	0.79	13.71	7.85	6.93
SD	0.16959777	0.08859591	0.05209636	0.1112907	0.07262862	0.06265955

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of methanol (MeOH) and various concentration of homoeriodictyol; OD – optical density, SD – standard deviation.

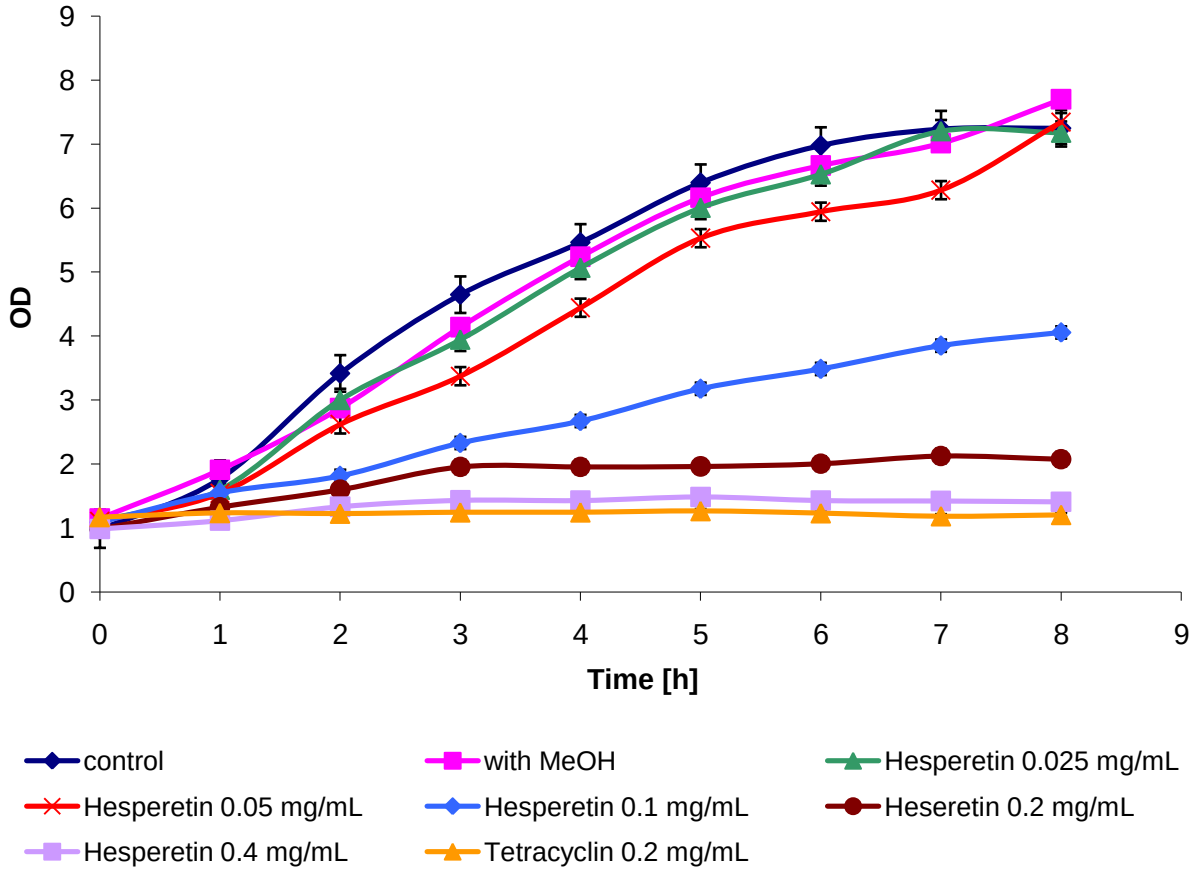


Time [h]	OD					
	Control	MeOH	Natamax 0.2 mg/mL	Homoeriodictyol		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.2127	0.2010	0.2333	0.2020	0.1733	0.2093
1	0.2553	0.2310	0.2357	0.2233	0.1767	0.2097
2	0.3127	0.2523	0.2280	0.2373	0.1780	0.2133
3	0.3827	0.2753	0.2213	0.2537	0.1793	0.2143
4	0.4567	0.3033	0.2160	0.2663	0.1790	0.2153
5	0.5313	0.3397	0.2113	0.2773	0.1793	0.2150
6	0.6037	0.3810	0.2077	0.2887	0.1800	0.2150
7	0.6677	0.4230	0.2040	0.3033	0.1803	0.2150
8	0.7203	0.4667	0.2013	0.3213	0.1807	0.2150
9	0.7733	0.5097	0.1983	0.3367	0.1810	0.2150
10	0.8533	0.5540	0.1960	0.3507	0.1820	0.2147
11	0.8793	0.5997	0.1937	0.3630	0.1830	0.2147

12	0.8933	0.6433	0.1913	0.3733	0.1850	0.2143
13	0.9153	0.6830	0.1903	0.3830	0.1883	0.2140
14	0.9383	0.7207	0.1883	0.3930	0.1910	0.2137
15	0.9613	0.7520	0.1873	0.4040	0.1903	0.2130
16	0.9820	0.7827	0.1860	0.4090	0.1897	0.2133
17	1.0023	0.8040	0.1853	0.4200	0.1887	0.2123
18	1.0163	0.8263	0.1843	0.4263	0.1887	0.2123
19	1.0293	0.8440	0.1840	0.4327	0.1877	0.2127
20	1.0380	0.8627	0.1833	0.4383	0.1873	0.2127
21	1.0480	0.8777	0.1827	0.4457	0.1867	0.2130
22	1.0557	0.8917	0.1827	0.4507	0.1867	0.2123
23	1.0590	0.9067	0.1823	0.4547	0.1870	0.2110
24	1.0660	0.9173	0.1823	0.4587	0.1857	0.2097
SD	0.01786773	0.02171292	0.01817688	0.05623998	0.020344	0.04851604

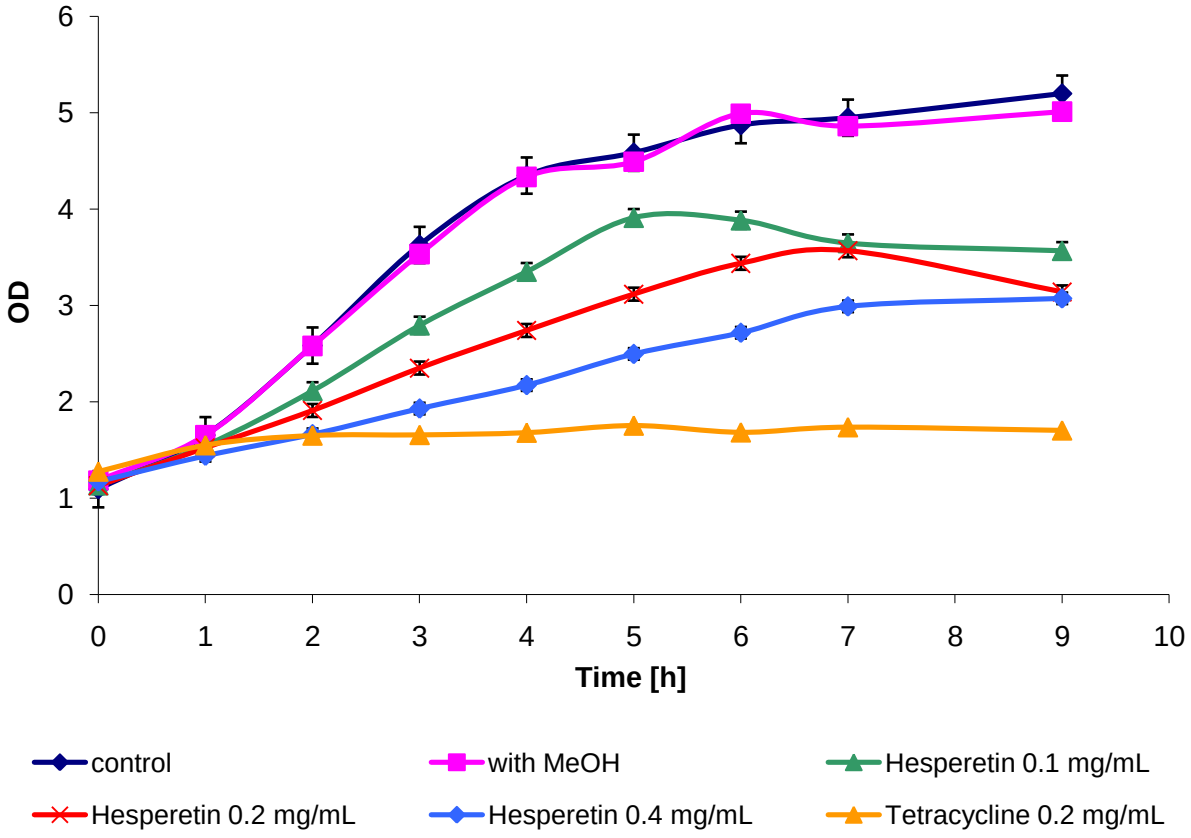
Hesperetin

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



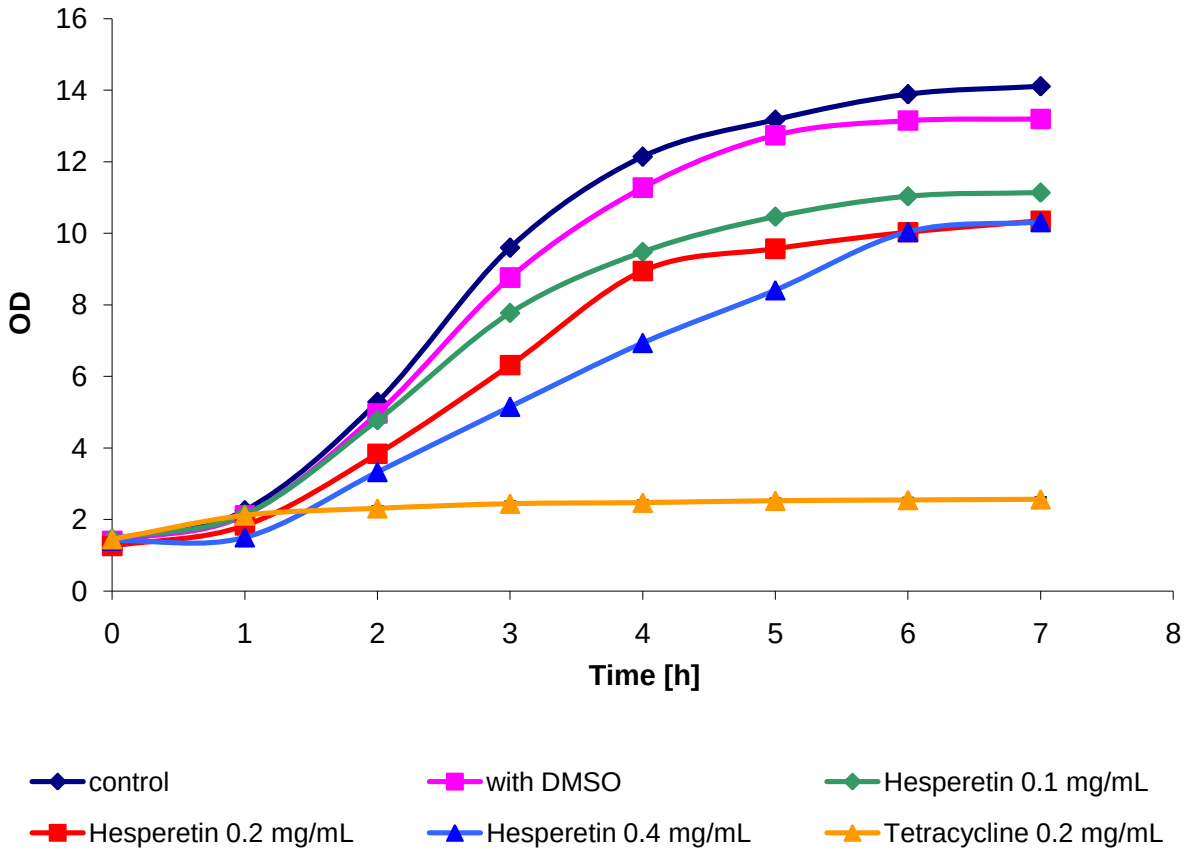
Time [h]	OD							
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperetin				
				0.025 mg/mL	0.05 mg/mL	0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.98	1.15	1.17	1.11	1.11	1.09	0.99	0.99
1	1.77	1.91	1.24	1.60	1.54	1.55	1.33	1.12
2	3.42	2.87	1.23	3.00	2.62	1.82	1.60	1.33
3	4.65	4.14	1.25	3.94	3.37	2.33	1.96	1.43
4	5.46	5.24	1.25	5.07	4.44	2.67	1.96	1.43
5	6.40	6.16	1.27	6.00	5.53	3.18	1.96	1.49
6	6.98	6.67	1.23	6.53	5.94	3.49	2.01	1.43
7	7.23	7.01	1.19	7.20	6.28	3.85	2.13	1.42
8	7.25	7.70	1.21	7.18	7.34	4.06	2.08	1.41
SD	0.285457	0.126878	0.030295	0.176428	0.14209	0.057219	0.111775	0.046932

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



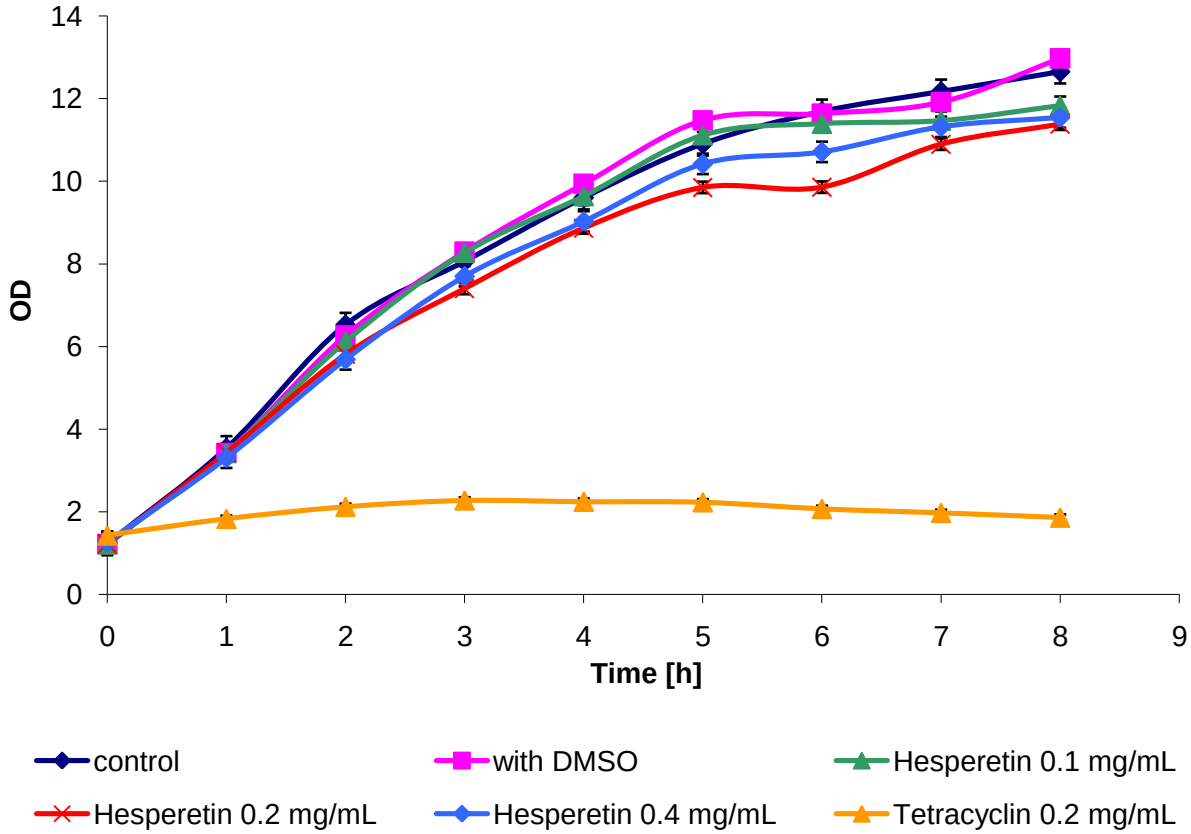
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Hesperetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.09	1.18	1.28	1.13	1.13	1.17
1	1.65	1.65	1.55	1.55	1.52	1.44
2	2.58	2.58	1.65	2.11	1.91	1.66
3	3.63	3.53	1.66	2.79	2.35	1.93
4	4.35	4.33	1.68	3.35	2.74	2.17
5	4.58	4.49	1.75	3.91	3.12	2.50
6	4.87	4.99	1.68	3.88	3.44	2.72
7	4.95	4.86	1.74	3.65	3.57	2.99
9	5.20	5.01	1.70	3.57	3.14	3.07
SD	0.18805956	0.07370752	0.05256633	0.08955168	0.06708303	0.05978532

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethzlsulfoxide (DMSO) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



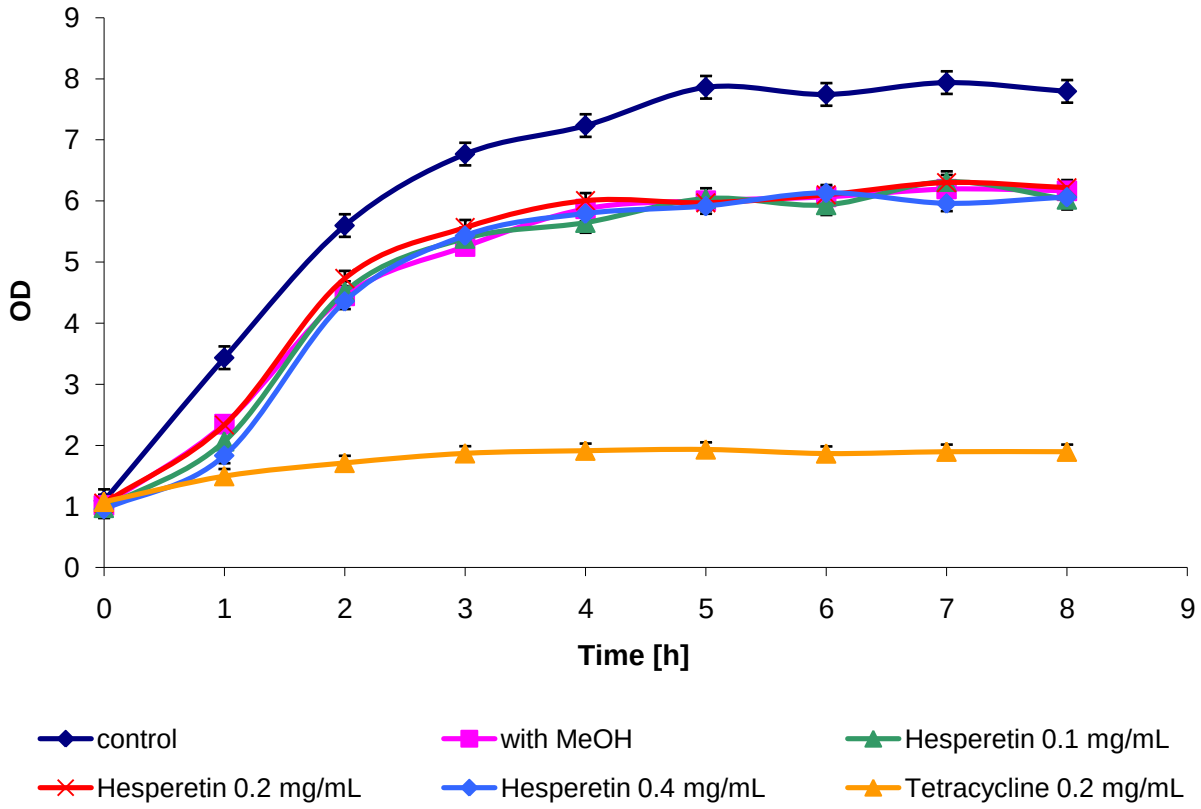
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Hesperetin 0.1 mg/mL	Hesperetin 0.2 mg/mL	Hesperetin 0.4 mg/mL
0	1.37	1.40	1.45	1.46	1.26	1.41
1	2.25	2.12	2.12	2.14	1.83	1.50
2	5.28	4.97	2.31	4.77	3.84	3.33
3	9.60	8.76	2.44	7.77	6.31	5.15
4	12.14	11.28	2.47	9.48	8.95	6.94
5	13.17	12.74	2.53	10.47	9.57	8.41
6	13.89	13.15	2.55	11.04	10.03	10.04
7	14.11	13.20	2.57	11.14	10.35	10.31
SD	0.24325339	0.20821501	0.05198393	0.17865882	0.16896394	0.21564296

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



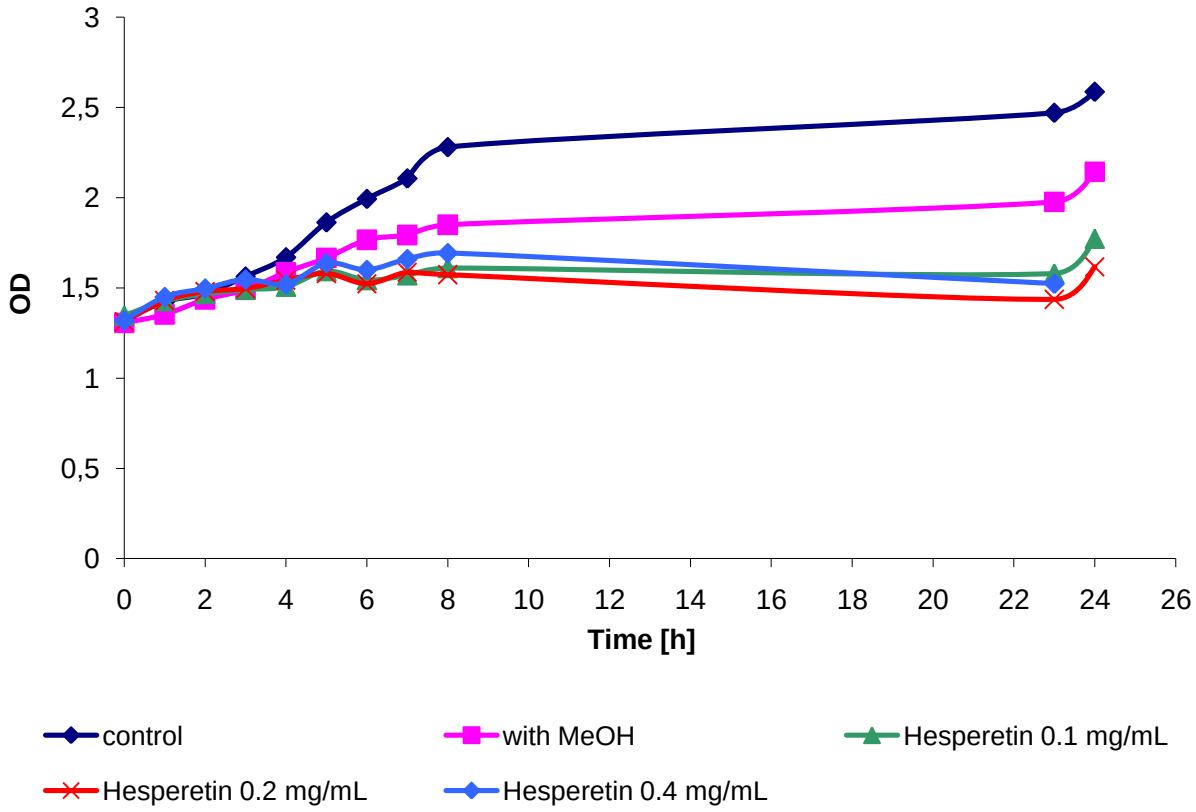
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Hesperetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.23	1.22	1.43	1.21	1.23	1.27
1	3.54	3.42	1.83	3.43	3.43	3.31
2	6.53	6.26	2.12	6.12	5.81	5.69
3	8.06	8.30	2.27	8.27	7.40	7.70
4	9.60	9.94	2.24	9.64	8.87	9.03
5	10.91	11.48	2.23	11.11	9.85	10.42
6	11.69	11.64	2.07	11.39	9.86	10.71
7	12.17	11.92	1.97	11.47	10.90	11.32
8	12.65	12.97	1.86	11.83	11.38	11.54
SD	0.28661737	0.16742039	0.07569628	0.22001901	0.13882933	0.24732234

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



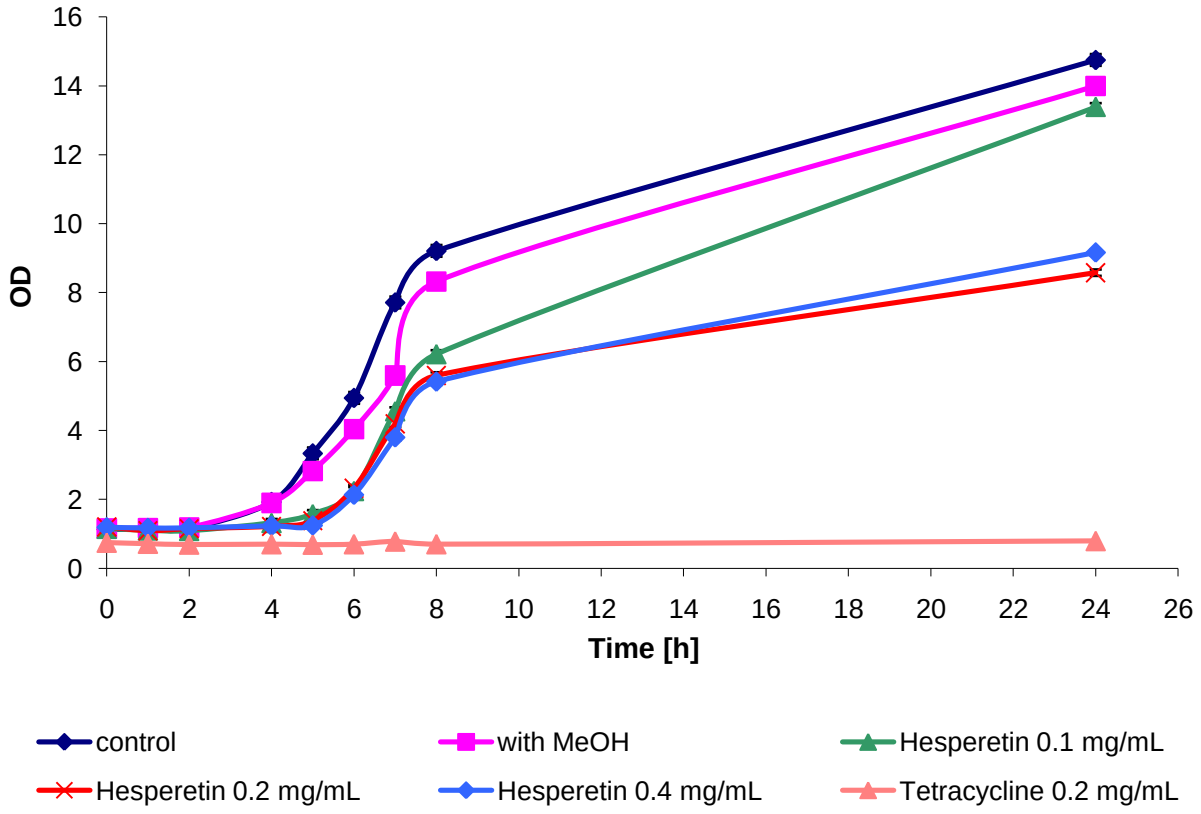
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperetin 0.1 mg/mL	Hesperetin 0.2 mg/mL	Hesperetin 0.4 mg/mL
0	1.09	1.02	1.08	0.98	1.05	0.96
1	3.43	2.35	1.50	2.07	2.33	1.83
2	5.60	4.44	1.71	4.52	4.73	4.36
3	6.77	5.25	1.87	5.39	5.56	5.43
4	7.23	5.87	1.91	5.65	6.00	5.79
5	7.86	6.00	1.93	6.04	5.97	5.92
6	7.74	6.07	1.87	5.94	6.10	6.13
7	7.94	6.19	1.90	6.32	6.30	5.96
8	7.79	6.17	1.90	6.03	6.22	6.06
SD	0.18522006	0.12798383	0.11445771	0.16742492	0.12475231	0.12698139

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



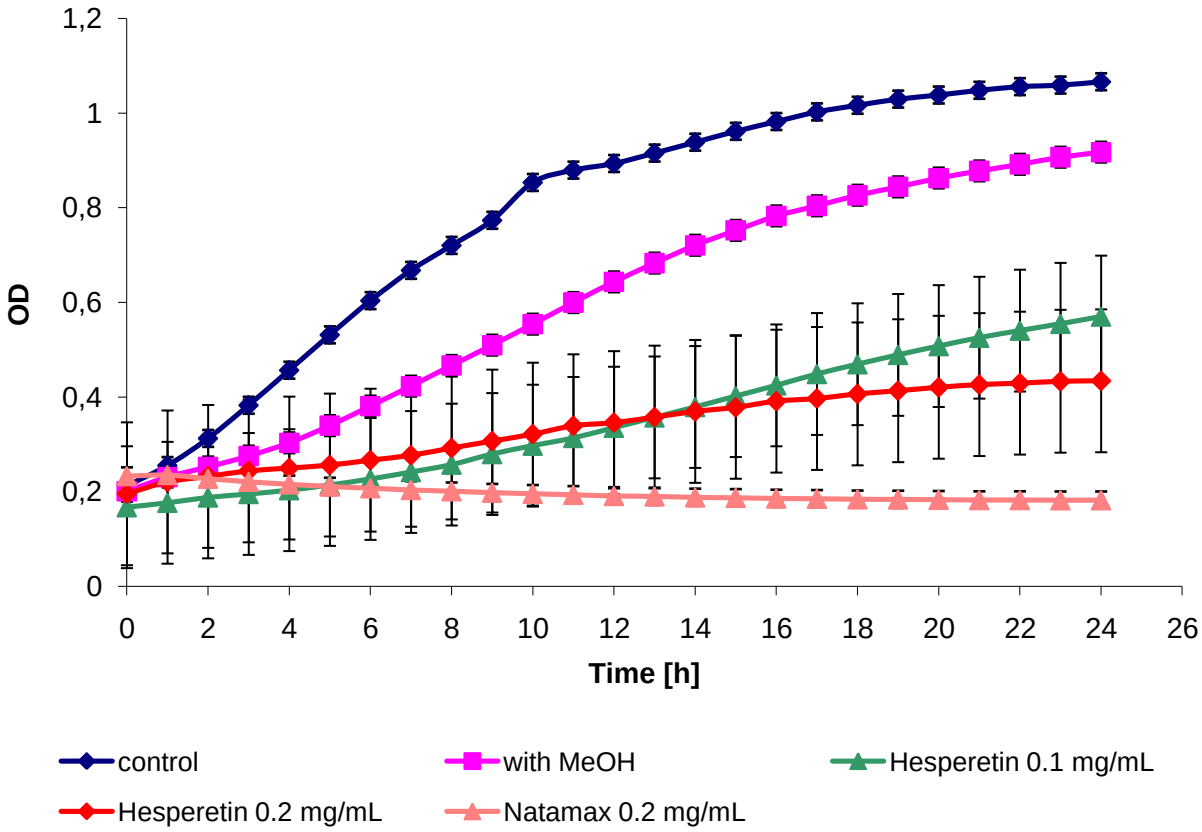
Time [h]	OD				
	Control	MeOH	Hesperetin		
			0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.32	1.31	1.35	1.31	1.32
1	1.42	1.35	1.43	1.43	1.45
2	1.47	1.44	1.47	1.48	1.50
3	1.56	1.49	1.49	1.50	1.55
4	1.67	1.59	1.51	1.54	1.52
5	1.86	1.67	1.59	1.58	1.64
6	1.99	1.77	1.54	1.52	1.60
7	2.11	1.79	1.57	1.59	1.66
8	2.28	1.85	1.61	1.57	1.69
23	2.47	1.98	1.58	1.44	1.52
24	2.59	2.14	1.77	1.62	1.75
SD	0.09303908	0.04113348	0.04254113	0.06191275	0.05777808

Growth curve of *Pseudomonas* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.13	1.15	0.75	1.15	1.20	1.19
1	1.13	1.17	0.72	1.10	1.09	1.17
2	1.16	1.18	0.69	1.09	1.15	1.17
4	1.92	1.90	0.70	1.32	1.22	1.23
5	3.33	2.82	0.69	1.58	1.38	1.24
6	4.94	4.04	0.70	2.25	2.32	2.13
7	7.71	5.59	0.78	4.56	4.19	3.80
8	9.21	8.32	0.70	6.22	5.59	5.41
24	14.75	14.00	0.79	13.39	8.58	9.16
SD	0.16959777	0.1625339	0.05209636	0.10920593	0.09246648	0.09134202

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of methanol (MeOH) and various concentration of hesperetin; OD – optical density, SD – standard deviation.

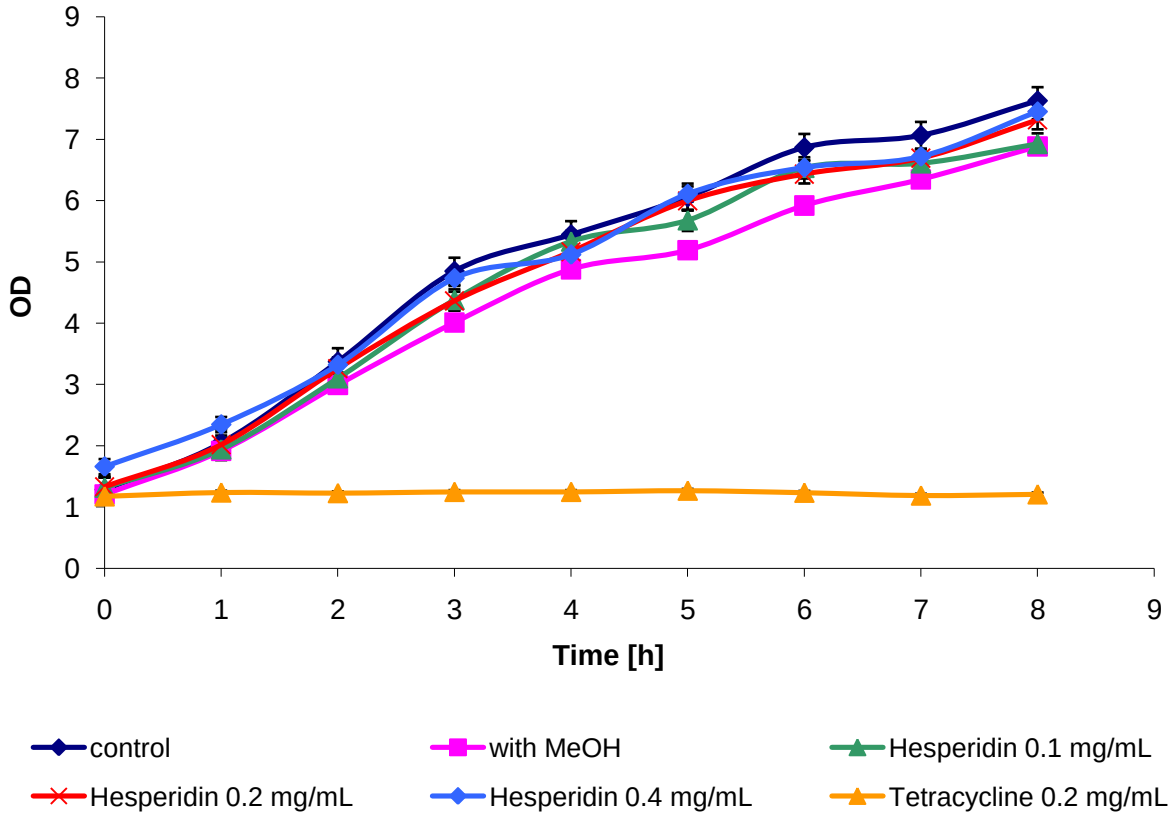


Time [h]	OD					
	Control	MeOH	Natamax 0.2 mg/mL	Hesperetin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.2127	0.2010	0.2333	0.1673	0.1957	0.2923
1	0.2553	0.2310	0.2357	0.1767	0.2207	0.3110
2	0.3127	0.2523	0.2280	0.1880	0.2323	0.3260
3	0.3827	0.2753	0.2213	0.1953	0.2440	0.3413
4	0.4567	0.3033	0.2160	0.2033	0.2500	0.3400
5	0.5313	0.3397	0.2113	0.2143	0.2563	0.3523
6	0.6037	0.3810	0.2077	0.2270	0.2667	0.3663
7	0.6677	0.4230	0.2040	0.2417	0.2770	0.3720
8	0.7203	0.4667	0.2013	0.2573	0.2923	0.3780
9	0.7733	0.5097	0.1983	0.2797	0.3070	0.3880
10	0.8533	0.5540	0.1960	0.2973	0.3217	0.4000
11	0.8793	0.5997	0.1937	0.3137	0.3393	0.4077

12	0.8933	0.6433	0.1913	0.3353	0.3460	0.4237
13	0.9153	0.6830	0.1903	0.3570	0.3577	0.4310
14	0.9383	0.7207	0.1883	0.3790	0.3697	0.4477
15	0.9613	0.7520	0.1873	0.4020	0.3783	0.4623
16	0.9820	0.7827	0.1860	0.4247	0.3913	0.4750
17	1.0023	0.8040	0.1853	0.4487	0.3970	0.4890
18	1.0163	0.8263	0.1843	0.4693	0.4067	0.5040
19	1.0293	0.8440	0.1840	0.4890	0.4133	0.5210
20	1.0380	0.8627	0.1833	0.5077	0.4207	0.5383
21	1.0480	0.8777	0.1827	0.5253	0.4263	0.5573
22	1.0557	0.8917	0.1827	0.5403	0.4293	0.5747
23	1.0590	0.9067	0.1823	0.5547	0.4333	0.5943
24	1.0660	0.9173	0.1823	0.5700	0.4343	0.6120
SD	0.01786773	0.02171292	0.01817688	0.12868813	0.15090393	0.05348847

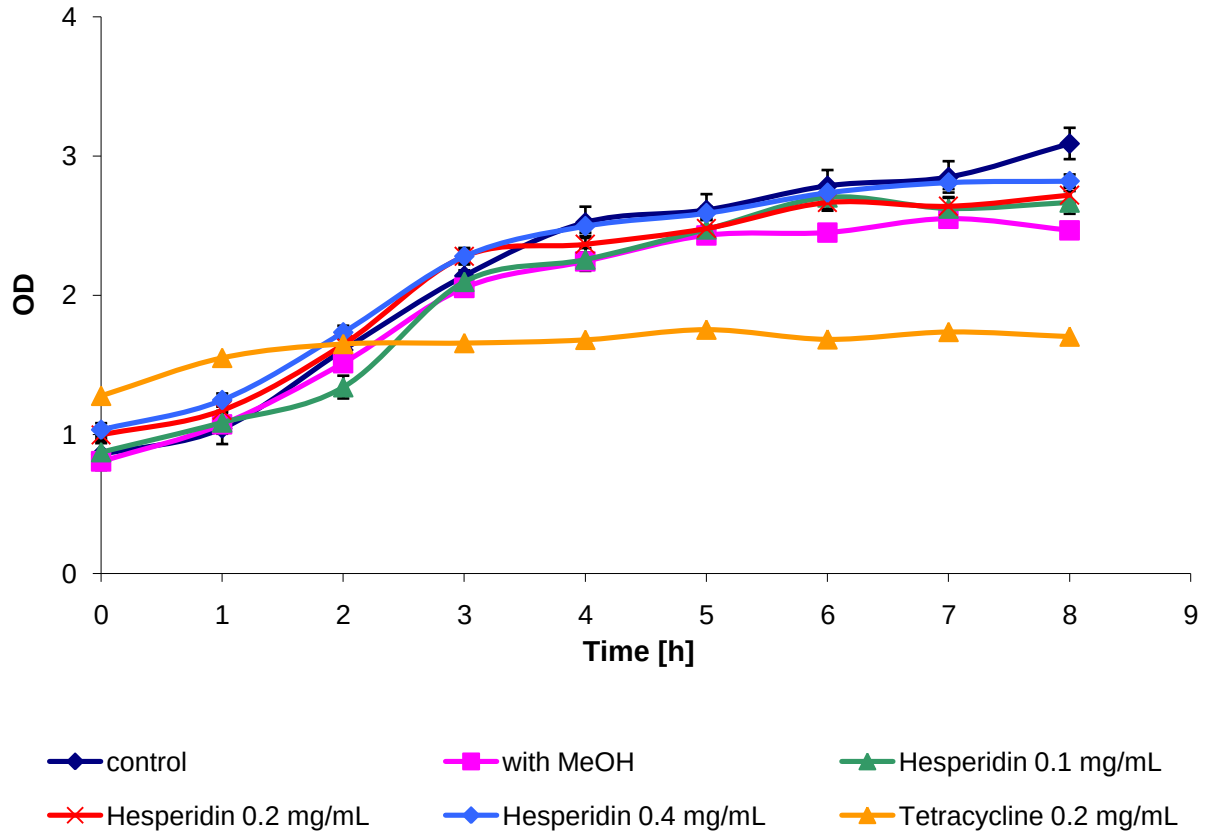
Hesperidin

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



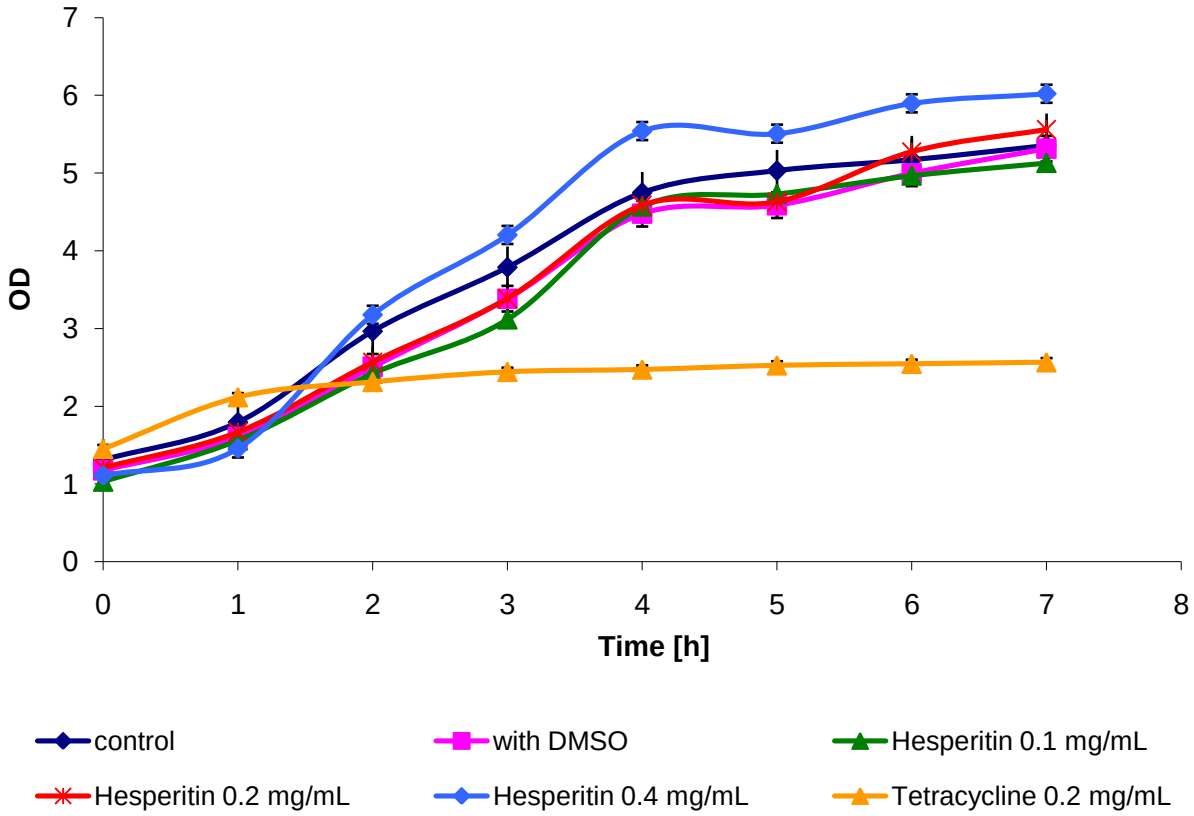
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperidin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.28	1.21	1.17	1.31	1.33	1.66
1	2.05	1.92	1.24	1.94	2.02	2.35
2	3.37	2.99	1.23	3.10	3.26	3.32
3	4.85	4.01	1.25	4.38	4.36	4.73
4	5.45	4.88	1.25	5.33	5.17	5.12
5	6.06	5.19	1.27	5.68	6.00	6.11
6	6.87	5.92	1.23	6.54	6.43	6.54
7	7.07	6.35	1.19	6.61	6.69	6.72
8	7.63	6.87	1.21	6.93	7.32	7.45
SD	0.22028176	0.11779816	0.03029522	0.17320847	0.15090383	0.1237931

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



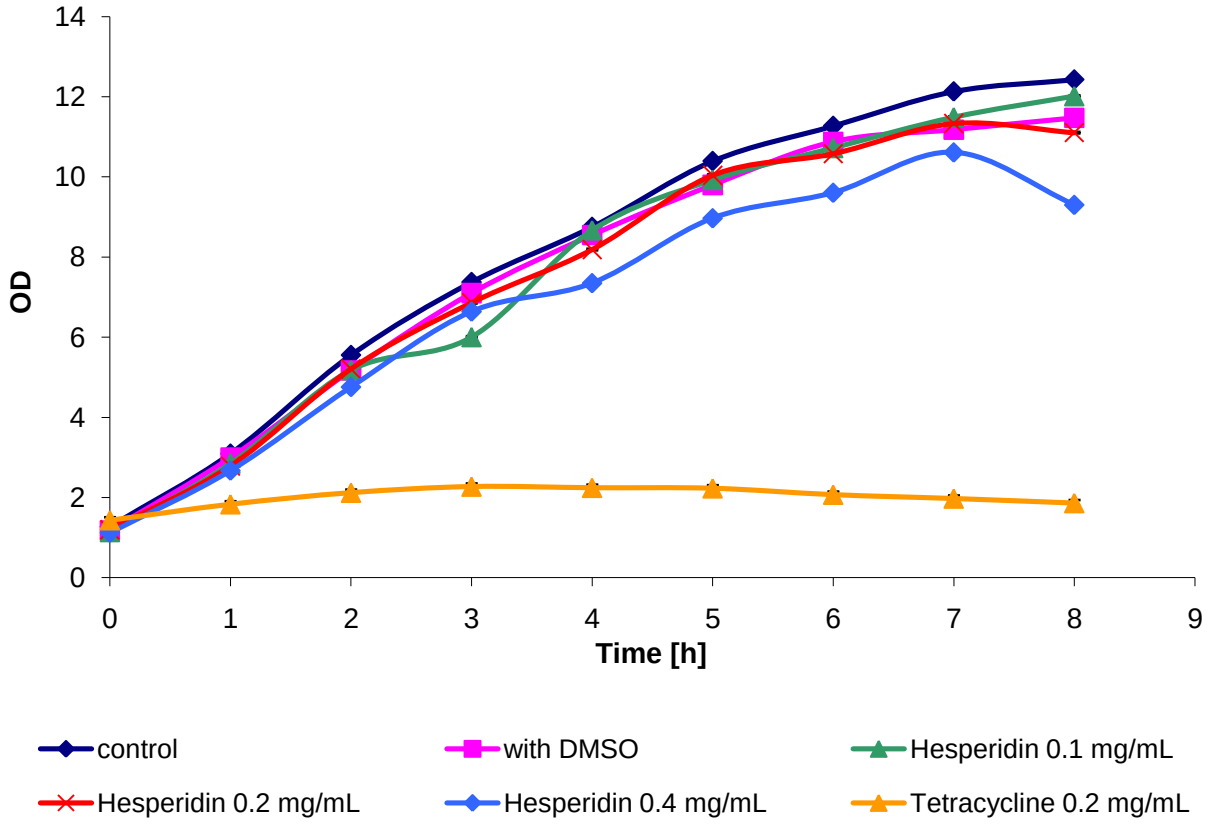
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperidin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.86	0.81	1.28	0.87	1.00	1.03
1	1.04	1.07	1.55	1.09	1.17	1.25
2	1.61	1.51	1.65	1.34	1.65	1.73
3	2.14	2.05	1.66	2.10	2.28	2.28
4	2.52	2.24	1.68	2.26	2.37	2.50
5	2.61	2.43	1.75	2.47	2.48	2.59
6	2.79	2.45	1.68	2.70	2.67	2.74
7	2.85	2.55	1.74	2.62	2.64	2.81
8	3.09	2.47	1.70	2.67	2.72	2.82
SD	0.11263633	0.05687601	0.05256633	0.08179199	0.05944335	0.04741458

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



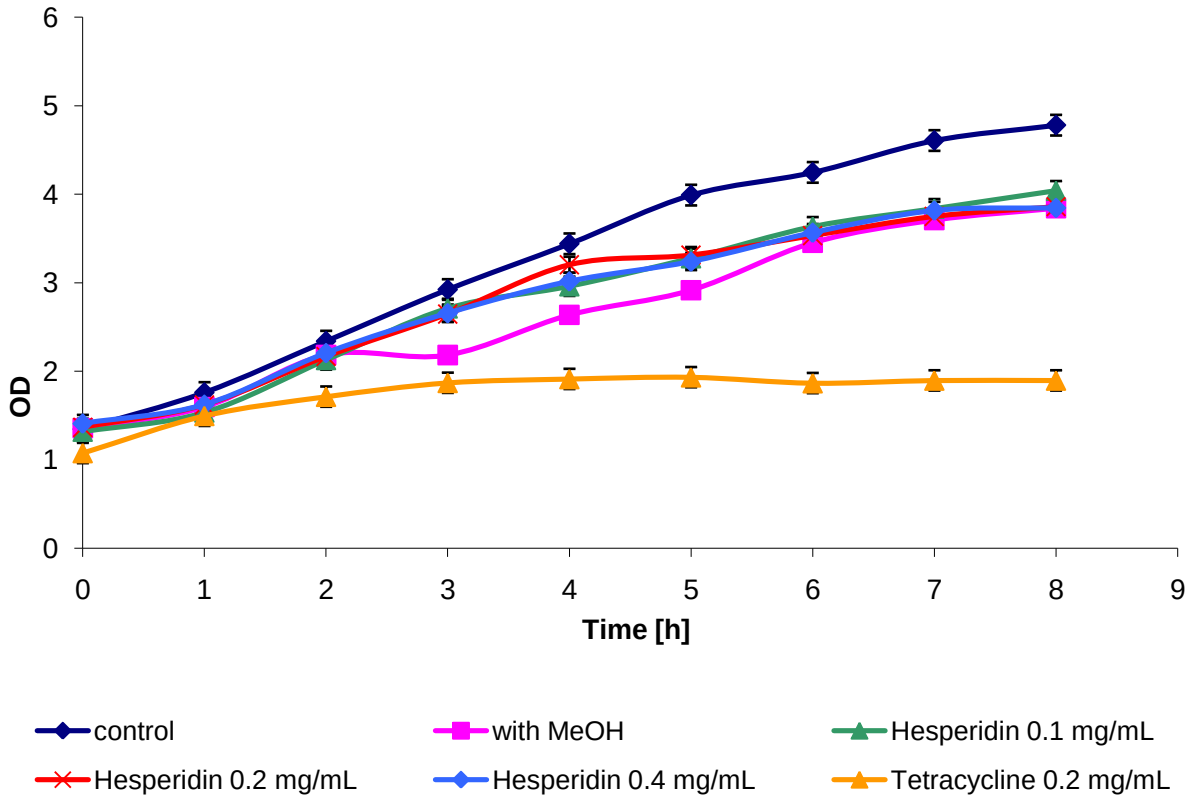
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Hesperidin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.31	1.18	1.45	1.03	1.21	1.11
1	1.79	1.61	2.12	1.56	1.67	1.46
2	2.96	2.51	2.31	2.42	2.57	3.18
3	3.79	3.38	2.44	3.12	3.39	4.20
4	4.75	4.48	2.47	4.57	4.59	5.54
5	5.03	4.59	2.53	4.73	4.63	5.51
6	5.17	5.00	2.55	4.97	5.27	5.90
7	5.35	5.31	2.57	5.13	5.56	6.02
SD	0.16334129	0.1813042	0.05198393	0.15629776	0.11981741	0.17339047

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of dimethylsulfoxide (DMSO) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



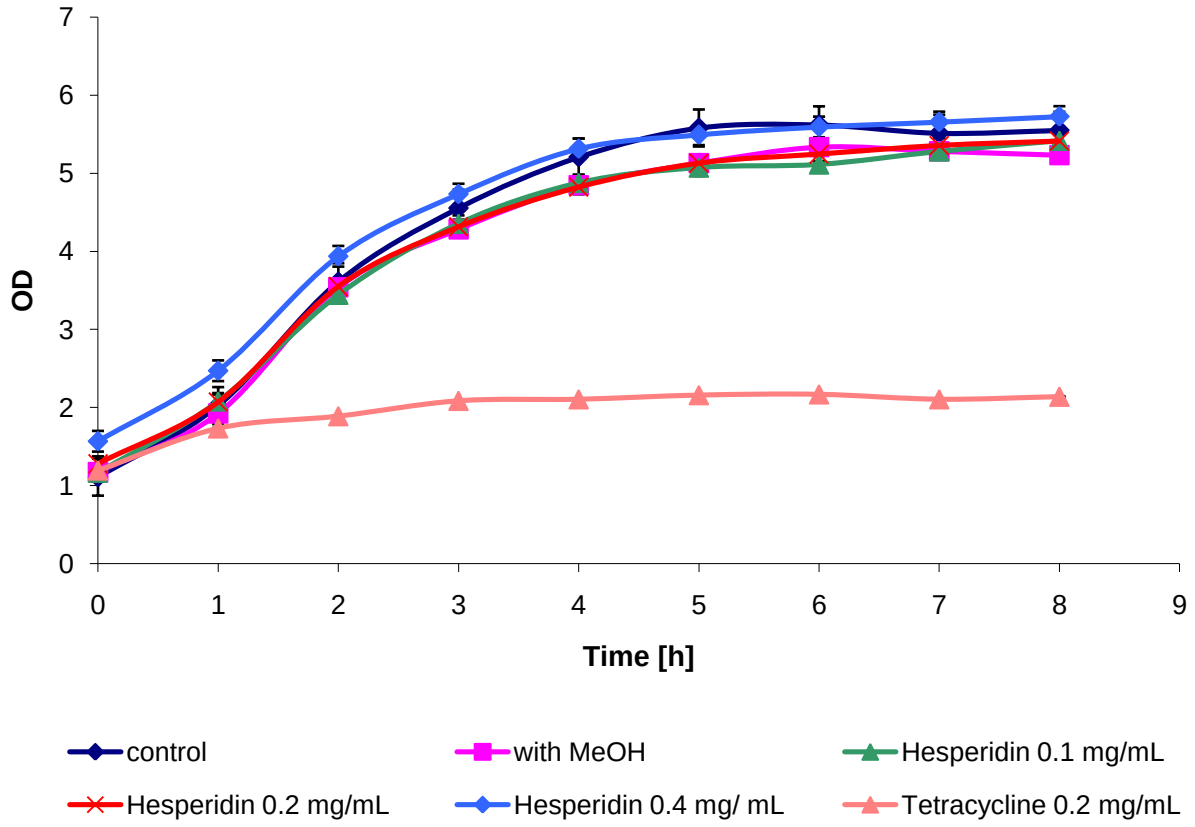
Time [h]	OD					
	Control	DMSO	Tetracycline 0.2 mg/mL	Hepseridin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	1.27	1.18	1.43	1.14	1.20	1.12
1	3.09	2.99	1.83	2.85	2.78	2.67
2	5.55	5.17	2.12	5.18	5.21	4.76
3	7.37	7.10	2.27	6.00	6.86	6.64
4	8.75	8.55	2.24	8.67	8.19	7.35
5	10.39	9.80	2.23	9.92	10.04	8.97
6	11.27	10.88	2.07	10.72	10.58	9.61
7	12.13	11.18	1.97	11.49	11.33	10.61
8	12.43	11.47	1.86	12.01	11.10	9.30
SD	0.02757578	0.01699112	0.07569628	0.02099836	0.01933617	0.03148424

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density. SD – standard deviation



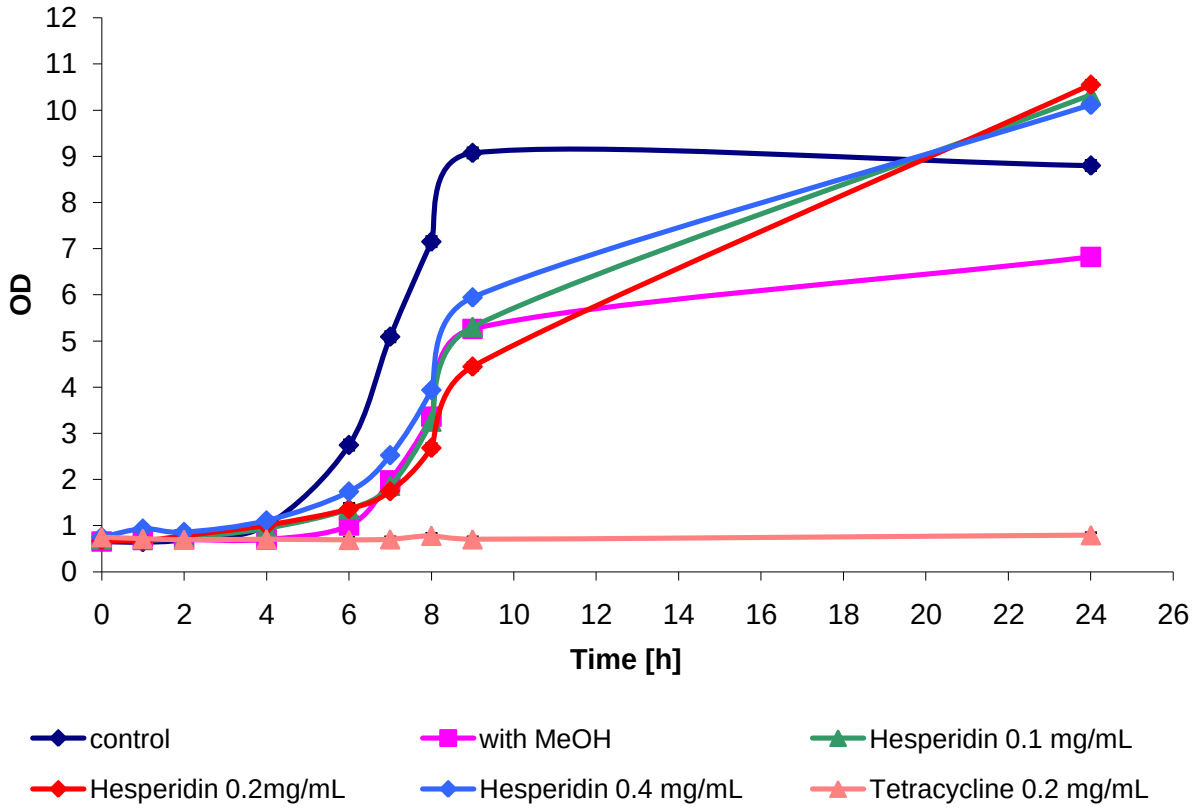
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperidin 0.1 mg/mL	Hesperidin 0.2 mg/mL	Hesperidin 0.4 mg/mL
0	1.35	1.36	1.08	1.32	1.37	1.41
1	1.76	1.60	1.50	1.53	1.62	1.62
2	2.34	2.18	1.71	2.13	2.17	2.21
3	2.92	2.18	1.87	2.71	2.65	2.66
4	3.44	2.64	1.91	2.96	3.20	3.02
5	3.99	2.92	1.93	3.28	3.31	3.24
6	4.25	3.45	1.87	3.63	3.53	3.57
7	4.61	3.71	1.90	3.84	3.75	3.82
8	4.78	3.84	1.90	4.04	3.86	3.85
SD	0.11657707	0.07600719	0.11445771	0.10875443	0.09034795	0.09698598

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



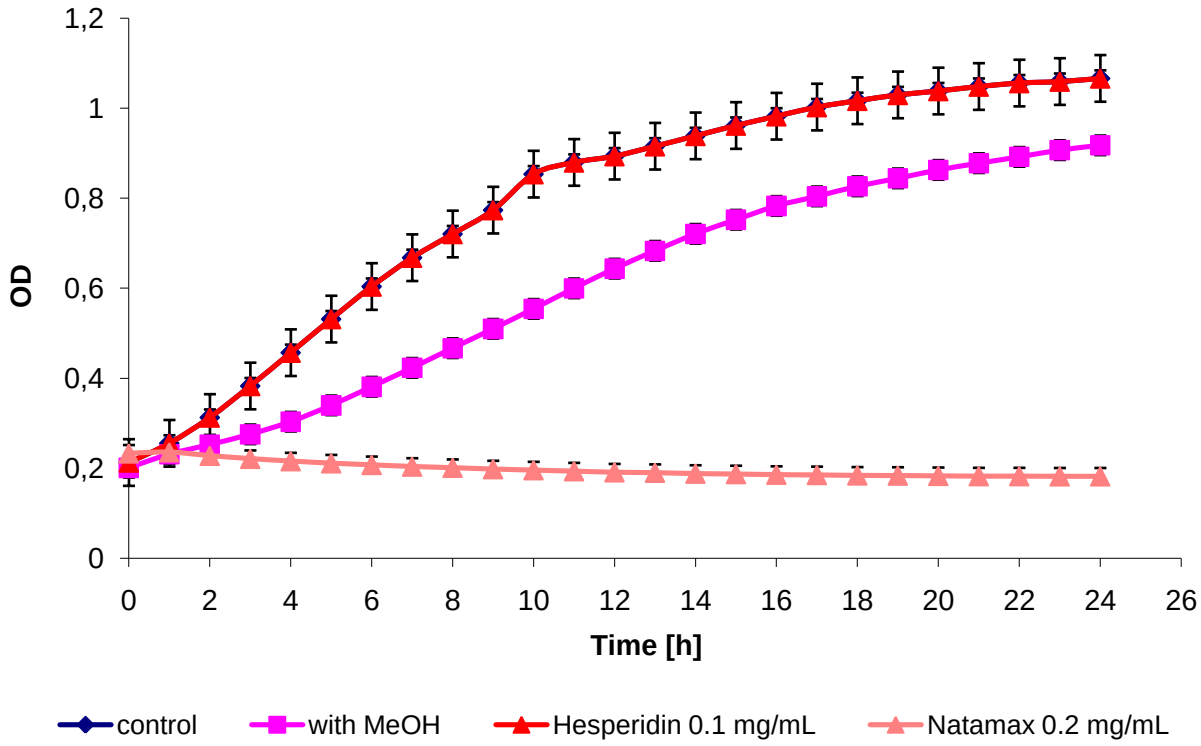
Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperidin 0.1 mg/mL	Hesperidin 0.2 mg/mL	Hesperidin 0.4 mg/mL
0	1.11	1.17	1.19	1.16	1.28	1.57
1	2.02	1.92	1.73	2.08	2.07	2.47
2	3.61	3.54	1.89	3.44	3.55	3.94
3	4.55	4.28	2.09	4.35	4.31	4.73
4	5.21	4.85	2.11	4.88	4.82	5.31
5	5.58	5.13	2.16	5.07	5.13	5.49
6	5.62	5.34	2.17	5.11	5.25	5.59
7	5.51	5.28	2.11	5.28	5.36	5.66
8	5.55	5.23	2.14	5.42	5.41	5.73
SD	0.23713693	0.09848988	0.03921362	0.10701442	0.09276078	0.13242711

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



Time [h]	OD					
	Control	MeOH	Tetracycline 0.2 mg/mL	Hesperidin		
				0.1 mg/mL	0.2 mg/mL	0.4 mg/mL
0	0.65	0.66	0.75	0.70	0.68	0.75
1	0.64	0.69	0.72	0.69	0.67	0.93
2	0.69	0.70	0.69	0.73	0.78	0.86
4	1.01	0.70	0.70	0.94	1.01	1.11
6	2.74	1.00	0.69	1.36	1.36	1.74
7	5.09	1.98	0.70	1.86	1.75	2.52
8	7.15	3.36	0.78	3.25	2.69	3.94
9	9.07	5.26	0.70	5.31	4.45	5.94
24	8.80	6.82	0.79	10.33	10.55	10.11
SD	0.11157338	0.12316401	0.05209636	0.12508866	0.08906318	0.07418101

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of methanol (MeOH) and various concentration of hesperidin; OD – optical density, SD – standard deviation.



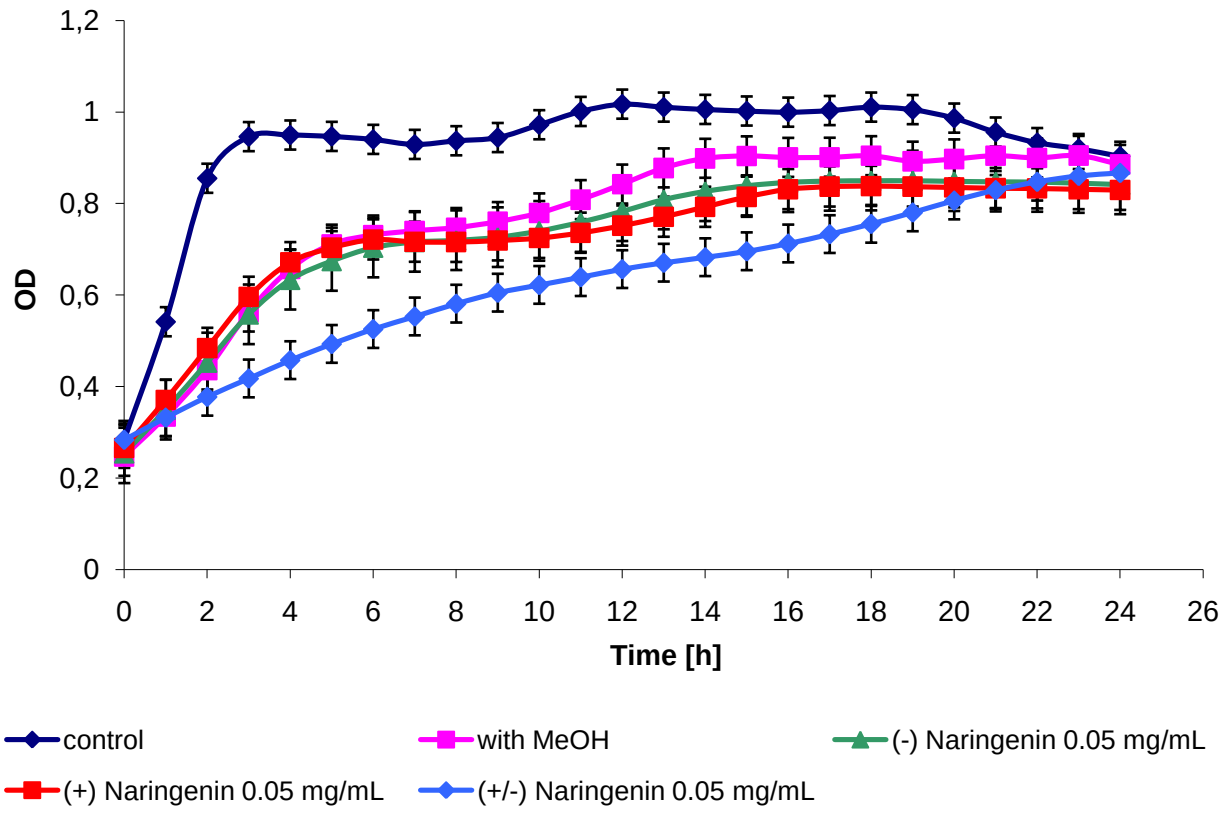
Time [h]	OD			
	Control	MeOH	Natamax 0.2 mg/mL	Hesperidin 0.1 mg/mL
0	0.2127	0.2010	0.2333	0.2127
1	0.2553	0.2310	0.2357	0.2553
2	0.3127	0.2523	0.2280	0.3127
3	0.3827	0.2753	0.2213	0.3827
4	0.4567	0.3033	0.2160	0.4567
5	0.5313	0.3397	0.2113	0.5313
6	0.6037	0.3810	0.2077	0.6037
7	0.6677	0.4230	0.2040	0.6677
8	0.7203	0.4667	0.2013	0.7203
9	0.7733	0.5097	0.1983	0.7733
10	0.8533	0.5540	0.1960	0.8533
11	0.8793	0.5997	0.1937	0.8793
12	0.8933	0.6433	0.1913	0.8933
13	0.9153	0.6830	0.1903	0.9153

14	0.9383	0.7207	0.1883	0.9383
15	0.9613	0.7520	0.1873	0.9613
16	0.9820	0.7827	0.1860	0.9820
17	1.0023	0.8040	0.1853	1.0023
18	1.0163	0.8263	0.1843	1.0163
19	1.0293	0.8440	0.1840	1.0293
20	1.0380	0.8627	0.1833	1.0380
21	1.0480	0.8777	0.1827	1.0480
22	1.0557	0.8917	0.1827	1.0557
23	1.0590	0.9067	0.1823	1.0590
24	1.0660	0.9173	0.1823	1.0660
SD	0.01786773	0.02171292	0.01817688	0.0518191

Annex II. Growth curves of the racemates and their enantiomers.

Naringenin

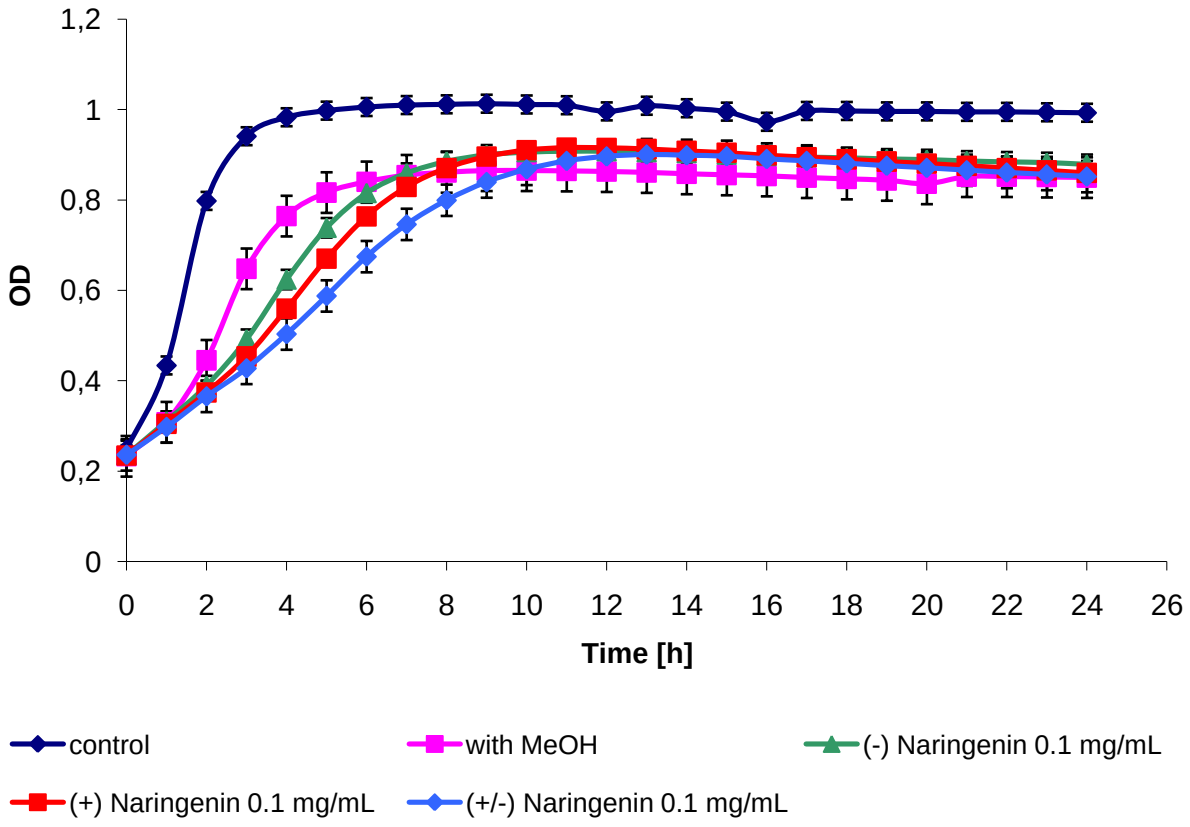
Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density. SD – standard deviation



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.05 mg/mL	(+) Naringenin 0.05 mg/mL	(+/-) Naringenin 0.05 mg/mL
0	0.2835	0.2473	0.2540	0.2660	0.2835
1	0.5415	0.3343	0.3493	0.3710	0.3320
2	0.8550	0.4360	0.4523	0.4843	0.3775
3	0.9460	0.5627	0.5577	0.5960	0.4175
4	0.9495	0.6570	0.6333	0.6717	0.4575
5	0.9465	0.7110	0.6743	0.7033	0.4930
6	0.9400	0.7310	0.7037	0.7213	0.5255
7	0.9290	0.7403	0.7160	0.7163	0.5530
8	0.9370	0.7473	0.7197	0.7160	0.5810
9	0.9440	0.7603	0.7263	0.7193	0.6050

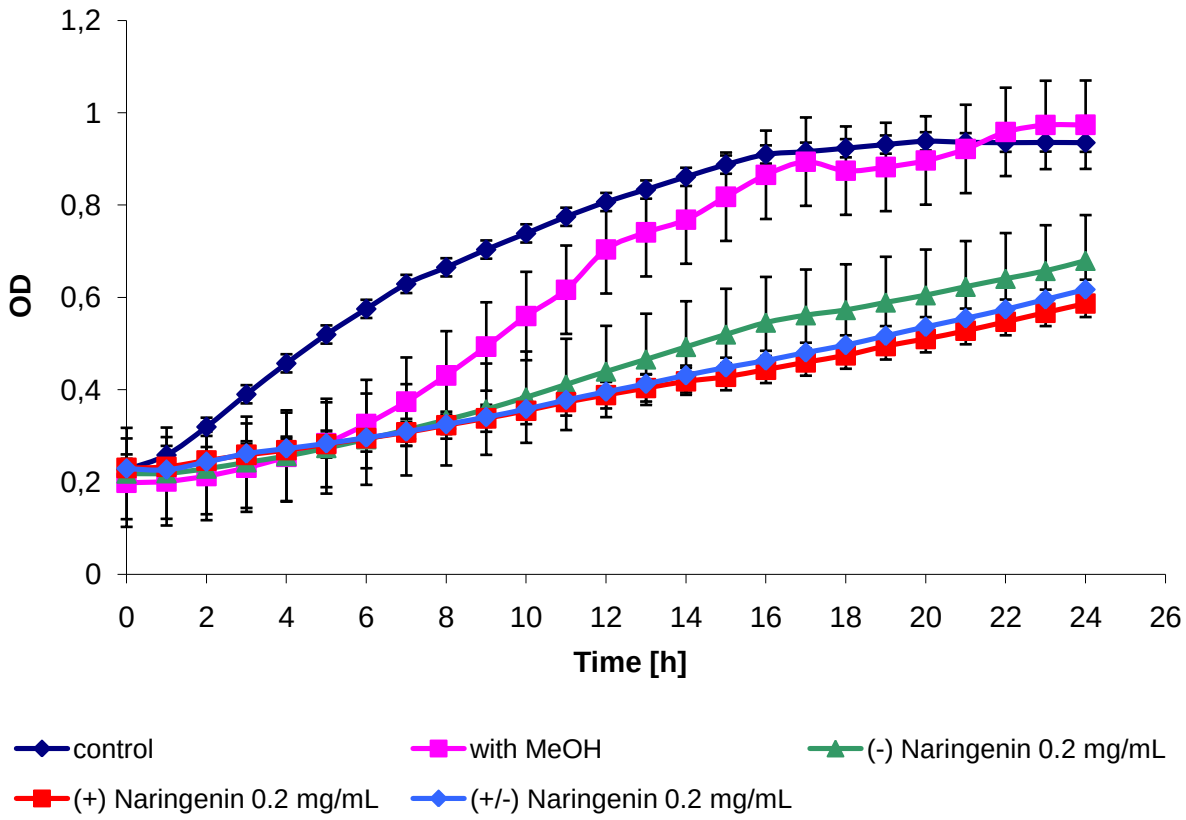
10	0.9720	0.7793	0.7400	0.7247	0.6220
11	1.0010	0.8083	0.7593	0.7360	0.6390
12	1.0170	0.8423	0.7830	0.7517	0.6565
13	1.0105	0.8777	0.8087	0.7710	0.6705
14	1.0055	0.8987	0.8267	0.7927	0.6825
15	1.0020	0.9040	0.8390	0.8147	0.6955
16	0.9995	0.9007	0.8463	0.8313	0.7125
17	1.0030	0.9010	0.8493	0.8370	0.7330
18	1.0105	0.9043	0.8500	0.8383	0.7555
19	1.0050	0.8923	0.8500	0.8370	0.7805
20	0.9865	0.8973	0.8487	0.8353	0.8065
21	0.9560	0.9047	0.8477	0.8337	0.8295
22	0.9330	0.8997	0.8470	0.8330	0.8475
23	0.9200	0.9050	0.8447	0.8313	0.8605
24	0.9030	0.8853	0.8417	0.8297	0.8670
SD	0.03181981	0.04260333	0.06516509	0.04385098	0.04123847

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.1 mg/mL	(+) Naringenin 0.1 mg/mL	(+/-) Naringenin 0.1 mg/mL
0	0.2477	0.2330	0.2360	0.2343	0.2360
1	0.4340	0.3083	0.3105	0.3047	0.2977
2	0.7980	0.4453	0.3895	0.3740	0.3653
3	0.9410	0.6477	0.4920	0.4537	0.4273
4	0.9830	0.7643	0.6240	0.5593	0.5033
5	0.9977	0.8163	0.7385	0.6700	0.5877
6	1.0057	0.8400	0.8155	0.7637	0.6747
7	1.0100	0.8547	0.8595	0.8287	0.7460
8	1.0117	0.8610	0.8855	0.8707	0.7993
9	1.0130	0.8647	0.9000	0.8960	0.8397
10	1.0113	0.8647	0.9060	0.9103	0.8677
11	1.0097	0.8640	0.9080	0.9157	0.8863
12	0.9960	0.8627	0.9080	0.9153	0.8963
13	1.0085	0.8607	0.9060	0.9127	0.9003
14	1.0030	0.8577	0.9040	0.9083	0.8987
15	0.9955	0.8553	0.9005	0.9043	0.8967
16	0.9730	0.8530	0.8975	0.8987	0.8907
17	0.9970	0.8493	0.8950	0.8947	0.8863
18	0.9970	0.8463	0.8935	0.8900	0.8810
19	0.9960	0.8433	0.8910	0.8850	0.8757
20	0.9960	0.8357	0.8895	0.8803	0.8710
21	0.9950	0.8515	0.8865	0.8757	0.8657
22	0.9950	0.8515	0.8845	0.8707	0.8607
23	0.9940	0.8505	0.8830	0.8653	0.8563
24	0.9930	0.8495	0.8790	0.8597	0.8517
SD	0.01983879	0.04494434	0.02155261	0.01386544	0.03468092

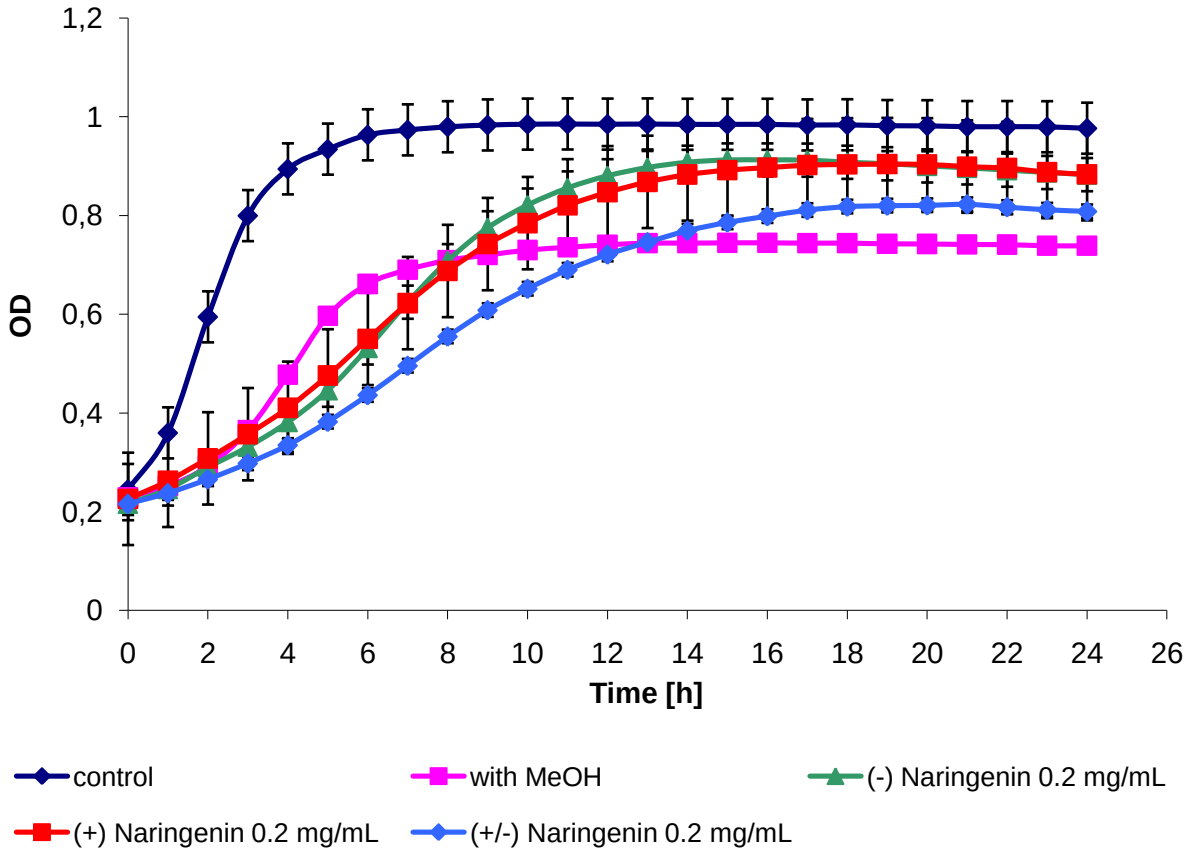
Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.2 mg/mL	(+) Naringenin 0.2 mg/mL	(+/-) Naringenin 0.2 mg/mL
0	0.2270	0.1985	0.2183	0.2305	0.2290
1	0.2585	0.2015	0.2190	0.2330	0.2265
2	0.3195	0.2130	0.2290	0.2465	0.2435
3	0.3900	0.2310	0.2427	0.2590	0.2615
4	0.4570	0.2545	0.2563	0.2690	0.2725
5	0.5195	0.2845	0.2737	0.2820	0.2835
6	0.5750	0.3255	0.2927	0.2950	0.2960
7	0.6290	0.3740	0.3130	0.3075	0.3090
8	0.6650	0.4310	0.3347	0.3230	0.3250
9	0.7035	0.4935	0.3577	0.3380	0.3405
10	0.7385	0.5595	0.3837	0.3545	0.3580
11	0.7745	0.6165	0.4113	0.3730	0.3775
12	0.8065	0.7040	0.4393	0.3885	0.3955

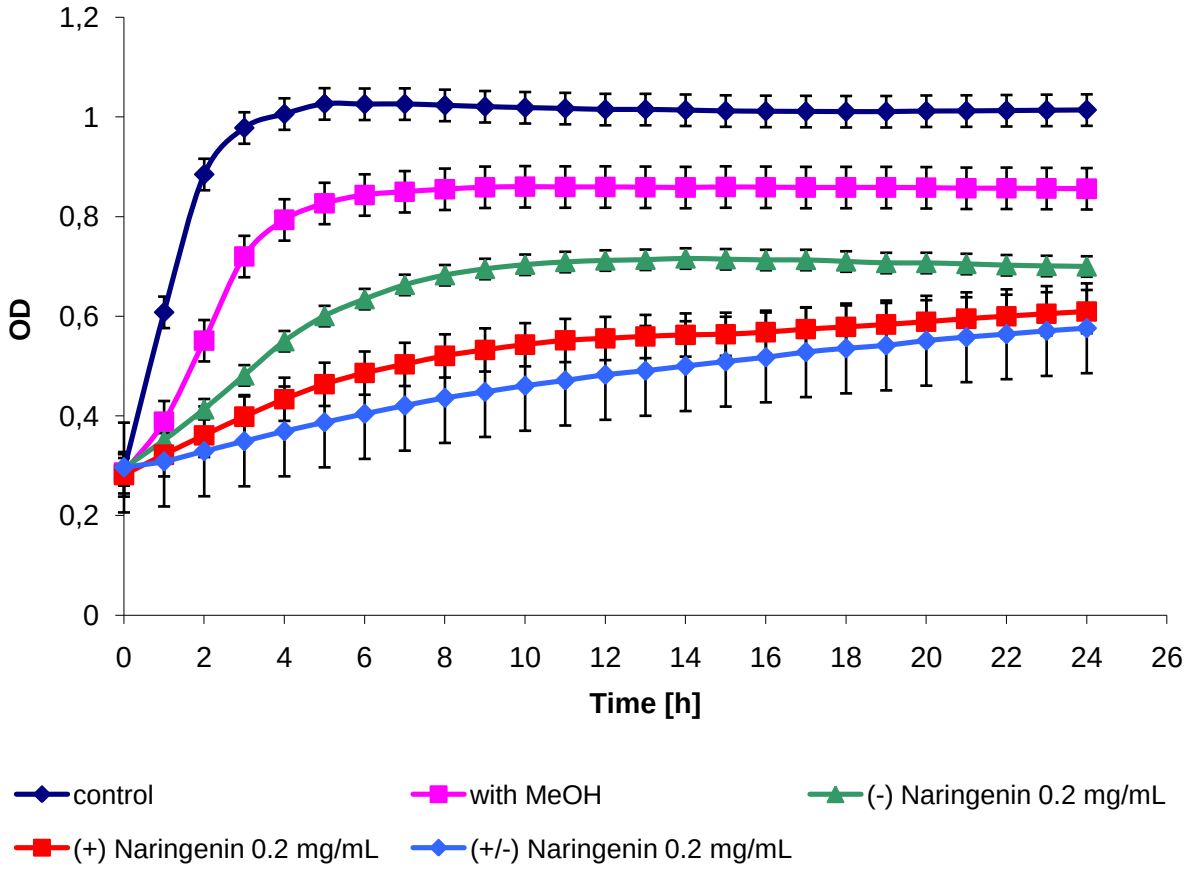
13	0.8335	0.7410	0.4657	0.4035	0.4125
14	0.8610	0.7685	0.4927	0.4180	0.4310
15	0.8875	0.8180	0.5197	0.4280	0.4480
16	0.9095	0.8655	0.5453	0.4435	0.4630
17	0.9155	0.8940	0.5613	0.4595	0.4805
18	0.9230	0.8745	0.5727	0.4745	0.4965
19	0.9310	0.8825	0.5890	0.4945	0.5165
20	0.9380	0.8965	0.6047	0.5100	0.5360
21	0.9360	0.9215	0.6230	0.5275	0.5545
22	0.9350	0.9585	0.6403	0.5470	0.5740
23	0.9355	0.9735	0.6573	0.5670	0.5955
24	0.9350	0.9740	0.6793	0.5865	0.6170
SD	0.01974242	0.09577054	0.09889814	0.02924594	0.0212132

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.1 mg/mL	(+) Naringenin 0.1 mg/mL	(+/-) Naringenin 0.1 mg/mL
0	0.2450	0.2293	0.2163	0.2260	0.2160
1	0.3597	0.2520	0.2460	0.2625	0.2377
2	0.5947	0.2923	0.2900	0.3080	0.2657
3	0.7997	0.3650	0.3317	0.3570	0.2980
4	0.8943	0.4773	0.3817	0.4105	0.3350
5	0.9343	0.5967	0.4460	0.4760	0.3823
6	0.9633	0.6613	0.5317	0.5500	0.4363
7	0.9733	0.6903	0.6243	0.6225	0.4957
8	0.9797	0.7093	0.7083	0.6875	0.5550
9	0.9833	0.7200	0.7750	0.7420	0.6083
10	0.9850	0.7300	0.8210	0.7845	0.6517
11	0.9853	0.7357	0.8557	0.8205	0.6900
12	0.9850	0.7407	0.8803	0.8470	0.7210
13	0.9853	0.7440	0.8970	0.8680	0.7460
14	0.9847	0.7440	0.9077	0.8830	0.7693
15	0.9847	0.7447	0.9123	0.8920	0.7860
16	0.9847	0.7447	0.9123	0.8970	0.7987
17	0.9833	0.7440	0.9120	0.9020	0.8110
18	0.9837	0.7440	0.9077	0.9035	0.8180
19	0.9820	0.7427	0.9047	0.9040	0.8200
20	0.9817	0.7423	0.9003	0.9035	0.8207
21	0.9800	0.7413	0.8963	0.8990	0.8227
22	0.9800	0.7410	0.8917	0.8960	0.8170
23	0.9797	0.7387	0.8870	0.8880	0.8117
24	0.9767	0.7387	0.8827	0.8835	0.8083
SD	0.05171719	0.01352508	0.0335989	0.09353609	0.01376227

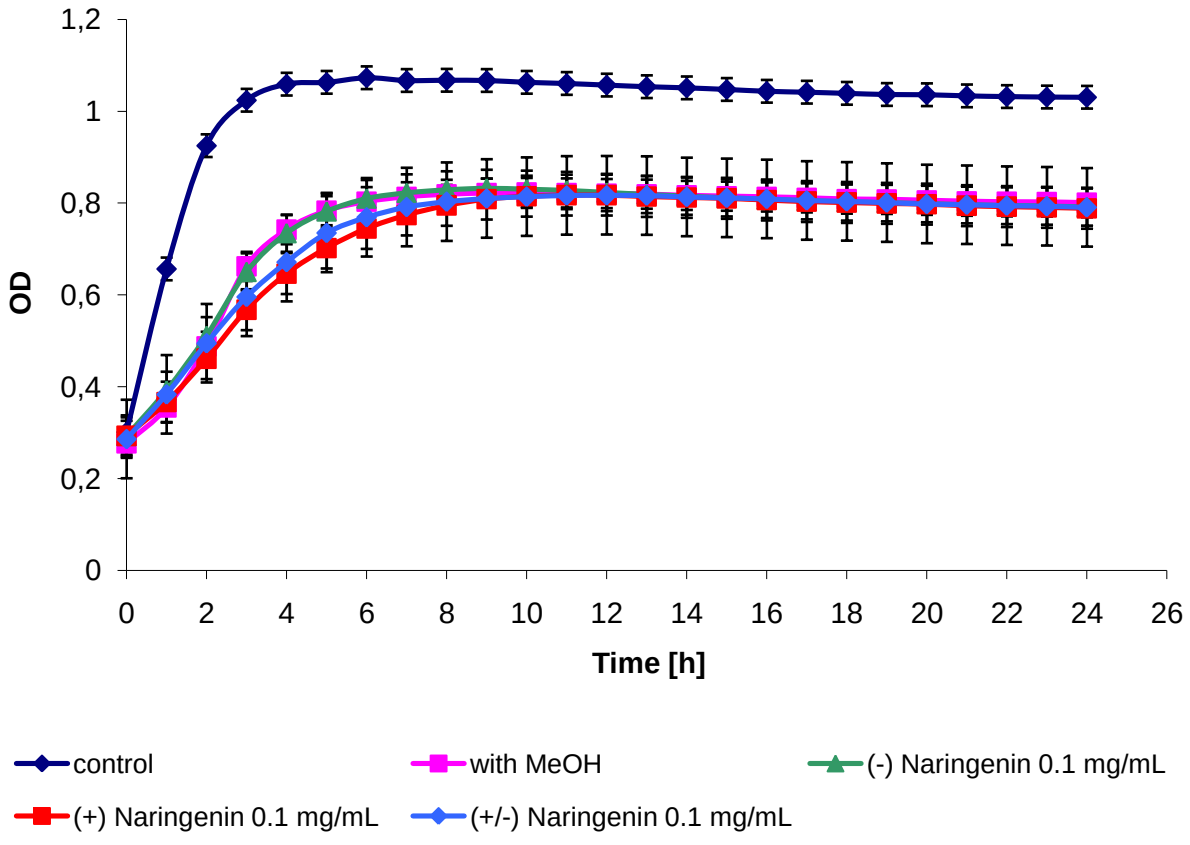
Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.2 mg/mL	(+) Naringenin 0.2 mg/mL	(+/-) Naringenin 0.2 mg/mL
0	0.2917	0.2860	0.2950	0.2815	0.2965
1	0.6080	0.3885	0.3510	0.3220	0.3085
2	0.8847	0.5510	0.4135	0.3610	0.3290
3	0.9780	0.7200	0.4815	0.3985	0.3490
4	1.0060	0.7935	0.5500	0.4335	0.3690
5	1.0263	0.8265	0.6005	0.4635	0.3870
6	1.0257	0.8435	0.6345	0.4860	0.4040
7	1.0260	0.8500	0.6630	0.5035	0.4205
8	1.0233	0.8550	0.6825	0.5205	0.4360
9	1.0207	0.8590	0.6950	0.5325	0.4480
10	1.0187	0.8600	0.7035	0.5430	0.4605
11	1.0170	0.8595	0.7090	0.5515	0.4710
12	1.0150	0.8595	0.7120	0.5555	0.4825

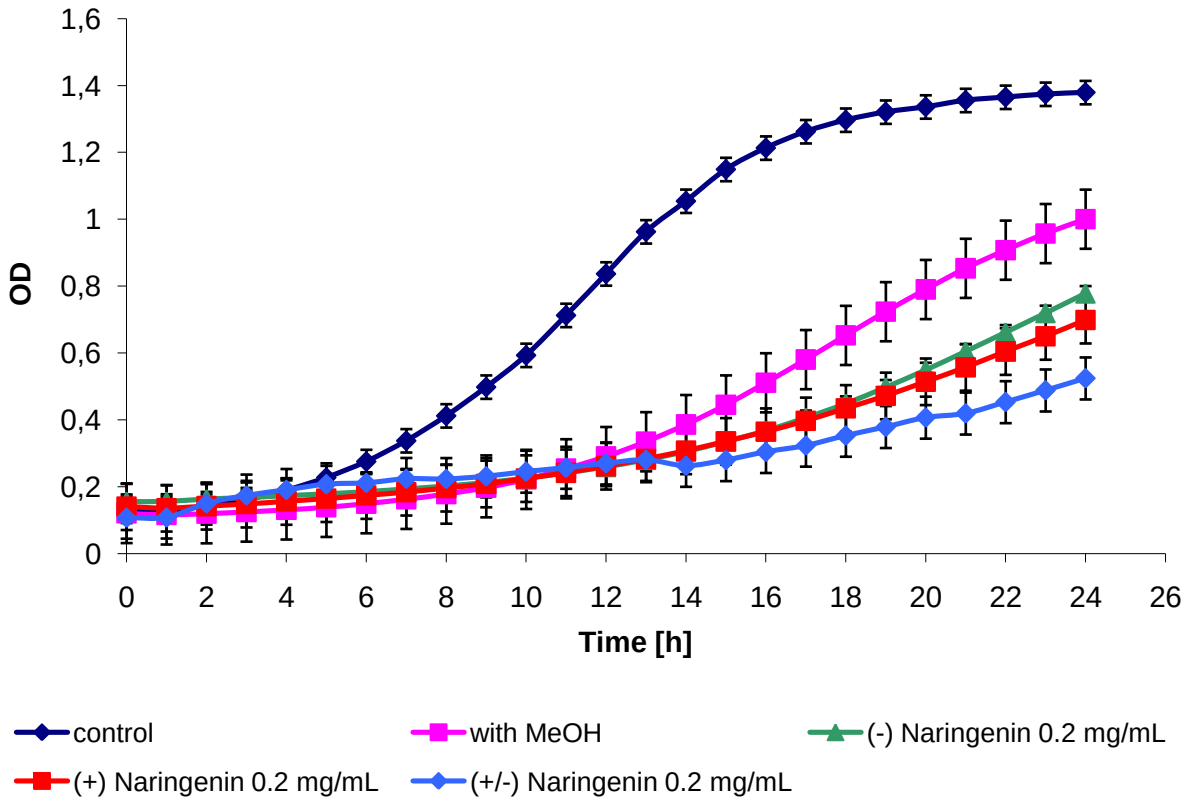
13	1.0150	0.8590	0.7135	0.5595	0.4905
14	1.0137	0.8585	0.7160	0.5625	0.5000
15	1.0120	0.8595	0.7145	0.5640	0.5090
16	1.0113	0.8590	0.7130	0.5680	0.5175
17	1.0110	0.8585	0.7130	0.5740	0.5280
18	1.0107	0.8585	0.7100	0.5785	0.5355
19	1.0107	0.8585	0.7070	0.5835	0.5415
20	1.0117	0.8580	0.7070	0.5890	0.5510
21	1.0120	0.8570	0.7050	0.5950	0.5580
22	1.0127	0.8570	0.7025	0.6000	0.5640
23	1.0133	0.8565	0.7010	0.6050	0.5705
24	1.0140	0.8560	0.7000	0.6095	0.5760
SD	0.03162462	0.04157788	0.02056267	0.04338807	0.09017026

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.1 mg/mL	(+) Naringenin 0.1 mg/mL	(+/-) Naringenin 0.1 mg/mL
0	0.3010	0.2767	0.2927	0.2930	0.2860
1	0.6565	0.3547	0.3920	0.3663	0.3833
2	0.9250	0.4880	0.5110	0.4610	0.4947
3	1.0240	0.6620	0.6500	0.5677	0.5957
4	1.0590	0.7420	0.7343	0.6463	0.6713
5	1.0630	0.7833	0.7817	0.7020	0.7350
6	1.0730	0.8030	0.8097	0.7447	0.7690
7	1.0670	0.8137	0.8220	0.7740	0.7913
8	1.0675	0.8190	0.8287	0.7950	0.8030
9	1.0670	0.8217	0.8320	0.8087	0.8100
10	1.0630	0.8230	0.8300	0.8150	0.8140
11	1.0605	0.8220	0.8273	0.8173	0.8167
12	1.0570	0.8207	0.8230	0.8173	0.8170
13	1.0535	0.8193	0.8187	0.8143	0.8163
14	1.0510	0.8167	0.8150	0.8123	0.8133
15	1.0475	0.8150	0.8113	0.8103	0.8113
16	1.0435	0.8133	0.8080	0.8067	0.8090
17	1.0415	0.8113	0.8050	0.8033	0.8057
18	1.0390	0.8087	0.8023	0.8013	0.8037
19	1.0365	0.8080	0.8007	0.7993	0.8010
20	1.0360	0.8060	0.7987	0.7977	0.7980
21	1.0335	0.8040	0.7963	0.7943	0.7963
22	1.0320	0.8027	0.7947	0.7923	0.7943
23	1.0310	0.8023	0.7933	0.7907	0.7930
24	1.0305	0.8007	0.7913	0.7887	0.7907
SD	0.02472045	0.03170895	0.04055199	0.04453407	0.08552419

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of naringenin racemate and its enantiomers; OD – optical density, SD – standard deviation.

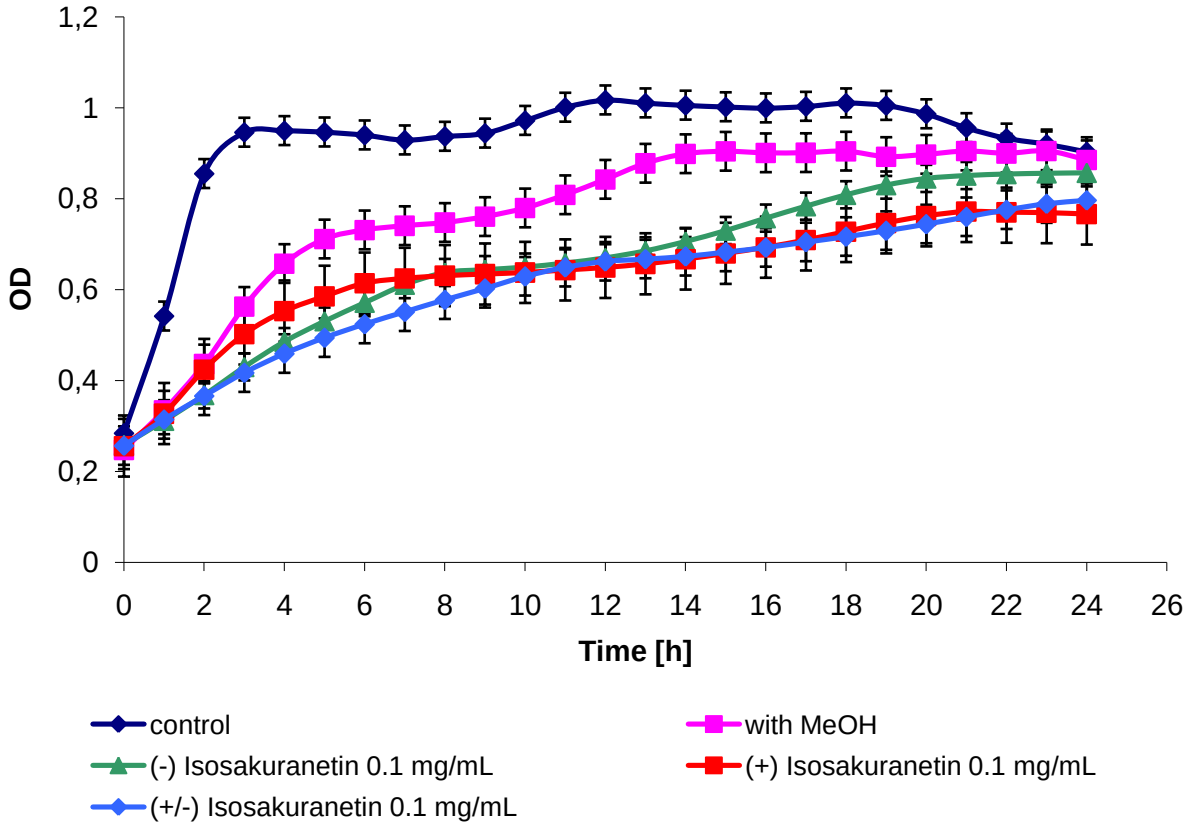


Time [h]	OD				
	Control	MeOH	(-) Naringenin 0.2 mg/mL	(+) Naringenin 0.2 mg/mL	(+/-) Naringenin 0.2 mg/mL
0	0.1265	0.1200	0.1550	0.1403	0.1073
1	0.1285	0.1157	0.1557	0.1357	0.1080
2	0.1420	0.1193	0.1627	0.1417	0.1497
3	0.1615	0.1243	0.1670	0.1480	0.1737
4	0.1895	0.1307	0.1727	0.1560	0.1903
5	0.2275	0.1383	0.1783	0.1643	0.2073
6	0.2755	0.1493	0.1847	0.1737	0.2113
7	0.3375	0.1623	0.1930	0.1837	0.2233
8	0.4120	0.1780	0.2017	0.1957	0.2230
9	0.4980	0.1970	0.2123	0.2083	0.2310
10	0.5930	0.2220	0.2253	0.2243	0.2450
11	0.7125	0.2537	0.2413	0.2417	0.2567
12	0.8365	0.2903	0.2590	0.2613	0.2697
13	0.9625	0.3347	0.2803	0.2833	0.2803
14	1.0540	0.3860	0.3050	0.3073	0.2627

15	1.1490	0.4447	0.3350	0.3357	0.2797
16	1.2130	0.5110	0.3673	0.3647	0.3040
17	1.2620	0.5803	0.4060	0.3970	0.3230
18	1.2965	0.6527	0.4483	0.4343	0.3527
19	1.3205	0.7237	0.4973	0.4717	0.3787
20	1.3360	0.7900	0.5490	0.5140	0.4067
21	1.3555	0.8533	0.6047	0.5570	0.4190
22	1.3650	0.9077	0.6617	0.6043	0.4530
23	1.3740	0.9573	0.7197	0.6497	0.4880
24	1.3790	1.0003	0.7783	0.6983	0.5240
SD	0.03504421	0.0884909	0.02198292	0.06968285	0.06281843

Isosakuranetin

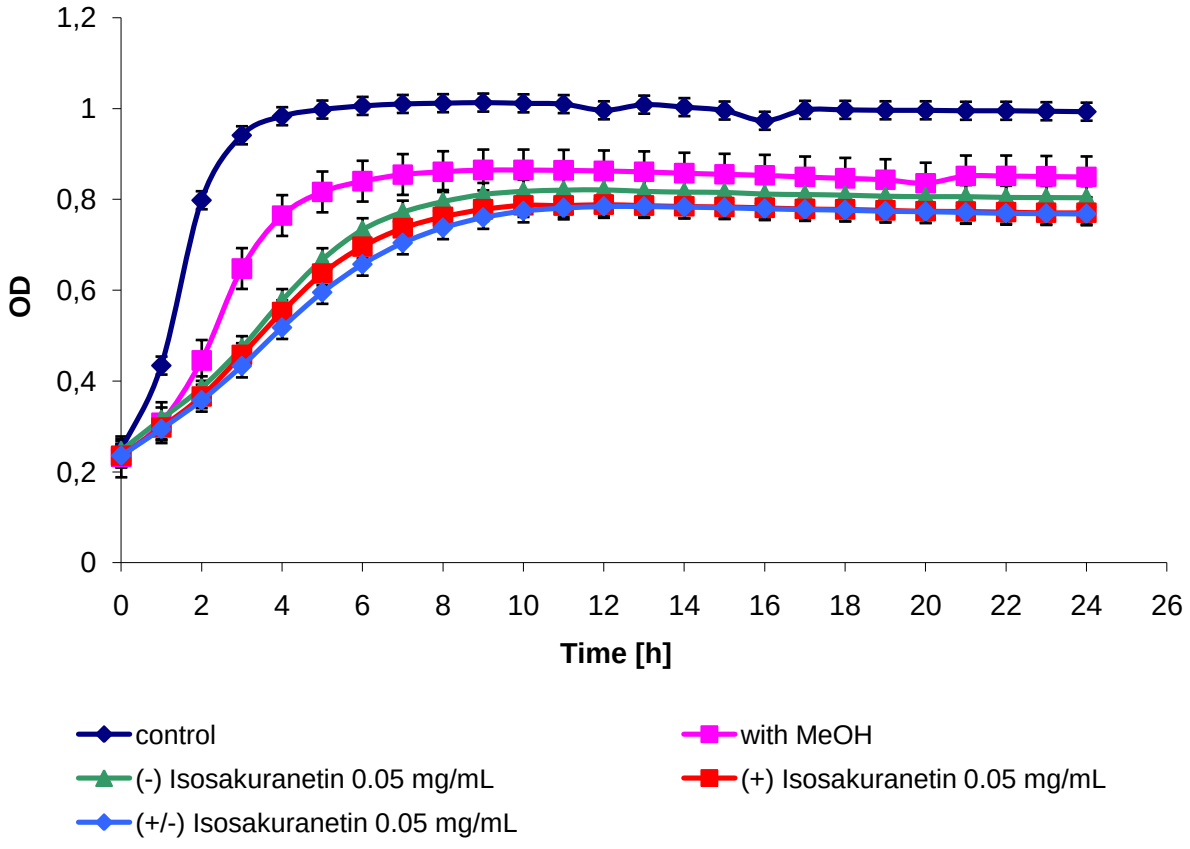
Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.1 mg/mL	(+) Isosakuranetin 0.1 mg/mL	(+/-) Isosakuranetin 0.1 mg/mL
0	0.2835	0.2473	0.2583	0.2555	0.2565
1	0.5415	0.3343	0.3113	0.3270	0.3140
2	0.8550	0.4360	0.3680	0.4240	0.3660
3	0.9460	0.5627	0.4297	0.5020	0.4170
4	0.9495	0.6570	0.4853	0.5525	0.4590
5	0.9465	0.7110	0.5303	0.5850	0.4940
6	0.9400	0.7310	0.5710	0.6140	0.5240
7	0.9290	0.7403	0.6107	0.6245	0.5510
8	0.9370	0.7473	0.6373	0.6305	0.5775
9	0.9440	0.7603	0.6437	0.6340	0.6025
10	0.9720	0.7793	0.6493	0.6375	0.6290
11	1.0010	0.8083	0.6583	0.6430	0.6490
12	1.0170	0.8423	0.6697	0.6485	0.6620

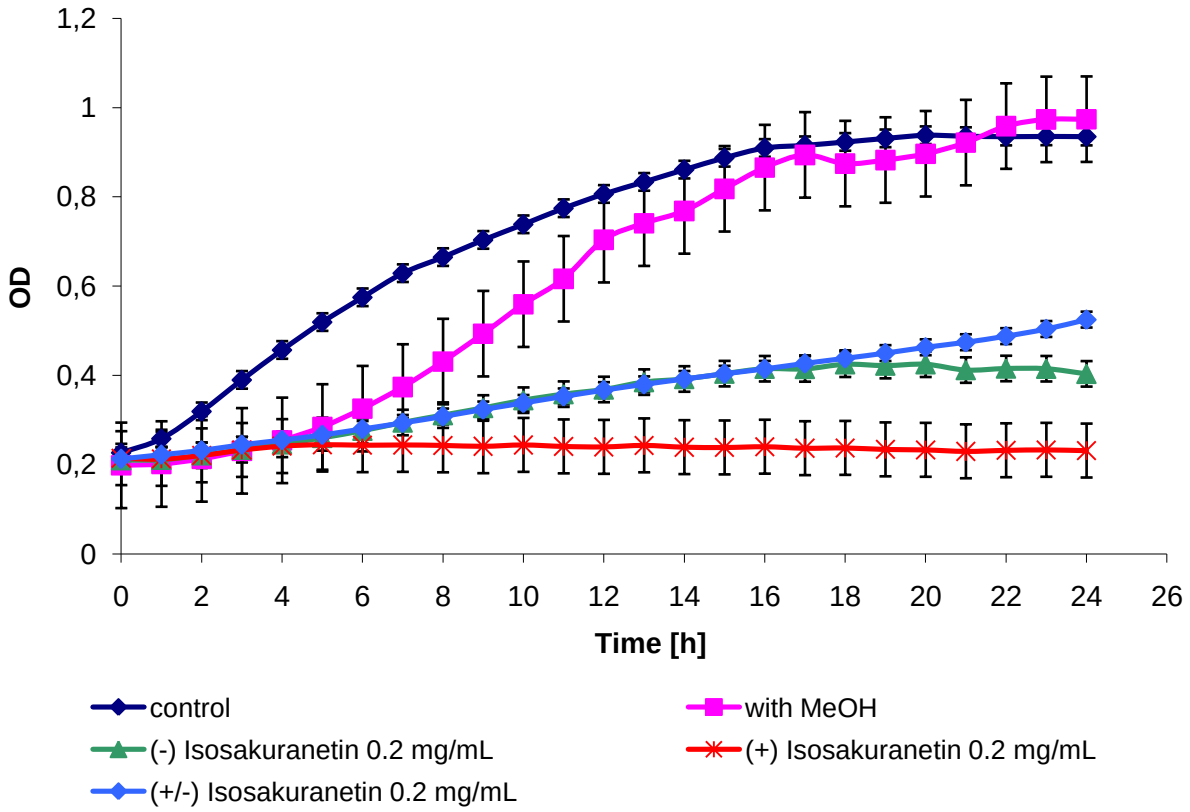
13	1.0105	0.8777	0.6850	0.6565	0.6670
14	1.0055	0.8987	0.7057	0.6670	0.6730
15	1.0020	0.9040	0.7297	0.6795	0.6825
16	0.9995	0.9007	0.7570	0.6930	0.6925
17	1.0030	0.9010	0.7833	0.7090	0.7045
18	1.0105	0.9043	0.8083	0.7275	0.7165
19	1.0050	0.8923	0.8297	0.7465	0.7295
20	0.9865	0.8973	0.8443	0.7620	0.7440
21	0.9560	0.9047	0.8507	0.7715	0.7600
22	0.9330	0.8997	0.8540	0.7700	0.7755
23	0.9200	0.9050	0.8557	0.7690	0.7890
24	0.9030	0.8853	0.8567	0.7660	0.7965
SD	0.03181981	0.04260333	0.02985659	0.06723663	0.04237179

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.05 mg/mL	(+) Isosakuranetin 0.05 mg/mL	(+/-) Isosakuranetin 0.05 mg/mL
0	0.2477	0.2330	0.2467	0.2355	0.2357
1	0.4340	0.3083	0.3160	0.2975	0.2943
2	0.7980	0.4453	0.3847	0.3660	0.3577
3	0.9410	0.6477	0.4730	0.4575	0.4330
4	0.9830	0.7643	0.5767	0.5520	0.5177
5	0.9977	0.8163	0.6667	0.6365	0.5950
6	1.0057	0.8400	0.7327	0.6960	0.6570
7	1.0100	0.8547	0.7713	0.7365	0.7040
8	1.0117	0.8610	0.7947	0.7615	0.7373
9	1.0130	0.8647	0.8103	0.7780	0.7600
10	1.0113	0.8647	0.8173	0.7870	0.7743
11	1.0097	0.8640	0.8200	0.7865	0.7810
12	0.9960	0.8627	0.8207	0.7885	0.7843
13	1.0085	0.8607	0.8173	0.7865	0.7843
14	1.0030	0.8577	0.8157	0.7845	0.7830
15	0.9955	0.8553	0.8150	0.7835	0.7817
16	0.9730	0.8530	0.8113	0.7815	0.7793
17	0.9970	0.8493	0.8103	0.7790	0.7777
18	0.9970	0.8463	0.8090	0.7780	0.7763
19	0.9960	0.8433	0.8067	0.7760	0.7740
20	0.9960	0.8357	0.8057	0.7740	0.7730
21	0.9950	0.8515	0.8057	0.7735	0.7713
22	0.9950	0.8515	0.8040	0.7720	0.7693
23	0.9940	0.8505	0.8037	0.7705	0.7687
24	0.9930	0.8495	0.8033	0.7705	0.7680
SD	0.01983879	0.04494434	0.02575888	0.02575888	0.02505015

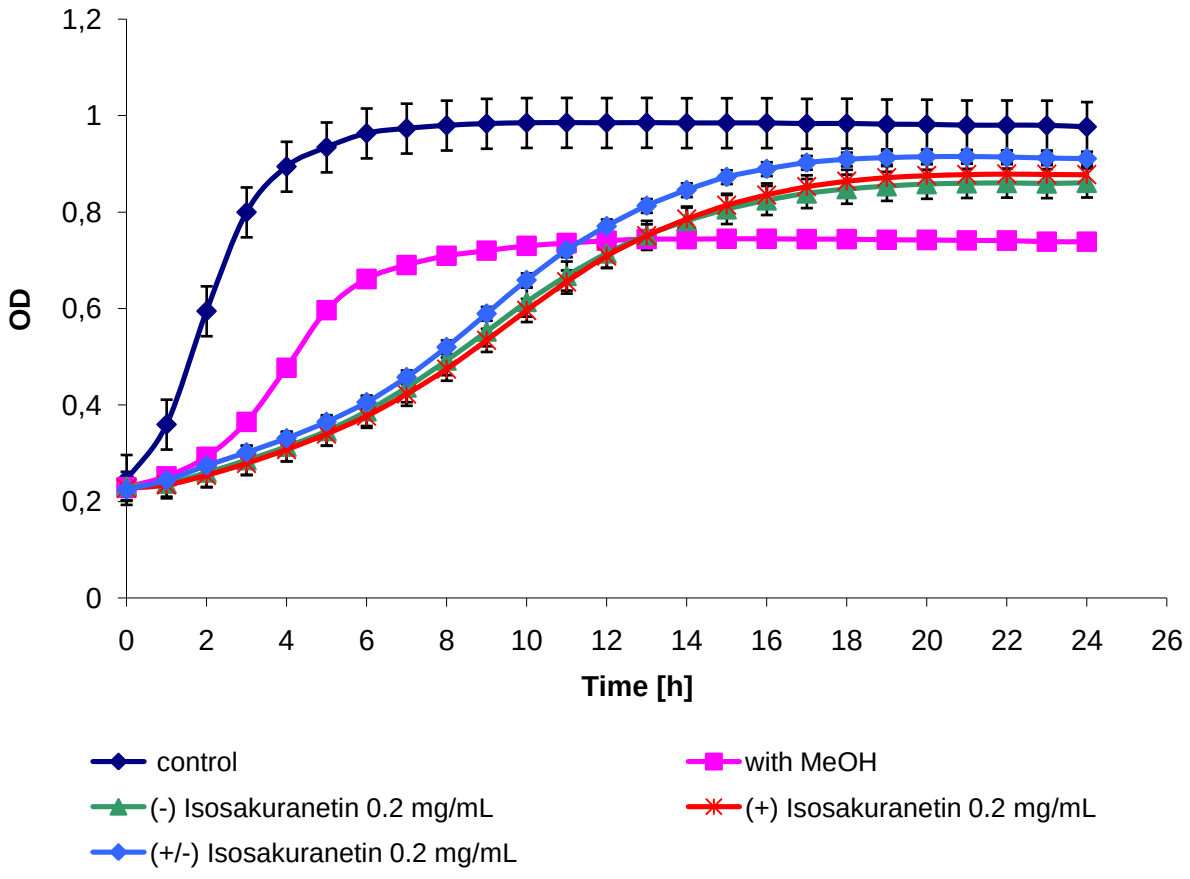
Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.2 mg/mL	(+) Isosakuranetin 0.2 mg/mL	(+/-) Isosakuranetin 0.2 mg/mL
0	0.2270	0.1985	0.2100	0.2147	0.2130
1	0.2585	0.2015	0.2110	0.2130	0.2223
2	0.3195	0.2130	0.2210	0.2210	0.2320
3	0.3900	0.2310	0.2335	0.2330	0.2447
4	0.4570	0.2545	0.2455	0.2417	0.2550
5	0.5195	0.2845	0.2600	0.2450	0.2667
6	0.5750	0.3255	0.2760	0.2437	0.2800
7	0.6290	0.3740	0.2945	0.2443	0.2937
8	0.6650	0.4310	0.3110	0.2433	0.3080
9	0.7035	0.4935	0.3270	0.2413	0.3237
10	0.7385	0.5595	0.3445	0.2443	0.3383
11	0.7745	0.6165	0.3580	0.2410	0.3530
12	0.8065	0.7040	0.3685	0.2400	0.3670

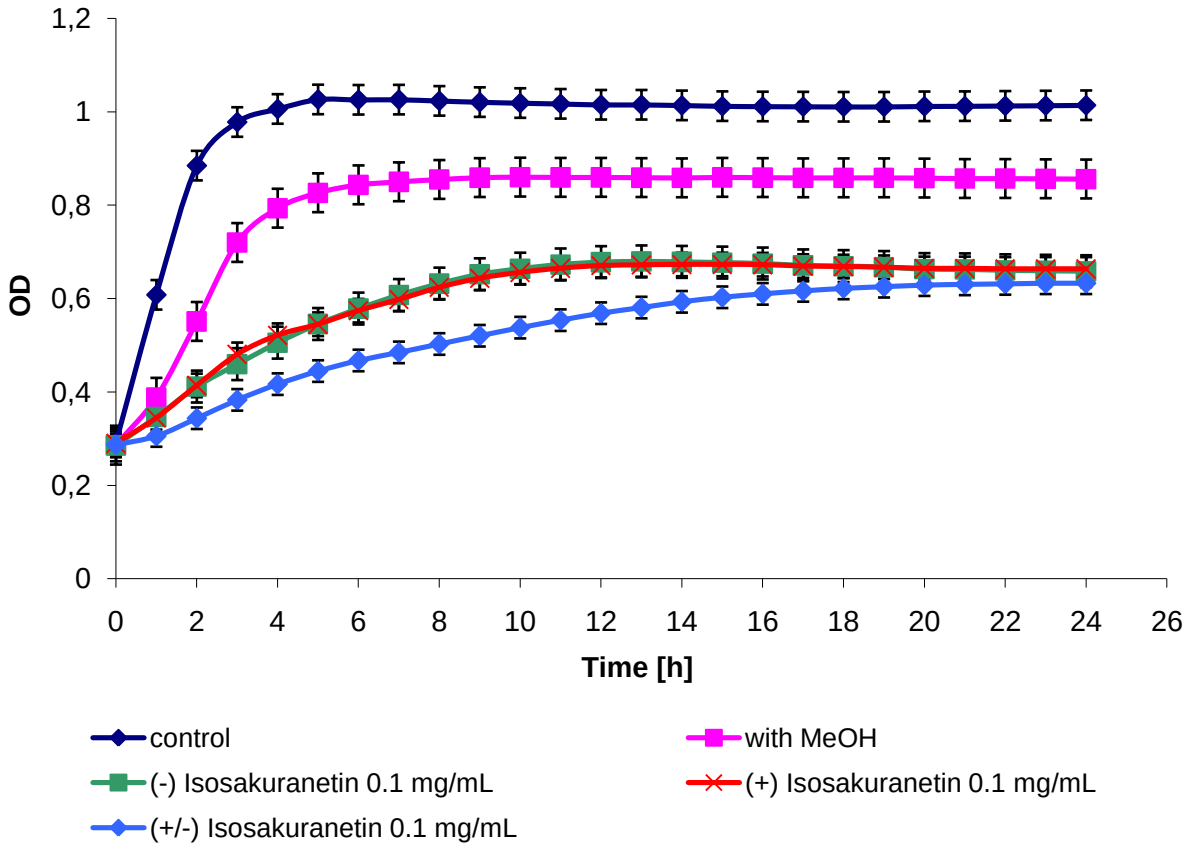
13	0.8335	0.7410	0.3850	0.2433	0.3800
14	0.8610	0.7685	0.3920	0.2393	0.3920
15	0.8875	0.8180	0.4040	0.2387	0.4037
16	0.9095	0.8655	0.4150	0.2403	0.4147
17	0.9155	0.8940	0.4145	0.2370	0.4270
18	0.9230	0.8745	0.4250	0.2377	0.4383
19	0.9310	0.8825	0.4220	0.2343	0.4500
20	0.9380	0.8965	0.4250	0.2333	0.4630
21	0.9360	0.9215	0.4120	0.2300	0.4743
22	0.9350	0.9585	0.4155	0.2323	0.4880
23	0.9355	0.9735	0.4150	0.2333	0.5040
24	0.9350	0.9740	0.4035	0.2317	0.5250
SD	0.01974242	0.09577054	0.02856711	0.06038452	0.01777264

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.1 mg/mL	(+) Isosakuranetin 0.1 mg/mL	(+/-) Isosakuranetin 0.1 mg/mL
0	0.2450	0.2293	0.2317	0.2270	0.2243
1	0.3597	0.2520	0.2370	0.2343	0.2440
2	0.5947	0.2923	0.2600	0.2543	0.2743
3	0.7997	0.3650	0.2857	0.2790	0.3017
4	0.8943	0.4773	0.3137	0.3077	0.3310
5	0.9343	0.5967	0.3460	0.3400	0.3650
6	0.9633	0.6613	0.3870	0.3770	0.4057
7	0.9733	0.6903	0.4363	0.4227	0.4577
8	0.9797	0.7093	0.4923	0.4747	0.5200
9	0.9833	0.7200	0.5523	0.5343	0.5893
10	0.9850	0.7300	0.6137	0.5963	0.6590
11	0.9853	0.7357	0.6677	0.6553	0.7210
12	0.9850	0.7407	0.7143	0.7090	0.7707
13	0.9853	0.7440	0.7520	0.7510	0.8130
14	0.9847	0.7440	0.7817	0.7857	0.8457
15	0.9847	0.7447	0.8057	0.8143	0.8727
16	0.9847	0.7447	0.8243	0.8357	0.8893
17	0.9833	0.7440	0.8387	0.8527	0.9023
18	0.9837	0.7440	0.8477	0.8640	0.9093
19	0.9820	0.7427	0.8537	0.8717	0.9127
20	0.9817	0.7423	0.8580	0.8757	0.9147
21	0.9800	0.7413	0.8597	0.8780	0.9150
22	0.9800	0.7410	0.8603	0.8790	0.9140
23	0.9797	0.7387	0.8593	0.8783	0.9120
24	0.9767	0.7387	0.8607	0.8777	0.9107
SD	0.05171719	0.01352508	0.03014472	0.02401512	0.0142547

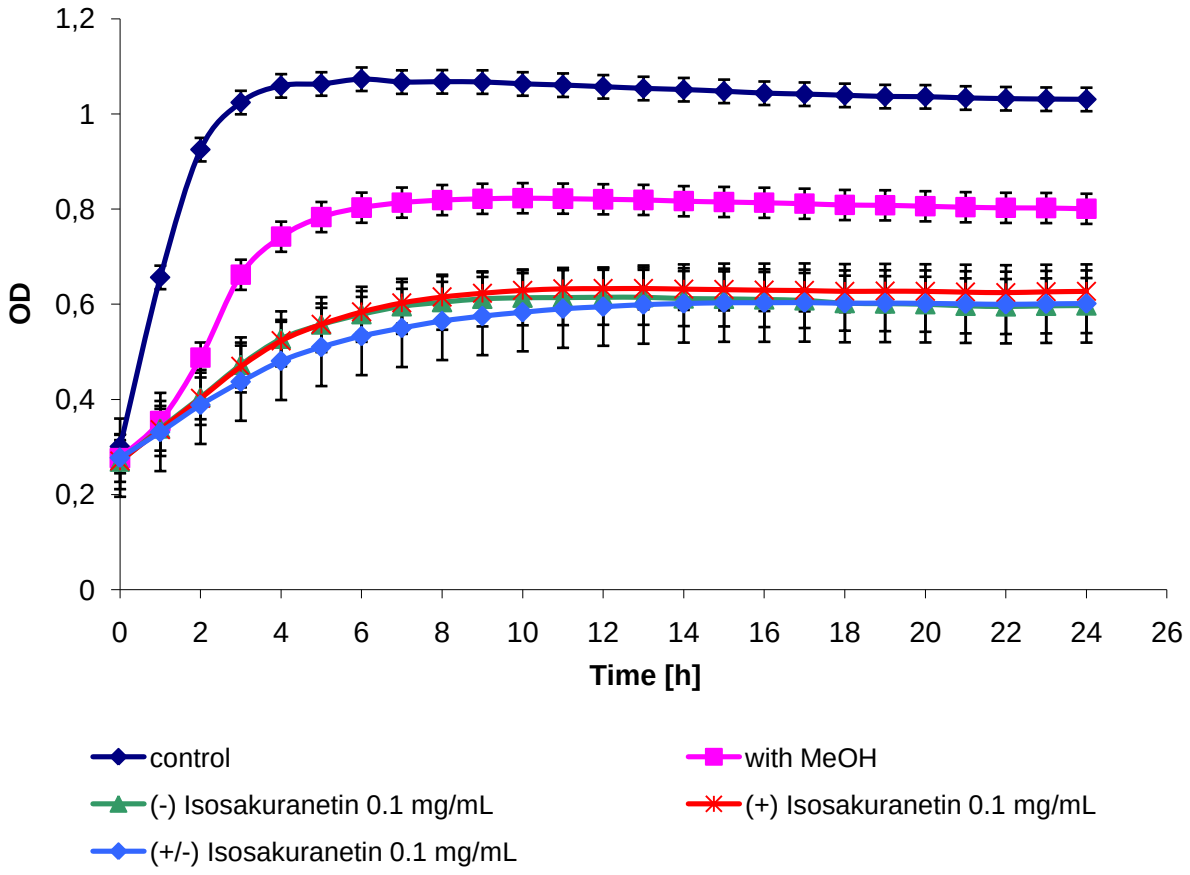
Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.1 mg/mL	(+) Isosakuranetin 0.1 mg/mL	(+/-) Isosakuranetin 0.1 mg/mL
0	0.2917	0.2860	0.2855	0.2900	0.2870
1	0.6080	0.3885	0.3465	0.3445	0.3057
2	0.8847	0.5510	0.4115	0.4140	0.3437
3	0.9780	0.7200	0.4595	0.4805	0.3830
4	1.0060	0.7935	0.5055	0.5215	0.4167
5	1.0263	0.8265	0.5455	0.5450	0.4447
6	1.0257	0.8435	0.5785	0.5740	0.4673
7	1.0260	0.8500	0.6075	0.5980	0.4847
8	1.0233	0.8550	0.6320	0.6240	0.5027
9	1.0207	0.8590	0.6520	0.6435	0.5203
10	1.0187	0.8600	0.6640	0.6560	0.5377
11	1.0170	0.8595	0.6730	0.6650	0.5537

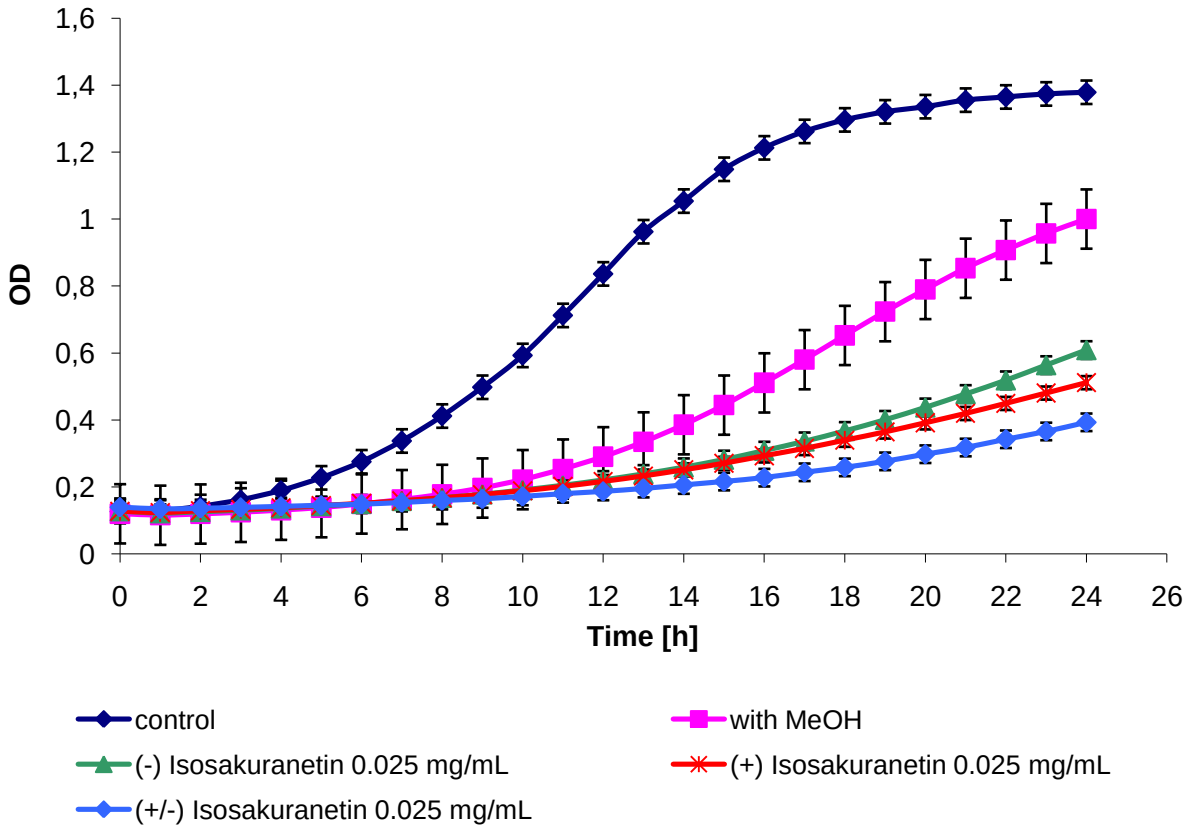
12	1.0150	0.8595	0.6780	0.6705	0.5687
13	1.0150	0.8590	0.6795	0.6720	0.5807
14	1.0137	0.8585	0.6785	0.6730	0.5930
15	1.0120	0.8595	0.6770	0.6730	0.6027
16	1.0113	0.8590	0.6750	0.6725	0.6100
17	1.0110	0.8585	0.6710	0.6695	0.6163
18	1.0107	0.8585	0.6695	0.6685	0.6217
19	1.0107	0.8585	0.6675	0.6670	0.6253
20	1.0117	0.8580	0.6630	0.6645	0.6287
21	1.0120	0.8570	0.6625	0.6645	0.6303
22	1.0127	0.8570	0.6605	0.6635	0.6313
23	1.0133	0.8565	0.6595	0.6635	0.6327
24	1.0140	0.8560	0.6585	0.6635	0.6327
SD	0.03162462	0.04157788	0.02056267	0.04338807	0.09017026

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.



Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.1 mg/mL	(+) Isosakuranetin 0.1 mg/mL	(+/-) Isosakuranetin 0.1 mg/mL
0	0.3010	0.2767	0.2693	0.2707	0.2777
1	0.6565	0.3547	0.3390	0.3363	0.3317
2	0.9250	0.4880	0.4043	0.4023	0.3887
3	1.0240	0.6620	0.4727	0.4693	0.4373
4	1.0590	0.7420	0.5273	0.5233	0.4810
5	1.0630	0.7833	0.5573	0.5580	0.5103
6	1.0730	0.8030	0.5790	0.5840	0.5333
7	1.0670	0.8137	0.5957	0.6030	0.5503
8	1.0675	0.8190	0.6043	0.6153	0.5650
9	1.0670	0.8217	0.6113	0.6233	0.5753
10	1.0630	0.8230	0.6137	0.6293	0.5833
11	1.0605	0.8220	0.6140	0.6327	0.5907
12	1.0570	0.8207	0.6147	0.6330	0.5950
13	1.0535	0.8193	0.6147	0.6333	0.5993
14	1.0510	0.8167	0.6120	0.6320	0.6017
15	1.0475	0.8150	0.6113	0.6310	0.6033
16	1.0435	0.8133	0.6100	0.6297	0.6033
17	1.0415	0.8113	0.6080	0.6290	0.6037
18	1.0390	0.8087	0.6023	0.6273	0.6023
19	1.0365	0.8080	0.6013	0.6277	0.6027
20	1.0360	0.8060	0.6000	0.6273	0.6020
21	1.0335	0.8040	0.5967	0.6257	0.6010
22	1.0320	0.8027	0.5953	0.6247	0.6000
23	1.0310	0.8023	0.5967	0.6263	0.6010
24	1.0305	0.8007	0.5973	0.6273	0.6017
SD	0.02472045	0.03170895	0.05781438	0.04385613	0.08218591

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of isosakuranetin racemate and its enantiomers; OD – optical density, SD – standard deviation.

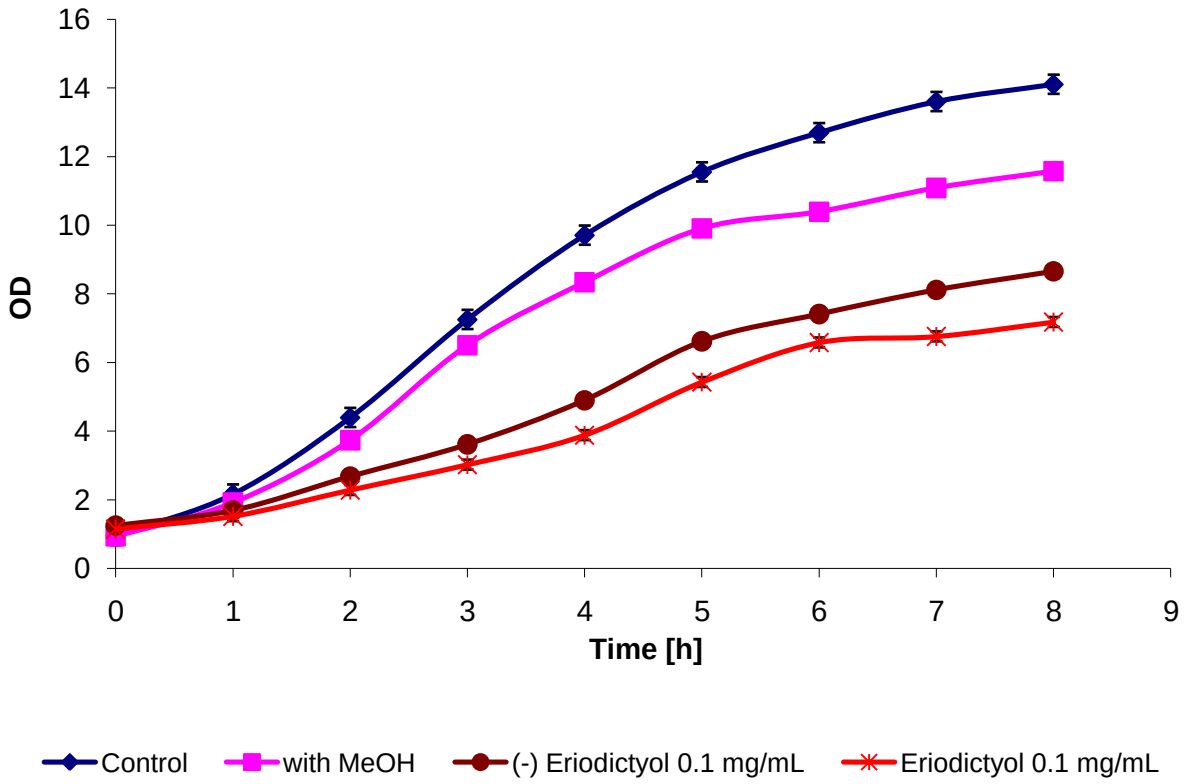


Time [h]	OD				
	Control	MeOH	(-) Isosakuranetin 0.025 mg/mL	(+) Isosakuranetin 0.025 mg/mL	(+/-) Isosakuranetin 0.025 mg/mL
0	0.1265	0.1200	0.1280	0.1273	0.1397
1	0.1285	0.1157	0.1220	0.1233	0.1343
2	0.1420	0.1193	0.1260	0.1287	0.1360
3	0.1615	0.1243	0.1307	0.1327	0.1387
4	0.1895	0.1307	0.1363	0.1377	0.1417
5	0.2275	0.1383	0.1433	0.1443	0.1450
6	0.2755	0.1493	0.1500	0.1507	0.1483
7	0.3375	0.1623	0.1587	0.1583	0.1533
8	0.4120	0.1780	0.1680	0.1673	0.1593
9	0.4980	0.1970	0.1783	0.1770	0.1643
10	0.5930	0.2220	0.1910	0.1883	0.1717
11	0.7125	0.2537	0.2050	0.2013	0.1797
12	0.8365	0.2903	0.2210	0.2163	0.1867
13	0.9625	0.3347	0.2390	0.2327	0.1953

14	1.0540	0.3860	0.2590	0.2510	0.2060
15	1.1490	0.4447	0.2820	0.2707	0.2163
16	1.2130	0.5110	0.3087	0.2930	0.2280
17	1.2620	0.5803	0.3363	0.3150	0.2440
18	1.2965	0.6527	0.3673	0.3397	0.2587
19	1.3205	0.7237	0.4007	0.3643	0.2767
20	1.3360	0.7900	0.4377	0.3917	0.2980
21	1.3555	0.8533	0.4777	0.4197	0.3180
22	1.3650	0.9077	0.5187	0.4497	0.3427
23	1.3740	0.9573	0.5640	0.4807	0.3660
24	1.3790	1.0003	0.6090	0.5117	0.3933
SD	0.03504421	0.0884909	0.02641592	0.01958191	0.02620772

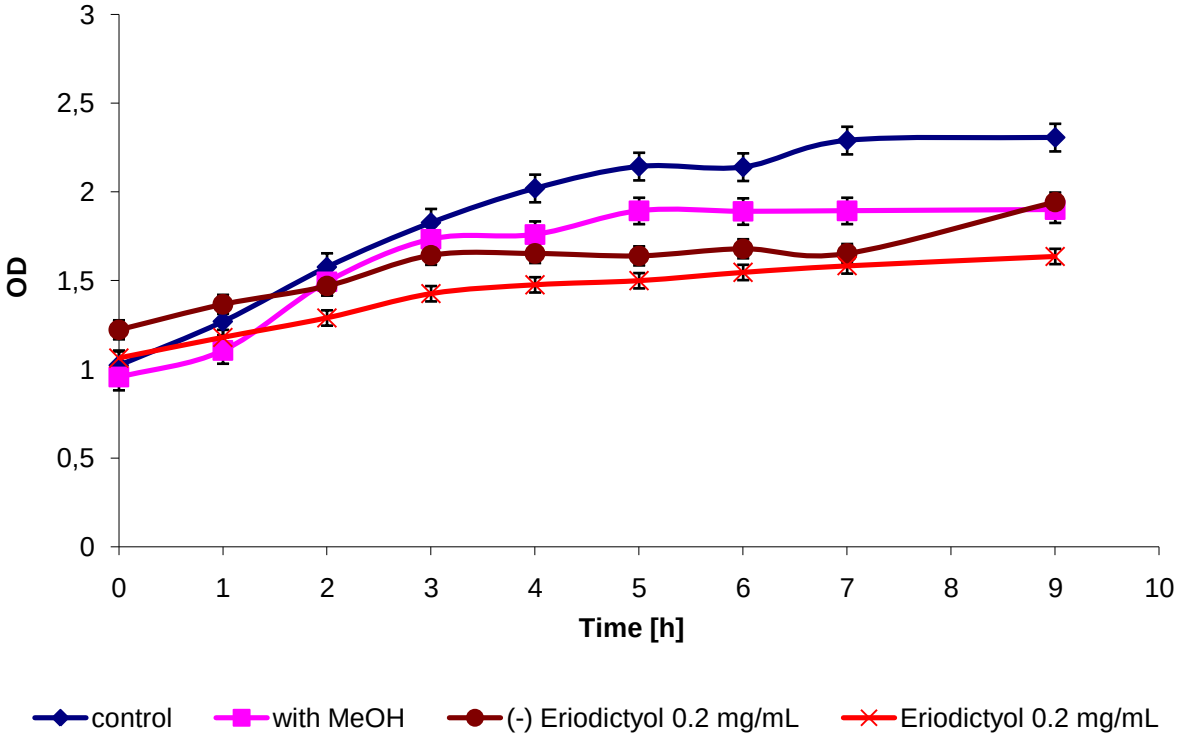
Eriodictyol

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



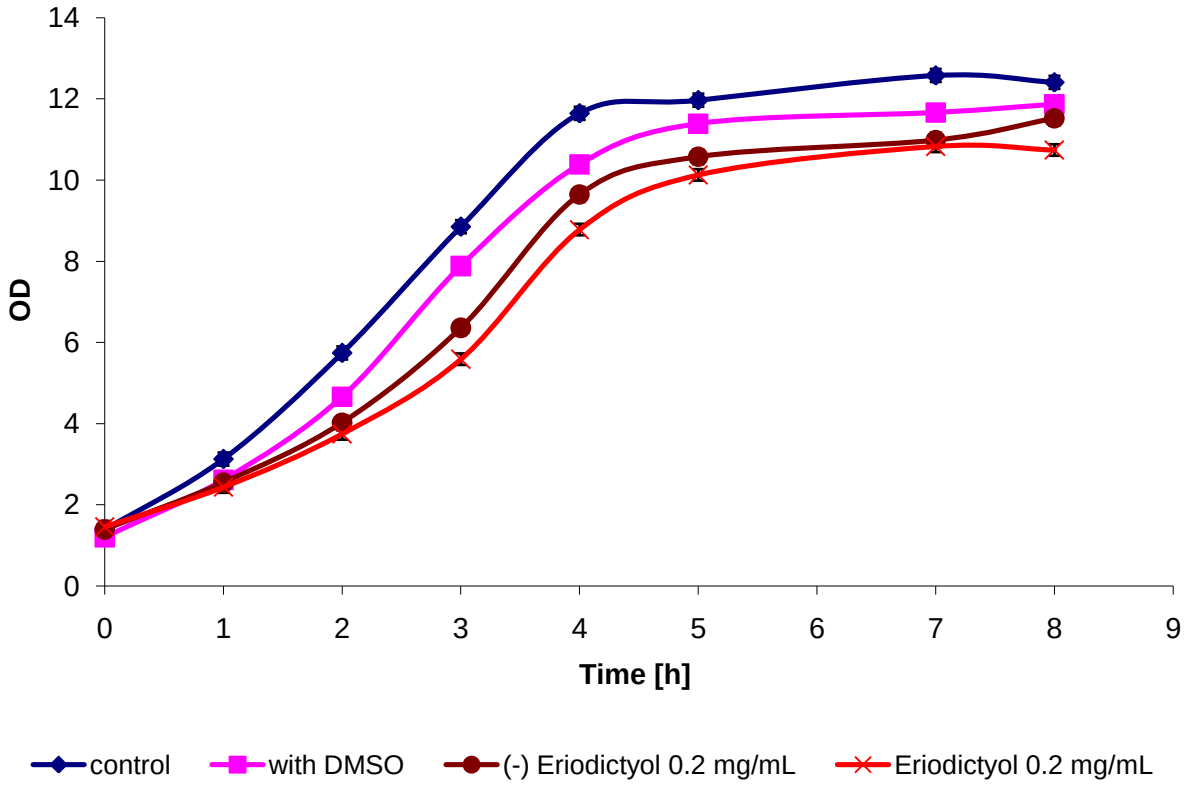
Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.1 mg/mL	(+/–) Eriodictyol 0.1 mg/mL
0	0.95	0.94	1.25	1.15
1	2.16	1.92	1.69	1.52
2	4.39	3.74	2.68	2.28
3	7.25	6.50	3.61	3.02
4	9.71	8.34	4.90	3.88
5	11.55	9.90	6.62	5.43
6	12.69	10.39	7.41	6.58
7	13.60	11.09	8.12	6.76
8	14.10	11.57	8.66	7.18
SD	0.27951377	0.13780487	0.17633222	0.14403671

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



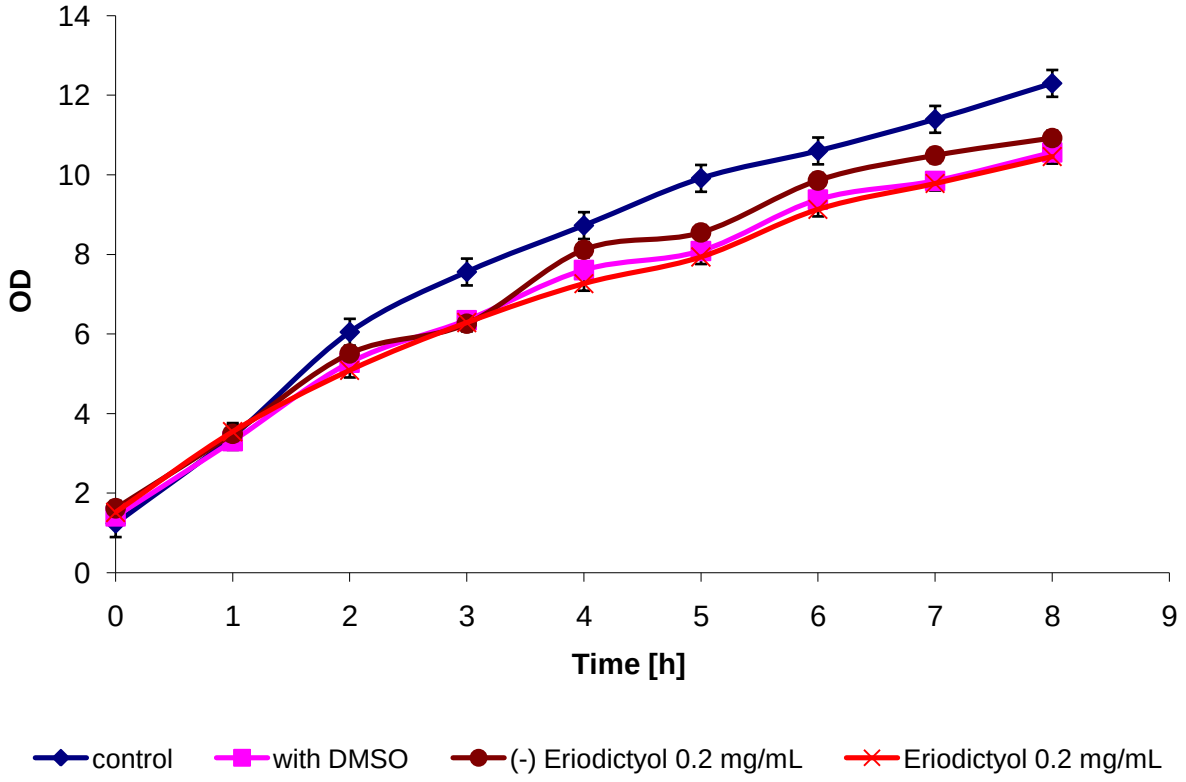
Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	1.02	0.96	1.22	1.06
1	1.27	1.11	1.37	1.18
2	1.58	1.49	1.47	1.29
3	1.83	1.73	1.64	1.43
4	2.02	1.76	1.65	1.48
5	2.14	1.89	1.64	1.50
6	2.14	1.89	1.68	1.55
7	2.29	1.89	1.65	1.58
9	2.31	1.90	1.94	1.64
SD	0.07790883	0.07439556	0.05371221	0.04317725

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



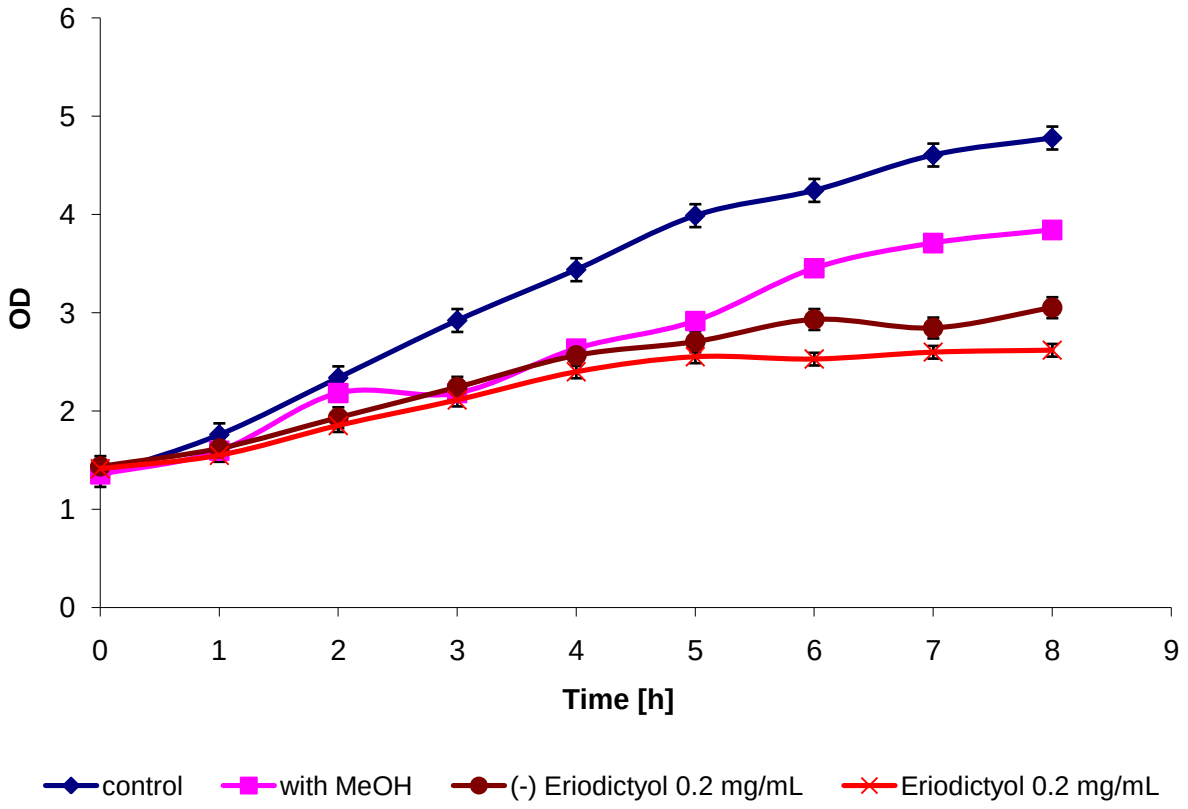
Time [h]	OD			
	Control	DMSO	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	1.39	1.20	1.39	1.46
1	3.13	2.61	2.55	2.44
2	5.74	4.66	4.02	3.75
3	8.85	7.88	6.36	5.59
4	11.64	10.39	9.64	8.78
5	11.97	11.39	10.57	10.13
6	11.37	10.44	10.20	9.72
7	12.58	11.66	10.98	10.83
8	12.41	11.87	11.52	10.74
SD	0.16110183	0.14637837	0.18080789	0.14140312

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



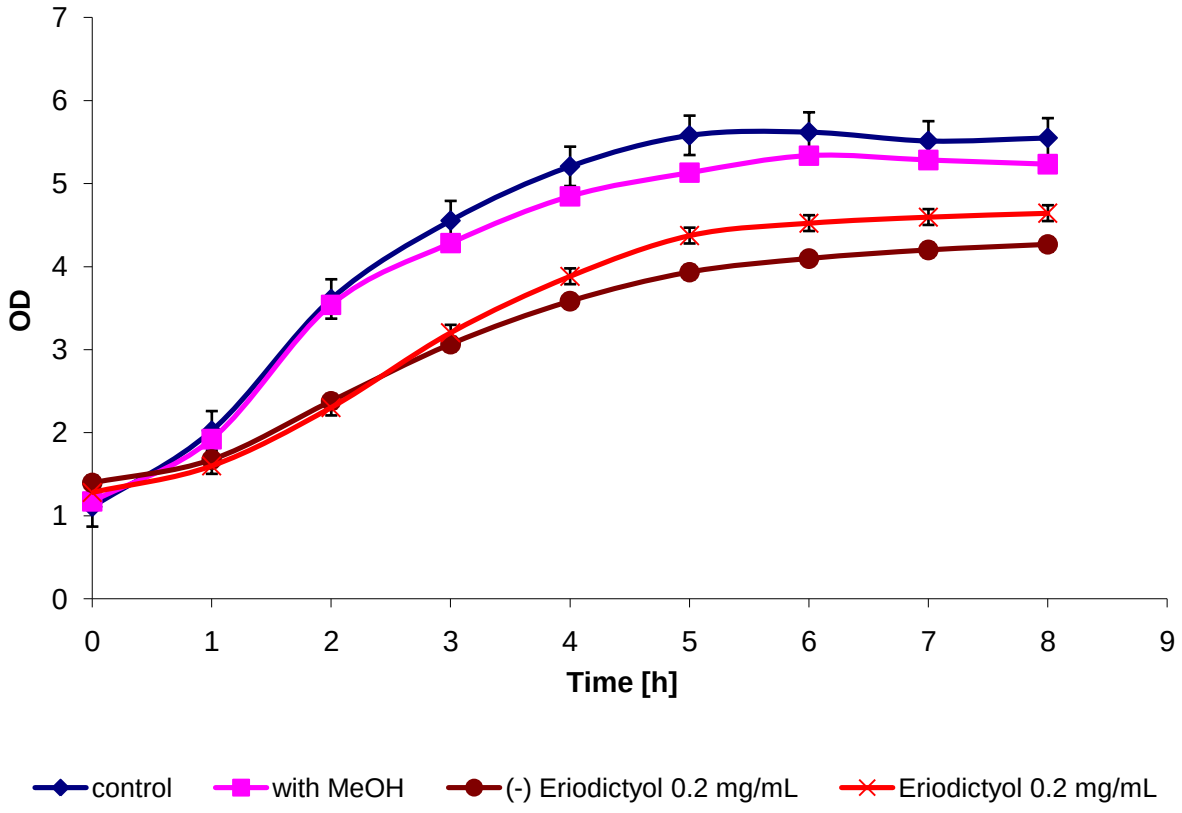
Time [h]	OD			
	Control	DMSO	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	1.24	1.41	1.62	1.52
1	3.43	3.31	3.49	3.54
2	6.05	5.28	5.51	5.09
3	7.56	6.35	6.26	6.29
4	8.73	7.61	8.12	7.27
5	9.91	8.09	8.55	7.94
6	10.60	9.38	9.86	9.13
7	11.40	9.85	10.49	9.78
8	12.30	10.56	10.92	10.46
SD	0.33715781	0.16619333	0.18723072	0.17614307

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



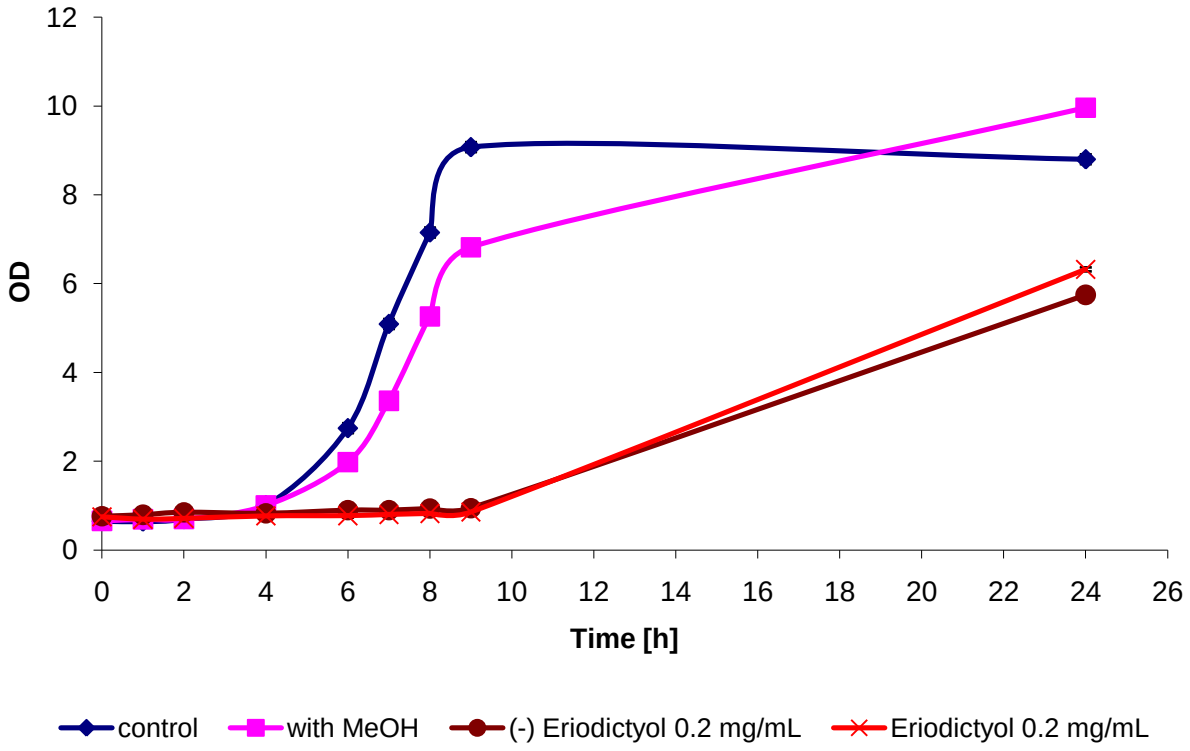
Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	1.35	1.36	1.44	1.41
1	1.76	1.60	1.62	1.55
2	2.34	2.18	1.93	1.85
3	2.92	2.18	2.24	2.11
4	3.44	2.64	2.57	2.40
5	3.99	2.92	2.71	2.55
6	4.25	3.45	2.93	2.53
7	4.61	3.71	2.85	2.60
8	4.78	3.84	3.05	2.62
SD	0.11657707	0.07600719	0.10635469	0.06442774

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



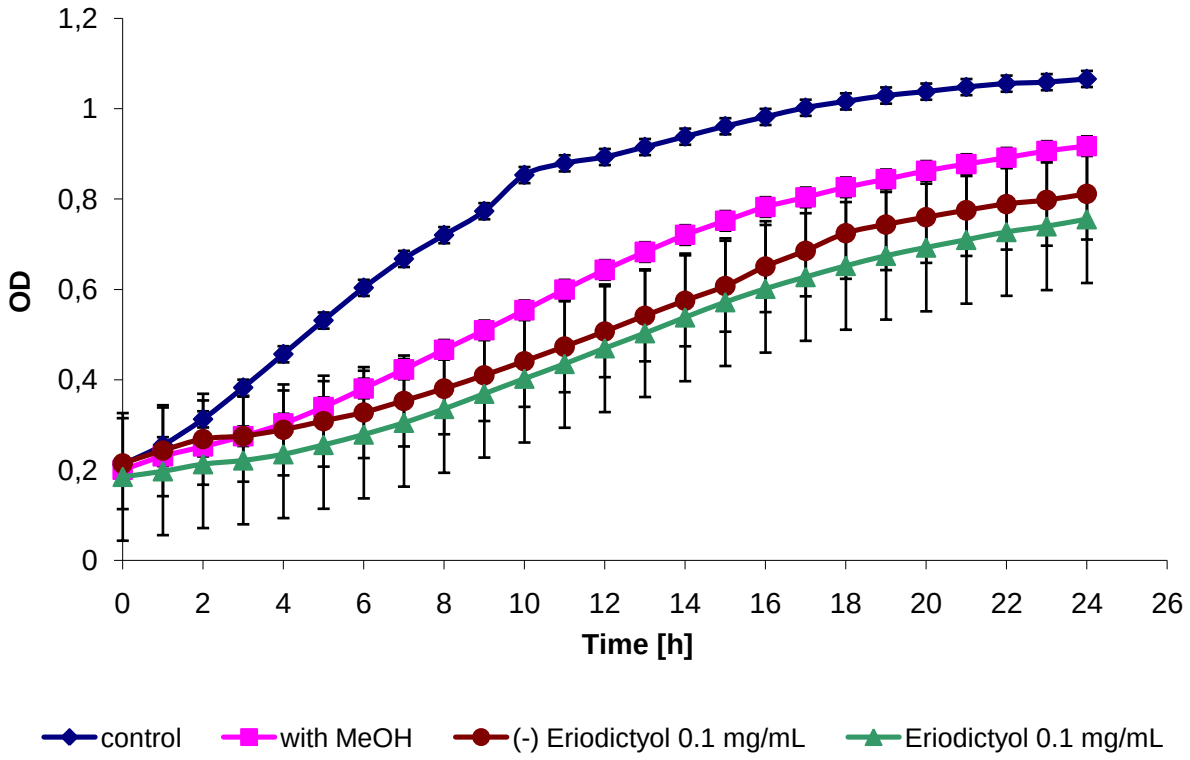
Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	1.11	1.17	1.40	1.28
1	2.02	1.92	1.68	1.60
2	3.61	3.54	2.38	2.30
3	4.55	4.28	3.07	3.20
4	5.21	4.85	3.58	3.88
5	5.58	5.13	3.93	4.37
6	5.62	5.34	4.10	4.52
7	5.51	5.28	4.20	4.60
8	5.55	5.23	4.27	4.64
SD	0.23713693	0.08796835	0.0847206	0.09513479

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.2 mg/mL	(+/–) Eriodictyol 0.2 mg/mL
0	0.65	0.66	0.76	0.75
1	0.64	0.69	0.79	0.69
2	0.69	0.70	0.85	0.71
4	1.01	1.00	0.83	0.77
6	2.74	1.98	0.90	0.77
7	5.09	3.36	0.90	0.80
8	7.15	5.26	0.93	0.82
9	9.07	6.82	0.94	0.85
24	8.80	9.96	5.75	6.32
SD	0.11157338	0.09769516	0.03974738	0.04471735

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of eriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.

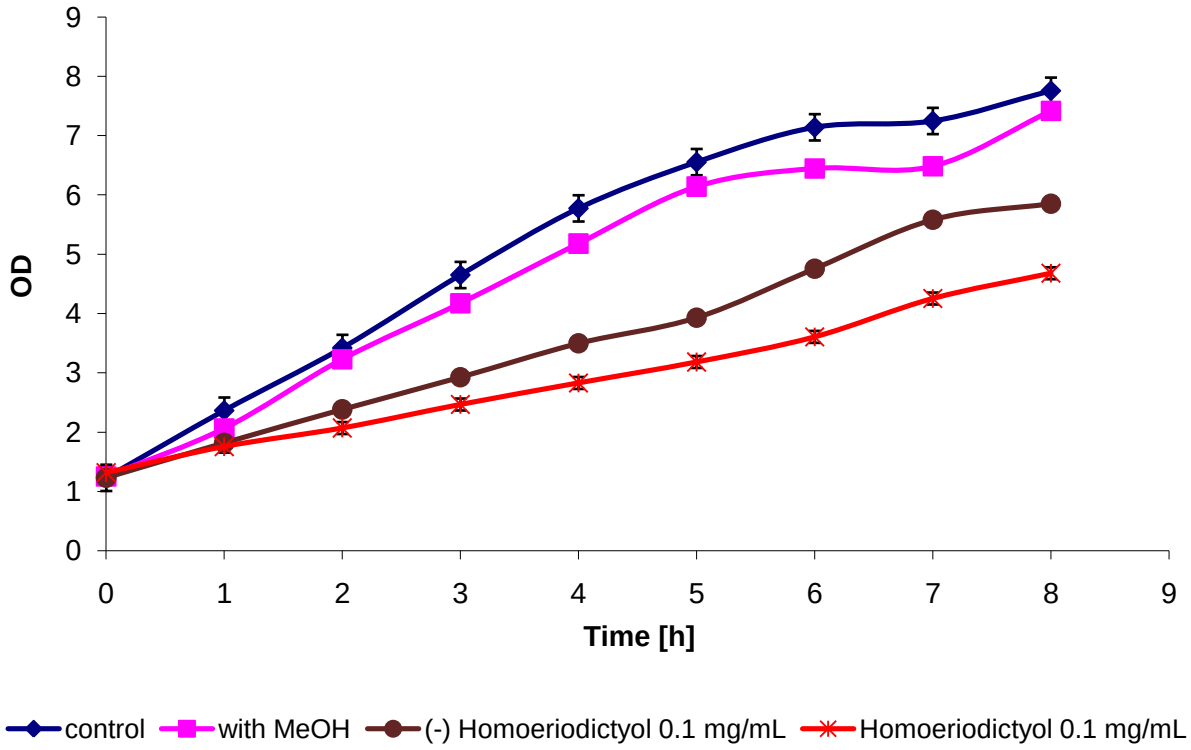


Time [h]	OD			
	Control	MeOH	(–) Eriodictyol 0.1 mg/mL	(+/–) Eriodictyol 0.1 mg/mL
0	0.2127	0.2010	0.2147	0.1853
1	0.2553	0.2310	0.2433	0.1977
2	0.3127	0.2523	0.2687	0.2133
3	0.3827	0.2753	0.2753	0.2217
4	0.4567	0.3033	0.2893	0.2353
5	0.5313	0.3397	0.3087	0.2560
6	0.6037	0.3810	0.3277	0.2790
7	0.6677	0.4230	0.3533	0.3050
8	0.7203	0.4667	0.3803	0.3357
9	0.7733	0.5097	0.4097	0.3693
10	0.8533	0.5540	0.4410	0.4027
11	0.8793	0.5997	0.4733	0.4353
12	0.8933	0.6433	0.5067	0.4700
13	0.9153	0.6830	0.5417	0.5033
14	0.9383	0.7207	0.5750	0.5383
15	0.9613	0.7520	0.6073	0.5720

16	0.9820	0.7827	0.6507	0.6017
17	1.0023	0.8040	0.6857	0.6277
18	1.0163	0.8263	0.7243	0.6523
19	1.0293	0.8440	0.7437	0.6747
20	1.0380	0.8627	0.7597	0.6930
21	1.0480	0.8777	0.7750	0.7100
22	1.0557	0.8917	0.7890	0.7273
23	1.0590	0.9067	0.7977	0.7400
24	1.0660	0.9173	0.8113	0.7557
SD	0.01786773	0.02171292	0.10063986	0.14125995

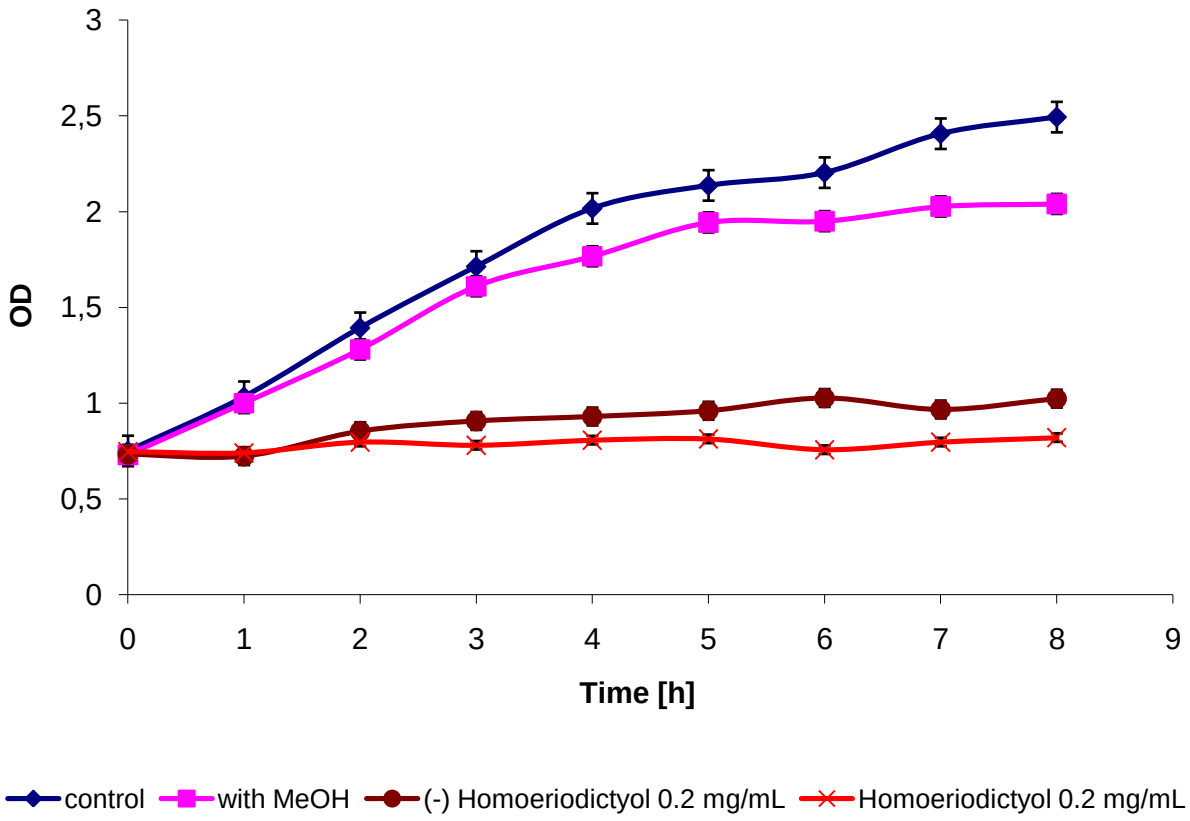
Homoeriodictyol

Growth curve of *Bacillus subtilis* ATCC 6633 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



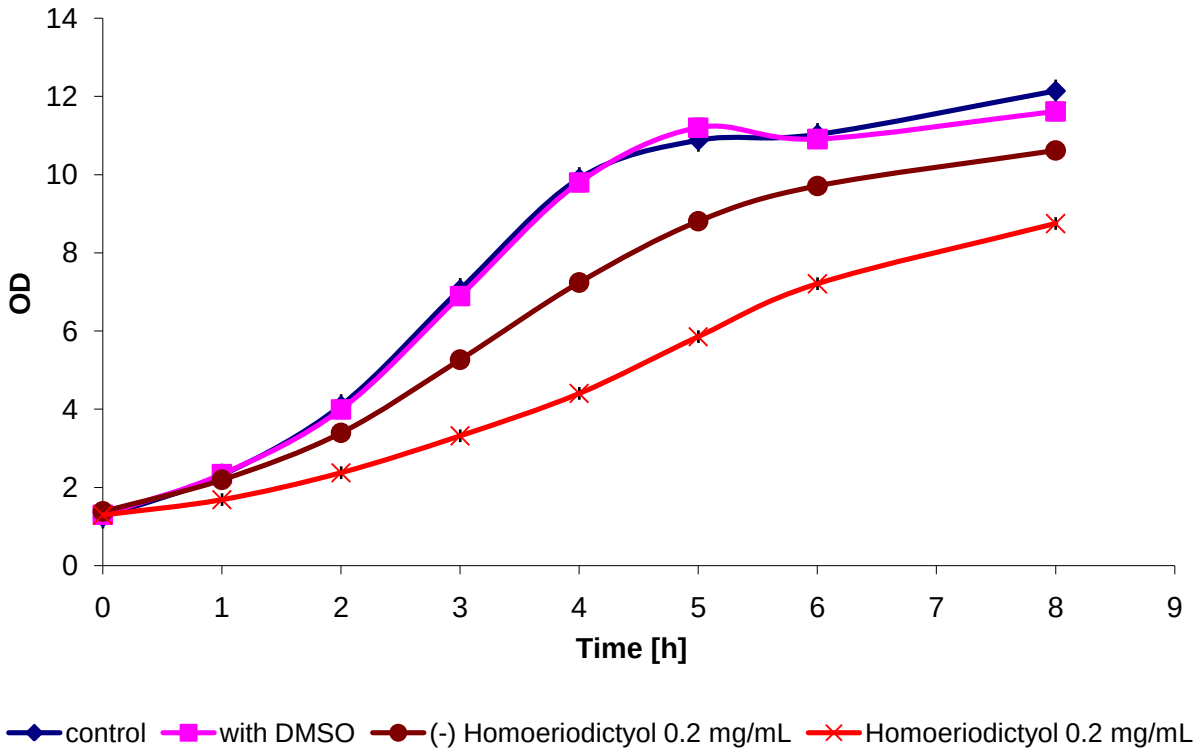
Time [h]	OD			
	Control	MeOH	(–) Homoeriodictyol 0.1 mg/mL	(+/–) Homoeriodictyol 0.1 mg/mL
0	1.23	1.25	1.23	1.32
1	2.36	2.06	1.82	1.76
2	3.42	3.23	2.38	2.07
3	4.65	4.17	2.93	2.47
4	5.77	5.18	3.50	2.83
5	6.55	6.14	3.93	3.18
6	7.14	6.45	4.76	3.61
7	7.25	6.48	5.58	4.25
8	7.76	7.42	5.85	4.68
SD	0.22157648	0.12373654	0.09471159	0.10078876

Growth curve of *Micrococcus luteus* ATCC 10240 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



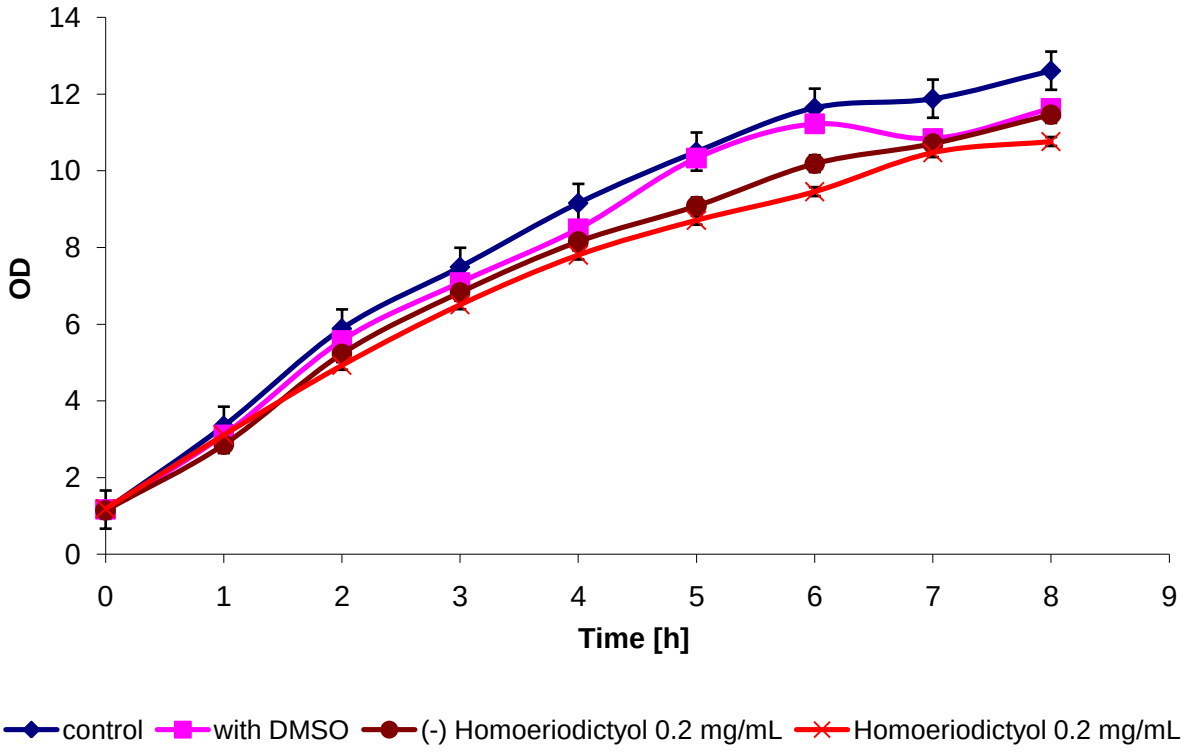
Time [h]	OD			
	Control	MeOH	(–) Homoeriodictyol 0.2 mg/mL	(+/–) Homoeriodictyol 0.2 mg/mL
0	0.75	0.73	0.73	0.75
1	1.03	1.00	0.72	0.74
2	1.39	1.28	0.85	0.80
3	1.71	1.61	0.91	0.78
4	2.02	1.77	0.93	0.81
5	2.14	1.94	0.96	0.81
6	2.20	1.95	1.03	0.76
7	2.41	2.03	0.97	0.80
8	2.49	2.04	1.02	0.82
SD	0.07961781	0.05228394	0.04662076	0.0222552

Growth curve of *Corynebacterium glutamicum* ATCC 13032 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



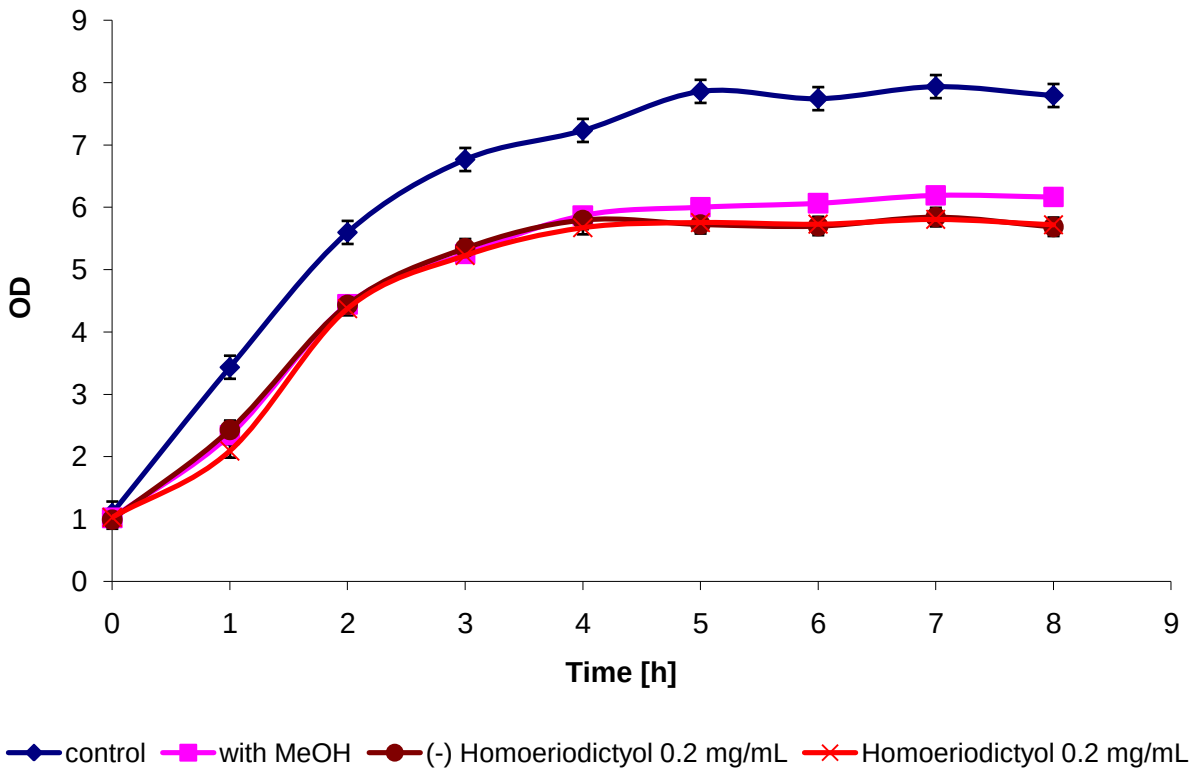
Time [h]	OD			
	Control	DMSO	(–) Homoeriodictyol 0.2 mg/mL	(+/–) Homoeriodictyol 0.2 mg/mL
0	1.24	1.30	1.39	1.29
1	2.31	2.34	2.20	1.69
2	4.09	4.00	3.40	2.38
3	7.06	6.90	5.27	3.32
4	9.90	9.80	7.24	4.40
5	10.88	11.20	8.81	5.86
6	11.03	10.91	9.71	7.21
8	12.14	11.62	10.62	8.75
SD	0.29079465	0.21741951	0.15174544	0.16190746

Growth curve of *Escherichia coli* ATCC 23716 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



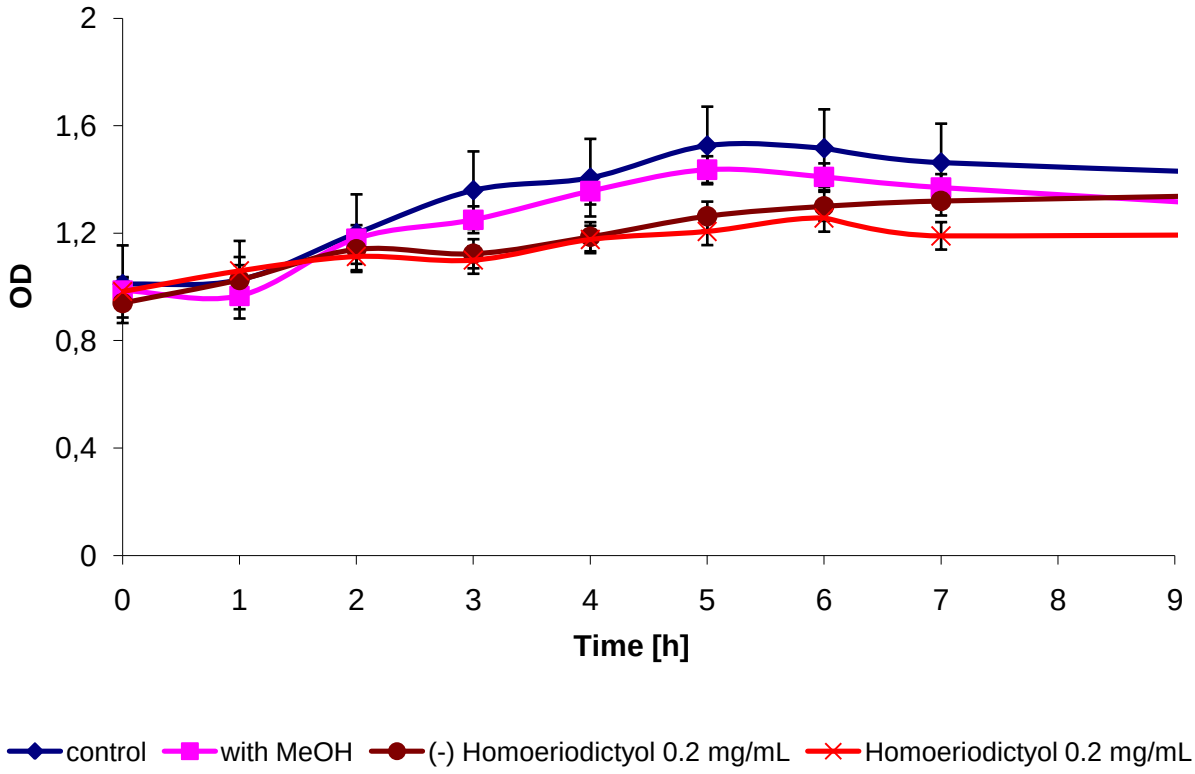
Time [h]	OD			
	Control	DMSO	(–) Homoeriodictyol 0.2 mg/mL	(+/–) Homoeriodictyol 0.2 mg/mL
0	1.16	1.17	1.14	1.19
1	3.35	3.11	2.86	3.11
2	5.89	5.58	5.23	4.93
3	7.49	7.09	6.83	6.51
4	9.16	8.49	8.15	7.80
5	10.50	10.33	9.08	8.71
6	11.64	11.23	10.19	9.46
7	11.88	10.85	10.71	10.47
8	12.61	11.63	11.47	10.76
SD	0.49803199	0.19264049	0.18429411	0.11667008

Growth curve of *Escherichia coli* ATCC 25922 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



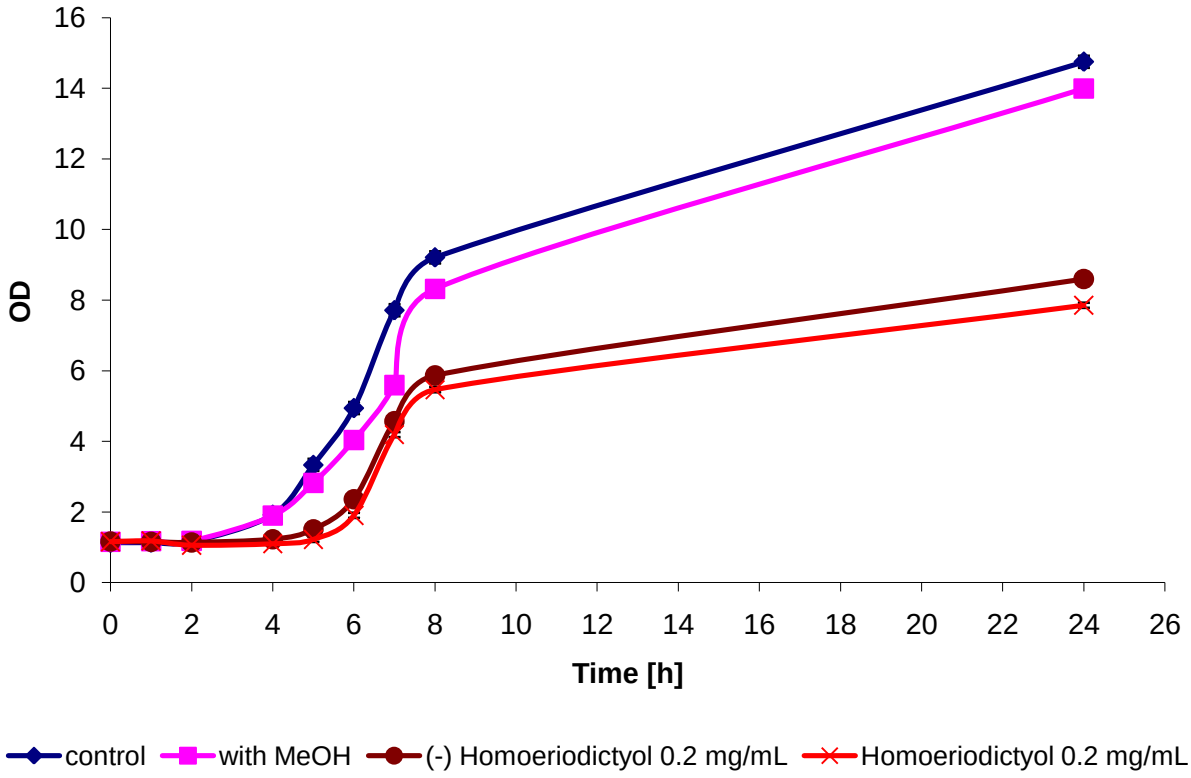
Time [h]	OD			
	Control	MeOH	(–) Homoeriodictyol 0.2 mg/mL	(+/–) Homoeriodictyol 0.2 mg/mL
0	1.09	1.02	0.99	1.02
1	3.43	2.35	2.43	2.09
2	5.60	4.44	4.44	4.37
3	6.77	5.25	5.34	5.23
4	7.23	5.87	5.80	5.67
5	7.86	6.00	5.73	5.76
6	7.74	6.07	5.70	5.73
7	7.94	6.19	5.84	5.81
8	7.79	6.17	5.69	5.72
SD	0.18522006	0.11445771	0.14823875	0.10739067

Growth curve of *Enterococcus faecalis* ATCC 19433 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



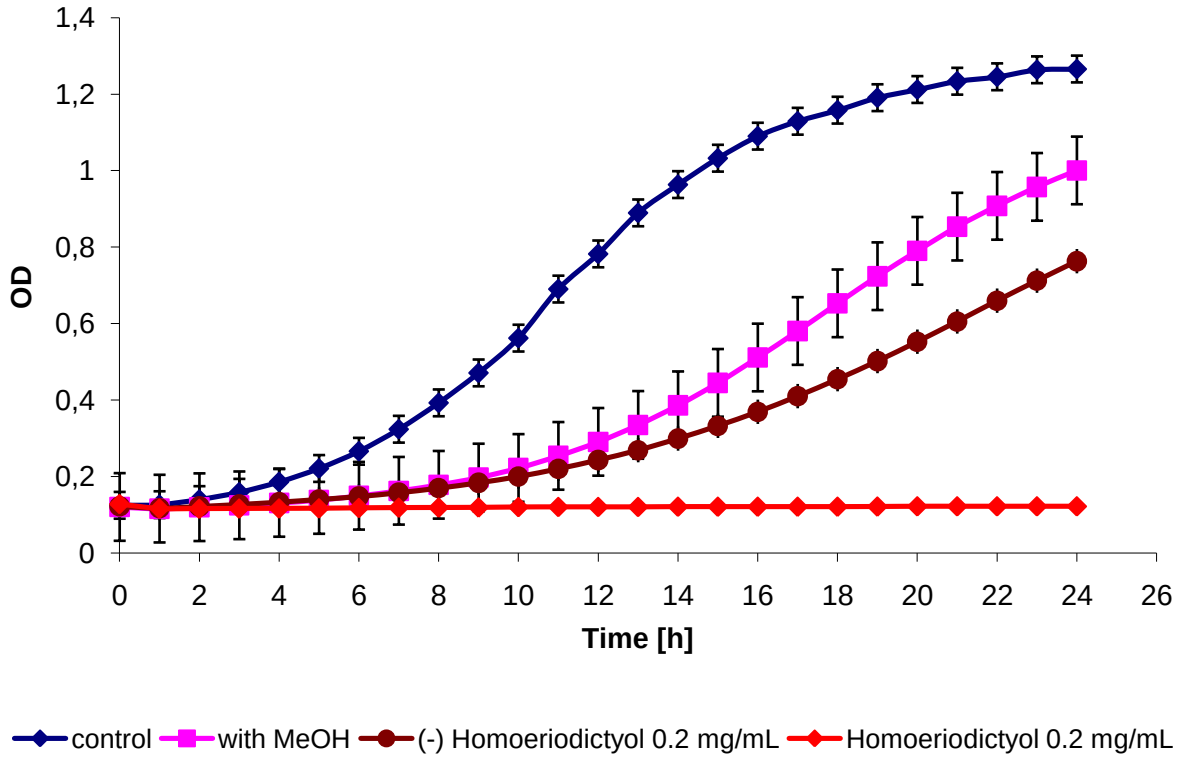
Time [h]	OD			
	Control	MeOH	(–) Homoeriodictyol 0.1 mg/mL	(+/–) Homoeriodictyol 0.1 mg/mL
0	1.32	1.31	1.35	1.38
1	1.42	1.35	1.52	1.47
2	1.47	1.44	1.52	1.55
3	1.56	1.49	1.55	1.56
4	1.67	1.59	1.57	1.59
5	1.86	1.67	1.57	1.63
6	1.99	1.77	1.61	1.70
7	2.11	1.79	1.62	1.65
8	2.28	1.85	1.67	1.66
SD	0.09303908	0.04113348	0.07829021	0.04293357

Growth curve of *Pseudomonas aeruginosa* ATCC 10145 on BHI medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



Time [h]	OD			
	Control	MeOH	(–) Homoeriodictyol 0.2 mg/mL	(+/–) Homoeriodictyol 0.2 mg/mL
0	1.13	1.15	1.17	1.16
1	1.13	1.17	1.15	1.18
2	1.16	1.18	1.14	1.06
4	1.92	1.90	1.23	1.10
5	3.33	2.82	1.51	1.22
6	4.94	4.04	2.36	1.90
7	7.71	5.59	4.57	4.19
8	9.21	8.32	5.87	5.46
24	14.75	14.00	8.60	7.85
SD	0.16959777	0.08859591	0.06878271	0.07262862

Growth curve of *Saccharomyces pasteurianus* on YNB medium with inhibitory effect of homoeriodictyol racemate and its (–) enantiomer; OD – optical density, SD – standard deviation.



Time [h]	OD			
	Control	MeOH	(-) Homoeriodictyol 0.2 mg/mL	(+/-) Homoeriodictyol 0.2 mg/mL
0	0.1243	0.1200	0.1217	0.1280
1	0.1260	0.1157	0.1170	0.1160
2	0.1393	0.1193	0.1213	0.1157
3	0.1583	0.1243	0.1267	0.1160
4	0.1853	0.1307	0.1327	0.1163
5	0.2207	0.1383	0.1397	0.1167
6	0.2657	0.1493	0.1473	0.1177
7	0.3233	0.1623	0.1573	0.1183
8	0.3923	0.1780	0.1693	0.1187
9	0.4707	0.1970	0.1833	0.1190
10	0.5617	0.2220	0.1997	0.1200
11	0.6900	0.2537	0.2200	0.1203
12	0.7820	0.2903	0.2430	0.1203
13	0.8893	0.3347	0.2687	0.1203

14	0.9633	0.3860	0.2990	0.1210
15	1.0323	0.4447	0.3323	0.1210
16	1.0900	0.5110	0.3690	0.1210
17	1.1290	0.5803	0.4100	0.1210
18	1.1580	0.6527	0.4543	0.1210
19	1.1907	0.7237	0.5017	0.1213
20	1.2120	0.7900	0.5523	0.1220
21	1.2337	0.8533	0.6053	0.1220
22	1.2453	0.9077	0.6597	0.1220
23	1.2637	0.9573	0.7120	0.1220
24	1.2657	1.0003	0.7630	0.1220
SD	0.11461544	0.0884909	0.03184472	0.00331632