KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

**Chandramohan Radhakrishnan**

# ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF NATURAL AND LAB TREATED *ASTRAGALUS GLYCYPHYLLOS* ISOLATED BY USING DIFFERENT METHODS

**Master's Degree Final Project**

**Supervisor**
Prof. Habil. Dr. Grazina Juodeikiene

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**Food Science and Safety (code 621E40001)**

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KAUNO TECHNOLOGIJOS UNIVERSITETAS
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# NATŪRALIŲ IR LAB APDOROTŲ *ASTRAGALUS GLYCYPHYLLOS* EKTRAKTŲ ANTIOKSIDACINĖS IR ANTIMIKROBINĖS SAVYBĖS

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"Antioxidant and Antimicrobial Activities of Natural and LAB Treated *Astragalus Glycyphyllos* Isolated by Using Different Methods "

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## SUMMARY

Liquor ice milkvetch *(Astragalus glycyphyllos, Fabaceae)* leaves have traditionally been used for medicinal purposes and sometimes for tea. It contains various bioactive compounds. The aim of this study was to evaluate the antioxidant activity of *Astragalus glycyphyllos* using different polarity solvents. It was grounded to 0.2 mm particle size fractions and extracted using solvents such as hexane, acetone, ethanol/water (70:30 %) in Soxhlet extraction, supercritical fluid extraction (SFE-$CO_2$) and pressurized liquid extraction (PLE) acetone, ethanol/water (70:30). The highest extract yield was obtained using ethanol-water as solvent and has a significant effect in most of cases. Total content of phenolic compounds (TPC) was measured by UV spectrophotometer using Folin-Ciocalteu reagent. The antioxidant capacity of extracts was determined by DPPH, ABTS, FRAP and ORAC assays. In general, the Ethanol/Water extracts possessed significantly higher antioxidant capacity and total phenolic content when compared to other solvents. The QUENCHER method was also employed to evaluate the antioxidant capacity (ABTS, ORAC and TPC) of un-extracted plant Material and (SFE-$CO_2$) residues after extraction. The un-extracted plant material possessed the highest total Phenolic Content, ABTS and ORAC with more effective antioxidant activity compared to residue obtained after SCE-$CO_2$ extraction (TPC, ABTS and ORAC). Characterization of polyphenolic compounds presented in the Soxhlet and SFE-$CO_2$ extracts were executed by the spectral data comparison obtained by UPLC/QTOF. The compounds were identified using different extracts: 12-oxooctadecanoic acid, m-coumaric acid, rutin and linoleic acid.

The second part of the experiment was dedicated to evaluate the antimicrobial activities of *Astragalus glycyphyllos* leaves extracts, obtained using Soxhlet extraction and different solvents (ethanol/water (70:30); acetone and hexane). Additional, the effect of plant leaves bio-treatment using *L. sakei* under solid state fermentation (SSF) conditions with the following extraction using different solvents has been studied. As control the leaves powder with/without treatment has been used. For this task, different extracts, LAB treated and extracted *Astragalus glycyphyllos* leaves were tested against five various bacteria (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis, Bacillus subtilis subspecies spizizenii)* and nine fungi *(Debaryomyces vanrijiae, Geotrichum fermentants, Candida pelliculosa, Pichia farinose, Pichia fermentans, Candida krusei, Kluyveromyces marxianus, Kluyveromyces lactis var lactis, Aureobasidum pullulans*).

The highest antibacterial activity showed extracts against *Bacillus subtilis* in the following order: ethanol/water (70:30) > acetone > hexane and antifungal - against *Debaryomyces vanrijiae* followed by: acetone > hexane > ethanol/water (70:30). *L. sakei* fermented in solid-state conditions (50 %) *Astragalus glycyphyllos* dry powder and extracted showed a wider zone of antibacterial inhibition against *Bacillus subtilis* as well as antifungal - against *Debaryomyces vanrijiae* in compare with non-fermented plant raw materials.

Thus, results showed, what *Astragalus glycyphyllos* leaves is a valuable source of antioxidants and their extracts and especially leaves after bio-treatment demonstrated antimicrobial activities; it confirms the perceptiveness of research.

Chandramohan Radhakrishnan, Natūralių ir LAB Apdorotų *Astragalus Glycyphyllos* Ektraktų Antioksidacinės ir Antimikrobinės Savybės: Baigiamasis magistro darbas. Darbo vadovė Prof. Habil. Dr. Gražina Juodeikienė. Cheminės technologijos fakultetas, Kauno technologijos universitetas.

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# SANTRAUKA

Saldžialapė kulkšnė *(Astragalus glycyphyllos*, Fabaceae) – gydomasis augalas, naudojamas daugelio tautų liaudies medicinoje. Šios žolės sudėtyje gausu bioaktyvių junginių, mineralinių medžiagų, vitaminų.

Šio darbo tikslas – nustatyti *Astragalus glycyphyllos* ekstraktų, gautų naudojant skirtingo poliariškumo tirpiklius (heksaną, etanolio/vandens (70:30 %) mišinį, acetoną), antioksidacinius aktyvumus ir įvertinti jų kokybinę bei kiekybinę sudėtį. Ekstraktai išgauti panaudojant daugiapakopę Soksleto ekstrakciją, superkritinę ekstrakciją anglies dvideginiu (SFE-$CO_2$) bei didelio slėgio ekstrakciją (PLE) etanoliu/vandeniu (70:30). Panaudojus etanolio/vandens mišinį, saldžialapės kulkšnės ekstraktų išeigos gautos žymiai didesnės nei Soksleto ar įprastos ekstrakcijos metu, naudojant heksaną ir acetoną.

Ekstraktų antioksidacinis aktyvumas įvertintas, naudojant gerai žinomus tyrimų metodus, tokius kaip DPPH (2, 2-difenil-1-pikrilhidrazilo) radikalų, ABTS (2, 2'-azino-bis-3-etilbenztiazolin-6-sulfono rūgšties) radikalų-katijonų surišimo įvertinimo, geležies (FRAP) jonų redukcijos antioksidantinės galios nustatymo ir deguonies radikalo surišimo gebos (ORAC). Ištyrus ekstraktų antioksidacinį aktyvumą, minėtais *in vitro* metodais nustatyta, kad didžiausiu antioksidaciniu aktyvumu pasižymėjo poliniais tirpikliais (etanoliu/vandeniu, 70:30%) išgauti ekstraktai.

QUENCHER metodu buvo tirtas netirpių išspaudų bei dervų antioksidacinis aktyvumas po atitinkamų ekstrakcijų (ABTS, ORAC bei TPC – netirpioms išspaudoms, SFE-$CO_2$ – dervoms). Netirpiose išspaudose po ekstrakcijų ABTS bei ORAC metodais, buvo nustatytas

likęs fenolinių junginių kiekis (Folin–Ciocalteu metodu), kuris buvo didesnis, nei likęs dervose po SCE-$CO_2$ ekstrakcijos.

Kokybinė ir kiekybinė saldžialapės kulkšnės išspaudų ekstraktų polifenolinių junginių analizė buvo atlikta, taikant ultra efektyviąją skysčių chromatografiją su kvadrupoliniu skriejimo laiko masių detektoriumi (UESCh-QTOF). Kiekybiškai įvertinti ekstraktų sudėtyje esantys junginiai: 12-okso-oktadekano, *m*-kumarino, linolo rūgštys ir rutinas.

Antroje eksterimento dalyje tirti *in vitro* antimikrobiniai aktyvumai tokių mėginių: (i) *Astragalus glycyphyllos* lapų ekstraktų, paruoštų įvairiais tirpikliais, (ii) *L. sakei* fermentuotų kietafazės fermentacijos sąlygose *Astragalus glycyphyllos* lapų ir (iii) augalinės žaliavos (be papildomo apdorojimo). Šio eksperimento metu *Astragalus glycyphyllos* lapų ekstraktai ir fermentuoti mėginiai bei kontrolė buvo išbandyti prieš įvairias bakterijas (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis* ir *Bacillus subtilis subspecies spizizenii)* ir grybus *(Debaryomyces vanrijiae, Geotrichum fermentants, Candida pelliculosa, Pichia farinose, Pichia fermentans, Candida krusei, Kluyveromyces marxianus, Kluyveromyces lactis var lactis, Aureobasidum pullulans).* Geriausias antibakterinis ekstraktų aktyvumas buvo pastebėtas prieš *Bacillus subtilis*, o priešgrybinis – prieš *Debaryomyces vanrijiae*. Fermentuoti *L. sakei* augalo milteliai (50 %), lyginant su nefermentuotais, rodė didesnį antibakterinį aktyvumą prieš *Bacillus subtilis*, o taip pat antibakterinį poveikį - prieš Debaryomyces *vanrijiae*.

Tokiu būdu, tyrimai patvirtino, kad *Astragalus glycyphyllos* ekstraktai pasižymi antioksidaciniu aktyvumu, o šio augalo ekstraktai ir ypač fermentuoti kietafazės fermentacijos sąlygomis šio augalo lapų milteliai - antimikrobiniu aktyvumu, tuo įrodant šios krypties tyrimų perspektyvumą.

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# ABBREVIATIONS

| | |
|---|---|
| TPC | Total phenolic content |
| ABTS | 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt |
| DPPH | 2, 2-diphenyl-1-picrylhydrazyl hydrate |
| FRAP | Ferric ion reducing antioxidant power |
| ORAC | Oxygen radical absorbance capacity |
| PLE | Pressurized liquid extraction |
| SFE- $CO_2$ | Supercritical fluid extraction carbon di oxide |
| TEAC | Trolox equivalent antioxidant capacity |
| UPLC | Ultra-performance liquid chromatography |
| ESI-MS | Electrospray ionization mass spectrometry |
| LAB | Lactic acid bacteria |
| DW | Dry weight |
| GAE | Gallic acid equivalents |
| TPC | Total plate count |
| YEPD | Yeast extracts peptone dextrose |
| SSF | Solid state fermentation |
| EX | Extractive yield |
| NaCl | Sodium chloride |
| ANOVA | Analysis of variance |
| V/V | Volume concentration |
| UV | Ultraviolet |
| V/W | Mass concentration |
| AO | Antioxidant |
| EX | Extract |
| E/W | Ethanol water |
| AX | Acetone |
| HX | Hexane |

# INTRODUCTION

Medicinal plants are generally used in food supplements as a source of bioactive compounds that have properties of food additives. Research has shown that plant extracts are effective bioactive compounds in food systems and can be used as additives, antioxidants and antimicrobials [1, 2].

Considering the application and increasing use of these bioactive compounds in foods, cosmetics and pharmaceuticals to replace synthetic bioactive compounds that might exhibit carcinogenic effects with extensive use, the discovery of natural compounds from medicinal plants have become a main focus of the current research [3].

Oxidation is one of the most common processes that occur in food systems. It reduces consumer acceptability of food by producing low molecular weight flavor compounds, as well as destroys essential nutrients and produces toxic compounds, dimers or polymers of lipids and proteins [4]. But, it can be minimized by removing the chemicals that are prone to oxidation such as free fats, metals. However completely removing those chemicals from food is complicated so antioxidants are added to minimize the oxidation. The naturally available antioxidants in vegetables can be processed and added to food products but it should be cheap, non-toxic, and stable at low concentrations, and also should not affect the taste and texture of the food. The amount of antioxidants is crucial to mitigate the oxidation of food compounds also the polarity, medium as well as presence of the antioxidants [5].

Natural products and antimicrobial agents are most important weapons in the fight against bacterial infections and have benefited greatly from the quality of life related to health since their introduction. New antibacterial compounds can be obtained naturally from medicinal plants which might provide distinct mode of action. And it has been widely studied in lot of plants with significant therapeutic promises in various parts of the world.

The World Health Organization estimates that around 80 % of the world population using herbal extracts or their active ingredients in traditional medicine. In the present-day scenario, the derivation of drugs and supplementary foods from plants are potential and effective in treating various diseases produced by microbial flora. Over the past decades a lot of natural phenolic compounds that shows promising antioxidant and antibacterial activity have been studied [6].

The main objective of our work was to study the plant *Astragalus glycyphyllos* leaves as a possible source of dietary antioxidants and antimicrobials. This plant is found in temperate regions in the northern hemisphere, the common names are milkvetch and is commonly used in Chinese traditional medicines and many other traditional medicines [7]. *Astragalus glycyphyllos* leaves has been chosen for the study for its potential antioxidant and antimicrobial activity and pharmaceutical application.

### 1.1.Aims

The following objectives were set in order to achieve the aim of the work:

- To produce and determine the yield of hexane, acetone and ethanol/water (70:30) in Soxhlet extraction, supercritical fluid extraction (SFE-$CO_2$) and pressurized liquid extraction (PLE) acetone, ethanol/water (70:30) extracts from *Astragalus glycyphyllos*.
- To evaluate the antioxidant activities of produced *Astragalus glycyphyllos* extracts using different radical scavenging assays.
- To determine the total amount of phenolic compounds in *Astragalus glycyphyllos* extracts.
- To evaluate of antioxidant capacity of solid substances by the QUENCHER method
- To identify polyphenolic compounds presented in extracts from *Astragalus glycyphyllos*.
- To evaluate the antimicrobial activity of various *Astragalus glycyphyllos* extracts against indicator bacterial and fungal strains.
- To investigate the antimicrobial effect of fermented *Astragalus glycyphyllos* leaves using LAB and solid state conditions.

### 1.2.Structure of the Thesis

- Chapter 2 is a literature review.
- Chapter 3 provides the methodology.
- Chapter 4 contains results, discussions and conclusions to the work carried out.
- Chapter 5 lists all the references used.
- Chapter 6 are annexes.

# REVIEW OF LITERATURE

## 2.1. *Astragalus glycyphyllos* as medicinal plant

*Astragalus glycyphyllos* belong to the Fabaceae family. This herbaceous perennial flower that is native to Europe, it is also located in North America and Asia. The herb is commonly known as licorice milkvetch, sweet milkvetch or wild liquorice. Generally, milkvetches are divided into two groups: medicinal and poisonous [7, 8]. It grows in the woods, slashes, flanks and shrubs. *A. glycyphyllos* is a perennial, 30-100 cm plant with thick, bare or overgrown with hairy stems. Root is short, thick and branched. The leaves are 10-20 cm long, newport pinnate, consisting of 15-40 mm sheets. Ankle leaves are elongated egg or elliptically and are, blunt tipped (Figure. 2.1).

**Figure 2.1.** *Astragalus* plant.

*Astragalus* plants are commonly used in traditional medicine. These plants have hepatoprotective, antiviral and immune system stimulant activity. Dried *Astragalus* plant roots are used as antiperspirants, diuretics and applied to treat diseases such as nephritis, diabetes, and hypertension. Some strains of *Astragalus* plant species grown in Asia are used for their distinguished-tragacanth gum, which is used in the production of a variety of drugs, as an emulsifier, stabilizer and viscosity regulator. *A. membranaceus* in Chinese medicine has been used for more than a thousand years. This plant known as an adaptogen, helps the body to adapt to stress, to improve heart function, to relieve the symptoms of heart disease and to reduce cholesterol level in blood [9, 10]. The *Astragalus* is one of the 50 crucial medicinal herbal plants in Chinese traditional medicines [11].

In traditional Bulgarian medicine *A. glycyphyllos* is used as an antihypertensive, anti-inflammatory agent and diuretic. The decoction of this plant is used in treating nerve damage as well as in dermatitis, venereal diseases, dysentery and digestive diseases [12].

*A. glycyphyllos* contains or synthesizes aliphatic nitro neurotoxic compounds such miserotoxin. It leads to microbial hydrolysis of miserotoxin releasing its glycone 3-nitropropanol which is then oxidized enzymatically to 3-nitropropionic acid. *A. glycyphyllos* has hypotensive and anticoagulation effects. A previous study reported with rats that has been assessed by *A. glycyphyllos* antioxidants and hepatoprotectives reduced carbon tetrachloride -induced liver infringement. It concluded that the extract of *A. glycyphyllos* has similar protection properties as Silymarin being source of flavonoids, saponins and polysaccharides [13, 11].

*Astragalus* is used to treat kidney associated diseases and improves the aging. It's generally taken together with other medicinal plants as concoction as *Ledebouriella seseloides* and *Atractylodes macrocephala [14]*. *Astragalus* root has a lot of biologically active ingredients, including saponins and flavonoids [15].

*Astragalus* has diverse medicinal application such as adaptogen which helps human body to resist various stresses, antipyretic to fight fever, diuretic agent and stimulant of uterine. Also, it is believed to fight against various cancers, uterine prolapse, chronic ulcers, to treat kidney inflammation like nephritis. Recent literatures show that the *Astragalus* root extracts enhances the production of immune cells like macrophage, thus helping to restore the normal function of the immune system of cancer patients [11, 16].

Most of the *Astragalus* genus are tested for the content of flavonoids, saponines, alkaloids and sterols. The antioxidant of *Astragalus* have cardioprotective effects. This is related to the weight of the phenolic compounds. As powerful antioxidants, these compounds reduce oxidative stress, induce damage of blood vessels, increase vascular contractility, smooth muscle proliferation, inflammation and endothelial dysfunction [17, 18].

Adapting the properties of these compounds, food supplements and teas with *A. membranaceus* material have already been developed and used. Their main indications are: strengthening immunity, stress reduction and cardio protection. Thus, this is helpful in plant

species with phenolic compounds and the anti-oxidant activity in order to judge the therapeutic benefits of this plant such as treating heart diseases [18, 19].

*Astragalus* has been used in the enhancement of the immune system for thousands of years. The study of Platikanov et al. showed the ability to control the T-helper cells 1 and 2, in principle to regulate the body's immune response. Although the complete medicinal potential of this plant is yet to be studied in detail, the existing literature proves that it can be used in many therapies to cure various illnesses [20].

Reactive oxygen species that forms free radical highly damage components of disease and aging, the bioactive components present in *Astragalus* fight these ROS and reduce the oxidative stress. It boosts the immune system and also improve the brain function, both of which increase the life expectancy [21].

Most recent reviews have shown the bioactive compounds from *Astragalus*, such as saponins, flavonoids seem to reduce the occurrence of tumor. *Astragalus* has potentially fight multi-drug resistance cancers such as liver cancer in traditional chemotherapy [22]. Also, it has been shown that *Astragalus* can help to recover after the chemotherapy. In cases of severe symptoms of chemotherapy, such as nausea, vomiting, diarrhea, and myelosuppression, *Astragalus* was given intravenously and combined with other Chinese herbal mixtures. Early studies suggest that it may reduce these symptoms and increase the effectiveness of chemotherapy treatment [20].

The flavonoids extracted from *Astragalus* act as powerful antioxidants which prevents the plaque formation and contraction of artery wall. One of the Chinese studies has reported that *Astragalus* injection has cured the heart disease along with conventional viral myocarditis treatment [23].

Medicinal applications of *Astragalus* has been well studied for diabetic patients which naturally alleviates the insulin resistance. The saponins, flavonoids and polysaccharides of this plant are all effective in the treatment and regulation of type 1 and type 2 diabetes. It improves the insulin sensitivity by protecting the cells that produce insulin [24]. It also slows down the kidney problems in diabetic patients and protects the renal system [25, 26].

Due to its anti-inflammatory properties, *Astragalus* has a long history in the treatment of wounds. Dried root of *Astragalus* has been used for the repair and regeneration of injured tissues and organs in traditional Chinese medicine. According to the study from Zhejiang

University *Astragalus* extracts treat the wounds and also increases the patient recovery rate in 2-3days and is promising against scarring [27].

*Astragalus* has anti-virus capabilities, it assists the body to fight viral flu and common cold. It's combined with other medicinal plant extracts like ginseng, angelica and licorice. It is also used as herbal supplement on regular basis that make the human health to protect from respiratory tract diseases. The *Astragalus* node before the colder months of the winter, is said to prevent or reduce cold and upper respiratory tract diseases in a number of people who catches this ailment during the season [28].

Abdulla et al. showed that aqueous extract of *Astragalus* plants has an anti-bacterial activity [29]. In addition, Balachandran et al. reported that *A. membranaceus* extracts were toxic to the pathogens such as *Escherichia coli, Bacillus cereus, Candida krusei* and *Aspergillus fumigatus* [30].

## 2.2. Plant phenolics as dietary antioxidants

### 2.2.1. Phenolic compounds

The phenolic compounds are a group of chemical compounds having antioxidant properties. This complex compounds are widespread in nature and are found in the roots, leaves and bark. They can be found in diet products such as olives, chocolate, cereals, tea, coffee and wine. In plants, phenolic compounds play a protective role by reducing UV radiation, pathogens, parasites and harmful predators [23].

The structure of phenolic compounds ranges from simple phenols to polymerized compounds such as condensed tannins. There are many phenolic compounds classified. Depending on the carbon chain length, phenolic compounds are divided into 16 classes. The main class of phenolic compounds is phenolic acids, stilbenes, lignans and flavonoids. According to the distribution of the wild, phenolic compounds can be divided into three classes: low spread (simple phenols), widely distributed (flavonoids, phenolic acids) and polymers (tannins and lignin) [24].

Flavonoids are low molecular weight bioactive compounds belonging to the group of polyphenols. The main feature of the flavonoid structure is 2-phenyl-benzo ring with two benzene rings, directed toward a heterocyclic pyrin ring. It's identified in more than 4000 flavonoids variations. The main classes of flavonoids are: flavanols, flavonols, flavones,

isoflavones, anthocyanidins, anthocyanins and flavanones. Individual compounds within each of these groups may be different positioned just like a hydroxyl group number and their alkylation and / or glycosylation. Flavonoids can be conjugated with monosaccharides, disaccharides and can form complexes with oligosaccharides, lipids and amino acid [21].

### 2.3. Pharmacological activities of phenolics

Phenolic compounds are consumed for cardiovascular related issues. According to the research data, products containing phenolic compounds can help to prevent coronary heart disease. These compounds also have an impact on the formation of atherosclerosis. Because phenolic compounds are inhibitors of LDL oxidation, they impede the occurrence of atherosclerosis. Having anti-inflammatory, antithrombotic effects on HDL-raising and the improvement of endothelial function, phenolic compounds are used for cardiovascular disease prevention [31].

The antioxidant activity of phenolic compounds has their importance in the human body. Acting as an antioxidant, they protect cells against oxidative stress. Safety mechanisms affect their ability to bind free radicals, to give a hydrogen atom or electron [24]. Antioxidant properties depend on the structure of the compounds. The most widely known phenolic compounds having these properties are flavonoids, phenolic acids, chalcones, tannins and coumarins [23].

Much research has been carried out with regard to the cardiovascular relations diseases (CVD) and the antioxidant status. Antioxidants may have a role as atherosclerotic cardiovascular disease component in protecting both PUFAs and LDL cholesterol from oxidation. Flavonoids have more potent antioxidant activity than vitamins C and E. The antioxidant activity of flavonoids exhibits through several mechanisms: free radicals are involved in producing enzymes such as glutathione S-transferase NADH oxidase inhibition of ROS adhesion and activation of antioxidant enzymes. Antioxidant activity of flavonoids in the mechanism depends on the layout of the hydroxyl groups and the quantity configuration of substituents [32].

Due to its antioxidant activity, flavonoid has a significant impact on the cardiovascular system. Oxygen radicals oxidize LDL, which is in violation of the endothelium and atherosclerosis. Flavonoids reduce the oxidation of LDL, increase HDL

concentrations, and inhibit the cardiac mast cell mediators [33]. A Japanese study observed an inverse correlation between the consumption of flavonoids and cholesterol concentration in blood plasma. Flavonoids by binding free radicals causes an antithrombotic effect, as supported by the endothelial prostacyclin and nitric oxide [21].

Flavonoids play an important role in hypertension control. They act as vasodilators, inhibit NADPH oxidase, inhibit superoxide production and support the NO emissions. Research suggests that long-term consumption of flavonoids might reduce cardiovascular risk of disease and its consequences [34]. At least three studies have suggested the role of flavonoids in prevention of coronary heart disease [35], studies of some medicinal plants have developed positive anti-atherogenic reactions due to the flavonoid components [36].

Studies of herbal plant hawthorn (*Crataegus oxyacantha*) used in cardiovascular medicine showed that oligomeric procyanidins and other flavonoid compounds with strong antioxidant properties fight against harmful effects of free radicals on the cardiovascular system, helping to minimizing risk atherosclerosis [37, 38]. The proposed cardio protective mechanism is that antioxidants directly protects human LDL from oxidative damage by ROS [39].

### 2.4. Oxidation processes and antioxidants

The oxidation process is the transfer of one or more electrons from the reducing agent (electron donor) to the oxidant (electron acceptor) due to a higher affinity of the latter. Electron transfer can cause chemical transformations of the two reactants and in some cases during chemical reaction an odd number of valence electrons may be produced. In such reactions, the radicals tend to be very unstable and therefore highly reactive. The free radicals produced by ROS compounds would create chain oxidation between them until the formation of stable products [40]. The hydroxyl, fluorine, chlorine and ozone are powerful oxidants [41].

The extensive oxidative damage on metabolites might cause various diseases in the human body such as coronary heart disease, atherosclerosis and also affect aging. It is believed that the health promoting effect of antioxidants from plants come from their protective effects by neutralizing ROS [42].

Oxidation in food usually occurs when food ingredients are exposed to oxygen in air or sunlight. This could be a very destructive process, leading to nutritional value losses and changes in the chemical composition. The organic compounds in foods (fats, proteins, carbohydrates) can oxidize forming products causing side reactions that could affect the sensibility and the nutritional value of food. In the oxidation reaction of lipid containing food products, primary oxidation products such as various peroxides and hydro peroxides are formed. These products can cause rancidity of lipids and makes them inedible. In fruits like apples it can lead to the formation of compounds that fades fruit. Oxidation is one of the most important processes that occur in food systems. Oxidation reduces consumer acceptability of food producing low molecular weight compounds flavor, as well as the destruction of essential nutrients and produces toxic compounds, dimers or polymers of lipids and proteins [4].

Metals, free fatty acids and oxidized compounds and similar pro-oxidants should be avoided to minimize food oxidation. Light is also one such pro-oxidant factor. Addition of oxygen scavenger or pressure reduced by air evacuation are methods adopted to reduce oxidation. Air anti-oxidants are increasingly added to foods to reduce the oxidation process as it is quite challenging to remove the entire pro-oxidants. The oxygen and reactive oxygen species are one of the main sources of primary catalyst used to initiate oxidation *in vivo* and *in vitro*. The oxidation process *in vivo* can accelerate the aging and can cause various diseases, including cancer and cardiovascular disease. Protection mechanisms in living cells and in food become weak, so it is necessary to strengthen them [43].

The oxidation of oxidizable antioxidant substrates retard significantly or inhibit at lower concentrations when compared to higher levels of lipids and proteins in foods. Antioxidants might not protect the cells once its introduced inside the cells, it has to be modified in a way to be in an usable form then it only can effectively protect the cells from oxidative damage. Also, the concentration of the antioxidants is crucial, so the available antioxidants should be absorbed into the body while digesting and into the cells [44].

The oxidation can be prevented or delayed by an antioxidant; such substances are organic molecules of synthetic or natural origin that can trap the oxygen free radicals involved in the oxidation. By mechanism, they are separated into primary and secondary antioxidants. Primary antioxidants are the chain breaking, scanning and decay of free

radicals, which do not trigger the reaction. The effectiveness of primary antioxidants is based on the inactivation of free radicals before they can attack the unsaturated fatty acids. Antioxidant radicals can terminate reactions when binding with lipid radical forms or non-radical derivatives.

$$LOO\cdot + AH \rightarrow LOOH + A\cdot$$

$$LO\cdot + AH \rightarrow LOH + A\cdot$$

$$L\cdot + AH \rightarrow LH + A\cdot$$

Secondary antioxidants do not allow free radicals, because of their stability. Therefore, they can act through different mechanisms that bind metal ions, reducing oxygen, and absorb ultraviolet light to inactivate singlet oxygen [40, 45].

$$LOO\cdot + A\cdot \rightarrow LOOA$$

$$LO\cdot + A\cdot \rightarrow LOA$$

$$L\cdot + A\cdot \rightarrow LA$$

$$A\cdot + A\cdot \rightarrow AA$$

Antioxidants must meet the main requirements for food additives. Firstly, they must be non-toxic. Antioxidants must be compatible with the substrate and effective at low concentrations. In addition, they should not affect the taste, aroma and color of foods [46].

A variety of antioxidants are available in the food industry to delay this oxidation of oils and fats, thereby preserving healthy aspects of the nature of the food. Food industry offers a wide range of antioxidants and it is importnt to choose the best one for each application. The most often used synthetic antioxidants are synthetic phenols, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) and gallic acid esters, e.g. propyl gallate (PG). There is some uncertainty, whether synthetic antioxidant consumption has no risk for health. Nevertheless, their use as food additives is restricted in several countries, because of the possible effect on the enzymes in the human body [47].

Natural antioxidants can be chemically synthesized in the industry and they are called identical natural antioxidants. These compounds are pure unlike antioxidants being extracted from the source material. Artificial antioxidants are relatively cheap and they combine the advantages of the synthetic and natural antioxidants [48].

Antioxidants that synthesis plants and a variety of micro-organisms, fungi and rare animals are known as natural antioxidants. They are important for normal growth of plants and the development of protection against infection, injury [49] and protecting against microorganisms, herbivores and pests [50].

Under natural conditions, animal and plant tissue contain its own antioxidant system of regulation. The main enzymatic antioxidants found are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-reductase (GR). In addition to these main enzymes, other antioxidants, including glutathione S-transferase (GST), heme oxygenase-1, and redox proteins, thioredoxins (TRX) peroxiredoxins (PRXs) and glutaredoxins are also found to play an essential role of antioxidant protection [51, 52].

Non-enzymatic antioxidants are represented by ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids and others.

### 2.5. Microbiological food safety control

One of the ongoing issues and of fundamental concern in relation to food production industry is microbial product safety. Few of the challenges affecting food security policy makers are mentioned here as follows: (1) complexity of food safety issues (2) different food processing treatments (3) food consumption patterns (3) microbial-host interactions (4) environment and pathogen interactions, and several other factors to be considered as well.

A complex combination of factors such as microbial interactions research need to be studied on a continuous basis to check food poisoning. New niches and vulnerable opportunities are created with increased chances of pathogens ability to evolve, victims of pathogens and the microbial environment. Based on the pathogen, host and environment in which it engages itself, a foodborne disease can be distinguished into three types. Sophisticated interactions among these three factors are essential for food borne diseases to occur. Let us consider an illustration, even a food item with innumerable number of microorganisms is consumed by a susceptible host. Food borne diseases will not occur if the organisms do not have the required features to cause an illness. Similarly, exposure factor can be minimized by cooking food and can make it safe. These three factors are important to reduce food borne illnesses. Washing vegetables may remove surface contamination (Fig. 2.2). But in many cases, safety can be ensured with more than one factor

since Food supply which is completely free of all pathogens is not possible with current techniques and advances.

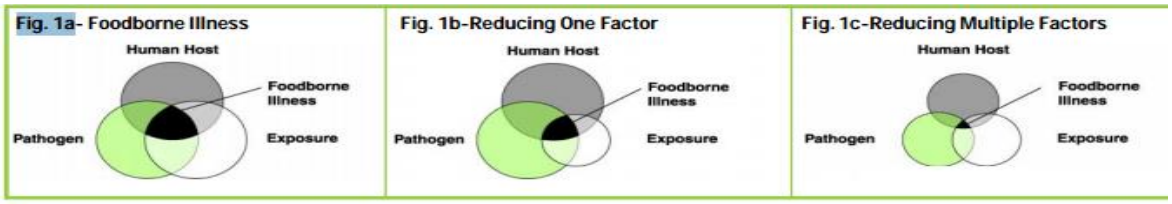


**Figure 2.2.** Trinity of factors.

When organisms are growing in detected media at increased levels, food components such as fats, vitamins and proteins are broken down by the enzymes produced. This causes changes in color, odor, taste, appearance and texture, making it inedible. This is called food deterioration. The causative organism's growth is detected in the range of $10^5$ to $10^7$ CFU /g (colony-forming units per gram) of food. Different alteration profiles are associated with different clarification of foods such as vegetables, red meat, fish, etc. It is because the fact that the organisms growth is dictated by it, causing that deterioration dominates. For example, gram-negative psychotropic, cold bodies blooming is associated with alteration of raw meat, as the former grows under refrigeration conditions. Acidic condition thriving organisms, yeasts and moulds, are associated with spoiling fresh fruits. In many of the cases, spoiled food is not responsible for food borne diseases, in fact, the high spoiling organisms are probably "out-competed" pathogens, while maintaining the growth of the pathogen test [53].

## 2.6. Antimicrobial compounds from plants

The component used against fungi are anti-fungal agents and those used against bacteria are anti-bacteria agents. Agents that inhibit microbial growth are called biostatic, while those that kill them are called microbicidal. Anti-microbial chemotherapy refers to treatment of microbial infections. Anti-microbial prophylaxis refers to using anti-microbial agents to avoid infection. Antimicrobials can be classified into disinfectants, antiseptics and antibiotics. Disinfectants prevent the spreading of disease and destroy a vast range of microbes. This includes anti-microbial agents such as bleach. Antiseptics applied on tissues during surgery help with the reduction of infection. Anti-biotics destroy microbes throughout the body. Though antibiotics originally refer to formulations derived from living beings, also covers synthetic anti-microbial agents such as sulfonamides or

fluoroquinolones. Anti-biotics can be either bactericidal, which wipe out microbes, or bacteriostatic, which show down microbial growth and development [54].

When plant cell membrane is damaged, it leads to anti-microbial action of plant essential oils (EO's). EO's react with the lipids of mitochondrial and cell membrane due to its hydrophobic nature, resulting in disrupting the structure and permaculture of the cell and mitochondrial membrane. Thus, EO's anti-microbial activity is higher at lower temperatures. At 7 ° C, the cytoplasmic membrane of phospholipids, allows EO to disperse easily in lipid bilayer as it makes the cell membrane more fluid. This is because, 7 ° C is a higher degree of unsaturation [55]. In addition, the antimicrobial activity is dependent on the extent to which oxygen is available. This may be because the oxygen is low, a number of essential oil oxidation can occur and / or the microbial cells being powered by the anaerobic metabolism making them susceptible to toxic effects. Oregano and thyme EO is highly active against *Salmonella typhimurium* and *Staphylococcus aureus*, where oxygen levels are low [56].

EO offer a viable alternative to synthetic preservatives used in food products. The previous report evaluated the antimicrobial activity of 6 EO against the 25 common microorganisms [57]. They concluded that EO's showed significant inhibitory effects against all tested bacteria. The report previously investigated the antimicrobial properties of 21 plant EO's against 5 common food-borne pathogens [58]. This work showed that gram-positive bacteria were more sensitive to the inhibition of plant EO's than conventional gram-negative bacteria.

Gupta et al. examined the 10 herbal EO antimicrobial activity against 10 common bacteria. They found that only cinnamon oil and clove oil has shown a wide range of antimicrobial activity. Minimum inhibitory concentration (MIC) of 1.25% (v / v) cinnamon oil against *Listeria monocytogenes* and *E. coli*, and 2.5% (v / v) for clove oil against *Staphylococcus epidermidis.* The previous report evaluated the antimicrobial activity of cloves, cinnamon and oregano EO's and applied these EO's as paraffin-based "active grip" for paper packaging materials [59]. It has been shown that inhibition effect of EO's against the tested gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus*) were observed and only the active cover with cinnamon EO's showed inhibition against gram-negative bacteria (*E. coli, Salmonella typhimurium*). It has also been shown that EO's added

hydrophobic starch to the coating materials of paperboard have a significant effect on the antimicrobial activity. It was previously reported that pure clove EO's have a better anti-microbial inhibition against the tested bacteria (*E. coli*, *Staphylococcus aureus*, *Bacillus cereus*) than clove EO's in starch matrix [60]. Natural plant EO's have shown promising anti-microbial activity against several bacteria and food-borne pathogens, including *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus* [61]. In addition, Burt has emphasized that gram- negative organisms are far less to essential oils than their gram-positive organisms such as *Campylobacter jejuni, Salmonella enteritidis* and *Escherichia coli,* which agrees with previous research [58].

Extracts from plant roots and seeds have also been reported to show extensive biological activity as antimicrobials. Arora and Kaur compared the sensitivity of some human pathogenic bacteria and yeast in a variety of spice extracts. They discovered that only clove and garlic extracts were found to have antimicrobial activity and inhibition of *Staphylococcus epidermidis* and *Salmonella typhimurium* [62]. Tiwari et al, analyzed the antibacterial susceptibility of various extracts of the Carica papaya root using the "well" diffusion method and found that the Carica papaya root extracts are characterized by a broad spectrum of activity against bacterial diseases [63]. Recently, Radfar et al. tested antibacterial and antifungal activity of various extracts of *Trianthema decandra's* roots and root callus were tested against both gram-positive and gram-negative bacteria [64]. Antimicrobial action of the seed extracts, particularly grape seed extract, has been shown in studies of Sivarooban et al. who investigated the antimicrobial properties of soy protein isolate (SPI) contains grape seed extract (1% w / w), nisin (10,000 lU/ g), ethylenediaminetetraacetic acid (EDTA, 0.16% w / w), and their combinations. Grape seed extract, nisin and EDTA connected to the SPI film were effective to inhibit the growth of *Listeria monocytogennes*, *E. coli* O157: H7 and *Salmonella typhimurium*, which has the potential application in ready to eat foods [65]. Anti-listeria effect of grape seed extract was evaluated using several tools and the results show that grape seed extract has a significant antimicrobial effect against *Listeria spp*. at relatively low concentrations [66].

### 2.7. Studies of Plant Extract Antibacterial Activity

Antibacterial agents generally destroy bacteria or suppresses its ability to reproduce. These products do not suppress the risk of symptoms of viral infectious diseases in healthy

persons. This does not involve the essential contribution of products to decrease symptoms of bacterial diseases. The microorganisms used in studies of *Astragalus glycyphyllos extract* antibacterial activities are described below.

### 2.7.1. *Escherichia coli*

The Enterobacteriaceae family member of intestinal bacteria are one of the most important bacteria of medical concern. *E. coli* is a human digestive tract settler (also *Enterobacter* and *Klebsiella*), but it can sometimes be associated with other diseases in humans. This optional anaerobic, motile, Gram-negative rod. *Escherichia* colonizes the gastrointestinal track of most warm-blooded animals within a few hours or a few days after birth. With the level of faecal contamination of water and food potential, you need to take common-sense steps to reduce the risk. Food poisoning ends up in hospitalization and most illnesses associated with *E. coli* respond to antibiotic therapy [70].

### 2.7.2. *Staphylococcus aureus*

This is a facultative anaerobic gram-positive coccal bacterium. It is found as part of the normal skin flora. However, it is the most common causes for staph infections. Catalase-positive S. aureus, can convert hydrogen peroxide ($H_2O_2$) to water and oxygen [66].

When food is stored in room or refrigerator temperature and is often contaminated through food handler, *Staphylococcus aureus is the causative microbe.* acute gastro-intestinal diseases may affect the consumers when food is infected with such microbes. The most recent study shows rhamnoside kaempferol isolated from *Persea lingue Nees* leaves (avocado tree typical sites) possess antibacterial activity against the coccus type [67].

### 2.7.3. *Bacillus cereus*

It is a Gram-positive, aerobic spore former. Its spores cannot swell the sporangium. These features, together with specific biochemical properties, is used to differentiate *B. cereus* from other species of the genus *Bacillus* (eg, *B. anthracis* and *B. thuringoemsos*). It is widely distributed in nature and in food. It is commonly found in soil, milk, grain, starch, herbs, spices and other dried foods. *B. cereus* is a very common and widespread. It is almost impossible to avoid fresh vegetables form their spores, as they naturally exist in soil. Chlorinated water treatment reduces the amount of the population, but it cannot be removed.

### 2.7.4. *Salmonella typhimurium*

Food poisoning is also done by Salmonella typhimurium, which is pathogenic for humans, causing enteric fever, gastroenteritis and Bacteremia. Isolated antigenic characteristics, sugar fermentation patterns and bacteriophage sensitivity basis are usually done here. Salmonella typhimurium, is genus of gram-negative, facultatively anaerobic, rod-shaped bacteria that utilizes citrate as a sole carbon source. Antigenic characteristics, sugar fermentation patterns and bacteriophage sensitivity basis can be isolated from organisms of this genus. There are about 2,200 known species of *Salmonella* of different kinds. *Salmonella enteric* serotype typhimurium is one of the most common *Salmonella serovars* causing salmonella poisoning. Recent studies suggest that the main mechanism of virulence of *Salmonella* may include the type III secretion system encoded by the plasmid and allow direct transfer of bacterial proteins into eukaryotic cells via a contact-dependent secretory mechanism. These effector proteins are able to improve noxious epithelial cell invasion [69]. *Salmonella typhimurium* can adapt to environmental stress inducing specific sets of genes termed stimulants [70]. Therefore, the only effective inactivation method is heat treatment.

### 2.7.5. *Bacillus subtilis sub species spizizenii*

The bacteria found in gastrointestinal tract of humans and ruminants is a gram-positive catalase bacterium which also is known as grass bacillus or hay bacillus. *Bacillus subtilis sub species spizizenii* is considered the best studied gram positive bacteria and a model organism to study the bacterial cell differentiation and chromosome replication. It has been historically categorized as binding aerobe, though evidence suggests it's not a binding aerobe. This is used in industrial scale biotechnological applications and is truly a champion among the secreted bacterial enzymes. *Bacillus subtilis* is a common intestinal microbe and is commonly found in the top layers of the soil. A study in the year 2009 revealed that it's spores are found in the soil in the composition of about $10^6$ spores per gram and in the human faeces in the composition of about $10^4$ spores per gram [71].

## 2.8. Studies of Plant Extract Antifungal Activity

Fungal infections like athlete's foot, ringworm are prevented or controlled by using anti-fungal agents. Fungal organism can be killed by working and exploiting the differences

between mammalian and fungal cells. The microorganisms used in studies of *Astragalus glycyphyllos extract* antifungal activities are described below.

### 2.8.1. *Aureobasidum pullulans*

*Aureobasidum pullulans* is a black yeast-like fungi that can be found in different environments (e.g. limestone). It is widely found in plant species such as apple, grape, cucumber, cabbage, water, phylloplane, wood and many other materials, plants, rocks, artifacts [72] ., without causing any symptoms of disease. It was found by *A. pullulans* to be able to control the growth of undesirable microorganisms and it has a high importance in biotechnology for the production of different enzymes. *A. pullulans* was also reported as a slime-producing impurity paper mills and can colonize optical lenses [73].

### 2.8.2. *Kluyveromyces lactis*

*K. lactis* is a creamy-white or pink, depending on their production of pigment pulcherrima, GC-content of the K. lactis genome is 40.3%, and the growth observed was between 25 ° C and 35 ° C. *K. lactis* includes 21 known strains that have been isolated from yoghurt, butter, milk, cheese (e.g. camembert) and other fermented dairy products. *K. lactis* has become one of the most important species of yeast, which is widely used for genetic studies and industrial applications. Its main ability is to assimilate lactose and convert it into lactic acid. It is also made rennet (chymosin) for cheese production [74].

### 2.8.3. *Kluyveromyces marxianus*

*Kluyveromyces marxianus* is an yeast of variety *Kluyveromyces* and is the sexual type of *Candida kefyr*. *K. marxianus* is utilized industrially to create the lactase catalyst like the utilization of other organisms, for example, those in the variety *Aspergillus* [75]. *K. marxianus* are able to isolate from, yoghurt, kefir, cheese, fermented milk, milk products, pozole (Mexican corn fermented dough), decaying plants, prickly pear, sorghum beer and insects. The study revealed that *K. marxianus* still involved in the fermentation of coffee.

K *marxianus* has also used for biotechnological applications and the production of inulinase or pectinase and beta-galactosidase [76].

### 2.8.4. *Pichia farinose*

Its yeast, from the family Saccharomycetaceae with circular, curved or elongated cells. It is teleomorph, and has a cap molded structure and forms round ascospores. *Candida species* are some anamorphic *Pichia* species and it is multi-laterally growing. It doesn’t

absorb lactose. The carbohydrate dependency varies with available species. Some of these species grows in milk products. [77].

### 2.8.5. *Candida krusei*

Candida krusei is a new fungal nosocomial pathogen. In particular, it has been found with malignant blood diseases and immune deficiencies [78]. It's natural resistance to fluconazole, an antifungal agent standard. It occurs in patients who have had conflicting evidence, sparking debate or fluconazole should be used prophylactically [79]. Two mechanisms of azole resistance have been described in *C. krusei*: excessive drug efflux pumps and reduced susceptibility target enzyme, the cytochrome P450 sterol 14-demethylase (CYP51 gene encoded) [80, 81]. *Candida krusei* is emerging yeast (species of fungus) involved in the manufacture of chocolate. Complex of different fatty acids have been shown as a metabolite when cultivated *C. krusei* medium containing lactose, it will also produce short chain carboxylic acids when grown in saliva supplemented with glucose; they include propionate, succinate, lactate, acetate, formate and pyruvate. The biological role of those, if any, is not yet known [78].

### 2.8.6. *Candida Pelliculosa*

*Candida Pelliculosa* yeast is often found in various fruits, tree exudates, soil, vegetables and other organic compounds [82]. It is sometimes reported fungaemia agents from both AIDS, including immunocompromised patients and immunocompetent. *C. pelliculosa* estimated endocarditis in intravenous drug users and nosocomial agents brain ventriculitis in low birth weight newborns [83, 84].

### 2.8.7. *Debaryomyces vanrijiae*

*Debaryomyces vanrijiae* is grown at 25˚C, the cells are spheroidal to short-ovoid and single in groups. Creeping pellicle are formed which look white wrinkled along with a sediment. *Debaryomyces vanrijiae* has been found to produce high levels of the extracellular alpha-glucosidase from the grape [85]. The enzyme can be used for food detoxification and several biotechnological applications, including biomass conversion, flavor enhancement with wine and other drinks [86].

# MATERIALS AND METHODS

## 3.1. Plant material

*Astragalus glycyphyllos* plants were selected from the collection of medicinal plants at Kaunas Botanical Garden (Vytautas Magnus University, Lithuania). The freshly cut leafy plants were settled, dried in the drying room with active ventilation at 30 ° C temperature and stored at room temperature in the dark before use. Dried leaves of the plant were ground and centrifuged by a ultra - centrifugal rotor mill Retsch ZM200 (Retsch GmbH, Germany). The ground sample was further sieved through a 0.2 mm sieve.

## 3.2. Extraction methods

### 3.2.1. Soxhlet extraction

The extraction was carried out in an automated extractor (Behr Labor - Technik, Germany), as a standard method [87], using hexane, acetone and ethanol/water (70:30). Twenty grams of ground *A. glycyphyllos* was stuffed into a cellulose thimble and inserted into the chamber of the apparatus. Firstly, the extraction was carried with 150 ml of hexane by applying 3 × 180 min extraction cycles. After the lipid fraction was removed from *A. glycyphyllos*. Secondly, the extraction was proceeded with 150 ml of acetone from the hexane extraction residues in a Soxhlet apparatus for 3 h with 3 extraction cycles. A rotary evaporator Büchi, R-114 (Donau, Flavil, Switzerland) at 40 °C was used to remove the solvents and then dried with a flow of nitrogen gas. The acetone residues were dried in an incubator at 50 °C. Finally, ethanol: water ET/W (70:30) was applied to extraction from the acetone extraction residue. Twenty grams of the acetone residue was taken in a 500-ml conical flask with 200 mL of solvent added and is shaken for 2 h on an orbital shaker at room temperature. The supernatant was separated by vacuum filtration and extraction cycles for 3 times. EtOH was removed by rotary evaporation at 40 °C and water removed by freeze-drying, and the dry extracts were stored in a freezer prior to further analysis.

### 3.2.2. Supercritical $CO_2$ extraction of *Astragalus glycyphyllos*

SFE–$CO_2$ was performed in a Helix extraction system (Applied Separation, Allentown, PA, USA) with 99.9% $CO_2$ (Gaschema, Jonava, Lithuania) from 14g of ground material placed in a 50 $cm^3$ cylindrical extractor (14 mm I.d. and 320 mm length). The extraction

vessel was lined with cotton wool on the top and bottom to prevent any leakages. The temperature of the extraction vessel is controlled by the surrounding heating jacket. The volume of consumed $CO_2$ was measured by a rotameter float ball and a digital counter mass flow in standard liters per minute (SL / min) in the standard state ($P_{CO2}$ = 100 kPa, $T_{CO2}$ = 20 °C, $\rho_{CO2}$ = 0.0018 g/mL). The $CO_2$ flow rate was maintained at, 2 L/min using standard conditions, the time for static extraction was 10 min. Extraction pressure and temperature (45 MPa, 60 °C) giving high extract yields were selected based on previously reported data for various plant materials [88, 89 and 90]. The extracts were collected into glass bottles at room temperature and atmospheric pressure. The extracts were stored at −20 °C until analysis.

### 3.2.3. Pressurized liquid extraction *of Astragalus glycyphyllos*

Pressurized liquid extraction (PLE) was carried out in an extraction apparatus by accelerated solvent ASE 350 (Dionex, Sunnyvale, CA, USA) for each sample consecutively applying different solvent polarity. 7 g of initial material were mixed with 12 g of diatomaceous earth and placed in a cell 66 ml Dionex stainless steel (2.9 cm diameter). The cells were equipped with a stainless-steel frit and a cellulose filter, with the ends of the cell being used to prevent the solid particles entering the collection vial. The extraction was performed sequentially with lower to higher polarity solvents, which were acetone and a mixture of ethanol/water (70/30, v/v) and water. Extraction time and pressure was 15 min and 10.3 Pa respectively, temperature was maintained at 70 °C and 140 °C. Evaporation of organic solvents was done in a rotary vacuum evaporator at 40 °C. The residual water was removed by using a freeze dryer. Dry extracts were kept under nitrogen flow for 20 min and stored in dark glass bottles at −18 °C. Choosing PLE parameters was based on the previous studies [91], which has shown that a higher temperature increases the yield of the extract, although the production time is reduced. In addition, the increase in temperature had no negative impact on phytochemicals and antioxidants power indicators extracts.

## 3.3. Measurements of antioxidant capacity

*Astragalus glycyphyllos* leaves extracts, unextracted plant material and residues remaining after extractions were estimated using model radical scavenging reaction systems with 2, 2-diphenyl-1-picrylhydrazyl (DPPH·). Furthermore, the antioxidant activity of

conventional *Astragalus glycyphyllos* were determined using 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS+·) radical, ferric reducing ability of plasma (FRAP) assays, oxygen radical absorbance capacity (ORAC) assays and total phenolic content (TPC). In additional, more recently developed QUENCHER analytical procedures to solids.

### 3.3.1. 2,2-Diphenyl-1-picrahydrazyl (DPPH) method

Radical scavenging activity of plant extracts against stable DPPH• (2, 2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically according to a modified [93] method. The solution of DPPH• in methanol (6×10-5 M) was prepared daily, before measurements by UV spectrophotometer (Spectronic Genesys 8, Rochester, USA). DPPH• working solution was diluted with methanol to an absorbance of 0.800 ± 0.030 at 515 nm (this solution is stable only for 1 day, so it need to prepare the same day), 1000 μL of A. glycyphyllos leaves extracts (or MeOH as blank) is added and 2000 uL of a DPPH methanolic solution. All mixtures were left in dark and absorbance was measured after 2 hours at 515 nm. Trolox solutions at the various concentration (0 – 60 μM/L MeOH) used for calibration and the final results are expressed in μM TE/g extract and g of sample DW. TEACDPPH was calculated by means of a dose–response curve for Trolox (y = 1.658x – 0.593; R2 = 0.995), as can be seen in (Annex 1). The experiments were carried out in triplicate. The capability to scavenge the DPPH• expressed as % of inhibition, was calculated using the following formula:

I% = (Abs blank -Abs sample)/Abs blank *100

These percentages were transferred on a graph against the concentration of the Trolox divided by the amount of initial DPPH.

### 3.3.2. 2,2-Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) method

Experiments were carried out with ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (Sigma-Aldrich Chemie, Steinheim, Germany) decolourisation test [92]. ABTS + was obtained by reacting ABTS radical cation potassium persulfate. Stock solution of ABTS (2 mmol/L PBS) was prepared by dissolving 50 ml of phosphate buffered saline (PBS) solution (75 mmol/L; 7.4 pH) is obtained by dissolving 0.27 g KH2PO4 (Jansen CHIMICA, Beerse, Belgium), 1.42 g Na2HPO4 8.18 g NaCl and 0.15 g of KCl (Merck, Darmstadt, Germany), 1 L of distilled water. Adjust pH 7.4 using

NaOH solution. ABTS + was obtained by reacting 50 ml ABTS stock solution with 200 µL K2S2O8 solution and keep the mixture in the dark at room temperature for 15-16 h prior to use. When stored in the dark at room temperature, the radical is stable in this form for more than 2 days. ABTS + solution was diluted with PBS to obtain from 0.700 ± 0.030 absorbance at 734 nm before the measurements are taken. A. glycyphyllos leaves extracts or Trolox solutions (50 µL) were reacted with 3000 µL of ABTS + solution mixture left in dark for 2 hours and absorbance was measured at 734 nm in spectrophotometer. Trolox solutions (0-2000 µM/L) were used for calibration.

The RSC of ABTS•+ was calculated using the formula: [(Abscontrol-Abssample)/ (Abscontrol)] ×100, where Abs-control and Abs-sample are the absorbance of ABTS•+ in control mixture with methanol and the extract respectively. RSC values were expressed in µmol Trolox equivalents (TE) per g extract (µM TE/g) and g of sample DW by means of a dose–response curve for Trolox (y = 0.044x + 1.856; R2 = 0.986), as can be seen in (Annex 2).

**3.3.3. Ferric ion reducing antioxidant power (FRAP) assay**

The ability of A. glycyphyllos leaves extracts to reduce ferric ion to ferrous one, FRAP assay is carried out by the method [95] with some modifications. By mixing 100 mmol/L TPTZ (in 40 mmol/L HCL), 200 mmol/L FeCl3.6H2o and 0.3 mmol/l sodium acetate buffer (pH 3.6), in ratio 1:1:10 FRAP reagent was prepared. For the analysis, 50µL of sample or MeOH (blank) was mixed with 150µL of distilled water and 1500µL of freshly prepared FRAP was kept in dark for 2 hrs. And absorbance of the optically clear supernatants was measured at 593 nm. Trolox solutions (50µL) at various concentrations (0-500 µM Trolox equivalents (TE) /L MeOH) are used for calibration. TEACFRAP is calculated by means of a dose–response curve for Trolox (y = 0.0014x +0.0805; R2 = 0.9956), as can be seen in (Annex 3).

**3.3.4. Oxygen radical absorbance capacity (ORAC) method**

The ORAC test advantage is that it uses a biological source with a radical. The reaction is carried out in 75 mM phosphate buffer (pH 7.4), fluorescein stock solution prepared according to [94]. Samples were prepared by dissolving in methanol Astragalus glycyphyllos leaves extracts. Prepared the plant samples or Trolox (25 µl) and fluorescein (150 µL; 14 µM) solutions were added to 96 wells of a black opaque microplates with

transparent flat bottom. The microplates were sealed and incubated for 15 minutes at 37 °C. After incubation, AAPH solution as peroxidation radical generator (25 µL 240 mM) was added manually with a multichannel pipette. Microplate was immediately placed in FLUO star Omega fluorescent reader. Plate with the samples shaken before each reading. Fluorescence measurements (excitation wavelength 485 nm; emission wavelength 510 nm) were read every 66 s, a total of 120 cycles. Fluorescein and AAPH solutions were prepared fresh every day. Aqueous solutions Trolox were used for calibration (6.5 to 160 µM). Antioxidants curve (fluorescence *vs* time) were normalized under the fluorescence decay curve (AUC) was calculated as $AUC = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$ the area where the initial fluorescence F0 0 minutes FI is fluorescence time i. The final ORAC values were calculated using a regression equation between Trolox concentration and the net area under the curve (AUC). Antioxidant activity was expressed in micromoles TE / g and g sample DW. $TEAC_{ORAC}$ was calculated using a dose - response curve Trolox (y = 0.1597x + 7.4387; R2 = 0.9953), as can be seen in (Annex 4).

### 3.3.5. Total phenolic content

The TPC was determined in medicinal plant extract using the method of [96] with slight modifications. 150 µL of appropriate dilutions of gallic acid solution or *A. glycyphyllos* leaves extracts were oxidized with 750 µL Folin-Ciocalteau's reagent (2M), solution in deionized water (1:9 v/v). The reagents were mixed, allowed to stand for 3 min and then neutralized with 600 µL of 7 % $Na_2CO_3$. The mixture was left in dark for 2 hours with the absorbance being measured at 765 nm with a spectrophotometer. Using gallic acid calibration curve, the TPC was calculated and expressed in mg gallic acid equivalents per g extract (mg GAE/g) and per g of sample dry weight (DW) using a dose–response curve for gallic acid (y = 0.003x + 0.067; R2 = 0.997), as can be seen in (see Annex 5).

### 3.3.6. Antioxidant activity assessment of solid substances by QUENCHER method

Total antioxidant capacity measurement with the modified ORAC, $ABTS^{\bullet+}$ and TPC are carried out by QUENCHER method [97]. Directly applied to the solid ground material and *Astragalus glycyphyllos* residue after SCE-$CO_2$ extraction.

For TPC, 10 mg of sample or solid dilutions in microcrystalline cellulose (black) were transferred to centrifugation tube, were mixed with 150 µL of distilled $H_2O$, 750 µL of

Folin–Ciocalteu's reagent (2 M), 600 µL of $Na_2CO_3$ solution, vortexed for 15 s, shaken at 250 rpm for 2 h in the dark, centrifuged (4500rpm, 5 min) and the absorbance of optically clear supernatant is measured at 760 nm. Gallic acid solutions (150 µL) are measured at various concentrations (0–80 µg/mL) were used for calibration. The samples of TPC are expressed as equivalents of gallic acid (mg GAE/g sample) using a dose–response curve for gallic acid (y = 0.012x + 0.052; R2 = 0.996), as can be seen in (Annex 6).

In $ABTS^{\bullet+}$ scavenging assay, ten mg of sample or solid dilutions in microcrystalline cellulose (blank) were transferred to a centrifugation tube, mixed with 25 µL of MeOH and 1500 µL of working $ABTS^{\bullet+}$ radical solution, vortexed for 15 s, the working solution was kept in shaker at 250 rpm for 2 h in the dark and centrifuged (4500 rpm, 5 min). The absorbance of optically clear supernatant was measured at 734 nm. Trolox solutions (25 µL) was at various concentrations from 0 to 1600 µmol/L MeOH) were used for calibration. Using a dose–response curve for Trolox, $TEAC_{ABTS}$ was calculated ($y = 0.050x - 2.533$; $R^2 = 0.997$), as can be seen in (Annex 7).

In ORAC assay, 10 mg of sample or solid dilutions in microcrystalline cellulose (blank) was transferred to a centrifuge tube, mixed with 150 µl of PBS (75 mmol / L) and 900 µl of a solution fluorescein (14 µmol / L PBS), vortexed for 15 seconds, stirring at 250 rpm for 30 min in the dark and centrifuged (4500 rpm for 5 min). Optically clear supernatant (175 µL) was transferred to the opaque black 96-well microplates, pre-incubated for 15 min at 37 ° C, followed by rapid addition of 25 µL AAPH solution (240 mmol/L) as a generator peroxyl radicals using a multichannel pipette. Fluorescence was recorded every 66s cycle, a total of 150 cycles. An experimental subsequent treatment and time was the same as those for the analysis of the extract. Trolox solutions (150 µL) at various concentrations (0 to 160 µmol / L of PBS) were used for calibration. TEAC ORAC was calculated using a dose - response curve to Trolox (y = 0.1662x + 0.6415, R2 = 0.9948), as can be seen in (Annex 8).

All methods, when samples are put too high antioxidant activity, they were diluted with the microcrystalline cellulose as an inert material. Results are given in micromoles TE / g.

### 3.4. Identification of compounds by UPLC-Q/TOF

An Acquity UPLC [98] system with a binary solvent system of distribution, an auto sampler with a sample loop 10 μL, a photodiode array (PDA) detector, a manager of the column and a data station execution of acquisition and data from Compass software (Waters, Milford, MA, USA) combined with a Bruker Maxis UHR –TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) has been used. Acquity BEH C18 column (1.7 μM, 50 × 2.1 mm inside diameter) was used for the separation of compounds at 25 ° C. Cell phase was initially composed of 95 % eluent A (0.4% v / v, formic acid solution of ultra-pure water), and 100 % B (acetonitrile), then linear gradient from 5% to 25% of eluent B for 8.5 min. During 1.5 min, eluent B was increased linearly from 25% to 100%, during which it was maintained for 2 min. Finally, the initial conditions were reintroduced for 1 min and equilibration time of 1 min (column was also balanced for 1 min before each race). With the effluents being monitored at 254 nm, the flow was observed at 0.3 ml / min. Effluents from the PDA detector were introduced directly in the mass spectrometer UHR - QTOF equipped with an ESI source. Instrument control and data acquisition were obtained using the Compass 1.3 software (HyStar 3.2 SR2). MRS experiments were performed in negative ionization mode. The end offset plate was 500 V with capillary voltage being maintained at 4500 V. Nitrogen was used as the drying and nebulizing gas at a rate of 10.0 L / min and a flow pressure 2.0 bar respectively. Nitrogen was introduced into the collision, the cell as a collision gas. Identification of the peaks was performed by comparing the retention times with those of the corresponding chromatograms peaks standards or by their accurate masses.

### 3.5. Antimicrobial activities of plant extracts

#### 3.5.1. Preparation of plant extracts

Dried leaves and blossoms of *A. glycyphyllos* leaves were ground in an ultra-centrifugal rotor mill. Ground sample was additionally sieved by using 0.2 mm sieve. Obtained plant material has been used for preparation of extracts using Soxhlet apparatus (87) and different solvents (hexane, acetone and ethanol/water (70:30). Additionally, plant material has been bio-treated using LAB solid state fermentation 50% with following Soxhlet extraction using the same solvents (see above). The antibacterial test all the extract was dissolved in the appropriate solvent having a concentration of 10 mg/ ml.

### 3.5.2. Preparation of LAB strain for anti-microbial activity

*Lactobacillus sakei* KTU 05-06, the strains are isolated from Lithuanian rye sourdoughs because of their preliminary inhibit properties [99] produced by the culture collection of Kaunas University of Technology, Department of Food Science and Technology. The LAB strains were usually grown in MRS (de Man Rogosa Sharpe). The optimum temperature is maintained between 25-30˚C depending upon the cultured LAB in the inoculum. Later, these inoculum seeds were propagated in MRS medium for another 24 hours. 2% LAB cells were inoculated into fresh medium and propagated 18 h. The obtained cells from the supernatant by centrifugation at 6000g for 10 minutes, at 4˚C, are subjected to 0.2 µm sterile Millipore filtration to remove cells and they were subjected to antimicrobial activity.

## 3.6. Cultured conditions

### 3.6.1. Indicator bacterial strains

Five bacterial isolates from different sources (vegetables, meat, fat, and water) were used as indicator organisms for testing of antimicrobial activities in this study and their isolation sources are listed in (Table 3.1.). These bacterial strains belong to the species *B. subtilis, Bacillus cereus, Staphylococcus aureus, Salmonella typhimurium and E. coli* ATCC 25922 were obtained from the collection of Kaunas University of Technology and food was used as indicator organisms for testing of antimicrobial activity.

**Table 3.1.** Indicator bacterial strains used in the study and their isolation sources.

| **MICRO ORGANISM** | **ISOLATION SOURCE** |
|---|---|
| *Escherichia coli ATCC 25922* | Water |
| *Salmonella typthimurium* | Daily products |
| *Staphylococcus aureus* | Daily products |
| *Bacillus suptilis* | Daily products |
| *Bacillus suptilis sub species spizizenii* | Daily products |

### 3.6.2. Indicator fungi strains

Nine indicator fungal strains were determined against *Debaryomyces vanrijiae, Geotrichum fermentants, Candida pelliculosa, Pichia farinose, Pichia fermentans, Candida*

*krusei, Kluyveromyces marxianus, Kluyveromyces lactis var latis, Aureobasidum pullulans* isolated from grain-based food, fruit, vegetables and meat in (Table 3.2.). These indicator fungi strains were obtained from the collection of the Institute of Botany of Nature Research Centre.

**Table 3.2.** Indicator antifungal used in the study and their isolation sources.

| **MICRO ORGANISM** | **ISOLATION SOURCE** |
|---|---|
| *Debaryomyces vanrijiae* | Cabbage |
| *Geotrichum fermentants* | Daily products |
| *Candida pelliculosa* | Wheat |
| *Pichia farinose* | Daily products |
| *Pichia fermentans* | Daily products |
| *Candida krusei* | Daily products |
| *Kluyveromyces marxianus* | Daily products |
| *Kluyveromyces lactis* | Daily products |
| *Aureobasidum pullulans* | Daily products |

### 3.6.3. Maintenance and growth of indicator strains

Indicator strains were propagated as follows*: S. aureus, S. typhimurium and E. coli* were cultured at 37˚ C in a nutritional medium (per 1 L used: 5 g peptone, by 1.5 g meat and yeast extract and 5 g NaCl); whereas *Bacillus sp*. were grown in a brain heart infusion (BHI) broth (OXOID) at 30˚ C. All fungi were cultured on YEPD medium (per 1 L: 10 g yeast extract, 20 g peptone, 20 g glucose and 18 g agar) at 25˚ C.

### 3.6.4. Determination of antimicrobial activity

The antimicrobial activity of LAB strains were tested against strong solid medium which serves as a nutritional factor for the isolated microbes. The ability of LAB strains to produce antimicrobial metabolites has been tested using an agar well diffusion test by [100]. The suitable solid medium was used for each microorganism indicator. Fungi and yeasts were growing in an incubator at 25˚C on YEPD media for days 3-7, while the bacteria were propagated 3 days at optimum temperatures, where the details are given above.

Spores and cells were harvested from cultures after growth tilted to prepare inoculants containing spores of ~$10^5$ spores/cells $ml^1$ of fungi and ~ $10^8$ spores/cells ml of bacteria. The

solid agar medium is prepared by adding agar to the broth medium. The 100 µl of agar medium is poured into the petri dish. Further, it is autoclaved for 20 minutes at 121˚C. The sterilized medium is taken under the laminar airflow chamber. The working area is sterilized and one should make sure the working area in the air flow chamber is fully free from harmful microorganisms. Now a sterile loop or micropipette is used to inoculate 100 µl of microbial strain obtained from the culture supernatant (10 µl) along with the fermented, non-fermented samples or extraction solvent for negative control. The above sample is loaded onto the well (6 mm) in diameter created on the agar medium using filter paper. The bacteria were incubated at 37˚C for 24 hours and all fungi incubated at 25˚C for 48 hours. After the incubation time, the zone of incubation is noted and measured to record the antimicrobial activities against inculcated fungi and bacterial strains. The clear zone of inhibition is measured in terms of mm.

### 3.7. Statistical analysis

All measurements were performed in triplicate and expressed as an average. Standard deviations were calculated for each result. The calculations of radical scavenging capacity and the concentration of total phenolic in the extracts were performed by using MC Excel software. Statistical analysis of the determined results were followed by one-way ANOVA, using Expert Design 8 (for optimization results) and SPSS Version20.0 IBM software. Significant differences among the extracts determination were analyzed by Tukey's range test $p < 0.05$, after the analysis of variance have been done. Antimicrobial activity: All experiments were performed at least in three independent experiences. The means and standard deviations of data were calculated.

# RESULTS AND DISCUSSION

## 4.1. Extraction yield of *Astragalus glycyphyllos* leaves

Plants biosynthesize different classes of organic compounds, which can be distinguished by using different polarity solvents, and the yield is highly dependent on the solvent used for the extraction of them. To develop this approach, a complete isolation of valuable components is an important task for the evaluation of plant antioxidant potential and in this case, can be applied by two methods: (I) extraction of all materials with a variety of solvents, and (ii) step-by-step extraction with different polarity solvents. Therefore, preparation of fractionation schemes can provide materials with different properties that can be further adapted to specific needs of a variety of products. Therefore, yield of components is very important, especially for the production processes of commercialization. For example, agrorefinery of *Tenacetum vulgare* was used hydrodistillation extraction methods obtained four different extracts yields 0.47, 2.15, 4.26 and 22.96%, having various antioxidant activities and consisting of different phytochemicals [101].

The first step of *Astragalus glycyphyllos* leaves fractionation was designed to isolate low polarity lipophilic molecules. Those molecules are an interest to use in the cosmetics industry, food and pharmaceutical. Extract yield to obtain non-polar solvents, pressurized liquid extraction (PLE), Soxhlet extraction and supercritical fluid extraction (SFE-$CO_2$)

The yield of the Soxhlet extractions have been found to be in the following order: hexane < acetone < ethanol/water (3.5% < 3.6% < 30%) respectively in Table 4.1.

The extraction yield with ethanol/water as solvent is almost 10-fold higher than average yields obtained by n-hexane and acetone. SFE - $CO_2$ experiments revealed that this method was 2.3 % of lipophilic yield extract. The yield of the PLE was 3.7% and 29.3% for acetone and ethanol/water respectively. Consequently, the yield of ethanol/water (70:30 v/v) extracts obtained from Soxhlet extraction, PLE was found to be similar, having the highest yield among all the extracts. However, the extraction process in PLE method was faster by 15 times.

**Table 4.1.** The yield of *Astragalus glycyphyllos* leaves extracts (mean ±SD; n=3), various superscripts in the same column show real significant differences among the extracts determination were analyzed by one way ANOVA and Tukey's test. $P < 0.05$.

| Extraction | Solvent | Extracts (Extractive yield, %) |
|---|---|---|
| Soxhlet extraction | Hexane | $3.56\pm0.10^{a}$ |
| Soxhlet extraction | Acetone | $3.66\pm0.14^{a}$ |
| Soxhlet extraction | Ethanol/Water 70:30) | $30.42\pm1.69^{b}$ |
| Supercritical fluid extraction (SFE) | $CO_2$ | $2.31\pm0.14^{a}$ |
| Pressurized liquid extraction (PLE) | Acetone | $3.77\pm0.21^{a}$ |
| Pressurized liquid extraction (PLE) | Ethanol/Water (70:30) | $29.32\pm0.86^{b}$ |

Previous Studies on *Astragalus brachypterus* Fischer reported the yield range of the extractions in water and ethanol (8.62% and 7.6%) respectively [102]. However, our study determines the extraction yield of Ethanol/ Water. (70:30 v/v) and from the table 4.1, it can be observed that the percentage of yield was greater in the Ethanol/Water method as compared to the individual yield percentages of ethanol and water.

The highest yield of extraction of the aqueous solution can be attributed to the increased polarity of the solvents by addition of water [103]. High temperature and solid-liquid ratio used in the aqueous extraction may also explain the increase in the extraction yield obtained. The polar solvent is often employed for the regeneration of plant polyphenols matrix.

## 4.2. Antioxidant activity of *Astragalus glycyphyllos* extracts

### 4.2.1. DPPH radical scavenging activity

DPPH method is used to analyses the antioxidant activity of *Astragalus glycyphyllos* extracts. DPPH is a stable free radical, which represents the maximum absorption at 517 nm in methanol. In the test, antioxidants were able to reduce the stable DPPH radicals

instead of 517 nm absorption. Yellow -colored diphenyl-picrylhydrazine is obtained after the stable radical DPPH gets reduced. The reduction of methanolic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction is the basis of this method [110].

Harmful role of free radicals (FR) in biological systems is checked by Scavenging of free radicals. A stable nitrogen targeted lipophilic FR, DPPH, is a widely used for free radical scavenging activity as the results can be obtained faster when compared with other methods. The antioxidant activity is estimated by usually using DPPH as a substrate.

The DPPH scavenging activity of different extracts of *Astragalus glycyphyllos* leaves determined in vitro. DPPH radical scavenging activity is indicated in Table 4.2.

**Table 4.2.** Radical scavenging effect on DPPH and FRAP of studied of *Astragalus glycyphyllos* leaves extracts (mean ±SD; n=3), various superscripts in the same column show real significant differences among the extracts determination were analyzed by one way ANOVA and Tukey's test. $P < 0.05$.

| Plant Name | Extraction | Solvent | Antioxidant Activity | | | |
|---|---|---|---|---|---|---|
| | | | DPPH | | FRAP | |
| | | | Extracts $\mu M\ TE/g$ | DW $\mu M\ TE/g\ DW$ | Extracts $\mu M\ TE/g$ | DW $\mu M\ TE/g\ DW$ |
| *Astragalus glycyphyllos* | Soxhlet extraction | Hexane | 2621±6.5[a] | 93.41±6.5 | 1086±22.28[a] | 38.70±22.28 |
| | | Acetone | 3572±11.27[b] | 98.58±11.27 | 2740±28.28[b] | 75.62±28.28 |
| | | Ethanol /Water (70:30) | 4128±34.44[c] | 1297. ±34.44 | 3117±21.38[c] | 979.36±21.38 |

The antioxidant activity (AO) values by DPPH method for the Soxhlet hexane, acetone and ethanol/water extracts are as follows: 2621±6.5 µM TE/g, 3572±11.27 µM TE/g and

4128±34.44 µM TE/g. DPPH values expressed in µM TE/g DW were: 93.41±6.5 (hexane), 98.58±11.27 (acetone), 1297±34.44(ethanol/water ).

The scavenging activities of extracts are ordered thusly: ethanol/water (70:30) > acetone > hexane. Scavenging free radicals is known to inhibit lipid oxidation that might otherwise be harmful to the functions and components of a cell [111]. Natural antioxidants were used for replacing the synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), which are currently used in the food industry, could have mutagenic activity [112]. With its radical scavenging potential, *Astragalus glycyphyllos* leaves extract may help protect the human body from oxidative damage that can lead to health related degenerative diseases.

**4.2.2. ABTS radical scavenging activity**

The radical scavenging activity of plant extracts and solid residues was measured using DPPH, ABTS assay by colorimetric methods. In ABTS test, the presence of antioxidants causes the conversion of the green / blue color ABTS radical cation to its colorless neutral form. This method is unique for its ability to assess the radical scavenging activity of hydrophilic antioxidants.

ABTS method provides a certain number of advantages over DPPH test, though both are relatively quick and easy to perform. While DPPH method is only limited to higher pH applications. ABTS method can be used over a wide pH range. Moreover, ABTS is soluble in aqueous and organic solvents used in the study and measurement of hydrophilic and lipophilic antioxidants conducting the investigation buffer media and organic solvents [113] or by partitioning antioxidant mixtures between hexane and aqueous solvent. However, water-soluble reactions seem to be preferred [114]. Only if they have a redox potential less than ABTS (0.68 V), Antioxidant compounds can react with ABTS. Most of the phenolic compounds have a lower redox potential as ABTS. The color interference is not the problem of ABTS, in contrast to DPPH method. Color interference DPPH samples containing anthocyanin leads to a lack of antioxidant activity.

The $ABTS^{\bullet+}$ scavenging capacities of the samples are presented in Table 4.3.

**Table 4.3.** Radical scavenging effect on ABTS and ORAC of studied of *Astragalus glycyphyllos* leaves extracts (mean ±SD; n=3), various superscripts in the same column show real significant differences among the extracts determination were analyzed by one way ANOVA and Tukey's test. $P < 0.05$.

| Plant Name | Extraction | Solvent | Antioxidant Activity | | | |
|---|---|---|---|---|---|---|
| | | | ABTS | | ORAC | |
| | | | Extracts $\mu m\ TE/g$ | DW $\mu m\ TE/g$ DW | Extracts $\mu mTE/g$ | DW $\mu mTE/g$ DW |
| *Astragalus Glycyphyllos* | Soxhlet extraction | Hexane | 1513±15.59[a] | 53.92±15.59 | 2746± 0.41[a] | 97.86± 0.41 |
| | | Acetone | 2683±93.14[c] | 74.05±93.14 | 3925± 2.04[b] | 108.33± 2.04 |
| | | Ethanol/ Water (70:30) | 3112±44.11[d] | 977.79±44.11 | 6167 ±5.08[d] | 1850.14±5.08 |
| | Supercritical fluid extraction (SFE) | $Co_2$ | 1628±38.64[b] | 36.57±38.64 | 4077 ± 2.27[c] | 91.58± 2.27 |
| | Pressurized liquid extraction (PLE) | Ethanol/ Water (70:30) | 3156 ± 73.11[d] | 856.60± 73.11 | 6393 ± 2.21[e] | 1874.42± 2.21 |

Values obtained for the hexane, acetone and ethanol/water (70:30) extracts from Soxhlet extraction were 1513±15.59 µM TE/g, 2683±93.14 µM TE/g and 3112±44.11 µM TE/g respectively. Similarly, the ABTS values for SFE – $CO_2$ extract was 1628±38.64 µM TE/g and PLE ethanol/water (70:30) extracts were found to be 3156 ± 73.11 µM TE/g. Again, ethanol/water extracts exhibited the highest $ABTS^{\bullet+}$ scavenging capacity expressed in µM TE/g DW, the antioxidant (AO) values are as follows: In Soxhlet extraction SE ethanol/water (70:30): 977±44.11, acetone (74.05±93.14), hexane (53.92±15.59), supercritical fluid extraction SFE-$CO_2$ (36.57±38.64) and pressurized liquid extraction PLE

ethanol/water (70:30) 856.60± 73.11. Antioxidant activity following ordered: SE (ET/W 70:30) > PLE (ET/W 70:30) > SE (ET) > SE (HX) > and SFE- $CO_2$.

Previous studies on *Astragalus psilocentros* roots revealed the antioxidant capacity by using various solvents such as hexane (4.56 ± 0.70) TE (g/mL), chloroform (15.29 ± 0.71) TE (g/mL), acetate (21.83 ± 1.15) TE (g/mL) and n-butanol (9.98 ± 1.05) TE (g/mL) [161]. Notable in our study, antioxidant capacity of *Astragalus glycyphyllos* of the hexane leaf extract was measured higher antioxidant activity than *Astragalus psilocentros* of the hexane root extract.

In addition, *Astragalus taipaishanensis* roots was investigated the antioxidant capacity by different solvents like ethanol (843.14 ± 4.58) μM TE/g of extract, ethyl acetate (994.50 ± 4.2) μM TE/g of extract, petroleum ether (823.09 ± 4.65) μM TE/g of extract, butanol (914.31 ± 5.78) μM TE/g of extract and water (333.93 ± 2.43) μM TE/g of extract [109]. Presented with two literatures, our studies on *Astragalus glycyphyllos* leaves extracts obtained higher antioxidant capacity in all solvent fractions.

**4.2.3. Ferric reducing antioxidant power assay (FRAP)**

FRAP assay developed by Benzie and Strain is used to determine the ability of the plants extracts to reduce ferric ions [95]. An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous-TPTZ (Fe (II)-TPTZ) complex which absorbs strongly at 593 nm.

The redox potentials of the compounds under study determines the antioxidant efficiency estimated by the present FRAP assay, its also characterized by the complexity of their molecules. The results obtained indicate that the reducing ability of *Astragalus glycyphyllos* and ET/W (70:30) extracts, as determined by the FRAP assay, could be dependent on the degree of hydroxylation and extent of conjugation of the phenolic compounds.

Similarly, the FRAP assay was also performed in Table 4.2 to estimate the antioxidant activity of the extracts: hexane: 1086±22.28 μM TE/g; acetone: 2740±28.28 μM TE/g and ethanol/water: 3117±21.38 μM TE/g. The DW in terms of μMTE/g for the FRAP assay for *Astragalus glycyphyllos* was found to be: 38.70±22.28 for Hexane, 75.62±28.28 for Acetone and 979.36±21.38 for Ethanol/water respectively. By performing FRAP assay it

can be observed that the effective AO concentration was found to be the highest in the ethanol/water gradient.

In this assay, Soxhlet Extraction (hexane, acetone and ethanol/water (70:30) extracts antioxidant compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before the complex is formed. Chelating agents reduce the redox potential by which, the oxidized form of the metal ions are stabilized, making Chelating agents to serve as secondary antioxidants. The present study reveals that the ET/W (70:30) extract of *Astragalus glycyphyllos* has a notable iron binding capacity in Table 4.2, suggesting that their action acts as a peroxidation protector. It is interesting that ET/W (70:30) extract is also quite a good ferric reducing agent, while its radical scavenging activity was quite low, in comparison with the rest of the extracts of this plant's aerial parts. Furthermore, ET/W (70:30) extracts were better reductants than the ones extracted with acetone and hexane.

The previous studies investigated from *Astragalus psilocentros* found the ferric reducing antioxidant capacity using different solvents such as hexane ((19.16 ± 0.92) TE (g/mL), chloroform (45.97 ± 0.54) TE (g/mL), ethyl acetate (56.89 ± 0.39) TE (g/mL) and n-butanol ((39.69 ± 0.52) TE (g/mL) [115]. It can be noted that the ferric reducing antioxidant capacity is higher in the hexane fraction from our studies on *Astragalus glycyphyllos* compared to the previously reported studies.

In addition, *Astragalus taipaishanensis* roots was investigated for their ferric reducing antioxidant capacity by using different solvents such as ethanol (355.85 ± 2.23) μM TE/g of extract, ethyl acetate (685.67 ± 3.21) μM TE/g of extract, petroleum ether (244.46 ± 2.49) μM TE/g of extract, butanol (395.02 ± 2.62) μM TE/g of extract and water (126.22 ± 1.98) μM TE/g of extract [109]. However, it can be observed from table 4.2 that the ferric reducing antioxidant capacity is greater in *Astragalus glycyphyllos* leaves as compared to the previously reported root investigations on *Astragalus taipaishanensis* roots.

**4.2.4. Oxygen radical absorbance capacity (ORAC)**

There are advantages and disadvantages of ORAC assay method. One of the main advantages of the adaptation of the ORAC procedure is that, the analysis includes an adjustable source peroxidation radical that mimic the reaction of lipids with antioxidants in

the food and physiological systems. It can therefore be used to detect both lipophilic and hydrophilic antioxidants in accordance with change radical source and a solvent [116].

The antioxidant mechanism and protection to fluorescein antioxidants mimics the critical biological surfaces, researchers suggested that, the ORAC procedure is carried out. Thus, real reaction of biological substrates may not be the outcome of the response mechanism between the antioxidant and fluorescein, and this procedure may not reflect the samples' real antioxidant capacity [117]. A biologically related radical source is the deciding factor for the advantages obtained by using the ORAC assay against other assays for the studying the radical scavenging capacity evaluation.

The following ORAC values for *Astragalus glycyphyllos* extracts were calculated in Table 4.3. In Soxhlet extraction: hexane (2746±0.47) μM TE/g, acetone (3925±2.04) μM TE/g, ethanol/water (70:30) (6167 $\pm$5.08) μM TE/g and $CO_2$ (4077 $\pm$ 2.27) μM TE/g, respectively. The AO value for ethanol/water (70:30 %) extract obtained by Pressurized liquid extraction was 6393 $\pm$ 2.21 μM TE/g. Again, ethanol/water values were found to be the highest. However, due to differences in extract yields, ORAC values expressed in μM TE/g DW were also varying in a wider range. The ORAC (AO) values for dry weight are 97.86±0.47 (HE)**,** 108.3±2.04 (AC), 1850.14±5.08 (ET/W), 91.58$\pm$ 2.27 (SFE-$CO_2$) and 1874.42$\pm$ 2.21 (PLE- ET/W 70:30).

One study showed high content of antioxidants in *Astragalus* roots. Using the ORAC value estimation about 17,773 μ mol TE/100g, the antioxidant activity is detected by using an aqueous methanol extract, indicates that *Astragalus* is water soluble mainly, rather than fat-soluble. The quantity of antioxidants in the roots suggests that there may be some immune boosting benefits which is long claimed by Chinese medicine. One study of *Astragalus membranaceus* used extracorporeal shock-wave Lithotripsy (ESWL), which deliberately caused oxidative kidney damage in rabbits [118]. However, when compared with the untreated rabbits, the renal morphological disorders were much milder in the 15 rabbits treated with the root extract. In our study, ORAC assay seemed to be effective in determining the AO property for *Astragalus glycyphyllos.*

### 4.2.5. Evaluation of total phenolic content of *Astragalus glycyphyllos* extracts

Phenolic compounds present in edible and nonedible plants considered to have significant antioxidant property. These compounds extracted from the natural sources preferred by food industry because it not only diminishes the oxidation of lipids also adds further nutritional value. Also, these compounds protect against various heart diseases and act against many cancers [104].

Thus, it being to consider an important the effects of many different solvents used for extraction on the phenolic content of *Astragalus glycyphyllos* leaf extracts. Hexane, acetone and ethanol/water (70:30) were used to estimate the effects of the total concentration of phenol extracted from *A. glycyphyllos*. Due to its ability to donate elections and hydrogen atoms, phenolic compounds use their ability to induce anti-oxidant activity. As presented in Table.4.2, the content of phenolic compounds (mg GAE/g) in Soxhlet SFE-$CO_2$ and ASE extracts varied between 120 – 642.2 mg GAE/g. The content of phenolic compounds was determined from the regression equation of a calibration curve ($y = 0.003x + 0.067$; $R2 = 0.997$) and is expressed as gallic acid equivalents (GAE).

Ethanol/water extracts possessed the highest TPC values (642±3.22 mg GAE/g), followed by hexane and acetone extracts (388±3.22; 444±5.75) from Soxhlet extraction. In PLE-method, the TPC values of ethanol/water (70:30) was 620±5.69, while SFE- $CO_2$ extracts were 120±2.72, respectively. Due to remarkably higher yields isolated with ET/W (70/30), the latter solvent was the most effective in extracting phenolic compounds from the sample matrix. Thus, the amounts of TPC isolated with ethanol (201±3.22 mg GAE/g DW) were about 15 times higher when compared to hexane (13.8±3.2 mg GAE/g DW), acetone (12.2±5.75mg GAE/g DW) and 75 times when compared to SFE-$CO_2$ extracts (2.69±2.72 mg GAE/g DW).

Previous studies report no data on the total phenolic contents extracted from other species of *Astragalus* [105, 106]. In our studies the Phenolic contents using different extraction methods performed on *A. glycyphyllos* can be observed in Table 4.4.

**Table 4.4.** Total phenolic content in the investigated of *Astragalus glycyphyllos* leaves extracts (mean ±SD; n=3), various superscripts in the same column show real significant differences among the extracts determination were analyzed by one way ANOVA and Tukey's test. $P < 0.05$.

| Plant Name | Extraction | Solvent | Antioxidant Activity<br>Total Phenolic Contents (TPC) | |
|---|---|---|---|---|
| | | | Extracts<br>mg GAE/g | DW<br>mg GAE/g DW |
| *Astragalus Glycyphyllos* | Soxhlet Extraction | Hexane | 388±3.22[b] | 13.82±3.22 |
| | | Acetone | 444.5±5.75[c] | 12.26±5.75 |
| | | Ethanol /Water (70:30) | 642.2±3.22[d] | 201.7±3.22 |
| | Supercritical fluid extraction (SFE) | $CO_2$ | 120±2.72[a] | 2.69±2.72 |
| | Pressurized liquid extraction (PLE) | Ethanol/Water (70:30) | 620±5.69[d] | 181.78±5.69 |

The previous studies investigated, the same plants of the genus *Astragalus* leaf extract of phenolics were higher, while comparing with raw material extract. Increased accumulation of phenolic compounds in *Astragalus glycyphyllos* sheets can be explained as leaf tissue protection against UV light. Plants that give fluorescence to UV-B light, starts producing phenolic compounds and antioxidant enzymes that protects the leaf tissues from photo oxidations damage [107,108].

The ground parts of *Astragalus squarrosus* root was determined the phenolic contents found (23.3 mg / g). [160]. In our studies (Table 4.2) we found that the total estimated phenolic contents in extracts of leaves were significant higher that in *Astragalus squarrosus* root extracts.

Furthermore, in previous studies total phenolic content in *Astragalus psilocentros* using different solvents like hexane ((16.76 ± 0.81) GAE mg/g, chloroform (47.32 ± 0.29) GAE mg/g, ethyl acetate (53.69 ± 0.68) GAE mg/g and n-butanol (39.73 ± 0.73) GAE mg/g has been studied [161]. While comparing in our studies, it can be noted that the total phenolic content is found to be higher in the hexane fraction from our studies *Astragalus glycyphyllos* compared to the previously reported studies.

Moreover, the total phenolic contents from *Astragalus taipaishanensis* roots extracted by different solvents like ethanol (15.20 ± 0.30) mmol GAE/100 g, ethyl acetate (46.41 ± 0.34) mmol GAE/100 g, petroleum ether (15.28 ± 0.17) mmol GAE/100 g, butanol (23.12 ± 0.62) mmol GAE/100 g and water (5.43 ± 0.23) mmol GAE/100 g [109]. Notably this study showed higher extraction rates in all the fractions in Table 4.4 from our studies on *Astragalus glycyphyllos*.

### 4.3. Evaluation of antioxidant capacity of solid substances by QUENCHER method

It is known that certain antioxidative insoluble active ingredients can be strongly bound to other components of the plant material matrix and are thus not extracted from the solvent. Previously, QUENCHER procedure was developed to evaluate the antioxidant activity plant material, including the insoluble fraction [119]. The results of antioxidant activity by QUENCHER approach of unextracted plant material and *Astragalus glycyphyllos* residue after SCE-$CO_2$ extraction are shown in Table 4.5.

The unextracted plant material possessed the highest total Phenolic Content. Total phenolic content of dried *A. glycyphyllos* leaves were (241.84±1.52 mg GAE/g DW) and supercritical fluid extraction (SFE-$CO_2$) residue was 198.16± 1.04 mg GAE/g DW. $ABTS^{\bullet+}$ scavenging values varied from 1379±5.04 mg TE/g DW (SCE-$CO_2$ residue) to 1547±5.87mg TE/g DW (unextracted plant material). ORAC values for *Astragalus glycyphyllos* was (8538±0.92 mg TE/g DW) dry leaves and SCE-$CO_2$ residue 6240±2.27 mg TE/g DW. Several reasons can be considered between extractions and QUENCHER approach findings. These findings can be explained with various reasons. In particular, by using a series of extraction processes, most active compounds were isolated in the previous steps of production series. Next reason, as already mentioned, is that some of the

antioxidatively active compounds may remain after extraction in plant matrix because they are attached to other components.

**Table 4.5.** Antioxidant characteristics of solid substances of *Astragalus glycyphyllos* leaves measured by QUENCHER method (mean ±SD; n=3), various superscripts in the same column show real significant differences among the solid substances determination were analyzed by one way ANOVA and Tukey's test. $P < 0.05$.

| Plant Name | QUENCHER | Solvent | Antioxidant Activity | | |
|---|---|---|---|---|---|
| | | | TPC Mg GAE/g DW | TEAC (ABTS) µmol TE/g DW | ORAC µmol TE/g DW |
| *Astragalus Glycyphyllos* | Supercritical Fluid Extraction (SFE) | $CO_2$ - Residue | 198.16± 1.04[a] | 1379±5.04[b] | 6240±2.27[c] |
| | Unextracted Plant Material | - | 241.84±1.52[a] | 1547±5.87[b] | 8538±0.92[c] |

Therefore, the antioxidant potential of plant material and the numerous separation processes for more efficiency can be described by the of antioxidant assays to the extracts by using traditional methods and to the solids by a QUENCHER procedure.

To the best of my knowledge, this method has not been applied to the overall evaluation of *Astragalus glycyphyllos* antioxidant properties. In addition, it was not reported earlier for *Astragalus glycyphyllos* as well.

### 4.4. Characterization of phytochemicals by using UPLC-Q- TOF-MS/MS

Identification of compounds was performed by the spectral data obtained by UPLC/Q-TOF. The compounds were organized on the basis precise mass and isotopic patterns using

KEGG ChemSpider and Met Fusion databases. Retention times (TA), the measured mass and molecular ion for each of the identified compounds are summarized in Table 4.6.

**Table 4.6.** Identification of polyphenolic compounds presented in extracts from *Astragalus glycyphyllos* by UPLC -Q- TOF-MS/MS.

| NO | Molecular Formula | RT on UPLC | Compound Names | m/z, [M-H] | Solvent |
|---|---|---|---|---|---|
| 1 | $C_8H_{14}O_4$ | 2.3 | Suberic acid | 173.0825 | Hexane |
| 2 | $C_{26}H_{36}O_6$ | 4.3 | Not identified | 443.2444 | |
| 3 | $C_{18}H_{30}O_3$ | 5.5 | Glanvillic acid B | 293.2121 | |
| 4 | $C_{16}H_{22}O_4$ | 5.9 | 2-[(2-Octanyloxy) carbonyl] benzoic acid | 278.1446 | |
| 5 | $C_{18}H_{34}O_3$ | 6.4 | 12-Oxooctadecanoic acid | 297.2435 | |
| 6 | $C_{22}H_{30}O_2$ | 7.7 | Not identified | 325.2173 | |
| 7 | $C_{18}H_{32}O_3$ | 8.2 | Not identified | 279.2330 | |
| 8 | $C_8H_{16}O_8$ | 0.3 | Not identified | 239.0768 | Acetone |
| 9 | $C_{20}H_{32}O_{10}$ | 1.9 | Not identified | 431.1916 | |
| 10 | $C_7H_{12}O_2$ | 2.1 | ρ -hydro benzoic acid | 127.0761 | |
| 11 | $C_9H_8O_3$ | 2.3 | m-Coumaric acid | 163.0396 | |
| 12 | $C_{37}H_{64}O_{13}$ | 3 | Not identified | 715.4265 | |
| 13 | $C_{18}H_{32}O_6$ | 2.8 | Not identified | 343.2119 | |
| 14 | $C_{37}H_{62}O_{13}$ | 3.4 | Not identified | 713.4105 | |
| 15 | $C_{18}H_{32}O_5$ | 3.6 | Not identified | 327.2172 | |
| 16 | $C_{18}H_{34}O_5$ | 3.9 | (-)-pinellic acid | 329.2328 | |
| 17 | $C_{16}H_{32}O_4$ | 4 | Glaurin | 287.2223 | |
| 18 | $C_5H_{10}O_5$ | 0.3 | L-(+)-Ribose | 149.0456 | Ethanol/ Water |
| 19 | $C_7H_{12}O_6$ | 0.4 | Not identified | 191.0562 | |
| 20 | $C_8H_{16}O_8$ | 0.5 | Not identified | 239.0772 | |
| 21 | $C_6H_8O_7$ | 0.8 | isocitric acid | 191.0197 | |
| 22 | $C_{33}H_{40}O_{21}$ | 1.5 | Not identified | 771.1989 | |
| 23 | $C_{27}H_{30}O_{16}$ | 2.1 | Rutin | 625.1407 | |
| 24 | $C_{39}H_{62}O_{13}$ | 3.3 | Not identified | 755.4223 | |
| 25 | $C_{38}H_{60}O_{11}$ | 3.7 | Not identified | 709.4166 | |
| 26 | $C_{42}H_{62}O_{16}$ | 3.8 | Glycyruhinic acid | 839.4081 | |
| 27 | $C_{16}H_{32}O_4$ | 4 | Glaurin | 287.2225 | $Co_2$ |
| 28 | $C_{18}H_{26}O_4$ | 4.7 | 2-[(Decyloxy)carbonyl] benzoic acid | 306.3975 | |
| 29 | $C_{18}H_{32}O_4$ | 4.9 | 9-Octadecenedioic acid | 311.2224 | |
| 30 | $C_{18}H_{30}O_2$ | 5.1 | Not identified | 309.2068 | |
| 31 | $C_{18}H_{28}O_3$ | 5.9 | Not identified | 291.1961 | |
| 32 | $C_{18}H_{32}O_2$ | 6.2 | Linoleic acid | 295.2277 | |

The chromatogram showed large number of recorded peaks which indicate the presence of complex mixtures in the extracts. However, the identification of compounds is difficult based on the exact mass data obtained by UPLC-QTOF-MS because the mass spectra libraries give too many candidate structures for the huge measured masses. It has further complicated the identification minor components in the extracts which was the scope of the present study to identify the novel constituents for the commercial applications.

Compound 1 (m/z 173.0825) corresponded to the molecular formula $C_8H_{14}O_4$, and was tentatively identified as Suberic acid. The analysis also revealed the presence of acid derivative compounds with the same molecular formula. Compound 3 – (m/z 293.2121) corresponded to the molecular formula $C_{18}H_{30}O_3$ and was therefore tentatively identified as glanvillic acid. The compound 4 – (m/z 278.1446) corresponded to the molecular formula $C_{16}H_{22}O_4$, was therefore tentatively identified as 2-[(2-Octanyloxy) carbonyl] benzoic acid. Compound 5 – (m/z 297.2435) corresponded to the molecular formula $C_{18}H_{34}O_3$ and was therefore tentatively identified as 12-oxooctadecanoic acid derivative and found in *Astragalus Glycyphyllos* hexane fractions. Compound 10 – (m/z 127.0761) corresponded to the molecular formula $C_7H_{12}O_2$, therefore was tentatively identified as ρ -hydro benzoic acid. Compound 11 – (m/z 163.0396) corresponded to the molecular formula $C_9H_8O_3$ and was therefore tentatively identified as m-coumaric acid. The compound 16 with m/z of 329.2328 corresponded to the molecular formula $C_{18}H_{34}O_5$ and was therefore tentatively identified as (-)-pinellic acid in acetone fraction. Compound 18 – (m/z 149.0456) corresponded to the molecular formula $C_5H_{10}O_5$ and therefore was tentatively identified as L- (+) - ribose. Compound 23 with m/z value of 625.1407 corresponded to the molecular formula $C_{27}H_{30}O_{16}$ and hence was tentatively identified as rutin in ethanol/water (70/30%) fraction.). Compound 28 – (m/z 306.3975) corresponded to the molecular formula $C_{18}H_{26}O_4$ was therefore tentatively identified as 2-[(Decyloxy) carbonyl] benzoic acid. Compound 29 – (m/z 311.2224) corresponded to the molecular formula $C_{18}H_{32}O_4$ was therefore tentatively identified as 9-octadecenedioic acid. The compound 32 – (m/z 280.4457) corresponded to the molecular formula $C_{18}H_{32}O_2$ and provisionally was identified as linoleic acid in $CO_2$ fraction. Linoleic acid was tentatively identified based on that mass 279.2312 and predicted with the molecular formulas $C_{18}H_{32}O_4$ along with matching data found in the literatures

(Farag et al., 2014 and Sun et al., 2015). Other compounds with their respective [M-H] – m/z values do not identify with their corresponding molecular formula.

One previous study, *Astragalus membranaceus* roots was reported among 30 phenolic compounds such as ρ-hydroxybenzoic acid, m-coumaric acid and rutin. The phenolic compound could be separated in three groups like phenolic acids, flavonoids and miscellaneous [120]. In our study, the acetone fraction was determined in the phenolic acids ρ-hydroxybenzoic acid, m-coumaric acid and ethanol/water (70:30) were rutin (flavonoid) in *Astragalus glycyphyllos* leaves.

In our study, due to the diversity and complexity of the mixture of natural phenolic compound from Astragalus *glycyphyllos* extracts, it was hard to describe each compound and to evaluate or compare the antioxidant activity. Different solvent extracts have generally various phenolic compounds and each of these compounds were different from each other antioxidant activity. So, in this experiment, we can assume that the identification of phenolic compounds presented in the table 4.6.

### 4.5. Antibacterial activities of plant extracts and fermented with LAB plant leaves

The antimicrobial activities of extracts (Soxhlet extraction) from *A. glycyphyllos* leaves using different solvents were studied against indicator strains (Table 4.7). Besides, the effect of plant bio-treatment using solid state conditions (before extraction) on antimicrobial activities of extracts have been analyzed.

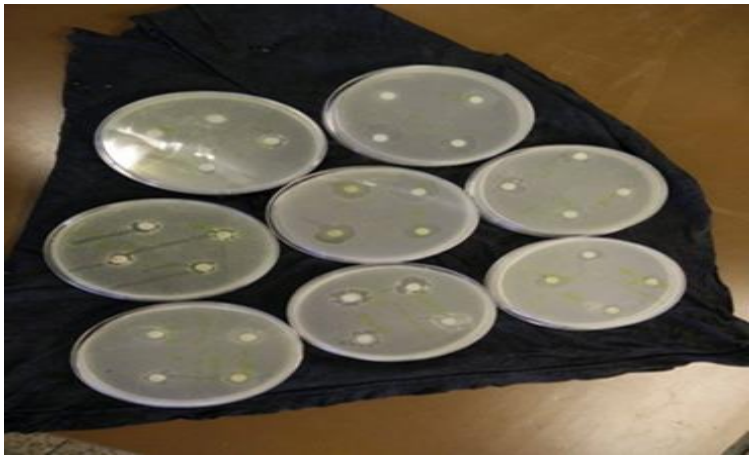

**Figure 4.1.** Zone of inhibition antibacterial

**Table 4.7.** Antibacterial activity of extracts and fermented using LAB and solid state conditions it's leaves, (-) no inhibition.

| Name of the Bacteria | Dried *Astragalus glycyphyllos* leaves powder | | Hexane Extract | | Acetone Extract | | Ethanol/Water (70:30) Extract | |
|---|---|---|---|---|---|---|---|---|
| | Non-fermented (mm) | Fermented (mm) | Non-fermented (mm) | Fermented (mm) | Non fermented (mm) | Fermented (mm) | Non-fermented (mm) | Fermented (mm) |
| *Escherichia coli* ATCC 25922 | 4±0.1 | 6±0.4 | - | 2.5±0.2 | 3±0.06 | 4±0.3 | - | - |
| *Salmonella typthimurium* | 5.5±0.5 | 7±0.3 | 3±0.2 | 6±0.4 | 5±0.2 | 6.5±0.4 | - | - |
| *Staphylococcus aureus* | 4±0.1 | 7.5±0.3 | - | 3±0.3 | 3±0.2 | 6.5±0.4 | - | - |
| *Bacillus subtilis* | 14±0.2 | 16.5±0.5 | 7±0.3 | 9±0.4 | 8±0.2 | 9±0.3 | 12±0.2 | 14.5±0.4 |
| *Bacillus suptilis sub species spizizenii* | 12±0.03 | 16±0.4 | 6±0.2 | 7±0.5 | 9±0.03 | 12±0.4 | 10±0.2 | 13±0.4 |

The influence of the type of solvents on the antimicrobial properties of plant extracts has been noticed. The highest antibacterial activity against *Bacillus subtilis* following by Soxhlet extraction using different solvent extracts: ethanol/water (70:30) > acetone > hexane has been determined. The ethanol extract, prepared by using lactic acid fermenting bacteria (LAB) for plant treatment, showed higher zone of inhibition against *Bacillus subtilis* than extracts without- plant fermentation (inhibition zone diameter - 14.5 ± 0.5 mm and 12 ±0.2 mm, respectively).

The positive influence of plant bio-treatment by using *L. sakei* on the extracts with antimicrobial properties was obvious. In all cases plant ethanol/water extracts showed higher zones of inhibition against *Bacillus subtilis sub spizizenii* than the extracts, prepared

without plant LAB fermentation: (inhibition zones diameters were 13 ± 0.4 mm and 10 ±0.2 mm, respectively) following by hexane (7 ± 0.5 mm and 6 ±0.2 mm) and acetone (12 ± 0.4 mm and 9 ±0.3 mm). Besides, LAB fermented plant extracts showed random clear zones of inhibition against *Salmonella typhimurium* and *Staphylococcus aureus*. The lowest zones of inhibition against *E. coli* by using fermented plant extracts have been also determined.

So, the higher level of inhibition zones occurs in the extracts, which were prepared by using plant bio-treatment and after an extraction procedure.

The previous studies determined that extracts, prepared using different solvents such as methanol, n-hexane, chloroform, ethyl acetate and butanol showed antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pasturell multocida* from *Astragalus psilocentros Fisch* [115]. In our study the obtained results of antimicrobial activity (Table 4.7) were significantly different in compare with those presented in the literature. Only extracts, obtained with hexane, showed the similar tendencies and exhibited the inhibitory activities against *Bacillus subitilis* (10 mm), *Staphylococcus aureus* (11 mm) and *Escherichia coli* (18 mm). Notably, *Astragalus glycyphyllos* leaf extract of hexane was showing very less the zones of inhibition against *Bacillus subitilis, Staphylococcus aureus* and *Escherichia coli*, while comparing with those presented in the literature.

In addition, *Astragalus atropilosulus subsp. Abyssinicus* were showing the zone of inhibition against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* by different extracts such as acetone, ethanol, methanol, 1/1 ethanol/acetone [121]. Compared to the literature only acetone fraction was similar. The zone of inhibition was shown against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli,* and *Pseudomonas aeruginosa* were 10.13±0.2, 17.0±0.89, 10.9±0.51 and 15.3±0.51 respectively from *Astragalus atropilosulus subsp. Abyssinicus* leaves. In our study, *Astragalus glycyphyllos* leaves acetone fraction was exhibiting less zone of inhibition activity comparing to the *Astragalus atropilosulus subsp. Abyssinicus* leaves.

### 4.6. Antifungal activities of plant extracts and fermented with LAB plant leaves

Antifungal activity has been studied against various fungal strains Table 4.8.

**Table 4.8.** Antifungal activity of extracts and fermented using LAB and solid state conditions it's leaves, (-) no inhibition.

| Name of the fungi | Dried *Astragalus glycyphyllos* leaves powder | | Hexane Extract | | Acetone Extract | | Ethanol/Water (70:30) Extract | |
|---|---|---|---|---|---|---|---|---|
| | Non-fermented (mm) | Fermented (mm) | Non-fermented (mm) | Fermented (mm) | Non-fermented (mm) | Fermented (mm) | Non-fermented (mm) | Fermented (mm) |
| *Debaryomyces vanrijiae* | 5±0.02 | 10±0.2 | 3±0.3 | 6±0.4 | 5±0.3 | 9±0.4 | 2±0.1 | 6±0.4 |
| *Geotrichum fermentants* | 3±0.2 | 6±0.3 | - | - | 3±0.02 | 7±0.3 | 2±0.03 | 5±0.4 |
| *Candida pelliculosa* | 4±0.01 | 8±0.2 | 2±0.3 | 6±0.4 | - | 3±0.2 | 2±0.3 | 6±0.4 |
| *Pichia farinose* | - | 6±0.3 | - | 3±0.3 | - | 2±0.3 | - | - |
| *Pichia fermentans* | 4±0.02 | 6±0.3 | - | 2±0.2 | - | - | - | - |
| *Candida krusei* | - | - | - | - | - | - | - | - |
| *Kluyveromyces marxianus* | - | - | - | - | - | - | - | - |
| *Kluyveromyces lactis* | - | - | - | - | - | - | - | - |
| *Aureobasidum pullulans* | 3±0.2 | 5±.01 | - | 2±0.3 | - | 3±01 | 2±0.2 | 4±.01 |

The extracts have shown significant activity against all tested fungal strains. From the results, the widest zone of inhibition against the fungus *Debaryomyces vanrijiae* followed by extracts: acetone > hexane > ethanol/water (70:30) has been found ((9 ± 0.4 mm and 5 ±0.3 mm) > (6 ± 0.4 mm and 3 ±0.3 mm) > (6 ± 0.4 mm and 2 ±0.3 mm)).Fermented plant extract using another indicator strain - *Pichia fermentants* shows a higher clear zone of inhibition (6±0.3 mm), while the antifungal activities of extracts without plant bio-treatment had lower activities against *Pichia farinose* (2 ± 0.2 mm). No antifungal activity against three strains such as *Candida krusei, Kluyveromyces marxianus* and *Kluyveromyces lactisvar latis* has been found by using all type of extracts in both cases (non-fermented and fermented with LAB plant material). The dry powder considered a higher zone of inhibition showed against *Debaryomyces vanrijiae* in compare with other antifungal. In case of *Candida pelliculosa*, showed wider zones of inhibition in hexane and ethanol extracts compare with acetone solvent. In addition to, LAB fermented plant extracts showed random clear zones of inhibition against *Geotrichum fermentants, Aureobasidum pullulans*.

The previous studies were reported on anti-fungal activity determined against *Alternaria alternatra, Candida sp, Drechslera halodes, Fusarium oxysporum, Macrophomina phaseolina, Pythium ultimum* and *Rhizoctoina solani* from *Astragalus atropilosulus subsp. Abyssinicus* leaves using different solvents such as acetone, ethanol, methanol and 1/1 ethanol/acetone mixture [121]. Hence, the zone of inhibition obtained from Candida spp by acetone, ethanol, methanol and 1/1 ethanol/acetone fractions were exhibited as 8.66±0.51mm, 8.83±0.25mm, 11.3±0.58mm and 8.73±0.41mm respectively but during case our study on determining the antifungal activity, the zone of inhibition is varied which is given in table 4.8. Remarkably, the zone of inhibition was shown very less antifungal activity against *Candida pelliculosa* by using different solvent extracts like hexane, acetone and ethanol /water (70:30) from *Astragalus glycyphyllos* leaves, while comparing with *Astragalus atropilosulus subsp. Abyssinicus* leaves. Compared to the literature only acetone fraction was similar. The zone of inhibition was shown against *Candida sp* (8.66±0.51mm), *Alternaria alternatra* (no inhibition), *Drechslera halodes* (11.6±0.45mm), *Fusarium oxysporum* (9.60±0.75mm), *Macrophomina phaseolina* (no inhibition), *Pythium ultimum* (19.6±0.36mm) and *Rhizoctoina solani* (no inhibition) from *Astragalus atropilosulus subsp*. *Abyssinicus* leaves [121]. In our study, *Astragalus glycyphyllos* leaves acetone fraction was exhibiting less zone of inhibition activity comparing to the *Astragalus atropilosulus subsp. Abyssinicus* leaves.

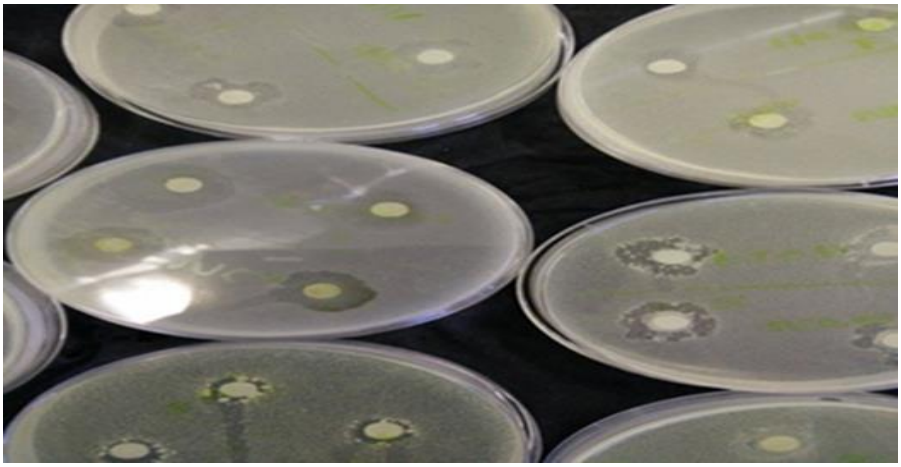

**Figure 4.2**. Zone of inhibition antifungal.

# CONCLUSIONS

1. The extraction yields of *Astragalus glycyphyllos* obtained by consecutive application of different polarity solvents, in Soxhlet extraction: hexane, acetone and ethanol/water, supercritical fluid extraction (SFE-CO2) and pressurized liquid extraction (PLE) acetone, ethanol/water (70:30) were compared. The yield of extract, obtained using ethanol/water (70:30), was higher in compare with the extracts isolated with hexane and acetone, (SFE-CO2) and pressurized liquid extraction (PLE) acetone.
2. The antioxidant activities, obtained by different assays such as the ethanol/water (70:30) extract, had higher antioxidant (AO) values than using TPC, ABTS, DPPH, ORAC and FRAP. Incidentally, by comparing the assays (TPC, ABTS, DPPH, and FRAP) employed to determine the antioxidant values, the ORAC assay values were significantly higher. Ethanol/water extracts obtained by conventional extraction and pressurized liquid extraction had similar properties as almost in all antioxidant assays where the values were also similar.
3. Important effects of many different solvents used for extraction of the phenolic content of *Astragalus glycyphyllos* leaf extracts were considered. In Soxhlet extraction: hexane, acetone and ethanol/water (70:30), supercritical fluid extraction (SFE-CO2) and pressurized liquid extraction (PLE) of ethanol/water (70:30) were used to estimate the effects of the total concentration of phenol extracted from *A. glycyphyllos*. In Soxhlet extraction, ET/W (70:30) extracts possessed the highest TPC values followed by hexane and acetone extracts (Soxhlet extraction), SFE-CO2 and PLE ethanol/water (70:30).
4. The antioxidant activities of non-extracted plant material and *Astragalus glycyphyllos* residue after SCE-CO2 extraction using QUENCHER approach (TPC, ABTS and ORAC) have been determined. The non-extracted plant material possessed the highest total Phenolic Content, ABTS and ORAC with more effective antioxidant activity compared to residue, obtained after SCE-CO2 extraction (TPC, ABTS and ORAC).
5. In addition, high-resolution UPLC-Q-TOF-MS enabled the identification of compounds, which have been isolated from Soxhlet extraction using different solvents: hexane, acetone, ethanol water (70:30) and SCE-CO2. In different extracts the following compounds were identified: 12-oxooctadecanoic acid, m-coumaric acid, rutin and linoleic acid.

6. The extracts from *Astragalus glycyphyllos* leaves, obtained in the Soxhlet extraction process (Hexane /acetone /ethanol: water 70:30), showed antibacterial and antifungal activities. The anti-bacterial action of the extracts was most effective against *Bacillus subtilis* and *Bacillus subtilis spizizenii* and similarly, anti-fungal action of the extract was most effective against *Debaryomyces vanrijiae*. The effect of extracts was inefficient against fungal species such as *Candida krusei, Kluyveromyces marxianus* and *Kluyveromyces lactis*.
7. The extracts, obtained after plant leaves bio-treatment using L. sakei under solid-state conditions (50 %), showed a wider zone of antibacterial inhibition against *Bacillus subtilis* as well as an antifungal behavior against *Debaryomyces vanrijiae* in compare with non-fermented plant raw powder (before extraction). These studies highlight the possibility that antimicrobial effect of extracts can be dramatically improved by using the LAB with antimicrobial activities as starter for plant fermentation.

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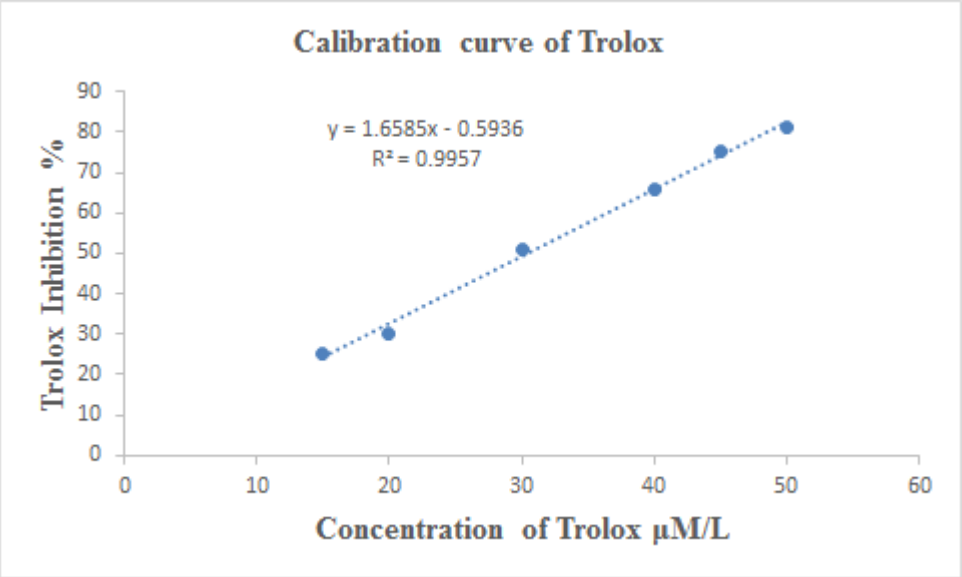
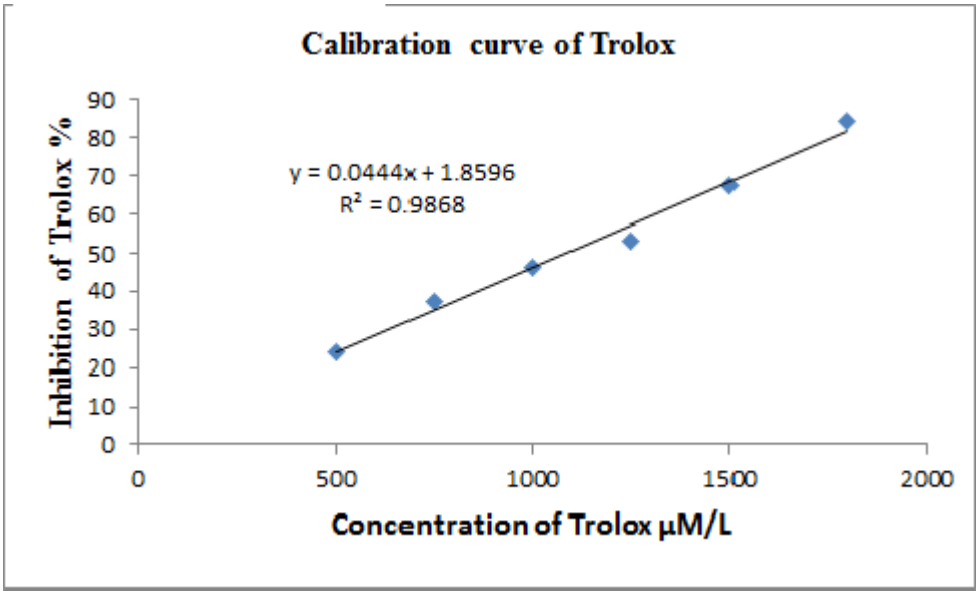
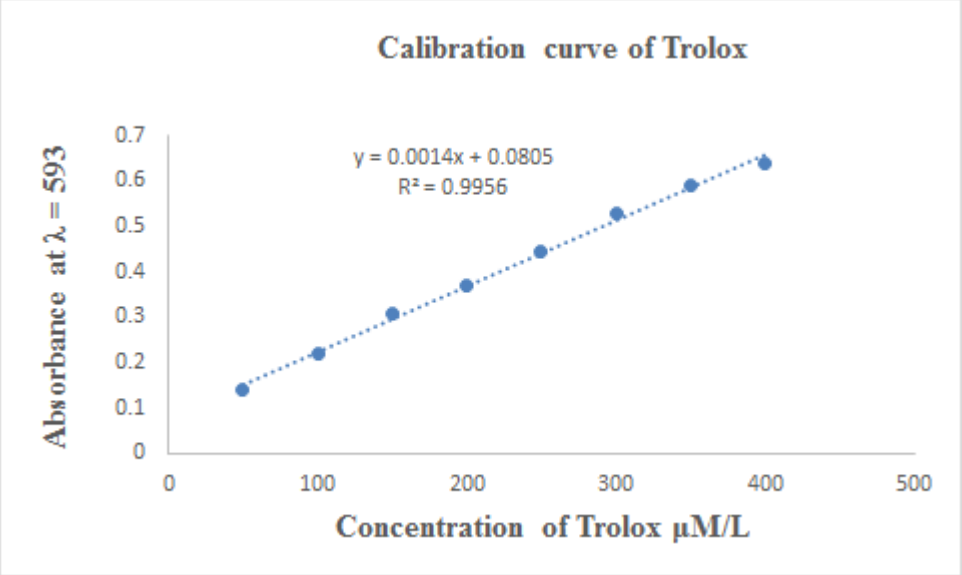
# ANNEXES



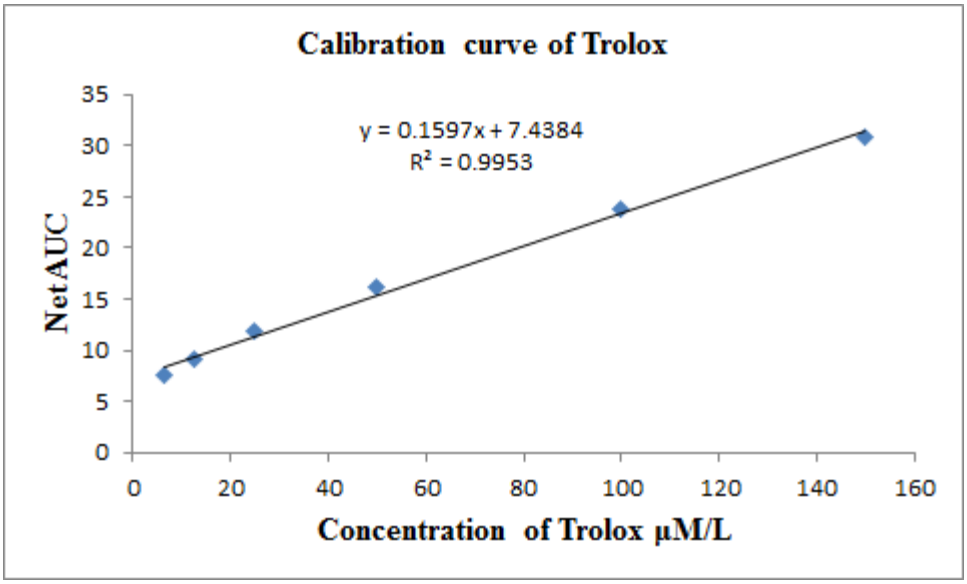

**Annex 1.** Trolox calibration curve for DPPH assay.



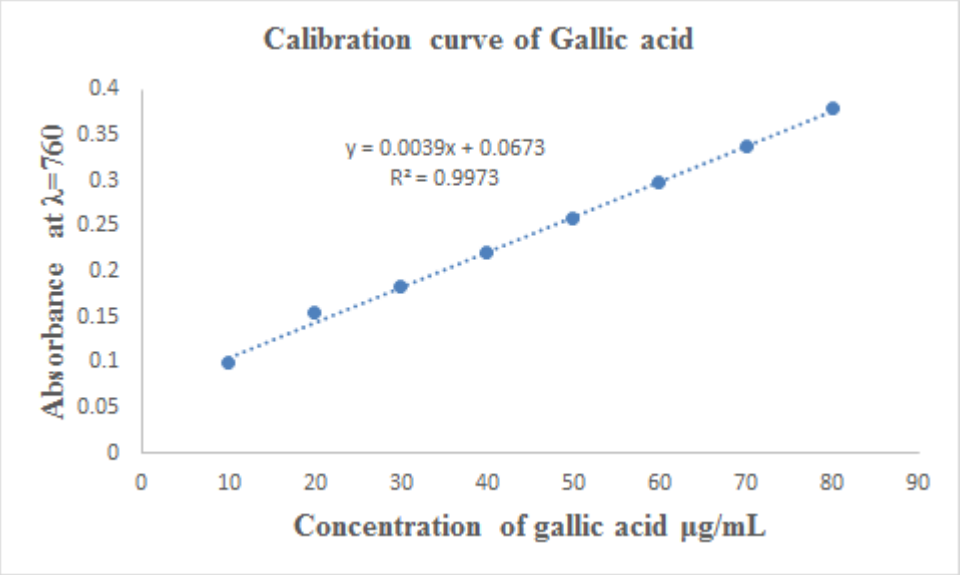

**Annex 2.** Trolox calibration curve for ABTS assay.



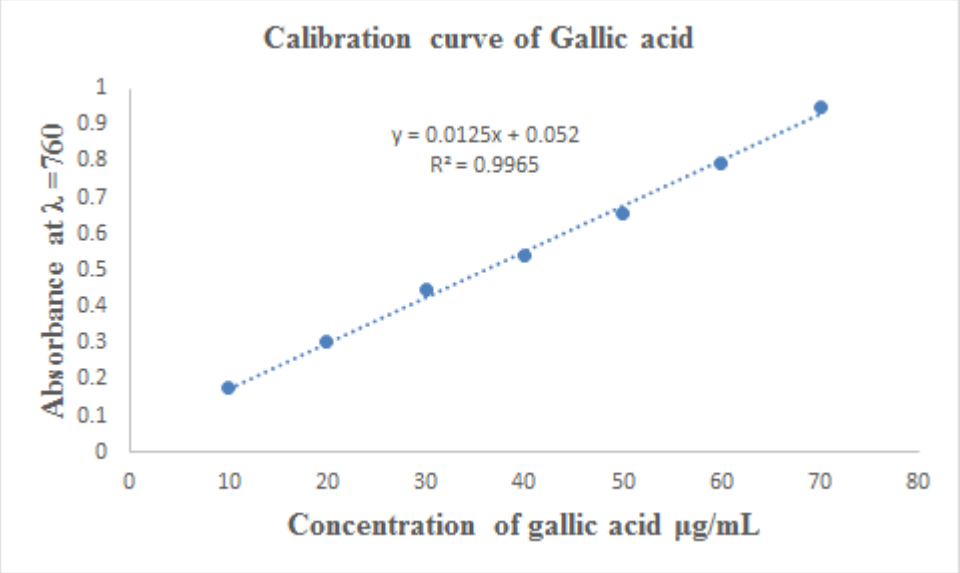

**Annex 3.** Trolox calibration curve for FRAP assay.



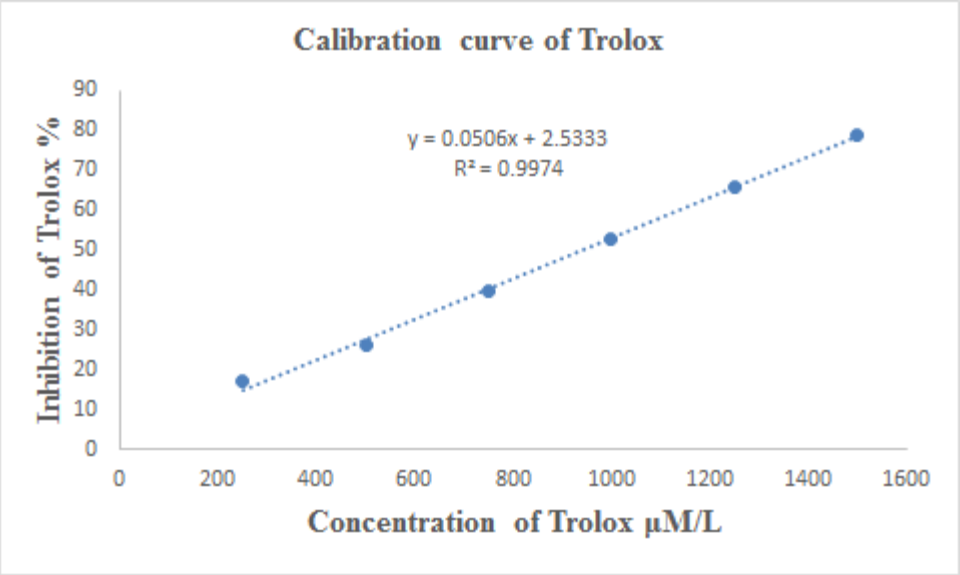

**Annex 4.** Trolox calibration curve for ORAC assay.



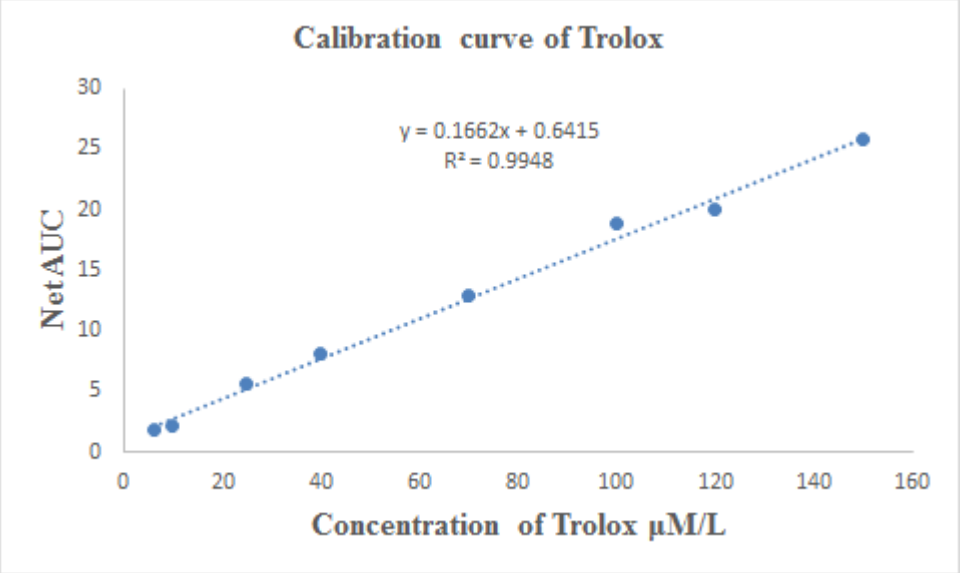

**Annex 5.** Gallic acid calibration curve for total phenol content determination.




**Annex 6.** Gallic acid calibration curve for total phenol content determination by QUENCHER.




**Annex 7.** Trolox calibration curve for ABTS assay by QUENCHER.




**Annex 8.** Trolox calibration curve for ORAC assay by QUENCHER.